Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Hibiscus sabdariffa extractivities on cadmium-mediated alterations of human U937 cell viability and activation

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ARTICLE INFO

Article history:
Received 15 June 2011
Received in revised form 15 July 2011
Accepted 15 August 2011
Available online 20 January 2012

Keywords:
Hibiscus sabdariffa
Cadmium
Macrophage
Quercetin dehydrate
Immunoprotective

ABSTRACT

Objective: To investigate the effect of the anthocyanin-rich extract of *Hibiscus sabdariffa* (*H. sabdariffa*) calyx on the viability of cadmium-treated U937 cells and cadmium-mediated activation of U937-derived macrophages. **Methods:** The macrophage cell line U937 was treated with cadmium (0.1 μ mol/L) and later incubated with the anthocyanin-rich extract and cell viability was assessed via trypan blue staining. In the other experiment, the U937 cells were transformed to the macrophage form by treatment with phorbol 12, myristate 13, and acetate and incubated with cadmium (10 μ mol/L). The anthocyanin-rich extract was added to the cells later and subsequently, the supernatant of each cell culture was analysed for the production of tumour necrosis factor-alpha (TNF- α), interleukin 1 (IL-1), interleukin 6 (IL-6), nitric oxide, and catalase activity as indices for the activation of macrophages. **Results:** It revealed that the anthocynanin-rich extract significantly ($P < 0.05$) increased the viability of the cells which was suppressed by cadmium when compared to quercetin dihydrate. The extract also reduced the cadmium-mediated production of the markers of macrophage-activation when compared to quercetin dihydrate. In both experiments, the activity of the extract was concentration-dependent ($P < 0.05$). **Conclusion:** The findings show that *H. sabdariffa* possesses significant immunoprotective effect. These corroborate the immense reported antioxidant and medicinal potential of the calyces of the plant which could be exploited for pharmacological and neutraceutical advantages.

1. Introduction

The consumption of plants for medicinal purposes has been an agelong tradition by mankind in many regions of the world. However, due to advances in pharmacology and medicine, attention shifted to the intake of synthetic drugs coupled with the lack of scientific basis for the then so-called medicinal plants[1]. Currently, there is a growing concern for the consumption of plants/plant products because they are cheap, affordable and abundant. This is further corroborated by the reported less frequent side effects following their consumption when compared to conventional chemotherapy[2]. This has led to vigorous research concerning the identification of plants of medicinal importance. Even though the scientific basis for the observed bioactivities of many plants has been in focus in recent times, there still exist a near-negative correlation between the reported bioactivities and the rate of consumption of these plants. One of the plants which is considered medicinal is *Hibiscus sabdariffa (H. sabdariffa)*. *H. sabdariffa* Linn (Malvaceae) is an annual dicotyledonous, erect and herbaceous plant that grows up to about 2.4 m tall. The tender leaves of the plant are eaten as salad and as an antedote for liver disorders, hypertension, and used as an antipurgative[3]. However, the red calyces are the major utilised part for medicinal purposes. The infusion of the calyces is consumed as a local energy-giving drink (Zobo drink) in Nigeria and has been shown to possess significant bioactivities such as antimicrobial, diuretic, anti-inflammatory, antihepatotoxic, and for the treatment of hypertention[4-6]. The calyces of *H. sabdariffa*

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have been shown to be rich in polyphenols especially anthocyanins whose bioactive potentials have been greatly explored[4,7]. This current study investigates the ability of the anthocyanin-rich extract of *H. sabdariffa* in reducing cadmium-induced modulation of raw human monocyte U937 cell line and U937-derived macrophages.

Cadmium is one of the most toxic heavy metal in our environment that causes significant metabolic and physiological abnormalities in humans[8]. One of its main targets is the immune system where it activates macrophages to the extent of causing significant inflammatory responses[9].

2. Materials and methods

2.1. Chemicals and reagents

Cadmium chloride, bovin serum albumin, methanol, Tween-20 and all antibodies used in the experiment were purchased from Merck Chemical Co. (UK). Foetal calf serum, L-glutamine, penicillin-streptomycin mixed antibiotic, and RPMI-1640 were purchased from Invitrogen (UK). Quercetin dihydrate and trypan blue were obtained from Sigma Chemical Co. (USA). All other chemicals and reagents were of analytical grade and were commercially available. All buffers and dilutions were made using 18.2 MΩ•cm water and stored under room temperature but buffers were used within 72 h following preparation.

2.2. Preparation of H. sabdariffa extract

Dried calyces of *H. sabdariffa* were purchased from a local market hence it could not be ascertained whether the calyces were from a single source. Preparation of the anthocyanin-rich extract was done according to the method of Ojeda *et al*[10] with modifications. The dried calyces were ground to a fine powder using a warring blender and soaked in distilled water overnight. The aqueous extract was lyophilised overnight after filtration. The resulting powder was dissolved in 80% methanol (3×100 mL). After 6 h, the solution was filtered and the filtrate concentrated using a rotary evaporator at 40 ℃. The residue was further vacuum dried to an anthocyanin-rich extract which was stored at 4 ℃. Recovery was 0.94%.

