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RESEARCH PAPER

Anti-inflammatory efficacy and skin irritation study of *Cleome gynandra* extract: an animal model and pre-clinical evaluation

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Abstract

Cleome gynandra, a kind of medicinal herb which has strong antioxidant properties has been traditionally used for treating various ailments including skin inflammatory diseases. To date, the anti-inflammatory efficacy of *C. gynandra* based cream has not been reported. The aim of the study was to evaluate the anti-inflammatory activities of *C. gynandra* leaf extract cream using an *in vivo* arachidonic acid-induced ear edema in the rat model. The expression of genes related to rat ear inflammation was measured to evaluate the topical anti-inflammatory activity of *C. gynandra* cream. The toxicity effect of *C. gynandra* cream was determined through a skin irritation test in rats and human cumulative skin irritation assessment. Data collected evidently showed a significantly higher (p<0.05) percentage of recovery rate (70.21±5.14%) in rats treated with *C. gynandra* cream when compared to the negative control (27.43±4.75%) and basal cream (55.8±4.61%), indicating that *C. gynandra* cream effectively reduced inflammation symptoms in rat ear edema model study. The expressions of all pro-inflammatory cytokines and acute inflammatory-related genes were significantly down-regulated in *C. gynandra* cream-treated group than untreated group (negative control). The dermatological safety aspect of *C. gynandra* cream was confirmed by human cumulative skin irritation assessment with the average cumulative irritation index of 0.046, indicating a non-irritating effect. The overall findings demonstrated that *C. gynandra* cream has great potential to be applied for treating skin-related inflammatory diseases.

1. Introduction

Inflammation is the pathophysiological reaction to harmful stimuli, such as pathogens, heavy metal compounds, or irradiation (Medzhitov, 2010). The five cardinal signs of inflammation include redness, swelling, heat, pain and loss of function (Ferrero-Miliani *et al*., 2007). Inflammation triggers the inflammatory signaling pathway to release active compounds such as prostaglandin, leukotriene, bradykinin, histamine, platelet-activating factor, and interleukin-1 (IL-1) (Chen *et al*., 2018). Inflammation can be easily occurred in the various parts of human body especially those vulnerable to external injuries such as skin, eye, ear, finger and mouth (Fuller, 2019).

Skin is made up of rough skin cells associated with protein called keratin and that makes skin tougher and waterproof. Skin is important in providing protection to body tissues, organs and structures from chemical irritants such as artificial detergents or natural irritants like poison. However, due to its function as a primary barrier of defence, skin is prone to injury or invasion of pathogens that will lead to inflammation. UV radiation and chemical irritants such as xylene, arachidonic acid, croton oil and 12-O-tetradecanoyl-13-phorbol acetate (TPA) are among the common stimuli that could cause inflammatory skin diseases (Lin *et al*., 2018). The

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incidence and prevalence of inflammatory skin diseases in the general population were estimated in between 15% to 25% (Chu *et al*., 2020). The occurrence of inflammatory skin diseases could be related to workplace factors such as alcohol-based disinfectants, detergents, and latex gloves.

Currently, numerous anti-inflammatory drugs especially non-steroidal anti-inflammatory drugs (NSAIDs) in a cream form are not suitable for their clinical use in inflammatory skin disease treatment due to adverse side effects such as allergic reaction, skin redness, itching, scaling, or peeling (Wang and Peng, 2020). Therefore, searching for safe, inexpensive and high-efficiency alternative medicine for skin disease treatment is necessary (Satyam *et al*., 2014). *Cleome gynandra*, or in Malay word known as 'Maman' is a kind of weed that has been commonly consumed by the local community in Malaysia as a traditional vegetable dish who believe *C. gynandra* leaf is enriched with vitamins A, C, and minerals such as calcium and iron (Chweya and Mnzava, 1997). Besides, the *C. gynandra* leaf is well known as a traditional remedy for curing ailments such as stomachache, epilepsy, constipation and other inflammation illnesses (Narendhirakannan *et al*., 2005). In addition, the phytochemicals contained in the leaf of *C. gynandra* such as rutin, terpenoids, flavonoids, carotenoids, tannins, and mineral contribute to its medicinal properties including antioxidant, anti-inflammatory, anti-diabetic, and anti-cancer (Chandradevan *et al*., 2020). Although there have been many studies evaluated the anti-inflammatory properties of *C. gynandra* leaf extract, however, none of those studies focused on the anti-inflammatory effect of *C. gynandra* in the form of topical cream. Hence, in this study, we aimed to investigate the effectiveness of *C. gynandra* cream in treating inflammation related to skin diseases.

2. Materials and methods

2.1 Chemicals and reagents

Arachidonic acid supplied by Sigma-Aldrich Chemical Co., Voren Plus gel (containing diclofenac sodium) manufactured by Y.S.P Industries (M) Sdn. Bhd. The other chemicals used were acetone and normal saline which were of analytical grade.

2.2 Cream preparation

The cream was prepared using virgin coconut oil as an oil base to give a moisturizing effect and to dissolve other oil ingredients such as vitamin E and fragrance. The aqueous phase consists of *C. gynandra* leaf extract (voucher specimen number MDI 12840), thickening agent, emulsifier and preservative agent. The oil phase was added slowly in a container containing mixture of aqueous solution to get coarse emulsion before subjected to high shear mixing in a homogenizer for 2 min to form cream. Two types of *C. gynandra* cream were prepared: Cream A (low dosage of C. *gynandra* leaf extract, 10 mg/g cream); b) Cream B (high dosage of *C. gynandra* leaf extract, 50 mg/g cream). The commercial anti-inflammatory cream, Voren Plus gel which contains diclofenac sodium (10 mg/g cream) was used as a positive control.

