# IDENTIFICATION, SEMI-SYNTHESIS AND EVALUATION OF ANTI-OVARIAN CANCER COMPOUNDS FROM PLANTS USED IN TRADITIONAL MEDICINES

**OKIEMUTE ROSA JOHNSON-AJINWO**

**Thesis submitted to Keele University for the**

**degree of Doctor of Philosophy**

**June 2017**

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## ABSTRACT

Ovarian cancer is the second leading cause of death among women in the gynaecological category of cancers. The current post surgery treatment which involves the use of platinum-based therapy with its attendant adverse drug-resistance often results in the return of the cancer. This research work, explored the role of natural products as the major source of new drugs by the evaluation of the anti-ovarian cancer activities of three selected medicinal plants and the semi-synthesis of analogues of an anti-cancer agent; thymoquinone from *Nigella sativa*. Using a bioassay-guided approach, an investigation of the cell growth inhibition of the extracts/fractions of these plants on four human ovarian cancer cell lines, A2780, OVCAR 4, OVCAR 8, and CIS-A2780 showed that *Acalypha wilkesiana, Margaritaria discoidea* and *Rutidea parviflora* had promising anti-ovarian cancer activities. This is the first report of the anti-cancer activities of *M. discoidea* and *R. parviflora*. The bioactive compounds of the plants were isolated by HPLC, identified by mass spectrometry/NMR spectroscopy and investigated for cytotoxicity. Gallic acid, ($IC_{50}$ range of 6.2±0.3 – 26.9±4.1 µM) was the active compound in *A. wilkesiana*. The significantly bioactive compounds of *M. discoidea* were securinine, ($IC_{50}$ range of 2.7±0.7 – 8.7±0.1 µM), betulinic acid, ($IC_{50}$ of 16.0±1.9 µM) and gallic acid. While palmatine, ($IC_{50}$ range of 5.5±0.9 – 7.9±0.5 µM) was the major active compound in *R. parviflora*. Twenty-one thymoquinone analogues including eleven semi-synthetic ones were evaluated for cytotoxicity. A synthetic 2-dimethylamino-5-methyl-1,4-benzoquinone demonstrated optimum activity ($IC_{50}$ range of 4.7±0.5 – 11.2±1.9 µM) and superior aqueous solubility. Palmatine, securinine and 2-dimethylamino-5-methyl-1,4-benzoquinone showed induction of apoptosis via increased caspase 3/7 activity. Palmatine demonstrated PAPR cleavage by western blot analysis. 2-dimethylamino-5-methyl-1,4-benzoquinone showed synergy with carboplatin and paclitaxel. These studies have provided scientific evidences for the potential treatment of ovarian cancer with these traditional medicinal plants and hit compounds for future optimization towards clinical trials.

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# ABBREVIATIONS

| TERM | ACRONYM |
|---|---|
| Acalypha wilkesiana | AW |
| America Tissue Culture Collection | ATCC |
| Applied Biological Materials | ABM |
| Betulinic Acid | BA |
| Carboplatin | CBPT |
| Combinational Index | CI |
| Dimethyl sulfoxide | DMSO |
| Fetal bovine serum | FBS |
| Gallic acid | GA |
| Gas chromatography mass spectrometry | GC-MS |
| Glacial acetic acid | GAA |
| High-performance liquid chromatography | HPLC |
| Human Ovarian Epithelial Cell Line | HOE |
| International Centre for Ethno medicine and Drug Development | INTERCEDD |
| Liquid Chromatography mass spectrometry | LC-MS |
| Margaritaria discoidea | MD |
| N,O-bis(trimethylsily)trifluoroacetamide | BSTFA |
| National Cancer Institute | NCI |
| Nuclear magnetic resonance | NMR |
| Palmatine | PALM |
| Phosphate buffered saline | PBS |
| Poly (ADP) ribose polymerase | PARP |
| Reactive oxygen species | ROS |
| Retention time | Rt |
| Room Temperature | RT |

| | |
|---|---|
| Roswell Park Memorial Institute | RPMI 1640 |
| Rutidea parviflora | RP |
| Securinine | SECU |
| Selectivity Index | SI |
| Stable Isotope Dilution Gas Chromatography−Mass Spectrometry | SID GC-MS |
| Standard deviation | SD |
| Standard error of the mean | SEM |
| Sulfordamine B | SRB |
| Thymoquinone | TQ |
| Trichloroacetic acid | TCA |
| Trimethylsilyl | TMSi |
| Trypan Blue Assay | TBA |
| Urs-12-en-24-oic acid 3-oxo-, methyl ester, | EA2 |
| 2-Dimethylamino-5-methyl-1,4-benzoquinone | COMPOUND **6** |

# PUBLICATIONS

## ***Journal Articles***

1. **Johnson-Ajinwo, O.R.,** Li, W.W. Stable Isotope Dilution Gas Chromatography-Mass Spectrometry for Quantification of Thymoquinone in Black Cumin Seed Oil. *J. Agric. Food Chem*. 2014, 62, 5466−5471 (Chapter 7 of Thesis)

2. **Johnson-Ajinwo, O.R.,** Richardson, A., Li, W.W. Cytotoxic effects of stem bark extracts and pure compounds from *Margaritaria discoidea* on human ovarian cancer cell lines. *Phytomedicine.* 2015 Jan 15;22(1):1-4. doi: 10.1016/j.phymed.2014.09.008. Epub 2014 Oct 22 (Chapter Three of thesis)

3. Li, W.W., **Johnson-Ajinwo, O.R.,** Uche, F.I. Advances of Plant-derived Natural Products In Ovarian Cancer Therapy. *International Journal of Cancer Research and Prevention*, Vol 9 No. 1, 2016 Nova Science Publishers Inc (Chapter One of Thesis)

## ***Book Chapter***

Handbook on Ovarian cancer: Risk Factors, Therapies and Prognosis, 2015. Editor-Bethany R. Colliers, Chapter 10: Potentials of Phytochemicals and Their Derivatives in the Treatment of Ovarian Cancer. Wen-Wu Li, **Okiemute Rosa Johnson-Ajinwo** and Fidelia Ijeoma Uche. Nova Science Publishers Inc. (Chapter One of Thesis)

## ***Conference Abstracts***

1. Li, W. W., **Johnson-Ajinwo, O. R.,** Siddique, M. R., Bajana, B., Sule-Suso, J., Richardson, A. Anti-cancer thymoquinone from *Nigella sativa*. *Planta Med*. 2013, 79, 1126
2. **Johnson-Ajinwo, O.R.**, Richardson, A., Li, W.W. Anti-Ovarian Cancer Activity of *Margaritaria discoidea* and other Nigeria Medicinal Plants. 2014, 93
3. **Johnson-Ajinwo, O.R.,** Richardson, A., Li, W.W. Design, Synthesis and Anti-ovarian Cancer Activity of Thymoquinone Analogues. APSGB. 2014, 3, 1

4. **Johnson-Ajinwo, O.R.,** Richardson, A., Li, W.W. Design, Synthesis, Drug-Likeness and Anti-ovarian Cancer Activity of Thymoquinone Analogues. PSNA. 2015, P19, 53

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# APPENDICES

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This research work is dedicated to the Almighty God, my source of Inspiration.

# Chapter One

## *Introduction*

## *1.1 Cancer*

Cancer is a health burden of global dimensions as it ranks amongst the deadliest diseases and is the leading cause of death in both developed and developing nations today. Cancer-related deaths claimed 8.2 million lives globally in 2012. The current global estimate of new cancer diagnosis made was 14 million in 2012. There is an immense cancer burden observed from the cancer statistics report for African, Asian, Central and South American populations; where about 70% of the cancer deaths reportedly occurred in these regions (Ferlay et al., 2017).

Cancer could be defined as a disease condition which is characterised by the uncontrollable proliferation of cells in any part of the body, leading to the destruction of tissues and organs. There are over 200 different types of cancer (UICC, 2017), of which ovarian cancer is the second most common gynaecologic cancer among women. Current treatment involves the use of radiotherapy, surgery and chemotherapy which usually poses some adverse effects to the patient.

## *1.2 Ovarian Cancer*

In the England, about 6000 women are diagnosed with ovarian cancer annually with a mortality of about 4000 deaths recorded (Trent Cancer Registry, 2012). This cancer is the fifth commonest cancer that affects women in the UK. In the United States, it was estimated that there would be about 22,000 new cases and 14,000 deaths from ovarian cancer in 2013 (NCI, Progress Report, 2012).

Ovarian cancer is the ninth most prevalent cancer in the US, and is classed under the gynaecologic cancers where it is the second major cause of death in this category of cancers in the UK and US (Siegel et al., 2012).

The symptoms of ovarian cancer include abdominal and pelvic pain, frequent urination, abdominal blotting, a feeling of fullness /loss of appetite, swollen abdomen, back ache, irregular menstruation and post-menopausal bleedings. This cancer forms in tissues of the ovary and there are several types of ovarian cancers, such as ovarian epithelial carcinoma and malignant germ cell tumours. The most prevalent of the ovarian cancers is the ovarian epithelial carcinoma with a prevalence of about 90% (Jelovac et al., 2011).

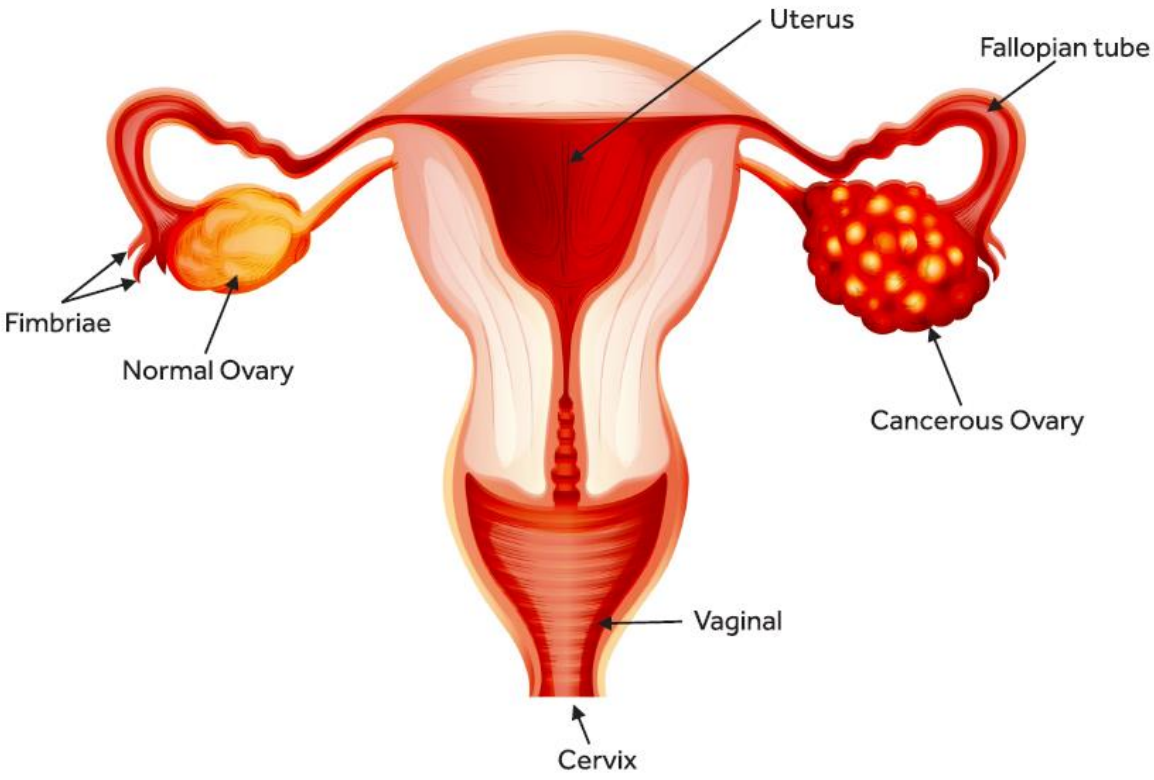



**Figure 1.1 An Illustration of Ovarian cancer**

The high mortality rate of ovarian cancer is largely due to poor diagnosis because the cancer lacks early detectable symptoms and thus there are no screening tests available for the cancer. About 70% of cases are not diagnosed until they have reached advanced stages due to ovarian cancer's ability to invade other surrounding tissues such as the bladder, bowel and uterus. These cancer- invaded tissues eventually develop new tumours; compounding the situation, whilst the cancer is yet undetected, thus making ovarian cancer poorly treatable (Jayson et al., 2014). Presently, the standard treatment for ovarian cancer entails surgical

cytoreduction aimed at removing most of the cancerous cells, followed by the administration of paclitaxel and carboplatin resulting in multiyear survival. However, use of platinum-based chemotherapy introduces drug resistance, which could result in ovarian cancer relapse leading to the death of the cancer patients (Petty et al., 1998; Xu et al., 2012). Thus, the investigations into the discovery and identification of new drug leads that may offer therapeutic benefits in ovarian cancer therapy is compelling and urgent in the light of the ovarian cancer menace.

## *1.3 Medicinal Plants-A Proven Source of Cancer Drugs*

The use of plants in the treatment of cancer, is not new, as there are documented evidences of the use of medicinal plants by indigenious communities for the treatment of cancer in several countries such as Nigeria (Sowemimo et al., 2010), Malaysia and Thailand (Lee et al., 2005). Hence, research into medicinal plants has once again aroused global attention and significance.

Historically, Mankind have long benefitted from the efficacy of medicinal plants, even before the advent of orthodox medicine. In the indigenous communities, it is still a common practice to use decoctions of medicinal plants for the treatment of diseases, which often has offered useful leads to the discovery of new drugs. For instance, the medicinal uses of the cinchona bark from which quinine was derived were first discovered by the Quechua people of Bolivia and Peru, who used the plant in the treatment of fever, before its antimalarial properties was validated (Motley et al., 2010). Similarly, *Artemisia annua*, from which artemisinin, a renowned antimalarial drug which has recently been shown to possess anticancer activity, has been in use in the traditional Chinese medicines (TCM) for a very long time (Tu1981).

Numerous drugs have been derived from plants. The discovery of the Vinca alkaloids (vinblastine and vincristine) in the 1950s marked the beginning of the search for anti-cancer agents from plants (Cragg et al., 2005). This was followed by the epipodophyllotoxin lignans (teniposide and etoposide), then the discoveries of paclitaxel and the camptothecins. These

four classes of plant derived antineoplastic agents are currently in use for various cancers (DeVita et al., 2008). An inventory of the anticancer drugs in use from 1981-2006 showed that 47% of the 155 clinically approved oncological drugs were derived from natural products, either as unmodified compounds, or semi-synthesised analogues, or synthesised based on natural product leads (Cragg et al., 2007). Interestingly it has been observed that secondary metabolites from natural products often have diverse structures with a high degree of biological suitability than compounds produced from chemical synthesis (Bindseil et al., 2001; Balunas et al., 2005). This unique quality of natural product-derived compounds have immense potentials as leads which could be further optimized by synthesis to obtain more potent analogues, which again underscores the importance of natural product as a rich source of drug remedies. Semi-synthesis offers several obvious advantages which makes it an indispensable tool in the drug discovery and production processes. Typically, the stem barks of about 2,000- 3,000 trees were required for the isolation of 1 kg of Taxol (paclitaxel), (Runowicz et al., 1993; Suffness et al., 1995). But through semisynthetic methods, comes the commercial production, which is geared towards meeting the herculean demand for the drug which otherwise would not be met by the natural sources. Paclitaxel is commercially produced based on semi-synthesis.

Another advantage of semi-synthesis is in the optimization of potent drugs. This use of semi-synthesis has been widely exploited in the development of analogues of a potent drug candidate, with a higher degree of potency, such as digitoxigenin, a synthetic analogue of digitoxin which has enhanced anticancer activity (Langenhan et al., 2005) and Vinorelbine a semi-synthetic analogue of the Vinca alkaloids (Goa et al., 1994). Also, the employment of semi-synthesis has been utilised in overcoming the problem of poor solubility and adverse reactions of potent drugs. For example, due to the low solubility and high toxicity of Camptothecin, semi-synthethic derivatives such as topotecan and irinotecan, with enhanced solubility and far-less toxicity were developed (Oberlies et al., 2004).

### 1.3.1 Natural Products Derived and Platin-derived Drugs Clinically Used in the Treatment of Ovarian Cancer

#### 1.3.1.1 Camptothecin

Camptothecin (Figure 1.2) is a cytotoxic compound isolated from the Chinese tree *Camptotheca acuminate* (family; Cornaceae) (Li et al 2005), by Drs' Monroe E. Wall and Mansukh C. Wani of Research Triangle Institute (Wall et al., 1966). The compound exhibited significant cytotoxicity against breast, pancreas, lung, stomach and ovarian cancers. The drug's mechanism of action is by the inhibition of DNA relaxation mediated by DNA topoisomerase I, through the formation of a complex between DNA and topoisomerase I (Thomas et al., 2004). Camptothecin is very hydrophobic, which have led to the semi-synthesis of some analogues of the drug. Two of these analogues; Hycamtin (topotecan) and Camptosar (irinotecan or CPT-11) (Figure 1.2) are clinically used in the treatment of ovarian and colon cancers and are marketed by GlaxoSmithKline and Pfizer respectively. The side effects of these drugs include severe myelosuppression, diarrhoea and increased susceptibility to infections (Oberlies et al., 2004).

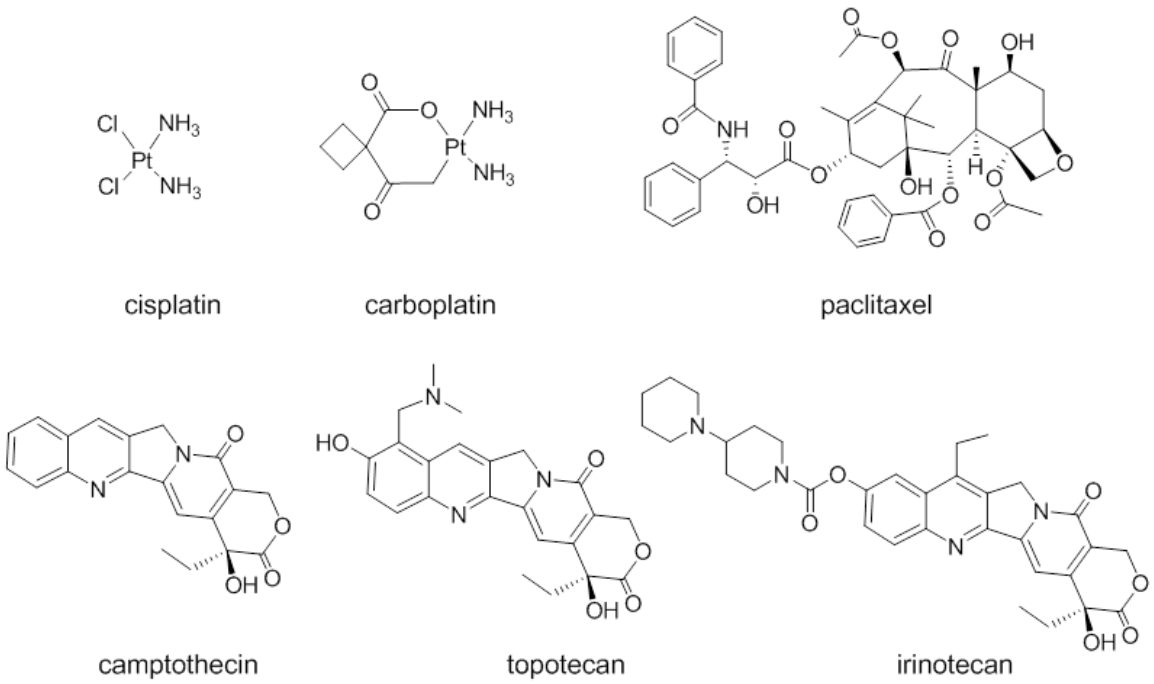



**Figure 1.2 The chemical structures of some natural products derived drugs and platin-derived drugs clinically used in the treatment of ovarian cancer.**

### 1.3.1.2 Paclitaxel

Paclitaxel (Figure 1.2) was originally discovered from the bark of the pacific yew tree, *Taxus brevifolia*, Nutt. in 1964 by Drs' Monroe E. Wall and Mansukh C. Wani of Research Triangle Institute (Wani et al., 1971). The use of paclitaxel in the treatment of cancers has been on for more than two decades. The mechanism of action of Paclitaxel involves the ability of the drug to bind onto beta-tubulin subunits of microtubule, thus stabilizing the microtubule and preventing a disassembly, causing defects in mitotic spindle assembly, segregation of chromosomes and cell division (Suffness et al., 1995; Lowe et al., 2001). Paclitaxel has been approved in the UK for use in the treatment of breast, lung, non-small cell lung and ovarian cancers. Paclitaxel is used in the first-line and second-line treatment of ovarian cancer (NICE 2003). The drug has a number of side effects which have necessitated its use in combination with other agents, such as carboplatin which are less toxic. These side effects include: shortness of breath, severe exhaustion, unusual bleeding, painful joints and vomiting (Ozcelik, et al., 2010). Several derivatives of paclitaxel have been developed. One of which is Abraxane; an albumin nano-particle-paclitaxel water-soluble formulation, with less side

effects, approved by the FDA in 2005 for the treatment of metastatic breast cancer (Chen et al., 2015). Another water soluble conjugate of paclitaxel and poly(L-glutamic acid); Paclitaxel-poliglumex has been assessed in pre-clinical pharmacokinetic studies and in phase I/II clinical trials for ovarian cancer. However the drug showed no significant advantage over the use of taxane-based regimen (Galic et al., 2011).

#### 1.3.1.3 Cisplatin

Cisplatin is one of the platinum-based drugs used in the treatment of cancers. This synthetic agent was the lead compound for the synthesis of other platin-based drugs. The mechanism of action (MOA) of cisplatin is by alkylation of the DNA. The drug is very toxic and could cause damage to kidneys, and nerves. Other adverse effects of cisplatin include loss of hearing, vomiting and bone marrow suppression resulting in anaemia. Cisplatin is used in the treatment of several cancers, such as testicular, small cell lung and ovarian cancers. The major drawback in the use of cisplatin is the adverse side effects, so it is used mainly in conjunction with other chemotherapeutic agents (Loehrer et al., 1984; Milosavlievic et al., 2010; CNRS 2010).

#### 1.3.1.4 Carboplatin

Carboplatin is another platinum-based drug in the treatment of some cancers. The compound is also a DNA alkylating agent but unlike cisplatin, carboplatin is less toxic and thus has fewer side effects compared with cisplatin. However, carboplatin is less effective than cisplatin. The drug is used in the treatment of lungs, ovarian, head and neck cancers. Also, as with all platinum drugs, there is the development of platinum resistance which may cause the cancer to re-emerge (Stordal et al., 2007; Wheate et al., 2010).

### 1.3.2 Plant natural products in Clinical Trials

#### 1.3.2.1 The Epipodophyllotoxin Lignans: Etoposide and Teniposide

The lignans, epipodophyllotoxin and podophyllotoxin (Figure 1.3) were isolated from the roots of the poisonous North American May Apple (Mandrake) plant, *Podophyllum peltatum* L. (family: Berberidaceae). The plant is highly toxic when consumed in large quantities but in small amounts has been used in folklore medicine for the treatment of a vast array of ailments such as typhoid fever, cholera, dysentery, venereal diseases, worm expulsion, prostate problems and cancer. The decoction has a strong purgative activity (Ernest et al., 1999). Podophyllotoxin is available as Podofilox (trade name is Condylox); an extract of the plant and is used for the treatment of genital warts (Xu et al., 2009). Podophyllotoxin's mechanism of action is through the formation of tubulin complex to forestall the production of microtubules, (Canel et al., 2000). The glycosidic derivatives; etoposide and teniposide possess anti-tumour activities, as topoisomerase II inhibitors. Both drugs are in clinical use with etoposide (VP-16) employed in the treatment of non-lymphocytic leukaemia, lymphoma, Kaposi sarcoma, Ewing's sarcoma, testicular and lung cancers (Hande 1998). Teniposide (Vumon) is used in the treatment of some brain tumours, Hodgkin's lymphoma and acute lymphocytic leukaemia in children (Jasek 2007).

Four clinical trials of etoposide in the treatment of ovarian cancer have been conducted. In the first study, the drug demonstrated activity in recurrent ovarian cancer previously treated with cisplatin, but showed a relatively short response and survival periods (Moosavi et al., 2004). Another trial observed positive outcomes from an alternating use of 50mg/day dose of orally administered etoposide and blotecan (a synthetic derivative of camptothecin) in platinum-resistant ovarian cancer patients, (Hwang et al., 2012). Other clinical trials for combinations of oral etoposide and irinotecan on recurrent ovarian cancers previously treated with platinum and taxane drugs were inconclusive, as one group observed high response rate in the combination but with significant un-anticipated haematological toxicity

recorded.A second group reported an insignificant moderate response rate in a study of 60 patients (Gronlund et al., 2005; Matsumoto et al., 2012, 2015). Further clinical trials are required to establish the potency and safety of etoposide in ovarian cancer treatment.

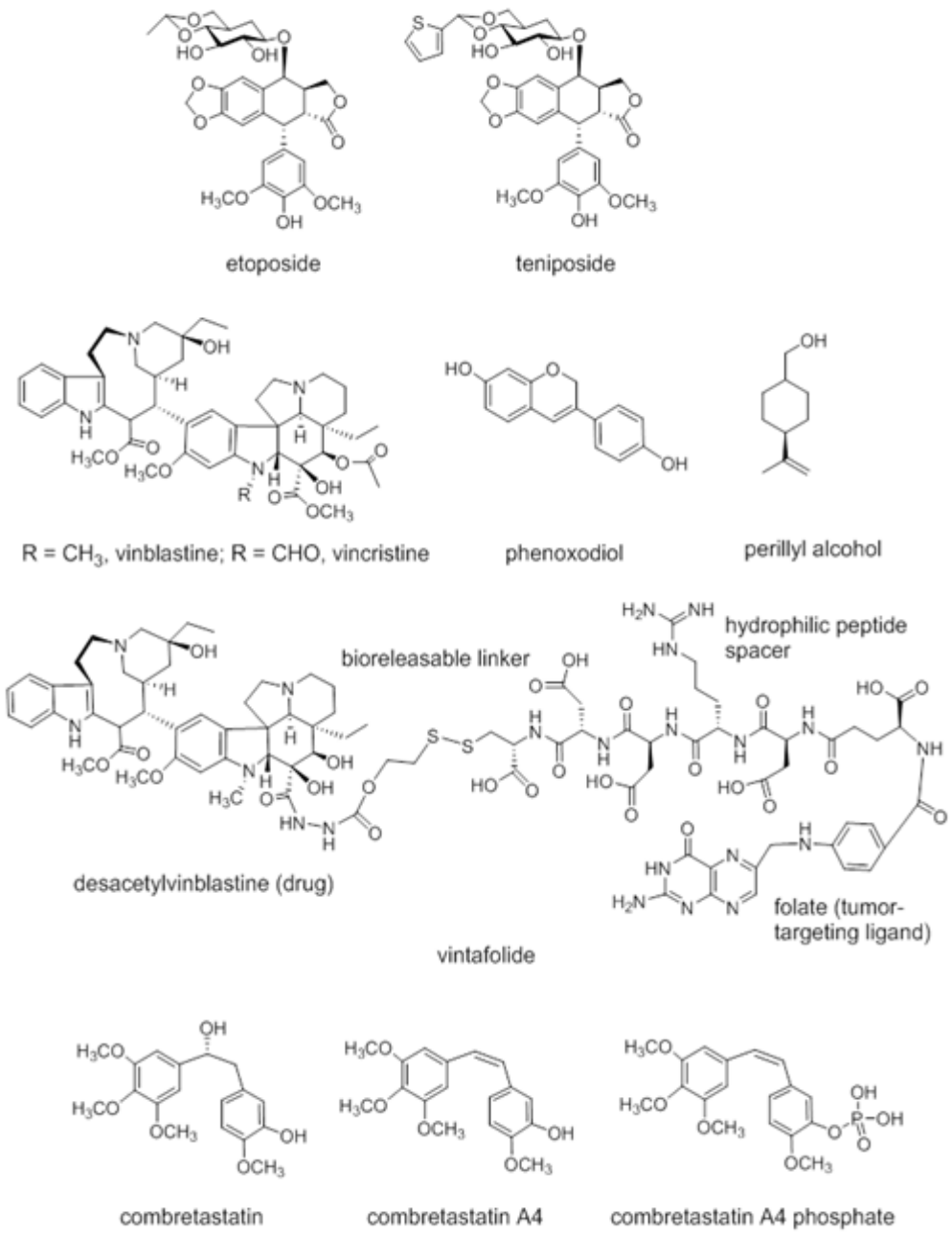



**Figure 1.3 Phytochemicals and their derivatives currently in clinical trials for the treatment of ovarian cancer.**

### 1.3.2.2 Vinca Alkaloids Derivatives

Two vinca alkaloids; vinblastine and vincristine (Figure 1.3) were obtained from *Catharanthus roseus* (L.) G. Don (Family: Apocynaceae), a Chinese plant. The compounds are microtubule inhibitors similar to podophyllotoxin (Capasso 2012) and are used as anti-leukaemic agents.

The overexpression of folate receptor (FR) is exhibited by ovarian cancer cells and has been explored as a diagnostic biomarker for the targeting of these cells by the development of a derivative of vinblastine; Vincafolide (EC145), which is a folic acid and desacetyvinblastine conjugate, (i.e. a FR-targeted vinca alkaloid agent) is being optimised for the treatment of epithelial ovarian cancer (Vergote et al., 2015).

Additionally, EC20, a radiopharmaceutical was developed for use as a non-invasive detection agent for active FR. The combined use of these two agents have demonstrated promise in ovarian cancer clinical trials, with vincafolide shown to possess a good safety profile in phase I trials. A comparative study involving the combination of vincafolide and pegylated liposomal doxorubicin (PLD) against the single use of PLD in a phase II trial of participants with platinum-resistant ovarian cancer observed statistically significant outcomes in progression-free survival in the drug combination group of participants. The Phase III Trial is currently on-going (Graybill et al., 2014).

### 1.3.2.3 Combretastatins

Combretastatins (Figure 1.3) were isolated from the South African bush willow tree, *Combretum caffrum* (Eckl. And Zeyh) Kuntze, (family: Combretaceae), (Petit et al., 1995). The compounds are stilbenoid phenols with antitumour activities against leukaemia, colon and lungs cancers. Combretastatins inducetumour necrosis by binding to the micro-tubulins (Tozer et al., 2002).An analogue, Combretastatin A-4 could be considered the most cytotoxic of the isolated combretastatins, (Petit et al., 1995; Ohsumi et al., 1998).

A Phase II clinical trial of a combination of combretastatin A4 phosphate, carboplatin and paclitaxel in the treatment of patients with platinum-resistant ovarian cancer, showed improved responses with an increase in tolerance compared with the administration of the chemotherapeutic agents in the absence of combretastatin A4 phosphate (Zweifel et al., 2011).

### 1.3.2.4 Perillyl Alcohol

Perillyl alcohol (Figure1.3) is a monoterpene derived from lavender, sage, peppermint, spearmint, lemon grass, cherries and celery seeds (Belanger et al., 1998; Crowell et al., 2001). The anti-cancer mechanism of action of perillyl alcohol may involve blocking signal transduction by the inhibition of post-translational isoprenylation of small G proteins, (Azzoli et al., 2003). A phase II clinical study of the compound in advanced ovarian cancer patients for a duration of six months observed no increase in the time-to-progression of the disease in the patients at a dose of 1200 to 1500mg/m2 (Bailey et al., 2008). The recent development in inhalation chemotherapy has been applied in the treatment of recurrent malignant glioma with perillyl alcohol and shown to be an effective and safe strategy (CO et al., 2013). There are several applications in clinical trials involving malignant brain tumour patients in Brazil (Chen et al., 2015).

### 1.3.2.5 Phenoxodiol

Phenoxodiol (Figure1.3) is a derivative of genistein, an isoflavone found in soybean, [*Glycine max* (L.) Merr; *Fabaceae*]. Recent findings seem to point to an inverse relationship between the dietary intake of isoflavones and the occurrence of cancer. Thus, investigations into the anti-cancer activities of these compounds have become necessary. Preclinical studies of phenoxodiol, indicated cytotoxic activities, with chemotherapeutic potentials in the treatment of solid carcinomas such as breast carcinoma, head and neck squamous carcinomas, (Constantinou et al., 2002; Aguero et al., 2005) and haematological-related cancers, (Patries et al., 2009).

The agent was shown to induce apoptosis in ovarian cancer cells and also act as a chemosensitizer, for many drugs, such as carboplatin, gemcitabine, paclitaxel, topotecan and docetaxel in chemo-resistant ovarian cancer cells, (Alvero et al., 2006).

Clinical trials of phenoxodiol on cervical, ovarian and prostate cancers have demonstrated that the agent is a promising drug candidiate for cancer treatment (Mor et al., 2006; Silasi et al., 2009). Further clinical evaluation of a combination of phenoxodiol and either cisplatin or paclitaxel on gynaecological cancers such as cancers of the ovary, fallopian tube and primary peritoneal cancer which have developed resistance to cisplatin or paclitaxel showed that a combination of phenoxodiol and cisplatin was the most active of the drug combinations. However a very recent study observed a lack of activity in the oral administration of phenoxodiol in combination with a weekly dose of AUC2-carboplatin in platinum-resistant ovarian cancer, (Fotopoulou et al., 2014).Further investigations on the drug candidate are relevant (Kelly et al., 2011).

### 1.3.3 Natural Products Derived Drugs in Pre-Clinical Trials for the Treatment of Ovarian Cancer

#### 1.3.3.1 Baicalin and Baicalein

Baicalin and baicalein (the aglycone of baicalin) (Figure 1.4), are flavones isolated from the Chinese medicinal plants; *Scutellaria baicalensis* Georgi (family; Lamiaceae) and *Scutellariae radix*. The anti-inflammatory, anti-cancer and neuroprotective activities of these compounds have recently being reported, (Ye et al., 2002). The compounds demonstrated cytotoxic activities *in vitro* on two ovarian cancer cell lines; OVCAR 3 and CP-70, at an $IC_{50}$ range of 25-40μM for baicalein and 45-55μM for baicalin, with mild toxicity on normal ovarian cancer cells, (Chen et al., 2013).

### 1.3.3.2 Betulinic Acid

Betulinic acid (Figure 1.4) a lupane-type pentacyclic triterpene was first isolated from the white birch, (*Betula pubescens*), is found in several plants, such as *Crossopteryx febrifuga* (family: Rubiaceae), *Diospros Leucomelas* (family: Ebenaceae) and *Ziziphus mauritiana* Lam. (family: Rhamnaceae), (Cichewicz et al., 2004; Zhang et al., 2015). Betulinic acid is a topoisomerase inhibitor, (Chowdhury et al., 2002).The compound has demonstrated cytotoxicity in leukaemia, (Ji et al., 2002) ovarian carcinoma and malignant brain tumour cell lines, (Zuco et al., 2002). The anti-cancer activities of betulinic acid has been reported in ovarian cancer *in vitro*, and the compound exhibited significant synergism with 5-fluorouracil on ovarian cancer cells, (Ying-Jian et al., 2015).

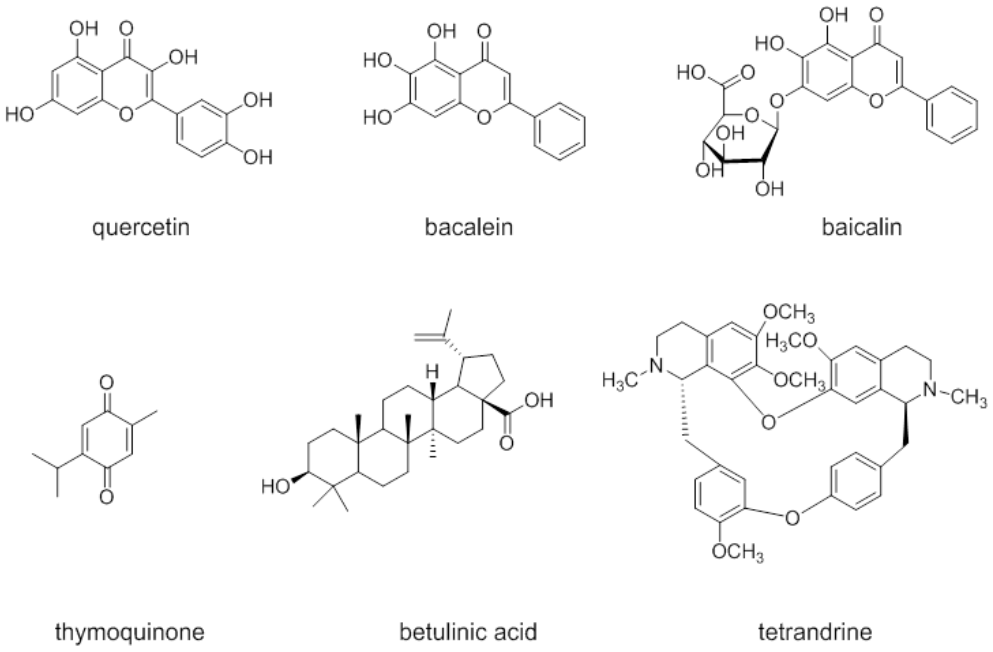



**Figure 1.4 Some plant-derived natural products in pre-clinical trials.**

### 1.3.3.3 Tetrandrine

Tetrandrine (Figure 1.4) is a bis-benzylisoquinoline-type alkaloid, isolated from the Chinese herb, *Stephania tetrandra* S Moore (family: Menispermaceae). The compound has been obtained from other Chinese and Japanese herbs. Tetrandrine, a known calcium channel

blocker has a broad range of bioactivities, which include anti-inflammatory, anti-allergenic, and anti-tumour activities, (Zhang et al., 2009) with the potentials for use in liver cancer treatment, (Liu et al., 2011). The *in vitro* and *in vivo* studies of combinations of cisplatin and tetrandrine on ovarian cancer demonstrated significant increase in cytotoxicity, in both studies compared to the single use of the agents (Zhang et al., 2011).

### 1.3.3.4 Quercetin

Quercetin (Figure 1.4) is a naturally occurring flavonoid obtained from vegetables like watercress, radish, fennel leaves, dill, red onion and capers. Quercetin has a wide range of pharmacological activities, such as anti-inflammatory, anti-allergic and anti-tumour activities. The compound's activity on ovarian cancer cell lines has been documented with the compound exhibiting synergism in combination with cisplatin, (Scambia et al., 1990). Quercetin has been shown to activate caspase 3 and 9 with the induction of apoptosis on A2780 ovarian cancer cell. Also, in xenograft studies of A2780 ovarian cancer tumour, the intravenous administration of a nano-formulation of quercetin encapsulated in mono-methoxy poly(ethylene glycol)-poly(ε-caprolactone) particles demonstrated a significant reduction in tumour growth (Gao et al., 2012). Further studies have examined the safety of quercetin and shown that the compound is less toxic and potentiates the therapeutic activity of cisplatin and paclitaxel in the treatment of ovarian cancer at low doses. The same effect was observed in a xenograft mouse model of ovarian cancer, involving the use cisplatin and quercetin (Yang et al., 2015). Quercetin's ability to reverse multi-drug resistance has been demonstrated in *in vitro* experiments (Maciejczyk et al., 2013).

### 1.3.3.5 Thymoquinone

Thymoquinone (TQ, Figure 1. 4), is a promising anticancer agent isolated from the volatile oil of *Nigella Sativa* L. (Family: Ranunculaceae) (El-Dakhakhany 1963; Schneider-Stock et al., 2014). The compound is a 2, 5-di-substituted benzoquinone and thymoquinone has shown activity against numerous cancers, which include leukaemia, osteosarcoma, breast

adenocarcinoma, lungs, colorectal, pancreatic, prostate and ovarian cancers (Gali-Muhtasib et al., 2006; Woo et al., 2011; Schneider-Stock et al., 2014).The compound has been shown to hinder the proliferation of ovarian cancer cells *in vitro*, with minimal toxicity on normal cells (Shoieb et al., 2003). Further studies on the mechanism of action of thymoquinone in breast cancer *in vitro* observed the induction of apoptosis and the activation of caspases 7, 8 and 9 (Woo et al., 2011). In a prostate cancer xenograft study, some researchers have reported the inhibition of tumour angiogenesis by thymoquinone. Further inhibition of tumour growth with minimal toxicity was also reported (Yi et al., 2008). More details about thymoquinone are discussed in later sections.

### 1.3.4 Recent Plant-Derived Compounds with Activities on Ovarian Cancer Cell Lines

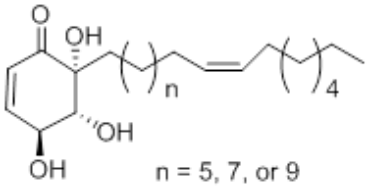
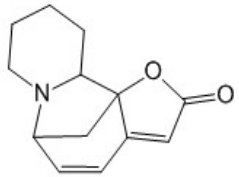
About 200,000 secondary metabolites have been reported in plants. Some of the important phytochemical constituents, found in plants, include alkaloids, flavonoids, tannins, terpenes, steroids, glycosides, saponins, fatty acids, and quinones. The bio-activities of medicinal plants have been linked to the presence of one or more of the various classes of phytochemicals. Alkaloids, phenolics and terpenes rank amongst the commonest class of biosynthesised compounds (Kinghorn et al., 2011).

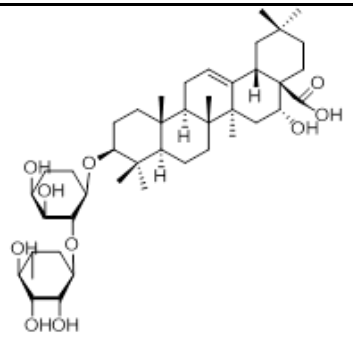
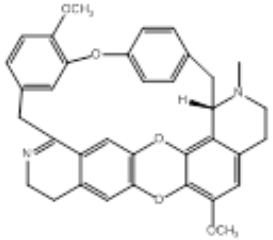
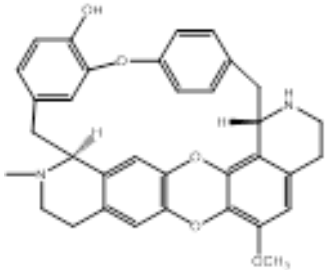
During the time-frame 1981-2010, about 50 anti-cancer drugs have been developed from natural products, with 5 drugs approved in 2010: romidepsin from the bacteria *Chromobacterium violaceum* and used in the treatment of T-cell lymphomas and peripheral T-cell lymphomas(NCI 2009); cabazitaxel a derivative of paclitaxel used in the treatment of prostate cancer [Jevtana (cabazitaxel) Injection](Sanofi-Aventis. 2010); eribulin, a synthetic macrocyclic analogue of halichondrin B which was obtained from species of *Halichondria*- a sea sponge and is used in the treatment of breast cancer (Towle et al., 2001; Yu 2005); mifamurtide, a synthetic derivative of muramyl dipeptide (MDP), isolated from Mycobacterium species used in the treatment of osteosarcoma (Bin et al., 2016); and vinflunine , a derivative

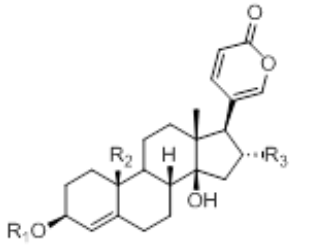
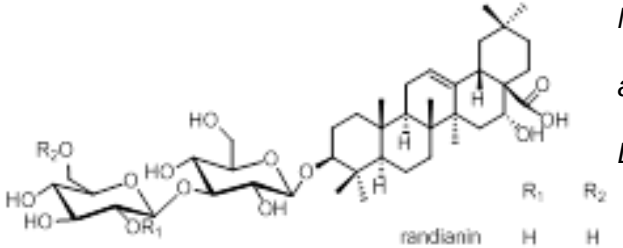
of vinca alkaloids used in the treatment of metastatic transitional cell cancer of the urothelial tract (Kruczynski et al., 1998). This underscores the importance of natural products as sources of new cancer chemotherapeutic (Newman et al., 2012).

However, no new plant-derived drug has been approved for ovarian cancer. In this review, some of the recently plant-derived anti-ovarian cancer compounds from 2001-2014 with $IC_{50}$≤ 10μM are presented in Tables 1.1.

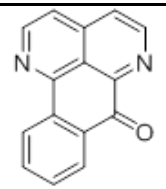
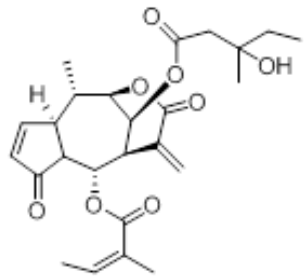
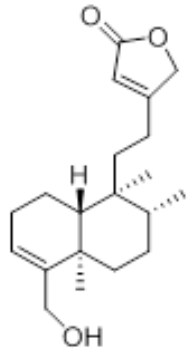
**Table 1-1 A list of novel and potent plant natural products against ovarian cancer cells discovered between 2001 and 2014 (the order of the compound is arranged according to the year in which they were reported).**

| Compound name | Structure | Plant name (family) | $IC_{50}$ (A2780 ovarian cancer cell line) | Reference |
|---|---|---|---|---|
| **trihydroxyalkylclohexenones** |   | *Pleiogynium timoriense (A. DC) Leenh* (Anacardiaceae) | 0.8, 0.7, and 0.8µM, respectively | Eaton et al., 2015a |
| **securinine** |   | *Margaritaria discoidea (Baill.) G. L. Webster* (Euphorbiaceae) | 3.16 µM (OVCAR8, A2780 and A2780cis[a] | Johnson-Ajinwo et al., 2015 |

| | | | | |
|---|---|---|---|---|
| **3-β-[O-aG55lpha-L-rhamnopyranosyl-(1-2)-αarabionopyranosyl)oxy]-16-α-hydroxyolean-12-en-28-oic acid** |  | *Polyscias duplicate (Thouars ex Bail.) Lowry and G.M. Plunkett (*Araliaceae*)* | 2.8µM | Eaton et al., 2015b |
| **(+)-1.2-dehydrotelobine and (+)-2'-norcocsuline** |  (+)-1.2-dehydrotelobine  (+)-2'-norcocsuline | *Anisocycla grandidieri Baill. (*Menispermaceae*)* | 4.1±0.3µM, respectively | Liu et al., 2013 |

| | | | | |
|---|---|---|---|---|
| **bufatrienolides**[b] |  <br>$R_1$ $R_2$ $R_3$<br>Glc CHO H<br>Glc CHO OAc<br>4'-Glc-Rha-3'-Rha $CH_3$ H<br>4'-Glc-Rha-3'-Rha CHO H | *Urginea depressa Baker (*Asparagaceae*)* | 24.1, 11.2, 111, and 40.6 nM respectively | Dai et al., 2013b |
| **randianin, 2''-O-acetylrandianin** |  <br>$R_1$ $R_2$<br>randianin H H<br>glycosides 2'-oacetylrandianin Ac H<br>6''-O-acetylrandianin H Ac | *Nematostylis anthophylla (A. Rich. ex DC.) Baill (*Rubiaceae*)* | 1.2, 1.7, and 2.2 µM, respectively | Dai et al., 2013a |

| | | | |
|---|---|---|---|
| **10-desoxygochnatiolide A** | *Gochnatia polymorpha (Less) Cabr. Ssp. Floccose Cabr. (Compositae)* | 2µM (OVCAR 3) | Strapasson, R., et al., 2012 |
| **tavinin A and epi-tavinin A** | *Sterculia taiva Baill.* (Malvaceae) EPI-TAVINI A | 5.5 and 6.7 µM, respectively | Dai et al., 2012 |
| **madagascarensilide A and madagascrensilide B** | *Leptadenia madagascariensis Decne.* (Apocynaceae) | 0.18 and 0.29 µM respectively | Pan et al., 2011a |

| | | | | |
|---|---|---|---|---|
| **sampangine** |  | *Ambavia gerrardii (Baill.) Le Thomas* (Annonaceae) | 0.58 µM | Pan et al., 2011b |
| **athrolide D** |  | *Athroisma proteiforme (Humbert) Mattf.* (Compositae) | 0.6 µM | Pan et al., 2011c |
| **16,18-dihydroxykolavenic acid lactone** |  | *Cyphostemma greveana Desc.* (Vitaceae) | 0.44 µM | Cao et al., 2011 |

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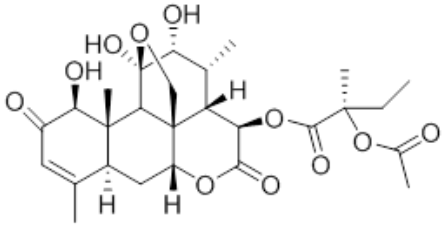
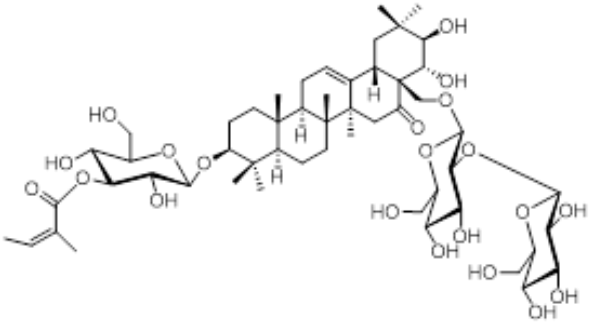
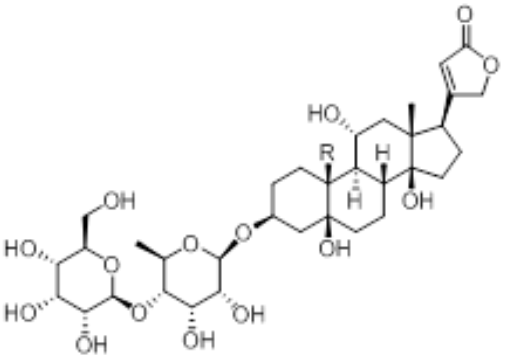
|---|---|---|---|---|
| **2'-(R)-O-acetylelaucarubinone** |  | *Quassia gabonensis Pierre [Syn. Odyendyea gabonensis (Pierre) Engl.]* (Simaroubaceae) | <18 nM (MSPC1, 2774-C10. HeyA8 Hoc7) | Usami et al., 2010 |
| **terminaliaside A** |  | *Terminalia tropophylla H. Perrier* (Combretaceae) | 1.2µM | Cao et al., 2010 |
| **cardenolide glycosides** |  | *Elaeodendron alluaudianum H. Perrier* | 70nM | Hou et al., 2009 |

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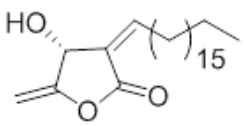
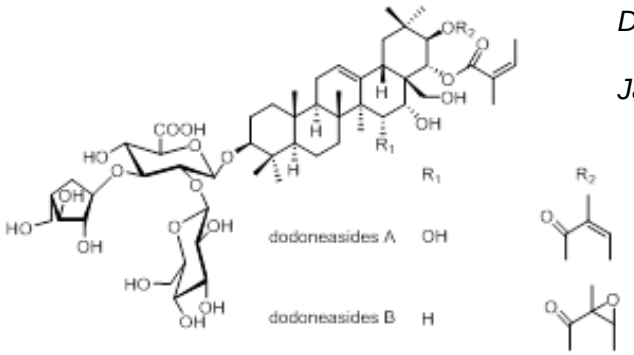
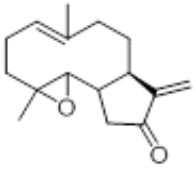
|---|---|---|---|---|
| **isomahubannolide-23** |  | *Machilus wangchiana Chun (Lauraceae)* | 2.66µ | Cheng, W., et al., 2009 |
| **dodoneasides A and B** |  | *Dodonaea wiscosa (L.) Jacq. (*Sapindaceae*)* | 0.79 and 0.70 µM, respectively | Cao et al., 2009 |
| **parthenolide** |  | *Magnolia kobus DC. (*Magnoliaceae*)* | 9.7 µM (SK.OV-3) | Park et al., 2010 |

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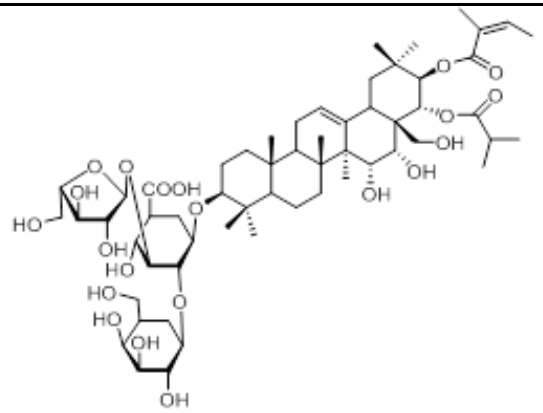
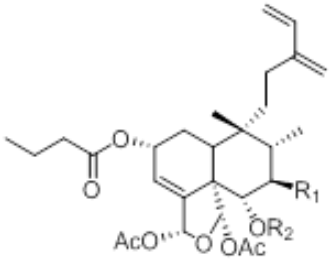
|---|---|---|---|---|
| **xanifolia-$Y_0$** |  | *Xanthoceras sorbifolia Bunge (*Sapindaceae*)* | 4.2±0.7 µM (OVCAR3) | Chan et al., 2008 |
| **caseanigrescens A-D** |   | *Casearia nigrescens Tul. (*Salicaceae*)* | 0.83-1-4 µM | Williams et al., 2007 |

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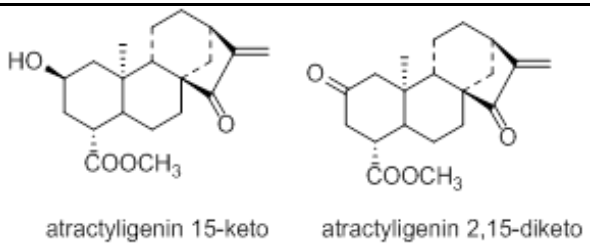
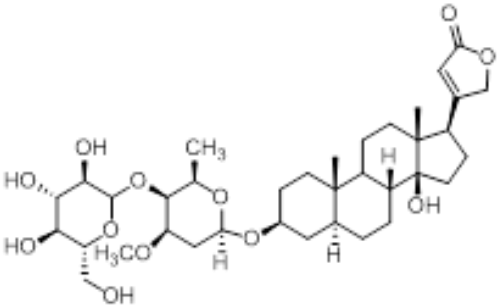
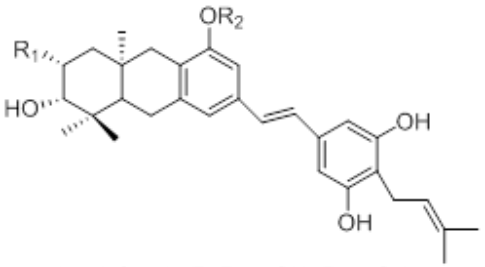
|---|---|---|---|---|
| **atractyligenin 15 keto and atractyligenin 2,15-diketo** |  atractyligenin 15-keto atractyligenin 2,15-diketo | *Atractylis gummiferaL.* *(*Asteraceae*)* | 0.3 and 0.2 µM (1A9), respectively | Rosselli et al., 2007 |
| **boivinide A** |  | *Roupellina (Strophanthus) boivinii Baill. (*Apocynaceae*)* | 0.17 µM | Karkare et al., 2007 |
| **vedelianin, achweinfurthin E** |  schweinfurthin E, $R_1$ = OH, $R_2$ = $CH_3$<br>vedelianin, $R_1$ = OH, $R_2$ = H | *Macaranga alnifolia* Baker *(*Euphorbiaceae*)* | 0.26 and 0.13 µM, respectively | Yoder et al., 2007 |

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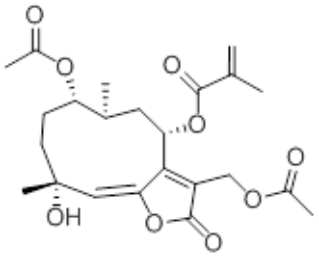
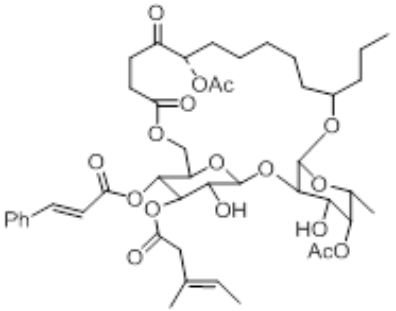
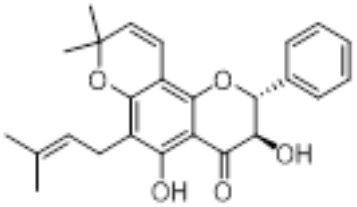
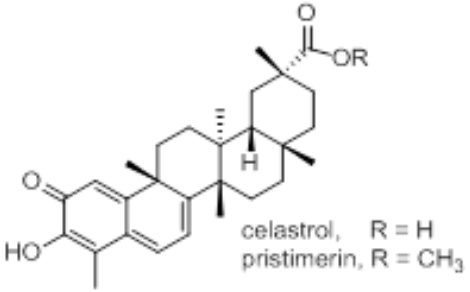
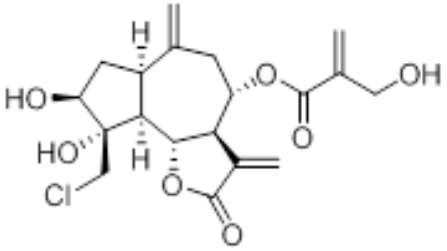
|---|---|---|---|---|
| **glaucolides M** |  | *Vernonia pachyclada* Baker *(*Compositae*)* | 3.3µM | Williams et al., 2005 |
| **Ipomoeassin D** |  | *Lpomoea squamosal Choisy (*Convolvulaceae*)* | 35nM | Cao et al., 2005 |
| **Isomundulinol** |  | *Mundulea chapelieri (Baill.) Du Puy and Labat (*Leguminosae*)* | 1.2µM | Cao et al., 2004 |

| | | | | |
|---|---|---|---|---|
| **molvizarin** |  | *Annonaceous plants* | 1.2 pM (1A9 cell) | Nakanishi et al., 2003 |
| **celastrol and pristimerin** |   | *Reissantia buchananii (Loes.) N. Hallé* (Celastraceae) | 0.17 and 0.22 µM (1A9), respectively | Chang et al., 2003 |
| **chlorojanerin** |  | *Centaurothamnus maximus (Forssk.) Wagenitz and Dittrich* (Compositae) | 15 µM (SK-OV-3) | Muhammad et al., 2003 |
| **cycloviolacin O2** | Cyclic peptide | *Viola odorata L.* (Violaceae) | 1.32 µM (OVCA) | Lindholm et al., 2002 |

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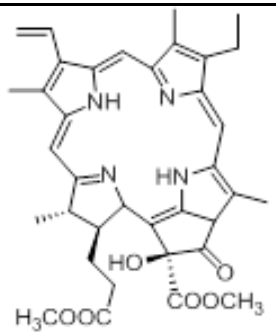
|---|---|---|---|---|
| **Methyl (10S)-hydroxypheophorbide a** |  | *Clerodendrum calamitosum L.* (Lamiaceae) | 0.43 µM (1A9) | Cheng et al., 2001 |

### 1.3.5 Some Other Compounds with Anti-cancer Activities

There are some recently discovered compounds which have shown promise on other cancers and have not being investigated on ovarian cancer cell lines. Some of these compounds are discussed in this review.

### 1.3.5.1 Curcumin

Curcumin (Figure 1. 5) is a polyphenol isolated from the South Asian plant *Curcuma longa* L. (Zingiberaceae). This plant is also called turmeric and has been shown to possess a host of bio-activities such as, anti-inflammatory, anti-microbial, anti-oxidant immunomodulatory and anti-tumour effects. Curcumin has been subjected to phase II trial in patients with advanced pancreatic cancer at the M.D. Anderson Cancer Centre in Houston, Texas (Dhillon et al., 2008). Currently, a Phase III Trial of Gemcitabine, Curcumin and celebrex in patients with advance or inoperable pancreatic cancer is on-going at the National Cancer Institute (NCI Clinical Trials 2013).

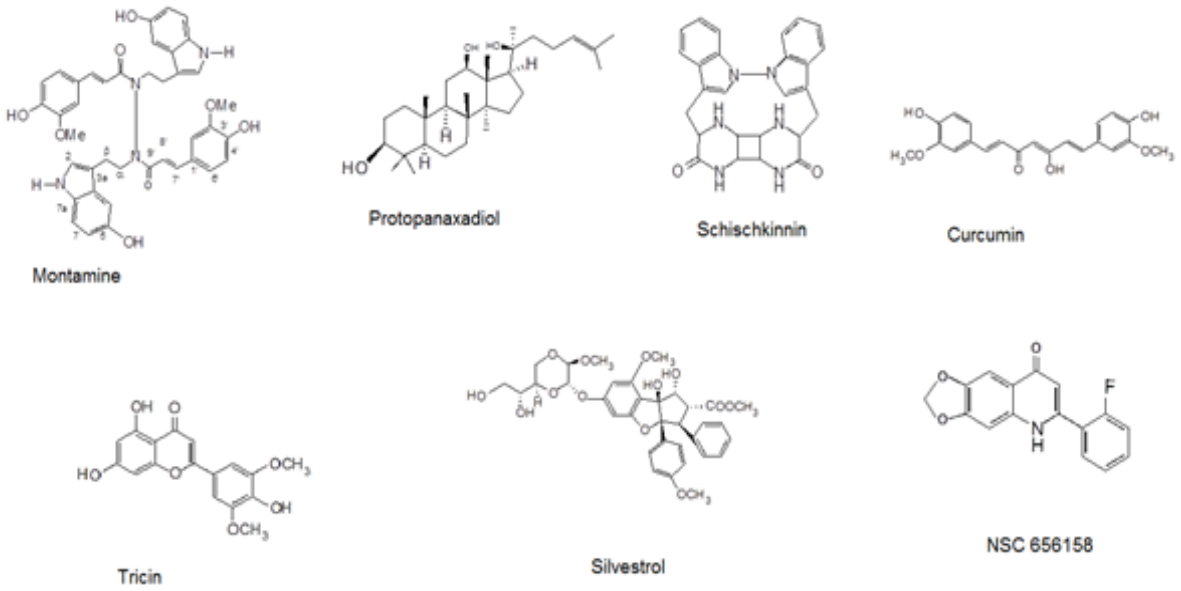



**Figure 1.5 Structures of some novel anti-cancer compounds with activities on other cancer cell lines.**

### 1.3.5.2 Phenylquinolones and Naphthyridinones: NSC-656158

Three naturally-occurring flavonoids with anti-leukemic activities were isolated from *Wikstroemia indica*. These are tricin (Figure 1.5), kaempferol-3-O-B-D-glucopyranoside and the lignin, (+)-nortrachelogenin. Synthetic modifications of 2-phenyl-4-quinolones which are structurally linked to the flavonoids had very active anti-mitotic and anti-tumour fluorinated quinolones. One of the fluorinated quinolone (NSC-656158) (Figure 1.5), at µM dose significantly inhibited hepatocyte growth factor-induced invasion of SK-Hep-1 human hepatocellular carcinoma cells by suppressing matrix metalloproteinase-9 expression. An analogue of these quinolones derived by the replacement of methylenedioxy with pyrolidine ring had the greatest activity with an *in vitro* tubulin inhibitory $IC_{50}$ of 0.46 µM (Lee 2010).

