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ANTIOXIDANT & ANTIMICROBIAL ACTIVITY OF *IN VIVO* AND *IN VITRO* GROWN PLANTS OF *Phyllanthus niruri* L.

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ABSTRACT

Since ancient time, *Phyllunthus niruri* L. are using in many herbal preparations. Increasing demand of *P. niruri* lead to adulteration, and thus there is a global need to develop quality plant material. In present investigation, an efficient *in vitro* technology was developed for a rapid and large-scale production of *P. niruri* via axillary bud proliferation using nodal segments of the mature plants. Elongated shoots were cultured on half strength MS medium fortified with various auxins such as NAA, 2, 4-D, IBA, IAA and at different concentrations (0.5 - 5.0 mg/l). *In vitro* developed plantlets were harden and acclimatized in natural environment. The antimicrobial activity (by well diffusion method) and antioxidant activity (by DPPH scavenging assay, reducing power and total antioxidant capacity methods) of *in vitro* developed plant, naturally grown plant and commercial available sample of *P.niruri* were determined. Results of antimicrobial activity of methanol extract from *in vitro* cultured *P.niruri* showed great potential as source of antimicrobial agent. In DPPH scavenging assay IC_{50} value of the three extracts was found 49.24µg/ml (*in vitro*), 41.66µg/ml (*in vivo*) and 36.14µg/ml (market sample) while the IC_{50} value of the reference standard ascorbic acid was 53.89 µg/ml. Results suggest *in vitro* developed plant posses highest antimicrobial and antioxidant activity and thus developed *in vitro* cultivation technology would provide quality plant material for medicine.

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KEY WORDS

Phyllunthus niruri, axillary bud proliferation, DPPH scavenging, antioxidant activity

INTRODUCTION

Since the use of complementary and alternative medicines has recently increased, this has lead to enhancing the market for herbal products worldwide[1]. But the purity of these herbal formulations exposes the human population to multiple risks and creates major concerns for various health agencies on both national and international levels [2].
Phyllanthus niruri L. (Euphorbiaceae) is a medicinal plant widely used in different regions in the world for the treatment of various diseases. An aqueous infusion of the whole plant, which is a typical preparation, is employed as a stomachic, aperitive, antispasmodic, laxative, diuretic, carminative, against constipation, fever including malaria, dysentery, gonorrhea, syphilis, tuberculosis, cough, diarrhea and vaginitis [3,4]. Interest in this plant was further enhanced due to reports of its anti-tumor and anti-carcinogenic activities and its potential as a remedy for hepatitis B viral infection[5]. Recently this plant is focused for its antioxidant activity and antibacterial activity[6,7,8].

P. niruri is grown as weeds in agricultural and waste lands. Most of the people collect this plant from different places without considering whether they are grown in polluted or unpolluted areas. The plants that were collected from polluted site were found to contain heavy metal or toxic components such as mercury, copper, lead, arsenic and many other toxic metals. *P.niruri* is often utilized by the human for local treatment and by the industries to make various formulations. Now in order to maintain sustainable supply of healthy, diseases free and quality plants for human consumption/drug preparation, *in vitro* propagation technique comes as a rescue that ensures a good quality plant. In present investigation we report is an *in vitro* method through which elite plants can be generated and evaluate their antimicrobial and antioxidant activity against the *in vivo* grown plant as well as commercial available sample.

MATERIALS & METHODS

(1) In vitro development of P. niruri:
Plant material:
Nodal segments (2 cm. long) were collected from 1.5 months old healthy dieses free mother plant growing in the Botanical garden, University of Sardar Patel University, V.V. Nagar, Anand- Gujarat.

Sterilization:
Leaves selected 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 0.05 % mercuric chloride ($HgCl_2$, w/v) for 5 minutes followed by repeated rinsing with sterile distilled water.

