FLAVONOIDS AND ESSENTIAL OILS OF *THYMUS VULGARIS AND T. SCHIMPERI*

Flavonoids and Essential Oils of *Thymus vulgaris* and

T. schimperi

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Solomon Hailu

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Abstract

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Solomon Hailu

Research advisor: Dr. Ermias Dagne

The essential oils of the leaves of *Thymus* species (*T. vulgaris* and *T. schimperi*) grown in different parts of Ethiopia were investigated using GC and GC-MS. The results indicated that *T. vulgaris* was rich in thymol (>40%) while the components of the oil of *T. schimperi* grown in different regions vary considerably. The major components of *T. schimperi* obtained from Dinshu, Maichew and Science Faculty Arobertum were thymol (42-50%) linalool (30%) and carvacrol (44%) respectively. Plant material purchased from Debre Sina was similar in its composition with that from Dinshu.

Solvent extraction of the aerial parts of *T. vulgaris* yielded the flavonoids naringenin and eriodicytol. Their structures were determined by spectroscopic methods.

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1.Introduction

A. Secondary Metabolites

Natural products are organic compounds of natural origin that are unique to one organism or common to a small number of closely related organisms. In most instances they appear to be nonessential to the plant, insect or microorganism producing them, in contrast to steroids, fatty acids, amino acids and the polymers derived from them, which are both essential structural role in most organisms. Same argue that substance that have no apparent utility to the organism that produces them are known as secondary metabolites.

The function of the natural product in living organism is a controversial issue for a long time. It has been suggested that secondary metabolites are formed in living organism as metabolic by products. Some investigator believed that the secondary metabolites are a measure of the fitness of the organism to survive. The ability to synthesize an array of secondary metabolites which may repel or attract other organism has evolved as one fact of the organisms strategy for survival.

Despite the vast number and structural diversity of secondary metabolites, almost all arise from one of three biosynthetic pathways or from a combination of two or more of these pathways. These are known as the acetate, mevalonate and shikmate. Many natural products find uses in medicine, agriculture, cosmetics, as food additives, etc. for the benefit of mankind. Therefore scientific investigations dealing with isolation and structural elucidation of natural products are of paramount importance [1, 2].

B. Essential Oils

Essential or ethereal oils are mixtures composed of volatile, liquid and solid compounds which vary widely with regard to their composition and boiling points [3]. They occur in various sites of a plant's anatomy and in some cases are found throughout the plant organs while sometimes they may be restricted to specific sites. They are found mainly in the flowers of plants, although smaller quantities are also present in fruits, stems, roots and leaves [4, 5]. In rare instances, different parts of a plant can give rise to oils which differ in chemical composition from each other [5].

The most abundant type of chemical compounds that are present in essential oils are terpenoids. Individual oils may also contain appreciable quantity of straight chain, aromatic or heterocyclic compounds. Trace of indole and anthranilic acid esters are found in orange oil, straight chain alcohols and aldehydes are recognized in oil of violet, while phenols and other compounds are common to many oils [3, 5, 6]. Among the terpenoids, monoterpenes and sesquiterpenes together with their oxygenated derivatives, such as alcohols, esters, aldehydes, ketones, are the main constituents of essential oils. Of these the oxygenated compounds are the principal odour carriers, although the non-oxygenated terpenes and sesquiterpenes, also, contribute to some degree to the total odour and flavour value of the oil. The oxygenated substances possess an advantage of better solubility in dilute alcohol and with the exception of some aldehydes, of greater stability against oxidizing and resinifying influences [3, 4, 7].

Due to their unsaturated character, the terpenes and sesquiterpenes oxidize and, resinify easily under the influence of air and light or under improper storing conditions. This means they bring about spoilage of odour and flavour, and lowenng of the solubility in alcohol. For many years, therefore, it has been the endeavour of the essential oil industry to supply the users with concentrated terpeneless and sesquiterpeneless oils. Such oils consist mainly of oxygenated compounds which have three major advantages. Firstly, the odour and flavour of the oil are better utilized yet it retains most of the odour and flavour characteristics of the original oil. Secondly, the oils are freed of any products of decomposition or resinification which might result from improper handling of the natural oils. Thirdly, in their better stability. There is same disadvantage of concentrated the oil to terpeneless and sesquiterpeneless which eliminate the natural fixatives such as waxes and stearoptenes which contribute to the retaining of the flavour on the natural oil. Elimination of mono and sesquiterpenes removes also a part of the characteristic odour and flavour of the natural oil. The application of heat undoubtedly has some effect on the delicate flavouring constituents of the oils. Heat treated oils may not display the freshness and bouquet of the original oil [3].

The essential oils are produced from a large number of different plants growing in many countries around the world. It is quite obvious that the variety within each of the species of plants has to be there and such variation may or may not influence the composition of the essential oils they produce. Thus, the quality and composition of essential oils depend on genetic type, geographical location, the deficiency and presence of the essentia! nutrient such as nitrogen, phosphorus, potassium etc. and the climatic condition, duration of storage and seasonal variation [8, 9].

Essential oils and their role in perfumery industry have close associations with the history of civilized man. They were utilized in ancient Egypt. Persia, China and India as archaeological finds have recently revealed. It is interesting to note that our distant ancestors used these selfsame plant extracts as perfumes to dispel the odour of unwashed humanity as well as flavour to obscure the test of putrid meat, etc [5, 1]. Generally the development of chemistry of terpenoids and application in perfumery have increased considerably due to three main factors. These are, the improvement of isolation techniques for isoprenoids from natural products, the increase of chemical and technological knowhow relevant for manufacture of such materials and the upgrading of raw materials from the paper, pharmaceutical and petrochemical industries. In general an increase in the chemical and technological knowhow in the field of isoprenoids has led to the manufacture of olfactively highly valued products of uniform quality [10]. Essential oils can be used as antibacterial, antiasthmatic, insecticides, deodorant and as additives in cosmetics, perfumes, soaps, detergents, etc. They are commonly used as flavours in candies, soft drinks and many other food products as well as in medicinal preparations, used to treat gastrointestinal upset and common cold [3, 7].

The relative share of synthetic essential oils in the consumption of fragrance and flavouring is increasing due to price fluctuation, irregular supplies and variation in quality of the natural essential oils. The supply of the oxygenated terpenoids which are minor constituent of natural essential oils can be increased by synthetic methods. The earliest method to these products are the chemical modification of non-oxygenated terpenes and sesquterpenes isolated from natural product. For example, pyrolysis of β-pinene obtained from natural product leads predominantly to myrcene which is the key substance for nerol, geraniol and linalool [7, 9].

The developing countries of Asia, Africa and Latin America constitute a vast area, which have almost every type of agroclimatic conditions capable of supporting growth of different types of essential oil bearing plants for almost all types of established use. Many of these countries also have vast resources, and if properly harnessed, wild plants can be adopted to useful crops generating a wide variety of products including essential oils and oleoresin of industrial value. However, the major consuming centre of essential oils are located in the developed countries. Many of high value plant raw materials are being transported to developed countries for extraction of their essential oils. Lack of proper know how and processing technology in the developing countries is the primary reason. Because of inadequacies in scientific inputs, the yields of these crops have been in most instances static, or have improved only marginally, and the volume of products has lacked consistency. It is these reasons that led the industry to seek synthetic alternatives for their requirements of perfumery and flavour chemicals [5, 11].

