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Original Research Article

Chemical Profiling, *In Vivo* Antioxidant Effect and Antimutagenic Evaluation of *Melilotus indicus* (L.) All. Extracts

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ABSTRACT

Melilotus indicus (L.) All. is used as a vegetable and known to possess valuable remedial uses. The whole plant was extracted using 70% methanol to give the total aqueous methanol extract (AME) which was defatted with petroleum ether to give petroleum ether extract (PEE) and defatted AME (DAME). PEE was analyzed by GC-MS and LC-ESI-MS. In addition, the chromatographic investigation of DAME led to the isolation of thirteen flavonoids and one phenolic acid. Their structures were elucidated through chemical and spectroscopic analysis (ESIMS, UV and NMR). Prospective activity of AME, PEE and DAME against alloxan-induced oxidative stress, diabetes and mutagenic effect in male rats was investigated. GC-MS analysis detected thirty-five non-polar compounds while the LC-ESI-MS revealed the presence of additional eight metabolites. AME and PEE significantly ($P < 0.01$) increased serum GSH content in rats (35.3 ± 0.8 and 34.9 ± 0.6 mmol/L) compared to diabetic rats (21.8 ± 0.3) and vitamin E (36.2 ± 1.1). Also, AME and DAME revealed a significant acute anti-hyperglycemic effect potentiated after 4 weeks of treatment with blood glucose levels of 96.9 ± 6.2 and 98.7 ± 6.1 mg/dL, respectively, compared to diabetic rats (263.4 ± 7.8) and Metformin group (81.9 ± 2.4) at $P < 0.01$. Additionally, AME revealed a significant inhibitory effect on the irregularity of bone marrow cells and sperm abnormalities appeared from higher inhibitory indices which were 75 and 76, respectively.

Keywords*:* *Melilotus indicus*, Phytoconstituents, Antioxidant, Antidiabetic, Antimutagenicity

Introduction

The genus *Melilotus* Mill. belongs to the family Fabaceae (Leguminosae). The members of the genus *Melilotus* are important crop due to their traditional therapeutic applications.[1] Several phytochemical studies proved that *Melilotus* species are rich in alkaloids, flavonoids, coumarins, triterpenes and saponins.[2] *Melilotus indicus* L. (All.) (=*Melilotus indica*) is one of seven species growing in Egypt; commonly named Indian sweet clover.[3] The seeds of *M. indicus* are used for curing heart diseases, bowel complaints, bronchitis, infantile diarrhea and leprosy. The plant has also been used as an anticoagulant, antipyretic, anthelmintic, astringent, emollient, laxative, narcotic and fomentation.[4-6] Earlier studies reported the presence of pterocarpan,[7] prenylated pterocarpan and methylene-dioxypterocarpan,[8] *C*-glycosides [9] as well as some flavone derivatives[4] from *M. indicus*. More recent study reported the detection of flavonol derivatives.[10,11] Type II diabetes mellitus (DM) incidence is rising globally and considered one of the major human health worries. Thus, intervention before the appearance of disease through edible vegetables may help in prevention and controlling its complications.[12] Oxidative stress and free radical formation intensively upswing in diabetes.[13]Accumulation of reactive oxygen species (ROS) due to hyperglycemia causes DNA damage and results in mutagenicity of bone marrow and sperms.[14] Potential antigenotoxicity was achieved by medicinal plants via their phenolic constituents through several mechanisms including their antioxidative stress effect.[15] No study has been carried out on the spectrometric analysis, *in vivo* anti-diabetic effect and antimutagenic evaluation of *M. indicus*. Therefore, the present study aims to identify and profiling the phytoconstituents of *M. indicus* extracts using GC-MS and LC-ESI-MS techniques. Also, their antioxidant, antidiabetic and antimutagenic effects in alloxan induced diabetic rats were evaluated

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Material and Methods

Plant material and extraction

The whole plants of *M. indicus* were freshly collected on 14 February 2016 as weeds of cultivation, 6th October City, Giza, Egypt. The plant sample was authenticated by Prof. Dr. M. M. Marzouk and the voucher specimen (N2_14216) was prepared and has been deposited in the herbarium of National Research Centre. The air-dried plant sample (650 g) was ground and extracted with 70% $MeOH/H_2O$ then 50% $MeOH/H_2O$ for several times till exhaustion, at room temperature. The combined extract was distilled off under reduced

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pressure and low temperature to obtain a residue of aqueous methanol extract (AME). The AME residue was dissolved in 500 mL H_2O and exhaustively extracted with light petroleum ether (40–60 °C). The petroleum ether extract (PEE) was dehydrated over anhydrous sodium sulphate, evaporated and preserved for analysis. The remaining defatted aqueous methanol extract (DAME) was also evaporated and kept for investigation.

Phytochemical study

General

NMR experiments were recorded on Bruker high performance digital FT-NMR spectrophotometer advance III 400 spectrometer and Jeol EX-500 spectrometer. The GC-MS system (Agilent Technologies) was furnished with GC (7890B) and MS detector (5977A). ESI-MS system was measured on HPLC (Waters Alliance 2695) and MS (Waters 3100). Shimadzu model-2401 CP spectrophotometer was used for recording the UV spectra. Rotary evaporator (HEIDOLPH 4000, Germany), water distiller (Hamilton, westwood industrial Estate) and water bath (Labtech) were used in the laboratory works. Column chromatography (CC) was carried out on Sephadex LH-20 (Pharmazia, Uppsala, Sweden) and Polyamide 6S (Riedel-De-Haen AG, Seelze Haen AG, Seelze Hanver, Germany). Whatman No. 1 and 3MM chromatographic sheets (descending) were used for paper chromatography techniques (PC). Solvents: distilled water (H_2O), methanol (MeOH, HPLC grade, ≥99.9%, Fisher Chemical, UK), petroleum ether (40–60°C) and acetic acid (AcOH) (Adwic, Cairo, Egypt) and n-butanol (n-BuOH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvent systems: 15% HOAc (H_2O: AcOH, 85:15), BAW (n-BuOH: AcOH: H_2O, 4:1:5, upper layer). Sugar authentic samples (E. Merck, Darmstadt, Germany), flavonoid authentic aglycones (Fluka AG, Bucns SG, Switzerland), while authentic glycosides were obtained from Phytochemical and Plant Systematics Department, NRC.

