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Original Article

Metabolite profiling by UPLC-PDA-ESI/HDMS and antibacterial activity of *Memecylon talbotianum* Brandis

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ABSTRACT

Introduction: UPLC based metabolite profiling was employed to evaluate the chemical constituents of *Memecylon talbotianum* Brandis extract and its antibacterial activity was studied *in vitro*. **Methods:** Methanol extracts of *M. talbotianum* was subjected to UPLC-PDA-ESI/HDMS metabolite profiling. The antibacterial activity was determined against human pathogens through disc diffusion, Minimum inhibitory concentration (MIC), Minimum biofilm inhibitory concentration (MBIC) test as visualized by Alamar blue and confocal laser scanning microscopy. **Results:** UPLC-PDA-ESI/HDMS analysis identified eighteen metabolites, synapoyl-hexose-formic acid, kaempferol 3-*O*-feruloylhexosyl rhamnoside, 6-C-arabinosyl-8-C-glucosyl-apigenin and isorhamnetin-3-O-glycoside-7-O-glycoside as the main constituents for the first time from this plant. A broad spectrum of antibacterial activity against to test human pathogens (MIC=54 mg/ mL; Gram-positive bacteria) causing lysis at 24 h incubation was reported, resulting in nearly a 4 $\log_{10}$ CFU / mL drop in cell viability at 1.6 X MIC (Gram-positive) for this extract. The extract at 2 fold MIC inhibited the bacterial biofilm formation and at 8 x MIC eradicated biofilms. However, a higher concentration of this extract was identified in this study for a similar effect on Gram-negative bacteria. **Conclusion:** The presence of these compounds could contribute to their *in vitro* inhibitory activities against pathogenic bacterial strains indicating its potential as medicinal agents in treatment and prevention of diseases of bacterial origin.

Key words: UPLC-PDA-ESI/HDMS, Bioactives, Anti-bacterial activity, Biofilm, MIC.

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INTRODUCTION

Over the last few decades, research into the traditional medicine system has gained importance in the field of herbal medicines. Traditional medicines are generally viewed as eco-friendly, profitable and comparatively safe due to their age-old usage compared to synthetic therapeutics. Ayurveda, Unani, Siddha and folk medicine are the chief healthcare systems in Indian society. These medicinal systems mainly depend on natural resources. Hence, medicinal plants have immense economic value due to the market for herbal medicine[1,2] However, there are several drawbacks in the growth of herbal medicine, including a lack of proper guidelines and information on medicinal plants, loss of biodiversity, computerization, certification and over-exploitation. Therefore a detailed study is very much important to authenticate such medicines for proper usage.[3,4] Plant based phytochemicals with known anti-microbial, pathogenic (antibiotic) against human pathogens, anti-bacterial activities.[5,6]

Several advanced method are available to confirm the mechanism of action and characterize bioactive compounds. Coupling of liquid chromatography/mass spectrometry (LC-MS/MS) with atmospheric pressure ionization techniques, i.e., electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), is one of the methods which has been demonstrated to be a powerful tool for the finger printing of total extracts of medicinal plants. These methods favor identification analysis of medicinal plant extracts for bioactives.[7-9]

Memecylon talbotianum Brandis, is a medicinal plant that is endemic to the Western Ghats, a biodiversity hotspot in India. This plant is used for the treatment of skin diseases in traditional therapeutic systems[10] and is used as a folk medicine.[11] In the last decades, this plant is mainly used in traditional preparations and there are no reports on any phytochemistry and pharmacological actions.

Recent investigations have revealed that methanol extracts of *M. talbotianum* have significant antioxidant and anti-inflammatory properties.[12] However, there is still no information on its chemical constituents responsible for the *in vitro* biological activities of this plant. This report describes the presence of eighteen metabolites including synapoyl-hexose-formic acid, kaempferol 3-*O*-feruloylhexosyl rhamnoside, 6-C-arabinosyl-8-C-glucosyl-apigenin and isorhamnetin-3-O-glycoside-7-O-glycoside compounds as major constituents. Further, a study was carried out to determine the antibacterial potential of methanol extracts of *M. talbotianum* based on disc diffusion assay, minimum inhibitory composition (MIC), Inhibition of bio-film formation as visualized by Alamar blue and confocal laser scanning microscopy. These data provide scientific evidence to support the folk medicinal utilization and *in vitro* pharmacological properties of *M. talbotianum* Brandis.

