Biological Activities of Synthetic Coumarin Derivatives

Submitted in partial fulfillment for the Degree of Master of Applied Sciences in Biotechnology in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

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REFERENCE DECLARATION

I, Mr. Kabange K– 21032182 and Prof Bharti Odhav do hereby declare that in respect of the following dissertation:

Title: **Biological Activities of Synthetic Coumarin Derivatives**

1. As far as we ascertain:

a) no other similar dissertation exists;

2. All references as detailed in the dissertation are complete in terms of all personal Communication engaged in and published works consulted.

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Signature of supervisor	**Date**
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Signature of co-supervisor	**Date**

AUTHOR'S DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof Bharti Odhav**, **Dr K.N. Venugopala** and **Dr Viresh Mohanlall**

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Student's signature

DEDICATION

I would like to dedicate this work to the Ilofo`s family : Salima Kahamba Prudence, Jean-Pierre Ilofo, Olivier Ilofo, Joel Ilofo and Christopher Ilofo, you are truly the inspiration of this work and I thank you for your encouragement, support throughout this study. It meant so much to me during the pursuit of my Masters`s degree and I am grateful for all that you have done for me, to each of you I offer my sincere thanks and deepest gratitude.

TABLE OF CONTENTS

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ABBREVIATIONS

15-LO	:	15-Lopoxygenase inhibitor
BSL	:	Brine shrimp lethality
CO_2	:	Carbon dioxide
COX	:	Cyclo-oxygenase
DEET	:	*N,N*- Diethyl-meta-toluamide
DMEM	:	Dulbecco`s Modified Eagle`s Medium
DMSO	:	Dimethyl sulphoxide
DPPH	:	2,2-Diphenyl-1-picrylhydrazyl
ELISA	:	Enzyme-linked Immunosorbent Assay
FCS	:	Foetal Calf Serum
FITC	:	Fluorescenisothiocyanate
IC_{50}	:	Concentration of inhibitor necessary for 50% inhibition
LOX	:	Lipoxygenase
MIC	:	Minimum inhibition concentration
MRC	:	Medical Research Council
MTT	:	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NaN_3	:	Sodium azide
PBMC	:	Peripheral Blood Mononuclear cells
ROS	:	Reactive oxygen species
RPMI	:	Roswell Park Memorial Institute
TLC	:	Thin- layer chromatography
WHO	:	World Health organization
UV	:	Ultraviolet

DBT : Durban University of Technology Culture collection reference based at the department of Biotechnology and Food Technology

PUBLICATION

Kasumbwe, K., Narayanaswamy, V. K., Gleiser, R. M., Aldhubiab, B. E., Attimarad, M. V., Odhav, B. (2014) Evaluation of Halogenated Coumarins for Antimosquito Properties. *The Scientific World Journal,*189824**,** 1-6.

INTERNATIONAL CONFERENCE PRESENTATIONS

Kasumbwe, K., Narayanaswamy, V. K., Odhav, B. (2013) Proprieties of 3-mono/dibromoacetyl, 6-halogenated coumarin analogues against *Anopheles arabiensis*. *6th Pan-African Malaria conference,* ICC Durban, South Africa, October 2013.

Kasumbwe, K., Narayanaswamy, V. K., Mohanlall, V., Odhav, B. (2013) Pharmacological activities of Halogenated Coumarin Analogues. South African Society for Microbiology Congress, Warm Bath Bela Limpopo, South Africa, November 2013.

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Coumarins are naturally occurring α-benzopyrone derivatives known for their pharmacological properties such as anticoagulant, antimicrobial, anticancer, antioxidant, anti-inflammatory and antiviral properties. The pharmacological, biochemical, and curative applications of coumarins depend on the substitution around the coumarin core structure. In the present study, seven halogenated coumarins **CMRN1 - CMRN7** were synthesized and evaluated for mosquito larvicidal, repellancy , and insecticidal activity against *Anopheles arabiensis*. Furthermore, the antimicrobial properties of compounds **CMRN1 - CMRN7** were evaluated by assessing the bacterial and fungicidal activities using the disc diffusion method. The anti-inflammatory properties were evaluated using the 5-lipoxygenase kit assay. The evaluation of the safe use of the compounds was determined using the Brine shrimp lethal test. The potential carcinogenic properties of the studied compounds was done using the *Salmonella* mutagenicity test. The anti-cancer property of the studied compounds was evaluated against UACC62 (Melanoma), MCF-7 (Breast cancer), and PBMC (Peripheral blood mononuclear) cell lines using of MTT assay. The apoptotic potential of the synthesized coumarin was evaluated against UACC62 (Melanoma) cell by assessing their morphological changes, membrane change, mitochondria membrane potential, and caspase-3 activity using the Annexin V-PI staining, JC-1, caspase-3 enzyme kits, respectively, on flow cytometer. The results were compared to a known anti-cancer drug, doxorubicin.

The results showed that compounds **CMRN1, CMRN2, CMRN4, CMRN5** and **CMRN7** exerted 100% larval mortality within 24 h of exposure. All halogenated coumarins reversibly knocked down adult mosquitoes but did not kill them after 24 h of exposure. Furthermore, the adulticidal activity of the compounds was considered only mild to moderate.

The antimicrobial activity of the synthetized coumarins **CMRN1 - CMRN7** were assessed against *E. coli, K. pneumoniae, S. marcescens, S. faecalis, B. cereus, B. coagulans, B. stearothermophilus, C. freundii, S. aureus and M. luteus* bacteria and three yeast cultures, *C. albicans, C. utilis, S. cerevisiae* as well as two fungal species, *A. flavus* and *A. niger*. Compounds **CMRN1** and **CMRN2** showed bacterial growth inhibition for all the tested species except *K. pneumonia* and *B. stearothermophilus*. Compounds **CMRN4** and **CMRN7** showed moderate bacterial inhibition against *B. cereus, M. luteus* and *S. aureus*.

The anti-inflammatory activity of the coumarins analogues showed that 1 mg/mL of the compounds **CMRN1, CMRN2, CMRN4** and **CMRN5** displayed moderate anti-inflammatory activity when compared to the positive control, 15-lapoxygenase.

The cytotoxicity results of the studied synthetized coumarins displayed selective activity towards the cancer cell lines used in this study. Our studies showed that **CMRN1, CMRN2, CMRN4,** and **CMRN5** had significant cytotoxity effect against UACC-62 (Melanoma) and MCF-7 (Breast) cancer cells with an inhibitory concentration (IC_{50}) which displayed significant cytotoxicity effect, in particular **CMRN4** and **CMRN5**. These compounds **CMRN1- CMRN7** showed no toxicity effect against PBMCs cell line.

The mechanism of cell death, that is, necrosis or apoptosis induced by the coumarins was investigated against UACC-62 (Melanoma). We found that **CMRN1, CMRN2, CMRN4, CMRN5** induced morphological changes, characteristic of apoptosis . Annexin V kit showed that **CMRN1**, **CMRN2** and **CMRN5** showed early apopotosis and late apoptosis was particularly higher for compound **CMRN4**. The disruption of the mitochondria membrane was noticed to be greater in **CMRN1** and **CMRN5** when compared to the positive control doxorubicin. Compound **CMRN4** produced high levels of caspase-3 positive compared to the control.

The coumarin compounds showed no mutagenicity and were also found to be non-toxic to brine shrimps.

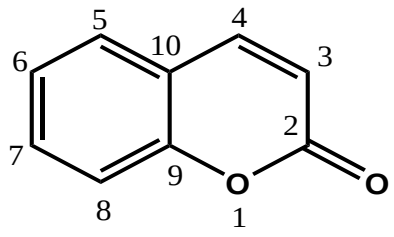
In conclusion, compounds **CMRN1, CMRN2**, **CMRN4, CMRN5** and **CMRN7** are potential larvicidal agents because they exhibited close to 100% activity within 24 h. Furthermore, the anti-cancer efficiency of compounds **CMRN1, CMRN2, CMRN4,** and **CMRN5**, is enough qualification for them to be optimized for increase anticancer potency.

1. INTRODUCTION

Throughout the world, many medicinal compounds are being discovered (Fakim, 2006). Scientists have learnt to modify the chemical structures of active compounds so that they can improved therapeutic activity and reduced the toxicity (Kostova, 2005). In view of the established low toxicity, relative cheapness, presence in the diet, and occurrence in various herbal remedies of coumarins, it appears prudent to evaluate their properties and applications further.

Coumarins were first isolated from the plant species *Dipteryx odorata Willd* (Family: Fabaceae) in 1820. The name coumarin is derived from 'Coumarou'. The French commonly called Tonka bean (*Dipteryx odorata Willd*, Fabaceae), from which coumarins were derived (Al-Bayati *et al*., 2010). Coumarins (2*H*-1-benzopyran-2-one) (Figure 1) are a class of phenolic substances found as secondary metabolites from plants, microbes, and are widely used as additives in cosmetics, food, perfumes, drugs, and optical brighteners (Narayanaswamy *et al*., 2014). Research on coumarins is important due to their biological properties (Malhotra *et al*., 2008). Owing to their significance in countless fields of everyday existence, their chemistry has been extensively investigated and countless natural and non natural coumarins have been synthetized (Kini *et al*., 2012).

Coumarin is a natural product isolated from plant and it is known for several biological activitites such as antibacterial (Kumar *et al*., 2013, Venugopala and Jayashree, 2008, Venugopala *et al*., 2008), antifungal (Al-Amiery *et al*., 2012, Shi and Zhou, 2011), antioxidant (Tyagi *et al*., 2005, Melagraki *et al*., 2009), analgesic (Venugopala and Jayashree, 2003), anti-inflammatory (Luchini *et al*., 2008, Venugopala *et al*., 2004) and anti-cancer proprieties (Velasco-Velázquez *et al*., 2003, Paul *et al*., 2013).



(**1**)

Figure 1: Chemical structure of 2*H*-chromen-2-one (α-benzopyrone) (1) (Jain and Joshi, 2012)

These studies are useful because they may form the backbone for developing novel therapeutic agents using coumarin compounds (Jain and Joshi, 2012), In addition, natural coumarins has shown very good activities such as antioxidants, enzyme inhibitors and precursors of toxic substances (Kostova, 2005).

As coumarins represent a large class of compounds (benzopyrones) throughout the plant kingdom, their dietary value is well known (Lake, 1999). They have high levels of essential oils such as cinnamon bark oil, cassia leaf oil and lavender oil. Lake (1999) also reported that coumarins are found in fruits (for example, bilberry, cloudberry), green tea and chicory.

The coumarin (**1**) and its derivatives can be synthesized by various methods that include Pechmann, Perkin, Witting and Knovenegal reactions (Shaabani *et al*., 2009; Smitha and Reddy, 2004; Valizadeh and Shockravi, 2005).

Since substitutions may arise at any of the six available sites (Figure 1) C3 - C8 on the basic molecular moiety of coumarin nucleus, the coumarins are exceptionally variable in structure. This structural variation leads to multiple biological properties (Kostova, 2005).

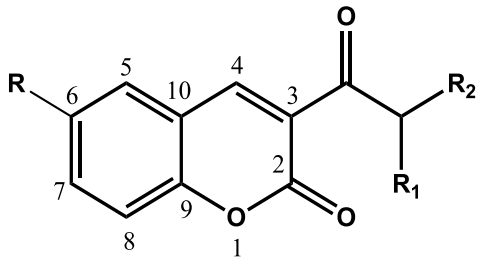
The aim of this study was to evaluate the pharmacological properties of seven synthetic halogenated coumarins (**CMRN1** - **CMRN7**) (Figure 2).

Research Objectives

1) To evaluate the pharmacological activities: insecticidal; antimicrobial; anti-inflammatory , antioxidant activity of the synthetic halogenated coumarins (**CMRN1 – CMRN7**).

2) To evaluate the cytoxicity of the synthetic halogenated coumarins (**CMRN1 – CMRN7**) against UACC62 (Melanoma), MCF-7 (Breast cancer) and PBMC (Peripheral blood mononuclear) using the MTT assay and determine their apoptotic potential using flow cytometry.

3) To determine the safe use and the toxicity of the synthetic halogenated coumarins (**CMRN1 – CMRN7**) by testing their mutagenic activity with the Ames mutagenicity test and the Brine shrimp.



CMRN 1 = R= H, R_1 = H, R_2 = Br

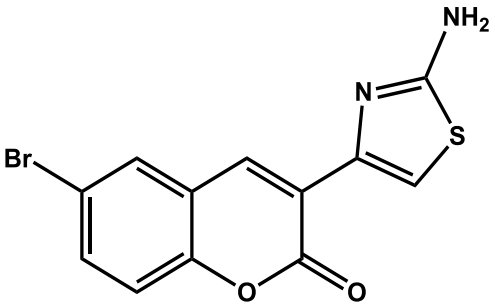
CMRN 2 = R= Br, R_1 = Br, R_2 = Br

CMRN 3 = R= Br, R_1 = H, R_2 = H

CMRN 4 = R= Br, R_1 = H, R_2 = Br

CMRN 5 = R= Cl, R_1 = H, R_2 = Br

CMRN 6 = R= Cl, R1 = H, R_2 = H



CMRN 7

Figure 2: Synthetic halogenated coumarins CMR1–CMR7 (Jayashree *et al*., 2004; Venugopala and Jayashree, 2003; Chopra *et al*., 2007; Venugopala *et al*., 2004; Venugopala and Jayashree, 2004; Jayashree *et al*., 2006; Jayashree *et al*., 2005, Chopra *et al*.,2006; Chopra *et al*., 2009)

2. LITERATURE REVIEW

2.1 Natural coumarins

Natural products from plants and microorganisms conventionally have endowed the pharmaceutical industry with one of its most important origins of lead compound find for new drugs and medicines (Haslam, 1996).

Many conventional drugs have since originated from plant sources, though about a century ago (due to the lack of modern biotechnological methods), very few effective drugs were plant-based. Due to technological advancements, companies have become able to pharmacologically screen plants (herbs) on a large-scale for their development into drugs that can be used to treat particular health conditions (Vickers *et al*., 2001).

Medicinal plants form the backbone from which current conventional drugs are developed for therapeutic purposes like cancer. Kamuhabwa *et al*. (2000) suggested that plants can be used for the development of anticancer agents. These anticancer agents, as listed in Cragg and Newman (2005), include: vinblastine, vincristine, taxol, etoposide, and the camptothecin .

Apart from these anticancer agents, natural products (like coumarins), serve as source of chemical structures, from which new drug compounds are synthesized. These coumarins, as mentioned previously, comprise a very large class of phenolic compounds, which are extensively distributed in the plant kingdom, particularly in angiosperms (Montagner *et al*., 2011).

Natural and synthetic coumarins exhibited broad pharmacological activities (Patel *et al*., 2011). Patel (2011) has suggested that recognizing the important structural features within the coumarin nucleus, can aid in designing and developing new analogues with better activity. In addition, characterization of the mechanism of action of coumarins, and their potential side effects, can be understood with clarity using the knowledge gained from their structural features. It has been found that the different substituent within the coumarin nucleus has a strong influence on the biological activity of the resulting derivatives (Sandhya *et al*., 2011). Some important coumarin members like novobiocin (**2**), coumermycin (**3**), and clorobiocin (**4**) were isolated from microbial sources such as *Streptomyces* and *Aspergillus* species (Cooke, 1998; Lacy and O'Kennedy, 2004; Yadagiri *et al*., 2014).

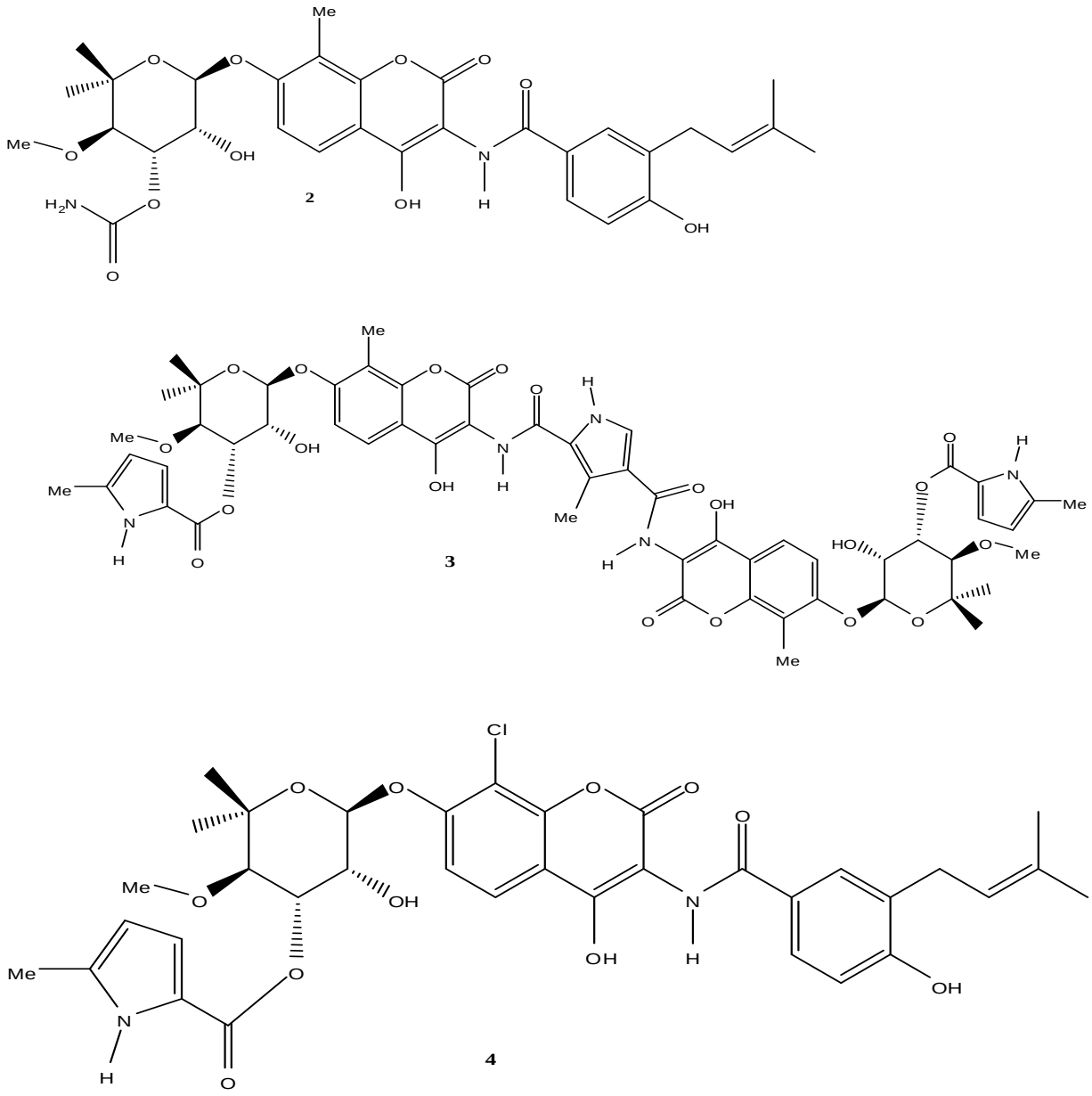


Figure 3: Examples of coumarin members isolated from microbial sources novobiocin (2), coumermycin (3), and clorobiocin (4) (Lacy and O'Kennedy, 2004).

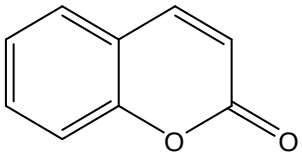
2.2 Classification of coumarins

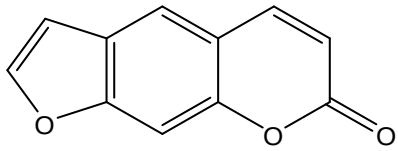
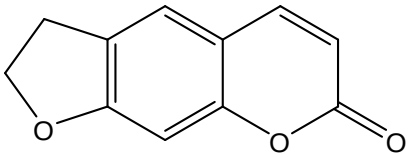
Fruits, vegetables, spices and herbs contain bioactive compounds that are valuable for human health (Almeida *et al*., 2011, Pinela *et al*., 2012). Coumarins are found as secondary metabolites in plants and constitute an important class of oxygen heterocycles.

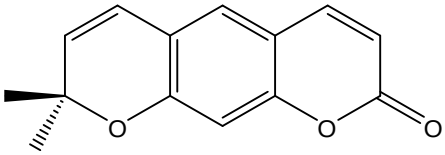
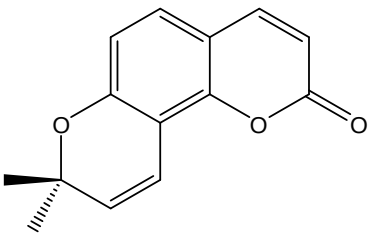
Natural coumarins are categorized into six types (Tables 1) based on their chemical structure, these are (i) Simple coumarins, (ii) Furanocoumarins, (iii) Dihydrofuranocoumarins, (iv) Pyranocoumarins, (v) Phenylcoumarins, and (vi) Bicoumarins (Venugopala *et al*., 2013). Simple coumarins are compounds which can undergo hydroxylation, alkoxylation and

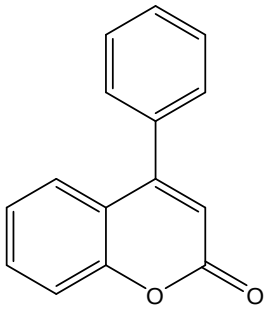
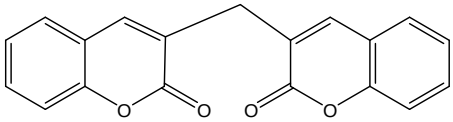
alkylation on the benzene ring to form coumarin derivatives. Furano coumarins, include of five-member furan ring attached to the benzene ring, linear or angular. Pyranocoumarins are compounds that have 6-membered pyran ring attached to a benzene ring. Pyrone-substituted coumarins are compounds that contain a substitution on pyrone ring usually at position C3 or C4 (Lacy and O'Kennedy, 2004).

Table 1: Types of coumarins and their biological activity (Venugopala *et al*., 2013)

Type of coumarin	General structure	Examples	Pharmacological activity
1.Simple Coumarins		Coumarin	Anti-inflammatory Anti-cancer
		Esculetin	Antiadipogenic Antioxidant Neuroprotective
		Ammoresinol	Antibacterial Antibacterial Antifungal
		Ostruthin	Anticancer Anticonvulsant Antioxidant
		Osthole	Antibacterial Antibacterial Antitumor Anticancer
		Novobiocin Coumermycin Chartreusin	Antiadipogenic Antioxidant
		Fraxin	Antitubercular Antiadipogenic
		Umbelliferone	Antihyperglycemic
		Fraxidin	Antitubercular
		Phellodenol A	Antiadipogenic
		Esculin, Fraxetin	
		Murrayatin Auraptene	

2.Furano Coumarins		Imperatorin Psoralen Bergapten Methoxsalan Marmalde, marmelosin	Anti-inflammatory Antibacterial Antifungal Antiviral Anticancer Anticonvulsant Antifungal Anti-TB Anti-TB Cyclochrome P450 inhibitor
3.Dihydrofurano coumarins		Anthogenol Felamidin Marmesin, rutartin	Antibacterial Antibacterial Anti-TB Anti-TB

4.Pyranocoumarins are of two types			
4a Linear type		Grandivittin Agasyllin Aegelinol Benzoate Xanthyletin	Antibacterial Antibacterial Antibacterial Anti-TB
4b Angular type		Inophyllum A,B,C,E,P,G_1 and G_2 Calanolide A,B, and F (+)-Dihydrocalanolide A and B Pseudocordatolic C	Antiviral Antiviral Antiviral Antiviral

5.Phenyl coumarins		IsodisparB, dispardiol B, mammea A/AB cyclo E, mammea A/AB Dioxalanocyclo F, disparinol D, disparpropylinol B	
6.Bicoumarins		Dicoumarol	Anticoagulant

2.3 Synthesis of coumarins

Coumarins can be produced through organic synthesis even though they can be discovered naturally in countless green plants. There have been many routes developed for the synthesis of coumarin and their derivatives. These routes include: (i) the Pechmann reaction, (ii) the Perkin reaction, (iii) the Knoevenagel reaction and, (iv) the Witting reaction (Shaabani *et al*., 2009; Smitha and Reddy, 2004; Valizadeh and Shockravi, 2005).

2.3.1 Synthesis of coumarins by Pechmann reaction

The Pechmann reaction is the most extensively used procedure for the synthesis of different coumarin compounds. It involves the condensation of phenols with β-keto esters with a variety of reagents and gives good yields of 4-substituted coumarins (**5**) (Figure 4). This synthetic method has been utilized to obtain coumarins and benzopyrones with biological or industrial importance (Borges *et al*., 2005, Bose *et al*., 2002).

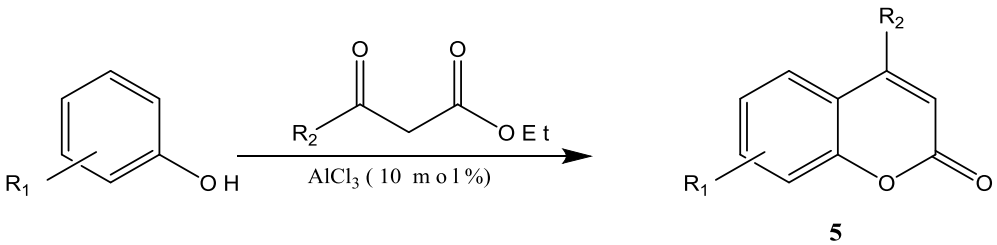


Figure 4: Synthesis of coumarins by Pechmann reaction (Bose *et al*., 2002).

2.3.2 Synthesis of coumarins by Knoevenagel condensation

Knoevenagel condensation is one of the most vital reaction for the preparation of substituted alkenes (Phadtare and Shankarling, 2012). Under the microwave irradiation, the knoevenagel condensation can be used for the synthesis of many coumarins. This easy and fast method was reported wherein the condensation of salicylaldehyde or its derivative including different derivatives of ethyl acetate in the presence of piperidine as catalyst under the solvent free condition leads to coumarin (6) derivative (Figure 5) (Bogdał, 1998).

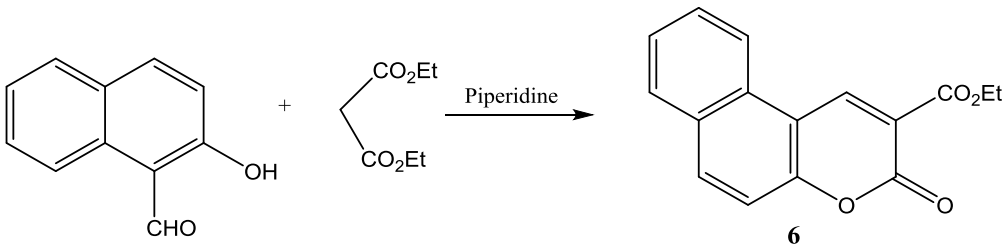


Figure 5: Synthesis of coumarin (6) by Knoevenagel condensation under microwave irradiation (Bogdał, 1998).

2.3.3 Synthesis of coumarin by Perkin Reaction

The Perkin reaction consists of the formation of a 7-hydroxy, 4-methoxy coumarin (**7**) by aldol condensation of aromatic *ortho*- hydroxybenzaldehyde and acid anhydrides, in the presence of an alkali salt of the acid (Figure 6). Different reports on the synthesis of coumarins through this method have been published (Borges *et al.*, 2005).

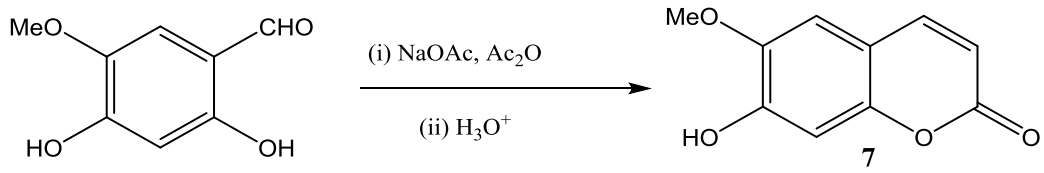


Figure 6: Synthesis of coumarin (7) by Perkin reaction (Borges *et al.*, 2005).

2.3.4 Synthesis of coumarin by Witting reaction

The Witting reaction consists of in situ coordinated carboxymethoxy methylene, triphenyl phosphine and salicylaldehydes supported on MgO for the quick and one-pot synthesis of simple coumarins in solvent-free status. The combination of salicylaldehydes (or its

derivatives), triphenyl phosphine, and chloroethyl acetate on MgO furnished on grinding, substituted coumarins (**8**) (Figure 7) (Shockravi *et al*., 2003).

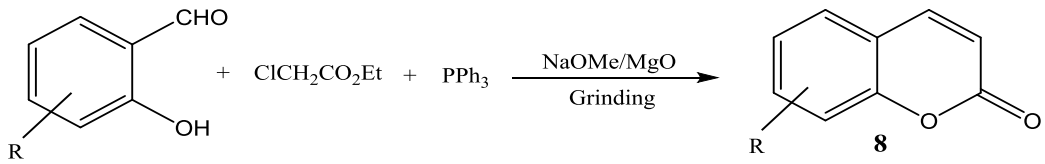


Figure 7: Synthesis of substituted coumarins (8**) by Witting reaction (Shockravi *et al*., 2003)**

2.3.5 Biosynthetic pathway of coumarins

Bourgaud *et al*. (2006) revised the biosynthesis of coumarin and reported that cinnamic acid is the starting precursor for the biosynthesis of simple coumarin and several mono and disubstituted coumarin with the help of several enzymes as shown in Figure 8.

