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Phytochemical Characterization and *in vitro* Antioxidant, *in vitro* Antidiabetic Activity of *Manilkara hexandra* Seed Extract

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Abstract

Manilkara hexandra (Roxb.) Dubard is a large evergreen tree species widely distributed throughout India and other countries and belongs to family Sapotaceae. Conventionally, the entire plant has been used for the treatment of various diseases. The present study has been carried out for identification of its bioactive constituents by high resolution-liquid chromatography-quadrupole-time-of-flight-mass spectrometry (HR-LC-QTOF-MS) and evaluation of *in vitro* antioxidant and *in vitro* antidiabetic activity of the methanolic extract of the seeds of the plant. Twenty-eight bioactive compounds from the methanolic extract of *Manilkara hexandra* seeds (MEMH) were identified using HR-LC-QTOF-MS. *In vitro* antioxidant activity of MEMH was evaluated using 1,1-diphenyl-2-picryl-hydrazyl, nitric oxide, superoxide, and hydroxyl radical scavenging assays and the IC_{50} values for MEMH were found to be similar to standard drug. *In vitro* antidiabetic activity was also evaluated using anti α-amylase and anti-α-glucosidase assays and the IC_{50} values for MEMH were found to be good as compare to standard drugs. The compounds identified by HR-LC-MS partly explain the various therapeutic effects of the seeds of the plant. Thus, it can be concluded that MEMH is rich in bioactive phytochemicals and possesses potential antidiabetic and antioxidant activity.

Keywords: Antidiabetic, Antioxidant, HR-LC-QTOF-MS, *Manilkara hexandra*, Seeds

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Introduction

Diabetes mellitus (DM) is a common metabolic disorder. It is characterized by hyperglycemia that results from an absolute or relative insulin deficiency and associated with long-term damage, and failure of various organs, especially the eyes, kidneys, nerves, hearts, and blood vessels.[1] There are two types of DM that are present – Type I DM which caused by the destruction of the pancreatic β-cells and Type II diabetes which 90% cases with the prevalence increasing with age.[2] The ultimate goal is to prevent microvascular and macrovascular complications associated with diabetes.[3] As many as 400 plants have shown antidiabetic activities.[4]

Free radicals are oxygen-containing molecules with unpaired electrons. The unpaired electron allows them to easily react with other molecules.[5] Hyperglycemia can increase the indicators of lipid peroxidation and oxidative stress, in which, free radicals have the main role in the pathogenesis of these complications. Therefore, antioxidants which combat oxidative stress should be able to prevent and repair free radicals which contribute to kidney damage, atherosclerosis, diabetes, heart disease, nephrotoxicity, hepatotoxicity, etc.[6]

Experimental diabetes in *in vitro* model has simulated the diabetic state extensively. In near future, some parts of plants may provide a possible source of hypoglycemic drugs because many plants and plant-derived compounds have been utilized in the treatment of diabetes. *Manilkara hexandra* (Roxb.) Dubard is a large evergreen tree species widely distributed with throughout India and other countries and belongs to the family Sapotaceae.[7] Conventionally, the entire plant has been used for the treatment of various diseases such as ulcer, bronchitis, jaundice, ulitis, fever, and arthritis.[8] Eskander *et al*., (2013) isolated saponin 1, 2, and 3 possessing protobassic acid and also three types of phenolic compounds such as gallic acid, myrecetin, and quercetin.[9] Gomathi *et al*., (2012) demonstrated that polysaccharides extracted from M. hexandra bark significantly stimulating macrophage function.[10] Kumar *et al*., (2010) found that methanolic leaf extracts of *M. hexandra* showed strong 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity.[11] Modi *et al*., (2012) demonstrated that acetone extract and its different fraction of *M hexandra* showed antiulcer activity.[12] Bindu *et al*., (2014) found that stem bark of *M. hexandra* contains triterpenoid saponin, β-sitosterol, etc.[13] The present study has been carried out for identification of its bioactive constituents by HR-LC-MS and evaluation of *in vitro* antioxidant and *in vitro* antidiabetic activity of the methanolic extract of the seeds of the plant.

