ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 10, Issue 6, 2017

Online - 2455-3891
Print - 0974-2441

Research Article

PHYTOCHEMICAL ANALYSIS, ASSESSMENT OF ANTIPROLIFERATIVE AND FREE RADICAL SCAVENGING ACTIVITY OF *MORUS ALBA* AND *MORUS RUBRA* FRUITS

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Received: 20 February 2017, Revised and Accepted: 15 March 2017

ABSTRACT

Objective: Mulberry is a nontoxic commonly eaten plant, belongs to the *Morus* and used in folk medicine in the remedy of dysentery, antiphlogistic, diuretic, expectorant, and antidiabetic. The purpose of this study is to evaluate the antiproliferative and radical scavenging activity of the total alcoholic and successive fractions thereof of *Morus alba* and *Morus rubra* fruits. In addition, the chemical composition of the bioactive fractions of each species was investigated.

Methods: The antiproliferative potential of 8 extracts on 4 human cancer cell lines, hepatocellular carcinoma (HepG2), Caucasian breast adenocarcinoma (MCF7), prostate (PC3), and colon carcinoma (HCT116) in addition to one normal cell line namely human normal immortalized skin fibroblast cells (BJ1) were carried out. Cell viability was determined using MTT assay. The potency was compared with the reference drug doxorubicin. These extracts were also assayed for 1,1-diphenyl-2-hydrazyl free radical scavenging activities. After saponification of the n-hexane fraction, unsaponifiable matter and fatty acid methyl esters were analyzed by gas liquid chromatography (GLC). The chemical composition of the bioactive fractions was investigated using gas chromatography/mass spectrometry (GC/MS) analysis.

Results: All the extracts showed significant free radical scavenging activity dose-dependently. The *n*-hexane and dichloromethane (DCM) fractions of *M. rubra* exhibited potent cytotoxic activity on almost cancer cell lines. In the same pattern, ethyl acetate (EtOAc) of *M. rubra* has moderate cytotoxic activity against all cell lines except HepG2. DCM fraction of *M. alba* possessed both radical scavenging and high potential antiproliferated activities against HCT116 and MCF7 with inhibitory concentration of 43.9 and 32.3 µg/ml, respectively, while it showed no cytotoxic effect on BJ1. GLC analysis showed the major hydrocarbons in *M. alba* and *M. rubra* were heptacosane and docosane, respectively. Sterols were similar in both species but with different ratios and cholesterol was the major one. Palmitic and margaric were the major saturated fatty acid while arachidonic was the major unsaturated fatty acid in both species. GC/MS analysis showed the main compound in DCM fraction of each *Morus* species was palmitic acid. Furthermore, 1,11-*bis*-(methoxycarbonyl-ethenyl)-10,2-dihydroxy-cycloeicosane and linolelaidic acid, methyl ester were the main compounds in the EtOAc fraction of each *Morus* species. Whereas, the main compounds in alcoholic extract of *M. alba* and *M. rubra* were methyl-14-methyl-pentadecanoate and 1,2-*O*-isopropylyidene-4-nonene-1,2,3-triol, respectively.

Conclusions: The results observed remarkable biological activity of the successive fractions of *M. rubra* more than those of *M. alba* and confirmed its importance as a natural bioactive source. *Morus* species are good candidates to be promising as possible sources for future antitumor and antioxidants in food and pharmaceutical formulations. The strong activity partly explains the potential effects of *Morus* species for the treatment of cancer and degenerative diseases caused by free radicals.

Keywords: *Morus alba*, *Morus rubra*, Radical scavenging, Antiproliferated activity, BJ1, Gas liquid chromatography, Gas chromatography/mass spectrometry.

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INTRODUCTION

Cancer is the leading cause of mortality worldwide and its incidence is still increasing especially due to diet, environment and carcinogenic virus infections [1]. Extensive damage to DNA, protein, and lipid could be related to the free radicals react with biomolecules, which are considered to be contributed to degenerative diseases of aging and cancer. Cancer, one major cause of death, is salient "free radical" disease. Antioxidant therapy is vital in scavenging free radicals and plays an important role in the later stages of cancer development [2,3]. Consumption of dietary and plant-derived antioxidant appears to be a suitable alternative preventing the formation of free radicals [4].

Resistance to several anticancer drugs has stimulated efforts to search for natural effective, nontoxic agents without adverse effects [5,6]. Many natural products have studied to find the new potent source of bioactive antitumor molecules [3,7]. The worldwide use of medicinal plants has become more and more important in primary health care especially in developing countries. Different extracts of natural products were found to possess antiproliferative effects in cancer. Moraceae plants showed a wide range of bioactive features including anti-inflammatory, antioxidative, and many useful antitumor agents developed from this family [7,8]. Mulberry is a fast-growing deciduous, nutritious, palatable, and nontoxic plant belongs to the *Morus*, a plant genus of the family Moraceae. The fruits may be eaten raw or cooked, and the herb has been used as a remedy for dysentery, antiphlogistic, anthelmintic, diuretic, expectorant, and antidiabetic in traditional medicine [2,8]. Few species of mulberry were evaluated for their edible fruits and reports indicate that mulberry fruits are a good source of micronutrients, stilbenes, polyhydroxylated alkaloids, flavonoids, and benzofurans [5,9]. These compounds have shown a wide range of bioactive features including hypoglycemic, neuroprotective functions, anti-inflammatory, and antioxidative effects [9-11].

The objective of the current exposition was to investigate the antiproliferative and radical scavenging effect of two *Morus* species involving purple mulberry (*Morus rubra*) and white mulberry (*Morus alba*). This work describes the identification of phytoconstituents of the total ethanol extract, dichloromethane (DCM) and ethyl acetate (EtOAc) fractions using gas chromatography/mass spectrometry (GC/MS) analysis for the first time along with the hydrocarbons, sterols and fatty acids of the *n*-hexane fraction (Hx) by gas liquid chromatography (GLC) analysis.

METHODS

Collection of plant material

Fresh fruits of white *M. alba* and purple *M. rubra* were collected in the Delta region, Egypt. The berries were selected according to uniformity of shape and color. The identification of the plant was confirmed by Therese Labib, Herbarium Section, El-Orman Botanical Garden, Giza, Egypt. The fresh fruit samples were cleaned, stored in polyethylene bags and frozen at −20°C, till further use.

Preparation of extracts for bioassays

The fresh fruits of each species (200 g) were extracted separately with 700 ml of 70% aqueous ethanol (EtOH) for 3 hrs, on an orbital shaker in the dark at room temperature. Each extract was separated by centrifugation (13,000 ×g, 10 minutes), the supernatant was taken, the residue was re-suspended in 50 ml of the same solvent, and the mixture was again separated by centrifugation. The two resulting supernatants were then combined and concentrated under reduced pressure at 40°C till dryness to get 2.63% and 2.50% of crude EtOH extract of *M. alba* and *M. rubra*, respectively. The residue was suspended in the least amount of water and transferred to a separator funnel, then successively fractionated with solvents of increasing polarity, Hx, DCM and EtOAc. Their residues were stored in the dark at −20°C. For antitumor bioassay, the residue of each extract was re-dissolved in 1 ml of dimethyl sulfoxide at a concentration of 100 mg/ml and diluted with tissue culture medium before use.