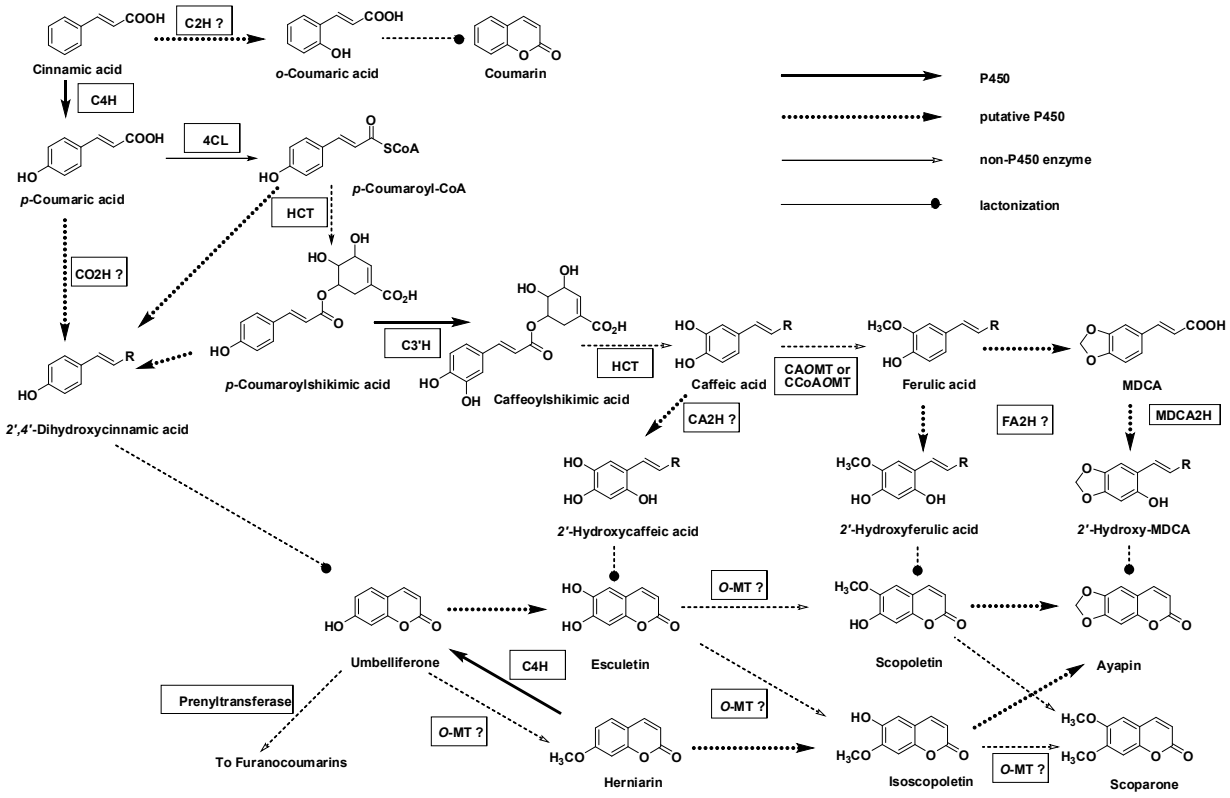


Figure 8: Biosynthesis of coumarins via phenylpropanoid pathway (Bourgaud *et al*.,2006)

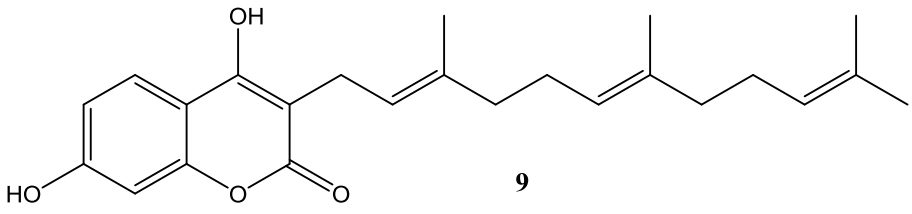
2.4 Biological activities of coumarins

Coumarins have been subjected to extensive studies because of their interesting biological activities and have been used as healing agents for handling of various diseases. Coumarins show diverse biological activities, including :

2.4.1 Antibacterial activity

For many years, coumarins have been recognized as well known naturally occurring oxygen-heterocyclic compounds extracted from various plants (Batra *et al*., 2012). Although coumarin (**1**) have been reported to show low antibacterial activity, however, other members, such as ammoresinol (**9**) and ostruthin (**10**) have exhibited significant antibacterial activity against a wide spectrum of bacteria such as *B. megaterium, M. luteus, M. lysodeikticus, S. aureus* due to long chain hydrocarbon substitutions on 7-hydroxy coumarins (Venugopala *et al*., 2013).

Compound anthogenol (**11**) obtained from green fruits of *Aegle marmelos* (Evans, 2009), was discovered to show an inhibitory effect on the growth of *Enterococcus* bacteria. Compounds agasyllin (**12**) and aegelinol (**13**) (Figure 9) has been rapported to demonstrate bacteria growth inhibition against clinically isolated bacteria such as *S. aureus, S. typhi, E cloacae*, and *E aerogenes*.



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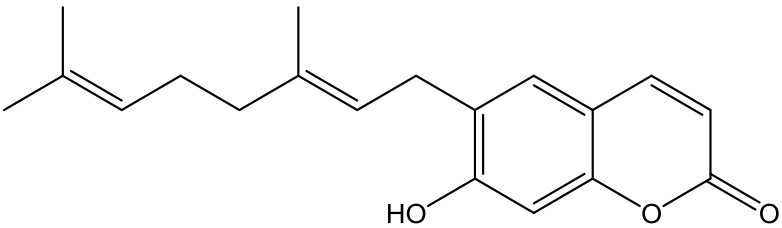

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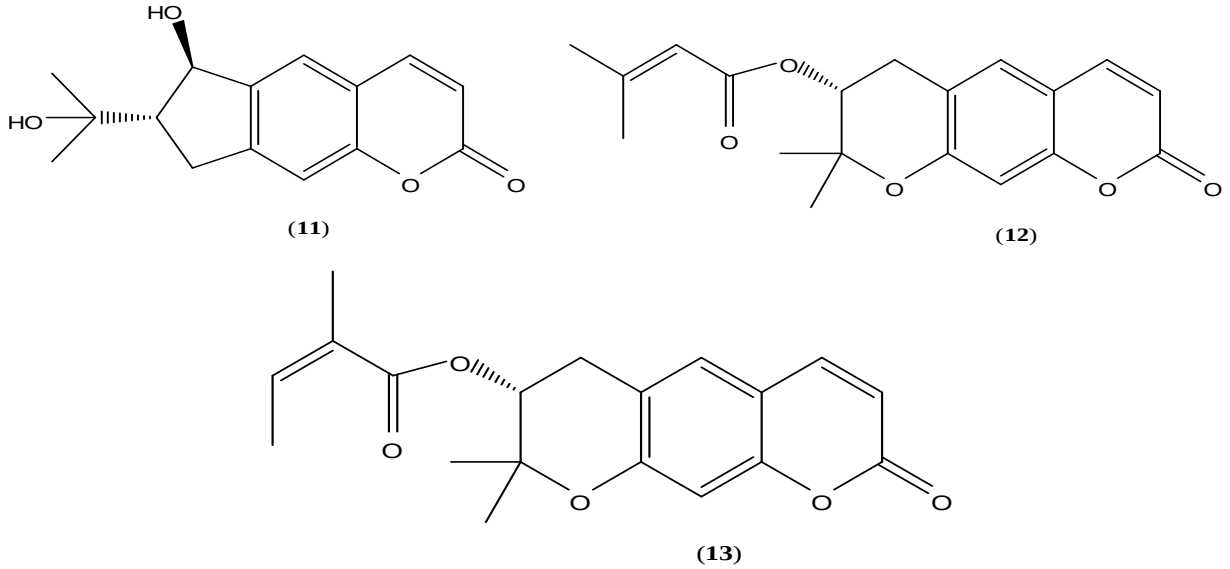


Figure 9: Anti-bacterial compounds (Razavi, 2013; Liu, 2005; Adhami *et al.*, 2013)

Coumarin compounds, particularly those that possess backbones comprising of 1, 2-benzopyrone (like the benzopyrone backbone of anti-infective quinolone drugs), have gained popularity because of their antibiotic potential (Shi and Zhou, 2011).

2.4.2 Antifungal activity

The bioactive coumarin derivative osthole (**14**) (Figure 10) was found to show antifungal activity against plant pathogens such as *R. solani*, *P. capsici*, *B. cinerea*, *S. sclerotiorum* and *F. graminearum* (Wang *et al*., 2009).

Many coumarins have been screened for antifungal activities, and the three most competent ones are the ostruthin (**10**), psoralen (**15**), and imperatorin (**16**) (Bourgaud *et al*., 2006).

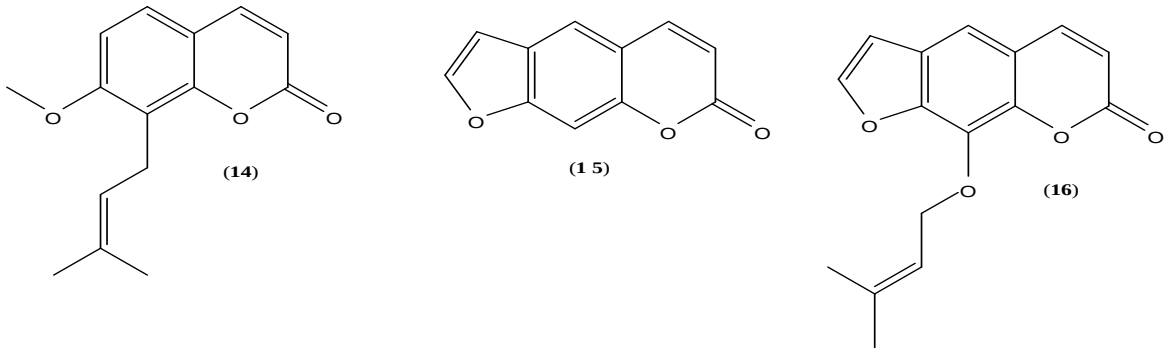


Figure 10: Antifungal compounds (Liu, 2005; Wei, 2009; Parast, 2011)

2.4.3 Anti-inflammatory activity

Inflammation, a dynamic process caused by the release of chemicals from the tissues and migrating cells, involves the excretion of pro-inflammatory cytokines such as tumor necrosis factor, α- interleukin (IL) -1β, and vascular endothelial growth factor (VEGF) from inflamed sites or within the body (Dinarello, 2010). Coumarin (**1**) as shown to have anti-inflammatory properties and is used in the treatment of edema, fluid from injured tissue by stimulating phagocytosis, enzyme production and thus proteolysis. The compound imperatorin (**16**) demostrated anti-inflammatory activity in lipopolysacharide-stimulated mouse macrophage (RAW264.7) *in vitro* using the carrageenan-induced mouse paw edema model. (Huang e*t al*., 2012).

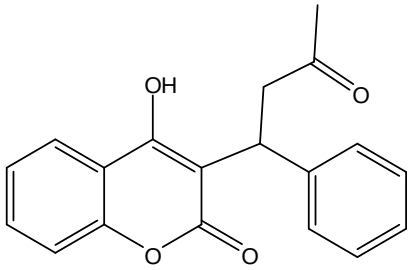
In 1975 (Pillar) reported that coumarins, in general, are used for the treatment of oedema since they stimulate phagocytosis during the oedema anti-inflammatory response. This treatment includes the removal of proteins and edema fluid from injured tissues, followed by the process of proteolysis.

2.4.4 Anti-mosquito activity

Mosquitoes are the major vectors of parasites and pathogens that cause malaria, filariasis, dengue fever, yellow fever, Japanese encephalitis, and other fevers affecting humans and domestic animals. The vector control programs mainly use four classes of chemical insecticides: organochlorines,organophosphates, carbamates, and pyrethroids. Bacterial insecticides and insect growth regulators have also become more widely used in recent years. However, use of chemicals on a vast and increasing scale has led to the wide spread development of resistance as a result of election for certain genes, and some species have even become resistant to multiple insecticides (Narayanaswamy *et al*., 2014).

Warfarin (**17**) (Figure 11) is a synthetic coumarin analogue (known as Coumadin) that is used as an anticoagulant and is known commercially with the trade name Coumadin. Essential oils and solvent extracts of plants containing coumarin have shown promising properties against mosquitoes. For example, coumarin extracted from southernwood (*Artemisia abrotanum* L.) and essential oil of carnation flowers (*Dianthus caryophyllus* L.) had a repellent effect against yellow fever mosquitoes (*A. aegypti* L.) and ticks (nymphs of *Ixodes ricinus* L.) (Tunón *et al*., 2006). Eight coumarin derivatives obtained from hexane extractions of the roots of *Esenbeckia grandiflora* Mart, were effective larvicides against *A. aegypti L* (De Oliveira *et al*., 2005).

The coumarin compounds imperatorin and osthole extracted from *Cnidium monnieri (L.) Cusson* fruit were effective against larvae of *Culex pipiens pallens Coquillett*, *Aedes aegypti L* (yellow fever mosquito) and against *C. p. pallens* larvae resistant to differents insecticides, suggesting that coumarins, pyrethroid and organophosphate insecticides do not have in common the same mode of action or cause cross-resistance (Narayanaswamy *et al*., 2014).

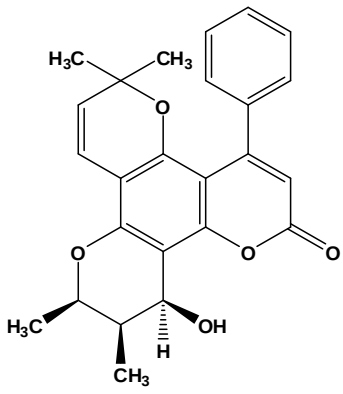


(**17**)

Figure 11: Anticoagulant agent (Narayanaswamy *et al*., 2014).

2.4.5 Antiviral activity

Natural products have been screened and reported possessing anti-HIV proprieties which include compounds with coumarin nucleus. The *inophyllums* and *calanolides* represent novel HIV inhibitory coumarin derivatives. Inophyllum *A* (**18**), inophyllum B (**19**), inophyllum C (**20**), inophyllum E (**21**), inophyllum P (**22**), inophyllum G1 (**23**) and inophyllum G2 (**24**) were isolated from giant African snail, *Achatina fulica*. Inophyllums B and P (**19** and **22**) (Figure 12) inhibited HIV reverse transcriptase (RT) with IC_{50} values of 38 and 130 nM, respectively, and both were active against HIV-1 in cell culture (IC_{50} of 1.4 and 1.6 µM) (Patil *et al* ., 1993).



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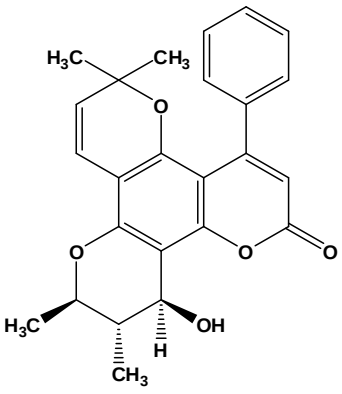

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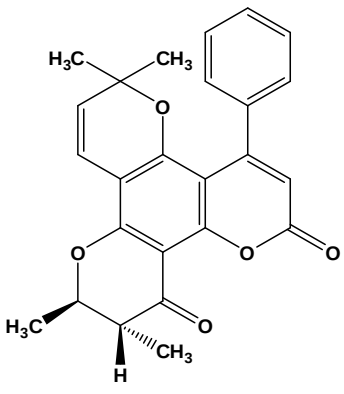

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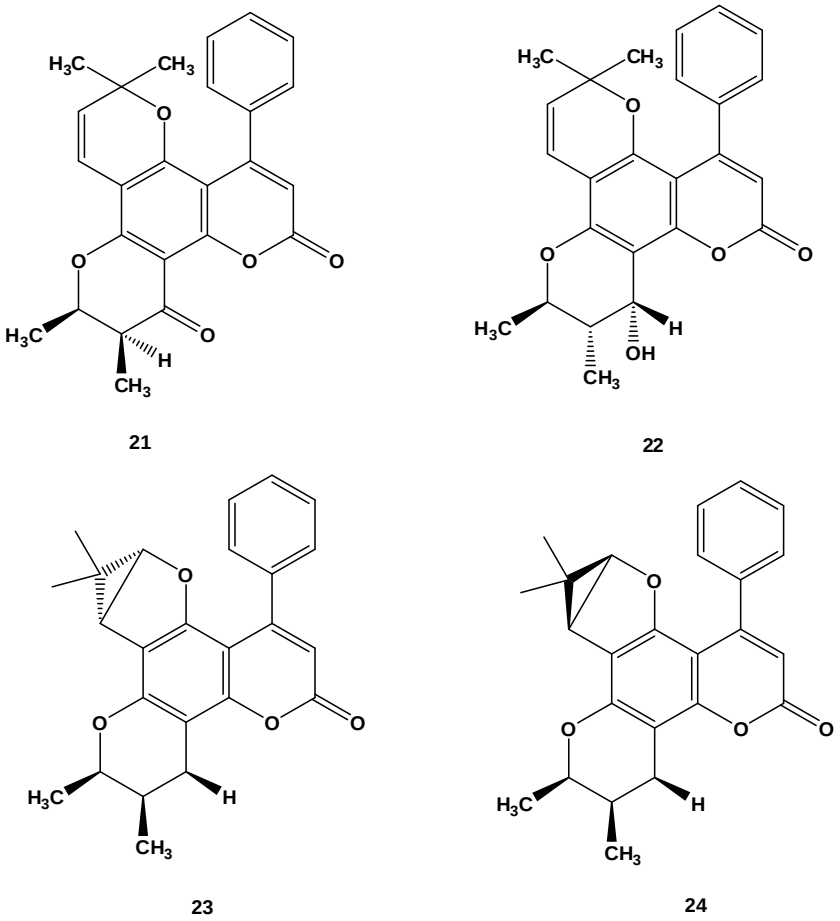
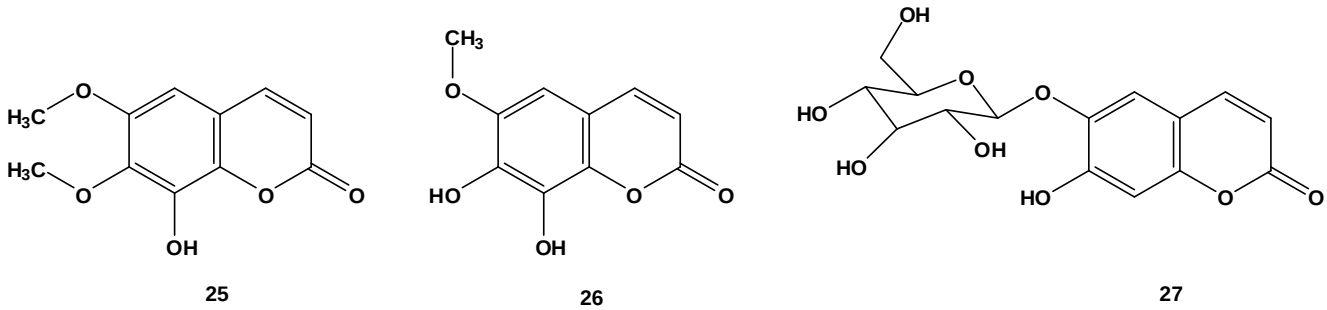
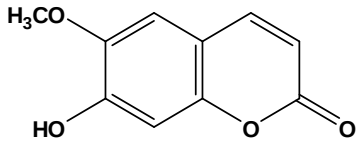


Figure 12: Antiviral compounds (Prasad *et al*., 2012; Ali, 1999; Yimdjo, 2004)

2.4.6 Antiadipogenetic Activity

A number of coumarins have been isolated from the stem barks of *Fraxinus rhynchophylla* DENCE (Oleaceae) such as Fraxidin (**25**), fraxetin (**26**), esculin (**27**), scopoletin (**28**), fraxin (**29**) and esculetin (**30**). These compounds as been screened *in vitro* and esculetin (**30**) (Figure 13) has been rapportated to show the most significant inhibitory effect against preadipocyte cell line, 3T3-L1, thus esculetin showed good antiadipogenic activity (Shin *et al*., 2010).





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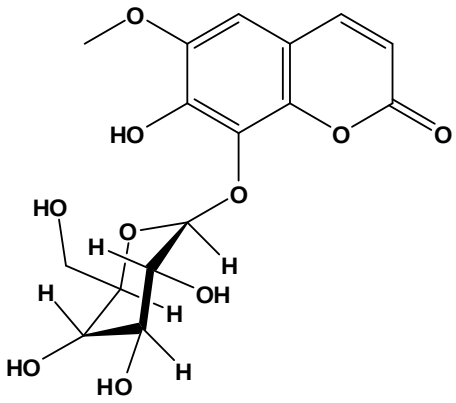

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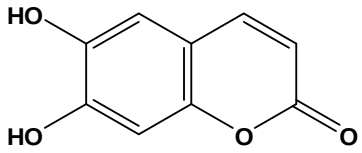

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Figure 13: Antiadipogenetic compounds (Zhuand Hou, 2008; Michalska and Kisiel, 2014; Hu *et al*.,2011; Oshima *et al*., 2013).

2.4.7 Antioxidant activity

The antioxidant activity of a compound is ascribed to the compounds ability of oxygen radical scavenging, inhibiting cellular microsomal P-450-linked mixed function oxidation (MFO) reaction, and prevent the formation of reactive oxygen species (ROS). Therefore, the lethal action of (oxygen) free radicals on cellular organelles and components can be an antioxidative. (Malhotra *et al*., 2008).

Fraxin (**29**) compounds was screened for antioxidant activity at the concentration (0.5 mM), this compound demostrated significant free radical scavenging effect and cell protective against H_2O_2 mediated oxidative stress (Whang, 2005).

Kadhum *et al*. (2011) synthesized two coumarins and screened them for antioxidant activity using (1,1-diphenyl-2-picrilhydrazyl) DPPH, nitric oxide and hydrogen peroxide, Kadhum *et al*. (2011) reported that these compounds showed signification antioxidant activity against the three methods used.

2.4.8 Safety of coumarin compounds

Although synthetic compounds may possess different pharmacological properties, they may also be toxic or mutagenic. Coumarin (**1**) is a natural compound originate in a variety of plants, such as cinnamon, lavender, and peppermint. Felter *et al*. (2006) suggested that exposure to coumarin usually ranges from 11mg/day from the consumption of natural food ingredients, to 7 g/day after clinical administration. To assess the safety of compounds, there are countless toxicity and cytotoxicity tests accessible.

The brine shrimp lethality assay is assumed to be a functional instrument for prelude evaluation of toxicity. It is additionally a functional tool for the isolation of bioactive compounds from plant extract. The method is appealing because it is easy, and inexpensive (Krishnaraju *et al*., 2005).

The identification of substances capable of investigating mutations has come to be a vital procedure in risk assessment. Carcinogenic and mutagenecity of chemicals could be modulated by supplementary chemicals. It is well understood that ingredients in dietary and supplementary plants, fruits and seeds can exert anti-carcinogenic and anti-mutagenic results (Hartman and Shankel, 1990, Hayatsu *et al*., 1988).

2.4.9 Anticancer activity

Amongst many diseases in the world, cancer is of high concern. The onset of cancer often occurs by mutation, ontogenesis, tumor suppressor genes or through the alteration of signaling pathways which often lead to programmed cell death (Ouyang *et al*., 2012). Coumarins are very significant in the treatment of cancer and is used in the treatment of prostate cancer, renal cell carcinoma and leukemia (Finn *et al*., 2002). Coumarins were found to have good maintenance therapy in the melanoma cell line and were also found to inhibit the spread of tumors. Coumarins are significant in cancer treatment due to their non-toxic, anticoagulant property. Coumarins have a cytotoxic mechanism and this allows them to have a therapeutic action against the renal cell carcinoma (Carter *et al*., 1977). The hormonal sensitivity and immune modulating effects of coumarins make it a potential treatment method for prostate cancer. There are various coumarin derivatives such as furanocomarins, pyronocoumarins, isoflavones, benzopyrones which are known to have a significant role in the treatment of a range of different cancers. Plant derived coumarins such as, agasyllin (**12**), aegelinolbenzoate (**13**), and osthole (**14**) from the plant Ferlagocampestris, exhibited marginal cytotoxicity against the A549 lung cancer cell line (Rosselli *et al*., 2009).

An integrated approach which uses the growing body of knowledge gained through scientific developments, is required for the treatment and management of cancer. One of these approaches is centered on developing therapeutic strategies that can target the induction of cell death processes like apoptosis.

2.4.9.1 Apoptosis (Programmed cell death)

Apoptosis (PCD) occurs in cells when cellular damage becomes irreparable due to cytotoxic and hormonal treatments. However, the efficacy of cancer treatments, depend on the cellular damage caused by cytotoxic and hormonal treatments, and ability of the cells to respond to the damage caused by these treatments by inducing the apoptotic machinery (Sjöström and Bergh, 2001). Coumarin (**1**) has been reported to induce cell cycle arrest and apoptosis in human cervical cancer cells (HeLa) (Chuang *et al*., 2007). Sporalidin compound has been reported to induce cytotoxicity against gastric (SNU-1, SNU-16), colon (HT-29), and breast (MCF-7) cancer cells. Furthermore, sporalidin has been also reported to induce apoptosis in androgen-dependent (LNCaP, C4-2B) and androgen-independent (DU-145, PC-3) prostate cancer cells and showed inhibitory effect against the growth of PC-3 xenograft tumor in nude mice (Bronikowska *et al*., 2012).

a) Characteristics of apoptosis

Apoptosis (PCD) is associated with different set of biochemical and physical changes. These changes involve the cytoplasm, nucleus, and plasma membrane (Kasibhatla and Tseng, 2003). The morphological and biochemical characteristics of an apoptotic cell include: (nuclear) chromatin condensation, membrane blebbing, mitotic body formation, nuclear and cellular shrinkage, DNA fragmentation (caused by the cleavage of DNA into 180 base pairs nucleosomal fragments) and cell debris engulfment by neighboring cells (Chinkwo, 2005, Weis *et al*., 1995). These characteristics are shown in Figure 14.

During apoptosis, the integrity of the mitochondria and Golgi bodies are maintained, while the nuclear membrane rests intact for an prolonged period. The cell breaks into membrane-bound apoptotic bodies which are then engulfed by macrophages and dendritic cells (Vermeulen *et al*., 2005). The morphological features of apoptosis result from the activation of cysteine proteases (caspases). These caspases are either activated through death receptor ligation or the release of apoptotic mediators from the mitochondria. Cell death through apoptosis requires energy in the form of ATP (Edinger and Thompson, 2004).

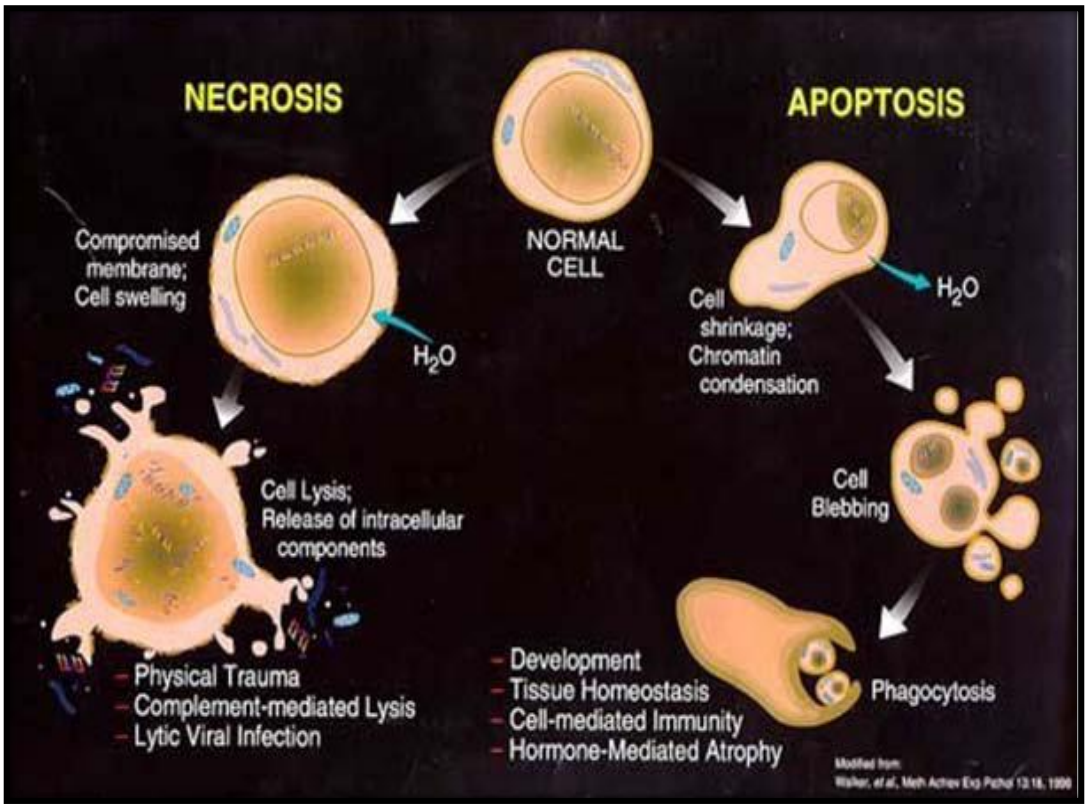

Figure 14: **Diagrammatic illustration showing the morphological distinctiveness occurring during apoptosis and necrosis (Van Cruchten and Van Den Broeck, 2002).**

b) Essential elements of apoptosis

Plasma membrane changes

Changes occurring in the plasma membrane of mammalian cells are the first features recognizable in apoptosis. During apoptosis, phosphatidylserine (PS) faces the outside of the cells and phospholipid flipping occurs resulting cell membrane changes that are visible in the cytoplasm (Figure 15). Cells experiencing apoptosis tend to lose phospholipid asymmetry in their plasma membranes. The apoptotic cells can be evaluated by determining the attachment of the Annexin V to phospatidylserine, that's exposed to the outside (Grosse *et al*., 2009).

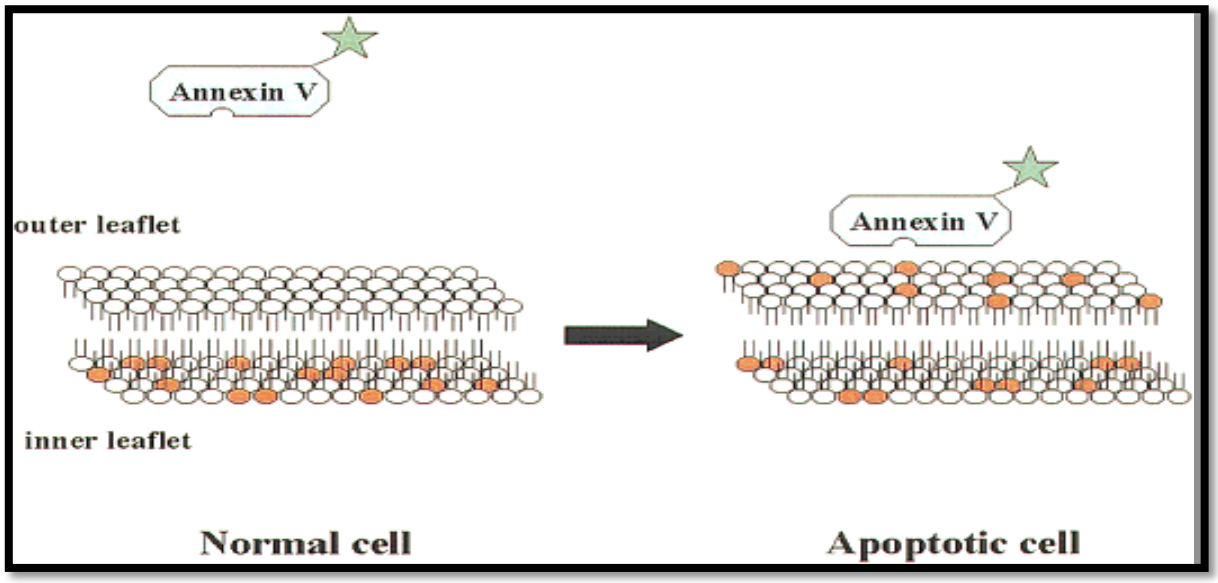

Figure 15: Schematic representation of the loss of membrane lipid asymmetry during apoptosis. During apoptosis, these PS molecule come to be exposed at the beyond membrane leaflet. Hapten-labeled annexin V can attach alongside elevated affinity to the exposed PS in the attendance of millimolar Ca^{2+} compression (Van Engeland *et al*., 1998).

