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Research Article

LC-MS study; antioxidant and antidiabetic potential of *Morusmacroura* Miq. leaves

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ABSTRACT

Morus leaves are used in folk medicine for hundreds of years as a hypoglycemic. However, leaves of *Morusmacroura* Miq. has not yet to be scientifically proven and confirmed their ability to treat diabetes. **Objective:** The recent work was aimed to investigate the phytochemical screening of *Morusmacroura*Miq. (*Moraceae*) leaves extracts and evaluate their chemical compositions as well as to overview the antidiabetic activities;total antioxidant capacity*via* digestive enzyme and free radical inhibitors which are used to prevent complications resulting from diabetes. The chemical composition was determined by Liquid Chromatography-Mass Spectrometry (LC-MS).The antioxidant activity of the extracts and isolated fractions were estimated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), phosphomolybdenum method (PM) assays, while digestive enzymes inhibitory activity was assessed using α-glucosidase inhibitory method as antidiabetic. The phytochemical screening showed that leaves methanol extract of *M. macroura*Miq. contain flavonoids, tannins, polyphenols, alkaloids and terpenoids. TheCH_2Cl_2 and MeOH extracts exhibited antidiabetic activity with IC_{50}20.56 and 17.65 µg/mL respectively. LC-MS analysis which showed the presence of different benzofuran derivatives, Diels–Alder type adducts,phenolic acids, quercetin derivatives and prenylated flavonoids, in which these compounds had high antioxidantand inhibitory activity against α-glucosidase or diabetes. LC-MS analysis revealed the presence of polyphenolic compoundswhich had high antioxidant and α-glucosidase inhibitory activities, might largely contribute in the antidiabetic activity.

Key words: Phytochemical screening, LC-MS, Anti-diabetic, *Morusmacroura*Miq.α-glucosidase, DPPH assay,PM assay.

[I] INTRODUCTION

Herbal medicines are now in great demand and still the mainstay of about 75 - 80% of the world populationmainlyin the developing world for primary health care not because they are inexpensive but also for better cultural acceptability, better compatibility with the human body and minimal side effects [1].

The mulberry family (Moraceae) is multicultural tree with the majority of species occurring in the Old-World tropics.Moraceae contain a large family of 60 genera and nearly 1400 species including genus *Morus*(mulberry) with more than 15 species of deciduous plants. It has been extensively cultivated due toits leaves, which serve as essential food for silkworms[2]. These are economic plants because the leaves are widely used in sericulture. In addition to this, many nutritional benefits and medicinal values are attributed to this plant. Different parts of the mulberry, from the root bark to the leaves have antioxidative, hypolipidemic, antihyperglycemic, and antiatherogeniceffects[2-4].

On revising the reviews and the previous phytochemical investigations on this genus revealed the presence of isoprenylated flavonoids, stilbenes, Diels–Alder type adducts, triterpenoids, and alkaloids[5]. Some *Morus* species are rich sources of phenolic compounds, which possess beneficial activities such as antioxidant and tyrosinase inhibitory activity,anticancer, anti-inflammatory properties, and postprandial hypoglycemic effects [6].*Morus* leaves are used in folk medicine for hundreds of years as a hypoglycemic[7-10],hypotensive[11]and antibiotic[12-14]. Recent researches proved that various phytochemical constituents of *Morus* plant possess antioxidant activity [15-17], anticancer activity [5, 18], anti-HIV [19],antihyperlipidimic[20],hepatoprotective [21].

Morusmacroura Miq. (*M. macroura*) (*Moraceae*) belongs to genus *Morus.* Its leaves have been an indispensable food source for silkworms, and its root barks have been used to treat diabetes, arthritis, and rheumatism. Previously, many compounds, which were regarded biogenetically as Diels-Alder adducts, were isolated from *M. macroura*[22].The biological important effects of many mulberry species are promising for the recent herbal medicine. Nearly all *Morus* species have been studied well except few of them including *M. macroura* Miq.

