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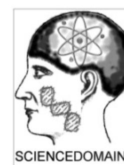

Bio-evaluation and Study of Anticancerous, Antibacterial and Antioxidant Properties of Explants of *Piper longum in vivo* and *in vitro*

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Authors' contributions

This work was carried out in collaboration between all authors. Author SB designed the study, wrote protocol and performed statistical analysis. Author GRP managed the analysis of study and guided in designing protocol. Author SB managed the analysis of study and literature searches and gave overall guidance. All authors read and approved the final manuscript.

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ABSTRACT

Piper longum is a medicinal plant of great importance. The present work is to compare anticancerous, antibacterial and antioxidant properties of different explants (leaf and stem) of *Piper longum in vivo* and *in vitro*. The anticancerous activity was measured in terms of percentage cytotoxicity and the cell line used was leukemic cell line K562. *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*, bacteria were used for determination of antibacterial activity. Solvent extracts were prepared from leaf and stem explants of *Piper longum* and their anticancerous, antibacterial and antioxidant activities were evaluated. Antioxidant activity was measured in terms of percentage (%) Total Phenolic Content (TPC) and percentage (%) 2,2– diphenyl 1-2-picryl hydrazy1 (DPPH) radical scavenging capacity. The anticancerous, antibacterial and antioxidant effects were found to be higher for hot extracts than cold extract. Further the results of *in vivo* explants were better than *in vitro* explants, in case of anticancerous activity and results of *in vitro* explants were better than *in vivo* explants, in case of antibacterial and antioxidant activity.

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Keywords: Piper longum; anticancerous activity; antibacterial activity; antioxidant activity; Leukemic cell line K562; TPC; DPPH.

1. INTRODUCTION

Piper longum is of south Asian Origin and is found almost all over the world including India, Asia and pacific islands, Europe, North East Africa and American continent. In India it is mostly found in forest and cultivated in Assam, central Himalayas, Khasi, Kerala, north east India and other parts of south India. It is a slender aromatic climber with perennial woody stems. Piper long is a dried fruit of *Piper longum*. Common name of *Piper longum* is pippali, Indian long pepper and pipal. *Piper longum* grows well is sandy loamy soils, which are well drained with rich organic matter and good moisture holding capacity. As the plant likes a humid and moist climate, it can be grown in areas of where there is heavy rain [1]. *Piper longum* is widely used in ayurvedic and unani systems of medicine [2] particularly diseases of respiratory tract most of them includes cough, bronchitis, asthma etc [3,4]. *Piper longum* is a medicinal plant having anticancerous, antibacterial and antioxidant properties [5,6,7]. Comparing these properties of explants of *Piper longum* i.e, leaves and stems *in vivo* and *in vitro* provides information of better medicinal values of different explants of this plant. Anticancerous activity was previously determined in fruits of *Piper longum,* by estimating the extent of its cytotoxicity on cancerous cell line [8]. *Piper longum* isolates are active against gram positive and moderately against gram negative bacteria. Antioxidant property of plant can scavange free radicals and protect the cell from oxidation.

2. MATERIALS AND METHODS

Piper longum plant was procured from botanical garden of National Research Institute of Basic Ayurvedic Sciences, Nehru Garden, Pune, Maharashtra, India. Cancerous cell line K562 was procured from National Centre for Cell Science(NCCS), Pune, Maharashtra, India. Chemicals and reagents used were Gallic acid, sodium carbonate, 2, 2 – diphenyl 1-2-picryl hydrazy1 (DPPH), ethyl acetate, hexane, methanol, Folin-Ciocalteu reagent, concentrated sulphuric acid, 3-4,5-Dimethylthiazol-2yl-2,5-Diphenyl Tetrazolium Bromide (MTT), and Phosphate buffer saline (PBS), Dimethyl sulphoxide(DMSO),Distilled water(DW), Indole acetic acid(IAA), Benzyl amino purine(BAP), Kinetin(KIN).

2.1 Preparation of Hot Extracts

10 gm leaf / stem powder of *Piper longum* was taken and mixed with 100 ml ethyl acetate it was then heated at 50℃ and kept on shaker overnight, next day it was dried in rotavapour, then filtered using whatman filter paper and was preserved at 4℃.

2.2 Preparation of Cold Extracts

10 gm leaf / stem powder of *Piper longum* was taken and mixed with 100ml hexane: water (1:1) and kept on shaker overnight, next day it was dried in rotavapour, then filtered using what man filter paper and was preserved at 4℃.

2.2.1 Determination of anticancerous activity of *Piper longum in vivo and in vitro*

2.2.1.1 Anticancerous activity of Piper longum in vivo

3-4, 5-Dimethylthiazol-2yl-2, 5-Diphenyl Tetrazolium Bromide (MTT) assay was performed to determine cytotoxicity this was done on ELISA reader plate.

Percentage cytotoxicity of plant's explants were checked on leukemic cell line K562 (cancerous cell line), so that its anticancerous effects can be studied. Explants of *Piper longum* were directly taken from the outer environment for *in vivo* analysis whereas explants which were inoculated in hormonal media by the process of tissue culture to obtain callus contributed to *in vitro* analysis, thus the extent of anticancerous activity of different concentrations of *in vivo* and *in vitro* leaf and stem explants of *Piper longum* was determined.

In 1st row of wells of microtiter plate 200µl of sterile Phosphate Buffer Saline (PBS) was pipetted in 9 wells, in 2nd row of wells 100 µl of cell suspension and100 µl of hot ethyl acetate extract of plant was pipetted. It was done in triplicate 200µg/ml, 400µg/ml and 800µg/ml concentration of plant extract was pipetted in 3 wells each, in the 3rd row of wells 100 µl of cell suspension and 100 µl of cold hexane: water extract of plant was pipetted. It was done in triplicate 200 µg/ml, 400 µg/ml and 800 µg/ml concentration of plant extract was pipetted in 3

wells each, in the 4th row of wells 200 µl of cell suspension was pipetted in 9 wells each it was taken as negative control. After pipetting the plate was incubated at 37℃ at 5% CO_2 for 24 hrs and then 48 hrs and then 72 hrs. After incubation media were removed from the wells without disturbing the cells. To each well 100 µl MTT solution was added. Plate was incubated in dark at 37℃ in CO_2 incubator for 4 hrs. Absorbance was taken at 570 nm using ELISA reader [8].

