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In Vitro evaluation of the antibacterial activity of *Petunia* leaf and callus extracts

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The antimicrobial activity of plant extracts were found in folk medicine as essential oils or isolated compounds. Some of these compounds were isolated or obtained by bio-guided isolation after previously detecting antimicrobial activity on the part of the plant. *Petunia* is an imporant medicinal and ornamental plant.The *in vitro* callus induction and antibacterial activity of *Petunia* leaf and callus extracts were studied against *Escherichia coli, Pseudomonas aeroginosa*, *Baccilus subtilis, Salmonella* spp., S*treptococcus* spp and *Staphylococcus aureus*. The antibacterial activities of different solvent extracts were determined by disc diffusion method. Among the five solvents used, leaf and callus extracted from chloroform were found to be more effective. The callus induction and antimicrobial activities of *Petunia* medicinal plants are discussed in the present paper.

Key words: *Petunia,* Callus induction*,* antibacterial activity, disc diffusion

Introduction

Use of plants as a source of medicine has been inherited and is an important component of the health care system. There are about 45,000 plant species in India with concentrated hotspot in the region of Eastern Himalayas, Western Ghats and Andaman and Nicobar Islands. The officially documented plants with medicinal potential are 3000 but traditional practitioners use more than 6000 plants. India is the largest producer of medicinal herbs and is appropriately called the botanical garden of the world (Ahmedulla and Nayar, 1999). Approximately 20% of the plants found in the world have been submitted to pharmacological or biological tests (Suffredini *et al.,* 2007). The systemic screening of antimicrobial plant extracts represents a continuous effort to find new compounds with the potential to act against multiresistant pathogenic bacteria and fungi.

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Medicinal plants are gifts of nature to cure limitless number of diseases among human beings. The abundance plants on the earth's surface has led to an increasing interest in the investigation of different extracts obtained from traditional plants as potential sources of new antimicrobial agents. Some medicinal plants used in traditional Indian medicine are effective in treating various ailments caused by bacterial diseases. New antimicrobial agents are needed to treat diseases in humans and animals caused by drug resistant microorganisms. In addition, there is a continuing consumer demand for "natural" and "preservative-free microbiologically safe foods and cosmetic products. As public demand for these products increases, an opportunity exists to satisfy consumer demands while providing wholesome and safe products.

Antimicrobial substances are substances that inhibit the growth and existence of microorganisms (Paul and Sainburg, 1991). These micro organisms could be pathogenic or non pathogenic, hence, antimicrobial substances are used in the treatment of various ailments. Quite a number of antimicrobial substances exist and they are gotten from diverse sources such as microbial, plant, animal and chemical sources (Ganellin and Roberts, 1999). Medicinal uses of these plants range from the administration of the plant's roots, bark, stem, leaves, fruits and seeds, to the use of extracts from the whole plant (Akujobi *et al.,* 2004). Plants have a great potential for producing new drugs of great benefit to mankind. There are many approaches to the search for new biologically active principles in higher plants (Jigna and Chanda, 2006).

This search for new antimicrobial properties of natural products cannot be ignored because this can be found in the most remote parts of the world where medical doctors are not present (Olukemi and Kandakai, 2004). Among the diseases that have been managed successfully by traditional (herbal) medicine include malaria, epilepsy, infertility, convulsion, diarrhoea, dysentery, gonorrhoea, flatulence, tonsillitis, bacterial and fungal infections, mental illness and worm infections (Sofowora, 1996).

Petunia belongs to the plant family *Solanaceae.* Several species of *Petunia* are ornamentals grown in gardens for their large, showy; multicoloured flowers and is also an important cut flower crop. Breeding of petunias began about 30 years after the plant's discovery. One of the early hybridizers was a Californian, Theodosia Burr Shepherd, who produced petunias with huge flowers. They were given the name Giants of California. Finally, some varieties feature fringed or ruffled petal edges instead of the usual smooth edged flower. *Petunia* is a mild-acting medicine possessing anti-microbial (Rahman *et al*., 2008) and shows the mildest anti-oxidation activity. Its leaves yield an important insecticide, widely used as natural insecticides, offer all the advantages of chemical compounds, that is, rapidity of action, activity against a

broad range of insects, and rapid biodegradability (Kays *et al.,* 1994). Here we report the result of our preliminary antimicrobial screening.

