Adama Science and Technology University

School of Applied Sciences

Department of Chemistry

Isolation and Characterization of Chemical Constituents from Roots of *Cyphostemma adenocaule* and Investigation of its Antibacterial and Antioxidant Activities

MSc Thesis Submitted to School of Applied Sciences, Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science in Organic Chemistry

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August, 2015
Adama

APPROVAL SHEET

ADAMA SCIENCE AND TECHNOLOGY UNIVERSITY

SCHOOL OF APPLIED NATURAL SCIENCES

CHEMISTRY PROGRAM

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Acknowledgment

I would like to express my deepest gratitude to my advisor, Dr. Hailemichael Tesso, for his advice starting from proposal preparation to final report of the thesis. My thanks also goes to my friend, Ms. Tsehay Kebede, for her support by searching the medicinal plant for my thesis, facilitating the condition for collecting the plant sample as well as for her support me financially for the success of my work and for her encouragement me to use most of my time to do this thesis.

Next, I like to thank Dr. Aman Dekebo for his support by giving me valuable advice and many materials like RP TLC and Sephadex LH-20 powder without which my work was not succeeded. Lastly, I would like to thank Ms. Marima Kufa, Lab Assistant, for her assist me in different direction in the lab with a great patience and wisely for 10 months.

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Abbreviations

CT: Condensed Tannin

Da: Dalton Atomic Mass Unit

DMSO: Dimethyl sulfoxide

EtOAc: Ethyl Acetate

Fig: Figures

$FeCl_{3:}$ Ferric Chloride

HCl: Hydrochloric Acid

$\lambda_{max:}$ Maximum Absorbance

HT: Hydrolyzable Tannins

IR: Infrared

KBr: Potassium Bromide

KOH: Potassium Hydroxide

KI: Potassium Iodide

MeOH: Methanol

NMR: Nuclear Magnetic Resonance

PA: Proanthocyanidins

PBS: Phosphate-Buffered Saline

PTLC: Preparative Thin Layer Chromatography

TLC: Thin Layer Chromatography

UV-Vis: Ultraviolet-Visible

WHO: World Health Organization

Abstract

The Vitaceae, or grape family, contains around 900 species and 15 genera, including cyphostemma, mainly having the liana growth form. C. adenocaule is one of over 6500 flowering plants and ferns found in Ethiopia. Its different parts are culturally used for different purposes in East African countries including Ethiopia like as a source of food, medicine, insecticide and fibers. Locally people use this plant for treating rabies. But, no detailed scientific investigation is available on the chemical composition of this plant. So, this research was aimed to isolate and characterize the chemical constituents from roots of the plant and to investigate its antibacterial and antioxidant activities. To achieve this objective, the root of the plant was extracted by methanol and successively suspended by hexane, chloroform and ethyl acetate. Phytochemical screenig was carried out on methanol extract. The result indicated the presence of alkaloid, saponnin, condensed tannin and flavanoid. The hexane, chloroform and ethyl acetate soluble portion was mixed and subjected to column chromatography based on their TLC profile and two compounds were selected for characterization namely $F(13\text{-}18)_2$ and $F(13\text{-}18)_5$. The condensed tannin, F(S), was purified from ethyl acetate insoluble portion by using sephadex LH-20 and the structures of all obtained compounds were elucidated by using UV, IR and NMR. The effect of F(S) on activities of four selected bacteria using different concentrations, 0.01, 0.1, 1, 10 mg\ml, was tested. The highest inhibition zone for Escherichia coli (8 mm) and Staphylococcus aurous (11 mm) were seen with 10 mg/ml. But, the highest inhibition zone for the other two bacteria, Salmonella thyphomurium (25 mm) and Streptococcus buvis (14 mm), were observed with 0.1 mg/ml. The antioxidant activity of the F(S) was evaluated by DPPH free radical using concentrations, 62.5, 125, 250, and 500µg/ml. Their % DPPH inhibition was 96, 95, 93, and 88 %, respectively. The purified condensed tannin was observed as it shwed good antibacterial activities and strong antioxidant activities when compared with condensed tannins reported from other plants. But, the concentration of condensed tannin in different maturity of the plant should be studied in detail.

Chapter One: Introduction

1.1 Background

Humans have relied on nature for their basic needs for the production of food stuffs, clothing, means of transportation, fertilizers, flavors, and fragrances, and not least, medicines (Romeo, 1999). Traditionally, humans have used plants as a medicine for a very long time, since 4000 BC (WHO, 1996, 2013; Bernhoft, 2010), and still continue to depend to the present day on plants, their extracts and plant derived drugs for various ailments (Maesen *et al*.,1996; Ahmad *et al*., 2006).

Traditional medicines are used by different countries in the world. As WHO (2013) report indicated, in Europe, the use of traditional medicine ranges from 42 % in Belgium to 90 % in the United Kingdom; in Africa, the range is from 70 % in Benin to 90 % in Burundi and Ethiopia. The main reason for using traditional medicines in developing countries (WHO, 2002) is its accessibility and affordability. In Uganda, for instance, the ratio of traditional medicine practitioners to population is between 1:200 and 1:400. This contrasts starkly with the availability of allopathic practitioners, for which the ratio is typically 1:20,000 or less. Traditional medicine is sometimes also the only affordable source of health care especially for the world's poorest patients. In Ghana, Kenya and Mali, research has shown that a course of pyrimethamine/sulfadoxine antimalarials can cost several dollars. Conversely, herbal medicines for treating malaria are considerably cheaper and may sometimes even be paid for in kind or according to the wealth of the client. However, to maximize the potential of traditional medicine

as a source of health care, a number of issues must be tackled those related to policy; safety, efficacy and quality; access; and rational use (WHO, 2000, 2002).

Ethiopia has over 6500 flowering plants and ferns (Getahun, 1976; Wondafrash, 2008). *C. adenocaule* is one of the wild medicinal plants in Ethiopia (Bosch, 2004). As Bosch, the different parts of this plant is used for different purposes in East African countries including Ethiopia for food, to cure ophthalmia, to remedy a sore throat, cough treatment, to reduce swellings, to cure pneumonia, to treat swollen abdomen, against tapeworm, to treat malaria, to treat syphilis, to prevent abortion, used as an insecticide, etc and also locally people are using this plant for treating rabies. But, no detailed scientific investigation is available on the chemical composition of *C. adenocaule.* Therefore, phytochemical investigation of this plant was very important.

1.2 Objective of the Study

1.2.1 General objective

The main objective of this research was to study the chemistry, antibacterial and antioxidant activities of the root of *C. adenocaule*

1.2.2 Specific objectives

- To extract the root of *C. adenocaule* using n-hexane, chloroform, EtOAc, and MeOH
- To fractionate and purify the MeOH crude extract of plant root using column chromatography or PTLC
- To characterize the structures of isolated compounds using different spectroscopic techniques (UV, IR, and NMR)
- To investigate the antibacterial activities of MeOH extract of the plant root and its fraction on four bacteria *(Escherichia coli, Staphylococcus aurous, Salmonella thyphimurium, and Streptococcus buvis)*
- To evaluate the antioxidant activities of MeOH extract of plant root and its fractions

Chapter Two: Review of Literature

2.1 The Vitaceous Plants

Vitaceae, or grape family, contains around 900 species and 15 genera mainly having the liana growth form. From this, the genera *Cissus* and *cyphostemma* contains large species that estimated to 350 and 250, respectively (Chen, 2009). In 1973 and 1990 Hegnauer reported (as cited in Kubitzki *et al*., 2007) Vitaceous plant contains gallic (**38**) and ellagic acids (**40**), proanthocyanins (PAs) (**102**) and catechin (**15** and **16**) and these are indicative of the presence of hydrolysable and condensed tannins, which are essential for the savor of wine, by precipitating protein (Ribereau-Gayon *et al.*, 2006). Adding, Kubitzki *et al.* discussed that in seeds and stalks of the fruits of grapevine, tannins amount to 7.3 %. Other widespread compounds include caffeic (**7**) and chlorogenic acids (**1**), and tartaric acid (**2**), accompanied by malic (**3**) and oxalic (**4**) acids. The fungitoxic stilbene resveratrol (**24**) is induced in the leaves as a phytoalexin, but is regularly present in the wood (Fig. 2, 7 and 11).

Jang *et al*. reported (as cited in Kundakovic *et al*., 2008), Vitaceous plants are known as a good source of stilbenoids and potent chemopreventive agents. In the same way, as Riviere *et al*. (2012) described, the most prominent stilbene containing plant family, the Vitaceae, represented by the famous wine producing grape vines *Vitis vinifera* , is one of the richest sources of novel stilbenes currently known, together with other families, such as Dipterocarpaceae, Gnetaceae and Fabaceae. Others reported that different part of Vitaceous plant can be used as a source of medicines. For example, Singh and Mishra recorded (as cited in Ojogbane *et al*., 2010) that the leaves of most plants in the family of Vitaceae are often used as medicinal herbs because they contain some bioactive compounds such as vitamins, proteins, carbohydrates, and phenols, among others.

2.2 *Cyphostemma adenocaule*

C. adenocaule (Fig. 1), (syn. *Cissus adenocaulis*) and local name *Aserkush tebetebkush* (Amharic), is in the family Vitaceae in the major group Angiosperms (Flowering plants) was given this name by Bernard M. Descoings in 1966, and is widespread in tropical Africa from eastern Senegal to Eritrea and south to Angola, Malawi and Mozambique. It is widespread in savanna and is found in gallery forests and fallow land as well (Bosch, 2004; Mengistu, 2004)

(a) (b)

Figure 1. *C. adenocaule* plant (a), its root (b)

2.2.1 Uses of *C. adenocaule*

Bosch (2004) described the different uses of *C. adenocaule* plant. The leaves and fruits of the plant are commonly eaten as a vegetable or soup in Ghana, DR Congo, Kenya and Uganda. In Uganda, the leaves are boiled with beans, pigeon peas, cowpeas, groundnut or sesame. The fruit is eaten raw in Côte d'Ivoire and Tanzania. The boiled roots are eaten in Ethiopia; and in Uganda sliced, dried and pounded roots are stored for famine periods. Although it has been reported that it is cultivated in Ethiopia, leaves and roots are normally collected from wild.

As Bosch mentioned, there are many medicinal uses recorded about the plant. Leaf sap is used to cure ophthalmia and it is applied to cuts. Leaves are chewed to remedy a sore throat, and macerated leaves are mixed with honey as a cough treatment. Leaves heated over a fire are applied as a compress to reduce swellings. An infusion of the leaves is taken as a purgative and to treat swollen abdomen. Leaves crushed in water are used as an insecticide against chicken lice. The roots were reported to contain tannins (Grubben & Denton, 2004). Macerated roots are taken against tapeworm. The root has been used to treat malaria. A paste made of the roots is applied topically to draw abscesses and reduce swellings. Water in which roots have been boiled is drunk to treat syphilis, abdominal pain (related to pregnancy or not) and to prevent abortion. Root and leaf are prescribed against diarrhea with blood. Cattle like to browse it and people cut the stems to obtain drinking water. Leaves and fruits contain oxalic acid that is responsible for the acrid taste. A fiber is obtained from the bark and string is made out of it. Dried stems are used for hut building.

2.3 Secondary Metabolites Isolated from Vitaceous Plants

2.3.1 Phenolic compounds

Different classes of phenolic compounds have been reported from different genus of Vitaceous plant (Fig. 2). For example, Nguyen *et al*. (2014) reported 12 phenolics from the stems of *Parthenocissus tricuspidata*, a Vitaceous plant. These are protocatechuic acid (**5**), benzoic acid (**6**), caffeic acid (**7**), methyl 3,4-dihydroxycinnamate (**8**), caffeoylglycolic acid (**9**), caffeoylglycolic acid methyl ester (**10**), 3,4',5-trihydroxybenzophenone (**11**), 5-(4-hydroxybenzyl)benzene-1,3-diol (**12**), 2R,3R-3,5,6,7,4′-pentahydroxy-flavanonol (**13**), acacetin (**14**), (+)-catechin (**15**) and (−)-catechin (**16**). From *Cissus adnata* plant, Laitonjam *et al*. (2011)

reported three compounds. From this, one, apigenin 8-C-*α*-D-glucopyranoside, adnatoside (**17**), represent phenolic compound. Others, Ahmadu *et al*.(2010) elucidated four flavonoids from Vitaceous plant called *Cissus ibuensis* and they were identified as quercitin 3-O-rutinoside (rutin) (**18**), kaempferol 3-O-*α*-rhamnopyranosy(1→6)-*β*-D-galcatopyranoside (**19**), kaempferol-3-O-rutinoside (**20**), and kaempferol-3-O-*α*-rhamnosyl-(1→6)-*α*-rhamnopyranosyl-(1→2)-*β*-galactopyranoside(**21**). The presence of five compounds have been reported by Croteau *et al*. (as cited in Sofiyanti *et al*., 2008) from *Tetrastigma leucostaphylum*, a Vitaceous plant. Among these two are phenolic compounds, catechin (**22**) and proanthocyanidin (**23**). Resveratrol (**24**), *ε*-viniferins (**25**), balanocarpol (**26**), and *β*- glucopyranosyl- 8'- balanocarpol (**27**) were reported from Vitis *vinifera* (Vitaceae) (Felicio *et al*., 2001).

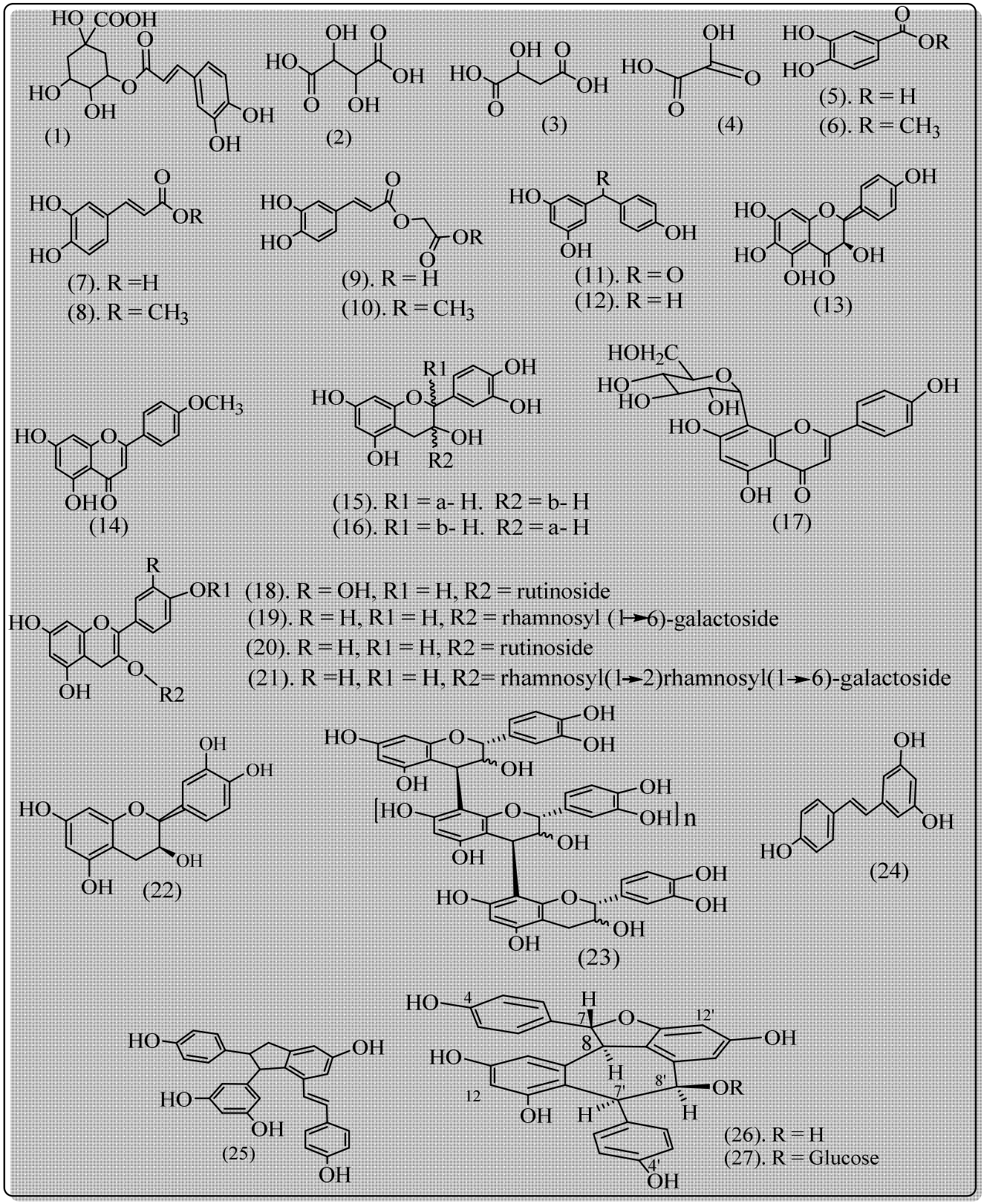


Figure 2. Some phenolic compounds and carboxylic acid derivatives from Vitaceous plants

2.3.2 Terpenoids

According to Harbome (1998), terpenoids are secondary metabolites that cover enormous range of plant substances and the term is used to indicate that all such substances have a common biosynthetic origin. Thus, terpenoids are all based on the isoprene molecule CH_2=C(CH_3)-CH=CH_2 and their carbon skeletons are built up from the union of two or more of these C_5 units. They are then classified according to whether they contain two (C_{10}), three (C_{I5}), four (C_{20}), six (C_{30}) or eight (C_{40}) such units (Fig. 3).

