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Pharmacological effect of methanolic and hydro-alcoholic extract of Coconut endocarp

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ABSTRACT: *Cocos nucifera* used as natural remedies in a wide variety of diseases. The current experiment aimed to determine the qualitative phytochemicals, anxiolytic, anti-diarrheal, anti-inflammatory, thrombolytic and cytotoxic actions of *C. nucifera* endocarp, which extracted by using methanol (MeOH-CNE) and the hydro-alcohol (HaE-CNE). The MeOH-CNE and HaE-CNE subjected to phytochemical screening, where both extracts showed the existence of secondary metabolites such as carbohydrates, flavonoids, cardiac glycosides, and proteins. The anxiolytic activity screened by elevated plus maze experiment, whereas the percentage of open arm accounts 74.55 ± 4.54%, 66.31 ± 4.41% at 400 mg/kg for HaE-CNE, MeOH-CNE respectively ($P < 0.05$) while the standard drug Diazepam (75.24 ± 3.91%). In the anti-diarrheal test, extracts (200 and 400 mg/kg) and standard drug loperamide (5 mg/kg) showed dose-dependent significant ($P < 0.05$) inhibition against castor oil-induced diarrhea. The MeOH-CNE and HaE-CNE exhibited 72.91 ± 4.20 %, 64.42 ± 5.50% inhibition of protein denaturation at 500 µg/ml, while in the thrombolytic test, HaE-CNE showed the highest and significant ($P < 0.05$) in clot lysis activity on human blood in comparison to water, whereas streptokinase used as standard. In brine shrimp lethality bioassay, the LC_{50} of HaE-CNE and MeOH-CNE ware 432.35 µg/ml and 1173.88 µg/ml respectively. The LC_{50} for standard vincristine sulfate was 43.15 µg/ml. The current results suggested that MeOH-CNE and HaE-CNE have promising pharmacological activity.

KEYWORDS: *Cocos nucifera*, endocarp, anxiolytic, antidiarrheal, cytotoxic activity.

INTRODUCTION

Cocos nucifera (L.) belongs to the family Arecaceae (palm family), which is frequently termed a tree of life. For many years, coconut products have used as folk medicine. In ayurvedic medicine, coconut products such as oil, milk, cream, and water are being castoff in the treatment of hair loss, burns and heart complications [1]. All parts of *C. nucifera* can be used traditionally in various pathological conditions such as shell fibers of *Cocos nucifera* are used in diarrhea [2], antipyretic, renal inflammation and as a cream for dermatitis, sores, and injuries. The fibers are also used in asthma and diabetes. Moreover, the leaves and roots parts are used in diarrhea and stomach pains. The solid albumen of coca extracted as oil, pulp, milk which are used in antipyretic, diarrheal treatment, preventing hair loss, wound healing [3, 4]; oral contraceptive, diarrheal treatment, aphrodisiac, get rid of from skin rash affected

by HIV infection, respectively. The white surface of the Coca is used to consider for fever and malaria. The water of *C. nucifera* is used in treatment of renal disease [4-7]. Several pharmacological and biological activities are identified in several reports, including analgesia; anti-inflammatory, antimicrobial, antioxidant, anti-osteoporosis, anti-diabetic, anti-neoplastic, anthelminthic, antihypertensive, vasodilation, defense of kidney, heart, and liver functions and anti-malarial activities [8-12].

Anxiety syndromes are the most usual mental, emotional, and behavioral complications [13] affecting 264 million peoples around the globe [14]. In terms of overall management and treatment, depression among mood disorders has always been problematic [15]. Stress, depression and anxiety might trigger insomnia. Medicinal plants have been taken as sleep aids in insomnia throughout the world [16]. Despite the development of more molecules against the treatment of depression, unfortunately this disease remains untreated in many patients because of the burden of their side effects [17]. Therefore, searching for new therapeutic agents having no adverse effects may be the option of improved pharmacological actions, higher efficacy and safety.

