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Determination of eugenol and its derivative isoeugenol in *Globularia alypum* using solvent system extraction and comparative study of their antioxidant activities with various oxidation conditions

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ABSTRACT

Globularia alypum leaves have been widely used for more years in gastrointestinal disorders as a traditional folk medicine. The aim of the present study was to determine the chemical composition of the petroleum ether extract and to evaluate its antioxidant activities in comparison with eugenol and its derivative isoeugenol. After phytochemical tests, a simple hydrodistillation was effectuated by Clevenger apparatus and the distillate was extracted with petroleum ether by decantation process. Gas chromatography mass spectroscopy was used to identify and quantify phenolic compounds in this extract. The antioxidant activity of petroleum ether extract of Globularia alypum was measured in vitro by 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. However, total antioxidant capacity (Molybdate phosphate test), hydrogen peroxide scavenging and reducing power antioxidant were estimated. The petroleum ether extract demonstrated a low free radical scavenging capacity compared to eugenol and isoeugenol extracted from Syzygium aromaticum. Furthermore, the analysis of this extract by CPG/ms led to the identification of new known phenol named eugenol and also its derivative isoeugenol with considerable amounts (10.56%, 0.87%), respectively. The antioxidant capacity of the petroleum ether extract is probably associated with phenolic compounds detected and its principle compounds indicate that this plant may be an important source of chemopreventive and chemotherapeutic natural products. The best of our knowledge is the combination of new detected compounds for the first time, eugenol and its derivative in this plant which have been tested separately as powerful antioxidant agents. However, further studies are required to determine if this is of clinical significance.

Keywords: *Globularia alypum*, Petroleum ether extract, CPG/ms, Eugenol, Isoeugenol, Antioxidant activities.

INTRODUCTION

The development of alternative antioxidants from natural origin has attracted considerable attention and many researchers have focused on the discovery of new natural antioxidants aimed at quenching biologically deleterious radicals [1]. Many natural compounds extracted from plants have exhibited biological activities including antioxidant potential [2]. Data bank assembles the chemical composition and the biological proprieties of plants which are in the course of constitution in certain countries of North Africa. These plants particularly studied are known by the local population for their benefits effects toward the human health. The genus *Globularia* consists of plants which are herbs, chamaephytes or perennial shrubs, found throughout the Mediterranean area, Europe and North Africa (Tunisia, Morocco, Libya and Algeria) [3]. *G. alypum*, named locally "Tasselgha" is mostly used in the indigenous system of medicine for a variety of purposes such as hypoglycemic agent, laxative, cholagogue,

stomachic, purgative and sudorific [4]. The essential oils or extracts obtained by chloroform treatment, ethyl acetate and water/methanol mixture (1:1) are proposed as treatment of infections caused by bacteria Gram+ and Gram– [5]. However, Ferhi and Aiache reported that aqueous extract exerts on the guinea pig an antiulcer activity against the gastric mucosal damages caused by indomethocin [6].

The global qualitative analysis of aqueous extract revealed principally the presence of tannins and flavonoids [7]. After the treatment of the aqueous phase with hexane followed by fractionation with methanol, Es-safi et al isolated the fraction responsible of the highest antioxidant power against DPPH radical (2,2-diphenyl-1-picrylhydrazyl) [8][9]. However, this substance has been used as the most crucial pathway to evaluate the free radical-scavenging activity of natural antioxidants [10]. The heterosides are for example frequently characterized by their diverse biological activities and made previously the subject of profound studies particularly in fruits such as grape and myrtle.

As known, phenolic derivatives like eugenol (4-allyl-2-methoxyphenol) and isoeugenol (2-methoxy-4-propenylphenol) are present also in a variety of plants such as *Syzygium aromaticum* (clove) [11]. Eugenol, o-methoxyphenol, is of interest for many recent researchers because of its anti-inflammatory and chemopreventive effects which stem from the antioxidant role contributed by its phenolic group [12]. It is considered non-mutagenic, non-carcinogenic and generally recognized as safe (GRAS) by Food and Drug Administration.

The purpose of this study was to determine the chemical composition of the petroleum ether extract from leaves of *G. alypum* and to evaluate the antioxidant activities of this fraction in comparison with eugenol and its derivative isolated from *Syzygium aromaticum*. However, the synergistic effect of eugenol and isoeugenol in the entire petroleum ether extract for the antioxidant activities was checked.

EXPERIMENTAL SECTION

2.1. Plant material

Fresh leaves of *Globularia alypum* were collected from remote areas in the suburbs of Souk ahras region (NE Algeria) during the flowering season (April 2012). The taxonomic identification was performed in Biology department, Badji mokhtar university, Annaba, Algeria. The plant material was then isolated from the other specimen and was dried in shade for a week at room temperature. The dried leaves were conserved for the extraction process.

