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In vitro bioactivity screening of methanolic extracts of *Artocarpus heterophyllus, Artocarpus altilis* and *Piper betle*

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Abstract
The aim of this work was to evaluate the antimicrobial, haemagglutination, cytotoxic, and phytotoxic activity of the selected plants namely *Artocarpus altilis*, *Piper betle* and *Artocarpus heterophyllus*. The leaves of *Artocarpus altilis* and *Artocarpus heterophyllus* and roots of *Piper betle* were subjected to methanolic solvent extraction and *in vitro* bioactivity screening for the presence of various bioactive constituents. The haemagglutination activity of the extracts were tested with human erythrocytes of Rh + and Rh – types of the blood groups A, B, AB and O. Haemagglutination was tested using tube agglutination method. Antimicrobial screening of the methanolic plant extracts was performed by the standard disc diffusion method. The organisms used in this assay were two Gram positive strains of microorganisms namely *Staphylococcus aureus*, *Bacillus subtilis* and two Gram negative strains namely *Escherichia coli*, *Pseudomonas aeruginosa* and four fungal strains were selected for antifungal screening: *Aspergillus niger*, *Aspergillus flavus, Penicillium* sp., *Rhizopus* sp. Brine shrimp larvae were used to determine the cytotoxicity activity of the selected methanolic plant extracts. Phytotoxicity screening was done by seed germination assay. The results indicated that all the selected plants possessed significant bioactivity and were not phytotoxic and cytotoxic.

Keywords: Bioactivity, Artocarpus altilis, Piper betle, Artocarpus heterophyllus, haemagglutination, antimicrobial

1. Introduction
Plants have served mankind since ages as they are reservoirs of important medicinal components and help to alleviate chronic diseases. The past was considered the synthetic era due to the commercial production of large varieties of synthetic drugs by pharmaceutical industries. Over time the continuous use of synthetic drugs caused severe side effects, and led to resistance of microbes. Also synthetic drugs are expensive and large populations cannot afford to get benefit from these drugs. During the last decades a global trend with focus on green medicines due to minimum side effects and cost effectiveness. Medicinal plants play an appreciable role in the development of modern herbal medicines as many diseases like cancer, liver diseases and arthritis find no complete cure in allopathy. The bioactive compounds of medicinal plants are used as chemotherapeutic, anti-inflammatory, anti-arthritic agents where no satisfactory cure is present in modern medicines [1].

Many plants have shown their immense potential to fight against dreadful diseases including cancer. Plants produce a wide variety of secondary metabolites with different functions like growth regulation, defense against infections and predations etc. Most important secondary metabolites include polyphenols, alkaloids, saponins, steroids etc. Many of these natural compounds have great medicinal importance due to their interesting biological and pharmacological actions. Polyphenols form one of the most common and widespread groups of compounds in flowering plants and occur in all vegetative organs as well as in flowers and fruits [2].

Herbal medicine is also called botanical medicine or phytomedicine. It refers to using a plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes. Herbalism has a long tradition of use outside of conventional medicine. It is becoming more main stream as improvements in analysis and quality control along with advances in clinical research show the value of herbal medicine in the treating and preventing disease. Plants had been used for medicinal purposes long before recorded history. Indigenous cultures used herbs in their healing rituals, while others developed traditional medical systems in which herbal therapies were used. Researchers found that people in different parts of the world tended to use the same or similar plants for the same purposes [3].

Piper betle, commonly called betel plant belongs to the piperaceae family and originated from South and South East Asia. It is valued both as a mild stimulant and for its medicinal properties. The betle plant is an evergreen and perennial creeper with glossy heart shaped leaves and white catkin. The betle plant is one of the highly investigated plants and phytochemical studies reveal that *Piper betle* contains a wide variety of biologically active compounds whose concentration is dependent on the variety of the plant, climate and season. The aroma of betle leaf is due to the presence of essential oils comprising of phenols and terpenes. For a very long time the leaves and the stalk of the betle plant have been used to treat various ailments in the traditional Indian system of medicine and also in various folk medicines in Southeast Asia. Preclinical experiments have shown that betle leaf possess immunomodulatory, anti-bacterial, anti-carcinogenic, anti-fungal, anti- larval, anti- protozoa, anti-filarial, anti-allergic, anti-diabetic, anti-inflammatory, hepatoprotective, anti-ulcer, anti-fertility, cardioprotective, anti hyperlipidaemic, anti-platelet, vasorelaxation effects [4].

