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Chemical compositions, antibacterial and antioxidant activities of essential oil and various extracts of *Geranium sanguineum* L. flowers

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ABSTRACT

This research presents the chemical composition, antibacterial and antioxidant activities of essential oils and various extracts from Geranium sanguineum L. flowers. The essential oil composition of Geranium sanguineum L. flowers were investigated by GC/MS with 77 identified volatile constituents. Phenyl butanone, linalool, benzyl alcohol, α-cadinol, globulol and viridiflorol were found to be the major components, respectively. The essential oil played a major role as a remarkable antimicrobial agent according to their inhibition action against all pathogenic bacteria followed by dichloromethane extract, hexane extract, and methanol extract, respectively. The essential oil were also evaluated to be superior to all extracts tested with an IC_{50} value of 85 µg/ml whereas other extracts showed their IC_{50} values ranging from 100 to 197.7 µg/ml.

Keywords: *Geranium sanguineum* L.; Essential oil; Antibacterial activity; Antioxidant activity.

INTRODUCTION

Most diseases in plants are caused from various pathogens including bacteria, fungi, nematodes and viruses [1]. Phytopathogenic bacteria and fungi could decrease the growth of many economically important crops [2]. Synthetic pesticides are currently the main solutions for microbial pathogenic diseases. Conversely, resistance of chemicals pesticides by various plant pathogenic bacteria and fungi is the primary cause with regard to poor disease control of agriculture [3, 4, 5]. Moreover, the use of chemical compounds could be dangerous to humans and the environment. Recently, the use of synthetic compounds is diminishing, thus the substitutions of their materials by natural products has increased significantly with regard to research, especially when considering the health and environmental benefits [6,7]. Alternative natural pesticides are necessary for use in the control of pathogenic bacteria diseases in plants. Essential oils and extracts from various parts of plants is one of the most promising groups of

natural compounds which may be developed for use as natural bactericide substitute the synthetic pesticides due to the presence of terpene constituents within differing functional groups found in the oils. There exists much evidence indicating that the essential oils and various extracts of plants were employed as biopesticide [8, 9, 10]. In addition, they were applied as antimicrobial [11, 12, 13] and antioxidant compounds [14, 15, 16]. *Geranium sanguineum* L., commonly called Bloody Cranesbill, is an herbaceous plant species in the Geraniaceae family. It is native from Europe and temperate Asia, and is cultivated as a garden subject, and a number of different cultivars exist. The flowers are purple and the name refers to the red color of the leaves in autumn. *G. sanguineum* L., has significant antioxidant activity and antiviral activity [17]. Its root extracts are used in traditional medicine to treat gastrointestinal disorders, infections and inflammatory conditions. It is also frequently used in folk medicine for the treatment of eruptive skin diseases and as a disinfectant bath and poultice for the affected area. However, there is no report describing the antibacterial and antioxidant activities of *G. sanguineum* L. flowers. In order to develop stable and safe antimicrobial sources, the aim of our research is to investigate the chemical composition of *G. sanguineum* L. flowers essential oil isolated by hydrodistillation. The antibacterial and antioxidant activities of essential oil and various extracts of *G. sanguineum* L. flowers were then investigated and discussed.

MATERIALS AND METHODS

Plant material and isolation of essential oils

For the extraction of essential oils, *Geranium sanguineum* L.was collected from the local area of Sfax (Tunisia, 35.23° N and 11.11° E). After the botanic identification of the species, a voucher specimen has been deposited in the herbarium of the laborartory (Institut de l'Olivier de Sfax) for future reference. Air-dried plant materials (200 g) were placed in a 5 l round-bottom distillation flask and 3 l double distilled water was added. The essential oils were obtained by steam distillation for 3 h). The isolated fractions of plant parts exhibited two distinct layers-an upper oily layer and the lower aqueous layer. Both the layers were separated and, after removing water traces with the help of capillary tubes and anhydrous sodium sulphate, the essential oils were stored at 4 °C in a clean amber glass bottle until used.

