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PURIFICATION AND CHARACTERIZATION OF BIOACTIVE COMPOUND FROM THE METHANOLIC LEAF EXTRACT OF *MILLINGTONIA HORTENSIS* LINN.

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ABSTRACT

Millingtonia hortensis is widely distributed tree which belongs to Bignoniaceae family, commonly used as a folk medicine, having anti epileptic, antioxidant, antimicrobial, larvicidal and anticancer activities. In the present study, purification and characterization of bioactive compound(s) from methanolic extract of the leaves of *Millingtonia hortensis* were carried out using silica gel column chromatography, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), FT-IR and mass spectrometry (LC-MS) detection. Structure of the bioactive compound was determined by nuclear magnetic resonance (^{13}C and ^{1}H NMR) spectroscopy. The S6 fraction obtained from silica gel column chromatography was identified to have potent antioxidant, antimicrobial and antiproliferative activities with a yield of 2090 mg. The Rf value of the isolated bioactive compound was found to be 0.41 using ethylacetate : methanol (9:1) as mobile phase. The molecular weight of the compound was determined using LC-MS was identified as 301 daltons. The FT-IR spectroscopy showed the presence of phenolic hydroxyl, aromatic and methoxy functional groups. The purified compound was identified as 5,7-dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4*H*-chromen-4-one (Hispidulin) by NMR spectroscopy. The isolated pure bioactive compound was named as MHH.

KEYWORDS: ***Millingtonia hortensis*, MHH compound, DPPH, HPLC, FT-IR, LC-MS, ^{13}C and ^{1}H NMR.**

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INTRODUCTION

Recently, the isolation of natural antioxidants from plant source has significantly increased because synthetic antioxidants such as Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT) and Tertiary Butyl Hydro Quinone (TBHQ) are suspected to be responsible for liver damage and carcinogenesis[1]. Plants contain a large variety of substances such as alkaloids, terpenoids, phenolics, flavonoids, vitamin C, vitamin E, xanthophylls, carotenes, glycosides, phytosterols and tannins possessing antioxidant, antimicrobial antiproliferative, anti-inflammatory, immunomodulatory and anti-diabetic activities[2,3,4,5]. The treatment of infectious diseases caused by microorganisms is based on the use of antibiotics, which lost their effectiveness due to the development of resistant strains[6]. Antibiotics also have been associated with adverse effects which include hypersensitivity, allergic reactions and immune-suppression[7]. In addition to known antimicrobial activities, a variety of antibiotics also have anti-cancer properties[8]. Chemotherapy and radiotherapy treatments have been followed for the treatment of cancer, but possess various side effects and found to have limited survival[9]. Thus, there is a need to develop alternative drugs and new anti-cancer drugs for treatment of infectious diseases from different sources such as medicinal plants[10]. Activity guided fractionation have been extensively used to separate bioactive compound(s) of medicinal plant extracts into different solvent fractions based on the polarity. The fractions obtained by liquid-liquid extraction still contain combination of various types of bioactive compounds. Purification of these compounds can be achieved by column chromatographic techniques. Silica gel column chromatography is widely used for the separation of phytochemicals with varying chemical nature. Multiple mobile phases with increasing polarity are therefore useful for good separation. The purity of bioactive compound(s) can be determined by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). Modern analytical spectroscopic techniques like Nuclear Magnetic Resonance Spectroscopy (NMR), Liquid Chromatography Electrospray Ionization Mass Spectroscopy (LC-ESI-MS) and Fourier Transform Infrared Spectroscopy (FT-IR) play a key role in identification and characterization of bioactive principles having antioxidant, antimicrobial and antiproliferative activities.

MATERIALS AND METHODS

Chemicals

Organic solvents such as hexane, chloroform, ethylacetate and methanol were of analytic grade and obtained from E. Merck, Mumbai, India. HPLC grade methanol and acetonitrile were obtained from Ranbaxy fine chemicals limited, India. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and precoated silica gel TLC sheets were obtained from Sigma Chemical, USA. Silica gel (230-400 meshsize) was obtained from Qualigens Fine Chemicals, India.

