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Steam distillation extraction and chemical composition of essential oils of *Toddalia asiatica* L. and *Eucalyptus camaldulensis* Dehnh.

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Abstract
Plant extracts and secondary metabolites such as essential oils (EOs) have a wide range of benefits and applications. Essential oils have been recognized as some of the most promising compounds in the development of novel products particularly in the pharmaceutical, agricultural, food and perfumery industries. The current research work aimed at extraction and chemical characterization of the essential oils from fruits of *Toddalia asiatica* L. and leaves of *Eucalyptus camaldulensis* Dehnh. Essential oil extraction was carried out by use of steam distillation method in a modified Clevenger-type apparatus, while the chemical composition of the oils was established by gas chromatography coupled with mass spectrometry (GC-MS). The oil yields were 0.5181 and 0.2514% (w/w) in *T. asiatica* and *E. camaldulensis*, respectively. The GC-MS analyses identified a total of 41 and 54 compounds corresponding to 90 and 95% of the total oil in *T. asiatica* and *E. camaldulensis*, respectively. Both oils were rich in monoterpenes and sesquiterpenes and their analogues namely; oxygenated monoterpenes and sesquiterpenes. The oils additionally contained non-terpenoid oxygenated hydrocarbons which accounted for 4.9 and 14.8% of all the identified components in *T. asiatica* and *E. camaldulensis*, respectively.

Keywords: Essential oil, steam distillation, *Toddalia asiatica, Eucalyptus camaldulensis*, terpenes.

1. Introduction
Toddalia asiatica L also known as the orange climber or wild orange is an evergreen woody liana that belongs to Rutaceae family. This plant is native to tropical Asia from India and Sri Lanka to Malaysia but has since been naturalized in many regions of the world [1]. The plant is currently found in the tropical and sub-tropical regions of Africa such as South Africa, East Africa, Mauritius and Madagascar where it grows mostly in forested riverine habitat [2]. *Toddalia asiatica* can grow up to a height of 15 m using other trees for support, aided by hooked thorns that are profusely found within its branched stems [3]. It produces small (5-7 mm) citrus-like fruits which are green in colour but turn orange on ripening. Both the fruits and leaves produce a lemony odour when crushed, with the fruits having a feel and taste comparable to that of an orange rind [4]. *Toddalia asiatica* is a plant of great medicinal importance in many parts of the world. In East Africa for example, the plant and its derivatives are used in preparation of folklore remedies for numerous ailments such as pain and inflammatory [5], malaria, stomachache and sore throat [6], and skin, respiratory and urinary tract infections [7]. In India, *T. asiatica* is used traditionally for treatment of cough, fever, epilepsy and dyspepsia [1] and also as an expectorant, analgesic, diaphoretic and anti-inflammatory [8]. Moreover, pharmacological studies have revealed promising biological activities of the plant such as antitumor activity [9], antimalarial activity [10], antifungal and antibacterial activity [11] and antiviral activity [12].
Eucalyptus (Myrtaceae) represents an important genus of about 800 species, hybrids and varieties that are native to Australia and Tasmania [13]. Most members of this genus have been successfully naturalized in other regions of the world where they are grown for various purposes [14]. Currently, *Eucalyptus* trees constitute the most extensively grown hardwoods in the temperate and subtropical zones, with members of the genus grown extensively for timbers and other construction materials, power transmission poles, pulp and paper, production of oils for perfumery and pharmaceutical industries and as ornamentals [15, 16]. In Kenya, *Eucalyptus* species were introduced by the colonial government in the early 1900s to provide building material for Kenya-Uganda railway construction and also to act a substitute to the declining natural forests [16]. Since then, these aggressive growers have become some of the most superior trees in the country in terms of species diversity, number of uses, and exceptionally robust growth. *Eucalyptus* species constitute a major

reservoir for a wide range of secondary metabolites many of which have been found to harbor a diverse range of biological activities [17, 18, 19]. A number of constituents isolated from *Eucalyptus* trees have been shown to have antibacterial, antifungal, antioxidant and repellent activities [20, 21]. *Eucalyptus camaldulensis* commonly known as the river red gum is widely grown in different agro-ecological zones of Kenya [22]. It is a medium-sized, fast-growing tree that can reach heights of 25- 30 metres and 1 metre d.b.h. but can sometimes grow to heights of up to 50 metres [23]. The tree has a generally smooth bark that can be grey, white or brown.