MATERIALS AND METHODS

Plant sample collection

Memecylon talbotianum leaves were collected from Sringeri region in Western Ghats of Karnataka State, India during April 2012. Leaves of the collected plant samples were carefully compared with the deposited voucher speciman of *M. talbotianum* at the herbarium (#IOE LP0002) at the Department of Studies in Biotechnology, University of Mysore and also authenticated by a plant taxonomist. The leaves were separated, washed under running tap water to remove adhering dust particles, followed by slow drying at regulated temperature of 30°C. The dried leaves were ground to a coarse powder using the mechanical grinder and stored at 4°C.

Preparation of extracts

Leaf powder (250 g) was extracted sequentially using 500 ml of non-polar, moderately polar and polar solvents (E. Merck, Bangalore, India) in increasing polarity (hexane < ethyl acetate <methanol <water) using

a Soxhlet apparatus by continuous hot percolation (boiling point, 52 to 62°C) until the solvent became colorless. The resultant solvent extracts were concentrated in a rotary evaporator (Speed Vac, Savant SPD 2010, Thermo Scientific, Germany) under controlled pressure. For the studies undertaken, the required amount of extracts were weighed and solubilized in dimethyl sulphoxide (1 mg/ml) and were further diluted as indicated in the sections below.

Qualitative screening of Phytochemicals

The screening for alkaloid, flavonoid, carbohydrates, glycosides, proteins and amino acids, steroids, fat and fixed oil was carried out according to the method described.[13]

Retention Time and Mass Spectrometric Profiling of Compounds in *Memecylon talbotianum*

For the qualitative analysis of the metabolites were analysed by Synapt G2 (UPLC separations with QuanTof) according to manufacturers protocol. The nebulizer pressure was 60 psi and the nitrogen flow rate 10 L/min at a drying temperature of 350°C. The methanol leaf extract was filtered (0.2 micron syringe filters, Millipore, U.S.A) and an aliquot (5 µl) was injected into the system. The mass spectra were acquired from m/z 100-1000 in negative ionization mode. Helium was used as the collision gas for the fragmentation of the isolated compounds in the ion trap. The detection conditions were as follows: capillary voltage, 3500 V; skimmer voltage, –40 V; cap exit voltage, –158.5 V; Oct 1 DC, –12 V; Oct 2 DC, –2.45 V; trap drive level, 45.0; Oct RF, 150 Vpp; Lens 1, 5.0 V; Lens 2, 60 V.

Antibacterial activity

The microbial strains used in the study were human pathogens procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. These microbes included the gram negative bacteria *Escherichia coli* (MTCC 724), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhi* (MTCC 733), and the gram positive bacteria *Staphylococcus aureus* (MTCC 96) and *Bacillus subtilis* (MTCC 441). All the aforementioned bacterial strains were cultivated in tryptic soy agar (TSA)/Luria Bertani (LB)/Muller-Hinton broth (MHB) medium from Difco, Becton Dickinson, at 37°C and were maintained at 4°C till further use.

The antibacterial properties of the extracts were determined by agar disc diffusion method. Individual colonies of the bacterial strains on TSA (Difco, Becton Dickson, Sparks, MD, USA) were grown for 16 h on MHB medium for 16 h at 37°C. Bacterial suspensions were prepared and adjusted to approximately 2.0×10^8 CFU / ml (optical density at 600 nm=0.2) and were spread evenly on the surface of MHB agar plates. A sterile blank disk (6 mm diameter, Difco, Becton Dicknson) was placed on the surface of the MHB. Extracts/standard antibiotic, ampicillin (20 ml) (Himedia, Bengaluru) were placed on these discs and incubated for 24 h at 37°C. DMSO (0.5%) were used as controls. The antibacterial activity was recorded as the average diameter of the zone of inhibition in millimeters. The absence of a zone of inhibition (<6 mm) was interpreted as absence of antibacterial activity.

