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ASSESSMENT OF ANTIBACTERIAL ACTIVITY OF *IN VITRO* AND *IN VIVO* GROWN GARLIC (*ALLIUM SATIVUM* L.)

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Abstract

Antibacterial activities of *In vitro* and *In vivo* grown garlic were compared against five bacterial strains *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter aerogenes* and *Staphylococcus aureus*. In 8-9 weeks, *In vitro* garlic bulblets were produced from shoot tip explants callus of garlic cloves cultured on modified MS medium. Ten µM BA induced somatic embryogenesis in soft, granular and dirty white to yellow colored clumps of calli, regenerated into plantlets, which eventually transformed into 9 or 10 mm dia bulblets on basal MS medium. Clear zones of inhibition were demarked by paper disc diffusion method. The content of micro-bulblets expressed greater antimicrobial activity than that of *In vivo* garlic cloves through wider diameter zone of inhibition against said bacterial strains. *In vitro* garlic extract formed 24mm and 22mm zones being the widest zones against *Klebsiella* and *Proteus* respectively.

Introduction

A vast knowledge, how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance (Diallo *et al.*, 1999). According to WHO, more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Plants used by traditional medicine practitioners contain a wide range of substances that can be used to treat chronic as well as infectious diseases. Many plant extracts have been shown to possess antimicrobial properties active against microorganisms *In vitro*. These extracts are nontoxic, non allergenic to the host (selective toxicity) and without undesirable side effects. They are able to reach the infectious parts of the human body and do not eliminate the normal flora of the host. Plant extracts are inexpensive and chemically stable.

Screening of medicinal plants for antimicrobial activities and phytochemicals is important for finding potential compounds for therapeutic use (Duraipandiyan *et al.*, 2006). The medicinal value of plants lies in some chemical substances that produce a definite physiological action in the human body. The most important of these bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compounds (Edeoga *et al.*, 2005). There has been a great shift from the prescription of antibiotics to the use of medicinal plants (Ekwenye & Elegalam, 2005) as if their biologically active principles e.g. flavones and flavonols are chemical compounds specifically active against microorganisms (Fessenden & Fessenden, 1982). Similarly, lipophilic flaonoids inhibit microbial activity by disrupting their membrane (Tsuchiya *et al.*, 1996).

Allium sativum, garlic, is a bulb-forming herb of the family Alliaceae, cultivated some thousands of years for use as a flavoring agent as well as a medicinal herb (Lewis & Elvin-Lewis, 2003). Its biological activities include antibacterial, antitumour, and antiartherosclerosis (Campbell *et al.*, 2001; Milner, 2001), cholesterol lowering (Yeh & Liu, 2001) and prevention of cardiovascular disorders (Rahman, 2001). It has been shown that garlic and garlic extracts have antioxidant activity in different *In vitro* models. The antioxidant activity of *Allium* plants has been mainly attributed to a variety of sulphur-containing compounds and their precursors (Kim *et al.*, 1997; Lampe, 1999). Allicin and allyl isothiocyanate are sulfur-containing compounds. Allicin, isolated from garlic oil, inhibits the growth of both Gram-Positive and Gram- Negative bacteria (Azzouz & Bullerman, 1982).

A plenty of work has been done on the garlic as such as folklore disease management and antimicrobial activity of garlic bulbs (Tyler, 1993; Foster, 1996). However, antimicrobial activity of *In vitro* grown garlic bulblets has not been reported so far. During micropropagation activities this aspect was given due consideration to ascertain that *In vitro* grown garlic bulblets as compared to *In vivo* ones are better source of antimicrobial agents that can be used to assist the primary health care. Antibacterial activity of extracts from *In vitro* and *In vivo grown* garlic bulblets were evaluated against five bacterial strains to compare the effectiveness of *In vitro* and *In vivo grown* garlic plants.

Materials and Methods

Tissue culture and bulblet regeneration: The bulbs of local cultivar of *Allium sativum* were obtained from the Punjab Seed Corporation, Lahore, Pakistan. Part of the material was subjected to micropropagation and regeneration studies, to compare the antimicrobial activity of active principles of *In vivo* and *In vitro* grown bulbs.

