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ANTIOXIDANT, ANTIBACTERIAL AND GUT MODULATING ACTIVITIES OF *KALANCHOE LACINIATA*

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Anstract: *Kalanchoe laciniata* (L.) DC. (Crassulaceae) is a widely distributed plant in Africa and Asia. Traditionally, various communities use this plant for the treatment of a variety of ailments such as gut problems, allergic conditions and wounds. The current study was designed to explore the antibacterial, antioxidant and gut modulating activities of *K. laciniata* in order to provide scientific rationale for its traditional uses. Phytochemical compounds were assessed through screening 70% crude methanolic extract of *K. laciniata*. Its gut modulatory activity was evaluated by *in vitro* tissue experiments on rabbit jejunum which yielded maximal spasmogenic response of 28.4 ± 4.6% (n = 4) at 3 mg/mL, while spasmolytic response was recorded with EC_{50} value of 3.2 mg/mL (2.8-3.5, 95% CI, n = 5). In antibacterial assays crude extract was found effective against *Staphylococcus aureus* and *Bacillus subtilis,* with MIC value of 5 and 2.5 mg/mL, respectively. The testing of the methanolic crude extract for antioxidants resulted in total phenolic contents of 27.8 ± 1.8 mg GAE/g DW and 22.7 ± 2.1 mg AAE/g DW total antioxidant activity. It also scavenged 17.3 ± 3.0% of DPPH free radical when compared with quercitin.

Keywords: spasmolytic, spasmogenic, calcium channel blocking, cholinergic

Kalanchoe laciniata (L.) DC. (local name: Zakhm-e-Hayat) is an erect branched herb of family Crassulaceae, which is predominantly found in Africa and tropical Asia. The leaves of *K. laciniata* are widely used in Asia for counter-irritant remedies because of their astringent and antiseptic properties (1). In India, Philippines, Cambodia, Vietnam and Malaysia its leaves are used topically on wounds, to relief headache and cough, treat ulcer and to reduce body temperature. The juice of its leaves is taken orally in case of diarrhea, while the whole plant can be taken as a tea to reduce heart discomfort and gastric pain (2-4). Traditional healers suggest this plant in skin allergy, bronchitis, asthma and impaired digestion. Southeastern Ethiopians use the root extract of fresh plant intranasally as a remedy for tonsillitis (5).

A plenty of pharmacological attributes have been described in different species of genus *Kalanchoe*, such as *Kalanchoe pinnata* which possesses antitumor, antimicrobial, antiviral, antiulcer, anti-inflammatory, antileishmanial, immunosuppressive, antioxidant and hepatoprotective activities (6). *Kalanchoe crenata* have analgesic, anticonvulsant (7), and antihyperglycemic activities (8), while *Kalanchoe brasiliensis* showed antithyroid and immunosuppressive activities (9, 10). This clearly indicates that the genus *Kalanchoe* is quite rich in medicinal properties. Despite the wide range of reported long-established uses of *K. laciniata*, there is no evidence of any pharmacological research over this plant. Hence, the study was aimed to investigate antimicrobial, antioxidant and calcium channel blocking activities of crude extract of *K. laciniata* to provide scientific justification for its conventional uses.

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MATERIALS AND METHODS

Plant material and extract preparation

The dried aerial parts of *K. laciniata* were purchased from the herbal market of Lahore. Its identity was confirmed by the botanist Mr. Abdul Hameed at Cholistan Institute of Desert Studies, the Islamia University of Bahawalpur, and a specimen having voucher no: KL-AP-07-10-008, was submitted at the herbarium of Faculty of Pharmacy and Alternative Medicine, the Islamia University of Bahawalpur. About 500 g of plant material was cleaned from dirt, coarsely crushed and then it was immersed three times in 70% methanol and filtered initially by muslin cloth followed by Whatman No. 1 filter paper. All the filtrates were accumulated together and evaporated under reduced pressure at 35-40°C. After removal of solvent, solidified sticky brownish crude extract of *K. laciniata* (Kl.Cr) was retrieved with a yield of about 17.51% and was stored at -20°C.

