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RESEARCH ARTICLE

In vitro photoprotective, antioxidant and antibacterial activity of *Vernonia squarrosa* (D. Don) Less

Adwaita Das[1], Sunanda Burman[2], Goutam Chandra[2] & Abhijit Bandyopadhyay[1*]

[1]Cytogenetics and Molecular Biology Laboratory, Department of Botany, The University of Burdwan, Burdwan-713 104, West Bengal, India
[2]Mosquito, Microbiology and Nanotechnology Research Units, Parasitology Laboratory, Department of Zoology, The University of Burdwan, Burdwan-713 104, West Bengal, India
*Email: palmajua@gmail.com

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ABSTRACT

Vernonia squarrosa (D. Don) Less. (Asteraceae) is an ethnomedicinally important plant of unexplored medicinal potential. The Hydro-Methanolic Leaf Extract (HMLE) reveals presence of alkaloids, terpenoid, tannins, phenols, flavonoids, saponins and also cardiac glycosides with notable amount of phenol, flavonoid, β carotene and lycopene. The high antioxidant activity of HMLE when assessed by 2, 2 diphenyl 1-picrylhydrazyl (DPPH) shows significant EC_{50} value of 11.63 ± 2.60 µg/ml. Photo-protection efficiency of HMLE was determined and expressed as Sun Protection Factor (SPF). Reasonably high SPF value of 38 (SPF=38 at 200 µg/ml) provides UV blockage up to 98%. HMLE extract was also screened for antibacterial activity against four human pathogenic bacterial strains, *Staphylococcus aureus* (MTCC 2940), *Pseudomonas aeruginosa* (MTCC 2453), *Bacillus subtilis* (MTCC 441), *Escherichia coli* (MTCC 739). Results showed prominent antibacterial impact on these tested bacteria. Lupeol, a pentacyclic triterpenoid was found to be present as a dominant member in Gas chromatography–mass spectrometry (GC-MS) analysis of HMLE. Lupeol is a pentacyclic triterpenoid compound with diverse pharmaceutical applications. This is the maiden report of bioactive compounds from *V. squarrosa* highlighting their antibacterial, photoprotective and antioxidant activities.

Introduction

Vernonia squarrosa (D. Don) Less. is an ethnomedicinally important green member of Asteraceae. *V squarrosa* is a shrub of 70 cm in height. Stems and branches are striate, glabrous or sparsely hairy. Leaves normally oblong to ovate narrowed at both ends, dentate. Inflorescence capitulum, 3-4 flowers are arranged in axillary or terminal position. It is a little known, unexplored plant found mostly in the Chotanagpur plateau (Adjacent parts of Odisha, West Bengal, Bihar and Chhattisgarh) in India. It has several ethnomedicinal properties, out of which antimicrobial, ascarcidal, luxation is established. However, majority of its medicinal properties is yet to be revealed in details. These plants belong to an evolutionary advanced family Asteraceae which has notable medicinally important members. Naturally, *V. squarrosa* stimulates interest to explore its medicinal attributes. Some of its closely related *Vernonia* Schreb species those are exhibit distinct bioactivity as anti-inflammatory, antidiabetic, antimicrobial and cytotoxic properties both under *in vitro* and *in vivo* (1). Fruits and or seeds of *V. squarrosa* plant were reported to be broadly used as the medicine of Diarrhea (2). The flower head of *V. squarrosa* is mostly used as ascaricidal agents. The mature leaves of the plant were reported as a popular medicine for the treatment of luxation, ulcer, dropsy and wounds (3). Delang already reported that its fruit powder boiled in water is efficient for removing back pain and kidney stone (4). Previous reports are there on antimicrobial efficacy of aqueous and methanolic leaf extracts of the plant on two pathogenic gram negative bacteria, *Vibrio cholerae* (MTCC 3904) and *Shigella dysenteriae* (Medical isolates) (5).

Generally, deadly pathogenic microorganisms mainly bacteria, acquire resistance properties against broad spectrum antibiotics due to their random misuses (6). Indiscriminate use of antibiotics not only created resistance issues of target microbes but lack of

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newly discovered antibiotics have compounded the problem leading to the serious concern about effectiveness of antimicrobial drugs. In this backdrop, plant derived secondary metabolites offer viable alternative as novel antimicrobial substances. Plant derived secondary metabolites like phenolics, flavonoids, terpenoids, alkaloids have an almost limitless opportunity for searching antimicrobial substances because of their diverse phytoconstituents. It is assumed that more than 250000-500000 plant species are existing with diverge array of phenols, tannins, terpenoids, coumarins, quinones and alkaloids (7). Phenolic acid and flavonoid have the tremendous free radical scavenging ability, antiviral, antimicrobial, antiproliferative and apoptotic activities (8). Flavonoids are the major pigments which can absorb harmful UV radiation strongly (9). The triterpenoid compounds derived from *Centella asiatica* and *Panax notoginseng* were also established as potential absorber for UV radiation (10, 11).

