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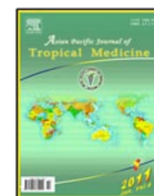

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Isolation and identification of bioactive antibacterial components in leaf extracts of *Vangueria spinosa* (Rubiaceae)

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ABSTRACT

Objective: The column chromatographic fraction of ethyl acetate (EA1, EA2, EA3, EA4 and EA5) leaf extracts of *Vangueria spinosa (V. spinosa)* were screened for antibacterial activity and phytochemical analysis. **Methods:** EA3 fraction was isolated and identified by column chromatography, thin layer chromatography, spectral data analysis and phytochemical screening were used for analysis. **Results:** EA3 fraction was significantly active at 4 to 64 mg/L against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* with minimum inhibitory concentration of 1.5625 to 3.1250 mg/mL. The active fraction (EA3) revealed the presence of flavonoid with retention factor value (R_f) of 0.39. The active antibacterial agent in the most potent fraction (EA3) was isolated and identified as flavonoid (-)-epicatechin-3-O-β-glucopyranoside by thin layer chromatography (TLC) and phytochemical screening. EA1 and EA2 show inhibitory activity at 4 to 64 mg/L against *Staphylococcus aureus* only where as fraction EA4 and EA5 do not shows any inhibitory activity within that range of concentration against any bacteria. **Conclusions:** The results support the ethnomedicinal use of leaf of *V. spinosa* for the treatment of bacterial diseases.

1. Introduction

There is global resurgence in the use of herbal preparations and in some developing countries like India; it is being gradually integrated into the primary and secondary health care systems. Nearly all societies have used herbal materials as sources of medicines and the development of these herbal medicines depended on local botanical flora. Several plants are indicated in folk and other traditional systems of medicines as anti-infective agents.

The extensive use of natural plants as primary health remedies due to their pharmacological properties is quite common[1]. The investigation of the efficacy of plant-based drugs has been paid great attention because of their few side effects, cheap and easy availability[2]. According to the World Health Organization 80% of the world population still relies mainly on plant drugs[3]. Growing misuse of antibiotics and chemotherapeutic agents leading to drug resistance[4] is now pushing a considerable proportion of people in both developed and developing countries to the use of herbal medicines. As a consequence of this in 1997, the 30th World Assembly adopted a resolution urging national governments of member nations to utilize their traditional systems of medicines with regulations suited to their national health care system.

Vangueria spinosa Roxb. (Rubiaceae)(*V. spinosa*) is a deciduous shrub or small tree that varies in height from 3-7 m, depending on the habitat. It can be single or multistemmed, but usually the latter. The bark is grayish to yellowish brown, smooth and peeling in irregular small strips. The leaves are single, oppositely arranged, as is typical of this family. The leaves are light green in colour, covered with soft, velvety short hairs and even more so when young. Antelope graze the leaves. This plant has some medicinal value too. An infusion of the roots and leaves has been used to treat malaria, chest ailments like pneumonia, as a purgative and to treat ringworms. An infusion of the leaves is used for the relief of toothache. For the treatment of swelling of the limbs the affected parts are bathed in a decoction of the pounded leaves and small twigs, especially in children[5-6]. It also possesses some antibacterial

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property[7,8]. Apart from some preliminary antibacterial effect, there is no reported phytochemical work identifying chemical constituents on *V. spinosa*.

Natural antimicrobial components in plants can inhibit the growth of bacteria by unknown mechanisms other than that of known antibiotics[9]. Resistance to almost all the known antibiotics has developed. For these reason, we suggest the continuation of the search for newer antibiotics. In an earlier work we reported the methanolic extract fraction of leaves of *V. spinosa* having higher activity than the aqueous extract on some selected bacterial isolates[7]. In present study, the *in vitro* antibacterial activity of the column chromatographic fractions of leaf extracts of *V. spinosa* was confirmed. Subsequently, the ethyl acetate extract of the leaf was fractionated using column chromatography and only the fraction (EA3) which tested positively for antibacterial activity was further isolated and identified. This study would contribute to the development of plant based antibacterial drugs.