2.2. Preparation of the plants extracts

The process adopted in this study to isolate the extract from *Globularia alypum* is the hydrodistillation according to the method recommended in the European Pharmacopeia [13]. Then, the distillate was successively extracted twice under occasional shaking with selected solvent using 10 ml of petroleum ether. Then, it was dried over anhydrous magnesium sulfate. The isolated residue was filtered and the solvent was eliminated using a rotary evaporator to obtain a dry extract. After evaporation, the extract was kept until the antioxidant bioassays. In the moment at utilization, the extract was diluted in 1ml of the same solvent of extraction. The extraction yield (%, w/w) was calculated as the ration of the weight of the extract to the weight of the crude leaves. Therefore, eugenol and its derivative were extracted from *Syzygium aromaticum* using a method adopted by Chae-Bin Yoo et al [14].

2.3. Reagents and chemicals

2.2-Diphenyl-1-picrylhydrazyl (DPPH), BHT (butylated hydroxytoluene), Gallic acid, Ascorbic acid (Vitamin C), EDTA, and Citric acid reagents were purchased from Sigma-Aldrich Chemical. All solvents and chemicals used in the experiments were of analytical grade and were of the highest purity needed for each application. The water used was purified and distilled.

2.4. CPG/ms analysis

The sample of petroleum ether extract isolated from *Globularia alypum* leaves was analyzed by CPG/ms using an HP 5890 series II gas chromatograph equipped with a flame-ionization detector and coupled to an HP 5972 mass spectrometer (Agilent Technologies) with electron-impact ionization (70eV) and an HP-5MS capillary column (30 m 9 0.25 mm coated with 5% phenyl methyl silicone, 95% dimethylpolysiloxane; a 0.25-µm film thickness were used). The operating conditions were as follows: The column temperature was programmed to rise from 50 to 240°C at a rate of 5°C /min, and the transfer-line temperature was 250°C. The carrier gas was helium with a flow rate of 1.2 ml/min and a split ratio of 60:1. The scan time and mass range were 1s and 40-300m/z, respectively. The mass-spectrometer conditions were the following: injection of 2µl aliquot of the sample and an HP-5MS capillary column (30 m × 0.25mm; coating thickness, 0.25µm).

The components of the extract were identified by a comparison of the fragmentation patterns in the mass spectra with those stored in the GPG/ms databases and other published mass spectra in relation to the retention time of a homologous series of alkanes (C-C) [15]. In addition, the percentages of the compounds were determined from their peak areas.

2.5. Antioxidant activities

2.5.1.DPPH radical-scavenging assay

The antioxidant activity of the isolated compound was evaluated through specrophotometric technique according to the method previously reported by Burits and Bucar [16]. Briefly, 50 ml of a methanolic solution containing the compound to be tested were added to 5 ml of 0.004% MeOH solution of DPPH. Then, the studied extract was tested with MeOH as control. The mixture was shaken vigorously and incubated in the dark and the absorbance at 517nm was determined after 30 min using a spectrophotometer. The absorbance (A) of the controls and samples was measured, and the DPPH scavenging activity in percentage was determined as follow: DPPH scavenging activity (%) = $(A_{control}-A_{sample})/A_{control}$ x100 where $A_{control}$ and A_{sample} are the absorbance of the control and the sample, respectively. The antioxidant reagent BHT, Gallic acid, Ascorbic acid (Vitamin C), EDTA and Citric acid were used as a positive control. The results are expressed as IC_{50}, the amount of antioxidant necessary to decrease the initial concentration of DPPH by 50%. The lower IC_{50} value indicated a higher antioxidant activity. The values are presented as the means of triplicate analysis.

2.5.2.Total antioxidant capacity (Molybdate phosphate test)

The purpose of Molybdate Phosphate test is the measure of the effectiveness of non enzymatic antioxidants. This method is based specially on the reduction of Molybdate (VI) into Molybdate (V) to estimate the formation of Molybdate Phosphate complex with green color and it was evaluated by spectrophotometric technique [17]. Then, 100µl of the solution containing the extract was added to 2ml of Molybdate Phosphate solution (0.6M sulfuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate). After 90min of incubation in 90°c, the absorbance was measured at 695nm. The antioxidants reagents BHT, Gallic acid, Ascorbic acid (Vitamin C), EDTA and Citric acid were used as a positive control. The total antioxidant capacity was expressed as the ration of mg of the antioxidant standard by mg of each extract.

2.5.3.Reducing power antioxidant

The ability of the extracts to reduce iron (III) was determined according to the Yildirim et al. method with some modifications [18]. An aliquot of 500µl of each sample was dissolved in ethanol and mixed with 1.25ml of reagent of 0.2M phosphate buffer (pH 6.6) and 1.25ml of 1% potassium ferracyanide. The mixture was incubated for 30 min at 50°C, followed by addition of 1.25ml of 10% (w/v) trichloroacetic acid. The mixture was then centrifuged at 1650g for 10 min. Finally, 1.25 ml of the supernatant solution was mixed with 1.25ml of distilled water and 250µl of 0.1% (w/v) ferric chloride. After 10 min, the absorbance was measured at 700nm.The antioxidants reagents BHT, Gallic acid, Ascorbic acid (Vitamin C), EDTA and Citric acid were used as positive controls. Increased absorbance of the reaction mixture indicated increased reducing power. The values are presented as the means of triplicate analysis.

