Phytochemical Screening, and Evaluation of the Toxicity, Antimicrobial and Anthelmintic Properties of the Different Extracts from the Air-dried Seeds of *Areca catechu* Linn.

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Abstract

The nuts of Areca catechu Linn. are used in India as an anthelmintic and according to Ayurvedic medicine, chewing Areca nut and betel leaf is a good remedy against bad breath and also said to have aphrodisiac properties. The main objective of this study is to determine the presence of the secondary metabolites and to evaluate the toxicity, antimicrobial, and anthelmintic properties of the different solvent extracts obtained from the crude ethanolic extract (CEE) from the air-dried seeds of Areca catechu Linn. The hexane (HE), ethyl acetate (EAE) and aqueous (AE) extracts obtained from the CEE reveal the presence of alkaloids, flavonoids, saponins, tannins and polyphenols. Presence of these secondary metabolites on EAE provide supplementary evidence of its antimicrobial effect against *E. coli, S. aureus*, and *C. albicans* which are 9.0 mm, 9.8 mm and 14.0 mm, respectively. The presence of tannins and polyphenols on HE confirm its anthelmintic property with a mean time for paralysis of 4.8 minutes and mean time for death of 38.8 minutes. In AE, all secondary metabolites are present except saponins, yet it shows the most pronounced lethal effect to brine shrimp nauplii with 60% - 100% mortality. One-way Analysis of Variance (ANOVA) is used to compare the means of the zones of inhibition of each of the treatments and it reveals that there is a significant difference on the effect of the different extracts. Duncan's Multiple Range Test (DMRT) shows that at each concentration, the different extracts reveal a significant different inhibitory effect.

Keywords: *Arecha catechu Linn*, Antimicrobial, Anthelmintic

Introduction

The Areca nut or Betel nut (*Areca catechu* Linn.) has been used for medicinal properties for more than 2000 years in South Asian countries. In old Indian scripts, such as Vagbhata (4th century) and Bhavamista (13th century), areca nut or betel nut has been described as a therapeutic agent for leucoderma, leprosy, anemia, obesity and helminthic infections (Amudhan *et al.*, 2012). During the past few decades, despite numerous advances made in understanding the mode of transmission and the treatment of parasites, there are still no efficient products to control certain helminthes and the indiscriminate use of some drugs has generated several cases of resistance (Nunomura *et al*., 2006). Furthermore, pathogens have developed immunity against many modern synthetic drugs.

These drugs may have shown distinct effectiveness in curing diseases but cause a number of side effects. Plant extracts, however, show less effectiveness but is relatively free of side effects. Furthermore, natural medicines have a fairly wider availability and were much cheaper than commercially available drugs. Some of the compounds present in plant extracts tend to inhibit the growth of certain microorganisms. Hence, the success of this study may help in developing new substances to combat these parasitic organisms and to seek potential natural and cheaper substitute to the commercially available drugs.

The main objective of this study is to test the absence or presence of secondary metabolites and to determine the toxicity, antimicrobial, and anthelmintic properties of the different solvent extracts obtained from the crude ethanolic extract from the air-dried seeds of *Areca catechu* Linn. Specifically, this study aimed: to obtain the different solvent extracts from the crude ethanolic extract from the air-dried seeds of *Areca catechu* Linn., to determine the possible presence of secondary metabolites in the hexane, ethyl acetate, and aqueous extracts from the air-dried seeds of *Areca catechu* Linn., to determine which among the hexane, ethyl acetate, and aqueous extracts showed significant toxic effect employing brine shrimp lethality test (BSLT), to evaluate which among the extracts showed antimicrobial property against the bacterial species, *Staphylococcus aureus* and *Escherichia coli* and fungal species, *Aspergillus niger* and *Candida albicans* and to determine which among the extracts has the highest anthelmintic potential against the test organism, *Eudrilus eugeniae*.

Methodology

Preparation of the crude ethanolic extract

Ripe fruits of *Areca catechu* Linn. were hammered to break the seed and remove it easily from the hard coating. The seeds were air-dried, ground then weighed. Six hundred eighteen grams of the ground seeds was soaked in ethanol for 72 hours. The soaked sample was then filtered through vacuum filtration followed by concentration *in vacuo* using a rotary evaporator. The residue was soaked twice in ethanol for 72 hours, filtered then concentrated *in vacuo* along with the first extract.

Solvent partitioning

One hundred fifty milliliters of the crude ethanolic extract was dissolved in 50 mL water and placed in a separatory funnel. Fifty milliliters of hexane was added to the separatory funnel. It was stoppered and the mixture was shaken gently while opening the stopcock after every shaking to dissipate the gas produced from shaking the mixture. The separatory funnel was fitted in an iron ring then clamped to an iron stand. The cover was removed and it was allowed to stand undisturbed until a clear separation between layers is observed. Then, the stopcock was opened to draw-off the bottom aqueous layer into a clean container. The upper organic layer was also transferred into a clean container. The aqueous layer was placed again in a separatory funnel and the same amount of hexane was added to collect the remaining organic portions and this was repeated three times. The hexane extracts were collected in one container. Then, the aqueous layer was further extracted with 50 mL ethyl acetate thrice. The hexane, ethyl acetate and aqueous extracts were then concentrated *in vacuo*.

Phytochemical screening

All crude extracts were subjected to phytochemical screening to determine the presence of secondary metabolites. This was carried out according to the method proposed by Harbone (1984).

Test for alkaloids

A quantity, 0.2 g of each of the extracts was added to 5 mL of 2% hydrochloric acid and heated on boiling water for 10 minutes. They were then allowed to cool and then filtered. To 1 mL of the filtrate in a test tube was tested with alkaloids reagent, Wagner's and Mayer's reagent and results compared to blank. Turbidity or precipitation indicated the presence of alkaloids.

Test for tannins and polyphenols

A quantity, 0.2 g of each of the extracts was boiled with 5 mL of 45% ethanol for 5 minutes. The hot mixture was filtered using a filter paper and the filtrate collected in a beaker. Two mL of the filtrate was mixed with 10 mL of distilled water and then a drop of iron chloride solution was added. A blue-black or blue-green precipitate indicates the presence of tannins and polyphenols.

Test for saponins

A quantity, 0.1 g of each of the extracts was measured into a beaker and 20 mL of distilled water was added, the beaker was heated in a water bath for over 5 minutes. The mixtures were filtered using a filter paper into another beaker to obtain a filtrate. Two mL of each of the filtrate was measured to another test tube and 10 mL of distilled water was added, it was shaken vigorously for over a minute. Frothing which persist on warming indicates the presence of saponin.

