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Original Article

Phytochemical Screening and Antibacterial Activity of Libyan *Globularia alypum*

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ABSTRACT

Aims. The aim of this study was to assess the phytochemical screening and antibacterial effect of the aerial parts of *G. alypum* cultivated in Libya. **Methods**. The methanolic extract of *G. alypum* (at concentrations 100 mg/ml, 300 mg/ml, 500 mg/ml and 700 mg/ml) were tested against 13 different strains of standard bacteria (ATCC) by four different methods; paper disc diffusion method, well diffusion method, broth dilution method and finally study the effect of extract on growth curve of bacterial cell were studied. **Results**. The results of phytochemical screening revealed the presence of flavonoids, phenols, reducing sugars, tannins, saponins, coumarins, steroids, terpenoids, carotenoids, antraquinones and glycosides. While the results of antibacterial tests showed remarkable inhibition of the bacterial growth, with maximum inhibition on growth of Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus aureus*. Moderate effect on growth of *Enterococcus faecalis*, *Bacillus subtilis*, *Aeromonas hydrophila* and *Salmonella poona*. While, the lowest inhibition showed in *Yersinia enterocolitica* and *Listeria monocytogenes*. However, the extract did not have any effect on *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhimurium* and *Klabsiella pneumonia*. **Conclusion**. The methanolic extract of *Globularia alypum*aerial parts contain the most important constituents with positive results during phytochemical screening, and have antibacterial activity against gram positive and gram-negative bacteria.

Keywords: *Globularia alypum*, Phytochemical Screening, Antibacterial Effect, Methanolic Extract.

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INTRODUCTION

Globularia alypum L. is a species medicinal plant belonging to the Globulariaceae family. It is known in Libya as "Azzriga" [1], and it is traditionally using in North Africa as folk remedy in the treatment of many illnesses [2]. In Libya, the decoction of leaves and branches are used in traditional medicine as a purgative and as a substitute for Senna. The leaves are also used as a cure for intermittent fever [1].
The excessive utilization of the antibacterial agents has prompted the presence of multidrug resistant bacterial strains [3]. Therefore, the major challenge is finding of new bioactive agents to use against multidrug-resistant bacteria. For this reason, the plant natural products represent promising sources to develop new antibacterial agents. In this context, the present

study aims to screen the phytochemical constituents of *G. alypum* extract and to evaluate their antibacterial activity.

MATERIAL AND METHODS

Reagents

Absolute methanol, and other used solutions were obtained from Fisher Scientific (Lough borough, UK). Nutrient agar, antibiotics were obtained from Oxford, England, UK.

Bacterial strains

Standard bacterial strains used in this study were ATCC. The strains were activated and cloned three successive times in nutrient agar and stored on nutrient agar slants at 4°C. The identification of the local bacterial isolates was confirmed using conventional biochemical test.

Plant Material

The aerial parts of *G.alypum* were collected from El-Rojban region of Eljabal Elgarbi, Libya, in March 2016 and a voucher number specimen (No. D 68411) was submitted at herbarium of Botany Department, Faculty of Science-University of Tripoli, for authentication. The collected plant was allowed to dry for about 15 days at room temperature. Then the air-dried plant material was finely ground using a grinder and approximately 450g were placed in a cellulose thimble and extracted using Soxhlet apparatus with methanol. The filtered extract was air dried at room temperature and stored in pre-weighed glass vials for further analysis [4].

Phytochemical Screening

Phytochemical screening of *G. alypum* aerial parts were carried out using a procedure that were based on those earlier reports by Harborne (1998), to detect the presence of saponins, tannins, phenols, alkaloids, flavonoids, terpenoids, steroids, glycosides, anthraquinones, coumarins, reducing sugars and carotenoids [4].

Test for Glycosides

5 mg of extract was treated with 2 ml of glacial acetic acid containing ferric chloride, sulfuric acid was added on the wall of test tube. Formation of red ring at the interface and the upper layer becomes blue to green indicated the presence of glycosides.

Test for Anthraquinones (Borentrager's Test)

5 mg of aqueous extract was boiled with dilute sulfuric acid and then filtered. To the cold filtrate equal volume of chloroform was added. Organic layer was separated and ammonia solution was added. The change of ammonia layer to pink or red color indicated the presence of anthraquinones.