Cell viability assay

Cell viability for four cancer cell lines, namely, human hepatocellular carcinoma (HepG2), MCF7, HCT116 and PC3 beside one normal cell line BJ-1, was assessed by the mitochondrial-dependent reduction of the yellow MTT assay to purple formazan [12]. All the following procedures were done in a sterile area using a laminar flow cabinet biosafety Class II level (Baker, SG403INT, Sanford, ME, USA). Cells were suspended in Roswell Park Memorial Institute 1640 medium for HepG2, MCF7, PC3, and HCT116. BJ-1 was maintained in Dulbecco's modified Eagle Medium (DMEM)-F12. All media was supplemented with 1% antibiotic-antimycotic mixture (10,000 µg/ml potassium penicillin, 10,000 µg/ml streptomycin sulphate and 25 µg/ml amphotericin B) and 1% L-glutamine at 37°C under 5% CO_2. Cells were batch cultured for 10 days and then seeded at a concentration of 10×10^3 cells per well in fresh complete growth medium in 96-well microtiter plastic plates at 37°C for 24 hrs under 5% CO_2 using a water-jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). The media were aspirated, fresh medium (without serum) was added, and cells were incubated either alone (negative control) or with different concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 µg/ml) to calculate the inhibitory concentration (IC_{50}) and IC_{90} of the promising extract/fraction. After 48 hrs of incubation, the medium was aspirated, 40 µl MTT salt (2.5 µg/ml) was added to each well and incubated for further 4 hrs at 37°C under 5% CO_2. To stop the reaction and to dissolve the formed crystals, 200 µl of 10% sodium dodecyl sulfate in deionized water was added to each well and incubated overnight at 37°C [13]. Doxorubicin was served as positive control. The absorbance was then measured using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595 nm and a reference wavelength of 620 nm. The percentage of change in viability was calculated according to the formula: ([Reading of extract/reading of negative control] - 1) ×100. All cell lines were generously provided by Professor Stig Linder, Oncology and Pathology Department, Karolinska Institute, Stockholm, Sweden. A probit analysis was performed for IC_{50} and IC_{90} determination using SPSS 11 program.

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The ability of different extracts to scavenge DPPH free radical were determined according to Goveas and Abraham method [14]. DPPH and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethanolic solution of DPPH (0.1 mM) was prepared, to give the initial absorbance value of 0.993 at 517 nm. The different concentration of samples in 0.1 ml of each sample (with appropriate dilution if necessary) was added to 3.0 ml of ethanolic DPPH solution. After incubation for 30 minutes in the dark, the absorbance was measured at 517 nm using ultraviolet/visible spectrophotometer (Lambda 25, PerkinElmer, USA). Ascorbic acid was used as standard. Control was prepared by mixing 2 ml of 0.1 mM DPPH solution with 1 ml of methanol. Statistical analysis is carried out using SPSS computer program (version 8), where unshared letters are significant at $P < 0.05$. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The percentage of scavenging was calculated using the following formula:

$$\text{Scavenging \%} = [1 - (A_{sample} - A_{blank}/A_{control})] \times 100\%.$$

Chemical characterization

Preparation of the lipoidal matter

Each fresh sample (10 g) was exhaustively extracted with Hx for 6 hrs using Soxhlet extractor apparatus (Sigma-Aldrich).

Investigation of the lipoidal matter

Determination of phytosterol and hydrocarbon contents

Each Hx was separately saponified [15] by refluxing with 50 ml alcoholic potassium hydroxide (10%) in a water bath for 2 hrs. After cooling, 50 ml of water was added, and the solution was extracted with chloroform. The organic phase was washed with water until it became alkali free and was then dried over anhydrous sodium sulfate. The solvent was evaporated to afford the unsaponifiable matters and the fatty acids.

Preparation of fatty acid methyl esters

The isolated free fatty acids were subjected to methylation by refluxing with absolute methanol and sulphuric acid for 2 hrs, extracted with ether, evaporated and analyzed by GLC [16].

Gas liquid chromatographic (GLC) analysis

Both the unsaponifiable and the saponifiable fractions of each *Morus* species were studied to identify their contents using GLC analysis. GLC conditions of the unsaponifiable matter and fatty acids were performed on Agilent Technologies (6890N)–Network-GC system equipped by a flame ionization detector. Nitrogen gas was used as a carrier gas. The analysis of the unsaponifiable matter was performed using capillary column (HP-5 phenyl methyl siloxane) and oven temperature at 80°C/8 minutes from 80 to 350°C. The operating conditions for fatty acid methyl ester analysis were capillary column HP-5% 5-phenyl methyl siloxane (30 m × 320 um × 0.25 um); column maximum temperature was 325°C. Detector and injection temperature was 250°C. Oven temperature was from 50 to 350°C with a rate 50°C/10 minutes.

GC/MS analysis

GC/MS analysis was performed using a Thermo Scientific, Trace GC ultra/ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30 m, 0.251 mm, 0.1 mm film thickness). An electron ionization system with ionization energy of 70 eV was used. Carrier gas was helium at a constant flow rate of 1 ml/minute. The injector and MS transfer line temperature was 280°C. The initial temperature oven was programed at 40°C (hold 3 minutes) to 280°C as a final temperature at an increasing rate of 5°C/minutes (hold 5 minutes). The constituents were identified by comparison of their mass spectral fragmentation patterns with

those of the available database libraries, Wiley (Wiley International, Colorado, USA) and National Institute of Standards and Technology, Colorado, USA, and/or published data [17].

RESULTS

Antiproliferative and safety to normal cells activities

In vitro cytotoxicity test was carried out using four human cancer cell lines and one normal cell line were used to assess the antiproliferation activity of eight fractions of the two *Morus* species. The data in Table 1 showed that 5 extracts with high cytotoxic activity (≥75%) against at least one cancer cell line. The results indicated that DCM of *M. alba* has potent activity against HCT116 and MCF7 with IC_{50} 32.3 µg/ml and 43.9 µg/ml, respectively. EtOAc fraction of *M. alba* with IC_{50} 56.5 µg/ml for HCT116 and IC_{50} 54.6 µg/ml for MCF7. The best results were found for DCM fraction of *M. alba* that has not any activity against BJ1 (normal cell line) at 100 µg/ml, while EtOAc fraction of *M. alba* has moderate activity on the same cell line (50% at 100 µg/ml). DCM fraction of *M. alba* has the same effect of doxorubicin on HCT116 but more safe where has not any effect on normal cell line.

DPPH scavenging activity

From the Table 2, DPPH scavenging activity it can be declared that EtOH exhibited potent antioxidant activity that increased with increasing amount of extract concentration, which was compared with standard ascorbic acid at different concentrations. The EtOAc, DCM and finally Hx of purple berry showed appropriate activities followed by the white berry in a dose-dependent manner (dose-dependent relationship).

Chemical characterization and phytochemical analysis

Extraction yield

The results showed that the extraction yields obtained was affected by the solvent used. Table 3 shows the extraction percent yields (w/w) which obtained in the following descending order; EtOH>EtOAc>DCM>Hx in each *Morus* species extracts.

GLC of lipoidal matter of *M. alba* and *M. rubra*

The Hx fraction was concentrated *in vacuo* to yield 130.5 and 110.0 mg of oily dry residue, representing 1.30% and 1.10% of the fresh fruit of *M. alba* and *M. rubra*, respectively. Percentages of the unsaponifiable matter were found to be 0.80% and 0.56%, while the total fatty acids were 27.3% and 23.5% of *M. alba* and *M. rubra*, respectively.

GLC analysis of the unsaponifiable fractions of white *M. alba* and purple *M. rubra* revealed the presence of 21 and 20 compounds in Hx fraction, respectively (Table 4). Hydrocarbon compounds were the major content (71.93% and 66.96%, respectively) in which heptacosane (13.46%) was the major one followed by the presence of octacosane (8.94%) in *M. alba* unsaponifiable fraction, whereas docosane (10.58%) was the major hydrocarbon followed by tricosane (7.67%) in *M. rubra* unsaponifiable fraction. Hx fraction of *M. alba* showed the presence of pentadecane which did not present in *M. rubra*.

