Anti-inflammatory activities of cucurbitacin E isolated from *Citrullus lanatus* var. *citroides*: Role of reactive nitrogen species and cyclooxygenase enzyme inhibition

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**ABSTRACT**

The *in vivo* and *in vitro* mechanistic anti-inflammatory actions of cucurbitacin E (CE) (*Citrullus lanatus* var. *citroides*) were examined. The results showed that LPS/INF-γ increased NO production in RAW264.7 macrophages, whereas L-NAME and CE curtailed it. CE did not reveal any cytotoxicity on RAW264.7 and WRL-68 cells. CE inhibited both COX enzymes with more selectivity toward COX-2. Intraperitoneal injection of CE significantly suppressed carrageenan-induced rat’s paw edema. ORAC and FRAP assays showed that CE is not a potent ROS scavenger. It could be concluded that CE is potentially useful in treating inflammation through the inhibition of COX and RNS but not ROS.

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**1. Introduction**

The attractive association between chronic inflammation and incidence of many diseases has been fertile land for the growth of ethnobiomedicinal research [1]. Traditionally, plants have been used to treat various human diseases including inflammation. Wild melon (*Citrullus lanatus* var. *citroides*; belongs to Cucurbitaceae family) is a low climbing, hairy and annual plant. In Northern Sudan, this plant is often used for rheumatism, swellings, gout and as laxative. Cucurbit plants were used actively as traditional herbal remedies for various diseases [2,3]. Cucurbit plants demonstrated anti-inflammatory, antitumor, hepatoprotective and immunoregulatory activities.

This family is also known to contain several bioactive compounds such as cucurbitacins, triterpenes, sterols and alkaloids. Plants containing cucurbitacins were early recognized in folk medicine to have biological values. Moreover, many plants used in folk medicine to treat inflammatory conditions have been found to contain cucurbitacins displaying potentially important anti-inflammatory actions, in different *in vivo* and *in vitro* assays [4]. In this regard, the root extract of *Wilbrandia ebracteata* (Cucurbitaceae) a plant commonly used in Brazil to treat rheumatic disease was reported to contain several cucurbitacins [5].

Epithelial cells express reactive nitrogen and oxygen species in response to inflammatory cytokines and the bacterial endotoxin, lipopolysaccharide (LPS). Depending on the cell type, various downstream signaling pathways are also involved in the transcriptional regulation of ROS and RNS.
This phenomenon of cellular inflammatory response implies also on other types of cells and is employed experimentally in inflammation models. Macrophages have been implicated in many of these experiments, as they are directly involved with the inflammatory response [6,7]. One of the important roles of macrophages is the production of various cytokines, reactive oxygen and nitrogen species, growth factors and chemokines as a response to activation signals such as chemical mediators, cytokines, and bacterial lipopolysaccharide (LPS) [8]. Although the bioactive molecules produced by macrophages have valuable outcomes in inflammation, these molecules were also shown to have unfavorable and damaging effects. Hence the modulation of these products provides a target for controlling inflammatory and malignant diseases [9]. Therefore, the current study was designed to investigate the mechanistic anti-inflammatory action of cucurbitacin E (Fig. 1) in vitro and in vivo. We have also tested the cytotoxicity of CE on human normal liver cells (WRL-68).

2. Materials and methods

2.1. Cell lines and reagents

All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, USA). Dulbecco’s modified Eagle’s medium (DMEM) both with and without phenol red, phosphate buffered saline and Hanks’ balanced salt solution (HBSS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), phosphate buffered saline and Griess reagent were from Sigma (St. Louis, USA). Interferon gamma (IFN-γ), dimethylsulfoxide (DMSO) and sodium nitrite (L-NAME), indomethacin, aspirin, indoleacetic acid, B-glucosylindole and phenol red were from Invitrogen (Carlsbad, USA). Fetal bovine serum (FBS) was from BD Biosciences (New Jersey, USA). All other chemicals and reagents used were of HPLC grade.

2.2. Plant

C. lanatus var. citroides was collected from AL-Musawarat Area, Northern Sudan, on February 2008. The voucher specimen (CL2-8) was identified by Dr. Wai’l S. Abdalla, a Senior Botanist at the Herbarium of Medicinal and Aromatic Plants Research Institute (MAPRI), Khartoum, Sudan.