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Methods

Plant Materials

The seeds of *M. hexandra* (Roxb.) Dubard were collected from 24 parganas (South), district of West Bengal, India and the plant was authenticated by the Botanical Survey of India, Howrah, India. The voucher specimen number is CNH/Tech.II/2021/2a.

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Preparation of Extract

Fresh seeds of *M. hexandra* (Roxb.) Dubard were properly washed with water; shade-dried; and crushed directly using an electrical grinder. The powdered dry seeds were stored in airtight containers for the future use. The powdered sample was extracted by soxhlet apparatus using three solvents, such as – petroleum ether (3.8% w/w, yield), chloroform (2.60% w/w, yield), and methanol (6.29% w/w, yield). The extract was collected and stored in desiccator for further study.

High Resolution-Liquid Chromatography-Quadrupole-Time-of-Flight-Mass Spectrometry (HR-LC-QTOF-MS) Analysis

The extract was prepared in methanol and then subjected to HR-LC-QTOF-MS analysis. The analysis was carried out at Sophisticated Analytical Instrument Facility, IIT Bombay, Powai, Mumbai. Agilent high resolution liquid chromatography and mass spectrometry model-G6550A with 0.01% mass resolution were used for the purpose. The separation was achieved using the reverse phase BEH C18 column (50 mm × 2.1 mm × 1.7 μm). The scan range was set to be 50 (m/z) to1000 (m/z). Acquisition time was 30 min and solvent system used was acetonitrile: Methanol: 95:5 v/v at a flow rate of 0.3 mL/min.

In Vitro Antioxidant Test

DPPH assay

The antioxidant activity of *M. hexandra* seed extract was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH.[14,15]

DPPH and methanol were added with methanolic seed extract of *M. hexandra*. The DPPH absorbance was measured at 517 nm. N-acetyl cysteine was used as the reference standard. The percentage (%) of DPPH scavenging activity was calculated using the following equation –

$$DPPH\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Hydroxyl radical scavenging activity assay

Hydroxyl radicals are highly reactive species and attack most of the organic molecules.[16] The reaction mixture consists of 2-deoxy-D-ribose (28 Mm KH_2PO_4-KOH buffer, pH 7.4), methanolic extract, EDTA (1.04 mM) and $FeCl_3$ (1:1 v/v), H_2O_2 (1.0 mM), and N-acetyl cysteine acid which is incubated at 37°C for 1 h. Thiobarbituric acid (1%) and trichloroacetic acid (2.8%) were added and incubated at 100°C for 20 min. After cooling, the absorbance was measured at 532 nm against a blank sample.

Superoxide radical scavenging activity

The scavenging activity against chemically generated superoxide radicals of the extracts and flavonoids was measured by spectrophotometric measurement of the product on reduction of nitro blue tetrazolium (NBT).[17] The samples were dissolved in DMSO and diluted in water to give a final concentration of 12% (v/v) for DMSO. Superoxide anions were generated in a non-enzymatic system. The reaction mixture contained test solution, 0.1 M phosphate buffer, pH 7.4, 20 μM PMS, 156 μM NADH, and 25 μM of NBT in phosphate buffer, pH 7.4. After 2 min of incubation at 25°C, the absorbance was measured at 560 nm. The percentage (%) of scavenging activity was calculated using the following equation –

$$Superoxide\ (\%) = 1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Nitric oxide scavenging assay

The assay of nitric oxide scavenging was used to evaluate the antioxidant potential of the extract.[18] 10 mM sodium nitroprusside dissolved in phosphate buffer saline (pH 7.4) was mixed with the extract. The mixture was then incubated at 25°C. After incubation, solution was withdrawn and mixed with Griess reagent. The mixture of sample was incubated at room temperature for 30 min. The absorbance was measured at 546 nm. The percentage (%) of nitric oxide activity was calculated using the following equation –

$$á - amylase (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ \text{control}} \times 100$$

