PHYTOCHEMICAL PROFILE, ANTIOXIDANT AND ANTIDIABETIC POTENTIAL OF ESSENTIAL OIL FROM FRESH AND DRIED LEAVES OF *Eucalyptus globulus*

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ABSTRACT

Pathogenesis of type 2 diabetes has been linked to oxidative stress. Synthetic drugs in use for the management of the stress and type 2 diabetes are expensive and their usage are associated with side effects. Some essential oils exhibited antioxidant and antidiabetic properties devoid of the side effects. Their activities are attributed to the type of phytochemicals whose presence in the oils of the fresh and dried samples are determined by environmental factors. This study therefore aimed at monitoring the effect of air-drying at ambient temperature on the phytochemical profile, antidiabetic and antioxidant potentials of leaf essential oils of *E. globulus.*

To achieve this, 500 g of fresh and dried leaves of *E. globulus* were separately pulverized and hydrodistilled for three hours. The leaves yielded oils in the range of 0.22–0.55% (w/w). GC and GC–MS analyses of the oils revealed predominance of α-phellandrene (4.6–10.5%), D-limonene (18.5%), β-myrcene (20.8%), m-cymene (5.0-29.8%), terpinen-4-ol (0.4-4.7%), globulol (3.1-10.5%) and spathulenol (4.9-18.8%). The antioxidant activity of the oils was determined by DPPH and FRAP assays using ascorbic acid as standard. α-Amylase and α-glucosidase inhibitory assays were used to determine the antidiabetic potential of the oils using acarbose as standard. The oils exhibited antioxidant and antidiabetic activities that compared favourably with the standards. Meanwhile, the oil obtained from the leaves air-dried for three days showed the highest activities with competitive mode of α-amylase and α-glucosidase inhibitions. The oil could serve as alternative for the management of oxidative stress and type 2 diabetes.

Keywords: *D-limonene, m-cymene, alloaromadendrene, β-myrcene, antioxidant, antidiabetic*.

INTRODUCTION

Type 2 diabetes is a chronic metabolic disorder characterized by hyperglycaemia due to low secretion of insulin in the pancreases[1,2]. Pathogenesis of the disease has been linked to oxidative stress in the living cells [3]. Miglitol, acarbose and voglibose are the synthetic drugs used for the management of diabetes while butylated hydroxytoluene, butylated hydroxyanisole and propyl gallate are used to ameliorate oxidative stress. However, the drugs are not affordable by the users and their usage are associated with vomiting, diarrhoea, flatulence, dizziness and weight gain [4]. Hence, efforts are on-going to find alternative antidiabetic and antioxidant drugs from plant source that could ameliorate these conditions with little or no side effect.

E. globulus belongs to the family Myrtaceae. It is native to the subtropical rain forest of South Australia and Tasmania [5]. Traditionally, fresh leaf of the plant is used for the treatment of cough, lung ailments, catarrh, flu, inflammation, cancer, diabetes and liver infections [6 – 8]. These activities justify its use in folklore medicine [8, 9]. Various extracts from the plant have been reported to possess antimicrobial, antidiabetic and antioxidant activities [10. 11]. Phytochemical analysis of the plant extracts revealed the presence of alkaloids, tannins, saponins, terpenoids, steroids, phenolic compounds and cardiac glycosides [12, 13]. The presence of these compounds is responsible for the biological and biochemical activities of the extracts.

Earlier work on the leaf essential oil of the plant revealed chemotypic variations from different parts of the world. For instance, the leaf oil of the plant grown in Ethiopia, Morocco, Algeria, Montenegro and China were of 1,8-cineole chemotype[14 – 18]. α-Phellandrene and α-terpineole chemotypes were reported for the leaf oil of the plant native to India and Nigeria, respectively [7, 15]. The chemotypic variations of the oils is due to differences in agro climatic conditions in locations where the plants were grown.

The leaf essential oil of the plant showed antioxidant activity against DPPH, β-carotene and superoxide radicals. The radical scavenging activity was linked to the presence of terpinen-4-ol, spathulenol, *p*-cymen-4-ol and 1,8-cineole in the oil [17, 19]. It has also been revealed that the oil inhibited the activities of α-amylase and α-glucosidase. Hence, it exhibits antidiabetic potential. It was reported that the activity is due to the presence of thymol, D-limonene, carvacrol, eucalyptol and globulol in the oil [14, 19, 20]. Their presence in the oils is determine by environmental factors that dictate the activity of terpene synthase required for the conversion of their precursors to various terpenic compounds [21 – 24]. It is on this basis that this study aimed at monitoring the effect of drying at ambient temperature on the phytochemical profile, antioxidant and antidiabetic potentials of essential oils from leaves of *E. globulus*.

EXPERIMENTAL

Sample Collection and Preparation

Leaves of *E. globulus* were harvested at the dam site of University of Ilorin, Ilorin, Kwara State, Nigeria. The plant was identified at the Herbarium of Plant Biology Department, University of Ilorin, Ilorin, where voucher specimen was deposited [UILH/002/1073]. The harvested leaves were air-dried at ambient temperature for five consecutive days.

Essential Oil Isolation

Fresh and dried leaves (500 g each) of *E. globulus* were separately pulverized and hydro-distilled for 3 hours in a Clevenger-type apparatus, according to the British Pharmacopoeia specification [25]. The oil from each sample was collected, preserved in a sealed sample tube and stored under refrigeration until the analysis was done.

Gas Chromatography (GC) Analysis of the Oils

GC analyses of the oils were performed separately on an Orion micromat 412 double focusing gas chromatography system fitted with two capillary columns coated with Cp- Sil 5 and Cp-Sil 19 (fused silica, 25m x 0.25mm, 0.15µm film thickness) and flame ionization detector (FID). The volume of each of the oils injected was 0.2 mL, and the split ratio was 1:30. Oven temperature was programmed from 50 – 230°C at 3°C/min using hydrogen as a carrier gas. Injection and detector temperatures were maintained at 200°C and 250°C, respectively. Calculation of peak area percentage was performed on the basis FID signal using the GC HP-chemstation software.

Gas Chromatography – Mass Spectrometry (GC/MS) Analysis of the Oils

A Hewlett – Packard HP 5890A GC, interfaced with a VG analytical 70-250s double focusing mass spectrometers was used. The MS operating conditions were: ionization voltage 70ev, ion source and transfer line temperature was maintained at 230°C. The GC operating conditions were identical with those of GC analyses. The MS data were acquired and processed by on-line desktop with a computer equipped with disk memory. The percentage composition of the oil constituents were computed in each case from GC peak areas.

The identification of the components was based on comparison of retention indices (determined relative to the retention times of series of n-alkanes) and mass spectra with those of authentic samples stored in NIST 08 library and with data from literature [26 – 28]. Component relative percentages were calculated based on normalization method.