Investigation of the unsaponifiable and saponifiable matters of PEE

Preparation of the unsaponified mattets

About 2 g of PEE of *M. indicus* was subjected to alkaline hydrolysis for saponification. PEE was refluxed with 100 ml of 0.5 N alcoholic KOH, at 100 °C for 6 h. The saponified extract was dried to one-third of its volume. The cooled reaction mixture was exhaustively extracted with ether after dilution with an equal volume of H_2O. The ether extract was extracted several times with H_2O till free from alkalinity. Then, it was dehydrated over anhydrous sodium sulphate, and then dried.[16] The dried residue (0.2g) was preserved for GC-MS analysis.

Preparation of the fatty acids methyl esters

After extraction of the unsaponifiable substance, the remaining alkaline aqueous solution was acidified with HCl to release the fatty acids and then was exhaustively extracted with ether. The combined ether extracts were washed with H_2O for several times till free from acidity, and then filtered over anhydrous sodium sulphate. The filtrate was dried and a residue of fatty acids was obtained. The fatty acid methyl esters were prepared by dissolving the fatty acids residue in 50 mL MeOH (HPLC grade) then mixed with 0.25 mL H_2SO_4 (refluxed for 3 h). The mixture was distilled off from MeOH, dissolved with H_2O (100 mL) and then extracted with ether for several times. The ether extract was washed several times with H_2O until free from acidity and then dehydrated over anhydrous sodium sulphate. The solvent was evaporated and the residue 0.17g (fatty acid methyl esters) was preserved for GC-MS analysis.[17]

GC-MS analysis

The unsaponified matter and fatty acid methyl esters were subjected to GC/MS analysis based on the method described by Salem et al. [18]

Investigation of DAME

Isolation and structure elucidation

DAME of *M. indicus* was applied to a polyamide column (100 x 5 cm) starting with H_2O as eluent then decreasing the polarity by increasing the concentration of MeOH. Collectively, 54 fractions were obtained, each of about 300 mL. Analogous fractions were collected according to their PC properties using BAW, H_2O and 15% AcOH as eluents to give four main fractions (A-D). Fractions A (20-30% MeOH/H_2O) and B (40-60% MeOH/H_2O) were separately applied to a Sephadex column (60x2.5 cm) using MeOH: H_2O (1:1). Fractionation gave rise to major sub-fractions. They were subjected to PC using BAW (several time), then on a Sephadex column (30x1.5 cm) using MeOH as eluent yielded compounds **5**, **6** and **12** from fraction A (100% H_2O-20% MeOH/H_2O). Similarly, compounds **4**, **11** and **14** were obtained from fraction B (40-60% MeOH/H_2O). Fraction C (70-90% MeOH/H_2O) yielded compounds **2**, **3** and **8**-**10** by separation on PC using 15% HOAc followed by BAW. Fraction D (100% MeOH) was chromatographed on PC using 15% HOAc (several times) to yield compounds **1**, **7** and **13**. All compounds were purified on a Sephadex column (30x1.5 cm) using MeOH as eluent.

LC-ESI-MS analysis

DAME was analyzed using the LC-ESI-MS system. Two solvents were used as mobile phase for gradient elution: solvent A (0.1% CH_2O_2 in H_2O) and solvent B (0.1% CH_2O_2 in CH_3CN/MeOH (1:1; *v*/*v*). The mobile phases were prepared daily by filtering through membrane disc filter (0.45 μm) then degassed by sonication. Linear gradient profile was applied as follows: 5% B for 5 min, 5-10% B for 5 min, 10-50% B for 35 min, 50-5% B for 10 min and 5% B for 5 min. The injection volume was 10 μL. The flow rate (0.6 mL/min) was split 1:1 before the MS interface. Desolvation gas flow (600 L/h), capillary voltage (3 kV), cone gas flow (50 L/h), cone voltage (50 eV), source temperature (150 °C), and desolvation temperature (350 °C) were the negative ion mode parameters. Spectra were recorded between 50-1000 *m/z* and analyzed using the MassLynx 4.1 SCN#14 software. Identified peaks were documented by comparing the retention time and MS fragments with the authentic samples (purity 95-98%, HPLC, UV, NMR and ESI) that were isolated and identified in the present study. The other standards were obtained from the research group of the Phytochemistry and Plant Systematics Department, NRC.[19,20]

Bioactivity evaluation

Drugs and biochemical kits

Vitamin E (*dl α*-tocopheryl acetate); gelatinous capsules, each containing 400 mg vitamin E, (Pharco Pharmaceutical Co) was used as a reference antioxidant drug. Alloxan (Sigma Co) was used for the induction of acute diabetes in rats. Metformin (Cidophage)® (Chemical Industries Development (CID), Giza, ARE) was used as standard antidiabetic drug. Biodiagnostic glutathione kit was used for assessment of antioxidant activity (Wak-chemie Medical Germany). BioMérieux kit (France) was used for assessment of blood glucose level. Doses of the drugs were administered orally by a gastric tube and were measured according to Paget and Berne's.[16]

Animals

Six albino mice (weighing 25–30 g) were used for determination of LD_{50}. About forty two male albino rats of the Sprague Dawely strain (10-12 weeks old, 150-200 g) were used for evaluation of antioxidant, antihyperglycemic and antimutagenic activities. All animals were obtained from National Research Centre (the breeding colonies, Dokki, Giza, Egypt), and acclimatized with free access to food (1% vitamins, 5% fats and20% protein) and tap water for at least one week at room temperature (23–25˚C). All animal procedures were performed after approval by the Medical Ethics Committee, National Research Centre (no.17158), according to the recommendations of the accurate care and usage of laboratory animals.