% of Cytotoxicity = 100 – [(O D of the treated cells/O D of the control cells) x 100]

2.2.1.2 Anticancerous activity of Piper longum in vitro

2.2.1.2.1 Tissue culture method for callus induction

2.2.1.2.1.1 Preparation of MS basal media for 1 litre

500 ml of DW was taken in a conical flask then 62.5 ml macronutrients was added then 1 ml of micronutrients were added after that 10 ml of iron source and 10 ml of organic supplement was added 30 gm of sucrose was added then volume was maintained up to 1 l then pH was maintained at 5.7 then 8 gm agar-agar was added and media was distributed in two parts and two different concentration of three growth regulators were used:

For the 1st, 500ml of MS media-IAA (Indole acetic acid) – 1 mg/l, BAP (Benzyl amino purine) - 2 mg/l, KIN (Kinetin)-1 mg/l concentration were used. For the 2nd 500 ml of MS media-IAA (Indole acetic acid) – 2 mg/l, BAP (Benzyl amino purine)-2 mg/l, KIN (Kinetin)-1 mg/l concentration were used.

2.2.1.3 Sterilization of the explants of Piper longum

At first the explants i.e. leaves and stems of *Piper longum* were washed with tap water 3-4 times. Then the explants were washed with 0.5% of tween-20 for 10-15 min. Then they were washed with tap water to remove the detergent completely. Then, it was washed with 0.5% of Bavastin for 10 min. Then again, explants were washed with tap water. Then, the explants were washed with distilled water. The explants were dipped in 0.1% $HgCl_2$ solution for 1 min. inside LAF. Finally the explants were washed with autoclaved distilled water for 5-7 times and then inoculated to the hormonal MS media, with three types of hormones: IAA, BAP, Kinetin. Callus induction was allowed to continue for 3-4 weeks.

In vitro grown callus were cut into pieces and crushed with the help of motor and pestle with distilled water and then 10 ml of solution was added to conical flask containing 90 ml of distilled water.

The steps for preparation of hot and cold extracts and protocol for detection of anticancerous activity were same as *in vivo.*

2.2.2 Determination of antibacterial activity of *Piper longum in vivo* and *in vitro*

2.2.2.1 Antibacterial activity of Piper longum in vivo

2.2.2.1.1 Nutrient broth preparation

A media of composition Tryptone - 10 gm/l, Sodium chloride - 10 gm/l, Yeast extract- 5 gm/l and Distilled water – 1000 ml was prepared and adjusted to a pH of 7.2.

2.2.2.1.2 Broth dilution technique

2.2.2.1.2.1 Method

Sample Dilutions:

Preparation of Dimethyl Sulphoxide (DMSO) solution: (10 ml)-1ml DMSO was added to 9 ml sterile nutrient broth in sterile tube. 10 mg leaf/stem extract was added to 1ml DMSO stock solution.

Serial dilutions:

For leaf extract it was diluted in the ratio 1:1(0.5 ml from stock solution was added to 0.5ml DMSO), 1:2(0.5 ml from stock solution was added to 1ml DMSO), 1:4 (0.5 ml from stock solution was added to 2 ml DMSO). Dilutions were separately prepared for hot extract and cold extract of leaf and stem.

Preparation of Bacterial cultures:

100 ml of nutrient broth was prepared in triplicate in 3 conical flask, autoclaved and then loopful of bacterial culture of *Escherichia coli*, *Bacillus subtillis*, *Staphylococcus aureus* was inoculated in three flasks. It was then incubated on shaker

for 2 hrs (O.D was 0.3 at 620 nm).Inhibition studies was done on ELISA plate.

A. Hot extract of leaf/stem and cell suspension of *Escherichia coli*, *Bacillus subtillis* and *Staphylococcus aureus*

In 1st row of plate, 100 µl of cell suspension and 100 µl of each dilutions (hot extract of leaf/stem) was pipetted in triplicate. In 2nd row of plate, 100 µl of cell suspension was added to 100 µl of ampicillin solution (1 ml sterile distilled water was mixed with10 mg antibiotic and stored at 4℃). It was pipetted in each well and it was taken as positive control. In 3rd row of plate 100µl of DMSO was added to 100 µl of cell suspension, it was taken as solvent control. In 4th row, 200 µl of cell suspension was added in each wells it was taken as negative control. The Plate was incubated at 37℃ for 48 hrs. Absorbance was taken at 620 nm on ELISA reader & extent of inhibition was interpreted.

B. Cold extract of leaf/stem and cell suspension of *Escherichia coli*, *Bacillus subtillis* and *Staphylococcus aureus*

In 1st row of plate 100µl of cell suspension and 100 µl of each dilutions (cold extract of leaf/stem) was pipetted in triplicate. In 2nd row of plate , 100 µl of cell suspension was added to100 µl of ampicillin solution(1 ml sterile distilled water was mixed with10 mg antibiotic and stored at 4℃) and it was pipetted in each well, it was taken as positive control. In 3rd row of plate 100 µl of DMSO was added to 100 µl of cell suspension it was taken as solvent control. In 4th row 200 µl of cell suspension was added in each wells it was taken as negative control. Plate was incubated at 37℃ for 48 hrs. Absorbance was taken at 620nm on ELISA reader & extent of inhibition was interpreted [8].

% inhibition =100- [(absorbance of the test sample/absorbance of control) x 100]

2.2.2.2 Antibacterial activity in vitro

In vitro grown callus were cut into pieces and crushed with the help of motor and pestle with distilled water and then 10 ml of solution was added to conical flask containing 90 ml of distilled water.

The steps for preparation of hot and cold extracts and protocol for detection of antibacterial activity were same as *in vivo.*

2.2.3 Determination of antioxidant activity of *Piper longum* in vivo and in vitro

2.2.3.1 Antioxidant activity in vivo

2.2.3.1.1 Total Phenolic Content (TPC) estimation in vivo

At first 9 test tubes were taken and autoclaved.1st test tube was kept as blank. In 2nd test tube-0.1 ml, 3rd test tube- 0.2 ml, 4th test tube-0.3 ml ,5th test tube- 0.4ml,6th test tube- 0.5 ml , 7th test tube- 1 ml, 8th test tube- 1 ml, 9th test tube- 1 ml gallic acid solution was added. In 7th test tube 200 µg/ml in 8th test tube, 400 µg/ml and in 9th test tube, 800 µg/ml of plant sample was added. Then in 1st test tube-2.5 ml. In 2nd test tube- 2.4 ml, 3rd test tube- 2.3 ml, 4th test tube-2.2 ml, 5th test tube- 2.1 ml, 6th test tube- 2 ml, 7th test tube- 1.5 ml, 8th test tube-1.5 ml, 9th test tube 1.5 ml DW was added, further 0.5 ml of folin reagent was added to each tube and were kept for 3 min at room temperature there after 1 ml of 20% sodium carbonate was added and incubated at room temperature for 90 min and absorbance of blue colour developed was read at 760 nm using spectrophotometer [8].

