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Research Article

COMPARISON OF ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL CONSTITUENTS OF *IN VIVO* AND *IN VITRO* GROWN *AMARANTHUS SPINOSUS* PLANTS

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ABSTRACT

Objectives: The antimicrobial activity of the extracts of *Amaranthus spinosus* were compared using *invitro* and *in vivo* systems.

Materials: The comparison was performed against five microbial strains including *Escherichia coli*, *Staphylococcus epidermidis*, *Staphylococcus aureus, Streptococcus mutans* and *Bacillus subtilis* using the agar well diffusion method. Acetone, benzene, methanol and cow urine were used as solvents for preparing the plant extracts.

Results: The highest callus induction (83.3±0.90) from leaf explants of *A.spinosus* was obtained in MS medium supplemented with 0.2 mg/l BA and 0.5 mg/l NAA while best direct adventitious shoot regeneration (86.6 ±0.49) from nodal explant was obtained on MS medium supplemented with 0.5 mg/l BA and 0.2 mg/l NAA. The best shoot regeneration and elongation MS medium was found to be supplemented with 1mg/l BA and 0.5 mg/l IAA. The rooting was obtained from elongated shoots was obtained in half MS medium containing 0.2mg/l IBA.

Conclusions: No significant difference was observed in antimicrobial activity of the plants regenerated through *in vitro* and *in vivo* grown plants. The antimicrobial activity and phytochemical constituents of plants regenerated through direct shoot regeneration from nodal explants and *in vivo* grown plants were also found comparable. The developed protocol for *in vitro* propagation of *A.spinosus* would provide quality plant material for medicine.

Keywords: *Amaranthus spinosus* L., Antimicrobial activity, Micropropagation, Callus, Direct shoot regeneration, Phytochemical constituents.

INTRODUCTION

Medicinal plants are the most important source of life saving drugs for majority of world population. In Indian subcontinent, plant oriented drugs have been used extensively from a very long time. According to a survey conducted by WHO, traditional healers treat 65% patients in Sri lanka, 60% in Indonesia, 75% in Nepal, 85% in Mayanmar, 60% in Pakistan, and 90% in Bangladesh. In India, 80% of the population especially in villages is getting health care by traditional practitioners (Hakims) who prescribe herbal preparations [1][2].Some plant based biological compounds isolated from herbs hence been explored for the growth inhibition of pathogenic microbes due to their antimicrobial potential [3].

Amaranthus spinosus is an annual herb which is widely distributed in tropical &sub-tropical regions of the world[4]. The various parts of plants are known to possess various pharmacological properties [5]. Extracts & leafs are used in treatment of menstrual disorders in women [6]. An infusion of powdered seeds of the plant is used for stomach problems and to alleviate labour pain in the pregnant women in Nepal [7]. Further it is used to treat several ailments like gonorrhea, inflammatory swelling, malaria, diabetes, leprosy [5][8]. Since global inclination towards herbal medicine is increasing, there is a large obligatory demand for a huge raw material of medicinal plants and also of right stage when the acute principles are available in optimum quantities at the requisite time for standardising of herbal preparations which is responsible when the medicinal plants are cloned through an *in vitro* strategy i.e micro propagation [9]. Micro propagation ensures not only continuous supply of plant throughout year but also prevent natural population and medicinal plants [10][11].

The selection of crude plant extract for screening programs has the potential of being more successful of initial steps than the screening of pure compounds isolated from natural products [12]. The available literature on the medicinal properties of the plant pertains mainly to the field grown plants. In our study the plants are successfully raised under *in vitro* tissue culture to study the antimicrobial activity of*Amaranthus spinosus* . The main aim of the present study was to standardize the procedure for proliferation, maintenance of *in vitro* grown plants, antimicrobial activity of *in vivo* and *in vitro* grown plants and their preliminary photochemical screening.

