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FREE RADICAL SCAVENGING ACTIVITY, TLC, HPTLC AND GC-MS ANALYSIS OF DRY FLOWER OF MICHELIA CHAMPACA LINN

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ABSTRACT

Free radicals contribute to more than one hundred disorders in humans. Free radical scavenging activity was observed in both the methanol extract (M.E) and aqueous extract (A.E) of Michelia champaca respectively. In this study significant free radical scavenging activity was determined by evaluating the inhibition concentration (IC50) in each test. In 2, 2-diphenyl-1-picrylhydrazyl (DPPH) model the extract displayed potential free radicals scavenging activity with IC50 of M.E is 270.80µg/mL and A.E is 395.77µg/mL.The nitric oxide model displayed IC50 of 282.74µg/mL in M.E and 470.80µg/mL in A.E. While the superoxide ion model showed IC50 of 269.62 µg/mL and 421.15µg/mL respectively, for both methanol and aqueous extract when compared to standard ascorbic acid. The presence of phenol, flavonoid and total antioxidant in both the extract justifies the antioxidant potential of the plant which brings about its free radicals scavenging potential.TLC plate results showed the five spots in methanol and its Rf values were 0.230, 0.307, 0.538, 0.641 and 0.948.whereas in aqueous extract shows only three bands and its Rf values were 0.4,0.7 and 0.9. GC-MS analysis showed the presence of 12 different phytochemicals with Carbamazepine 10,11 epoxide found to be the compound with maximum peak percentage 35.88% in methanol extract. HPTLC results confirmed that the extract contained several potential active components such as phenols, flavonoids, saponins and terpenoids as the slides revealed multi-coloured bands of varying intensities. This study confirmed that the M. champaca had multipotential antioxidant and free radicals scavenging activities. Thus may be due to the cumulative effect of the phytochemicals present in the flower which genuinely designate them as a free radical scavenger.

KEY WORDS Medicinal plants; Michelia champaca, Free radical scavenging activity, Phytochemical, Antioxidants, HPTLC, GC-MS.

INTRODUCTION

Free radicals are the main factor of oxidative damage in animals and humans. Reactive oxygen species (ROS) are formed by hydrogen peroxide or superoxide anions. The increased production of ROS can result in oxidative stress. The excess of ROS can cause DNA damage and be harmful to cells and tissues.[1] In vivo, some of these ROS play an important role in cell metabolism including energy production, phagocytosis and intercellular signalling.[2] However, they may also cause great damage to cell membranes and DNA, inducing oxidation that causes membrane lipid peroxidation, decreased membrane fluidity, and DNA mutations leading to cancer, various degenerative disorders such as cardiovascular diseases, aging and neurodegenerative diseases and other diseases.[3]

Antioxidant based drugs/formulations is used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease, and cancer have appeared during the last 3 decades. This has attracted a great deal of research interest in natural antioxidants. The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. Plants are potential sources of natural antioxidants. It produces various antioxidative compounds to counteract reactive oxygen species (ROS) in order to survive.[4] ROS, which include free radicals such as superoxide anion radicals (O2-), hydroxyl radicals (OH-) and non free-radical species such as H2O2 and singled oxygen (1O2), are various forms of activated oxygen. These molecules are exacerbating factors in cellular injury and aging process. The majority of the antioxidant activity is due to the flavones, isoflavones, flavonoids, anthocyanin, coumarin, lignans, [linens] catechins and isocatechins.[5] An improper balance between formation and destruction of free radicals may play a role in the occurrence of a number of ailments.Low levels of antioxidants or inhibition of the antioxidant enzymes, causes oxidative stress and may damage or kill cells. Medicinal plants are sources of certain bioactive molecules which act as antioxidants and[6] can protect the human body against cellular oxidation reactions. Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of a number of diseases.

The different Phyto constituents present in plants include anthraglycosides, arbutin, bitter drugs, flavonoids, alkaloids, saponins, coumarins, phenol, carboxylic acids terpenes and

valepotriates. In addition to the above compounds found in natural foods, vitamins C and E, β-carotene, and α-tocopherol are known to possess antioxidant potential.[7] The phytoconstituents confer specific characteristics and properties of plants. Therefore, the analysis of these constituents would help in determining various biological activities of plants. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug.Unlike synthetic drugs, herbal medicine is a complicated system of mixtures. Thus, the methods of choice for identification of 'botanical drug' are mainly intended to obtain a characteristic fingerprint of a specific plant that represent the presence of a particular quality defining chemical constituents. For such purposes, chromatographic techniques such as high performance liquid chromatography (HPLC), gas chromatography – mass spectrometry (GC-MS) and thin layer chromatography (TLC) were used widely[8]

Many Indian medicinal plants are considered potential sources of antioxidant compounds. In some cases, their active constituents are known. However, traditionally used medicinal plants awaits such screening. On the other hand, the medicinal properties of plants have also been investigated in the light of recent scientific developments throughout the world, due to their potent pharmacological activities, low toxicity and economic viability. So, in order to contribute further to the knowledge of Indian traditional plants, our present study is focussed on to determine their antioxidant and free radical scavenging properties of Michelia champaca dry flowers. The literature survey's related to active principle compounds present in it showed scanty information available on these plants and thus prompted us to analyze this common Ayurvedic plant.

Flowers have been used since as far back as 50,000 years in funeral rituals. Flowers are mainly used for religious purposes, after which they are thrown away into the environment. In modern times, people have sought ways to cultivate, buy, wear, or otherwise be around flowers and blooming plants, partly because of their agreeable appearance and smell. The other most important use of flowers is their use in medicine and cosmetics.

Michelia champaca L. (Family: Magnoliaceae) is a glorious ancient Indian medicinal plant, is a tall tree with yellow fragrant blossoms and aggregate fruits.Champaca flowers are diminutive, axillary, brachyblast, solitary or rarely in pairs, large, tepals 6-12, in 3-6 usually subequal whorls, white to yellow, stamens many anthers with a short to prominently elongated connective, gynoecium stipitate with spirally arranged, free or connate carpels

containing many ovules. Michelia champaca, a flower commonly known as Swarna Champa which is rich in antioxidants, flavonoids and possessing several folkloric uses, traditionally claimed of having CNS effects. Previous investigations on the plant have revealed that it possesses anti-inflammatory, antimicrobial and leishmanicidal activity.[9,11] anticancer activity,wound healing. Antidiabetic Cardio productive Locomotor, Antianxiety, antidepressant activity.[12,15]

Till present, the information on isolated constituents, chemical composition, biological activities and their medicinal values of genus M. champaca is still lacking in the literature. Therefore, this study was carried out to enrich the information of the medicinal property of M. champaca in terms of free radical scavenging (antioxidant) potential, as well as to evaluate the preliminary Phytochemicals by TLC, high performance thin layer chromatography (HPTLC), and GC-MS analysis of methanol extracts of Michelia champaca.L dry flower. However, most of the species of michelia are not well expoled yet, therefore, it is necessary and need of the hour to focus on phytochmical and biological studies of these plants in order to find out the role of this genus in the treatment of various diseases or disorders.