Artocarpus heterophyllus is commonly known as the jackfruit tree and belongs to the family Moraceae, originally native to the Western Ghats of India. The fruits are of dietary use and

are an important source of minerals, vitamins carbohydrate, protein and fat. The leaves, fruit, bark and roots have diverse medicinal properties and are used in the various traditional and folk systems of medicine to treat a variety of ailments. Preclinical studies have shown that jackfruit possesses antibacterial, anticariogenic, antioxidant, anti-inflammatory, antifungal, hypoglycemic and antineoplastic and wound healing. Clinical studies have also shown that the decoction of the leaves possesses hypoglycemic effects in both healthy individuals and non-insulin dependent diabetic patients [5].

Breadfruit (*Artocarpus altilis*) is a species of flowering tree belonging to the mulberry family, Moraceae. It grows throughout Southeast Asia, most Pacific Ocean islands and widely distributed over the tropical regions of Asia including Thailand. It is mainly grown for edible fruits and parts of this plant are also utilized as traditional medicine. The roots have been used as a component in folk remedies for cancer and venereal diseases, the stems and roots have been used traditionally for the treatment of hypertension and liver cirrhosis [5]. Recent interest in polyphenolic compounds and their bioactivities prompted this research work to explore the potential beneficial health properties of many indigenous medicinal plants. The present study demonstrates a modern strategy with combination of local and modern knowledge for evaluation of biological activity of medicinal plant species.

2. Materials and Methods

2.1 Collection of plant material and preparation of plant extracts

Middle aged leaves of *Artocarpus heterophyllus,* fruit of *Artocarpus altilis* and roots of *Piper betle* were collected from South Bangalore, Karnataka and used for the extraction of bioactive compounds. They were positively identified from the Botany Department of Mount Carmel College, Bengaluru. The plant materials were washed thoroughly with distilled water. They were shade dried, powdered and stored in air tight containers for extraction of phytochemicals. The plant materials used to prepare extract containing bioactive compounds were subjected to solvent extraction using 300mL of 80% methanol. The extract was then filtered and concentrated using rotary vacuum evaporator at 45-50 °C and stored at 4°C for further investigations.

2.2 Antimicrobial assays

Antimicrobial screening of the methanolic plant extracts was performed by the standard Disc Diffusion Method [6]. Following are the steps involved in this assay:

Evaluation of anti-bacterial activity of plant extracts

Bacterial strains

The organisms used in this assay were two Gram positive strains of microorganisms namely *Staphylococcus aureus*, *Bacillus subtilis* and two Gram negative strains namely *Escherichia coli*, *Pseudomonas aeruginosa*. The organisms were cultured and pure cultures were maintained in the Centre for research and advanced studies, Mount Carmel College, Bangalore.

Preparation of media

Mueller Hinton Agar medium (MHA) was used for determining antibacterial activity. The media was prepared as per the standard instruction provided by the HI-MEDIA Laboratories Pvt. Ltd., Mumbai. Mueller Hinton agar medium was prepared and sterilized and 20 mL of this pre autoclaved medium was poured into 90 mm diameter pre sterilized petriplates. The media was allowed to solidify at room temperature. To test the sterility the media, the plates were incubated overnight in oven at 37°C. The plates without any contamination were further selected for experiments.

Screening of antibacterial activity

1000 µL of freshly prepared microbial broth culture suspension was spread over the MHA using L- shaped sterilized glass spreader separately under aseptic condition in the laminar air flow. The different concentrations of the methanolic plant extracts were prepared by dissolving extracts in the range 5, 10, 15, 20, 25, 50, 75, 100 mg in 1mL double distilled water to get the concentrations in mg/ml. 10µl of each solution was applied on 6mm sterile discs. Thus the discs had 50, 100, 150, 200, 250, 750,500, 1000 µg/disc of plant extracts. The soaked discs were placed on the surface of the inoculated plate and allowed to dry in sterilized laminar hood. These plates were incubated at 37ºC for 24 hours in an inverted position. 500 µg/disc of antibiotic streptomycin was used as positive control and 1% DMSO was taken as negative control to compare the activity. The zone of inhibition of plant extracts was measured in (mm) and compared with the zone of inhibition of standard antibiotic streptomycin.