Fig 1: *Toddalia asiatica* leaves and fruits (A) and *Eucalyptus camaldulensis* leaves (B)

2. Material and Methods

2.1 Sampling of plant materials

Fruits of *T. asiatica* were collected from their natural habitat within Maseno area (0º0'10.39"S, 34º36'71"E; 1524 m.a.s.l), Kisumu County, Kenya while *E. camaldulensis* leaves were sampled from a tree plantation within the same area. Samples of collected plant materials were prepared, packaged and stored according to the herbarium rules and regulations. Identification and authentication of the collected plant samples was performed by a Plant Taxonomist at the School of Biological Sciences, University of Nairobi and voucher specimens deposited at the University's herbarium.

2.2 Essential oil extraction

Extraction of the essential oils was carried out at the Department of Chemistry, Maseno University. The essential oils were extracted from each of the plants separately. Leaves of *E. camaldulensis* were chopped into small pieces (about 10-15 cm) while for *T. asiatica*, whole fruits were distilled. Four kg of the plant material was weighed and loaded into the still of a flat-bottomed distillation tank that formed part of the modified Clevenger-type apparatus [24] (Figure 1). Eight liters of water was poured into the tank and the rid secured tightly. The plant materials were then subjected to steam distillation with the collection of the oils starting after a heating time of 50 minutes and continued until no more essential oil was obtained (5-8 hours). The volatile oils were collected from the top of the hydrosol and dried over anhydrous sodium sulphate (Na_2SO_4). The oils were filtered using Whatman filter paper (No.1), weighed and collected into 3 ml airtight glass vials. The essential oils were then stored at –20 °C in a freezer until when required for chemical analysis.

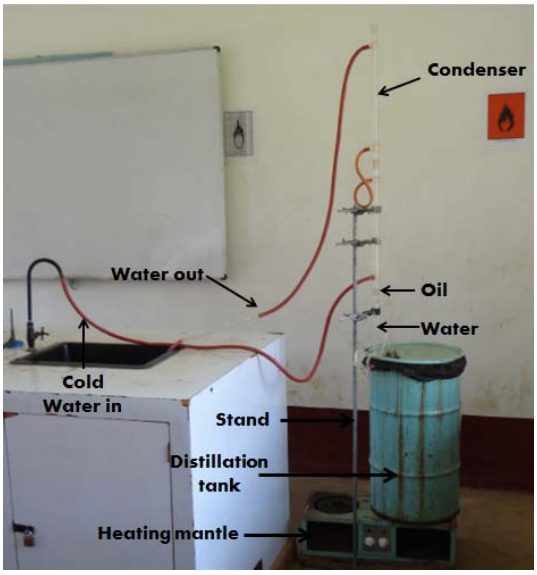

Fig 2: A modified Clevenger-type apparatus used to extract the essential oils of *T. asiatica* and *E. camaldulensis*

2.3 Gas chromatography-mass spectrometry

Samples of the essential oils were taken to the International Centre of Insect Physiology and Ecology (ICIPE) laboratories, Nairobi, Kenya for chemical analysis by Gas chromatography-mass spectrometry (GC-MS). Three replicates (each taken from a different extraction batch) of 1mg of *T. asiatica* and *E. camaldulensis* oils were separately weighed and diluted in 1ml volume of dichloromethane (DCM) to make a stock solution from which further dilutions were made to a final concentration of 100ng/µl which was then analyzed by GC-MS with the following conditions: HP-7890A (Agilent Technologies, Wilmington, USA) GC connected to an HP 5975C (Agilent, Wilmington, USA) MS. The GC equipment was fitted with HP-5MS capillary column; 30 m × 0.25 mm internal diameter; 0.25 µm film thickness with 5%-phenyl methyl silicone as the stationary phase (J & W Scientific, Folsom, USA). The carrier gas was Helium (1.2 ml min^{-1}) and the injector temperature was kept at 270 ºC. The oven temperature was programmed at 35 °C (for 5 min) to 280 °C at 10ºC min^{-1} and then held isothermal at 280 °C for 10.5 min.; injection mode was splitless. Mass spectra were acquired at 70 eV within a mass range of 38–550 Daltons (Da) with a scan time of 0.73 scans s^{-1} whereas the ion source was maintained at a temperature of 230 °C. Identification of the essential oil components was achieved on the basis of their retention indices (RI) (determined with reference to a homologous series of normal alkanes C_5-C_{31}) and calculated based on the equation of Van den Dool and Kratz and comparison with what is documented in literature [25, 26, 27].

