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RESEARCH ARTICLE

Antimicrobial, Antioxidant Activities, Phytochemical Evaluation and GC-MS Profiling of *Madhuca longifolia* Bark Extracts

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Highlights

- Different extracts of bark of *Madhuca longifolia* were prepared.
- Phytochemical screening, phenolics, flavonoids, tannins and sugars content as well as antibacterial and antioxidant activities were evaluated
- Hexane extract was analyzed by gas chromatography mass spectrometry, GCMS
- Greater amounts of phenolics, flavonoids, tannins and sugars were detected in methanol extract and showed potent free radical scavenging activity with DPPH
- Ethyl acetate, methanol and methanol-water extracts showed antibacterial activity
- Squalene, β-amyron, β-amyrin acetate, lupeol, lupeol acetate were identified by GCMS

Abstract

Madhuca longifolia, locally known as Mahua is a large to medium sized tree grows in Tarai region of Nepal. It is a medicinal plant of high economic value. Almost all parts of the plant are used by the indigenous people tocure various diseases.The bark is used to treat diabetes, cuts, wounds, itching and bleeding gums.Thescientific reportsaboutthe phytochemical analysis on the bark of M. longifolia is very limited. Here we report about the extraction based on solvent polarities, chemical screening of extracts, estimation of phenolics, flavonoids, gallotannins, condensed tannins and sugars in different extracts, their antioxidant and antibacterial activities as well as GC-MS profiling of hexane extract. In our findings, ethyl acetate, methanol and 50% aqueous methanol extracts showed the presence of most of the phytochemicals like terpenoids, phenolics, flavonoids, tannins and glycosides. The greater amountsof phenolics (196±15.28 mg gallic acid equivalent, GAE/g extract), flavonoids (975±13.31 mg catechin equivalent, CE/g extract), condensed tannins (980±10.75 CE/g extract)and sugars (126±8.83 mg glucose equivalent, GE/g extract) were detected in the methanol extract,greater amounts ofgallotannins were detected in the ethyl acetate extract (126±8.83 mg tannic acid equivalebt, TAE/g extract). In DPPH radical scavenging assay, methanol extract showedstrong radical scavenging activity with an IC_{50} value of 18.86± 1.07µg/mL. In antibacterial assay,ethyl acetate, methanol and 50% aqueous methanol extracts showed activity against S. aureusand E. coli with the inhibition zone ranged from 15-22 mm. GC-MS analysis of hexane extract showed the presence of more than nine compounds and squalene, β-amyron, β-amyrin acetate, lupeol, lupeol acetate and cis-3,14-Clerodadien-13-ol were tentatively identified by comparing mass fragmentation patterns with the standard NIST database. The finding of this study indicated that M. longifolia bark extracts can be used as a natural source of antioxidant, antibacterials and anti-inflammatory agents and supports its traditional use.

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Keywords: *Antioxidant, Antibacterial, Gas Chromatography-Mass Spectrometry, Phenolics, Flavonoids, Tannins, Sugars*

Introduction

Plants and plant-based products are an integrated part of most of the traditional and alternative systems of medicines worldwide. Such medicines may lead to the discovery and development of cost effective new drugs with enhanced performance [1]. *Madhuca longifolia* (J. konig) J.F. Macbr (synonmy: *Madhuca indica, Bassia longifolia, Sapotaceae family)* is very important medicinal plant, all plant parts are used to cure various diseases. It iscommonly known as English butter tree or honey tree and locally known as Mauwa. It is a large tree growing widely in dry tropical and sub tropical regions. The flowers are edible and it has good nutritional value. Seeds are the source of fat, commonly known as Mahua butter. Traditionally, bark has many medicinal values, it is used to treat ulcers, rheumatism, tonsillitis, decoction is used to treat diabetes, powder is used in itching and bleeding gums [2] and paste is applied externally to treat cuts, wounds and bleeding [3]. Several pharmacological potentials of bark extracts have been reported such as anti-hyperglycemic[4,5] anti-inflammatory, analgesic and anti-pyretic [6,7], anti-ulcer [8]. In our previous finding, the ethyl acetate extract of the bark showed immunomodulatory properties [9] and we have isolated flavan-3-ols with antibacterial and antidiabetic activites [10]. Some phytoconstituents like triterpenoids, saponins, steroids, flavonoids and their glycosides, tannins have been isolated from the bark [11,12].