Chemicals and reagents

All the chemicals used in this study were of analytical grade. NaCl, $CaCl_2$, KCl, $MgCl_2$, NaH_2PO_4, $NaHCO_3$, Na_2CO_3, Mueller Hinton agar and nutrient broth were purchased from Merck Millipore Darmstadt, Germany. Na_3PO_4, $(NH_4)_2MoO_4$, H_2SO_4, glucose, EDTA, 2,2-diphenyl-1-picrylhydrazyl (DPPH), verapamil, gallic acid, ascorbic acid, acetylcholine, Folin-Ciocalteu reagent and quercetin, were purchased from Sigma-Aldrich Co., while ampicillin and gentamicin antibiotic discs were purchased from Thermo Fisher Scientific Inc.

Animals and bacterial strains

Rabbits weighing 1-1.5 kg were purchased from local breeders, while albino mice having weight of 20-25 g, were obtained from National Institute of Health, Pakistan. The animals were kept at animal house under hygienic environment and provided with food and water. The experiments were performed in accordance with the guide for the care and use of laboratory animals (11). *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*, kindly provided by the Department of Biochemistry and Biotechnology, the Islamia University of Bahawalpur, were used to evaluate the antimicrobial activity of plant extract.

Phytochemical screening

Phytochemical contents of Kl.Cr were explored to detect the presence of saponins, alkaloids, flavonoids, tannins, anthraquinones, glycosides, steroids, ketones, monosaccharaides, carbohydrates and soluble starch by using standard methods as previously described by our research group (12).

In vitro isolated tissue experiments

In vitro tissue experiments were performed as described previously (13). On the day of experiment, a 24 h fasting rabbit was operated, its jejunum was removed and placed in pre warmed Tyrode solution (37°C), aerated with carbogen gas (5% CO_2 and 95% O_2). The jejunum tissue was cut into 1.5-2 cm pieces, cleaned from fatty tissues and fixed in 50 mL organ bath, filled with Tyrode solution (37°C) and continually aerated with carbogen gas. The jejunum tissue was equilibrated for 30 min and then treated with sub maximal strength of acetylcholine (0.3 µM) to stabilize the contractions. After stabilization, the effect of different concentrations of Kl.Cr on tissue was recorded by using student kymograph (MTA-786/1024). Spasmogenic effect was quantified by comparing with maximum contraction induced by high dose of acetylcholine (1 µM), while the spasmolytic response of Kl.Cr was observed by calculating the percentage reduction in spontaneous contractions. Spasmolytic response was further investigated by depolarizing jejunal tissue with high potassium (80 mM). After achieving sustained contractions, the tissue was treated with Kl.Cr in a cumulative manner to record relaxant effect on smooth muscles and percentage relaxation was calculated with reference to maximum contraction by high potassium. It is established fact that substances which inhibit high potassium induced contractions are also calcium influx blockers (14). To confirm that calcium channel blockade is the underlying mechanism for smooth muscle relaxing effect of Kl.Cr, we stabilized rabbit jejunum tissue in normal Tyrode solution, and then tissue was decalcified by incubating for 30 min in calcium-free Tyrode solution containing 0.1 mM EDTA. After 30 min, solution was changed with calcium-free, potassium-rich Tyrode solution and again allowed to stay for 30 min. Then, controlled $CaCl_2$-concentration curves were constructed and re-constructed until two superimposable curves were produced. The tissue was treated for 1 h with Kl.Cr, and again $CaCl_2$-induced curve was recorded. This whole procedure was performed with different concentrations of Kl.Cr and also with verapamil as a standard drug.

Agar disc diffusion assay

Antimicrobial effect of Kl.Cr was determined by the same method as previously described but

with some modifications (15). For agar disc diffusion assay, bacteria were cultured overnight in a nutrient broth at 37°C, then diluted to 1×10^8 colony forming units with sterile nutrient broth. This diluted bacterial culture was evenly spread on freshly prepared and solidified Mueller Hinton agar plates with the help of sterile cotton swab. By using sterile forceps, 6 mm filter paper discs each containing 10 mg of Kl.Cr, standard antibiotic discs of ampicillin (10 µg) and gentamicin (10 µg) were placed on agar plates at appropriate distance and incubated for 18-24 h at 37°C. On the next day, inhibition zones were measured and this experiment was repeated twice.