The identity of essential oil constituents was further verified by comparison of their mass spectral fragmentation patterns with those reported in the mass spectra library database (NIST05a and Adams MS HP, USA). To quantify terpenes in the essential oils, serial dilutions of authentic standard (1, 8-cineole; 99%; Gillingham, Dorset, England) (50-550 ng/µl) were analyzed by GC/MS in full scan mode to generate a linear calibration curve (peak area vs. concentration) with the following equation [$y = 7E+06x – 1E+07$ (R^2=0.9736)], which was used for the external quantification of the different terpenes.

3. Results and discussion

3.1 Essential oil yield

Four steam distillation batches yielded 0.5181% w/w essential oils from *T. asiatica* fruits, while a mean yield of 0.2514% w/w was obtained from the same number of distillation batches of *E. camaldulensis* leaves. In the case of *T. asiatica,* the percentage yield of the oil obtained in the current study was very much close to those reported previously i.e. 0.50 and 0.25% w/w from fruits and leaves of *T. asiatica*, respectively [28]. This could be attributed to the fact that the plant materials in these two studies were collected from the same geographical region, namely, Kisumu County, Kenya. For *E. camaldulensis*, much higher EO yields than those obtained in the current study have been reported in literature. For example, essential oil yield of 1.40% w/w was obtained from hydrodistillation of fresh leaves of *E. camaldulensis* [29] while in another study, adult fresh leaves, stems, and immature flowers of *E. camaldulensis* produced essential oil yields of 1.4, 0.57, and 0.46% (w/w), respectively [13].

These variations in essential oil yields are as a result of several biotic and abiotic factors involved in determining the amount and constituents of essential oils. Numerous studies have shown that it is possible for both yields and chemical profiles of essential oils from plants that are botanically identical to vary considerably [30, 31]. The variations in oil yields and chemical composition among plant belonging to similar taxonomic groups have been attributed to biotic and abiotic factors that exist in the external and internal environments of plants [32]. Moreover, other factors such as the stage of harvest [33]. Plant cultivation and/or harvesting procedures [18] plant parts used [34] extraction method employed [35, 36] and processing procedures such as drying of the plant materials [37, 38] have all been found to significantly affect the two essential oil qualities.

3.2 Some characteristics of the essential oils

The densities of the essential oils were obtained by taking the weight of 1ml of the each oil separately and the values obtained were used to calculate the oils' densities. *T. asiatica* oil had a density of 0.87 g/ml while *E. camaldulensis* has a density of 0.92g/ml. Both oils were less dense and insoluble in water. The oils were however soluble in ethanol, dichloromethane (DCM) and dimethyl sulfoxide (DMSO) at a level of 1:1(v/v). The oils had a watery viscosity and exhibited a pale yellowish color when observed against a white background (Figure 3). The oils were liquid at room temperature and maintained this state even on storage at -20 °C. *Eucalyptus camaldulensis* essential oil had a clear, sharp and very distinct minty/pine smell while *T. asiatica* had a strong lemony odour.

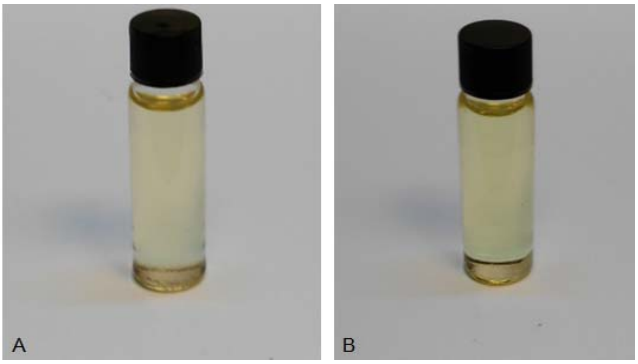

Fig 3: (A) *Toddalia asiatica* and (B) *Eucalyptus camaldulensis* essential oils in 3 ml airtight glass vials exhibiting a pale yellowish color