Median Lethal Dose (LD_{50}):

LD_{50} of AME of *M. indicus* was determined and calculated according to the method reported by Karber.[22]

Alloxan induced diabetic rats

DM was induced using intraperitoneal injection of a single dose of alloxan (150 mg/kg b.wt.), followed by an overnight fast.[23] After 72 h, blood samples of each rat were obtained from the retro-orbital venous plexus and the blood glucose level was measured using BioMérieux kit to confirm the induction of diabetes.[24] Rats with blood

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glucose levels greater than 200 mg/dL were considered as diabetic mellitus rats (DM_rats) and were included in the study.

Experimental design

Forty two male albino rats were classified into seven groups (six rats/group). The first group included healthy rats that received 1 mL saline (negative control) and groups 2-7 consisted of DM_rats. Group 2 was kept untreated, group 3 was DM_rats that received vitamin E (7.5 mg/kg) (positive control for antioxidant assay), group 4 was DM_rats that received Metformin (positive control for antihyperglycemic assay), while the fifth, sixth and seventh groups were DM_rats that received oral doses of AME, PE and DAME (100 mg/kg b.wt.), respectively.

Determination of serum reduced glutathione content (In vivo Antioxidant activity)

The *in vivo* antioxidant activities of *M. indicus* extracts were evaluated by determining the blood glutathione level (GSH) upon administration of AME, PEE and DAME for one week after oral administration of *M. indicus* extracts.[23,25] Blood samples were obtained and GSH was estimated using biodiagnostic kits as follow; GSH blood = A $_{sample}$* 66.66 mg/dL BW in an oral dose (100 mg kg^{-1} BW) for one week.[24]

Determination of blood glucose level (Antihyperglycemic assay)

The antihyperglycemic activities were evaluated by determining the blood glucose level (BGL) at zero, 2 and 4 weeks after oral administration of *M. indicus* extracts. BGL was determined using the OK glucometer (Lifescan, Milpitas, CA) instrument by the glucose-oxidase principle and the results were described as mg/dl. [26]

Antimutagenic assay

Chromosome evaluation in somatic cells

At 4 weeks after oral administration of DM_rats by *M. indicus* extracts, chromosome preparations from rat bone marrow (somatic cells) described based on the method of Yosida and Amano.[27] Hundred Well spread metaphases were analyzed per rat. Metaphases with different abnormalities in somatic cells were recorded under magnification (2500x) using a light microscope (Olympus, Saitama, Japan).

Sperm shape abnormalities

Sperms were prepared according to the suggested method of Wyrobek and Bruce [28] and smears were stained with 1% Eosin Y. A total of 1000 sperms were counted per animal (5000/each treatment), and different types of sperm abnormalities were scored (Head & Tail abnormalities). Sperm preparations were observed by light microscopy at magnification (1000x). Evaluation of the effect of *M. indicus* extracts to inhibit DNA damage induced in diabetic male rats was calculated according to the equation:

Inhibitory index (II)= [1- (diabetic rats+ plant extract–control) / (diabetic rats - control)] x 100.[29]

Statistical analysis

The results were expressed as mean ± standard error of the mean (S.E.M.). For cytogenetic analysis, the significance of the results from the negative control data and between *M. indicus* extracts plus diabetic group compared to diabetic group alone was calculated using t-test.

Results and Discussion

Phytochemical analysis

Investigation of the unsaponifiable matters and fatty acids esters of PEE (GC-MS analysis)

The characterization of the unsaponified compounds of PEE was accomplished by comparing their mass spectral data and retention times with those of the library and the published data described by Adams.[30,31] Peaks areas were used for the measurements of quantitative determinations. Thirty two compounds were identified and representing 99.6% of the total unsaponifiable content. The identified unsaponified components consist of 4.4% unoxygenated compounds, 60.7% oxygenated compounds and 34.5% miscellaneous compounds (Table 1). Three fatty acid methyl ester derivatives were identified in the saponified matters of PEE and representing 100% of the total compounds. Major fatty acids were 9, 12, 15-octadecatrienoic acid, methyl ester (59.53%) followed by, hexadecanoic acid, methyl ester (21.07%) and 9, 12-octadecadienoic acid, methyl ester (19.40%). Saturated fatty acids represented (21.07%) of the total fatty acids content, while di- and tri-unsaturated fatty acids represented (19.40 %) and (59.53%) of the total fatty acids content, respectively (Table 2).

