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Research article

Proton NMR and HR-LC/MS based phytochemical analysis of methanolic fraction of *Alectra parasitica* A. Rich. rhizomes

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ABSTRACT

Introduction: *Alectra parasitica* (Scrophulariaceae/Orobanchaceae) is a rarely occurring parasitic plant grows on roots of *Vitex negundo* L. (Verbenaceae). As per Indian system of medicine, Ayurveda, it can be used in treatment of various diseases. So far, this plant has not been explored phyto-chemically in detail. Non-small cell lung cancer (NSCLC) is mostly occurring type of lung cancer, which so far can be treated by chemotherapy approach including cisplatin.

Aim: Present research work was aimed towards preparation of methanolic fraction of *A. parasitica* rhizomes; its phytochemical analysis by 1H-NMR and HR-LC/MS; evaluation of its anti-cancer property against NSCLC-A549 cell line.

Methods: For preparation of methanolic fraction (MF), *A. parasitica* rhizome powder was defatted; extracted with combination of water and alcohol (1:1); added with lead acetate and then sulphuric acid; fractionation of ethyl acetate fraction with methanol. After phytochemical analysis of MF by preliminary chemical testing, TLC, ^{1}H-NMR and HR-LC/MS techniques, MF was screened for its anti - cancer property against NSCLC-A549 cell line by MTT assay.

Results: Detail phytochemical analysis reflected successful preparation of tannin-less MF of *A. parasitica* rhizomes. Different types of analytical techniques first time proved the proved the presence of various types of phyto-chemicals in this plant. On MTT assay, it was found that MF has anti-cancer property against NSCLC-A549 cell line with IC_{50} value, 306.51 μg/ml.

Conclusion: MF contains different phytochemicals like iridoids, flavonoids, steroid glycosides and also striga-lactones; which cumulatively exert anti-cancer effect on A549. Appearance of all these compounds is significant in chemotaxonomic surveillance of this rare plant and specially, strigalactones can be proved important in estab-lishing their parasitism with host plant.

1. Introduction

As such nirgundi, *Vitex negundo* L. (Verbenaceae) plant is found in various regions of India. But in few regions, it allows *Alectra parasitica* A. Rich. var. chitrakutensis (Scrophulariaceae/Orobanchaceae) (Figure 1), parasite plant to grow on its root. The plant is actually rare but so far previous researchers collected it from Chitrakut region of Madhya Pra-desh and Uttar Pradesh (Rau, 1961; Saxena et al., 1969) Bundi District of Rajasthan (Sharma and Bhutya, 2013) and Akola District of Maharashtra (Kakpure and Rothe, 2012). In Ayurveda, *Alectra parasitica* has been mentioned for treatment of disorders like rheumatism, constipation, fe-vers, swellings, paralysis and skin diseases like vitiligo and leprosy (Quattrocchi, 2012). So far, only azafrin and mannitol have been reported to be present in *Alectra parasitica* A. Rich rhizomes (Rajagopalan and Seshadri, 1964).

Around 1.8 million malignancy cases are diagnosed every year, most of which are lung cancer (WHO 2018). Approximately 80–85% of lung cancer cases are of non-small cell lung cancer (NSCLC) type. It may be squamous-cell carcinoma, adenocarcinoma, or large-cell carcinoma. Non-small cell lung cancer (NSCLC) is so fetal that only 15% of patients are able to survive after 5 years of initial diagnosis. Major etiologic factor for these lung cancers are smoking of cigarettes and chewing of tobacco. Treatments of unresectable NSCLC include adjuvant chemotherapy including intravenous administration of cisplatin with gemcitabine, etoposide or paclitaxel (Arriagada et al., 2004), which are the costlier affairs for more than 80% of NSCLC patients. Several research attempts

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Figure 1. *Alectra parasitica* A. Rich. var. chitrakutensis (Scrophulariaceae/Orobanchaceae).

have been made in search of molecule of herbal origin which can be used in treatment of NSCLC at affordable rates with same potency and easy route of administration. Since last 5 years, several plant extracts have been screened for their *in vitro* cytotoxicity against A549 (Table 1).

This research attempt was aimed towards preparation of tannin-less methanolic fraction of *A. parasitica* rhizomes, its phytochemical analysis by advanced techniques like ^{1}H-NMR and HR-LC/MS and evaluation of anti-cancer property against A549 cell lines of non-small cell lung cancer.

