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Inhibitory effects of flavonoids isolated from *Givotia rottleriformis* bark and *Cassia tora* leaves on the production of pro-inflammatory cytokines in LPS stimulated human whole blood

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The plants *Givotia rottleriformis* bark and *Cassia tora* leaves were used in indigenous medicine in the treatment of chronic inflammatory diseases like psoriasis. In previous work, three flavonoids namely Rutin (I), Luteolin-7-O-β-D-Glucuronide (II) and Kaempferol 3-O-[2-O-(6-O-feruloyl)-β-D-glucopyranosyl]-β-D-galactopyranoside (III) were isolated from *G. rottleriformis* bark and three flavonoids viz quercetin-3-*O*-β-d-glucuronide (IV), Luteolin-7-O-β-glucopyranoside (V) and Formononetin-7-O-β-D-Glucoside (VI) were isolated from *C. tora* leaves and evaluated for antipsoriatic activity. The cytotoxic effect of isolated compounds I-VI was evaluated using HaCaT cells, a rapidly multiplying human keratinocyte cell line, as a model of epidermal hyperproliferation in psoriasis. Among the isolated compounds, compound II, III and VI showed significant antiproliferant activity in HaCaT cells. Pro-inflammatory cytokines *viz*., IL-1α, IL-1β, IL-6, IL-8, IL-17 and TNF-*α* contributes to the pathogenesis of chronic inflammatory skin diseases such as psoriasis and has been a target for the development of the new anti-psoriatic drug. Hence the present study aimed to evaluate inhibitory effects of ethanol extract of selected plants and isolated compound II, III and VI (5-40 mg/mL) on lipopolysaccharide (LPS) induced pro-inflammatory cytokines *viz*., IL-1α, IL-1β, IL-6, IL-8, IL-17 and TNF-*α* production. The level of IL-1α, IL-1β, IL-6, IL-8, IL-17 and TNF-*α* pro-inflammatory cytokines were detected using enzyme-linked immunosorbent assay. The ethanol extract of both the plants was standardized by HPLC using chemical markers. Preincubation with isolated compounds II, III, VI and ethanol extract of selected plants strongly attenuated LPS-induced increase in the concentrations of IL-6 (50-73 %) and TNF-α (64-90 %). The compound II and III also showed significant inhibition (*$p \leq 0.05$) of IL-1β, IL-8 and IL-17. The results showed that the selected plants and isolated flavonoids have potential effects as anti-inflammatory agents by inhibiting the release of pro-inflammatory cytokines supporting the folkloric utilization.

Keywords: Anti-inflammation, *Cassia tora*, Cytokine, Flavonoids, *Givotia rottleriformis*, Interleukins, Psoriasis.

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Introduction

Psoriasis is a chronic inflammatory skin disease that can be considered as a T-cell mediated disease, with a complex role for a variety of cytokine interaction between keratinocytes and T-lymphocyte[1]. Keratinocytes secrete a number of cytokines and chemokines that either activate or suppress immune responses. In the psoriatic state, the epithelialization occurs in about 36 hours and so the division of keratinocytes which reduces to 10 days (240 hours). This phenomenon collectively leads to hyperkeratinized state[2]. The course of treatment available for treating psoriasis includes the use of the combinations of conventional methods like using coal tar preparations, dithranol, calcipotriol, topical corticosteroids and controlled UV radiations. However, serious side effects are associated with them. Systemic treatment is considered if extensive psoriasis fails to respond to local measures[3].

The serum TNF-*α*, IL-1, IL-6, IL-8, IL-12, and IL-17 levels were significantly higher in active psoriatic patients than in controls. Regulation of the inflammatory events initiated or perpetuated by keratinocytes could so represent an important strategy for the treatment of psoriasis and other chronic inflammatory skin diseases. Interestingly, recent advances by cellular immunologist have identified

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cytokines interleukins (IL) viz., IL-1α, IL-1β, IL-6, IL-8, IL-17 and TNF- α that play an essential pathogenic role in psoriasis. Thus inhibition of TNF-*α*, IL-1*α*, IL-1β, IL-6, IL-8, and IL-17 could be employed as criteria for the evaluation of antipsoriatic activity[4].

