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Original Article

Pharmcognostical and Phytochemical Investigations on *Stachytarpheta indica*

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ABSTRACT

Background: *Stachytarpheta indica* (Family: Verbenaceae), is a tropical plant useful to treat respiratory disorders, dropsy, rheumatic inflammation, intestinal worms and diarrhea. Pharmacological studies indicate antimicrobial, insecticidal and anthelmintic potential. **Objectives:** To establish quality control parameters, identify, isolate phytoconstituents and evaluate cytotoxic potential. **Materials and Methods:** *S. indica* was subjected to morphological, microscopical, phytochemical and cytotoxic studies. Flavonoid, scutellarin was identified by histochemical method and isolated; verbascoside was isolated, characterised by TLC of its hydrolytic products and UV spectroscopy. A new iridoid was isolated. Cytotoxicity of methanolic extract of *S. indica* was assessed on cancer cell lines (A549 for lung cancer, HT29 for colon cancer and MCF7 for breast cancer). **Results:** Transverse Section (TS) of the leaf include multiple collateral vascular bundles; stem TS features collenchymatous hypodermis and lignified fibres; while cork, phloem, and central wood represent root structure. Furthermore, a simple method for isolation of verbascoside from the plant would be beneficial as an alternative method for this important phenylpropanoid. Moreover, a new iridoid methyl 1-(4-ethoxybut-3-yn-1-yl)-4a-methyl-1,4a,5,6,7,7a hexahydrocyclopenta [c] pyran-4-carboxylate was isolated from aqueous fraction and characterized. The preliminary *in vitro* screening results of cytotoxicity study on cell lines indicated that the methanolic extract exhibited non-significant cytotoxicity on human lung cancer (A549) cells, colon cancer (HT29) and breast cancer (MCF7) cells. **Conclusion:** Identification parameters developed in the study would be useful tool for its quality control and analysis. Simple method for identification and isolation of important compounds is easy to employ for future research.

Keywords: Histochemical, Microscopy, Scutellarin, *Stachytarpheta indica*, Verbascoside.

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INTRODUCTION

Unfolding the chemical composition and bioactive components of plants and with techniques grounded in the traditional knowledge of local healers ensure both safety and efficacy of herbal medicines in health management.[1] The genus *Stachytarpheta* (Verbenaceae) includes more than 100 species distributed in tropical and subtropical parts of America, Brazil, Asia, and Australia.[2] *Stachytarpheta indica* (Syn.: *Verbena indica*) commonly known as Indian snakeweed is one of such an important indigenous plant used for digestive problems such as indigestion, acid reflux, ulcers, constipation, dyspepsia, as a pain reliever and anti-inflammatory.[3-6] Majorly sterols, β-sitosterol, lupeol, stigmasterol, ursolic acid, and flavone apigenin, iridoid ipolamide; verbascoside reported from entire plant of *S. indica*.[7-9] Plant has been reported to possess antimicrobial, insecticidal and anthelmintic activities.[10,11] In the view of very few scientific reports available on this plant, the present work is an effort to develop quality parameters, isolate and characterize scutellarin and verbascoside and evaluate cytotoxic potential.

MATERIALS AND METHODS

Identification, collection, authentication and preparation of plant samples

Fresh entire plants of *S. indica* were harvested in full bloom from a horticulture farm at Ahmedabad, Gujarat, India. The plant was identified and authenticated by the taxonomist of South Gujarat University, India. Voucher specimen has been deposited at the department of the authors. Fresh leaf, stem and root of the plant were used for pharmacognostical studies and for phytochemical evaluation plants were dried, powdered and passed through 60# sieve. The powdered drug was stored in an airtight container at room temperature.

