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Immunomodulatory Effect of Extracts of *Hibiscus sabdariffa* L. (Family Malvaceae) in a Mouse Model

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The immunomodulatory activity of water and alcohol extracts (including its fractions) of the dried calyx of the plant was evaluated in mice. The ability of the extracts to inhibit or enhance the production of two cytokines, namely tumor necrosis factor-alpha (TNF- α) and interleukin-10 (IL-10), respectively, implicated as proinflammatory and antiinflammatory interleukins were also evaluated.

The extracts at doses of 50 mg/kg were found to possess higher immunostimulatory activities in comparison with levamisole (positive control), with significant effects when compared with the vehicle-treated group ($p < 0.01$). Increased activity was observed with increase in doses of the 50% ethanol and absolute ethanol extracts. The insoluble fraction exhibited a significant dose-dependent immunostimulatory activity ($p < 0.05$), while the residual water-soluble fraction exhibited activity at 100 mg/kg body weight. The production of tumor necrosis factor-alpha (TNF-α), was low in all the extract groups tested, while the production of interleukin 10 (IL-10) was high compared with the control. The production of IL-10 was high in 300 mg/kg aqueous extract. The insoluble fraction exhibited a profound dose-dependent immunostimulatory activity higher than the positive control at 100 mg/kg.

This study established the immunoenhancing properties of the extracts of this plant confirming that the immunomodulatory activity is cell mediated and humoral. The insoluble fraction could find use as an immunostimulatory agent in humans. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: Hibiscus sabdariffa fractions; immunostimulatory activity; cytokines.

INTRODUCTION

The concept that immune responses are modulated to alleviate diseases has existed in many forms of traditional medicine beliefs, with plants being used in such systems to promote health and to maintain the body's resistance against infections by potentiating immunity. Some of these plants are specifically stimulatory or suppressive, and normalize or modulate pathophysiological processes, and are thereby termed 'immunomodulatory'.

The water infusion of the dried calyx of *Hibiscus sabdariffa* Linn. (Family Malvaceae) is taken in many parts of the world for the treatment or management of high blood pressure, liver diseases, fever, anemia (Odigie *et al.*, 2003; Herrera-Arrelano *et al.*, 2004; Falade *et al.*, 2005). The fruits and dried flowers are also used in the management of chronic wounds or ulcers (Inngjerdingen *et al.*, 2004). The pharmacological actions of the calyx extracts and some of the constituents include strong *in vitro* and *in vivo* antioxidant activity (Tseng *et al.*, 1997, 1998; Ali *et al.*, 2003; Lin *et al.*, 2003; Suboh *et al.*, 2004; Amin and Hamza, 2005), antimicrobial and antiinflammatory activity (Ogundipe *et al.*, 1998), inhibition of serum lipids with antiatherosclerotic activity (Chen *et al.*, 2003) and induction of apoptosis (Hou *et al.*, 2005; Chang *et al.*, 2005; Lin *et al.*, 2005).

There is, however, no report on the immunomodulatory activity of the extracts of the plant or its parts. The aim of this study is to therefore to evaluate the *in vivo* immunomodulatory activity of the extract of *H. sabdariffa* in an animal model.

METHODOLOGY

The flowers of *Hibiscus sabdariffa* were obtained from Bodija Market, in Ibadan and authenticated at the Forestry Research Institute of Nigeria (FRIN), Ibadan. A herbarium specimen of number FHI 106934 was made. The dried calyxes were further dried at 40 °C until a constant weight was obtained and pulverized to obtain a coarsely powdered material.

Extraction. One liter each of distilled water, water/ethanol mixture (50:50) and 100% ethanol were used to infuse 100 g each of the powdered plant material for 4 h. The extract obtained was decanted and the material was re-extracted with another 1 L of the appropriate solvent. The extract obtained was pooled, filtered and dried *in vacuo*. Ethanol was obtained from Thomas Baker Chemicals PVT Ltd (Mumbai, India).

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Extracts from the 50% ethanol/water mixture were further partitioned successively into dichloromethane (Merck KGaA, Darmstadt, Germany), ethyl acetate (Thomas Baker Chemicals PVT Ltd, Mumbai, India) and butanol (Thomas Baker Chemicals PVT Ltd, Mumbai, India).

