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PHYTOCHEMICAL AND ANTIMICROBIAL INVESTIGATIONS OF METHANOLIC EXTRACT & ETHYL ACETATE EXTRACT OF RICE HUSK (*ORYZA SATIVA*)

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Abstract

The crude methanolic extract of rice husk (*Oryza Sativa*) was investigated for possible phytochemical, antimicrobial activities. Thin layer chromatography and ultra-violet spectroscopy were used to detect the presence of various types of compound in methanolic extract of rice husk. The methanolic extract showed antibacterial activities. The crude methanolic extract was fractionated by vacuum liquid chromatography by differents seven polar solvent. The 7 fractions were identified by performing TLC and later Disc diffusion assay of the primary seven fractions were performed to show the antibacterial effect using gram positive, gram negative strains of bacteria and fungi. Among the fractions n-butanol demonstrated antimicrobial activity. Other six fractions did not demonstrate antimicrobial activity. The n-butanol fraction was further exploited VLC to isolate the active principle which exhibited the antimicrobial activity. Dichloromethane fraction showed good anti-microbial activity than other six fractions. DCM fraction also showed good result in the MIC and MBC using bacteria and fungi. Lethality index and Receptor binding activities of the DCM fraction was investigated by hemagglutination inhibition assay. DCM fraction showed the negligible amount of toxicity effect and showed moderate hemagglutination inhibition activities.

Key words: Oryza sativa, antioxidant, antibacterial, TLC, hemagglutination assay

Introduction

Rice is the seed of plants *(Oryza sativa).* Its one of the important foods of the world. Particularly, countries in Asia are popular eating rice daily and main food rather than in order regions of the world. Rice is sources of many bioactive non-nutrient compounds, known as phytochemicals [1].

Globally, approximately 600 million tons of rice paddy is produced each year. The total production of 120 million tones of rice husk is get on average 20% from rice paddy [2]. In majority of rice producing countries most of the husk produced from processing of rice paddy which is used either burnt or dumped as waste. The chemical and functional components in different parts rice seed (*Oryza sativa*) before and after germination. Rice was separated into hull, then analysed for crude protein, crude lipid, free sugars, fatty acids, phytic acid, vitamin E, γ-oryzanol and γ-aminobutyric acid (GABA). Before germination, the crude protein content of rough rice was 97.28 mg/g, whereas after germination, it increased to 105.14 mg/g [3].

The chemical compositions of RHA especially silica content, is not high (in the range of 85% to 95%). All the other constituents of RHA, Al2O3 , CaO , K2O except potassium and magnesium, are available in a very small range, i.e., less than 1%[4]. Rice can be used to treat skin conditions. The rice is boiled, drained and allowed to cool and mashed. The rice is made into a paste or moulded into balls and these can be applied to boils, sores, swellings and skin blemishes. Other herbs are sometimes added to the rice balls to increase their medicinal effects. Sticky glutinous rice is often taken to treat stomach upsets, heart-burn and indigestion[5].

Extracts from brown rice have been used to treat breast and stomach cancer and warts. They have also been used to treat indigestion, nausea and diarrhea[6].

Materials and Methods:

Plant collection and identification:

The fresh Rice and plant sample were collected from Comilla, under Chittagong division on 15th June, 2013 and identified by the taxonomist of the Bangladesh National Herbarium, Mirpur, Dhaka as *Oryza sativa*. A voucher specimen of the plant has been deposited (Accession No.: 39530) in the herbarium for further reference

Extraction of the plant material

700g of testa powder was measured using an

TLC Analysis

The extracts were analyzed by performing TLC to determine the composition of extract. TLC was done using three solvent systems. The best result was obtained from solvent system-2 (chloroform: ethyl acetate: formic acid- 5:4:1) [7]. After development of TLC plates, they were exposed to UV light. For charring the plates were sprayed with 10% sulphuric acid solution, dried and then heated to 80-90ºC. This allowed the spots to be visible. For detection of flavanoids the plates were dipped into 0.04% DPPH solution and dried while keeping in a dark place. For detection of polyphenols the plates were washed with Folin-ciocalteu reagent and dried.

Preliminary Phytochemical Investigation

The ethyl acetate extract of husk *Oryza sativa* was qualitatively tested for the detection of any kind of secondary metabolites like alkaloid (Wagner Test), anthraquinone (Borntrager's Test), cardiac glycosides (Keller-kiliani Test), flavanoids (NaOH Test), steroids and terpenoids (Liebermann- Burchardt Test) [8].