2. Materials and methods

2.1. Collection of plant material and extraction

The rhizomes and roots of *Alectra parasitica* was collected from Chitrakoot region of the state Uttar Pradesh, India and identified by botanist on submission of herbarium. Plant material was first washed under tap water, dried and pulverised to powder. Powdered material was first defatted using petroleum ether. Then, powder was extracted using mixture of ethanol and distilled water (1:1) in Soxhlet extractor for 3 h to get hydro-alcoholic extract, which further treated with lead acetate. Extract was then filtered to remove precipitate; added with little quantity of concentrated sulphuric acid and filtered again. Filtrate was then concentrated to 30% of its initial volume and fractionated with ethyl acetate. The separated ethyl acetate fraction was evaporated to dryness and fractionated with methanol. This methanolic fraction (MF) was finally taken for HR-LC/MS based phytochemical screening and evaluated for cytotoxicity on cancer cell lines.

Table 1. Plants screened for anticancer activity against A549 Lung cancer cell line.

Plant	Reference
Premna odorata	Tantengco and Jacinto, 2015
Artocarpus camansi	
Gliricidia sepium	
Turkish propolis	Demir et al., (2016)
Selaginella doederleinii	Sui et al., (2016)
Poria cocos	Chu et al., (2016)
Bidens pilosa	Shen et al., (2018)
Caesalpinia sappan	Bukke et al., (2018)
Curcumin longa	Vemuri et al., (2018)
Allium sativum	

2.2. Preliminary phytochemical screening of MF

Methanolic fraction (MF) was tested for the presence of different classes of phytocompounds like tannins, alkaloids, terpenoids, flavonoids, sterols, as described previously (Patil and Kumbhar, 2018). Presence of iridoids and lactones were tested by Trim-Hill reagent test (Harbone, 1998) and Feigel's test (Devika and Sajitha, 2007), respectively. Test specifically conducted for each phytochemical class was based on change in colour or formation of precipitate on addition of specific reagent. Based on results of these preliminary screens, MF was further studied with thin layer chromatographic technique, where MF was applied on F254 TLC plate (E. Merck) which was already coated with silica gel 60 in uniform thickness of 0.2 mm. Plate was allowed to run the mobile phase Toluene: Ethyl acetate (93: 07) through it in a twin trough chamber to a distance of 8 cm and then envisioned by spraying vanillin-sulphuric acid reagent and heating at 105 °C for 5–10 min.

2.3. ^{1}H-NMR analysis of MF

Facility for ^{1}H-NMR analysis was outsourced from Sophisticated Analytical Instrument Facility (SAIF) - North-Eastern Hill University (NEHU), Shillong, India where test sample of MF was dissolved in deuterated methanol (MeOD) where ^{1}H-NMR spectrum was generated on Brukar Avance II, 400 MHz instrument.

2.4. HR-LC/MS analysis of MF

For HR-LC/MS analysis of MF, sample was send to and facility was hired from Sophisticated Analytical Instrument Facility (SAIF) - Indian Institute of Technology, Bombay (IIT Bombay), India. Here, one-dimensional separation of phytochemicals present in MF was achieved using HPLC, comprising of a guard column (75 μm × 2 cm) and an analytical column (75 μm × 25 cm) which is packed with Thermo Acclaim Pepmap C18 (5 μm) material as a stationary phase and combination of water (A) and acetonitrile (90%) – 0.1 % formic acid in water (B) as mobile phase at flow rate of 100 μl/min. Then, LC-ESI-MS analysis of MF was performed in dual (positive and negative) ion mode using 1290 Infinity UHPLC System further coupled with 6550 iFunnel Q-TOF, Agilent technologies, USA where Agilent iFunnel technology generated ions by electrospray technique and focused them on Agilent Jet Stream technology with a hexabore capillary sampling array and dual-stage ion funnel for increased ion sampling and transmission. Here, iFunnel Q-TOF Mass Spectrometer segment of instrument had settings like capillary tension 3500 V, gas flow rate 13 L/min at a temperature of 250 °C, sheath gas flow rate 11 L/min at a temperature of 300 °C, and a 35-psi nebulizer gas flow pressure.