[II] MATERIALS AND METHODS

Plant material

Fresh leaves of *Morusmacroura*Miq. Were collected from Tenth of Ramadan City, September 2015. The plant was identified by Dr. Therese Labib Youssef, Ex- Manger and Taxonomist of Botanical Orman Garden, Giza, Egypt.The plants leaves were air-dried and reduced to No.36 powder and kept in tightly closed container. Voucher specimens (MM-02, 2015)were kept in Department of Pharmacognosy, Faculty of Pharmacy, Al -Azhar University.

Experimental:

Preparation of plant extracts

The air-dried powdered leaves of (1kg) were exhaustively extracted with 70%MeOH (6 x 2.5L), under reflux (70°C). The aqueous methanol extract was concentrated under reduced pressure and defatted with light petroleum ether (60 – 80 °C, 3×1.5 L), the marc left successively extracted with CH_2Cl_2(3×1.5 L), to give 50g CH_2Cl_2 extract then the remained viscous extract which was dissolved in water, and the water-insoluble residue was removed by filtration. The water-soluble portion was desalted by precipitation with excess MeOH to give a dry brown residue (100 g Methanol extract). It was suspended in water and chromatographed on a polyamide S (Fluka Hannover, Germany) column (5 X 110 cm) and eluted with H_2O followed by increased portions of MeOH to yield 40 fractions of 1L each. The fractions were concentrated under reduced pressure and monitored by paper chromatography (PC) using solvent systems S_1 and S_2 [S_1: 15% aqueous acetic acid and S_2: *n*-butanol-acetic acid-

water (4:1:5, top layer)] and UV-light to be combined into nine major collective fractions M_1-M_9.

Liquid Chromatography-Mass Spectrometry (LC-MS) studies

The chemical constituents of the methanol extract were determined using LC-MS. LC-MS analysis was performed using Mariner Bio spectrometry equipped with a binary pump. The HPLC was interfaced with a Q-TOF mass spectrometer fitted with an ESI source. Full-scan mode from m/z 100 to 1200 was performed with a source temperature of 140°C. HPLC column Phenomenex 5µ C8, (150 × 2 mm i.d.) was used for the analysis. Solvent was methanol with 0.3% formic acid. Solvents were delivered at a total flow rate of 0.1 mL/min. The solvent was run by isocratic elution. The MS spectra were acquired in the positive ion mode. The temperature of the drying gas (N_2) was 350^o C, at a gas flow rate of 6 mL/min, and a nebulizing pressure (N_2) of 25 psi. About 0.5 g of sample extracts was diluted with methanol and filtered with 0.22 µm nylon filterprior to analysis. A 5 µl volume of the extracts were injected onto the analytical column for analysis. The mass fragmentations were identified by using spectrum database for organic compounds in SDBS application and compared with the previous reported data.

Determination of total antioxidant capacity (TAC)

The antioxidant activity of each tested sample was determined according to phosphomolybdenum method (PM) using ascorbic acid as standard. This assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of a green colored [phosphate=Mo (V)] complex at acidic pH with a maximal absorption at 695 nm. In this method, 0.5 ml of each tested samle (100 µg /ml) in methanol was combined in dried vials with 5 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vials containing the reaction mixture were capped and incubated in a thermal block at 95°C for 90 min. After the samples had cooled at room temperature, the absorbance was measured at 695 nm against a blank. The blank consisted of all reagents and solvents without the sample and it was incubated under the same conditions. All experiments were carried out in triplicate. The antioxidant activity of the sample was expressed as the number of ascorbic acid equivalent (AAE) [23,24].

Evaluation of antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay *in vitro*[25,26]

150 µL of freshly prepared DPPH$^{\bullet}$ solution (0.4 M concentration) was carefully introduced into 96 round microplate wells each. 100 µL of sample/ standard (7.81–500 µg/mL) were added. Blanks contained only methanol and DPPH$^{\bullet}$. Ascorbic acid (dissolved in distilled water) was used as standard. The plate was left in the dark for 25 min to activate. Absorbance was noted at 517 nm. Tests were conducted in triplicate and the percentage inhibition of each sample/standard was calculated using the following equation:

$$(\%\ \text{DPPH inhibition}) = \frac{Ac - As}{Ac} \times 100$$

Where, Ac = absorbance of DPPH radical in MeOH, As = absorbance of DPPH radical in sample or standard.

IC_{50} values obtained from graphical plot from percentage inhibition against concentration were used to define the radial scavenging activity of each sample.