Four sterols (Fig. 1) were found in both *M. alba* and *M. rubra* (28.07% and 33.04%, respectively). Sterols were similar in both species unsaponifiable fractions but with different ratios. Cholesterol was the major one in both (14.54% and 11.96%) whereas β-sitosterol (2.45% and 7.23%) and campesterol (5.91% and 6.76%) were present in appropriate amount in both *M. alba* and *M. rubra* unsaponifiable fraction, respectively. Furthermore, stigmasterol was present with ratios 5.17% and 7.09%, respectively.

GLC analysis of fatty acid methyl esters fraction revealed the presence of 16 and 17 compounds; representing 99.50% and 99.0% of the total identified compounds *M. alba* and *M. rubra,* respectively (Table 5). 9 and 10 saturated fatty acids represented 50.81% and 47.84% of the total fatty acids content, while 7 of mono-, di-, tri-, and tetra-unsaturated fatty acids represented 48.69% and 51.16% of *M. alba* and *M. rubra,* respectively. Palmitic acid (19.54%) was the major saturated fatty acids

Table 1: Cytotoxicity of ethanol extract and successive fractions (100 µg/ml) of *M. alba* and *M. rubra* on four human tumor and normal cell lines

Extract	Cell lines													
	HCT116			PC3			HePG 2			MCF7			BJ1		
	LC_{50}	LC_{90}	Cytotoxicity (%)	LC_{50}	LC_{90}	Cytotoxicity (%)	LC_{50}	LC_{90}	Cytotoxicity (%)	LC_{50}	LC_{90}	Cytotoxicity (%)	LC_{50}	LC_{90}	Cytotoxicity (%)
EtOH of *M. alba*	-	-	19.2	-	-	21.5	-	-	22.8	-	-	15.6	-	-	15.2
EtOH of *M. rubra*	-	-	3.8	-	-	6.5	-	-	2.5	-	-	10.3	-	-	14.6
Hx of *M. alba*	43.9	70.9	100	-	-	27.1	-	-	11.3	74.3	115.1	75.3	57.2	96.2	86.3
Hx of *M. rubra*	9.6	17.6	100	94.4	144.3	51.5	-	-	3.5	23.6	37.6	100	7.4	15.6	100
DCM of *M. alba*	32.3	58.6	100	-	-	0	-	-	9.5	43.9	77.4	94.4	-	-	0
DCM of *M. rubra*	17.6	33.0	100	72.3	113.1	76.5	70.4	109.8	79.2	27.9	46.1	100	18.5	30.7	100
EtOAc of *M. alba*	56.5	100.8	79.1	-	-	39.6	-	-	0	54.6	106.3	77.3	-	-	47.3
EtOAc of *M. rubra*	-	-	47.3	82.9	128.6	64.2	-	-	2.3	-	-	53.2	43.2	71.1	97.6
Doxorubicin	37.6	65.1	100.0	23.8	40.1	100.0	21.6	37.8	100.0	26.1	48.9	100	31.6	54.9	100.0
DMSO	-	-	1	-	-	1	-	-	1	-	-	3	-	-	1
Negative control	-	-	0	-	-	0	-	-	-	-	-	0	-	-	0

M. alba: Morus alba, M. rubra: Morus rubra, HCT116: Colon cell line, PC3: Prostate cell line, HepG2: Human hepatocellular carcinoma cell line, MCF7: Human Caucasian breast adenocarcinoma, BJ1: Normal skin fibroblast, EtOH: Total ethanol extract, Hx: N-hexane fraction, DCM: Dichloromethane fraction, EtOAc: Ethyl acetate fraction, LC_{50}: Lethal concentration of the sample which causes the death of 50% of cells in 48 hrs (µg/ml), LC_{90}: Lethal concentration of the sample which causes the death of 90% of cells in 48 hrs (µg/ml), Remarks (%) at 100 ppm

Table 2: DPPH scavenging activity assay of *M. alba* and *M. rubra*

Extract/fraction	*Morus* species	Concentrations (µg)	Scavenging percentage	
			Ascorbic acid	DPPH
EtOH	*M. alba*	50	68.80±0.87^{i}	33.90±1.17^{ef}
		100	80.27±12.75^{g}	35.79±2.09^{j}
		150	85.13±4.54^{ef}	66.6±4.34^{d}
	M. rubra	50	75.22±4.00 ^{cd}	34.13±2.16^{ef}
		100	78.17±0.46^{bc}	54.34±3.47^{gh}
		150	80.03±4.56^{b}	69.88±3.11^{c}
EtOAc	*M. alba*	50	73.56±3.10^{de}	30.01±2. 07^{ef}
		100	76.45±0.12^{cd}	30.48±1.22^{ef}
		150	80.44±2.16^{b}	37.30±3.18^{j}
	M. rubra	50	69.80±1.26^{fg}	32.00±2.37^{ef}
		100	72.44±2.05^{efg}	34.06±1.23^{j}
		150	83.49±3.81^{dg}	46.43±1.85^{k}
DCM	*M. alba*	50	75.34±2.29^{fg}	25.10±0.37^{l}
		100	82.98±3.05^{dg}	31.22±0.87^{ef}
		150	87.49±3.81^{ef}	40.11±2.22^{cd}
	M. rubra	50	75.45±2.16^{fg}	27.11±1.22^{l}
		100	80.18±3.35^{dg}	33.23±2. 12^{ef}
		150	83.56±2.71^{dg}	45.00±1.85^{k}
Hx	*M. alba*	50	77.76±2.16^{fg}	20.00±1.00^{m}
		100	82.18±4.56^{dg}	31.46±1.21^{ef}
		150	87.22±3.34^{ef}	35.43±1.85^{j}
	M. rubra	50	74.11±2.66^{fg}	22.11±1.52^{m}
		100	82.00±3.05^{dg}	33.46±0. 87^{ef}
		150	87.49±4.00^{ef}	38.43±1.85^{cd}

M. alba: Morus alba, M. rubra: Morus rubra, EtOH: Total ethanol extract, Hx: N-hexane fraction, DCM: Dichloromethane fraction, EtOAc: Ethyl acetate fraction. Unshared letters between groups (b-m) are the significance values at $P<0.05$

Table 3: Percent yields (w/w) of extract/fractions of *M. alba* and *M. rubra*

Solvent	%	
	M. alba	***M. rubra***
EtOH	2.63	2.50
Hx	0.31	0.20
DCM	0.63	0.43
EtOAc	0.82	0.61

M. alba: Morus alba, M. rubra: Morus rubra, EtOH: Total ethanol extract, Hx: N-hexane fraction, DCM: Dichloromethane fraction, EtOAc: Ethyl acetate fraction

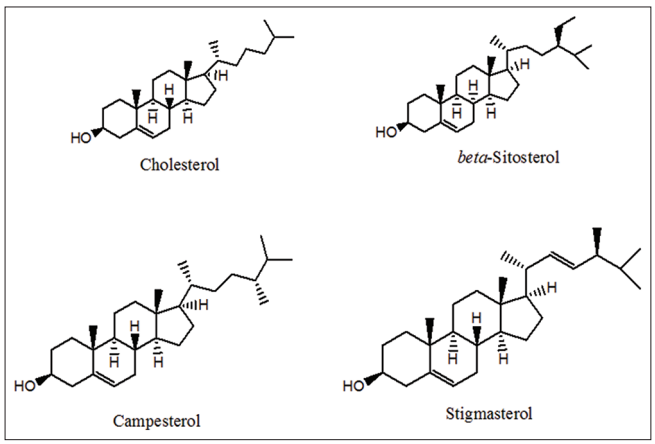

Fig. 1: Sterol compounds identified in Hx fractions of *Morus rubra* and *Morus alba*

in *M. alba,* but margaric acid (14.01%) was the major one in *M. rubra* saponified fraction. Arachidonic acid was the major unsaturated fatty acids presented in both *M. alba* and *M. rubra* (10.69% and 12.69%, respectively) and the lowest one in both fractions was myristoleic acid (0.85% and 0.86%, respectively).