Recent literature data continue to support the fact that polyphenolic compounds, found in most plants, can have a positive effect on many chronic diseases. Natural polyphenols, recognized as potent antioxidants, are multifunctional molecules that can act as anti-inflammatory and antiproliferative agents through the modulation of multiple signalling pathways[5]. This characteristic could be advantageous for the treatment of multi-causal diseases, such as psoriasis. Polyphenols are ubiquitous constituents of plants and possess a broad spectrum of biological activities such as immune system activities, oxygen radical scavenging, antimicrobial, anti-inflammatory and antitumor activities[6]. The flavonoid quercetin exhibits anti-inflammatory effects mediated by the inhibition of the proinflammatory cytokine TNF-α via modulation of NF-κβ1 and Iκβ[7]. The flavone chrysin isolated from *Potentilla evestita* Th. Wolf. Possess anti-inflammatory supported in silico by an interaction with COX-2 binding site[8].

Natural plant compounds which are able to suppress the production of inflammatory mediators can act as potential anti-inflammatory agents. Therefore, this study is aimed to explore and evaluate the anti-inflammatory potential of flavonoids from the ethanol extract of *Givotia rottleriformis* Griff. ex Wight (Euphorbiaceae) bark and *Cassia tora* L. (Caesalpinaceae) leaves. *G. rottleriformis* is a moderately sized tree. The bark and seeds of the tree are used in indigenous medicine in the treatment of rheumatism, dandruff and psoriasis[9]. *C. tora* is a wild crop and grows in most parts of India as a weed. The leaves and seeds of the plant have been traditionally used for the treatment of psoriasis and other skin diseases[10]. Previous studies have demonstrated that the plants *G. rottleriformis* bark[11] and *C. tora* leaves have antipsoriatic activity using *in-vivo* models[12]. These results prompted us to further investigate the species on the inhibitory effects on the IL-1α, IL-1β, IL-6, IL-8 and IL-17 and TNF-*α* biosynthesis.

Materials and Methods

Plant material

The bark of the plant *G. rottleriformis* was collected from the forest of Attur, Salem district, Tamilnadu during the month of January 2011. *C. tora* leaves were collected in Chennai, Tamilnadu during the month of October 2011. It was identified and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), Tambaram, Chennai. Voucher specimen No. PARC/2011/2140 and PARC/2011/2141, respectively, have been deposited in Vels University for further reference.

Extraction and isolation

About 500 g of the powdered *G. rottleriformis* bark was extracted using a Soxhlet apparatus with ethanol (70 % v/v) (18 hours). The extracted solution was filtered and concentrated in a rotary evaporator under reduced pressure (rotary vacuum flash evaporator). The crude ethanol extract (25 g) thus obtained was subjected to chromatography (Silica gel 120 mesh, 500 g) with gradient elution using solvents of increasing polarity, hexane, chloroform, ethyl acetate and methanol. Three flavonoid glycosides were isolated viz., Rutin (I), Luteolin-7-O-β-D-Glucuronide (II) and Kaempferol 3-O-[2-O-(6-O-feruloyl)-β-D-glucopyranosyl]-β-D galactopyranoside (III).

Phytochemical screening

Phytochemical screening was performed to assess the qualitative chemical composition of crude extract using commonly employed precipitation and colouration reactions to identify the major secondary metabolites like alkaloids, flavonoids, glycosides, proteins, phenolic compounds, saponins, starch, steroids, tannins and terpenoids. Concentrated ethanol (70 %) extract of *G. rottleriformis* bark and *C. tora* leaves were screened for the presence of various phytoconstituents using standard procedure[13].

Fingerprint analysis by HPLC

The Qualitative analysis of the sample was performed according to the method of Boligon *et al*[14]. The HPLC system of Jasco consists of a pump (model Jasco PU2080, intelligent HPLC pump) with injecting facility programmed at 20 µL capacity per injection was used. The detector consists of a UV/ VIS (Jasco UV 2075) model operated at a wavelength of 270 nm. The software used was Jasco Borwin version 1.5, LC-Net II/ADC system. The column was Thermo ODS Hypersil C_{18} (250 x 4.6 mm, 5 µm) in isocratic mode. The separation was achieved using a mobile phase of methanol, water and phosphoric acid (100:100:1, *v/v/v*) at a flow-rate of 1.5 mL/min. The effluent was monitored using UV detection at a wavelength of 270 nm. The mobile phase was filtered through 0.45µm nylon filter prior to use.

