

ANTIBACTERIAL ACTIVITY, *IN VITRO* ANTIOXIDANT ACTIVITY AND ANTHELMINTIC ACTIVITY OF ETHANOLIC EXTRACT OF *ANANAS COMOSUS L.* TENDER LEAVES.

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Summary

The antibacterial activity, *in vitro* antioxidant activity and anthelmintic activity from ethanolic extract of tender leaves of *Ananas comosus L.* were investigated in the present study. Antibacterial activity of the extract was evaluated against four bacterial strains at 50 and 100 mg/ml concentrations. A marked inhibitory effect was observed against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi* except *Bacillus subtilis*. In addition, reducing power assay, determination of total antioxidant activity and DPPH radical scavenging activity were performed to evaluate the *in vitro* antioxidant activity of the extract. The results indicated higher antioxidant activity of the extract than  $\alpha$ -tocopherol but lower than that of BHA (Butylated hydroxyanisole). At the concentration of 250  $\mu$ g/ml, the extract showed noticeable DPPH radical scavenging activity. Anthelmintic activity of the extract was evaluated using adult Indian earth worms (*Pheretima posthuma*). And the results indicated a dose dependent increase in anthelmintic activity of the extract at 25, 50 and 100 mg/ml concentrations.

**Keywords:** *Ananas comosus*, Antibacterial assay, Antioxidant activity, Anthelmintic activity, DPPH radical scavenging.

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### Introduction

Search for new natural antioxidant has grown over the past years because of developments in biomedical point to the involvement of free radicals in many diseases. Reactive Oxygen Species (ROS) production and oxidative stress theory have been shown to be linked to different diseases such as cardiovascular diseases, cancer, osteoporosis and degenerative diseases [1]. Free radicals attack the unsaturated fatty acid in the biomembranes resulting in membrane lipid peroxidation, a decrease in membrane fluidity, loss of enzyme, receptor activity and damage to membrane protein which lead to cell inactivation or death [2]. The harmful and pathologic action of the free radicals can be reversed or blocked by antioxidant substances. Antioxidant substances scavenge the free radicals and detoxify the physiological system. Current research in the field of free radicals biology has indicated a confirmation that foods and beverages mounted with antioxidants play an essential role in the prevention of cardiovascular and neurodegenerative diseases and cancer [3-5]. Although there are two existing types of antioxidants viz natural and synthetic, due to carcinogenic probability of synthetic antioxidants, natural antioxidants are more preferred compounds therapeutically and nutraceutically. Therefore, development and utilization of more effective antioxidants of natural origin are being considered [6].

Plants have been used for maintaining human health and improving the quality of human life since ancient times. As food and beverages as well as in cosmetics, dyes and medicines; plants have been serving the human community since time immemorial. A large number of plants have been screened as a viable source of antibiotics, antioxidants and other therapeutic compounds on the basis of their traditional folklore medicinal use in different communities throughout the world population. Plants are still a valuable source for new pure compounds or leads in process of drug discovery and development. There has been increasing focus in recent years that several plants derived polyphenolic compounds may possess antimicrobial, antioxidant, anticancer and apoptosis inducing properties [7]. Plants are considered as rich source of antimicrobial agents. A wide range of plants and their parts is used for their medicinal properties by local communities and folk healers. Random screening as tool in discovering new biologically active molecules has been most productive and successful in the area of antibiotics [8, 9].

*Ananas comosus* L. (Bromeliaceae) is a herbaceous perennial plant 2½ to 5 ft high with a spread of 3 to 4 ft. It is essentially a short, stout stem with a rosette of waxy, strap like leaves. They have long pointed leaves and are 20-72 inch in length, usually needle tipped and generally bear sharp, up curved spines on the margins. They may be all green or variously striped with red yellow or ivory down the middle or near the margins. As the stem continues to grow, it acquires at its apex a compact tuft or stiff, short leaves called the crown or tip. It has been one of the most popular tropical or subtropical fruit [10, 11]. It is grown extensively in Hawaii, Philippines, Caribbean area, Malaysia, India, Thailand, Australia, Mexico, Kenya, South Africa and Hainan province of China. This plant is also known for its folk medicinal utility, besides agricultural utilities such the fruit for nutritional food. *Ananas comosus* L. was used as an indigenous medicinal plant in Thailand for the treatment of dysuria [12, 13]. The cortices of *A. comosus* L. served as alexipharmic, antitussive and antidiarrhea agents in China and some other countries. Its leaves were also used as antidyspepsia or antidiarrhea agents in Chinese Traditional Medicine [14]. Paste of tender leaves has been used traditionally as oral anthelmintic medicine in some parts of north-east India [15].