Chemical analysis by UV spectroscopy

Ultraviolet (UV) spectroscopy scanning[9]. of the ethyl acetate extract of rice husk was performed within 200nm to 400nm using a Lambda UV spectrometer (Shimadzu, Japan). (Shimadzu,Japan).

DPPH Free Radical Scavenging Assay

The DPPH radical scavenging method was used for the determination of the antioxidant capacity of the sample [10]. Different concentrations of the ethyl acetate extract of husk *Oryza sativa* (10, 20, 30, 40 & 50µg/ml, in water) was prepared and 100 µl of DPPH solution was added. Different concentrations of L-Ascorbic acid (10-50 µg/ml) were used as the standard antioxidant. After 30 min at room temperature, the absorbance values were measured at 517 nm on a spectrophotometer and expressed into percentage of antioxidant activity using the following equation: DPPH antiradical scavenging capacity (%) = (Absorbance of sample – Absorbance of blank) × 100/Absorbance of blank. DPPH solution plus water was used as a control. IC_{50}values denote the concentration of the sample required to scavenge 50% of DPPH radicals. The results were expressed as mean ± standard deviations.

Determination of total phenolic content

The total phenolic content of ethyl acetate extract was determined using Folin-Ciocalteu method using

salicylic acid as standard [11] with some modifications. The samples were oxidized with 10% Folin-Ciocalteu reagent and were neutralized with 700 mM sodium carbonate solution. The absorbance of the sample was measured at 765 nm after 60 minutes. A calibration curve of salicylic acid was prepared. The total phenolic content was calculated as salicylic acid equivalent by the following equation: T = C x V /M, where, T is the total phenolic content in mg·g $^{-1}$ of the extracts as SAE, C is the concentration of salicylic acid established from the calibration curve in mg/ml, V is the volume of the extract solution in ml and M is the weight of the extract in g. The estimation of the phenolic compounds was carried out in triplicate. The results were expressed as mean ± standard deviations.

Antimicrobial Screening of ethyl acetate Extract of husk Oryza sativa

The antibacterial activity was carried out by the disc diffusion method [12] using 100µL of suspension containing ~10^3 CFU/mL of microorganism spread on nutrient agar medium (Himedia, India). Four different bacterial strains of gram positive, Eight different strains of gram negative bacteria and three different strains of fungi were used to carry out this assay. Dried and sterilized filter paper discs (6 mm diameter), ethyl acetate extract of husk, a stock solution of 10mg/ml was prepared and discs was soaked with solutions of 10µl of test samples and dried placed[13]. Standard disc of ciprofloxacin (30 µg/disc) was used as positive control. After incubation at 37^{0}C for 24 hours, the antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The results were expressed as mean ± standard deviations.

Minimum Inhibitory concentration of ethyl acetate Rice Husk

The minimal inhibitory concentration (MIC) values, which represent the lowest extract concentration that completely inhibits the growth of microorganisms. The sample in question is first prepared by producing a standard stock solution then subsequently diluting it to obtain different concentration[14]. Minimum Inhibitory concentration is carried out by using 100µL of suspension containing ~10^3 CFU/mL of microorganism spread on nutrient agar medium (Himedia, India). *S. aureus* and *S.cerevisiae* were used to carry this assay. Dried and sterilized filter paper discs (6 mm diameter), dried ethyl acetate extract of the husk, a stock solution of 60mg/ml was made in normalsaline. The plant samples were two fold serially diluted in eppendorf tubes to get different concentrations were prepared and discs were soaked with each solutions of 10µl of test samples were placed gently on the previously marked zones in the agar plates. After incubation at 37^{0}C for 24 hours, the antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The results were expressed as mean ± standard deviations.

Minimum Bacterial Concentration of ethyl acetate extract of Rice Husk

The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium [15]. 100µl of sample solution was placed on petridish. Nutrient agar was prepared by dissolving the appropriate amount of agar in distilled water in an agar bottle. All the necessary equipment and the nutrient agar were autoclaved at 121ºC for 45 minutes to ensure complete sterilization. After this all the equipment were shifted to the laminar airflow hood to prevent any contaminations. The sample of bacteria 1ml was taken in in an eppendrof tube containing 600µg ethyl acetate extract and then five times dilution in the five eppendrof tube to get different concentrations. The each tube were taken into vortex to ensure that the bacteria and ethyl acetate extract mixture out evenly. The agar was poured into the petridishes and left to cool and solidify. Using a sterile spreader the 100µl bacteria solution were taken and carefully swabbed on the agar plate so that the bacteria were uniformly distributed everywhere. The plates were left to rest for some time and then place in an inverted position in an incubator at 37ºC for 24 hrs[16**]**. After the prescribed incubation time the plates were removed from the incubator and the counting of the bacteria colony formation. Each experiment was carried out 2 times and was correlated against the controls.

