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Original Article

Anti-Psoriatic Activity of Flavonoids from the Bark of *Givotia rottleriformis* Griff. Ex Wight

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Abstract

Givotia rottleriformis has been used in the indigenous systems of medicine for the treatment of inflammatory diseases like rheumatism and psoriasis. In order to evaluate this information, antipsoriatic activity of three flavonoids isolated from the ethanol extract of the bark of *Givotia rottleriformis* were investigated using *in-vitro* and *in-vivo* model, 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). The extract was standardized by HPLC using chemical markers. *In vitro* antiproliferant assay of the ethanol extract and isolated flavonoids were done on HaCaT cell lines. Mouse tail test was used for the evaluation of antipsoriatic activity of ethanol extract (100, 200 and 400 mg/kg b.w.) and bioactive flavonoids (50 mg/kg b.w.) in Swiss albino mice. In the HPLC analysis, 4 flavonoids were identified by comparison with retention time of standard marker viz., Rutin, Quercetin, Kaempferol and Luteolin. Maximum antiproliferant activity was shown by isolated flavonoids II and III (56.50±12.84µg/ml and 76.50±8.60µg/ml). In mouse tail model, a significant reduction in epidermal thickness with respect to control was observed in groups treated with isolated flavonoids II, III and significant orthokeratosis was observed in groups treated with ethanol extract (200 and 400 mg/kg) and isolated flavonoids II, III.

Keywords: Bark, Flavonoids, *Givotia rottleriformis*, HaCaT cells, Perry's mouse tail, Psoriasis.

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1. Introduction

Psoriasis is a genetically determined chronic inflammatory skin disease characterized by red, scaly and raised patches that affects 2.3% of the population worldwide [1]. Psoriasis can be considered as a T-cell mediated disease, with a

complex role for a variety of cytokine interaction between keratinocytes and T-lymphocyte and epidermal hyperproliferation, abnormal keratinocyte differentiation, angiogenesis with blood vessel dilatation and excess Th-1 and Th-17 inflammation can be observed [2,3]. Affordability, availability, and side effects of prolonged use of allopathic drugs still remain a challenge and concern. In this context, flavonoids and polyphenols are proving to be highly effective and are therefore gradually emerging as viable alternatives to conventional drugs for various diseases. A large number of flavonoids have been shown to be potential immunomodulators, acting as anti-inflammatory, antistress, anticancer agents and in various skin diseases [4]. The therapeutic potential of flavonoids and the necessity for scientific validation in popular medicine have prompted increased interest in the field. In the present study, an attempt has been made to evaluate the anti-psoriatic activity of flavonoids from the bark of *Givotia rottleriformis*.

Givotia rottleriformis Griff. Ex Wight is a moderately sized tree of the family Euphorbiaceae. The bark and seeds of the tree are used in indigenous medicine in the treatment of rheumatism, dandruff and psoriasis [5]. The present study aimed to investigate the anti-psoriatic potential of standardized ethanol extract (70%v/v ethanol) and the flavonoids isolated from the bark of *Givotia rottleriformis* using *in-vitro* and *in-vivo* model to establish scientific evidence for its ethnobotanical uses.

2. Materials and Methods

2.1. General Experimental Procedures

UV spectra were recorded in Methanol using a Shimadzu UV-1601 PC spectrophotometer. IR spectra were recorded in KBr using a Perkin-Elmer spectrometer-1. ^{1}H NMR spectra were recorded on a Bruker Avance III 500 spectrometer (500 MHz) and ^{13}C NMR on a JEOL GSX 400 spectrometer (400 MHz). Melting points were determined with a Buchi B-540 instrument. Column chromatography was performed over Silica gel 60 (60-120 mesh, Merck).

2.2. Plant Material

The plant specimen for the proposed study was collected in the forest of Attur, Salem district, Tamilnadu. It was identified and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), Tambaram, Chennai. A voucher specimen No. PARC/2011/2140 has been deposited for further references.

2.3. Extraction and Isolation

About 500 gm of the bark of *Givotia rottleriformis* powder was extracted using a soxhlet apparatus with ethanol (70%v/v) (18 h). 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

Table 1. ^{1}H NMR (500 MHz, DMSO-d6) and ^{13}C NMR (100 MHz, DMSO-d6) data of compound I, II and III from *G. rottleriformis* bark.