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DPPH Antioxidant Assay of the Oils

The antioxidant activity of the oils was measured in terms of its hydrogen-donating or radical scavenging ability against DPPH, using the method reported by Ilhami, [29]. In the method, 1.5 ml of 2,2-diphenyl-1-picryl-hydrazil, DPPH, solution (10^{-4} M, in 95% Ethanol) was separately mixed with 1.5 ml of each of the oils at various concentrations (10-50 µl/ml). Each of the mixtures was shaken thoroughly and incubated in the dark for 30 minutes at ambient temperature. The control was prepared using the same procedure without the oil. Absorbance of the solution was measured at 517 nm using UV-spectrophotometer. The assay was carried out in triplicate and the results were expressed as mean values ± standard deviation. The concentration of the oil that gave 50% inhibition (IC_{50}) was calculated from the graph of percentage inhibition against the oil concentration. Ascorbic acid was used as standard. The percentage inhibitions were calculated using the equation:

$$\% \text{ Inhibition} = \frac{A_0 - A_T}{A_0} \times 100 \qquad \text{Eq. 1}$$

where, A_0 is the absorbance of the control sample (containing all reagents except the essential oil) and A_T is the absorbance of the essential oils.

Ferric Reducing Antioxidant Power (FRAP) Assay of the Oils

The ferric reducing antioxidant potential of the oils was determined by assessing its ability to reduce $FeCl_3$ to $FeCl_2$ as described by Oyaizu, [30]. In the method, 2.5 mL of the oils was separately mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 minutes, and then 2.5 mL of 10% trichloro acetic acid was added. The mixture was centrifuged at 3000 RPM for 10 minutes after which 5 ml of the supernatant was mixed with an equal volume of distilled water and 0.5 mL of 0.1% $FeCl_3$. The absorbance of the resulting solution was measured at 700 nm using UV spectrophotometer. The ferric-reducing antioxidant power was subsequently calculated and expressed as ascorbic acid equivalents using ascorbic acid as the standard.

α-Amylase Inhibition Assay of the Oils

The α-amylase inhibitory assay of the oils was performed using the method reported by Oboh *et al*., [31]. In the method, 20 µL of α-amylase solution was pre-mixed with 10 µL of the oil at different concentrations (5 – 20 µg/ml) and incubated for 15 minutes. Five hundred (500) µL of 1% starch solution in sodium phosphate buffer (pH 6.9, with 6 mMNaCl) was added to the incubated mixtures and stirred for 5 minutes at 37.5 °C. DNS reagent (500 µL) (1% 3,5-dinitrosalicylic acid, 12% Na-K tartarate in 0.4 M NaOH) was subsequently added to the mixture. The mixture was placed in a water bath for 15 minutes until boiling and then cooled to ambient temperature. The absorbance was measured at 540 nm and the inhibitory activity was expressed as percentage inhibition using the expression:

$$\% \text{ Inhibition} = \frac{A - B}{A} \times 100 \qquad \text{Eq. 2}$$

Where A is the absorbance of reaction blank, and B is the absorbance of reaction in the presence of the oil.

α-Glucosidase Inhibition Assay of the Oils

The α-glucosidase inhibitory potential of the oils was determined using the method described by Apostolidis *et al*., [32]. In the method, 100 µL of α-glucosidase solution was pre-mixed with 50 µL of the oils at different concentrations (5 – 20 µg/ml) and incubated for 10 minutes. Fifty (50)µL of 5 m M*p*-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH, 6.9) was added to the incubated mixtures and was mixed for 5 minutes at 25 °C.

The absorbance was measured at 405 nm. The control experiment was performed without the oil, and the α-glucosidase inhibitory activity was expressed as percentage inhibition using the equation:

$$\% \text{ Inhibition} = \frac{A - B}{B} \times 100 \qquad \text{Eq. 3}$$

Where A is the absorbance of reaction blank, and B is the absorbance of reaction in the presence of the oil.

Mode of α-amylase Inhibition

The mode of α-amylase inhibition by the most active essential oil (the oil with the lowest IC_{50} value) was determined according to the method described by Kim *et al*., [33]. In the method, 250 µL of the oil was pre-incubated with 250 µL of α-amylase solution for 10 mins at 37 °C in one set of test tubes. In another set of test tubes, 250 µL of phosphate buffer (pH 6.9) was also pre-incubated with 250 µL of α-amylase solution. Starch solution (250 µL) of increasing concentrations (0.30 – 5.0 mg/mL) was added to both sets of reaction mixtures to initiate the reaction. The mixture was then incubated for 10 min at 37 °C, and then boiled for 5 min after addition of 500 µL of DNS to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically at 405 nm using a maltose standard curve and converted to reaction velocities. A double reciprocal plot (1/V versus 1/[S]), where V is the reaction velocity and [S] is substrate concentration was plotted. The type (mode) of inhibition of the oils against activity of α-amylase was determined using double reciprocal (Line weaver-Burk) plot following Michaelis-Menten equation:

$$\frac{1}{V} = \left\{\frac{Km}{Vmax.[S]} + \frac{1}{Vmax}\right\} \qquad \text{Eq. 4}$$

Mode of α-glucosidase Inhibition

The mode of inhibition of α-glucosidase by the most active oil was investigated according to the method described by Kim *et al*., [33]. In the method, 50 µL of the oil was pre-incubated separately with 100 µL of α-glucosidase solution for 10 mins at 37 °C in one set of tubes. In another set of tubes, 50 µL of phosphate buffer (pH 6.9) was also pre-incubated with 100 µL of α-glucosidase solution. 50 µL of PNPG at increasing concentrations (0.30 – 5.0 mg/mL) was added to both sets of reaction mixtures to start the reaction. The mixture was then incubated for 10 min at 37 °C, and 500 µL of Na_2CO_3 was added to stop the reaction. Then, boiled for 5 min after addition of 500 µL of DNS to stop the reaction. The amount of reducing sugars released was determined spectrophotometrically at 405 nm using *p*-nitrophenol standard curve and was converted to reaction velocities. A double reciprocal plot (1/V versus 1/[S]), where V is the reaction velocity and [S] is substrate concentration was plotted. The type (mode) of inhibition of the oils against activity of α-glucosidase was determined using double reciprocal (Line weaver-Burk) plot following Michaelis-Menten equation.

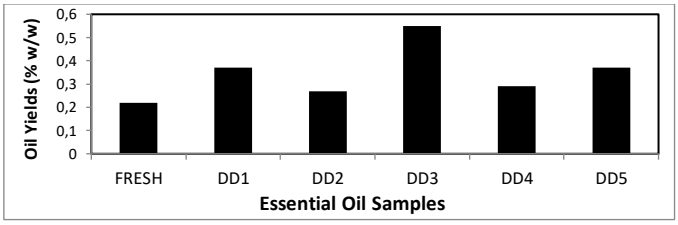
Statistical Analysis

Tests were carried out in triplicates. The mean values were calculated from the three values. The data for various biochemical parameters was expressed as mean ± SD (n = 3) and compared using one way analysis of variance (ANOVA) test, followed by Dunnett multiple comparison test with equal sample size test. Values were considered statistically significant at $p < 0.05$. The IC_{50} values were calculated by non-linear regression analysis from the mean values. Statistics was done using SPSS for windows version 10.