### 1.3.5.3 Protopanaxadiol

Protopanaxadiol (Figure 1.4) is derived from the hydrolysis of specific saponins from ginseng [*Panax ginseng* C.A. Mey.; Araliaceae]. The agent's MOA is by the induction of apoptosis in cancer cells through signalling pathways such as caspases 3, 8 and 9 (Saklani et al., 2008; Shin et al., 2015).

### 1.3.5.4 Schischkinnin and Montamine

Schischkinnin (Figure 1.5) and montamine are alkaloids isolated from the seeds of *Centaurea schischkinii* and *Centaurea Montana*, that have demonstrated moderate cytotoxicity against human colon cell lines (Shoeb et al., 2005; 2006).

### 1.3.5.5 Silvestrol

Silvestrol (Figure 1.5) is a derivative of a relatively recently discovered class of plants secondary metabolites called the rocaglate. Rocaglamide was the first member to be discovered in 1982 (King et al., 1992). Silvestrol was obtained from *Aglaia foveolata*, Pannell (Meliaceae), a tropical plant. The compound's anti-cancer activity competes favourably with that of paclitaxel and Camptothecin (Hwang et al., 2004). Silvestrol also showed β-cell

selectivity in both chronic lymphocytic leukaemia *in vitro* and acute lymphocytic leukaemia in *in vivo* models, (Lucas et al., 2009). Preclinical testing for silvestrol as an anti-leukemic agent is on-going (Pan et al., 2012).

## *1.4 Literature Survey of the Medicinal Plants Investigated in this study*

### 1.4.1 *Acalypha wilkesiana*- A Plant with Potential Chemotherapeutic Activities

*Acalypha wilkesiana* (AW), a specie of the genus *Acalypha*, belongs to the Euphorbiaceae family. This family is referred to as the 'spurge family' as a result of purgative use of these plants. Euphorbiaceae comprises of approximately 300 genera with 7500 species. Notable genera in the family are Jatropha, Manihot and *Acalypha*. The family is widely distributed in tropical climates; mainly in Indo-Malayan zones, North America, and some parts of Africa. Numerous species are found in the other non-tropical regions, which include the South of America, South Africa, Mediterranean basin and the Middle East.

**Figure 1.6 A picture of AW (Marginata Cultivar), taken at a Bioreserve.**

*Acalypha* is the fourth largest genus in Euphorbiaceae family, with about 450-570 species. A number of these species have long being used in ethno medicine by Africans and locals of Mauritius, Réunion and Rodrigues (Mascarene Islands). Most species of the genus are found in America and about 30% in Africa.

## Ethnopharmacological Uses of AW

AW is used in the treatment and management of various disease conditions, such as diabetes mellitus, malaria, hypertension and stomach problems in some indigenous communities (Quds et al., 2012). The plant is reputedly useful in the treatment of a wide range of skin infections in Nigeria (Oyelami et al., 2003) and the seeds are used for breast tumours in the western parts of Nigeria (Bussing et al., 1999). Other uses of the plant include the use of the aerial part as an abortifacient in Papua New Guinea (Kumar et al., 2012), the leaves for inflammations in Rodrigues, and the treatment of asthma, dysentery and diabetes mellitus in Mauritius (Gurib-Fakim et al., 1996).

## Research-Validated Bioactivities

AW has being investigated for pharmacological activities to validate the traditional uses of the plant. There is relatively little research into this area and these are presented in this review. A clinical study of the ointment formulation of AW demonstrated significant anti-fungal activities, with the achievement of a cure rate of 100% for infections of *Candida intetrigo*, *Pityriasis versicolor* and *Tinea pedis* (Oyelami et al., 2003). Similarly, the extracts of AW showed significant inhibition of MRSA (methicillin resistant*Staphylococcus aureus*), validating the plant's anti-bacterial activities (Akinyemi et al., 2005). The anti-malaria, analgesic (Udobang et al., 2010), cytotoxic and immunomodulatory activities of AW have also being reported (Bussing et al., 1999). The anti-emetic activity of AW was investigated in an *in vivo* study with chicks (Quds et al., 2012).

## Phytochemistry of the Plant

The phytochemical investigations of AW revealed the presence of a broad range of compounds, such as anthroquinine, alkaloids, saponin, tannin, and cardiac glycosides (Oladunmoye 2006). The isolation of corilagin, gallic acid and geraniin has been carried out in AW, (Adesina et al., 2000). The structures of these isolated compounds are shown in Figure 1.6.

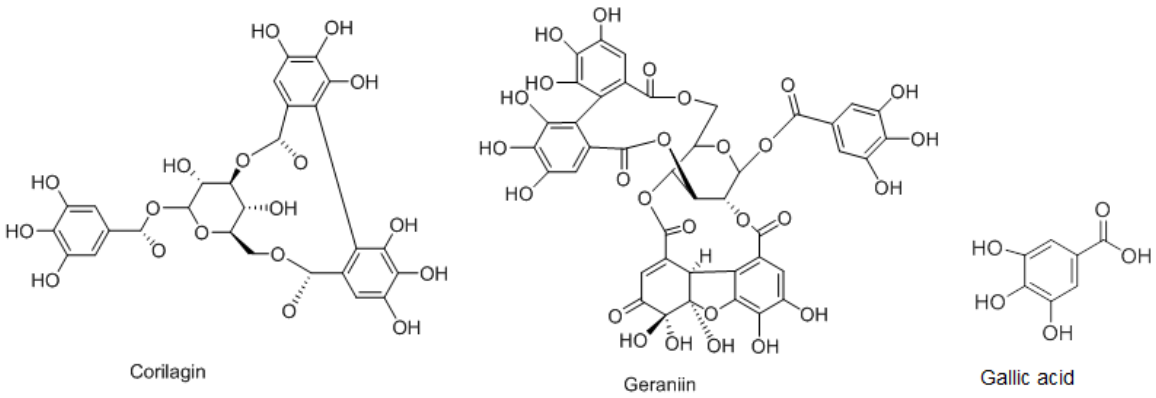



**Figure 1.7 The chemical structures of three compounds isolated from AW.**

### 1.4.2 *Margaritaria discoidea*- An unexplored plant for Anti-cancer Activities

*M. discoidea* (MD) also referred to as the pheasant berry or the bushveld peacock-berry is an average-growing tree, reaching about 30m in height. The plant is native to the tropics and sub-tropical climates, where it is often found in forests and near rivers. The stem is usually straight with a grey-brown to-red colour and the leaves in alternating positions. The plant is a flowering plant, with fragrant male and female flowers borne on separate plants, producing three-lobed fruit capsules which are brown in colour (Pooley 1993).

**Figure 1.8 Pictures of M. discoidea; showing a closer view of the leaves (Left) and M. discoidea with a height of about 30m (Right), taken at a bioreserve, Enugu state, Nigeria.**

*M. discoidea* has had a complicated taxonomy, with the plant being formerly placed in the *Phyllanthus* genus which is in the Phyllanthaceae family. The Phyllanthaceae family had previously being submerged into the Euphorbiaceae family together with Pandaceae, Picrodendraceae and Putranjivaceae families. Presently, these families have now being segregated from the larger family - Euphorbiaceae. Phyllanthaceae is rich in diversity with 60 genera and nearly 2000 species (Samuel et al., 2005). The largest genus of the Phyllanthaceae family is *Phyllanthus* which has 1270 species, and have been the focus of intense phytochemical and pharmacological research for decades with numerous pharmacologically important compounds isolated from the species (Peter 2001).

Presently, *M. discoidea* is a species of the genus *Margaritaria*, which belongs to the *Euphorbiaceae* family. The plant remains a species in the Phyllanthaceae family. *M. discoidea* is also named *Phyllanthus discoideus* (the name of the plant while it was place in the *Phyllanthus* genus) (Hoffmann et al., 2005). The plant is has a wide variety of uses, such

as medicinal, aphrodisiacs, pesticides and for domestic purposes. Additionally, the tree is used for shades in most farmlands. The pictures of MD are shown in Figure 1.8.

The genus *Margaritaria* is the smallest genus in the Phyllanthaceae family with thirteen species distributed in tropical and sub-tropical countries, such as Congo, Cuba, Ghana, Nigeria, India, Mexico, and Zaire. Most of the species in this genus have scanty mention in literature and are yet to be evaluated for biological activities. MD is of immense ethnopharmacological importance, as the plant has found uses in many indigenous communities. The commonly used parts of the plant are the stem bark and the leaves. MD is used in the treatment of infections of the skin and wounds in Ghana (Abbiw 1990). In Guinean traditional medicine, MD is used in the management of diabetes mellitus (Diallo et al., 2012), diarrhoea and stomach problems, worm infections, impotence (Carriere 2000), malaria (Traore et al., 2013) and wounds (Basilevskaia 1969). The stem bark is used in the treatment of inflammations in Malawi (Irvine 1961), and in Congo the plant is used in the treatment of kidney problems and stomach upsets. Again it is used in pregnancy to aid delivery and ease post-partum pains (Adedapo et al., 2009). Some indigenous communities in Zaire use the leaves and the roots in the treatment of blennorrhoea. While in some parts of West Africa the plant has been used as a purgative (Kerharo et al., 1974). Other uses of MD include the use of the dry leaves as a supplementary diet for sheep (Osakwe et al., 2004).The hard wood of the plant has been employed in furniture-making. Also, the plant's rapid growth and extensive height may be beneficial in agro forestation (Pooley 1993).

## Research-validated Bioactivities

A few reports about the bioactivities of MD have been documented. The plant has demonstrated significant anti-inflammatory activities in two independent studies (Adedapo et al., 2009; Dickon et al 2010). The antibacterial and anti-oxidant activities of MD have been reported (Dickson et al., 2010) and the plant shown to exhibit moderate anti-microbial and anti-protozoal activities *in vitro*. An investigation of the anti-HIV activities of MD, observed

that the plant had no measurable anti-HIV activities (Diallo et al., 2015). The free-radical scavenging activities (Ekuadzi et al., 2013) and the filaricidal activities (Cho-Ngwa et al., 2010) have also being reported. Additionally, MD possesses acaricidal properties, which have been investigated on farm animals (Kaaya et al., 1995). This suggested that MD would be a rich source from which novel medicinal compounds might be identified.

Median lethal dose ($LD_{50}$) studies of orally administered aqueous extract of MD, in mice recorded a lack of acute toxicity in all the doses used. Additionally, the treated mice were in stable behavioural conditions post-treatment, a demonstration of the high safety profile of MD (Adedapo et al., 2009).

## Phytochemistry of the plant

Earlier phytochemical studies on MD have reported the presence of alkaloids, triterpenes and lignans as the active constituents in the plant. The alkaloids in MD belong to the securinega-type of alkaloids characterised by a complex tricyclic back bone with anα, β-unsaturated-γ-lactone ring. The securinega alkaloids are divided into two classes. The first class is the securinane class, which derive their names from securinine and possess an indolizidine skeleton. The second class is the norsecurinane class with their parent name as norsecurinine and contains a pyrrolizidine skeleton (Snieckus 1975; Weinreb 2009).

The presence of eleven alkaloids has been established in the leaves of MD. These alkaloids are allo-securinine, 14, 15-dihydroallo-securinine-15-β-ol, dihydrosecurinine, norsecurinine, phyllabine, phyllocristine, phyllanthine, phyllanthidine, phyllantidine, securinine and viro-allo-securinine (Mensah et al., 1988; Calixto et al., 1998). Most of these alkaloids correspond to the securinega-type of alkaloids. Securinine is the first of the securinega alkaloids isolated from a *Phyllanthus* species, some fifty years ago (Quevauviller et al., 1959).

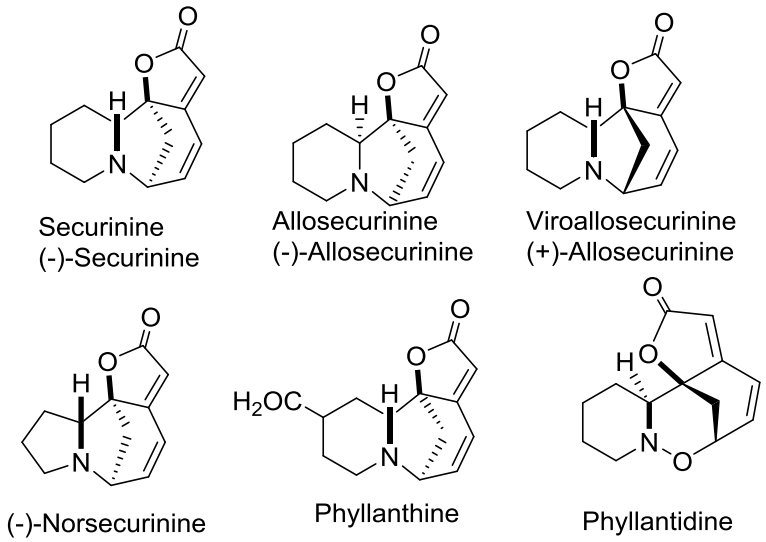



**Figure 1.9 Some securinega alkaloids and a lignan found in MD.**

The presence of a triterpene, betulinic acid and a lignan-phyllanthin has been reported in the aerial part of the plant (Calixto et al., 1998). The rootbark of MD has also been investigated for the presence of secondary metabolites. Five alkaloids, namely, allo-securinine, (Janot et al., 1958; Parello et al., 1963a; Bevan et al., 1964a; Rich 1973), phyllalbine, (Parello et al., 1963b), phyllanthidine, phyllanthine (Parello et al., 1965; Parello 1968; Horii et al.,1972) and securinine (Parello et al. 1963a; Parello 1966; Bevan et al., 1964b) were contained in the root bark. The Nigerian species of MD have been phytochemically investigated. Securinine has been documented as the major alkaloid present in these species. The presence of a small amount of allosecurinine was detected. A comparison of the amount of securinine obtained from the leaves, stembark and rootbark of the Nigerian species of MD, showed that the leaves had 0.06% securinine, the stembark had 0.2% securinine and rootbark had 0.4% securinine (Oliver-Bever 1986). Recently a flavonoid glycoside with significant anti-bacterial and free-radical scavenging activities has been isolated from MD (Ekuadzi et al., 2013).

### 1.4.3 *Rutidea Parviflora*- An un-researched Plant with Potentials for Cancer Treatment

*Rutideaparviflora* D.C. (RP) belongs to the genus *Rutidea* in the Rubiaceae family. Rubiaceae referred to as the coffee family or bedstraw family is the fourth largest family of the angiosperms. Rubiaceae has 611 genera and approximately 13500 species distributed in different regions of the world, but with greater diversity in the sub-tropical areas. Two important genera of this family are *Cinchona L*., from which the drug quinine used in the treatment of malaria was derived and *Coffea L*., which have provided the world with the most consumed beverage-coffee. Other genera are *Rubia*, a collection of dye plants used for decorative purposes, *Pavetta L*., *Uncaria* schreb*, Tarenna* Gaertn*, Oldenlandia* L., and *Galium* L. (Angiosperm Phylogeny).

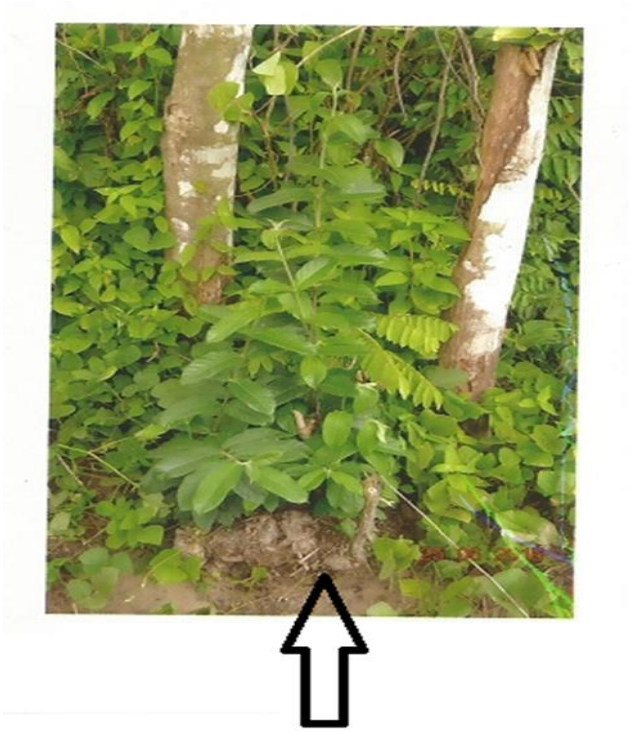


**Figure 1.10 A picture of Rutidea parviflora taken in a Bioreserve, with an arrow indicating the plant.**

### Ethnopharmacological Uses of RP

The plant has a variety of medicinal uses. The sap is used in the treatment of eye infections. The fruits are taken to induce vomiting and for the treatment of convulsions, epilepsy, spasm and paralysis (http://plants.jstor.org).

### Research-Validated Bioactivities

Presently, there is no report evaluating the pharmacological activity of RP. Thus there is a dire need for scientific investigations of the potential pharmacological activities of this plant to be carried out.

### Phytochemistry of the Plant

A phytochemical study of RP has not been reported so far. Also the phytochemistry of other species in the genus has not been investigated. Recently, the *in vitro* anti-ovarian cancer activities of *Tarenna grevei* (Drake) Homolle (Rubiaceae), obtained from Madagascar, on A2780 cell line was reported (Kingston et al., 2012). Further investigation of the ethanol extract of the fruit and leaves led to the isolation of two novel antiproliferative saponins; 23-hydroxylongispinogenin 3-o-β-D-glucpyranoside and longispinogenin 3-o-β-D-glucopyranosyl (1→2)-β-D-glucopyranoside with moderate activities.

## *1.5 Thymoquinone - A Potential Anticancer Lead*

*Nigella sativa*, a plant in the Ranunculaceae family is a popular Mediterranean herb. The genus *Nigella* is encomprised of about 14 species among which are *N. hispanica, N. integrifolia, N. orientalis, N. damascene, N. arvensis* and *N. cilliaris* (Aggarwal et al., 2008). *N. sativa* is the most important specie of the *Nigella* genus and has been employed by many indigenous communities for the treatment of various diseases such as diabetes, hypertension, fever, arthritis, inflammation, gastrointestinal disturbances, and cancer, (Padhye et al., 2008). The plant has a rich historical significance, which have paved the way

for scientific investigations into the pharmacological activities of the herb. *Nigella sativa* is popularly referred to as black cumin seeds (Figure 1.11) and kalonji seeds. The black seed oil (Figure 1.11) is well-known for its medicinal use and thus is a commercially available phytopharmaceutical product majorly used in the Mediterranean and other parts of the world including Europe. The most bioactive compound contained in the black cumin seed oil was found to be thymoquinone.

**Figure 1.11 The Seeds and oil of Nigella Sativa**

Thymoquinone (TQ), a promising anti-cancer agent is one of the compounds isolated from the volatile oil of *Nigella Sativa* (NS), in 1963 (El-Dakhakhny 1963), alongside two other compounds: thymol (THY), and dithymoquinone (DTQ) (Figure 1.12). It was discovered that the compound is a 2, 5-di-substituted benzoquinone.

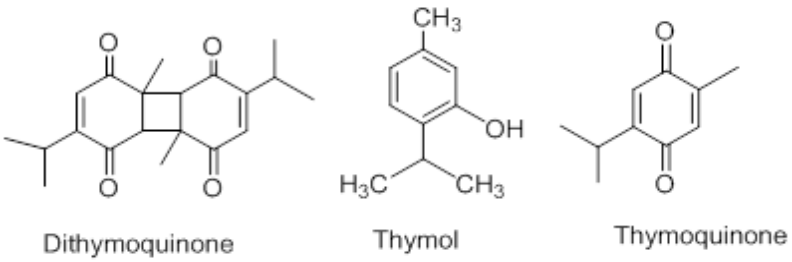



**Figure 1.12 Structures of dithymoquinone, thymohydrodquinone, and thymoquinones of Nigella sativa.**

Several investigations have shown that TQ inhibits the proliferation of a wide range of cancers such as breast adenocarcinoma, colorectal cancer, leukaemia, lung cancer, pancreatic cancer, prostate cancer and osteosarcoma (El-Mahdy et al., 2005; Rooney et al., 2005; Richards et al., 2006; Kaseb et al., 2007; Roepke et al., 2007;Alhosin et al., 2010; Jafri et al., 2010; Koka et al., 2010; Effenderger-Neidnicht et al., 2011; El-najjair et al., 2011; Woo et al. 2011). Studies on the mechanism of action showed that the agent exhibited antiproliferative activities, a reduction in cell viability and inhibition of DNA synthesis in prostate cancer cells while non-cancerous cells were apparently unaffected (Kaseb et al., 2007).

A 2009 study by Kimmel Cancer Centre in Philadelphia, Pennsylvania showed that TQ is both an antiproliferative and a chemopreventive agent. The study observed that TQ exerted significant cytotoxicity on pancreatic cancer cells, and also prevented the occurrence of this cancer, by increasing DNA acetylation which induces epigenetic changes in the cells (kimmel Cancer Centre 2009).The implication of this finding is that TQ could be exploited in chemoprevention.

## Toxicity of TQ

Studies on the $LD_{50}$ of TQ in mice and rats showed that the compound is relatively safe as reported from the intraperitoneal and oral administration of more than 15 and 150 times the doses investigated for *in vivo* antioxidant, anticancer and antiinflammation activities by intraperitoneal and oral routes respectively. The researchers reported that upon

intraperitoneal administration of TQ to mice and rats, $LD_{50}$ was determined to be 104.7mg/kg (95% CL) and 57.5mg/kg (95% CL) respectively. The $LD_{50}$ for oral administration was 870.9mg/kg (95% CL) and 794.3mg/kg (95% CL), respectively (Al-Ali et al., 2008).Also, a phase I study conducted on 21 cancer patients recorded no toxicities for doses of TQ ranging from 75 mg/day to 2600 mg/day (Al-Amri et al., 2009).

## Combinational Uses of TQ and Other Drugs

TQ has been found useful in ameliorating the adverse effects of other anti-cancer drugs from a number of studies on various combinational anti-cancer therapies.

### *Thymoquinone and Cisplatin Combinational Therapy*

A combination of 100 µMTQ and 5µM Cisplatin (the most active chemotherapeutic agent in lung cancer), was employed in a drug combination study for non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), *in vitro*. The researchers observed significant synergism in the combination of both drugs, than in either TQ or cisplatin on cell proliferation. Furtherxenograph study was conducted by use of a mouse model. Reduction of tumour volume was reported from a combination of 20mg/kg TQ and 2.5mg/kg of cisplatin, which was significant in comparison to the effects of the agents singly and minimal toxicity was observed (Jafri et al., 2010).

### *TQ and Doxorubicin Combinational Therapy*

Doxorubicin is one of the main cancer drugs with observed cardiotoxicity and a high incidence of multi-drug resistance. A recent study showed that an equimolar mixture of TQ and doxorubicin boosted the anticancer effect of doxorubicin in certain cancer lines. Also, cardiotoxicity and multi-drug resistance were greatly reduced by combination with TQ (Effenberger-Neidnicht et al., 2011).

*TQ and Gentamicin Combinational therapy*

An *in vivo* investigation into the chemoprotective effect of TQ when co-administered with gentamicinin rats showed that the compound ameliorated the nephrotoxicity of gentamicin, with minimal changes observed in the kidney tissues of the rats (Sayed-Ahmed et al, 2007).

Furthermore, the compound has demonstrated synergistic effect with cisplatin and oxaliplatin in ovarian cancer cells (Nessa 2011; Huq 2014; Wilson 2015).

## *1.6 Research Design*

This research work on 'Identification, Semi-synthesis and Evaluation of Anti-Ovarian cancer Compounds from Plants used in Traditional Medicines' was undertaken to identify novel therapeutics to treat ovarian cancer by investigation of three selected plants for *in vitro* anti-ovarian cancer activities and the semisynthesis of more potent analogues of thymoquinone; an anti-cancer compound from *Nigella sativa*. The selected plants were chosen based on the researcher's observation of the use of these plants in the treatment of cancer by some indigenous communities in Nigeria. This curiosity was further aided by the existing ethnopharmacological data on the uses of these plants for other disease conditions, except for *A. wilkesiana*, which had documented use in the treatment of cancer. Additionally, the scarcity of research-validated bioactivities (importantly anti-cancer activities) of the plants was also a reason for the selection of these medicinal plants. In this research work, two approaches were explored in the search for anti-ovarian cancer compounds.

Firstly, the selected medicinal plants; *Acalypha wilkesiana* Müll. Arg. (AW)*, Margaritaria discoidea (Baill.)* G. L. Webster (MD) also called *Phyllanthus discoideus* and *Rutideaparviflora* D.C. (RP) were screened for cell growth inhibitory activities on the ovarian cancer cell lines. Further bioassay-guided fractionation and isolation of anti-ovarian cancer compounds from the medicinal plants were conducted. The active compounds from the most active extracts were isolated and identified by both gas and liquid chromatography mass

spectrometry (GC-MS, LC-MS) and additionally by nuclear magnetic resonance (NMR) spectroscopy.

Secondly, semi-synthesis of eleven analogues of thymoquinone (TQ) was undertaken. Ten additional analogues were procured to enable the development of a SAR for the anti-ovarian cancer activities for TQ. These analogues were evaluated for cell growth inhibition on the ovarian cancer cell lines. Although a lot of studies on thymoquinone have been undertaken, there has been fewer studies on the synthesis of more potent analogues of TQ with anti-cancer activites despite the compound's *in vitro* and *in vivo* anti-cancer activities and good safety profile (Al-Ali et al., 2008; Al-Amri et al., 2009; Schneider-Stock et al. 2014). Additionally, thymoquinone, an anti-cancer agent is a small molecule with a molecular mass of 164, which makes it suitable as a lead compound for further modification (Schneider-Stock et al. 2014). Thus, there is a dire need for this unique compound with numerous bioactivities to be explored more closely, by semi-synthesis to obtain more potent analogues in order to harness the compound's vital benefits in the treatment of cancer.

The significantly active compounds obtained from the investigated medicinal plants and the most potent synthetic TQ analogue would be jointly administered with clinically used drugs in drug combination experiments. This is due to the increased clinical use of a combination of antineoplastic agents employed in the treatment of many cancers. The main benefits in this approach have been the need to increase the theraupectic effect, with an expected reduction in the side effects and the administered dose. Also, the prevention or minimization of the development of resistance is another therapeutic benefit offered by this approach (Chou 2006). A key factor in drug combination is the determination of synergy, which could potentially be explored in further studies. Synergy may not be predicated on the mechanism of action of the agents in the drug combination, as the MOA of many clinically used drugs, such as aspirin have not been clearly elucidated. Again most drugs tend to possess several mechanisms which would be difficult to investigate (Chou 2010). The current therapy for ovarian cancer involves a combination of paclitaxel and carboplatin, which have different

MOA, but have produced beneficial synergism in clinical outcome. Hence, investigations of the potential synergy of bioactive compounds with clinically used drugs by *in vitro* drug combination studies are worthwhile even though the MOA of these compounds are yet to be elucidated. Such studies hold promise in the early detection of the potential benefits of these compounds, which may spur further investigations.

The bioactive compounds from this research were investigated for potential synergy with carboplatin firstly. Further investigation of the combination of these compounds with paclitaxel were carried out, when significant synergy was demonstrated in the combination with carboplatin.

## *1.7 Aims and Objectives*

The main aim of this research is to characterize the anti-ovarian cancer activity and to identify the bioactive compounds of the three medicinal plants through bioassay-guided isolation, and to search better analogues of anti-cancer thymoquinone from *Nigella sativa*.

In the achievement of the aims of this research, three research questions were generated to assist the research.

The research questions were:

Could new compounds with potentials for the treatment of ovarian cancer be obtained from medicinal plants?

Could new compounds with potentials for the treatment of ovarian cancer be obtained semi-synthetically?

Could the Stable Isotope Dilution Gas Chromatography Mass Spectrometry method of quantification be developed for the quantification of Thymoquinone in Botanicals?

The following **hypotheses** were tested in this research:

**a)** These bioactive compounds would have single agent growth inhibition in a concentration-dependent manner

**b)** The growth inhibition activities of these agents would be due to apoptosis

**c)** Some of these agents may sensitize ovarian cancer cells to carboplatin or paclitaxel potentially.

The following objectives would be achieved in this research work:

(i) To investigate the *in vitro* anti-cancer activities of the selected medicinal plants

(ii) To obtain the bioactive fractions of the plant with optimum activity

(iii) To isolate the pure compounds with anti-cancer activities

(iv) To characterize the structure of the compounds using GC-MS, NMR and LC-MS

(v) To determine the selectivity index of these bioactive compounds

(vi) To investigate the route of cancer cell death exhibited by the potent bioactive Compounds

(vii) To investigate the synergistic potentials of these potent bioactive compounds with carboplatin firstly and with paclitaxel where significant synergy is demonstrated in the combination with carboplatin

(viii) To synthesise analogues of TQ with improved anti-cancer activities and obtain a SAR for the anti-ovarian cancer activity of TQ

(ix) To develop a stable isotope dilution method for the quantification of thymoquinone in commercially available *Nigella sativa* oils

(x) To investigate the cytotoxicity of some Gallic acid derivatives on human ovarian cancer cell lines

To test the **hypotheses**, the cell growth inhibition activities of the plants extracts, obtained fractions and the bioactive compounds were evaluated on the ovarian cancer cell lines. Similarly, the determination of the Selectivity index (SI) was by comparison of the obtained

$IC_{50}$s of the bioactive compounds on the cancer cell lines with the $IC_{50}$s of the respective compounds on a normal ovarian epithelial cell line in the cell growth assay. Two cell growth assays were employed; the Sulfordamine B (SRB) assay, (for determination of cell growth inhibition) and Trypan blue assay (TBA), for the determination of cell viability. Apoptosis studies were carried out by the evaluation of the Caspase3/7 activity of the bioactive compounds. Further investigations by Flow cytometry analysis of Annexin V/Propidium iodide labelling of cells pre-treated with these compounds respectively were undertaken. Western blot Immunochemistry assay was carried out where additional investigation was required. The synergistic potentials of the compounds were investigated by drug combination studies of the agents in combination with carboplatin firstly, then with paclitaxel, when significant synergism is demonstrated with carboplatin in the SRB and caspase 3/7 assays.

# Chapter Two

## *General Materials and Methods*

## *2.1 Materials*

### 2.1.1 Reagents

Ethyl acetate, methanol, n-hexane, n-butanol, and dichloromethane were purchased from Fischer Scientific, UK. N,O-Bis(trimethylsily) trifluoroacetamide (BSTFA) and pyridine were purchased from Sigma Aldrich.

### 2.1.2 Plant Materials

The following plants: *Acalypha wilkesiana* (Leaves), *Margaritaria discoidea* (Stem bark), and *Rutidea parviflora* (stem bark) were sourced from a bio reserve in Nigeria. The plants were authenticated by a botanist, Mr Alfred O. Ozioko (INTERCEDD) with expert advice offered by Prof. J. F. Bamidele of the Department of Plant Biology and Biotechnology, University of Benin, Nigeria. The voucher specimen numbers of the plants were:

*Acalypha wilkesiana* INTERCEDD/1571

*Margaritaria discoidea* INTERCEDD/1579

*Rutideaparviflora* INTERCEDD/1588

The voucher specimens were deposited at the International Centre for Ethno medicine and Drug Development (INTERCEDD), Nsukka, Nigeria.

The plants materials were dried under a shade at 25°C for 7-10 days before pulverization.

### 2.1.3 Cancer assay materials

Roswell Park Memorial Institute (RPMI) 1640 Media, L-gluthamine, 10% fetal bovine serum (FBS), PENSTREP (50 µg/ml penicillin/streptomycin), and phosphate buffered saline (PBS) were purchased from Lonza; trypsin-EDTA solution, trizma base, sulfordamine B sodium salt, glacial acetic acid (GAA), dimethyl sulfoxide (DMSO), trypan blue, carboplatin, and paclitaxel

were purchased from Sigma Aldrich; trichloroacetic acid (TCA) was purchased from Fischer Scientific, UK and Caspase-Glo 3/7 assay kit from Promega, UK.

Other requirements include: NAPCO water jacketed incubator (Precision Scientific), Grant JB Series water bath (Grant Instruments), Neubauer haemocytometer, Thermo Scientific Heraeus Megafuge 8 Centrifuge, Olympus CKX41 light microscope, BioTek Synergy 2 multi-mode microplate reader, Nuaire -86°C Ultralow freezer, pipettes, pipette gun, Industrial Methylated Spirit (IMS), Test-tubes, T-25 tissue culture Flasks, T-75 tissue culture flasks, multi-channel pipette, biological safety cabinet, 15 ml and 50 ml polypropylene tubes (Sarstedt), 6-wells, 12-wells and 96-wells plates (Sarstedt), 2 ml cryovials (Triple Red), "Mr Frosty" freezing container (Nalgene), liquid nitrogen storage tanks.

### 2.1.4 The ovarian cancer cell lines

The cell lines were ordered from America Tissue Culture Collection (ATCC). The cell lines A2780 was obtained from the ovarian carcinoma of a patient about to commence chemotherapy treatment (Eva et al., 1982). CIS-A2780 was derived by the exposure of A2780 cells to graded doses of cisplatin to render the cells cisplatin-resistant (Godwin et al., 1992).

The OVCAR 4 and OVCAR 8 cell lines were derived from human carcinoma of patients that were resistant to cisplatin and carboplatin, respectively (Hamilton et al., 1984).

### 2.1.5 The Human Ovarian Epithelial (HOE) Cell Line

HOE was ordered from Applied Biological Materials (ABM) Inc, Canada. The cells were from normal ovarian epithelium. The selectivity indexes of the drugs were investigated using HOE.

## *2.2 Methods*

### 2.2.1 Cell Growth Conditions

The ovarian cancer cell lines and the HOE were grown in a growth medium of RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 50 µg/ml penicillin/streptomycin (Pen strep). The CIS-A2780 cells were exposed to 1 µM cisplatin for 7 days every month to maintain chemo-resistant properties of the cells. The cell cultures were kept in the incubator at 37$^{o}$C under 5% $CO_2$ for 24h in a humidified atmosphere.

### 2.2.2 Resuscitation of cryopreserved cells

The preserved cells were collected from the liquid nitrogen storage tanks and thawed quickly in a water bath at 37$^{o}$C. Upon thawing, the cells were slowly added to 5 ml of media (pre-warmed in the water bath at 37$^{o}$C) in a 15 ml polypropylene tube. The cells were centrifuged at RT at a speed of 150g for 3 min, followed by removal of the media. The cell pellet was re-suspended in 8ml of growth media and transferred into a T25 tissue culture flask and kept in the incubator at a temperature of 37°C, for 24h. The growth media was changed to ensure the elimination of residual DMSO and dead cells and to enable the adherent live cells multiply to an appropriate number for experiments.

### 2.2.3 Trypsinization of Cells

Trypsinization was routinely carried out, when the cells were about 90% confluent as observed from a microscopic examination. The media was removed and the cells washed with 1 ml of phosphate buffered saline, PBS before exposure to 1ml of 0.01% trypsin in PBS to detach the cells. To facilitate detachment of cells, the T25 culture flask containing the cells was maintained at 37$^{o}$C in the incubator for 10 min, followed by gentle agitation of the flask. Once the cells were detached, the trypsin was neutralized by the addition of 1ml of growth media, and transferred into a 15 ml polypropylene tube for centrifugation at RT at a speed of 150g for 3 min. The media was gently aspirated and the cell pellet re-suspended in fresh growth media and transferred into T25 or T75 tissue culture flask as required for incubation.

Cells for experimentation were counted using the haemocytometer, to determine the number of cells, which was used in calculating the required volume of media and cell density required for each experiment.

### 2.2.4 Cryopreservation of Cells

Cryopreservation was carried out to preserve cells for further experiments without allowing the cells undergo much passage which could result in changes in their morphology and thus affect the results of the experiments. Typically, cells of passage <20 were cultured in T75 tissue culture flask to ensure 50% confluence. The cells were trypsinized, centrifuged and re-suspended in a 2 ml growth medium made of 1.8 ml of 10% FBS and 10% DMSO (0.2 ml). Aliquots of 0.5 ml were transferred into 2 ml cryovials and kept in a Mr Frosty freezing container (Nalgene), containing 200 ml isopropanol. The container was stored at -80$^{o}$C for 24h in a Nuaire -86$^{o}$C ultralow freezer before transfer into compartments in a liquid nitrogen tank for lasting storage until when required for experiments.

## *2.3 Cell Growth Assay*

Two cell growth assays were carried out; the Sulfordamine B (SRB) assay to determine inhibition of cell proliferation and the Trypan Blue Assay (TBA) to determine whether the effect in the cell growth assays reflected inhibition of proliferation or cell death.

The SRB protocol was used in the cancer assay of the plant extracts and the semi-synthesised compounds. This assay is one of the widely used assays for the determination of *in vitro* cytotoxicity and is also the method adopted by the American National Cancer Institute, NCI (Rubinstein et al., 1990; Monks et al., 1991; Boyd 1995; Vanicha et al., 2006).

The SRB assay was utilised for the evaluation of cell growth inhibition in the cell growth assays. OVCAR 4, OVCAR 8, A2780, CIS-A2780 and HOE were grown in RPMI, supplemented with 10% FBS, L-glutamine (2 mM) and penicillin-streptomycin (50 U/ml).

100 mg/ml concentrations of the plant extracts were prepared using 100% DMSO (organic extracts) and media (aqueous extracts) as stock solutions. 20 mM of thymoquinone and its analogues were prepared in 100% DMSO and used in the assay. Paclitaxel and carboplatin (the positive controls) in 100% DMSO were employed in the assays. The 0.1% DMSO in growth media was added to the cells; referred to as vehicle-treated cells (control). Nine concentrations of the drugs were prepared by using a two-fold serial dilution.

In the SRB assay, each well of the 96-well plates were seeded with 80µl of the ovarian cancer cells at a density of 2000 cells/well, except for OVCAR 4 which was plated at a density of 5000 cells/well. After 24h, 20 µl of plant extracts (1 mg/ml) after further 100-fold dilution of the stock solution in the medium or semi-synthetic quinones were added at the indicated concentrations and the cell cultures were kept in the incubator at 37$^{o}$C under 5% $CO_2$ for 72h in a humidified atmosphere. The medium was decanted and the cells fixed with 10% TCA on ice for 30 min and dried. The dried plates were stained with 0.4% SRB for 30 min, washed with 1% acetic acid and dried. 100 µL of Tris-base (10mM) were added to the plates and shaken for 10 min to solubilise the protein-bound SRB dye. The absorbance at 570 nm was measured using a spectroscopic plate reader. The recorded data was analysed by non-linear regression using the Graph pad PRISM software to fit a 4 parameter sigmoidal dose-response curve to determine $IC_{50}$ values and the Hill coefficient (Vidot et al., 2010).

Significant cytotoxicity was considered to be <10 µM; moderate cytotoxicity was in the range of 10-30 µM and low cytotoxicity was considered to be >30 µM.

Calculation of Selectivity Index

Based on the Mean $IC_{50}$s obtained the selectivity index (SI), was calculated for each bioactive compound using the formula:

$$SI = \frac{IC_{50}\ (\text{Normal Cell Line})}{IC_{50}\ (\text{Cancer Cell Line})}$$

(Koch et al., 2005).

The SI value obtained is an indication of the preferential selectivity in the cytotoxicity of the compound for cancer cells. A large value suggests that the compound would be more cytotoxic to cancer cells than normal cells.

### 2.3.1 Drug Combination Experiments

The drug combinations were carried out by a simultaneous exposure of the cells to a range of concentration of carboplatin (maximum dose of 100 µM) or paclitaxel (maximum dose 50 nM) obtained by two-fold serial dilution combined with a fixed concentration (indicated in the results chapters) of the potent compounds. At this fixed concentration, the test compounds were expected from $IC_{50}$ determination experiments with the single agents, to achieve a 5% cell growth inhibition on the OVCAR 4 cell line. The agents were also tested singly in this assay. The vehicle-treated cells were used as the negative control. The cells were incubated for 72h, followed by removal of supernatant and fixing in TCA for 30min. The cells were stained with SRB and same protocol repeated as described above. The combined effect of the drug combinations were evaluated by the calculation of the Combinational index (CI). The method employed for the determination of the CI was first described by Chou and Talalay (Chou et al., 1984).

The CI was calculated from the following equation:

$$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$$

Where $D_1$ and $D_2$ are the respective concentrations of drug 1 and drug 2 used in the combination that cause x% inhibition of cell growth, while $(Dx)_1$ and $(Dx)_2$ are the individual concentrations of drug 1 and drug 2 when used as single agents that cause cell growth inhibition by x%. The sum of the two drug fractions gave an indication of the effect.

The CI is a quantitative estimation of the drug combination effect, to determine whether the combined drugs produce synergistic, additive or antagonistic effect. Where a CI<1 is synergistic effect, CI=1, is additive and CI>1 is antagonism.

In this research work, a slight modification of this classification described by Giovanni and his co-researchers was used (Giovanni et al., 2011). In this classification, combinational index CI was classed as follows; CI <0.9, CI=0.9–1.2 and CI>1.2 indicated synergistic, additive, and antagonistic effect, respectively. The obtained CI results were compared to deviation from unity with a paired t-test.

### 2.3.2 Trypan Blue assay

The viability of the compounds was assessed by trypan blue assay. The cells were plated in 12 well plates at a seeding density of 1 $x10^5$ cells/well in 1 ml of media. The drugs were prepared at an initial concentration of 20 mM and diluted to a working concentration of 2mM. The cells were exposed to 40 µM (20 µl of drug), 20 µM (10 µl) and 10 µM (5 µl) concentrations of the drugs. 40 µM (10 µl) of carboplatin was the positive control, while 0.1% DMSO in growth media was the control. A combination of 40 µM carboplatin and the three drug concentrations was also carried out to investigate the combined effect of both compounds. The cells were incubated for 72h in the incubator at 37°C under 5% $CO_2$. The supernatant was removed, followed by detachment of the adherent cells with trypsin. The cells in the supernatant and the cells collected by trypsinization were combined, centrifuged at 150 x g for 3 min, and re-suspended in 0.2% trypan blue in PBS. The live and dead cells were counted by the haemocytometer.

The expected effect (R) of the drug combinations was calculated by the Bliss independence criterion (Goldoni et al., 2007; Yeh et al., 2009).

$$R(x, y) = [R (x) + R (y)] – [R (x) * R(y)]$$

Where R (x) = the effect of drug x, and R(y) = the effect of drug y

## *2.4 Apoptosis Studies*

Three apoptosis assays were carried out in this research to investigate the possible route of cell killing demonstrated by these drugs. The Caspase 3/7 activity was measured, as these

were the executor caspases involved in apoptosis. Flow cytometry analysis of cells stained with Annexin V and propidium iodide to investigate the effect of apoptosis were carried out.

Also the measurement of PARP [Poly (ADP-Ribose) Polymerase] (which is a DNA repair nuclear enzyme) cleavage by western blot immuno-staining was investigated.

### 2.4.1 Caspase 3/7 Assay

The cells were plated in 96 well plates at the same density used in the SRB assay, and exposed to 10μM concentration of the potent compounds or 10nM of paclitaxel/10μM carboplatin, supplemented with 10μl of growth media. A combination of 10μl of the potent compounds and paclitaxel or carboplatin respectively was also utilised in another simultaneous experiment. After 36h/48h incubation, 25 μl of Caspase 3/7 Glo-reagent (Promega) was added and the cells incubated in the dark at RT for 30min on a gentle rocker. The luminescence was measured by a BioTek Synergy 2 multi-mode microplate reader.

The expected effect (R) of the drug combinations was calculated by the Bliss independence criterion described above.

### 2.4.2 Flow Cytometry

The use of laser beam to analyse the characteristics of single cells in liquid suspensions has been employed clinically as a diagnostic tool for some diseases such as leukaemia (www.cancer.org) and is an important technique for studying apoptosis in cells, with the added benefit of quantification of the apoptosis. This technique involves the labelling of organelles and DNA with a fluorescent probe, for the detection of active mitochondria, which in this experiment was Annexin V. To further distinguish the activities of the cells, propidium iodide was added prior to analysis on the flow cytometer, to stain the DNA and RNA.

OVCAR 4 cells were seeded into 12-well plates at a density of $5 \times 10^4$ cells/well and incubated for 24h at $37^oC$ under 5% $CO_2$. The indicated drug concentrations were used

singly and in combination with 40 µM carboplatin. The control was 0.1% DMSO in growth media.

After 48h post-drug addition incubation, the supernatant was retrieved and the adherent cells were trypsinized. The cells obtained by trypsinization was combined with the supernatant, and then centrifuged for 3 min at a speed of 300g. The pellets were now ready for further preparative steps as directed by the Annexin V manufacturer's instructions.

Annexin V and Annexin V binding buffer were purchased from Miltenyibiotec. The manufacturer's instruction was adhered to in the sample preparation. The obtained pellets were re-suspended in 1ml of annexin V binding buffer and re-centrifuged for another 10min at the same speed. The supernatant was completely aspirated and the cells re-suspended in 100 µl of 1 X Annexin V binding buffer, followed by the addition of 10 µl of Annexin V fluorochrome. The solution was carefully mixed by pipetting and incubated in the dark for 15 min at RT. At the end of the incubation period, the cells were washed by an addition of 1ml of 1 X Annexin V binding buffer and centrifuged for 10 min at 300g. The supernatant was aspirated completely and the cells re-suspended in 500 µl of annexin V binding buffer. 1µg/ml of propidium iodide dissolved in PBS was added prior to flow cytometry analysis on a Beckman Coulter Cytomics 500 flow cytometer, equipped with CXP software for data acquisition.

The cell population was identified and gated by the forward scatter (FS) and side scatter (SS). The data was distributed in the FL1 (annexin V staining) and FL3 (propidium iodide staining) channels. The obtained data was analysed by the use of flowing software developed by Perttu Terho, Turku Centre for Biotechnology, University of Turku, Finland. The Bliss independent effect was calculated by the equation described above.

### 2.4.3 Methodology for Western Blot Assay

The measurement of PARP cleavage was carried out. Six wells plates seeded with OVCAR 4 cells at a density of 300,000/well and treated with 10 µM or 20 µM of palmatine (isolated from RP, see Chapter 5) or 40 µM of carboplatin and a combination of both agents for 48hr.The cells were collected, trypsinized, washed with cold PBS and lysed with RIPA buffer (3 ml) consisting of 2 mM EDTA, 150mM NaCl, 20 mM Herpes, 0.05 mM pepstatin, 0.12 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.5 % sodium deoxycholate and 1 % Nonidet-P40.

The protein concentrations were evaluated by the Bicinchoninic acid (BCA) Assay. The BCA protein kit obtained from Sigma-Aldrich was prepared by the addition of 4% copper (II) sulphate pentahydrate solution ($CuSO_4 \bullet 5H_2O$), to a solution of BCA in 0.1 M sodium hydroxide, supplemented with sodium bicarbonate, sodium carbonate and sodium tartate in a ratio of 1:50. Bovine serum albumin (BSA) standards were prepared for a range of concentrations 0.1 to 2 mg/ml.

A 100 µl of BCA was added to 10 µl of each concentration of the BSA standard or 5 µl of sample of cell lysate diluted with 5 µl of RIPA buffer, and then incubated at 37 $^{o}$C for 30 min, before the measurement of the absorbance at 570 nm by a spectrophotometer. The protein standard calibration curve was derived from the absorbance measurement and utilised in the calculation of the protein concentration for each sample of the lysate.

About 10 µg protein of each cell lysate sample was carefully added to NuPAGE sample buffer (obtained from Invitrogen) made up with 5% β-mercaptoethanol, before electrophoresis using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), for 15 min at a temperature of 70 $^{o}$C to denature the proteins. The denatured proteins were added to a 4-20% Nuview Tris-Glycine polyacrylamide gradient gel (NUSEP) in a Xcell surelock mini cell (the stationary phase) to fractionate the proteins from the cell

lysates. The mobile phase consisted of Hepes running buffer prepared from 100 mM Hepes, 100 mM Tris and sodium dodecyl sulphate (1%).

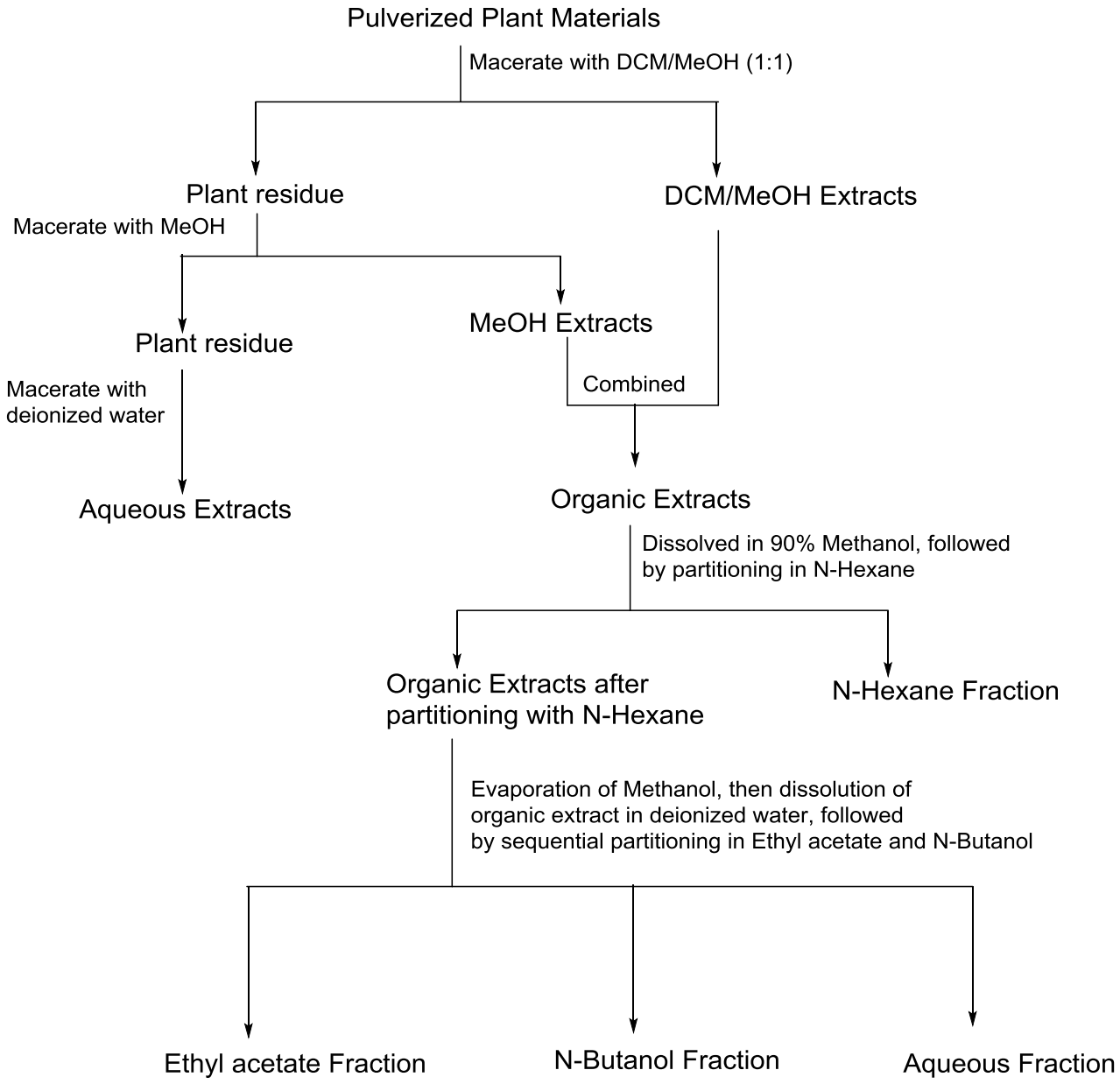
For the detection of the immunodetected proteins, pageruler plus pre-stained protein ladder was included on the gels to indicate the molecular weights of the separated proteins from the gels. These separated proteins were later transferred to Amersham Hybond P 0.45 µm polyvinylidene difluoride membrane and incubated in transfer buffer with a composition of 200 mM glycine, 25 mM Tris, 10% methanol and 0.075% SDS for 1.5hr. The membrane was again incubated in Tris Buffered Saline with Tween buffer (TBST) prepared by the addition of 150 mM NaCl, 5% skimmed milk powder and 0.1% Tween 20 to 50 mm Tris hydrochloride at pH 7.4, for 1.5h on a rocker at RT to achieve blocking of the membranes. The membrane was subsequently incubated at 4 $^{o}$C in the buffer with primary antibody and placed on a rocker overnight. The membrane was washed several times with TBST, before incubation in the buffer with secondary antibody for 1h at RT on a rocker. Following several washes in TBST, the membrane was analysed on a FluorChem M Imager, for the visualization of the protein bands using the UptiLight HRP chemiluminescent substrate according to the manufacturer's instructions (Uptima).

## *2.5 Extraction of Plant Materials*

The plant materials were extracted according to the NCI method of extraction (Thomas, G., 2010). Typically, 200g of 1 Kg of the pulverized plant material was macerated in a 1:1 mixture of 750 ml of dichloromethane and 750ml of methanol for 24h. The ratio of plant material to solvent used was 1:5. This ratio was maintained for all weighed amount of plant materials used.

The obtained solution containing the extracts was decanted off and 500 ml of methanol was added to the residue and allowed to stand for another 24h. The solution of the extract was collected by filtration and 1 litre of deionised water was added to the residue. The aqueous extract was collected after 24h of maceration. The methanol extraction was combined with

the 1:1 dichloromethane and methanol extraction to yield the organic extract. This extraction solution was evaporated to dryness on a rotary evaporator at a temperature of <40 °C. The obtained dry extracts were further dried in a desiccator to remove any trace of solvent. The aqueous extraction was dried on the lyophilizer after being solidified in a stream of liquid nitrogen. The percentage yields of the crude extracts of these plants were calculated as follows: Yield (%) = [extract obtained (g) × 100]/[Sample (g)]




NB: DCM is Dichloromethane
MeOH is Methanol

**Figure 2.1 A schematic representation of the extraction procedure of the plant materials to obtain the aqueous and organic extracts. Based on preliminary in vitro screening results, the organic extracts were subsequently fractionated as described in the diagram.**

### 2.5.1 Bioassay-guided fractionation of Plants

The organic extracts of the plants (typically 4-5g) with the potent anti-ovarian cancer activity were subsequently partitioned based on increasing polarity in n-hexane, ethyl-acetate, and n-butanol consecutively. The extracts were first dissolved in 90 % methanol in water (300 ml) and partitioned with n-hexane (150 ml × 3). The combined hexane solvent was evaporated to yield the n-hexane fraction. The 90% methanol fraction was evaporated and the resulting residue was dissolved in 300 ml water and partitioned with ethyl acetate (150 ml × 3), which upon evaporation, the ethyl acetate extract was obtained. Finally, partitioning in n-butanol (150 ml × 3) was carried out. After evaporation of the solventsthe n-butanol fraction and aqueous fraction were obtained respectively. These 4 fractions were investigated for growth inhibitory activities and the significantly active fractions were further fractionated; firstly by column chromatography and further purification by HPLC was carried out as described in the results sections of the chapters to yield the pure compound(s) responsible for the potential anti-ovarian cancer activity.

### 2.5.2 TMSi Derivatization of the Bioactive Fractions

1.0 mg of the bioactive fraction of these plant extracts were added to 10 µl of pyridine and 50 µl of N,O-Bis (trimethylsily) trifluoroacetamide (BSTFA). The obtained solution was incubated in the oven at a temperature of 37$^{o}$C for 2h to prepare the trimethylsilyl (TMSi) derivatives which were subsequently analysed on gas chromatography mass spectrometry (GC-MS) (Paulo de Andradea et al., 2012).

## *2.6 Gas chromatography Mass Spectroscopy*

The GC-MS system consisted of an Agilent 7890 A gas chromatography systems, coupled to an Agilent MS model 5975C MSD with triple axis detector (Agilent Technologies, US). The instrument's column is a HP5-MS column.

The gas chromatography began with an oven temperature of 60°C for 2 min, which increased to 300°C at the rate of 10°C/min, which was held at 300°C for 9 min to yield a total

run of 30 min under a constant helium pressure (10 psi). The mass spectrometry utilized an electron impact (EI) source at 70eV with an ion source temperature of 230°C. The mass spectra data were acquired in the scan mode in *m/z* range 16-600. The compounds were identified by GC-MS which is often used for identification as well as relative quantification and is comparable to GC-FID (Doddsa et al., 2005). These identified compounds were compared with standard compounds directly without measuring Kovat's indices.

## *2.7 High Performance Liquid Chromatography (HPLC) Analysis*

The bioactive plant fractions and semi-synthesised TQ analogues were firstly purified by column chromatography which utilized various solvent systems as specified in the chapters. The obtained sub-fractions with significant activities were subjected to preparative HPLC on the Agilent 1220 LC, USA instrument. A mobile phase composition made up of a mixture of solution A (0.1% TFA) and solution B (80% acetonitrile acidified with 0.1% TFA) was utilised on an Eclipse XDB-C18 reverse phase column with specifications 9.4 x 250 mm, particle size of 5 µm and pore size 300Å, (Agilent, UK) at a flow rate of 4 ml/min. The gradient and run times for the isolated compounds are specified in the preceding chapters. The purity of the isolated compounds were analysed using analytical HPLC C18 column (Phenomenex, UK). The flow rate was maintained at 1 ml/min. The column specifications were 4.6 x 250 mm, particle size of 5 µm and pore size 300Å.

## *2.8 Liquid chromatography mass spectrometry (LC-MS) Analysis*

The isolated compounds were analysed by LC-MS, to determine their molecular mass, and their retention times compared with purchased standard compounds. The Agilent technologies 1260 Infinity coupled to Agilent technologies 6530 Accurate mass Q-TOF LC-MS system was used. The gas temperature was 320 $^{o}$C, with a dry gas flow rate of 11 L/min. ESI was operated at a voltage of 4000V, an *m/z* range of 100-2000. The samples were injected at a volume of 5 µl and the run time was <3 min. The solvent systems were 20 % solvent A (a solution of 5 mM of ammonium formate and 0.1% formic acid in water) and 80%

solvent B (acetonitrile).The data was analysed by the Agilent mass-hunter qualitative analysis software.

## *2.9 NMR Spectroscopy*

$^{1}$H NMR spectra were obtained at 300 or 400 or 500 MHz with a Bruker 1D (DPX-300) NMR spectrometer, for 300 MHz; Bruker 1D (DPX-400) for 400 MHz and Bruker 1D (DPX-500) for 500 MHz. $^{13}$C and DEPT NMR spectra were obtained at 75 or 100 or 125 MHz with a Bruker NMR spectrometer and either Chloroform-D or Methanol-D was used as the solvent. Tetramethylsilane (TMS) served as the reference (δ=0 ppm) at a temperature of 25 °C. The obtained chemical shifts were expressed downfield from the reference, TMS.

ACD/Labs 10 Freeware (Advanced Chemistry Development Inc., Ontario, Canada) was used in the Analysis of the NMR Spectra.

## *2.10 Statistical analysis and Diagrams*

The absorbance at 570 nm for SRB assay was measured using a spectroscopic plate reader. The recorded data was analysed by non-linear regression using the Graph pad PRISM software to fit a 4 parameter sigmoidal dose-response curve to determine $IC_{50}$ values and the Hill coefficient. The $IC_{50}$ values of the extracts/pure compounds analogues were obtained from a plot of absorbance versus log of concentration in µg per ml/µM. The mean $IC_{50}$s were obtained from mean calculations using $IC_{50}$ values of three determinations of each plant extract. The standard error of the mean (SEM) was also determined.

Statistical significance of results was determined by one-way ANOVA and paired t-test using the Graph pad PRISM.

## *2.11 The Diagrams*

The chemical structures and Figure 2.1 were drawn using the ChemBioDraw Ultra 13.0 software, while the illustration of ovarian cancer, Figure 1.1 was drawn using Photoshop cc 2015. The pictures in the thesis were camera snapshots taken by the researcher.

# Chapter Three

## *Anti-Ovarian Cancer Compounds from Margaritaria Discoidea (Baill.) G. L. Webster*

## *3.1 Introduction*

*Margaritaria discoidea* (MD) is a plant in the *Margaritaria* genus. This genus has just thirteen species and is the smallest genus in the Phyllanthaceae family. Literatures on the scientific investigations of most of these species are lacking. Despite the rich Ethnopharmacological uses of MD in many indigenous communities the plant has received less-research attention and thus is a relatively un-explored species for identifying novel compounds with biological activities. Previous researchers have documented the anti-bacterial, anti-inflammatory and anti-oxidant activities of MD (Adedapo et al., 2009; Dickon et al 2010). However, there are no reported studies on the anti-cancer activities of the specie which has necessitated this research work on investigation of the anti-ovarian cancer activities of MD.