Evaluation of anti fungal activity of plant extracts

Fungal Strains

The following four fungal strains were selected for antifungal screening:

Aspergillus niger, Aspergillus flavus, Penicillium sp., *Rhizopus* sp. The organisms were cultured and pure cultures were maintained in the Centre for research and advanced studies laboratory, Mount Carmel College, Bangalore.

Preparation of media

Potato dextrose agar medium was used for screening the antifungal activity. 20 g of glucose, 20 grams of potato starch and 20 g of agar agar was dissolved in 1 L double distilled water in conical flasks, cotton plugged and autoclaved at 121°C for 15 minutes. The sterilized medium was then poured into petriplates and allowed to cool and solidify and incubated overnight at 37°C to check for contamination. The plates without any contamination were selected for further experimentation.

Screening of antifungal activity

Different concentration of the methanolic plant extracts were prepared as mentioned in the antibacterial assay. Inoculated fungal culture was poured into the medium. 6mm sterile filter paper discs with different concentrations of the plant extracts and 500 µg/disc of standard antifungal drug, fluconazole were placed on the medium. These plates were incubated at 37°C for 3 to 4 days. The zone of inhibition of plant extracts was measured in (mm) and compared with the zone of inhibition of standard antifungal drug, fluconazole. The experiment with each sample was carried out in triplicates and the average zone of inhibition was recorded in mm data was expressed as mean ± SD.

2.3 Detection of lectins and evaluation of haemagglutination activity

The methanolic plant extracts (200 mL) were mixed with 200 mL of NaCl solution (0.15 mol/ L) containing $CaCl_2$ (0.1 mol/ L) and $MnCl_2$ (0.1 mol/ L).Then, 100 mL of human erythrocytes (2%) were added to the mixture and incubated at 37ºC for 30 minutes and maintained at room temperature for 30 min. Reaction mixture was centrifuged at 8,000 x g and analyzed for the presence of erythrocyte agglutination. The haemagglutination activity of the extracts were tested with human erythrocytes of Rh + and Rh – types of the blood groups A, B, AB and O. Haemagglutination was tested using tube agglutination method. Haemagglutination activity was investigated serially in different dilutions of each extract against all the blood groups. 1 mL of each dilution was added to 1ml of 2% suspension of RBC taken in test tubes and incubated at 25°C for 15-20 minutes [7]. Controls were also tested simultaneously. Formation of smooth depositions of erythrocytes at the bottom of the tube indicates negative activity whereas granular depositions formed due to haemagglutination indicates a positive reaction. Strong, moderate and weak agglutination reactions were determined based on the amount of granular deposition.

2.4 Brine shrimp cytotoxicity assay

Brine shrimp larvae were used to determine the cytotoxicity activity of the selected methanolic plant extracts. Each extract was tested at 10, 100 and 1000 µg/mL concentration prepared in dimethylsulfoxide. Brine shrimp eggs (*A. salina*) were purchased aquarium stores and hatched in artificial sea water using a solution of 3.8% sodium chloride at room temperature in a small tray. Illumination was provided on one side to attract newly hatched larvae. After 48 hours, the larvae were collected and used for the bioassay. Ten Brine shrimp larvae, 5 mL sea water and 1 mL plant extracts were added to the vials. Two other vials were prepared with Potassium dichromate (23.289 µg/mL) as positive control and solvent as negative controls. After 24 hours, surviving larvae were counted and the percent deaths at each dose were determined [8]. LC_{50} values were determined using the probit analysis method. The experiment was repeated three times. Percentage of mortality was also calculated at all concentrations.

