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Original article

Phytochemical and antimicrobial efficacy of *in vivo* and *in vitro* tissues of *Aegle marmelos* (L.) Corrêa

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Abstract

A study was conducted with an aim to test the antimicrobial potential of *in vivo* and *in vitro* derived callus of *Aegle marrmelos* (L.) Corrêa against 12 phytopathogens, 5 benefical microbes and 4 clinical human pathogens. Antimicrobial efficacy of the chloroform and methanol extract of *A. marmelos* roots tested by well diffusion test showed effective reduction of growth of test pathogens. Among the phytopathogens tested, growth of *Alternaria helianthi and Sclerotium rolfsi* were inhibited. The chloroform extract of calli of *A. marmelos* showed the maximum antimicrobial activity (94.45 %) with an MIC of 2.0 µl. In clinical pathogens, methanol extract of the root bark showed high inhibition of *E.coli* over other organisms. The callus derived extracts showed similar response, however, *Klebsiella pneumonia* responded better to the callus extracts. The efficacy of the extracts derived from callus indicates their functional role in the chemical defence against microbial invasion. The present study suggests the possibility of using *in vitro* cultures for production of medicinally important bioactive compounds.

Keywords: Callus, bioefficacy, clinical pathogens, phytopathogens, bael, antimicrobial

1. Introduction

The World Health Organization (WHO) estimates the demand for medicinal plants at approximately US $14 billion per year. This is likely to increase to more than US $5 trillion in 2050 (Kala *et al*., 2006). Export market for herbal medicines is growing faster than the Indian domestic market. There is a consistent demand for more than 500 species both in the domestic and international markets. To meet this demand, rampant harvest of raw material from the wild is on. It is mostly as a destructive harvest, resulting in reduction in the genetic stocks. So, there is an urgency to manage and conserve these medicinal plant resources.

Conservation can be through management of wild populations and natural habitats. It can be propagated through seeds and cuttings. However, it takes time for the species to reach the harvestable age before it can be utilized for its pharmacologically active compounds. Modern pharmacopoeia contains at least 25% drugs derived from plants and many others which are synthetic analogues built on prototype compounds isolated from plants (Manoharachary and Nagaraju, 2016). Application of biotechnological tools can help to biosynthesize the compounds in low quantities. Well-known examples are artemisinin, paclitaxel, podophyllotoxin and vinca-alkaloids.

Secondary metabolite production can be manipulated by varying composition of the culture medium. This includes the types and amounts of plant growth regulators, mineral salts and carbon sources used, and environmental conditions, such as temperature, light, and gas composition in culture. The principal advantage of this technology is that it provides continuous, reliable source of pharmaceuticals. It could also be used for large-scale culture of plant cells from which these metabolites can be extracted. Production of many valuable secondary metabolites, namely; chlorogenic acid, taxoid, catharanthine and ginsenoside have been reported (Wang *et al*., 2003).

In traditional medicines, a large number of polyherbal formulations are used in the treatment of various chronic disease conditions (Nayanabhirama, 2016). These formulations have an appropriate cure against many diseases like jaundice, asthma, arthritis, diabetes, *etc*., where there is no complete cure in allopathy (Biradar, 2015; Subramoniam, 2016). However, quality assessment of herbal formulations is of paramount importance to justify their acceptability in modern system of medicine (Pushpangadan *et al*., 2018).

Aegle marmelos (L.) Corrêa is a sacred plant which belongs to the family Rutaceae. It is used in indigenous systems of medicine in India, China, Burma and Srilanka (Sivakumar *et al*., 2006). The bark of the roots finds use in the preparation of more than 75 medicinal formulations. Increasing demand of Ayurvedic medicines warrants more of plant material for preparations. Unscientific harvesting has resulted in a decline in this species in the wild. To check erosion of genetic stocks, and to ensure a continuous supply of the crude secondary metabolites, *in vitro* culture technology would enable production of these compounds in the callus or medium.

This paper focuses on the evaluation of the antimicrobial efficacy of the callus-derived tissues of *A.marmelos*. Efficacy was tested against phytopathogens, harmful and beneficial microbes and human pathogens, both bacteria and fungi (Figure 1).

