IJPSR (2019), Volume 10, Issue 8 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 17 November 2018; received in revised form, 14 February 2019; accepted, 28 February 2019; published 01 August 2019

POTENTIAL ANTIBACTERIAL AND ANTIOXIDANT PROPERTIES OF AQUEOUS, ETHANOL AND METHANOL EXTRACTS OF *TECTARIA MACRODONTA* C. CHR

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Keywords:

Antibacterial, Antioxidant, *Tectaria macrodonta*, GC-MS

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ABSTRACT: *Tectaria macrodonta* C. Chr. locally known as *Kaali negro* in Sikkim is a rhizome and spore-bearing terrestrial fern, traditionally used in folk medicine. In the present study, the aqueous, ethanol and methanol extracts of the rhizomes of *Tectaria macrodonta* C. Chr were evaluated for its potential antibacterial and antioxidant properties. The phytochemical analyses of different solvent extracts were done. Antibacterial activity was determined by the agar well diffusion method against some gram-positive and gram-negative bacteria. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the plant extracts against the test bacteria were determined using the broth dilution method. The antioxidant activity was evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, ferric reducing power assay (FRAP) and hydroxyl radical (OH) scavenging assay. Potential antibacterial and antioxidant activity were exhibited by the methanol and ethanol extracts of *Tectaria macrodonta.* The methanol extract was subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The results of the present study suggest the potential pharmaceutical importance of *Tectaria macrodonta* C. Chr. concerning its antibacterial and antioxidant properties.

INTRODUCTION: Plant-based medicines have always been described as the first line of defense against diseases and maintaining the health of people [1]. Due to the large spectrum of biologically active components present in the medicinal plants it has become a natural blueprint for the development of new drugs [2]. Medicinal plants are rich in a variety of secondary metabolites possessing antimicrobial and antioxidant properties such as saponins, tannins, alkaloids, alkenyl phenols, glycoalkaloids, flavonoids, sesquiterpenes, terpenoids and esters [3, 4].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(8).3785-94

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(8).3785-94

Although most of the plants have medicinal values, still not more than 16 % (4,478) of all the plants are being analyzed or cited in medicinal regulatory publication [5].

In Sikkim, a northeastern state of India, *Tectaria macrodonta* is locally known as *Kaali negro* and is used in the folk medicine [6]. *Tectaria macrodonta* C. Chr. belongs to the family of *Dryopteridaceae* and Genus *Tectaria* Cav consisting of about 200 species distributed in the tropical and subtropical regions of the world [7]. In India, there are about 26 species of *Tectaria* Cav. and in Sikkim 6 species are found [8]. In Sikkim, it is quite common up to an altitude of 1800 m [9]. *Tectaria macrodonta* is used by different ethnic communities to treat various diseases. In Eastern Nepal, the Limbu community use the rhizomes of the plant for dental problems, dysentery, diarrhea [10, 11] whereas the local people of Tanahun district, Western Nepal use the juice of

rhizomes against blood dysentery and the young shoots are eaten as a vegetable [12]. The Bheel tribes of Mount Abu use the leaves mixed with honey for asthma and bronchitis and also apply it to the place of irritation caused by the stings of the honeybee and the centipedes [13]. The Bhilla tribes of Maharashtra use the plant powder of *Tectaria macrodonta* for treating cough, cold and fever [14].

Decoctions of the root of *Tectaria macrodonta* are used in the Garhwal region for treating leucorrhoea, menorrhagia, and uterus infection [15]. The Chenchu tribes of Andhra Pradesh use the decoction of the whole plant for stomach ache [16]. The aqueous and alcoholic extracts from the leaves of *Tectaria macrodonta* have exhibited potential antibacterial activity [17].

It has been reported that the protein (Tma12) of *Tectaria macrodonta* finds its application in the genetically modified crops to control the life cycle of whitefly which damages the field crops [18]. Therefore, the present study aimed to evaluate the antibacterial and antioxidant properties of different solvent extracts of *Tectaria macrodonta*.

