Chemical and Biological Investigations of *Cerbera odollam* Gaertn

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ABSTRACT: The present article describes the isolation and characterization of secondary metabolites from the leaves of *Cerbera odollam* as well as bioactivities of the crude methanolic extract and its petroleum ether (PE), carbon tetrachloride (CTC), chloroform (CF) and aqueous (AQ) soluble fractions. In this investigation, successive chromatographic separation and purification yielded β-amyrin (CO-01), lupeol (CO-02), β-sitostenone (CO-03) and triticusterol (CO-04). The study also indicated the antioxidant, cytotoxic, antimicrobial, membrane stabilizing activities and poor thrombolytic activities. Thus, the present study endeavors to provide scientific basis to establish *Cerbera odollam* as a potential medicinal plant.

Key words: *Cerbera odollam*, triticusterol, antioxidant activity, cytotoxicity, antimicrobial activity, thrombolytic activity, membrane stabilizing activity.

INTRODUCTION

To date, plants have always been useful to us not only in the treatment of various diseases, but also as a potential lead for discovering bioactive molecules in drug development process. The scientific exploitation of plants that are traditionally used as folk medicine for pain relief, wound healing and abolishing fevers resulted in the identification of a wide range of compounds and consequently paved the way of new therapies for cancer, hypertension, diabetes and infectious diseases.[1] A dramatic increase is also observed in last decade in the demand of medicinal plant as new potential source for both traditional and contemporary medicine in both developing and developed countries.[2] It is estimated that, world's population will be more than 7.5 billion in the next 10 to 15 years. This increase in population will occur mostly in the southern hemisphere, where approximately 80% of the population rely on a traditional system of medicine based on herbal drugs for primary healthcare.[1] Despite such high demand of this traditional medicine, it is observed that only 1% of them are known by the scientists and accepted for commercial uses.[3] Therefore, in this article, an attempt has been described which was taken to isolate and characterize the chemical constituents of the medicinal plant *Cerbera odollam.* In addition, efforts were taken to evaluate the possible biological profiles of the plant.

C. odollam, sometimes called *C manghas* L, belongs to the poisonous Apocynaceae family. The plant is also known as pong-pong, buta-buta, nyan or yellow-eyed cerbera tree in various regions.[4] It mainly grows in coastal salt swamps and creeks in south India and also along the river banks in southern and central Vietnam, Cambodia, Sri Lanka, Myanmar and Malaysia.[5] The seeds have a long history as a

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poison, particularly in Madagascar, due to the presence of highly toxic cardiac glycoside in them. Apart from poisoning, the plant is also used traditionally as emetic, cathartic, in curing hydrophobia and rheumatism.[6] Previous phytochemical investigations of *C. odollam* showed the presence of triticusterol, 2,6-dihydroxy-4-methoxybenzoic acid, 2-hydroxy-4-methoxy-6-methyl benzoic acid,[7] vebioside, deacetyl-tanghinin, neriifolin, tanghinin, monoacetylneriifolin,[8] and cardenolide glycoside 3 beta-O-(2'-O-acetyl-l-thevetosyl)-15(14-->8)-abeo-5 beta-(8R)-14-oxo-card-20(22)-enolide (2'-O-acetyl cerleaside A).[9] Biological activity evaluation also revealed its potential in anticancer,[10] antinociceptive, anti-bacterial, diuretic,[11] cytotoxic,[12] neuropharmaco-logical[6] and antioxidant activities[7] of this plant.

The main objective of the present study was to isolate and characterize secondary metabolites of the leaf of *C. odollam* and evaluation of antioxidant, cytotoxicity, antimicrobial, thrombolytic and membrane stabilizing activities.

MATERIALS AND METHODS

Chemical investigation

Collection and preparation of plant material. For the investigation, leaves of the *C. odollam* were collected from the Sunderban area, Bangladesh. Identification was confirmed by an expert taxonomist of Bangladesh National Herbarium (Acc. No. 31302). After collection, the leaves were sun dried for eight days. For better grinding, the leaves were then subjected to oven drying for about 24 hours at considerably low temperature. A high capacity grinding machine was used for crushing the dried leaves into a coarse powder.