2. Materials and methods

2.1. Plant materials

The plant material used in this study consists of leaves of *V. spinosa*, collected from a village named Koshigram of Burdwan district (23°16′N, 87°54′E), WB, India during spring (mid-March to mid-April 2008) and taxonomically authenticated by Dr. Ambarish Mukherjee, Department of Botany, The University of Burdwan. Burdwan, West Bengal, India. Voucher specimen (voucher no. 112) was deposited in the herbarium of Department of Botany, The University of Burdwan. Burdwan, West Bengal, India. The leaves were initially rinsed with distilled water and dried on paper towels in laboratory at (37+1) ℃ for 24 h. Exposure to sunlight was avoided to prevent the loss of active components. After drying, the leaves were ground separately in a grinding machine (MX-110 PN, Japan) in the laboratory. It was sieved and stored in a sealed plastic container until required.

2.2. Test bacterial strains

Four bacterial strains were used during the study. Out of the four, three of the strains namely *Staphylococcus aureus* MTCC 2940, *Escherischia coli* MTCC 739 and *Pseudomonas aeruginosa* MTCC 2453 were obtained from Microbiology Laboratory of Burdwan Medical College, Burdwan, West Bengal, India and the remaining one, *Klebsiella pneumoniae* MTCC 432 was obtained from Institute of Microbial Technology, Chandigarh, India. The bacteria were grown in nutrient broth Hi-Media, M002 (Hi-Media Laboratories Limited Mumbai, India) at 37 ℃ and maintained on nutrient agar Hi-Media, M012 (Hi-Media Laboratories Limited Mumbai, India) slants at 4 ℃.

2.3. Other materials and chemicals

Most of the chemicals used were of analytical grade. Silica gel column (60 g, Si 60, 40-63 μm) was from Merck, Germany and commercially prepared TLC plate (Silica gel 60 F254 (Merck, Germany) plates, 20 × 20 cm, 1 mm thick) were used.

2.4. Extraction and fractionation procedure

The air dried powdered leaves (500 g) were soaked in $CHCl_3$: MeOH (1:1, v/v) and then in MeOH (100%). The solvent was removed under reduced pressure and the dried extracts were combined to give 23.5 g of crude green tar. This residue was suspended in 150 mL of water and successively partitioned between $CHCl_3$ and EtOAc to give 3.6 g and 7.1 g fractions, respectively. Evaporation of ethyl acetate to dryness in an oven at 40 ℃ yielded 30.2 % (w/w) residue. The procedure was repeated to obtain more residues. Part of the EtOAc fraction (7.1 g) was adsorbed on silica gel and subjected to chromatographic column. The fractions obtained were evaporated and the residues were screened for antibacterial activity.

2.5. Column chromatographic separation

The ethyl acetate fractions were chromatographed over silica gel column and eluted with ethyl acetate as described by Mbukwa *et al*,[10]. The residues obtained were: EA1 (1.3 g), EA2 (1.9 g), EA3 (2.9 g), EA4 (0.8 g) and EA5 (0.2 g). The column fractions were tested for antibacterial activity against four bacterial isolates (Table 1). The fraction(s) that exhibited significant activity was selected for minimum inhibition concentration (MIC) and the bioactive compound(s) in the most potent fraction (EA3) was further isolated and identified through TLC and phytochemical screening.

2.6. Isolation of flavonoids

10 g of the plant sample was extracted repeatedly with 100 mL of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper No. 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight[11].

2.7. TLC of isolated flavonoids

Ten microliters of the bioactive alkaloids were further fractioned by means of commercially prepared thin layer chromatography plates (Silica gel 60 F254 (Merck, Germany) plates, 20 × 20 cm, 1 mm thick) using acetone: water: 25% ammonium solution (90: 7: 3; v: v: v) as a mobile phase. Fifty milligrams of standard (-)-epicatechin (98% pure) obtained from Sigma Aldrich Chemical Co., St Louis, MO, USA was dissolved in 45 mL of 50% ethanol and 10 μL was applied as a band beside the sample solution. After the plate was

developed, the positions of the compounds were detected by spraying vaniline sulphuric acid. It was inspected immediately in day light. The R_f values of the bioactive fractions in the sample and the standard were determined[12].