2.3. Cell culture and treatment

The human cell line U937 was propagated in complete medium and washed using RPMI-1640. Complete medium is RPMI-1640 supplemented with foetal calf serum (50 mL), glutamine (0.02 mol/L) and penicillin-streptomycin mixed antibiotic (100 U/mL penicillin, 100 μg/mL streptomycin). Cells were thereafter maintained between 2.5×10^5-1×10^6 cells/mL in sterile tubes and cultured at 37 ℃ in a humidified incubator gassing up to 5% CO_2.

2.4. Measurement of cell viability

The U937 cells were seeded at 5×10^5 cells/mL in complete medium. Cadmium chloride (previously sterilised) was added to the cells to a final concentration of 0.1 μmol/L, gently swirled and incubated. After 24 h, the purified extract (2 mL) was added to the cells at different concentrations ranging from 0.25 μg/mL-10 μg/mL. Control experiment was not treated with $CdCl_2$. Seventy-two hours thereafter; viable cells were counted by trypan blue staining using a counting chamber. Cell viability was finally expressed as a percentage thus:

$$\%\ \text{viability}=\frac{\text{Cell count of cells treated with cadmium}}{\text{Cell count of control cells}}\times100\%$$

2.5. Cell transformation and activation assay

Two hundred microlitres of cells in complete medium was delivered into a 96-well coaster plate and transformed to the macrophage form via the treatment with phorbol 12, myristate, 13 acetate as reported[11]. Forty eight hours later, the supernatants were removed and 100 μL of cadmium chloride (10 μmol/L) was added to the transformed cells followed by the addition of 100 μL of the extract at different concentrations (0.25 μg/mL-10 μg/mL). A group of cells was treated with only cadmium chloride. All cells were incubated at 37 ℃ and the supernatant of each cell culture was subjected to biochemical analyses 24 h later.

2.6. Analysis of macrophage activation

The activation of macrophages analysed was determined by analysing catalase activity, nitric oxide secretion, and the release of the proinflammatory cytokines tumour necrosis factor alpha (TNF-α), interleukin 1 (IL-1), and interleukin 6 (IL-6). Catalase activity was determined according to Aebi[12] as modified by Okoko and Oruambo[11]. Nitric oxide inhibitory activity was analysed according to Hwang *et al*[13] as modified[14]. The levels of the proinflammatory cytokines were determined by antibody capture ELISA as reported[11]. The values for the cytokines secreted by the cells treated with the extract were converted to relative inhibitory activities with respect to the cells treated with cadmium chloride only (whose mean value was assigned 100% activation). Thus inhibitory activity was expressed as:

$$\%\ \text{Inhibitory activity}=\frac{\text{Amount of cytokine (cells treated with extract)}}{\text{Amount of cytokine (cells treated with cadmium only)}}\times100\%$$

2.7. Statistical analysis

Quercetin (20 μmol/L, as Quercetin dihydrate) was used as the reference standard because of its reported regulatory mechanisms in suppressing macrophage-induced cytokine

production[15]. In all cases, data were expressed as mean ± S.E.M. Significance of the results were tested by analysis of variance (ANOVA) followed by Duncan's multiple range test for comparison involving multiple groups, or two tailed student's *t*-test for comparison involving two groups. The level of statistical difference was set at $P < 0.05$.

3. Results

Table 1 shows the ability of the anthocyanin-rich extract in increasing the viability of the cadmium-treated cells. Cadmium reduced the viability of the cells by almost 86% (cell viability (14.14 ± 4.46)%. However, treatment with the extract significantly increased the viability of the cells from (25.56 ± 2.39)% (at a concentration of 0.25 μg/mL) to (71.67 ± 3.37)% (when treated with 10.00 μg/mL of the extract). The inhibitory activities of the extract on cadmium-induced activation of U937-derived macrophages are shown in Figures 1. The results revealed significant modulation of the cadmium-induced effects. In both cases, the extractivity was concentration-dependent and the ability of each group was significantly different from one another ($P < 0.05$).

Table 1
Viability of cells incubated with cadmium and *H. sabdariffa* extract.

Anthocyanin rich extract (μg/mL)	Cell viability (%)
0.00	14.14 ± 4.56
0.25	25.56 ± 3.39
0.50	33.05 ± 4.03
1.00	47.28 ± 4.23
2.00	54.78 ± 2.16
5.00	60.31 ± 3.22
10.00	71.67 ± 3.37

Values are expressed as mean±S.E.M. (n=5). Readings having different superscripts differ significantly (P<0.05). Viability of quercetin dihydrate (20 μmol/L) is (64.23 ± 2.12)%.