Culture media:
The surfaced sterilized explants were then aseptically inoculated on sterile MS medium[9] containing 3% sucrose as carbon source and 0.8% agar as solidifying agent. The medium was also supplemented with various growth regulators like auxins- (IAA, IBA, NAA, 2,4-D) and cytokinins (BA, Kn) and pH of the medium was adjusted to 5.8 before autoclaving at 15 lbs/square inch pressure (121 ^{0}C) for 15 min.. For multiple shoot induction different concentrations (0.5, 1.0, 2.0, 3.0, 4.0 mg/l) of

BA and Kin alone or in combination were used. However, various auxins such as NAA, 2, 4-D, IBA, IAA at different concentrations (0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0 mg/l) were examined for *in vitro* root induction.

Culture conditions:

The cultures were incubated at 25 ± 2°C under cool, white and fluorescent light of 2000-2500 lux and a relative humidity of about 60 %. Photoperiod of 16/8 hours of light and dark period was maintained in growth chamber, respectively. For each treatment, six replicates were used and every experiment was repeated at least thrice. Data on multiple shoot induction; elongation and rooting were taken and statistically analyzed. Observations were recorded periodically.

Induction of Multiple shoots:

The aseptic nodal segments were cultured on MS medium supplemented with 0.5, 1.0, 2.0, 3 .0, 4.0 mg/l of BA and Kn alone and/or in combination. Physiological conditions and number of shoots per explants were observed periodically.

Establishment of Root cultures, Hardening and Acclimatization:

In vitro elongated shoots (5-6 cm.) were taken out from the culture vials and transferred to half strength MS medium with different concentration (0.5-5.0 mg/l) of auxins like NAA, 2, 4-D, IBA, IAA 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 then transferred to polycups containing coco peat and vermiculite (1:1). Polycups were covered with plastic bag with 2-5 holes (2-3 mm) 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. Well acclimatized plants were grown in green house. One month old plants (leaves & stem) were used for antibacterial and antioxidant activity.

(2) Antimicrobial & Antioxidant activity:

Plant sample:

Aerial part (leaves+stem) of *P.niruri* were collected from three sources; 1> one month old tissue cultured plant (*in vitro*), 2> one month old wild grown (*in vivo)* plant from ISIBES campus, 3> dried sample from Petlad Mahal Arogya Mandal Pharmacy (market sample), Nadiad (Latitude: 22 42' 00" Longitude: 72 52' 00"), Gujarat-India. Plants were identified by Professor Minoo Parabia (VNSGU, Surat-India).

Extraction:

The shade-dried leaves were coarsely powdered and extracted with 95% methanol by a Soxhlet apparatus at 45 ^{o}C. The solvent was completely removed by rotary evaporator and obtained greenish gummy exudates. This crude extract was used for further investigation for potential antioxidant properties.

Antibacterial activity:

Procuring of bacterial strain:

Pure cultures of test organisms were collected from the Microbiology Department of Sardar Patel University, V.V.Nagar, Anand - Gujarat. Collected bacterial strains were *Staphylococcus aureus* (ATCC9144) (SA), *Klebsiella pneumoniae* (ATCC15380) (KP), *Pseudomonas aeruginosa* (ATCC25668) (PA), and *Salmonella typhi* (NCTC 8394) (ST). Purity of culture was maintained on Nutrient Agar by periodical transfers.

Agar diffusion assay:

Antimicrobial screening was done using agar well diffusion methods[10]. For this 25 ml of sterile Mueller–Hinton Agar No.2 (Hi-media), was poured in sterile autoclaved petri plates, before pouring 100µl of activated culture of bacteria was added, and then allowed to solidify completely. The wells were prepared with the help of sterile 10 mm diameter cork-borer. Then 100 µl of plant extract from stock (20mg dry powder extract/ml) solution were poured into the wells. The plates were sealed with plasticine and transferred to the

refrigerator to diffuse out for 30 mins. The plates were then incubated in the incubator at 37 °C for 24 hrs. Triplicate plates were prepared for each treatment and the average zone of inhibition excluding well, were recorded, 5.0% DMSO was used as negative control. Inoculum turbidity was maintained constant throughout the experiment to 0.8 OD at 660 nm. Level of turbidity is equivalent to approximately 1×10^8 CFU/ml. Gentamicin 30µg/ml was used as positive control.