2.5.4.H_2O_2 scavenging assay

The ability of the extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al. A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4). Extracts were dissolved (1mg/ml) in distilled water and added to a hydrogen peroxide solution (0.6 ml, 40mM). The absorbance of hydrogen peroxide at 230nm was determined 10 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging of the extracts and standard antioxidants were calculated: % scavenging [H2O2] = [(Ac– As)/Ac] x 100 Where Ac is the absorbance of the control and As is the absorbance in the presence of the sample of the extracts and the standards were: BHT, Gallic acid, Ascorbic acid (Vitamin C), EDTA and Citric acid [19].

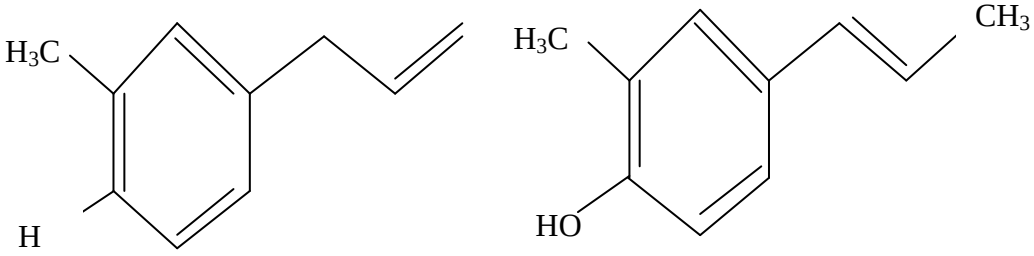
2.6. Statistical analysis

Data obtained from extracts and control reagents assays were expressed as mean values. Statistical analysis was performed with MINITAB 16 package. Differences were tested for significance by using the ANOVA procedure. Level of significance was: Significant when $P \leq 0.05$ (*); highly significant when $P \leq 0.01$ (**) and very highly significant when $P \leq 0.001$ (***). All data were reported as means ± SD standard deviation of at least triplicate of different assays.

RESULTS AND DISCUSSION

Medicinal plants are known to possess many different components which have various biological activities. The dried leaves of *Globularia alypum* were treated by hydrodistillation and the aqueous phase was exposed to fractionation by polar or non polar solvents. The chemical analysis of petroleum ether extract (11.73%, w/w) isolated from *Globularia alypum* leaves by CPG/ms showed 82.36% and 17.64% as major and minor total identified compounds, respectively, presenting high fluctuations in its chemical profile. All data are presented in **Table 1**. The results revealed relatively a high content of dehydroionone (18.13%), xylene (11.72%) and eugenol (10.56%) among 31 identified compounds. In addition, the other important components were present in scanty amounts, such as isoeugenol with 0.87%. The chemical structures of eugenol and its derivative were illustrated in **Figure 1.** In parallel, eugenol reached its highest concentration in *Syzygium aromaticum* (97%) where isoeugenol yield was

1,17%. A high number of phenols and phenolic ethers are produced in nature like thymol, safrole and eugenol which are presented in market as aromatic substances. For example, eugenol is characterized by its perfume in clove and it is the favorite antiseptic of dentists [20]. It is an interesting compound presented in many species where it reached 1,4 % in the essential oil of *Cinnamomum altissimum Kosterm* [21]. Thus, *Syzygium aromaticum* (*Eugenia caryophyllata*, Myrtaceae) is characterized also by high content of eugenol, acetyleugenol, chavicol, acetyl salicylate and humulenes [22]. In recent Algerian research, eugenol was detected also with little amounts in the essential oil of *Globularia alpyum* extracted by hydrodistillation in two different localities (3,06% and 0,22%) which had a significant antibacterial effectiveness [23]. A comparison between our plants content of eugenol and another species from various origins is illustrated in **Figure 2**. Owing to the value of eugenol in many fields, the researchers focused on the potential effect of eugenol as drug for preventing liver damage induced by hepatotoxins [24]. In addition, the synergistic interaction of eugenol and antibiotics was demonstrated against gram negative bacteria and it can combined also with cinnamaldehyde inducing a synergistic effects against wood decay fungi [25][26]. In another point of view, it was reported that eugenol could act as scavenger of peroxide anion and hydroxyl radicals [27][28]. However, the relationship structure-activity of eugenol was assessed to estimate its antioxidant activity [29].



Eugenol **Isoeugenol**

Figure 1: Chemical structures of eugenol (4-allyl-2-methoxyphenol) and isoeugenol (2-methoxy-4 -propenyphenol)

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Table 1: Chemical composition of the petroleum ether extract (PE) isolated from *Globularia alypum* leaves