Collection of plant material

Fresh leaves of *Millingtonia hortensis* were collected in sterile bags and carried to the laboratory. This plant was abundantly found in Seethampeta Village (Latitude-18^{o}69'N, Longitude-83^{o}8'E), Srikakulam District, North Coastal region of Andhra Pradesh and authenticated by Prof. M.Venkaiah, Department of Botany, Andhra University, Visakhapatnam. Specimen of the same was deposited in Botany Department Herbarium and voucher number was BDH-22079.

Preparation of plant material and Extraction of crude bioactive compound(s)

The fresh leaves were washed with tap water and then thoroughly cleaned with distilled water and shade dried for a week. Then the dried leaves were ground to a fine powder by using mortar and pestle. Shade dried and finely powdered leaves of *Millingtonia hortensis* (300 g) were extracted with methanol for 48 hours nearer to the solvent's boiling point with Soxhlet extractor yielding 45.6g of crude extract. The

crude methanolic extract was then concentrated to dryness using rotary evaporator (Superfit PBV-6) that resulted in 36.7 g dried extract.

Purification of bioactive compound(s) using Silica Gel Chromatography

The crude methanolic leaf extract of *Millingtonia hortensis* (25 g) was subjected to silica gel column chromatography to separate the extract into its component fractions. Silica gel was used as the stationary phase while varying solvent combinations of increasing polarity were used as the mobile phase[11]. The glass column (80cm in length and 5cm in diameter) was used for purification of bioactive compounds. A plug of cotton was placed at the bottom of the column and the column was fixed to stand vertically using clamps. Then 1/3 rd of the column was filled with n- hexane. Meanwhile, silica gel with mesh size 230-400 was activated by placing over night in hot air oven at 110°C and suspended in n-hexane to form slurry. The column was carefully packed with silica gel slurry with constant tapping. 25 g of methanolic extract was triturated with silica gel (1:2 w/w) and loaded onto the column. The column was covered with cotton plug and elution of the extract was done with solvent systems of gradually increasing polarity using hexane, chloroform, ethylacetate and methanol. The following ratios of solvent combinations were sequentially used in the elution process; n-hexane : chloroform 100:0, 75:25, 50:50 and 25:75; chloroform : ethyl acetate 100:0, 75:25, 50:50 and 25:75; ethyl acetate and methanol 100:0, 75:25, 50:50, 25:75 and 0:100. The 10 ml of fractions were collected and subjected to TLC. The eluted fractions with distinct spots and same Rf value in TLC analysis were pooled for each solvent system and condensed using rotary evaporator (Superfit PBV-6).

Thin Layer Chromatography

The homogeneity of the fractions was examined using thin layer chromatography. Aliquots of fractions were spotted on base line of precoated silica gel TLC sheets and allowed to dry for few minutes. Then the TLC plates were placed into TLC chamber saturated with solvent system. n-hexane-chloroform, chloroform-ethyl acetate and ethyl acetate-methanol were used as solvent systems in different ratios. TLC plates were sprayed with 20% H_2SO_4 in methanol to visualize the spots and allowed to air dry for 5min. Then the chromatographic plates were heated at 110^{o}C until the spots appeared.

Antioxidant activity

DPPH radical scavenging activity of all the pooled fractions was measured according to the method as described by Rajagopal et al 2008; Sivakumar et al, 2008; Cuendet et al, 1997[12,13,14].

Antimicrobial activity

Antimicrobial activity of all the pooled fractions was determined using agar well diffusion method as described by Olurinola et al., 1996, Murray et al., 1995, Perez et al., 1990[15,16,17].

Antiproliferative activity

Antiproliferative activity of all the pooled fractions was determined using MTT assay as described by Mosmann, 1983[18].

High Pressure Liquid Chromatography (HPLC)

HPLC (Agilent 1100 series) with LC solution software, PDA Detector (SPD-M 20 A) and Agilent TC C18-G column (4.6 x 250 mm) was used to determine the purity of all the pooled fractions with single spot in TLC analysis. 20 µl of sample was injected using Hamilton syringe (Bonaduz schweiz). Acetonitrile: water: formic acid (60:40:1) mixture was filtered through 0.2 micron membrane filter and used as mobile phase at a flow rate of 0.5 ml/min.

