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Isolation, Identification and Antibacterial and Wound Healing Studies of Quercetin-3-O- α-L- Rhamnopyranoside -2′′- Gallate

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Abstract: Flavonoids were isolated from flowers of *Salvia leucantha* using chromatography separation techniques. The structure of the isolated compound was analyzed by chemical tests and spectroscopic methods such as UV, ^{1}H-NMR, and ^{13}C-NMR. The spectral data's proved its structure confirmed as Quercetin-3-O- α-L- rhamnopyranoside -2′′- gallate. The present study was to evaluate the agar diffusion antibacterial against gram positive and gram negative microorganisms and wound healing activities to excision wound model in albino rats, respectively. The highest inhibitory zone was observed in *S. aurous* (28mm), and *E. coli* (24mm). The results revealed most valuable information and also support the continued sustainable use of Quercetin-3-O- α-L- rhamnopyranoside -2′′- gallate isolated from *Salvia leucantha* in traditional system of medicine for future development of new antibiotic agents.

Keywords: Quercetin-3-O- α-L- rhamnopyranoside -2^{II} - gallate; antibacterial activity; *Salvia leucantha*; wound healing.

1. Introduction

Flavonoids have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's reaction to allergens, viruses, and carcinogens [1]. Microbiological growth commonly induces undesirable organoleptic and appearance change. In classifying the antibacterial activity as gram- positive or gram-negative, it would generally be expected that a much greater number would be active against gram-positive than gram-negative bacteria [2]. However, treatment of infections has been remarkably effective since the discovery of antibacterial drugs, appearance of some resistant pathogens as well as undesirable side effect of certain antibiotics have led to the search for new antibacterial agents, in particular from medicinal plants [3-5]. Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases [6]. Numerous studies have reported that medicinal plants produce a large number of secondary metabolites with antimicrobial effects on pathogens [7, 8]. Therefore, the study of the antibacterial activity of some Indian traditional medicinal plants and the application in edible film has become an important research interest and a big challenge.

Wounds are any damage to or break of the skin or underlying tissues. The damage may be

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caused by accidents, incisions from surgery or others traumas [9]. The process of wound healing consists of integrated cellular and biochemical events leading to the reestablishment of structural and functional integrity with regain of strength of injured tissue. A therapeutic agent selected for the treatment of wounds should ideally improve one or more phases of healing without producing deleterious side effects [10]. By considering these above and other factors the present investigation was undertaken to evaluate the antibacterial and wound healing activities by using the chloroform extract of *Salvia leucantha.*

2. Materials and methods

2.1. Isolation and identification

Fresh flowers (2kg) of *S. leucantha* collected from the slopes of Kodaikanal, Dindugul District, Tamilnadu (India) and authenticated by Prof. N. Ramakrishnan, (Department of Botany) and voucher specimens (GACBOT-160) were deposited at the Herbarium of the Department of Botany, Government Arts College (Autonomous), Kumbakonam, Bharathidasan University, India. The flowers of *S. leucantha* were extracted with 90% methanol (MeOH) (4x500mL) under reflux. The alcoholic extract was concentrated *in vacuo* and the aqueous concentrate was fractionated with peroxide free ether (3x250mL) and chloroform (4x250mL).

The ether fraction was concentrated *in vacuo* and left in an ice-chest for a week. A yellow solid that separated was filtered and studied. On crystallization from MeOH, pale yellow needles were obtained [melting point: 313-315˚C]. It was readily soluble in organic solvents and sparingly in hot water. It reduced ammoniacal $AgNO_3$ in the cold and Fehling's solution on heating. It answered the Horhammer-Hansal, Wilson's boric acid and Gibb's tests. It chromatographic behavior and UV spectral data were all similar to those for the free aglycone flavonoid. It was identified as quercetin and the identity was confirmed by paper chromatography (p.c) and melting point (m.p) with an authentic sample of quercetin from *Physalisminima* [11, 12]. The R_f values are indicated in Table 1.

Table 1. R_f (X100) values of the constituents of the flowers of *salvia leucantha* (Whatmann no.1, ascending, 30±2˚C)

Compounds	*Developing solvents				
	a	b	c	d	e
[1]Aglycone	04	17	38	85	39
Quercetin (authentic)	04	17	38	85	39
[2]Glycoside	48	65	60	78	51
Glycoside (authentic)	48	65	60	78	51

Compounds

1) Aglycone - Quercetin

2) Glycoside - Quercetin-3-O- α-L-rhamnopyranoside-2^{II} -gallate

***Solvent key**

a) 15% aqueous acetic acid,

b) 30% aqueous acetic acid,

c) 60% aqueous acetic acid,

d) n-Butanol (B): Acetic acid (A): Water (W)=4:1:5 BAW (upper phase),

e) Phenol saturated with water.

