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Evaluation of Cytotoxic Activity by Brine Shrimp Lethality Bioassay of Ethanol Extract and Its Different Fractions of *Anogeissus acuminata* (Roxb.) Leave

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ABSTRACT

Background: The purpose of the investigation was to ascertain whether the leaf extracts of *Anogeissus acuminata* holds any significant medicinal properties.
Methods: Leaves of *A. acuminata* was extracted with pure ethanol (EEAA), then methanol extract fractioned with n-hexane (NHFEEAA), chloroform (CHFEEAA), ethyl acetate (EAFEEAA) and methanol (MFEEAA). Cytotoxic assay was done by using brine shrimp lethality bioassay for measuring LC_{50}.
Results: Ethanol extract and its different fractions of leaves of *A. acuminata* showed moderate to well cytotoxic effect. In brine shrimp lethality bioassay, MFEEAA and CHFEEAA revealed the highest cytotoxic activity having LC50 values 215.32 ± 3.02 µg/ml and 234.56 ± 2.01 µg/ml, respectively.
Conclusions: The overall results of the study indicated significant cytotoxic activity of ethanol extract and its different fractions of leaves of *A. acuminata*. Furthermore, this plant deserves further investigation for other paramount pharmacological activities and comprehensive research and isolation of the active constituents responsible for these activities and establishes the mechanism of action.

Keywords: *Anogeissus acuminata*, cytotoxic, brine shrimp, ethanol, fraction

GRAPHICAL ABSTRACT

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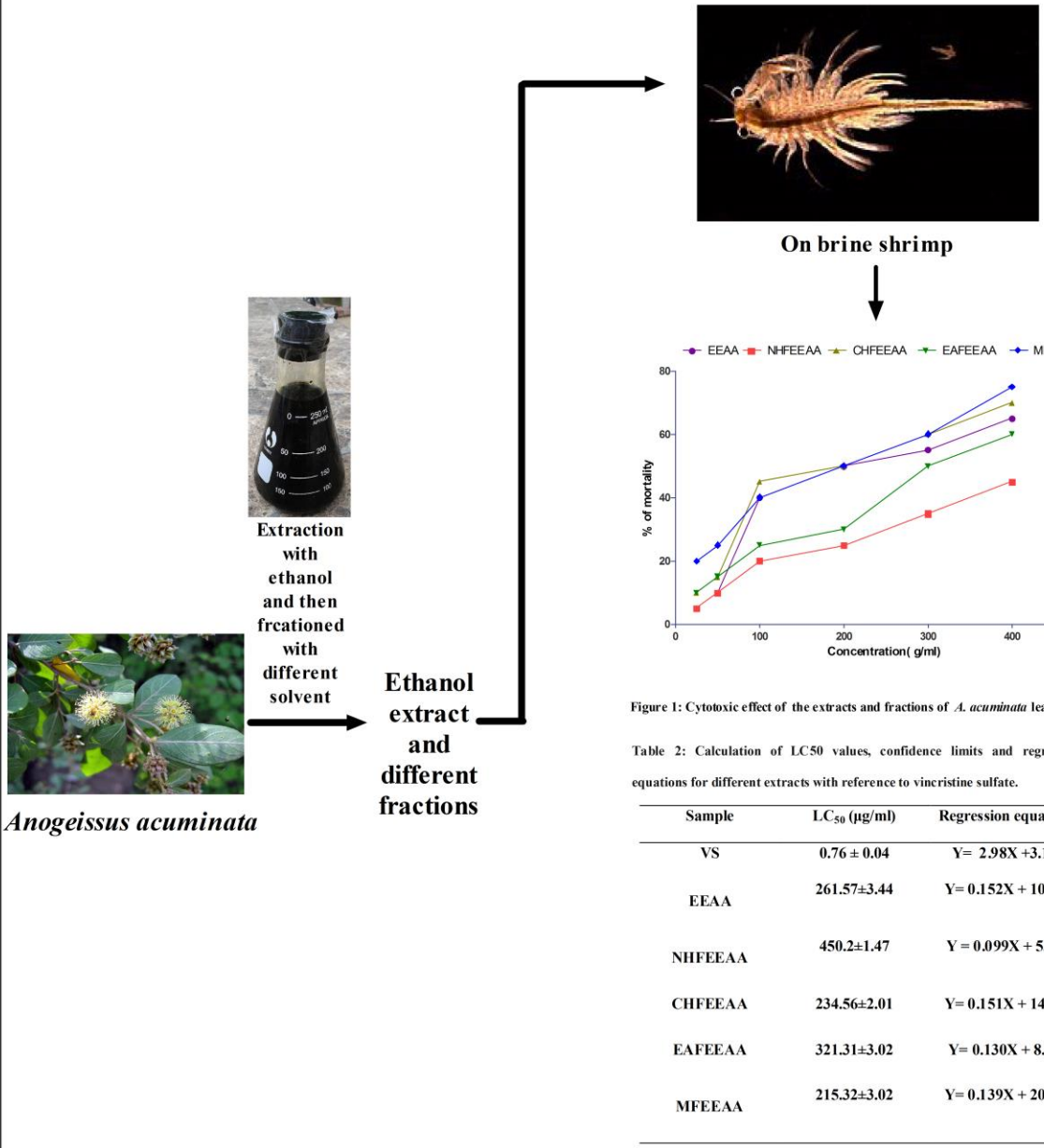