N°	Compounds	RT (min)	IR	Area (%)
1	Ethylbenzene	6.343	539	1.75
2	Xylene	6.567	576	11.72
3	D-Fenchone	12.923	1628	1.83
4	Camphor	14.585	1903	3.62
5	alpha-Terpineol	16.083	2151	1.2
6	Neohexane	18.246	2509	0.36
7	alpha-Fenchyl acetate	18.615	2570	1.15
8	n-Tetradecane	19.038	2640	1.44
9	Sabinyl acetate	19.702	2750	0.45
10	1-Ethyl-1,5-cyclooctadiene	20.482	2879	0.16
11	Eugenol	20.868	2943	10.56
12	Isoeugenol	20.971	2960	0.87
13	Diethylmethyl-borane	22.868	3274	1.19
14	Eicosane	23.225	3333	0.65
15	17-Pentatriacontene	23.829	3433	0.60
16	Nonadecane	23.992	3460	0.68
17	Pentadecane	24.119	3481	2.13
18	n-Octadecyl chloride	24.318	3514	4.79
19	Bicyclopentyl-2'-en-2-yl-dimethyl-amine	24.578	3557	0.50
20	3-Cyclopentyl-pentane	25.219	3663	0.63
21	Lignocerol	25.370	3688	1.42
22	Viridiflorol	26.403	3859	3.81
23	Cetane	26.475	3871	2.94
24	alpha-Cadinol	26.711	3910	4.92
25	Aromadendrene	27.212	3993	1.21
26	2,2-Dimethyl-6,10-dithiaspiro [4.5]decan-1-ol	27.309	4009	1.13
27	Hexatriacontane	27.545	4048	4.98
28	Neoclovene	28.360	4183	1.32
29	Dehydroionone	31.907	4770	18.13
30	Amphetamine oxime acetate	32.366	4846	4.56
31	Phthalic acid	44.136	6794	10.20
Total major identified compounds (>2%)				82.36
Total minor identified compounds (<2%)				17.64

The presence of different antioxidant components in the plant tissues makes it relatively hard to quantify each antioxidant component separately. Therefore, several intermediate extractions are used to ensure a maximum

extraction of the available antioxidants [30]. The isolation of petroleum ether extract was effectuated by different methods; the Tunisians used direct method of extraction by soxhlet aiming to obtain only non polar extract with high content of sterols [7]. In our case, the method adopted was the extraction from aqueous phase to isolate probably extracts with double affinity to polar or non polar solvents.

Four methods can be applied to determine the antioxidant activity: DPPH scavenging activity, total antioxidant capacity, reducing power activity and H_2O_2 peroxide scavenging. For instance, this study focused on the extracts to scavenge free radical DPPH which is commonly associated with the oxidative test. All data are presented in **Table 2**. Additionally, all plants extracts (eugenol, isoeugenol and petroleum ether extract) showed different antioxidant activities proving their capacity to scavenge the free radical DPPH when both of eugenol and its derivative presented a close activities. The IC_{50} values determined that eugenol (0,258±0,26 mg/ml) and isoeugenol (0,336±0,22 mg/ml) have higher antioxidant activity than the PE extract (0,460±0,16 mg/ml) indicating that there is no synergistic effect of both of them in the extract for the antioxidant activity. The decrease in absorbance at 517 nm for each extract in intervals of time is determined, and plotted **(Figure 3).**

Table 2: IC_{50} values of the extracts compared to the antioxidant controls with respect to the concentrations

Samples		IC_{50} values	R^2	AER	Order
Extracts	E	0,258±0,26	0,984	3,875±3,84	2
	IE	0,336±0,22	0,993	2,976±4,54	4
	PE	0,460±0,16	0,999	2,173±6,25	6
Positive controls	VC	0,241±0,29	0,992	4,149±3,44	1
	CA	0,352±0,23	0,999	2,840±4,34	5
	GA	1,454±0,54	0,989	0,687±1,85	8
	BHT	0,331±0,21	0,983	3,021±4,76	3
	EDTA	0,824±0,20	0,996	1,213±5,00	7

- *Standard deviations (SD) did not exceed 0,54 (IC_{50}) and 6,25 (AER)*
- *IC_{50} values were expressed as mg of extract or positive control/ml of methanol*
- *Antiradical efficacy relative (AER) was expressed as the inverse of IC_{50} (AER =1/IC_{50})*
- *The order of the DPPH scavenging activity is: VC> E> BHT> IE> CA> PE> EDTA> GA*
- *The statistical differences are expressed as:*

E Vs IE, PE, VC, CA, EDTA, GA, BHT: $^{***}P\leq0,001$; E Vs GA: $^{*}P\leq0,05$; E Vs EDTA: $^{*}P\leq0,05$; CA Vs GA: $^{*}P\leq0,05$; EDTA Vs BHT: $^{*}P\leq0,05$

In according with literature, the obtained results showed that the activity towards the DPPH free radical was probably due to the main constituents of the extract specially eugenol and isoeugenol which were separately most active free radical scavengers. In addition, it has been reported that there is a relationship between the content of phenolics in the extracts and their antioxidant activity [31].

