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Screening of Antibacterial, Thrombolytic, Membrane Stabilizing, Anti-inflammatory and Antitumor Activity of *Citrus assamensis* Leaf Extracts

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Abstract

The methanol, ethanol and chlorofom leaf extracts of Satkara, *Citrus assamensis* (family: Rutaceae), were subjected to *in vitro* anti-bacterial, thrombolytic, membrane stabilizing and *in vivo* anti-inflammatory and antitumor activity tests. The chloroform extract of *C. assamensis* showed the most important spectrum of activity against *Bacillus subtilis, Bacillus cereus*, *Sarcina lutea* among 6 gram positive and against 11 gram negative bacteria at the concentration of 1000 µg/disc, while the range of zones of inhibition were within 7-16 mm. Among the tested three extracts $CHCl_3$ extract showed potent thrombolytic activity and hypotonic solution induced haemolytic activity where the percentages of inhibition were found to be 35% and 55% respectively. All the extracts established significant ($p<0.05$) anti-inflammatory effect by regulating biphasic inflammatory process induced by carrageenan. The leaf extract dose-dependently and significantly decreases the number of EAC cell count and inhibition of cell growth in comparison to the EAC control and standard. The results obtained in the present study indicate that, *C. assamensis* leaf can be a potential source of anti-bacterial, thrombolytic, membrane stabilizing, anti-inflammatory and antitumor agents.

Keywords: *Citrus assamensis*; Anti-bacterial; Thrombolytic; Membrane stabilizing; Anti-inflammatory; Antitumor activity.

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1. Introduction

Medicinal plants are always very promising for the development of new drugs. To distinguish any plant possessing medicinal quality, proper scientific screening is essential. Traditionally different plants are known to have different efficacy for treating various types of diseases.

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Pathogenic bacteria have always been considered as a major cause of morbidity and mortality in humans. Even though pharmaceutical companies have produced a number of new antibacterial drugs in the last years, resistance to these drugs has increased and has now become a global concern [1]. Emergence of resistance to first-line antibiotics poses challenge in treatment of several human infections [2-4] and is prompting a revival in research of the antimicrobial role of plants against resistant strains due to comparable safety and efficacy. The use of crude extracts of plant parts and phytochemicals, of known antimicrobial properties, can be of great significance in their therapeutic applications. Plants are rich in a variety of phytochemicals including tannins, saponins, alkaloids, and flavonoids which have been found to have *in vitro* antimicrobial properties [5-7]. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant microbial pathogens.

Thrombosis is the fundamental pathophysiological process that underlies the acute coronary disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks; which are the main causes of morbidity and mortality in developed countries [8]. Thrombolytic therapy uses drugs called thrombolytic agents, such as alteplase, anistreplase, streptokinase, urokinase, and tissue type plasminogen activator (tPA) to dissolve clots. The purpose of a fibrinolytic drug is to dissolve thrombin in acutely occluded coronary arteries thereby to restore blood supply to ischemic myocardium, to limit necrosis and to improve prognosis [9]. For the treatment of myocardial infraction, many thrombolytic agents are used among which streptokinase is remarkable and widely used. Moreover, tPA is more effective and safer than either urokinase or streptokinase type activators. Because of the shortcomings of the available thrombolytic drugs, attempts are underway to develop improved recombinant variants of these drugs [10].

Inflammation is a very complex biological state. Chronic inflammation may be associated with aging, cancer, adipogenesis, diabetes, cardiovascular problems, lung disease, etc. [11-13]. Erythrocyte membrane stabilization is a process of maintaining the integrity of biological membranes, such as erythrocyte and lysosomal membranes [14,15], which highlights the effect of synthetic and herbal anti-inflammatory agents on erythrocyte membrane that is exposed osmotic and heat-induced lyses. Several herbal derived drugs have been demonstrated to contain principles that possess ability to facilitate the stability of biological membranes when exposed to induced lyses.

Inflammation and oxidative stress play an important role in various diseases. It involves a complex array of enzyme activation, mediator release, and extravasations of fluid, cell migration, tissue breakdown and repair [8]. Non-steroidal anti-inflammatory drugs (NSAID) or steroidal anti-inflammatory drugs (SAID) are commonly used to treat different inflammatory diseases. Conventional anti-inflammatory agents are generally disease modifying agents and can’t cure the diseases as well as cause greater toxic effects in organs like liver, kidney, etc. [16]. Therefore, the development of new anti-inflammatory drugs is still necessary to overcome the toxicity of NSAID and naturally originated agents as they have very little side effects which may be desirable to substitute chemical therapeutics [17,18].

Cancer is one of the most life-threatening diseases and serious public health problems in both developed and developing countries next to cardiovascular disorders. It can be characterized by a rapid and uncontrolled formation of abnormal cells which may mass together to form a tumor, or proliferate throughout the body indicating abnormal growth at other sites. Many antineoplastic agents produce serious chronic or delayed toxicities that may be irreversible, particularly in heart, lungs, and kidneys [19]. Hence, natural products now have been contemplated of exceptional value in the development of effective anticancer drugs. Medicinal plants are playing an important role as a source of effective anticancer agents and it is significant that 60% of currently used anticancer agents are derived from natural sources including plants [20]. Plant-derived products have been reported to exhibit potent antitumor activity against several rodent and human cancer cell lines [21-34]. Thus, cancer patients who already got crippled with this disease, and who are further burdened by drug-induced toxic side effects, have now turned to seek help from the complementary and alternative medicine hoping for a better cure [35].

Citrus genus belongs to the large family Rutaceae, containing 130 genera in the seven subfamilies with many important fruit and essential oil producers [36]. A genus of evergreen aromatic shrubs and small trees distributed in the Indo-Malay region, South-East Asia and China but cultivated throughout the topical and temperate regions of the world [37].

Citrus assamensis, locally known as Satkora in Bangladesh, is a small tree, 4.5 to 7.5 meter of height and moderately branched and thorny plant of the family Rutaceae, which is used as medicine by local tribes of Assam, India. Leaves, flowers, and fruits of *C. assamensis* are used for treating dysentery, indigestion, pimples and intestinal worms [38].