RESULTS AND DISCUSSION

Yields of Essential Oils of *E. globulus*

The yields of essential oil from the leaves of the plant were in the range of 0.22 – 0.55% (w/w). The yield increased from 0.22% in the fresh leaves to 0.37% in the leaves dried for one day. A decrease in the oil yield to 0.27% occurred in the leaves dried for two days. The quantity of the oil suddenly increased to 0.55% in the leaves dried for three days and later decreased to 0.29% in the leaves dried for four days. The yield subsequently increased to 0.37% in the leaves dried for five days. The lowest and the highest quantities of the oil were afforded by the fresh leaves and the leaves dried for three days respectively [Fig. 1].



Key: DD1 = Day one Dried leaf, DD2 = Day two Dried leaf, DD3 = Day three Dried leaf, DD4 = Day four Dried leaf, DD5 == Day five

Figure 1. Essential Oil Yields from Fresh and Dried Leaves of *Eucalyptus globulus.*

It has been reported that dried leaves afforded more oil than fresh leaves due to loss of moisture in the leaves during drying [34 – 37]. Hence, the higher oil yields from the dried leaves in this study could be attributed to the loss of moisture in the leaves during drying.

Chemical Composition of Essential Oils of *E. globulus*

The percentage composition, retention indices and identities of constituents of essential oils of the fresh and the dried leaves of *E. globulus* are presented in Table 1.

Table 1: Chemical Composition (%) of Essential Oils from the Fresh and the Dried Leaves of *E. globulus*

S/N	Compound	RI[a]	RI[b]	% Composition						Mass Spectra Data
				FR	DD1	DD2	DD3	DD4	DD5	
1.	α-Thujene	931	926	0.2	-	Tr	0.1	Tr	-	136,105,**93**, 77,50
2.	α –Pinene	939	933	0.5	-	-	0.3	Tr	-	135,105, **93**, 67, 55
3.	**β-Myrcene**	991	990	0.2	-	**20.8**	Tr	-	-	136,107, **93**, 79, 69
4.	2-Carene	1001	1001	0.4	-	0.1	0.2	0.2	0.2	150,121, **93**, 67, 53
5.	α-Phellandrene	1005	1005	8.5	6.9	6.1	4.6	7.6	10.5	136,105, **93**, 77, 65
6.	*p*-Cymene	1026	1022	-	0.4	-	-	-	-	**132**,105, 91, 77, 63
7.	**D-limonene**	1031	1027	**18.5**	-	-	-	-	-	136,107, 93, **68**, 53
8.	γ-Terpinene	1062	1057	1.9	0.5	0.8	0.5	1.0	1.7	136,105, **93**, 77, 65
9.	**m-Cymene**	1082	1078	-	14.5	5.0	**16.2**	**29.8**	**24.0**	134,**119**,103, 91,65
10.	6-Camphenone	1093	1092	-	4.6	-	-	-	-	150, 108, **93**, 79,59
11.	Linalool	1098	1098	0.6	-	0.3	0.4	0.3	0.3	136, 121, **93**, 71,55
12.	Isoamylisovalerate	1103	1104	0.6	-	0.2	0.5	0.7	0.5	129, 103, 85, **70**,57
13.	6-Camphenol	1109	1109	2.1	-	1.8	4.6	4.9	3.5	108, **93**, 79, 55, 50
14.	Cis-p-2-menthen-1-ol	1121	1121	Tr	-	Tr	0.9	0.3	-	154,139,111, **93**,69
15.	Pinene oxide	1159	1159	-	4.5	-	-	-	-	137, 109, **71**, 69,55
16.	Cis-verbenol	1140	1140	-	2.4	-	-	0.3	-	134,**119**,107, 93,69
17.	Cis-β-terpineol	1144	1141	-	0.3	0.2	0.3	0.3	-	154,139, 110,**79**,55
18.	Borneol	1165	1162	1.1	-	2.8	-	7.7	-	154, 110, **95**, 69,55
19.	Terpinen-4-ol	1177	1175	3.4	0.4	2.0	3.7	3.4	4.7	136, 111, 93, **71**,51
20.	α-Terpineol	1189	1188	1.1	0.4	0.3	0.8	0.8	0.9	136, 121, 93, **59**,51
21.	Trans-carveol	1217	1216	-	-	-	-	0.7	0.2	152, **109**, 84, 83,55
22.	n-Hexyl isovalerate	1234	1233	3.7	-	-	3.4	-	0.3	103, **85**, 81, 56, 50
23.	Ascaridol	1237	1237	0.1	-	Tr	0.4	0.3	-	168, 107,91, 69,**41**,
24.	Carvotanacetone	1246	1245	0.5	7.4	0.3	0.2	0.3	0.8	152, 109, **82**, 69,54
25.	Piperitone	1252	1250	1.8	3.4	4.7	8.9	7.3	2.4	152, 110, **82**, 67,39
26.	Carvone oxide	1277	1277	Tr	-	-	0.2	-	Tr	123, 109, 81, **43**,41
27.	Thymol	1290	1290	-	0.9	-	-	-	0.5	150, **135**,107,91,77
28.	Sabinyl acetate	1298	1298	1.7	1.0	1.8	1.7	1.5	2.8	175, 134, **92**, 81,43
29.	Carvacrol	1298	1299	1.8	1.4	1.8	1.5	1.0	1.7	150,**135**, 107,91,77
30.	2,3-Pinanediol	1320	1320	-	1.8	0.5	2.8	3.0	4.2	**126**, 111, 93, 71,55
31.	8-Hydroxy linalool	1338	1337	-	1.2	-	-	-	-	137, 93, 71, 67,**43**
32.	α-Ylangene	1371	1371	0.4	0.6	0.2	0.3	-	0.4	204, 189,**105**,85,55
33.	Isoledene	1376	1376	0.3	-	Tr	0.2	0.2	0.1	204, 161,105,**84**,79
34.	β-Elemene	1391	1391	1.5	0.1	-	-	-	0.1	189, 147, **91**, 81,67
35.	α-Gurjunene	1409	1409	0.6	0.5	0.5	0.9	Tr	0.8	204,161,**119**,105,91
36.	β-Caryophyllene	1418	1418	-	0.1	1.7	1.2	1.9	0.2	204, 133, **93**, 79,41
37.	β-Ylangene	1420	1420	-	-	0.2	-	0.3	0.2	202, **161**,133,91,79
38.	Trans-α-bergamotene	1436	1435	0.4	0.8	0.2	-	0.5	0.6	161, 119, **93**, 79,55
39.	α –Guaiene	1439	1438	1.4	0.3	-	-	0.7	-	204, 147,**108**,93,67
40.	**Alloaromadendrene**	1461	1460	4.8	**16.1**	5.1	4.6	4.0	9.2	204, 147,**105**,93,41
41.	Dehydroaromadendrene	1462	1462	1.7	1.5	1.6	1.5	-	1.6	202,159,**131**,105,91
42.	γ-Gurjunene	1471	1471	-	-	-	1.1	-	-	204,147, **105**,91,79
43.	Germacrene D	1480	1479	-	1.2	-	-	-	-	204, 136,**121**,93,79

