CHEMICAL COMPOSITION AND BIOLOGICAL ACTIVITIES OF THE ESSENTIAL OILS AND THE METHANOLIC EXTRACTS OF *BUNIUM INCRASSATUM* AND *BUNIUM ALPINUM* FROM ALGERIA

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ABSTRACT

In order to study the curing proprieties of endemic Algerian plants, it is evaluated the chemical composition and four biological activities of two Apiaceae species, which are *Bunium incrassatum* and *Bunium alpinum*. The essential oils (EO) obtained by hydro-distillation of dried aerial parts were analyzed by GC/MS. The antibacterial activity was investigated using the disc diffusion assay against ten (10) Gram-positive and Gram-negative bacteria. Antioxidant activity was evaluated by DPPH technique. Methanolic extracts (Me EXTs) were used in studying *in vitro* anti-inflammatory activity using egg albumin technique and *in vitro* anti-hemolytic activity using HBRC technique. The EO yield based on dried plant material was 0.09 % for *B. incrassatum* and 0.10 % for *B. alpinum*. Thirty-one compounds (corresponding to 97.19%) were identified for *B. incrassatum*. The main component was palmitic acid (18.39%). While twenty-four compounds (coresponding 87.33%) were identified for *B. alpinum.* The main component was caryophyllene oxide (33.84%). The study of antibacterial activity demonstrated that the EOs showed a modest antibacterial activity compared to gentamicin. The antioxidant activity revealed that EOs and Me EXTs demonstrated a very important anti-radical activity compared to standards (BHT, BHA, tocopherol, quercitin and rutin). The Me EXTs demonstrated also, significant antihemolytic and anti-inflammatory activities to those of standard (sodium diclofenac). It was found that all these activities are much related to the chemical composition of EO and Met EXTs. These activities could be exploited in the food industry for food preservation or in pharmaceutical industry.

Keywords : *Bunium*, anti-oxidant activity, anti-inflammatory activity, anti-hemolytic activity, antibacterial activity, essential oil.

1. INTRODUCTION

The increased use of drugs with over prescribing has led to the development of resistance to drugs especially antibiotics. Therefore, there is growing interest in the development of new types of natural effective and non toxic compounds "Research of effective plants agents". Herbal and botanical products, such EOs and plant extracts, have been studied for their biological activities. EOs have several activities ; other than antibacterial and antiviral effects, most of them possess insecticidal, antifungal, acaricidal, cytotoxic and antioxidant properties [1]. Therefore, they are intensely investigated in the fields of pharmacology, pharmaceutical botany, medical and clinical microbiology, phytopathology and food preservation.

Bunium genus possesses a range of compounds with many biological activities. It comprises more than 50 species [2] are distributed in Africa [3,4], in Europe [5] and in Asia [6-8]. *Bunium* species are used as additives in foods for their carminative, anti-dyspepsia and anti-spasmodic effects [9]. *B. persicum* is used as carminative, anti-obesity and lactogage [10], the roots of *B. paucifolium* are eaten like potatoes in eastern Anatolia [11] also that, its rhizomes are used in treatment of urinary inflammation[12].

Since the plant-derived became a source of novel therapeutics and in order to exploit local species, we aimed to study the biological activities of the essential oils (EOs) and methanolic extracts (Me EXT) of *Bunium alpinum* and *Bunnium incrassatum* knowing that the two species were never studied before.

2. MATERIAL AND METHODS

2.1. Chemical composition of essential oils

Plant Material

Flowering aerial parts of *B. incrassatum* were collected in Timellouka region (West Setif, Algeria) during Mai-July (2010-2014). However, flowering aerial parts of *B. alpinum* were collected at Megress Mountain (North Setif, Algeria) during the same period. The harvested plants material was air-dried in a shaded area at ambient temperature.

Extraction of Volatiles

EOs were obtained by hydro-distillation in a Clevenger-type apparatus [13]. Each isolated oil was stored in refrigerator at a steady Temperature of 4°C until use.

Essential Oil Analysis by Gas Chromatography-Mass Spectrometry

GC and GC-MS analysis were carried out using an Agilent 6890N gas chromatograph apparatus equipped with a flame ionization detector (FID) and coupled to a quadrupole Agilent 5973 network mass selective detector working in electron impact (EI) mode at 70 eV. The gas chromatograph was equipped with two fused silica capillary columns HP-1. The analytical parameters were the following: the carrier gas was helium at a flow rate of 1 ml/min. The oven temperature was programmed from 60 to 250 °C at 2 °C/min and held isothermal for 40 min. The injector (split mode, ratio 1/100) temperature was 250 °C. The FID temperature was set at 250 °C, and in the GC-MS analyses, the temperatures of the ion source and transfer line were 170 and 280 °C, respectively. The constituents of the essential oil were identified by comparison of their mass spectral pattern and retention indices (RI) with those of pure compounds registered in literature data and with a laboratory-made database built from authentic compounds[14].