Brine shrimp lethality test

Brine shrimp lethality test [17] was carried out to investigate the possible cytotoxic effect of the ethyl acetate extract of husk *Oryza sativa*. Brine shrimp eggs were hatched in a shallow rectangular dish filled with artificial seawater provided with light and

aeration. We allowed 2 days (48h) for the eggs to hatch and mature as nauplii. Samples were prepared by dissolving 20 mg of the husk ethyl acetate extract in the normalsaline Dilution of this stock solution gives the series of concentrations required for testing. 6 vials for each sample were taken. The no.6 vial was used as control (nauplii without sample). To each sample vial ten shrimps were transferred using a Pasteur pipette, and artificial seawater was added to make a total volume of 5 ml. The nauplii were counted against a lighted background. Counting for the chronic LC_{50} began 24 hour after initiation of tests [18]. Nauplii were considered dead if they were lying immobile at the bottom of the vials and the percentage of deaths at each concentration and at the control were determined.

Hemagglutination Inhibition Assay

Hemagglutination activity of ethyl acetate extract of rice husk was tested against human erythrocyte blood group A+ (positive) with some modifications[19]. Stock solution of the test samples was prepared at concentration of 10 mg/ml and each solution was serially diluted. Fresh blood was collected from healthy persons, centrifuged and the erythrocytes were separated. 2% erythrocyte suspension was prepared in phosphate buffer (pH 7.4). 100µl of sample was placed in the first well and then this was subsequently diluted two fold up to the 8th well. 100µl of the RBC suspension was added to all the wells and was incubated for one hour at 4°C. After incubation, the results were noted. Smooth button formation in bottom indicated negative activity, while a rough granular deposition at bottom showed positive activity. The intensity of hemagglutination was determined from the extent of deposition.

Results

TLC Analysis

TLC analysis was done as described in materials and methods. The plate was observed under UV light (indicated as 1). After charring of the TLC plate with sulfuric acid (indicated as 2) has visualized the three spots. After being soaked into DPPH and FC solution, plate 3 and 4 showed moderate yellow color.

Preliminary Phytochemical Investigation

Preliminary phytochemical screening showed the presence of alkaloid and Cardiac Glycoside in the ethyl acetate extract of husk *Oryza sativa.* Other phytochemicals were absent.

UV-Visible Spectrophotometric Sanning of ethyl acetate extract of Oryza sativa.

The spectrum of wavelength vs. absorbance for ethyl acetate extract of the husk was
obtained from the UV-Visible spectrophotometer and the values for the absorbance of the fraction was recorded. The graph is shown below.

DPPH Free Radical Scavenging Assay

From the analyses of Figure-2, we can conclude that the scavenging effect of ethyl acetate extract and ascorbic acid are very near to each other.

Total phenolic content assay of ethyl acetate extract

In case of polyphenolic content a standard curve was used where the equation is $y = 0.044x + 0.024$, $R^2 = 0.972$, From the standard curve, the total phenolic compounds as Salicylic acid equivalent (SAE) of the ethyl acetate extract was 40.9mg/g.

Antimicrobial Screening of ethyl acetate Extract

From the test conducted these results it is observed the husk of *Oryza sativa* possess antibacterial and antifungal activities.

Minimum Inhibitory Concentration of Ethyl Acetate Extract of Rice Husk.

The husk ethyl acetate extract was tested upon the Gram positive *S. aureus and* fungi *S.cerevisiae* the zones of inhibition were recorded. The results of this experiment are as follows

Minimum Bacterial Concentration of ethyl acetate Extract

The husk ethyl acetate extract was tested upon the Gram positive *S.aureus* the minimum bacterial concentration was recorded. The results of this experiment are as follows.

Brine shrimp lethality test

In brine shrimp lethality bioassay, percentage of mortality increased gradual with the increase in concentration of the test samples. Using the linear regression equation, $y = -12.28x + 104.6$ where $R^2 = 0.984$ the LC_{50} values of ethyl acetate extract was determined. From the results, it was revealed that, LC_{50} values was found to be 4.4.