Investigation of DAME

Isolation and structure elucidation

From DAME of *M. indicus,* thirteen flavonoids and one hydroxycinnamic acid glucoside (Figure 1) were isolated. Their structural patterns were elucidated through chemical and spectroscopic analysis (ESIMS, UV, 1D and 2D NMR) and comparison with reported data. They are identified as kaempferol (K) (**1**), [32] K 3-*O*-rhamnoside (**2**),[33] K 3-*O*-glucoside (**3**), [34] K 3-*O*-rutinoside (**4**), [35] K 3-*O*-(2″,6″ di α-rhamnopyranosyl)-*O*-β-galactopyranoside (**5**),[36] K 3-*O*-(2″,6″ di α-rhamnopyranosyl)-*O*-β-galactopyranoside-7-*O*-α-rhamnopyranoside (astrasikoioside) (**6**), [37] quercetin (Q) (**7**), [10] Q 3-*O*-glucoside (**8**), [10] Q 3-*O*-(6″-α-rhamnopyranosyl)-*O*-β-galactopyranoside-7-*O*-α-rhamno-pyranoside (clovin) (**9**),[38] isorhamnetin (I) 3-*O*-glucoside (**10**),[10] I 3-*O*-rutinoside (**11**) ,[10] I 3-*O*-(2″,6″ di α-rhamnopyranosyl)-*O*-β-galactopyranoside (**12**),[36] melimessanol B (**13**)[39] and *trans p*-coumaric acid-*O*-glucoside (**14**).[40] Except for compounds (**1**, **7**, **8**, **10** and **11**), all compounds were isolated for the first time from *M. indicus*.[10, 11] Also, compounds (**5** and **12**) were reported for the first time for the genus *Melilotus.* For knowledge, the triglycoside of quercetin (**9**) and the kaempferol tetraglycoside derivative (**8**) was previously identified in *Melilotus officinalis* (L.) Pall. and other leguminous species.[36-38]

ESI-MS analysis

The fourteen isolated compounds were used as reference samples and were observed in the base peak chromatogram as peaks 4, 5, 8, 10-13, 15-18, 20, 21 and 22 (Table 3, Figure 2). Other additional eight peaks were also observed and assigned as peaks 1-3, 6, 7, 9, 14 and 19. Peak 1 was tentative identified as asparagine, based on the molecular ion peak *m/z* 131 [M-H]$^-$ and product ion at *m/z* 113 [M-H-18 (H_2O)]$^-$.[41] Peak 2 with [M-H]$^-$ ion at *m/z* 293 and fragmentation pattern *m/z* 131 [M-H-162(hexose)]$^-$ and 113 [M-H-162(hexose)-18(H_2O)]$^-$, after loss of hexose unit and water molecule, respectively. It could be identified as asparagine-hexoside structure. Peak (3) is confirmed as quinic acid (*m/z* 191), based on comparison with reference sample and the presence of product ions *m/z* 129[M-H-44(CO_2)-18(H_2O)]$^-$ and 111[M-H-44(CO_2)-36(2H_2O)]$^-$.[42] Peaks 6 &7 were tentatively identified as 3-*O*-(rhamnosyl hexoside)-7-*O*-hexoside of quercetin and kaempferol, respectively. The loss of hexose unit at first suggested its substitution at position 7, also confirmed by presence of fragments indicating the mono hexoside derivative of quercetin and kaempferol at *m/z* 463 and 447 for peaks 6 and 7, respectively. Moreover, the presence of product ions at *m/z* 609 and 593 for peaks 6 and 7 with high intensity, indicating the occurrence of a disaccharide residue at the OH group at position 3, where the hexose unit is directly attached to the hydroxyl group and the rhamnose unit was terminal.[42] The substitution of such glycosylation pattern is the first detection in the genus *Melilotus*. Peak 9 (*m/z* 325) revealed product ions at *m/z* 163[M-H-162(hexose)]$^-$ and 119[M-H-162(hexose)-44(CO_2)]$^-$, indicating the presence of an additional coumaroyl hexoside isomer (isomer for compound **14**). Peak 14 (*m/z* 927) is suggested to be a mono acetyl derivative of the isolated compound **6** (peak 8) by the evidence with an extra 42 Da. Additionally, the acetyl moiety suggested to be attached to the rhamnoside unit at the OH group of position 7, supported the appearance of a product ion at *m/z* 473[kaempferol-H+188 (acetyl-rhamnose)]$^-$. Thus peak 14 is tentatively identified as kaempferol 3-*O*-(di-rhamnosyl)-*O*-galactoside-7-*O*-acetyl rhamnoside. This kind of structure is the first report in the genus *Melilotus*. Peak 19 (*m/z* 431) revealed fragment ions at *m/z* 285 and was identified as kaempferol 7-*O*-rhamnoside according to the retention time and mass fragments of standard.

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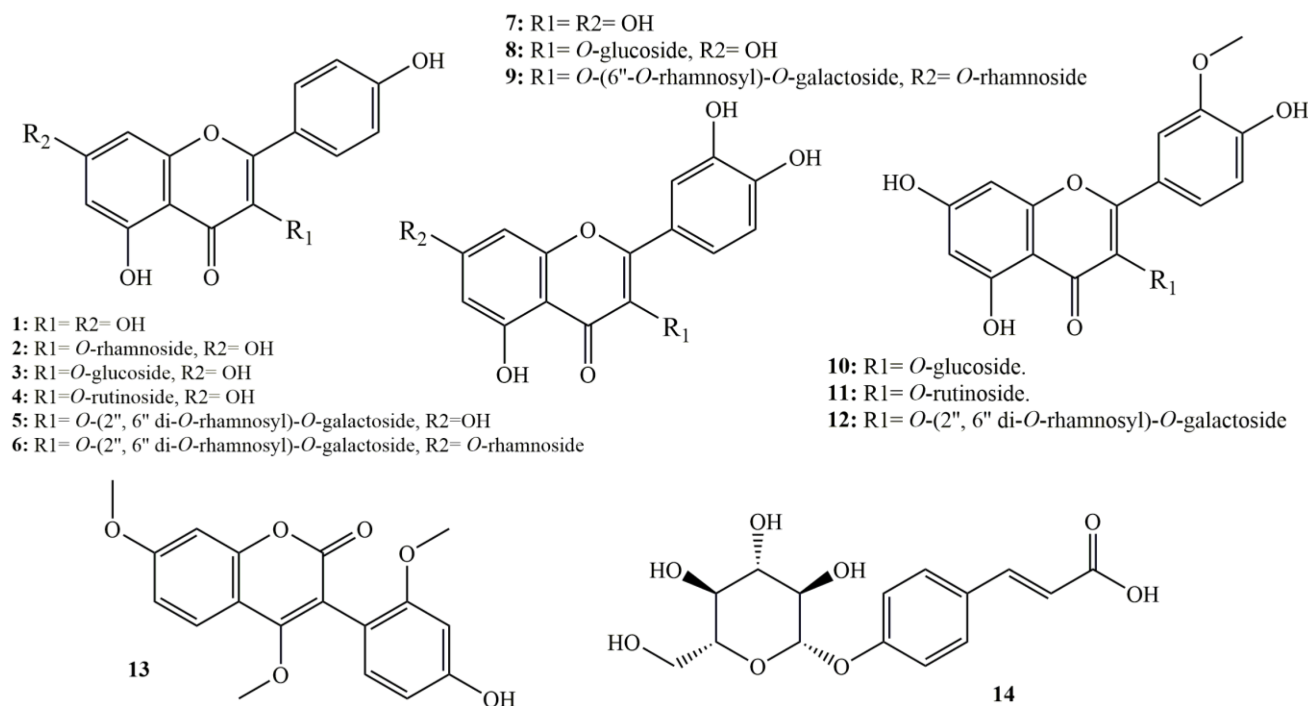