Preparation of the crude extracts

The dried flowers of *G. sanguineum* L. were agitated using a blender until very small particles (almost a powder) whereby 50 g of these samples was macerated individually with 200 ml of hexane, dichloromethane and methanol. Each extraction was performed at room temperature for 10 days. All extracts were filtered through filter paper and concentrated under a vacuum using a rotary evaporator. All crudes extracted were stored at 4°C for further analysis. The extracts obtained yields of 0.91%, 4.83% and 3.14% for hexane, dichloromethane and methanol extracts, respectively.

Gas chromatography–mass spectrometry (GC/MS)

The chemical compositions of *G. sanguineum* L. flower extracts obtained from different extraction solvents, were analyzed using a Hewlett Packard model HP6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP-5MS (5% phenyl-polymethylsiloxane) capillary column (30 m×0.25 mm i.d., film thickness 0.25 µm; Agilent Technologies, USA) interfaced to an HP model 5973 mass-selective detector. The oven temperature was initially held at 50°C and then increased by 2°C/min to 180°C. The injector and detector temperatures were 250 and 280°C, respectively. Purified helium was used as the carrier gas at a flow rate 1 ml/min. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 29–300. The electron multiplier voltage was 1150 V. The ion source and

quadrupole temperatures were set at 230 and 150°C, respectively. Identification of volatile components was performed by comparison of their Kovat retention indices, relative to C_8–C_{22} n-alkanes, and using a comparison of the mass spectra of individual components with the reference mass spectra in the Wiley 275 and NIST 98 databases. The quantity of all identified components was investigated by using a percent relative peak area.

Analysis of antibacterial activity of all extracts

Bacterial strains

The Bacterial strains used in this study were Gram positive bacteria (*Bacillus subtilis* ATCC 6051 , *Micrococcus luteus* LB 14110 and *Staphylococcus aureus* ATCC 6538) and Gram negative bacteria (*Ag. tumefaciens* C58, *Ag.rhizogene* CFBP 2408, *Ag. vitis* CFBP 2678^{T}, *Pseudomonas savastanoi* pv *savastanoi* IVIA 1628, *E. coli* ATCC 8739, *Pseudomonas aeruginosa* CIP 82.118, *Pseudomonas syringae*. pv. tomato and *Xanthomonas campestris* (kindly provided from the culture collection of The Centre of Biotechnology of Sfax). The bacterial strains were grown on Luria Bertani (LB) agar medium plates [containing (g/l): yeast extract 5, peptone 10, NaCl 10 and bacteriological agar 20].

Antibacterial assay

The agar disc diffusion method was employed to determine the antibacterial activity of the essential oils as described in the literature [18]. Briefly, a suspension of the tested bacteria (2×10^8 CFU/ml) was spread on the solid media plates. Filter paper discs (6mm in diameter) were individually impregnated with 15µL of the diluted oil samples (200 mg/ml, essential oil dissolved in Tween 80 of 0.5% and fractions dissolved in DMSO) and placed on the inoculated plates. Plates were kept at 4°C for 2 h, followed by incubating at 37°C for 24 h. Then the disc diameters of the inhibition zones (DDs) were measured in millimeters. Each test was performed in three replicates and repeated twice. Mean values were selected. Levofloxacin was used as positive control. 0.5% Tween 80 and DMSO were used as negative controls.

Determinations of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

A broth microdilution method was used to determine MIC and MBC as illustrated by [18, 19]. All tests were performed in Mueller Hinton broth supplemented with Tween 80 and DMSO at final concentration of 0.5% (v/v). Serial doubling dilutions of the essential oil and crude extracts of *G. sanguineum* L. were prepared in a 96-well microtiter plate ranging from 0.01 to 200.00 mg/ml, 0.01 to 6 mg/ml, respectively. The final concentration of each strain was adjusted to 5×10^4 CFU/ml. After staying at 4°C for 2 h, plates were incubated at 37°C for 24 h. The MIC is defined as the lowest concentration of the samples at which the bacterium does not demonstrate visible growth. The bacterium growth was indicated by turbidity. To determine MBC, broth was taken from each well and incubated in Mueller Hinton broth medium at 37°C for 24 h. The MBC was defined as the lowest concentration of the samples at which the incubated bacterium was completely killed. Each test was performed in three replicates and repeated twice. Levofloxacin was used as positive control.

