

Comparative study of the anti-inflammatory properties of Hibiscus Sabdarifa Calyx and Malus Domestica with Ibuprofen

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Abstract: Hibiscus Sabdarifa and Malus Domestica are well known and widely used herbs, which contains several interesting bioactive constituents and possesses health promoting properties. This study is made in order to determine the total phenolic and flavonoid contents, evaluate and compare the anti-inflammatory effects of ethanol extracts of the two extracts and a 1:1 blend of the extracts against the denaturation of proteins in vitro. The respective extracts were analyzed for their contents of polyphenols and flavonoids. The test extracts and reference drug (ibuprofen) of varying concentrations were also incubated with egg albumin under controlled experimental conditions and subjected to determination of absorbance to assess the anti-inflammatory property. The results obtained exhibited concentration-dependent inhibition of protein denaturation by both extracts, the blend as well as the reference drug. The EC₅₀ for extracts as well as those for the blend and the reference drug (ibuprofen) were determined by the dose- response curve using GraphPad Prism 5.0 software.
Keywords: Hibiscus Sabdariffa; Malus Domestica; flavonoids; ibuprofen; anti-inflammation; protein denaturation

I. INTRODUCTION

From the ancient past to the present day civilization and all over the world, man has consistently used plants or fruits in the treatment and prevention of many ailments [1]. According to Eni *et al.* [2], a plant becomes medicinal only when its biological activities have been enthnobotanically reported or scientifically established. Much of the medicinal uses of plant or fruits seem to have been developed through observation of wild animals and by trial and error. The world health organization [3] estimated that 80% of the world population uses medicinal plant for the treatment of disease, and in African countries, this rate is much higher. Protein denaturation has been described as a pathological process which involves the loss of configuration, and as a result, loss of functionality [4]. Ipomoea and Alstonia, Ipomoea Plamata, S. Subterrata. Used laboratory equipment and isolated lipoidal and phenolic compounds from these plants. The author confirmed that extract of Ipomoea palamata and S. subterrata produced significant anti-inflammatory activity [5].

Over 50% of presented used clinical drugs are of natural products based pharmaceutical industries [6]. Natural products have contributed significantly to the development of modern medicine. Recently traditional medicine worldwide is being re-evaluated by extensive research on different plant species and their active therapeutic principles [7,8]. *Passiflora edulis*, *Olex zeylanica*, *gymnema lactiferum*, *sesbania grandiflora*, *centella asiatica* and *cassia auriculata* L. Heat-induced hemolysis, proteinase inhibitory activity. Methanolic extracts of leafy vegetables were able to inhibit protein denaturation, in a conc dependent manner within the range from 3.8% to 23.1% at the concentrations of 25-100 μ g/mL.[9].

Inflammation is a bodily response to injury, infection, or destruction characterized by heat, redness, pain, swelling, and disturbed functions. Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical, or microbial agents. It is the body response to inactivate or destroy the invading organisms, to remove the irritants and set the stage for tissue repair. It is triggered by the release of chemical mediators from injured tissue and migrating cells [10]. Anti-inflammatory refers to the property of a substance or treatment that reduces inflammation. Anti-inflammatory drugs make up about half of analgesics, remedying pain by reducing inflammation as opposed to opioids, which affect the central nervous system [7,8,11,12].

Hydromethanol extracts of leaves of *C. auriculata*, *P. edulis*, *G. lactiferum*, *S. grandiflora*, *O. zeylanica*, and *C. asiatica* possess anti-inflammatory properties at varying levels. Leaves of *O zeylanica* and *P. edulis* showed higher hemolysis inhibition activity [13].

The numbers of animals required for the isolation of an anti-inflammatory prototype from complex natural product extracts using the activity-directed isolation pathway is alarming. It is for the above reason that the chick carrageenan response assay for the discovery of molecules with anti-inflammatory nociception properties was proposed [13,7,8].

The most commonly used drug for the management of inflammatory conditions is non-steroidal anti- inflammatory drugs (NSAIDs) and steroids which have several adverse effects especially gastric irritation leading to formation of gastric ulcers [14,7,8]. Grant et al. [15] have reported that one of the features of several non-steroidal anti-inflammatory drugs for example indomethacin, ibufenac, flufenamic acid and salicylic acid is their ability to stabilize (prevent denaturation) heat treated bovine serum albumin (BSA) at pathological pH (pH 6.2 - 6.5).