Test for flavonoids

A quantity, 0.1 g of each of the extracts was added a mixture of 10 mL of lead acetate solution (90% w/v) and 20 mL of 50% aqueous ethanol in a 200 mL conical flask. The mixtures were placed on boiling water for 2 minutes, cooled and filtered. A volume of 5 mL dilute ammonia was added to a portion of the aqueous filtrate followed by the addition of 1 mL concentrated sulfuric acid to 2 mL of potassium hydroxide solution and allowed to mix. Then into acid base mixture a small quantity of aqueous filtrate of the sample was added and observed for color change.

Brine shrimp lethality test

The brine shrimp lethality test was based on the method described by Guevara (2004) with slight modifications. Each crude extracts were prepared with 10 ppm, 100 ppm and 1000 ppm concentrations. From the concentrated extract, 0.05 g of the sample was dissolved in 5 mL of absolute ethanol to obtain a stock solution with a concentration of 10000 ppm. From the stock solution, 0.5 mL and 0.05 mL was placed in the first two test tubes. Then, from the stock solution, 0.05 mL was taken and diluted to 1 mL. From this 500 ppm solution, 0.1 mL was pipetted into the third tube for the 10 ppm concentration.

For liquid extracts, 0.05 mL of the sample was dissolved in 5 mL of absolute ethanol to obtain a 10000 ppm stock. Half milliliter and 0.05 mL were placed in the first two test tubes to obtain 1000 ppm and 100 ppm, respectively. Then, from the stock solution, 0.5 mL was dissolved in 1 mL absolute ethanol to obtain 5000 ppm. From

the second solution, to obtain 10 ppm, 0.01 mL was taken and placed in the third test tube. The test tubes were then set aside at room temperature until all solvents have evaporated.

A glass chamber was divided into two unequal compartments with the division having small holes. The brine shrimp eggs were sprinkled into the larger compartment and it was covered and remained dark; while the smaller compartment was illuminated with a light bulb. After 48 hours, the eggs that have been hatched transferred to the illuminated compartment through the holes in the division. After all solvents in the test tubes had evaporated, 2.5 mL of artificial seawater was added. However, for hexane extract, approximately 5 drops of dimethyl sulfoxide (DMSO) was initially added to dissolve the extract then 2.5 mL of artificial seawater was added. Then from the smaller compartment of the glass chamber, ten nauplii were pipetted into the test tubes and these were kept illuminated for 24 hours. A drop of yeast was added in each of the tubes as food. The number of dead nauplii was counted after 6 hours and after 24 hours.

Antimicrobial screening

In antimicrobial screening, 10000 ppm, 20000 ppm and 30000 ppm concentrations of each of the crude extracts and the standards, ampicillin and ketoconazole, were prepared.

Twenty three grams of nutrient agar and twenty three grams nutrient agar with 20 grams glucose were weighed and dissolved separately up to 1 liter of distilled water. Then, it was heated with constant stirring. The prepared culture media were poured into 20 mL test tubes and were sterilized for 15 minutes.

The filter paper discs, approximately 6 mm in diameter, were prepared and wrapped with an aluminum foil. These were placed inside the autoclave along with the forceps and the previously cleaned petri dishes for sterilization.

The nutrient broth culture of the different test microorganisms was thoroughly mixed with a vortex mixer then, aseptically, 250 microliter was pipetted into each test tube and the contents were poured into the petri dish. Petri plates were labeled and set aside to solidify. Each petri plate contains all treatments of the same concentrations for one test microorganism. Then, filter paper discs were picked using sterile forceps. The edge of the disc was dipped into the treatments until it became saturated with the solution. Then, aseptically, the impregnated disc was placed on the agar surface of each plate. The disc was gently pressed to ensure firm contact with the agar. Each plate represents one concentration with the three different treatments, the positive control, and the negative control. Three replicates were made for each concentration. All assay plates were incubated for 12 hours for the bacteria and 24 hours for the fungi at 37°C. Zones of inhibition were measured after the respective hours of incubation.

Anthelmintic assay

According to the method described by Patel *et al* (2011), 10000 ppm, 30000 ppm and 50000 ppm concentrations of the extracts were prepared by dissolving 500 milligrams, 1500 milligrams and 2500 milligrams of the extract in 50 milliliters of distilled water, respectively.

Ten milliliters of the solution was poured into previously cleaned and labeled petri dishes and five worms of approximately equal sizes and length were placed in it. Five replicates were done for every concentration. However, in the preparation of the positive standards, levamisole and combantrin tablets were separately powderized and dissolved in 50 mL of distilled water to obtain a concentration of 10000 ppm. Levamisole is a common anthelmintic for poultry and livestock while Combantrin is the commercial name for Pyrantel embonate that is used for curing helminthic infections in humans. Distilled water was used as the negative control in the assay.

Observations were made for the time taken to paralyze and death of individual test organism. Paralysis is marked by decreasing in vigorous wriggling of the worm. Slow movement of the worm after being pricked with pin indicates paralysis. No movement indicates death (Danquah *et al.*, 2012).

Time of paralysis was taken as soon as there is a test organism that exhibit paralysis until all of the test organisms were paralyzed. The start and the end of the time of paralysis were noted.

Same manner was done in noting the time of death of the test organisms, that is, as soon as there is no movement noticed in one of the test organisms and until all of them stops moving. Death was also confirmed by pricking the test organism with a needle and no response of the organism was considered dead. The change of the flesh color from light red to pale and a straight, relax pattern were also manifestations that the test organisms were dead.

Results and Discussion

Phytochemical screening

The possible presence of secondary metabolites in the different extracts from the crude ethanolic extract from the air-dried seeds of *Areca catechu* Linn. was carried out using the method described by Harbone (1984). The result for phytochemical screening of the different extracts is shown in **Table 2** below.

Table 2. The secondary metabolites present in the different crude extracts of the air-dried seeds of *Areca catechu* Linn.

Extracts	Alkaloids	Tannins and Polyphenols	Saponins	Flavonoids
Hexane	-	+	-	-
Ethyl acetate	+	+++	++	++
Aqueous	+	+	-	+

Legend: (-) absence, (+) slight appearance, (++) define appearance, (+++) heavy appearance

Hence, from the data obtained, the crude hexane extract contains only tannins and polyphenols and the crude ethyl acetate extract reveals the presence of flavonoids, alkaloids, saponins, tannins and polyphenols. The aqueous extract, however, has all the secondary metabolites tested except saponins. The presence of these secondary metabolites explains if there would be occurrence of bioactivity of the different crude extracts in the succeeding tests.

Brine shrimp lethality test

In the brine shrimp lethality test (BSLT), the average of the three trials were used in determining the % mortality of the brine shrimp nauplii after 24 hours. Percent mortality was taken from the number of dead nauplii over the total number of nauplii multiplied by 100. The table below shows the number of dead nauplii and % mortality.

Table 3. Number of dead nauplii and % mortality of each of the extracts after 24 hours.

