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ORIGINAL ARTICLE / ÖZGÜN MAKALE

IMMUNOMODULATORY POTENTIAL OF *CLAUSENA EXCAVATA* LEAVES FRACTIONS VIA DECREASING THE PRODUCTION OF REACTIVE OXYGEN SPECIES FROM IMMUNE CELLS

CLAUSENA EXCAVATA YAPRAK FRAKSİYONLARININ BAĞIŞIKLIK HÜCRELERİNDE REAKTİF OKSİJEN TÜRLERİNİN ÜRETİMİNİ AZALTARAK GÖSTERDİĞİ IMMÜNOMODÜLATÖR ETKİNLİK

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ABSTRACT

Objective: *Clausena excavata is known to possess anti-oxidant property. However, this property through which mechanism it affects the immune cells and suppresses the production of reactive oxygen species (ROS) has not been explored.*

Material and Method: *This study evaluated the immunomodulatory activities of ethyl acetate, petroleum ether, chloroform, and methanol C. excavata leaf extracts by decreasing the production of ROS from whole blood, polymorphonuclears (PMNs) cells and macrophages.*

Result and Discussion: *Among the fractions tested, ethyl acetate C. excavate extract (EACE) showed potent anti-oxidant property and significantly ($p < 0.001$) suppressed intracellular and extracellular phagocytic oxidative ROS burst produced by the zymosan and PMA-activated whole blood, PMNs, and macrophages cells with 50% inhibitory concentration (IC_{50}) values of 5.7 ± 0.01, 1.3 ± 0.01, and 0.7 ± 0.03 µg/mL respectively. This study provides information regarding the mechanism behind its anti-oxidant property and its herbal use in treating various higher oxidative stress associated diseases.*

Keywords: *Clausena excavata, inflammation, macrophage, ROS*

ÖZ

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Amaç: *Clausena excavate'ın anti-enflamatuvar etkinlik gösterdiği bilinmektedir. Bununla birlikte, bu özelliği, bağışıklık hücrelerine hangi mekanizma ile etki ettiği ve reaktif oksijen türlerinin (ROS) üretimini baskıladığı araştırılmamıştır.*

Gereç ve Yöntem: *Bu çalışmada, C. excavate yapraklarının etil asetat, petrol eteri, kloroform ve methanol ekstrelerinin tam kan, polimorfonkleer (PMN) hücreler ve makrofajlardan ROS üretimini azaltarak gösterdikleri immunomodülatör etki değerlendirilmiştir.*

Sonuç ve Tartışma: *Test edilen fraksiyonlar arasından, C. excavate etil asetat ekstrresi en güçlü antienflamatuvar etkiyi göstermiş ve zimosan ve PMA tarafından active edilmiş tam kan, PMN ve makrofaj hücrelerinde, intraselüler ve ekstraselüler fagositik oksidatif ROS üretimini anlamlı oranda ($p < 0.001$) baskılamış ve %50 inhibisyon konsantrasyonları (IC_{50}) sırasıyla 5.7 ± 0.01, 1.3 ± 0.01, and 0.7 ± 0.03 µg/mL olarak tespit edilmiştir. Bu çalışma, bitkinin antienflamatuvar aktivitesinin mekanizması ve çeşitli enflamatuvar rahatsızlıkların tedavisinde kullanımına dair bilgi sağlamaktadır.*

Anahtar Kelimeler: *Clausena excavata, enflamasyon, makrofaj, ROS*

INTRODUCTION

Prolonged inflammation plays detrimental role in the progression of various chronic diseases such as gastritis, atherosclerosis, cancer, diabetes, and other various diseases. Phagocytes, known for its role in first line of immune defense system provide protection in the site of inflammation by releasing sudden burst of reactive oxygen species so that it could counteract with various types of invading agents [1,2]. During higher oxidative stress, the antioxidant system of host cells and tissues could not counteract over produced ROS due to which excess ROS react with DNA, protein consequently leading to the progression of chronic inflammatory related diseases. In natural products, there are many compounds which possess antioxidant property therefore these can prevent oxidative stress and prevent damage to cells caused by foreign xenobiotics and thereby balancing oxidative stress level and prevent progression of inflammatory diseases [3-6].

