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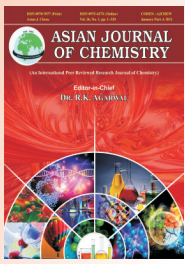

Anticancer and Antioxidant Activity of Ethanolic Extract of *Markhamia lutea* (Benth) K. Schum Stem Bark†

R. NARENDRAN[1,*], A. RAGAMANVITHA[2], K.P. ARUN[1] and P. BRINDHA[1]

[1]Centre for Advanced Research in Indian System of Medicine, SASTRA University, Thanjavur-613 401, India
[2]School of Chemical and Biotechnology, SASTRA University, Thanjavur-613 401, India

*Corresponding author: E-mail: carism.naren@gmail.com

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From time immemorial plants have been used to treat various diseases. Drug discovery from medicinal plants has played an important role in the treatment of cancer and, indeed, new clinical applications of plant secondary metabolites and their derivatives have been attempted towards combating cancer. In the present study a Bignoniaceae member, *Markhamia lutea* (Benth) K. Schum is selected and its chemical as well as anticancer efficacy were studied, with a view to get a lead to develop a safe and efficacious anticancer herbal drug. Ethanolic extract of stem bark of this plant have been prepared and subjected to chemical analysis. Active molecules present in the extract were identified using GCMS and LCMS/MS analysis. Antioxidant activity was assessed using DPPH method. Anticancer activity was evaluated against EAC cells by using MTT assay. The selected plant revealed good antioxidant activity with IC_{50} value of 169.0 µg/mL and anticancer activity with an IC_{50} value of 27.0 µg/mL.

Keywords: *Markhamia lutea*, GC-MS, LC-MSMS, Antioxidant, Anticancer.

INTRODUCTION

From time immemorial plants have been used for treating various ailments. More than 50 % of modern drugs existing in clinical use today are derived from natural products such as plants[1]. Literature review suggest that plants play a vital role in the prevention and treatment of cancer with the advancement in techniques and tools till date. Various anticancer molecules are identified from plants which evince their anticancer effect either by promoting apoptosis or by inhibiting cancer activating enzymes and hormones or by stimulating DNA repair mechanism or by promoting production of protective enzymes or by enhancing antioxidant status and immunity[2]. Such an anticancer molecule developed from a bignoniaceae member is β-lapachone and lapachol extracted from the bark of *Tabebuia impetiginosa* stand[3]. Some of the interesting bioactive compounds reported from this family are Iridoid glycosides, phenylethanoid glycosides and lapachol[4]. This prompted us to select a Bignoniaceae member *Markhamia lutea* (Benth) K. Schum available in and around Bangalore. After proper identification and authentication the ethanolic extract of the stem bark of the selected plant drug was evaluated for its antioxidant and anticancer potentials. Active molecules like terpenoids, flavonoids, glycosides and steroids have been reported from the leaves of this plant[4].

EXPERIMENTAL

The stem bark of *Markhemia lutea* was collected and air dried. The dried plant material was coarsely powdered using pulverizer. About 250 g of powdered stem bark was used for extraction. The defatted plant material was soaked in ethanol for 48 h at room temperature. Ethanol was removed by rotary vacuum evaporator. The extract obtained was collected and subjected to further analysis.

Preliminary phytochemical screening: Preliminary phytochemical screening of ethanolic extract of stem bark was done as per standard testual procedures[5] and the results were presented.

GC-MS analysis: GC-MS analysis of stem bark of *Markhamia lutea* ethanolic extract was carried out on a GC clarus 500 Perkin Elmer system interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-5 m fused silica capillary column with 30 m length and 0.25 µm film thickness composed of 5 % phenyl 95 % dimethyl polysiloxane, helium (99.999 % purity) was used as carrier gas at a constant flow of 1 mL /min and an

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injection volume of 1.0 µL was employed (split ratio of 10:1) with injector temperature 270 ºC; ion-source temperature 150 ºC. The oven temperature was programmed from 50 ºC, with an increase of 8 ºC/min, to 250 ºC hold for 5 min. Mass spectra were taken at 70 eV (electron ionization technique) at a scan interval of 0.2 s and fragments were scanned from 40-600 Da. Interpretation on mass spectrum GC-MS was conducted using the database of National Institute of Standard and Technology (NIST 2005) having more than 62,000 patterns. The spectrum of the separated components was compared with the spectrum of NIST library database. The identity of the spectra above 95 % was needed for the identification of components.

