



Phytochemical and chromatographic characterization of *Mimosa diplotricha* Wright ex. Sauvalle ethanolic leaf extract and assessment of antioxidant potential and anti-proliferative effect on colorectal cancer (HCT-116) cell lines

Sri Rashmy Madathil, Poornima Kannappan*, Devaki Kanakasabapathy, Sincy Varghese, Perumalsamy Muneeswari

Department of Biochemistry, Karpagam Academy of Higher Education, Coimbatore, Tamil Nadu, India



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ABSTRACT

Present study aims to evaluate phytochemical and chromatographic profile, along with antioxidant and anti proliferative properties of *Mimosa diplotricha* ethanolic leaf extract. Qualitative screening of phytoconstituents by consecutive solvent extraction in increased polarity basis and standardization of potential extract based on phytochemical elution profile was done. Selected secondary metabolites like phenols, flavanoids, tannins and alkaloids were quantified in ethanolic extract. Chromatographic profile was determined by HPTLC and GC-MS analysis. *In vitro* antioxidant potential was assessed by DPPH, superoxide, nitric oxide, hydrogen peroxide and hydroxyl radical scavenging assay. Anticancer potential was confirmed by cytotoxic screening in colorectal (HCT-116) cancer cell lines by MTT assay. Qualitative phytochemical analysis and chromatographic profile reveal a phytoconstituent rich profile for the ethanolic leaf extract. The amount of, phenols (56 ± 0.57 mg/g), flavanoids (27 ± 0.76 mg/g), tannins (33 ± 0.15 mg/g) were quantified as equivalent of gallic acid, quercetin and tannic acid standards respectively and alkaloids (2.51 ± 0.47 mg/g of extracted plant material) were expressed based on respective analysis. Results also reveal convincing antioxidant potential for respective extract. *In vitro* cytotoxicity confirmed by MTT assay represents an IC_{50} value of $97.82 \mu\text{g/ml}$. From the above results it can be concluded that *M. diplotricha* has got pharmacologically significant phytoconstituents and therapeutic active ingredients as evident in HPTLC and GC-MS analysis. This is further supported by considerable antioxidant and anti proliferative properties observed in respective assays.

*Corresponding Author

Name: Poornima Kannappan
Phone: +91-0422-6453777
Email: poorniovya@gmail.com

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INTRODUCTION

Plant kingdom with its medicinally significant herbs and pharmacologically active phytochemical principles constitute the foundation basis of therapeutics historically, traditionally and also in folk lore remedies. Literature reference of plants as cure to pathogenesis dates back to vedic times (*Rigveda*, 4500-1600 BC); Egyptian papyrus, 1550 BC and Hippocrates holistic system which sets the beginning of modern system of medicine (Agrawal and Paridhavi, 2007; Atanasov et al., 2015). Secondary metabolites of vegetable kingdom constituting phe-

nols, alkaloids, tannins, flavanoids, terpenoids, glycosides, steroids etc. manifest experimentally validated reports on antioxidant, antimicrobial, anti-inflammatory, antifungal, anticancer and antidiabetic potential and also secondary metabolites represent major raw material for drug development (Nasser *et al.*, 2019; Natarajan and Yadav, 2018).

These phytoconstituents represent the fundamental ingredients for developing and designing potent drugs from plants. According to World Health Organisation (WHO) herbal drugs serves as major source of primary curative medicine for about 80% of population of developing countries because of low cost and easy availability (Calixto, 2005). National Centre for Complementary and Integrative Health of National Institute of Health (NIH) maintains records and funds research based on phytotherapeutics (Burstein *et al.*, 1999).

Cancer is a serious menace globally in the present scenario and numerous phytochemicals are screened to develop potential anticancer drug. Abnormal proliferation of cells, ability to overcome apoptosis and metastasis is the main characteristic feature of cancer cells leading to tumorigenesis mediated by a cascade of molecular signaling pathways. Phytoconstituents especially polyphenols and isolated compounds like curcumin, resveratrol, catechin, genistein etc. have clinically proven to be beneficial in cancer therapy (Elvin-Lewis, 2001; Li, 2015). As per FDA (Food and Drug Administration) 60-75% phytochemicals and isolated active compounds are approved as valid chemo preventive agents (Jacobs, 2018).

