International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Chemical Analysis and Medicinal Activities of Volatile Components from the Seeds of *Croton Macrostachyus* Plant

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Abstract

One of the *Croton* species that is known for its medicinal use is *Croton macrostachyus* (Euphorbiaceae). It is one of the eight *Croton* species found in Ethiopia. The wide range medicinal uses of *Croton macrostachyus* led scientists to isolate compounds from its different parts. The primary objective of this research study was therefore to characterize the constituents of seeds of *Croton macrostachyus* (Euphorbiaceae) plant using GC-MS and to analyse the antimicrobial (medicinal) activity of the seed oil. 75 g of the powdered seeds of *Croton macrostachyus* was used for the extraction of the essential oil using hydrodistilation method. The oil was characterized using GC-MS and its biological activity was tested against some bacteria by Disk-diffusion method. GC-MS analysis of essential oils of *Croton macrostachyus* has shown over 40 components. From these, 19 compounds for each origin representing 82.86 % of *Croton macrostachyus* oil were identified as major components of the essential oil. 4-Hexen-1-ol, (E), Bis(2-ethylhexyl) phthalate, [1,1'-Biphenyl]-2-acetic acid, Epizonarene, Cyclopentene, 3-isopropenyl-5,5-dimethyl and 3-Carene were some of the major compounds identified from the oil. Antimicrobial results also showed that the essential oil was effective to control the growth of bacteria. So, collaborative works should be done between chemists, microbiologists and medical professionals to develop better medicinal drugs for the treatment of bacterial, fungal and viral infections.

Key words: *Croton macrostachyus;* disc diffusion method, essential oil; Gram negative bacteria; Gram positive bacteria; hydro distillation.

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1. Introduction

For thousands of years, several people depend on traditional medicines from flowers, bark, leaves and fruits of plants. Then synthetic drugs, most of which are similar to compounds identified in plants, come into use. In the past twenty five years, intensive efforts have been made to discover new clinically useful antibiotics. Recently, considerable research activities have focused on the identification of new antibacterial agents from plants, which led to the discovery of more than thousands of antibiotics out of which only dozens are promising and the search for new antibiotics among the lower plants has also increased [1]. Natural products are organic compounds that are formed by living systems. The elucidation of their structures and their chemistry, synthesis and biosynthesis are major areas of organic chemistry. Naturally occurring compounds may be divided into two broad categories. Firstly, there are those compounds which occur in all cells and play a central role in the metabolism and reproduction of those cells. These compounds include the nucleic acids, the common amino acids and sugars. They are known as primary metabolites**.** There are also the high-molecular-weight polymeric materials such as cellulose, the lignins and the proteins which form the cellular structures. Secondly, there are those compounds that are characteristic of a limited range of species. These are the secondary metabolites [2]. Use of natural products for curing human diseases is as ancient and universal as medicine itself. Most of the currently available drugs for treatment of different human and animal diseases obtained from natural products especially medicinal plants [3, 4, 5]. Such drugs have been discovered after observing the medicinal use of a particular plant or its parts (leaves, roots, barks, fruits or seed or whole plant) by herbalists, and subsequent isolation of bioactive compounds from the plant or part of the plant that was used traditionally for treatment of different human illnesses. Moreover, some compounds obtained from natural sources have also been used as leads or precursors that can be modified synthetically to improve their therapeutic activities [3, 4]. Introduction and development of several new and highly specific *in vitro* bioassay techniques, chromatographic methods, spectroscopic techniques and other standardized pharmacological methods have also made it much easier to screen, isolate and identify potential drug compounds quickly and precisely from natural sources to alleviate human illnesses [6, 7. 8]**.** Though natural products (e.g., medicinal plants) have many medicinal uses, there are several reasons that necessitate isolation and characterization of bioactive compounds from them. Some of the reasons are (i) distribution of medicinal plants is not uniform throughout the world to be used by people everywhere; (ii) most of the medicinal plants are under threat of extinction due to climate changes and population pressure; (iii) isolation and purification of compounds from natural sources is tedious, expensive and time consuming process; and (iv) the need to identify the chemical compounds that are responsible for the observed medicinal value of the plant [9]. *Croton* can be a tree, shrub or herbaceous plant which grows in tropical and warm regions. Some of the most popular uses include treatment of cancer, constipation, diabetes, digestive problem, dysentery, external wounds, intestinal worm, pain, ulcers and weight loss. Several *Croton* species are characterized by the presence of red sap [10]. One of the *Croton* species that is known for its medicinal use is *Croton macrostachyus* (Euphorbiaceae). The plant is native to some Eastern African countries such as Eritrea, Ethiopia, Kenya, Tanzania and Uganda. It is one of the eight *Croton* species found in Ethiopia [11]. In areas where it is native, the plant (or its parts) is used for treatment of several human health problems that include symptoms of diabetes [12], malaria [13], dysentery**,** stomach-ache, ascariasis and taeniasis [14], abdominal pain [15], gonorrhoea, wounds, ringworm infestation, hemorrhoids, venereal diseases, cough, rheumatismand as a

