Research Article


In vitro protective and anti-inflammatory effects of Capparis spinosa and its flavonoids profile

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Abstract: Multiple beneficial effects have been reported to numerous species of Capparis genus. Among these, Capparis spinosa has exhibited several biological benefits, including anti-inflammatory hepatoprotective, antioxidant, and anti-diabetic effects. Herein, C. spinosa was extracted with different solvents and the ability of these fractions to suppress nitric oxide (NO) production in RAW 264.7 cells were assessed via Griess reagent. The effects of C. spinosa fractions on different inflammatory markers were also determined in THP-1 and Human umbilical vein endothelial cells (HUVEC) using reverse transcription polymerase chain reaction. Additionally, high-performance liquid chromatography (HPLC) ultra-violet was employed to estimate the presence of three flavonoid compounds, namely, apigenin, kaempferol, and rutin. Our results indicate that chloroform (CHCl3) and ethyl acetate (EtOAc) fractions of C. spinosa exhibited a promising anti-inflammatory activity via in vitro inhibition of NO in lipopolysaccharide stimulated RAW 264.7 cells. Pretreated stimulated THP-1 cells with either CHCl3 or EtOAc fractions showed decreased expression of interleukin 1 beta (IL-1β) and tumor necrosis factor Alpha in a dose-dependent manner. In HUVEC cells, both fractions downregulate the expression of IL-1β, upregulate the peroxisome proliferator-activated receptor alpha PPAR-α while no significant impact was observed on PPAR-Y gene expression. The presence of apigenin, kaempferol, and rutin in the crude extract has been confirmed through HPLC method.

Collectively, these results support the ethnopharmacological usage of C. spinosa as a potential therapy for inflammation related conditions including atherosclerosis.

Keywords: anti-inflammatory, Capparis spinosa, nitric oxide, HUVEC

1 Introduction

Inflammation is a sophisticated process that participate in tissue maintenance and protection. It is well known that inflammation progression led to several illness including cancer, diabetes, and atherosclerosis [1]. Several studies have documented the role of inflammation in all stages of atherosclerotic process [2]. Hence, targeting inflammatory pathways may offer a promising strategy to delay and cure atherosclerosis [3]. Since the available anti-inflammatory medications are not entirely safe and efficient [4–6], there is an urgent necessity to develop new anti-inflammatory compounds. Medicinal plants have been successfully utilized in folk medicine to treat several inflammatory conditions and it is considered as a preferred option in these cases. Additionally, several reports have demonstrated their action in the molecular levels via gene expression regulation of several pro-inflammatory cytokines [7,8].

Capparis spinosa L. is widely distributed in different parts of the world and its pharmacological effects have received a lot of attention during the past two decades due to the fact that it contains diverse bioactive components. In general, C. spinosa contains numerous classes of bioactive substances, including terpenoids, alkaloids, flavonoids, steroids, and tocopherols [9]. The primary compounds that have been identified as crucial include derivatives of quercetin, kaempferol, and isorhamnetin. Additionally, myricetin, eriodictyol, cirsiamarin, and gallo catechin derivatives are noteworthy [10,11]. These bioactive compounds could be responsible for the various pharmacological activities of C. spinosa including antioxidant and anti-inflammatory activities [11,12]. The anti-inflammatory and antioxidant...
properties of *C. spinosa* may have implications for managing inflammatory conditions, such as cardiovascular disease. Extracts from various parts of *C. spinosa* have demonstrated an anti-inflammatory effect in both *in vitro* and *in vivo* studies and these effects have been attributed to the modulation of key inflammatory signaling pathways [13–15]. Moreover, rutin, tocopherols and kaempferol isolated from *C. spinosa* have demonstrated anti-inflammatory activity in different model [16]. An effective inflammatory response serves to protect living tissues against harmful stimuli. In the field of *in vitro* studies, the RAW 264.7 murine macrophage cell line is regarded as the most efficient anti-inflammatory cell type. Macrophage receptors can detect lipopolysaccharide (LPS), triggering inflammatory responses in these cells [17]. Additionally, endothelial cells play a crucial role in anti-inflammatory responses. Research conducted in human umbilical vein endothelial cells (HUVEC) has been recognized as a valuable model for studying the human endothelium [18].

