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MSc THESIS ON

ANALYSIS OF TOTAL PHENOLIC, TOTAL TERPENOID CONTENTS, AND ANTIOXIDANT ACTIVITY OF *Commiphora mollis (Oliv.) Engl.* RESIN

GUYO JILO

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JIMMA, ETHIOPIA

ANALYSIS OF TOTAL PHENOLIC, TOTAL TERPENOID CONTENTS, AND ANTIOXIDANT ACTIVITY OF *Commiphora mollis (Oliv.) Engl.* RESIN

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MSc THESIS APPROVAL SHEET

We, the undersigned, member of the Board of Examiners of the final open defense by **Guyo Jilo Mololе** have read and evaluated his/her thesis entitled "**Analysis of Total Phenolic, Terpenoid Contents, and Antioxidant Activity of *Commiphora mollis (Oliv.) Engl.* Resin**" and examined the candidate. This is therefore to certify that the thesis has been accepted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry (Analytical).

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ABSTRACT

Commiphora mollis (Oliv.) Engl. (Burseraceae) is a well-known medicinal plant and widely practiced by traditional healers in Borena Oromo society, Oromia regional state, Ethiopia for the treatments of wounds, stomach ache, and skin disorder. Therefore, this study aimed to investigate the total phenols, terpenoid contents, and antioxidant property of the C. mollis resinous exudate. C. mollis resin exudate was extracted with petroleum ether, chloroform, and methanol that yielded 27.46 ± 0.48, 46.56 ± 0.42, and 53.00 ± 1.39 % of the dry sample, respectively. Qualitative phytochemical analysis of the extracts revealed the presence of alkaloids, terpenoids, phenols, and saponins. The total phenolic contents of extracts were analyzed by the Folin-Ciocalteu redox method. Absorbances were measured at the experimentally determined maximum absorption wavelength (765 nm), after 30 of contact time between the extracts or standard and the reagent. The analysis showed that all the extracts are rich in phenolic compounds with the highest amount (112.24 ± 1.67 mg/g) observed in methanol extract. On the other hand, the total terpenoid content is high in chloroform (86.67 ± 3.82 %), and petroleum ether (75.0 ± 2.5%) extracts than the methanol extract (27.50 ± 2.50 %). This could be due to the less polar nature of terpenoids that can be extracted more by a less polar solvent such as chloroform and petroleum ether than methanol. Whereas the reverse could happen when it comes to phenolic compounds, as they are polar and thereby extracted more with methanol. The antioxidant activity and kinetics of the reaction was also determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay at 517 nm. It was observed that methanol extract and ascorbic acid displayed very fast reaction kinetics reaching the steady state of reaction at 0.288 to 0.545 min and 1.82 to 2.19 min, respectively, while chloroform and petroleum ether extracts showed low antiradical activity with the steady state time of 33.18 to 76.96 min and 25.74 to 47.21 min, respectively. At 60 min reaction time, methanol, chloroform, and petroleum ether extracts showed DPPH radical scavenging activity with IC_{50} values of 254.8 ± 1.08, 296.5 ± 2.76, and 316.70 ± 1.16 µg/mL, respectively. The high radical scavenging activity of the methanol extract than chloroform and petroleum ether extracts could probably be due to the high phenolic contents of methanol extract (as indicated by the phenolic analysis). For better understanding, further study accompanied by isolation and structure elucidation of pure compounds is recommended.

Keywords: *Commiphora mollis (Oliv.) Engl. resin, Phenol, Terpenoid, Folin-Ciocalteu, DPPH radical scavenging activity.*

ABBREVIATIONS AND ACRONYMS

AA	Ascorbic Acid
ABTS	2, 2`-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
ACNF	Acetonitrile fraction
AE	Aqueous extract
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BDE	Bond Dissociation energy
BHT	Butylated Hydroxytoluene
BUF	n-Butanol fraction
CHE	Cyclohexane extract;
CLE	Chloroform extract
DCMF	Dichloromethane fraction
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EAE	Ethyl acetate extract
EAF	Ethyl acetate fraction
EC	Effective Concentration
EE	Ethanol extract
EO	Essential oil
ET	Electron Transfer
F-C	Folin-Ciocalteau
FCP	Folin and Ciocalteau's Phenol reagent
F-CR	Folin-Ciocalteau’s Reagent
FRAP	Ferric reducing antioxidant power
GAE	Gallic Acid Equivalent
HAT	Hydrogen Atom Transfer
HF	Hexane Fraction
HPLC	High pressure liquid chromatography
IC	Inhibition Concentration
IOU	Inhibited Oxygen Uptake

IP	Ionization potential
LDL	Low-density lipoprotein
LOD	Limit of Detection
LOQ	Limit of Quantitation
MEE	Methanol fraction
MEF	Methanol fraction
ORAC	Oxygen Radical Absorbance Capacity
PEE	Petroleum ether extract
PUFA	Polyunsaturated fatty acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SET	Single Electron Transfer
SOD	Superoxide Dismutase
TBHQ	tert-butyl hydroquinone
TPC	Total Phenolic Content
TRAP	Total Radical Trapping Antioxidant Parameter
TTC	Total terpenoid content
UV	Ultraviolet

1. INTRODUCTION

Since antiquities, plants have been used in folks to treat oxidation induced diseases and food preservation [1]. In recent years, the studies of the antioxidant potential of plants have become an interesting topic. The oxidation process causes many dreadful diseases including cancer, diabetes, neurodegenerative disorders, and cardiovascular disease [2], and food deterioration [3]. To overcome these problems, the intake of antioxidants containing foods [2, 4] have been practiced. Synthetic antioxidants are commonly used for their performance, low costs, and wide availability [1]. Nevertheless, the use of synthetic antioxidants may have negative effects on the users' health; it can cause DNA damage and induce premature senescence [5], liver toxicity [6], and affect reproductive function, and cause developmental problems [7]. Moreover, very little is known about the environmental occurrence and fate of these compounds [1]. Although the toxicity profile of most medicinal plants has not been thoroughly evaluated, natural medicines derived from plants could be safer than the synthetic counterparts [8].

The antioxidant properties of plants arise from phytochemicals exhibiting antioxidant effects such as phenolics, flavonoids, carotenoids, and terpenoids, among other organic compounds [2, 9, 10]. Moreover, the potential of plants as an antioxidant is often investigated following their popular use in folk medicines to treat oxidation induced diseases. *Commiphora mollis* (Oliv.) Engl. family *Burseraceae,* commonly called Corkwood [11] is a non-spiny deciduous plant with a non-peeling stem and silky hairy leaves. It is used in traditional medicine as an anti-inflammatory, analgesic, and anti-parasite agent [12, 13].

Most of the plants from the genus *Commiphora,* produce resin, myrrh [14] which have long been used as a traditional medicine to treat inflammation, arthritis, wound, tumor, skin, and stomach disorders. Studies have also revealed diverse bioactivities of *Commiphora* species including antioxidant, anti-inflammatory, antitumor, analgesic, and antiulcer activities [15, 16]. Their antioxidant activity arises from antioxidant phytochemicals such as terpenoids and phenolic compounds. Antioxidative terpenoids including monoterpenes, oxygenated sesquiterpenes, furano-sesquiterpenes [17–20], and phenolic compounds such as muscanone [21] and ferulates [22] have been isolated from the resins *Commiphora* species. Besides, phenolic compounds such

as p-coumaric acid, ferulic acid, isoquercitrin, quercitrin, quercetin, rutin, kaempferol and apigenin [23], gallic, chlorogenic and protocatechuic acid [24], curcuminoid [12], and prenylated flavonoids [25] occur in stem bark and leaves of this plant species.

These compounds act as antioxidants either through electron transfer (ET) or hydrogen atom transfer (HAT) [26]. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a very sTable free radical which is most frequently used to test the free radical scavenging activity of plants. Upon reduction by antioxidant (either through HAT or ET), the deep purple-colored DPPH changes to hydrazine or substituted analogous hydrazine, and its absorbance decrease at λ_{max} (517 nm). The results are often expressed as IC_{50} (or EC_{50}), the concentration of the potential antioxidant needed to decrease the initial absorbance of the colored radical by 50% [27–29]. DPPH is a very sTable free radical which is unaffected by side reactions, like metal ion chelating and enzyme inhibition [30]. It does not dimerize, due to the delocalization of free radicals [29]. The test is simple, rapid and the assay showed good linearity and precision for evaluation of DPPH scavenging capacity of numerous antioxidants including gallic acid, catechin, quercetin, caffeic acid, and Trolox [27]. However, the results of the DPPH assay is dependent on the reaction mechanisms of antioxidants which affects the assay time [31]. Therefore, monitoring the reaction time could greatly improve the significance of the method [28].

Besides, the Folin-Ciocalteu (F-C) redox method determines the total reducing activity of plants as total phenolic content (TPC) [27, 28, 32, 33]. The Folin-Ciocalteu reagent reacts with antioxidants (phenolic compounds) and forms a blue complex that absorbs at about 765 nm. The concentration of phenolic antioxidants is proportional to the absorbance and can be estimated using gallic acid as standard. Although the test is claimed to provide the TPC of a sample, it is not specific for phenols and it can be used for estimation of total TPC and other oxidative substrates from plant tissues [28, 33]. This method is most commonly employed for its simplicity, precision, and reproducibility [27, 32–34]. However, the result could be affected by several factors. For instance, temperature affects the time of incubation for color development [32, 33, 35] while the variability in individual plants sampled affects the precision of the method [33]. Besides, the bathochromic shift promoted by the Folin-Ciocalteu reagent resulting from the oxyreduction reactions may present λ_{max} deviation capable of compromising the experimental responses by

underestimating the quantitative data [36]. Therefore, for new plant study, pre-analysis optimization of Folin-Ciocalteu and DPPH radical scavenging assays could be helpful to obtain the most likely result.

1.1. Statement of the Problem

In recent years, great interest has been focused on using natural antioxidants, due to studies indicating possible adverse effects that may be related to the use of synthetic antioxidants. Thus, the study of plants and their constituents for antioxidant activity is important. Studies showed that plants of *Commiphora* species exhibit antioxidant activity which may arise from phytochemicals such as phenols [22–24] and terpenoids [17–19]. *Commiphora mollis* (Oliv.) Engl. known by a local name *"Hagarsu"*, in Borana Oromo society, is well known by the surrounding traditional healers for its medicinal application. It produces a resinous exudate which is used in treatment of stomach and skin disorders, particularly in Camels and Cattle. However, information on antioxidant activity and phyto-content of *C. mollis* exudate has not been reported. Therefore, analysis of the antioxidant activity, total phenolic and terpenoids contents of resin of *C. mollis* was the aim of this study.

1.2. Objectives of the Study

1.2.1. General Objective

The main objective of this study was to analyze total phenolic, terpenoid contents, and antioxidant activity of *C. mollis* resinous exudate.

1.2.2. Specific Objectives

The specific objectives of this study were

- To extract the resin of *C. mollis*, using petroleum ether, chloroform, and methanol;
- To determine the total phenolic content of the extracts while optimizing the Folin-Ciocalteu method;
- To determine the total terpenoid contents of the extracts;

- To evaluate the *in-vitro* antioxidant activity and kinetic behavior of the extracts using DPPH radical scavenging assay;
- To evaluate the correlation between phytoconstituents and DPPH radical scavenging activity.

1.3. Significance of the Study

In the present work, the total phenolic, terpenoid contents, and antioxidant activity of *C. mollis* resin were investigated. The obtained results could be useful for those who want to engage in further study. The optimized Folin-Ciocalteu and DPPH radical scavenging assay methods could be helpful for other researchers who are interested to work on antioxidant property studies. Generally, the obtained information on the phytochemical contents, and antioxidant potential of *C. mollis* resin as well as the optimized experimental design could be important.

2. LITERATURE REVIEW

In recent years, there is an upsurge in the areas related to newer developments in the prevention of disease especially degenerative diseases that are related to oxidants (free radicals and reactive species). Antioxidants are the protection system to balance the healthy condition in organisms. Different synthetic antioxidants have been used in health care and food preservation to reduce the damage caused by free radicals and reactive species. But they have been reported to have toxicity. The use of natural products as an alternative for synthetic chemicals has been a reason for the increase in the search for natural antioxidants. This chapter contains a brief review on oxidants (free radicals, reactive species), oxidative stress, and antioxidants as a protective system against oxidative stress. The antioxidant activities of plant antioxidants such as phenolic compounds and volatile oils are also discussed. Moreover, the use, Phytochemistry, and antioxidative activity of the plants of the genus *Commiphora* will be reviewed. Finally, analytical methods for the antioxidant activity will be summarized.

2.1. The Role of Free Radicals (Oxidants) and antioxidants in Health

2.1.1. Free Radical

The concept of free radical was stressed during the events of World War II (1939-1945) where atom bombs in Japan led to massive deaths and the survivors had shortened life-span. There was speculation that the lethal effects of ionizing radiation might be ascribed to the formation of reactive oxygen species (ROS). Since then free radicals (atoms with an unpaired electron) such as ROS and reactive nitrogen species (RNS) have gained notoriety. In many scientific works of literature, the term ‘free radical’ is used in a broad sense and also includes related reactive species such as ‘excited states’ that lead to free radical generation or those species that result from free radical reactions. Indeed, free radicals are species with high reactivity and very short half-life [37]. The presence of unpaired electrons may result in definite familiar properties shared by most radicals. They are mainly derived from oxygen (reactive oxygen species/ROS) and nitrogen (reactive nitrogen species/RNS). Superoxide ($O_2^{\cdot -}$), hydroxyl radical (·OH) and lipid peroxy

radicals (LOO•) are derived from ROS while nitric oxide(NO), Peroxynitrite (ONOO-), nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3) are nitrogen derived oxidants [38, 39].