MATERIALS AND METHODS

Micropropagation

The fresh leaves of plant from nodal segments were removed and kept under running tap water for about 30 minutes to remove soil particles and then were rinsed with liquid detergent (1% Tween 20) (v/v)) for 10 minutes. Prior to inoculation, these explants were subsequently surface sterilized in the laminar air flow chamber with 0.5% sodium hypochlorite for 15-20 minutes followed by repeated rinsing with sterile distilled water.

Callus Induction and shoot regeneration

To achieve callus induction, leaf explants were cultured in 100ml flasks on MS [13] medium supplement with vitamins, 30 g l^{-1}(w/v)sucrose, 8 g l^{-1} (w/v) difcobacta agar (Lobachemie, Mumbai, India) and different combinations of BA and NAA. This medium was named as callus induction medium (CIM). The pH of medium was adjusted with 1N HCL and/or 1N NaOH to 5.8. The medium was autoclaved at 1.1 lbs/inch^{2}for 15 min at 121°C. The cultures were maintained at 24 ± 2°C with a 16-h photoperiod (50-60 µmol m^{-2} s^{-1}) provided by white, cool fluorescent lamps (40 wt each, phillips, India). After 4 week on CIM, the calli were sub cultured on the same medium for proliferation. The callus pieces (0.8-1 cm^2) were transferred to shoot generation medium containing MS medium supplemented with various concentration of plant growth regulators (PGRs) alone or with combination like BA andIndole-3- acetic acid (IAA). A total 20 concentration and combination of PGRs were used, although only the treatment that produced shoot ae represented here in table 2. The shoot elongated and multiplied on MS medium supplemented with the same medium used for shoot regeneration.

Direct Shoot regeneration

For direct shoot regeneration (DSR) the nodal explants were cultured on MS medium supplemented with supplemented with various concentration of plant growth regulators (PGRs) alone or with combination of BA and NAA. 30 g l^{-1} sucrose and solidified with 8 g l^{-1} agar (Difcobacto, LobaChemie, Mumbai, India). The pH of the

medium was adjusted to 5.8 with 1 N HCl and/or 1 NNaOH before autoclaving at 1.1lbs/inch^2 for 15 minutes. The shoots were transferred after two weeks to shoot elongation medium.

***In vitro* rooting**

In vitro elongated shoots (4-5 cm) were taken out from the culture vials and transferred to half strength MS medium with different concentration of IBA for root induction. The plantlets were taken out from culture vessel without damaging the delicate root system and rinsed with distilled water to remove adhering agar and thentransferred to plastic cups containing potting mixture (sterile sand, soil and FYM (1:1:1, v/v). Plastic cups were covered with plastic bag with 4-5 holes to maintain high humidity and to be kept in culture chamber. They were gradually exposed from artificial environmental conditions to natural conditions for their acclimatization. Four weeks old plants (leaves & stem) were used for antimicrobial activity. After four weeks, the observations were recorded for average number of roots/shoot and average root length.

Determination of antimicrobial activity

Procuring of bacterial strain

Pure cultures of test organisms were procured from IMTECH, Chandigarh, India. The bacterial and fungal strains used in the study were *Staphylococcus aureus* (ATCC96), *Staphylococcus epidermidis*(ATCC435), *Streptococcus mutans* (ATCC890), *E.coli*(ATCC483) and *Bacillus subtilis* (MTCC121). Purity of cultures was maintained on nutrient Agar(Hi media) by periodical transfers.

Determination of antimicrobial activity by agar well diffusion method

The leaves of *in vivo* and *in vitro* grown *Amaranthus spinosus* were air dried, powdered and macerated with organic solvents viz. acetone, benzene, methanol, cow urine (Sigma Aldrich ltd) for 3-7 days at room temperature. Filtration of soaked material was done by using what man filter paper. The aqueous part of the crude plant extract was dried by using a freeze-drier.