2.2. Preparation of methanolic extracts

Methanolic extracts (Me EXTs) were obtained using **Athamena method** [(15)]. The aerial parts of *B. alpinum* and *B. incrassatum* were separately pulverized into fine powder. Then 20g of each were macerated in 100 ml water-alcohol mixture at a ratio of 1/10 (v/v). Then the mixtures were swirled for 72 hours at ambient temperature. After that, these mixtures were individually filtered through filter paper and the entire extraction process was repeated (three times) on the residue obtained from the filtration process. The filtrates were individually pooled and methanol was removed from the filtrates under reduced pressure (Rotavapor). Finally ; Me EXTs were cooled in a desiccator before the yield of each extract was calculated.

2.3. Total phenolic content (TPC)

The TPC was obtained using the Folin-Ciocalteu reagent method [16]. A volume of 200 µl of each Me EXT was added to 1 ml of Folin-Ciocalteu reagent dissolved in water at a ratio of 1/10 (v/v). Solutions were mixed and incubated at room temperature. After 4 min, 800 µl of sodium carbonate (75 mg/ml) were added. The final mixture was shaken thoroughly and incubated for 2 hours in obscurity at room temperature.

Absorbance was measured at 765 nm using a UV/vis spectrophotometer. Triplicate measurements were made for each sample. A calibration curve of Gallic acid (GA) ranging from 0-250 µg/ml was prepared separately. Results were determined by the regression equation of the calibration curve and were expressed as milligram GA equivalents (GAE) per gram dry weight of draw material (DW).

2.4. Antibacterial activity of essential oils

Bacterial strains

Reference strains of all the bacteria were obtained from the collection of the American Culture Collection Type (ATCC) are presented in **table 1.**

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Table1. Strains of bacteria.

Bacteria	Gram	Origin	Code
Pseudomonas aeruginosa	-	ATCC	27853
Escherichia coli	-	ATCC	25922
Salmonella typhimurium	-	ATCC	13311
Citrobacter freundii	-	ATCC	8090
Klebsiella pneumonia	-	ATCC	700603
Proteus mirabilis	-	ATCC	35659
Bacillus cereus	+	ATCC	10876
Enterococcus faecalis	+	ATCC	49452
Lysteria monocytogenes	+	ATCC	15313
Staphylococcus aureus	+	ATCC	25923

Disc diffusion assay

Diffusion method was carried out according to **Bauer method** [17]**.** Briefly, a culture suspension of the tested bacteria (10^6 CFU/ml) was spread on the solid media plates (Muller Hinton ; MH). 6 mm's paper discs were impregnated with 10 µl of serial dilutions in dimethyl-sulfoxide (DMSO) and placed onto the solid media plates (MH). The antibacterial activity was evaluated by measuring the inhibition zones expressed in mm against the tested bacteria after 24 h of incubation at 37 °C. Gentamicin (10 µg/disc) was used as positive control.

2.5. Anti-oxidant activity using DPPH scavenging assay

The hydrogen atom's donation ability of chemical compounds was measured on the basis to scavenge the 2,2-diphenyl-1-picrylhydrazil free radical [18,19]. Fifty microliter (50 µl) of various concentrations of the EXT (in methanol) and EOs (in DMSO) were added to 1250 µl of DPPH solution (0.4 mM in methanol). After 30 min of incubation in the obscurity at room temperature, the absorbance was read against a blank at 517 nm using UV/vis spectrophotometer. DPPH free radical scavenging activity in percentage (%) was calculated using the following formula :

DPPH scavenging activity (%) = (A_{blank}-A_{sample}/A_{blank}) x 100

Where : A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC50) was calculated from the graph plotted of inhibition percentage against extract concentrations. Synthetics; BHT, BHA, tocopherol, quecitine and rutine were used as positive controls**.**

2.6. Evaluation of *In vitro* anti-inflammatory activity

Anti-inflammatory activity of Me EXTs was evaluated by protein denaturation method [20,21]. Diclofenac sodium, a powerful non-steroidal anti-inflammatory drug was used as a standard. The reaction mixture consisting of 2 ml of different concentrations of Me EXT (100-500 µg/ml) or standard diclofenac sodium (100 and 200 µg/ml) and 2.8 ml of phosphate buffered saline (pH 6.4) was mixed with 2ml of egg albumin (from fresh hen's egg) and incubated at (27±1) °C for 15min. Denaturation was induced by keeping the reaction mixture at 70 °C in water bath for 10 min. After cooling, the absorbance was measured at 660 nm using double distilled water as blank. Each experiment was done in triplicate and the average was taken. The percentage inhibition of protein denaturation was calculated using the following formula: **% Inhibition = (A_t-A_c)/A_c x 100,** Where: A_t is the absorbance of test sample; A_c is the absorbance of control.