The chloroform fraction was concentrated *in vacuo* and left in an ice-chest for a few days. A yellow solid [m.p. 316-318˚C] that separated was filtered and studied. It came out as pale yellow crystals on recrystallization from MeOH. It reduced ammoniacal $AgNO_3$ solution but not Fehling's solution. It appeared deep purple under UV that turned yellowish green on exposure to NH_3. It responded to Wilson's boric acid, Molisch and Gibb's tests, but did not answer the Horhammer-Hansal tests.

Supporting evidence for the structure of the flavonol glycoside is provided by the UV and NMR (100 MHz, DMSO-d_6 and TMS) spectral data were recorded on a Bruker AMX 400 NMR spectrometer.

^{1}H-NMR spectrum δ (400MHz, DMSO-d_6, TMS):δ ppm 12.64 (1H, s, 5-OH), 7.62 (1H, d, J=2.5Hz, H-2′), 7.60 (1H, dd, J=8.5, 2.5Hz, H-6′), 6.85 (1H, d, J=8.5Hz, H-5′), 6.80 (1H, d, J=2.5Hz, H-8), 6.43 (1H, d, J=2.5Hz, H-6), 5.49 (1H, d, J=6.9Hz, H-1″), 5.55 (1H, s, H-1‴), 5.4~3.0 (17H, m, glc and rha), 1.12 (3H, d, J = 6.0 Hz, -CH_3).

^{13}C- NMR(400 MHz, DMSO-d_6 and TMS):δ ppm 177.4 (C-4); 164.1 (C-7); 161.2 (C-5); 156.3 (C-2,9); 148.4 (C-4′); 144.8 (C-3′); 133.4 (C-3); 121.5 (C-6′); 121.2 (C-1′); 115.2 (C-2′,5′); 104.0 (C-10); 102.0 (C-6,1″); 93.5 (C-8); 71.9 (C-2″); 71.7 (C-3″); 71.3 (C-4″); 70.6 (C-5″); 17.6 (C-6″).

2.2. Hydrolysis of the glycoside

The glycoside dissolved in hot aqueous methanol was hydrolyzed with H_2SO_4 (5%) at 100˚C for about 2 hrs. The excess of alcohol was distilled off *in vacuo* and the resulting aqueous solution was extracted with ether. The residue from ether fraction was studied as described below. The glycoside was subjected to partial hydrolysis by treatment with 10% formic acid in cyclohexane and the resulting solution extracted with chloroform.

2.3. Antibacterial activity by agar diffusion method

The antibacterial activity of the isolated compound from chloroform extract was carried out by agar diffusion assay (National Committee for Clinical Laboratory Standards) using two different concentrations. The test microbe was taken from broth culture with an inoculating loop and transferred to a test tube containing 5.0mL sterile distilled water. The inoculum was added until the turbidity was equal to 0.5 McFarland standards. Cotton swab was then used to inoculate the test tube suspension onto the surface of Muller Hinton agar plate and the plate was allowed to dry. By using sterilized Whatmann paper disks (6mm in diameter) were transferred onto the agar surface. Two different concentrations of isolated compound were poured into the plates. The plates were incubated for 24 hours at 37°C and the zone of inhibition was measured in mm. Standard discs of the antibiotic Novobiocin (30µg), Chloramphenicol (30µg) served as the positive antibacterial controls.

2.4. Gel formulation

The chloroform extract thus obtained was evaporated at 40°C to dryness in a rotary evaporator in vacuum. The product thus obtained was used for gel formulation. 1g of the isolated compound was made up to 100g using Vaseline.

2.5. Wound healing activity by excision wound model

The animals were weight matched and placed into four groups (n=6 per group) for the experiment. They were anaesthetized and a full-thickness 2.5cm (width) x0.2cm (depth) excision wound was created. Group I animals served as normal control; animals in Groups II and III served as experimental animals with isolated drug at 100 and 200mg respectively and Group IV served as standard controls per day for sixteen days. Before starting the experiment on animals, the experimental protocol was subjected to the scrutiny of the Institutional Animal Ethics Committee (IAEC), Bharathidasan University, Trichirappalli, Tamilnadu, India (Approval No. BDU/IAEC/2011/31/29.03.2011). The treatment was done topically in all the cases. Changes in wound areas were calculated, giving an indication of the rate of wound contraction. Wound areas were measured on days 0, 4, 8, 12 and 16 for all the groups. Wound contraction was calculated as percentage of the reduction in wound area Percentage of wound contraction=[(Initial wound area-Specific day wound area)/Initial wound area]x100.

