ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF THE CHEMICAL CONSTITUENTS OF *TERMINALIA IVORENSIS* CHEV

BY

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DECLARATION

I hereby declare that this submission is my own work towards the MSc and that, to the best of my knowledge; it contains no material previously published by another person nor material which has been accepted for the award of any degree of the University, except where due acknowledgement has been made in the text.

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Dr. Sylvester K. Twumasi
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Dr. J. A. M. Awudza
(Head of Department)

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Date

DEDICATION

This work is dedicated to all mothers, especially Ms. Dorcas Aku Akpoley and Mrs. Regina Frimpong-Manso.

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ABSTRACT

Terminalia ivorensis is an indigenous plant which has found many uses in the road, housing, and carpentry industries, and is also used as phytomedicines. The bark of the plant was obtained from Kubease, a small town along the Kumasi-Konongo road in the Ashanti Region. The sample was washed, air-dried and milled. The moisture content of the milled sample was measured (10.33%). The remainder was extracted with water and ethanol, using soxhlet apparatus. The marc of the ethanol extraction was further extracted with water (herein referred to as water-after-ethanol extract). Portions of the extracts were screened for phytoconstituents and fractionated by bulk transfer methodology into groups of related polarity – chloroform fraction (i.e. acids and neutrals) and aqueous fractions (i.e. bases). The crude extracts and fractions were examined for antioxidant activities (hydrogen peroxide decomposition and Fe^{3+} reducing power) and also screened against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas earuginosa*, and S*treptococcus pneumoniae*, by the diffusion method.

The crude extracts were screened for the presence of some phytoconstituents. All the three extracts of *Terminalia ivorensis* were found to contain saponins, steroids and triterpenoids, and anthraquinones glycosides. In addition to these phytochemicals, tannins and polyphenols, and flavonoids were found in both crude aqueous and alcoholic extracts, and anthraquinones found only in the water-after-ethanol extract. None of the extracts contained carotenoids, general glycosides, alkaloids and coumarins.

From the IR spectra of the extracts and fractions, -OH, aliphatic CH_2 and CH_3 groups, primary amines, amides, conjugated C=C and C=O could be deduced

from the bark of the plant. This could confirm the presence of steroids and terpenes, polyphenols and some glycosides as indicated in the phytochemical screening.

All the extracts and their fractions (crude extracts, chloroform and aqueous fractions of the water, ethanol and water-after-ethanol extracts) of *Terminalia ivorensis* showed significant activities against *E. coli, S. pneumoniae, S. aureus,* and *P. aeruginosa* (MIC from 22.15 to 7937.04 µg/ml) compared to chloramphenicol (7.56 to 1881.64 µg/ml). The chloroform fraction of the water-after-ethanol extracts and the two fractions of the water extract however, did not show any activity against the test organisms.

The results from the antioxidant studies showed that the all extracts and fractions of *T. ivorensis*, to a large extent, have appreciable antioxidant activities, accomplished by their significant reducing power (with absorbance from 0.093 to 0.346) and decomposition of H_2O_2 (decreasing from 86.18 to 55.02mM). However, the crude water-after-ethanol extract and the chloroform extract of the aqueous extract however, could not decompose H_2O_2 (increasing from 86.18 to 588.49mM).

These antioxidant and antibacterial properties of the plant, to a large extent, are a significant factor in its usage for the management of wounds and age-related diseases (like cancer, trauma, stroke, asthma, hyperoxia, retinal damage, liver injury, and periodontis) and other bacterial-related diseases.

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Table A1: Results for zone of inhibition

Concentration (mg/ml)	**Average zone of inhibition** (mm)			
	Staphylococcus	*Streptococcus*	*E. coli*	*Pseudomonas*
Water extract				
CA				
40.00	10.60	10.5	6.75	6.75
20.00	10.33	9.00	6.75	6.75
10.00	8.00	7.75	6.75	6.75
5.00	7.33	7.00	3.50	3.50
AA				
16.80	6.75	NA	3.30	11.25
8.40	5.50	NA	3.00	9.50
4.20	6.25	NA	2.5	8.00
2.10	5.00	NA	NA	6.75
BA				
40.00	6.67	1.75	5.75	8.00
20.00	6.00	1.50	4.52	6.50
10.00	4.50	1.50	NA	5.50
5.00	3.00	NA	NA	NA
Ethanol extract				
CB				
40.00	12.33	13.50	7.75	9.75
20.00	11.00	10.00	5.00	7.00
10.00	8.67	9.25	5.50	7.00
5.00	5.33	7.50	3.75	7.00
AB				
40.00	5.50	NA	NA	3.75
20.00	NA	NA	NA	3.25
10.00	NA	NA	NA	2.25
5.00	NA	NA	NA	1.50
BB				
40.00	5.50	2.50	4.50	7.75
20.00	3.75	1.25	4.00	NA
10.00	2.00	NA	2.25	NA
5.00	NA	NA	NA	NA
Water-after-ethanol extract				
CC				
40.00	8.25	10.50	5.25	8.25
20.00	7.00	5.75	4.25	6.00
10.00	4.75	5.75	3.75	5.50
5.00	4.25	4.00	3.75	4.00
AC				
20.00	12.00	7.25	3.50	11.25
10.00	8.00	4.75	2..75	9.25
5.00	6.67	2.75	1.75	6.50
2.50	5.00	NA	NA	3.75
BC				
40.00	NA	2.00	NA	2.50
20.00	NA	NA	NA	2.00
10.00	NA	NA	NA	1.50
5.00	NA	NA	NA	NA
Chloramphenicol				
8.36	10.25	2.75	3.75	14.75
4.18	8.75	2.00	3.50	8.50
2.09	8.25	NA	3.00	8.00

Table A4: Hydrogen peroxide decomposition results

Time (s)	20mg/ml of sample			40mg/ml of sample			80mg/ml of sample		
	Titre (ml)	$n(Na_2S_2O_3)$ (mmol)	$c(H_2O_2)$ (mM)	Titre (ml)	$n(Na_2S_2O_3)$ (mmol)	$c(H_2O_2)$ (mM)	Titre (ml)	$n(Na_2S_2O_3)$ (mmol)	$c(H_2O_2)$ (mM)
Water extract									
CA									
60	5.60	0.2856	588.49	4.23	0.2155	532.77	3.30	0.1683	554.86
120	5.70	0.2907	599.01	4.30	0.2193	542.24	3.33	0.1698	559.91
180	5.60	0.2856	588.49	4.30	0.2193	542.24	3.30	0.1683	554.86
240	5.60	0.2856	588.49	4.18	0.2129	526.48	3.05	0.1556	512.86
AA									
60	8.15	0.2215	138.41	7.60	0.2065	154.87	5.30	0.1440	144.01
120	8.60	0.2337	146.05	8.15	0.2215	166.08	5.20	0.1413	141.29
180	8.40	0.2282	142.65	8.00	0.2174	163.02	5.35	0.1454	145.37
240	8.60	0.2337	146.05	8.00	0.2174	163.02	5.40	0.1467	146.73
BB									
60	4.70	0.2397	493.90	4.65	0.2378	586.35	3.10	0.1581	521.23
120	4.80	0.2448	504.42	4.75	0.2423	598.97	3.25	0.1658	546.46
180	4.50	0.2295	472.90	4.50	0.2295	567.46	3.25	0.1658	546.46
240	4.85	0.2474	509.70	4.85	0.2474	611.57	3.30	0.1683	554.86
Ethanol extract									
CB									
60	1.66	0.1156	72.23	1.81	0.0925	69.34	1.48	0.0755	75.51
120	2.30	0.1171	73.20	1.93	0.0986	73.96	1.45	0.0740	73.97
180	2.36	0.1202	75.12	1.84	0.0940	70.50	1.48	0.0755	75.51
240	2.45	0.1248	78.01	1.90	0.0971	72.81	1.51	0.0771	77.05
AB									
60	2.30	0.1171	73.20	1.96	0.1002	75.12	1.51	0.0771	77.05
120	2.33	0.1187	74.16	1.96	0.1002	75.12	1.54	0.0786	78.59
180	2.36	0.1202	75.12	1.90	0.0971	72.81	1.48	0.0755	75.51
240	2.30	0.1171	73.20	1.99	0.1017	76.28	1.48	0.0755	75.51
BB									
60	2.33	0.1187	74.16	1.90	0.0971	72.81	1.48	0.0755	75.51
120	2.27	0.1156	72.23	1.84	0.0940	70.50	1.54	0.0786	78.59
180	2.36	0.1202	75.12	2.02	0.1032	77.43	1.51	0.0771	77.05
240	2.30	0.1171	73.20	1.96	0.1002	75.12	1.48	0.0755	75.51
Water-after-ethanol extract									
CC									
60	3.90	0.10597	66.23	3.45	0.0937	70.30	2.80	0.0761	76.08
120	4.20	0.11412	71.33	3.50	0.0951	71.32	2.75	0.0747	74.72
180	4.10	0.11141	69.63	3.35	0.0910	68.27	2.55	0.0693	69.29
240	4.05	0.11005	68.78	3.35	0.0910	68.27	2.60	0.0706	70.65
AC									
60	5.95	0.16167	101.05	4.85	0.1318	98.83	3.80	0.1033	103.25
120	6.05	0.16439	102.74	5.05	0.1372	102.91	3.75	0.1019	101.90
180	6.05	0.16439	102.74	4.95	0.1345	100.87	3.70	0.1005	100.54
240	6.00	0.16303	101.90	5.25	0.1427	106.98	3.95	0.1073	107.33
BC									
60	3.50	0.09510	59.44	3.05	0.0829	62.15	2.50	0.0679	67.93
120	3.50	0.09510	59.44	2.95	0.0802	60.12	2.55	0.06929	69.29
180	3.55	0.09646	60.29	2.80	0.0761	57.06	2.30	0.06250	62.50

240	3.50	0.09510	59.44	2.70	0.0734	55.02	2.25	0.06114	61.14

CHAPTER ONE

1 INTRODUCTION

Medicinal plants constitute effective sources of antimicrobial and antioxidant natural products. The use of these plants all over the world predates the introduction of antibiotics and other modern drugs into the African continent. Plants have been used in traditional medicine for many centuries as arbotifacients, contraceptives, for menstrual regulation, fertility control, as well as for the treatment of ailments of both microbial and non-microbial origins. The Ghanaian flora is rich in medicinal plants which are usually exploited by herbalists otherwise called ´´native doctors´´ who administer such plants in the treatment of various ailments, including the treatment of wounds [1].

In vivo and in vitro investigations on extracts of some plants (including: *Mezoneuron benthamianum, Securinega virosa, Microglossa pyrifolia*, *Pterocarpus santalinus*) used in wound care have been done [2-5]. The bark decoction of *Terminalia ivorensis* (known in Akan as amire) for instance, is locally used as a lotion for the treatment of wounds, sores and cuts [6] and is believed to heal the wound in three days without a scar. The plant has been found to have anti-arthritic [7], anti-inflammatory activities [5, 8-11] and is known to be effective against *T. brucei rhoderienses* parasites, one of the major causative agents of trypanosomiasis [12]. The crude ethanolic extract of the plant has been found to be active against *E. coli*, *Pseudomonas aeruginisa*, and *Candida albicans* [13]. Its use as an ethonomedicine emanates from the fact that it is known to contain saponins, triterpenes, tannins, other phytochemicals and antioxidants [14].

The skin, like other integements, serves as protection from external environment [15-18]. A disruption in the continuity of the skin (normally as a result of

physical injuries [17]) exposes the epidermal and dermal layers, causing damage to the blood vessels and leading to bleeding [19-21]. This condition is referred to as wound.

The skin has its own natural mechanism of restoring the continuity-healing. This is a series of complex reactions and interactions among cells and "mediators" [21], which involves continuous and overlapping processes (phases) - inflammation, reconstruction (proliferation) and mutaration [19]. The healing process takes a few weeks but the presence of contaminants (infections) – organic/inorganic and most often bacteria (*Proprionibacterium acnes, Staphylococcus aureus, Streptococcus, E. coli, Proteus, Klebsiella, Pseudomonas earuginosa, Acinetobacter, Stenotrophomonas*) prolong and interfere with the natural healing process [2]. Interferences from reactive oxygen species (ROS) are also known to prolong the process [22].

The primary objective of care of wounds, then, is to prevent or minimize infection and promote healing [21]. Various materials and methods, especially antibacterial are employed in such an enterprise. Some of these wound care methods employed in Ghana include the use of ampicillin capsules, a combination of ampicillin and mebendazole, some plants [23] and herbal products (like “akuro bewu” ointment).

The continual use of the bark of *Terminalia ivorensis* in wound care and even its use in the preparation of some herbal products has just been a question of tradition, availability, affordability and most importantly, believe.

The medicinal action of a plant drug lies in its active constituents such as alkaloids, cardiac glycosides, saponins, flavonoids, tannins, volatile oils, steroids and terpenoids, resins and mucilage [24] etc. Plants are known to be rich in biologically active substances (flavonoids, phenolic acid, etc) many of which exhibit antioxidant

and antimicrobial activity known to aid wound healing [2, 22] and *Terminalia ivorensis* is no exception.

A work on the plant to ascertain its biochemical properties in wound care is of great importance to the chemist.

This project is generally aimed at determining the biochemical properties of the bark of *Terminalia ivorensis* that could be employed in the treatment of wounds.

The specific objectives of the work are:

- Extracting active chemical constituents of the plant sample with water, ethanol and the ethanol residue with water,
- Testing for the presence of phytochemical constituents in the extracts,
- Fractionating the extracts into chloroform and aqueous fractions,
- Performing thin layer chromatography on the extracts and fractions,
- Identifying the possible functional groups present in the extracts and fractions using infrared spectroscopy,
- Studying the antibacterial effects of the extracts against *Escherichia coli, streptococcus pneumoniae*, *staphylococcus aureus* and *Pseudomonas earuginosa*,
- Studying the antioxidant activities of the chemical constituents of the extracts and fractions and
- Making necessary recommendations for further work.

Injury leading to wounds has being one of daily occurrence and the management of these wounds with plants (like *Terminalia ivorensis*) has almost been a daily practice. The use of plant in the treatment of wounds dates back into history. They are believed to do the healing. Research has, however, revealed the skin's own natural way of healing with time, the presence of contaminants and bacterial

infections like *streptococcus, staphylococcus* and the *coliform* interfering and prolonging the healing process [17-21]. It is important therefore to cover the wound or prevent the wound from infection, hence the need of antibacterial and antioxidant agents in the healing process [20].

Various plants species have been found to show activity against these and other bacteria. One of these is *Terminalia ivorensis*, a member of the Combretaceae family and in the genus, Terminalia. Trees in this genus are known especially as a source of secondary metabolites like terpenes, and the aromatics. Some of these substances have antifungal, antibacterial, antioxidants and hepatoprotective properties.

Medicinal herbs have become indispensable part of the traditional medicine practiced all over the world due to easy access, low cost and ancestral experience [22]. With the increase in the production of herbal ointments on the Ghanaian market, which is believed to heal cuts and wounds, the use of these plants including *Terminalia ivorensis* for such purposes from their crude extract have declined considerably. Their use is now a question of affordability, accessibility and belief.

The process of oxidation causes the skin to lose its elasticity and develop wrinkles, thins hair, weakens muscles, impairs hearing and eyesight, decreases immunity, and increases degenerative diseases. Antioxidants stop this process and maintain the health of our bodies. They have been reported to play a significant role in the wound healing process [8]. Moseley *et al* [26] (2003) reported that polymorphonuclear leukocytes (PMN); which are reactive oxygen species, such as superoxide radical and hydroxyl radical species released in chronic wounds are believed to perpetuate inflammatory processes.

The WHO has also recommended (WHO 1980) that medicinal and scientific examination of such plants is undertaken. Launching its first comprehensive traditional medicine in 2002, the organisation among others encouraged countries to document traditional medicines [27]. A work, aimed at ascertaining the antioxidant and antimicrobial properties is a valuable national assert.

CHAPTER TWO

2 LITERATURE REVIEW

Terminalia ivorensis is an indigenous plant which has found many uses in the road, housing, and carpentry industries, and is also used as phytomedicines. Its medicinal use (especially in wound care) is of great importance to this work, and this chapter seeks to review various literature on the plant, phytochemicals, reactive oxygen species and antioxidants, the general description of the skin which would then lead to a discussion of cuts and wounds, wound contaminants, management and healing.

2.1 *Terminalia ivorensis*

Idigbo, as the plant is commonly known, is a common plant in Africa especially mostly distributed between Guinea and Cameroon. The plant is predominant in Cote d'Ivoire, no wonder it is known in many quarters as Ivory Coast almond. Other common names of *T. ivorensis* include: black afara, black bark, yellow terminalia, shingle wood, brimstone wood and satin wood. It is known in French as Framiré; terminalia in Spanish and mwalambe in Swahili.

In Ghana, *Terminalia ivorensis* has several names. It is known as Amire in Ashanti, Fanti, Wassa and Ahanta whilst it is Emere in Ga and Krobo. In Nzema, Brosa, Esa and Anyi, it is known commonly as (E) Frammire. The plant is known amongst Ewes as Dzogbedodo [16].

The taxanomony, morphology and general uses of the *Terminalia ivorensis* are discussed in the following subsections.

2.1.1 Taxanomony

Terminalia ivorensis is classified as follows:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Myrtales

Family: Combretaceae

Genus: Terminalia L

Species: *Terminalia ivorensis* chev

2.1.2 Morphology

Terminalia ivorensis is a tall, large and straight deciduous forest tree ranging in height from 15 to 46m, branchless for up to 30m. Its buttresses are blunt and extend up to the bole, frequently and narrowly fluted, with the bark becoming very dark with longitudinal fissures, flashing yellow [16]. The bark (figure 2.1) is smooth and light grey to dark brown when young and on branchlets and often blackish in mature trees; with deep longitudinal fissures, flaking off in long thin strips.

(a) Bark intact

(b) Part of bark removed

Fig 2.1: Bark of *Terminalia ivorensis*

The obovate, acuminate leaves (figure 2.2) measure up to 6.4 - 12.7cm x 2.5 - 6cm. They are whorled, simple, blunt tipped with orange-brown hairs below and on veins above, and are also found on the short stalks. The leaves have about 6 - 9 pairs widely spaced veins, which prominent below. The flowers are white, with nice fragrance and are in slender racemes of about 7.6 - 10.2cm long with bisexual flowers nearly to the apex [16].

Fig 2.2: Leaf of *Terminalia ivorensis*

Its fruits (which normally appear in January/February, June, or September/November) are winged and puberulous, elongated, narrow, emarginated and slightly decurrent with somewhat variable in size, especially in the width of the wing, measuring up to 10 x 2cm. They are finely tomentose with very short reddish or orange-brown hairs, and a pedicel of 7 - 11mm long. They are reddish-brown and woody when ripe, frequently having a weevil hole oval seed in the centre, 1.5 cm. It is from this (the habit of the leaves being crowded at the ends of the shoots) that the generic name, 'terminalis' (Latin) comes from [16].

The sapwood of *T. ivorensis* is normally yellow to light brown, whilst its heartwood is somewhat darker. The quality of the heartwood is, however, inferior when growth is too mature [16]. The wood is also of coarse and uneven texture with straight or wavy grains, and shrinks with seasoning. It is fairly resistant to fungi and insects except pin holes borers and powder post beetles and termites.

2.1.3 Uses of *Terminalia ivorensis*

T. ivorensis has found numerous uses in the building, construction, carpentry industries and even in household equipments due to special working properties such as: easy sawing, rapid drying, moderate wood bending, good gluing, good nailing and finishing (though filling is necessary) and no difficulty in machining [16].

In building and carpentry, the wood is used for ceiling as strip wood, and used to furnish and decorate interior walls as panels [7]. It is used in door and window frames, skirting, panelled and doors and stair treads [16]. The wood is much preferred for shingles because it splits easily and last very long (ca fifteen years). *Emire* wood is used for the construction of light bridges, mommy tracks (trotro), and as carriage fittings and as fencing materials. In the United States, *T. ivorensis* was used for radio cabinets [28].

In the South-western rain forest of Ghana, the wood is sometimes employed in the carving of canoes where the preferred wood does not grow. It is also used in carving mortars. The bark, which yields yellowish-red pigment, is employed in dyeing clothing [7]. This however, makes it unsuitable to be used as laundry equipment and draining boards [16].

The plant has also found a lot of uses as ethnomedicine. In Ghana, the bark decoction is used as a lotion for the treatment of wounds, sores and cuts [7] and for rheumatism [16]. The wound is believed to be healed without scar. The powdered bark is sprinkled on ulcerated wounds in various *in vivo* and *in vitro* studies and has also shown anti-inflammatory properties. *T ivorensis* is found to be effective against *T. brucei rhoderienses* parasites, one of the major causative agents of trypanosomiasis[13].

2.2 CHEMICAL CONSTITUENTS OF PLANTS

Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. It is well-known that plants produce these chemicals to protect themselves but recent research demonstrates that they can protect humans against diseases. A number of phytochemicals are known, some of which include: alkaloids, saponins, flavonoids, tannins, glycosides, etc. They do not only protect the plants but have enormous physiological activities in humans. These include cancer prevention, antibacterial, antioxidative, hormonal action, enzyme stimulation and many more.

The following subsections outline some of the biochemical properties (including the physiological activities) some of these phytochemicals.

2.2.1 Flavonoids

Flavonoids represent a very wide group of water-soluble derivatives of the basic compound shown in figure 2.3. Many of them are coloured – red, crimson purple or yellow [29]. They are polymeric compounds possessing fifteen carbon atoms, with two benzene rings joined by a linear three-carbon (3-C) chain as its basic structure [30]; the variation is the state of oxidation of the connecting 3-C moiety, which determines the properties and class of each compound.

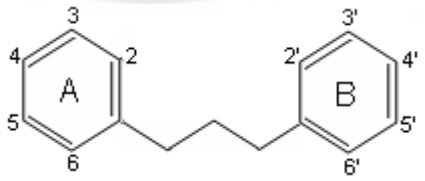


Fig 2.3: General structure of flavonoids

The classes include: Flavone, flavonol, flavonone, flavononol, chalcone, isoflavone, anthocyanidine, dihydrochalcone, aurone, catechine and flavan-3, 4-diol. Some of these structures are shown in figure 2.4 below.

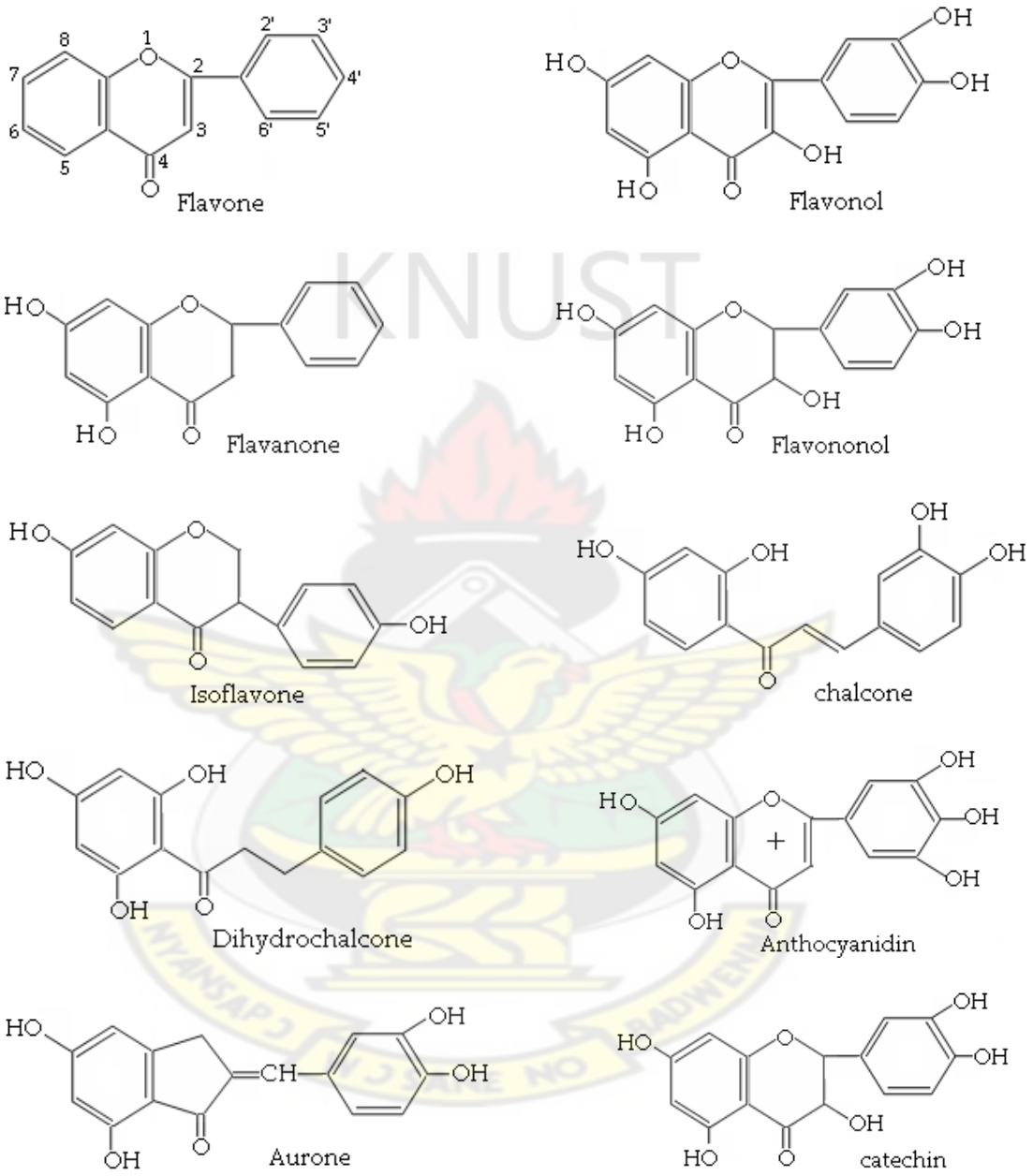


Fig 2.4: Classes of flavonoids

Flavonoids usually occur in plants (including: fruits, pollen, roots and heartwood) as glycosides in which one or more of the phenolic hydroxylic groups are combined with sugar [31]. Glycosides of flavonoid compounds may bear the sugar on

any of the available hydroxyl groups. The glycosides are generally soluble in water and alcohol but insoluble in other organic solvents.

The flavonoids dissolve in alkali giving a yellow coloured solution, which on addition of acid become colourless. Flavonoids are among three classes of micronutrients found in fruits and vegetables which are important in reducing the risk of atherosclerosis [32].

They are generally known to be physiologically active and these include: antioxidant, antimicrobial, anticancer, vasoprotective, anti-inflammatory, anti-viral, antithrombitic and antiallergenic effects activities. The flavonoid quercetin is known for its ability to relieve hay fever, eszema, sinusitis and asthma. Tea flavonoids have many health benefits [28, 30]. Tea flavonoids, for instance, reduce the oxidation of low-density lipoprotein, lowers the blood levels of cholesterol and triglycerides. Soy flavonoids, which are isoflavones, can also reduce blood cholesterol and can help to prevent osteoporosis. Soy flavonoids are also used to ease menopausal symptoms.
A few of the classes of flavonoids are briefly discussed in the subsections below.

2.2.1.1 Flavones

In flavones, the hydroxyl groups are nearly always found in positions 5 and 7 in ring A, while ring B commonly carries hydroxyl or alkoxyl groups at the 4'-position, or at both 3'- and 4'-positions, while ring C is basic and forms a pyrylium salt with hydrochloric acid. They are generally found in herbaceous families like Labiatae (*Umelliferae compositae*). The most widespread flavone is quercetin (figure 2.5). Others like primuletin (figure 2.5) and fisetin have only one hydroxyl group in ring A.

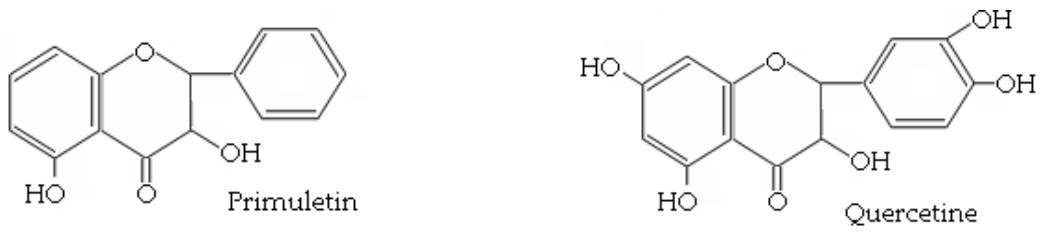


Fig 2.5: Examples of flavones

Numerous physiological activities have been attributed to flavones. Small quantities, for instance, may act as cardiac stimulants whilst highly hydroxylated one acts as diuretics and as antioxidants for fats. They are also believed to behave like auxins in stimulating the germinations of wheat seeds [31].

2.2.1.2 Flavanone

Flavanone has not been found in nature, even though their hydroxylated forms do occur in nature, either in the free form or as glycosides. Hisperidin (figure 2.6) and diosmin, for example, are found in the bark of *Zanthoxylum avicennae*, while rhoifolin and naringin (figure 2.6) in the peel of *Citrus aurantinum* [31]. Hisperidin appear to strengthen weak capillary blood vessels [30] and has been used for various treatment conditions characterized by bleeding.