purgative in cases of ascariasis [16]. The main objective of this research study was therefore to characterize the constituents of seeds of *Croton macrostachyus* plant and to examine the antimicrobial activity of the seed oil towards some bacteria.

2. Materials and Methods

2.1. Collection of Plant Materials

The seeds of the plant were collected from a farmland around Hadiya zone near, Hossana town, Ethiopia. After collection the seeds were washed repeatedly first with tap water and then with sterilized distilled water and were allowed to air dry completely for 72 hours at room temperature.

2.2. Experimental Methods

2.2.1. Preparation of the powder

About 500 grams of the seeds of the plant were air dried and ground using electric blender. The resulting powder was stored in polyethylene bag to avoid it from attack of certain environmental conditions (moisture, air and other surrounding dusts).

2.2.2. Hydro-distillation

About 75 g of the powdered seeds of *Croton macrostachyus* was placed in 1000 ml round bottom flask. Then 500 ml of distilled water was added and mixed thoroughly. The flask was fitted with Clevenger's apparatus, a glass condenser and heated using heating mantle and hydro distilled at atmospheric pressure for 3 hours. The oil moiety was separated from the aqueous layer by adding 100 ml of chloroform in separatory funnel. The small amount of aqueous liquid left with the chloroform was then dried by adding 5 g of anhydrous sodium sulphate and filtered using Whattman no1 filter paper. Finally, the mixture was concentrated using rotary evaporator and kept in refrigerator until required for analysis.

2.2.3. GC-MS analysis of the essential oil of Croton Macrostachyus

A GC-MS instrument from Agilent Technologies (Santa Clara, CA, USA) equipped with a 6890N network GC system, 5975 inert mass selective detector, 7683B series autosampler injector (10 μL in size), G1701DA GC/MSD Chem Station and HP5MS column (30 m length x 0.25 mm internal diameter x 0.25 μm film thickness) coated with 5% phenyl 95% methyl poly siloxane was used for analyzing the samples. 2 μL essential oil solution in chloroform was injected through autosampler and analyzed with HP5MS column. Column temperature was programmed as follows (55 to 120 °C at 20 °C/min, 120 to 150 °C at 1.5 °C/min, 150 to 250 °C at 20 ^{o}C/min, 250 °C (10 min) and 3 min solvent delay. Mass spectra transfer line temperature was 280 °C. Carrier gas was helium (1 mL/min) with a split ratio equal to 100:1. Injector, quadruple and detector temperatures were 220, 150 and 250 °C, respectively. The mass spectra were recorded in electron ionization (EI) mode at 70 eV with scanning from 50 to 500 amu at 0.5 s with the mass source being set at 230 °C. The identification of the compounds was based on retention time (tR) and by comparison with the spectral data available in the literature. Integration of peaks was performed using Hewlett Packard Chem Station software

(G1701BA Version B.01.00) for quantification of the peaks.

2.2.4. Bioactivity Test

The biological activity of the essential oil was tested against some bacteria by Disk-diffusion method. Reference drugs were also used side by side to compare the antimicrobial effectiveness. Four microorganisms (Gram negative bacteria like *Escherichia coli, Pseudomonas aruginosa* and Gram positive bacteria like *Staphylococcus aureus, Staphylococci saprophyticus*) were selected and administered the isolated oil.

2.2.4.1. Preparation of inoculums

The test bacterial strains, *Escherichia coli, Pseudomonas aruginosa* (Gram- negative) and *Staphylococcus aureus, Staphylococci saprophyticus* (Gram-positive) were transferred from the stock cultures and streaked on Mueller Hinton plates and incubated for 24 hrs at 37^0c. Well separated bacterial colonies were then used as inoculums. Bacteria were transferred using bacteriological/inoculating loop to autoclaved Mueller Hinton agar that was cooled to about 45°C in a water bath and mixed by gently swirling the flasks. The medium was then poured to sterile Petri dishes, allowed to solidify and used for the biotest [17].

2.4.4.2. Preparation of sample solutions

The oil (concentrated and diluted) was used for the test with two replications (10 µl and 20 µl). distilled water was used as a solvent for the preparation of the solutions.