The minimum inhibitory concentration (MIC) was tested using a standardAlamar blue (AB; *In vitro* gen, Life Technologies, Carlsbad, CA, USA) assay.[11] Serial dilutions of extracts in MHB (100 ml) with the test bacteria at a density of 8×10^6 CFU/ml were prepared in flat-bottom, polystyrene, non-tissue-culture-treated 96-well microtiter plates at 37°C. After 24 h, AB (10 ml ml) was added to each well and the plates were gently shaken and further incubated for 1 h at 37°C. After incubation, the absorbance was read at 570 and 600 nm using a microtiter plate reader (Varioskan Flash Top, Thermo Fisher Scientific, Germany). Controls included medium alone (blank) and the diluted medium with cells and AB (positive). The percent reduction of AB was calculated using the formula as per the manufacturers' instructions (absorbance difference between 570 and 600 nm). The average percent reduction was calculated from three independent experiments and used to determine MIC. The AB-MIC was defined as the lowest concentration of the extract resulting in <50% reduction of AB.

Anti-biofilm activity assay

The test bacteria were incubated at a density of 8×10^6 CFU/ml in 100 µL of MHB. The plates were incubated at 37°C for 24 h without shaking to allow the formation of biofilm. The extracts in MHB were prepared and added to the wells. After further 24 h incubation, the well contents were aspirated from the test and control wells, gently washed twice with sterile physiological saline. AB (10 ml) was added to the plates and analyzed as detailed earlier.

Confocal laser Scanning Microscopic (CLSM) analysis

For CLSM analysis, the test bacterial strains were allowed to form biofilms (control; only test bacterial strains and test; bacterial strains with methanol extracts) on the glass slides as described in the previous section. The biofilm formed in the glass slides were stained with 20 µL of 1% acridine orange (Sigma Aldrich, MO, U.S.A.). The excess stain was washed to remove it. These cover glasses were visualized under CLSM (LSM 710, Carl Zeiss, Germany) equipped with an excitation filter 515-560 and magnification at 20x.[12]

Statistical analysis

All experiment/measurements were made in triplicate, and all the values are expressed as the mean ± SE of three independent replicates. Data was subjected to ANOVA, and the significant differences were tested by post hoc comparison test (Student-Newman-Keuls) at $p<0.05$.

RESULTS AND DISCUSSION

Qualitative screening of *Memecylon talbotianum* for the presence of phytochemicals

Analysis of phytoconstituents from various solvent extracts of the leaves of Memecylon species

The phytochemicals are known to play an important role in bioactivity of medicinal plants. The dried powders of *M. talbotianum* were extracted

Table 1: Preliminary phytochemical screening of various extracts of the leaves of *Memecylon talbotianum* Brandis

Phytoconstituents	Solvent extract			
	Hexane	Ethyl acetate	Methanol	water
Alkaloids	+	+	-	-
Glycosides	+	-	+	-
Terpenoids	-	+	+	-
Carbohydrates	+	++*	+++*	+
Proteins	-	+	+	+
Phenols	+	++	+	+
Saponins	+	++*	+	-
aminoacids	-	+	-	-
Flavonoids	+	+	+++*	+
Steroids	+	++	+	-

Where no results (-), the particular phytochemical was not detected. The rating (+++) Present in high amount; (++) Present in medium amount; (+) Present in trace amounts are subjective estimates based upon relative quantities.
*significantly different from other ratings ($p<0.05$).

Table 2: Putative compounds identified by UPLC-PDA-ESI/HDMS

Putative Compound Identification[a]	*m/z* [M – H]$^-$	MS^2 [M – H]$^-$ [relative intensity (%)]
gallic acid	169.9322	153 (100), 125 (14), 57 (8)
cis-ferulicacid[b]	193.05	178 (28), 149 (56) 134 (100)
trans-sinapicacid[b]	223.0241	195 (8), 179 (100)
cis-sinapicacid[b]	223.0241	195 (8), 179 (100)
3,7 – dihydroxy – 4$^{'}$- methoxy flavones	284.1064	136 (100), 148 (70)
quercetin	301.0334	227 (10), 151 (10)
mono caffeoyl-quinic acid	353.1005	193 (100), 173.9625 (12)
feruloylsinapoyl glucose	355.0502	311 (100), 193 (12), 173 (6)
feruloylquinic acid	367.0012	193 (100), 191, 172.9 (5), 160.9 (2), 84.9 (2)
isoprenyl-1-5 – *O*- β – D – glucopyranosyl -2 (3*H*) – benzofuranone	379.0982	217 (100), 195 (14), 162 (8)
synapoylhexose + formic acid	431.1919	387 (80), 193 (100), 194 (42), 186 (10)
Cyanidin -3- *O*– malonyl glucoside	535.1555	449 (12), 287 (100)
6-C-arabinosyl-8-C-glucosyl-apigenin	545.2040	533 (16), 489 (38), 445 (70), 401 (90), 357 (100), 313 (35)
2$^{''}$ – *O* – pentosyl – 8C – hexosyl luteolin	579.1042	459 100), 449 (23), 429 (12), 357 (19), 327 (14), 309 (41), 297 (11), 285 (37)
isorhamnetin-3-O-glycoside-7-O-glycoside	639.2041	579 (100), 601 (41), 313 (52), 338 (31)
kaempferol 3-O-feruloylhexosyl rhamnoside[b]	769.1975	607 (100), 608 (44)
quercetin 3-*O*-sinapoyldihexose[b]	831.1941	787 (100), 641 (42), 625 (57), 607 (7), 479 (74)
quercetin 3-*O*-methoxy bezoyl-dihexoside[b]	921.2290	593 (25), 463 (100), 457 (37)