In addition to the materials of plant origin, there are a few animal products which have been used in perfumery for many centuries. The four such products of commercial importance are ambergris, castoreum, musk and civet. Musk and ambergris are expensive and are only used in the finest perfumes. The main function of animal products is as fixatives i.e. to stabilize the bouquet of blended perfumes. This ensures that the volatile components do not evaporate before the others, the perfume remain consistent over a long period [4, 10]. Plant material such as oak moss and benzoin resin are also used as fixatives but they are inferior to the animal products. A number of odourless organic liquids of high boiling point, such as benzyl benzoates have also been used for the same purpose, but this have a "flattening" effect on the bouquet [4].

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C. Flavonoids.

Flavonoids comprises a large secondary metabolites which are derived from sub-units supplied by the acetate and shikimate pathways. All flavonoids would have fifteen carbon atoms in their basic nucleus and these are arranged in a C_6-C_3-C_6 configuration, that is, two aromatic ring linked by a three carbon unit which may or may not form a third ring. Flavonoids occur almost exclusively in higher plants and responsible for much of the flavour of food and drinks of plant origin and for the colour of plants. Flavonoids commonly occur as flavonoid O-glycosides in which one or more hydroxyl groups is bound to a sugar units. The effect of glycosylation makes the flavonoids less reactive and more water soluble [1, 12].

Flavonoid compounds have attracted the attention of inquiring minds for many centuries. Certain flavones are among the earliest known natural dyestuffs. The importance of flavonoid compounds in the tanning of leather, the fermentation of tea, the manufacture of cocoa, in the flavour qualities of food stuff and insecticides have led to extensive investigation into the chemistry of derivatives of flavonoids [12, 13]. It was reported that flavonoids have a wide application as deodorant and antioxidative agents. Unsaturated fatty acids serve as building blocks of major membrane compounds such as phospholipids, glycolipids and triacyl glycerides. The oxidation of unsaturated fatty acid leads to decreasing structure and functions, coronary arteriosclerosis, diabetes mellitus as well as being associated with aging and carcinogenesis. Some flavonoids protect biological systems against various oxidative stress. Some antioxidants have been reported to prevent cancer and coronary heart disease and serve as a prophylactic agents against some neuronal symptoms of aging [14, 15].

1.1. Isolation, Separation and Characterization of Essential Oils and Flavonoids

Steam distillation, enfleurage and maceration are suitable to isolate natural flavours and essential oils from plant materials. Most essential oils are steam volatile, reasonably stable to action of heat and practically insoluble in water and hence are suitable for processing by steam distillation. However, water distillation suffers from hydrolysis of esters, polymerization and oxidation of some constituents of essential oils. Prolonged action of heat spoils odour and flavour. Beside this high boiling and water insoluble oil constituents cannot be completely vaporized from large quantities of water, therefore, the distilled oil will be deficient in regard to these constituents [3, 11, 16].

Both flavonoids and essential oils have been found to occur practically in all parts of plants. A number of solvents have been used to isolate flavonoids and essential oils from plant materials. The solvent penetrates the flowers, leaves and dissolves the natural perfumes, flavonoids together with some waxes, carotenoids and other coloured impurities. The solution is subsequently transfered into an evaporator and concentrated. The solvent normally used are petroleum ether, acetone, chloroform and alcohols. Petroleum ether is particularly advantageous in its selective power of dissolving the product relatively little wax and colouring matter, but corresponding more of the odoriferous compounds. Flavonoids can be isolated by relatively polar solvents [3, 11, 12, 16].

Since essential oils contain chiefly liquid and more or less volatile compounds of many classes of organic substances, separation of the individual components from the essential oil is accomplished by various methods. Fractional distillation under reduced pressure can be used to separate the constituents which boil within restricted temperature range. The essential oil should never be fractionated at atmospheric pressure, because the high temperature causes decomposition, polymerization and other side reaction [3 ,4, 16].

Column chromatography remains a useful technique for preliminary fractionation and purification of flavonoids and essential oils from crude plant extract. The column is usually eluted successively with solvents of increases polarity. The solvent used to elute the column are mixtures of petrol-EtOAc, C_6H_6-$CHCl_3$, $CHCl_3$-EtOAc to isolate essential oils and flavonoids from crude plant extract. The fraction obtained from the column of silica gel were further purified with sephadex LH-20 and PTLC to achieve a pure component of a natural product. Beside this, a visible spot was obtained for most flavonoid and phenolic compounds on spraying some selective spray reagent like ammonia, fast blue reagents etc. to a TLC of silica gel which gives a valuable information about the structure of the compound [3, 12, 13].

Gas liquid chromatography (GLC) is an extremely powerful analytical tool widely used to determine the qualitative and quantitative analysis of essential oils. The major success of the application of modern gas chromatography is due to the very high efficiency of separation which can be achieved with capillary columns, high sensitivity of the detection of even very small amounts of separated components, to the precision and accuracy of the data from quantitative analysis of very complex mixtures [17]. Quantitative information by GLC is obtained from a number of components separated and the amount of each components can be eluted from the chromatographic separation system. The qualitative information which can be derived from chromatograms is limited and in most cases insufficient for the identification of related compounds. However, chromatographic peaks are sometimes identified by comparison with relative retention values, or the chromatogram itself is sometimes used to identify an essential oil for quality control and determination of adulteration [16, 17, 18].

The complete essential oil mixture is often chromatographed without preliminary separation. There are several disadvantages to this type of chromatography. Complete separation of all compounds cannot usually be achieved at one column temperature, since the boiling point range for the hydrocarbons and oxygenated fraction is very wide. Sometimes the hydrocarbons constitute as the major fractions in an essential oil. For example, in lemon oil, the terpene hydrocarbons make up to 96% of the essential oil. The oxygenated portion, which gives the oil its distinctive flavour and aroma are minor constituent of the complete oil. When the complete oil is chromatographed, either the oxygenated fraction will be too dilute for good detection or, if a large sample is chromatographed, the hydrocarbons will overload the column and these peaks will mask the peaks of the oxygenated components. There are advantages to the use of the complete oil, if the limitation of the procedure are kept in mind. Firstly, this is a very rapid method, since no preliminary separation is necessary. Secondly, characteristic chromatograms, finger prints of an essential oil may be obtained if there are no major constituents present to mask the other components. This type of chromatogram is useful for quality control, finger printing of essential oils and determination of adulteration [16].

Direct GLC analysis is restricted to the few thermal stable natural occurring flavonoids which are sufficiently volatile, so a correlation can be made between flavone structure and the relative retention time [13]. Derivatization of flavonoids prior to GLC analysis is usually needed in order to increase their thermal stability and volatility. Flavonoids should be converted into trimethylsilyl derivatives using N,N-*bis*-(trimethylsilyl)trifloro acetamide as a silylating agent [19]. Other analytical tools like ^{1}H and ^{13}C-NMR, MS, IR, UV are also useful in identifying pure or enriched component of essential oils and flavonoids. Combination between a highly efficient and selective separation and highly specific methods of structural characterization, for example GC-MS, GC-IR, and GC-UV have proved to be very powerful tools in the analytical chemistry of complex mixtures [13, 16, 17, 18].

2. *Thymus* Species

The genus *Thymus* of the mint family (Labiate) includes about 350 species world wide, and is distributed mainly in temperate zones [20, 21]. *Thymus vulgaris* is a small, low-growing shrub with curled leaves, native to Southern Europe, Morocco and Algeria. The small, two-lipped purple of lilac coloured flowers are born in cluster at intervals along the flowers stalks. Since the plant has a tendency to become woody it has to be replanted after three or four years [20,21,22].