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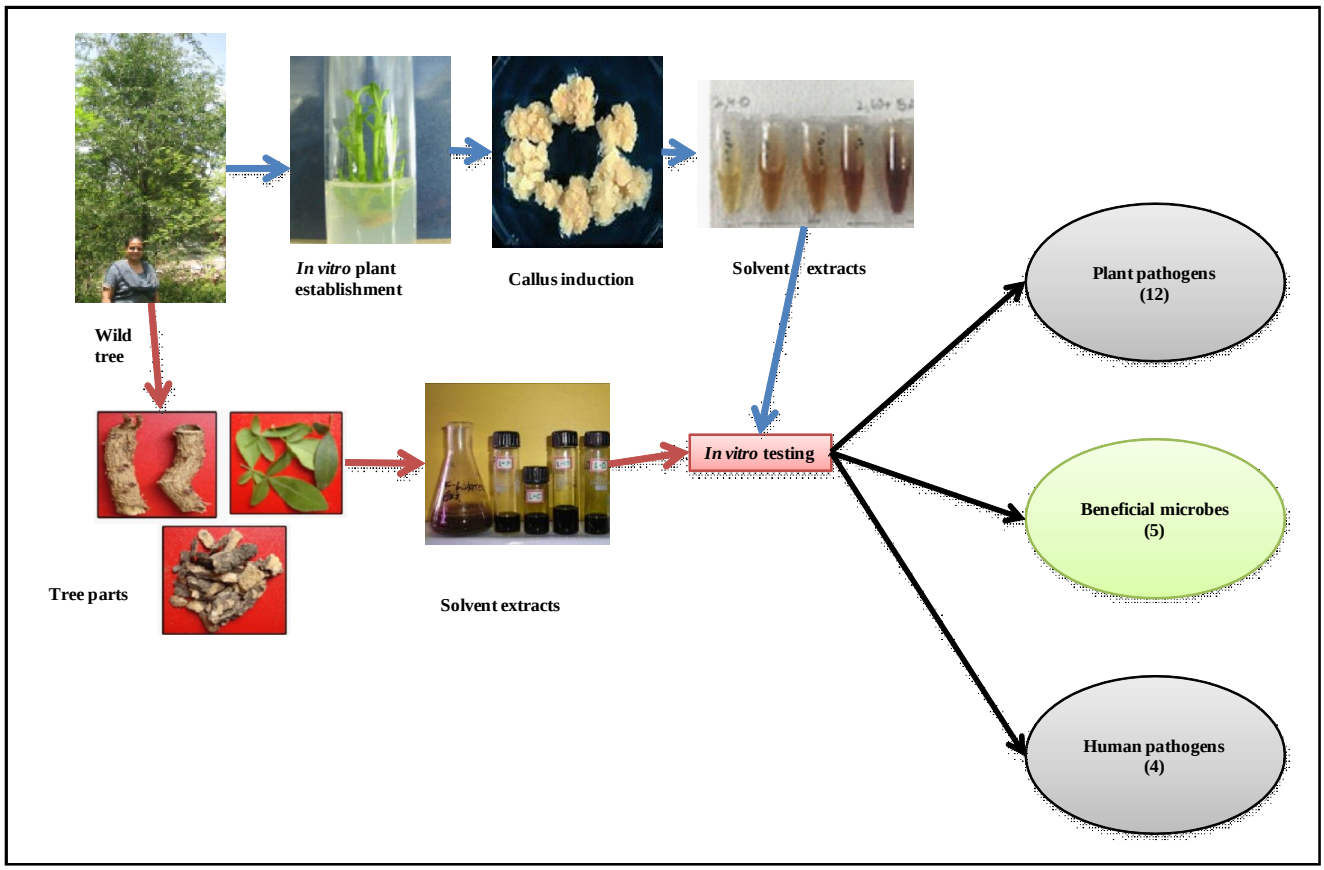

Figure 1: Flow chart illustrating the sequence of steps followed in the study.

2. Materials and Methods

2.1 Materials

Three types of organisms were selected for the study. They include phytopathogens, beneficial microorganisms and human pathogens. The test organisms were maintained on nutrient agar (Hi Media, India) at 4°C and sub-cultured before use. A schematic illustration of the work is presented in Figure 1.