Diarrheal syndromes are a key problem in underdeveloped countries and are accountable for the loss of a huge number of people every year [18]. Diarrhea is characterized by an increase in the water content, volume, or incidence of stools [19]. The research shown that several microbes like *Salmonella*, *Escherichia coli, Vibrio cholerae* and *Shigella* generate and release enterotoxins which are the major cause of diarrhea in developing countries [20]. Opioids and its derivatives such as difenoxin and loperamide are mostly used in the management of diarrhea [21]. Medicinal plants are an effective source for developing new antidiarrheal drugs. Therefore, the WHO has encouraged using medicinal plants in the management of diarrhea [22]. Currently available opioids and its derivatives are linked with several adverse effects for example abdominal pain, distention, bloating, nausea, vomiting, and constipation [23] and also cardiotoxicity has been testified by loperamide [24].

Endocarp of *C. nucifera* is the hardest part of the fruit and a rich source of phenolic and flavonoid content [25]. According to the literature review, *C. nucifera* shell has phenolic content [26]. The study aimed to investigate the *in vivo* anxiolytic, antidiarrheal and *in vitro* anti-inflammatory and thrombolytic, cytotoxic effects along with the phytochemical study of methanolic and hydro-alcoholic extract of *C. nucifera* endocarp. The present study investigates the *in vivo* anxiolytic, antidiarrheal and *in vitro* anti-inflammatory, thrombolytic, cytotoxic effects of *C. nucifera*, a Bangladeshi plant for the first time.

MATERIALS AND METHODS

Chemicals

Loperamide hydrochloride, castor oil (Sigma-Aldrich, MO, USA), Streptokinase (Beacon Pharmaceutical Ltd., Mymensingh, Bangladesh), Sea salt non ionized NaCl, vincristine sulphate (Sigma-Aldrich, MO, USA), ethanol, Tween 80 were used in this study. All other chemicals and reagent were used of analytical grade.

Preparation of plant extract

The moist endocarp of *C. nucifera* were collected from the Sitakunda local area of Chittagong, Bangladesh, which later validated by Professor Dr. Sheikh Bokhtear Uddin, Department of Botany, University of Chittagong. The *C. nucifera* endocarp layer was collected in a fresh condition. Then these are cut into small pieces if necessary to make it suitable for grinding purposes. Then the crude parts were rubbed for ten days and later grounded to powder by a mechanical drier (Ecocell, MMM Group, Germany) at 55-60 °C. By using another mechanical grinder (NOWAKE, Japan), the samples were ground to a coarse powder. The powder (500 g.) was soaked in 500 ml of methanol and 70% ethanol, respectively, for a week with occasional shaking and stirring on a shaker machine at room temperature. It was then filtered through a cotton plug followed by Whatman filter paper No. 1. After filtration, the filtrate was evaporated by using a rotary evaporator at 50 °C under reduced pressure to obtain the methanol crude extract (MeOH-CNE) (9.5 g) and hydroalcoholic extract (HaE-CNE) (11.48 g). The extracts were conserved at 4 °C in a refrigerator until further use.

Experimental animals

Swiss Albino mice (Six-seven weeks old) of either sex were purchased from the International Center for Diarrheal Diseases Research, Dhaka, Bangladesh (ICDDRB). The animals were adapted to the laboratory condition (25 ± 2 °C with a light/dark cycle of 12 h) for seven days before the study. The study was conducted following approval by the Institutional Animal Ethical Committee, Department of Pharmacy, International Islamic University Chittagong, Bangladesh, under the reference number (P&D-147/13-18).

Phytochemical screening

The qualitative phytochemical screening was executed according to the regular procedures [27] and the results shown presence or absence of secondary plant metabolites such as alkaloids, flavonoids, tannins, saponins, phenol, carbohydrate and glycoside.

Anxiolytic activity

Elevated plus maze test

Anxiolytic activity assessed by an elevated plus maze (EPM) apparatus, which used for the unlearned response. The EPM apparatus elevated from the floor at 40 cm, where it consists of two open arms and closed arms with a central square [28, 29]. The negative control received 1% Tween-80 (10 ml/kg, b.w), the reference drug Diazepam received (1 mg/kg, b.w) intraperitoneally, while the treatment group received 200 mg/kg and 400 mg/kg, b.w. by oral gavage. After sixty minutes, each mouse positioned in the center of the EPM facing towards the closed arm. Entry into open and closed arm recorded for 5 minutes and calculated the percentage by the resulting equation:

$$\%\ \text{of open arm entries} = \frac{\text{Number of entries in open arm}}{\text{Number of entries in open arm+number of entries in closed arm}} \times 100$$