2.7. Evaluation of *in vitro* anti-hemolytic activity using HRBC method

Preparation of Human Red Blood Cells (HRBC) Suspension

Fresh whole human blood was collected and mixed with equal volume of sterilized Alsever solution (2 % dextrose, 0.8 % sodium citrate, 0.05 % citric acid and 0.42 % sodium chloride in water). The blood was centrifuged at 3000 rpm for 10 min and packed cells were washed three times with isosaline (0.85 %, pH 7.2). The volume of the blood was measured and reconstituted as a percentage % (v/v) suspension with isosaline [22].

Heat Induced Hemolysis

The principle involved here is the stabilization of human red blood cell membrane by hypotonicity induced membrane lysis. The mixture contains 1 ml phosphate buffer (pH 7.4, 0.15 M), 2ml hyposaline (0.36 %), 0.5 ml HRBC suspension (10 % v/v) with 0.5 ml of Me EXT. This mixture, the standard drug diclofenac sodium (DC) of various concentrations (50, 100, 250, 500, 1000 and 2000 µg/ml) and the control (distillate water instead of hyposaline to produce 100 % hemolysis) were incubated at 37°C for 30 min and centrifuged respectively. The hemoglobin content in the suspension was estimated using spectrophotometer at 560 nm [22].

The percentage of hemolysis of HRBC membrane can be calculated using the formula:

% hemolysis = (A_2/A_1) ×100

The percentage of HRBC membrane stabilization can be calculated using the formula :

The inhibition of hemolysis or the protection = 100 × (1-A_2/A_1)

Where: A_1 is the absorption of the control sample. A_2 is the absorption of test sample solution [23].

3. RESULTS AND DISCUSSION

3.1. Essential Oil Analysis

EO obtained by hydrodistillation of the areal parts of *B. incrassatum* was isolated with a yield of 0.09 %. However, *B. alpinum* gave a yield of 0.1 % (based on dried plant material). The isolated EOs were yellowish clear limpids with strong aromatic fragrances and showed a high solubility in methanol and dimethylsulfoxyde (DMSO). The main components represented more than 97 % of the total of *B. incrassatum* EO and more than 87% of of *B. alpinum* EO.

B incrassatum (EO) had exhibited thirty one identified compounds **(table 2)** by comparison of their retention indexes and the mass spectra of each GC component with those of standards. The abundant components were: terpens and their derivatives, but, the major component was palmetic acid (18.39 %), followed by caryophyllene oxide (17.36 %), β-eudesmol (13.95 %), n-pentacosane (5.13 %), 10-epi-α-muurolol (4.36 %), hedycaryol (4.14 %) and spatuleneol (4.04 %).

Table 2 Chemical composition of *B. incrassatum* (EO).

Compound	RI	%	Retention time (min)
thymol	1289	0.65	18.93
carvacrol	1298	0.76	19.11
β-elemene	1389	0.28	22.08
(*E*)-caryophyllene	1417	2.62	22.98
(*E*)-β-farnesene	1440	0.25	23.48
α-humulene	1452	0.31	23.84
γ-muurolene	1478	0.67	24.29
D-germacrene	1484	0.55	24.5
α-muurolene	1500	0.27	24.86
β-bisabolene	1505	0.48	24.94
β-sesquiphellandrene	1521	0.65	25.33
1-endo-bourbonanol	1518	2.43	25.44
β-calacorene	1544	0.66	26
Hedycaryol	1546	4.14	26.1
spatuleneol	1577	4.04	26.98
caryophyllene oxide	1582	17.36	27.12
salvial-4(14)-en-1-one	1594	2.4	27.3
carvone	1595	0.71	27.6
humulene epoxide-I	1608	1.85	27.68
10-epi-α-muurolol	1640	4.36	28.36
β-eudesmol	1649	13.95	28.67
apiol	1677	0.94	28.98
α-bisabolol	1685	2.11	29.19
apritone	1708	2.98	29.63
myristic acid	1720	2.4	30.53
6,10,14-Trimethylpentadecan-2-one	1795	1.61	32.38
pentadecanoic acid	1820	1.8	32.66
palmitic acid	1984	18.39	34.87
linoleic acid	2173	1.09	38.22
n- tricosane	2300	1.35	41.9
n- pentacosane	2500	5.13	44.15

Table 3 shows the chemical composition of *B. alpinum* EO, twenty four compounds were identified, the most abundant one was caryophyllene oxide (II) (33.84 %), followed by humulene epoxide I (8.37 %), n-pentacosane (6.41 %), ledenoxide I (4.71%), 14-hydroxy-9-epi-(*E*)-caryophyllene (4.62 %) and 2α-hydroxy-amorpha-4,7(11)-diene (4.4 %).