Extracts	Conc. ppm	Number of dead nauplii			Mean	% Mortality
		1	2	3		
Hexane	10	3	1	0	1	10
	100	8	6	6	7	70
	1000	9	9	9	9	90
Ethyl acetate	10	3	4	6	4	40
	100	10	10	4	8	80
	1000	10	10	10	10	100
Aqueous	10	5	8	5	6	60
	100	10	8	10	9	90
	1000	10	9	10	10	100

Antimicrobial assay

The crude hexane extract, crude ethyl acetate extract and aqueous extract were subjected to antimicrobial assay against E*scherichia coli, Staphylococcus aureus, Aspergillus niger* and *Candida albicans*. Below are the results for the antibacterial and antifungal effects of the different extracts from the air-dried seeds of *Areca catechu* Linn.

Antibacterial effect

In determining the antibacterial potential, 10000 ppm, 20000 ppm, and 30000 ppm concentrations of the hexane, ethyl acetate and aqueous extracts and the standard ampicillin were tested against *E.coli* and *S.aureus*. The results are presented in **Table 4**.

Based on the results, it showed that varying the concentrations of ampicillin, ethyl acetate, and aqueous extracts can inhibit the growth of *E.coli* whereas the hexane extract suppressed the growth of bacteria. Upon statistical analysis, there is no significant difference in varying the concentrations of ampicillin and ethyl acetate; however, in the aqueous extract, as the concentration increases, its inhibition also increases, but there is no suppression in the growth of bacteria at 10000 ppm. Thus, the inhibitory effect of ethyl acetate is comparably the same to the inhibitory effect of ampicillin.

As shown in **Table 5,** by statistical analysis, although ethyl acetate extract is not significantly different from ampicillin, upon varying the concentration, there is a significant difference between each concentration on its imposed inhibition.

Distilled water and ten thousand ppm concentrations of hexane, and aqueous extracts did not inhibit the growth of *E.coli*. However, ampicillin and ethyl acetate did suppress its growth. Between ampicillin and ethyl acetate, the antibacterial standard ampicillin has a greater inhibitory capacity compared to ethyl acetate extract and they are statistically different.

At 20000 ppm, ampicillin has the greatest inhibitory capacity followed by ethyl acetate extract then the aqueous extract. Hexane extract and distilled water did not suppress the growth of the bacteria.

Ampicillin has the greatest inhibitory effect against *E.coli* at a concentration of 30000 ppm. It is followed by ethyl acetate and aqueous extract.

Table 4. Antibacterial effects of the hexane, ethyl acetate, and aqueous extracts of the air-dried seeds of *Areca catechu* Linn. against *Escherichia coli*

Treatments	Concentration (ppm)	Zones of inhibition (mm)					
		Escherichia coli					
		Replicates				P Values	Remarks
		1	2	3	Ave		
Ampicillin	10000	26.0	24.0	27.5	25.8	0.335	Not Significant
	20000	28.0	25.0	27.5	26.8		
	30000	27.5	28.0	27.5	27.7		
Hexane	10000	0.0	0.0	0.0	0.0		
	20000	0.0	0.0	0.0	0.0		
	30000	0.0	0.0	0.0	0.0		
Ethyl acetate	10000	8.0	8.0	9.0	8.3	0.451	Not Significant
	20000	8.0	10.0	9.0	9.0		
	30000	7.5	8.0	9.0	8.2		
Aqueous	10000	0.0	0.0	0.0	0.0^{B}	0.000	Significant
	20000	7.0	7.0	7.0	7.0^{A}		
	30000	9.5	8.0	9.0	8.8^{A}		
Distilled Water		0.0	0.0	0.0	0.0		Not Significant

Legend: P values ≥ 0.05 = Not significant. Means having the same letters are not significantly different at α = 0.05
A – D = highest inhibition – lowest inhibition

Based on statistical analysis, the inhibitory effect of ampicillin is significantly different from ethyl acetate and aqueous extracts and the latter two extracts are not significantly different from each other. Hexane and distilled water did not show any significant inhibition in its growth. In **Figure 10** was the graphical presentation of the mean of the zones of inhibition exhibited by the different crude extracts at various concentrations against *E.coli*.

Table 5. Antibacterial effects of the hexane, ethyl acetate, and aqueous extracts at a specific concentration against *Escherichia coli*

Treatments	*Escherichia coli*		
	Average zones of inhibition (mm)		
	10 000 ppm	**20 000 ppm**	**30 000 ppm**
Ampicillin	25.8^{A}	26.8^{A}	27.7^{A}
Hexane	0.0^{C}	0.0^{D}	0.0^{C}
Ethyl acetate	8.3^{B}	9.0^{B}	8.2^{B}
Aqueous	0.0^{C}	7.0^{C}	8.8^{B}
Distilled water	0.0^{C}	0.0^{D}	0.0^{C}

Legend: Means having the same letters are not significant at α = 0.05 DMRT. A – D = highest inhibition – lowest inhibition

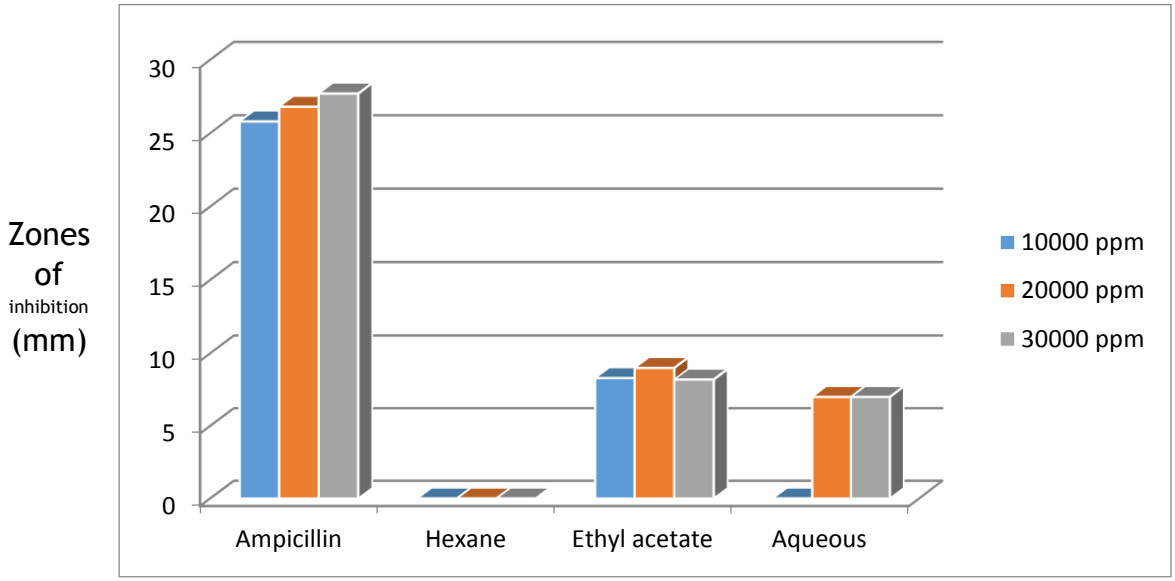

Figure 10. Graphical presentation for the mean of the zones of inhibition (mm) exhibited by the different crude extracts at various concentrations against *Escherichia coli*

For *S.aureus*, varying the concentration of ampicillin and ethyl acetate extract can inhibit its growth significantly but hexane and aqueous extracts did not exhibit suppression on its growth, see **Table 6**. Varying the concentrations of the ampicillin has no significant difference in inhibiting its growth, however, for ethyl acetate, 30000 ppm and 20000 ppm have greater zones of inhibition compared to 10000 ppm and they are significantly the same but significantly different compared to 10000 ppm.