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity:

Qualitative analysis:

The methanol extract was applied on a aluminum precoated 250 µm thick Silica Gel 60_{254} TLC Plates (Merck) (10 cm X 10 cm) as a spot (100 µg/ml) for chromatographic separation of the extract using the mobile phase methanol:chloroform (92:8, v/v). It was allowed to develop the chromatogram up to 9 cm length. After completion of the chromatogram the whole plate was sprayed with DPPH (0.10 % w/v) solution using an atomizer. The color changes (yellowish color development on pinkish background on the TLC plate) were noted as an indicator of the presence of antioxidant substances.

Quantitative analysis:

The free radical scavenging capacity of the extracts was determined using DPPH[11]. DPPH solution (0.004% w/v) was prepared in 95% methanol. Metahnol extract of *P. niruri* was mixed with 95 % methanol to prepare the stock solution (5 mg/ml). Freshly prepared DPPH solution (0.004% w/v) was taken in test tubes and *P. niruri* extracts was added followed by serial dilutions (1 µg to 500 µg) to every test tube so that the final volume was 3 ml and after 10 min, the absorbance was read at 515 nm using a spectrophotometer (Libra S11/S12 UV). Ascorbic acid was used as a reference standard and dissolve in distilled water to make the stock solution with the same concentration (5 mg/ml).

Control sample was prepared containing the same volume without any extract and reference ascorbic acid. 95 % methanol was served as blank. % scavenging of the DPPH free radical was measured by using the following equation.

$$\%\text{Scavenging Activity} = \frac{\text{Absorbance of the control} - \text{Absorbance of the test sample}}{\text{Absorbance of the control}} \times 100$$

The inhibition curve was plotted for duplicate experiments and represented as % of mean inhibition ± standard deviation. IC_{50} values were obtained by probit analysis[12].

Determination of total antioxidant capacity:

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure describe by Prieto et al.,[13]. The assay is based on the reduction of Mo (VI)–Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. A 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (Libra S11/S12 UV) against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of gram equivalents of ascorbic acid.

Reducing power:

The reducing power of *P. niruri* was determined according to the method previously described by Oyaizu[14]. Different concentrations of *P. niruri* extract (100– 1000 µg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml. 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the standard. Phosphate buffer (pH 6.6) was used as blank solution. The absorbance of the final reaction mixture of two parallel experiments was taken and is expressed as mean.

RESULTS AND DISCUSSION

In present research investigation, *in vitro* cultivation technology of *P. niruri* was achieved by nodal culture (Figure -1). Maximum number of shoots (3.16 ± 0.16) was obtained from nodal explants inoculated on MS medium supplemented with BA (3.0 mg/l) after 4 weeks. Further, increase in the concentrations of BA decreased the number of multiple shoots (Table 1). Similar observations were reported in other plant species such as Strawberry[15] and *Phyllanthus urinaria*[16].

Table: 1

Effect of growth regulators on shoot proliferation and number of shoots per culture from nodal explants. Data (Mean ± S.D.) were recorded after four weeks.