2.8. Analysis of the active ingredient

The bioactive fraction was subjected to spectral analysis by UV-Vis, IR and Mass spectroscopy. The UV-Vis analysis was carried out using (UV-1601 PC, Shimadzu spectrophotometer) with medium scan speed and sampling interval of 0.5 sec. The IR spectroscopy analysis of the active fraction was done using KBr plates (JASCO FT-IR Model- 420) with a scanning speed of 2 mm sec^{-1}. Mass spectroscopy was done using GCMS - Shimadzu - QP5050A with RT-1.3. All solvents and reagents used were of analytical grade and purchased from Merck, Mumbai, India.

2.9. Phytochemical screening

It was done using standard procedures of Trease and Evans[13] and El-Olemyl *et al*,[12]. The extract was dissolved in absolute alcohol and chromatographed using pre-coated and pre-heated (100 ℃ for 30 minutes) glass plates (eight glass plates), which were prepared with silica gel G using Unpoplan coating apparatus (Shadon, London). After 5 minutes of drying, each of the plates was placed in the separate glass chamber for TLC analysis, with different solvent systems as the mobile phase. After the movement of solvent at the top of the plates, each plate was removed from the glass chamber and separately air-dried. After 10 minutes each of plates was sprayed with a different spraying reagent for the identification of appropriate phytochemical. The phytochemicals included in the study were sapogenins, steroid, terpenoids, flavonoids, alkaloid, essential oils and phenolics Qualitative test was carried out to indicate the presence of saponins (frothing test); whereas remaining phytochemicals were determined using TLC analysis by the application of suitable solvents and spray reagents and, in each case, R_f values were recorded.

2.10. Antibacterial activity

The antibacterial activity was done using hole-in-plate bioassay procedures as reported by Vlietinck *et al*[5]. Pure cultures of the organisms were inoculated onto Müller-Hinton nutrient broth (Becton Dickinson, Cockeysville, Md), incubated for 24 h at 37 ℃, diluted with sterile nutrient broth to a density of 9×10^8 cfu/mL equivalent to MC-Farland test tube number 3. The suspension was used to streak for confluent growth on the surface of Müller-Hinton agar plates with sterile swab. Using a sterile cork-

Table 1
Antibacterial activity of column chromatographic fractions of ethyl acetate extract of *V. spinosa* leaf.

Fraction	Fractions concentrations (mg/L)	*Staphylococcus aureus*	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
EA1	4	6.20±0.19	–	–	–
	8	7.14±0.02	–	–	–
	16	7.50±0.11	–	–	–
	32	8.32±0.2	–	–	–
	64	10.20±0.17	–	–	–
EA2	4	6.54±0.33	–	–	–
	8	8.12±0.13	–	–	–
	16	9.34±0.03	–	–	–
	32	11.15±0.07	–	–	–
	64	14.12±0.13	–	–	–
EA3	4	15.30±0.05	13.11±0.12	17.35±0..32	16.20±0.13
	8	22.08±0.13	21.20±0.17	23.10±0.08	25.10±0.08
	16	31.50±0.33	25.10±0.06	28.60±0.14	30.12±0.10
	32	38.32±0.12	32.40±0.32	33.14±0.17	35.40±0.15
	64	42.03±0.03	35.27±0.01	39.70±0..20	40.10±0.16
EA4	4	–	–	–	–
	8	–	–	–	–
	16	–	–	–	–
	32	–	–	–	–
	64	–	–	–	–
EA5	4	–	–	–	–
	8	–	–	–	–
	16	–	–	–	–
	32	–	–	–	–
	64	–	–	–	–
Water	–	–	–	–	–
Tetracycline	10	12.0 ± 0.17	13.03 ± 0.12	11.16 ± 0.18	15.4 ± 0.21