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Materials and reagents used in cytotoxicity assay

To evaluate cytotoxic activity, A-549 (lung), HT29 (colon), and MCF7 (breast) cancer cell lines were used. Media (RPMI-1640, DMEM), L-glutamine, penicillin, and streptomycin were procured from Invitrogen, Mumbai, and FCS from Sigma-Aldrich (Gibco), India. Methanolic plant extract was prepared in DMSO (10 mg/mL) and filtered using 0.22 µm PVDF filters. Phenol red-free media were used for treatment. Doxorubicin (1, 10, 100 ng/mL) was used as a positive control. MTT reagent (0.5 mg/mL) was used to assess cell viability; formazan crystals were dissolved in DMSO, and absorbance was read at 570 nm (ref. 630 nm) using an ELISA reader.[12]

Pharmacognostical studies

Macro and microscopical studies for all parts of plant were studied separately by taking free hand transverse sections of leaf, leaf petiole, stem and root. Powder microscopy was performed for the dried powder of entire plant. The physio-chemical parameters such as loss on drying, determination of extractive and ash values were performed in accordance with the standard procedures described in Quality control methods for medicinal plant materials by World Health Organization.[13] Powder of plant was subjected to phytochemical screening separately for the presence of various phytoconstituents like carbohydrates, sterols, terpenoids, saponins, phenolics, flavonoids, phenylethanoids, coumarins compounds.[14] Major phytoconstituents present in the plant such as flavonoids, phenolics, tannins and saponins were estimated by various methods.[15-17]

Determination of scutellarin from leaf by histochemical screening

Small fragments of several leaves were placed in a vial containing a few mL of 5% hydrochloric acid.[18] After at least 24 hr the leaf-fragments were transferred to a large drop of lactophenol on a slide and covered with a cover slip. After clearing by gently warming the preparation was examined under microscope.

Isolation and extraction of *S. indica* entire plant

Dried powdered plant material (135 g) was defatted with petroleum ether (1L × 5) under reflux, yielding 0.740 g of extract (Ext A). The defatted material was then extracted with 95% ethanol (600 mL × 5) and tannins were removed using Polyvinyl Pyrrolidone (PVP), followed by filtration. The ethanol extract was concentrated and refrigerated for 24 hr, which on addition of excess acetone resulted in a yellowish-white precipitate of glycosidal fraction (Ext B). The precipitate was re-dissolved in alcohol, and acetone was added again, yielding 15 g of glycosidal fraction. Ext B was dissolved in 200 mL of 10% aqueous ethanol and washed with chloroform (4×100 mL) followed by extraction using ethyl acetate (6×100 mL), yielding residues of 70 mg (chloroform, Ext C), 400 mg (ethyl acetate, Ext D), and 12.7 g (aqueous soluble fraction Ext E). Chromatographic analysis (TLC) revealed the presence of lignans in the Ext D and flavonoids in the Ext E. Ext D was dissolved in a test tube containing a solvent system of ethyl acetate: water (1:1), shaken to partition compounds between the phases, and spotted onto a TLC plate. The TLC plate, developed with ethyl acetate: methanol: water (200: 33: 27, *v/v/v*) and visualized using ferric chloride (10%) followed by ammonia, showed a single spot (Compound A) in the lower phase. Further, compound A was subjected to mild acid hydrolysis with 1N HCl at 100°C for 15 min and complete acid hydrolysis with 1N HCl at 100°C for 2 hr. The hydrolysates were extracted with diethyl ether (3×20 mL), and the aqueous and organic phases were analysed by TLC for sugars and aglycones.

Further, aqueous fraction (Ext E) was subjected to the biphasic system, hexane: ethyl acetate: methanol: acetone: water (1:2:1:0.8:2). Non-polar upper phase showed the single fluorescent compound B (6.8 mg) on TLC performed using toluene: ethyl acetate (6:4, *v/v*) as a mobile phase. It was subjected to UV, IR, NMR, Mass spectral analysis.