2.2.1. Extraction and isolation of compounds

Three kilograms of the dried powdered fruit pulp was extracted using the method reported earlier [11] with some minor modifications. The dried powder was extracted sequentially using petroleum ether, chloroform and 90% ethanol. The ethanol extract (80 g) was re-dissolved in 200 ml of 90% ethanol and later 200 ml distilled water was added, to make an aqueous extract which was fractionated sequentially with ethyl acetate and butanol. The different extracts/fractions obtained were evaporated using Rotatory Evaporator and brought to complete dryness at room temperature. Chloroform extract and ethyl acetate fraction have shown the most active biological effects. Vacuum liquid chromatography was performed on crude extracts of chloroform (10 g) and ethyl acetate fractions (12 g), separately. Elution was carried out for the chloroform extract with petroleum ether:dichloromethane and dichloromethane:methanol mixtures of increasing polarity. Ten fractions were obtained by using TLC profile. Fraction three (0.54 g) was applied to Chromatotron to give cucurbitacin E (140 mg: 1.4%). The elution used to fractionate ethyl acetate fraction (12 g) was chloroform:methanol mixture of increasing polarity. Fraction five (5 g) was semi-pure and was subjected to column chromatography and crystallized to give cucurbitacin E (300 mg: 2.5%). Fraction six (3 g) was purified using column chromatography to give cucurbitacin L 2-O-β-glucoside (400 mg). The structures of the compound were established by spectroscopic method and by comparison with the previous reported works [10–12] and the purity (~98.8%) was checked using HPLC. All spectroscopic data are available as supplementary materials. The biological activities of cucurbitacin L 2-O-β-glucoside were not tested in this study.

2.2.2. Cell culture and stimulation

The murine monocytic macrophage cell line (RAW 264.7) was maintained in DMEM supplemented with 10% FBS, 4.5 g/l glucose, sodium pyruvate (1 mM), l-glutamine (2 mM), streptomycin (50 μg/ml) and penicillin (50 U/ml) at 37 °C and 5% CO2. Cells at confluency of 80–90% were centrifuged at 120 × g at 4 °C for 10 min and cell concentration was adjusted to (2 × 106) cells/ml, whereby the cell viability was always more than 90%, as determined by trypan blue exclusion. A total of 50 μl of cell suspension was seeded.
into a tissue culture grade 96-well plate (4×10^5 cells/well) and incubated for 2 h at 37 °C, 5% CO₂ for cell attachment. Then, the cells were stimulated by using 100 U/ml of IFN-γ and 5 μg/ml of LPS with or without the presence of CE tested at the final volume of 100 μl/well. DMSO was used as vehicle, where the final concentration of DMSO was maintained at 0.1% of all cultures. Cells were further incubated at 37 °C, 5% CO₂ for 20 h. The culture supernatant was subjected to Griess assay for nitrite determination and the cells remaining in the well were tested for cell viability assay by using MTT reagent.

2.3.2. Griess assay
To evaluate the inhibitory activity of CE on nitric oxide (NO) production, culture media were assayed using Griess reaction [13]. Briefly, an equal volume of Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride, dissolved in 2.5% H₃PO₄) was mixed with culture supernatant and color development was measured at 550 nm using a microplate reader (SpectraMax Plus, Molecular Devices Inc., Sunnyvale, CA, USA). The amount of nitrite in the culture supernatant was calculated from a standard curve (0–100 μM) of sodium nitrite freshly prepared in deionized water. Percentage of the NO inhibition was calculated by using nitrate level of IFN-γ/LPS-induced group as the control.

\[
\text{NO inhibitory (\%)} = \frac{[\text{NO}_2^-]_{\text{control}} - [\text{NO}_2^-]_{\text{sample}}}{[\text{NO}_2^-]_{\text{control}}} \times 100\%
\]

2.3.3. Cell viability of RAW 264.7 macrophage
The cytotoxicity of CE on RAW 264.7 cells was determined by assaying the reduction of MTT reagents to formazan salts [14]. After removing of supernatant, the MTT reagents (0.05 mg/ml dissolved in sterile PBS, pH 7.0) were added into each well. The remaining cells were incubated at 37 °C for 4 h and the formazan salts formed were dissolved by adding 100 μl of 100% DMSO in each well. The absorbance was then measured at 570 nm using SpectraMax Plus microplate reader (Molecular Devices, USA). The percentage of cell viability was calculated using the cell viability of IFN-γ/LPS-induced group as the control.

