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Anti-oxidative and Anti-inflammatory Effects of Carissa spinarum L., *Croton oblongifolius* Roxb and *Dioscorea bulbifera* L. in LPS-Induced RAW264.7 Cell

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ABSTRACT

The aim of this study was to evaluate the anti-inflammatory effect for ethanolic extracts of Carissa spinarum L. (C. spinarum), Croton oblongifolius Roxb. (C. oblongifolius) and Dioscorea bulbifera L. (D. bulbifera) in lipopolysaccharide (LPS)-induced RAW264.7 cell and anti-oxidative activity of these extracts by 1-Diphenyl-2-picryl hydroxyl (DPPH)-assay, Nitric Oxide (NO)-assay and Super Oxide (SO)-assay. Cell viability was done by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)-assay. Anti-inflammatory potential was assessed by evaluating the ability of the tested extracts to inhibit NO generating from LPS-stimulated RAW264.7 cells. The present work investigated that tested extracts showed the strong anti-oxidative activity on different forms of free radicals such as DPPH, NO and SO. Among them, C. oblongifolius Roxb. showed stronger anti-oxidative effects on NO and SO radicals than the other extracts. MTT-assay revealed that these tested extracts have no cytotoxic effects on RAW264.7 cell for concentration at and below 250 µg/mL. According to cytotoxic result, (62.5-250) µg/mL (cell viability>85%) were selected to determine NO inhibition experiment. Tested extracts significantly decreased the production of NO in LPS-stimulated RAW264.7 cells in a concentration-dependent manner. IC50 values of NO inhibition (generated by LPS) of C. spinarum L., C. oblongifolius Roxb. and D. bulbifera L. were 135.9 ± 10.8, 130.4 ± 7.8 and 159.1 ± 12.8 µg/m respectively. According to our results, C. oblongifolius Roxb. has both radicals scavenging and anti-inflammatory effects.

Keywords: Anti-oxidative; Anti-inflammatory; Lipopolysaccharide (LPS); Cell viability; RAW264.7

INTRODUCTION

Carissa spinarum L. (*C. spinarum*), *Croton oblongifolius* Roxb. (*C.oblongifolius*) and *Dioscorea bulbifera* L. (*D.bulbifera*) have been reported a wide range of phytochemicals, biological activities and their therapeutic effects on some diseases such as liver disease, epileptic disease, microbial disease, diabetes, cytotoxic and viral diseases [1-3]. An excessive amount of Reactive Oxygen Species (ROS) and insufficient anti-oxidant defense systems contributes to redox imbalance and is associated with harmful physiological consequences related to the pathogenesis of various important diseases [4].

The production of large amounts of pro-inflammatory cytokines, inflammatory mediators such as NO, prostaglandin (PG) E2, and ROS are contributed to oxidative stress and lead to acute and chronic inflammatory diseases. Therefore, the down-regulation of inflammatory mediators and ROS through the up-regulation of antioxidant enzymes is the way for the treatment of inflammation and oxidative-related diseases [5].

Macrophages involve the production of NO and several pro-inflammatory cytokines. RAW264.7, a murine macrophage cell line stimulated by (Lipopolysaccharide) LPS has been frequently used for the screening of the inhibition of NO production, a new therapeutic approach against inflammatory diseases [6].

Secondary metabolites from natural plant possess the potential antioxidant effect and considerable to inhibit NO production act as excellent anti-inflammatory agents based on protection of oxidative stress [7]. Our study approached to evaluate the anti-oxidative and anti-inflammatory effects of *C. spinarum* L., *C. oblongifolius* Roxb. and *D. bulbifera* L. in LPS-induced RAW264.7 cell.

MATERIALS AND METHODS

Materials

Dimethylsulfoxide, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl, 1,1-diphenyl-2-picrylhydrazyl, ascorbic acid, gallic acid, sodium nitrite, LPS, Griess reagent, sodium nitroprusside, riboflavin and Nitroblue Tetrazolium (NBT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Serum free Dulbecco Modified Eagle Medium (DMEM) was purchased from GenDEPOT Co. (Houston, TX, USA), fetal bovine serum, MTT, penicillin and streptomycin were purchased from GibcoBRL Co. (Grand Island, NY, USA)

Plant Materials

C. spinarum L., *C. oblongifolius* Roxb. and *D. bulbifera* L. were selected for this study depending on their traditional medicinal usage and also based on the literature [8] (The Wealth of India, 1951) (Table 1). Collection was made in Ta-soe & Katae village, Kyaukse Township, Mandalay Region, and Myanmar. Botanical identification was done by an authorized botanist of Mandalay University, Myanmar.