EA1 = Ethyl acetate fraction 1, EA2 = Ethyl acetate fraction 2, EA3 = Ethyl acetate fraction 3, EA4 = Ethyl acetate fraction 4, EA5 = Ethyl acetate fraction 5.

borer of 6 mm diameter, four holes were made in to the set agar in petri-dishes containing the bacterial culture. Concentrations of 4 to 64 mg/mL of the extracts were poured in to the wells. Tetracycline (10 mg/mL) was used as positive control. Antibacterial activity of (-)-epicatechin-3-O-β-glucopyranoside was also tested similarly. The plates were placed in the incubator at 37 ℃ overnight. Antibacterial activity was recorded if the zone of inhibition was greater than 9 mm. The significance of the difference of the antibacterial activities of the extracts was tested by one-way analysis of variance (ANOVA).

2.11. Determination of minimum inhibitory concentration (MIC)

The EA3 fraction of ethyl acetate extract that showed significant activity (P<0.05) was chosen to assay for MIC. This was determined by the standard method of Wariso and Ebong[14]. Nutrient broth was prepared and sterilized using autoclave. One mL of the prepared broth was dispensed in to the test tubes numbered 2-12 using sterile pipette. A stock solution containing 1 g of the extract in 10 mL of de-ionized water was prepared. Then 1 mL of the solution was dispensed into each of the tubes numbered 1 and 2. Subsequently, from tube 2, serial dilution was carried out and 1 mL from tube 2 was transferred up to tube number 10 and 1 mL from tube 10 was discarded. Tube 11 was control for sterility of the medium and tube 12 for viability of the organisms. An overnight culture (inoculums) of each of the test isolates was prepared in sterile nutrient broth 1: 100 (10^2 dilution of the broth). From this dilution, 1 mL of the inoculum was transferred into each tube from tube 2 to tube 12 with exception of tube 11, to which another sterile nutrient broth was added. The final concentration of the sample in each of the test tubes numbered 1-10 after dilution were 100; 50; 25; 12.5; 6.25; 3.125; 1.5625; 0.78125; 0.390625 and 0.1953125 mg/mL, respectively. Tetracycline was used as control. All tubes were incubated at 37 ℃ for 24-48 h and examined for growth. The last tube in which growth failed to occur was the MIC tube.

2.12. Statistical analysis

The data of the study was subjected to one way ANOVA. Statistical analysis was done using SPSS ver.11 software[15, 16].

2.13. (-)-epicatechin-3-O-β-glucopyranoside

Brown powder; m.p. 204 ℃. UV (MeOH) (λmax / nm): 212, 280. IR (KBr, cm^{-1}): 3431, 2923, 2922, 1650, 1600. ^{1}H-NMR (600 MHz, MeOH-d_4, δ/ppm): 5.10 (1H, *d*, J = 2.2 Hz, H-2), 2.75 (2H, *d*, J = 3.4 Hz, H-4), 6.03 (1H, *d*, J = 2.2 Hz, H-6), 5.89 (1H, *d*, J = 2.2 Hz, H-8), 6.78 (1H, br s, H-2′), 6.97 (1H, d, J = 10.0 Hz, H-5′), 6.54 (1H, br s, H-1′′), 4.83 (1H, br s, H-2′′), 4.65 (1H, *t*, J = 8.1 Hz, H-3′′), 4.34 (1H, *t*, J = 8.2 Hz, H-4′), 4.77 (1H, m, H-5′′), 4.20 (1H, *m*, H-6′′*a*), 4.46 (1H, *m*, H-6′′β). ^{13}C-NMR (MeOH-d_4, δ/ppm): 78.9 (C-2), 68.0 (C-3), 30.4 (C-4), 160.5 (C-5), 99.1 (C-6), 155.1 (C-7), 95.9 (C-8), 155.8 (C-9), 104.0 (C- 10), 132.9 (C-1′), 115.1(C-2′), 146.3 (C-3′), 146.4 (C-4′),116.0 (C-5′), 115.5 (C- 6′), 106.0 (C-1′′), 73.0 (C-2′′), 75.9 (C-3′′), 71.8 (C-4′′), 78.4 (C-5′′), 62.9 (C-6′′). EI-MS (*m/z*): 452 (M+), 256, 213, 170, 153, 125, 97. $C_{21}H_24O_{11}$.