2.2 Phytopathogens

Eleven fungal cultures used in this study were obtained from Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. The selected fungal pathogens, their major hosts and the diseases caused are given in the Table 1.

2.3 Beneficial microorganisms

Five beneficial microorganisms, commonly used for N_2 fixation, phosphorus solubilization and biocontrol in various crops were selected. Among these, one is a fungus and four are bacteria. The beneficial microorganisms chosen for the study and their major functions in plant ecosystem are given in the Table 2.

2.4 Human pathogens

Five human pathogens were tested at Microbiological Laboratory, Coimbatore, an NABL accredited laboratory, as it was considered unsafe carrying out the experiments with contagious organisms under general lab conditions (Table 3).

Table 1: Pathogenic fungi taken for study

S.No.	Plant pathogen used	Crop	Disease
1.	*Rhizoctonia solani*	Rice	Sheath blight
2.	*Macrophomina phaseolina*	Groundnut	Root rot
3.	*Fusarium oxysporum*	Cotton	Wilt
4.	*Scleorotium rolfsii*	Groundnut	Root rot
5.	*Alternaria solani*	Tomato	Early blight
6.	*Alternaria helianthi*	Sunflower	Leaf spot
7.	*Botridiplodia* spp.	Coconut	Bud rot
8.	*Colletotricum falcatum*	Sugarcane	Red rot
9.	*Trichosporium* spp.	Casuarina	Casuarina blister bark
10.	*Aspergillus niger*	Groundnut	Crown rot
11.	*Pyricularia oryzae*	Rice	Rice blast

Table 2: Beneficial microorganisms used in the study

S.No.	Organism	Major function
1.	*Trichoderma viride*	Antagonist
2.	*Azospirillum lipoferum*	Nitrogen fixer
3.	*Bacillus megaterium var. phosphaticum*	Phosphorus solubilizer
4.	*Gluconoacetobacter diazotrophicus*	Nitrogen fixer
5.	*Pseudomonas striata*	Phosphorus solubilizer
6.	*Bacillus subtilis*	Phosphorus solubilizer

Table 3: Human pathogens used against the extracts of *A. marmelos*

S.No.	Organism	Type	Infection caused
1.	*Klebsiella sp.*	Gram-negative	Pneumonia, urinary tract infections, septicemia, and soft tissue infections
2.	*Escherichia coli*	Gram-negative	Stomach disorders
3.	*Streptococcus pyogenes*	Gram-positive	Localised skin infections
4.	*Salmonella typhi*	Gram-negative	Typhoid/Enteric fever

2.5 Preparation of plant extracts (roots, leaves and stem bark extracts of *A. marmelos*)

Three different tissues of *A. marmelos* (leaves, stem bark and root bark) were collected and washed thoroughly with running tap water, followed by rinsing twice with distilled water. It was shade dried for a week and finely powdered and stored in a dark place for further use. About 30 g of each tissue was immersed in 150 ml of solvents with varying polarities, namely; chloroform, methanol, dichloromethane and petroleum ether separately (in the ratio 1:5) and kept overnight for digestion. The extract was filtered using Whatman No. 44 filter paper and concentrated using rotary vacuum evaporator to obtain dried extract, which was then stored under refrigerated condition.

Based on pilot studies, *S. rolfsii* and *A. helianthi* responded to methanol and chloroform extracts. These were selected for further studies.

2.6 Preparation of callus extracts

Different explants were tested under tissue culture conditions, using varying combinations of media and growth hormones. The combinations which produced the highest weight of callus was selected and used for the study. The processing of callus was similar to that of plant parts extracted.

2.7 *In vitro* testing of extracts against phytopathogens

To the PDA medium, 0.5, 1.0, 1.5, 2.0 per cent of chloroform and methanol extracts of roots and 1.5, 2.0, 2.5 per cent of chloroform and methanol extracts of callus obtained from roots was added. PDA without extracts served as control. A mycelial disc (9 mm) of test fungi taken from 3-5 days old culture was placed into each plate and incubated at 28°C (3 days for *S. rolfsii* and 5 days for *A. helianthi*). Clear zone of inhibition formed around wells were considered indicative of antimicrobial activity. The inhibitory activity was measured by calculating the area of inhibition zone.