Mitochondrial membrane potential

One of the first indicators of mitochondrial mediate apoptosis is a change in the mitochondrial membrane potential (Green and Reed, 1998). The mitochondrion is an organelle that's involved in gaseous exchange (ATP) in eukaryotic cells. This process is achieved when ADP and an inorganic phosphate undergo oxidative phosphorylation. This process transmits an electric charge across the mitochondrial inner membrane that provides the energy/power required for ATP synthesis to fuse ADP and PI (Christensen *et al*., 2013) which results in the mitochondrial membrane becoming polarized. Cytochrome C, an electron transport carrier, is vital for this process, is discharged throughout apoptosis, and this consequently prevents electrons from being transferred. This depolarizes of the mitochondrial membrane. After depolarization, Cytochrome C releases quickly. Because the mitochondrial membrane is negatively charged, it's potential can be measured using permeable cation dyes like JC-1 (Christensen *et al*., 2013).

Caspase -3

The normal executioners of apoptosis are the proteolytic enzymes called cysteinyl-aspartate-specific-proteases (caspases). Caspases are so categorized because cysteine is needed for induction of the catalytic mechanism and additionally because they cleave afterward an Aspartate residue (Shi, 2002). Caspases are grouped into initiator caspases (2, 8 and 9), which

are characterized by a long N-terminal, and effector caspases (3,6 and 7), which are characterized by a short N-terminal which result in the characteristic features of apoptotic cell death (Figure 16) (Huerta *et al*., 2007). The critical role of caspases in apoptosis was first observed in nematode worm *Caenorhabditis elegans* (Boatright and Salvesen, 2003). Once caspases are activated, they cleave other caspases. A previous study noticed that, in addition to their important role in the regulation of apoptosis, they are so implicated in cytokine activation of inflammatory responses (Zhang *et al*., 2005). In a previous study of Chandra and Tang (2003), it was reported that the existence of caspases in the mitochondrial intra-membrane space (procaspase-2, -3,-8 and -9), endoplasmic reticulum nucleus (procaspase-12) and Golgi apparatus (procaspase-2).

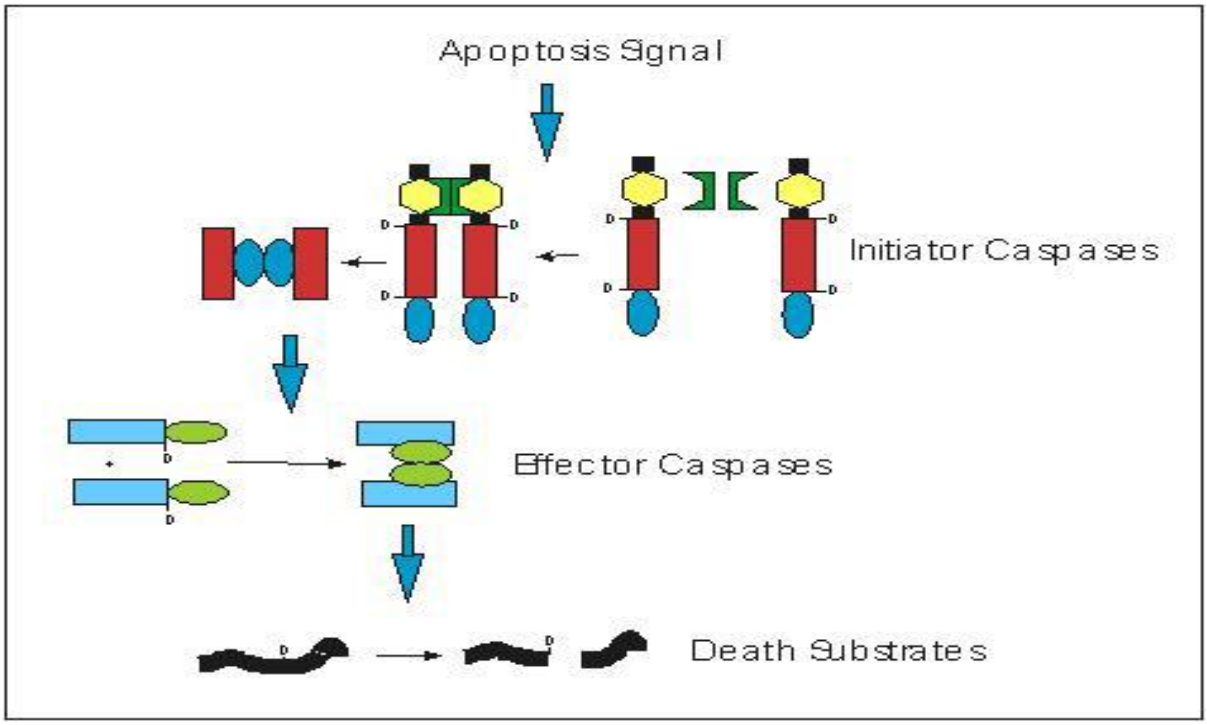

Figure 16: Schematic representation of the activation of caspase cascade. Apoptotic signals cause oligomerization of death adaptor proteins, which in turn oligomerize initiator pro-caspases. Oligomerized pro-caspases autoproteolytic activity result in active initiator caspase enzymes. Active initiator caspses then process and active effector pro-caspases. Active effector caspases cleave different substrates necessary for apoptosis to proceed (Stennicke and Salvesen, 2000).

2.4.9.2 Biochemical pathways

Programmed cell death is initiated through different mechanisms and detected by various methods. Two apoptotic pathways (intrinsic and extrinsic) have been studied in detail. The intrinsic and extrinsic mechanisms, both involve the activation of intracellular caspases (Kang and Reynolds, 2009). These caspases, as mentioned, belong to a family of cysteine-dependent aspartate-directed proteases (Vermeulen *et al*., 2005). Proteases, once activated, cleave other caspases and this forms a protease cascade that eventually results in apoptotic structural and morphological changes, which are recognized as (a cellular collapse or) apoptosis.

a) The Intrinsic Pathway

Mitochondria are fundamental regulator of intrinsic apoptotic pathways. Intrinsic apoptotic pathways are initiated inside cells. The most vital rotating point in the course of the intrinsic apoptotic process occurs in the mitochondria (Jin and El-Deiry, 2005).

The intrinsic (mitochondrial-dependent) pathway is induced by stress signals that cause a break of the mitochondrial membrane potential. Proapoptotic proteins such as cytochrome C, located within the mitochondria are subsequently released. This results in the activation of a cytosolic protein factor called apoptotic protease activating factor-1 (Apaf–1) (Figure17). Following activation, downstream caspases such as caspase 3 will be activated to initiate degradation of the cell (Hague and Paraskeva, 2004).

b) The extrinsic pathway

In the extrinsic pathway, apoptosis is induced by cell death receptors. These receptors are the death domains (DDs) and death effector domains (DEDs). The domains are induced so that signaling complexes can be assembled to initiate apoptosis (Natoni *et al*., 2005).

The extrinsic (death receptor-dependent) pathway, death receptors located on the cell membrane bind to their respective ligands on the target cell, triggering a series of events that eventually result in the activation of an adapter protein called FADD (Figure18) (Vermeulen *et al*., 2005; Hotchkiss *et al*., 2009). This results in the formation of the death inducing signal complex (DICS) (Adrain *et al*., 2002). Eventually, downstream caspases such as caspase 3 are activated, which initiates apoptosis of the target cell (Bridgham *et al*., 2003).

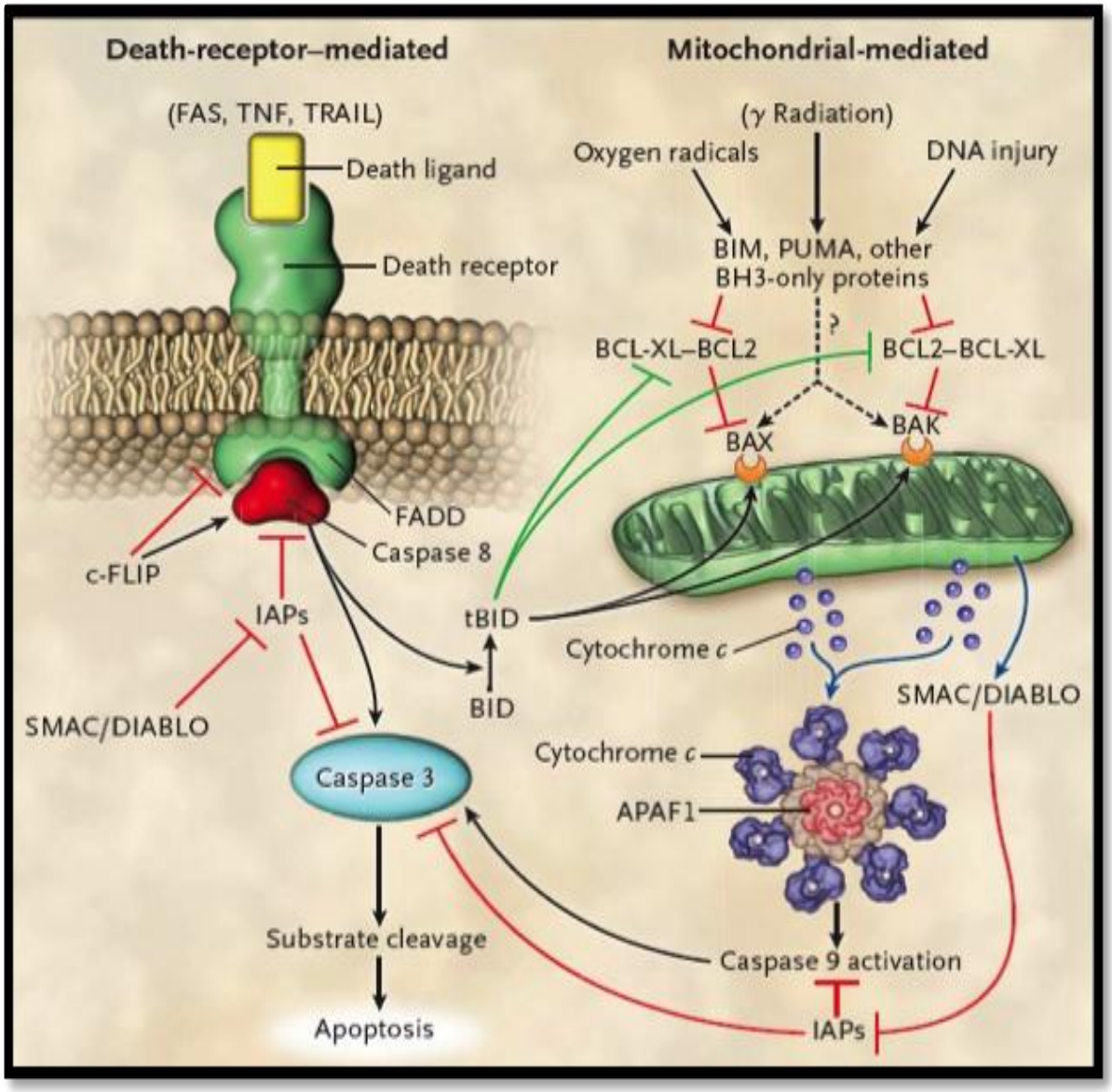

Figure 17: The intrinsic and extrinsic apoptotic pathways, The death-receptor pathway mediated by the activation of death receptors, and the BCL2-regulated mitochondrial pathway that is mediated by noxious stimuli causing mitochondrial injury (respectively) (Hotchkiss *et al*., 2009).

3. METHODOLOGY

3.1 Overview of the biological screening

A representation chart displaying the biological assays: antibacterial, antifungal, anti-inflammatory, anti-mosquito, salmonella mutagenecity, brine shrimp assay, and anti-cancer assays engaged in this study is shown in Figure 18

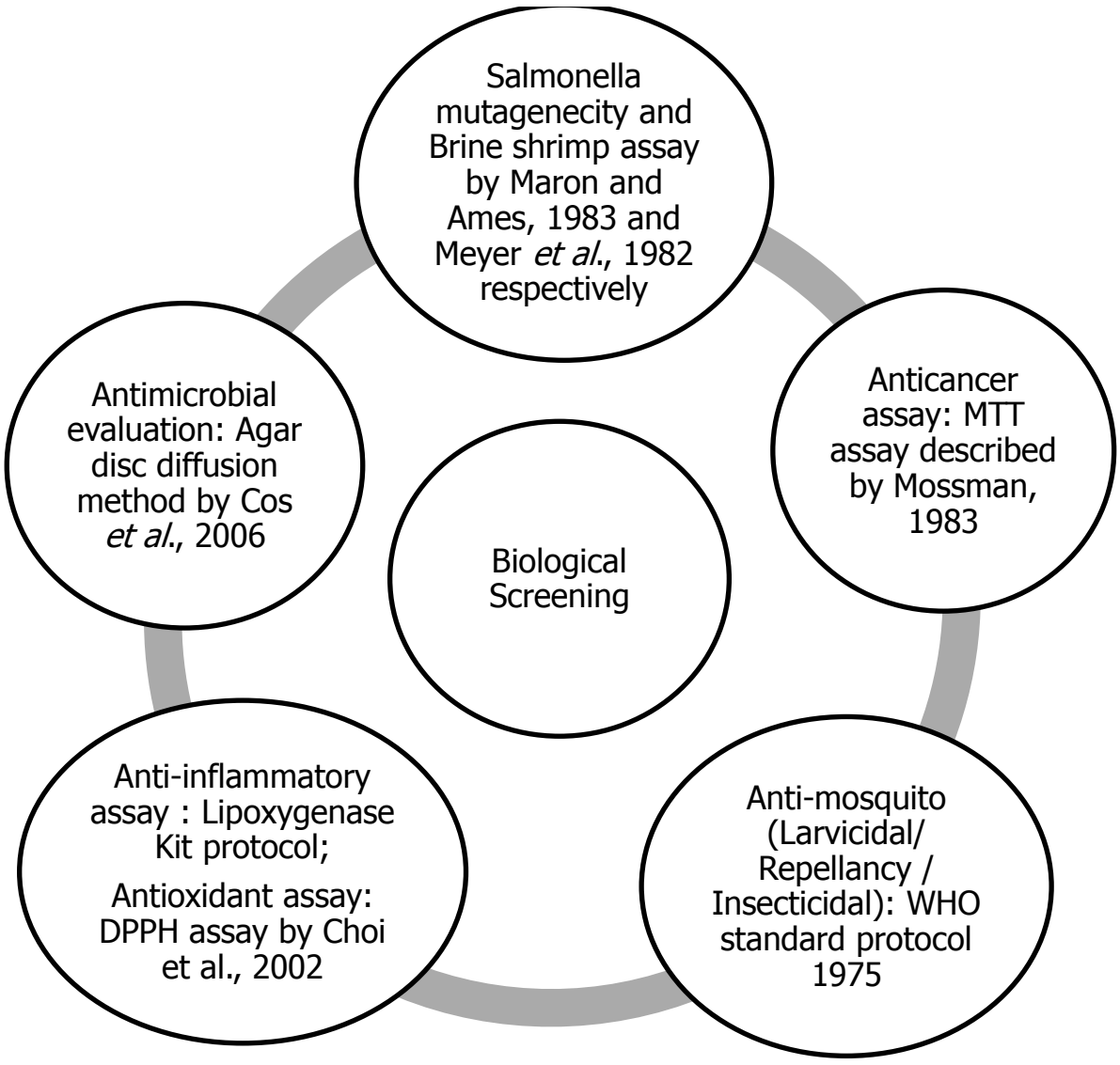

Figure 18: **Schematic diagram of biological assays engaged in this study.**

3.2 Synthetic routes of coumarins CMRN1- CMRN7 used in this study

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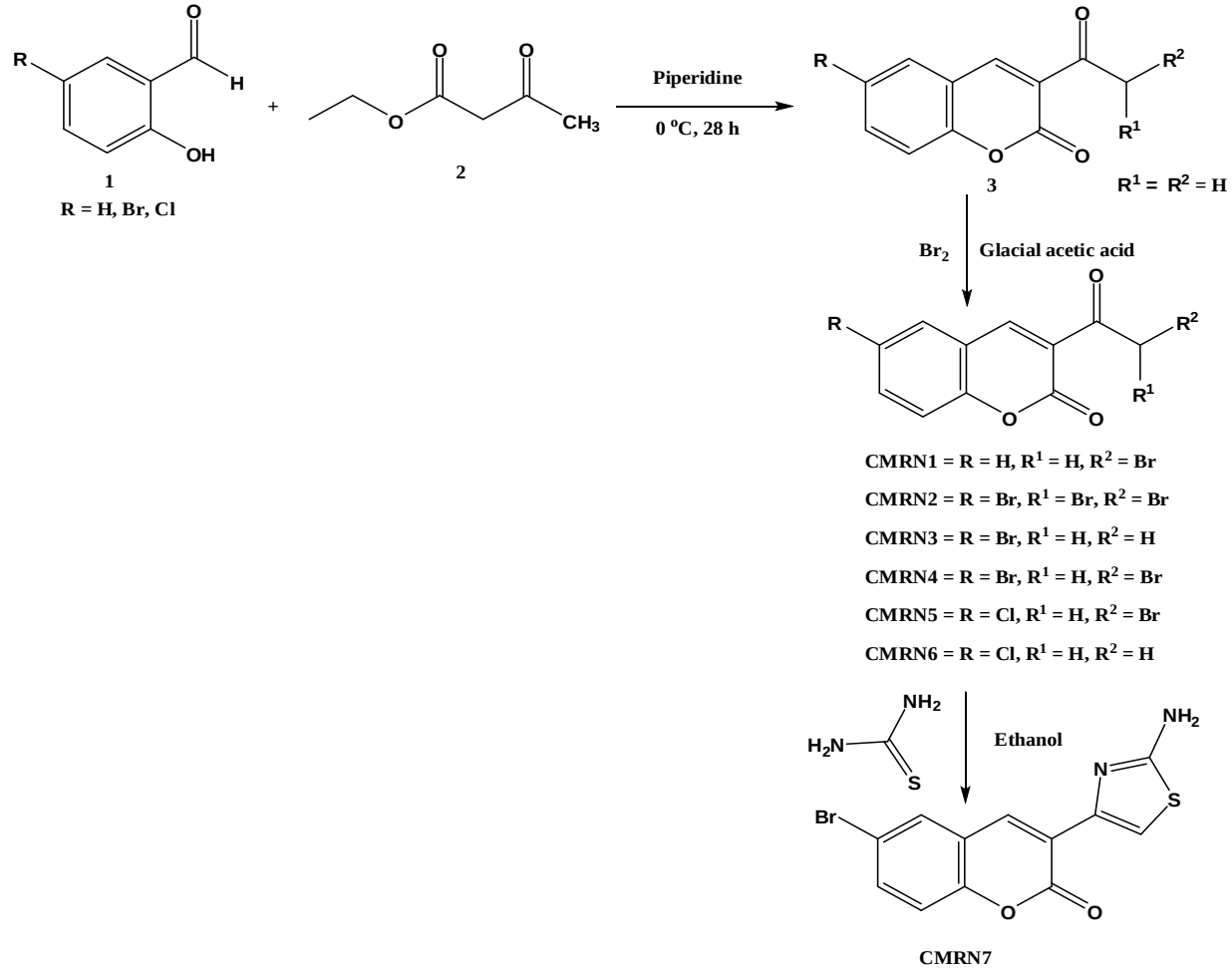

Scheme 1 General route for the synthesis of halogenated coumarins **CMRN1- CMRN 7.**

3.3 Synthesis of coumarins CMRN1- CMRN7 used in this study

3.3.1 Synthesis of 3-(2-bromoacetyl)-2*H*-chromen-2-one (CMRN1)

Compound **CMRN1** was synthesized using an earlier reported protocol of Jayashree *et al*. (2004). Twenty ml of alcohol-free chloroform was used to carry out bromination (0.1 mol) of 3-acetyl coumarin at 0.1 mol, with intermittent shaking. The reaction medium was heated for 20 min on a water bath to remove hydrogen bromide; the reaction completion was monitored on thin layer chromatography, and filtered. Almost pure product was washed with cold ether to obtain satisfactory yield of the pure product of **CMRN1** at 96% yield with melting point 121°C.

3.3.2 Synthesis of 6-bromo-3-(2,2-dibromoacetyl) -2*H*-chromen-2-one (CMRN2)

Compound **CMRN2** has been synthesized according to reported procedure by Venugopala and Jayashree, (2003); Chopra *et al*. (2007). Twenty mL of alcohol free chloroform was used to carry out bromination (0.1 mol) of **CMRN4** at 0.1 mol with intermittent shaking. The reaction medium was heated for 20 min on a water bath to remove hydrogen bromide. The reaction completion was monitored on thin layer chromatography, and filtered. The pure product was washed with cold ether to obtain pure product of **CMRN2** at 95% yield with melting point 147°C.

3.3.3 Synthesis of 3-acetyl-6-bromo-2*H*-chromen-2-one (CMRN3)

Compound **CMRN3** has been synthesized according to reported procedure by Venugopala *et al*. (2004). 5-Bromosalicylaldehyde (0.1 mol) was added to ethylacetoacetate (0.11 mol) in a conical flask with stirring on cold bath at 5-10 °C. To this reaction mixture, 1 g of piperidine was added. The reaction mixture was then kept at freezing temperature for 28 h, and then slowly a yellow colored solid product precipitated out. The precipitate obtained was stirred in cold ethanol and filtered. The solid product obtained was washed with ice-cold ethyl alcohol and dried. The product was recrystallized from acetic acid to obtain with 98% yield of **CMRN3** with a melting point of 221°C.

3.3.4 Synthesis of 6-bromo-3-(2-bromoacetyl) -2*H*-chromen-2-one (CMRN4)

Compound **CMRN4** has been synthesized according to the reported procedure by Venugopala and Jayashree, (2004). Twenty mL of alcohol free chloroform was used to dissolve 3-acetyl-6-bromo coumarin (0.1 mol) to carryout bromination at 0.1 mol, with intermittent shaking. The mixture was heated for 20 min on a water bath to remove the hydrogen bromide; the reaction completion was monitored on thin layer chromatography, cooled to room temperature and filtered. The solid product obtained was washed with cold ether to get satisfactory yield of **CMRN4** at 95% with melting point 205°C.

3.3.5 Synthesis of 3-(2-bromoacetyl) -6-chloro-2*H*-chromen-2-one (CMRN5)

Compound **CMRN 5** has been synthesized according to reported procedure by Jayashree *et al*.(2006). Twenty ml of alcohol free chloroform was used to dissolve **CMRN6** (0.1 mol) to carryout bromination at 0.1 mol, with intermittent shaking. The reaction medium was heated for 20 min on a water bath to remove the hydrogen bromide; the reaction completion was

monitored on thin layer chromatography, cooled to room temperature and filtered. The solid product obtained was washed with cold ether to get pure product of **CMRN5** at 94% yield with melting point 181°C.

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3.3.6 Synthesis of 3-acetyl-6-chloro-2*H*-chromen-2-one (CMRN6)

Compound **CMRN6** has been synthesized according to reported procedure by Jayashree *et al*., 2005, Chopra *et al*.(2006). 5-Chlorosalicylaldehyde (0.1 mol) was added to ethylacetoacetate (0.11 mol) in a conical flask with constant stirring at cold temperature. 1g of piperidine was added to this reaction mixture with intermittent shaking. The reaction mixture was then kept at freezing temperature for 28 h, and then a yellow colored product was precipitated out. The solid product was stirred in ice-cold ethanol and filtered. The product was washed with ice-cold ethanol and dried. Hot glacial acetic acid was used to recrystallize the product to obtain the yield of CMRN6 at 95% with melting point 219°C.

3.3.7 Synthesis of 3-(2-aminothiazol-4-yl)-6-bromo-2*H*-chromen-2-one (37)

Compound **CMRN7** has been synthesized according to reported procedure by Venugopala and Jayashree, (2004), Chopra *et al*.(2009). 0.1 mol of **CMRN4** was added to 17.5 ml of hot ethanol and 0.11 mol of thiourea was added. Exothermic reaction took place and the reaction medium turns to a clear solution that soon deposited as crystals. The product deposited was filtered, washed with ethanol and then boiled with 0.5g of sodium acetate in water to obtain **CMRN7**. The crude product obtained was recrystallized from hot absolute ethanol to get 87% yield with melting point 211°C.

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3.4 Biological screening

3.4.1 Anti-mosquito activity of coumarins CMRN1-CMRN7

The anti-mosquito proprieties of the coumarins derivatives were performed at the South African Medical Research Council (MRC), Durban, Malaria Research Unit

3.4.1.1 Sample preparation

Acetone was used in the preparation of the stock solution of coumarin compounds (**CMRN1-CMRN7**) by dissolving 1000 µg of each compound in to 1 ml of acetone. For each experiment, this assay was performed in triplicate (larvacidal, repellency and adulticidal assays).

3.4.1.2 Larvacidal assay of coumarins CMRN1-CMRN7

The *Anopheles arabiensis* was used in this study according to the protocol described by WHO (1975) guidelines in an insectary simulating the temperature (27.5°C), humidity (70%), and lighting (12/12) of a malaria endemic environment. One mL of test compound (1 mg/ml) was added to distilled water (250 ml) to obtain a final concentration of 4 μg/ml. Thirty instar larvae were introduced into a container. A negative control was set up using a solvent (acetone) and distilled water and a positive control included Temephos which is an active emulsifiable organophosphate larvicidal used by the malaria control program. Larval mortality was examined for each container separately for a duration of three days at 24 h intervals and fed specifically made cat food with less oil/fat content at regular intervals. Bioassays were performed in triplicate. The percentage mortality was determined relative to the initial number of exposed larvae.

3.4.1.3 Repellency assay of coumarins CMRN1-CMRN7

Repellent activity was evaluated by local application of each of the coumarin derivatives (**CMRN1-CMRN7**) to the skin. This was followed by exposing the treated surface of skin to the unfed female mosquitoes. Ethical authorization for the use of *Mastomys coucha* in this study was approved by the MRC Committee for Research on Animals. Individual adult *Mastomys* rodents were firstly weighed and afterward injected intraperitoneally with the sodium pentobarbital in considering to the weight of the animal for a specific concentration. The anesthetized rodents were then shaved on the ventral surface and one mililitre of the test compound was applied to each rodent's abdomen. The stock solution (1 mg/ml) was prepared using acetone as a solvent. Laboratory grade *N*, *N*-Diethyl-3-methylbenzamide and acetone

were employed as the positive and negative control respectively. Paper cups (500 ml) were modified by interchanging the basal part of the cup with mosquito net using a rubber band. A clear plastic sheet was used covered cup. Mosquitoes were then counted and a number of thirty unfed 4-day-old *A. arabiensis* females were released into the cup being held in contact with the treated part of each rodent (Figure 19A). The transparent sheet was used to view mosquito activity. After 2 min of exposure, mosquitoes were counted and recorded. The cups holding the mosquitoes were removed and mosquitoes were then observed for 24 h. All tests were done in triplicate for each sample. The rodent was given back to the animal facility and were observed for sometime until the recovering from anaesthetic. Repellency of the compounds was determined using the following below.

$$\text{Percentage mosquitoes repelled} = \frac{\text{number repelled}}{\text{number introduced}} \text{ X } 100$$

3.4.1.4 Animal preparation

For every coumarin compound, two mature *Mastomys coucha* rodents were weighed independently. The rodents were anesthetized with sodium pentobarbital (Figure 19B). The anesthetized rodents were injected with coumarin derivatives (1000 µg/ml) or a positive control (DEET – 97%) or a negative control acetone (Figure 19C). The number of mosquitoes on the net of the cup was counted and the others were knocked down in the presence of the compound (Figure 19D).

Figure 19: The repellency assays (A) Unfed *A. arabiensis* females, (B) Anaesthezided with sodium pentobarbital, (C) Compound applied to rodent's abdoments, (D) Unfed *A.arabiensis* females held in contact with treated ventral surface of rodent respectively.

3.4.1.5 Insecticidal assay of coumarins CMRN1-CMRN7

An insecticidal activity assessment was conducted by exposing susceptible adult mosquitoes to a treated surface, in accordance with WHO protocol (1975). One ml of test compound (1 mg/ml) was sprayed onto a clean, dry, nonporous ceramic tile by means of a precalibrated Potter's Tower apparatus (Figure 20A) . The tiles were then air-dried (Figure 20B) and the assay was initiated within 24h of spraying, by fixing a cone on the sprayed tile and introducing thirty non-blood-fed, 2–5-day-old adult *A. arabiensis* mosquitoes into the cone (Figure 20C). The activity of the test compounds was evaluated by determining the knock down rate, which was based on short paralysis of the mosquitoes throughout the 60 min exposure period, and

mortality 24h post exposure (Figure 20D). Deltamethrin (15 g/L; K-Othrine) was used as a active control and acetone as a negative control. The test were performed in triplicate.

Figure 20: **The insecticidal assay (A) Potters Tower, (B) Positive control sprayed on ceramic non-porous tiles, (C) *A. arabiensis* (30) females introduced into bioassay cone, (D) Transferred to olding cage containing nutrient solution overnight to check for mortality.**

3.4.2 Antibacterial activity of coumarins CMRN1-CMRN7

The antibacterial activity and the minimum inhibitory concentration (MIC) of the coumarin derivatives (**CMRN1-CMRN7**) were performed using the protocol described by Cos *et al.*(2006) using of agar disc diffusion assay. The bacterial strains used in the study were collected from the culture collection at the Department of Biotechnology and Food Technology, Durban University of Technology, South Africa. The strains include *S. aureus* (DBT*_E), *M. luteus* (DBT*_AR), *E. coli* (DBT*_L), *B. stearothermophilus* (DBT*_Q), *K.*

pneumoniae (DBT*_AM), *S. marcescens* (DBT*_ZM), *B. cereus* (DBT*_F), *B. coagulans* (DBT*_L), *C. freundii* (DBT*_L), *and S. faecalis* (DBT*_L).
DBT* : Durban University of Technology Culture collection reference strain based at the department of Biotechnology and Food Technology**.**

Stock cultures were sub-cultured to check their viability and stored using 50% of glycerol in micro bank vials (Davies Diagnostics, South Africa). For the assay, culture were plated on Nutrient Agar (Biolab) and kept in the incubator for 24 h at 37 ℃, then grown in Nutrient Broth (Biolab) at the same condition. MacFarland standard of 0.5 absorbance corresponding to 10^8cfu/ml was used to standardize the bacterial cell concentration. A suspension (100 µl of 10^8 cfu/ml) of the test bacteria was plated on Mueller Hinton Agar plates (Fluka, Biochemika). The whatman No. 1 filter paper was cut into 5 mm disks and dried in an open sterile petri-dishes biosafety chamber (LabtecBioflow II, South Africa). The disk were impregnated with 10 µl of each compounds (**CMRN1-CMRN7**) at the concentration of 3 mg/ml then placed onto the pre-inoculated bacterial agar plates and kept at 37 ℃ for 24 h. The assay was carried out in triplicate. Ciprofloxacin(Fluka, Biochemika) (3 mg/ml) was used as the active control while DMSO (100%) as a negative control.

The minimum inhibitory concentrations (MIC) of the coumarin derivatives were determined by broth micro dilution assay. The compounds were tested at 3, 1.5; 0.75; 0.37; 0.18; 0.09 mg/ml.