Preparation of Plant Extracts

The selected plant samples were collected, cleaned, air-dried, powdered and stored in air-tight containers for further use. Each of air dried powdered samples was percolated with 95% ethanol for one month. The solvents of each extract material were filtered and filtrates were concentrated by using rotary evaporator. The concentrated plant extracts were stored in refrigerator for further experiments.

Cell Culture

The murine macrophage RAW264.7 cell was purchased from Shanghai Institute of Cellular Biology of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin) and maintained at 37˚C in a humidified CO_2 incubator.

DPPH Radical Scavenging Assay

The different concentrations of tested extracts were made by diluting with DMSO to get the highest concentration of 200 µg/mL and the lowest concentration of 3.125 µg/mL. 100 µl of each extract was placed in well and then 100 µl of DPPH (0.2 mM in methanol) was added. DPPH in methanol without extract served as control. The mixture was allowed to react at room temperature for 30min. After 30min of incubation, the absorbance was measured at 517 nm using a microplate reader (SPECTRO Star Nano). The percentage of radical scavenging activity was calculated by following equation: [9].

% scavenging=[Absorbance of Control-Absorbance of Sample/Absorbance of Control] × 100

Graphical representation of radical scavenging activity (%) was plotted against concentrations of tested samples. IC_{50} value was calculated the inhibitory concentration at which radicals were scavenged by 50%. Ascorbic acid was used as positive control. The data were presented as mean values ± standard deviation (n=3).

Nitric Oxide Radical Scavenging Assay

The assay was performed in a standard flat-bottomed 96-well micro titer plate. The desired concentrations of extracts were diluted with DMSO from the stock 10 mg/mL (previous dilute with DMSO). Gallic acid was served as standard and it was diluted with distilled water in same concentration with tested extract concentrations. The reaction mixture containing of 10 µl different concentrations of each extract, 20 µl of potassium phosphate buffer (0.1 M) and 70 µl of sodium nitroprusside (10 mM) were added in each well and incubated at 25°C for (90-100) min. The same amount of reaction mixture with DMSO served as blank as control. After incubation, plate was pre-read at 540 nm by a microplate reader (SPECTRO Star Nano). And then 50 µl of [sulphanilic acid (0.33%) dissolved in 20% glacial acetic acid] was added to the reaction mixture. After that plate was stranded for 5 min to complete of diazotization. After 5 min, 50 ul of naphthalene diamine dihydrochloride (0.1%) was added and shaken. After that plate was incubated again at 25°C for 30 min. After complete incubation, plate was shaken again and final-measured at 540 nm by a microplate reader (SPECTRO Star Nano). Same procedure was done with gallic acid which was standard comparing with tested extracts. Percent inhibition was calculated by using the following equation:

% scavenging=[Absorbance of Control-Absorbance of Sample/Absorbance of Control] × 100

IC_{50} which is an inhibitory concentration of each extract that reduce 50% of NO formation was determined. IC_{50} was calculated from the plot of inhibition percentage against extract concentration [10,11]. Tests were carried out in triplicate.

Superoxide Radical Scavenging Assay

Assay was assessed by Winterbourne et al. [12]. The assay was based on the ability of extracts to inhibit formazan formation by scavenging the super oxide radicals generated in riboflavin-light-nitroblue tetrazolium (NBT) system.

For this research work, it was a little modified in a reagents concentration and test volume for uses 96-well micro titer plate. The reaction mixture containing 10 ul of different concentration of tested extracts, 15 µl of EDTA (12 mM), 10 µl of NBT (1.2 mM), 5 µl of riboflavin (0.5 mM) and it was diluted up to 200 µl with phosphate buffer (50 mM). Then DMSO was added instead of extracts for control. Plate was shacked and pre-measured at 560 nm in a microplate reader (SPECTRO Star Nano). After that the plate was illuminated for 30 min and measured again for final read at 560 nm [13]. Super oxide scavenging activity was indicated that the difference in absorbance before and after illumination value. Gallic acid was used as standard control. IC_{50} values for each extract as well as Gallic acid were calculated. Scavenging activity was calculated by following equation:

% scavenging=[Absorbance of Control-Absorbance of Sample/Absorbance of Control] × 100

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] Assay for Cytotoxicity

RAW264.7 macrophages, cells (3×10^4 cells/well) were treated with or without various concentrations of tested extracts in the presence or absence of LPS (1 µg/mL), and cell viability was evaluated using MTT assay [14]. Briefly, cells were treated with various concentrations of tested extracts and then incubated with or without LPS (1 μg/mL). After 24 h of incubation, MTT was added and the cells were incubated for 3 h at 37°C and. The medium was then removed and the formazan precipitate was solubilized in DMSO. The absorbance was measured at 550 nm on a microplate reader (SPECTRO Star ^{Nano}) (data not shown).