The research in this chapter aims to investigate the anti-cancer activities of MD on ovarian cancer cell lines. Through bioassay-guided fraction, the active compounds were isolated and characterised. Further growth inhibition evaluation using cell-growth assay were carried out to validate the antiproliferative activities of the bioactive compounds. Selectivity index (SI), of the bioactive compounds with potent activitieswas undertaken. Through the apoptosis studies, the possible route, by which the potent bioactive compound kills cancer cells, was investigated. Drug combination studies were carried out to identify potential synergy between the bioactive compounds and clinically used ovarian cancer drugs both in cell growth assays and in the apoptosis studies.

## *3.2 Results and Discussion*

### 3.2.1 Extraction of plant materials

The pulverised stembarks of MD were extracted using a combination of dichloromethane and methanol to obtain the organic extract, followed by extraction with deionised water to yield the aqueous extract, according to the procedures (Figure 2.1) used by National Cancer Institute (NCI, USA). The amounts of the extracts and their yields were obtained (Table 3.1).

**Table 3-1 Percentage Yield of the MD Extracts obtained from the extraction with dichloromethane/methanol (organic extract) and extraction with deionized water (aqueous extract).**

| SAMPLE AMOUNT (G) | ORGANIC EXTRACT (G) | AQUEOUS EXTRACT (G) | % YIELD OF TOTAL EXTRACT OF PLANT |
|---|---|---|---|
| 1000 | 9.8 | 1.3 | 1.11 |

From the data in Table 3.1, it was observed, that the stembarks of MD yielded relatively low amounts of solid extract. Also, the organic extract was more than the aqueous extract. This may be due to the method of extraction, which was sequential; organic extraction, followed by aqueous extraction.

### 3.2.2 Screening of anti-ovarian cancer activity of the plant extracts

The organic and aqueous extracts of MD were screened for their inhibition of cell growth on OVCAR 8 and A2780 ovarian cancer cell lines using a cell growth assay in which cell number was estimated by staining with SRB. The results of the $IC_{50}$ values of the preliminary anti-cancer investigations of the plant extracts (Table 3.2).

The $IC_{50}$ values of organic extracts of MD had the most significant growth inhibitory activity, as observed from the plant extracts' $IC_{50}$ values of 14.2 and 14.4 µg/mL on the A2780 and OVCAR 8 ovarian cancer cell lines, respectively, (Table 3.2). This $IC_{50}$ is below the threshold

which is considered to be significant from the perspective of the American national Cancer institute criteria, which from a screening of about 20,000 plant extracts, identified 452 cytotoxic extracts with $IC_{50}$<50µg/mL (Thomas 2010). Thus, the organic extract of MD with $IC_{50}$s <50µg/mL on the two cell lines tested warranted further purification and investigation for anti-cancer activity.

**Table 3-2 Results of Preliminary Screening of the Extracts of MD on ovarian cancer cell lines in cell growth assays. The cells were treated for 72h with the indicated extract, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments.**

| EXTRACT | $IC_{50}$ OF A2780 (µg/ml) | $IC_{50}$OFOVCAR8 (µg/ml) |
|---|---|---|
| ORGANIC EXTRACT | 14.2±3.0 | 14.4±1.2 |
| AQUEOUS EXTRACT | 35.3±1.8 | 66.3±0.6 |

These findings are consistent with the reported observations of several studies that have shown that some related species of *Margaritaria*, such as *Phyllanthus pectinatus* inhibited ovarian cancer cells, (Sujatha et al., 2011). *Phyllanthus amarus* has been shown to retard the proliferation of liver cancer *in vitro* (Jeena et al., 1999). Also, from an *in vivo* study of *Phyllanthus urinaria,* the reduction of tumour growth in lung carcinoma has been reported (Huang et al., 2006). *Phyllanthus emblica* has demonstrated cytotoxic activities on gastric and uterine cancers *in vitro* (Zhang et al., 2004), and the cytotoxic activites of *Phyllanthus polyphyllus* on breast, colon and liver cancer cells has also been documented (Rajkapoor et al., 2007).

## *3.3 Bioassay-guided fractionation of* M. discoidea

Further solvent fractionation of 4.0 g of the crude organic extract of MD, using various solvents was perfomed (mass of solid compound recovered is shown in parentheses: n-hexane (0.5 g), ethyl acetate (0.8 g), n-butanol (1.5 g) and aqueous (0.9 g) fractions respectively. These fractions were further evaluated in cell growth assays (Table 3.3).

**Table 3-3 The Results of the growth inhibition evaluation of the fractions of MD on ovarian cancer cell lines in cell growth assays. The cells were treated for 72h with the indicated fraction, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments.**

| Fraction | Abbrev | $IC_{50}$ of OVCAR8 (µg/ml) | $IC_{50}$ of A2780 (µg/ml) |
|---|---|---|---|
| **n-Hexane** | MD-N-HEX | 33.7±2.2 | 27.0±1.3 |
| **Ethyl Acetate** | MD-EA | 11.6±1.5 | 7.3±0.7 |
| **n-Butanol** | MD-N-BU | 95.4±15.2 | 68.2±0.9 |
| **Aqueous** | MD-AQ | 63.9±7.2 | 91.2±0.9 |

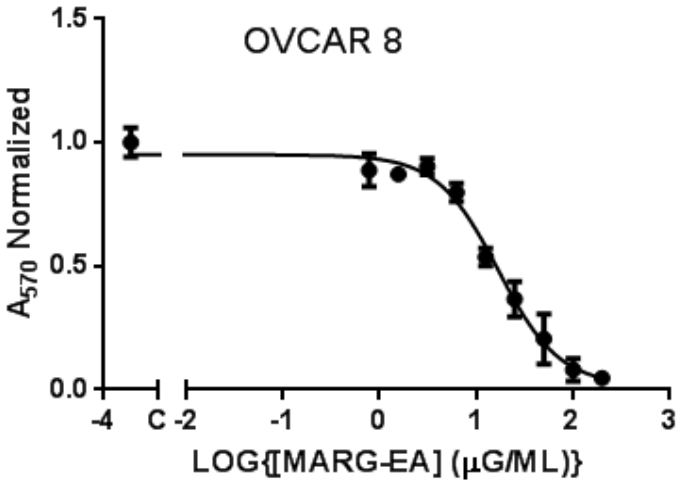



**Figure 3.1 Mean concentration-response curve of the ethyl acetate fraction of Margaritaria discoidea on the OVCAR 8 ovarian cancer cell line. The cells were treated for 72h with the indicated compound, followed by an estimation of the number of surviving cells by SRB staining. The results were expressed as mean IC50 ± SEM, n=3 experiments.**

The ethyl acetate fraction showed lower $IC_{50}$ values both in the OVCAR8 and A2780 cell lines and was the most potent of the fractions. Thus, ethyl acetate fraction merited further chemical characterization.

A similar finding has also been observed in a study on the growth inhibition of human gynaecologic and colon cancer cells, which showed that the anti-cancer activity of *Phyllanthus watsonii* specie, a related specie of the *M. discoidea* plant family was in the middle range polarity phase of the solvent system as reported by the authors (Ramasamy et al., 2012).

## *3.4 Chemical characterization of active fraction of* M. discoidea

In order to find out the anti-cancer components in the ethyl acetate fraction, their TMSi derivatives were prepared and analysed by GC-MS.

The TMSi derivatives of the ethyl acetate fraction of *M. discoidea* was obtained by the treatment of BSTFA and pyridine and subjected to further analysis on the GC-MS to determine the potential bioactive compounds that could be responsible for the activity of the fraction.

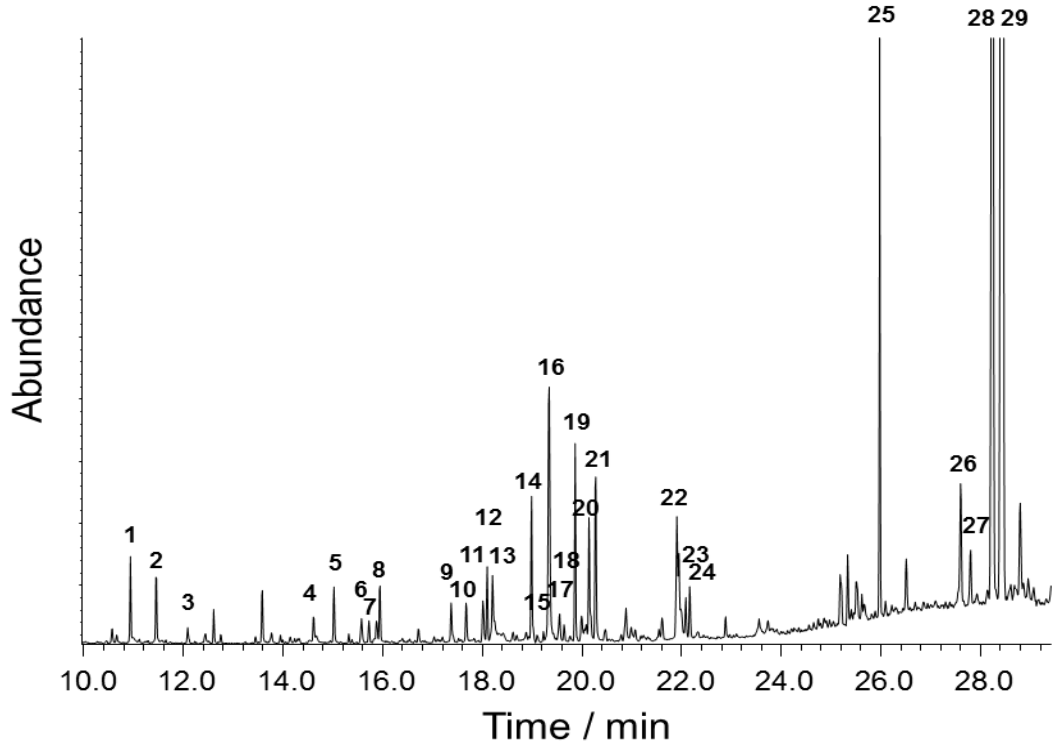



**Figure 3.2 GC Chromatogram of TMSi derivatives of Ethyl Acetate Fraction of M. discoidea**

The GC chromatogram of the ethyl acetate fraction of *M. discoidea* is displayed in Figure 3.2. Twenty-nine compounds were detected; some of them could be identified by comparison of their mass spectra with those in the NIST library. For example, Figure 3.3 shows the identification of a peak at 19.37 min as securinine, an alkaloid. There were also many unknown compounds. As a result, many compounds such as 2-(2-hydroxyphenyl)acetic acid, 4-hydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, 4-(2-hydroxyethyl)-2-methoxyphenol, (E)-3-(4-hydroxy-3-methoxyphenyl) acrylaldehyde, 3,4-dihydroxybenzoic acid, ethyl 2-hydroxy-2-(2-hydroxyphenyl)acetate, 3-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol, 3,4,5-trihydroxybenzoic acid (gallic acid), nonanedioic acid (azelaic acid), and securinine could be identified (Table 3.4) and their chemical structures are shown in Figure 3.4.

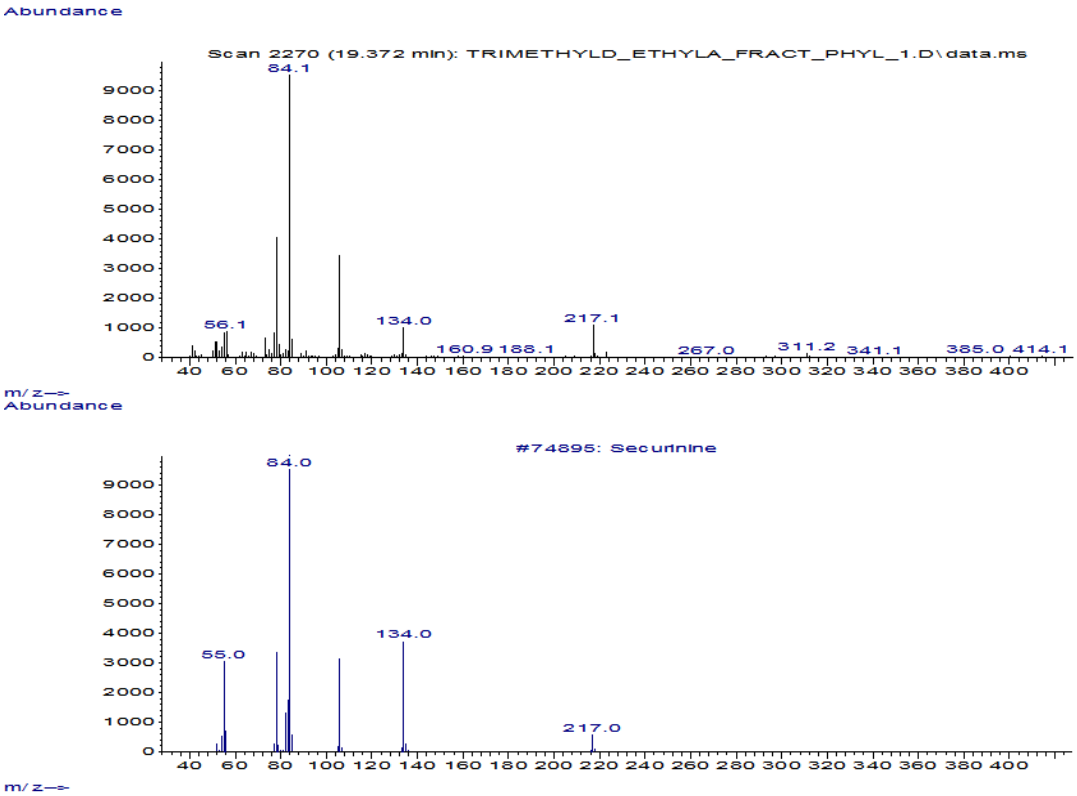



**Figure 3.3 Mass spectrum of a peak at 19.372 min for securinine in the ethyl acetate fraction of M. discoidea (top) and mass spectrum of securinine from NIST library (bottom).**

Securinine, an indolizidine alkaloid (Figure 3.4) is the major alkaloid of *Securinega suffruticosa*, plant. Securinega suffruticosa, yī yè qiū (presently known as *Flueggea*

*suffruticosa*) is one of the fifty important herbs used in the traditional Chinese Medicine (TCM). The shrub is a dioecious plant, belonging to the Phyllanthaceae family as MD, but classed under the genus; *flueggea* (Wong et al., 1976). The plant is rich in pharmacologically important alkaloids, such as securinine, which was isolated in 1956 by the Russian scientist-Muraveva and Bankovski; and its analogues, allosecurinine, virosecurinine and viro-allosecurinine*.* *F. suffruticosa* had historically been used in the treatment of a wide range diseases and ailments, such rheumatism, neuroparalysis, impotence and infant paralysis (Kee 1999). Similarly, another anti-tumour compound, corilagin, has been reportedly found in *Phyllanthus niruri*, a related specie of *M. discoidea* (Jia et al., 2013).

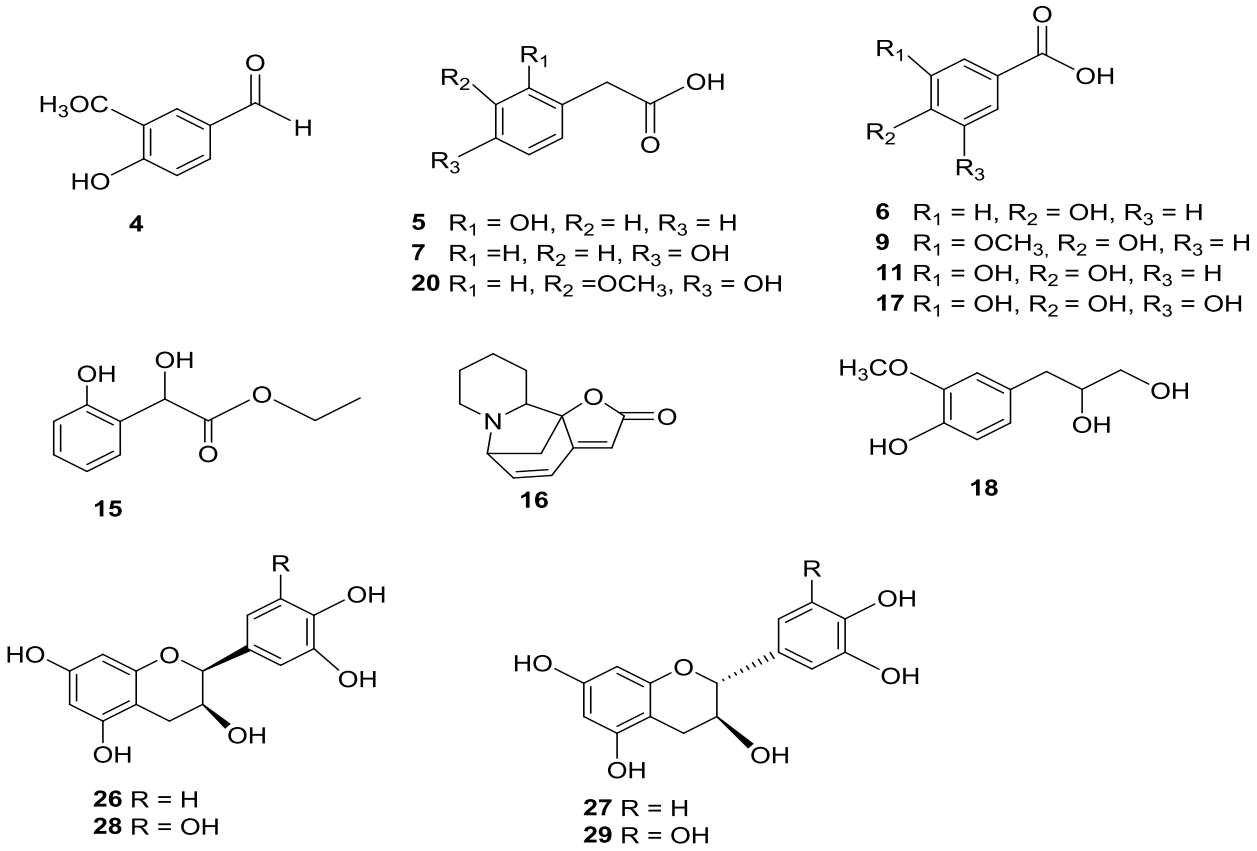



**Figure 3.4 Chemical structures of some compounds found in the ethyl acetate fraction of *M.discoidea* by GC-MS.**

**Table 3-4 TMSi derivatives detected in the ethyl acetate fraction of *M. discoidea* by GC-MS.**

| peak | retention time (min) | TMSi derivatives | parent molecules in plants | area of peak (%) (n =3) |
|---|---|---|---|---|
| **1** | 10.94 | glycerol, tris(trimethylsilyl) ether | glycerol | 0.47 ± 0.07 |
| **2** | 11.49 | butanedioic acid, bis(trimethylsilyl) ester | butanedioic acid | 0.44 ± 0.07 |
| **3** | 12.13 | nonanoic acid, trimethylsilyl ester | nonanoic acid | 0.11 ± 0.03 |
| **4** | 14.61 | 3-hydroxy-4-methoxy benzaldehyde, trimethylsilyl ether | 3-hydroxy-4-methoxy benzaldehyde | 0.21 ± 0.03 |
| **5** | 15.02 | acetic acid, [o-(trimethylsiloxy)phenyl]-, trimethylsilyl ester | 2-(2-hydroxyphenyl)acetic acid | 0.37 ± 0.08 |
| **6** | 15.72 | benzoic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester | 4-hydroxybenzoic acid | 0.17 ± 0.01 |
| **7** | 15.87 | Benzene acetic acid, 4-[(trimethylsilyl)oxy]-, trimethylsilyl ester | 4-hydroxybeneacetic acid | 0.16 ± 0.03 |

| | | | | |
|---|---|---|---|---|
| **8** | 15.95 | dodecanoic acid, trimethylsilyl ester | dodecanoic acid | 0.43 ± 0.03 |
| **9** | 17.37 | benzoic acid, 3-methoxy-4-[(trimethylsilyl)oxy]-, trimethylsilyl ester | 4-hydroxy-3-methoxybenzoic acid (vanillic acid) | 0.31 ± 0.06 |
| **10** | 17.67 | azelaic acid, bis(trimethylsilyl) ester | azelaic acid | 0.24 ± 0.09 |
| **11** | 18.01 | benzoic acid, 3,4-bis[(trimethylsilyl)oxy], trimethylsilyl ester | 3,4-dihydroxybenzoic acid | 0.37 ± 0.06 |
| **12** | 18.09 | D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether | D-(-)-fructofuranose | 0.46 ± 0.06 |
| **13** | 18.19 | D-(-)-Fructopyranose, pentakis(trimethylsilyl) ether | D-(-)-fructopyranose | 0.89 ± 0.11 |
| **14** | 18.99 | beta.-D-Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)- | beta-D-glucopyranose | 1.04 ± 0.11 |
| **15** | 19.22 | 2-hydroxymandelic acid, ethyl ester, di-TMS | ethyl 2-hydroxy-2-(2-hydroxyphenyl)acetate | 0.06 ± 0.02 |
| **16** | 19.34 | securinine | securinine | 2.06 ± 0.76 |

| | | | | |
|---|---|---|---|---|
| **17** | 19.55 | benzoic acid, 3,4,5-tris(trimethylsiloxy)-, trimethylsilyl ester | 3,4,5-trihydroxybenzoic acid (gallic acid) | 0.20 ± 0.06 |
| **18** | 19.63 | 3-vanil-1,2-bis(trimethylsilyloxy)propane | 3-(4-hydroxy-3-methoxyphenyl)propane-1,2-diol | 0.12 ± 0.01 |
| **19** | 19.86 | D-(+)-galactopyranose, pentakis(trimethylsilyl) ether | D-(+)-galactopyranose | 1.32 ± 0.09 |
| **20** | 20.13 | Benzeneacetic acid, 3-methoxy-alpha,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester | 3-methoxy-4-hydroxybeneneacetic acid | 0.92 ± 0.18 |
| **21** | 20.27 | hexadecanoic acid, trimethylsilyl ester | hexadecanoic acid | 1.42 ± 0.04 |
| **22** | 21.89 | 9,12-octadecadienoic acid (Z,Z)-, trimethylsilyl ester | (Z,Z)-9,12-octadecadienoic acid | 2.05 ± 0.17 |
| **23** | 22.08 | 2,2-bis(4-trimethylsioxy)phenyl]propane | 2,2-bis(4-hydroxyphenyl]propane | 0.25 ± 0.09 |
| **24** | 22.15 | octadecanoic acid, trimethylsilyl ester | octadecanoic acid | 0.41 ± 0.06 |
| **25** | 25.97 | sucrose, octakis(trimethylsilyl) ether | sucrose | 7.16 ± 1.06 |

| | | | | |
|---|---|---|---|---|
| **26** | 27.59 | 2H-1-benzopyran, 3,4-dihydro-2-[3,4-bis[(trimethylsilyl)oxy]phenyl]-3,5,7-tris[(trimethylsilyl)oxy]-, (cis)- | cis-catechin | 1.23 ± 0.14 |
| **27** | 27.79 | 2H-1-benzopyran, 3,4-dihydro-2-[3,4-bis[(trimethylsilyl)oxy]phenyl]-3,5,7-tris[(trimethylsilyl)oxy]-, (trans)- | trans-catechin | 0.89 ± 0.22 |
| **28** | 28.23 | 2H-1-benzopyran, 3,4-dihydro-2-[3,4,5-tris[(trimethylsilyl)oxy]phenyl]-3,5,7-tris[(trimethylsilyl)oxy]-, (cis)- | cis-gallocatechin | 21.6 ± 0.95 |
| **29** | 23.53 | 2H-1-benzopyran, 3,4-dihydro-2-[3,4,5-tris[(trimethylsilyl)oxy]phenyl]-3,5,7-tris[(trimethylsilyl)oxy]-, (trans)- | trans-gallocatechin | 43.0 ± 1.83 |

## *3.5 Column Purification of the Ethyl Acetate Fraction of MD*

Nine sub-fractions were obtained from a solvent-polarity based column chromatography fractionation eluted with n-hexane/ethyl-acetate (gradient of polarity) based on the optimum TLC profile obtained from a mobile phase of n-hexane: ethyl acetate (1:1). The fractionation began with a 100% n-hexane and ended with 100% methanol. The obtained sub-fractions were evaluated for growth inhibition in the cell growth assays (Table 3.5).

**Table 3-5 The $IC_{50}$ Values of the Sub-Fractions of MD-EA on A2780 cell line. Three independent experiments were carried out. The results are expressed as Mean±SEM, n=3 (for sub-fractions with activities <30 µg/mL).**

| Sub-fractions of MD-EA | A2780 Cell Line $IC_{50}$ Value (µg/ml) | Solvent Fraction |
|---|---|---|
| **F1** | >30 | N-hexane |
| **F2** | >30 | N-hexane/ethyl acetate |
| **F3** | >30 | " |
| **F4** | **7.5±0.4** | **"** |
| **F5** | **6.3±0.7** | **"** |
| **F6** | 13.7±0.9 | " |
| **F7** | >30 | Ethyl acetate |
| **F8** | >30 | Ethyl acetate/Methanol |
| **F9** | >30 | Methanol |

Two of the sub-fractions; F4 and F5 showed notable activities, and were subjected to further purification, while no activity was measured in the other fractions.

**Isolation of Compound MPHYF4**

Sub-fraction F4, was treated with hot methanol and recrystallized at a temperature of 0-4$^{o}$C, to yield white needle crystals henceforth referred to as MPHYF4. The LC-MS analysis identified an ion with *m/z* of 457.36 $[M + H]^{+}$, therefore the mass of this compound was 456.36 with a match quality score of 99.78%.

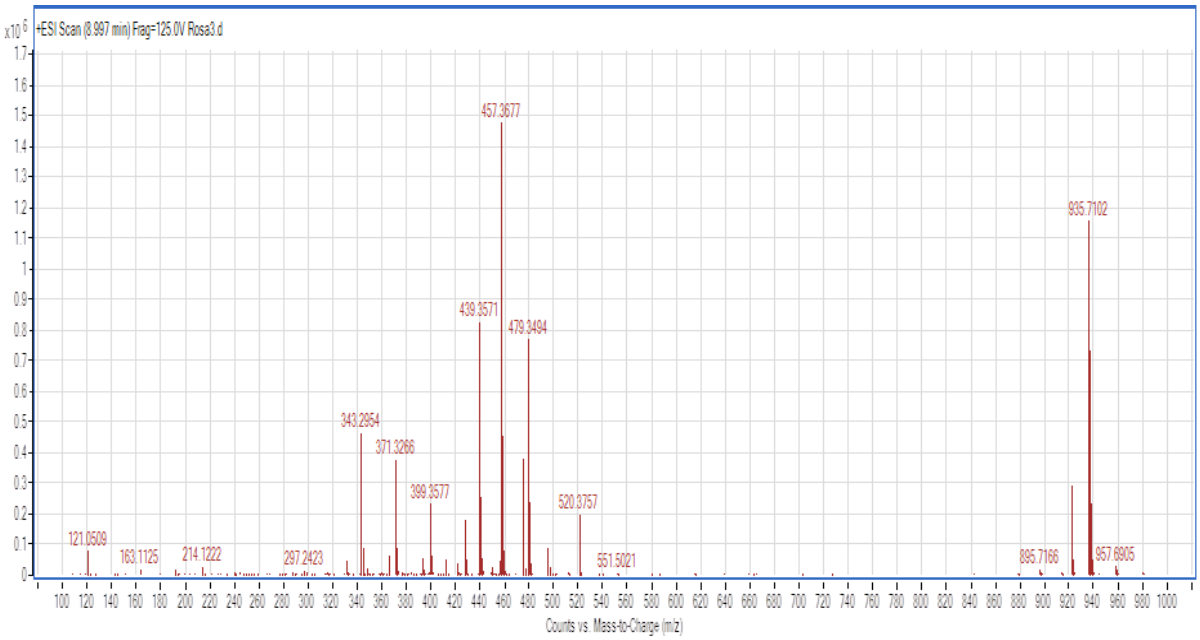



**Figure 3.5 The LC-MS mass spectrum of MPHYF4, isolated from MD-EA**

The $^{1}$H NMR spectrum of MPHYF4 was obtained at 300 MHz with a Bruker 1D (DPX-300) NMR spectrometer in $CDCl_3$[δ 3.50(s, 12H), 1.96(s, 3H), 1.72(s, 3H), 1.43(s, 3H), 1.26(s, 3H), 0.95(m, 3H), 0.80(d, 3H, 4.8Hz)] and the data was consistent with the reported data in literature for betulinic acid (Rodrigues et al., 2008). The compound was tentatively identified as betulinic acid.

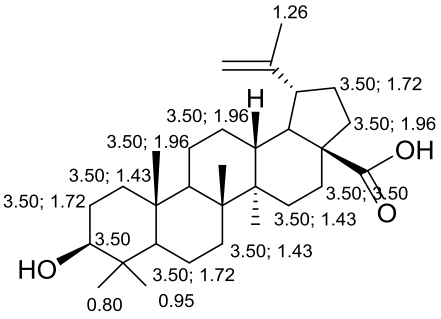



**Figure 3.6 The $^{1}$H NMR assignment of Betulinic acid**

The $^{13}C$ NMR spectrum of MPHYF4 was obtained at 400 MHz with a Bruker 1D (DPX-400) NMR spectrometer in $CDCl_3$ [δ 150.43, 109.68, 97.39, 79.02, 55.40, 50.57, 49.33, 46.91, 42.48, 38.88, 38.41, 37.24, 29.73, 28.00, 19.39, 18.32, 16.15, 16.05, 15.35, 14.72. This obtained NMR data was comparable with the data in literature, (Satiraphana et al., 2012). The compound was determined as betulinic acid.

GC-MS analysis of the TMSi derivatized sample of MPHYF4 was carried out. The compound could not be detected when the GC oven temperature was set at 60$^{o}$C to 300$^{o}$C for 30min run time. However, when the oven temperature was raised to 250$^{o}$C as the initial temperature and allowed to increase to 300$^{o}$C at a run time of 65min, the compound was detected as a single peak with Retention time of 34.49min. The derivatized *m/z* was 585.5. Further confirmation of the compound was carried out by the GC-MS analysis of the derivatized standard betulinic acid, which also had the same Retention time. Again both compounds were mixed together in equal proportion and re-analysed on the GC-MS to observe the co-elution of the compounds at the same retention time as shown in Figure 3.7

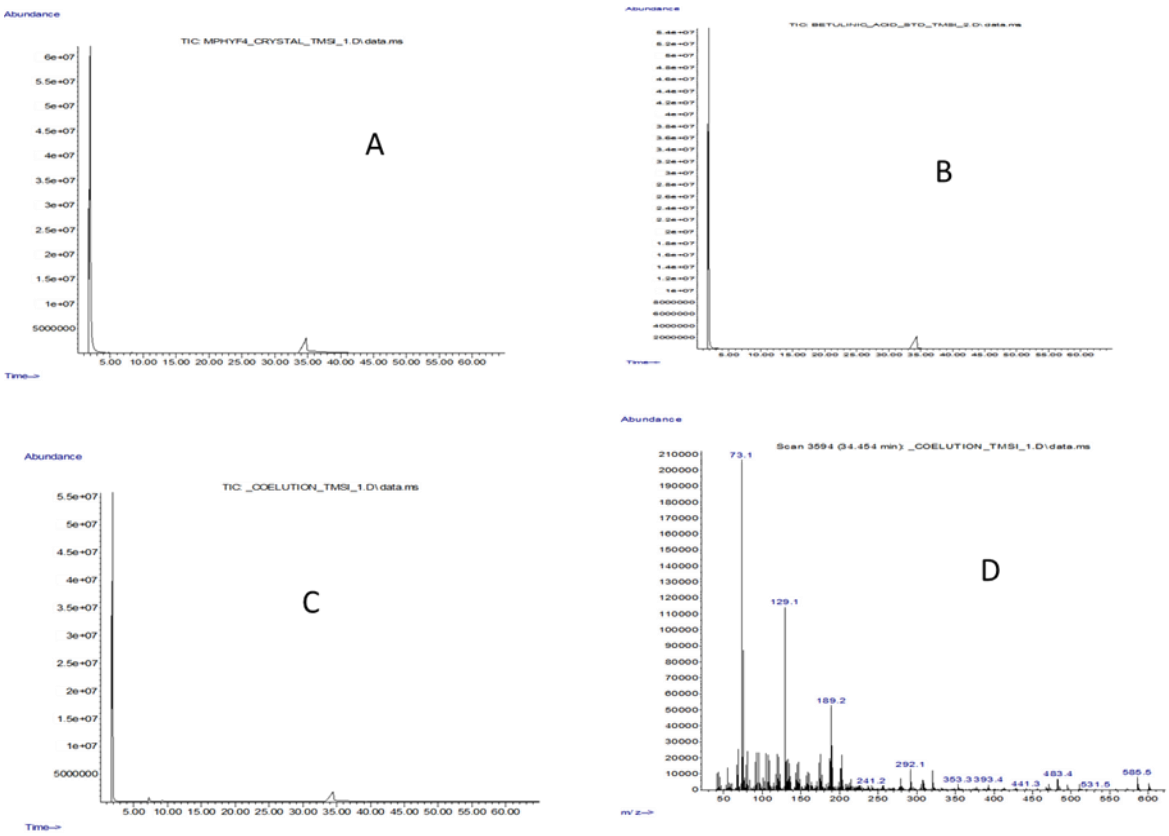



**Figure 3.7 The GC-MS analysis of the TMSi derivatized MPHYF4 and standard betulinic acid. GC-MS chromatograms of MPHYF4 (A), betulinic standard (B), the co-elution of both compounds (C), and the MS of the co-eluted compound (D).**

The chromatograms of MPHYF4 and the standard betulinic acid procured were exact matches with the chromatogram of the compounds when co-eluted. The retention time on the GC-MS was the same for the compounds singly and when co-eluted. Additionally, the spectrum shown in Figure 3.7(D),was identical for both MPHYF4 and the standard betulinic acid. Thus, MPHYF4 was unequivocally determined as betulinic acid (BA), from the LC-MS, $^{1}$H NMR,$^{13}$C NMR and GC-MS analysis. This compound was not initially detected in the GC-MS analysis of the derivatized MD-EA fraction due to the low start-off temperature and short run time of the analysis.

## Isolation of Compound F(10)4 - Securinine

The most potent sub-fraction, F5 was subjected to HPLC purification; with a mobile phase composition of 100% water acidified with 0.1% Trifluoroacetic acid (TFA) as solvent A and 80% acetonitrile acidified with 0.1% TFA as solvent B. The gradient began with 100% of A for

5 min and increased to 80% B during 20 min of the run time, and then maintained at 100% B for 5 min of the analysis; making the total run time 30min. A yellow amorphous powder was obtained from the HPLC purification and the isolated compound was henceforth referred to as F(10)4, which upon GC-MS analysis was tentatively identified as securinine with *m/z* of 217 and a retention time of19.34min. The GC-MS chromatogram and spectrum are presented in Figure 3.8.

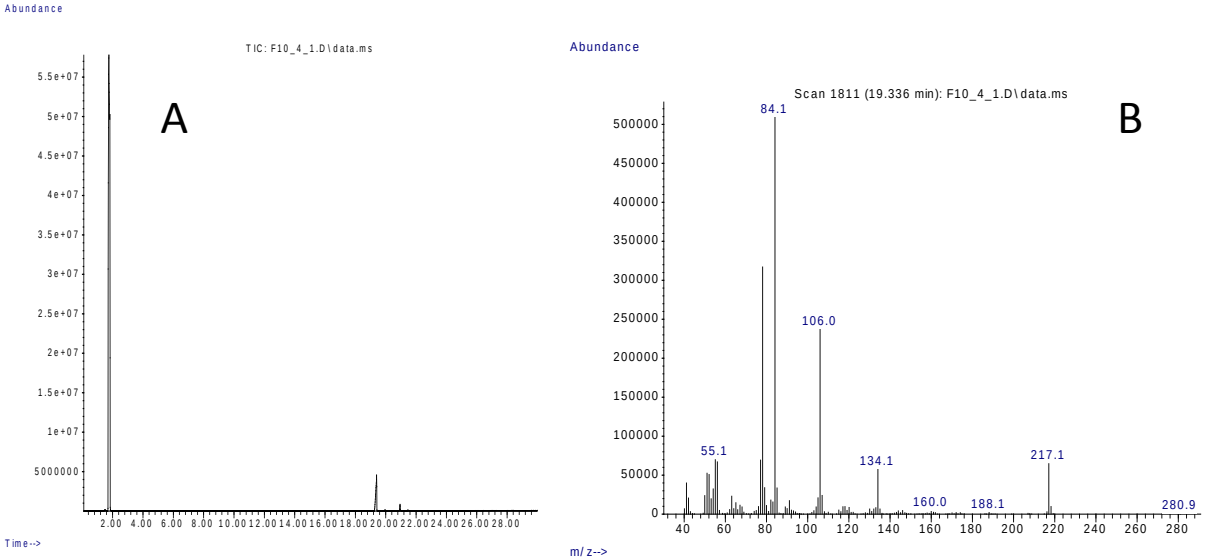



**Figure 3.8 The GC-MS chromatogram of compound F(10)4 in (A) and the MS spectrum of the compound F(10)4 (B).**

Additional confirmation was carried out by the $^{1}$H NMR spectrometry of F(10)4 on a 300 MHz with a Bruker 1D (DPX-300) NMR spectrometer in $CDCl_3$ [δ 6.62(m, 1H), 6.45 (m, 1H), 5.57(s, 1H), 3.83(q, 1H), 2.97(m, 1H), 2.51(m, 2H), 2.17(s, 1H), 1.86(m, 1H), 1.77(dd, 1H), 1.59(m, 2H), 1.28(s, 2H), 0.83(m. 1H)], which was consistent with the reported data of securinine in literature (Diallo et al., 2015).

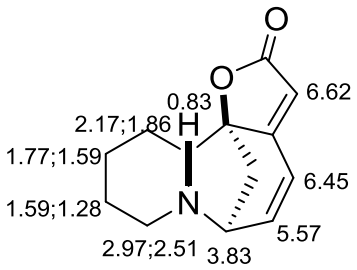



**Figure 3.9$^{1}$H NMR Assignment of Securinine**

Thus, F(10)4 was determined as securinine.

## Identification and Confirmation of the Compounds (-)-Catechin and (-)-Gallocatechin

The TMSI-derivatized sample of sub-fraction 7 was analysed on the GC-MS, four compounds were identified; (-)-Catechin (cis-Catechin) and the structural isomer (+)-catechin (trans-catechin), which in the derivatized form gave a mass of 650.30 at a retention time of 27.59min and 27.79 min respectively. The other two compounds were (-)-gallocatechin (cis-Gallocatechin) and (+)-gallocatechin (trans-Gallocatechin) with a derivatized mass of 738.31 at a Retention time of 28.22min and 28.42min respectively. However, the cis and trans-compounds (26 (28) could be 27 (29), vice versa) and perhaps exchangeable. The spectra of these compounds are contained in Figure 3.10.

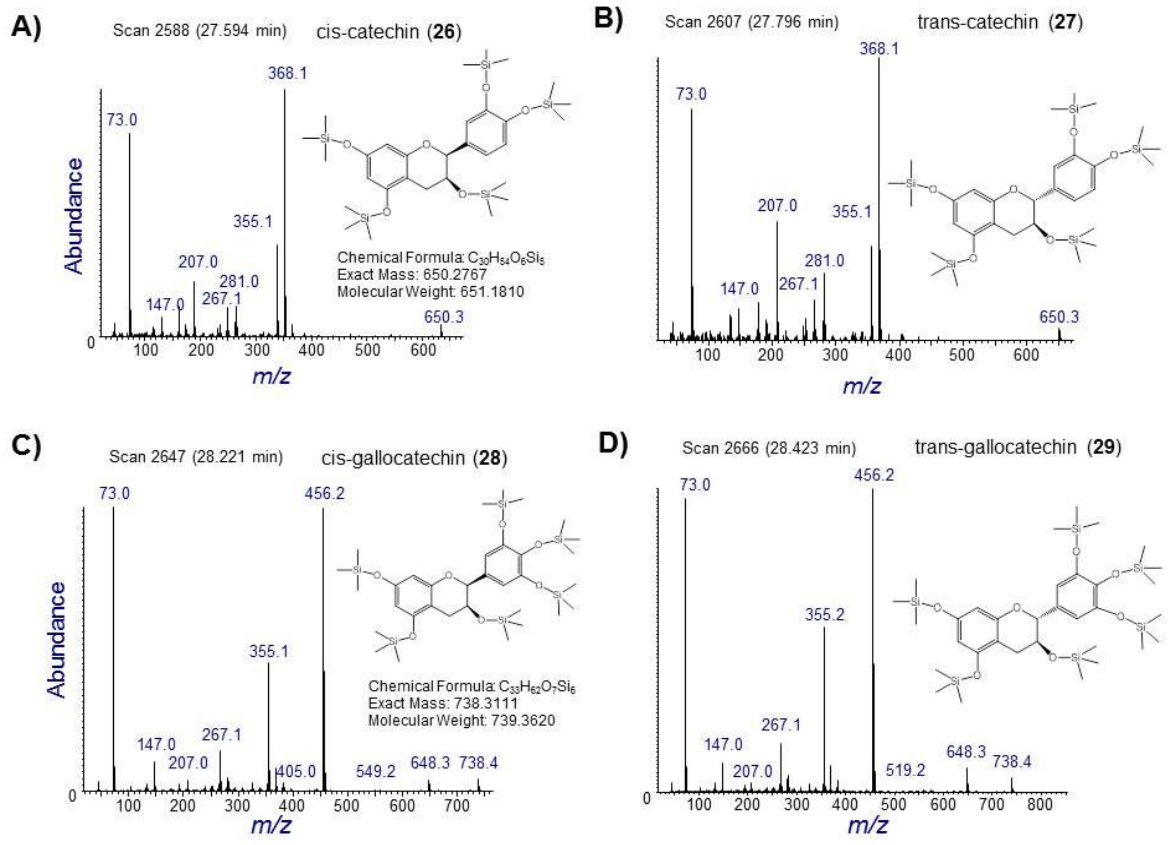



**Figure 3.10 Full EI mass spectrum of TMSi derivative of cis-catechin (TMS-cis-catechin, 26) (A), TMSi derivative of trans-catechin (27) (B), TMSi derivative of cis-gallocatechin (TMS-cis-gallacatechin, 28) (C), and TMSi derivative of trans-gallocatechin (29) (D).**

Additional confirmation of (-)-Catechin and cis-Gallocatechin was made by a comparison of the Chromatograms and spectra of the GC-MS analysis of the procured derivatized standards of these compounds. A schematic representation of the possible electron impact fragmentation pathways for the TMSi derivative of cis-catechin (26) and the TMSi derivative of cis-gallocatechin (28) is described in Figure 3.11.

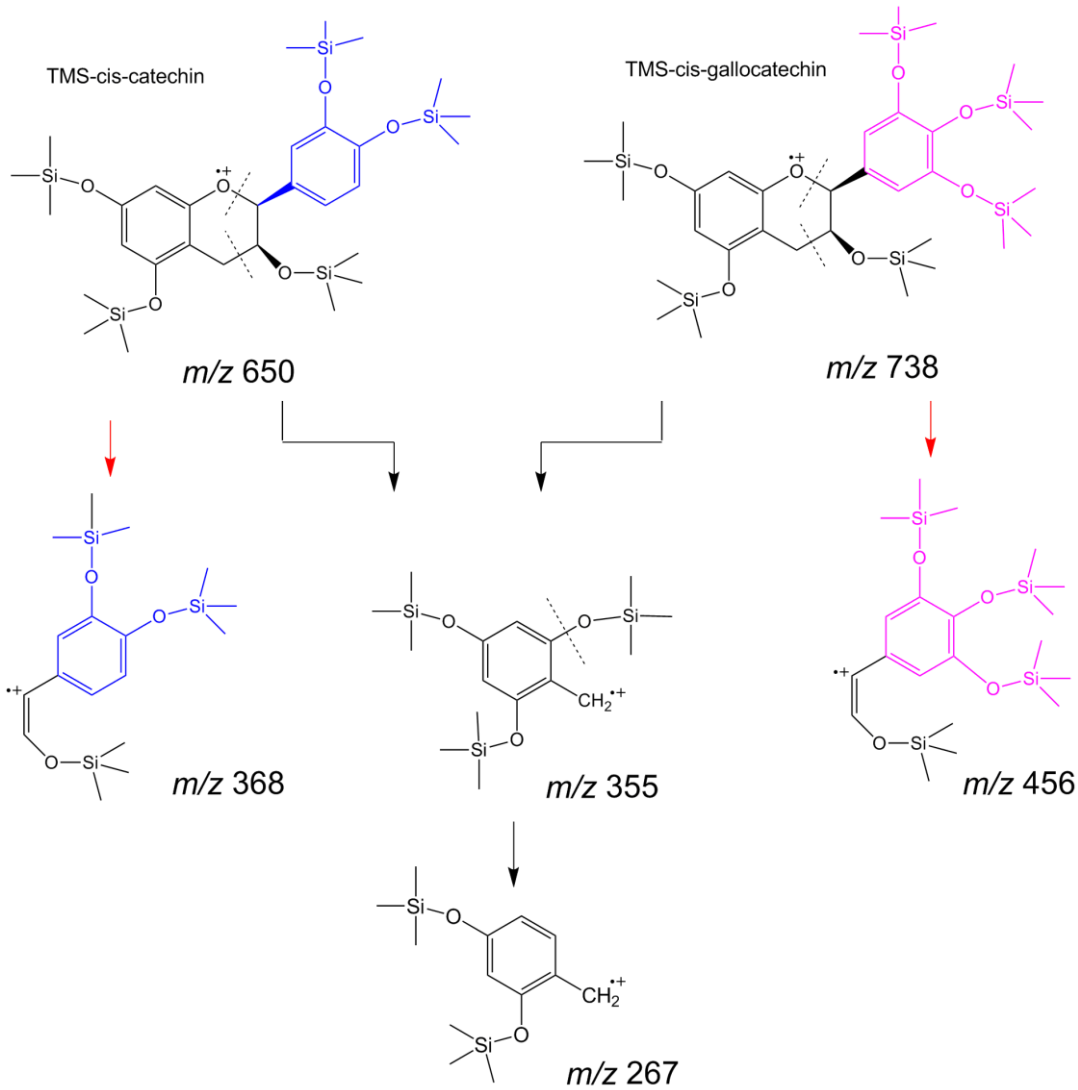



**Figure 3.11 Possible electron impact fragmentation pathways for the TMSi derivative of cis-catechin (26) and the TMSi derivative of cis-gallocatechin (28)**

### Identification of Gallic Acid

Gallic acid was one of the compounds putatively identified by the GC-MS analysis of the MD-EA fraction and shown in Figure 3.4 above. The identity of the compound was confirmned by

comparison of the GC-MS obtained spectrum with that of a standard gallic acid purchased from Sigma Aldrich. The spectra of both compounds were indistinguishable as shown in Figure 3.12 below. Thus, the identity of gallic acid was confirmed as a compound present in MD.

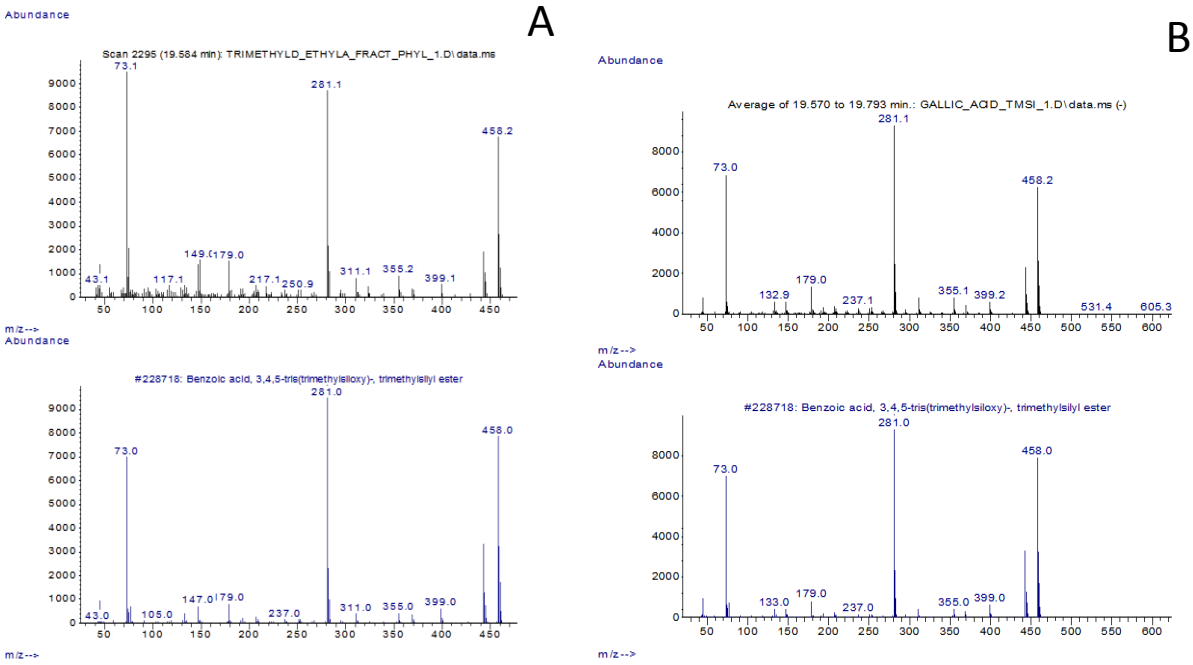



**Figure 3.12 The GC-MS spectrum of the gallic acid contained in the TMSi derivatized sample of MD-EA (A) and the spectrum of the TMSi derivatized gallic acid standard (B)**

## *3.6 Investigations of the anti-cancer activity of isolated compounds from MD-EA*

The five identified bioactive compounds were investigated for their cell growth inhibition on the ovarian cancer cell lines and on HOE (Table 3.6 and Figure 3.13).

**Table 3-6 The growth inhibition evaluation obtained by measuring the $IC_{50}$ (μM) of the bioactive compounds of MD-EA on ovarian cancer cell lines in cell growth assays.** The cells were treated for 72h with the indicated compound, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments.

| Cell Line | Securinine | Gallic Acid | (-)-Catechin | (-)-Gallocatechin | Betulinic Acid | MPHYF4 |
|---|---|---|---|---|---|---|
| **A2780** | 2.7±0.7 | 6.2±0.3 | 14.2±3.1 | 14.0±1.1 | ND | ND |
| **OVCAR 8** | 5.2±0.1 | 16.2±0.5 | 17.6±2.5 | 18.9±2.0 | ND | ND |
| **A2780CIS** | 5.4±0.3 | 6.5±0.4 | 56.6±4.6 | 13.7±2.1 | ND | ND |
| **OVCAR 4** | 8.7±0.1 | 26.9±4.1 | ND | ND | 16.0±1.9 | 19.1±1.1 |
| **HOE** | 10.7±1.2 | 73.5±3.8 | ND | ND | 25.9±2.1 | ND |

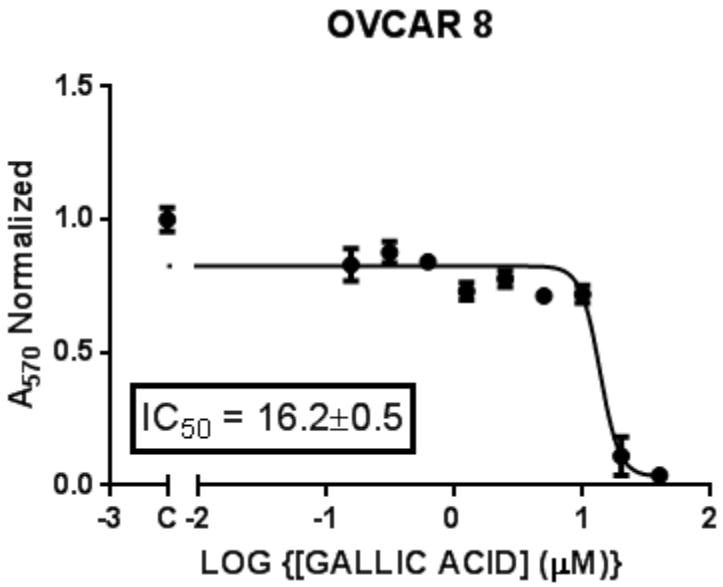



**Figure 3.13 The Mean concentration-response curves of A bioactive compounds of MD-EA; Gallic acid on OVCAR 8 cell line. The cells were treated for 72h with the indicated compound, followed by an estimation of the number of surviving cells by SRB staining. The results were expressed as mean IC50 ± SEM, n=3 experiments.**

The results of the growth inhibition evaluation of the identified bioactive compounds in MD-EA showed that the five compounds possessed antiproliferative activities on the ovarian cancer cell lines, which when ranked in increasing order was shown to be securinine > gallic acid > betulinic acid> (-)-gallocatechin> (-)-catechin. **Hypothesis a** was tested by these results. Securinine had more potent activities in comparison with the other bioactive compounds on all the cell lines tested. This finding was in agreement with the previous report on the anti-cancer properties of this compound against colon cancer SW480 cell (Yong-hui et al., 2011). Further studies on securinine were carried out to validate these findings.

## *3.7 Results of Further Evaluation and Drug Combination Experiments on Betulinic Acid (BA)*

The trypan blue assay, TBA was carried out to determine whether the effect in the cell growth assays reflected inhibition of proliferation or cell death induced by by bioactive compounds of MD. The OVCAR 4 cell line was selected for this assay as this cell line is among the top five most resistant human ovarian cancer cell lines which typifies very serious ovarian cancer and thus is a suitable model for the investigation of *in vitro* anti-ovarian cancer activities (Domcke et al., 2013).

BA was investigated at three concentrations singly and in combination with carboplatin. The observation from Figure 3.14 is that the antiproliferative activities of BA in the SRB are replicated in the trypan blue assay. Again, this activity is in a concentration-dependent manner, with the maximum concentration of 40µM exhibiting the most cytotoxicity. The effect of the combined use of BA and carboplatin on the cells were also investigated. The agents combined demonstrated significant synergism ($p<0.05$ for 40, 20 and 10µM BA) because all three concentrations elicited effects which exceeded that expected for the combination assuming additivity and calculated by the Bliss independence criterion. Furthermore, these results suggest potential synergism between BA and carboplatin.

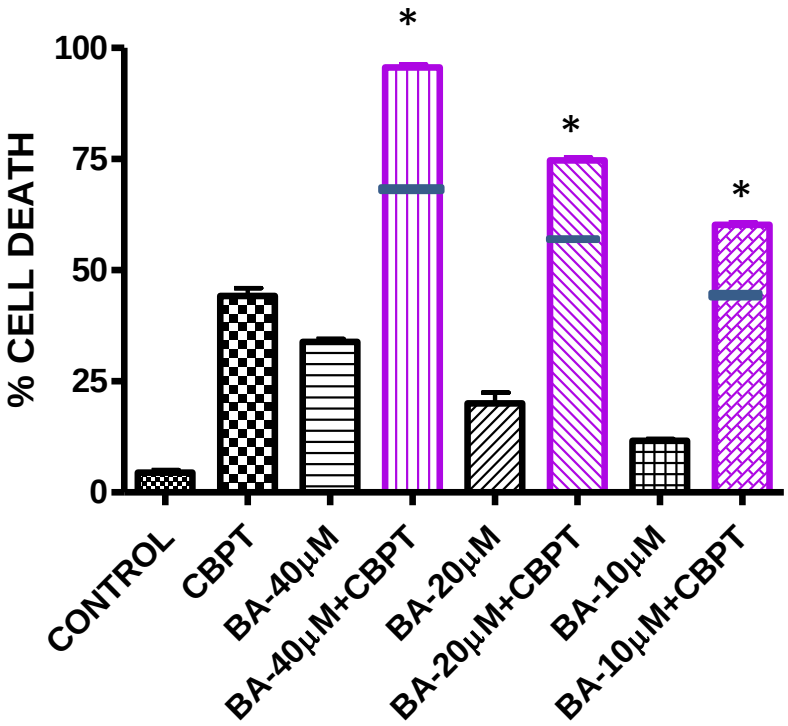



**Figure 3.14 The effect of betulinic acid (BA) at the indicated concentrations and in combination with carboplatin (CBPT), (40 µM) at 48h on cell viability. The cells were stained with trypan blue and the percentage of dead cells was obtained by microscopy. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. * denotes that the result is significantly different (P<0.05, paired t-test) from the effect expected from the Bliss independence criterion. The results were expressed as mean ±SD, n=3.**

Further studies to investigate the synergistic potentials in the growth inhibition activities of the drug combination between BA and carboplatin on OVCAR 4 cells were carried out (Figure 3.15).

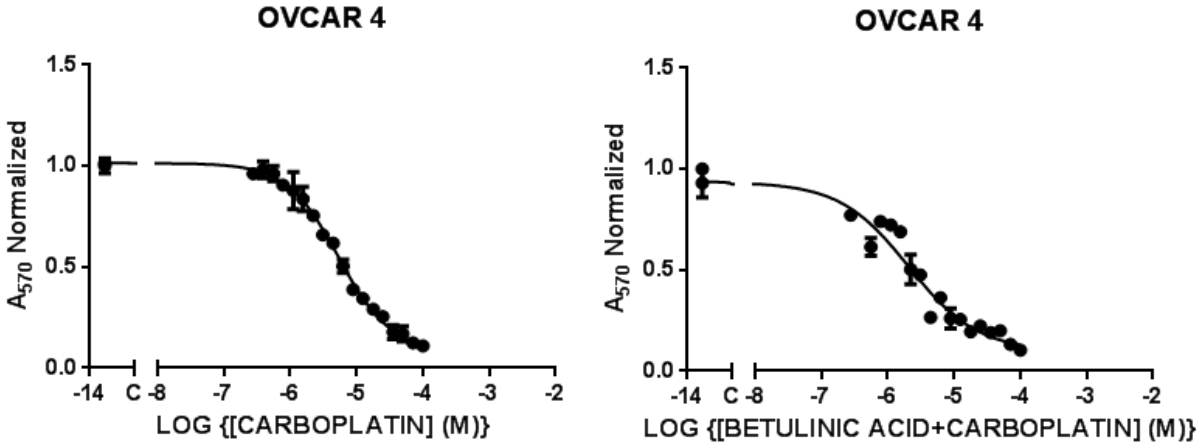



**Figure 3.15 Mean concentration-response curves of untreated cell carboplatin-treated cells (maximum concentration of 100 µM) and the drug combination of betulinic acid at a fixed concentration of 6 µM and Carboplatin (Maximum concentration of 100 µM) on the OVCAR 4 cell line determined after 72h of treatment. Results were expressed as mean of three experiments, Mean± SEM.**

The results contained in Figure 3.15, showed that the activity of carboplatin is increased when in combination with betulinic acid. This results revelaed a synergistic effect in the drug combination of carboplatin and betulinic acid, with a CI = 0.87±0.1, which was not significant from synergism) at $p<0.05$. This observation may be due to the 6µM fixed concentration of betulinic acid used. It was expected that at this concentration the compound would cause 5% growth inhibition of the cancer cells, judging from the activity of the compound from single agent studies. An alternative would be to repeat these studies with different betulinic acid concentrations to see if significant synergy is observed.

These results obtained from this present study suggest that betulinic acid could be used in chemoprevention and as a combinational agent for standard cancer drugs in the treatment of ovarian cancer.

## *3.8 The Results of Securinine TBA and Drug Combination Studies*

The growth inhibitory activities of securinine in cell growth assay was further assessed using the TBA to determine if these observed activities where due to the anti-proliferation or cell death induced by securinine (Figure 3.14).

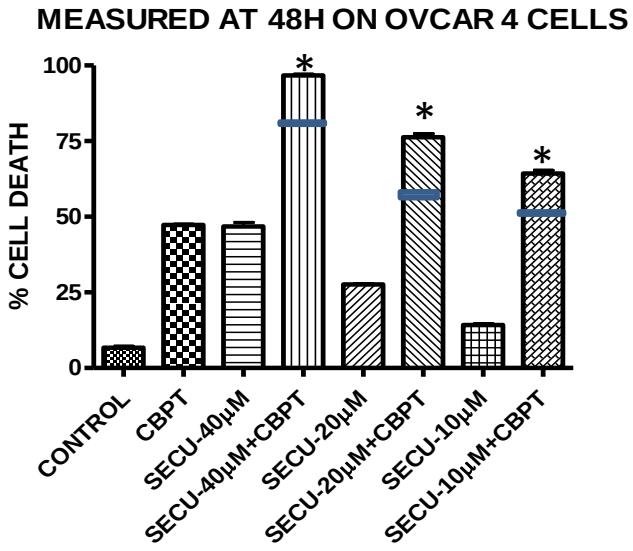



**Figure 3.16 The Effect of securinine (SECU) at the indicated concentrations and in combination with carboplatin (CBPT) (40 µM) at 48h on cell viability. The vehicle-treated cells served as the control. The cells were stained with trypan blue and the percentage of dead cells was obtained by microscopy. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. * denotes that the result is significantly different (P<0.05, paired t-test) from the effect expected from the Bliss independence criterion. The results were expressed as mean ±SD, n=3.**

The results are in consonance with the observed inhibitory activities of securinine in the SRB assay and in a concentration-dependent manner, with the maximum concentration of 40µM exhibiting the most activity. The effect of the combined use of securinine and carboplatin on the cells were also investigated. The agents combined demonstrated significant synergism (*p<0.05* for 40, 20 and 10µM securinine) as all three concentrations elicited effects which exceeded that expected for the combination assuming additivity and calculated by the Bliss independence criterion.

Further studies on the effect of drug combination between securinine and carboplatin on OVCAR 4 cells were carried out to investigate the synergistic potentials of securinine in increasing the growth inhibition activities of carboplatin (Figure 3.17).