In Vitro Antidiabetic Assay

α-Amylase inhibition assay

The assay was carried out following the standard protocol.[19] The seed extract of *M. hexandra* was dissolved in 10% DMSO and further dissolved in buffer (pH 6.9). Then, α-amylase solution was mixed with the extract and incubated at 30°C for 10 min. After that, starch solution was added and incubated for 3 min. After reaction, 3,5-dinitrosalicylic acid reagent was added and boiled at 85–90°C for 10 min in a water bath. Then the mixture was cooled and diluted with distilled water. The absorbance was measured at 540 nm. The percentage (%) of α-amylase activity was calculated using the following equation –

$$á - amylase (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ \text{control}} \times 100$$

α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity of *M. hexandra* seed extract was carried out according to the standard method.[20] Standard and methanolic extract of *M. hexandra* seeds (MEMH) were prepared in test tubes. Moreover, then, phosphate buffer and α-glucosidase were added in test tubes and incubated at 37°C for 20 min. The absorbance was measured at 405 nm. The percentage (%) of α-glucosidase activity was calculated using the following equation –

$$á - glucosidase\ (\%) = 1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Results

Twenty-eight bioactive compounds from MEMH were identified using HR-LC-QTOF-MS. The identified compounds belong to diverse classes of phytochemicals such as tannins, phenols, flavonoids, steroids, terpenes, alkaloids, and glycosides. The list of identified compounds has been given in Table 1. *In vitro* antioxidant activity of MEMH was evaluated using DPPH, nitric oxide, superoxide, and hydroxyl radical scavenging assays and the IC_{50} values for MEMH were found to be 116 μg/ml, 229.4 μg/ml, 147 μg/ml, and

Table 1: Bioactive compounds identified in the methanol extract of *Manilkara hexandra* seeds