Ext E was hydrolysed under reflux for 2 hr with a 1:1 mixture of 2N HCl and after neutralizing with sodium carbonate, was extracted with ethyl acetate (3×50 mL), resulting in a yellow residue of compound C (190 mg). Its identification was done by preparative TLC and chemical reactions with Mg- HCl, $FeCl_3$ and NH_3 and also by UV spectral analysis using various reagents such as aluminium trichloride, sodium acetate, boric acid and sodium methoxide.[19]

Cytotoxic activity of plant (MTT Assay)

The cytotoxicity assay was performed as per the method described by Gupta SK.[20] The cytotoxic potential of the methanolic plant extract was evaluated using the MTT assay on A-549, HT29, and MCF7 cancer cell lines. Exponentially growing cells (1×10^4/well) were seeded in 96-well plates and incubated for 24 hr. Cells were then treated with different concentrations of the extract (0.1, 10, 50, and 100 µg/mL) and incubated for 72 hr. After treatment, MTT reagent was added and incubated for 3 hr. Formazan crystals formed were dissolved, and the optical density was measured at 570 nm with a reference wavelength of 630 nm. Cell viability was calculated as a percentage of untreated control, and IC_{50} values were determined.

RESULTS

Pharmacognostical studies

Macroscopical study

The plant has simple, ovate leaves with serrate margins, reticulate-pinnate venation, and a size of 10.0 cm × 4.8 cm. Its stem is quadrangular, hairy, and measures 1.5-2 m in length, with the upper part dark green and the lower part woody and light brown. The root is cylindrical, light brown, and marked by

longitudinal wrinkles. Flowers are purplish, bisexual, and borne on spikes (Figure 1).

Microscopical study

Leaf

The Transverse Section (TS) of the leaf reveals a convex structure with dorsal and ventral elevations. The midrib consists of collenchymatous cells encircling a central arc-shaped vascular bundle, accompanied by 2-3 developing bundles above it. The leaf is distinctly divided into the lamina and midrib. The shape of the leaf petiole differs slightly from that of the leaf. Vascular bundles present on the two-lateral side of central vascular bundle in leaf petiole. The TS of the leaf shows greater elevations on both the dorsal and ventral sides compared to the leaf petiole (Figure 1).

Surface preparation of leaf

The surface preparation of the leaf reveals the upper and lower epidermis consisting of wavy-walled, polygonal epidermal cells with diacytic stomata. The lower epidermis contains a higher number of stomata compared to the upper epidermis. Various types of simple multicellular covering trichomes and glandular trichomes are also observed.

Stem

The transverse section of the stem shows a quadrangular outline with a single-layered epidermis covered by a thin cuticle, composed of tangentially elongated cells bearing multicellular trichomes. In angular regions, the epidermis includes 2-3 layers of thick-walled, square-shaped cells. Beneath it, are 2-3 layers of collenchymatous hypodermis and 3-4 layers of parenchymatous cortex containing patches of lignified sclerenchymatous pericyclic fibers. The bicollateral vascular bundle features lignified, radially arranged medullary rays and peri-medullary phloem underneath the xylem. The pith comprises pitted parenchymatous cells with occasional calcium oxalate prisms (Figure 2).

Root

The transverse section of the root is circular in outline, with an outermost cork layer and a central wood region occupying the majority of the section, surrounded by phloem and a narrow cortex. A detailed TS of the root reveals 2-3 layers of tangentially elongated, non-lignified, suberized cork cells. In the cortex, lignified small stone cells and 7-8 layers of parenchymatous cells are present. The vascular bundle consists of phloem and xylem elements, with irregular phloem cells located below the cortex. The xylem comprises vessels and fibers arranged radially and traversed by bi- to tri-striate medullary rays (Figure 2).

Powder study

A brownish-green fine powder with a slightly bitter taste and a faint odour exhibit following diagnostic characters. It contains both covering trichomes and glandular trichomes. The covering trichomes are very occasional, uniseriate, conical, and composed of four or five cells. The glandular trichomes are of two types: one with a uniseriate, multicellular stalk made up of two to three cells and a multicellular head of one to two cells, and the other being sessile glandular trichomes with a bicellular head. The powder displays abundant fragments of the upper and lower epidermis with diacytic stomata in surface view, lignified and single-pitted xylem vessels, lignified fibres with blunt tips, and occasional fragments of dark brown suberized cork cells in surface view (Figure 3).

Results of quantitative microscopy and physicochemical parameters are given in Tables 1 and 2 respectively. Preliminary phytochemical screening identified presence of flavonoids, phenolics, saponins, steroids, triterpenoids and carbohydrates. Flavonoids were found to be the major constituents and data is entered in Table 3.