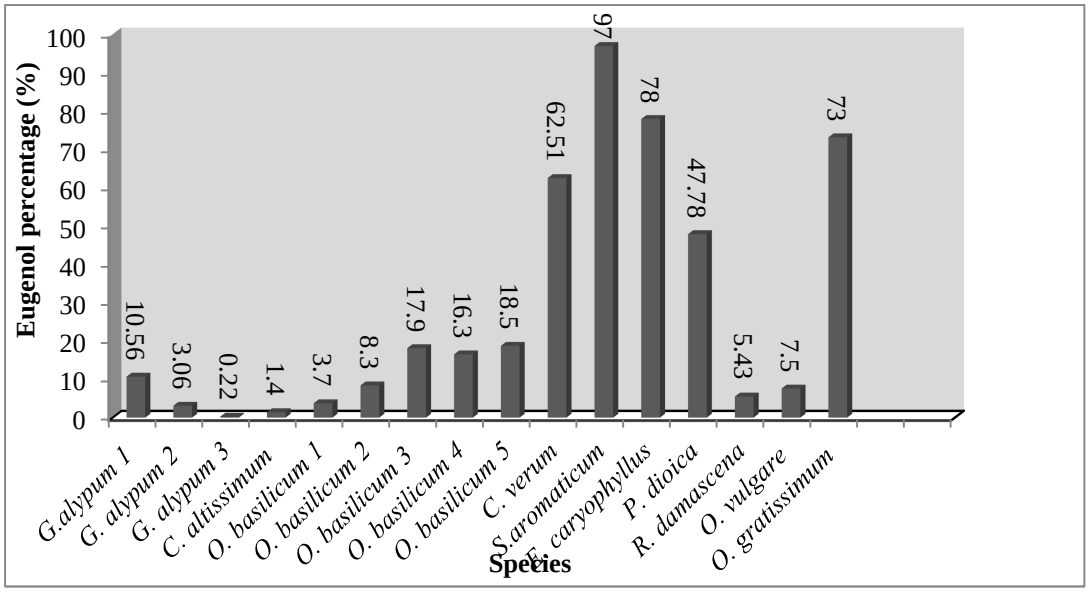

Figure 2: Investigation of eugenol percentage (%) of different species from various origins. *Globularia alypum* 1 (Algeria, Soukahras, sample studied), *Globularia alypum* 2 (Algeria, Boutaleb), *Globularia alypum* 3 (Algeria, Khenchla), *Syzygium aromaticum* (sample studied), *Ocimum basilicum* 1 (Turkey, Location 1), *Ocimum basilicum* 2 (Turkey, Location 2), *Ocimum basilicum* 3 (US, Location 1), *Ocimum basilicum* 4 (US, Location 2), *Ocimum basilicum* 5 (Greece), *C. altissimum Kosterm* (Malaysia), *Cinnamomum verum* (Madagascar), *Eugenia caryophyllus* (Madagascar), *Pimenta dioica* (Antilles), *Rosa damascene*(Iran), *Origanum vulgare L* (Croatia), *Ocimum gratissimum* (India). The order of the abundance: EC>OG>CV>PD>OB_5>OB_3>OB_4>GA_1>OB_2>OV>RD>OB_1>GA_2>CA>GA_3

[21, 23, 36, 44, 45, 46, 47]

Similarly, Es-Safi et al have isolated a phenolic compound, 6-hydroxy-luteolin-7-laminaribioside, from the aerial parts of *G. alypum* which displays an important antioxidant activity and subsequently they estimated the relationship between structure-activity of this potent fraction [32].

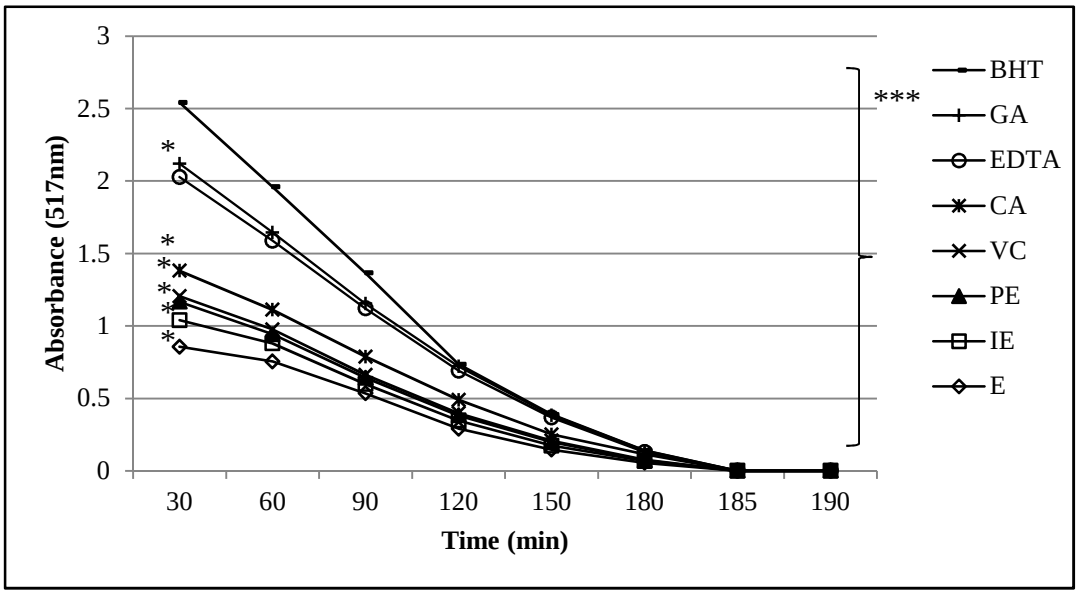

Figure 3: Plot of absorbance at 517nm of the extracts compared to the antioxidant controls as a function of time (30-180min). Extracts: Eugenol, Isoeugenol and Petroleum ether extract. Controls: Vitamin C, Citric acid, EDTA, Gallic acid and BHT (butylated hydroxytoluene).

