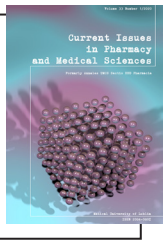
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In vivo and *in vitro* antidiabetic potential of *Taraxacum officinale* root extracts

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ABSTRACT

Taraxacum officinale F.H. Wigg (Asteraceae) root is traditionally used to treat diabetes, dyspepsia, heartburn, anorexia and hepatitis. In this work, petroleum ether, chloroform, methanol and aqueous extracts of T. officinale root were evaluated for their antidiabetic activity in normoglycemic and alloxan-induced diabetic mice at two concentrations (200 and 400 mg/kg) using antidiabetic and subcutaneous glucose tolerance tests. Herein, *in vitro* glucose uptake assay was performed using HepG2 and 2-NDBG, while LC-MS/MS was employed for the phytochemical study of the main active constituents in the active extract. In the experiments, *T. officinale* root aqueous extract (400 mg/kg) showed a significant decrement in blood glucose level (62.33%, $p \leq 0.05$), while other extracts ($p > 0.05$) showed insignificant activity – in alloxan-induced diabetic mice with no apparent effect on the normoglycemic model. The extracts also showed an insignificant reduction in glucose levels ($p > 0.05$) in the subcutaneous glucose tolerance test. However, a significant glucose uptake enhancement (149.6724%, $p \leq 0.05$) was exhibited by the aqueous extract. Phytochemical study of the aqueous extract showed higher total phenolic than total flavonoid content, in which chlorogenic acid, protocatechuic acid, and luteolin-7-glucoside were identified.

INTRODUCTION

Diabetes mellitus is a crucial worldwide health problem characterized by elevated blood glucose levels owning to the lack of insulin secretion or resistance to insulin at the cellular level [1]. Attempts to discover new drugs have resulted in considerable progress in the treatment of diabetes by synthetic hypoglycemic oral drugs. However, there are several limitations. Hepatorenal disturbance and hypoglycemia are the common side effects associated with synthetic drugs [2]. Botanically derived drugs such as *Acacia arabica* (Lam.) Muhl. ex Willd. (Family: Mimosaceae), *Aegle marmelos* (L.) *Correa ex Roxb*. (Family: Rutaceae), *Brassica juncea* (L.) Czern. (Family: Brassicaceae), *Catharanthus roseus* (L) G. Don. (Family: Apocynaceae), *Camellia sinensis Kuntze* (Family: Theaceae) play important role in the traditional medicine for managing diabetes by various mechanisms such as enhancing glucose uptake, increasing insulin secretion, inhibiting hepatic glucose production and intestinal absorption [3-5]. The scientific investigation of the phytochemical nature and ethnobotanical information of plant material support the correct usage of herbal remedies [6,7].

Taraxacum officinale F.H. Wigg. (Asteraceae), commonly known as dandelion [8,9] is a well recognizable weed widely distributed in the warm temperate zones of the Northern Hemisphere [9,10]. *T. officinale* is a herbaceous perennial plant with an average height of 15-30 cm and roots of 60-100 cm in length that bears small yellowish flowers [9,11]. Traditionally, *T. officinale* root has been considered to be a valuable remedy used as infusions and decoctions to treat various diseases such as kidney diseases, dyspepsia, heartburn, spleen and liver ailments, anorexia and hepatitis [9,12,13]. Furthermore, *T. officinale* was held to have aperient, diuretic, stimulant, stomachic, tonic, antidiabetic and detoxicant activities [14].

The main reported active constituents of *T. officinale* roots are taraxacerin, taraxacin, inulin, gluten, gum, and potash. *T. officinale* is considered an important natural source for the beta-carotene from which vitamin A is generated. The plant thus has great value for both medicinal and culinary purposes [14,15].

T. officinale roots are used traditionally in Erbil, Iraq as an effective remedy for the management of diabetes and

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other ailments. The aim of this study is *in vivo* and *in vitro* evaluation of the antidiabetic potential of *T. officinale* root extracts and identification of the main active constituents.

MATERIAL AND METHODS

IN VIVO ANTIDIABETIC STUDY

1. Plant material

Roots of *T. officinale* were collected during May 2016 from discrete regions of Erbil, Iraq. The plant was authenticated by Assistant Professor Alaadin Naqishbandi, the roots were cleaned, dried in shade for 4-6 days and kept in closed containers at 25°C. The voucher specimen (L-21) is stored at Pharmacognosy Department, Pharmacy College, Hawler Medical University, Erbil, Kurdistan Region/Iraq.

