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IN VITRO SCREENING OF ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL ANALYSIS OF *PUNICA GRANATUM* LINN. (FLOWERS)

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ABSTRACT

The search for new substances with antimicrobial properties has become necessary due to the microbial capacity of resistance. Medicinal plants have become an important substitute since many plants exhibit antimicrobial activity. In this study, the ethanolic flower extracts of *Punica granatum* were assessed for their phytoconstituents and the antimicrobial property. A total of 21 microorganisms (19 bacteria and 2 fungi) were used for antimicrobial activity by disc diffusion method, broth dilution method and a standard procedure was employed to identify the Phytoconstituents. The Ethanolic flower extract showed significant inhibitory activity against *Salmonella paratyphi* A (25.6 mm), *Salmonella typhimurium*, *Salmonella enterica* (22.8 mm), *Yersinia enterocolitica* (22 mm), *Staphylococcus epidermidis* (21 mm), *Salmonella brunei* (20.6mm), *Escherichia coli*, *Pseudomonas aeruginosa* (20 mm) and *Staphylococcus aureus*, *Burkholderia cepacia* (19.6 mm) mean values were documented. Based on the broth dilution method, the ethanolic flower extract showed the MIC value 256µg/ml against *Salmonella paratyphi* A with a significant inhibitory activity. Ethanolic flower extract of *P. granatum* showed the highest antibacterial activity when compared to the antifungal activity could be due to the presence of some active phytoconstituents.

KEY WORDS: ***P. granatum*, Phytoconstituents, MIC, Antibacterial and Antifungal activity**

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INTRODUCTION

Medicinal plants have been known for millennia and are highly esteemed all over the world as a rich source of therapeutic agents for the prevention of various ailments. Antimicrobial resistance to antimicrobial agents has led to treatment failure and the shift of medical care from orthodox to herbal medicine [1]. This development has led to increased search to unfold new, broad spectrum, potent antimicrobial agents [2]. And also, the overuse of synthetic drugs, which results in higher incidence of adverse drug reactions, has motivated humans to return to nature for safe remedies. Therefore, it is of great interest to carry out a screening of these plants in order to validate their use in folk medicine and to reveal the active principle by isolation and characterization of their constituents. Systematic screening of them may result in the discovery of novel active compounds [3]. *P. granatum* belongs to the family punicaceae, commonly known as pomegranate, is a shrub or small tree with several upright, thorny stems, the leaves are elliptic, roughly 2×1 inches, flowers white or red, double-flowered races, native of Asia and Mediterranean Europe[4]. For centuries, the barks, leaves, flowers, fruits and seeds of this plant have been used to ameliorate diseases [5]. The potential therapeutic properties of pomegranate are wide-ranging and include treatment and prevention of cancers, cardiovascular disease, diabetes, dental conditions, erectile dysfunction and prevention of ultraviolet (UV) radiation. *P. granatum* flowers are used as an astringent, haemostatic, antifungal and as a remedy for cut wounds, bronchitis, diarrhoea, digestive problems, men sex power reconstituent and dermal infected wounds [6]. The dried flowers are used in hematuria, hemorrhoids, hemoptysis and dysentery. The powdered flower buds are used in bronchitis [7]. Flower juice is recommended as a gargle for sore throat, in leucorrhea, hemorrhages, and ulcers of the uterus and rectum. In addition, *P. granatum* is reported to have antioxidant [8,9], anti-atherosclerotic [10,11], antibacterial [12-15] and antiviral [16,17,18] properties. The present study has extensively investigated on phytochemical analysis and antimicrobial property of its flower ethanolic extract against respiratory tract infection, urinary tract infection, wound infection and diarrhoeal infection causing microorganisms.

MATERIALS AND METHODS

Collection of plant materials

Fresh flowers of *P. granatum* (L.) were collected from the natural habitat of Tiruchengode in Namakkal district, Tamil Nadu. The plant species was preliminarily identified in Department of Botany, Vivekanandha College of Arts and Sciences for Women, Elayampalayam, and Tiruchengode. Further, it was authentified by the Scientists of the Botanical Survey of India (BSI) in Coimbatore. A voucher specimen was preserved in our laboratory for future reference.