2. MATERIALS AND METHODS

Drugs and chemicals Gallic acid, quercetin, ascorbic acid, sodium nitroprusside, phenazonium methosulphate, NADH and DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) were purchased from Sigma Aldrich. Co. (St. Louis, USA). All the other chemicals, which were obtained from Sisco Research Laboratory (Mumbai, India), were of analytical grade.

2.1 Collection and Identification

Flowers of Michelia champaca were collected from the local markets from Chennai, and was authenticated by Dr. D Aravind, Department of Medicinal Botany. Voucher specimens have been deposited in the Herbarium of National institute of Siddha, Reg no: NIS/MB/94/2013. The collected flowers were cleaned, dried under shade at room temperature. Then the flower was ground into a coarse powder with the help of a suitable grinder. The powder was stored in an airtight container and kept in a cool, dark and dry place until the analysis commence.

2.2 Extraction Procedure

150 grams of M. champaca dry flowers were extracted with 500 mL of sterile distilled water and methanol using the Soxhlet apparatus. The extracts were then filtered with Whatman No

1 filter paper and then freeze dried, stored at 4°C for further investigation. The extraction efficiency was quantified by determining the weight of each of the extracts (Table-1) and the percentage yield was calculated.

2.3 Determination of total phenolic content

Total phenolic content of the extract was determined by the Folin-Ciocalteu reagent method.[16] One milliliter of the plant extracts/standard of different concentration solutions was mixed with 5 mL Folin-Ciocalteu reagent (previously diluted with water 1:10 (v/v)) and 4 mL of sodium carbonate (7.5%). The mixtures were vortexed for a few seconds and allowed to stand for 30 min at 20°C for color development. Absorbance of samples and standard was measured at 765 nm using spectrophotometer against blank. The total phenolic content of the plant extract was calculated as the gallic acid equivalent.

2.4 Determination of total flavonoids content

Total flavonoids content was determined by aluminum chloride method.[17] One milliliter of the plant extracts was mixed with 3 mL of methanol, 0.2 mL of aluminum chloride, 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water. The mixture remained at room temperature for 30 min, and the absorbance of the reaction mixture was measured at 415 nm with spectrophotometer against blank. The total flavonoids content of the plant extract was calculated as the quercetin equivalent.

2.5 Total antioxidant capacity

The total antioxidant capacity of various extracts was evaluated as per the method described by Prieto et al.[18] Extracts were dissolved in a mixture of 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. After the samples were cooled to ambient temperature, the absorbance of the solution was measured at 695 nm against reagent blank containing only the respective solvents. The total antioxidant content of the plant extract was calculated as the ascorbic acid equivalent.

2.6 2, 2-diphenyl- 1-picrylhydrazyl (DPPH) free radical scavenging assay

The free radical scavenging capacities of the extract were determined using DPPH.[19] The mixture of DPPH (0.1 mM) in methanol was prepared and 4 mL of this solution was added to 1 mL of sample solution in methanol at different concentrations (50 to 250 µg). Thirty minutes later, the absorbance was measured at 517 nm and at room temperature using a

spectrophotometer Shimadzu UV-1800A. Lower absorbance of the reaction mixture indicates a higher free radical scavenging activity. Ascorbic acid was used as a standard. DPPH scavenged (%) = [(Abs control – Abs test) / Abs control] × 100% Where Abs control is the absorbance of the control reaction and Abs test is the absorbance of the extract/standard.

2.7 Nitric oxide scavenging assay

The nitric oxide scavenging assay was carried out according to the method reported by Sreejayan et al.[20]In this assay 200 µL of 10 mM sodium nitroprusside and 50-250 µg of various concentrations of the sample were incubated at room temperature for 150 min. Following incubation, 500 µL of Griess reagent (1% sulfanilamide in 5% orthophosphoric acid and 0.1% N-(1-napthyl ethylenediaminedihydrochloride) in the ratio of 1:1 was added and incubated for 10 min at room temperature. Ascorbic acid was used as a standard. The absorbance was measured at 546 nm. Controls were run devoid of samples and the inhibition rate was calculated as follows. Nitric oxide scavenging effect = O.D (blank) – O.D (sample) × 100% (Inhibition rate (%)) O.D (blank)

2.8 Superoxide anion scavenging assay

Superoxide anion scavenging was carried out according to the method described by Liu et al.[21] In this method, superoxide radicals were generated in 3 mL of Tris HCl buffer (16 mM, pH–8.0) containing 1 mL of nitroblue tetrazolium (NBT; 50 µM) solution, 1 mL of nictonamide adenine dihydrogen salts (NADH; 78 µM) solution and 50-250 µg of the samples was added, whereas 1mL of distilled water was added to the control tubes. The reaction started by adding 1 ml of phenazonium methosulphate (PMS) solution (10 µM) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. Superoxide anion scavenging effect = O.D (blank) – O.D (sample) × 100% (Inhibition rate (%)) O.D (blank)

2.9 Reducing power capacity assessment

Reducing power was carried out using the method reported by Yildirim et al.[22] One milliliter of the extract and its sub-fractions (final concentration 50-250 µg/mL) were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide. The mixture was then incubated at 50°C for 20 min. To this mixture, 2.5 mL of trichloroacetic acid was added, then centrifuged at 3000 rpm for 30 min. Finally, 2.5 mL of the supernatant solution was collected and mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride;

absorbance was measured at 700 nm. Ascorbic acid was used as a standard and phosphate buffer as the blank solution.

2.10 Thin layer chromatography (TLC)

The TLC was performed on precoated 20×20 cm and 0.25 mm thick plates. The plates were prepared by using silica gel G for TLC, were left overnight for air drying. These plates were activated by hot air oven at 100ºc for 1hr.The plates were dried and developed in suitable solvents for rapid screening toluene: chloroform**:** methanol. The plates were run in the above solvent systems and dried at room temperature. The spots were identified both in the UV light at 254nm, and in the iodine chamber. Different bands were observed and corresponding Rf values are determined. Rf value of each spot was calculated as.

Rf = Distance travelled by the solute / Distance travelled by the solvent.

2.11 HPTLC

For HPTLC fingerprinting analysis 3, 6 and 9 µL of the plant samples were loaded in precoated HPTLC plates [silica gel 60 F 254 (E. MERCK KGaA) and plate size- 5x10 cm]. The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phases (flavonoids and phenols), solvent system [chloroform: methanol: formic acid: acetic acid (80:15:2.5:2.5)], solvent front position 50.0 mm and volume 10 mL. The developed plate was dried by hot air at 60°C to evaporate solvents from the plate. The plate was kept in photo documentation chamber (CAMAG TLC Scanner 3) and the images were captured at UV 254 nm and UV 366 nm. The retention factor (Rf) values at fingerprint data were recorded by WINCATS software.

2.12 GCMS Analysis

Gas chromatography

An Agilent 6890 gas chromatograph equipped with a straight deactivated 2 mm direct injector liner and a 15m Alltech EC-5 column (250µ I.D., 0.25µ film thickness). A split injection was used for sample introduction and the split ratio was set to 10, 1. The oven temperature program was programmed to start at 35°C, hold for 2 minutes, then ramp at 20°C per minute to 260°C and hold for 5 minutes. The helium carrier gas was set to 2 ml/minute flow rate (constant flow mode).