Since solar radiation consists of electromagnetic wave associated with infrared, visible and UV light and as a consequence direct exposure of ultraviolet radiation through solar light has both positive as well as negative health effect on the human skin. Ultraviolet B spectrum is crucial for Vitamin D_3 synthesis as it penetrates the human skin to convert 7-dehydrocholestrol into vitamin D (12). UVR (Ultra Violate Radiation) ranges from 100 nm to 400 nm and can be divisible into three regions (UV C 100 nm-280 nm, UV B 280 nm-315 nm, UV A 315-400 nm). UV A region of the spectra declines strongly when it enters into the earth surface and drops to near zero to 290 nm (13). Longer exposure to UV light cause photo aging, pigmentation erythrema and oxidative skin damage as DNA molecules readily absorb UV radiation and are also become targets of mutation. UV B is a shorter length of radiation that can cause melanomas as well as basal and squamous cell carcinomas. The denaturation temperature of type I collagen is decreased when the intensity of UV radiation is increased and triple helical structure is destroyed (14). Exposure to various environmental factors leads free radical formation. The most common free radicals are oxygen or ROS (Reactive Oxygen Species), singlet oxygen and hydroxyl radical (15, 16). When they reach electron deficit condition they snatch electron from other molecules mainly DNA, that results DNA damage and mutagenesis. Plants epidermal cell, cuticular wax, flavonoids, phenolic acid, pigments can absorb incoming UV radiation up to 90-99 % (17). In modern lifestyles, sunbathing and holiday habit increases daily exposure of UV light to the skin. Some cosmetic products (Sunscreen, Shampoo) are used to absorb or filter the harmful effect of UV radiations without hampering the normal Vitamin D synthesis process. The photoprotective ability of a sunscreen is measured as a function of their SPF (Sun Protection Factor) value. To protect such oxidative damage, several naturally occurring food and dietary components like flavonoids, phenols, terpenoids and β carotene that are well recognized antioxidant as well as anti cancerous agents, are mixed to fortify pharmaceutical formulations of commercial sunscreens. This study was carried out to evaluate the bioactivity of *V. squarrosa* because to the best of the knowledge of the authors, no investigation was executed till now regarding phytochemical value, antioxidant potentiality and photoprotective ability. The authors analysed the potential antibacterial properties of HMLE as well, of two gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) and two gram negative (*Pseudomonas aeruginosa* and *Escherichia coli*) bacteria.

Materials and Methods

Plant material collection and authentication

V. squarrosa was collected (September, 2018) from Joypur forest area (23.0540 N & 87.4345 E) of Bankura district of West Bengal where the plant has been naturally inhabited. The plant sample was identified and authenticated by Dr. Bandana Bhattacharjee and Dr. Avishek Bhattachaejee, Botanical Survey of India. A voucher specimen was deposited in Central National Herbarium of Botanical Survey of India with the reference no. CAL0000031671.

Preparation of Hydro-methanolic leaf extracts

The fresh leaves were cleaned properly under running tap water and then shade dried in room temperature for 15 days. Then the material was ground to powder with a grinder. For preparation of Hydro-Methanolic Leaf Extract (HMLE), 20 gm of dried powdered leaves was loaded in Soxhlet apparatus containing 200 ml of 80% methanol maintaining 1:10 ratios. Extraction period was set for 8-10 h per day with total 90 h of extraction period by following the standard method with few modifications (18). Thereafter, solvents were collected and excess solvent was evaporated by using rotary evaporator (Buchi type) Residue thus obtained was collected and stored in refrigerator (4 °C) for further use.

Preliminary phytochemical screening

Preliminary phytochemical analysis was executed using the protocols by (19) for testing the presence of alkaloids, terpenoids, phenols, flavonoids, cardiac glycosides, tannins, saponins, steroids, anthroquinones in HMLE of *V. squarrosa*.

Determination of Total Phenols Content in the leaf extract

The phenolic content of HMLE was determined spectrophotometrically according to Folin-Ciocalteu method (20) with little modification. A stock solution of 1 mg/ml extract was prepared by dissolving the HMLE in distilled water. At first 1 ml of sample was taken and mixed with 5 ml of 1:10 Folin-Ciocalteu reagent and shaken gently. After 5 min 4 ml of 7.5% Na_2CO_3 solution was added to the mixture. After 60 min of dark incubation the absorbance of reaction mixture was measured at 765 nm (in triplicate). All values were expressed as µg/mg Gallic Acid Equivalent (GAE).

Estimation of Total Flavonoids

Total flavonoid content was determined by spectrophotometrically by using aluminium nitrate (21). At first, 1 ml of extract (HMLE) (1 mg/ml) was added with 0.1 ml of 10% aluminium nitrate. Then

0.1 ml of 1 M potassium acetate was added to mixture and allowed to stand for 2 min at room temperature. Then 3.8 ml ethanol was added to the mixture and was kept for 40 min. The absorbance was measured at 415 nm (in triplicate) and the absorbance value expressed as µg/mg Quercetin Equivalent (QE).