GC/MS analysis of total ethanol extracts and successive fractions

This study gives a detailed analysis for the first time to the composition of the total EtOH, Hx, DCM and EtOAc of purple *M. rubra* fruits in comparing with those of white *M. alba* through GC/MS analysis. The structures of the identified compounds I-XXIX in the DCM and EtOAc fractions by GC/MS were illustrated in Figs. 2 and 3. A total of 29 compounds were identified in *M. alba* and *M. rubra* fruits.

GC-MS analysis of the DCM fractions of *M. alba* and *M. rubra* showed the presence of 12 and 9 compounds, respectively. Palmitic acid, methyl ester, VI (Fig. 2) was the major compound in both DCM fractions of *M. alba* and *M. rubra* (66.55% and 48.51%) which followed by linolelaidic acid, methyl ester, XI (22.02%) in *M. alba* and methyl-9-cis,11-trans-octadecadienoate, X (32.00%) in *M. rubra* (Table 6 and Fig. 2). The analysis revealed that the identified components of DCM fraction of each of *M. alba* and *M. rubra* consist of 99.77% and 99.87% oxygenated compounds; whereas, the unoxygenated compounds were 0.23% and 0.13%, respectively. Esters were the major content in both fractions (96.32% and 98.81%, respectively).

GC-MS analysis of the EtOAc fractions of *M. alba* and *M. rubra* showed the presence of 12 and 14 compounds, respectively (Table 7). 1,11-bis-(Methoxycarbonyl-ethenyl) 10,2-dihydroxycycloeicosane, XXVIII (67.96%), palmitic acid, methyl ester VI (10.46%), and 1-benzyloxy-2,4-dimethyl-5-hexen-3-ol, XVI (6.25%) were the major compounds in the EtOAc fraction of *M. alba,* whereas linolelaidic acid, methyl ester, XI (70.00%), palmitic acid, methyl ester, VI (12.15%) and dioctyladipate, XXVII (4.10%) are the main compounds in the EtOAc fraction of *M. rubra* (Table 7). The analysis of EtOAc fraction of each of *M. alba* and *M. rubra* revealed the presence of oxygenated compounds 99.94% and 95.66%, respectively. Whereas, the unoxygenated compounds represented 0.06% and 4.43%, respectively. It was noticed that esters represented 25.58% and 95.53%, respectively. Pregnane-3,20-diol, XXIX was found only in *M. rubra* with a total percent 2.08%. The corresponding compounds with their retention times, concentrations (%) and their nature are shown in Table 7 (Figs. 2 and 3).

GC/MS analysis of total alcoholic EtOH extract of *M. alba* and M. rubra fruits were carried out, and the structures of the identified compounds

Table 4: GLC analysis of the unsaponifiable fractions of *M. alba* and *M. rubra*

RT	Compound name	Compound concentration (%)	
		M. alba	*M. rubra*
11.13	Pentadecane	0.51	-
12.25	Hexadecane	1.07	1.14
13.63	Heptadecane	0.53	0.59
14.85	Octadecane	1.91	2.98
16.52	Nonadecane	1.11	2.51
16.99	Eicosane	2.40	2.51
17.30	Heneicosane	2.93	2.05
19.55	Docosane	5.01	10.58
20.38	Tricosane	2.17	7.67
21.64	Tetracosane	5.62	5.96
22.66	Pentacosane	1.78	7.37
23.57	Hexacosane	8.33	7.35
24.52	Heptacosane	13.46	1.22
25.36	Octacosane	8.94	6.94
26.24	Nonacosane	8.30	5.70
27.04	Triacontane	7.86	2.39
28.61	Cholesterol	14.54	11.96
29.45	Campesterol	5.91	6.76
30.39	Stigmasterol	5.17	7.09
31.22	β-Sitosterol	2.45	7.23

M. alba: Morus alba, M. rubra: Morus rubra, RT: Retention time in minutes, GLC: Gas liquid chromatography

Table 5: GLC analysis of the fatty acid fractions of *M. alba* and *M. rubra*

RT	Compound name	Compound concentration (%)	
		M. alba	*M. rubra*
19.26	Tridecanoic acid (13:0)	1.34	0.90
21.30	Myristic acid (14:0)	1.73	1.14
21.94	Myristoleic acid (14:1)	0.85	0.86
23.77	Pentadecanoic acid (15:0)	3.38	2.82
29.02	Palmitic acid (16:0)	19.54	10.56
30.58	Margaric acid (17:0)	15.87	14.01
31.32	Palmitoleic acid (16:1)	4.81	6.22
34.24	Stearic acid (18:0)	4.28	2.54
35.26	Oleic acid (18:1)	5.48	3.41
36.61	Linoleic acid (18:2)	9.77	8.06
37.52	Linolenic acid (18:3)	6.54	12.45
42.53	Arachidic acid (20:0)	0.79	1.09
43.55	Arachidonic acid (20:4)	10.69	12.69
45.84	Behenic acid (22:0)	1.45	3.90
46.66	*cis*-13-Docosenoic acid (22:1)	10.55	7.47
48.82	Tricosanoic acid (23:0)	2.43	9.26
50.49	Tetracosanoic acid (24:0)	-	1.62

M. alba: Morus alba, M. rubra: Morus rubra, RT: Retention time in minutes, GLC: Gas liquid chromatography

(XXX-XLVII) were illustrated in Figs. 4 and 5. A total of 18 compounds were identified through GC/MS analysis of EtOH extract of *M. alba* with a total percentage 95.00% and 10 compounds with 97.11% in *M. rubra* fruits (Table 8).

The analysis revealed that the identified components of the total ethanol extract of each of the *M. alba* and *M. rubra* consist of 89.42% and 97.00% oxygenated compounds; in addition, the unoxygenated compounds were 5.58% and 0.11%, respectively. Hydroxylated compounds, ketones, esters and miscellaneous compounds represented 30.34%, 3.47%, 42.98% and 18.21%, respectively, in *M. alba* and represented 94.96%, 0.04%, 1.40% and 0.71%, respectively, in *M. rubra*. Methyl-14-methyl-pentadecanoate, XLIII (Fig. 5) and 1,2-O-isopropylyidene-4-nonene-1,2,3-triol, XXXV (Fig. 4) were the major compounds in each extracts (25.86% and 84.94%, respectively).

phenol,4,6-*bis* (1,1-dimethylethyl)-2-methyl, I (Fig. 2), cyclomulberrin XXXVI (Fig. 4) and nonacosane, XXIII (Fig. 3) were found in EtOH extract of *M. alba* with more ratios (7.60%, 1.59% and 3.84%, respectively than in *M. rubra* 9.00, 1.02 and 0.11, respectively).

Palmitic acid methyl ester, VI (Fig. 2) was found only in EtOH extract of *M. rubra* with a ratio 0.66%. Eaters were the major content in total ethanol extract of *m. alba* (30.34%), whereas the hydroxylated compounds represented the major content in *M. rubra* (94.96%).