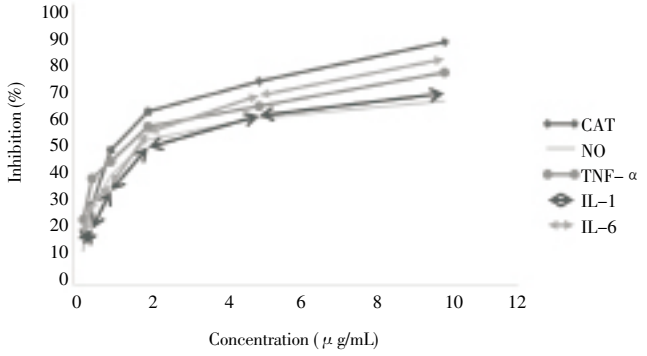

Figure 1. Inhibitory activity of *H. sabdariffa* calyx extract in cadmium-induced activation of macrophages.
Inhibitory activities of quercetin dihydrate (20 μmol/L) are (54.23 ± 1.57)%, (35.44 ± 2.36)%, (53.27 ± 4.11)%, (37.78 ± 3.91)%, and (71.18 ± 2.67)% for CAT, NO, TNF-α, IL-1, and IL-6 respectively. CAT is catalase activity, NO is nitric oxide, TNF-α is tumour necrosis factor alpha, IL-1 is interleukin 1 and IL-6 is interleukin-6.

4. Discussion

Though being a highly toxic heavy metal, the basis of the toxicity of cadmium is not very well understood however it has being suggested to inhibit crucial biochemical processes which principally involve calcium transport. It also inflicts damage to the mitochondrial structure[8]. Cadmium is thought to block calcium uptake by tissues by inhibiting Ca^{2+}-ATPase, thus competing with Ca^{2+} for binding sites. This may lead to significant disruption of calcium homeostasis in organisms[16]. Other signal transduction cascades are also thought to be sensitive targets of cadmium including the inhibition of DNA damage repair. It also causes significant lipid peroxidation which is considered as the main cause of its deleterious activities on membrane-dependent functions[17]. This may be responsible for the significant reduction in cell viability of the U937 cells when incubated with cadmium only. However at the gene level, small amounts of cadmium induce the expression of metallothionein-a cysteine rich protein that binds cadmium in the liver and kidney for detoxification. This could protect the tissues from the deleterious activities of the toxic metal[18]. However due to continual exposure to cadmium, the bioconcentration level may overwhelm the endogenous concentration of metallothionein and other detoxification systems. At this point, the heavy metal could exert significant toxic insult. It has been suggested that cadmium enhances the production of reactive oxygen species such as superoxide ion, hydrogen peroxide and hydroxyl radicals by depleting the levels of glutathione, and other protein bound sulfhydryl groups[19]. Lipid peroxidation could be one of the responses that reduced the viability of the cells in the presence of cadmium. Previous work shows that cadmium exerts its effect on various cell lines by triggering apoptosis[20]. Even though the mechanism of apoptosis is poorly understood, it may be as a consequence of the peroxidation of membrane lipids especially in the mitochondrion.

The release of the cytokines TNF-α, IL-6, and IL-1 reveals that cadmium causes the activation of macrophages[21,22] and triggers the release of nitric oxide[23]. Nitric oxide is an important signal molecule involved in a lot of responses to maintain vascular homeostasis. It is synthesised from L-arginine in a reaction catalyzed by nitric oxide synthase (NOS). Catalase is an important enzyme of the antioxidant system that catalyses the degradation of hydrogen peroxide (a strong reaction oxygen species) to water and molecular oxygen. It has been reported that catalase induces the transcription of inducible NOS (the isoform in the macrophage) which may lead to the production of nitric oxide by the macrophage[24]. This clearly shows that the cadmium-mediated induction of nitric oxide may be partly due to the activity of catalase.

The anthocyanin rich extract significantly reduced the cadmium-mediated effects in both the U937 cells and the macrophages when compared to quercetin dihydrate. Several reports linked the antioxidant effects of *H. sabdariffa* calyces to the presence of anthocyanins[10,25]. Flavonoids

have been shown to prevent apoptosis by triggering reactions that tend to block the formation of a permeability transition pore in mitochondria[26]. Flavonoids also inhibit the activity of protein kinase and also exert possible effects on phosphatases. This correlates positively with the inhibition of the proinflammatory cytokine release induced by cadmium. Flavonoids especially anthocyanins protect cells by the upregulation of phase II enzymes in addition to improving endothelial function by lowering oxidative stress and improving coronary function[11].

Anthocyanins are thought to be the major chemical constituents of *H. sabdariffa* calyces. Anthocyanins isolated from the calyces include delphinidin-3-O-glucoside, delphinidin-3-O-sambubioside, and cyanidin-3-Osambubioside which are suggested to be the most abundant chemical constituents in the extract[10]. Thus the observed bioactivities are attributed principally to the presence of these anthocyanins. The presence of *H. sabdariffa* plant is indeed a blessing of creation thus could be exploited for pharmacological and nutritional advantages. Further work to characterise various bioactive compounds in various parts of the plant is in progress.

Conflict of interest statement

The authors declare there is no conflict of interest

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