2.12.1 Mass Spectrometry

A JEOL GC mate II bench top double-focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-2000 software used for all analyses. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 700 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 750 at 1 second per scan.

2.12.2 Mass spectrometry library search

Identification of the components of the purified compound was matching their recorded spectra with the data bank mass spectra of NIST library V 11 provided by the instruments software.

3. RESULTS AND DISSCUSSION

The recent growth in the knowledge of free radicals and reactive oxygen species (ROS) in biology is producing a medical revolution which promises a new age of health and disease management. Plant products have been part of phytomedicine since time immemorial. These can be derived from any part of the plant which may contain active components. Knowledge of the chemical constituents of plants is desirable because such information will be of value for the synthesis of complex chemical substances. Such phytochemical screening of various plants is reported by many workers. The interest in the physiological role of bioactive compounds present in plants has increased dramatically over the last decade, particularly in relation to human health. The pharmacological effects exerted by polyphenols on the human body are thought to be strongly related to their high antioxidant capacity.Plants possess active ingredients with many non-vitamin antioxidants that can also prevent ROS production.[24] Therefore, more and more attention has been focused on natural products. In the present study, the result for percentage yield of extraction was quantified by determining the weight of each of the extracts and the percentage yield (Table-1) was calculated for methanol and aqueous extract was 30% and 26% respectively. Several biologically active substances from M. Champaca have been isolated that have been identified and contributing to its observed medicinal effect.

EXTRACTION YIELD

Table-1 (Weight of dry extracts in grams /Initial dry plant extracts) × 100

S.No	Plant extract of Michelia champaca	Weight of dry extracts in grams	Initial dry plant extracts in grams	Extraction Yield % After freeze drying
1.	Methanol extract	7.5	25	30 %
2.	aqueous extract	6.7	25	26.8%

Table-2 Total antioxidant, phenol and flavonoid content of Michelia champaca plant extracts Values are the mean of duplicate experiments and represented as mean ± SD.

Samples	Total phenol content (mg/g Gallic acid equivalent)	Total flavonoid content (mg/g quercetin Equivalent)	Total antioxidant Capacity (mg/g ascorbic acid equivalent)
Michelia champaca methanol extract	272.33 ± 2.08	109 ± 2.0	342.33 ± 23.50
Michelia champaca aqueous extract	165.33 ± 2.08	101.33 ± 3.78	282.66 ± 27.63

In our study both methanol and aqueous extract of M.champaca displayed a considerable antioxidant activity which is justified by the content of polyphenol like phenol and flavonoids (table-2). As reported by Mahakunakorn et al.,[25] these phenolics and flavonoids, compounds present in extracts are believed to intercept the free radical chain of oxidation and donate hydrogen from the phenolic hydroxyl groups, thereby forming stable free radicals, which do not initiate or propagate further oxidation.

Total phenolic contents of the aqueous and methanol extracts of M. champaca were determined using the Folin-Ciocalteu reagent and expressed as Gallic acid equivalent per gram of plant extract. The total phenolic contents of the test fractions were calculated using the standard curve of gallic acid (y = 0.087 x + 0.035; R2 = 0.997). Methanol and aqueous extract of M. Champaca was found to have 272.33 ± 2.08, 165.33 ± 2.08 mg/g phenolic content. Phenolic compounds are well known as antioxidative and scavenging agents against oxidative damage associated with free radicals. A direct relationship between antioxidant activity and phenolic content of plant extracts has been reported. Epidemiological studies have shown that the consumption of foods and beverages rich in phenolic content can reduce the risk of heart disease.[26] Aluminium chloride colorimetric methods were used to determine the total flavonoid contents of the aqueous and methanol extracts of M. champaca. Total flavonoid contents were calculated using the standard curve of quercetin (y = 0.480 x –

0.395; R2 = 0.995) and expressed as quercetin equivalent per gram of the plant extract. Methanol and aqueous extract of M. champaca was found to have 109 ± 2.0, 101.33 ± 3.78 mg/g flavonoids. Flavonoids are also known to have a wide array of therapeutic activities as antihypertensive, antirheumatism, antimicrobial, diuretic, antioxidative and chemopreventive.[27] Role in the cancer through their effect on signal transduction in cell proliferation and angiogenesis. Total antioxidant capacities of aqueous and methanol extract of M. Champaca were evaluated by the phosphomolybdenum method and were expressed as ascorbic acid equivalent per gram of plant extract. Total antioxidant contents of the test samples were calculated using the standard curve of ascorbic acid (y = 0.0014x + 0.0098; R2= 0.9983), and found to be 342.33 ± 23.50 and 282.66 ± 27.63 mg/g.

DPPH free radical scavenging activities

The proton-radical scavenging action has been known as an important mechanism of anti-oxidation. DPPH radical is a stable organic, free radical which has been extensively used for evaluating the free radical scavenging potential of natural antioxidants. In the present study, The free radical scavenging activities of aqueous and methanol extract of M. Champaca were studied by its ability to reduce DPPH, a stable free radical; As DPPH picks up one electron in the presence of a free radical scavenger, the absorption decreases and the resulting discoloration is stechiometrically related to the number of electrons gained.[28] DPPH is a purple color dye with absorption maxima at 517 nm, and upon reaction with a hydrogen donor the purple color fades or disappears due to conversion of it to 2, 2diphenyl-1-picryl hydrazine resulting in decrease in absorbance. The DPPH scavenging effect was found to increase with increased concentrations. At 250µg/mL, methanol extracts showed maximum activity of 50% and aqueous extract showed maximum activity of 36.08% whereas ascorbic exhibited 90.02% inhibition. IC50 values were calculated and expressed (Table 3). Percentage inhibition indicates a better scavenging activity or antioxidant potential.

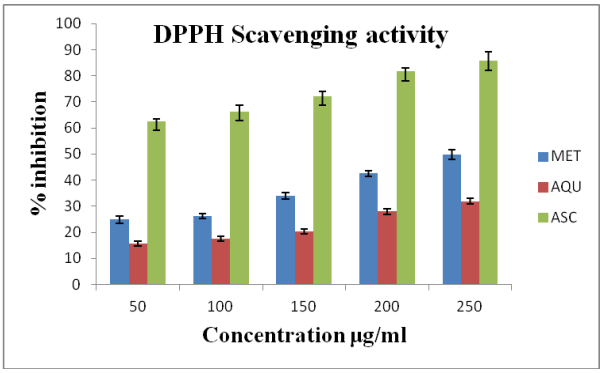


Fig. 1: Comparative DPPH scavenging activities of M.champaca extract and ascorbic acid. Values are the mean of duplicate experiments and represented as mean ± SD.

NO radical scavenging activities

Nitric oxide is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumour activities.[29] Suppression of released NO may be partially attributed to direct NO scavenging .The extents of NO radical scavenging at concentrations (50-250 µg/mL) of M. champaca aqueous and methanolic extract were measured, with ascorbic acid as a standard. The radical scavenging effect was found to increase with increased concentrations. At 250 µg/mL, aqueous extracts showed maximum activity of 29.12% and methanol extract 42.08% whereas ascorbic exhibited 91.66% inhibition. IC50 values were calculated and expressed (Table 3).