The present study was designed to examine antimicrobial and *in vitro* antioxidant activities of ethanolic extract of tender leaves of *Ananas comosus* L. Preliminary phytochemical screening of the ethanolic extract was also done along with the determination of total phenolic content. It was also of interest to find the anthelmintic activity of the extract against adult Indian earth worms to confirm the traditional folklore use of this plant in worm infestation.

### Materials and methods

**Plant material:** Tender leaves of *A. Comosus* L. were collected from Tinsukia district of Assam (India) and authenticated by Department of Botany, Doomdooma College, Dibrugarh University. A voucher specimen herbarium was deposited in the Pharmacognosy department of Abhilashi College of Pharmacy, Mandi, Himachal Pradesh for further reference.

**Drugs and chemicals:** Chemicals, such as Folin-Ciocalteu reagent, trichloroacetic acid (TCA), ethanol, ammonium thiocyanate, Dimethylsulphoxide (DMSO), Gallic acid, Tween 20,  $\alpha$ -tocopherol, Butylated hydroxyanisole (BHA) were purchased from E. Merck (India) Limited. 1, 1 Diphenyl- 2-picryl-hydrazyl (DPPH) was procured from Sigma, USA. Albendazole (BANDY, Mankind Pharma Ltd., New Delhi) was used as a standard antihelmintic drug. Petroleum ether, Tween 80 and all other chemicals and solvents used were of analytical grade available commercially (SRL Mumbai, Himedia, E.Merck India).

**Preparation of extracts:** The fresh and tender leaves of *Ananas Comosus* L. were shade dried followed by drying in hot air oven for 30 minutes at low temperature and then powdered. The powder was used for the ethanolic extraction. Ethanol 70% was used for the extraction. In the process of extraction, 500 gm of dried powder was extracted with 700 ml of ethanol 70% at 50 °C. A total of 60 cycles were run to obtain thick slurry. This slurry was then evaporated to yield a solid extract. The percentage yield of ethanolic extract was found to be 3.5 %.

**Preliminary Phytochemical screening:** Identification of the chemical constituents were carried out on the powdered drug and on the ethanolic extract using chemical methods according to the methodology proposed by Harborne 1984 [16].

**Determination of total phenolic content:** Total soluble phenolics in the leaf extract of *Ananas comosus* L. were determined with Folin–Ciocalteu reagent according to the method using gallic acid as a standard phenolic compound. About 1.0 ml of extract solution containing 10 mg extract in a volumetric flask was diluted with 46 ml of distilled water. About 1.0 ml of Folin–Ciocalteu reagent was added and mixed thoroughly. Three minutes later 3.0 ml of 2% sodium carbonate was added and the mixture was allowed to stand for 3 h with intermittent shaking. The absorbance of the mixture was measured at 760 nm in a spectrophotometer (UV -1601 Shimadzu, Japan). The concentration of total phenols was expressed as mg/g of extract [17]. The concentration of total phenolic compounds in the extract was determined as gram of gallic acid equivalent using an equation obtained from the standard gallic acid graph:

$$Y = 0.0026 x + 0.1203, R^2 = 0.9066$$

Where, Y was the absorbance and x was the concentration.

### Antibacterial assay

**Microorganisms:** The microbial strains used for testing antimicrobial activities included the Gram positive bacteria *Bacillus subtilis*, *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli* and *Salmonella typhi*. All strains were clinical isolates obtained from the Microbiology Laboratory of the Biotechnology Department, Abhilashi College of Life Sciences, Mandi, Himachal Pradesh, India. The bacteria were cultured on nutrient agar slants. The cultures were maintained by subculturing periodically and were preserved at 4 °C prior to use.