Figure 1: Chemical compounds isolated from DAME of *Melilotus indicus*

Table 1: Components identified by GC/MS of the unsaponified matter of *M. indicus*

No.	R_t	*RR_t	Compound	Mol. Formula	M.Wt	Relative area %
1	10.83	0.45	2,5-cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-hydroxy-4-methyl-	$C_{15}H_{24}O_2$	236	0.7
2	11.61	0.48	Benzene, (1-butylhexyl)-	$C_{16}H_{26}$	218	0.57
3	11.72	0.48	Benzene, (1-propylheptyl)-	$C_{16}H_{26}$	218	0.68
4	11.95	0.49	Benzene, (1-ethyloctyl)-	$C_{16}H_{26}$	218	0.55
5	12.38	0.51	Benzene, (1-methylnonyl)-	$C_{16}H_{26}$	218	0.88
6	12.72	0.53	Benzene, (1-pentylhexyl)-	$C_{17}H_{28}$	232	0.80
7	12.76	0.53	Benzene, (1-butylheptyl)-	$C_{17}H_{28}$	232	1.99
8	12.87	0.53	Benzene, (1-propyloctyl)-	$C_{17}H_{28}$	232	1.69
9	13.12	0.54	Benzene, (1-ethylnonyl)-	$C_{17}H_{28}$	232	2.23
10	13.54	0.56	Benzene, (1-methyldecyl)-	$C_{17}H_{28}$	232	2.80
11	13.80	0.57	Benzene, (1-pentylheptyl)-	$C_{18}H_{30}$	246	1.52
12	13.86	0.57	Benzene, (1-butyloctyl)-	$C_{18}H_{30}$	246	1.69
13	13.99	0.58	Benzene, (1-propylnonyl)-	$C_{18}H_{30}$	246	1.29
14	14.23	0.59	Benzene, (1-ethyldecyl)-	$C_{18}H_{30}$	246	1.48
15	14.64	0.61	Benzene, (1-methylundecyl)-	$C_{18}H_{30}$	246	2.51
16	14.85	0.61	Benzene, (1-pentyloctyl)-	$C_{19}H_{32}$	260	1.96
17	14.92	0.62	Benzene, (1-butylnonyl)-	$C_{19}H_{32}$	260	1.27
18	15.01	0.62	2-Pentadecanone, 6,10,14-trimethyl-	$C_{18}H_{36}O$	268	2.09
19	15.05	0.62	Benzene, (1-propyldecyl)-	$C_{19}H_{32}$	260	1.62
20	15.30	0.63	Benzene, (1-ethylundecyl)-	$C_{19}H_{32}$	260	1.34
21	15.70	0.65	Benzene, (1-methyldodecyl)-	$C_{19}H_{32}$	260	1.93
22	17.62	0.73	Phytol	$C_{20}H_{40}O$	296	1.54
23	19.84	0.82	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	281	4.44
24	20.78	0.86	Behenic alcohol	$C_{22}H_{46}O$	326	1.19
25	20.83	0.86	1-Hexadecanol, 2-methyl-	$C_{17}H_{36}O$	256	0.98
26	22.33	0.93	Heptacosane	$C_{27}H_{56}$	380	5.63
27	23.99	1	1-Dodecanol, 2-octyl-	$C_{20}H_{42}O$	298	36.34
28	28.32	1.18	Campesterol	$C_{28}H_{48}O$	400	1.65
29	28.86	1.20	Stigmasterol	$C_{29}H_{48}O$	412	1.52
30	29.95	1.24	gamma-Sitosterol	$C_{29}H_{50}O$	432	12.26
31	30.62	1.27	α -Amyrin	$C_{30}H_{50}O$	426	0.48
32	31.57	1.31	Lupeol	$C_{30}H_{50}O$	426	1.95
Total identified content						99.6

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Table 2: Components identified by GC/MS of the fatty acids methyl esters of *M. indicus*

No.	R_t	*RR_t	Compound	Mol. Formula	M.Wt	Relative area %
1	10.68	0.80	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	21.07
2	13.10	0.99	9,12-Octadecadienoic acid, methyl ester, (E,E)-	$C_{19}H_{34}O_2$	294	19.40
3	13.22	1	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	$C_{19}H_{32}O_2$	292	59.53
Total identified content						100