UV-Visible Absorption Spectroscopy

The determination was carried out on an UV-1800 UV-Vis spectrophotometer (Shimadzu). The isolated bioactive compound with single peak in HPLC analysis was dissolved in methanol (1mg/ml) and the absorbance was measured ranging from 200nm to 700nm at an interval of 5nm against methanol blank.

Liquid Chromatography Electrospray Ionization Mass Spectroscopy (LC- ESI-MS)

LC-ESI-MS was used to determine the molecular weight of the compound. The purified bioactive compound was analyzed by Agilent 1100 series LC-MSD with electro spray ionization (ESI) and quadrupole mass analyzer. Ammonium hydroxide (0.75 M) was used as buffering reagent. The analysis was performed at positive ion mode under following conditions: flow rate 0.5 ml/min, nebulizer pressure-25 psi, capillary voltage-3 kV, fragmentor voltage-75V and drying gas temperature- 350ºC. Spectrum of the purified compound was scanned over a mass range of m/z (0-500).

FT-IR Spectroscopy

The functional groups of the purified bioactive compound were analyzed using Bruker alpha FT-IR instrument (Software opus 6.5). 1 mg of purified bioactive compound was mixed with 100 mg of dry potassium bromide (KBr) and the mixture was compressed to prepare as small pellet. Then this pellet was analyzed under FT-IR spectrophotometer in the range of 4,000 - 500cm^{-1} at room temperature.

NMR Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was performed to elucidate the structure of purified bioactive compound. About 20 mg sample dissolved in 0.5 ml $CDCl_3$ which was used for recording the spectra. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX500 NMR instrument operating at 500 MHz, for 6 hours at room temperature using deuterated chloroform ($CDCl_3$). The region from 0 to 12 ppm for ^{1}H NMR and 0 to 200 ppm for ^{13}C NMR was employed for scanning. Trimethyl silane (TMS) was used as an internal standard. Chemical shifts (δ) were expressed in parts per million (ppm) and coupling constants (j) were indicated in hertz (Hz).

RESULTS AND DISCUSSION

Silica gel column chromatography has been frequently used for the purification of bioactive compounds such as phenolic compounds[19]. In the present study, the methanolic leaf extract of *Millingtonia hortensis* was subjected to silica gel column chromatography for further purification of bioactive compound(s).

Table1

Silica gel column chromatography of methanolic leaf extract of Millingtonia hortensis

S. No.	Fraction No.	Ratioof solvents	TLC Spot& Rf Value	DPPH radical scavenging activity (%)	Antimicrobial activity		Anti proliferative activity
					Antibacterial	Anti fungal	
1	1-40	n-hexane (100)	Spot1-0.52	13.31	-	-	-
			Spot2-0.34	17.73	-	-	-
2	41-80	n-hexane:Chloroform (75:25)	Spot1-0.61	24.18	+	-	-
			Spot2-0.42	27.34	+	-	-
3	81-120	n-hexane:Chloroform (50:50)	Spot1-0.67	18.54	+	-	-
			Spot2-0.39	16.53	+	-	-
4	121-195	n-hexane:Chloroform (25:75)	Spot1- 0.81	42.62	+	+	-
			Spot2 - 0.64	41.68	+	+	-
5	196-285	Chloroform (100)	Spot1- 0.84	34.48	+	+	+
			Spot2- 0.74	37.59	+	+	+
6	286-360	Chloroform: Ethyl acetate (75:25)	Spot1- 0.82	61.43	+	+	+
			Spot2- 0.76	64.76	+	+	+
7.	361-430	Chloroform: Ethyl acetate (50:50)	Spot1- 0.75	72.94	+ +	+ +	+ +
8	431-490	Chloroform: Ethyl acetate (25:75)	Spot1- 0.68	68.86	+ +	+ +	+
9	491-555	Ethyl acetate (100)	Spot1- **0.41**	**92.54**	**+ + +**	**+ + +**	**+ + +**
10	556-615	Ethyl acetate: Methanol (75:25)	Spot1- 0.58	44.56	+ +	+	+
11	616-675	Ethyl acetate: Methanol (50:50)	Spot1- 0.81	36.87	+	+	+
			Spot2- 0.77	33.57	+	+	-
			Spot3- 0.59	38.65	+	-	-
			Spot4- 0.35	41.63	+	-	-
12	676-725	Ethyl acetate: Methanol (25:75)	Spot1- 0.79	49.58	+	+	+
			Spot2- 0.74	52.69	+	-	-
			Spot3- 0.58	53.54	+	-	-
13	726-765	Methanol (100)	Spot1- 0.79	22.9	-	-	-
			Spot2- 0.74	25.43	-	-	-