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\text{Cell viability (\%)} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100\%
\]

2.4. In vitro evaluation of COX-1 and COX-2 inhibitory activity
The inhibition of the enzymes COX-1 and COX-2 is considered as one of the mechanisms of anti-inflammatory actions; therefore, CE was tested for COX-1 and COX-2 inhibitory activity using a COX-inhibitor screening kit (Catalog No. 560101, Cayman Chemical, USA) according to the manufacturer’s instructions. The EIA kit to determine the COX-1 and COX-2 inhibitory activity has been used earlier [15]. COX is involved in the biosynthesis of prostaglandins, thromboxanes, and prostacyclins. COX catalyzes the conversion of arachidonic acid to PGH₂. This assay measures the production of PGF₂αx generated by SnCl₂, in the presence of PGH₂. The initial reactions take place in test tubes heated at 37 °C. In the background tubes reaction buffer and heme are mixed. In the 100% activity tubes reaction buffer, heme, the enzyme in question and solvent are added. In the sample tubes reaction buffer, heme, inhibitor at different concentrations and enzyme are added. The tubes are incubated for 15 min at 37 °C. Then arachidonic acid is added and the tubes are incubated for 2 min. HCl 1 M was used to stop the reaction and stannous chloride traps the reaction product and reduces it to a more stable form. The tubes are incubated a final time for 5 min at room temperature. The tubes are then diluted while the backgrounds left as they are. A 96 well plate coated with mouse anti-rabbit IgG is provided. In the well plate nonspecific binding, maximum binding, standards, and the inhibitor dilutions are added with tracer and antiserum. The plate is incubated at room temperature for 18 h, washed 5 times with wash buffer, developed with Ellman’s reagent and read on a microplate reader at 410 nm. The stock solution of the compound was dissolved in DMSO and the final concentration was 0–100 μM. The percentage of inhibition for the respective COX enzyme was graphically determined from three-point curves. Indomethacin was used as reference standard.

2.5. Paw edema induced by carrageenan

Male Balb/c mice (6 weeks of age) were obtained from Animal House, University of Malaya, Kuala Lumpur, Malaysia. The animals were first acclimatized for at least 2 weeks before the experiment and were only used once throughout the experiments. All the experiments were conducted in accordance with the ethical guidelines on animal experimentation approved by the Animal Care Unit Committee, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia [Ethics No. PM 07/05/2008 MAA (a)(R)]. Animals were fasted 12 h prior to experiment. Mice (N = 40) were randomly divided into four groups, and thus each group consisted of 10 animals. CE was intra-peritoneally administered to mice at the dose of 30 and 60 mg/kg. To induce acute phase inflammation in the paw, rats were injected subcutaneously into the right hind paw with a 1% solution of carrageenan dissolved in saline 30 min after vehicle or CE treatment. The paw volumes were measured up to 5 h after the injection at intervals of 1 h. The hind paw volume was determined volumetrically using a plethysmometer (Letica, Rochester, MI, USA).

2.6. Antioxidant capacity of cucurbitacin E

2.6.1. Oxygen radical absorbance capacity
The oxygen radical absorbance capacity (ORAC) assay was done to test the antioxidant capacity of CE based on the procedure described earlier with slight modifications [16]. Briefly 175 μl of the sample/blank was dissolved with PBS at concentrations of 160 μg/ml, pH 7.4, 75 mM and serial dilutions for the Trolox standards were prepared accordingly. ORAC assay was performed in a 96-well black microplate with 25 μl of samples/standard/positive control and 150 μl of fluorescent sodium salt solution, followed by 25 μl of 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) solution after 45 min incubation at 37 °C (200 μl total well volume). Fluorescence was recorded until it reached zero (excitation
wavelength at 485 nm, while emission wavelength at 535 nm) in a fluorescence spectrophotometer (Perkin-Elmer LS 55), equipped with an automatic thermostatic autocell-holder at 37 °C. The positive control was quercetin and the negative control was blank solvent/PBS. Data were collected every 2 min for a duration of 2 h. Results are calculated using the differences of areas under the fluorescein decay curve (AUC) between the blank and the sample and are expressed as Trolox equivalents.

2.6.2. Ferric reducing/antioxidant power assay

The determination of the total antioxidant activity (FRAP assay) is a modified method of Benzie and Strain [17]. The stock solutions included 300 mM acetate buffer (3.1 g C₂H₃NaO₂·3H₂O and 16 ml C₂H₄O₂), pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM FeCl₃·6H₂O solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml FeCl₃·6H₂O. The temperature of the solution was raised to 37 °C before use. Cucurbitacin E (10 μl) was allowed to react with 300 μl of the FRAP solution in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 100 and 1000 μM FeSO₄. Results are expressed in μM Fe (II)/g dry mass and compared with that of ascorbic acid and quercetin.