Petri plates containing 20ml of nutrient agarwere inoculated with 100µl of diluted bacterial and fungal cultures by the spread plate technique and were allowed to dry in a sterile chamber. A well of about 7.0mm was aseptically punctured with sterile cock borer. A 150µl of the extract (200mg/ml) were loaded into the wells and were allowed to dry completely. Methanol was used as a negative control whereas streptomycin was used as positive control. Plates were incubated at 37ºc for 24hrs. The antibacterial activity was assessed by measuring the zone of inhibition.

Phytochemical Analysis of Extract

The solvent extracts of different plants were subjected to preliminary phytochemical screening to identify the chemical constituents[14].

RESULTS AND DISCUSSION

In present investigation, *invitro* micro propagation of *A. spinosus*was achieved by direct and indirect shoot regeneration method (Fig 1). Highest callus induction (83.3±0.2724) was achieved from leaf explants inoculated on MS medium supplemented with 0.2 mg/l BA and 0.5 mg/l NAA after 4 weeks of inoculation. Further increase in growth hormone concentration decreased the percent callus induction (Table 1). Similar reports were also reported in other plant species such as *Phyllanthusuri naria*[15].

Table 1: Effect of growth regulators on percentage callus induction from leaf explants of *Amaranthus spinosus L.* Data (Mean ± S.E.) were recorded after four weeks.

Medium	PGR (BA+ NAA) mgl^{-1}	Percent callus induction
CIM1	0.0+0.0	0
CIM2	0.2+0.1	16.6±0.24
CIM3	0.2+0.2	41.6±0.33
CIM4	0.2+0.5	83.3±0.90
CIM5	0.5+0.1	58.3±0.33
CIM6	0.5+0.2	75.6±0.56
CIM7	0.5+0.5	33.3±0.12
CIM8	1.0+0.1	16.6±0.98
CIM9	1.0+0.2	16.6±0.19
CIM10	1.0+0.5	5.7±0.11

The fully developed callus was transferred on shoot regeneration medium. Shoot induction from callus, multiplication of shoots and effect of different combinations of BA and IAA on shoot regeneration has been summarised in table 2. Maximum number of shoots (8.66± 0.32) was obtained on MS medium supplemented with 1.0 mg/l BA and 0.5 mg/l IAA (Fig 1). Combination of BAP and IAA has been reported to be better for shooting and shoot elongation in other plant species also [16]. Highest shoot induction percentage and elongation of shoots was also obtained in the same medium composition. Maximum number of shoots, highest shoot regeneration percentage and elongation of shoots was also achieved in the same medium in chrysanthemum by [17].With the decrease and increase in hormone concentration, the percent shoot regeneration was found to decrease.

Direct regeneration of adventitious shoots on the explant provides a fast and dependable method for the production of large quantities of uniform plantlets in a short time. Direct shoot regeneration without any intervening callus phase apprehends any chance of somaclonal variation. Thus ensuring genetic fidelity[18]. Direct regeneration of shoots from nodal segments of *A. spinosus* was achieved on MS medium supplemented with 0.5 mg/l BA and 0.1 mg/l NAA (Table 3). The results are in accordance with the result of[19].The shoots regenerated were multiplied and elongated in the same media as described above(Fig1 C).