However, so far, there is no report demonstrating the *in vitro* and *in vivo* biological activities of *C. assamensis* plant extract, which prompted us to design the present study to evaluate the *in vitro* antibacterial, thrombolytic and membrane stabilizing activity as well as *in vivo* anti-inflammatory and anti-tumor activity on Swiss albino mice by using the organic soluble materials of the leaf extracts for the first time.

2. Materials and Method

2.1. *Collection, identification and processing of plant samples*

Leaves of *C. assamensis* were collected from Jaintyapur, Sylhet, Bangladesh and the plant was taxonomically identified with the help of the National Herbarium of Bangladesh, Mirpur, Dhaka (DACB; Accession Number- 38759*).* Leaves were sun dried for seven days. The dried leaves were then ground in coarse powder using high capacity grinding machine (Jaipan Designer Mixer Grinder, India) which was then stored in air-tight container with proper labeling for identification and kept in cool, dark and dry place for investigation.

2.2. *Extraction procedure*

The powdered plant parts (30 g) were successively extracted in a soxhlet extractor at elevated temperature using 500 mL of distilled methanol at (40-60) °C which was followed by ethanol and chloroform. After extraction all extracts were kept in refrigerator at 4°C for further investigations with proper labeling for identification.

2.3. *Anti-bacterial activity*

The antimicrobial screening, which is the first stage of antimicrobial drug discovery, was performed by the disc diffusion method against Gram positive and Gram negative bacteria (Table 1) collected as pure cultures from an authentic source. Standard disc of ciprofloxacin (5 µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. The antibacterial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm [39].

2.4. *Streptokinase (SK)*

Commercially available lyophilized altepase (streptokinase) vial (Popular Pharmaceutical Ltd.) of 15, 00,000 I.U., was collected and 5 mL sterile distilled water was added and mixed properly. This suspension was used as a stock from which 100µl (30,000 I.U.) was used for *in vitro* thrombolytic activity evaluation.

2.5. *Blood sample*

Blood samples (n=6) was drawn from healthy human volunteers without a history of oral contraceptive or anticoagulant therapy and 1 mL of blood was transferred to the previously weighed micro centrifuge tubes and was allowed to form clots.

2.6. *Thrombolytic activity*

The thrombolytic activity of all extracts of the plants was evaluated by the method developed by Prasad *et al*. [40] using, streptokinase (SK) as the standard [41].

2.7. *In vitro* membrane stabilizing activity

The erythrocyte membrane resembles to thelysosomal membrane and as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of the lysosomal membrane [42]. The membrane stabilizing activity of the extractives was assessed by using the hypotonic solution-induced method [43]. To prepare the erythrocyte suspension, whole blood was obtained from healthy human volunteer and was taken in syringes containing anticoagulant EDTA (3.1% sodium citrate). The blood was

centrifuged and blood cells were washed three times with solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min. at 3000 g.

2.7.1. *Hypotonic solution induced hemolysis*

The test sample consisted of stock erythrocyte (RBC) suspension (0.50 mL) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extracts (1.0 mg/mL) or acetyl salicylic acid (0.1 mg/mL). The control sample consisted of 0.5 mL of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min. at room temperature, centrifuged for 10 min. at 3000 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either hemolysis or membrane stabilization was calculated using the following equation:

Percentage inhibition of hemolysis = $100 \times (OD_1 - OD_2/OD_1)$

Where, OD_1= optical density of hypotonic-buffered saline solution alone (control)
OD_2= optical density of test sample in hypotonic solution

2.7.2. *Heat induced hemolysis*

Isotonic buffer containing aliquots (5 mL) of the different extractives were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 56 °C for 30 min. in a water bath, while the other pair was maintained at 0-5 °C in an ice bath. The reaction mixture was centrifuged for 5 min at 2500 g and the absorbance of the supernatant was measured at 560 nm. The percentage inhibition or acceleration of hemolysis in tests and was calculated according to the equation:

Percentage inhibition of hemolysis = $100 \times [1 - (OD_1 - OD_2)/ (OD_3 - OD_1)]$

Where, OD_1= optical density of unheated test sample
OD_2= optical density of heated test sample
OD_3= optical density of heated control sample

2.8. *Experimental animal*

Swiss albino mice of either sex, 4-5 weeks of age, weighing between10-24 g were collected from icddr,b, Dhaka. Animals were maintained under standard environmental conditions and free access to feed and water. The animals were acclimatized to laboratory condition for one week prior to experiments. All protocols for animal experiment were approved by the institutional animal ethical committee.

2.9. ***In vivo anti-inflammatory activity***

2.9.1. *Carrageenan induced rat paw edema*

Swiss albino mice were divided into five groups (n= 6). Acute inflammation was produced by the subplantar administration of 0.1 mL of 1% Carrageenan in normal saline in the left hind paw of the Swiss albino mice. The paw volume was measured at 0 h and 2 h after Carrageenan injection using plethysmometer [44,45]. The first group received normal saline (3 mL kg^{-1} body weight p.o.) and the second group received phenylbutazone (100 mg/kg body weight p.o.). While the third, fourth and fifth groups were treated with methanol, ethanol and chloroform extracts at a concentration of (250 mg/kg body weight p.o.) respectively. The animals were pre-treated with the drug 1 h before the administration of Carrageenan.

Final paw volume (mL) = Mean paw volume after 2 h of induction - Mean paw volume at the time of induction

Percentage inhibition = (Final paw volume of control group - Final paw volume of drug treated group) / Final paw volume of control group × 100

2.10. ***In vivo anti-tumor activity***

Anticancer potentiality of methanol, ethanol and chloroform extract of *C. assamensis* leaf was evaluated by measuring tumor cell growth inhibition.

2.10.1. *Cell growth inhibition*

In vivo tumor cell growth inhibition was carried out by the method as described by Islam *et al*. [46] with slight modification. For this study, eight groups of Swiss albino mice (6 in each group) were used. For therapeutic evaluation 136×10^5 cells/mouse were inoculated into each group of mice on the first day. Treatment was started after 24 h of tumor inoculation and continued for 7 days. The first group received bleomycin (0.3 mg/kg of body weight) intra-peritoneally (i.p.) and the second group was treated with the vehicle (normal saline) and was considered as untreated control. Groups 3, 4 received methanol extract, groups 5, 6 received ethanol extract and groups 7, 8 received chloroform extract of *C. assamensis* at the dose of 100 mg/kg and 200 mg/kg of body weight (i.p.) respectively per day per mouse. In each case the volume of the test solution injected (i.p.) were 0.1 mL/day per mouse. The mice were sacrificed on the 8th day after transplantation and tumor cells were collected by repeated intra-peritoneal wash with 0.9% saline. Viable tumor cells per mouse of the treated group were compared with those of control (Ehrlich Ascites Carcinoma, EAC untreated) group.