Extraction of plant materials

The flowers of *P. granatum* (L.) were shade dried and pulverized. 250 g of powdered material was packed in Soxhlet apparatus and subjected to continuous hot percolation for 8 h using 450 ml of ethanol (75% V/V) as solvent. The ethanol extract was concentrated under vacuum and dried in a desiccator. The obtained extracts were preserved in the refrigerator and dissociated in dimethyl sulfoxide (DMSO) prior to use.

Phytochemical screening

The preliminary phytochemical tests of ethanolic flower extract of *P. granatum* (L.) were screened for the presence of carbohydrates, glycosides, fixed oils and fats, protein and amino acids, saponins, tannins, phenolic compounds, phytosterols, terpenoids, alkaloids and flavonoids using standard procedures [19-22].

Antimicrobial Screening

Source of microbial strains

Strains of human pathogenic microorganisms used in this study as follows, three gram positive bacteria *Staphylococcus aureus* (MTCC 96), *Staphylococcus epidermidis* (MTCC 435) and *Streptococcus mutans* (MTCC 890), sixteen gram negative bacteria,

Escherichia coli (MTCC 739), *Klebsiella pneumoniae* (MTCC 432), *Enterobacter aerogenes* (MTCC 111), *Proteus vulgaris* (MTCC 742), *Proteus mirabilis* (MTCC 425), *Salmonella typhi* (MTCC 733), *Salmonella paratyphi* A (MTCC 735), *Salmonella typhimurium* (MTCC 98), *Salmonella infantis* (MTCC 1167), *Salmonella enterica* (MTCC 660), *Salmonella brunei* (MTCC 1168), *Pseudomonas aeruginosa* (MTCC 424), *Burkholderia cepacia* (MTCC 1617), *Vibrio parahaemolyticus* (MTCC 451), *Haemophilus parahaemolyticus* (MTCC 1776) and *Yersinia enterocolitica* (MTCC 80); two fungus, *Candida albicans* (MTCC 183) and *Cryptococcus neoformans* (clinical isolate). The microorganisms were originally obtained from Microbial Type Culture Collection Centre (MTCC), Institute of Microbial Technology, Chandigarh, India. Cultures were maintained as respective agar slants in screw-capped bottles and stored at 4˚C. All cultures were checked for viability and purity by regular plating.

Minimum inhibitory concentration (MIC) - Disc diffusion method

The antimicrobial activities of the successive ethanolic extracts of flower were tested by disc diffusion method [23]. The culture plates were prepared by pouring 20 ml of sterile Hi-sensitivity (Hi media - M 486) agar medium. The depth of the medium was approximately 4 mm. Three to four similar colonies of pure cultures were inoculated with tryptone soy broth (Hi media - M 323), further, it was incubated at 37˚C for 2-8 h and inoculum size was adjusted to yield uniform suspension containing 10^5-10^6 cells/ml (McFarland's standard). The agar surface of the plates was swabbed in three directions, turning the plates at 60˚ between each swabbing. Confluent growth is desirable for accurate results. The sterile discs (6 mm - Hi media) were used for the loading ethanolic flower crude extract. Five different concentrations were prepared (250, 500, 750, 1,000 and 1,250 µg) and loaded with appropriate discs. The impregnated discs were incubated at 37˚C for an hour. The dried discs were placed over the surface of swabbed medium with equal distance to avoid the overlapping of the zones of inhibition. Then discs were pressed gently on the surface of the medium. Allowed the plates to stand in refrigerator for 30 min (Pre-diffusion time). The plates were incubated at 37˚C for 16-18 h during which the activity was evidenced by the presence of zones of inhibition surrounding the discs. Each experiment was done in triplicate. A panel of antibiotics was used against each microbial strain and which antibiotic given sensitive with particular organism is used as a control.

Minimum Inhibitory concentration- Broth dilution method

Tube dilution method was used to determine the minimum inhibitory concentration (MIC) of the extracts in Muller Hinton broth (Hi media - M 391) and Sabouraud Dextrose Broth (Hi media - M 033) as specified by the National Committee for Clinical Laboratory Standard [40]. A total of 10 ml of each broth was dispensed into separate test tubes and was sterilized at 121˚C for 15 min and then allowed to cool. Two-fold serial dilutions of the extracts in the broth were made from the stock concentration of the extracts to obtain 8- 4,096 µg/ml of ethanolic flower extracts. About 0.1 ml of the standardized inoculums of the microbes was inoculated into the different concentration of the extracts in the broth. The test tubes of the broth were incubated at 37 ˚C for 24 h and 30 ˚C for 1-2 days for bacteria and fungi respectively and observed for turbidity. The lowest concentration that showed no turbidity in the test tube was recorded as the MIC.