3.3 Chemical composition of the essential oils of *Toddalia asiatica* and *Eucalyptus camaldulensis*

Gas chromatography - mass spectrometry analysis identified forty-one compounds corresponding to 90% of the total oil of *T. asiatica* (Table 1). The oil contained majorly a mixture of monoterpenes and sesquiterpenes. The most abundant monoterpenes was Sabinene (15.8%) followed by β-Pinene (9.5%) while the least was δ-3-Carene (3.6%). Sesquiterpenes concentration on the other hand ranged from 0.3-1.6% with geranyl acetate and β-Copaene topping the list at 1.6% and 1.2%, respectively. The most abundant classes of compound from the EO were non-oxygenated monoterpenes and sesquiterpenes, which accounted for 31.7% and 24.4%, respectively, of all the identified constituents. Other classes of compounds included oxygenated sesquiterpenes (22%), oxygenated monoterpenes (14.6%), non-terpenoid oxygenated hydrocarbons (4.9%) and oxygenated diterpene (2.4%). Most of the components identified in EOs of *T. asiatica* in the this study such Linalool, Phytol, α- Phellandrene, α-Pinene and Myrcene have been cited in literature as some of commonest constituents in *T. asiatica* essential oils [39,40].

Table 1: Compounds identified in the essential oil of *Toddalia asiatica* along with the retention indices and concentration percentage.

No[a]	RT (min)	Compound Name	RI[a]	Concentration mean (%) ±SE
1	9.574	α-Thujene	910	2.6±0.08
2	9.65	α-Pinene	913	1.4±0.09
3	10.581	Sabinene	955	15.8±0.06
4	10.996	Myrcene	974	4.4±0.06
5	11.218	α- Phellandrene	984	2.8±0.08
6	11.312	δ-3-Carene	988	3.6±0.10
7	11.47	δ -2-Carene	996	5.7±0.05
8	11.733	β-Pinene	1010	9.5±0.01
9	11.844	(Z)-β-Ocimene	1016	2.8±0.08
10	12.043	(E)-β-Ocimene	1028	2.4±0.08
11	12.265	ɣ-Terpinene	1042	5.6±0.04
12	12.371	(E)-(IPP vs OH)-Sabinene hydrate	1048	0.8±0.02
13	12.745	Terpinoline	1048	3.2±0.08
14	13.043	Linalool	1089	8.3±0.01
15	13.324	(Z)-p- Menth-2-en-1-ol	1106	0.5±0.03
16	13.435	Allo- Ocimene	1112	4.3±0.05
17	13.704	(E)-pMenth-2-en-1ol	1128	0.7±0.10
18	14.284	Terpinen-4-ol	1162	6.3±0.06
19	14.617	Decanal	1182	1.3±0.09
20	14.705	(Z)-Piperitol	1187	0.6±0.10
21	14.956	Nerol	1202	0.5±0.13
22	15.372	Linalool propanoate	1229	4.5±0.05
23	15.822	Bornyl acetate	1259	0.4±0.10
24	16.822	Neryl acetate	1328	0.9±0.10
25	17.086	Geranyl acetate	1347	1.6±0.09
26	17.279	β-Elemene	1361	0.5±0.10
27	17.402	Decylacetate	1370	0.8±0.10
28	17.682	β-Copaene	1390	1.2±0.10
29	17.694	(E)-Caryophyllene	1391	0.6±0.02
30	18.004	Germacrene D	1414	0.4±0.12
31	18.133	α-Humulene	1424	1.0±0.10
32	18.238	(E)-Muurola-4(14),5-diene	1432	0.5±0.10
33	18.384	ɣ- Amorphene	1443	0.4±0.23
34	18.671	α- Muurolene	1466	0.8±0.10
35	18.858	ɣ-Cadinene	1480	0.4±0.10
36	18.952	α-Cadinene	1487	0.7±0.10
37	19.361	(E)-Nerolidol	1520	0.6±0.09
38	19.66	Spathulenol	1545	0.4±0.09
39	19.976	Ledol	1570	0.3±0.09
40	20.075	iso- Leptospermone	1579	0.9±0.06
41	25.059	Phytol	2082	0.3±0.10