MATERIALS AND METHODS:

Chemicals and Reagents: All the chemicals, reagents and media used in the experiment were obtained from Sigma-Aldrich, USA, Merck Germany, and HiMedia India.

Collection and Identification of the Plant Samples: *Tectaria macrodonta* C. Chr. was collected from Padamchey, East Sikkim, India. Taxonomic identification of the plant sample was done, and the voucher specimen was deposited at the herbarium of the Plant Taxonomy Division, Department of Botany, University of North Bengal, Siliguri, West Bengal, India with accession number 09738.

Preparation of the Plant Extracts: The dried rhizomes of *Tectaria macrodonta* were pulverized into a fine powder using Waring blender (Cole Parmer, RZ-04245-21). Powdered material (10 gm) was extracted with 100 ml of different solvents (aqueous, ethanol and methanol) for 24 h.

The extracts were concentrated using Rotary evaporator (Buchi, Switzerland, R-3) and kept for further use in sterilized vials at 4 ºC [19].

Test Bacteria: The test bacteria were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh India. In the present study, the test bacteria used were *Bacillus cereus* (MTCC-6840), *Staphylococcus aureus* (MTCC-7443), *Bacillus subtilis* (MTCC-3384), *Klebsiella pneumonia* (MTCC-3384), *Pseudomonas aeruginosa* (MTCC-1034), *Proteus vulgaris* (MTCC-742), and *Escherichia coli* (MTCC-3384).

Preparation of the Inoculum: Test bacteria were maintained at 4 ºC on nutrient agar slants. Each bacterial test isolates were inoculated into the nutrient broth medium and grown overnight at 37 ºC. Turbidity was assessed by Spectrophotometer (Lambda 25 UV/Vis /Perkin Elmer, L600-00BB) by measuring the absorbance of the bacterial suspension. The absorbance was in the range of 0.08-0.13 OD at 625 nm corresponding to 1×10^8 CFU/ ml (McFarland standard 0.5) [20].

Phytochemical Analysis: The aqueous, ethanol and methanol extracts of the plant sample was subjected to standard phytochemical analyses to examine the phytoconstituents namely phenol, flavonoid, tannin, saponin, steroid, anthocyanin, alkaloid, glycoside, carbohydrate, protein and fat [21].

Antibacterial Assay: The antibacterial activity of the aqueous, ethanol and methanol extracts of *Tectaria macrodonta* was estimated by using agar well diffusion technique [22]. Various concentrations (20 mg/ml, 100 mg/ml, 200 mg/ml and 400 mg/ml) of the plant extracts (aqueous, ethanol and methanol) were dissolved in 0.25% dimethyl sulphoxide (DMSO). Gentamicin (0.1 mg/ml) [23] was used as positive control. DMSO was used as negative control. Antimicrobial activity was determined by measuring the diameter of inhibition zone [24] inclusive of the well diameter of 8 mm. All the experiments were performed in triplicate, and the data represent the mean values ± SD.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC): The minimum inhibitory concentration (MIC) was determined by the broth dilution method. The MIC is the lowest concentration of the plant extract at which the bacterial growth was inhibited after 24 h

[25]. For determination of MIC, double fold dilution of extracts was made in the range of 400-0.048 mg/ml. MBC was the lowest concentration of the substance at which survival of any bacterial cell was not possible after incubation for 48 h. For determination of the MBC, 100 µL broth cultures that showed no visible growth was reinoculated on Mueller Hinton agar plates and was incubated at 37 ºC for 24 h [26].

Determination of Antioxidant Properties:

Total Phenolic and Flavonoid Contents: The total phenolic and flavonoid contents present in the different extracts of the plant were determined by Folin - Ciocalteu method as described by Lister and Wilson [27] and aluminum chloride colorimetric method as described by Chang *et al*., [28] respectively. The total phenolic and flavonoid contents were expressed as mg of Gallic acid equivalent (GAE) per gram of extract and mg of Rutin equivalent (RE) per gram of extract respectively.