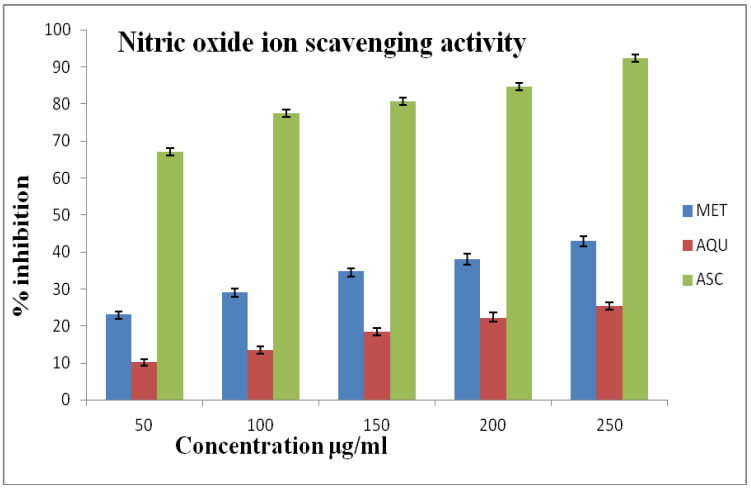


Fig. 2: Nitric oxide scavenging activities of M.champaca extract and ascorbic acid. Values are the mean of duplicate experiments and represented as mean ± SD.

Superoxide anion scavenging activities

The extents of superoxide anion scavenging at concentrations (50-250 µg/mL) of M. champaca aqueous extract were measured, with ascorbic acid as a standard. The radical scavenging effect was found to increase with increased concentrations. At 250 µg/mL, aqueous extracts showed maximum activity of 30% and methanolic extracts showed maximum activity of 46.02 % whereas ascorbic exhibited 90.02% inhibition. IC50 values were calculated and expressed (Table 3).

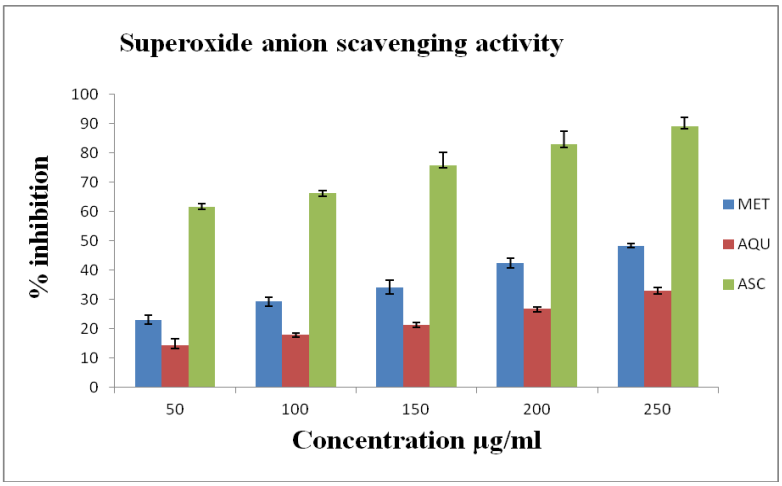


Fig. 3: Superoxide anion scavenging activities of M.champaca extract and ascorbic acid. Values are the mean of duplicate experiments and represented as mean ± SD.

Reducing power capacity

The antioxidant can donate an electron to free radicals, which leads to the neutralization of the radical. Reducing power was measured by direct electron donation in the reduction of Fe3+(CN−)6–Fe2+(CN−)6.[30] The product was visualized by forming the intense Prussian blue color complex and then measured at λ700nm. As shown in Fig. 4, a higher absorbance value indicates a stronger reducing power of the samples. M. champaca extract showed concentration dependent reducing power. The reducing power of aqueous extract (0.305) and methanol (0.416) whereas ascorbic acid (0.572) at 250 µg/mL. Higher absorbance indicates more reducing power. Antioxidant compounds are able to donate electrons to reactive radicals, reducing them into more stable and unreactive species.

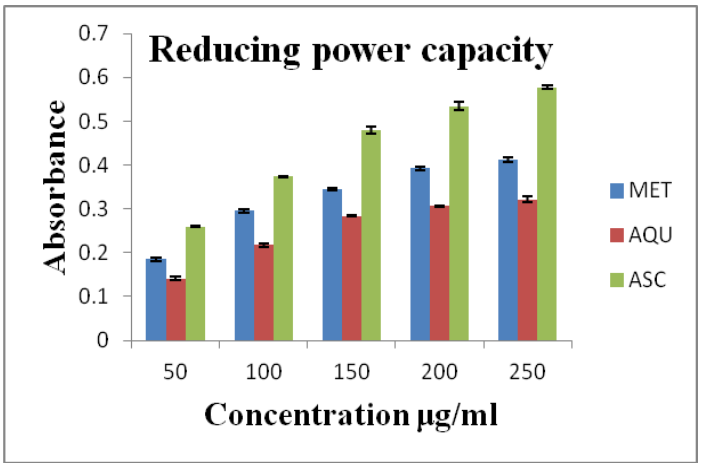


Fig. 4: Comparative reducing power capacity of M. champaca extract and ascorbic acid. Reducing power compared between methanol, aqueous extract of M. champaca and ascorbic acid respectively.

The extract displayed a creditable DPPH scavenging (Fig-1) nitric oxide radical scavenging (Fig-2) and superoxide ion scavenging activity (Fig-3) for their respective extract. However, in the entire three tests methanol extract displayed better scavenging activity than aqueous extract for this study. These finding suggest that the plant extract could have contained phytochemicals that is capable of donating hydrogen to a free radical in order to remove the odd electron which is responsible for the radicals reactivity.

The IC50 value for both the extract in DPPH, nitric oxide and superoxide ion scavenging model were tabulated in table-3. It is observed that M. champaca extracted with methanol significantly has the enhanced ability to scavenge free radicals, and this could be attributed to the fact that methanol being a polar solvent proved better than the nonpolar aqueous solvent for extraction. The inverse correlation between total phenol and flavonoid content and IC50 values in methanol and aqueous were also justified, because with higher total phenolic and flavonoid content in the plant, the lower the amount of extract is required to reduce the DPPH, nitric oxide and superoxide ion scavenging activity. These results were also found to be concomitant with the total antioxidant activity observed in this study for both the extract (table-2).

Table-3 IC_{50} values of methanol extract of Michelia champaca in DPPH, Nitric oxide and superoxide ion scavenging assay.

Extract	IC 50 µg/ml		
	DPPH	**NO**	**O_2^-**
Methanol extracts	270.80	282.74	269.62
Aqueous extracts	95.77	470.80	421.15
Ascorbic acid	39.87	37.85	41.05

Herbal medicines are composed of many phytoconstituents and, therefore, variation in their chemical composite has been always present. TLC, HPTLC and GCMS analysis has also found that M. Champaca contains not a single compound, but a mixture of compounds, so it is established that the pharmacological activity shown by them is due to the cumulative effect of all the compounds present.

