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Research Article

In Vitro Anti-Inflammatory Potential of Twelve Myanmar Medicinal Plant Extracts via Anti-Protein Denaturation and Membrane Stabilization Effect

Mya Thida[1,*], Hay Mar Aung[1], Thandar Aung[2], Zar Ni Linn[2], The Su Moe[2]

1 Cell Culture Laboratory, Department of Biotechnology Research, Ministry of Science and Technology, Kyaukse 05151, Myanmar
2 Pharmaceutical Research Laboratory, Department of Biotechnology Research, Ministry of Science and Technology, Kyaukse 05151, Myanmar

ABSTRACT

This study is conducted to investigate the anti-inflammatory potential in collected plant extracts. The cytotoxicity activity of the test sample was evaluated by hemolysis assay, and antioxidant potency was measured by radical scavenging assays and total phenolic and flavonoid contents examination. The anti-inflammatory activity of test samples was carried out by antiglycation, protein denaturation inhibition, proteinase inhibition, and membrane stabilization. The hemolysis effect of tested extracts expressed in a percent range of 5.38 ± 0.18 to 29.77 ± 1.47, representing non-cytotoxic activities. *Tamarindus indica* (leaf) and *Croton oblongifolius* showed significant radical scavenging effects and antiglycation potency. Most of the tested extracts had higher phenolic and flavonoid contents. In addition, tested extracts have a potential anti-protein denaturation effect. According to the results, *Benincasa cerifera*, *Tamarindus indica* (leaf), *Croton obligifolius*, and *Rumex maritimus* represented significant inhibition to protein denaturation action at all test inducers to compare against their standard group. Moreover, *Tamarindus indica* (leaf), *Croton oblongifolius*, and *Rumex maritimus* exhibited significantly higher membrane stabilization activities. *Tamarindus indica* (leaf), *Croton oblongifolius*, and *Rumex maritimus* demonstrated the protein and membrane stabilization effect with higher antioxidant potency. Therefore, these tested extracts could be used as therapeutic supplements in the treatment of chronic inflammatory diseases.

Keywords:
Anti-inflammatory; Antioxidant; Anti-protein denaturation; Cytotoxicity; Membrane stabilization.

1. INTRODUCTION

Chronic inflammation is one of the non-communicable diseases caused by excessive stress on certain tissues or parts of the body, which has serious consequences on the body and leads to chronic degenerative diseases such as obstructive pulmonary disease, arthritis (osteoarthritis, rheumatoid arthritis), inflammatory bowel diseases, psoriatic arthritis and gout, heart disease, diabetes, and many types of cancer. Inflammatory, external, and internal biochemical inducers increased the production of free radicals, oxidative stress, mitochondrial dysfunction, production of advanced glycation end products (AGEs), uric acid (urate) crystals, oxidized lipoproteins, and others. Moreover, the denaturation of proteins initiates inflammation, a well-documented cause of rheumatoid arthritis[1]. Similarly, red blood cell membrane lysis and destabilization triggered by hemolysis and oxidation of hemoglobin due to injurious substances lead to inflammation-related diseases. Therefore, anti-protein denaturation and red blood cell (RBC) membrane stabilization are responsible for preventing anti-inflammatory agents through the inhibition of the protein

***Corresponding author:**
* Mya Thida Email: myathida@dbr.gov.mm

denaturation and the hemolysis processes, respectively[2]. Low-glycemic diet, reduced intake of saturated fat and trans fats, fruits and vegetables, fiber, micronutrients, physical exercise, anti-inflammatory agents, and nonsteroidal anti-inflammatory drugs (NSAIDs) or dietary supplements alleviate inflammation triggers and reduce chronic inflammation[3]. The natural products of plant extracts are well-known for being widely used, validated, sensitive, and reliable for evaluating anti-inflammatory activity by *in vitro* technique[4]. Indigenous plants are growing mainly in Southeast Asia, such as *Coccinia indica*, *Croton oblongifolius*, *Clerodendrum indicum*, *Phyllanthus urinaria*, *Acacia concinna*, *Tamarindus indica*, *Rumex maritimus*, *Hydrocotyle astatica*, *Caralluma umbellata,* and *Benincasa cerifera*, which are reported for many medicinal activities (e.g., antioxidant activity, anti-inflammation potency) with less toxicity. Among them, *Croton oblongifolius, Phyllanthus urinaria,* and *Tamarindus indica* have mostly been revealed to possess anti-inflammatory effects and reduce subjective pain due to containing secondary metabolites such as anthraquinones, flavonoids, stilbenes, diterpenes, alkaloids, terpenes, lignans, and tannins in these plants[5-14].

This study has mainly focused on the anti-inflammatory activity of twelve test extracts from natural sources against oxidative stress, preventing protein denaturation and membrane destruction function.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Chemicals and reagents used for this study were analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of plant extracts

Tamarindus indica (DBR.CC.002), *Phyllanthus urinaria* (DBR.CC.012), *Acacia concinna* (DBR.CC.013), *Clerodendrum indicum* (DBR.CC.025), *Coccinia indica* (DBR.CC.045), *Rumex maritimus* (DBR.CC.051), *Croton oblongifolius* (DBR.CC.054), *Hydrocotyle astatica* (DBR.CC.062), *Caralluma umbellata* (DBR.CC.063) and *Benincasa cerifera* (DBR.CC.065) were collected from Katae village with a geographical location 21˚35'41.9"N, 96˚07'07.7" E, Kyaukse Township, Mandalay Region, Myanmar, and identified by an authorized botanist from the Botany Department, Mandalay University, Mandalay, Myanmar. Tested plant samples (**Table 1**) were cleaned, air-dried, powdered, and percolated with 90% ethanol, then the solvent was filtered and filtrates were concentrated by a rotary evaporator (IKA RV 10, Germany) to get the extracts. The concentrated plant extracts were dried and evaporated at room temperature and then used as tested samples for further experiments.