Antioxidant Assay: The potential antioxidant property of the rhizome of *Tectaria macrodonta* was determined by the DPPH radical scavenging assay using Thin Layer Chromatographic Method described by Bartholomew *et al*., [29] DPPH radical scavenging assay by using the Spectrophotometer Method described by Braca *et al*., [30] Ferric Reducing Power Assay (FRAP) described by Klein *et al*., [31], Hydroxyl Radical Scavenging Activity (HRSA) as described by Kostic *et al*. [32]

Thin Layer Chromatography (TLC): The aqueous, ethanol and methanol extracts of *Tectaria macrodonta* was subjected to thin layer chromatography (TLC) using pre-coated TLC plates (Silica gel 60 F 254 Merck) to separate bioactive components. The chromatography was performed using different solvent mixture as A: Methanol: chloroform: hexane (7:2:1), B: Methanol: chloroform: hexane (3:5:2), C: Methanol: chloroform: hexane (5:2:1), D: Ethyl acetate: benzene (1:5), E: Butanol: acetic acid: water (4:1:5), F: Ethanol: benzene: ethyl acetate (3:5:2). The developed chromatogram was observed under visible light and UV light (254 and 366 nm). R_f values were determined corresponding to the bands observed.

R_f = Distance Travelled by the Solute / Distance Travelled by the Solvent [33].

Fluorescence Spectroscopy: The methanol extract of *Tectaria macrodonta* was subjected to Fluorescence Spectroscopy, and its spectra were recorded using Perkin Elmer LS55 spectrometer using the excitation wavelength of 365 nm [34].

GC-MS Analysis: The methanolic extract was analyzed by GC-MS on a GCMS- QP210Plus (Shimadzu, Kyoto, Japan) system with the headspace sampler (AOC-20s) and auto-injector (AOC-20i), equipped with mass selective detector, having an ion source temperature of 230 °C, interface temperature of 270 °C, a solvent cut time of 3.50 min threshold of 1,000 eV and mass range of 40 to 500 m/z. Helium gas was used as a carrier at a linear velocity of 40.9 cm/s with a split ratio of 10:0. The oven temperature was programmed from 100 ºC (2 min), rising at the rate up to 10 ºC/min with 5 min hold, raising at the rate of 15 ºC /min up to 280 ºC with 26 min hold. The injector temperature and volume of injected samples were maintained at 270 °C and 1 µl respectively. Compounds of the test plant extracts were identified by comparison of their mass spectra with those in the National Institute of Standards and Technology (NIST11) and WILEY8 library.

Statistical Analysis: The statistical analysis was done using one-way analysis of variance (ANOVA), followed by Turkey test and student t-test using Graph Pad Prism version 5.01. The mean and SD values were calculated using Microsoft Office Excel 2007. A p value of <0.05 was considered to be statistically significant. All experiments were done in triplicates.

RESULTS AND DISCUSSION: The present study investigated the antibacterial and antioxidant activity of *Tectaria macrodonta* C. Chr. collected from the eastern district of Sikkim, India.

Phytochemicals are stored in different organs of plants like the rhizomes, stems, leaves, tubers, bulbs or whole plants. Therefore, different extraction methods are employed to get the various active phytochemical components of plants whose determination largely depends on the solvent being used [35, 36.] In the present study, phytochemical analyses of the aqueous, ethanol and methanol extracts of *Tectaria macrodonta* revealed the presence of saponin, tannin, anthocyanin,

flavonoid, phenol, carbohydrate and glycosides. Both the phenolic and flavonoid compounds comprise the largest group of secondary metabolites having multiple biological effects including antioxidant activity [37]. The total phenolic content in the methanol extract was 146.4 mg GAE/g; ethanol extract was 76.2 mg GAE/g and aqueous extract was 31.3 mg GAE/g. The total flavonoid content was 7.95 mg RE/g, 9.15 mg RE/g, 6.6 mg RE/g for methanol, ethanol and aqueous extracts of *Tectaria macrodonta* respectively.