S. No.	*Name of the compound*	*Molecular formula*	*Molecular weight (m/z)*	*Retention time (min)*	*Compound nature*	*Activity*
1.	2′-Hydroxy-3,4′,5′,7,8-pentamethoxyflavone	$C_{20}H_{20}O_8$	388.118	1.098	Flavonoid	Antioxidant and anti-cancer activity.
2.	2,6-Dihydroxybenzoic acid	$C_7H_6O_4$	154.024	3.179	Organic acid	DPPH scavenging activity and microbial growth inhibition.
3.	Valproic acid glucoronide	$C_{14}H_{24}O_8$	320.145	3.683	Fatty acid	Antineoplastic and antiangiogenesis activities.
4.	m-hydroxybenzoic acid	$C_7H_6O_3$	138.029	3.921	Organic acid	Antioxidant activity.
5.	(+)- Taxifolin	$C_{15}H_{12}O_7$	304.056	6.087	Flavonoid	Anti-inflammatory, antioxidant, antitumor, anti-microbial, and anti-cancer activity.
6.	Quercetin	$C_{15}H_{10}O_7$	302.041	7.965	Flavonoid	Antioxidant, anti-inflammatory, antibacterial, and antiviral, anticarcinogenic.
7.	Acetylsoyasaponin A_3	$C_{60}H_{92}O_{27}$	1244.552	8.541	Triterpene	Antioxidant activity.
8.	Shikimate-3-phosphate	$C_7H_{11}O_8P$	254.019	9.17	Monoalkyl phosphate	No activity reported.
9.	Petroselinic acid	$C_{18}H_{34}O_2$	282.253	18.876	Fatty acid	No activity reported.
10.	11-Hydroxy-9-tridecenoicacid	$C_{13}H_{24}O_3$	228.170	0.84	Fatty acid	No activity reported.
11.	Dihydrocaffeic acid 3-o-glucuronide	$C_{15}H_{18}O_{10}$	358.088	1.05	Phenolic glycosides	Antioxidant activity.
12.	2-o-a-L-Fucopyranosyl-galactose	$C_{12}H_{22}O_{10}$	326.120	1.691	Fatty acyl glycoside	Alzheimer's disease and non-toxic therapy for the treatment of resistant nephrotic syndrome.
13.	1, 5-Dibutyl methyl hydroxycitrate	$C_{15}H_{26}O_8$	334.163	3.333	Tricarboxylic acid	No activity reported.
14.	N-Acetyl-L-Phenylamine	$C_{11}H_{13}NO_3$	207.089	4.313	Aromatic amine	Anti-inflammatory, antidepressant, and antioxidant activity.
15.	Flurandrenolide	$C_{24}H_{33}FO_6$	436.227	4.788	Corticosteroid	Anti-inflammatory and anti-allergic activity.
16.	Dihydrodioscorine	$C_{13}H_{21}NO_2$	223.157	5.009	Alkaloid	Antifungal activity.
17.	Kolanone	C33H42O8	502.297	5.288	Monoterpenoid	Antimicrobial activity.
18.	Tropacocaine	$C_{15}H_{19}NO_2$	245.141	5.645	Alkaloid	Inhibited sodium-dependent choline uptake and acetylcholine synthesis.
19.	3-O-Methylniveusin A	$C_{21}H_{28}O_8$	408.177	6.489	Terpene lactones	No activity reported.
20.	Triethyl citrate	$C_{12}H_{20}O_7$	276.12	6.49	Tri-carboxylic acid	No activity reported.
21.	Manumycin A	$C_{31}H_{38}N_2O_7$	550.260	8.998	Fatty amide	Anti-cholinergic, and antibiotic, anti-cancer activity.
22.	Sulfadimidine	$C_{12}H_{14}N_4O_2S$	278.086	10.209	Sulphonamide	Inhibit the growth of bacteria.
23.	Moracin A	$C_{16}H_{14}O_5$	286.086	11.276	Benzofuran	Anti-cancer, antimicrobial, immunomodulator, antioxidant, and anti-inflammatory activity.
24.	Fluticasone propionate	$C_{25}H_{31}F_3O_5S$	500.182	13.081	Steroid esters	Anti-inflammatory, anti-allergic, and anti-asthmatic activity.
25.	Majoroside F_3	$C_{42}H_{72}O_{14}$	800.487	13.958	Iridoid glucoside	No activity reported.
26.	Convallasaponin A	$C_{32}H_{52}O_9$	580.357	14.116	5 beta-spirostanol triglycoside	Cytotoxic activity.
27.	(ent-2b, 4s, 9a)-2,4,9-Trihydroxy-10 (14)-oplopen-3-one 2-(2-methlybutanoate) 9-(3-methyl-2E-pentenoate	$C_{22}H_{38}O_5$	448.279	14.267	Sesquiterpenoid	No activity reported.
28.	Mycinamicin VII	$C_{29}H_{47}NO_7$	521.346	16.182	Macrolide	Antibiotic activity.

166 µg/ml, respectively. The results of *in vitro* antioxidant activity are represented in Figure 1a and b. *In vitro* antidiabetic activity was also evaluated using anti α-amylase and anti-α-glucosidase assays and the IC_{50} values for MEMH were found to be 880 µg/ml and 204 µg/ml, respectively. The results of *in vitro* antidiabetic activity are represented in Figure 2a and b.

DPPH, nitric oxide, superoxide, and hydroxyl radical scavenging activity of MEMH and the standard N-acetylcysteine was performed as per standard protocol and the data was represented in percentage inhibition. Each point represents the values obtained from three experiments and performed in triplicate (mean ± SEM).

Alpha amylase, Alpha glucosidase activity of MEMH and the standard Acarbose was performed as per standard protocol and the data was represented as a percentage inhibition. The data represent the percentage of alpha amylase and alpha glucosidase inhibition. Each point represents the values obtained from three experiments and performed in triplicate (mean ± SEM).