LC MS/MS analysis: Molecular screening of compounds present in the extract was carried out using LC-HR-ESI-MS/MS analysis (UHPLC Dionex C18 RP Acclaim 120 Å, 2.1 mm × 150 mm, 3.0 µm column, USA and MSMS Bruker Q-II TOF). Polar and semi polar compounds were separated and identified at 325 nm, 0.2 mL/min flow rate, gradient mobile system start with 0.2 min at 1 % acetonitrile and 99 % water (1 % acetic acid) to 75 % ACN at 16th min, this was brought to 100 % acetonitrile at 19th min to 5 % acetonitrile at 21st min and was maintained at same condition till run ends at 23rd min. ESI in negative mode using following conditions: Nebulizer 30.5 psi with 6.0 L/min N_2 flow, *m/z* range: 50-1000 *m/z*, Capillary voltage 4500 V, dry heater temperature at 280 ºC were performed to identify the molecules based on their monoisotopic masses.

Free radical scavenging activity

DPPH method: Free radical scavenging activity of ethanolic extract of *Markhamia lutea* stem bark was carried out using 2,2-diphenyl-1-picryl hydrazyl radical (DPPH) assay. 0.1 mM solution of DPPH and 1mg/mL concentration of extract in methanol was prepared. Prepared the sample in various concentrations and added DPPH solution to all the test solutions. An equal amount of DPPH was used as negative control. Butylated hydroxytoluene (BHT) was used as positive control. After adding DPPH to all the tubes, the set up kept in a dark condition at room temperature. Absorption was taken after 20 min at 515 nm. The percentage inhibition was calculated using the following formula.

$$\text{Inhibition } (\%) = 100 - \left(\frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100$$

A linear graph between concentrations *vs.* % inhibition was plotted and IC_{50} value was calculated[6].

Cytotoxicity studies: *In vitro* anticancer activity was carried out for ethanolic Extracts of *Markhamia lutea* against EAC cells (Ehrlich Ascites Carcinoma). EAC cells were grown in RPMI-1640 media (2nM L-glutamine, 10 % heat-inactivated fetal bovine serum (FBS), 1 % penicillin/streptomycin) at 37pC and 5 % CO_2 to reach 80 % confluence. 0.1 × 10^6 EAC cells were treated with different concentration (1000-1.95 µg/mL) of ethanolic extract of selected plant in a 96well plates for 48 h. MTT assay was used to evaluate the cytotoxicity potential of plant drug[7]. Formazan formed was then dissolved using 0.1 % DMSO. The optical density (OD) of each well was measured using Epoch microplate spectrophotometer (BioTek, USA) set at 590 nm filter[7].

RESULTS AND DISCUSSION

Phytochemical screening of ethanolic extract of *Markhamia lutea* stem bark revealed the presence of flavonoids, coumarins, phenols, sugars and terpenes (Table-1).

TABLE-1
PRELIMINARY PHYTOCHEMICAL SCREEING OF ETHANOLIC EXTRACT

Test	Procedure	Ethanolic extract
Saponin	Extract + water	–
Tannins	Extract + lead acetate solution	–
Sugars	Extract + Fehling's A and B solution + heating	+
Coumarins	Extract + 10 % NaOH	+
Alkaloids	Extract+ acetic acid + Dragondroff reagent	–
Quinones	Extract + conc. H_2SO_4	+
Sterols	Extract+ $CHCl_3$ + glacial acetic acid + heat and cool then add conc. H_2SO_4	–
Flavonoids	Extract + 10 % NaOH	+
Phenols	Extract + alcoholic $FeCl_3$	+
Gums	Extract + water	–
Proteins	Extract + picric acid	–
Terpenes	Extract + tin + thionyl chloride	+

Preliminary screening gives the platform to understand the nature of compounds present in the extract. Fig. 1 shows the GCMS spectrum of ethanolic extract. There are 25 compounds identified from the stem bark which are presented in the Table-2. Among these compounds bezofuran 3,5-dihydro, cinnamaldehyde, 4*H*-pyran-4-one-2,3-dihydro-3,5-dihydroxy-6-methyl, 2-furancarboxaldehyde-5-(hydroxymethyl) are known to possess antitumor, antiproliferative and antimicrobial activity[8].