The diameter of mycelial colony was measured after the incubation period and per cent inhibition was calculated using the formula (Vincent, 1927), Inhibition (%) = (C – T) / C × 100, where C and T were mycelial growth (cm) of test microbe in control and treatments, respectively.

The experiment was repeated thrice and the average values were calculated for antimicrobial activity.

2.8 Determination of minimum inhibitory concentration (MIC) for effective extracts

Minimum inhibitory concentration was taken as the lowest concentration of extract that completely inhibited the bacterial growth, indicated by the lack of visual turbidity. *Trichoderma viride* was used as the test microorganism.

2.9 Compatibility studies-effective plant and callus extracts versus beneficial microorganisms

One day old (24 h) culture of actively growing beneficial organism was streaked on the medium impregenated with specific plant extract and suitable concentration and respective controls (without plant extracts). The plates were incubated for 2 to 5 days (depending on the growth of organisms) and observed. The plates were scored – for no growth, + for very less growth, ++ for moderate growth and +++ for growth compared to control. MICs of plant extracts were used to fix the inhibitory concentrations. Growth pattern was studied with an intention to check whether they retain their original morphological characteristics.

2.10 Evaluation of the effect of plant extracts on the growth rate and growth pattern of human pathogens

All the test strains were maintained on nutrient agar slopes and were sub-cultured once in every two weeks. These bacteria served as test pathogens for antibacterial activity assay. Antibiogram was done by disc diffusion method using plant extracts. The test quantity of extracts was dissolved in distilled water since it did not affect the growth of microorganisms, in accordance with control experiments. The surfaces of media were inoculated with bacteria from a broth culture. High potency biodiscs were placed on the agar. After 24 h of incubation at 37°C, the plates were examined and the diameters of the inhibition zones were measured to the nearest millimeter.

Standard antibiotics of ampicillin-10 µ/disc, kanamycin-30 µ/disc, nalidixic acid-30 µ/disc, were used as positive controls. After 24 h of incubation, diameter of the inhibition zone was recorded in mm. Clear zone of inhibition formed around wells were considered indicative of antimicrobial activity. The experiment was repeated thrice and the average values were calculated for antibacterial activity.

2.11 Statistical analysis

All the data were analyzed by analysis of variance (ANOVA), followed by Duncan's Multiple Range Test to separate the treatment means at $p \leq 0.05$, using SPSS. Data are expressed as mean and variation as standard deviation (SD).

3. Results

3.1 Identification of effective extracts

Among the twelve plant extracts studied for antimicrobial activity, only *A. marmelos* roots showed antimicrobial activity against *S. rolfsii and A. helianthi*. The roots extracted in methanol, chloroform and dichloromethane showed inhibition of mycelial growth of *S. rolfsii and A. helianthi*. The other plant extracts exhibited either nil or slight antimicrobial activity. Extracts which exhibited antimicrobial activity were taken up for further studies.

3.1.1 *S. rolfsii*

The chloroform extract of *A. marmelos* roots showed maximum mycelial inhibition (100 per cent) (Figure 2). Chloroform extract of the callus also showed significant inhibition (93.4 per cent) of mycelial growth of *S. rolfsii* (Figure 3).

3.1.2 *A. helianthi*

Methanol extract of *A. marmelos* roots and callus checked mycelial growth of *A.helianthi* (Figures 4 and 5) (100 and 94.45 per cent, respectively).

3.2 Minimum inhibitory concentration (MIC) for pathogens

The effective root and callus extracts subjected to varying concentrations showed that MIC was 1.5 per cent in case of chloroform extract of *A. marmelos* roots against *S. rolfsii*, 2 per cent in case of methanol extract of roots against *A.helianthi*, 2.5 per cent in case of chloroform extract of root callus against *S. rolfsii* and 2.5 per cent in case of methanol extract of callus against *A. helianthi* (Table 4).