Position Aglycone	Compound I		Compound II		Compound III	
	δC	δH	δC	δH	δC	δH
2	157.5	-	165.7	-	157.4	-
3	133.6	6.74s	103.9	6.74 s	134.6	-
4	177.9	-	182.4	-	177.6	-
5	160.8	12.74	161.7	-	161.8	12.5 s
6	99.8	6.26 d	100.4	6.43 d	99.8	6.14 br s,
7	164.7	-	164.1	-	163.4	-
8	94.1	6.4 d	97.9	6.77 d	95.2	6.32 br s,
9	156.4	-	157.6	-	155.4	-
10	104.1	-	105.2	-	105.8	-
1′	121.6	-	119.9	-	121.4	-
2′	115.5	7.48 d	112.6	7.43 d	131.8	8.13 d
3′	145.3	-	146.2	-	116.6	6.85 d
4′	148.6	-	149.7	-	160.8	-
5′	116.9	6.87 d	115.5	7.30 d	116.6	
6′	122.6	7.54 d	121.5	7.46 dd	131.8	
	Glu		**Sugar**		**β-Galc**	
1″	101.8	5.30 d	99.2	5.14 d	99.2	5.46 d
2″	74.2		72.8	3.24 -3.58	81.3	4.43 m
3″	76.4	3.30-3.80	76.7	3.24-3.58	73.3	4.34 m
4″	70.8		70.8	3.30 t	67.3	4.26
5″	75.7	3.72 dd	75.2	3.00 m	76.4	4.28
6″	67.6		172.3	3.74 d	59.8	3.73
	Rham			3.49 d	**β-Glu**	
1‴	101.4	4.41 d			104.2	4.73
2‴	70.9				74.8	3.26
3‴	70.6	1.20 d			75.6	3.18
4‴	72.4				69.6	3.10
5‴	68.6				74.2	2.76
6‴	18.4				63.3	2.43
					β-ferulolyl	
1⁗					125.7	7.45 d
2⁗					110.6	7.14 d
3⁗					147.3	3.73 s
4⁗					149.2	6.24 d
5⁗					115.2	6.77 d
6⁗					122.3	6.87 dd

elution using solvents of increasing polarity, hexane, chloroform, ethyl acetate and methanol. Totally 58 fractions were eluted. Shinoda test was carried out for confirming the presence of flavonoids and TLC studies was carried out using Benzene: Methanol: Ammonia (9:1:0.1) solvent system. The spot was visualized by spraying with ammonia, a reagent specific for flavonoids. The eluates chloroform: ethyl acetate (25:75), ethyl acetate: methanol (50:50) and methanol (100) gave positive response for flavonoids producing pink colour with shinoda

test. The fractions 1-19 did not exhibit any spots. The fractions 20-31, 34-48 and 49-54 exhibited single spot with Rf values of 0.18, 0.61, 0.76. Fractions with similar spots and positive test for flavonoids were pooled together and concentrated to obtain compound I, II, III. The yield of each compound was I (130 mg), II (360 mg), III (325 mg).

2.4. Acid Hydrolysis of Compound

Each compound was refluxed in 2N HCl (5 mL) for 1 h. The aglycones were extracted with ethyl acetate and identified by co-TLC with authentic standards and UV spectral analysis with the usual shift reagents. Sugar moieties were detected on cellulose TLC plates with ethyl acetate-methanol-water-acetic acid (13:3:3:4) using aniline phthalate as spraying reagent.

2.4.1. Rutin (Compound I): A yellow amorphous powder; mp 192-196 °C; Mol. formula $C_{27}H_{30}O_{16}$, Mol wt. 610. TLC R*f*: 0.18 (Benzene: Methanol: Ammonia).

UV λ_{max} (MeOH) 358, 298sh, 266sh, 258 nm, (NaOMe) 410, 328, 202 nm, ($AlCl_3$) 432, 302sh, 272nm, ($AlCl_3$/HCl) 402, 360sh, 300, 270 nm (NaOAc) 385, 327, 274 nm, (NaOAc/H_3BO_3) 376, 290, 264.

IR (KBr) λ_{max} 3340 (OH), 1634 (C=C), 1507, 1374 (aromatic ring) cm-1.

^{1}H NMR (500 MHz) and ^{13}C NMR (100 MHz): Table 1.

2.4.2.Luteolin-7-O-β-D-Glucuronide (Compound II): A yellow amorphous powder; m.pt- 260-262^{o}C; Mol. formula $C_{21}H_{18}O_{12}$ Mol wt 462.36. TLC R*f*: 0.53 (Benzene: Methanol: Ammonia).

UV λ_{max} MeOH nm: 254, 268, 346; + NaOMe: 263, 309 sh, 398; + NaOAc + H_3BO_3: 268, 293 sh, 370; + $AlCl_3$: 276, 298 sh, 386; + $AlCl_3$ /+ HCl: 270, 289 sh, 353, 387; + NaOH: 243 sh, 270, 304 sh, 392.