The oil content and components of *T. vulgaris* are affected by various factors, such as fertilizer, weather condition, seasonal variation, storage, method of harvesting etc. Maximum oil yield with rich in phenolic compounds were obtained from fresh material harvested at the beginning of blooming and distilled immediately [23, 24, 25]. Comparison between the effect of cultivation method, stage of ontogenesis and storage on the component of the essential oil was examined and found that most of the variations were due to different plant stages at the time of harvesting and storage of the oil for a long time [24, 25, 26, 27]. In cultured thyme, the total oil content was higher, however *p*-cymene and 1,8-cineole content were lower in summer variety as compared to winter variety [28]. When fertilizer containing 40% potassium salt and superphosphate were used during and after blooming, a favourable result in the oil content were obtained [29]. Analysis of the composition of the essential oil of *T. vulgaris* in relation to leaf age and season of the year shows that the monoterpenes in the oil change so much in the course of a season that definite components which dominate in the oil at one time may even fall to a level below detection. It was also found that the essential oil components of the bud and leaf of the same plant were not comparable due to difference in the state of the secreting glands of the buds from those in mature leaves [30, 31]. Many of the morphological characteristic and chromosome number of the hybrid obtained from *T. serphyllum* and *T. vulgaris* were intermediate between the parent species. Essential oil components of the hybrid contained nearly all compounds found in the parent species. In many cases, phenols which were abundant in *T. vulgaris*, were minor component in the hybrid [32]. Detailed studies of the distribution of the chemical forms in the essential oil of *T. vulgaris* shows that it was possible to draw up a map based on association of the two chemical forms most abundant in the various sectors. This fact seems to indicate that the chemical forms are able to indicate the environmental condition in certain situation. Thus, with the use of intraspecific units, micro ecological studies can be effected [33].

Many species of the *Thymus* yield the commercially important thyme oil. In Spain alone, the major producing country of this oil, there exist numerous species and varieties of the wild growing *Thymus*. Spanish thyme oil is distilled from the flowering subshurbs of *T. vulgaris* and *T. zygis*, both of which grow wild on vast area. Inferior qualities of Spanish thyme oils find their way in the market because of insufficient care exercised in the selection and collection of the plant material, sometimes because of adulteration with thyme oils derived from the plants other than those indicated above. Natural thyme oil distilled in Spain or Morocco possesses usually a yellowish red, sometimes deep red colour, caused by the action of phenols upon iron condensers in the field stills. An almost colourless oil can be obtained by redistilling the red oil. The process is accompanied by some loss of oil and by a corresponding increase in the phenol content of white thyme oil [22, 34]. White thyme oil contains amyl alcohol (**1**), 3-hexenol (**2**), 4-hexenol (**3**), ß-pinene (**4**), camphene (**5**), *p*-cymene (**6**), γ-terpinene (**7**), linalool (**8**), l-borneol (**9**), 4-terpineol (**10**), geraniol (**11**), thymol (**12**), carvacrol (**13**), caryophyllene (**14**) and sesquiterpene alcohol (**15**) [34, 35].

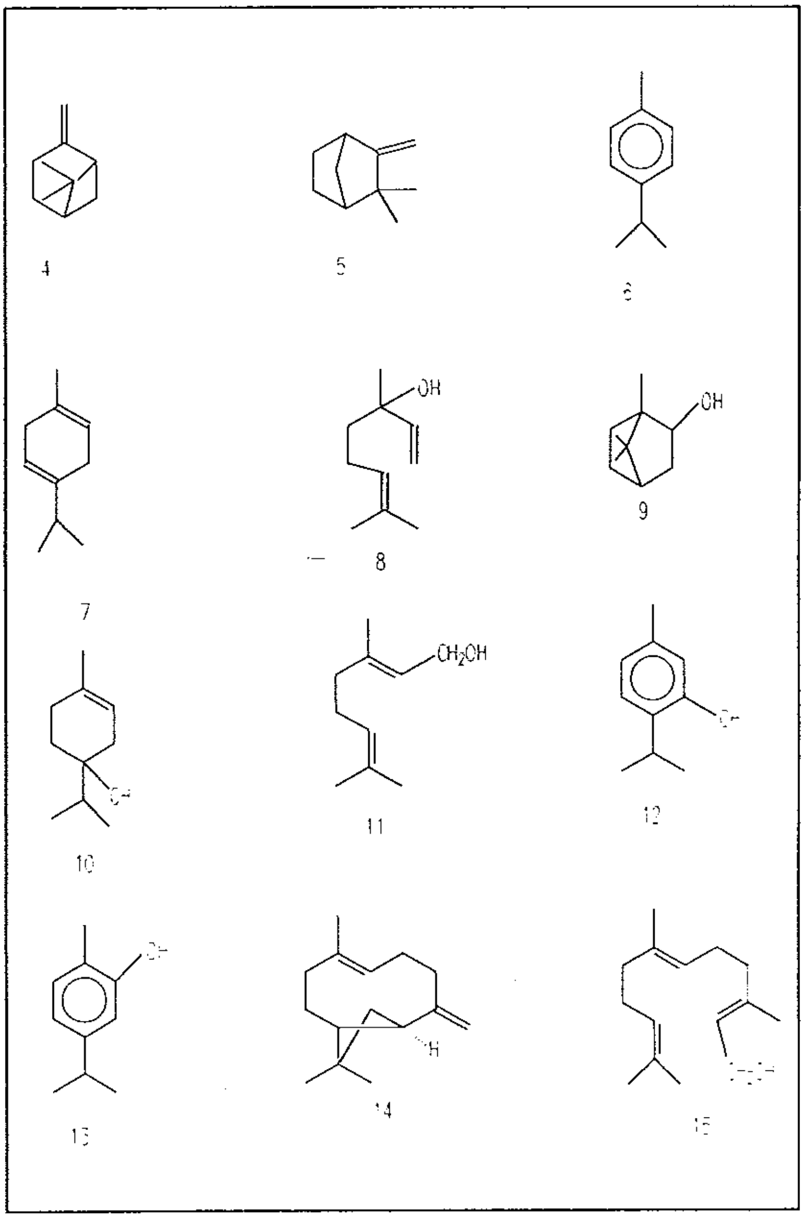


Fig. 1: Some constituents of thyme oil that were reported from essential oils of *Thymus* species

For practical purposes thyme oil can be classified into three groups based on its composition. Firstly, thyme oil (thymol type) contains 42-60% of phenol derivatives mainly thymol (**12**). Secondly, origanum oil (carvacrol type) contains 63-74% of phenol derivatives mainly carvacrol (**13**). Thirdly, lemon thyme oil contains citral (**15**) which accounts for its somewhat lemon like odour. Thyme oil is occasionally adulterated by adding terpenes or the commercial product called "thymine", which consists mainly of p-cymene (**6**), some camphene (**5**) and γ-terpinene (**7**) which are also natural constituents of thyme oil. Due to high content of thymol (**12**) in thyme oil the oil crystallizes at room temperature, a most important feature that helps to distinguish it from origanum oil and adulterated thyme oil [34]. The quality of a liquid extracted thyme oil should be evaluated not only by secondary criteria (sp.gr., solid residue, etc.) but also by the content of essential oil and its phenol fractions (thymol and carvacrol) [36].