Table1. The Collected Test Plants

No.	Botanical Name	Myanmar Name	Family	Part of uses
1	*Coccinia indica* W.&A.	Kin-bon	Cucurbitaceae	Leaves
2	*Croton oblongifolius* muell.arg	Thet-yin-gyi	Euphorbiaceae	Bark
3	*Clerodendrum indicum* Linn.	Nga-yan-padu	Lamiaceae	Leaves
4	*Phyllanthus urinaria* Reta.	Taung-zee-byu	Phyllanthaceae	Fruit
5	*Acacia concinna* DC.	Kin-mun-gyin	Fabaceae	Leaves
6	*Tamarindus indica* Linn.	Magyi	Fabaceae	Fruit
7	*Tamarindus indica* Linn.	Magyi	Fabaceae	Seed
8	*Tamarindus indica* Linn.	Magyi	Fabaceae	Leaves
9	*Rumex maritimus* Linn.	Taw-dauk-gwa	Polygonaceae	Leaves
10	*Hydrocotyle astatica* Linn.	Myin-khwar-ywat	Apiaceae	Leaves
11	*Caralluma umbellata* Haw.	Ta-zaung-ga	Apocynaceae	Whole part
12	*Benincasa cerifera* Savi.	Kyauk-pha-yone	Cucurbitaceae	Fruit

2.3. Phytochemical qualitative analysis

Ethanolic aqueous solutions of tested plant extracts were carried out for screening the presence or absence of alkaloids, glycosides, tannins, saponins, phenolics, flavonoids, amino acids, carbohydrates, terpenoids, and cyanogenic glycosides. The phytochemical analysis was done by standard methods[15].

2.4. Hemolysis assay

The hemolytic activity of test extracts, positive control: Triton X-100 (100% lysis), and negative control: phosphate buffer saline (PBS) (0% lysis), were carried out by using blood[16] [blood sample was provided from a healthy volunteer]. For preparation of blood suspension, 3 mL of blood was added into a 0.85% NaCl

solution in a 15 mL sterile Falcon tube and centrifuged at 850 × g for 5 min. Then, the blood pellet was washed with chilled PBS three times, RBCs were re-suspended in 0.85% NaCl solution, and 2% erythrocyte suspension was prepared for further hemolysis assays. Briefly, 20 µL (1 mg/mL) of sample extracts were placed in 1.5 mL microtubes and gently mixed with 180 µL of blood suspension, and then incubated at 37°C for 30 min. After that, tubes were agitated for 10 min, placed on ice for 5 min, and centrifuged again for 5 min at 1310 g. Add 100 µL of supernatant to 900 µL cooled PBS and then keep on ice and each sample was placed in a 96-well to measure at 576 nm (BMG Labtech, SPECTRO Star Nano). Hemolysis (%) was calculated as follows:

$$\% \text{ Lysis} = \frac{\text{OD of sample}}{\text{OD of positive control}} \times 100$$

2.5. Radical scavenging capacity of the tested extract

This study was evaluated by measuring the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging effects at a percent inhibition on a 96-well microtiter plate[17]. Briefly, 100 µL of each extract in desired concentrations were added to 100 µL (0.3 mM) DPPH solution, and the reaction mixture was incubated for 30 min at dark and room temperature. After incubation, the reaction mixture has measured the absorbance at 517 nm. Ascorbic acid was used as a positive control. The inhibition (%) on radical scavenging activity was calculated as follows:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

2.6. Antiglycation assay

Advanced glycation end-product inhibition activity of tested extracts was determined using the BSA-glucose models following Lunceford and Gugliucci[18] with slight modifications. Antiglycation effects were measured at excitation and emission wavelengths of 360 and 420 nm to explore the inhibition ability of tested samples. Briefly, BSA (10 mg/mL) was dissolved in phosphate buffer (pH 7.4) containing 0.01% of sodium azide to prevent microbes. The glucose solution (90 mg/mL) was made with the same buffer. The desired concentrations of each sample in different doses were mixed with BSA and glucose solution in the same volume. Glucose solution and BSA solution without sample were used to serve as blanks, and rutin was used as a positive control. The reaction mixture was incubated for 72 h at 37°C. After incubation, AGE formation was measured at the fluorescence intensity excitation (360 nm) to emission (420 nm) [Cary Eclipse Fluorescence Spectrofluorometer]. The percentage of AGE inhibition was used to calculate the following equation:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

2.7. Nitric oxide radical scavenging assay

The reaction mixture containing [sample extract in different concentrations, potassium phosphate buffer (0.1 M), and sodium nitroprusside (10 mM)] was added to a 96-well plate and incubated at 25°C for (90-100) min. DMSO is used in blank control instead of extract. The reaction mixture was pre-read at 540 nm after incubation at a microplate reader (SPECTRO Star Nano). Then, 50 µL of sulphanilic acid (0.33%) with 20% glacial acetic acid was added to the reaction mixture, and the plate was stranded for 5 min. After that, 50 µL of naphthalene diamine dihydrochloride (0.1%) was added and shaken. The plate was incubated again at 25°C for 30 min. In complete incubation, the plate was shaken and measured for a final read at 540 nm. Percent inhibition was calculated by using the following equation[19]:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

2.8. The total phenolic content (TPC) determination

The amount of TPC in the tested extracts was estimated by the Folin-Ciocalteu method. Briefly, 100 µL of each tested extract was prepared and mixed with 500 µL of 10% Folin-Ciocalteu reagent. After 5 min, 500 µL of sodium carbonate (7.5%) was added. The solution mixture was shaken and incubated at 37˚C for 1 h. The absorbance was measured at the wavelength of 765 nm using the SPECTRO Star Nano microplate reader. The TPC amount in samples was calculated as Gallic acid equivalent (GAE) by the following equation:

T= CV/M, T is the total phenolic content in mg g^{-1} of the extracts as GAE, C is the concentration of Gallic acid established from the calibration curve in mg ml^{-1}, V is the volume of the extract solution in ml, and M is the weight of the extract in g.

2.9. The total flavonoid content (TFC) determination

This assay was evaluated by using aluminum chloride ($AlCl_3$). The same volume of plant extract and methanol was mixed with 2% of aluminum chloride and then the reaction mixture was incubated at room temperature for 60 min. After that, the absorbance of the reaction mixture was measured at 420 nm using a spectrophotometer (SPECTRO Star Nano microplate reader). Methanol was placed instead of plant extract used as a blank. A standard curve of rutin was done and the results were expressed as mg of rutin equivalent per gram of extract[20].