Hemagglutination Inhibition Assay

Ethyl acetate extract exhibited hemagglutination inhibition activity potentially from highest concentration 4 mg/ml to 0.5 mg/ml i.e. it has potential binding capacity with human erythrocytes

Discussions

Single chromatogram observed under UV light in TLC indicated the presence of different compounds in that sample. Spraying of DPPH solution on the TLC plate have shown significant formation of plate yellow color. This provides us a preliminary idea of the various types of compounds that may be present in the ethyl acetate extract of the husk of *Oryza sativa*. From the preliminary Phytochemical screening it was concluded that the ethyl acetate extract might be alkaloid and cardiac glycosides because it gave positive result for the alkaloid test only.

The spectrum of wavelength vs. absorbance for ethyl acetate extract of the husk was obtained from the UV-Visible spectrophotometer and the value for the absorbance of the extract was recorded. 232nm, 285nm & 316 nm were found to be the λ max for the husk ethyl acetate extract of *Oryza sativa.*

To evaluate the antioxidant activities of ethyl acetate extract of husk DPPH Free Radical Scavenging Assay was used. The results show that the IC50% of ascorbic acid is 36.38 µg/µl and husk ethyl acetate extract is 45.99 µg/µl. These results show that the husk of *Oryza sativa* possess antioxidant free radical scavenging activity. This indicates that there are many compounds present in them that have antioxidant potential. A previous study showed that, antioxidant activity measured in ethanolic extract of Rice paddy (µmol Ascorbic acid equivalent/g fresh mass) were 67.09 and 15.55 as determind by DPPH radical –scavenging acitivity respectively **[20]** . So the isolated compound could be a future drug interest for antioxidant activity.

A study reported that the total polyphenolic content found in methanolic extract of boro and amon ,compared to boro rice and amon rice contained more phenolic compounds. Aman rice TPC ranged between 13.58 mg/g eq (BR22) to 25.30 mg/g eq and BRRI dhan37 had the TPC value of 21.14 mg/g[21]. In our study showed that the phenolic content of ethyl acetate extract of rice husk was 40.09 mg/g eq. of salicylic acid which was higher than reported the phenolic content of methanolic extract extract of aman and boro rice.

In antimicrobial screening, ethyl acetate extract husk *Oryza sativa* showed good result against antibacterial and antifungal activities. The positive control was used Ciprofloxacin. Ethyl acetate extract was showed zone of inhibition up to 20mm antibacterial activity at the concentrations used against *S. aureus, A. niger, B. subtilis, Saprophyti* than other strain. A previous study showed that the oils possessing antimicrobial activity can be employed against human pathogens[22]. The intake of rice bran oil might promote human health by preventing bacterial pathogenesis The result found in our study might have correlation with this. Methanol extract of Rice husk of *Oryza sativa* was further exploited in an attempt to isolate the active principle which exhibited the different antimicrobial activity. Due to the good result minimum inhibitory concentration test was carried out with ethyl acetate extract against the Gram positive *S. aureus* and fungi *S.cerevisiae* From the results we see that the MIC of the ethyl acetate extract of the husk of *Oryza sativa* for *S. aureus* and *S.cerevisiae* is 3.75µg/disk. Therefore, the dichloromethane fraction of methanolic extract of *Oryza sativa* may be considered as a useful source for discovering a safe and novel antimicrobial compound.

The MBC test determines the lowest concentration at which an antimicrobial agent will kill a particular microorganism. The MBC test was carried out with the ethyl acetate extract of rice husk since it exhibited good antimicrobial activity against microbes. The concentration of 600µg, 300µg and 150µg husk ethyl acetate extract exhibited good result against *B .cereus.* A study showed that, 500 µg/ml methanolic crude extracts of *Anabaena* BT2 produced 72-78 % growth inhibition of test bacteria [23].

The brine shrimp lethality assay was used for the evaluation of cytotoxic effects of the ethyl acetate extract of husk *Oryza sativa*. The LC_{50} values of ethyl acetate extract was determined respectively. The LC_{50} values for ethyl acetate extract was 6.3 mg/ml.

Hemagglutination inhibition assay was performed to investigate the receptor binding affinity of the compounds present in the husk ethyl acetate extract on human erythrocytes. It was observed that the extract has different binding affinity to the different receptors of erythrocytes and prevent agglutination. Hence the results showed a possible benefits of *Oryza sativa* Ethyl acetate extract as an antiviral therapeutics.