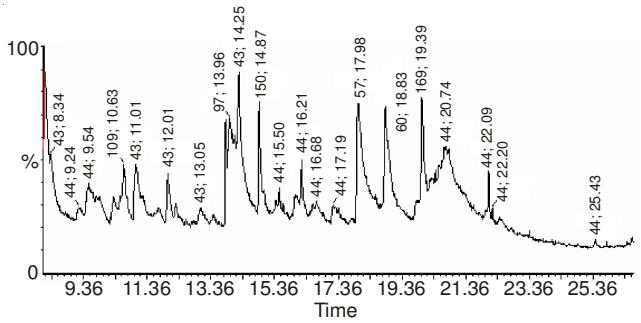

Fig. 1. GC-MS spectrum of ethanolic extract of *M. lutea*

LCMS/MS analysis suggested the presence of some anticancer molecules in the ethanolic extract. UV and MS spectrum of the extract was given in Figs. 2 and 3. Fig. 4 shows MS/MS pattern of the identified molecules. Molecules identified from LCMSMS are well known anticancer molecules such as oleanolic acid (*m/z* 456.7), pomolic acid (*m/z* 472.69), musambin A $[M-H]^-$ (*m/z* 503) and β-sitosterol-3-O-β-D-glucopyranoside (*m/z* 667.82). Among these compounds, pomolic acid is reported to induce apoptosis in HL-60 cells (Human promyelocytic leukemia cells) by activating caspases 3 and 9, it also alters mitochondrial transmembrane energy[9]. Pomolic acid activates the cyclic AMPK, inhibits cell proliferation and induces the apoptosis in MCF7 breast cancer cells[10]. Oleanolic acid inhibits the tumor cell proliferation of human colon cancer cell line

TABLE-2
LIST OF COMPOUNDS IDENTIFIED FROM GC-MS ANALYSIS

Peak name	Retention time	Peak area	Peak area (%)
Name: Ethane, 1,1-diethoxy; m.f.: $C_6H_{14}O_2$ m.w. 118	3.32	1186446	0.7957
Name: Propanoic acid, 2-oxo-, methyl ester; m.f.: $C_4H_6O_3$, m.w.: 102	4.57	1604345	1.0759
Name: 1-Pentanol, 4-methyl; m.f.: $C_6H_{14}O$, m.w.: 102	7.19	1176745	0.7892
Name: Phenol; m.f.: C_6H_6O, m.w.: 94	9.24	2924907	1.9615
Name: Diglycerol; m.f.: $C_6H_{14}O_5$, m.w.: 166	9.54	10589547	7.1017
Name: 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone; m.f.: $C_6H_8O_3$, m.w.: 128	10.31	4783433	3.2079
Name: Mequinol; m.f.: $C_7H_8O_2$; m.w.: 124	10.63	13055027	8.7552
Name: 1-Deoxy-d-altritol; m.f.: $C_6H_{14}O_5$, m.w.: 166	11.71	2576385	1.7278
Name: 2-Amino-octadec-7-ene-1,3-diol butaneboronate; m.f.: $C_{22}H_{44}BNO_2$, m.w.: 365	11.01	7660588	5.1375
Name: 4*H*-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl; m.f.: $C_6H_8O_4$, m.w.: 144	12.01	7687722	5.1557
Name: Cinnamaldehyde, (E); m.f.: C_9H_8O, m.w.: 132	12.26	1703663	1.1425
Name: Benzoic acid, 2-hydroxy-, methyl ester; m.f.: $C_8H_8O_3$, m.w.: 152	12.57	294570	0.1975
Name: 1,4:3,6-Dianhydro-α-d-glucopyranose; m.f.: $C_6H_8O_4$, m.w.: 144	13.45	1234557	0.8279
Name: Benzofuran, 2,3-dihydro; m.f.: C_8H_8O, m.w.: 120	13.83	3753516	2.5172
Name: 2-Furancarboxaldehyde, 5-(hydroxymethyl); m.f.: $C_6H_6O_3$, m.w.: 126	13.96	3850044	2.5820
Name: 2-Methoxy-4-vinylphenol; m.f.: $C_9H_{10}O_2$, m.w.: 150	14.87	8750419	5.8684
Name: Endo-2,3-O-ethylidene-α-d-erythrofuranose; m.f.: $C_6H_{10}O_4$, m.w.: 146	15.63	205054	0.1375
Name: 1,4-Benzenediol, 2-methoxy; m.f.: $C_7H_8O_3$, m.w.: 140	17.19	3441297	2.3079
Name: 1,3;2,5-Dimethylene-l-rhamnitol; m.f.: $C_8H_{14}O_5$, m.w.: 190	17.98	33773640	22.6498
Name: D-Allose; m.f.: $C_6H_{12}O_6$, m.w.: 180	18.83	22093308	14.8166
Name: Benzoic acid, 4-hydroxy-3-methoxy; m.f.: $C_8H_8O_4$, m.w.: 168	19.86	848391	0.5690
Name: Phenol, 3,4,5-trimethoxy; m.f.: $C_9H_{12}O_4$, m.w.: 184	19.99	9842982	6.6011
Name: *n*-Decanoic acid; m.f.: $C_{10}H_{20}O_2$, m.w.: 172	21.98	146605	0.0983
Name: 3-Eicosene, (E); m.f.: $C_{20}H_4O$, m.w.: 280	22.09	2530213	1.6969
Name: 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol; m.f.: $C_{10}H_{12}O_3$, m.w.: 180	22.43	1183675	0.7938