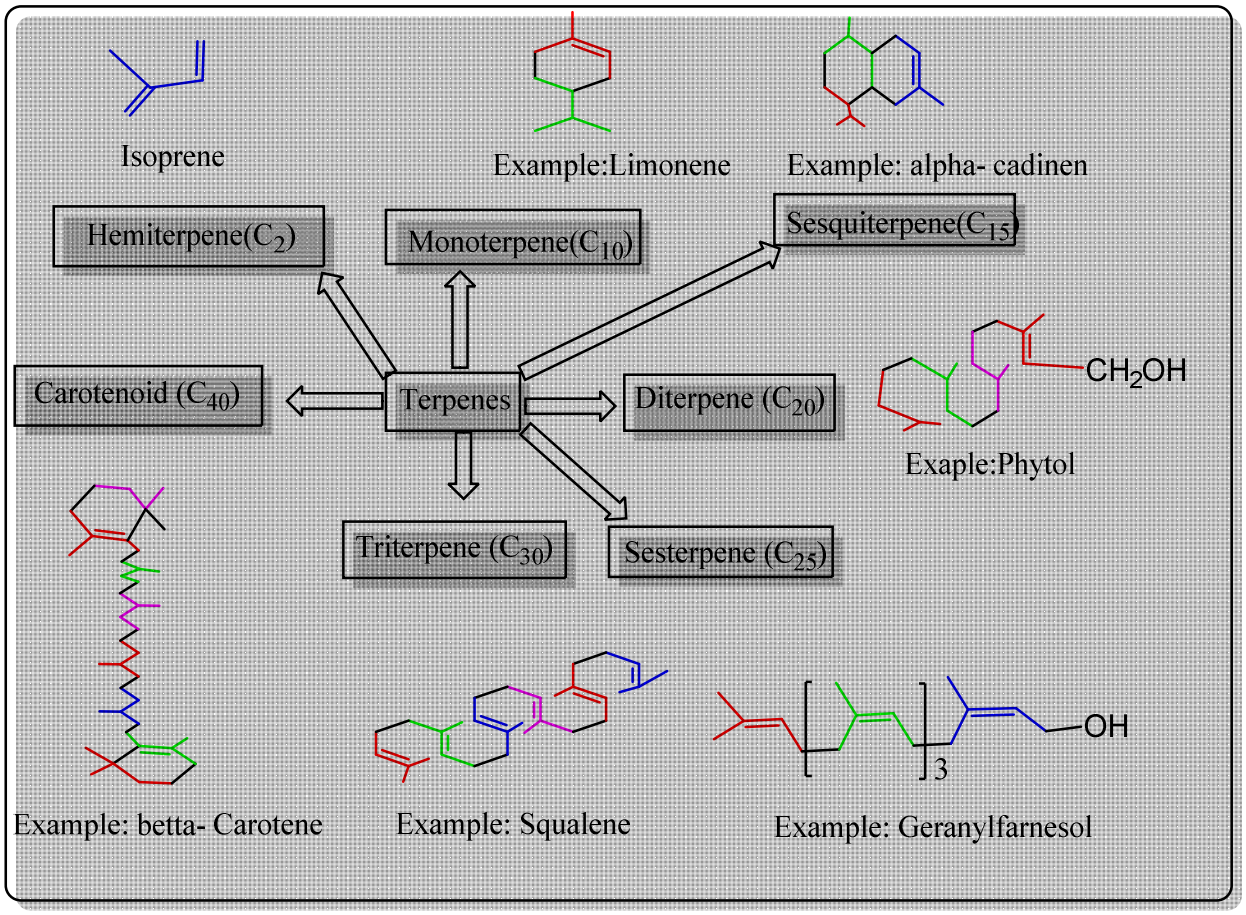


Figure 3. Classes of terpenoids with examples

Some terpenoids were reported from Vitaceous plants (Fig. 4). Jain *et al.* (2009) elucidated the structure of five compounds from *Cissus quadrangularis.* Among these two are

tepenoids class namely *α*-amyrin (**28**) and *β*-sitosterol (**29**). Together with one phenolic compound, Laitonjam *et al*. (2011) elucidated two terpeniod compounds from *Cissus adnata* leaves, other Vitaceae genera. These are onocer-7-ene-3*α*, 21*β*-diol (**30**) and β-amyrin [olean-12 (13)-en-3-ol] (**31**). Cao *et al*. (2011) reported that bioassay-guided fractionation of the ethanol extracts obtained from *C*. *greveana (*Vitaceae*)* led to the identification of three sesquiterpenoids, 12-hydroxy-15-oxo-selina-4,1l-diene (**32**), 1*β*, 6*α*-dihydroxyeudesm-4(15)-ene (**33**), and (7*R*)-opposit-4(15)-ene-1*β*, 7-diol (**34**), and the new diterpenoid, 18- hydroxykolavenic acid lactone (**35**).

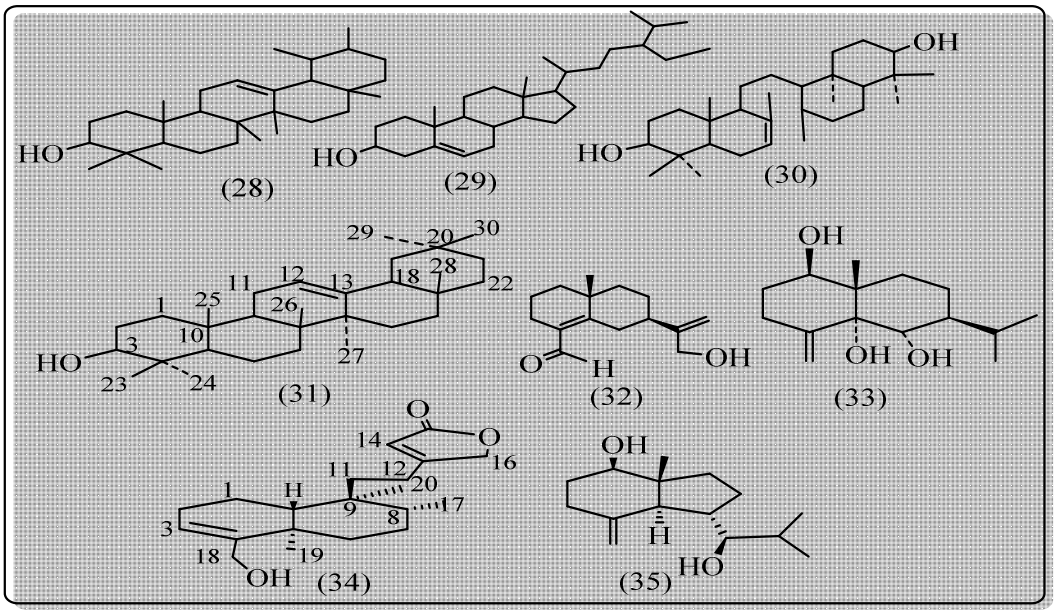


Figure 4. Some terpenoids of Vitaceous plants

2.3.3 Nitrogen containing compounds

Nitrogenous compounds (Fig. 5, Kar, 2007) which though only 2 % of the dry weight of plants, compared to 40 % for carbon; there are still a very large number of different nitrogen-containing organic substances known in plants. Amino acids are involved in the biosynthesis of practically all the other nitrogenous plant compounds, from the proteins to the alkaloids, amines,

cyanogenic glycosides, porphyrins, purines, pyrimidines and cytokinins (Harbome, 1998; Cseke, *et al*., 2006).

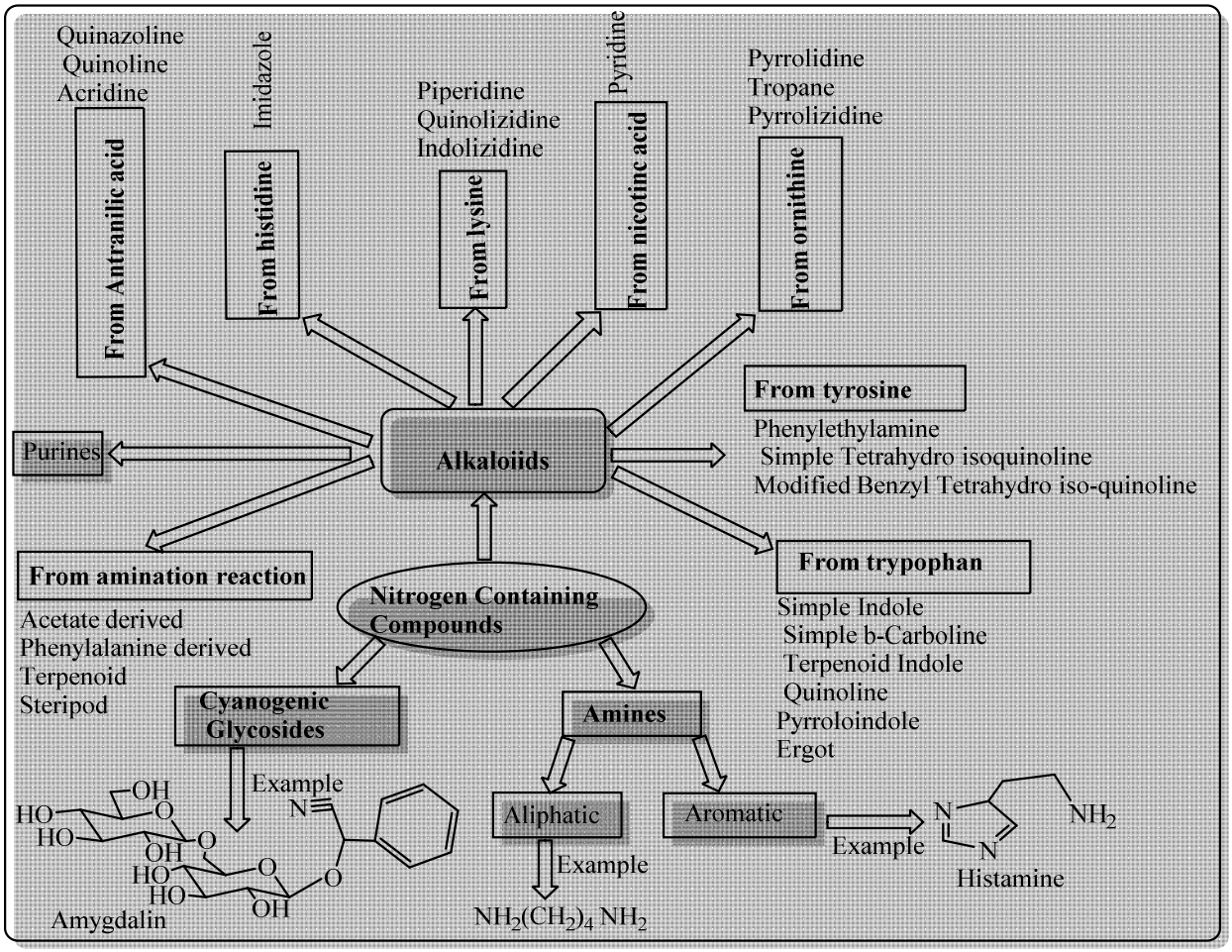


Figure 5. Classes of nitrogen containing compounds with examples

In addition to three phenolic compounds (as cited in Sofiyanti *et al*., 2008) Croteau *et al*. reported nicotine (**36**) and caffeine (**37**) from *Tetrastigma leucostaphylum* (Fig. 6).

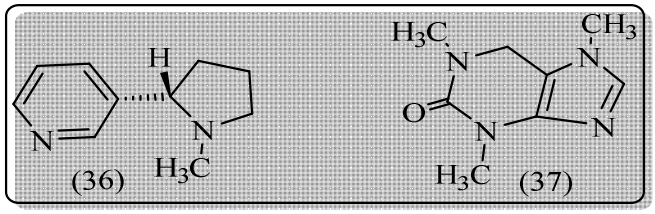


Figure 6. Nitrogen containing compounds from Vitaceous plants

2.4 Tannins

Tannins can be defined differently by different scholars as Hagerman (2002) and Kuete (2013) noted. Accordingly, tannins can be defined as water-soluble phenolic compounds having molecular weights between 500 to 27,000 Da and having special properties such as the ability to precipitate alkaloids, polysaccharides, proteins, lipids and minerals in addition to their intrinsic reactions which are of scientific interest as the basis of their biological effects and uses in the manufacture of leather, foodstuffs and beverages, herbal medicines, and in chemical defense and pigmentation in plant (Naumann *et al*., 2013). In the same way, Sampietro *et al*. (2009) defined tannins as water-soluble phenolics of molecular weight between 500 and 3,000, which, in addition to displaying the classic reactions of phenols, can precipitate alkaloids and gelatin, and can react with proteins, forming stable water-insoluble co-polymers. Others also defined tannins as it possesses some 12-16 phenolic groups and 5-7 aromatic rings per 1000 relative molecular mass in terms of structural and polyphenolic character.

Structurally, tannins can be classified into two main classes. These are hydrolysable tannins (HTs) derived from galloyl and hexahydroxydiphenyl (HHDP) esters and proanthocyanidins (PAs) (Hagerman, 2002; Kuete, 2013). As Vermerris and Nicholson (2006) discussed, HTs are derivatives of gallic acid (3, 4, 5-trihydroxyl benzoic acid) (**38**). Gallic acid is esterified to a core polyol, and the galloyl groups may be further esterified or oxidatively cross linked to yield more complex HTs (Fig. 7). Based on this, HT can be gallotannins (**41**), which is a polyol core, substituted with 10-12 gallic acid residues. The other is ellagitannins (example, **42**) which is derived from ellagic acid (**40**) that is formed spontaneously from HHDP acid (**39**) in aqueous solution via an intra-molecular esterification reaction.

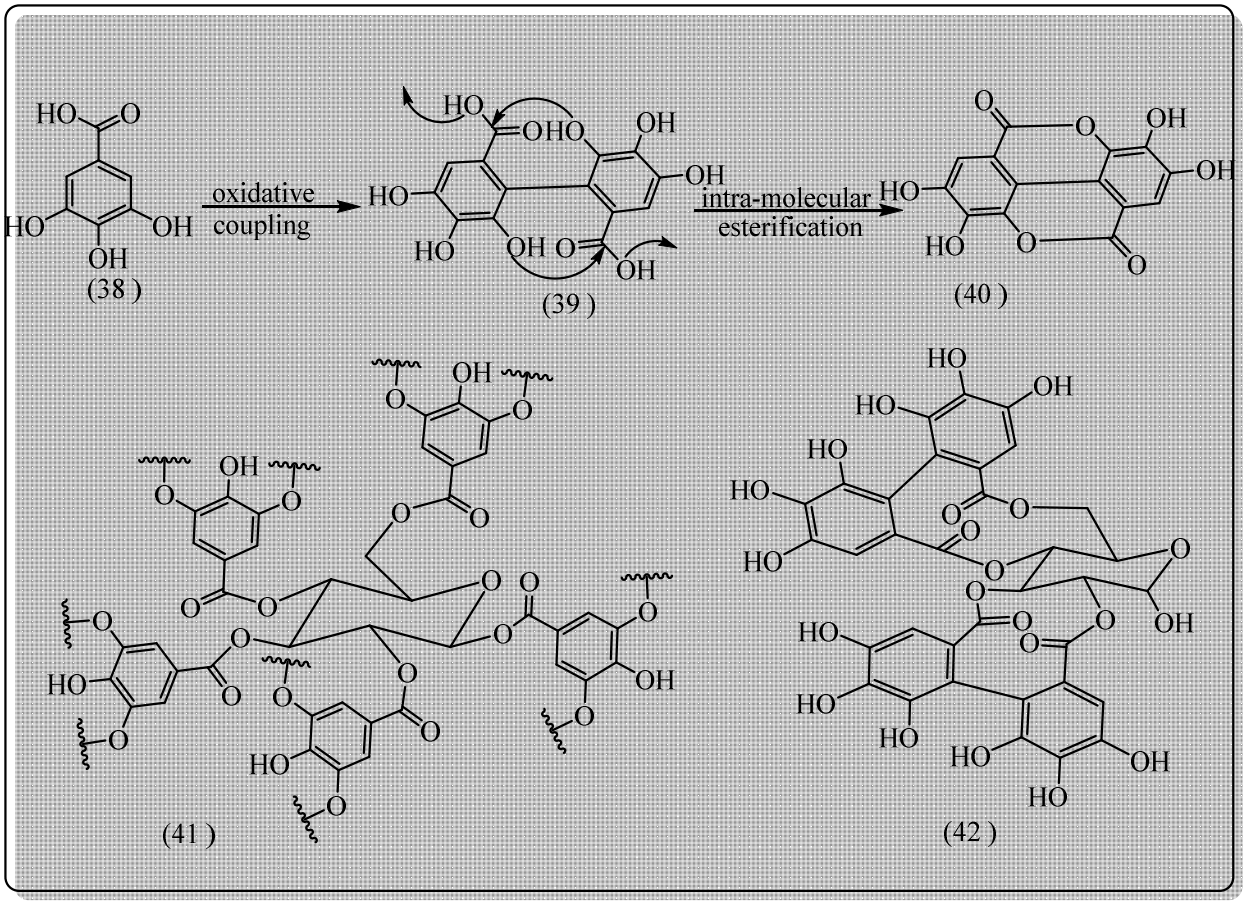


Figure 7. Monomers and some structures of hydrolysable tannins

2.4.1 Condensed tannins

Condensed tannins (synonym: PAs) comprise a group of polyhydroxyflavan-3-ol oligomers and polymers linked by carbon-carbon bonds between flavanol subunits (Makkar, 2013). PAs (condensed tannins, CTs) are polymeric flavanoids. The flavanoids are a diverse group of metabolites based on a heterocyclic ring system derived from phenylalanine (B) and polyketide biosynthesis (A) as indicated on Fig. 8 (**43)**.

2.4.1.1 Nomenclature of condensed tannin

As Kuete (2013), Hemingway and Karchesy (1989) discussed; PAs are named in a way similar to polysaccharides, where C-4 of the flavan monomer unit is equivalent to C-1 of a

monosaccharide in an oligo- or polysaccharide chain. The system assumes that the flavonoid skeleton is drawn and numbered in the usual way, as illustrated on fig.8 (**43**).The inter flavonoid linkage is indicated in the same way as polysaccharides, the bond and its direction being contained in parentheses (4→ or 2→) that can be (4→ 8 or 4→6 and 2→O→ 5 or 7). The stereochemistry of the C2-C3 linkage may be either *trans* (2R, 3S and 2S, 3R) or *cis* (2R, 3R and 2S, 3S). All flavan-3-ol with *cis* configuration are prefixed with "*epi*" and those with 2S-configuration are distinguished by *enantio (ent*-) prefix. The configuration of the interflavonoid bond at C-4 is indicated by *α* or *β* nomenclature in parentheses like (4*β* or *α*→).