ROS and RNS are both produced in a well-regulated manner to help maintain homeostasis at the cellular level in normal healthy tissues and play an important role as signaling molecules. Most cells can produce superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and nitric oxide (NO) on-demand [40]. In humans, about 1–3% of the oxygen consumed by the body is converted into superoxide and other ROS under physiological conditions [41]. Free radicals perform many important functions in physiological processes (e.g., microbial killing, cell signaling, or gene transcription [40] and generation of molecules of adenosine triphosphate (ATP) in aerobic organisms [42].Recently, the role of H_2O_2 as it could mimic the action of the insulin growth factor and that of enzymatically produced NO• in vasodilation and neurotransmission have been reported [43]. In some cases, the amount of oxidants can be more than demand due to altered metabolism or external contaminants exposition. Mitochondrial electron transport chain, respiratory burst by phagocytes, beta-oxidation of fat in the peroxisome, auto-oxidation of amino acids, catecholamines, hemoglobin and ischemia-reperfusion injury are endogenous sources While electromagnetic radiation, cosmic radiation, cigarette smoke, car exhaust, UV light, ozone (O_3), and low wavelength electromagnetic radiations are external sources of ROS [44]. In our body, pro-oxidants also promote the formation of free radicals as of H_2O_2 which is easily converted to HO•. Moreover, other oxidants such as nitric oxide promote chain reactions [39, 45, 46].In the human brain metal ions like iron(II)/copper(I) and iron(III)/copper(II) ions are also reported to be involved in the conversion of $O_2^{-\bullet}$ and H_2O_2 into HO• via the Fenton reaction [47].

$$Fe^{n} + H_2O_2 \longrightarrow Fe^{n+1} + HO^{\bullet} + HO^{-} \quad (\mathbf{1})$$

$$Fe^{n+1} + H_2O_2 \longrightarrow Fe^{n} + HOO^{\bullet} + H^{+} \quad (\mathbf{2})$$

In plants, one major source of free radical is the photosystem since chlorophyll may act as photosensitizer forming singlet oxygen. But also in other compartments like mitochondria, microsomes, peroxisomes, and others, the formation of ROS may occur [41].

2.1.2. Antioxidants

Antioxidants are compounds capable of slowing or retarding the oxidation of an oxidizable material, even when used in a very modest amount (<1%, commonly 1-1000 mg/L) as compared to the amount of material they have to protect [48]. They are used to normalize the amount of oxidants in biological system. These antioxidants can be endogenous and exogenous (enzymatic and non-enzymatic) [46, 49, 50].Various endogenous enzymatic antioxidants such as Superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, thioredoxin, thiols, and disulfide bonding are buffering systems in every cell. α-tocopherol (vitamin E) is an essential nutrient that functions as a chain-breaking antioxidant that prevents the propagation of free radical reactions in all cell membranes in the human body. Ascorbic acid (vitamin C) is also part of the normal protecting mechanism. Other non-enzymatic antioxidants include carotenoids, flavonoids, and related polyphenols, α-lipoic acid, glutathione, etc. [51].

Antioxidants, capable of neutralizing free radicals or their actions, act at the levels of prevention, interception, and repair. Preventive antioxidants attempt to stop the formation of ROS (e.g., SOD-which catalyzes the dismutation of superoxide to H_2O_2 and catalase-that breaks it down to water [52]. The interception of free radicals is mainly by radical scavenging, while at the secondary level scavenging of peroxyl radicals is affected. The effectors include various antioxidants like vitamins C and E, glutathione, other thiol compounds, carotenoids, flavonoids, etc. At the repair and reconstitution level, mainly repair enzymes are involved [37].

2.1.3. Oxidative Stress

Under normal conditions, there is a mechanism of balance between oxidants and antioxidants in biological systems. In some cases, undesirable conditions may cause the production of the excess of free radicals[44].The imbalance between oxidants and antioxidants in favor of the oxidants leads to the so-called oxidative stress which may occur in animals and plants. This results in the damage

of the DNA, proteins, or lipids. In food, because food tissues are (or were) living and free radicals, reactive oxygen species, and pro-oxidants generated both exogenously (heat and light) and endogenously (H_2O_2 and transition metals) cause their deterioration [54].

2.1.4. Effects of Free Radicals in Biological Systems

All the biological molecules present living body including plants are at risk of being attacked by free radicals. Such damaged molecules can impair cell functions and even lead to cell death eventually resulting in diseased states. Free radicals have been implicated for causing several human diseases for the development of several chronic ailments, for example, Alzheimer's disease, atherosclerosis, cancers, as well as ageing [1, 2, 55].

One of the damages induced by free radical is lipid peroxidation which is a free radical chain reaction initiated by the hydrogen abstraction or addition of oxygen radical from/to polyunsaturated fatty acids (PUFAs) [56]. It can occur in membrane lipids and the brain due to the presence of a large amount of PUFAs. The reaction produces a large number of toxic byproducts that can have effects at a site away from the area of generation, behaving as 'second messengers'. The damage caused by lipid peroxidation is highly detrimental to the functioning of the cell [37].

The initiation of a peroxidative sequence is due to the attack by any species, which can abstract a hydrogen atom from a methylene group (CH_2), leaving behind an unpaired electron on the carbon atom (•CH). The resultant carbon radical is stabilized by molecular rearrangement to produce a conjugated diene, which then can react with an oxygen molecule to give a lipid peroxyl radical (LOO•). These radicals can further abstract hydrogen atoms from other lipid molecules to form lipid hydroperoxides (LOOH) and at the same time propagate the reaction further. The peroxidation reaction can be terminated by several reactions. The major one involves the reaction of LOO• or lipid radical (L•) with a molecule of antioxidant such as vitamin E or α-tocopherol (α-TOH) forming more sTable tocopherolphenoxyl radical that is not involved in further chain reactions. This can be 'recycled' by other cellular antioxidants such as vitamin C. The following equations illustrates lipid peroxidation reaction [37].

$$LH + {}^{\bullet}OH \longrightarrow L^{\bullet} + H_2O \quad (3)$$

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet} \quad (4)$$

$$LOO^{\bullet} + LH \longrightarrow L^{\bullet} + LOOH \quad (5)$$

$$LOO^{\bullet} + \alpha\text{-}TOH \longrightarrow LOOH + \alpha\text{-}TO^{\bullet} \quad (6)$$

When an organism is exposed to a high concentration of ROS, the endogenous antioxidant system is compromised and, consequently, fails to guarantee the complete protection of the organism [57, 58]. Additional antioxidants are important in counteracting the deleterious effects of free radicals in biological systems [1, 49]. The intake of fruits and vegeTables for humans [4] and the incorporation of antioxidants in food products have been suggested [1]. Natural antioxidants, easily obtained from natural sources, possess great potential to reduce oxidative stress caused by free radicals.

2.2. Plants Secondary Metabolites as Antioxidants

Plants produce two classes of metabolites named after their metabolism as primary or secondary. Primary metabolites are compounds produced through primary metabolism, for normal growth, development, and reproduction which include amino acids, carbohydrates, proteins, etc. [59]. Plants also produce secondary metabolites the major of which are nitrogen-containing (alkaloids) and non-nitrogen containing(phenols and terpenoids) to revive harsh environmental conditions such as UV radiation and wounding [60, 61].to defend themselves against predators and pathogens and to get a reproductive advantage as intraspecific and interspecific attractants. To date, thousands of different types of secondary metabolites have been identified in plants. Alkaloids are accumulated in approximately 20% of plant species which mainly include terpenoidindole alkaloids, tropane alkaloids, and purine alkaloids. However, under *in vitro* antioxidant measurement assay conditions, the radical scavenging potential of alkaloids is reportedly moderate to nonexistent [62]. According to Brewer [54], the major antioxidative plants contain phenolics and volatile oils which is mainly composed of terpene and terpenoids. On top of this, supporting evidence, in vivo, and in vitro antioxidative analysis of terpenoids and volatile oils have been reported. Monoterpenes [55], sesquiterpenes, furano-sesquiterpenoids [17–19] and diterpenes [55]

have been found to possess notable antioxidant activity in different *in vitro* assays. Tetraterpenes and carotenoids have been shown to possess potent antioxidant activity within both *in vivo* and *in vitro* studies; however, some valuable carotenoids such as beta-carotene showed pro-oxidant effects at high concentration and oxygen pressure [62].

Among all secondary metabolites, phenolic antioxidants appear to be the most important since they have shown promising antioxidant activity in both *in vivo* and *in vitro* investigations. The intense antioxidant activity of plant-derived chemicals such as phenolic compounds and essential oils of many spice plants have been reported. Their significance is higher because it has been found that many synthetic antioxidants exhibit side effects and toxicity after prolonged use [62].These biomolecules exhibit their activity through removing free radicals, binding metal ions, inhibiting enzymatic systems that produce free radical forms, increasing the concentration of biologically important endogenous antioxidants, and inducing the expression of a variety of genes responsible for the synthesis of enzymes that inhibit oxidative stress [63].

2.2.1. Phenolic Compounds

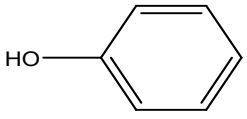
Phenolic compounds are considered to be the most abundant constituents of plants and synthesized partly as a response to ecological and physiological pressures such as pathogen and insect attack, UV radiation, and wounding [64]. They are most commonly studied for they are antioxidants to prevent heart disease, reduce inflammation, and lower the incidence of cancers and diabetes. They can show their antioxidant activity in plants, foods, and humans due to their reducing properties and the ability to interact with metal ions and proteins. These enabled them to inhibit lipid oxidation and reduce oxidative stress. Phenolic compounds exert their antioxidant activity by direct scavenging of ROS, inhibition of enzymes involved in oxidative stress, regeneration of another antioxidant (α-tocopherol), and chelation of metal ions that are responsible for ROS production and, finally, stimulation of endogenous antioxidant defense systems [65].

The basic structural feature of phenolic compounds is an aromatic ring bearing one or more hydroxyl groups [64]. In the plant kingdom, they vary from the simplest phenol (C_6H_5OH) (**1**) to polyphenols based on the number of phenol units in the molecule [66]. Polyphenols can occur as

free and conjugated forms. They can be classified as non-flavonoids and flavonoids. Phenolic acids and flavonoids are the most abundantly existing phenolic compounds in plants [67].

Non-flavonoids phenolics can be further classified into four main groups: phenolic acids, stilbenes, lignans, and the polymeric lignins [67]. From these, phenolic acids are reported to be the most abundant and also reported to show antioxidative properties. Structurally, they contain carboxyl group with one or more hydroxyl groups grafted onto a benzene nucleus [64].

Phenolic acids occur in large amounts (30%) in our diets and are found in different forms in plants, including aglycones (free phenolic acids), esters, glycosides, and/or bound complexes [64, 66, 68]. Based on the position of the hydroxyl group, phenolic acids can be divided into two main types, benzoic acid (C_1– C_6) and cinnamic acid derivatives (C_3–C_6) [67]with general structures of (**2 and 3**) respectively.



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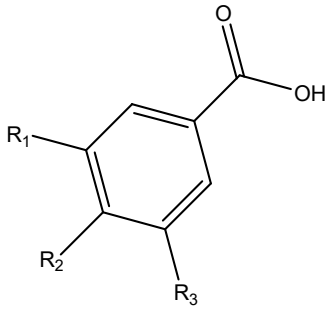

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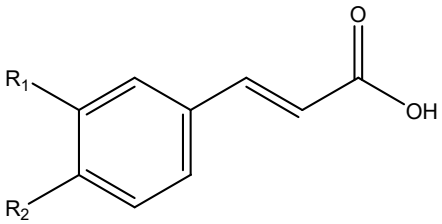

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3. p-hydroxybenzoic acid (R_2 = OH)
4. Gallic acid (R_1, R_2, R_3 = OH)
5. Vanillic acid (R_2 = OH, R_3 = OCH_3)
6. Syringic acid (R_1, R_3 = OCH_3, R_2 = OH)

8. Ferulic acid (R_1 = OCH_3, R_2 = OH)
9. Caffeic acid (R_1, R_2 = OH)
10. p-coumaric acid (R_2 = OH)

Figure 1. Chemical structure of simple phenol, (1); hydroxybenzoic acids,(2); hydroxycinnamic acids (7).

The most common hydroxybenzoic acids are gallic (**4**) vanillic (**5**), syringic (**6**), and acids. Among hydroxycinnamic acids, ferulic (**8**) and caffeic (**9**) acids are the most abundant compounds in foods. Ferulic acid is mainly found from dietary fiber, sources of which include wheat bran and caffeic acid occurs mainly as esters (chlorogenic acid) and is largely obtained from coffee, fruits, and vegetables [69].

Flavonoids together with carotenoids and chlorophyll give color to many species of flowers and fruits. In plants, they can occur as free a glycones and as glycosidic conjugates in which one or more hydroxyl groups of phenols are combined with reducing sugars [68]. Flavonoids are polyphenolic compounds of the basic structure C_6-C_3-C_6 in which the two phenolic benzene rings A and B are linked by a pyran ring C (2-phenyl-1-benzopyran) (**11**) [67]. They are classified according to the substitution profile of the heterocyclic ring taking into account the oxidation state of the heterocyclic ring as well as the position of the secondary aromatic ring. A total of about 12 subgroups of flavonoids are distinguished. The secondary (B) ring may be in position 2 (flavones, flavonols, dihydroflavonols, catechins, flavans, and anthocyanidins), position 3 (isoflavonoids), or position 4 (4-phenyl-coumarins, neoflavonoids). In a few cases, the six-membered heterocyclic ring occurs in an open isomeric form (chalcones and dihydrochalcones) or is replaced by a five-membered ring [70].