*E Vs IE, PE, VC, CA, EDTA, GA, BHT:*** $P \leq 0,001$; *E Vs IE:* * $P \leq 0,05$; *E Vs PE:* * $P \leq 0,05$; *E Vs VC:* * $P \leq 0,05$; *E Vs CA:* * $P \leq 0,05$; *E Vs GA:* * $P \leq 0,05$

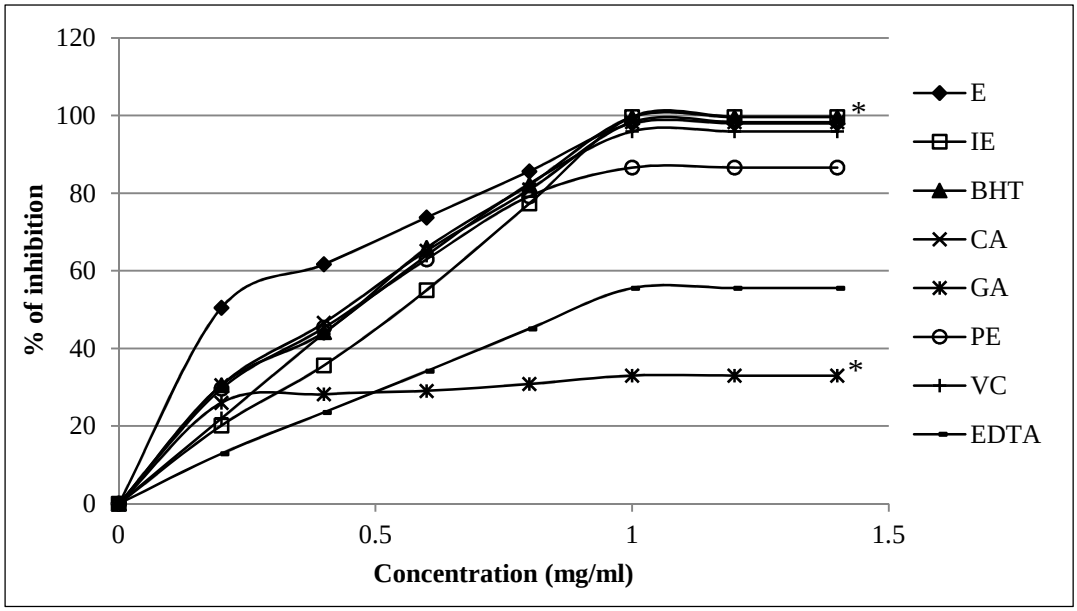

Figure 4: Dose-dependent DPPH radical scavenging activity of the extracts compared with the antioxidant controls with respect to the concentrations. Extracts: Eugenol, Isoeugenol and Petroleum ether extract. Controls: Vitamin C (AA), Citric acid, EDTA, Gallic acid and BHT (butylated hydroxytoluene). E Vs GA: * $P \leq 0,05$

Results of DPPH free radical activity indicated that eugenol sample exhibited significant activity with dose dependent in resemblance with isoeugenol sample but better than of petroleum ether extract sample **(Figure 4).** However, DPPH activity of eugenol could be due to its phenolic components [33]. The petroleum ether extract of *G. Alypum* was characterized by estimation of total phenolics and flavonoids present in it using CPG/ms. As known, aromatic compounds including eugenol and isoeugenol are a class of antioxidant agents which act as free radical terminators [34][35]. Besides, the Indian researchers indicated that the significant increases in DPPH free radical scavenging power of eugenol were observed in concentration dependent fashion (IC_{50} value was 242,47ug/ml) and ascorbic acid; the power antioxidant had lower IC_{50} value than eugenol [36]. In assessment of antioxidant activities of eugenol by *in vitro* and *in vivo* methods, eugenol as lipophilic compound investigated its ability to scavenge chain

propagating radicals which form during lipid peroxidation [37]. In another point of view, the antioxidant efficacy of encapsulated eugenol in chicken noodles was also evaluated as the best inhibitor of the DPPH radical during successive days of storage with significant variation of percentage of inhibition in comparison with chitosan and EDTA [38].