Estimation of Total Tannin Content

Vanillin hydrochloride method by Burns 1971 was used to quantify the total tannin content (22). Vanillin hydrochloride reagent (4%) was prepared by 4 gm vanillin in 100 ml methanol with 8% hydrochloric acid. This reagent was always freshly prepared just before use. To 5 ml reagent and 1 ml HMLE (1 mg/ml) was mixed and incubated for 20 min in room temperature. Absorbance was measured at 500 nm (in triplicate), Keeping vanillin reagent as blank. The absorbance value was expressed as µg/mg Phloro-Glucinol Eqivavalent (PGE).

Estimation of β carotene and Lycopene Content

Amount of β carotene and lycopene were determined by following protocol of (23, 24) with little modification. In this process, 100 mg of extract was shaken with 10 ml acetone-hexane mixture (4:6) for 1 min and absorbance was measured at 453, 505 and 663 nm. Consecutively by using the following formula lycopene and β carotene contents were computed (in triplicate).

β-carotene (mg/100 ml): $0.216\ A_{663} – 0.304\ A_{505} + 0.452\ A_{453}$

Lycopene (mg/100 ml): $–0.0458\ A_{663} + 0.372 A_{505} – 0.0806\ A_{453}$

Estimation of Antioxidant Activity by DPPH Method

DPPH assay: The traditional 2, 2 diphenyl 1-picrylhydrazyl free radical method was used in this study (25). A stock solution of 1 mg/ml of HMLE was prepared and diluted to following concentration *viz*. 10, 20, 40, 60, 80 and 100 µg/ml and mixed with 0.004 % methanol solution of DPPH in 1: 1 ratio. Scavenging process of DPPH was carried out at room temperature under dark condition for 30 min. Subsequently, gradual reduction in absorbance of the mixture was recorded at 517 nm keeping ascorbic acid as standard (in triplicate). EC_{50} value was taken a potent concentration where 50 % of scavenging of DPPH radicals by HMLE was computed within given incubation period. The scavenging efficacy is express by the following formulae

$$\% \text{ DPPH scavenging} = \{(A_0 - A_1)/A_0\} \times 100$$

A_0=Absorbance of control A_1=Absorbance of test sample

ABTS Radical Scavenging Assay

The ABTS free radical scavenging efficiency was evaluated by following previous method described by (26). Firstly, ABTS free radical was generated by the addition of 7 µM ABTS solution with 2.45 µM potassium persulfate solution in 1:0.5 proportion and the mixture was incubated in dark for 12-16 hr at room temperature for free radical conversion before use. Then the prepared $ABTS^{*+}$ solutions were diluted with ethanol up to OD of 0.7 ± 0.05 at 734 nm at 30° C. To evaluate the scavenging efficiency of HMLE, 10 µl sample (100-1000 µg/ml) was mixed with 1 ml of diluted $ABTS^{*}$. The absorbance was measured after 10 min of dark incubation at 734 nm using ascorbic acid as standard (in triplicate). Scavenging was finally expressed using following formulae.

$$\% \text{ ABTS scavenging} = \{(A_0 - A_1)/A_0\} \times 100$$

A_0=Absorbance of control A_1=Absorbance of test sample

Reducing Power Assay

Ferric ion reducing power of HMLE of *V. squarrosa* was determined by following the method of Oyaizu *et al.* 1986 with few changes (27). A stock solution of 1 mg/ml of HMLE was prepared and diluted with methanol following concentration *viz*. 10, 20, 40, 60, 80, 100 µg/ml. To 1 ml each of diluted extracts, 1 ml of 0.2 M phosphate buffer pH 6.6 was added in separate tubes, to which 1 ml of 1% (w/v) potassium ferricyanide was further mixed to each of the reaction mixture and was then incubated for 20 min at 50° C. Then 10 % 1 ml Trichloroacetic acids (TCA) were added in each reaction mixtures. After that, 2 ml of each reaction mixture was diluted with 2 ml of distilled water followed by addition of 0.4 ml ferric chloride solution (0.1% w/v). Absorbance was measured at 700 nm. Ascorbic acid was used as internal standard.

In Vitro Assessment of Sun Protection Factor of HMLE

SPF properties of the HMLE were examined as per the standard method (28). A stock solution of 1 mg/ml of crude leaf extract was prepared and diluted to following concentration *viz*. 50, 100, 200, 400, 600, 800, 1000 µg/ml. Absorption spectra of sample solution were obtained in the range of 200 nm to 400 nm taking distilled water as blank. The absorption data in triplicate were recorded by UV-Vis spectrophotometer (Shimadzu UV-1800) at every 5 nm interval from 290 to 320 nm. SPF value was expressed as a function of erythemal effect spectrum as per following formulae.

$$SPF = CF \times {}^{320}\Sigma_{290}\ EE\ (\lambda) \times I\ (\lambda) \times Abs\ (\lambda)$$

EE(λ)=erythemal effect spectrum. Abs(λ)=absorbance of sunscreen product. CF=correction factor (10). The values of EE × I remain constant (29).