FIG. 1: A. *TECTARIA MACRODONTA* C. CHR. B. RHIZOMES C. DRIED SAMPLE OF RHIZOMES D. POWDERED SAMPLE

The plants and plant products are the endless generators of phytochemicals and can be a good alternative to the antimicrobials [36]. The effectiveness of the plant extracts depends upon the chemical composition of the extracts, membrane permeability of the microbes for the chemicals and their metabolism [3]. Agar well diffusion test is a qualitative technique and is used as a preliminary screening for antibacterial activity. In the present study, the aqueous, ethanol and methanol extracts of *Tectaria macrodonta* exhibited significant antibacterial activity. Methanol and ethanol extracts were more efficient in inhibiting the growth of the test bacteria than the aqueous extract. At the lower concentration of 20 mg/ml, the methanol extract inhibited the growth of all the test bacteria followed by the ethanol extract which inhibited the growth of all the test bacteria except *Staphylococcus aureus*. However, the aqueous extract inhibited the growth of all the test bacteria except *Proteus vulgaris* at the concentration of 400 mg/ml. In case of methanol extract the maximum diameter of the zone of inhibition was observed against *Bacillus subtilis* (15.66 ± 0.57 mm), for ethanol extract the maximum diameter of the zone of inhibition was observed against *Bacillus cereus* (17 ± 1.73 mm) while the maximum diameter of the zone of inhibition for aqueous extract was observed against *Staphylococcus aureus* (12 ± 0.57 mm) at a higher concentration of 400 mg/ml. Among the three different solvent extracts, the ethanol extract of *Tectaria macrodonta* showed the maximum diameter of the zone of inhibition at 400 mg/ml. However, all the solvent extracts of *Tectaria macrodonta* did not inhibit the growth of *Pseudomonas aeruginosa* **Fig. 1** which may be due to the permeability barrier of the outer membrane [37].

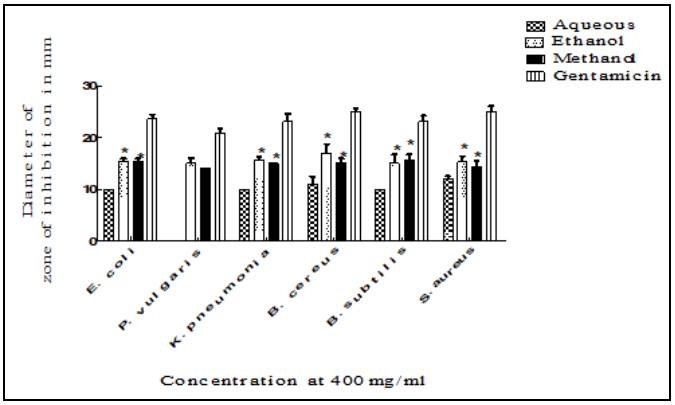


FIG. 2: DIAMETER OF THE ZONE OF INHIBITION IN MM WITH 400 mg/ml OF METHANOL, ETHANOL AND AQUEOUS EXTRACTS OF *TECTARIA MACRODONTA* AGAINST GRAM POSITIVE AND GRAM-NEGATIVE BACTERIA.*p<0.05 represent significant changes with the aqueous *vs.* methanol and ethanol extracts. *E. coli- Escherichia coli; P. vulgaris- Proteus vulgaris; K. pneumonia- Klebsiella pneumonia; B. cereus- Bacillus cereus; B. subtilis- Bacillus subtilis; S. aureus- Staphylococcus aureus*