The shoot tips were taken from healthy cloves of garlic and after a brief treatment with 0.1% $HgCl_2$ solution for 5-7 min, inoculated on MS (Murashige & Skoog, 1962) medium containing 4.5 µM dichlorophenoxy acetic acid (2,4-D) and 4.42µM indole butyric acid (IBA) as described by Fatima *et al.*, (2006). The pH of the medium was adjusted to 5.8 and autoclaved at 121°C for 20 min. Cultures were kept in a growth room at 25±2°C under a 16h photoperiod and a light intensity of 72 µmol m^{-2} s^{-1}. The calli produced after 2 months were transferred onto MS media containing various concentrations and combinations of growth regulators, *viz.* benzyle adenine (BA), naphthalene acetic acid (NAA) and kinetin (Kin) to induce regeneration (Table 1). The somatic embryos were transformed into plantlets with roots and shoots, after 4 weeks on basal MS medium. The regenerated plantlets with highest shoot count were again transferred onto basal MS medium for bulblets formation. Average 1-2 bulblets with few roots were produced per culture within 4-6 weeks.

Table 1. Effect of MS medium supplemented with growth regulators on garlic callus cultures and plantlet regeneration through somatic embryogenesis.

Growth regulators (µM)		Regeneration/culture			Callus characteristics
BA	**NAA**	**Kin**	**Shoot count**	**Root count**	**Color & texture**
9.06	-	-	$12.67^{a} \pm 1.70$	$6.00^{b} \pm 0.82$	Green Soft, friable, Shiny & granular
13.59	-	-	$7.67^{b} \pm 1.25$	$8.33^{b} \pm 0.94$	Lush green to yellowish green Soft, granular, friable & shiny
9.06	2.69	-	$3.00^{c} \pm 0.82$	$33.00^{a} \pm 1.41$	Dark green to yellowish green Soft, friable & granular
18.12	-	2.32	$3.00^{c} \pm 0.82$	$5.67^{b} \pm 1.25$	Green with yellow patches Soft, granular & shiny
-	-	2.32	$3.0^{c} \pm 0.82$	$7.00^{b} \pm 2.16$	Green Soft, nodular & friable
-	2.69	3.48	$9.33^{ab} \pm 1.25$	$8.67^{b} \pm 1.25$	Yellow with green patches Nodular, friable & soft

* = Mean separation in columns by Duncan's Multiple Range Test, p = 0.01

Extraction of bioactive material: The *In vivo* grown garlic bulbs were washed with deionized water to reduce the extraneous materials. Then air-dried and removed outer coverings manually. *In vivo* and *In vitro grown* bulblets were sliced separately. Materials were placed in hot air oven for drying at 65ºC for 72 hours and pulverized with pestle and mortar. Weighed 1.0 g powders of each samples, dissolved in 40 ml of 80% ethanol individually, and vigorously stirred with a sterile glass rod. Extracts were occasionally shaken during 24 h and then filtered through Whatman No.1 filter paper (Azoro, 2000) discarding the precipitates. Yellow colored filtrates were evaporated to dryness on steam bath at 100ºC. The dried extracts were sterilized in UV light for 24h. Each of the alcoholic extracts was reconstituted by adding 2 ml of dimethyl sulphoxide (DMSO). Paper disc diffusion method was applied to test the antimicrobial activity of the extracts. Filter paper (Whatman No.1) discs of 5 mm dia. were prepared wrapped in tinfoil and sterilized by hot air oven. Normal strength nutrient agar medium (OXOID, England) was prepared and autoclaved at 121ºC for 15 min. at 15 psi for culture growth and determination of antibacterial activity.

Test organisms: Prior to inoculation five bacterial strains *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter aerogenes* and *Staphylococcus aureus* were subcultured thrice onto the fresh nutrient agar media to obtain a more vigorous population. The stock cultures were incubated at 37ºC for 24 h.