2.10. Determination of protein denaturation inhibitory activity using heat-induced bovine serum albumin (BSA) method

The methods used for assessing the anti-protein denaturation effects of tested samples in this study. A protein denaturation assay was examined by using a fluorescent method with slight modification[21]. Briefly, a stock solution of 0.2% (w/v) of BSA was prepared in tris-buffer saline with pH (8.53 to 6.74). The reaction mixture contains BSA stock solution and test extract dilution with methanol. The control contained BSA with methanol without extract. The reaction mixture was incubated at 37°C for 20 min and then heated at 72°C for 5 min and then cooled at room temperature, and absorbance was read using a spectrofluorometric wavelength parameter of Ex 480/Em 520.

The percentage inhibition of protein destabilization was estimated on a percentage in an equation as follows:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

2.11. Determination of protein denaturation inhibitory activity using heat-induced egg-albumin method

In this assay, the reaction mixture contained egg albumin, phosphate buffer, and different concentrations of tested extracts. For the control, double distilled water was added instead of the test extract. The reaction mixture was incubated at 37°C for 15 minutes and afterward heated to 70°C for 5 minutes. The absorbance was measured at 660 nm. Diclofenac sodium was taken as a standard. The protein denaturation inhibition percentage was determined by the following formula. Where OD of control is the control absorbance and OD of sample is the test absorbance[22].

$$\% \text{ Inhibition} = \left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

2.12. Determination of protein denaturation inhibitory activity using protease-induced bovine serum albumin (BSA) method

This experiment was performed according to the method[23] with slight modifications. Trypsin was used as a protease enzyme, and BSA was used as a protein. The reaction mixture includes trypsin, 20 mM Tris HCl buffer (pH 7.4), and a test sample/standard drug, diclofenac sodium. The reaction mixture is incubated at 37°C for 5 min, and then 1 ml of 0.8% BSA is added. The reaction mixture is incubated for 20 min. Then, 5% trichloroacetic acid (TCA) is added to stop the reaction. The suspension mixture was centrifuged at 3000 rpm for 10 min, and the supernatant was read at the spectrofluorometric wavelength parameter of Ex 480 / Em 520. The experiment is performed in triplicate. The proteinase inhibitory activity (%) is calculated using the following equation.

$$\% \text{ Inhibition} = \left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

2.13. Red blood cells (RBCs) membrane stabilization assay using hypotonic-induced method

The investigation of the hypotonicity-induced erythrocyte membrane hemolysis assay was performed following the Shinde and Oyedapo method[24]. RBCs suspension was done by the previous section of a hemolysis assay method, and red blood cells (10% v/v) were prepared in a 0.85% NaCl solution. Briefly, the reaction mixture contained sodium phosphate buffer (pH 7.4, 10 mM), hypotonic solution (0.36% w/v NaCl), and 10% blood cell suspension (10%, v/v) with the desired concentration of sample extracts and diclofenac sodium (reference drug). Distilled water is replaced to induce 100% hemolysis, used as a control. The reaction mixture was incubated at 37°C for 30 min and then centrifuged at 3000 rpm for 10 min. After that, the supernatant was estimated by measuring the absorbent (OD) at 560 nm for membrane stabilization activity of the tested sample and standard by calculating the following equation:

$$\text{Membrane Stabilization (\%)} = \left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

2.14. Red blood cells (RBCs) membrane stabilization assay using heat-induced method

This assay was done by using a heat-induced model for membrane destabilization. The reaction mixture included test samples of desired concentrations and diclofenac (standard), sodium phosphate buffer (pH 7.4, 10 mM), and 10% erythrocyte suspension. Instead of tested extract, a vehicle is used in the reaction mixture for control. Then, the reaction mixture is incubated in a water bath at 60ºC for 30 minutes. After incubation, the tubes are cooled at room temperature. After that, the reaction mixture was centrifuged at 3000 rpm for 5 min, and the supernatant was estimated by measuring the absorbent (OD) at 560 nm for membrane stabilization. The percentage inhibition of membrane destruction is calculated as follows:

$$\text{Membrane Stabilization (\%)} = \left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

2.15. Red blood cells (RBCs) membrane stabilization assay using H_2O_2-induced method

The membrane stabilization assay was done according to the modified method described by Ebrahimzadeh et al[25]. In this study, hydrogen peroxide (H_2O_2) is used as an oxidizing agent to damage the erythrocyte membrane. RBC suspension was mixed with tested extract and PBS. After 5 min of incubation at room temperature, 0.3% H_2O_2 was added, and the mixture was incubated at 37°C for 240 min. A control was prepared by omitting the extracts. At the end of incubation, the reaction mixture was centrifuged at 3000 rpm for 10 min, and the supernatant was measured at the absorbance wavelength of 540 nm. The membrane stabilization of tested samples was calculated as follows:

$$\text{Membrane Stabilization (\%)} = \left(1 - \frac{\text{OD of sample}}{\text{OD of control}}\right) \times 100$$

2.16. Statistical analysis

The results were performed in triplicate and the data were expressed as the mean ± SD. Values for each sample were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests ($P \leq 0.05$) using GraphPad Prism ver. 7.00 (GraphPad Software, La Jolla, California, USA).

3. RESULTS AND DISCUSSION

3.1. Screening the phytochemical constituents of tested samples

The qualitative phytochemical examination of tested extracts represented the absence of cyanogenic and the presence of alkaloids, carbohydrates, glycosides, phenolics, flavonoids, steroids, and tannins in all tested plant extracts, especially those that can contain phenolics and flavonoids **(Table 2).**

These secondary metabolites in the tested samples might be responsible for the obvious anti-inflammatory activities through anti-protein denaturation and membrane stabilization effects due to their antioxidant potential and related therapeutic effects[26]. Some flavonoids have significant inhibitory potential in acute inflammation against the production of inflammatory mediators and perform their anti-inflammatory action by inhibiting nitric oxide production by decreasing iNOS expression[27].