2.4.1.2 Classification of condensed tannin

Condensed tannins are classified on the basis of the hydroxylation patterns of the A- and B-rings (Hemingway & Karchesy, 1989; Ribereau-Gayon *et al*., 2006; Kuete, 2013).

*Procyanidins***.** Procyanidins are members of the proanthocyanidin class of flavonoids. They are oligomeric compounds, formed from (+, -)-catechin (**44, 50**) and (-, +) - epicatechin (**45, 51**). They yield cyanidin when depolymerized under oxidative conditions and they are found most widely in the plant kingdom with 2R, 3R *(*2, 3-cis) chain extender units. It can be classified as B-type, C-type and A-type.

B-type consists of B1 to B8. B1 to B4 is the most common and characterized by the 4→8 linked dimmers. The 4→8 bonds can be in *α* or in *β* position. The 4*β*→8 includes [epicatechin-(4*β*→ 8)-catechin (procyanidin B1) (**66**), epicatechin-(4*β* →8)-epicatechin (procyanidin B2) (**67**), catechin-(4*β*→8)-catechin (procyanidin B3) (**68**), and catechin-(4*β*→8)-epicatechin (procyanidin B4) (**69**)]. B5 to B8 dimers are occurs in lower concentrations by linking as 4→6 in *α* or in *β* position. Some of the 4*β*→ 6 are [epicatechin-(4*β* →6)-epicatechin (procyanidin B5)

(**70**), catechin-(4$\beta \rightarrow$6)-catechin (procyanidin B6) (**71**), epicatechin-(4$\beta \rightarrow$6)-catechin (procyanidin B7) (**72**), catechin-(4$\beta \rightarrow$6)-epicatechin (procyanidin B8) (**73**)].

C-types are trimers linked as like epicatechin-(4$\beta \rightarrow$8)-epicatechin-(4$\beta \rightarrow$8)-epicatechin (procyanidin C1) (**74**) and catechin-(4$\beta \rightarrow$8)-catechin-(4$\beta \rightarrow$8)-catechin (procyanidin C2) (**75**).

Type- A are a doubly linked type in the same molecule as 4$\beta \rightarrow$8 (B-type) and the second between C-2 of the "upper" unit and O-7 of the lower unit of a dimer. Such type of linkage is a less common feature in proanthocyanidins. This can be epicatechin-(2$\beta \rightarrow$O$\rightarrow$7; 4$\beta \rightarrow$8)-epicatechin (proanthocyanidin A-2) (**76)**; proanthocyanidin A-1 (**77**), assigned as epicatechin-(2$\beta \rightarrow$ O$\rightarrow$7; 4$\beta \rightarrow$ 8)-catechin.

Prodelphinidin. Procyanidins is a name for the polymeric tannins composed of (+, -)-gallocatechin (**46, 52**) and (-, +)-epigallocatechin (**47, 53**). It yields delphinidin during depolymerisation under oxidative conditions. They are the major constituents in the leaves of conifers.

Propelargonidins. Propelargonidins are a type of condensed tannins formed from (+, -) - afzelechin (**48, 54**) and (-, +) - epiafzelechin (**49, 55**). They yield pelargonidin when depolymerized under oxidative conditions and are comparatively rare PA class.

*Prodistenidin***.** Prodistenidin are other classes of PAs composed of distenin (**56**) monomer unit. They yield distenidin when depolymerized under oxidative conditions.

*Procassinidin***.** Procassinidi*n* are PAs composed of cassiaflavan (**57**) monomer. They yield cassinidin when depolymerized under oxidative conditions.

*Proapigeninidin***.** Proapigeninidin is PAs which is a polymer of apigeniflavan (**58**). They yield apigeninidin when depolymerized under oxidative conditions.

*Proluteolinidin***.** Proluteolinidin is PAs with hydroxyl pattern 3', 4', 5, 7 and is a polymer of luteoliflavan (**59**). It yields luteolinidin when depolymerized under oxidative conditions.

Protricetinidin. Protricetinidin is other PA type composed of tricetiflavan (**60**). It yields tricetinidin when depolymerized under oxidative conditions

*Proguibourtinidin***.** Proguibourtinidins are a type of condensed tannins formed from guibourtinidol (**61**). They yield guibourtinidin when depolymerized under oxidative conditions.

*Profisetinidin***.** Profisetinidin is a type of condensed tannins formed from fisetinidol (**62**). They are among the best known proanthocyanidins and represent a wide range of structures like procyanidin. They yield fisetinidin when depolymerized under oxidative conditions.

Prorobinetidins. Prorobinetidins are other type of condensed tannins formed from robinetinidol (**63**). They form robinetinidin when depolymerized under oxidative conditions.

Proteracacinidin. Proteracacinidin is PA which is the polymer of oritin (**64**). They form teracacinidin when depolymerized under oxidative condition.

*Promelacacinidin***.** Promelacacinidin is the other PAs formed from mesquitol (**65**) that has 3, 3', 4', 7, 8 hydroxylation pattern. It yields melacacinidin when depolymerized.

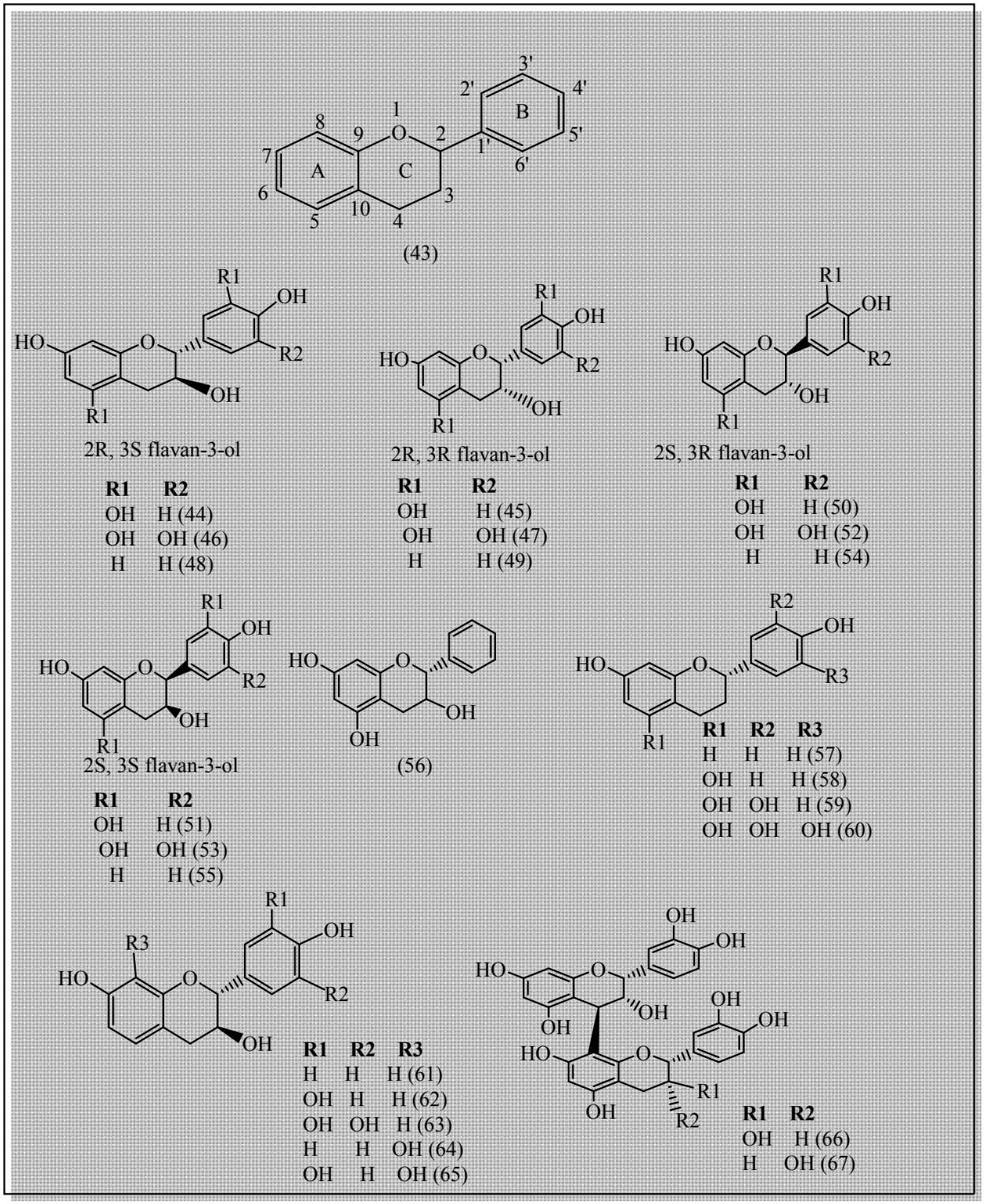


Figure 8: Some structures of condensed tannin and their monomer

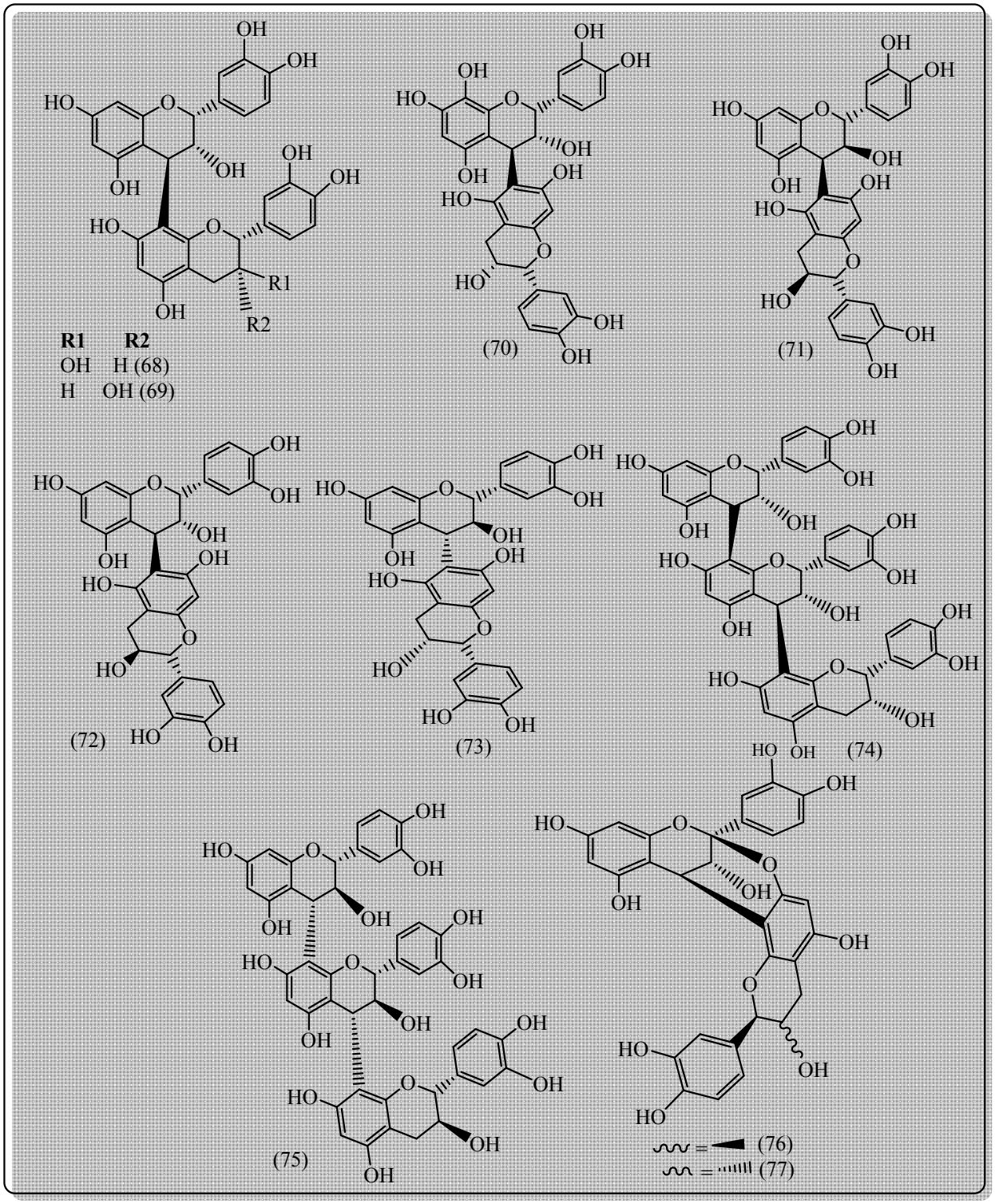


Figure 8: Cont'd

2.4.1.3 Analytical methods for characterization of condensed tannins

Different methods are used to test tannins in crude extracts (Harbome, 1998; Shah & Seth, 2010).

Goldbeater's skin test. Goldbeater's skin is a membrane produced from the intestine of an Ox. It behaves just like untanned animal hide. A piece of goldbeaters skin previously soaked in 2 % hydrochloric acid and washed with distilled water is placed in a solution of tannin for 5 minutes. It is then washed with distilled water and transferred to 1 % ferrous sulphate solution. A change of the color of the goldbeater's skin to brown or black indicates the presence of tannin.

Gelatin Test. To a 1 % gelatin solution, add little 10 % sodium chloride. If a 1 % solution of tannin is added to the gelatin solution, tannins cause precipitation of gelatin from solution

Vanillin-hydrochloric acid test. Drug shows pink or red color with a mixture of vanillin: alcohol: dilute HCl in the ratio 1:10:10. The reaction produces phloroglucinol which along with vanillin gives pink or red color.

Once the presence of tannins is identified, the next step is purification using sephadex after checking the solvent system on TLC (Hemingway & Karchesy, 1989; Hagerman, 2002).

Thin Layer Chromatography. Proanthocyanidins in their free phenolic form can be analyzed by TLC on silica using acidified solvents. The solvent system ethyl acetate-water-formic acid (90:5:5) is used to monitor isolations by column chromatography. The solvent system toluene-acetone-formic acid (30:30:10) can be used with silica TLC to obtain a visual pattern of the molecular weight distribution of procyanidin oligomer mixtures. In this case, the Rf values of the compounds is found to be in reverse order of molecular weight. Resolution is

obtained for monomers through hexamers, and C-4 → C-6 linked dimers are also distinguished from C-4 → C-8 linked dimers. A useful method of spot detection of these phenolic compounds on silica has is the sulfuric acid-formalin (40: 1) spray reagent. Such reagents are not always necessary, however. After TLC development with the toluene-acetone-formic acid solvent system, easily oxidized compounds such as the procyanidins and catechins will slowly appear as brown spots as the plates are dried.TLC of acetylated or methylated proanthocyanidin derivatives is frequently carried out on silica as a means of purification. A benzene-acetone (8:2 v / v) solvent system is used frequently to isolate procyanidin dimer and trimer acetates, whereas, chloroform-methanol (500: 1 and 40: 1) is useful for isolation of their respective methyl ether derivatives.

Column Chromatography. Sephadex LH -20 sorbs tannins in alcohol, and releases them in aqueous acetone. Chromatography on Sephadex is very useful for separating tannin from non tannin phenolics, or for fractionating hydrolysable tannins. Size-based separation is not achieved on any Sephadex for tannins, since phenols sorbs to the packing.

High performance liquid chromatography (HPLC). HPLC is increasingly used for both analysis and preparative isolation or purification of proanthocyanidins. A general trend has been to use reversed-phase columns with acidified methanol-water or similar acidified solvent systems, which leads to better peak resolution than with non acidified solvent systems. However, because of the concern for acid catalyzed reactions during workup, many prefer to use neutral solvents for preparative isolations, although this has not always been done. The successful chromatographer must ascertain whether or not a suggested acidified solvent system will alter the compounds of interest.

Structural characterization of condensed tannin can be possible by two methods. The first is chemical methods (Fig.9) mainly based on depolymerization, relied on color measurement (Gros *et al*., 1999; Hagerman, 2002; Sampietro *et al*., 2009)

Acid Butanol Assay. Proanthocyanidins like epicatechin$_2$ 4$\beta\rightarrow$8 catechin (**78**) are compounds that yield two cyanidin , extender, and one catechin (**79**), terminal, upon oxidative cleavage in hot alcohols . Although the assay is simple and gives good indication of the presence of condensed tannins, chemical characteristics of the tannins such as position of the interflavan bond and oxygenation pattern affect color yield significantly.

Vanillin Assay. The vanillin reaction involves reaction of an aromatic aldehyde, vanillin (**81**), with the meta substituted ring of flavanols like catechin (**80**) to yield a red adduct (**82**). Although the vanillin reaction has been widely used to estimate condensed tannin (proanthocyanidin), the reaction is not specific for condensed tannins. Any appropriately substituted flavanol reacts in the assay. Thus the formal "monomer" of the condensed tannins, catechin, also reacts to yield a red colored adduct. Furthermore, because the vanillin reacts only with meta-substituted flavanoids, the 5-deoxy proanthocyanidins (e.g. quebracho) do not produce much color with vanillin.

Modified Vanillin Assay **(**for molecular weight estimation**)**. One major problem with the vanillin assay (methanol) is the different kinetics of the reaction of catechin and tannin, which makes it difficult to use catechin as a valid standard for determining tannin. When the vanillin reaction is run with glacial acetic acid as the solvent, tannin (proanthocyanidin) and catechin react with similar kinetics. The similar kinetics is probably the result of the specificity of the reaction between vanillin and condensed tannin in glacial acetic acid. Only the terminal units of

the tannin react with vanillin in glacial acetic acid. The site-specificity of the reaction can be exploited to estimate the degree of polymerization of purified condensed tannins. The absorbance obtained in the vanillin (glacial acetic acid) assay for equal weights of tannin and of tannin monomer is directly compared to estimate degree of polymerization of the tannin. If the tannin monomer and polymer solutions do not dissolve in glacial acetic acid, they can be dissolved in a minimal volume of methanol and then diluted with glacial acetic acid.