PGR mg^{-1}	Shooting Frequency (%)	No. shoot/node	Length (cm)
BA			
0.0	40.6	1.0 ± 0.0	2.23 ± 0.06
0.5	50.2	2.12 ± 0.17	2.76 ± 0.06
1.0	58.4	2.28 ± 0.30	3.12 ± 0.12
2.0	75.0	2.30 ± 0.02	3.18 ± 0.08
3.0	95.4	3.16 ± 0.16	4.12 ± 0.33
4.0	60.0	1.88 ± 0.06	3.32 ± 0.14
Kn			
0.5	45.0	1.18 ± 0.12	2.12 ± 0.23
1.0	64.2	2.22 ± 0.04	3.12 ± 0.24
2.0	78.0	2.43 ± 0.23	3.18 ± 0.02
3.0	60.8	2.64 ± 0.11	3.78 ± 0.16
BA + Kn			
1.0 + 0.5	65.0	2.12 ±0.02	3.12 ±0.08
1.0 + 1.0	60.0	2.24 ± 0.18	3.78 ± 0.04
2.0 + 0.5	85.6	2.13 ± 0.32	3.88 ± 0.24
2.0 + 1.0	65.0	2.18 ± 0.28	3.85 ± 0.36
3.0 + 0.5	96.4	2.85 ± 0.04	4.45 ± 0.24
3.0 + 1.0	78.6	2.33 ± 0.16	4.03 ± 0.54

These well developed shoots were separated from the explants and sub-cultured on the same medium for their elongation in in-vitro conditions in case of size of shoots were less than 3 cm. Elongation of shoots were also reported in other plant species like *Costu speciosus* [17] on the same medium in which they were initiated.

Shoot induction, multiplication and rooting, the effect of cytokinins and auxins on morphogenesis of nodal segment explant is presented in Tables–1, 2 and Fig.1. The effect of cytokinins at various concentrations on axillary shoot induction from nodal explants is presented in table -1. BA and Kn both promote shoot induction significantly as compared to control.

Table: 2

Effect of different concentration and combination of auxins on adventitious root formation from the in vitro grown micro-cutting cultured on ½ MS medium. Data (Mean ± S.D.) were recorded after four weeks.

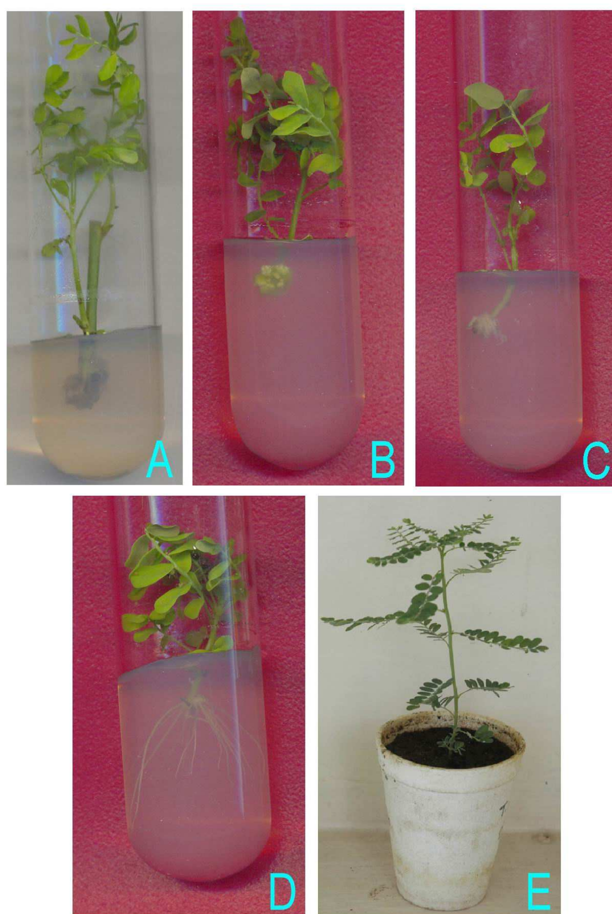
PGR mg $^{-1}$	Rooting Frequency (%)	No. of Root/node	Root Length (cm)	Remarks
MS medium full strength	0	0	0	Nil
MS medium half strength	0	0	0	Nil
IBA				
0.5	99	20.5 ± 0.04	4.33 ± 0.07	Roots were well developed, smooth white, thin, without branched and healthy.
1.0	85.4	18.6 ± 0.08	5.25 ± 0.11	
1.5	70.6	12.2 ± 0.12	4.02 ± 0.27	
2.0	50.4	10.3 ± 0.36	3.38 ± 0.02	
2.5 – 5.0	0	0	0	Nil
2,4-D				
0.5	50.4	09. 4 ± 0.12	3.48 ± 0.12	Roots were well developed, smooth white, thick, without branched and healthy.
1.0	60.0	12.6 ± 0.22	3.30 ± 0.06	
1.5	48.1	08.4 ± 0.20	3.04 ± 0.26	
2.0	30.6	05.7 ± 0.04	3.17 ± 0.24	
2.5 – 5.0	0	0	0	Nil
NAA				
0.5 – 5.0	0	0	0	Nil
IAA				
0.5 -5.0	0	0	0	Nil