2.4.4.3. Testing for antibacterial activity

Paper discs of about 6 mm in diameter were cut from whatman-No.1 filter paper with an office paper punch and placed in a beaker covered with aluminum foil and sterilized in an oven at 180 ^{o}C for 1h. Then concentrated oil, 10 µl and 20 µl of solution of essential oil were pipetted to the discs. After allowing the solvent to evaporate, the paper discs impregnated with the sample solutions were then transferred with sterile forceps to Muller Hinton Agar (MHA) seeded with spore suspension of test bacteria as described above. The Petri dishes were incubated at 37^{o}C for 24hours. The antibacterial activity was evaluated by measuring the zone of inhibition against the test organism and Ceftriaxone as a control drug.

2.3. Statistical data analysis

The data obtained from GC-MS were recorded, organized and summarized. The compounds were identified by means of their retention times, mass spectral fragmentation patterns and by comparing their mass spectra with the NIST 2005 library of mass spectra. Antimicrobial activities of the essential oil were investigated by comparing the inhibition zones of the oil with the inhibition zone of reference drug (Ceftriaxone) and control solvent. Three samples of the essential oil at different concentrations were assayed on their medicinal activities. Each sample was analyzed individually in triplicate against different microorganisms for its antimicrobial activities and data is reported as mean ± standard deviation.

3. Results and Discussion

3.1. Results

3.1.1. Percentage yield of the Essential Oil

The hydrodistilation of the air dried powder from the seeds of *Croton macrostachyus* (75 g) collected from Hadya zone surrounding Wachemo University yielded a clear colorless essential oil of 4.34 g (5.78% w/w). From the total amount of the sample extracted by hydrodistillation only 2 µl of the essential oil was used for characterization by GC-MS while the majority oil was used for antimicrobial examination.

3.1.2. GC-MS display of essential oil of Croton macrostachyus

GC-MS analysis of essential oils of *Croton macrostachyus* has shown over 40 components (Figure 1). From these, 19 compounds for each origin were identified as major components of the essential oil. The compounds were identified by means of their retention times, by comparison with the spectra data in the literature and mass spectral fragmentation patterns and by comparing their mass spectra with the NIST 2005 library of mass spectra. Unidentified components were present in such low amounts that either no mass spectrum could be recorded or the spectrum was too poor for interpretation.

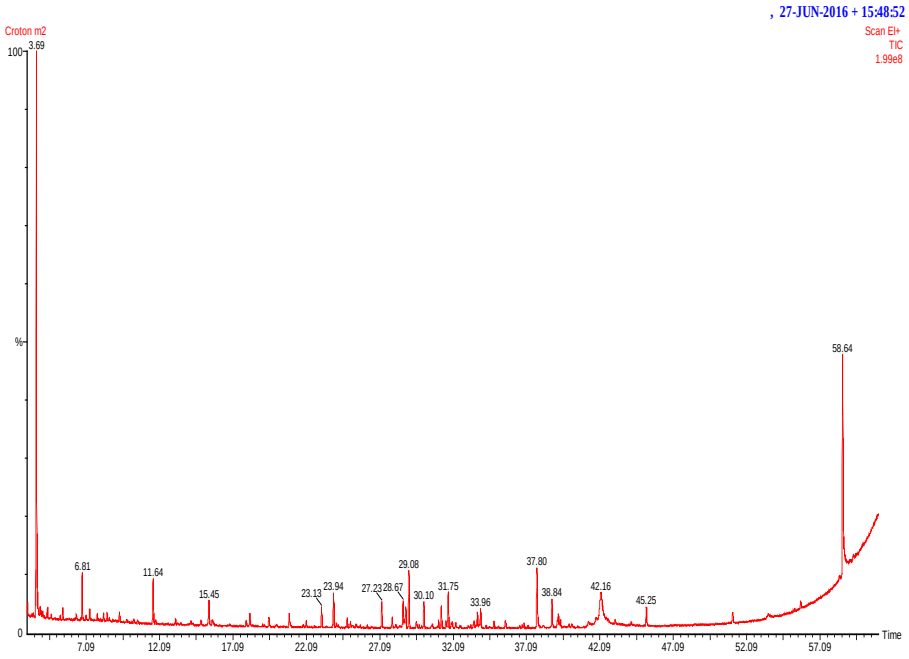


Figure 1: Typical GC-MS chromatogram of *Croton macrostachyus* essential oil showing the separation of chemical components

3.1.3. Investigation of medicinal activities of Croton macrostachyus

The antimicrobial activities of *Croton macrostachyus* essential oil extracts against the microorganisms examined in the present study, and their potency, were qualitatively and quantitatively assessed by the presence or absence of inhibition zones and zone diameter. The results are given in Tables 1 and 2.