IR (KBr) λ_{max} 3420 (OH), 1634 (C=O), 1620, 1507, 1374 (aromatic ring) cm-1.

^{1}H NMR (500 MHz) and ^{13}C NMR (100 MHz): Table 1.

2.4.3. Kaempferol-3-O-[2-O-(6-O-feruloyl)-β-D-glucopyranosyl]-β-D-galactopyranoside (Compound III): A dark yellow amorphous powder; mp 210-214°C; Mol. formula $C_{37}H_{38}O_{19}$, Mol wt. 786. TLC R_f: 0.64 (Benzene: Methanol: Ammonia).

UV (MeOH) λ_{max} 269, 328 nm; + NaOAc: 273, 369; + NaOAc + H_3BO_3: 270, 339; + $AlCl_3$: 259 sh, 278, 295 sh, 351, 387 sh; + $AlCl_3$ + HCl: 256 sh, 280, 293 sh, 345, 387 sh; + NaOH: 269, 302 sh, 379.

IR (KBr) ν_{max} cm^{-1}: IR 3410 cm^{-1} (OH) 3500-3300, 2925 (C-H) 2930-2853, 1610 (C=O in flavon), 1610-1444 (Aromatic rings).

^{1}H NMR (500 MHz) and ^{13}C NMR (100 MHz): Table 1.

2.5. Fingerprint Analysis by HPLC

The Qualitative analysis of the sample was performed according to the method of Boligon *et al* 2012 [6]. 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 C18 (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 0.45 µm nylon filter prior to use.

Powdered drug of *Givotia rottleriformis* bark was weighed 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 100mL volumetric flask. About 20mL of methanol was added to the 250mL flask, sonicated for 30 min, filtered and the filtrate was transferred to the 100mL volumetric flask, the residue was washed on the filter with methanol. The washing was collected in the same 100mL volumetric flask and diluted to volume. Identification is based on retention times and on-line spectral data in comparison with authentic standards.

2.6. In Vitro Anti-Psoriatic Activity Using Hacat Cell Inhibition Assay

In vitro antipsoriatic activity was carried out in HaCaT human keratinocyte cell line. Human HaCaT keratinocytes were obtained from NCCS, Pune, India. The cells were seeded at a concentration of 1.0×10^5 cells/ml in a 96 well microtitre plate and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (BioWest). After 24 h, the supernatant was decanted and the monolayer was washed once. Then 100µl of test substance, ethanol extract of *Givotia rottleriformis* bark and isolated flavonoids I, II and III of various concentration was added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere. Antiproliferant activity was assessed by performing the Sulphorhodamine B (SRB) assay [7].

Cells were fixed by adding 25µl of ice-cold 50% trichloroacetic acid on top of the growth medium and the plates were incubated at 4◦C for 1 h, after which plates were washed to remove traces of medium, drug and serum. SRB stain (50µl; 0.4% in 1% acetic acid) (Sigma) was added to each well and left in contact with the cells for 30 min after which they were washed with 1% acetic acid, rinsing 4 times until only dye adhering to the cells was left. The plates were then dried and 100µl of 10mM Tris buffer

(Sigma) added to each well to solubilise the dye. The plates were shaken gently for 5min and absorbance read at 550 nm using a micro plate reader (Biorad, USA).

2.7. Animals

Healthy Male Swiss albino mice (25-30 g) obtained from the institutional animal house were used for the study. Animals were housed in polypropylene cages and were left 7 days for acclimatization to animal room maintained under controlled condition (a 12 h light–dark cycle at 22±2◦C) on standard pellet diet and water ad libitum. All animals were taken care of under ethical consideration as per the guidelines of CPCSEA with due approval from the Institutional Animal Ethics Committee. The Institutional Animal Ethics Committee (IAEC) approved the protocol (Registration no. 52 CPCSEA dated 12.8.2012). Animals were used for acute toxicity study and mouse tail test for psoriasis.

2.8. Acute Toxicity

Acute toxicity studies were carried out using mice as per Economic Co-operation and Development OECD 425 guidelines [8]. Mice (6/group) were divided into three groups. A limit test at a dose of 2000 mg/kg body weight was carried out of ethanol extract and 500 mg/kg of isolated flavonoids II and III. The animals were observed for clinical signs and mortality for a period of 15 days and body weight changes were recorded every week. The tested samples were found to be safe and did not produce any mortality after 15 days.