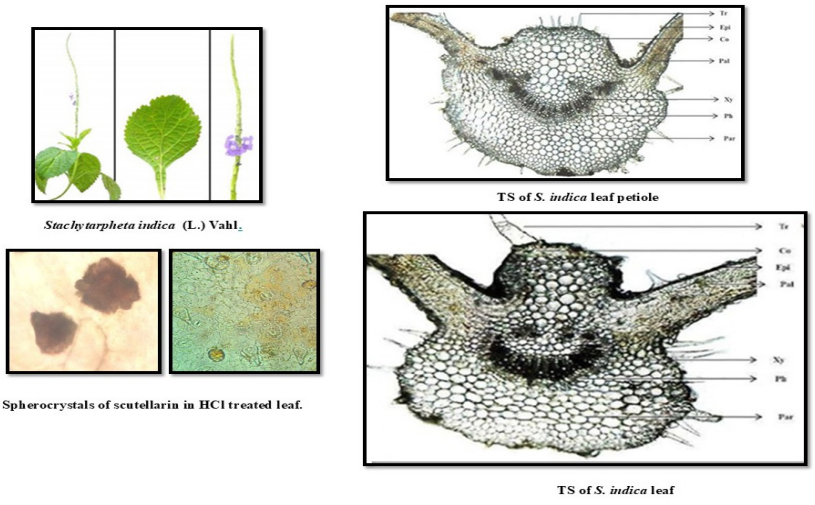


Figure 1: *Stachytarpheta indica* (L.) Vahl., TS of *S. indica* leaf petiole, TS of *S. indica* leaf and Spherocrystals of scutellarin in HCl treated leaf.

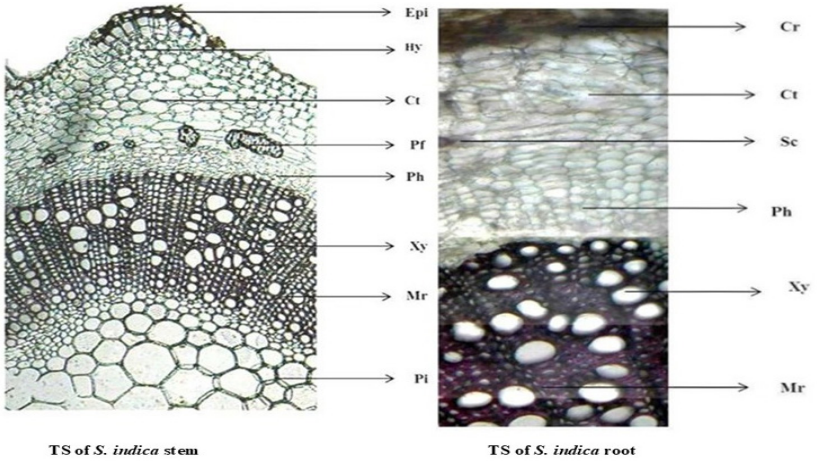


Figure 2: TS of *S. indica* stem and TS of *S. indica* root.