Thyme essential oils exhibit high antimicrobial effect compared to the oils of other plants. These antimicrobial properties are related to the chemical composition of the oils, which varies within the different species of the genus *Thymus*. The work of Lattaouii and Tantaoui-Elaraki [37] on the antimicrobial activity of the essential oils of *T. broussonettii*, *T. zygis* and *T. satureioides* on the *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger* related to the chemical composition of the oils. They found that the oil of *T. broussonettii* had the highest efficiency on the three microorganisms while the oil of *T. zygis* showed higher potency than *T. satureioides*. The major component of the essential oil of *T. broussonettii* was carvacrol (**13**) reaching 63.3% of the total oil. The high content of carvacrol (**13**) in *T. broussonettii* oil accounts for its antimicrobial activity. The oil of *T. zygis* is dominated by *p*-cymene (**6**) (50.6%), while *T. satureioides* has borneol (**9**) (31.2%) as major component. However in *T. zygis* the oxygenated products may play an important role in the antimicrobial activity; thymol (**12**) and carvacrol (**13**) reach more than 13% and borneol (**9**) represents 5.8%. While *T. satureioides* has 45% hydrocarbons (camphene and pinene), which dilute borneol (**9**), and thus probably reduce its antimicrobial action.

The pharmaceutical investigation of thyme oil shows that the petrol insoluble fraction has a greater effect on relieved coughing than has the petrol soluble fraction, but the total effect of both were greater than for either alone which indicates that the petrol soluble potentiates the petrol insoluble. Even though thymol (**12**) and carvacrol (**13**) were slightly secrolytic, neither thymol (**12**) nor carvacrol (**13**) relieved coughing [38]. The spasmolytic activity (against $BaCl_2$ and acetyl choline) of the major component of several *Thymus* species were examined in rat duodenum tissue. It was found that thymol (**12**), carvacrol (**13**) and caryophyllene (**14**) showed antagonistic action against both spasmogens while myrcene (**16**) showed antagonistic action against acetyl choline. But borneol (**9**) revealed partial antagonistic action against both spasmogen while camphor (**17**) showed non antagonistic action against either spasmogen [39]. Thyme oil inhibit germination and root growth of pea and the effect was reversed by washing with water. This germination inhibiting effect of thyme oil was attributed to the action on the protein portion of the plant enzyme [40]. The *Thymus* species with high content of thymol (**12**) and carvacrol (**13**) were not advisable as food additives for animals [41].

Thyme oil increase tyrpsin activity with the active components being thymol (**12**), carvacrol (**13**) and β-pinene (**4**). It was also observed that the *ortho* and *meta* derivatives of phenols strongly enhanced trypsin activity than the *para* derivatives [42, 43]. Thyme oil is used to flavour a wide range of food products, such as soup, vegetables, meats, etc.[44].

The effect of primary antioxidant on the activity of methanol extract of thyme oil were examined. It was observed that when the extract was combined with butylated hydroxy anisole, butylated hydroxy toluene or ascorbyl palmitate it showed an additive antioxidant effect. Beside this, it was found that both petroleum ether soluble and insoluble fraction of thyme oil showed strong antioxidant effect [45, 46]. Thymol (**12**) which is the major constituent of thyme oil was originally introduced as a disinfectant in lieu of carbolic acid, having the advantage of a more pleasant odour. Thymol (**12**), however, has the drawbacks of producing a strong local irritant effect and of being only sparingly soluble in water. Thymol seems to possess more antibacterial properties than carbolic acid. Thymol (**12**) is employed in many antiseptic mixtures intended for use upon mucous cavities, especially in gargle mouth washes and as a local anaesthetic in toothache. Thymol is also used as a gastrointestinal disinfectant in fermentative gastritis, against intestinal parasites especially hook worm and as antifungal. Like thymol (**12**), carvacrol (**13**) is also used widely as a powerful antiseptic, as germicide in all kinds of medicinal and oral preparations, as disinfectant and as local anaesthetic in tooth ache [47, 48].

In addition to essential oils, biphenyls and flavonoids isolated from *Thymus* species shows spasmolytic activity, deodorant and antioxidative properties. Crisilineol (**18**), thymonin (**19**) and 8-methoxy crisilineol (**20**) isolated from *T. saturicioides* and *T. vulgaris* showed more spasmolytic activity than thymol (**12**) and carvacrol (**13**) in guina pig ileum and trachea. The compounds caused a dose dependent depression of maximum response to carbinol, histamine, 1,1-dimethyl-phenyl piperazinium iodide and $BaCl_2$ without any shift along the dose axis [49, 50, 51]. 3,4,3',4'-Tetrahydroxy-5,5'-diisopropyl-2,2'-dimethylbiphenyl (**21**) and 3,4,4'-trihydroxy-5,5'-diisopropyl-2,2'-dimethyl biphenyl (**22**) isolated from *T. vulgaris* showed strong deodorant effect than rosmanol and carmosol which are known to be effective deodorant compounds [15]. 3,4,3',4'-Tetrahydroxy-5,5'-diisopropyl-2,2'-dimethylbiphenyl (**21**) and eriodicytol (**23**) isolated from *T. vulgaris* are strong antioxidative compounds which strongly inhibited the production of lipid peroxides induced by microsomal NADPH oxidation, mitochondrial lipid peroxidation supported by NADPH and NADPH in the presence of ADP and protect red cells against any oxidation stress. It was observed that the biphenyl were more antiperoxidative than the flavonoid. This may be due to the stabilizing effect of the electron donating alkyl groups on the *ortho* phenoxy radicals generated in the process of antiperoxidation [14].

Table 1. The composition of the oil reported in the literature of *Thymus vulgaris* grown in Chile (C), Italy (I) , Poland (P) and Spain (S).

Composition	C	I	P	S
α-pinene	x	x	x	x
β-pinene	x		x	
α-thujene				x
camphene	x	x	x	x
Δ-3-carene				x
α-phellandrene				x
limonene	x		x	x
1,8-cineol			x	x
γ-terpinene	x			x
p-cymene	x	x	x	x
fenchone	x			
linalool		x	x	x
borneol	x	x		x
linalyl acetate		x		x
α-terpineol		x		x
terpine-4-ol		x		x
geraniol			x	
citral	x			
citronellal	x			
anethole	x			
thymol		x	x	x
carvacrol	x	x	x	x
thymol methyl ether			x	
carvacrol methyl ether			x	
References	52	22	53	22

Table 2 : Components of an essential oils of some *Thymus* species

Species	Compounds	Ref.
T. fomini	α-pinene, camphene, β-pinene, limonene, γ-terpinene, *p*-cymene, 4-terpineol, thymol, carvacrol.	54
T. chamaedris	p-cymene, 2,8-dimethyl-2-decene, 2,9-dimethyl-2-decene, β-cubinene, α-cubinene, β-diabolene, α-farnesene, β-selinene, methyl thymol ethere, camphor, 4-terpineol, linalool, borneol, α-terpineol, geraniol, p-cymene-8-ol, nerolic acid, isopropyl benzoic acid, thymol, carvacrol.	55
T karamarianiaccus	α-pinene, β-pinene, camphene, limonene, sabinene, myrcene, p-cymeme, citronellol, citral, caryophyllene, borneol, geraniol, geraniol acetate.	54
T. magnus	γ-terpinene, α-pinene, β-caryophyllene, β-bisabolene, p-cymene, thymol, carvacrol, terpinene-4-ol, borneol.	56
T. sarureioides	α-pinene, camphene, β-pinene, myrcene, α-terpinene, limonene, 1,8-cineol, carvacrol methyl ester. p-cymene, trans-4-thjanol, linalool, camphor, bornyl acetate, γ-terpinene, terpinene-4-ol, β-caryophllene, α-terpineol, borneol, thymol, carvacrol.	57
T. serphylleum.	thymol, linalool, p-cymene, α-pinene, d-borneol, geraniol, geraniol acetate, terpineol, terpineol acetate, citronellol, borneol acetate, linalyl acetat, nerolidol, citral, 1,8-cineol, carvacrol, neryl acetate, citronellal, camphene.	55, 58, 59, 60
T. Trancaucasicus	thymol, 1,8-cineol, borneol, carvacrol, limonene, geraniol, geranyl acetate, linalool, α-pinene, 4-terpinolene, γ-terpinene, p-cymene. myrcene, β-pinene, camphene.	54, 61, 62
T. trautvetteri	α-pinene, β-pinene, caryophyllene. caryophyllene oxide, linalool, limonene, borneol, p-cymene, geraniol, geranyl acetate, sabinene, myrcine, camphene, thymol.	63
T. pulegioides	borneol, geraniol, bornyl acetate, geranyl acetate, linalool, linalyl acetate, α-pinene, cineole, thymol, thymol methyl ether, carvacrol.	64

T. vulgaris is a species recently introduced into Ethiopia. It is cultivated by the Essential Oils Research and Development Centre (EORC) in Wonndo Genet.

while that from tha Arboretum (S-658) had high content of carvacrol (**13**) (44%) with insignificant amount of non-oxygenated monoterpenes.