% TPC = (Observed concentration/Actual concentration) x 100

2.2.3.1.2 DPPH radical Scavenging Capacity in vivo

At first 6 test tubes (sterile) were taken.1st tube was marked as blank. In 2nd test tube- 1 ml in 3rd test tube- 2 ml, in 4th test tube- 3 ml, in 5th test tube 4 ml and in last test tube 5 ml of methanolic extract of plant leaf/stem was added. In 1st test tube- 5 ml, in 2nd test tube- 4 ml, in 3rd test tube- 3 ml, in 4th test tube- 2 ml in the 5th test tube- 1 ml methanol was added, no methanol was added to the last tube then 5ml DPPH was added to each tube and kept for 20 min at 27℃. Using methanol as blank OD of sample was measured at 517 nm [8].

% DPPH radical scavenging activity = (control OD – sample OD/control OD) X 100

2.2.3.2 Antioxidant activity in vitro

In vitro grown callus were cut into pieces and crushed with the help of motor and pestle with methanol and then 10 ml of solution was added to conical flask containing 90 ml of methanol.

The steps for preparation of hot and cold extracts and protocol for detection of antioxidant activity (TPC and DPPH radical scavenging capacity) were same as *in-vivo.*

3. RESULTS

The anticancerous, antibacterial and antioxidant effects were found to be higher for hot extracts than cold extracts. Further the results of *in vivo* explants were better than *in vitro* explants in case of anticancerous activity and results of *in vitro* explants were better than *in vivo* explants, for antibacterial and antioxidant activity, moreover results of stem explants were better than leaf explants, the *in vivo* and *in vitro* percentage in each case was calculated by taking mean of triplicate sample's optical density the result obtained was further applied in the formula of anticancerous, antibacterial and antioxidant activities which is shown as *in vivo/in vitro* and are presented in (Table 1, Table 2 and Table 3 and Fig. 1 to Fig. 10). For antibacterial activity among three bacteria used, percentage inhibition were found better in *Escherichia coli* and *Bacillus subtilis* as compared to *Staphylococcus aureus*. According to anticancerous, antibacterial and antioxidant activity the results of *Piper longum* are presented as follows:

3.1 Anticancerous Activity

For leaf, hot extract showed better results than cold extract for both *in vivo* and *in vitro* cases similarly, for stem, hot extract showed better results than cold extract for both *in vivo* and *in vitro* cases and highest % cytotoxicity was shown by hot extract of leaf. Further, *In vivo* results were better than *In vitro* results for both leaf and stem of *Piper longum*. In this case PBS was taken as positive control. The % of positive control in case of *in vivo* was 7.78% and 6.28% for leaf and stem respectively and in case of *in vitro* it was 8.36% and 7.19% for leaf and stem respectively. % cytotoxicity incase of *in vivo* where 5.03% and 3.84% for hot extract of leaf and stem respectively. The % cytotoxicity incase of *in vitro* where 4.91% and 3.45% for hot extract of leaf and stem respectively. The % cytotoxicity incase of *in vivo* where 3.94% and 3.29% for cold extract of leaf and stem respectively. The % cytotoxicity incase of *in vitro* where 3.65% and 3.16% for cold extract of leaf and stem respectively.

3.2 Antibacterial Activity

For *Escherichia coli*, the results indicated that hot extract of stem of *Piper longum* showed highest % of inhibition thus, has high antibacterial activity than cold extract of stem of *Piper longum*. On comparing the % of inhibition of both hot & cold extract, hot extract of stem shows highest % of inhibition than hot extract of leaf. *In vitro* results were better than *in vivo* results, % inhibition was highest in hot extract than cold extract.

For *Bacillus subtilis*, the result shows that hot extract of stem shows better result than cold extract of stem for both *in vivo* and *in vitro* cases and the hot extract of *in vitro* grown callus of leaf shows highest antibacterial activity against *Bacillus subtilis*.

In vitro results were better than *in vivo* in each case except in case of *Staphylococcus aureus*. Hot and cold extract of plant showed good antibacterial activity against bacteria *Escherichia coli* and *Bacillus subtilis* as compared to *Staphylococcus aureus* for which it was least effective.

In the present work, in case of antibacterial activity there was dose dependent increase in %inhibition, Ampicilin was taken as positive control, this antibiotic has antibacterial activity for all three bacteria used. It is used against *Piper longum* as control to check the extent of antibacterial activity of its explants. Comparative results of control and test sample are presented below.

The results of positive control for *Escherichia coli* and hot extract of leaf and stem explants used as test sample are as follows, % inhibition of control (ampicilin) were 74.47% and 21.97% in case of *in vivo* respectively and for *in vitro* case it was 74.71% and 25.87% respectively. On the other hand for test samples the % inhibition for *in vivo* case were 4.32% and 5.36% for leaf and stem respectively and *in vitro* case were 4.54% and 6.79% respectively. The results of positive control for *Escherichia coli* and cold extract of leaf and stem explants used as test sample, the % inhibition for control were 65.47% and 25.54% respectively in the case of *in vivo* and for *in vitro* case, it was 66.34% and 30.60%.On the other hand for test samples the % inhibition were 3.36% and 4.34% for *in vivo* and in case of *in vitro* it was 4.37% and 6.10% respectively. The results of positive control in case of *Bacillus subtilis* and hot extract of leaf and stem explants

the % inhibition for control were 71.23% and 68.74% in case of *in vivo* and for *in vitro* case it was 69.16% and 69.54% respectively. On the other hand for test samples the % inhibition for *in vivo* case were 5.18% and 4.23% and for *in vitro* case it was 5.99% and 4.92% respectively. For cold extract % inhibition for control was 23.56% and 23.20% in case of *in vivo* and 22.07% and 25.12% in case of *in vitro* respectively. On the other hand for test samples the % inhibition were 3.36% and 4.34% in case of *in vivo* and 4.98% and 4.43% in case of *in vitro* respectively. The results of positive control in case of *Staphylococcus aureus* and hot extract of leaf and stem explants used as test sample the % inhibition for control were 24.0% and 15.76% respectively in case of *in vivo* and for *in vitro* case it was17.78%/17.79% respectively. On the other hand for test samples the % inhibition for *in vivo* case were 1.64% and 1.79% and for *in vitro* case it was 1.27% and 1.24%, for cold extract % inhibition for control was 12.20% and 12.90% in case of *in vivo* and 13.56% and 23.72% respectively in case of *in vitro.* On the other hand for test samples the % inhibition were 1.41% and 1.68% in case of *in vivo* and 1.05% and 1.49% in case of *in vitro* respectively.