Table 6. Antibacterial effects of the hexane, ethyl acetate, and aqueous extracts of the *Areca catechu* seeds against *Staphylococcus aureus*

Treatments	Concentration (ppm)	Zones of inhibition (mm) *Staphylococcus aureus* Replicates 1	2	3	Ave	P Value	Remarks
Ampicillin	**10000**	30.0	32.5	32.0	31.5	0.365	Not Significant
	20000	33.0	32.0	35.0	33.3		
	30000	31.0	34.0	32.5	32.5		
Hexane	**10000**	0.0	0.0	0.0	0.0		
	20000	0.0	0.0	0.0	0.0		
	30000	0.0	0.0	0.0	0.0		
Ethyl acetate	**10000**	7.0	8.5	7.0	7.5^{B}	0.027	Significant
	20000	9.5	11.0	9.0	9.8^{A}		
	30000	10	10.0	9.0	9.7^{A}		
Aqueous	**10000**	0.0	0.0	0.0	0.0		
	20000	0.0	0.0	0.0	0.0		
	30000	0.0	0.0	0.0	0.0		
Distilled Water		0.0	0.0	0.0	0.0		

Legend: P values ≥ 0.05 = Not significant. Means having the same letters are not significantly different at α = 0.05
A - D = highest inhibition - lowest inhibition

For *S.aureus* at 10000 ppm, 20000 ppm, and 30000 ppm, only the ethyl acetate extract and ampicillin did inhibit its growth; and distilled water, hexane and aqueous extracts did not show any inhibition in its growth.

At all concentrations, in **Table 7,** ampicillin has a greater inhibitory effect compared to ethyl acetate extract and they are significantly different from each other.

Table 7. Antibacterial effects of the hexane, ethyl acetate and aqueous extracts at a specific concentration against *Staphylococcus aureus*

Treatments	*Staphylococcus aureus*		
	Average zones of inhibition (mm)		
	10000 ppm	20000 ppm	30000 ppm
Ampicillin	31.5^{A}	33.3^{A}	32.5^{A}
Hexane	0.0^{C}	0.0^{C}	0.0^{C}
Ethyl acetate	7.5^{B}	9.8^{B}	9.7^{B}
Aqueous	0.0^{C}	0.0^{C}	0.0^{C}
Distilled water	0.0^{C}	0.0^{C}	0.0^{C}

Legend: Means having the same letters are not significant at α = 0.05 DMRT.A - D = highest inhibition - lowest inhibition

Figure 11 depicts the graphical presentation of the mean of the zones of inhibition exhibited by the different crude extracts at various concentrations against *S.aureus* while **Figure 12** and **Figure 13** show the extract's zone of inhibition against *E.coli* and *S.aureus*, respectively.

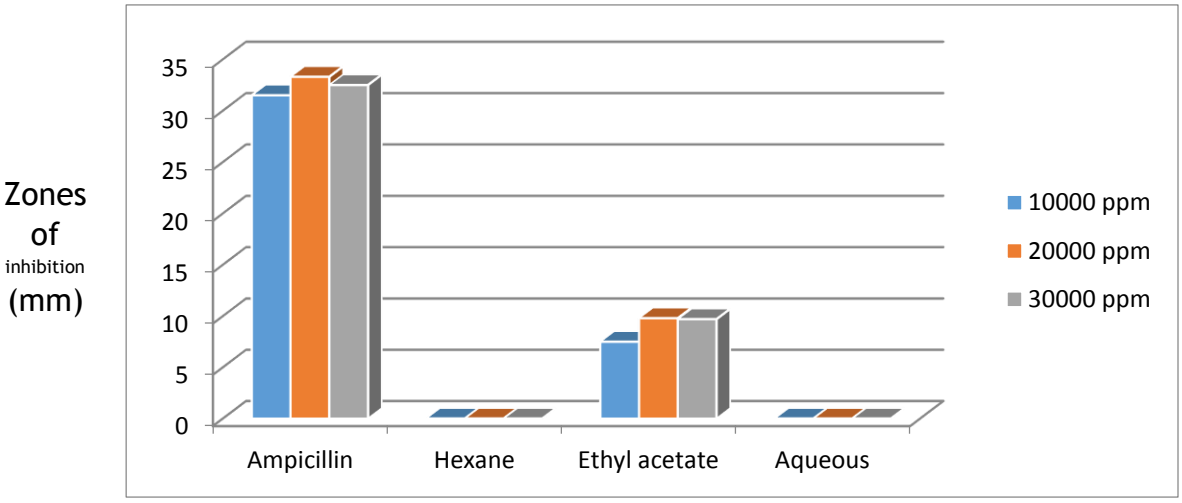

Figure 11. Graphical presentation for the mean of the zones of inhibition (mm) exhibited by the different crude extracts at various concentrations against *Staphylococcus aureus*