Antioxidant activity

DPPH radical scavenging assay

The radical scavenging abilities of *G. sanguineum* L. flowers oil and various extracts were analyzed compared to a standard butyl hydroxyl toluene (BHT) and α-tocopherol based on the reaction with 2,2-diphenyl-2-picrylhydrazyl radical (DPPH$^{\cdot}$). This method was evaluated using a spectrophotometric method followed similar to the modified method described by [20]. One milliliter of various concentrations of each sample in methanol were added to 1 ml of a 0.003%

methanol solution of DPPH and the reaction mixture was shaken vigorously. The tubes were allowed to stand at room temperature for 30 min. Each reaction mixture was then placed in the cuvette holder of a Perkin Elmer-Lamda 25 UV/Vis spectrophotometer and monitored at 517 nm against blank which used methanol as the baseline correction. The scavenging ability was calculated as follows: Scavenging ability (%) = 100 × [Absorbance of control-Absorbance of sample/Absorbance of control]. The antioxidant activity of all samples was expressed as IC_{50} which was defined as the concentration (in µg/ml) of oil required to inhibit the formation of DPPH radicals by 50%. The experiment was carried out in triplicate.

Determination of total phenolic contents

Total phenolic content of all samples obtained from *G. sanguineum* L. flowers was determined using the Folin–Ciocalteu reagent according to the modified method reported by [21] with gallic acid as the standard. The solution (0.2 ml) was mixed with 1.0 ml of Folin–Ciocalteu reagent, 1.0 ml of an aqueous solution of 7% Na_2CO_3 and 5.0 ml of distillated water, respectively. The mixture was then vortexed vigorously. The reaction mixtures were allowed to stand for 30 min before absorbance at 765 nm was measured. The same procedure was also applied to the standard solutions of gallic acid. The calibration equation for gallic acid was y = 0.00515x - 0.00400 (R^2 = 0.999) where y is the absorbance and x is the concentration of gallic acid in mg/ml.

RESULTS AND DISCUSSION

Chemical composition of essential oil of *G. sanguineum* L.

GC/MS analyses of the oil led to the identification of 77 different components, representing 90.59 % of the total oil. The volatile components identified by GC/MS, their relative area percentages and their retention indices are summarized in Table 1. The essential oil of *G. sanguineum* L. flowers contained high percentages of the group of monoterpenes and sesquiterpene. The dominant components were 1-phenyl butanone (22.43%), linalool (8.42% %), benzyl alcohol (6.65%), α-cadinol (5.21), globulol (4.82%) and viridiflorol (3.65%). Pulegone (3.29%), epi-α-cadinol (3.05%), terpinen-4-ol (2.64%), germacrene A (2.28%) and paramethyl anisole (2.09%) were also found to be minor components of the *Geranium sanguineum* L.flower oil.

Table 1 : Chemical constituents of *G. sanguineum* L. flower oil with the percentage of content obtained by hydrodistillation

No.	*Compound*	*LRI*	**%**
1	Furfural	829	0.25
2	α Thujene	919	0.22
3	α-Pinene	925	0.15
4	Camphene	940	0.17
5	Sabinene	964	0.18
6	β-Pinene	968	t
7	Myrcene	982	0.15
8	Mesitylene	988	0.11
9	α-Phellandrene	999	t
10	α-Terpinene	1010	0.18
11	*para*-methyl Anisole	1016	2.09
12	β-Phellandrene	1021	0.37
13	δ-3-Carene	1028	0.15
14	Z-β-Ocimene	1039	0.52
15	Benzyl alcohol	1042	6.65
16	γ-Terpinene	1050	0.15
17	Acetophenone	1061	0.15
18	Z-Sabinene hydrate	1062	0.12