3.3 Antioxidant Activity

In case of TPC and DPPH, the results of hot methanolic leaf extract were better than results of hot methanolic stem extract for both *in vivo* and *in vitro* cases. Further the results of *in vitro* were better than results of *in vivo.* In case of TPC positive control was gallic acid. The %TPC for gallic acid was 40.69% and 46.32% for *in vivo* case for leaf and stem respectively. The % TPC for gallic acid was51.32% and48.91% for *in vitro* case for leaf and stem respectively. % TPC for *in vivo* case it was 43.75% and 41.42% for leaf and stem respectively and for *in vitro* case it was 44.75% and 43.24% for leaf and stem respectively.

% DPPH radical scavenging capacity for hot methanolic leaf and stem extract in case of *in vivo* was 10.40% and 7.2% respectively and in case of *in vitro* it was 12.40% and 10.85% respectively. In this case positive control was considered in which there was no methanol but only sample. The % of positive control in case of *in vivo* was 1.60% and 0.8% for leaf and stem respectively and in case of *in vitro* it was 5.10% and1.55% for leaf and stem respectively.

3.3.1 Bio-evaluation and study of anticancerous properties of explants of *Piper longum in vivo* and *in vitro*

3.3.1.1 MTT assay results

In the following table, the first percentage shows results obtained in the case of *In vivo* and the second percentage gives the result obtained in the case of *in vitro* for both leaf and stem

3.3.2 Bio-evaluation and study of antibacterial properties of explants of *Piper longum in vivo* and *in vitro*

In the following table, the first percentage shows results obtained in the case of *In vivo* and the second percentage gives the result obtained in the case of *in vitro* for both leaf and stem.

3.3.3 Bio-evaluation and study of antioxidant properties of explants of *Piper longum in vivo* and *in vitro*

In the following table, the first percentage shows results obtained in the case of *In vivo* and the second percentage gives the result obtained in the case of *in vitro* for both leaf and stem.

4. DISCUSSION

Anticancerous activity is very important medicinal property of plants. Plants possessing this property exhibits antitumor effects on immortal cell lines, thus is beneficial for human beings as studied earlier [9,10,11].Two types of extract were prepared hot extract and cold extract. Ethyl acetate is highly polar and is used as solvent for preparation of hot extract as it has high boiling point, it is less toxic and easily evaporates the elutant. Hexane is non polar it is used as solvent for preparation of cold extract as it as low boiling point between 50℃- 70℃. It is easy to use as it is less toxic. Cytotoxicity was determined by MTT assay, it is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Addition of a detergent results in the liberation of the crystals, which are solubilized. The colour can be spectrophotometrically measured. The level of the coloured formazan products is directly proportional to the number of surviving cells.

On calculating % cytotoxicity results were obtained, by the help of which the difference between effects of *in vivo* and *in vitro* explants (leaf and stem) for hot and cold extracts on leukemic cell line K562 were checked. Control sample was considered, which was only cell line sample and no plant sample was combined with it and according to formula of % cytotoxicity no result was obtained in case of negative control. On comparing these results of different explants (leaf and stem), it was seen that there was dose dependent increase in % cytotoxicity as presented in Fig. 1. Previous studies which was carried on fruits of *Piper longum* to check its anticancerous activity also showed dose dependent increase in the % cytotoxicity but it was only for *in vivo* studies [8]. In case of cytotoxicity activity highest % obtained for both control and test sample were compared in each case i.e for leaf and stem for both *in vivo* and *in vitro*. Same was done in case of antibacterial activity and antioxidant activity.

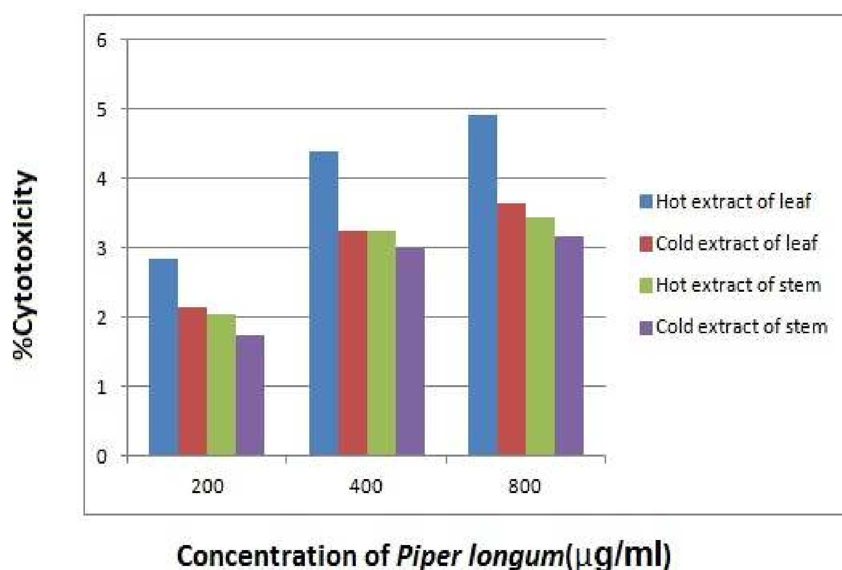

Fig. 1. Comparison of *in vivo* results of anticancerous activity of *Piper longum*