α-Glucosidase inhibition test[27,28]

A 250 µL solution of p-nitrophenyl-α-D-glucopyranoside 5 mM and 495 µL phosphate buffer 0.1M pH 7 was added to the reaction tube containing 5 µL of the sample solution in DMSO with concentrations variation of 100, 50, 25, and 10 µg/mL. After homogeneous, the solution was pre incubated for 5 min at 37°C, the reaction was initiated by the addition of 250 µL α-glucosidase solution (0.062 units), incubation was continued

for 15 min. The reaction was stopped by the addition of 1 ml of Na_2CO_3 0.2 M. The activity of the enzyme was measured, based on the reading of p-nitrophenol absorbance at λ 400 nm. Quercetin was used as a reference standard with concentration of 10, 7.5, 5, 2.5, and 1 µg/mL. % inhibition was measured by using the equation:

% Inhibition = $\frac{B - S}{B} \times 10$(B = Absorbance of blank (DMSO) &S = Absorbance of sample).

[III] STATISTICAL ANALYSIS

Results were given as mean±standard deviation of 3 replicates. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with α=0.05. This treatment was carried out using SPSS v.16.0 (Statistical Program for Social Sciences) software.

[IV] RESULTS AND DISCUSSION

The phytochemical screeningof leaves methanol extractof *M. macroura*Miq. leavesaccording to Jyothiprabha and Venkatachalam 2016[29] reveled presence of carbohydrates and / or glycosides, flavonoids, alkaloids and / or nitrogenous bases, tannins,saponins, steroland / or triterpenes; while absence of volatile oil, anthraquinones, coumarins and iridoids, (Table 1). However, the plant extractsprovide a different bioactivity and give positive results against antidiabetic. This is led it proceeds with the analysis of phytoconstituents using Liquid Chromatography-Mass Spectrometry (LC-MS) studies. LC-MS analysis of methanol extracts *Morusmacroura*Miq. had detected tentatively identifiedtwenty-one peaks. Then each peak was fragmented, resulting fragmentation spectra with candidates' mass (m/z) of proposed compounds. The results of spectrum interpretation of *Morusmacroura*Miq. methanol extracts, indicating that there are different benzofuran derivatives likeDihydro- Moracin J and Mulberrofuranat RT=0.735 and 6.555 respectively; Diels–Alder type adducts as Kuwanon E at RT=18.215; phenolic acids as Quinic and chlorogenic acids at RT=1.545 and 4.865 respectively; quercetinderivatives as quercetindihexosideat RT=10.495 and prenylated flavonoidsas TetrahydroMongolicin Dat RT=8.225 and other different compounds (Table 2). The LC-MS spectrums interpretation was performed using a spectrum database for organic compounds in SDBS application and comparison with reported literature review. These results were confirmed by each of the fragmentation patterns.

The spectrophotometric method has been developed for the quantitative determination of antioxidant capacity**.** Antioxidant capacity assays may be broadly classified as single electron transfer (SET) and hydrogen atom transfer (HAT) based assays. Majorities of HAT assays are kinetics based and involve a competitive reaction scheme in which antioxidant and substrate compete for free radicals thermally generated through the decomposition of azo compounds.

Antioxidant capacity assays due to single electron transfer (SET) that measure the capacity of an antioxidant in the reduction of an oxidant which changes colorwhen reduced like phosphomolybdenum method (PM). PM is routinely applied in the laboratory to evaluate the total antioxidant capacity of plant extracts[23]. PM measures the reduction degree of Mo (VI) to Mo (V): PM assay is a quantitative method to investigate the reduction reaction rate among antioxidant, oxidant and molybdenum ligand. It involves in thermally generating auto-oxidation during prolonged incubation period at higher temperature and it gives a direct estimation of reducing capacity of antioxidant.

Other antioxidant tests may be based on the measurement of free radical scavenging potency (hydrogen donating ability or hydrogen atom transfer). The radical scavenging donates hydrogen to free radicals (HAT), leading to nontoxic species. The use of DPPH radical provides an easy, rapid and suitable method to evaluate the antioxidant and radical scavenging.