2.5. MTT assay for determination of mitochondrial synthesis in A549 cell line of non-small cell lung cancer

2.5.1. Preparation of DMEM and MEM

Commonly used Dulbecco's modified Eagle Medium, DMEM containing 10 % Fetal bovine serum, FBS was prepared as per Current Protocols in Immunology (Appendix) 1999, which suggested composition of

1% nonessential amino acids, 2 mM/L-glutamine, 50 μM 2-mercaptoethanol (2-ME), 100 U/ml penicillin, 100 μg/ml streptomycin sulfate and 4500 g/L glucose. To this about 10% heat-inactivated 1 h at 56 °C FBS was added. Eagle's Minimum Essential Medium, MEM was comprised of 12 different essential amino acids, glutamine, 8 different water-soluble vitamins of B-complex group, choline, inositol and some basic inorganic salts.

2.5.2. MTT assay for cytotoxicity

The cell suspension of non-small cell lung cancer cell line A549 was harvested by centrifugation and cell count achieved was 1.0×10^5 cells/ml by few serial dilutions, using DMEM containing 10% FBS. Then, around 100 μl of this diluted cell suspension was added to each of 96 well flat bottom micro titre plates (approximately 10,000 cells/well). After 24 h, cell pellets collected by centrifugation, were suspended in 100 μl of different test sample concentration, prepared in same media. Then, plates were incubated at 37 °C for 48 h under 5% CO_2 atm pressure and 20 μl of MTT (2 mg/ml) in MEM lacking in phenol red was added. Plates were then shaken and incubated for 2 h at 37 °C under 5% CO_2 atm pressure. To solubilize formazan synthesized and liberated about 100 μl of DMSO was added. Optical density of 96 well plate was recorded using microplate reader at wavelength of 540 nm.

3. Results and discussion

3.1. Extraction and preliminary phytochemical screening of MF

After entire extraction process, MF was obtained as brown viscous extract, preliminary phytochemical screening of which showed the presence of flavonoids, steroids, iridoids. Treatment of initial hydro-alcoholic extract with lead acetate made the final MF free from tannins. Further, TLC analysis of MF (Figure 2), carried out in given mobile phase and visualised by treatment with anisaldehyde-sulphuric acid and subsequent heating at 105 °C indicated the presence of a wide range of phytochemicals including terpenoids, steroids, flavonoids (Wagner and Bladt, 2007).)

3.2. Proton-NMR spectrum analysis

Present ^{1}H-NMR spectrum of MF (Figure 3) indicated the occurrence of several types of phytochemicals, like, **flavonoid, iridoid, steroid,** particularly in their glycosidic forms**.** Doublet signal at δ 5.42 could be assigned to H-2 while doublet at δ 6.18 represented H-6 and H-8 of the A and C rings of flavonoid. Doublets at δ 2.47 and 3.07 exposed two hydrogens at H-3 (typical AMX system). Double doublet at 7.31 could be assigned to hydrogens at *ortho*-position i.e.H-2′ and H-6' of B ring. All these assignments of peaks to respective hydrogens could be claimed to presence of **flavanone** type of flavonoid (Maltese et al., 2009). Another type of phytochemicals, **iridoids** where oxygen containing six-membered ring bound to a cyclopentane ring; cyclopenta[c]pyran could also be predicted to be present in MF from ^{1}H-NMR spectrum. Doublet at δ 5.41 indicate H-1 of C-1 of iridoid moiety. At the same time occurrence of doublet at δ 6.3 indicate H-3 of C-3, when there is no attachment to C-4. However, attachment of methyl ester, $COOCH_3$ to C-4 can also be confirmed by singlet at 3.7 for hydrogens of terminal methyl group. In this case, H-3 of C-3 was observed as singlet at 7.23. Hence, the presence of iridoids with and without –$COOCH_3$ substitution at C-4 can be claimed (El-Naggar and Beal, 1980; Dinda et al., 2011). Several peaks could be assigned to hydrogens present in **steroidal** moiety. Triplets at δ 5.16 and 4.29 could be assigned to H-1 and H-3 of ring A; doublets at δ 1.88 and 1.71 represented H-8 and H-9 at the joining of ring B and C of steroids. Singlet at δ 3.02 indicated single hydrogen (H-17) of C-17 of ring D, proving substitution at C-17 and singlet at δ 0.84 exposed 3 hydrogens of methyl group (C-18) attached to C-13. Double doublet at δ 4.72 and singlet at δ 5.59 were characteristic peaks for H-21 and H-22 of five