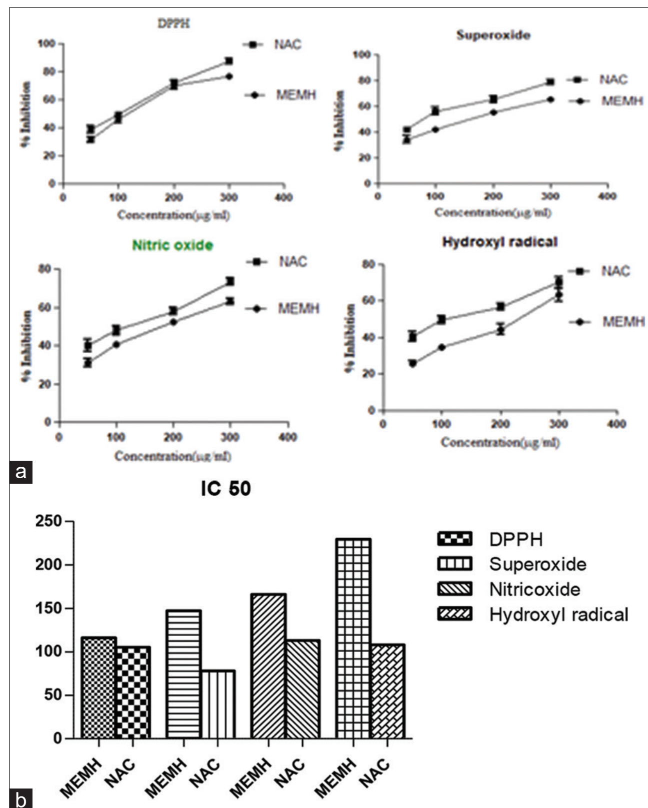

Figure 1: (a) *In vitro* antioxidant activity and (b) DPPH, nitric oxide, superoxide, and hydroxyl radical scavenging activity of MEMH and the standard N-acetylcysteine. Values represent mean of triplicate (mean ± SEM) analysis

Discussion

The screening of phytochemical characterization and *in vitro* antioxidant and *in vitro* antidiabetic activity of natural products was performed in this experimental study. Preliminary phytochemical profiling of MEMH using HR-LC-QTOF-MS showed the presence of alkaloid, tannins, phenols, flavonoids, steroids, terpenes, and glycosides. Alkaloids are known for their anti-inflammatory, antioxidant, anti-malarial, anti-fungal, anti-HIV, and analgesic activities.[21] The basic activities of flavonoids include antioxidant, anti-bacterial, antiviral, anti-inflammatory, and anti-cancer activity.[22] The presence of flavonoids and benzoic acid derivatives in the MEMH explains the *in vitro* antioxidant activity of MEMH. Steroids are known for their anti-cancer, anti-inflammatory, antineoplastic, antisecretoric, antieczematic, antihypercholesterolemic, and antipsoriatic activities.[23] The pharmacological activities of terpenes are antitumor, anti-inflammatory, antibacterial, antiviral antimalarial, etc.[24] The activities of glycosides are anti-inflammatory, analgesic, antipyretic, antioxidant activity, etc.[25] Thus, phytochemical screening using HR-LC-QTOF-MS of MEMH showed that it contains many bioactive compounds and justifies the myriad therapeutic potential of *M. hexandra*. The IC_{50} values of MEMH as obtained in DPPH, hydroxyl radical, superoxide, and nitric oxide radical scavenging assays when compared to that of standard compound N-acetyl cysteine indicated that MEMH possesses good *in vitro* antioxidant properties. The IC_{50} values of MEMH as obtained in α-glucosidase and anti-α-amylase inhibitory assays when compared to that of standard compound acarbose indicated that MEMH possesses good *in vitro* antidiabetic activity.