3. Results

Fractionation of the ethyl acetate extract of leaf of *V. spinosa* by column chromatography on silica gel yielded five fractions (EA1, EA2, EA3, EA4 and EA5). The *in vitro* antibacterial activity of ethyl acetate column chromatographic fractions against four pathogenic bacteria was presented in Table 1. The ethyl acetate extract fraction (EA3) of the leaf has shown significant (F=224.39, P<0.05) inhibitory activity against the bacterial isolates tested at 4 to 64 mg/mL. Minimum inhibitory concentration (MIC) value of bioactive fraction (EA3) was presented in Table 2. The fraction indicated MIC of 1.5625 to 3.125 mg/mL against the isolates used. EA1 and EA2 show inhibitory activity at 4 to 64 mg/L against *S. aureus* only where as fraction EA4 and EA5 does not shows any inhibitory activity within that range of concentration on all the isolates used.

As such, different solvent extracts of some plants may have different pharmacological properties. Chatterjee *et al*,[7] reported methanolic leaf extracts of *V. spinosa* to have higher antibacterial activity than aqueous extract. Isolation and identification of the active constituents (flavonoid) in EA3 fraction on TLC indicated R_f value of 0.39 for EA3 fraction and 0.30 for the reference standard. Fraction EA3 gave a yellow color with vaniline sulphuric acid reagent indicating its flavonoid nature and its Mass revealed a molecular ion peak$[M]^+$ at m/z 452 corresponding to the molecular formula $C_{21}H_{24}O_{11}$.

The IR spectrum showed bonded OH at (3431 cm^{-1}) and an aromatic group at 1600 and 1650^{-1}. The 1H-NMR spectrum showed a pair of doublets at δ 2.7 and 2.8 ppm, assigned to the H-4 protons (coupled to each other with J = 16.7 Hz and to H-3 with J = 4.5 and 2.5 Hz), a doublet at 5.10 ppm (J = 2.2 Hz, H-2), a dtd signal at 4.45 ppm (J = 2.2, 3.4 Hz, H-3) and a pair of meta coupled doublets (J = 2.2 Hz) at 6.0 ppm (H-6) and 5.89 ppm (H-8). The ^{1}H-NMR spectrum showed a resonance due to an anomeric proton at 6.54 ppm (br, s, H-1′′), a broad signal at 4.80 ppm (H-2′′) and four other peaks, indicating that the glucose moiety is a β-D-glucopyranosyl group[17]. Also, the ^{13}C-NMR signals at C-2 and C-3 confirm that the compound suggested is (-)-epicatechin with a glucose moiety at C-3[18]. Based on the results mentioned above, it was concluded that EA3 is (-)-epicatechin with a β-D-glucopyranosyl at C-3.

The *in vitro* antibacterial activity of (diameters of the inhibitory zones) of (-)-epicatechin-3-O-β-glucopyranoside (4 mg/mL) against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* were (18.20±0.17) mm, (14.80±0.06) mm, (20.50±0.17) mm (17.60±0.19) mm, separately. (-)-epicatechin-3-O-β

Table 2
Minimum inhibitory concentration of bioactive ethyl acetate column chromatographic fraction (EA3) of *V. spinosa* leaf.

Fractions concentrations (mg/mL)	*Staphylococcus aureus*		*Escherichia coli*		*Klebsiella pneumoniae*		*Pseudomonas aeruginosa*	
	V. spinosa	TC	*V. spinosa*	TC	*V. spinosa*	TC	*V. spinosa*	TC
100	–	–	–	–	–	–	–	+
50	–	–	–	–	–	–	–	+
25	–	–	–	–	–	–	–	+
12.5	–	+	–	+	–	–	–	+
6.25	–	+	–	+	–	–	–	+
3.125	–	+	–	+	–	+	–	+
1.5625	–	+	–	+	+	+	+	+
0.78125	–	+	+	+	+	+	+	+
0.39625	+	+	+	+	+	+	+	+
0.1953125	+	+	+	+	+	+	+	+
Water (Negative control)	–	–	–	–	–	–	–	–

–= No growth of test organism, + = Growth of test organism, TC = Tetracyclin

Table 3
Phytochemical analysis of ethyl acetate column chromatographic fractions of *V. spinosa* leaf.