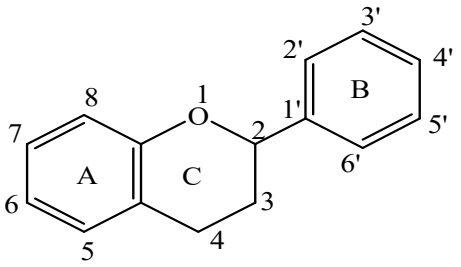

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Figure 2. Basic chemical structure of flavonoids

Phenolic compounds contain hydroxyl group substituents with transferable electron or hydrogen atom and aromatic ring which contribute to the delocalization of the electron. Due to this, the transfer of a hydrogen atom and/or electrons to oxidants are the main chemical mechanisms of the

antioxidant activity of phenolic compounds [71]. In Hydrogen Atom Transfer (HAT) **(equation 7)** [63] phenol antioxidant donates H-atom to an unsTable free radical (R•).

$$R^{\bullet} + ArOH \longrightarrow RH + ArO^{\bullet} \quad (7)$$

This results in the formation of the corresponding phenoxyl radical which is stabilized by delocalization of the unpaired electron throughout the aromatic ring [48]. Due to its stability, the product phenoxyl radical will not propagate the radical chain, but rather "wait" for a second peroxyl radical and quench it in a very fast radical-radical reaction (**equation 8**).

$$ArO^{\bullet} + R^{\bullet} \xrightarrow{fast} \text{nonradical products} \quad (8)$$

The number n of peroxyl radicals (or oxidative chains) quenched by one molecule of antioxidant is called the "stoichiometric factor" (n = 2 for phenols such as guaiacol or α-tocopherol) [48]. In the Electron Transfer (ET), mechanism, the phenoxyl radical is produced by single-electron oxidation of the phenol antioxidant, followed by rapid deprotonation of the resultant radical cation **(equation 9)**.

$$R^{\bullet} + ArOH \underset{-R^-}{\xrightarrow{-1e^-}} ArOH^{\bullet +} + H_2O \xrightleftharpoons{\text{Deprotonation}} ArO^{\bullet} + H_3O^+ \quad (9)$$

These reducing effects are characterized by two important physicochemical parameters, the bond dissociation energy (BDE) of the O–H bond and the ionization potential (IP) of the phenolic compounds that quantify the HAT and ET, respectively. The lower the BDE and the IP, the stronger is the reducing activity of a phenolic compound [72]. In previous studies, the major antioxidative phenolic acids were reported to be gallic acid (**4**), caffeic acid (**9**), p-coumaric acid (**10**), protocatechuic acid (**12**), and rosmarinic (**13**) acid [54].

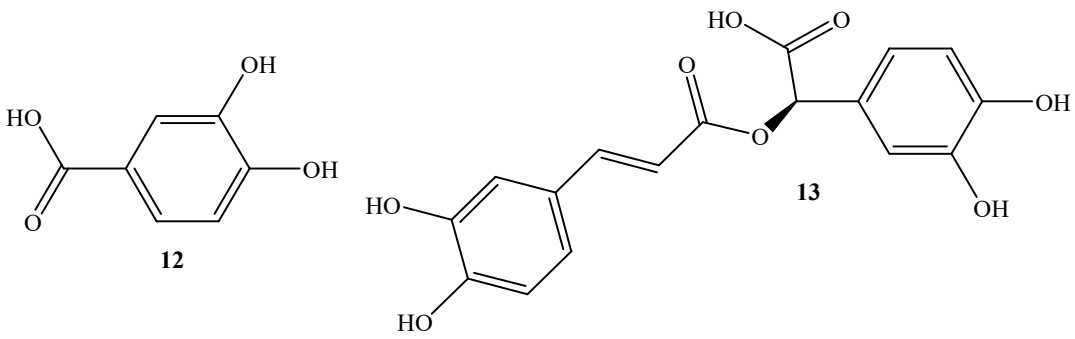

Figure 3. Chemical structure of protocatechuic acid (12), and rosmarinic (13) acid

Antioxidant activity of polyphenols has been proven to be dependent on position and number of -OH group. Concerning the position the *ortho*- position was found to be the more active one, due to its ability to form intramolecular hydrogen bonding, followed by *para*-position and then *meta*-position of compounds [72–74]. The study on free radical scavenging activity of a group of polar compounds was reported to decrease in order of ferulic acid, coumaric acid,propyl gallate, and gallic acid. In another case, the free radical-scavenging activity of a group of nonpolar compoundswas reported for rosmarinic acid, BHT, tert-butyl hydroquinone (TBHQ), α-tocopherol in decreasing order. Only propyl gallate, TBHQ, gallic acid, and rosmarinic acid inhibited lipid oxidation in an oil-in-water emulsion that may reflect the ability of these compounds to orient at the interface of the oil droplet in the emulsion[75].

From flavonoids, quercetin (**14**), catechin (**15**), Epicatechin (**16**) and rutin (**17**) shows promising antioxidative property [54].Most of the time, quercetin and rutin are used as standards in different assays. Considering their structure, classes of flavonoids (**14-17**) differ in the level of saturation of the C ring. Individual compounds within a class differ in the substitution pattern of the A and B rings that influence the phenoxyl radical stability and the antioxidant properties of the substances.

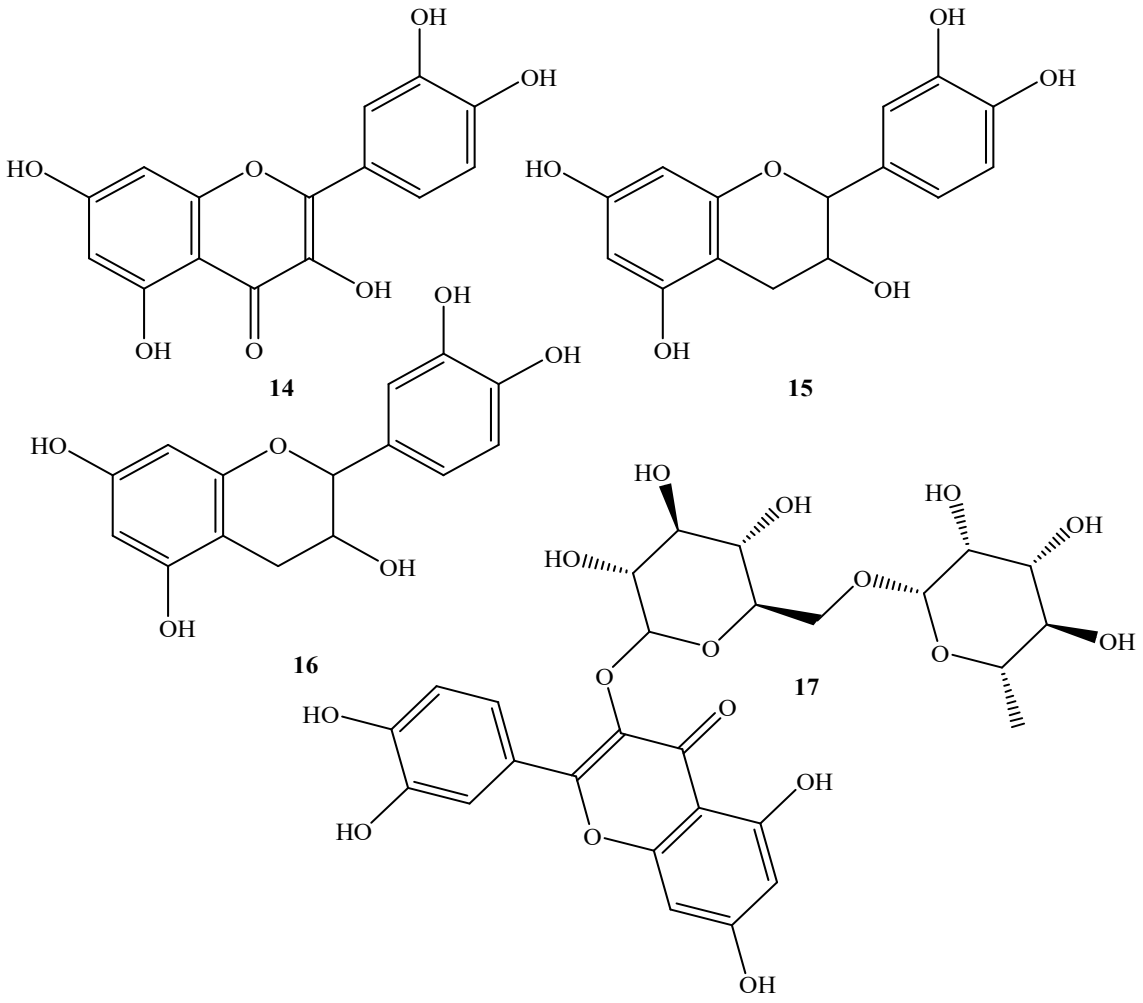

Figure 4. Chemical structure of quercetin (14), catechin (15), Epicatechin (16) and rutin (17)

Antioxidant potential of natural flavonoids appears to depend on the number and position of the free hydroxyl groups. The study on the effect of the B-ring substitution pattern of four flavonols (galangin, kaempferol, quercetin, and myricetin) on their free radical-scavenging ability to quench the intrinsic fluorescence of bovine serum albumen, reported their activity in decreasing order from myricetin, quercetin, kaempferol galangin. The authors also demonstrated the hydrogen bond force to play the main role [76].

Flavonoids with multiple hydroxyl groups are more effective antioxidants than those with only one. The presence of the ortho-3,4-dihydroxy structure increases the antioxidative activity [77].

Flavonoids can also reduce the risk of oxidation by transition metals by donating hydrogen atom, rendering them less proxidative. Besides, flavones and some flavanones (naringenin) can preferentially bind metals at the 5-hydroxyl and 4-oxo groups [78].

2.2.2. Essential Oils

Essential oils (EOs) are liquid mixtures of volatile compounds obtained from aromatic plants, most commonly by steam distillation. They constitute what is called the "essence" of a plant and usually have pleasantly scented fragrances. Aromatic plants and EOs have been used for millennia for their health benefits, well documented in ancient literature. Hundreds of compounds (secondary metabolites) with relatively low boiling points have been identified in EOs, and the large chemical diversity of their constituents influences the oxidative stability of EOs. On the other hand, several essential oils have been attributed to good antioxidant properties, which can be exploited to protect other materials, such as food, from rancidity. These attributes are due to the inherent ability of some of their components, particularly phenols, to stop or delay the aerobic oxidation of organic matter, although the procedure by which the oil is obtained from the raw material (distillation) limits the content of phenolics in the final matrix because many such compounds are nonvolatile. Nevertheless, there are phenol-free EOs that express antioxidant behavior which contains terpenoids and other volatile constituents [48].

Despite the observed large chemical diversity, the main components of common essential oils can be classified into two structural families concerning the hydrocarbon skeleton. The major constituent, terpenoids, are formed by the combination of isoprene unit (**18**).

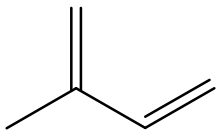

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Figure 5. Chemical structure of isoprene unit

Moreover, most natural terpenoids hydrocarbon have the general formula $(C_5H_8)_n$ Therefore, terpenoids can be classified based on the number of carbon they contain in addition to the number

of isoprene units. The single isoprene unit, represents the most basic class of terpenes, the hemiterpenes. When an isoprene unit bound to a second isoprene a monoterpene is formed (10-C). Sesquiterpenes contain three isoprene units (15-C), while diterpenes (20-C) and triterpenes (30-C) contain two and three terpene units respectively. Tetraterpenes consist of four terpeneunits (40-C), polyterpenes more than four such units (> 40-C)[41]. From these, mono-and sesquiterpenes are most commonly found in essential oils of different plants. The second constituent of essential oil is phenylpropanoids. Both terpenoid and phenylpropanoid families comprise phenolic compounds, sometimes accounted among principal components of several EOs[48]. Some examples of common volatile oils constituents include rosmarinic acid (**13**) and phenolic terpenoids such as carnosic acid (**19**), rosmanol (**20**), carnosol (**21**), eugenol (**22**), carvacrol (**23**), thymol (**24**), conyferil alcohol (**25**) and guaiacol (**26**)) [48, 54].