Anthocyanin determination. The anthocyanin content of the dried calyx was determined calculating for cyanindin-3-glucoside using a colorimetric method based on the ability of anthocyanins to produce a color at pH 1.0 that disappears at pH 4.5. This characteristic reaction is produced by a pH dependent structural transformation of the chromophore. The colored oxonium ion predominates at pH 1.0, while the non-colored hemiketal is present at pH 4.5 allowing the accurate and fast determination of total anthocyanins, still with the presence of polymeric pigments and other compounds (Wrolstad *et al.*, 2005). Briefly, 1 g of the plant material was extracted with the appropriate solvent and diluted with buffer solutions at pH 1.00 and pH 4.5. The difference in the absorbance at 510 and 700 nm at the different pH was used in calculating the monomeric anthocyanin pigment present in 1 g of the plant material using the following equation:

$$\text{Total anthocyanins (mg/L)} = A \times MW \times DF \times \sum \times 1 \quad (1)$$

where A, absorbance =
$(A_{510nm} - A_{700nm})$pH 1.0 – $(A_{510nm} - A_{700nm})$pH 4.5;
MW, molecular weight; DF, dilution factor;
$\sum$ = molar extinction coefficient($L \times mol^{-1} \times cm^{-1}$).

Immunomodulatory activity. This activity was evaluated using red blood cell-induced immunostimulation in a mouse model. Swiss albino mice weighing between 18 g and 24 g consisting of five animals per group were used for the study. Aqueous (A), 50% ethanol (AE) or absolute ethanol (E) extracts were administered at 5, 50 or 300 mg/kg body weight dose to albino mice with an oral gavage needle daily for 28 days. In a separate experiment, the insoluble fraction (EAC) obtained from the 50% ethanol extract were administered at 50 and 100 mg/kg and the residual water-soluble fraction at 100 mg/kg body weight. Levamisole (0.6818 mg/kg) orally was used as a positive control, while the vehicle-control group was administered 0.5 mL of water daily. The negative control group was administered intraperitoneal cyclophosphamide (200 mg/kg) on day 5.

On day 7, 200 µL of 10% of New Zealand rabbit red blood cell (RRBC) suspension in normal saline was administered intraperitoneally. This was repeated 14 days later as a booster dose. The body weight of the animals was recorded weekly. On day 28, all the animals were bled and the serum collected was stored at –20 °C for further studies.

Haemagglutination test was performed by adding 100 µL of rabbit RBC (6×10^3 cells per mL) to 25 µL of the serial two fold dilutions of the serum in Alsever's solution in U-bottom microtitre plates, shaken and allowed to stand for 4 h at 25 °C. Rabbit RBC-setting patterns were then read. The haemagglutination (HA) titer was expressed as the reciprocal of the highest dilution of the serum showing definite agglutination formation as opposed to smooth dot in the center of the well.

Cytokine determination. Due to a prior study (Ogundipe *et al.*, 1998) showing that extract of the dried calyx possess antiinflammatory activity in carrageenan-induced rat paw model, two cytokines, namely tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) implicated as proinflammatory and antiinflammatory interleukins, respectively, were evaluated using enzyme-linked immunosorbant assay (ELISA) for the quantitative measurement of mouse TNF-α, and IL-10 (Endogen$^{(R)}$ Mouse ELISA kit, Pierce Biotechnology, Inc, Rockford). Sera from animals in the same group were pooled for the assay.

To 50 µL of pooled serum or standard concentrations of lyophilized recombinant mouse tissue necrosis factor-alpha, biotin-labelled detecting antibody reagent was added in tumor necrosis factor-alpha precoated wells. Incubation was done for 2 h at 37 °C. Thereafter, a 100 µL dilution of 1:400 streptavidin conjugated with highly purified horseradish peroxidase was added to each well and further incubated for 30 min.

For determination of IL-10, to each endogen mouse interleukin-10 pre-coated well was added 50 µL assay buffer. The same amount of standards (containing known concentrations of lyophilized recombinant mouse IL-10) or samples from the pooled sera was added to the wells and incubated at 37 °C for 3 h. After washing the plates, 50 µL of biotin-labelled detecting antibody reagent was added to each well, incubated for 1 h, after which 100 µL of 1:400 diluted streptavidin-horseradish peroxidase was added and incubated for 30 min. To both IL-10 and TNF-α plates, 100 µL of premixed 3,3′,5,5′-tetremethylbenzidine substrate solution was added to each well and developed in the dark for 30 min. The blue color reaction obtained was stopped with 100 µL of 0.18 M sulphuric acid. The difference in absorbance was measured using a spectrophotometer plate reader (Versamax, Molecular Devices, USA) at 450 nm and 550 nm. The difference in absorbance was read off the calibration curve obtained from the standard concentrations. The concentration of each cytokine was determined in picogram per milliliter of serum.

Statistical analysis. The effects of the extracts and fractions on haemagglutination titer and other parameters were compared with the controls using the one-way analysis of variance with Dunnett's post hoc test. The level of significance was set at $p < 0.05$ using GraphPad Instat$^{(R)}$ statistical analytical software.

RESULTS AND DISCUSSION

The yield of the extracts was found to be 18.54% with ethanol (E), 15.18% with water (A) and 15.0% in 50% ethanol (AE). Ethanol was observed to extract the least anthocyanin (as cyanidine-3-glucoside) of 1.23 mg/g plant material, while the highest was found with 50% ethanol (3.83 mg/g) and water (3.22 mg/g).