Therefore, the selected extracts of *Morusmacroura* (Methanol extract, methylene chloride extracts and isolated fractions: M_5, M_7-M_9) were evaluated for their antioxidant activity by PM and DPPH methods.

The reducing capacity of antioxidant was coined in a single measure as "Total Antioxidant Capacity" (TAC)[30].

TAC of each sample was estimated based on its reducing capacity by different methods such as DPPH and PM assays. Absorbance reflects directly to the reducing power in the two methods.

Analysis of the antioxidant activities of *Morusmacroura* extracts and fractions at concentration (100 µg/mL) on DPPH radical with their corresponding IC_{50} values is shown in Table 3 with ascorbic acid (AC), and quercetin (QC) as reference standards. The methanol and methylene chloride extracts displayed a promising free radical scavenging activity against $DPPH^{\bullet}$. M_7-M_9 and M_5 fractions obtained from the methanol extract showed the highesttotal antioxidant capacity (347.96 ± 2.57& 256.90 ± 3.44 mg AAE/g dry sample)and radical inhibitory activity with the lowest IC_{50} values of 9.30 ± 1.73 and 10.65 ± 1.29µg/mL respectively, while the methanol and methylene chloride extract exhibited significant total antioxidant capacity (183.81 ± 2.67 & 94.38 ± 2.75 µg/ml) , with IC_{50}=17.75 ± 2.34 and 38.23 ± 1.83 µg/mL respectively (Figure 1). The strong free radical effect of the all tested samples was attributable to the presence of phenolic compounds in significant amounts. Similar activity of their methanol extracts has been previously reported for *Morusalba*[16]. The outcome of this assay showed that M_7-M_9 and M_5 are the most active fractions and therefore isolation of compounds was carried out on these fractions.

So, on the basis of results obtained from different antioxidant capacity assays, each tested sample of *Morusmacroura* has shown a significant total antioxidant capacity. The reducing capacity depends on phenolic contents which can be estimated with the help of LC/MS assay. Over viewing the reducing capacity, the use of *Morusmacroura* might contribute a certain level of health protection against oxidative damage.

In the light of results, we can conclude that the plant extracts screened herein showed inhibitory activity against α-glucosidase enzyme in dose dependent manner.Dichloromethane extract has lower α-glucosidase inhibition activity than the methanol extract. Based on theantidiabetic test, quercetin solution at concentrations of 10, 7.5, 5, 2.5, 2 ,1.5 and 1 µg/mL had % inhibition of80.66%, 65.52%,49.52%,45.33%, 40.21%, 30.45 % and 8.77% respectively Table 4 , Figure 2. The methanol extracts of *Morusmacroura*Miq leaves at concentrations of 300,200, 100, 50, 25 and10 µg/mL had % inhibitions of 99.45%, 99.45%, 98.23%, 92.23%, 81.39%, 57.42% and 24.87%respectively. While, the methylene chloride extracts of *Morusmacroura*Miq leaves at concentrations of 300, 200, 100, 50, 25 and 10 µg/mL had % inhibitions of 85.33%, 84.5%, 83.4%, 80.2%, 71.09%, 48.02%, 38.25% and 35.99% respectively. Based on the results of antidiabetic test in Table 2, Figure 3, it can be seen that both methanol and methylene chloride extracts of *Morusmacroura*Miq.displayedhigh antidiabetic activity in concentration- dependent activitywith IC_{50} of 17.65 and 20.56 µg/mLrespectively.In comparison with that of IC_{50} of quercetin that was 5.3µg/mL (Figure 2). Quercetin had high α-glucosidase inhibitor activity and often used as a standard on antidiabetic test[31,32].

The results found in this study indicate that *Morusmacroura* Miq. extracts had hypoglycemic properties; therefore, it can be recommended for its efficacy in the treatment of diabetes in humans.

Polyphenols were often present in polar glycosides and were easily soluble in polar solvents. The antioxidant and antidiabetic properties of these compounds were related to the presence of phenolic groups that can donate hydrogen atoms to a free radical so that the radicals become less reactive.