Phytochemicals currently practiced as anticancer chemo drugs include; taxol, vinblastine, vincristine, camptothecin derivatives, topotecan, etoposide and irinotecan. Phytochemicals are potentially rich in antioxidant bioactive principles and oxidative stress is directly correlated to cancer onset and progression (Narmadha and Devaki, 2012; Perumal *et al.*, 2012). Damage to biomolecules like DNA, lipids and proteins as a result of oxidative stress contributes to the onset of various types of malignancies.

Mimosa diplotricha, a species which occupy subfamily Mimosoideae of legume family (Fabaceae), comprises 40 genera and more than 2000 species. Distribution is pantropical with few species occupying temperate region (Aguiar *et al.*, 2012). It is a perennial shrub, highly invasive and characterized by scrambling or straggling habit. Economically *M. diplotricha* is significant as green manure and frequently used in crop rotation due to its nitrogen fixing potential. Information regarding phytochemical

composition and medicinal uses of *M. diplotricha* is scanty. In Formozan folk remedies roots of the plant is used as an antidote, analgesic, tranquilizer, anticancer supplement and hemostatic agent (Chirumbolo, 2012).

Plant is rich in polyphenols as peculiar to other Fabaceae members and polyphenols are reported to have antioxidant, antimicrobial, anticancer and anti-inflammatory properties (Lin *et al.*, 2011).

There are reports of few isolated compounds like pinoresinol, salicifoliol, quercetin, deoxyflavones, chalcones and diterpenoids from aerial parts and roots of *M. diplotricha* (Chiou *et al.*, 2016; Trease and Evans, 1996).

Present study is focused to evaluate phytochemical profile by qualitative and quantitative means, chromatographic evaluation by HPTLC & GC-MS analysis, antioxidant and *in vitro* antiproliferative properties of *M. diplotricha* ethanolic leaf extract on colorectal cancer (HCT-116) cell lines.

EXPERIMENTAL PROCEDURE

Preparatory phase

Collection of sample, purchase of chemicals and cell line

Samples of *M. diplotricha* were procured from Palakkad District, Kerala, South India in the month of July-August. The specimens were identified, authenticated (Botanical Survey of India) and the respective voucher number is BSI/SRC/5/23/2018/Tech./ 2040. All chemicals required for study were obtained from Sigma-Aldrich (St. Louis, MO) and HiMedia laboratories (India) and of analytical grade. Colon cancer cell lines (HCT-116) were obtained from National Centre for Cell Science (NCCS), Pune.

Qualitative Phytochemical studies

Collected plant specimens were washed in distilled water and shade dried. The powdered sample is then subjected to extraction in organic solvents sequentially in ascending order of polarity; petroleum ether, chloroform, ethyl acetate, ethanol and water respectively. Each consecutive extract is then analysed for characteristic phytochemical profile including, alkaloids, flavanoids, phenols, tannins, steroids, cardioglycosides, carbohydrates, aminoacids/proteins, saponins, oils/fats and terpenoids according to (Harborne, 1989; Trease and Evans, 1996).

Estimation of secondary metabolites

50g of powdered leaf sample was extracted with 250ml of ethanol by mild maceration for 72 hours.

Table 1: Qualitative phytochemical profile of ethanolic leaf extract of *M. diplotricha*

Phytochemical constituents	Solvents				
	Petroleum ether	Chloroform	Ethyl acetate	Ethanol	Water
Alkaloids	+	-	+	+	+
Steroids	+	-	-	+	+
Flavonoids	-	+	+	++	+
Tannins/phenols	-	-	++	++	+
Aminoacids and Proteins	-	+	+	+	+
Oil & fats	-	-	-	-	-
Carbohydrate	-	+	+	+	-
Glycosides	-	+	-	+	+
Saponins	+	-	-	+	+
Terpenoids	-	+	+	+	-

Table 2: IC₅₀ value of antioxidant assay

Antioxidant assay	IC ₅₀ (µg/ml)	
	Sample	Standard
DPPH radical scavenging assay	63±0.63	66±0.18
Superoxide radical scavenging assay	58±0.24	53±0.39
Nitric oxide radical scavenging assay	57±0.08	66±0.14
Hydrogen peroxide radical scavenging assay	66±0.55	51±0.67
Hydroxyl radical scavenging assay	50±0.95	64±0.03

Values are expressed as Mean ± SD for triplicate

Extract concentrated by evaporation of solvent and stored in air tight bottle for further phytochemical and *in vitro* free radical scavenging and cytotoxic assays.