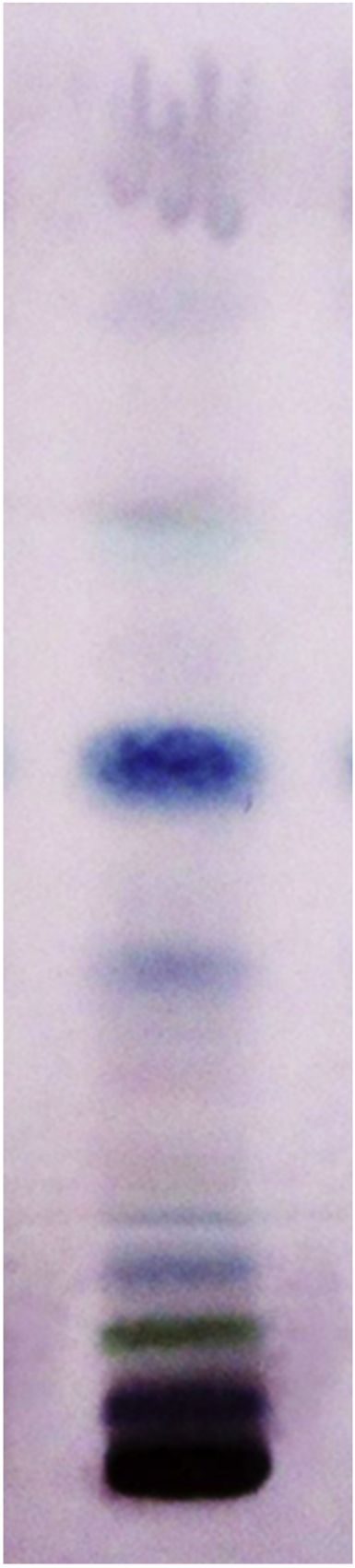

Figure 2. Thin Layer Chromatographic (TLC) profile of MF, viewed after spraying with anisaldehyde-sulphuric acid and subsequent heating at 105 °C. It showed various coloured spots indicating the presence of terpenoids (blue-violet), steroids (green) and flavonoids (pink).

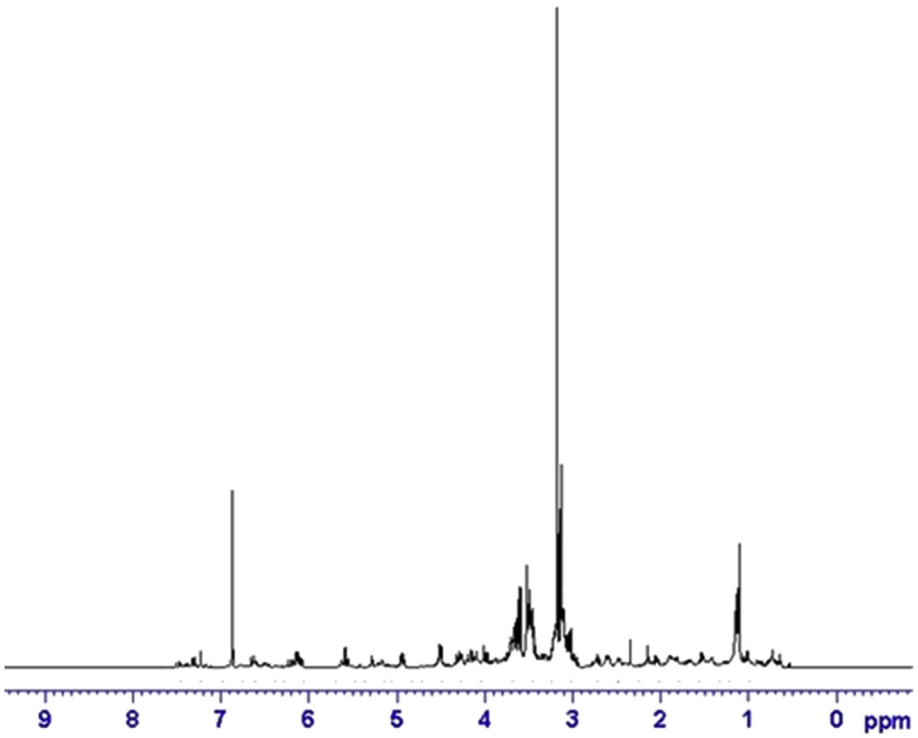

Figure 3. ^{1}H-NMR spectrum of MF showing various peaks, few of which represented hydrogen of more than one phyto-compounds. For example, singlet at δ 5.59 represents hydrogen of five membered lactone ring attached to steroid moiety and also the hydrogen of C-4 substituted tricyclic lactone ring of strigolactone.