THIN LAYER CHROMATOGRAPHY OF METHANOLIC EXTRACT OF M. CHAMPACA

In our study, the most suitable TLC system for analysis was shown to be toluene: chloroform: methanol with the largest discriminating power. Five spots were found in methanol extract

and its Rf values were 0.230, 0.307, 0.538, 0.641 and 0.948.whereas in aqueous extract shows only three spots and its Rf values were 0.4,0.7 and 0.9.

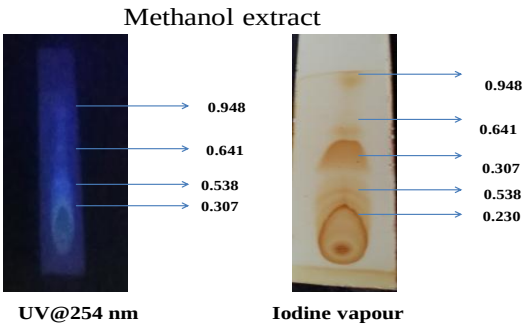


Figure 5: It shows various bands of secondary metabolites separated on TLC by using solvent system toluene: chloroform: methanol, Rf values of all bands are marked with solvent front and sample spot.

In the present study based on the presence of spots in the TLC plates phytochemical screening was done and the results were revealed the presence of phenols, flavonoids, saponins and terpenoids in methanol extract, which was confirmed by performing HPTLC separation technique with H2SO4 spraying reagents. HPTLC showed a clear separation of components present in the methanol extract of M. champaca. This method was applied to identify the plant of M. champaca from other species. HPTLC fingerprint enables a particular plant to be identified and distinguished from closely related species.[31] The presence or absence of a chemical constituent has been found useful in the placement of the plant in taxonomic categories.

Various solvent compositions of the mobile phase for HPTLC analysis were examined in order to achieve high resolution and reproducible peaks. Chromatogram was developed in M. champaca methanol extract under chamber saturation conditions using chloroform: methanol: formic acid: acetic acid (80:15:2.5:2.5) as the mobile phase or solvent system. methanol extracts of M. champaca were subjected to chromatographic finger printing. After chromatographic deprivation with anisaldehyde H2SO4, M. champaca methanol extract at different concentration displayed the chromatogram in 3D at 254 nm (Fig. 5). The HPTLC fingerprinting of M. champaca revealed several peaks, 22 spots at 3 µL and 19 spots at 6 µL and 9 µL of extract samples (Fig.6,7and8). There were seventeen polyvalent phytoconstituents and the corresponding ascending order of Rf values ranged from 0.05 to

1.01 (Table 2). The highest area (%) of the phytoconstituents was found to be 23.40% and its corresponding Rf value was 0.15.

Exposure of the spotted and developed HPTLC plate to UV 254 nm showed the presence of numerous organic compounds as dark and light bands in a green background (Fig. 9A). The developed HPTLC plate at UV 366 nm exposure of slides revealed multi-colored bands with varying intensities (Fig. 9B). HPTLC developed plates at 366 nm exposure displays light and dark blue, light yellow, bluish purple and fluorescent sky blue color bands. HPTLC fingerprint analysis of methanol extract of M. champaca flowerss revealed the presence of phenols, flavonoids, saponins and terpenoids, tannins, steroidal terpens, phenols and anthroquinonesence. It corresponds to several polar and non-polar compounds. Blue and light brown color zone detected in UV after derivatization in the chromatogram confirmed the presence of polyphenols. Light yellow colored fluorescent zone peak observed in the chromatogram after derivatization confirmed the presence of flavonoid and saponin.[32] Blue-violet colored zones were detected from the chromatogram after derivatization, which confirmed the presence of flavanoids.[33] Purple and bluish purple observed in the chromatogram after derivatization confirmed the presence of terpenoids. More components were identified from methanol extract of M. champaca. The results obtained in this study suggest that the identified phytochemical compounds might be the bioactive constituents responsible for the efficacy of the flowers of the plants studied.

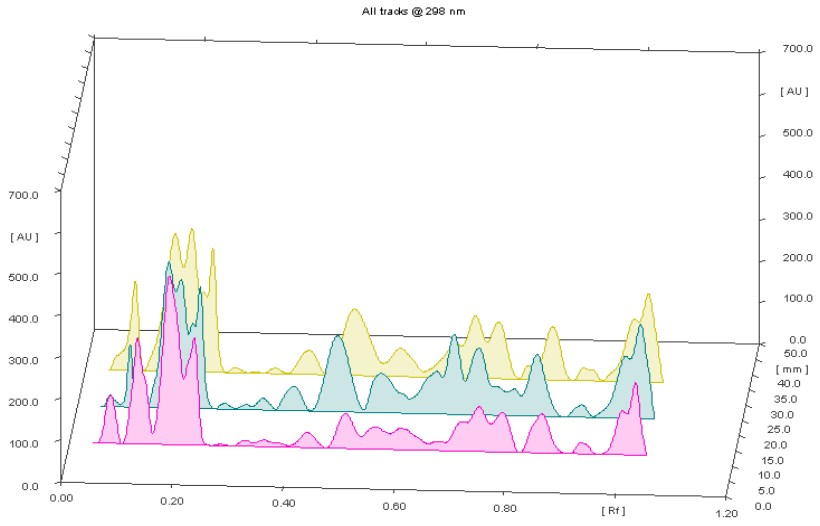


Fig:6 3D Chromatogram of Michelia champaca extract at different concentrations3,6,9,µl injection volume at 254nm

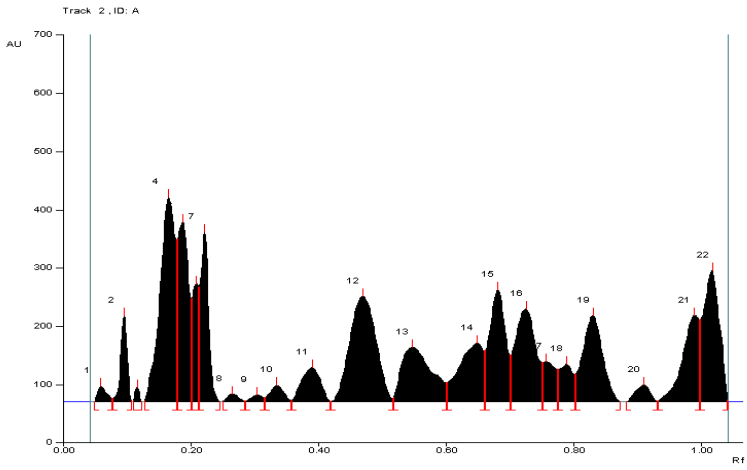


Fig: 7 Chromatogram: HPTLC of methanol extract of michelia champaca at 3 µl volume injected