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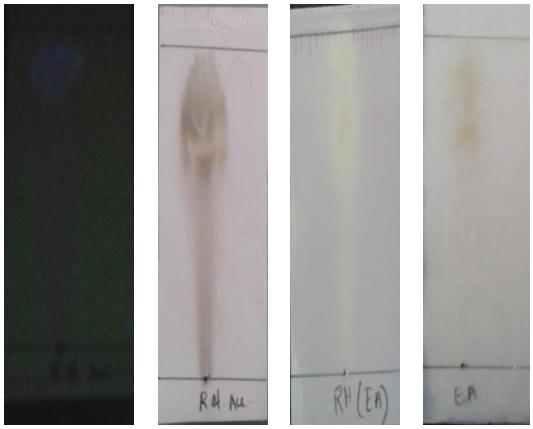
Figure 1:



1 2 3 4

Table1. Results of preliminary Phytochemical Investigation of secondary metabolites

Phytochemical Test	Result
Alkaloid	+
Anthra-quinone	-
Cardiac Glycoside	-
Flavonoid	-
Steroid & Terpenoid	-

Figure 2. The graph of Wavelength vs. Absorbance for husk ethyl acetate extract of *Oryza sativa*

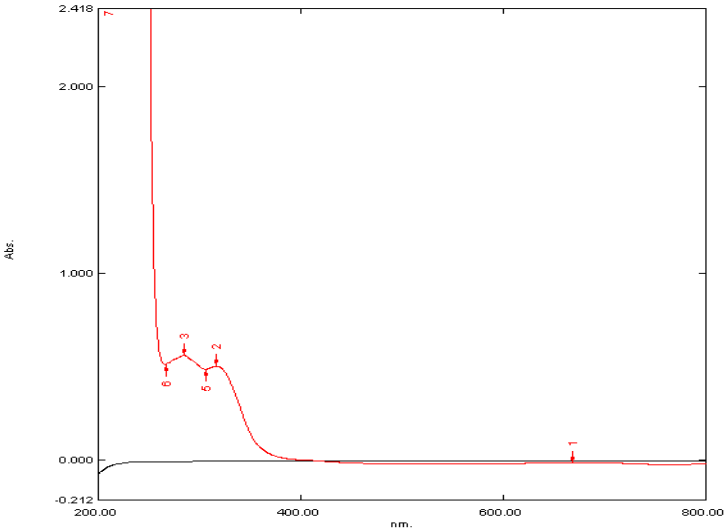


Table2. Ethyl acetate results of the Total Phenolic Content Assay

Sample Name	Total phenolic content (mg/g SAE)
Ethyl acetate	40.9 ±2.0

Table 3: Antibacterial Activity of ethyl acetate extract on the microorganisms test

Species	Zone of inhibition (mm)		
	Ethyl acetate extract of rice husk(10mg/ml)	Ciprofloxacin	Negative control
Vibriomimicus	15.5±0.7	20.5±0.7	-
S. cerevisae	18±2.8	22.5 ±2.1	-
S. pyrogen	20±0.0	22.5±3.5	-
Shigella boydii	20±0.0	23±1.4	-
S. typhi	16.5±2.1	19.5±2.1	-
C. albicans	17±0.0	19±1.4	-
Saprophyti	21±2.8	23.5±2.1	-
B. subtilis	22.5±2.1	26.5±2.1	-
S. aureus	23±1.4	23.5±2.1	-
S. dysentry	10.5±0.7	17.5±0.7	-
A.niger	22±1.4	23±1.4	-
Klebshiella	17.5±0.7	20±2.8	-
E.coli	17.5±2.1	21.5±3.5	-
Beta Hemolyte	17±1.4	22±0.7	-

Figure 3. Zone of inhibition for Ethyl acetate extract of rice husk. The values were expressed as mean ± standard error of mean. Blue bars represent values of Ethyl acetate extract; red bars represent values of positive control ciprofloxacin.

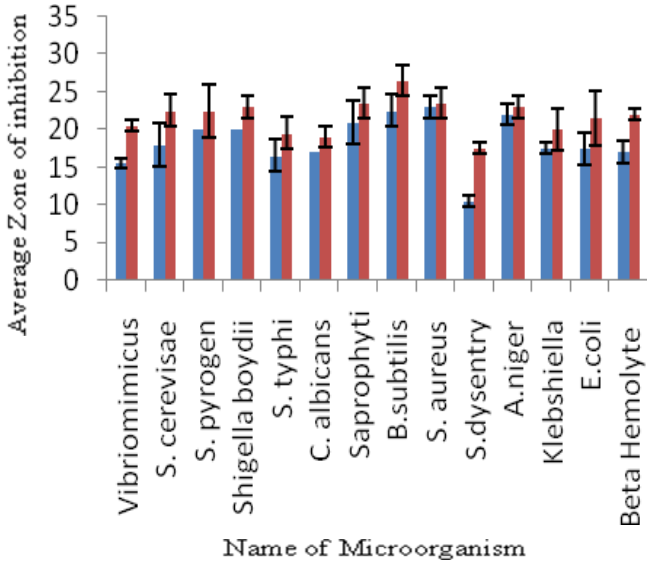


Table 4. Zones of Inhibition of *S. aureus* and *S.cerevisiae* when tested with the husk Ethyl acetate extract