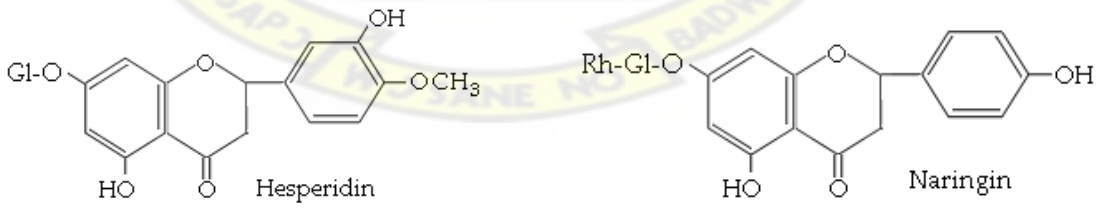


Fig 2.6: Example of flavanones

Flavanones also decompose into benzaldehyde, acetic acid and phenol in alkalis under drastic conditions [31].

2.2.1.3 Isoflavone

Isoflavones are 3-phenylchromones, which are known to have estrogenic, insecticidal and antifungal activities; some are also known to be potent fish poisons. There are about thirty-five known isoflavone, with daidzein (figure 2.7), genistein and tlanlancuayin (figure 2.7) being common [31].

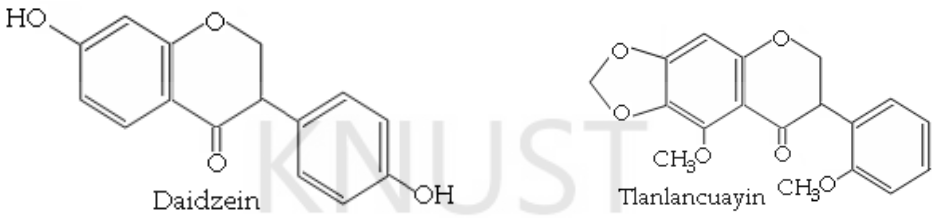


Fig 2.7: Examples of Isoflavone

2.2.2 Alkaloids

Most of the known alkaloids contain a basic nitrogen atom. An alkaloid containing plant almost never contains one alkaloid but rather a whole range of closely related components [31, 33]. Alkaloids are very difficult to define. The term alkaloid is commonly applied to basic nitrogenous compounds that are physiologically active. They nearly contain their nitrogen as part of a heterocyclic system [29, 33] and are often quite complex in structure. Alkaloids usually show specific pharmacological activity. Amongst the pharmacologically active ingredients found in plants, alkaloids are the most arguably important [30]. More than thirty are used therapeutically and they cover a broad spectrum of pharmacological effects. Some alkaloids have further use; quinine (commonly found in *chinchona* spp), for example, is used in the food industry for bitter flavouring and as an antimalaria drug [34]. They are known to have antioxidant and antimicrobial activities [1, 35].

The basicity of alkaloids depends on the structure of the compound and the position of the functional groups [30].

The classification of alkaloids is usually based on the chemical structure from which they are derived. The biological functions of alkaloids in plants are not well understood. Many alkaloids are extremely toxic. They evolved as a defence mechanism that protects plant against predators [31, 33].

Some examples of alkaloids are nicotine (from *Nicotiana tabaccum*), quinine (from *Cinchona officinalis*), dopamine, anabasine (from *Anabasis aphylla*), edamine, cocaine, morphine (from *Papaver samniferum*), strychnine and conetine [30]. A few of them are shown in figure 2.8 below.

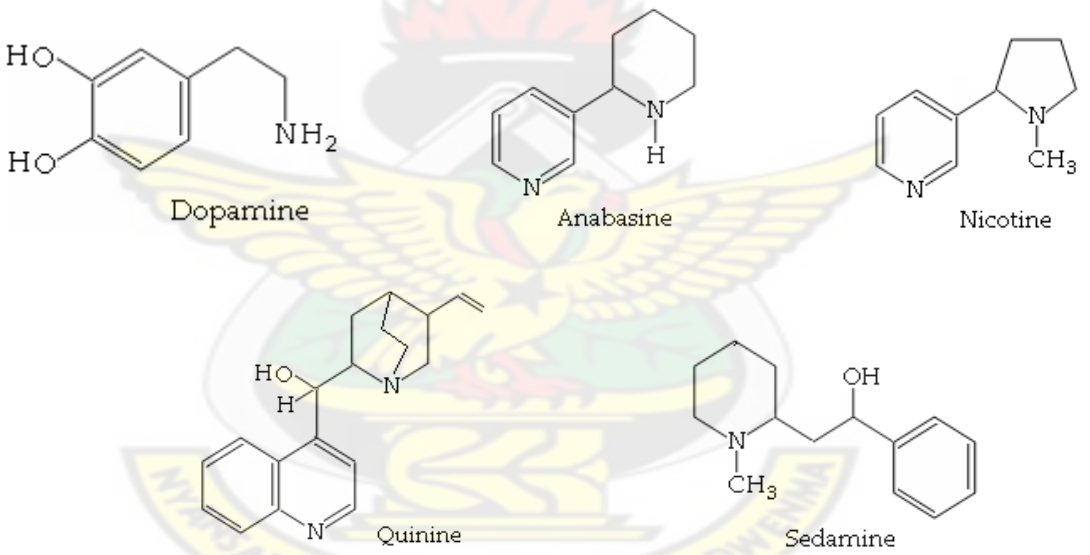


Fig 2.8: Some examples alkaloids

2.2.3 Saponins

Saponins are special glycosides with distinctive foaming characteristics; they form froth or foam when shaken with water. They can be said to be natural detergents found in plants because they contain both water-soluble and lipid-soluble components. They consist of a lipid-soluble nucleus, having either a steroid or

triterpenoids saponins with one or more side chain of water-soluble carbohydrate (sugar). Their physiological action depends on the fact they break up the red blood cells – Haemolysis. Saponins have a bitter and acidic taste [36]. They are highly toxic to cold-blooded animals because of their haemolytic properties [28]. They are, however, comparatively harmless when taken by mouth. Upon hydrolysis of saponins a product known as sapogenin is produced. The highly toxic sapogenins are called sapotoxins. A simple test for sapotoxins in saponins is to shake up an aqueous alcoholic extract in a test tube. The formation of persistence foam above the alcoholic extract in test tubes is a reliable evidence to show their presence [36].

Saponins are thought to act as resistant compounds against plants pathogens and are membrane active agents. The properties of saponins include antioxidant effect, direct and select cytotoxicity of cancer cell, immune-modulation, acid and neutral sterol metabolism and regulation of all proliferation. Scientists are now looking at how they can help humans fight fungal infection, combat microbes and viruses and boost the effectiveness of certain vaccines. Their natural tendency to inhibit the growth of microbes may prove to be especially useful for treating those difficult to control fungal and yeast infections [36]. Among the chemical properties of saponins, their polarity, hydrophobicity and nature of the reactive groups seem to be important determinants of their biological properties. They are known to have antibacterial, antitumour and cytotoxic, fungucidal and molluscicidal activities [1, 36].

Sarsapirilla is rich in saponins, but is widely used in the preparation of non-alcoholic beverages. Saponins have a high molecular weight and their isolation in a state of purity presents some problems. As glycosides they are hydrolyzed by acids to give aglycone (sapogenin) and various sugars and related uronic acids. According

to the structure of aglycone, two kinds of saponins are recognized - the steroidal (commonly tetracyclic triterpenoids) and pentacyclic triterpenoids.

2.2.4 Tannins

The name 'tannin' is used for various types of polyphenols obtained from plants and used to convert animal hides and skins to leather [33]. It is also used for the polyphenols that combine with or precipitate the protein of skins to increase their stability to water, micro organisms, heat, and abrasion. Chemically, tannins are complex substances, which usually occur as mixtures of polyphenols that are difficult to separate because they do not crystallize. Complex tannins are generally considered to have arisen from simple polyphenols by polymerization [28].

Tannins are one of the many types of secondary compounds found in plants. They are oligomeric compounds with multiple structure units with free phenolic groups. Their molecular weight ranges from 500 to 20,000 or more [32]. They are soluble in water, with exception of some high molecular weight structures, and are able to bind proteins forming insoluble or soluble tannin-protein complexes. Tannins are distributed in vascular plants, their occurrence in angiosperms being particularly associated with wood tissues. They have astringent property and are used as astringents, healing agent for gonorrhoea, burns, piles, etc. Tannins solution is used in the putrefaction of animal hides by converting them to leather. Many extracts with high tannin content are used to promote wound healing. This is achieved through encouragement of the formation of new tissues under the leathery layer formed on broken mucosal surface by the action of tannins. Considerable epidemiological and experimental evidence suggests that prolonged utilization of certain tannins rich plant material may be hazardous owing to their carcinogenic potential [37].

There are two main types of tannins which are distributed unevenly throughout the plant kingdom. These are: Hydrolysable tannins (HT) and Proanthocyanidins (PA) (often called Condensed Tannins).

2.2.4.1 Hydrolysable tannins

Hydrolysable tannins are molecules with a polyol (generally D-glucose) as a central core. The hydroxyl groups of these carbohydrates are partially or totally esterified with phenolic groups like gallic acid (gallotannins) or ellagic acid (ellagitannins). Hydrolysable tannins are usually present in low amounts in dicotyledonous plants. They are composed of gallotannins, which upon hydrolysis decompose into gallic acid and sugar, and ellagitannins; which upon hydrolysis yield hexahydroxydiphenic acid, which is rapidly converted to its stable dilactone, ellagic acid and sugar [37]. Some authors define two additional classes of hydrolysable tannins: taragallotannins (gallic acid and quinic acid as the core) and caffetannins (caffeic acid and quinic acid) [28].

Gallotannins: The phenolic groups that esterifies with the core are sometimes constituted by dimers or higher oligomers of gallic acid (each single monomer is called galloyl). Each Hydrolysable tannin molecule is usually composed of a core of D-glucose and six (6) to nine (9) galloyl groups.

In nature, there is abundance of mono and di-galloyl esters of glucose (MW about 900). They are not considered to be tannins. At least three hydroxyl groups of the glucose must be esterified to exhibit a sufficiently strong binding capacity to be classified as tannin. The most famous source of gallotannins is tannic acid obtained from the twig galls of *Rhus semialata*. It has a penta galloyl-D-glucose core and five more units of galloyl linked to one of the galloyl of the core.

Ellagitannins: The phenolic groups consist of hexahydroxydiphenic acid, which spontaneously dehydrates to the lactone form, ellagic acid. Its molecular weight ranges from 2000 to 5000 [31].

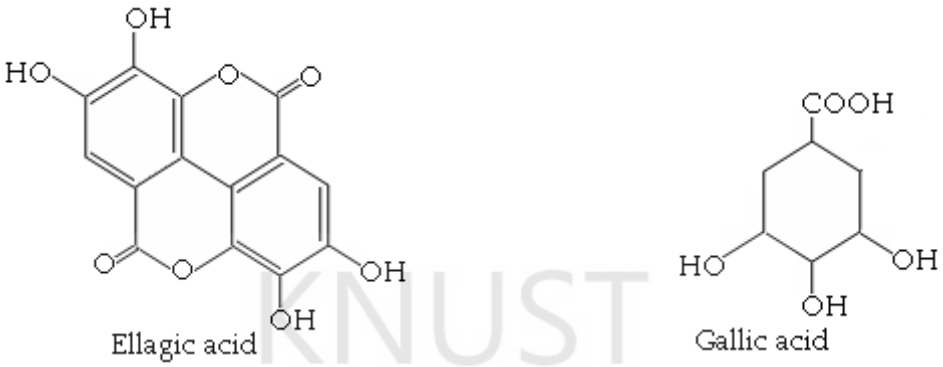


Fig 2.9: Gallotannins and Ellagitannins:

Hydrolysable tannins are hydrolysed by mild acids or mild bases to yield carbohydrate and phenolic acids. However, proanthocyanidins (condensed tannins) do not hydrolyse under the same conditions. They are also hydrolyzed by hot water or enzymes (tannase).

2.2.4.2 Proanthocyanidins (Condensed Tannins)

Proanthocyanidins are more widely distributed than hydrolysable tannins and are widespread in ferns and angiosperms, especially in wood species. They are oligomers or polymers of flavan units (i.e. flavan-3-ol) linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis [30, 33].

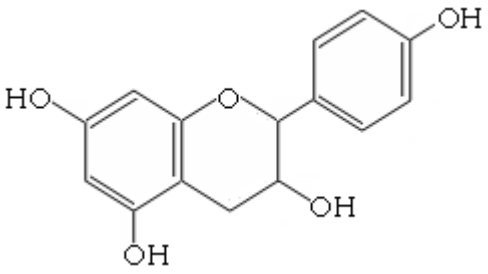


Fig 2.10: Flavan-3-ol

Proanthocyanidins are more often called condensed tannins due to their condensed chemical structure. However, hydrolysable tannins also undergo condensation reaction. The term, condensed tannins, is therefore potentially confusing. The term, proanthocyanidins, is derived from the acid catalysed oxidation reaction that produces red anthocyanidins upon heating in acidic alcohol solutions. The most common anthocyanidins produced are cyanidin (flavan-3-ol, from procyanidin) and delphinidin (from prodelphinidin) are shown in figure 2.11 below.

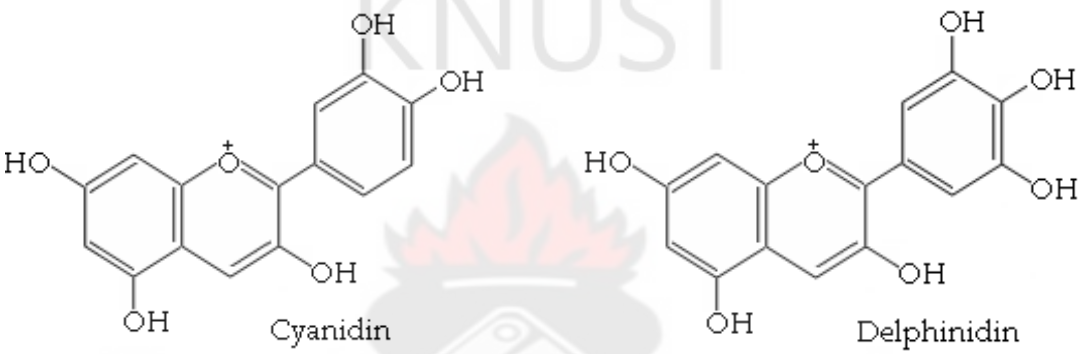


Fig 2.11: Products of proanthocyanidins

Proanthocyanidins may contain from two to fifty or greater flavan units; proanthocyanidin polymers have complex structures because the flavan units can differ for some substituents and because of the variable sites for interflavan bonds. Anthocyanidin pigments are responsible for the wide array of pink, scarlet, red, mauve, violet, and blue colours in flowers, leaves, fruits, fruit juices, and wines. They are also responsible for the astringent taste of fruit and wines [33, 33]. Proanthocyanidin carbon-carbon bonds are not cleaved by hydrolysis. Depending on their chemical structure and degree of polymerization, proanthocyanidins may or may not be soluble in aqueous organic solvents [33].

2.2.5 Coumarins

Coumarins are phytochemicals widely distributed in several plants, including: Tonka beans, lavender, liquorice, strawberries, apricots, cherries, cinnamon, and sweet clover. They are unsaturated aromatic lactones, which occur either in the free state or combined with the sugar glucose (Coumarin glycoside) [31].

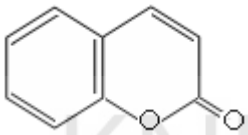


Fig 2.12: General structure of coumarins

They possess characteristic odour (vanilla-like flavour) and have aromatic bitter taste. Coumarins were formally used as flavouring agents but due to their therapeutic qualities they have been banned. Coumarins have blood-thinning, anti-fungal and anti-tumour activities. They are known to increase the blood flow in the veins and to decrease capillary permeability. Coumarin can be toxic when used at high doses for a long period. Coumarin seems to work as a pesticide in the plants that produce it. Plants also use them as growth inhibitors (anti-auxins) as well as defence compounds. Certain types of coumarins, especially 4-hydroxycoumarins (figure 2.13), are used as medicines in strictly controlled form [30].

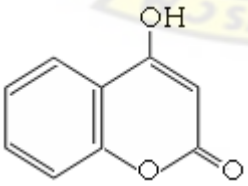
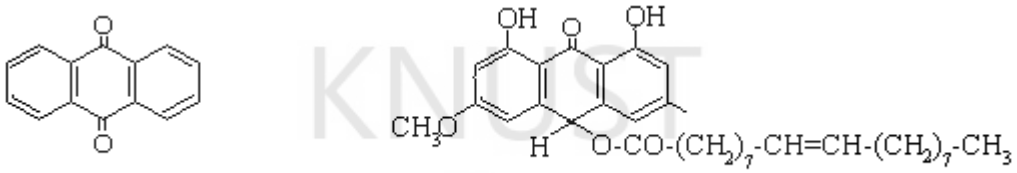


Fig 2.13: 4-Hydroxycoumarin

2.2.6 Anthraquinones

Anthraquinones (figure 2.14) occur in various types of plant materials and may occur as free anthraquinones, or as glycosides. Natural products have also been found to contain reduced derivatives of anthraquinones. They are oxanthrones (an ether form shown in figure 2.14), anthranols and anthrones and compounds formed by union of two conthrone molecules.



Anthraquinone Revandchinone-1

Fig 2.14: Anthraquinone and an oxanthrone

A number of glycosides with aglycone related to anthracene are present in drug as *cascara sangrada*, frangula and chrysarobin. With the exception of chyrarobin, which is too irritating, these drugs are employed as cathartics [38].

Anthraquinone derivatives are sometimes orange-red compounds that may be obscured in sight. They are usually soluble in hot water and dilute alcohol and are known to have antibacterial and antifungal properties [38]. Bomtrager's test is used for their detection [39].

2.2.7 Glycosides

Glycosides are compounds that yield one or more sugars among the products of hydrolysis. They are acetals in which the hydroxyl of sugars is condensed with a hydroxyl group of a non-sugar component, and a secondary hydroxyl is condensed within the molecule itself to form an oxide ring. More simply, glycosides can be considered as sugar-ethers [28] consisting of non-sugar and a component sugar in the

same molecule [39]. The sugar and the non-sugar components are known as *aglycone* and *glycone* respectively. Since sugars exist in isomeric α- and β-forms, both α- and β-glycosides are theoretically possible depending on the stereoconfiguration of the glycosidic linkage. However, all natural glycosides are practically of the β-type, even though the α-linkage is found in some carbohydrates like sucrose, glycogen and starch. The type of glycosides described above (involving oxygen linkages) occurs most in nature and are normally known as O-glycosides. Other glycosides do, however, occur in which the linkage is through sulphur (S-glycoside), nitrogen (N-glycoside) or carbon (C-glycoside) [39].

Glycosides play many important roles in the life of the plant and are involved in its protective, regulatory, and sanitary functions. They are used as therapeutic active agents as they contribute to almost every therapeutic class [31, 39].

Some glycosides contain more than one saccharide group, possibly as di- or trisaccharides. The most common sugar is the D-glucose, although the presence of other sugars as rhamnose and rutinose are quite possible. Rare sugars, such as digtose, digitalose and cymarose are also occasionally found. All natural glycosides are hydrolysed into a sugar and another organic compound by boiling with mineral acids; however, they vary widely in ease with which these hydrolyses are performed. Upon proper conditions, one or more of the saccharide groups can be removed from such compounds, resulting in glycosides of simpler structures [28]. An example is the conversion of amygalin to mandelonitrile as shown below.

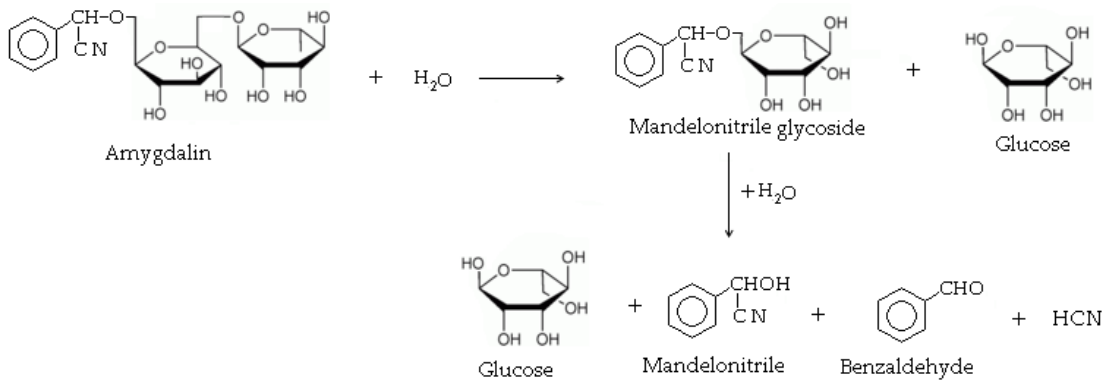


Fig 2.15: Hydrolysis of Amygdalin

These groups of compounds are so large that they are very difficult to classify. They may be classified by the glycone, by the type of glycosidic bond, or by the aglycone. For the classification based on the glycone, for instance, if the glycone group of a glycoside is glucose, then the molecule is a glucoside; if it is fructose, then the molecule is a fructoside. The molecule is a glucuronide, if the glycone group is glucuronic acid. In the body, toxic substances are often bonded to glucuronic acid to increase their water solubility; the resulting glucuronides are then excreted [30].

Depending on whether the glycosidic bond lies "above" or "below" the plane of the cyclic sugar molecule, glycosides are classified as *α-glycosides* or *β-glycosides*. This classification is based on the type of glycosidic bond. Some enzymes such as α-amylase can only hydrolyze α-linkages; others, such as emulsin, can only affect β-linkages [28].

When the chemical nature of the aglycone group is used as the basis of systematization, the classification is as follows: anthraquinone group, cardioactive group, tannins, aldehyde group, alcohol group, saponins group, lactone group, cyanophore group, isothiocyanate group, phenol group, flavonol group [28]. A few of these groups are discussed briefly below:

2.2.7.1 Anthraquinone glycosides

These glycosides contain an aglycone group that is a derivative of anthraquinone (figure 2.14). The glycosides, upon hydrolysis yield aglycones that are di-, tri- or tetrahydroxyanthraquinones or modifications of these compounds. A typical example is fragulin, which hydrolyses to form emodin (1, 6, 8-trihydroxy-3-methylanthrquinone) and rhamnose. Anthraquinone glycosides are mostly present in senna, rhubarb and aloes, frangula, chrysarobin and *caseara sagrada*; they have laxative effects and some are employed as cathartics [28].

Glycoside of anthranols and anthrons, reduced derivatives of anthraquinones, also occur in plants materials, and they make significant contributions to the therapeutic actions of these natural products. The free anthraquinone aglycones exhibit little therapeutic activities. The sugar residue facilitates absorption and translocation of the aglycone to the site of action [28].

2.2.7.2 Saponins glycosides

This group of glycosides is widely distributed in the higher plants, including the rhizome and root of *Glycyeehiza glabra* Linne (Spanish licorice). Various species of Dioscorea (Mexican yams) also present rich sources of principles used as cortisone precursors [36]. Saponin glycosides are divided into two types based on the chemical structure of their aglycones (sapogenins) – neutral and acid saponins [32]. The neutral saponins are derivatives of steroids with spiroketal side chains. The acid saponins possess triterpenoid structures.

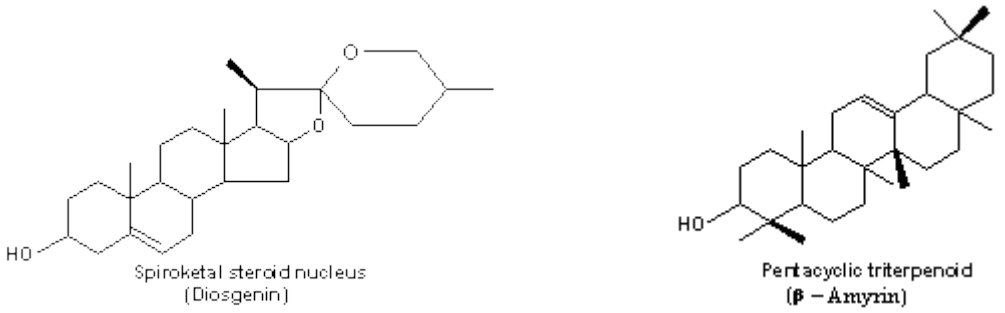


Fig 2.16: Structural types of sapogenins

A typical and common example, as mentioned above, is Glycyrrhiza (figure 2.17), which is the dried rhizome and roots of *Glycyrrhiza glabra* (licorice). Glycyrrhiza contains a saponin glycoside called Glycyrrhizin which is the Ca^{2+} and K^{+} salts of glycyrrhizinic acid.

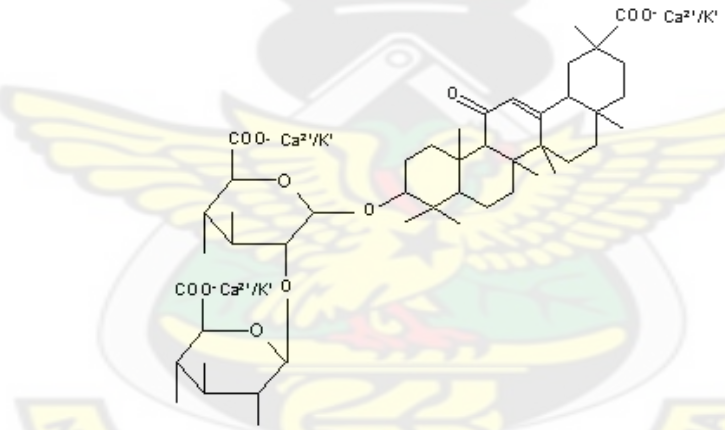


Fig 2.17: Glycyrrhiza

Glycyrrhizinic acid is 50 times sweeter than sugar (sucrose). The glycoside loses its sweet taste upon hydrolysis, and is converted to the aglycone, glycyrrhetinic acid plus two molecules of glucuronic acid. Glycyrrhetinic acid is a pentacyclic triterpenoid derivative of the beta-amyrin type. It is used considerably as a flavouring agent and is frequently employed to mask the taste of bitter drugs such as aloe, quinine etc and has expectorant and antitussive properties. Glycyrrhetinic acid inhibits the enzymes (15-hydroxyprostaglandin dehydrogenase and delta 13-

prostaglandin) that metabolise the prostaglandins, PGE_2 and PGF_{2alpha} to their respective 15 keto-13, 14-dihydro metabolites which are inactive.

2.2.7.3 Cyanophore (Cyanogenic) glycosides

The aglycone of this group contains a cyanide group, and the glycoside can release the poisonous hydrogen cyanide if acted upon by some enzyme. They yield hydrocyanic acid on hydrolysis. The group is mostly represented by amygdalin (figure 2.18), found in large quantities in bitter almonds. Cyanogenic glycosides can be found in the fruits (and wilting leaves) of the rose family (including cherries, apples, plums, almonds, peaches, apricots, raspberries, and crabapples) and many seeds of the *Rosacae* [29]. Cyanogenic glucosides are also common in cassava, and therefore have to be washed and ground under running water prior to consumption. Another example is prunasin (figure 2.18), which occurs in *Prunus serotina*.

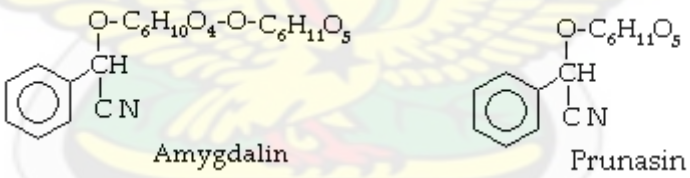


Fig 2.18: Cyanogenic glycosides

2.2.7.4 Isothiocyanate glycosides

The aglycones of this group are isothiocyanates and are present in the seeds of several cruciferous plants. These aglycones may be either aliphatic or aromatic derivatives. Principal among these are sinigrin (from black mustard, shown in figure 2.19), sinalbin (from white mustard, shown in figure 2.19) and gluconapin (from rape seed) [28]. Most of these glycosides are known to have anticancer properties. Sinalbin,

for instance, is believed to prevent cancer of the colon if foods containing it are eaten regularly.

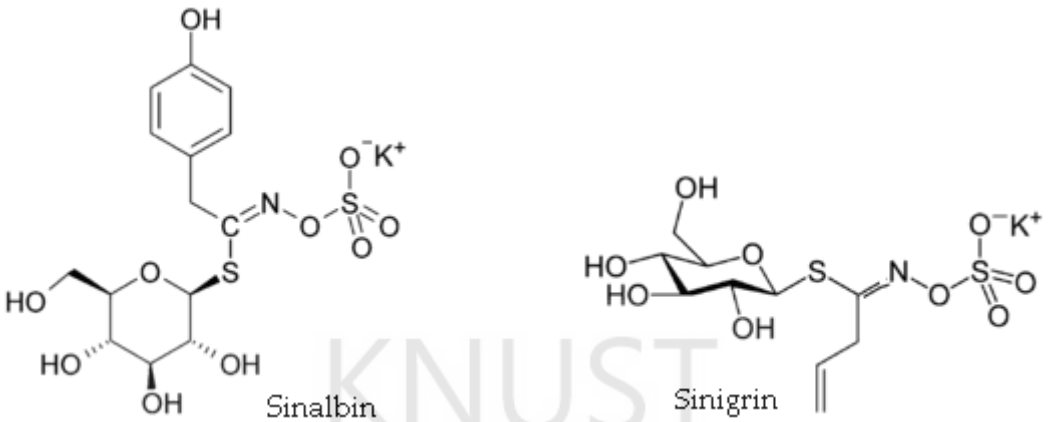


Fig 2.19: Isothiocyanate glycosides

2.2.7.5 Flavonol glycosides

Here the aglycone is a flavonoid. A large number of different flavonol glycosides occur in nature, and these are widely distributed throughout the higher plants. The best known flavonol glycosides include: Hesperidin (Hesperetin and Rutinose), Naringin (Naringenin and Rutinose), Rutin (Quercetin and Rutinose), Quercitrin (Quercetin and Rhamnose) [29].