Determination of Activity Index

The activity index of the crude plant extract was calculated as;

$$\text{Activity Index (AI)} = \frac{\text{Zone of inhibition of the extract}}{\text{Zone of inhibition obtained for standard antibiotic}}$$

RESULTS

The screening of phytochemical analysis of *P. granatum* revealed that the presence of carbohydrates, glycosides, protein, amino acids, tannins, phenolic compounds, phytosterols, terpenoids and flavonoids in the ethanolic flower extract of *P. granatum* whereas fixed oils and fats, saponins and alkaloids were absent in the ethanolic flower extract of *P. granatum* (Table 1).

Table 1

Phytochemical analysis of ethanolic flowers extract of P. granatum (L.)

S.No	Name of the Phytoconstituents	Flowers (EtOH)
01.	Carbohydrates	+
02.	Glycosides	+
03.	Fixed oils and fats	-
04.	Protein and amino acid	+
05.	Saponins	+
06.	Tannins and phenolic compounds	+
07.	Phytosterols and terpenoids	+
08.	Alkaloids	-
09.	Flavonoids	+

+ = present - = absent

The antimicrobial activity of the ethanolic flower extract at different concentrations was screened by the disc diffusion method and the mean value of zone of inhibition was assessed in millimeter diameter. The data pertaining to the antimicrobial potential of ethanolic extracts of flowers of *P. granatum* were presented in table- 2 and figure- 1. The minimum inhibitory concentration (MIC) was determined by the broth dilution method and the results are given in table 3. The ethanolic extract of flowers of *P. granatum* showed maximum inhibitory activity against *S. paratyphi* A (25.0 ± 1.08 mm and AI- 0.94), *S. typhimurium* (22.8 ± 1.66 mm and AI- 0.78), *S. enterica* (22.8 ± 1.66 mm and AI- 0.78), *Y. enterocolitica* (22.0 ± 1.09 mm and AI- 0.75), *S. epidermidis* (21.0 ± 2.19 mm and AI- 0.77), *S. brunei* (20.6 ± 2.35 mm and AI- 0.71), *E. coli* (20.0 ± 1.72 mm and AI- 0.66), *P. aeruginosa* (20.0 ± 1.06 mm and AI- 0.62), *S. aureus* (19.6 ± 1.08 mm and AI- 0.49), *B. cepacia* (19.6 ± 1.59 mm and AI- 0.67), *S. typhi* (19.2 ± 1.93 mm and AI- 0.66), *S. mutans* (18.2 ± 1.53 mm and AI- 0.56), *K. pneumoniae* (18.2 ± 1.15 mm and AI- 0.62), *P. vulgaris* (18.2 ± 1.15 mm and AI- 0.70), *H. parahaemolyticus* (17.4 ± 1.28 mm and AI- 0.66), *S. infantis* (16.8 ± 1.04 mm and AI- 0.57), *E. aerogenes* (16.8 ± 0.66 mm and AI- 0.56) and *P.mirabilis* (15.6 ± 0.96 mm and AI- 0.62) were documented. Ethanolic extracts of flowers exhibit significant antibacterial activity when compared than the antifungal activity and their results also nearer to the zones produced by the control antibacterial agents. The results of the ethanolic extract of flowers revealed that 256 µg/ml was observed as a MIC value against *S. paratyphi* A whereas 512 µg/ml against *S. epidermidis*, *E. coli*, *S.typhi*, *S.typhimurium*, *S.enterica* and *Y. enterocolitica*, 1,024µg/ml against *S. aureus*, *S. mutans*, *K. pneumoniae*, *E. aerogenes*, *P. vulgaris*, *S. infantis*, *S. brunei*, *P. aeruginosa*, *B. cepacia* and *H. parahaemolyticus* and the 2048 µg/ml against *P. mirabilis* were documented. The result revealed that the ethanolic flower extract possesses efficient inhibitory activity against the maximum number of bacteria when compared as fungus.

Table 2
Antimicrobial activity of ethanolic flowers extract of P. granatum (L.)