Table 3 Chemical composition of *B. alpinum (*EO).

Compound	RI	%	Retention time (min)
geraniol	1255	0.74	18.07
β-elemene	1375	0.9	22.7
β-caryophyllene	1417	1.96	23.8
α-humulene	1452	0.35	24.92
Sesquicineole	1471	0.42	25.14
γ-muurolene	1477	0.65	25.55
β-selinene	1485	0.32	26.03
γ-gurjunene	1473	0.19	26.3
β-bisabolene	1509	0.32	26.45
Elemicin	1554	0.41	27.75
ledenoxide I	1560	4.71	28.24
1,5-epoxy salvial-4(14)-ene	1571	1.42	28.77
Spathulenol	1576	2.55	29.19
caryophyllene oxide	1581	33.84	29.47
salvia-4(14)-en-1-one	1594	3.45	29.72
humulene epoxide (II)	1608	8.37	30.26
caryophylla-4(12),8(13)-dien-5α-ol	1639	1.12	31.16
allo-aromadendrene epoxide	1639	2.62	31.34
14-hydroxy-9-epi-(*E*)-caryophyllene	1668	4.62	32.28
α-bisabolol	1683	3.31	32.58
2α-hydroxy-amorpha-4,7(11)-diene	1775	4.4	32.82
palmitic acid	1984	2.01	41.38
n-tricosane	2300	2.24	51.3
n-pentacosane	2500	6.41	51.45

Thus, terpens and terpens alcohols such as spathulenol, humulene, humulene epoxide I, salvial-4(14)-en1-one and caryophyllene oxide are quantitatively abundant in the two species. The essential chemical groups represented in both EOs are represented in **figure 1.**

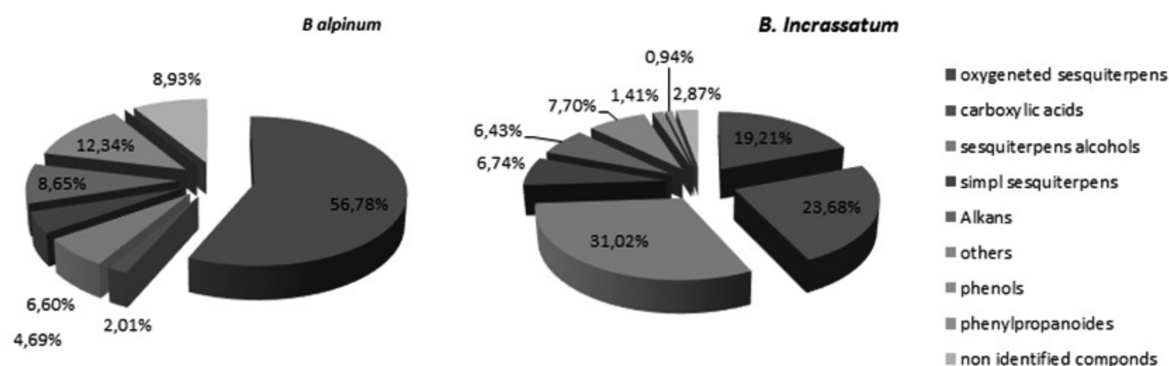


Fig. 1 Yields of chemical groups of components present in EOs

Interestingly, there were significant differences between the yields and the composition of the two EOs, although they belong to the same genus. These properties in EOs from one species are dependent on several factors; growth location, soil, climate (seasonal variations)[24], vegetation period, and sunlight [25]. Drying and improper storage can reduce the amount of EO in plants [26]. Many researchers have determined that the maximum oil content is obtained when all the flowers have reached full maturity, because the cups contain the largest number of secretory glands per unit area[27]. To obtain an EO with a constant composition, it must be extracted under the same conditions from the same organ of the plant which was growing on the same soil under the same climate and was harvested in the same season [28]. Also, the composition and the yield of EO can be attributed to the method of extraction [29].

3.2. Methanolic extracts

Me EXTs were obtained with yields of 1.82 % and 0.89 % for *B. incrassatum* and *B. alpinum* respectively.