2.5 Phytotoxicity assay

Solutions of each extract were tested at a concentration level of 500 µg/mL and 1000 µg/mL prepared using double glass distilled water. Whatman no: 1filter papers were placed in petri plates, moistened with 5mL of each concentration of extracts. Filter paper was dried at room temperature for reducing extra solvent. To each Petri plate, 5mL double distilled water was added. Ten pea seeds surface sterilized with 0.1% mercuric chloride were placed on these petriplates. The plates were tightly sealed with parafilm to avoid moisture loss and incubated at 25°C. 5mL of double distilled water was used as the control [9]. The experiment was repeated in triplicates. After ten days the germination percentage was determined and root, shoot length were measured. The experiments were carried out in strict aseptic conditions.

3. Results and Discussion

3.1 Evaluation of antimicrobial activity of plant extracts

There are many important reasons to screen for novel alternative antimicrobial substances from natural sources mainly plants. The toxicity and side effects of the drugs presently used in health care and medicine being a major area of concern. The generation of drugs in plenty from natural sources with more efficacy, low cost of production and low or negligible side effects has become a prime focus of the pharmacological industry. All over the world and also in the developing countries, most infectious bacterial diseases have resulted in the death of individuals. These organisms include Gram positive and Gram negative bacteria like different species of *Staphylococcus, Bacillus, Pseudomonas and Salmonella* which cause severe infections in humans [13]. The development of synthetic antibiotics have been successful in eliminating these organisms to an extent but pose the limitations like development of drug resistance by microorganisms, high cost and adverse side effects on the host. The continuous emergence of novel infections and the microorganisms developing resistance make already existing antibiotics less effective. In such scenarios, natural products which are a part of our daily diet serve as the best candidates for new antibacterial drug discovery. Phenolic compounds have various defensive functions in plants which include cell wall strengthening and repair and antimicrobial activities.

The methanolic extract of *Artocarpus heterophyllus* exhibited significantly higher growth inhibition of microorganisms compared to *Artocarpus altilis* and *Piper betle* extracts at different concentrations and showed maximum inhibition zones at 1000 µg /disc (Table1). Zone of inhibition was found highest against *B. Subtilis* (35.000±1.000 mm) followed by *E. coli and S. aureus* (30.000±1.000 mm). Moderate antibacterial potential was observed against *P. aeruginosa* (26.000±1.000 mm). The methanolic extract of *Artocarpus altilis* significantly inhibited the growth of microorganisms at different concentrations and showed maximum inhibition zones at 1000 µg /disc. Zone of inhibition was found highest against *B. Subtilis* (31 ± 1.0 mm) followed by *E. coli* (24.333±0.577).

Moderate antibacterial potential was observed against *S. aureus* (20.000±1.000 mm zone of inhibition) and *P. aeruginosa* (18.000±1.000 mm). The methanolic extract of *Piper betle* significantly inhibited growth of microorganisms at different concentrations and showed maximum inhibition zones at 1000 µg/disc. Zone of inhibition was found highest against *B. Subtilis* (32.000±1.000 mm) followed by *S. aureus* (28.000±1.000 mm) and *E.coli* (28.000±1.000 mm). Moderate antibacterial potential was observed against *P. aeruginosa* (25.000±1.000 mm). These results were compared with a standard antibiotic and it revealed that the antibacterial activities of the conventional antibiotics were higher than that of the plant extracts at the same concentration in accordance to literature [14].

Table 1: Antibacterial activity of *Artocarpus altilis, Artocarpus heterophyllus* and *Piper betle* extracts