Vanillin/Acid Butanol Ratio for Degree of Polymerization. Without pure proanthocyanidin, the modified vanillin method cannot be used to directly estimate degree of polymerization. In that case, the relative degree of polymerization for the crude extracts can be estimated by comparing the absorbance obtained with the modified vanillin assay in which only terminal units react to produce colored product (**84**) with the absorbance obtained with the acid butanol assay in which all units except the terminal unit react to produce colored product (**83**) .The best application of this method is probably monitoring changes in tannins in a single species. This method is only useful for comparisons of structurally identical tannins, since the reactivity in the acid butanol assay is a function of the reactivity of the interflavan bond. The assumption made for this method is that all of the flavanoid units are released and form anthocyanidin pigments in the acid butanol assay; if some interflavan bonds are not broken, then the color yield in the acid butanol assay will be low and the apparent degree of polymerization will be underestimated. For each extract, the ratio of the absorbance (acid butanol assay/vanillin assay) describes the relative degree of polymerization. The absolute degree of polymerization cannot be determined using crude extracts.

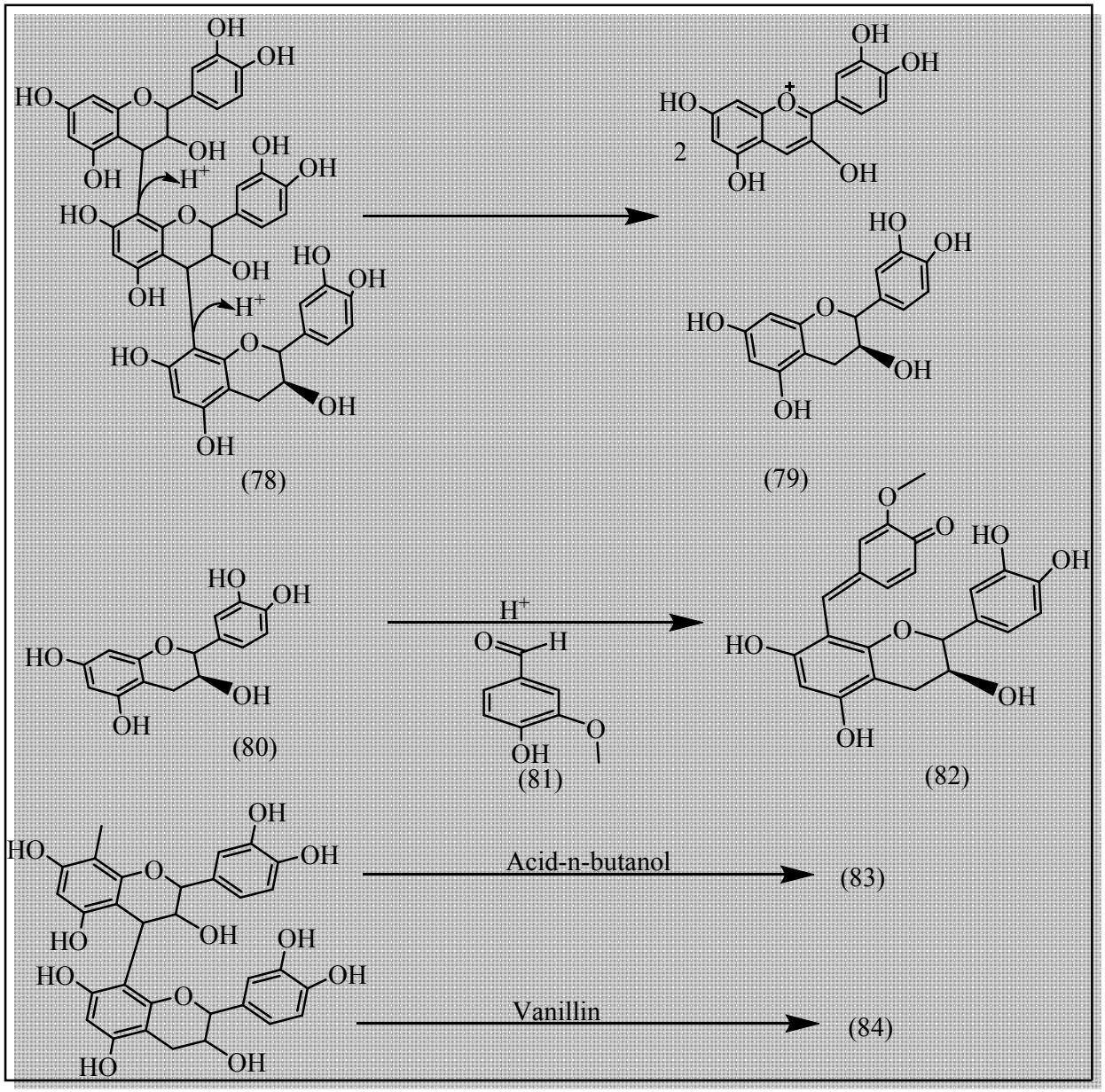


Figure 9: Some analytical methods of condensed tannin

The second methods for structural characterization of condensed tannis are physical methods that are non-degradative (Hemingway & Karchesy, 1989; Gros *et al*.1999).

NMR. A ^{13}C-NMR method was applied to tannins from various origins that have been used to define the hydroxylation patterns of the A- and B-rings, the stereochemistry of the

heterocyclic rings, the degree of branching within the polymer, and the number average molecular weight. It is based on the distinction of the chemical shifts and relative signal areas of C-3 of the lower flavanol units from C-3 of the extension units. The former are located between 65 and 68ppm, whereas the latter are between 72 and 73 ppm. Degree of polymerization is directly obtained by the ratio of the signal areas. Nevertheless, the method is limited by the accurate observation of the C-3 signal of the terminal units, which is all the less intense as degree of polymerization is higher. Therefore, polymers presenting molecular weight above 8,000 cannot be precisely characterized according to this procedure. In comparison with chemical degradation, the ^{13}C-NMR method has the advantage of being nondestructive. Moreover, it gives precise information on the stereochemistry of the heterocycles of the constitutive units and also on the hydroxylation pattern of the B nuclei (procyanidins/prodelphinidins ratio). In some favorable cases, it may also provide information on the proportion of C4-C6 interflavanic linkages and on the existence of chain branching in the polymers. ^{1}H-NMR is used in measuring degree of polymerization with combination of thiolytic degradation. The H-2 signals of the two classes of reaction products (i.e., flavan-3-o1 and thioether derivatives corresponding, respectively, to terminal and extension units) were well separated and identified on the ^{1}H-NMR spectra, and the degree of polymerization could be estimated.

Mass spectrometry (MS). MS constitutes a technique of real benefit in the field of condensed tannin analysis and for other polyphenolic oligomers and polymers. However, until now, it does not allow measurement of degree of polymerization of procyanidin fractions, but has been largely employed to obtain accurate assessment of molecular size of purified procyanidins. At present, electro spray ionization seems to give the best results in its application to high molecular weight polyphenolic compounds.

2.4.1.4 Pharmacological activity of condensed tannins

Kuete (2013) and Colegate and Molyneux (2008) discussed the uses of tannins for treating a range of ailments and disorders.

*Antioxidant Activity***.** Procyanidins B1 and B3 were demonstrated to have stronger antioxidant activity for linoleic acid in aqueous systems than ascorbic acid or *α*-tocopherol. It was seen that the dimeric procyanidins could trap eight peroxy radicals from a study of their radical scavenging properties toward radicals induced from methyl linoleate. The higher the degrees of polymerization, the more radicals are scavenged per molecule. Condensed tannins are also able to stabilize DPPH radicals. DPPH (1, 1-diphenyl-2-picrylhydrazil) (**85**) is a stable free radical having maximum absorbance in between 515- 528 nm. DPPH is changed to pale yellow from its deep violet when reacting with hydrogen donating molecules or antioxidant (**86**) which gives the reduced form diphenylpicrylhydrazine **(87)** as indicated on figure 10. This assay is technically simple and rapid and it only requires a UV–Vis spectrophotometer that might explain its widespread use in antioxidant screening. An important limitation of this method is the interpretation of the role of hydrophilic antioxidants, because DPPH can only be dissolved in organic media. (Gross *et al*., 1999; Lewis, 2012; Nollet &Toldra, 2012).

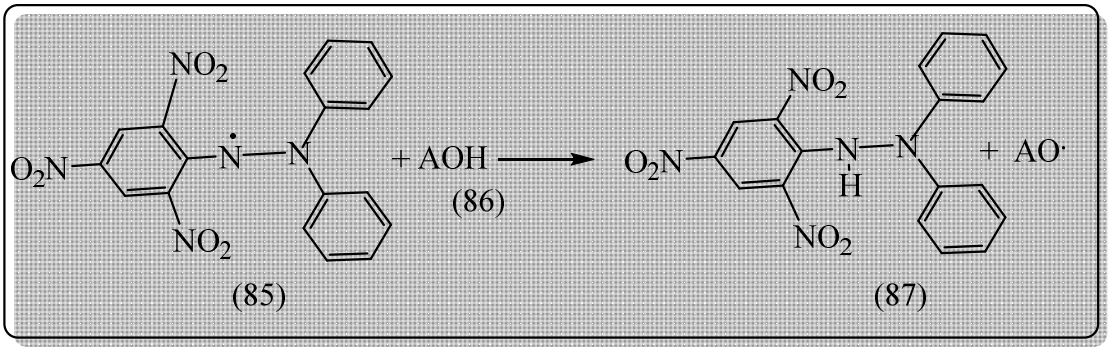


Figure 10. Reaction mechanism of DPPH free radical and antioxidant molecules

Antiviral Activity. Phenolic compounds may bind to and inactivate proteins and thus may affect receptor binding and enzyme assays. Tannins can be present in high concentration in some plant extracts, and they have been shown to inhibit HIV replication and infection of cells. Even though the antiviral activity of condensed tannins depends on the extent of oligomerization, the difference in the interflavan linkage type and the stereochemistry of the 3-ol function strongly influence the inhibitory capacity by inhibiting virus cell interactions. Galloylated CTs showed antiviral activity against the herpes simplex virus (HSV-1, HSV-2); their effects were due to inhibition of virus adsorption. Procyanidin and prodelphinidin-type B-ring moieties exhibit antiviral activity against the respiratory syncytial virus (RSV), influenza A virus (FLU-A), and parainfluenza virus (PIV) comparable to that of ribavirin.

Antibacterial Activity. CTs also showed inhibitory effects on the growth of several species of rumen bacteria, including *Streptococcus bovis, Eubacterium spp*., and *Prevotella bryantii*, as well as bacteria in the gastrointestinal tract. Condensed tannins exhibit very good antibacterial activity against the Gram-positive organisms as compared to Gram-negative bacteria like *Euscherchia coli* and *Salmonella paratyphoid* B (Sulaiman *et al*., 2011) since plant extracts have little effect against Gram-negative bacteria because of less susceptibility of their outer membrane surrounding their cell wall (very rich with lipopolysaccharide) to antibacterial activity.

Enzyme-Inhibitory Activity. Tannins usually inhibit the activity of enzymes at relatively high concentration. However, at low concentration they often stimulate enzymatic activity. The correlation between inhibition and stimulation varies depending on the enzyme and the structure and concentration of the tannins.

2.4.1.5 Biosynthetic pathway of proanthocyanidins

The first step of the pathway is the condensation and subsequent intramolecular cyclization of three malonyl –CoA (**89**) molecules with one 4-coumaroyl-CoA (**88**) molecule to produce a naringenin chalcone (**90**) Fig. 12. The second step of the path way is the isomerization of the naringenin chalcone to the naringenin (**92**), which can occur spontaneously, without enzymatic activity. However, chalcone isomerase (CHI) stereospecifically directs and greatly accelerates the intramolecular cyclization of chalcones to form the flavonones in the cytoplasm of plant cells. Flavonoid 3'-hydroxylase (F3'H) or flavonoid 3', 5'-hydroxylase (F3'5'H) can catalyze the conversion of naringenin into eriodictyol (**91**) or pentahydroxyflavonone (**93**), respectively. However, dihydrokaempferol (**95**) can also be the potential substrate of F3'H or F3'5'H, which can convert it to dihydroquercetin (**94**) or dihydromyricetin (**96**), respectively. Thus, both flavonones and dihydroflavonols can be hydroxylated by F3'H and F3'5'H. In one pathway branch, leucoanthocyanidin (**97**) molecules are oxidized by the catalysis of anthocyanidin synthase (ANS) to form colored anthocyanidins (**100)**.These unstable anthocyanidins could be further converted into colorless (2R, 3R)-flavan-3-ols (**101**) ((-)-epiafzelechin (**49**), (-)-epicatechin (**45**), and (-) epigallocatechin (**47**), respectively) by the action of anthocyanidin reductase. In another pathway branch, leucoanthocyanidins can be converted into (2R, 3S) - flavan-3-ols ((+)-afzelechin, (+)-catechin, and (+)-gallocatechin, respectively) by leucoanthocyanidin reductase (LAR) (**98**) (Kuete, 2013). Finally proanthocyanidin (**102**) is formed.

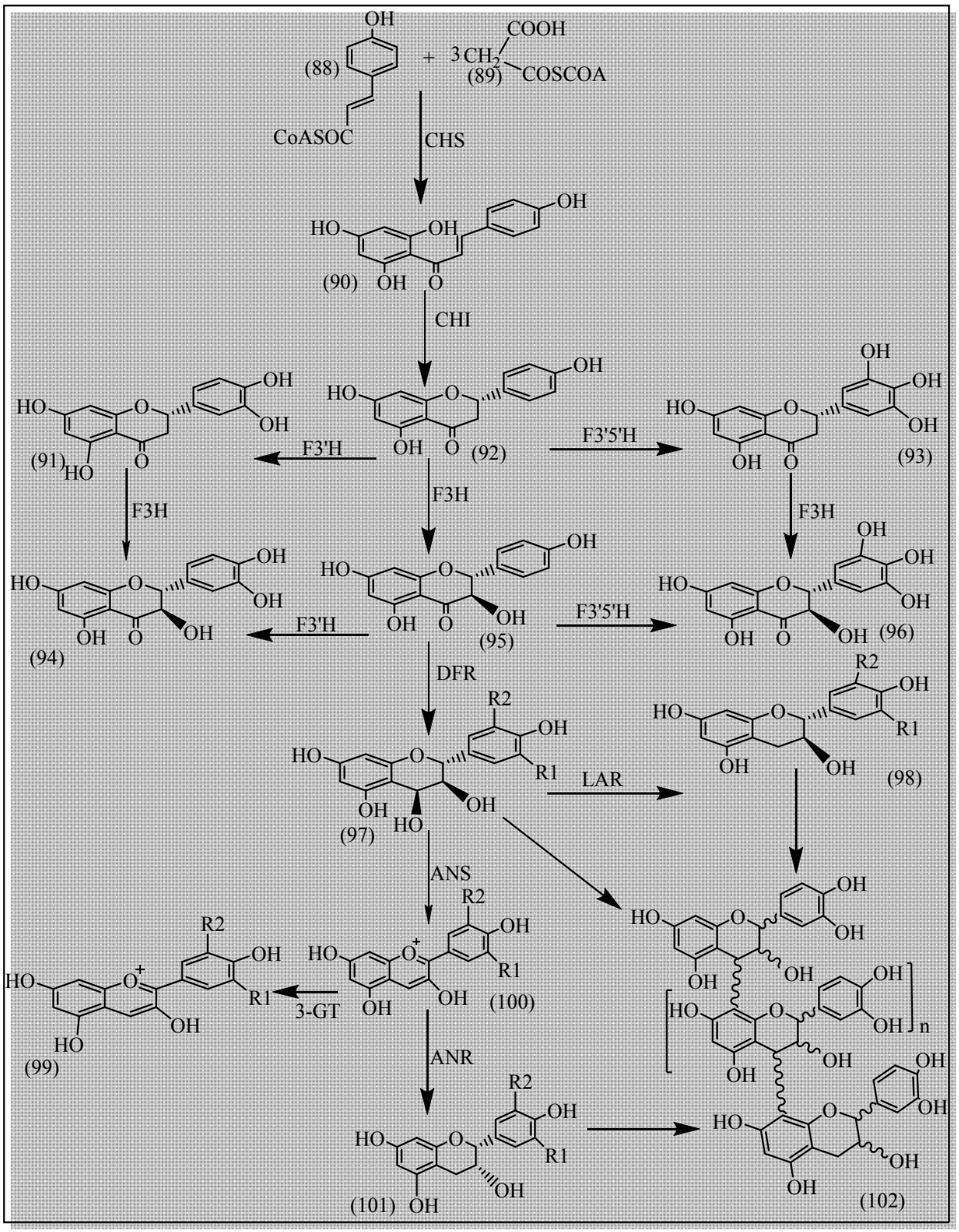


Figure 11: Biosynthesis of proanthocyanidins

Chapter Three: Materials and Methods

3.1 Plant Materials Collections, Identifications and Preparation

The roots of *C. adenocaule* were collected from Wonji area which is 10 Km to south east from Adama town, Ethiopia, in the month of July and October 2014. The plant material was taxonomically identified with the help of available literature and authenticated by Mr. Melaku of the National Herbarium, Biology Department, Addis Ababa University, Ethiopia. The collected plant material was immediately brought to Chemistry laboratory of Adama Science and Technology University and was allowed to dry at room temperature under shade. The dried plant material was then pulverized using an electric grinder for further analysis (Harbome, 1998).