MIC and MBC determination

The minimum inhibitory concentration (MIC) was determined for bacterial strains which were sensitive to Kl.Cr in agar disc diffusion assay, by using previously described method (16). Briefly, 12 h old bacterial culture was adjusted with nutrient broth at 0.5 McFarland standard. Kl.Cr was solubilized in sterile water (20 mg/mL), followed by two fold serial dilutions ranging from 10 to 0.3 mg/mL. Then, 95 µL of nutrient broth and 5 µL of pre-diluted bacterial culture were added in 96 well plates under sterile conditions. A 100 µL of Kl.Cr initially prepared at concentration of 20 mg/mL was added into first well followed by 100 µL of each of serial dilutions added to the next wells so that final volume in each well became 200 µL. Ampicillin/gentamicin were used as standards, while nutrient broth and pure bacterial culture served as negative and positive control, respectively. For each bacteria separate plate was used to avoid the cross contamination. The plates were covered with lid, shook for 5 min on shaker, and then incubated for 24 h at 37°C. On the next day, plates were observed and confirmation of growth inhibition was done by sub-culturing 5 µL from clear wells, on nutrient agar for 24 h at 37°C. Experiments were performed three times and MIC was described as minimum concentration of Kl.Cr which caused inhibition of bacterial growth. Minimum bactericidal concentration was the lowest concentration which exhibited no growth after this re-plating.

Estimation of total phenolic contents

Total phenolic contents of Kl.Cr were estimated by the same procedure as described earlier (17). Pre-diluted Kl.Cr (125 µL) was mixed with Folin-Ciocalteu reagent (125 µL) and water (500 µL). Then, 1250 µL of Na_2CO_3 (7%) was added and the mixture was shaken. Final volume was made up to 3 mL with distilled water, and incubated in dark at 23°C for 90 min. After incubation, absorbance was measured at 760 nm, and phenolic contents were determined through calibration curve of gallic acid (0-500 µg/mL). The experiment was repeated three times.

Estimation of total antioxidant capacity

Phosphomolybdenum method was used to determine the total antioxidant activity of Kl.Cr as described (18). Briefly, 3 mL of reagent solution (28 mM sodium phosphate, 4 mM ammonium molybdate and 0.6 M sulfuric acid) was mixed with 0.3 mL of properly diluted Kl.Cr and incubated for 90 min at 95°C. After cooling it down to room temperature, absorbance was measured at 695 nm, while ascorbic acid served as standard. The experiment was performed three times.

DPPH radical scavenging assay

Kl.Cr was evaluated for its radical scavenging ability by using previously described method (19), with minor modifications. Ninety µL of DPPH solution (100 µM) was mixed with 10 µL of pre-diluted crude extract in 96-well plate and incubated for 30 min, at room temperature in dark place. After incubation, absorbance was measured at 517 nm, while quercitin served as standard antioxidant. The experiment was performed in triplicate. Percentage radical scavenging was calculated by applying the following formula:

Scavenging activity (%) = 100 – [Absorbance of test compound/Absorbance of control] ×100

Acute toxicity testing

To determine the toxicity of Kl.Cr we followed the preceding method (13). Mice were randomly divided into four groups and each group contained five animals. Group 1, 2, and 3 were orally given increasing concentrations of Kl.Cr; i.e., 0.3, 1 and 3 g/kg, respectively, while group 4 was given normal saline (0.9% NaCl, 10 mL/kg). The animals were observed for 24 h and allowed to have food and water *ad libitum*.

Statistical analysis

Statistical analysis was done by using Graph PAD prism 5.01 software. Results were presented as the mean ± standard error of the mean (SEM), and EC_{50} at 95% confidence interval (CI), with p value equal or lower than 0.05, while "n" shows number of experiments performed.

RESULTS

Phytochemical tests performed on Kl.Cr indicated that it contains flavonoids, saponins, glyco-