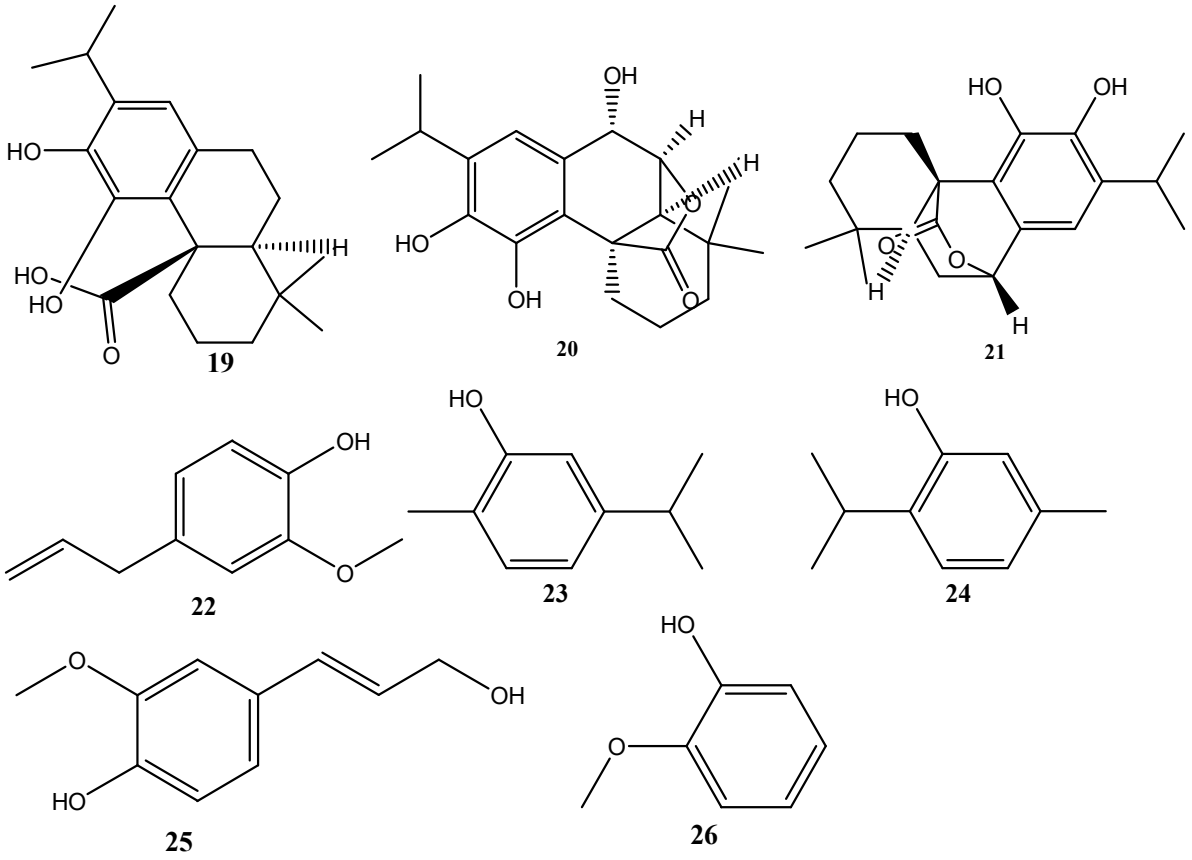


Figure 6. Chemical structure of carnosic acid (19), rosmanol (20), carnosol (21), eugenol (22), carvacrol (23), thymol (24), conyferil alcohol (25) and guaiacol (26)

Since they contain aromatic ring and hydroxyl group these phenolic terpenoids can acts as antioxidants just like other polyphenols. Moreover, other no-phenolic terpenoids have been studied for their antioxidant activities. They can achieve their antioxidant activity through the transfer of hydrogen atom to free radicals [55].The studies based on DPPH and ABTS assays have shown that terpenes with conjugated double bonds can lose allylic hydrogen atoms, be resonance-stabilized, and able to terminate the chain reaction [79].

Wojtunik and others [55] studied antioxidant activity of common terpenoid constituents of essential oils employing DPPH assay. In this reaction, π bonds are responsible for the chain-breaking antioxidant activity of monoterpenes. Among monoterpenes with conjugated π bonds, citral (**27**), carvone (**28**), myrcene(**29**), γ-terpinene (**30**), and pulegone (**31**) has been found to scavenge DPPH• radical very quickly and with higher percentage with α-terpinene and α-phellandrene (**32**) exerted lower scavenging activity in methanol. However, those monoterpenes lacking conjugated π bonds (α-pinene, p-cymene, eucalyptol, menthol, and terpinene-4-ol) have been found inactive or of weak activity in the same solvent.

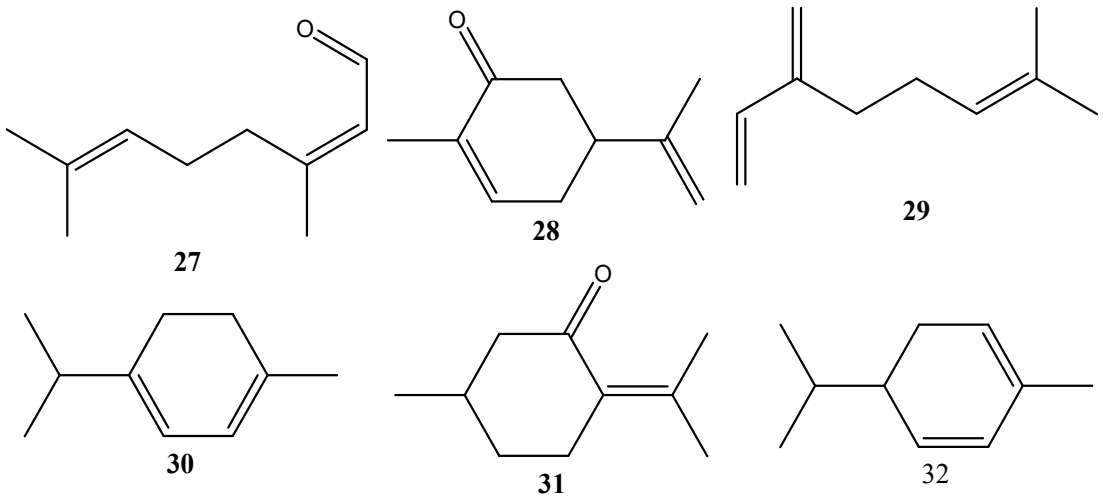


Figure 7. Chemical structure of citral (27), carvone (28), myrcene(29), γ-terpinene (30), and pulegone (31) and α-phellandrene (32)

In other experiments, the π bonds of monoterpenes were blocked with the use of silver ions as the Ag (I)-diene complexes are easily formed. The addition of silver nitrate leads to the decrease of

chain-breaking antioxidant activity of terpenes. The decrease is more significant for terpenoids possessing conjugated double bonds (citral, myrcene, α-phellandrene) than for those without conjugation of double bonds, for example, citronellal. It can be concluded that π bonds are responsible for the chain-breaking antioxidant activity of terpenes. It has been proved, for the first time, that blocking conjugated double bonds leads to inhibition of the chain-breaking antioxidant activity of terpenes [55].

Raw essential oils also have been studied for their antioxidative properties. Because natural essential oils are mixtures of several components, the different types of antioxidants or oxidizable terpenoid components previously described often coexist. When a natural essential oil is used to protect some material, one could expect that the most effective antioxidant components dominate, and the overall oxidative protection offered by the oil is mostly that due to such components. This is true in some cases, but many exceptions have been observed. The overall performance as an antioxidant is, in fact, the result of the complex interplay among components and the oxidizable material to be protected [48].

2.3. Introduction to the Genus Commiphora

The name *Commiphora* originated from Latin *commis* and Greek *kommi* ‘gum’ and *phoros* means bearing which implies gums, oils, and resin extracted from some species[80]. It belongs to family *Burseraceae* which consists of approximately 700 species from 18 genera. The genus Commiphora with more than 150 plant species, is distributed in the tropical and subtropical regions, especially in Southern Africa [13], northeastern Africa including mainly Somalia, Eastern Ethiopia, and Kenya [81].The plants of Commiphora species are characterized as small trees or shrubs with spinescent branches, pale-gray bark, and fragrant reddish-brown resinous exudates. With *C. myrrha* known to be a source of true myrrh, it is also obtained from *C. abyssinica* and *C. schimperi*, both of which may attain a height of 10 m. The drug is chiefly collected in Somaliland and Ethiopia [14]. Collect gum, the natives make incisions into the bark, causing the exudation of a yellowish oleoresin. Exposed to the air, this dries, hardens and turns reddish-brown. Myrrh is partly soluble in ethanol (~ 30 % alcohol-soluble material) and is also partly soluble in water and ether [15, 82].

2.3.1. *Commiphora mollis* (Oliv.) Engl.

Commiphora mollis (Oliv.) Engl. is one of the species of genus *Commiphora,* known by the common name "*fluweelkanniedood*" in South Africa, "*mukunguu"* in Tanzania and velvet corkwood or velvet-leaved corkwood in English. The name *Commiphora mollis* originated from Latin *commis* and Greek *kommi* 'gum' plus *phoros* 'bearing' [11] and *mollis* – referring to the soft, silky hairs on the leaves [83]. *Commiphora mollis* is a tree that is not spiny and the bark does not peel. The bark differs in structural appearance; it may be wrinkled, smooth, or fragmented and is silvery when burnt by the sun. The trunks are sometimes knobbly or angular. The young branchlets are sparsely pilose to densely pubescent. The leaves are compound, pinnate, with 3 – 7 leaflets present. They are greyish-green dorsally and a paler green ventrally, and are densely covered or scattered with velvety hairs. The flowers are small and are found in groups on long, red slender stalks [83].

2.4. Uses of Commiphora Species in Medicine

Commiphora species are acknowledged as one of the oldest house-hold remedies and its traditional use is now supported by several scientific studies. Besides the use of the resinous exudates in incense and perfumes, they have been traditionally used for the treatment of various disease symptoms. Members of the genus have been traded extensively in ancient Egypt, Greece, India, and China [80]. The resinous exudates of the *Commiphora* species, known as 'myrrh', are used in traditional Chinese medicine for the treatment of trauma, arthritis, fractures, and diseases caused by blood stagnation. Myrrh has also been used in the Ayurvedic medical system because of its therapeutic effects against inflammatory diseases, coronary artery diseases, gynecological disease, obesity, etc. The recognition of the therapeutic and medicinal value of myrrh (known as *guggul* in India, the resinous exudates of *C. mukul*) in Ayurvedic medical system dates from 3000 years ago. Guggul is regarded as the most important herb for the treatment of obesity, stomach disorder, and is used as a hypolipidemic agent to treat lipid disorder. Moreover, in Greece, *C. myrrh* has been reported to be used in treatments of wounds, worms, and sepsis, cough, and snakebite, infections in mouth, teeth, and eyes. In eastern Africa gum resin of Commiphora species have been used for

the treatment of microbial infection and to kill and repel tick pest populations on camel and cattle [16].

2.5. Pharmacological Activities and Phytochemistry of Commiphora Species

Plant-derived substances have recently become of great interest owing to their importance and broad applications. Recently, different studies have revealed the medicinal importance of plants from Commiphora species by application of various assays. Accordingly, they have been studied for their anti-oxidative, antimicrobials, anticancer, analgesic, and anti-inflammatory activities. Table 1 shows the reported pharmacological activities of some Commiphora species. Different parts including leaf, stem (stem bark), root, and a resinous exudates of these plants have been studied.

Plants tend to synthesize secondary metabolites that constitute alkaloids, phenolic compounds, terpenoids, and the others. In many cases, these substances serve as plant defense mechanisms against predation by higher animals, infestation by microorganisms and insects [61]. They are also studied for their different activities in human health which emanates from their chemical constituents. Previous studies on plants from genus *Commiphora* have proven the presence of terpenoids (monoterpenes, diterpenes, and sesquiterpenes) [17–19] and phenolics [12, 21, 22, 24, 25] for the majority of the species, and alkaloids. Moreover, essential oil, which contains complex mixtures of volatile organic compounds has been extracted from the leaf, stem bark and oleo gum-resin exudate of many of the species.

Table 1 shows the reported pharmacological activities of some Commiphora species. Different parts including leaf, stem (stem bark), root, and a resinous exudates of these plants have been studied.

Table 1. Pharmacological activities of different Commiphora species

Species	**Studied plant parts**	**Pharmacological Activity**	**Ref.**
C. mollis, C. tenuipetiolata, C. neglecta, C. schimperi, C. marlothii, C. glandulosa, C. viminea, C. pyracanthoides	Leaf and stem Bark extracts	Antioxidant, anti-inflammatory, anticancer, antimicrobial	[13]
C. mollis	Leaf extract	Analgesic and Anti-Inflammatory	[12]
C. Africana	Leaf and stem bark	Antioxidative, anti-inflammatory	[23]
C. myrrha, C. confusa, C. baluensis, C. pseudopaolii, C. guidotti, C. holtziana and C. kataf	Oleo-gum resins	Antimicrobial	[80]
C. mukul	Gum resin	Antihyperglycemic and antioxidant	[84]
C. wightii	Guggul exudates	Antioxidant	[85]
C. erythraea	Resin extracts and essential oil	Anti-inflammatory, antioxidant and antifungal	[18]
C. myrrh	Resin	Antioxidant and antimicrobial	[19]

2.6. Antioxidant Activity of Genus *Commiphora*

The study on the leaf, stem bark, and resins of Commiphora species for antioxidant activity while being accounted for the presence of certain metabolites have been reported. The essential oil of *C. myrrha* was reported to exhibit potent singlet oxygen quenching activity better than the control α-tocopherol. This effect was attributed to the reaction between the furan ring of *C. myrrha* constituents (particularly the furanosesquiterpenoids) and singlet oxygen [86]. Fraternale et al., [18] reported furanosesquiterpenoids from *C. myrrha* showed DPPH radical scavenging activity with EC_{50}values of 1.08, 4.29, and 2.56 mg/mL. Moreover, the stem extracts of *C. tenuipetiolata*, *C. neglecta* and *C. mollis* showed antioxidant activity in ABTS assay with IC_{50} values of 5.10, 7.28 and 8.82 μg/mL [13] while the stem extract of *C. schimperi, C. neglecta, C. tenuipetiolata, C. edulis, C. berryi and C. caudate* exhibited antioxidant effect in the DPPH assay, with IC_{50} values between 7.31 and 26.92 μg/mL. The antioxidant potential of methanol, ethyl acetate crude extracts and essential oil of *C. myrrh* resin was studied in DPPH radical scavenging and Fe^{2+} chelating assays. In both assays, the methanolic extract exhibited the highest activity compared to ethyl acetate extract and essential oil. In this study the major constituents of the essential oil identified from the resin of *C. myrrh* were α-elemene (12.86%), 7-isopropyl-1,4-dimethyl-2-azulenol (12.22%), curzerene (11.64%), and germacra-1(10)7,11-trien-15-oicacid,8,12-epoxy-6-hydroxy-c ,-lactone (6.20%). The authors demonstrated that the higher ferric reducing power of essential oil was due to the presence of hydrogen atom donating monoterpenoids and sesquiterpenoids [19].