Nitric Oxide Inhibition Assay in LPS-Induced Raw 267.4 Cell

The amount of nitrite in the culture supernatants were estimated by a microplate assay. The assay was carried out as described previously with slight modification [15]. RAW264.7 cells were treated with different concentrations of tested extracts, for 30 min prior to LPS treatment for 24 h. After incubation, the amount of NO in the cultured medium was measured by the Griess reagent. Briefly, 100 μL of cell culture medium with 100 μL of Griess reagent in a 96-well plate was incubated at room temperature for 10 min and then the absorbance at 540 nm was measured in a microplate reader (SPECTRO Star ^{Nano}). The amount of nitrite in the samples was calculated with the standard curve of sodium nitrite ($NaNO_2$).

Statistical Analysis

The data were expressed as mean ± SD. The statistical analysis was carried out by one way analysis of variance (ANOVA) using SPSS Statistics (version 20.0, IBM Corporation, Chicago, USA) and GraphPad Prism (version 5). Values of $p<0.05$ were considered as statistically significant.

Table1. Selected Medicinal Plants

No	Botanical Name	Family Name	The Parts Used	Myanmar Name
1	*Carissa spinarum L.*	Apocynaceae	Bark	Khan, khan-sat
2	*Croton oblongifolius Roxb.*	Euphorbiaceae	Bark	That -Yin
3	*Dioscorea bulbifera L.*	Dioscoreaceae	Fruit	Myauk- nwe

RESULTS AND DISCUSSION

Effect of Tested Extracts on DPPH Radical

The radical scavenging effects (%) of *C. spinarum* L., *C. oblongifolius* Roxb. and *D. bulbifera* L. showed 80.9%, 71.2% and 78% at 200 µg/mL with their IC_{50} values of 33.8 µg/mL, 86.2 µg/mL and 36.6 µg/mL respectively. From

this observation indicated that all of tested extracts have radical scavenging activity. The effect of ascorbic acid (standard) that expressed as 84.1% inhibition to DPPH radical with IC_{50} value of 5.2 µg/mL (Figure 1A and Table 2). This result clearly showed that the standard and texted extracts exhibited concentration-dependent radical scavenging manner. The highest DPPH radical scavenging effect was found in *C. spinarum* L. extracts with its lowest IC_{50} values.

Effect of Tested Extracts on Nitric Oxide Generated by Sodium Nitroprusside

The maximum inhibition percent of *C. spinarum* L., *C. oblongifolius* Roxb. and *D. bulbifera* L., were 56.7%, 63.8% and 59.7% with IC_{50} values of 47.5 µg/mL, 6 µg/mL and 20 µg/mL respectively at 200 µg/mL concentration. For standard gallic acid, the inhibition percent exhibited 91.98% at maximum concentration with its IC_{50} value of 3.5 µg/mL. All tested extracts exhibited the scavenging effects on free radical by a dose-dependent manner (Figure 1B). Among them, *C. oblongifolius* Roxb, possessed the most powerful scavenging effects on the nitric oxide radical as the significant IC_{50} values of 6 µg/mL (Table 2).

Effect of Tested Extracts on Super Oxide Radical

The ability of radical scavenging percent of *C. spinarum* L., *C. oblongifoliu* Roxb. and *D. bulbifera* L. were 81.2%, 96.6% and 93.86% at the maximum concentration of 200 µg/mL. The IC_{50} values for these extracts were 21.6 µg/mL, 13.5 µg/mL and 20 µg/mL respectively while the standard gallic acid scavenging activity was 91.8% at 50 µg/mL with the IC_{50} value of 1.79 µg/mL (Figure 1C and Table 2). It was recorded that *C. oblongifoliu* Roxb has powerful scavenging effect with the lowest IC_{50} value.

Table 2. IC_{50} Values for radical scavenging activities of tested extracts

Tested Sample	DPPH (µg/mL)	NO (µg/mL)	SO (µg/mL)
Carissa spinarum L.	33.85 ± 2.1	47.5 ± 0.17	21.75 ± 0.77
Croton oblongifoliu Roxb.	86.2 ± 6	6 ± 0.4	13.5 ± 0.02
Dioscorea bulbifera L.	36.6 ± 0.4	20 ± 1.6	20 ± 3
Ascrobic Acid	5.2 ± 0.9	-	-
Gallic Acid	-	3.5 ± 0.1	1.79 ± 0.01