Table 3: Yield and physicochemical properties of the essential oils of *T.Schimperi* and *T. vulgaris.*

specie	yield	sp.gr.	refractive index	optical rotation
T. schimperi	0.93%	-	1.5003	-1.190
T. vulgaris	0.8-1%	0.93991	1.4966	0.940

*The physicochemical properties of the neat oils were measured at 20^{0}C.

Table 4: Percentage composition of essential oil components of *T.schimperi* and *T.vulgaris*

Component	*T. schimperi* (S-913)	*T.schimperi* (S-658)	*T. vulgaris*	Method of identification
3-thujene	1.1			
cis-ocimene	0.3			
octen-3-ol			0.72	
β-myrcene	1.6		1.44	
3-octanol	0.3	0.6		
α-terpinene	1.4		2.33	
3-octanone	1.4	3.3		
limonene	0.3			
p-cymene	10		6.1	PE, NMR
1,8-cineol			1.3	
γ-terpinene	12.1		13.2	PE, NMR
linalool	6.6	8.3	5.0	PE
borneol			1.7	
α-terpineol	0.3	0.3	trace	
terpine-4-ol	0.3	0.3		
thymol	49.1	33	46.5	PE, NMR
carvacrol	10.2	44	1.9	PE
trans-caryophyllene	1.2	0.2	2.00	

* Components are arranged in order of their elution from a DB-17 capillary column.

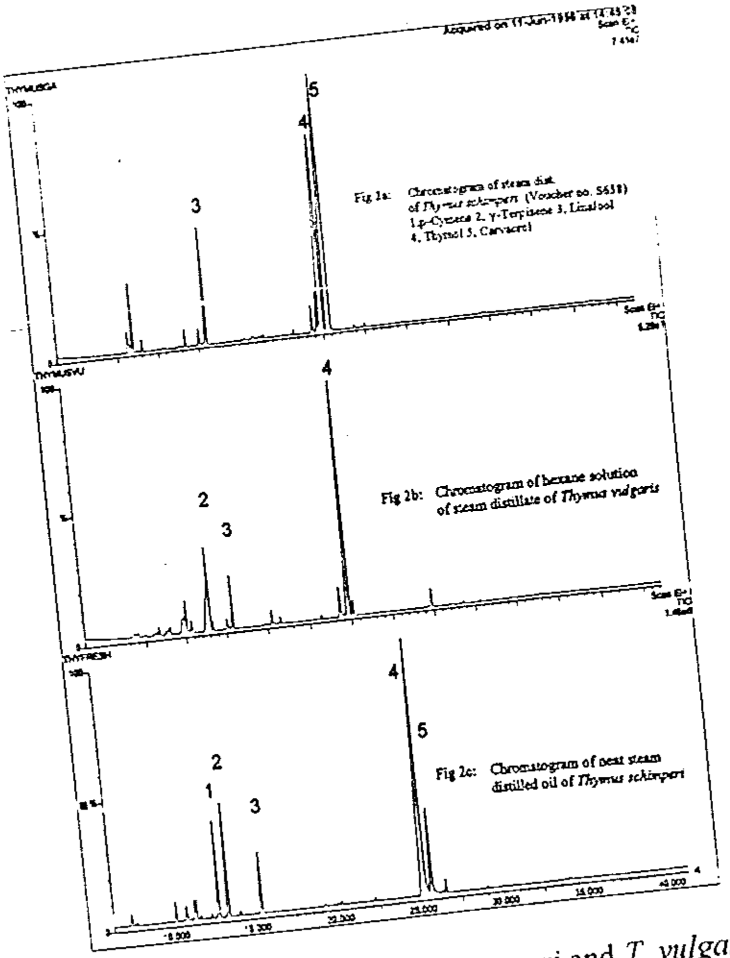


Fig. 2: Gas Chromatography of the *T.schimperi* and *T. vulgaris*.

4.2 The Chemical Composition of Oils of *T. schimperi* of Different Origin.

The essential oils of *T. Schimperi* collected from Dinshu, Debre Sina , Addis Ababa Univerist Arboretum (S-658) and Maichew were analyzed separately so as to check their difference physicochemical properties (Table 5) and in composition (Table 6). It was found that the obtained from species grown in the Arboretum was dominated with phenolic monoterpene

The oils isolated from the samples of *T.schimperi* collected from Dinshu, Debre Sina, Maic were dominated with thymol (**12**), carvacrol (**13**), linalool (8), *p*-cymene (**6**) and γ-terpi

(7). Thymol (**12**) (45-50%) was the major component of the oil of this species grown in Debre Sina and Dinshu. However linalool was the major component of the oil of this plant from Maichew. Seasonal varation, enviromental factors and geographical location may affect the content of the oil of this plant. The varation in the stage of the development of the plant material at the time of harvesting or the existence of different chemotypes might be the reason for the absence of non-oxygenated monoterpenes in the oil of the same plant from the Arboretum. During the young stage it is known that the percursors of the phenolic compounds such as γ-terpinene (7) and *p*-cymene (**6**) may be the major components in such plants..

Table 5 :The yield and physicochemical properties of *T.schimperi.*

Location	Yield	Refractive index	Optical rotation
Debre Sina (dry)	1.67%	1.500	0.13
Dinshu (fresh)	0.93%	1.500	-1.19
Dinshu (dry)	1.5%	1.497	-0.27
Maichew (fresh)	0.37%	1.478	-5.98
Arboretum (fresh)	0.52%	1.510	-

* Measurements of the physicochemical properties of the neat oils were conducted at 20^0C.

Table 6 : Percentage composition of the major constituents of the essential oils of *Thymus schimperi*

Substance	Percentage composition				
	Debre Sina (dry)	Dinshu (fresh)	Dinshu (dry)	Maichew (fresh)	Arboretum (fresh)
thymol	42.3	49.1	43.3	14.8	33
p-cymene	22.0	10.2	20.0	15.0	-
γ-terpene	8.7	12.1	10.4	7.5	-
carvacrol	14.8	10.2	7.8	4.8	44
linalool	2.1	6.6	5.5	33.8	8.3

4.3 Characterization of Some of the Constituents of Essential Oil

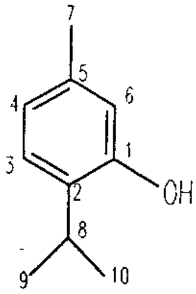
The essential oils were obtained from the plant materials by hydrodistillation at atmospheric pressure. Isolation of compounds was conducted using chromatography and characterization of the enriched compounds was also conducted with IR, ^{1}H-NMR and ^{13}C-NMR