Phenolics was quantified according to the standard protocol, (Singleton and Rossi, 1965). Experimental analysis is based on the principle that phenolics in alkaline medium react with phosphomolybdic acid of Folin-Ciocalteu reagent resulting in intense blue colour which is estimated colorimetrically at 650nm. Gallic acid was used as the reference standard. Phenolic concentration is calculated from calibration graph and represented as gallic acid equivalents in milligrams per gram of dry sample weight (mg/g).

Flavonoids

Total flavanoids were determined according to (Soong and Barlow, 2004). To sample and standard (quercetin) aliquots, 0.5ml of 2% aluminium chloride was added and kept for 1 hour at room temperature. Flavonoids gives yellow colour with ethanolic aluminium chloride which was colorimetrically quantified at 420nm. From calibration curve flavanoid concentration in sample was calculated

and expressed as quercetin equivalent in mg/g of dry matter.

Tannins

Tannins were determined according to the method described by (Amadi et al., 2004). Tannins give yellow colour by reduction of phosphomolybdic acid in alkaline medium which can be measured spectrophotometrically at 700nm. Tannin concentration expressed as tannic acid equivalent in mg/g of dry matter.

Alkaloids

Alkaloids were quantified according to the standardized protocol of (Harbone, 1973). About 50g of powdered leaf sample dissolved in 40ml of 10% acetic acid prepared in ethanol and kept aside for 4 hours. The mixture was then filtered and reduced to one quarter volume. Concentrated ammonium hydroxide is added to the extract, precipitate formed is filtered, washed in dilute ammonium hydroxide and weight recorded in a pre weighed vial. Total alkaloid content was estimated as follows;

$$\text{Total alkaloid content} = \frac{W_x - W_y}{W} \times 100$$

Table 3: GC-MS profile of bioactive compounds present in ethanolic extract of *M.diplotracha* leaves

S No	RT	Name of the compound	Molecular formula	Molecular weight	Peak area %
1	25.533	alpha.-d-mannofuranoside, 1-O-decyl- hexofuranoside	$C_{16}H_{32}O_6$	320	5.28
2	25.932	1,5-anhydro-d-mannitol	$C_6H_{12}O_5$	164	4.09
3	30.826	Ledol	$C_{15}H_{26}O$	222	1.13
4	31.262	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	6.32
5	34.020	2-Hexadecen-1-ol, 3,7,11,15-tetra E,E,Z-1,3,12-nonadecatriene-5,14-diol	$C_{20}H_{40}O$	296	8.25
6	34.414	Cyclopropaneoctanoic acid	$C_{22}H_{38}O_2$	334	7.65
7	34.812	Ethyl (9Z,12Z)-9,12-octadecadien	$C_{20}H_{36}O_2$	308	6.19
	42.968	Methyl (Z)-5,11,14,17-eicosatetraenoate	$C_{21}H_{34}O_2$	318	7.55
9	45.246	2,6,10,14,18,22-Tetracosahexaene	$C_{30}H_{50}$	410	5.70
10	48.414	.gamma.-Tocopherol	$C_{28}H_{48}O_2$	416	1.09
11	49.853	.alpha.-Tocopherol-.beta.-d-mannoside	$C_{35}H_{60}O_7$	592	4.68
12	52.446	Stigmasterol	$C_{29}H_{48}O$	412	8.01

Where W_x is the weight of empty vial, W_y weight of vial having extract and W initial weight of powdered sample.