The cell growth inhibition was calculated by using the formula:

Percentage cell growth inhibition = $(1 - Tw/Cw) \times 100$

Where, Tw = Mean of number of tumor cells of the treated group of mice and Cw = Mean of number of tumor cells of the control group of mice

2.11. ***Statistical analysis***

Data was expressed as mean ± SEM (Standard error of mean). The results were analyzed statistically by ANOVA followed by Dunnet's test. Results with $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered statistically significant.

3. Results and Discussion

3.1. ***In vitro anti-bacterial activity***

The antibacterial activities of different extracts of *C. assamensis* leaf are depicted in Table 1. The results indicated that the extracts showed antibacterial activities at variable degrees against both Gram positive and Gram negative bacteria. The methanol extracts of *C. assamensis* did not show antibacterial activity against the majority of the bacteria tested, their inhibitory effect being noted against *Staphylococcus aureus, S. epidermidis, Sarcina lutea, Salmonella typhi, Shigella dysenteriae* and *S. sonnei* only at the concentration of 1000 µg/disc, whereas ethanol extract produced antibacterial activity only against *Bacillus megaterium*. However, the chloroform extract displayed the most important spectrum of activity against *Bacillus subtilis, Bacillus cereus*, *Sarcina lutea* and all tested Gram negative bacteria at the concentration of 1000 µg/disc, whereas the range of zone of inhibition was within 7-16 mm, but *S. aureus* and *S. epidermidis* showed resistance at the same concentration.

The use of crude extracts of plants parts and phytochemicals, of known antimicrobial properties, can be of great significance in the therapeutic treatments. In this present study, preliminary screening for antimicrobial activity showed that, chloroform extract displayed profound antibacterial activity on at least 4 of 6 Gram positive and 11 out of 11 Gram negative bacterial strains tested. While the methanol and ethanol extracts of *C. assamensis* showed least inhibitory activity. For instance, ciproflaxacin showed the maximum zone of inhibition against all bacterial strains tested. From the literature review it was observed that the methanol extract of *C. microcarpa*, *C. reticulate* and *C. sinensis* at 20 mg/mL showed better inhibition compare to *C. aurantifolia* and *C. limon*a gainst *S.aureus* and *E. coli* [47].

Hydroalcoholic extract of *C. aurantifolia* leaf exhibit antibacterial activity on *K. pneumonia, Pseudomonas* sp., *S. aureus* [48]. The antibacterial activity of ethanol extracts of *C. sinensis* leaves showed little zones of inhibition against *E. coli, P. aeruginosa, K. pneumoniae,* and *S. aureus* (1-3mm in diameter) [49].

Table 1. Antibacterial screening of different leaf extracts of *C. assamensis* by disc diffusion assay.

Test Microorganisms	Zone of inhibition in diameter (mm)						
	Methanol Extract		Ethanol Extract		Chloroform Extract		Ciprofloxacin
	500 µg/disc	1000 µg/disc	500 µg/disc	1000 µg/disc	500 µg/disc	1000 µg/disc	5 µg/disc
Gram Positive Bacteria							
S. aureus	-	7	-	-	-	-	32
S. epidermidis	-	9	-	-	-	-	34
B. subtilis	-	-	-	-	12	16	38
B. cereus	-	-	-	-	11	12	35
B. megaterium	-	-	-	9	13	12	36
Sarcina lutea	-	6	-	-	-	10	35
Gram Negative Bacteria							
S. typhi	-	8	-	-	10	14	38
S. paratyphi A	-	-	-	-	-	7	36
S. paratyphi B	-	-	-	-	-	9	36
S. boydii	-	-	-	-	12	13	34
S. dysenteriae	-	11	-	-	10	11	31
S. sonnei	-	7	-	-	13	15	30
Pseudomonas aeruginosa	-	-	-	-	-	8	31
Escherichia coli	-	-	-	-	10	12	42
V. cholerae	-	-	-	-	-	10	31
V. mimicus	-	-	-	-	-	11	32
Klebsiella pneumoniae	-	-	-	-	11	12	36

According to Dulay and De Castro [50], ethanol extract of *C. microcarpa* significantly recorded the highest diameter zone of inhibition of 13.53 mm, which statistically comparable with the ethanol extract of *C. aurantium* and *C. maxima* having the diameter zone of inhibition of 12.93 mm and 11.26 mm respectively. However in the in the present study, differences were also observed between antibacterial activities of the extracts. Adnan *et al*. [51] reported that the methanol extracts of *C. sinensis, C. paradisi* and *C. jambhiri* leaf showed no antimicrobial activity at higher concentrations (4000 µgmL^{-1}) against *S. aureus, M. luteus, E. coli, B. bronchiseptica, S. typhimurium* and *E. aerogens*; which is similar with the present study. Citrus plants are known for their antibacterial properties due to their strong bioactive components. For example, the 2-hydroxypropane-1,2,3-tricarboxylic acid, active compound of *C. microcarpa*, exhibited *C. freundii*, *A. hydrophila*, *P. aeruginosa*, *S. agalatiae*, *E. tarda*, *E. coli*, and *Y. enterocolitica* [52]. In addition, mycelial growth of *A. flavus* decreased in increasing concentration of essential oils of *C. maxima* and completely inhibited the growth at 500 ppm. This oil contained DL-limonene (31.83%), E-citral (17.75%), 1-hexene-4-methyl (15.22%) and Z-citral (13.38%) as the major components [53]. On the other hand, extract of *C. aurantium* contain 8 flavonoids namely, isonaringin, naringin, hesperidin, neohesperidin, naringenin, hesperitin, nobiletin and tangeritin, which are known to exhibit antibacterial property [54]. These differences could be due to the differences in the chemical composition of these extracts as the secondary metabolites of plants have many effects including antibacterial and antiviral properties.