DISCUSSION

Various plants have been found to inhibit tumor growth and cause a phenotype reversion in certain cancers. Recently, natural plants have received much attention as sources of biological active substances including antioxidants, antimutagens, and anticarcinogens [18]. The fruits are fast-growing known as Tuta in Sanskrit and Tuti in Marathi and it is also known as Tut in Egypt [19]. *Morus* comprises 10-16 species of deciduous trees commonly known as mulberries, growing wild and under cultivation in many temperate world regions. Local people in these regions traditionally believe that deep-colored fruits, especially black and purple mulberry fruits, are healthier for the human body [20].

Morus species are well known to contain natural colorant anthocyanins. Anthocyanins, such as cyanidin-3-glucoside and cyanidin-3-rutinoside, were reported that they may have antioxidant capacities and decrease the invasiveness of cancer cells [21]. These compounds exhibited an inhibitory effect on the migration and invasion of a human lung cancer cell line.

The antitumor activity testing was performed as follows: A modified MTT assay was used to examine the antiproliferative activity of the 8 extracts of the two investigated *Morus* species against 4 human cancer cell lines, PC3, HCT-116, HepG2, and MCF-7.

This study showed antiproliferative and radical scavenging activity of organic solvent fractions (Hx, DCM, and EtOAc) as well as the total ethanol extract EtOH. It was reported that organic extracts may induce higher antiproliferative activity than the water extract in HepG2 cells [19]. This activity may be related to a depression in the levels of AFP, a carcinoembryonic protein and a very important marker of primary HepG2, in HepG2 cells treated with organic solvent extracts. Moreover, the antiproliferative and free radical scavenging potential of *M. rubra* fruits were established here for the first time.

The data in Table 1 showed five plant extracts with high cytotoxic activity (≥75%) against at least one cancer cell line. Bioactive phytoconstituents as flavonoids, flavones, flavanones, alkaloids, benzophenones, coumarin derivatives, stilbenes, and terpenoids were present in *M. alba* and *M. rubra* [4,20] and may be responsible about the antiproliferaive and radical-scavenging activities [8,21,22]. These components are known for their ability to improve human health or decrease the effect of disease [20,23].

The cytotoxic activity assay against almost all cancer cell lines, in this study, showed that the most potent antiproliferative extract was reported for the organic extracts of mulberry (n-hexane from both species as well as the DCM fraction from *M. rubra*). They showed inhibition of HepG2 hepatoma cells and this activity may be through G2/M phase arrest, induction of apoptosis and inhibition of topoisomerase IIα activity [24]. Furthermore, these results were in agreement with the previous work on leukemia cells, declared the plant leaf extract was able to inhibit HL-60 human leukemia cells and B16 mouse melanoma cells [8]. In the current study, the purple mulberry showed higher activity. Deep-colored fruits were reported as a good source of phenolics, including flavonoids, anthocyanins and carotenoids, and mulberries are rich in phenolics [25].

Free radical-scavenging act by preventing the onset of cancer during carcinogenesis, and they are generally beneficial to cells, unlike the

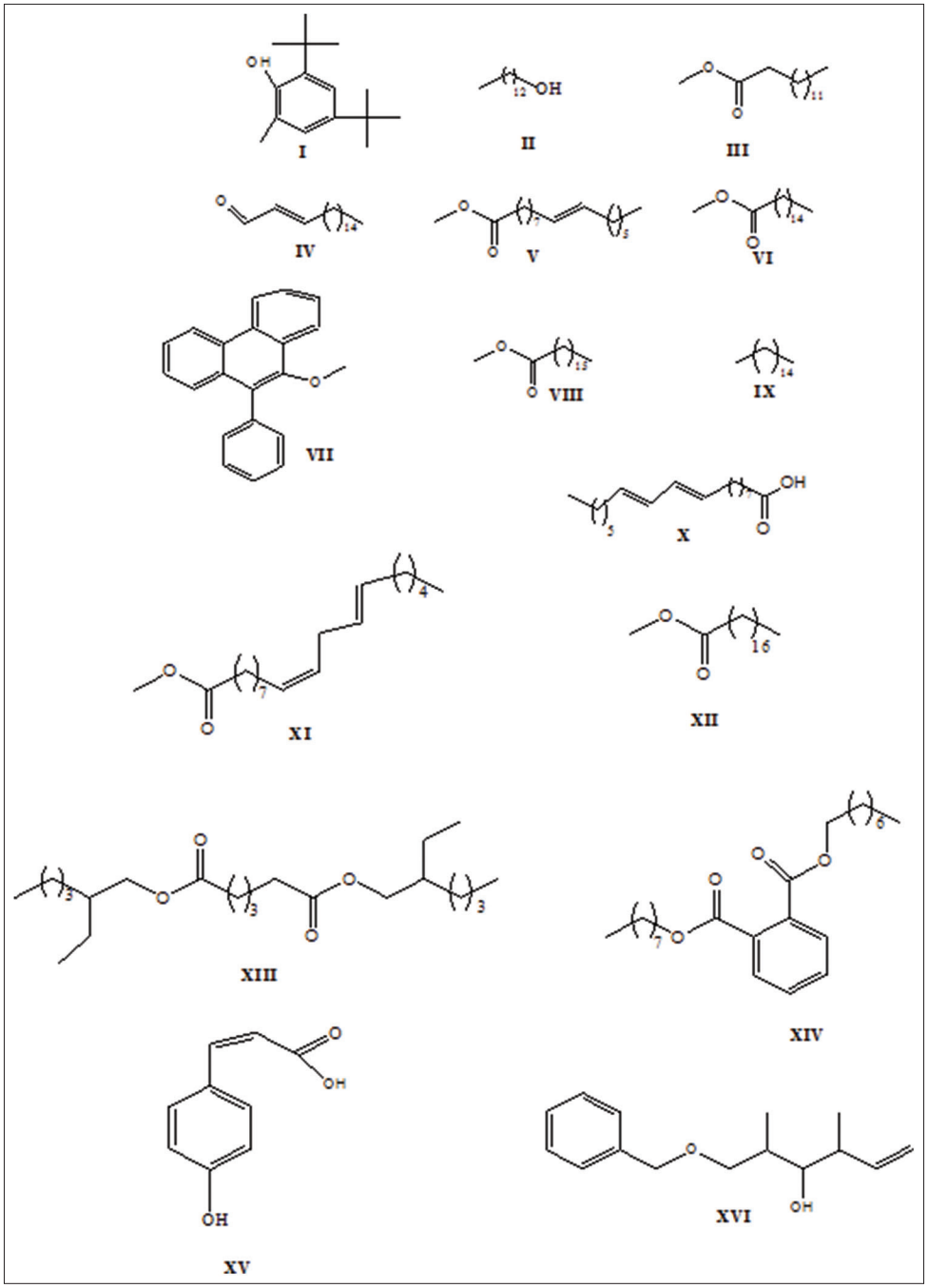

Fig. 2: Structures of the identified compounds (I-XVI) in the dichloromethane and ethyl acetate fractions by gas chromatography/mass spectrometry

cytotoxic agents that damage tumor cells [26]. In recent years, interest has grown in the use of natural antioxidants for the prevention or treatment of cancer and different diseases [27]. A cytotoxic activity included in the cancer chemopreventive agent would be beneficial to eradicate any microscopic cancer lesions that might evolve in early stages. In addition, the cytotoxic extracts were tested on normal human cells to select for extracts safe to normal cells and consequently are expected to be safe on prolonged usage.

Radical-scavenging plays an important role in different stages of cancer development [19]. There is increasing evidence that oxidative processes promote carcinogenesis, although the mechanisms for this are not well understood. Limiting or inhibiting free radical reactions should be able to reduce the rate of cancer incidence.