**Screening for antibacterial activity:** Antibacterial activity was tested by the agar-well diffusion method. Different concentrations of the extracts (50 and 100 mg/ml) were prepared by reconstituting the extract in dimethyl sulphoxide (DMSO). The test microorganisms were seeded into the medium by gently mixing 0.5 ml (For determination of antibacterial activity, bacterial cultures were adjusted to # 0.5 McFarland turbidity standards) of the 24 h fresh cultures with 20 ml sterile melted agar cooled to about 45 °C, in sterile Petri plates. After hardening, four 6 mm diameter wells were made using a sterile borer. The wells were filled with 100 µl of the sample extracts or solvent blanks. The antibacterial assay plates were incubated at 37 °C for 24 h. The standard antibiotic streptomycin (500 µg/mL) served as positive antibacterial control. The diameter of the zones of inhibition around each of the wells (well diameter included) was taken as measure of the antimicrobial activity. Each experiment was carried out in triplicate and the mean diameter of the inhibition zone was recorded [18].

**Determination of Minimum Inhibitory Concentration (MIC):** The extracts, which showed antibacterial activity in the agar-well diffusion method, were subjected to the MIC assay. The minimum inhibitory concentration (MIC) of the extracts was determined for each of the test organisms in triplicates. To a 0.5 ml of varying concentrations of the extracts (20.0, 18.0, 15.0, 10.0, 8.0, 5.0, 1.0, 0.5, 0.05 and 0.005 mg/ml), 2 ml of nutrient broth was added (so the extracts were dilute by a factor of 5). Therefore the final concentrations were 4, 3.6, 3, 2, 1.6, 1, 0.2, 0.1, 0.01, 0.001, and 0 mg/ml as a control.), and then a loopful of the test organism previously adjusted to # 0.5 McFarland turbidity standard for bacteria was introduced to the tubes. The procedure was repeated on the test organisms using the standard antibiotic (Streptomycin 500 µg/mL). A tube containing nutrient broth only was seeded with the test organisms as described above to serve as control. Tubes containing bacterial cultures were then incubated at 37°C for 24 hr period. After incubation the tubes were then examined for microbial growth by observing the turbidity present in the tubes [19].

### *In vitro* antioxidant activity

**Reducing power:** The reducing power of extract was determined according to the method of Oyaizu. The different concentrations of extract (50-250µg/mL) in 1ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [ $K_3Fe(CN)_6$ ] (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 for 10 min at 3000 rpm. The upper layer of 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 in a spectrophotometer (UV -1601 Shimadzu, Japan). Higher absorbance of the reaction mixture indicated greater reducing power [20].

**Total antioxidant activity:** The total antioxidant activity of the extract was determined according to the thiocyanate method. Ten milligrams of extract was dissolved in 10 ml water. Different concentration of extract (50-250 µg/mL) or standard samples in 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.0) was added to linoleic acid emulsion (2.5 ml) in potassium phosphate buffer (0.04 M, pH 7.0). Five millilitres linoleic acid emulsion consists of 17.5g Tween-20, 15.5µl linoleic acid, and 0.04M potassium phosphate buffer (pH 7.0). On the other hand, 5.0 ml control consists of 2.5 ml linoleic acid emulsion and 2.5 ml potassium phosphate buffer (0.04 M, pH 7.0). The mixed solution was incubated at 37°C in a glass flask and in the dark. After the mixture was stirred for 3 min, the peroxide value was determined by reading the absorbance at 500 nm in a spectrophotometer (UV -1601 Shimadzu, Japan), after reaction with FeCl<sub>2</sub> and thiocyanate at intervals during incubation. During the linoleic acid oxidation, peroxides formed. These compounds oxidize Fe<sup>2+</sup> to Fe<sup>3+</sup>. The latter Fe<sup>3+</sup> ions form complex with SCN<sup>-</sup>, which had maximum absorbance at 500 nm. Therefore, high absorbance indicates high linoleic acid oxidation. The solutions without extract or standards were used as blank samples. All data about total antioxidant activity are the average of triplicate analyses. The inhibition of lipid peroxidation in percentage was calculated by following equation:

$$\text{Inhibition (\%)} = (A_0 - A_t / A_0) \times 100$$

Where A<sub>0</sub> was the absorbance of the control reaction and A<sub>t</sub> was the absorbance in the presence of the sample [21].