The search for new natural antioxidant and antimicrobial agents from plants remains a potential area of investigation.This can be done by conducting phytochemical analysis.Therefore, the present study was carried out to estimate the total content of phenolics, flavonoids, tannins and sugars in different extracts, their antioxidant and antibacterial activities and GC-MS profiling of hexane extract. To the best of our knowledge, this is the first systematic investigation of the bark extracts of *M. longifolia.*

Materials and methods

Plant materials

The bark of *M. longifolia* was collected from Kailali district of western Nepal. It was identified by comparison with the herbarium species deposited at Central Department of Botany, Tribhuvan University. The voucher specimen (SB-19-MR) was deposited at Research Center for Applied Science and Technology, RECAST, Tribhuvan University. The collected plant materials were washed properly, chopped into pieces and shade dried. The dried samples were ground to fine powder and stored in air tight plastic bags.

Extraction

About 80 g of the sample was extracted first with 300 ml hexane in a Soxhlet apparatus. The extraction process was continued for 6-7 hours. The residue was then allowed to dry and extracted with 300 ml dichloromethane, followed by 200 ml of ethylacetate and finally by 200 ml of methanol. The remaining residue was then refluxed with 100 ml of aqueous methanol (1:1) for 1 hour, allowed to cool and filtered. Then the solvents were evaporated separately using rotary evaporator under reduced pressure. The extracts were kept in the fridge which was used for further analysis.

Phytochemical screening

The method described by Culie [13] was adopted for the phytochemical screening.The extracts were reacted with different reagents and visible colors were noted.

Total phenolic content

By using Folin-Ciocalteu reagent, the total phenolic content in different extracts was quantified. Calibration curve was constructed by using different concentrations of gallic acid. Total phenolic content is represented as milligrams gallic acid equivalent per gram dry extract [14].

Total flavonoids content

By using aluminium chloride colorimetric assay, the total flavonoids content in different extract was quantified. Calibration curve was constructed using different concentrations of catechin. Total flavonoid content is represented as milligrams catechin equivalent per gram dry extract [15].

Total gallotannins content

By using Folin-Ciocalteu method, the total tannins content in different extracts was quantified. Calibration curve was constructed using different concentrations of tannic acid. Total tannin content is represented as milligrams tannic acid equivalent per gram dry extract [16].

Total condensed tannins content

By using vanilin-HCl colorimetric method, the total condensed tannins content in different extracts was quantified. Calibration curve was constructed using different concentrations of catechin. Total condensed tannin content is represented as milligrams catechin equivalent per gram dry extract [16].

Total sugars content

By using anthrone reagent, the total carbohydrate/sugar content in different extracts was quantified. Calibration curve was constructed using different concentrations of D-glucose. Total sugar content was represented as milligrams glucose equivalent per gram dry extract [17].

Antioxidant activity using DPPH

DPPH free radical was used to determine the antioxidant activity of extracts [18]. DPPH solution (2.5 ml, 0.10 mM in methanol) and plant extracts (0.5 ml) of different concentrations were mixed together. A control was prepared by mixing 2.5 ml DPPH solution and 0.5 ml methanol instead of extracts. The resulting solutions were incubated by keeping in the dark room for 30 min. Then the absorbance was recorded at 517 nm against methanol. The percentage of DPPH radical scavenging activity was calculated using the equation I, where Ac represents the absorbance of control and As represents the absorbance of sample. IC_{50}values is defined as the concentration of the sample that scavenges 50% of DPPH radical.

$$\% \text{ of radical scavenging } = \frac{Ac-As}{Ac} \times 100 \text{ ----------[I]}$$

Antimicrobial assay

For antimicrobial assay, one Gram positive bacteria *Staphylococcus aureus*(ATCC 25923) and one Gram negative bacteria *Escherichia coli* (ATCC 25922) were selected. Agar well diffusion method was adopted to determine the antibacterial activity [19]. The extracts were prepared in two different concentrations in 50% DMSO, 100 and 200 mg/ml. 50 µl extract of each concentration was introduced into agar well of 6 mm diameter so each well contain 5 mg and 10 mg extract respectively. Chloramphenicol was used as a positive control and 50 µl of 50% DMSO was used as a negative control. The plats were incubated for 18 hours. After that, growth inhibition of bacterial around the wells in the presence of different extracts, standard antibiotic and 50% DMSO were visualized. The inhibition zones were measured.

