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Investigation of the influence of tree ages and locations on essential oil yield of *Eucalyptus globulus* leaves in Ethiopia

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Abstract
The aim of this study is to investigate the influence of two experimental factors: tree ages of (2, 3, 4, 5, 6, 7 and 8 years) and growing locations (i.e. Entoto, Fiche, Debresina and Jida) on Essential oil content (EOC) and Essential oil yield (EOY) of *Eucalyptus globulus* leaves. The experiment was carried out to determine the Moisture content (MC), Essential Oil Content (EOC) and Essential Oil Yield (EOY) which were expressed by MC (%), EOC (%) and EOY (kg/ha) basis of dry leaf weight. The oil was extracted by hydro distillation from *E. globulus* leaves using Clevenger apparatus for 3hr. The MC, EOC and EOY were highly significantly ($p \leq 0.0001$) affected by the experimental factors: tree ages, its growing locations and their corresponding interactions. It has been observed that in case of crossways at all locations the mean maximum value of MC was recorded as 52.57% on tree of age 5 and minimum 42.02% on tree of age 8. Moreover, the mean value of EOC content has revealed significantly higher value for leaves of age 4 (2.49%) and leaves of age 6 (2.5%), followed by leaves of age 5 (2.39%). The least EOC content was recorded for leaves of age 8 (1.84%), which yielded 26.4% lower than the maximum. The mean maximum value of EOY was also obtained from leaves of age 6 (4254 kg ha $^{-1}$) in *Fiche*, and the next higher value of EOY was recorded on leaves of age 8 (2666 kg ha $^{-1}$) and leaves of age 2 (2674 kg ha $^{-1}$), respectively at the same growing site. The crossways at all growing sites per age on mean values of EOY content has shown that significantly higher value for leaves of ages 6 (1643.75 kg ha $^{-1}$) and leaves of age 2 (1711.38 kg ha $^{-1}$). The least value of EOY was recorded for leaves of age 3 (1045.50 kg ha $^{-1}$), which yielded 38.91% lower than the maximum value. Therefore, it can be concluded that tree leaves of age 6, which was grown at Entoto have significantly high EOC with value of 3.89% in dry biomass (DB) than matured tree leaves of ages 7 and 8, which were grown at Fiche, Debresina and Jida. The tree leaves of age 6, which was grown at Fiche have had significantly high EOY with value of 4254 kg ha $^{-1}$ in dry biomass (DB) than young leaves of ages 2-5, which were grown at Entoto, Debresina and Jida.

Keywords: Essential oil yield; essential oil content; *Eucalyptus globulus;* growing location; moisture content; tree age.

1. Introduction
Eucalyptus globulus, which is known as '*blue gum*', is one of the tree species belongs to the family of Myrtaceae [1]. It is native to Australia; and normally disseminated in Tasmania as well as mainly grown and cultivated in tropical and sub-tropical regions across the globe. Moreover, it is predominantly planted, grown and distributed in most areas of Ethiopian highlands, in Swaziland, Lesotho and South Africa [2, 3]. The genus, *Eucalyptus globulus* species is a drought-tolerant, grows fast in low nutrient areas, adaptable, provides high yield per unit area, coppicing and short-rotation tree, and it provides opportunity for high economic benefits [4, 5]. It is an evergreen tree species, cylindrical in shape with diameter up to 200 cm, bole straight with height up to 70 m [2]. The species of *E. globulus* has been cultivated and grown predominantly in highland regions of Ethiopia (i.e. in the Central, eastern and western areas of highlands). In Ethiopia, it is known as '*Nech-Bahir-Zaf*' (*In Amharic*) [5, 6].
E. globulus is the most valuable tree species that provides a number of advantages for the whole society particularly, in construction industries, fuel wood and charcoal making, wood processing industries, making particleboard and fiberboard, pallets, boats and ship building, railway sleepers, building vehicle bodies, poles, tools and furniture making, pulp and paper production [2, 6, 7]. However, commercial and local producers and growers of *E. globulus* tree species were emphasizing on its potential utilization as construction materials, fuel wood and poles; while they were wasting or underutilize barks and leaves (i.e. leaves with high potential source of essential oil) of the tree.