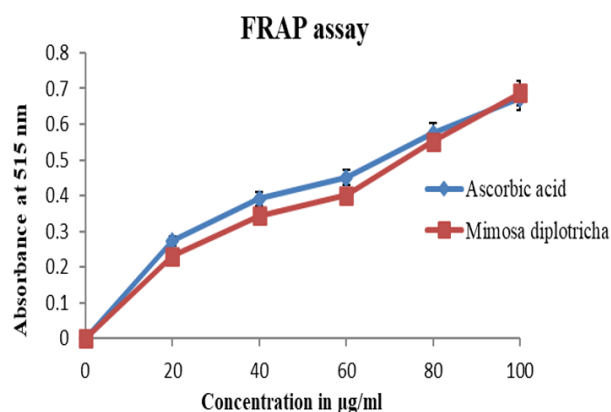


Figure 1: Graphical representation of FRAP assay of *M.diplotracha* ethanolic leaf extract

In vitro free radical scavenging assays

Extract dissolved in 95% of ethanol and concentration fixed as 1mg/1ml. From this stock solution appropriate dilution is carried out and sample concentration is standardized to 20µg, 40µg, 60µg, 80µg and 100µg.

Ascorbic acid is used as standard. Antioxidant potential of the plant sample was quantified by executing the following free radical scavenging assays; DPPH, superoxide, nitric oxide, hydrogen peroxide, hydroxyl, reducing power and ferric reducing

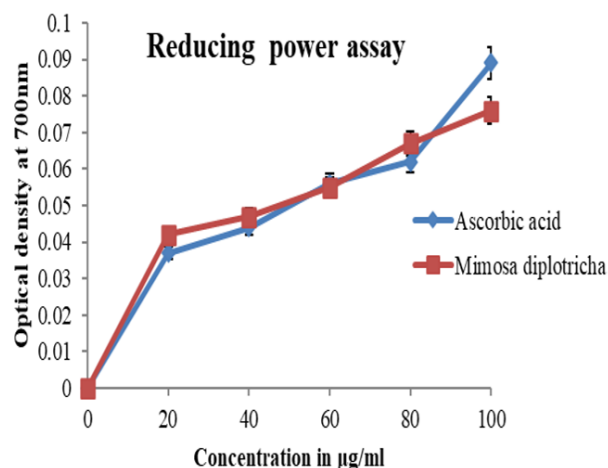


Figure 2: Graphical representation of reducing power assay of *M.diplotracha* ethanolic leaf extract

antioxidant potential (FRAP) by means of standard cited protocols (Blois, 1958; Liu *et al.*, 2000; Klein *et al.*, 1981; Ruch *et al.*, 1989; Garrat, 1964; Oyaizu, 1986; Benzie and Strain, 1996).

Percentage of inhibition or radical scavenging is directly proportional to antioxidant ability of respective extract. For superoxide, nitric oxide, DPPH, and hydrogen peroxide radical scavenging assay inhibition percentage was evaluated by the

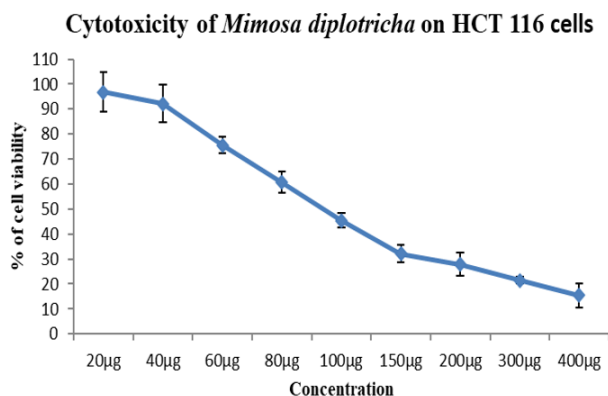


Figure 3: In vitro cytotoxicity activity (MTT assay) of *M.diplotricha* ethanolic leaf extract in HCT 116

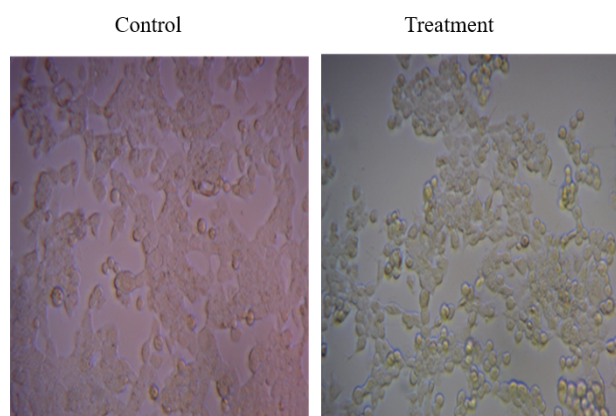


Figure 4: Morphology of HCT 116 colon cancer cells

formula as represented,

$$\text{Percentage of radical scavenging} = \frac{C_A - S_A}{C_A} \times 100$$

where C_A is absorbance of control and S_A is the absorbance of sample. For hydroxyl radical scavenging assay percentage inhibition was calculated as ratio of difference in absorbance of sample and control. The value obtained is then subtracted by one and expressed graphically.