2.6. Statistical analysis

The experimental results were expressed as multiple comparisons of Mean±SEM were carried out by one way analysis of variance (ANOVA) followed by Dunnet Multiple Comparisons Test and statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1. Chemical constituents

The flowers of *S. leucantha* have been found to contain quercetin and its Quercetin-3-O- α-L-rhamnopyranoside -2′′- gallate (Figure 1). The UV spectrum of the aglycone exhibited two major peaks at 370 nm (band-I) and 255 nm (band-II), to reveal a flavonoid skeleton. Decomposition was observed on the addition of NaOMe to the aglycone. Since flavonoid which has free hydroxyl groups at the 3, 3′ and 4′-positions are unstable in NaOMe and the absorption peaks degenerated in a few minutes, it was inferred that there was free -OH group at C-3, C-3′ and C-4′ in the compound. A shift of +58nm on the addition of $AlCl_3$-HCl showed the presence of a free 5-OH in the A-ring. A shift of +30nm was observed in the case of $AlCl_3$ without acid, which also revealed a B-ring showed O-dihydroxyl group. The presence of a free -OH at C-7 was ascertained by a shift of +20nm (band-II) on the addition of NaOAc. The catechol type of dihydroxyl group in B-ring was further evidenced by the bathochromic shift +16nm on the addition of H_3BO_3.

The UV spectrum of the flavonol glycoside from the chloroform fraction showed two absorption peaks at 255nm and 340nm. A bathochromic shift of 54nm (band-I) showed that the NaOMe spectrum indicated the presence of a free -OH at C-4′. The $AlCl_3$/HCl spectrum showed a bathochromic shift of +60nm ascertained the presence of a free 5-OH. The band-I absorption in $AlCl_3$ spectrum is +30nm more than that showed on the addition of $AlCl_3$/HCl. This indicates that O-dihydroxyl group presents in the B- ring. The presence of a free -OH at C-7 was evident from the bathochromic shift of +17nm (band-II) on the addition of NaOAc. The dihydroxyl group in B- ring was further evidenced by the bathochromic shift of +27nm on the addition of H_3BO_3.

In the ^{1}H-NMR spectrum of the isolated compound appears at δ 5.90ppm and δ 6.20ppm corresponding to C-6 and C-8 protons respectively. The 5-OH proton resonates at δ 12.70ppm

and the signals at δ 7.30ppm correspond to the protons at C-2′ and C-6′. The protons at C-5′ appear at δ 6.80ppm and H-1″ of rhamnose resonates at δ 4.40ppm respectively. The three methyl protons of the sugar rhamnose appear at δ 0.90ppm. The rest of the sugar protons appear in the range δ 3.20-3.90ppm. The ^{13}C NMR data indicated that were 27 carbons in this structure, 15 of which were typical for a flavone skeleton, and the others were assigned to glycoside. Analysis of ^{1}H and ^{13}C-NMR data revealed that the aromatic signals are close to those reported for quercetin moiety [13, 14]. The sugar signals ^{13}C-NMR, δ ppm 102.0, 71.9, 71.7, 71.3, 70.6 and 17.6 are comparable with those reported for α-L-rhamnopyranoside [15], ^{1}H showed the presence of anomeric proton signal at , δ ppm 5.30 (1H, brs) indicated the presence of α- Linked sugar. The sugar moiety was proved to be acylated at C-3 of the aglycone as deduced from the correlation between the anomeric proton at δ ppm 5.30 and the C-3 at δ ppm 133.4.

Based on the above mentioned physical and chemical evidences the aglycone and glycoside obtained from *S. leucantha* flowers has been characterized as the quercetin-3-O- α-L-rhamnopyranoside -2″ -gallate with those reported for similar compounds [11-13].

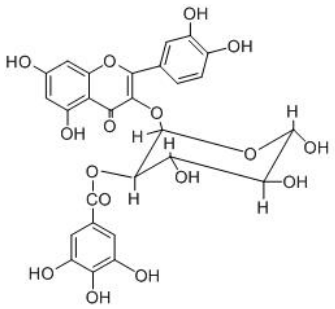

Figure 1. Quercetin-3-O- α-L- rhamnopyranoside -2″ -gallate

3.2. Antibacterial activity study

Many plants and their constituents have been investigated for their antibacterial activity [16]. Quercetin seems to exert antibacterial activity against almost all the strains of bacteria known to cause respiratory, gastrointestinal, skin and urinary disorders [17]. In the present study we investigated the flowers of *S. leucantha* for their antibacterial properties using agar diffusion method. The clinically important strains at three different concentrations at 50, 100 and 200µg of chloroform extract from *S. leucantha* were tested. The antibacterial activity against clinical isolates of *Staphylococcus aureus, Bacillus subtillis, Sarcina lutea* gram positive group and *E. coli*, *Pseudomonas aeruginosa* and *Candida albicans* gram negative group. These bacteria were chosen to be studied as they are important pathogens and also due to rapidly developed antibiotic resistance as antibiotic use increases.