Some plants, vegetables and fruits are reported to be rich sources of antioxidants, such as vitamin A, vitamin C, Vitamin E, carotenoids, polyphenolic compounds, flavonoids and hydrolysable tannins, which can significantly destroy the free radicals, reactive oxygen species responsible for degenerative diseases [16,17] or prevent free radical damage, thus reducing the risk of chronic diseases. These plants include *Ocimum basilicum* (basil), *Prunolla vulgaris* (self-heal) *Cinnamomum cassia*, *Ginkgo bioba* L., *Camellia sinensis* Lin., *Aloe vera*, *Malus domestica*, *hibiscus sabdariffa* calyx and quite a number of others. This study is carried out to investigate and compare the possible anti- inflammatory effects of ethanol extracts of *Malus domestica* and *Hibiscus sabdariffa* calyx against the denaturation of protein in vitro.

Malus domestica is a fruit of the genus *Malus* (about 25 species) belonging to the family Rosaceae, the most widely cultivated tree fruit which is well known the world over for its delicious and nutritious fruits. The skin of ripe apples is generally red, yellow, green or pink, although many bi- or tri-colored varieties may be found [18]. The skin may also be wholly or partly russet i.e. rough and brown and may be covered in a protective layer of epicuticular wax [19]. The fruits are usually eaten fresh and raw except for the seeds, making the nutritional values fully available for the body [20-23]. Evaluation of the antimicrobial, antimutagenic, anticarcinogenic, antioxidant, antidiabetic/osteoporosis properties and related qualities of apple fruits and juices have been reported [22]. Various models including, but not limited to carragenan-induced paw oedema, human red blood cell membrane stabilization, lipoxygenase inhibition, as well as protein denaturation have been used to confirm anti-inflammatory activities of *C. cajan* (Gungo), 17-19 *MM. indica* (Julie mango) 23-25. *Clinical* observations have shown that apple consumption is associated with reduction in risk of cancer [24-26]. Antioxidant activity of apple components is known to inhibit cancer cell proliferation, decrease lipid oxidation and lower cholesterol [1, 20-22]. Apples are rich source of photochemical that have been reported to reduce risk of cardiovascular diseases, asthma, dysentery, heart problem, diabetes, cataracts, Alzheimer's disease/cognitive decline and pulmonary functions [1, 21].

Hibiscus Sabdariffa (Roselle or Sour Tea) is a tea where the usually dark colored flowers are used to brew. It appears to inhibit carbohydrate absorption to a degree and appears to be effective in reducing blood pressure. *Hibiscus sabdariffa* calyx is commonly called Roselle in English, Yakuwa in Hausa. However, the fresh calyx is commonly called soboroto in Nigeria. It belongs to the family Malvaceae. *Hibiscus sabdariffa* is "an herbaceous and perennial plant cultivated in the tropical and subtropical regions. It has limited traditional medicinal use and tends to be a treatment for high blood pressure, gastrointestinal disorders, diaphoresis, and anuria [27]. The red flowers have sometimes been used traditionally for their pigmentation as a dye, which may have a role in histological studies [28]. *Hibiscus sabdariffa* extract protected human erythrocytes against lipid peroxidation [29]. The vegetable is widely grown and commonly used as port herb or soup in the northern part of Nigeria. In many countries of the world, it is eaten raw in salads, cooked and used as flavouring in cakes and making jellies, jams, soups, sauces, pickles and puddings. It is also used as colouring to herb teas and can be roasted as coffee substitute. Herbal drink made with roselle, scent leaf, garlic and ginger could be a home-made cure for diarrhea and preumonna. In recent times, it has been commercialized because of its touted medicinal potentials. Zobo is the hot water extract of *Hibiscus Sabdariffa* calyx and has been shown to possess medicinal/pharmaceutical properties including anti-diabetic, anti- hypertensive and anti-inflammatory properties [30]. A study [31] published in 2007 compared *Hibiscus sabdariffa* L. to the drug lisinopril on people with hypertension. *Hibiscus* "decreased blood pressure (BP) from 146.48/97.77 to 129.89/85.96 mmHg, reaching an absolute reduction of 17.14/11.97 mmHg (11.58/12.21%, $p < 0.05$)". Blood pressure "reductions and therapeutic effectiveness were lower than those obtained with lisinopril ($p < 0.05$)".