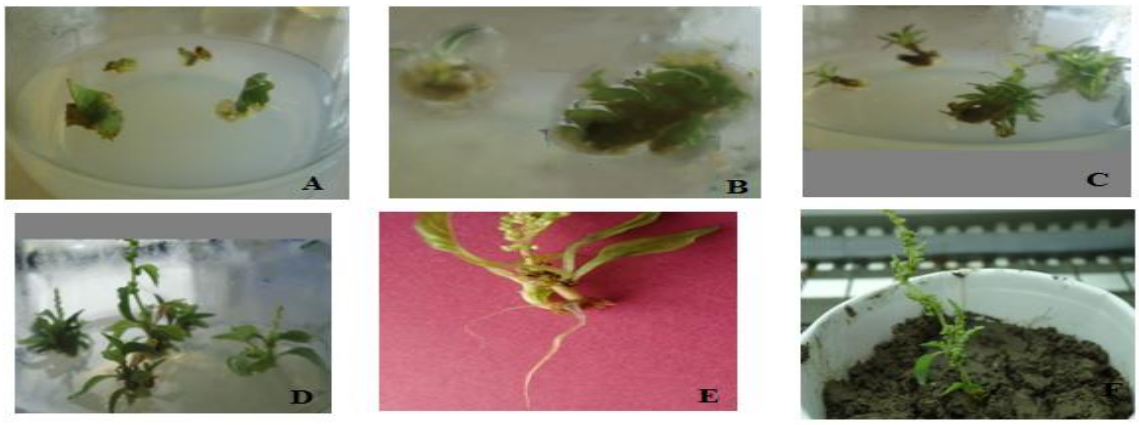

Fig. 1: Plant regeneration studies in *Amaranthus spinosus* : (A). callus induction from leaf explants after 4 weeks(B). Shoot regeneration from callus after 4 weeks (C). Direct adeventitious shoot regeneration from nodal explant after 6 weeks (D). Shoot multiplication and elongation (E).Rooting in elongated shoots after 4 eeek.(E). *invitro* plantlets transferred to the garden pot containing FYM.

Table 2: Effect of growth regulators on shoot regeneration and number of shoots per callus from leaf explants of *Amaranthus spinosus* .Data(Mean ± S.E.) were recorded after four weeks.

Medium	BA+IAA	Percent shoot regeneration	No. of shoots/ explant	Length (cm)
SRM1	0.5+0.25	53.3±0.27	2.56±0.16	1.16±0.30
SRM2	0.5+0.5	73.3±0.34	3.93±0.26	4.49±0.18
SRM3	0.5+1.0	86.6±0.56	6.98±0.78	7.76±0.28
SRM4	1.0+0.25	66.6±0.78	5.76±0.09	6.96±0.03
SRM5	1.0+0.5	93.3±0.11	8.66±0.32	7.54±0.16
SRM 6	1.0+1.0	54.6±0.39	5.15±0.98	5.54±0.23
SRM7	2.0+0.25	46.6±0.54	4.09±1.09	3.13±0.33
SRM8	2.0+0.5	33.3±0.19	3.32±0.21	2.56±0.57
SRM9	2.0+1.0	26.6±0.21	1.19±0.19	0.96±0.69

Table 3: Effect of different growth regulators on direct shoot regeneration from nodal segment of *Amaranthus spinosus*

Medium	BA+ NAA	Percent shoot regeneration	Shoot length (cm)
DRM1	0.0+0.0	0	0
DRM2	0.2+0.1	20±0.47	2.99±0.23
DRM3	0.2+0.2	40±0.69	4.64±0.49
DRM4	0.5+0.5	66±0.71	5.34±0.73
DRM5	0.5+0.2	86.6±0.49	5.96±0.38
DRM6	0.5+0.1	93.3±0.27	6.78±0.21
DRM7	1.0+0.1	33.3± 0.72	3.34±0.64
DRM8	1.0+0.2	40±0.41	3.96±0.43
DRM9	1.0+0.5	13.3±0.74	1.78±0.29

Root formation was induced in *in vitro* regenerated shoots by culturing them on half strength MS media with different concentrations of IBA (Table 4). Highest root induction (66.6±0.57) was achieved with 0.2 mg/l IBA (Fig 1 D). The roots observed were smooth white, healthy. Maximum root length (5.67±0.35) was observed in 0.5 mg/l IBA. These findings are in agreement with those observed in other plant species [20][21]. The *in vitro* derived plants acclimatize better under *in vivo* condition when they were transferred on polycups containing FYM mixture at 1:1:1 ratio as potting mix and moistened uniformly when covered with plastic bag was good enough to maintain required humidity. After periodic intervals, by taking special care without damaging the roots, plants were further transferred to soil for establishment. Nearly 60 % of the regenerated plantlets could tolerate and survive under field conditions (Fig1E). A number of plantlets were lost due to damping off & necrosis during acclimatization in field condition and damage to stem during acclimatization.