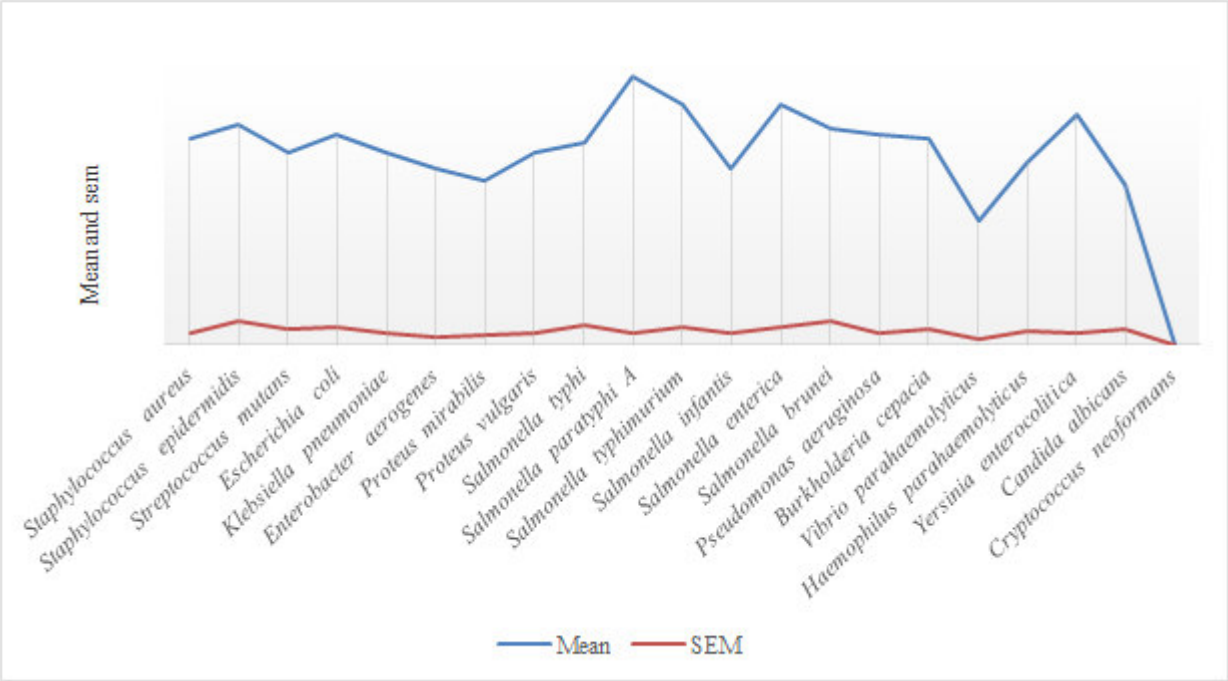
S.No	Name of the organism	Zone of inhibition in mm					
		Minimum	Maximum	Mean ± SEM	Standard Antibiotic µg/disc	Zone in mm	Activity Index (AI)
01.	*Staphylococcus aureus*	16	23	19.6 ± 1.08	Amoxycillin (30)	40	0.49
02.	*Staphylococcus epidermidis*	14	27	21.0± 2.19	Cloxacillin (5)	27	0.77
03.	*Streptococcus mutans*	14	23	18.2 ± 1.53	Ciprofloxacin (5)	32	0.56
04.	*Escherichia coli*	15	26	20.0 ± 1.72	Ciprofloxacin (5)	30	0.66
05.	*Klebsiella pneumoniae*	15	22	18.2 ± 1.15	Nalidixic acid (30)	29	0.63
06.	*Enterobacter aerogenes*	12	22	16.8 ± 0.66	Ciprofloxacin (5)	30	0.56
07.	*Proteus mirabilis*	13	19	15.6 ± 0.96	Lomefloxacin (10)	25	0.62
08.	*Proteus vulgaris*	15	22	18.2 ± 1.15	Lomefloxacin (10)	26	0.70
09.	*Salmonella typhi*	13	26	19.2 ± 1.93	Chloramphenicol (30)	29	0.66
10.	*Salmonella paratyphi A*	22	29	25.6 ± 1.08	Chloramphenicol (30)	27	0.94
11.	*Salmonella typhimurium*	17	28	22.8 ± 1.66	Chloramphenicol (30)	29	0.78
12.	*Salmonella infantis*	14	21	16.8 ± 1.04	Chloramphenicol (30)	29	0.57
13.	*Salmonella enterica*	17	28	22.8 ± 1.66	Chloramphenicol (30)	29	0.78
14.	*Salmonella brunei*	17	25	20.6 ± 2.35	Chloramphenicol (30)	29	0.71
15.	*Pseudomonas aeruginosa*	17	24	20.0 ± 1.06	Amikacin (10)	32	0.62
16.	*Burkholderia cepacia*	15	25	19.6 ± 1.59	Amikacin (10)	29	0.67
17.	*Vibrio parahaemolyticus*	11	14	11.8 ± 0.52	Ciprofloxacin (5)	29	0.40
18.	*Haemophilus parahaemolyticus*	14	22	17.4 ± 1.28	Nalidixic acid (30)	26	0.66
19.	*Yersinia enterocolitica*	19	26	22.0 ± 1.09	Ciprofloxacin (5)	29	0.75
20.	*Candida albicans*	11	20	15.2 ± 1.56	Nystatin (100)	24	0.63
21.	*Cryptococcus neoformans*	-	-	-	Ketoconazole (10)	35	-