"+++" High "++" Moderate "+" Traces & "-" Absent

As shown in table-1, out of 765 fractions, 53 fractions (493 - 546) eluted with 100% ethyl acetate showed significant antioxidant, antimicrobial and antiproliferative activities. These fractions were pooled and named as S6. TLC analysis of S6 exhibited single spot with an Rf value of 0.41. The S6 fraction was further subjected to HPLC analysis. This specific separation of bioactive compound(s) may be due to increased polarity of n-hexane with chloroform, chloroform with ethyl acetate and ethyl acetate with methanol[20].

Determination of purity by HPLC

The purity of the bioactive compound was analyzed by HPLC and the chromatogram was

shown in Figure 1. A sharp single peak was obtained at the retention time of 4.910 min with purity of about 94%. Single peak in HPLC profile indicates the homogeneity of pooled fractions. Hence, the isolated pure bioactive compound was named as MHH and characterized using various spectroscopic studies.

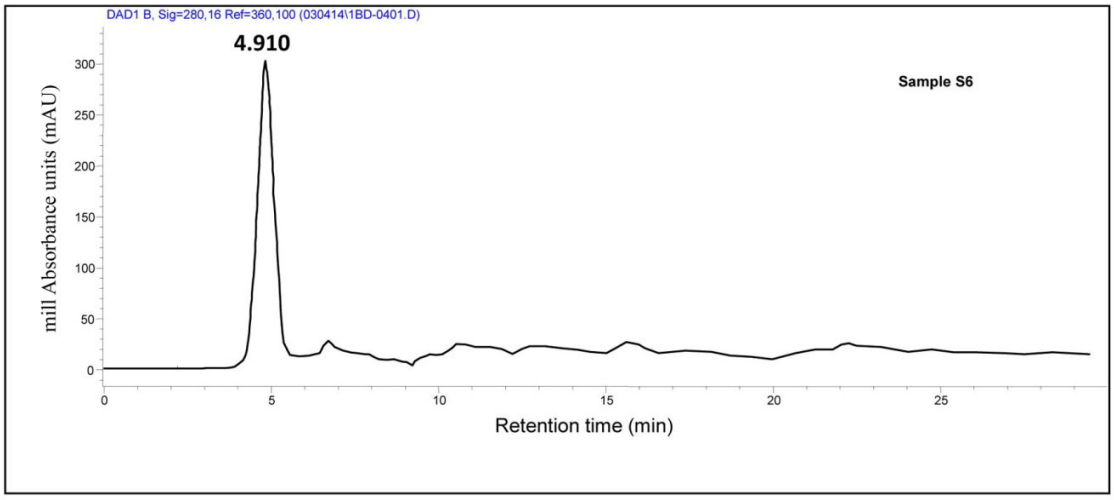

Figure 1
HPLC profile of the purified MHH compound

Properties, characterization and structural elucidation of MHH compound

The purified MHH compound was obtained as green in color. It is soluble in water, dimethyl sulfoxide, methanol and ethanol. The melting point of the purified MHH compound was found to be 180^{o}C (Capillary method).

UV absorption spectrum

The UV-Visible absorption spectrum of the purified MHH compound was shown in figure 2. The purified MHH compound showed two characteristic peaks one at 278 nm and the other at 327 nm. Kajdzanoska *et al*. (2010) reported that the absorption maxima at 280 indicates flavanols[21]. Sisa *et al*. (2010) reported that the absorption maxima at 255, 280, 330 and 350 nm indicating the presence of flavone ring system[22]. From this data, it was observed that the purified MHH compound contain a flavonoid ring or its derivative.