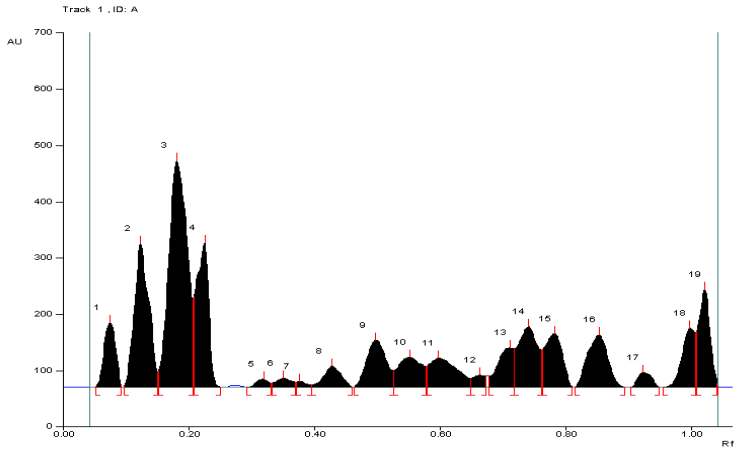


Fig: 8 Chromatogram: HPTLC of methanol extract of michelia champaca at 6 µl volume injected

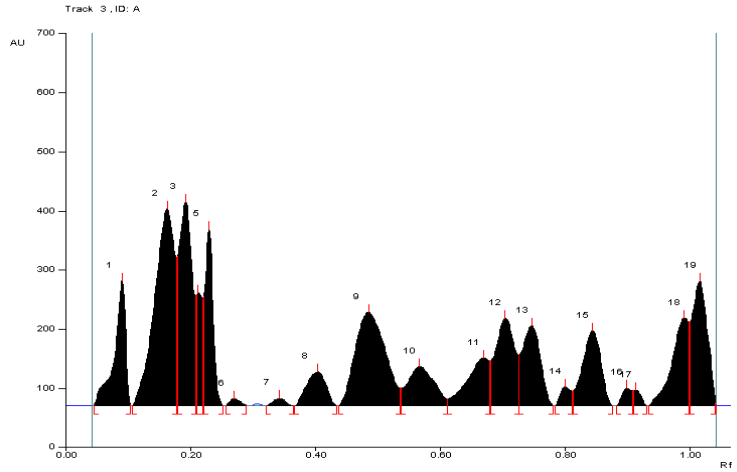


Fig: 9 Chromatogram: HPTLC of methanol extract of michelia champaca at 9 µl volume injected

HPTLC at 254nm **HPTLC at 366nm**

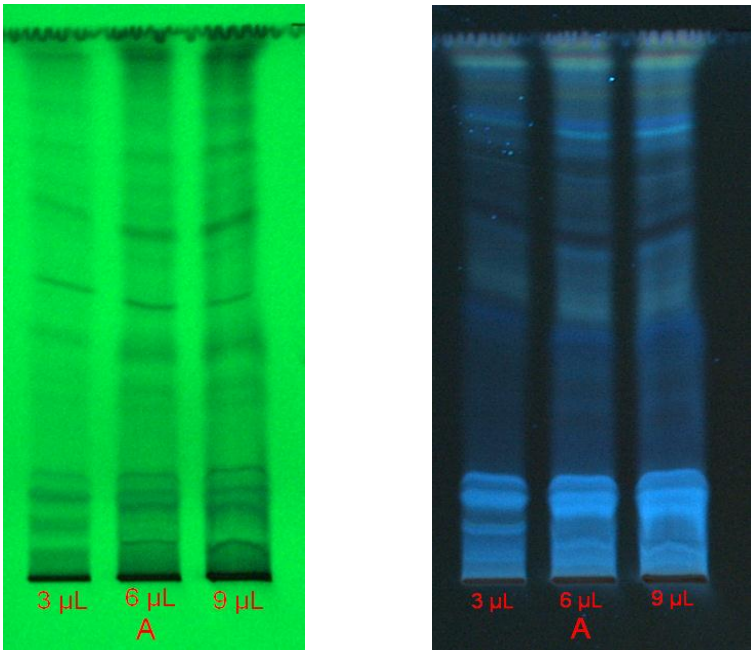


Fig: 10 Photo documentation of methanol extract of michelia champaca

Table No: 4 HPTLC profile of methanol extract of M. champaca at 3µL, Rf values of the peak and peak height

Peak	Start RF	Start height	Max RF	Max %	End RF	Max height	End hight	Area	Area %
1	0.05	0.8	0.07	114.8	5.78	0.09	2.4	1821.0	4.18
2	0.10	2.1	0.12	255.1	12.85	0.15	25.5	5055.5	11.61
3	0.15	28.4	0.18	403.0	20.31	0.21	155.8	10190.3	23.40
4	0.21	160.6	0.23	256.8	12.94	0.25	0.1	4278.4	9.82
5	0.29	0.0	0.32	13.8	0.70	0.33	7.5	241.6	0.55
6	0.33	7.6	0.35	16.3	0.82	0.37	9.3	345.6	0.79
7	0.37	9.3	0.38	9.9	0.50	0.39	3.7	138.9	0.32
8	0.40	3.9	0.43	37.0	1.87	0.46	0.1	892.5	2.05
9	0.46	0.3	0.50	83.4	4.20	0.53	29.6	2251.6	5.17
10	0.53	29.9	0.55	52.9	2.67	0.58	36.9	1715.9	3.94
11	0.58	37.5	0.60	51.8	2.61	0.65	15.2	1983.7	4.56
12	0.65	15.4	0.66	21.1	1.06	0.67	20.1	349.7	0.80
13	0.68	19.8	0.71	69.9	3.52	0.72	68.6	1534.4	3.52
14	0.72	68.3	0.74	107.0	5.39	0.76	66.6	2842.4	6.53
15	0.76	66.8	0.78	94.7	4.77	0.81	0.1	2282.9	5.24
16	0.81	0.3	0.85	92.6	4.67	0.89	0.1	2596.9	5.96
17	0.90	0.1	0.92	26.4	1.33	0.95	0.1	506.8	1.16
18	0.95	0.0	1.00	104.5	5.27	1.01	97.6	1854.7	4.26
19	1.01	99.1	1.02	173.2	8.73	1.04	5.7	2665.9	6.12