The *E. globulus* leaves are the major source of essential oil worldwide, and *Eucalyptus* oils can be obtained or extracted by steam distillation process [8]. The essential oil extracted from *E. globulus* leaves contain 1, 8 cineol (i.e. known as eucalyptol), approximately 70% rich in volatile oil. The oil has a number of advantages and extensive usage in pharmaceutical, perfumery and food processing industries [9]. Furthermore, the oil do possess a broad spectrum of several bio-chemical activities and medicinal values, such as anti-insecticidal, anti-microbial, nematicidal, anti-fungicidal, herbicidal [10], anti-inflammatory activity, anti-viral activity, bronchodilator effects, ant-tissue effects [11-14]. Moreover, the eucalyptol oil has been used as disinfectant, hemostat, anti-septic, antiperiodic, sedative stimulant, anti-phlogistic, fumigant, vermifuge, anesthetic, diaphoretic, expectorant, preventive and insect-repellant [15, 16].

The influence of leaf types and leafage mass of eucalyptus species, and the composition of extracted oil and its concentration extremely affected by a number of complicated factors such as geographical locations of species (i.e. growing sites of species), species variety (i.e. in terms of genotype), characteristics of soil, harvesting season, wilting period, tree ages, moisture content and various climatic conditions [17, 18]. Therefore, it is very much important to give careful consideration and investigate the most convenient *E. globulus* growing areas of Ethiopia with suitable climatic conditions, stand history and suitable factors of growing sites, while insuring adequate supply of the tree leaves for commercial production of essential oil. The rational of this investigation was to make comparisons of essential oil potential of the *E. globulus* leaves with various tree age groups (i.e. 2, 3, 4, 5, 6, 7 and 8 years) that were collected from the main growing sites of Ethiopia namely, Entoto, Fiche, Debresina and Jida. Particularly, the main objective of this study was to identify the influence of tree ages and its locations on essential oil yield and essential oil content of *Eucalyptus globulus* leaves.

2. Material and Method

2.1. Description of the study areas: Leaves of *E. globules* tree was collected indiscriminately from each site, namely Entoto, Debresina, Fiche and Jida, with an altitudes between 2600 - 3100 m, 2630 - 2830 m, 1300 and 2500 m, and 1000 - 3000 m above sea level, respectively. The annual average rainfall of the these areas are Entoto (1200 mm), Debresina (989 mm), *Fiche* (840 mm) and *Jida* (1026 mm), and the annual maximum and minimum temperatures are Entoto (14 ^{0}C and 20^{0}C), *Debresina* (12.8 ^{0}C and 17.6 ^{0}C), *Fiche* (15 ^{0}C and 19 ^{0}C) and *Jida* (24.4 ^{0}C and 29.3 ^{0}C), respectively [19-21].

2.2. Collection of *E. globulus* leaves: A total of 84 treatment combinations were used for the study, constituting 4 levels of tree growing site (Entoto, Jida, Fiche and Debresina) and 7 different tree ages (2, 3, 4, 5, 6, 7 and 8 years) were designed in the experiment with three replications [22]. During the experimental process, Completely Randomized Design (CRD) was used for laboratory analysis. From each site, measurements of leaf biomass were taken in pear for each harvested tree and were taken into account for evaluation of essential oil yield (kg ha $^{-1}$). The leaf samples of 1.5 kg for each treatment have been collected and placed in a plastic bag over ice and taken to Essential Oils Research Laboratory (EORC) in order to conduct extract the desired oil [23].

