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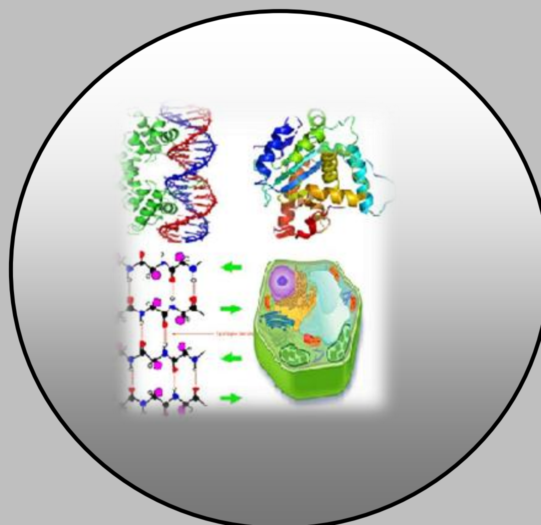

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In vitro Evaluations of Antibacterial and Antifungal activity of the Unsaponified Components (β-sitosterol) of *Pelargonium graveolens L.*

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ABSTRACT

The present investigations was aimed to study the antimicrobial activity (antibacterial and antifungal) of the unsponified matter β-sitosterol from the plant Pelargonium graveolens L. of family Geraniaceae. The dried plant compound was successively extracted from ethanol, methanol, petroleum ether and water as solvent and extract were assessed for their antimicrobial activity against human pathogens like bacteria: Bacillus spp, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and fungi: Aspergillus niger, Aspergillus flavus and, Rhizopus stolonifer. The potential antibacterial and antifungal activity against bacteria and fungi was examined by MIC and zone of inhibition and MBC and MFC analysis by using agar well diffusion and micro dilution method respectively. All the extract shows the significant activity against the microorganism. But the alcoholic extracts show the maximum zone of inhibition and minimum inhibitory concentration. The minimum for zone of inhibition and comparatively maximum inhibitory concentration was determined in petroleum ether and water extracts of compound showing the least antimicrobial activity against all the experimental strains. Hence, this compound from the plant of Pelargonium graveolens L. could be source to obtain new and effective herbal medicine to treat infection and may be exploited for the future antimicrobial drugs.
***Keywords**: Antibacterial activity, antifungal activity, MIC, MBC, MFC, Pelargonium graveolens L., E. coli, A. niger, P. aeruginosa and Zone of inhibition.*

INTRODUCTION

The use of plant and its product has a long history that began with medicine through the year has been incorpated into medicine. Many plant species reported to have pharmacological properties as they are known to posses various secondary metabolites like glycosides, saponins, flavonida, steroids, tannins, alkaloids, trepenes which is therefore should be utilized to combat the disease causing pathogen.[1]

With the advancement in science and technology, remarkable progress has been made in the field of medicine with the discoveries of many natural and synthetic drug medicinal plant have been used as traditional treatment for numerous human disease for thousand of years and in many parts of the world. Hence, researchers have recently paid attention to biologically active compound isolated from plant species used in herbal medicine with acceptable therapeutic index to for the development of novel drugs.

The plant *Pelargonium graveolens* was shade dried, powered and extracted with petroleum ether in a soxhlet extract for about 70 hrs at (40 -60 ˙c). After extraction was concentrated to a get yellow viscous mass. Which was saponified by refluxing with alcoholic potassium hydroxide. The soap obtained was dissolved in water and the unsaponified matter was recovered by extraction with ether in separating funnel. The compound was identified as β-sitosterol (Chopra, 1980). After showing literature its medicinal important and the extraction process of the compound the present protocol has been outlined regarding the antimicrobial activity on these selected plant compound using different extract. It is in view of this, that the present research was the set up to evaluate the antimicrobial activity of compound β-Sitosterol from the plant *Pelargonium graveolens* (Linn). Using different plant extraction against bacteria and fungi (Murray et al., 1995).

MATERIAL AND METHOD

1. **Selection of medicinal plant**: The medicinal plant Pelargonium graveolens L. was collected in curing several aliments and its ethnomedicinal importance. Healthy and disease free plants were selected for the antimicrobial screening (Akinyemi et al., 2005).

2**. Extraction Procedure**: Powered material of compound was extracted with different solvents (Like Ethanol, Methanol, Petroleum Ether and Water) according to their increasing polarity successively for 8-10 hrs in the soxhlet apparatus at a temperature not exceeding the boiling point of the respective solvents. After extraction excess solvent was removed by distillation and the concentrated extract so obtained were further dried in incubator at 40^0c.The residual extract after drying were dissolved in 50% DMSO and stored in refrigerator at 4^0c in small and sterile glass tubes.