Methanol extracts of *Morusmacroura* Miqwas useful as an antidiabetic through several mechanisms depended mainly on the content of the compounds therein:

1. Methanol extracts of *Morusmacroura*Miq leaves were rich in flavonoids which have hypoglycemic[34]and other phenolic compounds. Phenolic compounds can donate their hydrogen atoms and function as free radical inhibitors, where phenolic compounds will protect organs such as the pancreas from free radical'sattack[33].
2. Another mechanism was by competitively inhibiting α-glycosidase activity, these inhibitors help to prevent the fast breakdown of sugars and thereby control the blood sugar level[35, 36].*Morusmacroura* was reported to contain many ingredients such as polyphenols, tannins and flavonoids (for example, quercetin). The tannins were found to restore the function of pancreatic beta cells and enhance their release of insulin. Quercetin is an antioxidant thatacts by several mechanisms including oxygen radicals scavenging; hence, it protects against lipid peroxidation and metal ions chelation[37]. The presence of these substances with their anti-oxidant properties may explain the anti-diabetic effect of this plant as indicated by previousstudies[6].Also, this in agree with the previous report that chlorogenic acid and rutin play a major role in the *in vivo* anti-diabetic activity of *Morus alba* leaf extract on type II diabetic rats[10, 38]Our results need further support for the proposed mechanism of *Morusmacroura* anti-diabeticeffect. It isrecommended to investigate the effect of the plant extract on pancreatic B-cells by histopathological examination

Table 1: Phytochemical screening of *Morusmacroura*Miq. Leaves methanol extract.

Constituents	Result
Volatiles	-
Carbohydrates and/or glycosides	+
Alkaloids and/or nitrogenous bases	+
Flavonoids	+
Tannins	+
Anthraquinones	-
Saponins	+
Coumarins	+
Unsaturated sterols and/or terpenes	+
Iridoids	-

(+): positive (-): negative

Table 2: LC-MS profile of tentatively identified compounds of*Morusmacroura* Miq leaves methanol extract.

Peak no.	RT	Mode	MF	MW	$[M+H]^+$	Ms/ Ms	Proposed compounds (Tentative identification)
1.	.735	-	$C_{15}H_{14}O_5$	274	273	273,159	Dihydro- Moracin J
2.	0.92	+	$C_{30}H_{46}O_3$	454	455	455,381,365,293,266,219,176	Betulinic acid
3.	1.545	-	$C_7H_{12}O_6$	192	191	191,161,137,155,111,87	Quinic aid
4.	3.495	+	$C_{18}H_{34}O_5$	329	328	291, 209, 197, 155	Trihydroxy-octadecenoic acid
5.	4.865	-	$C_{25}H_{24}O_{12}$	516	515	353,223,191.179,173	Chlorogenic acid
6.	6.025	-	$C_{16}H_{18}O_9$	354	353	707,353,191	Dimer of chlorogenic acid
7.	6.555	-	$C_{29}H_{34}O_4$	446	445	445	Mulberrofuran W or Z

8.	6.925	-	$C_{20}H_{18}O_5$	338	337	337,285,241,191	Moracin H
9.	7.595	-	$C_{35}H_{30}O_{11}$	626	625	625,579,523,415,379,329,278,215	Kuwanon L or Guangsangon K
10.	7.755	-	$C_{45}H_{40}O_{11}$	756	755	755,565,482,461	Dehydro- Kuwanon W
11.	8.025	-	$C_{19}H_{16}O_4$	308	307	307,261	Morunigrol C orMoracin D or E
12.	8.225	-	$C_{45}H_{40}O_{10}$	740	739	739,693,583,394,377,217	TetrahydroMongolicin D
13.	8.545	-	$C_{35}H_{30}O_{10}$	610	609	609,463	Guangsangon M or N
14.	8.565	+	$C_{27}H_{30}O_{16}$	610	611	633 ($[M+Na]^{+)}$,611,465,303	Rhamnosylhexosyl quercetin
15.	9.165	-	$C_{35}H_{30}O_9$	594	593	593,515,490,447,427,405	Guangsangon G
16.	9.665	+	$C_{30}H_{44}O_3$	452	453	453,355,330,241,197	Dehydro keto ursolic acid
17.	10.495	-	$C_{27}H_{30}O_{17}$	626	625	625,579,480,463,431	Quercetin dihexoside
18.	12.775	-	$C_{15}H_{16}O_2$	228	227	249,227	Dihydro-chalcone
19.	18.215	+	$C_{25}H_{28}O_6$	424	425	425,403,329	Kuwanon E
20.	19.115	-	$C_{20}H_{18}O_6$	354	353	353	Isolicoflavonol orglyasperin F or Sanggenon F or H
21.	20.605	-	$C_{20}H_{20}O_5$	340	339	339	Morachalcone A or 3',5'-dihydroxy-6-methoxy-7-prenyl-2-arylbenzofuran