The detected compounds by their m/z values ([M–H)]$^-$) and the MS^2 fragments in the extract of *Memecylon talbotianum* methanol leaves extract. [a]identified by *m/z* [M – H]$^-$; [b]identified by *m/z* [M – H]$^-$ using in-house database.

Table 3: Minimum inhibitory concentration (MIC) of extracts on the test pathogens as detected by the Alamar blue method

	Mean MIC[a] (mg / mL) value				
	Gram-positive bacteria		Gram-negative bacteria		
Plant extracts / standard	***Staphylococcus aureus* (+)**	***Bacillus subtilis* (+)**	***Salmonella typhi*(-)**	***Escherichia coli*(-)**	***Pseudomonas aeruginosa* (-)**
Ampicillin	12.5[b]	11[b]	20[c]	15[c]	18[c]
Methanol	54[d]	63[d]	864[e]	1008[f]	918[f]
Ethyl acetate	89[d]	126[e]	1043[f]	>2500[g]	1503[f]
Water	1540[f]	>2500[g]	>2500[g]	>2500[g]	>2500[g]

[a]MICs were determined by the Alamar blue assay. MIC is defined here as the lowest concentration of the extract resulting in <50% reduction of AB (average of three independent experiments). Results with lower case letters indicate significantly different values (p<0.05). MIC values >2500 indicates that antimicrobial activity for the particular extract was not detected. Test strains were inoculated at 5 × 10^6 CFU / ml in each well.

Table 4: Minimum biofilm inhibitory concentration (MBIC) against its biofilm formation and preformed biofilm reduction

Methanol extract	MIC[a] (mg / ml)	MBIC[ab] (mg / ml)
Staphylococcus aureus (+)	110*	~ 0.3*
Bacillus subtilis (+)	130*	~ 0.5*
Salmonella typhi(-)	1750#	~ 6.7#
Escherichia coli(-)	5 (mg / mL)	~ 20
Pseudomonas aeruginosa (-)	1850#	~ 7.5#

Minimum inhibitory concentration (MIC; identified as a <50% reduction of AB) or minimum biofilm inhibitory concentration (MBIC) was visualized as preformed biofilm represented a as dot at the bottom of the well upon addition of AB after exposure to methanol extract. Significant differences in the concentration of methanol extract required for Gram-positive#/Gram-negative* bacteria.