3.4.3 Antifungal activity of coumarins CMRN1-CMRN7

The antifungal activity of the coumarin derivatives (**CMRN1-CMRN7**) was carried out using 3 yeast cultures, *C. albicans* (DBT*_AB), *C. utilis* (DBT*_AB), *S. cerevisiae* (DBT*_R) and 2 species of fungi, *A. flavus* (DBT*_AR) and *A. niger* (DBT*_AR). Sabouraud Dextrose broth was used to grow the yeast culture at 37°C for 24 h (see section 3.4.2), and fungi incubation were carried out at 28°C until sporulation for about 4-7 days using the same media. The spores that were produced were collected in a sterile distille water (10ml), the concentration was adjusted to 10^6 spores/ml and then counted in a Neubauer counting chamber. Hundred microliter of sterile distille water was inoculated with the fungal spores (10^6 spores/ml), then poured on the Sabouraud Dextrose Agar plate. The filter disks and samples were prepared as described earlier in section 3.4.2. Inoculated disks were placed on the inoculated agar plates and incubated for 72 h at 25°C. The assay was carried out in triplicate for each sample. Each

reaction contained 3 mg/ml of sample, amphotericin B (Fluka, Biochemika) was used as the active control and 10 µl DMSO (100%) as negative control.

3.4.4 Anti-inflammatory activity of coumarins CMRN1-CMRN7

Anti-inflammatory activity was measured using the Lipoxygenase Kit protocol. The kit that was used for this test was the Cayman`s Lipoxygenase Inhibitor Screening Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA), which detects and measures hydroxides that are produced during lipoxygenation (lipoxygenation uses purified LO. This kit is very sensitive in the detection of hydroxides and its sensitivity is often compared with its detection of hydroperoxides at various positions within fatty acids. This assay works with fatty acids of any carbon length. It is thus the general detection method for LO, and can be used to screen libraries of compounds that inhibit LO enzymes.

The protocol used for anti-inflammatory activity involved the addition of 100 µl assay buffer to blank wells. The activity of coumarins **CMRN1-CMRN7** was determined at 1 mg/ml using 15-lipoxygenase as a standard positive control, 90 µl of assay buffer was added to 2 wells, while for the 100% initial activity wells, 90 µl lipooxygenase enzyme and 10 µl of solvent (the same solvent used to dissolve the inhibitor) was added to 2 wells. In contrast, the 2 inhibitor wells contained 90 µl lipoxygenase enzyme and 10 µl of **CMRN1-CMRN7**. The anti-inflammatory reaction was initiated by adding 10 µl linoleic acid (substrate) to all wells. Thereafter, they were placed on a shaker for 5 minutes. To stop enzyme catalysis and to develop the reaction, 100 µl chromogen was added to each well. The plate was thereafter covered and placed on a shaker for 5 minutes. Plate covers were then removed and read at a wavelength of between 490 and 500 nm using and ELISA plate reader (Mark elyza). All analyses were performed in triplicate and the results were presented as mean (±SD). Lipoxygenase results are depicted in Table 10 and the percentage inhibition was determined using the following equation

$$\% \text{ Inhibition} = \frac{\text{Initial activity} - \text{Inhibitor}}{\text{Initail activity}} \text{ x } 100$$

3.4.5 Antioxidant activity of coumarins CMRN1-CMRN7

The decolouration protocol earlier reported by Choi *et al*.(2002) for measuring the radical the radical scavenging activity of different compounds using the stable free radical scavenger, DPPH (2, 2 diphenyl-2-picryl hydrate), was performed (Choi *et al*., 2002). Methanol was used to dilute the stock solutions of the coumarins derivatives (**CMRN1-CMRN7**) to the final concentrations of 1, 20, 40, 60, 80, 100, 250, 500, and 1000 μg/ml. A comparison of the results obtained with Qeucertin-3-rutinoside that is an effective anti-oxidant was carried out. The radical scavenging was evaluated as the decolourization percentage of the test sample with formula below

$$\text{Scavenging capacity (\%)} = 100 - \frac{\text{absorbance of sample} - \text{absorbance of blank}}{\text{Absorbance of negative control}} \text{ X } 100$$

3.5 Cytotoxicity of coumarin compound against cancer cells

3.5.1 Cell line

The MCF-7 (Breast cancer) (Figure 21A), UCAA-62 (Melanoma) (Figure 21B) and PBMC (human peripheral blood mononuclear) (Figure 21C) cell lines were studied. Both cancer cell lines (MCF-7 and UCAA-62) were supplied by Natasha Kolesnikova from the Council for Scientific and Industrial Research (Bioscience, CSIR). These cells were stored in 25 cm^2 tissue culture flasks and incubated at 37^oC in a humidified incubator (SnjidersHepa, United Scientific, Cape Town, South Africa) that contained a 5% CO_2 environment. Upon arrival, the cells were then transferred to 2 different 75 cm^2 flasks (Greiner, Germany) after 3 days or once the cells reached 80% confluency in each flask.

3.5.2 Cell maintenance

All cell culture maintenance experiments were conducted in the laminar flow cabinet (Scientific Engineering, INC) in order to maintain an aseptic/sterile environment. The laminar flow cabinet was sterilized by exposing it to UV-light as well as swabbing frequently with 70% ethanol (Merck, South Africa) prior to performing cell culture experiments. MCF-7 (Breast cancer) (Figure 22A) and UCAA-62 (Melanoma) (Figure 22B) cells were grown as 2 separate monolayers in Dulbecco`s Modified Eagle Medium (DMEM) (comprised of glucose (4.5 gl^{-1}),

1 mM L-glutamine and 1 mM sodium pyruvate) (Sigma-Aldrich, Inc). The DMEM was supplemented with 10% heat inactivate foetal calf serum (FCS) and 1% antibiotic (penicillin/streptomycin) solution (Sigma-Aldrich, Inc). MCF-7 and UCAA-62 (Melanoma) cell lines were subcultured every 2-3 days, once the flasks had become 80% confluent in order to ensure they were in the exponential growth phase.

During sub-culturing, supplemented DMEM was removed from flasks and the monolayer of cells was washed with 5 mL Phosphate Saline Buffer (PBS). Thereafter, an aliquot of 1 mL trypsin was added to the flasks. The monolayer of cells were incubated at 37^{o}C in the humidified incubator with a 5% CO_2 environment for 3 minutes. In order to detach the monolayer, the flasks were then tapped on the side for 30 seconds. Ten ml of supplemented-DMEM was added and 1 ml of cell culture was thereafter transferred to each flask. A final aliquot of 20 ml DMEM was added to each flask and this was followed by incubation at 37^{o}C in a humidified incubator that contained a 5% CO_2 atmosphere. The cells were monitored for contamination on a daily basis by noting medium colour and turbidity changes during the incubation. Cell growth was examined with an inverted microscope (Nikon, Japan).

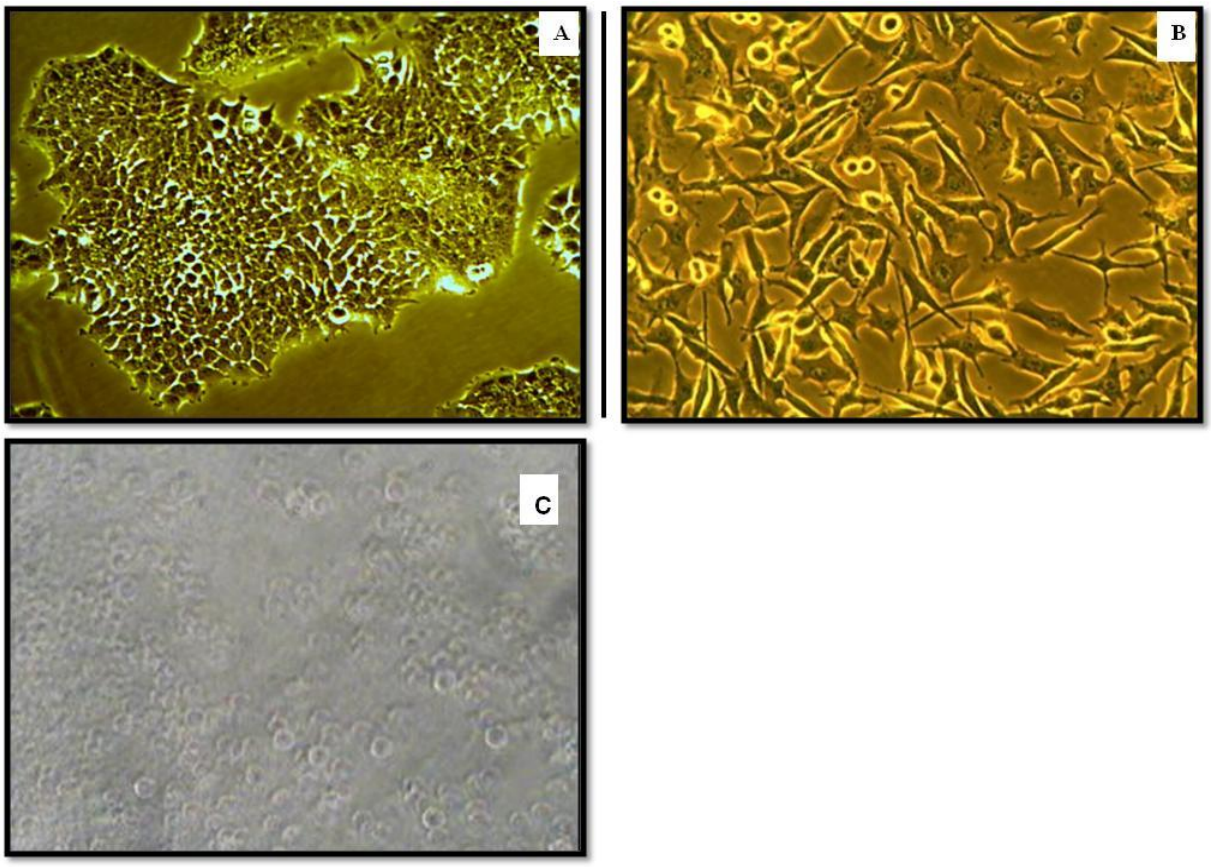

Figure 21: **(A) Microscopic observation of the morphology of MCF-7, (B) UCAA-62(Melanoma) , and (C) human peripheral blood mononuclear (PBMC) cells lines, respectively. The monolayer shows spindle-shaped cells at 100x.**

3.5.3 Storage of cells

Cell culture flasks that were 80% confluent were washed with 5 ml PBS and thereafter subjected to treatment with trypsin, as described during sub-culturing. An aliquot of 10 ml DMEM was thereafter added to each flask, and the cells were subsequently transferred to 50 mL tubes. The 50 ml tubes were then centrifuged at 1500 rpm for 10 min in order to collect pellets of cells. 2 ml of cryo-protective medium (comprised of 10% DMSO, 20% FBS and 70% DMEM) were added to re-constitute the pellet. An aliquot of 1 ml cryo-protective/cell-containing solution was thereafter added to the cryotubes (Corning, South Africa). These cryotubes were transferred to a biofreezing vessels and stored in a – 80^{o}C biofreezer.

3.5.4 Cell regeneration

When there were no freshly incubated cells available for immediate use, cells were removed from storage at – 80^{o}C and quickly thawed. Thereafter, the cells were transferred to 20 ml pre-warmed supplemented-DMEM in 75 cm^{2} tissue culture flasks. These cells-containing flasks were then incubated at 37^{o}C in a humidified incubator containing a 5% CO_2 atmosphere.

3.5.5 Cell Enumeration

Cells were enumerated by means of trypan blue, an exclusion dye used for counting viable cells. This staining technique is based on the principle that viable (or intact) cells would not use the trypan blue dye, while non-viable cells (cells in which membrane integrity is altered) would. Trypan blue staining allows scientists to visualize the morphology of cells since viable cells remain translucent, while non-viable cells appear blue in colour. In this study, 100 µl trypan blue (Bio Whittaker, Wakersville, USA) was mixed with 100 µl cell suspension cultures in centrifuge tubes. These tubes were then incubated at room temperature approximately 1 minute. Thereafter, an aliquot of 10 µl trypan-suspended cell cultures were loaded into both chambers of the Neubauer haemocytometer. The cells within the middle square and in the four 1 mm corner squares of the two chambers were then counted. The following equation was used to determine the number of cells in suspension:

Total cell count = 16 squares x 4

= Cell counts in 4 sets of 16 squares

16 squares = 2×10^4/ml

Therefore, cells per ml = $\frac{\text{total cell count}}{4}$ x 2×10^4 per ml

= cells per ml

3.5.6 Isolation of monocytes from the whole blood (PBMCs)

The collection, separation and isolation of peripheral blood mononuclear cells (PBMCs) in the present study followed the prescribed methods that have been described in Böyum (1967) with some modifications. Aseptic techniques were strictly being employed during the isolation of monocytes from PBMCs. The materials used were stored within a Class II Biological Safety Cabinet. Furthermore, the reagents were sprayed with 70% ethanol prior use within the laminar hood.

In order to isolate the monocytes from PBMCs, 50 ml of Buffy coat sample was obtained from the South African National Blood Services (SANBS). Thereafter, the Buffy coat sample was diluted with an equal volume (50 ml) of pre-warmed Hanks' Balanced Salt Solution (HBSS) (Bioscience, country). This mixture was then carefully layered, in a 5:4 ration, onto pre-warmed Histopaque 1077 (Sigma), within sterile 15 ml centrifuge tubes (Greiner). This mixture was centrifuged (at room temperature) at 2000 rpm for 30 min and the centrifugation revealed 4 separate different layers (Figure 22). This mixture was then carefully aspirated using a sterile Pasteur pipette from the interface between the HBSS and the pre- warmed Histopaque. Once aspirated, monocytes were thereafter transferred to a sterile centrifuge tube. The obtained mononuclear cells was then washed in HBSS twice, followed by centrifugation after each eash for 15 min at 1200 rpm at room temperature in order to remove any residual Histopaque. Cell pellets were re-suspended in 1 ml RPMI 1640 (supplemented with 2 mM glutamine, 10% unheated foetal calf serum, 100 IU/ml penicillin and 100 Ig/ml streptomycin (Bioscience) (Böyum, 1967).

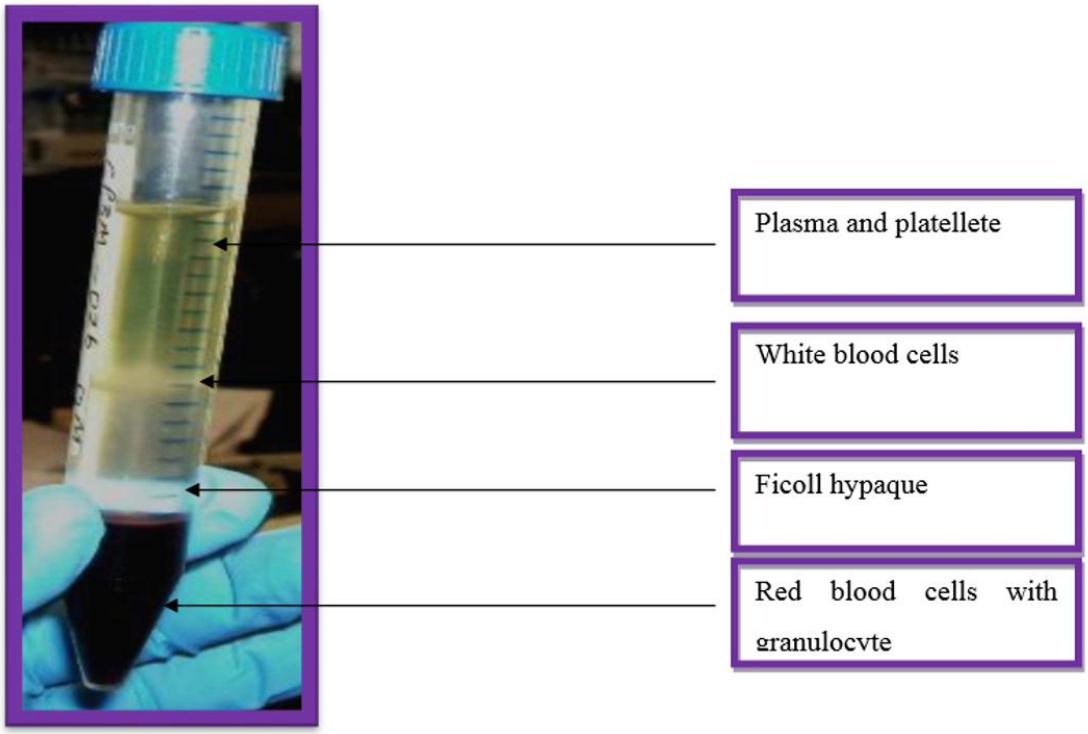

Figure 22: Isolation of PBMC (human peripheral blood mononuclear) cells (Hurinanthan,2009).

3.6 Cytotoxicity assay of coumarins CMRN1-CMRN7

The MTT (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) cytotoxicity assay was used to evaluate the cytotoxic effect of the synthetic coumarins derivatives against PBMC, MCF-7 (Breast cancer) and UCAA-62 (Melanoma). This method (MTT) is simple, accurate and reproducible, and by principle, it's centered on the ability of a specific type of mitochondrial dehydrogenase – known as succinate dehydrogenase to cleave the tetrazolium ring structure of diphenyltetrazolium bromide in viable (living) cells. The cleavage reaction produces formazan crystals by reducing yellow coloured MTT to purple-coloured formazan crystals generated from this reduction reaction is taken as being a measure of the number of metabolically active (or surviving) cells (Mosmann, 1983). This implies that an increase or decrease in the number of viable cells is (or would be) a determinant of the amount of formazan crystals produced. Mossman (1983) has indicated that the amount of formazan would be formed in turn, an indication of the degree of cytotoxicity induced/caused by a compound.

In this study, the MTT assay was evaluated referring to the protocol described by Mossman (1983). Cells were pipetted (90 µl of cell culture – 1×10^5 cells/ml) in each well of 96-well microtitre plates, and the outer wells were filled with PBS (phosphate buffer saline) in order to prevent the medium from evaporating during incubation. Thereafter, plates were incubated at

37^{o}C for 24 h. This provided sufficient time for the cells to attach to the wells of the plate. A serial dilution of the stock solution (50 µg/ml) prepared with growth medium was performed to ensure that all sample solutions were made consistently. Each well of the plate was then treated with 10 µL of varying concentration (50, 45, 40, 35, 30, 25, 20, 15, 10, 5 and 1 µg/ml) of the sample solution.

The negative control in this experiment was DMSO, it was added to wells to determine if it had been cytotoxic against MCF-7, PBMC and UCAA-62 (Melanoma) cell lines. The test compounds, solvent, and negative control were each tested in triplicates. After, the cell-containing microtitre plates were incubated for 2 days at 37^{o}C in a humidified incubator that contained a 5% CO_2 atmosphere. After the incubation time, a specific amount of MTT reagent (5 mg/ml) was futher added to individual well. The plate was then incubated for further 4 h at 37^{o}C (5% CO_2 incubator). The media was then removed after incubation, and an aliquot of 100 µL DMSO (Dimethyl Sulfoxide) was added to each well in order to dissolve the formazan crystals that were formed in metabolically active cells. Thereafter, plates were incubated for an extra hour. The absorbance of the formazan was evaluated at 590-630 nm using an ELISA plate reader. The inhibitory concentration of the tested halogenated compounds (IC_{50}) derivatives was determined using Graph Pad Prism6 software.

A) % Cell viability = $\frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}}$ X 100%

B) % Cytotoxicity = 100% - % Cell viability

Further test compounds that exhibited over 70% cell growth inhibition on UACC-62 (Melanoma) and MCF-7 (Breast) tumor cell lines were used to determine their inhibitory concentration (IC_{50}).

3.6.1 Quantitative determination of apoptosis using flow cytometry

3.6.1.1 Principle

Flow cytometry is a procedure that is used to evaluate and simultaneously measure the physical properties of particles (such as cells) as they flow through a fluid stream through a beam of light (Sgonc and Gruber, 1998). The light scattering properties of cells are used to analyze changes in cell size, granularity, internal complexity, and relative fluorescence intensity. The

flow cytometry results are based on cellular morphological changes, membrane loss, and DNA fragmentation. In principle, flow cytometry is a very rapid and objective technique for enumerating apoptotic cells (Sgonc and Gruber, 1998).

Flow cytometer experiments were carried out by growing UCAA-62(Melanoma) cells in 25 cm^2 flasks at a concentration of $5x10^5$. The cells were allowed to attach to the flasks for 24 h before adding the drug. Thereafter, 1 mL of coumarin compound derivatives was added and the flasks were incubated for an additional 24 h. After 24 h, the cells were removed and washed with PBS. The floating and adherent cells were then collected. The floating cells were collected by centrifugation, while the adherent cells were harvested by trypsinisation using the cell dissociation buffer. Both cell suspensions were pooled.

3.6.1.2 Membrane changes using Annexin V kit

The Annexin V apoptosis detection Kit (BD Biosciences) was used as per manufacturer's protocol. Cell pellets were suspended in 100 µl of staining solution that was supplied with the Annexin-V Staining Kit. The stained cell pellet was incubated at room temperature for 15 min. Then, 400 µl of binding buffer was added to the stained pellets and the cells were analyzed directly by flow cytometry. Early apoptotic cells were indicated by localization in the lower right quadrant of a dot-plot graph using Annexin-Versus PI (propidium iodide). The controls used were: doxorubicin**,** DMSO, DMEM medium, unstained cells, cells stained with FITC, Annexin V (no PI) and cells stained with PI (no FITC Annexin V).

3.6.1.3 Apoptosis detection using caspase-3 kit

The PE caspase apoptosis Kit (BD Pharmagen) was used as permanufacture`s protocol. Cells ($1x10^6$) were re-suspended in 0.5 mL of BD cytofix/Cytoperm and incubated for 20 min on ice**.** Cells were then pelleted and the BD Cytofix/Cytoperm solution was discarded. Cells were then washed twice with 0.5 ml BD Perm/Wash™ buffer (1X) at room temperature and then re-suspended in BD Perm/Wash buffer (1X) plus antibody. The re-suspended cells were incubated for 30 min at room temperature. Following incubation, the cells were pelleted and the BD Perm/Wash buffer (1X) plus the antibody solution was discarded. The cells were then washed with one mL of BD Perm/Wash™ buffer (1X) and then re-suspended in 0.5 mL of BD Perm/Wash™ buffer (1X). The cells were then analyzed by flow cytometry.

3.6.1.4 Mitochondrial membrane potential using JC-1 kit

The JC-1 apoptosis detection Kit (BD Bioscience) was used as per manufacturer's protocol. To each pellet 0.5 ml of JC-1 (Mitochondrial membrane potential assay kit) working solution was added and vortexed gently to break clumps. Thereafter cells were incubated in JC-1 working solution for 10-15 min at 37 ℃ in a CO_2 incubator. The cells were then centrifuged and the supernatant was discarded. Thereafter cells were washed twice with 1 × Assay Buffer and the pellets were gently re-suspended in 0.5 ml 1 × Assay Buffer. The cells were then analyzed by flow cytometry.

3.7 Evaluation of safety of compounds

3.7.1. Ames Mutagenicity Test

The *Salmonella* mutagenicity experiment was carried out using the modified Maron and Ames (1983) protocol. *Salmonella typhimurium* TA 98 and TA 100 strains, was obtained on disc cultures from Medical Research Council Durban (MRC). Both strains were retrieved from broth cultures that were initially supplied as frozen disc cultures. With the aid of a flamed bacteriological needle, the culture disks were aseptically removed and then inoculated into a sterile 250 ml flask that contained 25 ml of nutrient broth (Oxoid) as well as 78 µl of Ampicillin (8 mg/ml). The flask was kept on a shaking incubator (150 rpm) at 37 ℃ for 16 h. This was performed to acquire an optical density ranging between 1.2 to 1.4 at 660 nm.

Salmonella TA 100 and TA 98 (100 µl) was added to 2 mL of 0.05 mM containing histidine/0.05 mM biotin top agar and mixed. After vortexing the mixture was plated onto a minimal glucose agar plate and incubation at 37 ℃ for 48 h. Well separated colonies from plates were used as primary broth cultures. The cultures were obtained by inoculating nutrient broth together with the master plate colonies. These cultures were then placed on a shaker (150 rpm) at 37 ℃ for 24 h.

Compounds **CMRN1** – **CMRN7** were prepared for anti-mutagenicity tests by dissolving them in DMSO so that respective concentrations of 10, 100 and 1000 µg/ml were obtained. Sodium azide (NaN_3), a potent mutagenic compound was used as the active control (Maron and Ames, 1983). The NaN_3 was dissolved in DMSO (at 5 µg/ml, 10 µg/ml and 20 µg/ml). The negative control used was the DMSO.

For each concentration of test compound, three plates were used. In a sterile test tube, 100 µl bacterial culture, 100 µl test compound, and 2.9 ml soft agar at 45 ℃ were added. The tube was briefly mixed using a vortex and poured onto glucose minimal agar plates. Once the agar

overlay became solid, plates were inverted and incubated at 37 ℃ for 48 h. Thereafter, revertant colonies (i.e. histidine dependant) were counted and the mutant frequency was determined and recorded. The mutant frequency was calculated as a fraction of the number of revertant colonies and the number of colonies in the negative control. The formula is shown below (Maron and Ames, 1983):

$$\text{Mutant Frequency} = \frac{\text{number of revertant colonies}}{\text{No.of colonies negative control}}$$

3.7.2 Brine Shrimp Lethality Assay

The Brine Shrimp toxicity test was carried out using the Brine Shrimp (*Artemia salina*) bioassay. Artificial seawater was filled in a 1 L conical-shaped flask (33 g/l sea salt adjusted to a pH of 8.5 using 1N NaOH solutions), in which the Brine Shrimp eggs were allowed to hatch. This occurred under constant aeration for 48 h so that sufficient time was available for all the active nauplii to become free from their egg shells. The active nauplii were then collected from the brighter portion of the hatching chamber and used in the bioassay. The protocol involved drawing 10 shrimp through a glass capillary and placing them in a 6-well plate. Each well of the plate contained 4.95 ml Brine solution. In each experiment, 50 µl prepared DMSO-compound solution (10, 100, 500 µg/ml) was added to the Brine solution-containing (4.95 ml) wells. The plate was maintained at room temperature (37^{o}C) under visible light for 24h. The dead larvae were counted and percentage death determined (Meyer *et al*., 1982).

4. RESULTS

4.1 Synthetized coumarin compounds (CMRN1 - CMRN7) used in this study

The test compounds **CMRN1 - CMRN7** were synthesized as described previously using chemicals from Aldrich and Merck chemical company. The products synthesized listed in section 3.3, were purified and characterized in our laboratory at the Durban University of Technology South Africa.

The physicochemical characteristics of the synthetic coumarin analogues **CMRN1- CMRN7** are summarized in Table 2.

Table 2: Physicochemical characteristics of coumarin analogues CMRN1 - CMRN7

Compound	Molecular formula (MolecularWeight)	Yield (%)[a,b]	m.p. (℃)		Mass (*m/z*)	*clog P*[c]
			reported	found		
CMRN1	$C_{11}H_7BrO_3$ (265)	96	120-122	121	266(M +1)	1.4023
CMRN2	$C_{11}H_5Br_3O_3$ (421)	95	146-148	147	422 (M +1)	3.4753
CMRN3	$C_{11}H_7BrO_3$ (265)	98	220-222	221	266 (M +1)	2.0193
CMRN4	$C_{11}H_6Br_2O_3$ (343)	95	204-206	205	344 (M +1)	2.2723
CMRN5	$C_{11}H_6BrClO_3$ (299)	94	180-182	181	300 (M +1)	2.1223
CMRN6	$C_{11}H_7ClO_3$ (222)	95	218-220	219	223 (M +1)	1.8693
CMRN7	$C_{12}H_7BrN_2O_2S$(321)	87	210-212	211	322 (M +1)	2.6992

[a]All of the products were characterized by spectral and physical data
[b]Yields were on isolated basis
[c]*c log p* was calculated using ChemBioDraw Ultra 13.0v.

4.2 Anti-mosquito activity of coumarins CMRN1-CMRN7

Generalized linear model assuming a Gaussian distribution was considered to evaluate differences between treatements registerd in larval mortality (larvicidal assay), adulticidal effect, and knockdown (in repellence assay) for anti-mosquito assays. LSD Fisher test was carried out for post hoc analyses. In all cases, a value of $p < 0.05$ was considered statistically significant.

There were a significant effect of treatment on larval mortality, significant differences between treatment times and significant interactions between treatment and exposure times (Table 3).

The highest activity were detected with compounds **CMRN**1**, CMRN2, CMRN4, CMRN5**, and **CMRN**7 showing close to 100% mortality after 24 h of exposure, which was the same as for the positive control Temephos. Compounds **CMRN3** and **CMRN6** showed a statistically significant higher mortality compared to the negative control that increased after 48 h however, mortality values were low from a practical point of view (5 to 28.3%).

Table 3: Larvicidal effects: Mortality of *Anopheles arabiensis larvae* expose to coumarins (CMRN1-7) at 4 µg/mL and their negative (acetone) and positive (Temephos) controls.

Comp Code	Assessment time (h)		
	24	**48**	**72**
CMRN1	98.7 ±1.3[a]	100.0 ±0.0[a]	100.0 ±0.0[a]
CMRN2	97.7 ±2.3[a]	100.0 ±0.0[a]	100.0 ±0.0[a]
CMRN3	1.0 ±1.0b[b]	6.3 ±2.0[c]	7.3 ±1.3[c]
CMRN4	98.7 ±1.3[a]	100.0 ±0.0[a]	100.0 ±0.0[a]
CMRN5	97.7 ±2.3[a]	100.0 ±0.0[a]	100.0 ±0.0[a]
CMRN6	1.0 ±1.0[b]	2.0 ±1.0[b]	5.0 ±1.0[c]
CMRN7	98.7 ±1.3[a]	100.0 ±0.0[a]	100.0 ±0.0[a]
Water	0.0±0.0 [A]	0.0±0.0 [A]	0.0±0.0 [A]
Acetone	0.0±0.0 [A]	0.0±0.0 [A]	0.0±0.0 [A]
Temephos[C]	100.0±0.0 [C]	100.0±0.0 [C]	100.0±0.0 [C]

[A-C] Assessment times and treatments not sharing a capital letter differ significantly ($p<0.05$).

A repeated measures ANOVA test on adult mosquito mortality data showed that there were significant effects of the compound, exposure time, and their interaction on mosquito mortality. The positive control K-Othrine showed 100% knockdown/mortality from the first 30 min of exposure, while the synthetic compounds and negative control did not knockdown mosquitoes throughout the 24 h observation period.

All components tested (except the positive and negative controls) knocked down mosquitoes within the 2 min exposure time. **CMRN1, CMRN2, CMRN5** and **CMRN6** were the most potent, knocking approximately 100% of them (Table 4). However, 24 h after exposure all mosquitoes recovered. The few mosquitoes exposed to the coumarin analogues that were not knocked down did not attempt to bite; due to the low number of mosquitoes remaining active no further statistical analyses were carried out.

Table 4: Adulticidal effects: Mortality of *Anopheles arabiensi*s and acetone and temephos of the larvae exposed to halogenated coumarins at 4 µg/ml.