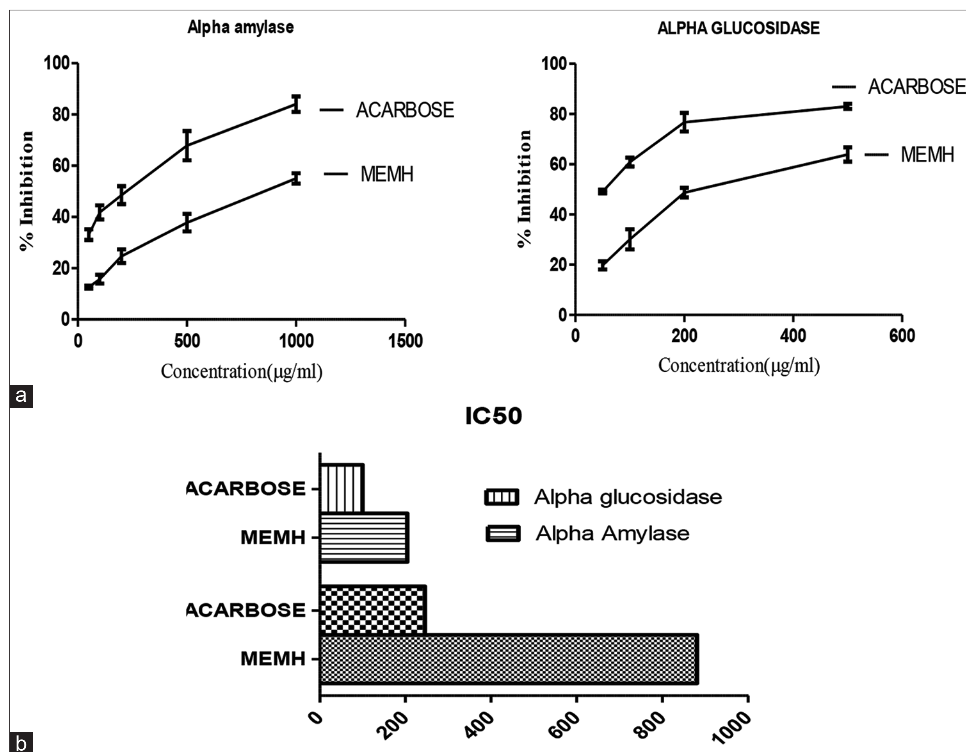

Figure 2: (a) *In vitro* antidiabetic activity and (b) Alpha amylase, Alpha glucosidase activity of MEMH, and the standard Acarbose. Values represent mean of triplicate (mean ± SEM) analysis

Conclusions

This research article focused on phytochemical characterization of MEMH using HR-LC-QTOF-MS and also evaluated it's *in vitro* antioxidant and *in vitro* antidiabetic activity. Phytochemical profiling of MEMH showed that many bioactive compounds are present in it which partly explains its myriad therapeutic potential. Moreover, the different *in vitro* antioxidant and *in vitro* antidiabetic assays carried out pointed out conclusively that MEMH possesses good antioxidant and antidiabetic potential. Thus, MEMH has great potential as phytomedicine for treating diabetes as well as for reversing associated oxidative damage.

Declarations

Funding

NSHM Knowledge Campus Kolkata Group of Institutions funded the entire study including purchase of reagents, chemicals, and also provided the necessary infrastructure.

Competing Interests

We declare that we do not have any competing interests.

Availability of Data and Material

All the relevant data and material are available with the authors for reference.

Code Availability

GraphPad Prism was used for statistical analysis.

Authors' Contributions

NS, BM, and SF were responsible for the collection, authentication of the plant, and carrying out the study. NS was also involved in drafting the manuscript. MC carried out the experimental design and calculations. MM was responsible for HR-LC-QTOF-MS data interpretation and manuscript correction. All the authors have read and approved the manuscript for final submission.

Ethics Approval and Consent to Participate

Not Applicable.

Consent for Publication

Not Applicable.

Plant Authentication

The plant specimen (NSHM/BM 01) was authenticated from Botanical Survey of India, Kolkata, India.

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