3.2. *In vitro thrombolytic activity*

As a part of discovery of cardio protective drugs from natural resources, the extractives of *C. assamensis* were assessed for thrombolytic activity and the results are presented in Fig. 1. An *in vitro* thrombolytic method was used to investigate the thrombolytic activity of plant extracts in blood sample from healthy human volunteers, along with streptokinase as a standard and sterile distilled water as a control. Addition of 100 µL streptokinase (30,000 I.U.), to the clots and subsequent incubation for 90 min. at 37 °C, showed only 12% of clot lysis. On the other hand, clots when treated with 100 µL sterile distilled water showed only negligible clot lysis (11%). The significant average percent of clot lysis (35%) of chloroform extract of *C. assamensis* was found.

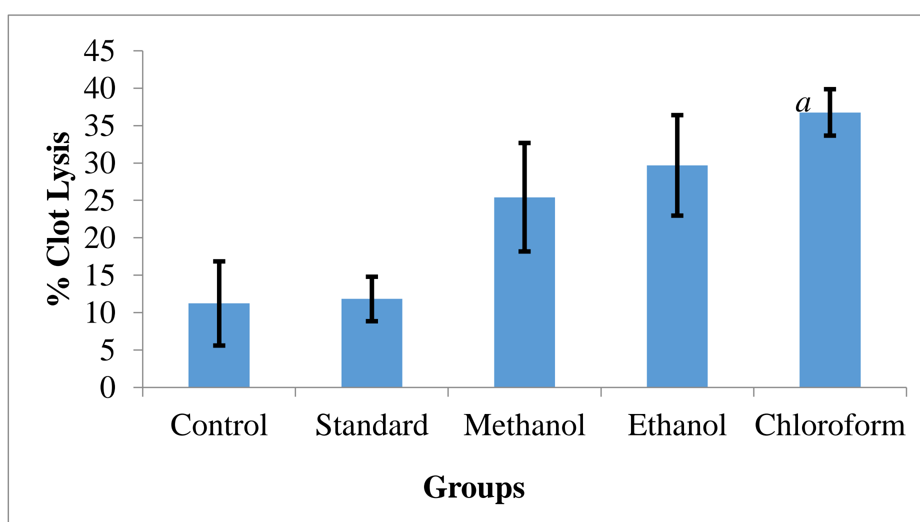

Fig. 1. Percent of clot lysis by different leaf extracts of *C. assamensis.*
Values are mean ± SEM (n=6), [a]*(p< 0.05) significantly different when compared with the corresponding value of control group, done by independent sample t-test*

Thrombosis or blood clot formation is a critical event in which the damaged regions of the endothelial cell surface or blood vessel are blocked by the deposition of platelets, tissue factor and fibrin [55]. Various thrombolytic agents are used to dissolve the clots that have already formed in the blood vessels; but these drugs have limitations and can lead to serious and sometimes fatal consequences [56-59]. A number of research works have been conducted to discover the plants and natural food sources and their supplements having antithrombotic effect and there is indication that consuming such food leads to prevention of coronary events and stroke [60-66]. From the literature review it was also observed that the *C. hystrix (Leaf), C. macroptera* (Fruit), *C. sinensis* (Peel oil) has a significant thrombolytic effect [67-70]. From the present study, it can be concluded that the different leaf extracts of *C. assamensis* is able to lyse clot by disrupting the fibrinogen and fibrin

contained in a clot. In addition, positive result in thrombolytic activity test led us to the interference that the plant extract may contain bioactive compounds, which may aid ongoing cardiovascular drug discovery from the floristic resources.

3.3. *In vitro membrane stabilizing activity*

The leaf extracts of *C. assamensis* at concentration of 1.0 mg/mL, were tested against lysis of human erythrocyte membrane induced by hypotonic solution as well as heat, and compared with the standard acetyl salicylic acid (0.10 mg/mL) (Table 2). For hypotonic solution induced hemolysis, at a concentration of 1.0 mg/mL, the chloroform extract inhibited 55.68% hemolysis of RBCs as compared to 91.81% produced by acetyl salicylic acid (0.10 mg/mL). On the other hand, during heat induced condition different leaf extracts of *C. assamensis* like methanol, ethanol and chloroform extracts demonstrated 29.91, 23.82 and 27.10% inhibition of hemolysis of RBCs, respectively whereas acetyl salicylic acid inhibited 70.75%.

Table 2. Effect of extractives of *C. assamensis* leaf on hypotonic solution & heat induced hemolysis of human erythrocyte.

Samples	Concentration (mg/mL)	Per centinhibition of hemolysis	
		Hypotonic solution induced	Heat induced
Control	50 mM	-	-
Acetyl salicylic acid	0.1	91.81±0.69	70.75 ± 0.76
Methanol Extract	1	52.25 ± 4.96^a	29.91 ± 0.66^a
Ethanol Extract	1	32.91 ± 3.29^a	23.82 ± 0.78^a
Chloroform Extract	1	55.68 ± 4.86^a	27.10 ± 0.73^a

Values are mean ± SEM (n=6), ^{a}*(p< 0.05) significantly different when compared with the corresponding value of control group, done by independent sample t-test*

Membrane stabilizing activity is related to anti-inflammatory activities preliminary [42]. During inflammatory events, lysosomal enzymes and hydrolytic constituents are released from phagocytes into the extracellular space that produce a variety of disorders. Hypotonic solution and heat induced erythrocyte membrane lysis can be taken for the *in vitro* determination of anti-inflammatory activity of drugs or plant extracts by stabilizes the erythrocyte membrane. Exposure of red blood corpuscles to injurious substances such as; hypotonic medium, heat and methyl salicylate results in the lysis of the membranes, accompanied by haemolysis and oxidation of haemoglobin [71]. Membrane stabilization leads to the prevention of leakage of serum protein and fluids into the tissues during a period of increased permeability caused by inflammatory mediators [72]. In the present study, the mean difference in percentage inhibition of haemolysis between standard and tested extracts were found to be significant ($p < 0.05$). *C. assamensis* leaf extract perhaps stabilized the red blood cell membrane by preventing the release of lytic enzymes and active mediators of inflammation. Ali *et al.* [67] investigated the membrane stabilizing activity of *C. hystrix* using the hypotonic solution induced haemolysis method where the

ethanol extract inhibited 74.40% haemolysis of RBCs, compared to 93.24% inhibited by acetyl salicylic acid (0.10 mg/mL). While studies have shown flavonoids to exert stabilizing effects on lysosomes [73], tannin and saponins have been reported as being capable of binding cations, thereby stabilizing the erythrocyte membrane [74].