**Determination of DPPH (1-1-diphenyl- 2-picryl hydrazyl) radical scavenging activity:** The free radical scavenging activity of extract was measured by DPPH• using the method of Shimada et al. A 0.1mM solution of DPPH• in ethanol was prepared and 1ml of this solution was added to 3ml of extract solution in water at different concentrations (50-250 µg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer (UV -1601 Shimadzu, Japan). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percent DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH}^{\bullet} \text{ scavenging effect (\%)} = 100 - [(A_0 - A_t / A_0) \times 100]$$

Where A<sub>0</sub> was the absorbance of the control reaction and A<sub>t</sub> was the absorbance in the presence of the standard sample or extract [22].

### Anthelmintic activity

**The Worms:** Indian adult earth-worms (*Pheretima posthuma*) were collected from moist soil and washed with normal saline to remove all faecal matter. The earth-worms of 4-5 cm in length and 0.1-0-2 cm in width were selected for the study of anthelmintic activity. Indian adult earth-worms were selected due to its anatomical and physiological resemblance with intestinal round worm parasite of human beings [23]. Indian adult earth-worms have been used for the initial evaluation of anthelmintic compounds *in vitro* due to its easy availability [24].

**Screening of anthelmintic activity:** The screening of anthelmintic activity was performed as per the method described by Nirmal et al., 2007 [25]. The extract was dissolved in a minimum volume of 1% w/v of Tween 80 solution and the volume was adjusted to 10 ml with saline water to make desired concentrations. Five groups of Indian earth-worms consisting of six earth-worms in each group were released into the 50 ml of formulations of different concentration prepared. These concentrations i.e. 25, 50 and 100 mg/ml of extract

were prepared and used for the study. Observations were made for the time taken to paralyse or death of individual worm. Paralysis was said to occur when the worms did not revive even in normal saline. Death was confirmed when the worms lost their motility followed by slight discolouration of their normal body colour.

**Statistical analysis:** Tests were carried out in triplicate for antibacterial assays and data were expressed as mean. The amount of extract needed to inhibit free radicals concentration by 50% (IC<sub>50</sub>) was graphically estimated using a linear regression analysis. Data for anthelmintic study was expressed as mean  $\pm$  SEM for 6 worms. All other data were expressed as mean of three independent analysis.

## Results and discussion

**Preliminary Phytochemical screening:** Preliminary phytochemical screening of ethanolic extract of *Ananas comosus* L. indicated the presence of carbohydrates, alkaloids, saponins, flavanoids, tannins and phenolic compounds and proteins and amino acids. Presence of flavanoids, phenolic compounds and tannins were very prominent.

**Determination of total phenolic content:** Most antioxidant activities of plant sources are derived from phenolic-type compounds [26]. But the antioxidant effects do not necessarily always correlate with the presence of large quantities of phenolics. The *Ananas comosus* L. ethanolic extract was evaluated for total phenolic content. The amount of total phenolics in this study was found to be 6.565 mg GAE/g extract. The present study did not show any correlation between phenolic content and antioxidant activity.

**Antibacterial assay:** The antibacterial activity of ethanolic extract of *Ananas comosus* L. was studied by the agar – diffusion method and the results are shown in table 1. Clear zone of inhibitions were observed against all the bacterial strains except *Bacillus subtilis*. Inhibition zones were ranging from 13 to 21 mm. Greater inhibition was observed for higher concentration of extract. As observed in the study, all the bacterial strains, except *Bacillus subtilis*. were sensitive towards the *Ananas comosus* L. extract. In the long run, it can be concluded that the *Ananas comosus* L. extract showed antibacterial activity.

**Determination of Minimum Inhibitory Concentration (MIC):** The MIC values of *Ananas comosus* L. extract were determined by the broth dilution method against all the susceptible bacterial strains. Results are shown in table 2. The MIC values against all the susceptible bacterial strains such as *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhi* of the extract of *Ananas comosus* L. ranged from 0.3125 to 1.25 mg/ml. The MIC values were 0.3125, 0.625, 1.25 mg/ml for *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli* respectively. The inhibition of bacterial growth at concentration as low as 0.3125 mg/ml indicated considerable antibacterial activity of the *Ananas comosus* L. extract against *Staphylococcus aureus*.