membered lactone ring attached to steroid moiety (Faloye et al., 2018; McIntyre et al., 1990).

One or more **strigolactones (SLs)**, unusually reported, germination stimulants for parasitic weed could also be detected from ^{1}H-NMR spectrum of MF (Figure 3). In all SLs, there is tricyclic lactone (ABC-unit) connected through an enol ether linkage to α, β-unsaturated lactone (D ring). The hydrogen at this enol ether linkage can be confirmed by doublet at δ 7.49. Hydrogens of two methyl groups attached C-8 could be exposed by singlets at δ1.11 and 1.14. In most of SLs, there is no substitution to carbon at 5th, 6th and 7th position in tricyclic lactone ring, here two hydrogens attached to each of these three carbons could be demonstrated by multiplate peaks in region of δ 1.83–1.92, 1.64–1.72 and 1.20–1.30, respectively. In few cases, there is substitution at C-4 of tricyclic lactone ring, where single hydrogen attached could be detected by singlet at δ 5.59. Carbon at 2' position of α, β-unsaturated lactone (D ring) has hydrogen attached to it, which can be identified by triplet at δ 6.23 (Ueno et al., 2015; Xie et al., 2013).

3.3. HR-LC/MS analysis

On liquid chromatographic separation by using 1290 Infinity UHPLC System, extract showed around 32 peaks (Figure 4) suggested

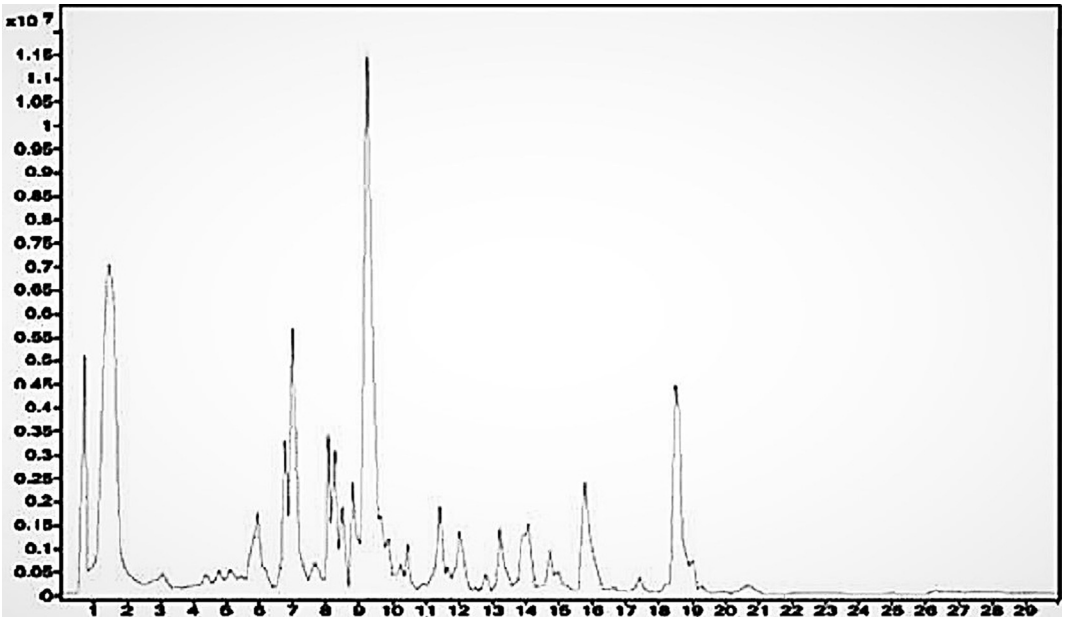

Figure 4. Liquid chromatographic separation of iridoids (0–6 min), strigalactones (6:30 to 10 min), steroidal glycosides (10:30 to 12 min) present in MF.