3. Culture and Maintenance of microorganism: Pure culture of all experimental bacteria and fungi were obtained. The pure bacterial culture were maintained no nutrient agar medium (NA) and fungal culture on Potato dextrose medium (PDA).

4. **Microorganism use for test:** The bacterial strains used for antibacterial screening were;-

a) Bacillus Cereus b) Staphylococcus aureus c) Escherichia coli d) Pseudomonas aeruginosa

The fungal strains used for antifungal screening were;-

a) *Aspergillus niger* b) *Aspergillus flavus* and c) *Rhizopus stolonifer*

5. **Media preparation and its sterilization:** For agar well diffusion method (Muclay et.al 1995 later modified by ocurinola, 1996) antimicrobial susceptibility was tested on solid (agar-agar) media in petriplates for bacterial assay nutrient agar (NA) (40 gm/l) and fungus PDA (39gm/l) was used for developing surface colony growth. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) value were determined by serial micro dilution assay. The suspension culture, for bacterial cell growth 2.4%w/v and PDA for fungus cell growth was taken for evaluation. All media prepared was then sterilized by autoclaving the media at (125°c) for 20 min (Ahmad and Beg, 2001).

6. **Agar Well diffusion method:** Agar Well diffusion method was followed to determine the antimicrobial activity. Nutrient agar (NA) and potato Dextrose Agar (PDA) plates were swabbed with 8 hrs old broth culture of respective bacteria and fungi. Wells (10 mm diameter and about 2 cm a part) were made in each of these plates using sterile cork borer. Stock solution of each plant extract was prepared at a concentration of 1mg/ml in different plant extract viz. methanol, ethanol, Petroleum ether, water. About 100ul of different concentration of plant solvent extract was added sterile syringe into the well and allowed to diffuse at room temperature for 2hrs. Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37oc for 18-24hr for bacterial pathogen and 280c for 48 hr fungal pathogen. The diameter of the inhibition zone (mm) was measured and the activity index was calculated. Triplicate were maintained and the was repeated for each pelicates the reading were taken in three different direction and the average value was recorded.

7. Minimum Inhibitory concentration: the minimum inhibitory concentration is defined as the lowest concentration able to inhibit any visible bacterial growth on the culture plates. This was determined from reading on the culture plates after incubation. The most commonly methods are tube dilution method and agar dilution methods. Serial dilution is made of the products in bacterial and fungal growth media. The test organism is then added to the dilution of the products, incubated and scored for growth. This procedure is a standard assay for antimicrobials. Clinically, the minimum inhibitory concentrations are used not only to determine the amount of antibiotic used that the patient will receive but for microbial resistance to specific antimicrobial agents.

8. Test for antibacterial activity: The antibacterial assay was carried out by micro dilution method in order to determine the antibacterial activity of compound tested against the pathogen bacteria. The bacterial suspension was adjusted with sterile saline to a concentration of $1 * 10^{7}$CFU/ml. The inocula were prepared and store at 4^{0}c until use. Dilution of the inocula was cultured on solid medium to verify the absence of contamination and to check the validity of incolum. All the experiments were done induplicate and repeated three times.

9. Test for antifungal activity: The antifungal assay was carried out by micro dilution method in order to determine. The fungi l spores were washed from the surface of the agar plates with sterile .85% saline containing o.1% Tween 80 (v/v).

The spore suspension was adjusted with sterile saline to a concentration of 1 $*10^{7}$ in as final volume of 100ul per well. The inocula were prepared and store at 4^{0}c until use. Dilution of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of incolum

10. Determination of MBC: The MBC was determined by serial sub-cultivation of 2ul into micro liter plates containing 100ul of broth per well and further incubation 72hours at 28^{0}c. The lowest concentration with no visible growth was defined as MBC, indicating 99.5% killing of the original inoculums. The reading was taken and compared with the standard streptomycin for bacteria as the positive controls. All the experiments were performed in duplicate and repeated three times.

11. Determination of MFC: The MFC was determined by serial sub-cultivation of 2ul into micro liter plates containing 100ul of broth per well and further incubation 72hours at 28^{0}c. The lowest concentration with no visible growth was defined as MFC, indicating 99.5% killing of the original inoculums. The standard Greisofluvin was used as the positive controls.(1-3000ug/ml). All the experiments were performed in duplicate and repeated three times.

Table 1. Antimicrobial activity (zone of inhibition,mm) of various extracts of B-sisoterol against pathogen (Bacterial and Fungal).