3.2 Chemicals, Apparatus and Instruments

3.2.1 Chemicals

Chemicals used in this research were n-hehane, dicloromethane, ethyl acetate, chloroform, acetone, methanol, silica gel, sephadex LH-20, Wagner's reagent, Keller's reagent, Keller Kiliani reagent, Salkowski reagent, distilled water, ferric chloride, potassium hydroxide, lead acetate, gelatin solution, sodium hydroxide, DMSO, KBr, PBS solution, agar and DPPH free radicals.

3.2.2 Apparatus and instruments

Apparatus and instruments used in this research were electronic balance (Model № ESJ200-4) , electrical grinder, drying oven, measuring cylinders, bottles, shaker, ruler, filter papers, funnels, round bottom flasks, rotary evaporator, conical flasks, vials, TLC Chamber, TLC plates, capillary tubes, UV-lamp , glass rod, column (size 34/35 and 19/26), PTLC plates,

PTLC Chamber, heating plate (the laboratory model), sterilizer, micro pipettes, Petri dishes, incubator and microscope.

UV-Vis spectroscopy. λ_{max} for each purified compounds were recorded on PG instrument UV-Vis double beam spectrometer(T80+ model) having scanning range from 190-2100 nm . For all isolated compounds methanol was used for dissolving sample as well as used as blank solution. The instrument was on and heated for 30 minutes. After the necessary setting carrying out, the dark current was corrected by black block. Using quartz cuvettee the baseline was carried out by pure methanol solvent. The samples were dissolved in methanol and small volume of it was taken by capillary tube and transferred to quartz cuvettee half filled with methanol. Then, scanning was done on the selected wave length range. From the spectral data obtained, the type of absorption was determined.

NMR-spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded on 400 MHz Bruker NMR spectrometer using DMSO, $CDCl_3$ and CD_3OD solvents. The chemical shifts were reported in δ unit (ppm).

IR-spectroscopy. FT-IR spectra were obtained on a Perkin-Elmer (spectrum 6) apparatus. The spectra were measured in KBr pellets and presented in cm^{-1}. The sample was mixed with KBr and pressed to a pellet. The pellet was immediately put into the sample holder of Perkin Elmer Spectrophotometer and operated in the range 400 – 4000 cm^{-1}. From the spectral data obtained, the functional groups were detected.

3.3 Extraction

As indicated in Figure 14, 300 g powdered plant root was soaked in three liters of methanol and kept for 72 hrs. This was filtered and concentrated under a reduced pressure using rotary evaporator at 60 ℃ to yield 26 g (8.7 %) brown color crude extract (Fig. 12). This was suspended by hexane and filtered. The hexane soluble portion was concentrated by using rotary evaporator and yielded 0.1 g. The hexane insoluble portion of the Methanol extract was again extracted with chloroform and filtered. The filtrate was concentrated using rotary evaporator at 40 ℃ giving 0.2 g. The chloroform insoluble portion was extracted with ethyl acetate, filtered and concentrated to yield 1.3 g. The ethyl acetate insoluble portion was found to be 15 g.

Figure 12: Methanol extract of the *C. adenocaule*

3.4 Phytochemical Screening of the Methanol Crude Extract

The phytochemical screening of the plant root extracts was carried out using the standard procedures reported in literature (Mallik & Nayak, 2011; Tiwari *et al*., 2011; Tacio, 2012).The

phytochemical screening was conducted on methanolic extract in order to detect the classes of compounds available in it (sections 3.4.1- 3.4.7).

3.4.1 Detection of alkaloids

Solvent free extract, 50 mg was stirred with few ml of dilute HCl and filtered. The filter was tested carefully with various alkalodial reagents as follow:

Wagner's test. To 2-3 ml of filtrate, few drops of Wagner's reagent (1.27 g of iodine and 2 g of KI was dissolved in 5 ml of distilled water and made up to 100 ml with distilled water) was added by the side of the test tube. A reddish brown precipitate was used as the test is positive.

Hager's test. 2-3 ml filtrates were treated with 2 ml Hager's reagent (saturated picric acid solution). Presence of alkaloids was confirmed by the formation of yellow colored precipitate**.**

3.4.2 Detection of glycosides

Modified Borntrager's test. Extracts were treated with ferric chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. The presence of anthranol glycosides were confirmed by the formation of rose-pink color in the ammonical layer.

Keller Kiliani test. 0.5 g of extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This would then be underlayed with 1 ml of concentrated sulphuric acid. A brown ring obtained at the interface and a violet ring may appeared below the brown ring while, in the acetic acid layer a greenish ring may form just above the brown ring and

gradually spread throughout this layer would be taken as the indication for the presence of a deoxysugar characteristic of cardenolides.

Salkowski test. 0.5 g of the extract was dissolved in 2 ml of chloroform. Sulfuric acid was then carefully added to form a lower layer. Formation of a reddish-brown color at the interface was used as indication for the presence of a steroidal ring (i.e. aglycone portion of the cardiac glycoside).

3.4.3 Detection of saponins

Foam test. 0.5 gm of extract was shaken with 2 ml of water. The presence of saponin was confirmed by the persistence of foam for ten minutes.

3.4.4 Detection of phytosterols

Salkowski's test. Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of concentrated sulphuric acid, shaken and allowed to stand. The presence of triterpenes was confirmed by appearance of golden yellow color.

3.4.5 Detection of phenols

Ferric Chloride test. 50 mg of extract was dissolved in 5 ml of distilled water. To this, few drops of neutral 5 % $FeCl_3$ solution was added. A dark green color was taken as confirmation for the presence of phenolic compound**s.**

3.4.6 Detection of tannins

Gelatin test. Little 10 % sodium chloride was added to a 1 % gelatin solution. Then a 1 % solution of the crude extract was added to the gelatin solution, and a white precipitation of gelatin from solution was observed, which was indicative of the presence of tannins.

Ferric chloride test. 0.2 gm of extract was mixed with10 ml of distilled water and heated on water bath. The mixture were filtered and to a filtrate 5 % (w /v) solution of ferric chloride was added and the formation of dark green solution was taken as an indication for the presence of condensed tannins.

3.4.7 Detection of flavonoids

Alkaline reagent test. Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, was used as the indication for the presence of flavonoids.

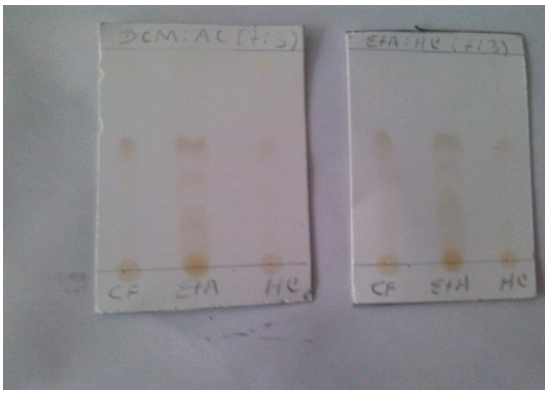
Lead acetate test. Extracts were treated with few drops of lead acetate solution. Formation of yellow color precipitate was used as conformation for the presence of flavonoids.

3.5 Isolation of Compounds from the Methanol Crude Extract

The hexane, chloroform and ethyl acetate soluble of the methanol extract was analyzed with TLC. Based on their TLC profile, the hexane, chloroform and ethyl acetate soluble were mixed together (totally1.6 g crude) because they showed similar TLC profile under UV light and when treated with iodine vapor (**d**) Fig.13. Then, this amount of crude extract was subjected to column chromatography (**e**). Several fractions were collected using increasing polarity of hexane and ethyl acetate and chloroform and methanol. Totally 33 fractions were collected. By analyzing the composition of each fraction using TLC, fractions (6-8), fractions (10-12), fractions (13-18) and fractions (22-30) were combined (Fig. 14).

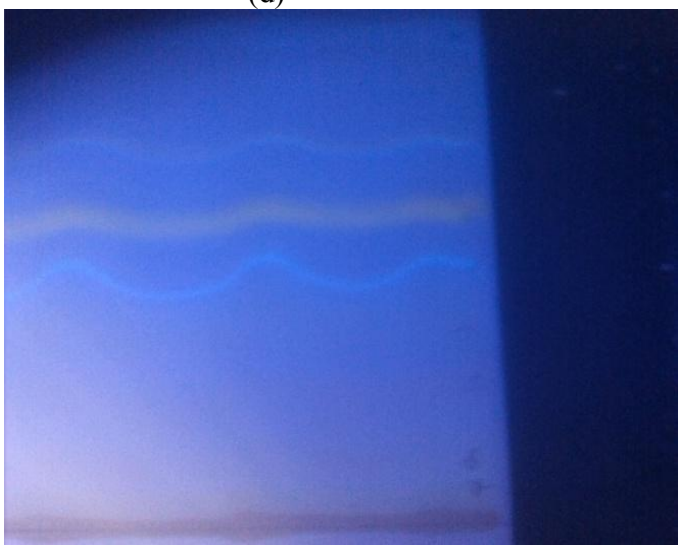
Fractions 13-18 was applied on PTLC (**f**, Fig.13) by using solvent system hexane: chloroform: ethyl acetate: methanol (2:3:4:1) (. Six compounds showing different colors under UV and Vis light were isolated as f $(13\text{-}18)_2$, f $(13\text{-}18)_4$, f $(13\text{-}18)_5$, f $(13\text{-}18)_6$ and f $(13\text{-}18)_7$ (Fig.14).

The ethyl acetate insoluble portion of the methanol extract was dissolved in water and filtered. 60 ml of filtrate was mixed with 10 g sephadex LH-20 and stored overnight at 4 ℃. Then it was filtered. Next, the non-tannin phenol compounds were started to be removed by water until absorbance was near to zero at 280 nm and continued by 50 % aqueous methanol until the absorbance was again near to zero at 280 nm that was also confirmed by clear filtrate. Finally, condensed tannin was recovered by 70 % aqueous acetone (g*)* Fig. 13 and was named as F(S). The acetone was removed by rotary evaporator at 40 ℃ (Diouf *et al*., 2013) and the aqueous part was dried gradually by heating at 40 ℃ over heating plate.



(d)

(e)



(f)

(g)

Figure 13: Purification process

3.6 Antibacterial Activity of Methanol Crude Extract and F(S)

Four bacterial pathogens, two gram negative (*Escherichia coli* and *Salmonela thyphimurium*) and two gram positive (*Staphylococcus aurous* and *Streptococcus buvis*) were used for test. They were obtained from the Department of Microbiology, Faculty of Veterinary, Addis Ababa University. Bacterial cultures were maintained on nutrient CMO337 Muller-Hinton agar at 37 ºC and the cultures were kept in appropriate media slants and stored at 4 ºC until used.

The antibacterial activities were tested by the disc-diffusion agar method (Sulaiman *et al*., 2011). 1 mL, diluted by PBS, of bacterial cells from 24 hour old culture was placed in sterile Petri-dishes and then 15 ml of molten nutrient agar for bacteria were poured into the plates. The plates were shaken gently to allow evenly mixing of bacteria cells and agar. 20 mg of F(S) was dissolved in 2 ml of methanol to obtain 10 mg/ml of the extract. The other concentration 1, 0.1, and 0.01 mg/ml were prepared by dilution law from 10 mg/ml by using distilled water. In the same way, 200 mg of methanol extract, from which F(S) was purified, was dissolved in 10 ml methanol to give 100 mg/ml. The other concentrations 10 mg/ml, 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml were prepared by dilution law from 100 mg/ml by using distilled water. For each sample, 20 µl of each concentration saturated with discs (6.00 mm diameter disc, Whatman filter paper) was placed on plate and incubated at 37 $^{\circ}$C for 24 hours. The diameters of the inhibition zones were calculated. Clear inhibition zones that formed around the discs indicated the presence of antimicrobial activity (Sulaiman *et al*., 2011).

3.7 Antioxidant Activity of Methanol Crude Extract and its Fractions

The antioxidant activity of the methanol extract and its fractions were evaluated by DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay by using procedures indicated in literature with little modification (Molyneux, 2004; Lewis, 2012; Garcia, 2012; Grujic *et al*., 2014). Thus, 4 mg DPPH was dissolved in 100 ml methanol to obtain 40 µg/ml and stored in dark bottle at 4 ℃ by covering with aluminum foil. 5 mg of each of the methanol extract, F(S), F $(13\text{-}18)_2$ and F $(13\text{-}18)_5$ were dissolved in 5 ml methanol to obtain 1 mg/ml and were diluted to 500, 250, 125, 62.5 μg/ml concentrations to 4 ml final volume by using methanol. 4 ml DPPH in 4 ml methanol (control) and 4 ml DPPH with each prepared concentration (experimental part) were placed at 37 ℃ in oven for 30 min. The absorbance of both control and experimental part was taken by UV-Vis spectrometer at 517 nm using methanol as blank. The percent (%) inhibition of DPPH free radical by the sample was calculated as:

$\%\ \text{Inhibition} = \left(\frac{\text{Ac} - \text{As}}{\text{Ac}}\right) \text{x}\ 100\%$. Where A_c: absorbance of control and A_s: absorbance of sample. IC_{50} (the concentration of sample that inhibit 50 % of DPPH free radical) was calculated by using graph pad prism software from regression line (Khlifi *et al*., 2011). A lower value of IC_{50} indicates greater antioxidant capacity (Castro *et al*., 2014).

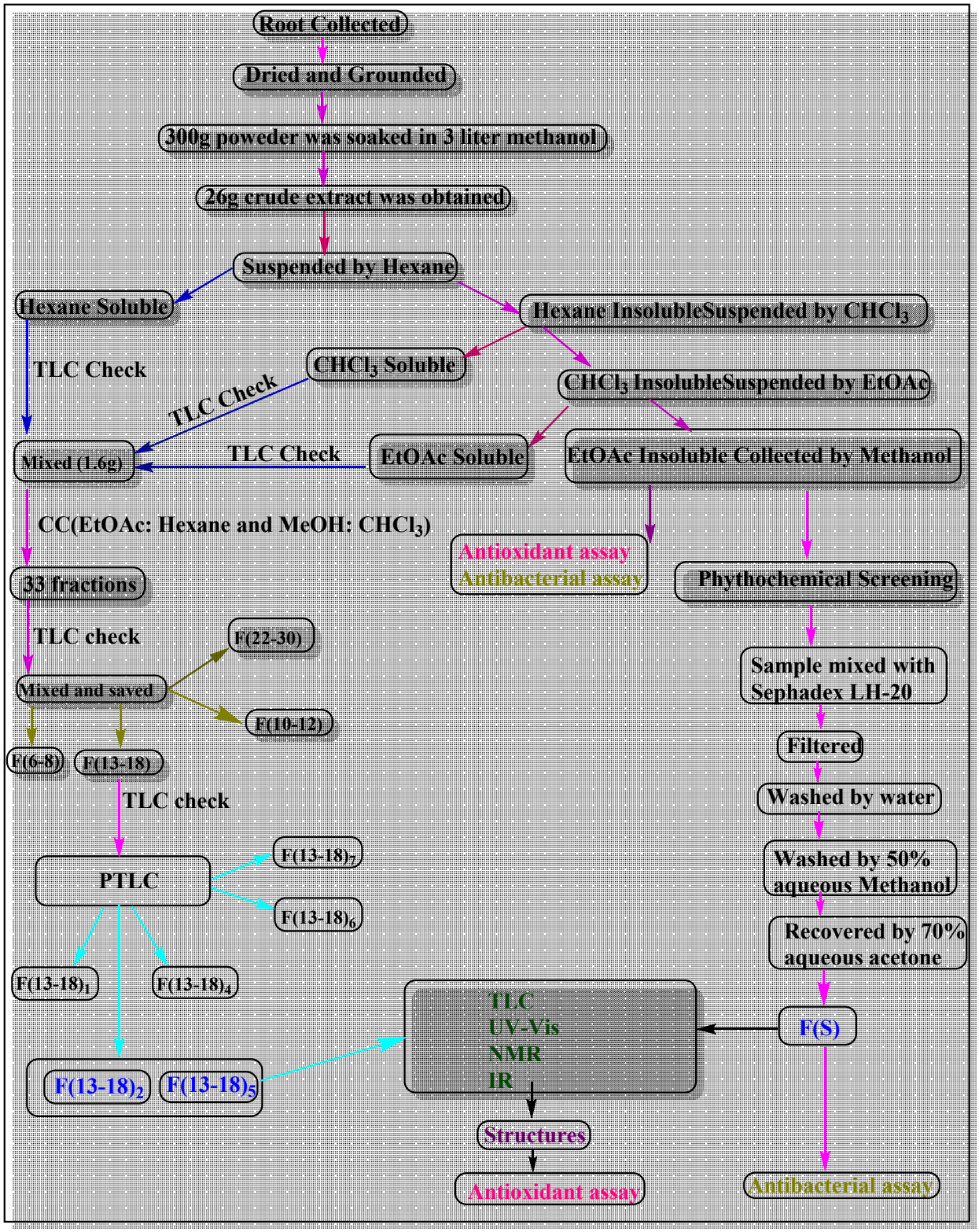


Figure 14: General experimental procedure

Chapter Four: Results and Discussion

4.1 Phytochemical Screening Result of the Methanol Crude Extract

The Pyhythochemical screening tests (Fig. 15) indicated the presence of alkaloids, saponins, flavonoids and tannins. The latter gave deep green (**h***)* with ferric chloride and white precipitate (**i**) (not reacted with water but with acetone (**j**), Makkar, 2013) with gelatin solution (Tiwari *et al*., 2011**)** that was identified as condensed tannin. The result was shown in Table I.

(h) (i) (j)

Figure 15: Phythochemical screening of the MeOH extract

Table I: *Phytochemical Screening of MeOH Crude Extract*

	Phytochemical	Method of Test	Result
1	Alkaloids	Wagner's	+
		Hager's	+
		Modified Borntrager's	-
		Keller Killen's	-
2	Glycosides	Salkowiski 's	-
3	Saponin	Foam	+
4	Phytosterol	Salkowiski's	-
5	Tannins	Gelatin solution	+
		Iron Chloride	+
6.	Flavonoids	NaOH	+

Note: '+' indicates presence '-' indicates absence

4.2 Characterization of Compound F(S)

4.2.1 UV-Vis spectral data

On appendix A, λ max of F(S) was seen near 220 nm and also showed peaks at 281 with no band broadening beyond 300 nm. This is a confirmation for that the compounds are condensed tannin. This is supported by the data reported by Chen *et al*. (2014) and Kardel *et al*. (2013).