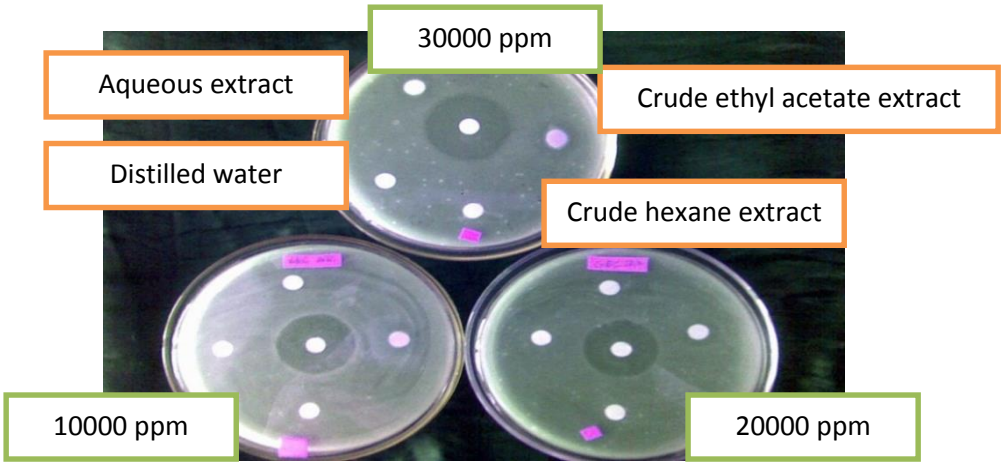

Figure 12. Antibacterial assay against *Escherichia coli*

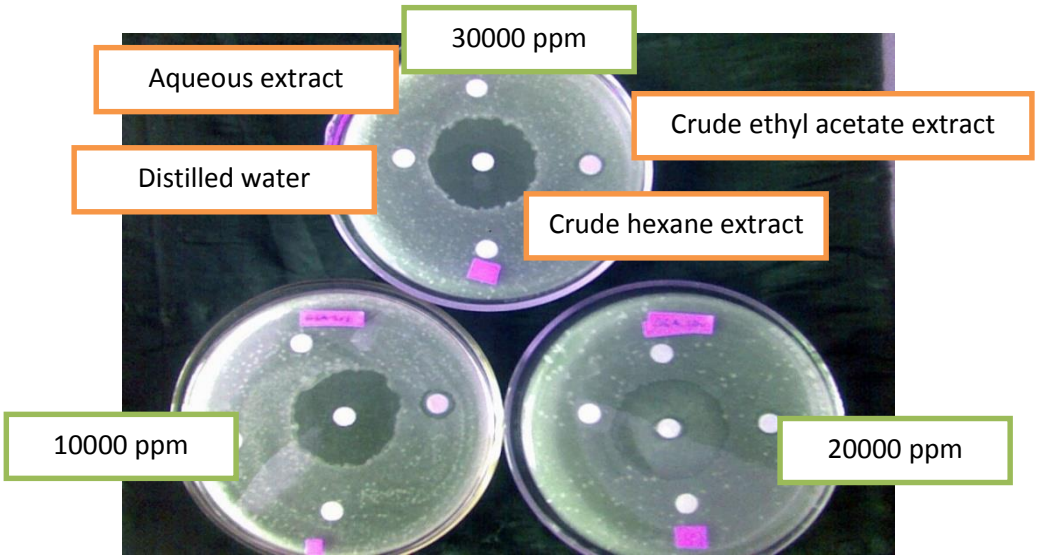

Figure 13. Antibacterial assay against *Staphylococcus aureus*

Antifungal effect

The test microorganisms for the determination of the antifungal potential of the different extracts from the seeds of *Areca catechu* Linn. were *Aspergillus niger* and *Candida albicans*. The antifungal standard ketoconazole was prepared in three concentrations, 10000 ppm, 20000 ppm, and 30000 ppm. **Table 8** shows the diameters of the zones of inhibition by the hexane, ethyl acetate and aqueous extracts against *Candida albicans*.

For C.*albicans*, varying concentrations of ketoconazole, ethyl acetate and aqueous extracts can significantly inhibit its growth while hexane extract did not show any inhibition. Using ketoconazole, 30000 ppm exhibited the greatest inhibition compared to 10000 ppm and 20000 ppm which are not significantly different from each other. At 30000 ppm and 20000 ppm of the ethyl acetate extract, they are not significantly different from each other and they have a greater inhibition compared to 10000 ppm. However, in aqueous extract, the greater the concentration, the higher its zones of inhibition which means that 30000 ppm has the greatest inhibitory capacity among the concentrations.

Table 8. Antifungal effects of the hexane, ethyl acetate and aqueous extracts from the seeds of *Areca catechu* Linn. aginst *Candida albicans*

Treatments	Concentration (ppm)	Zones of inhibition (mm)					
		Candida albicans					
		Replicates				P values	Remarks
		1	2	3	Ave		
Ketoconazole	10000	14.0	15.0	14.0	14.3^{B}	0.003	Significant
	20000	15.0	16.0	16.0	15.7^{A}		
	30000	11.0	12.0	13.0	12.0^{C}		
Hexane	10000	0.0	0.0	0.0	0.0	0.422	Not significant
	20000	0.0	0.0	0.0	0.0		
	30000	8.0	0.0	0.0	2.7		
Ethyl acetate	10000	9.0	9.0	9.0	9.0^{C}	0.015	Significant
	20000	14.0	15.0	11.0	13.3^{B}		
	30000	12.0	15.0	15.0	14^{A}		
Aqueous	10000	0.0	0.0	0.0	0.0^{C}	0.000	Significant
	20000	8.0	9.0	10.0	9.0^{B}		
	30000	11.0	11.0	11.0	11.0^{A}		
Distilled water		0.0	0.0	0.0	0.0		

Legend: P values ≥ 0.05 = Not significant. Means having the same letters are not significantly different at α = 0.05
A – D = highest inhibition – lowest inhibition

Varying the concentrations of the different extracts has different inhibitory capacity. At 10000 ppm, only ketoconazole and ethyl acetate did inhibit its growth while distilled water, hexane and aqueous extracts did not. Ketoconazole has a greater inhibitory effect compared to ethyl acetate and they are significantly different.

At 20000 ppm, the ketoconazole, ethyl acetate, and aqueous extracts did inhibit its growth while hexane extract and distilled water did not. Among the ampicillin, ethyl acetate, and aqueous extracts, ampicillin has the greatest inhibitory capacity followed by ethyl acetate and aqueous extracts, respectively. They are significantly different from each other.

At 30000 ppm, ampicillin, aqueous and ethyl acetate extracts did inhibit the growth of the bacteria while distilled water and hexane extract did not. At this concentration, ethyl acetate exhibited the greatest inhibitory capacity followed by ampicillin and aqueous extract. The latter are not significantly different from each other and ethyl acetate is significantly different from the rest. A summary of these values was presented in **Table 9.**

Table 9. Antifungal effects of the hexane, ethyl acetate and aqueous extracts at a specific concentration on *Candida albicans*

Treatments	*Candida albicans*		
	Average zone of inhibition (mm)		
	10000 ppm	20000 ppm	30000 ppm
Ketoconazole	14.3^{A}	15.7^{A}	12.0^{A}
Hexane	0.0^{C}	0.0^{D}	2.7^{C}
Ethyl acetate	9.0^{B}	13.3^{B}	14.0^{B}
Aqueous	0.0^{C}	9.0^{C}	11.0^{B}
Distilled water	0.0^{C}	0.0^{D}	0.0^{C}

Legend: Means having same letters are not significant at α = 0.05 DMRT. A – D = highest inhibition – lowest inhibition

Figure 14 shows the graphical presentation of the inhibitory effect of the different solvent extracts to the fungal species *Candida albicans*.