Sample preparation

Powdered drug of *G. rottleriformis* bark was weighed (1 g) and transferred to a 250 mL flask fitted with a reflux condenser. About 78 mL of extraction solvent (Alcohol: Water and Hydrochloric acid (50:20:8) was added, refluxed on a hot water bath for 135 minutes, cooled at room temperature and transferred to a 100 mL volumetric flask. About 20 mL of methanol was added to the 250 mL flask, sonicated for 30 min, filtered and the filtrate was transferred to the 100 mL volumetric flask, the residue was washed on the filter with methanol. The washing was collected in the same 100 mL volumetric flask and diluted to volume. Identification is based on retention times and on-line spectral data in comparison with authentic standards. The procedure was repeated with powdered leaves of *C. tora.*

Cytokine inhibition assay

Endotoxin (LPS) from *Escherichia coli* 055:B5 was obtained from Difco (Detroit, MI). Heparin was purchased from Takeda (Osaka, Japan) and ELISA kits from RayBio® (RayBiotech, Inc.).

Blood collection

About 20 mL of blood collected from healthy human volunteers after an overnight fast of 10–12 h. containing 20U heparin/mL by venapuncture and 30 % solution is prepared by suspending in supplemented RPMI-1640 medium containing 100 U/mL penicillin and 100 mg/mL streptomycin.

Procedure

Lipopolysaccharide stimulated human peripheral mononuclear cells (LPS) (1 mg/mL) was dissolved in the supplemented RPMI-1640 media at a concentration of 3 mg/mL. The test samples, ethanol extract of *G. rottleriformis* bark and ethanol extract of *C. tora* leaves were dissolved in DMSO at concentrations of 5, 10, 20 and 40 mg/mL and each of these concentrations was diluted with the supplemented RPMI-1640 media (1:100). Only DMSO was contained in control suspension. Equal volumes from each of three solutions (whole blood, LPS and test sample) were mixed and the mixture was incubated at 37 °C in a humidified atmosphere of 95 % air, 5 % CO_2 for 18-24 hours. The supernatant of culture prepared by centrifugation was stored at -20 °C until the assay of cytokine. The concentrations of the human cytokines (IL-lα, IL-1β, IL-6, IL-8, IL-17, TNF-α) were assayed using an ELISA kits[15]. The assays were performed as described in the manufacturer's instructions. The ratio (%) of inhibition of the cytokine release was calculated by the following equation:

Inhibition (%)= 100 × (1-T/C)

where, T represents the concentration of the cytokine in the culture supernatant with the test compound, and *C* represents the concentration of the cytokine in the culture supernatant with the solvent (control).

Statistical analysis

The values of three separate sets of experiments are expressed as mean±S.D. The significance of differences between the respective controls was tested using Student's t-test for each paired experiments. $P \leq 0.05$ was considered as significant.

Results and Discussion

About 500 g of *G. rottleriformis* bark and *C. tora* powder was extracted using a soxhlet apparatus with ethanol (70 %) and the yield of ethanol extract of *G. rottleriformis* bark and *C. tora* powder was about 12.50 % w/w and 11.36 % w/w.

Phytochemical screening

Preliminary phytochemical screening of ethanol extract of *G. rottleriformis* bark showed positive results for alkaloids (Dragendorff's test), steroids (Liebermann-Burchard test), flavonoids (Shinoda's test), tannins (Ferric chloride test), terpenoids (Salkowski's test), and saponins (Foam test). The ethanol extract of *C. tora* leaves showed positive results for steroids (Liebermann-Burchard test), flavonoids (Shinoda's test), tannins (Ferric chloride test), terpenoids (Salkowski's test), carbohydrate (Molisch's test) and anthraquinone glycoside (Borntrager's test).

HPLC analysis

Qualitative analysis of the ethanol extract of the *G. rottleriformis* showed 7 components (Fig. 1). The main difference was in peak eluted at 3.96, 11.52, 16.68 and 30.76 min, respectively. In the present investigation, 4 flavonoids were identified at 254 nm using peak area by comparison to a retention time of the standard marker, rutin, quercetin, kaempferol and luteolin (Fig. 2). Similarly, the ethanol extract of the *C. tora* leaves showed 13 components (Fig. 3). The main difference was in peak eluted at 12.07, 17.15, 19.62 and 31 minutes, respectively and 4 flavonoids were identified at 254 nm using peak area by comparison to a retention time of the standard marker, luteolin, quercetin and formononetin (Fig. 4).