The authors [31] and others [32-34] concluded that *hibiscus* “exerted important antihypertensive effectiveness with a wide margin of tolerability and safety, while it also significantly reduced plasma ACE activity and demonstrated a tendency to reduce serum sodium (Na) concentrations without modifying potassium (K) levels”. They attributed the blood pressure reducing effect of *hibiscus* to its diuretic effect and its ability to inhibit the angiotensin-converting enzyme through the presence of anthocyanins [34,35].

II. Materials and Methods

II.1 Methods

The method used for assessing the anti-denaturation effects of natural products with anti-inflammatory properties is simple and inexpensive as has been reported by several authors [7,8,15,36,37]

II.2 Chemicals and drugs

The standard reference drug, Ibuprofen Emzor brand was purchased from Sulhu Pharmaceutical Drug store, Yarkutungu along old Katsina township stadium. Other reagents (hydrochloric acid, sodium dihydrogenphosphate, disodiumhydrogenphosphate, sodium chloride, glycerol) used were of analytical grades from BDH, M&B, Sigma or, Fluka.

II.3 Plant materials

The *Malus domestica* fruit and *Hibiscus sabdariffa* calyx were obtained from Yarkutungu Market in Katsina metropolis, and were identified at the herbarium, Department of Biological Sciences of Umaru Musa Yar’adua University, Katsina.

II.4 Preparation of extracts

The *Malus domestica* fruit material was washed and chopped into bits and air-dried for 14 days. The *Hibiscus sebdariffa* calyx were purchased dried and used as. The crude samples were separately ground in a laboratory grinder until a smooth powder was obtained in order to ensure high surface area for increased diffusion. About 440g of each plant powdered sample were packed in a Soxhiet extractor and extracted with 1 .5liters of ethanol for one week. This cycle was repeated for 7 days. After extraction, the solvent was removed, typically by means of a rotary evaporator, yielding the crude extracts. And the non-soluble portion of the extracted plants remains in the thimble and were discarded. The extracts were dried on water bath at 70°C for 72hours. The dry extracts of *Malus domestica* fruit and *Hibiscus sabdariffa* calyx were kept in a vacuum desiccator until when ready for use.

II.5 Estimation of total phenolic compounds and flavonoids

The total phenolic contents of the two extracts were determined spectrophotometrically by applying the Folin-Ciocalteu assay with gallic acid as standard [8, 38]; while a slight modification of the method of Chang *et al.*[8, 39] was used for estimation of flavonoids contents of the test extracts with rutin as standard.

II.6 In-vitro anti-inflammatory activity bioassay

The screening for anti-inflammatory activity was carried out according to a modification of the in vitro protein denaturation bioassay methods described by Jagtap *et al.*[40], Sakat *et al.* [41] and Shallangwa *et al.*[8] Anti-inflammatory bioassay in vitro consist of reaction mixture (5mL) which are 0.2mL of egg albumin (from fresh hen's egg), 2.8mL of phosphate buffered saline (PBS, pH 6.4) and 2mL of varying concentrations of apple extract (of final concentrations 50, 100, 200, 400, 800, 1600 $\mu\text{g/mL}$, respectively). Similar composition was made where no drug or test sample was added but only doubly distilled water to serve as control.

Then the mixtures were incubated at $(37 \pm 2^\circ\text{C})$ in Corsair Heating & Catering Limited incubator for 15 mm. Denaturation was induced by keeping the reaction mixtures at $60 \pm 2^\circ\text{C}$ in water bath for 10 mm. After cooling, the turbidity was measured at 660 nm (UV-Visible U2800 Spectrophotometer, Hiatachi Ltd). Percentage of inhibition of denaturation was calculated from control where no drug was added. Each experiment was done in triplicate and the average taken. Similar procedures were applied to *Hibiscus sabdariffa* calyx extract too. The Ibuprofen tablet at the final concentrations of 50, 100, 200, 400, 800, 1600 $\mu\text{g/mL}$, respectively were also used as reference drug and treated similarly for determination of absorbance. Each experiment was done in triplicate and the average taken. The percentage inhibition of denaturation was calculated by using following formula:

$$\% \text{ Inhibition} = 100 \times \left(\frac{V_t}{V_c} - 1 \right)$$

Where,

V_t = mean absorbance of test sample.