2.3. Laboratory Activities

2.3.1. Determination of Essential Oil Content and Essential Oil yield: The harvested samples of *E. globulus* tree leaves from each site were weighed and subjected to hydro-distillation using Clevenger Apparatus [8]. Two hundred gram of fresh leaf material was chopped into small pieces and placed in the distillation flask. Then, water was poured into the distillation flask until the leaves completely submerged. The distillation flask was placed on heating mantle at temperature of (> 100ºc) for 3 hours and allowed to boil in the water until the distillation process was accomplished successfully. The distillate was collected in a separatory funnel in which the aqueous portion of the distillate is separated from the volatile oil. The water layer (bottom) was drained slowly until the oil layer remains. Then, the distilled oil was measured and the essential oil content (EOC) was calculated following the formula explained by *Daniel* [24]. The essential oil yield was also calculated by: {essential oil content in kg of dry biomass (DB) x Biomass leaves weight (kg ha $^{-1}$)}, and the unit was expressed as kg ha $^{-1}$ [15, 25].

2.3.2. Laboratory materials: Pieces of equipment used the laboratory were include: Analytical balance to weigh distilled sample; Round bottom flask of 2L capacity to hold samples during distillation; Condenser with length of 300 mm to condense the volatile substance; Clevenger apparatus with capacity of 12 ml to separate oil from water; The heating mantle for the distillation process, Ice box to ship the test composite samples to the laboratory [25]; and Amber glass vials to collect the separated essential oil.

2.3.3. Data Analysis: The statistical data analysis was carried out with the statistical analysis system (SAS) software version 9.0 and SAS Studio (which is free university license and very good for assumption checking). The classical general linear model with two-way analysis of variance (ANOVA) fits the data very well as per demonstrated in results. The mean separations were carried out using the least significant difference (LSD) at (P<0.001).

3. Results

3.1 Variation in Essential Oil Content, Moisture Content and Essential Oil Yield of *E. globulus*: The Essential Oil Content (EOC), Moisture Content (MC) and Essential Oil Yield (EOY) of *E. globulus* leaves were highly significantly ($P < 0.001$) affected by tree ages and locations (i.e. growing sites) of tree. The interaction effect between tree ages and its growing sites (i.e. locations) has also shown highly significant effect on essential oil content, moisture content and essential oil yield of *E. globulus* leaves (Table 1).

Table 1: Analysis of variance (ANOVA) for various yield parameters of *E. globulus* leaves collected from different locations and different tree ages.

Source of Variation	DF	Mean Square		
		Moisture content (%) (MC)	Essential Oil Content (%) (EOC)	Essential Oil yield (kg ha $^{-1}$) (EOY)
Age	6	182.99***	0.46***	1621488.25***
Location	3	708.22***	2.42***	12802422***
Age*Locations	17	187.99***	1.12***	1477060***

Error	54	5.14	0.064	29764.46
CV		4.83	11.07	13.54
R^2		0.96	0.90	0.98

*** = Significant at $P < 0.001$; ** = Significant at $P < 0.01$; * = Significant at $P < 0.05$;
ns = Non-significant at $P < 0.05$ MC - Moisture content; EOC - Essential oil content; EOY - Essential oil content;
CV - coefficient of variation; and R - Regression factor

3.2. The interaction effect of tree age and its growing site on essential oil content (EOC) and essential oil yield (EOY) of *Eucalyptus globulus*

3.2.1. Moisture content: Except for *Debresina* growing site, the interaction effect of average moisture content (MC) of *Eucalyptus globulus* leaves is significantly uniform between Entoto, Fiche and Jida growing sites with values of 51.15%, 50.31% and 50.75%, respectively (Table 2). The overall mean value of the moisture content (MC) has revealed significantly higher value for leaves of age 5 tree (52.57%) and followed by the statistically similar value of moisture content on leaves of age 4 tree (51.12%). The least value of moisture content (MC) was recorded for leaves of age 8 (42.02%), which yielded 20.07% lower than the maximum value (Table 2).