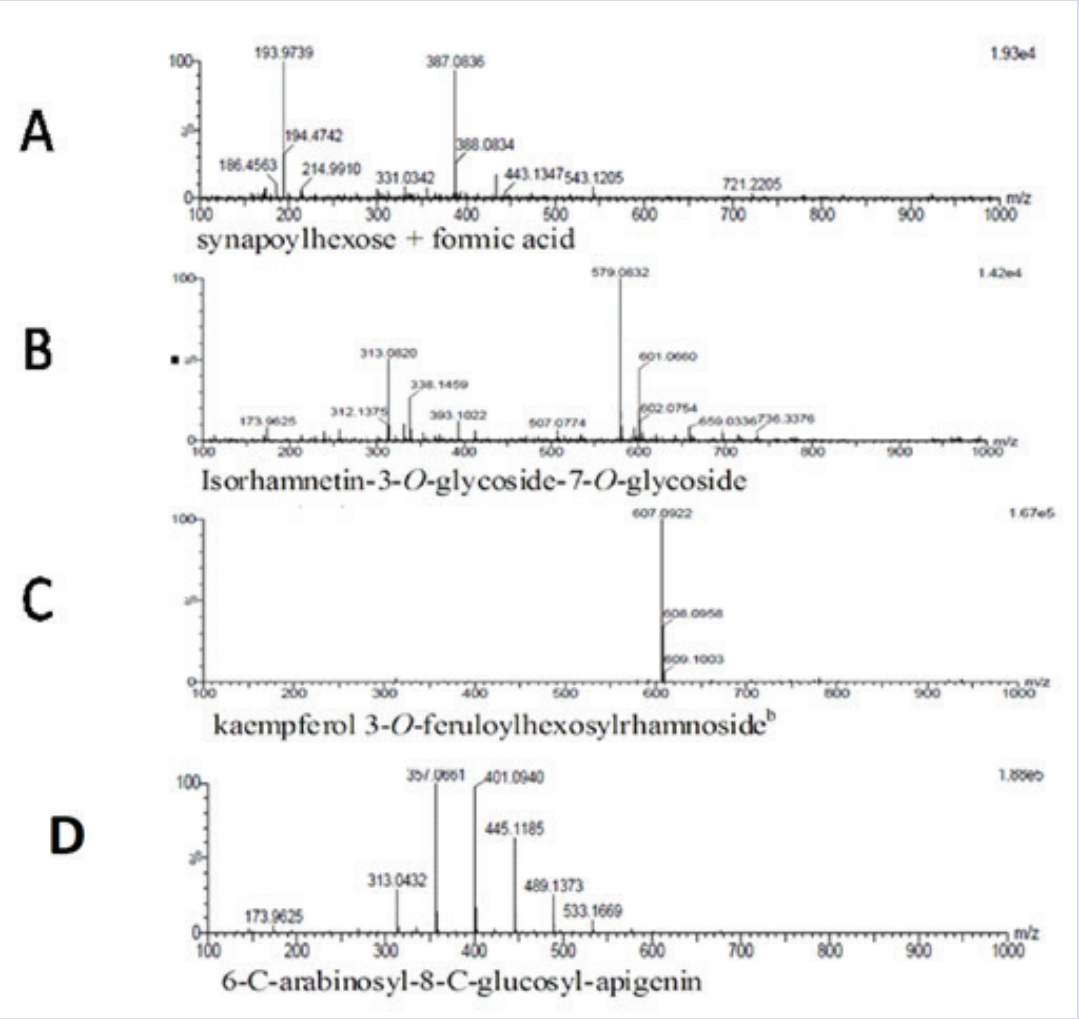

Figure 1: Mass spectrometric profile of phytochemicals in the methanol extract of *Memecylon talbotianum* determined by UPLC-PDA-ESI/HDMS. Hydroxyl-benzoic acid (gallic acid) and hydroxycinnamic acid (ferulic acid and isomers of sinapic acid), A; Isorhamnetin-3-O-glycoside, B; 3-O-methoxy bezoyl-dihexoside, C; 6-C-arabinosyl-8-C-glucosyl-apigenin (D).

with hexane, ethyl acetate, methanol and water in the Soxhlet apparatus. The yield from these solvents for different *Memecylon* species was; hexane (~5.37 g); ethyl acetate (~6.2 g); methanol (~8.23 g) and water (~2.32 g) from 250 g dry weight of leaf. Preliminary phytochemical screening of various extracts of the leaves revealed the presence of terpenoids, triterpenes, steroids, carbohydrates, glycosides, alkaloids and saponins where the highest amount of phytochemicals were found in the methanol extracts of *M. talbotianum* whereas lowest amount of phytochemicals was observed in hexane, ethyl acetate and water extracts (Table 1). The alkaloids, phenolic compounds, tannins, flavonoids have been associated with various degress of biological activities such as anti-oxidant, anti-inflammatory and anti-microbial activities.[16-18]

Profiling of Compounds in methanol extract of M. talbotianum leaves determined by UPLC-PDA-ESI/HDMS