V_c =mean absorbance of control.

Summary of Advantages of methods used in this research

- a) **Preparation of extract:-** Washing with of fruits and air-drying ensures that dust particles, impurities and all germs are removed, and air drying instead of using oven to dry would ensure no decomposition of the bioactive nor loss of useful component in the fruits agents present in the fruit occurs.
The grinding process for the crude sample ensures larger surface area which the use of soxhlet extractor with ethonal of solvent ensure cleanliness, rapidity of extraction and minimal loss of material, and ensure greater extract yield.
- b) Estimation of total phenolic compounds and flavonoid (Bioactive constituents) using the spectromatric method ensured accuracy and precision of result. it gives result readiness faster.
- c) The evaluation of the Anti-inflammatory effects of both the plant extract on protein denaturation was done in vitro instead of in vitro to abide by all ethical issues of not having feeling for human lives if any animal is being killed or poisoned for the sake of research secondly the in-vitro method takes into account the cost implication of the research. In-vitro also ensure easier manipulates of variables of research, it ensures as contaminations by animal antibodies, germs or blood contaminations. In-vitro also ensures non-interference by outside thermodynamic parameters as the research was in progress.

II.7 Statistical analysis

All the pharmacological test data are expressed as the Mean \pm Standard Error of the Mean (SEM). The 50% Inhibitory Concentrations (IC₅₀) expressed as 50% Effective Concentrations (EC₅₀) were determined after logarithmic transformation of the concentration-response curve using GraphPad Prism 5.0 software. The Efficiency Index (EI) was expressed as EC₅₀/E_{max}.

III. Results and Discussion

III.1 Total phenolic compounds and flavonoids (Bioactive Constituents)

The overall extraction yield of *Malus domestica* fruit and *Hibiscus sabdariffa* calyx were 42.05% and 39.82% dry weight materials. The study also revealed that the extracts were rich in phenolic and flavonoids. This result is in agreement with previous studies done by Jelodarian *et al.* [42]; Yang *et al.* [43]. Total polyphenols of the extract of *Malus domestica* fruit and *Hibiscus sabdariffa* calyx were estimated by the Folin-Ciocalteu method as 8.4 \pm 4.1 μ g GAE/mg and 21.87 \pm 3.5 μ g GAE/mg, respectively, dry extract weight. The total flavonoid content of the plant ethanolic extracts were estimated and presented in Table 1.

Table 1: Extraction yield, total polyphenols and total flavonoids contents of extracts from *Malus domestica* and *Hibiscus sabdariffa* calyx

Parameter	M. domestica	H. sabdariffa
Total yield (%W/W)	42.05	39.82
Total polyphenols contents (μg GAE/mg dry extract weight)	8.4 \pm 4.1	21.87 \pm 3.5
Total flavonoids contents (μg GAE/mg dry extract weight)	4.8 \pm 3.2	12.6 \pm 2.2

GAE: Gallic acid equivalents and RE: Rutin equivalents

It has been reported widely that phenolic and flavonoids content show significant antioxidant action on human health [7,42-45]. The polyphenolic compounds like flavonoids, phenolic acids and tannins are known to have an ideal chemical structure for effective free radical-scavenging activities that have shown to be more effective as antioxidants in vitro than vitamins E and C. This fact, has been established by many researches [46,47]. Furthermore, a number of previous studies have demonstrated a strong correlation between phenolic content, antioxidant properties and anti-inflammatory properties [7,8,48-50].