It is important to note that the effectiveness of *C. spinosa* extracts in treating inflammatory related conditions may vary, and more research is needed to establish definitive therapeutic effects. Therefore, more investigations are required to understand the specific mechanisms of action of the bioactive compounds found in *C. spinosa* and their potential therapeutic applications. Herein, the effect of *C. spinosa* on LPS induced inflammation in macrophage cells (THP-1) and tumor necrosis factor alpha (TNF-α)-induced vascular inflammation in endothelial cells (HUVEC) were explored. We also quantified three flavonoid compounds using high-performance liquid chromatography (HPLC) analysis. Our findings showed a better understanding of how *C. spinosa* extracts inhibit the expression of inflammatory mediators and subsequently its potential benefit in atherosclerosis prevention.

## 2 Materials and methods

### 2.1 Cell culture

Human THP-1 (ATCC-TIB202), HUVEC endothelial cells (ATCC-6549) as well as RAW 264.7 (ATCC TIB-71) cells were cultured in RPMI-1640 media complemented with 10% FBS (Invitrogen), 2 mM L-glutamine, and penicillin/streptomycin.

### 2.2 Plant collection and extraction

The aerial parts of *C. spinosa* were collected from Al Diriyah region, north-west of Riyadh, Saudi Arabia. The plant was identified by a field taxonomist and a voucher specimen with the voucher number-210 was stored in the herbarium of Pharmacognosy Department, College of Pharmacy, King Saud University. The aerial parts of *C. spinosa* were crushed using a herb grinder and the derived plant powder underwent a Soxhlet extraction process which is considered an exhaustive method of extraction. In this method, percolation and maceration techniques are combined to guarantee that all the analytes are extracted from the plant materials. In brief, 500 mL of n-hexane, chloroform (CHCl₃), ethyl acetate (EtOAc), and butanol were sequentially employed for the extraction and each performed over a 24 h period. Subsequently, the obtained extracts were concentrated under vacuum conditions utilizing a rotary evaporator.

### 2.3 Cell viability assay

Cell survival was evaluated after extracts exposure using MTT assay, as previously described [19]. The cells were grown on 24-well culture plates in which 5 × 10⁴ cells were seeded in each well. After 24 h, the cells were exposed to different concentrations (200, 100, 50, and 25 µg/mL) of *C. spinosa* fractions as well as vehicle (0.1% DMSO). After incubation period (24 h), 100 µL of MTT solution (5 mg/mL) was added. Afterward, formazan crystals were solubilized with acidified isopropanol (1 mL) and the absorbance was taken at 570 nm using plate reader (BioTek, USA). Percentage of cell survival was calculated as follows: % Inhibition = (O.D. of treated cells/O.D. of non-treated) × 100. Inhibitory concentration (IC₅₀) was calculated by OriginPro software.

### 2.4 Nitric oxide (NO) assay

Total nitrate concentration as an indicator of NO production was estimated using commercial Griess reagent (Cat. No. ab234044, Abcam, UK) following manufacturer guidelines. Briefly, RAW 264.7 (5 × 10⁴ cells/mL) cells were plated in 24-well plate and incubated for 24 h. Next cells were then pretreated with CHCl₃ and EtOAc fractions (for 4 h before being stimulated with LPS (Sigma, USA) at 1 µg/mL and further incubated for 24 h. Thereafter, 100 µL of culture supernatant was transferred to 96-well plate and mixed with an equal amount of fresh Griess reagents. The absorbance at 490 nm was measured using a microplate reader (BioTek, USA) after a 15 min incubation period. Nitrite levels were measured using a nitrite standard reference curve.
2.5 Isolation of total RNA and reverse transcription polymerase chain reaction (RT-PCR)

Cells were treated with either LPS or TNF-α for 3 h after being exposed for 2 h to various fractions of CHCl₃ and EtoAc extract in macrophage THP1 or HUVEC cells. THP1 cells were utilized to study the levels of interleukin 1 beta (IL-1β) and TNF-α, while PPAR-γ and IL-1β were quantified by RT-PCR in HUVEC cells. Using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), total RNA was isolated from treated cells or control according to manufacturer's protocol. Nanodrop spectrophotometer was employed to determine the quality and quantity of RNA. Next 1 μg of extracted RNA was used to synthesize c-DNA with SuperScript Vilo cDNA synthesis kit. To measure the level of TNF-α, IL-1β, and PPAR-γ expression in THP1 and HUVEC cells, a semiquantitative PCR was used as described in our previous work [20].