Values of EE (λ) x I at different wavelength	Value of EE x I
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0837
320	0.0180

Determination of Antimicrobial Potentiality

Test Microorganism Antibacterial assay was conducted by agar well diffusion method (30) on four human-pathogenic bacterial strains *Staphylococcus aureus* MTCC 2940, *Bacillus subtilis* MTCC 441, *Escherichia coli* MTCC 739 and *Pseudomonas aeruginosa* MTCC 2453. Inoculum of each tested bacteria was prepared in Müeller-Hinton broth and turbidity was adjusted to 0.5 Mcfarland turbidity standards. Uniform wells of 6 mm in diameter were made on solidified agar. Each well was filled with 50 µl of HMLE (5000 µg/ml). Tetracycline (30 µg/ml) was kept as positive control. Plates (sets in triplicate) were then

incubated overnight at 37 °C. Wells filled with sterile distilled water was taken as the negative control. Antibacterial activity of the extract (HMLE) was determined by assessing the diameter of the clear zones around each well.

Determination of Minimum Inhibitory Concentration (MIC)

MIC is the lowest concentration of antibacterial agent that inhibits the growth of bacteria after 18-24 hr incubation. MIC of the tested extracts was evaluated by serial broth dilution technique (31, 32) in which overnight cultures of test bacterial strains grown in nutrient broth cultures were diluted 100 folds. Different concentrations (100-1000 µg/ml) of HMLE were prepared in distilled water and were added to each test tube containing the bacterial cultures to evaluate the MIC of leaf extract (HMLE) against all the strains. Test tubes were then incubated at 37 °C for about 24 hrs. Test bacteria inoculated inoculums were kept as negative control for each test batch. MIC of Tetracycline (positive control) was also assessed. The lowest concentration i.e. the highest dilution of the extract (HMLE) that produced no visible bacterial growth i.e. no turbidity in comparison to the relative turbidity (OD) of the positive control was considered as MIC value.

FT-IR Analysis

FT-IR (Fourier Transform Infrared Spectroscopy) is a powerful approach to identify the type of chemical bond (functional group) present in the compounds. The Hydro-Methanolic Leaf Extract (HMLE) of the plant mixed with KBr salt using a hydraulic press and compressed into thin tablets and IR spectra and peaks were recorded on a FT-IR (Jasco, FT/IR- 4700), with a scan range from 400 to 4500 cm^{-1}. Each analysis was done two times for confirmation. FT-IR spectroscopy reveals various functional groups present in the sample.

GC-MS Analysis

The purified fraction (filter sterilize by 0.22 µ syringe filter) was subjected to GC-MS analysis using TRACE GC Ultra method coupled with POLARIQ MS (MFd-thermo scientific with ion trap technology). Stationary phase used was DBMS column. GC-MS analyses were done with ionization energy of 70 eV. The initial oven temperature was programmed to 40 ˚C for 2 min, and was gradually increased by 3 ramps, first at a rate of 3 ˚C/min (hold time 1 min) to 130 ˚C, second at 2˚C/min (hold time 2 min) to 180 ˚C and 3rd at a rate of 3˚C/min until reaching the final temperature of 280 ˚C and holding for 15 min. The total run time was 60 min. Helium (He) was the carrier gas used at a linear flow-rate of 1.0 ml/min (99.999%). The scan range was at a rate of 0.7 scan/s from 50-900 m/z. Purity of each GC peak was checked by taking MS (m/z ratio) at various parts of each peak. All compounds were identified via mass spectral database search of National Institute of Standard Technologies (NIST, 2014) by the matching of MS data.

Statistical Analysis

The values were analyzed by using MS Excel 2007 and presented as mean ± SD of three replicates.

Results and Discussion

Qualitative and Quantitative Estimation of Secondary Metabolites of HMLE

Aqueous extraction is generally used for pharmacological screening but, since the aromatic organic compounds are generally active on microbial growth and behave as free radical scavengers, they are often dissolved in polar organic solvents like methanol or ethanol (33). Hydro-Methanol Leaf Extract (HMLE) was used for the study of antioxidant, photoprotective and antimicrobial and also for presence of bioactive organic compounds. Preliminary phytochemical investigations revealed the presence of phenols, flavonoids, tannins, cardiac glycosides, alkaloids (Table 1). The major chemical component found to be phenols (272.44 ± 9.78 µg/mg) as Gallic Acid Equivalent (GAE), followed by flavonoids (77.50 ± 1.41 µg/mg) and tannins (27.97 ± 3.15µg/mg). Whereas small quantity of β-carotene (53 ± 0.0009 µg/mg) and Lycopene (28 ± 0.003 µg/mg) were also detected (Table 2).