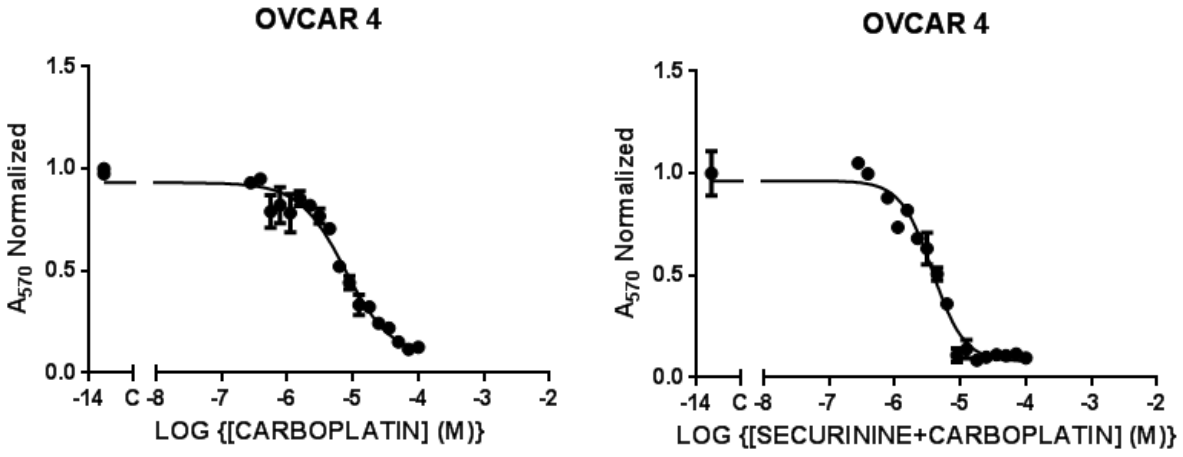



**Figure 3.17 Mean concentration-response curves of vehicle-treated cells, carboplatin-treated cells (Maximum concentration of 100 µM) and the drug combination of securinine at a fixed concentration of 6 µM and Carboplatin (Maximum concentration of 100 µM), on the OVCAR 4 cell line determined after 72h of treatment. The surviving cell number was estimated by staining with SRB. The results were expressed as mean of three experiments, Mean± SEM.**

The results showed that the activity of carboplatin was amplified by a combination of 6µM of securinine. The obtained CI for the drug combination of securinine, was 0.9±0.2 which indicates synergy but these results were not statistically significant from synergism at $p<0.05$. It is possible that at higher concentrations of securinine, significant synergy between securinine and carboplatin would be achieved.

## *3.9 Apoptosis studies of Securinine*

In order to investigate, the possible route of cell death induced by securinine, experiments were conducted to evaluate the activity of securinine on caspase 3/7. In this experiment, the effect of drug combination between securinine and carboplatin/paclitaxel respectively were further examined (Figure 3.18).

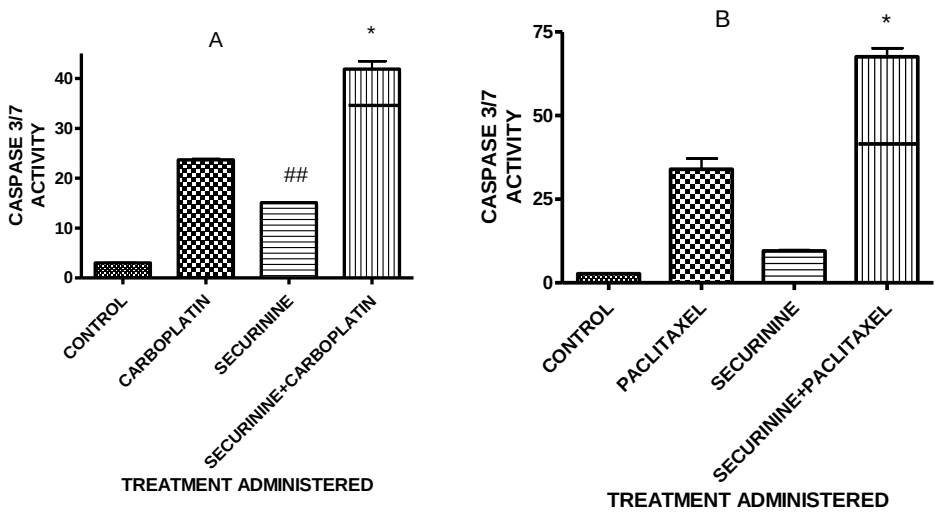



**Figure 3.18 The Effect of securinine (10 µM) combined with paclitaxel (10 nM) or carboplatin (10 µM), on Caspase 3/7 activity at 48h for carboplatin (A) combination and 36h for paclitaxel (B) combination. The caspase activity was measured and normalized with corresponding SRB-stained cells to estimate the surviving cell number. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. * denotes that the result is significantly different (P<0.05, paired t-test) from the effect expected from the Bliss independence criterion. While ## denotes significant difference (P<0.001) from the control, one-way ANOVA. The results were expressed as mean ±SD, n=3.**

The results demonstrated that securinine induced significant caspase activity at a concentration of 10µM with both carboplatin and paclitaxel. The combination of securinine and carboplatin (10µM) or paclitaxel (10nM), yielded an increase in caspases 3/7 activity which was greater than the bliss expected effect. A further comparison of the caspase activities of securinine and the vehicle-treated cells (control), showed that at 48h, securinine induced significant caspase activity *p<0.001* significance. Although, at 36h, the induced caspase activity of securinine was not statistically different from the control, however this is an indication that securinine induces significant apoptosis in cells after 36h of treatment at a concentration of 10µM.

Further investigation of the observed potential synergism between securinine and carboplatin was carried out by means of Annexin V/Propidium Iodide labelling, followed by flow cytometry analysis (Table 3.7, Figures3.19 and 3.20).

**Table 3-7 The Effect of Securinine and carboplatin Combinations was assessed (for the apoptotic cells) after 48h of treatment by Annexin V/PI Staining of the cells followed by flow cytometry analysis.** *denotes that the result is significantly different (*P<0.05, paired t-test*) from the effect expected from the Bliss independence criterion. The results are expressed as mean ±SD, n=3.

| TREATMENT | PERCENTAGE OF APOPTOTIC CELLS MEAN±S |
|---|---|
| CONTROL | 3.2±0.4 |
| CARBOPLATIN(20µM) | 14.4±1.1 |
| SECURININE(5µM) | 4.0±0.1 |
| CARBOPLATIN+SECURININE | 19.7±0.8* |

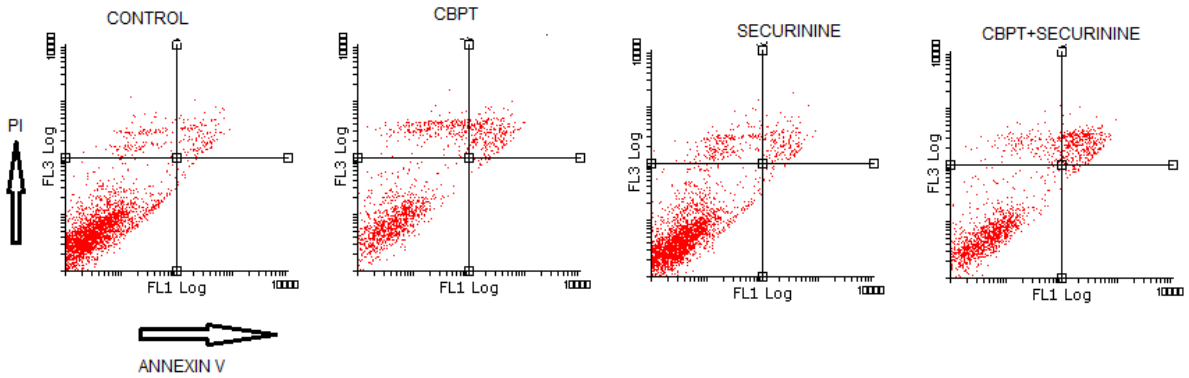



**Figure 3.19 The effect of Securinine (5 µM) in combination with carboplatin (20 µM) and the agents singly on OVCAR 4 cells treated for 48h was assessed by Annexin V/PI staining, followed by flow cytometry analysis. The vehicle-treated cells were used as the control. The results were expressed as a representative of three experiments.**

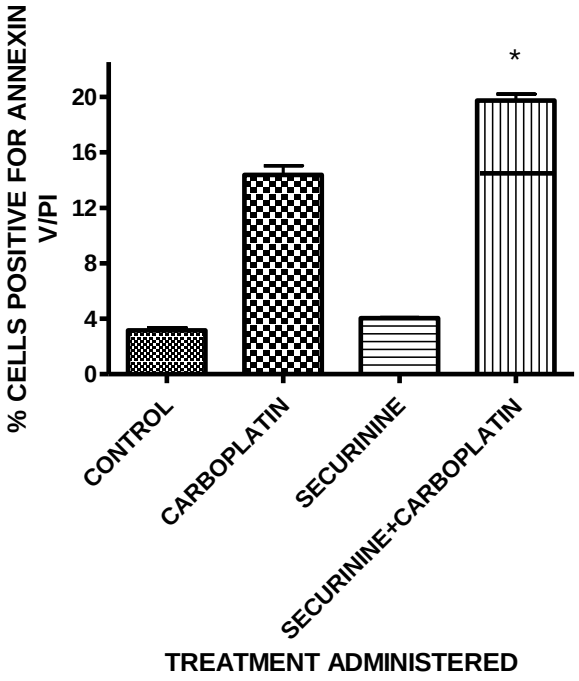



**Figure 3.20 The effect of securinine (5 µM) combined with carboplatin (20 µM), and the agents singly on Annexin V/PI staining on OVCAR 4 cells analysed by flow cytometry at 48h. A representation of the quantification of the combined early and late phase apoptotic cells is shown. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. *denotes that the result is significantly different (P<0.05, paired t-test) from the effect expected from the Bliss independence criterion. The results were expressed as mean ±SD, n=3.**

The results of the Annexin V/PI studies (Table 3.7 and the Figures 3.19 and 3.20), showed that securinine in combination with carboplatin led to increased apoptosis. The results further demonstrated a statistically significant synergism between securinine and carboplatin in the Annexin V /PI assay, by which **hypotheses b** and **c were tested**. This result further confirms the observed synergism in the TBA, drug combination experiments in the cell growth assays and in the caspase 3/7 assay. Again, these results had huge significance at $p<0.05$, in comparison with the calculated bliss expected effect.

## *3.10 Discussions*

The investigations of the anti-ovarian cancer activities of MD have been undertaken in this research work. The results showed that the organic extract of the plant had more potent inhibition than the aqueous extract. Further purification of the extract led to the isolation of betulinic acid and securinine. While the identities of gallic acid, cis-catechin and cis-gallocathecin where authenticated by the obtained standard compounds. Evaluation of the growth inhibition of these bioactive compounds was carried out and the activity of the compounds on the ovarian cancer cells showed that the compounds had a range of activity from 2.7-56.6µM, with securinine being the most potent compound. The results showed that gallic acid had notable antiproliferative activities on all the ovarian cancer cell lines with a SI>3. Similarly, the inhibitory effect of the leaf extract of *Toona sinensis*, of which the major bioactive compound is gallic acid has been, demonstrated in a panel of human oral squamous carcinoma cell lines. The study indicated that gallic acid may be responsible for the observed anti-cancer activities of *Toona sinensis* (Chia et al., 2010). The inhibitory activities of gallic acid has been observed in cervical, gastric and prostate cancer (Komal et al., 2008; Bing et al., 2013; Hsieh-Hsun et al., 2013). An in-depth *in vitro* and *in vivo* study demonstrated significantly the apoptosis-inducing activities of gallic acid with indications that gallic acid could be exploited as a chemopreventive agent (Sharad et al., 2013). The results of this present study suggest that gallic acid, a phenolic acid which is found in many plants and is used as an anti-oxidant food supplement possess antiproliferative activities against cancer cells with very minimal cytotoxicity towards normal cells and thus would potentially be useful in the treatment and prevention of ovarian cancer. Further investigations of gallic acid are presented in chapter 4.

Betulinic acid, a naturally occurring pentacyclic triterpenoid which was first isolated from the stem bark of *Melaleuca cajuput* in 1995, is a well-known anti-cancer agent, with significant cytotoxicity recorded on several human cancer cell lines which include, brain tumours, breast, colon, melanoma, prostate, head and neck, colorectal, lungs, ovarian, cervical

cancers and leukaemia (Alakurtti et al., 2006; Fulda 2008; De Melo et al., 2009; Bache et al., 2011). The results obtained from this study also demonstrate that betulinic acid is a potent inhibitor of ovarian cancer *in vitro* and is a promising candidate for cancer therapeutics. The agent was previously evaluated in a Phase I/II clinical trial of twenty-eight participants for the treatment of high risk dysplastic nevi; a condition indicative of skin cancer. The trial was assigned an identification number: NCT00346502, (Udeani 2010). Further updates of the outcome of this trial are not currently available.

Drug combinations between betulinic acid, BA and carboplatin were carried out in this study. The results suggest potential synergy between the agents. However this result was not significant from unity, which may be due to the concentration of BA used in the study. Similar drug combination studies involving BA and 5-Fluorouracil on OVCAR 432 ovarian cancer cells, reported synergism between the agents in western blot and flow cytometry experiments (Ying-Jian et al., 2015). These researchers also observed the involvement of caspase in the MOA of this combination with a significant increase in apoptotic cells. Again, this finding was further correlated by another study of BA and other cancer drugs by Fulda et al., in 2005, with observations of caspase activation and increased apoptosis (Fulda et al., 2005). The role of BA as an apoptosis inducer has been investigated on melanoma and neuro-ectodermal tumour cells. The investigators also observed an improvement in the treatment of tumours by radiotherapy in hypoxic conditions with the administration of BA (Vadivelu et al., 2012).

The green catechins, a group of poly-phenolic compounds have long being recognised for their vast health benefits mainly due to their anti-oxidants and free radical scavenging properties, with studies indicating that the most abundant compound (-)-epigallocatechin gallate (EGCG) is the most bioactive. In this study two catechins; (-)-catechin or cis-catechin and (-)-gallocatechin or cis-gallocatechin were identified and investigated. The results presented in Table 3.7 showed that the (-)-gallocatechin was more bioactive than (-)-catechin. This result is further strengthened by the findings of other researchers who

reported that (-)-gallocatechin gallate was the next more active catechin after EGCG in the inhibition of the growth/attachment of *Porphyromonas gingivalis* on the epithelial cells of the buccal cavity. The same investigators equally observed that (+)-catechin and (+)-gallocatechin had lesser activities (Sakanaka et al., 1996). Studies on the synergism between catechins and anti-cancer drugs have been undertaken. The results from a study of 42 *in vitro* and 13 xenograft mouse models drug combination experiments showed synergy between catechins and the standard treatment drugs (Hirota et al., 2015).

The most potent bioactive compound obtained from this study of MD is securinine. Recently, the anti-cancer properties of L-securinine (a designation assigned to securinine when compared with its optical isomer D-securinine), against colon cancer SW480 cell has been reported (Yong-hui et al., 2011). Securinine has also been shown to exhibit antiproliferative activities against acute myeloid leukaemia, AML both in *in vitro* and *in vivo* studies (Gupta et al., 2011).

Securinine's MOA was identified as a GABA-antagonist by the compound's ability to reverse GABA inhibition of cat spinal neurons. The compound stimulates the central nervous system and was used clinically for the treatment of neurological disorders in Russian pharmacopoeia in 1968, but was later deleted from subsequent editions (Raj et al., 2008). The anti-plasmodial activities against *plasmodium falciparum* has been reported, also the compound exhibited significant activities against *T. gondii* and has demonstrated anti-bacterial activities. Securinine's potential as a promising agent in the treatment of acute myeloid leukaemia (AML) has been evaluated (Gupta et al., 2011). Studies have shown that securinine is a pharmacologically important compound, owing to the numerous bioactivities, such as anti-malarial (Weenen et al., 1990) anti-bacterial (Mensah et al., 1990) and anti-Toxoplasma (Holmes et al., 2011) activities. The compound has previously being reported in *M. discoidea* obtained from Nigeria, and according to Bevan, the stembark of MD obtained from Nigeria, contains 0.2% securinine and minute quantities of allosecurinine and phyllanthine (Oliver-Bever 1986).The findings from this present research showed potential synergism between

securinine and carboplatin/paclitaxel in the caspase 3/7 experiments, which was similarly demonstrated in the drug combination of securinine and carboplatin evaluated in the trypan blue assay and in the Annexin V/PI labelling assay.

Similarly, observations of the inhibition activities of securinine on MCF-7 breast cancer cells in a time and dose dependent pattern have been documented. The results of the referenced study further observed that a concentration range of 0-40µM of securinine for 48h incubation resulted in a significant increase in the quantified apoptosis and an acute decline in the G1 phase of the cell cycle (Li et al., 2014). Also, a related study on the effect of securinine on Acute Myeloid Leukaemia (AML), showed that securinine synergised with ATRA and decitabine, standard AML drugs (Gupta et al., 2011).

Some researchers have reported that securinine exhibited selective cytotoxicity towards cancer cells by apoptosis through the p73-independent pathway (Rana et al., 2010), which was also demonstrated in the caspase 3/7 and Annexin V experiments carried out in this study.

These findings strengthen the observations of the anti-cancer activities of securinine. Furthermore, an evaluation of the confirmed GABA receptor antagonist MOA of several securinine-type alkaloids on mice induced with tonic seizures, showed a very high $IC_{50}$ of about 50µM, with a 7-fold decrease in the activity of securinine in comparison to bicuculline. A weak GABA antagonism was exhibited by securinine, according to the researchers (Beutler et al., 1985). It is most likely that the GABA antagonism exhibited by securinine may be as a secondary MOA, while the compound's main MOA is yet to be elucidated. The view that securinine is majorly a GABA receptor antagonist, has resulted in the mis-application of the drug as a CNS stimulant, and led to the drug's failure to deliver significant therapeutic benefits. This may be the reason while the use of securinine as a drug in the 1968, 10th Edition of the Russian Pharmacopeia, was discontinued, as the drug must have failed in its application as a CNS stimulant in the treatment of neurological diseases.

Subsequentlysecurinine nitrate was used as a stimulant and an anti-spasmodic in USSR. Again, the use of the drug was discontinued in the 1990s', possibly due to lack of therapeutic benefits (Chirkin et al., 2015). This is also the view shared by a team of American researchers who evaluated the potentials of securinine in the treatment of Amytropic Lateral Sclerosis, ALS in nine patients and found the drug of no effect. A quote from these researchers is shown below:

"*To the Editor-In December 1971, E. G. Dimond made reference to research in China on the use of securinine in the treatment of poliomyelitis (218:1552, 1971). Spurred by this reference, we obtained a small amount of the drug and treated one case of amyotropic lateral sclerosis (ALS) with securinine (Pa Med 76:36, 1973). Subsequently, with the permission of the Food and Drug Administration (IND 8929), the drug was administered to eight additional patients, affording us sufficient experience to report our impressions. We do not believe that securinine has any significant effect on the progression of ALS. Not one of our patients experienced a remission, nor did any of our patients show a substantial slowing of the rate of progression. In this respect, our experience probably is in agreement with what must have been the negative experience of the Russian investigators who discovered the drug and who make no reference*" (Copperman et al., 1974).

The conclusions from this present study are that securinine possess anti-cancer activities, with apoptosis as the route of cell killing. The compound has demonstrated very significant synergism with carboplatin, a standard drug in the treatment of ovarian cancer and also with paclitaxel another first-line ovarian cancer drug. The potency of securinine is similar to that of carboplatin. Securinine does show preferential selective cytotoxicity for cancer cells, with less toxicity observed on normal cells *in vitro*. This compound is the lead bioactive compound in *Margaritaria discoidea*.

It may be suggested that the confirmed anti-cancer activities of MD could be as a result of these identified bioactive compounds. The anti-inflammatory activity of MD has been

documented and the acute toxicity of the plant was also investigated in an *in vivo* study. The researchers observed a lack of acute toxicity in the oral administration of 3200mg/kg of the aqueous extract in rats (Adedapo et al., 2009). This finding is of immense significance as the plant is usually taken as a decoction in ethno medicine. Again, the investigated bioactive compounds, all displayed selective inhibition activities towards cancer cells in comparison to normal cells.

## *3.11 Conclusion*

In conclusion, an investigation of the anti-cancer activities of *Margaritaria discoidea*, MD, on ovarian cancer cell lines has been carried out. Previous studies have investigated the anti-inflammatory and the analgesic effect of the aqueous extract of the stem back of *M. discoidea*in rats. The results of this referenced study, observed significant activities comparable with the effect of indomethacin, the standard drug employed in the study (Adedapo et al., 2009).

This present study showed that the organic extract had the notable anti-cancer activity. Furthermore the bioassay-guided fractionation of this extract showed that the ethyl acetate fraction had the most potency. The identified/isolated bioactive compounds of this fraction were securinine, gallic acid, betulinic acid, (-)-catechin and (-)-gallocatechin presented in the order of decreasing potency. The observed cytotoxic activities of these compounds were further compared with the observations of other researchers, which validated these finding. The lead compound in MD is securinine and the results of these studies have demonstrated that securinine is a promising agent for ovarian cancer treatment. The agent merits further investigation into the anti-cancer MOA of this pharmacologically important compound, which would guide the synthesis of more-potent securinine analogues.

Furthermore, this study justifies and intensifies the necessity to revisit the libraries of drugs that are deemed to have failed in a particular activity evaluated. It is hoped that such drugs for instance, securinine would be found of immense therapeutic use for other activities.

Finally, this is the first report of the anti-cancer activities of *Margaritaria discoidea*, a plant in the Phyllanthaceae family, which could be used both as a chemopreventive decoction and in the treatment of cancer.

# Chapter Four

## *Anti-Ovarian Cancer Activities of Acalypha Wilkesiana Müll. Arg.*

## *4.1 Introduction*

*Acalypha wilkesiana* Müll. Arg. (AW) popularly called copperleaf, Jacob's coat and Fire dragon is a perennial plant belonging to the Euphorbiaceae family. This plant specie has several cultivars, such as Godseffiana, Macrophylla, Marginata, Obovata cristata and Hoffmannii. The genus *Acalypha* has 450-570 species, widely distributed in tropical climates. *Acalypha* species have been utilised in ethno medicine by indigenous communities. Literature of the pharmacological studies of this genus showed that investigations have been carried out in about forty species, with a focus on the anti-microbial and anti-malarial activities of the species (Seebaluck et al. 2015). Phytochemical investigations have also been carried out in some species, with the identification of bioactive compounds. There are apparently very few anti-cancer studies carried out on many of the species. Literature search revealed that there are five taxonomically identified species of *Acalypha* native to Nigeria; *A. alnifolia*Klein ex Willd. (*A. capitata* Willd.), *A. fimbriata*Schumach.&Thonn*., A. torta* Pax & K. Hoffm., *A. racemosa* Wall. Ex Baill.and*A. wilkesiana* Mull. Arg. (AW). Documented evidence has shown that AW has been employed in the indigenous treatment of breast tumours in the western parts of Nigeria (Quds et al., 2012). The ethyl acetate extract was shown to induce apoptosis by causing DNA breaks in human glioma and lung carcinoma cells (Lim et al., 2011).There are scanty data on the anti-cancer activities of AW, and demonstrated studies that have identified the active principles responsible for the observed anti-cancer activities of AW. Hence this research work on the investigation of the anti-ovarian cancer activities of AW was undertaken.

The research in this chapter aims to investigate the anti-cancer activities of AW on ovarian cancer cell lines. Through bioassay-guided fraction, the active compounds would be isolated and characterised. Further growth inhibition evaluation on cell-growth assay would be carried

out to validate the antiproliferative activities of the bioactive compounds. Selectivity index of the bioactive compounds with optimum activities would be undertaken. Preliminary SAR studies of the derivatives of the obtained bioactive compound would be carried out in order to guide future synthesis of more potent analogues and determine the anti-cancer activities of derivative compounds with potential therapeutic application in chemotherapy.

## *4.2 Results and Discussion*

### 4.2.1 Extraction of Plant Materials

The pulverised leaves (inclusive of flowers and seeds) of AW (Marginata) were extracted using a combination of dichloromethane and methanol to obtain the organic extract, followed by extraction with deionised water to yield the aqueous extract, according to the procedures (Figure 2.1) used by National Cancer Institute (NCI, USA). The amounts of the extracts and their yields obtained are shown in Table 4.1

**Table 4-1 Percentage Yield of the AW Extracts obtained from the extraction with dichloromethane/methanol (organic extract) and extraction with deionized water (aqueous extract).**

| SAMPLE AMOUNT (g) | ORGANIC EXTRACT (g) | AQUEOUS EXTRACT (g) | % YIELD OF TOTAL EXTRACT OF PLANT |
|---|---|---|---|
| **1000** | 12.5 | 2.0 | 1.5 |

From the data in Table 4.1, it was observed, that the leaves yielded relatively low amounts of solid extract. But in comparison with the yields from the stembark of MD and RP repectively, this yield of 1.5% was much. Also, the organic extract is more than the aqueous extract. This may be due to the method of extraction, which was sequential; organic extraction, followed by aqueous extraction.

### 4.2.2 Screening of anti-ovarian cancer activity of the plant extracts

The organic and aqueous extracts of AW were screened for their inhibition of cell growth on OVCAR4, OVCAR8 and A2780 ovarian cancer cell lines using a cell growth assay in which cell number was estimated by staining with SRB. The results of the $IC_{50}$ values of the preliminary anti-cancer investigation of the plant extracts are presented in Table 4.2.

**Table 4-2 Results of Preliminary Screening of the Extracts of AW on ovarian cancer cell lines in cell growth assays.** The cells were treated for 72h with the indicated extract, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments

| **Extract** | $IC_{50}$ in µg/mL | | |
|---|---|---|---|
| | **A2780** | **OVCAR 8** | **OVCAR 4** |
| Organic Extract | 35.4±1.5 | 42.6±1.9 | 46.7±1.8 |
| Aqueous Extract | 54.7±2.2 | 150.7±3.5 | 73.9±1.3 |

The results presented in Table 4.2 showed that AW extracts caused growth inhibition on the three ovarian cancer cell lines tested. The organic extract was more potent than the aqueous extract on all the tested cell lines. An important observation was that the $IC_{50}$ of the organic extract was <50µg/mL on all three ovarian cancer cell lines. This $IC_{50}$ is below the threshold which is considered to be significant from the perspective of the American national Cancer institute criteria, which from a screening of about 20,000 plant extracts, identified 452 cytotoxic extracts with $IC_{50}$<50µg/mL(Thomas 2010). Thus, the organic extract of AW with $IC_{50}$s <50µg/mL warranted further purification and investigation for anti-cancer activity. Ethnopharmacological data showed that AW has been employed as an essential ingredient in a complex herbal preparation used in the indigenous treatment of breast tumour in the western part of Nigeria (Ikewuchi et al., 2010; 2013). The antiproliferative activity of AW has been reported (Madlener et al., 2009).

### 4.2.3 Bioassay-guided fractionation of AW

Further solvent fractionation of 5.0 g of the crude organic extract of AW was carried out, by the sequential solvent partitioning of the organic extract in n-hexane, ethyl acetate, n-butanol and water, as described in the scheme presented in Figure 2.1 in the Materials and Methods chapter. The following yields were obtained (mass of solid compound recovered is shown in parentheses): n-hexane (2.5g), ethyl acetate (1.0g), n-butanol (0.7g) and aqueous (0.5g) fractions respectively. These fractions were investigated for growth inhibition on the OVCAR4 cell line (Table 4.3).

**Table 4-3 The results of the evaluation of the growth inhibition of the fractions of AW on OVCAR 4 cell line in cell growth assays.** The cells were treated for 72h with the indicated fraction, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments**.**

| FRACTION | ABBREV. | $IC_{50}$ (µg/ml) |
|---|---|---|
| N-HEXANE | N-HEX | 95.2±5.6 |
| ETHYL ACETATE | EA | 28.9±1.9 |
| N-BUTANOL | N-BUT | 111.0±12.2 |
| AQUEOUS | AQ | 84.9±7.6 |

The observations from the results were that the ethyl acetate fraction had the most significant antiproliferative activities on the OVCAR 4 cell line. The aqueous, the n-hexane and the n-butanol fractions had moderate activities. Similar findings have been observed from previous studies, on the anti-microbial activities of the methanol extract and the obtained fractions of AW. The researchers reported that the ethyl acetate fraction had potent inhibitory activities on the bacteria and fungi tested than the other fractions (Haruna et al., 2013). Another study observed that the ethyl acetate extract of AW showed significant inhibitory activities on A549

human lung carcinoma and U87MG human brain glioblastoma with minimal inhibitory effects on the MRC5 normal human lung fibroblast (Lim et al., 2011).

### 4.2.4 Chemical characterization of AW

The TMSi derivatives of the organic extract, the aqueous extract and ethyl acetate fraction were prepared and analysed by GC-MS. The TMSi derivatives of the extracts and fraction of AW were obtained by the treatment of BSTFA and pyridine and subjected to further analysis on the GC-MS to determine the potential bioactive compounds that could be responsible for the observed activities. The GC-MS chromatograms of the TMSi derivatives gave a number of compounds, with the most compounds detected in the organic extract. A significant finding was the presence of gallic acid in the derivatized organic and aqueous extracts and in the ethyl acetate fraction. This observation suggests that the activity of the aqueous extract in this study is largely due to the presence of gallic acid.

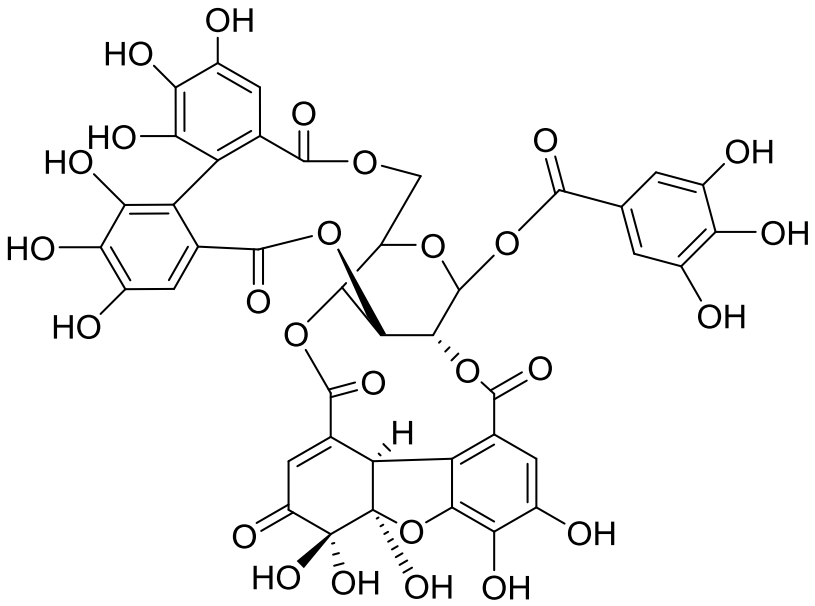
The GC chromatogram of the ethyl acetate fraction of AW displayed a large number of compounds. Twelve compounds with a >80% quality match with the NIST library were detected. These compounds are shown in Table 4.4.

Three of the twelve identified compounds had a match quality of 99%. These were gallic acid, protocatechuic acid (PCA), and shikimic acid. Shikimic acid and PCA are compounds produced in the biosynthesis of gallic acid. Previous studies have documented the presence of gallic acid, together with geraniin and corilagin (Figure 4.1) as the bioactive compounds present in AW (Adesina et al., 2000). The anti-cancer activities of these other two compounds have been reported in literature, with investigations of corilagin carried out on SKOv3ip Hey and HO-8910PM ovarian cancer cell lines. The documented $IC_{50}$ value on these cell lines for corilagin was <30µM (Jia et al., 2013). In a related study, geraniin has demonstrated anti-cancer activities on breast cancer MCF-7 cells with the observation of a range of $IC_{50}$s from 9.9-42.3 µM in a time-dependent manner (Zhai et al., 2016).

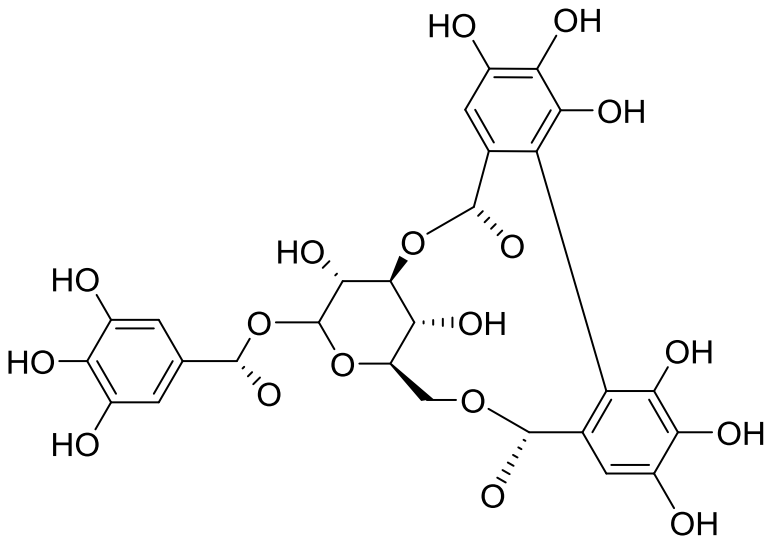
**Table 4-4 TMSi derivatives detected in the ethyl acetate fraction of AW by GC-MS.**

| PEAK | RETENTION TIME (MIN) | TMSi DERIVATIVE | PARENT MOLECULES IN PLANT | MATCH QUALITY |
|---|---|---|---|---|
| **1** | 3.43 | Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester | Butanedioic acid | 96 |
| **2** | 5.99 | 1-Cyclohexene-1-carboxylic acid, 3,4,5-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester, [3R-(3.alpha.,4.alpha.,5.beta.)]- | Shikimic acid | 99 |
| **3** | 6.11 | D-(-)-Fructofuranose, pentakis(trimethylsilyl) ether (isomer 1) | D-(-)-Fructofuranose | 89 |
| **4** | 6.16 | Benzoic acid, 3,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester | Protocatechuic acid | 99 |
| **5** | 6.29 | D-(-)-Fructopyranose, pentakis(trimethylsilyl) ether (isomer 1) | D-(-)-Fructopyranose | 93 |
| **6** | 6.71 | D-Pinitol, pentakis(trimethylsilyl) ether | D-Pinitol | 90 |
| **7** | 6.95 | .beta.-D-Glucopyranose, 1,2,3,4,6-pentakis-O- | .beta.-D-Glucopyranose | 95 |

| | | | | |
|---|---|---|---|---|
| | | (trimethylsilyl)- | | |
| **8** | 7.46 | **Benzoic acid, 3,4,5-tris(trimethylsiloxy)-, trimethylsilyl ester** | **Gallic acid** | 99 |
| **9** | 7.62 | Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-, D-chiro- | Inositol | 97 |
| **10** | 7.72 | Glucopyranose, pentakis-O-trimethylsilyl- | Glucopyranose | 93 |
| **11** | 12.86 | Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester | Hexadecanoic acid | 91 |
| **12** | 13.69 | alpha-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)- | alpha-D-Glucopyranoside | 94 |




Geraniin




Corilagin

**Figure 4.1 The chemical structures of two previously identified bioactive compounds in AW (Adesina et al., 2000).**

The anti-cancer activities of PCA have been reported on human leukaemia cells (Babich et al., 2002). Gallic acid has demonstrated significant antiproliferative activities on the A549 human lung adenocarcinoma *in vitro* in a dose-dependent manner at an experimental time of 24h. Further investigations showed that the compound induced the activation of caspase 3; a strong indication of apoptosis (Dharmendra et al., 2011).

### 4.2.5 Bioassay-Guided Fractionation of Ethyl Acetate fraction of AW

Further bioassay-guided fractionation of the ethyl acetate (EA) fraction of AW was carried out. A trial of three different solvent combinations was first undertaken on TLC to ascertain the most suitable solvent system for the silica gel column fractionation of the EA fraction. Upon TLC of the $CHCl_3$: Methanol (10:1), 2 spots were visualised on the TLC plate. While for EA: n-hexane (2:1) solvent systems, 4 spots were detected. Then for EA: n-hexane (1:1) solvent system, 7 spots were observed. Eighteen sub-fractions were obtained from a solvent-polarity based fractionation which utilised various combinations of n-hexane/ethyl acetate. The fractionation began with a 100% n-hexane and ended with 100% methanol. TLC of the collected sub-fractions showed that some sub-fractions had identical TLC characteristics, and thus these sub-fractions were bulked together resulting in eight sub-fractions. The obtained sub-fractions were evaluated for cell growth inhibition on OVCAR 4 cell line (Table 4.5).

**Table 4-5 The Results of the evaluation of the growth inhibition activity of the Sub-Fractions of the ethyl acetate fraction of AW on OVCAR 4 cell line in cell growth assays.** The cells were treated for 72h with the indicated fraction, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ± SEM, n=3 experiments.

| Sub-fractions of EA | OVCAR4 cell line (µg/ml) |
|---|---|
| **F1** | >200 |
| **F2** | 34.5 ±1.8 |
| **F3** | **15.7±0.1** |
| **F4** | 50.7±3.9 |
| **F5** | 47.2±6.8 |
| **F6** | **17.8±1.8** |
| **F7** | >200 |
| **F8** | >200 |

The results of the cell growth inhibition evaluation of the obtained sub-fractions showed that sub-fractions 3 (F3) and 6 (F6) were potent with significant inhibitory activities on the OVCAR4 cell line. TMSi derivatization of F3 was carried out to detect the possible bioactive compounds in that sub-fraction. The detection of eleven compounds with a match quality of >80% was made. Some of these compounds were also present in the parent ethyl acetate fraction. The GC chromatogram of the EA fraction 3 is shown in Figure 4.2.

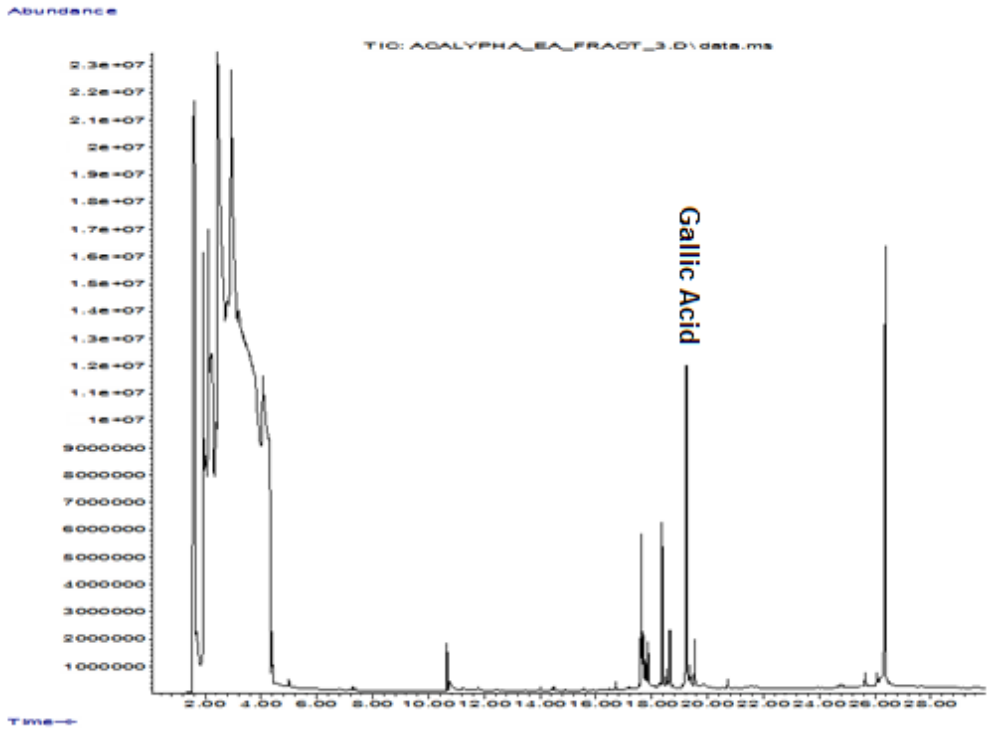



**Figure 4.2 The GC-MS chromatogram of the TMSi derivatized ethyl acetate fraction F3, showing several peaks, with the gallic acid peak highlighted.**

The MS of gallic acid in the fraction and the corresponding spectrum of standard gallic acid in the NIST library are presented in Figure 4.3.The detected compounds are listed in Table 4.6.

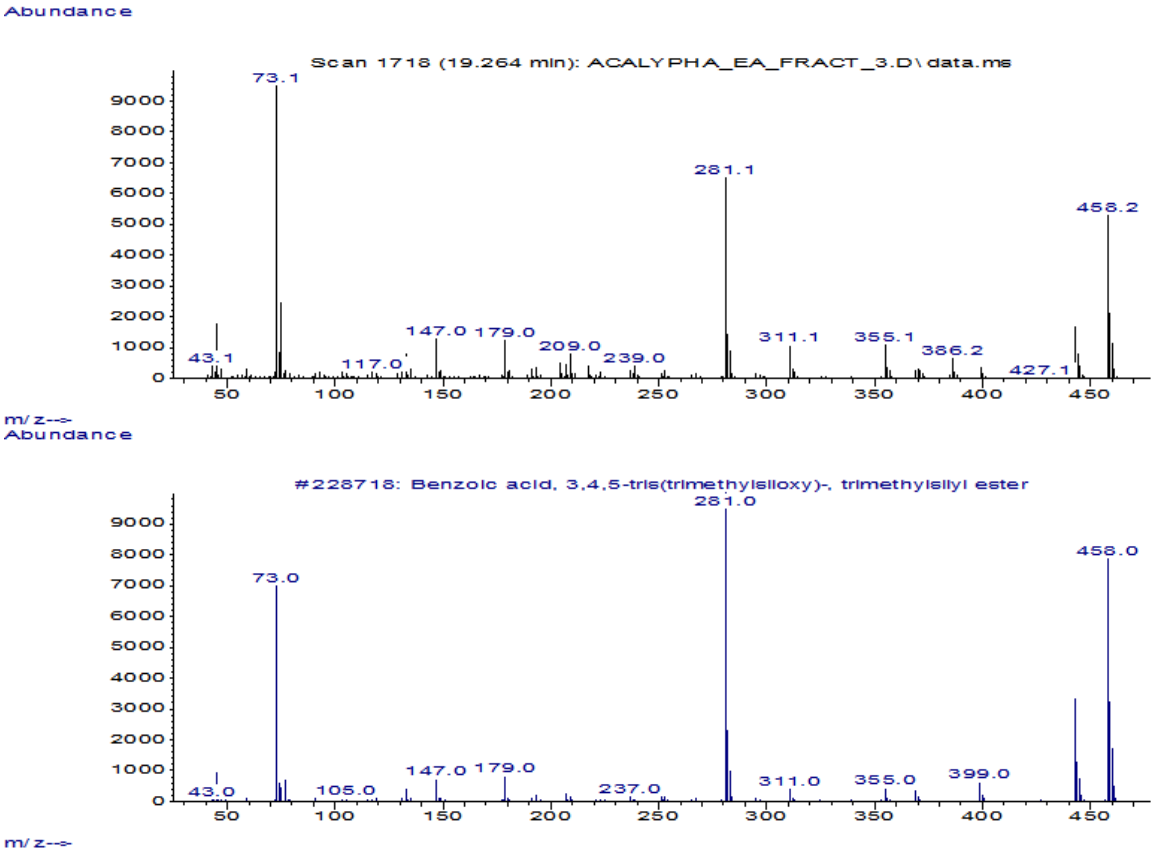



**Figure 4.3 The mass spectrum of the derivatized gallic acid contained in ethyl acetate fraction F3 (above) and the spectrum of gallic acid obtained from the NIST Library of the GC-MS (below).**

**Table 4-6 TMSi derivatives of Compounds detected in F3 sub-fraction of ethyl acetate fraction of AW by GC-MS.**

| PEAK | RETENTION TIME (MIN) | TMSi DERIVATIVE | PARENT MOLECULES IN PLANT | MATCH QUALITY | AREA OF PEAK (%) (N=3) |
|---|---|---|---|---|---|
| **1** | 10.67 | Glycerol, tris(trimethylsilyl) ether | GLYCEROL | 90 | 3.2±0.5 |
| **2** | 17.63 | 1-Cyclohexene-1-carboxylic acid, 3,4,5-tris[(trimethylsilyl)oxy]-, trimethylsilyl ester, [3R-(3.alpha.,4.alpha.,5.beta.)]- | SHIKIMIC ACID | 99 | 14.5±1.5 |
| **3** | 17.76 | D-Psicofuranose, pentakis(trimethylsilyl) ether (isomer 1) | D-PSICOFURANOSE | 91 | 3.4±0.8 |
| **4** | 17.87 | D-(-)-Fructopyranose, pentakis(trimethylsilyl) ether (isomer 1) | D-(-)-FRUCTOPYRANOSE | 95 | 5.1±0.6 |
| **5** | 18.39 | D-Pinitol pentakis(trimethylsilyl) ether | D-PINITOL | 90 | 12.5±1.1 |
| **6** | 18.65 | .beta.-D-Glucopyranose, 1,2,3,4,6-pentakis-O- | BETA-D- | 94 | 4.6±0.4 |

| | | | | | |
|---|---|---|---|---|---|
| | | (trimethylsilyl)- | GLUCOPYRANOSE | | |
| **7** | 19.24 | Benzoic acid, 3,4,5-tris(trimethylsiloxy)-, trimethylsilyl ester | GALLIC ACID | 99 | 28.7±1.2 |
| **8** | 19.36 | Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-, D-chiro- | INOSITOL | 87 | 2.7±0.2 |
| **9** | 19.53 | Glucopyranose, pentakis-O-trimethylsilyl- | GLUCOPYRANOSE | 95 | 4.2±0.4 |
| **10** | 20.71 | Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)- | MYO-INOSITOL | 95 | 0.8±0.1 |
| **11** | 25.66 | alpha-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)- | ALPHA-D-GLUCOPYRANOSIDE | 91 | 1.4±0.3 |

The prime compounds gallic acid and shikimic acid were among the detected compounds in the F3 sub-fraction of ethyl acetate fraction of AW. Again these compounds were detected with a match quality of 99%.

A calculation of the peak area was made and used as a measurement of the amount of the compounds present in the fraction. This quantification showed that gallic acid was 28.7% and shikimic acid was 14.5%. These two compounds were the most abundant compounds contained in the F3 sub-fraction of the ethyl acetate fraction of AW. This finding strongly suggests that the significant anti-cancer activity of AW is attributable to the vast amount of gallic acid contained in the plant. This finding could be exploited in the commercial sourcing of natural gallic acid for both food and pharmaceutical industries. TMSi derivatization of Fraction 6 was carried out and analysed on the GC-MS, but no significant compound was detected. Fraction 6 was subjected to hplc purification, but effective separation of the compounds contained in the fraction could not be achieved as there were too many peaks with very close retention times. Given the insufficient amount of the fraction, further investigation of this fraction was discontinued. In order to identify the potential bioactive compounds in F3, further purification of F3 was carried out by semi-prep HPLC on the C-18 column with a mobile phase composition of 100% water acidified with 0.1% Trifluoroacetic acid (TFA) as solvent A and 80% acetonitrile acidified with 0.1% TFA as solvent B. The gradient began with 100% of A for 5 min and increased to 100% B for 25 min of the run time. and maintained for 5 min of the analysis; making the total run time 35 min.

Ten hplc-fractions were isolated upon separation and these hplc fractions were further investigated for inhibitory activities on OVCAR 4 cell line (Table 4.7).

**Table 4-7 The Results of the evaluation of the growth inhibition of the HPLC fractions of F3 on the OVCAR 4 cell line in cell growth assays.** The cells were treated for 72h with the indicated fraction, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments.

| HPLC FRACTIONS OF F3 | Retention time (min) | $IC_{50}$ (µg/ml) |
|---|---|---|
| **1** | 2.2-2.4 | 83.3±6.2 |
| **2** | 2.4-2.5 | 38.0±2.5 |
| **3** | 2.5-2.7 | 14.9±0.4 |
| **4** | 2.7-2.8 | 31.9±4.9 |
| **5** | **2.8-3.4** | **10.9±0.7** |
| **6** | 7.4-9.2 | 54.4±4.2 |
| **7** | 10.9-11.3 | 49.6±1.9 |
| **8** | 11.4-12.3 | 29.8±4.6 |
| **9** | **12.4-13.0** | **11.9±0.9** |
| **10** | 18.8-19.4 | 48.1±7.8 |

The results of the screening of the HPLC fractions of F3 presented in Table 4.7 showed that the fraction 5 at 2.8-3.4 min was the most potent fraction. HPLC fraction 5 was a white amorphous powder. Documented phytochemical studies of the red-*Acalypha*, a cultivar of AW (closely-related to the Marginata cultivar used in this study), recorded the presence of gallic acid and geraniin from n-butanol fraction, obtained from an aqueous ethanol extract. The fractionation procedure, used by these researchers by-passed the use of ethyl acetate. The researchers further reported the effect of seasonal variation on the constituents of the plant. According to the study, AW collected in the month of July (Peak Rainy Season in Nigeria), contained corilagin. While AW collected in the month of November (Dry Season in Nigeria) contained gallic acid (Adesina et al., 2000). This finding may explain while the presence of corilagin was not detected in this current study of AW. The collection period of the plant materials utilised for this research was during the Dry season in Nigeria (December-March). The presence of geraniin, with an *m/z*of 952.64 could not be detected by the GC-

MS, as upon TMSi derivatization, the detectable *m/z*, would have been above the detection limit of the GC-MS. LC-MS is needed for identification of such compounds.

The HPLC fraction 5 was derivatized and analysed on the GC-MS using a modified program, with an increase start-off oven temperature. This was necessary in order to detect compounds which could not be detected at lower temperatures. The GC-MS, chromatogram (Figure 4.5) below showed a massive peak with a retention time of 7.43-7.48min and > 95% purity, corresponding to gallic acid. The presence of an unknown peak accounted for <3% of the isolated compound, while a minute amount of shikimic acid (<0.6%) was present in the isolated gallic acid obtained from the GC-MS analysis. The proton NMR analysis carried out in methanol-$d_4$ showed a single peak [δ (ppm) =7.04 (s, 2H)].$^{13}$C NMR ($CD_3OD$),[δ (ppm) = 170.51, 146.59, 139.85, 121.86, 110.20]. The NMR spectra were comparable with the reported spectra in literature for gallic acid, (Kamatham et al., 2015).Thus, hplc fraction 5 was unequivocally determined as gallic acid.

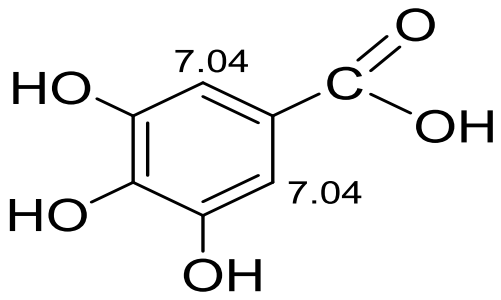



**Figure 4.4$^1$H NMR Assignment of HPLC fraction 5 (Gallic acid)**

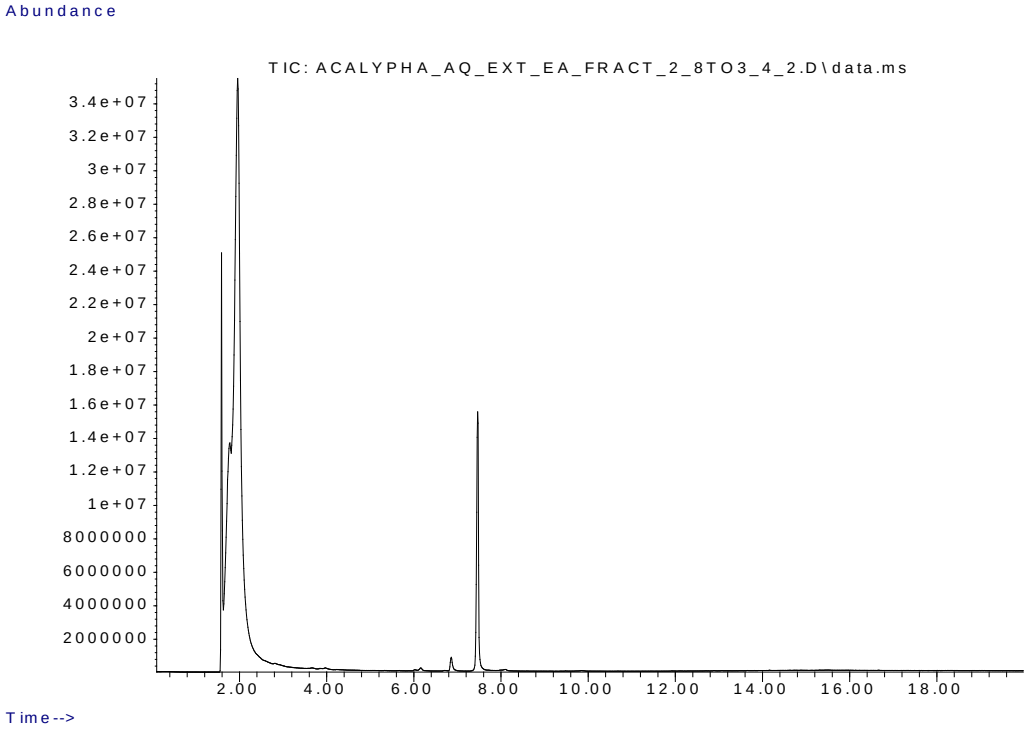



**Figure 4.5 GC-MS chromatograms of hplc fraction 5 (retention time 2.8-3.4) after TMSi derivation.**

The mean $IC_{50}$ curves of Acalypha EA-fraction, the sub-fraction F3 and the HPLC fraction 5 (retention time 2.8-3.4 min) are shown in Figure 4.6.The results showed that there is a progressive increase in the potency of the fractions from the Acalypha EA-fraction to HPLC fraction 5, with the purification of the sample. Further investigation into the selectivity index of HPLC fraction 5 was carried out by investigation of the growth inhibition of the compound on HOE (Table 4.8 and Figure 4.6).

**Table 4-8 The Mean $IC_{50}$ Values of HPLC Fraction 5 on OVCAR 4 and HOE Cell Lines, and the Calculated Selectivity Index (SI).** The results are expressed as Mean IC50 ± SEM, n=3.

| FRACTION | $IC_{50}$ OF OVCAR 4 (µg/ml) | $IC_{50}$ OF HOE (µg/ml) | SI |
|---|---|---|---|
| HPLC FRACTION 5 (gallic acid) | 10.9±0.7 | 39.3±1.6 | 3.6 |

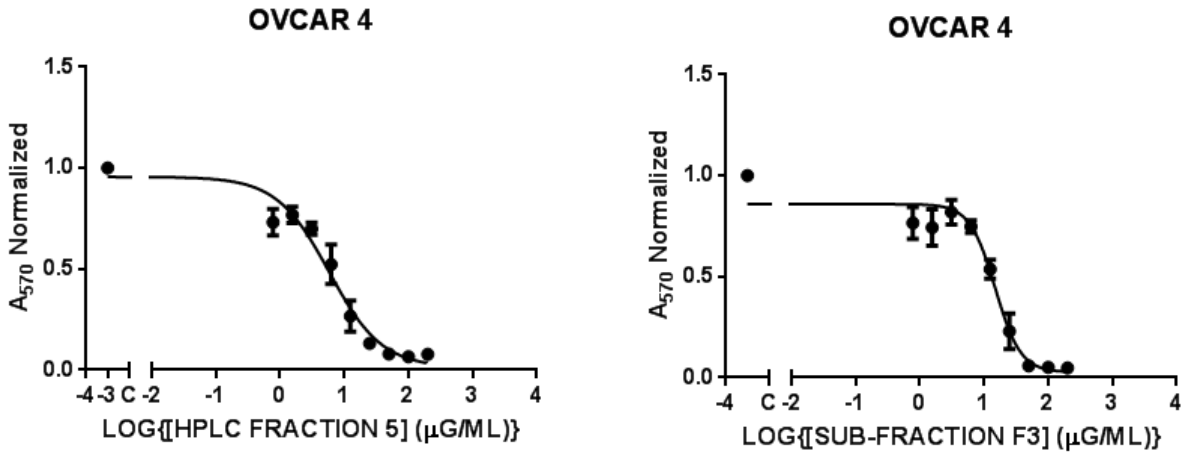



**Figure 4.6 The mean concentration-response curve of the ethyl-acetate fraction of AW, the sub-fraction of EA, F3 and the hplc fraction 5 on OVCAR 4 cell line. The cells were treated for 72h with the indicated compound, followed by an estimation of the number of surviving cells by SRB staining. The results were expressed as Mean $IC_{50}$± SEM, n=3.**

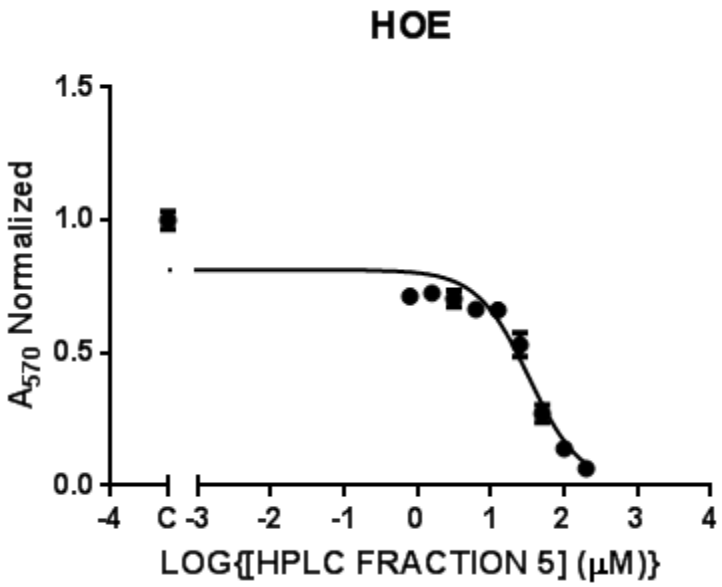



**Figure 4.7 The mean concentration-response curve of hplc fraction 5 (gallic acid) on the human ovarian epithelial cell line (HOE). The cells were treated for 72h with the indicated compound, followed by an estimation of the number of surviving cells by SRB staining. The results were expressed as Mean $IC_{50}$ ± SEM, n=3.**

The selectivity index of gallic acid (hplc fraction 5), was obtained as 3.6, which showed a very significant preferential selectivity in the killing of cancer cells, with minimal inhibition on normal cells. Similar observations have been reported by Lim and his co-researchers, from a study of the evaluation of the cytotoxicity of the ethyl acetate extract of AW on A549 lung

cancer cell line and the corresponding normal lung cell line, MRC5. The researchers observed potent antiproliferation on the cancer cells, without any significant inhibition on the normal cells (Lim et al., 2011).

### 4.2.6 Investigations of the Synergistic Potentials of Gallic Acid (GA) and Clinically Used Ovarian Cancer Drug

Further investigation to determine whether the effect in the cell growth assays reflected inhibition of proliferation or cell death was undertaken by TBA. Also, the synergistic potentials of gallic acid with carboplatin was investigated in this assay. The cells were plated in 12 well plates at a seeding density of 1 $x10^5$ cells/well. The cells were exposed to 40µM, 20µM and 10µM concentrations of gallic acid. 40µM Carboplatin was the reference standard, while the vehicle-treated cells were used as the control. A combination of 40µM Carboplatin and the three gallic acid concentrations was also carried out to investigate the combined effects of both compounds and an assessment of the viability of the cells was carried out after 48h incubation, by haemocymeter counting of the live and dead cells, pre-stained with trypan blue(Figure 4.8).

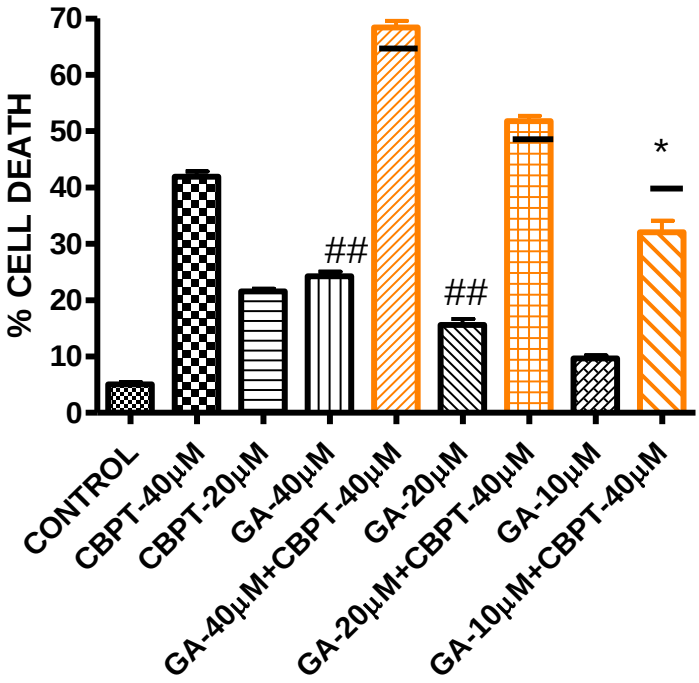



**Figure 4.8 The effect of gallic acid at the indicated concentrations and in combination with carboplatin (40 µM) at 48h on cell viability. The vehicle-treated cells served as the control. The cells were stained with trypan blue and the percentage of dead cells was obtained by microscopy. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. * denotes that the result is significantly different (P<0.05, paired t-test) from the effect expected from the Bliss independence criterion. While ## detones significant difference from the control (P<0.001, one-way ANOVA).**

The results of the trypan blue assay, TBA carried out for gallic acid, GA at concentrations; 10-40µM, and their respective combinations with carboplatin (40µM), (Figure 4.8) showed that the activity of GA was in a concentration-dependent manner, with the lowest concentration of 10µM exerting the least antiproliferative effect. The 40µM concentration of GA had increased activity than the standard drug, carboplatin (20µM).Similar results have been reported from an evaluation of the cytotoxicity of GA combined with flutamide on AR+ LNCaP human prostate cancer cells with the observation that the activity of 50µg/ml of gallic acid was significantly greater than the activity of the standard drug flutamide at a concentration of 25µM (Larry et al., 2011).In comparison to the control, the 40µM and 20µM concentrations had statistically significant *p<0.001* significance, while the 10µM didn't attain

a *p<0.05* significance which may be indicative of the effective concentration of gallic acid being above 10µM.

Further examination of the results of the combination of the different concentrations of gallic acid and carboplatin (40µM) showed that40µM and 20µM GA combinations with carboplatin had better activity than the single use of the agents alone. However, the results were not significant from that which would be expected for additivity with the bliss expected effect. It was observed from the results that the 10µM gallic acid combination with carboplatin did not attain the bliss expected effect criterion at *p<0.05*. The results demonstrate the possibility of antagonism between carboplatin and GA at the 10µM concentration.