Test for Alkaloids

5 mg of extract were boiled with 1 ml of hydrochloric acid and heated in a water bath for 10 minutes. 1 ml was taken and drops of Dragendroff's reagent/ Wagner's reagent/ Mayer's reagent were added separately, and mixed. The appearance of orange precipitate/ brownish-red precipitate/ creamy precipitate respectively, indicated the presence of alkaloids.

Test for Flavonoids

5 mg of extract was treated with few drops of 20% sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute hydrochloric acid, indicated the presence of flavonoids.

Test for Saponins (Foam Test)
6 ml of distilled water was added to 1 mg of extract in a test tube. The mixture was shaken vigorously and observed for the formation of persistent foam that confirmed the presence of saponins.
Test for Coumarins
2 mg of methanolic extract was dissolved in 2 ml of sodium hydroxide, spotted on a Wattman's filter paper and examine under long UV lamp. The appearance of blue fluorescence indicated the presence of coumarins.
Test for Tannins (Braymer's Test)
2 mg of extract was treated with 3% alcoholic ferric chloride solution and observed for formation of blue or greenish color solution.
Test for Reducing Sugars
5 mg of extract was dissolved in 10 ml distilled water and the mixture was boiled for 5 mint. The mixture was filtered while hot. 5 ml of Fehling's solution was added to 2 ml of the filtrate in test tube. The resulted mixture was boiled for 2 minutes. Appearance of brick red precipitate at the bottom of test tube indicated the presence of reducing sugars.
Test for Phenols
10 mg of dried powder of plant was boiled with 10 ml of distilled water for 5 minutes and filtered while hot. 1 ml of ferric chloride solution was added. Formation of blue-black coloration indicated the presence of phenols.
Test for Carotenoids
5 mg of extract was treated with 10 ml of chloroform in a test tube with vigorous shaking, the resulted mixture was filtered, and then 35% sulfuric acid was added. Appearance of blue color at the interface indicated the presence of carotenoids.
Test for Steroids
1 mg of extract was mixed with 2 ml of glacial acetic acid, few drops of concentrated sulfuric acid were added. The change in color indicated the presence of steroids.
Test for Terpenoids
1 mg of extract was mixed with 2 ml of chloroform. 1 ml of concentrated sulfuric acid was guardedly added. A reddish-brown color of the interface indicated the presence of terpenoids.
Determination of Antibacterial Activity
In vitro antibacterial screening of methanolic extract of *G. alypum* were carried out using four different methods; paper disc diffusion method, well diffusion method, determination minimum inhibitory concentration using broth dilution method (Macrodilution assay), and finally, determination the effect of the extract on bacterial growth curve.
Paper Disc Diffusion Method
Antimicrobial susceptibility was tested using paper disc diffusion method [5]. *G.alypum* extract was dissolved in 20% (v/v) DMSO solution to get different concentrations: 100 mg/ml, 300 mg/ml, 500 mg/ml and 700 mg/ml, then filtered using 0.22 μm Millipore filters for sterilization. DMSO was previously tested for antibacterial activity against all test bacteria and found to have no antibacterial activity. Paper discs (6 mm in diameter, Wattman's No.1) were sterilized by autoclave and soaked in extract solutions. Bacterial strains were first grown on nutrient broth at 37 °C for 18 to 20 hours.
Bacterial suspension of each strain was prepared using sterile saline (0.9% NaCl) and turbidity had been adjusted to equal to that of No.0.5 McFarland standard (108 CFU/ml).

The suspension was used to inoculate Mueller Hinton agar Petri-dishes using a sterile cotton swab. Paper discs at varying concentrations were placed separately in the plate under aseptic conditions. Ciprofloxacin 10 µg/disc was used as a standard. The controls were prepared using the 20% DMSO without extract. Triple plates were used for each concentration. All Petri dishes were stored in the dark at 4 °C for 1 hour, to allow the diffusion of the extract from discs to medium without microbial growth and then all inoculated plates contain the papers and standard discs were incubated at 37 °C for 24 hours [5]. The zones of inhibition were subsequently measured in millimeters [6].