III.2 Anti-inflammatory activity bioassay in controlled environment

In the present study, the evaluation of anti-inflammatory effects was undertaken using the effect of *Malus domestica* and *Hibiscus sabdariffa* calyx extracts on protein denaturation. Denaturation of proteins is well documented and is caused by inflammation process, mostly in conditions like arthritis [40,49,50]. Therefore, using agents that can prevent protein denaturation would be worthwhile for anti-inflammatory drug development. Several anti-inflammatory drugs have shown dose dependent ability to inhibit heat induced protein denaturation [8]. Protection or inhibitory effect against protein denaturation, which is the main mechanism of action of NSAIDS, plays an important role in the anti-inflammatory activity of NSAIDS [7]. Studies of anti-inflammatory activities in controlled environment has its own advantages compared to using animals in experimental pharmacological research, because it addresses the ethical issues and the lack of rationale for the use of live animals when other suitable methods could be employed [7,8].

The ability of *Malus domestica* and *Hibiscus sabdariffa* calyx extracts to inhibit protein denaturation may contribute to their anti-inflammatory properties. In the present investigation, the in vitro anti-inflammatory effect of *Malus domestica* and *Hibiscus sabdariffa* calyx extracts, blend of their extracts, were evaluated against denaturation of egg albumin. The results are as presented in Table 2.

Table 2: Anti-inflammatory data on *M. domestica*, *H. sabdariffa*, extracts blend of *M. domestica* and *H. sabdariffa* and, Ibuprofen

Concentration ($\mu\text{g/mL}$)	<i>M. domestica</i> (% inhibition)	<i>H. sabdariffa</i> (% inhibition)	Blend of <i>M. domestica</i> and <i>H. sabdariffa</i> (% inhibition)	Ibuprofen (% inhibition)
Control	--	--	--	--
1600	551.9 \pm 7.1	549.2 \pm 15.7	534.2 \pm 3.3	278.2 \pm 13.4
800	523.1 \pm 20.4	529.6 \pm 13.1	462.8 \pm 3.3	151.4 \pm 8.4
400	326.4 \pm 14.6	463.3 \pm 7.3	339.9 \pm 11.7	122.6 \pm 3.2
200	285.6 \pm 6.5	422.0 \pm 7.9	336.1 \pm 9.3	118.5 \pm 13.6
100	270.4 \pm 9.8	421.2 \pm 5.2	288.9 \pm 2.7	97.2 \pm 5.6
50	205.4 \pm 7.6	407.3 \pm 13.6	285.1 \pm 4.9	78.8 \pm 6.2

(Values are expressed as SEM of 3 readings)

The study showed a concentration-dependent inhibition of protein (egg albumin) denaturation by *Malus domestica*, *Hibiscus sabdariffa* calyx and the blend of the two extracts, within the concentration ranges of 50.0 to 1600.0 $\mu\text{g/mL}$. The reference drug, Ibuprofen, also exhibited concentration-dependent inhibition of protein denaturation. Table 2 showed that at low concentrations (50-400 $\mu\text{g/mL}$), the *Hibiscus sabdariffa* calyx was more effective than the blend extract, *Malus domestica* and Ibuprofen, in that order. At the moderate concentration of 800 $\mu\text{g/mL}$ there was slight change in the trend of percentage inhibition of denaturation; *Hibiscus sabdariffa* calyx extract (529.6 \pm 13.1) did just a little better than the *Malus domestica* extract (523.1 \pm 20.4), and the two extract were both separately better than blend (462.8 \pm 3.3) with Ibuprofen (151.4 \pm 8.4) being the least effect in preventing denaturation. But at higher concentration (1600 $\mu\text{g/mL}$) the *Malus domestica* extract (551.9 \pm 7.1) showed that it is more effective than the *Hibiscus sabdariffa* calyx extract (549.2 \pm 15.7) in inhibiting denaturation of protein, followed by blend of extracts (534.2 \pm 3.3) and Ibuprofen (278.2 \pm 13.4), in that order, respectively.

The extract concentration for 50% inhibition expressed as effective concentration (EC_{50}) was determined by the dose-response curve using GraphPad Prism 5.0 software and values are as presented in Table 3.