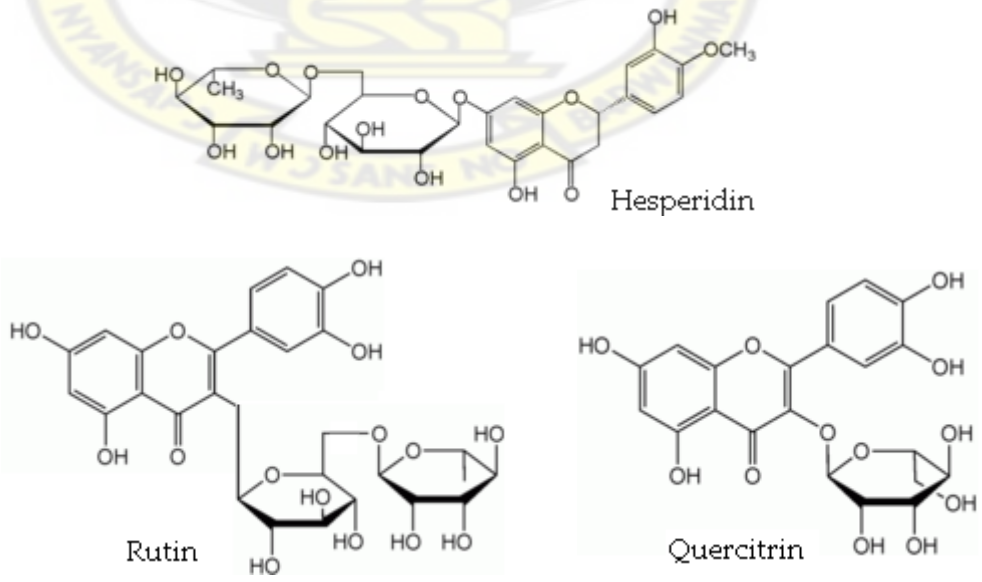


Fig 2.20: Flavonol glycosides

Among the important effects of these glycosides is their antioxidant effect. They are also known to decrease capillary fragility [30].

2.2.7.6 Cardio active (Cardiac) Glycosides

The aglycone here is part of a steroidal nucleus. Two classes have been observed in nature - the cardenolides and the bufadienolides (figure 2.21). The cardenolides have an unsaturated butyrolactone ring while the bufadienolides have an α-pyrone ring [30].

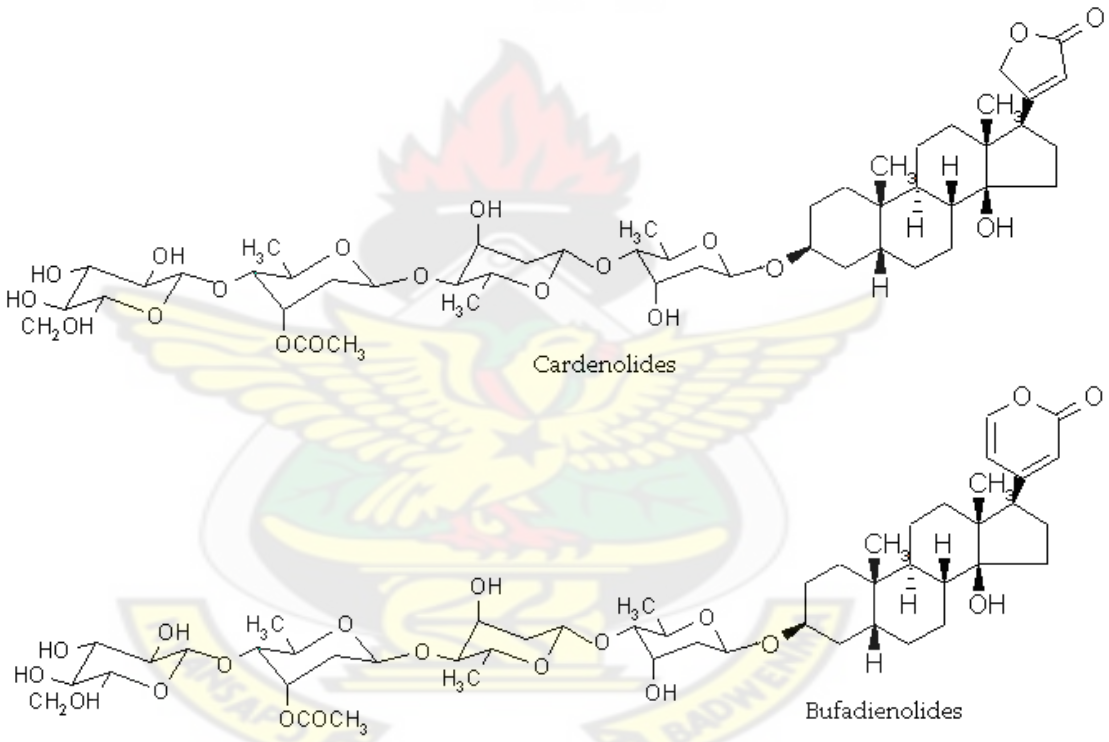


Fig 2.21: Classes of cardiac glycosides

The cardiac glycosides are found in the plant genera *Digitalis, Scilla,* and *Strophanthus*. They occur mainly in plants from which the names have been derived. *Digitalis purpurea*, *Digitalis lanata*, *Strophanthus grtus*, and *Strophanthus kombe* are the major sources of the cardiac glycosides. Some common examples include: digitoxin (figure 2.22), digoxin and gitoxin [30].

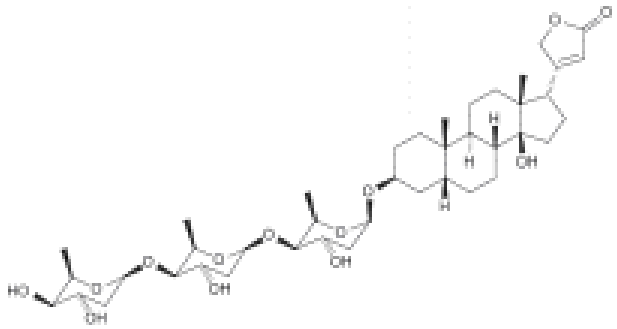


Fig 2.22: Digitoxin

The cardiac glycosides are an important class of naturally occurring drugs whose actions include both beneficial and toxic effects on the heart. Plants containing cardiac steroids have been used as poisons and heart drugs at least since 1500 B.C. Throughout history these plants or their extracts have been variously used as arrow poisons, emetics, diuretics, and heart tonics. Cardiac steroids are widely used in the modern treatment of congestive heart failure and for treatment of arterial fibrillation and flutter.

2.2.7.7 Alcohol glycosides

The aglycones of these glycosides are alcohols. An example of an alcoholic glycoside is salicin (figure 2.23) which is found in several species of *Salix* and *Popurea* but the principal sources are *salix pupurea* and *salix fragilis*. Salicin is converted in the body into salicylic acid, which is closely related to aspirin and has analgesic, antipyretic and anti-inflammatory effects. Populin (benzoyl salicin) is also associated with salicin and is found in the bark of the *salicaeae* [28].

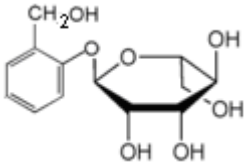


Fig 2.23: Salicin

2.2.8 Terpenoids and Steroids

Terpenes are derived biosynthetically from units of isoprene, which has a molecular formula of C_5H_8. The basic molecular formulae of terpenes, then, are multiples of it, $(C_5H_8)_n$, where n is the number of linked isoprene units [31].

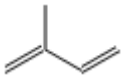


Fig 2.24: Isoprene unit

The isoprene units may be linked together "head-to-tail" to form linear chains or they may be arranged to form rings.

Terpenoids are relatively volatile, this has several important consequences. Firstly they are easily isolated by distillation from volatile plant materials. Secondly, they have distinctive and sometimes pleasant aromas. For this reason they have been extensively used in the flavour and fragrance industries. The biological functions of terpenoids are not known, in some cases they seem to protect the plant from the animals through their disagreeable taste and odour, but no general role has been established [32].

Terpenes are subdivided or classified into – monoterpenes, sesquiterpenes, diterpenes, sesterpenes, triterpenes, tetraterpenes, and rubber – depending upon the total number of carbon atoms or isoprene units in the molecule [28-34].

Monoterpenes consist of two isoprene units and have the molecular formular $C_{10}H_{16}$. They may be linear (acyclic) or may contain rings. Monoterpenes include: linalol, nerol, geraniol, myrcene, ocimene, alloocimene, menthol, limonene (see figure 2.25 below)

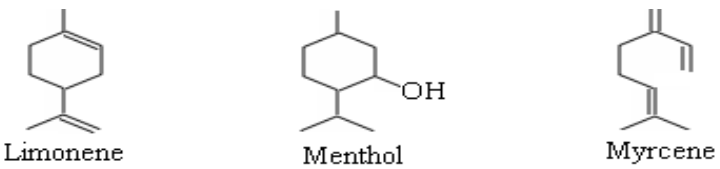
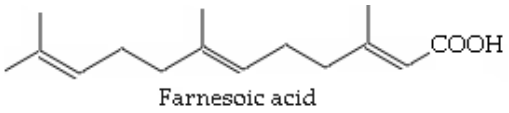


Fig 2.25: Monoterpenes

Sesquiterpenes are fifteen (15) carbon compounds derived by the assembly of three (3) isoprenoids units and are found mainly in higher plants but also in several invertebrates, with a molecular formula of $C_{15}H_{24}$. They present several acyclic, mono-. di-, tri- and tetracyclic systems. Some of the natural sesquiterpenes are: farnesoic acid, methyl farnesoate, juvenile hormone III, farnesol and nerolidol.

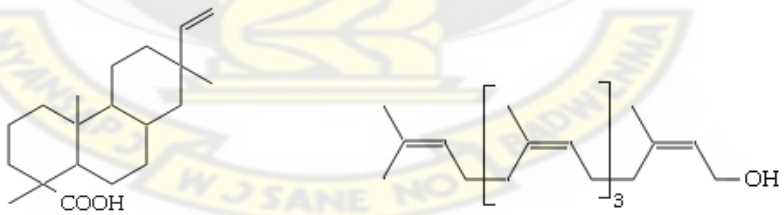


Farnesoic acid

Fig 2.26: Sesquiterpene

Diterpenes are composed of four isoprene units and have the molecular formula $C_{20}H_{32}$. They are derived from geranyl pyrophosphate. Examples of diterpenes are cembrene, taxadiene, abietic acid, kaurene, marrubin, and taxisin. Diterpenes also form the basis for biologically important compounds such as retinol, retinal and phytol.

Sesterpenes are also derived from geranyl pyrophosphate and have 25 carbon atoms. They were isolated from insect protective waxes and from fungal sources. They include gascardic acid, geranylfarnesol and pimeric acid.



Pimeric acid Geranylfarnesol

Fig 2.27: Examples of sesterpenes

Triterpenes form a large group of natural substances which include steroids and consequently sterols. They are made up of six isoprene units having 30 carbon atoms. Squalene is the immediate biological precursor of all triterpenoids. Other examples are cholesterol and lanosterol (figure 2.28).

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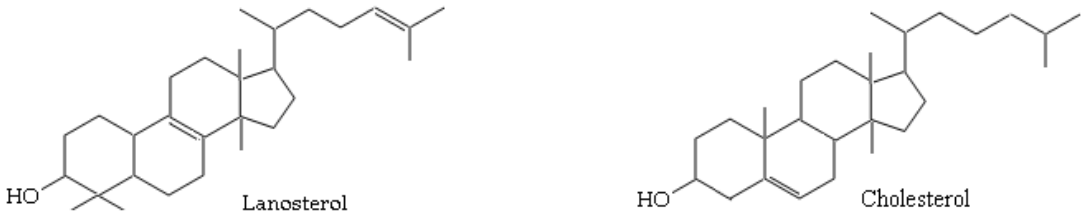


Fig 2.28: Triterpenes

Steroids are modified triterpenoids which are also derived from squalene by cyclisation, unsaturation and substitution. The nucleus of all steroids is the tetracyclic C17 hydrocarbon 1, 2 cyclopentanoperhydrophenanthrene (gonane or sterane) substituted by methyl groups at C10 and C13, as well as alkyl side-chain at C17. Steroids may possess a nucleus derived from the former one by one or more C-C bond scissions or ring expansion or contractions.

The diversity of biologic activities of steroids includes the development and control of the reproductive tract in humans, the moulting in insect (ecdysis) and the induction of sexual reproduction in aquatic fungi (antheridiol). In addition steroid contributes to a varied range of therapeutic applications such as cardiotonics (digitoxin), Vitamin D precursors (ergosterol), oral contraceptive agents (some synthetic estrogens and progestins), anti-inflammatory agents (corticosteroids) and anabolic agent (androgens) [30, 31].

2.2.9 Carotenoids

Carotenoids are a class of yellow, red or red natural fat-soluble pigments [31] (lipochromic pigments) found principally in plants, algae, and photosynthetic bacteria, where they play a critical role in the photosynthetic process. They also occur in some non-photosynthetic bacteria, yeasts, and molds, where they may carry out a protective function against damage by light and oxygen. In animal organism,

carotenoids are either dissolved in fats or combined with protein in the aqueous. In higher plants, the carotenoids are found in leaves together with chlorophyll [31]. Although animals appear to be incapable of synthesizing carotenoids, many animals incorporate carotenoids in their diet. Within animals, carotenoids provide bright coloration, serve as antioxidants, and can be a source for vitamin A activity [30].

Carotenoids are responsible for many of the red, orange, and yellow hues of plant leaves, fruits, and flowers, as well as the colors of some birds, insects, fish and crustaceans, and of many microorganisms [28]. In general, the greater the intensity of colour, the higher the level of carotenoids. In green leafy vegetables, beta-carotene is the predominant carotenoid. In the orange-coloured fruits and vegetables - such as carrots, apricots, mangoes, yams, winter squash - beta-carotene concentrations are high, but other pro-vitamin A carotenoids typically predominate. Yellow vegetables have higher concentrations of yellow carotenoids (xanthophylls), hence a lowered pro-vitamin A activity; but some of these compounds, such as lutein, may have significant health benefits, potentially due to their antioxidant effects. The red and purple vegetables and fruits - such as tomatoes, red cabbage, berries, plums, flamingoes and salmon [30] - contain a large portion of non-vitamin A–active carotenoids. Legumes, grains, and seeds are also significant sources of carotenoids. Carotenoids are also found in various animal foods, such as salmon, egg yolk, shellfish, milk, and poultry. A variety of carotenoids is also found in carrot juice and "green drinks" made from vegetables, dehydrated barley greens, or wheat grass [30].

Carotenoids are defined by their chemical structure. The majority of carotenoids are derived from a 40-carbon polyene chain, which could be considered the backbone of the molecule. This chain may be terminated by cyclic end-groups (rings) and may be complemented with oxygen-containing functional groups. The

hydrocarbon carotenoids are known as *carotenes*, while oxygenated derivatives of the carotenes are known as *xanthophylls*. The carotenes which are soluble in petroleum ether [28] are α-carotene, β-carotene and γ-carotene (lycopene). These are shown below.

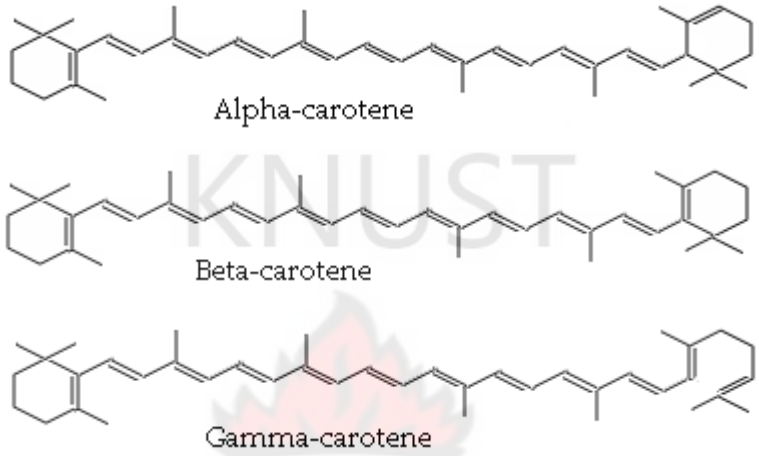
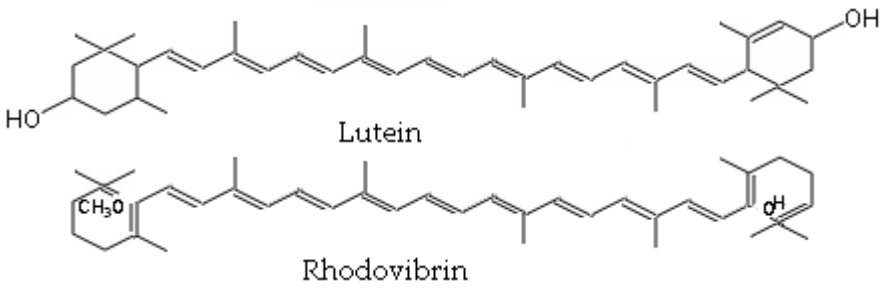


Fig 2.29: Some Carotenes

The xanthophylls, which are alcohols, aldehydes, ketonesepoxides,a and acids are soluble in ethanol. They comprise: *hydroxylated Carotenoids* (crytoxanthin, lutein, the major yellow pigment of marigold petals and zeaxanthin); *Methoxylated Carotenoids*, which are lycopene derivatives (e.g. rhodovibrin); *Oxocarotenoids* (capsanthin and rhodoxanthin); *epoxycarotenoids*, which exist in nature as 5, 6- and 5, 8-epoxides or both (examples, violaxanthin, flavoxanthin and luteochrom), and *Carboxycarotenoids* (bixin and crocetin) [30].



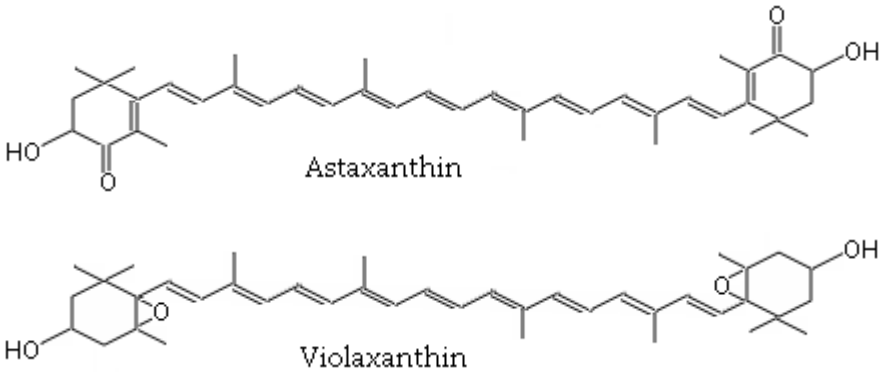


Fig 2.30: Structures of selected Xanthophylls

The structure of a carotenoid ultimately determines what potential biological function(s) that pigment may have. The distinctive pattern of alternating single and double bonds in the polyene backbone of carotenoids is what allows them to absorb excess energy from other molecules, while the nature of the specific end groups on carotenoids may influence their polarity. The former may account for the antioxidant properties of biological carotenoids, while the latter may explain the differences in the ways that individual carotenoids interact with biological membranes [30].

In human beings, carotenoids can serve several important functions. The most widely studied and well-understood nutritional role for carotenoids is their provitamin A activity. Deficiency of vitamin A is a major cause of premature death in developing nations, particularly among children. Vitamin A, which has many vital systemic functions in humans, can be produced within the body from certain carotenoids, notably beta-carotene. Dietary beta-carotene is obtained from a number of fruits and vegetables, such as carrots, spinach, peaches, apricots, and sweet potatoes [29]. Other provitamin A carotenoids include alpha-carotene (found in carrots, pumpkin, and red and yellow peppers) and cryptoxanthin (from oranges, tangerines, peaches, nectarines, and papayas).

Carotenoids also play an important potential role in human health by acting as biological antioxidants, protecting cells and tissues from the damaging effects of

free radicals and singlet oxygen. Lycopene is particularly effective at quenching the destructive potential of singlet oxygen. Lutein and zeaxanthin, xanthophylls found in corn and in leafy greens such as kale and spinach, are believed to function as protective antioxidants in the macular region of the human retina [31] and hence improving vision. Astaxanthin, a xanthophyll found in salmon, shrimp, and other sea foods, is another naturally occurring xanthophyll with potent antioxidant properties[29].

Other health benefits of carotenoids that may be related to their antioxidative potential include enhancement of immune system function, protection from sunburn, and inhibition of the development of certain types of cancers. They have also been shown to prevent colds and flu, support successful pregnancy, and ward off diabetes. Their antioxidant effects enable these compounds to play a crucial role in protecting plants against damage during photosynthesis [28, 34].

2.3 REACTIVE OXYGEN SPECIES AND ANTIOXIDANTS

The important roles of reactive oxygen species in diseases related to ageing and the necessity and benefits of nutraceuticals in the prevention of diseases and the promotion of healthy ageing have been an area of interest in recent years. This section reviews what they are and some examples.

2.3.1 REACTIVE OXYGEN SPECIES (ROS) [40]

Oxygen derived species such as superoxide radical, hydrogen peroxide, singlet oxygen and hydroxyl radical are well known to be cytotoxic and have been implicated in the etiology of a wide array of human diseases. ROS are also implicated in diabtes and neurodegenerative diseases.

Reactive oxygen species (ROS) are derived from the metabolism of molecular oxygen. ROS include superoxide anion radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($^{\bullet}OH$). The deleterious effects of oxygen are said to result from its metabolic reduction to these highly reactive and toxic species. ROS normally exist in all aerobic cells in balance with biochemical antioxidants. Oxidative stress occurs when this critical balance is disrupted because of excess ROS, antioxidants depletion, or both. To counteract the oxidant effects and to restore redox balance, cells must reset important homeostatic parameters.

In living cells, the major sources of endogenous ROS are hydrogen peroxide and superoxide anion, which are generated as by-products of cellular metabolism such as mitochondrial respiration. Alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase. Variability or inductive changes in the expression of these enzymes can significantly influence cellular redox potential. ROS can cause tissue damage by reacting with lipids in cellular membranes, nucleotides in DNA, sulphydryl groups in proteins and cross-linking/fragmentation of ribonucleoproteins. The relatively unreactive superoxide anion radical is converted by superoxide dismutase (SOD) into H_2O_2, which in turn take part in the "Fenton reaction", with transition metal ion (copper or iron) as catalysts, to produce the very reactive hydroxyl radical.

There is growing awareness that oxidative stress plays a role in various clinical conditions such as malignant diseases, diabetes, atherosclerosis, chronic inflammation, viral infection, and ischemia-reperfusion injury. ROS can cause oxidative DNA and protein damage, damage to tumor suppressor genes and enhanced expression of proto-oncogenes and oxidative stress has been shown to

induce malignant transformation of cells in culture. Diseases associated with oxidative stress such as diabetes mellitus and cancer show a pro-oxidative shift in the redox state and impaired glucose clearance suggesting that muscle mitochondria is the major site of elevated ROS production. This condition may be referred to as 'mitochondrial oxidative stress'.

Chronic hepatitis B (HBV) and hepatitis C virus (HCV) infections are associated with an increased production of ROS within the liver that is responsible for the oxidation of intracellular macromolecules. Infection with these viruses can also affect the host cell pro-/antioxidant balance by increasing cellular pro-oxidants such as iron and nitric oxide and also by inhibiting the synthesis of antioxidant enzymes. Antioxidants, together with agents interfering with the harmful effects of cytokines and lipid mediators, may have a role in the treatment of viral diseases. ROS may facilitate or even promote replication of many viruses, depending on the cell and type of virus involved. Enhanced oxidative stress modulates the HCV RNA replication and hepatic cell survival via activation of oncogenic transcription factors that leads to the generation of hepatocellular carcinoma. Redox-sensitive kinases, Src, JAK, PI3K-Akt and MAPK (Erk, JNK) regulate transcription factors through phosphorylation of the protein modules. Chronic HBV infection results in an increased total intrahepatic iron and/or increase in the pro-oxidant lowmolecular weight iron compartment of the liver. Previously, a strong correlation between the presence of HBV surface antigen and iron deposition in the Kupffer cells and spleens of infected individuals has been reported.

HIV-seropositive humans exhibit decreased concentrations of naturally occurring antioxidant reductants such as total acid-soluble thiols, cysteine, and glutathione in plasma, peripheral blood monocytes, and lung epithelial-lining fluids.

In addition, elevated levels of hydroperoxides and malondialdehyde are found in plasma of HIV-infected individuals. In cell culture system, ROS promotes replication of HIV, and antioxidants such as NAC inhibit the replication of the virus. Oxidative stress has been reported to affect the cellular protein kinase/phosphatase balance, which is described in a number of tumors. The exogenous oxygen radical load is contributed by a variety of environmental agents (inhaled smoke and polluted air) and dietary antioxidants. Mutagens, tumor promoters and a variety of carcinogens including benzene, aflatoxin and benzo(a)pyrene may be exerted partly by generating ROS during their metabolism.

Free radicals are atomic or molecular species with unpaired electrons on an otherwise open shell configuration. These unpaired electrons are usually highly reactive, so they are likely to take part in chemical reactions. Radicals play an important role in combustion, atmospheric chemistry, polymerization, plasma chemistry, biochemistry, and many other chemical processes, including human physiology. Some of these are discussed in the subsections below.

2.3.1.1 Hydroxyl radical ($^{\cdot}OH$)

Fenton's reaction for production of hydroxyl radicals by oxidation of Fe^{2+} ions is well known [41]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}.$$

It is known to be the most reactive free radical and has the highest 1-electron reduction potential (2310mV). In general, aromatic compounds or compounds with carbon-carbon multiple bonds undergo addition reactions with hydroxyl radicals resulting in the hydroxylated free radicals. When it reacts with aromatic compound, it adds across a double bond, resulting in hydroxycyclohexadienyl radical [42]. In

saturated compounds, a hydroxyl radical abstract a hydrogen atom from the weakest C-H bond to yield a free radical [43]. The resulting radicals can react with oxygen and generate other free radicals. Hydroxyl radicals also react with lipid, polypeptides and DNA and also especially with thiamine and gaunosine [42].

2.3.1.2 Superoxide anions

Superoxide is the anion $\cdot O_2^-$. It is important as the product of the one-electron reduction of dioxygen, which occurs widely in nature. With one unpaired electron, the superoxide ion is a free radical, and, like dioxygen, it is paramagnetic.

Superoxide anion is a reduced form of molecular oxygen created by receiving one. It is formed from mitochondrial electron system. Mitochondria generates energy using four electron chain reaction, reducing oxygen to water. Some of the electrons escaping from the chain reaction of mitochondria directly react with oxygen and form superoxide anions [40].

$$O_2 \xrightarrow[H^+]{e^-} HO_2 \xrightarrow[H^+]{e^-} H_2O_2 \xrightarrow[H^+]{e^-} OH + H_2O \xrightarrow[H^+]{e^-} 2H_2O$$

The superoxide plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical or single oxygen in living systems.

$$2O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$$

The superoxide anion can react with nitric oxide ($NO^{\cdot}$) and form peroxynitric oxide ($OONO^-$), which can generate toxic components such as hydroxyl radical and nitric dioxide.

$$OONO^- + H^+ \longrightarrow {}^{\cdot}OH + NO^{\cdot}{}_2$$

2.3.1.3 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a very pale blue liquid which appears colourless in a dilute solution, slightly more viscous than water. It is a weak acid. It has also found use as a disinfectant, as an oxidizer, and in rocketry (particularly in high concentrations as high-test peroxide (HTP) as a monopropellant), and in bipropellant systems.

Hydrogen peroxide can be generated through a dismutation reaction from superoxide anion by superoxide dismutase. Enzymes such as amino acid oxidase and xanthine oxidase also produce hydrogen peroxide from superoxides. Hydrogen peroxide is highly diffusible and crosses the plasma membrane easily [40].

Hydrogen peroxide is the least reactive among the reactive oxygen species and is stable under physiological pH and temperature in the absence of metal ions. It is a weak oxidizing and reducing agent and thus regarded as being poorly reactive. Hydrogen peroxide can generate the hydroxyl radical in the presence of metal ions and superoxide anion.

$$\cdot O_2 + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2$$

It can produce singlet oxygen through reaction with superoxide anion or with HOCl or chloroamine in living system [44]. Hydrogen peroxide can degrade certain heme proteins such as haemoglobin, to release iron ions.

2.3.1.4 Singlet oxygen

Singlet oxygen is a nonradical and excited species, with the electrons in the π-antibonding orbitals paired. It can be formed from hydrogen peroxide as described under section 2.3.1.3 above.

Singlet oxygen is rather mild and non-toxic for mammalian tissues compared with other ROS [44]. However, it has been known to be involved in cholesterol oxidation, which results in the formation of 5α-OOH (3β-hydroxy-5α-cholest-6-ene-5-hydroxyperoxide)

2.3.1.5 Peroxyl and alkoxyl radicals

Peroxyl radicals ($ROO^{\cdot}$) are formed by a direct reaction of oxygen with alkyl radicals ($R^{\cdot}$), for example, the reaction between lipid radicals and oxygen. Decomposition of alkyl peroxides (ROOH) also result in peroxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals. Irradiation of UV light or the presence of metal can cause decomposition of peroxides to produce peroxyl and alkoxyl radicals.

$$ROOH \xrightarrow{uv} ROO^{\cdot} + H^{\cdot}$$

$$ROOH + Fe^{3+} \longrightarrow ROO^{\cdot} + Fe^{2+} + H^{+}$$

Peroxyl and alkoxyl radicals are good oxidizing agents, having more than 1000mV of standard potential. They can abstract hydrogen from other molecules with lower standard potential. This is frequently observed in the propagation state of lipid peroxidation. Very often, the alkyl radical formed from this reaction can react with oxygen to form another peroxyl radical, resulting in chain reaction. Some peroxyl radicals break down to liberate superoxide anion or can react with each other to generate singlet oxygen [45]. Aromatic alkoxyl and peroxyl radicals are less reactive than respective open chain radicals because of the delocalization of electrons in the ring.