Anti-diarrheal activity

Castor oil-induced diarrhea

The castor-oil induced diarrhea was followed to evaluate the antidiarrheal according to the method described by Taufiq et al. and Bellah et al. [30, 31]. Mice of either sex fasted for 24 hours. The negative control received 1% Tween-80 (10 ml/kg, b.w), the reference drug loperamide (5 mg/kg, b.w; as oral suspension), while the treatment group received 200 mg/kg and 400 mg/kg, b.w. by oral gavage. After 60 minutes, each mouse received 0.5 ml castor oil orally by gavage and individually placed in cages consist of transparent paper. The transparent paper changed in every hour and counted dry and wet feces in every 60 min for 4 hours. The equation calculated the level of % inhibition of defecation:

$$\%\ \text{inhibition of defecation} = \frac{A - B}{A} \times 100$$

Where, A = average eradication feces number of the control group; B = average eradication feces number of the text group.

Anti-inflammatory activity

Inhibition of protein denaturation method

The anti-inflammatory activity of the extracts determined using the protein denaturation method [32, 33]. The constituents of the reaction solution were 100 µl of the extracts (final concentration 62.5-500 µg/ml) and 100 µl of 5% aqueous bovine serum albumin. Then the pH was adjusted using glacial acetic acid. The samples were incubated at 37 °C for 20 min and then heated to 70 °C for 10 min. After incubation, the mixture was allowed to cool for 10 min, and a turbid solution found. Then the turbidity was measured at 660 nm. The blank consist of the sample and distilled water. Distilled water was used as negative control. The positive control was diclofenac sodium. Percentage inhibition was calculated using the formula:

% of protein denaturation = [(absorbance of control - absorbance of test sample) / (absorbance of test control)] × 100

Thrombolytic activity

The clot lysis activity performed as described by Prasad et al. [34]. A 3 ml venous blood is withdrawn from the ten healthy volunteers who did not have any previous history of taking NSAID or contraceptives. The withdrawn blood was distributed in nine different pre-weighed sterile micro centrifuge tubes (0.5 ml/tube) and incubated for 45 min at 37 °C. After the formation of the clot, completely removed the serum without disturbing the clot and each tube reweighed for calculating the clot weight. The plant extracts ware added to each micro centrifuge tube separately containing pre-weighed clot. 100 µL of streptokinase and distilled water were added separately to the positive and negative control group. All the tubes were then incubated at 37 °C for 90 min and observed for clot lysis. The released fluid was removed and reweighed the tube to calculate the difference in weight after clot disruption.

% of clot lysis = (weight of clot after remove of fluid/clot weight) × 100

Brine shrimp cytotoxicity

The brine shrimp lethality bioassay experiment was followed by the previous reported method [35]. In the artificial seawater (3.8% NaCl solution), the shrimp eggs hatched for 48 hours for maturing the shrimp called nauplii. The shrimp egg collected from the Katabon, Dhaka. The crude extract was dissolved in DMSO to obtain a solution of 5 mg/ml, which was subjected to serially diluted concentrations of 20 to 100 µg/ml, followed by the addition of 5.0 ml of artificial seawater in each test tubes. Ten of the living nauplii applied to

each of all experimental vials and control vials. Following 24 hours, all vials inspected by an amplifying glass, and the number of living nauplii in each vial was observed and recorded. Experiments were conducted in a set of triplicate manner along with reference drug vincristine sulfate. The lethal concentration (LC_{50}) that would kill one-half of the nauplii was determined from a linear regression equation.

$$\% \text{ of mortality} = (N_0\text{-}N_1/N_0) \times 100$$

Where, N_0= the number of nauplii taken; N_1= the number of nauplii alive.

Statistical analysis

Result represented as Mean ± Standard Error Mean (SEM). P (< 0.05 and < 0.001) were measured as statistically significant while the Dunnet's test was used to describe the significance using GraphPad Prism Version 6.0 (GraphPad software Inc., San Diego, CA).