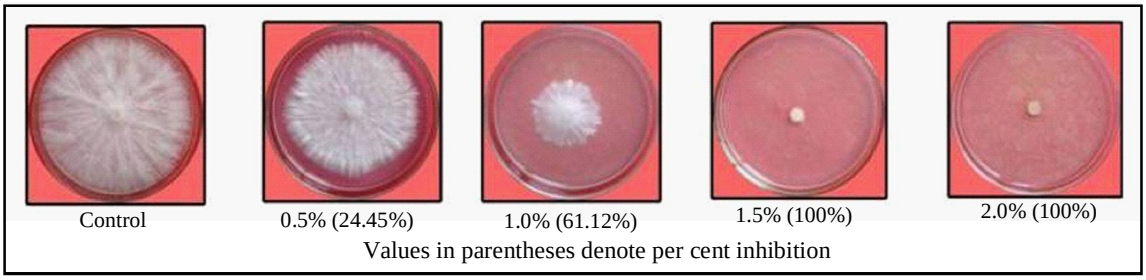

Figure 2: Effect of different concentrations of chloroform extract of *A. marmelos* root bark on *S. rolfsii*.

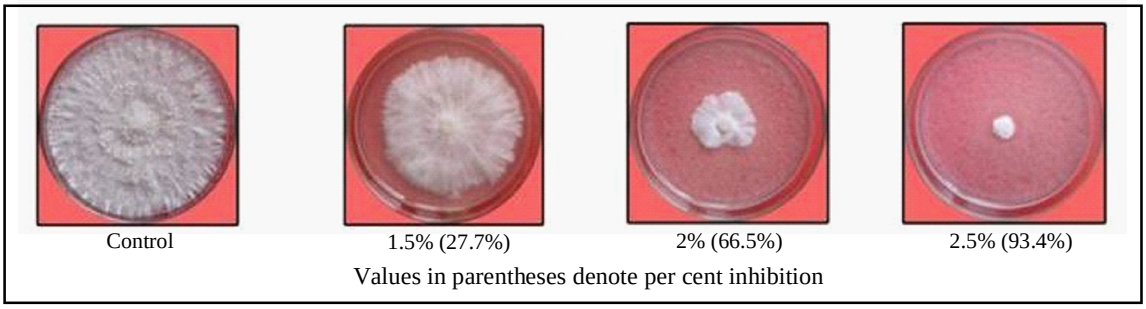

Figure 3: Effect of different concentrations of chloroform extract of *A. marmelos* callus on *S. rolfsii*.

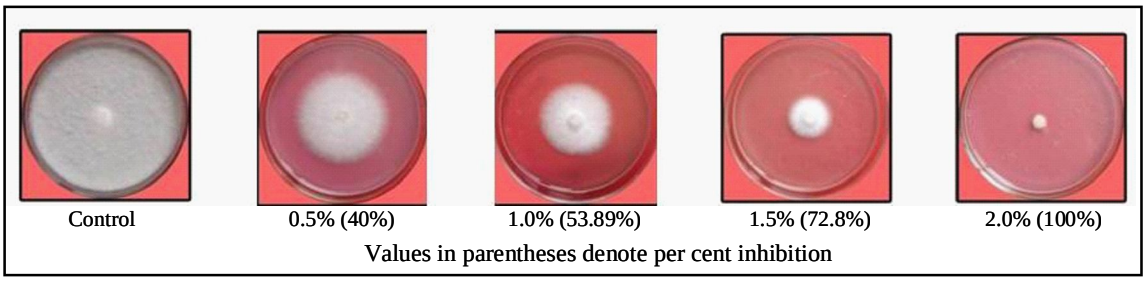

Figure 4: Effect of different concentrations of methanol extract of *A. marmelos* root bark on *A. helianthi*.