Table 1: Zone of bacterial growth inhibition (mm) of essential oil of *Croton macrostachyus* against Gram negative (*Escherichia coli and Pseudomonas aruginosa*) bacteria

Sample		Bacteria	
Dose	*Escherichia coli*	*Pseudomonas aruginosa*	
Oil	concentrated	9.2± 0.42	9.24± 0.48
10 µl	7.4±0.42	7.6±0.67	
20 µl	7.1± 0.42	6.75±0.39	
Ceftriaxone		26.40±0.21	28.50±0.39
Distilled water		-	-

Values are represented in terms of mean of the three trials ± SD; - stands for no inhibition

Table 2: Zone of bacterial growth inhibition (mm) of essential oil of *Croton macrostachyus* against Gram positive (*Staphylococcus aureus, Staphylococci saprophyticus*) bacteria

Sample		bacteria	
Dose	*Staphylococcus aureus*	*Staphylococci saprophyticus*	
Oil	concentrated	8.8± 0.22	9.39± 0.42
10 µl	7.94±0.22	8.20±0.68	
20 µl	-	7.45±0.32	
Ceftriaxone		-	19.57±0.73
Distilled water		-	-

Values are represented in terms of mean of the three trials ± SD; - stands for no inhibition

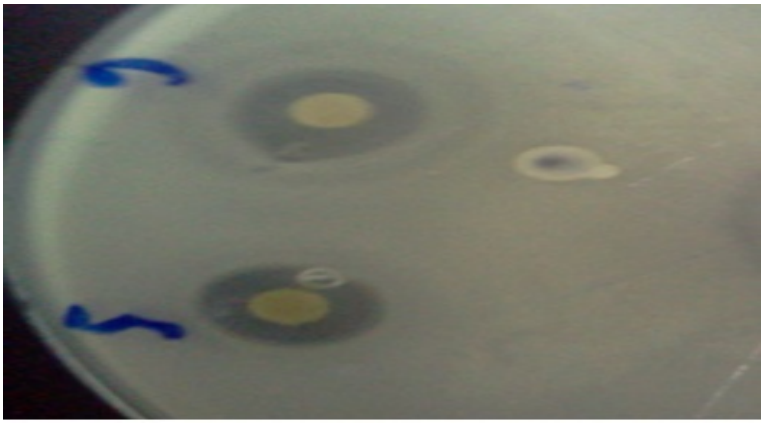


Figure 2: Inhibition zone of *Croton macrostachyus* essential oil against *Pseudomonas aruginosa(5*=oil diluted with 10 µl distilled water, 6=concentrated oil*)*

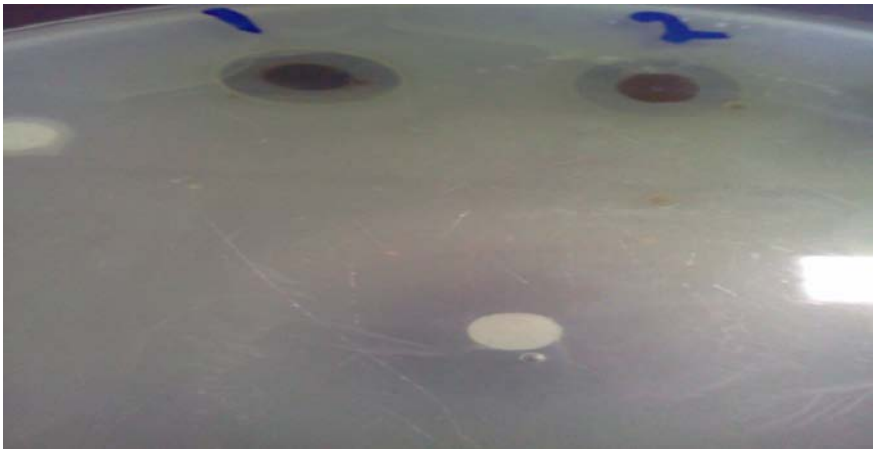


Figure 3: Inhibition zone of *Croton macrostachyus* essential oil against *Staphylococci saprophyticus(1=concentrated oil, 2*=oil diluted with 10 µl distilled water*)*

3.2. Discussion

3.2.1. Characterization of Croton macrostachyus Essential Oils

The yield of the essential oil were too undersized than it is expected (5.78 %). This is because seeds of many plants are rich of seed oils than essential oils and seed oils can evaporate at higher temperature which is too difficult to carry out with hydrodistillation.