Screening for antibacterial activity: Bacterial cultures were serially diluted in normal saline solution. From 10^{-3} dilution, a sterile swab stick was used to seed the nutrient medium culture plates in the inoculating chamber. Extract impregnated discs of known strength were placed on pre-inoculated culture media and incubated at 37ºC for 24 h. The zone of inhibition in each case was measured as the diameter of the clear zones around the discs.

Control experiment using antibiotics*:* This was done to compare the diameter zones of inhibition of the extracts and already standardized antibiotics. This could help for the prescription of either antibiotics or plants extracts with antimicrobial activities. The antibiotics used were erythromycin (Abbott, Pakistan), tetracycline (Pfizer, Australia) and ampicillin (SmithKline Beecham, England). The concentration of erythromycin used was 5 mg/ml and that of tetracycline or ampicillin was 6 mg/ml, individually.

Statistical analysis: The results obtained in present study were statistically analyzed with one-way analysis of variance in completely randomized design. The means were separated by Duncan multiple range test at 1% and 5% level of significance as described by Steel & Torrie (1980).

Results and Discussion

The best garlic calli were produced on MS medium with 2,4-D (4.53 µM) alone or additionally supplemented with BA (2.22 µM) or IBA (4.42 µM). The calli produced were soft, nodular and yellow. Clumps of calli were transferred to MS medium supplemented with growth regulators for regeneration (Table 1). After 4 weeks, the yellowish or white nodular calli turned yellowish green to green randomly and exhibited distinct morphogenetic changes resembling embryoid like structures (Fig. 1-B). The globular embryos subsequently developed shoot and root apices giving tuft-like appearance generally within initial four weeks which thereafter germinated to give plantlets during next 4 weeks. The similar observations have been also reported by Fereol *et al.,* (2002). The highest shoot count obtained on MS supplemented with 9.06 µM BA was 12.67 ± 1.70^{a} while the root count was less (6.00 ± 0.82^{b}). This result matched with the findings of Choi *et al*., (1993) who reported that BA was the most effective stimulator for shoot formation and increased percentage of shoot regeneration. BA in combination with NAA and Kin gave low shoot count as compared to BA alone and high root count as shown in Table 1. Regenerated shoots from MS medium were transferred on growth regulator (GR) free MS basal medium. 80% healthy bulblets were produced in 4-6 weeks. Average 1-2 bulblets with few roots were produced per culture (Fig. 1-D). Haque *et al.,* (2003) reported that the bulblet growth was significantly active on the GR-free medium supplemented with 3% sucrose. This study contradicted the findings of Khan *et al.,* (2004) who reported that GR-free MS medium was favourable for only root induction in case of garlic cultures.