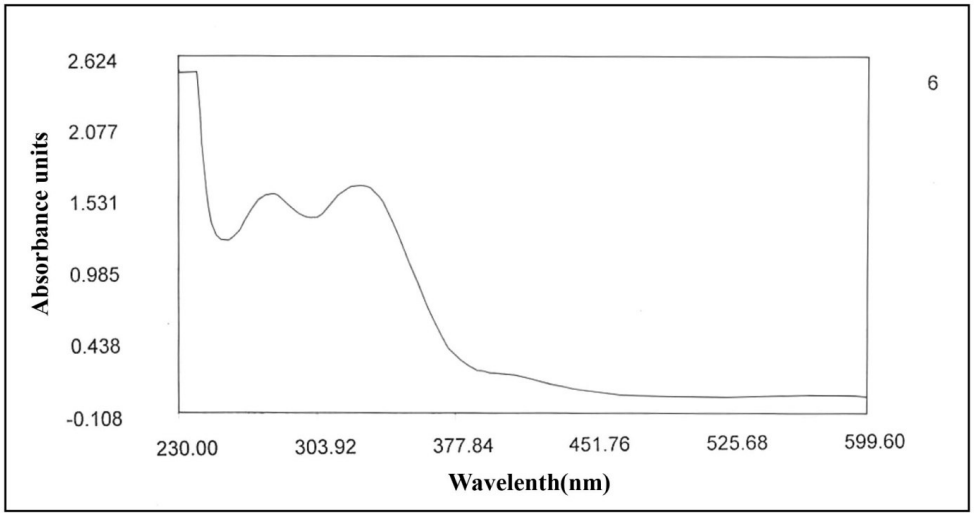

Figure 2
UV-Visible Absorption spectrum of the purified MHH compound

FT-IR absorption spectrum

The FT-IR absorption spectrum of the purified MHH compound was shown in figure 3. The purified MHH compound exhibited absorptions at 3398.28, 2854.69, 1656.96, 1461.06, 1249.03, 1249.03, 1175.81 cm^{-1}. The two characteristic peaks at 3398.28 and 1461.06 cm^{-1} indicated the presence of phenolic hydroxyl and aromatic groups respectively. Further, peaks at 1656.96 cm^{-1} and 1249.03, 1175.81 cm^{-1} suggests the presence of carbonyl group(C=O) and hetero atom bond (O–C), respectively. The major signal at 2854.69 cm^{-1} indicates the presence of methoxy group ($O–CH_3$)[23]. These FT-IR results supported the presence of the aromatic flavone ring and other functional groups of flavonoids in the purified MHH compound.