2.3.1.6 Nitric oxide and nitric dioxide

Nitric oxide ($NO^{\cdot}$) is a free radical with a single unpaired electron. It is formed from L-arginine [46]. Nitric oxide itself is not very reactive free radical, but the over production of $NO^{\cdot}$ in ischemia reperfusion causes neurodegenerative and chronic inflammatory disease such as rheumatoid arthritis and inflammatory bowel disease.

2.3.1.7 Peroxynitrite

Reactions of nitric oxide and superoxide generate peroxynitrite.

$$O_2^{\cdot} + NO \longrightarrow OONO^-$$

Peroxynitrite is a cytotoxic species and oxidizes low-densidty lipoprotein (LDL). It appears to be an important tissue-damaging species generated at the sites of inflammation and has been shown to be involved in various neurodegenerative disorders and several kidney diseases [40].

Peroxynitrite can cause direct protein oxidation and DNA base oxidation and modification acting as a “hydroxyl radical-like” oxidant. The significance of peroxynitrite as a biological oxidant comes from its high infusibility across cell memebrane. Nitrotyrosine, which can be formed from peroxynitrite-mediated reactions with amino acids, has been formed in age-associated tissues.

2.3.2 ROS IN PLANTS [35]

In plants, reactive oxygen species (ROS) are continuously produced as by-products of various metabolic pathways that are localized in different cellular compartments. Under physiological steady-state conditions, these molecules are scavenged by different antioxidative defence components that are often confined to

particular compartments. The balance between production and scavenging of ROS may be perturbed by a number of adverse environmental factors. As a result of these disturbances, intracellular levels of ROS may rapidly rise. External conditions that adversely affect the plants can be biotic, imposed by other organisms, or abiotic, arising from an excess or deficit in the physical or chemical environment. In the presence of transition metal ions, hydrogen peroxide may be reduced by superoxide to hydroxyl radicals. Since there are no known scavengers of hydroxyl radicals, the only way to avoid oxidative damage through this radical would be to control the reactions that lead to its generation. Thus, cells had to evolve sophisticated strategies to keep the concentrations of superoxide, hydrogen peroxide, and transition metal ions such as Fe^{2+}, Fe^{3+}, and Cu^{2+} under tight control. These include:

(a) Nonenzymatic antioxidants such as ascorbate and glutathione (GSH), but also tocopherol, flavonoids, alkaloids, and carotenoids. Ascorbate and GSH are major cellular redox buffers. Mutants with decreased ascorbic acid levels or altered GSH content are hypersensitive to stress.

(b) Enzymatic ROS scavenging mechanisms in plants like superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). SODs act as the first line of defense against ROS, dismutating superoxide to H_2O_2. APX, GPX, and CAT subsequently detoxify H_2O_2.

Among the different ROS, only H_2O_2 can cross plant membranes and can therefore directly function in cellto-cell signalling. Moreover, cells have evolved several mechanisms for rapid and controllable ROS production and removal.

2.3.3 ANTIOXIDANTS

As the name implies, antioxidants are substances (including nutrients, phytochemicals and enzymes) that are capable of counteracting the damaging, but normal, effects of the physiological process of oxidation caused by free radicals in animal tissue. They are believed to play a role in preventing the development of such chronic diseases as cancer, heart disease, stroke, Alzheimer's disease, Rheumatoid arthritis, and cataracts [47].

The process of oxidation causes the skin to lose elasticity and develop wrinkles, thins hair, weakens muscles, impairs hearing and eyesight, decreases immunity, and increases degenerative diseases. Antioxidants stop this process and maintain the health of our bodies. Antioxidants serve as a skin barrier to prevent free radicals from penetrating the living dermal layer. The highest concentration of antioxidants in the body is found in the outermost layer of skin, the epidermis. As we age, the concentration of antioxidants in the epidermis starts decreasing considerably. This is one reason that skin starts showing signs of damage [47].

2.3.3.1 The Antioxidant Process

Antioxidants block the process of oxidation by neutralizing free radicals. In doing so, the antioxidants themselves become oxidized. That is why there is a constant need to replenish our antioxidant resources.

How they work can be classified in one of two ways:

- *Chain-breaking* - When a free radical releases or steals an electron, a second radical is formed. This molecule then turns around and does the same thing to a third molecule, continuing to generate more unstable products. The process continues until termination occurs -- either the radical is stabilized by a chain-breaking antioxidant

such as beta-carotene and vitamins C and E, or it simply decays into a harmless product.

- *Preventive* - Antioxidant enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase prevent oxidation by reducing the rate of chain initiation. That is, by scavenging initiating radicals, such antioxidants can thwart an oxidation chain from ever setting in motion [47]. They can also prevent oxidation by stabilizing transition metal radicals such as Cu^{2+} and Fe^{3+}.

There are also varieties of antioxidants that, by reacting with oxygen free radicals, nullify their effects. These include α-tocopherol (vitamin E), tocotrienol, ascorbic acid (vitamin C), uric acid, sulphydryl containing compounds as cystein and glutathione, bilirubin, ubiquinol and carnosine [40].

L-Ascorbic acid is a 6-carbon lactone ring structure with 2, 3-enediol moiety. The antioxidant activity of ascorbic acid comes from the 2, 3-enediol. L-ascorbic acid first changes to semi-dehydroascorbic acid through donating one hydrogen and three electrons [47].

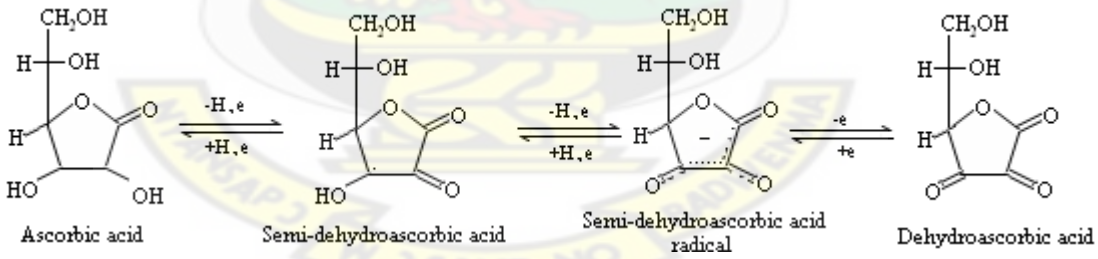


Fig 2.31: Sequential 1-electron oxidation of L-ascorbic acid

The antioxidant mechanisms of ascorbic acid are based on hydrogen atom donation to lipid radicals, quenching of singlet oxygen, and removal of molecular oxygen. Scavenging of aqueous radicals and regenerating of α-tocophenols from the

tocopheroxyl radical species are also known antioxidant mechanisms of ascorbic acid.

Tocopherols consist of a chroman ring and a long, saturated phytal chain. They are 2-methyl-2-(4?, 8?, 12?-trimethyl tridecyl) chroman-6-ols (figure 2.3).

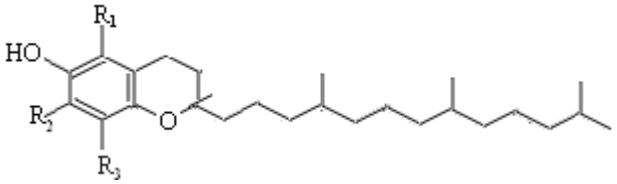


Fig 2.32: General structure of Tocopherols

Their antioxidant mechanisms also include the transfer of hydrogen atom at 6-hdroxy group on the chroman ring and scavenging of free singlet oxygen and other reactive species [48].

Again, carotenoids are the most efficient singlet oxygen quenchers in biological systems. The rate of singlet oxygen quenching by carotenoids is dependent on the number of conjugated double bonds and on the type of functional group on the ring structure of the molecule. To act as an efficient singlet oxygen quencher, at least seven conjugated bonds are required and as the number of conjugation increases, quenching efficiency also increases [49]. Singlet oxygen mechanisms by carotenoids are physical quenching without generating oxidizing products.

$$^{1}O_2 + {}^{1}\text{Carotenoid} \longrightarrow {}^{3}O_2 + {}^{3}\text{Carotenoid}$$

The effectiveness of any given antioxidant in the body depends on which free radical is involved, how and where it is generated, and where the target of damage is. Thus, while in one particular system an antioxidant may protect against free radicals, in other systems it could have no effect at all. Or, in certain circumstances, an antioxidant may even act as a "*pro-oxidant*" that generates toxic oxygen species [40].

2.3.3.2 Determination of antioxidant properties

The antioxidant activities of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging [50]. Various methods are used in determining the antioxidant properties of plant samples. These include: measurement of total phenolic content, reducing power, hydrogen peroxide decomposition / consumption, DPPH scavenging and Fe^{2+} chelation. Yildirim *et al* (2001) [50] has suggested non-linear correlation between total antioxidant activity and the individual measurement and that the antioxidant activity of any species is a cumulative effect of most (if not all) of the measurements. This subsection gives an overview of some these methods.

2.3.3.2.1 Reducing power determination

Heavy metals like Fe^{3+} and Cu^{2+} are known to catalyse oxidative process in living organisms. Fe^{3+}, for instance, is reduced to Fe^{2+} in the process. It follows that if the +2 state does not aid the oxidative process, then the process does occur. A species ability to reduce Fe^{3+} to the +2 state is known as it reducing power and it is an indication of its antioxidant property [50]. The Fe^{3+} reducing power of the samples is determined based on the chemical reaction of

Fe (III) → Fe (II).

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [50]. Oyaizu (1986) has described a dose-dependent method (which was modified by Yildirim *et al* [50] in 2001) for the determination of the reducing capacity of samples. In his method, various concentrations (100-1000

µg/ml) of the plant samples are prepared and mixed with phosphate buffer and 1% w/v of potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture is incubated at 50^oC for 30 minutes, after which 10% w/v trichloroacetic acid is added and centrifuged at 3000 rpm for 10 minutes. To about 2.5ml of the supernatant layer of the solution is added 2.5 ml distilled water and 0.5 ml of 0.1% w/v $FeCl_3$. The sample's ability to reduce the Fe (III) to Fe (II) is determined by measuring the amount of the Fe (II) spectroscopically; the absorbance of the reaction mixture is measured at 700 nm. Increased absorbance indicates increased reducing power [51].

2.3.3.2.2 Hydrogen peroxide decomposition/consumption [52]

The ability of plant samples to decompose or consume hydrogen peroxide is studied through iodometric titration. The assay mixture contains a known amount of H_2O_2 solution and 2ml of 1% w/v of the sample. The mixture is rapidly mixed by gentle swirling motion and about 1ml portion of the reaction mixture is allowed to run into a large volume (25ml) of water to slow down the consumption of H_2O_2 by the extracts at 60 minutes intervals for a period of four minutes. About 2g of KI and 5ml of 3M HCl are added and the resulting iodine solution is allowed to stand for about thirty minutes to get maximum I_2 generation and is then titrated with a standard solution of sodium thiosulphate.

The process is repeated for 4ml and 8ml of the 1% w/v of the samples. The decomposition/consumption of H_2O_2 is back calculated using the equations below.

$$H_2O_2 + 2KI + 2H^+ \longrightarrow I_2 + 2H_2O + K^+$$

$$I_2 + 2S_2O_3^{2-} \longrightarrow S_4O_6^{2-} + 2I^-$$

The effects of the concentration of the sample on the consumption of H_2O_2 are also examined by plotting the concentration of H_2O_2 against concentration of samples at the times at which the measurements were made.

2.3.3.2.3 DPPH scavenging ability

The antioxidant ability of a sample can also be estimated be determining the hydrogen donating ability of the samples in the presence of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical at 517 nm on the basis of the method of Hatano *et al* (1988) [53]. The determination is based on the discolouration of the purple coloured methanolic solution of DPPH free to yellow by free radical scavengers.

Fig 2.33: 1, 1-diphenyl-2-picrylhydrazyl (DPPH)

Representing the DPPH radical by $Z^{\bullet}$ and the donor molecule by AH, the primary reaction is

$$Z^{\bullet} + AH \rightarrow ZH + A^{\bullet}$$

Where ZH is the reduced form and $A^{\bullet}$ is free radical produced [54].

In the experiment, 1.0ml volume of the extract (of different doses) dissolved in 70% methanol is added to 3.0ml of DPPH in a test tube. The reaction mixture is kept at 25^{o}C for thirty minutes and the absorbance of the residual DPPH is determined at 517nm after the thirty minutes. The methanol solution is used as the blank, with n-propyl gallate (of different doses) as the standard.

The DPPH scavenging effect (% of control) is calculated using the equation:

$$\text{DPPH scavenging Effect (\% control)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{test}}}{\text{Abs}_{\text{control}}} \times 100$$

2.3.3.2.4 Chelation of Fe^{2+}

Another way of determining the antioxidant ability of a test sample is by ascertaining its iron chelating property. 1, 10 - phenanthroline is used as the chelating agent for the determination of free irons ions (Fe^{2+}) using a standard Fe^{2+} solution containing about 1ml conc. H_2SO_4.

1, 10 - phenanthroline chelate with Fe^{2+} through donation of the lone pair on the nitrogen as shown below

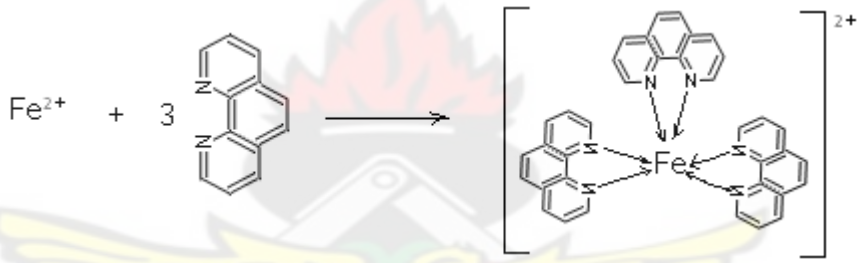


Fig. 2.34: 1, 10 - phenanthroline chelation of Fe^{2+}

To reduce all possible Fe^{3+} ions to Fe^{2+}, 2ml of 10% hydroxyl ammonium chloride solution is added to the Fe^{2+} solution. Different concentrations of the sample are mixed with the Fe^{2+} solution and then 4ml of 0.1% 1, 10-phenanthroline solution is added to chelate the free Fe^{2+} ions (left unchelated by the extracts) at a pH of 3.5. The pH is maintained at by using 10% sodium acetate buffer. Absorbances of ferrous-1, 10-phenanthroline complex are measured at 510.0nm against blanks devoid of ferrous sulphates [52].

2.4 THE HUMAN SKIN AND WOUND HEALING

This section reviews the structure of the human skin and its functions, wounds and cuts, and the healing mechanism.

2.4.1 THE HUMAN SKIN

The skin is the covering or integument of the body's surface that both provides protection and receives stimuli from external environment. It is believed to be the most accessible of all organs [18] in the human body. It is very elastic and sturdy [17] and known to be one of the largest organs in the body [18, 55] having a surface area of 1.8 m^2 and making up to about 16% of the body's weight [19] that of an average adult male weighs 4.5 to 5 kg. It covers the surface of the body at a thickness of just 1.4 to 4.0 mm. The skin is thickest on areas of the body that regularly rub against objects, such as the palms of the hands and the soles of the feet [55].

The skin is essential to a person's survival. Like other integumetary systems, it forms a barrier that helps prevent harmful micro organisms and chemicals from entering the body, and it also prevents the loss of life-sustaining body fluids. It protects the vital structures inside the body from injury and from the potentially damaging ultraviolet rays of the sun. The skin also helps regulate body temperature, excretes some waste products, and is an important sensory organ. It contains various types of specialized nerve cells responsible for the sense of touch and gives communication with the environment. It also cushions and insulates the body. Both delicate and resilient, the skin constantly renews itself and has a remarkable ability to repair itself after injury[17-19, 55].

2.4.2 WOUND HEALING

This subsection appraises what wounds and cuts are - the various classifications/types, ways of contamination and contaminants and leads to a short discussion on micro organisms and finally the healing mechanism itself.

2.4.2.1 Wounds and cuts

A wound is a disruption in the continuity and regulatory process of tissue cells; wound healing is the restoration of the continuity [17]. A cut (in contrast to puncture wounds) usually lies open, bleeds easily as in less likely to be come infected. Such a wound is caused by a knife, a razor, broken glass or any sharp edge. Wounds are normally classified as:

Incised wounds: these are made by a clean cut of sharp instrument, such as surgical incision with a scalpel.

Contused wounds: are those made by blunt force that typically does not break the skin but causes considerable tissue damage with bruising and swelling.

Lacerated wound: are made by an object that tears tissues producing jagged, irregular edges; e.g. include: glass, jagged wire, and blunt knife.

Puncture wounds: are also made by a pointed instrument, such as ice pick, bullet or nail [16, 21].

Contamination of a wound may occur at the moment of wounding or at anytime thereafter until healing is complete. These include nonbacterial contaminants, such as organic substance which tends to be more irritating. The critical factor in this case is the extent of the contamination. The other, which is of great importance – bacterial contaminant - are infections caused by virulent bacteria nourished by dead tissue and organic foreign material. It has three forms. They include gas gangrene; arising from almost exclusively in damaged muscle tissue and spreading with alarming rapidity to cause death if unchecked by surgical or medical treatment, infection by *streptococcus*, *staphylococcus*, coli form, other bacteria and finally, tetanus [16, 17].

2.4.2.2 Bacterial contaminants of wounds

It has been established that the presence of contaminants such as bacteria like *Proprionibacterium acnes, Staphylococcus aureus, Streptococcus, Escherichia coli, Proteus, Klebsiella, Pseudomonas earuginosa, Acinetobacter,* and *Stenotrophomonas* interfers and prolongs the healing process [16-20]. This section reviews some of these contaminants and their activities.

2.4.2.2.1 Streptococcus pneumoniae

Streptococcus pneumoniae are Gram-positive lacet-shaped cocci (elongated cocci with a slightly pointed curvature). They do not display an M protein, they hydrolyze insulin, and their cell wall composition is characteristic both in terms of their peptidoglycan and their teichoic acid) [56, 57].

2.4.2.2.2 *Staphylococcus aureus*

Staphylococci are Gram-positive spherical bacteria that occur in microscopic clusters resembling grapes. They are found primarily on mammalian skin, including the anterior nares of humans but can also be found, although infrequently, on the other body sites such as mouth, throat, mammary glands and intestinal tract [57]. In 1884, Rosenbach described the two pigmented colony types of staphylococci and proposed the appropriate nomenclature: *Staphylococcus aureus* (yellow) and *Staphylococcus albus* (white) [56]. More than 20 species of *Staphylococcus* are described but only *Staphylococcus aureus* and *Staphylococcus epidermidis* (known as *Staphylococcus albus*) are significant in their interactions with humans. *S. aureus* colonizes mainly the nasal passages, but it may be found regularly in most other anatomical locales. *S epidermidis* is an inhabitant of the skin [57].

Staphylococcus aureus causes a variety of suppurative (pus-forming) infections and toxinoses in humans. It causes superficial skin lesions such as boils, sties and furunculosis; more serious infections such as pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections; and deep-seated infections, such as osteomyelitis and endocarditis. *S. aureus* is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices. *S. aureus* causes food poisoning by releasing enterotoxins into food, and toxic shock syndrome by release of super antigens into the blood stream [56].

2.4.2.2.3 ***Escherichia coli***

Escherichia coli (*E. coli*) is one of many species of bacteria living in the lower intestines of mammals, known as gut flora. When located in the large intestine, it actually assists with waste processing, vitamin K production, and food absorption. Discovered in 1885 by Theodor Escherich, a German pediatrician and bacteriologist, *E. coli* are abundant: the number of individual *E. coli* bacteria in the faeces that a human defecates in one day averages between 100 billion and 10 trillion [56]. However, the bacteria are not confined to this environment, and specimens have also been located, for example, on the edge of hot springs [57].

2.4.2.2.4 ***Pseudomonas earuginosa***

Pseudomonas aeruginosa is a Gram-negative, aerobic rod measuring 0.5 to 0.8µm by 1.5 to 3.0µm, belonging to the bacterial family *Pseudomonadaceae.* These bacteria are common inhabitants of soil and water. They occur regularly on the surfaces of plants and occasionally on the surfaces of animals [57].

Pseudomonas aeruginosa is an opportunistic pathogen, meaning that it exploits some break in the host defenses to initiate an infection. It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed [56]. *Pseudomonas aeruginosa* infection is a serious problem in patients hospitalized with cancer, cystic fibrosis, and burns. The case fatality rate in these patients is 50 percent [17].

2.4.2.2.5 ***Klebsiella pneumoniae***

Klebsiella pneumoniae is one of the most common Gram-negative bacteria seen by physicians worldwide. Pneumonias that are caused by *Klebsiella pneumoniae* are difficult to control; mortality rates have even been reported as up to 50% after antibiotic treatment. They are opportunistic pathogens found in the environment and in mammalian mucosal surfaces and are commonly passed by hands of hospital personel. Common sites for nosocomial *Klebsiella* infections include: the urinary tract, lower respiratory tract, biliary tract, and surgical wound sites [56].

2.4.3 Determination of microbial activities

Much of microbiology depends on the ability to grow and maintain micro organisms in the laboratory, and this is possible only if suitable culture media are available [58]. Knowledge of a micro organism's normal habitat often is useful in selecting the appropriate culture medium because its nutrients requirements reflect its natural surroundings.

This subsection reviews the nutritional requirements of micro organisms, the various culture media used and the antimicrobial activity tests undertaken in the laboratory.

2.4.3.1 Culture media

For any bacterium to be propagated for any purpose it is necessary to provide the appropriate biochemical and biophysical environment. The biochemical (nutritional) environment is made available as a culture medium, and depending upon the special needs of particular bacteria (as well as particular investigators) a large variety and types of culture media have been developed with different purposes and uses [56].

Culture media may be classified into several categories depending on their composition or use. A *chemically-defined (synthetic) medium* is one in which the exact chemical composition is known. A *complex (undefined) medium* is one in which the exact chemical constitution of the medium is not known. Defined media are usually composed of pure biochemicals off the shelf; complex media usually contain complex materials of biological origin such as blood or milk or yeast extract or beef extract, the exact chemical composition of which is obviously undetermined. A defined medium is a *minimal medium* if it provides only the exact nutrients (including any growth factors) needed by the organism for growth [56, 57].

Other concepts employed in the construction of culture media are the principles of selection and enrichment. A *selective medium* is one which has a component(s) added to it which will inhibit or prevent the growth of certain types or species of bacteria and/or promote the growth of desired species. One can also adjust the physical conditions of a culture medium, such as pH and temperature, to render it selective for organisms that are able to grow under these certain conditions [57].

2.4.3.2 Antimicrobial activity tests

Antimicrobial activity is measured in vitro in order to determine the potency of an antibacterial agent in solution, its concentration in body fluids and tissues, and to determine the sensitivity of a given microorganism to known concentration of the drug. The determination of these quantities may be undertaken by one of the principal methods – dilution or diffusion [59].

Dilution tests are carried out by incorporating antimicrobial substances in graded amounts in liquid or solid bacteriological media. The media are subsequently inoculated with test bacteria and incubated. The end point is taken as that amount of bacterial substance required to inhibit or to kill the test bacteria.

In the diffusion method, a filter disk, a porous cup or a bottomless cylinder containing measured quantities of drug is placed on a solid medium which has been heavily seeded with the test organisms. After incubation, the diameter of the clear zone of inhibition surrounding the deposit of drug is taken as a measure of the inhibitory power of the drug against the particular test organism [59]. This is normally referred to as minimum inhibitory concentration (MIC). An antibiotic's MIC is the smallest amount per unit of volume that will inhibit the growth of a certain organism[60].

2.2.2.3 Wound healing mechanism

The skin's thinness and position on the outside of the body make it vulnerable to injury, but the skin has a remarkable ability to repair itself. The process of wound healing depends on how deep the wound is - whether just the superficial epidermis or both epidermis and dermis are involved [59]. Normal wound healing is of two forms [61].

The first, *healing by primary intension* occurs in clean surgical wounds with exposed margin (e.g. cuts). It occurs in several phases: blood fills the defect and coagulates, forming a scab (i.e. a meshwork composed of fibrin and fibronectin). In the second stage, macrophases remove cell debris and secrete growth factors that stimulate angiogenesis and the in growth of fibroblasts and myofibrioblasts, leading to the formation of granulation tissue. The epithelium then regenerates, covering the surface defects. At this stage, deposition of an extracellular matrix, composed initially of collagen type III and later of collagen type I, occurs, resulting in fibrous union. By the end of the first week, 10% of the preoperative strength is regained and sutures can be removed safely. The last phase, scar maturation, is a protracted phase during which cross – linking of collagen takes place. By the end of three months, 80% of the normal tensile strength of the tissue has been restored [16, 61].

The second form of healing, *healing by secondary intension*, occurs in large gaping or infected wounds. These wounds typically show a more pronounced and prolonged inflammatory phase in which neutrophils may persist for days, more abundant granulation tissue and wound contraction by myofibroblasts, which help to draw the margins of the margins of the wound closer to one another [16, 61].

The process may be delayed, as a result of infection or contamination, mechanical factors (e.g. trauma, tension, and foreign bodies), malnutrition, poor circulation, or advanced age and drugs (e.g. corticosteroids and cytotoxic drugs). The treating of small wound leaves no trace, but in more severe wounding the damage is not completely repaired. Instead the tissue contract and the remaining space are filled by scar tissue, which is imperfect in comparison to the original: it may have an incomplete blood supply, and lack innervations [21, 47, 61].

2.5 THIN LAYER CHROMATOGRAPHY (TLC)

Thin layer chromatography is a simple, quick, and inexpensive procedure that gives the chemist a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound (preferably both run on the same TLC plate).

Generally, a solvent or a solvent mixture of the lowest polarity consistent with a good separation is employed. The strength with which an organic compound binds to an adsorbent depends on the strength of the following ion-dipole, hydrogen bonding, dipole-dipole, hydrogen bonding, van der Waals forces, and dipole induced dipole interactions. With silica gel, the dominant interactive forces between the adsorbent and the materials to be separated are of the dipole-dipole type. Highly polar molecules interact fairly strongly with the polar Si-O bonds of these adsorbents and will tend to stick or adsorb onto the fine particles of the adsorbent while weakly polar molecules are held less tightly. Weakly polar molecules thus generally tend to move through the adsorbent more rapidly than the polar species [62].

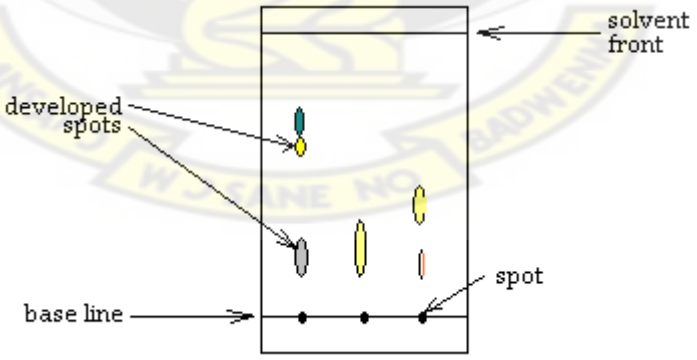


Fig 2.35: Chromatograph

The retention factor or R_f is defined as the distance travelled by the compound divided by the distance travelled by the solvent [62, 63].

R_f = distance travelled by the compound
Distance travelled by the solvent front

The R_f for a compound is a constant from one experiment to the next only if the chromatography conditions like Solvent system, adsorbent, thickness of the adsorbent, amount of material spotted and temperature are also constant. These factors are, however, difficult to keep constant from experiment. Relative R_f values are generally considered. "Relative R_f" means that the values are reported relative to a standard, or it means that you compare the R_f values of compounds run on the same plate at the time [62, 63].

The larger an R_f of a compound, the larger the distance it travels on the TLC plate. When comparing two different compounds run under identical chromatography conditions, the compound with the larger R_f is less polar because it interacts less strongly with the polar adsorbent on the TLC plate.

The R_f can provide corroborative evidence as to the identity of a compound. If the identity of a compound is suspected but not yet proven, an authentic sample of the compound, or standard, is spotted and run on a TLC plate side by side (or on top each other) with the same compound. If two substances have different R_f values, they are definitely different compounds. This identity check must be performed on a single plate, because it is difficult to duplicate all the factors which influence R_f exactly from experiment to experiment [63].

CHAPTER THREE

3 MATERIALS AND METHODS

This chapter outlines the materials used and the various methods adopted in solving the set-out goals.

3.1 MATERIALS

This subsection lists the chemicals, equipments and glass wares used in performing the various experiments

3.1.1 chemicals

- Conc. sulphuric acid (AR, BDH)
- Conc. hydrochloric acid (AR, BDH)
- 96% ethanol (GPR, BDH)
- Chloroform (AR, Meirch)
- Sodium chloride (AR, BDH)
- Sodium hydroxide (AR, BDH)
- Magnesium turnings
- Ferric chloride (AR, BDH)
- Potassium hydroxide (AR, BDH)
- Hydrogen peroxide (AR, BDH)
- Benzene
- Ammonia
- Potassium iodide (AR, BDH)
- Trichloroacetic acid
- Sodium phosphate (AR, BDH)
- Potassium ferric cyanide (AR, BDH)
- Methanol (GPR, BDH)
- Nutrient agar (Meirch)
- Anhydrous sodium sulphate (AR, BDH)
- Lead acetate
- Diethyl ether (AR, BDH)
- Mayer's reagent (potassium mercuric iodide)
- Hager's reagent (picric acid solution)
- Fehling's solution [copper (II) sulphate, potassium sodium tartarate and sodium hydroxide mixture]

- Sodium thiosulphate
- N-propyl gallate

3.1.2 Equipments and glass wares

- Soxhlet apparatus (with 1L round bottom flask)
- Büch Rotavapour R-114 Rotary evaporator
- 500ml separating funnel
- Filter papers (Whatman)
- Beakers (250ml, 600ml, 1L)
- Test tubes
- Capillary tube
- 50ml burette
- 1ml pipette
- 10ml dropping pipette
- Sanyo Mistral 3000E Centrifuge
- Gallenkamp illuminated cooled Incubator
- Hexious 1.7 UV spectrophotometer
- Petri dishes
- 10mm cork borer
- Heating mantle
- Electric oven
- 250ml conical flask
- Watch glasses
- Shimadzu FTIR-8201A single beam Infrared spectrometer

3.2 METHODS

The samples collected were prepared and the following procedures: solvent extractions, phytochemical screening, antioxidant and antimicrobial tests as well as fractionation, thin layer chromatography and infrared investigations were undertaken to achieve the set objectives.