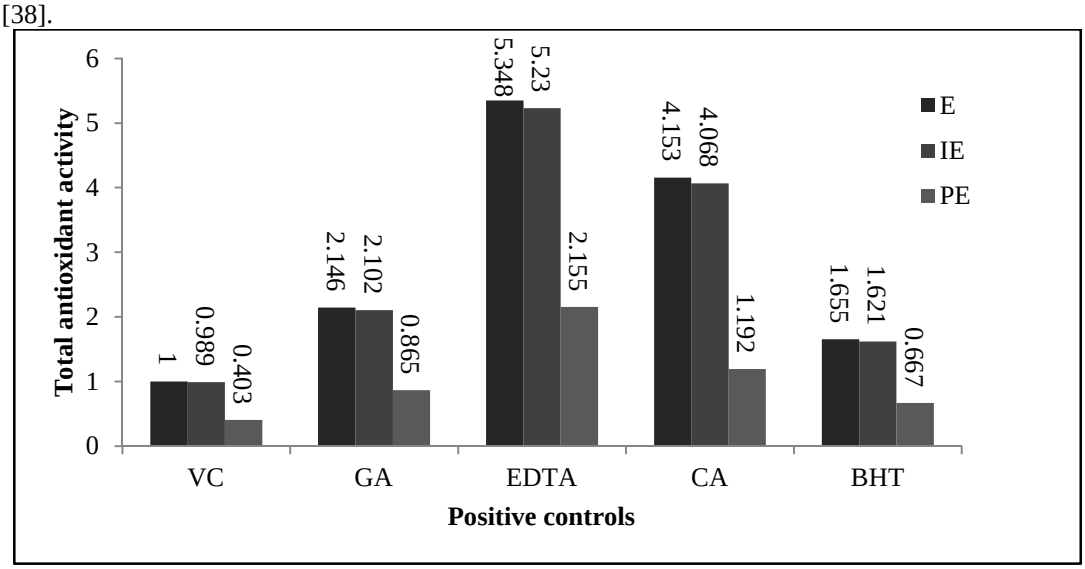

Figure 5: Comparison of the total antioxidant capacity of the extracts as the ratio of mg positive control/mg extract. Extracts: Eugenol, Isoeugenol and Petroleum ether extract. Controls: Vitamin C (AA), Citric acid, EDTA, Gallic acid and BHT (butylated hydroxytoluene)

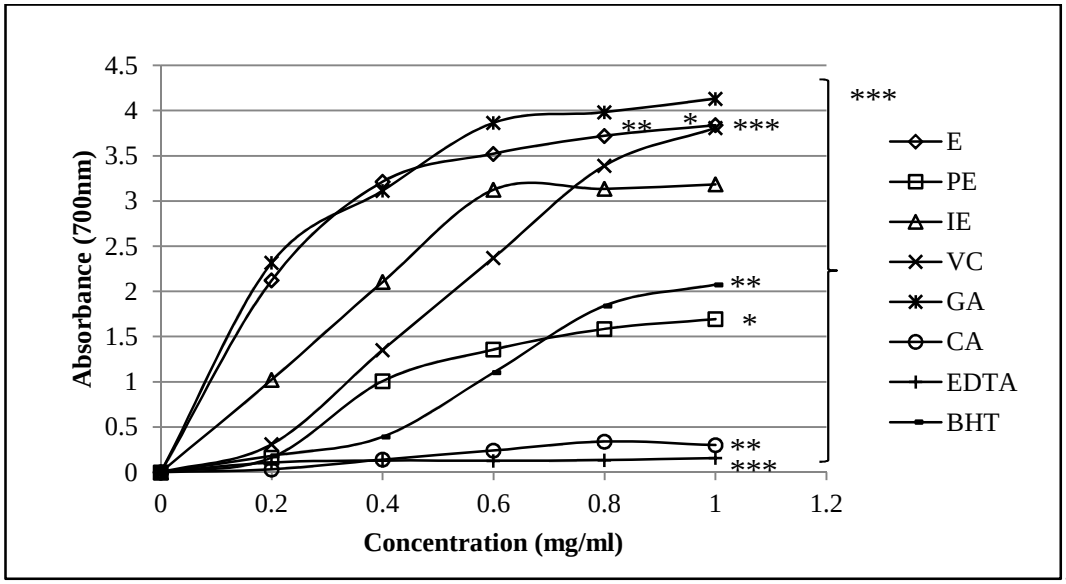

Figure 6: Plot of reducing power antioxidant of the extracts compared to the antioxidant controls with respect to the concentrations (mg/ml). Extracts: Eugenol, Isoeugenol and Petroleum ether extract. Controls: Vitamin C (AA), Citric acid, EDTA, Gallic acid and BHT (butylated hydroxytoluene)

E Vs IE, PE, VC, CA, EDTA, GA, BHT: $^{***}P\leq0,001$; E Vs PE: $^{*}P\leq0,05$; E Vs CA: $^{**}P\leq0,01$; E Vs EDTA: $^{***}P\leq0,001$; E Vs BHT: $^{**}P\leq0,01$

As seen on **Table 2,** it can be concluded that the positive controls using high polarity solvents (methanol) were considerably more effective radical- scavengers than were those using low to null polarity solvents (ether and petroleum ether respectively). Change in solvent polarity alters its ability to dissolve a selected group of antioxidant compounds and influences activity estimation [31]. Among the best inhibitors, *G. alypum* is almost more potent than ascorbic acid which classed as a potent standard antioxidant [39].