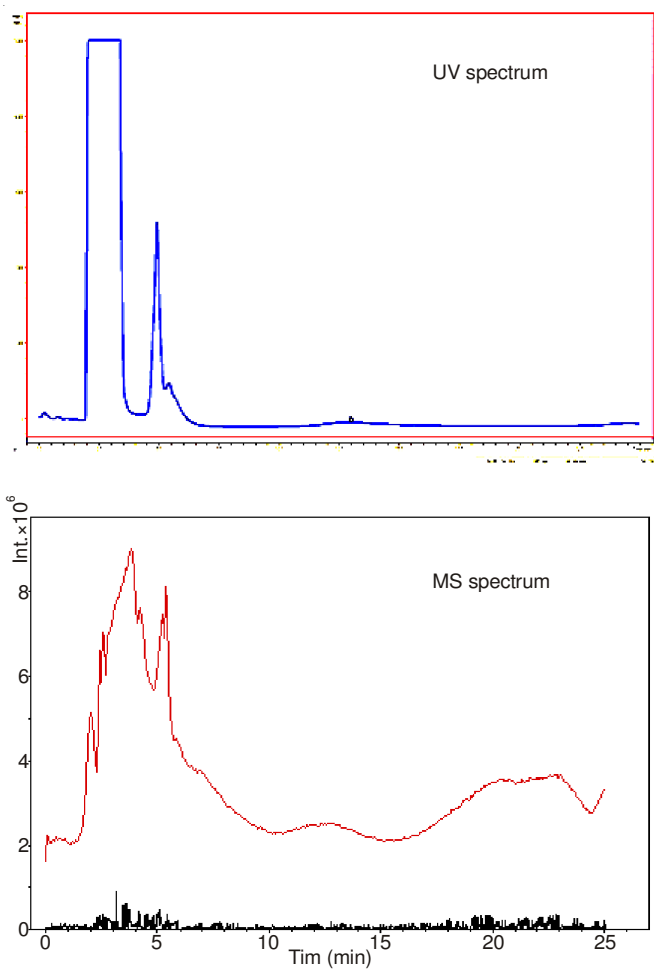

Fig. 2. UV and MS spectrum of ethanolic extract of *markhamia lutea*

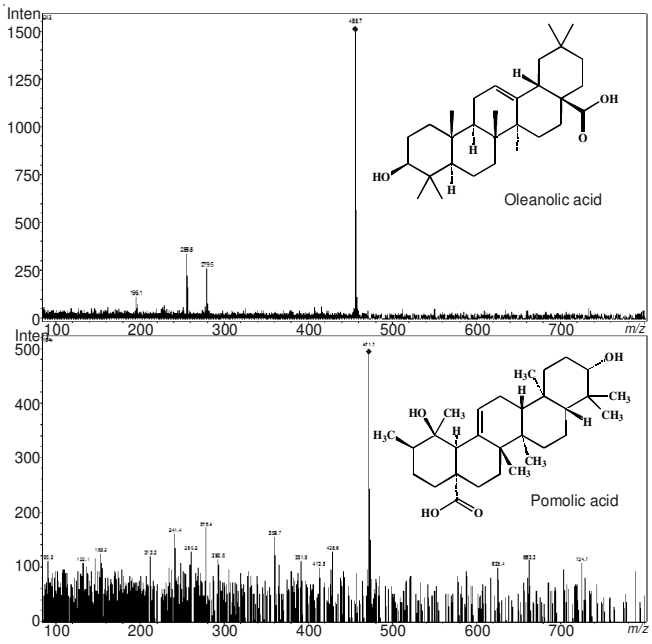

Fig. 3. MSMS pattern of oleanolic and pomolic acids

HCT15 by cell cycle arrest[11]. It also shows good angiogenic activity by strongly inhibiting the embryonic angiogenesis and capillary formation in chick embryo chorioallantoic membranes[12]. A report on anticancer activity of musambin A on human fetal lung fibroblast (MRC-5) cells is also available[4].