RESULTS

Phytochemical *analysis* revealed the presence of secondary metabolites

The qualitative phytochemical screenings of MeOH-CNE and HaE-CNE have performed to find out the presence or absence of secondary plant metabolites. In our results, both extracts showed the presence of carbohydrates, flavonoids, cardiac glycosides, and protein. Whereas, only HaE-CNE showed the presence of alkaloids (Table 1).

Table 1. Qualitative phytochemical screening of MeOH-CNE and HaE-CNE.

Phytochemical class	Test performed	Observations	Results	
			MeOH-CNE	HaR-CNE
Alkaloids	Dragendorff's Test	Turbidity/precipitation	−	+
Carbohydrates	Molisch Test	Formation of a purple product at the interface of the two layers	+	+ +
Flavonoid	Ferric chloride test	Formation of yellow color which changed to colorless on acid addition	+ + +	+ + +
Steroids	Liebermann Burchard test	Green to pink color was absent	−	−
Terpenoids	Liebermann Burchard test	Appearance of reddish brown-deep red color	−	−
Cardiac Glycosides	Keller Killanic test	Lower reddish-brown layer & upper acetic acid layer which turns bluish green	+	+
Protein	Burette's Test	Appearance of purple color	+ +	+ +

(-) = not present; (+) = present in low concentration; (++) = present in moderately high concentration; (+++) = present in very high concentration. Where, MeOH-CNE: methanolic extract Cocos nucifera endocarp and HaE-CNE: hydro-alcoholic extract of Cocos nucifera endocarp.

Both the extracts reveled significant anxiolytic activity in EPM test

In elevated plus-maze (EPM) test, both extract reveled significant (P < 0.05) anxiolytic activity compared to the control group. The HaE-CNE treated mice at dose of 400 mg/kg showed significant (P < 0.05) increase of the percentage of open-arm entries (74.55 ± 4.54) compared to the control group, whereas the MeOH-CNE treated mice showed 66.31 ± 4.41 percentage of open-arm entries at the same dose (Figure 1). On the other hand, the reference drug diazepam at a very small dose 1 mg/kg, b.w treated mice produced a noticeable increase in the percentages of open-arm entries (75.24 ± 3.91).

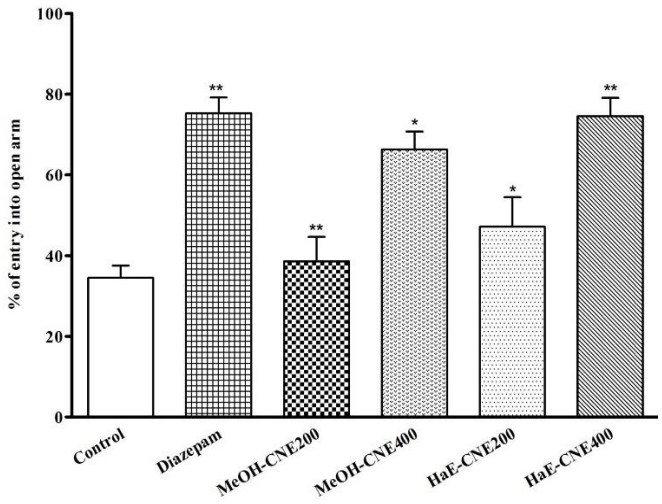

Figure 1. Effect of MeOH-CNE and HaE-CNE on percentage of entry into open arm in elevated plus maze test in mice. Values are mean ± S.E.M. **P* < 0.05 and ***P* < 0.01, significantly different from control; ANOVA followed Dunnett's test (n = 6, per group). Where, MeOH-CNE: methanolic extract *Cocos nucifera* endocarp and HaE-CNE: hydro-alcoholic extract of *Cocos nucifera* endocarp.

Both the extracts reduces the rate of defecation in a time-dependent manner

In the castor oil-induced diarrheal experiment, both extracts were found to be effective and significant (*P* < 0.05) in a dose dependent manner on experimental mice at all tested doses. At the dose of 400 mg/kg the HaE-CNE showed 56.79 ± 2.33% reductions in the rate of defecation in albino mice. Whereas, the MeOH-CNE extract showed 51.23 ± 3.26% reductions at the same dose. This condition was markedly reduced (62.49 ± 1.11%) by standard drug Loperamide at a dose of 5 mg/kg Figure 2.