Table 2. Phytochemical Screening of Tested Samples, [(+) Present, (-) Absent]

No.	Tested Samples	Alkaloid	Glycoside	Reducing sugar	Phenolic	Flavonoid	Saponin	Cyanogenic glycoside	Terpenoids	Carbohydrate	Tannins
1	*Coccinia indica* &A.	+	-	+	+	+	+	-	+	+	-
2	*Croton oblongifolius* muell. arg	+	+	+	+	+	+	-	+	-	+
3	*Clerodendrum indicum* Linn.	+	+	+	+	+	+	-	+	-	+
4	*Phyllanthus urinaria* Reta. (Fruit)	+	+	+	+	+	+	-	+	-	+
5	*Acacia concinna* DC.	+	+	+	+	+	+	-	+	+	+
6	*Tamarindus indicca* Linn. (fruit)	+	+	+	+	+	+	-	-	+	+
7	*Tamarindus indica* Linn (seed)	+	+	+	+	+	+	-	+	+	+
8	*Tamarindus indica* Linn. (leaf)	+	+	+	+	+	+	-	+	+	+
9	*Rumex maritimus* Linn.	+	+	+	+	+	-	-	+	+	+
10	*Hydrocotyle asiatica* Linn.	+	+	+	+	+	+	-	+	+	+
11	*Caralluma umbellata* Haw.	+	+	+	+	+	-	-	+	-	-
12	*Benincasa cerifera* Savi.	+	+	+	+	+	+	-	+	-	+

3.2. Cytotoxic activity of tested samples

The hemolysis activities of tested extracts and positive control Triton X-100 compared with PBS were expressed in **Figure 1**. The hemolysis is an indicator of general cytotoxicity, and the assay is used to determine whether the effect of the tested samples is toxic or non-toxic to normal cell membranes and functions. The red blood cell (RBC) revealed a useful model for finding out the toxicity of natural or synthetic drugs on cellular metabolism and functions. This study determined the safety assessment of the tested extracts for the therapeutic purposes of herbal-based related drugs. Plant extracts were considered to have less than 30% of the hemolysis activity on red blood cells, revealing a non-cytotoxic effect[28].

In this study, all tested extracts performed less than 30% lysis on the RBCs and percentages in a range of 5.38 ± 0.18 to 29.77 ± 1.47 at 1 mg/mL of each sample concentration, which represented the safety of tested extracts for further therapeutic use (**Figure 1**).

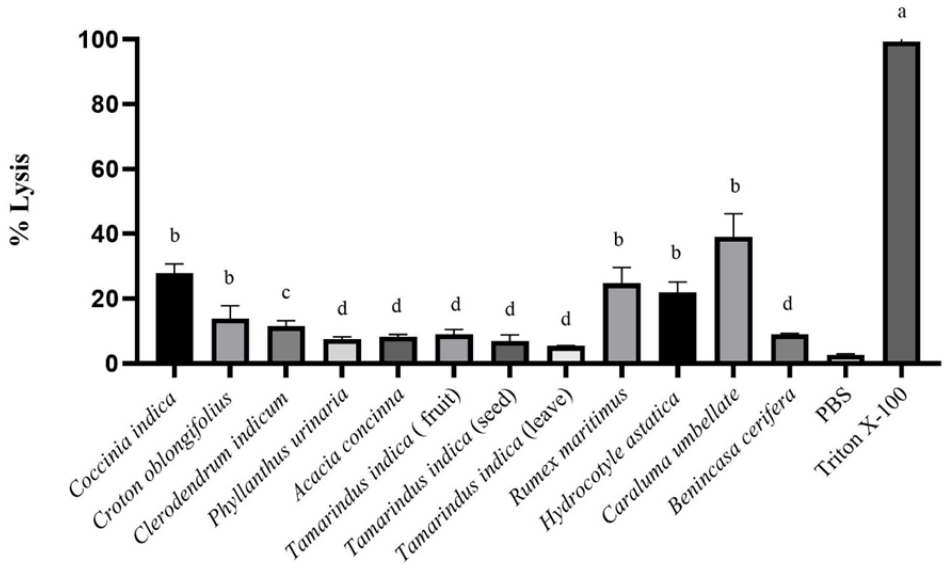

Figure 1. Comparison on Cytotoxicity Activity of Test Extracts, Positive Control; Triton X-100 and Negative Control; PBS. Results were performed in triplicate and the data were expressed as the mean ± SD, $^{d}p < 0.05$, $^{b}p < 0.001$, $^{c}p < 0.01$, $^{a}p < 0.0001$, Test Extracts and Positive Control vs Negative Control.

3.3. Inhibition activity of tested samples on DPPH radical

In this study, the free radical scavenging activity of tested extracts was evaluated by measuring DPPH scavenging activity on a 96-well microtiter plate. For this investigation, all of the test samples showed a higher free radical inhibitory capacity, represented with IC_{50} values in a range between 33 µg/mL and 483 µg/mL by dose-dependent appearance. Ascorbic acid is used as the standard. The IC_{50} values of tested extracts and the standard for their antioxidant effects on DPPH were presented in **Figure 2**. Tested extracts in this study showed radical scavenging activity with lower IC_{50} values responsible for their antioxidant potency.

3.4. Inhibitory activity of tested samples on glycated end-product

In this investigation, the BSA-glucose assay was used to evaluate the antiglycation potency of tested extracts (**Figure 2**). The significant effects were observed in the *Tamarindus indica* leaf, and the IC_{50} value represented 23.82 µg/mL. Rutin was served as a positive control with an IC_{50} value of 41 µg/mL. Antiglycation activity of *Clerodendrum indicum*, *Phyllanthus urinaria*, and *Croton oblongifolius* also showed higher inhibition to AGE with IC_{50} values of 72.54 µg/mL, 101.07 µg/mL, and 160.19 µg/mL, respectively (**Figure 2**).

The higher amount of reactive oxygen species (ROS) increases AGE formation due to intracellular oxidative stress. AGEs that bind to RAGE on the endothelial cell surface lead to a signaling cascade, stimulating increased ROS production. The ligand-RAGE interaction may stimulate signaling targets to NF-κB translocation to the nucleus linked to inflammations and increase transcription of several proteins and tissue factors. AGE formation alters cell structure and affects extracellular and intracellular functions[29]. AGEs-dependent RAGE (Receptor for Advanced Glycation End Products) expression is proinflammatory and expressed on endothelial cells, fibroblasts, mesangial cells, and macrophages, leading to a loss of protein function, mitochondrial dysfunction, impaired elasticity of tissues such as blood vessels, skin, tendons, and cell damage[30]. AGEs-mediated inflammation, including tissue injury, protein denaturation, and cell membrane destruction, stimulates gene expressions of proinflammatory molecules and triggers oxidative stress and apoptosis in many cells, such as neurons, endothelial cells, lung cells, and muscle cells[31]. Therefore, herbal-based therapeutic agents such as AGE inhibitors and breakers containing antioxidants, natural substances, and anti-inflammatory molecules are needed to develop.