[a]No = Peak numbers referring to Figure 4
[a]RI = Retention index
SE = Standard error of the mean

Eucalyptus camaldulensis essential oils showed a more complex composition with fifty-four compounds representing 95% of the total leaf oil identified (Table 2). Like was the case with *T. asiatica* oil, *E. camaldulensis* EO contained majorly a mixture of monoterpenes and sesquiterpenes hydrocarbons. The most abundant monoterpene was 1,8-Cineole (16.2%) followed closely by α-Pinene (15.6%) while the least was δ-2-Carene 0.2%. Sesquiterpenes concentration on the other hand ranged from 0.2 -2.1% with iso-Leptospermone and (E)-Caryophyllene being the most abundant within this group of compounds accounting for 2.2% and 1.6% respectively. In summary, the most abundant classes of compound from this oil were oxygenated monoterpenes and sesquiterpenes which accounted for 29.7% and 22.2%, respectively, of all the identified components. Other classes of compounds were non oxygenated monoterpenes (18.5%), non-terpenoid oxygenated hydrocarbons (14.8%), oxygenated sesquiterpenes and non-terpenoid hydrocarbons each of which accounted for (7.4%) of all components identified in the oil. The major component in *E. camaldulensis* EO was 1,8-cineole, an oxygenated monoterpene that has been cited as the principal component in EOs of *E. camaldulensis* [18, 19, 41]. α-Pinene, a non-oxygenated monoterpene which was the second most abundant constituent has also been reported in literature as one of the most abundant constituent in the EOs of *E. camaldulensis* [17, 19, 42].

Table 2: Compounds identified in the essential oil of *Eucalyptus camaldulensis* along with the retention indices and concentration percentage

No[b]	RT (min)	Compound Name	RI[b]	Concentration mean (%) ±SE
1	4.427	Isopentyl formate	714	1.1±0.01
2	6.211	2,4-dimethyl-3-Pentanone	779	0.3±0.02
3	9.411	2-methylpropyl- 2-methylpropanoate	903	0.9±0.01
4	9.838	α-Pinene	922	15.6±0.18
5	10.054	Camphene	932	1.8±0.01
6	10.645	β-Pinene	958	4.6±0.04
7	10.961	Myrcene	972	0.6±0.02
8	11.224	α-Phellandrene	984	10.0±0.11
9	11.476	δ-2-Carene	996	0.2±0.02
10	11.669	p-Cymene	1006	8.1±0.08
11	11.809	1,8-Cineole	1014	16.2±0.19
12	12.037	(E)-β- Ocimene	1028	0.2±0.02
13	12.143	dihydro-Tagetone	1034	0.2±0.02
14	12.277	ɣ-Terpinene	1042	4.4±0.03
15	12.500	2,6-dimethyl-3,5-Heptanedione	1056	0.4±0.02
16	12.739	Terpinolene	1070	1.9±0.01
17	12.777	α-Terpinene	1317	0.5±0.02
18	12.915	3,7-dimethyl-1,6-Octadien-3-ol	1081	0.5±0.02
19	12.997	3-methyl-Butanoic acid-3-methylbutyl ester	1086	0.6±0.02
20	13.044	n-Amyl isovalerate	1089	0.4±0.02
21	13.190	endo-Fenchol	1098	1.6±0.01
22	13.377	2,6-dimethyl-2,4,6-Octatriene	1109	1.4±0.01
23	13.634	(E) - Pinocarviol	1124	2.3±0.01
24	13.699	Camphor	1128	0.3±0.02
25	13.757	Camphene hydrate	1131	0.3±0.02
26	13.980	Pinocarvone	1144	0.8±0.01
27	14.079	Borneol	1150	2.9±0.01
28	14.237	Terpinen-4-ol	1159	2.0±0.01
29	14.483	α-Terpineol	1174	4.4±0.03
30	14.541	Myrtenol	1177	0.9±0.01
31	14.834	(E)-Carveol	1194	0.5±0.02
32	15.155	Cumin aldehyde	1215	0.3±0.02
33	15.202	Carvone	1218	0.2±0.02
34	15.267	Carvotanacetone	1222	0.6±0.02
35	15.840	(E)- Isosafrole	1261	0.3±0.02
36	15.980	Carvacrol	1270	0.7±0.02
37	16.536	1,5,5-Trimethyl-6-methylene-cyclohexene	1308	0.5±0.02
38	17.033	Isoledene	1343	0.2±0.02
39	17.080	α-Copaene	1347	0.3±0.02
40	17.361	Methyl eugenol	1367	0.3±0.02
41	17.548	α-Gurjunene	1380	0.6±0.02
42	17.700	(E)-Caryophyllene	1391	1.6±0.01
43	17.788	β-Gurjunene	1398	0.2±0.02
44	17.952	β-Selinene	1410	1.0±0.01
45	18.233	α-Guaiene	1432	0.7±0.01
46	18.350	Zonarene	1441	0.3±0.02
47	18.572	Δ-Selinene	1458	0.5±0.02
48	18.654	Viridiflorene	1464	1.1±0.01
49	18.859	ɣ- Muurolene	1480	0.2±0.02
50	18.952	δ-Cadinene	1487	0.5±0.02
51	19.555	ɣ-Eudesmol	1536	0.3±0.03
52	19.672	Spathulenol	1546	0.7±0.02
53	19.754	Globulol	1553	0.7±0.01
54	20.093	iso-Leptospermone	1581	2.2±0.01