Antimicrobial activity of this plant species has not been reported earlier. The principle aim of the present work was to study the antimicrobial activity of *Petunia* leaf and callus extracts in different solvents like, petroleum ether, chloroform, *Ethyl acetate*, ethanol and aqueous extract against both human and plant pathogenic bacteria including *Escherichia coli, Pseudomonas aeroginosa*, *Baccilus subtilis, Salmonella* spp., S*treptococcus* spp and *Staphylococcus aureus*.

Materials and methods

The leaves are collected from the green house of the Karpagam University garden. The explants were washed thoroughly under running tap water for 30 min, followed by 0.2% bavistin for 15 minutes. Bavistin treated explants were washed with sterile distilled water, treated with 2.5% Tween 20 for 5 min, and washed repeatedly with sterile distilled water. The explants were then surface sterilized with 1.0% mercuric chloride for 2 min and washed with sterile distilled water for 3 times each under the laminar airflow followed by 70% ethanol for 1 min. After washing in sterile distilled water, the explants were inoculated aseptically in MS medium.

The MS nutrient medium (Murashige and Skoog, 1962) was used for callus induction and suspension culture studies. The Millipore water was used for the preparation of culture media. After addition of all the media constituents, the pH was adjusted to 5.8 using 0.1 N KOH or 0.1 N HCl. Gelling agent (agar-agar) at a concentration of 0.8% was added and the medium was steamed to melt the gelling agent. It was then dispensed into test tubes (10 ml per tube) or screw capped bottles (25 ml per bottle). The medium was autoclaved at 121° C at a pressure of 1.1 $kg.cm^{-2}$ for 20 min. All the plant growth regulators used during the course of the present work were added before autoclaving the medium.

The working table of the laminar airflow chamber was first surface sterilized with 70% ethanol. Sterile Petri dishes and tools (forceps, scalpels, sterile cotton, and sterile paper towels) that were used for inoculation were kept in the laminar airflow chamber. The ultra violet light was switched on for 20 min. prior to inoculation; hands were sterilized with 70 % ethanol. The forceps and scalpels were dipped in 70% ethanol and flamed, cooled and used for inoculation. For callus induction studies, the leaves were trimmed into pieces of about 1 cm^2 and inoculated on to the medium. All these cultures were incubated at 25±2ºC. Each experiment had 10 replicates with three explants each. A

photoperiod of 16/8 h light and dark was maintained for all experiments; except for callus induction where one set of replicates were maintained in dark.

MS medium supplemented with the varying concentration of the auxin, 2, 4-D was used for callus induction studies. The effect of different concentration of the auxin (0.5, 1.0, 1.5 and 2.0 mg/ml), 2, 4-D on callus induction was studied on explants. The effect of light on callus induction was studied by maintaining 10 replicates under a photoperiod of 16/8 h and 10 replicates were maintained in dark.

All the calli obtained were subculture after 30 days and the friable callus obtained from best concentration were transferred to MS medium supplemented with 1.0 mg/L BAP for regeneration. A photoperiod of 16/8 h light and dark was maintained.

Fresh leaves of *Petunia* (Fig. 1) free from diseases were collected from Karpagam University Garden (India), washed thoroughly 2-3 times with running tap water shade-dried, powdered and used for extraction.

A known quantity of one month fresh calluses was taken and oven dried at 50^{O}C to constant weight. The callus was finely ground and extracted with following extraction. The crude extraction obtained was used for antimicrobial studies. The dried leaves and callus of *in vitro* and *in vivo* raised *Petunia* were subjected to different extraction methods, using various solvents viz. petroleum ether, chloroform, *Ethyl acetate*, ethanol and aqueous extract.

Escherichia coli, Pseudomonas aeroginosa, *Baccilus subtilis, Salmonella* spp., S*treptococcus* spp and *Staphylococcus aureus* stock cultures were collected from the Department Microbiology, Karpagam University, Coimbatore. The stock was maintained on nutrient agar slant and subculture in nutrient broth for incubation at 37°C prior to each antimicrobial testing. Inoculation of the test organisms on nutrient agar-prepared plates was achieved by flaming a wire loop on a spirit lamp, cooling the wire loop (air cooling) and fetching the test organisms. A 24 h fresh culture was prepared in Nutrient Broth and was used for the antimicrobial testing.

The agar diffusion method was used for the antimicrobial activities (Okeke *et al.,* 2001). The antibacterial activity was tested by Agar Diffusion Method. Briefly 20 ml of Muller Hinton agar (Hi Media Pvt LTD) was poured into the Petri-dish and 8 mm well bored in the agar. 100 µL of concentrations of extracts was poured into the wells. The plates were incubated for 24 h at 37°C and the zone of inhibition was measured in mm. Commercially available chloramphenicol disc are used as positive control and respective solvents acting as a negative control. All the tests were done in triplicates.