As a supplement, 3 mg/l BA showed the best performance of proliferation that produced shoots in 100% of cultured explants. Explants produced the highest number of 3.16 ± 0.16 shoots per culture on the medium with 4.12 ± 0.33 cm maximum length of shoots. When the explants were cultured on Kn based medium, only 45 -78 % of them proliferated. In this particular treatment the highest number of shoots per explants and average shoot lengths were 2.64 ± 0.11 and 3.78 ± 0.16 respectively.

Root formation was induced in *in vitro* regenerated shoots by culturing them on half strength of MS with auxines at different concentration as shown in Table 2. Among the four types of auxin, IBA & 2,4-D were found to be most effective at different concentrations tested for root production on cut margins of the elongated shoot (Table 2). Shoot cultured in medium fortified with IBA were developed, smooth white, thin, and healthy roots, and same morphology of roots were observed in medium with 2,4-D. Both IBA & 2,4-D at 0.5 – 2.0 mg/l were produced morphologically identical roots, but they were different in length, number and root producing frequency. At higher concentration (< 2.0 mg/l) both IBA & 2,4-D failed to produce roots. IBA at 0.5 mg/l concentration produced maximum number (20.5 ± 0.04) and maximum length (4.33 ± 0.07) of roots per shoot cutting margin. These findings are in agreement with those observed in other plant species *Phyla nodfolia*, *Leptadenia reticulata*[18] and *Lins culinaris* Medik[19]. Half strength MS medium support root production, while full strength MS medium did not produce any roots from cutting margin. NAA and IAA at 0.5 – 5.0 mg/l concentration failed to develop rooting.

Figure 1

Regeneration of plantlets in vitro from the nodal explants obtained from field grown Phyllunthus niruri L. A – Development of shoot from nodal explant, B – Elongation of shoot, C – Initiation of roots, D – Well developed roots, E – In vitro developed plant ready to field transfer.



The *in vitro* derived plants acclimatize better under *in vivo* condition when they were transferred on polycups containing coco-peat and vermiculite mixture at 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 90 % of the regenerated plantlets could tolerate and survive under field conditions. A number of plantlets were lost due to damping off & necrosis during acclimatization in field condition and damage to stem during acclimatization.

Due to increase health consciousness in society in past few years, there has been growing interest in the involvement of reactive oxygen species (ROS) in several pathological situations. ROS produced *in vivo* include superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). H_2O_2 and O_2^- can interact in the presence of certain transition metal ions to yield a highly-reactive oxidizing species, the hydroxyl radical (^-OH)[20]. Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals[21]. There is growing interest in natural triterpinoids caused as much by the scientific aspects extraction and structural analysis of these compounds, as by the fact of their wide spectrum of biological activities, they are bactericidal, fungicidal, antiviral, cytotoxic, analgesic, antiinflammatory, anti-cancer and antiallergic[22].