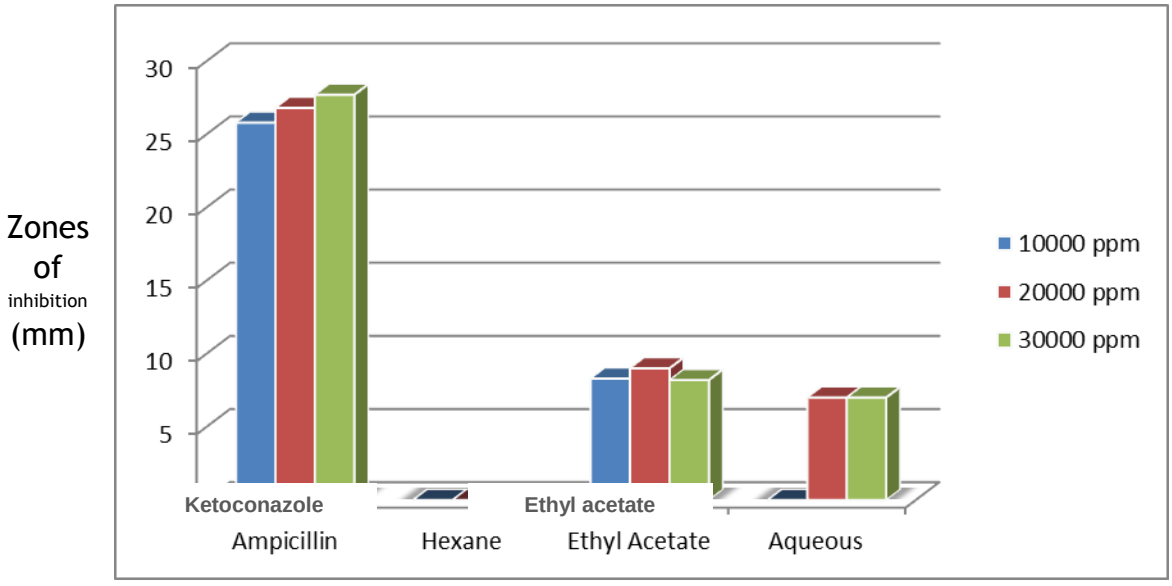

Figure 14. Graphical presentation for the zones of inhibition (mm) exhibited by the different crude extracts at various concentrations against *Candida albicans*

As depicted in **Figure 15**, the ethyl acetate extract shows zones of inhibition against *Candida albicans* at all concentrations but the aqueous extract only showed inhibition at 20000 ppm and 30000 ppm concentrations. While in **Figure 16**, no inhibition was observed.

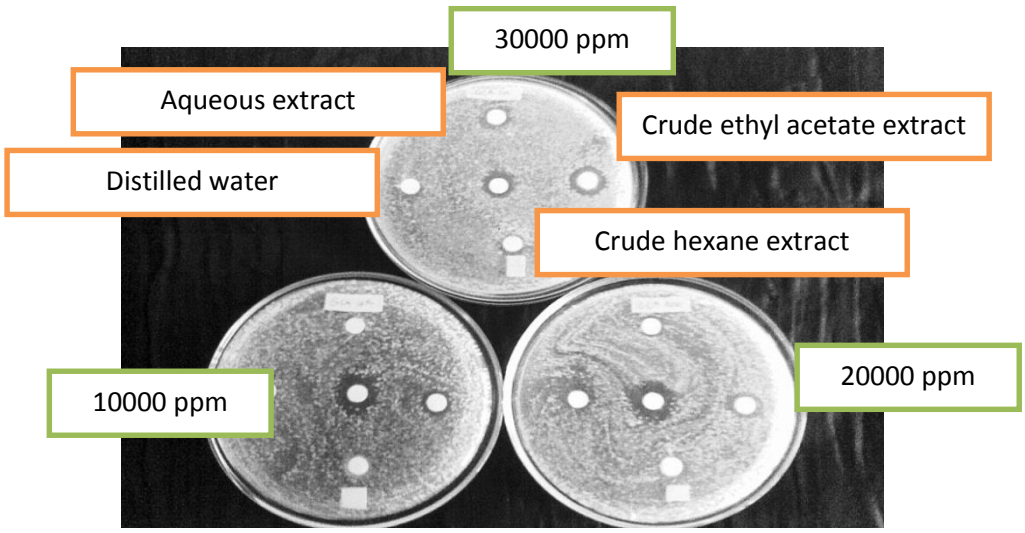

Figure 15. Antifungal assay against *Candida albicans*

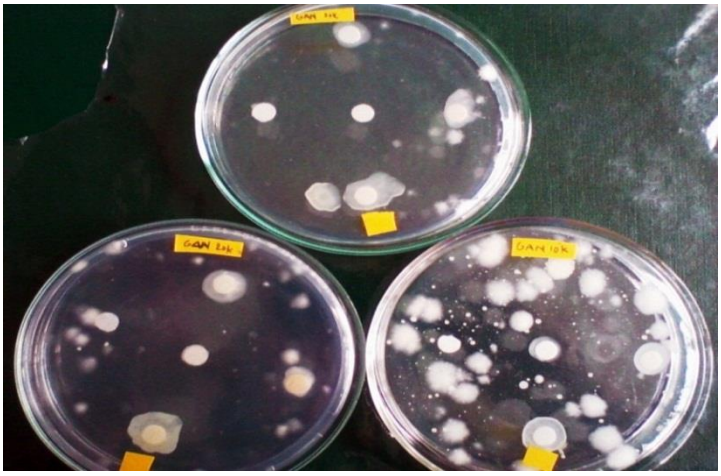

Figure 16. Antifungal assay against *Aspergillus niger*

Upon statistical analysis as shown in **Table 10,** the susceptibility of the test microorganisms was determined to the different treatments and relative to the antibacterial and antifungal standards.

Table 10. Susceptibility of the test microorganisms towards the different treatments

Test microorganisms	Ampicillin/ Ketoconazole	Hexane	Ethyl acetate	Aqueous	Distilled water
Staphylococcus aureus	A	C	B	C	C
Escherichia coli	A	D	B	C	D
Candida albicans	A	D	B	C	D
Aspergillus niger	-	-	-	-	-

Legend: Test microorganisms having same letters are not significant at α = 0.05 DMRT

In terms of the sensitivity of the different organisms towards the different extracts, it is discussed as follows: The growth of S.*aureus* is greatly inhibited by ampicillin and then followed by ethyl acetate. Crude hexane extract, aqueous extract and distilled water show no comparable effect. *E.coli* is most susceptible to ampicillin, followed by the crude ethyl acetate extract, then the aqueous extract but it is not sensitive to crude hexane and distilled water. However, inhibition of the growth of *C.albicans* is greatly observed in the ketoconazole treatment, followed by the crude ethyl acetate extract, then the aqueous extract but not in the crude hexane extract and

distilled water. All extracts and the standards ampicillin and ketoconazole did not show any inhibition towards *A.niger* and hence *A.niger* is not sensitive to all treatments.