Clausena excavata Burm .f. can be found in tropical and subtropical Asian regions [7]. In many countries, still the leaves of *C. excavata* are being practiced in traditional medicine to treat wound, abdominal pain, headache, diarrhea, and snake-bite [8]. Some of the major bioactive compounds like alkaloids, flavonoid, carbazole, glycosides, coumarins etc are reported to be present in the leaves of *C. excavata* [9]. Even though, many biological activities of *C. excavata* like anticancer, antiinflammatory, antioxidant, and antiulcer properties has been reported but till now [10], the immunomodulatory ability of this plant to decrease oxidative stress has not been reported yet. In pursuance to this unreported activity, we investigated the ROS inhibiting ability of *C. excavata* by using chemiluminescence assay and further gave more evidence regarding its potent antioxidant property.

MATERIAL AND METHOD

Extraction of *C. excavata*

C. excavata plant was collected and submitted to Biodiversity Unit, Institute of BioScience, Universiti Putra Malaysia. It was thoroughly evaluated by Dr. Shamsul Khamis, botanist and after authentication, a specimen voucher no: TI-013201-CE was issued. At room temperature, fresh leaves were dried, powdered, and extracted according to the procedure described previously [11]. Briefly, the extraction was done using 5:1 petroleum ether: dried plant (weight to volume) suspension for 4 days. Thus, the filtrate collected was subjected to further extraction with chloroform, ethyl acetate, and methanol. Filtrates of all extracts were dried by evaporating in a rotary evaporator under reduced pressure to obtain the crude extract.

Immunomodulatory studies

Estimation of intracellular ROS production

Fresh human blood approximately 2-3 mL was withdrawn from a healthy person with consent which was approved from independent ethics committee with protocol no (IEC-047-HB-2019 /PROTOCOL/1.0), University of Karachi. Ficoll-hypaque density gradient centrifugation method was used to isolate polymorphonuclear cells (PMNs). Equal volume (2 mL) of blood, lymphocytes separation medium, and Hank's Balance Salt solution (HBSS--) were added and mixed. RBCs were lysed using hypotonic solution for 1 min incubation so that lymphocytes purified will be freed from contaminating RBCs. Lysis process was stopped by adding HBSS solution. Thus, obtained PMNs were resuspended in (HBSS++) solution and adjusted to $0.5\text{-}1\times10^6$ cells/ mL [12].

Estimation of extracellular ROS production

One Balb/c mice (25 g) was taken from animal house facility of ICCBS, with approved animal study protocol from animal house facility of ICCBS, University of Karachi. It was important to immunize mice therefore 1 mL of fetal bovine serum (FBS) was injected intraperitoneally. After 3 days, animal was sacrificed by cervical dislocation. Whole animal was dipped in 70% ethanol to sterilize whole body. After sterilization process, 5 mL of 10% complete RPMI medium was injected into the peritoneal cavity, massaged for 2 min and peritoneal cavity was exposed by cutting abdominal skin from lower side. Previously injected RPMI containing peritoneum exudate cells having macrophage was collected and centrifuged at 500 rpm for 5 min at 4°C. Cell pellet was formed at bottom, discarded supernatant and again cells were resuspended in incomplete RPMI medium containing HBSS++ solution. Equal volume (10 µL) of macrophages and trypan blue was mixed and counted cells using hemocytometer, and cells concentrations adjusted to $2x10^6$ cells/mL [13].

Chemiluminescence assay

The protocol of Mesaik et al., 2012 was performed for luminol enhanced chemiluminescence assay. 1 mL of whole blood was diluted 20 times in sterile HBSS++. Briefly, 25 µL of this diluted blood

suspension, 25 µL of PMNs ($1x10^6$ cells/mL), 25 µL of mice macrophage ($2x10^6$ cells/mL) in different white 96 well plate, were mixed with 25 µL of different concentration of plant extract (1, 10, and 100 µg/mL) in triplicate and incubated at 37ºC. Ibuprofen was used as a drug control. After 15 min incubation, 25 µL of 0.3% serum opsonized zymosan and 25 µL of luminol dye ($7x10^{-5}$ M) were added to those wells containing PMNs and whole human blood. Whereas 25 µL of PMA dye and 25 µL lucigenin dye were added to those wells containing macrophages. The final volume in each well became 100 µL. Plate was inserted inside the luminometer and chemiluminescence was monitored as relative light units (RLU). The level of the ROS was recorded and inhibition of ROS production (%) was calculated using following formula and IC_{50} values were determined. Experiment was carried out in six wells per concentration and was done in triplicate in three different days.

$$\% \text{ inhibition of ROS production} = 100 - \frac{\text{Average reading of test plant extracts}}{\text{Average reading of positive control}} \text{X } 100.$$

Statistical analysis

One way (ANOVA) with Tukey's post-hoc test ($p<0.05$) was applied to compare the data (mean ± SD) of treated samples with untreated control. All calculations were calculated by using GraphPad prism 6.0 statistical software.