GC-MS Analysis

The hexane extract was analyzed by an analytical GC-MS. The instrument used was JEOL AccuTOF GCX Time of Flight Mass-spectrometer. It was fitted with Agilent 7693A type GC injector and a ZB-5MS plus capillary column (28.9 m x 0.25 mm i.d., film thickness 0.25 µm). For GC analysis, the ion chamber temperature was maintained at 250 °C, GC interface temperature and inlet temperature was kept at 300 °C. The initial oven temperature was maintained at 120 °C with a hold time of 30 seconds. During analysis, the temperature was programmed so that the oven temperature was gradually increased to 320 °C at a rate of 25 °C/minute with hold time of 10 minutes. The maximum oven temperature was maintained at 325 °C and kept at final temperature for 5 minutes. The diluted sample was injected at 300 °C in the split less mode. Helium was used as a carrier gas. Flow rate was maintained at 1.2 ml/min. Mass spectrometer was operated in electron impact mode with an ionization energy of 70 eV. Full scan mass spectra were acquired from 25-600 amu. The total run time was 18.5 minutes. The detected compounds in the gas chromatogram were identified by processing the raw GC-MS data with MSAxel software (Version 1.1.6.17127 Copyright 2014-2015 JEOL Ltd.). The obtained MS spectral data were compared with the mass spectral database of the National Institute of Standard and Technology, NIST mass spectral library 2.2. The compounds were identified based on fragmentation patterns.

Results and Discussion

Extractive values in different solvents

Different amounts of extracts were obtained by using solvents of different polarities which is presented in Table 1. 50% aqueous methanol provided the highest amount of extract (8.10 g) while ethyl acetate provided the lowest amount of extract (1.02 g). The yield generally depends on the type of solvents used. On the other hand, extraction time, temperature and physical nature of the sample also play some role [20].

Phytochemical screening

Screening of phytochemicals present in plant extracts provides general information about different classes of bioactive compounds. The results of the phytochemical screening of different extracts of bark of *M. longifolia* are shown in Table 1. Except terpenoids, all the tested phytochemicals were absent in hexane and dichloromethane extracts. In general, non-polar solvents extracts non polar compounds like fatty acids, hydrocarbons and terpenoides. However, in ethyl acetate, methanol and 50% aq. methanol extracts, all the tested phytochemicals like alkaloids, phenolics, flavonoids, gallo and condensed tannins were present. This indicated that the bark extracts are good source of various classes of polar to highly polar secondary metabolites. These compounds are known to exhibit wide range of biological activities.

Table 1. Yield of extracts from 80 g of bark and phytochemical screening of *B. longifolia* extracts

Extracts	Hexane	CH_2Cl_2	EtOAc	MeOH	50% aq. MeOH
Yields in gram	5.76	3.95	1.02	7.53	8.10
Alkaloids	-	-	+	+	+
Terpenoids	+	+	+	+	+
Flavonoids	-	-	+	+	+
Phenolics	-	-	+	+	+
Glycosides	-	-	+	+	+
R.Sugars	-	-	+	+	+
Saponins	-	-	+	+	+
Tannins	-	-	+	+	+
Quinones	-	-	+	+	+

Total phenolic content

Plant phenols are one of the common natural products, found in wide range of plant based foods. They have beneficial effects on human health. They lowered the risks of many diseases of heart, nervous system and many others originated due to oxidative stress [21, 22]. Regression equation of calibration curve ($Y = 0.013x$, $R^2 = 0.999$) was used to calculate the phenolics content in plant extracts. It was written as mg of GAE per gram of dry extract. The TPC values ranged from 64.0 mg GAE/g dry extract in 50% aq. methanol extract to196.0 mg GAE/g dry extract in methanol extract. The results are shown in Table 2. The total phenolic content in flower, fruit, leaf and bark of *M. longifolia* were reported. All the plant parts contained relatively low amounts of phenolics [23, 24, 25] than our sample. The Folin-Ciocalteu method gives a general measurement of phenolic content. It is not specific for all phenolic compounds [26].