### *In vitro* antioxidant activity

**Reducing power:** Figure 1 shows the reductive capability of the extract compared to  $\alpha$ -tocopherol and BHA. For the measurement of the reductive ability, it was investigated the  $Fe^{3+}$ -  $Fe^{2+}$  transformation in the presence of extract samples using the method of Oyaizu, 1986. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity has been attributed to various mechanisms. The reducing power of the extract samples was increased with increasing

concentration. At all the studied concentrations, the plant extract showed higher activity than  $\alpha$ -tocopherol. Although reductive power of the extract samples was lower than BHA. Reducing power of extract and standard compounds followed the order:  $\alpha$ -tocopherol < extract < BHA.

**Total antioxidant activity:** Total antioxidant activity of the extract was determined by the thiocyanate method as described by Mitsuda et al., 1996. The plant extract exhibited effective and powerful antioxidant activity at 250  $\mu$ g/mL concentration as shown in figure 2. The studied concentration of the extract (250 $\mu$ g/mL) showed higher antioxidant activity than that of 250  $\mu$ g/mL  $\alpha$ -tocopherol (26 %) but the antioxidant activity of the extract was lower than that of 250  $\mu$ g/mL BHA (96.8%). Percentage inhibition of 250  $\mu$ g/mL concentration of the extract in linoleic acid system was 57.2%. On the other hand, percentage inhibition of 250  $\mu$ g/mL concentrations of  $\alpha$ -tocopherol and BHA (butylated hydroxyanisole) was found as 26% and 96.8% respectively. The antioxidant activity of the extract and standard compounds followed the order:  $\alpha$ -tocopherol < extract < BHA.

**Determination of DPPH (1-1-diphenyl- 2-picryl hydrazyl) radical scavenging activity:**

The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. DPPH• is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Hence, DPPH• is usually used as a substrate to evaluate antioxidant activity of antioxidants. Figure 3 shows a decrease in the concentration of DPPH radical due to the scavenging ability of the extract and standard compounds. BHA and  $\alpha$ -tocopherol were used as standard radical scavengers. The scavenging effect of extract and standards on the DPPH radical decreased in the order of BHA >  $\alpha$ -tocopherol > extract and were 87.15, 71.95 and 56.36% at the concentration of 250  $\mu$ g/mL, respectively. The IC<sub>50</sub> values were found to be 124.70, 160.31 and 214.96  $\mu$ g/ml for BHA,  $\alpha$ -tocopherol and extract respectively. From the results it was indicated that the extract have a noticeable effect on scavenging free radicals. Based upon the data obtained from this study, it is evident that the plant extract is a good free radical inhibitor or scavenger. It was also reported that oxidative stress, which occurs when free radical formation exceeds the body's ability to protect itself, forms the biological basis of chronic condition [27]. As a promising antioxidant, the plant extract reacts with free radicals, which may limit free radical damage occurring in the human body.

**Anthelmintic activity:** From the results for anthelmintic activity the predominant effect of albendazole and piperazine citrate was to cause a flaccid paralysis of the worms. Albendazole by inhibiting micro tubule polymerization and thereby inducing immobilization produced paralysis and death of worms [28]. Data in table 3 and figure 4 and 5 reveals that all the three concentrations of extract of *Ananas comosus* L. showed significant dose dependent anthelmintic property at 25, 50 and 100 mg/ml concentrations. Results clearly indicated that 100 mg/ml concentration of the extract has the highest potency as an anthelmintic (took least time to cause paralysis and death of worms) when compared to standard drug piperazine citrate and albendazole. The present study clearly indicated the traditional ethno medical claim that the leaves of the plant *Ananas comosus* L. has the potential to be an anthelmintic.

Table 1. Antibacterial activity of ethanolic extract of *Ananas comosus* L.

Microorganisms	Zone of Inhibition (mm)		
	Ethanolic Extract 50 mg/mL	Ethanolic Extract 100 mg/mL	Streptomycin 500µg/mL
<i>Bacillus subtilis</i>	-	-	26
<i>Staphylococcus aureus</i>	16	21	31
<i>Escherichia coli</i>	13	19	27
<i>Samonella typhi</i>	10	17	25

-: no activity. Each zone of inhibition is an average of three independent determinations and the solvent (DMSO) did not show any inhibition.

Table 2. Minimum Inhibitory Concentration (MIC) of *Ananas comosus* L. extract.

Microorganisms	MICs of extract (mg/mL)
<i>Bacillus subtilis</i>	Nt
<i>Staphylococcus aureus</i>	0.3125
<i>Escherichia coli</i>	1.25
<i>Samonella typhi</i>	0.625

nt: not tested.