In the present study, the DCM extract of *M. alba* possessed both free radical scavenging and high potential antiproliferated activities against human cancer cells HCT116 and MCF7 with IC_{50} 43.9 and 32.3 µg/ml, respectively. While it, fortunately, showed no cytotoxic effect on BJ1. This organic extract of white mulberry fruits may also inhibit the growth of HepG2 cells through suppressing the activity of nuclear factor kappa B gene expression and modulate the biochemical markers [19].

The present results showed that the extraction yields obtained was affected by the solvent used. Difference in yields of extracts affected with the polarity of solvents and various compounds present. The extraction yield was obtained in the following descending order; EtOH>EtOAc>DCM>Hx. Polarity of the solvent, nature of the

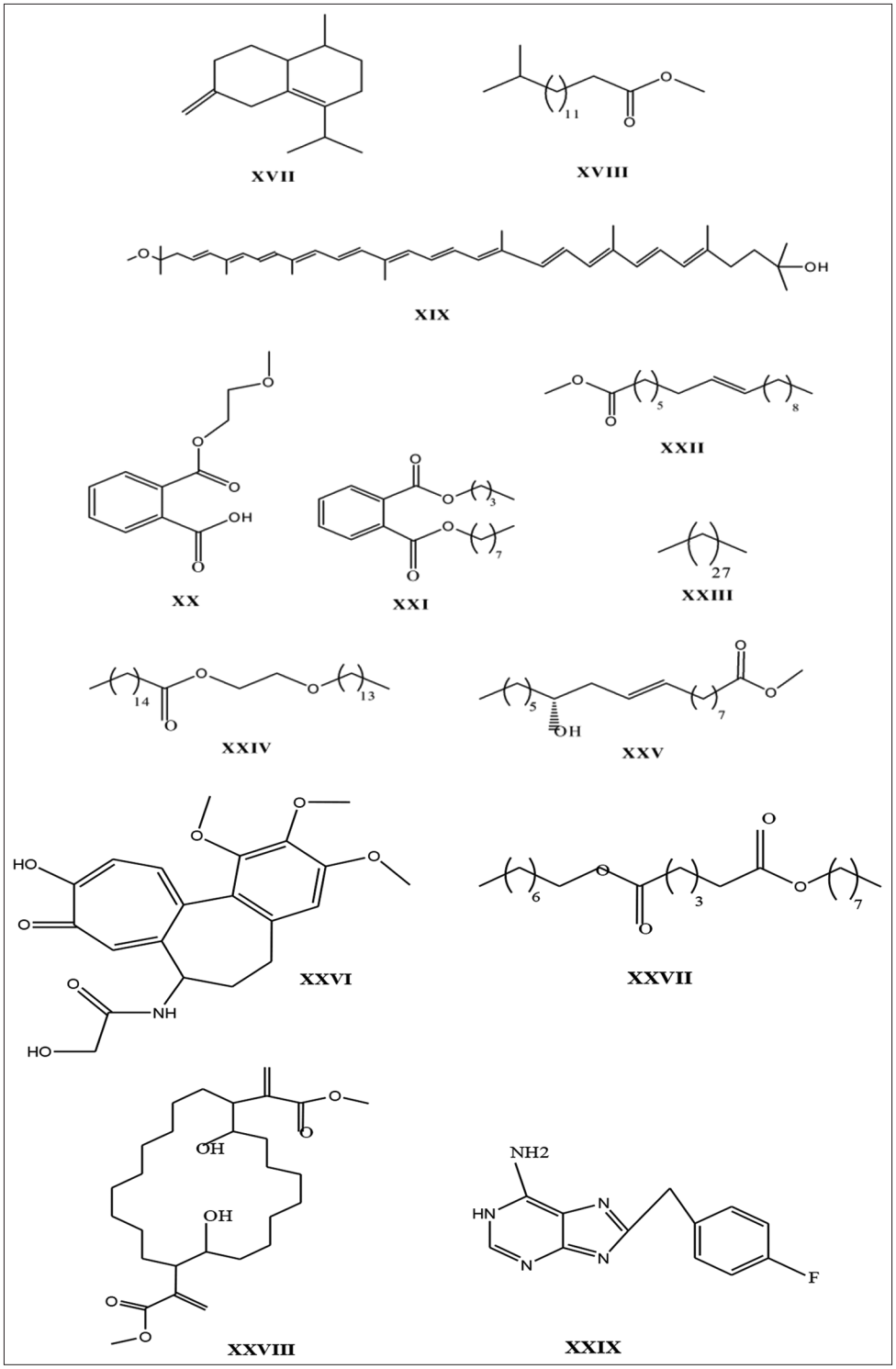

Fig. 3: Structures of the identified compounds (XVII-XXIX) in the dichloromethane and ethyl acetate fractions by gas chromatography/ mass spectrometry

extracted compounds and extraction process highly affects antitumor activity [18].

In the current work, the cytotoxic activity of the Hx fraction of *M. rubra* against PC3 (51.5%) and MCF7 (100%) cancer cells could be slightly attributed to the presence of sterols and unsaturated fatty acids in high content compared with the white mulberry. The sterol content in *M. rubra* was 33.04% compared with *M. alba* (28.07%), and the unsaturated fatty acids were 51.16% compared with *M. alba* (48.69%). These compounds have been reported to exhibit cytotoxicity against various cancer cells [30].

Palmitic and margaric were found to be the major saturated fatty acids in both current investigated *Morus* species. Linoleic acid, C18:2, as a representative example (8.06%) in *M. rubra* has been reported to possess significant antiproliferative activities [31]. The cytotoxic

Table 6: GC/MS analysis of DCM fractions of *M. alba* and *M. rubra*

RT	Identified compounds	Number of compound	Concentration (%)	
			M. alba	*M. rubra*
31.05	Phenol, 4,6-di (1,1-dimethylethyl)-2-methyl	I	0.45	0.65
33.35	n-tridecan-1-ol	II	0.12	-
37.08	Methyl myristate	III	1.42	3.55
38.86	2-Octadecenal	IV	0.95	0.41
41.72	Methyl palmitoleate	V	0.75	-
42.33	Palmitic acid, methyl ester	VI	66.55	48.51
43.90	10-Methoxy-9-phenyl-phenanthrene	VII	1.93	-
43.92	Ethyl palmitate	VIII	-	10.76
44.20	2-Hexadecene	IX	0.23	0.13
46.35	Methyl-9 cis, 11-trans-octadecadienoate	X	-	32.00
46.40	Linolelaidic acid, methyl ester	XI	22.02	-
47.00	Methyl stearate	XII	2.97	-
52.73	Adipic acid, bis-(2-ethylhexyl) ester	XIII	0.31	1.58
55.67	Dioctyl phthalate	XIV	2.30	2.41

M. alba: Morus alba, M. rubra: Morus rubra, RT: Retention time in minutes, GC/MS: Gas chromatography/mass spectrometry, DCM: Dichloromethane