Studies on the effect of drug combination between gallic acid and carboplatin on OVCAR 4 cells were carried out to investigate the synergistic potentials of gallic acid in increasing the growth inhibition activities of carboplatin by the fixed dose method (Figure 4.9).

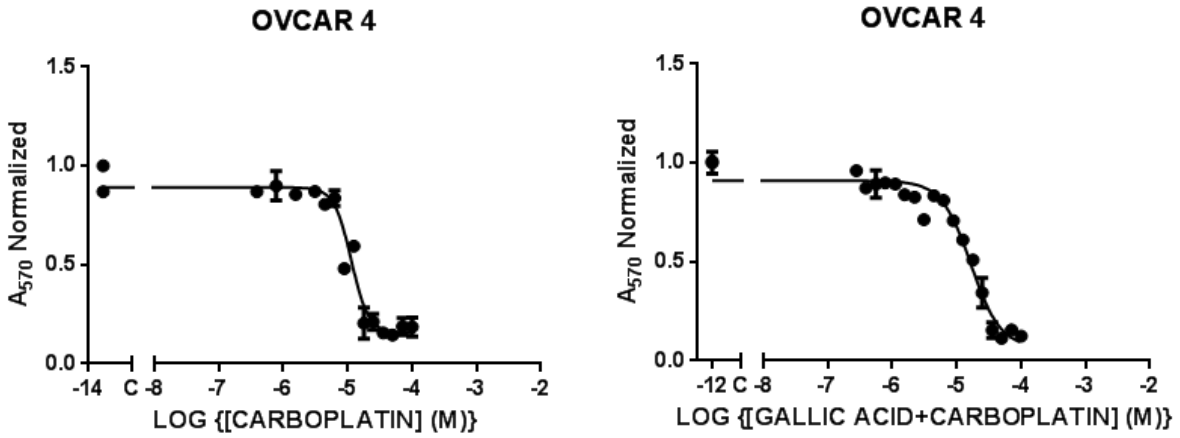



**Figure 4.9 Mean concentration-response curves of vehicle-treated cells carboplatin-treated cells (Maximum concentration of 100 µM) and the drug combination of gallic acid at a fixed concentration of 10 µM and Carboplatin (Maximum concentration of 100 µM), on the OVCAR 4 cell line determined after 72h of treatment. The surviving cell number was estimated by staining with SRB. The results were expressed as mean of three experiments, Mean± SEM.**

The results shown in Figure 4.9 revealed an antagonistic effect in the drug combination of 10µM of gallic acid and carboplatin. This result was statistically significant from antagonism at $p<0.05$. This observation may be due to the 10µM fixed concentration of gallic acid used which was expected that at this concentration the compound would cause 5% growth inhibition of the cancer cells, judging the activity of the compound from single agent studies. Importantly, this finding supports the observed antagonism shown in the TBA results of carboplatin and gallic acid at the 10µM concentration.

## *4.3 Discussions of Acalypha wilkesiana and Gallic Acid*

The anti-ovarian cancer activities of *A. wilkesiana*, AW were investigated in this research, by the screening of the extracts of the plant. The results (Table 4.2) showed that the organic extract had more potent inhibition activities than the aqueous extract. Other studies have documented anti-hypertensive and hypoglycaemic activities of the aqueous extract of AW (Ikewuchi et al., 2013), with no mention of an organic extract. Similarly, the ethanol extracts of AW has been shown to possess significant anti-microbial activities both from *in vitro*

Haruna et al., 2013) and *in vivo* (Oyelami et al., 2003; Chukwuemeka et al., 2013) studies. These documented researches, suggest that the active principles in AW are of moderate polarity, hence when the extraction is carried out by water or ethanol and methanol, the extracts have been found to be active. This finding thus justify the indigenous use of the water extract of AW in the treatment of neonatal jaundice, diabetes, skin infections and stomach problems (Soladoye et al., 2008; Iniaghe et al., 2013). However, when the plant is extracted sequentially by a polar organic solvent, followed by aqueous extraction, then the organic extract possesses a better activity than the aqueous extract, as seen in the results contained in Table 4.2 above.

Fractionation of the organic extract by solvent partitioning of the extract sequentially in n-hexane, ethyl acetate, n-butanol and methanol to obtain four fractions was carried out. The evaluation of these fractions on the ovarian cancer cell lines demonstrated that the ethyl acetate fraction had notable activities. GC-MS analysis of the Tmsi derivatized sample of the ethyl acetate fraction showed that gallic acid constituted 28.7% of the fraction in comparison to the other identified compounds. Further bioassay-guided purification carried out by both column chromatography and hplc, led to the isolation of gallic acid.

Gallic acid (Figure 4.10) is a phenolic acid, which belongs to the tannin class of compounds. The compound is widely distributed in many plants such as *Belamcanda chinensis* (Liu et al., 2012), *Boswellia dalzielii* (Alemika et al., 2007), *Camillia sinensis* (Zhu et al., 2013), *Phyllanthus emblica* (Calixto et al., 1998), and *Toona sinensis* (Yi-Chen et al., 2010). The compound is contained in some fruits such as berries, grapes, mango, pomegranate and gallnuts.

Phenolic compounds have long being recognised for their beneficial roles in the prevention of diseases due to their oxidation-reduction properties. Importantly, gallic acid, one of the phenolic compounds had received much scientific interest owing to the presence of three hydroxyl groups and a carboxylic acid in the chemical structure. Additionally, the compound

possesses a small molecular mass of 170, and has the propensity to prevent oxidative damages triggered by reactive oxygen species, ROS. Therefore, gallic acid has immense benefits in cardio protection (Priscilla et al., 2009), hepato protection (Jagan et al., 2008), nephron protection (Vijaya et al., 2011) and neuroprotection (Lu et al., 2006). The anti-oxidant and free-radical scavenging activities of gallic acid, has being documented, with the compound shown to be the major compound responsible for the anti-oxidant, anti-radical and in some cases anti-cancer compound of many plants (Agarwal et al., 2006; Yi-Chen et al., 2010; Palafox-Carlos et al., 2012). The compound has potent anti-inflammatory, (Kroes et al., 1992; Tsang et al., 2016), anti-microbial (Borges et al., 2013), anti-ulcer, (Siddaraju et al., 2007), and anti-viral activities, (Jadel et al., 2008).

Recently, investigations into the anti-cancer activities of Gallic acid have being documented for several cancers *in vitro*. Gallic acid has been found to exert cytotoxic activities on breast (García-Rivera et al., 2011; Wang et al., 2014), cervical, (Zhao et al., 2013), colon (Yoshioka et al., 2000; Salucci et al., 2002; Jebakkan et al., 2010; Aruna et al., 2016), glioma (Lu et al., 2010), stomach (Yoshioka et al., 2000), leukaemia (Chandramohan et al., 2012), lung (You et al., 2011), pancreatic (Cedó et al., 2014), and prostate (Agarwal et al., 2006; Veluri et al., 2006) cancers.

The investigation of the growth inhibition activity of gallic acid in cell growth assays demonstrated that the agent had selective potent antiproliferative activities on ovarian cancer cells with minimal inhibition observed on normal cells. Further studies were carried out to determine whether the effect in the cell growth assays reflected inhibition of proliferation or cell death. The findings from this study showed that the compound may be cytotoxic on cancer cells. Additionally it was observed that the effective concentration for the activity of gallic acid on OVCAR 4 cells was >10µM. This is not unusual as a significant drawback of polyphenols is their observed poor bioavailability, which from a review of 97 kinetic studies of 18 major polyphenols showed that plasma concentrations of the polyphenols were between 0-4µmol/L from 50mg consumption of the equivalent aglycone. The researchers observed

that Gallic acid and isoflavones were the most absorbed of the polyphenols, followed by the catechins (Manach et al., 2005).

Importantly, these results suggest that gallic acid is cytotoxic to the OVCAR 4 cancer cells; a cell line that is ranked among the most resistant cell lines of the human ovarian cancer, and which typifies serious ovarian cancer. By implication, these results have provided some evidence that gallic acid would be cytotoxic on most ovarian cancer cell lines. This view is further supported by the cytotoxic studies of gallic acid on two ovarian cancer cell lines; OVCAR 3, A2780/CP70 (a cisplatin-resistant cell line) and a normal ovarian cell line, ISOE-364. The researchers reported that gallic acid significantly inhibited cell viability on the ovarian cancer cell lines, without a commensurate inhibition on the ISOE-364 cells. It was also noted that 40µM concentration of gallic acid had better inhibitory effects on the ovarian cancer cell lines than 20µM cisplatin (Zhiping et al., 2016).

Diverse studies into the mechanism of action of gallic acid have been undertaken by different researchers. The major consensus is that gallic acid perturbed the G2/M phase resulting in cell cycle arrest (Salucci et al., 2002; Ou et al., 2010). The agent has been shown to be an apoptosis inducer (Agarwal et al., 2006; Veluri et al., 2006). Gallic acid has been implicated in the generation of ROS, which are cytotoxic to cancer cells and resistant to normal cells. The agent has been shown to trigger an elevation of intracellular calcium level (Isuzugawa et al., 2001).

Further evaluation of the synergistic potentials of gallic acid in enhancing the growth inhibition activities of carboplatin was undertaken by drug combination experiments in this study. The findings suggest antagonism between gallic acid and carboplatin which was also observed in the trypan blue assay. Other researchers have also observed significant antagonism in the combined use of the gallic acid and three other cancer drugs; 5-fluorouracil, mitomycin C and gemcitabine (Peter et al., 2007). It maybe suggested that

higher concentrations of gallic acid (>40μM) may be required in order to achieve synergism between gallic acid and carboplatin.

A significant finding from this study is that gallic acid selectively inhibits cancer cells with very minimal inhibitory effect towards normal cells, which shows that gallic acid potentially could be employed as a chemopreventive agent and also in the treatment of ovarian cancer. Further investigations on gallic acid were not deemed worthwile as the compound has been shown to induce apoptosis from several other studies.

Given the renewed interest in the pharmacological investigations of phenolic compounds as potential sources of chemotheraupeutic agents, this research evaluated the cell growth inhibition activities of some common gallic acid derivatives in order to obtain some structure activity relationship (SAR) information that would guide the synthesis of more potent gallic acid derivatives for anti-ovarian cancer activities.

## *4.4 Investigations of Gallic Acid Derivatives*

Derivatives of gallic acid are currently in use as food additives, which may offer added advantages such as anti-oxidants which could hamper the peroxidation of lipids and enhance the prevention of a number of diseases such as cardio vascular diseases and hypertension. One study has shown that some gallic acid derivatives; methyl gallate, propyl gallate and lauryl gallate possess free-radical scavenging properties and were better inhibitors of the peroxidation of phospholipids in ox brain than parent gallic acid (Aruoma et al., 1993).

This research work further investigated the growth inhibition of some common gallic acid derivatives on ovarian cancer cell lines, in order to obtain some preliminary SAR information that would guide the synthesis of more potent gallic acid analogues (Table 4.9 and Figure 4.10).

**Table 4-9 The growth inhibition evaluation of Gallic Acid Derivatives in cell growth assays.** The cells were treated for 72h with the indicated compound, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean $IC_{50}$ ±SEM, n=3 ($IC_{50}$ values are in μM).

| CELL LINE | GALLIC ACID (GA) Dose range (0.8-40μM) | LAURYLGALLATE (L-GAL) Dose range (0.02-5μM) | ETHYLGALLATE (E-GAL) Dose range (0.6-160μM) | 3,4,5-TRIMETHOXYBENZOIC ACID (345-MBA) Dose range (1.25-320μM) | PROTOCATECHUIC ACID (PCA) Dose range (0.2-50μM) | CARBOPLATIN Dose range (0.2-40μM) |
|---|---|---|---|---|---|---|
| **A2780** | 6.2±0.3 | 0.8±0.0 | 61.8±11.6 | 30.3±0.3 | 0.6±0.2 | ND |
| **OVCAR 8** | 16.2±0.5 | 0.8±0.1 | 58.1±3.1 | 81.2±0.7 | 3.3±0.5 | 10.8±1.3 |
| **CIS-A2780** | 6.5±0.4 | 0.3±0.05 | 60.0±0.05 | 34.2±1.6 | 3.7±0.9 | ND |
| **OVCAR 4** | 26.9±4.1 | 2.5±0.1 | 63.2±1.2 | ND | ND | 11.3±0.7 |
| **HOE** | >100 | 2.3±0.05 | ND | ND | ND | ND |

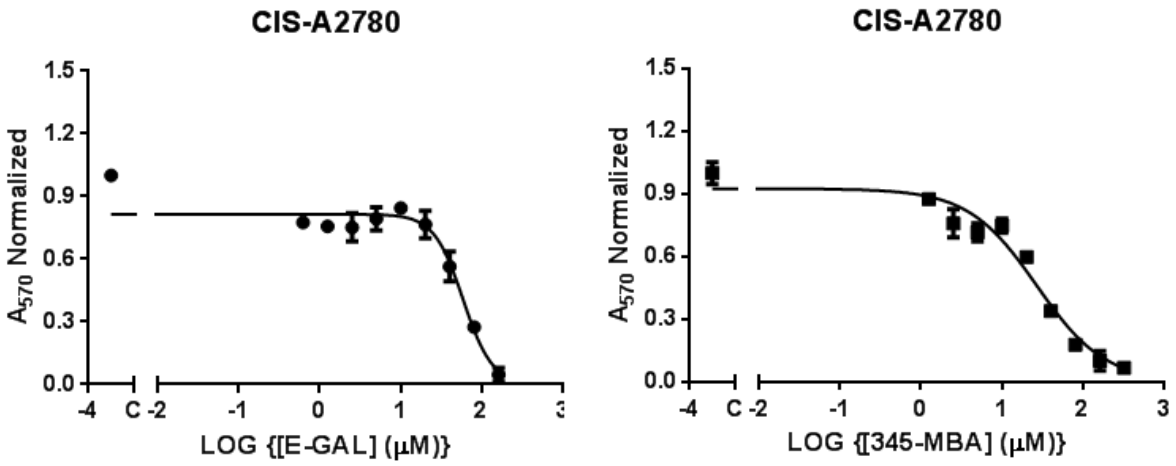



**Figure 4.10 The mean concentration-response curves of two gallic acid derivatives ethyl gallate (E-GAL) on CIS-A2780 cell line (A) and 3,4,5-TRIMETHOXYBENZOIC ACID (345-MBA) on CIS-A2780 cell line (B). The cells were treated for 72h with the indicated compound, followed by an estimation of the number of surviving cells by SRB staining. The results were expressed as Mean $IC_{50}$ ±SEM (n=3).**

The structures of the evaluated gallic acid derivatives shown in Figure 4.11 below.

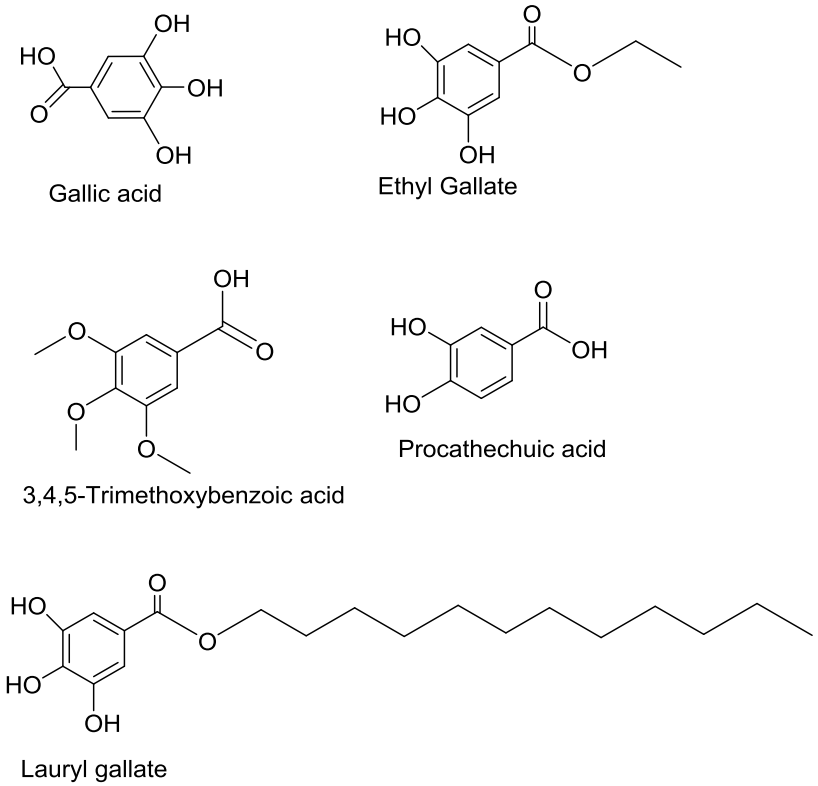



**Figure 4.11 Chemical structures of gallic acid derivatives investigated for growth inhibition on ovarian cancer cell lines.**

## *4.5 Discussion of Gallic acid Derivatives*

The results in Table 4.9 and in Figure 4.10 above showed that all the gallic acid derivatives demonstrated inhibitory activities on the ovarian cancer cell lines. A ranking of the order of activity in increasing antiproliferative activities is given as:

L-GAL> PCA> GA>345-MBA >E-GAL

Significant inhibition activities were demonstrated by L-GAL and PCA, which was≥ 10-fold increase in potency for L-GAL on all the cell lines, while for PCA, a 10 fold increase was showed on A2780, and a 4-fold increase on OVCAR 8. Similar findings have been reported from a study of phenolic acids, caffeic and gallic acid derivatives on three human cancer cell lines; HeLa cervical cancer cell line, MDA-MB-231 breast cancer adenocarcinoma and MOLT-3 lymphoblastic leukaemia. Among the compounds investigated were gallic acid, PCA and some catechols. The study concluded that all the phenolic compounds were antiproliferative on all three cancer cell lines (Gomes et al., 2003).

A closer look at the structures of the compounds showed that PCA has two hydroxyl groups on the ring, while parent GA has three hydroxyl groups on the benzene ring. The loss of one hydroxyl group, may have contributed to the significant increase in activity of PCA. Several researchers have reported the effect of the number of hydroxyl groups of gallic acid esters on anti-cancer activity. Their observation was that anti-cancer activity is enhanced by an increase in the number of hydroxyl groups on the aromatic ring (Gomes et al., 2003; Fiuza et al., 2004; Locatelli et al., 2008). In particular, one group that investigated a series of tri-hydroxyl and di-hydroxyl gallic esters noted that the tri-hydroxyl esters exhibited better inhibitory activities on the human cervical cancer cell line HeLa (Fiuza et al., 2004). However, the result of this present study did not observe an enhancement of anti-cancer activity due to an increase in the number of hydroxyl groups' on the aromatic ring in the ovarian cancer cell lines A2780, CIS-A2780 and OVCAR 8 evaluated. Another observation is the diminished activity of 345-MBA. This finding has been demonstrated in two studies. One research group

observed that at a concentration of 100µM, the compound had no inhibition on L-1210 leukaemia cells (Locatelli et al., 2008).The results from this present study further suggested that a short-chain alkyl substituent on the ring may result in decreased activity, while a long-chain alkyl substituent would significantly increase the activity of the compound as observed from the extremely low $IC_{50}$ values of L-GAL in comparison with E-GAL. This observation has being documented by the above-referenced researchers and in particular the effect of alkyl-chain length was observed in both di-hydroxyl and tri-hydroxyl gallic acid esters on all the human cancer cell lines investigated. This increase in activity exhibited by long alkyl-chain substituent may be attributed to the required interaction of a drug in the hydrophilic (cytoplasm) and the lipophilic (membrane) environments that enable the process of drug uptake in the body. The ability of a drug to interact optimally with these two environments is a bonus to activity. The long-chain alkyl substituent signified increased lipophilicity of the molecule, which may be of therapeutic benefit (Lu et al., 2006). The anti-cancer activity of L-GAL has been reported in a study involving three diverse human breast cancer cells: MCF7, MDA-MB-231 and MCF7 ADR which were significantly inhibited by L-GAL. Importantly, the MCF7 ADR cell line is a multi-resistant cancer cell line. The report further observed low cytotoxicity on normal cells and an increase in apoptosis on all the cancer cell lines (Calcabrini et al., 2006). The results from this study showed that L-GAL has demonstrated significant growth inhibition on cancer cells *in vitro*, with a significantly much lower $IC_{50}$ value compared to carboplatin, a first-line drug used in the treatment of ovarian cancer and has displayed preferential selectivity for cancer cells in comparison to normal cells on three ovarian cancer cell lines. Additionally, the agent has been shown to prevent the peroxidation of mitochondrial lipid (Kubo et al., 2002), which has immense therapeutic potentials in cancer therapy and other diseases (Kubo et al., 2003; Rogério et al., 2015). Furthermore L-GAL is officially a food additive (E312) and anti-oxidant since 1976and is currently used as a food additive for the prevention of bacterial and fungi growth in oils and fatty food products. Studies on the safety profile of the agent documented in the European Food Safety Authority, EFSA Journal reported that the agent hampered tumour growth in a long-term study with no

sign of genotoxicity, a further assessment of L-GAL carried out by the EFSA Panel in 2015, recommended the continued use of L-GAL as a food additive, (EFSA, 2015; 2014). Further studies on L-GAL are merited as this agent could be a potential candidate for ovarian cancer prevention and treatment.

## *4.6 Conclusion*

In conclusion, the investigation of the anti-cancer activities of *Acalypha wilkesiana* on ovarian cancer cell lines has been carried out. The study showed that the main active compound isolated from the plant is gallic acid, which can be extracted by polar organic solvents. Cytotoxic investigations of the plant showed that the AW possesses anti-cancer activities *in vitro*, which may provide evidence for the indigenous use of the plant for the treatment of cancer.

Further findings from this present research are the potential existence of significant antagonism between gallic acid (10µM) and carboplatin, as observed in the trypan blue assay and drug combination experiments.

The investigations of four derivatives of gallic acid were further conducted, and the results demonstrated that all four derivatives; ethyl gallate, lauryl gallate, 3, 4, 5 -trimethoxybenzoic acid and protocatechuic acid were inhibitory on the cell lines. Significant inhibition was observed in lauryl gallate, a food additive. This agent was more potent than carboplatin; a standard first-line drug employed in the clinical treatment of ovarian carcinoma, and thus is a promising hit compound for future development.

# Chapter Five

## *Anti-Ovarian Cancer Compounds from Rutidea parviflora DC.*

## *5.1 Introduction*

*Rutidea parviflora* DC (RP) belongs to the Rubiaceae family, in the Angiosperm category of plants. A review of the Rubiaceae family published in 2015 showed that there were no reported phytochemical studies on the genus *Rutidea* (Martins et al., 2015).This genus has 21 species and there are no documented investigations on the bioactivities of the species. *R. parviflora* is thus a; relatively un-exploredspecie for biological activities and this has prompted this research work, as the first reported investigations of the anti-ovarian cancer activities of RP.

The research in this chapter aims to investigate the anti-cancer activities of RP on ovarian cancer cell lines. Through bioassay-guided fraction, the active compounds were isolated and characterised. Growth inhibition evaluation using cell-growth assays were carried out to investigate the antiproliferative activities of the bioactive compounds. An estimation of the selectivity index of the bioactive compounds was undertaken. Through the apoptosis studies, the possible route, by which the most potent bioactive compound kills the cancer cells, was investigated. Drug combination studies were carried out to identify potential synergy between the most potent bioactive compound and clinically used ovarian cancer drugs both in cell growth assays and in apoptosis studies.

## *5.2 Results and Discussions*

### 5.2.1 Extraction of plant materials

The pulverised stembarks of RP were extracted using a combination of dichloromethane and methanol to obtain the organic extract, followed by extraction with deionised water to yield the aqueous extract, according to the procedures (Figure 2.1) used by National Cancer

Institute (NCI, USA). The amounts of the extracts and their yields obtained are shown in Table 5.1.

**Table 5-1 Percentage Yield of the RP Extracts obtained from the extraction with dichloromethane/methanol (organic extract) and extraction with deionized water (aqueous extract).**

| SAMPLE AMOUNT (G) | ORGANIC EXTRACT (G) | AQUEOUS EXTRACT (G) | % YIELD OF TOTAL EXTRACT |
|---|---|---|---|
| 1000 | 7.2 | 1.5 | 0.9% |

From the data in Table 5.1, it was observed, that the stembark yielded relatively low amounts of solid extract. Also, organic extract is more than the aqueous extract. This may be due to the method of extraction, which was sequential; organic extraction, followed by aqueous extraction.

## 5.2.2Screening of Anti-ovarian cancer of the plant extracts

The organic and aqueous extracts of RP were tested for their inhibition of the growth of OVCAR8, OVCAR4, A2780, and CIS-A2780 ovarian cancer cell lines using a cell growth assay in which cell number was estimated by staining with SRB (Table 5.2).

**Table 5-2 Results of Preliminary Screening of the Extracts of RP on ovarian cancer cell lines in cell growth assays. The cells were treated for 72h with the indicated extract, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments ($IC_{50}$ is in µg/ml).**

| EXTRACT | A2780 | OVCAR8 | CIS-A2780 | OVCAR 4 |
|---|---|---|---|---|
| ORGANIC EXTRACT | 3.2±0.3 | 8.7±0.5 | ND | 6.6±1.6 |
| AQUEOUS EXTRACT | 2.2±0.5 | 5.9±0.03 | 3.7±0.03 | ND |

The aqueous and organic extracts of RP, had very significant inhibition activities, as observed from the plant extracts' $IC_{50}$ values of CIS-A2780, A2870, OVCAR4 and

OVCAR8ovarian cancer cell lines, respectively presented in Table 5.2. These $IC_{50s}$ were below the threshold which is considered to be significant from the perspective of the American national Cancer institute criteria, which from a screening of about 20,000 plant extracts, identified 452 cytotoxic extracts with $IC_{50}$<50µg/mL(Thomas 2010). Thus, both the organic and aqueous extracts of RP with $IC_{50}$s <50µg/mL on the investigated cell lines were deemed worthy of further purification and investigation for anti-cancer activity.

These findings are consistent with the reported observations of a study on *Tarennagrevei*, a plant native to the Madagascar dry forest in the Rubiaceae family, belonging to the genus *Tarenna*, which exerted a moderate cytotoxicity on the A2780 ovarian cancer cell line (Kingston et al., 2012). Presently, research on the anti-cancer activities of the *Rutidea* genus is lacking.

### 5.2.3 Bioassay-guided fractionation of RP

Given that both the aqueous and organic extracts were active, further solvent fractionation of 5.0 g of the crude organic extract of RP, using four solvents was perfomed (mass of solid compound recovered is shown in parentheses); n-hexane (2.0g), ethyl acetate (1.3g), n-butanol (0.9g) and aqueous (0.5g) fractions. The fractionation of the aqueous extract could not be undertaken as more intricate and advanced techniques such as extractions with buffers, ion exchange chromatography, gel filtration and desalting would be required inorder to achieve a separation of the constituents (Shimizu et al., 2006). This situation was further compounded by the low yield of the extract.

The obtained organic fractions were further evaluated in cell growth assays (Table 5.3).

**Table 5-3 The Results of the growth inhibition evaluation of the fractions of the organic extract of RP on ovarian cancer cell lines in cell growth assays.** The cells were treated for 72h with the indicated fraction, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments ($IC_{50}$ is in µg/ml).

| Fraction | OVCAR4 | OVCAR8 | A2780 |
|---|---|---|---|
| n-Hexane | 23.3±1.0 | 18.3±0.3 | 7.3±0.8 |
| **Ethyl Acetate** | **5.4±0.3** | **5.8±0.4** | **2.5±0.2** |
| **n-Butanol** | **2.6±0.1** | **2.6±0.3** | **1.7±0.2** |
| Aqueous | 22.9±1.3 | 22.1±1.1 | 12.8±1.3 |

The results of the investigation of the antiproliferative activities of the fractions of the organic extract of RP showed that the ethyl acetate and the n-butanol fractions were the most bioactive fractions. Further fractionation of these fractions was undertaken.

### 5.2.4 Chemical characterization of bioactive organic extract of RP

In order to find out the anti-cancer components in RP organic extract, the TMSi derivatives was prepared and analysed by GC-MS.

The TMSi derivative of RP was obtained by the treatment of BSTFA and pyridine and subjected to further analysis on the GC-MS to determine the potential bioactive compounds that could be responsible for the activity of the extract. The identified compounds with a match quality >80%, are presented in Table 5.4, and Figure 5.1 is the obtained GC-MS chromatogram for the compounds listed in Table 5.4 below.

**Table 5-4 The Compounds Identified by the GC-MS Analysis of the Organic extract of RP.**

| RP-dcmm-tmsi | Quality match | Retention time |
|---|---|---|
| Glycerol, tris(trimethylsilyl) ether | 91 | 10.97 |
| 3-Pyridinecarboxylic acid, trimethylsilyl ester | 94 | 11.19 |
| Butanedioic acid, bis(trimethylsilyl) ester | 98 | 11.48 |
| Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester | 91 | 11.82 |
| meso-Erythritol, tetrakis(trimethylsilyl) ether | 91 | 14.24 |
| Benzoic acid, 4-ethoxy-, ethyl ester | 95 | 14.50 |
| L-Threonic acid, tris(trimethylsilyl) ether, trimethylsilyl ester | 95 | 14.83 |
| Benzenepropanoic acid, .alpha.-[(trimethylsilyl)oxy]-, trimethylsilyl ester | 87 | 15.29 |
| D-Ribose, 2,3,4,5-tetrakis-O-(trimethylsilyl)- | 89 | 15.76 |
| D-Ribose, 2,3,4,5-tetrakis-O-(trimethylsilyl)- | 91 | 16.14 |
| Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)- | 93 | 17.06 |
| Ribitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)- | 91 | 17.12 |
| Benzoic acid, 3,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester | 99 | 18.07 |

| | | |
|---|---|---|
| .beta.-D-(-)-Tagatopyranose, pentakis(trimethylsilyl) ether | 81 | 18.23 |
| D-(-)-Fructopyranose, pentakis(trimethylsilyl) ether (isomer 1) | 93 | 18.21 |
| alpha.-D-Galactopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)- | 91 | 19.14 |
| **Benzoic acid, 3,4,5-tris(trimethylsiloxy)-, trimethylsilyl ester** | 99 | 19.58 |
| Glucopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)-, D- | 94 | 19.89 |
| Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)- | 89 | 20.17 |
| D-Gluconic acid, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, trimethylsilyl ester | 93 | 20.23 |
| Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)- | 99 | 21.10 |
| beta.-Gentiobiose, octakis(trimethylsilyl) ether | 80 | 21.38 |
| D-(+)-Cellobiose, octakis(trimethylsilyl) ether (isomer 2) | 87 | 21.51 |
| Glyceryl-glycoside TMSether | 87 | 22.05 |
| Stigmasterol trimethylsilyl ether | 99 | 26.15 |
| **Urs-12-en-24-oic acid, 3-oxo-, methyl ester, (+)-** | 99 | 27.33 |

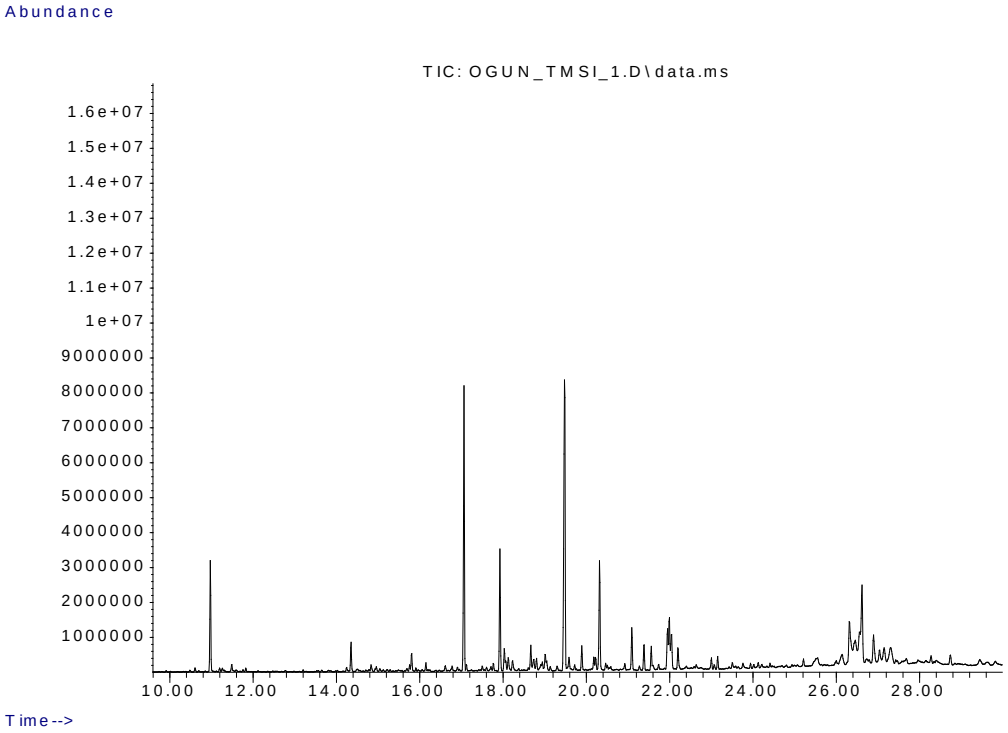



**Figure 5.1 The GC-MS chromatogram of the organic extract of RP for the 10-30min range of compounds.**

The GC-MS analysis of the organic extract of RP detected fifty-eight compounds, twenty six of which, had a match quality >80% and were identified by comparison of their mass spectra with those in the NIST library as shown in Table 5.4 and in Figure 5.1. Importantly two bioactive compounds were identified from the GC-MS analysis. Gallic acid, a bioactive compound with demonstrated cytotoxicity on many cancer cell lines (already discussed in Chapter 4), and Urs-12-ene-24-oic acid, 3-oxo-methyl ester; a ursolic acid derivative. Ursolic acid from previous studies has been shown to exert cytotoxic activities on a number of cancer cell lines, which includes A2780 and SK-OV3 ovarian cancer cell lines (Song et al., 2012), HT29 colon cancer cell line and PC-3 prostate cancer cell line (Bai et al., 2012). Recently the anti-cancer activities of some ursolic acid derivatives have been reported (Shao et al., 2011; Chen et al., 2015).

### 5.2.5 Fractionation of the Ethyl Acetate Fraction of RP (RP-EA)

Ten sub-fractions of RP-EA were obtained from a silica gel column chromatography which utilised different combinations of n-hexane/ethyl acetate. The elution began with a 100% n-hexane, followed by several combinations of n-hexane and ethyl acetate, then with 100% ethyl acetate. The column was finally eluted with 100% methanol.

The obtained sub-fractions were further evaluated in cell growth assays (Table 5.5).

**Table 5-5 The Results of the growth inhibition evaluation of the sub-fractions of RP-EA on OVCAR 4 cell line in cell growth assays.** The cells were treated for 72h with the indicated fractions, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments.

| Sub-fractions of RP-EA | OVCAR 4 (µg/ml) | Solvent Fraction |
|---|---|---|
| **F1** | >200 | N-hexane |
| **F2** | **4.2±0.2** | **N-hexane/Ethyl acetate** |
| **F3** | 65.9±10.1 | " |
| **F4** | >200 | " |
| **F5** | 36.2±1.4 | " |
| **F6** | 90.6±9.6 | " |
| **F7** | 17.4±2.3 | " |
| **F8** | 11.6±1.0 | " |
| **F9** | 37.3±6.5 | " |
| **F10** | >200 | Ethyl acetate |
| **F11** | **3.6±0.8** | **Methanol** |

Two of the sub-fractions (F2 and F11) showed potent activities. Fraction 2, which was obtained from the elution of the chromatographic column by a solvent system of n-hexane and ethyl acetate, 1:1, appeared white in colour. This fraction was treated with hot methanol and recrystallized at a temperature of 0-4$^{o}$C to yield white crystals (12.8%). The identity of this compound was investigated by GC-MS. A single peak with a mass of 468 was identified as Urs-12-ene-24-oic acid, 3-oxo-methyl ester, by the NIST-Library with a retention time of 27.33 min. The compound was tentatively determined as Urs-12-ene-24-oic acid, 3-oxo-methyl ester, and was henceforth referred to as EA2, with the structure shown in Figure 5.2.This compound has been identified from the GC-MS analysis of the ethanolic extract of the whole plant; *Canscora perfoliata* used in the treatment of poisonous bites (Thanga et al., 2012).

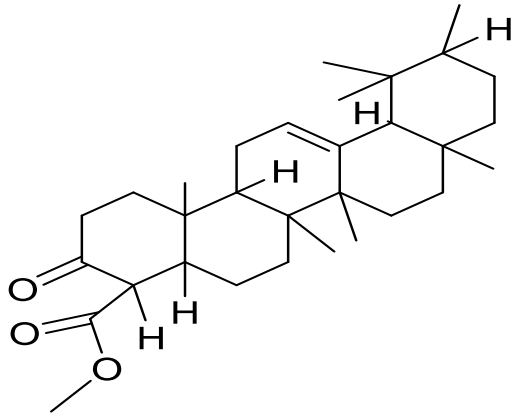



**Figure 5.2 The structure of URS-12-ENE, 24-OIC ACID, 3-OXO-METHYL ESTER (EA2)**

Fraction 11 was obtained from 100% methanol elution of the chromatographic column, which suggests a high polarity of the compounds present. This fraction was not subjected to further purification, as the isolation of the potential bioactive compounds in the fraction may require more elaborate techniques as with the aqueous extract mentioned above.

$^{1}$H NMR spectra of EA2 was obtained at 300 MHz with a Bruker 1D (DPX-300) NMR spectrometer in $CDCl_3$ δ: 3.57(s, 3H), 2.4(m, 3H), 1.83(s, 3H), 1.62(s, 3H), 0.95(s, 3H)}. The $^{13}$C NMR was obtained at a 400 MHz Bruker NMR in $CDCl_3$ δ: 31.96, 31.46, 30.24, 29.72, 29.49, 29.38, 29.00, 27.63, 24.90, 22.73, 21.32, 16.52, 14.15. Attempts to obtain the

literature reported $^{1}H$ and $^{13}C$ NMR for this compound proved abortive, as the data could not be obtained from NIST, PUBMED, Chemspider and Chembl databases and from literature search. The identity of the compound was assigned tentatively as Urs-12-ene-24-oic acid, 3-oxo-methyl ester. However, given the GC-MS match quality of 99% and the proton NMR data which had some similarities with the proton NMR of ursolic acid (Seebacher et al., 2003), there is some evidence to suggest that EA2 would most likely be Urs-12-ene-24-oic acid, 3-oxo-methyl ester.

### 5.2.6 Investigation of the cell growth inhibition of EA2, the isolated Bioactive Compound of RP-EA

The identified bioactive compound, EA2 was further investigated in cell growth assays (Table 5.6).

**Table 5-6 Results of growth inhibition evaluation of EA2 on ovarian cancer cell lines and HOE in cell growth assays.** The cells were treated for 72h with EA2, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments.

| Cell Line | A2780 | OVCAR8 | OVCAR 4 | HOE |
|---|---|---|---|---|
| **$IC_{50}$ in µM** | 31.6±3.3 | 48.9±2.0 | 85.4±2.4 | >200 |

The results of the inhibition experiments showed that EA2 exhibited moderate inhibition of the growth of ovarian cancer cell cultures. The compound also showed apparently no effect on normal cells, which is an indication of advantageous safety profile.

Further investigation of the compound's antiproliferative activities was carried by the trypan blue assay (TBA), to determine whether the effect in the cell growth assays reflected inhibition of proliferation or cell death (Figure 5.3).

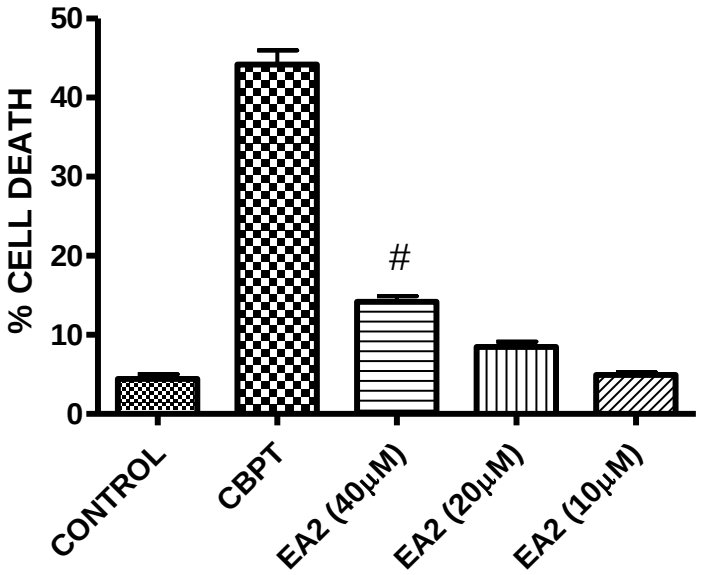



**Figure 5.3 The Effect of EA2 at the indicated concentrations and carboplatin (CBPT), (40 μM) at 48h on cell viability. The cells were stained with trypan blue and the percentage of dead cells was obtained by microscopy. # denotes significant difference from the control, (*P<0.001, one-way ANOVA*). The results were expressed as mean ±SD, n=3.**

The results obtained from the TBA study showed that EA2 exerted antiproliferative activities on cancer cells in a concentration-dependent manner. The 40μM concentration had significant activity, (*P<0.001*) in comparison to the control. The activity of the compound is moderate and these results suggest that the activity of the extracts and the ethyl acetate fractions in the cell growth assay was at least in part due to cell death. This implies that EA2 maybe cytotoxic to ovarian cancer cells. However the compound was less active than carboplatin, the reference drug. Previous studies have reported the anti-bacterial and anti-microbial activities of plants' fractions that contain Urs-12-ene-24-oic acid, 3-oxo-methyl ester (Natarajan et al., 2012; Adebayo et al., 2014).

The observation made from the investigation of the growth inhibition of EA2 is that this compound is partly responsible for the activity of RP-EA, but there might be other bioactive compounds present in the fraction, which may have contributed to the activity of RP-

EA2possibly through synergism with EA2. However, due to insufficient quantity of this fraction, other compounds could not be isolated.

### 5.2.7 Apoptosis studies of EA2

In order to investigate, the possible route of cell death caused by EA2, the effect of the compound was measured in caspase 3/7 experiments (Figure 5.4).

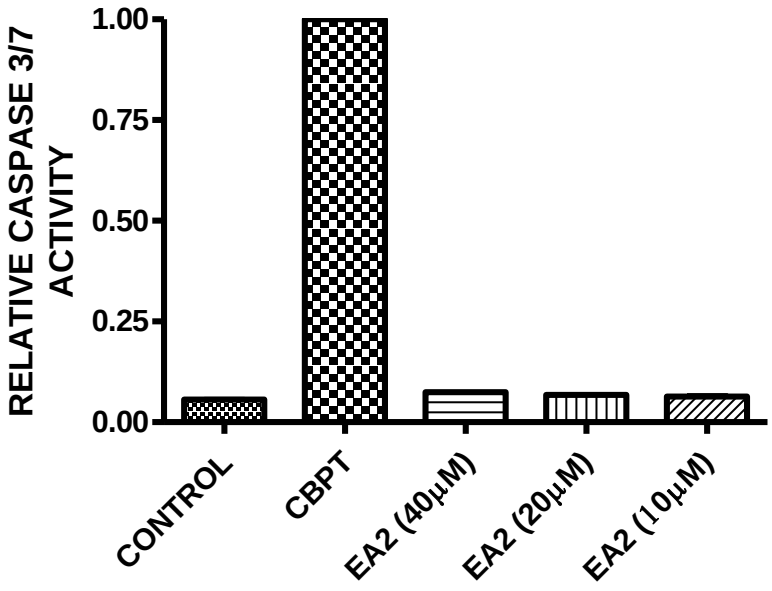



**Figure 5.4 The Effect on caspase 3/7 activity measured after 48h on OVCAR 4 cell line by the vehicle-treated cells (control), carboplatin (CBPT), EA2 at 40 μM, 20 μM and 10 μM concentrations. The results were expressed as mean ±SD, n=3, which were obtained as fractions of the treated cells when compared with the reference standard; the carboplatin-treated cells after normalization with corresponding SRB-stained cells to estimate the surviving cell number.**

The results of the caspase 3/7 study observed that EA2 exhibited slight increase in caspase 3/7 activity in comparison to the control and carboplatin. There is the possibility that at increased concentrations of the compound, the caspase 3/7 activity would be more evident. However it is noteable that 40μM EA2 was sufficient to cause antiproliferation and cell death (measured by trypan blue staining) without substantially increasing caspase 3/7 activity.

Further studies on EA2 were not deemed worthwhile given the moderate activity of the compound.

Previous researchers have documented the anti-asthma, anti-arthritic, anti-inflammatory, anti-microbial and diuretic activities of Urs-12-ene-24-oic acid, 3-oxo-methyl ester (Ramalingam et al., 2015). It may be suggested that EA2 could be benefical in chemoprevention given the other activities of the compound.

### 5.2.8 Results of the fractionation of the n-butanol fraction of RP (RP-BUT)

Nine sub-fractions were obtained from the column fractionation of RP-BUT by means of a solvent-polarity based fractionation which utilised various combinations of ethylacetate/ methanol which entailed a ratio-based decrease in the volume of ethyl acetate, with a progressive increase in the volume of methanol. The fractionation began with a 100% ethyl-acetate and ended with 100% methanol. The obtained sub-fractions were further evaluated in the cell growth assays (Table 5.7) below.

The results in Table 5.7 showed that five of the RP-NBUT fractions were active against the OVCAR 4 cell line. This cell line is ranked among the top five most resistant human ovarian cancer cell lines which typifies very serious ovarian cancer and thus is a suitable model for the investigation of *in vitro* anti-ovarian cancer activities (Domcke et al., 2013). A closer look at these results showed that sub-fraction 3 and 7 were the sub-fractions with optimum inhibitory activities on the cancer cells. Further purification of these two sub-fractions was undertaken.

**Table 5-7 The Results of the growth inhibition evaluation of the sub-fractions of RP-BUT on ovarian cancer cell line in cell growth assays.** The cells were treated for 72h with the indicated fraction, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments.

| SUB-FRACTION OF RP-NBUT | $IC_{50}$ (µg/ml) | Solvent Fraction |
|---|---|---|
| RP-NBUT-1 | >200 | Ethyl acetate |
| RP-NBUT-2 | >200 | Ethyl acetate/Methanol |
| RP-NBUT-3 | **1.9±0.3** | **“** |
| RP-NBUT-4 | 4.8±1.6 | “ |
| RP-NBUT-5 | >200 | “ |
| RP-NBUT-6 | >200 | “ |
| RP-NBUT-7 | **2.8±0.1** | **“** |
| RP-NBUT-8 | 8.0±0.0 | “ |
| RP-NBUT-9 | 18.9±0.4 | Methanol |

### 5.2.8.1 Purification of RP-NBUT-7

This sub-fraction was subjected to HPLC separation on a semi-preparative column with a mobile phase composition of 100% water acidified with 0.1% Trifluoroacetic acid (TFA) as solvent A and 80% acetonitrile acidified with 0.1% TFA as solvent B. The gradient began with 100% of A for 5 min and increased to 80% B for 25 min of the run time. Then the gradient was increased to 100% B and maintained for 6 min of the analysis; making the total run time 36 min. Fifteen hplc-fractions were collected. The yield of eight of these fractions were <2%, and thus could not be evaluated in the cell growth assays. The remaining seven fractions were evaluated in the cell growth assays (Table 5.8).

**Table 5-8 The growth inhibition evaluation of the HPLC-fractions of RP-BUT-7 on OVCAR 4 cell line in cell growth assays and the masses found by LC-MS for the most potent fractions.** The cells were treated for 72h with the indicated fraction, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments.

| SUB-FRACTION OF RP-NBUT-7 (RETENTION TIME) | Code for the sample | $IC_{50}$ OF OVCAR 4 CELL LINE (µgG/mML) | Mass found by LC-MS |
|---|---|---|---|
| 2.2-2.6 | - | >100 | |
| 2.6-3.1 | - | >100 | |
| 19.0-19.6 | - | 3.5±0.3 | |
| 19.7-21.3 | nbut-7-1 | **2.0±0.4** | 544.3469, $C_{26}H_{48}N_4O_8$ |
| 27.4-28.2 | nbut-7-2 | **0.7±0.2** | 330.2194, $C_{21}H_{30}O_3$ |
| 28.5-31.5 | nbut-7-3 | **1.5±0.6** | 297.3394 |
| 32.2-33.2 | - | >100 | |

Three bioactive hplc-fractions were identified out of fifteen hplc-fractions. These fractions were henceforth referred to as nbut-7-1 at retention time (Rt) of 19.7-21.3, nbut-7-2 with Rt 27.4-28.2, and nbut-7-3 with Rt 28.5-31.5 min on the hplc (Table 5.8).

The LC-MS was carried out and the following findings were made.

For **nbut-7-1**

The mass of a major compound peak in this chromatogram was given as 544.3469 with a match quality score of 99.34%. The Retention time was 8.705min. The predicted chemical structure of this compound was given as $C_{26}H_{48}N_4O_8$. Several other peaks were identified. These included 352.2459 and 358.2138. This fraction was impure and further hplc purification was not feasible due to insufficient quantity of the sample.

For **nbut-7-2**

The LC-MS detected a small peak with a mass of 330.2194 (predicted chemical formula as $C_{21}H_{30}O_3$) with a match quality score of 99.7%. The Retention time of the peak was 11.729min. The fraction contained many other peaks and thus was impure. The identity of this compound could not be established, as further purification could not be undertaken due to insufficient quantity of the fraction.

For **nbut-7-3**

The LC-MS detected a tiny peak with mass 297.3394 and a score of 99.67% at a retention time of 15.412min.Also other peaks were detected. The peak signals from these chromatograms were very weak. Further purification efforts were not feasible due to insufficient quantities.

### 5.2.8.2 Purification of RP-NBUT-3

This sub-fraction was subjected to HPLC separation on a semi-preparative column; with a mobile phase composition of 100% water acidified with 0.1% Trifluoroacetic acid (TFA) as solvent A and 80% acetonitrile acidified with 0.1% TFA as solvent B. The gradient began with 100% of A for 5 min and increased to 80% B for 25 min of the run time. Then the gradient was increased to 100% B and maintained for 6 min of the analysis; making the total run time 36 min. Eighteen hplc fractions were collected, but due to insufficient quantities of eleven of

the fractions with yields <2%, only seven of these fractions were subjected to further evaluation in the cell growth assays (Table 5.9)

**Table 5-9 The Results of the growth inhibition evaluation of the HPLC-fractions of RP-BUT-3 on OVCAR 4 cell line in cell growth assays.** The cells were treated for 72h with the indicated fraction, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments.

| SUB-FRACTION OF RP-NBUT-3 (CODED BY THEIR HPLC RETENTION TIME) | $IC_{50}$ OF OVCAR 4 CELL LINE (µgG/mML) |
|---|---|
| 2.2-2.5 | >100 |
| 2.5-3.4 | >100 |
| 11.3-11.9 | 1.2±0.3 |
| **15.4-17.0** | **0.7±0.3** |
| **21.6-22.6** | **0.9±0.4** |
| **26.8-27.4** | **0.02±0.3** |
| 32.1-32.6 | 31.4±2.7 |

Three bioactive hplc-fractions were identified out of eighteen hplc-fractions. These fractions were nbut-3-1 at Retention time of 15.4-17, which had a characteristic yellow colour, nbut-3-2 with Retention time of 21.6-22.6, and nbut-3-3 with Retention time of 26.8-27.4 min on the hplc. The hplc subfractions obtained from RP-NBUT-3 were analysed by LC-MS (Table 5. 10).

**Table 5-10 The Identified Peaks from the LC-MS analysis of the bioactive hplc subfractions of RP-NBUT-3.**

| HPLC SUBFRACTIONS | IDENTIFIED PEAKS | LC-MS RETENTION TIME |
|---|---|---|
| **NBUT-3-1** | 352.1548 | 7.7835 |
| **NBUT-3-2** | 338.3421 | 11.6742 |
| | 311.106 | 13.965 |
| **NBUT-3-3** | 374.2466 | 9.2590 |
| | 312.1135 | 10.0962 |

Further hplc purification of nbut-3-1 was carried out to improve the purity of the compound as the yield was sufficient (23.8%) by a slight modification of the hplc purification method. The hplc retention time of the purified compound was found to be 15.2 min. The proton NMR analysis was carried out and the compound tentatively determined as Palmatine.

Palmatine:

$^{1}$H NMR (500 MHz, $CD_3OD$), δ 9.76 (1H,s), 8.80 (1H, s), 8.12 (1H, d, J = 9.0 Hz), 8.01 (1H, d, J = 9.1 Hz), 7.67 (1H, s), 7.05 (1H, s), 6.88 (1H, s), 5.29 (2H, t, J = 6.5Hz), 4.21 (3H, s), 4.11 (3H, s), 3.99 (3H, s), 3.94 (3H, s), 3.28 (2H, t, J =6.4 Hz). HSQC: 144.9, 118.3, 126.3, 122.6, 108.1, 110.1, 118.8, 60.9 ($OCH_3$), 55.5 ($OCH_3$), 54.9 ($OCH_3$), 54.5 ($CH_2$), 54.6 ($OCH_3$), 26.2 ($CH_2$). $^{1}$H NMR (300 MHz, $CDCl_3$), δ 8.42 (1H, s), 7.88 (2H, dd), 7.56 (1H, s), 7.04 (1H, s), 6.88 (1H, s), 5.29 (2H, t), 4.33 (3H, s), 4.11 (3H, s), 4.09 (3H, s), 4.02 (3H, s), 3.36 (2H, t).$^{13}$C NMR (500 MHz, $CD_3OD$), δ 152.45, 150.52, 149.54, 145.00, 144.37, 138.45, 133.90, 128.68, 126.69, 123.03, 119.9, 116.52, 110.82, 78.15, 77.88, 77.63, 73.92, 56.25, 50.54.

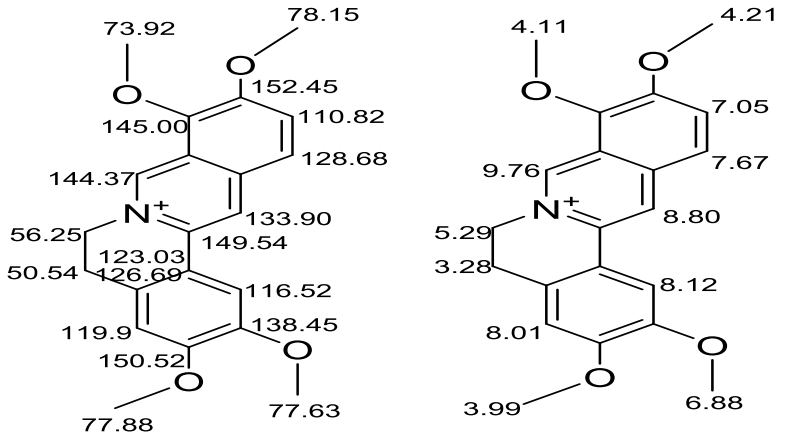



**Figure 5.5$^{1}$H NMR (left) and $^{13}$C NMR (right) Assignment of Palmatine**

The LC-MS analysis of nbut-3-1 (Figure 5.6) was carried out. The identity of the nbut3-1 hplc-fraction was unequivocally identified to be palmatine by comparing with the standard compound. The comparison showed that the mass of the standard palmatine was 352.1549, while the isolated palmatine had a mass of 352.1548 which was consistent with the compound being palmatine. Further hplc analysis of the standard palmatine gave a Retention time of 15.2min consistent with the isolated compound.

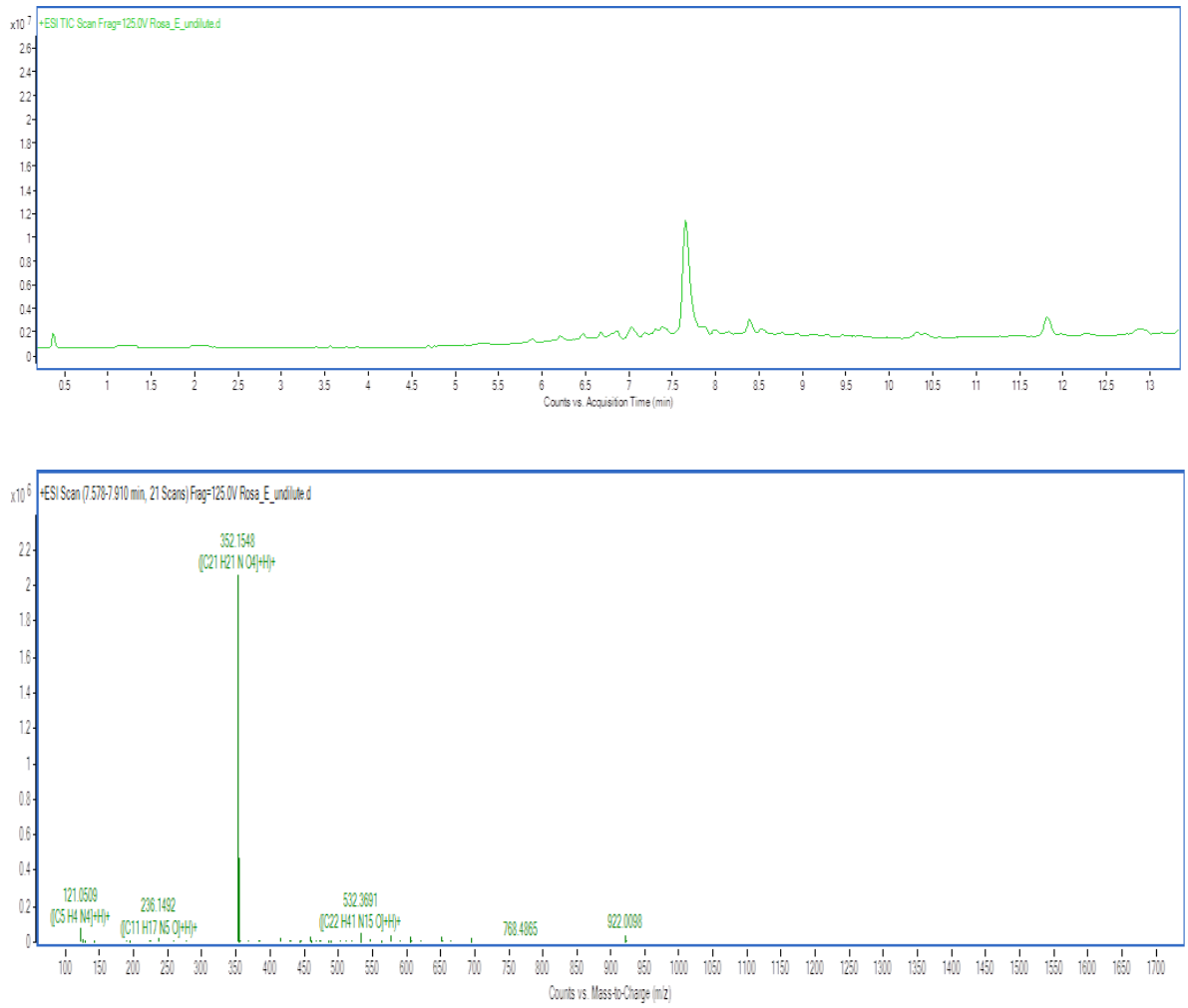



**Figure 5.6 LC-MS Chromatogram (above) and MS of nbut-3-1 (below).**

Palmatine which is intense yellow in colour is a quaternary protoberberine alkaloid that is present in *Rhizoma coptidis*, an important medicinal plant commonly used in the Traditional Chinese Medicine, TCM, (Ding et al., 2012). The compound has been previously isolated from several medicinal plants such as *Rhizoma coptidis*, (Zhao et al., 2010*), Fibraurea tinctoria*, (Su et al., 2007), *Jateorhiza macrantha* (PROTA 2008), *Chasmanthera dependens* (Iwu 2014) and *Enantia chlorantha* (Vennerstrom et al., 1988).

Studies on *Rhizoma coptidis* and *Fibraurea tinctoria* have documented the presence of berberine, coptisine, epiberberine, jatrorrhizine and palmatine in these plants (Ding et al., 2012;Suet al., 2007).Other phytochemical studies on *Jateorhiza macrantha* and *Chasmanthera dependens*, showed that palmatine, has been obtained with columbamine

and Jatrorrhizine; two other protoberberine alkaloids. Additionally, three diterpenes; palmarin, columbin and chasmanthin were found (PROTA2008; Iwu 2014).Columbin and chasmanthin are bitter non-nitrogenous compounds. Chasmanthin and Palmarin have the same mass of 374. The 358.2138 peak identified in the RP-N-BUT-7-1 subfraction may be that of columbin, given the mass of columbin is 358. It is very likely that the peak with mass 374 in nbut-3-3 may be either chasmanthin or palmarin.

The minor peak found in nbut3-2 with a mass of 338.3421could be either jatrorrhizine with mass 338.38 or columbamine whose mass is 338.3771. The occurrence of both compounds and palmatine has been observed in *Jateorhiza macrantha* and *Chasmanthera dependens* (Prota 2008;Iwu 2014).

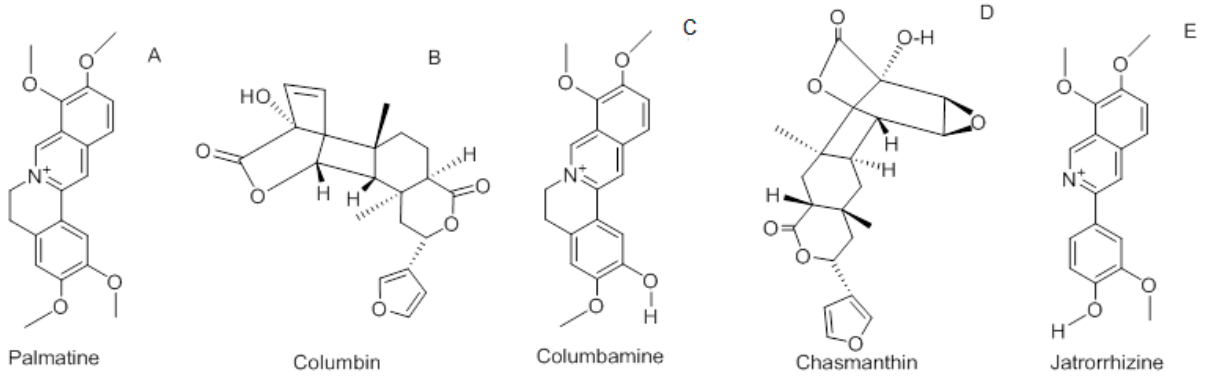



**Figure 5.7 Structure of palmatine, a bioactive compound isolated from RP and the possible structures of some compounds of the Identified Peaks in the LC-MS Analysis of RP.**

## 5.2.9 Investigation of the Cytotoxicity of Palmatine

Growth inhibition studies were carried in cell growth assays for palmatine on the ovarian cancer cell lines and on HOE (Figure 5.8and Table 5.11).