Plant Extracts	Zone of inhibition in mm				
	Concentration (µg/disc)	***Staphylococcus aureus***	***Bacillus subtilis***	***Escherichia coli***	***Pseudomonas aeruginosa***
Artocarpus altilis	250	14.000±1.000	16.000±1.000	16.333±0.764	11.000±1.000
	500	17.000±1.000	18.000±1.000	18.000±1.000	14.000±1.000
	750	19.000±1.000	22.667±1.528	20.000±1.000	17.000±1.000
	1000	20.000±1.000	31.000±1.000	24.333±0.577	18.000±1.000
Artocarpus heterophyllus	250	22.000±1.000	19.000±1.000	16.000±1.000	14.000±1.000
	500	27.000±1.000	22.000±1.000	19.000±1.000	19.000±1.000
	750	29.000±1.000	28.000±1.000	24.000±1.000	21.000±1.000
	1000	30.000±1.000	35.000±1.000	30.000±1.000	26.000±1.000
Piper betle	250	20.000±2.000	17.000±1.000	17.000±1.000	17.000±1.000
	500	22.000±2.000	21.000±1.000	19.833±0.764	18.000±1.000
	750	26.000±1.000	24.000±1.000	27.733±0.643	23.000±1.000
	1000	28.000±1.000	32.000±1.000	28.000±1.000	25.000±1.000
Standard antibiotic	500	35.333±1.528	40.000±2.082	35.333±2.000	33.333±2.517

Values are mean ± SD of triplicate analysis

All the three plant extracts showed antifungal activity at different concentrations and showed maximum inhibition zones at 1000 µg /disc (Table 2). *Artocarpus heterophyllus* extract strongly inhibited the growth of tested fungal strains at all concentrations compared to *Artocarpus altilis* and *Piper betle* extracts. *Artocarpus heterophyllus* extract demonstrated excellent antifungal potential and inhibited the growth of *Aspergillus niger* (29.000 ± 1.000 mm zone of inhibition), *Aspergillus flavus* (26.000 ± 1.000 mm zone of inhibition), *Penicillium* sp. (24.000± 1.000 mm zone of inhibition), *Rhizopus* sp. (22.667 ± 0.577 mm zone of inhibition) at the concentration of 1000 µg / disc.

Piper betle extract was second most effective antifungal extract and showed zone of inhibition of 24.000±1.000, 23.000±1.000, 21.000±1.000, 20.000±1.000 mm against *A.niger, A.flavus, Penicillium* sp., *Rhizopus* sp. respectively. *Artocarpus altilis* extract demonstrated moderate activity and was found to be comparatively the least effective with a zone of inhibition of 20.000± 1.000, 18.000± 1.000, 17.000± 1.000, 16.000 ±2.000 mm against *A.niger, A.flavus, Penicillium* sp., *Rhizopus* sp. respectively. These results were compared with a standard antifungal drug and it revealed that the antifungal activities of the conventional antifungal drugs were higher than that of the plant extracts at the same concentration in accordance to literature [15].

Table 2: Antifungal activity of *Artocarpus altilis, Artocarpus heterophyllus* and *Piper betle* extracts

Plant Extracts	Zone of inhibition in mm				
	Concentration (µg/ disc)	***Aspergillus niger***	***Aspergillus flavus***	***Penicillium* sp.**	***Rhizopus* sp.**
Artocarpus altilis	250	13.000 ± 2.000	11.000 ± 2.000	10.000 ± 2.000	10.000± 2.000
	500	16.000 ± 2.000	14.000 ± 2.000	13.000 ± 2.000	12.000 ±1.000
	750	17.000 ± 2.000	16.000 ± 2.000	15.000 ± 2.000	14.000± 1.000
	1000	20.000± 1.000	18.000± 1.000	17.000± 1.000	16.000 ±2.000
Artocarpus heterophyllus	250	18.000± 2.000	16.000± 2.000	14.000 ± 2.000	15.000 ± 1.000
	500	23.000 ± 1.000	18.000 ± 1.000	17.000 ± 2.000	18.000 ±1.000
	750	27.000 ± 1.000	22.000 ± 1.000	20.000 ± 2.000	20.000 ± 2.000
	1000	29.000 ± 1.000	26.000 ± 1.000	24.000± 1.000	22.667 ± 0.577
Piper betle	250	14.167±0.289	10.000±1.000	10.000±1.000	12.000±1.000
	500	16.500±0.500	17.000±1.000	14.000±1.000	16.000±1.000
	750	20.667±0.764	20.000±1.000	17.000±1.000	18.000±1.000
	1000	24.000±1.000	23.000±1.000	21.000±1.000	20.000±1.000
Standard antifungal drug	500	35.000±2.000	30.000±2.000	28.000±1.000	38.000±1.000