The data generated from the various experiments were subjected to statistical analysis by using the statistical software AGRES, in completely

randomized design (CRD). Percentage values were transformed to arcsine values before statistical analysis, wherever necessary. Each experiment had 10 replicates with three explants each (callus culture).

Fig. 1. Petunia Explant.

Results and discussion

Effect of four different concentrations of 2, 4-D (0.5, 1.0, 1.5, and 2.0 mg/L) on callus initiation and callus mass were assessed in leaf explants. Callus initiation and proliferation was observed at weekly intervals. The percentage of callus initiated was recorded 4 weeks after inoculation. The increase in mass as gain in weight was recorded as the proliferation rate of callus after 6 weeks of inoculation (Fig. 2).

Fig. 2. Petunia Callus.

The effects of 2, 4-D on callus induction in leaf are summarized in Table 1. A significant difference is observed between treatments. A 100 ± 0.26% callus induction was observed for leaf explants in 1.5 mg/L 2, 4-D followed by 2.0, 1.0 and 0.5 mg/L. The callus induction on MS medium supplemented with 2.0 mg/L 2,4-D was 86 ± 0.12 % and 76 ± 0.13 % in 1.0 mg/L while the callus response was lowest (22 ± 0.11%) in 0.5 mg/L 2,4-D.

Chand and Basu 1998 studied the effect of growth regulators on culture response in *Petunia*, the results indicated that MS medium supplemented with 2.0 mg/L 2, 4-D was the optimal media for callus induction, which is on par with our studies on callus induction. Datta *et al*. (2006) achieved high callus response in *Taxus wallichiana* on half WPM supplemented with 1.0–2.0 mg/L 2,4-D which was in accordance to our results for both leaf and petal explants.

The effect of 2, 4-D on callus proliferation is summarized in Table 1. A significant difference was observed between treatments. The proliferation rate was found to follow an increasing trend with increase in 2, 4-D concentration. The proliferation rate was minimum in media supplemented with 0.5 mg/L 2,4-D (24 ± 0.05 mg) for leaf callus and maximum at 1.5 mg/L 2,4-D (46 ± 0.11 mg) supplementation followed by 1.0 mg/L 2,4-D (33 ± 0.07 mg) and 2.0 mg/L 2,4-D (29 ± 0.12 mg).

Our results are supported by studies of Bonfill *et a*l., (2002) where the influence of auxins on callus production was significant and good calli was obtained from both leaf and stem segments on MS basal media supplemented with 2.0 mg/L 2,4-D within 2 weeks of culture. Similarly, supplementation of 2, 4-D gave rise to large callus mass in pepper (Rubluo and Barroso 1992).

The result obtained in the present study relieved that the tested *Petunia* plant extracts posses potential antibacterial activity against *Escherichia coli, Pseudomonas aeroginosa*, *Baccilus subtilis, Salmonella* spp., S*treptococcus* spp and *Staphylococcus aureus* (Table 2). The extracts showed significant inhibition zone against microbes in comparison to positive control. The negative control no zone of inhibition was found.

Table 1. Effect of 2, 4-D Concentration on Callus Induction.

Concentration of 2,4-D mg/L	Callus induction Percentage ± SE	Callus Proliferation in mg ± SE
0.5	22 ± 0.11	24 ± 0.05
1.0	76 ± 0.13	33 ± 0.07
1.5	100 ± 0.26	46 ± 0.11
2.0	86 ± 0.12	29 ± 0.12
SEd	0.17	0.08
CD (0.05)	0.42	0.16

Table 2. Antimicrobial activity of leaf and callus extracts.