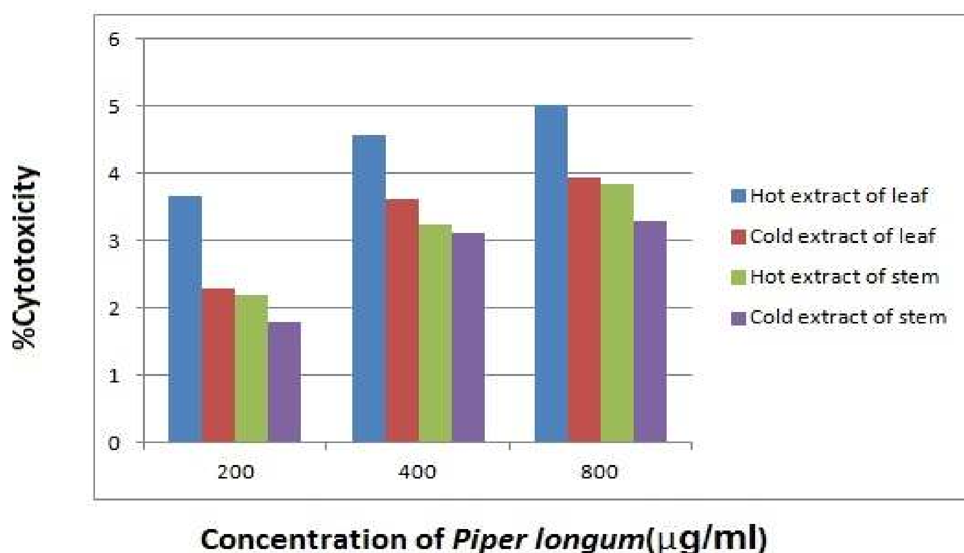

Fig. 2. Comparison of *in vitro* results of anticancerous activity of *Piper longum*

Table 1. Comparision of % cytotoxicity of hot and cold extract of leaf/stem *in vivo* and *in vitro*

	Hot extract and leukemic cell line K562 (*In vivo/In vitro)*			Cold extract and leukemic cell line K562 *(In vivo/In vitro)*		
	200 µg/ml	**400 µg/ml**	**800 µg/ml**	**200 µg/ml**	**400 µg/ml**	**800 µg/ml**
Leaf	3.68%/2.84%	4.58%/4.40%	5.03%/4.91%	2.28%/2.13%	3.63%/3.24%	3.94%/3.65%
Stem	2.18%/2.04%	3.25%/3.25%	3.84%/3.45%	1.80%/1.74%	3.12%/2.98%	3.29%/3.16%

Table 2. Comparision of % inhibition of hot and cold extract of leaf/ stem *in vivo* and *in vitro*

		Hot extract and cell suspension *(in vivo / in vitro)*			Cold extract and cell suspension (*in vivo/in vitro)*		
	Dilutions	**1:1**	**1:2**	**1:4**	**1:1**	**1:2**	**1:4**
	Bacteria						
Leaf	*E. coli*	4.32%/4.54%	3.69%/3.80%	2.25%/2.55%	3.36%/4.37%	2.19%/3.19%	1.56%/2.40%
	B. subtilis	5.18%/5.99%	3.17%/3.85%	2.17%/2.54%	4.49%/4.98%	2.81%/3.32%	2.08%/2.25%
	S. aureus	1.64%/1.27%	1.36%/0.14%	1.17%/0.71%	1.29%/1.05%	1.41%/0.18%	1.14%/0.76%
Stem	*E. coli*	5.36%/6.79%	3.68%/4.28%	2.23%/2.75%	4.34%/6.10%	2.95%/4.04%	1.89%/2.33%
	B. subtilis	4.23%/4.92%	3.45%/3.80%	2.72%/3.15%	3.93%/4.43%	3.21%/3.30%	2.41%/3.08%
	S. aureus	1.79%/1.24%	1.53%/0.53%	1.21%/0.31%	1.68%/1.49%	1.34%/0.18%	1.24%/1.32%

Table 3. Comparision of % TPC and % DPPH radical scavenging capacity of hot methanolic leaf/stem extract *in vivo* and *in vitro*

	Hot methanolic extract(µg/ml) *in vivo/in vitro* % TPC			Hot methanolic extract(ml) *in vivo/in vitro* % DPPH				
	200 µg/ml	**400 µg/ml**	**800 µg/ml**	**1 ml**	**2 ml**	**3 ml**	**4 ml**	**5 ml**
Leaf	37.83%/38.78%	42.42%/43.53%	43.75%/44.75%	10.40%/12.40%	9.60%/10.85%	4.0%/8.52%	3.20%/4.65%	1.60%/5.10%
Stem	36.64%/37.86%	39.77%/41.83%	41.42%/43.24%	6.4%/10.85%	7.2%/9.30%	2.4%/6.20%	1.6%/2.32%	0.8%/1.55%

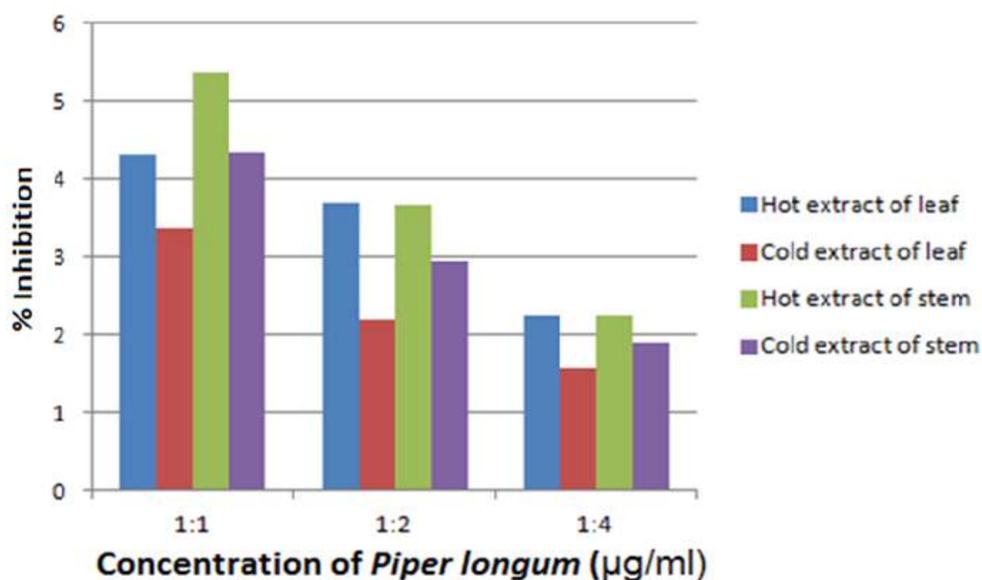

Fig. 3. Comparison of *in vivo* results of antibacterial activity of leaf and stem for *Escherichia coli*