2.3 Arachidonic acid-induced ear edema in rat model

The study of arachidonic acid-induced ear edema in rats was performed as described by Mulla *et al*. (2010) with slight modifications. A total of 40 Male Sprague – Dawley rats (weighed approximately 20 – 270 g) were obtained from animal house at Universiti Kebangsaan Malaysia (UKM) and were acclimatized for 10 days under standard housing conditions (24±1°C and 55±10% relative humidity with 12:12 h light/dark cycle). The rats were habituated in laboratory conditions for 48 hrs prior to the experimental protocol to minimize any non specific stress. All protocol used in this study was approved by the Institutional Animal Ethics Committee of the Malaysian Agricultural Research and Development Institute (MARDI) (Ethic No: 20180810/ R/MAEC00031) and animal were maintained under standard conditions in the animal house.

The rats (n = 8) were divided into five groups i.e. normal control, negative control (induced by arachidonic acid (AA) only), positive control (Voren Plus gel), vehicle (basal cream), and treatment (*C. gynandra* cream A). The 5% of arachidonic acid was dissolved in acetone and 10 µL of the mixture was applied to both the anterior and posterior surfaces of the left ear of each rat. After 30 mins, 100 mg of each *C. gynandra* cream and commercial diclofenac sodium cream (Voren Plus gel) were applied topically on rat ears as treated and positive control group, respectively. The basal cream without *C. gynandra* leaf extract was applied as a vehicle group of rats by the same mode of application. The ear thicknesses of every rat group were measured using a micrometre screw gauge at specific treatment intervals (i.e. 15, 30, 45, and 90 mins) upon the application of inducer (5% of arachidonic acid). The differences of the ear thickness were then calculated to obtain the degree of ear edema, of which was expressed as the increment in the ear thickness in mm. The percentage of ear edema recovery (%) was subsequently calculated using the following formula:

$$Ear\ edema\ recovery\ (\%) = \frac{\text{net reduction degree of edema}}{\text{degree of edema}} \times 100$$

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2.4 Quantitative real-time polymerase chain reaction analysis

The rat ear tissue samples were collected from treated and control rats (three individual rat samples per group). Approximately 25 mg of each rat ear tissue sample was homogenized with FastPrep-24™ 5G Instrument (MP Biomedicals, USA) in the presence of FastPrep® lysing matrix (MP Biomedicals, USA) and 500 µL NucleoZOL lysis buffer (Macherey-Nagel, Germany). The total RNA was extracted according to NucleoZOL manufacturer instructions. The extracted total RNA was then purified using an RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. Complementary DNAs were synthesized using QuantiNova Reverse Transcriptase kit (Qiagen, Germany). Predesigned PrimeTime Assay Std Probes 5' FAM/ZEN/3' IBFQ were synthesized by Integrated DNA Technology, IDT, Singapore) as follows: Glyceraldehyde 3-phosphate dehydrogenase (GADPH) (Rn.PT.58.35727291), interleukin 1α (IL-1α) (Rn.PT.58.35304224), interleukin 1β (IL-1β) (Rn.PT.58.38028824), interleukin 6 (IL-6) (Rn.PT.58.13840513), TNF-α (Rn.PT.58.11142874), C-C motif chemokine ligand 2 (CCL2) (Rn.PT.58.17897665), C-X-C motif chemokine ligand 1 (CXCL1) (Rn.PT.58.9564322), nitric oxide synthase 2 (NOS2) (Rn.PT.58.9699876), nuclear factor kappa B subunit 1 (NFKB1) (5'-/56-FAM/T ACAGCGCC/ZEN/ ATCTCACCGCG/31ABkFQ/-3'). The qPCR assay was performed in a StepOnePlus real-time PCR system (Applied Biosystems, USA). The PCR cycling conditions used for sample analysis were as follows: 1 cycle of 95°C/3 mins for DNA polymerase activation, 40 cycles of 95°C/5 s for denaturation, and 60°C for 30 s for annealing and extension. The fluorescent dye ROX served as an internal reference for normalization of the FAM fluorescent signal. Gene expression was normalized to the expression of reference genes of GADPH and relative gene expression analysis was performed using the 2–[delta][delta]Ct method.

2.5 Skin irritation test

The skin irritation test was prepared according to the Dandagi *et al*. (2020) procedure with minor modifications. The test was carried out according to OECD guidelines for *in vitro* skin irritation test No. 439. A total of 20 male Sprague–Dawley rats were used to carry out skin irritation tests. The rats were randomly segregated into 4 groups: control and three treatment groups (basal cream, Cream A and Cream B). Each group comprised of 5 rats. The back skin area of 5 cm^2 of each rat was shaved one day prior to skin irritation study. After 24 h, the control group was applied with normal saline and treatment groups were applied with Cream A, Cream B, and basal cream, respectively. Rats were then examined for any signs of irritation. The irritation scores were given according to the severity of skin irritation based on a grading scale as 0 = no visible reaction, 1 = weakly positive reaction (usually characterized by mild erythema and/ or dryness across most of the treatment site), 2 = moderately positive reaction (usually distinct erythema or dryness, possibly spreading beyond of the treatment site), and 3 = strongly positive reaction (strong and often spreading erythema with edema and/or eschar formation). On day 28, the rats were sacrificed. Blood samples were collected *via* cardiac puncture for biochemical and haematological analyses.

2.6 Blood haematological analysis

Whole blood sample was obtained from each rat by using the cardiac puncture method (under anaesthesia) and placed on ice. The lavender cap blood tube containing anticoagulant, ethylene diamine tetra-acetic acid (EDTA) was used to keep blood samples. Hematological analysis was performed by using an automated haematology analyser (Beckman Coulter, Brea, CA). The examined parameters were erythrocytes (RBC), white blood cells (WBC), platelet count (PLT), haemoglobin (Hgb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).

2.7 Blood biochemical analysis

The blood sample for biochemical analysis was gently placed in a yellow cap serum-separating tube containing a gel separator to avoid hemolysis of blood cells. Blood serums were taken by centrifugation of the blood samples at 15 mins at 2200 rpm (International Organization for Standardization (ISO), 2010). Biochemical analysis was performed by using an automated biochemical analyser (Beckman Coulter, Brea, CA). Biochemical studies were carried out using standard methods for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein, albumin, globulin, total bilirubin, urea, and creatinine.