RESULT AND DISCUSSION

Since many centuries, different medicinal plants present in different geographical locations had been used locally as medicinal agent to treat various diseases, therefore, medical practitioners, chemical and biological scientists have been focusing in the area of ethnopharmacology to further explore undiscovered benefits of those plants, especially natural drug with potent immunomodulation activities by inhibiting the production of ROS from immune cells in inflammatory processes [14-24]. Previous studies reported that *C. excavata* can modulate the immune cell through different mechanism [7, 10]. In pursuance of those studies, this current study intends to evaluate the immunomodulation effects of *C. excavata* that could be potential treatment for chronic inflammatory diseases. Activator agents such as PMA/zymosan are always used for preliminary screening of the immunomodulatory efficiency of a product. Luminol and lucigenin chemiluminescence dye are used in measuring extracellular and intracellular ROS, respectively [12-14].

Methanol, chloroform, and ethyl acetate extracts exhibited a significant oxidative burst inhibition in whole blood, PMNs and macrophages. Table 1 shows the potent inhibition of whole blood and PMNs cells generated ROS. Among the tested extracts on whole blood for inhibiting ROS production, the ethyl acetate extract showed potent activity ($p < 0.01$,**) with the lowest IC_{50} of < 10 µg/mL, followed by

methanol (20.2 ± 0.3 µg/mL) (p <0.05,*) and chloroform (27.9 ± 0.4 µg/mL) (p <0.05,*) fractions. In another experiment, ethyl acetate and chloroform showed significant (p < 0.001,***) inhibition of oxidative burst generated from zymosan-activated PMNs at lower concentrations (IC_{50} of 1.3 ± 0.3 and 2.1 ± 0.1 µg/mL respectively).

Table 1. Intracellular ROS production after treatment with *C. excavata* fractions.

Fraction/Drug	WB ROS/IC_{50} ± SD (µg/mL)	PMNs ROS/IC_{50} ± SD (µg/mL)
Methanol	20.2 ± 0.3 (*)	7.1 ± 0.05 (*)
Ethyl acetate	<10 (**)	1.3 ± 0.3 (***)
Chloroform	27.9 ± 0.4 (*)	2.1 ± 0.1 (***)
Petroleum ether	93.1 ± 0.5	33.1 ± 2.1
Standard (Ibuprofen)	10.1 ± 1.7	3.0 ± 0.5

WB: Whole Blood, ROS: Reactive Oxygen Species, PMNs: Polymorphonuclear cells (*p<0.05, **p<0.01, ***p<0.001)