2.7. Analytical Methods for Antioxidant Activity of Plant Extracts

Antioxidant activity is one of the important indexes for estimating the medicinal value of plants. Various assay techniques for antioxidant activity are used in the literature. Analysis of the antioxidant capacity of plant extracts has been made in correlation to the total concentration of certain metabolites such as phenolic compounds [27], flavonoids [87–91], and terpenoids [17, 18, 41, 92]. These natural compounds can exert the antioxidant action through quenching of singlet oxygen, transfer of hydrogen, or transfer of an electron [63] These may result in the formation of complex compounds, another free radical or free radical cation. The formed free radical can be terminated by reaction with other free radicals or release of thermal energy. Referring to their

chemical reactions or reaction mechanisms, antioxidant capacity can be studied through two main mechanisms by which antioxidants can play their protective role. These are Hydrogen Atom Transfer (HAT) based assay and Single Electron Transfer (SET) based assay. The potential and kinetics for side reactions vary while the result for both reactions is the same. Moreover, information about the antioxidant efficiency of the antioxidant compound can be obtained from two important parameters; the bond dissociation energy (BDE) and the ionization potential (IP). This can be varied with the structure of antioxidant as the presence and number of hydroxyl groups, and double bond conjugation and resonance effects are the reason behind the antioxidant activity of phenolics [93].

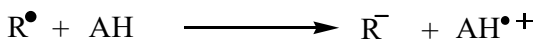
Hydrogen Atom Transfer (HAT)-based methods: measure the ability of an antioxidant to suppress free radicals by transfer of hydrogen atom to produce sTable compounds (**Equation 10**)

$$R^{\bullet} + AH \longrightarrow RH + A^{\bullet} \qquad (10)$$

In this mechanism, the free radical removes a hydrogen atom from the antioxidant (AH) that itself becomes a radical. The HAT-based procedures consist of an antioxidant, a synthetic generator for free radicals, and an oxidizable molecular probe. Mostly, the HAT-based assays employ a competitive scheme, through which both substrate and the antioxidant content for peroxyl radicals that are thermally produced through azo-compounds decomposition. HAT reactions do not depend on pH and solvent and generally accomplished in a duration of seconds to minutes [93]. Moreover, an important parameter here is the bond dissociation energy (BDE) of the A-H bonds; the weaker the A-H bond the easier will be the reaction of free radical inactivation [72]. The following assays are based on Hydrogen Atom Transfer (HAT):

1 ORAC (Oxygen Radical Absorbance Capacity)
2 TRAP (Total Radical Trapping Antioxidant Parameter)
3 DPPH (Diphenylpicrylhydrazyl)
4 IOU (Inhibited Oxygen Uptake)
5 Linoleic oxidation inhibition.
6 Low-density lipoprotein (LDL) oxidation inhibition

Single-electron transfer (SET) based methods: these methods detect the ability of a potent antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals. In this mechanism, the antioxidant can give an electron to the free radical becoming itself a radical cation [63, 93].



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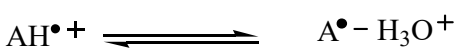

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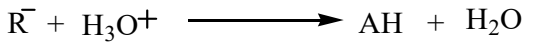

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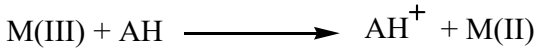

(14)

The SET-based assay procedures comprise of one redox reaction with the oxidant as a probe for reaction monitoring as the reaction endpoint identifier. These assays analyses antioxidant ability to hold an oxidant that alters color upon reduction. The intensity of the color is interrelated with the concentration of the antioxidant present in the sample [93]. Besides, the lower the ionization potential (IP), the easier is the electron abstraction [72].

In the methods based on this mechanism, reactions depend on pH, slower, and need a longer time to be completed. In contrast to the HAT, SET relies on the solvent, can produce new antioxidants using phenolic compounds polymerization, and could undervalue the antioxidant potential when reactions are not approaching completion. SET reaction mechanisms are involved in different assays, among few are ABTS (2,2`-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), FRAP (Ferric Ion Reducing Antioxidant Parameter), DPPH (Diphenylpicrylhydrazyl), Copper (II) reduction capacity, TPC (Total Phenol Content, using Folin-Ciocalteu reagent).

It should be pointed out that the single electron transfer and hydrogen atom transfer could take place simultaneously. On top of this, the dominating mechanism in the system can be identified by the antioxidant properties [93]. The most commonly used assays are presented below

Total Phenolic Content (TPC): The Folin-Ciocalteu (F-C) method is based on a single electron transfer mechanism and is used to quantify the contents in total phenolic compounds in plant

extracts using gallic acid as a standard. The F–C method provides the reducing capacity of a sample, which has normally been expressed in terms of phenolic content. Since its mechanism is an oxidation/ reduction reaction, the F-C method can be considered also a method for quantification of the antioxidant capacity. The F-C method involves the reduction of the molybdenum component in the phosphotungstic-phosphomolybdic complexing reagent according to the following reaction [35, 94]:

$$Mo^{6+}(\text{yellow}) + ArOH \longrightarrow Mo^{5+} + [ArOH]^{\bullet +} \qquad (\mathbf{15})$$

Numerous reducing compounds could interfere in the quantification of polyphenols by the F-C method, vitamin C is supposed to have the major contribution, but other reducing substances such as some sugars and amino acids could also interfere. Consequently, vitamin C quantification should be concomitantly performed in this method to get appropriate values for polyphenol contents [95].

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging method: DPPH is a very sTable free radical. Unlike *in vitro* generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition. A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. This purple color generally fades when antioxidant molecules quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into a colorless/bleached product (i.e. 2,2-diphenyl-1-hydrazine, or substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm band [27, 31].

3. MATERIALS AND METHODS

3.1. Sample Collection

The resin of *C. mollis* was collected from *Gorile,* in *Das district,* Borana Zone, Oromia Region State, Ethiopia in February 2020. The collected sample was air-dried, manually cleaned, and double-sealed with a plastic bag and transported to Analytical Chemistry Postgraduate Laboratory, Jimma University, Ethiopia, for further treatment and analysis.

3.2. Chemicals and Instruments

Solvents (petroleum ether, chloroform, and methanol) were purchased from Loba Chemie, India. The 2, 2-diphenyl-1-picrylhydrazyl, (85%, Alpha Chemika), Folin-Ciocalteau's Phenol (FCP) Reagent (2 N, Sisco), sodium carbonate anhydrous, (99.5%, *Blulu*x), L-Ascorbic acid (99%, *NICE*), and gallic acid (99.5%, Indiamart) were the major chemicals used during the experimental work. Double-beam UV/Vis spectrophotometer (SPECORD 200 PLUS, Analytik jena, Germany) was used for quantitative measurements.

3.3. Preparation of Resin Extracts

The pulverized resin (15 g) was extracted with petroleum ether, chloroform, and methanol, 200 mL each. The extractions were performed by shaking the mixed samples for 15 min and kept it at room temperature for 24 h. The extracts were filtered through Whatman No. 1 filter paper and the residue was re-extracted with an equal volume of the same solvent in the same way. Finally, the filtrates were combined and evaporated using a rotary evaporator at 40 ^{o}C. The obtained extracts were allowed to dry at room temperature and the yield of each extract was calculated and reported as a percent of dry extract per weight of the dry sample [91].

3.4. Qualitative Analysis

Crude extracts were qualitatively evaluated for the presence of alkaloids, flavonoids, phenolics, saponins, and terpenoids. The screening tests were carried out using standard qualitative procedures as described in the following sections

3.4.1. Detection of Alkaloids (Wagner's test)

2 g KI and 1.27 g I_2were dissolved in 100 mL distilled water and 0.1 g of the solvent-free extract was dissolved in a few drops of this prepared reagent. The formation of a brown colored precipitate indicates the presence of alkaloids [96].

3.4.2. Detection of Flavonoids (Alkaline reagent test)

0.2 g of extract was treated with six drops of 2% sodium hydroxide solution. The formation of intense yellow color, which develops into a colorless solution on the addition of dilute HCl is an indication of the presence of flavonoids in the extracts [96].

3.4.3. Detection of Phenolics (Folin-Ciocalteu Test)

A solvent-free extract, 0.1 g, was dissolved in 3 mL of methanol and then 0.5 mL of the resulting solution was mixed with 2 mL of F-C reagent (10%) and vortexed for 15 s. After 5 min, 4 mL of Na_2CO_3 (7.5%) was added and allowed to stand for 30 min at room temperature for color development. The appearance of blue color indicates the presence of phenols [97].

3.4.4. Detection of Terpenoids (Salkowski test)

The crude extract (0.1 g) was taken in a test tube and dissolved in 0.5 mL chloroform and 1 mL of concentrated H_2SO_4 was added to the content along the side of the test tube. The formation of a reddish-brown precipitate indicates the presence of terpenoids in the extracts [96].

3.4.5. Detection of Saponins (Foam test)

0.2 g of the extract was dissolved in 6 mL of distilled water in a graduated cylinder. The resulting solution was vigorously shaken for 15 min. The formation of bubbles that persist for 10 min or more indicated the presence of saponins [96].

3.4.6. Qualitative test of antioxidant activity

The antioxidant activity of the extracts was carried out using standard procedure [97]. Briefly, 1000 µg/mL of the crude extracts and 40 µg/mL DPPH were separately prepared in methanol. Then, equal volumes (1 mL) of each of the prepared solutions were mixed and incubated in a dark region for 30 min. After the incubation period, the observed color change from purple to yellow and purple to pale pink indicated strong and weak positive antioxidant activities, respectively. Finally, the extracts having antioxidant activity were subjected to further quantitative analysis [97].

3.5. Analysis of Total Phenolic Contents (TPC)

A colorimetric assay was used for the estimation of total phenolic content and other oxidation substrates in plant tissues, as described by Ainsworth and Gillespie [33] and S. Adusei et al. [98], with slight modifications. The method employed Folin-Ciocalteu reagent (F-CR), a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$), Na_2CO_3, and gallic acid [33, 34]. Methanol (95% v/v) was used to dissolve standard and extracts.

3.5.1. Preparation of Samples and Standard Solutions

A 0.05 g of each dry extract was separately placed in 15 mL centrifuge tubes, and 10 mL of methanol was added. Then, the resulting mixture was sonicated to dissolve the samples. The obtained solution was filtered using Whatman filter paper into a 50 mL volumetric flask. Then, the volumetric flask was filled to the mark with methanol (95% v/v) to obtain a stock solution containing 1000 µg/mL.

Gallic acid stock solution: A stock solution, 1000 µg/mL, was prepared by dissolving 0.1 g gallic acid in 100 mL methanol (95 % v/v).

Sodium carbonate: 7.5% (w/v) Na_2CO_3 was prepared in a 500 mL volumetric flask by dissolving 37.5 g of the salt in a half-filled flask with distilled water. After complete dissolution of the salt, the flask was filled to the mark with distilled water.

Note: *All the prepared stock solutions were stored in the refrigerator at 4 ^{0}C until use.*

Folin-Ciocalteu reagent was freshly prepared on daily basis by dilution of 2 N stock solution with distilled water (1:10).

3.5.2. General Procedure of the Folin-Ciocalteu Assay

0.5 mL standard sample or blank was added to 2 mL of Folin-Ciocalteu reagent (10%) and mixed using a vortex for few seconds. Then, after 3 min, but before 8 min, 4 mL of Na_2CO_3 (7.5%, w/v) was added. Finally, absorbance was measured at 765 after 30 min of incubation at room temperature [98].

3.5.3. Pre-analytical Optimization

For the optimization of the Folin-Ciocalteu redox method, the maximum absorption wavelength, and reaction time were studied at room temperature. To obtain the maximum absorption wavelength 500 µg/mL of the sample and 50µg/mL standard were scanned over the wavelength range from 400 to 900 nm, after 30 min of addition of the last reagent, i.e Na_2CO_3, described in the general procedure of the Folin-Ciocalteu assay. The spectral data were used to choose the maximums and for evaluation of the samples' dilutions [46]. The variation in absorbance values were tested at the local maximums. Then, calibration curves were construed at local maximums (745, 750, 760, 765 nm) using absorbances of calibration solution containing 0, 25, 50, 75, 100, 125 and 150 µg/mL gallic acid. The slopes and intercepts were compared using one-way analysis of variance.

The reaction time was studied from the absorbance *versus* reaction times for the samples and standard. The measurements were conducted after 5 min from addition of the last reagent, for 1 h at 5 min intervals [46].