19	Z-linalol oxide (furanoid)	1066	0.26
20	meta-Cymenene	1077	0.1
21	α-Terpinolene	1079	0.15
22	Methyl benzoate	1088	0.11
23	Linalool	1098	8.42
24	1.3.8-*para*-Menthatriene	1109	0.3
25	1.3.8-*ortho*-Menthatriene	1113	0.55
26	Z-*para*-menth-2-en-1-ol	1118	0.85
27	1-Terpineol	1136	0.8
28	Ethyl benzoate	1163	0.23
29	Terpinen-4-ol	1171	3.12
30	Geraniol	1183	0.57
31	α-Terpineol	1188	1.26
32	E-Piperitol	1203	0.3
33	Pulegone	1235	4.15
34	δ-Elemene	1326	0.22
35	α-Cubebene	1337	t
36	Isoledene	1361	0.52
37	α-Copaene	1366	0.22
38	β-Bournonen	1372	0.33
39	β-Cubebene	1378	0.24
40	α-Gurjunene	1397	0.22
41	Z-Caryophyllene	1411	0.87
42	β-Duprezianene	1416	0.24
43	α-Guaiene	1424	0.17
44	γ-Elemene	1425	0.26
45	Aromadendrene	1430	0.7
46	1-Phenyl butanone	1438	22.43
47	α-Humulene	1443	0.15
48	allo-Aromadendrene	1448	0.45
49	E-Cadina-1(6).4-diene	1464	0.14
50	Germacrene D	1472	0.42
51	β-Selinene	1477	0.24
52	*E*-Muurola-4(14).5-diene	1478	0.17
53	Bicyclogermacrene	1483	0.6
54	Viridiflorene	1485	0.18
55	Germacrene A	1491	3.24
56	E-β-Guaiene	1495	0.58
57	γ-Cadinene	1504	0.16
58	δ-Cadinene	1512	0.14
59	β-Sesquiphellandrene	1518	0.17
60	Germacrene B	1548	0.36
61	Ledol	1562	1.59
62	Germacrene D-4-ol	1564	0.19
63	Spathulenol	1575	0.42
64	Globulol	1581	4.82
65	Viridiflorol	1588	3.65
66	5-epi-7-epi-α-Eudesmol	1596	0.13
67	Sesquithuriferol	1607	0.2
68	1.10-di-epi-Cubenol	1615	1.44
69	10-epi-γ-Eudesmol	1621	0.27
70	Eremoligenol	1625	0.13
71	epi-α-Cadinol	1634	3.95
72	epi-α-Muurolol	1635	0.1
73	α-Cadinol	1649	5.21
74	Z-methyl epijasmonate	1671	0.14
75	Acorenone B	1675	0.26
76	Z-α-bisabolene epoxide	1733	0.69
77	Benzyl benzoate	1755	1.2
Total			90.59

LRI, linear temperature program retention index on DB-5 column.
%, compound percentage; t, trace amounts <0.1%

Antibacterial activities of essential oils and crude extracts of *G. sanguineum* L.

The DDs, MICs and MBCs of essential oil of *G. sanguineum* L. for the bacteria tested are shown in Table 2. The data obtained from agar disc diffusion method indicated that essential oil of *G. sanguineum* showed a broad spectrum of antibacterial activity that was effective against Gram positive and Gram negative bacteria. The results of MIC indicated that the oil inhibited all bacteria tested and the strongest inhibitory activity was against *Ag.vitis* CFBP 2678^T. The oil was demonstrated to have good bactericidal effects. The essential oil had the best bactericidal activity against *Ag. vitis* CFBP 2678^T with the lowest MBC of 0.01mg/mL, and the weakest activity was observed against *Bacillus subtilis* ATCC 6051with the highest MBC of 12.50 mg/ml. The inhibotory action of the essential oil could be attributed to the occurrence of high proportions of monoterpenes and sesquiterpenes in the oil as indicated by the study of [22, 23] due to their different chemical composition. Antimicrobial properties of action might be related to these compounds which have a high potential in strongly inhibiting microorganism pathogens. The following components present are believed to play an important role as antibacterial agents, linalool, α-cadinol, globulol and viridiflorol, pulegone, epi-α-cadinol, terpinen-4-ol, germacrene A and *para*-methyl anisole corresponding to the amounts present in the essential oil. Conversely, [22] also reported that there was no significant correlation between the activity and the percentage of the identified compounds.