The results in Table 5.11 and Figure 5.7 examined the growth inhibition of the standard palmatine and the isolated palmatine by the SRB assay. The $IC_{50}$s of both compounds were indistinguishable, a further confirmation that the two compounds possess antiproliferative activities *in vitro*, and that the purification processes had not compromised the activity of the

isolated palmatine. This result is in agreement with the reported cytotoxicity of palmatine on MCF-7 breast cancer cell line evaluated by the MTT assay (Wu et al., 2016b).

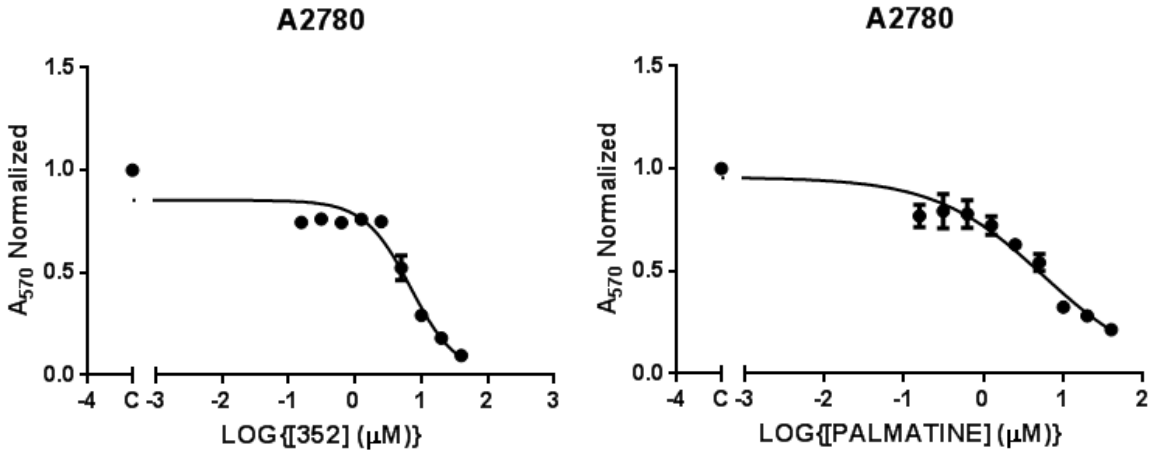



**Figure 5.8 The mean $IC_{50}$ concentration-response curves of the isolated Palmatine (352) and palmatine standard investigated on A2780 ovarian cancer cell. The cells were treated for 72H and the cell number obtained by SRB-staining of the surviving cells. The results were expressed as mean ±SEM, for n=3 experiments.**

**Table 5-11The evaluation of the growth inhibition of standard palmatine and the isolated palmatine (352) on ovarian cancer cell lines in cell growth assays. The cells were treated for 72h with the indicated compound, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments.**

| CELL LINE | Commercial PALMATINE ($IC_{50}$ in μM) | ISOLATED PALMATINE ($IC_{50}$ in μM) |
|---|---|---|
| A2780 | 5.6±1.0 | 6.6±0.5 |
| CIS-A2780 | 5.2±1.3 | 5.5±0.9 |
| OVCAR4 | 7.6±1.4 | 7.4±0.3 |
| OVCAR8 | 6.8±0.4 | 7.9±0.5 |
| HOE | 25.1±5.0 | ND |
| SI (against A2780) | 4.5 | - |

Another important observation is that the compounds demonstrated selective growth inhibition towards cancer cells, with less effect on normal cells.

Further evaluation of the cytotoxicity and synergistic effects of palmatine were carried out and discussed below.

## 5.2.10 The Drug Combination Studies of Palmatine

The antiproliferative activities of palmatine was further investigated by trypan blue assay, to determine whether the effect in the cell growth assays was as a result of inhibition of proliferation or cell death. The synergistic potentials of palmatine in combination with carboplatin, was also investigated (Figure 5.9).

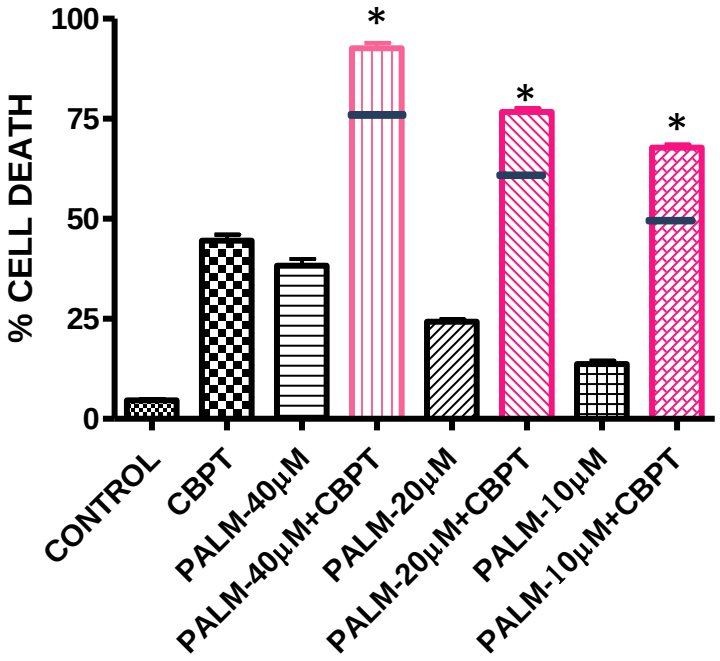



**Figure 5.9 The Effect of palmatine at the indicated concentrations and in combination with carboplatin (CBPT) (40 µM) at 48h on OVCAR 4 cell viability. The cells were stained with trypan blue and the percentage of dead cells was obtained by microscopy. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. * denotes that the result is significantly different (*P<0.05, paired t-test*) from the effect expected from the Bliss independence criterion. The results were expressed as mean ±SD, n=3.**

The results of the TBA showed that palmatine maybe cytotoxic to cancer cells in a concentration-dependent manner, with the maximum concentration of 40µM exhibiting the most cytotoxicity. The effect of the combined use of palmatine and carboplatin on the cells

were also investigated. The agents combined demonstrated significant synergism (*p<0.05*) because all three concentrations used in the study elicited effects which exceeded that expected for the combinations assuming additivity and calculated by the Bliss independence criterion, by which **hypothesis a** was tested. These results showed potential synergism between palmatine and carboplatin. Also these results are a confirmation of the observed inhibition of cancer cells by palmatine in the SRB assay.

Drug combination studies between palmatine and carboplatin on OVCAR 4 cells were carried out to investigate the synergistic potentials of securinine and carboplatin by the fixed dose method on the cell growth assay using the SRB protocol (Figure 5.10).

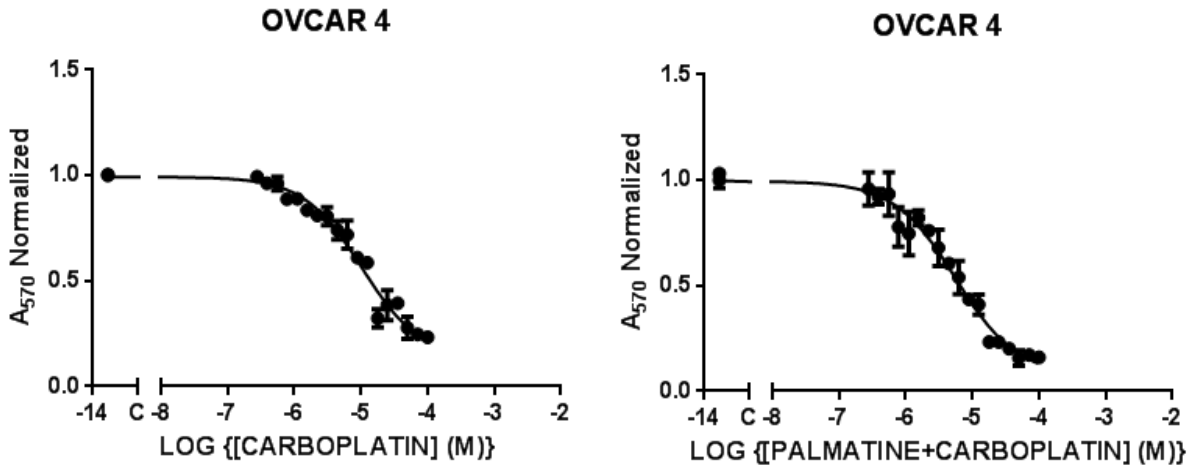



**Figure 5.10 Mean concentration-response curves of vehicle-treated cells, carboplatin-treated cells (Maximum concentration of 100 µM), and the drug combination of palmatine at a fixed concentration of 6 µM and Carboplatin (Maximum concentration of 100 µM) on the OVCAR 4 cell line determined after 72h of treatment. Results were expressed as mean of three experiments, mean±SEM.**

An examination of the results presented in Figure 5.10revealed an additive effect in the drug combination of Carboplatin and Palmatine which was not statistically significant from additivity. This observation may be due to the 6µM fixed dose of palmatine used. It was expected that at this dose the compound would cause 5% growth inhibition of the cancer cells, judging the activity of the compound from single agent studies. An alternative would be to repeat these studies with different palmatine concentrations to see if synergy is observed.

This observed activity of the agent, as a result of the concentration used is further shown from a study of palmatine on the induction of tumour suppressor miRNA miR-200c in MCF7 breast cancer cells. These researchers noted that palmatine could induce the MiRNA miR-200c at a dose of 10μM, and exerted antiproliferative activities on the breast cancer cells (Keitaro et al., 2015).

## 5.2.11 Apoptosis studies of Palmatine

### Caspase 3/7 activity

In order to investigate the possible route of cell death, experiments were conducted to evaluate the activity of palmatine on caspase 3/7. In these experiments, the effect of drug combination between palmatine and carboplatin was further examined. Additionally, the effect of palmatine and paclitaxel was also investigated (Figure 5.11).

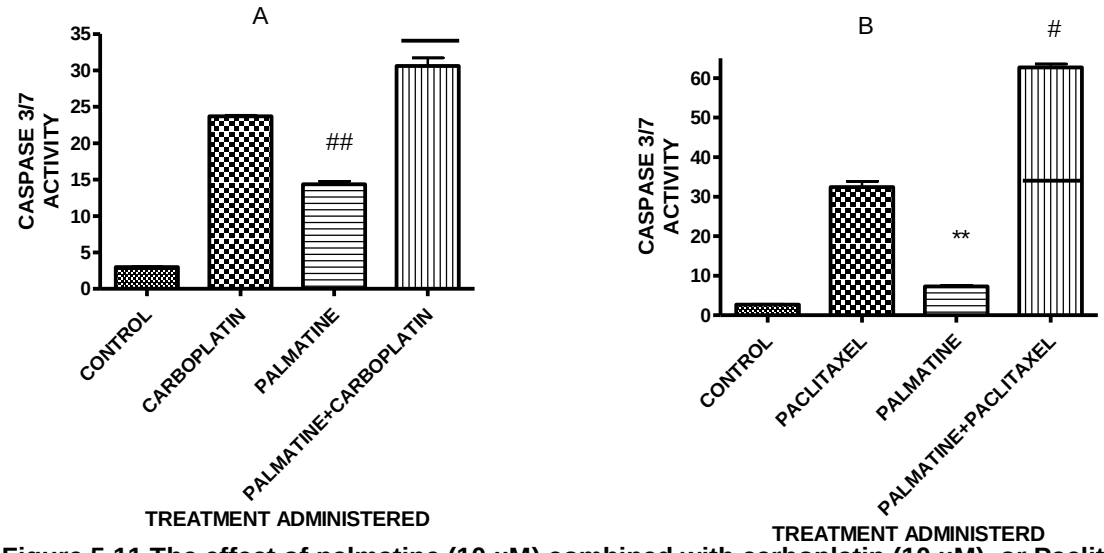



**Figure 5.11 The effect of palmatine (10 μM) combined with carboplatin (10 μM), or Paclitaxel (10 nM) on Caspase 3/7 activity at 48h for carboplatin (A) combination and 36h for paclitaxel (B) combination on OVCAR 4 cell line. The caspase activity was measured and normalized with corresponding SRB-stained cells to estimate the surviving cell number. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. * denotes that the result is significantly different (P<0.05, paired t-test) from the effect expected from the Bliss independence criterion. While ** and ## denotes significant difference (P<0.05; P<0.001) respectively from the control, one-way ANOVA. The results were expressed as mean ±SD, n=3. from the Bliss independence criterion. While ** and ## denotes significant difference (*P<0.05*; *P<0.001*) respectively from the control, *one-way ANOVA*. The results were expressed as mean ±SD, n=3.**

The results contained in Figure 5.11, demonstrated that palmatine significantly increased caspase 3/7 activity at greater at *p<0.05* in comparison to the vehicle-treated cells (control) at a concentration of 10µM for an experimental period of 36-48h. The combinations with paclitaxel also showed statistically significant caspase 3/7 activity, greater at *p<0.001*than that which would be expected for additivity with the bliss expected effect. However, it was observed that the combination of palmatine and carboplatin did not attain the bliss expected effect and the result was not statistically significant. These findings suggest potential synergy between palmatine and paclitaxel. While the results from the combination of palmatine with carboplatin may be indicative of mild additivity which was also demonstrated in the drug combination studies. It may be that the observed synergy in the trypan blue assay was due to the use of an increased concentration of carboplatin 40µM in the combination with 10µM palmatine.

## Flow Cytometric analysis

Further investigation of the observed apoptosis of palmatine, and potential synergism between palmatine and carboplatin was carried out by means of Annexin V/Propidium iodide labelling, followed by flow cytometry analysis (Table 5.12, Figure 5.12and 5.13).

**Table 5-12 The Effect of Palmatine and carboplatin Combinations was assessed (for the apoptotic cells) after 48h of treatment by Annexin V/PI Staining of the OVCAR 4 cells followed by flow cytometry analysis.** Results are expressed as mean±SD, n=3 experiments.

| TREATMENT | PERCENTAGE OF APOPTOTIC CELLS MEAN±SD |
|---|---|
| CONTROL | 6.2±0.7 |
| CARBOPLATIN(20µM) | 17.0±1.8 |
| PALMATINE(10µM) | 10.0±1.0 |
| PALMATINE + CARBOPLATIN | 22.8±1.1 |

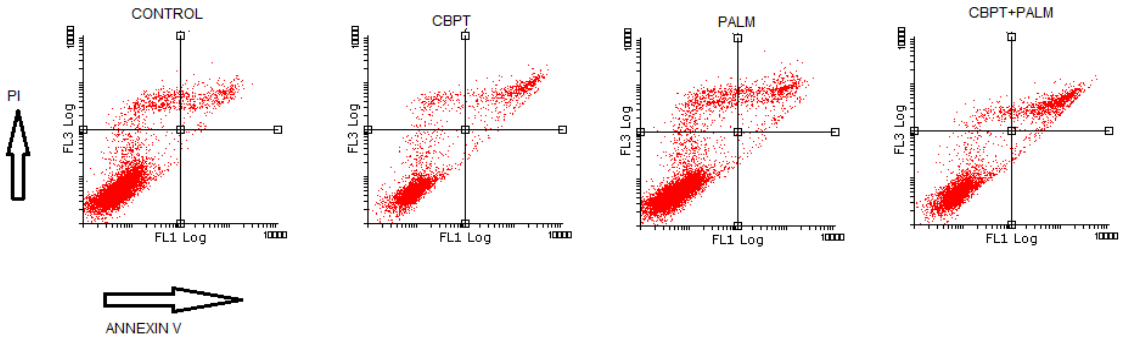



**Figure 5.12 The effect of palmatine (10 µM) combination with carboplatin (20 µM) and the agents singly on OVCAR 4 cells treated for 48h was assessed by Annexin V/PI staining, followed by flow cytometry analysis. The vehicle-treated cells were used as the control. The results were expressed as a representative of three experiments.**

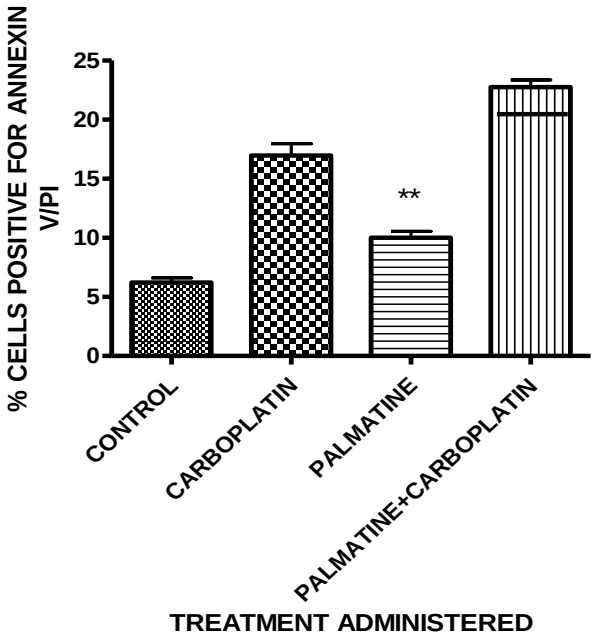



**Figure 5.13 The Effect of palmatine (10 µM) combined with carboplatin (20 µM) and the agents singly on Annexin V/PI staining on OVCAR 4 cells analysed by flow cytometry at 48h. It was assumed that at a concentration of 20 µM, carboplatin caused a maximum effect. However, this was not the case. A representation of the quantification of the combined early and late phase apoptotic cells is shown. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. ** denotes that the result is significantly different (P<0.05, paired t-test) from the control. The results were expressed as mean ± SD, n=3.**

The results of the Annexin V/PI studies shown in Table 5.12 and Figure 5.13 showed that palmatine induced significant increase in apoptosis compared to the control. Again these results did not demonstrate synergism between palmatine and carboplatin at the 10µMwhich

further confirmns the mild additivity observed in the caspase 3/7drug combination experiments and in the SRB cell growth assay. An important observation is that palmatine's route of cell death may be by apoptosis. This view is again amplified, by the flow cytometry analysis of palmatine-treated MCF-7 cells, which showed significant apoptosis and an increase in intracellular ROS level, as reported by Wu and co-workers (Wu et al., 2016b).

Further investigations of the combination of Palmatine (20µM) and carboplatin (40µM) was undertaken to determine the possible existence of synergism between the agents was carried out by means of Annexin V/Propidium iodide labelling, followed by flow cytometry analysis (Figure 5.14).

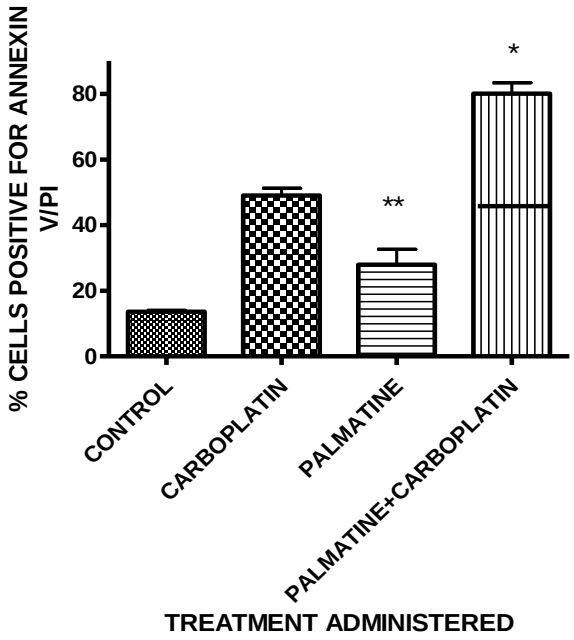



**Figure 5.14 The Effect of palmatine (20 µM) combined with carboplatin (40 µM) and the agents singly on Annexin V/PI staining on OVCAR 4 cells analysed by flow cytometry at 48h. A representation of the quantification of the combined early and late phase apoptotic cells is shown. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. * denotes that the result is significantly different (P<0.05, paired t-test) from the effect expected from the Bliss independence criterion. While ** denotes that the result is significantly different (P<0.05, paired t-test) from the control. The results were expressed as mean ± SD, n=3.**

The results presented in Figure 5.14demonstrated synergism between palmatine and carboplatinat an increased concentration of both agents. The induced apoptosis of palmatine was significant from the control. By means of these results **hypotheses b** and **c** where tested. Furthermore, these results have shown that significant synergism between palmatine and carboplatin may be observed at concentrations ≥10μM for palmatine and for concentrations of carboplatin ≥40μM. The lack of synergism in the drug combination of the caspase 3/7 and in the SRB cell growth assay was due to the concentrations of the agents employed in the assay.Further investigations of the apoptosis induced by palmatine were monitored microscopically for 48-72h.

## Morphological change

The morphological changes of cells treated with palmatine were monitored microscopically at 48-72h (Figure5.15).

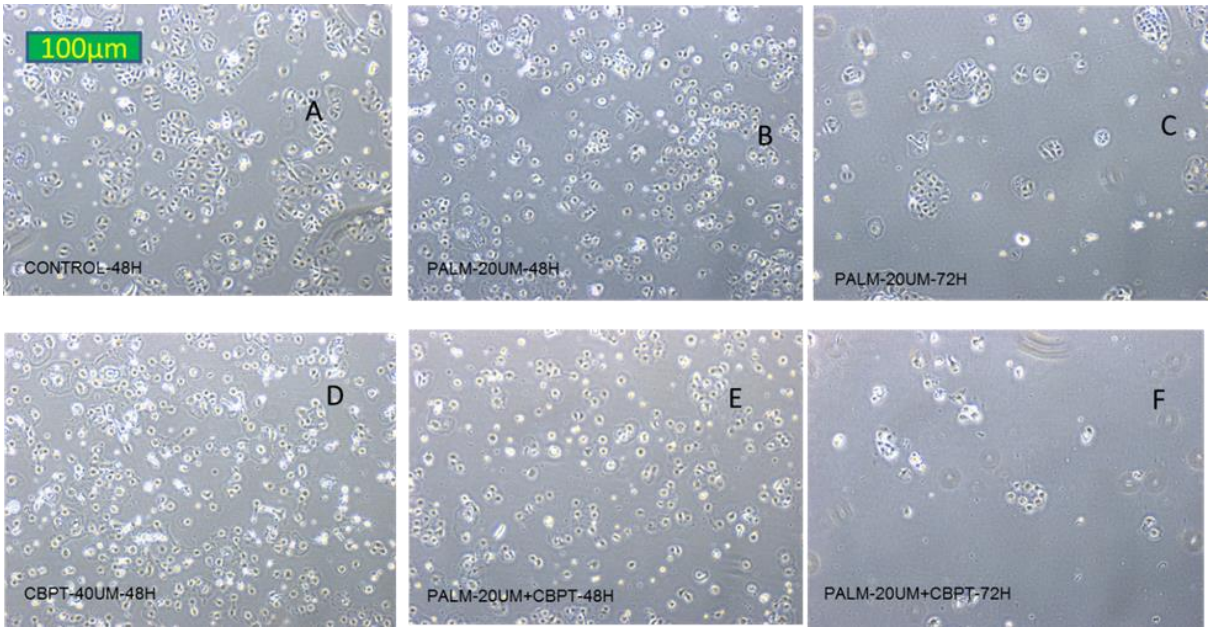



**Figure 5.15 The effects of palmatine (20 μM) on cell morphology of OVCAR 4 cells monitored by light microscope (100 μm) for 48-72h after treatment. The Morphological changes observed in the vehicle-treated OVCAR 4 cells which was used as the control (A), OVCAR 4 cells treated with 20 μM palmatine at 48h (B), cells treated with 20 μM palmatine at 72h (C), cells treated with 40 μM carboplatin at 48h (D), cells treated with a combination of carboplatin and palmatine at 48h (E) and at 72h (F) respectively. The results were expressed as a representative of three experiments.**

The characteristic features of apoptosis such as blebbing and shrinkage of cells were clearly observable in the pictures of the cell morphology (Figure 5.15). The finding from the caspase and the Annexin V/PI staining was consistent with the observed morphological changes and indicative of apoptosis.

### Western Blotting

To confirm these results, measurement of PARP [Poly (ADP-Ribose) Polymerase] (which is a DNA repair nuclear enzyme) cleavage (Figure 5.16) below was assessed by immuno-staining.

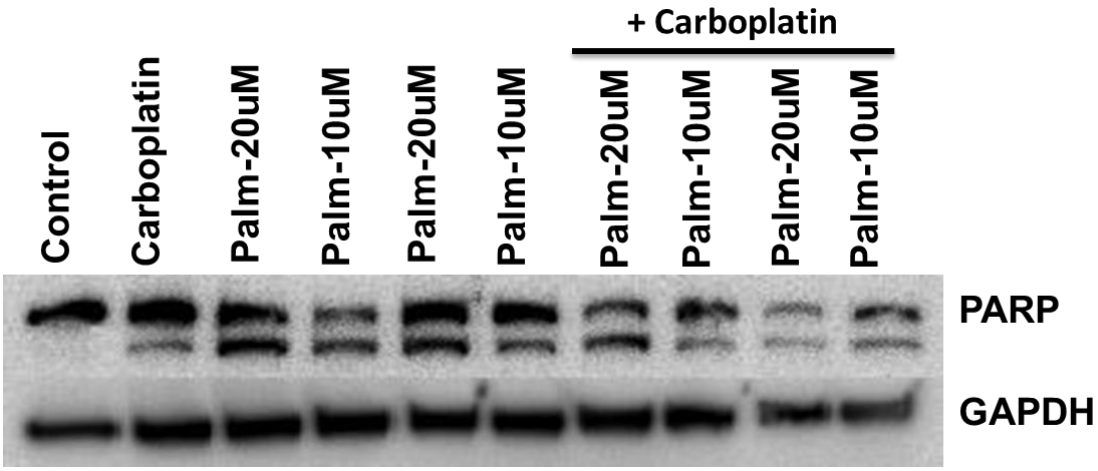



**Figure 5.16 PARP Cleavage by the indicated concentrations of Palmatine and carboplatin (40 µM), singly and in combination on OVCAR 4 cell line at 48h post treatment, determined by immunoblotting. The vehicle-treated cells served as the control. The results were expressed as a representative of three experiments.**

PARP cleavage is a well-established method of apoptosis investigation, as PARP is a substrate for caspase 3, which is cleaved in the course of apoptosis into two fragments (D'Amours et al., 1998). PARP is a DNA repair nuclear enzyme which detects DNA fragmentation (Trucco et al., 1998). The cleavage of PARP-1, often referred to as PARP is a confirmation of apoptosis. It is also considered that caspase 7 is involved in the *in vivo*

cleavage of PARP (Germain et al., 1999).Significant PAPR-1 cleavage was observed after treatment of palmatine or a combination of palmatine with carboplatin.

These results were consistent with the induced apoptosis activities of palmatine in the caspase 3/7 assay, the Annexin V/Propidium Iodide staining analysed by flow cytometry and the microscopic examinations of treated cells at 48h and 72h respectively.

The findings from this present research showed significant synergism between palmatine and carboplatin which was demonstrated in the TBA cell growth drug combination. But in the caspase 3/7 and SRB cell growth drug combination studies this synergy was not observed which may be attributed to the concentration of the agents employed in these assays.

## *5.3 Discussions of Rutidea parviflora and Palmatine*

The research in this chapter is the results of the investigations of the anti-cancer activities of *R. parviflora* (RP) on ovarian cancer cells. The preliminary screening of the plant for growth inhibition showed that the crude extracts of the plant had significant antiproliferative activities. Fractionation of the organic fraction showed that the potent compounds in the extract were in the ethyl acetate and n-butanol fractions. Further purification resulted in the isolation of two bioactive compounds: Urs-12-en-24-oic acid, 3-oxo-, methyl ester, which was determined by NMR and GC-MS analysis, and Palmatine which was confirmned by NMR and LC-MS. The third bioactive compound; Gallic acid was determined by GC-MS. Evaluation of the inhibitory activities of these compounds demonstrated that palmatine was the most potent bioactive compound. The anti-cancer activities of palmatine have been reported from several studies. An evaluation of the cytotoxicity of palmatine on three human cancer cell lines; CEM (acute lymphoblastic leukaemia), CEM/VCR(vincristine-resistant CEM) and SMMC7721, and two rat cancer cell lines; Lewis (lung carcinoma) and K III a melanoma showed that the compound was cytotoxic at micromolar concentration in all the cell lines investigated. Further *in vivo* study of the compound on a murine sarcoma, S180 xenograft model recorded a tumour inhibition rate of 34.09% at a dose of 2.5mg/kg (Lei et al., 2012). Similar findings were made

in a study of osteoporosis using mouse preosteoclastic cell line (RAW 264.7) (Shintaro et al., 2016). The compound has been found active against prostate cancer *in vitro* (Hambright et al., 2015). Palmatine has also been shown to demonstrate significant photo-cytotoxicity in colon cancer HT-29 cells (Wu et al., 2016a) and MCF-7 breast cancer cells, (Wu et al., 2016b). The anti-malarial activities of berberine, palmatine and jatrorrhizine which are also berberine-type alkaloids have been reported *in vitro*. The compounds exhibited similar activities comparable to that of quinine (Vennerstrom et al., 1988).

Further investigations of the cytotoxic activities of palmatine by trypan blue assay were undertaken in the cell growth assay. The results demonstrated that palmatine was cytotoxic to ovarian cancer cells. These results confirmed the antiproliferative activities of the compound observed in the SRB cell growth assay. Apoptosis studies were carried out to investigate the possible route of cell death induced by palmatine. The results showed that palmatine induced significant apoptosis both in the caspase 3/7 assay and in the Annexin V/Propidium iodide labelling, which was analysed by flow cytometry.

There have been conflicting reports about the apoptosis-inducing action of palmatine. One Group had earlier reported a lack of apoptosis in a DNA interaction study of HL-60 leukaemia cells, in which 25µg/mL of the agent was used. The experimental time was 24h (Kuo et al., 1995). Recently, the apoptosis inducingactivity of palmatine on RAW 264.7, a mouse pre-osteoclastic cell line was observed at concentrations of 10µM, 40µM and 100µM for a treatment period of 24h (Shintaro et al., 2016).

The results of this present studies is in line with the findings of the later researchers. This current research has investigated the apoptotic activities of palmatine at a concentration of 10-20µM at an experimental duration of 36-48h. The observations from these studies were that palmatine is an apoptosis inducer and potentially synergises with the standard ovarian cancer drug-carboplatin.

Previous studies have showed that berberine and coptisine, which are classed together with palmatine as protoberberine cationic alkaloids, due to the presence of a quaternary nitrogen atom, bearing a positive charge are capable of inhibiting mitochondrial respiration and induce

cancer cell death by causing alterations in the mitochondrial bioenergetics(Barreto et al., 2003).

Palmatine has been shown to accumulate mitochondrial potential, Δψm, which has been exploited in the targeting of anti-oxidants into the mitochondria (Félix et al., 2015).Mitochondria have being implicated in cellular death, especially by apoptosis, through the intrinsic pathway(Galluzzi et al., 2010). This research work shows that palmatine is an apoptosis inducer and merits further studies on the agent's mechanism of action in cancer cells and *in vivo*.

## *5.4 Conclusion*

An investigation of the anti-cancer activities of RP on ovarian cancer cells has been undertaken. This research is the first pharmacological and phytochemical study of this plant. Ethno medicinal use of this plant in folklore is limited, and no research has reportedly been carried out to validate these traditional medicinal uses. RP, a member of the Rubiaceae family from the genus *Rutidea* which has 21 species require further investigation for possible bioactivities.

This research work has shown that RP has significant cytotoxic activities on cancer cells. Activity was demonstrated in both the aqueous and organic extracts of the plant, with the focus of this research work on the organic extract. Three bioactive compounds: Palmatine, Urs-12-ene-24-oic-3-oxo-methyl ester and Gallic acid were identified and isolated/authenticated. The most bioactive compound isolated in this study was palmatine, which showed significant activity both in the cell growth assays and in the apoptosis assays.

In conclusion, this present study has shown that palmatine possess anti-cancer activities, with apoptosis as the route of cell killing. The compound has demonstrated very significant synergism with carboplatin, a first-line clinically used drug in the treatment of ovarian cancer in the TBA experiment. However, a lack of synergy with carboplatin was observed in the SRB

cell growth drug combination experiment, caspase 3/7 and Annexin V/PI assays as a result of the low concentrations used. Further apoptosis studies undertaken observed significant increase in apoptosis induced by palmatine singly and in combination with paclitaxel in the caspase 3/7 assay. At an increased concentration of palmatine and carboplatin, an investigation for potential synergy between the agents by Annexin V/Propidium iodide labelling, followed by flow cytometry analysis was carried out. The obtained results demonstrated increased apoptosis of the combined agents, which was significant from the bliss expected effect at $P<0.05$; an indication of the existence of a possible synergism between the agents. Again, there is some evidence from the caspase experiments that this compound may synergise with paclitaxel, another first-line ovarian cancer drug. Palmatine does show some preferential selective cytotoxicity for cancer cells, with minimal inhibition on normal cells. This study has shown that palmatine is a potential agent for the treatment of ovarian cancer and the agent merits further investigations.

Finally, this is the first report of the anti-ovarian cancer activities of *Rutideaparviflora*, a medicinal plant, identified from folk medicine.

# Chapter Six

## *Anti-Ovarian Cancer Investigations of Synthetic Thymoquinone Analogues.*

## *6.1 Introduction*

Thymoquinone (TQ), the most bioactive compound in *nigella sativa* (NS) is a benzoquinone that has received intense scientific attention evidenced by the numerous pharmacological researches on the compound, since its isolation and characterization in 1963 by El-Dakhakhny. Documented activities of TQ abound with the compound shown to be a potent inhibitor of several cancer cell lines such as prostate, ovarian, colon, breast, and pancreatic cancers, including leukaemia and osteosarcoma (El-Mahdy et al., 2005; Rooney et al., 2005; Richards et al., 2006; Roepke et al., 2007; Kaseb et al., 2007; Alhosin et al., 2010; Jafri et al., 2010; Koka et al., 2010; Effenberger-Neidnicht et al., 2011; El-najjair et al., 2011; Woo et al., 2011). TQ is reported to possess Chemo preventive activity against pancreatic cancer. Other bioactivities of TQ include anti-inflammatory and anti-oxidant activities. Also, investigations into the anti-microbial, anti-asthmatic, immunostimulant, anti-hyperglycemic, anti-histaminic and anti-parasitic have been carried out (Al-Ali et al., 2008).

While most researches have focused on the investigation of TQ's anti-cancer activities, less emphasis have been placed on the development of novel TQ-analogues, with more potent anti-cancer activities. The need for more potent TQ analogues is further heightened by the moderate anti-cancer activities of TQ, despite the compound's apparent safety as suggested by the large $LD_{50}$ values obtained from both oral and intraperitoneal administration of the compound in mice and rats reported in literature. These values obtained from intraperitoneal injection and oral administration of the compound respectively were 10-15 times and 100-150 times more than the doses required for the induction of anti-cancer, anti-oxidant and anti-inflammatory activities (Al-Ali et al., 2008).

The research in this chapter aims to synthesise more potent analogues of TQ, investigate the growth inhibition of TQ analogues on A2780, OVCAR 8, CIS-A2780, and HOE cell lines. In order to generate a SAR for TQ, ten commercially available analogues were procured and evaluated together with eleven synthesised analogues on OVCAR 8, A2780, and CIS-A2780 ovarian cancer-cell lines. Further investigation into the selectivity index of these analogues was carried out on the human ovarian epithelial (HOE) cells to determine the preferential selectivity in the inhibitory activities of the analogues for cancer cells in comparison with normal cells. Solubility studies were carried out to determine the solubility of TQ, the most promising synthesised analogue and the most potent procured analogue. The most promising synthesised analogue was further investigated for cytotoxicity by the trypan blue assay. Apoptosis investigations were further carried out by measurement of caspase 3/7 activity and flow cytometry to determine the possible route of cell death induced by the most potent synthesised analogue. Drug combination experiments of the most potent synthesised analogue and clinically used first line ovarian cancer drugs were also undertaken.

## *6.2 Experimental methods*

### 6.2.1 Design of TQ analogues

Previous synthesis of TQ analogues with anti-cancer activities, have been carried out by some researchers. One group conjugated TQ with terpenes such as fatty acids found in the *nigella sativa* oil and betulinic acid. Their observation was that activity was dependent on the length of the spacer group between the quinone and the terpene. Analogues with a four-carbon spacer exhibited better cytotoxicity on the four cancer cell lines tested. The range of $IC_{50}$s obtained in this study was, 7-79.9μM for the cervical carcinoma cell line, KB-V1/Vbl (Effenberger et al., 2010). Banerjee and his co-workers synthesised 27 TQ analogs in a SAR study of TQ for Pancreatic Cancer. This study investigated a series of 2, 5-bis (alkyl/aryl-amino) 1,4-benzoquinones amines. The researchers observed from the study that bulkier substituents had $IC_{50}$s >10μM, while the $IC_{50}$s of compounds with smaller substituents were

<10μM on pancreatic cancer *in vitro* (Banerjee et al., 2010). This same researchers recently synthesised anti-cancer TQ analogues investigated on pancreatic cancer, published in 2013 (Yusufi et al., 2013). The referenced paper reported the moderate activities of 3-aminothymoquinone which was the building block for some of the analogues which were not disclosed. Also, the researchers observed from the comparison of the cytotoxic effect of one hydroxylated TQ analogue and one fluorinated TQ analogue that the hydroxylated TQ analogue had better activities than the fluorinated derivative.

In designing the TQ analogues to be synthesised, three objectives were considered:

Synthesis of six amino-TQs aimed at investigating the role of the position of amino substituent group on the aromatic ring on anti-cancer activity;

An investigation of the effect of bulky substituent groups on the anti-cancer activity;

Synthesis of five hydroxylated TQs aimed at a comparison of the activities of the corresponding benzoquinones and these synthesised hydroxylated TQs.

These considerations formed the basis of the synthesis of the eleven TQ analogues.

The ten procured analogues were selected based on four aims:

The investigation of the effect of halogen atoms on the aromatic ring on anti-cancer activity

The effect of chain length of alkyl substituents on the aromatic ring on anti-cancer activity

Comparison of the activity of an isopropyl group and a tertiary butyl group on the aromatic ring.

A further comparison of the activity of a 2,5-substituent group and a 2,6-substituent group on the aromatic ring.

This study employed these seven aims to derive a SAR for the anti-ovarian cancer activities of TQ.

## 6.2.2 Materials and Methods of Synthesis

## 6.2.2.1 Materials

Procured TQ Analogues; 2-Isopropyl-5-methyl-1,4-benzoquinone (TQ), 2-Methyl-1,4-benzoquinone, 2,5-Dibromo-6-Isopropyl-3-Methyl-1,4-Benzoquinone, 2-Tert-butyl-3,6-dichloro-5-methyl-1,4-benzoquinone, 2,5-Di-tert-butyl-1,4-benzoquinone, 2-Tert-butyl-1,4-benzoquinone, 2,6-Di-tert-butyl-1,4-benzoquinone, 2,6-Dimethylbenzoquinone, 2,5-Bis-(1,1-Dimethyl-propyl)-1,4-benzoquinone, 2-Tert-butyl-5-methyl-1,4-benzoquinone and 2,5-Di-tert-octyl-1,4-benzoquinone were purchased from Sigma Aldrich.

Reagents and solvents; 4-fluoroaniline, sodium azide, sodium hydrosulphite and deuterated chloroform were procured from Sigma Aldrich. Dimethyl amine, diethyl amine, glacial acetic acid, n-hexane, ethyl acetate, ethanol, methanol, trichloroacetic acid, hydrochloric acid, and sodium hydrogen carbonate were purchased from Fischer Scientific.

**Gas chromatography Mass Spectroscopy**

The GC-MS system consisted of an Agilent 7890 A gas chromatography systems, coupled to an Agilent MS model 5975C MSD with triple axis detector (Agilent Technologies, US). The instrument's column is a HP5-MS column.

The gas chromatography began with an oven temperature of 60°C for 2min, which increased to 300°C at the rate of 10°C/min, which was held at 300°C for 9 min to yield a total run of 30min under a constant helium pressure (10psi). The mass spectrometry utilized an electron impact (EI) source at 70eV with an ion source temperature of 230°C.

**$^{1}$H NMR Spectroscopy**

$^{1}$H NMR spectra were obtained at 300 or 400 MHz with a Bruker 1D NMR spectrometer. $^{13}$C and DEPT NMR spectra were obtained at 75 MHz or 100MHz with a 300 or 400 Bruker NMR spectrometer respectively.

### 6.2.2.2 Methods of Synthesis of the TQ Analogues

*6-Isopropyl-2-methylamino-3-methyl-1,4-benzoquinone* (**3**). 164mg of TQ was added to 0.5ml of dimethyl amine in a round-bottom flask. 1ml of water was added into the mixture. The mixture was stirred for 2h at RT, followed by evaporation of the water on a rotary evaporator. The dried product was subjected to TLC in a 2:1 n-hexane: ethyl acetate solvent system, upon which four spots were obtained. Column chromatography yielded four fractions. One of which gave one-spot with an rfof 0.5, fractions 2 (compound **3**, 11.8mg, yield of 7.2%) a bright red amorphous powder was obtained. GC-MS, Retention time (Rt)=16.116 min. MS [electron ionization (EI)] *m/z* (relative intensity, %): 193.1 (100) $[M]^{\bullet+}$, 178.1 (35) $[M\text{-}CH_3]^{\bullet+}$, 165.1 (50) $[M - CH_2CH_3]^{\bullet+}$, 136.1, 122.1, 108.1, 94.1, 69.1, 53.0, 41.1.$^1$H NMR (300MHz, $CD_3OD$), δ 6.36 (s, 1H), 3.19 (s, 3H), 3.09 (m, 1H), 2.08 (s, 3H), 1.13 (d, 6H, J =7.0 Hz).$^{13}$C NMR (300 MHz, , $CD_3OD$), δ 133.22, 117.69, 113.03, 32.73, 27.34, 26.85, 26.48, 22.67, 21.34, 15.38, 9.97.

Fraction 5 contained compound **3a** **(***6-isopropyl-2-dimethylamino-3-methyl-1,4-benzoquinone***)**and several impurities, (compound **3a**, 27.6mg, yield of 15.3%), GC-MS, Retention time (Rt)=14.713 min. MS [electron ionization (EI)] *m/z* (relative intensity, %): 207.1 (100) $[M]^{\bullet+}$, 192.1 (90) $[M\text{-}CH_3]^{\bullet+}$, 177.1 (40) $[M - CH_3CH_3]^{\bullet+}$, 164.1, 149.1, 136.1, 122.1, 108.1, 94.1, 69.1, 53.0, 41.1.$^1$H NMR (300MHz, $CDCl_3$), δ 6.30 (s, 1H), 3.00 (s, 6H), 3.00 (m, 1H), 1.95 (s, 3H), 1.10 (d, 6H, J =7.0 Hz). $^{13}$C NMR ($CDCl_3$), δ 188.43, 185.91, 152.42, 151.54, 130.41, 122.80, 108.2, 43.42, 26.62, 21.4,14.43. Column chromatography purification was carried out, but the compound could not be obtained without the impurities. Further attempts to purify the compound on the HPLC, resulted in the presence of more impurities in the fraction. This compound was considered impure (<90% purity) and was excluded in the cytotoxicity assays.

*2-Ethylamino-6-isopropyl-3-methyl-1,4-benzoquinone* (**4**). 164mg of TQ was added to 1ml of Diethylamine in a round-bottom flask equipped with a stirrer, 1ml of water was introduced into the reaction mixture to act as catalyst, The reaction was heated for 2h at a temperature of <30$^{o}$C with stirring. The resultant solution was dried on a rotary evaporator at a temperature <40$^{o}$C. The obtained dry product was subjected to TLC in a 2:1 n-hexane: ethyl acetate solvent system, upon which three spots were obtained. The dry product was purified on a column using various combinations of the n-hexane and ethyl acetate solvent system. 19 Fractions were obtained and subjected to TLC, of which fraction 4(compound **4**, 73.5mg, 32.0%), a bright red amorphous powder gave a single spot with an rfof 0.5.GC-MS, Rt=14.521 min. MS [electron ionization (EI)] *m/z* (relative intensity, %):207.1 (70) $[M]^{\bullet+}$, 192.1 (100) $[M - CH_3]^{\bullet+}$, 178.1 (20) $[M - CH_2CH_3]^{\bullet+}$,164.1,150.0,136.1. $^{1}$H NMR (300MHz, $CDCl_3$) *δ*: 6.34 (s, 1H), 3.47 (q, 2H, J = 7.0 Hz), 2.89 (m, 1H), 2.01 (s, 3H), 1.19 (t, 3H, J = 7.2Hz), 1.03 (d, 6H,J =6.8 Hz). $^{13}$C NMR ($CDCl_3$), δ 186.94, 184.72, 149.67, 144.40, 133.23, 108.11, 40.02, 26.53, 21.34, 16.22, 10.11.

Fraction 6 contained **4a**, (*2-Diethylamino-6-isopropyl-3-methyl-1,4-benzoquinone*, another compound **4b**,*2-Diethylamino-6-isopropyl-3-methyl-1,4-hydroquinone* and three other unknown impurities (compound **4a**and **4b**, 32.6mg, yield of 14.2%), GC-MS, Retention time was (Rt)=17.223 min and 17.511min respectively for **4a** and **4b**. MS [electron ionization (EI)] *m/z* (relative intensity, %) for **4a**: 235.2 (30) $[M]^{\bullet+}$, 220.2 (100) $[M\text{-}CH_3]^{\bullet+}$, 206.1 (10) $[M - CH_2CH_3]^{\bullet+}$, 192.1, 176.1,162.1, 150.0, 133.1, 121.1, 103.0, 91.1, 77.0, 67.0, 53.1, 41.1.MS [electron ionization (EI)] *m/z* (relative intensity, %) for **4b**: 237.2 (70) $[M]^{\bullet+}$, 222.2 (90) $[M - CH_3]^{\bullet+}$, 208.2 (100) $[M - CH_2CH_3]^{\bullet+}$, 194.1, 178.1, 164.1, 152.0, 135.1, 123.1, 105.0, 93.1, 79.0, 67.0, 55.1, 43.1.

Column chromatography and HPLC purifications were carried out, but these compounds could not be resolved and thus were considered impure and excluded from further cytotoxicity assays.

*2-Dimethylamino-5-methyl-1,4-benzoquinone* (**6**) and *6-Dimethylamino-5-methyl-1,4-benzoquinone* (**7**). 122mg of 2-methyl-*p*-benzoquinone was added to 0.5µl of dimethyl amine in a round-bottom flask.1ml of water was added to catalyse the reaction. The reaction was stirred for 2h at room temperature. The resultant solution was extracted with petroleum ether and the petroleum extract fraction dried on the rotary evaporator at a temperature of <40°C. The obtained product was subjected to TLC in a solvent-system of 2:1 n-hexane to ethyl acetate. Five spots were observed from the TLC. Subsequently, the dried product was purified by column chromatography. Six fractions were collected of which fraction 5 gave one spot upon TLC investigation. Fraction 6 was further purified on column to obtain three sub-fractions. TLC investigation showed that one of the sub-fractions (sub-fraction 3) gave one spot. Fraction five and sub-fraction 3 were further subjected to GC-MS and $^{1}$H NMR investigation which showed that the fractions were isomers of the same compound. Fraction 5(compound **6**, 49.5mg, yield of 30.00%) is a dull red amorphous powder. GC-MS, Rt =14.666 min.MS [electron ionization (EI)] *m/z* (relative intensity, %): 165.1 (100) $[M]^{\bullet+}$, 150.1 (38) $[M - CH_3]^{\bullet+}$, 137.1 (30) $[M - CHCH_3]^{\bullet+}$, 122.1, 108.1, 94.1, 82.1, 69.1, 53.1, 42.1. $^{1}$H NMR (300MHz, $CDCl_3$), δ 6.40 (s, 1H), 5.50 (s, 1H), 3.11 (s, 6H), 1.95 (s, 3H). $^{13}$C NMR ($CDCl_3$), δ 185.92, 185.64, 151.22, 142.74, 134.5, 104.53, 42.41, 15.82.

Sub-fraction 3 (compound **7**, 9.76mg, yield of 8.0%); a dull red amorphous powder. GC-MS, Rt= 14.479 min.MS [electron ionization (EI)] *m/z* (relative intensity, %): 165.1 (43) $[M]^{\bullet+}$, 150.1 (65) $[M - CH_3]^{\bullet+}$, 137.1 (11)$[M - CHCH_3]^{\bullet+}$, 122.1, 108.1, 96.1, 82.1, 69.1, 53.1, 42.1. $^{1}$H NMR (300MHz, $CDCl_3$), δ 6.30 (s, 1H), 5.52 (s, 1H), 3.10 (s, 6H), 1.98 (s, 3H). $^{13}$C NMR ($CDCl_3$), δ 185.44, 185.43, 150.62, 147.41, 131.13, 104.24, 42.33, 15.82.

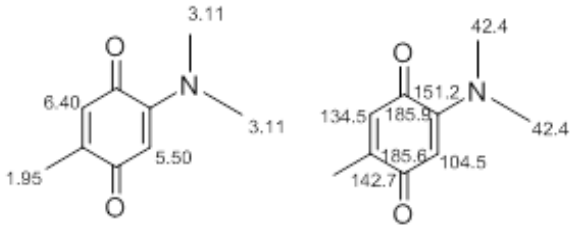



**Figure 6.1$^{1}$H NMR and $^{13}$C NMR Assignment for Compound 6**

*2-(4'-Fluoroanilinyl)-5-methyl-p-benzoquinone* or *2-(4'-Fluoroanilinyl)-6-methyl-p-benzoquinone* (**8**). 0.48ml of HCl and 0.48ml of iced deionised water were placed in a round-bottom flask equipped with a stirrer. 0.11ml of fluoroaniline was added drop wise. The mixture was stirred for 2h at 0-5$^{o}$C and yielded a clear solution which was filtered. The filtrate was added drop wise to a mixture of 120mg of 2-methyl-*p*-benzoquinone, 200mg of sodium bicarbonate and 70ml of water in a flask. The reaction mixture was stirred at 8-12$^{o}$C for 2h and then at room temperature for 2h. A brown solution was obtained. This was subsequently extracted with 200ml of ethyl acetate to yield the reaction product. Upon TLC, three spots were visualised. The reaction product was purified by column chromatography. Ten fractions were obtained using a solvent-system of different combinations of n-hexane and ethyl acetate. Fractions 5gave one spot in the TLC (compound **8**, 6.2mg, yield of 2.7%); a brownish red amorphous powder. GC-MS, Rt=19.771min.MS [electron ionization (EI)] *m/z* (relative intensity, %): 231.1 (100) $[M]^{\bullet+}$, 202.1 (30) $[M -CH_2CH_3]^{\bullet+}$, 184.1 (15),162.1 (40),135.1,108.1,89.1,68.1,41.1.$^{1}$H NMR (300MHz, $CDCl_3$), δ: 7.17(s, 4H), 6.58(s, 1H), 6.05(s, 1H), 2.11(s, 3H), 1.67(s, 1H).$^{13}$C NMR (400 MHz, $CDCl_3$), δ:166.55, 164.59, 152.83, 147.19, 127.56, 117.73, 117.13, 102.27, 101.73, 100.88, 92.21, 68.34, 32.73.

*2-Methyl-1,4-hydroquinone* (**10**) and *2-Isopropyl-5-methyl-1,4-hydroquinone* (**11**). Reduction of 2-methyl-*p*-benzoquinone and TQ respectively by the addition of Sodium hydrosulphite dissolved in 2ml of water in a ratio of 1:2 (TQ/MBQ dissolved in 2 ml of Methanol: $Na_2S_2O_4$). The reaction mixture was stirred continuously for 4h, during which the reaction was monitored by TLC for completion. This was followed by the evaporation of the

solvent. The obtained product was extracted severally by ethyl acetate. The combined ethyl acetate extracts was washed with water (3 × 10 mL) and then dried over sodium sulphate. After evaporation of ethyl acetate, the pure hydroxylated quinones were obtained as: (compound **10,** red crystals(103.8 mg, yield of 70%), GC-MS, Rt = 12.165 min.MS [electron ionization (EI)] *m/z* (relative intensity, %): 124.1 (53) $[M]^{\bullet+}$, 107.1 (100) $[M - CH_3]^{\bullet+}$, 95.1 (40) $[M - CH_2CH_3]^{\bullet+}$, 77.1, 67.1, 55.1, 41.1.$^1$H NMR (300MHz, $CDCl_3$), δ: 6.60, 6.50, 6.40, 2.55 (s, 3H), 2.05 (s, 3H).$^{13}$C NMR (δ, 149.19, 147.76, 117.83, 117.63, 115.78, 113.32, 15.95.

Compound **11,** white-yellow coloured crystals (139.7mg, 83.8%), GC-MS, Rt = 14.873min. MS [electron ionization (EI)] *m/z* (relative intensity, %): 166.1 (43) $[M]^{\bullet+}$, 151.1 (100) $[M - CH_3]^{\bullet+}$, 137 (4), 123.1 (11), 95.1 (10), 77.1 (10), 53.0 (3), 41.1.$^1$H NMR (300MHz, $CDCl_3$) δ:6.56 (s, 1H), 6.47 (s, 1H), 3.06 (m, 1H), 2.10 (s, 3H), 1.14 (d, 6H, J = 7.2 Hz). $^{13}$C NMR (400 MHz, $CDCl_3$), δ 147.75, 146.30, 133.12, 121.62, 117.71, 113.06, 26.87, 22.66, 21.05, 15.36

*2-Isopropyl-5-methyl-3-amino-1,4-benzoquinone* (**12**). 50mg of TQ was reacted in 65 mg of sodium azide dissolved in 1ml of 10% TCA. The reaction was maintained at 60-70$^o$C for 4h. The reaction was further monitored on the GC-MS to obtain the desired product. On completion of the reaction, the reaction mixture was extracted with ethyl acetate to obtain the crude product which was subjected to TLC and subsequently to column chromatography using a solvent system of n-hexane: ethyl acetate in a ratio of 1:1. Five fractions were obtained, of which fraction 3 had one spot on the TLC which yielded Compound **12**(Compound **12**, 9.6 mg, yield of 11.6%), an orange coloured amorphous powder. GC-MS, Rt=15.032 min. *m/z*: 179, 164, 151, 136, 124, 108, 91, 81, 67, 53, 41.$^1$H NMR (300 MHz $CDCl_3$) δ: 6.54(s, 1H), 2.98(s, 2H), 1.77(s, 3H), 1.53(m, 1H), 1.19(d, 6H).$^{13}$C NMR (300 MHz, $CDCl_3$) δ, 150.08, 122.50, 21.80, 8.20.

*2,5-Dibromo-6-Isopropyl-3-Methyl-1,4-hydroquinone* (**20**), *2,6-Dimethyl-1,4-hydroquinone* (**21**) and *2-Tert-Butyl-5-methyl-1,4-hydroquinone* (**22**). Reduction of 2,5-

Dibromo-6-Isopropyl-3-Methyl-1,4-benzoquinone, 2,6-Dimethyl-1,4-benzoquinone and 2-Tert-Butyl-5-methyl-1,4-benzoquinone respectively by the addition of sodium hydrosulphite dissolved in 2ml of water in a ratio of 1:2 (parent compound dissolved in 2 ml of Methanol: $Na_2S_2O_4$). The reaction mixture was stirred continuously for 4h, during which the reaction was monitored by TLC for completion. This was followed by the evaporation of the solvent. The obtained products were extracted severally by ethyl acetate. The combined ethyl acetate extracts was washed with water (3 × 10 mL) and then dried over sodium sulphate. After evaporation of ethyl acetate, the pure hydroxylated quinones were obtained as:

Compound **20**, reddish-yellow crystals(183.5 mg, yield of 85.0%). GC-MS, Rt=18.158 min.MS [electron ionization (EI)] *m/z* (relative intensity, %): 324.1 (65) $[M]^{\bullet+}$, 308.9 (100) $[M - CH_3]^{\bullet+}$, 228.1 (33), 121.1, 79.1, 67.1, 51.1.$^1$H NMR (300MHz, $CDCl_3$) δ: 3.49(m, 1H), 2.37(s, 3H), 1.29(d, 6H, *J*=7.2Hz).$^{13}$C NMR (300 MHz, $CDCl_3$) δ: 144.56, 144.15, 130.46, 122.26, 114.92, 19.83, 16.99.

Compound **21**, white crystals (134.8 mg, yield of 86.0%), GC-MS, Rt=12.914 min.MS [electron ionization (EI)] *m/z* (relative intensity, %): 138.1 (55) $[M]^{\bullet+}$, 123.1 (100) $[M - CH_3]^{\bullet+}$, 109.1 (64), 95.1, 77.1, 65.1, 53.0, 41.1.$^1$H NMR (300MHz, $CDCl_3$) δ: 6.47(s, 2H), 2.17(s, 6H).$^{13}$C NMR (300 MHz, $CDCl_3$) δ: 187.90, 145.84, 133.32, 16.17.

Compound **22,**brownish crystals (128.3mg, yield of 75.6%), GC-MS, Rt=15.682 min.MS [electron ionization (EI)] *m/z* (relative intensity, %): 180.1 (40) $[M]^{\bullet+}$, 165.1 (100) $[M - CH_3]^{\bullet+}$, 137.1 (80), 97.1, 65.1, 41.1.$^1$H NMR (300MHz, $CDCl_3$) δ: 6.73(s, 1H), 6.45(s, 1H), 2.15(s, 3H), 1.37(d, 9H). $^{13}$C NMR (400 MHz, $CDCl_{3)}$ δ: 147.76, 147.14, 135.46, 134.84, 131.56, 121.72, 118.85, 114.14, 34.19, 29.68, 15.13.

### Determination of aqueous solubility of TQ (1), compounds 6 and 16

The aqueous solubility of TQ (**1**), compounds **6** and **16** were investigated by the method described by Salmani and co-workers (Salmani et al., 2014). 10mg of each compound was

added to 1 ml of phosphate buffer (pH=7.4) in sealed amber glass bottles to prevent photo degradation and maintained on a shaker for 24h at a temperature of 37°C. The solutions were filtered through a membrane filter of size 0.45µm, and subsequently diluted before analysis of the dissolved solutes by HPLC. Quantification was carried out by calibration curves derived from five concentrations of the compounds prepared from a stock solution of 1mg of the respective compounds dissolved in 1mL of ethanol which was diluted with the phosphate buffer (pH=7.4) to yield 10, 20, 40, 60 and 80µg/mL concentrations. These experiments were replicated three times.

## *6.3 Results and Discussion*

The synthesised TQ analogues are described in the schematic representation (Figure 6.1). It could be seen that the hydroxylated analogues (reduced hydroquinones) had very high product yields, this could be as a result of the simple reduction reaction utilised in the synthesis of these compounds. Another observation is that the one-pot synthesis approach utilised less toxic chemicals, the method is economical with less environmental impact. Additionally, from this method, two new compounds were synthesised. A library search showed that compounds **4** and **8** have not being previously synthesised. While compounds **3** (Zincke 1881) and **6** (Kallmayer et al., 1986) had been synthesised previously but with no reported pharmacological activity. Previous syntheses of the hydroxylated compounds (**10, 11, 20, 21,** and **22**) were not taken into account as the possibility that these compounds may have been synthesised by others could not be ruled out. The aim of the synthesis of these hydroxylated analogues was to compare their antiproliferative activities with the corresponding quinones and in the determination of a SAR for the anti-ovarian cancer activities of TQ. The purity of the synthesised compounds were >93% as confirmed from the GC-MS quantification of the peaks.

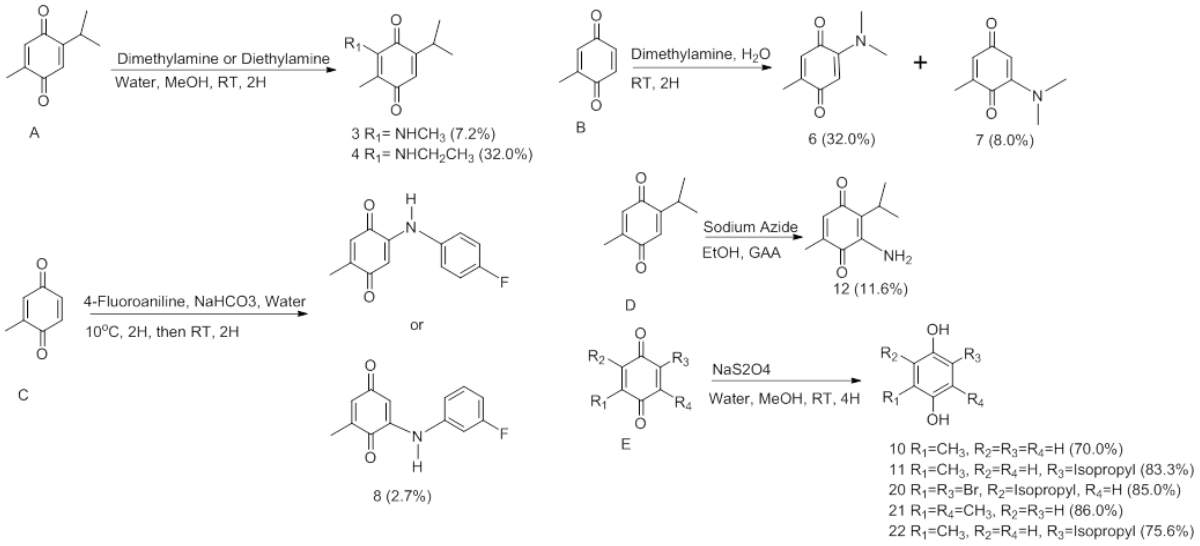
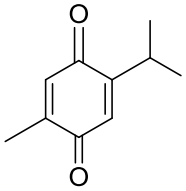
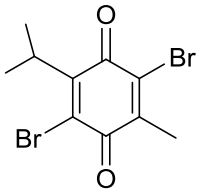



**Figure 6.2 The schemes for the synthesis of TQ analogues 3 and 4 (A), compounds 6 and 7 (B), compound 8 (C), compound 12 (D), and compounds, 10, 11, 20, 21 and 22 (E).**

Synthesis of **3, 4, 6** and **7** was carried out using a modified green protocol to obtain the aminobenzoquinones (Jhillu et. al., 2008). The benefits of carrying out amine addition to benzoquinones using the green protocol, is enormous. The protocol is cost- effective, has a high degree of environmental friendliness and additionally is a one-pot synthesis. Synthesis of 2-(4'-Fluoroanilinyl)-5-methyl-p-benzoquinone or 2-(4'-Fluoroanilinyl)-6-methyl-*p*-benzoquinone (structure **8**), was undertaken using a method described for the synthesis of poly (aryl ether ketones) from new bisphenol monomers (Guang et. al., (2010). This synthesis was carried out in order to obtain a secondary amine analogue of TQ that has a bulky substituent. This was necessary as all the other synthesised analogues, were secondary amine analogues, with a two-carbon side chain. Structure 12 was synthesised according to a modified method described by Moore and Shelden (Moore et al., 1968). Structures **10, 11, 20, 21** and **22** were synthesised by the reduction of the corresponding benzoquinone to hydroquinones. The GC-MS and $^{1}$H NMR characterizations were carried out for all the synthesised compounds.