Total flavonoid content

Flavonoids are a group of low-molecular-weight polyphenolic compounds found in a wide variety of plant-based foods. They possess diverse biological activities. Regression equation of calibration curve ($Y = 0.004xR^2 = 0.995$) was used to calculate the flavonoids content in plant extracts. It is written as mg catechin equivalent (CE) per gram of dry extract. The total flavonoid content ranged from 220.0 mg CE/g dry extract in 50% aq. methanol extract to 975.0 mg CE/g dry extract in methanol extract. The total flavonoid content of different extracts is shown in Table 2. The total flavonoid content in methanol extract of bark (368.16 mg QE/g extract) has been reported [25] which is lower than our finding. In our case, we have used catechin as the

standard but in the literature, quercetin was used as the standard. It could be the reason for the difference in flavonoid content in two experiments. Again we found that the total flavonoid content is greater than total phenolic content. This is in agreement with the literature data [25].

Table 2. Total phenolic, flavonoid, tannin and sugar content and antioxidant activity

Extracts	EtOAc	MeOH	50% aq. MeOH
Total phenolic content (mg GAE/g dry extract (Mean ± S.D) (n=3)	81.00 ± 6.22	196.00 ± 15.28	64.00 ± 5.68
Total flavonoids content (mg CE/g extract) (Mean ± S.D) (n=3)	490.00±25.72	975.00±13.31	220.00±17.29
Total condensed tannin content (mg CE/g extract) (Mean ± S.D) (n=3)	460.00±15.30	980.00±10.75	198.00±13.31
Total gallotannins content (mg TAE/g extract)(Mean ± S.D)(n=3)	189.00±10.24	145.00±20.40	163.00±21.48
Total sugar content (mg GE/g extract) (Mean ± S.D)(n=2)	66.00±10.70	126.00±8.83	70.00±10.65
IC_{50} µg/ml against DPPH free radical assay(Mean ± S.D)(n=2)	37.17±1.86	18.86±1.07	29.13±1.69

Total condensed tannin content

Condensed tannins are polymers of flavan-3-ol. They are present in many plants as bioactive principle [27, 28]. Regression equation of calibration curve ($Y = 0.0028x+0.035$, $R^2 = 0.962$) was used to calculate the tannins content in plant extracts. It is written as mg catechin equivalent (CE) per gram dry extract. The total condensed tannin content in different extracts ranged from 198.0 mg CE/g dry extract in 50% aq. methanol extract to 980.0 mg CE/g dry extract in methanol extract.The results are shown in the Table 2.The high content of condensed tannin indicated that almost all flavonoids present in methanol extract are condensed tannin. In our previous work, we have estimated tannin content using FC reagent and found that the methanol extract of bark contained 42.2% tannins [10]. Here, we used vanillin reagent assay that is largely specific for condensed tannins. The reaction with vanillin takes place at 6 or 8 position of A ring of any proanthocyanidine. Vanillin condensed with proanthocyanidine in the presence of mineral acid to give a pink colored complex that absorbed at 500 nm [29]. However, the results obtained with colourimetric methods are highly empirical.

Total gallotannin content

Gallo tannins are common in many plant families. They also have wide range of biological properties [30]. Regression equation of calibration curve ($R^2 = 0.998$) was used to calculate the tannins content in plant extracts. It is written as mg tannic acid equivalent (TAE) per gram dry extract. The total gallo tannin content in different extracts ranged from 145.0 mg TAE/g dry extract in methanol extract to 189.0 mg TAE/g dry extract in ethyl acetate extract.The results are shown in the Table 2.