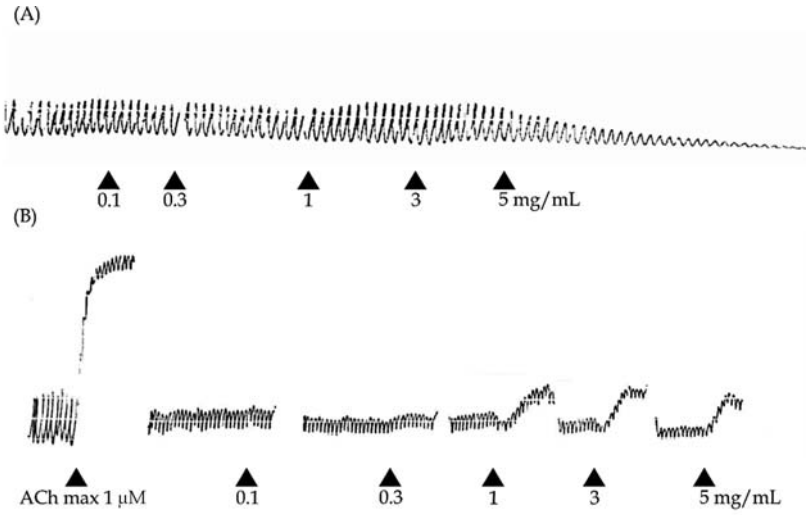

Figure 1. (A) Spontaneous contractions and spasmolytic activity of *K. laciniata* crude extract checked in the presense of atropine. (B) spasmogenic response of this extract checked in the absence of atropine

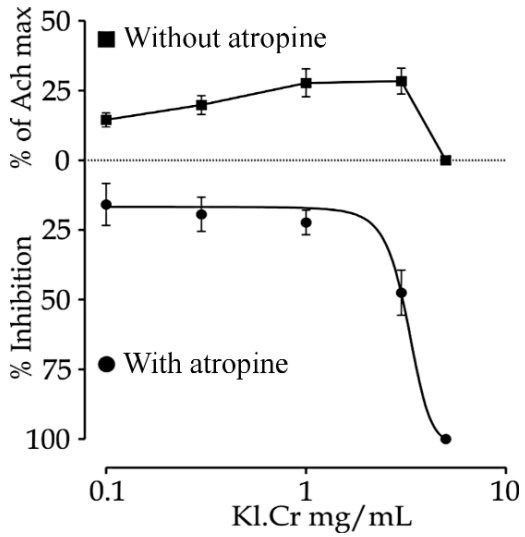

Figure 2. Spasmogenic and spasmolytic effect of *K. laciniata* crude extract on isolated rabbit jejunum tissue with (n = 5) and without atropine (n = 4)

sides, tannins, carbohydrates, steroids, soluble starch and pentoses. The crude extract of *K. laciniata* exhibited both spasmogenic and spasmolytic response when it was checked on spontaneously contracting rabbit jejunum tissue (Fig. 1). At concentrations of 1, 3 and 5 mg/mL, initially spasmogenic response was observed, which was followed by spasmolytic activity, the maximum contraction was induced at 1 and 3 mg/mL of Kl.Cr. The contractile response was assessed by comparing it with maximal dose of acetylcholine (1 µM), which was 27.7 ± 4.9% and 28.4 ± 4.6% (n = 4) of acetylcholine induced maximum contraction for 1 and 3 mg/mL, respectively (Fig. 2). To investigate the underlying mechanism of spasmogenic response we atropinized the jejunal tissue with 1 µM atropine. In atropinized tissue, spasmogenic response was abolished and Kl.Cr exhibited spasmolytic activity only, having EC_{50} value of 3.2 mg/mL (2.8-3.5, 95% CI, n = 5) as shown in Figure 2. The possible mechanism behind the spasmolytic effect of Kl.Cr was investigated by treating the jejunal tissue with high potassium (80 mM) to generate the sustained contractions, and then Kl.Cr was added in cumulative manner. The sustained contraction was completely relaxed at concentration of 5 mg/mL with EC_{50} value of 3 mg/mL (2.6-3.4, 95% CI, n = 4). It has been already established that calcium channel blockers can relax potassium induced contractions. So, spasmolytic activity was further investigated by constructing calcium-response curves in calcium-free and potassium-rich Tyrode solution. Kl.Cr shifted the calcium-response curve towards right at concentrations of 0.3 and 1 mg/mL (n = 4). This shift in curve was similar when compared with verapamil at concentration of 0.03 and 0.1 µM (n = 4) as shown in Figure 3, which confirmed calcium channel blocking activity of Kl.Cr.