MF: Molecular Formula; RT: Retention Time; MW: Molecular weight

Table 3. Total antioxidant capacity (TAC) and free radical scavenging antioxidant activity (DPPH) of *Morusmacroura*Miq. leaves extracts and isolated compounds.

Sample	Total antioxidant capacity (mg AAE/g dry sample)[1,2]	DPPH IC_{50} (µg/ml)[3]
MeOH Extract	183.81 ± 2.67	17.75 ± 2.34
CH_2Cl_2 Extract	94.38 ± 2.75	38.23 ± 1.83
M_5	256.90 ± 3.44	10.65 ± 1.29
M_7- M_9	347.96 ± 2.57	9.30 ± 1.73
Ascorbic acid	-	6.25 ± 1.45

[1]Results are (means ± S.D.) (*n* = 3).

[2]AAE: Ascorbic acid equivalent.

[3]IC_{50}: concentration from sample required for scavenging of 50% of radical.

Table 4: α-Glucosidase inhibitory activity (mean ± SEM, n = 3) of methanol and dichloromethane extracts of *Morusmacroura* Miq.

Extract/ standard	Concentrations (µg/ml)	Inhibition (%)	IC_{50} (±SEM, µg/ml)
Methanol extract	300	99.45±0.23	IC_{50}= 17.65±**0.58**
	200	99.45±1.01	
	100	98.23±1.12	
	50	92.23±0.14	
	25	81.39±0.23	
	20	57.42±0.44	
	15	24.87±1.01	
	10	13.09±1.12	
Dichloromethane	300	85.33±1.01	IC_{50}= 20.56±**1.01**

extract	200	84.5±1.89	
	100	83.4±0.45	
	50	80.2±1.5	
	25	71.09±0.56	
	20	48.02±0.24	
	15	38.25±0.12	
	10	35.99±0.98	
Quercetin standard	10	80.66±0.12	IC_{50}= 5.3±**0.23**
	7.5	65.52±0.24	
	5	49.52±0.54	
	2.5	45.33±1.02	
	2	40.21±1.04	
	1.5	30.45±1.03	
	1	8.77±1.00	