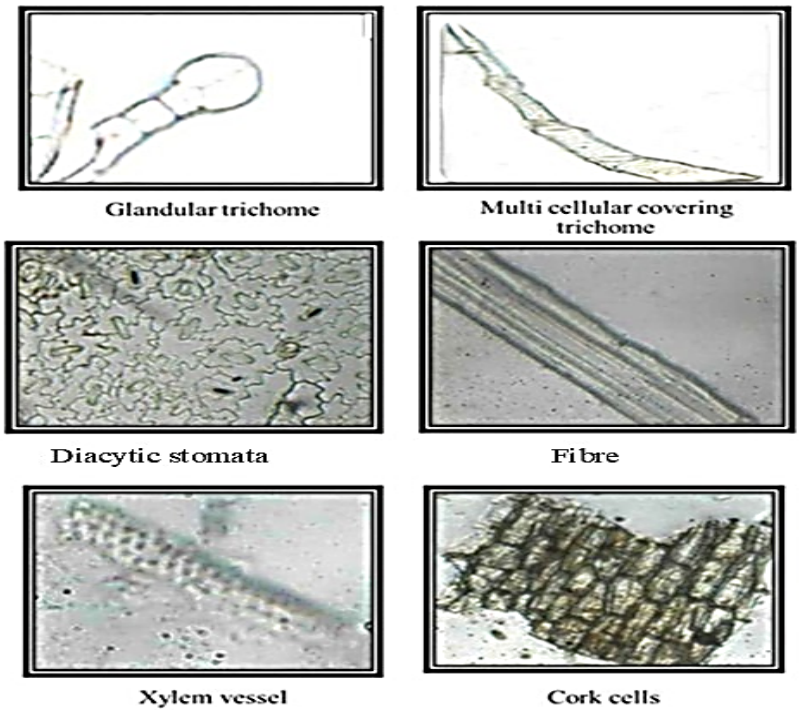


Figure 3: Powder study of *S. indica* whole plant.

Histochemical identification of scutellarin from leaves of *S. indica*

HCl treated leaf fragments showed large, yellowish brown spherocrystals throughout the lamina indicating the presence of scutellarin (7-glucuronide of scutellarin). Here HCl transforms the soluble salts of scutellarin into the highly insoluble free acid form (because of carboxylic group of glucuronic acid) and thus induces crystallization of the compound (Figure 1).[21]

HCl-test already used by Molisch and Goldschmiedt[22] and by Strecker.[23] It is specific for scutellarin. The formation of spherocrystalline masses of scutellarin by immersion of leaf fragments in cold HCl is a useful feature to characterize it in whole plant part.

Table 1: Quantitative microscopy of leaf.

Sl. No.	Parameters	Results (*n*=3)
1	**Stomatal number**	
	Upper Surface	3.5±0.70
	Lower surface	17.91±0.11
2	**Stomatal index**	
	Upper Surface	5.60±0.21
	Lower surface	17.75±0.08
3	Palisade Ratio	4.65±0.17
4	Vein islet number	7.25±0.09
5	Vein termination number	31.75±0.87
6	Trichrome length	227.2µm±3.1272

Spectral analysis of verbascoside

The spectral analysis of the isolated compound A revealed it to be verbascoside (acetoside), a light yellowish amorphous powder with a melting point of 108-109°C. Under UV, it exhibited a blue fluorescence, turning green-yellow on exposure to NH_3 and which on further treatment turned dark blue with 1% $FeCl_3$ solution. Mild acid hydrolysis in 1N HCl released rhamnose, while complete hydrolysis (1N HCl, 100°C, 2 hr) yielded rhamnose (R_f 0.41), glucose (R_f 0.5), and caffeic acid (R_f 0.36), consistent with standards. TLC analysis revealed presence of verbascoside at R_f 0.7 in sample matching with reference standard. UV spectra revealed λ_{max} at 332, 290, 250, and 220 nm, while in methanol it showed λ_{max} at 212, 250, and 332 nm. The IUPAC name for verbascoside is 2-(3,4-dihydroxyphenyl)-ethyl-(30-O-L-rhamnopyranosyl)-(40-O-caffeoyl)-*β*-D-glucopyranoside. IR spectra (KBr) displayed peaks at 3380 cm^{-1} (OH), 2935 cm^{-1} (C=C), 1694 cm^{-1} (C=O), and 1602, 1515, 1385 cm^{-1} (aromatic ring). Mass spectrometry showed M-H^- at 623.1 and [M+Na]$^+$ at 647.1. All data matched literature reports, confirming the compound's identity.[24,25]

Spectral analysis of iridoid compound

Compound B obtained from aqueous fraction as white crystals (12 mg), when subjected for TLC using mobile phase toluene: ethyl acetate: formic acid (5:4:1, *v/v/v*) silica gel as stationary phase revealed single spot at R_f 0.89 with bright blue fluorescence under UV light.