Species	Minimum Zones of Inhibition (mm)				
	30µg	15µg	7.5µg	3.75µg	1.88µg
S. aureus	17±1.4	13.5±0.7	13±1.4	10 ±0.0	6.5 ±0.7
S.cerevisiae	15.5±0.7	12 ±1.4	9.5 ±2.1	7.5 ±2.1	-

Figure 4. Graph of zones of inhibition of *S. aureus a*nd *S.cerevisiae* when tested with the husk Ethyl acetate extract.

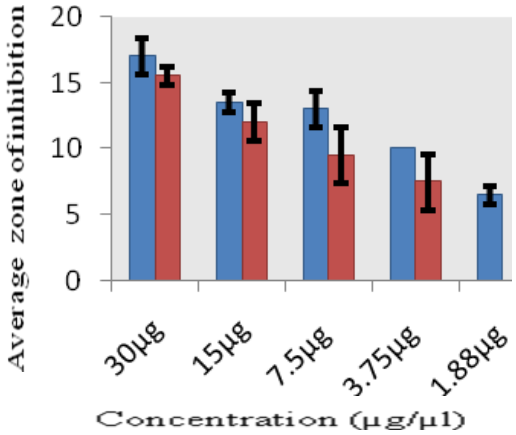


Table5. Minimum bacterial concentration of *S.aureus* when tested with the husk Ethyl acetate extract.

Species	Minimum bacterial count (CFU)				
	600µg	300µg	150µg	75µg	Control
S.aureus	4±4.24	4 ±2.83	7±4.24	169±4.24	177.5±10.61

Figure 5. Graph of the Minimum bacterial concentration of *S.aureus* tested with the husk ethyl acetate extract

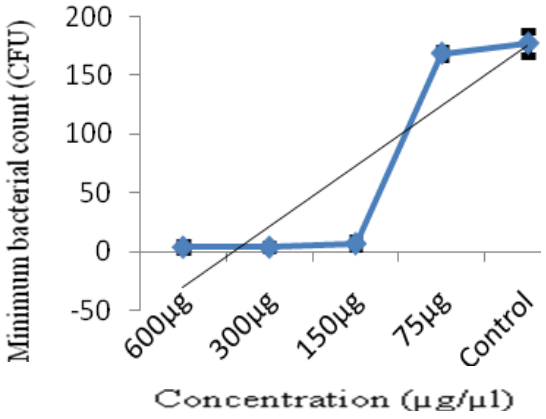


Figure 6. Concentration vs. percentage of lethality graph of Ethyl acetate extract

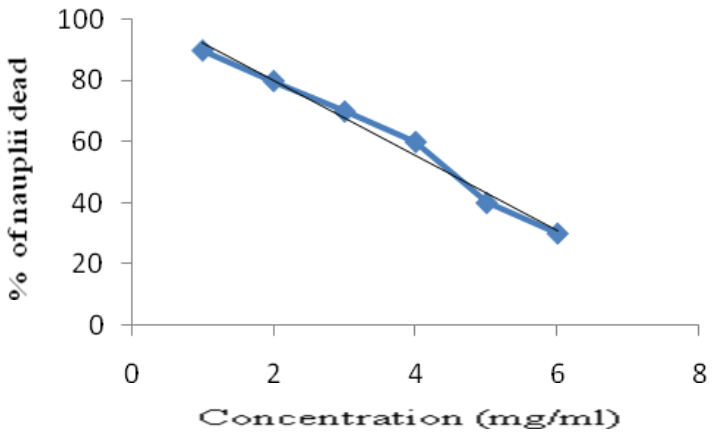


Table 6. Hemagglutination Inhibition, Test for ethyl acetate extract

Sample Name	Sample concentrations (mg/ml)							
	4	2	1	0.5	0.25	0.125	0.0625	0.03125
Ethyl acetate extract	+++	+++	+++	+++	-	-	-	-