In the phosphomolybdenum assay, the petroleum ether extract, eugenol and its derivative exhibit different degrees of antioxidant activity which increase with same concentrations and same conditions **(Figure 5).** The reducing power assay is often used to evaluate the ability of antioxidant to donate electron [18]. To fulfil our goal, a direct correlation between the antioxidant activities and the reducing power of some bioactive compounds was reported. However, a higher absorbance indicated a higher antioxidant activity and the reducing power is proportionally increase with the concentration. Hence, the Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action. In the reducing power assay, the ability of

our antioxidant agents to reduce Fe3+ to Fe2+ was checked. **Figure 6** showed the reducing power activities of eugenol, isoeugenol and petroleum ether extract. Indeed, the presence of antioxidants in the herbal products would result in the reducing of Fe3+to Fe2+by donating an electron [40].

In agreement with an Indian research, the purified and isolated eugenol from *O. gratissimum* has the reductive potential activity that might act as an electron donor, terminated the radical chain reaction but it possesses lower antioxidant power than ascorbic acid where the reducing power of eugenol was found to increase with the increasing concentration which was comparable with the standard antioxidant ascorbic acid [36]. For the last method, H_2O_2 scavenging activity of each extract was ranged from strong (92,81%, VC) to moderate (32,65%, EDTA) effect in comparison between the extracts and the antioxidant controls **(Figure 7).**

Many factors may be effected the increasing of reducing power capability such as the decrease in the absorbance of the eugenol after esterification and also the high concentrations used [12] in according with Loo et al who reported that an increase in the number of both hydroxyl and methoxy groups in the phenyl ring increases the reducing ability of hydroxybenzene and methoxyphenols [41]. The difference in our results and the literature can be attributed to the different plant parts used, the different methods of extraction and finally, the climate differences between Morocco, Tunisia and Algeria, geographical origin, harvesting time and growing conditions [9]. On the other hand, Djeridane et al reported that the hydromethanolic extract of *G. alypum* has also an antioxidant activity evaluated by the DPPH assay [8]. Among the factors responsible of the different degrees of the activities, the chemical structure illustrated by the configuration of atoms where the position of the double bond in the flavonoid C-ring, the number and the relative position of hydroxyl groups (–OH) in the aromatic ring are the most important parameters to explain the change in the activity of the phenolic extracts [42]. Therefore, Foti et al proposed that the antioxidant activity of flavonoids was especially dependent on the presence of ortho phenolic functions [43]. In the light of the facts cited above, many reasons can be proposed to explain which factor may intermediate exactly in the antioxidant activities but the real mechanisms of action of this plant compounds still unclear.

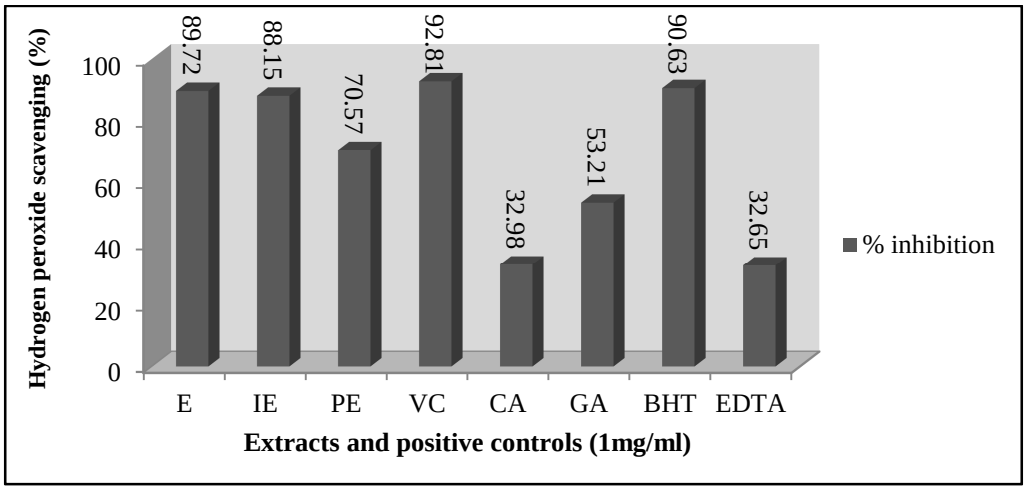

Figure 7: Plot of H_2O_2 scavenging activity of the extracts compared to the antioxidant controls with respect to the concentrations (mg/ml). Extracts: Eugenol, Isoeugenol and Petroleum ether extract. Controls: Vitamin C, Citric acid, EDTA, Gallic acid and BHT (butylated hydroxytoluene)
The order of the activity: VC>BHT>E>IE>PE>GA>CA>EDTA.

CONCLUSION

Chemotherapeutic approaches using non-toxic plant derived substances may be one strategy to defeat many diseases. However, several physiological activities have been described for both substances eugenol and isoeugenol presented in different known varieties. This fact prompted us to determine their presence also in *Globularia alypum* leaves (10.56% and 0.87% respectively). We therefore studied the antioxidant activities of both substances separately and synergistically in the Petroleum ether extract. Finally, it is concluded that the petroleum ether extract characterized by high content of phenolic compounds exhibited an antioxidant activity. Taken together, these results give reason to assume that the both eugenol and isoeugenol in this plant might act as such chemotherapeutic agents.

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