Total carbohydrate/sugar content

Plants are an important source of carbohydrates. In addition to provide calorie, they have many biological functions [31, 32, 33]. So their estimation is vital. A colorimetric assay using anthrone was used to determine the total sugar content in plant extracts. Plant extracts were first hydrolysed with dilute hydrochloric acid. In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. An enol form of anthrone, anthronale then reacts with hydroxymethyl furfural and forms a green coloured product. The absorbance was measured at 630 nm against blank. Regression equation of calibration curve () was used to calculate the total sugar content. It was written as mg glucose equivalent (GE) per gram of dry extract. The total sugar content ranged from 66.0 mg GE/g dry extract in ethyl acetate extract to 126.0 mg GE/g dry extract in methanol extract which are presented in Table 2.

DPPH free radical scavenging activity

Phenolics are powerful antioxidants. They are more potent antioxidants than Vitamin C, E and carotenoids [34, 35]. Antioxidants generally scavenging free radicals and reduce oxidative stress. Oxidative stress is an imbalanced condition which is responsible for development of chronic diseases [36]. The antioxidant activity of plant extracts is determined by using a stable DPPH free radical. In DPPH assay, in the presence of hydrogen donor, DPPH is reduced and violet color of DPPH is changed to pale yellow. In DPPH radical scavenging assay, methanol extracts showed the lowest IC_{50} values (18.86± 1.07 µg/ml) and the ethyl acetate extract showed the highest IC_{50} value (37.17± 1.86 µg/ml). The results are shown in Table 2.The stronger antioxidant activity of the methanol extract could be due to the presence of higher amounts of phenolics/flavonoids/tannins as evidenced by phytochemical analysis (Table 2).The methanol extract of bark collected from Karnataka, India showed IC_{50} value of 12.50 µg/ml[25].

Antibacterial activity

It is well known that phytochemicals are the sources of anti-bacterials drugs. Therefore, testing of plant extracts for antibacterial activity may help to find new antibiotics with new mode of action. This is very crucial step in drug development as the existing antibiotics develop resistance [37]. The different extracts were tested against *S. aureus* and *E. coli* by agar well diffusion method. The results of antibacterial assay revealed that ethyl acetate, methanol and 50% aqueous methanol extracts, which are polyphenol containing extracts, showed antibacterial activity against *S. aureus* and *E. coli.*The results are given in Table 3. In our previous work, we have reported the antibacterial activity of condensed tannin enriched fraction of *M. longifolia* bark extract [10]. It is well known that polyphenols have antibacterial property and it depends on bacterial strain and interactions between polyphenols and bacterial cells surface such as hydrogen bonding of the phenolic compounds to enzymes. This elevates lipophilic character of phenolic compounds and enhances the antimicrobial character [38].

Table 3. Antibacterial activity of different extracts

Extracts	Inhibition zone produced by different extracts in mm (mg/well)									
	Hexane		Dichloromethane		Ethyl acetate		Methanol		50% aq. methanol	
	5 mg	10 mg	5 mg	10 mg	5 mg	10 mg	5 mg	10 mg	5 mg	10 mg
S. aureus	-	-	-	-	17	20	22	23	21	23
E. coli	-	-	-	-	15	16	18	19	19	21

GC-MS analysis of hexane extract

GC-MS technique was used to determine the chemical composition of hexane extract of *M. longifolia*. Altogether, nine peaks were detected in gas chromatogram. However, only six compounds were tentatively identified by mass spectrum. The identified compounds are given in Table 4. Fig 1 shows the gas chromatogram. Fig 2. (upper) shows the mass spectrum of unknown compound eluted between 11.0984-11.1384 min in gas chromatogram. Fig 2 (lower) shows the mass spectrum of β-Amyrone available in NIST library. The fragmentation pattern of unknown compound is similar to the fragmentation pattern of β-Amyrone. In this way, β-Amyrone was tentatively identified.