Figure 1: Cytotoxic effect of the extracts and fractions of *A. acuminata* leaves.

Table 2: Calculation of LC50 values, confidence limits and regression equations for different extracts with reference to vincristine sulfate.

Sample	LC_{50} (µg/ml)	Regression equation
VS	0.76 ± 0.04	Y= 2.98X +3.16
EEAA	261.57±3.44	Y= 0.152X + 10.24
NHFEEAA	450.2±1.47	Y = 0.099X + 5.43
CHFEEAA	234.56±2.01	Y= 0.151X + 14.58
EAFEEAA	321.31±3.02	Y= 0.130X + 8.23
MFEEAA	215.32±3.02	Y= 0.139X + 20.07

INTRODUCTION

Medicinal plants are the richest natural sources of medicinal products used in traditional and orthodox medicine [1]. The search for medicinal values of different plants has attracted increasing interest in the past couple of decades, presumably because of their potential as sources of potent pharmacological activities, convenience to users, economic viability, as well as low toxicity [2]. Plant-derived drugs proved to be relatively safer and dependable even in long-term use, where synthetic drugs are always feared in chronic cases [3]. Plants have also formed the basis of sophisticated traditional medicine systems in different therapeutic areas for thousands of years in many countries [4]. Therefore, continuous efforts in the search for newer sources of traditional medicines as well as screening of existing ones for newer therapeutic indications are inevitably urgent.

The study of plant materials is an imperative area which elucidates us about their functional characteristics and is one of the richest sources for various different chemical compounds exerting high medicinal value. Due to their potent pharmacological activities, low toxicity and economically suitable an extensive research can be processed on plants [5].

Cytotoxicity is a detrimental effect of cells which upon exposure of such toxic chemicals can result in necrosis of cell losing membrane integrity as a result of cell lysis and also can be followed by genetically program of controlled cell death known as apoptosis [6,7]. In this present study ethanol extract and fractions of the plant is collected and were tested for *in vitro* for their cytotoxic effect against the brine shrimp [8].

The plant under investigation *Anogeissus acuminata* (Family: Combretaceae). It is an indigenous plant distributed in Chittagong Hill Tracts and Cox's Bazar and with tribal name Phul jhumuri gaas (Chakma). The plant is rich in tannins and flavonoids. The plant material used for this study was collected from Bandarban district, Bangladesh. The tannoid principles of the plant possess antioxidant activity which was proven to reduce microbial infection [9,10].