Table 1. Qualitaive phytochemical screening

Phytochemicals	HMLE
Alkaloid	+ Ve
Terpenoid	+ Ve
Phenols	+ Ve
Flavonoids	+ Ve
Cardiac glycosides	+ Ve
Steroides	- Ve
Saponins	+ Ve
Anthraquinones	- Ve

Table 2. Quantitative value of secondary metabolites

Parameters	HMLE
Phenol (µg GAE/mg of extract)	272.44 ± 9.78
Flavonoid (µg QE/mg of extract)	77.50 ± 1.41
Tannin (µg PG/mg of extract)	27.97 ± 3.15
β-carotene (µg/mg of extract)	53 ± 0.0009
Lycopene (µg/mg of extract	28 ± 0.003

Antioxidant Activity of Hydro-Methanol Leaf Extract (HMLE)

The stable free radical, DPPH (2, 2-diphenyl-1-picrylhydrazyl) when dissolved in methanol it produces violate color which contains N_2-centered free radical. The violet color of the solution turns yellow when the free radical was scavenged by the antioxidants agents. Hydro-Methanol Leaf Extract

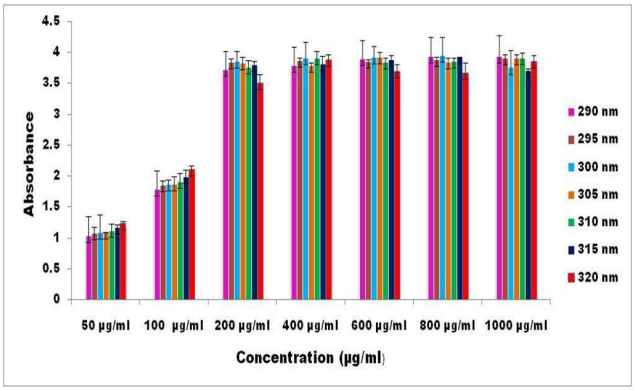

Fig. 2. Effect of HMLE concentration on the UV absorption capacity.

(HMLE) of *V. squarrosa* exhibited significant free radical quenching ability at the rate of 32.16, 51.12, 92.26, 95.76, 92.36 and 96.50 % at 10, 20, 40, 60, 80 and 100 µg/ml respectively (Fig. 1 A). Previously, (supplementary material Table S1-S7), which is concentration dependent too. The absorbance of the extract did not alter even when the concentration of extract was raised beyond 200 µg/ml. Consequently

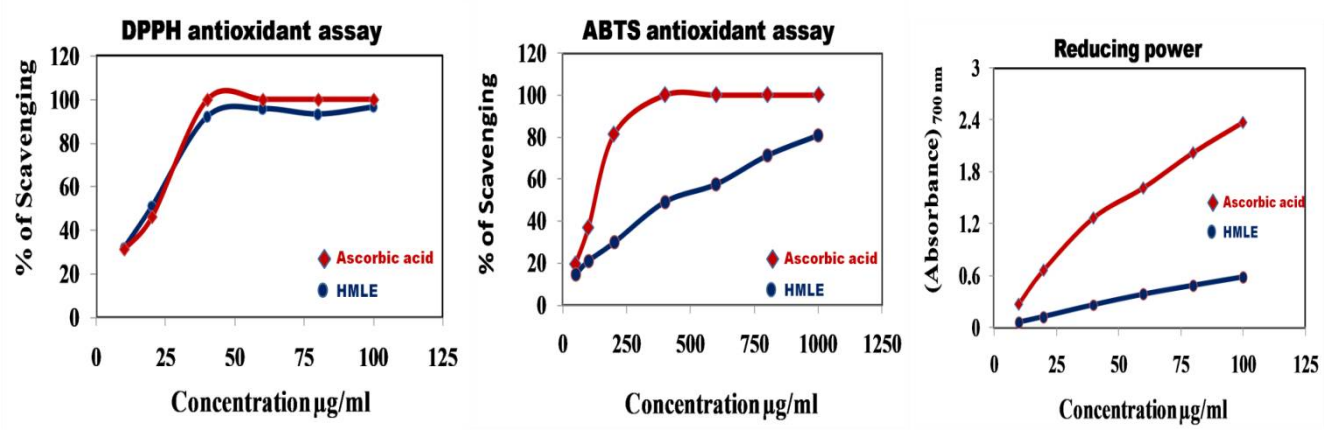

Fig. 1. Antioxidant activity of crude HMLE of *V. squarrosa* A) DPPH free radical scavenging ability B) ABTS free radical scavenging potentiality C) Reducing power of HMLE.

reported that methanolic (90%) leaf extract of *Vernonia cinerea* demonstrated DPPH free radical scavenging potentiality with 56 ± 6.92 EC_{50} Value (34). Whereas *V. squarrosa* (HMLE) exhibits far better antioxidant capabilities with EC_{50} value of 11.63 ± 2.60 µg/ml (Table 3). The free radical cation of ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) was generated after addition of potassium persulfate which leads to a blue coloration. The scavenging percentage of ABTS free radical was found to be