This investigation provided that *Tamarindus indica* (leaves), *Clerodendrum indicum* (leaves), *Phyllanthus urinaria* (fruit), and *Croton oblongifolius* (bark) scavenged the protein glycation with a significant IC_{50} value responsible for preventing ROS formation and reducing the loss of protein function.

3.5. Nitric oxide inhibition potency of tested samples

In this study, the most effective extract in reducing NO production was *Croton oblongifolius*

(bark), with an IC_{50} value of 30.52 µg/mL, followed by *Tamarindus indica* (leaves), *Coccinia indica* (leaves), *Tamarindus indica* (seed), *Phyllanthus urinaria* (fruit), and *Clerodendrum indicum* (leaves), which exhibited the effective IC_{50} values (**Figure 2**).

NO, an inflammatory mediator, plays a vital role in the pathogenesis of inflammation due to overproduction in abnormal situations. It can cause tissue damage and consequences for inflammatory-related diseases, such as rheumatoid arthritis, cardiovascular and respiratory diseases, autoimmune diseases, and cancer. Flavonoid compounds can inhibit the mechanism of inflammation action attributed to decreased nitric oxide production[32].

According to the nitric oxide scavenging activity of tested extracts, the flavonoids and other active components present in the samples may prevent inflammatory mediators and reduce the overproduction of nitric oxide.

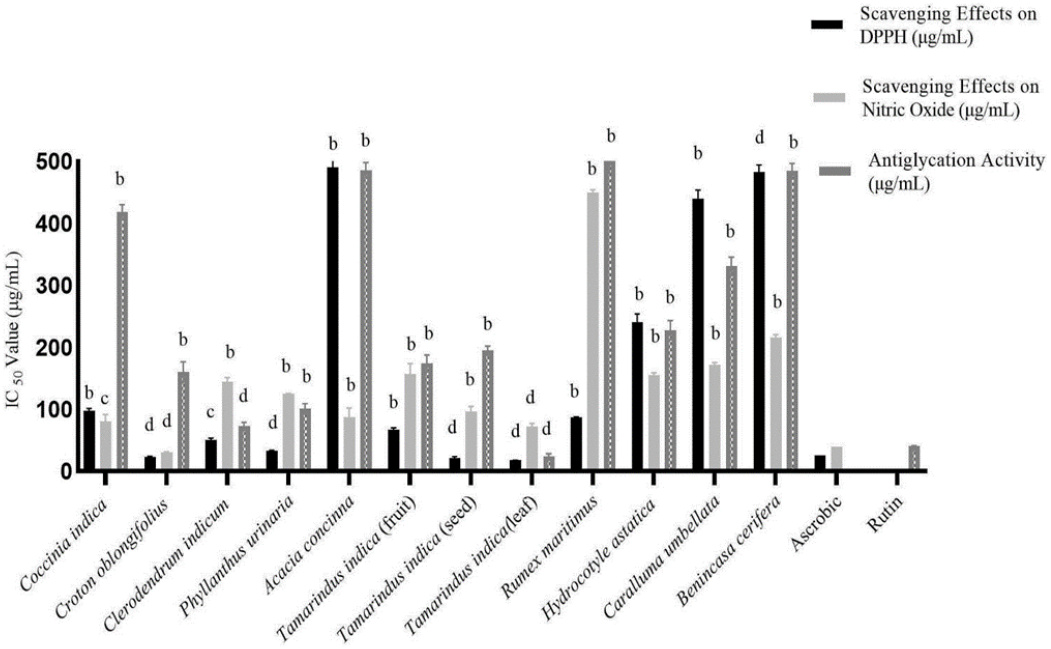

Figure 2. Comparison on Radicals (DPPH and Nitric oxide) Scavenging Effects and Anti-Glycation Ability of Test Extracts and Positive Control; Ascorbic acid and Rutin, respectively. Results were performed in triplicate and the data were expressed as the mean ± SD, $^{d}p < 0.05$; $^{c}p < 0.01$; $^{b}p < 0.001$, Test Extracts vs Positive Control.

3.6. Quantitative estimation of total phenolic content in tested sample

The presence of phenolic content in the tested extracts was shown in **Table 3**. In this study, moderate to higher phenolic content was found in tested samples, and the maximum phenolic content was shown in *Phyllanthus urinaria* (fruit) (244.75 mg GAE/g of extract), *Hydrocotyle astatica* (leaves) (226.83 mg GAE/g of extract), *Tamarindus indica* (leaf) (181.9 mg GAE/g of extract), and *Croton oblongifolius* (bark) **(**174.5 mg GAE/g of extract).

Phenolic compounds are one of the major contributors to the antioxidant potency of medicinal plants, food, and vegetables that can prevent most chronic diseases[33]. According to the values of total phenolic contents, *Phyllanthus urinaria* (fruit)*, Hydrocotyle astatica* (leaves)*, Tamarindus indica* (leaf), *and Croton oblongifolius* (bark) possessed the higher phenolic content, which contributed to antioxidant capacity. A large amount of total phenolic contents represented the antioxidant ability of tested extracts.

3.7. Quantitative estimation of total flavonoid content in tested sample

The total flavonoid contents of tested extracts expressed in a ranged from 63.72 to 144.81 mg GAE/g of extract at a tested concentration of 1 mg/mL (**Table 3**). Flavonoids have been assumed to be super antioxidants for their prevention effect on free radicals and cure of the associated oxidative stress-related diseases[34]. This study showed most of the tested samples explored with the highest content of flavonoids indicate the correlation to their antioxidant capacity.

Table 3. Estimation of Total Phenolic and Total Flavonoid Content of Tested-Extracts

No.	Tested-Extracts	Total Phenolic	Total Flavonoid
		(mg GAE/g of extract)	(mg Rutin Equivalent/ g of extract)
1	*Coccinia indica*	119.7 ± 6.44	179.07 ± 2.13
2	*Croton oblongifolius*	174.5 ± 0.88	67.44 ± 1.64
3	*Clerodendrum indicum*	143.43 ± 2.12	127.44 ± 3.35
4	*Phyllanthus urinaria*	244.73 ± 3.36	126.2 ± 4.67
5	*Acacia concinna*	101.1 ± 1.04	86.51 ± 1.23
6	*Tamarindus indica* (fruit)	189.27 ± 1.66	67.98 ± 2.44
7	*Tamarindus indica* (seed)	104 ± 2.55	191.24 ± 4.3
8	*Tamarindus indica* (leaf)	181.9 ± 2.51	97.36 ± 1.36
9	*Rumex maritimus*	106.57 ± 0.86	28.53 ± 9.74
10	*Hydrocotyle astatica*	226.83 ± 8.61	144.81 ± 6.12
11	*Caralluma umbellata*	64.87 ± 2.7	94.49 ± 0.82
12	*Benincasa cerifera*	97.57 ± 1.8	63.72 ± 0.46

3.8. Anti-inflammatory activity of tested samples

Protein denaturation inhibition and membrane stabilization capacities were determined for the anti-inflammatory activity of tested extracts.