Both the synthesised TQ analogues and the procured TQ analogues were investigated for growth inhibition on three ovarian cancer cell lines. The results of these investigations were used in obtaining a SAR for the anti-ovarian cancer activities of TQ. Further investigation of the Selectivity index, SI was carried out by comparing the $IC_{50}$ of the analogues on the CIS-A2780 cell line with the compounds $IC_{50}$ on the human epithelial ovarian cell line (HOE). The Selectivity index, SI of these compounds were determined (Table 6.1 and in Figure 6.3).

**Table 6-1 The Results of the growth inhibition evaluation on ovarian cancer cell lines and calculated SI and log P values of the TQ Analogues. The cells were treated for 72h with the indicated compound, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments**

| No. | Compound | LOG P | A2780(μM) | OVCAR8(μM) | CIS-A2780(μM) | HOE (μM) | SI |
|---|---|---|---|---|---|---|---|
| **1** |  2-Isopropyl-5-methyl-1,4-benzoquinone (TQ) | 1.04 | 7.9 ± 0.2 | 11.6 ± 0.3 | 7.8 ± 0.2 | 17.8±0.4 | >2 |
| **2** |  2,5-Dibromo-6-Isopropyl-3-Methyl-1,4-Benzoquinone | 1.66 | 4.9 ± 0.2 | 3.6 ± 0.3 | 5.3 ± 0.5 | 7.3±0.4 | 2.1 |

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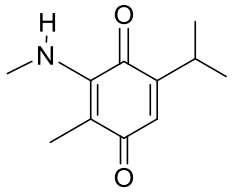
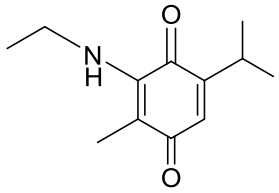
|---|---|---|---|---|---|---|---|
| **3** |  6-Isopropyl-2-Methylamino-3-Methyl-!,4-Benzoquinone | 0.01 | 14.0 ±0.4 | 12.9 ±0.6 | 7.3 ± 0.3 | 36.8±3.0 | >7 |
| **4** |  2-Ethylamino-6-Isopropyl-3-Methyl-1,4-Benzoquinone | 0.35 | 12.6 ± 0.2 | 16.0±0.7 | 9.6 ± 0.6 | 30.3±2.0 | >2 |

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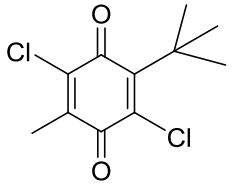
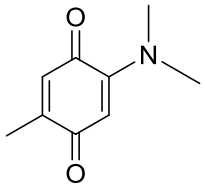
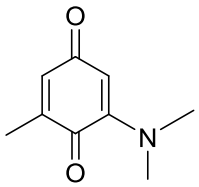
| **5** |  <br>2-Tert-Butyl-3,6-Dichloro-5-Methyl-1,4-Benzoquinone | 1.59 | 4.5±.05 | 6.0±0.4 | 4.2±0.6 | 3.1±0.3 | <2 |
| **6** |  <br>2-Dimethylamino-5-Methyl-1,4-Benzoquinone | -0.71 | 6.2 ± 0.2 | 5.6 ± 0.4 | 4.7 ± 0.5 | 7.9±0.9 | <2 |
| **7** |  <br>6-Dimethylamino-5-Methyl-1,4-Benzoquinone | -0.71 | 16.7±2.0 | 35.6±6.6 | 20.5±.3.0 | 28.2±3.9 | <2 |

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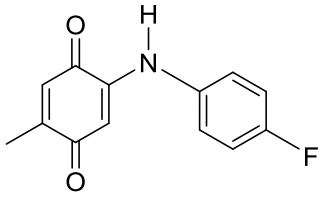
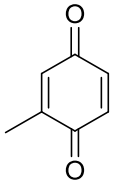
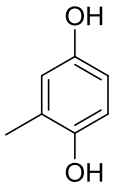
| **8** |  <br>2-(4'-Fluoroanilinyl)-5-Methyl-*p*-Benzoquinone | 0.74 | 15.0 ± 0.5 | 32.0 ± 3.9 | 21.8 ± 0.3 | 20.2±2.1 | <2 |
| **9** |  <br>2-Methyl-1,4-Benzoquinone | -0.06 | 12.1±0.4 | 13.4±2.5 | 11.8±0.5 | 6.0±0.3 | <2 |
| **10** |  <br>2-Methyl-1,4-Hydroquinone | 1.74 | 5.7±0.4 | 6.2±0.2 | 4.7±0.2 | 6.3±0.7 | <2 |

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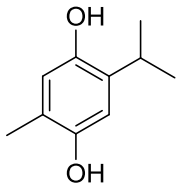
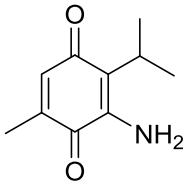
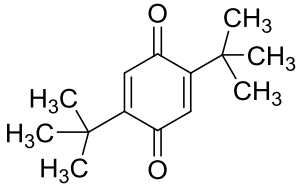
| **11** |  <br>2-Isopropyl-5-Methyl-1,4-Hydroquinone | 2.98 | 3.1±0.2 | 8.9±0.7 | 9.8±0.3 | 14.0±2.4 | 4.5 |
| **12** |  <br>2-Isopropyl-5-Methyl-3-amino-1,4-Benzoquinone | -0.51 | 19.9±0.6 | 35.1±0.4 | 19.3±1.5 | 138.9±5.0 | >7 |
| **13** |  <br>2,5-Di-tert-butyl-1,4-Benzoquinone | 2.73 | 34.0±5.6 | 51.2±0.4 | 42.5±1.9 | 117.4±1.6 | <5 |

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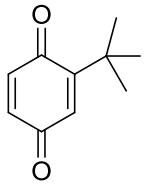
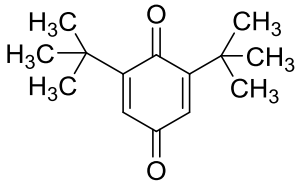
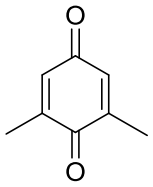
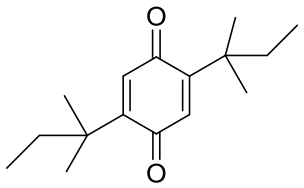
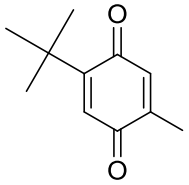
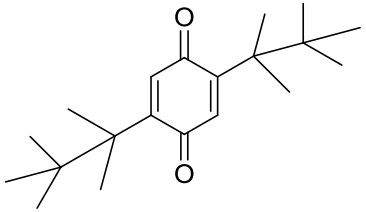
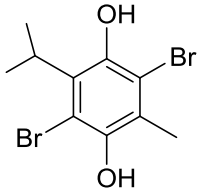
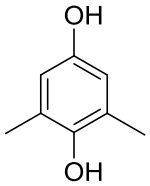
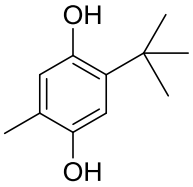
|---|---|---|---|---|---|---|---|
| **14** |  <br>2-Tert-butyl-1,4-Benzoquinone | 1.16 | 5.7±0.5 | 10.0±0.5 | 4.9±1.2 | 10.3±1.1 | >2 |
| **15** |  <br>2,6-Ditert-butyl-1,4-Benzoquinone | 2.73 | 24.6±0.6 | 37.4±1.5 | 31.1±4.1 | 51.5±0.5 | >2 |
| **16** |  <br>2,6-Dimethylbenzoquinone | 0.29 | 3.2±0.2 | 5.2±0.2 | 2.9±0.3 | 5.3±0.5 | 1.8 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **17** |  2,5-Bis-(1,1-Dimethyl-propyl)-(1,4)-Benzoquinone | 3.56 | 36.1±0.1 | 54.2±1.6 | 48.8±5.4 | In-active | >100 |
| **18** |  2-Tert-Butyl-5-Methyl-1,4-Benzoquinone | 1.51 | 7.5±1.2 | 13.5±1.5 | 7.9±2.0 | 55.8±6.2 | 7.5 |
| **19** |  2,5-Di-tert-octyl-1,4-Benzoquinone | 6.06 | 44.2±0.3 | 56.5±0.6 | 51.8±3.1 | In-active | >100 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **20** |  2,5-Dibromo-6-Isopropyl-3-Methyl-1,4-Hydroquinone | 4.63 | 3.4±0.4 | 11.6±0.5 | 4.8±0.4 | 11.0±2.1 | >2 |
| **21** |  2,6-Dimethyl-1,4-Hydroquinone | 2.23 | 3.6±0.4 | 8.3±0.7 | 3.6±0.6 | 7.8±1.6 | >2 |
| **22** |  2-Tert-butyl-5-Methyl-1,4-Hydroquinone | 3.45 | 6.4±1.5 | 12.2±0.5 | 11.5±0.3 | 85.6±5.2 | |

| | | | | | |
|---|---|---|---|---|---|
| **23** | Cell Culture Media | >10,0000 | >10,0000 | >10,0000 | - |
| **24** | DMSO 0.1% (vehicle) | >10,0000 | >10,0000 | >10,0000 | - |
| **25** | Carboplatin (clinical drug) | ND | 10.8±1.3 | ND | - |
| **26** | Paclitaxel (clinical drug) | 0.009± 0.0009 | 0.01 ± 0.004 | ND | - |

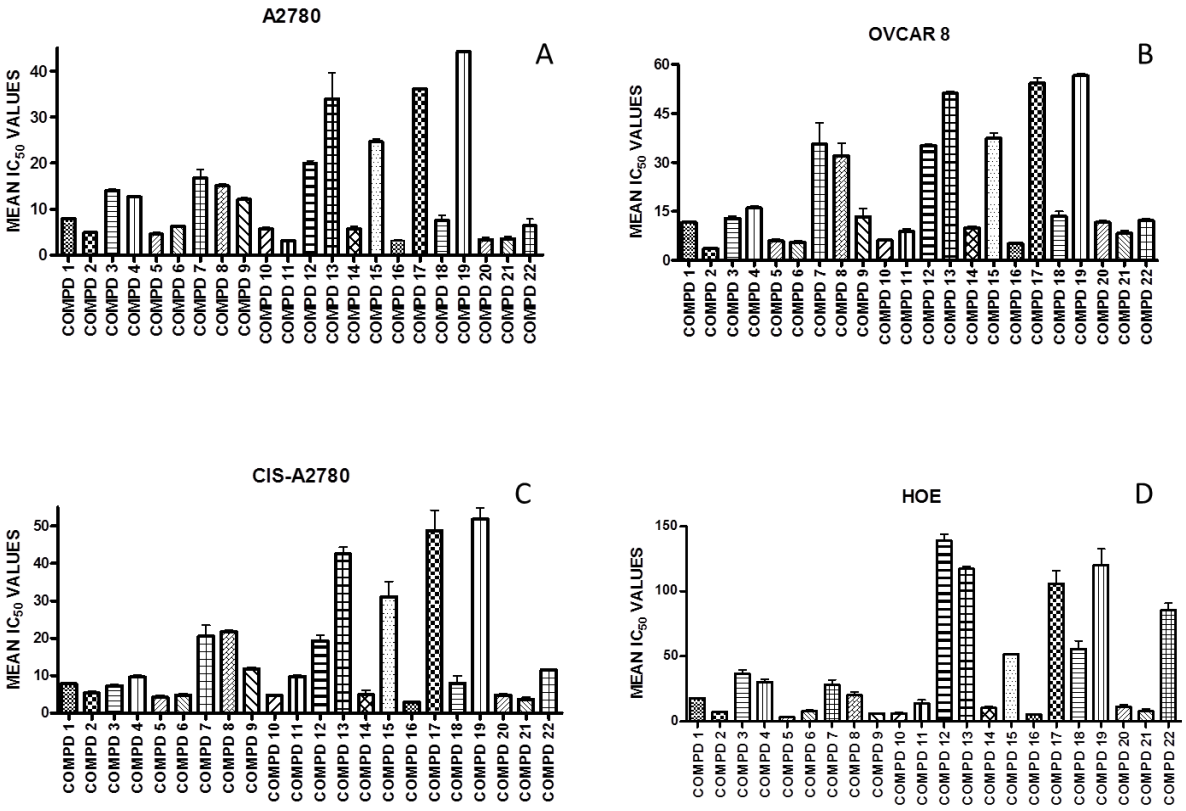



**Figure 6.3 The Graphical Representation of the growth inhibition evaluation of the 21 TQ analogues on A2780 (A), OVCAR 8 (B), CIS-A2780 (C) and HOE (D). The treated cells were incubated for 72h, followed by SRB staining and the surviving cell numbers estimated. The results are for three individual experiments with the Mean IC50s calculated. (Mean ±SEM).**

The Figure 6.3 contain the results of the TQ analogues evaluated on the three ovarian cancer cell-lines and the human ovarian epithelial cell line (HOE). The results showed the antiproliferative activities of the twenty-one TQ analogues and TQ. The compounds had more inhibitory effects on the A2780 cell line with the $IC_{50}$ of eleven compounds being <10µM. A ranking of the activities of the twenty-one analogues is shown below:

**11>20>21>16>5>2>10>14>6>22>18>9>4>3>8>7>12>15>13>17>19**

Further observation, showed that three of the hydroxylated TQ analogues (compounds **11**, **20, 21**) exhibited potent antiproliferative activities; these compounds were followed by **16**, a procured analogue. The next potent compound was **5**, a chlorinated procured analogue which exhibited more potent activity than compound **2**, the brominated analogue. Compound **10** another hydroxylated analogue was more potent than **14**, a procured analogue which had

a single substituent at position two (tertiary butyl). This compound had slightly more potency than compound **6**. Two other compounds which were more potent than parent TQ were compound **22**, another hydroxylated analogue and compound **18**, which is structurally different from **14**, by an additional substituent (methyl group) at position five. Importantly, all the analogues induced inhibitory activities within an $IC_{50}$ range of 3.1-56.5µM on the A2780 cell line.

The activities of the analogues on the OVCAR 8 cell line showed similar trend as in the A2780, but with eight analogues having $IC_{50}$s of <10µM. It could be seen that the analogues had less inhibitory effects on this cell line, an indication that OVCAR 8 is a more resistant cell line. A ranking of the activities of the analogues is shown:

**2>16>6>5>10>21>11>14>15>20>22>3>9>18>4>8>12>17>18>21>13**

The ranking of the activities of the analogues on the OVCAR 8 cell line showed that compounds **2, 6** and**16** were the most potent of the analogues. Further observations showed that only two of the hydroxylated analogues had $IC_{50}$s<10µM. Also among the synthesised analogues, compound **6** was the most potent. The results showed that eight analogues were more potent than TQ on the OVCAR 8 cell line.

Again from the results in Table 6.1 and Figure 6.3, the analogues were all potent on the CIS-A2780 cell line. The compounds exhibited similar activities as in the A2780 cell line. Ten analogues had $IC_{50}$s <10µM. A ranking of the activity of the analogues is shown:

**16>21>5>6>10>20>14>2>3>18>11>15>22>9>4>12>7>8>13>17>19**

The ranking of the activities of the analogues showed that the most potent analogue was **16**, followed by the compound's corresponding hydroxylated analogue. Importantly it was observed again that the chlorinated analogue was more active than the brominated analogue. Also, the next more potent analogue was compound **6**. Three of the hydroxylated analogues had $IC_{50}$s<10µM and were more antiproliferative than their corresponding

benzoquinones. This result is a confirmation that hydroxylated TQ analogues were more potent than the corresponding benzoquinone analogues. As observed from the evaluation of the potency of the analogues on the OVCAR 8 cell line, it could be seen that 9 analogues were more potent than TQ on the CIS-A2780 cell line.

The growth inhibition of the analogues was evaluated on the HOE cell line, with the observation that most of the analogues had less inhibition on the HOE cells than on the cancer cells. The calculated SI contained in Table 6.1 showed that the chlorinated analogue (5) had no selectivity. Further observations showed that more potent compounds had SI <2, while the less potent compounds had SI >2. The results also showed that the analogues with bulkier groups had very high SI which for compounds **17** and **19** were >100.

An estimation of the Log P values of the TQ analogues was carried out by means of the ChemBioDraw software in order to investigate the correlation between the inhibition of the analogues and their lipophilicity. This data is shown in Table 6.1. The observation from the estimated Log P values of the TQ analogues showed that most of the analogues had Log P values <2. A comparison of the Log P values with the obtained $IC_{50}$ values (Table 6.1) which is a measure of the antiproliferative activities of the compounds; showed that compounds which had smaller $IC_{50}$s also had smaller Log P values, while compounds such as **13**, **17** and **19** with higher $IC_{50}$s, equally had higher Log P values. An exception was observed in the hydroxylated compounds, which had higher Log P values and smaller $IC_{50}$s when compared to the corresponding benzoquinones. However, the Log P values was<5 for the hydroxylated compounds. These results suggest that higher lipophilicity as a result of bulkier substituents on the aromatic ring may diminish inhibitory activities in benzoquinones. But for hydroxylated compounds, the presence of the OH group enhances activity; thus, higher lipophilicity does not result in diminished inhibitory activity.

## Aqueous solubility studies of TQ, compounds 6 and 16

Further solubility studies of TQ, compounds **6** and **16** were undertaken in order to determine the solubility of these compounds in a physiological buffer, which could be indicative of the bioavalability of the compounds and thus the potency of the compounds. In order to achieve this, phosphate buffer (pH=7.4) was used and the experiment was carried out at 37°C. A comparison of the obtained solubility data was compared with the estimated Log P values obtained by the use of the ChemBioDraw software. A correlation between the activities of the compounds and their solubility was made. The results of these experiments are demonstrated below (Table 6.2) and compared with the Log P values.

**Table 6-2The solubility study of TQ, Compounds 6 and 16 obtained from the dissolution of the individual compounds in phosphate buffer (pH 7.4).** The measurements were obtained at 37 °C, after 24h. The results are expressed as mean±SD, n=3 independent experiments. The Log P values were obtained by the use of ChemBioDraw.

| Compound | Water Solubility (µg/mL) | Log P Value |
|---|---|---|
| **TQ (1)** | 467.8±2.2 | 1.04 |
| **6** | 8320.7±0.7 | -0.71 |
| **16** | 5043.0±6.5 | 0.29 |

The results presented in Table 6.2, showed that the more potent analogues were more soluble than TQ. The obtained solubility of TQ is consistent with the previously documented solubility of TQ (Salmani et al., 2014). The results in Table 6.2 correlate with the estimated Log P values of the compounds which showed that TQ had the highest value, and was the most lipophilic of the compounds. Further evaluation of the results in Table 6.2, demonstrated that compound **6** which had the least Log P value, was about 16 times more soluble than TQ. While compound **16** was about 8 times more soluble than TQ. This finding is significant as poor aqueous solubility is a major deterrant to the clinical advancement of almost 50% of potent compounds classed as new chemical entities (NCEs). Low aqueous solubility could impair absorption, posing challenges in the bioavailability of drugs, with a

resultant effect in the diminished potency of the agents (Savjani et al., 2012). The low solubility of TQ may be responsible in part for the reduced potency of the compound in comparison with the more potent analogues as observed from this study.

## *6.4 Derivation of the Anti-ovarian cancer SAR for the TQ Analogues*

The results of the growth inhibition of the TQ analogues on the three ovarian cancer cell lines were analysed to derive a structure activity relationship (SAR). It could be seen that the isopropyl group or an isopropyl-like group enhances the cytotoxic activity of a compound, as demonstrated by **2,5,6,14**, and **18**. Similar finding has been reported from a study that evaluated the pharmacological potentials of five structurally related para-benzoquinones to verify the pentylenotetrazol-induced seizures model in order to establish the structural characteristics that influence the anti-convulsant activity of TQ. It was further observed that the di-substituted benzoquinones having two alkyl groups were active. However, activity was enhanced when the alkyl group at position 2 was replaced with an isopropyl group (Sousa et al., 2011).

Again, additional substituent on the side of the isopropyl group diminishes activity as seen in **13, 17, 19** and **8** where a bulky aromatic substituent is present. This phenomenon has previously been reported from a structure activity study of TQ analogues (Banerjee et. al., 2010) which showed that 2,5-dimethylamino-6-isopropyl-3-methyl-1,4-benzoquinone and other TQ analogues with phenyl substituent groups attached to the aromatic ring had no significant activity. An interesting observation, from the results, showed that the presence of two methyl groups at positions 2 and 6 on the ring, tends to favour activity, Also, when a comparison of compound **13** and **15** was made, there was a significant difference in the activities of these two compounds on all the cell lines. The key difference in the structures of these compounds, showed that compound **13**, has two substituents at positions 2 and 5. While compound **15**, has these same substituents at positions 2 and 6. Further observations were that the presence of electronegative groups, such as bromine and chlorine on the ring

could contribute significantly to the increase in activity. Again the presence of nitrogen-containing group in a substituent attached to the ring would boost activity, when para to a methyl group as demonstrated in compound **6**.

The SAR studies further observed that reduction of the ketone group to a hydroxyl group impacts activity to a compound. This finding was clearly manifested in five compounds:**10, 11, 20, 21,** and**22** in comparison with their keto-counterparts:**1, 2,9,16** and **18**. This finding has been reported in a study of alkylated hydroquinones, in which two compounds: Violaceiod C and D were evaluated on five leukaemia cell lines. The study reported that the range of $LD_{50}$ was 5.9-8.3µM. A third compound Violaceiod F was cytotoxic on HCT116 cell line at a concentration of 6.4µM (Yusuke et al, 2014). This enhanced cytotoxicity exhibited by hydroxylated compounds may not be unconnected with the generation of reactive oxygen species (ROS) which is known to inflict severe damages to the cells. The OH radical is a short-lived radical which is very reactive and thus affects a number of macromolecules (Hoyt et al., 2011).

The activities of compound **12**, which is 3-amino-thymoquinone, was in the $IC_{50}$ range reported in a similar study of the structure activities of the potentials of several synthesised amino-thymoquinone on pancreatic cancer. The results of the cited study recorded $IC_{50}$ values of >10µM for eight compounds (Banerjee et al., 2010).

The results of the evaluation of the analogues on HOE are contained in the Table 6.1 and in the Figure 6.3 above. The aim of this investigation was to determine the selectivity index of the analogues.

The observation made is that the less active analogues had the most selectivity, while the more active analogues had the least selectivity with the SI values averaged around 2. Similar values were found in literature for some currently used drugs, such as digoxin (Calderon-Montano et al., 2014) and paclitaxel (Marcos et al., 2015).

A comparison of the activities of the synthesised analogues observed that three of the hydroxylated analogues (**11, 20** and **21**), performed better than the benzoquinones on the A2780. However, on the OVCAR 8 cell line compound **6** was the most potent. Similarly, on the CIS-A2780 cell line, compound **21** was the most potent, with compound **6** being the second most potent compound. The hydroxylated analogues were not considered for further investigation. Compound **6** was chosen for further cytotoxic investigations based on the activity of the analogue on the three cell lines evaluated. An evaluation of the antiproliferative activities of TQ and compound **6** was carried out on the OVCAR 4 cell line which was chosen for further cytotoxic and apoptosis studies.

**Table 6-3The growth inhibition evaluation of TQ and 6 on OVCAR 4 cell line in cell growth assays. The cells were treated for 72h with the indicated compound, followed by an estimation of the number of surviving cells by SRB staining. The results are expressed as mean ±SEM, n=3 experiments**

| **Compound** | **Mean $IC_{50}$ on OVCAR 4 Cell Line** |
|---|---|
| **TQ** | 23.1±2.3 |
| **6** | 11.2±1.9 |

The result of the comparison of the activities of TQ and compound **6** on the OVCAR 4 cell line is presented in Table 6.3. It could be seen that compound **6** displayed a two-fold increase in potency than parent TQ on the OVCAR 4 cell line. Similar results were observed from the $IC_{50}$ values of the compounds on the A2780, CIS-A2780 and OVCAR 8 cell lines presented in Table 6.1 and Figure 6.3. The increased activities of compound **6** over TQ was statistically significant at $p<0.05$ for the four ovarian cancer cell lines. The mean concentration-response curves of TQ and compound **6** is presented in Figure 6.4.

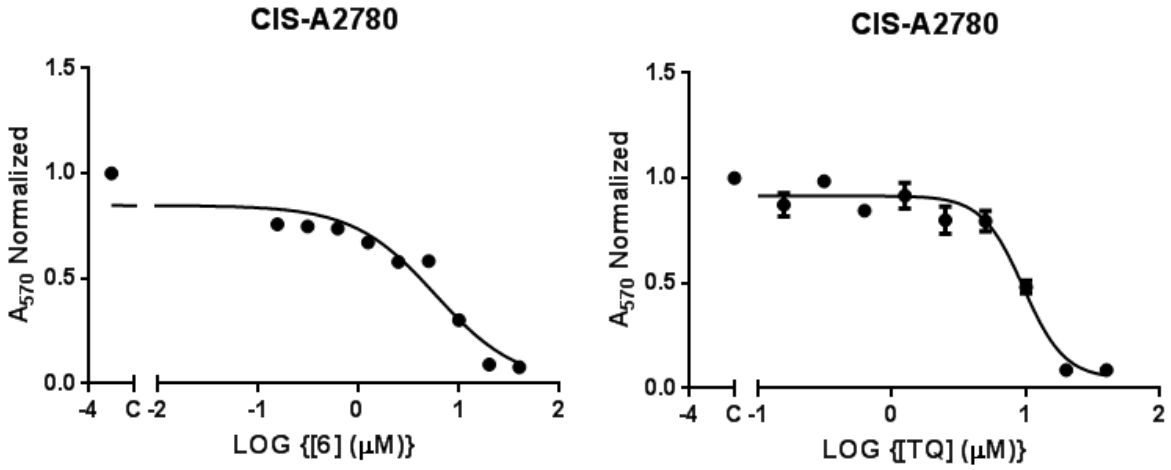



**Figure 6.4 The mean concentration-response curves of CIS-A2780 cells treated with either TQ or 6 for 72h. The surviving cell number was estimated by staining with SRB. The vehicle-treated cells served as the control. The mean was calculated from three independent experiments, (Mean±SEM).**

The evaluation of the inhibition of **6** and TQ determined on the CIS-A2780 cell line showed that **6** is more potent than TQ as observed in Figure 6.3. It could be seen that **6** exhibited more activity than TQ in seven out of the nine concentrations in the dose-response curve; a confirmation that the compound possessed better inhibitory activities than TQ. Further investigation of possible literature-reported bioactivities of **6** was warranted.

A library search of compounds, by use of Reaxys search engine was undertaken to find out reported bioactivities of **6** by other researchers. An in-depth search revealed that compound **6**was synthesised in 1986 by two researchers in Germany, while investigating quinone-amine reactions. The compound's Reaxys number is 6192682. The method of synthesis employed was by the reaction of desipramine: an anti-depressant drug(which is a known inhibitor of the uptake of serotonin and norepinephrine) and 2,5-dimethyl-1,4-benzoquinone, with copper (II) acetate and atmospheric oxygen used as catalyst (Kallmayer et al.,1986). This synthesis was aimed at studying the physiochemical properties of the synthesised amino-quinones, without

further investigation for pharmacological activities. The literature search revealed that there has been no report of the bioactivities of compound **6** from previous researches.

A schematic representation of the literature-reported method of synthesis of **6** is shown in Figure 6.5 below:

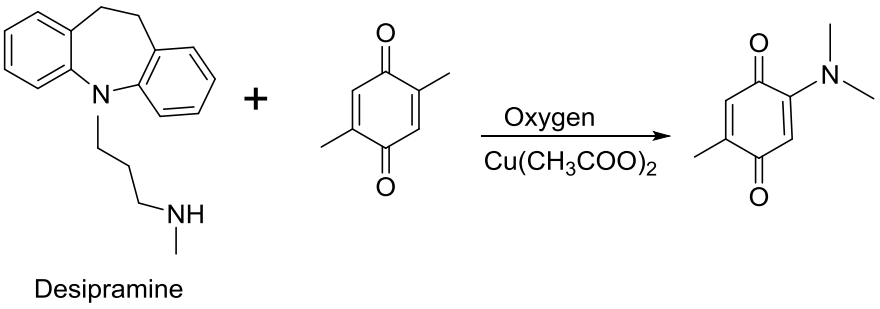



**Figure 6.5 Schematic representation of synthesis of compound 6 by reaction of desipramine with 2,5-dimethyl-14-benzoquinone.**

In contrast to the reported method of synthesis shown in the scheme above, the method of synthesis employed in this research, followed a guide: an existing literature of some synthesised TQ analogues evaluated for pancreatic cancer. This research work was further motivated by a drive to achieve TQ analogues with increased potency on human ovarian cancer cell lines. Furthermore, this research has obtained a simplified method for the synthesis of **6**, which is very economical and has less impact on the environment. The possible electron impact fragmentation pathway of **6**in the EI-MS is shown in Figure 6.6.

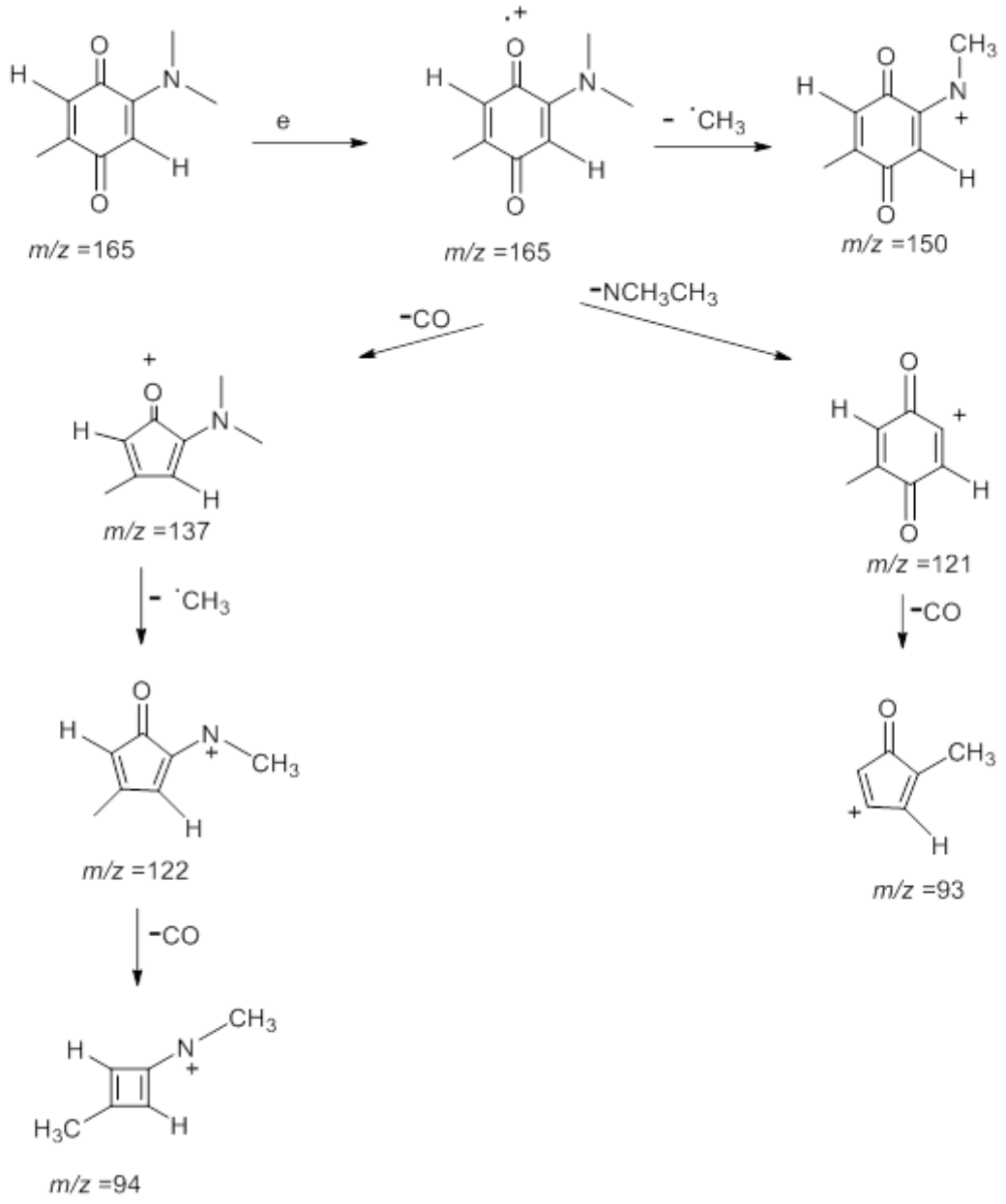



**Figure 6.6 Possible electron impact fragmentation pathways for Compound 6.**

Further evaluation of the cytotoxicity of **6** was assessed by the TBA to determine whether **6** induced cytotoxicity and antiproliferative activities on the cancer cells. Also the compound's synergistic potentials with carboplatin and paclitaxel were investigated on drug combination studies in the TBA, caspase 3/7 and by flow cytometric analysis.

## *6.5 The Results of the Drug combination Studies of Compound 6*

The cytotoxicity of **6** on cell growth assay was further assessed using the TBA (Figure 6.7).

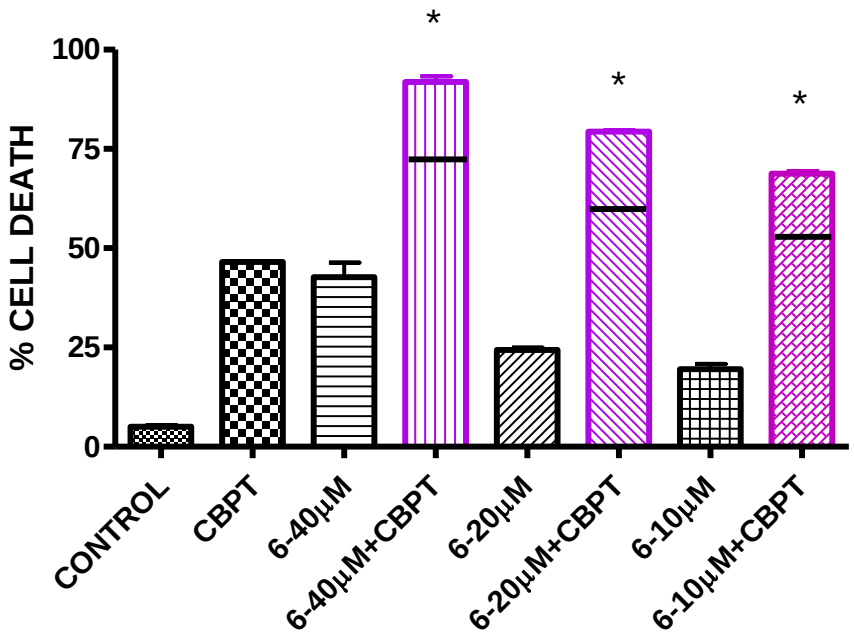



**Figure 6.7 The effect of 6 at the indicated concentrations and in combination with carboplatin (CBPT) (40 µM) at 48h on cell viability. The cells were stained with trypan blue and the percentage of dead cells was obtained by microscopy. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. * denotes that the result is significantly different (P<0.05, paired t-test) from the effect expected from the Bliss independence criterion. The results were expressed as mean ±SD, n=3.**

The results of the TBA showed that **6**is cytotoxic to cancer cells in a concentration-dependent manner, with the maximum concentration of 40µM exhibiting the most cytotoxicity. The effect of the combined use of **6** and carboplatin on the cells were also investigated. The agents combined demonstrated significant synergism (*p<0.05*) because all three concentrations used in the study elicited effects which exceeded that expected for the combinations assuming additivity and calculated by the Bliss independence criterion. From these findings **hypotheses a** and **c** were tested. Further cytotoxic investigations of the treated cells stained by TBA at 72h incubation(results not shown) observed that 40µM of**6** had very scanty cells, the 20µM and 40µM of**6** drug combinations with carboplatin were almost void of cells; an indication that virtually all the cells were death. These results in

Figure 6.6 showed potential synergism between **6** and carboplatin. Again these results are a confirmation of the observed inhibition of cancer cells by **6** in the SRB assay.

Further studies on the effect of drug combination between **6** and carboplatin on OVCAR 4 cells were carried out to investigate the synergistic potentials of these agents in cell growth assays by the fixed dose method using the SRB protocol (Figure 6.8).

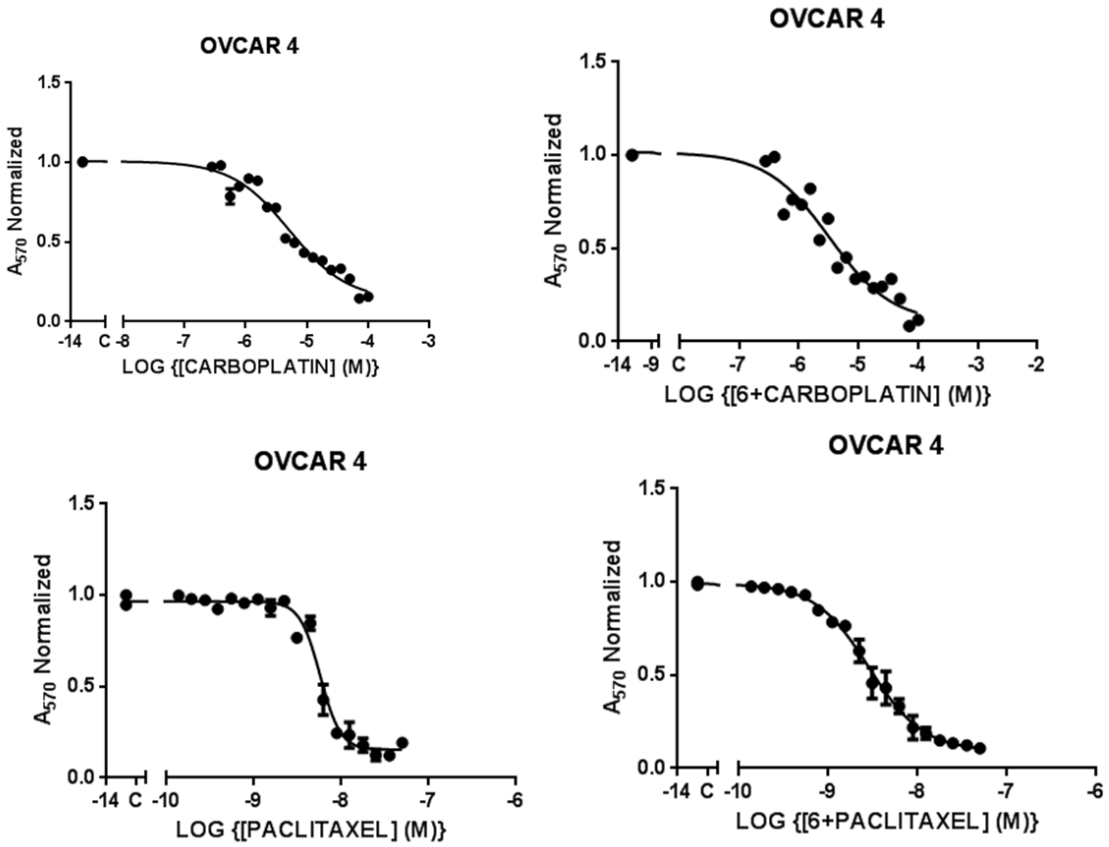



**Figure 6.8 Mean concentration-response curves of 6 and carboplatin (TOP), the vehicle-treated cells, carboplatin-treated cells (maximum concentration of 100 µM), and the drug combination of 6 at a fixed concentration of 6 µM with carboplatin. Mean concentration-response curves of 6 and paclitaxel (DOWN), Vehicle-treated cells, Paclitaxel-treated cells (Maximum concentration of 50 nM), and the drug combination of 6 at a fixed concentration of 6 µM and paclitaxel (Maximum concentration of 50 nM) on the OVCAR 4 cell line determined after 72h of treatment, followed by an estimation of the number of surviving cells by SRB staining. Results were expressed as mean of three experiments ± SEM.**

An evaluation of the results of the drug combinations showed that there was significant synergy between**6** and carboplatin at *p<0.001*,which was stastistically significant from

synergism. Also, the combination with paclitaxel demonstrated similar synergistic significance greater than $p<0.05$. Again these results were statistically significant from synergism.

Similar findings have been observed in a drug combination of carboplatin and silibinin; a quinone. The researchers reported significant synergy in the combination of both agents on the MCF-7 breast cancer cell line in both cell growth assay and in the quantification of apoptosis (Tyagi et al., 2004).

## *6.6 Apoptosis studies of compound 6*

In order to investigate the possible route of cell death caused by **6**, the effects of **6** on caspase 3/7 activity were measured. In these experiments, the effect of drug combination between **6** and carboplatin was further examined. Additionally, the effect of **6** and paclitaxel was also investigated. Previous apoptotic studies of combinations of carboplatin and silibinin; another quinone compound, that has documented cytotoxicity on cancer cells, have indicated synergism in a range of concentration (25-100μM) of silibinin with 2-20μg/mL of carboplatin (Cheung et al., 2010; Tyagi et al., 2004). For this present study, 10μM of **6** was used in combination with 10μM of carboplatin and 10nM paclitaxel (Figure 6.9).

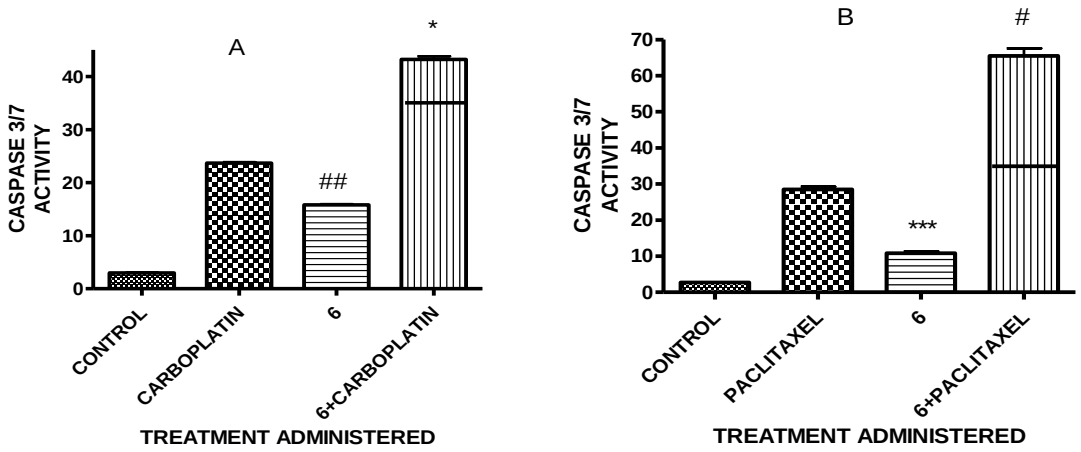



**Figure 6.9 The Effect of 6 (10 µM) combined with carboplatin (10 µM) or Paclitaxel (10 nM), on Caspase 3/7 activity at 48h for carboplatin (A) combination and 36h for Paclitaxel (B) combination. The vehicle-treated cells served as the control. The caspase activity was measured and normalized with corresponding SRB-stained cells to estimate the surviving cell number. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. * and # denotes that the result is significantly different (P<0.05 and P<0.001 paired t-test) from the effect expected from the Bliss independence criterion. While ***, ## denotes significant difference (P<0.01 and P<0.001) from the control, one-way ANOVA. The results were expressed as mean ±SD, n=3.**

The results contained in Figure 6.9, observed that **6** significantly increased caspase 3/7 activity at greater than *p<0.001* in comparison to the vehicle-treated cells (control) at a concentration of 10µM for an experimental period of 36-48h. The combination with carboplatin and paclitaxel also showed statistically significant caspase 3/7 activity, greater at *p<0.05* and *p<0.001* respectively than that which would be expected for additivity with the bliss expected effect. These results tested **hypotheses b** and **c**.

Further investigation of the observed apoptosis of **6**, and potential synergism between **6**and carboplatin was carried out by means of Annexin V/Propidium iodide labelling, followed by flow cytometry analysis (Table 6.4, Figure 6.10 and 6.11).

**Table 6-4 The Effect of 6 and carboplatin Combinations was assessed (for the apoptotic cells) after 48h of treatment by Annexin V/PI Staining of the cells followed by flow cytometry analysis.** * denotes that the result is significantly different (P<0.05, paired t-test) from the effect expected from the Bliss independence criterion. The results are expressed as mean ±SD, n=3.

| TREATMENT RECEIVED BY THE CELLS | PERCENTAGE OF APOPTOTIC CELLS MEAN±SD |
|---|---|
| **CONTROL** | 3.2±0.4 |
| **CARBOPLATIN (20μM)** | 14.4±1.1 |
| **6 (10μM)** | 5.8±0.3 |
| **CARBOPLATIN +6** | 22.7±0.7* |

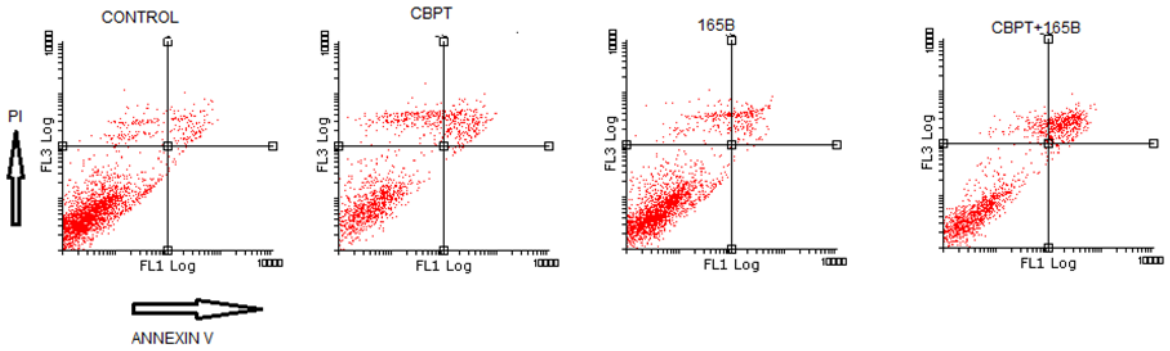



**Figure 6.10 The effect of 6 (10 μM) combination with carboplatin (20 μM) and the agents singly on OVCAR 4 cells treated for 48h was assessed by Annexin V/PI staining, followed by flow cytometry analysis. The vehicle-treated cells were used as the control. The results were expressed as a representative of three experiments.**

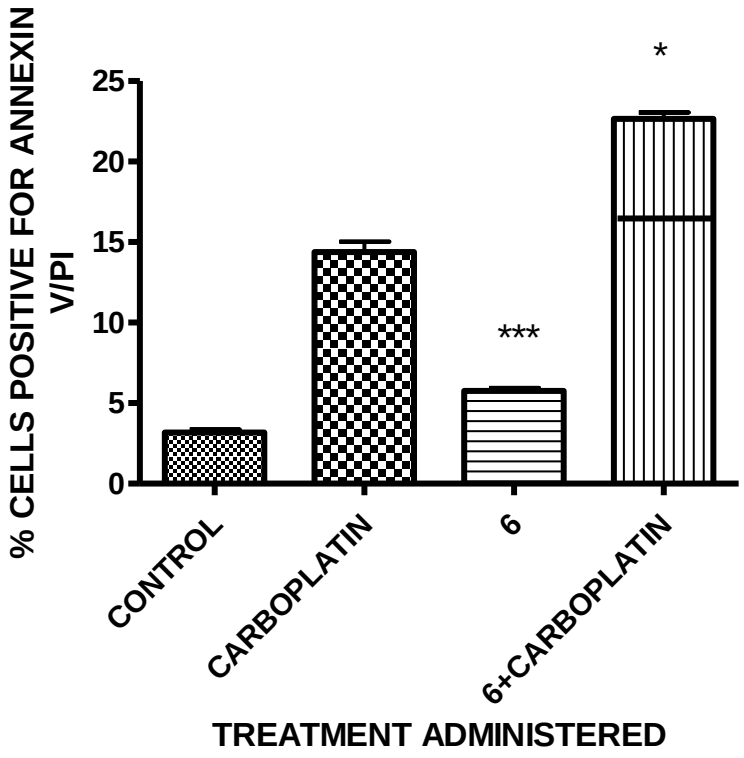



**Figure 6.11 The effect of 6 (10 µM) combined with carboplatin (20 µM), and the agents singly on Annexin V/PI staining on OVCAR 4 cells analysed by flow cytometry at 48h. A representation of the quantification of the early and late phase apoptotic cells is shown. The horizontal blue bars indicate the expected effect obtained from the bliss independent effect calculation comparing the effect of the combination and the single agents. * denotes that the result is significantly different (P<0.05, paired t-test) from the effect expected from the Bliss independence criterion. While *** denotes significant difference (P<0.01) from the control, one-way ANOVA. The results were expressed as mean ±SD, n=3.**

The results of the Annexin V/PI studies shown in Table 6.4 demonstrated significant synergism between **6** and carboplatin in the Annexin V/PI apoptosis assays. These results had huge significance at *p<0.05*, when compared with the calculated bliss expected effect. Also, this observation supports the inhibition observed in the TBA results (Figure 6.7 above).

Similarly, these results demonstrate that **6** is an apoptosis inducer as seen from the significantly induced apoptosis in comparison with the control at *P<0.01*. These results further confirmed the observed apoptosis of the agent in the caspase 3/7 assay. Again the demonstrated synergism between 6 and carboplatin in the cell growth assays and in the caspase 3/7 activity was again exhibited in the Annexin V/PI assay analysed by the flow

cytometer. Thus **hypotheses a, b** and **c** were tested. Further confirmation is seen from the morphological features of the cells monitored by light microscopy at 72h (Figure 6.12).

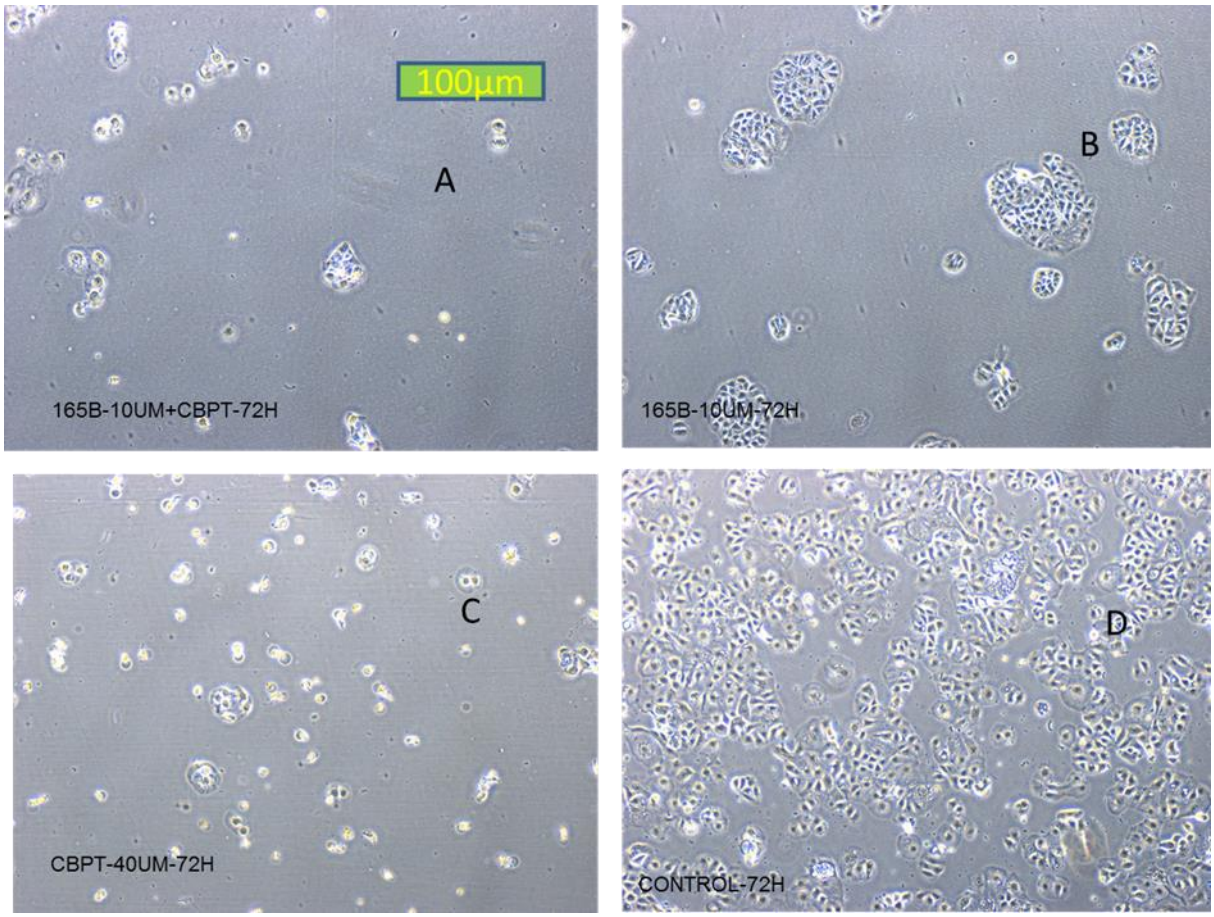



**Figure 6.12 The effects of 6 on cell viability of OVCAR 4 cells monitored by Light microscope (100 µm) at 72h after treatment. The Morphological changes observed in OVCAR 4 cells treated with 10 µM 6 and carboplatin (40 µM) (A), OVCAR 4 cells treated with 10 µM 6 (B), cells treated with carboplatin 40 µM (C), and vehicle-treated cells (D) respectively. These results are representative of three experiments.**

The observations from Figure 6.12 showed that **6** had significant cytotoxicity on OVCAR 4 cells. This implies that the agent kills cancer cells and does not only inhibit the proliferation of the cells. Also, it could be seen that the compound synergises with carboplatin as shown in Figure 6.12(A). The characteristic features of apoptosis such as blebbing and shrinkage of cells were clearly observable in the pictures of the cell morphology (Figure 6.11). The finding from the caspase and the Annexin V/PI staining was consistent with the observed

morphological changes and indicative of apoptosis. These results strongly suggest that the route of cell killing exhibited by **6** is by apoptosis.

A number of quinone-containing drugs are clinically used in chemotherapy for the treatment of cancers such as doxorubicin, mitomycin C and mitoxantrone. These agents have been shown to manifest several mechanism of action, MOA such as topoisomerase inhibition, (Tewey et al., 1984), redox-cycling properties with the generation of reactive oxygen species, ROS (Pelicano et al., 2004; Le et al., 2007) and DNA alkylation (Begleiter 1983; Li et al., 1996). However, a complete elucidation of the MOA of quinone drugs has not been demonstrated as the drugs often exhibit multi-mechanisms. An example is doxorubicin hydrochloride which is used in the treatment of ovarian cancer. The agent is both a DNA intercalating agent and a topoisomerase II inhibitor (Lu et al., 2005; Brayfield 2013).

## *6.7 Conclusion*

An evaluation of the anti-cancer activities of TQ and twenty one of its analogues has been carried out. The existing literature of a SAR obtained from the investigation of the cytotoxicity of some synthesised TQ analogues on pancreatic cancer was employed in the design and synthesis of 11 analogues, while 10 additional analogues were procured. The aim of this research was to obtain more potent TQ analogues and derive a SAR for the anti-ovarian cancer activities of TQ analogues.

The results of the study demonstrated that all the TQ analogues possess antiproliferative activities on the A2780, OVCAR 8 and CIS-A2780 ovarian cancer cell lines screened. Further findings, showed that compound **16** of the procured analogues and compound **6** of the synthesised analogues had significant inhibitory activities that doubled that of TQ. In total, eight analogues were more potent than parent TQ.

The SAR investigation revealed that the isopropyl group or a similar group was relevant for cytotoxic activity. Also, less bulky substituents boosted activity, while bulky substituents on the ring resulted in diminished activity.

Further investigation of the twenty one analogues and TQ on HOE was undertaken. The SI for the analogues was obtained. The values compared well with the reported values for some clinically used drugs. Statistical evaluation for significance of the activity of compound **6** in comparison to TQ showed that, compound **6** was significantly more active than TQ at $p<0.05$.The results of the trypan blue assay (TBA) conducted for compound **6** correlated with the observed inhibition of this compound in the SRB assay

Drug combination studies were carried using two clinically used first-line ovarian cancer drugs: Carboplatin and paclitaxel. The CI showed statistically significant synergism between **6** and both drugs in the cell growth assays.

The route of cell killing exhibited by **6** was investigated by caspase3/7 experiments, with the results indicative of apoptosis as the route of cell death. The results of this experiment demonstrated a very significant synergism between **6** and paclitaxel, at a $p<0.001$ value with a $p<0.05$ significance recorded for the combination of **6** and carboplatin.

Further investigation of the apoptosis of **6** was carried out by Annexin V/PI labelling of the cancer cells pre-treated with this agent singly and in combination with carboplatin followed by flow cytometry analysis. The obtained results confirmed that **6** is an apoptosis inducing compound. The results further observed a significant synergism between **6** and carboplatin, greater at $p<0.05$.

In conclusion, **6** is a more potent analogue than parent TQ, with a two-fold increase in potency. Apoptotic studies carried out demonstrated that the compound induces apoptosis and showed significant synergism with two clinically used ovarian cancer drugs: carboplatin and paclitaxel in both the two cell growth assays and in the apoptosis assays. Further investigations of the mechanism of action of **6** are relevant.

# Chapter Seven

## *Stable Isotope Dilution Gas Chromatography−Mass Spectrometry for Quantification of Thymoquinone in Black Cumin Seed Oil*

## *7.1 Introduction*

Black cumin seed (*Nigella sativa* L.) is a widely used spice in Mediterranean countries, Pakistan, and India (Ramadan et al., 2002; Kiralan 2012). It is also used as a traditional medicine for the treatment of a range of diseases, such as diabetes, hypertension, fever, arthritis, inflammation, gastrointestinal disturbances, and cancer (Padhye et al., 2008). The black cumin seed oil is a highly sought after medicinal product which have resulted in the establishment of numerous botanical/phytochemical-based pharmaceutical firms. Many commercially available brands of the black cumin seed oils abound in the Mediterranean countries and in parts of Europe. Studies into the biological activities of this plant have revealed that the volatile essential oil components, predominantly thymoquinone (1) (Figure 7.1) (El-Dakhakhny et al., 1963) possess anti-oxidant(Burits et al., 2000) anti-inflammatory (Hajhashemi et al., 2004), anti-diabetic (El-Mahmoudy et al., 2005), immunomodulatory(Salem 2005), and anti-tumor (Worthen et al., 1998; Sethi et al., 2008;Woo et al., 2011) activities. There is the possibility that the amount of thymoquinone contained in the black cumin seed oil may vary based on the source and this may affect the pharmacological activities of the oil.

Aim of Study

Thymoquinone was previously quantitated by means of high performance liquid chromatography (HPLC) (Ghosheh et al., 1999; Hadad et al., 2012), high-performance thin-layer chromatography (HPTLC) (Velho-Pereira et al., 2011) and a differential pulse polarographic method (Michelitsch et al., 2003). These methods could achieve high sensitivities and reproducibility, but they often need multi-steps of sample preparation and external calibrations; thus, the results could be influenced by complex matrices. Gas chromatography (GC) and/or gas chromatography−mass spectrometry (GC−MS) are

preferred techniques for the analysis of volatile and semi-volatile compounds and have also been used for the quantification of thymoquinone (Houghton et al., 1995) and identification of the composition of black cumin seed oil (Burits et al., 2000; Liu et al., 2012).

A stable isotope dilution assay/analysis (SIDA) should overcome the problem of the matrix effects, such as compound discrimination during extraction, chromatographic separation, and ultraviolet (UV)/mass spectrometry (MS)/electrochemical detection (Stanford et al., 2007; Allen et al., 1994) when a deuterated thymoquinone is used as an internal standard. Stable isotope dilution coupled with GC−MS (SID GC−MS) has widely been used for determination of various compounds, e.g., methoxypyrazines in red wines (Allen et al., 1994), dietary lignans in food (Penalvo et al., 2005), natural estrogens in vegetables and fruits (Lu et al., 2012, 2013), and alkypyrazines in coffee (Pickard et al., 2013).

The aim of this research is to synthesis stable deuterium (D or $^{2}H$)-labelled thymoquinone (Figure 7.1) for the first time, and apply this compound as an internal standard for the quantification of thymoquinone in black cumin seed oil by means of SID GC-MS.