Table 7: GC/MS analysis of EtOAc fractions of *M. alba* and *M. rubra*

RT	Identified compounds	Number of compound	Concentration (%)	
			M. alba	*M. rubra*
14.33	p-coumaric acid	XV	0.07	-
22.44	1-Benzyloxy-2,4-dimethyl-5-hexen-3-ol	XVI	6.25	1.01
31.04	2-Isopropyl-5-methyl-9-methylenebicyclo[4.4.0]dec-1-ene	XVII	0.06	0.21
37.07	Methyl myristate	III	-	0.82
37.09	Pentadecanoic acid, 14-methyl, methyl ester	XVIII	2.26	-
37.30	Rhodovibrin	XIX	-	0.14
40.85	Phthalic acid, mono-2-methoxyethyl ester	XX	-	0.03
41.40	Methyl palmitoleate	V	-	0.26
42.30	Palmitic acid, methyl ester	VI	10.46	12.15
43.20	Phthalic acid, butyloctyl ester	XXI	-	0.17
46.31	Linolelaidic acid, methyl ester	XI	5.74	70.00
46.32	Methyl-9-cis, 11-trans-octadecadienoate	X	0.14	8.00
46.46	8-octadecenoic, acid methyl ester	XXII	6.05	-
50.67	Nonacosane	XXIII	-	4.13
50.83	Palmitic acid, 2-(tetradecyloxy) ethyl ester	XXIV	0.88	-
52.02	Ricinoleic acid, methyl ester, acetate	XXV	0.05	-
52.73	Colchifoleine	XXVI	0.08	1.00
52.79	Dioctyl adipate	XXVII	-	4.10
55.94	1,11-bis-(Methoxycarbonyl-ethenyl)-10,2-dihydroxycycloeicosane	XXVIII	67. 96	-
56.11	Pregnane-3,20-diol	XXIX	-	2.08

M. Alba: Morus Alba, M. Rubra: Morus Rubra, RT: Retention time in minutes, GC/MS: Gas chromatography/mass spectrometry, EtOAc: Ethyl acetate

activity of *Morus* Hx fraction could be attributed to the presence of palmitic acid (a major component) which has been reported as a potential anticancer drug [32].

On the other hand, the activity of the DCM of white *M. alba* extract may be attributed to the bioactive phytoconstituents as geranyl-substituted flavanones. These compounds were reported as cytotoxic agents to selected human cancer cell lines and normal human fibroblasts [18]. The anti-metastatic effect of resveratrol, a phenolic compound previously isolated from many *Morus* species, on the pulmonary metastasis of murine B16 melanoma cells was evaluated [7]. The inhibitory effect of the same compound on the growth of cancer cells and induces apoptosis by acting at multiple cellular targets was reported. In addition, the resveratrol-mediated chemoprevention of rat liver carcinogenesis may be related to alteration of proinflammatory cytokines [33]. Ahmed *et al*. [6] suggested that the antitumor activity may be due to the presence of phytosterols such as stigmasterol and β-sitosterol.

The current work reported that margaric acid, palmitic acid, and arachidonic acids were the major fatty acids in purple and white mulberry, whereas cholesterol, stigmasterol, *β*-sitosterol and campesterol were the main sterols identified by GLC and the *M. alba* fruits had the higher total fat content. Palmitic acid was reported as a selective cytotoxic substance [32]. Moreover, the oil containing saturated aliphatic hydrocarbons, as octacosane and triacontane, could induce apoptosis on B16F10-Nex2 melanoma cells and displays antitumor activity [34]. Furthermore, polyunsaturated fatty acids were reported to have cytotoxicity against cancer cells [9]. Fractions containing fatty acids such as linoleic, myristic, and palmitic acids were evaluated for their cytotoxic activity [30]. Consequently, the high cytotoxic activity of Hx successive fraction of purple *M. rubra* more than *M. alba* reported in this study could be explained on the basis of the presence of a high percentage of unsaturated fatty acids (51.16%) and phytosterols (33.04%) found in Hx fraction of *M. rubra* more than those present in *M. alba* (48.69% for unsaturated fatty acids and 28.07% for phytosterols).

The GC-MS analysis of DCM and EtOAc extracts, where the corresponding compounds with their retention times, concentrations (%) and their nature are shown in Tables 6 and 7. The results indicated the presence of compounds of different categories such as phenols, hydrocarbons, esters, alcohols, alkaloids, and other aromatic components in the different extracts.

It was reported that the most of the identified compounds by GC-MS analysis of different successive fractions of *M. alba* L. were hydrocarbons and fatty acids, where palmitic acid was common compound [35].

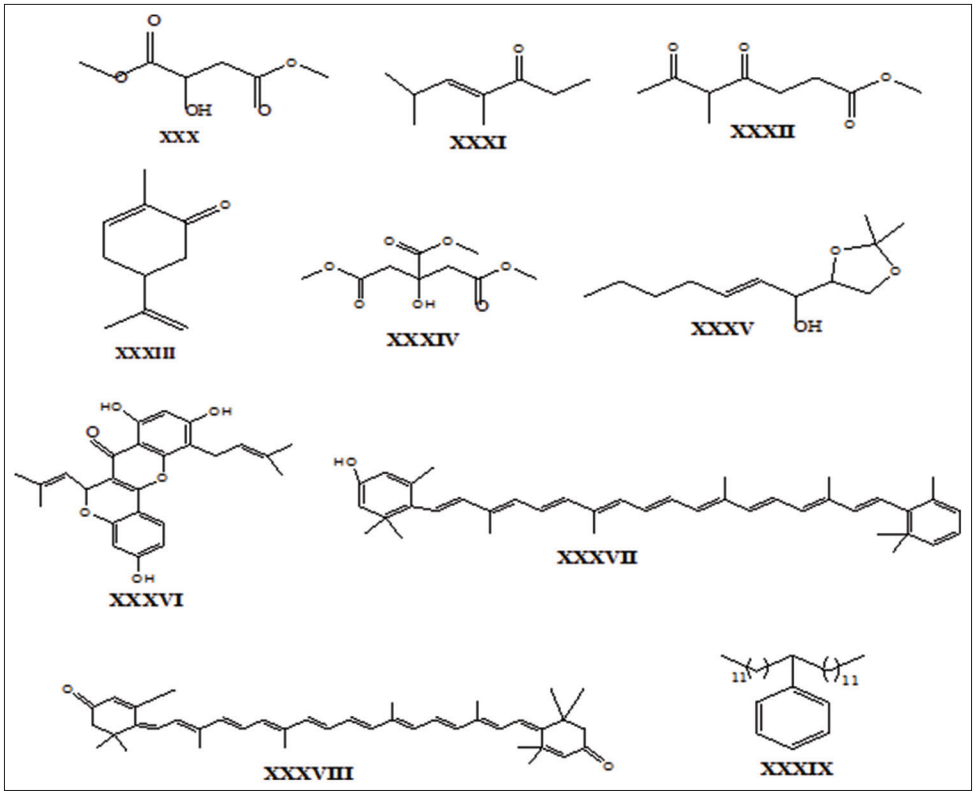

Fig. 4: Structures of the identified compounds (XXX- XXXIX) in the aqueous ethanol extract by gas chromatography/mass spectrometry

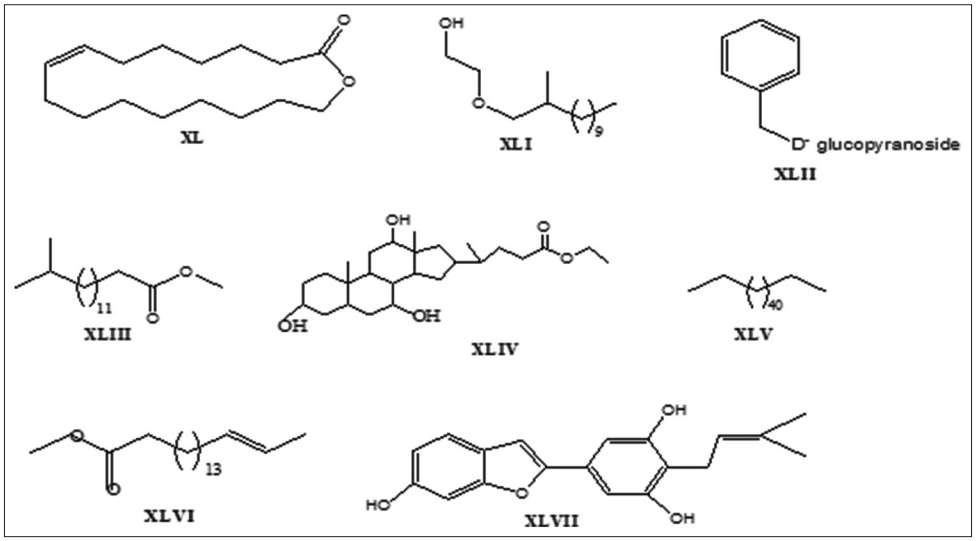

Fig. 5: Structures of the identified compounds (XL-XLVII) in the aqueous ethanol extract by gas chromatography/mass spectrometry