3.2.1 SAMPLE COLLECTION AND PREPARATION

Leaves of the *Terminalia ivorensis* were collected from Kubease, a small town along the Kumasi-Konongo road in the Ashanti Region and were identified by

Mr. Alhassan Mohammed of the Department of Horticulture, KNUST and further authenticated by Mr. Ntim, Herbarium of the Forestry Commission, Kumasi. The bark of the authenticated plant was then collected in September, 2006 by cutting with cutlass for preparation and further study.

The bark was washed, cut in pieces (ca 6cm x 8cm) and air-dried for eight weeks to obtain constant weight. The dried sample was again cut into smaller pieces (ca 2cm x 4cm) and then milled into coarse particles with a hammer miller at the Department of Soil Science of the Faculty of Agriculture at the KNUST, Kumasi. The powdered sample was bagged in black plastic bags and stored in an air-tight container for further work.

3.2.2 MOISTURE CONTENT

0.60g of the powdered air-dried sample was weighed into a dried crucible of known weight. It was then dried at 105^{o}C for three days to a constant weight in an oven. The loss in weight on drying was then expressed as a percentage with reference to the air-dried sample to obtain the moisture content.

3.2.3 EXTRACTION

150g portion of the powdered sample was extracted with 800ml of 96% ethanol. The extraction was carried out in a soxhlet apparatus (with a 1L round bottom flask). Four extractions were done and the extracts were concentrated to about one-sixth of the original volume at 60^{o}C under reduced pressure using a rotary evaporator. About half of the extract was dried to constant weight in an oven at 50^{o}C and kept in air-tight containers for further work, while the other half was kept to be fractionated into groups of similar polarities.

The same weight of the sample was also extracted with distilled water in the same way as described above. The marc of the ethanol extraction was further extracted with the same quantity of water and the extract was indicated as 'water-after-ethanol extract'. The dried aqueous, alcoholic and water-after-ethanol extracts were labelled as CA, CB and CC respectively**.**

3.2.4 PERCENTAGE YIELD

To determine the percentage yield of extracts from the various solvents, the averages of the masses of the various dried extracts were taken and expressed as percentages of the masses of the powdered air-dried samples taken.

3.2.5 PHYTOCHEMICAL SCREENING ON CRUDE EXTRACTS

This section outlines the methods employed to investigate the phytochemicals present or otherwise in the crude samples. The description of the methods used in the determination of the phytochemicals in various extracts (water, ethanol and water-after-ethanol extracts) investigated are presented in the following subsections.

3.2.5.1 Test for Alkaloids

About 0.5g of the dried aqueous extract was redissolved in 10ml chloroform and then shaken with 15ml 5% HCl in a separating funnel. The lower chloroform layer was drained off. 8ml of 5% NaOH was added to the remaining aqueous layer and shaken with diethyl ether and the aqueous layer was screened for alkaloids. In screening for alkaloids, about 2ml of the basified aqueous solutions were placed in two different test tubes and tested with Mayer's and Hager's reagents (three drops each) separately.

A confirmation was done using the method described by Akinyemi *et al* (2006) [1]. In this case, 5ml 1% HCl was added to 0.5g of the extracts, boiled, and then filtered. Three drops of Mayer's and Hager's reagents were then added to about 2ml of the filtrates.

The same procedure was performed with the dried extracts of ethanol and water-after-ethanol.

3.2.5.2 Test for Tannins and polyphenols

3ml of hot distilled water was added to 0.3g of the dried aqueous extract, stirred and then allowed to cool to room temperature. 2ml of 10% sodium chloride was added to salt out the non-tannin compounds. The mixture was filtered and divided into two different test tubes. Four drops of lead acetate solution was added to one of the test tubes and four drops of ferric chloride to the other.

This was repeated for the dried extracts of ethanol and water-after-ethanol extracts.

3.2.5.3 Test for Saponins

In determining the presence or absence of saponins, 0.2g of the dried aqueous extract was placed in a test tube and 3ml of distilled water added and boiled for fifteen minutes. The content was filtered and the filtrate shaken vigorously.

The same procedure was repeated for the dried extracts of ethanol and water-after-ethanol.

3.2.5.4 Test for Flavonoids

About 0.5g of the dried aqueous sample was redissolved in 4ml ethanol and 2ml of the resulting solution was put in test tubes. Then, 0.5ml of concentrated HCl and two pieces of magnesium turnings added.

The same procedure was repeated for the dried extracts of ethanol and water-after-ethanol.

3.2.5.5 Test for Steroids and Triterpenoids

0.5g of the dried aqueous extract was dissolved in 10ml chloroform and washed with two portions of 5ml equal volumes of distilled water. The aqueous layer was discarded and chloroform layer dried using anhydrous sodium sulphate. 2ml portions of each of the dried chloroform extracts were placed in two test tubes. Two drops of concentrated H_2SO_4 was added to one of the test tubes and five drops of acetic anhydride followed by five drops of conc. H_2SO_4 were added to the other test tube for confirmation.

The same procedure was repeated for the dried ethanol and water-after-ethanol extracts.

3.2.5.6 Test for Carotenoids

In testing for carotenoids, 4ml of ethanol was added to redissolve 0.3g of the dried aqueous extract. The solution was filtered and 3ml of antimony trichloride was added to about 2ml of the filtered extract. For confirmation, 0.5g of the extract was extracted with 25ml ether and 1ml of concentrated sulphuric acid was added to 2ml of the ethereal extract to form a layer under the ethereal solution.

The same procedure was repeated for the dried extracts of ethanol and water-after-ethanol.

3.2.5.7 Test for General Glycosides

About 0.5g of each the three extracts was redissolved in their respective solvents and 2ml of the resulting solutions were put in separate test tubes. About 1ml of Fehling solution was added to each solution and heated in a water bath for about fifteen (15) minutes.

The same procedure was repeated for the dried extracts of ethanol and water-after-ethanol.

3.2.5.8 Anthraquinones

To test for anthraquinones, 0.5g of the aqueous extract was boiled with 25ml of 0.5M KOH and 4ml of 1M H_2O_2. The mixture was cooled, filtered, acidified with four drops of acetic acid and the resulting solution was extracted with 15ml benzene. The resulting yellowish benzene layer was separated and then shaken with 4ml of ammonium hydroxide. The same procedure was repeated for the dried extracts of ethanol and water-after-ethanol.

3.2.5.9 Anthraquinone glycosides

0.5g of the aqueous extract was boiled with 25ml of dilute sulphuric acid and filtered hot. The filtrate was allowed to cool and about 10ml portion was shaken with an equal volume of benzene. The benzene layer was separated and shaken with about half its volume of dilute ammonia solution.

The same procedure was repeated for the dried ethanol and water-after-ethanol extracts.

3.2.5.10 Coumarins

0.2g of the dried aqueous extract was placed in a test tube and moistened with water. The test tube was then covered with a piece of filter paper moistened with dilute NaOH solution and then placed in a hot water bath. The filter paper was removed after fifteen minutes and exposed to UV light.

The same procedure was repeated for the dried extracts of ethanol and water-after-ethanol extracts.

3.2.6 FRACTIONATION

The three concentrated extracts were fractionated by bulk transfer methodology into groups of related polarity as illustrated in Fig 3.1.

About 150ml portions of the concentrated extracts were measured separately into a 500ml separation funnel. 50ml of 5% HCl was added and shaken well to mix and 150ml of chloroform was then added. The solution was then shaken vigorously, opening and closing the valve intermittently to release the pressure built in the funnel. The separating funnel was hanged for several minutes for separation to occur. Two portions were obtained, the $CHCl_3$ and the aqueous fraction. The aqueous portions were extracted twice with the chloroform, with the chloroform fractions also washed twice with water. The chloroform fraction was then concentrated and dried at 50^oC. To 100ml portions of the aqueous fractions were added about 8ml of 6N NaOH. The deprotonated aqueous fraction was evaporated to about three-quarter of its volume and was also oven-dried at 50^oC until they attained constant weight.

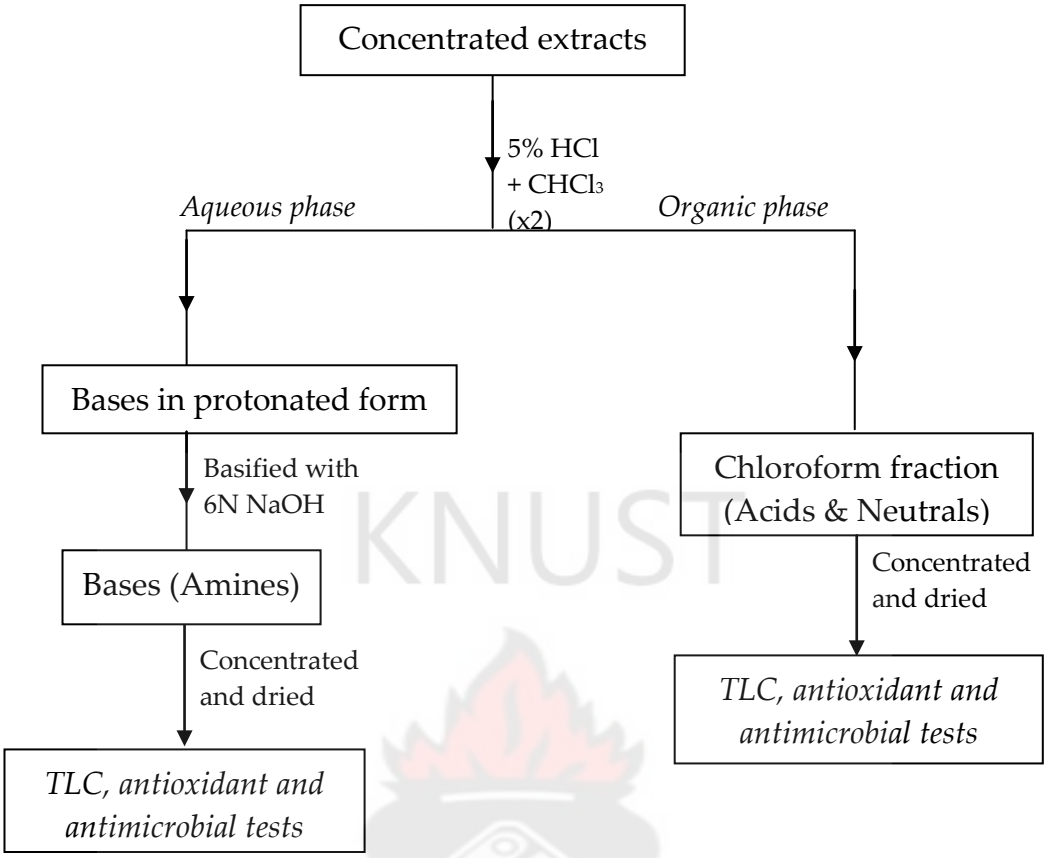


Fig 3.1: Bioactivity-guided fraction of *Terminalia ivorensis.*

The dried fractions were kept in air-tight containers for further work. The chloroform fractions were labelled as AA, AB, and AC for the aqueous, ethanolic and water after ethanol extracts respectively; while the aqueous fractions were also labelled as BA, BB and BC respectively.

3.2.6 THIN LAYER CHROMATOGRAPHY.

Thin layer chromatography was performed on the crude extracts, chloroform and aqueous fractions using precoated thin layer chromatographic plates (DC – Fertigplatten Sil G – 25 UV_{254}) manufactured by Macherey-Nagel, Germany. The plates (50mm x 100mm) had a thickness 0.25mm of silica gel.

About 0.5g each of the crude extract and fractions of the aqueous extract was redissolved in 2ml of water separately in a beaker. 1cm was measured from the base of the TLC plate, marked with a pencil and labelled. Capillary tube was used to spot the plates with the crude extract and fractions. Small quantities of the concentrated solutions were collected with capillary tube by dipping it in the solution. They were then used to spot the plates; three spots were made on each plate (Fig. 3.2). Labelling of the samples was done with a pencil. 15ml of methanol was measured and poured into a chromatank (with a 20cm x 20cm base area). The plates were placed in a chromatank and covered, ensuring that the solvent was just below the spots. Apparatus was placed on a level surface for the solvent to rise.

The plate was removed after about two hours when the solvent had risen close to the top edge, marking the distance travelled by solvent with a pencil. It was then dried at room temperature. The dried plate was then placed in a container, with iodine vapour to develop the spots on the plates. The spot moved by solvents and shape of spots were also marked out with a pencil. The distances moved by the solvents and the spots were measured, in millimetres, with a rule. The retention factors of the samples were then determined.

The retention factor, R_f is defined as the distance travelled by the compound divided by the distance travelled by the solvent [62, 63].

i.e. $$R_f = \frac{\text{distance travelled by the compound}}{\text{distance travelled by the solvent}}$$

The same procedure was repeated for the crude extracts and fractions of ethanol and water-after-ethanol using the following solvent systems: acetone, chloroform, methanol: acetone (1:1), methanol: water (1:1) and chloroform: acetone (1:4).

3.2.7 ANTIOXIDANT ACTIVITY TESTS

Antioxidant activity tests were carried out on the dried crude extracts, chloroform and aqueous fractions. The procedures used are described in this section.

3.2.7.1 Hydrogen peroxide decomposition

Hydrogen peroxide consumption/decomposition was studied through iodometric titration method. The assay mixtures contained 8ml of 80mM H_2O_2 solution and 2ml of 1% w/v *Terminalia ivorensis* extracts and fractions. Each mixture was rapidly mixed by gentle swirling motion. 1ml portions of the reaction mixture were allowed to run into 25ml volumes of distilled water to slow down the decomposition of H_2O_2 by the extracts and fractions. This was done at sixty seconds (60s) intervals for a period of two hundred and forty seconds (240s).

About 2g of KI and 5ml of 3M HCl were added to each mixture and the resulting iodine solutions were allowed to stand for about thirty minutes to get maximum I_2 generation. They were then titrated with a standard solution of sodium thiosulphate, until the yellow iodine colour becomes faint. About 1ml of freshly prepared starch solution was then added and the titration was continued to the end point. The end point was determined by the disappearance of the blue colour.

The process was repeated for 4ml and 8ml portions of the 1% w/v extracts and fractions. The decomposition/consumption of H_2O_2 was back calculated using the equations below.

$$H_2O_2 + 2KI + 2H^+ \longrightarrow I_2 + 2H_2O + 2K^+$$

$$I_2 + 2S_2O_3^{2-} \longrightarrow S_4O_6^{2-} + 2I^-$$

The effects of the concentration of the *Terminalia ivorensis* extracts and fractions on the consumption of H_2O_2 were also examined by plotting the concentration of H_2O_2

consumed against concentration of extracts and fractions at the times at which the measurements were made.

3.2.7.2 Fe^{3+} Reducing Power

The reducing power of all extracts and fractions of *Terminalia ivorensis* and n-propyl gallate (standard) were determined using the method described by Oyaizu (1986) and Yildirim *et al* (2001) [50]. Four different doses of the test samples (0.1, 0.3, 1.0, and 3.0mg/ml) were used.

1.0ml portion of a selected dose of the test sample was mixed with 2.5ml of 0.2M sodium phosphate buffer (pH 6.6) and 2.5ml of 1% potassium ferric cyanide [$K_3Fe(CN)_6$] solution in a centrifuge tube. The mixture was then incubated at 50^oC for 20 minutes. 1.5ml of 10% trichloroacetic acid was added to the incubated mixture and centrifuged at 3000rpm for 10 minutes.

2.5ml of the supernatant solutions were then mixed with 2.5 ml of distilled water and 0.5ml of 0.1% ferric chloride in centrifuge tubes. The absorbance of the resulting solution was measured at 700nm in a Hexious 1.7 spectrometer. For the blank, a 1.0ml volume of distilled water was added to 2.5ml sodium phosphate buffer and 2.5ml potassium ferricyanide in a test tube and resulting mixture was taken through the same process as done for the test samples. The determinations were performed at the Department of Biochemistry, KNUST, Kumasi.

3.2.8 ANTIMICROBIAL ACTIVITY TEST

The crude extracts and fractions were tested against 24 hour broth cultures of *Escherichia coli, Streptococcus pneumoniae, Staphylococcus aureus,* and *Pseudomonas aeruginosa*. The various steps taken in determining the antimicrobial

activities of the test samples are briefly described in the next subsections. The tests were performed at the Microbiology Laboratory of the Faculty of Pharmacy, KNUST, Kumasi.

3.2.8.1 Bacterial Susceptibility Test

Susceptibility tests were performed on the crude extracts to ascertain their activity or not against *Escherichia coli, streptococcus pneumoniae, Staphylococcus aureus,* and *Pseudomonas aeruginosa*. Higher concentrations of the extracts were used (50mg/ml, using methanol as solvent). In the test tube, 20ml nutrient agar (in a test tube) was melted at 100^{o}C and stabilized at 45^{o}C for about 15 minutes. About 0.1ml inoculums were added from culture tubes to the agar in the test tube by the use of a loop [60]. The test tube containing the agar and the inoculums was then rolled in between the palms gently to mix the inoculums thoroughly with the agar [60, 64]. The loop was flamed before it was used each time.

The content of the test tube was poured into a Petri dish and allowed to set. The Petri dishes were then labelled with the respective organism (inoculums) and date. By means of a 10mm cork borer, three cups were bored, well separated and equidistant from each other in the agar. The cups were labelled with the three crude extracts. Each cup was filled with its corresponding extract to about three-quarters full. They were kept on a bench at room temperature for about 60 minutes (for the extracts to diffuse into the agar). The plates were then incubated aerobically at 37^{o}C and examined for any zone of inhibition after 24 hours.

3.2.8.2 Determination of Minimum Inhibitory Concentration.

Four different concentrations of the antimicrobial agents were prepared (40, 20, 10 and 5mg/ml) from the crude extracts, aqueous and also from the chloroform fractions of the various extracts. The working area was disinfected with phenol before the start of the work.

20ml nutrient agar was melted at 100 ^{o}C and stabilized at 45 ^{o}C for about 15 minutes in a test tube. About 0.1ml *staphylococcus aureus* was added from culture tubes to the agar in the test tube by the use of a loop [60]. The test tube containing the agar and the inoculums was then rolled in between the palms gently to mix the inoculum thoroughly with the agar [60, 64]. The loop was flamed before it was used each time.

The content of the test tube was poured into a Petri dish (which was previously autoclaved at a pressure of 15 lb/in^2 for 20 min) and allowed to set. The Petri dish was then labelled with the name of the inoculum and date. By means of a 10mm sterile cork borer, four cups were bored well separated and equidistant from each other in the agar. The cups were labelled with the four concentrations of the crude aqueous extract. Each cup was filled with its corresponding extract to about three-quarters full.

The Petri dish was quickly covered and then kept on a bench at room temperature for about 60 minutes (for the extracts to diffuse into the agar).

The same procedure was followed for the different extracts and fractions, with the same organism and the other organisms. Thus, each extract and fraction was tested against each of the test organism, using chloramphenicol as the control for each organism.

The plates were incubated aerobically at 37^{o}C for 24 hours and examined for any zone of inhibition. The reading was done against a dark background under reflected light [60]. The diameters of the zones of growth of inhibition were measured with the help of a pair of dividers and a rule from the underside of the covered plates for spots with inhibitions. The average of the diameters was taken. The actual zones were calculated by subtracting the diameter of the cups (10mm) from the total zone of growth.

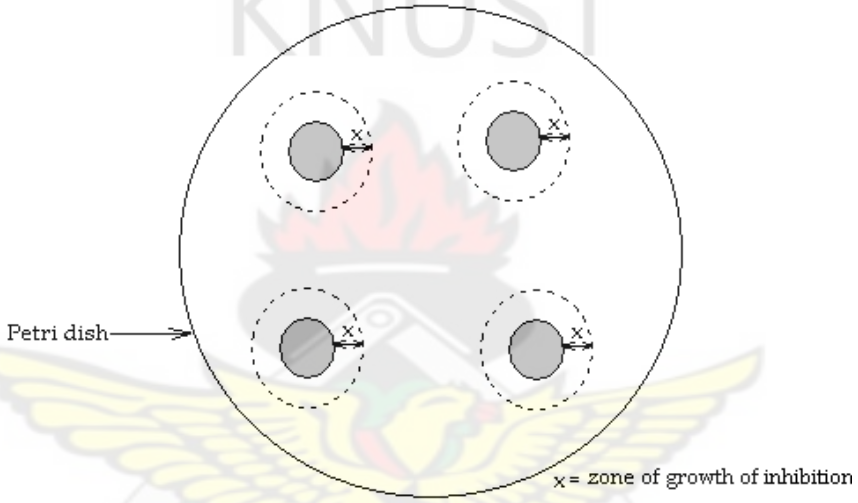


Fig. 3.3: Measurement of zone of growth of inhibition

The zones of inhibition obtained were plotted against the log of concentrations to determine the minimum concentrations at which these extract can inhibit the growth of the test organisms. The minimum inhibitory concentrations were obtained by determining the concentration at which the zone of inhibition was zero.

3.2.9 INFRARED SPECTROSCOPY ANALYSIS

About 1.5g of the oven-dried samples were put in sample tubes and further dried in a desiccator containing concentrated sulphuric acid in other to remove all

moisture. They were then covered tightly to prevent any further reabsorption of moisture and used for the infrared analysis. These extracts were then analyzed for the presence of possible functional groups that may be present in them.

The dried solid samples were pressed into pellets (with KBr) and the functional groups were determined using Shimadzu FTIR-8201A single beam laser Infrared Spectrometer at the Chemistry Department of the University of Cape Coast, Cape Coast.

CHAPTER FOUR

4 RESULTS AND DISCUSSION

This chapter appraises the findings, analyses and discusses the results obtained from the experiments as described in the previous chapter. In the following subsections, the results obtained for the phytochemical screening, moisture content, thin layer chromatographic analyses, antibacterial tests, infrared analyses and antioxidant properties of the extracts of *T. ivorensis* are presented and discussed.

4.1 RESULTS FROM PHYTOCHEMICAL SCREENING

The phytochemical screening performed indicates the presence of saponins, anthraquinone glycosides, steroids and triterpenoids in all the three extracts, with tannins and polyphenols, and flavonoids present in only the aqueous and ethanolic extracts. Anthraquinones were present only in the water-after-ethanol extract. General glycosides, alkaloids and coumarins were absent in all the three extracts. These have being summarised in the table below.

Table 4.1**:** summary of inferences from phytochemical screening

Secondary metabolites	INFFERENCE		
	Water	Ethanol	Water-after-ethanol
Saponins	+	+	+
Anthraquinones glycosides	+	+	+
Steroids and Triterpenoids	+	+	+
Flavonoids	+	+	-
Tannins and polyphenols	+	+	-
Anthraquinones	-	-	+
Carotenoids	-	-	-
General Glycosides	-	-	-
Alkaloids	-	-	-
Coumarins	-	-	-

NB: (+) and (-) indicate presence and absence respectively.

Detailed descriptions of observations and inferences made in the phytochemical tests (section 3.2.3) are presented in the following subsections.

4.1.1 Alkaloids

Clear yellowish solutions (with no precipitates) were observed in the water, ethanol and water-after-ethanol extracts test for the alkaloid (see page 64) indicating the absence of alkaloids in the three extracts. Similar observations were made in the confirmatory test, confirming their absence.

4.1.2 Tannins and polyphenols

White precipitate (with lead acetate) and bluish-green colouration (with ferric chloride) were observed in only the water and ethanol extracts (see page 65). No

white precipitate was observed in the water-after-ethanol extract; deep yellow and black solutions with the addition of lead acetate and ferric chloride respectively. This indicates the presence of tannin and polyphenols in the water and ethanol extracts but absent in the water-after-ethanol extracts.

4.1.3 Saponins

Persistent foamy layers were observed on all the filtrates indicating the presence of saponins in all the three extracts (see page 65).

4.1.4 Flavonoids

Pink colourations with the evolution of effervescences were observed in the water and ethanol extracts (see page 65). There was however, an effervescence of a gas with no pink colouration in the water-after-ethanol extract. Flavonoids can be inferred to be present in the water and ethanol extracts but absent in the water-after-ethanol extract.

4.1.5 Steroids and Triterpenoids

Reddish brown rings were observed in the chloroform layers of all the three extracts (see page 66). This indicates the presence of steroids and triterpenoids in them.

4.1.6 Carotenoids

No dark-blue or violet colours in any of the acid layers of the three extracts were observed (see page 66). This indicates the absence of carotenoids in the extracts.

4.1.7 General Glycosides

Greenish solutions were observed in all extracts (see page 67), giving indication of the absence of general glycosides in all the extracts.

4.1.8 Anthraquinones

A yellowish alkaline layer was observed in only the water-after-ethanol extract (see page 67), while colourless layers were observed in the case of the water and ethanol extracts indicating the presence of anthraquinones in only the water-after-ethanol extract.

4.1.9 Anthraquinones glycosides

Coloured ammoniacal layer were observed in all the three extracts, with pink in both water and ethanol extracts and yellow in the water-after-ethanol (see page 65). The results indicate the absence of anthraquinone glycosides in all the three extracts.

4.1.10 Coumarins

No fluorescence was observed in any of the extracts on exposure to UV light (see page 68). Coumarins may be absent in all the three extracts.

4.2 PERCENTAGE YIELD AND MOISTURE CONTENT

In the determination of the percentage yield; 9.80g, 31.34g and 4.11g of crude extracts were obtained for the aqueous, ethanolic and water-after-ethanol extracts respectively, which correspond to 6.53%, 20.89% and 2.74% yields respectively from these extracts.

A mass of 0.538g of the powdered air-dried sample was obtained after oven drying (as described in section 3.2.2), which means that the sample contained 0.062g of moisture indicating 10.33% moisture content. The value, thus, obtained is moderately below the highest permitted moisture content limit for good crude drug of 20% at 96% humidity, which indicates a longer expected shelf life [65].

4.3 THIN LAYER CHROMATOGRAPHY

The results obtained (distances travelled by solvent front and extracts) from performing the thin layer chromatography as described in section 3.2.4.2 have been analysed and the various R_f values are displayed and discussed in this section.

The results obtained for the TLC analyses of the aqueous extract in Table 4.2a below. Methanol was observed to be a better solvent (among the solvents used in this TLC) for the chloroform fraction (AA) of the aqueous extract, as it gave four separations. The mixture of methanol and water (1:1) was also better for the aqueous fraction (BA), giving three separations, even though it gave one separation in the crude (CA) and chloroform (AA) fractions. Although the mixture of methanol and acetone (1:1) gave only two separations for the crude extract, both solvents separately gave three separations each. They would be considered better solvents for the separation of the crude extract (CA).

Table 4.2a: R_f values for Aqueous extract

Solvent system	R_f values		
	Crude (CA)	Acids (AA)	Bases (BA)
Methanol	0.08, 0.22, 0.53	0.10, 0.22, 0.43, 0.56	0.20, 0.55
Acetone	0.20, 0.40, 0.63	0.19, 0.32, 0.61	0.15
Methanol: acetone (1:1)	0.51, 0.77	0.43, 0.78	0.10, 0.74
Methanol: water (1:1)	0.08	0.06	0.07, 0.34, 0.83
Chloroform: acetone (1:4)	0.07, 0.42	0.05, 0.22, 0.42	0.00
Chloroform	0.00	0.16	0.00

Table 4.2b below, summarises the results for the TLC analyses performed on the ethanolic extract and its fractions.

Table 4.2b: R_f values for Ethanolic extract

Solvent system	R_f values		
	Crude (CB)	Acids (AB)	Bases (BB)
Methanol: water (1:1)	0.09, 0.56, 0.79	0.05	0.05, 0.46, 0.70
Methanol	0.09, 0.43, 0.57	0.10	0.18, 0.47
Acetone	0.22, 0.40, 0.72	0.05	0.07
Methanol: acetone (1:1)	0.67, 0.74	0.07, 0.59, 0.79	0.00
Chloroform: acetone (1:4)	0.14, 0.24, 0.45	0.07, 0.97	0.00
Chloroform	0.18	0.16	0.00

The solvent systems used generally produced very poor separation for the acids (AB) and bases (BB) fractions of the ethanolic extract; for the crude ethanolic extract (CB) relatively good separations were obtained.

With the exception of chloroform and methanol: acetone (1:1) solvent system; where one and two separations respectively were obtained, three separations were obtained for the other solvents

The separation situation in the water-after-ethanol extract (table 4.2c) was similar (if not worse) to that of ethanolic extract. Methanol and water (1:1) mixture separated the acidic (AC) and basic (BC) fractions more than any other solvent system. In addition to the methanol and water (1:1) mixture, acetone and methanol also gave three separations each in crude extract (CC). Chloroform gave no separation in any of the extract and fractions, with chloroform: acetone (1:4) having only one separation in acid fraction (AC).