2.8 Human cumulative irritation test

The human cumulative irritation test was conducted by an authorized lab, Biocompatible and Clinical Laboratory Healthmedic Research under the Centre for Research and Instrumentation Management (CRIM) of Universiti Kebangsaan Malaysia (Ethic no: HMREC-HMR-08-2020). A total of thirty healthy subjects, free from skin disease, were recruited for the study. Age

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range of the study subjects was in between 22 to 55 years (43.23±10.15). The irritation scores were given according to the severity of skin irritation based on the classification mean of cumulative irritation index (MCII) (Dandagi *et al*., 2020). The mean of cumulative irritation index (MCII) was calculated based on four classifications which were non-irritating (<0.25), slightly irritating (0.25-1; non-inclusive), moderately irritating (1 -2; non-inclusive), and very irritating (2-3; non-inclusive).

2.9 Patch application

The *C. gynandra* cream (10 mg/g cream), corresponding control normal saline (negative control) and sodium dodecyl sulfate (SDS) in water (positive control) were prepared separately and applied using filter paper onto the skin. Patch application of *C. gynandra* cream was made repeatedly onto the skin of the upper area of the back (scapular area) under occlusive conditions. Patches were applied for 24 hrs (7 times per week) for three weeks. The sites were scored at baseline (day 1) and at each study visit, week 1 (day 3 - 7, inclusively), week 2 (day 8 - 14, inclusively), and week 3 (day 15 - 22, inclusively). When an irritation reaction related to the product was scored as Grade 3 for any site, the product application was discontinued for the incriminated site. When an irritation reaction was related to the application of an adhesive patch, the wearing of a patch particular site was prohibited, all patch applications were discontinued for the subject and the subject was withdrawn from the study. The test was evaluated for reaction after 48, 72, and 96 hrs post-application. Treatment sites were examined for any sign of irritation at baseline and at each study visit. The degree of severity cutaneous reaction was graded according to ISO 10993-10 (ISO, 2010).

2.10 Statistical analysis

All data are presented as mean ± standard error mean (SEM) of replicated samples and subjected to one-way analysis of variance using statistical software, IBM SPSS Statistic 22.0 (IMB Corp., USA). The comparisons were performed by Tukey post-hoc test with statistical significance set at the level of $P<0.05$.

3. Results

3.1 Arachidonic acid-induced ear edema in rat

Topical application of 5% arachidonic acid was performed to induce edema formation in the left rat ear at 0 min. The peak of edema formation was observed to occur after 30 mins post-induction, which marked the initiation of treatments. The thickness of the rat ear was measured at specific intervals (i.e. 15, 30, 60, and 90 mins) post-treatment with *C. gynandra* cream, Voren Plus cream and basal cream, respectively. Generally, it was observed that all cream treatment rat groups showed increased recovery percentages in a time-dependent manner (Figure 1). During the treatment period, the negative control group (AA only) showed the lowest percentage of recovery rate (<30%) which was consistently observed at each time point. The rat group treated with basal cream initially displayed no significant differences ($P>0.05$) in the percentage of recovery rate at the first 15 mins (7.73±2.17%) and 30 mins (24.85±5.67%) post-treatment. However, the percentage of recovery rate was found significantly higher ($P<0.05$) than negative control after post-treatment at 60 mins (35.9±5.64%) and 90 mins (55.8±4.61%). In contrast, treatment with *C. gynandra* cream showed an earlier recovery effect on rat ears with a significantly higher percentage of recovery rate at 15 mins (40.5±7.91%), 60 mins (63.89±6.74%) and 90 mins (70.21±5.14%) when compared to negative control ($P<0.05$). Although, the percentage of recovery rate under *C. gynandra* cream and Voren plus cream treatment at 30 mins (45.32±7.52%) was insignificant when compared to negative control, the subsequent percentage of recovery rates were found to significantly different at 60 and 90 min, indicating the recovery took place after 30 mins post-treatment. Interestingly, the recovery rate of *C. gynandra* treated group was comparable to the positive control (Voren plus cream) at every interval suggesting the cream was efficacious as the commercial cream. On the other hand, the negative control group (AA) only showed a minor consistent recovery of ear edema throughout the experimental duration (15-90 mins).

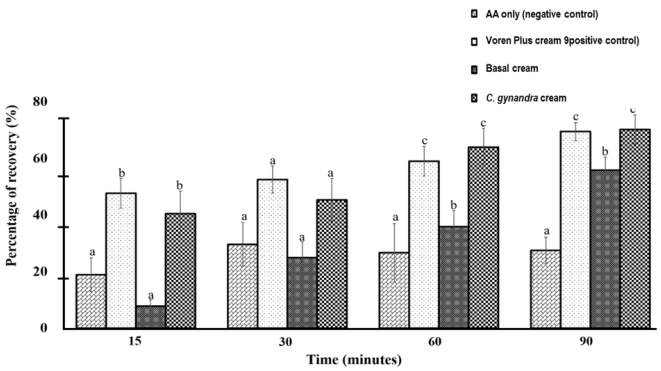

Figure 1. The percentage of recovery (%) of arachidonic acid-induced rat ear edema treated with the topical cream of *C. gynandra*, Voren Plus (positive control) and basal (vehicle). Data are indicated as mean of percentage of recovery ± SEM (n = 8). Bars with different notations are statistically significantly different analysed using one-way ANOVA, followed by Tukey post-hoc test, ($P< 0.05$).