3.5.4. Determination of Total Phenolic Content (TPC)

Determination of TPC was carried out at the previously determined wavelengths (765 nm) and contact time (30 - 40 min) between Folin-Ciocalteu reagent and extracts/standard. Firstly, calibration standards containing 0, 25, 50, 75, 100, 125 and 150 µg/mL of gallic acid were

prepared. Each extract (1000 µg/mL) was used for measurements. Following the procedure, standards, samples, or blank (95%, v/v methanol) were mixed with F-CR and Na_2CO_3. The reaction mixture was incubated at room temperature for 30 min for color development. Then, absorbances of the calibration standards and samples were measured at 765 nm using a spectrophotometer. The calibration curve was construed as absorbance versus the concentration of the standard solution. The validity of the results and the method was determined employing coefficient of determination (R^2), limit of detection (LOD) and limit of quantitation (LOQ) calculated from the calibration curve. Finally, the TPC of the extract (1000 µg/mL) was calculated using the following formula and reported as milligram of gallic acid equivalent per gram of dry extract [99].

$$C = C_1 \text{ x } V/m$$

Where C is total phenolic content in mg/g, in GAE (gallic acid equivalent), C_1 is the concentration of gallic acid established from the calibration curve in mg/mL, V is a volume of extract in mL, and m is the weight of the plant extract in g.

3.6. Determination of Total Terpenoid Content (TTC)

The total terpenoid content of different extracts of *C. mollis* resin was determined using the method described by Truong et al. [100] with slight modification. The dried plant extract, 100 mg was soaked in 9 mL of ethanol for 24 h. The extract was filtered and extracted with 10 mL of petroleum ether using a separating funnel. The ethanol part was again re-extracted with 10 mL of petroleum ether. The ether extracts were combined in a pre-weighed container and waited for its complete drying and the yield (%) of total terpenoids contents was calculated by the following formula.

$$\text{TTC}(\%) = \left[\frac{\text{initial weight of dry extract - weight of petroleum ether extract}}{\text{initial weight of the dry extract}} \right] \times 100$$

3.7. Antioxidant Activity

The *in vitro* antioxidant activity of the extracts was determined by a spectrophotometric method based on the reduction of a methanol solution of 2,2-diphenyl-2-picrylhydrazyl (DPPH)[101].

3.7.1. Preparation of Samples and DPPH Solutions

A stock solution, 400 µg/mL of DPPH, was prepared by dissolving 0.01 g of DPPH in 25 mL methanol. The solution was kept at room temperature for 60 min for its stabilization and then kept in the refrigerator at 4 ^{0}C when not used. 0.05 g of each extract were dissolved in 50 mL of methanol to prepare sample stock solutions (1000 µg/mL).

3.7.2. Pre-analytical Optimization

The wavelength of maximum absorbance and time for completion of the reaction between DPPH and the antioxidants were studied. Firstly, DPPH standard solutions of 5, 10, 15, 20, 25, and 30 µg/mL were prepared. These solutions were scanned in a spectrophotometer over a wavelength range of 400 - 600 nm against methanol (blank). The same standards were used to prepare the calibration curve.

To determine DPPH radical scavenging activity at a steady-state reaction time for the reaction between DPPH and the antioxidants available in the extract as well as ascorbic acid, 1 mL of ascorbic acid (200 µg/mL) and extract (1000 µg/mL) was mixed with DPPH(40 µg/mL) and transferred to a quartz cuvette. Then, the absorbance of the solution was measured at the maximum wavelength in 3 min intervals for the first 10 min and subsequently, at 5 min intervals until the difference between two subsequent absorbance readings is ≤ 0.001. Then, the concentration of DPPH before and after reaction with the samples at instants was calculated using a calibration curve. Finally, the remaining percent of DPPH was calculated from the following equation.

$$\%[\mathrm{DPPH}]_R = \frac{[\mathrm{DPPH}]_{t=T}}{[\mathrm{DPPH}]_{t=0}} \times 100$$

Where %[DPPH]$_R$ represents the percent of remaining DPPH concentration, [DPPH]$_{t=T}$ was the concentration of DPPH at time T, and [DPPH]t=0 was the initial concentration of DPPH [102].

A kinetic study was carried out by fitting the calculated values of %[DPPH]$_R$ against the time using the Levenberg–Marquardt method [103] implemented in Graph pad prism v 8.0.2 software for Windows, choosing the preferable exponential model.

3.7.3. DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of extracts was assessed at a previously measured λ_{max} and the steady-state reaction time. Solutions containing 500, 400, 300, 200, and 100 µg/mL of the extract were prepared from the stock solution. 1 mL of each of these solutions mixed with 1 mL of 40 µg/mL DPPH. The mixture was then incubated in the dark for 1 h at room temperature. Finally, the absorbance was recorded at 517 nm. The concentration of DPPH at steady state was calculated from the calibration curve and the percent of remaining DPPH concentration was calculated as

$$\%[\mathrm{DPPH}]_R = \frac{[\mathrm{DPPH}]_f}{[\mathrm{DPPH}]_i} \times 100$$

Where % [DPPH]$_R$ represents the percent of remaining DPPH concentration, [DPPH]$_i$ was the initial concentration of DPPH, and [DPPH]$_f$ was the concentration of DPPH at the steady-state. The IC_{50} values were estimated from nonlinear regression by plotting %[DPPH]$_R$ versus concentration of test sample [101].

3.8. Statistical Analysis

All the experiments and measurements were conducted at least in triplicates and the final results were reported as mean ± SD. Analysis of variance (ANOVA) with Tukey's multiple comparison test was performed to compare the obtained results. The correlation analysis for antioxidant activity with total phenolic and terpenoid contents was evaluated using Pearson's correlation

coefficients. The values were considered significant at $P < 0.05$. Graph Pad prism v 8.0.2 and Microsoft Excel was used for statistical calculations.

4. RESULTS AND DISCUSSION

4.1. Extraction Yield

Investigation of phytoconstituents and pharmacological activities of plant passes through different steps of sample preparation including size reduction, homogenization, and extraction [104]. Extraction is the first crucial step to isolate natural compounds and extraction efficiency affects analysis results for pharmacological activity and phytoconstituents [105, 106]. The extraction yield of the conventional solvent extraction method depends on the pH, temperature, extraction time, and composition of the sample. Under the same extraction time and temperature, solvent and composition of a sample are the most important parameters [104]. In the present work, methanol, chloroform, and petroleum ether were studied for their effects on the extraction yield of *C. mollis* resin. The result showed a significant difference in the extraction yield using different solvents.

Table 2. The extraction yields of the solid residue obtained from triplicate extraction of resin.

Weight of dry resin (g)	Extraction solvents	%Yield per gram of dry resin
15	Methanol	53.00 ± 1.39
15	Chloroform	46.56 ± 0.42
15	Petroleum ether	27.46 ± 0.48

As can be seen from **Table 2**, methanol resulted in the highest extraction yield, followed by chloroform, and petroleum ether. The yield of extracTable solids increased with the polarity of the extraction solvent. Furthermore, methanol and chloroform gave reddish-yellow extracts and a small amount of whitish insoluble materials. Petroleum ether, however, gave a golden extract. According to literatures, myrrh is an oleo-gum resin obtained from *Commiphora* species which dissolve in alcohol, chloroform and ether. When extracted with alcohols, it yields not less than 30% alcohol-soluble matter and whitish mass of gum and impurities remains [14]

4.2. Qualitative Analysis

The qualitative analysis carried out on methanol, chloroform and petroleum ether extracts of *C. mollis* resin showed the presence of phytochemicals including alkaloids, phenols, terpenoids, and saponins in at least one of the extracts while flavonoids were absent in all the extracts (**Table 3).** Alkaloids were detected in methanol and chloroform extracts but not in petroleum ether extract. Alkaloids are important antioxidant, anticancer [107], and insecticide [108] agents. Phenols and terpenoids were detected in all of the three extracts while saponins were absent in methanol extract. The study on methanol leaf extracts of *C. mollis* [12] revealed the presence of terpenoids, saponins, and flavonoids.

Table 3. Qualitative phytochemical analysis of *C. mollis* resin extracts

Phytochemicals	**Tests**	**Extracts**		
		Methanol	***Chloroform***	***Petroleum ether***
Alkaloids	Wagner's test	+	++	-
Flavonoids	Sodium hydroxide test	-	-	-
Terpenoids	Salkowski test	++	++	++
Saponins	Foam test	-	++	+
Phenols	Folin-Ciocalteu's test	++	++	++
Antioxidant	DPPH test	++	++	++

++, strong presence; +, presence; -, absence

In previous studies, extracts of resin of other *Commiphora* species showed the presence of phenols [22, 25], and terpenoids[17–19]. Qualitative antioxidant test with DPPH and Folin-Ciocalteu reagent displayed positive results in all extracts.

4.3. Total phenolic contents (TPC)

4.3.1. Pre-analytical optimization

The Folin-Ciocalteu method is an electron transfer (ET) based assay and gives reducing capacity, which has normally been expressed as phenolic contents [109]. The assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid ($H_3PW_{12}O_{40}/H_3PMo_{12}O_{40}$) complexes to form blue complexes (possibly $(PMoW_{11}O_{40})^{4-}$) that are determined spectroscopically at approximately 765 nm [33, 34]. The Folin-Ciocalteu method provides an aggregate analysis of phenols using a single standard which is often more informative than reams of data difficult to summarize from various techniques, such as high-performance liquid chromatography (HPLC) that separate a large number of individual compounds[35]. Furthermore, it is fast, precise, reproducible, easy to perform, and does not require expensive reagents or sophisticated instrumental devices [32–34].

The original Folin-Ciocalteu method was modified to minimize variability in experimental conditions including optimal reaction time, stated in 2 h [110]. Over the past years, it has been subjected to many modifications[109] and different markers have been employed. For example, the absorbance of the sample was measured at 760 nm, after 1 h of dark incubation [23], at 765 nm, after 30 min with intermittent shaking [98]; at the same wavelength, after 2 h reaction at room temperature [111]; at 750 nm, after 15 min at room temperature [112]. Most of the authors did not explain why they selected those wavelengths or reaction time except a few [32, 36, 113, 114]. Thus, the selection of a particular method asks for further optimization or validation to ensure the reliability of the results.

In this work, maximum wavelength and time were optimized for the determination of TPC from the resin of *C. mollis.* The shifting of the position of λ_{max} can compromise the experimental responses by underestimating the quantitative data [36]**. Figure 8,** showed the absorption spectra for products of the reaction of F-CR with gallic acid and the extracts. The maximum absorption wavelength lies between 700 and 800 nm around 750 nm for both standard and samples.

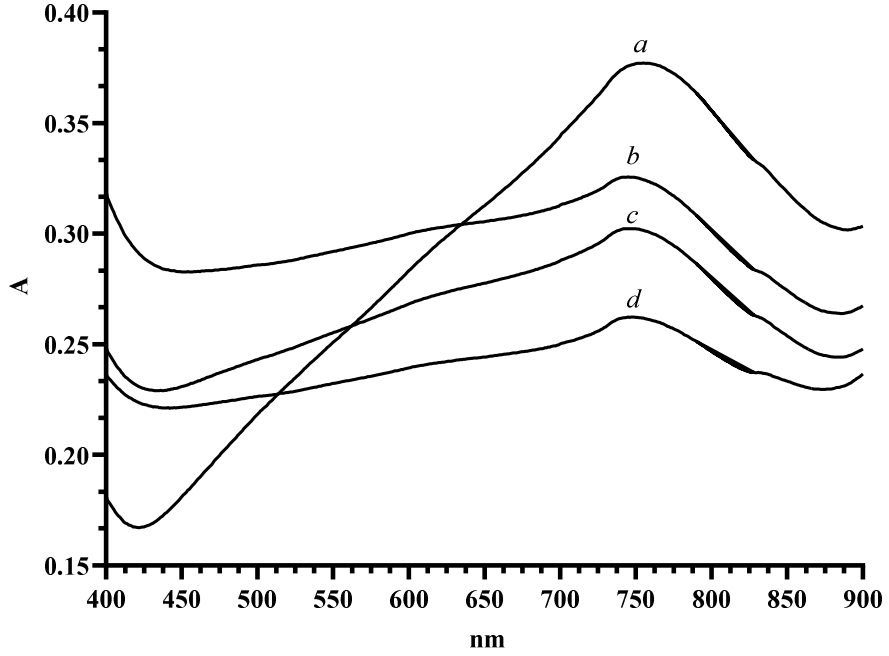

Figure 8. Absorption spectra (400 nm to 900 nm) of 50 µg/mL gallic acid (*a*), methanol (*b*), chloroform (*c*), and (*d*) petroleum extracts of *C. mollis* exudate (500 µg/mL) after reaction with F-CR for 30 min.

The variation in absorbance data was evaluated at 745, 750, 755, 760 and 765 nm. The statistical analysis showed absorbances that were not significantly different in all cases. i.e., the calculated F-values were less than the critical value ($F_{critical} = 3.478$) and the calculated P-values were greater than 0.05 (gallic acid, $F_{4,10} = 0.485$, $P = 0.747$; methanol extract, $F_{4,10} = 1.871$, $P = 0.192$; chloroform extract, $F_{4,10} = 3.063$, $P = 0.069$; petroleum extract, $F_{4,10} = 1.980$, $P = 0.174$). Thus, further study on the gallic acid calibration curve was considered to select one of the wavelengths for analysis of TPC.