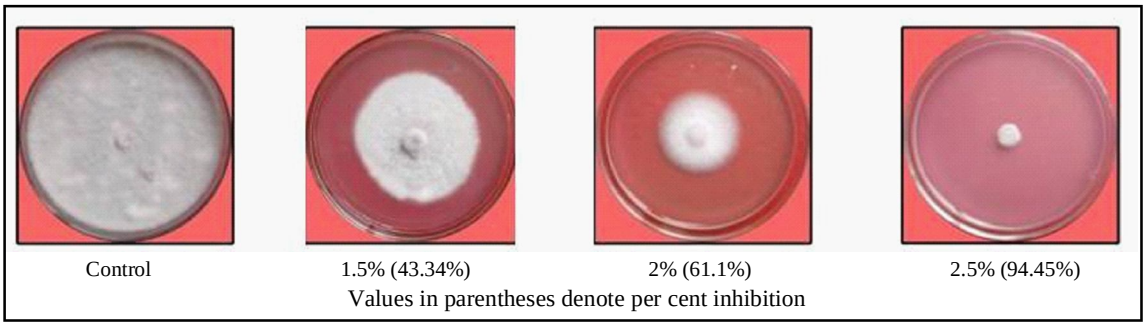

Figure 5: Effect of different concentrations of methanol extract of *A. marmelos* callus on *A. helianthi*.

3.3 Compatibility studies - extracts *versus* beneficial microflora

Both methanol and chloroform extracts of *A. marmelos* roots and callus were fully compatible with *A.lipoferum, B.megaterium* and *B.subtilis* and partially compatible with *G. diazotrophicus, P. striata* (Table 5). To evaluate the effect of plant extracts on the growth rate and growth pattern of beneficial organisms, colorimetric testing was done. No such deviation from normal growth was reported. All the colorimetric readings were found to be on par with each other. The plating tests conducted did not show any change in growth pattern of the tested organisms (Table 5).

3.4 Compatibility of *Trichoderma viride* with effective root and callus extracts

It was found that the methanol extracts of roots and callus was found to be fully compatible with *T. viride* and partially compatible with chloroform extracts of roots and callus (Table 6).

3.5 Evaluation of the effect of plant extracts on the inhibition pattern of human pathogens

3.5.1 *Escherichia coli*

Among all the effective extracts tested, methanol extract (2.5%) of root bark showed maximum inhibition zone of *E. coli* (12 mm). The methanol extract of the callus showed an inhibition zone of 8.0 mm for the same organism.

3.5.2 *Klebsiella pneumonia*

Low concentrations of the methanolic extract showed inhibitory effect on *K. pneumonia*. Callus extract was observed to mimic the action similar to 2.0% extract.

3.5.3 *Salmonella typhi*

Among all the effective extracts tested, methanol extract of callus did not show a similar response to its inhibition.

Table 4: Testing of different concentrations of effective root and callus extracts of *A.marmelos* on pathogenic microbes (n=3)

Conc.(%)	Particulars	AMR/ Chloroform/ SR	AMC/ Chloroform/ SR	AMR/ Methanol/AH	AMC/ Methanol/AH
0.0	Colony dia. (mm)	9.00	9.00	9.00	9.00
	% inhibition	0.00	0.00	0.00	0.00
0.5	Colony dia.(mm)	6.80	-	5.40	-
	% inhibition	24.45	-	40.00	-
1.0	Colony dia. mm	3.50	-	4.15	-
	% inhibition	61.12	-	53.89	-
1.5	Colony dia. mm	0.00	6.35	2.45	5.10
	% inhibition	100.00	27.70	72.80	43.34
2.0	Colony dia.(mm)	0.00	3.10	0.00	3.41
	% inhibition	100.00	66.50	100.00	61.10
2.5	Colony dia.(mm)	-	0.60	-	0.50
	% inhibition	-	93.40	-	94.45
	SE_d	**0.12**	**0.12**	**0.11**	**0.11**
	CD	**0.26**	**0.29**	**0.25**	**0.26**

*AMR : *A. marmelos* roots; SR : *S.rolfsii;* AH : *A.helianthi* *AMC : *Aegle marmelos* callus

Table 5: Compatibility studies and growth rate of beneficial bacteria with effective root extracts* (n=3)

Name of the organism	AMR/C	AMC/C	AMR/M	AMC/M	Control	SE_d	CD(0.05)
Azospirillum spp	+++(0.14)	+++(0.14)	+++(0.12)	++(0.12)	+++(0.15)	0.01	0.02
Gluconoacetobacter	+++(0.36)	+++(0.36)	+++(0.38)	++(0.39)	+++(0.39)	0.004	0.011
Bacillus megaterium var. phosphaticum	+++(0.58)	+++(0.58)	+++(0.56)	+++(0.56)	+++(0.58)	0.015	0.035
Pseudomonas striata	+++(0.50)	+++(0.50)	+++(0.53)	+++(0.54)	+++(0.55)	0.015	0.031
Bacillus subtilis	++(0.44)	++(0.44)	++(0.45)	++(0.45)	+++(0.45)	0.018	0.039