Compound	Knockdown		Mortality
	30 min	60 min	24 h
CMRN1	2.0 ±2.0 ^{D}	2.0 ±2.0 ^{D}	7.7 ±3.9 ^{DE}
CMRN2	2.0 ±1.0 ^{D}	3.0 ±1.7 ^{D}	7.3 ±1.3 ^{DE}
CMRN3	1.0 ±1.0 ^{D}	2.0 ±0.0 ^{D}	13.3 ±3.3 ^{AE}
CMRN4	1.0 ±1.0 ^{D}	1.0 ±1.0 ^{D}	10.7 ±2.3 ^{E}
CMRN5	0.0 ±0.0 ^{D}	0.0 ±0.0 ^{D}	19.5 ±3.5 ^{AE}
CMRN6	2.0 ±2.0 ^{D}	2.0 ±2.0 ^{D}	7.7 ±3.9 ^{DE}
CMRN7	2.0 ±1.0 ^{D}	3.0 ±1.7 ^{D}	7.3 ±1.3 ^{DE}
Water	3.0 ±0.0 ^{D}	3.0 ±0.0 ^{D}	10.0 ±0.0 ^{AE}
Acetone	0.0 ±0.0 ^{D}	0.0 ±0.0 ^{D}	6.0 ±0.0 ^{DE}
K-Othrine	100.0 ±0.0 ^{F}	100.0 ±0.0 ^{F}	100.0 ±0.0 ^{F}

$^{A-F}$ **Treatments and pos-treatment times not sharing a capital letter differ significantly ($p<0.05$).**

Repellency effects were all in the range of 87-97% except for compound **CMRN7** which was only 18% when compared to positive control DEET (Table 5). **CMRN1-CMRN6**, knocked down mosquitoes within 2 min exposure. However, 24 h after exposure, all the mosquitoes recovered, the few mosquitoes exposed to the coumarin analogues that were not knocked down did not attempt to bite ; due to low number of mosquitoes remaining active, no further stastical analyses were carried out.

Table 5: **Repellency effects: Mortality of *Anopheles arabiensis* and acetone and temephos of the larvae exposed to halogenated coumarins at 4 µg/ml.**

Comp. code	Repelled*	Knockdown*	Corrected repellence	Repelled or knocked
CMRN1	61.0 ±30.5	33.3 ±33.3	91.7 ±1.7 ^{AB}	94.4 ±2.9 ^{AB}
CMRN2	59.7 ±30.1	33.3 ±33.3	90.0 ±6.7 ^{B}	93.3 ±5.1 ^{AB}
CMRN3	58.7 ±29.5	38.4 ±31.0	96.7 ±3.3 ^{A}	92.2 ±4.8 ^{AB}
CMRN4	64.3 ±32.0	35.3 ±32.4	100.0 ±0.0 ^{A}	97.8 ±2.2 ^{AB}
CMRN5	61.0 ±30.9	33.3 ±33.3	91.7 ±8.3 A^{B}	94.4 ±5.5 ^{AB}
CMRN6	54.3 ±27.2	33.3 ±33.3	81.7 ±1.7 ^{B}	87.8 ±6.2 ^{B}
CMRN7	18.00 ±2.0	0.00 ±0.00	18.0 ±2.0 ^{BC}	18.3 ±1.7 ^{C}
Water	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0 ^{C}	0.0 ±0.0 ^{C}
Acetone	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0 ^{C}	0.0 ±0.0 ^{C}
DEET	100.0 ±0.0	0.0 ±0.0	100.0 ±0.0 ^{A}	100.0 ±0.0 ^{A}

$^{A-C}$ **Within same column, treatments not sharing a capital letter differ significantly ($p<0.05$).**

*** No significant differences ($p<0.05$) between treatments.**

4.3 Antimicrobial activity of coumarins CMRN1-CMRN7

The antimicrobial activities of the synthetized coumarins derivatives (**CMRN1-CMRN7**) were screened using the disc diffusion method against ten bacterial strains, 3 yeast and 2 fungi. The antimicrobial activity is shown as zones of inhibition of the synthetized coumarins in Table 6 and 7. The minimum inhibition concentrations (MIC) were determined by serial dilutions of the test compounds to levels where no inhibition of growth of bacteria were noticed, the results are summarized in Table 8. The inhibitory zone of compound: **(A) CMRN1, (B) CMRN2, (C) CMRN4, (D) Ciprofloxacin** against *B. coagulans, S. aureus* and *B. cereus* are also shown in Figures 23, 24 and 25.

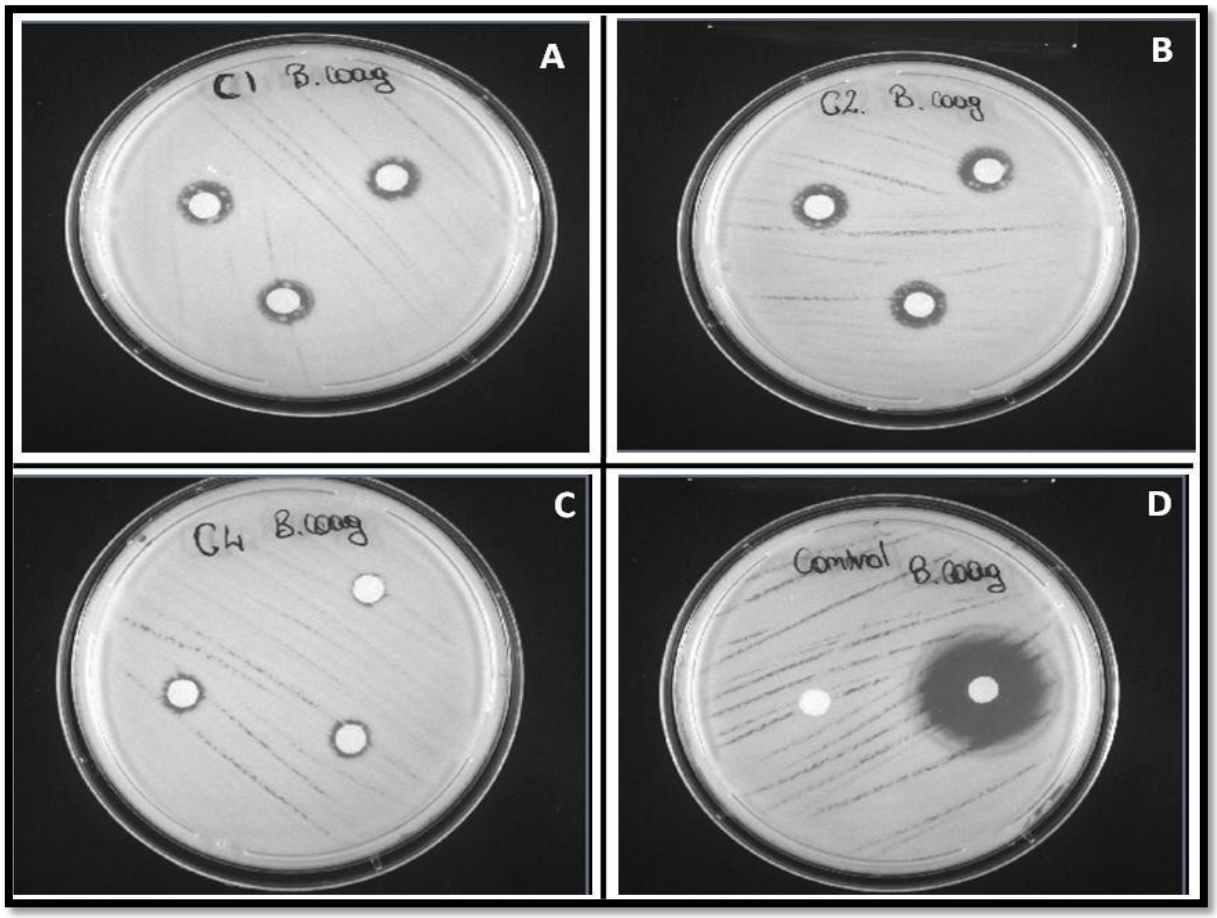

Figure 23: Antibacterial screening showing zones of inhibition produced by compounds: (A) CMRN1, (B) CMRN2, (C) CMRN4, (D) Ciprofloxacin (positive control) against *B. coagulans.*

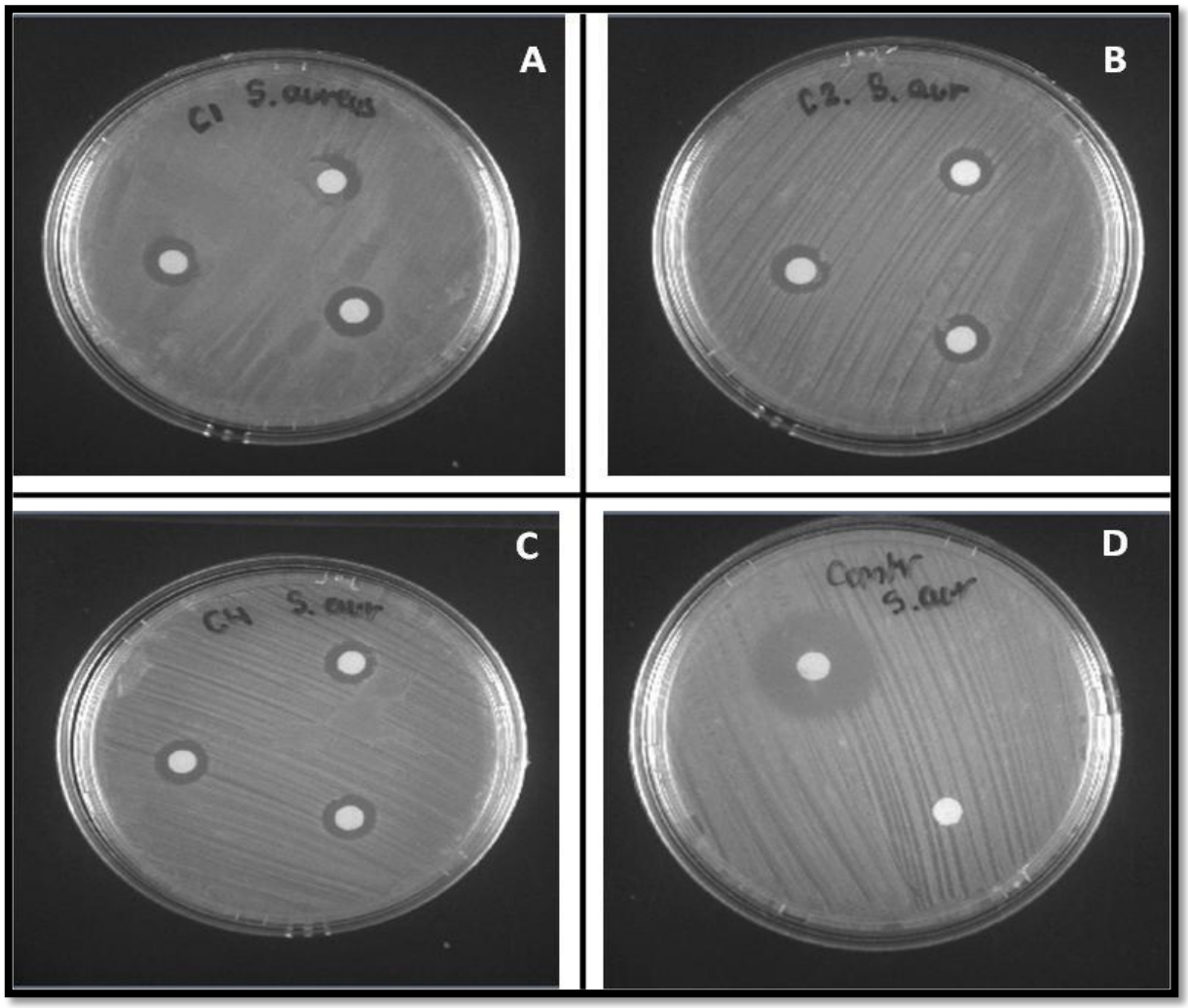

Figure 24: Antibacterial screening showing zones of inhibition produced by compounds: (A) CMRN1, (B) CMRN2, (C) CMRN4, (D) Ciprofloxacin (positive control) against *S. aureus.*

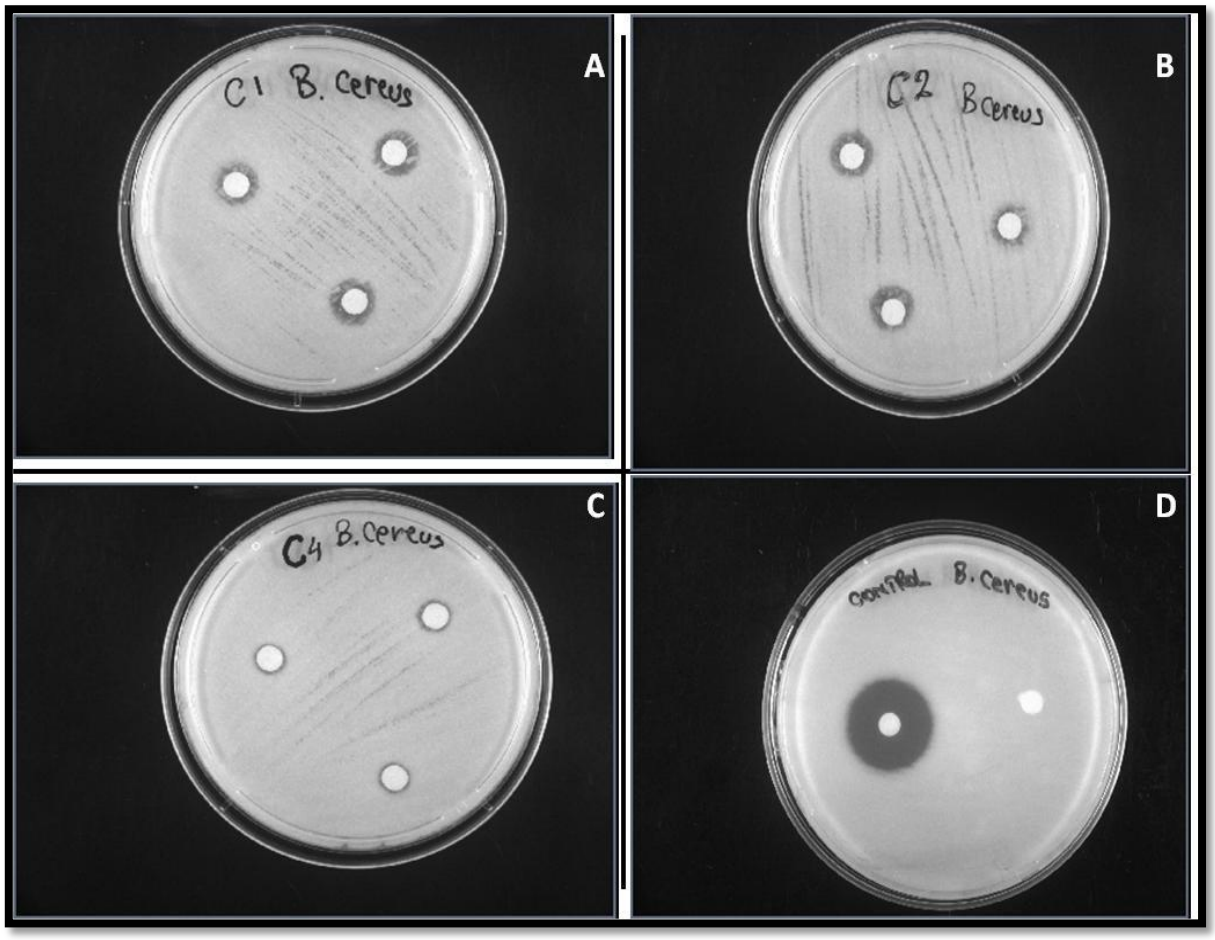

Figure 25: Antibacterial screening showing zones of inhibition produced by compounds: (A) CMRN1, (B) CMRN2, (C) CMRN4, (D) Ciprofloxacin (positive control) against *B. cereus.*

Compound **CMRN1** induced an inhibition zone of 10 mm around *B. cereus, B. coagulans,* and *S. faecalis*, (Table 6) with an MIC of 0.75 mg/ml (Table 8). Compound **CMRN2** induced an inhibition zone of 10 mm around *B. Cereus and B.coagulans*, 11 mm against *S. aureus* and *S. faecalis* (Table 6), with MIC of 0.75 mg/ml (Table 8). These two compounds showed activity against most of the tested bacteria used in this study.

The inhibition zone for compound **CMRN4** was 8 mm and 10 mm while the MIC was 1.5 mg/mL against *M. luteus* and *S. aureus.* Compounds **CMRN1** and **CMRN2** showed an inhibition zone of 8 mm with an MIC of 1.5 mg/ml against *E. coli* and *S. aureus.* Compound **CMRN4** showed an inhibition zone of 10 mm (Table 6) with an MIC of 1.5 mg/ml against *S. aureus* (Table 8). Compound **CMRN7** showed a slight activity against *B. cereus, S. aureus, M. luteus and C. freundii* (Table 6). All of the tested compounds displayed no antifungal activity against *A. niger, A. flavus* and against the yeast except for compounds **CMRN**7 and **CMRN5** that demonstrated a slight activity against *C. albicans* and *C. utilis* while compounds **CMRN2** and **CMRN4** were slightly active against *S. cerevisiae* (Table 7).

Table 6: Antibacterial activity of synthetized coumarins CMRN1 - CMRN7

Bacteria	1	2	3	4	5	6	7	Control Ciprofloxacin
B. cereus	10 ±0.00	10.0 ±0.0	0	7.02 ±0.11	0	0	6.03 ±0.05	29.1 ±0.17
E. coli	8 ±0.00	8.0 ±0.0	0	0	0	0	0	35.04 ±0.06
M. luteus	6.06 ±0.11	6.07 ±0.13	0	8.0	0	0	7.07 ±0.12	29.06 ±0.01
S. aureus	10	11.0 ±0.0	0	10	0	0	7.04 ±0.06	25.06 ±0.01
S. marcescens	6.06 ±0.10	6.03 ±0.05	0	0	0	0	0	35.0 ±0.0
C. freundii	6.03 ±0.05	6	0	0	0	0	7.06 ±0.1	30.0 ±0.00
B. coagulans	10.0 ±0.0	10.0 ±0.0	0	7.06 ±0.2	0	0	0	28.05 ±0.08
B. stearothermophilus	0	0	0	0	0	0	0	30.1 ±0.28
S. faecalis	10 ±0.00	11 ±0.00	0	0	0	0	0	22.03 ±0.05
K. pneumoniae	0	0	0	0	0	0	0	36.07 ±0.12

Values are mean ±SD (n=3), 0: no activity, 1-7 = Compounds CMRN1 - CMRN7

Table 7: Antifungal activity of synthetized coumarin CMRN1 - CMRN7

Yeast	1	2	3	4	5	6	7	Control Amphotericin
C.albicans	0	0	0	6 ±0.00	0	0	6.0 ±0.00	30.04 ±0.08
C.utilis	0	0	0	6 ±0.00	0	0	6.0 ±0.00	28.0 ±00
S.cerevisiae	0	6 ±0.00	0	6.0 ±0.00	0	0	0	28.0 ±00
Fungi								
A.flavus	0	0	0	0	0	0	0	19.03 ±0.05
A.niger	0	0	0	0	0	0	0	19.1 ±0.17

Values are mean ±SD (n=3), 0= no activity, 1-7 = Compounds CMRN1 - CMRN7

Table 8: **Minimum inhibition concentration (MIC) of coumarin derivatives**

Bacteria	**MIC (mg/mL)**		
	CMRN1	**CMRN2**	**CMRN4**
B.cereus	0.75	0.75	na
E.coli	1.5	1.5	na
M.luteus	na	na	1.5
S.aureus	0.75	1.5	1.5
B.coagulans	0.75	0.75	na
S.faecalis	0.75	0.75	na

Values are mean ±SD (n=3), standard deviation = 0, number of sample (n=3), na: not applicable

4.4 Anti-inflammatory activity of coumarins CMRN1-CMRN7

The anti-inflammatory activity of halogenated coumarins derivatives **CMRN1- CMRN7** (at the concentration of 1 mg/ml) was investigated with the 5-Lipoxygenase Kit Assay. The protocol supplied with the kit was followed. The analyses were performed in triplicate and the results were represented as mean value of the percentages of the triplicate assays of each sample. The results was expressed as mean of the % and SD % as mean (±SD) and summarized in Table 9. The 15-lipoxygenase inhibitor was used as the positive control and displayed activity of 100% of lipoxygenase ezyme inhibition. Compared to this, in our study only compounds **CMRN1, CMRN2, CMRN4** and **CMRN5** exhibited lipoxygenase enzyme inhibition activity of 63.19, 60.74; 63.39 and 61.55% respectively while, compounds **CMRN3**, **CMRN6**, **CMRN7** showed activity of 45.81 ; 43.97 and 48.47% respectively.

Table 9: Anti-inflammatory activity of coumarin CMRN1- CMRN7

Compounds	Lipoxygenase inhibition (%) at 1 mg/mL
CMRN1	63.19 ±0.005
CMRN2	61.55 ±0.005
CMRN3	45.81 ±0.003
CMRN4	60.74 ±0.006
CMRN5	63.39 ±0.002
CMRN6	43.97 ±0.001
CMRN7	48.47 ±0.007
15-LO	100.00 ±0.0

Values are mean ±SD (n=3), 15-LO: 15-Lopoxygenase inhibitor

4.5 Antioxidant activity of coumarins CMRN1-CMRN7

The results of antioxidant activities of the compounds **CMRN1- CMRN7** are shown in Table 10. The results found revealed that compounds **CMRN1** (56%), **CMRN4** (86%), **CMRN7** (61%) exhibited free radical-scavenging activities. Compounds **CMRN2, CMRN3, CMRN5**, and **CMRN6** displayed low antioxidant activity between the range of 23 - 36% at 1 mg/ml while the active control was 95%. The results are also depicted in Figure 26.

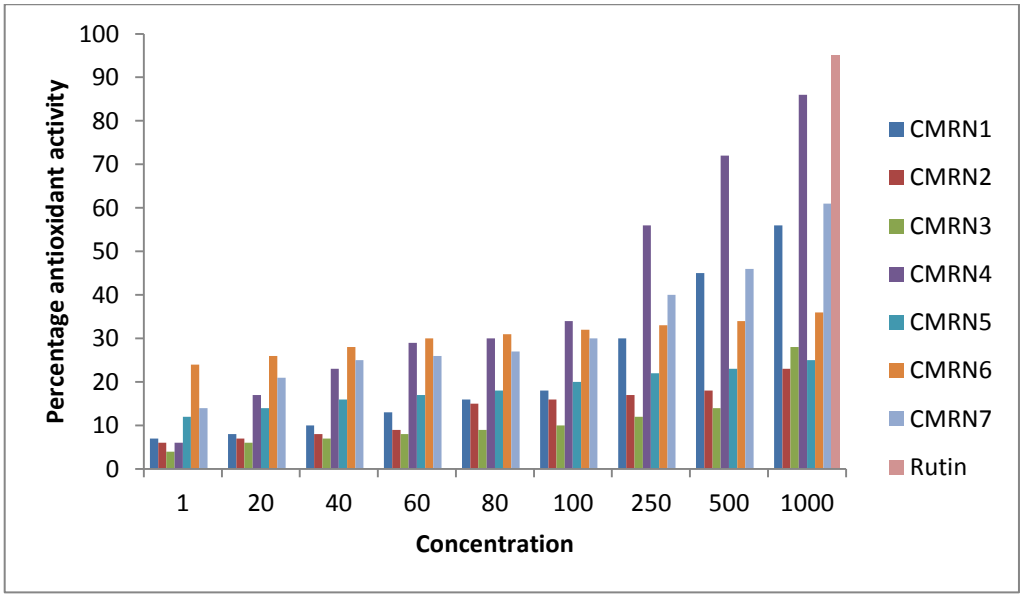

Figure 26: Percentage antioxidant activity of CMRN1-CMRN7

Table 10: Percentage antioxidant activity of CMRN1-CMRN7.

Comp Code	Concentration (µg/mL)								
	1	20	40	60	80	100	250	500	1000
CMRN1	7±0.011	8±0.022	10±0.003	13±0.006	16±0.011	18±0.007	30±0.019	45±0.022	56±0.012
CMRN2	6±0.005	7±0.013	8±0.009	9±0.009	15±0.002	16±0.003	17±0.003	18±0.012	23±0.021
CMRN3	4±0.005	6±0.007	7±0.007	8±0.003	9±0.003	10±0.001	12±0.005	14±0.005	28±0.001
CMRN4	6±0.005	17±0.024	23±0.005	29±0.051	30±0.037	34±0.006	56±0027	72±0.006	86±0.011
CMRN5	12±0.004	14±0.002	16±0.002	17±0.003	18±0.013	20±0.013	22±0.003	23±0.002	25±0.014
CMRN6	24±0.029	26±0.002	28±0.006	30±0.006	31±0.004	32±0.005	33±0.004	34±0.008	36±0.007
CMRN7	14±0.005	21±0.004	25±0.004	26±0.004	27±0.004	30±0.007	40±0.003	46±0.003	61±0.032
- Control (Methanol)	-	-	-	-	-	-	-	-	0
+control (Rutin)	-	-	-	-	-	-	-	-	95

Values are mean ±SD (n=3)

4.6 Cytotoxicity assay of coumarins CMRN1-CMRN7

The coumarin derivatives **CMRN1 - CMRN7** were screened for toxicity against UACC-62 (Melanoma), MCF-7 (Breast Cancer) and PBMC (Peripheral blood mononuclear) cell lines using MTT assay. Highly toxic coumarin compounds were tested for their IC_{50} values.

Compounds **CMRN1, CMRN2, CMRN4** and **CMRN5** showed the highest toxicity against UACC-62 (Melanoma) and MCF-7 (Breast Cancer), The cytotoxicity effect on UACC-62 (Melanoma) was 97.4; 97.9; 94.7 and 90.8%, and for MCF-7 (Breast Cancer) was of 96.8; 90.9; 92.9; and 94.2%, at the concentration of 50 µg/ml. These results are summarized in Table 11 and 12. There was very low or no activity against the PBMC (Peripheral blood mononuclear) cells (Table 13) which was used as reference to indicate the effect of the compounds on normal cell.

Table 11: Cell inhibition (%) of the synthetized coumarins against UACC-62 (Melanoma) cell at the concentration of 100 µg/mL and 50 µg/ml

Compounds	100 µg/mL	50 µg/mL
CMRN1	98.5 ±0.003	97.4 ±0.010
CMRN2	91.5 ±0.012	90.8 ±0.009
CMRN3	50.0 ±0.08	25.1 ±0.28
CMRN4	98.0 ±0.003	97.9 ±0.004
CMRN5	95.9 ±0.017	94.7 ±0.006
CMRN6	50.8 ±0.06	19.4 ±0.12
CMRN7	29.6 ±0.06	22.6 ±0.11
Doxorubicin	--	92.8 ±0.009
Untreated	--	2.8 ±0.195

Values are mean ±SD (n=3)

Table 12: Cell inhibition (%) of the Synthetized coumarin against MCF-7 cells at the concentration of 100 µg/mL and 50 µg/ml

Compounds	100 µg/mL	50 µg/mL
CMRN1	97.3 ±0.001	96.8 ±0.003
CMRN2	95.7 ±0.008	94.2 ±0.011
CMRN3	32 ±0.04	26 ±0.07
CMRN4	91.1 ±0.007	90.9 ±0.008
CMRN5	94.3 ±0.009	92.9 ±0.002
CMRN6	31.4 ±0.043	28.6 ±0.03
CMRN7	15.1 ±0.08	14.2 ±0.06
Doxorubicin	--	90.3 ±0.008
Untreated	--	1.2 ±0.02

Values are mean ±SD (n=3)

Table 13: Cell inhibition (%) of the Synthetized coumarin against PBMC cells at the concentration of 100 µg/mL and 50 µg/ml

Compounds	100 µg/mL	50 µg/mL
CMRN1	-151.53 ±0.006	-95.95 ±0.005
CMRN2	-154.08 ±0.016	-98.23 ±0.005
CMRN3	0.26 ±0.002	-2.53 ±0.002
CMRN4	-10.97 ±0.011	-9.11 ±0.02
CMRN5	-169.64 ±0.011	-98.23 ±0.004
CMRN6	4.85 ±0.002	0.25 ±0.002
CMRN7	7.14 ±0.004	9.37 ±0.006
Doxorubicin	18.11 ±0.002	21.66 ±0.001
Untreated	--	-1.79 ±0.001

Values are mean ±SD (n=3)

The IC_{50} values of the synthetized compounds **CMRN1**, **CMRN2**, **CMRN4** and **CMRN5** were determined using UACC-62 (Melanoma) and MCF-7 cell lines. The results obtained in Table 14, indicate that all of the compounds had selective activity towards the cancer cell lines, with the **CMRN2** which had an IC_{50} ranging from 28.78 to 30.93% on both cell lines. Each of the other compounds **CMRN1**, **CMRN4**, **CMRN5** produced significant cytotoxicity activity which was observed to be greater in UACC-62 (Melanoma) cancer cells, particulary for compounds **CMRN4** and **CMRN5** when compared to the positive control doxorubicin.

Table 14: Table showing the IC_{50} (µg/ml) of compounds CMRN1, CMRN2, CMRN4, CMRN5 against UACC-62 (Melanoma) and MCF-7 cancer cells

Compounds	UCAA-62(Melanoma)	MCF-7
CMRN1	19.12 ±0.01	20.73 ±0.02
CMRN2	30.93 ±0.05	28.78 ±0.02
CMRN4	7.282 ±0.03	15.39 ±0.01
CMRN5	1.772 ±0.01	21.96 ±0.04
Doxorubicin	0.09 ±0.01	1.61 ±0.01

Values are mean ±SD (n=3).

The inhibitory concentration that killed cells by 50% (IC_{50} µg/ml) data of compounds **CMRN1**, **CMRN2**, **CMRN4** and **CMRN5** against UACC-62 (Melanoma) and MCF-7 cancer cells are also presented in Figure 27 and Figure 28 respectevely.

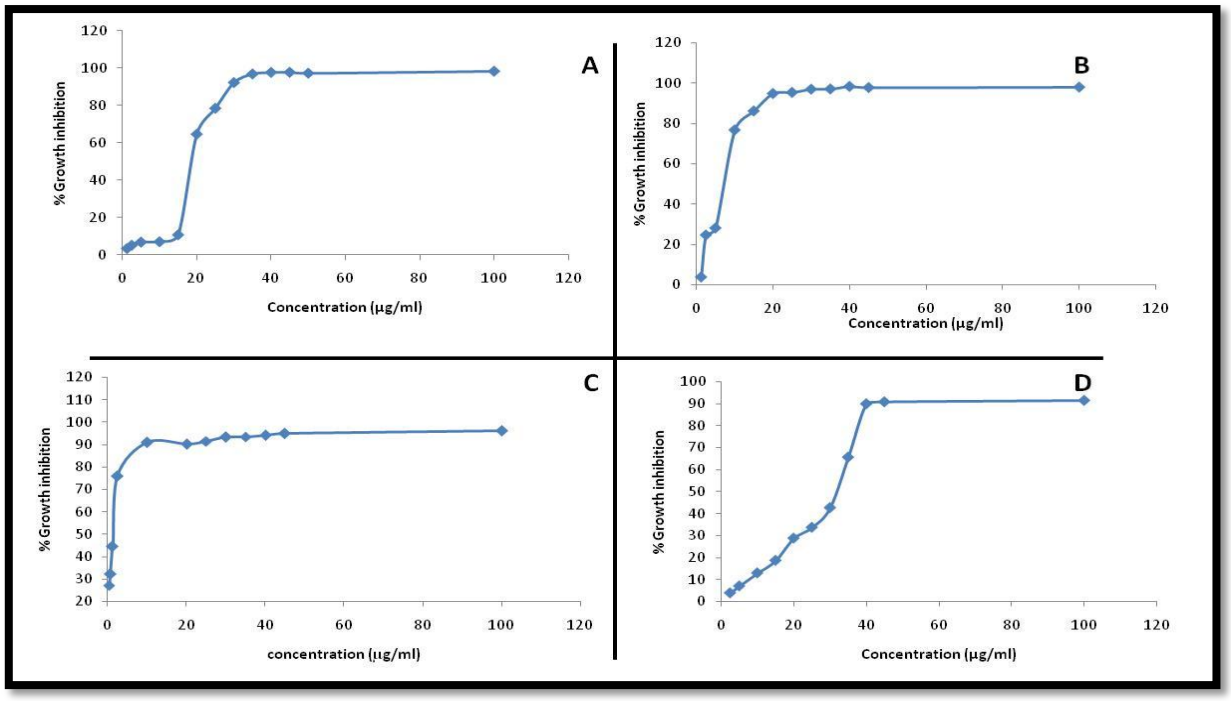

Figure 27: Inhibitory concentration of the substituted halogenated coumarins that killed cells by 50% (IC_{50}): (A) CMRN1; (B) CMRN4; (C) CMRN5; (D) CMRN2 against UACC-62 (Melanoma) cell line.