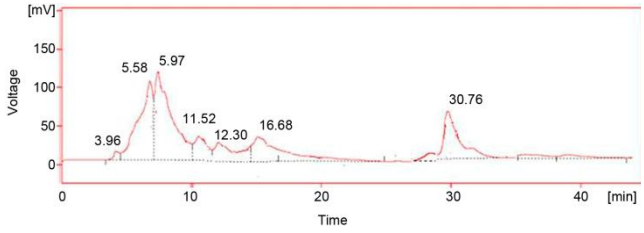

Fig. 1 — HPLC fingerprint of ethanol extract of *Givotia rottleriformis* bark

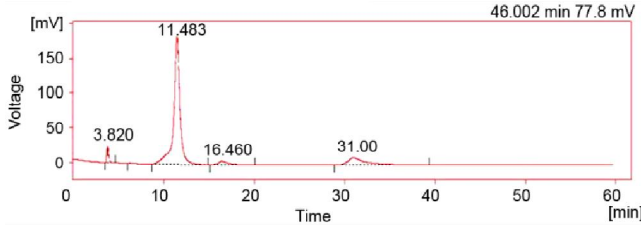

Fig. 2 — HPLC fingerprint of reference standards 1: Rutin 2: Quercetin 3: Kaempferol 4: Luteolin.

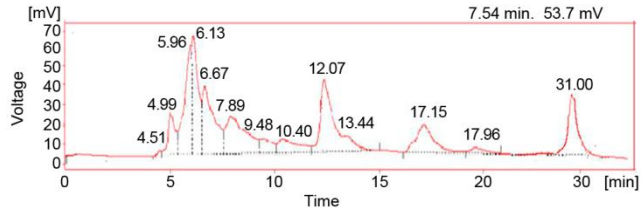

Fig. 3 — HPLC fingerprint of ethanol extract of *Cassia tora* leaves

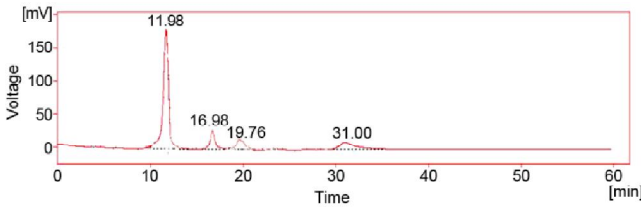

Fig. 4 — HPLC fingerprint of reference standards 1: Quercetin 2: Kaempferol 3: Formononetin 4: Luteolin

The results above showed, therefore, that the bark of *G. rottleriformis* and *C. tora* leaves was a rich source of the important biologically active flavonoids. The described HPLC procedure could be useful for the qualitative analysis of flavonoids in plant materials.

Cytokine inhibition assay

Inhibitory effects of the test samples on IL-lα, IL-1β, IL-6, IL-8, IL-17 and TNF-α biosynthesis were given in Fig. 5 as the inhibitory percentages. For the interpretation of the results, percentage values were classified under four groups; an inhibition between 70 and 100 % is accepted as high, values between 40 and 69 % as moderate, 20 and 39 % as low and an inhibition less than 20 % is considered to be insignificant.

Psoriasis can be described as a T-cell-mediated disease, with a complex role for a variety of cytokines and other factors. The interaction between T lymphocytes and keratinocytes, via cytokines, is likely to play a pivotal role in the pathogenic process in psoriasis. The Th1 cytokines (TNF-*α*, IFN-*γ*, and IL-12) and some proinflammatory cytokines (such as IL-6, IL-8, and IL-18) are influenced in the serum of psoriatic patients[16]. The exact role of TNF-*α* in the pathomechanism of psoriasis is still unclear, but anti-TNF-*α* therapy is highly effective in psoriasis indicating that this cytokine has, together with IFN-*γ*, a central role in the pathogenesis. IFN-*γ* and TNF-*α* induce IL-6, IL-8, IL-12, and IL-18 and constitute an important link in the cytokine network in the pathogenesis of psoriasis[17]. Moreover, the intradermal administration of IFN-*γ* into the nonlesional skin of psoriatic patients causes the appearance of lesions at the inoculation site[18]. IL-6 mediates T cell activation, stimulates proliferation of keratinocytes[19] and, at the beginning of acute inflammation, mediates the acute phase responses[20]. The IL-17 family is a key pathogenic messenger in the development of psoriasis. Binding of IL-17 to its receptor stimulates several activities on both structural skin cells as well as circulating and resident skin immune cells. In the case of psoriasis, keratinocyte hyperproliferation, maturation of myeloid dendritic cells, and recruitment and activation of neutrophils and macrophages are all IL-17 dependent activities. Collectively, these activities initiate and propagate the inflammation and architectural changes in the skin that manifest clinically as psoriatic lesions[21,22]. In fact, data currently available suggest that this cytokine exerts a critical role as a potent chemo-attractant for neutrophils and T lymphocytes, as well as a factor prompting keratinocyte proliferation. Weseler *et al.* reported that the flavones fisetin, morin, or tricetin attenuated LPS induced increases in concentrations of TNF-α in blood from COPD patients (chronic obstructive pulmonary disease) and IL-6 in blood from T2D patients (Type 2 diabetes), indicating a potential application as nutraceutical agents for this patient groups[23]. In an earlier work, Vijayalakshmi *et al.*[24] reported that the flavonoid quercetin showed a significant reduction in epidermal thickness with respect to control in Perry's mouse tail model.