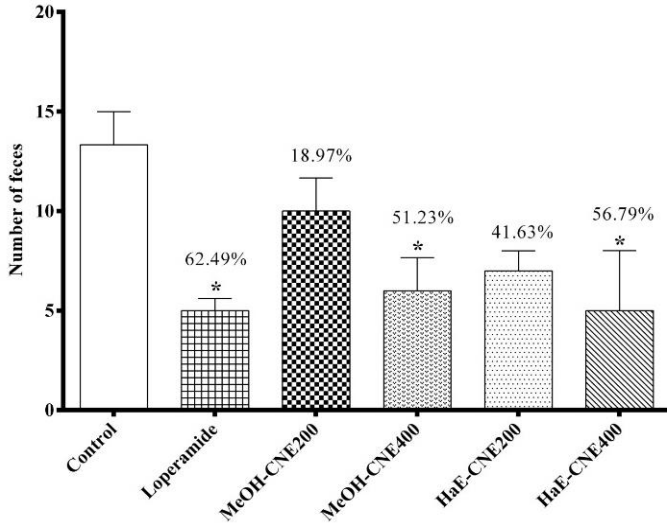

Figure 2. Antidiarrheal activity of MeOH-CNE and HaE-CNE against castor oil-induced diarrhea in mice. All values are expressed as mean ± SEM (n = 6); Data were analyzed by one way analysis of variance using GraphPad Prism for Windows, Version 6.0 (GraphPad software Inc., San Diego, CA, USA) followed by Dunnett's test for multiple comparisons,. Values with * *P* < 0.05 were considered as significant. MeOH-CNE: methanolic extract *Cocos nucifera* endocarp and HaE-CNE: hydro-alcoholic extract of *Cocos nucifera* endocarp.

Both the extracts increases the inhibition of protein denaturation

The results of anti-inflammatory activity of the extract are displayed in Figure 3. The result showed a dose dependent and significantly (*P* < 0.05) increased the inhibition of protein denaturation by MeOH-CNE, HaE-CNE and Diclofenac-Na throughout the concentration range (62.5-500 µg/ml). The crude hydro alcoholic extract (HaE-CNE) demonstrated maximum inhibition of protein denaturation (72.91 ± 4.20%) at 500 µg/ml, whereas the methanolic extract (MeOH-CNE) showed 64.42 ± 5.50% inhibitions at the same concentration.

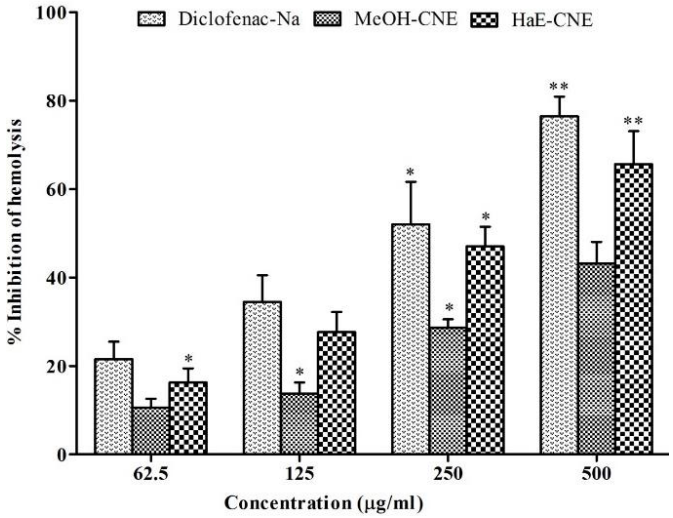

Figure 3. Percentage inhibition of protein denaturation by MeOH-CNE, HaE-CNE. Results are mean ± SEM (n = 3). ANOVA followed by Dunnett's test. Where, MeOH-CNE: methanolic extract *Cocos nucifera* endocarp and HaE-CNE: hydro-alcoholic extract of *Cocos nucifera* endocarp.

Both the extracts significantly increases clot lysis activity

The results of clot lysis activity of extracts are shown in Figure 4. After addition of 100 µl of Streptokinase a positive control (15,00,000 I.U.) to the clots along with 90 minutes of incubation at 37 °C, showed 73.44 ± 1.87% clot lysis. When clots were treated with negative control (sterile distilled water), a negligible clot lysis (8.87 ± 1.32) has observed. After the treatment of clots with HaE-CNE and MeOH-CNE, the significant (*P* < 0.05) clot lysis was observed. The clot lysis activity of MeOH-CNE and HaE-CNE ware 35.73 ± 3.21% and 55.74 ± 2.78% respectively.