2.9. In-Vivo Anti-Psoriatic Activity Using Perry Scientific Mouse Tail Model

This is accepted as a screening method for measuring anti psoriatic activity of drugs [9]. The mouse-tail model is based on the induction of orthokeratosis in those parts of the adult mouse-tail, which have normally a parakeratotic differentiation. Animals were divided in to nine groups of six. The control group received normal saline (10 ml/kg, p.o.) and standard group received retinoic acid (0.5 mg/kg, p.o.). Remaining groups received the test samples (ethanol extracts at 100, 200 and 400 mg/kg, p.o and isolated flavonoids II and III 50 mg/kg, p.o.). All test solutions were prepared in water. Animals received their treatment once in a day for five days a week for 2 weeks. Two hours after the last treatment animals were sacrificed, longitudinal sections of the tail skin were made and prepared for histological examination (hematoxylin- eosin staining) as an indicator of orthokeratosis the number of scale regions with a continous granular layer is counted and expressed as a percentage of the total number of scale regions per section. Drug activity is defined by the increase in percentage of orthokeratotic regions. Ten sequential scales were examined for the presence of a granular layer induced in the previously parakeratotic skin areas. The induction of orthokeratosis in those parts of the adult mouse tail, which have

normally a parakeratotic differentiation, was quantified measuring the length of the granular layer (A) and the length of the scale (B). The proportion (A/B)×100 represents the % orthokeratosis per scale, and the drug activity (DA) was calculated as follows:

$$DA = \frac{\text{mean OK of treated group - mean OK of control group}}{100 - \text{mean OK of control group}} \times 100$$

where OK = orthokeratosis:

The measurements were carried out at the border of the scale with a semiautomatic image evaluation unit [10].

2.9.1. Measurement of Epidermal Thickness

It was also examined the vertical epidermal thickness between the dermoepidermal junction and the lowest part of the stratum corneum (n = 3 measurements per scale, n = 3 scales per animal, n = 6). The percentage relative epidermal thickness of all the groups was calculated in comparison to the control group (100%; n = 54 measurements per treatment).

2.10. Statistical Analysis

Level of significance of all the parameters was expressed as the arithmetic mean ± SE and was analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's "t" test. P value less than 0.05 ($P < 0.05$) was the critical criterion for statistical significance.

3. Results and Discussion

The ethanol extract of *Givotia rottleriformis* bark defatted with petroleum ether and subjected to column chromatography over silica gel to yield 57 fractions. Three flavonoids were isolated.

3.1. Isolation of Compound

Compound I gave yellow colour with alkalis, pink colour with Mg-HCI, olive green with Fe^{3+} and answered Molisch's test. Acid hydrolysis of I afforded aglycone - quercetin and sugars-rhamnose and glucose. The UV spectrum of the aglycone in methanol and changes observed after the addition of shift reagents indicated that there is a free hydroxyl group presented at C-5 and C-7 position and the 3-hydroxyl group was substituted.

The ^{1}H NMR spectrum of the compound I exhibited a characteristic proton signal at δ H 12.74 corresponding to a chelated hydroxyl group at C-5. In addition to this, the presence of five aromatic protons were seen in the ^{1}H NMR spectrum; two ortho coupling protons assignable to H-6' (δ 7.54, 1H, d, J = 8.4 Hz) and H-5' (δ 6.87, 1H, d, J = 8.4 Hz); two-meta coupling protons at H-6 (δ 6.26, 1H, d, J =1.6 Hz) and H-8 (δ 6.40, 1H, d, J = 1.6 Hz); a singlet aromatic proton at H-2' (δ 7.48, 1H, d). The ^{1}H NMR spectrum also supported the presence of rhamnose and glucose moieties with the rhamnose anomeric proton signal at δ H 4.41 and glucose H-1 signal at δ H 5.30. A doublet of methyl group of rhamnose was observed at high

field *δ H* 1.20 (3H, d, J = 6 Hz). The rest of protons in the sugar moiety resonated between 3.30 and 3.80 ppm.

The ^{13}C NMR spectrum (100 MHz, DMSO-*d*6) showed 27 carbon signals which indicated the presence of 15 carbon signals due to the flavonol skeleton. In the aliphatic region of ^{13}C NMR, 12 carbon resonances are assigned for a rutinoside moiety among which the most downfield signals at 101.4 and 101.8 are assigned for the two anomeric carbons C1''' and C1'' of rhamnose and glucose, respectively. The cross-peak between the δ H 4.41 (H-1''', rhamnose) and the δ *C* 67.67 (C6'' of the glucose) confirmed that the glycosylation of the glucose unit by the rhamnose took place on the 6″-hydroxyl. The chemical shift of each carbons of the isolated compound in the ^{13}C NMR spectrum (Table 1) was assigned by comparing with the literature data [11]. The structure of the compound I was further evidenced by mass spectrum. The mass spectrum of the glycoside shows prominent peaks at *m*/*z* 609 $[M-H]^-$ and a typical fragments due to the loss of diglycoside moiety (glucose and rhamnose) at *m/z* 301 aglycone-H. The molecular formula was determined as $C_{27}H_{30}O_{16}$, *m*/*z* 610 by Negative ESI-MS. Therefore, the compound I was established as Rutin.