Table 3. *In vitro* antioxidant ability of HMLE of *V. squarrosa*

Antioxidant parameters	EC_{50} value of HMLE (µg/ml)	EC_{50} value of Ascorbic acid (µg/ml)
Scavenging ability of DPPH free radical	11.63 ± 2.60	13.33 ± 0.88
Scavenging ability of ABTS free radical	497.85 ± 12.38	86.18 ± 0.99
Reducing power	96.53 ± 0.83	14.93 ± 0.98

dependent on concentration of HMLE with a scavenging ability of 25.07, 31.40, 45.34, 58.10, 69.41 and 80.24 % at 100, 200, 400, 600, 800, and 1000 µg/ml concentration with EC_{50} value of 497.85 ± 12.38 µg/ml (Fig. 1 B, Table 3). Similarly, assay for reducing power of HMLE was carried out to evaluate electron donating capabilities of the HMLE for the reduction of potassium ferricyanide, which was also found to be concentration dependent. The EC_{50} value for the conversion of Fe^{3+}/ferricyanide to the ferrous form (Fe^{2+}) was recorded as 96.53 ± 0.83 µg/ml (Fig. 1 C, Table 3).

Estimation of Sun Protection Factors of Hydro-Methanol Leaf Extract (HMLE)

The SPF values were evaluated by the standard method (28). The SPF value of HMLF of *V. squarrosa* was determined by taking absorbance at 5 nm interval from 290 nm to 320 nm on different concentrations *viz*. 50, 100, 200, 400, 600, 800 and 1000 µg/ml (Fig. 2). The evaluated photoprotective activity (SPF value) recorded from 11.04-38.87 SPF value did not fluctuate too much and it stuck around a value of 38. The SPF value above 6 is appropriate for sunscreen formulation according to the Brazilian law, RDC 30 from June 1, 2012 (35, 36). According to SPF reference scale, sunscreen products can be classified as low protection (SPF<12), moderate (12-30) which can protect around 93% from UV B rays, SPF (30-50) high protection which can block harmful UV B radiation up to 97% (37, 38) (Fig. 3, Table 4). There are reports on the essential oil of *Calendula officinalis* (Asteraceae) possessed good photoprotective ability (SPF 14.84 ± 0.16) (39). Crude *Rosa kordesii* extract (SPF 20.15 ± 0.05) showing significant amount of UV ray blocking potentiality (40). The results of Hydro-Methanol Leaf Extract

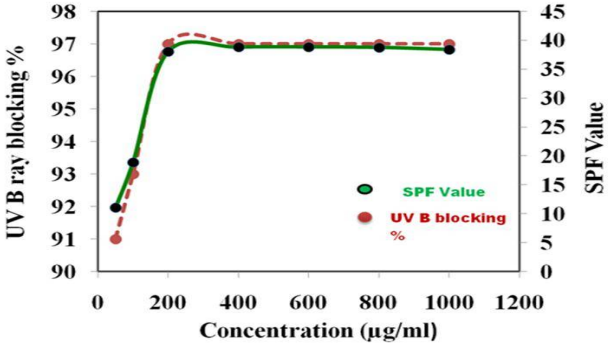

Fig. 3. Concentration dependent SPF value with its protection levels and UV B light blocking percentage.

Table 4. Concentration dependent SPF value with its protection levels

Concentration (µg/ml)	SPF value	Protection level (UV B blocking)
50	11.04 ± 3.04	Low protection
100	18.90 ± 0.54	Moderate
200	38.08 ± 1.60	High protection
400	38.47 ± 0.62	High protection
600	38.87 ± 0.28	High protection
800	38.80 ± 0.39	High protection
1000	38.44 ± 0.70	High protection

(HMLE) clearly indicate that *V. squarrosa* possesses amazing photoprotective ability with SPF 38 at minimum concentration of 200 µg/ml which appears to be significant finding. It's not difficult to envisage that such plant extract would be commercially sourced to extract such high potency SPF compounds to develop next generation sunscreens.

Antibactertial Activity of Hydro-Methanol Leaf Extract (HMLE)

Plants are endowed with significant antibacterial ability and are sourced to cure ailments to human civilization. Presence of several phytoconstituents is imparting bioactive efficacies of plant extracts. In the present study antibacterial effect of hydro methanolic leaf extract of *V. squarrosa* has been assessed (Table 5). The HMLE is effective against both gram positive and gram negative pathogenic strains and highest inhibition zone (20.33 ± 0.33 mm) was recorded in *B. subtilis* (MTCC 441) followed by *P. aeruginosa* (MTCC 2453), *E. coli* (MTCC 739) and *S. aureus* (MTCC 2940). Minimum inhibitory concentration ranged between 125-250 µg/ml (Table 5). Thus the results of MICs clearly indicate that MIC values of *V. squarrosa* were significantly lesser compared earlier reports of similar plant *Vernonia amygdalina* which had MIC values ranged between 12.5 and 50 mg/ml (41). When tested with standard broad spectrum antibiotic Tetracycline (30 µg/ml), results indicated that HMLE was more effective than Tetracycline in terms of the size of inhibition zone. These findings signify that this plant has potential source of antibacterial agents.