3.2.2. Essential oil content: According to the data shown in Table 1, the interaction effect of the tree ages and its locations (i.e. its growing sites) on MC, EOC and EOY of *E. globulus* leaves is highly significant. The results in the experiment indicated that high yield of EOC was recorded on leaves of age 6 (3.89%) and statistically similar with value on leaves of age 7 (3.62%) at Entoto tree growing sites. The next higher value of EOC was recorded on leaves of age 5 (2.99%) at Entoto; and followed by statistically with, no significant variation, leaves of age 2 (2.97%) and leaves of age 4 (2.79%) at Depressing and Fiche growing sites, respectively. It was observed that statistically the least value of EOC was recorded on leaves of age 8 (1.32%) at Jidda and age 7 (1.30%) at Debresina growing sites (Table 2). The overall mean values of EOC has revealed that significantly higher values were observed from leaves of age 4 (2.49%) and age 6 (2.5%), followed by leaves of age 5 (2.39%). The least value of EOC was recorded for leaves of age 8 (1.84%), which yielded 26.4% lower than the maximum value (Table 2).

3.2.3. Essential oil yield: High value of essential oil (kg/ha) of *E. globulus* leaves was recorded on leaves of age 6 (4254 kg ha $^{-1}$) at Fiche tree growing site, followed by leaves of age 8 (2666 kg ha $^{-1}$) and leaves of age 2 (2674 kg ha $^{-1}$), respectively, at the same growing site. It was observed that statistically the least values of EOY were recorded on leaves of age 2 with value of 259 kg ha $^{-1}$ and leaves of age 3 with value of 215 kg ha $^{-1}$ at Entoto growing site (Table 2). The overall mean value of EOY has shown significantly higher value was observed from leaves of age 6 (1643.75 kg ha $^{-1}$) and leaves of age 2 (1711.38 kg ha $^{-1}$). The least value of EOY was recorded on leaves of age 3 (1045.50 kg ha^{-1}), which yielded 38. 91% lower than the maximum value Table 2.

Table 2: The interaction effect of different ages and growing locations on yield parameters of *Eucalyptus globulus* leaves

Age	Moisture Content					Essential Oil Content					Essential Oil Yield				
	Entoto	Finche	D/sina	Jida	Mean	Entoto	Finche	D/sina	Jida	Mean	Entot	Finche	D/sina	Jida	Mean
2	61.55^{a}	57.72^{a-c}	37.27^{hi}	47.05^{d-g}	50.90^{ab}	1.69^{ijkl}	2.27^{defg}	2.97^{bc}	1.96^{hij}	2.22^{bc}	259^{mn}	2674^{b}	2324^{c}	1589^{e}	1711.8^{a}
3	45.15^{e-h}	55.03^{a-d}	28.59^{ij}	53.73^{a-f}	45.63^{dc}	2.40^{de}	2.64^{bcd}	1.83^{hij}	1.96^{hij}	2.21^{bc}	215^{n}	2316^{c}	1253^{f}	398^{klmn}	1045.0^{c}
4	45.15^{e-h}	52.70^{a-f}	53.23^{a-f}	53.42^{a-f}	51.12^{ab}	2.36^{def}	2.79^{bc}	2.60^{d}	2.21^{efh}	2.49^{a}	547^{jkl}	2220^{cd}	1104^{g}	503^{jklm}	1093.0^{c}
5	48.88^{b-g}	48.78^{b-g}	58.30^{ab}	54.31^{a-e}	52.57^{a}	2.99^{b}	1.83^{hij}	2.35^{def}	2.38^{def}	2.39^{ab}	700^{hij}	909^{ghi}	1042^{fg}	311^{lmn}	740.50^{d}
6	52.86^{a-f}	44.94^{e-g}	25.37^{j}	49.27^{b-g}	43.11^{cd}	3.89^{a}	2.39^{de}	1.43^{klm}	2.28^{d-g}	2.50^{a}	918^{ghi}	4254^{a}	664^{hij}	739^{hij}	1643.5^{a}
7	51.49^{b-f}	48.70^{b-g}	41.50^{gh}	48.73^{b-g}	47.60^{bc}	3.62^{a}	2.00^{fghi}	1.30^{m}	1.54^{jklm}	2.12^{c}	582^{jkl}	1943^{d}	953^{gh}	737^{hij}	1053.75^{c}
8	52.98^{a-f}	44.32^{f-h}	22.36^{j}	48.43^{c-g}	42.02^{d}	2.31^{defg}	1.96^{hig}	1.77^{ijk}	1.32^{lm}	1.84^{d}	2080^{cd}	2666^{b}	499^{jklm}	710^{hij}	1488.7^{b}
Mean	51.15^{a}	50.31^{a}	38.08^{b}	50.75^{a}		2.75^{a}	2.27^{b}	2.04^{c}	1.95^{c}		757^{c}	2426^{a}	1120^{b}	712.4^{c}	