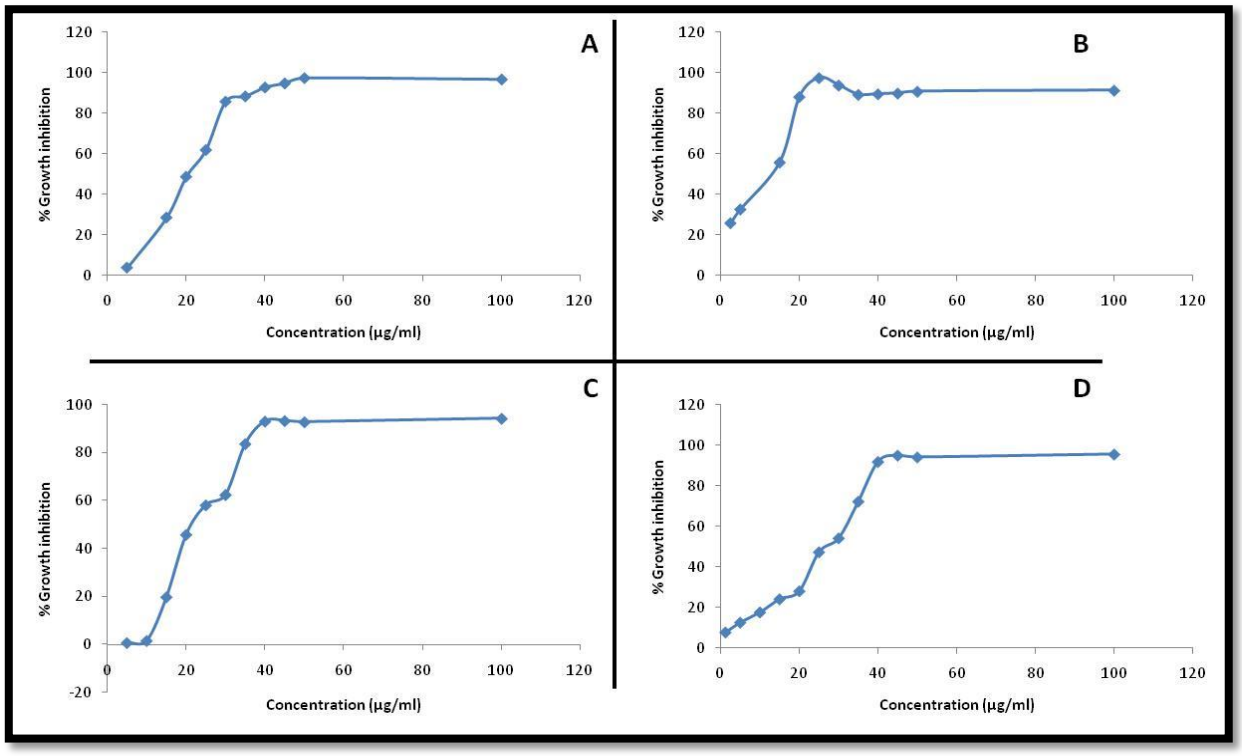

Figure 28: Inhibitory concentration of the substituted halogenated coumarins that killed cells by 50% (IC_{50}): (A) CMRN1; (B) CMRN4; (C) CMRN5; (D) CMRN2 against MCF-7 (Breast) cell line.

4.7 Flow cytometry evaluation of apoptosis

In order to assess whether the tested coumarin compounds induced apoptosis, apoptotic cells were identified. This evaluation involved determining the (1) characteristic morphological changes associate with apoptosis, (2) plasma membrane changes using Annexin V kit, (3) changes in mitochondrial membrane potential using the JC-1 kit, and (4) caspase activation using the caspase-3 kit. The UACC-62 (Melanoma) cancer cell line was investigated further for apoptotic potential.

4.7.1 Morphological Examination

Apoptosis, or programmed cell death, is a process involving many typical morphological characteristics that include plasma membrane blebbing, cell shrinkage, chromatin condensation and fragmentation (Vermeulen *et al*., 2005).

Light microscopy observations of UACC-62 (Melanoma) treated with compounds **CMRN1**, **CMRN2**, **CMRN4**, **CMRN5** and Doxorubicin showed that these compounds have the potential to increase cell death at treatment concentrations of 50 µg/ml. The results obtained in figure 29 showed that the untreated UACC-62 (Melanoma) cell (Figure 29A) and the DMSO

control (negative control) (Figure 29B), treated cells appear normal, while those treated with the synthetized coumarin compounds and the doxorubicin control, exhibited the characteristic features of apoptosis. These features are cell shrinkage, detachment and change in shape (rounded).

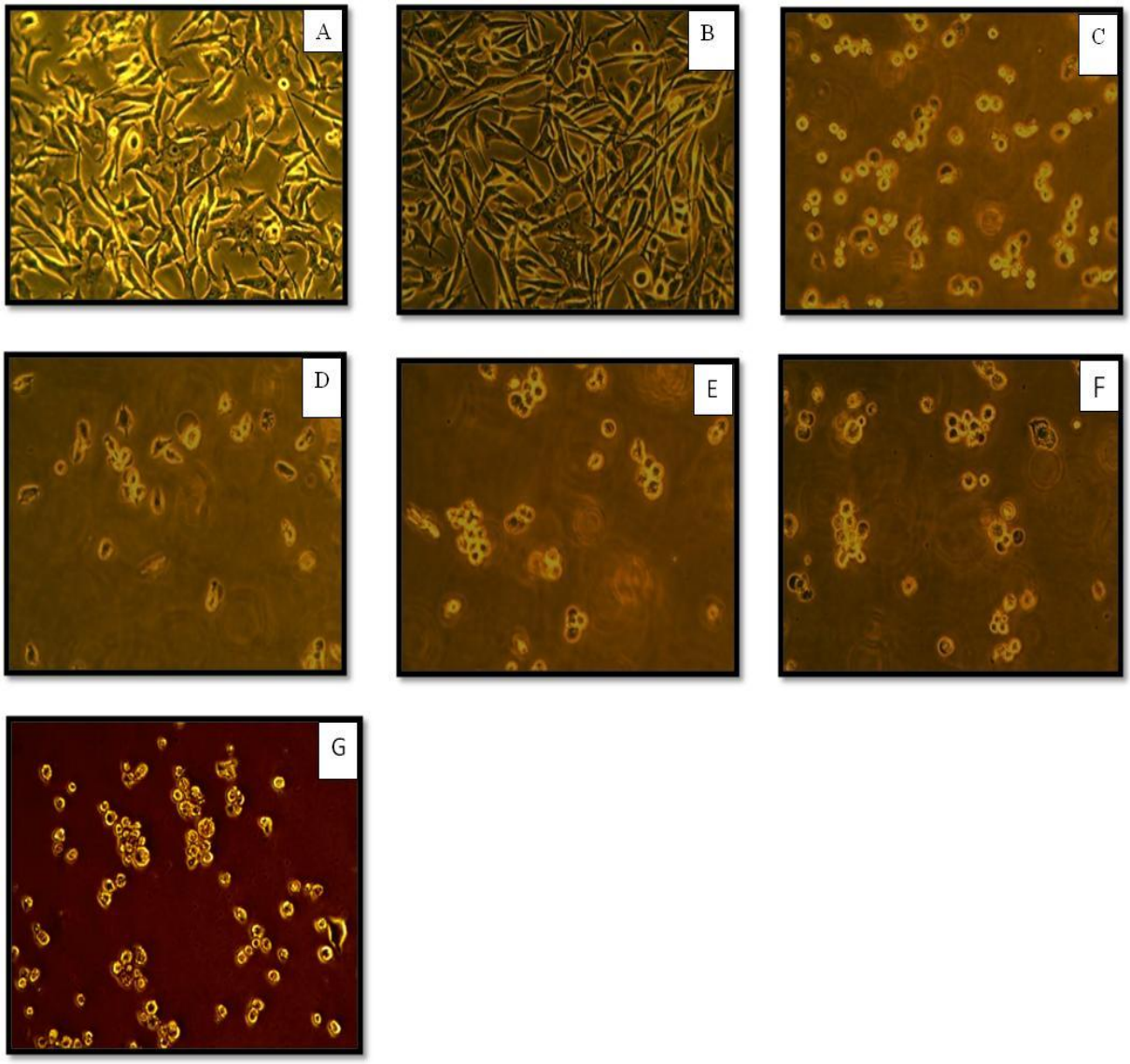

Figure 29: Microscopic observation of the morphology of UACC-62(Melanoma) cells: (A) In the absent of the tested compounds, (B) Treated with 50 µg/ml of DMSO, treated with compounds (C) CMRN1; (D) CMRN2; (E) CMRN4; (F) CMRN5 and (G) Doxorubicin 6 µg/ml respectively.

4.7.2 Membrane changes using Annexin V kit

The Annexin V Apoptotic kit was used according to the manufacturer's protocol (BD Biosciences). Annexin V-FITC was used to quantitatively define the percentage of cells within a population that are actively undergoing programmed cell death. The cell fluorescence was measured by flow cytometry using an FL2 channel (565 to 605 nm) and a minimum of 10 000 events were acquired per sample. The results are depicted in Figure 30.

The percentage Annexin positive cells that were observed are shown in Table 15. Compounds **CMRN1, CMRN2** and **CMRN5** induced early apoptosis in UACC-62 cells at apoptotic percentages of 13.1; 12.1and 15.1%, respectively. These were the results obtained by subtracting the DMSO value received (7.75%). Late apoptosis was also induced by compounds **CMRN1, CMRN2, CMRN4,** and **CMRN5**. The apoptotic percentages were 25.4; 28.5, 67.9; and 27.3%. The percentage of apoptotic activity induced by the DMSO control that was subtracted was 10.4%. These results indicated that coumarins compounds have the potential to produce apoptosis in UACC-62 (Melanoma) cell lines. The results of annexin V were acceptable since doxorubicin, administered at 6 µg/ml, induced late apoptosis in the UACC-62 (Melanoma) cell line by 46%.

Table 15: UACC-62 (Melanoma) treated with coumarin and stained with Annexin V-FITC/PI

Treatment	Viable (%)	Apoptosis (%)	Late apoptosis (%)	Necrotic (%)
Untreated Cells unstained	100	0	0	0
Untreated stained cells Annexin/PI	51.9	0	23.9	7.98
Annexin only	58.3	33.45	0	0.
DMSO	80.5	7.75	10.4	3.62
Doxorubicin	3.18	0	46	39.9
CMRN1	28.9	13.1	25.4	14.5
CMRN2	27.7	12.1	28.5	17.5
CMRN4	14	0	67.9	2
CMRN5	23.6	15.1	27.3	15.9

Apoptosis of UACC-62 (Melanoma) cell line, plot of Annexin-Vis presented in the Figure below

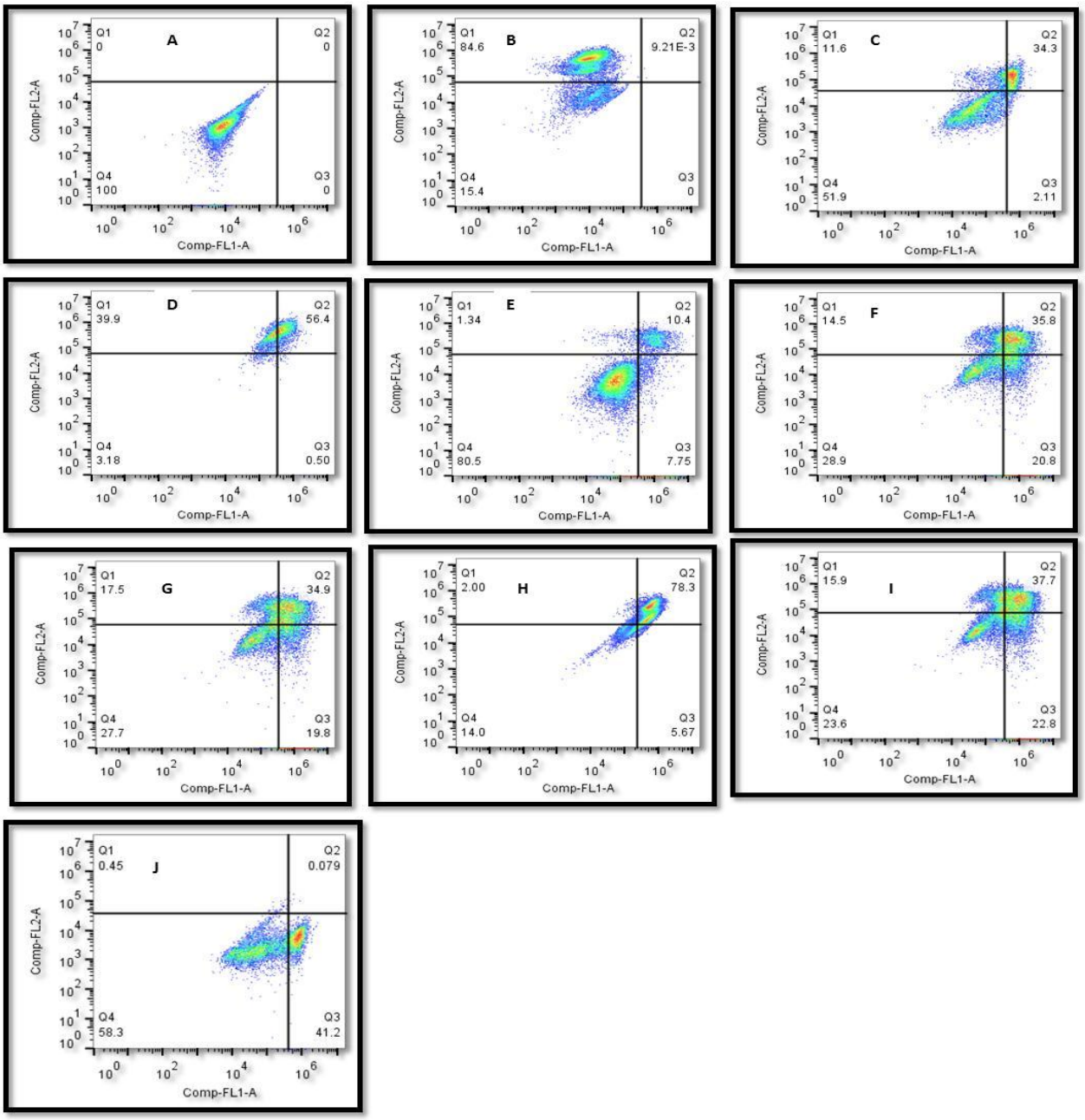

Figure 30: Apoptosis evaluation of compounds using Annexin V–FITC

Apoptotic cells (%) are localized in the lower right quadrant (Q3) and the late apoptotic (%) are located in the high right quadrant (Q2). (A) Untreated Cells unstained, (B) PI Control, (C) Untreated stained cells annexin/PI, (D) Cells treated with 6 µg/ml of doxorubicin, (E) Cells treated with 50 µg/ml of DMSO, Cells treated with 50 µg/ml of (F) **CMRN1,** (G) **CMRN2,** (H) **CMRN4,** (I) **CMRN5,** (J) treated with annexin only.

4.7.3 Mitochondria membrane potential

Changes in mitochondrial membrane are one of the early marker for apoptosis. The monomers and aggregates of JC-1 have different emission spectra, changes in mitochondria membrane potential (BD Bioscience). Cell fluorescence was measured using the flow cytometer by comparing that ratio of fluorescence between the FL1 and FL2 channels and a minimum of 10 000 events was acquired per sample.

Compounds which showed mitochondrial membrane potential and induced apoptosis (Figure 31), compounds **CMRN1, CMRN5** had an apoptosis activity of 27.8; 25.8 and compounds **CMRN2, CMRN4** displayed apoptotic activity of 8.5, 16.2% respectively (Table 16) after subtracting the background of the DMSO which was 6.5%. These results were also validated by running the control doxorubicin that had 49.5% activity after subtraction of the DMSO control.

Table 16: Table of the mitochondrial membrane potential results

Treatment	Stained Viable cell (%)	Apoptosis (%)
Untreated unstained Cells	100	0
Untreated stained cells	93.6	4.58
DMSO	92.3	6.50
Doxorubicin	27.6	49.5
CMRN1	65.6	27.8
CMRN2	77.2	16.2
CMRN4	84.9	8.5
CMRN5	67.5	25.8

The plot of mitochondrial membrane potential UACC-62(Melanoma) cell line are also depicted

In figure below

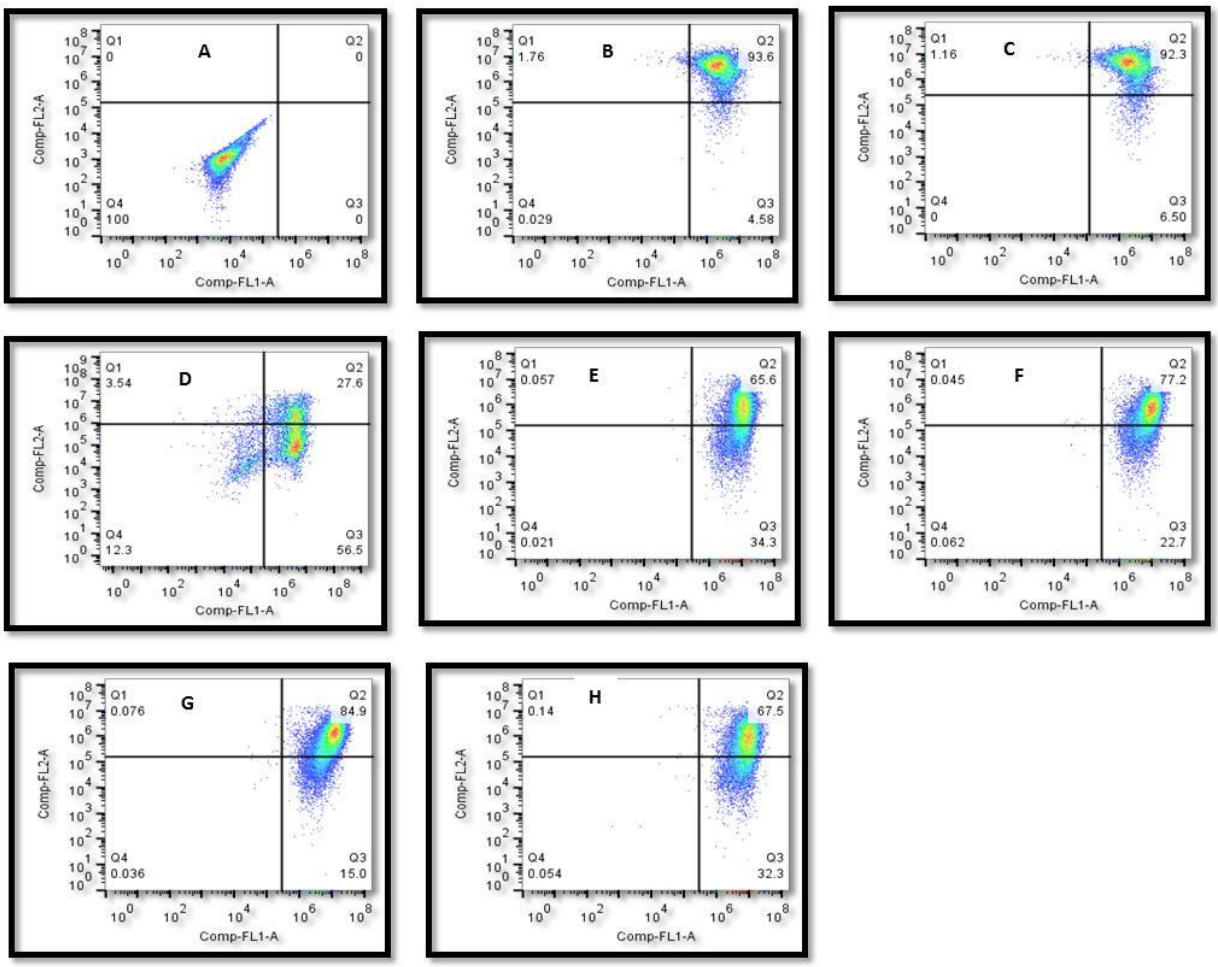

Figure 31: Plot of mitochondrial membrane potential.

Apoptotic cells (%) are localized in the lower right quadrant (Q3) and the stained alive cells are located in the high right quadrant (Q2). (A)Untreated unstained Cells, (B) Untreated stained cells, (C) Cells treated with 50 µg/ml DMSO, (D) Cells treated with 6 µg/mL Doxorubicin, Cells treated with 50 µg/mL of (E) **CMRN1**, (F) **CMRN2**, (G) **CMRN4**, (H) **CMRN5**.

4.7.4 Apoptosis detection using caspase-3 kit

The caspase-3 kit successfully detected apoptosis by identifying the activation of caspases in treated UACC-62 (Melanoma) cells. As mentioned, these caspases cleave several proteins, resulting in cell death, particularly since the cleavage of these proteins causes cell structure (and hence function) disruption. Caspase-3 activity is known to be a common effector molecule in most apoptotic pathways (Jeong *et al*., 2007).

Using the caspase-3 kit, the UACC-62 (melanoma) cells were first successfully treated and stained with PE-conjugate polyclonal active caspase-3 antibody. The analyzed flow cytometer data is represented in Figure 32. In the treated cells, coumarin compounds induced a significant percentage of caspase-3 activity. The most significant caspase-3 activity was observed for

compound **CMRN4** (97.64%), (Table 17) while considerable activity was found to be exhibited by compounds **CMRN1, CMRN2** and **CMRN5** (70.64, 74.24, and 77.84%). The background caspase-3 activity by DMSO (2.06%) was excluded from all reported finding. The doxorubicin (6 µg/mL) control induced a caspase-3 activity of 97.74%. This validated the caspase-3 results for all samples.

Table 17: **Caspase-3 activity of coumarins CMRN1, CMRN2, CMRN4, CMRN5**

Treatment	Caspase negative PE-A-	Caspase positive PE-A+
Negative control	97.9	2.06
Doxorubicin	0.21	97.74
CMRN1	27.3	70.64
CMRN2	23.7	74.24
CMRN4	0.25	97.64
CMRN5	20.1	77.84

The Histogram of caspase activity of UACC-62(Melanoma) cell line are also presented in the below.

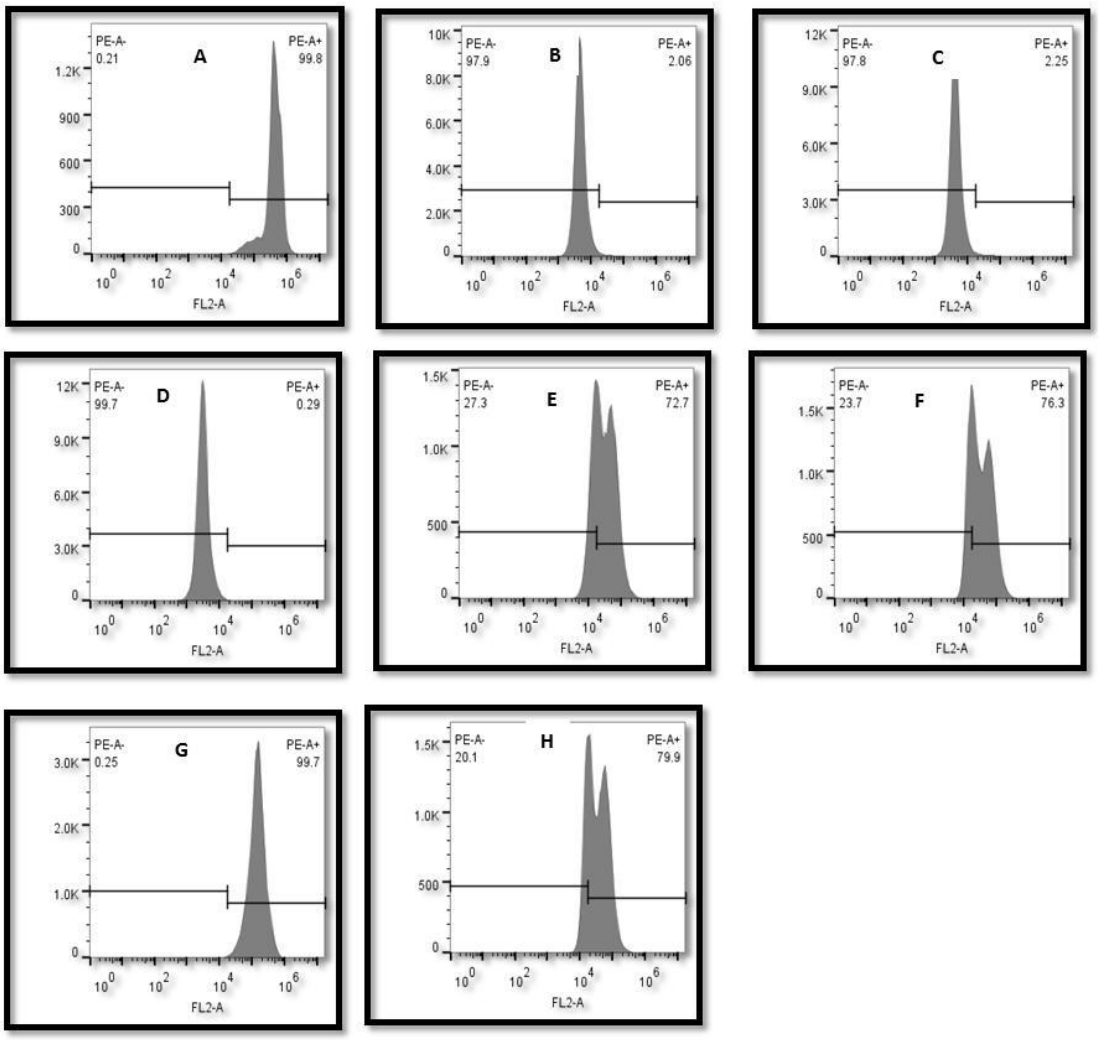

Figure 32: Histogram of caspase activity, Apoptotic cells (%) are localized at the right PE-A+. (A) Cells were treated with 6 µg/ml of doxorubicin, (B) 50 µg/ml DMSO, (C) Untreated stained cells, (D) Untreated unstained cells, Cells treated with 50 µg/ml of (E) CMRN1, (F) CMRN2, (G) CMRN4, (H) CMRN5.

4.8 Safety Assay of coumarins CMRN1- CMRN7

4.8.1 Ames Assay

The mutant frequency caused by the synthetized coumarin compounds in *S. typhimurium* TA 98, TA 100 is shown in Table 18. The mutant frequency was expressed as a fraction of the quotient of the number of revertant colonies divided by the number of colonies in the negative control sample. Mutant frequency greater than two was considered mutagenic. This study indicated that the coumarin compounds had no mutagenic activity against *S. typhimurium* TA 98, TA100 strain (Table 18). None of the tested synthetized compounds induced any significant increase in the number of revertant colonies in comparison to the control (sodium

azide). Sodium azide (NaN_3), a mutagen, was used to confirm the revertant colonies, if any, in the experiment. In this experiment, it was observed that an increase in the number of revertant colonies was in direct proportion to the concentration. A plates showing the revertant colonies of *S. typhimurium* TA 98, TA 100 is shown in Figure 33.

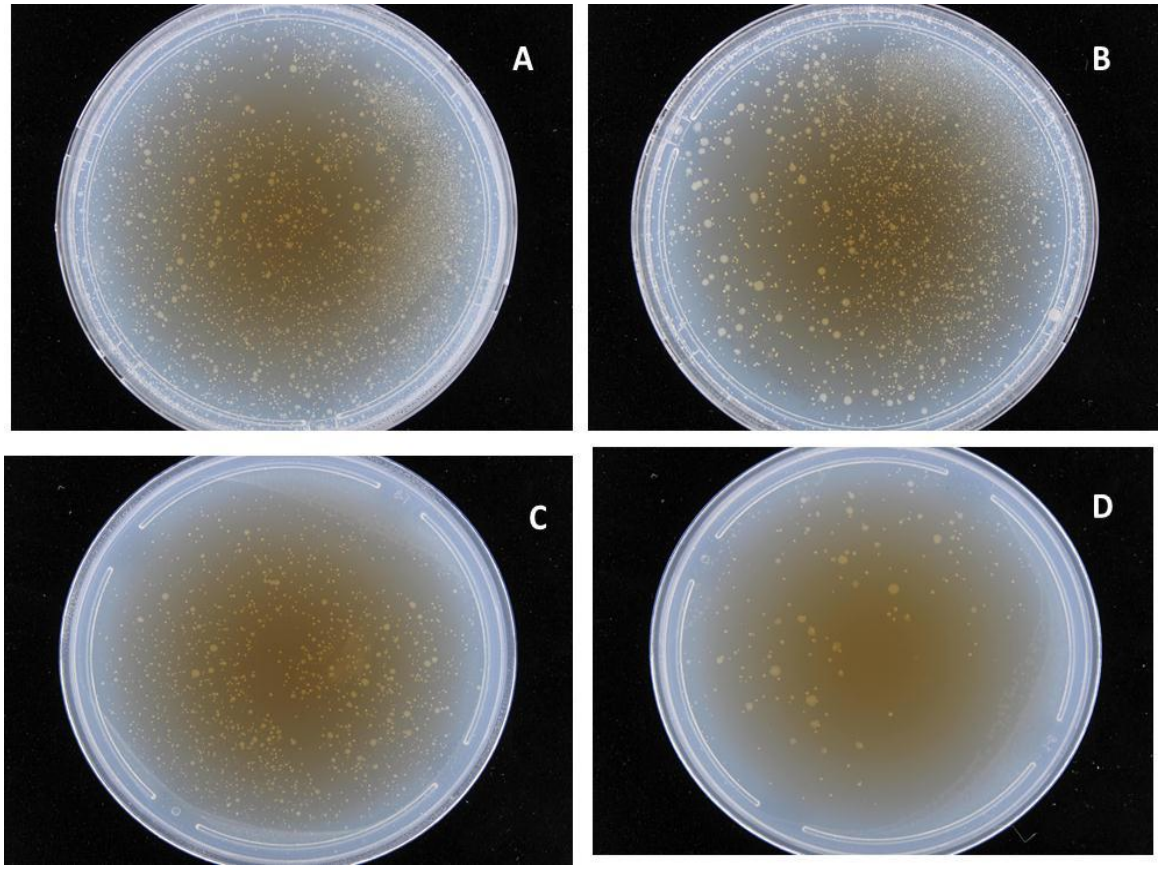

Figure 33: (A) Plate with T 100 showing revertant colonies, (B) Plate with TA98 showing revertant colonies, (C) Plate showing revertant colonies of compound CMRN2 against TA100 at 1 mg/mL, (D) Plate showing revertant colonies of compound CMRN4 at 1 mg/ml against TA98.