++Less than control;+++ Equivalent to control. *AMR : *A.marmelos* roots; *AMC : *A.marmelos;* C : Chloroform; M : Methanol. * MICs of effective root extracts taken for study

Table 6: Compatibility testing of effective plant extracts* with *Trichoderma viride*(n = 3)

Plant extract	Concentration (%)	Colony diameter (mm)	Per cent inhibition
Callus/chloroform	2.0	8.15	9.45
Callus/methanol	2.5	8.65	3.89
Root/methanol	2.0	8.35	7.23
Root/chloroform	1.5	7.90	12.23
Control	0.0	9.00	0.00

SE_d 0.15, CD (0.05) 0.38 * MICs of effective root extracts taken for study.

Table 7: Testing of different concentrations of effective root and callus extracts* of *A. marmelos* on human pathogens (n=3)

Extracts	Inhibition zone (mm)			
	E. coli	*K. pneumonia*	*S. typhi*	*S. pyogenes*
1.5 % Root Bark Methanol	6 ± 0.1	11 ± 0.1	11 ± 0.1	6 ± 0.05
2.0 % Root Bark Methanol	6 ± 0.1	10 ± 0.1	11 ± 0.1	10 ± 0.1
2.5 % Root Bark Methanol	12 ± 0.15	7 ± 0.05	10 ± 0.1	7 ± 0.05
2.0 % Callus - Methanol	8 ± 0.05	9 ± 0.1	6 ± 0.05	7 ± 0.05
Control#	28 ± 0.05(K)	20 ± 0.05(A)	20 ± 0.05(Na)	18 ± 0.05(A)

SE_d 0.10, CD (0.05) 0.27; * MICs of effective root extracts taken for study;
#A: Ampillicin; K:Kanamycin; Na:Naldixic acid

3.5.4 *Streptococcus pyogenes*

The methanol extract of the callus showed the same result as the 2.5% methanolic extract of the root bark. Both showed an inhibition zone of 7.0 mm. The results have been presented in Table 7.

4. Discussion

Synthesis of secondary metabolites on a commercial scale is not economically viable, because they often have complex quaternary structures essential for biological activity. Tree species being continuously exploited from the wild, these natural resources are becoming rare and even extinct. To overcome these bottlenecks, cell and tissue culture technology can be adopted for extraction of certain secondary metabolites (Rajendra and D' Souza, 2000). The cultured cells obtained from any part of the plant are expected to yield secondary metabolites similar to those of the plant grown *in vivo* under suitable culture conditions.

The capacity for plant cells to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been well recognized. The rising demand for natural products has shifted focus on *in vitro* plants as potential factories for secondary phytochemical products (Karuppusamy, 2009).

Many workers have demonstrated the capability of *in vitro* induced callus to produce phytochemicals similar to the parent plant. Srividya *et al.* (1998) reported that the production of azadirachtin and nimbin was higher in cultured shoots and roots of *Azadirachta indica*, compared to field-grown plants. Examples of production of secondary metabolites from tree species include azadirachtin from *Azadirachta indica* (Sujanya *et al.*, 2008*);* sennosides from *Cassia senna* (Shrivastava *et al.*, 2006); podophyllotoxin from *Podophyllum hexadrum* (Chattopadhyay *et al.*, 2002).

Studies hypothesize *A.marmelos* to have a broad spectrum antibacterial activity (Saradha Jyoti and Subba Rao, 2010). In the present study, about 90 per cent inhibition of mycelial growth of *S. rolfsii* and *A. helianthi* was shown by 1.5 per cent chloroform extract of *A. marmelos* roots and 2.0 per cent methanol extract of *A. marmelos* roots, respectively. A 2.5 per cent of chloroform / methanol extract of *A. marmelos* callus could inhibit the growth of *S. rolfsii* and *A. helianthi*, respectively. These results indicate that the callus contains some potent antimicrobial principles similar to the wild plants.