Extraction. About 600.0 gm of powdered plant material (leaves) was soaked in about 2.00 liter of methanol in a 4.00 liter capacity amber glass container. It was kept for two weeks with occasional shaking and stirring. At the end of two weeks, the whole mixture was subjected to filtration through cotton plug and then through Whatman No. 1 filter paper. For evaporating the solvent a Buchii Rota evaporator was used at low temperature and pressure to provide about 36.00 gm crude dry extract.

Isolation of chemical constituents. For the initial rapid fractionation, the extract was subjected to vacuum liquid chromatography (VLC).[13] The whole crude extract was fractionated over silica gel powder (Silica gel 60H) by using different solvents of increasing polarities. Initially, 100% pet ether was used as the solvent system, which was followed by a mixture of pet ether and ethyl acetate and then ultimately 100% ethyl acetate. At the end, small amount of methanol was also used in combination with ethyl acetate for better separation of the crude extract. A total of 23 fractions (each 100 ml) were eluted .

For the assessment, elutes were spotted on Thin Layer Chromatography (TLC) plates . Based on the TLC plate behavior, some of the fractions were then subjected to gel permeation chromatography over lipophilic Sephadex (LH-20).

A total of 121 fractions, each 100 ml were collected. Then the fractions were examined using TLC and on the TLC features, potential fractions were selected for further purification. At this stage, the fractions were subjected to TLC over silica gel PF_{254} and the developed plates were then examined under UV lamp (both at 254 nm and 366 nm) and also by spraying the plate with vanillin-sulfuric acid reagent followed by heating at 110ºC for few minutes to locate promising spots. Further purification of fractions 11, 101, 02 and 107 by preparative TLC provided CO-04, CO-01, CO-02 and CO-03 respectively.

NMR spectroscopy. A Brucker (400 MHz) instrument was used for recording ^{1}H NMR and ^{1}H-^{1}H COSY spectra in $CDCl_3$ and the chemical shift values were determined with respect to the residual non-deuterated solvent signal.

Biological investigation of *Cerbera odollam*. For biological investigation, the crude methanolic extract of *C. odollam* was subjected to solvent-solvent partitioning using the protocol designed by Kupchan and modified by Van Wagenen *et al.*[14] According to this method, about 5 mg crude extract

was dissolved in 10% aqueous methanol. Then the methanolic solution was extracted with petroleum ether followed by carbon tetra chloride and finally with chloroform. Then each of the fractions were subjected to biological investigations such as evaluation of antioxidant, cytotoxic, antimicrobial, thrombolytic and membrane stabilizing activities.

CO-01(β-amyrin): White amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$), δ 0.77 (3H, s, H-24), 0.78 (3H, s, H-28), 0.85 (6H, s, H-29, H-30), 0.92 (3H, s, H-25), 0.95 (3H, s, H-23), 0.98 (3H, s, H-26), 1.23 (3H, s, H-27), 3.21 (1H, dd, *J*= 10.8, 5.2 Hz, H-3), 5.11 (1H, dd, *J*= 3, 6 Hz, H-12).

CO-02 (Lupeol): White amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$), δ 0.77 (3H, s, H-28), 0.78 (3H, s, H-23), 0.81 (3H, s, H-24), 0.94 (3H, s, H-25), 0.99 (3H, s, H-26), 1.05 (3H, s, H-27), 1.66 (3H, s, H-30), 3.20 (1H, m, H-3), 4.54 (1H, br, s, H-29), 4.66 (1H, br, s, H-29).

CO-03 (β–sitostenone): White amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$), δ 0.67 (3H, s, H_3-18), 0.80 (3H, d, *J* = 6.8 Hz, H_3-27), 0.82 (3H, d, *J* =6.4 Hz, H_3- 26), 0.82 (3H, t, *J* = 7.6 Hz, H_3-29), 0.90 (3H, d, *J* = 6.4 Hz, H_3-21), 1.16 (3H, s, H_3-19), 5.70 (1H, s, H-4).