Values are mean of three replicate determinations (n=3) ± standard deviation

Effect of Tested Extracts on Nitric Oxide (NO) Production by LPS-Induced RAW264.7

To determine the cytotoxicity of tested extracts on RAW264.7 macrophages, cells were treated with or without various concentrations of extracts in the presence or absence of LPS, and cell viability was evaluated using MTT assay. According to the cytotoxicity results, the tested concentrations (at or below 250 µg/mL) did not effect to cell viability (>85%) (data not shown). Therefore, the extract concentration of (62.5-250) µg/mL was used for NO experiment. As shown in Figure 2, all of tested extracts inhibited LPS-induced NO production in a dose-dependent manner. The IC_{50} values of *C. spinarum* L., *C. oblongifoliu* Roxb. and *D. bulbifera* L. were 135.9 ± 10.8, 130.4 ± 7.8 and 159.1 ± 12.8 µg/mL respectively. MTT cell viability assay revealed that the inhibitory effect of tested extracts on NO production by LPS-stimulated RAW264.7 cell were not due to cell damage.

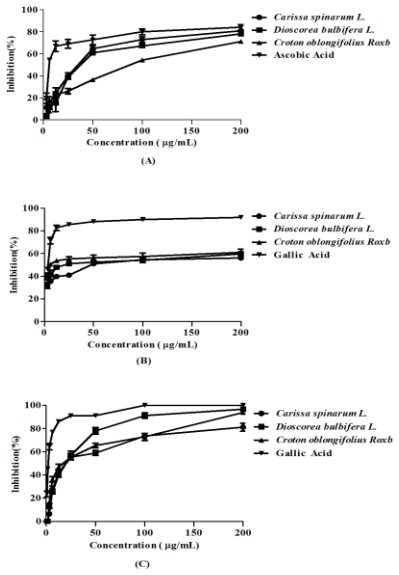

Figure 1. Radical scavenging activities of tested extracts on (A) DPPH (B) NO and (C) SO

Values are mean of three replicate determinations (n=3) ± standard deviation.

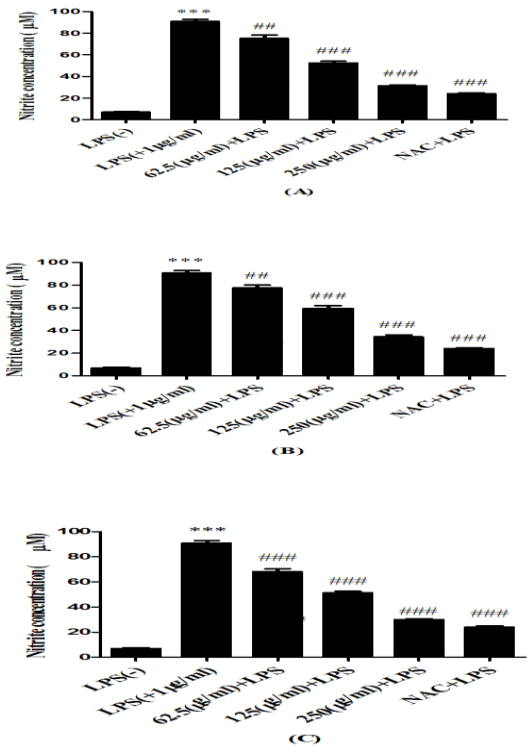

Figure 2. Inhibitory effects of tested extracts on NO production by LPS-induced RAW264.7 cells (A) *C. spinarum* L. (B) *D. bulbifera* L. and (C) *C. oblongifoliu* Roxb. RAW264.7 cells were stimulated with 1 µg/mL of LPS.

Notes: Data are expressed as mean M ± SD; n=3. $^{***}p<0.001$ compared with the control; #$p<0.05$, ##$p<0.01$ and ###$p<0.001$ compared with the LPS (+1 µg/mL).

DISCUSSION

In vitro and *in vivo* studies revealed that *C. spinarum, C. oblongifoliu* and *D. bulbifera* have enormous ethno medicinal uses because of their bioactive compounds and several biological functions. Since antiquity, these plants have been used in folklore medication and significant applications as traditional herbal medicine. In addition, different kind of extracts, fraction and active compounds from these plants was focused for several biological activities, including antiherpetic, antidiabetic, cytotoxic, antioxidant, hepatoprotective and antibacterial effects [16-18]. Therefore, present study aimed to select the above plants as tested plants and exploring their anti-oxidative and anti-inflammatory activities of these extracts.