4. Discussion

The tree age has become into consideration in order to have the most pronounced effect on leaves oil content of *E. globulus* according to the description of *Boland* [26]. In the present study, the tendency of overall mean of interaction effects between the *Eucalyptus globulus* ages and its growing sites on the essential oil content (EOC) has shown the slightly increment the oil content for leaves of age 4 (2.49%) to leaves of age 6 (2.50%) and then down, when observed across for leaves of age 7 (2.12%) to leaves of age 8 (1.84%). This value is in conformity with value observed by *List* [27], and the variation among the observed values might be permissible due to the regulation of oil accumulation by the specialized oil gland (i.e. glandular trichomes) and the oil gland capacity that was connected to leaf thickness and leaf area with leaf maturation may regulate oil content [28]. Moreover, the observed results are in agreement with previous reports regarding essential oil extracted from another medicinal plant's leaves that were influenced by various extraction factors as per explained in ontogenesis stages [29-32]. Hence, in this research finding it has been observed that the maximum value of essential oil was recorded in young leaf than matured leaf of the subjected tree species. Another studies also described that the essential oil content in young leaves increases continuously as they grow and oils accumulates in their specialized glands. However, it has been indicated that once a leaf is completely expanded oil accumulation terminates, and oil content may indeed decreases, thenceforth via evaporation and leakages. Furthermore, according to Daoran [32] it has been reported that the percentage oil content of leaves up to age of 5 months were significantly affected by the physiological variations related with maturation of leaves. The overall mean value of interaction effects between the tree ages and its locations (i.e. growing sites) has shown variation in EOY. Among these, leaves of age 2 (1711.38 kg ha $^{-1}$) showed the highest value of EOY followed by leaves of age 6 (1643.75 kg ha $^{-1}$) and leaves of age 8 (1488.75 kg ha $^{-1}$), whereas the lowest value of EOY was obtained from leaves of age 5 (740.50 kg ha $^{-1}$) (Table 2). Consequently, the variations in biomass yield of leaves (leafages yield) with ages of leaves happened due to the existence of variations in geographical locations of the concerned tree (i.e. variation in latitudes and longitude), and variations in mean annual rainfall, relative humidity and mean annual temperatures. The obtained

percentage values of essential oil content and essential oil yields were in conformity with the values in previous studies on essential oil content and foliage yield of *Eucalyptus pellita* [33].

5. Conclusion

The main aim of this study was to determine influence of tree ages and its growing sites on the essential oil content and the essential oil yield of *Eucalyptus globulus* while making comparison of essential oil content and essential oil yield of *E. globulus* leaves collected from different growing sites (i.e. Entoto, Debresina, Jida and Fiche). Based on observed results, it has been clearly shown the impact of tree age and its growing sites on EOC and EOY of *E. globulus* leaves and the tree is growing effortlessly at different sites with various potential source of essential oil content under the Ethiopian climatic conditions. Therefore, it can be concluded that the *E. globulus* tree leaves of age 6, which is grown at Entoto do possess significantly high EOC of value 3.89% in dry biomass (DB) than the EOC in matured tree leaves of ages 7 and 8, which is grown at Fiche, Debresina and Jida. Moreover, tree leaves of age 6, which was grown at Fiche have had significantly high EOY with value of 4254 kg ha $^{-1}$ in dry biomass (DB) than young leaves of ages 2 - 5, which were grown at Entoto, Debresina and Jida. This study provided the substantial information about the essential oil extracted from the *E. globulus* tree for these interested organization (s) who are willing to commercialize the oil.

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