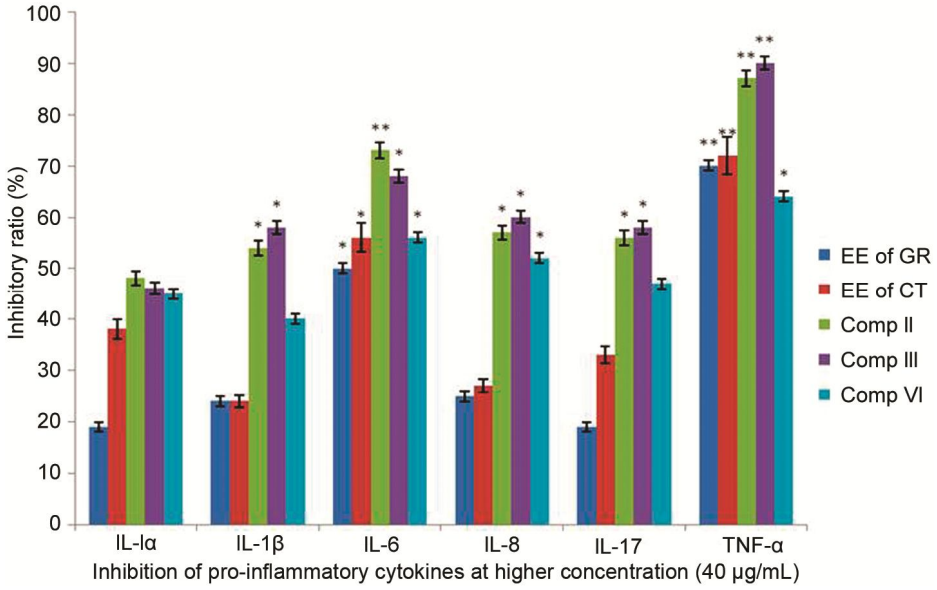

Fig. 5 — Inhibitory effects of the isolated flavonoids and ethanol extract of *Givotia rottleriformis* and *Cassia tora* on IL-lα, IL-1β, IL-6, IL-8, IL-17, TNF-α Biosynthesis; EE of GR - Ethanol extract of *Givotia rottleriformis* bark; EE of CT - Ethanol extract of *Cassia tora* leaves; Data are given as mean±SD from three sets of independent experiments. *$p \leq 0.05$, **$p \leq 0.01$ represent significant difference compared with cells treated with LPS alone

Jadranka Skuric *et al.*[25] reported that the flavonoids from propolis offer some protection against psoriatic complications through their roles as inhibitors of inflammation and as free radical scavengers on animal model psoriasis, induced by the Di-n-Propyl Disulfide iritant (PPD).

In the present study, the ethanol extract of the bark of *G. rottleriformis, C. tora* leaves showed remarkable inhibition of IL-6 and TNF-α release at higher concentration and flavonoid glycosides Luteolin, Kaempferol and Formononetin showed high percentage of inhibition of IL-1β, IL-6, IL-8, IL-17 and TNF- α, key cytokines involved in the pathogenesis of psoriasis in *ex vivo* LPS-stimulated blood.

Conclusion

In conclusion, the study demonstrated that the ethanol extract of the plants *G. rottleriformis, C. tora* and flavonoid glycosides Luteolin, Kaempferol and Formononetin were able to attenuate LPS-induced cytokine release and may provide safe and effective treatment options for a variety of inflammation-mediated diseases such as psoriasis. However, additional clinical investigation of these compounds is indicated to evaluate the efficacy and safety of their application as dietary supplements with health benefits to psoriatic patients.

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