The potential activity of the ethanolic and methanolic extracts can be attributed to the presence of a higher amount of polyphenols as compared to the aqueous extract [38]. As per the reports, the plant compounds showing anti-microbial activities are aromatic or saturated organic compounds which are mostly extracted with ethanol and methanol [39, 40]. Interestingly, it was found that the extract of *Tectaria macrodonta* inhibited the growth of *Escherichia coli, Bacillus subtilis, Bacillus cereus* and *Staphylococcus aureus* which are the causative agents for gastrointestinal disorder and food poisoning and also Gram-negative bacteria *Klebsiella pneumonia* which causes pneumonia [41]. This might be due to the presence of various phytochemicals present in the plant. Mori *et al*., [42] have reported the presence of polyphenols and tannin in *Tectaria* which have antimicrobial and anti-diarrhoeal activity [43, 44].

Accordingly, in the present study phytochemical analysis revealed the presence of tannin in all the solvent extracts of *Tectaria macrodonta*. All the extracts of *Tectaria macrodonta* were able to inhibit the growth of both the Gram positive and Gram-negative bacteria. The lowest MIC value of 6.25 mg/ml and the lowest MBC value of 12.5 mg/ml were exhibited by the methanol extract of *Tectaria macrodonta* against *Bacillus cereus* and *Staphylococcus aureus* respectively **Table 1**. The test results indicated all the plant extract to be bactericidal as MIC index was ≤4. According to Kone *et al*., [45] if the MIC index is ≤4, the extract is bactericidal, and when the MIC index is >4, the extract is bacteriostatic. Therefore, the anti-microbial activity of different solvent extracts of *Tectaria macrodonta* may be due to the presence of various phytochemical components. It has also been reported that the various phytochemical components may probably interfere with the protein synthesis and DNA functioning of bacterial cells [46].

TABLE 1: MINIMUM INHIBITORY CONCENTRATION, MINIMUM BACTERICIDAL CONCENTRATION IN mg/ml AND MIC INDEX OF THE EXTRACTS AGAINST TEST BACTERIAL STRAINS

Microorganisms	Extracts	MIC (mg/ml)	MBC (mg/ml)	INDEX
	Methanol	25	25	1
Escherichia coli	Ethanol	12.5	25	2
	Aqueous	100	400	4
Proteus vulgaris	Methanol	25	100	4
	Ethanol	50	100	2
	Aqueous	-	-	-
Klebsiella pneumonia	Methanol	25	50	2
	Ethanol	50	100	2
	Aqueous	200	400	2
Bacillus cereus	Methanol	6.25	12.5	2
	Ethanol	12.5	50	4
	Aqueous	200	400	2
Bacillus subtilis	Methanol	50	50	1
	Ethanol	50	50	1
	Aqueous	100	400	4
Staphylococcus aureus	Methanol	6.25	12.5	2
	Ethanol	50	100	2
	Aqueous	100	400	4

The antioxidant activity of methanol, ethanol and aqueous extracts of *Tectaria macrodonta* was analyzed by using different methods. In the TLC-DPPH radical scavenging assay, a yellow spot present on the TLC plate indicated the presence of an active antioxidant compound [47]. All the extracts of *Tectaria macrodonta* developed a yellow spot on the TLC plate indicating the presence of the potent antioxidant compound. The DPPH radical scavenging method is mainly for lipophilic antioxidants while FRAP is more appropriate for hydrophilic antioxidants [48]. DPPH radical scavenging activity is expressed in terms of 50% inhibitory concentration (IC_{50} value). The IC_{50} value is defined as the concentration (in µg/ml) of the extract that scavenges the DPPH radicals by 50%. The higher value of the percentage inhibition indicates higher antioxidant activity [48]. There was a significant concentration-dependent increase in DPPH radical scavenging activity. The IC_{50} value of methanol extract was 30 µg/ml with percentage inhibition of 53.26 ± 0.33%. For ethanol and aqueous extracts, the IC_{50} value was 50 µg/ml and 60 µg/ml with percentage inhibition of 53.92 ± 0.81% and 52.909 ± 0.60% respectively **Fig. 1**. The maximum reducing power concerning FRAP assay was shown by the ethanol extract followed by methanol and aqueous extracts **Fig. 2**. The hydroxyl radical is an extremely reactive species formed in the biological systems. The hydroxyl radical is known to cause DNA damage by degradation of deoxyribose moiety, and the antioxidant capacity of particular substances is directly proportional to its scavenging capacity [49].