Well Diffusion Method

This method was performed using freshly prepared Mueller Hinton agar with overnight culture of bacteria (108 CFU/ml), on each plate wells were made by sterile cork borer. Each well was filled with 50 µl of *G. alypum* extract (100 mg/ml, 300 mg/ml, 500 mg/ml and 700mg/ml) separately, ciprofloxacin 10 µg/disc was used as a standard. The controls were prepared using the same solvent without extract. The plates were then incubated for 24 hours at 37 °C. Triple plates were used for each concentration. The zones of inhibition were measured [7].

Determination of Minimum Inhibitory Concentration

Inoculate were prepared by growing each strain of tested bacteria in nutrient broth, its turbidity had been adjusted as that of a No.0.5 McFarland standard (To get the bacteria number about $1x10^8$ CFU/ml). The concentrations of the extract were prepared by taking the calculated weight of the extract, and dissolved in 2 ml nutrient broth. Two-fold serial dilutions were prepared, to reach concentrations ranging from 700 mg/ml to 2.7 mg/ml. A blank (Media only) and a control growth (Media and bacteria) tubes were also performed [8].

Ciprofloxacin tablets (16 µg) was dissolved in 2ml nutrient broth. Two-fold serial dilutions were prepared, to reach concentrations ranging from 4 µg/ml to 0.125 µg/ml (Standard tubes). The tubes were incubated with 1 ml of bacterial suspension (1x108 CFU/ml) at 37 °C for 24 hours. Control tubes were tested after 24 hours to determine whether the extract-containing tubes were ready to be read, this was accomplished by adding 0.02 ml of Alamar blue reagent at concentration (0.0125% (w/v) resazurin salt in phosphate-buffered saline solution) to the control tube and incubating it for 10 minutes at 37 °C. If the color in the control tube changed from blue to pink after 10 minutes of incubation, then Alamar blue will be added to the extract-containing tubes and standard tubes. These tubes were incubated for 30 minutes at 37 °C [8]. The lowest concentration at which color change occurred was taken as the minimum inhibitory concentration (MIC) value [9]. The mean value was calculated from three separate experiments. The absorbance was measured using UV spectrophotometer at 600 nm. The concentration that gave the 50% inhibition (IC50) was calculated by Probit analysis.

Determining the Growth Curves of Bacterial Cell Exposed to G. alypum Extract

Liquid culture of bacteria was prepared by inoculated a single colony into 20 ml sterile nutrient broth medium in a sterilized conical flask and then it was incubated for 24 hours at 37 °C. The culture was diluted (1:50) into 0.4 ml for 20 ml. 1 ml of plant extract (500 mg/ml) was added and then incubated at 37 °C in a water bath with shaking. The absorbance of zero time was read at 600 nm using UV spectrophotometer. Subsequent absorbance reading was recorded at 1-hour intervals for 5 hours of inoculation. Furthermore, the absorbance was recorded after 24 and 25 hours of inoculation. The test was performed in Triplicate. The

results were transferred to Excel program. Mean absorbance values were calculated, logarithmic graphs were drawn [10].

Statistical Analysis

All data were expressed as means ± standard errors of the mean (SEM), n=3. Using the Statview® version 5.0.1 software package (SAS Institute Inc, Abacus Concept, Inc., Berkeley, CA, USA). A p value of < 0.05 was considered significant. Excel 2007 program was used to draw the figures shown in the results.

RESULT AND DISCUSSION

Phytochemical Screening

Phytochemical analysis of methanolic extract of *G. alypum* was summarized in table 1. It revealed the presence of flavonoids, phenols, reducing sugars, tannins, saponins, coumarins, steroids, terpenoids, carotenoids, anthraquinones and glycosides. However, absence of alkaloids. These compounds have been shown to be active against potentially significant pathogens including those that are responsible for enteric infections [11].

These finding are in agreement with previous study reported that the methanol extract of aerial parts of Moroccan *G. alypum* revealed the presence of phenols, flavonoids, tannins, saponins, coumarins, terpenoids and glycosides. And absence of alkaloids, amino acids and proteins [12].