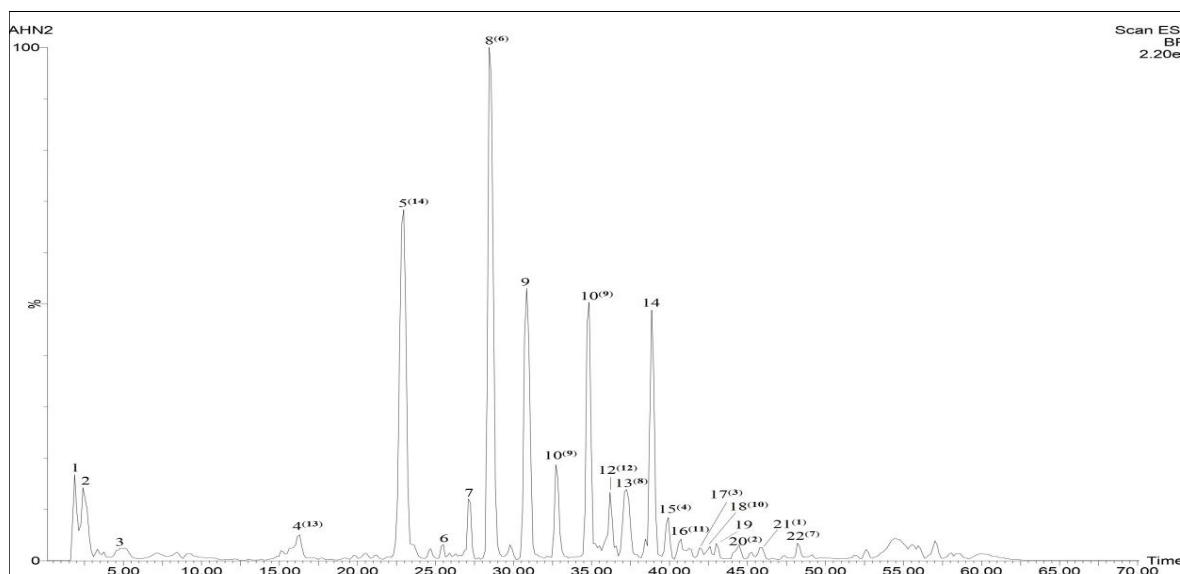

Figure 2: LC-ESI-MS chromatogram of DAME of *M. indicus*, numbers with no superscript are peak no., numbers with superscript are isolate no. (Compounds isolated and identified using chromatographic technique)

Table 3: Negative LC-ESI-MS analysis of chemical constituents of *M. indicus*

Peak no. (Isolate no.)	*Rt* (min)	$[M-H]^-$	*m/z* fragments	Tentative identification
1	2.01	131	113, 87,70	Asparagine
2	2.54	293	131, 113, 87	Asparagine-hexoside
3	4.48	191	129,111, 85	Quinic acid [b]
4 (**13**)	16.15	327	193, 176, 147, 133, 109	Melimessanol B [a,b]
5 (**14**)	22.91	325	163, 119	*trans p*-Coumaric acid-*O*-glucoside [a,b]
6	25.36	771	609, 463, 301	Quercetin 3-*O*-(rhamnosyl hexoside)-7-*O*-hexoside
7	27.2	755	593, 447, 285	Kaempferol 3-*O*-(rhamnosyl hexoside)-7-*O*-hexoside
8 (**6**)	28.4	885	739,593, 431, 285	Kaempferol 3-*O*-(2″,6″ di α-rhamnopyranosyl)-*O*-β-galactopyranoside-7-*O*-α-rhamnopyranoside [a,b]
9	30.97	325	163, 119	Coumaroyl glucoside isomer
10 (**9**)	32.18	755	609, 447, 301	Quercetin 3-*O*-(6″-α-rhamnopyranosyl)-*O*-β-galactopyranoside-7-*O*-α-rhamnopyranoside [a,b]
11 (**5**)	34.8	739	593, 285	Kaempferol 3-*O*-(2″,6″ di α-rhamnopyranosyl)-*O*-β-galactopyranoside [a,b]
12 (**12**)	36.18	769	315, 314	Isorhamnetin 3-*O*-(2″,6″ di α-rhamnopyranosyl)-*O*-β-galactopyranoside [a,b]
13 (**8**)	37.25	463	301	Quercetin 3-*O*-glucoside [a,b,c]
14	38.85	927	781, 473, 431, 430, 285	Kaempferol 3-*O*-(di-rhamnosyl)-*O*-hexoside-7-*O*-acetyl-rhamnoside
15 (**4**)	39.95	593	285	Kaempferol 3-*O*-rutinoside [a,b]
16 (**11**)	41.25	623	315	Isorhamnetin 3-*O*-rutinoside [a,b,c]
17 (**3**)	42.08	447	285, 284	Kaempferol 3-*O*-glucoside [a,b]
18 (**10**)	42.45	477	315, 314	Isorhamnetin 3-*O*-glucoside [a,b,c]
19	42.99	431	285	Kaempferol 7-*O*-rhamnoside [b]
20 (**2**)	44.17	431	285, 284	Kaempferol 3-*O*-rhamnoside [a,b]
21 (**1**)	45.9	285	151, 145, 117	Kaempferol [a,b,c]
22 (**7**)	48.2	301	179, 151,121	Quercetin [a,b,c]

[a] Compounds isolated and identified in the present study, [b] Compounds identified by comparing their retention times and mass spectrum with the authentic, [c] Compounds isolated previously from *M. indicus*.

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Biological activities

Median Lethal Dose (LD_{50})

LD_{50} of the AME was found to be 4.7g extract/kg b.wt. This indicated that *M. indicus* could be considered as safe.