A total of 18 compounds were identified from the methanol extract of *M. talbotianum*. These compounds were putatively identified based on the mass spectral analysis and was confirmed by comparison with literature values. Their retention times and product ions from the negative ion mode EPI scan are listed (Table 2). Identification of metabolites based on chromatographic and in-house MS data identified compound A as hydroxyl-benzoic acid (gallic acid) and hydroxycinnamic acid (ferulic acid and isomers of sinapic acid) (Figure 1A). Compound B was identified as isorhamnetin-3-O-glycoside (Figure 1B).[19] The compound C is identified as 3-O-methoxy bezoyl-dihexoside (Figure 1C), an acylated flavonol glycoside of quercetin.[20] Compound D was identified as 6-C-arabinosyl-8-C-glucosyl-apigenin (Figure 1D) by comparison of retention time and MS/MS data.[21]

Anti-bacterial activity of *Memecylon talbotianum*

Anti-bacterial activity of the extracts was initially screened as reported earlier[22] by the standard agar disc diffusion method. Ampicillin inhibited the growth of test bacterial samples.

The extracts were further subjected to record MIC against the above said pathogenic bacteria. In general, the methanol extract exhibited better inhibition of bacterial growth than the other fractions. The MIC ranged from 54-63 mg/mL and 864-1008 mg/mL for gram-positive/negative bacteria respectively. The ethyl acetate fraction exhibited MIC of 89–126 mg/ml for Gram-positive and 1043–1503 mg / ml for Gram-negative strains of test microbes. Thus the concentrations of extracts required to

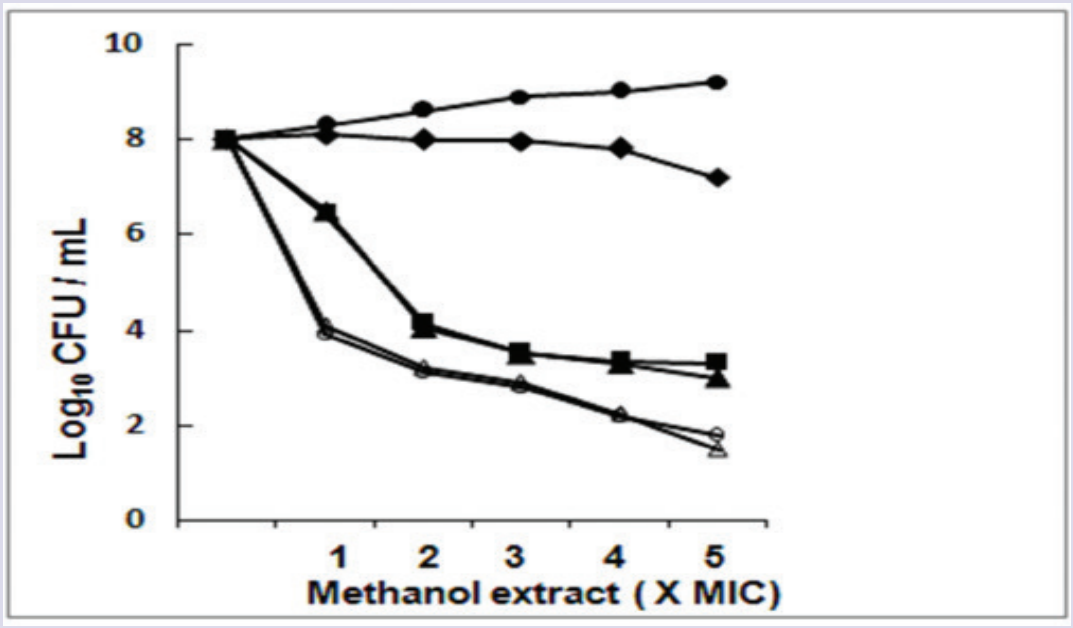

Figure 2: Evaluation of lytic action of methanol extract against the human pathogenic bacteria in MHB.