## *7.2 Materials and Methods*

Thymoquinone (99%), $D_2$-sulfuric acid ($D_2SO_4$, 99.5%), deuterated water [deuterium oxide ($D_2O$), 99.5%], sodium hydrosulphite ($Na_2S_2O_4$), and chromium trioxide ($CrO_3$) were purchased from Sigma-Aldrich. Hexane (analytical grade) was purchased from Fisher-Scientific, U.K. The following black cumin seed oils were purchased from Amazon: Manako black cumin oil, CEBRA*Nigella sativa* oil, Avena black cumin oil, Iman VERGIN black cumin oil, and This Health black seed oil.

### $^{1}H$ NMR Spectroscopy

$^{1}H$ NMR spectra were recorded on a Bruker DPX 300 MHz spectrometer using $CDCl_3$ as the solvent. Chemical shifts are quoted on the δ scale in parts per million (ppm) using $CDCl_3$ as the internal standard. NMR data were analyzed using ACD/Laboratories 12 software.

Multiplicities of signals are described as singlet (s), doublet (d), triplet (t), quadruplet (q), and multiplet (m).

## GC−MS

The GC−MS system consisted of an Agilent 7890A GC system with split injection (250 °C; 1:10), coupled to an Agilent MS model 5975C mass selective detector (MSD) with a triple axis detector (Agilent Technologies, Santa Clara, CA). A 30 m HP5-MS column [(5% phenyl)-methylpolysiloxane, 0.25 mm inner diameter, film thickness (df) = 0.25 µm, Agilent Technologies, Santa Clara, CA] was used for the quantification of thymoquinone. The GC heating program was as follows: initially at 80°C, raised to 160 °C at 20 °C/min, then raised to 300 °C at 50 °C/min, and finally held at 300 °C for 3.2 min under a constant helium pressure (10 psi). MS was fitted with an EI source operating at an electron energy of 70 eV held at a temperature of 230 °C. Mass spectra and selected ion monitoring (SIM) were acquired using MSD ChemStation (Agilent Technologies, Santa Clara, CA). Full-scanmode was used to identify thymoquinone in the black cumin oil and characterize the synthetic compounds **2**, [$^2H_2$]−**2** and [$^2H_2$]−**1**. After observation of the major ions for thymoquinone (**1**) and doubly deuterated thymoquinone ([$^2H_2$]-**1**), SIM mode (m/z 164.1 for compound 1 and *m/z* 166.1 for deuterium-labelled compound [$^2H_2$]-**1**) was performed for quantification of thymoquinone using SID GC−MS analysis. SIM mode (three selected ions at *m/z* 164.1, 149.1, and 136.1 for compound **1**) was applied for the quantification of thymoquinone using SIM GC−MS with standard addition and external calibration methods.

## 7.2.1 Synthesis

The doubly deuterated thymoquinone ([$^2H_2$]-1) (Figure 7.1) was prepared and purified according to a similar method for preparation of deuterated 1, 4-benzoquinones (Charney et al., 1965; Lu et al., 2010) with modification.

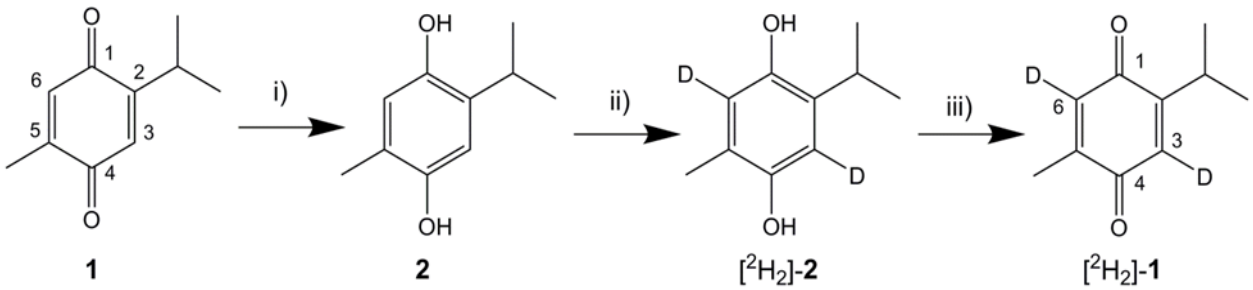



**Figure 7.1 Scheme for synthesis of [$^2H_2$]-thymoquinone ([[$^2H_2$]-1). i) $Na_2S_2O_4$ in $H_2O$/MeOH, RT, 2 h; ii) a) 3.4 M $D_2SO_4$ in D2O, reflux 48h; b) Cool down to room temperature and work-up with $H_2O$; iii) $CrO_3$ in 60 % HOAc in $H_2O$, at -4 °C for 2h.**

*2-Isopropyl-5-methyl-1, 4-benenediol* (**2**). A solution of sodium hydrosulphite (348 mg, 2.0mmol) in 2.0 mL of water was added to a solution of thymoquinone (164 mg, 1.0 mmol) in 2.0 mL of methanol. The mixture was stirred for 2h at room temperature. The methanol was evaporated, and the resultant solution was extracted with ethyl acetate (3 × 10 mL). The combined organic phase was washed with water (3 × 10 mL) and then dried over sodium sulphate. After evaporation of ethyl acetate, 2-isopropyl-5-methyl-1, 4-benenediol (thymohydroquinone, 2, 70 mg, yield of 42%) was obtained. MS [electron ionization (EI)] m/z (relative intensity, %): 166.1 (43) $[M]^{\bullet+}$, 151.1 (100) $[M - CH_3]^{\bullet+}$, 137 (4), 123.1 (11), 95.1 (10), 77.1 (10), 53.0 (3). Proton nuclear magnetic resonance ($^1$H NMR) (300 MHz, $CDCl_3$) δ: 6.56 (s, 1H), 6.47 (s, 1H), 3.06 (m, 1H), 2.10 (s, 3H), 1.14 (d, 6H, J = 7.2 Hz, $-CH(CH_3)_2$).

*2-Isopropyl-5-methyl-1, 4-dideuterobenenediol* ([$^2H_2$]-**2**). 2-Isopropyl-5-methyl-1,4-benenediol (**2**, 68 mg, 0.40 mmol) was refluxed for 48h in 3.4 M $D_2$-sulfuric acid ($D_2SO_4$, 0.9 mL) in deuterated water ($D_2O$, 4.2 mL). After cooling to room temperature, water ($H_2O$, 10 mL) was added and the mixture was extracted with ethyl acetate (3 × 10 mL). The ethyl acetate extract was washed with water ($H_2O$, 3 × 10 mL) and then dried over sodium sulphate. After

evaporation of the solvent, 2-isopropyl-5-methyl-1, 4-dideuterobenenediol ([$^2H_2$]-**2**, 45 mg) was obtained. MS (EI) *m/z* (relative intensity, %): 168.1 (44) $[M]^{\bullet+}$, 153.1 (100) $[M - CH_3]^{\bullet+}$, 139.1 (3), 125.1 (11), 97.0 (10), 79.1 (8), 55.0 (3).

*2-Isopropyl-5-methyl-1, 4-dideuterobenzoquinone* ([$^2H_2$]-thymoquinone ([$^2H_2$]-**1**). 2-Isopropyl-5-methyl-1, 4-dideuterobenenediol ([$^2H_2$]-**2**, 42 mg, 0.25 mmol) was dissolved in 60% acetic acid (2 mL) at −4 °C. Chromium trioxide (150 mg, 1.5 mmol) in 0.5 mL of 30% acetic acid was added, and the mixture was stirred at −4 °C for 2h. Water (10 mL) was then added to the mixture, followed by extraction with ethyl acetate (2 × 10 mL). After drying over sodium sulphate, the solvent was removed under vacuum to give crude doubly deuterated thymoquinone, which was sublimed to give pure **2**- isopropyl-5-methyl-1, 4-dideuterobenzoquinone ([$^2H_2$]-thymoquinone, [$^2H_2$]-**1**, 28.0 mg, 43% overall yield based on thymohydroquinone (**2**)). The purity of compound [$^2H_2$]-**1** was determined to be 97.7% by GC−MS using full-scan mode (Figure 7.2).

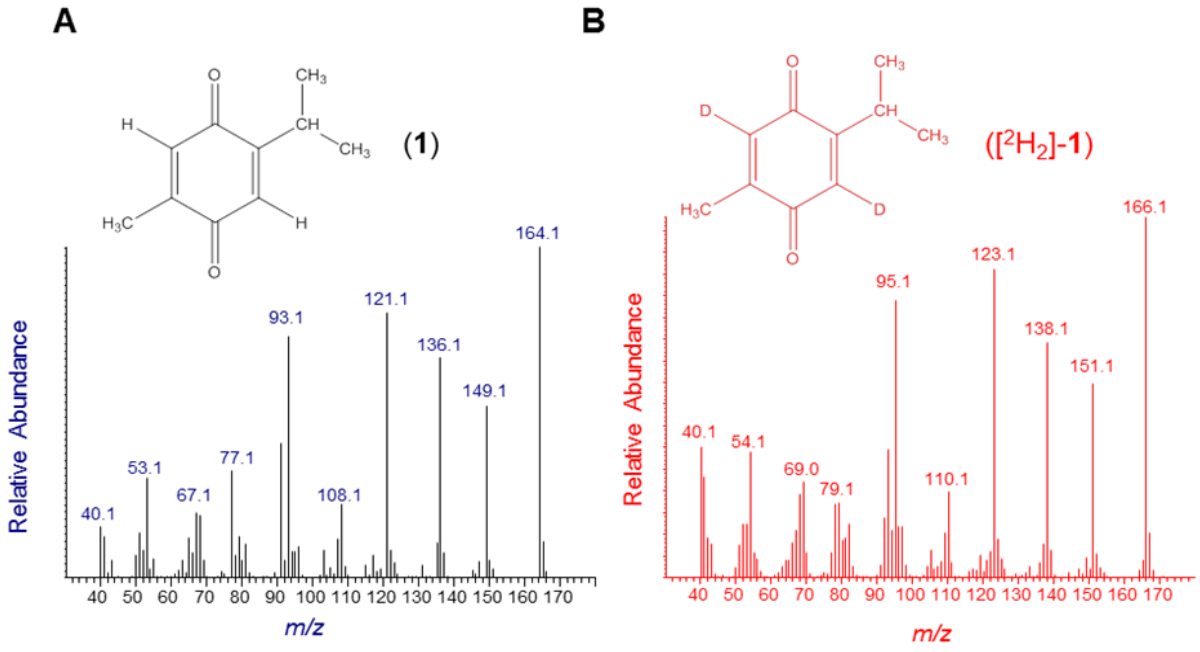



**Figure 7.2 Full EI mass spectrum of thymoquinone (1) (A) and the doubly-deuterated thymoquinone ([2H2]-1) (B).**

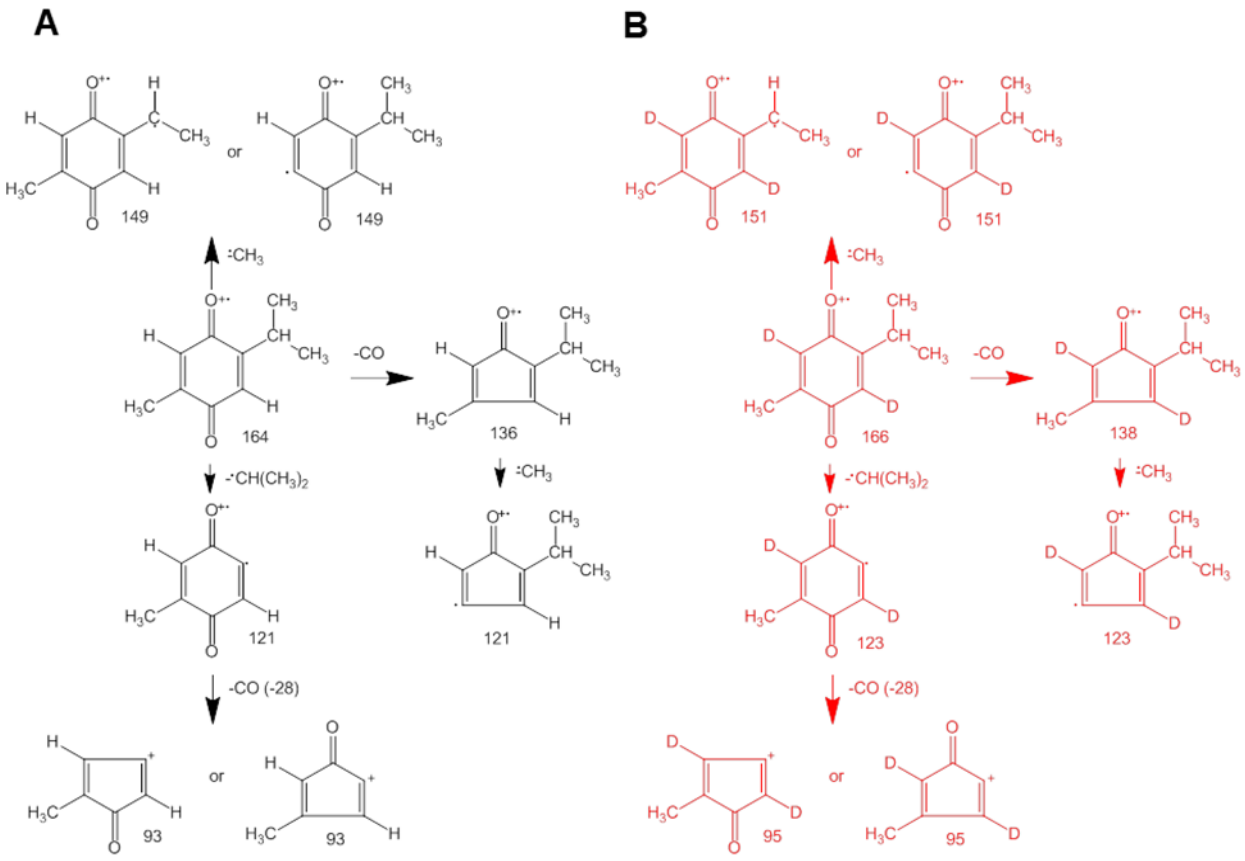



**Figure 7.3 Possible electron impact fragmentation pathways for thymoquinone (1) (A) and for the doubly-deuterated thymoquinone ([$^2H_2$]-1) (B).**

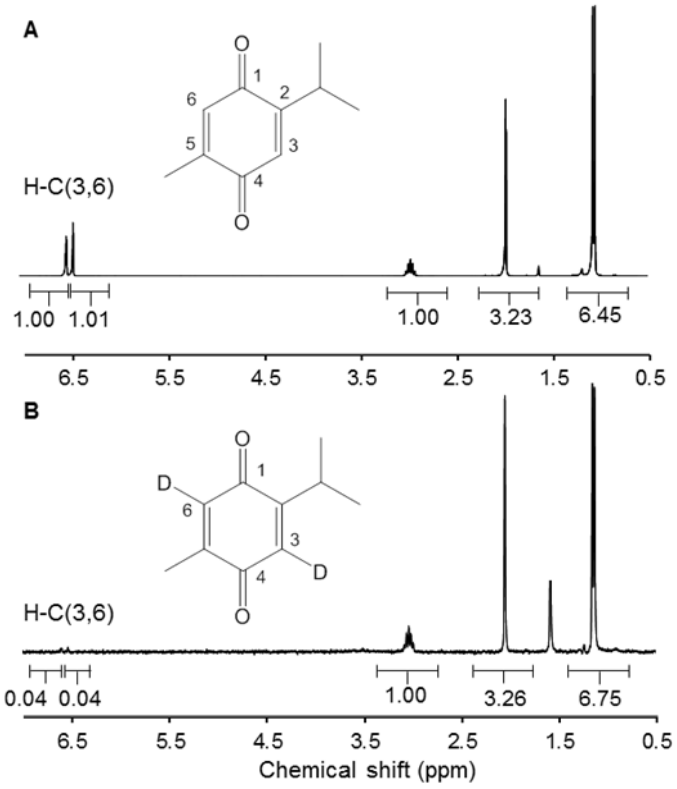



**Figure 7.4 $^{1}$H -NMR spectra (300 MHz; $CDCl_3$) of thymoquinone (1) (A) and [$^{2}H_2$]-thymoquinone ([$^{2}H_2$]-1) (B)**

The disappearance of the signals at 6.52 and 6.45 ppm for H-3 and H-6 in the spectrum of [$^{2}H_2$]-**1** indicates > 93% double substitution of deuterium for the two protiums of **1**. The extent of double deuteration according to $^{1}$H NMR (Figure 7.4) and MS (see Figure 7.2) was greater than 93%. MS [electron ionization (EI)] m/z (relative intensity, %): 166.1 (100) $[M]^{\bullet+}$, 151 (54) $[M - CH_3]^{\bullet+}$, 138.1 (62) $[M - CO]^{\bullet+}$, 123.1 (73) $[M - CH_3CHCH_3]^{\bullet+}$, 110.1 (19), 95.1 (63), 79.1 (19), 69.1 (22), 54.1 (27) (see Figures 7.2 and 7.3). $^{1}$H NMR (300 MHz, $CDCl_3$) δ: 3.06 (m, 1H, $-CH(CH_3)_2$), 2.10 (s, 3H, $CH_3$), 1.10 (d, 6H, $-CH(CH_3)_2$) (Figure 7.2B).

## 7.2.2 Development of a SID GC−MS Method for Quantification of Thymoquinone

### Calibration

The analyte thymoquinone (**1**) and the internal standard [$^2H_2$]-thymoquinone ([$^2H_2$]-**1**) were mixed in six ratios from 0.1 to 10 (5−500 µg/mL in hexane) and analyzed by means of GC−MS in triplicate. Calibration solutions were kept at 4 °C until analysis. The ratios of the area under the curve (AUC) of ion *m/z* 164.1 (AUC164) for compound **1** to the AUC of ion *m/z* 166.1 (AUC166) for compound [$^2H_2$]-**1** were plotted against the concentration ratio of compound 1 to compound [$^2H_2$]-**1** (Figure 7.5). The linearity of the calibration regression line was $R^2$ = 0.99. The equation obtained was y = 0.843x+ 0.2297 (**1**/[$^2H_2$]-**1**). The limit of detection (LOD) was 0.16 µg/mL with a signal-to-noise ratio of 3:1, and the limit of quantification (LOQ) (10:1)26 was 0.50 µg/mL respectively. When using splitless injection mode, LOD and LOQ for thymoquinone were 0.04 and 0.16µg/mL, respectively.

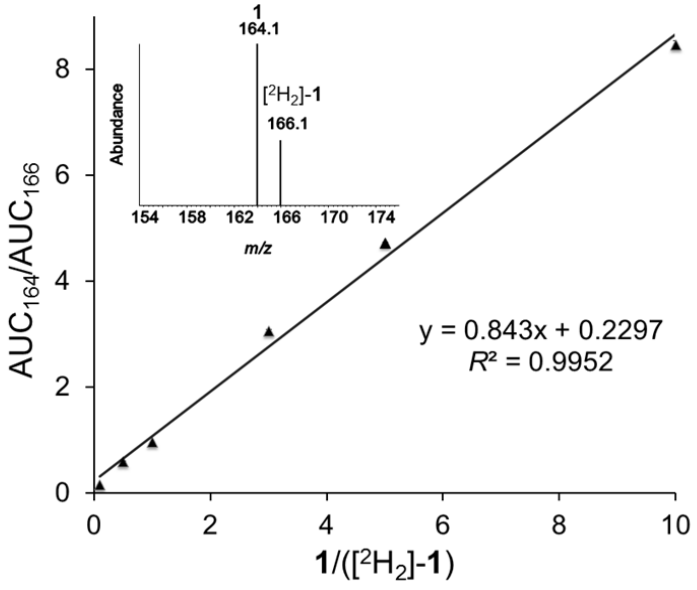



**Figure 7.5 Calibration curve for the SID GC-MS method (Inset shows the mass spectrum of the selected ion at m/z: 166.1 and 164.1 for 1 and [$^2H_2$]-1, respectively). Results are the means ± SD for n = 3 independent experiments.**

Quantification of Thymoquinone in the Black Cumin Seed Oils by SID GC−MS

A sample of black cumin seed oil (50 µL) was added to a 10 mL volumetric flask, and hexane was added to the mark. Aliquots of the diluted oil (200-fold) in hexane (980 µL) was spiked and mixed with a solution of compound [$^2H_2$]-**1** in hexane (20 µL, 1.0 mg/mL). A total of 1 µL of sample was injected into the GC−MS instrument. Each black cumin seed oil sample was prepared 3 times and analysed with three replicates.

### Method Validation

Recovery experiments were performed with a sample of Manako black cumin seed oil. An aliquot of the diluted Manako oil (200-fold) in hexane (920 µL) was spiked with an increasing amount (20, 40, and 60 µL) of compound 1 dissolved in hexane (1.0 mg/mL) and mixed with 20 µL of compound [$^2H_2$]-**1** in hexane (1.0 mg/mL). Finally, hexane was added to give 1.0 mL of black cumin seed oil solution. Aliquots (1 µL) of the diluted oils were analyzed using the SID GC−MS method described above. Three replicates were performed. To study the intra- and inter-day imprecisions of the SID GC−MS method, diluted Manako oil in hexane (200-fold) was analyzed. Relative standard deviations (RSDs) were determined for intra- and inter-day variations based on a set of 9 (in 1 day) and 15 (on 5 different days) measurements SIM

## GC−MS Analysis of Thymoquinone Using Standard Addition and External Calibration

### Standard Addition

An aliquot of the diluted (200-fold) Manako and Avena oil (800 µL) was added with an increasing amount of compound 1 in hexane to make oil solutions (1.0 mL) that contained an additional 40, 60, 80, 100, 120,140, and 160 µg/mL of compound **1**. Linear calibration curves ($R^2$>0.99) were obtained by plotting the AUC for the three selected ions (*m/z* 164.1, 149.1, and 136.1) versus the concentration. Each measurement was repeated in triplicate.

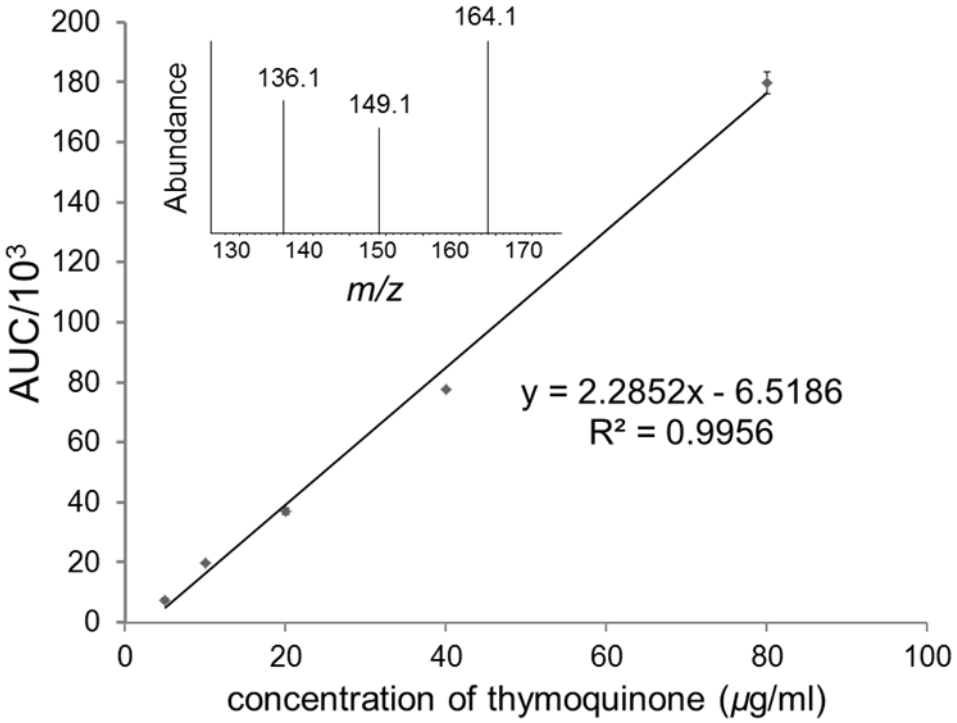



**Figure 7.6 External calibration curve for thymoquinone (1) using SIM GC-MS method (Inset shows the mass spectrum of the selected ions for 1 at m/z 166.1, 149.1, and 136.1). Results are the means ± SD for n = 3 independent experiments.**

## External Calibration

Quantification of thymoquinone using external calibration was carried out by comparing the AUC for the three selected ions (*m/z* = 164.1, 149.1, and 136.1) of compound **1** in the diluted oil samples to those defined standard solutions of compound **1** dissolved in hexane. A sample of oil (50 μL) was diluted with hexane (10 mL) in a 10 mL volumetric flask, and an aliquot (1μL) of the diluted samples were analyzed via SIM GC−MS. Each measurement was performed in triplicate. The quantity of thymoquinone was determined using a five-point response curve based on the analysis of standard solutions. Those solutions contained the defined amount of thymoquinone in different concentrations ranging from 5 to 80μg/mL. The calibration curve (Figure 7.6) obtained was y = 2.2852x − 6.5186 ($R^2$ =0.99), where y is the AUC/103 and x is the concentration of thymoquinone (μg/mL). The LOD was 0.10 μg/mL

with a signal-to noise ratio of 3:1, and the LOQ (10:1) was 0.31 µg/mL. When using splitless injection mode, LOD and LOQ were 0.02 and 0.08 µg/mL, respectively.

## *7.3 RESULTS AND DISCUSSION*

### Synthesis of a Stable Isotope-Labelled Thymoquinone

The scheme of synthesis of the doubly deuterated thymoquinone ([$^2H_2$]-**1**) is shown in Figure 7.1. Thymoquinone (**1**) was first reduced to thymohydroquinone (**2**) by sodium hydrosulphite. The two protiums (H-3 and H-6) of compound **2** were then exchanged by deuterium in 3.4 M $D_2SO_4$ in $D_2O$ under refluxing for 48h. After cooling to room temperature, the deuterated thymohydroquinone ([$^2H_2$]-**2**) was obtained after workup with $H_2O$ rather than expensive $D_2O$. Finally, compound [$^2H_2$]-**2** was oxidized with $CrO_3$ in 60% HOAc to yield compound [$^2H_2$]-**1** (17% overall yield). GC−MS shows a single peak with high purity (97.7%) based on GC−MS (Figure 7.2) and $^1$H NMR (Figure 7.4) for compound [$^2H_2$]-**1**. A comparison of the EI−MS spectrum of thymoquinone (1) to that of compound [$^2H_2$]-**1** (Figures 7.2 A and 7.2 B) showed that a mass increase of the molecule ion m/z 164.1 by two deuterium atoms to *m/z* 166.1, which confirmed [$^2H_2$]-**1** was the predominant isotopologue. The electron impact fragmentation pathways for compounds **1** and [$^2H_2$]-**1** are very similar because of the substitution of H-3 and 6 of compound 1 by two deuteriums (Figure 7.3). The isotopic distribution of compound [$^2H_2$]-1 detected by GC−MS was found to be 2.0% natural [$^2$H] abundant compound 1, 5.0% compound [$^2$H1]-**1**, and 93.0% compound [$^2H_2$]-**1**. Further comparison of the 1H NMR of compound 1 (Figure 7.4 A) to that of compound [$^2H_2$]-**1** (Figure 7.4 B) shows the disappearance of peaks for H-3 and H-6 because of the protium/deuterium exchange, again confirming the presence and position of two deuterium atoms in 2-isopropyl-5-methyl-1,4-dideuterobenzoquinone ([$^2H_2$]-**1**). It shows only minor protium abundance of 4.0% at position C-3 and 4.0% at position C-6 of compound [$^2H_2$]-**1** (Figure 7.4), which is consistent with a high degree of double deuteration of compound [$^2H_2$]-**1** found by GC−MS (Figure 7.2). Because only 2.0% natural [$^2$H] abundant compound 1 was present in the

synthetic compound [$^2H^2$]-1 and less than 2.0% of the third isotopic peak of compound 1 (*m/z* 166.1) was detected, the ratios of the AUC for ion m/z 164.1 for compound 1 to the AUC for ion *m/z* 166.1 for compound [$^2H_2$]-1 were used for the calibration and quantification of thymoquinone (1) using the SID GC−MS method. Compound [$^2H_2$]-1 was found to be stable in hexane for more than 6 months in the dark and, therefore, is suitable to be used as an internal standard for the SIDA.

## Establishment of a SID GC−MS Method

The conditions for gas chromatographic separation were optimized to quantify thymoquinone (1) in black cumin seed oil within only 10 min (Figure 7.6). Figure 7.6A shows a typical total ion current (TIC) gas chromatogram of the Manako oil sample spiked with compound [$^2H_2$]-1 using full-scan mode. A number of compounds can be detected. SIM (*m/z* 164.1 and 166.1 for compounds **1** and [$^2H_2$]-**1**, respectively) mode was used to quantify compound 1 in the oil using SID GC−MS (chromatogram not shown). Panels B and C of Figure 7.7 shows the reconstructed ion chromatograms of compounds **1** (*m/z* 164.1) and [$^2H_2$]-1 (m/z 166.1) in the Manako black cumin seed oil. Compounds **1** and internal [$^2H_2$]-1 were co-eluted at 4.81 min. A calibration graph (Figure 7.6) was plotted from a mixture of known mass ratios of compounds 1 and [$^2$H2]-1 and their corresponding peak area ratios in GC−MS by linear regression analysis. This calibration curve showing a linear response ($R^2$ =0.99) could enable the determination of the quantity of thymoquinone in the samples based on their measured ion intensities.

Recovery experiments were performed to check the trueness of the SIDA method. Specified quantities of thymoquinone (1) were added to the diluted Manako oil sample in three different concentrations before using SID GC−MS analysis. The quantity of compound **1** was measured and compared to those found in the blank diluted Manako oil sample as the control (Table 7.1). The mean recovery rate was determined to be 99.1 ± 1.1% RSD based on the amount of compound **1** added to the diluted oil sample. These results demonstrated

that the developed SID GC−MS is a reliable, rapid, and accurate method for quantification of compound **1**. Thymoquinone in five marketed oil products was quantified, and their results are listed in Table 7.2. The concentration of thymoquinone ranged from 3.34 to 10.8 mg/mL. Manako oil has the highest content of thymoquinone, while CEBRA oil has the lowest content of thymoquinone.

**Table 7-1 The Recovery Rates for the Quantification of Thymoquinone in Black Cumin Seed Oil (Manako) Determined by a SID GC-MS Method (Data were expressed as the Mean±RSD, n=3)**

| Amount of **1** added [µg/mL] | Conc. Calculated [µg/mL] | Conc. Determined [µg/mL] | Recovery [%] | Average Recovery [%] |
|---|---|---|---|---|
| | | 50.6±0.64 | | |
| 20.0 | 70.6±0.64 | 71.6±0.98 | 101.4±1.7 | |
| 40.0 | 90.6±0.64 | 85.2±0.59 | 94.5±0.93 | 96.8±2.1 |
| 60.0 | 110±0.64 | 104.7±0.51 | 94.7±0.7 | |

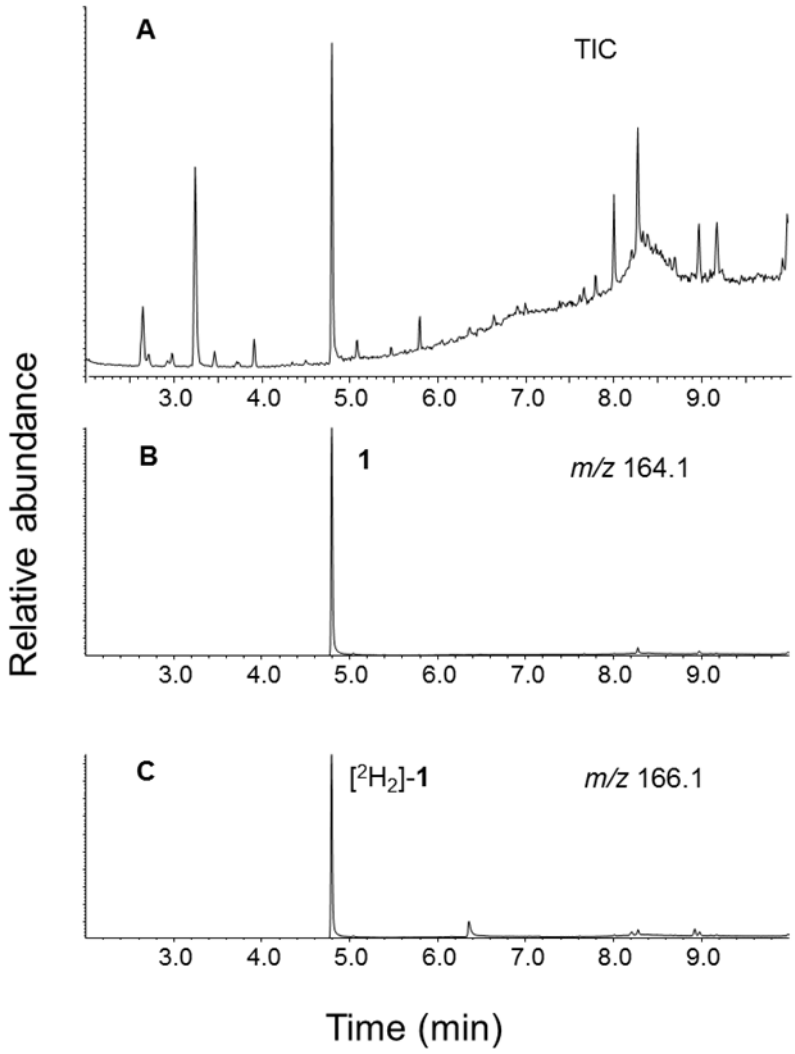



**Figure 7.7 Typical total ion current chromatogram (A) and reconstructed ion chromatograms of thymoquinone (1) (B) and deuterium-labelled thymoquinone ([$^2H_2$]-1) (C) in Manako black cumin seed oil spiked with [$^2H_2$]-1.**

**Table 7-2 Quantification of Thymoquinone in Black cumin Seed Oils Via SIDA GC-MS, External Calibration, and Standard Addition Methods (Data were expressed as the Mean± RSD, n-=3).**

| Black cumin seed oil | Concentration (mg/mL) (n=3) | | |
|---|---|---|---|
| | SIDA GC-MS | External calibration | Standard addition |
| **Manako** | 10.78±0.14 | 8.95±0.03 | 10.60±0.45 |
| **Avena** | 7.27±0.14 | 7.33±0.08 | 7.17±0.13 |
| **CEBRA** | 3.34±0.07 | 2.96±0.18 | * |
| **Iman** | 7.23±0.05 | 6.02±0.09 | * |
| **This Health** | 7.74±11 | 7.03±0.10 | * |

* Concentration not determined.

## Cross-Validation of the SID GC−MS Method

The standard addition method can also overcome the matrix effects and is often used for the validation of the SIDA method (Stanford et al., 2007; Stark et al., 2011).

To validate the SID GC−MS method developed above, the data obtained using SID GC−MS were compared to those found using standard addition and external calibration (Table 7.2). Three characteristic ions (*m/z* 164.1, 149.1, and 136.1) for compound 1 were selected for the quantification of thymoquinone using standard addition and external calibration. An external calibration curve with a linear response ($R^2$ = 0.99) (Figure 7.6) was attained by linear regression analysis of the AUC versus the concentration. Quantification based on the standard addition and external calibration showed 98.1 ± 1.2 and 82.9 ± 0.3% of compound **1** in Manako oil and 98.6 ± 0.4 and 100.8 ± 1.1% of compound **1** in Avena oil (calculated from data in Table 7.2) when compared to the results obtained by means of SID GC−MS, respectively.

The standard addition and SID GC−MS results revealed very similar concentrations of compound **1** in Manako and Avena oils (differences are less than 2%), therefore validating the accuracy and advantage of the SIDA method. The concentrations of thymoquinone in all five oils determined by external calibration generally agreed with those determined from SIDA and/or standard addition methods (differences range between **1** and 17%). The slight discrepancies are probably due to matrix effects from the different preparations of these black cumin seed oils (Stanford et al., 2007). The intraday precision of the SID GC−MS method for compound 1 showed a RSD of ±1.3% (n = 9) by analysing diluted Manako oil in hexane in nine independent measurements in 1 day. Inter-day precision studies with diluted Manako oil revealed a RSD of ±5.9% (n = 15) in three independent measurements in each day for 5 days. These data again demonstrate that the SID GC−MS method is a reliable, rapid, and accurate analytic means for thymoquinone in black cumin seed oils.

In previous reports, thymoquinone was quantified by several other analytical techniques (Houghton et al., 1995; Ghosheh et al., 1999; Michelitsch et al., 2003; Velho-Pereira et al., 2011; Hadad et al., 2012). HPLC was used for quantification of thymoquinone in commercial black cumin seed oil (Ghosheh et al., 1999) and in different phytopharmaceuticals with a LOD of 0.006 µg/mL (10 µL of injection volume, ~60 pg), (Hadad et al., 2012). Quantification of thymoquinone in herbal extracts and oil was also achieved using HPTLC with LOD of 50 ng/spot (Velho-Pereira et al., 2011). A differential pulse polarographic method was developed for the determination of thymoquinone in black seed oil, where LOD was found to be 0.05 µg/mL (10 mL of volume, ~500 ng) (Michelitsch et al., 2003). The content of thymoquinone in black cumin seed oils was also found to be in the range of 0.13−0.17% (w/v) of the oil by GC (Houghton et al., 1995). In our GC−MS methods, where split injection mode (1:10) was used to avoid overloading samples, LODs of thymoquinone for SIM GC−MS and SID GC−MS were found to be 0.10 and 0.16 µg/mL (1 µL of injection volume, ~100 and 160 pg), respectively. However, for trace analysis of thymoquinone, LODs can be further improved using splitless injection mode and range from 0.02 to 0.04 µg/mL (1 µL of

injection volume, ~20−40 pg). Therefore, the sensitivities of GC−MS methods are comparable to that of HPLC and are higher than those of HPTLC and differential pulse polarographic methods. Furthermore, GC−MS and/or SID GC−MS methods have advantages of identifying analytes and overcoming matrix effects over other analytical methods (Allen et al., 1994; Stanford et al., 2007).

In summary, synthesis of a highly pure deuterated thymoquinone has been achieved for the first time, which enables us to develop a SIDA of the biologically important thymoquinone in black cumin seed oil using GC−MS. Cross validation using standard addition confirms the accuracy and reliability of the SIDA method, which can quantify a large number of black cumin seed oil products for their quality control in high-throughput means. Synthetic deuterated thymoquinone and the SID GC-MS method should also be useful in the studies on the mechanism of binding thymoquinone to proteins, (Charney et al., 1965; MacDougall et al., 1980; Li et al., 2005, 2006; Lu et al., 2010; El-Najjar et al., 2011; Stark et al., 2011) *in vitro* and *in vivo* pharmacokinetics of thymoquinone, which are ongoing in our laboratory (Li et al., 2013).

# Chapter Eight

## *General Discussions, Conclusions and Outlook*

## *Significant Findings*

This research work on 'Identification, Semi-synthesis and Evaluation of Anti-Ovarian cancer Compounds from Plants used in Traditional Medicines' have been carried out. Two approaches were employed in the research: Investigation of the anti-ovarian cancer activities of three selected medicinal plants and semi-synthesised TQ analogues. The study reported the presence of a number of anti-cancer compounds, some of which have previously being identified. The synthesis of two novel anti-cancer compounds; 2-Ethylamino-6-isopropyl -3-methyl- 1,4-benzoquinone (compound **4**) and 2-(4'-Fluoroanilinyl)-5-methyl-*p*-benzoquinone (compound **8**) were achieved in this research. While the anti-cancer activities of two previously synthesised compounds; 6-Isopropyl-2-methylamino-3-methyl-1,4-benzoquinone (compound **3**) and 2-Dimethylamino-5-methyl-1,4-benzoquinone (compound **6**) were reported for the first time in this research work. Evaluation of the cytotoxicities of the bioactives compounds obtained in this studies showed that palmatine, securinine and **6** were the most potent compounds with potentials as monotherapeutic agents and in combination with carboplatin or paclitaxel. Other likely drug candidates which also possess chemopreventive potentials identified include; lauryl gallate (which is more potent than carboplatin), betulinic acid and gallic acid. Three potentially chemopreventive compounds were also identified; Urs-12-en-24-oic acid, 3-oxo-, methyl ester, cis-Catechin and cis-Gallocatechin. The anti-cancer activities of two of the medicinal plants (*M. discoidea* and *R. parviflora*) investigated is being reported for the first time. Also, the findings of this study support the traditional use of *A. wilkesiana* for the treatment of cancer. Importantly, the research on *R. parviflora* is the first time report about the phytochemical and pharmacological investigations of a specie of the genus; *Rutidea*. Thus, this work is a ground-breaking research into the investigations of the genus; *Rutidea*.

## *Limitations of the study*

The main limitation of this study was the insufficient amounts of the plant fractions, which limited the possibility of identifying other bioactive compounds. It was expected that 1kg of pulverized plant materials would be adequate for the research work. However, this was not the case.

Secondly, the fractionation of the aqueous extracts and highly polar fractions could not be undertaken as more intricate and advanced techniques such as extractions with buffers, ion exchange chromatography, gel filtration and desalting would be required inorder to achieve a separation of the constituents (Shimizu et al., 2006), which due to time and resources constraints were not deemed feasible.

Other challenges encountered in this research were time and resources constraints. For instance, some of the LC-MScompound peaks obtained from the bioactive hplcsubfractions of *R. parviflora* couldnot be unequivocally identified or authenticated with standard compounds.

## *Future Work*

Further phytochemical studies would be carried out on *Rutideaparviflora* in order to establish the presence of the un-identified compounds in the plant. Other species of the *Rutidea* genus would be investigated for potential bioactivities. Similarly, other un-researched species of *Margaritaria* and *Acalypha* would be investigated for potential bioactivities

Semi-synthesis of more potent thymoquinone analogues would be carried out. The aim of this study would be the conjugation of some bioactive compounds with thymoquinone. The gallic acid pharmacophore would be exploited in semi-synthesis.

The *in vivo* investigation of the anti-cancer activities of the identified bioactive compounds in this research would be carried out.

## *Prospects and Outlook*

"Life is Green" is a popular term, which emphasizes the pivotal role played by plants in the existence of mankind. Plants are an integral part of the human race, and is the principal source of the three basic necessities of life; food, shelter and clothing, without which the continued existence of the human race on planet earth may be questionable. A fourth need of man has been identified as the need for medicine which cannot be over-emphasised. Historical records of 2600 BC, documented in clay tablets showed that plants have been used as medicine. Examples of such plants were *papaver somniferum*, *commiphora* species and *glycyrrhiza glabra*, which are still being used as remedies in modern times (Newman et al., 2000).

Further documentation has also shown that diverse ethnic groups have employed medicinal plants in the treatment of ailments, for instance, the ancient civilizations of China, Egypt, India and Greek also had written evidences of the use of natural products as remedies for disease conditions. An inventory, such as, "*Ebers Papyrus*", an Egyptian record, dated 1500 BC, documented more than 700 drugs. Also, the Chinese "*Materia Medica*" dated 1100 BC, recorded over 600 medicinal plants (Cragg et al., 1997). But the isolation of active constituents from natural products began with the isolation of morphine from *Papaver somniferum* in 1806 by Friedrich Serturner (Cragg et al., 2005).

These available documentations of medicinal plants have been beneficial in the isolation of pharmacologically important compounds, such as artemisinin, which have contributed to the decrease in the death toll from malaria. Even more the medicinal plants have provided a vast number of leads for the pharmaceutical industries in the development of chemotherapy. Paclitaxel, camptothecin and the Vinca alkaloids are some of plant leads with many synthetic analogues.

Literature has shown that less than 20% of the 15% taxonomically classified plants (which is about 500,000 species) have been investigated for biological activities. Nearly 200,000

secondary metabolites obtained from plants have been documented in the Natural products dictionary (Kinghorn et al., 2011). These research efforts have yielded 170,000 diverse structures. This is an indication that plant-based drug discovery is very relevant. Despite the availability of this rich archive of plant-derived structures, clinical trials have utilised just 15% of this number and a greater majority (close to 60%) (CHEMnetBASE, 2013) of the drugs used were obtained from just 10 taxonomically classified families, with 235 trials conducted for anti-cancer activities (Sharma et al., 2013). This situation may be a lack of compelling evidence of the pharmacological activities of some of the plant-derived structures. This underscores the invaluable contributions of pharmacological studies in harnessing the potentials of medicinal plants. Another possibility is the reluctance of the scientific community to probe other genuses within a family, once some pharmacological compounds have been obtained from a particular genus in the family. Researchers, tends to focus on a genus that have been shown to hold some promise at the expense of the seemingly irrelevant genuses. A true case scenario is the Phyllanthaceae family. Much of the pharmacological investigations were carried out on species of the genus *Phyllanthus*. This is evident from the reviews and textbooks on the *Phyllanthus* genus (Calixto et al., 1998; Patel et al., 2011; Kuttan et al., 2012). Whereas other genuses, such as *Martretia*, and *Meineckia* scarcely have any mention in literature. *Margaritaria*, one of the genus in the Phyllanthaceae family, was not investigated for anti-cancer activities until this present research work demonstrated the anti-cancer potentials of *M. discoidea*; one of the thirteen species of this genus. Given the above-mentionedpercentage of researched plants, it is very likely that there exist a vast majority of plant families and genuses (for instance, the genus; *Rutidea*, whose specie *R. parviflora* was investigated in this research) that have not been brought to the search light of the scientific community. This class of un-researched plants could be latent sources of desirable pharmacological activities.

Ethnopharmacological database is a viable means of discovering medicinal plants. Most successful plant-based Investigations have relied on the traditional medicine information of

indigenous communities, for instance, the medicinal uses of the cinchona bark from which quinine was derived were first discovered by the Quechua people of Bolivia and Peru, who used the plant in the treatment of fever, before its anti-malarial properties were, validated (Motley 2010). Similarly, *Artemisia annua*, from which artemisinin, a renowned anti-malarial drug which has recently been shown to possess anti-cancer activity, has been in use in the traditional Chinese medicines (TCM) for a very long time (Tu1981). Again, a comparison of the Ethnopharmacological uses and research-validated activities of plant-derived drugs showed that 80% of 122 drugs had activities related to the traditional medicinal uses of the plants (Fabricant et al., 2001).

In this present research, the documented traditional use of *Acalypha wilkesiana* was employed in the selection of the plant for anti-ovarian cancer activity, with the aim of finding the bioactive compound responsible for this anti-cancer activity. It is worthy to note, that most ethnopharmacological information are undocumented in many developing countries, but keen researchers, could collaborate with other researchers in such countries and obtain these important ethnopharmacological clues. It may be said that tropical and sub-tropical climates are key locations for medicinal plants, given the body of existing knowledge of medicinal plants from these regions. Studies have also shown that the tropical rainforests have been the most harnessed locations for the collection of medicinal plants, as a result of the rich and very diverse floral present in these areas. However, it is pertinent to note that the rainforests which are the habitats of most of the endemic species are threatened by rapid deforestation occasioned by population explosion in these regions (Burslem et al., 2001; Pitman et al., 2002).

Regions such as parts of Africa, Asia, South and Central America need to be combed in search for un-researched plants, as some plants are known to be endemic to specific locations. This fact has been established in Africa. The documented record showed that a vast majority of African plants could be found solely in the continent (White 1983), of which Madagascar, an extensive island country situated off the southeast coast of Africa, is a

unique region for medicinal plants. 4,900 of the 12,000 identified plant species found in the unexplored rainforest regions on this island country are endemic to the country; with the percentage of endemism documented as 82% (McNeely et al., 1990). An interesting observation is that close to 90% of the animals on this island, such as lemurs and fossa are found only in Madagascar (Hobbes et al., 2008). The country has been classified as a 'biodiversity hotspot' by Conservation International (Conservation International 2007). The uniqueness of Madagascar has made some ecologists refer to the island as the 'eighth continent' (Hillstrom et al., 2003). A number of novel anti-ovarian cancer compounds were recently isolated from two Madagascar plants; *Tarenna grevei* (Drake) Homolle (Rubiaceae) and *Tarenna Madagascariensis* (Baill.) Homolle by two groups of researchers: Kingston, D. and co-workers in 2012 and the research team of Salmoun, M. in 2007 (Kingston et al., 2012; Salmoun et al., 2007).

Planned investigations of the anti-cancer activities of medicinal plants commenced about 60 years ago, with the discovery of the vinca alkaloids (vinblastine and vincristine) in the 1950s (Cragg et al., 2005). Thus anti-cancer researches on plants are relatively recent, with fewer plants investigated, compared to studies on other bioactivities (Tan et al., 2006; Cragg et al., 2009). A compilation of the anti-cancer drugs in use from 1981-2006 showed that 47.1% of the 155 clinically approved oncological drugs were derived from natural products, either as unmodified compounds, or semi-synthesised analogues, or synthesised based on natural product leads (Cragg et al., 2007). A further 5 drugs were approved in 2010: romidepsin from the bacteria *Chromobacterium violaceum* and used in the treatment of T-cell Lymphomas and Peripheral T-cell lymphomas(NCI 2009); cabazitaxel a derivative of paclitaxel used in the treatment of prostate cancer(Sanofi-Aventis 2010); eribulin, a synthetic macrocyclic analogue of halichondrin B which was obtained from species of *Halichondria*- a sea sponge and is used in the treatment of Breast cancer (Towle et al., 2001; Yu 2005); mifamurtide, a synthetic derivative of muramyl dipeptide (MDP), isolated from Mycobacterium species used in the treatment of osteosarcoma (Bin et al., 2016); and vinflunine , a derivative of vinca

alkaloids used in the treatment of metastatic transitional cell cancer of the urothelial tract (Kruczynski et al., 1998). This underscores the importance of plants as sources of new cancer chemotherapeutic (Cragg et al., 2013).

The role of chemical synthesis, in the drug discovery process, is of great importance. Most of the obtained plant-derived drugs were found in insufficient amounts in the wild. For instance, the stem barks of about 2,000- 3,000 trees were harvested to enable the isolation of 1 kg of Taxol (paclitaxel), (Runowicz et al., 1993; Suffness et al., 1995). The continued reliance on medicinal plants for the provision of paclitaxel for clinical use would have been unsustainable. But through semi-synthetic methods, paclitaxel is commercially produced. Secondly, the need to optimize the activity of the plant-derived compound is another compelling reason, why semi-synthesis is required. It could be observed that many synthetic analogues have been derived for paclitaxel, camptothecin and the Vinca alkaloids. The combination of natural product and chemical synthesis in the drug development process could be likened to the two sides of the same coin.

In plant-derived compounds for anti-cancer research, much emphasis has been given to the discovery of novel or more potent compounds. An area, which should be explored, is the identification of chemopreventive compounds. Most studies target compounds with very low $IC_{50}$s in the range of nanomolar- single digit micromolar. While this is commendable, as more potent drugs with less-side effects are required. Another strategy should be geared towards discovery or identification of chemopreventive compounds, which may be present in plants that have been used as food for generations. Chemopreventive compounds are necessary, as these compounds could be beneficial in the prevention of cancers. One such compound that has demonstrated chemopreventive activity is thymoquinone, which is a popular mediterranean herb that has served both culinary and medicinal uses. The agent was shown to inhibit the development of pancreatic cancer by researchers at the Kimmel Cancer Centre at Jefferson, USA (Kimmel Cancer Centre 2009).

Gallic acid is another compound with potentials for chemoprevention. The compound is found in many fruits and vegetables. The cytotoxicity of gallic acid has been evaluated in a panel of cancer cell lines and in this present study. The compound showed moderate activities, and warrants further investigations.

Also, the gallic acid pharmacophore is another entity for exploration in semi-synthesis, this is because several studies including this present study have shown that some gallic acid analogues such as laurylgallate, demonstrated enhanced activity on cancer cells, with $IC_{50}$ in the nanomolar-single digit micromolar range. Thus the synthesis of more bioactive compounds with the gallic acid pharmacophore is potentially a noble pursuit.

Drug combination is a new approach to disease treatment, which is been explored in the treatment and management of cancer. Currently, cancer regimens often employ a combination of two or more drugs, to improve therapeutic outcomes and minimize side effects. A significant drawback of clinically used cancer drugs is the very serious side effects, such as heart damage, myelosuppression, hair loss, nausea and vomiting (Tacaret al., 2013), unusual bleeding, painful joints and vomiting (Ozcelik et al., 2010). A key goal of drug combination studies is to discover new agents which could sensitize cancer cells to clinically used drugs, thus result in a reduction of the drug dosage and an attenuation of side effects. A number of moderately active compounds reported in literature and from this study would be suitable candidates for this desired synergism. Agents such as securinine, palmatine, 2-dimethylamino-5-methyl-1,4-benzoquinone, and betulinic acid, warrants further pharmacological investigations and elucidation of their mechanism of action, as these compounds possess potentials as drug combination candidates and also as monotherapeutic agents.

It is pertinent to note that medicinal plant researches are not without some challenges. An often cited disadvantage has been the repeated isolation of previously known compounds and the isolation of bioactive compounds in minute quantities. These observations and

experiences should not hinder plant-based research, as seen in this present study. The discovery of new sources of a previously identified compound is also a novel achievement, as this new finding adds to the existing body of literature about the compound. Also, an alternative source of a bioactive compound has been discovered, which may be beneficial for further studies. The isolation of minute amounts of bioactive compounds is another obstacle in plant-based research. However, these challenges present the opportunities for collaborative research, which could help resolve most of these issues.

Finally, this research work has undertaken the anti-cancer investigations of two plant genuses; *Margaritaria* and *Rutidea*. Most of the *Rutidea* species are endemic to Africa and there are no documented phytochemical or pharmacological data until this present study, which have brought the genus into the pharmacological spotlight of the scientific community. It is hoped that similar studies of previously unknown medicinal plants would be embarked on, as it is glaring from this study that medicinal plant researches are merited. Thus, a scientific expedition into the vast reserves of plants is very relevant and timely in unlocking the tremendous potentials yet untapped.

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# Appendices

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Short communication

## Cytotoxic effects of stem bark extracts and pure compounds from *Margaritaria discoidea* on human ovarian cancer cell lines

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ABSTRACT

*Margaritaria discoidea* (Baill.) G. L. Webster (Euphorbiaceae) is a well-known medicinal plant in Africa used for the treatment of various diseases. So far, no cytotoxic effects of plant extracts on cancer cell lines have been reported.

*Aim of the study:* To evaluate the cytotoxicity against human ovarian cancer cells of extracts of *M. discoidea* and characterize the major bioactive compounds.

*Methods:* Both organic and aqueous extracts of this plant were obtained by maceration. The sulforhodamine B cell proliferation assay was used for evaluation of their cytotoxic activities and the potential bioactive compounds were characterized by gas chromatography–mass spectrometry.

*Results:* The organic extract of *M. discoidea* showed stronger cytotoxicity than the aqueous extract with $IC_{50}$ values of 14.4 ± 3.0, 14.2 ± 1.2 and 34.7 ± 0.5 μg/ml on OVCAR-8, A2780 and cisplatin-resistant A2780cis ovarian cancer cells, respectively. The organic extract was further subjected to bioassay-guided fractionation by partitioning with n-hexane, ethyl acetate, and n-butanol in water. The ethyl acetate fraction was the most potent on the three ovarian cancer cell lines. A GC–MS analysis of trimethylsilyl derivatives of this fraction indicated the presence of phenolic compounds such as gallic acid and the alkaloid securinine. The $IC_{50}$ values of these two compounds were determined to be in the range of 3–16 μM, which indicated that they could contribute to the cytotoxic activity of the extract of *M. discoidea*.

*Conclusions:* This study has evaluated the cytotoxicity of stem bark extracts of *M. discoidea* against ovarian cancer cells and provided a basis of further development of this plant for the treatment of ovarian cancer.



### Introduction

Ovarian cancer is the second most common gynaecologic cancer among women and the ninth most prevalent cancer in the US (Siegel et al., 2013). The standard treatment for ovarian cancer is the use of paclitaxel and platinum-based therapy after aggressive surgical reduction and this has improved survival. However, the re-emergence of cancer in a drug-resistant form frequently results in a poor overall survival rate. Natural products have played an important role in the development of new medicines. Currently, more than 50% of all approved drugs (not limited to cancer) are natural products and their derivatives (Cragg et al., 2009).

*Margaritaria discoidea* (Baill.) G. L. Webster [synonym *Phyllanthus discoideus* (Baill.) Müll. Arg.)] (Euphorbiaceae) is a tree native to Africa and has been traditionally used for the treatment of onchocerciasis, wounds and skin infections (Mensah et al., 1990), and oedema (Obiri et al., 2014). Pharmacological studies have shown that it has uterotonic, anti-malarial (Weenen et al., 1990), antibacterial (Mensah et al., 1990), anti-inflammatory and analgesic, filaricidal, free-radical scavenging (Ekuadzi et al., 2014), anti-allergic, and anti-arthritic activities (Obiri et al., 2014). Many *Phyllanthus* species which are closely related to the genus *Margaritaria* (both in the Euphorbiaceae family) have been shown to inhibit the growth of human breast, lung, gynaecologic, colon and ovarian (Jia et al., 2013) cancer cells. The *Phyllanthus* species have been shown to be a source of alkaloids, phenolics and terpenoids (Patel et al., 2011). Previously, alkaloids such as viroallosecurinine and securinine (Mensah et al., 1990; Weenen et al., 1990), and

*Abbreviations:* BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; EI, electron ionization; FBS, foetal bovine serum; GC–MS, gas chromatography–mass spectrometry; NCI, National Cancer Institute; SRB, sulforhodamine B; TCA, trichloroacetic acid; TMCS, chlorotrimethylsilane; TMSi, trimethylsilyl.

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Article

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# Stable Isotope Dilution Gas Chromatography–Mass Spectrometry for Quantification of Thymoquinone in Black Cumin Seed Oil

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Supporting Information

ABSTRACT: Black cumin seed (*Nigella sativa* L.) is a widely used spice and herb, where thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) is the major bioactive compound. Here, a stable isotope dilution (SID) gas chromatography–mass spectrometry (GC–MS) technique was developed for the quantification of thymoquinone. A doubly deuterated thymoquinone ([$^2H_2$]-thymoquinone) was synthesized for the first time with more than 93% deuteration degree shown by mass spectrometry and proton nuclear magnetic resonance ($^1$H NMR). This compound was used as an internal standard for the quantification of thymoquinone using a SID GC–MS method. The validation experiment showed a recovery rate of 99.1 ± 1.1% relative standard deviation (RSD). Standard addition and external calibration methods have also been used to quantify thymoquinone, which cross-validated the developed stable isotope dilution assay (SIDA). In comparison to external calibration and standard addition methods, the SIDA method is robust and accurate. The concentration of thymoquinone in five marketed black cumin seed oils ranged between 3.34 and 10.8 mg/mL by use of SID GC–MS.

KEYWORDS: *Nigella sativa, thymoquinone, stable isotope dilution, GC–MS, quantification*

## INTRODUCTION

Black cumin seed (*Nigella sativa* L.) is a widely used spice in Mediterranean countries, Pakistan, and India.[1,2] It is also used as a traditional medicine for the treatment of a range of diseases, such as diabetes, hypertension, fever, arthritis, inflammation, gastrointestinal disturbances, and cancer.[3] Studies into the biological activities of this plant have revealed that the volatile essential oil components, predominantly thymoquinone (1) (Figure 1),[4] possess antioxidant,[5] anti-inflammatory,[6] antidiabetic,[7] immunomodulatory,[8] and anti-tumor[9−11] activities.

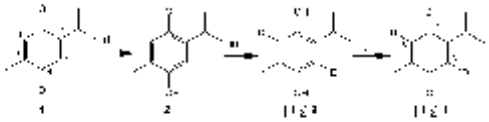

Figure 1. Scheme for synthesis of [$^2H_2$]-thymoquinone ([$^2H_2$]-1): (i) $Na_2S_2O_4$ in $H_2O$/MeOH at room temperature for 2 h, (ii) (a) 3.4 M $D_2SO_4$ in $D_2O$ with reflux for 48 h and (b) cool to room temperature and workup with $H_2O$, and (iii) $CrO_3$ in 60% HOAc in $H_2O$ at −4 °C for 2 h.

Thymoquinone was previously quantitated by means of high-performance liquid chromatography (HPLC),[12,13] high-performance thin-layer chromatography (HPTLC),[14] and a differential pulse polarographic method.[15] These methods could achieve high sensitivities and reproducibility, but they often need multi-steps of sample preparation and external calibrations; thus, the results could be influenced by complex matrices. Gas chromatography (GC) and/or gas chromatography–mass spectrometry (GC–MS) are preferred techniques for the analysis of volatile and semi-volatile compounds and have also been used for the quantification of thymoquinone[16] and identification of the composition of black cumin seed oil.[5,17] A stable isotope dilution assay/analysis (SIDA) should overcome the problem of the matrix effects, such as compound discrimination during extraction, chromatographic separation, and ultraviolet (UV)/mass spectrometry (MS)/electrochemical detection,[18,19] when a deuteriated thymoquinone is used as an internal standard. Stable isotope dilution coupled with GC–MS (SID GC–MS) has widely been used for determination of various compounds, e.g., methoxypyrazines in red wines,[19] dietary lignans in food,[20] natural estrogens in vegetables and fruits,[21,22] and alkypyrazines in coffee.[23] Here, we report synthesis of a stable deuterium (D or $^2$H)-labeled thymoquinone (Figure 1) for the first time and its application as an internal standard for quantification of thymoquinone in black cumin seed oil by the means of SID GC–MS.

## MATERIALS AND METHODS

Thymoquinone (99%), $D_2$-sulfuric acid ($D_2SO_4$, 99.5%), deuterated water [deuterium oxide ($D_2O$), 99.5%], sodium hydrosulfite ($Na_2S_2O_4$), and chromium trioxide ($CrO_3$) were purchased from Sigma-Aldrich. Hexane (analytical grade) was purchased from Fisher-Scientific, U.K. The following black cumin seed oils were purchased from Amazon: Manako black cumin oil, CEBRA *Nigella sativa* oil, Avena black cumin oil, Iman VERGIN black cumin oil, and This Health black seed oil.

**Synthesis.** The doubly deuteriated thymoquinone ([$^2H_2$]-1) (Figure 1) was prepared and purified according to a similar method

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