Table 4.2c: R_f values for water-after-ethanol extract

Solvent system	**R_f values**		
	Crude (CC)	Acids (AC)	Bases (BC)
Methanol: water (1:1)	0.08, 0.54, 0.82	0.08, 0.56, 0.77	0.05, 0.53, 0.75
Acetone	0.09, 0.32, 0.64	0.09, 0.32	0.05
Methanol	0.05, 0.26, 0.56	0.15	0.06, 0.43
Methanol: acetone (1:1)	0.10, 0.78	0.12, 0.36	0.04
Chloroform: acetone (1:4)	0.00	0.05	0.00
Chloroform	0.00	0.00	0.00

4.4 ANTIMICROBIAL TEST

Micro organisms are major contaminants in wounds; they prolong the healing of wounds and even make the victim susceptible to other diseases. All these contaminants also cause other serious ailments. The phytoconstituents present in the

various extracts and fractions of *T. ivorensis* are known to have antimicrobial activity[13]. It is important then, to ascertain particular micro organisms for which they are active. The microbial activity of the various extracts and fractions against *Streptococcus pneumoniae, Escherichia coli, Pseudomonas earuginosa,* and *Staphylococcus aureus* was determined (described under section 3.2.8) and the results are outlined and discussed in this section.

4.4.1 Susceptibility Test

Susceptibility tests performed on the crude extracts showed that all the three extracts of *T. ivorensis* have some antimicrobial activity against the test organisms as indicated in the table 4.3 below.

Table 4.3: Summary of susceptibility test on crude extracts

Extract	***Staphylococcus***	***Streptococcus***	***E. coli***	***Pseudomonas***
Water	+	+	+	+
Ethanol	+	+	+	+
Water-after-ethanol	+	+	+	+

4.4.2 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations (MICs) of the crude extracts, the fractions and the standard (chloramphenicol) were determined from plots of log of concentrations of the extract and the standard against their zones of inhibition using MS Excel® (see appendix 2).

The crude extracts, generally, had a high antimicrobial activity against the test organisms than the fractions (see tables 4.4, 4.5 and 4.6). This may be as a result of the combined effect of the activities of the fractions; a probable reason for the usage of the crude extracts in Ghanaian herbal treatments.

The crude sample of the water extract (CA), for instance, inhibited the test organisms to a greater extent than its fractions; with a minimum inhibitory concentration as low as 79.93µg/ml for *S. aureus* as shown in table 4.4. The crude samples had a higher activity against *S. pneumoniae* than chloramphenicol (90.54, 342.75, 1403.09µg/ml for the crude extracts of water, ethanol and water-after-ethanol respectively as against 1881.64µg/ml for the chloramphenicol).

The chloroform fractions of the water and ethanol extracts, however, had no activity against *S. pneumoniae*.

Table 4.4: Minimum inhibitory concentration of water extract

Test organism	**Minimum Inhibitory Concentration (µg/ml)**			
	crude	*$CHCl_3$*	*aqueous*	*Chloramph*
S. aureus	79.93	1147.60	3546.39	7.71
S. pneumoniae	90.54	NA	2948.61	1881.64
E. coli	207.75	1370.73	6244.34	7.56
P. earuginosa	911.41	1445.30	3535.52	492.14

*NA = not active

The fractions of the alcoholic extracts generally exhibited very low microbial activity against the test organisms with even no activity against *S. pneumoniae* and *E. coli* (see table 4.5). The crude, on the other hand, was moderately active but with a relatively high activity against *P. earuginosa* (MIC of 22.15µg/ml), which was even more than the broad spectrum antibiotic, chloramphenicol (MIC of 492.14 µg/ml).

Table 4.5: Minimum inhibitory concentration of ethanol extract

Test organism	**Minimum Inhibitory Concentration (µg/ml)**			
	Crude	*$CHCl_3$*	*aqueous*	*Chloramph*
S. aureus	883.76	7937.01	4859.57	7.71
S. pneumoniae	342.75	NA	6729.51	1881.64
E. coli	513.81	NA	4168.77	7.56
P. earuginosa	22.15	1278.27	7937.01	492.14

*NA = not active

Even though the water-after-ethanol extract generally had a low activity compared to the other extracts and the chloramphenicol (see table 4.6), a few showed better activities against some organisms; the crude extract showed a better activity against *E. coli* (with MIC of 39.07µg/ml) than all the extracts and fractions. The aqueous fraction was however; inactive against *S. aureus* and *E. coli*, and its activity against the other organisms was very low.

Table 4.6: Minimum inhibitory concentration of water-after-ethanol extract

Test organism	**Minimum Inhibitory Concentration (µg/ml)**			
	Crude	*$CHCl_3$*	*Aqueous*	*Chloramph*
S. aureus	741.00	605.53	NA	7.71
S. pneumoniae	1403.09	2410.03	7937.04	1881.64
E. coli	39.07	2486.78	NA	7.56
P. earuginosa	633.25	865.50	3855.47	492.14

*NA = not active

The results of phytochemical analyses indicated that all the crude extracts contained saponins and tannins, plants metabolites known to have antimicrobial activity [1]. These may be responsible for the antibacterial activity observed and thus justifying its traditional use as medicinal plants for the treatment of wounds and other bacterial related diseases.

Again, it is known that most microbes that utilise oxygen produce hydrogen peroxide, which is toxic to their growth. Their survival in the presence of this antimetabolite is possible because they produce catalase and peroxidase, which converts the hydrogen peroxide to water and oxygen [64]

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2.$$

The high hydrogen peroxide decomposition in all the ethanolic extracts and both the crude and aqueous fractions of the water-after-ethanol extract observed in section 4.6.1 possibly explains why the antimicrobial activities of the extracts and

fractions are low (with high MICs). The comparative high activities of the aqueous extracts and fractions and the chloroform fraction of the water-after-ethanol extract may be attributed to their inability to reduce H_2O_2 as a result the possible absence of catalase and peroxidase.

4.5 INFRARED (IR) SPECTROSCOPY

The IR spectra obtained for the three crude extracts namely crude water extract, crude ethanol extract and crude water-after-ethanol as well as their fractions (see section 3.2.9) are presented and discussed in the following subsections.

4.5.1 Infrared spectra of Crude Extracts

This subsection presents the IR spectra (Figures 4.1 to 4.3) obtained for the crude extracts (see section 4.5); deductions made from the spectra are also presented and discussed.

The spectrum below (figure 4.1) was obtained for the crude aqueous extract, which was found to contain saponins, anthraquinone glycosides, steroids and triterpenoids, tannins and polyphenols and flavonoids in the phytochemical analysis.

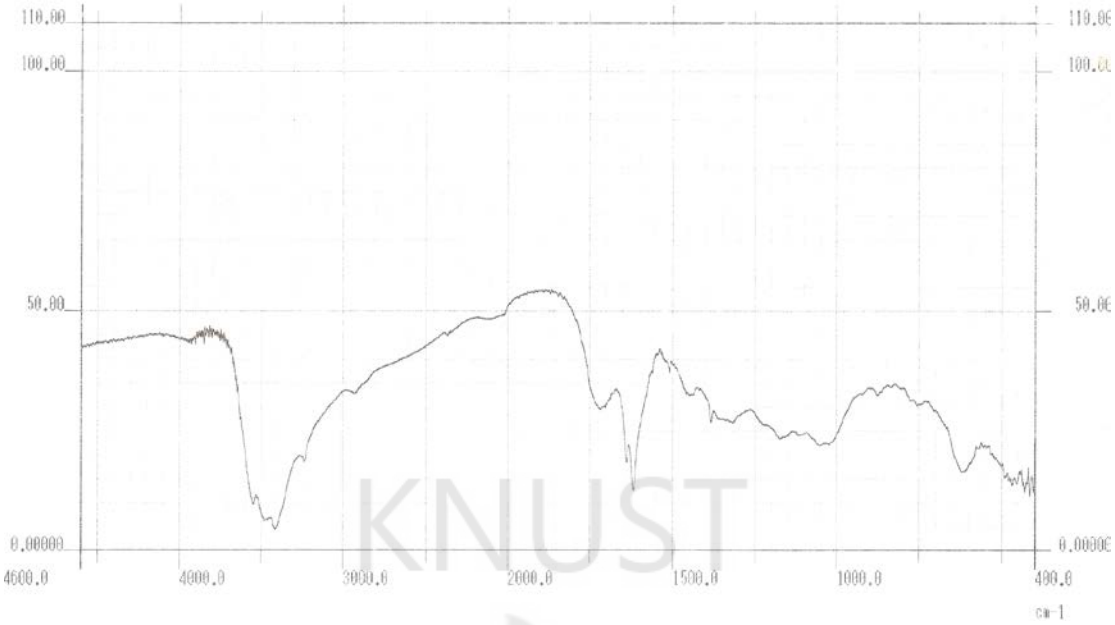


Fig 4.1: IR spectrum of crude water extract

The following functional group were observed from the IR spectrum of the crude water extract (see table 4.7).

Table 4.7: Deductions made from the absorption bands of IR Spectrum of crude water extract.

Frequency, cm^{-1}	**Functional group**	**Remarks**
$3550cm^{-1}$ - $3300cm^{-1}$	Free –OH, -NH	Broad band with absorbance at 3550, 3500, $3400cm^{-1}$
3230	OH stretch	Sharp band.
1725	C=O for normal ester	Broad band
1638	>C=C<	Strong band
1613, 1510	Aromatic C=C	Strong bands
1125	C-O stretching	Weak. Associated with esters
1050	C-O stretch	Weak band.

The ethanolic extract, which was found to contain saponins, anthraquinone glycosides, steroids and triterpenoids, flavonoids, tannins and polyphenols gave the spectrum shown in figure 4.2 below.

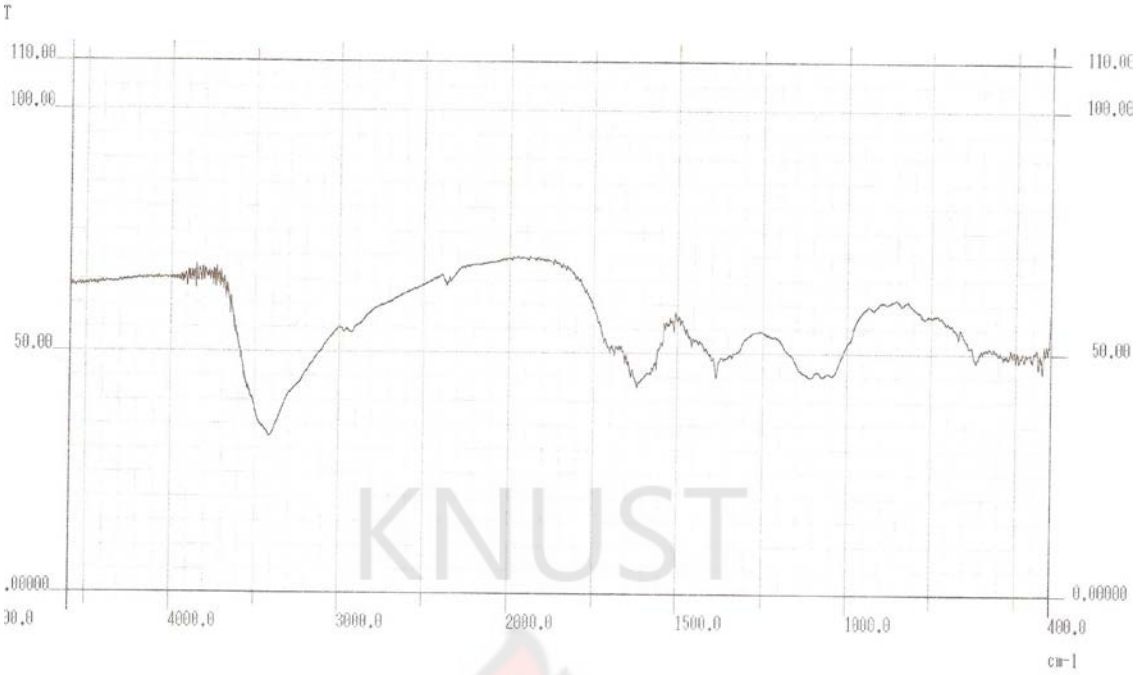


Fig 4.2: IR spectrum of crude ethanol extract

The following functional groups were observed from the IR spectrum of the crude ethanolic extract (table 4.8).

Table 4.8: Deductions made from the absorption bands of IR Spectrum of crude ethanol extract.

Frequency, cm^{-1}	**Functional group**	**Remarks**
3400	O-H stretch	Broad band
2960	$>CH_2$ stretching	Weak band
1725	C=O for normal ester	Broad band
1638	>C=C<	Sharp intense band
1613	Aromatic C=C	intense band
1510	Aromatic C=C	Weak sharp band
1125, 1050	C-O stretching	Weak band.

The IR spectrum of the extractive obtained from the water-after-ethanol extraction, which was found to contain saponins, steroid and triterpenoid and anthraquinone glycoside is shown in figure 4.3 below.

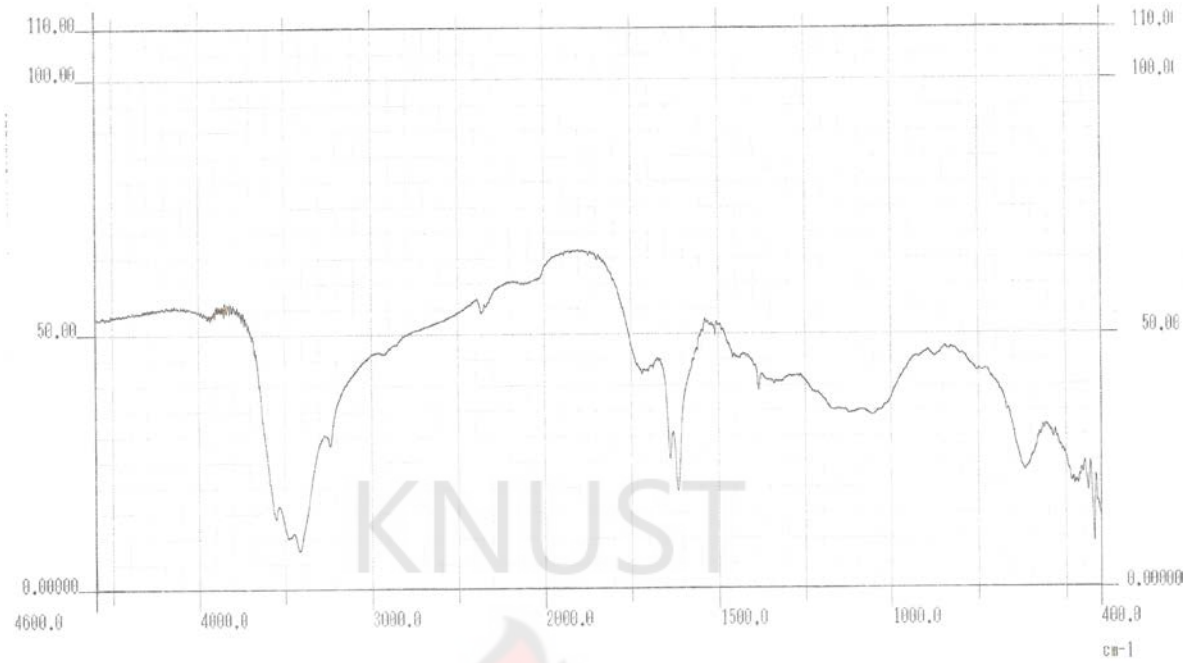


Fig 4.3: IR spectrum of crude water-after-ethanol extract

The following functional groups were observed from the IR spectrum of the crude water-after-ethanol extract (see table 4.9).

Table 4.9: Deductions made from the absorption bands of IR Spectrum of crude water-after-ethanol extract.

Frequency, cm^{-1}	**Functional group**	**Remarks**
3550 - 3400	Associated –OH, -NH	Broad band with absorbance at 3550, 3500, 3400cm^{-1}
3230	OH of acid	Sharp band
1725	C=O for normal ester	Sharp band
1715	C=O stretch. conjugation	Sharp band
1700	C=O stretch for acid	Broad band
1638	>C=C<	Sharp intense band
1613	C=C of benzene	Sharp intense band
1510	Aromatic C=C	Weak sharp band
1125, 1050	C-O stretch	Weak band.

A careful look at these spectra above reveals close similarities amongst them. The differences in these spectra, as is always the case, are particularly observed in the fingerprint region (which is characteristic for a particular moiety). A broad

absorption band was observed from 3300cm^{-1} to 3550cm^{-1} observed in all the spectra above, with three absorption bands at 3550, 3500 and 3400cm^{-1} (for both water and water-after-ethanol extracts) and only one peak at 3400cm^{-1} for the ethanolic extracts. These absorption bands are attributable to –OH [66], which normally overlaps –NH stretching.

The IR spectra for the extracts above (figure 4.1 – 4.2) showed a C=O band observed around 1725cm^{-1} attributable to α, β-unsaturated esters, which appears from 1730 - 1715cm^{-1} [67] and 1125 cm^{-1} (C-O stretch for esters). Even though there is sharp band at 3500 cm^{-1} (likely to be associated with –NH stretch, for amides), the possibility of it associated with an ester is confirmed by several bands between 1300 and 1000cm^{-1} (especially at around 1125cm^{-1}) attributable to C–O stretches of esters (generally within 1300-1000 cm^{-1}). The presence of an amide in the water and water-after-ethanol extracts cannot be totally overruled since N-H is further supported by absorption bands at about 1630cm^{-1} and 3500cm^{-1} [66].

Again, the weak symmetric C-H band at about 2930cm^{-1} and CH_3 symmetric deformation at around 1390cm^{-1} are indicative of the presence of aliphatic compounds. The corresponding C=C stretching frequency between 1650 – 1600cm^{-1} (ca 1630cm^{-1}) of low intensity indicates the presence of a compound with aliphatic characteristics [34]. Unsaturated C=C aromatic ring absorption is also observed between 1640 and 1500cm^{-1} (in all three crude extracts) and an amide (in the water and water-after-ethanol). Although the aryl-H stretching mode is overshadowed, as usually the case, by the strong and broad band characteristic of associated -OH band.

The –OH groups (observed around 3400 and 3230cm^{-1}) may be attached to a benzene ring (which has absorbance around 1613 and 1510cm^{-1}) suggests the presence of phenolic groups like tannins, polyphenols and flavonoids found in the

phytochemical analysis. The –OH group may also be attached to a carbonyl (C=O) arising from carboxylic acid (with absorbance at 3230 and 1700cm^{-1} for C=O of acids) which may either be aliphatic (due 1700cm^{-1}) or phenolic (due 1613 and 1510cm^{-1}) [67]probably due to the polyphenols, tannins and flavonoids or carboxylic.

4.5.2 Infrared spectra of fractions

This subsection presents the IR spectra for the fractions (aqueous and chloroform) of the various extracts, with the deductions and discussions made from them.

4.5.2.1 Infrared spectra for chloroform fractions

The IR spectra of the chloroform fractions of the three extracts are presented in figures 4.4 – 4.7 below, the deductions made from them and the discussion are presented in this subsection.

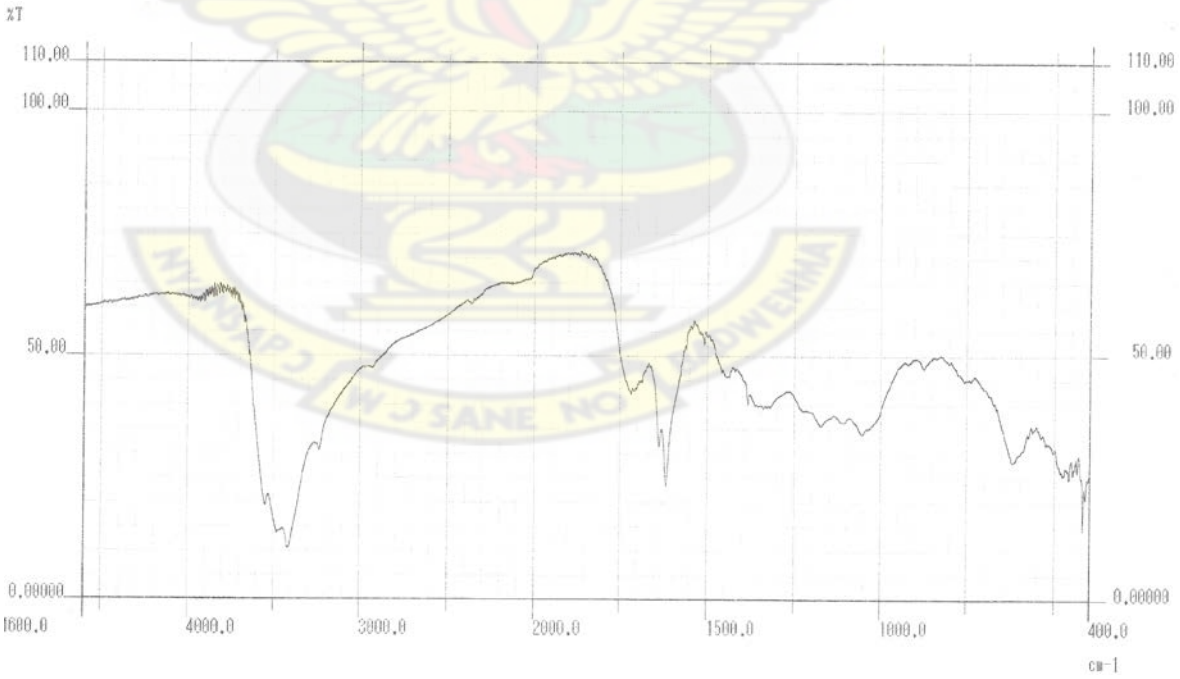


Fig 4.4: IR spectrum of chloroform fraction of water extract.

The following functional groups can be identified from the chloroform fraction of the water extract (see table 4.10)

Table 4.10: Deductions made from IR band of chloroform fraction of water extract

Frequency, cm^{-1}	Functional group	Remarks
3550 - 3000	–OH, -NH	Broad band with absorbance at 3550, 3500, 3400cm^{-1}
3230	-OH stretch	Weak band
1725	C=O for normal ester	Broad band
1638	>C=C<	Sharp intense band
1613, 1510	Aromatic C=C	Sharp bands
1125	C-O stretching	Weak. Associated with esters
1050	C-O stretch	Weak band.

The IR spectrum of the chloroform fraction of the ethanol extract is shown figure 4.9

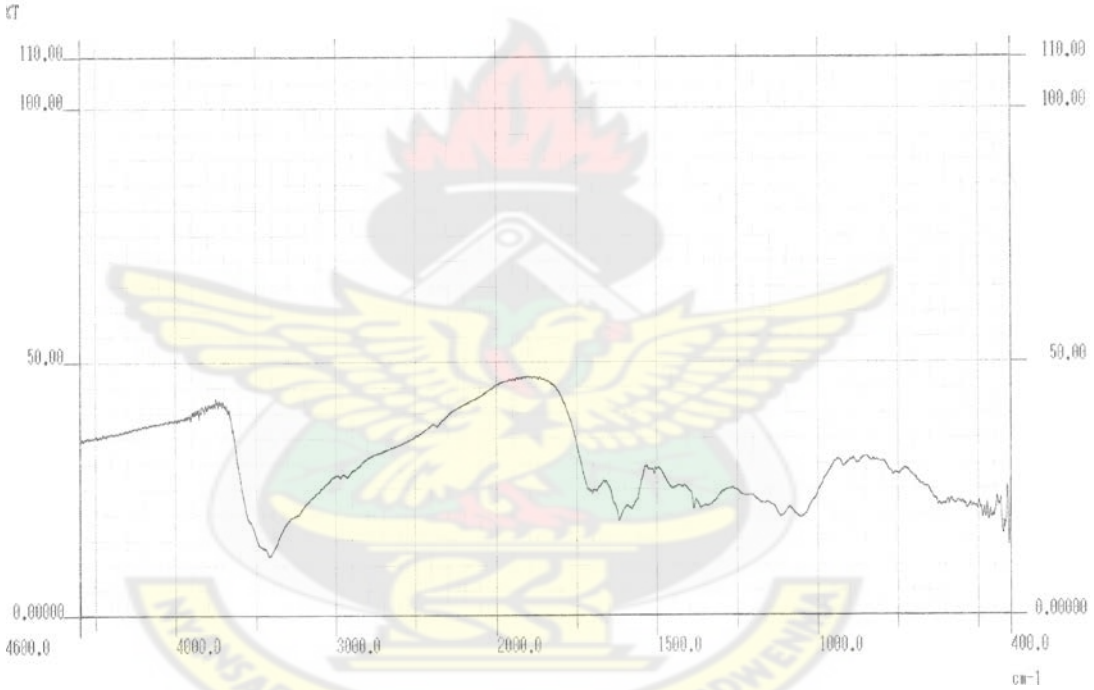


Fig 4.5: IR spectrum of chloroform fraction of ethanol extract

The following functional groups can be identified from figure 4.9 (see table 4.11)

Table 4.11: Deductions made from IR band of chloroform fraction of ethanol extract

Frequency, cm^{-1}	Functional group	Remarks
3400	O-H stretch	Broad band
2930	C-H stretch	Weak band
1710	C=O stretch	Broad band
1613	C=C of benzene	Sharp intense band
1125, 1050, 920	C-O stretching	Weak band.

The IR spectrum for the chloroform fraction of the water-after-ethanol extract is shown in figure 4.6

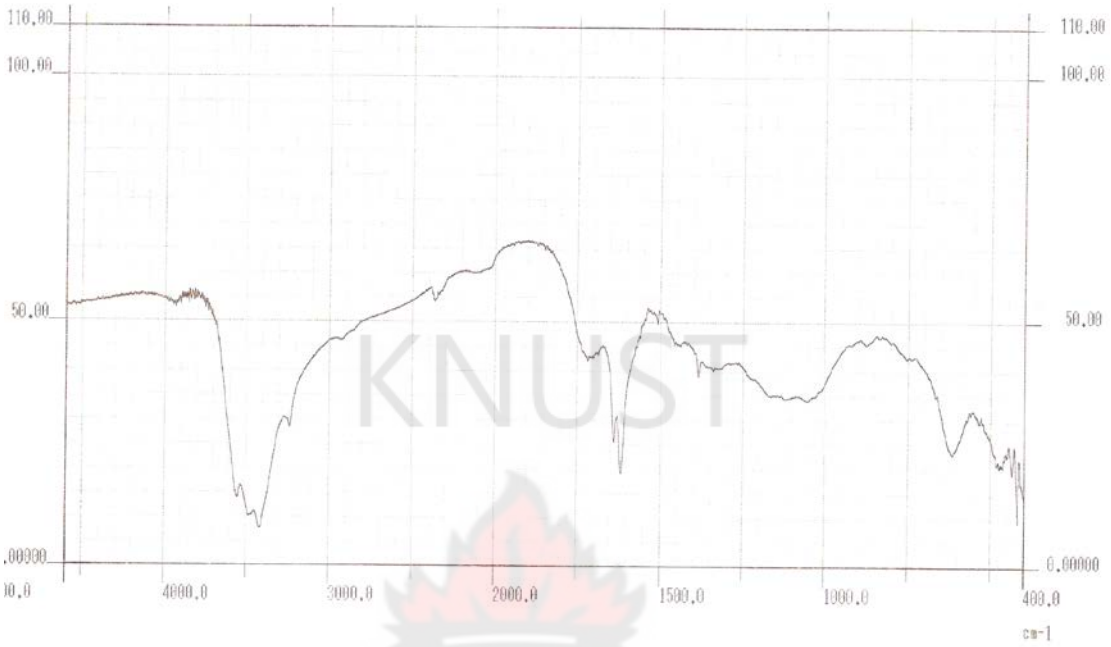


Fig 4.6: IR spectrum of chloroform fraction of water-after-ethanol extract.

The functional groups identified in the above spectrum (figure 4.5), are summarised in the table 4.12 below.

Table 4.12: Deductions made from IR band of chloroform fraction of water-after-ethanol extract

Frequency, cm^{-1}	**Functional group**	**Remarks**
3550 - 3000	Free –OH, -NH	Broad band with absorbance at 3550, 3500, 3400cm^{-1}
3230	OH of acid	Weak band
1725	C=O for normal ester	Broad band
1638	>C=C<	Sharp intense band
1613, 1510	Aromatic C=C	Sharp bands
1125, 1050	C-O stretch	Weak band.

From the spectra of the chloroform fractions above (figure 4.4 – 4.6), a broad peak was observed at 3600 - 3250 cm^{-1} and 1050 cm^{-1} indicating hydroxyl group and one absorption band at 1650 cm^{-1} indicating carbon-carbon double bonds [34, 66]. The prominent peak of 3200-3400 cm^{-1} (for carboxylic acid -OH) and strong signal at

1720 cm^{-1} due to carbonyl absorption, 1640cm^{-1} (associated with C=C), including absorbance at 1465, 1380, 1050 cm^{-1} (associated with C-C, C-H and C-O) tallied well with the olefinic and aromatic acid ring structure.

There are absorbing frequencies around 1720cm^{-1} (in all the spectra) which corresponds to aryl and αβ-unsaturated esters and lactones [67]. Again, the absorbance at about 3550cm^{-1}, 3240cm^{-1} and 1650cm^{-1} presupposes the presence of –NH with carbonyl groups (at around 1700cm^{-1} and 1630cm^{-1}) quickly suggests the presence of an amide (1750cm^{-1} – 1700cm^{-1}). The absorbance at about 1050cm^{-1} and 1330cm^{-1} confirm the presence of esters and/or alcohols [34]. The C-O stretching at 1110cm^{-1} may be attributed to the glycosidic bonds/linkages (from anthraquinone glycoside in crude extracts) which are sp^3 C-O (1625cm^{-1}).