It was reported that β-Amyrone has anti-inflammatory activity [39] and β-amyrin acetate showed antioxidant, cytotoxicity and anti-inflammatory activities [40, 41]. Lupeol possesses anti-inflammatory, anti-hyperglycemic, anti-dyslipidemic and anti-mutagenic activities [42]. Lupeol acetate showed to slow down the progression of rheumatoid arthritis by inhibiting the activation of macrophages and osteoclastogenesis [43]. Furthermore, lupeol acetate reduced rheumatoidal arthritis symptoms by inhibition of inflammatory cytokine expression. This anti-inflammatory activity is very important for skin regeneration process [44]. The presence of these bioactive compounds in hexane extract supports its traditional use in inflammation process and wound healing.

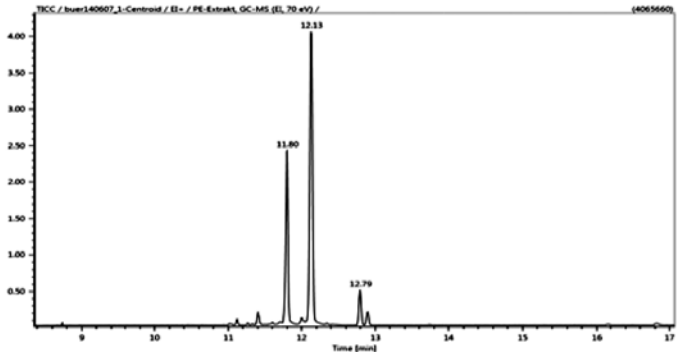

Fig 1. Gas chromatogram of hexane extract of *M. longifolia*

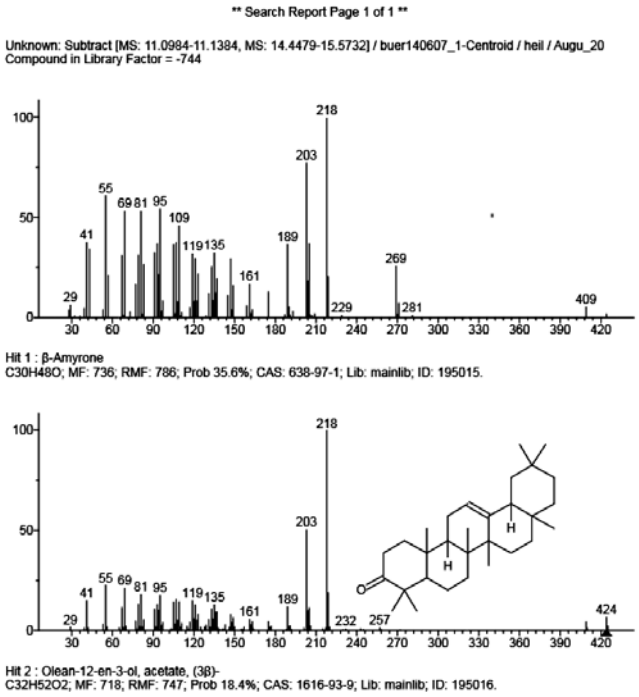

Fig 2. Mass spectrum of unknown compound eluted between 11.0984-11.1384 min (above) and mass spectrum of β-Amyrone (below)

Table 4. Results of GCMS of hexane extract of *M. longifolia*

S. No	RT	Compounds
1	8.7312-8.7478	Squalene
2	11.0984-11.1384	β-Amyrone
3	11.7710-11.8209	Olean-12-en-3-ol, acetate, (3β) (β-amyrin acetate)
4	11.9874-12.0173	Lupeol
5	12.0973-12.1572	Lup-20(29)-en-3-ol, acetate, (3β) (Lupeol acetate)
6	12.7731-12.8131	cis-3,14-Clerodadien-13-ol

Conclusions

The present research highlighted the variation in extraction yield, phenolic, flavonoid, condensed tannin, gallo tannin, sugar content, antioxidant and antimicrobial activities based on solvent polarity during extraction of *M. longifolia.* The methanol

extract is a good source of flavonoids, mainly condensed tannins with high antioxidant activity. Antibacterial compounds are mostly accumulated in medium polar to highly polar extracts. Hexane extract is a good source of anti-inflammatory compounds. Thus, this plant could be the source for obtaining many pharmacologically active secondary metabolites that might be used as natural antioxidants antibacterial and anti-inflammatory agents. These results could justify the traditional use of this plant.

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