Table 5. Antibactertial activity of HMLE of *V. squarrosa* and its minimum inhibitory concentration

Bacterial strains	Zone of Inhibition (mm) of HMLF (5000 µg/ml)	Zone of Inhibition (mm) of Tetracycline(30 µg/ml	MIC (µg/ml) of HMLE	MIC (µg/ml) of Tetracycline
Staphylococcus aureus (MTCC 2940)	14.33 ± 0.33	21.00± 0.33	250.00±0.67	2.00± 0.88
Pseudomonas aeruginosa (MTCC 2453)	18 .67±0.88	16.00 ±0.67	125.00±0.33	16.00± 0.58
Bacillus subtilis (MTCC 441)	20.33 ± 0.33	15.00± 0.00	125.00±0.00	2.00 ±0.00
Escherichia coli (MTCC 739)	15.00 ± 0.00	16.00± 0.33	250.00±0.00	64.00±0.33

FTIR Analysis Leaf Extract (HMLE)

FTIR analysis (Fig. 4) of the HMLF revealed broad band at 3292.86 $cm^{-1,}$ implied to the hydrogen bonded O-H stretching. Another two peaks at around 2923 cm^{-1} and 2853 cm^{-1} indicated the existence of aliphatic C-H stretching of methylene (42, 43). The peak at 1593.88 cm^{-1} assigning the C=N and C-N-H stretching (Amide II) whereas band at 1032.69 cm^{-1} confirming the presence of alcoholic -OH group (44).

GC-MS Analysis of Leaf Extract (HMLE)

GC MS is a powerful technique to identify and quantify volatile compound like terpenoids, ester. In our study, HMLE exhibited eight peaks on GC MS chromatogram (Fig. 5). Those peaks denote the existence of medicinally important bioactive

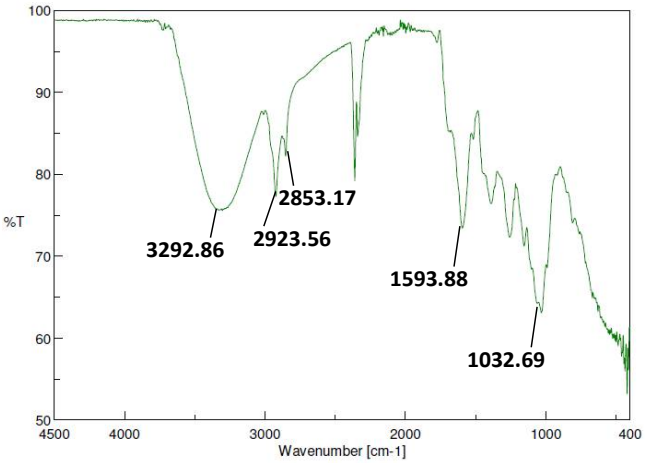

Fig. 4. FTIR spectra of HMLE of *V. squarrosa.*

compounds. The molecular structures of those identified compounds are listed in the (Table 6 and supplementary material Fig. S1-S8). Among eight compounds, 2-Thio-2,4-oxazolidinedione ($C_3H_3NO_2S$, RT 5.77) and its derivative are actively used for the synthesis of anti-cancer agents (45). Benzaldehyde, 3-(2,4,6-trichlorophenoxy methyl-4-methoxy ($C_3H_3NO_2S$,

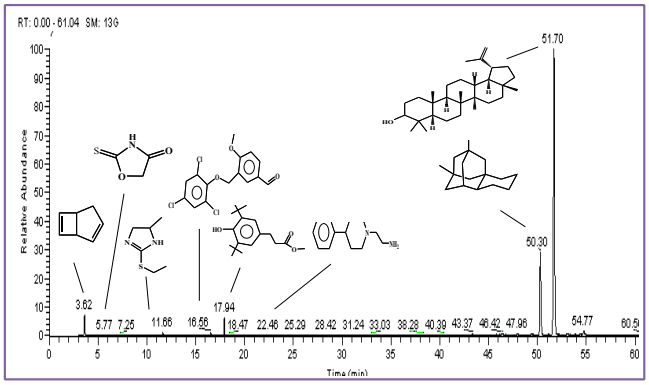

Fig. 5. GC MS chromatogram of hydro-methanolic leaf extracts (HMLE) of *V. squarrosa.*