3.4. *In vivo anti-inflammatory activity*

Table 3 shows the results on anti-oedematous effect of intra-peritoneally administered *C. assamensis* leaf extracts on Carrageenan paw edema in mice. At doses of 250 mg/kg body weight, different leaf extracts caused a significant reduction in paw edema (52.81%, 67.78% and 87.20% respectively). In this model, phenylbutazone, a commonly used anti-inflammatory drug, produced a significant inhibition by 95.70%. As shown in Table 3, all leaf extracts exhibited anti-inflammatory activity higher than that of the standard phenylbutazone.

Table 3. Anti-inflammatory activity of extracts of the leaf of *C. assamensis* on acute model of inflammation by Carrageenan-induced edema in left hind paw of mice.

Treatment	Doses (mg/kg)	Volume in ml of the left hind paw of mice before and after carrageenan injection		
		0 h	2 h	% Inhibition
Control		0.26±0.005	0.56±0.005	-
Phenylbutazone	100	0.22±0.06	0.26±0.01	10.56
Methanol Extract	250	0.28±0.015	0.41±0.012	52.81[a]
Ethanol Extract	250	0.29±0.01	0.35±0.005	67.78[a]
Chloroform Extract	250	0.25±0.01	0.55±0.001	87.20[a]

Values are mean ± SEM (n=6), [a](p< 0.05) significantly different when compared with the corresponding value of control group, done by independent sample t-test

Carrageenan induced hind paw edema test has been extensively used to evaluate the anti-inflammatory effect of new pharmaceutical agents [75]. All the extracts of *C. assamensis* established significant anti-inflammatory effect by regulating biphasic inflammatory process induced by carrageenan (Table 3). *C. medica* peel extract is reported to have significant potential in carrageenan induced inflammatory pain in rats [76]. Methanol extract of *C. limon* fruit rind produced significant inhibition of paw edema [77]. Ethyl acetate peel extract of *C. decumana* at the dose of 300 mg/kg produced a significant decrease in paw volume and pain when compared to diclofenac and morphine [78]. Our results indicated that oral dose of *C. assamensis* repressed the edema throughout all stages of inflammation, which is perhaps due to inactivation of different chemical mediators of inflammation.

3.5. *In vivo anti-tumor activity*

Effect of different leaf extracts of *C. assamensis* at the doses of 100 and 200 mg/kg body weight (i. p.) and bleomycin at dose 0.3 mg/kg body weight (i. p.), on growth of EAC

cells in mice on day 8 after tumor transplantation is represented in Table 4. Treatment with *C. assamensis* extracts resulted in a dose-dependent tumor growth inhibition except methanol extract. Among the three extracts, chloroform extract resulted in maximum cell growth inhibition of 75.04% at the dose 200 mg/kg body weight, which was 56.05% at dose 100 mg/kg body weight. Treatment with bleomycin at dose 0.3 mg/kg body weight showed cell growth inhibition by 76.67%.

Table 4. Effect of different leaf extracts of *C. assamensis* on EAC cells in mouse.

Treatment group	Dose (mg/kg body weight, i.p.)	No. of EAC cells in mouse on day 8 after tumor cell inoculation	Percentage of cell growth inhibition
Control	-	$9.15 \times 10^8 \pm 1.9 \times 10^8$	-
Bleomycin	0.3	$2.07 \times 10^7 \pm 4.3 \times 10^6$	76.67 ± 2.89
Methanol Extract	100	$4.47 \times 10^8 \pm 8.3 \times 10^{7c}$	57.58 ± 3.48
	200	$4.48 \times 10^8 \pm 2.2 \times 10^{7c}$	52.47 ± 4.16
Ethanol Extract	100	$6.70 \times 10^8 \pm 2.1 \times 10^{7c}$	7.08 ± 2.30^b
	200	$4.90 \times 10^8 \pm 6.2 \times 10^{7b}$	45.46 ± 2.53
Chloroform Extract	100	$4.18 \times 10^8 \pm 2.9 \times 10^{7c}$	56.05 ± 2.47
	200	$2.42 \times 10^8 \pm 2.2 \times 10^{7b}$	75.04 ± 1.51

Values are mean ± SEM (n=6), ^{b}(p< 0.01), ^{c}(p< 0.001) significantly different when compared with the corresponding value of control group, done by independent sample t-test

Segura *et al*. [79] reported that, as a spontaneous murine mammary adenocarcinoma, the Ehrlich tumor is a rapidly growing carcinoma with very aggressive behavior and is able to grow in the majority of mouse strains and is accepted as a transplantable tumor model used to evaluate the anticancer effects of numerous substances. Ehrilich ascites carcinoma, β-cell lymphoma proliferative diseases and chronic lymphocytic leukemia diseases perform their mechanism by uncontrolled blood cell regulation. Kinase inhibitor enzyme are needed to treat them as it binds with kinase protein, whereas flavonoids with base neucleosides in presence of nitrogenous base, it form intermolecular bond with target protein kinase and stop cell further growth [80-82]. Results of the present study indicate that *C. assamensis* dose-dependently and significantly decrease number of EAC cell count and inhibition of cell growth when compared to those of EAC control mice and standard. Kundusen *et al*. [83] observed that the intra-peritonial administration of methanol leaf extracts of *C. maxima* in EAC treated mice showed to increase the life span, nonviable tumor cell count and decrease in the tumor volume. From the literature review it was observed that the fruit and peel extracts of *C. sinensis*, *C. reticulata*, *C. limon*, *C. aurantifolia has potential anticancer activity* [84-90]. Moreover, the citrus secondary metabolites such as flavonoids, hesperetin, limonoids, nobiletin, naringenin and coumarins were studied for anticancer activity, which are associated with a reduced risk of cancer, including gastric cancer, breast cancer, colon cancer, lung tumorigenesis, colonic tumorigenesis, hepatocarcinogenesis, and hematopoietic malignancies, etc. [21-34].