The GC-MS spectrum of the essential oil is shown in (Figure 1) and the essential oil contents and components identified from the seeds of *Croton macrostachyus* are tabulated in Table 3. A total of 19 compounds representing 82.86 % of *Croton macrostachyus* oil were identified. 4-Hexen-1-ol, (E), Bis(2-ethylhexyl) phthalate, [1,1'-Biphenyl]-2-acetic acid, Epizonarene, Cyclopentene, 3-isopropenyl-5,5-dimethyl, 3-Carene, 3-n-Hexylthiane, S,S-dioxide, Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl), Caryophyllene andcis-2-Methyl-4-n-butylthiane, S,S-dioxide were identified as the major compounds.

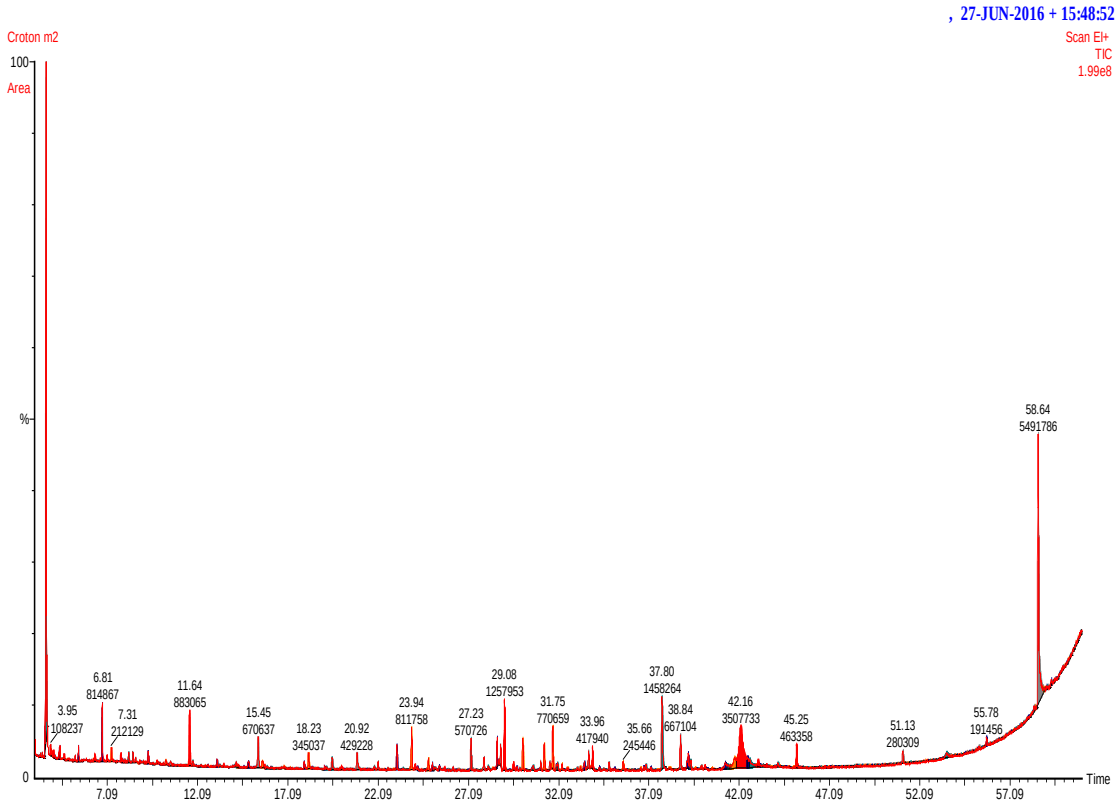


Figure 4: Area of *Croton macrostachyus* essential oil peaks at the specific t_R

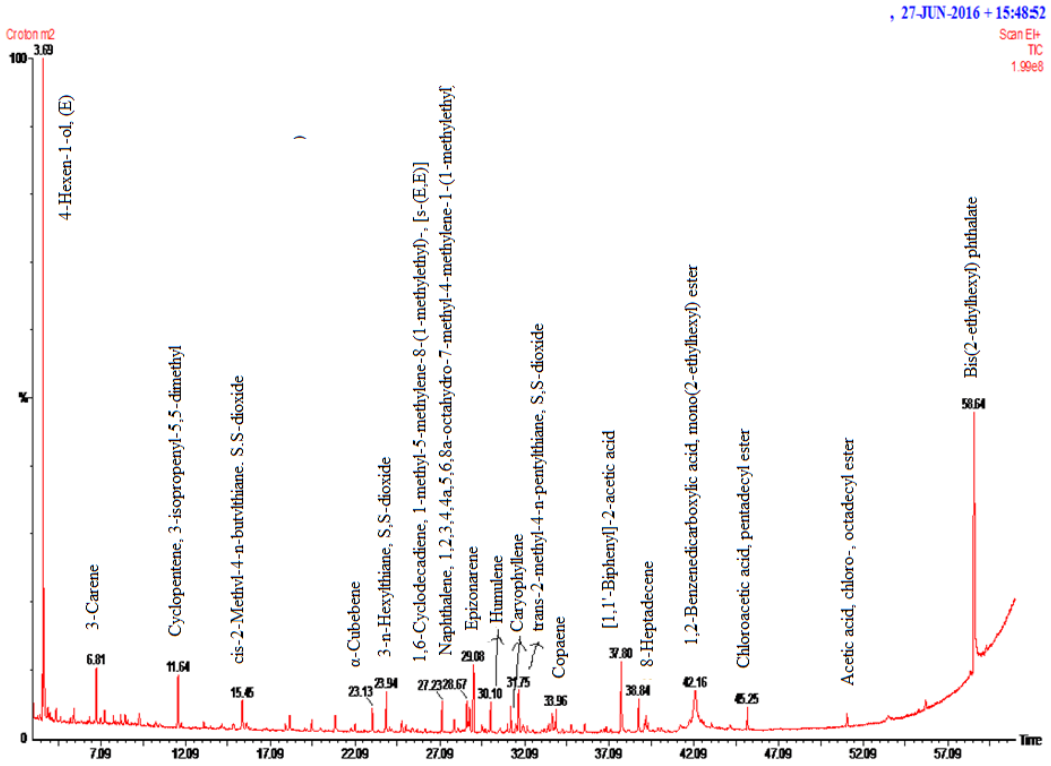
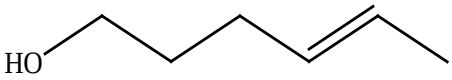


Figure 5: Typical Gas chromatogram of *Croton macrostachyus* essential oil with respect to its branded compounds