RT 16.56) was reported as an anti-microfouling agent (46). Benzenepropanoic acid, 3, 5-bis (1, 1-dimethylethyl)-4-hydroxy-methyl ester ($C_{18}H_{28}O_3$ RT 17.94) is a well established anti-oxidant agent (47). Lupeol ($C_{30}H_{50}O$, RT 50.70) with 61.77 % peak area is the major compound identified from *V. squarrosa*. Lupeol possesses diverse pharmacological activities under *in vitro* and *in vivo* conditions such as anti-inflammation, anti-cancer, anti-arthritis, anti-microbial, anti-angiogenic, cardiovascular disease and it is also used as a cholesterol lowering agent (48). Antioxidant properties of lupeol had been extensively reviewed (49). Moreover, Tchimene *et al.* in 2016 revealed strong antioxidant activity of lupeol isolated from methanolic leaf extract of *Crateva adansonii* Oliv. (Capparidaceae) (50). Antibacterial activity of lupeol has also been established from *Mesua ferrea* stems (51). In our study, we also found potential photoprotective, antibacterial and antioxidant activities of the leaf extract *V. squarrosa* and from these findings an inference can be made that the presence of lupeol and other compounds may responsible for these biological properties of *V.*

Table 6. Chemical compounds of HMLF identified by GC MS

Compound	RT	% peak Area	Mol. weight	Chemical formula
Bicyclo [3,2,0] hepta-2,6-diene	3.62	2.65	92	C_7H_8
2-Thio-2,4-oxazolidinedione	5.77	0.25	177	$C_3H_3NO_2S$
2-Ethylthio-5-Methylimidazoline	11.66	0.32	144	$C_6H_{12}N_2S$
Benzaldehyde,3-(2,4,6-trichlorophenoxy methyl-4-methoxy	16.56	0.3	344	$C_3H_3NO_2S$
Benzenepropanoic acid,3,5-bis(1,1-dimethylethyl)-4-hydroxy-methyl ester	17.94	1.95	292	$C_{18}H_{28}O_3$
2-(4-phenyl-piperidine-1-yl)-ethylamine	22.46	0.15	204	$C_{13}H_{20}N_2$
9,11-Dimethyletracyclo [7,3,1,0(2,7),1(7,11)] tetradecane	50.30	16.15	218	$C_{16}H_{26}$
Lupeol	50.70	61.77	426	$C_{30}H_{50}O$

squarrosa plant extract. This is the first time we report the exploration of bioactive compounds from Hydro Methanolic Leaf Extract of *V. squarrosa*, particularly for their photoprotective, antibacterial and antioxidant activities.

Conclusion

This study was carried out to evaluate the bioactivity of *V. squarrosa*. To the best of our knowledge, no investigation has been carried out till now regarding bioactive compounds, antioxidant potential and photoprotective property of this plant. The preliminary investigation revealed that HMLE exhibits relatively good potential in DPPH, ABTS radical scavenging and reducing power mostly due to high phenol, flavonoid and terpenoid content. The HMLE exhibits amazing photoprotective ability and antimicrobial properties against two gram positive (*Staphylococcus aureus*, *Bacillus subtilis*) and two gram negative (*Pseudomonas aeruginosa*, *Escherichia coli*) bacteria tested. The presence of lupeol (pentacyclic triterpenoid) was identified in significant quantity by GC MS analysis which is a momentous finding of our study. The above findings underline the significance of *V. squarrosa* as storehouse of a variety of herbal medicines.

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Authors' contributions

AD, SB carried out all the experiments. AD, SB wrote and formatted the manuscripts. AB hypothesized and designed all above the experiments. AB, GC read and made critical changes.

Conflict of interests

The authors have no conflict of interest to disclose.

Supplementary files

Table S1. Table showing the Determination of SPF Value at 50 µg/ml extracts (HMLE) concentration

Table S2. Table showing the Determination of SPF Value at 100 µg/ml extracts (HMLE) concentration

Table S3. Table showing the Determination of SPF Value at 200 µg/ml extracts (HMLE) concentration

Table S4. Table showing the Determination of SPF Value at 400 µg/ml extracts (HMLE) concentration

Table S5. Table showing the Determination of SPF Value at 600 µg/ml extracts (HMLE) concentration

Table S6. Table showing the Determination of SPF Value at 800 µg/ml extracts (HMLE) concentration

Table S7. Table showing the Determination of SPF Value at 1000 µg/ml extracts (HMLE) concentration

Fig. S1. The mass spectrum of Bicyclo [3,2,0] hepta-2,6-diene, RT 3.62

Fig. S2. The mass spectrum of 2-Thio-2,4-oxazolidinedione, RT 5.77

Fig. S3. The mass spectrum of 2-Ethylthio-5-Methylimidazoline, RT 11.66

Fig. S4. The mass spectrum of Benzaldehyde,3-(2,4,6-trichlorophenoxy methyl-4-methoxy, RT 16.56

Fig. S5. The mass spectrum of Benzaldehyde,3-(2,4,6-trichlorophenoxy methyl-4-methoxy, RT 17.94

Fig. S6. The mass spectrum of 2-(4-phenyl-piperidine-1-yl)-ethylamine, RT 22.46

Fig. S7. The mass spectrum of 9,11-Dimethyletracyclo [7,3,1,0(2,7),1(7,11)] tetradecane, RT 50.30

Fig. S8. The mass spectrum of Lupeol, RT 50.70

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