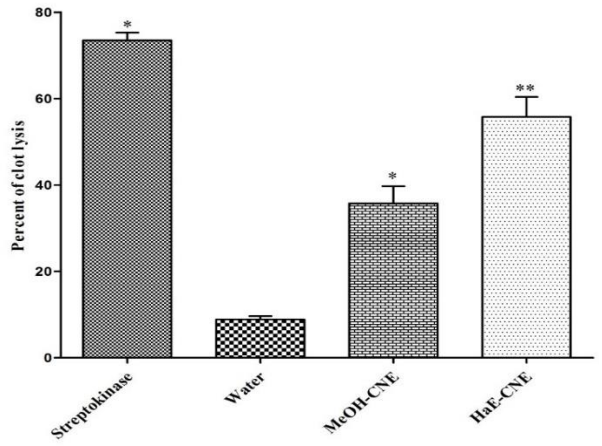

Figure 4. Clot lysis by streptokinase, MeOH-CNE, HaE-CNE and water *in vitro*. Values are expressed as mean ± S.E.M. $P < 0.05$, significantly different from control; ANOVA followed by Dunnett's test. Where, MeOH-CNE: methanolic extract *Cocos nucifera* endocarp and HaE-CNE: hydro-alcoholic extract of *Cocos nucifera* endocarp.

Both the extracts significantly decreases the mortality of brine shrimp

In brine shrimp lethality bioassay, the rate of mortality of nauplii was presented in Figure 5. The degree of lethality shown by the extracts was found to be directly proportional to the concentration of the extract ranging from the lowest concentration (20 µg/ml) to the highest concentration (1000 µg/ml). Both extract virtually non-toxic on the brine shrimp. They showed very low toxicity, giving LC_{50} values greater than 100 µg/ml. The LD_{50} values of MeOH-CNE, HaE-CNE and vincristine sulfate (VCS) were 432.35 µg/ml 1173.88 µg/ml and 43.15 µg/ml, respectively.