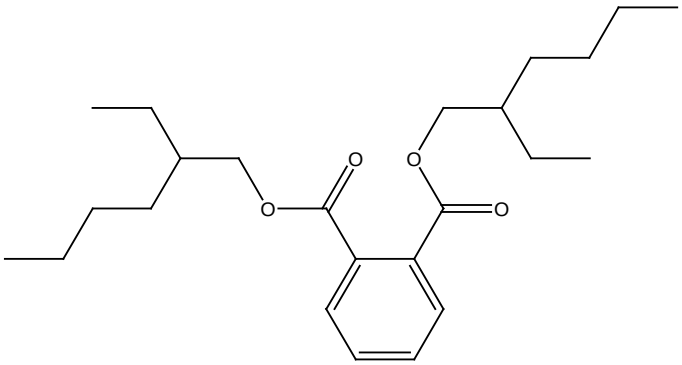
4-Hexen-1-ol, (E) (32.57%) which comprised of the majority of the essential oil is a colorless, oily liquid with an intense grassy green odor of freshly cut green grass and leaves. It is produced in small amounts by most plants and it acts as an attractant to many predatory insects. It is a very important aroma compound that is used in fruit and vegetable flavors and in perfumes.

Bis(2-ethylhexyl) phthalate (16.28%) which holds the second most composition of the essential oil is an organic compound with the formula $C_8H_4(C_8H_{17}COO)_2$. It is the most common member of the class phthalates which are used as plasticizers. It is the diester of phthalic acid and the branched chain2-ethylhexanol.



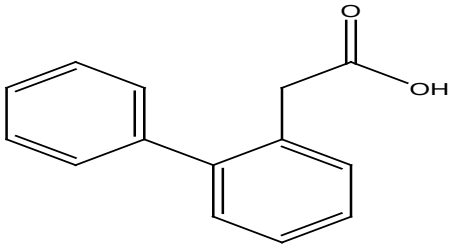
4-Hexen-1-ol, (E)

(A)



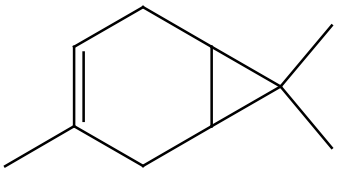
Bis(2-ethylhexyl) phthalate

(B)