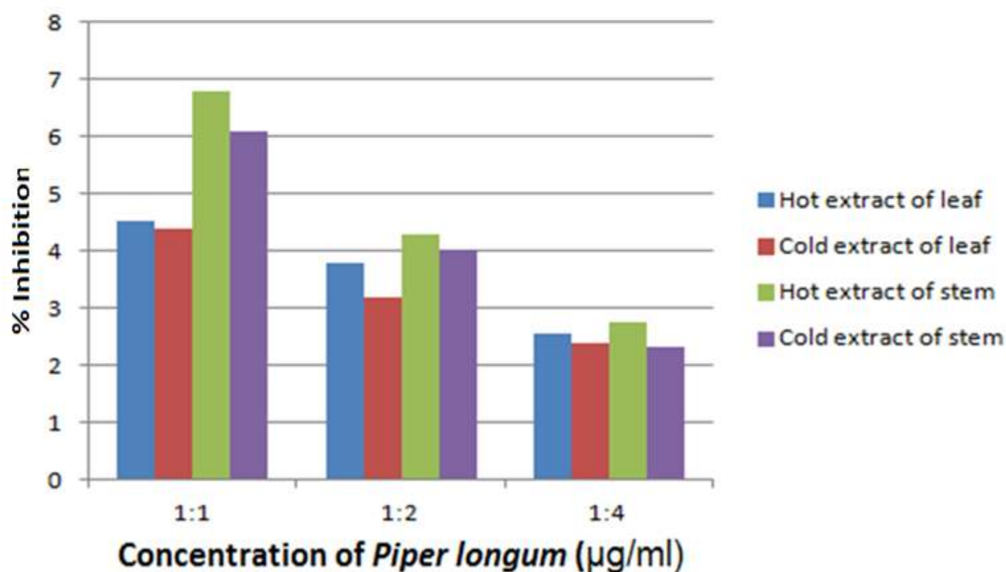

Fig. 4. Comparison of *in vitro* results of antibacterial activity of leaf and stem for *Escherichia coli*

Previous studies which was carried on fruits of *Piper longum* to check its antibacterial activity also showed dose dependent increase in the % of inhibition, but in case of fruit extract there was no *in vivo* and *in vitro* comparison [12,13,14].

In the present work, in case of antibacterial activity there was dose dependent increase in % inhibition, Ampicillin was taken as positive control, this antibiotic has antibacterial activity for all three bacteria used. It is used against *Piper longum* as control to check the extent of antibacterial activity of its explants. Comparative results of control and test sample are presented in result section.

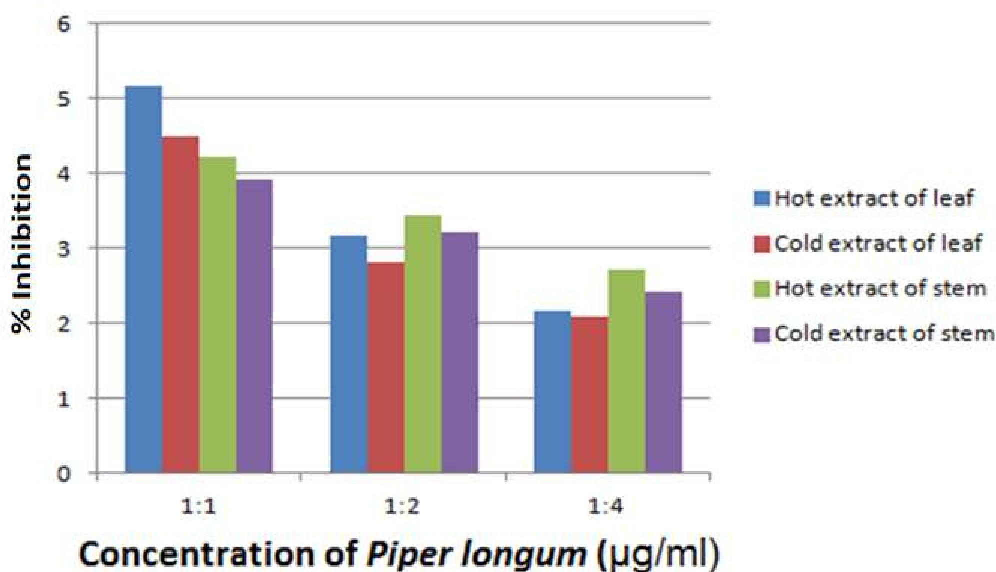

Fig. 5. Comparison of *in vivo* results of antibacterial activity of leaf and stem for *Bacillus subtilis*

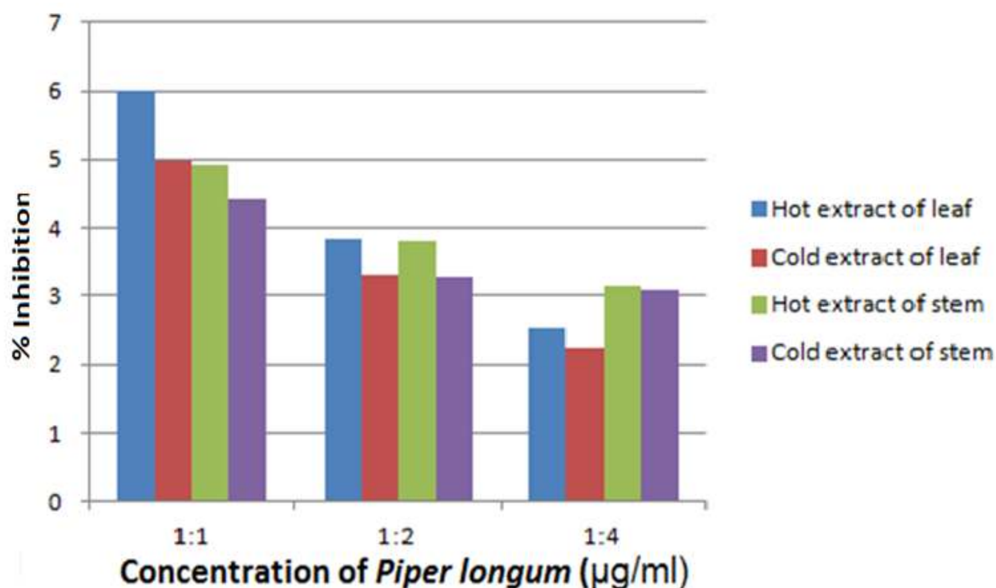

Fig. 6. Comparison of *in vitro* results of antibacterial activity of leaf and stem for *Bacillus subtilis*