Values are mean ± SD of triplicate analysis

3.2 Detection of lectins and evaluation of haemagglutination activity

Lectins are a class of proteins of that bind carbohydrates without modifying them. Originally, the term lectin was restricted to soluble, multivalent proteins capable of agglutination and historically was limited to proteins of plant origin. However, today the term lectin is used in a broad sense to denote all types of carbohydrate-binding proteins that do not catalyze reactions with their ligands. There are numerous lectins available in the nature, and the fact that they can be easily prepared in the purified form and accessible for chemical manipulation makes them potential tools for biological research. The applications of lectins ranges from identification of microorganisms, cell surface biology studies to cancer research and they serve as probes for the characterization and isolation of simple and complex sugars [16]. The studies on detection of lectins revealed that in the methanolic extracts of *Artocarpus heterophyllus*, the lectins

were present in abundance; *Artocarpus altilis* extract had lectins in trace amounts whereas it was absent in *Piper betle* extract (Table 3). The haemagglutination activity tests done on the human blood groups A, B, AB and O showed that the methanolic extracts of *Artocarpus heterophyllus* exhibited strong haemagglutination activity, *Artocarpus altilis* extract showed weak agglutination and *Piper betle* extract showed no agglutination (Table 4).

Table 3: Detection of lectins in plant extracts

***Artocarpus heterophyllus* extract**	***Artocarpus altilis* extract**	***Piper betle* extract**
+++	+	---

+ = Trace, +++ = Abundant, --- = Absent

Table 4: Evaluation of haemagglutination activity of plant extracts

Blood Groups	**Dilutions**											
	Artocarpus heterophyllus				***Artocarpus altilis***				***Piper betle***			
	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16
A +	+++	++	+	-	+	-	-	-	-	-	-	-
A -	+++	++	+	-	+	-	-	-	-	-	-	-
B +	+++	++	+	-	+	-	-	-	-	-	-	-
B-	+++	++	+	-	+	-	-	-	-	-	-	-
AB +	+++	++	+	-	+	-	-	-	-	-	-	-
AB -	+++	++	+	-	+	-	-	-	-	-	-	-
O +	+++	++	+	-	+	-	-	-	-	-	-	-
O -	+++	++	+	-	+	-	-	-	-	-	-	-

-: No agglutination, +: Weak agglutination, ++: Moderate agglutination, +++: Strong agglutination

The results therefore clearly confirm the presence or absence of lectins in the extracts. Various studies have shown that plants that are rich in anti-nutritional compounds like lectins possess antimicrobial activity against a number of microorganisms. Antimicrobial activity of lectins is an interesting and important topic of study because of the abundant prevalence of pathogenic microorganisms in the environment. Plant sources can be used to reduce the pathogenic effect of such microorganisms as they are eco-friendly and do not cause toxicity to the environment.

3.3 Brine shrimp cytotoxicity assay

The brine shrimp lethality assay represents a simple, rapid and inexpensive bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxicity and anti tumor properties. The assay is considered a useful tool for preliminary assessment of toxicity and it has been used for the detection of plant extract toxicity, fungal toxins, heavy metals, pesticides etc [17]. In the present study the brine shrimp lethality of extracts of the three extracts was determined using the procedure of McLaughlin [9]. The extracts with LD_{50} values higher than 1000 µg/mL were considered to possess low toxicity whereas those with LD_{50} values lower than 1000 µg/mL were thought to possess significant toxicity. All the three extracts showed LD_{50} values greater than 1000 µg/mL thereby indicating that they do not possess cytotoxic activity (Table 5).

Table 5: Percentage mortality of brine shrimps at different concentrations of plant extracts and respective LC_{50} values

Crude methanolic plant extracts	**Mortality (%)**			**LC_{50} (µg/ml)**
	1000 µg/ml	**100 µg/ml**	**10 µg/ml**	
Artocarpus heterophyllus extract	26%	18.3%	10%	>1000
Artocarpus altilis extract	15.8%	12%	5.2%	>1000
Piper betle extract	32%	22%	14%	>1000

3.4 Phytotoxicity Assay

Pea seeds have been used in general toxicity studies because of their sensitivity to phytotoxic compounds and as a standard assay in allopathic studies. All the three extracts exhibited no toxicity in pea seed bioassay at 1000 µg/mL. The present study showed that there was no adverse effect on growth stimulation or inhibition. There was no pronounced effect on the shoot growth and root growth by the extracts indicating that the present extracts non-phytotoxic (Figs. 1, 2 and 3).