***Table 1**: Qualitative phytochemical evaluation of methanol extract of Globularia alypum.*

Tests	Observations	Results
Test for flavonoids	Formation of intense yellow color.	+ve
Test for phenols	Appearance of blue-black color.	+ve
Test for reducing sugars	Appearance of brick-red precipitate.	+ve
Test for tannins	Formation of a blue or greenish color.	+ve
Test for saponins	Formation of persistent foam.	+ve
Test for coumarins	The appearance of blue fluorescence under long UV lamp 366nm.	+ve
Test for steroids	Formation of black color.	+ve
Test for terpenoids	Formation of reddish-brown color.	+ve
Test for carotenoids	Appearance of blue color at the interface.	+ve
Test for anthraquinones	Formation of pink or red color.	+ve
Test for glycosides	Formation of red ring at the interface.	+ve
Test for alkaloids: **Dragendorff's reagent** **Wagner's reagent** **Mayer's reagent**	 No change No change No change	-ve

Antibacterial Screening

Paper Disc Diffusion Method and Well Diffusion Method

Tables 2 and 3 shows the antibacterial activity of *G. alypum* methanol extract by paper disc diffusion method and well diffusion method, against 13 different bacterial strains. Where the antibacterial activity of the extract was evaluated based on the diameters of clear inhibition zone surrounding the paper discs and the wells. If there are no inhibition zones, it is assumed that there is no antimicrobial activity.

***Table 2**: The antibacterial activity of Globularia alypum methanolic extract using paper disc diffusion method.*

Bacterial strains	The concentration (mg/ml)				Ciprofloxacin 10 µg/disc (Standard)
	100	300	500	700	
	Mean ± SEM of the inhibition zones				
S. aureus **ATCC 29213**	5±0.33	7±0.45	8±0.57	11±0.5	22±0.4
MRSA ATCC 43300	7±0.33	9±0.57	11±0.3	12±0.5	23±0.5
B. subtilis **ATCC 6633**	5.5±0.33	6.5±0.3	8±0.57	9±0.57	20±0.3
E. faecalis **ATCC 29212**	4±0.85	5±0.55	7±0.75	9±0.33	19±0.5
A. hydrophila **ATCC7966**	3±0.55	4.5±0.33	6±0.57	8.5±0.33	20±0.57
S. poona **ATCC 4840**	2±0.75	4±1.10	6±0.57	8±1.20	19±1.45
L. monocytogenes **ATCC 35152**	1.5±0.85	3±0.57	5±0.45	7±0.57	23±0.33
Y. enterocolitica **ATCC 9610**	1±0.33	3±0.55	4±0.85	7±0.33	20±0.55
E. coli **ATCC 25922**	0±0	0±0	0±0	0±0	26±0.33
P. aeruginosa **ATCC 9027**	0±0	0±0	0±0	0±0	23±0.57
P. mirabilis **ATCC 14153**	0±0	0±0	0±0	0±0	15±0.30
S. typhimurium **ATCC 14028**	0±0	0±0	0±0	0±0	19±0.55
K. pneumonia **ATCC 13883**	0±0	0±0	0±0	0±0	17±0.57

***Table 3:** The antibacterial activity of Globularia alypum methanolic extract using well diffusion method.*

Bacterial strains	The concentration (mg/ml)				Ciprofloxacin 10 µg/disc (Standard)
	100	300	500	700	
	Mean ± SEM of the inhibition zones				
S. aureus **ATCC 29213**	11±0.55	13±0.57	14.5±0.33	18±0.50	23±0.42
MRSA ATCC 43300	12±0.85	15±0.57	17.5±0.44	19±0.57	22±0.50
B. subtilis **ATCC 6633**	10±0.45	12±0.33	14±0.57	15±0.57	21±0.33
E. faecalis **ATCC 29212**	9±0.85	11±0.75	13±0.88	15±0.88	19±0.57
A. hydrophila **ATCC7966**	8.5±0.57	10±0.33	12±0.57	14.5±0.33	23±0.57
S. poona **ATCC 4840**	7±0.33	9.5±1.15	11±0.57	13±1.20	21±1.45
L. monocytogenes **ATCC 35152**	6±0.33	7±0.85	8±0.57	9±0.55	23±0.33
Y. enterocolitica **ATCC 9610**	5±0.45	6±0.57	7±0.75	9±0.88	20±0.55
E. coli **ATCC 25922**	0±0	0±0	0±0	0±0	25±0.33
P. aeruginosa **ATCC 9027**	0±0	0±0	0±0	0±0	25±0.57
P. mirabilis **ATCC 14153**	0±0	0±0	0±0	0±0	17±0.30
S. typhimurium **ATCC 14028**	0±0	0±0	0±0	0±0	16±0.55
K. pneumonia **ATCC 13883**	0±0	0±0	0±0	0±0	19±0.57