The presence of carbon-carbon double bond (C=C) at 1510cm^{-1} and 1620cm^{-1} which may be aliphatic or aromatic [34, 67]. There is the high probability of these C=C been part of an aromatic system (arising from absorbance at around 1613 and 1510cm^{-1}), which may be have substitution (as indicated around 600cm^{-1}).

4.5.2.2 Infrared spectra of aqueous fractions

The spectra obtained from the aqueous fractions of the three extracts are shown from figures 4.7 to 4.9 below, with deductions made from them. They are subsequently discussed.

The IR spectrum for the aqueous fraction of the water extract is shown in figure 4.7 below.

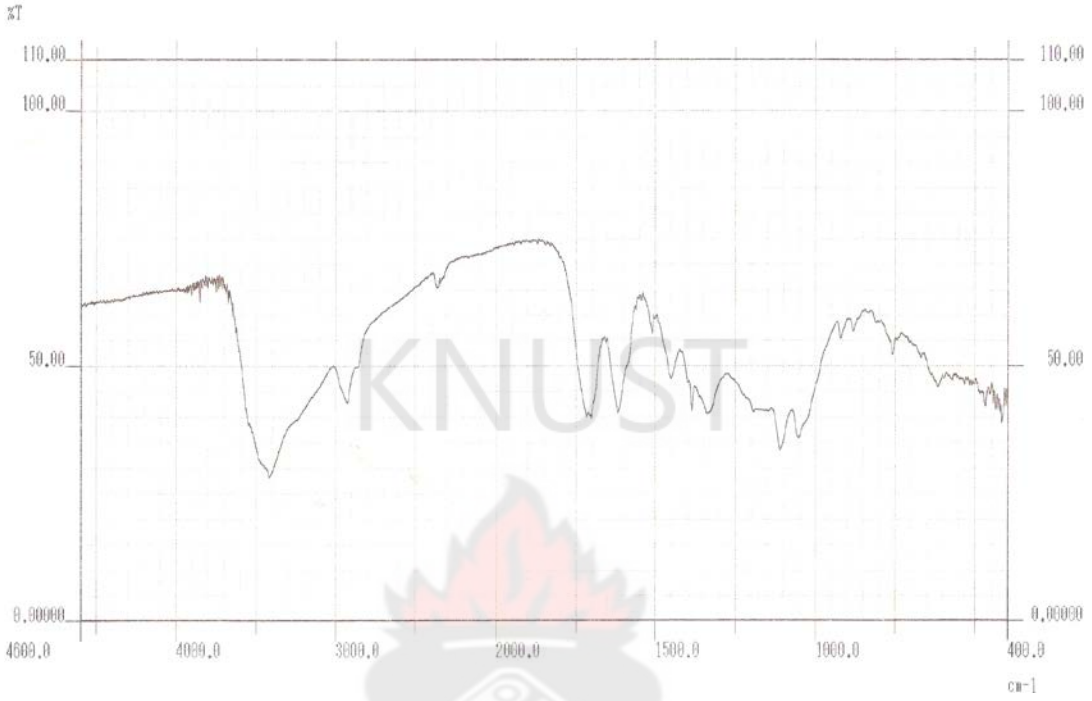


Fig 4.7: IR spectrum of aqueous fraction of water extract

The following important functional groups can be identified from the IR spectrum of the aqueous fraction of the water extract (see table 4.13)

Table 4.13: Deductions made from IR band of aqueous fraction of water extract

Frequency, cm^{-1}	**Functional group**	**Remarks**
3400	O-H, -NH stretch	Broad band
2370	$>NH_2$	Weak and intense
1613	N-H scissoring	Strong and intense
1510	Aromatic C=C	Weak band
1350	C-N stretch	Intense band
1125, 1050	C-O stretching	Weak band.
800	N-H bending	Weak and intense

The IR spectrum for the aqueous fraction of the ethanolic axtract is shown in figure 4.8 below.

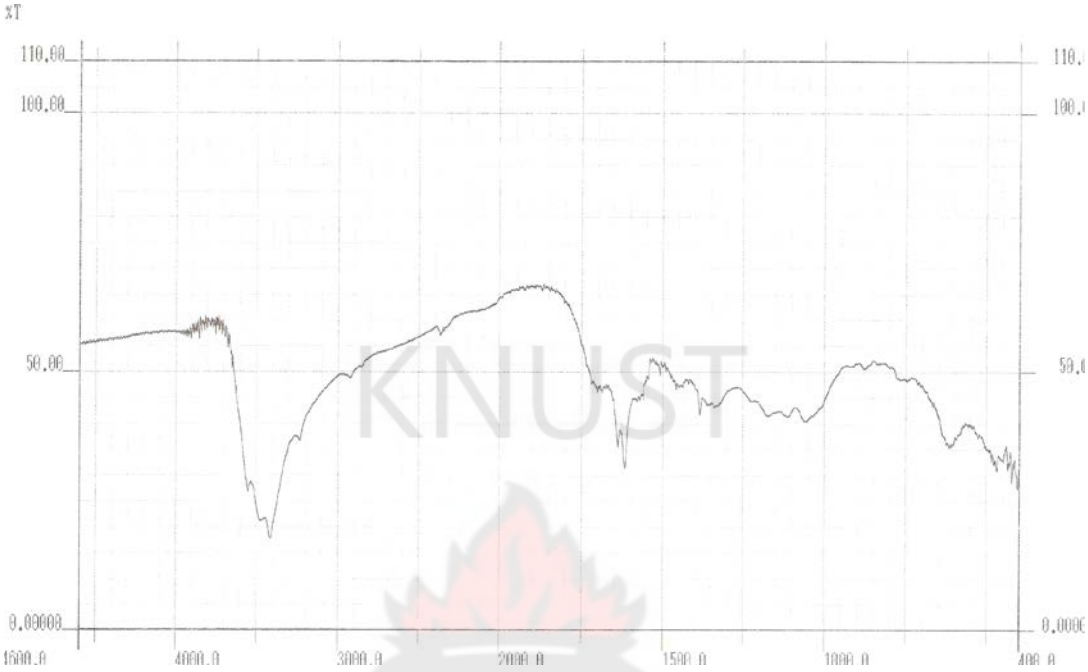


Fig 4.8: IR spectrum of aqueous fraction of ethanol extract

The IR spectrum for the aqueous fraction of the water-after-ethanol extract is shown in table 4.9 below.

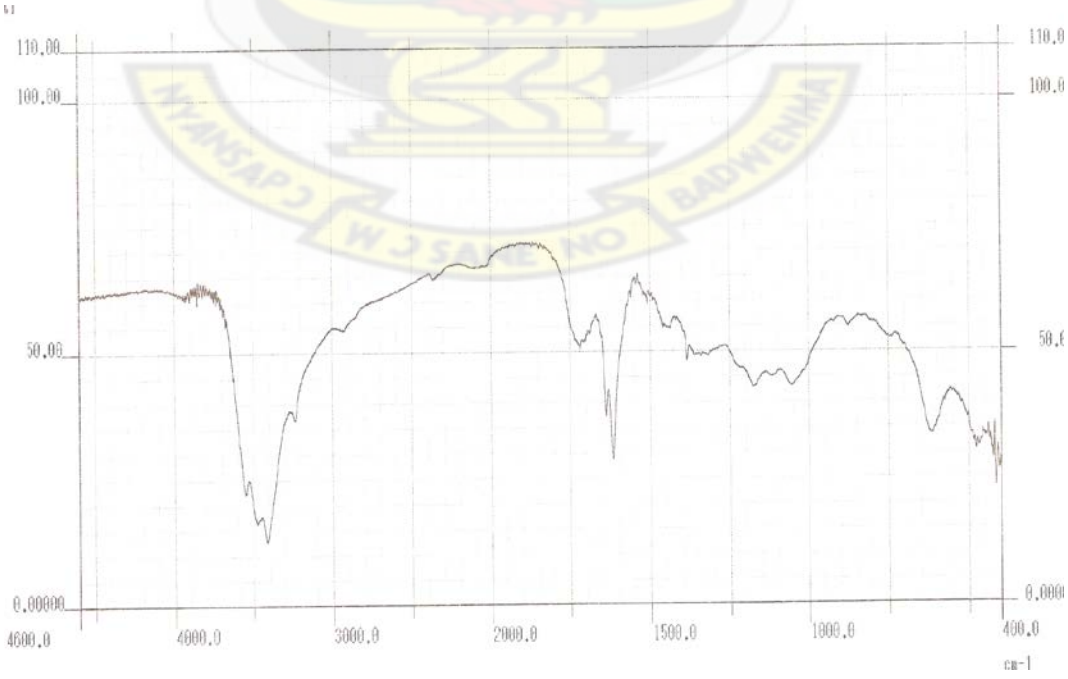


Fig 4.9: IR spectrum of aqueous fraction of water-after-ethanol extract

The functional groups identified from the above spectra (figures 4.8 and 4.9) are summarised in table 4.14 below.

Table 4.14: Deductions made from IR band of aqueous fraction of ethanol and water-after-ethanol extracts

Frequency, cm^{-1}	**Functional group**	**Remarks**
3550 - 3000	Free –OH, -NH	Broad band with absorbance at 3550, 3500, 3400cm^{-1}
3230	-OH stretch	Weak band
1725	C=O stretch	Broad band
1638	>C=C<	Sharp intense band
1613	N-H scissoring	Sharp intense band
1510	Aromatic C=C	Weak sharp band
1125, 1050	C-O stretching	Weak band.
800	N-H bending	Weak band

The aqueous fractions were found to contain associated OH groups (3500 and 3400cm^{-1}). These may be attached to a benzene ring with the observed absorbance at 2930cm^{-1} and 1510cm^{-1}. Again, a strong and sharp band at about 1620cm^{-1} in figure 4.7 indicates the presence of conjugated C=C, which may also result from a phenyl group [66]. There are also absorbance at frequencies 1700cm^{-1}, 1115cm^{-1} and 1050cm^{-1} which are identical to carbonyls, typically for esters [66, 67].

Broad absorption band(with absorptions at 3550, 3500 and 3400cm^{-1}) from 3000 – 3600 cm^{-1} were observed in the aqueous fraction of the ethanolic and water-after-ethanol extracts as shown in figure 4.7 and 4.8 above. The broad band in the aqueous fraction of the water extract, however, had a peak at 3400cm^{-1}. The broad band in this region (3000 – 3600 cm^{-1}) is attributable to associated –OH, which normally overshadows the bands for Aryl-H and -NH [66]. The absorption intensities (at 3470cm^{-1} and 3400cm^{-1}) can therefore, be assigned to them. The sharp band of medium intensity at about 1620cm^{-1} (for conjugated C=C) and 1510cm^{-1} confirms

the presence of conjugated aryl (phenyl) groups [34]. This may be substituted rings (having absorbance at 625cm^{-1}) [67] probably with –OH.

Amines and immines can also be seen to be present, with weak absorption bands at 3470 and 3230cm^{-1} [66]. Sharp signal at about 1640cm^{-1} and 1620cm^{-1} due to carbonyl absorption and absorption between 1050cm^{-1} and 1125cm^{-1} for C-O [68] suggest the presence of esters, amides and ketones. These functional groups observed are probably because of the presence of saponins, anthraquinones and steroids as indicated crude extract. The presence of aliphatic compounds indicated by weak symmetric C-H band at around 2920cm^{-1} and CH_3 symmetric deformation around 1390cm^{-1} is also worth mentioning.

There is however, the absorbance at around 1613cm^{-1} and 1515cm^{-1} which confirms the presence of nitrogenous moiety in the form of –NH and >NH [66]. Again, weak absorbance around 1720cm^{-1} which falls within the carbonyl region of ketones[66, 67] probably due to anthraqunones (as found in crude extract of the water-after-ethanol).

4.6 ANTIOXIDANT PROPERTIES

Various methods are known to be used in determining the antioxidant properties of plant species, two of these were used as described under section 3.2.7 and the results obtained are discussed in this section

4.6.1 Hydrogen Peroxide Decomposition

Hydrogen peroxide is a weak oxidizing agent and can inactivate thiol (-SH) groups. It can cross cell membrane rapidly, once inside the cell. Hydrogen peroxide

reacts with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this may be an origin of its toxic effects as shown by Fenton's reaction

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

The trends of the decomposition of hydrogen peroxide by the ethanolic extract and its fraction at various concentrations have been shown in figure 4.10 below. It can be observed that 20mg/ml of the extract and its fractions are just the minimum investigated concentrations that are able to produce effective decomposition within a reasonable time of 60s. The decompositions increased uniformly with time up to about 60s and became irregular onwards.

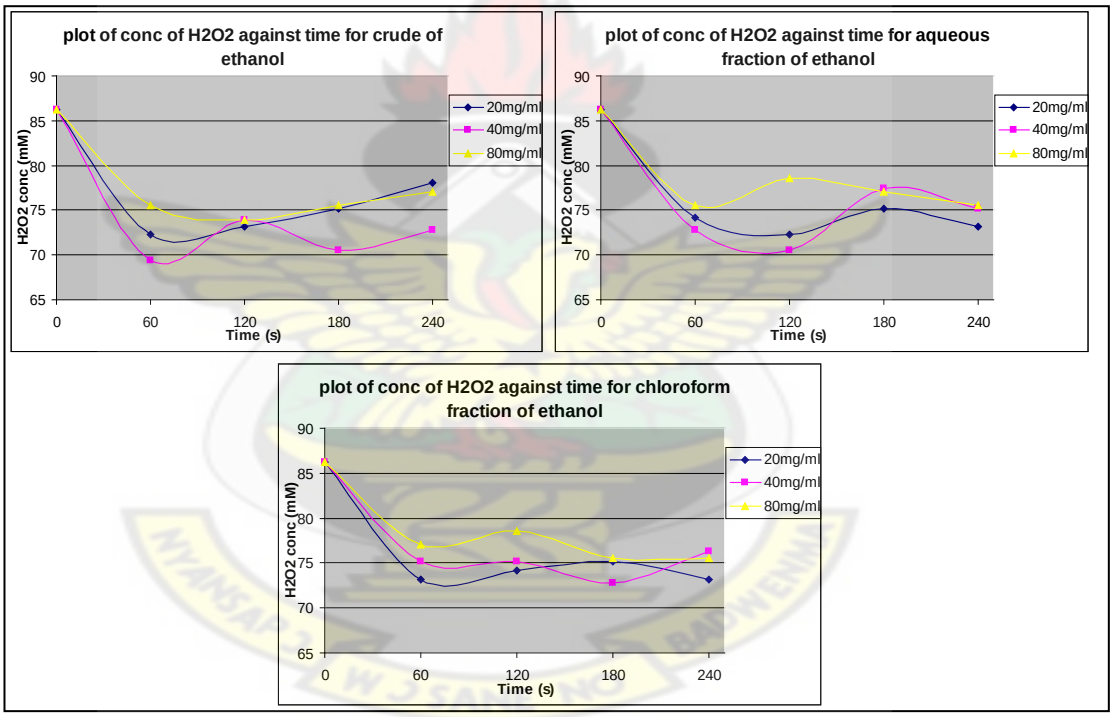


Fig 4.10: hydrogen peroxide decomposition of ethanolic extract

Even though the decomposition of the ethanolic extract of *Terminalia ivorensis* is not really time dependent, it could be observed to cause decomposition in a dose dependent manner (but not at very high concentrations). This is clearly depicted in figure 4.11 below. The chloroform and crude extracts were effective even

at 40mg/ml of the extracts' concentration; the decomposition of H_2O_2 a found to be low.

It is biological advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. The catalase and peroxidases are the two types of enzymes that exist to remove hydrogen peroxide (to ground state oxygen without any singlet oxygen) within cells [64]. The extracts may have decomposition of peroxide activity partly due to these enzymes.

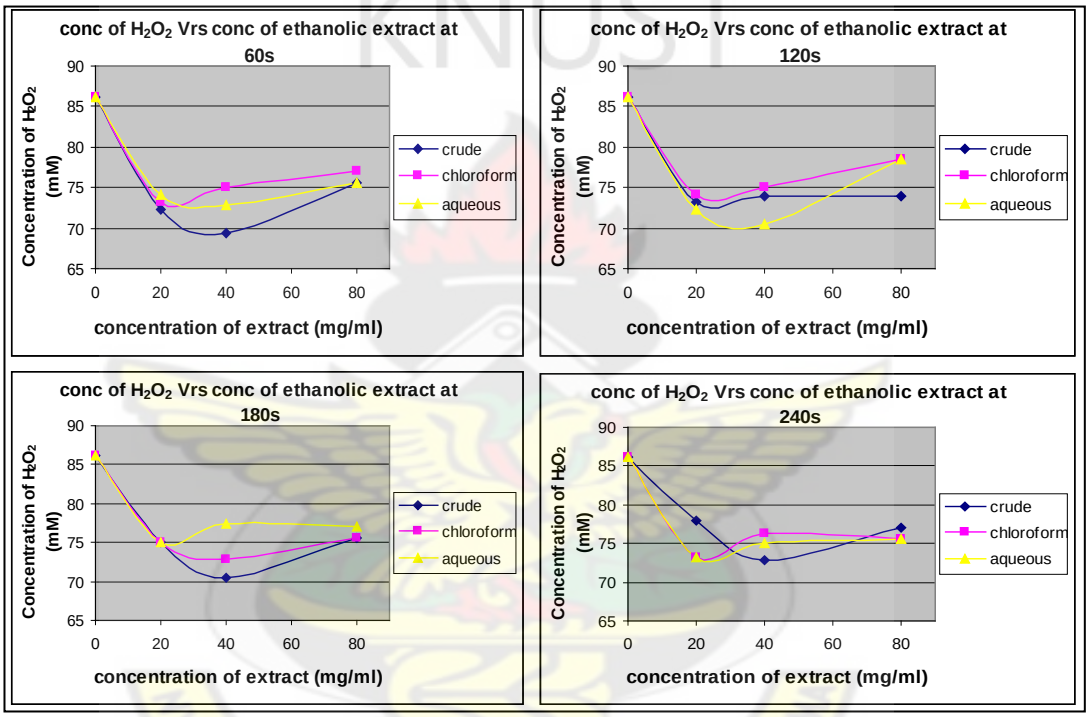


Fig 4.11: Effect of concentration of *Terminalia ivorensis* ethanolic extract on H_2O_2 decomposition

The crude extract and aqueous extract of the water-after-ethanol extract of *T. ivorensis* also caused decomposition of hydrogen peroxide with time up to 40mg/ml of the samples (shown in figure 4.12). The peroxide decomposition was greater in aqueous fraction. The chloroform fraction however, caused an increase the concentration (composition) of peroxide in a dose dependent manner throughout time.

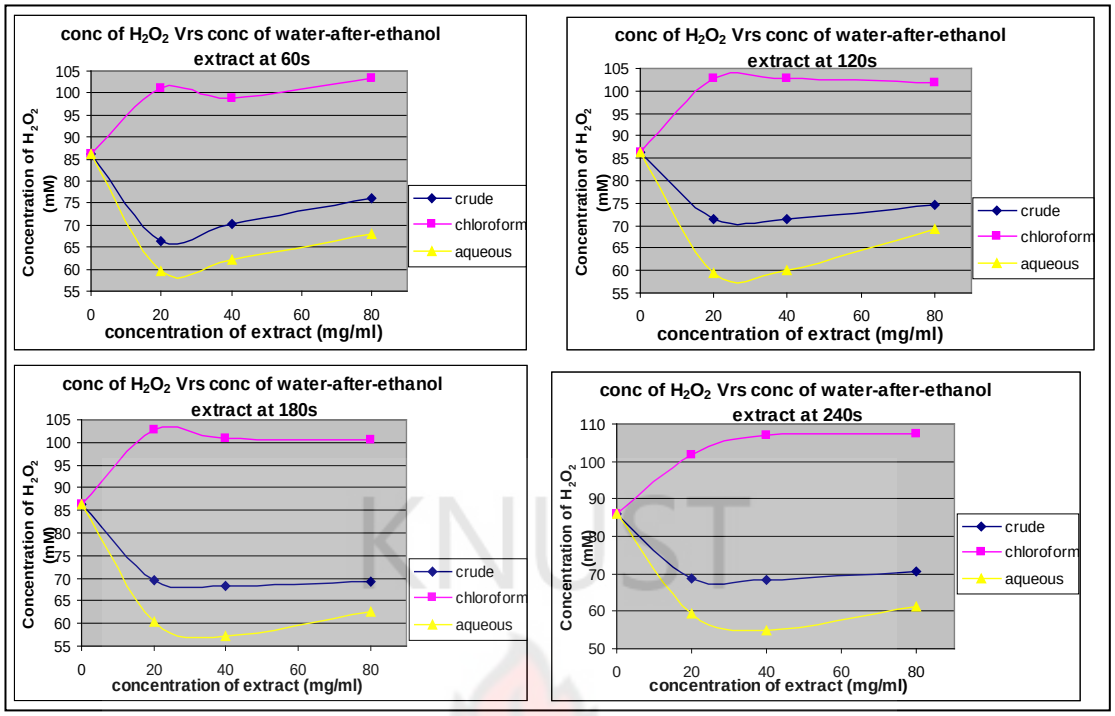


Fig 4.12: Effect of concentration of water-after-ethanol extract of *Terminalia ivorensis* on H_2O_2 decomposition

The trends of composition/decomposition of hydrogen peroxide as a function of time at various concentrations of the water-after-ethanol extract and of its fractions are shown in figure 4.13 below. It could be observed from the graphs that 20mg/ml of each of the extracts and fractions is required to produce an effective decomposition (for the crude and aqueous fraction) and composition (in the chloroform extract) within a reasonable time of 60s. After 60s, the concentration of the peroxide decreased appreciably in the crude and aqueous fraction (for 20mg/ml and 80mg/ml of the sample) throughout the time within which the reaction was observed.

The composition of H_2O_2 increased drastically within 60s for the chloroform fraction at all measured concentrations. The increase levelled up after 60s at 40mg/ml, with a further increase in the 20mg/ml and 80mg/ml with time as observed in the crude and aqueous fractions.

The composition / decomposition of H_2O_2 activity of the extracts and fractions may be due to activity of catalase and peroxidase [57, 64]. The composition of the peroxide in the chloroform extract may be attributed to a low or even no activity (absence) of these enzymes.

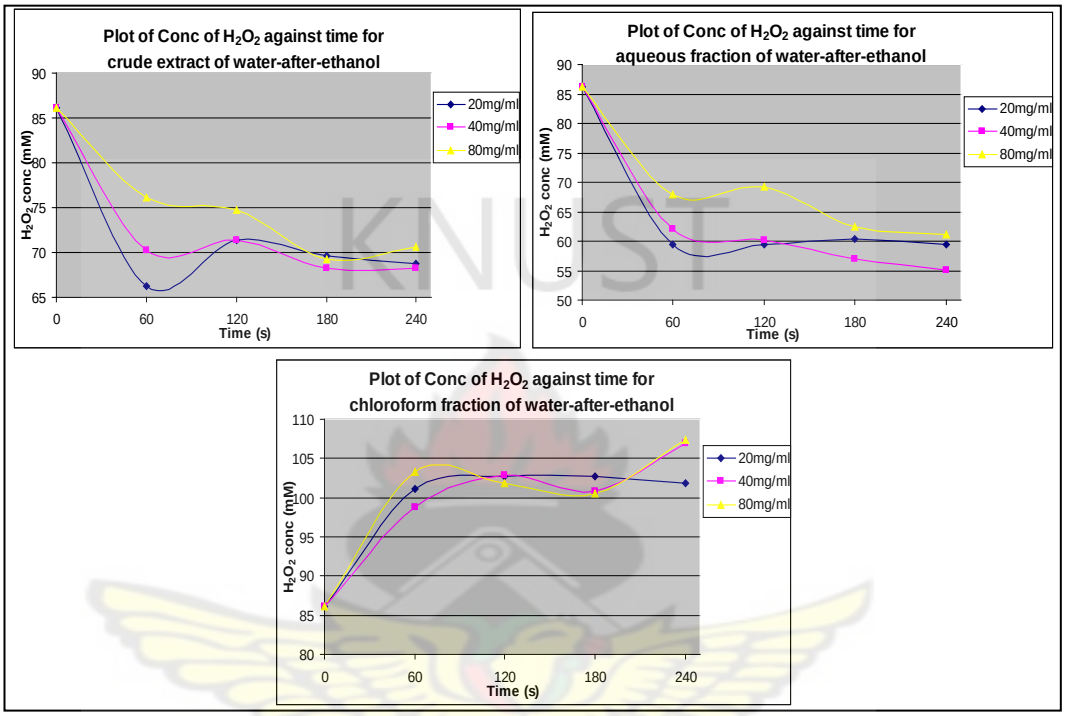


Fig 4.13: Hydrogen peroxide decomposition of water-after-ethanol extract

The decomposition of the peroxide was increased in a dose dependent manner throughout time for the crude extract and the aqueous fraction at a minimum concentration of 20mg/ml. with the effect of decomposition in aqueous fraction significantly high.

The decomposition H_2O_2 of water extract is however, virtually the opposite. The increase in the concentration of the peroxide was rapid in within 60s and then levelling up or increasing with time (figure 4.14). The increase in the concentration of H_2O_2 for the chloroform extract was marginal, though. A concentration of 20mg/ml gave minimal oxidation in both the aqueous and chloroform fraction within 60s, while it gave a maximum in the crude extract.

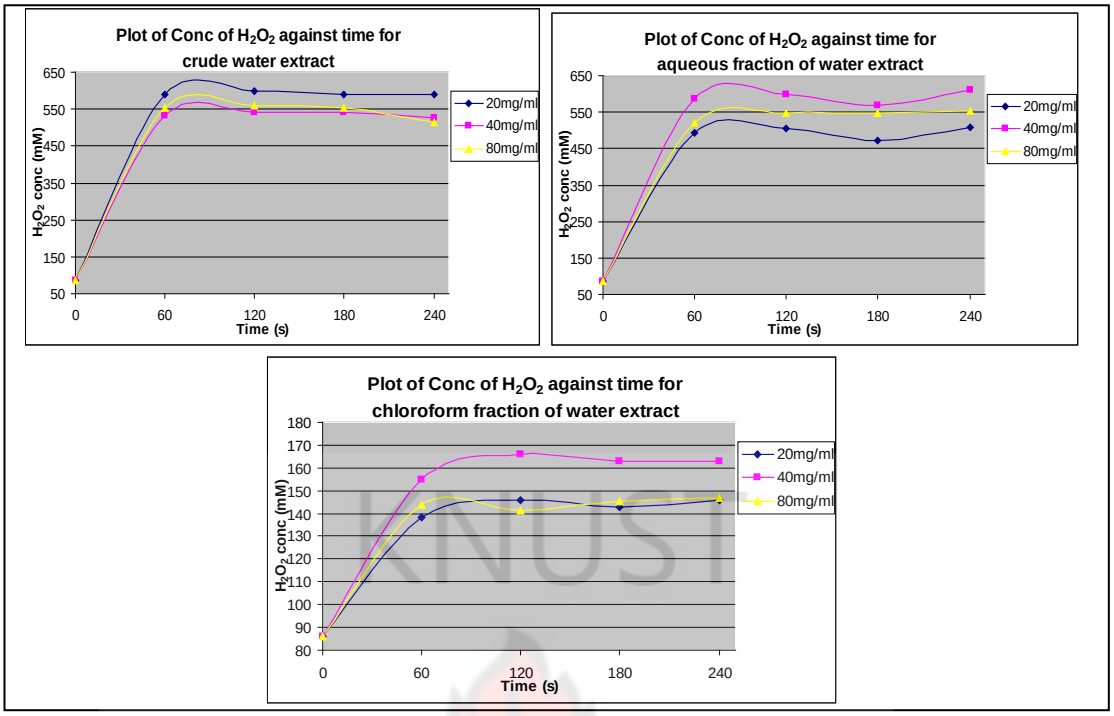


Fig 4.14: Hydrogen peroxide decomposition in aqueous extract

The trend of the increase in the peroxide concentration is the same throughout time with increase in the concentration of the extract (as shown in the graphs in figure 4.13). There is however, a somewhat dose dependent decrease in the peroxide concentration. The oxidative activity of the aqueous extract may be due low or no enzymic activities of catalase and peroxidase. The minimal oxidative action of the in the chloroform fraction man be attributed to an appreciable activities of these enzyme.

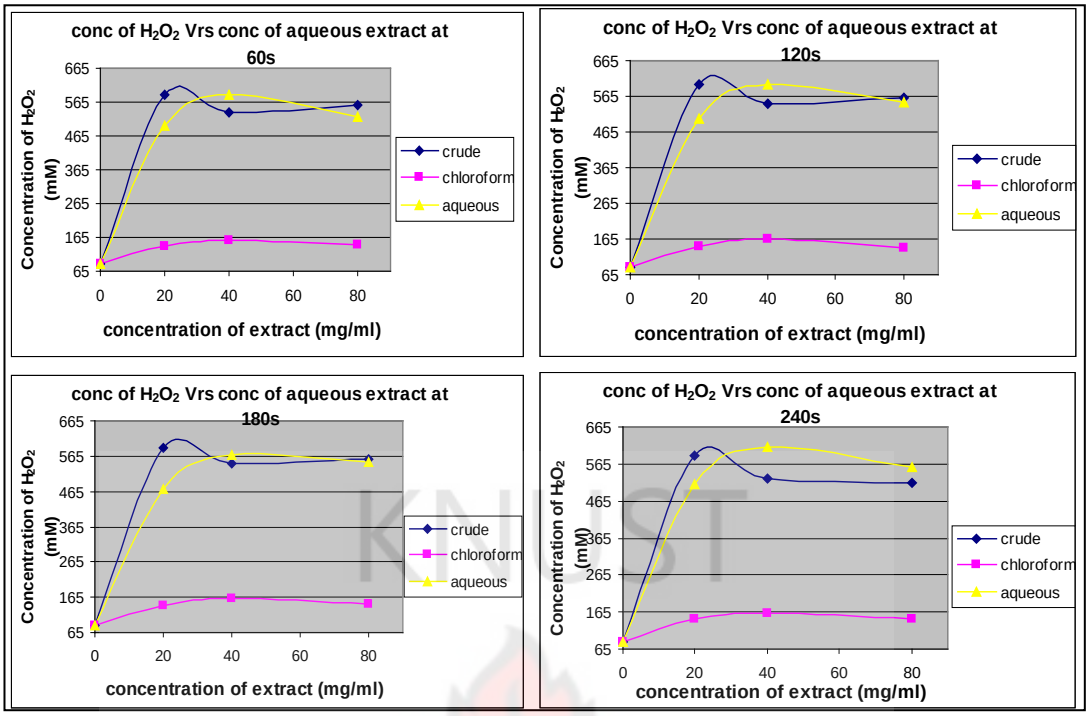


Fig 4.15: Effect of concentration of *Terminalia ivorensis* aqueous extract on H_2O_2 decomposition

4.6.2 Reducing Power

The absorbances obtained for the various concentrations of the extracts and fractions as described in Section 3.2.7.2 were plotted against the concentrations and are shown in figure 4.6.