Note: SEM- Standard Error of Mean

Table 3
Minimum Inhibitory Concentration of ethanolic flowers extract of P. granatum (L.)

S.No	Name of the organism	Concentration of extracts (in µg/ml)											MIC in (µg/ml)
		4096	2048	1024	512	256	128	64	32	16	8	Control	
		Ethanolic flowers extract											
01	*Staphylococcus aureus*	-	-	-	+	+	+	+	+	+	+	+	1024
02	*Staphylococcus epidermidis*	-	-	-	-	+	+	+	+	+	+	+	512
03	*Streptococcus mutans*	-	-	-	+	+	+	+	+	+	+	+	1024
04	*Escherichia coli*	-	-	-	-	+	+	+	+	+	+	+	512
05	*Klebsiella pneumoniae*	-	-	-	+	+	+	+	+	+	+	+	1024
06	*Enterobacter aerogenes*	-	-	-	+	+	+	+	+	+	+	+	1024
07	*Proteus mirabilis*	-	-	+	+	+	+	+	+	+	+	+	2048
08	*Proteus vulgaris*	-	-	-	+	+	+	+	+	+	+	+	1024
09	*Salmonella typhi*	-	-	-	-	+	+	+	+	+	+	+	512
10	*Salmonella paratyphi A*	-	-	-	-	-	+	+	+	+	+	+	256
11	*Salmonella typhimurium*	-	-	-	-	+	+	+	+	+	+	+	512
12	*Salmonella infantis*	-	-	-	+	+	+	+	+	+	+	+	1024
13	*Salmonella enterica*	-	-	-	-	+	+	+	+	+	+	+	512
14	*Salmonella brunei*	-	-	-	+	+	+	+	+	+	+	+	1024
15	*Pseudomonas aeruginosa*	-	-	-	+	+	+	+	+	+	+	+	1024
16	*Burkholderia cepacia*	-	-	-	+	+	+	+	+	+	+	+	1024
17	*Vibrio parahaemolyticus*	-	+	+	+	+	+	+	+	+	+	+	4096
18	*Haemophilus parahaemolyticus*	-	-	-	+	+	+	+	+	+	+	+	1024
19	*Yersinia enterocolitica*	-	-	-	-	+	+	+	+	+	+	+	512
20	*Candida albicans*	-	+	+	+	+	+	+	+	+	+	+	4096
21	*Cryptococcus neoformans*	+	+	+	+	+	+	+	+	+	+	+	-

Figure 1
Antimicrobial activity of ethanolic flowers extract of P. granatum (L.)