TABLE: 5

Peak	Start RF	Start height	Max RF	Max %	End RF	Max height	End hight	Area	Area %
1	0.05	0.1	0.06	26.8	0.93	0.08	6.1	323.7	0.56
2	0.08	6.3	0.09	148.1	5.15	0.11	1.1	1285.7	2.22
3	0.11	2.1	0.12	24.3	0.85	0.12	0.2	141.8	0.25
4	0.13	0.8	0.17	351.0	12.21	0.18	278.2	7218.6	12.47
5	0.18	279.9	0.19	308.5	10.73	0.20	176.9	4485.5	7.75
6	0.20	177.7	0.21	202.9	7.06	0.21	196.0	1747.6	3.02
7	0.21	197.0	0.22	291.4	10.14	0.25	0.2	3165.9	5.47
8	0.25	0.0	0.26	13.2	0.46	0.28	1.6	199.1	0.34
9	0.29	1.7	0.30	11.8	0.41	0.31	7.1	177.1	0.31
10	0.32	7.6	0.33	28.2	0.98	0.36	2.0	518.6	0.90
11	0.36	2.5	0.39	58.4	2.03	0.42	0.7	1486.0	2.57
12	0.42	0.0	0.47	180.8	6.29	0.52	5.4	6437.5	11.12
13	0.52	6.5	0.55	93.3	3.25	0.60	32.9	3822.7	6.61
14	0.60	33.1	0.65	101.1	3.52	0.66	87.6	3273.3	5.66
15	0.66	88.4	0.68	191.6	6.67	0.70	79.9	4133.0	7.14
16	0.70	80.0	0.73	158.9	5.53	0.75	67.4	4425.1	7.65
17	0.75	67.6	0.76	69.2	2.41	0.77	55.7	1145.3	1.98
18	0.78	55.8	0.79	64.3	2.24	0.80	47.5	1148.0	1.98
19	0.80	47.9	0.83	147.2	5.12	0.87	0.3	4002.8	6.92
20	0.88	0.2	0.91	29.3	1.02	0.93	0.8	567.9	0.98
21	0.93	1.0	0.99	148.4	5.17	1.00	141.1	3331.3	5.76
22	1.00	141.4	1.02	225.0	7.83	1.04	3.0	4835.9	8.36

TABLE: 6

Peak	Start RF	Start height	Max RF	Max %	End RF	Max height	End hight	Area	Area %
1	0.05	1.0	0.09	211.3	8.06	0.10	1.0	3254.7	5.75
2	0.11	0.1	0.16	332.6	12.69	0.18	250.9	9244.7	16.34
3	0.18	252.6	0.19	344.7	13.15	0.21	186.4	6367.0	11.25
4	0.21	187.9	0.21	191.5	7.30	0.22	180.0	1680.3	2.97
5	0.22	184.8	0.23	298.1	11.37	0.25	0.4	3247.3	5.74
6	0.26	0.3	0.27	11.9	0.45	0.29	0.2	171.4	0.30
7	0.32	0.1	0.34	12.4	0.47	0.36	0.0	202.6	0.36
8	0.37	0.1	0.40	57.1	2.18	0.43	0.4	1543.4	2.73
9	0.44	0.5	0.49	158.2	6.03	0.54	29.8	6577.9	11.63
10	0.54	29.9	0.57	66.3	2.53	0.61	11.6	2416.8	4.27
11	0.61	11.7	0.67	81.0	3.09	0.68	75.7	2490.0	4.40
12	0.68	76.3	0.70	148.2	5.65	0.73	85.8	3858.9	6.82
13	0.73	85.9	0.75	135.2	5.16	0.78	0.4	3376.0	5.97
14	0.78	0.1	0.80	31.4	1.20	0.81	24.6	475.0	0.84
15	0.81	25.2	0.84	126.9	4.84	0.88	0.3	3194.8	5.65
16	0.88	0.3	0.90	29.7	1.13	0.91	25.3	389.3	0.69
17	0.91	25.4	0.91	26.0	0.99	0.93	0.5	287.0	0.51
18	0.93	0.5	0.99	148.5	5.66	1.00	141.8	3390.3	5.99
19	1.0	142.1	1.02	210.6	8.03	1.04	4.6	4413.1	7.80

GC-MS is one of the best techniques to identify the constituents of volatile matter, long chain, branched chain hydrocarbons, alcohols acids and esters, etc. Knowledge of the chemical constituents of plants is desirable not only for the discovery of new therapeutic agents, but also disclosing new sources of economic phytocompounds for the synthesis of complex chemical substances and determining the actual significance of folkloric remedies.The GC-MS analysis was carried out on the methanol extract of Michelia champaca flowers revealed the presence of twelve compounds (phytochemical constituents) (fig:10) were identified through mass spectrometry attached with GC. Molecular weight, molecular formula and structure of the isolated compounds were ascertained.

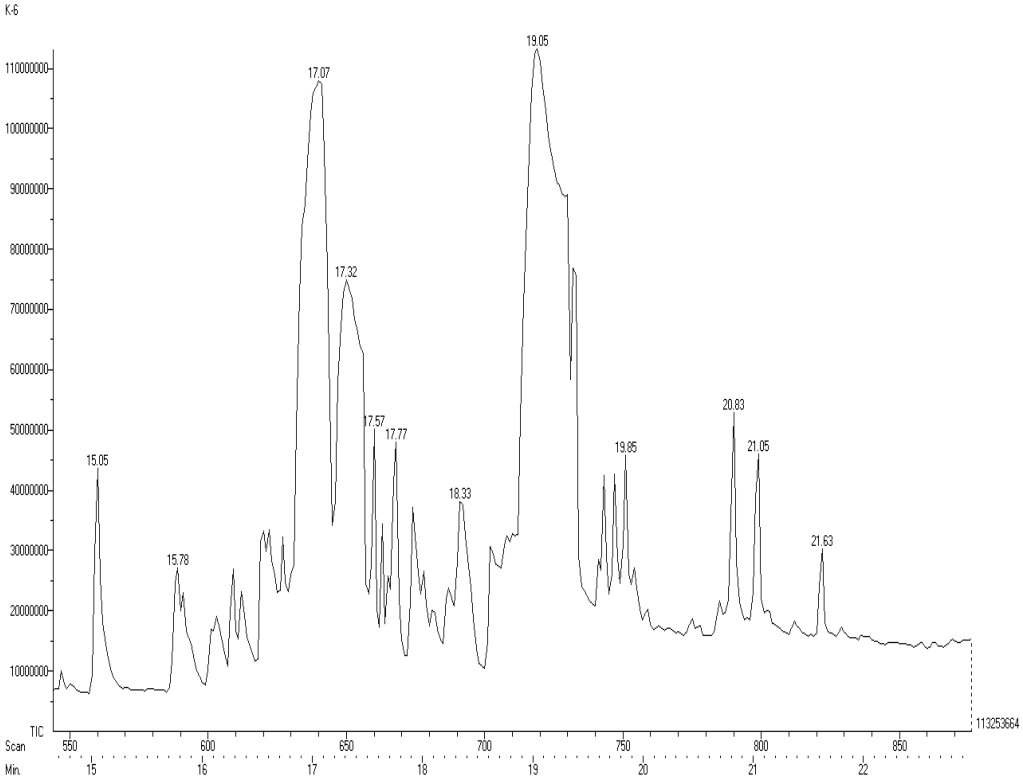
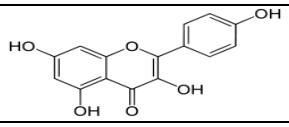
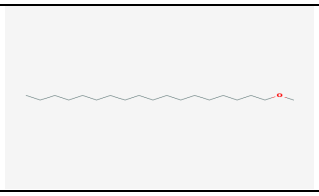
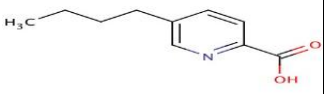
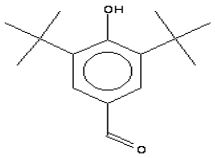
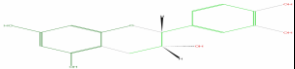
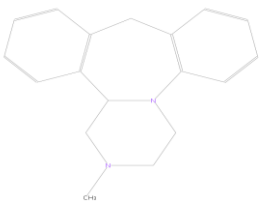
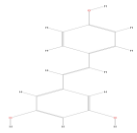