In vivo Antioxidant activity

Oxidative stress and free radical formation may act as a common passageway to diabetes and to its later complications. Diabetes is associated with a decrease in the level of glutathione (GSH). GSH is a tripeptide that forms the main reducing capacity of the cytoplasm and its intracellular present at high concentrations. It is known to neutralize reactive oxygen species (ROS) and defend the cellular system against toxic effects of lipid peroxidation [13]. In the current study, the lower levels of GSH in DM_rats were significantly restored by AME, PEE and DAME with the percentage of change 11%, 12 % and 15%, respectively, compared to 9% for DM_rats treated with vitamin E and 45 % for the untreated DM_rats (Table 4).

Antihyperglycemic assay

This comprehensive *in vivo* study is established in view of earlier *in vitro* preliminary antidiabetic researches on the plant under study.[43] Significant antihyperglycemic activities were exhibited by all extracts as indicated by inhibition of the blood glucose level (BGL) in DM_rats, the highest activity exhibited by both AME and DAME (62%) followed by PEE (54%), in comparison with Metformin (69%) (Table 5).

Table 4: Effect of *M. indicus* extracts and reference drug (vitamin E) on the blood GSH of alloxan induced diabetic male rats (n = 6)

Animal Group	Blood glutathione (mg/dL)	% of change
Healthy rats (Control)	39.9 ± 1.2	-
DM_rats	21.8 ± 0.3*	45
DM_rats + vitamin E	36.2 ± 1.1**	9
DM_rats + AME	35.3 ± 0.8**	11
DM_rats + PEE	34.9 ± 0.6**	12
DM_rats + DAME	33.9 ± 0.8**	15

DM_rats; Diabetes mellitus rats; AME ; 70% aqueous methanol extract, PEE; Petroleum ether extract, DAME; defatted aqueous methanol extract. Data are expressed as mean_SEM of 6 rats/group. *Statistically significant difference from control at P < 0.01. **Statistically significant difference from diabetic at P < 0.01.

The activity of most of the flavonoids identified in DAME was proven in earlier investigations to normalized blood glucose level, such as kaempferol, quercetin, [44,45] quercetin-3-*O*-glucoside,[46] kaempferol 3-*O*- rhamnoside [47] and isorhamnetin 3-*O*-glucoside**.**[45] Based on the structure/activity relationship and mechanism of actions performed by flavonoids to regulate plasma glucose concentrations, former study suggested a certain structural requirements to be antidiabetic candidate, as found in the isolated quercetin derivatives with free OH groups at position 3′ and 4′.[48] Also, kaempferol derivatives with rhamnosyl groups in either position 3 or 7 are important structural requirements for hypoglycemic activity**.**[47]

Table 5: Effect of *M. indicus* extracts and reference drug (Metformin) on BGL of alloxan induced diabetic male rats (n=6)

	Blood glucose level (mg/dl)			% change		
	zero	**2 weeks**	**4 weeks**	**zero**	**2 weeks**	**4 weeks**
Healthy rats	82.3 ± 0.4	81.6 ± 0.2	82.4 ± 0.5	-	-	-
DM_rats	243.6 ± 7.4	256.3 ± 6.5	263.4 ± 7.8	-	-	-
DM_rats + AME	257.4 ± 8.1	216.5 ± 7.8*	96.9 ± 6.2*	-	15	62
DM_rats + PEE	261.2 ± 8.9*	205.4 ± 8.1*	119.3 ± 6.7*	-	21	54
DM_rats + DAME	265.1 ± 9.7	192.9 ± 7.8*	98.7 ± 6.1*	-	27	62
DM_rats + Metformin	265.4 ± 8.2*	191.5 ± 6.8*	81.9 ± 2.4*	-	27	69

DM_rats; Diabetes mellitus rats; AME ; 70% aqueous methanol extract, PEE; Petroleum ether extract, DAME; defatted aqueous methanol extract. *significantly different from control group at P < 0.01.

Antimutagenic activity

3.2.3.1 Percentage of inhibition of aberrations in bone marrow cells.

The current study demonstrates different number and percentage of abnormalities in all treated groups. DM_rats induced a high percentage of chromosomal aberrations (p < 0.01) in rat bone marrow cells reaching 12.20±0.72 compared to 3.4 ± 0.40 for the negative control. DM_rats orally administered AME, PEE and DAME of *M. indicus* (100mg/kg b.wt) for 4 weeks had the ability to decrease the percentage of aberrations in a statistically significant manner (p < 0.01). AME (75%) offered the highest percent of DNA preservation followed by PEE (54%) then DAME (43%) (Table 6). Chromosomal abnormalities of bone marrow cells in diabetic rat are shown in Figure (3).

Table 6: Effect of *M. indicus* extracts on the abnormalities of bone marrow cells in alloxan induced diabetic male rats (n = 5)

Treatments (mg/kg b.wt)	Total abnormal metaphases		No. of different types of abnormal metaphases				Inhibitory index (II)
	No.	**Mean(%) ± SE**	**Gap**	**Fragment and/or Break**	**Deletion**	**Polyploidy**	
Healthy rats	17	3.40±0.40	7	9	1	0	-
DM_rats	61	12.20±0.72 [a]	11	36	7	7	-
DM_rats + AME	28	5.60±0.74 [ab]	9	10	4	5	75
DM_rats + PEE	37	7.40±0.89 [ab]	11	21	5	0	54
DM_rats + DAME	42	8.40±0.87[ab]	8	25	7	2	43

DM_rats; Diabetes mellitus rats; AME ; 70% aqueous methanol extract, PEE; Petroleum ether extract, DAME; defatted aqueous methanol extract. Number of examined metaphases = 500 (100 metaphase/animal, 5 animals/ group), a; significant compared to negative control (p < 0.01), b; Significant compared to DM group (p < 0.01); t-test.