Table 3: EC_{50} values for *M. domestica*, *H. sabdariffa*, extracts blend and ibuprofen

	<i>M. domestica</i>	<i>H. sabdariffa</i>	Extracts blend (<i>M. domestica</i> / <i>H. sabdariffa</i>)	Ibuprofen
IC_{50} ($\mu\text{g/mL}$)	510 \pm 3.1	481.1 \pm 2.2	692.6 \pm 4.1	571.3 \pm 2.1

(Values are expressed as SEM of 3 readings)

This is defined as the concentration sufficient to obtain 50% of a maximum inhibition of protein denaturation. In this study the EC₅₀ values showed that the *Hibiscus sabdariffa* calyx extract is more effective than the *Malus domestica*, followed by the reference drug, Ibuprofen; with the blend extract being the least effective. This was almost the same trend of efficiency observed at the moderate concentration of 800µg/mL (Table 2), only that the reference was a little better than the blend.

The increments in the absorbance of the test samples with respect to the control indicated that there was stabilization of protein. That is, there was inhibition of protein (egg albumin) denaturation, which is also a measure of the anti-inflammatory effect of test extracts, blend and the reference drug, ibuprofen [7,8,15,36].

IV. Conclusion

It has been reported that one of the features of several non-steroidal anti-inflammatory drugs is their ability to prevent denaturation of protein. From the results of this study, it can be concluded that *Malus domestica* and *Hibiscus sabdariffa* calyx as well as their blend possessed marked anti-inflammatory effects as they can limit the denaturation of protein process in vitro. This can contribute to the validation of the anti-inflammatory activity of these plants and may provide some evidence for their folk uses separately and in combinations. These results also provide the motivation for further planning of clinical nutrition and anti-inflammatory research studies on bioactive compounds of the plants and development of anti-inflammatory products.

The objective of this research was first of all to determine the total bioactive constituents in two dried plant extracts namely Hibiscus Sabdariffa and Malus Domestica using spectroscopy method, and secondly to compare the protein denaturation inhibition capabilities of these extracts separately to those of synthetic drug Ibuprofen. This has expectedly provided information on which of them possess the best anti-inflammatory action with less side effect upon consumption.

It was found out in this research that the protein Denaturation inhibition capabilities using incubated egg-albumin + extracts of both plants extracts reveals the Hibiscus Sabdariffa has the highest capability values then both the synthetic drug and the Malus Domestica. The protein Denaturation inhibition capability of each of the plant extracts suggest their various capabilities in helping to conserve or resist protein loss by the body. The results further revealed that the plants and the extracts possess anti-inflammatory properties at various concentrations and this could be due to the presence of bioactive compounds in them, which has also good antioxidants that can act effectively on radicals that may cause inflammation on tissues.

Contributions of research to knowledge

Protein as sourced in egg-albumin are essentially required for the of injured, damaged or worn-out tissues in the body. This injury normally result in pains of inflammation as a result of inhibition of red blood cell hemolysis in the affected areas. The consumption of synthetic drugs (NSAIDS) help in providing relief by inhibiting protein denaturation in the body so that loss of protein, which could have been used in repairing injured tissues can be avoided. However, the excessive intake of consumption of these pain-relieving drugs is capable of triggering the formation of some other diseases in the human body.

This research has revealed that bioactive agents (phenolic compounds and flavonoids) that are present in the extracts of the used plants on play more effective healing roles against pains using similar mechanism to that of the NSAID drugs as Ibuprofen. The spectrophotometric method of measuring and recording various absorbance values of various concentrations of the extracts as provided by the incubated egg-albumin extracts of these bioactive agents has experimentally revealed the higher potency and inhibition of protein denaturation of Hibiscus sabdariffa at EC_{50} of 692.6 ± 4.1 , followed by Ibuprofen 571.3, then Malus Domestica 510 mg/L. Thus, regular consumption of these plant extracts (Malus Domestica and Hibicus Sabdariffa), provides the body with more of the antioxidant bioactive compound (flavonoids polyphenoly) which could help in the healing process for injuries to tissues due to their protein denaturation as well as their anti-inflammatory roles.

These would contribute significantly to the protection of cell membrane against injurious substances by taking care of, or arresting all forms of progression of inflammation. It is possible that there is interaction of the plant extracts with the cell membrane, the plant extracts could affect the ratio of surface area/volume of the cells by an expansion of membrane or the shrinkage of cells or by the interaction with membrane protein as a mechanism for their anti-inflammatory roles.

V. References

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