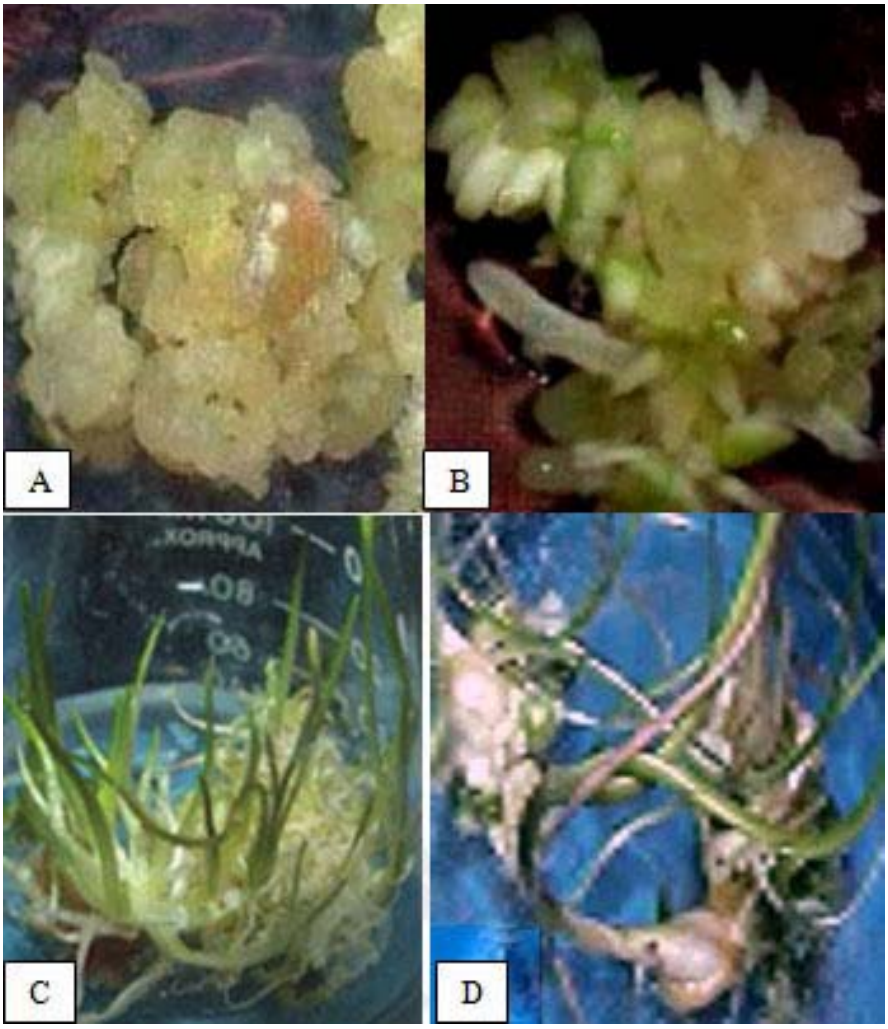

Fig. 1 *In vitro* regeneration and bulblet formation via somatic embryogenesis from shoot tip nodular callus of garlic (*Allium sativum* L.). A. Friable and nodular callus from shoot tip explants on MS medium (2,4-D+IBA). B. Nodular callus showing well developed embryos. C. Multiple plantlet formation. D. Bulblets formation from *In vitro* regenerated shoots of garlic on growth regulator free MS medium 300Dpi pic.

The sensitivity of different microorganism with the ethanolic extracts of both *In vitro* and *In vivo* grown garlic is shown in Table 2. Paper disc method was used for this purpose. Both samples inhibited the growth of all test organisms. However, *In vitro grown* garlic extract exhibited a greater degree of antibacterial activity and wide diameters of zones of inhibition were observed (Table 3). White *et al.,* (2007) made similar observation that extract of micropropagated *Peperomia tetraphylla* plant had high antimicrobial activity.

E. aerogenes and *K. pneumoniae* were found to be more sensitive to *In vitro* garlic extract and their diameter of zones of inhibition were $22.33^{a} \pm 1.77$ mm and $24.00^{a} \pm 3.74$ mm with high level of significance as compared to those of *E. coli, P. mirabilis* and *S. aureus* which gave the diameter of zone of inhibition $11.00^{b} \pm 1.22$ mm, $9.00^{bc} \pm 0.70$ mm and $17.66^{b} \pm 1.77$ mm respectively. Similar observations were also noted by Onyeagba *et al.,* (2004), who assayed the antimicrobial effect of aqueous and ethanolic extracts of garlic, ginger and lime against *Staphylococcus aureus*, *Bacillus* spp., *E. coli* and *Salmonella* spp. and observed highest inhibition zone of 19 mm with a combination of the aforesaid three extracts on *Staphylococcus aureus*. Significant level of differences among all bacterial species in respect of their sensitivity was assessed in *In vivo* grown garlic extract. *E. coli*, *P. mirabilis* and *S. aureus* were not sensitive with *In vivo grown* garlic extract. The widest zone of inhibition was $7.33^{b} \pm 1.77$ mm with *In vivo* grown garlic extract on *K. pneumoniae* and $6.66^{b} \pm 2.04$ mm on *E. aerogenes* (Table 3).