Antioxidant activity of ethanolic extract of *Markhamia lutea* stem bark was measured using DPPH assay. Fig. 5 shows the graph between the concentration and % inhibition which suggests an IC_{50} value of 169 µg/mL revealing a good antioxidant activity of the ethanolic extract.

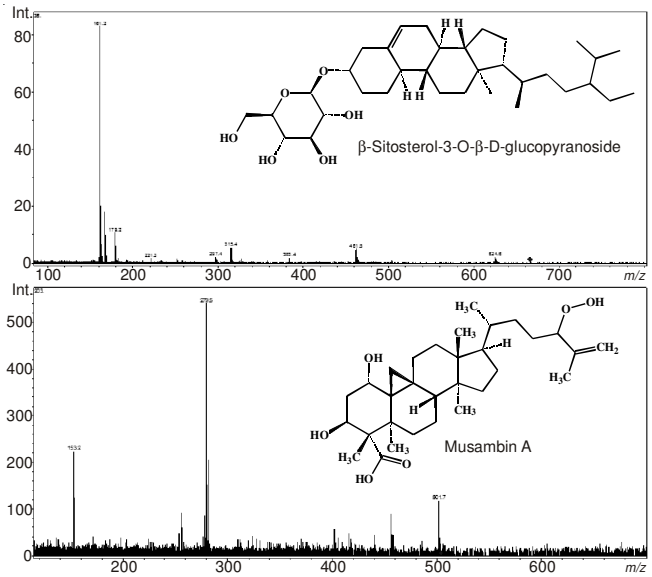

Fig. 4. MSMS pattern of β-sitosterol-β-glycopyranoside and musambin A

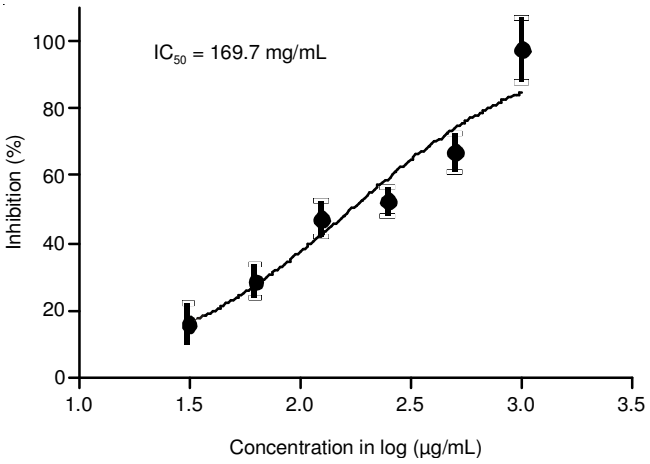

Fig. 5. DPPH assay for Antioxidant activity of ethanolic extract of *Markhamia lutea* stem bark

in vitro Anticancer activity of ethanolic extract against EAC cell lines was performed and the IC_{50} value is 27 µg/mL. Fig. 6 indicates the plot of concentration and % inhibition of the EAC cell growth. A drug with IC_{50} value below 30 µg/mL is suggested as a good anticancer agent.

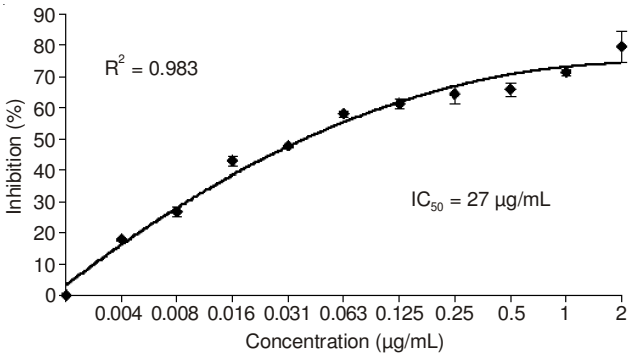

Fig. 6. MTT assay for anticancer activity of ethanolic extract of *Markhamia lutea* stem bark

Conclusion

The data depicted that the ethanolic extract of stem bark of *Markhamia lutea* posses good anticancer and antioxidant activity. This may be probably due to the presence of anticancer molecules with antioxidant potentials such as oleanolic acid, pomolic acid, β-sitosterol-3-O-β-D-glucopyranoside and musambin A present in the stem bark. Taking lead from the present work futher in-depth studies using this extract can be planned which can contribute significantly in the development of a novel herbal anticancer drug based on the Holistic approach.

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