In the present study, all the extracts of *Tectaria macrodonta* exhibited free radical scavenging activity which increased significantly ($p<0.05$) with increasing concentrations of the plant solvent extracts. The scavenging activity of methanol extract was significantly higher than the aqueous and ethanol extracts **Fig. 3**.

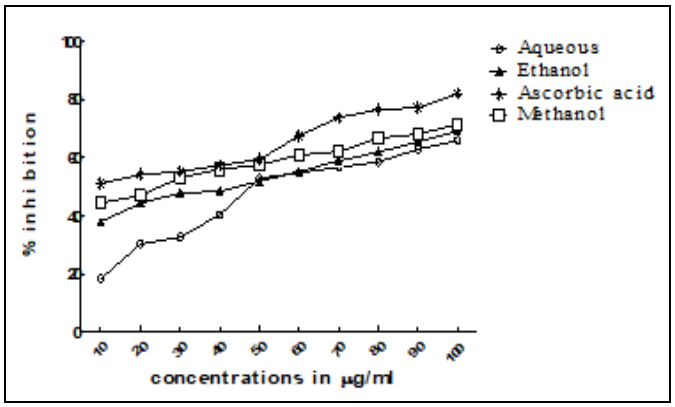


FIG. 3: DPPH RADICAL SCAVENGING ACTIVITY OF DIFFERENT SOLVENT EXTRACTS OF *TECTARIA MACRODONTA* AT DIFFERENT CONCENTRATIONS OF THE SOLVENT EXTRACTS. Each value represents mean ± SD (n=3).

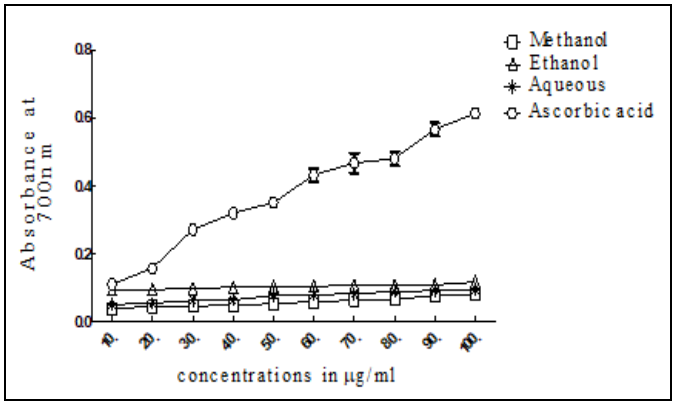


FIG. 4: FRAP ACTIVITY OF DIFFERENT SOLVENT EXTRACTS OF *TECTARIA MACRODONTA* AT DIFFERENT CONCENTRATIONS. Each value represents mean ± SD (n=3).