IR spectra showed peaks at 2921, 2858, 2350, 1730, 1458 and 1274 cm^{-1} indicating the presence of methyl (CH_3), aliphatic (-CH2-), acetylenic (C⏲C), carbonyl (C=O), aromatic (C=C) and ether (C-O) functional groups. It also showed presence of methyl ester (terminal - CH_3) which is also confirmed by ^{1}H NMR. The IUPAC name of this iridoid compound is methyl 1-(4-ethoxybut-3-yn-1-yl)-4a-methyl-1,4a,5,6,7,7a hexahydrocyclopenta[c]pyran-4-carboxylate (Figure 4).

Isolation and characterization of flavonoid

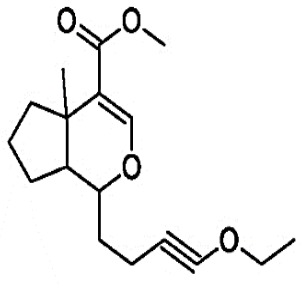
Compound C revealed as flavonoid scutellarin, as being glucuronide (present in plants as salts) was extracted by boiling water and subsequently precipitated from the aqueous extract by HCl. It was subjected to TLC using ethyl acetate: formic acid: water (5: 1: 1, *v/v/v*). It showed a yellow prominent band at R_f 0.56. They later gave yellowish brown colour with Mg- HCl, reddish brown with $FeCl_3$ and also changed from yellow to green with NH_3 (characteristic of vicinal trihydroxy flavone). It was purple under UV and UV/ NH_3, and it was identified by UV spectral shifts. Moreover, it was hydrolysed and tested for glucuronic acid using Tollen's naphtha resorcinol reagent. UV spectral shifts using chemical reagents were assessed on TLC developed using above mobile phase. It showed bathochromic shift of 18 nm in $A1C1_3$ and absence of any shift with $NaOAc/H_3BO_3$ in band I indicating the absence of *o*-dihydroxyl in B ring. The presence of band III at 320 nm in NaOMe spectrum revealed the presence of 6,7-oxygenation.[21]

Cytotoxicity of extracts on human cancer cell lines

The methanolic extract of *S. indica* entire herb when tested for cytotoxic activity on human lung cancer (A-549), colon cancer (HT-29) and breast cancer (MCF7) cell lines showed IC_{50} values

Table 2: Physico-chemical parameters.

Sl. No.	Quality parameters	(%w/w±S.D) (*n*=3)
1	Loss on drying	80.03±1.02
2	Total ash	89.33±0.05
3	Acid insoluble ash	10.89±0.63
4	Water soluble ash	97.48±0.24
5	Alcohol soluble extractive value	11.4±0.01
6	Ether soluble extractive value	2.9±0.12
7	Water soluble extractive value	13.6±0.16



IUPAC Name: methyl 1-(4-ethoxybut-3-yn-1-yl)-4a-methyl-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-4-carboxylate
Chemical Formula: $C_{17}H_{24}O_4$
Molecular Weight: 292.37

Figure 4: Structure of compound B (Iridoid compound).

Table 3: Estimation of phytoconstituents.

Sl. No.	Phytoconstituents	%w/w (*n*=3)
1	Flavonoids	3.15±0.14
2	Phenolics	0.8±0.32
3	Tannins	0.041±0.04
4	Saponins	0.95±0.18

of 49.20, 100.37, and 90.66 µg/mL respectively indicating its nonsignificant cytotoxic potential in the cell lines used.