Table 4: Effect of growth regulators on percent root regeneration and number of roots per shoot of *Amaranthus spinosus* . Data (Mean ± S.E.) were recorded after four weeks.

RIM	IBA	Percent root induction	Root length (cm)
RIM1	0.0	0	0
RIM2	0.05	0	0
RIM3	0.1	33.3± 0.23	1.33±0.09
RIM4	0.2	66.6± 0.45	5.67±0.35
RIM5	0.5	33.3± 0.56	4.02±0.77
RIM6	1.0	33.3± 0.86	3.41±0.43
RIM7	2.0	0	0

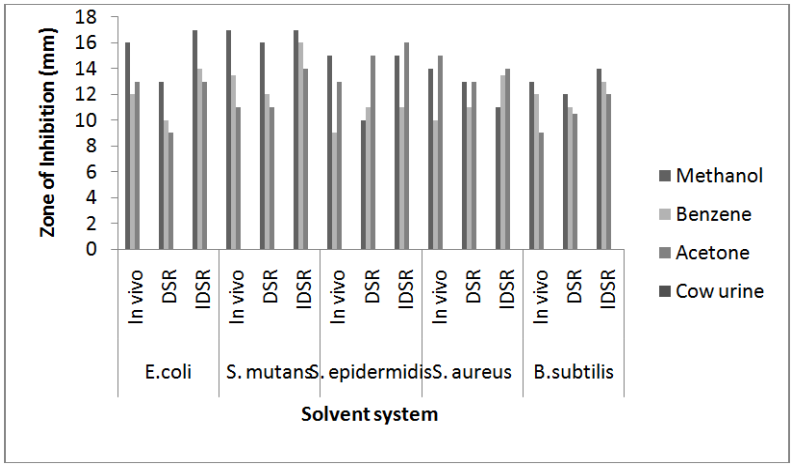

Fig. 2: The antimicrobial activity of *in vivo* and *in vitro* grown plants of *A. spinosus*

In vivo : Plants grown under *in vivo* conditions.

DSR: Plants grown from direct adventitious shoot regeneration

IDSR: Plants grown through callus.

The crude leaf extracts of *Amaranthus spinosus* in different solvent system proved to be very effective antimicrobial agent. The methanolic extract was the most effective against all the bacteria and fungus. The activity was similar to antibiotic tested. Among the bacterial species maximum inhibition was found in *S. aureus*, followed by *Streptococcus mutans, Staphylococcus epidermidis, B. Subtilis* and *E. coli* (Table 3). Maximum inhibition on comparative scale was found in *S. aureus* , *S. mutans* , *B. subtilis* followed by *E. coli* on the basis of the zone of inhibition. This is in accordance to the results of [22]. The activity of the extract were found to be comparable with 1% concentration of antimicrobial used (streptomycin).

When the growth of the micro-organisms against the solvents (without leaf extract of *Amaranthus spinosus*) was evaluated, no marked inhibition of growth was observed. Our two of the four solvent extracts (acetone and methanol extracts) presented significant antimicrobial activity against almost all the strains through agar well diffusion technique. In the present study, the methanol and acetone leaf extracts of *Amaranthus spinosus L.* exhibited maximum antibacterial activity against *E. coli and Staphylococcus aureus* with 16 mm and 14.5 mm zone of inhibition respectively. This is in conformity with the results of [23] who found maximum antimicrobial activity in 10 Indian weeds including *Amaranthus spinosus* .The cow urine extract was not found effective against all the microorganisms tested. However antimicrobial activity of cow urine has been reported[24].

The results (Fig 2) showed that *Amaranthus spinosus* leaves which were produced through the *in vitro* system had no increased ability with those which were applied from the *in vivo* system to act as an antibacterial agent. [25]found antimicrobial activity of *in vivo* and *in vitro* grown plants comparable. In general, most of the extracts evaluated for antimicrobial activity were active against microbial strains which were employed for the test. However, the antimicrobial activity of plants grown through callus mediated phase was more than that regenerated directly from nodal explants.