In the case of control, two percentages were shown in the above data as two different explants that is leaf and stem were considered, for which the positive control were pipette two times in different plates, one for hot extract of leaf and other for hot extract of stem. Similarly, it is done for cold extract of leaf and stem for all the three bacteria. In the case of test sample two percentages were shown in the above data as two different explants that is leaf and stem were considered in the case of hot and cold extract for all the three bacteria. The results indicated that hot and cold extract of explants of *Piper longum* for both *in vivo* and *in vitro* cases for all three bacteria that is *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* showed less % of

inhibition then their respective positive control. Further *in vitro* results of test samples were better than *in vivo* results, moreover the results of *Escherichia coli* and *Bacillus subtilis* were better than that of *Staphylococcus aureus* which is presented in Figs. 3, 4, 5, 6, 7 and 8.

Antioxidant property was determined by estimation of Total Phenolic Content (TPC) [15]. In the present work, control sample was considered which was methanol and no plant sample was combined with it and according to formula of % TPC no result was obtained in this case this was negative contol. Same condition was in case of DPPH that is no result was obtained for control. There was dose dependent increase in %TPC for hot methanolic leaf and stem extract. In previous studies there was also increase in % TPC [8]. Antioxidant property was also determined on the basis of the ability of DPPH to scavenge free radicals [16]. In present work, results showed that at low

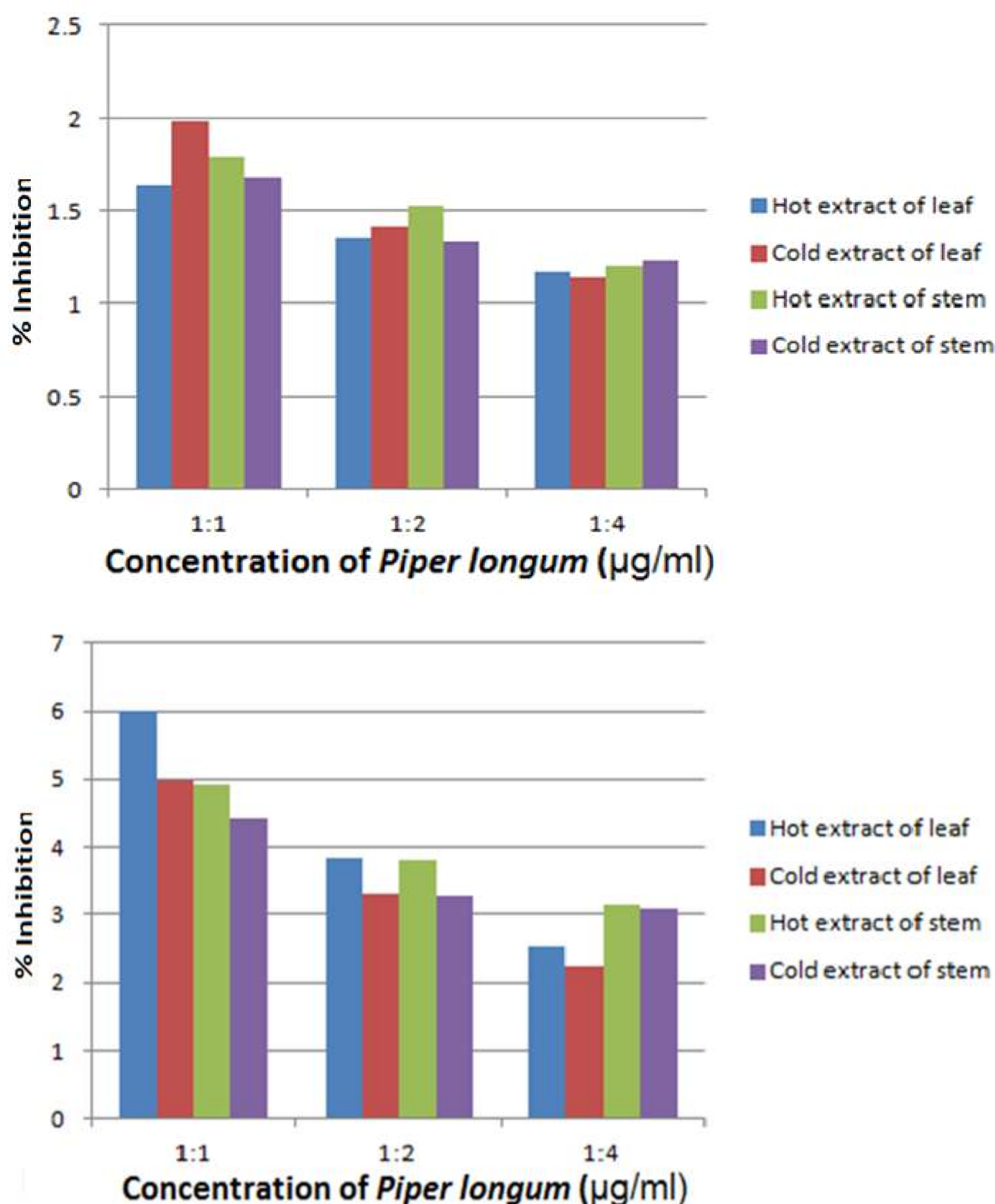

Fig. 7. Comparison of *in vivo* results of antibacterial activity of leaf and stem for *Staphylococcus aureus*