Compound II was isolated as yellow amorphous powder, gave yellow colour with alkalis, pink colour with Mg-HCI, olive green with Fe^{3+} and answered Molisch's test. Acid hydrolysis of II afforded aglycone - luteolin and sugar - glucuronic acid. The UV spectrum of the aglycone in methanol and changes observed in UV shift with AlCl3/HCl relative to MeOH indicated that there is a free hydroxyl group presented at C-5 position and the 7-hydroxyl group was substituted.

The ^{1}H NMR spectrum showed the pattern of a luteolin-O-glucoside. Thus, the signals located at δ H 6.43 (1H, d, J=1.7 Hz) and 6.77 (1H, d, J=1.9 Hz) could be attributed to H-6 and H-8 of ring A, respectively, while the signals at δ 6.89 (d, J=8 Hz), 7.46 (d, J=2 Hz) and 7.43 (dd, J=2 and 8.7 Hz) was consistent with the signals of a 3′,4′-disubstituted ring B of a flavonol. Further features were signals corresponding to the anomeric proton δ 5.14, (d, J=7.6 Hz) of a β-D-glucuronic unit.

The identity of the sugar was confirmed by ^{13}C NMR (Table 1) was assigned by comparing with the literature data [12]. The ^{13}C NMR spectrum of II showed the presence of signals at δ C 172.3 for C-6″ of the glucuronic acid, that supported the FAB-mass data (The fragmentation pattern showed a peak at m/z 285 $[M-H-176]^-$ due to the loss of glucuronic acid). The structure of the glycoside luteolin 7-O-substituted aglycone was further evidenced by mass spectrum. The mass spectrum of the glycoside shows prominent peaks at *m*/*z* 461 $[M-H]^-$ and at 285 $[M-H-176]^-$ aglycone-H. The molecular formula was determined as $C_{21}H_{18}O_{12}$, *m*/*z* 462 by Negative ESI-MS. Therefore, the compound II was established as Luteolin-7-O-β-D-glucuronide.

Compound III gave yellow colour with alkalis, pink colour with Mg-HCI, olive green with Fe^{3+} and answered Molisch's test. Acid hydrolysis afforded aglycone - kaempferol and sugars - glucose, ferulic acid, galactose. The aglycone was yellow under UV and UV/NH_3, characteristic of a flavonol with free 5-OH and had λ_{max} (MeOH) 259, 266, 365nm. The UV spectrum of the aglycone in methanol and changes observed after the addition of shift reagents indicated that there is a free hydroxyl group presented at C-5 position and the 3-hydroxyl group was substituted.

Its ^{13}C NMR spectrum showed 37 carbon signals. The UV and ^{1}H NMR spectra were suggestive of flavonol glycosides. The ^{1}H NMR spectrum revealed H-3′, 5′ and H-2′,6′ protons at δ 6.85 (2H, d, *J*= 8.6 Hz) and 8.03 (2H, d, *J*= 8.6 Hz), respectively, suggesting a *para*-substituted B ring. In addition, a 5,7-dihydroxy-substituted A ring was evident from the two broad singlets at δ 6.14 and 6.32 for H-6 and H-8, respectively. These data together indicate that the aglycone moiety is kaempferol, and the ^{13}C NMR spectrum was comparable to that of kaempferol itself. The presence of a *trans* feruloyl moiety was deduced from the ^{1}H NMR spectrum, which displayed three additional 1,2,4-trisubstituted aromatic protons (δ 7.14, 6.87, and 6.77), a pair of double bond protons (δ 6.24 and 7.45), and a methoxy proton (δ 3.73). The fragment at *m*/*z* 449 [aglycone + hexose + $H]^+$ suggested the existence of an acyl moiety since it indicated loss of hexose and acyl moieties from the molecule. A disaccharide unit was suggested by the pair of anomeric carbon resonances at δ 98.4 and 104.5. Together the anomeric protons (δ 5.46 and 4.73) and anomeric carbon signals suggested the δ-configuration of the glycoside bonds. The ^{13}C NMR chemical shifts of the carbohydrate moiety of III were very similar to those of kaempferol 3-*O*-[2-*O*-(6-*O*-caffeoyl)-β-D-glucopyranosyl]-β -D-galactopyranoside isolated from *Hedyotis diffusa*,[13] indicating that the sugar part is a [2-*O*-(6-*O*-feruloyl)-β-D-glucopyranosyl]- β-D-galactopyranoside. The sites of sugar and acyl linkages were further supported by the glycosylation shift (ca. 9.2 ppm) of C-2″ in galactose and acylation shift (ca. 2.4 ppm) of C-6‴ in glucose, respectively.