DISCUSSION

Quality control of herbal drugs is important for their use in herbal medicine and formulations to ensure their safety, efficacy, and consistency. *Stachytarpheta indica* (L.) Vahl. (Family: Verbenaceae), indigenous to tropical America and Eastern Africa is documented in traditional medicinal literature to be useful to treat respiratory disorders, ulcers, and fevers, rheumatic inflammation, intestinal worms, and venereal diseases. *S. indica* was subjected to morphological, microscopical and physicochemical screening. Microscopic features showed collenchymatous cells and vascular bundles in leaf; collenchymatous hypodermis, lignified fibers, and a parenchymatous pith in stem; while cork, phloem, and central wood in the root. Powder microscopy revealed diagnostic features like uniseriate covering and sessile glandular trichomes, diacytic stomata, pitted xylem vessels, lignified fibers, and cork cells. Physiochemical parameters developed for the entire plant parts separately and for powdered *S. indica* gave clarity for its identification. Flavonoid, scutellarin was confirmed through histochemical study in HCl treated leaf which was further isolated and characterised by TLC of its hydrolytic products and UV spectroscopy method. Based on the chemo-taxonomical aspects, the isolation method for verbascoside was focused and accordingly the plant material was defatted with petroleum ether (Ext A), followed by preparation of alcoholic extract that was used to get glucosidal fraction (Ext B) after removal of tannins. Ext B was taken in 10% aqueous alcohol and fractionated to get chloroform (Ext C) and ethyl acetate (Ext D) fractions. Remaining aqueous alcohol extract was hydrolysed and successively extracted with toluene and ethyl acetate (Ext E). Verbascoside was isolated from Ext D and characterized by TLC of its hydrolytic products and spectral analysis. Aqueous fraction revealed presence of a new iridoid methyl 1-(4-ethoxybut-3-yn-1-yl)-4a-methyl-1,4a,5,6,7,7a hexahydrocyclopenta [c] pyran-4-carboxylate. Cytotoxicity assay of methanolic extract of *S. indica* was carried out on cancer cell lines (A549 for lung cancer, HT29 for colon cancer and MCF7 for breast cancer). The preliminary screening results showed that the methanolic extract exhibited negligible cytotoxicity on human lung cancer (A549) cells but showed non-significant activity on colon cancer (HT29) and breast cancer (MCF7) cell lines. Thus, the present investigation offers a complete monograph on *S. indica* with its quality parameters and cytotoxic activity. Moreover, a method developed for verbascoside isolation from the plant is easy to apply and would serve as a promising alternative for this important phenylpropanoid.

CONCLUSION

The phytopharmacognostical study was performed on the plant *Stachytarpheta indica* and concluded that the Pharmacognostical, Physiochemical and Phytochemical features which no doubt can be proved beneficial and serve as scientific background for further isolation steps to obtain the scutellarin and verbascoside along with methyl 1-(4-ethoxybut-3-yn-1-yl)-4a-methyl-1,4a,5,6,7,7a hexahydro cyclopenta[c]pyran-4-carboxylate using simple techniques. The results of cell line studies indicated that the methanolic extract *S. indica* does not possess cytotoxic potential in human lung cancer (A-549), colon cancer (HT-29) and (MCF7) cell lines.

ABBREVIATIONS

HCl: Hydrochloric acid; **EXT:** Extract; **Na_2CO_3:** Sodium carbonate; **Mg-HCl:** Magnesium in hydrochloric acid; **$FeCl_3$:** Ferric chloride; **NH_3:** Ammonia; **$AlCl_3$:** Aluminum trichloride; **$NaOAc/H_3BO_3$:** Sodium acetate/ Boric acid; **NaOMe:** Sodium methoxide; **DMEM:** Dulbecco's Modified Eagle Medium; **RPMI:** Roswell Park Memorial Institute; **FCS:** Fetal Calf Serum; **DMSO:** Dimethyl sulfoxide; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PVDF:** Polyvinylidene fluoride; **ELISA:** Enzyme-Linked Immunosorbent Assay.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

As a basic approach to explore a medicinal plant with least reported data, in-depth analysis was focused to develop quality parameters in a monograph form for the authentication of *Stachytarpheta indica*. Moreover flavonoids, phenolics, tannins, and saponins were estimated Scutellarin was identified by a histochemical chemical test. Verbascoside and methyl 1-(4-ethoxybut-3-yn-1-yl)-4a-methyl-1,4a,5,6,7,7a hexahydro cyclopenta[c]pyran-4-carboxylate were isolated by simple method and confirmed by TLC, IR and NMR. The methanolic extract of the plant did not show cytotoxic action on the cell lines tested human lung cancer (A-549), colon cancer (HT-29) and breast cancer (MCF7) cell lines.

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