Table 18: Mutagenic response to *S. typhimurium* strain TA98 and TA 100

Compound	Mutant frequency of revertants at different concentrations (µg/mL)				
	5	10	20	100	1000
CMRN1	na	0.470 ±0.045	na	0.577 ±0.037	0.613 ±0.031
		0.350 ±0.30		0.352 ±0.052	0.458 ±0.017
CMRN2	na	0.549 ±0.21	na	0.565 ±0.155	0.560 ±0.421
		0.361 ±0.564		0.381 ±0.235	0.390 ±0.321
CMRN3	na	0.719 ±0.562	na	0.720 ±0.124	0.725 ±0.126
		0.597 ±0.201		0. 615 ±0.414	0.623 ±0.215
CMRN4	na	0.60 ±0.120	na	0.78 ±0.343	0.80 ±0.222
		0.462 ±0.100		0.470 ±0.320	0.486 ±0.018
CMRN5	na	0.312 ±0.029	na	0.6 ±0.016	0.720 ±0.027
		0.280 ±0.138		0.552 ±0.020	0.635 ±0.014
CMRN6	na	0.193 ±0.021	na	0.278 ±0.036	0.345 ±0.018
		0		0	0.230 ±0.24
CMRN7	na	0.04 ±0.360	na	0.05 ±0.423	0.16 ±0.100
		0		0	0.123 ±0.210
Sodium Azide	1.034 ±0.48	2.026 ±0.600	3.786 ±0.21	na	Na
	1.059 ±0.21	2.153 ±0.341	4.20 ±0.200	na	Na

Values are expressed as mean (±SD), n=3, in black represent TA100 and in red indicate TA 98, na= not applicable, 0= no activity.

4.8.2 Brine Shrimp Assay

The brine shrimp lethality assay preliminarily assessed the toxicity of the compounds. In addition, the method was found to be rapid, simple and reproducible. According to Meyer *et al*, (1982) for a test to be considered not lethal, it has to demostrate the brine shrimp death of lower than 50%. This study, found that the synthetized coumarins derivatives had less toxicity effect against *Artemia* sp (Table 19) at 1, 2, 3, 4, 20, 24 h and were consider not lethal. The analyses were performed in triplicate at the concentration of 500, 100, and 10 µg/ml and the results were expressed as mean (±SD).

Table 19: Table showing the percentage death of Brine shrimp

Compound	Concentration (µg/mL)	% Death (hr)					
		1	2	3	4	20	24
CMRN1	10	0	0	0	0	0	0
	100	0	0	0	0	0	6 ±0.57
	500	0	0	0	0	0	**41 ±2.08**
CMRN2	10	0	0	0	0	0	0
	100	0	0	0	0	0	3 ±0.57
	500	0	0	0	0	0	**32 ±1.52**
CMRN3	10	0	0	0	0	0	0
	100	0	0	0	0	0	0
	500	0	0	0	0	0	**23 ±0.5**
CMRN4	10	0	0	0	0	0	0
	100	0	0	0	0	0	6 **±1.15**
	500	0	0	0	0	0	**37 ±1.55**
CMRN5	10	0	0	0	0	0	0
	100	0	0	0	0	0	3 ±0.57
	500	0	0	0	0	0	**14 ±1.52**
CMRN6	10	0	0	0	0	0	0
	100	0	0	0	0	0	0
	500	0	0	0	0	0	**33 ±0.55**
CMRN7	10	0	0	0	0	0	0
	100	0	0	0	0	0	0
	500	0	0	0	0	0	**0**

Values are expressed as mean (±SD), n=3

5. DISCUSSION

The present study describes the synthesis and characterization of 3-mono/dibromoacetyl-6-halogenated coumarin analogues **CMRN1 - CMRN6**, and thiazolyl bromo coummarin **CMRN7.** These coumarin analogues were evaluated for anti-mosquito, antibacterial, antifungal, anti-inflammatory, and antioxidant. Their safe use was established by using ames mutagenicity, the brine shrimp assay, and anti-cancer properties.

5.1 Anti-mosquito activity of compounds CMRN1 - CMRN7

In the evaluation of larvicidal, adulticidal and repellent effects against the malaria vector *A. arabiensis*, five of the seven coumarin analogues exhibited 100% larvicidal effect, which was comparable to the positive control, Temephos. This promising activity is attributed to the presence of electron withdrawing halogen atoms (bromine and chlorine) on acetyl group at the third and sixth position of the coumarin nucleus (Figure 2). Larvicidal and ovicidal activity of 4- methyl-7-hydroxy coumarin derivate against vectors *A. aegypti* and *C. quinquefasciatus* have also been attributed to bromine atoms present at C-5 and C-8 positions (Deshmukh *et al*., 2008). Coumarin and mainly furanocoumarins can alter the detoxication capability of an organism, by reversibly or irreversibly inhibiting cytochrome P450 detoxication enzymes (Letteron *et al*., 1986, Neal and Wu, 1994).

A short exposure to freshly applied coumarin analogues knocked down mosquitoes, but they recovered in the next 24 h. An insect immobilization effect of coumarins has been reported for other insects (Moreira *et al*., 2007, Nicholson and Zhang, 1995), and a slowly developing paralysis eventually leading to death has been a major feature of insect poisoning by coumarins such as surangin B (Nicholson and Zhang, 1995). Bioenergetic disruption of muscle has been determined as a prominent mechanism underlying the insecticidal action of surangin B (Zheng *et al*., 1998). However, knockdown on mosquitoes resulting from short exposure to synthetized coumarins **CMRN1 - CMRN7** was fast (2 min or less) and reversible, and no adulticidal effects were detected from 60 min exposure. This may be partly explained by differences in experimental design, since topical application or injection were common procedures in previous reports on insecticidal properties (Moreira *et al*., 2007, Nicholson and Zhang, 1995). Besides mortality, coumarins may exert other negative effects on insect populations such as decreasing their reproductive potential (Kaur and Rup, 2002).

5.2 Antimicrobial activity of CMRN1 - CMRN7

The antimicrobial activities of the seven synthetized coumarin compounds were screened by determining their minimum inhibitory concentrations (MICs) using the disc diffusion method. The antimicrobial were measured against ten strains of gram-positive and gram-negative bacteria, three yeast cultures and two fungus species (see Tables 6 and 7).

The results shown in Table 6, revealed that compounds **CMRN1, CMRN2, CMRN4** and **CMRN7**, are inhibitory on the growth of some of the tested strains. Thus, these compounds exhibit antibacterial potencies, which affect the growth of gram-positive and gram-negative bacteria used. Among the active coumarin compounds, compounds **CMRN1** and **CMRN2** induced bacterial growth inhibition in most of the tested microorganisms. The exceptions were for *B. stearothermophilus* and *K. pneumoniae*. The MIC was defined as the lowest concentration of a compound that inhibits bacterial growth Table 8. Compound **CMRN1** showed an inhibition zone of 10 mm around *B. cereus, B. coagulans,* and *S. faecalis*, (Table 6) with a minimum inhibitory concentration of 0.75 mg/ml (Table 8). Compound **CMRN2** induced an inhibition zone of 10 mm around *B. cereus and B.coagulans*, 11 mm against *S. aureus* and *S. faecalis* (Table 6), with an MIC of 0.75 mg/ml (Table 8). The inhibition zone for compound **CMRN4** was 8 mm and 10 mm while the MIC was 1.5 mg/ml against *M. luteus* and *S. aureus.*

The antifungal activity of the coumarin derivatives (**CMRN1-CMRN7**) was carried out using 3 yeast cultures, *C. albicans, C. utilis, S. cerevisiae* and two species of fungi, *A. flavus* and *A. niger* (Table 7). The results showed that compound **CMRN4** exhibited slight inhibiton effect against the three tested yeast species; compound **CMRN7** displayed a slight activity against *C. albicans* and *C. utilis*, whilst compound **CMRN2** displayed a slight activity against *S. cerevisiae.* Compounds **CMRN2, CMRN4,** and **CMRN7** were not antifungal against *A. niger* and *A. flavus.*

These antibacterial and antifungal results of all seven synthetized coumarin **CMRN1 - CMRN7** had low effects on the bacterial and fungal species tested in comparison to the controls, Ciprofloxacin and Amphotericin (at 3 mg/ml, same as compounds). Venugopala *et al,* (2013) reported that coumarin has low antibacterial activity, with the exception of compounds that possess long-chain hydrocarbon substitutions such as ammoresinol and andostruthin. These substituents result in coumarins displaying activity against gram-positive bacteria such as *B. megaterium, M. lysodeikticus,* and *S. aureus*.

Brahmbhatt *et al.* (2010) also Synthetized 3-[4-(3-aryl-1-phenyl-1*H*-pyrazol-4-yl)-6-aryl-pyridin-2-yl]and4-methyl-3-phenyl-6-(4-(3-aryl-1-phenyl-1 *H*-pyrazol-4-yl)-6-aryl-pyridin-2-yl] coumarin derivative and screened for antibacterial (gram-negative, *E. coli* and gram-positive, *B. utillis*) and antifungal (*C. albicans*) activity. Streptomycin was used in the antimicrobial tests as an antibacterial standard, while cotromazole was used as an antifungal standard. Both standards were used at 1000 μg/ml. Brahmbhatt *et al*. (2010) showed that the synthesized compounds had a much lower antimicrobial and antifungal activity compared to the standards. It has been suggested that antibacterial activity is attributed to bromoacetyl being present on carbon three because this substituent enhances binding to the bacterial enzyme, there by inhibiting growth.

Comparing the anti-bacterial activities of the synthesized coumarins, compounds **CMRN1** and **CMRN2** showed the highest anti-bacterial activity against *B. cereus, B. coagulans, S. faecalis, S. aureus* and *E. coli* (Table 8). This enhanced anti-bacterial activity could be ascribed to the presence of bromine in the acetyl group at position C3 of the coumarin nucleus (Figure 2). This observation is based on the fact that compounds **CMRN3** and **CMRN6** have bromine and chlorine atoms at position C6, respectively but they do not have a bromoacetyl group at position C3 of the coumarin nucleus. Compounds **CMRN4** and **CMRN7** have bromine at position C6 of the coumarin nucleus as well as a bromoacetyl and aminothiazol groups respectively, at position C3. These structural differences contributed to the reduced activity of the compounds. The absence of bromoacetyl group at the postion C3 of compounds **CMRN3** and **CMRN6** makes them to have no activity although they have bromine and chlorine atoms at position C6, respectively. In the case of compound **CMRN5,** although it has a bromoacetyl group at position C3 of the coumarin nucleus, but it has no activity. This is due to the chlorine atom at position C6.

5.3 Anti-inflammatory Activity of CMRN1 - CMRN7

Anti-inflammatory activity of each synthesized coumarin compound was studied using the Lipoxygenase Inhibitor assay. This study shows that the synthesized compounds **CMRN1, CMRN2, CMRN4,** and **CMRN5** exhibited moderate anti-inflammatory activity compared to the standard drug 15-Lipoxigenase (Table 9). The rest of the compounds exhibited weak anti-inflammatory responses, which is less then 50% of enzyme inhibition. Bolakatti *et al*. (2007) synthesized a novel sequence of coumarinyl Mannich based by reacting 3-acetyl coumarin with different substituted secondary amines in the presence of paraformaldehyde. Among the compound tested 3-(3-(diethylamino) propanoyl)-2*H*- chromen-2-one and 3-(3-(piperidine-1-

yl) propanoyl)-2*H*-chromen-2-one demonstrated 63.1 and 66.7% inhibition when compared with the standard drug diclofenac 68.8%.

Kontogiorgis and Hadjipavlou-Litina, (2005) reported that coumarins possess anti-inflammatory activity which include the inhibition of matrix metalloproteinase actions, phagocytosis, as well as the transcription factors that are used in the expression of inflammatory protein mRNAs. These authors have also suggested that coumarins also inhibit the expression of mRNA and the activities of enzymes, and cyclooxygenase-2 (COX-2).

The high anti-inflammatory activity (60% - 63%) (Table 9) (section 4.4) of the compounds **CMRN1, CMRN2, CMRN4** and **CMRN5** may be ascribed to the presence of bromoacetyl group at position C3 of the coumarin nucleus (Figure 2) because Compounds **CMRN3** and **CMRN6** which do not have bromoactetyl group at position C3 showed low activity (43% - 45%). Likewise, compound **CMRN7** which has a 2-aminothiazol group at position C3 of the coumarin nucleus showed low activity (48%).

5.4 Antioxidant activity of CMRN1 - CMRN7

The results of the DPPH radical scavenging activities of the coumarin derivatives are shown in Table 17. The free radical scavenging activities of coumarin derivatives are related to the number and position of the hydroxyl group on the benzenoid ring of the coumarin system. However the anti-oxidant activity has been mainly attributed to the presence of free hydroxyl groups, high anti-oxidant effect has been discovered for compounds where these groups are acetylated or benzoylated (Qi *et al*., 2006, Wang *et al*., 2009) .

These results showed that compounds **CMRN1** (56%), **CMRN4** (86%) , **CMRN7** (61%) demonstrated antioxidant activity , this can be explained by considering the structural relationships amongst the coumarin analogues. Compounds **CMRN1** and **CMRN4**, which have a bromoacetyl group at position C3 of the coumarin nucleus and compound **CMRN7** which has a 2- aminothiazol group on position C3 of the coumarin nucleus exhibit over 50% of antioxidant activity.

However considering the relative antioxidant activities **CMRN2**, **CMRN3**, **CMRN5** and **CMRN6** the lack of bromoacetyl group at position C3 reduced the antioxidant activity considerably in compounds **CMRN2**, the dibromoacetyl group at position C3 futher reduced antioxidant activity (23%, the least activity) (Figure 2). Although **CMRN2** has bromine position C6 like **CMRN4**, its activity was not enhanced significantly like **CMRN4**. Therefore,

the presence bromoacetyl group at position C3 is a critical pharmacophore in all the synthesized coumarins.

5.5 Cytotoxicity Assay of CMRN1 - CMRN7

The cytotoxicity activity was carried out according to (Mossman, 1983), the cytotoxic standard used in the MTT cytotoxicity assay was doxorubicin. The compounds **CMRN1 -CMRN7** and standard doxorubicin were screened against the following cell lines, UACC-62 (Melanoma), MCF-7 (breast cancer) and PBMC (Peripheral blood mononuclear cell) cell line.

From the screening results, it was found that compounds, **CMRN1**, **CMRN2**, **CMRN4**, and **CMRN5** were most effective against UACC-62 (Melanoma) and MCF-7 cells. These synthetized compounds showed a significant inhibitory activity (above 70% cell inhibition) at 50 μg/mL as the standard used (see Table 11 and 12). Compounds **CMRN3**, **CMRN6** and **CMRN7** did not display a significant inhibitory activity on these cell types under the same used dose concentration and treatment times. Lopez-Gonzalez *et al*. (2004) reported that the cytotoxicity effects induced by coumarin compounds rely on the cell type and the concentration tested.

The inhibitory concentration (IC_{50}) that killed UACC-62 (Melanoma) and MCF-7 cancer cell lines are shown in Table 14. It was observed that **CMRN4** and **CMRN5** displayed strong inhibitory effects against these cell lines with IC_{50} values of 7.282 and 1.772 μg/mL respectively. The inhibitory activities of **CMRN1**, **CMRN2** and **CMRN4** were in an IC_{50} range of between 15.39 to 30.93 μg/ml against both cell lines. In a study, Kini *et al*. (2012) tested the cytotoxic activity of synthesized benzothiazole derivates against MCF-7 cell lines using the MTT method and found that the cytotoxicity of this derivate increased after 48 h (this means that the response was time dependent).

All the active compounds contain bromoacetyl or dibromoacetyl groups at position C3. Compounds **CMRN4** and **CMRN5** showed significant anticancer activity this is attributable to the presence of chlorine and bromine at position C6 of their structures.

Riveiro *et al*. (2008) and Pan *et al*. (2011) have shown that studying the anticancer potential of coumarins can reveal their mechanism of action during apoptosis and their role in anti-angiogenesis.

5.5.1 Morphological changes in cell during apoptosis

Apoptosis, also known as programmed cell death, is a major form of cell death. It generally causes a change in cell morphology by inducing cell blebbing, cell shrinkage, chromatin condensation and DNA fragmentation (Green, 2011).

In the MTT assay, it was found that coumarins significantly suppressed UACC-62 (Melanoma) viability. It is, thus, possible for these coumarin compounds to induce morphological changes that are characteristic of apoptosis. The results obtained in Figure 30 show that the untreated UACC-62 (Melanoma) cell and the DMSO negative control appear to have normal cell features. On treatment with coumarin compounds, this cell line was found to exhibit characteristic feature of apoptosis, such as cell shrinkage and detachment.

(Häcker, 2000) has also indicated that a striking feature of apoptosis is when cell undergoing apoptosis round up and severe all contacts with other cells in tissues.

5.5.2 Membrane change in cell during apoptosis

The Annexin V binding assay in combination with PI vital staining is a quick method for detection of early apoptosis (Sgonc and Gruber, 1998).

The redistribution of phosphatidylserine (PS) to the external side of the cell membrane occurs due to perturbation in the cellular membrane. This is regarded as one of the biochemical features of apoptosis (Bratton *et al*., 1997). Annexin V, a recombinant phosphatidylserine binding protein, interacts mainly with phosphatidylserine residues, and it is used in conjunction with fluorochromes for detecting apoptosis using flow cytometry (Arur *et al*., 2003; Huerta *et al*., 2007).

In this investigation, it was found that compounds **CMRN1, CMRN2, CMRN4, CMRN5** inhibited UACC-62 (Melanoma) by inducing apoptosis. The percentage of apoptosis quantified by flow cytometer analysis, showed that the vast majority of UACC-62 (Melanoma) cells in the untreated setup were healthy and remained unstained with Annexin V, while cells treated with coumarin compounds and doxorubicin, increased in the Annexin positive cells (**CMRN1, CMRN2, CMRN4, CMRN5,** and doxorubicin), inducing apoptosis Table 15.

It was found that the cell population and the synthized coumarin subjected to Annexin V underwent early apoptosis (13.1-15% of cell population) or cell death with an exception of **CMRN4**. Compound **CMRN4** induced a significant percentage of late apoptosis (67.9%), while **CMRN1**, **CMRN2** and **CMRN5** induced late apoptosis by 25.4 - 28.5%.

5.5.3 Mitochondria membrane potential in cell during apoptosis

Mitochondria play an essential role in inducing apoptosis triggered chemical agents. The mitochondrial responses during apoptosis include the release of cytochrome C in the cytosol, presumably through an outer membrane pore (transition permeability pore) (Goel *et al*., 2007).

In the cytosol, this cytochrome C binds to Apaf-1 (Apoptotic protease activating factor 1), allowing caspase-9 and apoptosome complex formation. This results in caspase-3 activation and the execution of cell death (Green and Reed, 1998).

In the present study, the mitochondrial membrane potential of apoptotic cells was determined by flow cytometry. It was found that they was a decrease in membrane potential (Table16) when the cells were treated with **CMRN1** (27.8% apoptosis), **CMRN2** (16.2% apoptosis), **CMRN4** (8.5% apoptosis) and **CMRN5** (25.8% apoptosis). It's known that changes in mitochondrial membrane potential have originally been postulated to be early, obligate events in the apoptotic signaling pathway. The nuclear features of apoptosis are preceded by changes in mitochondrial structure and membrane potential resulting in apoptosis (Ly *et al*., 2003). Chuang *et al*. (2007) reported that factors such as the production of reactive oxygen species, Ca^{2+}concentrations, and dose-dependently-induced depolarization of mitochondrial membrane potential, affects the apoptotic potential of coumarins. Furthermore, Chuang *et al*. (2007) found that coumarin treatment has the potential to decrease G0/G1-associated proteins expression, leading to G0/G1 arrest and an induction of anti-apoptotic proteins (Bcl-2 and Bcl-xL), as well as an increasein the expression of pro-apoptotic protein, Bax. In that study, coumarin decreased mitochondrial membrane potential and promoted the release of cytochrome C and thus activation of caspase-3, before leading to apoptosis (Chuang *et al*., 2007).

5.5.4 Apoptosis detection using Caspase 3 kit

Caspase-3 can acts as an indicator for the induction of apoptosis since active caspase-3 is present in viable cells in both extrinsic and intrinsic apoptotic pathways (Ghavami *et al*., 2009).

In mammalian cells, caspase-3 is presumed to be a major executor caspase which cleaves the majority of cellular substrates. Caspase-3 can be processed and activated by initiator caspases or by executioner caspases such as caspase-7 and caspase-3 (Pop and Salvesen, 2009).

CMRN4 exhibited significant caspase-3 inducing ability of 97.64% (Table 17) when compare to the result obtained for the positive control, doxorubicin 97.74%. **CMRN1, CMRN2** and **CMRN5** exhibited a high caspase-3 inducing potential that ranged between 70.64 - 77.84%.

5.6 Safety of coumarin derivatives (CMRN1 - CMRN7)

Mutations are an important early step in carcinogenesis. In a previous study, a short-term genetic test called the *Salmonella*/reversion assay was successfully used to detect the presence of mutagens/carcinogens and antimutagenes/ anticarcinigens (Ben Rhouma *et al*., 2012).

In principle, the greater the amount of revertant colonies, the better frequency of mutations the compound cause. Maron and Ames (1983) reported that, mutagenic potential can be expected if the mutant frequency is greater than 2. However other possibilities by these authors indicate that a probable mutagenic potential can be assumed if the mutant frequency ranges between 1.7 and 1.9; and that no mutagenic potential can be considered if the mutant frequency is lower than 1.6. From the results presented in Table 18, compounds **CMRN1-CMRN7** were consider to be non mutagenic. Sodium azide, the positive control, exhibited a mutagenic potential with increasing concentration. Furthermore, with an increase in concentration, there was an increase in the number of revertant colonies.

Finn *et al*. (2002) screened some selected coumarin compounds and found that their mutagenic potential using the Ames test was negative against the tested colonies and that this was probably attributed to either base-pair substitution or frame shift mutation. This indicated that the metabolites were also non-mutagenic.

In this study, the Brine shrimp larvae (*Artemia salina*) were hatched in sea water for 24-48 h prior to being used in the test. 5 mL sea water and ten brine shrimp were added to each vial and treated with 500 µg/mL of the coumarin compounds. The dead ones were counted after 24h (Meyer *et al*., 1982). According to Meyer *et al*. (1982), the compound has to reveal brine shrimp death of less than 50% in order for a test compound to be considered not lethal.

It was noticed in the present study that at the concentration of 500 µg/ml, coumarin compounds did not show significant toxicity effects on larvae Table 19. In a study of Morabito *et al*. (2010), the author reported that when 4-methyl coumarin was screened for its toxicity against the brine shrimp, Among the tested coumarin, none of them affected the viability of the brine shrimp significantly, even at a concentration of up to 500 µM.

6. CONCLUSION

In this study, seven halogenated coumarins (**CMRN1** – **CMRN7**) were synthesized in good yields (87-98%). Purity of the compounds was ascertained by HPLC and it was found to be over 99%. Characterization of the compounds was achieved by GC-MS analysis and molecular mass of each compound was in good agreement with calculated mass.

This study have shown that compounds **CMRN1, CMRN2**, **CMRN4, CMRN5** and **CMRN7** are potential larvicidal agents. However, they did not show appreciable adulticidal activity against mosquitoes, the repellence effect of this compounds should be further explored because the compounds knocked down mosquitoes within two minute of exposure and thereafter they recovered.

The antimicrobial screening results revealed that the compounds **CMRN1** and **CMRN2**, showed antibacterial activity against both Gram-positive and Gram-negative bacteria used. Based on the results, functional substitutions on the benzene ring as well as the acetyl group of the coumarin nucleus affect the activity of the coumarin compound negatively or positively. For example, mono-bromo substitution of the acetyl group increased antimicrobial activity whereas bromo or chloro substitution at position C6 of the coumarin nucleus reduced activity.

Compounds **CMRN1, CMRN2, CMRN4,** and **CMRN5** exhibited moderate anti-inflammatory activity compare to the standard drug 15-Lopoxigenase. The antioxidant results indicated that compounds **CMRN1, CMRN4, CMRN7** showed free radical-scavenging activities. The coumarin derivatives **CMRN1-CMRN7** showed no toxicity against Brine shrimp larvae (at 500 µL) and PBMC cells. These compounds were also found to be non- mutagenic against the *S. typhimurium* cultures TA100 and TA98.

The *in vitro* cytotoxicity screening of all the compounds revealed that compounds **CMRN1, CMRN2, CMRN4, CMRN5** were more active against UACC-62 (Melanoma) and MCF-7 (Breast) cancer cells than compounds **CMRN3, CMRN6** and **CMRN7**. These compounds were found to induce apoptosis by causing the morphological changes, membrane change, distruption of the mitochondria membrane and most importantly, they were found to activate the caspase-3 and induce apoptosis against UACC-62 (Melanoma).

Finally, this study provides new information, with respect to the potential use of coumarins compounds in producing new larvicidal agents. Compounds **CMRN1, CMRN2**, **CMRN4, CMRN5** and **CMRN7** showed larvicidal activity close to 100% within 24 h. Furthermore, the

synthetic coumarins have anti-cancer activity as shown by the death of UACC-62 (Melanoma) and MCF-7 (Breast) cancer cells, and the protection of non caner cells, for exemple, PBMC-(normal) cells. Compounds **CMRN1, CMRN2, CMRN4, CMRN5** killed cancer cell by apoptosis in UACC-62 (Melanoma) which is a hallmark of cancer. This property as well as the inflammatory property qualify these coumarin compounds as leads for anticancer drug development.

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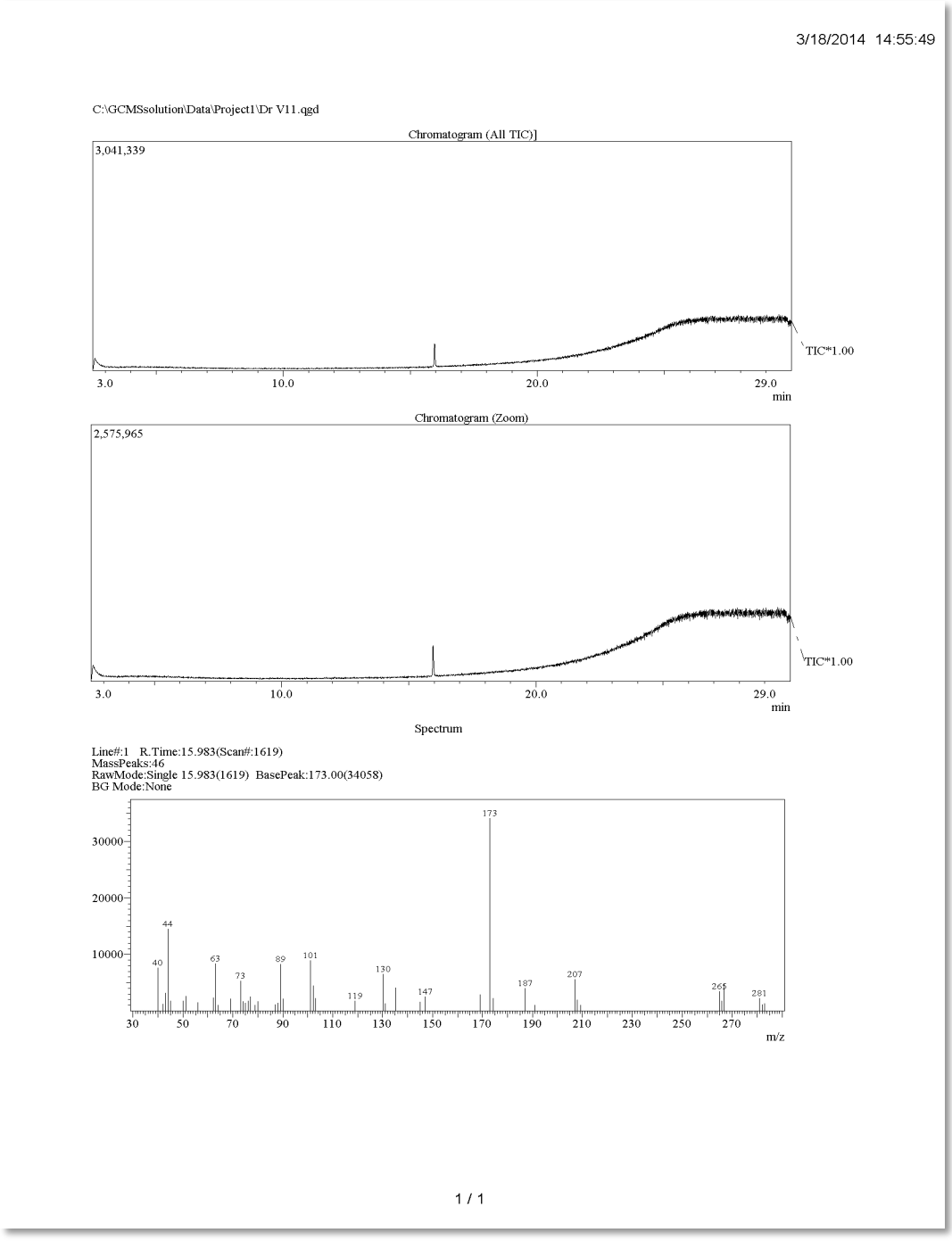
APPENDIX

Gas chromatography mass spectrometry of halogenated coumarins CMRN1, CMRN3, CMRN4, CMRN6 and CMRN7.