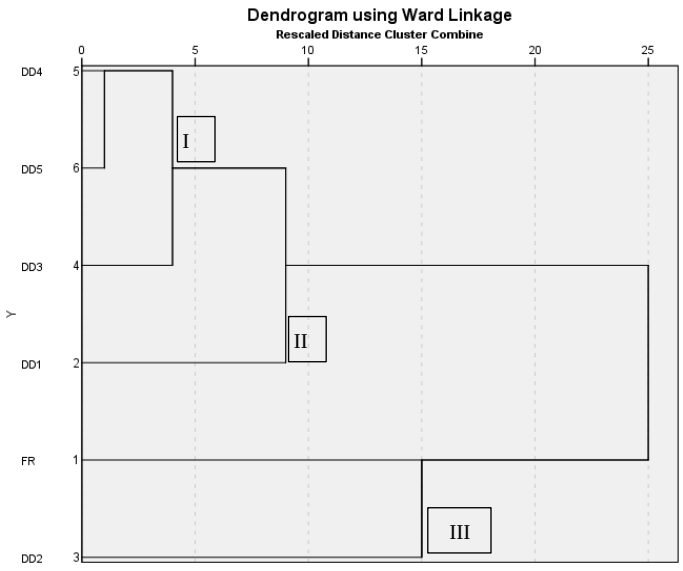
44.	Phenylethylisovalerate	1487	1488	0.8	-	0.2	0.4	0.6	0.9	152, 120,**104**,77,57
45.	Diosphenol			0.4	7.2	1.5	1.0	-	1.2	168, 124, **98**, 69,41
46.	β-Guaiene	1490	1489	0.4	-	0.7	0.1	-	0.5	204,161,119,**105**,81
47.	Ledene	1493	1493	2.4	1.7	2.8	2.8	-	2.5	204, 161,1**07**,93,55
48.	α-Selinene	1494	1494	0.8	-	0.2	0.3	0.2	0.5	204, 189,**107**,93,81
49.	α-Bulnesene	1505	1505	-	-	-	-	-	0.6	204, 161,**105**,91,67
50.	β –Cadinene	1519	1518	0.2	-	0.2	-	-	-	204, 161,143,91,67
51.	δ-Cadinene	1524	1523	0.2	-	0.3	0.5	0.1	0.3	204, 161,**119**,91,55
52.	Elemol	1547	1547	-	-	-	1.6	-	-	204, **149**, 93, 59,55
53.	γ-Elemene	1555	1555	0.5	0.3	-	-	2.8	-	161, **121**,107,93,67
54.	Calamenene	1559	1559	-	-	-	0.2	0.1	-	202, **159**,131,77,50
56.	Globulol	1576	1582	4.4	3.1	10.5	7.5	7.0	8.1	204, 161,111,**93**,67
57.	Spathulenol	1578	1575	7.0	5.5	18.8	15.9	4.9	7.5	220, 147,**119**,91,69
58.	Isoaromadendrene oxide	1579	1579	Tr	-	0.4	0.6	0.2	0.3	220, **107**, 93, 81,55
59.	Rosifoliol	1600	1600	1.4	-	-	2.2	-	2.0	204, 164,**149**,93,59
60.	β-Eudesmol	1649	1648	0.4	-	-	0.8	0.4	0.9	204, 149, 93, **59**,55
61.	α-Eudesmol	1652	1651	-	-	0.9	-	1.4	-	204, 164,**149**,93,81
62.	α –Cadinol	1653	1653	0.3	-	0.7	1.1	0.2	0.3	204, 121, **95**, 81,55
63.	Shyobunol	1689	1689	-	-	-	1.2	-	-	207, 121, 93, 81,55
64.	1,3-Dimethyl-1-cyclohexene			1.5	5.8	1.9	0.9	0.9	1.7	112, **95**, 82, 67, 41
	Total (%)			**99.3**	**98.1**	**99.0**	**98.9**	**99.1**	**99.8**	

Key:FR = Fresh leaf, DD1 = Day one Dried leaf, DD2 = Day two Dried leaf, DD3 = Day three Dried leaf, DD4 = Day four Dried leaf, DD5 == Day fiveDired leaf,RI^a = Literature Retention Indices, RI^b = Calculated Retention Indices, Retention indices on fused silica capillary column coated with CP-Sil 5; Tr (< 0.1 %), **Bolded names = Chemotypes.**

In the Table, 31 – 45 compounds that represented 99.0 – 99.3% of the oils were identified from their mass spectra. Monoterpenoids constituted 36.5 – 75.5% of the oils. The percentage composition of sesquiterpenoids in the oils ranged from 24.5 to 57.8. The principal constituents of the oils were; α-phellandrene, (4.6 – 10.5%), piperitone (1.8 – 8.9%), alloaromadendrene (4.0 – 16.1%), globulol (3.1 – 10.5%) and spathulenol (4.9 – 18.8%). Other major compounds included; D-limonene (18.5%) in the oil of the fresh leaves, β-myrcene (20.8%) in the oil of the leaves air dried for two days, m-cymene (5.0 – 29.8%) in the oils of the leaves air dried for one, three, four and five days, borneol (2.8 – 7.7%) in the oils of the leaves air dried for two and four days, carvotanacetone (7.4%) and diosphenol (7.2%) in the oil of the leaves air dried for one day. Terpinen-4-ol (0.4 – 4.7%), α-terpineol (0.3 – 1.1%), sabinyl acetate (1.0 – 2.8%), γ-terpinene (0.5 – 1.9%) and carvacrol (1.0 – 1.8%) were detected in significant amount in the oils.

Compounds that were identified in appreciable quantities were; 6-camphenone (4.6%), 8-hydroxy linalool (1.2%), germacrene D (1.2%) and pinene oxide (4.5%) in the oil of the leaves air dried for one day; 6-camphenol (1.5 – 4.9%) in the oils other than the oil of the leaves dried for one day; cis-verbenol (0.3 and 2.4%) in the oils of the leaves air dried for one and four days; 2,3-pinanediol (0.5 – 4.2%) in the oils of the dried leaves; dehydroaromadendrene (1.5 – 1.7%) and ledene (1.7 – 2.8%) in the oils of the oils of the fresh leaves and the leaves air dried for one, two, three and five days; and γ-gurjunene (1.1%), elemol (1.6%) and shyobolol (1.2%) in the oil of the leaves air dried for three days. Ascaridol (0.1 – 0.4%) in the oils of the fresh leaves and the leaves air dried for three and four days, α-thujene (0.2%) and α–pinene (0.3%) in the oil of the leaves air dried for three days, 2-carene (0.1 – 0.4%), carvotanacetone (0.2 – 0.8%) and linalool (0.3 – 0.6%) in the oils of the fresh leaves and the leaves air dried for two, three, four and five days and α-ylangene (0.2 – 0.6%) in the oils other than the oil of the leaves air dried for four days were detected in minor quantities.

Other compounds that existed in minor quantities were; p-cymene (0.4%) in the oil of the leaves air dried for one day, cis-β-terpineol (0.2 – 0.3%) in the oils of the leaves air dried for one, two, three and four days and carvone oxide (tr – 0.2%) in the oils of the fresh leaves and the leaves air dried for three and five days. The correlation between the constituents of the oils is shown in the Dendrogram in Figure 2.