[b]No = Peak numbers referring to Figure 5
[b]RI = Retention index
SE = Standard error of the mean

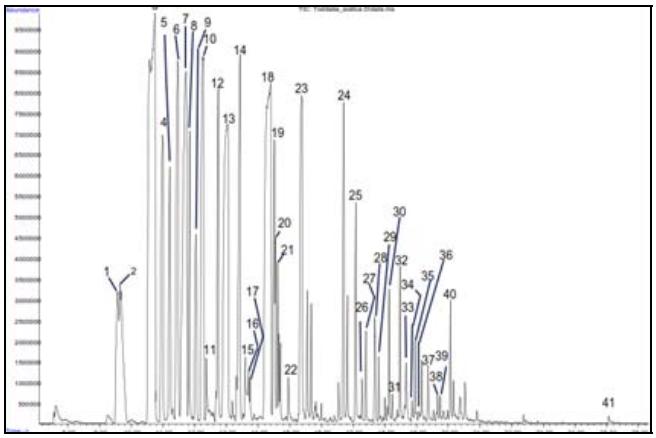

Fig 4: Representative total ion chromatogram of *Toddalia asiatica* essential oil

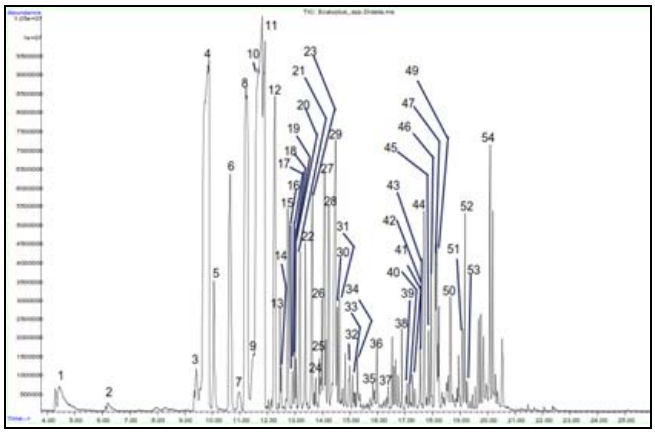

Fig 5: Representative total ion chromatogram of *Eucalyptus camaldulensis* essential oil.

4. Conclusion

The results obtained revealed that that the chemical profiles of the two steam-distilled essential oils were quite different. However, both oils were rich in monoterpenes and sesquiterpenes and their analogues namely; oxygenated monoterpenes and sesquiterpenes. Furthermore, some chemical constituents such as α-Pinene, β-Pinene, Myrcene, (E)-β- Ocimene and (E)-Caryophyllene were found in both oils though in varied concentrations.

5. Acknowledgement

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6. Compliance with ethics guidelines

Conflict of interest

The author declares that there is no conflict of interest

Human and animal rights, informed consent

This article does not contain any studies with human or animal subjects.

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