3.3. Total phenolic content (TPC)

TPC expressed in terms of GA and yield (%) of EXT was found to be 268.2 µg EQ/mg (w/w) for *B. alpinum* EXT and 236.6 µg EQ/mg (w/w) for *B. incrassatum* EXT. TPC was calculated using the following linear equation based on the calibration curve of Gallic acid;

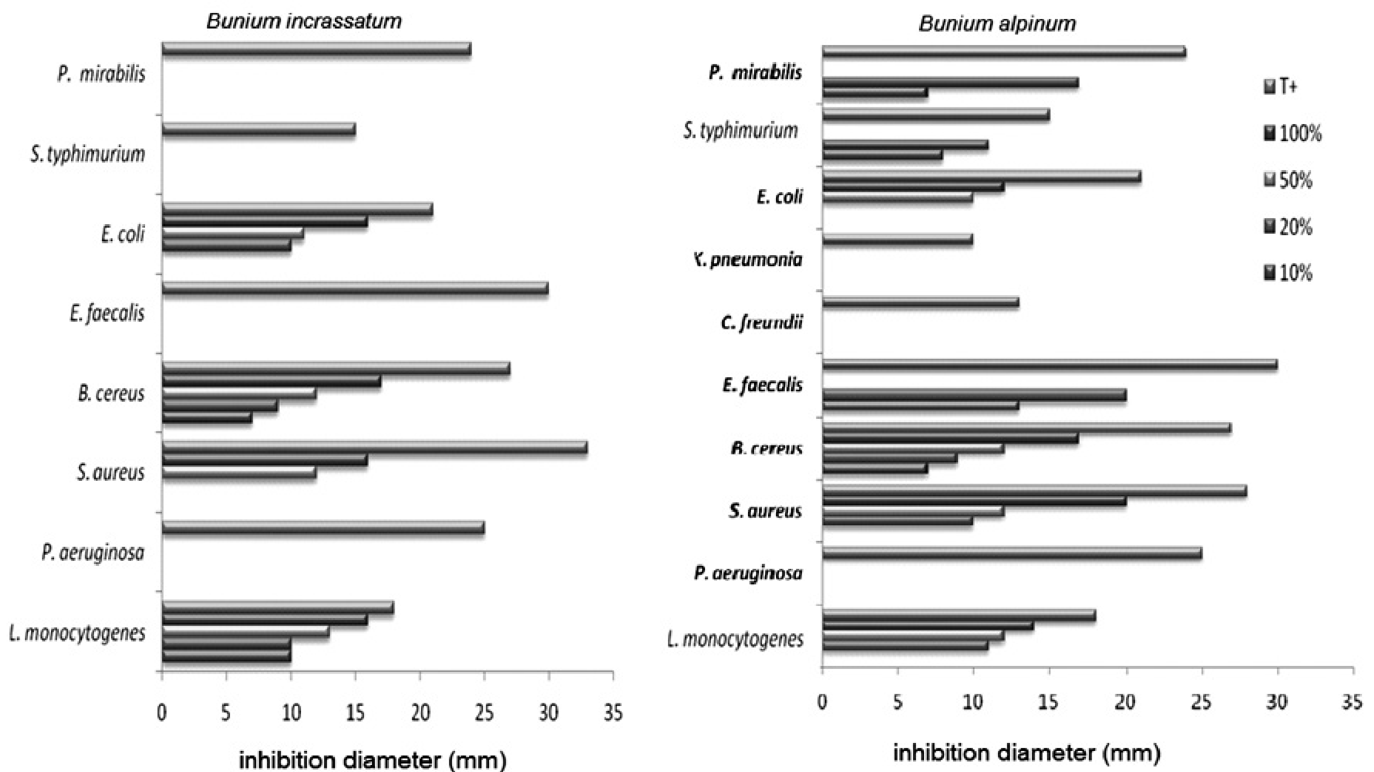
$\mathbf{y = 0.005\,x - 0.06,\ R^2 = 0.99}$, were y is the absorbance and x is the amount of Gallic acid in µg. It is necessary to estimate the TPC in the Me EXTs, because, they were thought to be responsible of different biological activities [30].

3.4. Antibacterial activity

It was reported that herbs and spices containing essential oils (EOs) in the range of 0.05-0.1% have demonstrated potential activity against bacteriae, such as *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus*, in food systems [31].

It is clearly seen in **figure 2**, that the antibacterial potential is inversely proportional with the dilution of the EOs, and it is well noted that Gentamicin (T^+) inhibited the growth of all bacteria tested by forming halos varied from 7 mm against *B. cereus* to 33 mm against *S. aureus.*

EOs of *B. incrassatum* and *B. alpinum* showed wide array of antibacterial activity. The application of *B. incrassatum* EO on most bacteria at 10 %, 20 % and 50 % dilution demonstrated a total resistance. But, concentrations of 100 % gave remarkable inhibition zone diameters with 15 to 20 mm. However, *B. alpinum* EO presented a good activity against most of bacteria tested, mostly, when using dilution of 50 % and 100 % EO.