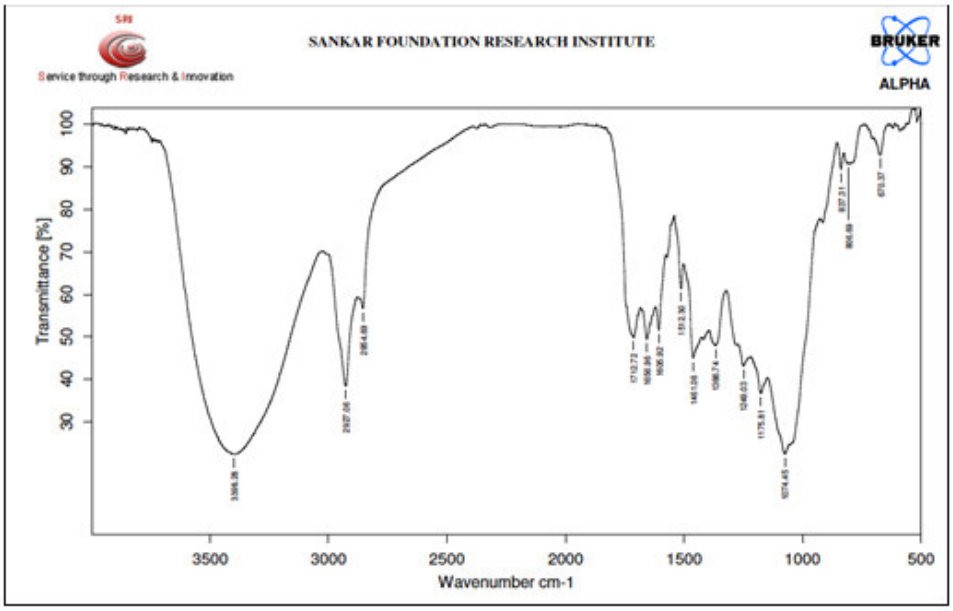

Figure 3
FT-IR spectrum of the purified MHH compound

Mass spectrum

The mass spectrum of the purified MHH compound was shown in Figure 4. The results of the present study of the purified MHH compound at positive ion mode and the ESI spectrum showed major ($M+H^+$) peak at 301(m/z). The molecular mass of the purified MHH compound was found to be 301 daltons.