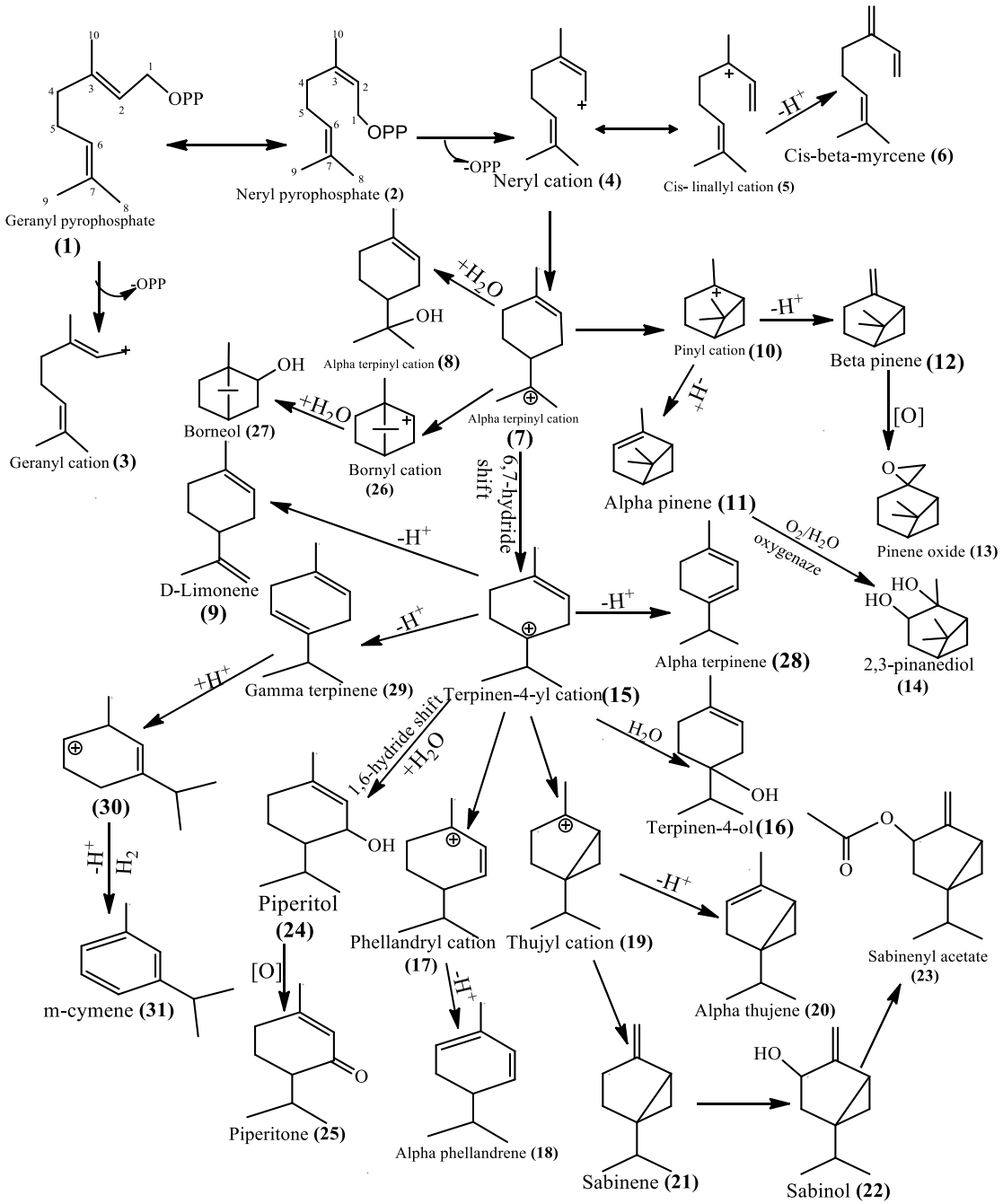


Key: FR = Fresh leaf, DD1 = Day one Dried leaf, DD2 = Day two Dried leaf, DD3 = Day three Dried leaf, DD4 = Day four Dried leaf, DD5 == Day five.

Figure 2. Dendrogram obtained by the Hirachical Cluster Analysis of the Percentage Composition of Constituents of Essential Oils of the Fresh and the Dried Leaves of *E. globulus*, based on Euclidean Distance.

The predominance of D-limonene in the oil of the fresh leaves, alloaromadendrene in the oil of the leaves air dried for one day, β-myrcene in the oil of the leaves air dried for two days and m-cymene in the oils of the other air dried leaves revealed that the oils were of D-limonene, β-myrcene, alloaromadendrene and m-cymene chemotypes. Akolade *et al*., [19], reported D-limonene chemotype for the oil of the fresh leaf of the plant.

It has been established that the synthases of the most abundant mono- and sesquiterpenoids mediate the biosynthesis of all mono- and sesquiterpenoids from their precursors in the leaf [37 - 39]. The predominance of m-cymene, D-limonene, β-myrcene, alloaromadendrene, spathulenol and globulol in the oils signified that the synthases of these terpenoids facilitated the transformation of geranyl, neryl, and farnesyl pyrophosphates to various cationic intermediates in the presence of divalent metal ions. The intermediate ions undergo series of cyclization and rearrangements until the reactions are terminated by deprotonation or hydration to form various terpenic comopounds (Reaction Scheme 1) (39-41).



Reaction Scheme 1. Bigenesis of Monoterpenoids in the Oils of the Fresh and the Dried Leaves of *E. globulus* Mediated by D-limonene, m-cymene and β-myrcene Synthases (38 – 41).