3.9. Anti-protein denaturation effects of tested samples on protein denaturation by heat and proteinase injurious substances

In the investigation of the inhibition effect of heat-induced bovine serum albumin denaturation, the best prevention effect is shown in *Tamarindus indica* (seed), followed by *Phyllanthus urinaria* (fruit), *Coccinia indica* (leaves), *Tamarindus indica* (leaves), *Croton oblongifolius* (bark), and *Benincasa cerifera* (fruit), decreasing order of IC_{50} value (µg/mL) as: 122.35 ± 3.75, 154.63 ± 12.55, 170.11 ± 12.55, 174.7 ± 7.29, 175.81 ± 23.33, and 195.53 ± 8.51, respectively (**Figure 3**). The rest of the tested extracts described moderate inhibition of protein denaturation at a concentration under 0.5 mg/mL. The standard drug diclofenac showed an IC_{50} value of 78.35±1.14 µg/mL.

In the inhibition potency for egg-albumin denaturation by heat experiment, *Caralluma umbellata* (whole part) and *Hydrocatyle astatica* (leaves) were effective in inhibiting albumin denaturation, with represented IC_{50} values of 38.64 ± 0.93 µg/mL and 45.4 ± 7.85 µg/mL. *Acacia concinna* (leaves), *Coccinia indica* (leaves), *Tamarindus indica* (seed), *Rumex maritimus* (leaves), and *Benincasa cerifera* (fruit) exhibited higher prevention effects of protein denaturation in the IC_{50} ranges between 72.33 µg/mL to 151.73 µg/mL. On the other hand, the rest of the tested extract showed lower activity compared with the IC_{50} value of diclofenac, showing 20.33 ± 4.3 µg/mL (**Figure 3**).

In the study on inhibition of proteinase-induced bovine serum albumin denaturation, trypsin was used as a proteinase enzyme for the protein denaturation. The tested extracts showed significant anti-protease activity. The maximum inhibition was observed in *Clerodendrum indicum* (leaves), *Phyllanthus urinaria* (fruit), *Benincasa cerifera* (fruit), *Hydrocatyle astatica* (leaves), *Tamarindus indica* (leaf), and *Croton obligifolius* (bark) in decreasing order of IC_{50} (µg/mL) values as 169.34, 176.63, 201.03, 241.58, 264.56, and 279.03, respectively. The standard diclofenac expressed the proteinase inhibitory action of IC_{50} value at 162.83 µg/mL.

Chronic inflammation is known to release pro-inflammatory markers, which respond to a biochemical reaction of protein denaturation resulting in loss of tissue function[35]. External stress or compound exposure causes protein denaturation, leads to the loss of protein structures, and alters their biological functions, a consequence of inflammatory and arthritic diseases. The release of extracellular lysosomal proteinases due to endogenous abnormalities causes further tissue inflammation and damage[36]. For this reason, tissue protein denaturation inhibitors may essentially be examined as target drugs for inflammatory and arthritic diseases. This study aims to evaluate the anti-protein denaturation effects of tested samples for their stabilization potency as non-steroidal anti-inflammatory drugs (NSAIDs)[37].

This evaluation indicates that most tested extracts have potential anti-inflammatory agents showing an anti-protein denaturation effect. Among them, *Benincasa cerifera* (fruit), *Tamarindus indica* (leaf), *Croton obligifolius* (bark), and *Rumex maritimus* (leaves) expressed their significant inhibition to protein denaturation action at all kinds of test inducers (**Figure 3**). The prevention effect of protein denaturation capacity of the tested extracts could partly be attributed to the antioxidant effect of the phenolics, flavonoids, and other components present in the extracts.