As a part of relentless scrutinization of different valuable medicinal plants in Bangladesh the methanol extract of *A. acuminata* and its aqueous and organic soluble fractions were studied for the potential of cytotoxic effect.

MATERIALS AND METHODS

Plant materials

The leaves of *A. acuminata* were collected from Bandarban, Bangladesh in March, 2015 at mature stage. The leaves were cut into small pieces and then dried in shade at 21-30ºC for 7 days. Then the materials were dried in an oven at low temperature to improve grinding. Then the pieces were ground by a mechanical grinder and then passed through a size 60 mesh screen to obtain a fine powder of the leaf material. This was stored in an air-tight container.

Preparation of sample

The fine powder of leaves of *A. acuminata* (800 g) was taken in a clean round-bottom flask (5 L) and soaked in 4 L of Ethanol for 15 days at room temperature with occasional shaking and stirring. Then the mixture was first filtered with cotton plug followed by Whatman No. 1 filter paper. The filtrate is evaporated to dryness in Heidorph rotary evaporator at 45ºC to obtain a concentrated extract. This was then air dried to obtain solid residue. Thus the Ethanolic extract of the leaves of *A. acuminata* was prepared and then four solvents chloroform, n-hexane, ethyl acetate and methanol was used for solvent-solvent partitioning from ethanol solution.

Chemicals and reagents

The chemicals used were: ethanol, methanol, n-hexane, ethyl acetate, chloroform (Merck, Germany). Dimethylsulfoxide (DMSO) was from Sigma-Aldrich and rests of the chemicals used were analytical grade.

Cytotoxicity assay

Brine shrimp bioassay was carried out with the method as described by Meyer et al. to investigate the cytotoxicity of the extracts and fractions [11-13]. The dried extract preparations were re-dissolved in DMSO to obtain a solution of 10 mg/ml which was subjected to serial dilution to get the concentrations between 25 µg/ml- 1000 µg/ml. Standard drug Vincristine Sulphate (VS) was used as positive control at concentrations of 5 µg/ml - 0.312 µg/ml. A 5.0 ml of artificial sea water was added into all the test tubes. Simple zoological organism (*Artemia salina*) was used as a convenient monitor for cytotoxic screening. The eggs of the brine shrimps were collected from local aquarium shop (Dhaka, Bangladesh) and hatched in artificial seawater (Prepared by using sea salt 38 g/L and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 24 h under the light. The hatched shrimps were allowed to grow by 48 h to get shrimp larvae called Brine shrimp. After 48 h, active Brine shrimp were attracted to one side in a glass petri dish by using a micropipette. The Brine shrimp were then separated from the eggs by allocating them in another glass petri dish containing artificial sea water and used for the assay. Suspension containing 10 Brine shrimp was added into each test tube and was incubated at room temperature (25 ± 1°C) for 12 h under the light. The tubes were then examined after 24 h and the number of surviving larvae in each tube was counted with the aid of a 3X magnifying glass. Experiments were conducted along with VS in a set of three tubes per dose. The concentration that would kill 50% of the Brine shrimp (LC_{50}) was determined from a linear regression equation using the software "Microsoft excel 2007" [14].

Statistical analysis

Data are expressed as mean ± SEM. LC50 values of VS, extract and fractions were calculated by Microsoft Excel 2007. GRAPHPAD PRISM® (version 6.00; GraphPad Software Inc., San Diego, CA, USA) was used for graphical presentation.

RESULTS

Cytotoxic activity

The regression analysis for brine shrimp bioassay was presented in Table 1. Comparative mortality of brine shrimps and LC50 values for different extract and fractions was shown in Figure 1, respectively. No extract and fractions was found to be significantly toxic compared to positive control.