2.6 HPLC instrumentation and operating parameters

HPLC chromatographic analysis was conducted using an Alliance chromatographic system (Waters Instruments, Inc., USA), equipped with dual wavelength absorbance detectors. For reverse phase analysis, an Agilent HC-C18 column with dimensions of 4.6 × 250 mm and a particle size of 5 μm was utilized. The mobile phase consisted of two solutions: Solution A, which contained water with 1% formic acid, and Solution B, which was a mixture of methanol and acetonitrile in a ratio of 70:30. The gradient flow rate was set at 1 mL/min, with the following gradient profile: 0–7 min, increasing from 5 to 20% B; 7–15 min, increasing from 20 to 35% B; 15–30 min, increasing from 35 to 55% B; 30–47 min, increasing from 55 to 83% B; 47–60 min, maintaining 83% B. Before injection into the system at a volume of 20 μL, all samples were filtered through a 0.45 μm membrane filter from Millipore. The identification and detection of the three C. spinosa flavonoid compounds were performed at a wavelength of 280 nm by comparing their retention times with those of the corresponding commercial standards.

2.7 Statistical analysis

The results were presented as mean value ± standard deviation (SD), and all data were subjected to Student’s t-test analysis of variance using OriginPro software. A significant difference was considered at a significance level of P < 0.05.

3 Results

3.1 Cytotoxicity evaluation

The MTT cell survival assay was utilized to assess the cells viability after the exposure to various concentrations of C. spinosa for 24 h. The obtained results from MTT assay are presented in Figure 1. As displayed in Figure 1, the C. spinosa CHCl₃ and EtoAc fractions showed promising effect compared to n-hexane and butanol fractions. On the basis of the results, noncytotoxic concentrations (25 and 50 μg/mL) of each fraction was used for subsequent experiments in this study.

![Figure 1: Effects of C. spinosa on HUVEC, THP-1, and RAW 264.7 cell viability determined by the MTT assay. The bars show the percentage of cells viability after being exposed to various concentrations of each fraction (0–200 μg/mL) for 24 h. DMSO (0.1%) was used as a vehicle control. Data from three different experiments are provided as average ± SD.](image-url)
3.2 Effects of *C. spinosa* fractions on inhibiting NO production

NO production by RAW 264.7 cells in response to inflammatory stimulation is one of their main characteristics. In this study, we utilized Griess reagent to quantify the presence of NO in the culture supernatants as a first step in determining if *C. spinosa* fractions have anti-inflammatory activity. We found that LPS-treated cells remarkably released NO to about 53.9 ± 1.6 µM compared to the untreated cells (15.5 ± 0.61 µM) (Figure 2). However, NO production was significantly reduced (24 ± 1.17 and 16.8 ± 1.2 µM) in LPS-treated cells incubated with CHCl3 at 25 and 50 µg/mL, respectively. Similarly, EtOAc fraction significantly reduced the produced NO to 21 ± 1.79 and 17 ± 1.19 µM at the indicated concentration, respectively (Figure 2).

3.3 *C. spinosa* fractions decrease the expression of TNF-α and IL-1β in stimulated THP-1 cells

To explore the anti-inflammatory effects of *C. spinosa* fractions, the THP-1 cells were preincubated with CHCl3 and EtOAc for 2 h and then stimulated with 1 µg/mL LPS for 3 h. After 3 h of LPS, stimulation cells were harvested and looked for the expression of proinflammatory cytokines, namely, TNF-α and IL-1β which are involved in promoting inflammation. As shown in (Figure 3), the expression of TNF-α and IL-1β dramatically increased upon the treatment with stimuli LPS (1 µg/mL), whereas pretreated THP-1 cells with either CHCl3 or EtOAc fractions showed decreased expression of IL-beta and TNF-α in a dose-dependent manner on LPS-stimulation. The result of our study shows that CHCl3 and EtOAc fraction reduced the LPS stimulated mRNA for TNFα and IL-1β.