DISCUSSION

The steadily increasing microbial resistance to existing drugs is a serious problem in antimicrobial therapy. Recently, the acceptance of traditional medicine as an alternative form for health care and the development of microbial resistance to the available antibiotics have led authors to investigate the antimicrobial activity of medicinal plants [24]. Plants and plant derived agents have long history to clinical relevance as source of potential chemotherapeutic agents [25].Thousands of plant species have been screened for their antimicrobial activity, but relatively few were found to be sufficiently active and non-toxic to humans [26]. Plants have a highly ability to synthesize aromatic products, most of which are phenols. These are secondary metabolites and in many cases, these products serve as plant security mechanisms against predation by microorganisms, insects, and so forth [27]. Generally, herbal products cause to changes in microorganism's cell, such as cytoplasm granulation, cytoplasmic membrane disruption, inactivation or inhibition of enzyme activity within cell and outside cell, and cell lapse [28-30]. An antimicrobial activity of pomegranate extract is related to attendance of antibiotic compounds [31,32]. The tree of *P. granatum* has been used for centuries to confer health benefits in a number of diseases. Based on its usage in Ayurvedic and Unani medicine, dietary supplements containing pomegranate extract are becoming popular for the treatment and prevention of arthritis and other diseases. The flower has numerous pharmacological activities such as an astringent, haemostatic, antidiabetic, antioxidant and hepatoprotective. Flowering part of this plant has been recommended in Unani literature as a remedy for diabetes. In the recently years,*P. granatum* has been the subject of much scientific research which have showed its pharmacological property. In the present study, crude ethanolic extract of the flowers of *P. granatum* showed the presence of the phytochemicals such as carbohydrates, glycosides, protein, amino acids, tannins, phenolic compounds, phytosterols, terpenoids and flavonoids whereas fixed oils, fats and alkaloids was absent in the ethanolic flower extract of *P. granatum*. The presence of saponins, flavonoids, phenolic compounds and tannins in the ethanolic flower extract was reported previously [33]. In the previous studies the phytochemical analysis of flower extract

revealed that it contains ursolic acid, oleanolic acid, maslinic acid, Asiatic acid [34,35]. The flowers contain compounds also found in peels (e.g. gallic acid) and seed (e.g. ursolic acid), and quite possibly unique, distinctive compounds as well [6]. The phytochemical analysis of flower extract also revealed that it contains flavonoids characterized as 5,6,7,8,2′,3′,5′-heptahydroxy-4′-methoxyflavanone (punicaflavanol) and 5,6,7,8,2′,5′-hexahydroxy-4′-methoxyflavanone -7-*β*-D- xylopyranoside (granatumfla vanyl xyloside) [36]. Flavonoids are well known for their ability to inhibit pain perception. Flavonoids also have anti-inflammatory properties due to their inhibitory effects on enzymes involved in the production of the chemical mediator of inflammation [37]. Phytochemicals like polyphenols, saponins, tannins and vit-C may play a vital protective role against oxidative stress induced damage [38]. The presence of these phytoconstituents make the plant useful for treating different ailments and have a potential of providing useful drugs of human use. Establishing standards is an integral part of establishing the correct identity and quality of a crude drug. In the previous studies *P. granatum* flowers were tested only for its antifungal property but not for the antibacterial effect. But it evident from the present study that flowers of *P. granatum* also possesses the antibacterial activity. The ethanolic flowers extract inhibited the growth of *S. paratyphi* A, *S.typhimurium*, *S.enterica*, *Y. enterocolitica*, *S. epidermidis*, *S.brunei*, *E. coli*, *P. aeruginosa*, *S. aureus*, *B. cepacia*, *S. typhi*, *S. mutans* , *K. pneumoniae*, *P. vulgaris*, *H. parahaemolyticus*, *S. infantis*, *E. aerogenes* and *P. mirabilis*. *C. albicans* are also inhibited with minimum zones of inhibition. Maximum inhibition zones of flower, peel, stem and leaf extracts against *C. albicans* were obtained in 200 µl concentration respectively [39]. Contraindicatedly, in the present study *C. albicans* was weakly inhibited by the ethanolic flower extract. An explosion of interest in the various therapeutic values of *P. granatum* over the last decade has led to numerous *in vitro*, animal and clinical trials. Extracts of *P. granatum* L. flowers in this study demonstrated a broad-spectrum of activity against both bacteria and fungi with different diameter zone of inhibition.

CONCLUSION

The broad-spectrum antimicrobial activities of the plant extract possibly may be due to the presence of secondary metabolites such as tannins, phenolic compounds or flavonoids that were abundant in this plant. Among the various microorganism tested, *Salmonellae* sp., have been inhibited significantly by the ethanolic extract of *P. granatum* flowers. On the other hand, the unknown minor components present have not been elucidated in terms of their broad-spectrum activity. The ethanolic extract of *P. granatum* flower effect on 19 bacterial and two fungal pathogens only have been tested *in vitro*. This study paves the way for further attention and research to identify the active compounds responsible for the plant biological activity. Further studies should be promoted on to reveal the exact mechanism of action by which extracts exert their antimicrobial effect.

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CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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