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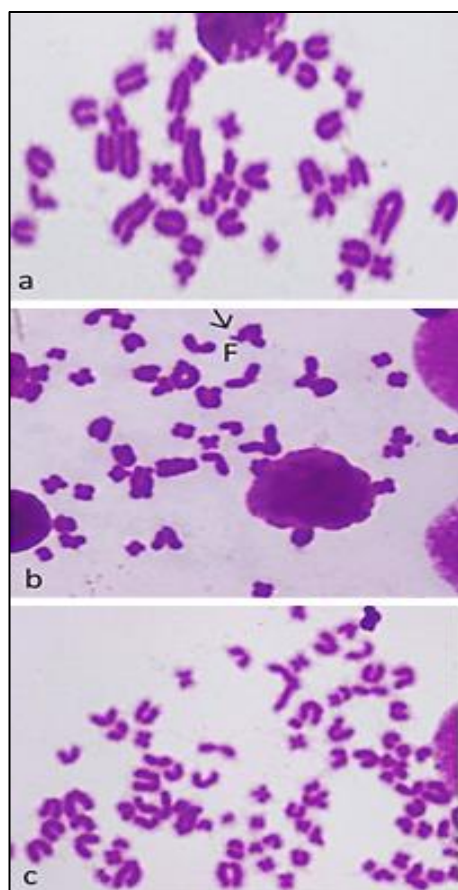

Figure 3: Chromosomal abnormalities of bone marrow cells in diabetic rat treated with *M. indicus* extracts showing: (a) Normal, (b) Fragment, and (c) Polyploidy

Percentage of inhibition of sperm abnormalities

DM_rats group induced a high percentage ($p < 0.01$) of sperm abnormalities reaching 13.82 ± 0.65, compared to 3.04 ± 0.47 for the negative control (Table 7). Administration of AME, PEE and DAME of *M. indicus* (100 mg/kg b.wt, 4 weeks) minimized the sperm abnormalities in a statistically significant manner ($p < 0.01$) with percentage of inhibition reaching 76 %, 55 % and 45 %, respectively (Table 7). The shape of sperm abnormalities in diabetic male rat are shown in Figure (4).

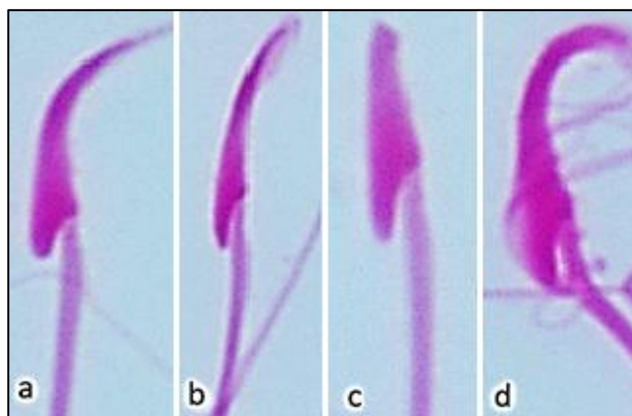

Figure 4: Sperm abnormalities in diabetic male rat treated with *Melilotus indicus* extracts showing: (a) Normal, (b) Banana, (c) Straight and without hook, and (d) Amorphous

The obtained data proved elevation in DNA damage induced by DM causing significant increase in chromosomal abnormalities of somatic and germ cells in DM_rats compared with untreated ones ($p < 0.01$), which is compatible with previous findings. [49,50] Promotion in the frequency of chromosomal abnormalities might be precious by oxidative stress and GSH concentrations in DM_rats. [51]

Phenolics, especially flavonoids employ their genoprotection by reducing oxidative stress and modulation of enzymes responsible for bioactivation of genotoxic agents and detoxification of their reactive metabolites. Certain phenolics either isolated or detected in the present study are well recognized for their antigenotoxic potential in prior literature, such as some quercetin, kaempferol and hydroxycinnamic acid derivatives.[15,50]

Table 7: Effect of *M. indicus* extracts on the sperm abnormalities in alloxan induced diabetic male rats (n = 5)

Treatments (mg/kgb.wt)	Abnormal sperms		No. of different types of sperm abnormalities						Inhibitory index (II)
	No.	Mean % ± SE	Straight	Banana shape	Amorphous	Without hook	Big head	Coiled tail	
Healthy rats	152	3.04 ± 0.47	49	60	14	25	1	3	-
DM_rats	691	13.82 ± 0.65^{a}	192	233	42	189	6	29	-
DM_rats + AME	280	5.60 ± 0.82^{ab}	43	123	28	73	7	6	76
DM_rats + PEE	391	7.82 ± 0.75^{ab}	101	162	42	69	4	13	55
DM_rats + DAME	450	9.00 ± 0.66^{ab}	119	207	24	83	2	15	45

DM rats; Diabetes mellitus rats; AME ; 70% aqueous methanol extract, PEE; Petroleum ether extract, DAME; defatted aqueous methanol extract. Number of examined metaphases = 500 (100 metaphase/animal, 5 animals/group); a: significant compared to negative control ($p < 0.01$); b: significant compared to DM group ($p < 0.01$); t-test.

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Conclusion

The lipoidal content of *M. indicus* was investigated for the first time through GC/MS analysis to identify thirty five non polar compounds. Also, fourteen compounds have been isolated from the polar content (DAME); nine of them were reported for the first time from *M. indicus.* Moreover, the polar extract was further analyzed using LC-ESI-MS which led to the tentative identification of extra eight compounds, which showed the majority of kaempferol and coumaric acid glycosides. The significant antioxidant, antidiabetic and antimutagenic activities shown by *M. indicus* extracts can be attributed to the abundant fatty acids and flavonoid glycosides. The current study suggests *M. indicus* as a source of a long term natural drug designed to keep diabetic condition under control and lower the risk of diabetes complication. Further *in vivo* studies are necessary to determine its viability in antidiabetic clinical trials.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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