3.4 *C. spinosa* fractions modulate the expression of PPAR-α, PPAR-Y, and IL-1β in HUVEC endothelial cells

We have preincubated the HUVEC cells with and without CHCl3 and EtOAc fractions for 3 h prior to stimulating with and without TNF-α in order to investigate the impact of *C. spinosa* on the regulation of PPAR-α, PPAR-Y, and IL-1β. It was found that TNF-α-stimulated HUVEC cells dramatically downregulated PPAR-α which is crucial for regulating inflammation. In HUVEC cells, PPAR-α levels were downregulated and inversely correlated with IL-1β levels. But when CHCl3 and EtOAc fraction were added to the HUVEC cells, this effect was neutralized, and the expression of the PPAR-Y gene was not significantly affected (Figure 4). TNF-α stimulation increases the expression levels of IL-1β proinflammatory cytokine, however pretreatment of HUVECs with *C. spinosa* fractions reduced the proinflammatory cytokine’s up-regulation induced by TNF-α (Figure 4).
3.5 Flavonoid components levels in *C. spinosa* fractions

The flavonoids composition of *C. spinosa* fractions were thoroughly investigated using HPLC ultra-violet (HPLC-UV). The goal was to identify and quantify some specific flavonoid compounds present in the extracts. The analysis involved comparing the retention parameters of each assay with standard controls and evaluating peak purity using UV-visible spectral reference data. A reversed-phase gradient system was employed to quantitatively determine the levels of three bioactive flavonoids, namely rutin, apigenin, and kaempferol (Figure 5) in CHCl₃ and EtOAc fractions. The extracts, at a concentration of 10 mg/mL, were analyzed. These three flavonoids showed a high UV absorption at 280 nm; therefore, this wavelength was used for their quantitative determination. The results of the analysis revealed the presence of additional compounds, alongside rutin, apigenin, and kaempferol, in smaller quantities (Figure 6). The levels of these compounds exhibited variations among different fractions, as observed through the HPLC analysis (Table 1). Among the fractions, the EtOAc fraction allowed for the quantification of all three compounds, while only rutin was identified in the CHCl₃ fraction. Notably, there were statistically significant differences in the levels of rutin between the EtOAc and CHCl₃ fractions, with values of 5.58 ± 4.13 and 1.94 ± 3.85 µg/mg, respectively.

4 Discussion

Inflammation is considered as a crucial protective defense mechanism in response to tissue injury. However, tissue damage can result as a consequence of irregularity of this process [1]. Several evidences indicated that inflammatory responses are closely related to cardiovascular diseases. Hence, inhibiting the inflammatory mediators could be an important tactic in the early stages of cardiovascular development [21]. The ubiquitous occurrence of NO within the living organism implies its significant role in maintaining body health. Emerging as a vital source for novel
drug development, the rich content of flavonoids and other polyphenolic compounds found in vegetables and fruits is thought to underlie their advantageous impact on health. In fact, many compounds within this group have been shown to stimulate the production of NO while others inhibit its activation during instances of inflammation and infection [22]. In this study, *C. spinosa* fractions demonstrated an inhibition capacity of NO production in LPS-stimulated RAW 264.7 supporting the anti-inflammatory properties of this plant species. Previously, it was also reported

Figure 4: Effects of *C. spinosa* fractions on the levels of PPAR-γ, PPAR-α, and IL-1β in TNF-α-stimulated HUVEC cells assayed at 25 and 50 µg/mL: (a) CHCl₃ fraction and (b) EtOAc fraction. Data represent the SD of three experiments. Mean values were analyzed by student t-test. *p ≤ 0.05 and **p ≤ 0.01 indicate a significant difference between control and treated cells.