The mass spectrum of the glycoside shows prominent peaks at *m*/*z* 787 $[M+H]^+$ and typical fragments due to the loss of glucose at *m*/*z* 577 [M-Glc]+, 449 [(M - feruloyl glucose) + H]+, and at 287 [aglycone + H, kaempferol]+. The molecular formula was determined as $C_{37}H_{38}O_{19}$, *m*/*z* 786. Therefore, the compound III was established as Kaempferol 3-*O*-[2-*O*-(6-*O*-feruloyl)-β-D-glucopyranosyl]-β-D-galactopyranoside.

3.2. HPLC Analysis

The HPLC chromatograms of the bark of *Givotia rottleriformis* showed 7 components (Fig. 1). The main difference was in peak eluted

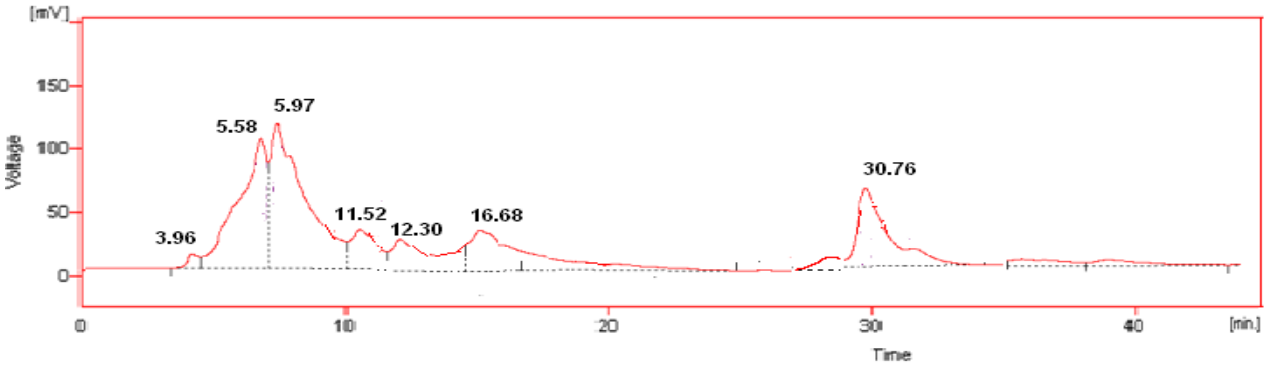

Figure 1. HPLC Profile of ethanol extract of *Givotia rottleriformis* bark.

at 3.96 min, 11.52 min, 16.68 min and 30.76 min respectively. In the present investigation, 4 flavonoids were identified at 254 nm using peak area by comparison to a retention times of the standard marker rutin, quercetin, kaempferol and luteolin (Fig. 2). The results above showed, therefore, that the bark of *Givotia rottleriformis* is a rich source of the important biologically active flavonoids, rutin, quercetin, kaempferol and luteolin. The described HPLC procedure could be useful for the qualitative and quantitative analysis of flavonoids in plant materials.

3.3. HaCaT Cell Inhibition Assay

The cytotoxic effect of ethanol extract and isolated flavonoids I, II and III were evaluated using HaCaT cells, a rapidly multiplying human keratinocyte cell line, as a model of epidermal hyperproliferation in psoriasis. Among the tested flavonoids, II and III showed appreciable antiproliferant activity in HaCaT cell line. The results were validated using asiaticoside as positive control. Flavonoid III was found to have more potent antiproliferant activity