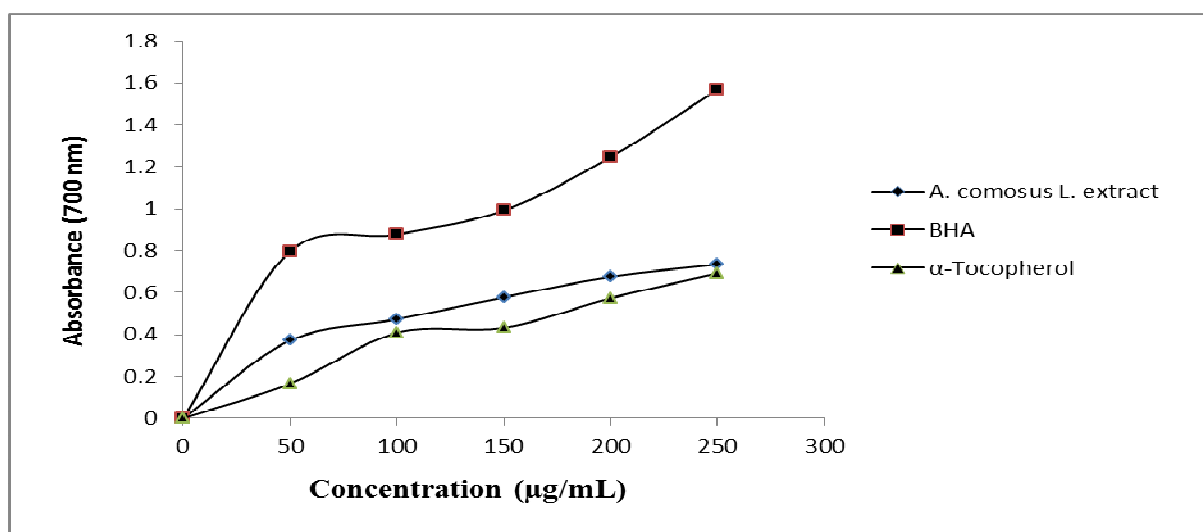
Figure 1. Reducing power of  $\alpha$ -tocopherol, BHA and *Ananas comosus* L. ethanolic extract



Figure 2. Total antioxidant activity  $\alpha$ -tocopherol, BHA and *Ananas comosus L.* ethanolic extract at 250  $\mu$ g/ml concentration