3-(2-Bromoacetyl)-2*H*-chromen-2-one (CMRN1)



3-Acetyl-6-bromo-2*H*-chromen-2-one (CMRN3)

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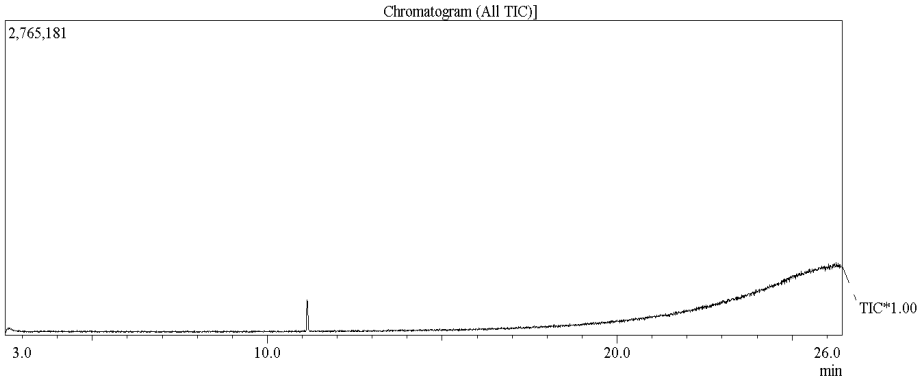

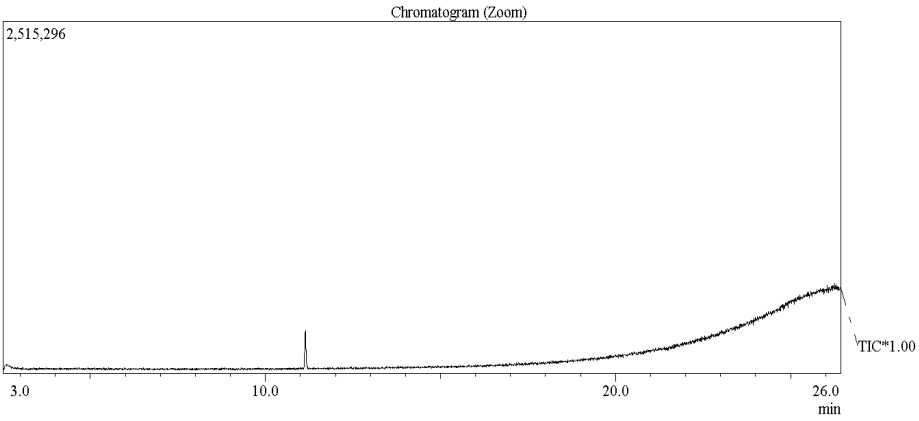

Spectrum

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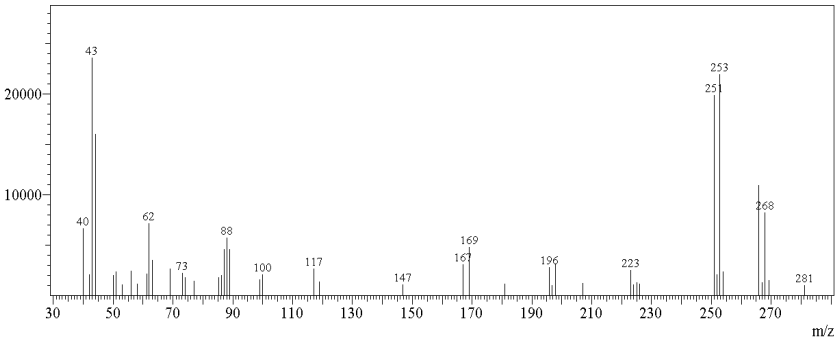
RawMode:Single 11.125(1036) BasePeak:43.05(23615)
BG Mode:None



6-Bromo-3-(2-bromoacetyl)-2*H*-chromen-2-one (CMRN4)

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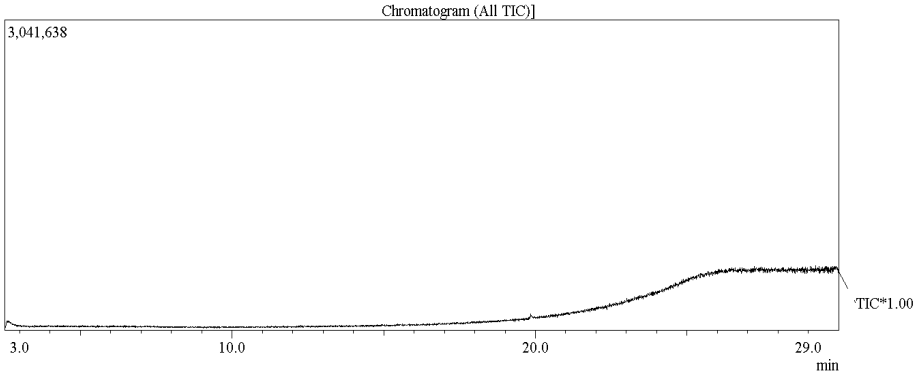

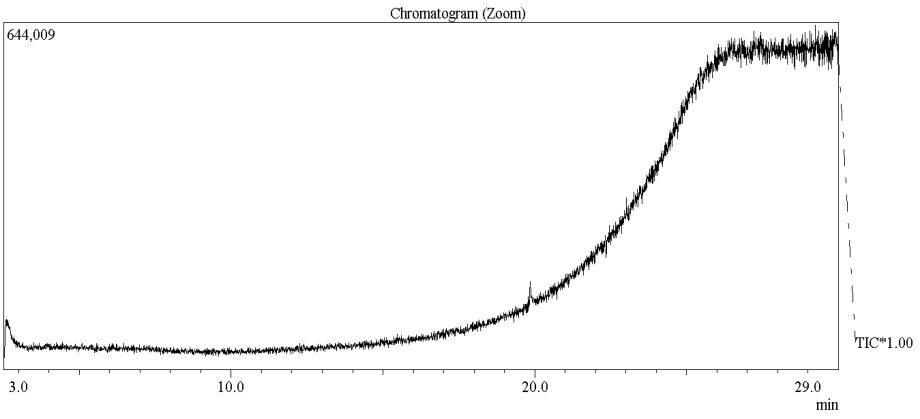

Spectrum

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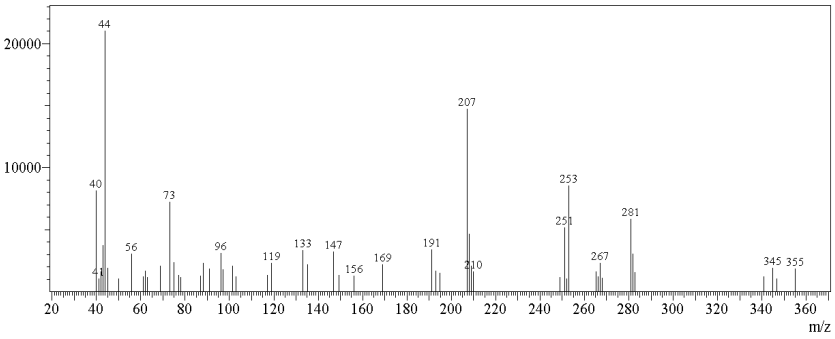

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(3-Acetyl-6-chloro-2*H*-chromen-2-one (CMRN6)

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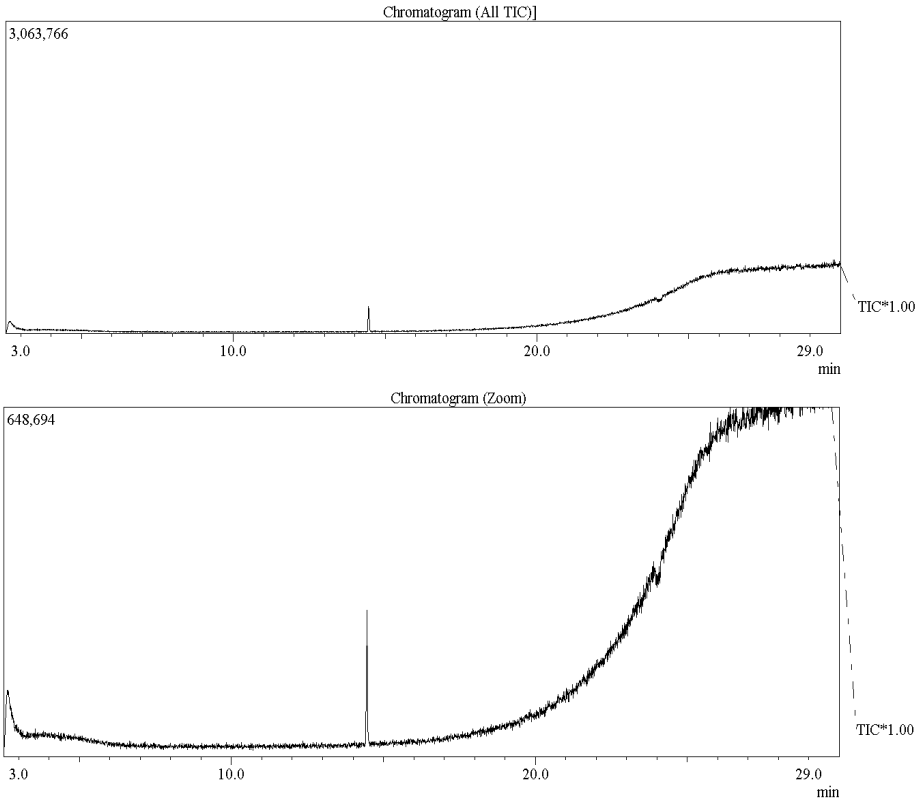

Spectrum

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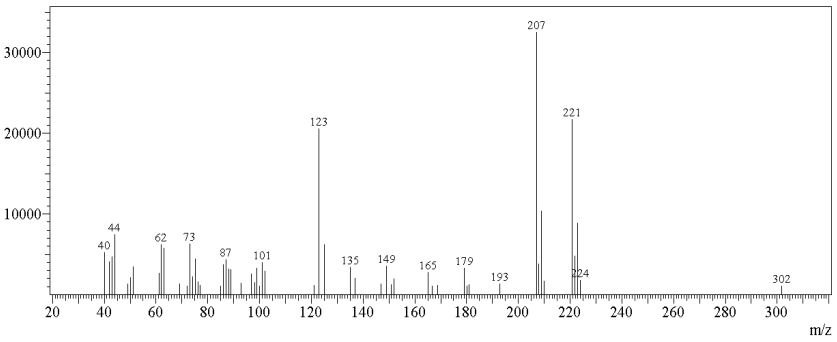

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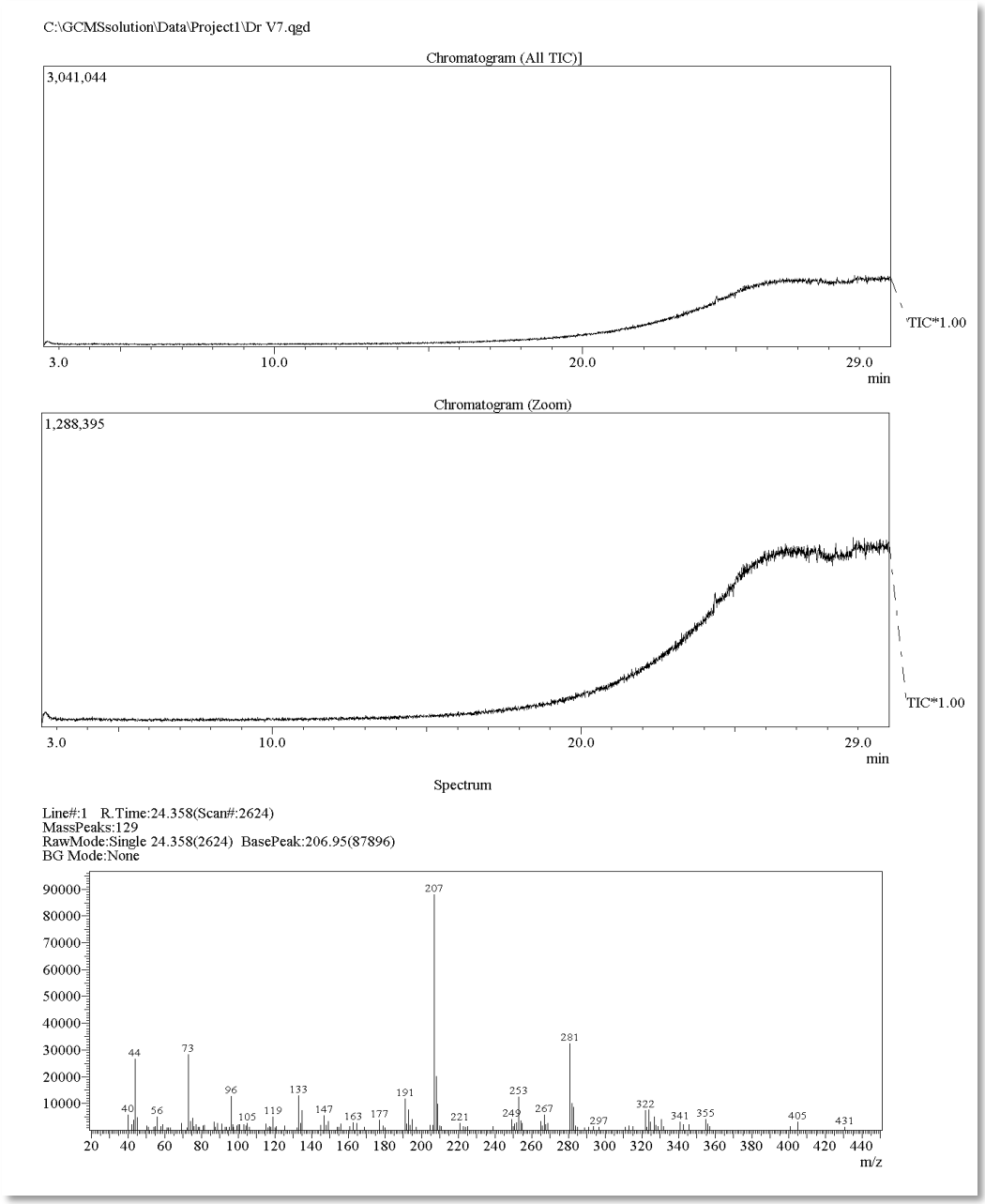
3-(2-Aminothiazol-4-yl)-6-bromo-2*H*-chromen-2-one (CMRN7)



PREPARATIONS OF REAGENTS

Solution and media for ames test

1. 0.5 Mm histidine/ 0.5Mn biotin for top agar

Ingredients	Per 250ml
D-Biotin	30.5 mg
L-Histidine	26.2mg
Double distill water (ddH_20)	250ml

Biotin and histidine were dissolved in warm water and filter sterilised

2. Top Agar

Ingredients	Per 100ml
Bacteriogical Agar	6g
NaCl	5g
Double distill water (ddH_20)	1000ml

3. Vogel-Bonner medium E stock for minimal agar base

Ingredients	Per 1000ml
Warm ddH_20	670ml
$MgSO_4$. $7H_2O$	10g
Citric acid monohydrate	100g
K_2HPO_4	500g
$NaHNH_4PO_4$. H_2O	175g

4. 40% Glucose solution

Ingredients	Per 1000ml
Glucose	400g
ddH_2O	1000ml

5. Minimal glucose agar plates

Ingredients	Per 1000ml
Bacteriological Agar	15g
ddH_2O	930ml
Vogel-Bonner medium E	20ml
40% glucose solution	50ml

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Research Article

Evaluation of Halogenated Coumarins for Antimosquito Properties

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Mosquitoes are the major vectors of parasites and pathogens affecting humans and domestic animals. The widespread development of insecticide resistance and negative environmental effects of most synthetic compounds support an interest in finding and developing alternative products against mosquitoes. Natural coumarins and synthetic coumarin analogues are known for their several pharmacological properties, including being insecticidal. In the present study halogenated coumarins (3-mono/dibromo acetyl, 6-halogenated coumarin analogues) were screened for larvicidal, adulticidal, and repellent properties against *Anopheles arabiensis*, a zoophilic mosquito that is one of the dominant vectors of malaria in Africa. Five compounds exerted 100% larval mortality within 24 h of exposure. All coumarins and halogenated coumarins reversibly knocked down adult mosquitoes but did not kill them after 24 h of exposure. Repellent properties could not be evidenced. Five compounds were considered potential larvicidal agents for further research and development, while adulticidal activity was considered only mild to moderate.

1. Introduction

Mosquitoes are the major vectors of parasites and pathogens that cause malaria, filariasis, dengue fever, yellow fever, Japanese encephalitis, and other fevers affecting humans and domestic animals [1]. Vector control programs mainly use four classes of chemical insecticides: organochlorines, organophosphates, carbamates, and pyrethroids. Bacterial insecticides and insect growth regulators have also become more widely used in recent years. However, use of chemicals on a vast and increasing scale has led to the widespread development of resistance as a result of selection for certain genes [2], and some species have even become resistant to multiple insecticides [3]. Mosquito resistance to at least one insecticide used for malaria control has been identified in 64 countries [3]. Besides, synthetic organic insecticides, such as those currently used to control mosquitoes, affect nontarget organisms and result in negative environmental effects [4]. Thus, there is an interest in the finding and development of alternative antimosquito products.

Coumarins (2*H*-1-benzopyran-2-one) are a class of phenolic substances found as secondary metabolites from plants, bacteria, and fungi, widely used as additives in food, perfumes, cosmetics, pharmaceuticals, optical brighteners, and dispersed fluorescent. Natural coumarins are known for their several pharmacological properties and have been recently reviewed by Venugopala et al. [5], also reported for synthetic coumarin analogues, such as analgesic, anti-inflammatory [6, 7], anticoagulant [8], antibacterial [9–11], antifungal [12], antiviral, anticancer [13], antihypertensive [14], antitubercular [15], antihyperglycemic [16], and antioxidant [17] properties. Warfarin is a synthetic coumarin analogue (known

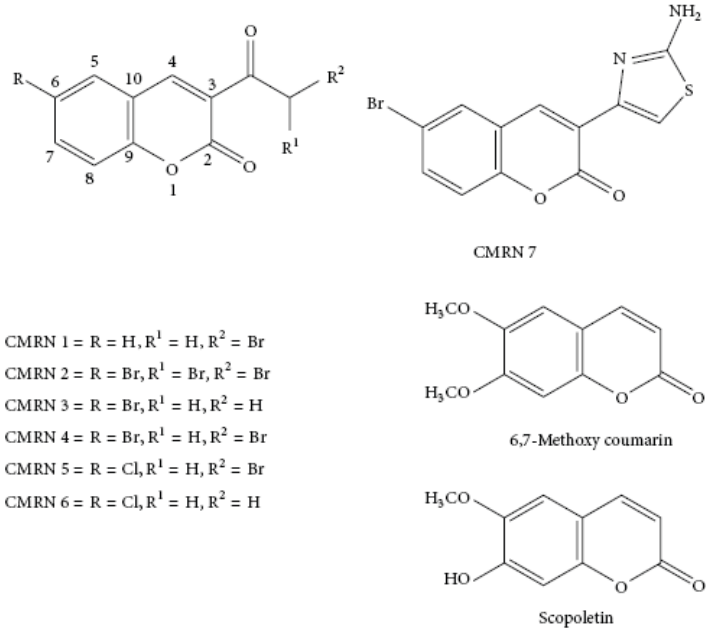

FIGURE 1: Synthetic halogenated coumarin compounds CMRN 1–CMRN 7, 6,7-dimethoxy coumarin, and scopoletin tested for antimosquito properties.

as Coumadin) that is used as an anticoagulant and is commercially available in the market with a trade name Coumadin. Essential oils and solvent extracts of plants containing coumarin have shown promising properties against mosquitoes. For example, coumarin extracted from southernwood (*Artemisia abrotanum* L.) and essential oil of carnation flowers (*Dianthus caryophyllus* L.) exerted a repellent effect against yellow fever mosquitoes (*Aedes aegypti* L.) and ticks (nymphs of *Ixodes ricinus* L.) [18]. Eight coumarin derivatives obtained from hexane extractions of the roots of *Esenbeckia grandiflora* Mart. were effective larvicides against *Ae. aegypti* [19]. The coumarin derivative pachyrrhizine, a compound from tubers of *Neorautanenia mitis*, showed larvicidal and adulticidal activities against *Anopheles gambiae* and *Culex quinquefasciatus* Say that were comparable to deltamethrin and alpha-cypermethrin, two standard mosquitocides [20]. A component of *Tagetes lucida* Cav. hexane extract, 7-methoxy coumarin, showed larvicidal activity against *Ae. aegypti* [21]. The linear furanocoumarin imperatorin and the coumarin osthole extracted from *Cnidium monnieri* (L.) Cusson fruit were effective against larvae of *Culex pipiens pallens* Coquillett and *Ae. aegypti* and against *C. p. pallens* larvae resistant to various insecticides, suggesting that these coumarins and the pyrethroid and organophosphate insecticides do not share a common mode of action or elicit cross-resistance [22]. In this context, and in continuation of our search for novel chemical agents with antimosquito properties [23, 24], in the present study we undertake the screening of halogenated coumarins (3-mono/dibromo acetyl, 6-halogenated coumarin analogues) for larvicidal and repellent properties against *Anopheles arabiensis*, one of the dominant vectors of malaria in Africa [25].

2. Materials and Methods

2.1. Chemicals Tested. The test compounds halogenated coumarin analogues (CMRN 1–CMRN 7) were synthesized as described previously [6, 7, 9–11, 26–28], using chemicals from Aldrich and Merck chemical company without further purification. Compounds 6,7-methoxy coumarin and scopoletin were obtained from Sigma-Aldrich. The products assessed were 3-(2-bromoacetyl)-2*H*-chromen-2-one (CMRN 1) [7, 11] and 6-bromo-3-(2,2-dibromoacetyl)-2*H*-chromen-2-one (CMRN 2) [28]; 3-acetyl-6-bromo-2*H*-chromen-2-one (CMRN 3) [6], 6-bromo-3-(2-bromoacetyl)-2*H*-chromen-2-one (CMRN 4) [9], and 3-(2-bromoacetyl)-6-chloro-2*H*-chromen-2-one (CMRN 5) [10]; 3-acetyl-6-chloro-2*H*-chromen-2-one (CMRN 6) [26]; 3-(2-aminothiazol-4-yl)-6-bromo-2*H*-chromen-2-one (CMRN 7) [27]; 6,7-methoxy coumarin; and scopoletin (Figure 1). The physicochemical characteristics of the halogenated coumarin analogues (CMRN 1–CMRN 7) are summarized in Table 1.

2.2. Larvicidal Activity. The *Anopheles arabiensis* used were from a colonized strain from Zimbabwe which had been reared according to the WHO (1975) guidelines [1] in an insectary simulating the temperature (27.5°C), humidity

Table 1: Physicochemical characteristics of 3-mono/dibromoacetyl-6-halogenated coumarin analogues CMRN 1–CMRN 7.

Code	M. F (M. Wt.)	Yield (%)[a,b]	m.p. (°C) reported	m.p. (°C) found	Mass (m/z)	$c \log P$[c]
CMRN 1	$C_{11}H_7BrO_3$ (265)	96	120–122	121	266 ($M+1$)	1.4023
CMRN 2	$C_{11}H_5Br_3O_3$ (421)	95	146–148	147	422 ($M+1$)	3.4753
CMRN 3	$C_{11}H_7BrO_3$ (265)	98	220–222	221	266 ($M+1$)	2.0193
CMRN 4	$C_{11}H_6Br_2O_3$ (343)	95	204–206	205	344 ($M+1$)	2.2723
CMRN 5	$C_{11}H_6BrClO_3$ (299)	94	180–182	181	300 ($M+1$)	2.1223
CMRN 6	$C_{11}H_7ClO_3$ (222)	95	218–220	219	223 ($M+1$)	1.8693
CMRN 7	$C_{12}H_7BrN_2O_2S$ (321)	87	210–212	211	322 ($M+1$)	2.6992

[a]All of the products were characterized by spectral and physical data.
[b]Yields were on isolated basis.
[c]$c \log P$ was calculated using ChemBioDraw Ultra 13.0 v.

Table 2: Mortality of *Anopheles arabiensis* larvae exposed to coumarins and halogenated coumarins at 4 μg/mL and their negative (acetone) and positive (Temephos) controls and adult knockdown activity after 2-minute exposure to halogenated coumarins at 1000 μg/mL and their negative (acetone) and positive (DEET) controls against repellence assays (adjusted means and standard errors).

Compound code	Larval mortality	Knocked down
CMRN 1	98.9 ± 3.1[a]	100.0 ± 10.9[a]
CMRN 2	97.8 ± 3.1[a]	96.7 ± 10.9[ab]
CMRN 3	1.1 ± 3.1[b]	91.1 ± 10.9[b]
CMRN 4	98.9 ± 3.1[a]	91.1 ± 10.9[b]
CMRN 5	97.8 ± 3.1[a]	97.8 ± 10.9[ab]
CMRN 6	1.1 ± 3.1[b]	95.6 ± 10.9[ab]
CMRN 7	98.9 ± 3.1[a]	81.1 ± 10.9[c]
6,7-Methoxy coumarin	3.3 ± 3.1[b]	96.7 ± 10.9[ab]
Scopoletin	0.0 ± 3.1[b]	96.7 ± 10.9[ab]
Acetone (control)	0.0 ± 3.1[b]	0.0 ± 10.9[d]
Temephos	100.0 ± 3.1[a]	
DEET		0.0 ± 10.9[d]

[a–d]Within each column, compounds not sharing a letter differ significantly ($P < 0.05$).

(70%), and lighting (12/12) of a malaria endemic environment. One mL of test compound (1 mg/mL) was added to distilled water (250 mL) producing a final concentration of 4 μg/mL. Thirty 3rd instar larvae were placed in the container. A negative control was set up using a solvent (acetone) and distilled water and a positive control included Temephos (Mostop; Agrivo), an effective emulsifiable organophosphate larvicidal used by the malarial control program. Each container was monitored for larval mortality at 24 h intervals for a period of three days and fed specially made cat food with reduced oil/fat content at regular intervals. Bioassays were triplicated. The percentage mortality was calculated relative to the initial number of exposed larvae. The larvicidal results are tabulated in Table 2.

2.3. Insecticidal Activity. Insecticidal activity assessment was conducted by exposing susceptible adult mosquitoes to a treated surface, in accordance with WHO protocol (1975) [1]. One mL of test compound solution (1 mg/mL) was sprayed onto a clean, dry, nonporous ceramic tile using a precalibrated Potter's Tower apparatus [8]. The tiles were then air-dried and the assay was initiated within 24 h of spraying, by fixing a cone over the sprayed tile and introducing thirty non-blood-fed, 2–5-day-old susceptible adult *A. arabiensis* mosquitoes into the cone. The effect of the test compounds was measured by determining the knockdown rate, which was based on temporary paralysis of the mosquitoes during a 60 min exposure period, and mortality 24 h postexposure. Deltamethrin (15 g/L; K-Othrine) was used as a positive control and acetone as a negative control. All bioassays were triplicate to ensure validity of results.

2.4. Repellence Assays. Repellent activity was assessed by topical application of the compound to skin and subsequent exposure of the treated areas of skin to unfed female mosquitoes. Ethical approval for the use of *Mastomys coucha* in these trials was approved from the Medical Research Council's Ethics Committee for Research on Animals. Adult *Mastomys* rodents were weighed individually and injected intraperitoneally with the correct concentration of sodium pentobarbital in comparison to the weight of the animal. The anesthetized rodents were then shaved on the ventral surface and a test compound (1 mL) was applied to each rodent's abdomen. Acetone was used as a solvent for the preparation of stock solution (1 mg/mL). Laboratory grade DEET (IUPAC: *N*,*N*-Diethyl-3-methylbenzamide) was used as the positive control and plain acetone was used as negative control.

Paper cups (500 mL) were modified by replacing the base of the cup with mosquito netting held in place with a rubber band and covering the mouth of the cup with transparent plastic film. Thirty unfed 4-day-old *A. arabiensis* females were introduced into the cup that was held in contact with the treated ventral surface of each rodent. Mosquito activity was observed through the transparent plastic film. After a period of 2 min, the numbers of mosquitoes probing were recorded. The cups holding the mosquitoes were removed and mosquitoes were then observed for 24 h. All tests were triplicated. The rodent was then returned to the animal facility and allowed to recover from anaesthetic. No adverse reactions to the applied components were observed on any of the *Mastomys* rodents during the 3 days they were monitored.

Repellence of the extracts was calculated using the following formula;

$$\text{Percentage mosquitoes repelled} = \frac{\text{Number repelled}}{\text{Number introduced}} \times 100. \quad (1)$$

2.5. Statistical Analysis. Generalized linear models assuming a Gaussian distribution were used to determine differences between treatments registered in larval mortality (larvicidal assays), adulticidal effects, and knockdown (in repellence assays). LSD Fisher test was used for post hoc analyses. In all cases, a value of $P < 0.05$ was considered statistically significant.

3. Results and Discussion

The title compounds obtained were in good yields (87–98%) and characterization was completed by GCMS analysis. Purity of the compounds was confirmed by HPLC and it was more than 99%. *c* Log *P* of the compounds was calculated using ChemBioDraw Ultra 13.0 v and values were in the range of 1.8693–3.4753.

There was a significant effect of treatment on larval mortality ($P < 0.001$) (Table 2). The highest activity was detected with CMRN 1, CMRN 2, CMRN 4, CMRN 5, and CMRN 7 showing close to 100% mortality after 24 h of exposure, which was the same as for the positive control Temephos. Compounds CMRN 3, CMRN 6, scopoletin, and 6,7-methoxy coumarin showed a statistically lower mortality that was equivalent to the negative control.

The promising larvicidal activity of CMRN 1, CMRN 2, CMRN 4, CMRN 5, and CMRN 7, which was comparable to the positive control Temephos, may be attributed to the presence of electron withdrawing halogen atoms (bromine and chlorine) on acetyl group at the third and sixth positions of the coumarin nucleus. Larvicidal and ovicidal activity of 4-methyl-7-hydroxy coumarin derivate against vectors *Aedes aegypti* and *Culex quinquefasciatus* have also been attributed to bromine atoms present at C-5 and C-8 positions [29]. Coumarin and mainly furanocoumarins can alter the detoxication capability of an organism, by reversibly or irreversibly inhibiting cytochrome P450 detoxication enzymes [30, 31].

On adulticidal assays, adult mosquito mortality of positive control K-Othrine showed 100% knockdown/mortality from the first 60 min of exposure, while the natural coumarin, synthetic compounds, and negative control did not knockdown mosquitoes throughout the 24 h observation period.

All components tested for repellence (except the controls) knocked down mosquitoes within the 2 min exposure time, and CMRN 1, CMRN 2, CMRN 5, CMRN 6, 6,7-methoxy coumarin, and scopoletin were the most potent, knocking approximately 100% of them ($P < 0.001$) (Table 2). However, 24 h after exposure, all mosquitoes recovered. The few mosquitoes exposed to the coumarin analogues that were not knocked down did not attempt to bite; because of the low number of mosquitoes remaining active, no further statistical analyses were carried out.

An insect immobilization effect of coumarins has been reported for other insects [32, 33], and a slowly developing paralysis eventually leading to death has been a major feature of insect poisoning by coumarins such as surangin B [32]. Bioenergetic disruption of muscle has been determined as a prominent mechanism underlying the insecticidal action of surangin B [34]. Moreover, homology modeling and docking studies indicate that coumarin, as well as other terpene compounds, may act as acetylcholinesterase inhibitors and can block the octopamine receptor pathway and thus be neurotoxic against mosquitoes [35]. However, knockdown of mosquitoes resulting from short exposure to 3-mono/dibromoacetyl-6-halogenated coumarin analogues CMRN 1–CMRN 7 was fast (2 min or less) and reversible, and no adulticidal effects were detected from 60 min exposure. This may be partly explained by differences in experimental design, since topical application and injection were common procedures in previous reports on insecticidal properties [32, 33]. Besides mortality, coumarins may exert other negative effects on insect populations, such as decreasing their reproductive potential [36], which were not assessed in the present study.

Because no (active) mosquitoes were observed attempting to bite the rodent treated with coumarin analogues, further assessment of the repellent properties of these compounds after different treatment times and concentrations merits further testing.

4. Conclusions

The present study evaluates 3-mono/dibromoacetyl-6-halogenated coumarin analogues CMRN 1–CMRN 7, scopoletin, and 6,7-methoxy coumarin for larvicidal and repellent effects against the malaria vector *A. arabiensis*. Compounds CMRN 1, CMRN 2, CMRN 4, CMRN 5, and CMRN 7 were considered potential larvicidal agents for further research and development, because these compounds exerted close to 100% mortality within 24 h of exposure. Adulticidal activity on the other hand was considered negligible and repellence should be further explored. Of particular interest are the bromo- and chloroanalogues of CMRN 2, CMRN 4, and CMRN 5 that have potential to be used to prevent and control malaria by controlling the vector *A. arabiensis*.

Conflict of Interests

The authors declare that they have no conflict of interests.

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