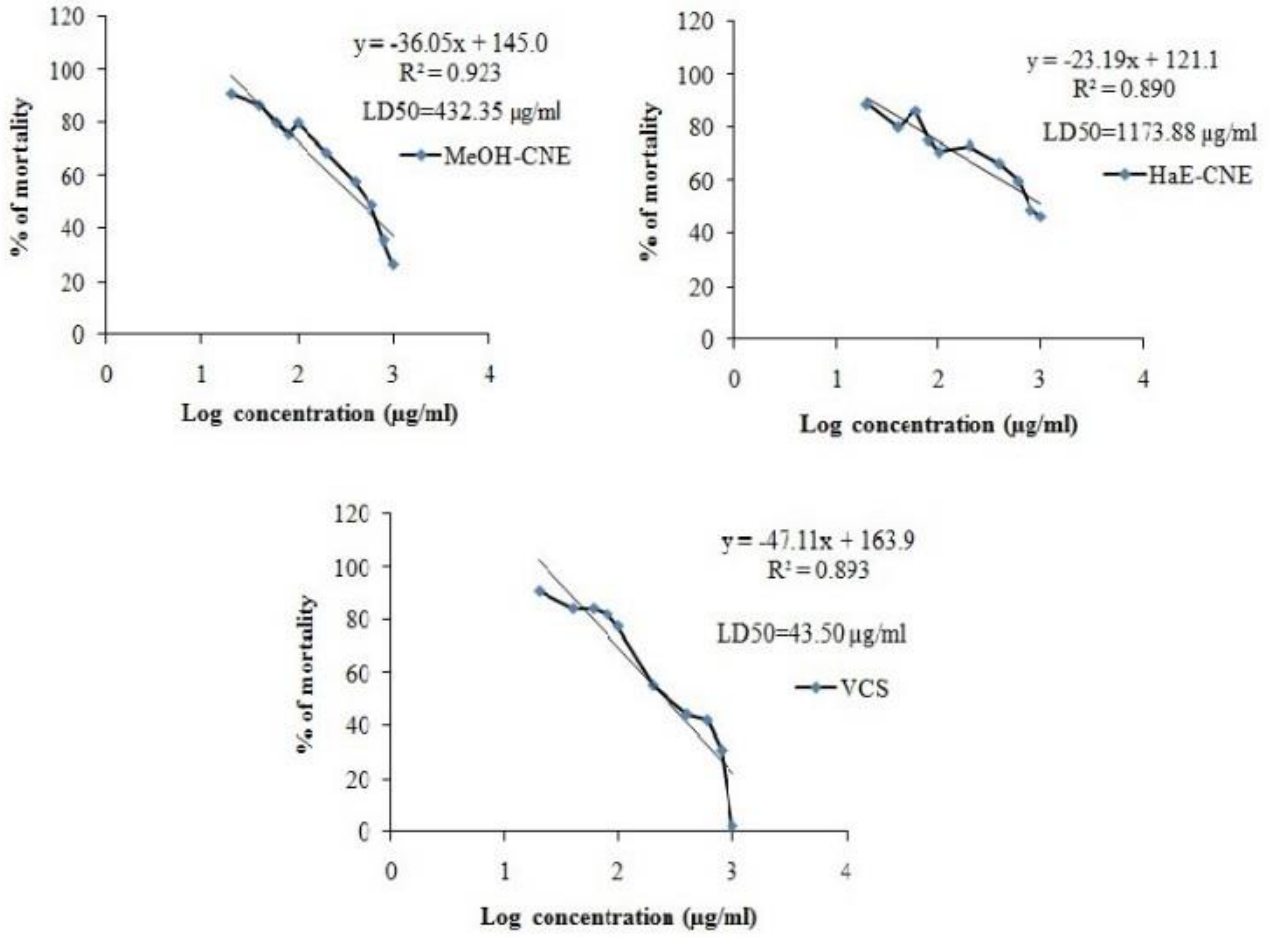

Figure 5. Cytotoxic effects of MeOH-CNE and HaE-CNE. Extrapolation of inhibition concentrations of both MeOH-CNE, HaE-CNE and reference cytotoxic agent vincristine sulfate through regression analysis. Data are shown as mean ± SEM of 10 shrimps for each concentration. MeOH-CNE: methanolic extract *Cocos nucifera* endocarp and HaE-CNE: hydro-alcoholic extract of *Cocos nucifera* endocarp.

DISCUSSION

The phytochemical analysis led on the plant extracts shown the presence of compound which is referred to show therapeutic as well as biological activities. Analysis of plant extract shows the presence of alkaloids, carbohydrates, flavonoids, cardiac glycosides, and protein. The phytochemical investigation led on the plant extracts shown the presence of compound which is referred to show therapeutic as well as physiological activities [36]. The current findings are similar to the previous study of the ethanolic extract of mesocarp [4].

The elevated plus maze (EPM) test is considered to be a good test procedure to evaluate the anxiety-like behavior [28, 37]. The increasing number of entries in the open arm indicates the anxiolytic effects and the increasing number of entries at the closed arm indicates the anxiogenic effects [37, 38]. Administration of MeOH-CNE, HaE-CNE showed an increased amount of entries in the open arms, an indication of anxiolytic-like behavior. Similar observations found to the reference drug (diazepam) which significantly increased the percentage of open arm entries. The current findings are similar to the previous study of ethanolic extract of *C. nucifera* endocarp, whereas the 500 mg/kg dose exhibited 74.77 ± 6.86 percentages of open arm entries [39].

The assessment of *C. nucifera* endocarp on castor oil-induced diarrheal mice to exhibit the dose-dependent manner, especially HaE-CNE showed a significant ($P < 0.05$) amount of reduction of diarrhea in comparison to the negative control. Similar observations found to the reference drug (Loperamide) which significantly increased the reduction of diarrheal faces. Castor oil is a recognized diarrheal agent due to the presence of ricinoleic acid which causes changes in the intestinal mucosa, as a result, fluid and watery luminal content that flow rapidly by intestine [40]. Due to the release of ricinoleic acid, it produces different inflammatory mediators such as prostaglandins, nitric oxide, platelet-activating factor, cAMP, and histamine [41, 42]. The present result validating the traditional use and the presence of secondary metabolites flavonoids [43].