4.2.2 NMR spectral data

The ^{1}H-NMR and ^{13}C-NMR spectra was given on appendix C.1. The ^{1}H-NMR of F(S) showed a signal at δ 5.8. This was assigned to H-6 of structure indicated on Fig.16 and H-8 of its extender unit. Signals around δ 6.8 were assigned to H-2', H-5' H-6'. Signals observed around δ 9 were due to OH on C-3', C-4' and C-5. The ^{13}C-NMR spectrum showed signals at δ 116 which we assigned to C-2' and C-5'. Signals at δ 120 and 131 were assigned to C-6' and C-1', respectively. Signals around δ 145 was for C-3' and C-4'. Signals collected around δ 155 were representing C-5, C-7 and C-8a. Chen *et al*. (2014) reported similar data of ^{13}C-NMR for proanthocyanidin from *Caryota ochlandra.* In the same way*,* Diouf *et al*. (2013) had reported the assignment of ^{13}C- NMR data of condensed tannin isolated from *Picea mariana* bark.

4.2.3 IR spectral data

IR data of F(S) confirms the presence of condensed tannin where a strong absorption is occurred at 3433 cm^{-1} with a wide band (appendix B). This band is assigned to the hydroxyl groups (OH) stretching vibrations due to the wide variety of hydrogen bonding between OH in the compound which lies in the range of 3570–3200 cm^{-1} (Coates, 2000). In this spectrum a

sharp and weak signal is observed at 2924 and 2851 cm^{-1} representing –C-H- asymmetric and symmetric stretching vibrations of CH_2, respectively. The signals lies in the range of 1615 – 1450 cm^{-1} indicates C=C-C aromatic ring stretch. The band seen around 1385 cm^{-1} represents the O-H bend of phenols. Signals from 1225-950 cm^{-1} indicates aromatic in-plane C-H bending. But, a signal shown at 1116 cm^{-1} may be represented by C-O stretch of secondary alcohols. Signals from 900- 670 cm^{-1} are assigned for aromatic C-H out of plane. In this spectrum, no signal shows the presence of carbonyl groups that lies in the range of 1630- 2100 cm^{-1} (Coates, 2000) establishing the compound as tannin (Castroa & Rodrígueza, 2012; Santhanakrishnan *et al*., 2014, Abdul Rahim *et al*., 2013). All IR signals were listed in Table II.

Table II*: Infrared Data of Compound F(S)*

Frequency (cm-1)	Assignment
3433.59	O-H(s., H–bonded alcohol and phenol)
2924.86	-CH_2(C-H, asym. s.)
2851.03	-CH_2(C-H, sym. s.)
1621.44	C=C-C (aromatic ring s.)
1525.02	C=C-C (aromatic ring s.)
1445.21	C=C-C (aromatic ring s.)
1385.36	Phenol, O-H bend
1205.81	C-H (Aromatic ip. bend)
1116.04	C-H (Aromatic ip. bend or-secondary alcohol, C-O s.)
1069.48	C-H (Aromatic ip. bend)
973.059	C-H (Aromatic ip. bend)
879.957	C-H(Aromatic oop. bend)
820.107	C-H(Aromatic oop. bend)
786.856	C-H(Aromatic oop. bend)

Note: asym= asymmetric, sym= symmetric, s= stretch, oop= out of plane, ip = in plane

By combining the data from phytochemical screening, UV-Vis spectral data, NMR data, Infrared data and literature review (Kuete, 2013); the structure of compound F(S) was identified as condensed tannin as indicated on Fig. 16 (**103**). But, the size of the chain, the type of interflavan bond, the stereochemistry of C-2 and C-3 and the type of C-3' can be identified from

clear C-13 NMR data by comparing with reported data of Kuete (2013), Diouf *et al*.(2013) and Chen *et al*.(2014) .

4.3 Characterization of Compound F $(13\text{-}18)_2$ and F $(13\text{-}18)_5$

4.3.1 UV-Vis spectral data

As can be seen from the spectrum given in Appendix A, λ max of F $(13\text{-}18)_2$ was near 220 nm. It showed another peak at 325 nm. Similarly, λ max of F $(13\text{-}18)_5$ appeared near 220 nm with additional peaks at 323 nm and near 280 nm. In both compounds, the absorption peak near 220 indicative of the presence of benzene rings and the peak near 320 nm are due to the presence of heteroatom (Pretsch *et al*., 2000).

4.3.2 NMR spectral data

The ^{1}H-NMR and ^{13}C-NMR spectra of both compounds were shown in Appendix C.2 and C.3. Compound $(13\text{-}18)_2$ gave proton signals at δ 5.7 (N-H), 7.9 (H-3) and 8.4 (H-2). The ^{13}C-NMR of this compound was observed at δ 123.10 (C-3), 134.52 (C-2) and 173.28 ppm (C-4). In the same way, compound $(13\text{-}18)_5$ showed proton signals at δ 8.1 (H-3), 8.7 (H-2) and 10.17 (H-O). In the same way its ^{13}C-NMR were indicted at δ 123.80 (C-3), 134.80 (C-2) and 173.8 (C-4).

4.3.3 IR spectral data

IR data of compound $(13\text{-}18)_2$ and $(13\text{-}18)_5$ (Appendix B) showed broad signal at 3433 cm^{-1} corresponds to N-H or O-H stretch and showed signal at 1641 that may be C=O affected by near heteroatom (Pavia *et al*., 2001). Based on the data collected and interpreted data, the

structure of F (13-18)$_2$ proposed as pyridin-4(1H)-one (**104**) and F (13-18)$_5$ proposed as 1-hydroxypyridin-4(1H)-one (**105**) as indicated on Fig 16.

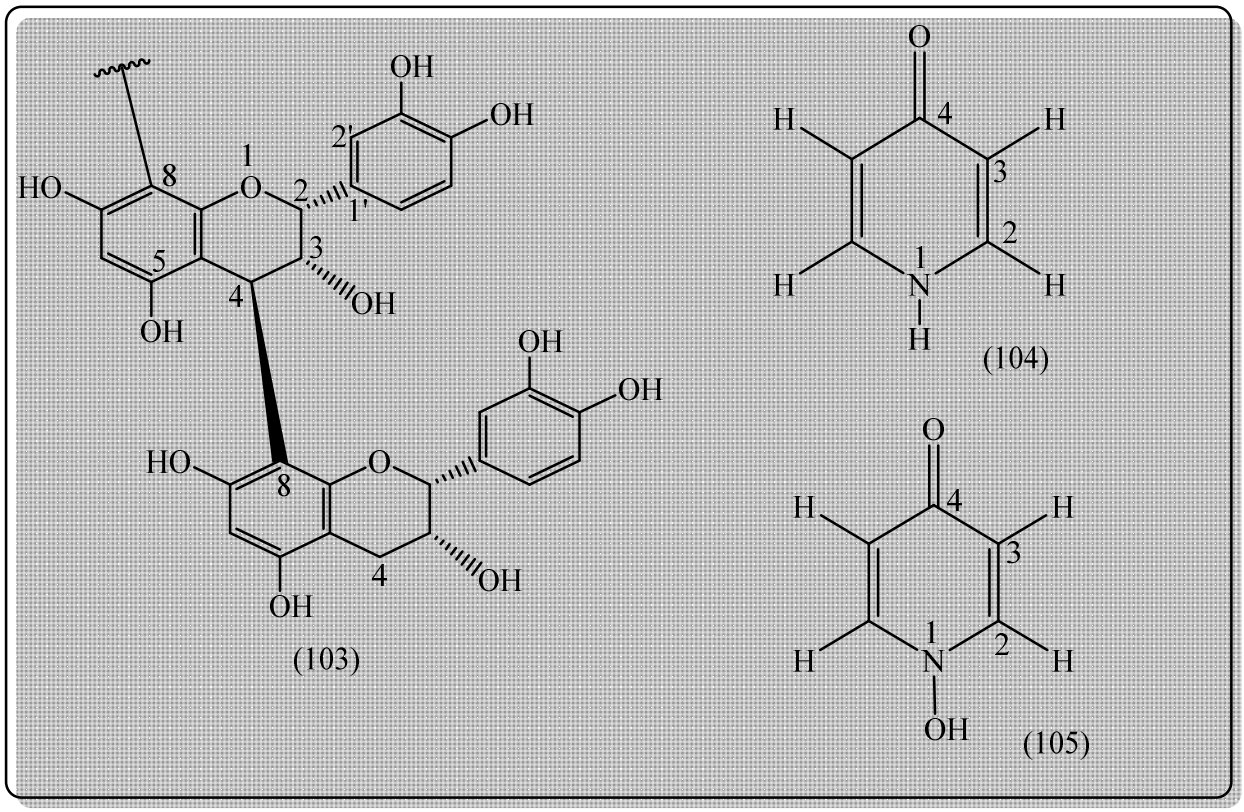


Figure 16: The proposed structures

4. 4 Antibacterial and Antioxidant Activity of Methanol Crude Extract and its Fractions

4.4.1 Antibacterial activity of methanol crude extract and F(S)

The effect of crude extract and F(S) on four selected bacteria using different concentrations 0.01, 0.1,1,10,100 mg\ml was shown in Table III and Appendix D. Except activity of F(S) fraction on *Escherichia* coli and *Staphylococcus aurous*, the relationship between concentration of the sample and their activities on selected bacteria were not linear. For crude extract the inhibition zone for *Escherichia* coli bacteria was 0 mm, 0 mm, 6 mm, 0 mm and 0 mm, respectively, from high concentration to low concentration. For *Staphylococcus aurous* the

inhibition zones were 20 mm, 11 mm, 0 mm, 8 mm and 9 mm. For *Salmonella thyphomurium* the zones were 15 mm, 10 mm, 7 mm, 5 mm and 7 mm. In the same way for *Streptococcus buvis* the zones were 15 mm, 0 mm, 8 mm, 0 mm and 7 mm. The effect of F(S) on activities of four selected bacterial using different concentrations 0.01, 0.1, 1,10 mg\ml was explained in the same way. The inhibition zone for *Escherichia* coli bacteria was 8 mm, 7 mm, 7 mm and 6 mm, respectively, from high concentration to low concentration. For *Staphylococcus aurous* the inhibition zones were 11 mm, 9 mm, 8 mm and 7 mm. For *Salmonella thyphomurium* the zones were 7 mm, 6 mm, 25 mm and 8 mm. In the same way for *Streptococcus buvis* the zones were 7 mm, 7 mm, 14 mm and 0 mm. From this data, F(S) identified as condensed tannin showed comparative antibacterial activity as some of reported data for condensed tannin extracted from different plant species. For example, Al-Ani *et al*. (2008) reported the inhibition zone of condensed tannin extracted from *Pimpinella anisum* for *Escherichia* coli 14, 11 and 7 mm for 100, 10 and 1 mg/ml, respectively. For *Staphylococcus aurous* the inhibition zones were 12 mm, 9 mm and 7 mm for the same concentration, respectively.

Table III: *Inhibition Zone of Antibacterial Activity of MeOH Crude Extract and F(S)*

		Inhibition Zone Diameter(mm)								
		Compound F(S)				Crude Extract				
Bacteria	Conc.	10	1	0.1	0.01	100	10	1	0.1	0.01
Escherichia coli		8	7	7	6	0	0	6	0	0
Staphylococcus aurous		11	9	8	7	20	11	0	8	9
Salmonela thyphimurium		7	6	25	8	15	10	7	5	7
Streptococcus buvis		7	7	14	0	15	0	8	0	7

4.4.2 Antioxidant activity of methanol crude extract and its fractions

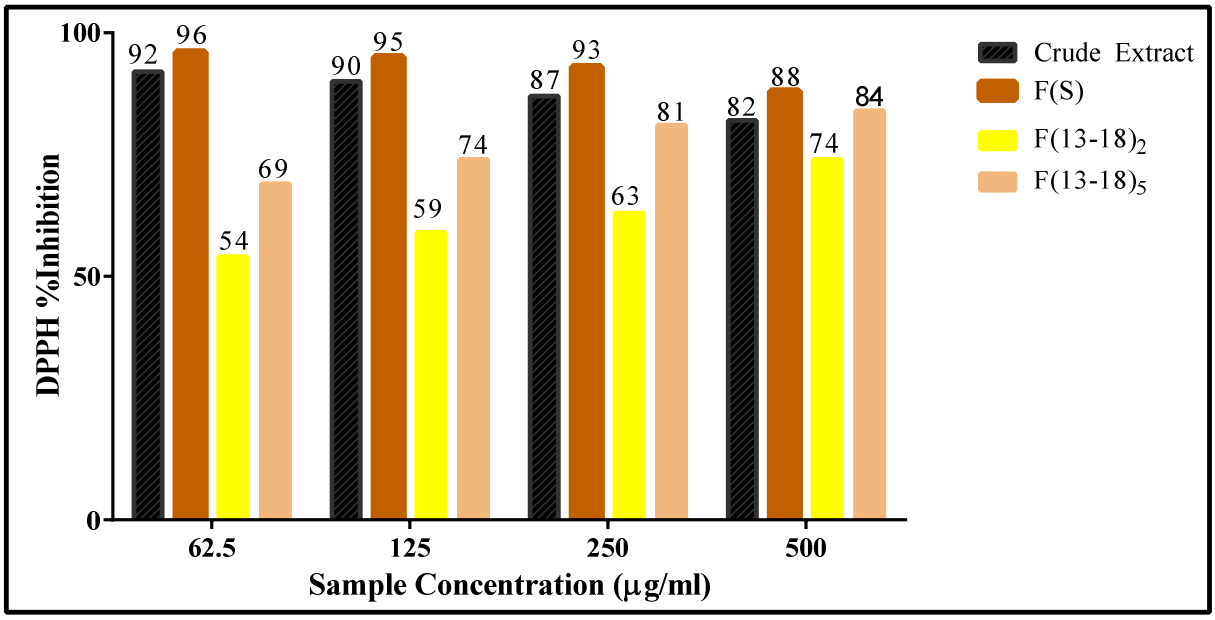
The result of the antioxidant activity of the crude extract and the purified compounds evaluated by DPPH (Appendix E) was shown as DPPH % inhibition as indicated on Fig. 17. This was calculated from absorbance of each concentration for all methanol extracts and purified compounds in terms of the absorbance of the control solution at 517 nm as shown on Table V.

Table IV*: Antioxidant Activities of the Crude MeOH Extract and its Fractions*

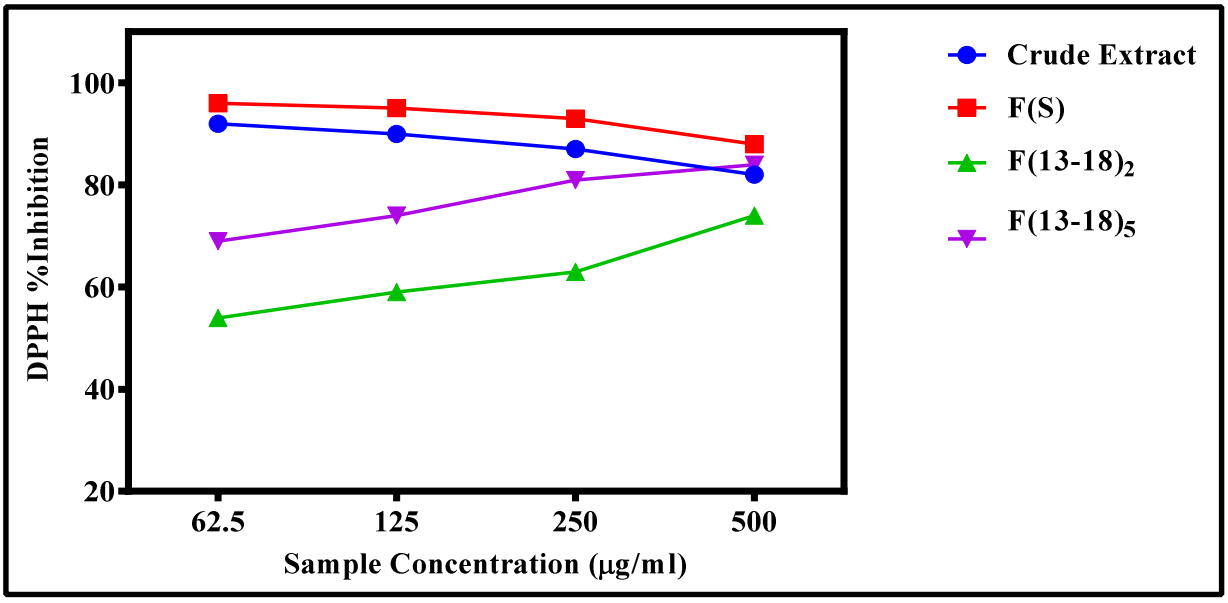
Concentration	Absorbance			
	Methanol extract	F(S)	F(13-18)2	F(13-18)5
Control (40 µg/ml)	0.496			
62.5 µg/ml	0.042	0.020	0.227	0.154
125 µg/ml	0.047	0.022	0.206	0.127
250µg/ml	0.063	0.034	0.183	0.094
500 µg/ml	0.089	0.059	0.130	0.078

The vertical axis on Fig 17 (**k**) represents the DPPH % inhibition (sample's capacity to inhibit DPPH) and the horizontal axis represents the four concentrations of the samples. DPPH % inhibition of methanol extract, condensed tannin, F $(13\text{-}18)_2$ and F $(13\text{-}18)_5$ at 500 µg/ml was 82.1, 88.1, 73.8 % and 84.3 %, respectively, at 250 µg/ml 87.3 %, 93.1 %, 63.1 % and 81 %, respectively, at 125 µg/ml 90.5 %, 95.6 %, 58.5 % and 74.4 %, respectively. In the same way at 62.5 µg/ml it was 91.5 %, 96 %, 54.2 % and 69 %, respectively. The reverse relationship between % inhibition of DPPH radical and concentration of crude extract and F(S) is due to reversible reaction of the reduced DPPH radical at the end of the reaction (Bondet *et al*., 1997 & Molyneux, 2004**).** Sulaiman *et al*. (2011) have been reported that the capacity of condensed tannin from *Rhizophora apiculata* barks to inhibit free radical DPPH is greater than the standard antioxidant ascorbic acid. At 500 and 250 µg/ml % inhibition of this condensed tannin was around 65 % and 25 %, respectively and for ascorbic acid with the same concentration the inhibition capacity was around 35 % and 20 %, respectively. In other way Amarowic &Troszyñska (2003) compared the scavenging effect of pea crude extract and the condensed tannin fractionated from this crude on DPPH free radical. The condensed tannin showed more scavenging activity towards DPPH free radical. When compared with this reported data, the activity of condensed tannin in this study could be taken as strong antioxidant. . IC_{50} was

calculated from regression line as shown on Fig 17(**l**). Therefore, IC_{50} value for F $(13\text{-}18)_2$ was 46.90 µg/ml and for F $(13\text{-}18)_5$ was 10.07 µg/ml. The result indicated F $(13\text{-}18)_5$ has more antioxidant activities (Castro *et al*., 2014).