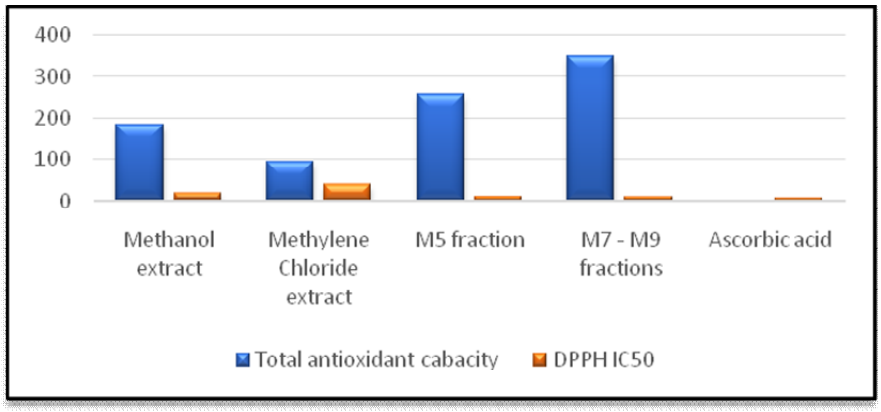
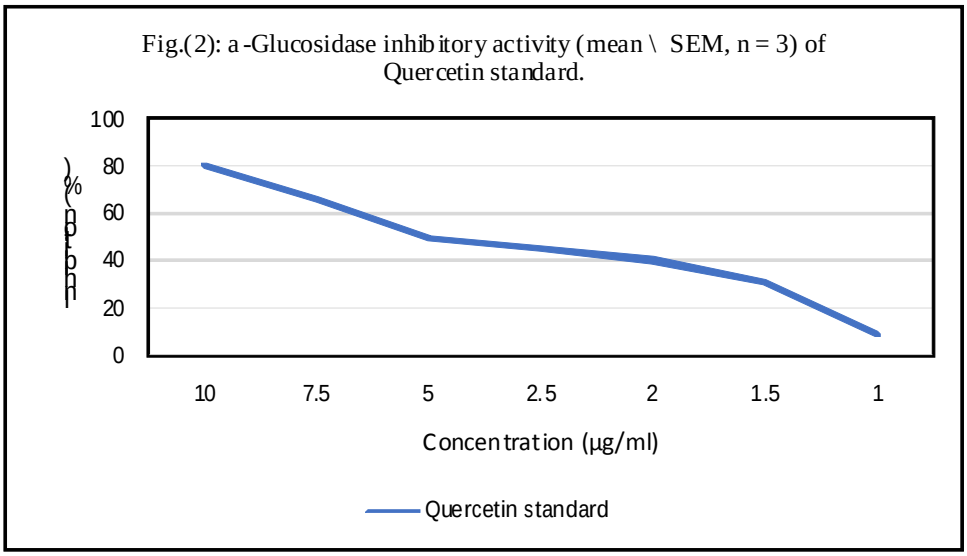
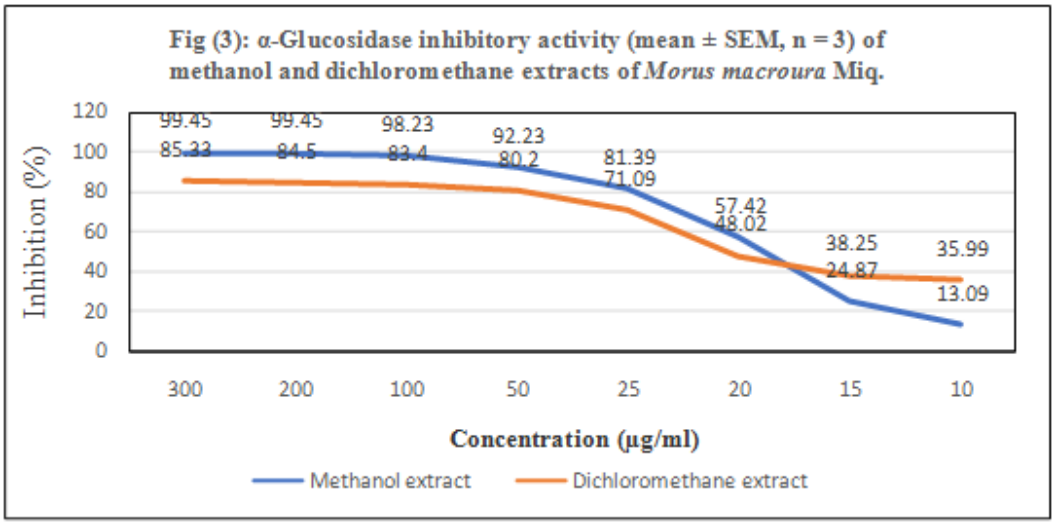


Fig. (1): Total antioxidant capacity (TAC) and Free radical scavenging antioxidant activity(DPPH) of *Morusmacroura*Miq. leaves extracts and fractions





[V] CONCLUSION.

Based on the LC-MS analysis which was interpreted by SDBS application, it can be identified that some of the compounds in the methanol extracts were benzofuran derivatives, Diels–Alder type adducts,phenolic acids, quercetin derivatives and prenylated flavonoids. From these results, it can be concluded that methanol extracts of *Morusmacroura*Miq. leaves had great potential as biomedicine for diabetes diseases.further structural elucidation using different spectroscopic means and characterization methodologies have to be carried out in order to identify the bioactive constituents.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest. **Abbreviations** LC-MS: Liquid Chromatography Mass Spectrometry; MS: Mass Spectrometry; PM: phosphomolybdenum method; DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate; RT: Retention time; MW Molecular weight: MF: Molecular formula.

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