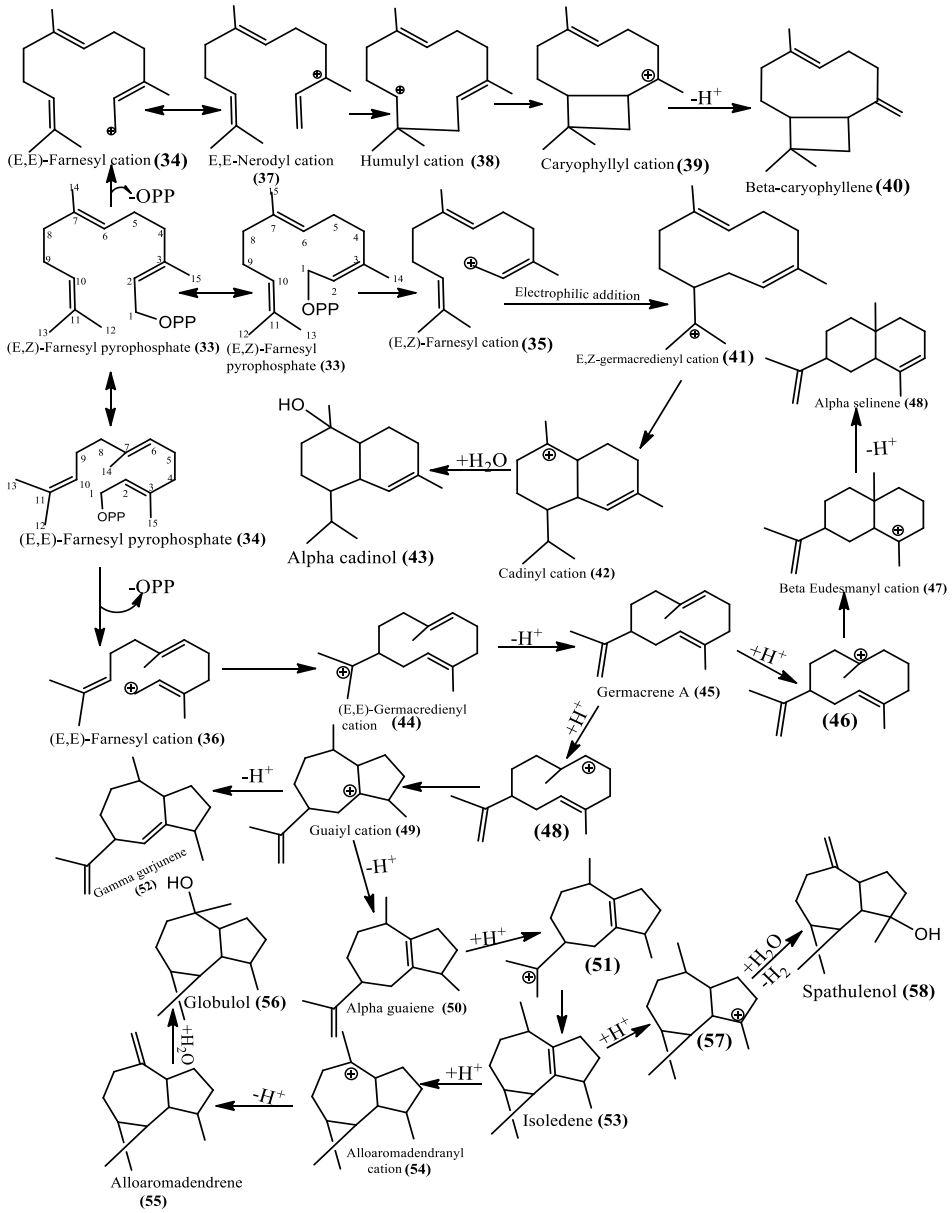
In the Scheme, the m-cymene, D-limonene and β-myrcene synthases facilitated the ionization of geranyl and neryl pyrophosphates **(1 and 2)** to geranyl and neryl cations **(3 and 4)** in the leaves. Isomerisation of neryl cation **(4)** formed *cis*-linalyl cation **(5)**. D-limonene and β-myrcene synthases catalyzed deprotonation of neryl cation **(4)** at C_{10} to form cis-β-myrcene **(6)** in the oils of the leaves air dried for three and four days. Electrophilic addition of the *cis*-linalyl cation to C_6-C_7 double bond formed α- terpinyl cation **(7)**. α-Terpineol **(8)** was formed in the oils by hydration of the cation **(7)**. D-limonene synthase mediated deprotonation of the ion **(7)** at C_8 and formed D-limonene **(9)** in the oil of the fresh leaves. Folding of the terpinyl cation followed by its electrophilic attack on the C_2-C_3 double bond via C_2 produced pinyl cation (**10**). Deprotonation of the ion **(10)** at C_4 gave α-pinene (**11**) in the oils of the fresh leaves and the leaves air dried for three and four days. The formation of the compound in the oils was facilitated by D-limonene and m-cymene synthases.

Deprotonation of the pinyl cation **(10)** at C_{10} formed β–pinene **(12)**. M-cymene synthase aided epoxidation of β-pinene to form pinene epoxide **(13)** in the oil of the leaves air dried for one day. Hydroxydation of α–pinene at C_3 and C_4 produced 2,3-pinanediol **(14)** in the oils of the air dried leaves. The reaction was facilitated by β–myrcene and m-cymene synthases. The activity of the D-limonene

synthase did not favour the formation of the compound in the oil of the fresh leaves. Terpinen-4-yl cation **(15)** was formed via 6, 7-hydride shift of α- terpinyl cation. Hydration of the ion **(15)** formed terpinen-4-ol **(16)** in the- oils. 1,6-hydride shift in terpinen-4-yl cation followed by delocalization of the pi electrons from C_2 to C_1formed phellandryl cation **(17)**. Subsequent deprotonation of the ion **(17)** at C_4 produced α-phellandrene **(18)** in the oils. Electrophilic attack of the terpinen-4-yl cation **(15)** on the C_2-C_3 double bond via C_2 gave thujyl cation **(19)**. Loss of proton by the ion **(19)** at C_4 formed α-thujene **(20)** in the oils of the fresh leaves and the leaves air dried for two, three and four days.

Deprotonation of the thujyl cation **(19)** at C_{10} gave sabinene **(21)**. Allylic hydroxylation of sabinene at C_4, aided by the synthases in the leaves produced sabinol **(22)**. Acetylation of the compound **(22)** by acetyl CoA formed sabinyl acetate **(23)** in the oils. 1,6-hydride shift of terpinen-4-yl cation followed by its hydration formed piperitol **(24)**. The compound **(24)** was oxidized to piperitone **(25)** in the oils. Folding of the α–terpinyl cation towards C_2 – C_3 double bond followed by anti-Markovniff's addition to the C_2 – C_3 double bond formed bornyl cation **(26)**. The synthases catalysed hydration of the ion **(26)** to form borneol **(27)** in the oils of the fresh leaves and the leaves air dried for two and four days. Deprotonation of the terpinen-4-yl cation **(15)** at C_1 and C_5 formed α–terpinene **(28)** and γ-terpinene **(29)** in the oils respectively. Protonation of the C_3-C_4 double bond via C_3 followed by 2,3-methyl shift gave an intermediate ion **(30)**. Deprotonation of the ion **(30)** at C_2 and subsequent dehydrogenation at C_4-C_5 sigma bond formed m-cymene **(31)** in the oils of the leaves air dried for one, three, four and five days.

The abundance of spathulenol and alloaromadendrene in the oils revealed that their synthases aided the biosynthesis of all sesquiterpenoids in the oils via ionization of their precursors (E,Z-farnesyl and E,E-farnesyl pyrophosphates) to various cationic intermediates (Reaction Scheme 2).



Reaction Scheme 4.2: Biogenesis of Sesquiterpenoids in the Essential Oils of the Fresh and the Dried Leaves of *E. globulus* (38 – 41).