4. Conclusion

Some chemical and biological investigations have been carried out so far on *C. assamensis* plant in this study mainly focusing on the leaf of the plant but those are considered to be preliminary. As a result more elaborated research may be necessary to reach concrete conclusion about the findings of the present study. On the basis of above result and available reports, all three leaf extracts of *C. assamensis* had potent *in vitro* anti-bacterial, thrombolytic, membrane stabilizing as well as*in vivo*anti-inflammatory and antitumor activity. However, isolating new bioactive compounds and evolution of their extracts mode of action and chronic toxicity profile might be the next steps to be followed to eventually find new led compounds. The plant can be further screened against various diseases in order to find out its unexplored efficacy and can be a potential source of chemically interesting and biologically important drug candidates.

References

1. G. Adwan and M. Mhanna, Middle-East J. Sci. Res. **3**, 134 (2008).
2. L. J. Soma, M. Shahriar, S. N. Narjish, and M. A. Bhuiyan, Dhaka Univ. J. Pharm. Sci. **13**, 193 (2014). https://doi.org/10.3329/dujps.v13i2.21900
3. R. Ferdousi, S. N. Narjish, D. Chowdhury, and M. Shahriar, Int. J. Pharm. **5**, 1079 (2015).
4. S. Das, M. Shahriar, S. N. Narjish, and R. Akhter, Int. J. Med. Health Res. **2**, 33 (2016).
5. K. I. Siddique, M. M. N. Uddin, M. S. Islam, S. Parvin, and M. Shahriar, J. Appl. Pharm. Sci. **3**, 94 (2013). https://doi.org/10.7324/JAPS.2013.30318
6. D. Parvin, M. M. N. Uddin, M. S. Islam, S. Parvin, and M. Shahriar, Am. J. Res. Commun. **1**, 49 (2013).
7. S. Das, R. Akhter, S. Khandaker, S. Huque, P. Das, M. R. Anwar, K. A. Tanni, S. Shabnaz, and M. Shahriar, J. Coast Life Med. **5**, 360 (2017). https://doi.org/10.12980/jclm.5.2017J7-65
8. R. Bhowmick, M. S. Sarwar, S. M. R. Dewan, A. Das, B. Das, M. M. N. Uddin, M. S. Islam, and M. S. Islam, Biol. Res. **47**, 56 (2014). https://doi.org/10.1186/0717-6287-47-56
9. D. R. Laurence and P. N. Bennett, Clinical Pharmacology, Churchill Livingstone, 7th Edition (New York, 1992).
10. D. H. Wu, G. Y. Shi, W. J. Chuang, J. M. Hsu, K. C. Young, and C. W. Chang, J. Biol. Chem. **276**, 15025 (2001). https://doi.org/10.1074/jbc.M005935200
11. M. Olszanecka-Glinianowicz, B. Zahorska-Markiewicz, P. Kocelak, J. Janowska, E. Semik-Grabarczyk, T. Wikarek, W. Gruszka, and P. Dabrowski, Mediators Inflamm. 2009, 439107 (2009). http://dx.doi.org/10.1155/2009/439107
12. N. Khansari, Y. Shakiba, and M. Mahmoudi, Drug Discov. **3**, 73 (2009). https://doi.org/10.2174/187221309787158371
13. A. Sjoholm and T. Nystrom, Lakartidningen **101**, 1716 (2004).
14. O. O. Oyedapo, B. A. Akinpelu, and S. O. Orefuwa, J. Trop. Med. Plants. **5**, 161 (2004).
15. O. O. Oyedapo, B. A. Akinpelu, K. F. Akinwunmi, M. O. Adeyinka, and F. O. Sipeolu, Int. J. Plant Physiol. Biochem. **2**, 46 (2010).
16. H. P. Rang, M. M. Dale, J. M. Ritter, and R. J. Flower, Pharmacology, 6th Edition (Elsevier, 2008).
17. M. Yonathan, K. Asres, A. Assefa, and F. Bucar, J. Ethnopharmacol. **108**, 462 (2006). https://doi.org/10.1016/j.jep.2006.06.006
18. E. Boakye-Gyasi, E. Woode, G. K. Ainooson, D. D. Obiri, C. Ansah, M. Duwejua, and A. Donkoh, Afr. J. Pharm. Pharmacol., **2**, 191 (2008).
19. B. Nitha, C. R. Meera, and K. K. Janardhanan, Amala Res. Bull. **25**, 165 (2005).