(k)



(l)

Figure 17: Antioxidant activity assay results.

Chapter Five: Conclusion

5.1 Conclusions

Carrying out phytochemical investigation on *C.adenocaule* is the first time. For the root collected on October, the phytochemical screening result, UV-Vis spectral data, IR spectral data and the NMR spectral data confirmed the presence of condensed tannin in the plant. So, the presence of condensed tannin was reported for the first time from this plant. The antibacterial activity of the F(S) isolated in this research indicated comparative antibacterial activities with some reported results and also considered as strong ant oxidizing agent. But, since people use this plant for treatment of rabies most probably due to high concentration of condensed tannin in the plant, the consequence of the extract from different maturity of the plant on user should be studied in detail. Because of the extract of the plant root collected on July and October has no the same color.

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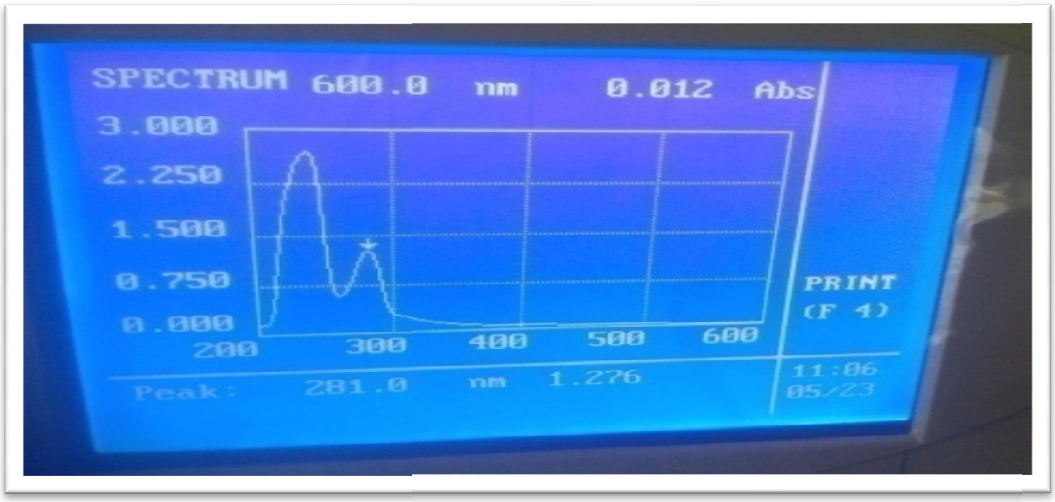
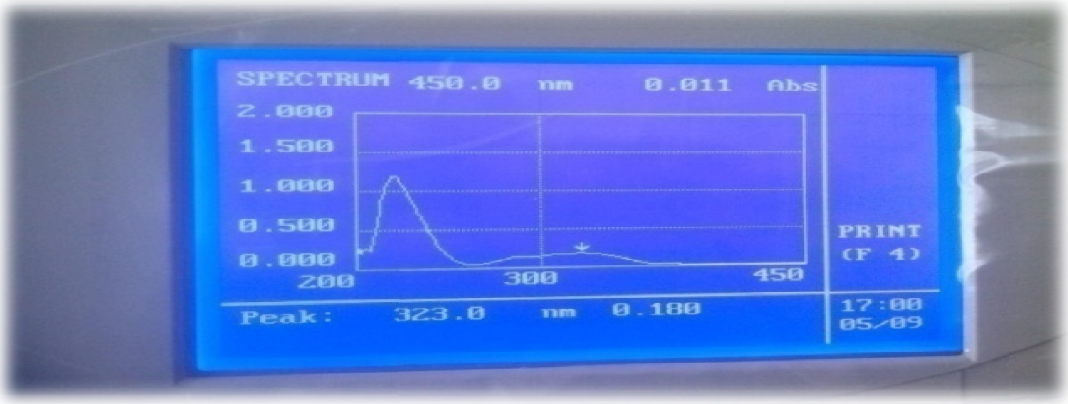
Appendix A
UV-Vis Spectral Data

F(S)



F $(13\text{-}18)_2$



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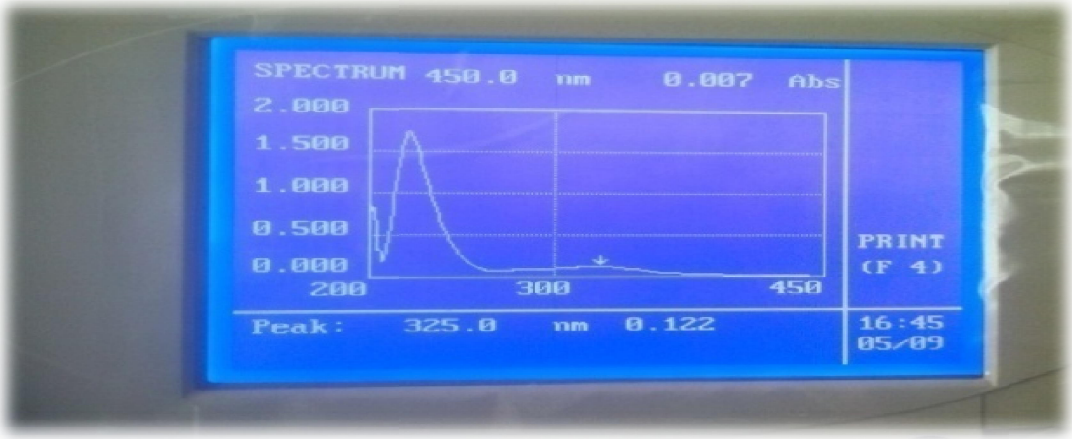
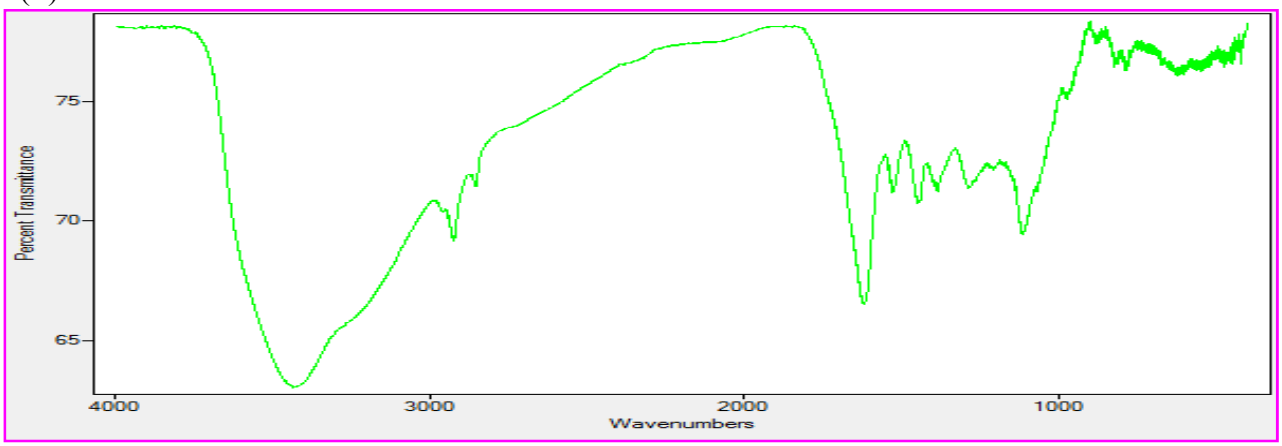
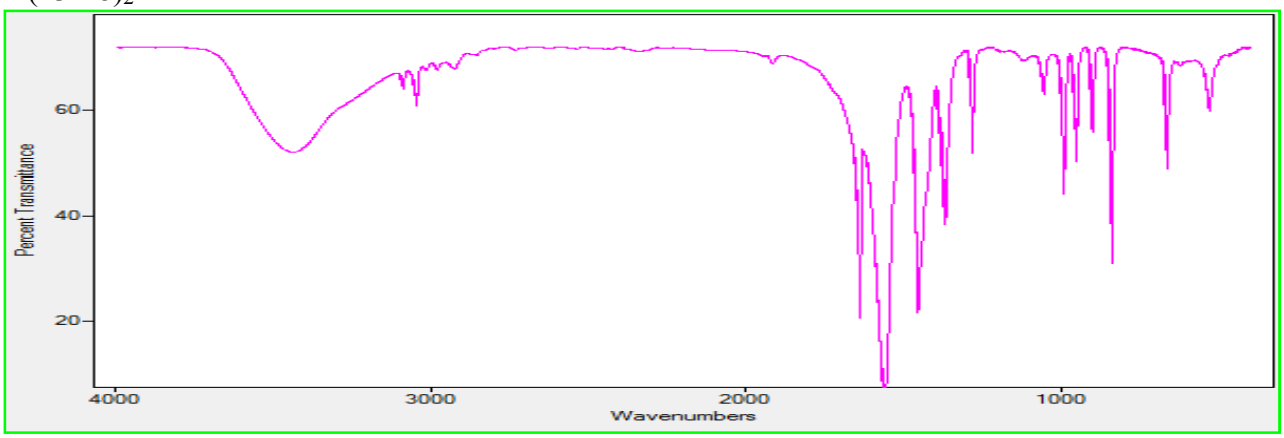


Appendix B
IR Spectral Data

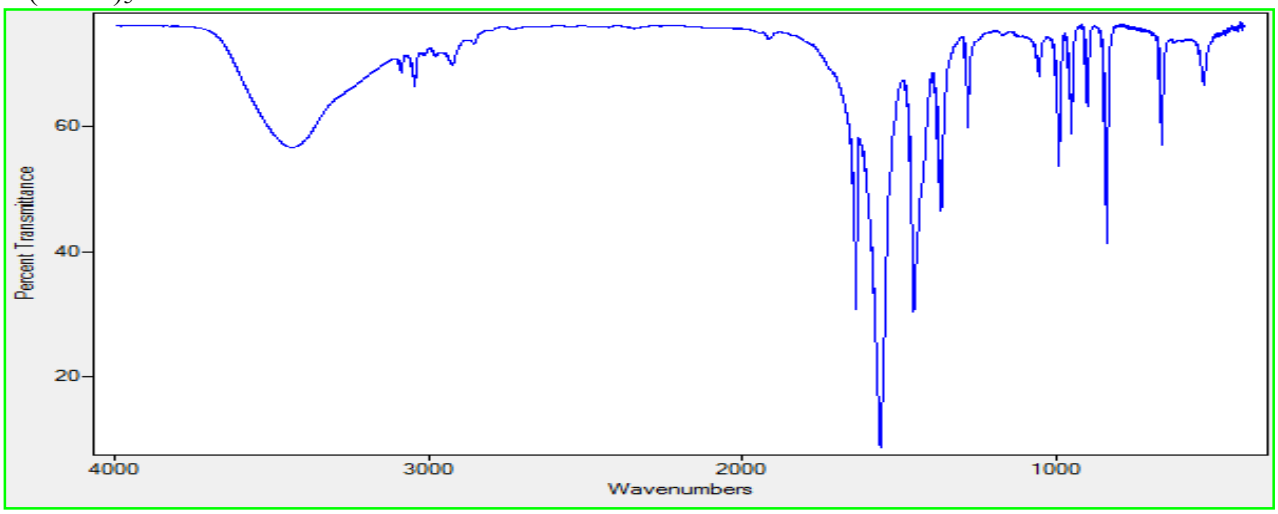
F(S)



F $(13\text{-}18)_2$

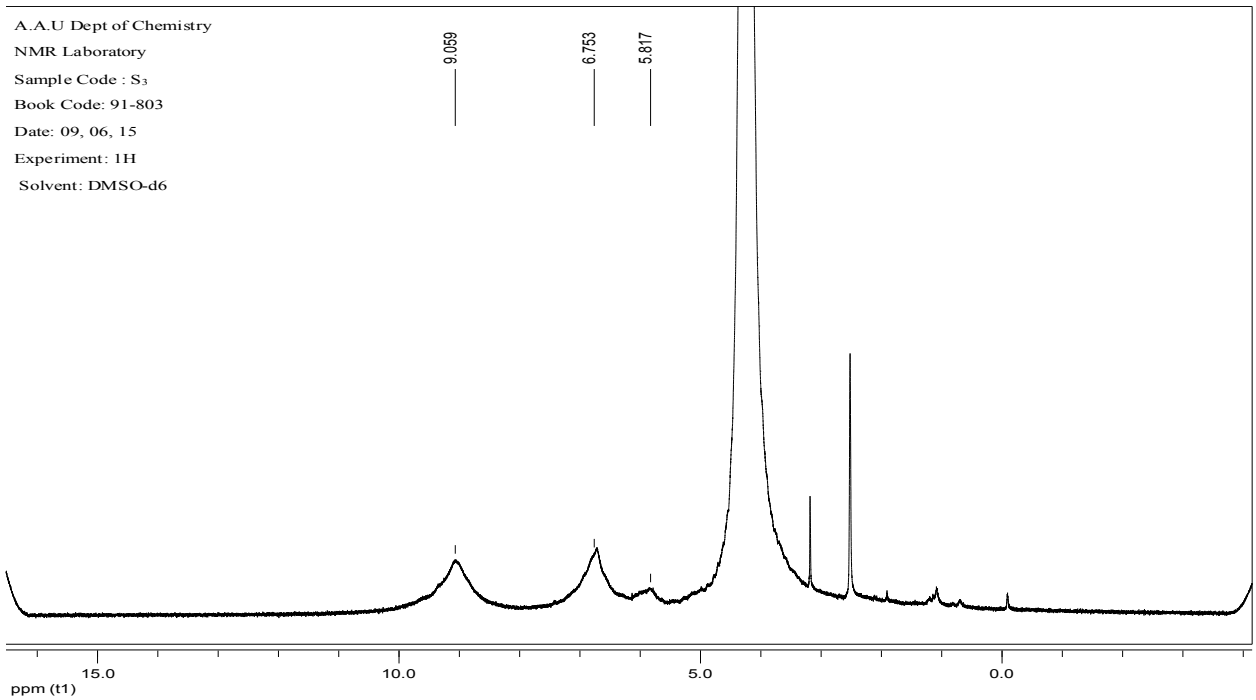


F $(13\text{-}18)_5$

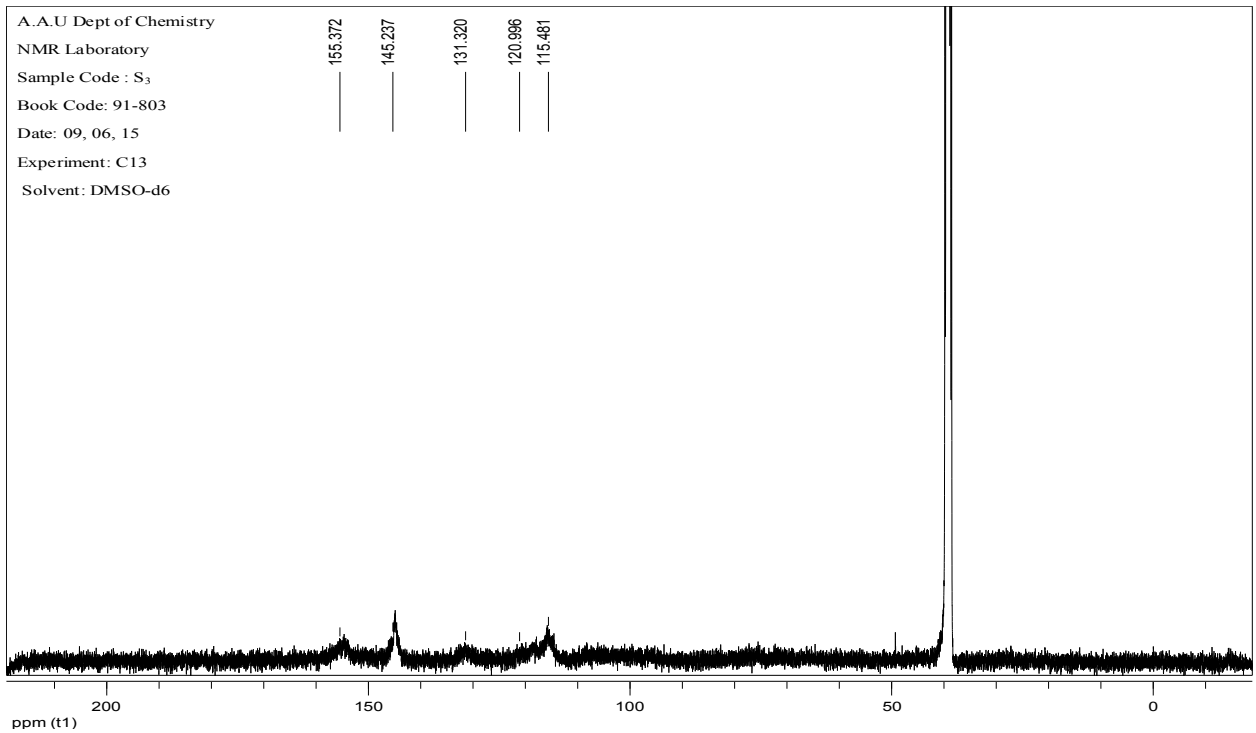


Appendix C
NMR Spectral Data
C.1: NMR Data of F(S)

