Research Article

Antibacterial and Wound Healing Activities of Quercetin-3-O-A-L-Rhamnopyranosyl-(1→6)-β-D-Glucopyranoside Isolated from *Salvia leucantha*

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ABSTRACT

Flavonoids were isolated from *Salvia leucantha* using column and thin layer chromatography separation techniques. By comparing their melting points, R_f values and UV, ^{1}H-NMR, ^{13}C-NMR spectral data proved it to be quercetin – 3 – O – α - L-rhamnopyranosyl - (1 → 6) – β – D – glucopyranoside. The present study is aimed to investigate the agar diffusion antibacterial and wound healing activities of isolated compound against different microorganisms and excision wound model in albino rats, respectively. The results revealed most valuable information and also support the continued sustainable use of quercetin glycoside isolated from *Salvia leucantha* in traditional system of medicine.

Keywords: Antibacterial activity, Quercetin – 3 – O – α - L-rhamnopyranosyl - (1 → 6) – β – D – glucopyranoside, *Salvia leucantha*, Wound healing.

INTRODUCTION

Medicinal plants are important sources for the study of their traditional uses through the verification of pharmacological effects and can be natural composite sources that act as new anti-infectious agents.[1] Chemicals act as antimicrobial compounds which inhibit the growth of undesirable micro-organisms. Though a number of traditional or regulatory approved antimicrobials exist, they have many limitations.[2,3] Researchers are increasingly turning their attention to natural products looking for new leads to develop better drug against cancer as well as viral and microbial infections.[4,5] 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 [6, 7] have led to the search for new antibacterial agents, in particular from medicinal plants.[8-10] In 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.[11]

Wounds are any damage to or break of the skin or underlying tissues. The damage may be caused by accidents, incisions from surgery or others traumas.[12] 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.[13] Therefore, the aim of treating a wound is to either shorten the time required for healing or to minimize the undesired consequences.[14]

By considering these above and other factors the present investigation was undertaken to evaluate the antibacterial and wound healing activities by using the ethyl acetate extract of *Salvia leucantha.*

MATERIALS AND METHODS

Plant material

Fresh flowers (2 kg) of *Salvia leucantha* were collected from the slopes of Kodaikanal, Tamilnadu (India) in the month of Feb - March 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 *Salvia leucantha* were extracted with 90% MeOH under reflux. The alcoholic extract was concentrated *in vacuo* and the aqueous concentrate successively fractionated with peroxide - free ether and ethyl acetate (Sigma Aldrich Co., India).

Phytochemical screening of plant extract

A small amount of the dry extract was used for the phytochemical tests[15] for compounds which include alkaloids, flavonoids, tannins, saponins, glycosides, phenol and terpenoids while steroids, coumarin and cardiac glycosides are absent in all the crude extracts.

Isolation and identification

The isolation of the methanol extract was subjected to column chromatography with silica gel (60-120 mesh) as the stationary phase. The charged column was then eluted with different mobile phases with gradual increase in polarity. The fractions were collected and the solvent recovered by simple distillation. All the concentrated fractions were subjected to TLC for the identification of the desired bands.

TLC was performed on the 20 × 20 cm plates precoated with silica gel (Sigma Aldrich Co., India). TLC analysis of

ether and ethyl acetate extracts were performed using three different developing solvent systems (BuOH - AcOH – H2O, 4 : 1 : 5 (in volume); EtOAc – HCOOH – H2O, 10 : 2 : 3 (in volume); 15% AcOH. Based on the R_f - value, number of fractions were obtained and the one with good resolution was visualized under ultraviolet (UV) light, indicating that it was a pure compound, was selected.

Supporting evidence for the structure of the flavone and glycoside is provided by the UV and NMR (125 MHz, CDCl3) spectral data that were recorded on a Bruker AMX 400 NMR spectrometer. Chemical shifts were referenced to the respective residual solvent peaks and the values were recorded in δ.

Ether fraction

The ether fraction was concentrated *in vacuo* and left in an ice-chest for a week. The residue from the ether fraction of the hydrosylate was taken up in acetone and left under chilled conditions for a few days when yellow needles (m.p. 316˚C) were obtained. It answered the Horhammer-Hansal, Wilson's boric acid and Gibb's tests. TLC chromatograms observed in the above three solvent systems of the ether extract spots were characterized by R_f - values and color under UV light. It had λ_{max}^{MeOH} nm 255, 269 sh, 370; +NaOMe 262 sh, 322, 420; +$AlCl_3$ 267, 303, 458; + ($AlCl_3$ - HCl) 267, 303, 351, 428; +NaOAc 275, 328, 390 and + (NaOAc - H_3BO_3) 262, 303 sh, 386 nm. Its R_f were 0.81, 0.94, 0.28.