The antibacterial activity of chloroform extract was compared with the standard antibiotics such as Novobiocin and Chloramphenicol in Table 2. In general, the mean zone of inhibition produced by the commercial antibiotics was between 26.0 and 36.0mm at 50µg and the inhibition produced by quercetin glycosides which was between 2.0 and 28.0mm. Based on the results, the chloroform extract at 200µg showed the maximum zone of inhibition when compared with the commercial antibiotic against all the tested microorganisms and also it was more active against *S. aureus* and *E. coli,* no growth was observed in both organisms. A slight decrease inhibition zone was observed for *S. aureus* at 200µg when compared with other tested microorganisms. However, the quercetin glycosides did not exhibit any activity against *C. albicans*.

Table 2. Anti-microbial effects of quercetin glycoside drug determined by agar diffusion method (zone of inhibition in mm)

Microorganisms	Quercetin glycoside (50µg)	Quercetin glycoside (100µg)	Quercetin glycoside (200µg)	Standard-1 novobiocin (50µg)	Standard-2 chloramphenicol (50µg)
S. aureus	16	22	28	33	-
B. subtillis	6	13	17	36	-
S. lutea	8	15	19	-	27
E. coli	9	17	24	-	28
P. aeruginosa	2	5	10	31	-
C. albicans	-	-	-	-	26

Note: (-) indicates no inhibition.

3.3. Wound healing activity

The classical system of Indian medicine especially Ayurveda, Siddha and Unani employ a large number of medicinal plants for treatment of skin disease, which includes cut wounds and burns [10]. The present investigation revealed that the quercetin-3-O- α-L- rhamnopyranoside -2″ -gallate extracted from *S. leucantha* was investigated for its wound healing activity by comparing it with the standard soframycin ointment. Albino rats were used as animal models where a control is used for the purpose of deciding the healing property. The clinical observations on wound healing were discussed below. The studies on excision wound healing model reveals that all the four groups showed decreased wound area day by day. On complete wound closure, epithelization was observed on the 16th day (Table 3). All readings are found to be statistically significant and comparable with control. However, on 16th post wounding day, the period of epithelization 62.46±2.29 was found to be control animals (which may be due to self-immunity) whereas soframycin treated animals showed 18.93±2.21. On the other hand, the quercetin treated groups showed 46.18±1.79 (Group-II) and 31.50±1.56 (Group-III) respectively. On the 12th day, the standard and chloroform extract at 200mg treated animals showed significantly greater wound closure when compared to control animals.

Table 3. Effect of drug on excision wound [wound area (mm^2)]

Oral treatment	Wound contraction (mm^2/rat) (M±SD)				
	0 day	4th day	8th day	12th day	16th day
Group I	365.83±1.52	274.00±2.89	260.30±1.23	124.14±1.08	62.46±2.29
Group II	348.87±2.17	251.67±1.58	182.87±2.03	98.02±2.26	46.18±1.79
Group III	378.10±3.02	236.80±2.16	164.11±2.98	78.45±1.68	31.50±1.56
Group IV	386.43±3.27	258.43±1.54	134.10±3.61	48.23±1.64	18.93±2.21

Values are expressed by mean ± stranded deviation (M±SD)
One-way ANOVA (Dunnetts method) Means for groups in homogeneous subsets are displayed.
Subset for alpha=0.05 level
Group (I): No treatment and served as controlled.
Group (II): Test group with wound and treated with isolated Quercetin glycoside (100mg/day).
Group (III): Test group with wound and treated with isolated Quercetin glycoside (200mg/day).
Group (IV): Test group with standard drug ointment (Soframycin).

4. Conclusions

The present investigations our results demonstrate that quercetin-3-O- α-L-rhamnopyranoside -2′′- gallate extracted from *S. leucantha p*ossess significant antibacterial and wound healing activity. The drugs are capable of inhibiting the growth of the organism. The highest inhibitory zone was observed in *S. aureus* (28mm), and *E. coli* (24mm). Thus the study ascertains the value of plants used in ayurveda, which could be of considerable interest to the development of new drugs.

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