In antibacterial activity, growth of *S. aureus* and *B. subtilis* was inhibited by Kl.Cr, but it was not effective against *E. coli*. The growth inhibitory effect was more pronounced on *B. subtilis* as compared to that of *S. aureus*. In agar disc diffusion assay, the zone of inhibition for *B. subtilis* was 14.6

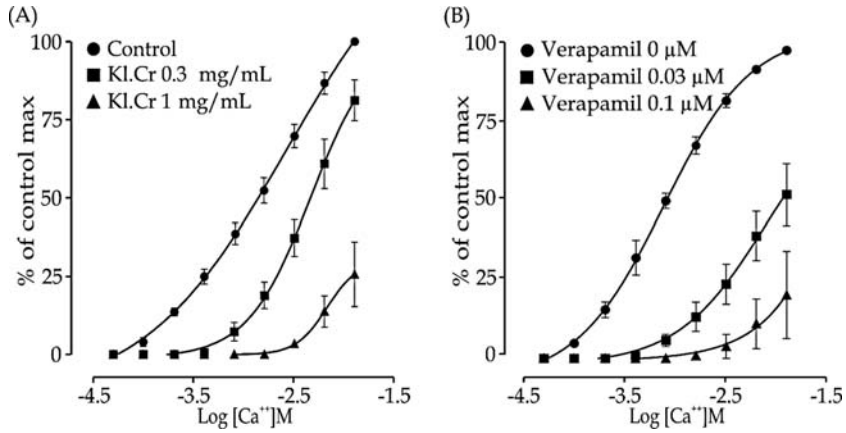

Figure 3. (A) Calcium-response curves constructed on isolated rabbit jejunum tissue in the presence and absence of different concentrations of *K. laciniata* crude extract. (B) Calcium-response curves in isolated rabbit jejunum tissue with and without verapamil. Values are shown as the mean ± SEM of 4 experiments

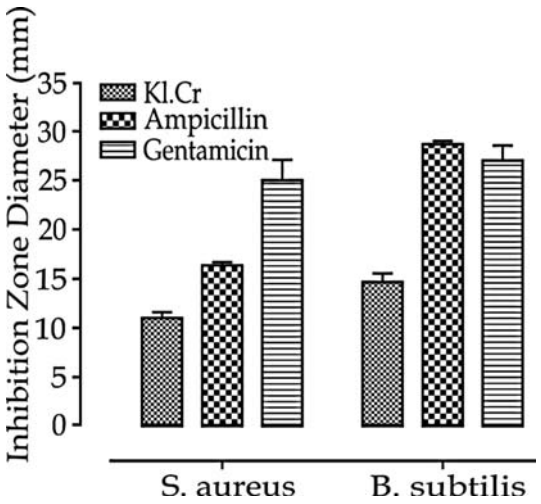

Figure 4. Comparison of inhibition zone diameter against *S. aureus* and *B. subtilis* by the *K. laciniata* crude extract, ampicillin and gentamicin. Values are shown as the mean ± SEM of three replicates

± 1.5 mm and for *S. aureus* it was 11 ± 1 mm (Fig. 4). Minimum inhibitory concentration of Kl.Cr was also determined, which was 5 and 2.5 mg/mL for *S. aureus* and *B. subtilis*, while 10 and 5 mg/mL were minimum bactericidal concentrations for *S. aureus* and *B. subtilis*, respectively.

In antioxidant assays, total phenolic contents of Kl.Cr were found 27.8 ± 1.8 mg equivalents of gallic acid per gram of crude plant extract while total antioxidant activity of Kl.Cr was 22.7 ± 2.1 mg ascorbic acid equivalents per gram of dry extract, which is comparable with total phenolic contents. So, antioxidant activity of Kl.Cr might be considered due to its phenolic contents. Finally, the radical scavenging potential of Kl.Cr was 17.3 ± 3.0% and 95.6 ± 0.01% for 0.1 mg/mL of Kl.Cr and quercitin, respectively (Fig. 5). Kl.Cr did not exhibit any lethal effect on mice in acute toxicity testing, so it was considered safe till the dose of 3 g/kg *p.o.*