Table 2 : Antibacterial activities of the essential oil of *Geranium sanguineum* L. flowers

Microorganisms	*Essential oil*			**Levofloxacin**		
	DD[a]	MIC [b]	MBC[b]	DD[c]	MIC[d]	MBC[d]
Gram positive bacteria						
Bacillus subtilis ATCC 6051	15	12.5	12.5	22	6.25	12.5
Micrococcus luteus LB 14110	18	6.25	6.25	11	200	NA
Staphylococcus aureus ATCC 6538	21	3.13	6.25	10	50	100
Gram negative bacteria						
Ag. vitis CFBP 2678^T	38	0.01	0.01	30	0.05	0.05
Ag.rhizogene CFBP 2408	25	0.78	1.56	18	12.5	12.5
Ag. tumefaciens C58	34	0.02	0.39	25	0.78	0.78
Pseudomonas savastanoi . pv. *savastanoi* IVIA 1628	36	0.01	0.01	22	3.13	6.25
E. coli ATCC 8739	19	6.25	6.25	10	200	NA
Pseudomonas aeruginosa CIP 82.118	24	1.56	1.56	13	50	50
Pseudomonas syringae. pv. *tomato*	36	0.02	0.39	20	6.25	6.25
Xanthomonas campestris	23	1.56	1.56	18	12.5	12.5

DD, diameter of zone of inhibition (mm) including disc diameter of 6 mm. NA, no active.
[a] Tested at a concentration of 3mg/disc.
[b] Values given as mg/ml.
[c] Tested at a concentration of 3µg/disc.
[d] Values given as µg/ml.

The antibacterial activity of solvent fractions of *G. sanguineum* L. was also evaluated against the pathogenic bacteria are shown in Table 3. The results of MIC and MBC indicated that all the fractions had the strongest antibacterial activities against *Ag. vitis* CFBP 2678^T (MIC and MBC of 0.25–2.00 mg/ml), whereas weakest activities against *E. coli* ATCC 8739 and *Pseudomonas aeruginosa* CIP 82.118 (MIC and MBC of 6.00 mg/ml). As a control, the solvent did not effect the growth of the pathogenics bacteria at the concentration used in this study. It appeared that the flower oil exhibited greater border antibacterial activity than the flower extracts of dichloromethane, hexane and methanol. The results indicated that the flower dichloromethane extract was found to be the remarkable antibacterial extract in this study according to their inhibition action against all tested pathogenic bacteria followed by the hexane and the methanol extract. This was considered by the intermediary polarities of dichloromethane which can extract higher numbers of the intermediary polarity compounds with different polarity which may

involve the bacterial inhibition in the sample rather than hexane and methanol which can extract only non-polar and polar components, separately.

Table 3 :Antibacterial activities of solvent fractions of *Geranium sanguineum* L. flower

Microorganisms	dichloromethane extract		hexane extract		methanol extract	
	MIC[a]	MBC[a]	MIC[a] MBC[a]		MIC [a]	MBC[a]
Gram positive bacteria						
Bacillus subtilis ATCC 6051	0.5	2	2	3	2	4
Micrococcus luteus LB 14110	1	2.5	2	4	6	> 6
Staphylococcus aureus ATCC 6538	1	2.5	6	6	6	> 6
Gram negative bacteria						
Ag. vitis CFBP 2678^T	0.25	0.25	0.5	1	1	2
Ag.rhizogene CFBP 2408	0.5	2	0.5	2	6	> 6
Ag. tumefaciens C58	0.5	2	0.75	2	2	6
Pseudomonas savastanoi . pv. *savastanoi* IVIA 1628	0.25	0.5	1	2	6	> 6
E. coli ATCC 8739	6	6	6	6	6	6
Pseudomonas aeruginosa CIP 82.118	6	6	6	6	6	6
Pseudomonas syringae. pv. *tomato*	0.5	2	2	6	6	> 6
Xanthomonas campestris	0.5	2	2	6	6	> 6