CO-04 (triticusterol): 0.89 (3H, s, H-1), 0.91 (3H, d, *J*=6.4 Hz, H-7), 0.98 (3H, s, H-8), 0.98 (3H, d, *J*=6.4 Hz, H-9), 1.01 (3H, d, *J*= 6.4 Hz, H-11), 1.02 (3H, d, *J*= 6.4 Hz, H-16), 3.20 (1H, m, H-18), 4.69, 4.64 (2H, s, H-24).

Evaluation of antioxidant activity. For the evaluation of antioxidant activity, DPPH assay, designed by Brand-Williams *et al*.[15] was selected for the current investigation. In this investigation, evaluation of scavenging activities of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was used as the basis for assessing the antioxidant activities of the test samples taking butylated hydroxyl toluene (BHT) as the reference standard.

Evaluation of cytotoxicity. Brine shrimp lethality test was selected for screening cytotoxic activity of the plant.[16] In this investigation, DMSO (Dimethyl sulfoxide, Bioreagent, for molecular biology: Sigma-Aldrich, India) was used as solvent and negative control, whereas anticancer drug vincristine sulphate (Techno Drugs Ltd., Bangladesh), was used as positive control. Statistical analysis of concentration-mortality data was performed by using linear regression by using a simple IBM-PC program.

Screening of antimicrobial activity. For the current study, the method described by Bauer *et al*.[17] was selected for screening antimicrobial activity against six gram positive bacteria and eight gram negative bacteria. The strains of bacteria were collected as pure culture from Institute of Nutrition and Food Science, University of Dhaka. Sterile blank discs and standard antibiotic discs (Ciprofloxacine 30 µg/disc) were as negative and positive controls respectively. The test samples were loaded on the discs at a concentration of 400.0 µg/disc. The antimicrobial activity of the test samples were than evaluated by measuring the zone of inhibition with a digital slide calipers.[17,18]

Evaluation of thrombolytic activity. Thrombolytic activity of the test samples were evaluated by following established protocol.[19] Streptokinase (SK) was used as a standard for this investigation. For the preparation of positive control, commercially available lyophilized Altepase (Streptokinase) Eppendorf tube of 15, 00, 000 I.U. was collected from Beacon Pharmaceuticals Ltd, Bangladesh. Distilled water was used in the investigation as negative control.

Evaluation of membrane stabilizing activity. Membrane stabilizing activity of methanolic fraction of *C. odollam* leaf extract as well as other fractions of it was evaluated by following previously described method.[20]

RESULTS AND DISCUSSION

Chemical investigation of *Cerbera odollam*. The methanolic extract of the leaves of *C odollam*

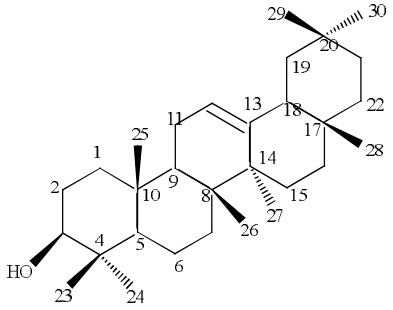
(Family: Apocynaceae) was investigated for isolation of the potent secondary metabolites from this plant. Successive chromatographic separation and purification yielded a total of ten compounds. Among them, the structures of four compounds were solved as β-amyrin, lupeol, β-sitostenone and triticusterol (Figure 1). Except triticusterol, this is the frist report of isolation of β-amyrin, lupeol and β-sitosterol from this plant.

The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound CO-01 showed the presence of eight methyl singlets at δ 0.78 (3H, s, H-28), 1.23 (3H, s, H-27), 0.98 (3H, s, H-26), 0.92 (3H, s, H-25), 0.77 (3H, s, H-24), 0.95 (3H, s, H-23), 0.85 (6H, s, H-29 and H-30). This suggested CO-01 to be a pentacyclic triterpenoid. A characteristic double doublet of one intensity at δ 5.11 (1H, t, *J*=, 3.6 Hz) was assigned to H-12, on an olean-12-ene skeleton. An oxymethine proton at δ 3.21 (1H, dd, *J*=10.8, 5.2 Hz) could be assigned to H-3. The coupling constant of this methane proton indicated that the hydroxyl function must be in axial position. The above features were similar to the ones reported for β-amyrin. On this basis, CO-01 was identified as β-amyrin.[21]