In the Scheme, the alloaromadenrene, spathulenol and globulol synthases aided the ionization of E,Z-farnesyl and E,E-farnesyl pyrophosphates **(32 and 33)** to E,Z-farnesyl and E,E-farnesyl **(34 and 35)** cations in the leaves. Delocalization of C_2-C_3 pi electrons in E,Z-farnesyl cation **(34)** formed E,E-nerolidyl cation **(36)**. Electrophilic attack of the ion **(36)** on the C_{10}-C_{11} double bond via C_{11} formed humullyl cation **(37)**. Markovnikoff's addition of the ion **(37)** to the C_2-C_3 double bond via C_2 formed caryophyllyl cation **(38).** Deprotonation by the ion **(38)** at C_{15} gave β-caryophyllene **(39)** in the oils of the air dried leaves. The activity of spathulenol synthase did not favour the formation of the compound in the oil of the fresh leaves. Electrophilic addition of the E,Z-farnesyl cation to the C_{10}-C_{11} double bond via C_{10} gave E,Z-germacredienyl cation **(40)**. 10,11- and 1,10-hydride shifts of the ion **(40)** followed by

electrophilic attack of the intermediate cation on the C_6-C_7 double bond via C_6 formed cadinyl cation **(41)**. Hydration of the ion **(41)** formed α-cadinol **(42)** in the oils of the fresh leaves and the leaves air dried for two, three, four and five days. The spathulelol synthase was not active to catalyse the biosynthesis of both δ-cadinene and α-cadinol in the oil of the leaves dried for one day.

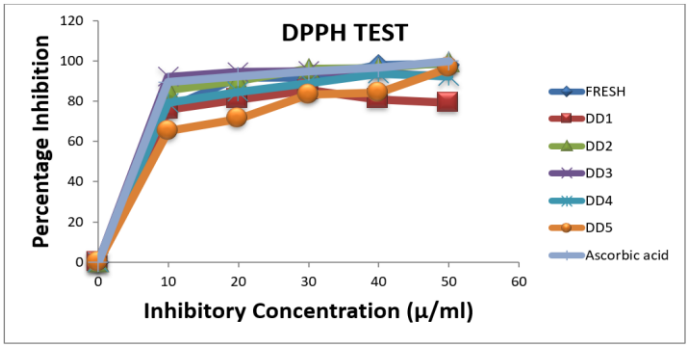
Electrophilic attack of the E,E-farnesyl cation on the C_{10}-C_{11} double bond via C_{10}produced E,E-germacredienyl cation **(43)**. Deprotonation of the ion **(43)** at C_{12} produced germacrene A **(44)**. Protonation of the C_6-C_7 double bond of the compound **(44)** via C_7 formed an intermediate cation **(45)**. Electrophilic attack of the ion **(45)** at C_2-C_3 double bond via C_2 formed eudesmanyl cation **(46)**. Deprotonation of the ion **(46)** at C_4 gave α-selinene **(47)** in the oils of the fresh leaves and the leaves air dried for two, three, four and five days. Alloaromadendrene synthase was inactive and could not aid the biosynthesis of the compound in the oil of the leaves air dried for one day. Protonation of the C_6-C_7 double bond of germacrene A **(44)** via C_6, followed by C_7 - C_6 hydride shift formed a cationic intermediate **(48)**. Electrophilic addition of the ion **(48)** to the C_2-C_3 double bond at C_2 formed guaiyl cation (**49**). Deprotonation of the ion **(49)** at C_2 produced α-guaiene **(50)** in the oils of the fresh leaves and the leaves air dried for one and four days.

Deprotonation of the guaiyl cation **(49)** at C_1 formed α-gurjunene **(52)** in the oil of the leaves air dried for three days. Protonation of the C_{11}-C_{12} double bond of α-guaiene formed an intermediate cation (**51**). Deprotonation of the ion (**51**) at C_1 followed by its electrophilic addition on the deprotonated carbon gave isoledene **(53)** in the oils of the fresh leaves and the leaves air dried for two, three, four and five days. Protonation of isoledene **(53)** followed by 6,7-hydride shift formed alloaromdendryl cation **(54)**. Loss of proton by the ion **(54)** at C_{15} formed alloaromadendrene **(55)** in the oils. Hydration of the ion **(54)** formed globulol **(56)** in the oils. Protonation of isoledene **(53)** and subsequent 2,3-hydride shift gave an intermediate cation **(57)**. Hydration of the ion **(57)** followed by dehydrogenation at C_{14} and C_{15} formed spathulenol **(58)** in the oils.

ANTIOXIDANT ACTIVITY OF THE OILS

DPPH Radical Scavenging Activity of the Oils

The oils were active against DPPH radical and their activity was concentration dependent. The activity of the oils from the fresh leaves and the leaves dried for two and five days increased steadily with increase in concentration. The highest activities of the oils of the leaves dried for one and three days were obtained at 30 µl/ml. However, the oil of the leaves dried for four days has the highest activity at 40 µl/ml. The activity of ascorbic acid also increased steadily with an increase in concentrations (Figure 3).



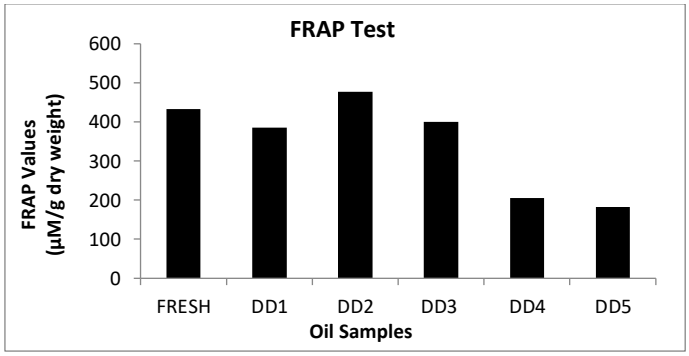
Key: DD1 = Day one Dried leaf, DD2 = Day two Dried leaf, DD3 = Day three Dried leaf, DD4 = Day four Dried leaf, DD5 == Day five.

Figure 3: DPPH Radical Scavenging Activity of Essential Oils from the Fresh and the Dried Leaves *E.globulus.*

The DPPH radical scavenging potential of leaf essential oils of *Melaleuca alternifolia*, *Hyptis crenata*, *Origanum rotundifolium* and *Thymus vulgaris* was due to the predominance of terpine-4-ol, borneol, carvacrol, thymol and 1,8-cineole in the oils [36, 40, 41]. The IC_{50} of 8.70, 10.64, 6.68, 4.11, 9.18 and 14.42 µg/ml were obtained for the oils of the fresh leaves and the air dried leaves respectively. Meanwhile, the oil of the leaves air dried for three days with the least IC_{50} was the most active. Its activity was higher than that of the standard, ascorbic acid (IC_{50} = 5.710 µl/ml). The oil contained greater amount of oxygenated monoterpenoids than the other oils. Thus, the higher DPPH radical scavenging activity of the oil from this leaf could be attributed to synergistic actions of the oxygenated constituents in the oil.

Ferric Reducing Antioxidant Activity of the Oils

The oils showed ferric reducing antioxidant activity and the activity varied with concentrations and period of drying of the leaves. The oil of the fresh leaves has FRAP activity of 460.17±0.491 µM/g dry weight. The activity decreased in the oil of the leaves air dried for one day and later increased steadily from the oil of the leaves air dried for two days to the oil of the leaves air dried for three days. Suddenly, the activity decreased in the oil of the leaves air dried four days and subsequently increased in the oil of the leaves air dried for five days (Figure 4).



Key: DD1 = Day one Dried leaf, DD2 = Day two Dried leaf, DD3 = Day three Dried leaf, DD4 = Day four Dried leaf, DD5 == Day five.