The summary of the results for the immunomodulation tests are shown in Tables 1 and 2. The extracts did not cause significant weight changes in the animals when compared with the vehicle group. Cyclophosphamide caused a reduction in the weight of the animals as can be seen in Table 1. As expected, there was a great

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Table 1. The immunostimulatory effects of the extracts of *Hibiscus sabdariffa* in a mouse model (mean ± SD, $n = 5$)

	Treatment group		Parameter			
	Weight changes in animals	RBC (10^6) in animals	WBC (10^3) of animals	HA titer of animals	TNF-α (pg/mL)	IL-10 (pg/mL)
A 5 mg/kg	16.30 ± 2.99	7.67 ± 1.78	6.14 ± 2.25	384.00 ± 215.46[a]	262	1315
A 50 mg/kg	12.53 ± 7.50	8.32 ± 0.76	7.22 ± 2.46	768.00 ± 147.80[b]	294	1151
A 300 mg/kg	7.87 ± 5.79	7.96 ± 1.31	12.10 ± 4.05[a]	384.00 ± 73.90[a]	191	4321
AE 5 mg/kg	3.75 ± 12.34	9.21 ± 1.76	7.93 ± 3.72	264.00 ± 98.09[a]	207	1314
AE 50 mg/kg	19.82 ± 7.30	7.54 ± 1.92	6.85 ± 4.06	640.00 ± 384.00[b]	215	2086
AE 300 mg/kg	16.61 ± 3.39	7.59 ± 0.77	8.99 ± 3.35	768.00 ± 147.80[b]	814	2187
E 5 mg/kg	14.88 ± 7.43	7.75 ± 1.47	7.41 ± 3.70	213.33 ± 42.67[a]	396	3150
E 50 mg/kg	16.27 ± 9.09	7.62 ± 0.49	5.63 ± 1.90	512.00 ± 258.00[a]	302	1026
E 300 mg/kg	2.86 ± 8.91	6.33 ± 0.86	6.27 ± 2.58	597.33 ± 198.12[b]	246	3299
Levamisole	20.99 ± 8.62	7.31 ± 0.50	11.29 ± 4.30[a]	448.00 ± 73.90[a]	396	2872
Cyclophosphamide	–9.83 ± 5.98[b]	6.04 ± 0.33	5.24 ± 1.32	28.00 ± 4.00	1270	1006
Vehicle group	10.16 ± 6.21	7.00 ± 0.72	6.71 ± 1.03	53.33 ± 37.33	1263	1138

A, aqueous extract; AE, 50% ethanol extract; E, ethanol extract.
Significant difference [a] $p < 0.05$; [b] $p < 0.01$ in comparison with the vehicle control, water.

Table 2. The immunostimulatory activities of the fractions of 50% ethanol extract in a mouse model (mean ± SD, $n = 5$)

	Treatment		Parameter		
	Weight changes in animals (%)	WBC (10^3) of animals	HA titer of animals	TNF-α (pg/mL)	IL-10 (pg/mL)
EAC 50 mg/kg	8.57 ± 2.02	2.63 ± 0.44[b]	144.00 ± 123.59	261	6 747
EAC 100 mg/kg	5.20 ± 2.57[a]	12.60 ± 1.35[b]	928.67 ± 619.00[b]	7	14 190
RWSF 100 mg/kg	–2.85 ± 2.84[b]	8.80 ± 0.80	223.50 ± 32.50	233	5 219
Levamisole	11.19 ± 2.04	8.48 ± 2.04	597.33 ± 225.97	240	9 631
Cyclophosphamide	2.56 ± 2.44	4.33 ± 0.30	0.00	1108	3 902
Vehicle group	16.31 ± 3.96	7.22 ± 0.25	0.00	1090	4 132

EAC, ethylacetate-insoluble fraction; RWSF, residual water-soluble fraction.
[a] $p < 0.05$; [b] $p < 0.01$ when compared with the vehicle-treated group.

reduction in the leukocyte count of cyclophosphamide-treated animals, but not for the extracts.