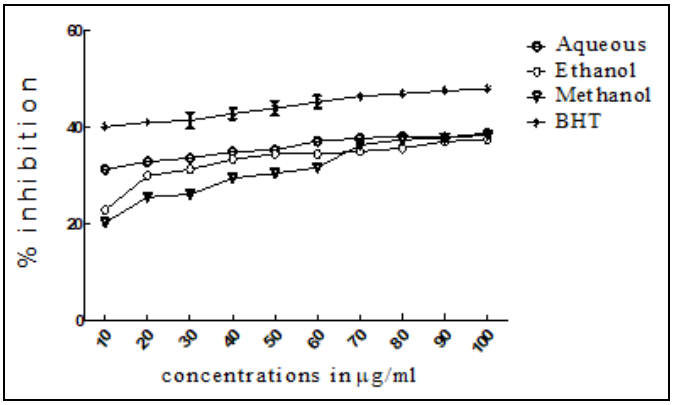
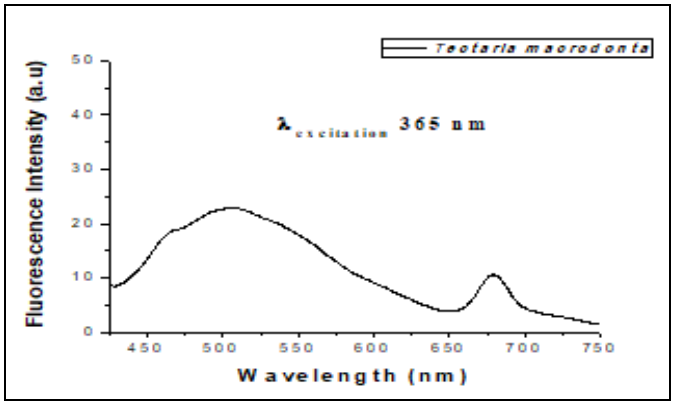


FIG. 5: HYDROXYL RADICAL SCAVENGING ACTIVITY OF DIFFERENT SOLVENT EXTRACTS OF *TECTARIA MACRODONTA* AT DIFFERENT CONCENTRATIONS. Each value represents mean ± SD (n=3).

According to Katalinic *et al*., [50] three types of kinetics have been described for the reaction between DPPH radicals and phenolics as rapid, intermediate and slow. This kinetics differs along with variable flavonoid and phenolic content in all the samples. Thus the Coefficient determination value (R^2) was considered very high if $R \geq 0.90$, high if $0.75 \leq R < 0.90$ and moderate if $0.50 \leq R < 0.75$. All R^2 values below 0.50 were considered weak correlation and negative values correspond to inverse relationships [51].

In the present study, the correlation between total phenolic, flavonoid contents and DPPH radical scavenging activity of methanol, ethanol and aqueous extracts of *Tectaria macrodonta* were analyzed. The result suggested that 84.4% phenol and 96.5% flavonoid of methanol extract, 96% phenol and 96.8% flavonoid of ethanolic extract and 91.9% phenol and 85.3% flavonoid of aqueous extract contributed to the DPPH radical scavenging activity.

Thin Layer Chromatography (TLC) is a powerful technique for isolation and identification of bioactive components in crude plant extracts [52]. TLC was performed with a mixture of solvents namely methanol and chloroform which showed a prominent fluorescent band under a UV lamp. The UV-VIS spectrophotometer shows an absorption maximum at around 365 nm. Therefore, on the evaluation of the same fraction of crude extract with UV-VIS spectrofluorimeter by exciting at 365 nm emission in the range of 450 to 550 nm was obtained. This emission is consistent with other literature reports [35] and is attributed to phenolic compounds.



Further, GC-MS analysis of methanol extract of *Tectaria macrodonta* was carried out to identify the bioactive compounds responsible for antibacterial and antioxidant activities. The analysis separated and identified a total of 53 known compounds belonging to different chemical classes (Figure 6). The major compounds included pentadecanoic acid (13.97%), 5-hydroxymethylfurfural (13.62%), 2, 3-dihydro-3, 5-dihydroxy-6-methyl-4h-pyran-4-one (7.03%), stigmast-5-en-3-ol, (3.Beta.) (6.07%), D-allose (4.55), 1, 3-benzenediol, 4-propyl (4.10%), 9, 12-octadecadienoic acid (Z, Z) (3.71%), heptadecane-(8)-carbonic acid-(1) (3.10%), octadecanoic acid (3.96%), methyl 4, 6-O-nonylidenehexopyranoside (3.75%).