2.7. Effect of cucurbitacin E on the viability of human normal hepatic cells (WRL-68)

This colorimetric assay, is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan derivatives by mitochondrial succinate dehydrogenase in viable cells, and was used to determine any potential cytotoxicity. Human normal hepatic cells (WRL-68) were obtained from American Type Cell Collection (ATCC), maintained in a 37 °C incubator with 5% CO₂ saturation and maintained in Dulbecco’s modified Eagle’s medium (DMEM). Medium was supplemented with 10% fetus calf serum (FCS), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. For measurement of cell viability, cells were seeded at a density of 1 x 10⁵ cells/ml in a 96-well plate and incubated for 24 h at 37 °C and 5% CO₂. Cells were treated with CE and incubated for 24 h. After 24 h, MTT solution at 2 mg/ml was added for 1 h. Absorbance was measured at 570 nm. Results were expressed as a percentage of control giving percentage cell viability after 24 hour exposure to test agent. The potency of cell growth inhibition for CE was expressed as an IC₅₀ value, defined as the concentration that caused a 50% loss of cell growth. Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

2.8. Statistical analysis

The data obtained was statistically analyzed using one-way ANOVA. This was followed by Dunnett’s or Tukey’s post hoc tests when the ANOVA produced significant results. All data were expressed as the mean±SEM. The tests were performed using the GraphPad Software ver 5.01 (GraphPad Software Inc., San Diego, CA). Differences are considered significant when P<0.5.

3. Results

3.1. Effect of CE on NO₂⁻ production and RAW cells’ viability

The induction of RAW 264.7 cells into an inflammatory state by treatment with LPS/IFN-γ caused a significant increase in NO as shown in Fig. 2. The breakdown product of secreted NO namely NO₂⁻ was detected in media at a mean concentration of 38.40±4.11 μM. Cells that were not induced released trace amounts of NO. CE showed a dose-related inhibition of NO production in which significant inhibition was still evident at 0.78 μM. The IC₅₀ was calculated at 17.6±2.42 μM. L-NAME, a standard NOS inhibitor, was used as a positive control and caused a significant inhibition (84.54±5.77%) of NO at 250 μM. Fortunately, CE did not affect cell viability at 50 μM as assessed

![Figure 2](image_url)

*Fig. 2.* The effect of CE on NO production: murine macrophage cells were left untreated or pretreated with the indicated concentrations of CE. The cells were then either left in medium or were pretreated with LPS/IFN-gamma. Percentage NO inhibition was then compared between the untreated cells and the different concentrations of CE to cells treated with LPS/IFN-gamma (0 μg/ml) using ANOVA. The data is average of 3 independent experiments. *Significant at 0.05 and ** at 0.01 as compared to induced cells.

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by mitochondrial reduction of MTT following a 17–20-h treatment. Viability was always >82% (Fig. 3).

3.2. Effects of CE on cyclooxygenase-1 and 2

CE was evaluated for in vitro COX-1 and COX-2 inhibitory activity in a COX catalyzed prostaglandin biosynthesis assay. As shown in Fig. 4, CE was observed to inhibit both enzymes with more selectivity toward COX-2. Whereby, the IC_{50} values of CE on COX-1 and COX-2 are 90 and 69 \mu M, respectively. Indomethacin (100 \mu M) as a non-selective COX-1 and COX-2 inhibitor shows inhibition of 81.37±5.5 and 92±6.5, respectively.

3.3. Carrageenan-induced rat paw edema

The anti-inflammatory activity of CE was also measured at the doses of 30 and 60 mg/kg b.w against acute paw edema induced by carrageenan. At these doses, CE inhibits inflammation significantly (P<0.05) from the fourth hour (Fig. 5). These
results demonstrate that CE was able to regress paw edema through the inhibition of COX-2 but not the scavenging of ROS.

3.4. ORAC antioxidant activity assay

To evaluate the antioxidant capacity of CE and its role in the anti-inflammatory effect of this natural compound, ORAC assay was used and the potency of this natural compound was compared with the positive control; quercetin. The area under the curve (AUC) was calculated for the CE, Trolox and quercetin. CE at 20 $\mu$g/ml is equivalent to a concentration of 40.25±1.56 $\mu$M of Trolox. Quercetin at 5 $\mu$g/ml is equivalent to a concentration of 160.32±2.75 $\mu$M of Trolox.