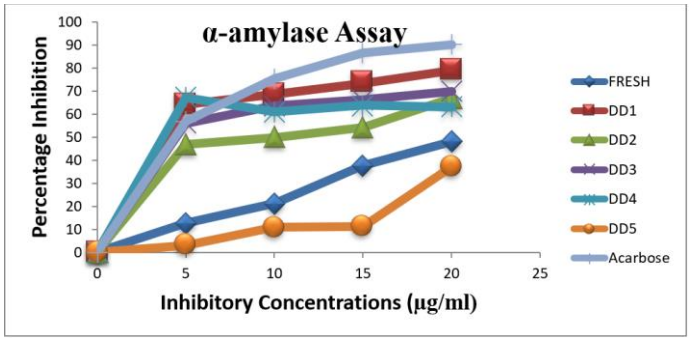
Figure 4. Ferric Reducing Antioxidant Potential of Essential Oils from the Fresh and the Dried Leaves of *E. globulus.*

The standards, BHT and BHA, were more active than the oils. However, the oil of the leaves air dried for three days was the most active. Irrespective of level of dryness, the oil from this study showed higher ferric reducing antioxidant potential and was richer in oxygenated compounds than the oil of the leaves of the plant native to Pakistan [42].

ANTIDIABETIC ACTIVITY OF THE OILS

α-Amylase Inhibitory Activity

The oils inhibited the activity of α-amylase. The activity of the oils was concentration dependent and increased steadily with concentrations. The oils of the fresh leaves and the leaves air-dried for five days showed less than 50% inhibition of the enzyme activity at all concentrations. Meanwhile, more than 50% inhibition of the enzyme activity were shown by the other oils [Figure 5].



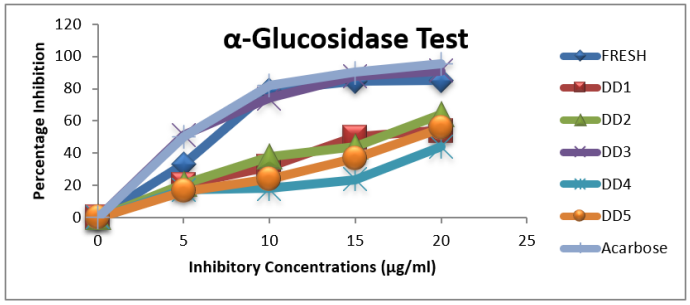
Key:DD1 = Day one Dried leaf, DD2 = Day two Dried leaf, DD3 = Day three Dried leaf, DD4 = Day four Dried leaf, DD5 == Day five

Figure 5. α-amylase Inhibition of Essential Oils from the Fresh and the Dried Leaves *E.globulus.*

The oils of the leaves air dried for three and five days with IC_{50} of 12.015 and 32.78 µg/ml were the most, and least active.

α-Glucosidase Inhibitory Assay

The oils were active against α-glucosidase and their activity increased steadily with concentration. Thus, the highest activity of the oils was obtained at the highest concentration of 20 µg/ml. The oil of the leaves dried for three days has more than 50% activity at minimum inhibitory concentration of 5 g/ml [Figure 6].



Key: DD1 = Day one Dried leaf, DD2 = Day two Dried leaf, DD3 = Day three Dried leaf, DD4 = Day four Dried leaf, DD5 == Day five.

Figure 6. α-glucosidase Inhibition by the Essential Oils from the Fresh and the Dried Leaves *E. globulus* and Acarbose

Essential oils of the fresh leaves and the leaves air dried for three days have comparable IC_{50} (8.574 µg/ml and 7.523 µg/ml) with that of the standard (IC_{50} = 7.048 µg/ml). Hence, the oil of the leaves dried for three days was again the most active.

Mode of α-amylase and α-glucosidase Inhibitions

The mode of inhibition of α-amylase and α-glucosidase by the active constituents of the oil from the leaves air dried for three days was investigated using Line weaver-Burk plot [Figures 7 and 8].

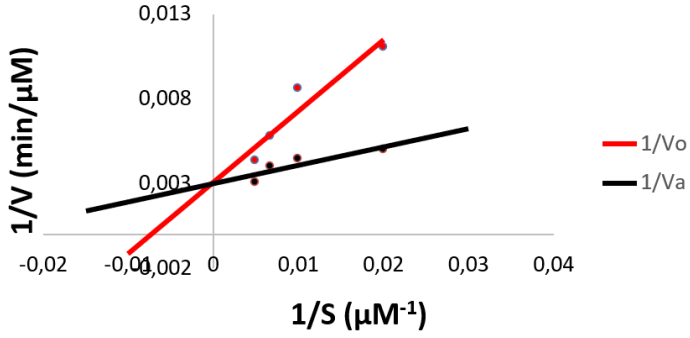


Figure 7. Lineweaver-Burk Plot of α-amylase Inhibition by the Oil of the Leaves Air-dried for Three Days.

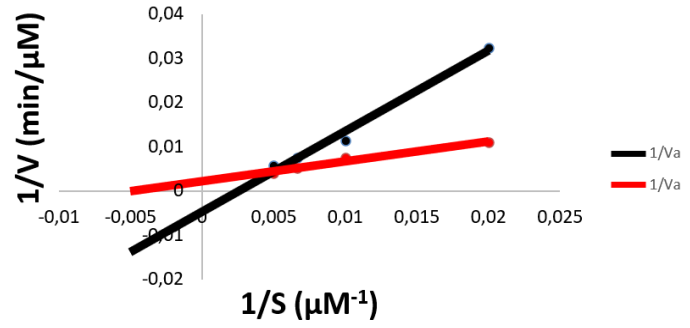


Figure 8. Lineweaver-Burk Plot of α-glucosidase Inhibition by the Oil of the Leaves Air-dried for Three Days.

The graphs portrayed in the figures showed competitive mode of inhibition for both α-amylase and α-glucosidase. This implies that the active constituents in the oil compete with the substrate for binding to the active site of the enzymes, thereby, reducing or preventing hydrolysis of starch to disaccharides, oligosaccharides, and subsequently monosaccharaides.

The dendrogram portrayed in Figure 2 showed a correlation between the constituents of the oils from the leaves dried for three, four and five days. The oils were richer in m-cymene, 6-camphenol, terpinen-4-ol, piperitone and 2,3-pinanediol than the other oils. Despite the correlation, the oils of the leaves dried for three and five days showed the highest and the lowest antioxidant and antidiabetic activities respectively. Thus, the higher activity of the oil of the leaves air-dried for three days might be due to the synergistic action of all constituents in the oil.

CONCLUSIONS

The period of drying at ambient temperature affected the yields and composition pattern of essential oil from leaves of *E. globulus*. Irrespective of the period of drying, the oils showed comparable antioxidant and antidiabetic activities with the standards. Interestingly, the leaves air-dried for three days afforded more oil than other leaves. The oil also showed higher antioxidant and antidiabetic activities than other oils due to the abundance of oxygenated monoterpenoids. Hence, the oil could serve as better candidate for the management of oxidative stress and type 2 diabetes.

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