As can be noted from these tables, the average diameter of growth inhibition zone ranged from 1 mm to 12 mm by disc diffusion method and from 5 mm to 19 mm by well diffusion method. Particularly, the largest diameters of growth inhibition zone were in *Methicillin Resistant Staphylococcus aureus* (MRSA) and *Staphylococcus aureus*. While, moderate diameters of inhibition zone were observed on growth of *Bacillus subtilis*, *Enterococcus faecalis*, *Aeromonas hydrophila* and *Salmonella poona*. However, the lowest diameters were in *Yersinia enterocolitica* and *Listeria monocytogenes* cultures. Inhibition zone size increased at a higher extract concentration (700 mg/ml) for all sensitive bacteria, which indicate that the *G. alypum* methanolic extract is more effective at high concentration. Nevertheless, there was not any

effect of the extract on *Escherichia coli*, *Proteus mirabilis*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Klabsiella pneumonia*.

Consequently, the methanolic extract of *G. alypum* exhibited antibacterial activity with some differences depending upon the concentration of the extract and the targeted bacterial strain. Where increasing the concentration of extract will lead to increase the inhibition effect on bacterial cells [13].

It is clear from the data above that, generally, the zones of inhibition produced by positive control (Ciprofloxacin) were larger than that produced by methanolic extract of *G. alypum*, this may be attributed to the fact that the plant extracts contains smaller concentration of bioactive compounds [14].On the other hand, there were highest growth inhibition zones by well diffusion method in comparison with disc diffusion method, probably this might attributed to that the paper discs retained the active components and did not allow it to diffuse into the Muller Hinton agar.

In general, the agar diffusion methods (Paper disc and well) allow to simultaneously testing a large number of antimicrobial agents in a relatively easy and inexpensive manner [15]. However, the results are considered as qualitative, because it can only reveal the susceptibility of antimicrobials against tested bacteria, which described as susceptible, intermediate and resistant correlated with diameters of the inhibition zone. Indeed, the major disadvantages of these methods are unable to generate the MIC values and difficult to examine the susceptibility of fastidious and slow-growing bacteria [16]. Besides, it is labor-intensive and time-consuming [17].

Furthermore, other disadvantages of these methods to determine antimicrobial effect, are that the antimicrobial activity may be affected by the agar type, salt concentration, incubation temperature and molecular size of the antimicrobial components. Moreover, they do not distinguish between bactericidal and bacteriostatic effects [18].

Minimum Inhibitory Concentration (MIC)

As can be seen from table 4, the MIC values ranging from 87.5 mg/ml to 700 mg/ml, a dose range which was dramatically high comparing to standard control ciprofloxacin. Even though, it cannot be neglected. This could be attributed to the fact that the phytochemicals have a low antibacterial effect and need very high concentrations to be clinically valuable. Additionally, IC50 (Half maximal inhibitory concentration) values range was from 75 mg/ml to 630 mg/ml. These finding supported the data obtained from the agar diffusion methods of this study. Whereby, MIC and IC50 values also depending on the targeted bacterial strain. Particularly the most sensitive bacteria were *MRSA* and *S. aureus*, with lowest MIC values 87.5 mg/ml and 116 mg/ml, respectively. While, the least sensitive bacteria were *L. monocytogenes* and *Y. enterocolitica* with the highest MIC values 595 mg/ml and 700 mg/ml, respectively.

Table (4): The MIC and IC50 values of Globularia alypum methanolic extract.

Bacteria	MIC of the extract (mg/ml)	IC50 of the extract (mg/ml)	MIC of ciprofloxacin (µg/ml)
	Mean ± SEM		
MRSA ATCC 43300	87.5±0.0	75±2,20	0.125±0.04
***S. aureus* ATCC 29213**	116±13.20	99±2.15	0.25±0.10
***B. subtilis* ATCC 6633**	150±15.83	130±2.89	0.25±0.08
***E. faecalis* ATCC 29212**	189±16.37	153±3.35	0.5±0.22
***A. hydrophila* ATCC 7966**	204±17.45	170±3.58	0.29±0.11
***S. poona* ATCC 4840**	290±18.25	240±3.77	0.41±0.08
***L. monocytogenes* ATCC 35152**	595±19.10	501±4.50	1±0.16
***Y. enterocolitica* ATCC 9610**	700±0.0	630±3.92	0.87±0.50

Growth Curves of Bacterial Cell Exposed to G. alypum Extract

In present study, the results were plotted as optical density values recorded at 600 nm wavelength, as well as the growth curves of treated bacteria were compared to untreated bacteria, shown in figures (1-8), which shows that the methanol extract (500 mg/ml) of *G. alypum* reduced the absorbance (Growth) of eight tested bacteria, during 25 hours of inoculation. Therefore, the extract exhibited antibacterial activity with bacteriostatic nature.