inhibit Gram-negative bacteria ranged from 5-15 times higher than that required for Gram-positive strains (Table 3). Methanol extract was further evaluated for bacterial lysis. Bacterial lysis showed that a 1.6 x MIC and 1.63 x MIC of methanol extract caused a reduction (50%) in culture turbidity over a period of 24 h (Figure 2) for *S. aureus* and *B. subtilis* respectively. A reduction in cell density to 1.8 and 1.49 $\log_{10}$ CFU/mL at 5 x MIC from an initial concentration of 8 $\log_{10}$ CFU/mL was recorded. However, for the Gram-negative bacteria a higher concentration (x MIC) was determined to decrease the bacterial density significantly. Studies on inhibition of biofilm formation and resistance of preformed biofilm to methanol extract identified that the former property required within 2-fold dilutions higher than MICs, whereas concentrations of at least 8-fold higher than MICs was identified to reduce the preformed biofilms (Table 4). For example, the MIC for *E. coli* was 1008 mg/mL and the minimum concentration that inhibited biofilm formation was 2016 mg/mL. However, against preformed biofilm this value was as high as 8 mg/mL. These findings indicate that bioactives in methanol extract were able to inhibit bacteria biofilm formation at sub-MIC concentration, without killing them. However, to further eradicate the preformed biofilm, a higher concentration of the extract was required (Figure 3). The results of the present study indicated antimicrobial properties for the bioactives (phenols and flavonoids) corroborating earlier reports[20-21] in methanol extract attributed to their substituted hydroxyl groups.[22] The membrane interaction of these bioactives (phenolics being partially hydrophobic) augment their interaction with the lipid bilayer (bacterial cytoplasmic membrane and lipo-polysaccharide interfaces) more effectively causing a decrease in membrane stability.[23] The penetration into this phospholipid bilayer results in profound outcome on a wide range of cell functions. This capacity could result in intercalation with essential intracellular macromolecules, interfering with normal metabolism, resulting in cell death. The ability of the phytochemicals to cause cell lysis and as a result compromise the integrity of the bacterial membrane may explain their capacity to eradicate *S. aureus*. Furthermore, the limited activity of this extract on Gram-negative bacteria could be due to the limited capacity to cause perturbation of the bacterial cell membrane[24] leading to limited ingress across the outer membrane. Its inability could be related to another intrinsic mechanism reported involving efflux and multidrug efflux pumps reportedly associated with insusceptibility of *E. coli* to various biocidal agents.[25] The bioactives were found to prevent biofilm formation at lower concentrations in the present study as well as other reports[26,27] indicating a membrane-perturbing capacity. The increasing attraction for the use of phenolics as antibacterial agents maystimulate the use of these extracts as natural food preservatives, potential antibiotic replacements, and as a potential natural source of antimicrobial drugs which will provide novel or lead compounds that may be employed in controlling some infections.[28-31]

CONCLUSION

UPLC based metabolite analysis was employed to evaluate the chemical constituents of Eighteen metabolites were identified were synapoyl-hexose-formic acid, kaempferol 3-*O*-feruloylhexosyl rhamnoside, 6-C-arabinosyl-8-C-glucosyl-apigenin and isorhamnetin-3-O-glycoside-7-O-glycoside reported for the first time from this plant. Its methanol extract showed antibacterial activity was studied *in vitro*. The presence of these compounds may contribute to their *in vitro* biological activities of *M. talbotianum,* which could be used as marker compounds in standardization of the traditional medicine.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