4.3.1. Thymol (12).

This compound was obtained from the EtOAc fraction and by fractional distillation of *Thymus vulgaris*. In addition to this the compound was isolated from petrol and EtOAc extract of *Thymus schimperi*. This compound was identified from its retention time, IR, ^{1}H and ^{13}C-NMR. The IR spectrum indicated a broad band at 3398.9 cm^{-1} and 1619 cm^{-1}, 1584.4 cm^{-1}, 1513.9 cm^{-1} shows the presence of hydroxyl group and aromaticity respectively. The ^{1}H-NMR shows that the two methyl signals occurs as a doublet at δ 1.25, an isolated methyl signal occur as a singlet at δ 2.25, The methine as a septet at δ 3.15 and a singlet at δ 5.15 for the hydroxyl proton. In addition to this the ^{1}H-NMR shows three aromatic protons at downfield with different chemical environment, the doublet at δ 7.07 for the *meta* proton, the doublet at δ 6.71 for the *para* proton and a singlet at δ 6.55 for the *ortho* proton to the hydroxyl group. This can be explained by the fact that the hydroxyl group act as an electron withdrawal to the *meta* position which decrease the electron density so that it shifts to the down field where the resonance participation of the lone pair of the hydroxyl oxygen increase the electron density on the *ortho* and *para* position which leads to a signal in the upfield as compared to the signal of the *meta* proton [67]. The ^{13}C-NMR with DEPT spectrum displayed 10 carbon resonances, corresponding to three primary, four tertiary and three quaternary carbon atoms.

Table 7: ^{13}C-NMR data of thymol (22.5 MHz, $CDCl_3$).

C	δ,ppm
1	152.8
2	131.7
3	126.1
4	121.4
5	136.2
6	116.2
7	21.2
8	34.5
9	23.9*
10	22.7*



* : Interchangeable

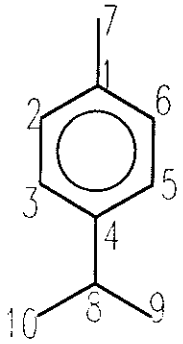
4.3.2. γ-terpinene (7)

This compound was isolated from the hydrocarbon fraction of *T. schimperi*. The identity of this compound was further established by comparison with authentic sample using GC and ^{13}C-NMR. The ^{13}C-NMR with DEPT spectrum displayed 10 carbon resonance, corresponding to three primary, two secondary, three tertiary and two quaternary.

δ 126.2 suggest that the aromatic ring was symmetrically disubstituted. The presence of two methyl groups with identical chemical environment were evident from an intense signal at δ 24.1.

Table 9: ^{13}C-NMR data of p-cymene (22.5 MHz, $CDCl_3$).

C	δ,ppm
1	135.00
2	129.00
3	126.20
4	145.80
6	129.00
7	20.90
8	33.70
5	126.20
9	24.10
10	24.10



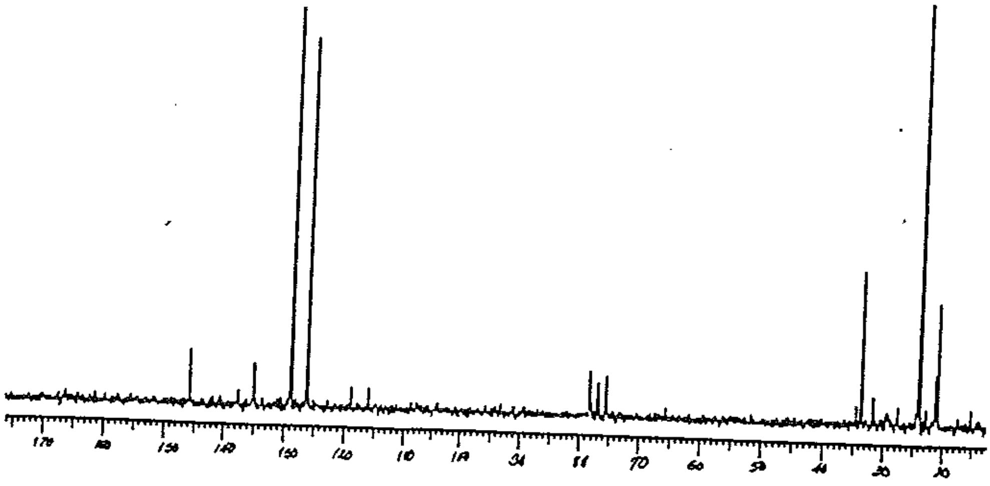


Fig. 3: ^{13}C-NMR spectrum of *p*-cymene measured in $CDCl_3$ at 22.5 MHz

4.4 Identification of Flavonoids.

The dried leaves of *T. vulgaris* were extracted with acetone-methanol (1:1) mixture. Purification of the crude extract with column chromatography and sephadex LH-20 of the extracts resulted in the isolation and identification of naringenin (**1**), eriodicytol (**2**) and a third compound tentatively identified as rosmrinic acid (**3**). Characterization of the compounds were conducted with IR, EIMS, ^{1}H-NMR and ^{13}C-NMR.

4.4.1 Naringenin (1)

A yellow crystal of compound **1** was obtained from the extract of acetone-methanol (1:1) mixture after its fractionated of the crude extract in column of silica gel by eluting using mixtures of petrol and increase the amount of ethyl acetate as detailed in the experimental section. The fraction was further purified with sephadex LH-20 by eluting with $CHCl_3$-CH_3OH (1:1) mixtures. Its phenolic nature was indicated by the red colour on spraying the silica gel TLC spot with fast blue reagent. The EIMS of compound **1** showed a molecular ion peak at m/z 272.2. The IR spectrum of **1** suggested the presence of a broad band shift to lower frequency for the hydroxyl group (3317cm^{-1}) and carbonyl group (1633cm^{-1}) due to intramolecular hydrogen bonding. The ^{1}H-NMR spectrum displayed a doublet of doublet at δ 5.44 for H-2 coupled with axial and equatorial protons attached to C-3. The multiplate signals at δ 2.7-3.3 attributed for the methylene protons at C-3 confirm that the protons have different chemical environment which were coupled with each other and furtherly coupled with proton at C-2. This suggested the presence of a flavanone skeleton. The signal at δ 5.95 indicates for H-6 and H-8. The ^{1}H-NMR revealed a doublet signal at δ 7.39 attribute to H-2' and H-6' and at δ 6.90 to H-3' and H-5' confirm the B-ring was symmetrically disubstituted. The ^{1}H-NMR spectrum of **1** measured in Me_2CO-d_6 shows a singlet at δ 12.16 which disappear when it was measured in MeOH-d_4 confirm the presence of chelated hydroxyl group. The presence of 13 carbon signals was evident from the ^{13}C-NMR with DEPT spectrum, corresponding to one secondary, five tertiary and seven quaternary. The B-ring carbon C-2',6' and C-3',5' are clearly visible as intense signal at 129.9 and 117.0 which support for the symmetrically disubstituted B-ring. Beside this the ^{13}C-NMR spectrum suggested the presence of a flavanone skeleton with peaks for C-2 and C-3 at 80.8 and 44.4 respectively.