Table 4 summarizes a comparison of regression parameters. Linear regression is one of the most widely used mathematical techniques to compare two data strings. For good reliability between two strings, R^2 must be more than 0.995 [115]. By plotting calibration curves, the obtained R^2 value at the investigated wavelengths is relatively better at 760 and 765 nm (0.9979). The other calibration curves also respects the condition of $R^2 > 0.995$ ($R^2 = 0.9977$).

Table 4. Comparison of linear regressions on working standard solutions of gallic acid (0, 25, 50, 75, 100, 125, 150) at wavelengths of 745, 750, 760, and 765 nm ($P < 0.05$)

		Regression Parameters				
		R^2	**Slope**	**σ_{slope}**	**y-intercept**	**$\sigma_{intercept}$**
Regression at λ (nm)	745	0.9977	0.0077	0.0002	0.0582	0.0151
	750	0.9977	0.0077	0.0002	0.0594	0.0148
	760	0.9979	0.0076	0.0002	0.0612	0.0142
	765	0.9979	0.0075	0.0002	0.0620	0.0139
ANOVA	-	-	$F_{3, 20} = 0.355$, P = 0.786		$F_{3, 20} = 0.0139$, P = 0.998	
	$F_{critical} = 3.098$					

R^2, Coefficient of determination; σ, standard error; ANOVA, Analysis of variance

The comparison of the regression curves and intercepts can clarify if the calibration curves are parallel. The statistical analysis (**Table 4**) showed no significant differences between either of the slopes and intercepts. Measurement of absorbance at 765 nm was mentioned by different protocols [114, 33, 34] and therefore, it was chosen for analysis of TPC.

The second step was the determination of time for completion of the reaction of F-CR with gallic acid and the extracts. The plot of absorbance versus time (**Figure 9**) showed that absorbance for standard and the extracts increased from the start up to 30 min of contact with the reagent. However, it did not decrease between 30 and 40 min. The absorbance values were then decreased after 45 min were passed. The F-C method measures the intensity of the blue color (absorbance) the development and stability of which is influenced by conditions such as temperature and alkali level. Higher temperature and alkali levels speed color development and its fading [33, 35]. Thus, one may obtain a reliable result by monitoring the kinetics of the reaction between reagent and substrate.

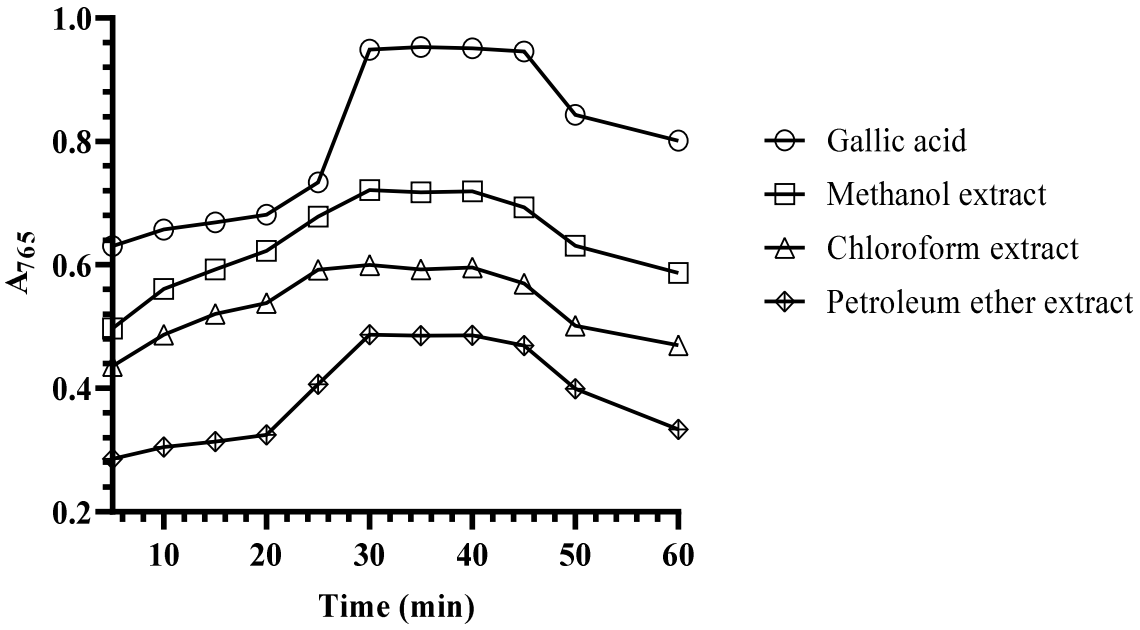

Figure 9. A Plot of Absorbance versus time for 100 µg/mL gallic acid and 500 µg/mL crude extracts of *C. mollis* exudate in response to incubation time at 765 nm.

The statistical analysis indicated no significant difference at 30-40 min (gallic acid, $F_{2,6} = 0.186$, $P = 0.835$; methanol extract, $F_{2,6} = 0.242$, $P = 0.793$; chloroform extract, $F_{2,6} = 1.404$, $P = 0.316$; petroleum extract, $F_{2,6} = 0.108$, $P = 0.899$). These results suggested a period of 30 - 40 min for the spectrophotometric measurement of gallic acid and the extracts. A number of validation studies of F-C method, for determination of TPC from plant extracts justified 30 min of incubation for absorbance measurement [32, 36, 114]

4.3.2. Determination of Total Phenolic Contents

The linear calibration curve was carefully prepared from gallic acid calibration standards (0-150 µg/mL) (**Figure 10**). The high coefficient of determination ($R^2 = 0.999$) shows that 99.91 % of the variation of absorbance can be explained by variation in the concentration of the standard solutions confirming the linearity of the method. The LOD and LOQ were 3.45 and 10.47µg/mL, respectively.

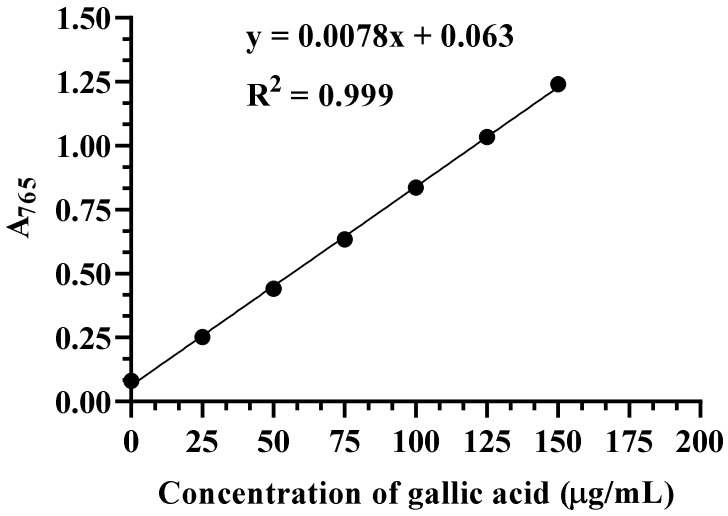

Figure 10. Gallic acid calibration curve

The TPC of resin of *C. mollis* extracts is expressed as a milligram of gallic acid equivalent per gram of dry extract (mg GAE/g) (**Table 5**). Among the three extracts, the highest concentration was measured in methanol extracts (112.24 ± 1.67 mg GAE/g) while a significant amount was obtained from both chloroform (71.50 ± 1.33 mg/g), and petroleum ether (56.05 ±1.33 mgGAE/g) extracts. The extraction of phenolic contents from different samples is influenced by the polarity of extraction solvents and solubility of phenolic compounds in the solvents [104]. The TPC of the extracts increased with increasing polarity of extraction solvents showing a strong positive The highest phenolic content in methanol extract results from the higher solubility of these compounds in methanol than the other solvents [100, 104]. However, the phytochemical screening of the extracts did not show the presence of flavonoids. This indicated these phenolic contents could be other classes of compounds containing phenols.

The result of the analysis was compared with the values reported for plants from the same genus. As can be seen from **Table 5**, *C. mollis* resin extracts showed higher TPC than the reported values except *C. africana* leaves extracts.

Table 5. TPC of *C. mollis* resin compared to the values obtained for other *Commiphora* species.

Plant	Analyzed part	Extracts	TPC	Reference
C. africana	Leaves	HF	5.86 ± 0.64	[23]
		DCMF	150.51 ± 2.40	
		ACNF	819.39 ± 10.56	
		EAF	719.45 ± 2.78	
		MEF	260.73 ± 4.50	
		BUF	424.90 ± 12.55	
C. leptophloeos	Bark	AE	33.64 ± 0.5	[24]
		MEE	20.3 ± 0.78	
		CLE	12.54 ± 0.55	
		CHE	13.8 ± 0.53	
		EAE	13.7 ± 0.04	
C. molmol	Oleo-gum resin	EO	3.3[a]	[116]
		EE	3.9[a]	
C. mukul	Leaves	MEE	83.61 ± 1.82	[117]
C. mollis	Resin	MEE	112.24 ± 1.67	Current study
		CLE	71.50 ± 1.34	
		PEE	56.05 ± 1.33	

TPC, Total phenolic content; **HF**, n-Hexane fraction; **DCMF**, Dichloromethane fraction; **ACNF**, Acetonitrile fraction; **EAF**, Ethyl acetate fraction; **MEF**, Methanol fraction; **BUF**, n-Butanol fraction; **AE**, Aqueous extract; **MEE**, Methanol extract; **CLE**, Chloroform extract; **CHE**, Cyclohexane extract; **EAE**, Ethyl acetate extract; **EO**, Essential oil; **EE**, Ethanol extract; **PEE**, Petroleum ether extract; **[a]**, these values were expressed as % gallic acid/dry extract and others were expressed as mg gallic acid equivalent/gram (mgGAE/g)

The determination of plants is strongly supported due to the vital role of polyphenols in health. The beneficial effects of phenolic contents measured by the F-C assay in human biological samples were demonstrated. For example, a decrease in cardiovascular risk parameters such as blood pressure, decrease in DNA oxidation, and reduced fragility, halting a decline in physical performance was reported [118]. The TPC of these *Commiphora* species were correlated with anti-inflammatory, antioxidant [23], and antimicrobial [24] activities.

4.4. Total Terpenoid Content (TTC)

The TTC was expressed as the percentage per gram of dry extract for different extracts of *C. mollis* resin. Among the three extracts, chloroform (TTC = 86.67 ± 3.82%) extracted the highest percentage of terpenoids followed by petroleum ether (TTC = 75.0 ± 2.50%). The lowest amount was measured in methanol extracts (TTC = 27.50 ± 2.50%). Terpenoids occur in aromatic plants as simple terpenes or in combination with other structures or functional groups; for example, as phenolic terpenes [119] or terpenoid-alkaloids [120] and thus, include polar and non-polar compounds. The high concentration of terpenoids in non-polar solvents means the resin of *C. mollis* contains a high percentage of non-polar terpenoids.

Terpenoids are useful in the prevention and therapy of several diseases, including cancer, and also to have antioxidant [41, 92, 121] antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, and anti-inflammatory properties [122]. In a previous study, furano-sesquiterpenoids isolated from *C. erythraea* resin showed anti-inflammatory, antioxidant, and antifungal activities[18]. Sesquiterpene alkaloids isolated from *Resina commiphora* exerted accepTable cytotoxicity toward normal cells and displayed high selectivity in cancer cells [123]. The furano-sesquiterpenoids isolated from *C. myrrh* exhibited promising DPPH radical scavenging activity [17, 19].

4.5. Antioxidant Activity

Oxidation is one of the most important processes that induce diseases [2] and food deterioration [44]. Antioxidants may play a protective role through ET or HAT to scavenge free radical species [50]. A DPPH assay measures a free radical scavenging activity of a test sample. DPPH is a stable nitrogen-centered free radical that exhibits a deep purple color and shows an absorption maximum at 517 nm. Its color changes from deep purple to yellow upon reduction and substances which can perform this reaction can be considered as radical scavengers [31]. The percent decrease in absorbance at 517 nm as a function of the concentration of the test sample in the mixture is employed to determine antioxidant capacity [29].

4.5.1. Pre-analytical optimization

The test is simple, rapid, linear, and precise with different antioxidants [27]. The measurement of absorbance at ambient temperature eliminates the risk of thermal degradation of the molecules in the tested sample [124]. The maximum peak of DPPH solution, 517 nm, can be confirmed by scanning solutions of DPPH with a UV-Vis spectrophotometer.

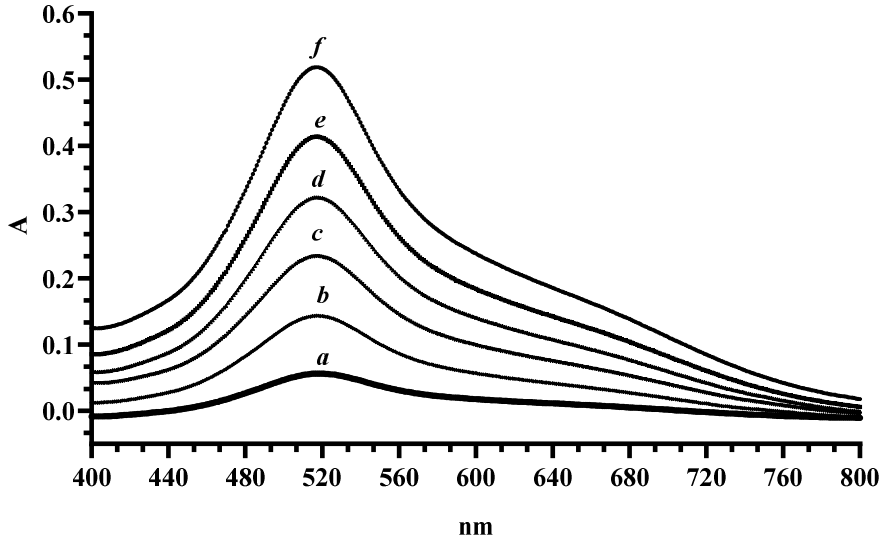

Figure 11. Absorption spectra (400 to 800 nm) of methanol solutions containing 5, 10, 15, 20, 25, and 30 μg/mL of DPPH, going bottom-up from ***a*** to ***f***.