T+: Gentamicine (10 ug/disc). (100, 50, 20 and 10) %: Percentages of dilution of EOs in DMSO (v/v)

Fig. 2 Antibacterial activity of the two EOs

The bioactive molecules thought to be responsible for antibacterial activity are terpens and sesquiterpens [32] which have been obtained in large amounts in *B. incrassatum* and *B. alpinum* EOs (more than 50 % in their chemical composition). The antibacterial activity is also attributed to the presence of fatty acids in the composition [33,34], and the chemical analysis of *B. incrassatum* EO shows the presence of palmitic acid with 18.39 %.

The mode of the antibacterial action may be due to surface interaction of these active molecules with the bacterial cell wall and membrane leading to their alteration[1]. Plasmic membrane perturbation, rupture induced by ionic forces and coagulation of cytoplasmic matters are some of the other mechanisms involved in the EOs antibacterial activities[35].

3.5. Anti-oxidant activity using DPPH

Reduction of DPPH radical (blue), which accepts an electron of hydrogen radical to become a stable molecule (yellow), was estimated by the decrease in its absorbance at 517 nm by antioxidants. The antioxidant capacity is more potential when IC_{50} is smaller [36,37].

Figures 3 and **4** show the scavenging effect of EOs, Me EXTs and standard antioxidants (BHT, BHA, tocopherol, rutine and quercitine).