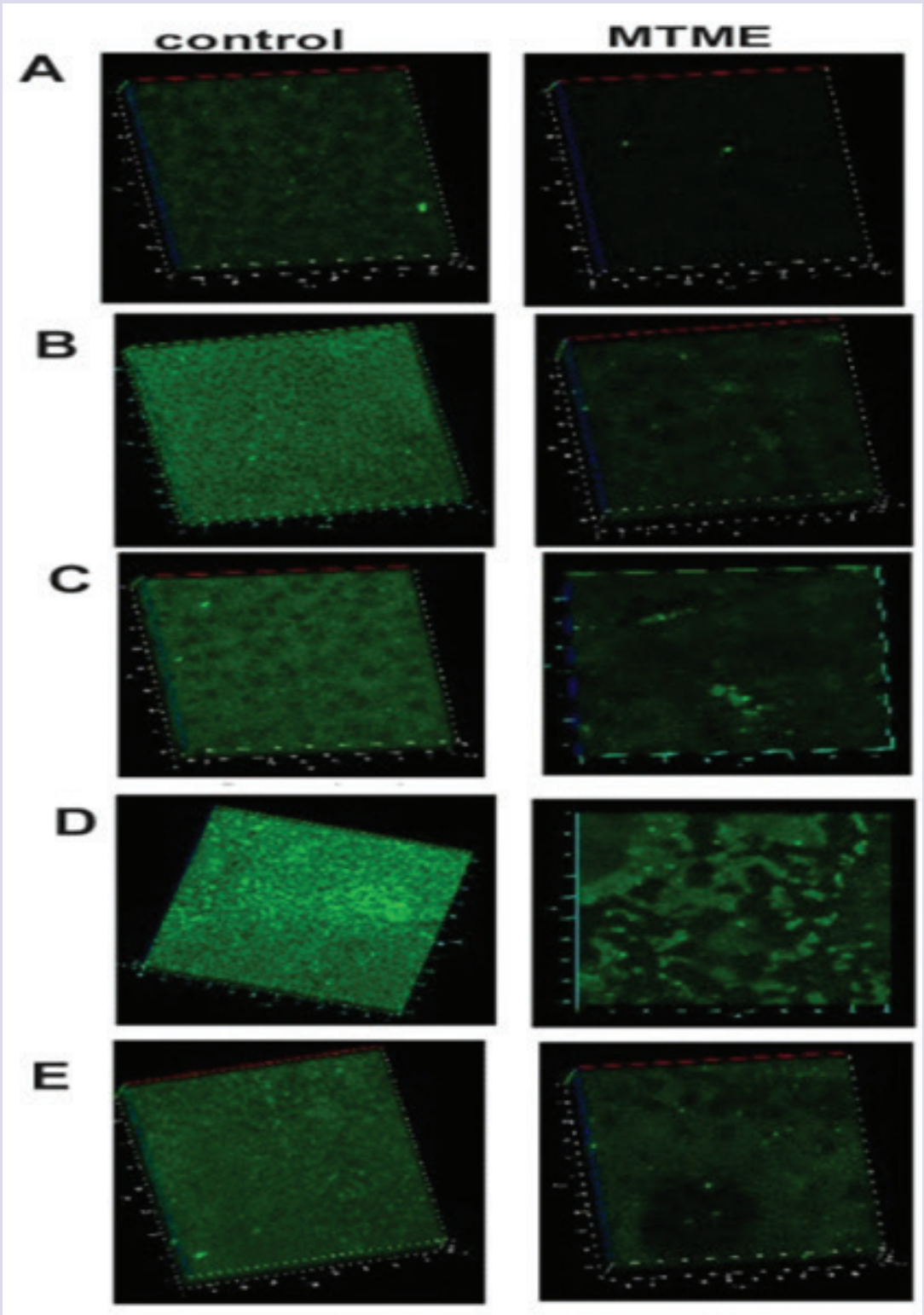

Figure 3: Inhibition of biofilm formation by methanol leaf extract of *Memecylon talbotianum*. The methanol extract was tested at 1 mg/ml concentration as described in materials and methods. Data expressed n = 3 experiments. The representative confocal laser scanning micrographs and presented. (A) *S. aureaus* (B) *B. subtillis*, (C) *S. typhi*, (D) *E.coli*, (E) *P.aeruginosa*.

ABBREVIATION USED

UPLC PDA ES/HDMS: Ultrapure liquid chromatography photo diode array electron spin high definition mass spectrometry; **MIC:** Minimum inhibitory concentration; **MBIC:** Minimum biofilm inhibitory concentration.

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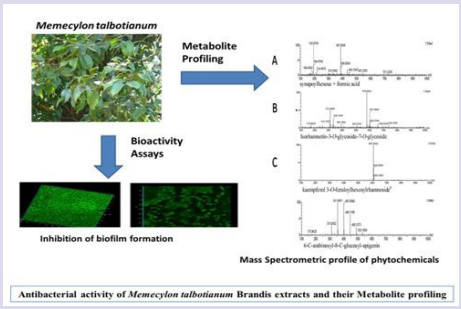
PICTORIAL ABSTRACT



SUMMARY

- UPLC based metabolite profiling was employed to evaluate the chemical constituents of *Memecylon talbotianum* Brandis extract and its antibacterial activity was studied *in vitro*.
- Eighteen metabolites, synapoyl-hexose-formic acid, kaempferol 3-O-feruloylhexosyl rhamnoside, 6-C-arabinosyl-8-C-glucosyl-apigenin and isorhamnetin-3-O-glycoside-7-O-glycoside as the main constituents were reported for the first time from this plant.
- A broad spectrum of antibacterial activity against to test human pathogens (MIC = 54 μg/ mL; Gram-positive bacteria) causing lysis at 24 h incubation was reported, resulting in nearly a 4 log10 CFU/mL drop in cell viability at 1.6 X MIC (Gram-positive) for this extract.

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