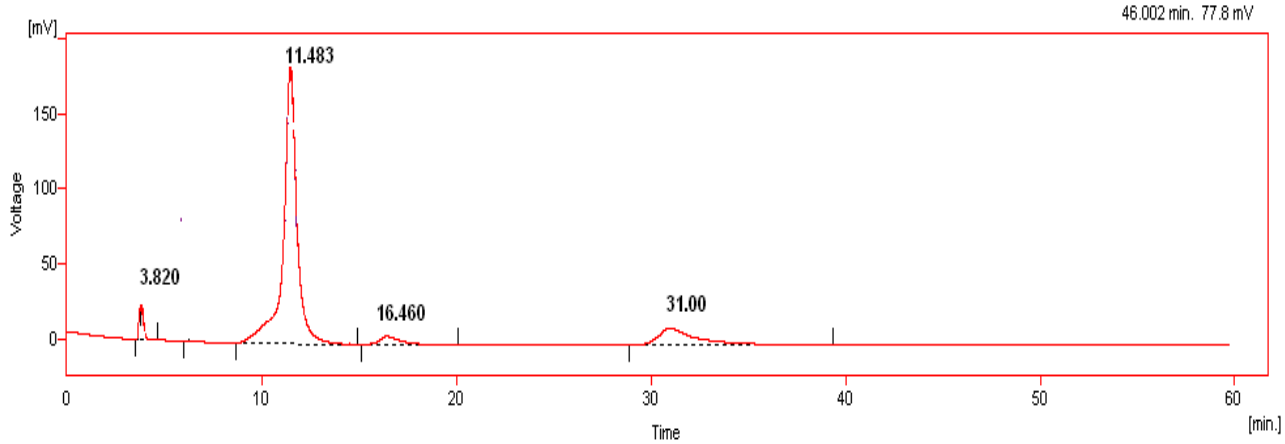

Figure 2. HPLC of reference standards 1: Rutin 2: Quercetin 3: Kaempferol 4: Luteolin.

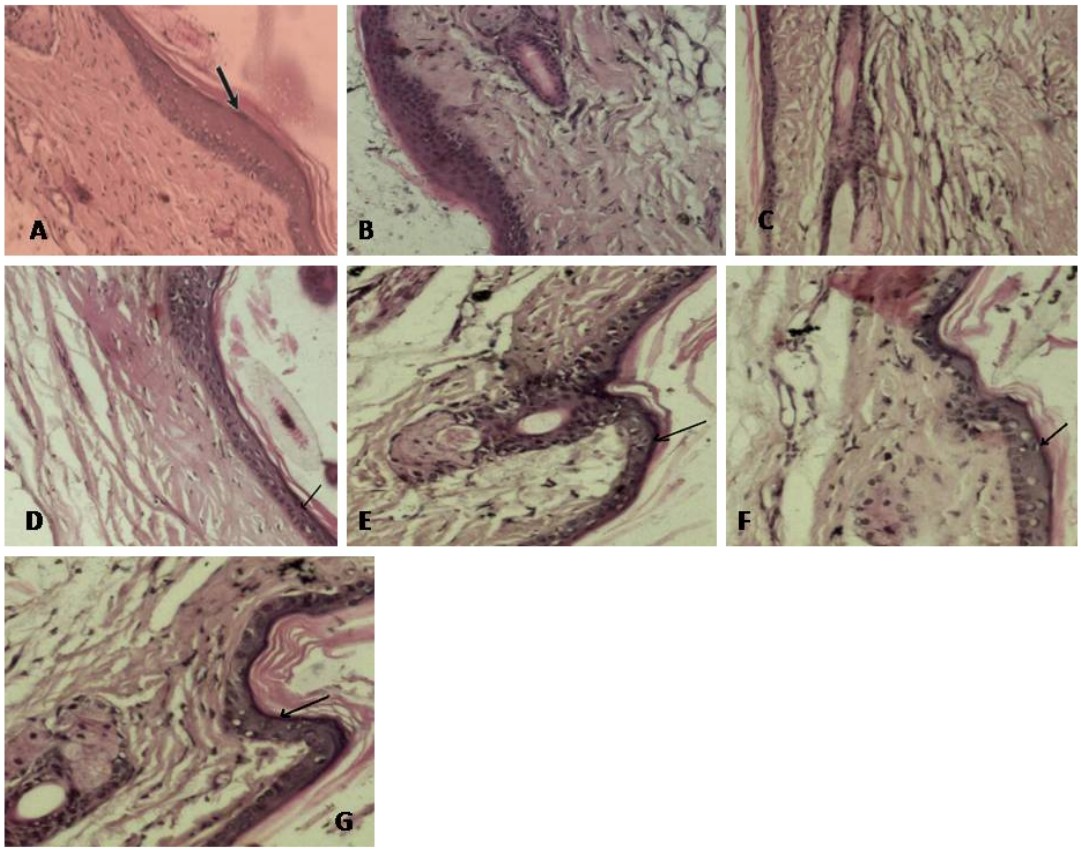

Figure 3. Longitudinal histological sections through the skin of mouse tails treated orally for 2 weeks, HE staining (original magnification 40×).
(A) Standard retinoic acid 0.5 mg/kg; (B) Vehicle control; (C) Ethanol extract 100 mg/kg; (D) Ethanol extract 200 mg/kg; (E) Ethanol extract 400 mg/kg; (F) Compound II 50 mg/kg; (G) Compound III 50 mg/kg.
(Note that the retinoic acid induced orthogranulosis (indicated by an arrow) are clearly seen over the whole horizontal length of the scale (b), whereas a granular layer is missing in most parts of the control specimen (a))