DISCUSSION

Gut motility is the coordinated movement of gastrointestinal smooth muscles. This coordinated movement is responsible for normal processing of food we eat, while any abnormality in gastrointestinal movements results in disorders such as spasm, constipation and diarrhea. *K. laciniata* has been traditionally known for its use in treating dysentery, diarrhea and dyspepsia, so it was evaluated to provide pharmacological basis for its conventional uses. *In vitro* tissue experiments of Kl.Cr performed on isolated rabbit jejunum exhibited both spasmogenic and spasmolytic response. Spasmogenic action of crude plant extracts can be of cholinergic or non-cholinergic type, which can be differentiated by testing the plant extract in the presence of a relatively high concentration of the anti-cholinergic drug (20). The spasmogenic effect of Kl.Cr was completely abolished by the presence of atropine indicating the cholinergic mechanism. It has already been described that gastrointestinal smooth muscle contractions are mediated by M_3 receptors which are main muscarinic receptors in GIT (21), so it might be possible that Kl.Cr induces contractile response by interacting with M_3 receptors. Spasmolytic activity of crude plant extract is usually mediated by blockade of calcium channels (22, 23). Calcium channel blockers constitute an important

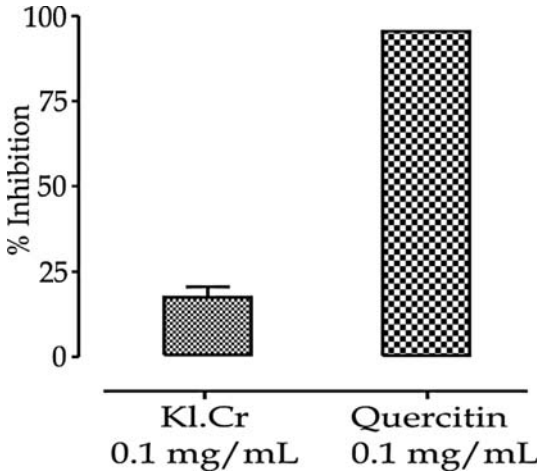

Figure 5. DPPH free radical scavenging activity of *K. laciniata* crude extract and quercetin. Values are shown as the mean ± SEM of three replicates

therapeutic group, they characteristically inhibit the slow entry of calcium in a dose dependent manner, which is reversed by calcium (24). Calcium-response curve constructed with Kl.Cr was almost identical to that of verapamil, which confirmed that Kl.Cr possesses calcium channel blocking activity. Both the cholinergic and calcium channel blocking activities of Kl.Cr can be considered due to the presence of saponins and flavonoids as these substances are reported to exhibit cholinergic and calcium channel blocking activities (13, 25).

Kl.Cr contained flavonoids, saponins, and tannins as indicated by its phytochemical analysis. Flavonoids and related polyphenols play an important role in protecting plants against microbial invasion (26), and nowadays polyphenols are gaining much focus due to their wide biological activities. Several recent studies have reported that flavonoids and flavonones isolated from plants were found to possess antimicrobial activity (27, 28) and the possible mechanism is thought to be metal ion deprivation, interaction with enzymes and destruction of microbial membranes (29). Tannins also exhibit toxicity to microorganisms by complexing various enzymes such as cellulases, pectinases, xylanases, peroxidases, laccase, and glycosyl transferase (30, 31), while Gram negative bacteria were found to be less sensitive than Gram positive bacteria, due to the coating of lipopolysaccharides on the surface of Gram negative bacteria which may repulse the phenolic compounds (32). Saponins also take part in plant defense systems and it was described before that they possess antimicrobial activity (33). Their mechanism of antibacterial action is not well understood yet, but it is known that Gram positive bacteria are more susceptible to saponins as compared to Gram negative bacteria, which is attributed to glucosidase enzyme synthesized in Gram negative bacteria that may degrade the saponins (34). In view of above discussion, it can be proposed that Kl.Cr exerts antimicrobial activity due to the presence of tannins, flavonoids and saponins and it is more active against Gram positive bacteria. Tannins and flavonoids also possess antioxidant activity and metal chelating properties. They are used therapeutically for their antioxidant, anti-inflammatory, wound healing, and anti-microbial activities (35).

CONCLUSION

It is concluded from the experimental data that crude extract of *K. laciniata* contains both concentration dependent cholinergic and calcium channel blocking activity, which accounts for its spasmogenic and spasmolytic response. It also possesses antibacterial activity against common skin pathogen *S. aureus* and antioxidant activity which provides reason for its traditional use in wound healing and skin problems.

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Declaration of interest

The authors declare no conflict of interest.

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