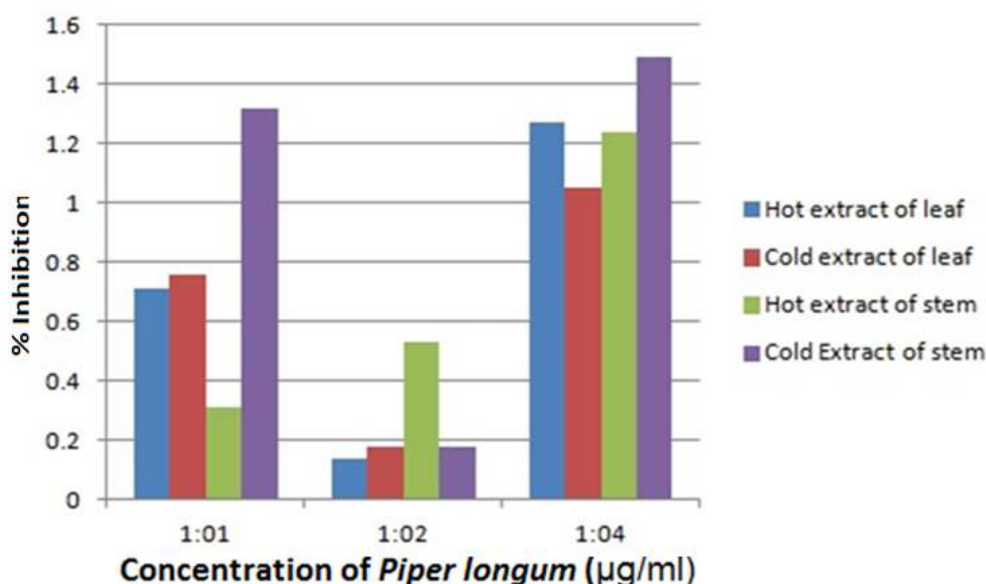

Fig. 8. Comparison of *in vitro* results of antibacterial activity of leaf and stem for *Staphylococcus aureus*

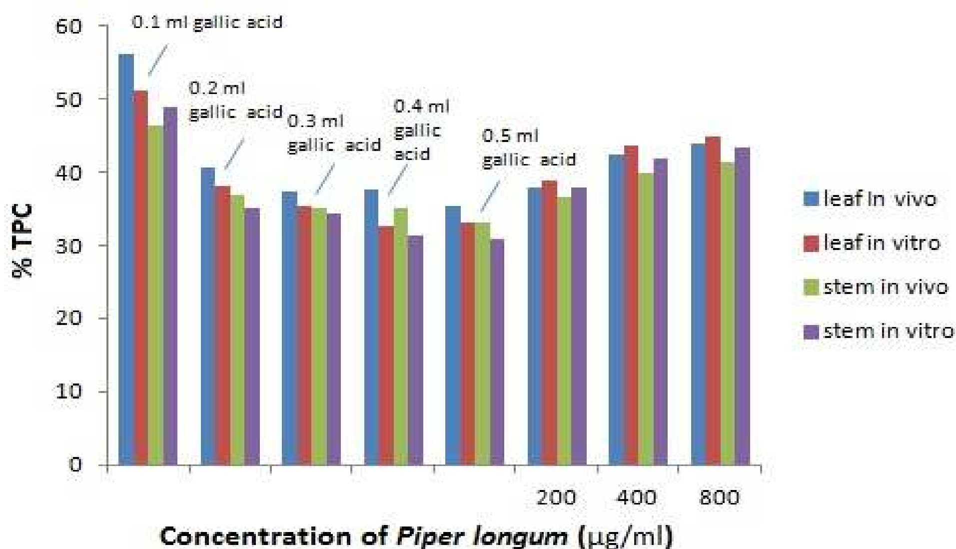

Fig. 9. Comparison of *in vivo* and *in vitro* results of antioxidant activity of TPC for *Piper longum*

concentration of leaf and stem extract, % DPPH radical scavenging capacity is more as compared to high concentration of leaf and stem extract. Comparison between %TPC and % DPPH *in vivo* and *in vitro* results shows that *in vitro* results were better than *in vivo* results which is presented in Fig. 9 and Fig. 10, but it was different in case of previous studies carried on fruits of *Piper longum*, in that there was dose dependent increase in % DPPH radical scavenging capacity [17,18].

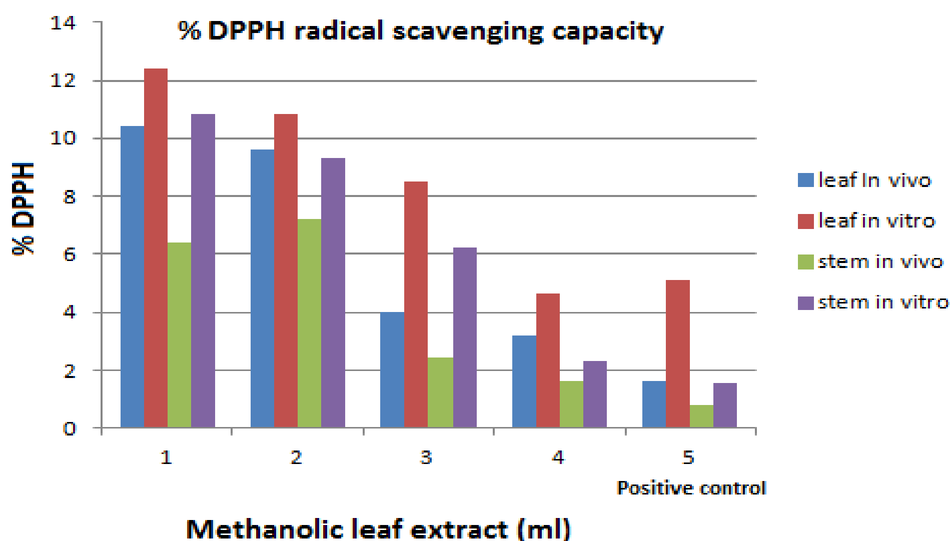

Fig. 10. Comparison of *in vivo* and *in vitro* results of antioxidant activity of DPPH radical scavenging capacity for *Piper longum*

5. CONCLUSION

It was concluded by the findings that the plants possessing medicinal properties are of great importance to mankind, as by determining its anticancerous, antibacterial and antioxidant properties many diseases can be treated, information about all the above properties of *in vivo* and *in vitro* explants (leaf and stem) of *Piper longum* for hot and cold extracts helps to know about plant's medicinal uses in each case, further the difference in, *in vivo* and *in vitro* results makes clear that there is importance of tissue culture, i.e *in vitro* grown callus obtained, yield better results for all above medicinal properties .Results concluded that there is dose dependent increase in percentage for all the properties in both *in vivo* and *in vitro* cases except DPPH radical scavenging capacity. In my research work there was low % cytotoxicity in case of anticancerous activity and low % of inhibition in case of antibacterial activity thus minor results were obtained. This protocol can further be used to determine the above mentioned properties to obtain better results.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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