3.2 Quantitative real-time polymerase chain reaction analysis

Six genes encoding for pro-inflammatory cytokines

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(IL-1α, IL-1β, IL-6, TNF-α, CCL2 and CXCL1) and two genes related to acute skin inflammation (NOS2 and NFKB1) were measured for their gene expressions in each of the control and *C. gynandra* cream A treatment group. It was found that the expressions of all pro-inflammatory cytokines were significantly down-regulated (P<0.01) in treated groups compared to the untreated group (negative control) in the range of 1.9-3.2 -fold (Figure 2A-F). The degrees of down-regulation in gene expression were comparable to the positive control group, particularly for pro-inflammatory cytokines of IL-1α, IL-1β, IL-6 and TNF-α. Significant reductions of acute inflammatory-related gene expression of NOS2 and NFKB1 by 7.4- and 1.8-fold were also shown in the treatment group compared to the negative control, respectively (Figure 2G-H). Additionally, the altered levels of gene expression for most of the pro-inflammatory and acute inflammatory-related genes were able to be restored to normal levels after treatment except for IL-6, CCL2 and CXCL1.

3.3 Skin irritation test

Skin irritation test was carried out by a 28-day course of topical application in order to determine the toxicity effect of *C. gynandra* cream on the skin. It was observed that the control group (saline) showed no sign of irritation such as erythema, dryness, red rash, or red bumps upon the completion of 28 days with a mean

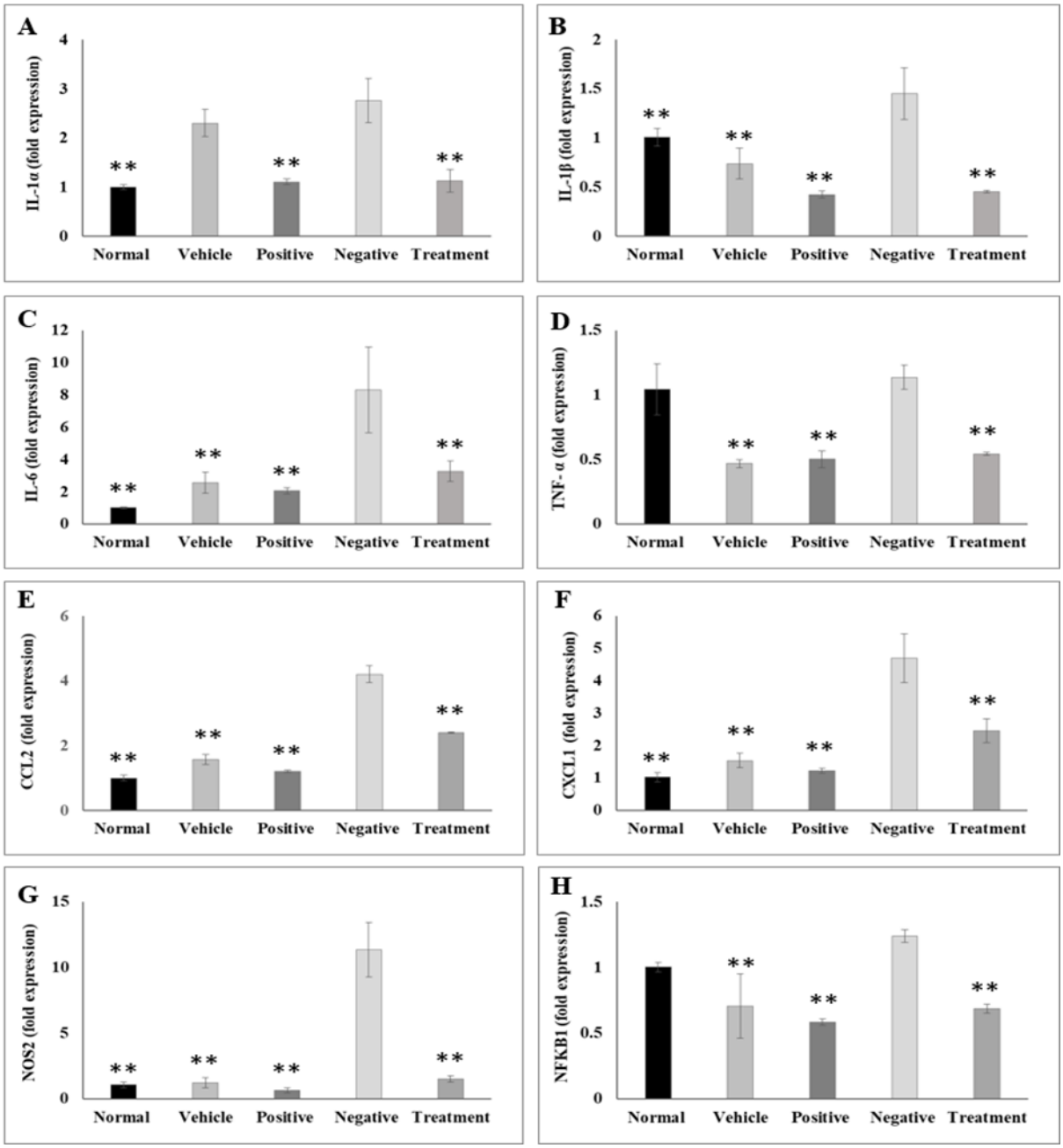

Figure 2. Expression of gene markers related to skin inflammation of treated rat with the topical cream of *C. gynandra.* Gene expression of IL-1α, IL-1β, IL-6, TNF-α, TGF-β1, CCL2, CXCL2, NOS2 and NFKβ1 (A-H) in controls (Vehicle, Positive and Negative controls) and treatment (1% *C. gynandra* extract cream) relative to Normal control. Data are indicated as mean of percentage of recovery ± SEM (n = 8). Bars with different notations are statistically significantly different analysed using one-way ANOVA, followed by Tukey post-hoc test, (P< 0.05).

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irritation score value of 0 observed (Figure 3). As expected, the positive control was highly irritant to the subjects with an observed mean irritation score value of 3. To our interest, the mean irritation score values for both treatments with *C. gynandra* creams either at low dosage (Cream A, 10 mg/g) or high dosage (Cream B, 50 mg/g) remained below 0.25 throughout the course, indicating these creams as well as their basal cream was non-irritating to the tested subjects.