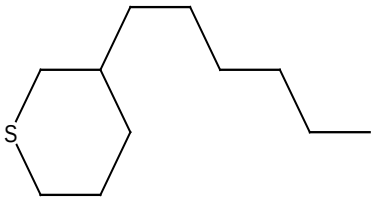
[1,1'-Biphenyl]-2-acetic acid

(C)



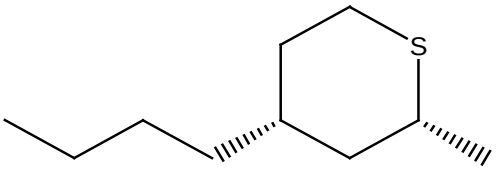
3-Carene

(D)



3-n-Hexylthiane

(E)



cis-2-Methyl-4-n-butylthiane,

(F)

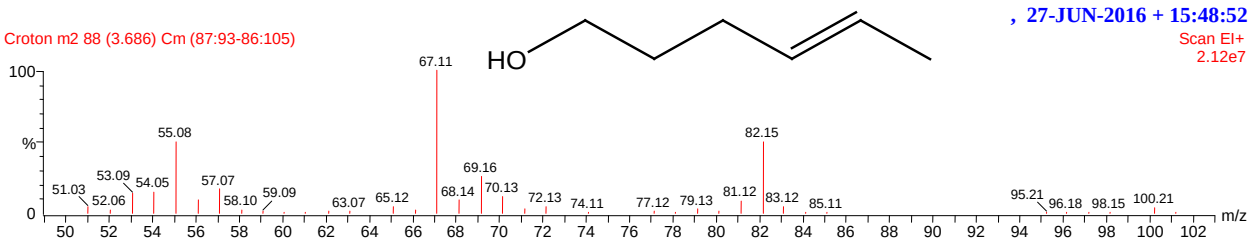
Figure 6: Proposed structures of some of the major components of *Croton macrostachyus* essential oil

Table 3: GC-MS analysis of the hydrodistilled seeds of *Croton macrostachyus* essential oil

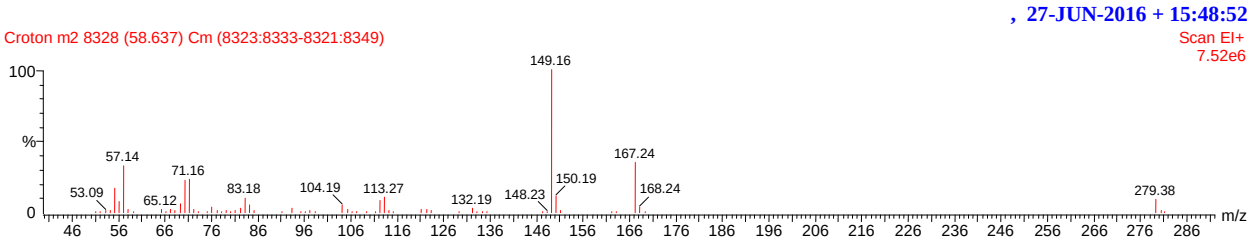
No.	Retention time	Compound name	Relative percentage (%)
1	3.69	4-Hexen-1-ol, (E)	32.57
2	6.81	3-Carene	2.42
3	11.64	Cyclopentene, 3-isopropenyl-5,5-dimethyl	2.62
4	15.45	cis-2-Methyl-4-n-butylthiane, S,S-dioxide	1.98
5	23.13	α-Cubebene	1.88
6	23.94	3-n-Hexylthiane, S,S-dioxide	2.41
7	27.23	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)-, [s-(E,E)]	1.69
8	28.67	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)	2.07
9	29.08	Epizonarene	3.73
10	30.10	Humulene	2.08
11	31.28	Caryophyllene	2.07
12	31.75	trans-2-methyl-4-n-pentylthiane, S,S-dioxide	2.28
13	33.96	Copaene	1.24
14	37.80	[1,1'-Biphenyl]-2-acetic acid	4.32
15	38.84	8-Heptadecene	1.98
16	42.16	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	1.04
17	45.25	Chloroacetic acid, pentadecyl ester	1.37
18	51.13	Acetic acid, chloro-, octadecyl ester	0.83
19	58.64	Bis(2-ethylhexyl) phthalate	16.28
Total			82.86%

There is lack of plenty works on the extraction and identification of bioactive ingredients from the volatile components of the seeds of the plant and probably this could be the first research to extract essential oils from the seeds of *croton macrostachyus* and characterizing its constituents using GC-MS. However, some considerable researches have been performed about the volatile and non volatile components of the seeds and areal parts of *Croton* species other than *croton macrostachyus.*

The yield of essential oil obtained by hydrodistillation of dried seeds of *Croton macrostachyus* was fairly comparable with those previously reported for other species of *Croton* in different parts of the world. Hydrodistillation of dried leaves of *Crotonheliotropiifolius* and *Crotonpulegiodorus* produced 0.2% and 5% of oil, respectively [18]. While fresh branches of *Crotonadamantinus* gave 0.6% of oil [19] and fresh leaves and branches of *Crotoncampestris* yielded 0.04% and 0.02% of oil, respectively [20]. Additionally, [21] reported a 3.15% yield of essential oil following steam distillation of the dried aerial parts of *Crotonzehntneri*.