Microorganism bacteria	**Ethanol**	**Methanol**	**Petroleum ether**	**Aqueous (water)**	**Standard (streptomycin)**
B. cereus	13.4	18.9	10.1	14.5	23..5mm
S. aureus	12.6	16.5	11.4	13.6	18.3mm.
E.c oli	18.9	14.6	11.7	ND	21.4mm
P. aeruginosa	13.2	14.3	12.4	ND	20.5mm
fungi	**Ethanol**	**Methanol**	**Petroleum ether**	**Aqueous (water)**	**Standard (griesoflvin)**
A. niger	16.4	13.1	12.4	ND	17.53mm
A. flavus	13.7	15.5	14.7	11.3	15.33mm
R. stolonifer	12.9	13.3	11.4	12.3	13.76mm

ND: not detected the growth of microorganism

Observation

In the present investigation, the inhibitory effect of different extracts (methanol, ethanol, petroleum ether and water) of β–sisoterol form plant Pelargonium graveolens L. evaluated against both fungicidal and bacterial strains. The antimicrobial activity was determined using agar well diffusion method and micro dilution method summarized in the table. The activity was quantitatively assessed on the basis of inhibition zone and their index is also calculated along with minimum inhibitory concentration (MIC).

Measurement of antimicrobial activity

The antimicrobial potential of the experimental compound of the plant was evaluated according to their zone of inhibition against various pathogens and the results were compared with the activity of the standard streptomycin (1mg/disc) and griseofluvin (1mg/disc). The result reveals that all the extracts are potent antimicrobial agent against the entire microorganism [Ah] studied among the different solvent extract studied

Minimum inhibitory concentration MIC (µg/ml) and MBC of the different various extracts of β-sisoterol

Micro organism bacteria	Ethanol		Methanol		Petroleum Ether		Aqueous (water)		Standard	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. cereus	26.4	43.8	22.4	45.7	32.4	55.4	19.8	54.6	39.5	58.9
S. aureus	18.8	51.3	34.5	65.8	21.6	45.3	14.4	53.7	25.6	62.3
E. coli	19.8	38.7	13.7	42.1	17.3	33.7	22.10	47.6	31.8	65.6
P. aeruginosa	11.2	53.6	17.4	48.3	19.4	55.8	20.2	58.3	27.3	72.1

Minimum inhibitory concentration MIC (µg/ml) and MFC of the different various extracts of β-sisoterol

Micro organism Fungi	Ethanol		Methanol		Petroleum Ether		Aqueous (water)		Standard	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. niger	19.2	39.5	21.6	42.7	18.7	45.3	16.5	32.4	22.7	47.8
A. flavus	22.3	31.3	16.4	53.4	25.3	58.9	22.1	43.6	26.5	59.6
R. stolonifer	14.2	32.5	11.2	21.7	16.3	28.0	17.3	25.1	19.9	34.4

RESULT AND DISCUSSION

In the present investigation showed that all ethanol, methanol, petroleum ether, and aqueous extract of the β-sitosterol of plant *Pelargonium graveolens L*. were active against the bacterial and fungal strain. Susceptibility of each extract was tested by serial dilution method (MIC) and agar well diffusion method was determined.

The alcoholic viz. ethanol and methanol extract of *Pelargonium graveolens L.* shows the significant antimicrobial activity against the different strains of alcoholic extract is due to difference between extract compounds in this two extracts. These extract show maximum activity against in E. coli, B. cereus and Scores strain in alcoholic extract and more activity in methanol extract. The study also revealed that Petroleum Ether extract show moderated activity against different bacterial and fungal strain and aqueous show the minimum antimicrobial activity. But it has same antibacterial and fungal activity against some strains.
The observation of the MIC study has been tabulated in table and it was found to be varying in different extract. In the present study the bacterial and fungal strain which show the maximum value of MIC and MBC/MFC for the different extracts show the less antimicrobial activity and the different bacterial and fungal strain which shows the minimum value of MIC and MBC/MFC show the maximum antimicrobial activity. It the organism which shows maximum value has the less potential to develop the antimicrobial agent and which show the low MIC and MBC/MFC value for the other bacteria is an indication of the efficacy of the extract. The analysis suggest that the extract were bacterostatic at lower concentration but bactericidal at higher concentration.

CONCLUSION

In conclusion, of the present study contain the potential antimicrobial component that may be of great use for the therapy against various disease .The study indicates that can be studied for the further assay to evaluate effectiveness as antimicrobial agent. Further, the potential of this plant must be explore more and more, in order to develop an alternate therapy for the treatment of infection cause by different bacterial and fungal organism. By developing new and more effective antibacterial and antifungal agent.

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