GC/MS analysis of the EtOH extracts of *M. alba* and *M. rubra* indicated the presence of compounds of different categories such as esters, ketones, carotenoids, hydrocarbons, and aromatic amines. Alcohol as 1,2-O-isopropylyidene-4-nonene-1,2,3-triol XXXV, phenol as 4,6-bis-(1,1-dimethylethyl)-2-methyl I, flavones as cyclomulberrin XXXVI and moracin C XLVII were also identified in these extracts. Cyclomulberrin and moracin C were previously isolated from the root bark of *Morus* plants of *M. alba* [9].

Mulberry fruit contains essential fatty acids that humans cannot synthesize and must be obtained through diet. Essential fatty acids are long-chain polyunsaturated fatty acids derived from linolenic, linoleic, and oleic acids, and they are necessary for the formation of healthy cell membranes, the proper development and functioning of the brain and nervous system [30].

Most of the compounds (Figs. 2-5) detected in Tables 6-8 were related to different categories that have been previously reported to possess anticancer potentials. Phenol-4,6-di-(1,1-dimethylethyl)-2-methyl, I and the phenanthrene derivative, 10-methoxy-9-phenyl-phenanthrene VII were identified in EtOH extract and DCM fraction of *M. alba,* and they were reported to have antitumor activity and were considered as new pharmacological agents with appropriate biological activity for the treatment of various diseases [36]. The cytotoxic potential effect of many fatty acid methyl esters was reported against three human tumor cell lines [37].

Table 8: GC/MS analysis of total ethanol extracts of *M. alba* and *M. rubra*

RT	Identified compounds	Number of compound	Concentration (%)	
			M. alba	*M. rubra*
19.43	Malic acid dimethyl ester	XXX	6.92	-
20.15	4,6-Dimethylhept-4-en-3-one	XXXI	-	0.04
22.34	Methyl -5-methyl-4,6-dioxoheptanoate	XXXII	-	0.06
22.60	d-Carvone	XXXIII	2.17	-
30.06	Trimethyl citrate	XXXIV	-	0.59
30.55	1,2-O-Isopropylyidene-4-nonene-1,2,3-triol	XXXV	6.20	84.94
31.05	Phenol, 4,6-bis (1,1-dimethylethyl)-2-methyl	I	7.60	9.00
32.33	Cyclomulberrin	XXXVI	1.59	1.02
34.59	Anhydrolutein II	XXXVII	1.36	-
36.41	Rhodoxanthin	XXXVIII	1.00	-
39.79	Pentacosane, 13-phenyl	XXXIX	1.74	-
40.68	Oxacycloheptadec-8-en-2-one [Ambrettolid]	XL	1.30	-
41.13	1-(2-Hydroxyethoxy)-2-methyl-dodecane	XLI	3.22	-
41.89	Benzyl- β- D- glucopyranoside	XLII	11.45	-
42.21	Palmitic acid, methyl ester	VI	-	0.66
42.28	Methyl-14-methyl- pentadecanoate	XLIII	25.86	-
44.00	Ethyl iso-allocholate	XLIV	4.58	-
46.31	n-tetratetracontane	XLV	4.91	-
46.39	16-octadecenoic acid, methyl ester	XLVI	5.62	-
46.90	Methyl stearate	XII	-	0.09
55.64	Colchifoleine	XXVI	5.36	0.60
55.71	Moracin C	XLVII	0.28	-
58.42	Nonacosane	XXIII	3.84	0.11

M. alba: Morus alba, M. rubra: Morus rubra, RT: Retention time in minutes, GC/MS: Gas chromatography/mass spectrometry

In several diseases including cancer, the metabolism of essential fatty acids as *cis*-linoleic and linolenic acid is altered. Thus, these acids and their derivatives have significant clinical implications [30]. The antitumor activity of unsaturated fatty acids may be related to the disturbance provoked on gene expression and protein activity by disrupting cell cycle progression [38]. Unsaturated fatty acids such as oleic (18:1), linolenic acid (18:3), arachidonic (20:4), and cis-13-docosenoic (22:1) acids were identified in the Hx of both mulberry species in the current study. Moreover, the presence of these types of fatty acids could enhance the fluidity of cell membranes in the structures of membrane lipids and resulted in facilitating the entry of anticancer drugs into the cell [30,38]. These acids were undergoing lipid peroxidation, yielding free radicals that they react with reactive oxygen species, harming various cell targets.

Colchifoleine, XXVI was related to nitrogenous agents that were reported to have cytotoxic effect in *vitro* and in *vivo* experiments [1,5]. Rhodovibrin, XIX (0.14%) was presented only in EtOAc fraction of *M. rubra* (Table 7 and Fig. 3). Whereas, anhydrolutein, XXXVII (1.36%) and rhodoxanthin, XXXVIII (1.00%) presented in the EtOH extract of *M. alba* (Table 8 and Fig. 4), were related to carotenoids. These class of compounds were reported to have anticancer effects in some specific animal models, using specific carcinogens and may be likely responsible for the bioactivities of the *Morus* plants [5,20,39].

The white mulberry aqueous alcoholic had an effective inhibition effect on the growth of HepG2 cells and may be able to inhibit cell proliferation. The activity may be related to the substantial levels of phenolics, flavonoids, and carotenoids acid present in mulberry species [38].

Decades of research on phytochemistry of genus *Morus* have led to the identification of various compounds, such as stilbenes, benzofurans, and flavonol glycosides that showed a wide range of bioactive features [4,9,39]. In the present study, the radical scavenging activity of ethanolic extract of fruits was concentration-dependent. The fruits of *M. rubra* in particular exhibit higher antioxidant activity than the white mulberry. *M. rubra* is one of the most important species of the genus Morus, the fruits containing substantial levels of phenolic compounds [39].

GC-MS and biological assays of organic extracts of the *M. rubra* fruits established their unique constituents. The deep-colored fruits are a rich source of these compounds, including flavonoids, anthocyanins, and carotenoids [4,39]. The radical scavenging activity may be correlated with phenolic constituents of mulberry fruits. Based on the results obtained, *Morus* fruits were found to serve as a potential source of food diet and natural antioxidants.

CONCLUSION

The results observed remarkable biological activity of the extracts of *M. rubra* more than those of *M. alba* and confirmed its importance as natural bioactive source. *Morus* species are good candidates to be promising as possible sources for future novel antitumor and antioxidants in food and pharmaceutical formulations. The strong activity partly explains the potential effects of *Morus* species for the treatment of cancer and degenerative diseases caused by free radicals. Due to their active contents, it seems that these plants are worth investigating for further studies to evaluate the antioxidant, anticancer efficacy and general toxicity of the active extracts, especially the promising of DCM fraction of *M. alba*, in animal models. However, further merit investigations including clinical study are necessary in the future to confirm this hypothesis.

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