3.2 Effects of treatment on blood haematological profile

The haematological parameter values for all treatments, including control, were within the standard reference ranges of Sprague Dawley rats (Table 1). It was revealed that the haematological parameters for RBC, WBC, Hgb, MCV and MCHC were not significantly different between treatment groups, except for slight differences for PLT, HCT, and MCH. The value of PLT for basal cream (902.33±2.85 10^9/L) was significantly lower than the control group (969.67±5.24, 10^9/L). In addition, treatment *C. gynandra* cream at high dosage (1114.67±7.42 10^9/L) showed a significantly higher PLT value than control (969.67±5.24 10^9/L) ($P<$ 0.05). On the other hand, treatment of *C. gynandra* cream at low dosage showed PLT (868.00±6.08 10^9/L) and MCH (17.67±0.09 pg), which were significantly lower than PLT (969.67±5.24 10^9/L) and MCH (18.27±0.07 pg) of the control group. In contrast, the values of parameter HCT of treatment *C. gynandra* cream at low dosage (52.23±1.47%) showed a significantly higher than control (45.20±0.96%).

3.3 Effects of treatment on blood biochemical profile

All blood biochemical parameter values for all treatments and control were within the normal range of blood serum of Sprague Dawley rats (Table 2). No significant differences (P>0.05) were found for AST, albumin, globulin, and creatinine between all treatment groups. However, slight differences were observed for ALT, ALP, total protein, total bilirubin, and urea between treatment groups. When compared to the control group, it can be seen from the data in Table 2, that the value of a parameter for ALT for all treatment groups including the *C. gynandra* creams at low dosage (84.33±3.18, U/L) and high dosage (83.67±2.96 U/L), as well as basal cream (84.00±2.31 U/L) were significantly lower than control (98.67±0.67 U/L). Besides, results showed that ALP values between treatment groups of high dosage *C. gynandra* cream (370.00±2.08 U/L) and basal cream (458.33±1.45 U/L) were significantly higher compared to control (369.33±1.33 U/L). In addition, *C. gynandra* cream at high dosage (4.8±0.20 mmol/L) and basal cream (6.2±0.03, mmol/L) showed higher urea levels compared to control (4.0±0.23 mmol/L). Meanwhile, only the basal cream group (73.57±0.26, g/L) showed a significantly higher value of total protein than the control (68.27±0.17, g/L). Lastly, total bilirubin for all treatment groups including *C. gynandra* cream at low dosage (15.54±0.47, µmol/L), high dosage (15.37±0.60, µmol/L), and basal cream (16.11±0.87, µmol/L) were significantly higher than control (12.47±0.27, µmol/L).

3.4 Human cumulative irritation test

A human cumulative irritation test was carried out to determine the toxicity effect or allergic reaction of *C. gynandra* cream in human for 21 days of application. It is always important to cross check and confirm the

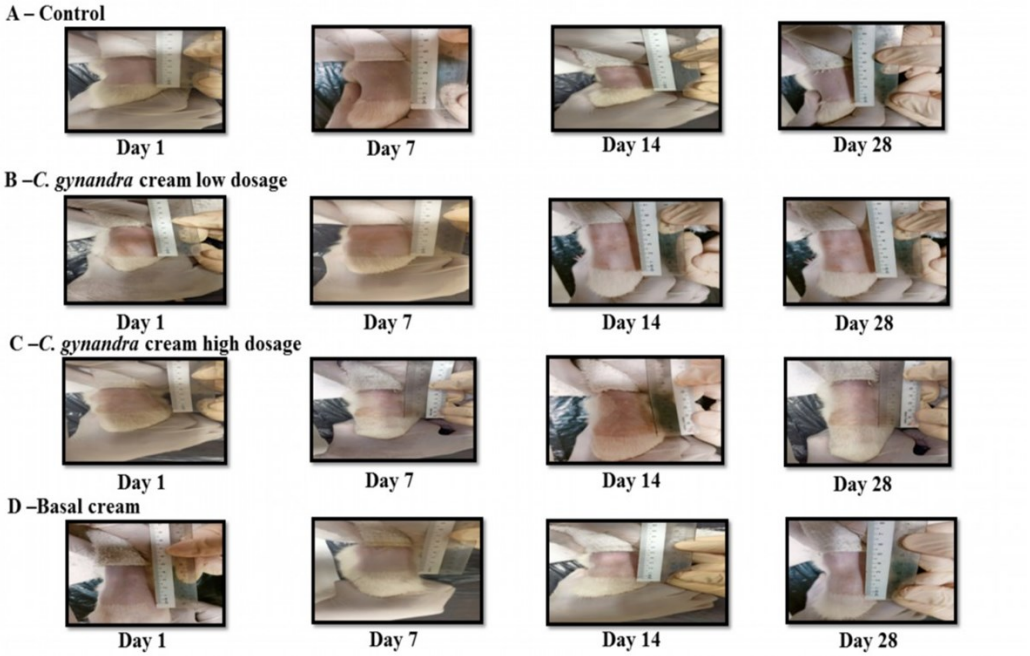

Figure 3. Skin irritation test of control and test group after treatment application with the topical cream of *C. gynandra.* Abbreviations: *C. gynandra* cream low dosage (10 mg/g cream) and *C. gynandra* cream high dosage (50 mg/g cream).