Cytotoxicity in HCT-116 cell lines

MTT assay

Inhibition of tumor cell proliferation by ethanolic leaf extract of *M.diplotricha* was assessed by MTT (3-(4) 5-Dimethyl-thiazol-Zyl) - 2, 5 biphenyl tetrazolium bromide) assay. Colorectal cancer cell lines were seeded in 96 well plate.

Sample fraction in 0.1% DMSO diluted in accordance to get appropriate concentration. Cell lines treated with varying concentration of extract were incubated for 24 hours. Control group left alone having media and 0.1% DMSO. Plates were washed with

PBS and MTT dye was added. Absorbance recorded at 540nm. Cell viability was calculated as follows,

$$\text{Percentage of viability} = \frac{100 - [(A_o - A_t)]}{A_o} \times 100$$

Where A_o is the absorbance of control and A_t is the absorbance of cells subjected to treatment. IC_{50} value was calculated from calibration graph.

GC-MS chromatographic analysis

GC-MS is a combined chromatographic technique that facilitates separation of volatile constituents and fragmentation of separated entities on the basis of molecular mass and detection of compounds by means of associated mass spectrophotometer utilizing the principle of charge to mass ratio.

Gas chromatogram and mass spectra of the components in sample were detected and recorded by Thermo GC-Trace Ultra Version: 5.0, Thermo MS DSQ II and mass with those of Wiley library and NBS (National Institute Standard and Technology) database available in computer library.

HPTLC analysis

HPTLC (High performance thin layer chromatography) is a precise and reliable chromatographic technique for the documentation of phytochemical profile. It combines the principle of TLC and scanning densitometry.

The sample loaded plate was kept in saturated TLC chamber with specific mobile phase for alkaloids (Ethyl acetate : Methanol : Water; 8 : 1 : 1), flavanoids (Toluene : Ethyl acetate : Methanol, 7:3:1), phenols (Toluene : Ethyl acetate : Acetic acid, 6 : 3 : 1) and tannins (Toluene : Ethyl acetate : Methanol : Formic acid, 6:3:0.8:0.2).

The plate after development was air dried and scanned at 366nm using Camag Scanner with WIN-CATS software. The retention factor of each compound and peak area percentage was also recorded.

Statistical evaluation

All the experimental observations were performed in triplicates and data were recorded as mean \pm standard deviation of experimental triplicates.

RESULTS AND DISCUSSION

Phytochemical parameters

Qualitative phytochemical profile of ethanolic leaf extract revealed phyto compounds like alkaloids, flavanoids, steroids, glycosides, saponins, aminoacids, carbohydrates, terpenoid, tannins and phenols (Table 1).