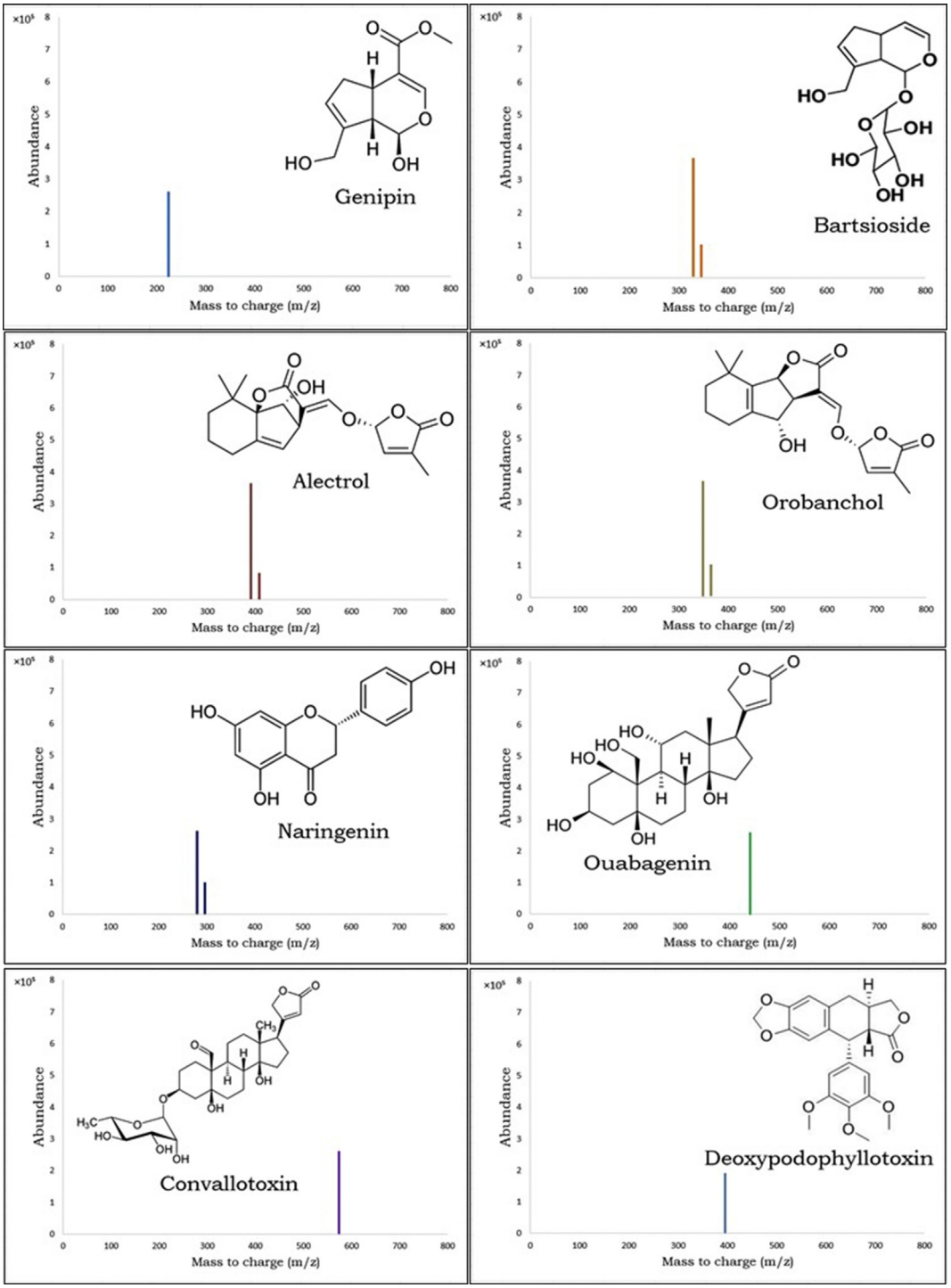

Figure 5. Mass fragmentation of Genipin (A), Bartsioside (B), Alectrol (C), Orobanchol (D), Naringenin (E), Ouabagenin (F), Convallatoxin (G), Deoxypodophyllotoxin (H) present in MF.