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Table 1. Blood hematological analysis of rats treated with the topical cream of *C. gynandra.*

Treatment	Parameter							
	RBC	WBC	PLT	Hgb	HCT	MCV	MCH	MCHC
	(10^{12}/L)	(10^9/L)	(10^9/L)	(g/dL)	(%)	(fl)	(pg)	(g/dL)
Reference Values	7.8 - 9.9	4.8 - 20.1	370.0-1418.0	13.9-17.3	38.9-55.2	46.5-60.0	16.3-20.2	29.4-38.3
Control	9.27±0.13^a	5.37±0.28^a	969.67±5.24^c	17.20±0.35^a	45.20±0.96^a	50.50±0.15^a	18.27±0.07^b	35.30±0.30^a
C. gynandra low dosage	9.61±0.42^a	5.27±0.68^a	868.00±6.08^a	17.10±0.70^a	52.23±1.47^b	49.33±0.69^a	17.67±0.09^a	35.37±0.23^a
C. gynandra high dosage	9.86±0.20^a	6.23±1.49^a	1114.67±7.42^d	17.10±0.38^a	51.80±1.57^{ab}	50.57±0.09^a	18.17±0.03^b	35.47±0.38^a
Basal cream	9.82±0.10^a	6.20±0.56^a	902.33±2.85^b	17.03±0.09^a	49.90±1.95^{ab}	50.70±0.12^a	18.20±0.15^b	36.37±0.07^a

Values are presented in mean±SEM of triplicate measurement for three whole blood samples of rat. Value with different superscripts within the same column are statistically significantly different analysed with one-way ANOVA followed by Tukey post-hoc test (P<0.05).

Table 2. Blood biochemical analysis of rats treated with the topical cream of *C. gynandra.*

Treatment	Parameter								
	ALT	AST	ALP	Total Protein	Albumin	Globulin	Total Bilirubin	Urea	Creatinine
	(U/L)	(U/L)	(U/L)	(g/L)	(g/L)	(g/L)	(umol/L)	(mmol/L)	(umol/L)
Reference Values	1.0-223.3	0.2-838.3	160.0-838.3	60.0-83.0	37.0-53.0	2.0-3.5	5.1-19.0	2.82-8.2	0.2-1.2
Control	98.67±0.67^b	85.33±1.33^a	369.33±1.33^a	68.27±0.17^a	38.98±0.12^a	3.50±0.21^a	12.47±0.27^a	4.0±0.23^a	1.0±0.06^a
C. gynandra low dosage	84.33±3.18^a	80.67±2.73^a	482.67±3.71^a	70.27±1.08^a	37.35±0.85^a	2.88±0.06^a	15.54±0.47^b	5.6±0.35^{ab}	0.9±0.06^a
C. gynandra high dosage	83.67±2.96^a	81.00±1.15^a	370.00±2.08^c	68.87±1.62^{ab}	37.57±0.16^a	3.21±0.14^a	15.37±0.60^b	4.8±0.20^{bc}	0.7±0.09^a
Basal cream	84.00±2.31^a	85.00±1.53^a	458.33±1.45^b	73.57±0.26^b	38.02±1.02^a	2.76±0.30^a	16.11±0.87^b	6.2±0.03^c	0.7±0.16^a

Values are presented in mean±SEM of triplicate measurement for three whole blood samples of rat. Value with different superscripts within the same column are statistically significantly different analysed with one-way ANOVA followed by Tukey post-hoc test (P<0.05).

dermatological safety aspect of *C. gynandra* cream *via* human clinical trial following animal study. The results on human cumulative irritation test are depicted in Table 3 and Figure 4. Treatment with SDS in water (positive control) showed a mean cumulative irritancy index (MCII) score of 2.349, which was close to a mean irritation score value of 3.0 of which the value represents a highly irritating agent. In contrary, *C. gynandra* cream treatment was classified as non-irritating with a MCII score value of 0.046, which was far below than that of mean irritation score baseline (0.25). Treatment using *C. gynandra* cream gave no irritation symptoms throughout the experiment similar to what was observed in the negative control group where normal saline was used.

Table 3. Cumulative score (CS) and mean cumulative irritancy index (MCII) treated with the topical cream of *C. gynandra.*

	Material	CS	MCII	Classification
A	*C. gynandra* cream	29	0.046	Non-irritating
B	Negative control (Normal saline)	16	0.025	Non-irritating
C	Positive control (SDS in water)	1480	2.349	Very irritating

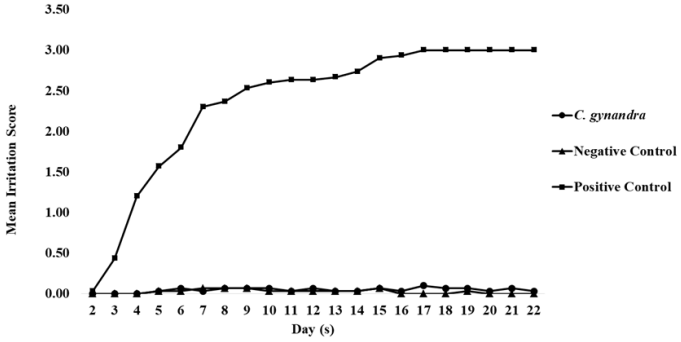

Figure 4. Human cumulative irritation test of *C. gynandra* cream. Data are shown as daily mean irritation score of subject's skin ± SEM (n = 30) treated with the *C. gynandra* cream, negative control (Normal Saline) and positive control (SDS in water). The dotted line marked on the baseline is the mean irritation score at 0.25, where values below 0.25 are classified as non-irritating.

4. Discussion

4.1 Arachidonic acid-induced ear edema in rat

In this study, arachidonic acid was used to induce edema on the left rat ear where the edema formation started after 30 min post-induction. Since then,

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treatments on edema ear rats were conducted using respective cream i.e. *C. gynandra* cream, Voren Plus cream and basal cream, and the recovery on edema was evaluated based on the ear thickness measured every 15 min until 90 mins post-treatment. As expected, the negative control group (AA only) exhibited the lowest percentage of recovery rate (<30%) with a minor consistent recovery of ear edema throughout the experimental period (15-90 mins). This slow and auto-recovery phenomenon is likely to be due to the body's innate immune response against invaders that cause inflammation in humans or animal (Chowdhury *et al*., 2020). For instance, the rat body immune response in rat helps in maintaining homeostasis and activating the healing process which contributes to a slow gradual recovery in the untreated rat negative control.