A) 4-Hexen-1-ol, (E)



B) Bis(2-ethylhexyl) phthalate

Figure 7: Mass Spectra of the two major components of the essential oil of *Croton macrostachyus*

The characteristic features of the essential oil of *croton macrostachyus* obtained in the present study moderately resembled to those reported by [22] in which a total of 57 compounds, mainly mono- and sesquiterpenoids, were identified by GC-MS analysis of the freshly isolated essential oil of *Croton rhamnifolioides*, and these components represented more than 92% of the total oil. According to this report the major constituents of the oil was the oxygenated sesquiterpene sesquicineole (16.79%), followed by the monoterpene α-phellandrene (12.83%), the oxygenated monoterpene 1,8-cineole (7.24%), and the sesquiterpene (*E*)-caryophyllene (6.33%).

Reference [23] reported presence of 15 different compounds identified from the volatile components of the different *Croton* species. According to [24] in the essential oil of Croton isabelli, 14 compounds were identified, representing 98.2 % of the essential oil, which was characterized by the exclusive presence of sesquiterpenes.

The oil composition studied, significantly differs from the oil of the same species studied in other places. This is due to the fact that the chemical compounds of any plant essential oil can vary greatly depending upon geographical region, the age of the plant, local climate; seasonal variations, experimental conditions and genetic difference are responsible for the changes in the types of chemical compounds.

3.2.2. Analysis of antimicrobial effectiveness of Croton macrostachyus essential oil

The strongest inhibition zone of *croton macrostachyus* essential oil was observed against *Staphylococci saprophyticus* and *Pseudomonas aruginosa* (9.39± 0.42 mm and 9.24± 0.48 mm respectively). Moreover, the inhibition zone of the control drug (Ceftriaxone) was higher in *Pseudomonas aruginosa* and *Escherichia coli*

(28.50±0.39 mm and 26.40±0.21 mm respectively). Results also showed that as the concentration of the essential oil is minimized, the killing strength of the oil also diminished proportionally.

There was also a lack of plenty works on the antimicrobial activities of essential oils from the seeds of *C. macrostachyus* which could be due to the specific location of the plant. The plant is not distributed worldwide. Ethiopia, Eritrea, Tanzanya, Uganda and Nigeria are the five African countries with a broad distribution of the plant.

The results of this study are in a good agreement with [25] whoreported analgesic and anti-inflammatory properties of the aqueous and methylene chloride/methanol extracts of the stem bark of *Croton macrostachyus*. A recent report by [26] showed larvicidal activity of *Croton macrostachyus* against *Anopheles arabiensis* Patton (a potent malaria vector). Its crude extract was found to demonstrate high activity against reference strain of *N. gonorrhoeae* and mitogenicactivity on human lymphocytes and mice spleenlymphocyts.

Recent reports also indicated that essential oils from *Croton macrostachyus* possess antibacterial activities [27] and antileishmanial activities [28]. The oils that were obtained from berries of the plant were tested for their antileishmanial activities (against *L. donovani*and *L. aethiopica* promastigotes and axenic amastigote stages) and were found to have high efficacy.

4. Conclusions

More compounds can be isolated from the essential oils of the plant grown in different geographical location using GC-MS and other spectroscopic methods. The medicinal activities of essential oils from *C. macrostachyus* may be due to the presence of high content of biologically active ingredients (phytochemicals).

5. Recommendations

Essential oils from the seeds of the plant could replace synthetic antimicrobial agents in the future. Studies should also be extended to evaluating the practical effectiveness of essential oil against the growth of different food borne and spoiling microbes under the specific environmental, storage, and food processing conditions. Moreover, the use of this plant and its derivatives for the primary purpose of flavoring will be of interest for further study.

Acknowledgements

The authors thank to the Department of Chemistry, Ambo University for cooperation in running the GC-MS results of the samples. We would also like to be grateful for the staff members of Hossana regional laboratory for voluntarily assisting us during antimicrobial experiments.

Conflict of interest

None declared

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