the presence of different compounds. Due to addition of formic acid to mobile phase, peak resolution was improved. Here, it was noted that each resolved peak may correspond to more than one phyto-compound because of exhibiting same retention time, as a function of similarity in their polarity and chemical properties. After separation of compounds Agilent iFunnel technology generated their different fragments ions by electrospray technique and focused them on Agilent Jet Stream technology for increased ion sampling and transmission. For detection of phytochemical which can accept proton and those which can donate proton, both positive $[M+H]^+$ and negative ion modes and $[M-H]^-$ were selected for ions. The formation of alkali adducts (with Li+, Na+ and K+) were characterised by increased in mass values of $[M+H]^+$ ions by 6, 22 and 38 Da, respectively. Finally, phyto-compounds were predicted to be present on the basis of fragmentation at particular mass ion and it's matching with already available database, METLIN.

As per METLIN database and detail literature search, major compounds predicted were belonging to different types of secondary metabolites like flavonoid (naringenin); steroid glycosides (ouabagenin and convallatoxin); iridoids (genipin and bartsioside); lignans

Table 2. Compounds predicted on the basis of mass fragments.

tR (min.)	Mass fragments (m/z)	Ion	Compound predicted
0.97	226.95	$[M+H]^+$	Genipin
5.06	353.12	$[M+Na]^+$	Bartsioside
9.23	411.20	$[M+Na]^+$	Alectrol
9.82	369.15	$[M+Na]^+$	Orobanchol
10.32	295.05	$[M+Na]^+$	Naringenin
11.16	438.22	$[M+H]^+$	Ouabagenin
11.75	573.25	$[M+H]^+$	Convallatoxin
12.02	398.13	$[M+H]^+$	Deoxypodophyllotoxin

(deoxypodophyllotoxin) and also special for root parasitic plant, strigalactones (alectrol ad orobanchol) (Figure 5, Table 2).

Genipin, naringenin, ouabagenin were detected in their aglycone forms while convallatoxin and bartsioside were appeared as *O*-glycoside, indicating that addition of concentrated sulphuric acid in preparation of MF brought up partial hydrolysis of glycosides present in MF.

The presence of phytocompounds predicted after HR-LC/MS analyses was supported by preliminary as well as ^{1}H-NMR analysis. These are the secondary metabolites; most of which have been reported to be present in plants belonging to family Scrophulariaceae or Orobanchaceae. The iridoid bartsioside was already detected from another species of same plant, *Alectra vogelii* (Rank et al., 2004). Alectrol and orobanchol are the strigolactones (SLs) which are active as seed germination stimulants for parasitic weeds of *Striga, Orobanche,* and *Alectra* spp, in the family Orobanchaceae. These SLs are biosynthesized and released by host plants and for exhibition of parasitism with host, absorbed by roots of parasitic plant species (Joel et al., 1995; Sato et al., 2005). Genipin was isolated as glycoside from roots of *Cistanche deserticola* Y. C. Ma (Orobanchaceae) (Liu et al., 2011), *Scrophularia ningpoensis* Hemsl (Ji et al., 2014), *Rehmannia glutinosa* Libosch. (Scrophulariaceae) (Fu et al., 2011). Not ouabagenin and convallatoxin, but similar steroidal glycosides are present in *Digitalis* species, belonging to family Scrophulariaceae. Similarly, so far, naringenin and deoxypodophyllotoxin have not been reported to isolated or detected from any plant of Scrophulariaceae.

3.4. MTT assay for cytotoxicity

The MTT assay was carried out to determine the cytotoxicity of the MF against A549 cell line of non-small cell lung cancer. Figure 6 showed the cytotoxicity effect of MF against of A549 cell line of non-small cell lung cancer. The IC_{50} value for MF was found to be 306.51 μg/ml.