Phytochemical screening of the leaf and stem extracts of plants under study revealed the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins, saponins, phenol and reducing sugars (Table 3). These compounds have significant application against human pathogens, including those that cause enteric infections and are reported to have curative properties against several pathogens and therefore could suggest their use in the treatment of various diseases[26]. Methanol extract of all plants showed the presence of flavonoids and tannins while saponins and phenols were absent. This is in confirmation with [27]who reported better phytochemical extraction in methanol. Tannins have been found to form irreversible complexes with proline rich protein resulting in the inhibition of cell protein synthesis[28]. Tannins are known to react with proteins to provide the typical tanning effect which is important for the treatment of inflamed or ulcerated tissues[29]. Herbs that have tannins as their main components are astringent in nature and are used for treating intestinal disorders such as diarrhoea and dysentery[30].

Another secondary metabolite compound observed in the stem and leaf extracts of plants was alkaloid. One of the most common biological properties of alkaloids is their toxicity against cells of foreign organisms. These activities have been widely studied for their potential use in the elimination and reduction of human cancer cell lines[31]. Alkaloids which are one of the largest groups of phytochemicals in plants have amazing effects on humans and this has led to the development of powerful pain killer medications [32].

Apart from tannin and alkaloids compounds, other secondary metabolite constituents of all the plants included flavonoids, glycosides, carbohydrates, steroids and aldehyde. Flavonoids are potent water-soluble, antioxidants and free radical scavengers which prevent oxidative cell damage[33]. Acetone and benzene extracts showed presence of only few phytochemicals while cow urine extracts failed to show presence of any phytochemical constituent. However antimicrobial activity and presence of phytochemical constituents in cow urine extracts have been reported by many researchers[24][34] .

Table 5: Phytochemical analysis of *in vivo* and *in vitro* grown plants of *A. spinosus*

Phytochemical constituents	Solvent systems											
	Acetone			Benzene			Methanol			Cow urine		
	In vivo	DSR	IDSR	*In vivo*	DSR	IDSR	*In vivo*	DSR	IDSR	*In vivo*	DSR	IDSR
Aldehyde	_	_	+	_	+	_	+	_	+	_	_	+
Alkaloids	_	+	+	+	_	_	+	+	_	_	_	_
Carbohydrates	+	_	_	+	+	+	_	+	+	_	+	_
Flavonoids	+	+	+	_	+	+	+	_	+	_	_	+
Glycosides	+	_	_	_	+	_	+	+	+	_	_	_
Phenol	_	+	+	_	+	+	+	+	+	_	+	+
Steroids	+	_	_	+	_	_	+	+	_	_	_	_
Terpenoids	_	+	+	+	+	_	+	_	+	_	_	_
Tannins	+	+	+	+	_	+	+	+	+	_	_	_

The inhibitory activity of plant extracts generally depends upon the concentration, type of parts used and microbes tested. The accumulation and concentration of secondary metabolites which are responsible for inhibitory activity is varied according the plant parts. It is quite possible that some of the plants extracts that were ineffective in this study were just not in sufficient concentrations so as to be effective. It is also possible that the active chemical constituents were not soluble in used solvents. It may be a reason for the variation in the inhibitory activity of extracts of *A. spinosus*.

CONCLUSION

The present study presents the protocol for direct and indirect regeneration of *A. spinosus* plants. The comparison between the antimicrobial activity and phytochemical constituents of *A. Spinosus* shows that there is no significant difference between *in vitro* and *in vivo* grown plants. The plants grown from callus were found to have higher antimicrobial activity than those grown from direct adventitious shoot regeneration from nodal explant. The advances in plant tissue culture will be helpful in improving the quality and quantity of raw material of *A.spinosus* and thereby will be helpful in improving the drug quality.

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