One unanticipated finding was that basal cream (vehicle) showed significantly higher in the percentage of recovery compared to negative control after 60 mins onwards of treatment, most probably because of the presence of virgin coconut oil in the preparation of basal cream. It has been well known that virgin coconut oil gives a moisturizing effect and retains hydration to human skin (Noor, 2013). Besides, virgin coconut oil was found to have anti-inflammatory activities, promoting the reduction of ear edema significantly higher than the negative control (Amin *et al*., 2020). These results are in agreement with Danby *et al*. (2022) who also found that the vehicle for topical cream application was more than just a placebo. However, in this study, at 60 and 90 mins after treatment application, basal cream (vehicles) showed a significantly lower recovery rate when compared with *C. gynandra* cream and Voren Plus cream (positive control). This indicated that a lower efficacy of basal cream (vehicle) in treating rat ear edema compared to both *C. gynandra* cream and Voren Plus cream (positive control). Another interesting finding was a continual increment in the recovery rate of *C. gynandra* cream-treated rat group which was comparable to Voren Plus (positive control) cream-treated group that contained diclofenac sodium drug (10 mg/g cream) (Figure 1). These results corroborate the findings of many previous studies that *C. gynandra* extract possesses anti-inflammatory properties, with its potency as good as the commercially available drugs such as diclofenac sodium (Adhikari and Paul, 2018). The anti-inflammatory activity of the *C. gynandra* cream was suggested as the synergistic effect of active constituents, i.e., rutin, glutamic acid, valine, phenylalanine, succinic acid, glutaric acid, lactic acid, and cytosine, present in the *C. gynandra* plant extract (Chandradevan, Simoh, Mediani, Ismail and Abas, 2020). While other reports have shown that this herb contains rutin, a potent anti-inflammatory bioactive compound (Batiha *et al*., 2020) that could be the key contributor to reducing inflammation of ear edema in this study.

4.2 Quantitative real-time polymerase chain reaction analysis

In this study, a fluorescent probe-based qPCR approach for measuring the expression of six genes encoding for pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, TNF-α, CCL2 and CXCL1) and two genes related to acute skin inflammation (NOS2 and NFKB1) was used to evaluate the anti-inflammatory activity of *C. gynandra* cream. At the onset of acute skin inflammation induced by an irritating agent such as arachidonic acid, it was expected that there was induced expression of IL-1α in keratinocytes (Lisby and Baadsgaard, 2006). Upon the activation of IL-1α, further downstream stimulations of proinflammatory cytokines and chemokines such as IL-1β, TNF-α, IL-6, and CXCL8 (IL-8) by surrounding epidermal and dermal cells were triggered (Zepter *et al*., 1997). A homolog chemokine to human interleukin-8 (IL -8; CXCL8) which is known as chemokine (C-X-C motif) ligand 1 (CXCL1) or cytokine-induced neutrophil chemoattractant type-1 (CINC-1) in rats was used in this study (Zagorski and DeLarco, 1993).

On the other hand, the upregulation of expression TNF-α in the skin has been previously reported following the application of irritants such as dimethyl sulfoxide (DMSO), phorbol myristate acetate (PMA), formaldehyde and tributyltin (Corsini *et al*., 1996). In this current study, the gene expression of NO2 and NFKβ1 were also tested, as these two genes were reported to be highly associated with acute skin inflammation. For instance, NO2 has been considered a key factor in regulating the apoptosis process of inflammatory cells, while NFKβ1 is activated by responding to TNF-α and IL-1 signallings (Atmanto, 2019). The treatment of *C. gynandra* cream successfully repressed the upregulation of inflammatory cytokines as observed in the untreated negative group, particularly for IL-1α, IL-1β, IL-6 and TNF-α, achieving at least a 2-fold reduction in the respective gene expression level, comparable to results obtained from Voren Plus cream treatment. While acute skin inflammatory genes, the expression levels of NO_2 were markedly reduced after treated with both *C. gynandra* and Voren Plus creams (more than 7-fold), in comparison to a subtle reduction for NFKβ1. Although the vehicle group (base cream containing VCO only) was able to reduce gene expression levels for most of the pro-inflammatory and acute inflammatory-related genes, the degrees of reduction were somehow not as high as *C. gynandra* cream or Voren Plus cream (positive control) treatment,

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particularly for IL-1α and IL-1β. These two genes are known to be the key pro-inflammatory cytokines responding to skin irritants. Clearly, the overall gene expression data results corresponded well with the percentage of recovery of rat ear edema, where both *C. gynandra* and Voren Plus creams could significantly reduce rat ear edema induced by AA, as supported by the significant repressions in the expression levels of inflammatory cytokines and acute inflammatory-related genes.

4.3 Skin irritation test

The non-toxic effect of *C. gynandra* cream on the skin is an important criteria parameter as it has been a part of basis in public health and regulatory decisions that concern the safety of herbal medicine uses (Arome and Chinedu, 2013). Therefore, the present study conducted a skin irritation test using rat model for a duration time of 28 days of application to evaluate the toxicity effect of *C. gynandra* creams. All rats treated with *C. gynandra* cream at low dosage (Cream A, 10 mg/g), high dosage (Cream B, 50 mg/g) and even for the basal cream showed no sign of irritation at all upon the completion of the treatment course. The results indicated that *C. gynandra* cream either at a low dosage (Cream A) or high dosage (Cream B) is free from toxic substances that could irritate rats' skin. This finding is in agreement with the outcome of toxicity studies on *C. gynandra* by other researchers (Ahouansinkpo *et al*., 2016). Based on a toxicity study conducted by Ahouansinkpo *et al*. (2016), the value of the lethal dose of *C. gynandra* leaves extract was LC_{50}=3,125 mg/mL, thus suggesting that both high and low dosages of *C. gynandra* creams used in this study were safe and non-toxic. Similar toxicity work done by Karimulla and Ravindhranath (2013) reported no sign of toxicity when a dose of *C. gynandra* treatment of 2000 mg/kg was used on rats. Besides, our finding was also supported by a recent study conducted by Chandradevan *et al*. (2020) which reported that 100% ethanolic extract of *C. gynandra* at a dosage of 1 mg/mL achieved as high as 90% cell viability of murine macrophage cell, RAW 264.7 cell *via* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium (MTT) assay, indicating no toxicity effect on the cells. The presence of virgin coconut oil as an oil base in cream formulation could provide moisturizing effect to reduce irritation symptoms. Moreover, virgin coconut oil neither causes toxicity nor inflammation to the skin (Alam *et al*., 2020). Other ingredients in the basal cream such as vitamin E, fragrance, thickening agent, emulsifier, and preservatives agent also did not give any irritation effect on the skin as confirmed in a basal cream skin irritation study (Sango *et al*., 2016).