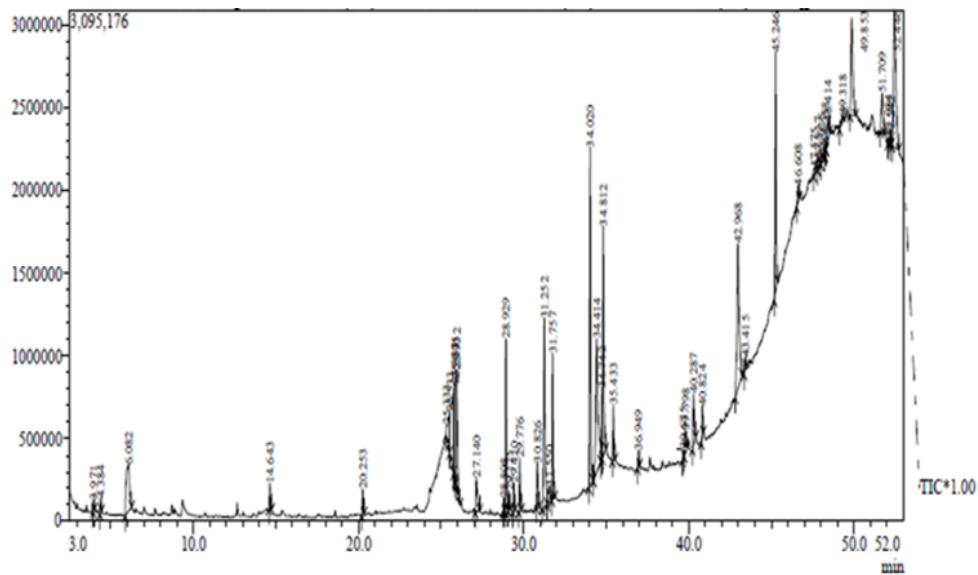


Figure 5: GC-MS chromatogram of ethanolic leaf extract of *M. diplotricha* Wright ex. Sauvalle

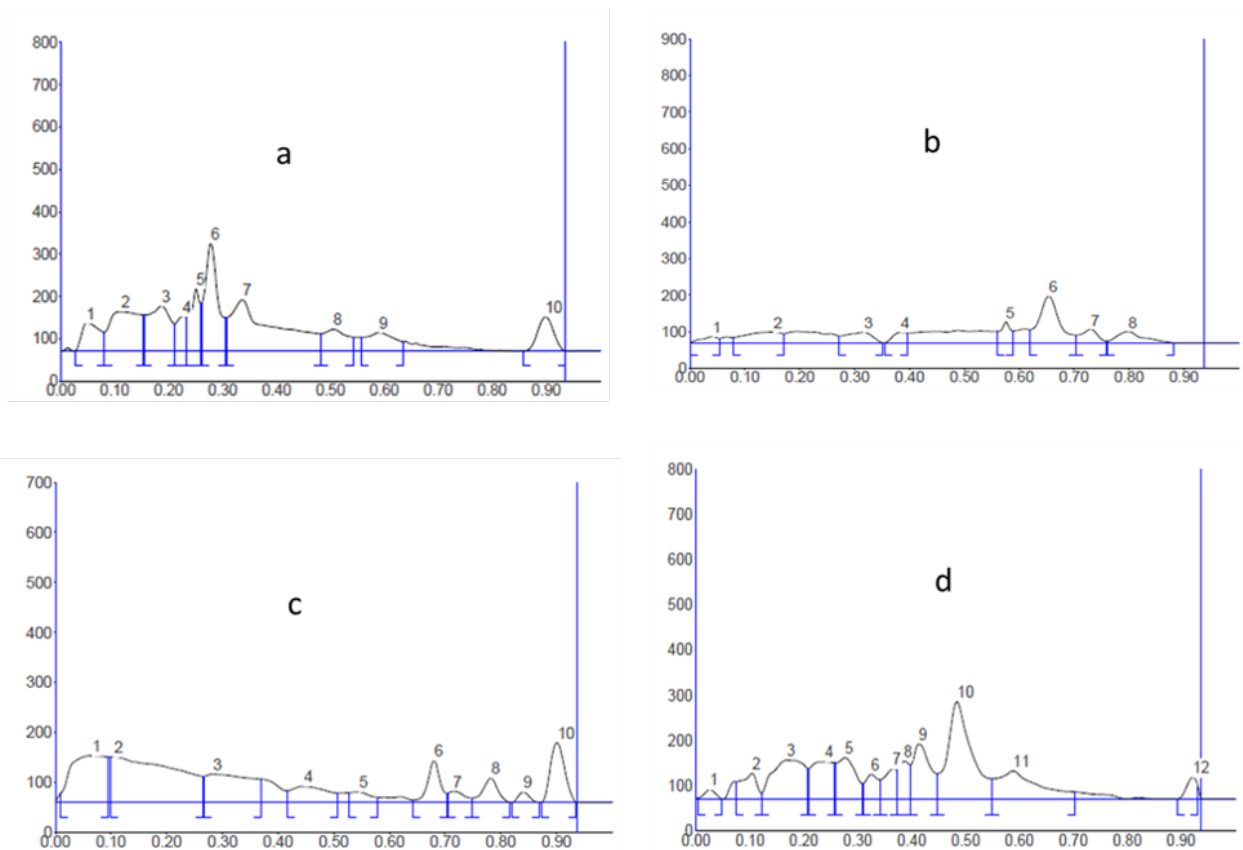


Figure 6: a: HPTLC densitogram of flavanoid profile; b: HPTLC densitogram of alkaloid profile; c: HPTLC densitogram of phenol profile and d: HPTLC densitogram of tannin profile for ethanolic leaf extract of *M. diplotricha*

