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In vitro anti-inflammatory properties of methanolic extract of *Hibiscus sabdariffa* flowers

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Abstract
The present study aims to investigate the *in vitro* Anti-inflammatory activity of *Hibiscus sabdariffa* mithanolic flowers extract. *In vitro* anti-inflammatory activity such as inhibition of albumin denaturation and membrane stabilization assay were performed in methanolic extract. Anti-inflammatory activity of *Hibiscus sabdariffa* was confirmed. The results obtained indicate that the extract possessed significant level of activity; the highest concentration of extract was high effective as an anti-inflammatory agent. However, these effects need to be confirmed using *in vivo* models and clinical trials for its effective utilization as therapeutic agents.

Keywords: Anti-inflammatory, albumin denaturation, membrane stabilization, *Hibiscus sabdariffa*

1. Introduction
Traditional folk medicine is well known since thousand years ago. Commonly the ailment incidence in the rural area is treated with local plants that contain many pharmaceutical constituents (Sofowora, 1983) [20]. Among the 120 active compounds currently isolated from the higher plants are widely used in modern medicine, today 80 percent show a positive correlation between their modern therapeutic use and the traditional use of the plants from which they are derived (Fabricant and Daniel, 2001) [4].
The bioactive compounds of medicinal plants are used as anti-diabetic, chemotherapeutic, anti-inflammatory, anti-arthritic agents where no satisfactory cure is present in modern medicines. Medicinal plants have been used as dietary adjunct and in the treatment of numerous diseases without proper knowledge of their function. Although physiotherapy continues to be used in several countries, few plants have received scientific or medical scrutiny (Dinesh *et al*., 2009) [3].
Inflammation is considered as a primary physiologic defense mechanism that helps body to protect itself against infection, burn, toxic chemicals, allergens or other noxious stimuli. An uncontrolled and persistent inflammation may act as an etiologic factor for many of these chronic illnesses. Although it is a defense mechanism, the complex events and mediators involved the inflammatory reaction can induce, maintain or aggravate many diseases. Currently used synthetic anti-inflammatory drugs are associated with some severe side effects. Therefore, the development of potent anti-inflammatory drugs with fewer side effects is necessary from medicinal plants origin (Sofowora 1993) [20].
Inflammation is a bodily response to injury, infection or destruction characterized by heat, redness, pain, swelling and disturbed physiological functions. It is triggered by the release of chemical mediators from injured tissue and migrating cells (Sofowora 1993) [20].
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Hibiscus sabdariffa belongs to (family: Malvaceae) has been used in folk medicine as a diuretic, laxative, and treatment for cardiac and nerve diseases, hypertension and cancer (Chewonarin *et al.,* 1999; Akindahunsi and Olaleye, 2004) [2, 1]. The heated leaves are applied to cracks in the feet and on boils and ulcers to speed maturation. A lotion made from leaves is used on sores and wounds. In several countries, it is used as a natural medicine for treating hypertension, pyrexia and liver disorders and microorganism growth limitation (Oboh and Elusiyan, 2004) [14], as well as a diuretic, digestive and sedative (Akindahunsi and Olaleye, 2004) [1]. Pharmacological studies of anthocyanins in hibiscus have shown that they have antioxidant activity in patients with atherosclerosis. The plant grows as an annual and sometimes biannual shrub with straight branches and small ramifications, with yields that can reach 0.5 to 2 depending on the variety.

Cultivated plants reach between 1 and 3 m in height depending on the location and season of sowing. The crop is susceptible to the attack of various plant pathogens which can infect plants at early development stages, when competition from weeds can also be deleterious (Garbi *et al.,* 2016) [5]. The present study aims to investigate the *in vitro* Anti-inflammatory activity of *Hibiscus sabdariffa* mithanolic flowers extract.

2. Materials and methods

2.1 Plant collection

The *H. sabdariffa* (calyxes) was collected from local market Khartoum in January 2017. The plant was identified and authenticated at the Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), Khartoum, Sudan. Flowers of *H. sabdariffa* were air dried, under the shade and pulverized and stored prior to extraction.

2.2 Preparation of crude extracts

Extraction was carried out according to the method described by Harbone (1984) [6]. Calyx was separated from the plant in order to prepare extract for screening of the antibacterial activity. Specific weight of sample about 50 g of the powdered sample was successively extracted with methanol (80%) using soxhlet extractor apparatus. Methanol extraction carried out for six-to-eight hours whereas methanol solvent was removed by using rotary evaporator. Extract was allowed to air dryness the percentage yield was calculated. The extract were kept and stored at 4 °C until use.

2.3 Anti-Inflammatory (Membrane Stability) Activity Assay

Principle

The lysosomal enzyme released during inflammation produces a variety of disorders. The extracellular activity of these enzymes is said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane. Since HRBC (human red blood cell) membrane is similar to lysosomal membrane, the study was undertaken to check the stability of HRBC membrane by the extracts to predict the anti-inflammatory activity *in vitro*. The various extracts at the concentration of 100, 50, 25 and 12.5 μg/mL, respectively, were incubated separately with HRBC solution (Varadarasu *et al*., 2009) [21].

2.4 HRBC Membrane Stabilization Method

The anti-inflammatory activity of various extracts of leaves of *Gardenia coronaria* was assessed by *in vitro* HRBC membrane stabilization method. Blood was collected from healthy volunteers. The collected blood was mixed with equal volume of Alsever solution (dextrose 2%, sodiumcitrate 0.8%, citric acid 0.05%, sodium chloride 0.42%, and distilled water 100 mL) and centrifuged with isosaline. To 1mL of HRBC suspension, equal volume of test drug in three different concentrations, 100, 50, 25 and 12.5 μg/mL, was added. All the assay mixtures were incubated at 37 ºC for 30 minutes and centrifuged. The haemoglobin content in the supernatant solution was estimated by using spectrophotometer at 560 nm (James *et al.,* 2007) [8]. The experiment was carried out in triplicates. The percentage of haemolysis was calculated then by the formula as given below:

Percent of hemolysis = OD of test OD of control × 100.