20. D. J. Newman, G. M. Cragg, and K. M. Snader, J. Nat. Prod. **66**, 1022 (2003). https://doi.org/10.1021/np030096l
21. M. Pan, S. Li, C. Lai, Y. Miyaucho, M. Suzawa, and C. Ho, Food Sci. Hum. Wellness. **1**, 65 (2012). https://doi.org/10.1016/j.fshw.2012.09.001
22. S. Aranganathan and N. Nalini, Phytother. Res. **27**, 999 (2013). https://doi.org/10.1002/ptr.4826
23. M. K. N. Chidambara, G. K. Jayaprakasha, and B. S. Patil, Food. Funct. **4**, 803 (2013). https://doi.org/10.1039/c3fo30325j
24. N. Yoshimizu, Y. Otani, Y. Saikawa, T. Kubota, M. Yoshida, T. Furukawa, K. Kumai, K. Kameyama, M. Fujii, M. Yano, T. Sato, A. Ito, and M. Kitajima, Aliment. Pharmacol. Ther. **20**, 95 (2004). https://doi.org/10.1111/j.1365-2036.2004.02082.x
25. K. Gao, S. M. Henning, Y. Niu, A. A. Youssefian, N. P. Seeram, A. Xu, and D. Heber, J. Nutr. Biochem. **17**, 89 (2006). https://doi.org/10.1016/j.jnutbio.2005.05.009
26. G. Ekambaram, P. Rajendran, V. Magesh, and D. Sakthisekaran, Nutr. Res. **28**, 106 (2008). https://doi.org/10.1016/j.nutres.2007.12.002
27. D. Arul and P. Subramanian, Biochem. Biophys. Res. Commun. **434**, 203 (2013). https://doi.org/10.1016/j.bbrc.2013.03.039
28. D. Lee, K. Park, H. Park, S. Kang, A. nagappan, J. Kim, E. Kim, W. Lee, Y. Hah, H. Chung, S. An, and G. Kim, Evid. Based Complement Alternat. Med. **2012**, 515901 (2012). http://dx.doi.org/10.1155/2012/515901
29. T. Tanaka, T. Tanaka, M Tanaka, and T. Kuno, J. Biomed. Biotechnol. **2012**, 516981 (2012). https://doi.org/10.1155/2012/516981
30. H. Xiao, C. S. Yang, S. Li, H. Jin, C. Ho, and T. Patel, Mol. Nutr. Food Res. **53**, 398 (2009). https://doi.org/10.1002/mnfr.200800057
31. H. Jin, W. S. Lee, J. W. Yun, J. H. Jung, S. M. Yi, H. J. Kim, Y. H. Choi, G. Kim, J. M. Jung, C. H. Ryu, S. C. Shin, and S. C. Hong, Oncol. Rep. **30**, 2336 (2013). https://doi.org/10.3892/or.2013.2711
32. C. Lai, S. Li, C. B. Liu, Y. Miyauchi, M. Suzawa, C. Ho, and M. Pan, Mol. Nutr. Food Res. **57**, 551 (2013). https://doi.org/10.1002/mnfr.201200606
33. D. Arul and P. subramanian, Biochem. Biophys. Res. Commun. **434**, 203 (2013). https://doi.org/10.1016/j.bbrc.2013.03.039
34. A. Ghorbani, M. Nazari, J. Mahmood, and H. Zand, Eur. J. Nutr. **51**, 39 (2012). https://doi.org/10.1007/s00394-011-0187-2
35. G. V. Rao, S. Kumar, M. Islam, and Mansour, Cancer Ther. **6**, 913 (2008).
36. J. Lucker, M. K. Tamer, W. Schwab, F. W. A. Verstappen, L. H. W. V. Plas, H. J. Bouwmesster, and H. A. Verhoeven, Eur. J. Biochem. **269**, 3160 (2002). https://doi.org/10.1046/j.1432-1033.2002.02985.x
37. J. Sidana, V. Saini, S. Dahiya, P. Nain, and S. Bala, Int. J. Pharm. Sci. Rev. Res. **18**, 20 (2013).
38. A. J. Das, R. Kumar, M. Athar, D. S. Rawat, M. Kumar, M. A. Khan and J. Prakash, Int. Res. J. Pharm. **4**, 146 (2013). https://doi.org/10.7897/2230-8407.04741
39. A. W. Bauer, W. M. Kirby, J. C. Sherries and M. Tuck, Am. J. Clin. Pathol., **45**, 493 (1966). https://doi.org/10.1093/ajcp/45.4_ts.493
40. S. Prasad, R. S. Kashyap, J. Y. Deopujari, H. J. Purohit, G. M. Taori, and H. F. Daginawala, Thromb. J. **4**, 14 (2006). https://doi.org/10.1186/1477-9560-4-14
41. M. H. Kawsar, M. A. Sikder, M. S. Rana, I. Nimmi, and M. A. Rashid, Bang. Pharm. J. **14**, 103 (2011).
42. U. A. Shinde, A. S. Phadke, A. M. Nair, A. A. Mungantiwar, V. J. Dikshit, and M. N. Saraf, Fitoterapia. **70**, 251 (1999). https://doi.org/10.1016/S0367-326X(99)00030-1
43. A. Braca, N. D. Tommasi, L. D. Bari, C. Pizza, M. Politi, and I. Morelli, J. Nat. Prod. **64**, 892 (2001). https://doi.org/10.1021/np0100845
44. C. A. Winter, E. A. Risley, and G. W. Nuss, Proc. Soc. Exp. Biol. Med. **111**, 544 (1962). https://doi.org/10.3181/00379727-111-27849
45. E. Sugishita, S. Amagaya, and Y. Ogihara, J. Pharmacodynamics **4**, 565 (1981).