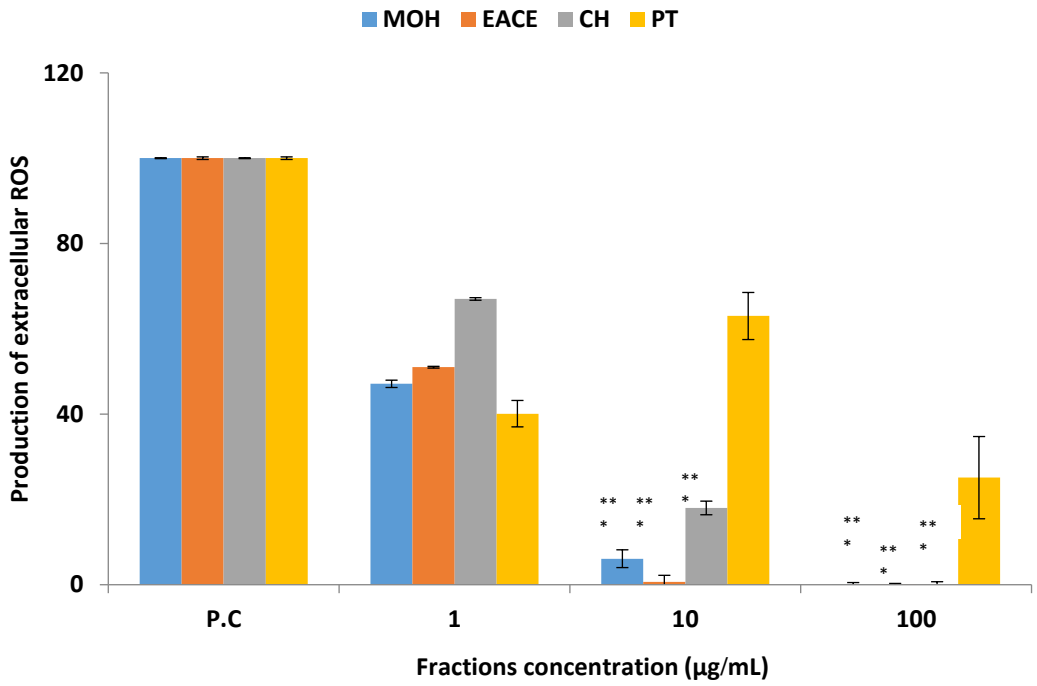

Figure 1. Extracellular ROS production after treatment with *C. excavata* fractions

Abbreviations: P.C, positive control; MOH, methanol; EA, ethyl acetate; CH, chloroform; PT, petroleum ether. (*p<0.05, **p<0.01, ***p<0.001)

The immunomodulatory activity of different fractions of *C. excavata* leaves to suppress extracellular ROS production from macrophages is presented in Fig. 1. Chloroform, methanol and ethyl acetate of *C. excavata* showed significant (p < 0.001) decreasing of oxidative burst generated from PMA-activated macrophages (IC_{50} 2.3 ± 0.01, 1.7 ± 0.01, and 0.7 ± 0.03 µg/mL respectively).

The inhibited level of different extracts might be related to high phenolic contents that could be main contributor to antioxidant and immunomodulatory capacity of the extract, as previously reported publication [11]. The phenolic compounds can inhibit protein kinase C (PKC) and complement system; therefore, it is possible that these compounds present in this plant decrease ROS production by inhibiting NADPH oxidase enzyme [21-28]. Phenolic compounds also block the mitochondrial respiratory chain and ATPase [29]. Different polyphenolic compounds like curcumin [30], resveratrol [31], quercetin [32], ellagic acid [33], chlorogenic acid [34], exert their antioxidant properties by regulating the antioxidant enzyme genes through PKC signaling. The phytochemical analysis of essential oil obtained from the leaves of *C. excavata* through GC-MS showed anethole, and estragole as the major constituents and these are reported for their strong antioxidant properties [35]. Current findings of this study are in agreement with a previous outcome [11,36,37], in which LCMS/MS analysis of methanolic and ethyl acetate leaf extracts of *C. excavata* contained higher total phenolic contents, total flavonoid content, quercetin, furocoumarin, 8-geranyloxy psoralen, myricetin glucoside conjugate, kaempferol conjugate, caffeic acid and showed anti-inflammatory and antioxidant activities. Based on the outcomes in this study and those reported earlier, the leaves extract of *C. excavata* especially ethyl acetate extract has strong therapeutic to be developed into a controlling inflammation agent.

In conclusion, the ethyl acetate, chloroform, and methanol extract possess significant immunomodulatory activity as evidenced from decreased ROS production from activated immune cells. Thus, these extracts have potential to retard the progression of acute and chronic inflammatory conditions, support, and encourage for its traditional use in the folklore herbal medicine to treat inflammatory-related conditions.

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AUTHOR CONTRIBUTIONS

Conception: *S.F.A.A.*; Design: *S.F.A.A.*; Supervision: *S.F.A.A.*, *R.M.*; Resources: *S.F.A.A.*, *R.M.*; Data Collection and/or processing: *S.F.A.A.*; Analysis and/or interpretation: *S.F.A.A.*; Literature search: *S.F.A.A.*; Writing manuscript: *S.F.A.A.*, *R.M.*; Critical review: *S.F.A.A.*, *R.M.*; Other: *R.M.*

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

ICCBS/IEC-028-HB-2017/PROTOCOL/1.0, University of Karachi

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