Source	Solvents	Zone of Inhibition in mm					
		Escheri chiacoli	*Pseudomonas aeroginosa*	*Baccilus subtilis*	*Salmonella* spp	*Streptococcus* spp	*Staphylococcus aureus*
Leaf extract	**PE**	19±0.21	7±0.24	13±0.23	12±0.22	12±0.31	7±0.52
	CH	23±0.33	9±0.35	20±0.35	16±0.34	15±0.44	14±0.24
	EA	21±0.34	8±0.41	18±0.34	15±0.41	13±0.51	12±0.35
	ET	22±0.26	7±0.26	10±0.35	14±0.27	13±0.21	12±0.41
	AQ	16±0.31	6±0.42	9±0.36	8±0.28	10±0.22	9±0.25
Callus extract	**PE**	13±0.41	6±0.33	11±0.44	9±0.34	10±0.32	6±0.41
	CH	20±0.22	8±0.43	15±0.27	12±0.41	13±0.42	10±0.43
	EA	16±0.32	7±0.37	14±0.38	10±0.40	12±0.51	8±0.21
	ET	12±0.35	6±0.34	8±0.42	9±0.24	11±0.34	7±0.33
	AQ	10±0.27	6±0.24	7±0.26	7±0.35	9±0.32	6±0.41
Positive control (Chloramphenico)		25±0.41	23±0.35	24±0.32	24±0.43	23±0.27	23±0.32

PE- Petroleum ether, CH- Chloroform, EA- Ethyl Acetate, ET- Ethanol, AQ- Aqueous

The highest antibacterial activity in chloroform leaf extract of *Escherichia coli* (23±0.33 mm) and least activity recorded in callus aqueous extract of *Pseudomonas aeroginosa* (6±0.42 mm). When tested by the disc diffusion method, the leaf methanolic extract of this plant showed highest activity against *Escherichia coli* and *Baccilus subtilis*. All other extracts also showed inhibition against all the other microbes but chloroform extracts only showed maximum inhibition against the test microorganism followed by *Ethyl acetate*, ethanol, petroleum ether and aqueous extracts. The results were also correlated with the earlier study carried out by the researchers for the ethanolic extracts of Eqyptian propolis which was reported by Hegazi *et al.* (2001).

The antimicrobial activity of all the five extracts was carried out by determining the zone of inhibition. Methanolic extracts showed higher zone of inhibition than all other extracts against the entire microorganism. Chloroform extracts showed higher zone of inhibition than against *Escherichia coli* (20±0.22 mm) followed by *Baccilus subtilis*, S*treptococcus* spp., *Salmonella* spp., *Staphylococcus aureus* and *Pseudomonas aeroginosa* at 100mg/ml concentration.

Where as compare to the all the extracts very highest activity were showed in methanolic followed by *Ethyl acetate*, ethanol, petroleum ether and aqueous extraction. The highest growth inhibition was found in the callus extract of *Catha edulis* (Elhag *et al.,* 1999) but in our plant extract showed lowest activity compare to the leaf extract it showed that activity was less in callus extract.

In all the extracts, leaf extracts only showed highest activity against the test organism compare to the callus extract. The leaf methanolic extract of *Petunia* shows maximum inhibition compare to other extracts. Many studies have reported the antimicrobial activities of such have reported the

antimicrobial activities of such sealers, but the results have not always been concordant (Siqueira *et al.,* 1996; Abdulkadar *et al.,* 1996 and Mickel *et al.,* 2003). Singh and Sudarshana (2003) tested the aqueous and ethenolic extract of *Baliospermum axillare* leaf and callus against bacteria like *X. campestris*, *P. syringae*. Similar results were also reported by Hegazi *et al.* (2001).However, studies have indicated that certain bioflavonoid have inhibitory activity against human pathogen bacteria (Lin *et al.,* 2001).

All these studies indicate that plants have potential antimicrobial activity and even if plants of the species are collected from different regions, they exhibit different activities. Because of both the differences in species and the differences in the parts that are extracted, it is natural that there are differences in their antimicrobial activities. The important thing is the determination of the antimicrobial activities that plants have and their usability in the preparation of new drugs. Effective compounds to be obtained by the determination of the active compound in the plant can account for new resources for chemotherapeutics to be synthesized. Even at a trace level, presence of antibacterial active agents in the plant will allow for the preparation of new drugs with new biological agents as a result of obtaining that active agent from the plant through different methods and purification process. The extracts of higher plants can be very good source of antibiotics (Fridous *et al.,* 1990) against various bacterial pathogens. Plant based antimicrobial compounds have enormous therapeutical potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials. Higher plants have also made important contributions in the areas such as cancer therapies.

This study demonstrated that the activity of the extracts depend on the solvent employed in their extraction from the plant samples, while the leaf extracts indicated strong antibacterial effects compared to the callus. This indicates that the antibacterial constituents are more active in the leaf than in the callus. Further work is needed to isolate the active principle from the plant extracts and to carry out pharmaceutical studies. The secondary metabolites identified in the *Petunia* could be responsible for antimicrobial activity exhibited by this plant.

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