The percentage of protection can be hence calculated from the equation as given below:

Percent of protection = 100 − OD of test OD of control × 100.

Here "OD of test" is optical density or the test sample's absorbance and "OD of control" is optical density or absorbance of the negative control. Here, the negative control used was Alsever's solution with blood in it and standard (diclofenac) was taken as a positive control.

2.5 Inhibition of albumin denaturation

Inhibition of protein denaturation was evaluated by the method of (Mizushima and Kobayashi 1968) [12] and (Sakat *et al.,* 2010) [18] with slight modification. 500 µL of 1% bovine serum albumin was added to 100 µL of plant extract with deferent concentrations. This mixture was kept at room temperature for 10 minutes, followed by heating at 51 °C for 20 minutes. The resulting solution was cooled down to room temperature and absorbance was recorded at 660 nm. Standard (diclofenac) was taken as a positive control. The experiment was carried out in triplicates and percent inhibition for protein denaturation was calculated using:

$$\% \text{ Inhibition} = 100 - ((A_1 - A_2)/A_0) * 100)$$

Where A_1 is the absorbance of the sample, A_2 is the absorbance of the product control and A_0 is the absorbance of the positive control.

3. Results and discussion

In this study the yield percentage of *H. sabdariffa* methanol extract was 19.1grams.

3.1 Membrane stabilization

The HRBC membrane stabilization has been used as a method to study the *in vitro* anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. The extra cellular activity of these enzymes are said to be related to acute or chronic inflammation. The non-steroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing the lysosomal membrane the extract was shown inhibiting the heat induced hemolysis compare with standard diclofenac. These results provide evidence for membrane stabilization as an additional mechanism of their anti-inflammatory effect. The extract inhibited the heat induced hemolysis of RBCs to varying degree (Table1).

Meanwhile the methanol extract showed 35.79%, 14.31%, 13.88% and 7.65% at concentrations 100 μg/mL, 50 μg/mL, 25 and 12.5 μg/mL respectively. Diclofenac a standard anti-inflammation drug showed the maximum inhibition 86.75% at the concentration of 200 µg/ml.

3.2 Inhibition of albumin denaturation

Inflammation is a bodily response to injury, infection or destruction characterized by heat, redness, pain, swelling and disturbed physiological functions. Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents. Protein Denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. Most biological proteins lose their biological function when denatured. Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation on the mechanism of the anti-inflammation activity, ability of extract protein denaturation was studied (Sangita *et al.,* 2012) [19]. The results have been summarized in (Table 2). Maximum inhibition 51.2 % was observed from 100 μg/mL concentrations followed by 34.55%, 28.67% and 18.62% in concentrations 50 μg/mL, 25 and 12.5 μg/mL respectively. Diclofenac a standard anti-inflammation drug showed the maximum inhibition 76.04 % at the concentration of 200 µg/ml.

Table 1: Effect of *H. sabdariffa* mithanolic flowers on inhibition of haemolysis

Concentration (µg/ml)	Absorbance at 560nm	% inhibition of haemolysis
Diclofenac	0.061	86.75
100	0.299	35.79
50	0.399	14.31
25	0.401	13.88
12.5	0.430	7.65
Control -ve	0.465	0.00

Table 2: Effect of *H. sabdariffa* mithanolic flowers on heat induced protein denaturation

Concentration (µg/ml)	Absorbance at 660nm	% inhibition of Protein denaturation
Diclofenac	0.097	76.04
100	0.199	51.22
50	0.267	34.55
25	0.291	28.67
12.5	0.332	18.62
Control -ve	0.408	0.00

A recent review stated that specific extracts of *H. sabdariffa* exhibit activities against atherosclerosis, liver disease, cancer, diabetes and other metabolic syndromes (Lin *et al.,* 2011) [9]. The plants are rich in anthocyanins, as well as protocatechuic acid. The dried calyces contain the flavonoids gossypetin, hibiscetine and sabdaretine. The major pigment, formerly reported as hibiscin, has been identified as daphniphylline. Small amounts of myrtillin (delphinidin 3-monoglucoside), chrysanthenin (cyaniding 3-monoglucoside), and delphinidin are also present. *H. sabdariffa* seeds are a good source of lipid-soluble antioxidants, particularly gamma-tocopherol (Mohamed *et al.,* 2007) [13].

This *in vitro* method was more time saving, flexible, and convenient in other ways. The investigation suggested good ability of the Methanolic extract to resist the cell lysis in large concentrations as compared to the standard drug diclofenac at 200 µg /mL. *H. sabdariffa* is known to have ascorbic acid as one of its phytochemicals which has been proposed to have an anti-inflammatory activity (Mahadevan *et al*, 2008) [11]. Apart from this phytochemical, it also consists of flavonoids such as hibiscitrin and hibiscetin and polyphenols (Lin *et al*., 2007) [10] and other minerals that have been shown to used in the treatment of hypertension (Wang *et al,* 2000; Rimm and Stamfer, 2000) [22, 16], also having hypocholesterolemic effect (Olaleye, 2007) [15], anti-oxidative and hepatoprotective effect (Wang *et al*, 2000) [22].

4. Conclusion

H. sabdariffa showed that the various degree of Anti-Inflammatory. Further investigations regarding the mode of action and other related pharmacological studies such as *in vivo* investigation, drug formulation and clinical trials are highly recommended.

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