46. F. Islam, H. Khatun, S. Ghosh, M. M. Ali, and J. A. Khanam, Asian Pac. J. Trop. Biomed. **2**, 394 (2012). https://doi.org/10.1016/S2221-1691(12)60063-X
47. M. Sekar, Int. J. Curr. Pharm. Res. **5**, 32 (2013).
48. R. K. Pathan, P. R. Gali, P. Pathan, T. Gowtham, and S. Pasupuleti, Asian Pac. J. Trop. Dis. **2**, S328 (2012). https://doi.org/10.1016/S2222-1808(12)60176-5
49. U. N. Ekwenye and O. V. Edeha, Int. J. Pharma. Bio. Sci. **1**, 742 (2010).
50. R. M. R. Dulay and M. E. G. De Castro, Pharm. Lett. **8**, 167 (2016).
51. M. Adnan, A. Umer, I. Ahmad, K. Hayat, and S. N. Shakeel, Sains Malays. **43**, 185 (2014).
52. S. W. Lee and M. Najiah, Agr. Sci. China. **8**, 880 (2009). https://doi.org/10.1016/S1671-2927(08)60291-6
53. P. Singh, R. Shukla, B. Prakash, A. Kumar, S. Singh, P. K. Mishra, and N. K. Dubey, Food Chem. Toxicol. **48**, 1734 (2010). https://doi.org/10.1016/j.fct.2010.04.001
54. X. He, L. Lian, L. Lin, and M. W. Bernart, J. Chromatogr. A **791**, 127 (1997). https://doi.org/10.1016/S0021-9673(97)00779-6
55. B. Furie and B. C. Furie, N. Engl. J. Med. **359**, 938 (2008). https://doi.org/10.1056/NEJMra0801082
56. J. M. Wardlaw, E. Berge, G. D. Zoppo, and T. Yamaguchi, Stroke **35**, 2914 (2004). https://doi.org/10.1002/14651858.CD000213.pub3
57. T. Capstick and M. T. Henry, Eur. Respir. **26**,864 (2005). https://doi.org/10.1183/09031936.05.00002505
58. D. B. Baruah, R. N. Dash, M. R. Chaudhari, and S. S. Kadam, Vascular Pharmacol. **44**, 1 (2006). https://doi.org/10.1016/j.vph.2005.09.003
59. A. Mannan, M. J. Kawser, A. M. A. Ahmed, N. N. Islam, S. M. M. Alam, M. A. E. K. Emon, and S. D. Gupta, J. App. Pharm. Sci. **1**, 56 (2011).
60. W. D. Ratnasooriya, T. S. P. Fernando, and P. P. Madubashini, J. Natn. Sci. Foundation Sri Lanka **36**, 179 (2008). https://doi.org/10.4038/jnsfsr.v36i2.151
61. K. J. Joshipura, A. Ascherio, J. E. Manson, M. J. Stampher, E. B. Rimm, and F. E. Speizer, J. Ama. Med. Assoc. **282**, 1233 (1999). https://doi.org/10.1001/jama.282.13.1233
62. S. Liu, J. E. Manson, I. M. Lee, S. R. Cole, C. H. Hennekens, W. C. Willett, and J. E. Buring, Am. J. Clin. Nutr. **72**, 922 (2000). https://doi.org/10.1093/ajcn/72.3.856a
63. L. A. Bazzano, J. He, L. G. Ogden,C. M.Loria, S. Vupputuri, L. Myers, and P. K, Whelton, Am. J. Clin. Nutr. **76**, 93 (2002). https://doi.org/10.1093/ajcn/76.1.93
64. S. K. Verma and A. Bordia, Indian J. Med. Sci. **55**, 83 (2001).
65. A. Bordia, S. K. Verma, and K. C. Srivastava, Prostaglandins Leukot. Essent. Fatty Acids **58**, 257 (1998). https://doi.org/10.1016/S0952-3278(98)90034-5
66. H. Sumi, H. Hamada, H. Tsushima, H. Mihara, and H. Muraki, Experimentia. **43**, 1110 (1987). https://doi.org/10.1007/BF01956052
67. M. Ali, R. Akhter, S. N. Narjish, M. Shahriar, and M. A. Bhuiyan, Int. J. Pharm. Sci. Res. **6**, 2367 (2015).
68. K Aktar and T. Foyzun, Evid. Based Complement. Alternat. Med. **2017**, 9789802 (2017). https://doi.org/10.1155/2017/9789802
69. N. Karim, M. H. Sakib, M. Y. Sarkar, M. S. Hossain, M. S. Hossain, M. A. Ansari, and R. Dhar, SAS J. Med. **1**, 16 (2015).
70. M. Vinodhini, M. Kalaiselvi, R. Amsaveni, and V. Bhuvaneshwari, WJPMR **3**, 164 (2017).
71. M. Feirrali, C. Signormi, L. Ciccolili, and M. Comporti, Biochem. J. **285**, 295 (1992). https://doi.org/10.1042/bj2850295
72. R. Chaitanya, S. Sandhya, B. David, K. R. Vinod, and S. Murali, Int. J. Res. Pharm. Biomed. Sci. **2**, 256 (2011).
73. M. M. Hossain, S. K. Ahamed, S. M. R. Dewan, M. M. Hassan, A. Istiaq, M. S. Islam, and M. M. R. Moghal, Biol. Res. **47**, 45 (2014). https://doi.org/10.1186/0717-6287-47-45
74. I. Khan, M. Nisar, F. Ebad, S. Nadeem, M. Saeed, H. Khan, Samiullah, F. Khuda, N. Karim, and Z. Ahmad, J. Ethnopharmacol. **121**, 175 (2009). https://doi.org/10.1016/j.jep.2008.10.009
75. K. Araruna and B. Carlos, Phytopharmacology **1**, 1 (2010).

76. S. Sood, S. Bansal, A. Muthuraman, N. Gill, and M. Bali, Res. J. Med. Plant **3**, 123 (2009). https://doi.org/10.3923/rjmp.2009.123.133
77. N. Sivakumar and R. Venkataraman, Pharm. Sin. **1**, 1 (2010).
78. S. Sood, B. Arora, S. Bansal, A. Muthuraman, N. S. Gill, R. Arora, M. Bali, and P. D. Sharma, Inflammopharmacology **17**, 267 (2009). https://doi.org/10.1007/s10787-009-0015-y
79. J. A. Segura, L. G. Barbero, and J. Márquez, Immunol. Lett. **74**, 111 (2000). https://doi.org/10.1016/S0165-2478(00)00208-X
80. P. Sur, S. P. Bag, and J. A. Khanam, Neoplasma. **44**, 197 (1997).
81. R. Zahan, B. Alam, S. M. Islam, G. C. Sarker, N. C. Chowdhury, S. B. Hosain, M. A. Mosaddik, M. Jesmin, and E. Haque, Int. J. Cancer Res. **7**, 254 (2011). https://doi.org/10.3923/ijcr.2011.254.262
82. B. Alam, I. Sajid, J. Rashid, M. Islamand, and B. K. Karmaker, Glob. J. Pharmacol. **8**, 47 (2014).
83. S. Kundusen, M. Gupta, U. K. Mazumder, P. K. Haldar, P. Saha, and A. Bala, ISRN Pharmacol. **2011**, 138737 (2011). https://doi.org/10.5402/2011/138737
84. N. A. E. Zawawy, Int. J. Curr. Microbiol. Appl. Sci. **4**, 298 (2015).
85. J. R. Patil, G. K. Jayaprakasha, K. N. C. Murthy, M. B. Chetti, and B. S. Patil, Microchem. J. **9, 4**, 108 (2010). https://doi.org/10.1016/j.microc.2009.09.008
86. J. R. Patil, G. K. Jayaprakasha, K. N. C. Murthy K, S.E Tichy, M. B. Chetti, and B. S. Patil, Food Chem. **114**, 1351 (2009). https://doi.org/10.1016/j.foodchem.2008.11.033
87. J. R. Patil, G. K. Jayaprakasha, J. Kim, K. N. C. Murthy, M. B. Chetti, S. Y. Nam, and B. S. Patil, Planta Med. **79**, 219 (2013). https://doi.org/10.1055/s-0032-1328130
88. M. Gharagozloo, M. Doroudchi, and A. Ghaderi, Phytomedicine **9**, 475 (2002). https://doi.org/10.1078/09447110260571751
89. B. Adina, F. A. Goenadi, F. F. Handoko, D. A. Nawangsari, A. Hermawan, R. I. Jenie, and E. Meiyanto, Iran. J. Pharm. Res. **13**, 919 (2014).
90. G. Castillo-Herrera, L. Farias-Alvarez, J. Garcia-Fajardo, J. Delgado-Saucedo, A. Puebla-Perez, and E. Lugo-Cervantes, J. Supercrit. Fluids **101**, 81 (2015). https://doi.org/10.1016/j.supflu.2015.02.026