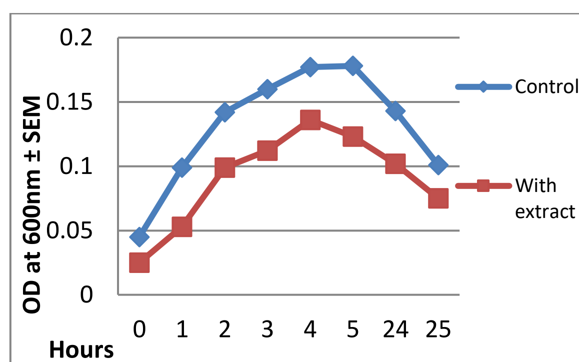

***Figure 1**: Growth curves of Methicillin Resistance Staphylococcus aureus ATCC 43300.*

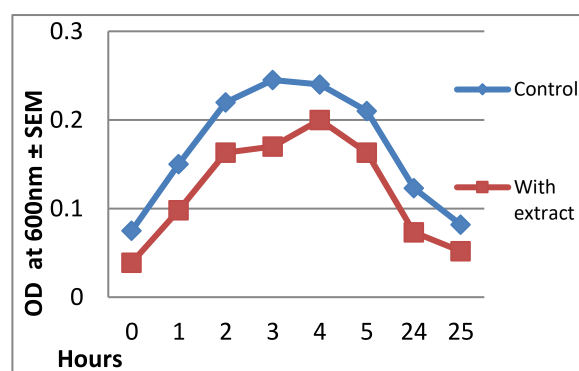

***Figure 2**: Growth curves of Staphylococcus aureus ATCC 29213.*

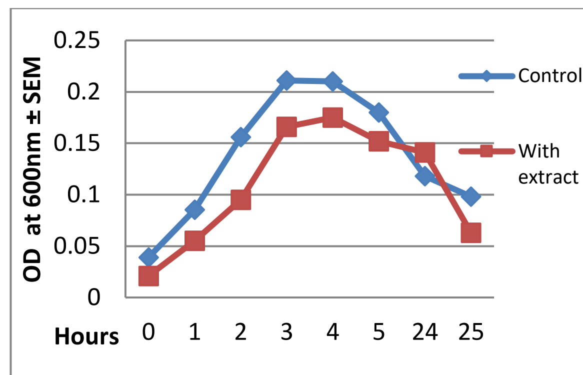

***Figure 3**: Growth curves of Bacillus subtilis ATCC 6633.*

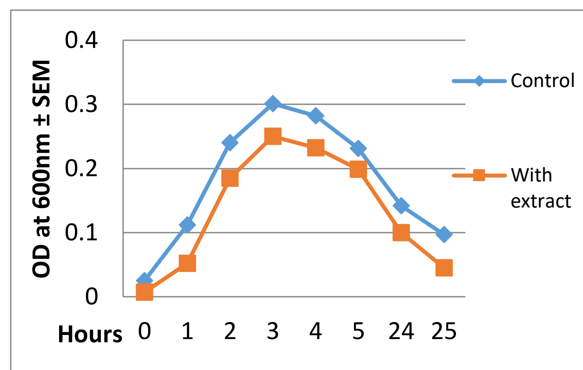

***Figure 4**: Growth curves of Listeria monocytogenes ATCC 35152.*

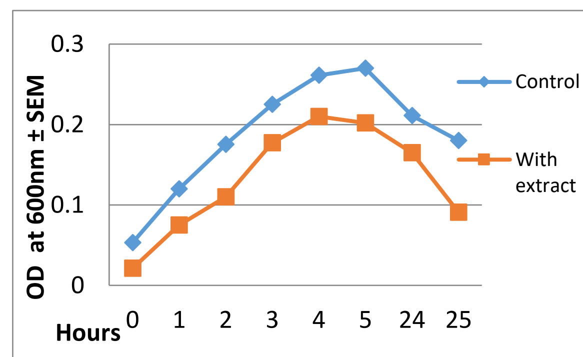

***Figure 5**: Growth curves of Enterococcus faecalis ATCC 29212.*

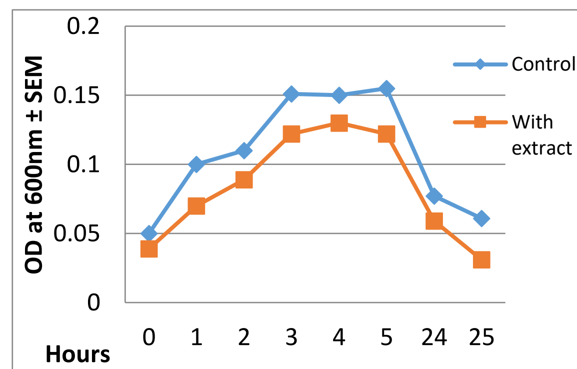

***Figure 6**: Growth curves of Aeromonas hydrophila ATCC 7966.*

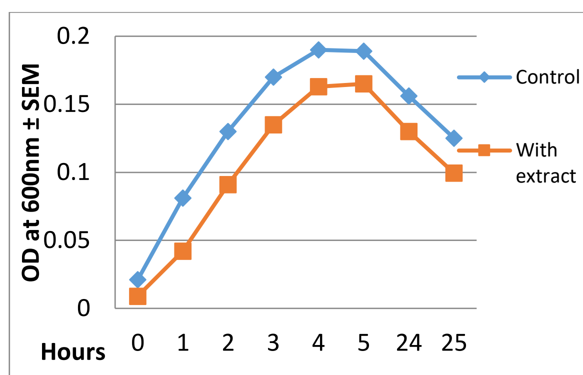

Figure 7: Growth curves of *Salmonella poona* ATCC 4840.

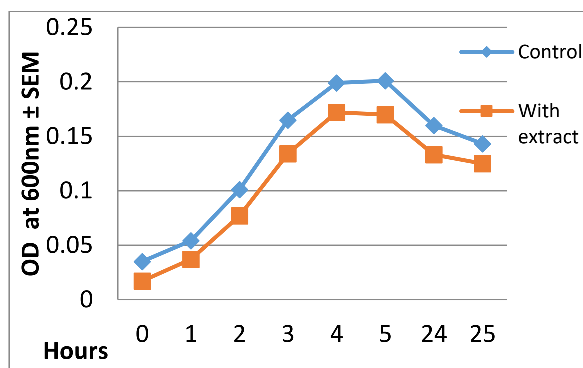

***Figure 8**: Growth curves of Yersinia enterocolitica ATCC 9610.*

CONCLUSION

The present work shows that methanol extract of aerial parts of *Globularia alypum* contain the most important constituents with positive results during phytochemical screening. Furthermore, this methanolic extract have antibacterial activity against gram positive and gram-negative bacteria. However, some gram-positive bacteria, especially *Methicillin Resistant Staphylococcus aureus* and *staphylococcus aureus*, are found more susceptible (With MIC values 87.5 mg/ml and 116 mg/ml, respectively), than gram negative bacteria such as *Aeromonas hydrophila*, *Salmonella poona* and *Yersinia enterocolitic*, which showed MIC values 204 mg/ml, 290 mg/ml and 700 mg/ml, respectively. While, other gram-negative bacteria as *Escherichia Coli*, *Proteus Mirabilis*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Klabsiella pneumonia* are resistance to the methanolic extract of this plant.

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Competing interests

Authors have declared that no competing interests exist.

Authors' Contributions

This work was carried out in collaboration between all authors. Suad and Abdulruzag designed the study, wrote the protocol and the first draft of the manuscript. Suad and

Basma managed the literature searches; analyses of the study, performed the spectroscopy analysis. Suad and Basma did the analyses of the study with help of statisticians. All authors read and approved the final manuscript.

Consent
All authors declare that verbal informed consent was obtained from the participate for publication of this study.

Ethical approval
The study protocol was reviewed and approved by the Ethical Committees of National Authority for Scientific Research (NASR) of Libya in December 2016 by University of Tripoli.

Disclaimer
The article has not been previously presented or published.

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