[a] *Values given as mg/ml.*

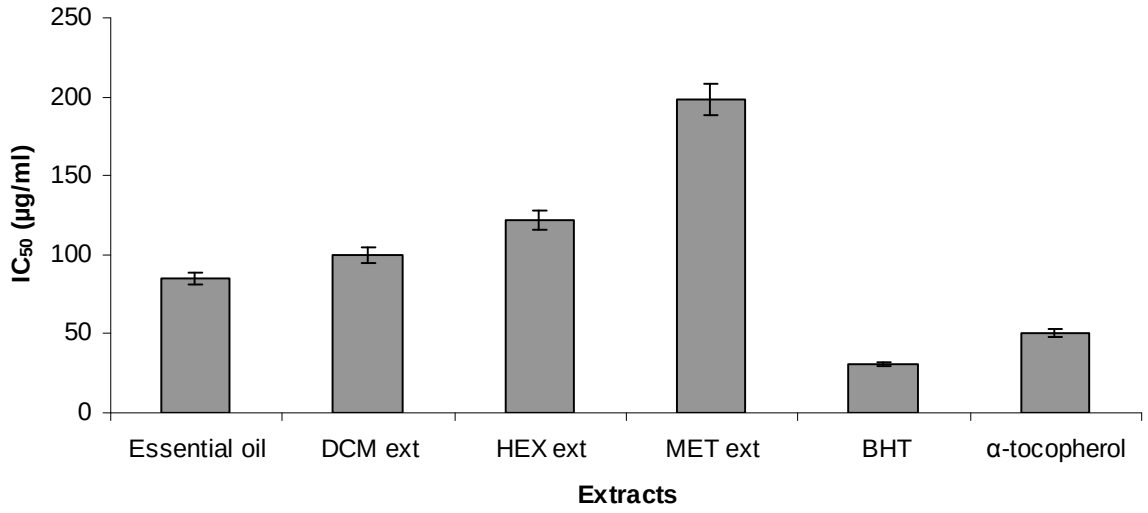

Figure 1. Antioxidant activities (IC_{50}) of different *G. sanguineum* L. flower extracts.
Values represent averages ± standard deviations for triplicate experiments. HEX ext; hexane extract, DCM ext; dichloromethane extract, MET ext; methanol extract and BHT; butyl hydroxyl toluene.

Antioxidant activity

According to various extracts, the antioxidants properties are considered to be different. Antioxidant activities of the extracts were tested by the DPPH radical scavenging. The violet color of the radical disappeared when mixed with the substances in the sample solution that donate a hydrogen atom. Antioxidant activities of all samples, standard BHT and α-tocopherol are presented in Figure 1 in which lower IC_{50} values indicate higher antioxidant activity. The flower oil, dichloromethane, hexane and methanol extracts were able to reduce the stable free-radical DPPH with an IC_{50} of 85, 100, 122.50 and 197.70 µg/ml whereas IC_{50} of standard BHT

and α-tocopherol were lower at 30.53 and 50.23 µg/ml, respectively. In this study, the flower oil exhibited greater antioxidant activity than other extracts. As seen, the essential oil contained high levels of monoterpenes and sesquiterpenes showing a highly antioxidant activity. It was shown that these terpene hydrocarbons, whose antioxidant activity is closed to that of phenolic compounds, break free-radical chain reactions, which could be accompanied by their irreversible oxidation into inert compounds as reported by [24, 25] as well as [26]. In addition, [15] reported that the essential oils which contain monoterpene hydrocarbons, oxygenated monoterpenes and/or sesquiterpenes have greater antioxidant properties. More evidence was reported by the work of [27]. The monoterpene hydrocarbons had a significantly protective effect possessing several variants due to their different functional groups.