^{1}H-NMR



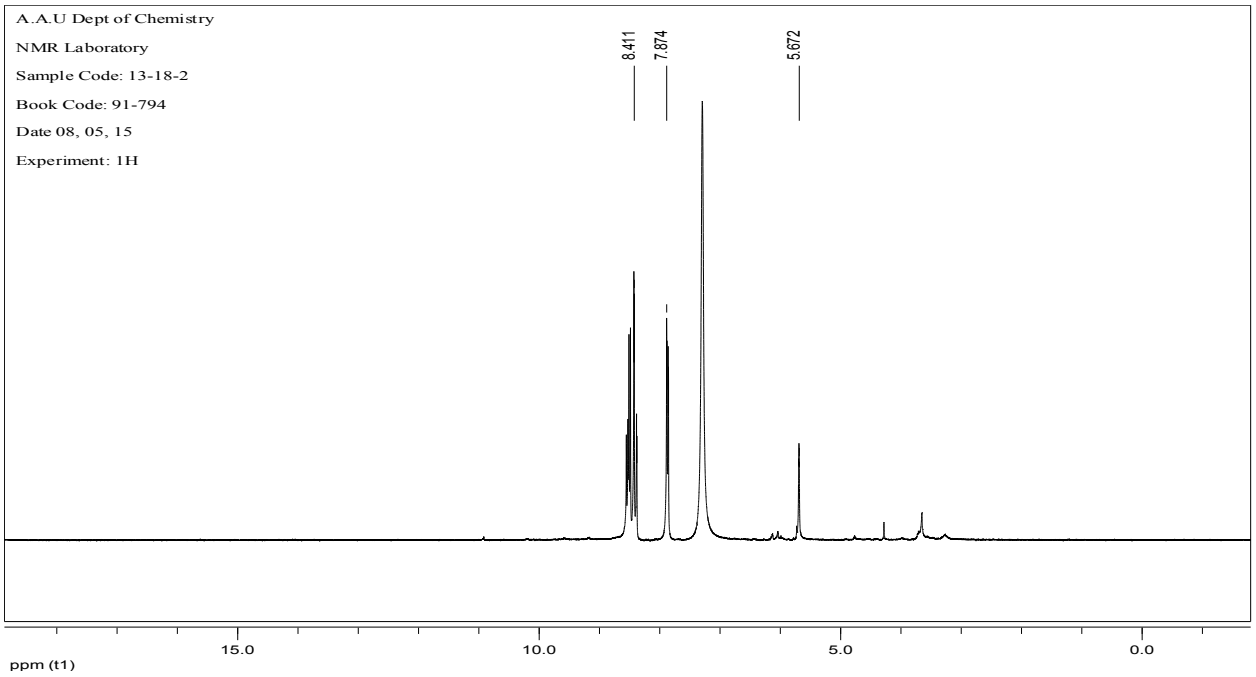
^{13}C-NMR



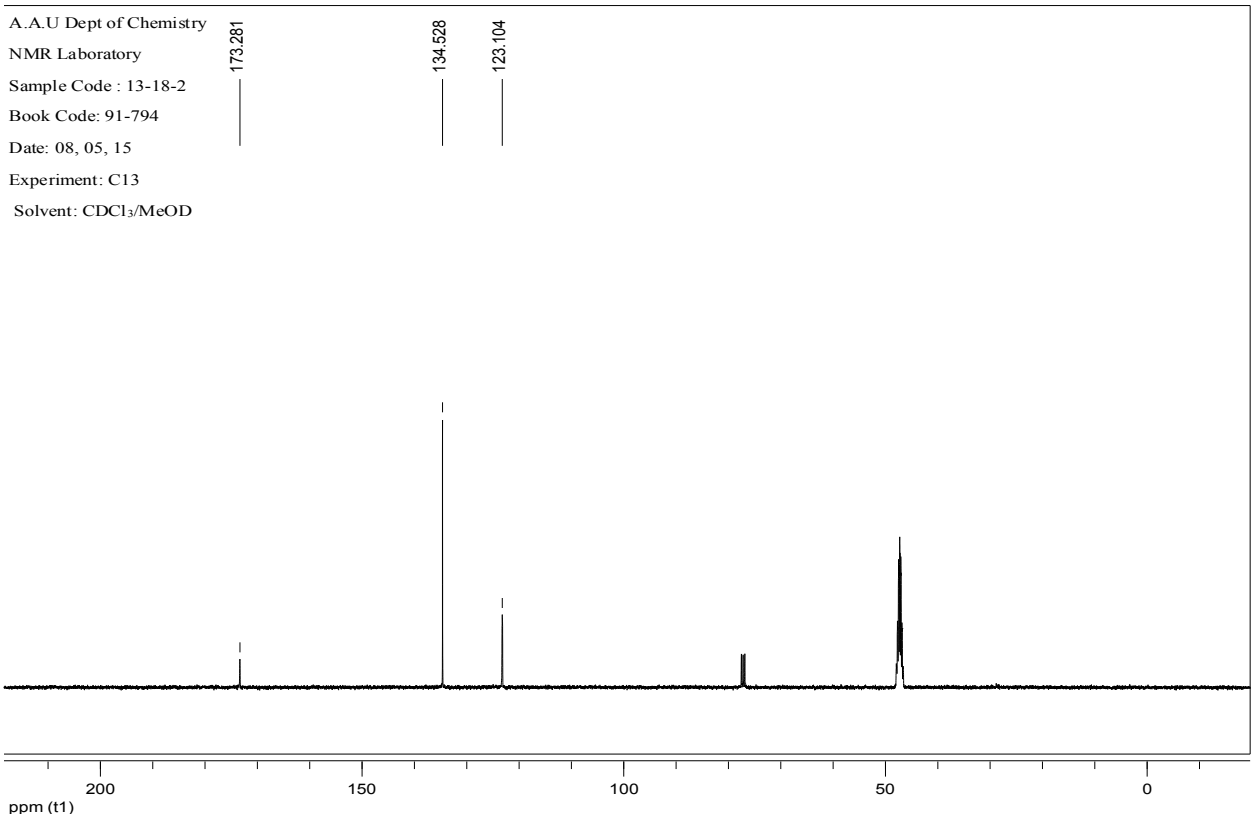
Appendix C: Cont'd

C.2: NMR data of F (13-18)$_2$

^{1}H-NMR



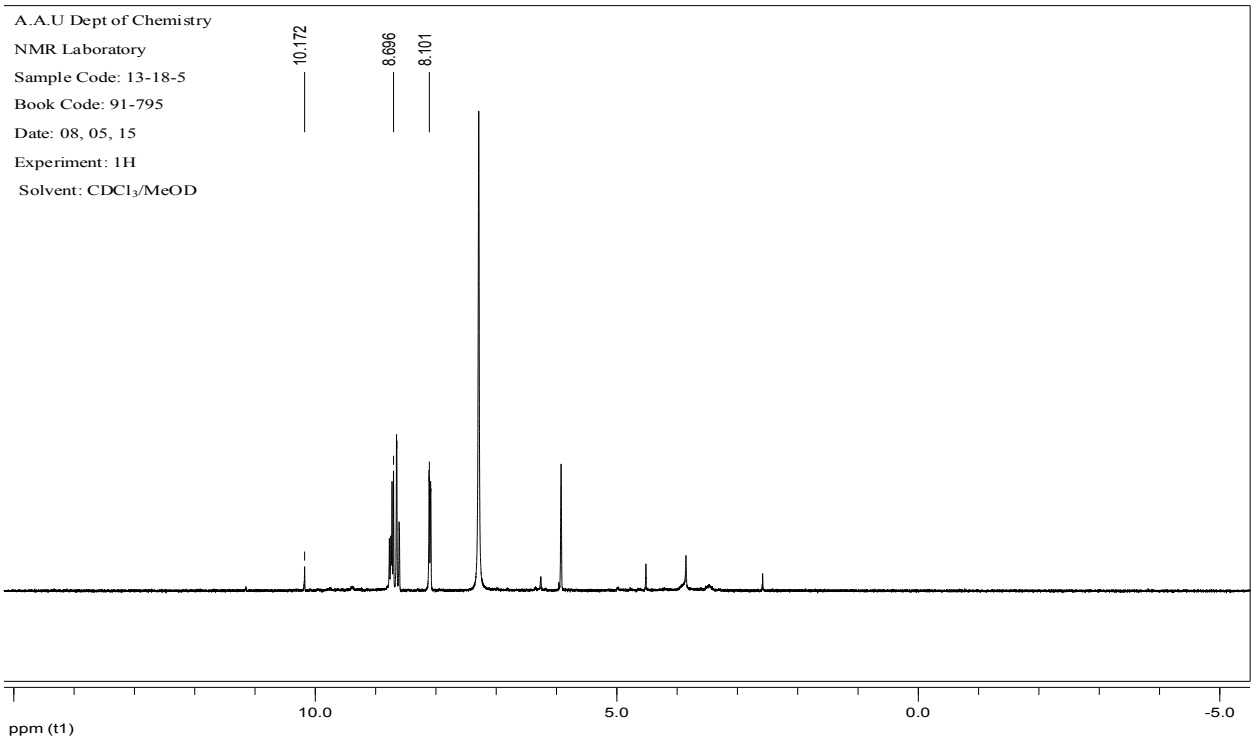
^{13}C-NMR



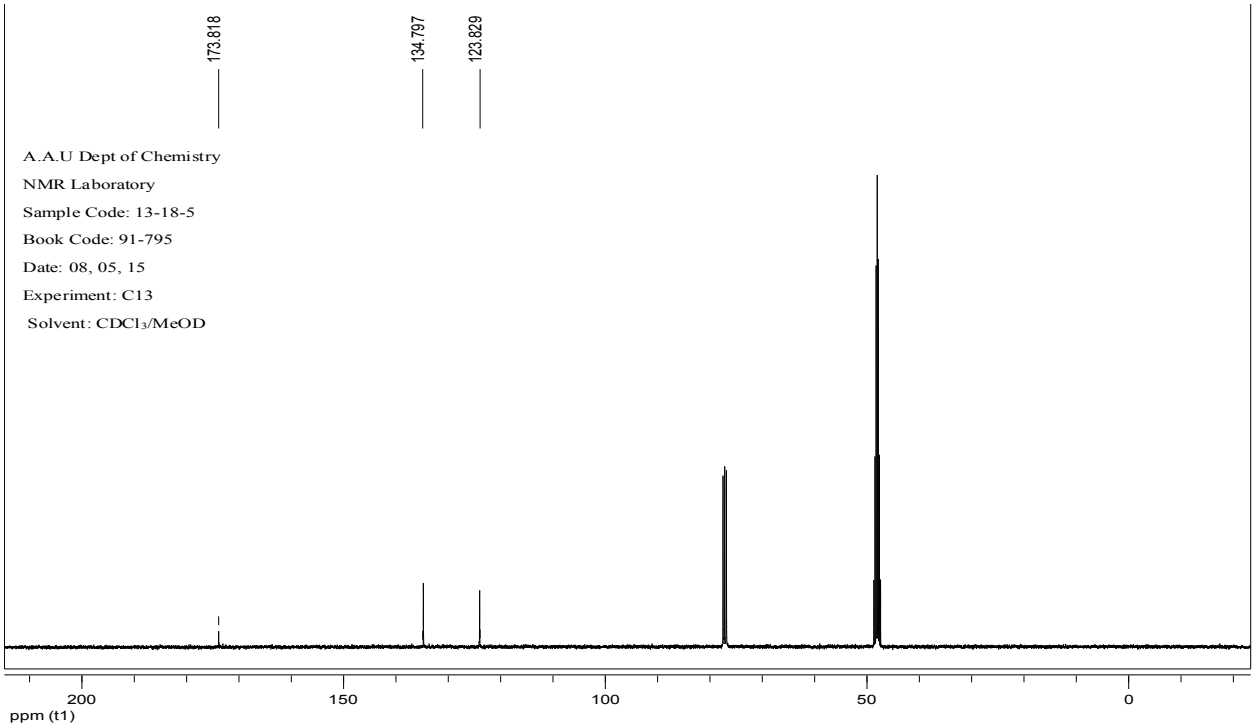
Appendix C: Cont'd

C.3: NMR data of F (13-18)$_5$

^{1}H-NMR

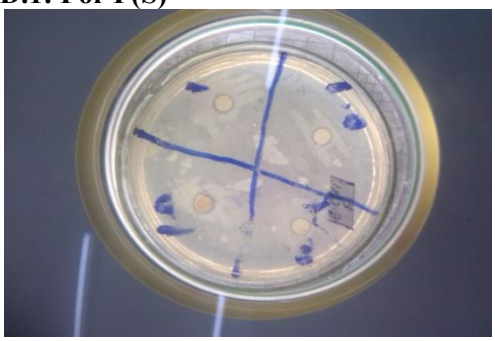


^{13}C- NMR



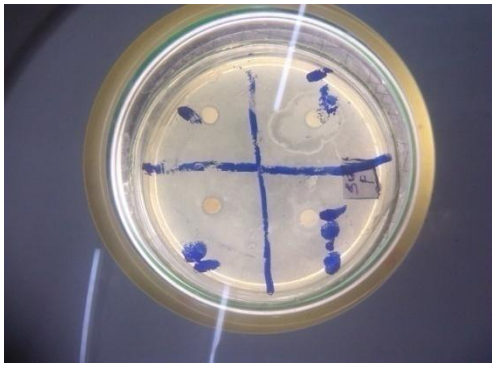
Appendix D: Antibacterial Assay Result

D.1: For F(S)



Escherichia coli

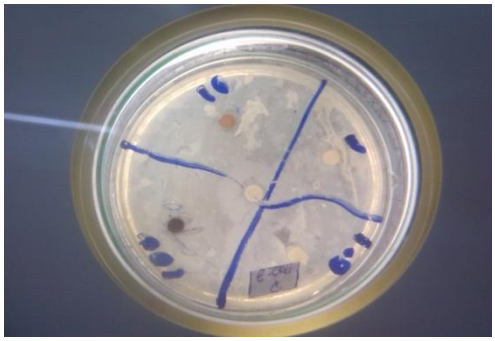
Staphylococcus aurous



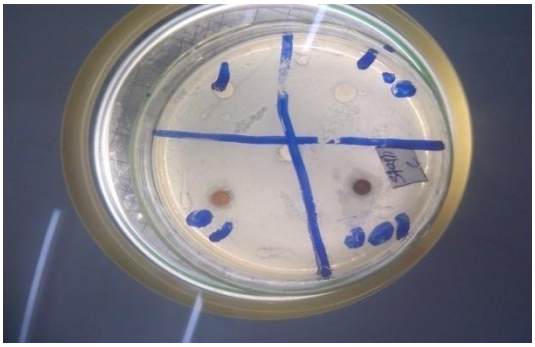
Salmonella thyphimurium

Streptococcus buvis

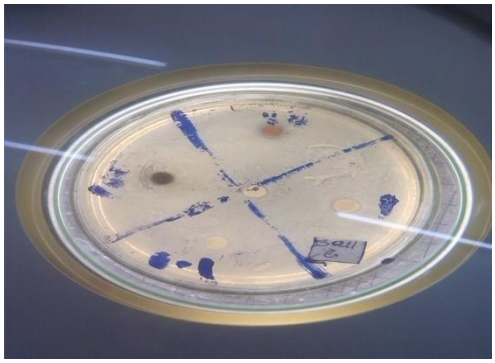
D.2: For Crude Extract



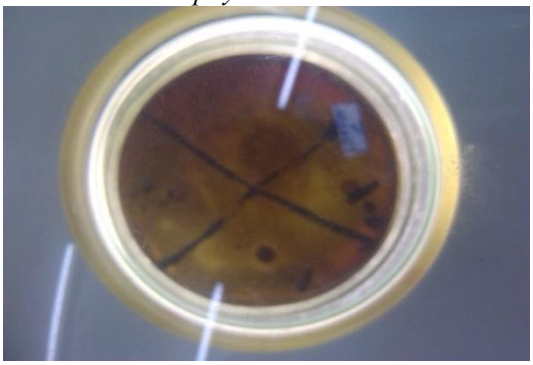
Escherichia coli



Staphylococcus aurous



Salmonella thyphimurium

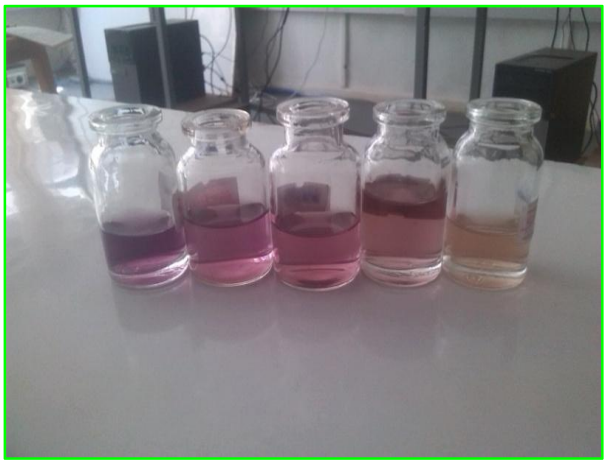


Streptococcus buvis

Appendix E: Antioxidant Assay Result

F(S)

Crude Extract



F $(13\text{-}18)_2$

F $(13\text{-}18)_5$