The assay of anti-inflammatory effects was the possible effect of MeOH-CNE, HaE-CNE on protein denaturation assay. Inflammation causes lysis of lysosomes which release their enzyme and produces a variety of diseases. NSAIDs exhibit their activity by inhibiting the release of lysosomal enzymes [44]. Denaturation of proteins is initiated by inflammatory processes [45]. Thus, NSAIDs provide protection against protein denaturation. So, the ability of MeOH-CNE, HaE-CNE to inhibit the protein denaturation may provide a significant contribution to its anti-inflammatory properties. The present result might be responsible for the presence of secondary metabolites such as alkaloid and flavonoid [46].

The available thrombolytic drugs in the market, particularly streptokinase, convert plasminogen to plasmin and increased clot lysis. According to the literature review explained that flavonoids, among the plant metabolites, affect thrombosis and cardiovascular disease by interfering with platelet activation. *Cocos nucifera* endocarp, especially HaE-CNE, showed a significant ($P < 0.05$) amount of clot lysis in comparison to the negative control. This result might be because of the presence of flavonoids which influence embolus and cardiovascular disorder by interfering with platelet actuation [47].

Brine shrimp lethality assay is used for the cytotoxicity study. Ideally, the potential agent for the treatment of cancer should be nontoxic to a normal cell. However, anticancer agents are every so often lethal to normal cells, particularly towards rapidly growing cells. It is important to test this extract in low concentration to assess its potency [48]. The results observed in 24 h were found to be nontoxic for extracts. Generally, the smaller the LC50, the higher the toxicity, and vice versa. The value of LC50 over 1000 µg/ml is considered to be nontoxic, ranging from 500 -1000 µg/ml is weakly toxic, moderately toxic for 100 - 500 µg/ml while less than 100 µg/ml is considered as highly toxic [49-69]. No extracts were found to be toxic compared to Vincristine sulfate. The presence of alkaloids and saponins are present in this study, which can be effective as cytotoxic agents [27].

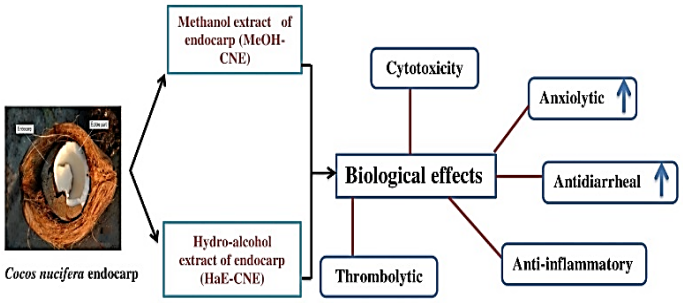

Figure 6. Graphical representations of pharmacological activities of *Cocos nucifera* endocarp.

CONCLUSIONS

Coconut endocarp extract can be regarded as a promising candidate with high therapeutic potential for drug preparation (Figure 6). The current study may provide useful data concerning the different medicinal properties in coconut shells for protecting humans against common diseases. From the above results, it may

be concluded that the endocarp extract of *C. nucifera* exhibited secondary metabolites with a dose-dependent manner anti-inflammatory activity and moderate thrombolytic activity with lower cytotoxicity. Moreover, significant anxiolytic and antidiarrheal effects observed by both methanolic and hydro-alcoholic extract of *C. nucifera* endocarp. Therefore, further work, especially bioassay-guided fractionation, is warranted in order to isolate and characterize the active constituents responsible for the specific biological property.

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AUTHOR CONTRIBUTIONS

SA, MS, AMT, MSN, MAR, ASMAR, ZMB and MAH together planned and designed the research. ASMAR, MAR and TBE arranged the whole facilities for the research and supervised the whole research. SA, MS, AMT, MSN and MJR conducted the entire laboratory works with ZMB and MAH. MAR, ASMAR and TBE imparted in study design and interpreted the results putting efforts on statistical analysis and also participated in the manuscript draft and has thoroughly checked and revised the manuscript for necessary changes in format, grammar and English standard. All authors read and agreed on the final version of the manuscript.

CONFLICTS OF INTEREST

Authors declared that they have no conflict of interest.

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