MF exhibiting cytotoxicity actually composed of several compounds as predicted by phytochemical analyses. Most of these chemicals, except alectrol and orobanchol, were also reported to have anticancer property against A549 or general cytotoxicity. Convallatoxin induces of autophagy and apoptosis in various cancer and normal cell lines by inhibiting Na+/K+-ATPase, thereby exerts anti-angiogenic or cytotoxic activity (Yang et al., 2014) which may not require functional p53 genes (Anderson and Barton, 2017). It also inhibits KLK gene expression by down regulation of specific transcription factors, such as c-MYC and c-FOS (Prassas et al., 2008). It has been reported that convallatoxin (Schneider et al., 2016) and naringenin (Chang et al., 2017) exhibit anti-proliferative activity by decreasing migration and invasion of lung cancer cells A549 by suppressing matrix metalloproteinase (MMP-2 and MMP-9) expression. Yang et al., 2014 reported that genipin increased Bax levels as a reply to p38MAPK signalling, resulting in the activation of caspace-9 and -3 causing apoptosis, initiated through mitochondrial death cascade in non-small-cell lung cancer. Deoxypodophyllotoxin was also reported to possess *in vitro* inhibitory effect on human lung cancer A-549 (Khaled et al., 2013). Moreover, bartisoside was called as cytotoxic (Rank et al., 2004). Hence, it can be quoted that cytotoxicity showed by MF was cumulative effect of these pharmacologically active phytocompounds.

4. Conclusion

Alectra parasitica is a very rare plant, grows as parasite on roots of *Vitex negundo* (Verbenaceae). It can be concluded that several phytochemicals, specific to plants belonging to family Scrophulariaceae/ Orobanchaceae are present in it's rhizomes. This was first time, the plant was collected for detailed phytochemical analysis by advanced techniques like NMR and HR-LC/MS spectrometry and subsequent METLIN and literature based prediction resulted to detection of several phytochemicals like flavonoid (naringenin); steroid glycosides (ouabagenin and convallatoxin); iridoids (genipin and bartsioside); lignans (deoxypodophyllotoxin) and strigalactones (alectrol ad orobanchol). The extent of cytotoxicity exhibited by MF was the cumulative impact of detected phytochemicals. This first time detection of various chemicals from this plant could definitely be helpful in chemotaxonomic study of this plant. Also, strigalactones detected from this species of genus *Alectra* laid down the foundation of research that can be initiated for the study of parasitism between *A. parasitica* and *V. negundo*.

5. Future prospects

As such the plant *Vitex negundo* is widely available in India, but parasite *Alectra parasitica*, which grows on its roots is much rare. Ethnic evidence revealed use of *Alectra parasitica* rhizomes in treatment of leprosy. Present research attempt indicate the presence of various types of phytochemicals in it. Now, research attempts could be made using this plant on several interdisciplinary fronts including quantification and isolation of detected phytochemicals and their

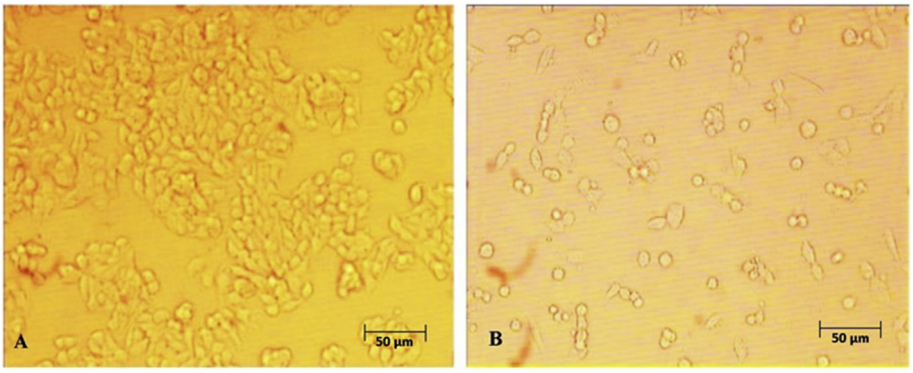

Figure 6. Viable cells as functions of cytotoxicity of MF against A549 cells, A: Normal culture without MF; B: Culture treated with MF.

structural elucidation. Then, biological activities of extracts prepared using different solvents and/or isolated compounds, like azafrin could be evaluated using suitable pharmacological models, both *in vitro* and *in vivo*, mainly for antimicrobial potentials against variety of microbes. Furthermore, for newly isolated, biologically active chemical entity, molecular docking and *in silico* studies could be performed for determination of mechanism of biological action and pattern of receptor or enzyme binding. In order to use this plant in modern medicine, toxicological studies shall be important. Biologists could focus on establishment of parasitic relationship with *Vitex negundo* via strigalactones.

Declarations

Author contribution statement

S. Patil: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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