Some literature shows that the antibacterial property of a substance may be enhanced by the presence of fatty acids, procyanidins and hydrolysable tannins which include tannic acids (De Miranda *et al.*, 1996). The results of the antimicrobial screening reveal that the crude ethyl acetate extract generally exhibits the most antimicrobial effect. And based on **Table 2,** the crude ethyl acetate extract shows a highly positive result on tannins and polyphenols which could be the reason behind its antimicrobial property.

Anthelmintic assay

The test organism that was used for anthelmintic evaluation was *Eudrilus eugeniae*. The time for paralysis and death of the test organisms were noted in minutes. **Table 11** and **Table 12** show the results of the mean paralysis and mean death time comparison among the different extracts, respectively.

As clearly depicted in the table, the hexane extract has the highest effect in the paralysis of the test organisms with an average paralysis time of 4.8 minutes relative to the levamisole standard with an average paralysis time of 3.8 minutes.

Table 11. Mean paralysis time using comparison among the different crude extracts

Treatments	Mean (min.)
Ethyl acetate	27.3
Aqueous	39.6
Hexane	4.8
Levamisole	3.8
Combantrin	539.6

Legend: Values are based on the average of five readings of five organisms per treatment per trial

Table 12. Mean death time using comparison among the different crude extracts

Treatments	Mean (min.)
Ethyl acetate	173.3
Aqueous	203.4
Hexane	38.8
Levamisole	9.4
Combantrin	619.4

Legend: Values are based on the average of five readings of five organisms per treatment per trial

From the statistical evaluation of data, levamisole exhibits the least average time to kill all test organisms with 9.4 minutes average death time; followed by hexane, ethyl acetate, aqueous and combantrin with 38.8, 173.3, 203.4 and 619.4, respectively. Statistical approach was employed to the mean values on the tables above showing the significant difference between the anthelmintic effects of the extracts; refer to **Table 13** and **Table 14.**

Based on the results of One-Way ANOVA and Duncan's Multiple Range Test (DMRT), the relationship between time of paralysis and death of test organisms and each extracts at various concentrations is discussed as follows:

Table 13. Tabulated mean of time of paralysis of *Eudrilus eugeniae* exposed to the different crude extracts

Treatment	Concentration (ppm)			P value	Remarks
	10 000	30 000	50 000		
	Time, minutes				
Hexane			4.8		
Ethyl acetate	37.8^{C}	27.4^{BC}	16.8^{A}	.011	Significant
Aqueous	61.6^{C}	41.6^{B}	15.6^{A}	.000	Significant
Levamisole	3.8	-	-	-	
Combantrin	539.6	-	-	-	
Distilled water	-	-	-	-	

Legend: Values are based on the average of five readings of five organisms per treatment per trial. Means having the same letter are not significantly at $\alpha = 0.05$ DMRT

Table 14. Tabulated mean of time of death of *Eudrilus eugeniae* exposed to the different crude extracts

Treatment	Concentration (ppm)			P value	Remarks
	10 000	30 000	50 000		
	Time, minutes				
Hexane			38.8		
Ethyl acetate	197.0^{A}	96.0^{C}	240.0^{B}	.000	Significant
Aqueous	175.4^{A}	180.0^{B}	198.0^{B}	.005	Significant
Levamisole	9.4				
Combantrin	619.4				
Distilled water					

Legend: Values are based on the average of five readings of five organisms per treatment per trial. Means having the same letter are not significantly at α = 0.05 DMRT

At 50000 ppm, the aqueous and ethyl acetate extracts showed no significant difference in the time taken for the paralysis of the test organisms. The two extracts are
significantly the same but the hexane extract showed significance relative to the two. For the time of death, there is no significant difference between the aqueous extract and the ethyl acetate extract. However, the hexane extract took the least time for the test organism to experience death.

With respect to the anthelmintic standards, levamisole and combantrin, there is no significant difference in the time taken for the test organisms to experience paralysis between the hexane extract and levamisole but there is a significant difference between hexane extract and combantrin. The same is true for the analysis of the time of death of the test organisms.

For ethyl acetate, there is no significant difference between 50000 ppm and 30000 ppm and between 30000 ppm and 10000 ppm for the time taken for the paralysis of test organisms. But there is a significant difference between 50000 ppm and 10000 ppm. However, for the time of death, a significant difference can be observed among the concentrations.

For the time taken for paralysis of test organisms by the aqueous extract, all concentrations each had exhibit a significant difference among each other. However, in the time of death, there is a significant difference between 10000 ppm and 30000 ppm and 10000 ppm and 50000 ppm; but no significant difference between 50000 ppm and 30000 ppm.

Figure 17, Figure 18 and **Figure 19** show the appearance of the test organisms in the plates before paralysis, in time of paralysis and death, respectively.

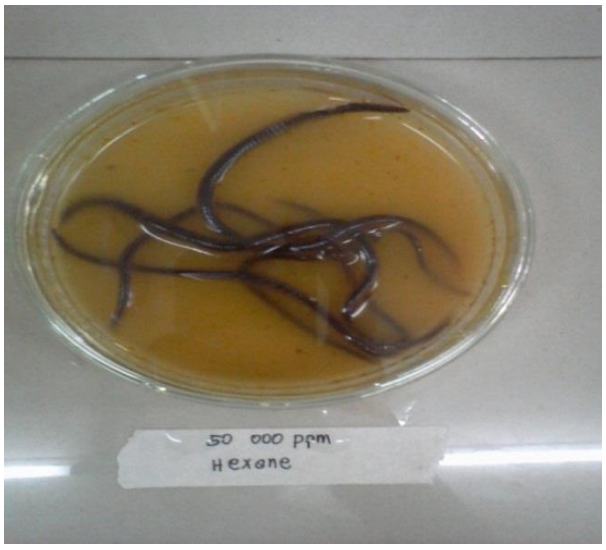

Figure 17. Before paralysis of *Eudrilus eugeniae*

Figure 18. Paralyzed *Eudrilus eugeniae*

Figure 19. Dead *Eudrilus eugeniae*

The potent bioactivity of each of the various crude extracts from the air-dried seeds of *Areca catechu* Linn. may be due to the presence of bioactive compounds isolated and distributed into the different extracts. The assumption may be supported by the results of phytochemical screening of the secondary metabolites: alkaloids, flavonoids, saponins, tannins and polyphenols.

The anthelmintic potential of the crude hexane extract is relatively higher among all other extracts at 50000 ppm concentrations with a mean time for paralysis of 4.8 min. which is significantly comparable to the positive control levamisole with a mean paralysis time of 3.8 min. Phytochemical screening of the crude hexane extract reveals the presence of tannins and polyphenols which could be a supplementary factor for its anthelmintic property. According to Atnasiadou *et al*. (2001), tannins have been shown to produce anthelmintic effects. The anthelmintic effect of plants containing tannins actually depends on the type and content of tannins in the plant. Furthermore, some synthetic phenolic anthelmintics example niclosamide, oxyclozanide and bithiol are known to interfere with energy generation in helmintic parasites by uncoupling parasite specific fumarate reductase mediated oxidative phosphorylation reaction. Another feasible effect of tannins is that they can bind to free proteins in the gastro intestinal tract of the host animal as glycoprotein on the cuticle of the parasite and cause death to it (Thompson and Geary, 1995). In this study, the crude hexane extract may have components that produce similar effects.