Extract fractions	Phytochemicals	Solvent used	Spraying reagent	R_f values	Conclusions
EA1	Sapogenins	Acetone–hexane (4:1)	Antimony chloride in concentrated hydrochloric acid	–	Absent
EA2				–	Absent
EA3				–	Absent
EA1	Alkaloids	Methanol–concentrated ammonium hydroxide(200:3)	Dragendroff	–	Absent
EA2				–	Absent
EA3				–	Absent
EA1	Steroids	Chloroform	Libermann–Buchard	–	Absent
EA2				–	Absent
EA3				–	Absent
EA1	Flavonoids	Chloroform–acetic acid–water (90:45:6)	Vanillin–sulphuric acid	0.34	Present (+)
EA2				0.30	Present (+)
EA3				0.39	Present (+++)
EA1	Essential oil	Chloroform–benzene (1:1)	Saturated alcoholic sodium acetate	–	Absent
EA2				–	Absent
EA3				–	Absent
EA1	Phenolics	Ethyl acetate–benzene (1:1)	Folin reagent	–	Absent
EA2				–	Absent
EA3				–	Absent
EA1	Terpenoids	Impregnation with chloroform treated with silver nitrate	Antimony chloride in chloroform	–	Absent
EA2				0.89	Present (+)
EA3				–	Absent

–glucopyranoside also has antibacterial potentiality which is slightly higher than our active fraction (EA3) in the same concentration (4 mg/mL). Phytochemical analysis of chloroform column chromatographic fractions of *V. spinosa* leaves were presented in Table 3. Presence of flavonoid in the active fraction (EA3) indicates that it is the principal antibacterial agent in the leaf extract of *V. spinosa*.

4. Discussions

Drug resistance in human pathogenic microorganism has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This condition has forced scientists to search for new antimicrobial substances from various sources[19, 20]. The antibacterial properties of leaf extracts of *V. spinosa* were shown to be an interesting field for application in pharmaceutical industry[7, 8]. The present study represents a systematic study on isolation and identification of bioactive compounds and antibacterial properties of *V. spinosa* against four pathogenic bacteria. The results support the view that *V. spinosa* is a potent antibacterial agent.

The results of the present study showing significant ($P<0.05$) inhibition of the bacterial isolates by the EA3 fraction only have really contradicted assertion. It is probable that the bioassay guided fractionation employed in the extraction, is responsible for the inactivity of the EA4 and EA5 chloroform fractions. The MIC studies show that *V. spinosa* ethyl acetate extract (EA3) fraction was the most potent against the pathogenic bacteria employed at very low doses. Hence, this re–validates its pharmacological and therapeutic potentials. This underscores the ethnobotanical evidence for

the selection of *V. spinosa* in the discovery of new array of bioactive compounds.

Present findings of low MIC values are comparable to those of[19,20] who indicated ethanolic extract of *Aloe vera* leaf gel, although a different family, with antibacterial activity against pathogenic bacteria at very low doses. Flavonoids in the EA3 fraction may be responsible for the antibacterial actions of *V. spinosa*. Flavonoids isolated from different plant species having antibacterial activities were also reported[21-23]. The mechanism of action of the active components (EA3) may be due to the inhibition of nucleic acid synthesis[24], inhibition of cytoplasmic membrane function[25] and inhibition of energy metabolism[26]. Flavonoids are also known for their radical scavenging activities[27]. Results of the present study revealed that the flavonoids isolated from *V. spinosa* leaves possess antibacterial activity. Further studies in our laboratory are in progress to elucidate the structure of the active compound.

Conflict of interest statement

We declare that we have no conflict of interest.

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