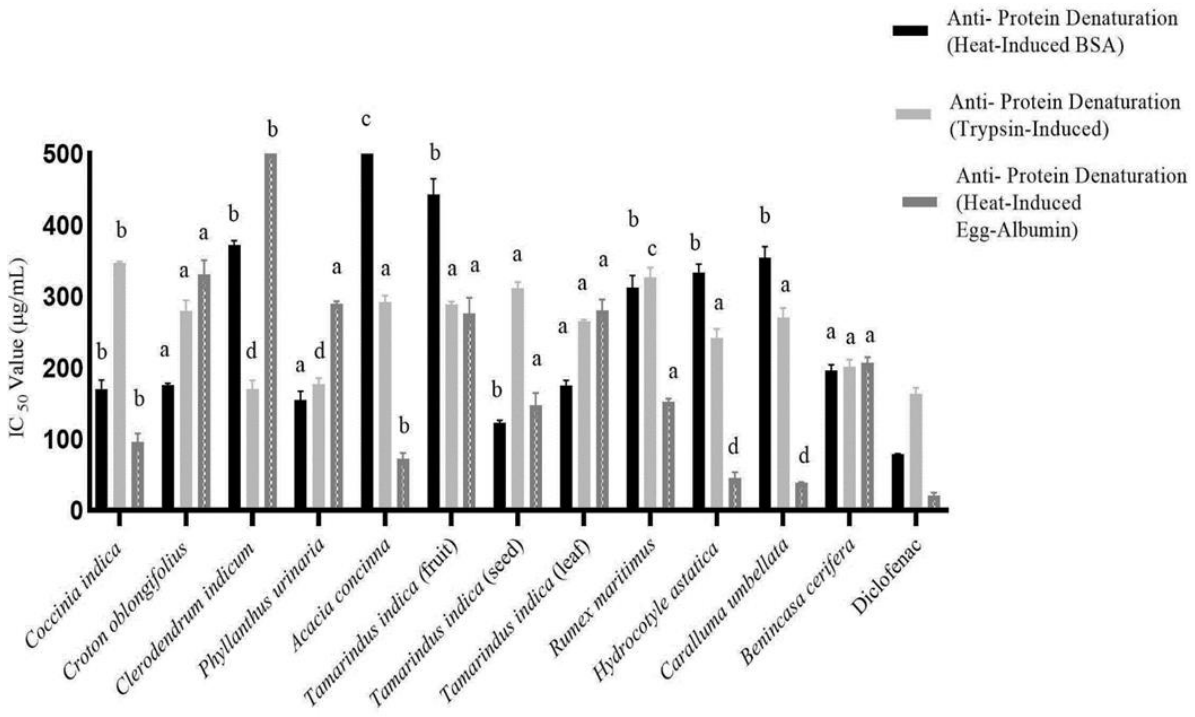

Figure 3. Comparison on Anti-Protein Denaturation Capacity of Test Extracts and Positive Control; Diclofenac by IC_{50}. Results were performed in triplicate and the data were expressed as the mean ± SD, $^{d}p < 0.05$; $^{c}p < 0.01$; $^{b}p < 0.001$, $^{a}p < 0.0001$, Test Extracts vs Positive Control.

3.10. Membrane stabilization effect of tested extracts on RBCs destruction by hypotonic, heat and H_2O_2

In this investigation, the inhibition percent tested samples of RBCs membrane destabilization treated with different inducers are shown in **Figure 4**. The results indicate that tested plant extracts have potential as an anti-inflammatory agent. Significant membrane stabilization activity was observed in *Tamarindus indica* (leaf) and *Rumex maritimus*, with the prevention effect on hypotonic-induced membrane destabilization by percent inhibition of 77.52% and 76.7%, respectively, compared with diclofenac; inhibition is 54.35%. In addition, membrane stabilization activity of tested extracts on hypotonic-induced membrane distraction was shown in decreasing order as *Hydrocotyle astatica* (leaves) > *Benincasa cerifera* (fruit) > *Phyllanthus urinaria* (fruit) > *Croton oblongifolius* (bark) and their inhibition percent as 59.55% > 55.89% > 55.2% > 51.02%. The rest of the tested extracts showed lower stability effects in hypotonicity **(Figure 4**).

Heat-induced membrane destabilization action was effectively reduced by *Croton oblongifolius* and *Rumex maritimus* with inhibitions of 70.97% and 70.03%, respectively, at an extract concentration of 0.5 mg/mL. These results expressed a higher capacity than the standard diclofenac; inhibition percent showed 68.91% at the same concentration. *Tamarindus indica* (leaf), *Phyllanthus urinaria* (fruit), and *Clerodendrum indicum* (leaves) have also shown their effective prevention on the heat-induced membrane in stability by respective inhibition percent, 68.73, 67.97, and 6.79 (**Figure 4**).

The evaluation of the membrane stabilization ability of tested extracts on erythrocyte membranes was explored using an oxidant agent (H_2O_2). The maximum inhibition of cell membrane oxidation induced by H_2O_2 indicated in *Croton oblongifolius* (bark) at 80.49% and *Rumex maritimus* (leaves) at 67.75%. These results confirmed that most of the tested extracts possess membrane stabilization activity, especially in *Croton oblongifolius* (bark), *Rumex maritimus* (leaves) and *Tamarindus indica* (leaves) showed their inhibition with significant effect (**Figure 4**).

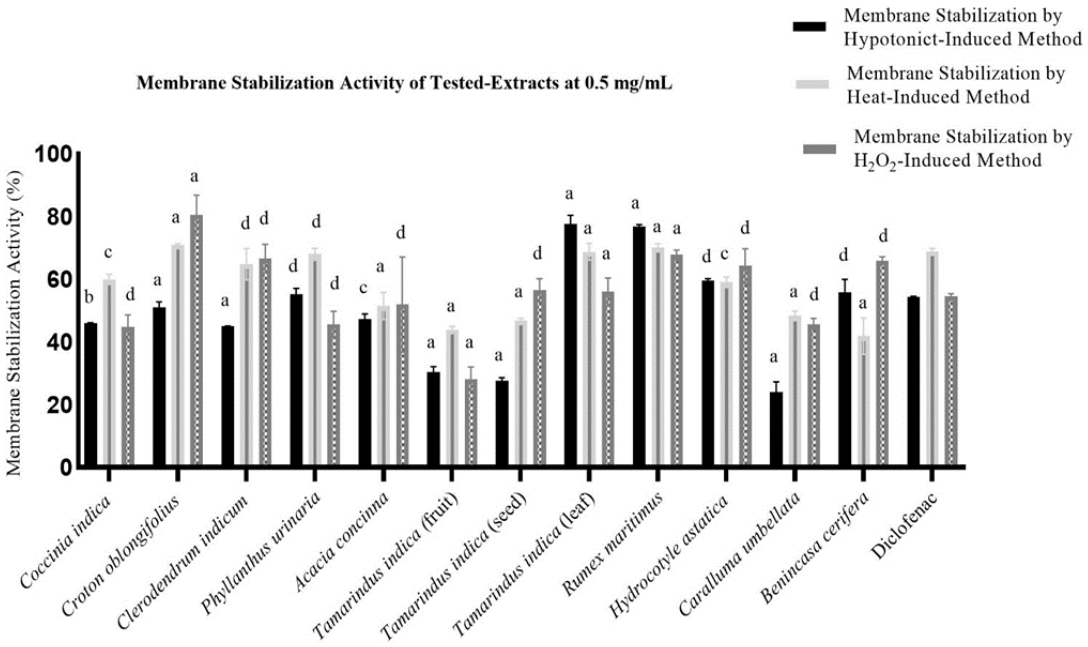

Figure 4. Comparison on Membrane Stabilization Activities of Test Extracts and Positive Control; Diclofenac by IC_{50}. Results were performed in triplicate and the data were expressed as the mean ± SD, $^{d}p < 0.05$; $^{c}p < 0.01$; $^{b}p < 0.001$, $^{a}p < 0.0001$, Test Extracts vs Positive Control.