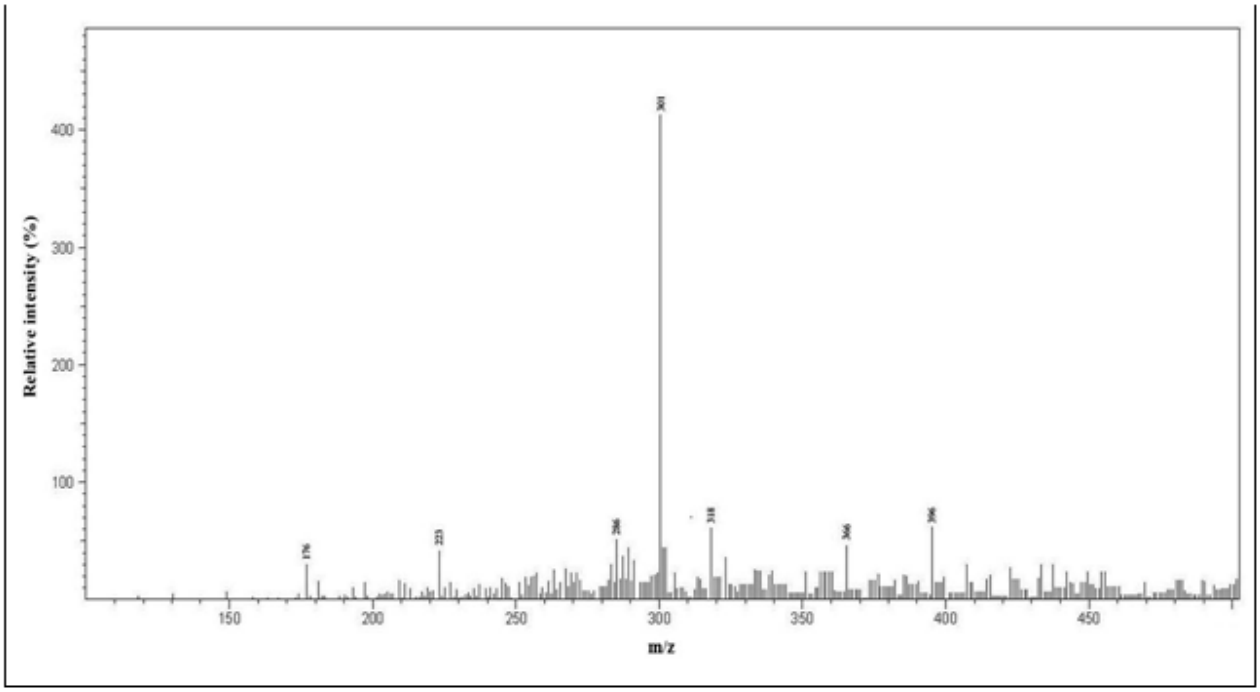

Figure 4
LC-ESI-MS spectrum of the purified MHH compound

NMR analysis

^1H NMR of the purified MHH compound was obtained at 400.13 MHz and ^{13}C NMR spectra was obtained at 100.5 MHz and were shown in figure 5 and figure 6 respectively. From the ^1H NMR spectra the chemical shifts were observed at 3.82 (3H, s, OCH3-3), 12.09 (1H, s, OH-5), 6.40 (1H, d, H-3), 10.9 (1H, s, OH-7), 7.8 (1H, d, H-8), 7.60 (1H, d, H-2'), 10.74 (1H, s, OH-3'), 9.6 (1H, s, OH-4'), 7.6 (1H, d, H-5'), 6.8 (1H, dd, H-6'). From the C^{13} NMR spectra, the δ values of carbon atoms were 152.57 (C-2), 103.35 (C-3), 180.42 (C-4), 145.68 (C-5), 138.61 (C-6), 159.20 (C-7), 114.50 (C-8), 118.77 (C-9), 105.3 (C-10), 137.14 (C-1'), 129.78 (C-2'), 126.07 (C-3'), 159.84 (C-4'), 125.98 (C-5'), 127.02 (C-6'), 60.35 (OCH3). These ^1H and ^{13}Cdata were consistent with the literature[24].