Results showed that all three antibiotics were more effective against *S. aureus* and less effective against *P. mirabilis* with significant levels of difference (Table 3). Ampicillin is more effective with non-significant differences against all testing microbes except *E.coli.* Similarly, *P. mirabilis* is less sensitive against Erythromycin as compared to other bacterial species with non-significant differences. *E. coli* and *P. mirabilis* showed significant differences ($10.00^{b} \pm 1.41$ mm and $6.33^{c} \pm 1.08$ mm respectively) in case of tetracycline while strong action was observed on remaining three bacterial species.

Table 2. Antimicrobial activity of the ethanolic extract of *In vitro, In vivo* grown garlic & antibiotics.

Samples	Escherichia coli	Klebsiella pneumoniae	Proteus mirabilis	Enterobacter *aerogenes*	Staphylococcus *Aureus*
In vitro garlic extract	++	++	++	++	++
In vivo garlic extract	-	++	-	++	-
Erythromycin	++	++	++	++	++
Tetracycline	++	++	++	++	++
Ampicillin	++	++	++	++	++

- Key: ++ = Inhibition>6.00 mm diameter; = No inhibition

Table 3. Diameter zone (mean ± S.E.) of inhibition (mm)*.

Samples	Escherichia coli	Klebsiella pneumoniae	Proteus mirabilis	Enterobacter *aerogenes*	Staphylococcus *aureus*
In vitro garlic	$11^{b} \pm 1.22$	$24.00^{a} \pm 3.74$	$9.00^{bc} \pm 0.70$	$22.33^{a} \pm 1.77$	$17.66^{b} \pm 1.77$
In vivo garlic	$6.33^{c} \pm 1.63$	$7.33^{b} \pm 1.77$	$6.66^{d} \pm 2.04$	$6.66^{b} \pm 2.04$	0.00
Erythromycin	$17.33^{a} \pm 2.16$	$24.00^{a} \pm 1.22$	$12.00^{b} \pm 2.12$	$21.66^{a} \pm 2.48$	$25.00^{a} \pm 1.41$
Tetracycline	$10.00^{b} \pm 1.41$	$27.00^{a} \pm 1.87$	$6.33^{c} \pm 1.08$	$21.00^{a} \pm 3.24$	$25.00^{a} \pm 2.12$
Ampicillin	$10.33^{b} \pm 1.08$	$26.66^{a} \pm 2.16$	$16.66^{a} \pm 1.08$	$16.00^{a} \pm 2.54$	$23.00^{a} \pm 2.54$

* = Mean separation in columns by Duncan's Multiple Range Test, p = 0.05

From the results, it is evident that zone of inhibition of *In vitro* grown garlic bulblet extract was even greater than those of antibiotics in some cases. This clearly indicates that antibacterial effect of *In vitro* grown garlic bulblet extract was more pronounced than that of *In vivo* grown garlic. Higher antibacterial activity of *In vitro* grown garlic bulblet extract may be due to altered cultural condition such as GRs provided in the culture medium for plantlet regeneration. Allicin being the main constituent and having high antimicrobial activity would be increased within the *In vitro* grown garlic bulblets due to these altered cultural conditions. Effects of phytohormones for production of secondary metabolites has been reported by Fett-Neto *et al.,* (1993) and Goleniowski & Trippi (1999), which supports present studies. Several products were found to be accumulating in cultured cell at a higher level than those in native plants through optimization of cultural conditions. For example, ginsenoside by *Panax ginseng* (Choi *et al.*, 1994), shikonin by *Lithospermum erythrorhizon* (Takahashi & Fjita, 1991), were accumulated in much higher levels in cultured cells than in the intact plants.

That is why, *In vitro* grown garlic bulblet contents showed wider zones of inhibition as compared to those of *In vivo* grown garlic cloves. It can be conferred from the result that the use of *In vitro* grown garlic could be a better substitute of commonly used antibiotics due to the presence of strong bioactive compounds active against microbes.

The experiments were repeated with same samples of the ethanolic extracts of *In vitro* and *In vivo* garlic bulblets after 45 days of storage at 4°C. The results showed that the antibacterial property of these garlic extracts retained their molecular specificity during storage.

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