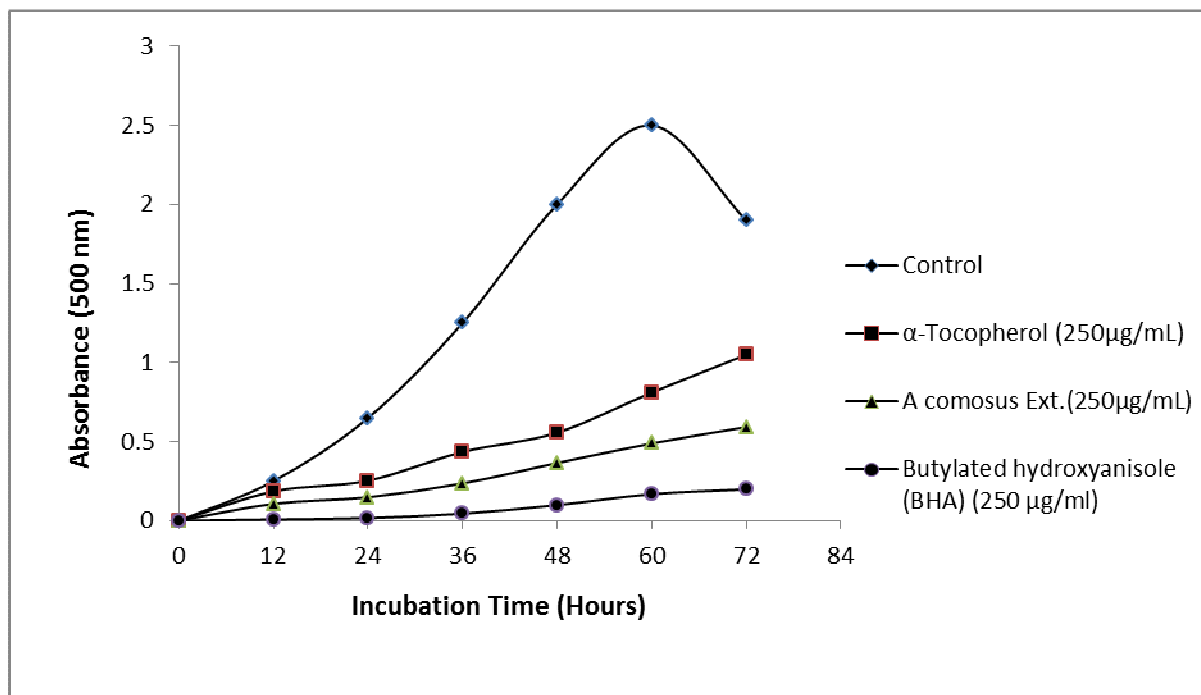


Figure 3. DPPH radical scavenging activity  $\alpha$ -tocopherol (Ascorbic acid), BHA and *Ananas comosus L.* ethanolic extract

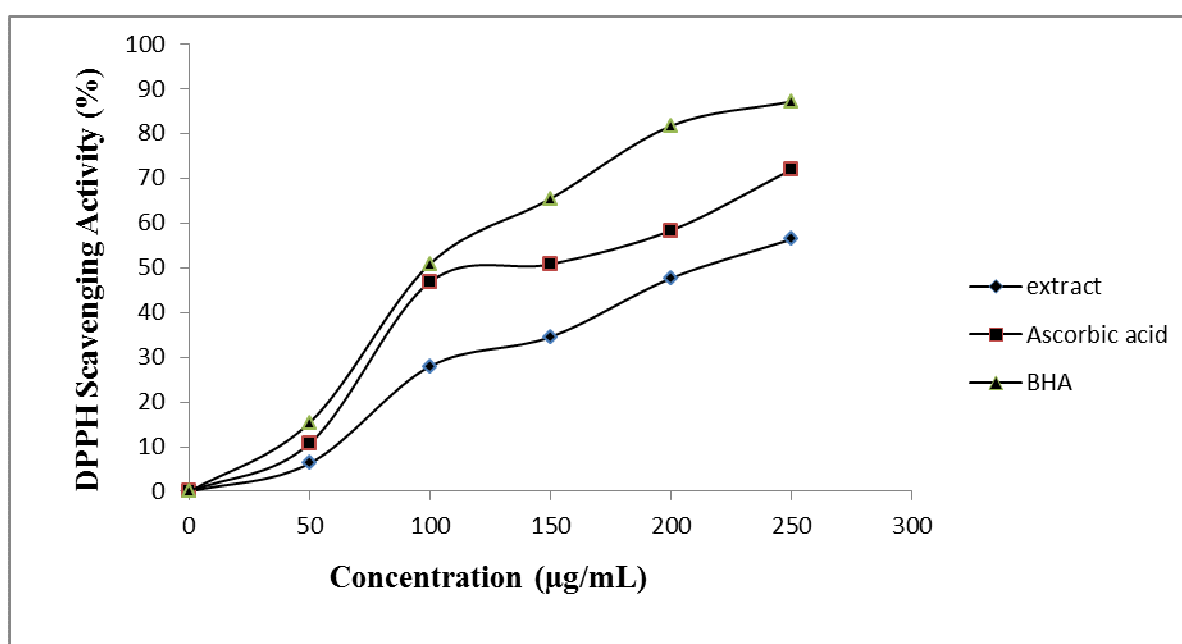
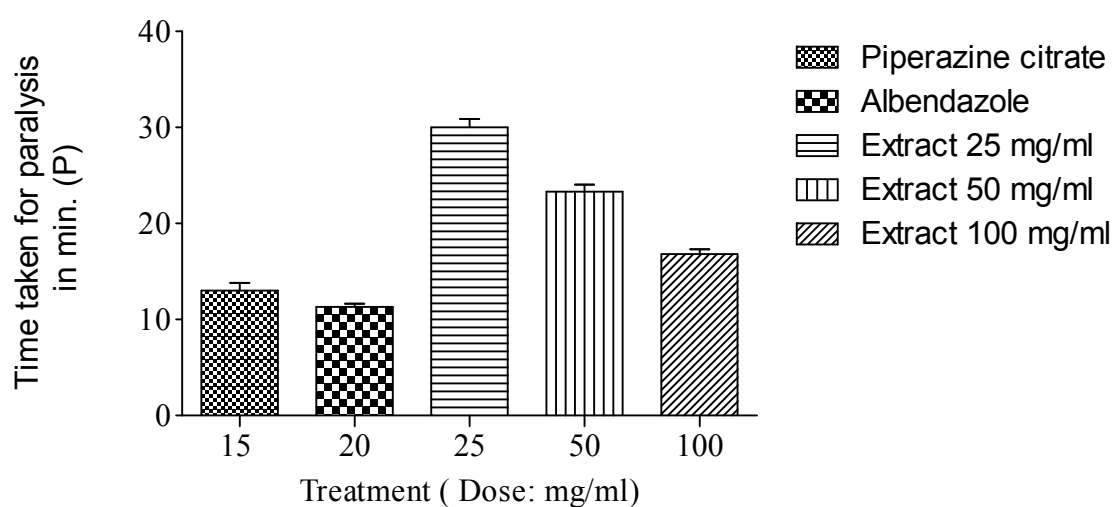