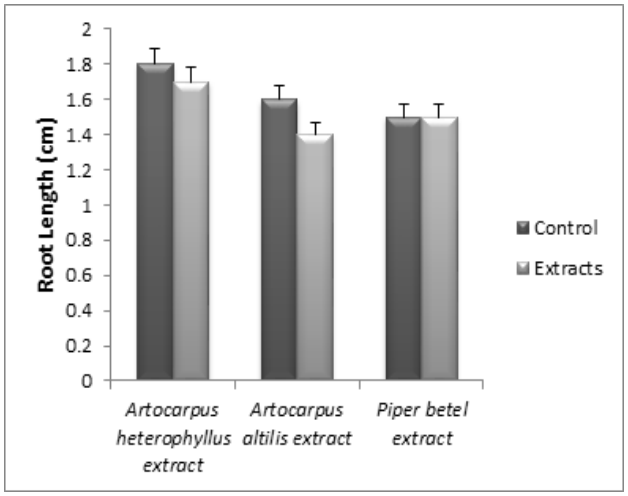

Fig 1: Effect of plant extracts on root elongation (Values are expressed as mean ± SD, n = 3)

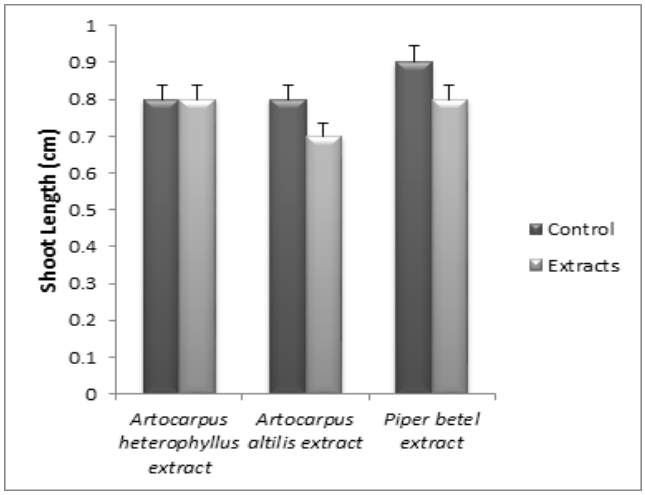

Fig 2: Effect of plant extracts on shoot elongation (Values are expressed as mean ± SD, n = 3)

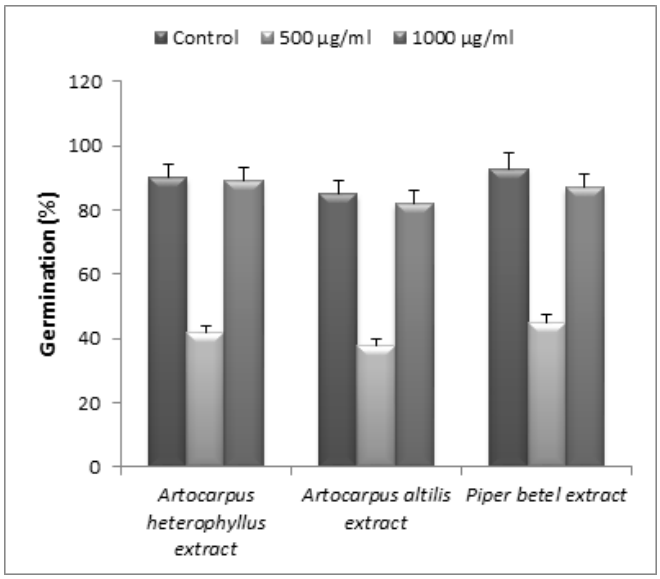

Fig 3: Effect of plant extracts on seed germination (Values are expressed as mean ± SD, n = 3)

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