The obtained absorption wavelength, 517 (**Figure 11**) is in agreement with the literature report [17, 23, 29, 125]. Within the range of 5 – 30 µg/mL, the concentration of DPPH solutions and the absorbance values showed a strong positive correlation ($r = 0.9999$, $P < 0.0001$) which is in analogy to Beer's Law.

The kinetic studies of the DPPH-extract reaction were carried out to estimate the scavenging activity at the steady-state reaction time. The initial concentration of DPPH just before mixing with sample or standard and the remaining ones at different time intervals were calculated using the calibration curve of DPPH ($y = 0178x – 0.00915$, $R^2 = 0.9998$). The remaining percent of DPPH was calculated taking into account the initial concentration. The percentage of the remaining concentration of DPPH against time was expressed using Levenberg–Marquardt exponential models [103]. Over the entire time of the experiment, the initial concentration of DPPH free radical in the control sample was measured and it remained constant (**Figure 12)**.

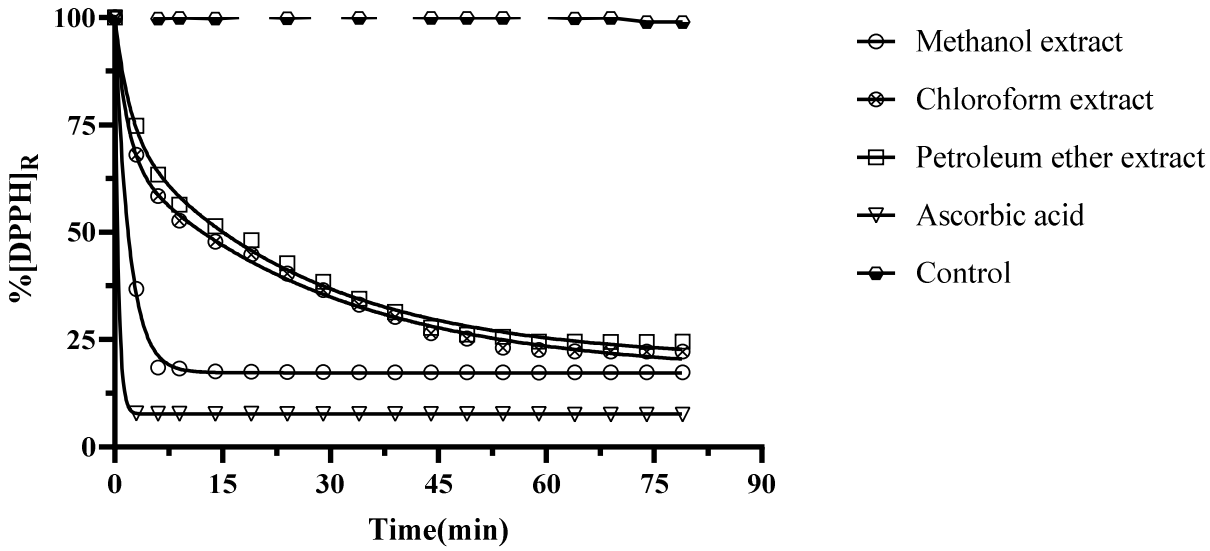

Figure 12. The percentage of remaining concentration of DPPH versus time for reaction of DPPH with ascorbic acid and crude extracts of C. mollis resin.

As it can be seen in **Figure 12**, the percent depletion of DPPH against time in ascorbic acid and methanol extract fitted one phase exponential model **(a)** while chloroform and petroleum ether extracts fitted biphasic exponential model **(b).** This could suggest a different free radical scavenging behavior. High determination coefficients (R^2) were obtained for all models (Ascorbic acid, 0.9999; methanol extract, 0.9985; Chloroform extract, 0.9971; petroleum ether extracts, 0.9961).

$$Y = y_0 + Ae^{-kt} \quad \text{(a)}$$

$$Y = y_0 + A_1e^{-k_1t_1} + A_2e^{-k_2t_2} \quad \text{(b)}$$

Where Y is the remaining percent of DPPH at any time, A is the amplitude (the difference between the initial and steady-state DPPH concentrations), y_0 represents the steady-state concentration of DPPH, k is rate constant and t is reaction time. The subscript 1 and 2 for A, k, and t in equation **(b)** shows fast and slow phases of the reaction.

The equilibration of the reaction between DPPH and antioxidant is influenced by the structure of occurring antioxidant which determines the dominant reaction mechanism that exhibits different reaction rates [31]. Ascorbic acid and methanol extract showed an extremely fast, one-phase depletion of DPPH concentration. Among plants' antioxidants, phenolic compounds display fast kinetic behavior as a result of fast abstraction of H-atom and/or ET reactions [126]. Similar behavior was observed for pure compounds such as gallic acid [127] and δ-tocopherol [101], and lemon juice extract [127]. Terpenoids, other group of compounds which is also constituted by *C. mollis* resin reacts via a fast HAT transfer process [55, 79]. Chloroform and petroleum ether extract reaction with DPPH resulted in a fast initial decrease followed by a slow subsequent disappearance of DPPH until the reaction reached the steady-state. In previous studies, the initial rapid depletion of DPPH was ascribed to a fast abstraction of H-atom while the followed slow decay could be the result of the reactions with the products of dimerization or with the products of the reaction of the antioxidants [126]. Pure compounds such as ferulic acid and eugenol [101, 124] and rosemary essential oil [127] displayed similar reaction behavior. For plant extracts, complex mixtures of antioxidants could lead to complex reaction mechanisms.

Table 6. Kinetic data of radical scavenging activity.

Samples	Half-life $t_{1/2}$ (min)		Time at the steady-state (min)
Ascorbic acid	0.21 to 0.370		0.288 to 0.545
Methanol extract	1.30 to 1.46		1.82 to 2.19
	Fast	***Slow***	
Chloroform extract	0.82 to 1.70	23.00 to 53.34	33.18 to 76.96
Petroleum ether extract	0.75 to 2.12	17.84 to 32.72	25.74 to 47.21

For ascorbic acid, the reaction endpoint was reached within half a min (**Table 6**). This confirms the fast DPPH radical inhibition of this compound reported in previous studies [102, 127]. Regarding the extracts of *C. mollis* resin, methanol extract exhibited relatively high efficiency of antiradical potential. It showed rapid reaction kinetics with the times of reaction at steady-state spanning 1.82 to 2.19 min. Chloroform and petroleum ether extracts; however, displayed lower efficiency **(Table 6).** The antiradical efficiency was also expressed in half-life ($t_{½}$) which was shorter for methanol extract. Chloroform and petroleum extract, showed 60.31 and 67.07% of the slow phase reaction, respectively. Their reactions with DPPH displayed a longer half-life relative to methanol extract.

4.5.2. DPPH radical scavenging activity

The radical scavenging activity of different extracts of *C. mollis* resin was expressed in terms of IC_{50} values (**Figure 13**). It is the concentration of antioxidants necessary to decrease the initial concentration of DPPH by 50%, which is inversely proportional to the antioxidant capacity of the sample, with a lower value of IC_{50} indicates greater antioxidant activity.

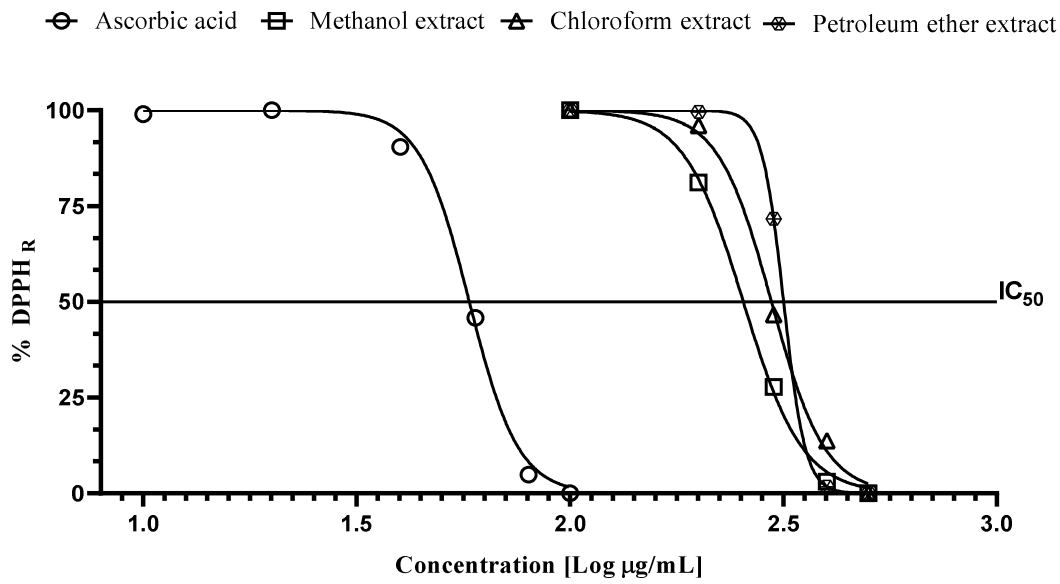

Figure 13. DPPH radical scavenging activity**.**

The results indicated that methanol extract exhibited the highest DPPH radical scavenging activity (IC_{50} = 254.80 ± 1.08 µg/mL) relative to the chloroform (IC_{50} = 296.50 ± 2.76 µg/mL), and petroleum ether (IC_{50} = 316.70 ± 1.16) extracts. This could be due to the larger amount of phenolic contents in the methanol extract. However, it gave low DPPH radical scavenging activity compared to L-ascorbic acid (IC_{50} = 58.07 ± 2.01 µg/mL).

The correlation study of the antioxidant activity of *C. mollis* resin extracts to TPC and TTC was carried out. It was observed that the IC_{50} showed strong negative correlation with TPC ($r = -0.998$, $P < 0.03$) and strong positive correlation with TTC ($r = 0.871$, $P < 0.03$). Phenolics and terpenoids compounds possess diverse biological activities that might also be related to their antioxidant activity [128, 129]. The results of this study showed that the antioxidant capacity of resin extracts could be mostly attributed to phenolic content. Methanol extract which contained the highest TPC and lowest IC_{50} showed very much less terpenoid content than chloroform and petroleum ether extracts. The significant correlation of phenolics to DPPH radical scavenging activity conformed to previous data that indicated the important contribution of phenolics [130, 131].

Previous studies on the related resin of other *Commiphora* species showed the potential of radical scavenging activity. For *C. myrrh* resin, the methanol, ethyl acetate, and essential oil extract exhibited IC_{50} values 320 µg/mL, 930 µg/mL, and 1133 µg/mL, respectively [19]. The authors implicated the highest activity of methanol extract due to terpenoids concentration. Similarly, *C. erythraea* resin extracts showed DPPH radical scavenging activity with IC_{50} values between 1080 and 4290 µg/mL [18]. In both cases, the obtained values were lower compared to the current study. However, the fraction of *C. wightii* ethyl acetate extract showed IC_{50} values of 16.0 µg/mL[22], which is much higher than the obtained value for *C. mollis.* The authors also isolated the mixture of two phenolic compounds (ferulates).

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

In the present study, the resinous exudate of *C. mollis* was extracted with methanol, chloroform, and petroleum ether. The result showed that the highest extractable solid was obtained by using methanol as an extraction solvent. The qualitative study revealed the presence of phytochemicals such as alkaloids, terpenoids, phenols, and saponins.

The total phenolic content was determined by the Folin-Ciocalteu redox method. The maximum absorption wavelength, 765 nm, and the time of interaction of the standard/extract with the F-CR, 30 min were experimentally justified as optimum conditions for the TPC determination. The resin exhibited significant content of total phenol content with the highest value obtained from methanol extract. Furthermore, the total terpenoid content was determined for each extract. In this case, methanol extract exhibited the lowest TTC while chloroform exhibited the highest value.

The *in vitro* antioxidant activity was carried out by the DPPH free radical scavenging method. Besides, the radical scavenging activity of the extracts was applied to determine their kinetic behavior. The methanol extract showed very fast kinetic action compared to the chloroform and petroleum ether extract. Furthermore, methanol extract fitted a one-phase reaction model while the other two extracts showed a biphasic model. This could probably be a result of the different kinetic behavior of antioxidants present in *C. mollis* resin. The IC_{50} value was determined for each extract. It was found that methanol extract displayed the highest antioxidant activity. Furthermore, the correlation study showed that the antioxidant activity of *C. mollis* resin extracts showed a positive correlation with TPC.

5.2. Recommendation

Depending results obtained, the researcher recommended that an approach for the elaboration of the analytical procedure for the analysis of TPC and DPPH radical scavenging could be used to ensure the reliability of results. For a better understanding of the antioxidant activity of *C. mollis* resin, further *in vitro* and *in vivo* studies supported by isolation and elucidation of individual component compounds could be important. Moreover, owing to my knowledge about its use in the community, toxicological analysis of this resinous exudate is also recommended.

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