Table 3. Anthelmintic activity of *Ananas comosus L.* ethanolic extract.

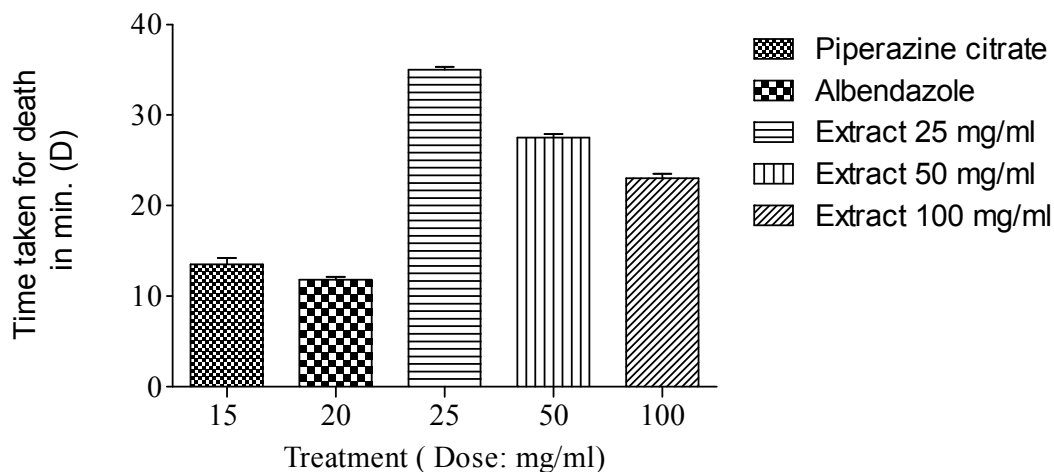
S. No.	Groups	Concentration (mg/ml)	<i>Pheretima posthuma</i>	
			Time taken for paralysis (P) in min. (Mean $\pm$ SEM)	Time taken for death (D) in min. (Mean $\pm$ SEM)
I	Control	1 % w/v Tween 80	No paralysis occurs	No death occurs
II	Piperazine citrate	15 mg/ml	13.000 $\pm$ 0.8165	13.500 $\pm$ 0.7188
III	Albendazole	20 mg/ml	11.333 $\pm$ 0.3333	11.833 $\pm$ 0.3073
IV	Extract	25 mg/ml	30.000 $\pm$ 0.8944	35.000 $\pm$ 0.3651
V	Extract	50 mg/ml	23.333 $\pm$ 0.7149	27.500 $\pm$ 0.4282
VI	Extract	100 mg/ml	16.833 $\pm$ 0.4773	23.000 $\pm$ 0.5164

Data were expressed as mean  $\pm$  SEM for 6 worms (n = 6)

Figure 4. Anthelmintic activity of ethanolic extract of *Ananas comosus L.* (Time taken for paralysis)

Control group worms were alive up to 24 hours of the experiment.

Co

**Figure 5: Anthelmintic activity of ethanolic extract of *Ananas comosus L.* (Time taken for death)**

Control group worms were alive up to 24 hours of the experiment.

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