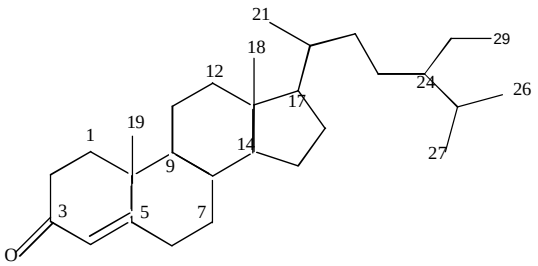
The ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound CO-02 displayed signals for a major and a minor compound. However, careful analysis allowed us to identify the major compound as lupeol. Two olefinic protons at δ_H 4.66 and 4.54 (1H, br. s each), a vinylic methyl at δ1.66 (3H, s, H-30) and six tertiary methyl signals at δ 1.05 (3H, s, H-27), 0.99 (3H, s, H-26), 0.93 (3H, s, H-25), 0.81 (3H, s, H-24), 0.78 (3H, s, H-23) and 0.77 (3H,s,H-28). Further the spectrum showed an oxymethine proton signal at δ 3.20 (1H, m), attributable to H-3. On this basis, CO-02 indicates the presence of lupeol.[22]

The ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of compound CO-03 displayed a broad singlet for an olefinic proton at δ 5.70 (H-4). The spectrum also revealed singlets at δ 1.16 and 0.67 (each 3H, s) assignable to two tertiary methyl groups at C-10 (H_3-19) and C-13 (H_3-18), respectively. The doublets at δ0.90 (*J* =6.4 Hz), 0.82 (*J*=6.4 Hz) and 0.80 (*J*=6.4 Hz) of three proton intensity each were demonstrative of methyl groups at C-20 (H_3- 21) and C-25 (H_3- 26, H_3- 27), respectively. A three proton triplet (J=7.6 Hz) at δ_H 0.82 was observed for another methyl group at C-28 (H_3-29). On this basis, the structure of the compound CO-03 was resolved as β-sitostenone, an isomer of β-sitosterone. The identity of compound CO-03 as β-sitostenone was confirmed by comparison of its spectral data with published values.[23]

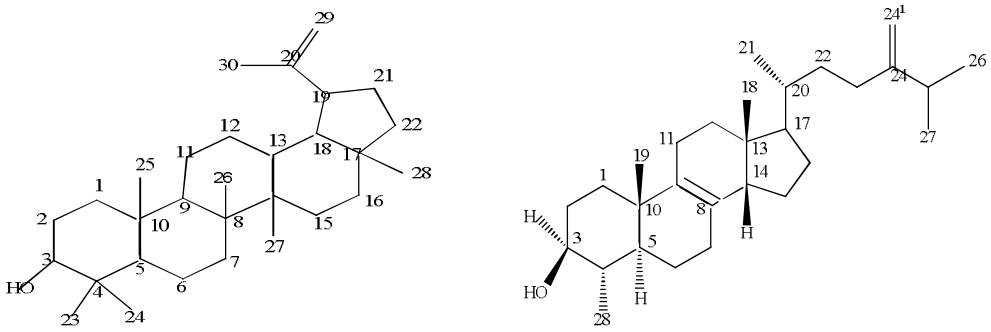
The ^{1}H NMR spectrum (400MHz, $CDCl_3$) of compound CO-04 also showed signals for a major and a minor compound. However, careful analysis allowed us to identify the major compound as triticustrerol. Two signals at $\delta_=$ 0.89 and 0.97 ppm for angular methyl groups at C-13 and C-10, respectively. It also revealed four doublets centered at δ0.92 (3H, d, *J*=6.4 Hz); 0.98 (3H, d, *J*=6.4 Hz); 1.01 (3H, d, *J*=6.4 Hz) and 1.02 (3H, d, *J*=6.4 Hz) which could be attributed to the methyl substituents at C-20, C-4, and C-25 respectively. The spectrum further demonstrated a one proton multiplet at δ3.2, indicative of H_α-3 of the steroid nucleus. Two downfield singlets were appeared at δ 4.64 and 4.69 ppm suggesting the presence of two exomethylene protons (H_2-24[1]). These features are in close agreement to those observed for triticustrerol.[7]



CO-01(β-amyrin)



CO-03 (β-sitostenone)



CO-02 (Lupeol) CO-04 (Triticusterol)

Figure 1. Structures of CO-01, CO-02, CO-03 and CO-04.