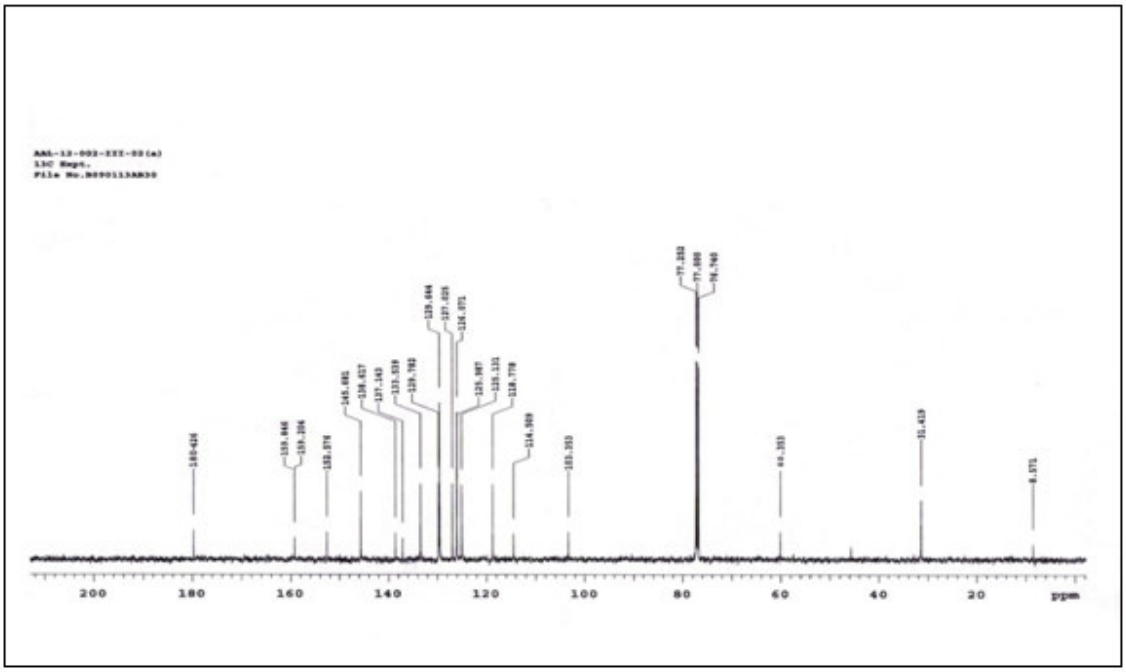

Figure 5
^{13}C NMR spectrum (100 MHz, $CDCl_3$) of the purified MHH compound

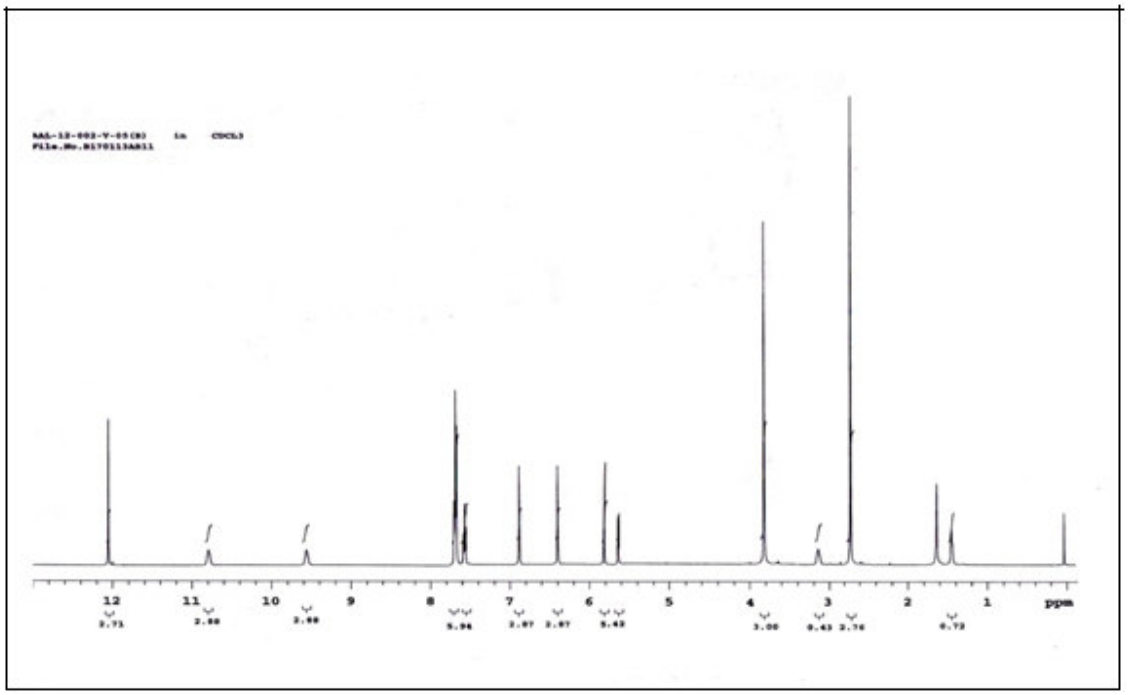

Figure 6
^{1}H NMR spectrum (400 MHz, $CDCl_3$) of the purified MHH compound

These chemical assignments obtained from the UV, FT-IR, LC-ESI-MS and NMR Spectroscopy suggested the bioactive compound MHH as 5,7-dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4*H*-

chromen-4-one (Hispidulin) and the proposed structure was shown in Figure-7 using chemdraw ultra 12.0. The molecular formula of the purified bioactive compound MHH was determined to be $C_{16}H_{12}O_6$.

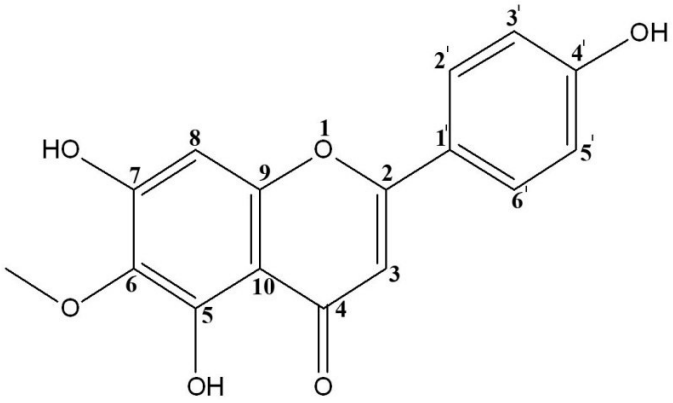

Figure 7
Proposed structure of the purified MHH compound (5,7-dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4H-chromen-4-one)

CONCLUSION

In the present study, the methanolic leaf extract of *Millingtonia hortensis* was subjected to silica gel column chromatography for purifying bioactive compounds. The purity of obtained fractions was analyzed by TLC and HPLC. The antioxidant, antimicrobial and antiproliferative activities of the methanolic fractions were determined by using DPPH assay, agar well diffusion assay and MTT assay respectively. The fraction that showed potential antioxidant, antimicrobial and antiproliferative activities was designated as S6. The S6 was further characterized by UV-Vis, FT-IR, LC-ESI-MS and NMR spectroscopic techniques. From the analysis of the above data, the compound structure was proposed as 5,7-dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4*H*-chromen-4-one (Hispidulin, $C_{16}H_{12}O_6$) and named as MHH.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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