A large number of free radicals including nitric oxide and superoxide can damage healthy cells related to ROS and contribute to the degenerative processes leading to aging and diseases such as cancer, cardiovascular disease, and neurodegenerative disorders [19]. Natural plant constituents ‘‘plant chemicals’’ or ‘‘phytochemicals’’ possess the potential antioxidant effect which can protect from free radical’s generation in human being. For this reason, there has been a considerable interest in finding natural antioxidants from plant materials to replace synthetic ones [20].

The present investigation for the antioxidant property of tested extracts on DPPH radical by using spectrophotometer with 96-well plate was investigated. The radical scavenging activity of the standard and tested extracts exhibited concentration-dependent manner (Figure 1A). The DPPH radical scavenging effect of *C. spinarum* showed the highest with IC_{50} values of 33.8 µg/mL (Table 2).

Nitric oxide radical inhibition was estimated by Griess’ reagent. Sodium nitropursside decomposes in potassium phosphate buffer at pH (7.4) producing NO. This is reacting with oxygen to produce stable products, nitrite ion during aerobic condition. The quantities of nitrite can be determined using Griess reagent. Scavenger of nitric oxide competes with oxygen to reduce the production of nitrite oxide [21]. In this study, NO scavenging assay was based on the scavenging ability (%) of tested extracts measured at 540 nm using a microplate reader (SPECTRO Star ^{Nano}). Ethanolic extracts of tested plants were examined with standard gallic acid at same concentration. In this results, all tested extracts were exhibited the scavenging effects on free radical by a dose-dependent manner (Figure 1B). Among them, *C. oblongifolius* possessed the most powerful scavenging effects on the nitric oxide radical as the significant IC_{50} values of 6 µg/mL (Table 2).

Superoxide (SO) radical is the most reactive oxygen species among the free radicals. Redox imbalance associated with harmful physiological consequences is related to the overproduction of superoxide radical [22]. SO scavenging effect of tested extracts were analyzed in terms of inhibition the formation of blue formazan measured at 560 nm. This method is based on generation of superoxide by auto oxidation of riboflavin in presence of light [23]. In this observation, tested extracts had ability to inhibit SO radical especially at highest concentration 200 µg/mL (Figure 1C). Among the tested extracts, *C. oblongifolius* indicated that the significant scavenging effect of IC_{50} value at 13.5 µg/mL (Table 2) while the standard gallic acid scavenging activity was 91.8% for maximum concentration at 50 µg/mL with the IC_{50} value of 1.8 µg/mL (Figure 1C and Table 2).

Accumulation of reactive oxygen species (ROS) associated with oxidative stress leading to chronic diseases. NO, a free radical derived from the interaction with oxygen or ROS involved in regulation of various beneficial

physiological processes in body [24]. However, inappropriate amount may lead to the chronic diseases including cancer, inflammatory diseases such as juvenile diabetes, arthritis and ulcerative colitis [25].

RAW264.7, a macrophage cell activated by LPS plays a useful model for anti-inflammatory agent nowadays and pivotal roles in inflammatory diseases *via* excess production of inflammatory mediators, such as NO and pro-inflammatory cytokines. Among them, NO cause toxicity to cells and directly concern to the pathogenesis of inflammation process [26]. In the present study, in order to investigate the effects of tested extracts on cell viability and NO production using LPS-stimulated RAW264.7 cells. The cytotoxicity test was evaluated by MTT assay and cell viability was not affected by tested extracts up to 250 μg/mL (data not shown). Therefore, concentrations of tested extracts (cell viability>85%) were used for NO experiments. In these results, all tested extracts significantly decreased nitrite accumulation in LPS-stimulated RAW264.7 cells in a concentration-dependent manner (Figure 2). Therefore it was indicated that the tested extracts potentially inhibited the NO production. The IC_{50} values of *C. spinarum*, *C. oblongifoliu* and *D. bulbifera* were 135.9 ± 10.8, 130.4 ± 7.8 and 159.1 ± 12.8 μg/mL respectively. According to this data, *C. oblongifoliu* significantly suppressed the NO production in LPS-activated RAW264.7 cell. The present investigations indicated that the inhibition of free radicals may be the presence of antioxidant molecules in tested extracts and that would be responsible for the inhibitory action of NO production in LPS-stimulated RAW264.7 cells.

CONCLUSION

In conclusion, *C. oblongifolius* had powerful antioxidant effects on NO and SO radicals than *C. spinarum* and *D. bulbifera* as well as it can suppress NO generation in LPS-activated macrophage cell. As cell viability was not affected by tested extracts in MTT assay, the inhibition of NO production by the extracts was not due to cytotoxic effects. Further studies are needed to clarify the isolated compound on inhibition of NO production in cellular and molecular levels. It will be beneficial in therapeutic agents for inflammation-mediated diseases.

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