The results (Tables 1 and 2) implied that the mechanism of immunostimulation with extracts of *Hibiscus sabdariffa* may not be mainly through activity of the white blood cells. There seems to be an interplay between humoral (B-cells) and cell-mediated (T cells) immunity since the administration of the extracts and the fractions led to changes in the production of two cytokines, TNF-α and IL-10 (cell mediated immunity) and antibodies titer (humoral immunity). However, the evaluation of immunoglobulin G and M, another humoral immunity response parameter, could not be determined due to unavailability of the reagents. The haemagglutination, HA, titer of the extracts was found to be significantly different when compared with the vehicle group. An increase in antibody production was observed with increase in doses of both the 50% ethanol and ethanol extracts. For the aqueous extract however, there was a decline of activity at 300 mg/kg. Failure to have a dose-dependent, immunostimulatory activity could have been due to the fact that with plant extracts, immune response is not always directly related with the immunomodulator concentration. This phenomenon has been noticed in other studies (Rezaeipoor *et al.*, 2000; Tiwari *et al.*, 2004), and has been proposed to be due to different constituents present at different concentrations in the fractions/extracts thereby affecting saturation of cells responsible for the immune response differently. It is not impossible that some of the constituents have immunosuppressive activity while the others have immunostimulant activity, an event usually observed with extracts of plant origin. However, all the extracts at different doses showed better immunostimulatory activity than observed either with the vehicle control, water, or with the negative control, cyclophosphamide, with some having better activity than the positive control, levamisole.

The fractions, however, exhibited a different profile. The insoluble fraction (EAC) exhibited a dose-dependent activity. The group administered with 100 mg/kg body weight exhibited more than a 6-fold increase in immunostimulatory activity when compared with the 50 mg/kg dose using the HA titer as a response parameter (Table 2). The residual water-soluble fraction (RWSF) showed activity at 100 mg/kg body weight. There were significant changes in the white blood cell (WBC) profile of the fractions. The WBC count was significantly higher for EAC 100 mg/kg ($p < 0.01$) and lower for the EAC 50 mg/kg ($p < 0.05$) than the control. The RWSF led to weight loss ($p < 0.01$), while the EAC doses led to weight gain which was found to be significantly different with the 100 mg/kg ($p < 0.05$).

The extracts generally led to low TNF-α and high IL-10 production. It is not unusual to find both pro- and antiinflammatory types simultaneously produced

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in situ though with tightly controlled and balanced activity (Fujiwara and Kobayashi, 2005). Concentrations of the TNF-α, though not exhibiting dose-dependence in any of the extract group, were lowest in the aqueous extract at 300 mg/kg. With the ethanol extract, there was a noticeable reduction in the amount of TNF-α as the dose of the extract increases. The fractions also showed a significant reduction in the production of TNF-α when compared with the negative control and the vehicle control group. In none of the groups, however, was TNF-α concentration as high as in the negative or vehicle groups (Tables 1 and 2). Previous studies have shown that the extracts and some of its constituents have *in vitro* activity against some carcinoma cell lines (Lin *et al.*, 2005; Hou *et al.*, 2005) and *in vivo* activity against atherosclerosis in animal models (Chen *et al.*, 2003). Previous studies have also implicated high levels of TNF-α in clinical diagnoses of atherosclerosis (Bruunsgaard *et al.*, 2000) suggesting that low TNF-α may lead to reduction of calcification observed with atherosclerosis (Tintut *et al.*, 2000), an activity that has been confirmed with extracts of *Hibiscus sabdariffa* in an atherosclerotic animal model (Chen *et al.*, 2003) though prolonged exposure to TNF-α may lead to some antiinflammatory effects.

Interleukin 10 (IL-10), usually referred to as an anti-inflammatory cytokine, was high in the serum of animals on 300 mg/kg of extracts comparable to levamisole (the positive control). Animals administered with A300 mg/kg had the highest concentrations bearing out the results of an earlier study by Ogundipe *et al.* (1998) that the water extract of the dried calyx of *H. sabdariffa* has antiinflammatory activity in carrageenan-induced rat paw oedema. The insoluble fraction produced high amount of IL-10 in a dose-dependent manner, and this may well be one of the fractions responsible for the immunostimulatory activity. The residual water-soluble fraction at 100 mg/kg body weight also caused an increase in IL-10 concentration at 100 mg/kg. IL-10 has been known to down-regulate factors present in inflammation and tumors. An extract or chemical compound that could increase antiinflammatory interleukin, for example, IL-10, and reduce the formation of a pro-inflammatory factor such as TNF-α may be found to be of great usefulness in preventing or reducing formation of atherosclerotic plaques, may also help in reduction of stress, and may also possess antitumour activity.

The low level and high level of TNF-alpha and IL-10 respectively confirmed that the extracts and the fractions tested, apart from having these biological properties, may also stimulate immunomodulation through the activities of cytoleukines.

CONCLUSION

The study revealed that, to some extent the extracts, and to a large extent, two fractions of the plant possess the ability to stimulate the immune system *in vivo*. The activity may be as a result of interplay between the production of interleukin 10, inhibition of tumor necrosis factor-alpha and the effect of B-cells responsible for antibody production.

One of the fractions showed good possibilities of being developed into a drug entity that may be used to stimulate immunity as an adjunct to therapy in immunosuppressed disease conditions. However, the extract, as it is taken in humans as a beverage may be of benefit in enhancing immunity. Further studies need to be done to evaluate the exact mechanism of action.

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