(56.50±12.84µg/ml) which was followed by flavonoid II (76.50±8.60µg/ml), flavonoid I (180.70±15.60µg/ml) and ethanol extract (220.30±7.40µg/ml). Except flavonoid I, II and III showed appreciable antiproliferant activity in HaCaT cell line. Asiaticoside showed a potent activity with IC_{50} value of 31.50 µg/ml.

3.4. Acute Toxicity Study

During the acute toxicity study, the ethanolic extract and compound I, II and III was administered orally and animals were observed for mortality and behavioral responses. There was no mortality observed even at 2000 mg/kg for the extract and at 500 mg/kg for the compounds. All the animals were found to be

Table 2. Effect of ethanol extract and isolated flavonoids on the degree of orthokeratosis, epidermal thickness and the 'drug activity' in the mouse tail test.

Treatment	Orthokeratosis (%)	Activity (%)	Relative epidermal thickness (%)
Saline	18.47±4.86	-	100
Retinoic acid (0.1 mg/kg)	68.06±1.09**	60.9**	57.60±2.28**
Ethanol extract (100 mg/kg)	26.76±3.90	9.7	75.18±1.26
Ethanol extract (200 mg/kg)	34.86±5.60**	19.5**	85.07±3.82
Ethanol extract (400 mg/kg)	45.93±7.12**	34.1**	70.50±5.37
Compound II (50 mg/kg)	66.26±5.76**	58.5**	53.83±8.09**
Compound III (50 mg/kg)	57.18±6.24**	47.5**	64.26±1.28

Values are mean ± SEM of 6 parallel measurements.

Statistical significant test for comparison was done by ANOVA, followed by Dunnet's 't' test (n=6). ***P*< 0.01, **P*< 0.05 when compared against control.

normal and there were no gross behavioral changes till the end of the observation period.

3.5. Perry's Scientific Mouse Tail Model

Psoriasis is characterized by complex and striking alterations in epidermal growth and differentiation [14]. Granular layer of the epidermis is greatly reduced or absent in psoriatic lesions. Parakeratotic condition is seen in the adult mouse tail which is one of the hallmarks of psoriasis. Induction of orthokeratosis in the adult mouse tail is the basis behind the mouse tail test [15,16]. The healing process of psoriasis includes increase in orthokeratotic portion which is non nucleated region lies on uppermost skin layer. i.e. epidermis. Many drugs presently used in the treatment of psoriasis have been evaluated by the mouse tail test and were found to have shown good efficacies. The ethanolic extract and isolated flavonoid II and III from the ethanol extract of *Givotia rottleriformis* bark were screened for their possible antipsoriatic activity using Perry's scientific mouse tail model. Drug activity is defined by the increase in percentage of orthokeratotic regions (These are the regions in a cell having no nucleus and involved in protection from invaders like micro-organisms, UV rays, weak acids & bases). Ethanolic extract (100, 200 and 400 mg/kg) has increased the orthokeratotic regions by 26.76%, 34.86 and 45.93 % respectively, where as 57.18% and 66.26% by isolated flavonoid II and III (50 mg/kg) in comparison to normal. The standard drug Retinoic acid (0.1 mg/kg) showed the increase by 68.08% (Table 2 and Fig. 3).

These results suggest that the ethanol extract of *Givotia rottleriformis* bark and the flavonoid

kaempferol and luteolin showed maximum antiproliferant activity and significant orthokeratosis and has antipsoriatic activity through the inhibition of keratinocyte proliferation. Overall, the results of this study support the use of *Givotia rottleriformis* in traditional Indian medicine and show that extracts and isolated flavonoids of these plants can be used as an easily accessible source of natural antipsoriatic agent and can be useful in some skin problems.

4. Conclusion

The present investigation aims at the development of potent phytomedicine from *Givotia rottleriformis* bark for the treatment of psoriasis. The result of the study authentifies the folk lore claim in the use of *Givotia rottleriformis* bark in traditional medicine for the treatment of psoriasis. Further studies are in process for identification of possible mechanisms of its anti-psoriatic property.

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