3.5. FRAP assay

CE exhibited weak FRAP value, with a value of 38±2.61 $\mu$mol/l, while the positive control used in this study exhibited values of 350±9.5 and 251±5.7 for ascorbic acid and quercetin, respectively (Fig. 6).

3.6. Effect of CE on human normal hepatic cells (WRL-68)

The IC$_{50}$ of CE on WRL-68 was observed to be very high with the value of 51.91±5.22 $\mu$g/ml which indicates the safety of this natural compound. Paclitaxel, a positive cytotoxic control, has shown an IC$_{50}$ of 0.10±0.05 $\mu$g/ml.
4. Discussion

The cucurbitacins are of great interest because of the wide range of biological activities they exhibit in plants and animals [18]. In the present study, we investigated for the first time the in vitro and in vivo anti-inflammatory effects of cucurbitacin E (CE) and the involvement of reactive oxygen and nitrogen species and COX enzymes. The inhibitory effects of CE on RNS and COX enzymes but not on ROS are suggested to be the mechanism of the anti-inflammatory activities of this natural compound.

Nitric oxide (NO) is a free radical gas with important immune, cardiovascular and neurological second messenger functions that are implicated in sepsis, cancer and inflammation. This molecule is synthesized from the amino acid L-arginine by a family of enzymes, the nitric oxide synthases (NOS). NO in tissues is susceptible to manipulation by proinflammatory cytokines [19,20]. The obtained results suggest that CE has dose-dependent anti-inflammatory activities related with its inhibition of NO production in macrophages without affecting the viability of these cells. Our results are in line with previous findings which showed that cucurbitacin compounds are able to inhibit the production of NO [5]. Similarly, COX-2 is an inducible enzyme that catalyzes the production of prostaglandins, which contribute to the inflammatory process and tissue damage. It is reported that COX-2 can also be activated by high concentrations of nitric oxide, contributing toward more intense inflammatory responses as seen in many chronic inflammatory disorders. Several natural products of plant origin have been shown to transmit their anti-inflammatory activities through suppression of COX-2, however, for that suppression of nitric oxide production is critical [21]. In the current study, it was observed that CE was able to inhibit COX-2 when tested using EIA kit (Fig. 2).

The injection of carrageenan in mice produces a typical biphasic edema associated with the production of several inflammatory mediators, such as COX enzymes, prostaglandins, nitric oxide, and cytokines. The carrageenan test is highly sensitive to non-steroidal anti-inflammatory drugs, and has long been accepted as a useful phlogistic tool for investigating new drug therapies [22]. The degree of swelling of the carrageenan injected paws was maximal at the 3rd hour after injection. Statistical analysis revealed that CE (30 and 60 mg/kg) significantly inhibited the development of edema at the fourth hour after treatment (P<0.05; Fig. 5). It is well known that the third phase of the edema induced by carrageenan, in which the edema reaches its highest volume, is characterized by the presence of prostaglandins and other compounds of slow reaction [23,24].

The acute inflammatory response is associated with the production of reactive oxygen species (ROS) [25]. In a number of pathophysiological conditions associated with inflammation or oxidative stress, these ROS have been proposed to mediate cell damage via a number of independent mechanisms including the initiation of lipid peroxidation, the inactivation of a variety of antioxidant enzymes, and depletion of glutathione [26,27]. It was found that the antioxidant activities of CE were weak in both ORAC and FRAP assays, which probably stems from the fact that the mechanisms involved in these two assays are different. ORAC represents a hydrogen atom transfer reaction mechanism, while FRAP method is an electron transfer based assay. Given the importance of the oxidative status in the formation of edema [28], the anti-inflammatory effect exhibited by CE might not be related to its antioxidant properties [8,27].

In conclusion, the current study demonstrated that the cucurbitacin E (CE) isolated from C. lanatus var. citroides inhibits the production of NO in LPS/IFN-γ-stimulated macrophages. One of the possible mechanisms principally involved in the anti-inflammatory effects of this natural compound seems to be the inhibition of RNS and COX but not ROS. Fortunately, the compound did not affect normal human liver cells. Therefore, wild melon crude is a valuable source for potential anti-inflammatory compounds. Cucurbitacin E may have therapeutic potential and a possible effective treatment for a variety of inflammation-mediated diseases. Therapeutic potential of cucurbitacin E could be limited by the bitter taste of this compound but special pharmaceutical formulation is highly recommended to overcome this issue.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.fitote.2011.08.002.

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