Many of these identified components are known to possess several pharmacological activities. 5-hydroxymethylfurfural, a sugar compound is a major phytoconstituent of methanolic extract of *Tectaria macrodonta*, with known antioxidant and antiproliferative properties [53]. Similarly, fatty acid compound Octadecanoic Acid and flavonoid compound 2, 3-dihydro-3, 5-dihydroxy-6-methyl-4h-pyran-4-one are reported to exhibit anti-microbial activity [54].

Further, pentadecanoic acid, fatty acid compound, D-allose sugar compound, 2-furan methanol furan compound are known to possess antioxidant properties [55]. The natural coumarin derivatives are considered to have a wide range of biological activities, such as anti-inflammatory, anticancer [56, 57] anti-coagulant, anti-oxidant, anti-HIV and antibacterial [58]. Coumarins are known to possess antithrombotic, anti-inflammatory, antiviral, antitumor and antioxidant properties [59]. Esculetin, a class of coumarins, possesses anti-adipogenic, antioxidant and neuroprotective properties.

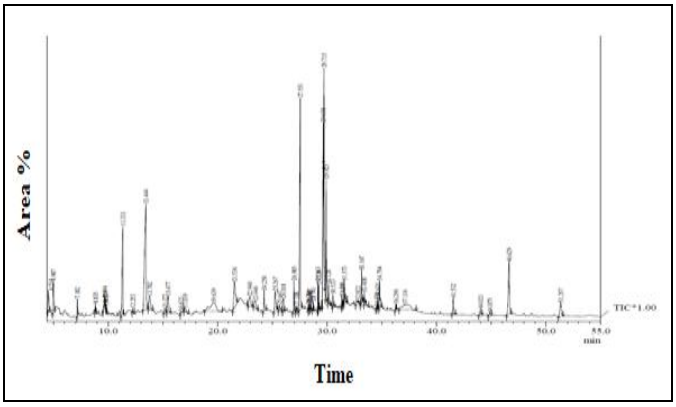


FIG. 6: GAS CHROMATOGRAPHY - MASS SPECTROMETRY CHROMATOGRAM OF METHANOL EXTRACT OF *TECTARIA MACRODONTA*

Esculetin is a blue fluorescence compound [60] and since the methanol extract of *Tectaria macrodonta* showed blue fluorescent band on TLC plate, this compound might be a coumarin derivative, Esculetin. Therefore, the occurrence of all these bioactive compounds in the methanolic extract of *Tectaria macrodonta* can be correlated with its various biological activities.

CONCLUSION: In the present study, the aqueous, ethanolic and methanolic extracts of *Tectaria macrodonta*, showed potential antibacterial activity against all the test bacteria except *Pseudomonas aeruginosa*. The antibacterial activity of the extract could be due to the presence of various bioactive compounds responsible for the antibacterial property as detected by the GC-MS analysis. The different solvent extracts of *Tectaria macrodonta* also exhibited potential antioxidant property. Further analysis, including isolation and characterization of the different components in the solvent extracts of *Tectaria macrodonta,* have to be done to reveal the pharmaceutically important components.

ACKNOWLEDGEMENT: The authors would like to acknowledge the University Grants Commission (UGC) Rajiv Gandhi National Fellowship (RGNF), Government of India, for providing financial support to Ms Bhumika Poudyali and the Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim for providing all the laboratory facilities for this research work. The authors also acknowledge Dr. Sudarsan Tamang, Assistant Professor, Department of Chemistry, Sikkim University for the analysis of the fluorescence spectroscopy data. The Advanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi, India, is gratefully acknowledged for the GC-MS analysis.

COMPETING INTEREST: The authors declare that they have no competing interests.

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How to cite this article:
Poudyali B and Singh B: Potential antibacterial and antioxidant properties of aqueous, ethanol and methanol extracts of *Tectaria macrodonta* C. Chr. Int J Pharm Sci & Res 2019; 10(8): 3785-94. doi: 10.13040/IJPSR.0975-8232.10(8).3785-94.