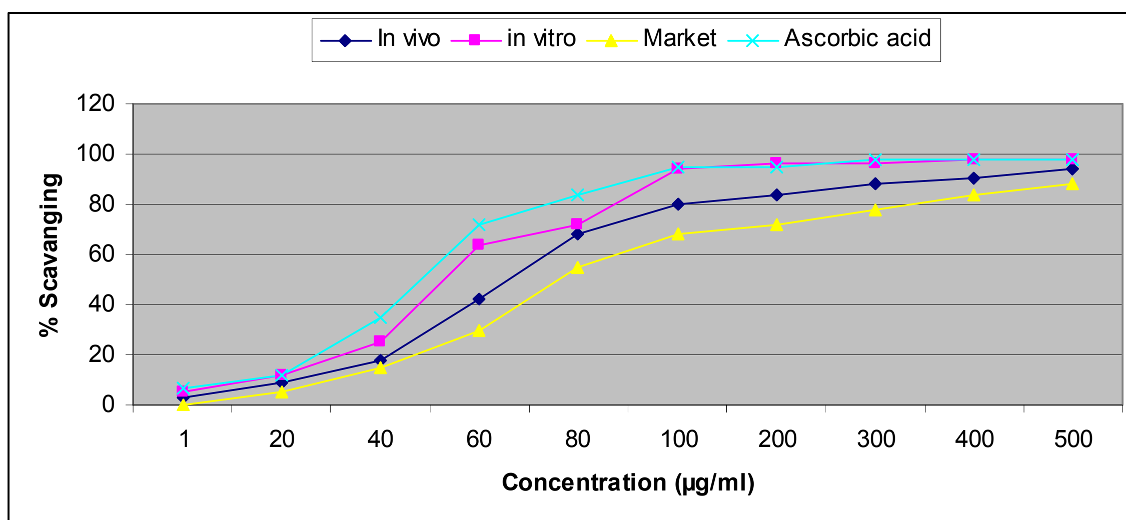


Fig. 2

DPPH radical scavenging activity of the methanol extracts of Phyllunthus niruri (in vitro, in vivo and market sample). Values are average of duplicate experiments and represented as mean value.

The DPPH radical scavenging activity of three source of *P.niruri* is given in Fig. 2. This activity was increased by increasing the concentration of the sample extract. DPPH antioxidant assay is based on the ability of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is

decolorized, which can be quantitatively measured from the changes in absorbance. The IC_{50} value of the *in vitro* plant extract was 49.24µg/ml, followed by *in vivo* plant extract (41.66µg/ml) and extract of market sample (36.14µg/ml). While opposed to that in ascorbic acid IC_{50} value was 53.89 µg/mL, which is a well known antioxidant. Results indicate that *in vitro* cultured plants contain high concentration of antioxidant compounds.

Table 3

Results of Total antioxidant capacity of the plant extracts Phyllanthus niruri. Values are average of duplicate experiments and represented as mean ± SD value.

Material	Concentration (µg/ml)	Equivalent to ascorbic acid
In vivo	100	0.25±0.10
	200	0.54±0.12
	400	1.23±0.06
	600	1.76±0.08
	800	2.12±0.14
In vitro	100	0.54±0.12
	200	1.23±0.07
	400	2.44±0.12
	600	3.82±0.14
	800	4.29±0.04
Market sample	100	0.17±0.02
	200	0.27±0.11
	400	0.36±0.14
	600	0.78±0.16
	800	1.12±0.08

Total antioxidant capacity of the *P.niruri* extract, expressed as the number of gram equivalents of ascorbic acid, is shown in Table 3. By comparing activity of total antioxidant activity to ascorbic acid extracts from *in vitro* culture was best among other extract. *Phyllunthus* sp. Contain Alkaloids[23], Hydrolysable tannins, Phenolics, Polyphenols and Flavonoids[24]. The antioxidative effect is mainly due to phenolic components, such as phenolic acids, and phenolic diterpenes[25]. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides[26]. The reducing properties of plant extracts are generally associated with the presence of reductones[27], which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom[28]. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Figure-3 shows the reductive capabilities of the plant extract compared to ascorbic acid. The reducing power of extract of in vitro developed plants of *P.niruri* was found remarkable and the reducing power of the extract was observed to rise as the concentration of the extract gradually increased. Among three extracts of *P.niruri*, extract from commercial market sample has least antioxidant activity. Perhaps the antioxidant compounds may deteriorate during storage, drying at collection place.