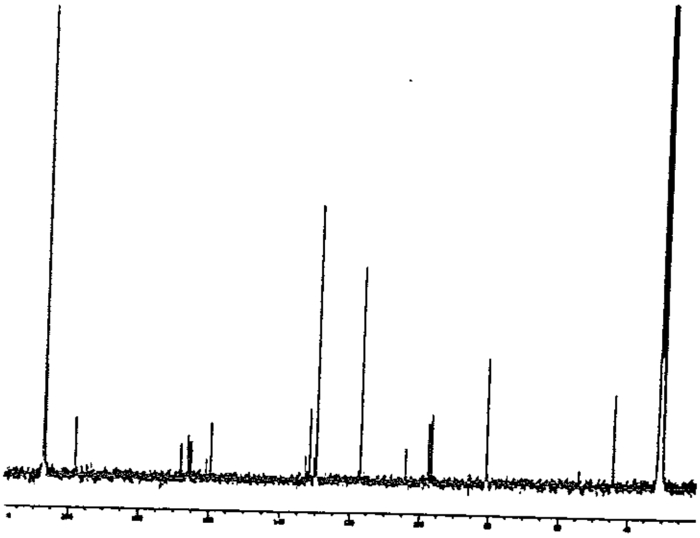
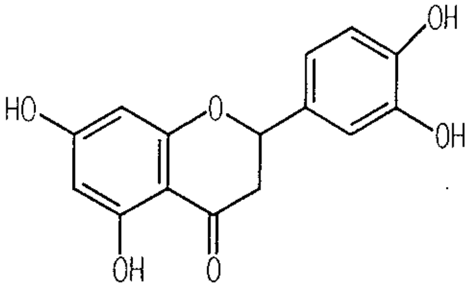


Fig. 5: ^{13}C-NMR specctrum of naringenin measured in Me_2CO-d_6 at 75MHz

4.4.2 Eriodicytol (2)

A yellow amorphous powdered of compound **2** was obtained from the extract of methanol-acetone (1:1) mixture. Its phenolic nature was indicated by the red colour on spraying the silica gel TLC spot with fast blue reagent. TLC analysis shows that compound 2 is more polar than compound **1**. The EIMS of compound **2** showed a molecular ion peak at m/z 288.2 and abundant OE$^{\cdot}$ ion $(M-18)^{+\cdot}$ is a strong evidence that the two hydroxyl groups in the B-ring are ortho to each other. The IR spectrum of compound **2** suggested the presence of hydroxyl group (3365cm^{-1}) and chelated carbonyl group (1637cm^{-1}). The ^{1}H-NMR revealed a doublet of doublet at δ 5.45 and 2.65-3.2 which is characteristic of a flavanone skeleton and is assignable to H-2 and H-3 respectively. The ^{1}H-NMR spectrum of compound **2** displayed a down field singlet at δ 12.15 for the proton of chleated hydroxyl group and an A-ring singlet at δ 5.95 attributed to H-6 and H-8. furthermore, the ABC pattern at δ 6.85 integrate for two protons which are superimposed and at δ 7.02 for one proton indicates the presence of three aromatic protons in B-ring.



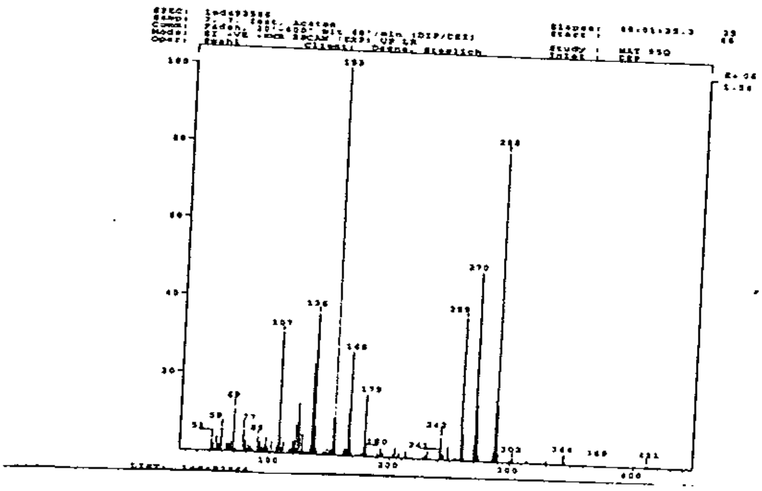


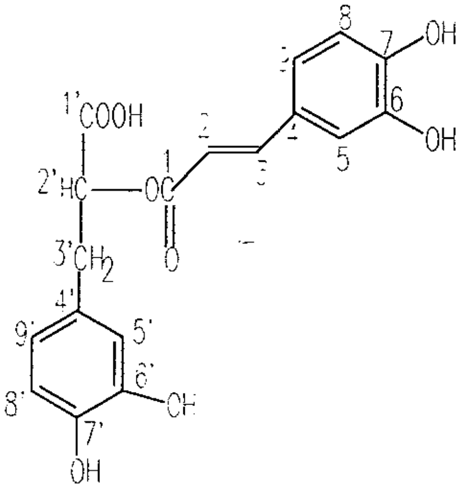
Fig. 6: EIMS of eriodicytol measured in 70 eV.

4.4.3 Rosmrinic acid (**3**).

A yellow oily substance of compound (**3**) was obtained from the mixture of acetone: methanol (1:1) extract after fractionated using column of silica gel followed by sepadex LH-20. The phenolic nature of the compound was confirmed by intensification of the brown colour on spraying the silica gel TLC spot with solution of fast blue reagent. The ^{1}H-NMR spectrum displayed a doublet at δ 7.4 for H-3 and at δ 6.16 for H-2 confirm the presence of an α,β-unsaturated carbonyl group in the skeleton of the compound. The 1H-NMR revealed the presence of six aromatic protons assignable to a doublet of doublet at δ 6.92 for H-9, δ 6.80 for H-5, δ 6.64 for H-8 and H-9', δ 6.53 for H-5' and H-8'. The ^{1}H-NMR spectrum of **3** measured in MeOH-d_4 show a doublet of doublet at δ 4.95 for methine proton at C-2' coupled with metylene protons at C-3' which are *diasterotopic* protons which have different chemical shift in any environment. The multiplate signals at δ 2.85-3.01 attributed for the methylene protons at C-3' confirm that the protons have different chemical environment which were coupled with each other and furtherly coupled with proton at C-2' which is a chiral center of the compound. The presence of 18 carbon atoms with one secondary, nine tertiary and eight quaternary carbon was evident from ^{13}C-NMR with DEPT spectrum.

Table 11: ^{13}C-NMR data of rosmrinic acid (75 MHz, MeOH-d_4)

C	δ
1	169.1
2	117.5[a]
3	146.8
4	131.0c
5	115.1[a]
6	149.5[b]
7	146.0[b]
8	116.2[a]
9	121.7[d]
1'	178.6
2'	77.5
3'	37.6
4'	127.8[c]
5'	115.4[a]
6'	146.9[b]
7'	144.8[b]
8'	116.5[a]
9'	123.0[d]



a, b, c, d: interchangeable

5. Conclusion

We conclude from this study together with literature that the oils obtained from *T. schimperi* and *T. vulgaris* were rich in phenolic monoterpenes which clearly indicate their medicinal value and as food additives. The principal components of the oils of these plants were thymol and carvacrol, which are known to have antimicrobial activities. These compounds are also reported toa have antioxidant properties when added to food products.The chemical investigation of the oils of *T. schimperi* from three locations showed significant difference in physicochemical and chemical composition. This indicates that there is no consistency on the quantitative and qualitative composition of the oil of this species.

The flavonoids isolated from *T. vulgaris* have also medicinal value to protect tissues and cells against various oxidative stresses.

6. Experimental.

6.1 General

The essential oils from the plant materials were obtained by steam distillation at atmospheric pressure using 4 l. round bottom flask with clevenger apparatus and a glass condenser. The plant material was chopped to a suitable size and entirely covered with water in the still which was heated by an electrical mantle. Distillation was continued until no more oil was collected. The oils were separated from the distillate and dried over anhydrous sodium sulphate. Sodium sulphate and other suspension impurities were removed from the oils by filtration and the drying agent was washed by volatile solvents to avoid loss in samples.