Data as per consecutive solvent extraction, it was evident that ethanolic extract have maximum phytochemical profile. Quantitative estimation of secondary metabolites indicates that the plant is comparatively rich in phenolic group of compounds.

The total phenol, flavanoid, tannin and alkaloid content of the plant sample was found to be 56 ± 0.57 (mg/g gallic acid equivalent), 27 ± 0.76 (mg/g quercetin equivalent), 33 ± 0.15 (mg/g tannic acid equivalent) and 2.51 ± 0.47 (mg/g of extracted plant material) respectively.

***In vitro* free radical scavenging assay**

Antioxidant ability of plants is mainly due to non-nutritive phytochemicals classified as secondary metabolites. Each plant species is characteristic in having its own unique phytochemical profile which contributes to specific pharmacological attributes of medicinal herbs.

From the results of antioxidant assays it is evident that the plant is having significant free radical scavenging potential. IC₅₀ value of DPPH, superoxide, nitric oxide, hydrogen peroxide and hydroxyl radical scavenging assay are shown in (Table 2). FRAP and reducing power assay also exhibit increased percentage of scavenging with increase in concentration (Figures 1 and 2).

Antiproliferative activity in HCT-116 cell lines (MTT assay) Antiproliferative effect of ethanolic leaf extract of *M.diplotricha* is evaluated by metabolic assay, based on the reduction of MTT dye by mitochondrial oxido reductase to purple colored formazan which can be quantified colorimetrically.

From the results it can be evaluated that the plant exhibit inhibition of cell viability in a concentration dependent manner. IC₅₀ value of the corresponding extract is $97.82 \mu\text{g/ml}$ at 24 hours of incubation (Figure 3). Morphology of colon cells are shown in Figure 4.

GC-MS analysis

GC-MS chromatographic profile of ethanolic leaf extract of *M.diplotricha* revealed 45 peaks corresponding to 45 volatile Phytoconstituents. GC-MS chromatogram is depicted in Figure 5.

Among 45 secondary metabolites, compounds having comparatively higher peak area and having reports on pharmacological activity are depicted in Table 3.

HPTLC analysis

Based on the evaluation of phytochemical quantitative estimation data HPTLC analysis was carried out specifically for alkaloids, flavanoids, phenols and

tannins to validate a preliminary documentation of respective phytochemical profile.

HPTLC densitogram of secondary metabolites are depicted in Figure 6, which confirms that the ethanolic leaf extract of *M.diplotricha* is having significant phytoconstituents of which polyphenols being comparatively abundant.

Statistical Analysis

The data are expressed as Mean \pm Standard deviation from three independent experiments.

Recent innovative strategy in pharmacological research constitutes experimental evaluation of herbal extracts for their therapeutic potency and further isolation of bioactive ingredients and biological drug interactions.

Cardiovascular diseases, cancer and diabetes can be recognized as ubiquitous global menace and all over world phytochemical or herbal products based research is steering to find an alternative for aggressive synthetic therapeutic drugs.

Understanding of phytochemical constituents of species under concern is the preliminary step in pharmacological research. As per findings of present study *M.diplotricha* is having all the major class of characteristic secondary metabolites and ethanolic extract is comparatively rich in almost all phytochemicals except fats & oils.

Literature reports classify *M.diplotricha* as a nutrient rich fodder plant abundant in nitrogen and amino acids. The species also have reports on non protein amino acid mimosine which is reported to have inhibitory effect on Cyclin E associated kinases thereby putting a check on abnormal cell division (Jayasree, 2005).