Several external and internal factors, such as toxicants, chemotherapeutic drugs, radiation, oxidative stress, and bone marrow failure, trigger the destruction of cell integrity, including erythrocyte cell membrane disruption, swelling, shrinkage, etc., which may lead to pathological conditions in humans and inflammation-related diseases[38]. For this reason, the membrane stability of tested extracts is an important tool to explore in this study. HRBC membrane stabilization is one of the indicators of the anti-inflammatory effect, and *in vitro* membrane stabilization methods have been used as parameters for the anti-inflammatory process. Membrane destabilization induced the release of lysosomal constituents, leading to tissue inflammation and proceeding to chronic inflammatory diseases[39]. Hypotonic-related excessive fluid accumulation into cells becomes swelling and then rupture of the cell membrane[40]. Loss of red blood cells is responsible for anemia and other inflammation-related diseases, including arthritis. Phytochemical compounds known as natural antioxidants reveal powerful pharmacological activities and protective effects on red blood cells and cellular functions[41]. Flavonoids have been assumed to scavenge free radicals, enhance reducing power, and inhibit lipid peroxidation, as well as protect erythrocyte membranes from damage and prevent hemolysis[42]. Most phenolic-based compounds have a wide range of medicinal properties due to their antioxidant capacity. The use of plant-derived products emerges as an interesting and, in many cases, a safer approach to prevent cellular membrane disruption[43]. The edible plant, *Tamarindus indica* L., has been recognized as having multiple medicinal properties and medicinal uses for all parts of the plant. Previously, experiments confirmed that *Tamarindus indica*, consisting of varieties of chemical constituents such as essential oils, fatty acids, β- sitosterol, polyphenols, flavonoid derivates, triterpenoids, lupanone, lupeol, and more specific flavone types, are major compounds with a high medicinal value for therapeutics essential. The other study reported the traditional folk uses and pharmacological properties for *Tamarindus indica*, mostly described in antioxidant potency, antimicrobial, hypolipemic, hepatoprotective, and anti-inflammatory activities associated with the presence of flavonoid and/or phenol[44]. The genus *Croton* species has been reported to be rich in active constituents and containing the predominant of different classes of alkaloids, diterpenoids, phenolic substances, flavonoids, lignoids, proanthocyanidins, and volatile oils containing mono and sesquiterpenoids, indicating for many biological activities considerably the importance of the medicinal point of view. The previous studies revealed that they are rich in alkaloids, flavonoids, phytosterols, and phenolic substances and their reported biological activities such as lower blood glucose and triglyceride in rats, insect growth inhibition, anti-inflammatory, anticancer, antibacterial, antifungal, anti-diabetic, and anti-hyperlipidemic[45]. *Rumex* species have been recommended for use in global folk care medicine of Southeast Asia, India, China, and southern Africa for their therapeutic potency for bacterial infections, inflammation, tumors, and skin diseases through several chemical profiles and bioactivities[46]. It was revealed that

all parts of *T. indica* extracts exhibited the highest nitric oxide inhibition in LPS-induced RAW 264.7 macrophages, promising anti-inflammatory properties related to the presence of triterpenoids and steroids. Many reports from the *Croton* species provided a significant clue of the chemical constituents, which considering ethnomedicinal uses. *C. oblongifolius* Roxb., grown in Asia, and the previous reported pharmacological therapeutic use in chronic hepatitis, gastric ulcers and gastric cancers, fever, and wounds. The main biologically active compounds were isolated from *Rumex* species, including quinones, flavonoids, tannins, terpenes, alkaloids, and large quantities of anthracene derivatives, responsible for anti-inflammatory and anticancer properties. An isolated compound of *Rumex maritimus* was reported for anthraquinone, chromone and flavone derivatives, and 2-methoxystypandrone, and the root of the plant represented stronger antioxidant activity and antidiarrhoeal activity in an animal model[47-49].

In this study, *Tamarindus indica* (leaf), *Croton oblongifolius,* and Rumex *maritimus* exhibited significant anti-protein denaturation potency. In addition, *Tamarindus indica* (leaf), *Croton oblongifolius,* and *Rumex maritimus* have demonstrated membrane stabilization activities by stabilizing the cell membrane against lysis with a higher antioxidant content and antiglycation effect. Moreover, the higher content of secondary metabolites such as phenolic and flavonoid in these tested plant extracts could reduce tissue inflammation and decrease access to harmful molecules, such as oxidants, thus protecting injury to the structure and function of membranes[50]. Therefore, this study indicated that protein and membrane stabilization potency have contributed to the anti-inflammatory ability of tested extracts, which may be associated with the concentration of polyphenols and flavonoids effect.

4. CONCLUSIONS

This research work indicated that the ethanolic extracts of *Tamarindus indica* (leaf), *Croton oblongifolius,* and *Rumex maritimus* exhibited significant inhibition of protein denaturation and expressed potent membrane stabilization activities. These activities were confirmed by comparing them with the standard drug diclofenac. *Tamarindus indica* L. leaf extract was reported to have anti-inflammatory and analgesic effects via the ability to inhibit inflammatory mediators. This efficacy might come from the several bioactive compounds such as alkaloids, flavonoids, tannins, phenols, saponins, and steroids of *Tamarindus indica* [51]. In a previous study, *C. oblongifolius* Roxb. (Bark) expressed the anti-inflammation potency through the suppressing capacity of nitric oxide generation in lipopolysaccharide (LPS)-induced macrophage cells (RAW264.7). Various biological activities of *C. oblongifolius* have been reported scientifically and may be based on the major components of monoterpenes and sesquiterpenes from the stem bark of *C. oblongifolius*[52]. It has been reported that a crude extract of *Rumex* spp. is a potentially effective medicine for many diseases, including inflammation, to become a clinical medicine, and anthraquinone derivatives are assumed to have anti-inflammatory actions via various inhibitory mechanisms[53]. Our study had practical implications with the previously reported above that confirmed the anti-inflammatory potentials of *Tamarindus indica* (leaf), *Croton oblongifolius* (bark), and *Rumex maritimus* (the whole part). Moreover, from the antioxidant and antiglycation determination, these tested extracts showed significant activities with non-cytotoxic effects. Anti-protein denaturation and membrane stabilization potency of tested extracts could be promising for anti-inflammatory activity. The study assumed that *Tamarindus indica* (leaf), *Croton oblongifolius,* and Rumex *maritimus* could provide as anti-inflammatory agent for use in inflammation-related diseases for their ethnomedicinal claims. Future studies should focus on the isolation of pure compounds and the evaluation of pharmacological activities under the possible mechanisms of action.

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Author contribution

Mya Thida, designed the study, analyzed data, prepared the manuscript and The Su Moe have read and approved the manuscript. All authors performed the experiments, read and approved the study.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Ethics approval

The authors state that there have “none to declare” in this section

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