Biological investigation of *Cerbera odollam*

Evaluation of antioxidant activity. The methanolic extract of *C. odollam* and its different organic fractions were subjected for antioxidant activity evaluation by the method suggested by Brand-willium *et al.*[15] Here, BHT was used as reference for which 23.50 ±1.00 of IC_{50} value was obtained. In the evaluation, chloroform fraction of the plant showed the least inhibitory concentration of all the fractions with IC_{50} value of 40.00 ±1.00 µg/ ml, then carbon tetrachloride fraction and the methanolic extract showed the significant free radical scavenging activity with IC_{50} value of 72.01 ± 1.00 µg/ml and 75.02 ±1.00 µg/ml, respectively. On the other hand, less significant activity was observed for non-polar fractions (Petroleum ether and aqueous fractions) (Figure 2). Comparing the IC_{50} values obtained from the polar fractions with the reference, it may be suggested that the plant may act as a potential source of antioxidant.

Evaluation of cytotoxicity. In the investigation, the lethal concentrations LC_{50} of the test samples were calculated after 24 hours of observation by plotting the percentage of the shrimp died against the logarithm of sample concentration. The best fit line was taken from the curve by means of regression analysis. In this study, vincristine sulfate (VS) was used as a positive control for which LC_{50} was found to be 0.37 µg/ml. The LC_{50} values of PEF, CTCF, CF, MF & AQF were found to be 12.80 µg/ml, 8.49 µg/ml, 7.67 µg/ml, 10.77 µg/ml, and 6.28 µg/ml respectively (Figure 3). Comparing these values with the value obtained from standard, it was observed that all the fractions were quite lethal for the brine shrimp nauplii. From this observation, it may be proposed that this plant might act as a potential source of anticancer agents.

Antimicrobial screening. In the microbiological investigation, Ciprofloxacine was used as standard. The methanolic extract fraction (MEF) of *C. odollam* demonstrated moderate activity (zone of inhibition 8 mm) against *Bacillus megaterium* & *Shigella flexneri*, Pet ether fraction (PEF) was moderately active (zone of inhition between 7-8 mm) against *Bacillus cereus & Shigella sonni*. Carbon tetrachloride fraction (CTCF) showed moderate activity (zone of inhibition between 7-9 mm) against maximum bacterias: *Bacillus subtilis, Bacillus megaterium, Bacillus cereus, Staphylococus aureus* & *Sacina lutea*. Chloroform fraction (CF) showed moderate activity (zone of inhibition between 7-8 mm) against *Bacillus subtilis, Bacillus polymyxa, kleb Species* & aqueous fraction (AQF) demonstrated moderate activity (zone of inhibition between 7 mm) against *Bacillus subtilis.* It can be predicted from the observations that the plant is primarily active against gram positive bacteria. Thus, it may be suggested that the plant might act as a potential source of antimicrobial agents.