The amounts of total phenolic compounds in all extracts were also investigated spectrometrically according to the Folin–Ciocalteu procedure, calculated as gallic acid equivalents shown in Figure 2. The total phenols of all extracts ranged from 54.43 to 88.25 µg/ml. The highest phenolic concentration was observed in the essential oil followed by the dichloromethane extract, hexane and methanol extract. Similar results were obtained from the study of total phenolic content and free-radical scavenging activity. The measurements of phenols in various *G. sanguineum* L flower extracts may be related to their antioxidant properties as reported by [28]. Various extracts of *G. sanguineum* L. flowers exhibited the border antibacterial and antioxidant activities. The antibacterial and antioxidant activity may be related to the presence of the terpene components especially monoterpenes. These activities may be attributed to the presence of linalool, α-cadinol, globulol and viridiflorol, pulegone, epi-α-cadinol, terpinen-4-ol, germacrene A and *para*-methyl anisole.

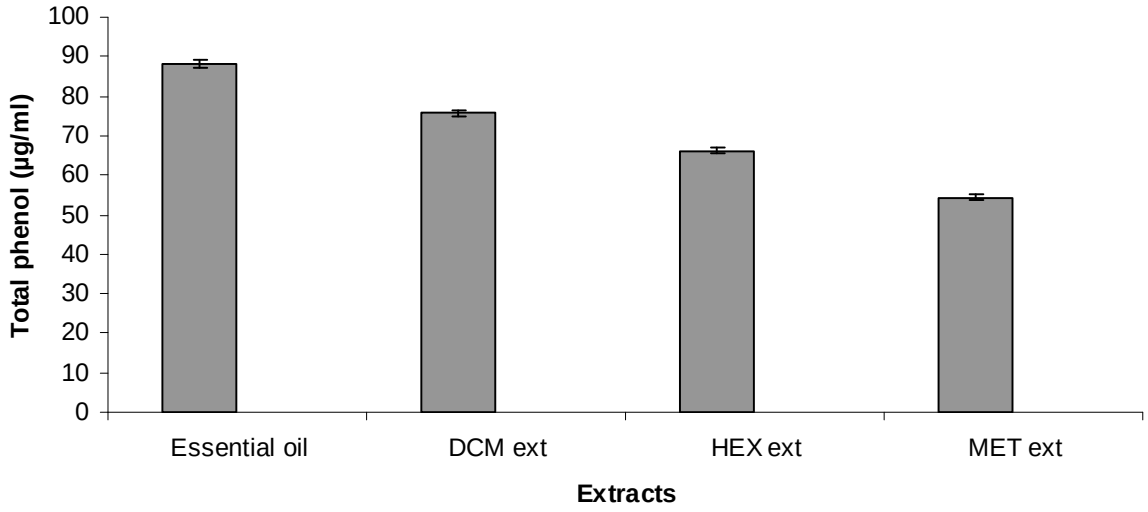

Figure 2. Total phenols of different *G. sanguineum* L. flower extracts
Values represent averages ± standard deviations for triplicate experiments. DCM ext; Dichloromethane extract, HEX ext; hexane extract, and MET ext; methanol extract

CONCLUSION

In the present study, it was found that the essential oil obtained from flowers of *G. sanguineum* L. possessed antibacterial and antioxidant activities. To the best of our knowledge, this is the first report of such activities activities from *G. sanguineum* L. flowers. It can be concluded that the essential oil and the dichloromethane extract and of *G. sanguineum* L flowers could be

considered as alternative natural bactericides for use in screening and developing the natural compounds for the biocontrol of many agricultural plant pathogens. Further studies are needed to evaluate the in vivo potential of this oil as a natural disinfectant and its effect on seeds vigour response.

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