The study also tested the extracts against beneficial microbes used as biofertilizers and antagonists. Elslahi *et al.* (2014) reported that fungicides could be applied without restriction when using inocula based on growth promoting bacteria such as symbiotic nitrogen fixers (*Rhizobium meliloti*), non-symbiotic nitrogen fixers (*Azospirillum braziliense*) or potassium solibilizers (*Bacillus circulans*). Plant leaf extracts had little inhibitory effect on the mycelia growth of *Trichoderma harzianum* (Sharma and Chandel, 2016).

Several studies have revealed the antibacterial activity of *A. marmelos* (Dey and De, 2012; Gautam *et al.*, 2013; Prasannabalaji *et al.*, 2012). Aqueous and ethanol extracts of *A. marmelos* have been postulated to have strong inhibitory effect on the growth of some bacteria causing common human diseases including *S. aureus*, *P. aeruginosa* and *E. coli* (Chattopadhyay *et al.*, 2009). Maity *et al.* (2009) have suggested *A. marmelos* to have therapeutic potential for developing novel antimicrobials. Antimicrobial potential of *A.marmelos* root extracts against *E. coli, B. subtilis, P. aeruginosa, S. aureus, A. brasiliensis* and *C. albicans* revealed high MIC, indicating the presence of wide range of phytochemicals like flavanoids, alkaloids, phenolic contents (Dahiya *et al.*, 2016). Roots of *A.marmelos* contain coumarins such as scoparone, scopoletin,

umbelliferone, marmesin and skimmianine, alkaloids, halopine and terpenes responsible for protective functions (Sharma and Dubey, 2016).

Sharma and Joshi (2011) reported that the active antioxidant compounds of *A. marmelos* are better extracted in methanol. Chloroform extract of the leaves of *A. marmelos* has been reported to have a higher activity against *Proteus mirabilis* and *K. pneumoniae*, whereas the methanol extracts have a better action against *S. typhi* (Kothari *et al.*, 2011). The aqueous and ethanol extracts of *A. marmelos* have been shown to have significant antimicrobial activity against ten species of multidrug resistant strains of enteropathogenic bacteria (Rath and Padhy, 2012). Volatile oil obtained from the leaves of plant has been documented to have potent antifungal activity against various strains of fungi (Rana *et al.*, 1997).

Methanol extract (2.5%) of root bark and callus showed an inhibition of *E.coli* and *S.pyogenes* in the present study. The demonstration of inhibitory activity against both gram-positive and gram-negative bacteria is an indication of broad spectrum activity of these plant extracts which can be explored for drug development. Higher concentrations of callus extract inhibited *K. pneumonia*. *S. typhi*, though responded to wild plant extracts, was not inhibited by callus extracts. Petroleum ether extract obtained from callus culture of *A. marmelos* has been documented to have an inhibitory effect on the growth of *S. typhi* under *in vitro* conditions (Thangavel *et al.*, 2008).

The efficacy of the metabolites produced under *in vitro* conditions have been tested. Ethanol extract of *in vitro* regenerated plantlets of *Orthosiphon stamineus*, showed strong scavenging activity with per cent inhibition higher than the wild plant extracts (Sheena and Jothi, 2015).

In vitro derived calli and calli mediated shootlet extract demonstrated significant antibacterial activity in *Balisospermum montanum* and *Alternanthera sessilis* (Johnson *et al.*, 2005; 2010).

5. Conclusion

Based on the present study, it could be concluded that the extracts from *A. marmelos* roots possess strong fungitoxic and antibacterial properties. The ability of the plant extracts of *A. marmelos* to inhibit growth of bacteria and fungi is an indication of its broad spectrum antimicrobial activity which could be a potential source for development of novel antimicrobial agents (Meena *et al.*, 2016). The callus extracts from *A. marmelos* possess strong biocidal properties as is evident from the antifungal and antibacterial activities presented in the study. These activities mimicked similar activities observed in the wild plants. The resultant extracts, thus obtained could replace wild plant parts, thereby enabling us to achieving our aim of conservation of the genetic resources. Further, studies are needed to test thermostability, stability to storage and phytotoxicity.

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Conflict of interest

The authors declare that no conflict of interest exists in the course of conducting this research. All authors had final decision regarding the manuscript and the decision to submit the findings for publication.

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