Fig: 11 GCMS

Table No: 7 Bioactive components identified in methanol extract of Michelia champaca [GC MS study]

Peak No.	RT (Min.)	Compound Name	Compound Nature	Peak area (%)	Structure
1	15.05	Kaempferol MW- 286.23 MF- $C^{15}H^{10}O^{6}$	Flavonoid	3.62%	

2	15.78	Methyl octadecyl ether [1-ethoxyoctadecane] MW - 284.30 MF - $C_{19}H_{40}O$	Alkane hydrocarbon	1.81%	
3	17.07	Fusaricacid[5-Butylpicolinic acid] MW – 179.22 MF – $C_{10}H_{13}NO_2$	Picolini acid derivative,pyridine compound	27.34%	
4	17.32	Nonanedioic acid, dibutyl ester [azelaic acid] MW- 300.4336 MF - $C_{17}H_{32}O_4$	Aliphatic esters	18.34%	
5	17.57	3,5 di-tert-butyl-4-hydroxybenzaldehyde MW - 234.33 MF – C15H22O2	Phenol salicylaldehyde derivative	1.95%	
6	17.77	Ranitidine MW-314.4 WF- C13H22N4O3S	h2 receptor antagonist	2.76%	
7	18.33 (F)	(-) Epicatechin MW-290.3 MF- C15H14O6	Flavonoids	2.83%	
8	19.05	Carbamazepine 10,11 epoxide MW-252.27 MF-C15H12N2O2	Dibenzazepines (major metabolite of carbamazepine)	35.88%	
9	19.85	Fenofibrate MW-360.83 MF-C20H21C1O4	fibrate	2.67%	
10	20.83	Mianserin[(±)-2-methyl-1,2,3,4,10,14b-hexahydrodibenzo [c,f]pyrazino [1,2-a]azepine] MW-264.365 MF-C18H20N2	Tetracyclic	2.09%	

11	21.05	Resveratrol MW-228.24 MF-C14H12O3	Natural phenol	2.67%	
12	21.63	Benserazide MW-257.243 MF-C10H15N3O5	Aromatic Lamino acid	1.61%	

GC-MS analysis shows the presence of different phytocompounds in methanol extract (Table-4) namely fusaric acid (27.34%) Has been reported to have potent anti-proliferative activity[34] inhibition of cell proliferation and DNA synthesis and also relaxes the blood vessel through Ca++ depletion[35] Michael N Diringer et al stated that aggression and motor activity were reduced by the highest doses of fusaric acid , and treatment with fusaric acid at doses of 30 to 60 mg/kg decreased brain norepinephrine and dopamine, inhibits Dopamine betahydroxylase.

Azelaic acid (18.34%), it has been reported to have Antioxidant,Antimicrobial, Antibacterial, Anti inflammatory [36] Anti cancer, Anti proliferative activity and also used in skin disorders. Carbamazepine 10,11epoxide (35.88%), were found as the major components in the methanol extract ,plays essential role in Analgesics, Anticonvulsant, Antimanic agent, mainly used to control grand mal and psychomotor or focal seizures, and also used in the treatment of epilepsy ,pain associated with true trigeminal neuralgia, psychiatric disorders including manic depressive illness and aggression due to dementia, post traumatic stress disorder and attention deficit hyperactivity disorder[37] the other minor components such as Kaempferol (3.62%), also possess Antioxidant, Anti microbial, Anti inflammatory, Anti cancer activity[38] Productive effect on cardiovascular diseases[39] Protective role in atherosclerosis and reduces relative risk of type2 diabetes[40] and also posses Antiobesity effect . Methyl octadecyl ether (1.81%)- has anticarcinogenic activity.[41] 3,5 Di –tert-butyl-4-hydroxybenzaldehyde (1.95%), also has Antioxidant prooxidant, antibacterial and antiinflammatory activity.[42] Ranitidine (2.76%), has been reported to have Hepatoprotective Antinociceptive, Antioxidant activity,Exerts neuroprotective actions on ischemic neural cell death[43]Protects against neural death induced by oxygen, glucose deprivation, Prevention of stress related mucosal disease[44] its analog Jws-usc-751x has important role in enhancement of memory. (-)Epicatechin (2.83%), plays important role in enhancement of Memory and also enhances angiogenesis[45]Activates endothelial cell, NO,ENOS related signaling pathways,improves

mitochondrial related protein levels & ameliorates oxidative stress[46] it also enhances fatigue resistance and oxidative capacity and Prevents onset of type 1 diabetes. Fenofibrate (2.67%), Reduce cholesterol levels in patients at risk of cardiovascular disease and also reduces LDL, VLDL, Tg levels, Increase HDL.[47] Minaserin (2.09%), has Antidepressant,Anxiolytic, Hypnotic, Antiemetic, Orexigenic,Anti histamine effects, also used in the treatment for parkinsons disease,in schizophrenia it reduces negative symptoms & cognitive symptoms.[48] Resveratrol (2.67%), has been reprted to have antioxidant anti angiogenic and hepatoprotective[49] activity and act as an acetyl cholinesterase inhibitor[50] also has Neuroprotective effect[51] mainly effective against neuronal cell dysfunction & cell death,effective in huntingtons disease,alzheimers disease[52]Inhibit the progression of cardiac fibrosis, increase natural testosterone production from being both a selective estrogen receptor modulator and an aromatase inhibitor activity and also has Cardioprotective, Antidiabetic effect[53] antihyperglycemic,Skin conditioning and Antidepressant effect in animal models, it reverses the restraint stress-induced cognitive dysfunction.[54]Benserazide (1.61%), is a Dopa decarboxylase inhibitor, mainly used in the management of parkinson's disease[55] and it has antihypertensive effect.[56]

These compounds activity could contribute the medicinal quality of the plant (Table 4). the identification of the phytochemical compounds were confirmed based on the peak area, retention time and molecular formula. The result of the preliminary phytochemical screening was carried out on aqueous and methanolic extracts revealed the presence of a wide range of phytoconstituents including alkaloids, flavonoids, phenolic, terpenoids, tannins, saponins supporting the reason for its wide range of biological activities which may be responsible for the neuroprotective activity.

4. CONCLUSION

In the present study, methanol extract of M. champaca showed free radical scavenging activity on NO, O2- and DPPH models. The preliminary study showed the presence of phenols and flavonoids, indicating its potent antioxidant activity and HPTLC analysis confirmed the presence of phenols, flavonoids, saponins and terpenoids. Thus summarizing these results, it is evident that methanol proved superior when compared to aqueous extract in this particular study. However, both exhibited free radical scavenging activity and this may have occurred due to the synergic effect of their chemical constituents. Further, in depth toxicity and dosage may reveal its efficacy as an herbal drug. This investigation on M.

champaca could serve as a primary basis for further pharmacological and drug designing studies.

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