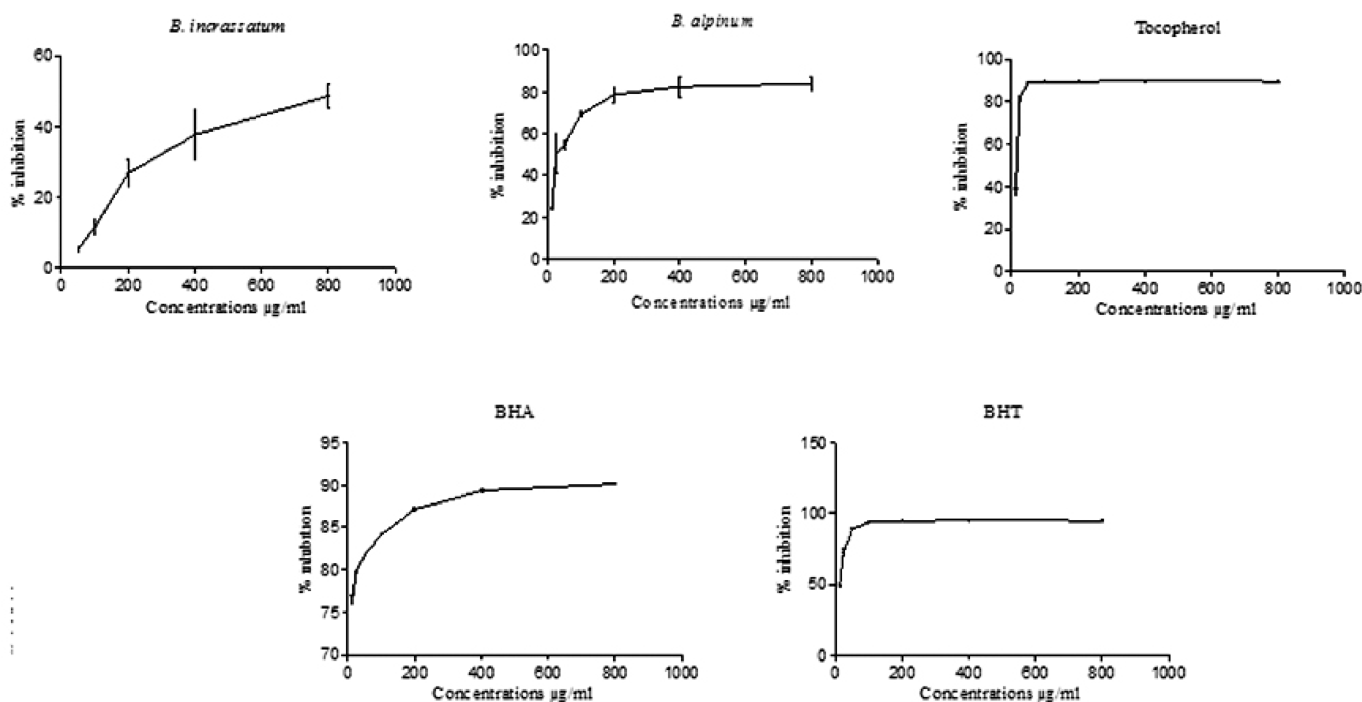


Fig. 3 Scavenger effect of EOs, Tocopherol, BHA and BHT

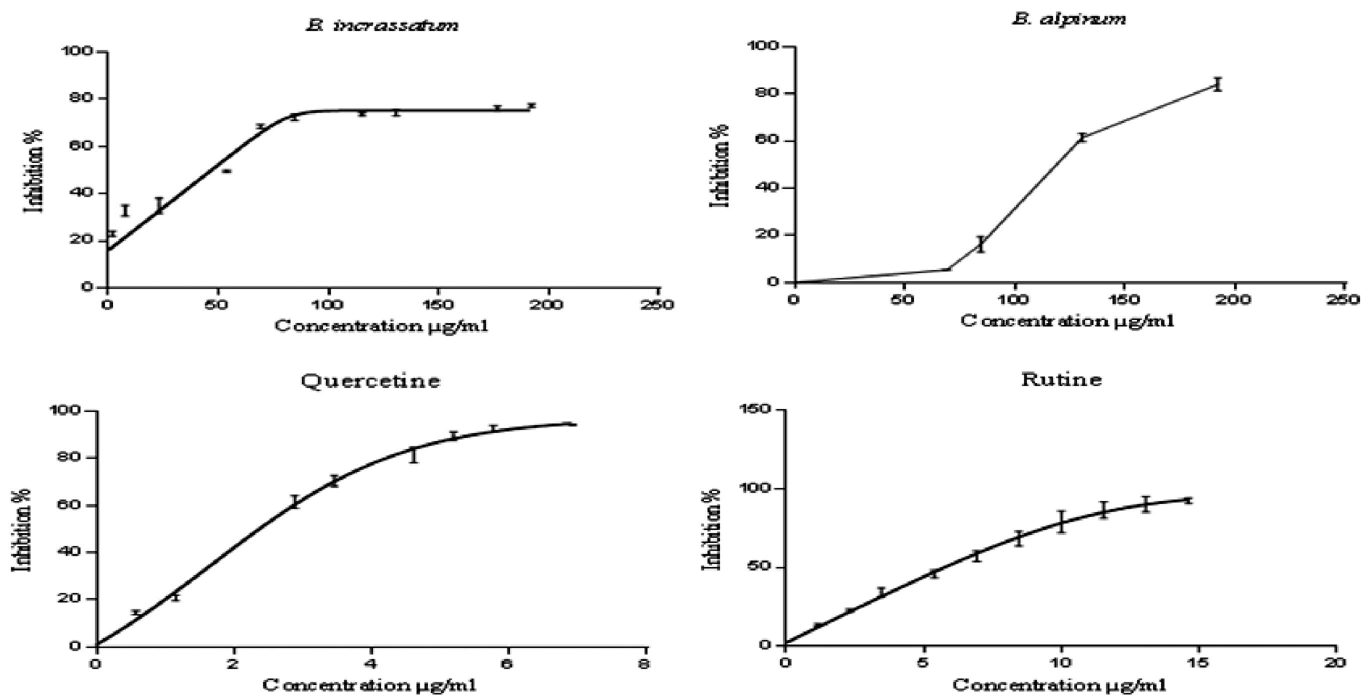


Fig. 4 Scavenger effect of Me EXTs, Quercetine and rutine

Methanolic extracts exhibited the highest scavenging of the DPPH radical, they showed significant results compared to the standards. However, EOs gave the lowest values, meaning not at a level comparable with the standards. IC_{50} values are presented in **table 4**.

Table 4. IC_{50} values for EOs and Me EXTs

Samples	IC_{50} *(µg/ml) ± SD*	*Standards*	IC_{50} *(µg/ml) ± SD*
B. incrassatum (EO)	38.52 ± 2.40	BHT	12.99 ± 0.41
B. alpinum (EO)	> 800	BHA	6.14 ± 0.43
B. alpinum (Met EXT)	21.85±1.32	Tocopherol	13.02 ± 5.17
B. incrassatum (Met EXT)	55.77±3.25	Quercitine	2.63 ± 1.26
		Rutine	5.94 ± 2.00

Many studies on the anti-oxidant activity of a wide variety of EOs showed that this activity is related to the chemical composition. However the presence of some molecules (even in minority) such as; geraniol, bisabolol (*B. alpinum* contains 4.05 %), carvacrol and thymol (*B. incrassatum* contains 1.41 %) were found to attribute a high degree of anti-oxidant property by synergy against free radicals [38,39] and they were found to be as strong reductors of DPPH and inhibitors of the lipidic peroxidation. It was proved that, the anti-oxidant effect is due to the presence of hydroxyl groups in the chemical structure such as quercitine and carnosol which are well known to be good free radical scavengers [40-42].