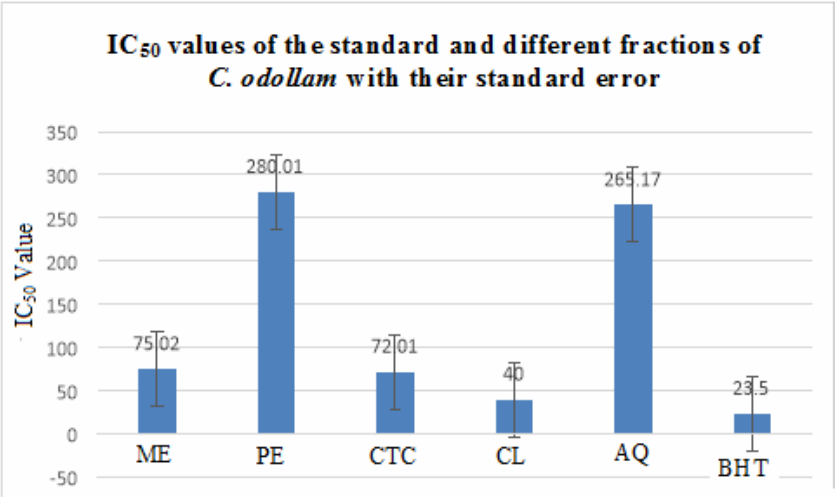
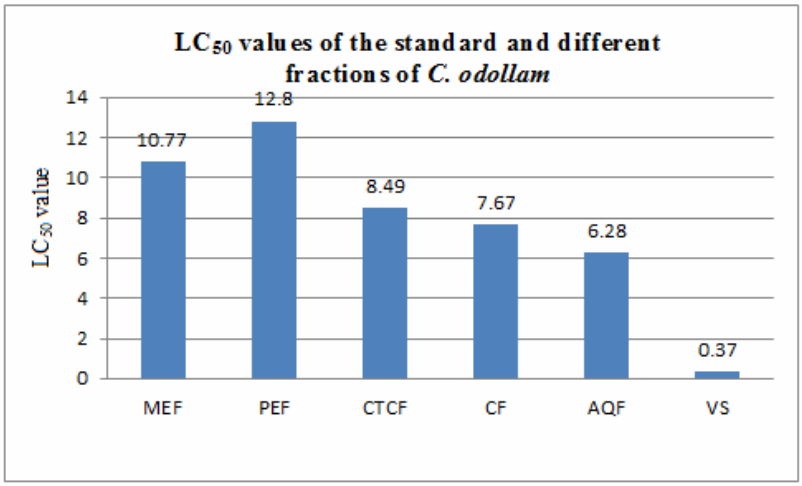


Figure 2. IC_{50} values of the standard and different fractions of *C. odollam (*ME= Methanolic Extract of the leaf, PE = Pet-ether fraction of the leaf, CTC = Carbon tetrachloride fraction of the leaf, CL= Chloroform fraction of the leaf, AQ = Aqueous fraction of the leaf, BHT = *tert*- butyl-1-hydroxytoluene)



Test samples	Regression line
MEF	y=26.01x+23.15
PEF	y = 25.29x+21.99
CTCF	y = 27.50x+24.44
CF	y = 30.40x+23.12
AQF	y = 25.73x+29.47
VS	y = 31.40x+63.68

Figure 3. LC_{50} values of the standard and different fractions of *C. odollam (*MEF = Methanolic Extract of the leaf, PEF = Pet-ether fraction of the leaf, CTCF = Carbon tetrachloride fraction of the leaf, CF = Chloroform fraction of the leaf, AQF = Aqueous fraction of the leaf, VS= Vincristine sulphate)

Thrombolytic activity. In thrombolytic activity evaluation, the standard Streptokinase (SK), that was used as a positive control showed 65.50 ±1.00% lysis of clot. On the other hand, distilled water was treated as negative control which exhibited a negligible percentage of lysis of clot (8.20 ±1.00%). The mean difference in clot lysis percentage between positive and negative control was found very significant. In this study, the methanolic extract of *C. odollam* (MESF) exhibited thrombolytic activity 8.88 ± 1.00%, the petroleum ether fraction (PEF) exhibited 0.41 + 1.00%, carbon tetrachloride soluble fraction (CTCF) exhibited 14.10 ± 1.00%, chloroform fraction (CF) exhibited 11.80 ± 1.00% and the aqueous soluble fraction (AQF) exhibited 8.34 ± 1.00% thrombolytic activity (Figure 4). By comparing the test results with the standard agents, it may be proposed that at higher dose, this plant might act as a potential thrombolytic agent.

Evaluation of membrane stabilizing activity. The methanolic extract and its different fractionates of leaves of *C. odollam* were effective in the

membrane stabilizing activity as the extractives prevented the lysis of erythrocytes induced by hypotonic solution. The petroleum ether fraction (PEF) inhibited 58.63%, methanolic extract fraction (MEF) inhibited 55.43%, chloroform fraction (CF) inhibited 47.03%, carbon tetrachloride fraction (CTCF) inhibited 42.42% and aqueous fraction inhibited (AQF) 36.87% of haemolysis of RBC. For membrane stabilizing activity Acetyl Salicyalic Acid was used as standard drug for which 71.90% inhibition of haemolysis was observed at normal condition.