Conclusion

The crude hexane, crude ethyl acetate and crude aqueous extracts from the crude ethanolic extract from the air-dried seeds of *Areca catechu* Linn. were investigated for the absence or presence of the secondary metabolites: alkaloids, flavonoids, saponins, tannins and polyphenols, for its lethality by the brine shrimp lethality test at 10 ppm, 100 ppm, and 1000 ppm concentrations, for its antibacterial and antifungal properties by the filter paper disc diffusion method at 10000 ppm, 20000 ppm and 30000 ppm concentrations of the extracts and the standards: ampicillin and ketoconazole, for its anthelmintic potential against *Eudrilus eugeniae* at 10000 ppm, 30000 ppm, and 50000 ppm concentrations of the extracts and the positive controls: levamisole and combantrin.

Phytochemical screening of the extracts revealed that crude ethyl acetate extract contains alkaloids, saponins, flavonoids, tannins and polyphenols; while aqueous extract has all except saponins and the crude hexane extract has tannins and polyphenols only. This explains why the crude ethyl acetate extract, having a positive result to all phytochemical treatments, showed the most significant antimicrobial effect on the test microorganisms except *A. niger*. The aqueous extract, however, showed the highest lethality to *Artemia salina* with 100% mortality at 1000 ppm, 90% at 100 ppm, and 60% at 10 ppm. And the crude hexane extract, with a significantly positive result on tannins and polyphenols, exhibited the highest anthelmintic effect on *Eudrilus eugeniae* with a mean paralysis time of 4.8 minutes and mean time of death of 38.8 minutes.

References

1. Amudhan, M. Senthil, V. Hazeena Begum and K. B. Hebbar. (2012). *Review on Phytochemical and Pharmacological Potential of Areca catechu L. seed*. International Journal of Pharmaceutical Sciences and Research. Vol. 3(11): 4151-4157
2. Artero, V. and V. Santos. *Betel-nut Palm Care*.Fruit Production Publication. 2000.
3. Atnasiadou S, Kyriazakis I, Jackson F and Coop RL (2001). *Direct anthelmintic effects of condensed tannins towardsdifferent gastrointestinal nematodes of sheep: In vitro and In vivo studies*. Vet. Parasitol, 99. 2205-219
4. Cyriac, M.B., V. Pai, I. Varghese, M. Shantaram, M. Jose (2012). *Antimicrobial properties of Areca catechu (Areca nut) husk extracts against common oral pathogens.* International Journal of Research in Ayurveda Pharmacy. 3(1). 81-84.
5. De Miranda, CM, CW van Wyk, P van der Biji, NJ Basson (1996). *The effect of Areca nut on salivary and selected oral microorganisms*. 46(4). 350-6.
6. Ekeanyanwu, RC and OF Etienajirhevwe (2012). *Phytochemical analysis and in vitro anthelmintic potentials of Xylopia aethiopica (Dunal) A. rich (Annonacea) from Nigeria*. International Journal of Biology, Pharmacy and Applied Sciences. 1(3). 322-330.
7. Guevara, Beatrice Q. A Guidebook to Plant Screening: Phytochemical and Biological. Manila, Philippines: University of Santo Tomas, 2004.
8. Harbone, JB. Phytochemical methods, 2nd ed. 279. 1984.
9. Harwich, J. and Scott, P. (1971). Brine shrimp (Artemia nauplii L.) larvae as a screening system for fungal toxins. Applied Microbiology, 21: 1011-1016
10. Hill AF. (1972).Economic Botany. 2nd Ed. Tata McGraw Hill Publishing Company Pvt. Ltd, New Delhi. pp 275-276
11. Kale, J RD and Nair P (2012). *Earthworm muscle cells as models to study anthelminitic properties of plant extracts*. International Journal of Research in Pharmaceutical and Biomedical Sciences. Vol. 3. Pp. 489-496.
12. Laguna, CJB. (2013). *Anthelmintic Evaluation of the Different Extracts and Determination of the Presence of Tannins of the Crude Ethanolic Extract From the Seeds of Areca catechu Linn*.
13. Mondal, S., S. Bhattacharya and M. Biswas (2012). *Antidiabetic activity of Areca catechu leaf extracts against streptozotocin induced diabetic rats.* Journal of Advanced Pharmacy Education and Research. 2(1). 10-17.
14. Nethravathi HR*, Pallavi SS, Shruthi MN, Nandini HR, Prashith Kekuda TR,
15. Mukunda S (2010), *Antibacterial and anthelmintic activity of Chogaru (Betel nut concentrate)*, Research and Reviews in Biomedicine and Biotechnology.Volume 1, Issue 1, Pp. 20-23.
16. Pandey, K.N., S. G. Sharathchandra, G. S. Sarin, N. K. Ajmani, H. K. Chuttani, (1971). *Pyrantel Embonate in Treatment of Hookworm Infestation*.British Medical Journal. Vol.4. Pp. 399-400.
17. Sorgeloos, P. M. Baeza-Mesa, M. Benijts and G. Persoone (1976). *Current research on the culturing of the brine shrimp Artemia salina L. at the State University of Ghent, Belgium*. In Proceedings 10th European Symposium on Marine Biology. Vol.1. Pp. 473-495.
18. Tangalin, MG (2011). *Anthelmintic Effects of Processed Mature Betel Nut as Dewormer to Native Chicken and Small Ruminants (Sheep and Goats).* International Peer Reviewed Journal. Vol.1. No.1. Pp.230-243.
19. Temjenmongla and Yadav, A.K., (2005). Anticestodal efficacy of folklore medicinal plants of Naga tribes in Northeast India. Vol. 2(2): 129-133]

20. Thompson, DP and TG Geary (1995). *The structure and function of helminth surfaces*. Biochemistry and Molecular Biology of Parasites 1st edition. 203-32.
21. Vijaya, TM, Sushil Kumar M 1 2 iddha, 3Talambedu Usha, 1H.K. Aruna, 1R.Bharathi, 2Deepti Saini and 4G. Govindaraj (2012), *Morphological and Histological Studies on the Vermicomposting Indian Earthworm Eudrilus eugeniae,* World Journal of Zoology 7 (2): Pp. 165-170.
22. Williams S., Malik A. Chowdhury S. and Chauhan S (2002). *Sociocultural aspects of areca nut use*. Addict Biol; Vol.7:147–154.