Figure 5: Chemical structure of the determined flavonoid compounds.
C. spinosa exhibited an anti-inflammatory effect through reducing the production of NO induced by IL-1β on human chondrocytes [23]. IL-1β and TNF-α are considered among the most important proinflammatory cytokines that involved in the initiation of other proinflammatory mediators [24,25]. As a part of the inflammatory cascade, TNF-α has been shown to have a vital role in the development of atherosclerosis. In light of these, targeting TNF-α may arise as a potential therapeutic strategy to counteract the onset cardiovascular diseases [26]. Herein, we found that C. spinosa extracts remarkably decreased the expression of IL-β and TNF-α in a dose-dependent manner upon LPS-stimulation which suggest that both C. spinosa fractions protect treated cells against LPS-induced cell damage. In fact, previous study reported that C. spinosa extract was found to decrease the expression levels of IL-β and TNF-α in LPS-stimulated microglia cells [15]. The methanolic extract of another caper family species (Capparis ecuadorica) mediated anti-inflammatory effect in LPS-stimulated RAW 264.7 cells via downregulation of TNF-α, IL-β, and mRNA levels [27]. Inflammation in the endothelial cells causes the release of inflammatory cytokines which led to initiation of atherosclerosis [28]. PPARα and PPARγ are the main members of peroxisome proliferator activated receptor family that

Table 1: Flavonoid contents in CHCl₃ and EtOAc fractions from C. spinosa

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Conc. (µg/mg) ± RSD</th>
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<tr>
<td></td>
<td>EtOAc fraction</td>
</tr>
<tr>
<td>1 Rutin</td>
<td>5.58 ± 4.13</td>
</tr>
<tr>
<td>2 Apigenin</td>
<td>0.81 ± 3.23</td>
</tr>
<tr>
<td>3 Kaempferol</td>
<td>1.37 ± 5.04</td>
</tr>
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RSD = relative SD. Data represent the mean value ± RSD of three determinations.

Figure 6: HPLC-UV analysis (at 280 nm) of (a) standard flavonoid compounds, (b) EtOAc fraction, and (c) CHCl₃ fraction obtained from leaves of C. spinosa.
exhibits a broad effect within the cardiovascular system. The involvement of TNF-α in the pathogenesis of atherosclerosis, and inflammation is linked to its ability to inhibit the activity of these receptors [29–31]. In this study, the inhibitory effects of *C. spinosa* fractions was mediated by enhancing the expression of PPAR-α in HUVEC endothelial cells. The pivotal role of PPAR-α in endothelial cell functions and control of inflammation as well as decreasing the development of atherosclerosis have been reported [30,32].

It has been reported that flavonoids possess a promising capacity to reduce many inflammatory cytokines, including TNF-α, IFN-γ, and numerous interleukins which play a pivotal role in mitigating diseases associated with inflammation. Moreover, flavonoids found in natural herb not only counteract oxidative stress but also exhibit anti-inflammatory and anti-adhesion properties when used for endothelial dysfunction treatment [33]. Flavonoids consumption also have shown to lower the development of cardiovascular disease [34]. Among these flavonoid compounds, apigenin, kaempferol, and rutin have documented to modulate the expression and activation of key cytokines [35]. Previously, the impact of rutin on the TNF-α and IL-1β in arthritic rats showed dose-dependent decreasing of these cytokines [36]. Reduced production of pro-inflammatory cytokines (TNFα and IL-1β) was also reported in LPS-stimulated peripheral blood mononuclear cells following *in vitro* exposure to the flavonoids apigenin [37]. Kaempferol has been documented to inhibit the production of NO induced by LPS in RAW 264.7 cells, resulting in a reduced inflammatory response. Additionally, kaempferol treatment has been observed to mediate inflammatory response via reductions in IL-1β and TNF-α [38]. In this study, HPLC analysis confirmed the presence of these flavonoids in *C. spinosa* EIOAc fraction. In fact, previous reports have reported that *C. spinosa* is rich in antioxidants, including rutin, and kaempferol that contribute to the antioxidant properties of this valuable plant and its potential health benefits [10,39].

It would be valuable to explore alternative inflammation pathway to confirm the obtained results. Moreover, further investigations using in *vivo* models are also required to provide a more comprehensive understanding.

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**Ethical approval:** The conducted research is not related to either human or animal use.

**Data availability statement:** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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