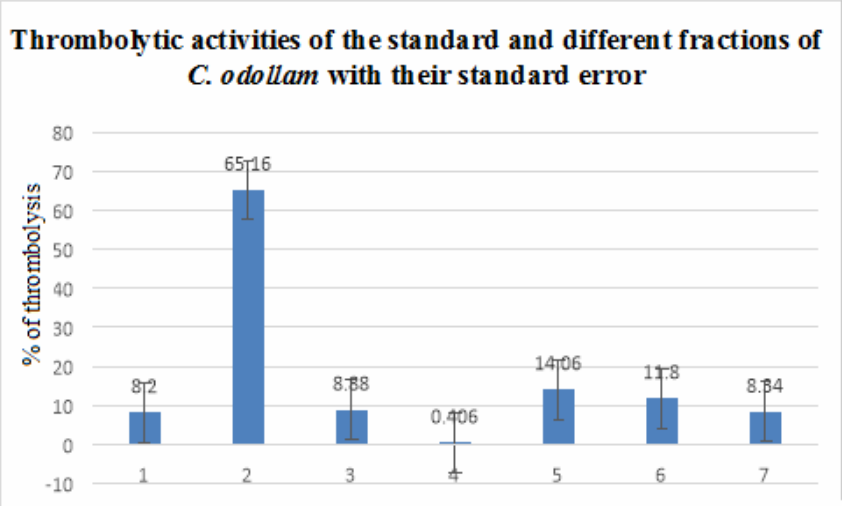


Figure 4. Thrombolytic activity of the standard and different fractions of *C. odollam* (1= Blank, 2= Streptokinase, 3= Methanolic extract of the leaf, 4= Pet ether fraction of the leaf, 5= Carbon tetrachloride fraction of the leaf, 6= Chloroform fraction of the leaf, 7= Aqueous fraction of the leaf)

But, the methanolic extract and its different fractionates of leaves of *C. odollam* were mildly effective in the heat induced membrane stabilizing activity. In the experiment, the petroleum ether fraction (PEF) inhibited 38.74%, metahanolic extract (ME) inhibited 35.41%, chloroform fraction (CF) inhibited 23.76%, carbon tetrachloride fraction (CTCF) inhibited 23.34% and aqueous soluble partitionate inhibited (AQSF) 14.81% of haemolysis of RBC. For membrane stabilizing activity Acetyl Salicyalic Acid was used as standard drug that exhibited 42.20 % inhibition of haemolysis at normal condition. Comparing the test results with the standard, the plant concerned might be suggested to be a potential source of anti-inflammatory drug.

CONCLUSION

Successive chromatographic separation and purification of methanolic extract of the leaves of *C. odollam* yielded a total of ten compounds. Among them the structures of four compounds were elucidated, viz., β-amyrin, lupeol, β–sitostenone and triticusterol. In free radical scavenging assay, chloroform soluble fraction showed the least inhibitory concentration among all the fractions with IC_{50} value of 40.00 µg/ml. In brine shrimp lethality bioassay, all the fractions showed good cytotoxic activity. In the microbiological investigation, the carbon tetrachloride soluble fraction of methanolic extract demonstrated mild activity against most of the bacteria with zone of inhibition 7-8 mm. During evaluation of thrombolytic property, the extractives of *C. odollam* showed very poor clot lysis activity as compared to the standard substance, streptokinase (SK). Only the carbon tetrachloride fraction showed little thrombolytic activity (14.06%). In membrane stabilizing activity assay, the methanolic extract of leaves of *C. odollam* at a concentration of 1.00 mg/ml, moderately protected the lysis of human erythrocyte membrane induced by hypotonic solution

and heat, as compared to the standard acetyl salicylic acid (0.10 mg/ml). Due to the presence of medicinally important secondary metabolites, *C. odollam* has significant biological importance. So, further study concerning this plant, there might be a new pathway for the mitigation of human sufferings.

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