All the extracts and fractions reduced Fe (III) significantly. The reducing ability of the chloroform fractions increased uniformly with increasing concentrations in the ethanol and water extract but with a reduction at 3mg/ml. The reducing ability at 1mg/ml was even higher than the n-propyl gallate (absorbance of 0.23).

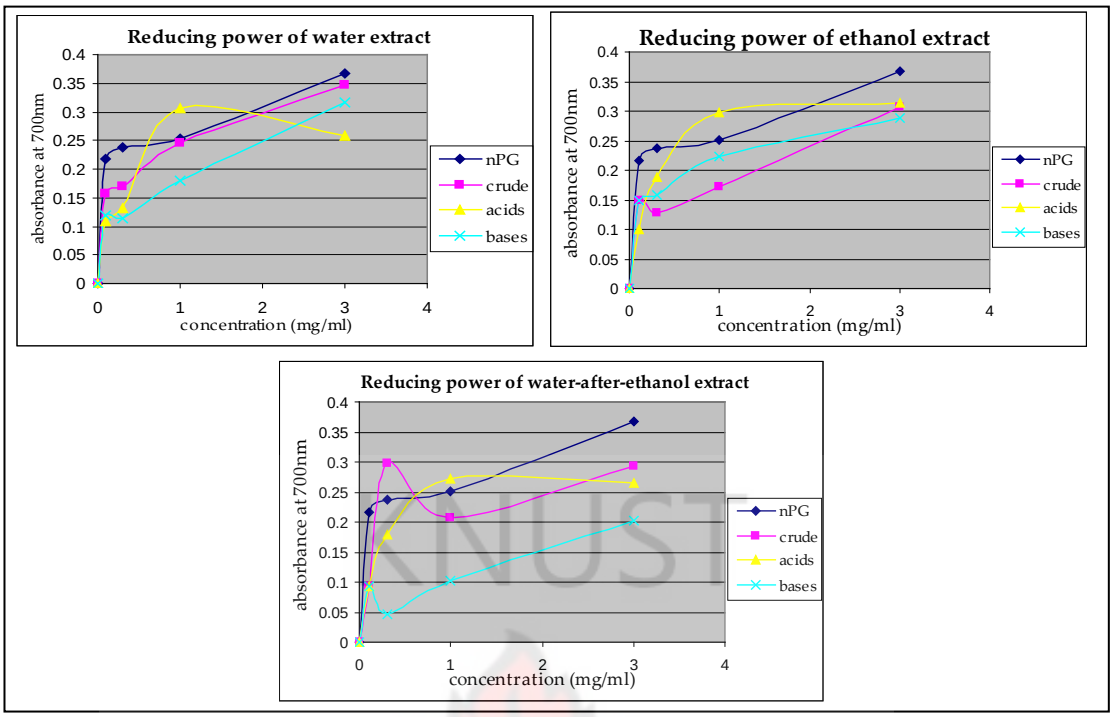


Fig 4.16: Graphs depicting the trend of reducing powers of extracts and fractions

The reducing abilities and the peroxide decomposition of the plant can also be attributed to the type of functional groups present, aside the enzymic action. Rutin (a flavonol glycoside), for instance, is known to combine with cations, supplying nutrients from the soil to the cells in plants. In humans, it attaches to the iron ion Fe^{2+}, preventing it from binding to hydrogen peroxide which would otherwise create a highly reactive free radical that may damage cells. It is also an antioxidant, and therefore plays a role in inhibiting some cancers [40].

CHAPTER FIVE

5 CONCLUSIONS AND RECOMMENDATIONS

Having outlined the methods and findings of the work in the previous chapters, this chapter presents the summary of the major findings of the study and give suggestions and recommendations for future work in the same area.

5.1 Conclusions

From the results obtained, the following conclusions can be made:

The moisture content in *Terminalia ivorensis* is 10.33% whilst the percentage yields obtained were 6.53%, 20.89% and 2.74% respectively for the aqueous, ethanolic and water-after-ethanol extracts.

All the three extracts of *Terminalia ivorensis* contain saponins, steroids and triterpenoids, and anthraquinones glycosides; while tannins and polyphenols, and flavonoids are present in both aqueous and alcoholic extracts with anthraquinones present in only the water-after-ethanol extract. None of the extracts contained carotenoids, general glycosides, alkaloids and coumarins.

From the IR spectra of the extracts and fractions, -OH, aliphatic CH_2 and CH_3 groups, primary amines, amides, conjugated C=C and C=O could be deduced from the bark of the plant. This could confirm the presence of steroids and terpenes, polyphenols and some glycosides as found in the phytochemical screening.

All the extracts and their fractions (crude extracts, chloroform and aqueous fractions of the water, ethanol and water-after-ethanol extracts) of *Terminalia ivorensis* show significant activities against *E. coli, S. pneumoniae, S. aureus*, and *P. aeruginosa* (MIC from 22.15 to 7937.04 µg/ml) compared to chloramphenicol (7.56 to

1881.64 μg/ml). The chloroform fraction of the water-after-ethanol extracts and the aqueous and the chloroform fractions of the water extract however, do not show any activity against the test organisms.

The results from the antioxidant studies showed that the extracts of *T. ivorensis*, to a large extent, have appreciable antioxidant activities, accomplished by their significant reducing power and decomposition of H_2O_2. The water-after-ethanol extract and the chloroform extract of the aqueous extract however, did not decomposed H_2O_2. This may be due to their chemical constituents (like phenol, saponins and terpenes) and the enzyme activities of catalase and peroxidase. These antioxidant and antimicrobial properties of the bark of *T. ivorensis*, to a large extent, is a significant factor in its usage for the management of wounds, age-related diseases (like cancer, trauma, stroke, asthma, hyperoxia, retinal damage, liver injury, and periodontis) and other bacterial-related diseases.

5.2 Recommendations

- More solvent systems (mixtures) must be tried (using thin layer and column chromatograpgies) to obtain the best solvent for the separation of the fractions especially.
- Even though the crude extracts have been found to inhibit the growth of *Escherichia coli, streptococcus pneumoniae, Staphylococcus aureus,* and *Pseudomonas aeruginosa* considerably, minimum bacterialcidal concentrations (MBC) must be performed on the plant to ascertain whether it is just inhibitory or can also kill these organisms and other organisms.
- Since the antioxidant property of any species is known to be the cumulative properties of most of the antioxidant methods [50], other antioxidants methods

like total phenol, Fe^{2+} chelation and DPPH scavenging properties should be performed to ascertain the total antioxidant property of the plant.

- *Terminalia ivorensis* is known to have antifungal activity; further work should be done on various extracts and fractions to ascertain its activity against particular fungi.
- The phytoconstituents found to be present must be isolated to determine their antioxidant, antimicrobial and antifungal activities.

REFERENCES

1. Akinyemi KO, Oluwa OK and Omomigbehin EO (2006), *Antimicrobial activity of crude extracts of three medicinal plants used in South-West Nigerian folk medicine on some food borne bacterial pathogens,* Afr. J. Trad. CAM 3(4): 13 – 22.

2. Biswas TK, Maity LN, Mukherjee B., (2004), *Wound healing potential of Pterocarpus santalinus linn: a pharmacological evaluation,* Int J Low Extrem Wounds.;3(3):143-150. http://www.ncbi.nlm.nih.gov/pubmed/15866805?ordinalpos=3&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum (assessed 2006 November 16).

3. Trombetta D, Puglia C, Perri D, Licata A, Pergolizzi S, Lauriano ER, De Pasquale A, Saija A, Bonina FP (2006), *Effect of polysaccharides from Opuntia ficus-indica (L.) cladodes on the healing of dermal wounds in the rat,* Phytomedicine; 13(5):352-358. www.ncbi.nlm.nih.gov/pubmed/16635743?ordinalpos=2&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum (assessed 2006 November 15).

4. Dickson RA, Houghton PJ, Hylands PJ, Gibbons S. (2006), *Antimicrobial, resistance-modifying effects, antioxidant and free radical scavenging activities of Mezoneuron benthamianum Baill., Securinega virosa Roxb. &Wlld. and Microglossa pyrifolia Lam,* Phytother Res; 20(1): 41-45.

5. Kubo M, Matsuda H, Tanaka M, Kimura Y, Okuda H, Higashino M, Tani T, Namba K, Arichi S (1984), *Studies on Scutellariae radix. VII. Anti-arthritic and anti-inflammatory actions of methanolic extract and flavonoid components from Scutellariae radix,* Chem Pharm Bull (Tokyo); 32(7):2724-2729.

6. Abbiw, D. K. (1990), *Useful plants of Ghana: West African uses of wild and cultivated plants*, short Run Press, England, pp. 7, 84-86, 88, 92, 195, 203, 225.

7. Iwu MM, Anyanwu BN (1982), *Phytotherapeutic profile of Nigerian herbs,* I: *Anti-inflammatory and anti-arthritic agents,* J Ethnopharmacol.; 6(3):263-274.

8. Duwiejua M, Zeitlin IJ, Waterman PG, Gray AI. (1994), *Anti-inflammatory activity of Polygonum bistorta, Guaiacum officinale and Hamamelis virginiana in rats,* J Pharm Pharmacol. 46(4):286-290.

9. Gokhale AB, Damre AS, Kulkami KR, Saraf MN (2002), *Preliminary evaluation of anti-inflammatory and anti-arthritic activity of S. lappa, A. speciosa and A. aspera,* Phytomedicine; 9(5):433-437.

10. Cyong J, Otsuka Y, (1982), *A pharmacological study of the anti-inflammatory activity of Chinese herbs. A review*, Acupunct Electrother Res.; 7(2-3):173-202.
http://www.ncbi.nlm.nih.gov/sites/entrez (assessed 2006 November 17)

11. Fernandez PB, Iglesias PI, Villar del Fresno AM, (1997), *Anti-inflammatory and antiulcer activity of Teucrium buxifolium*, J Ethnopharmacol; 55(2):93-8.

12. Agedahusi, JM; Anao, I; Adewunmi CO; Craft, SL (2006), *Trypanocidal properties of Terminlia ivorensis. Chev (Combrataceae)*, AJTCAM, 3(2): 57-73.

13. Olipoh, G (2003), *antimicrobial properties of ethanol extracts of Terminalia ivorensis and Panda oleosa*, BSc thesis, Chemistry Department, KNUST, pp 34-37.

14. Shukla A, Rasik AM, Dhawan BN (1999), *Asiaticoside-induced elevation of antioxidant levels in healing wounds*, Phytother Res; 13(1):50-54.

15. Irvine FR (1961), *Woody plants of Ghana*, Oxford University Press, London, pp 133, 134.

16. Safra J. E (2003), *wound*, in: the new Encyclopaedia Britannica, 5th Edition, Encyclopaedia Britannica Inc, USA, pp 762, 763.

17. Gawkrogder, D. J. (1992), *an illustrated colour text: Dermatology*, Longman group, UK, p 4.

18. Junqueira LC, Carneiro J. , Kelley, R. O, (1998), *Basic Histology*, 9th Edition, Appleton and Hange, pp 347 – 359.

19. Oratidiya, L. O; Fakoya, F. A, Agbani E. O, Iwakwa E. O (2005), *Vascular permeability increasing effect of the leaf essential oil of ocimum gratissimum linn as a mechanism for its wound healing property*, Afr. J. Trad. CAM 2 (3): 253 – 288.

20. Shryock H and Swarttout H. O. (1965), *Your Health and you*, Pacific Press Pub Assoc, pp 21, 24.

21. Nettina, S. N (2001), *Manual of Nursing Practice*, 7th edition, Lippincott Williams and Wikins, USA, pp 127-129.

22. Mensah AY, Houghton PJ, Agare C, Komlaga G, Menah MLK, Fleischer TC, Sarpong K (2006), *Investigation of activities related to wound healing of secamon afzelii*, J. Sci and Tech 26(3): 83 – 89.

23. Broughton G 2nd, Janis JE, Attinger CE. (2006), *Wound healing: an overview*, Plast Reconstr Surg; 117(7 Suppl):1e-S-32e-S.

24. Jagtap SD, Deokule SS, Bhosle SV. (2006), *Some unique ethnomedicinal uses of plants used by the Korku tribe of Amravati district of Maharashtra, India*, J Ethnopharmacol; [Epub ahead of pub].

25. Mossa J. S., Al-Yahya M.A., and Lieber D. C. (1987), *Medicinal Plants of Saudi Arabia*, vol 1King Saudi Univ. Press, pp 1, 2, 198.

26. Moseley R, Walker M, Waddington RJ, Chen WY (2003), *Comparison of the antioxidant properties of wound dressing materials--carboxymethylcellulose, hyaluronan benzyl ester and hyaluronan, towards polymorphonuclear leukocyte-derived reactive oxygen species*, Biomaterials, 24(9):1549-1557.

27. Houghton, P. J. and Mensah A. Y, *Herbal practitioners and pharmacists in Ghana*, The Pharmaceutical Journal, 271(7258): 93-9.

28. Tyler VE, Brady LR and Robbers JE (1995), *Pharmacognosy*, Lea and Febiger, PA (USA), pp 57, 58, 70-73.

29. Goodwin T. W, Mercer E. I. (1983), *Introduction to plant Biochemistry*, Pergamon Press, pp 480 – 480, 541.

30. Ikan R (1991), *Natural products: a laboratory guide*, 2nd Ed., Academic press Inc., pp 3-7, 105, 226.

31. Mann J, Davidson R. S, Hobbs J. B, Banthorpe and Harborne J. B (1994), *Natural products: their chemistry and biological significance*, Longman Group UK Ltd., pp 56-62, 205, 206.

32. Caret RL, Dennisto KJ, Topping JJ (1997), *Principles and application of Inorganic, Organic and Biological Chemistry*, 2nd edition, Tinges Mirror higher Education Grp Inc., pp 217, 420.

33. Browning BL (1775), *The chemistry of wood*, Robert K. Krieger Pub Co., pp 333, 334, 340, 352, 353, 626-627.

34. Schmid, G. H. (1996), *Organic Chemistry*, Mosby-Year Book Inc., pp 179 – 191, 975, 976.

35. Pitzschke A, Forzani C, and Hirt H (2006), *Reactive Oxygen Species Signaling In Plants*, Antioxidants & Redox Signalling 8, (9 & 10): 1757–1764. www.liebertonline.com/doi/pdf/10.1089/ars.2006.8.1757 (assessed 2007 August 3).

36. Hostettman K and Marston A (1995), *Saponins*, Cambridge Univ. Press, pp 1, 233.

37. Rockett F. H. (1960), *McGraw-Hill Encyclopaedia of Science and Technology*, Vol 14, 6th Ed, McGraw-Hill Book Inc., NY, pp 72.

38. Babu KS, Srinivasa PV, Praveenb B, Kishoreb KH, Murtyb US, Raoa JM, (2003), *Antimicrobial constituents from the rhizomes of Rheum emodi*, Phytochemistry, 62: 203–207.

39. Trease GE and Evans WC (1983), *Pharmacognosy*, 12th ed., Bailliere Tindall, pp 273, 277, 383.

40. Gulam W and Haseeb A (2006), *Reactive oxygen species: role in the development of cancer and various chronic conditions*, J. Carcinogenesis, 5:14. www.biomedcentral.com/content/pdf/1477-3163-5-14.pdf (assessed 2007 August 3).

41. Matysik, J, Alia, Bhalu B, and Mohanty P. (2002) *Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants*, Current Science, 82(5): 525-532.

42. Ashok B, Ali R (1999), *The ageing paradox: free radical theory of ageing*, Exp gerontol 34:293-302.

43. Korycha-Dahl MB, Richardson T (1978), *Activated oxygen species and oxidation of food constituents*, Crit rev, Food Sci. Nutri 10:209-241

44. Steif TW (2003), *The physiology and pharmacology of singlet oxygen*, Med. Hypoth, 60: 567-572.

45. Halliwell B (1997), *Antioxidants and human disease: a general introduction*, Nutr Rev, 55: 544-549.

46. Fang YZ, Fang S and Won G (2002), *Free radicals, antioxidants and nutrition*, Nutrition 18: 872 – 879.

47. Holliday R (1995), *Understanding ageing*, Cambridge Univ. Press, pp 27, 28, 34, 35, 56-59.

48. Watkins TR, Bierenbaum ML, Giampalolo A (1999), *Tocotrienols: Biologicals and health effects*, in: *Antioxidant status, diet, nutrition and health* Papas AM (editor), CRC Press, pp 479 – 496.

49. Boff J, Min DB (2002), *Chemistry and reaction of singlet oxygen in food*, Food Sci. Saf 1:58-72.

50. Yildirim, A; Oktay, M and Bülaloúlu, V (2001), *The Antioxidant Activity of the Leaves of Cydonia vulgaris*, Turk J Med Sci 31: 23-27. http://mistug.tubitak.gov.tr/bdyim/abs.php?dergi=sag&rak=9910-2 (assessed 2007 June 4).

51. Blázovics A, Lugasi A, Szentmihályi K, Kéry A (2003), *Reducing power of the natural polyphenols of Sempervivum tectorum in vitro and in vivo+*, Acta Biol Szeged 47(1-4):99-102.

http://www.sci.u-szeged.hu/ABS/2003/ActaHP/4799.pdf (Accessed, 2007 June 4).

52. Badu-Tawiah, KA (2005), *Characterisation, hypoglycaemic effects and antioxidant properties of the chemical constituents of portulaca oleracea* linn, MSc Thesis, Chemistry Department, KNUST, p 59.

53. Hagymási K, Kocsis I, Lengyel G, Sipos P, Fehér J, Blázovics A (2003), *Further evidence of altered redox status of hyperbilirubinaemic patients: role of bilirubin in Gilbert syndrome*[+], Acta Biol Szeged 47(1-4):131-134. http://www.sci.u-szeged.hu/ABS/2003/ActaHP/47131.pdf (Accessed, 2007 June 4).

54. Molyneux, P. (2004), *The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity*, Songklanakarin J. Sci. Technol., 26(2) : 211-219. www.psu.ac.th/PresidentOffice/EduService/journal/26-2.pdf/07-DPPH.pdf (assessed 2007 November 25).

55. Edelson RL (2006), *Skin*, Microsoft® Student 2007 [DVD]. Redmond, WA: Microsoft Corporation.

56. Prescott LM, Harley JP and Klein DA (1996), *Microbiology*, 3rd Ed, McGraw-Hill Comp. Inc., pp 66-70.

57. Atlas RM (1995), *Microbiology in our world*, Mosby-year Book Inc., pp 296-306

58. Boyd R. F. (1984), *General Microbiology*, Time Mirror/Mosby College Pub, pp 52, 81, 468, 469, 628, 643, 644.

59. Jawetz E, Melnick JK and Adeleberg EA (1976), *Review of medical microbiology*, 12th Ed., Lange Medical Pub (Canada), p110.

60. Smith JR, Laudicina RJ, Rafo RD (1985), *Learning guides to the medical microbiology laboratory*, John Wiley and Sons Inc., Canada, pp 53, 57, 59, 372-376.

61. Damjanov, I (2000), *High-yield pathology*, Lippincott Williams and Wikins, USA, pp 10, 11.

62. Pavia DL, Lampman GM, and Kriz GS (1995), *Introduction to Organic laboratory Techniques: A microscale approach*, Saunders College Publisher (USA), pp 754-768.

63. Stahl, E (1990), *Thin layer chromatography: A laboratory handbook*, Springer-Verlin NY, p127.

64. Benson HJ (1998), *Microbiological Applications: Laboratory Manual in General Microbiology*, 7th Ed., McGraw-Hill Inc (USA), pp 55, 78-80, 83, 87, 124-129, 137, 139,155-162, 445.

65. Singh GK and Anil B (2000), *Textbook of Pharmacognosy*, CB Publisher, Indiana, pp34-37.

66. Williams DH and Fleming I (1966), *Spectroscopic methods in organic chemistry*, 4th ed., rev, McGraw-Hill Inc (UK), pp 29 – 62

67. Carey FA (2003), *Organic Chemistry*, 5th Ed., McGraw Hill, NY, pp 559 – 564.

APPENDICES

APPENDIX 1: CHEMICALS PREPARED FOR PHYTOCHEMICAL SCREENING

The methods for the preparation of the various reagents used in the phytochemical screening are outlined in the section

1. *Fehling's solution*

(a) Copper (II) sulphate solution
6.9g of $CuSO_4.5H_2O$ was dissolved in distilled water and diluted to 100ml

(b) Alkaline tartarate solution
17.3g of potassium sodium tartarate and 5g of NaOH were dissolved in 50ml water.

For use, equal volumes of solutions (a) and (b) (0.5ml each) were mixed

2. *Lead acetate solution*

13.9g of $Pb(C_2H_3O_2)_{2.}3H_2O$ was dissolved to make up to 100ml of solution

3. *Ferric chloride solution*

13.5g of $FeCl_3.6H_2O$ was dissolved in 100ml of distilled water containing 20ml conc. HCl.

4. *Mayer's reagent (Potassium mercuric iodide)*

1.358g of $HgCl_2$ dissolved in 60ml of distilled water and added to a solution of 5g KI in 10ml distilled water, the mixture was made up to 100ml with distilled water.

5. *Hager's reagent (saturated picric acid solution),*

1g of picric acid was dissolved in 100ml distilled water.

6. *Antimony chloride*

3.6g of $SbCl_3$ was dissolved in 100ml distilled water.

APPENDIX 2: DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION

Below is a table for the average of the zones of inhibition obtained for the various extracts and their fractions and chloramphenicol (standard).

The graphs below were obtained by plotting the average zones of inhibition against the Log of concentration of the various extracts and sample using MS Excel. The minimum inhibitory concentrations were obtained by finding the values of the Log of concentration where the ordinate is zero.

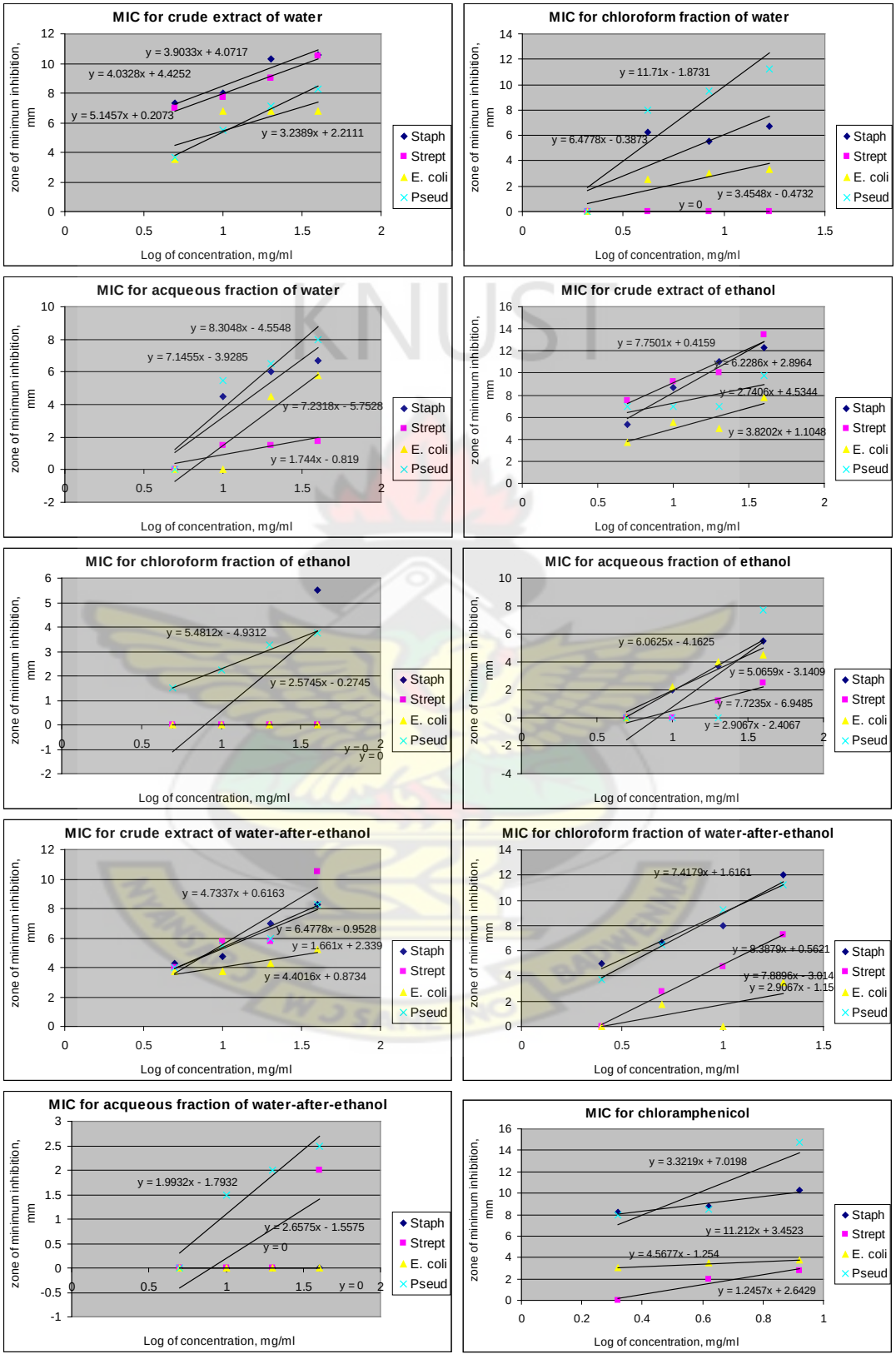


Fig A2: minimum inhibitory concentrations of extracts and fractions

APPENDIX 3: REDUCING POWER

The absorbance of the various extracts and fractions at different concentrations are shown in the table below

Table A2: Absorbance of extracts and fractions at 700nm

concentration	Absorbance at 700nm									
(mg/ml)	nPG	*CA*	*AA*	*BA*	*CB*	*AB*	*BB*	*CC*	*AC*	*BC*
0.0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.1	0.217	0.158	0.109	0.119	0.149	0.101	0.149	0.093	0.094	0.094
0.3	0.238	0.148	0.131	0.114	0.128	0.189	0.157	0.298	0.179	0.047
1.0	0.252	0.246	0.306	0.181	0.172	0.298	0.224	0.208	0.271	0.103
3.0	0.368	0.346	0.259	0.317	0.306	0.314	0.288	0.294	0.266	0.202

APPENDIX 4: THIN LAYER CHROMATOGRAPHY

The table below shows the distances moved by the various extracts and fractions in their respective solvent systems

Table A3: TLC results for extracts and fractions

Solvent system	Distance moved (cm)									
	Solvent font	*CA*	*AA*	*BA*	*CB*	*AB*	*BB*	*CC*	*AC*	*BC*
Methanol	8.8	0.7 1.9 4.7	0.9 1.9 3.8 4.9	1.8 4.8	0.8 3.8 6.3	0.9	1.6 4.1	0.4 2.3 4.9	1.3	0.5 3.8
Acetone	8.8	1.8 3.5 5.6	1.7 2.8 5.4	1.3	1.9 3.5 6.3	0.4	0.6	0.8 2.8 5.6	0.6 2.1	0.4
Chloroform	5.7	0	0.9	0	1.0	0.9	0	0	0	0
Methanol:water (1:1)	8.7	0.7	0.5	0.6 3.0 7.2	0.8 4.9 6.9	0.4	0.4 4.0 6.1	0.7 4.7 7.1	0.7 4.9 6.7	0.4 4.6 6.5
Chloroform:actone (1:4)	9.2	0.6 3.9	0.5 2.0 3.9	0	1.3 2.2 4.1	0.6 8.9	0	0	0.4	0
Methanol:acetone (1:1)	8.1	4.1 6.2	3.5 6.3	0.8 6.0	5.4 6.0	0.6 4.8 6.4	0	0.8 6.3	1.0 2.9	0.3

APPENDIX 5: HYDOGEN PEROXIDE DECOMPOSITION

The following were obtained for the iodometric titrations performed to ascertain the hydrogen peroxide decomposition of the various extracts and fractions using 51.00mM sodium thiosulphate and 86.18mM hydrogen peroxide standard solutions.

APPENDIX 6: CALCULATION OF PERCENTAGE YIELD AND MOISTURE CONTENT

$$\text{Moisture content} = \frac{(\text{Initial weight} - \text{Final weight})}{\text{Initial weight}} \times 100$$

Codes used:

CA = Crude extract of water extract
AA = Chloroform fraction of water extract
BA = Aqueous fraction of water extract
CB = Crude extract of ethanol extract
AB = Chloroform fraction of ethanol extract
BB = Aqueous fraction of ethanol extract
CC = Crude extract of water-after-ethanol
AC = Chloroform fraction of water-after-ethanol
BC = Aqueous fraction of water-after-ethanol
NA = not active