3.6. *In vitro* Anti-inflammatory activity

There are certain problems in using animals in experimental pharmacological research, such as ethical issues and the lack of rationale for their use when other suitable methods are available or could be investigated [43].

The reason for choosing the albumin is that albumin is the most abundant protein in the blood and its concentration drop in cases of inflammation (denaturation) [44,45] , also, albumin acts as primary antioxidant in these cases [46, 47].

The present findings exhibited a concentration dependent inhibition of protein (albumin) denaturation. Also, it is noted that among all samples, at the concentration of 800 µg/Ml, showed a significant anti-inflammatory activity and more than 70 % protection of HRBC. Results were compared with standard (Diclofenac Sodium) which showed ≈ 88 % protection. These results are presented in **figure 5**. Denaturation inhibitory values were calculated and found to be 49.74 ± 0.90 mg/ml for *B. incrassatum* EXT*;* 49.66 ± 0.94 for *B. alpinum* EXT. and 49 .98 ± 0.45 mg/ml for DS.

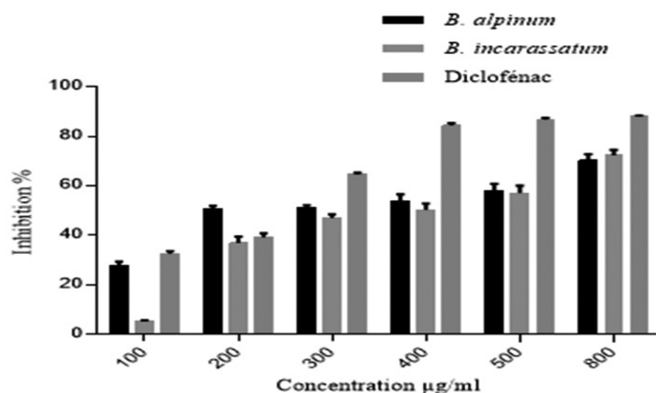


Fig. 5 Protein denaturation inhibitory of *B. alpinum* and *B. incrassatum* Me EXTs compared to Diclofenac Sodium.

It was reported that, the presence of secondary plant metabolites such as alkaloids, flavonoids, glycosides, tannins, steroids and sesquiterpens in the composition of the Me EXTs can be responsible for a significant anti-inflammatory response [21, 44, 48].

3.7. Anti-hemolytic activity

The inhibition of hypotonicity induced HRBC membrane lysis or stabilization of HRBC membrane was taken as a measure of the anti-inflammatory activity. Me EXTs are effective in inhibiting the heat induced hemolysis of HRBC at different concentrations (50-2000 µg/ml) as shown in **figure 6**. Results showed the maximum inhibition ≈ 90 % for both extracts at 2000 µg/Ml. The inhibition of membrane hemoslysis (membrane stabilization/protection) increased proportionally with the increase of concentration. Hence, anti-inflammatory activity of the extracts was concentration dependent.

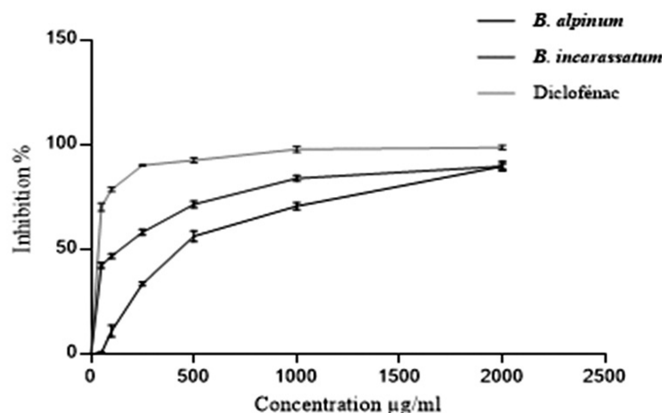


Fig. 6 Effect of *B. alpinum*, *B. incrassatum* EXTs and diclofenac sodium on membrane protection.

It has been found that certain chemicals or antioxidants, having ability to generate radicals, attack the erythrocyte membrane, inducing the chain oxidations of lipids and proteins and eventually causing membrane damage leading to hemolysis (during inflammatory process) [46]. Therefore**,** when HRBC were treated with Me EXTs; a marked reduction in hemolysis and potential anti-inflammatory phenomena were induced. That could be due to their antioxidant potential and free radical scavenging activity [50-52]**.**

CONCLUSION

Our results suggested that EOs and Met EXTs of the selected plants can be exploited as effective antibacterial, antioxidant, anti-inflammatory agents. These findings provide scientific basis to justify their traditional use. But, further studies on their cytotoxicity and *in vivo* applications, must be done for rounding off the evaluation of the potential therapeutic of these plants.

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