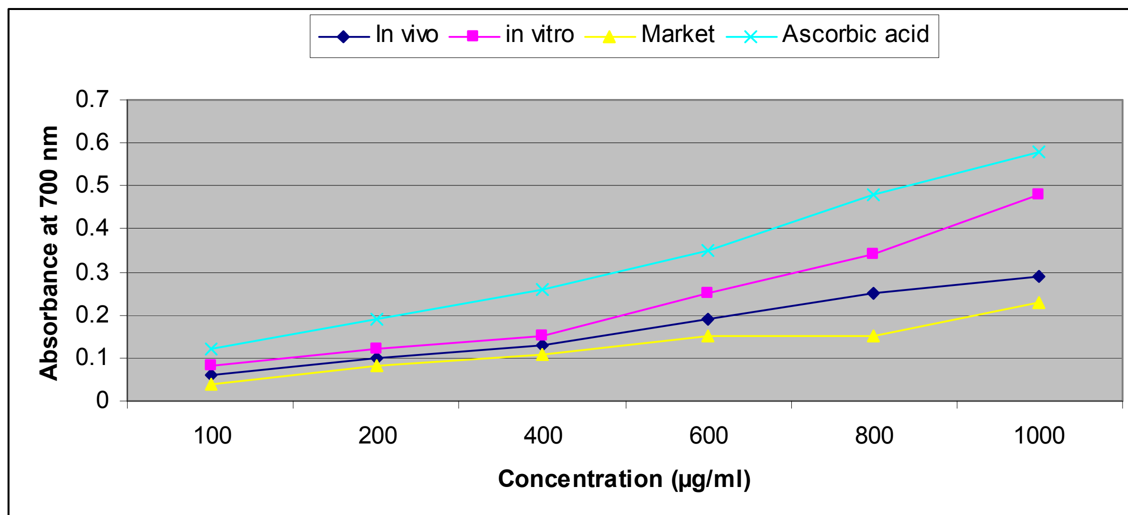


Fig. 3
Reducing power of the crude plant extract of Phyllunthus niruri (in vitro, in vivo and market sample). Values are average of duplicate experiments and represented as mean value.

Table: 4
Antimicrobial activity of the crude plant extract of Phyllunthus niruri (in vitro, in vivo and market sample). Values are average of triplicate experiments and represented as mean value.

Microorganism	*In vitro*	*In vivo*	Market sample	Gentamicin	5 % DMSO
	Methanol extract 20 mg/ml			(30µg/well)	(100 µl/well)
	Inhibition zone (mm)				
Staphylococcus aureus (ATCC9144),	14	12	10	10	--
Klebsiella pneumoniae (ATCC15380)	08	07	05	16	--
Bacillus subtilis (ATCC 6051)	12	11	10	10	--
Pseudomonas aeruginosa (ATCC25668)	12	12	07	12	--
Salmonella typhi (NCTC 8394)	10	10	05	16	--

Along with antioxidant activity if plant have antibacterial activity it prefer first in health supplements. Results of antimicrobial activity of all three extracts of *P.niruri*, expressed as zone of inhibition, and are shown in Table 4. The over all highest antimicrobial activity was observed in extract from *in vivo* cultured plants followed by *in vivo* and market sample. Comparison of antimicrobial activity to Gentamicin indicates *in vitro* cultured plant has great potential to develop/isolate antimicrobial agent.

CONCLUSION

From the above results and discussion it can be concluded that the methanol extract of *Phyllunthus niruri* possesses the potent antioxidant & antimicrobial substances and a which may be responsible for its anti-tumor, anti-carcinogenic and remedy for hepatitis B viral infection mechanism as well as justify the basis of using this plant's extract as folkloric remedies. In vitro cultivation technology would improve quality of raw material and so ultimately the efficiency of developed drug from them.

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