4.4 Effects of treatment on blood haematological profile

Due to many chemicals that can cross the skin barrier and be absorbed into the bloodstream, screening on the blood hematological profile is helpful in determining any cellular toxicity effect of a particular drug or chemical substances contained in any plant extract or cream made from that extract. In this study, topical application of *C. gynandra* cream at low and high dosages as well as the basal cream for 28 days produced no significant change in all blood parameters except for PLT, HCT and MCH. Changes of those specific blood parameters were not harmful and importantly as their values were in within a normal range of blood for male Sprague Dawley rat (Han *et al*., 2010). For example, although the PLT value for treatment *C. gynandra* cream at high dosage (1114.67±7.42 10^9/L) was found to be significantly higher than low dosage treatment and control (Table 1), all the values observed for treatments and control were well within the references range of 370.00-1418 10^9/L for laboratory rats, indicating no compromise of PLT value (Han *et al*., 2010).

4.5 Effects of treatment on blood biochemical profile

Screening on the serum biochemical profile is important in xenobiotics' toxicological assessment (Bariweni *et al*., 2018). The condition of the liver can be analyzed and predicted by using a serum liver function test. For example, the level of liver enzymes i.e. ALT, AST, and ALP indicate its cellular integrity, while albumin, globulin, total protein, and total bilirubin can explain its functionality (Adeoye and Oyedapo, 2004). For instance, in respond to an injury, liver cell produces higher levels of AST and ALT enzymes to the blood serum which is an indicator for hepatocellular toxicity (Brautbar and Williams, 2002; Adedapo *et al*., 2004).

Our findings showed there was a significant lower ALT for all treatment cream groups when compared to control group. However, values for both all treatment and control groups were well within the reference range of 1.0 – 223.3 U/L for laboratory rats (Han *et al*., 2020). Therefore, treatment creams may be inferred as nontoxic with no harmful effect to livers of all treatment group of male Sprague Dawley rat. A decrease in serum concentration of globulin, albumin, total protein, and total bilirubin represents lowered synthetic function, then apparent in liver damage or disease. A rise in these parameters is observed generally in malignant or increasing protein intake levels (Levine *et al*., 2014). This study showed a significantly increased total protein value for the treatment of basal cream and a significantly increased total bilirubin value for all treatment creams compared to the control. However, values for both all treatment and control groups were well within the

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reference ranges of total protein (60 – 83 g/L) and total bilirubin (5.1 – 19.0 µmol/L) for laboratory rats (Han *et al*., 2010). The kidney functions could be indicated by determining the levels of blood urea and creatinine. The urea concentration of all treatments and controls in this study were well within the normal range (Han *et al*., 2010). A high urea concentration shows that the kidney might not be running efficiently, or the experimental animal is in thirsty conditions. In contrast, low urea concentration can be observed in common diseases of acute liver failure or overhydration (Slack *et al*., 2010). One of the indicators of glomerular filtration rate is by measuring the creatinine clearance. The treatment cream did not trigger any significant change in creatinine levels compared to the control, indicating that the treatment cream may neither be poisonous nor toxic to the kidney.

4.6 Human cumulative irritation test

A human cumulative irritation test was important to confirm the dermatological safety aspect of *C. gynandra* cream. SDS was used as positive control due to its high similarity to allergens and chemicals that commonly irritate humans (Austoria *et al.,* 2010). The irritating intensity caused by SDS can be classified as a 'very-irritating' level and therefore it was included as a positive control for the human cumulative irritation test in this study. The study also used SDS in water as their positive control for a high mean irritation score given (Basketter *et al*., 2004).

The human irritation test results of *C. gynandra* cream confirmed that this cream was dermatological safe with no skin irritation occurred throughout the 21-day observation. This finding has once again validated the results obtained from a skin irritation study using a rat model. The need to conduct a clinical study on human skin is essential as human and primate skin due to differences in terms of absorption and permeability to test chemicals between human and rodent skin (Chandra *et al*., 2015). Besides, there might be differences in the aspect of the immune response, allergic reaction, or other complications between animal and human skin reactions exposed to the same types of irritants (Akhtar, 2015). For instance, previous studies reported that results of Draize test eye on rabbit is not always predictive of human response as rabbit is much more easily get irritant by chemical compared to human skin (Calvin, 1992).

5. Conclusion

From the current study, based on the rat ear edema recovery assessment and qPCR results, it was concluded that *C. gynandra* in a form of topical cream was effectively treating skin inflammation with its efficacy comparable to the commercial topical anti-inflammatory drug containing diclofenac sodium (Voren plus gel). Both skin irritation and human cumulative irritation tests were evident that *C. gynandra* cream is safe in regards to its dermatological aspect. Further study should be carried out to investigate the underlying molecular mechanisms related to the anti-inflammatory properties of *C. gynandra* in order to find out more medicinal benefits of this natural remedy.

Conflict of interest

The authors declare no conflict of interest.

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