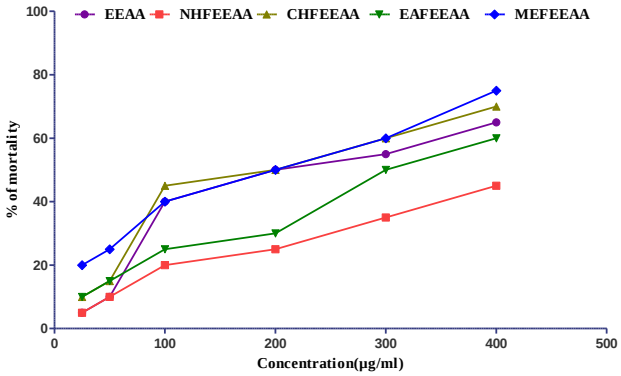

Figure 1: Cytotoxic effect of the extracts and fractions of *A. acuminata* leaves. EEAA = Ethanol extract of *A. acuminata* leaves; NHFEEAA = n-hexane fraction; CHFEEAA = Chloroform fraction; EAFEEAA: Ethyl acetate fraction and MFEEAA: Methanol fraction of ethanol extract of *A. acuminata* leaves

Table 1: Calculation of LC50 values and regression equations for the extract and fractions with reference to Vincristine sulfate (VS)

Sample	LC_{50} (µg/ml)	Regression equation
VS	0.76 ± 0.04	Y= 2.98X + 3.16
EEAA	261.57 ± 3.44	Y= 0.152X + 10.24
NHFEEAA	450.2 ± 1.47	Y = 0.099X + 5.43
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Each value represents a mean ± SEM (n = 3). EEAA = Ethanol extract of *A. acuminata* leaves; NHFEEAA = n-hexane fraction; CHFEEAA = Chloroform fraction; EAFEEAA: Ethyl acetate fraction and MFEEAA: Methanol fraction of ethanol extract of *A. acuminata* leaves.

DISCUSSION

Plants are potential sources of new drugs for human benefit. Plants used in traditional medicine contain a vast array of substances that can be used to treat chronic and even infectious diseases [15]. Also medicinal plants have recently drawn much attention of the scientists due to the strong healing effect with no or less adverse effects [16].

Brine shrimp lethality is a general bioassay which is indicative of cytotoxicity, antibacterial activities, pesticidal effects and different pharmacological activities [17,18]. Therefore, the isolation of bioactive compounds from natural sources and the use of plant extracts require toxicity information on the constituent of interest in order to delineate the effect of toxicity on both the host cells and target cells of pharmacological uses. Lethal concentration (LC50) from the regression and probit analysis in 24 h of our study showed that LC50 value of MFEEAA was 215.32 ± 3.02 µg/ml (confidence limit 95%) where the Regression equation was Y= 0.139X + 20.07 [19,20]. Comparison of this result with the standard Vincristine sulfate (0.76 ± 0.04 µg/ml) indicated that the lethality of *A. acuminata* extract and fractions are statistically significant ($P<0.05$) suggesting the notable clinical importance of the extract against tumor cells, pesticides etc. because the brine shrimp assay is considered as a convenient probe for a preliminary assessment of toxicity, detection of fungal toxins, pesticidal and anti-tumor effect and other pharmacological actions.

The plant extract is considered to be significantly cytotoxic when LC_{50} value obtained in brine shrimp lethality test is 250 µg/ml or less [21] and the result Figure 1 and Table 1 of our present study clearly indicates that the plant is not significantly cytotoxic. No extract or fractions was found to be significantly toxic compared to Vincristine sulfate. Among the fractions, n-hexane showed lower toxicity compared to others. So these fractions may not be harmful for the body.

CONCLUSIONS

In conclusion, this well informed study evaluated significant cytotoxic activity of Ethanol extract and its different fractions of *A. acuminata*. Although, All pharmacological profile were not performed for a single plant further improvements on such techniques and advancements in the procedure could be acquired through careful and systemic methods and hence requires advance new supplementary approaches. More precise studies are needed to elucidate their mechanism of actions for individual phytochemical if possible.

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