The dried and powdered aerial parts of the plant materials were subjected to a solvent extract at room temperature. The extracts were filtered and concentrated to dryness under reduced pressure every 24 hr. while the residues were further extracted with fresh solvents for three days.

Gas chromatographic analysis of the oils, fractions, standard samples and mixtures was performed on varian model 3700 gas chromatographed equipped with flame ionization detector (FID) using DB-5 non polar capillary column with 30m×0.53mm internal diameter and 1.5 micron film thickness. Analysis were performed by programming the column temperature from 60-170°C at 4°C/min. Injector and detector temperature were 220°C and 270°C respectively. N_2 at 25ml/min. was used as a carrier gas. Peak areas were determined with the aid of varian 4290 integrator. The composition of the essential oils of the three samples were recorded using perkin Elmer FTIR spectrometer with NaCl cell. The EIMS were measured with MAT 95Q on a 70eV.

6.2 Plant Materials.

Thymus vulgaris L. (S-864) was collected from the Essential Oils Research and Development Centre (EORC) plantation site at Wondogenet. Fresh samples of *Thymus schimperi* R. (S-913, S-954) were collected from Dinshu and Maichew. Dry samples (S-885, S-914) of this plant were also purchased from Debre Sina and Dinshu respectively. *Thymus Serrulatus* H. ex Benth (S-658) was collected from Addis Ababa University Science Campus. The plant materials were identified by Dr. Sebsibe Demissew. The voucher specimens are deposited at the National Herbarium Addis Ababa Univeristy.

6.3 Steam distillation.

The plant materials were chopped to a suitable size and subjected to steam distillation at atmospheric pressure using 4 l round bottom flask fitted with cleavenger apparatus and a glass condenser.

6.4 Isolation of Thymol.

2.5 g of the oil of *T. vulgaris* was run on 40 g of column of silica gel. This was eluted successively with petrol and ethyl acetate to obtain the hydrocarbon and oxygenated fraction respectively. The oxygenated fraction was further fractionated on a column of silica gel by increasing the polarity from petrol to petrol-EtOAc (95:5, 9:1, 8:2) and EtOAc. A total of 24 fractions each 25 ml were collected. The fractions were then concentrated using rotary evaporator. Each fraction is subjected to gas chromatography. GC analysis of fraction 21 obtained by eluting with petrol-EtOAc (9:1) shows 85% of the mixture was attributed from thymol. 25 ml of the oil of *T. vulgaris* was subjected to fractional distillation under reduced pressure so that 9 fractions were collected. The gas chromatography of the last fraction was found to be enriched by thymol.

This compound was also isolated from petrol and ethyl acetate extract of *Thymus schimperi*. 10 g of each petrol extract and ethyl acetate extract were adsorbed with silica gel and applied to a column of silica gel. The petrol and ethyl acetate extract were fractionated by increasing polarity from petrol to petrol-EtOAc (9:1, 8:2, 6:4, 4:6, 2:8) and EtOAc. A total of 24 fractions each from both extract were collected. The fractions were then concentrated using rotary evaporator. Fraction 11 from petrol extract and fraction 2 from EtOAc obtained by eluting with petrol-EtOAc (8:2) shows a single spot on the TLC (petrol : EtOAC; 95:5). The ^{1}H-NMR data obtained for fraction 11 and 2 agree very well with that reported for thymol in the literature [68]. The compound was furtherly confirmed by its IR and ^{13}C-NMR.

The IR ν_{max} cm^{-1}: 3398.9, 3010, 2961, 1584.4, 1513.9, 1423.4, 1251, 1166, 1115.4, 942.2, 810.6; ^{1}H-NMR (90 MHz, $CDCl_3$): 1.25 (6H, d, J=7.7, 8-CH_3); 2.25 (3H, s, 5-CH_3); 3.15 (1H, m, 8-H); 5.15 (1H, s,1-OH); 6.55 (1H, s, 6-H); 6.71 (1H, d, J=7.7, 4-H); 7.07 (1H, d, J=7.7, 3-H); ^{13}C-NMR (22.5 MHz, $CDCl_3$): see table 7.

petrol-EtOAc (6:4) containing a mixture of compound **2** was chromatographed on a sephadex LH-20 eluting with a mixture of $CHCl_3$-MeOH (1:1) and four fractions each 25 ml were collected. The last fraction shows a single spot on TLC (C_6H_6-petrol-EtOAc, 3:4:3 and $CHCl_3$-MeOH, 9:1) afforded 2.5 mg of eriodicytol (**2**). Fraction 21 was obtained by eluting with EtOAc-MeOH (1:1) was purified with sephadex LH-20 and collected ten fractions each 25 ml. Fraction 7-9 shows a single spot on TLC ($CHCl_3$:MeOH, 1:1) afforded 200 mg of rosmrinic acid (**3**).

Naringenin (**1**): Yellow crystal mp. 210-215^{0}C. IR $v_{max}cm^{-1}$: 3316.7,3116.7, 3011.7, 2933.3, 2850, 1633.3, 1600, 1525, 1500, 1450, 1316.7, 1250, 1183.3, 1150, 1079, 1066.7, 833.4, 733.1; ^{1}H-NMR (300MHz, Me_2CO-d_6): δ 12.2 (1H, s, 5-OH), δ 7.39 (2H, d, J = 8.58Hz, H-2' and H-6'), δ 6.90 (2H, d, J = 8.58Hz, H-3' and H-5'), δ 5.95 (2H, s, H-6 and H-8), δ 5.45 (1H, dd, J=2.97Hz and 12.84Hz, H-2), δ 2.7-3.3 (2H, br, H-3). MS m/z (%): 272 (M^+, 100), 271 (41.2), 179 (22.7), 166 (25.6), 154 (11.5), 153 (68.5), 152 (13.3), 149 (10.3), 120 (43.1), 107 (14.8). ^{13}C-NMR (75MHz, Me_2CO-d_5) see table 10.

Eriodicytol (**2**): yellow amorphous substance. IR $v_{max}cm^{-1}$: 3853.9, 3365.3, 2958.9, 2856.9, 1636.9, 1603.06, 1450.8, 1272.2, 1158.9, 1083.4 826.6 737.1; ^{1}H-NMR (90 MHz, Me_2CO-d_6): δ 12.15 (1H, s, 5-OH), δ 7.05 (1H, br, H-6'), 6.85 (2H, br, H-2' and H-5'), δ 5.95 (2H, s, H-6 and H-8), δ 5.45 (1H, dd, H-2) δ 2.65-3.2 (2H, m, H-3); MS m/z (%): 288 (M^- 79.2), 287 (15.5), 271 (14.4), 270 (48), 259 (36.3), 179 (15.7) 166 (26.5), 165 (10.8), 153 (100), 136 (35.9), 134 (23), 123 (13), 107 (30.8).

Rosmrinic acid (**3**): yellow oily substance. ^{1} H-NMR (300 MHz, MeOH-d_4): δ 7.4 (1H, d, J = 15.9 Hz, H-3), δ 6.92 (1H, dd, J = 1.83 Hz and 8.13 Hz, H-9), δ_5 6.80 (1H, dd, J = 1.86 Hz, H-5) δ 6.64 (2H, dd, J= 7.11Hz and 1.8Hz, H-8 and H-9'), δ 6.53 (2H, dd, J= 7.68Hz and 1.8Hz, H-5' and H-8'), δ 4.95 (1H, dd, J= 3.5Hz and 9.1Hz, H-2') δ 2.85-3.01 (2H, br, H-3'). ^{13}C-NMR (75MHz, MeOH-d_4) see table 11.

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