Ethyl acetate fraction

The ethyl acetate fraction was concentrated *in vacuo* and left in an ice-chest for a few days. A yellow solid that separated was filtered and studied. It came out as pale yellow crystals (m.p. 242˚C) on recrystallization from methanol. 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. TLC chromatograms observed in the above three solvent systems of the ethyl acetate extract spots were characterized by R_f - values and color under UV light. UV spectra had λ_{max}^{MeOH} nm 255, 266sh, 299sh, 340; +NaOMe 270,326, 394; +$AlCl_3$ 275, 302 sh, 330,430; + ($AlCl_3$ / HCl) 272, 303 sh, 352, 400; +NaOAc 272, 320sh, 370; and + (NaOAc/ H_3BO_3) 259, 301 sh, 367, Its R_f were 0.64, 0.33, 0.60**.**

Antibacterial activity by agar diffusion method

The antibacterial activity of the isolated compound from ethyl acetate 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 5.0 mL 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 (6 mm 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 (30 µg), Chloramphenicol (30 µg) served as the positive antibacterial controls.

Wound healing activity by excision wound model

Experimental Animals

Healthy albino rats (Wistar strain) of either sex and of approximately the same age, weighing about 200-250 g were used for the study. They were fed with standard diet and water adlibitum. They were individually housed, maintained in polypropylene cages under standard conditions. 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 animals were weight matched and placed into three groups (n = 6 per group) for the experiment. They were anaesthetized and a full-thickness 2.5 cm (width) X 0.2 cm (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 and standard controls per day for sixteen days respectively. 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.

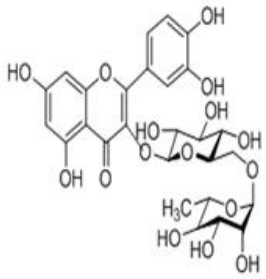

Figure 1. Quercetin - 3 - O - α - L-rhamnopyranosyl - (1 → 6) - β - D - glucopyranoside

RESULTS AND DISCUSSION

Chemical constituents

The flowers of *Salvia leucantha* have been found to contain Quercetin-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside. The UV spectrum of the aglycone from the ether fraction exhibited two major peaks at 370 nm (band-I) and 255 nm (band-II), to reveal a flavonoid skeleton. The UV spectrum of the flavonol glycoside from the ethyl acetate fraction exhibited two absorption peaks at 255 nm and 340 nm indicating a flavonol skeleton. The ^{1}H and ^{13}C NMR spectra (Table 1) showed the expected signals in the aromatic region for the quercetin moiety in the flavonoids. The ^{13}C NMR data indicated that were 27

carbons in this structure, 15 of which were typical for a flavone skeleton, while others were assigned to glycoside. In the ^{1}H - NMR spectrum, A-ring protons at C-6 and C-8 appear separately at δ 5.90 ppm and δ 6.20 ppm respectively. The signal at δ 7.30 ppm corresponds to the protons at C-2^{I} and C-6^{I}. The protons at C-5^{I} appear at δ 6.80 ppm. The two signals were observed in the region characteristic for anomeric protons of sugars. Doublets at δ 5.32 ppm were assigned to glucose β-linked to the aglycone. Signals at δ 4.43 ppm corresponded to the anomeric proton of α – linked rhamnose[15]. The methyl protons of the sugar rhamnose appear at δ 0.98 ppm and rest of the sugar protons appear in the range δ 3.04 - 3.39 ppm. In ^{13}C NMR signals corresponding to the anomeric carbon of glucose were found at 100.58 ppm and those corresponding to rhamnose were seen at 101.02 ppm. The attachment of the rhamnose to C-6 of the glycosyl moiety was evidenced by the downfield shift of the glycosyl C-6 carbon resonance to δ 66.84 ppm and accompanying up field shift of the resonances of the adjacent carbons C-5 to 76.32 ppm. The chemical shift values of all the recorded sugar carbon resonances confirmed the pyranose form of the two sugar moieties in the Quercetin-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (Figure 1). 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 5.32 and the C-3 at δ ppm 133.4. By comparing their UV, ^{1}H-NMR and ^{13}C-NMR data it was proved to be Quercetin-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside with those reported for similar compound.[16]

Table 1: ^{13}C NMR and ^{1}H NMR spectroscopic data for quercetin-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside

^{13}C NMR (125 MHz,$CDCl_3$, δ in ppm, J in Hz)		**^{1}H NMR (500 MHz, $CDCl_3$, δ in ppm, J in Hz)**	
Aglycone	**Quercetin glycoside**	**Proton**	**δ_H**
4	177.40	6’	7.30 dd (2.26; 10.00)
2	156.30	2’	7.30 dd (2.26; 10.00)
7	164.10	5’	6.80 d (8.59)
5	161.20	8	6.20 d (1.98)
9	156.30	6	5.90 d (2.11)
4’	148.40	5-OH	12.70
3’	144.80	Glc-1”	5.32 d (7.60)
1’	121.20	Rha-1’”	4.43 d (1.26)
6’	121.50	Glc-6”	3.70 br d (9.17)
5’	115.20	Rha-Me	0.98 d (6.2)
2’	115.20	2”,3”,4”,5”,2’”,3’”,4’”,5’”	3.39 - 3.04 m
10	104.00		
3	133.40		
6	98.50		
8	93.50		
Glc 1”	100.58		
2”	73.92		
3”	75.74		
4”	70.44		
5”	76.32		
6”	66.84		
Rha 1”’	101.02		
2’”	70.21		
3’”	69.86		
4’”	71.74		
5”’	68.14		
6’”	17.61		

Table 2: Anti-microbial effect of quercetin glycoside drug at 200 µg /ml and 100 µg /ml by agar diffusion method

	Zone of inhibition in mm			
Microorganisms	**Quercetin glycoside 100 µg**	**Quercetin glycoside 200 µg**	**Standard-1 Novobiocin (30 µg)**	**Standard-2 Chloramphenicol (30 µg)**
Staphylococcus aureus	21	27	30	-
Bacillus subtillis	14	18	33	-
Sarcina lutea	17	19	-	24
Escherichia coli	19	22	-	26
Pseudomonas aeruginosa	6	11	32	-
Candida albicans	-	-	-	25

Note: (-) indicates no inhibition.

Antibacterial activity

Antibacterial activity of plant origin is effective in the treatment of several infections. The action of compounds containing phenolic hydroxyl groups may be related to the inhibition of hydrolytic enzyme or other interactions to inactivate microbial adhesions.[17] In this study, the ethyl acetate extract was studied for its antibacterial activity by using different clinically important strains at two different concentrations of 100 and 200 µg/disc by agar diffusion method. The microorganisms chosen to be studied were gram positive, *Staphylococcus aureus, Bacillus subtillis, Sarcina lutea* and gram negative *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. 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 activity of isolated quercetin double glycosides was compared with the standard antibiotics, as mentioned in Table 2. In general, the mean zone of inhibition produced by the commercial antibiotic Novobiocin and Chloramphenicol was between 24.0 and 33.0 mm and the inhibition produced by quercetin glycosides which was between 6.0 and 27.0 mm. Based on the results, the quercetin glycosides showed the maximum zone of inhibition when compared with the commercial antibiotic against all the tested microorganisms, followed by the quercetin glycosides extract which was more active against *Staphylococcus aureus* and *Escherichia coli* when compared with other tested microorganisms. In a research conducted using the isolated compound from *Salvia leucantha* higher range of zone of inhibition against *Staphylococcus aureus* and *Escherichia coli* bacteria's at 200 µg/ml was found. However, the quercetin glycosides did not exhibit any activity against *Candida albicans*.

Wound healing activity

The wound healing property of the yellow pigments is a common feature frequently observed in literature.[18] The present investigation revealed that the quercetin glycoside extracted from *Salvia leucantha* was proved to possess 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 three groups showed decreased wound area day by day. The wound control showed a time dependent increase in percent (Table 3). On complete wound closure, epithelization was observed on the 16th day. All readings are found to be statistically significant and comparable with control. On the 12th day, the standard and quercetin treated animals showed significantly greater wound closure when compared to control animals. However, on 16th post wounding day, the control animals showed 83.33% of healing (which may be due to self-immunity) whereas soframycin treated animals showed 97.66% healing. On the other hand, the quercetin treated group showed 91.83 % of wound healing. It was evident that the number of neutrophils, lymphocytes and macrophages were moderate at the level of studies.

Table 3: Effect of quercetin glycoside on excision wound [%wound closure]

Day	Group I	Group II	Group III
0	0%	0%	0%
4	24.30%	37.50%	33.33%
8	29.00%	58.33%	65.07%
12	66.66%	79.85%	87.50%
16	83.33%	91.66%	95.83%

CONCLUSION

On the basis of the results obtained in the present investigation, it is possible to conclude that the Quercetin glycoside has significant wound healing activity and also the drug was found to possess antibacterial activity for both gram positive and gram negative organisms. Our results indicate the potential usefulness of *Salvia leucantha*, in the treatment of various bacterial infections. Thus, these studies ascertain the value of plants used in ayurveda, which could be of considerable interest to the development of new drugs.

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