Quantitative estimation and HPTLC data confirms that the plant is rich in polyphenols a characteristic feature of Fabaceae. Chemical profiling by GC-MS analysis elucidate 45 volatile bioactive phytochemicals comprising sugar alcohols, terpenoids, fatty acid esters and steroids.

Among the identified compounds ledol, sesquiterpene is having reports on antimicrobial, anti-inflammatory and anti-cancer properties (Kumar et al., 2007; Matos et al., 2006).

Stigmasterol a steroid component is characteristic in having antioxidant and anti-inflammatory attributes (Matos et al., 2006). Tetracohexane, gamma-tochopherol, n-hexadeconoic acid, alpha-D-mannofuranoside, 1, 5-anhydro-d-mannitol is having reports on antioxidant and antimicrobial properties (Gomathi et al., 2015; Kumar et al., 2007).

For most of the degenerative diseases like

atherosclerosis, Alzheimer's, Parkinsonism, Ischemic disease, cancer and diabetes, free radical imbalance constitute as a major risk factor (Shirwaikar *et al.*, 2006).

Natural or herbal products based antioxidants is being widely exploited in the present era. Most of the phytoconstituents and isolated compounds have valid reports on antioxidant potential. The present species under consideration is having considerable antioxidant potential.

IC₅₀ value of DPPH, hydroxyl, nitric oxide, superoxide and hydrogen peroxide in comparison with standard ascorbic acid proved that the plant is having significant radical scavenging activity.

FRAP and reducing power assay further confirms the result. Effective antioxidant potential of the plant is attributed to the presence of potent bioactive phytoconstituents, confirmed by phytochemical and chromatographic profiling data.

M.diplotricha exhibit convincing results on anti-neoplastic activity. At 97.82 μg concentration of extract 50% of cell viability is encountered at 24 hours treatment. Natural products especially herbal formulations and drugs are ever increasing in demand in pharmaceutical market particularly in oncology therapeutics as alternative to synthetic cancer drugs which are highly toxic to biological system.

Colorectal carcinoma is a typical variant of gastrointestinal cancer, ranks third in incidence and fourth in mortality rate. Due to westernized diet and changed environmental conditions colorectal cancer is being evident as a frequently encountered neoplasm in persons above 50 (Kolligs, 2016).

Finding a herbal alternative for colorectal carcinoma is a demanding prerequisite of current scenario. Anti-cancer property of phytochemicals is mainly due to antiapoptotic, antiangiogenic and cytostatic due to microtubule disorganization as vinca alkaloids/taxol or cell cycle arrest (altering the expression of cell cycle checkpoint proteins) (Alam *et al.*, 2018).

Respective plant is rich in bioactive constituents like tochopherol, ledol, stigmasterol having reports on anti-inflammatory and *in vitro* antiproliferative activity. Stigmasterol and ledol is having anti-inflammatory and anticancer activity (Kumar *et al.*, 2007; Matos *et al.*, 2006).

Inflammatory mechanism has got a direct correlation in colorectal neoplasm by mediating NF- κ B inflammatory pathway. Anti-inflammatory activity directly contributes as a preventive measure in the onset of colorectal cancer. Tochopherol stim-

ulates the expression of wild type tumor suppressor (p53) gene and results in down regulation of mutant (p53) gene. Tochopherol also exhibit anti-angiogenic effect (Kumar *et al.*, 2007).

Quercetin a significant and effective antineoplastic and anti-inflammatory agent is reported to be present on aerial parts of *M.diplotricha*. Quercetin has experimental evidence on inhibitory effect on colon, gastric, endometrial, liver, leukemia, ovary and non-small-cell lung cancer through mediating regulation of expression of p53 and p21 (Chirumbolo, 2012).

CONCLUSIONS

Based on the results it can be concluded that *M.diplotricha* ethanolic leaf extract have a rich phytochemical profile representing all major class of secondary components. *In vitro* radical scavenging and cytotoxic screening in HCT-116 cell lines indicates significant antioxidant and antineoplastic activity of the plant. These cumulative findings paves way to further explore the species for isolation of pharmacologically active ingredients and assessing anticancer potential by *in vitro* and *in vivo* models and consequent elaboration into molecular mechanism of action in biological system.

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Conflict of Interest

The authors declare no conflict of interest.

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