2. Plant extract preparation

Powdered plant material (1000 g) was introduced for successive extraction using petroleum ether, chloroform, methanol and water in increasing polarity manner at a ratio of 1:3 (one part of the dried plant material to three parts of the solvent) as described by Mona *et al*. (2012) [16] by means of applying the ultrasonic assistant extraction method (200 kHz, 1h, 40°C) [17]. The liquid extracts were dried using rotary vapor machine (Buchi Rotavator®, Switzerland) for petroleum ether, chloroform and methanol extracts and freeze drier (Martin Christ Alpha 1-2 LD plus, Germany) for water extract yielding [1.49, 0.58, 19.42, 12.07% (w/w) extract to plant material weight] to afford the corresponding extracts TOPEF, TOCF, TOMF, and TOAF, respectively. All extracts were kept at 4°C until used for antidiabetic evaluation. For acute toxicity and *in vivo* study, petroleum ether and chloroform extracts were reconstituted in tween 80 (20%) and normal saline used as a solvent for reconstitution of methanol and aqueous extracts.

3. Animals

Adult Swiss albino mice (mean weight 25 g/mouse) were obtained from the animal house of the Medicine College, Hawler Medical University. They were housed in groups of 6 animals per polypropylene cage at a temperature 22±3°C under 12h light/12h dark cycle. The animals were allowed to access commercial food and tap water *ad libitum*. The experimental procedure was approved by the pharmacy college, Hawler medical university ethic committee (code number 160302/20).

4. Acute toxicity test

The experiment was conducted in accordance with the guidelines of the Organization for Economic Cooperation and Development (OECD) for the testing of chemicals [18]. Twenty-four female albino mice divided randomly into four groups each of six mice were administered a single oral dose (2000 mg/kg of body weight BW) each of petroleum ether, chloroform, methanol and aqueous extracts, respectively. All animals were kept under close observation for 24 h. Monitoring continued for 7 days until any changes either in general behavior or physical activity were noticed.

5. Experimental protocol

The antidiabetic test methods described by Khalid *et al*. (2013) were adopted [19], with slight modification. Diabetes was induced by intraperitoneal injection of 200 mg/kg BW of alloxan (Sigma Aldrich Chemical Co., UK) as an initial dose, followed by 150 mg/kg BW as the second dose after 72h to ensure the appearance of diabetic symptoms. After 16h fasting, mice were examined for hyperglycemia using a counter blood glucose meter (Bayer). Mice with blood glucose levels higher than 200 mg/dL were included in the study as diabetic mice.

6. Antidiabetic test

Sixty-six mice each of normal and diabetic-induced models were divided randomly into 11 groups (n=*6*). Group (1-8) received 0.5 mL each of extracts (200 and 400 mg/kg BW), group (9) received 0.5 mL metformin (500 mg/kg BW in normal saline), group (10) received 0.5 mL normal saline, and group (11) received 0.5 mL tween 80 (20%).

In all groups, oral administration was performed by a specific mouse feeding needle. Blood was collected from the tail vein at 0, 1, 2, 3, 5 and 7h for glucose level measurements.

7. Subcutaneous glucose tolerance test (SGTT)

A similar protocol of animal distribution in the antidiabetic test was applied for SGTT. Fifteen minutes after oral drug administration, glucose (50 mg/mL) was subcutaneously administered 1.5 mL to all groups. Blood samples were collected from the tail vein for glucose level measurements at 0 min (before drug administration) and at 15, 30, 45, 60, 90 and 120 min after glucose administration.

8. Statistical analysis

Mean of hypoglycemic results were expressed as mg/dL ± standard error of the mean (SEM) for groups of six animals. The comparison was performed via one-way analysis of variances using (ANOVA), and for intergroup comparison – Dunnett's test post hoc. SPSS software version 23 was employed for data analysis. P *values* ≤0.05 are considered as statistically significant.

IN VITRO HYPOGLYCEMIC STUDY

Glucose uptake assay was performed on the antidiabetic extract (TOAF) using fluorescence glucose analog 2-(n-(7-nitrobenz-2-oxa-1,3-diazol- 4-yl) amino)-2-deoxyglucose (2-NBDG) and liver carcinoma cell HepG2 obtained from the tumor bank of the German Cancer Research Center (Heidelberg, Germany).

1. Cell culture

Human liver carcinoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with Gluta Max (Invitrogen, Germany) containing 10% fetal bovine serum (FBS, Invitrogen, Germany) and 1% penicillin/streptomycin (Invitrogen, Germany), incubated in 5% CO_2 atmosphere at 37°C.

2. Cytotoxicity assay

Resazurin reduction assay was conducted to evaluate the cytotoxic effect of the antidiabetic active extract of *T. officinale* (TOAF) on HepG2 cells. The principle of the assay is based on the conversion of the resazurin to its reduced form resorufin by viable cells with blue colour intensity detected in non-viable cells [20]. In brief, an aliquot of 100 µL contained (5×10^3) cells per well were seeded in a 96-well plate maintained for 24h incubation at 37°C, 5% CO_2. After the incubation period, 100 µL of the reconstituted extract was used to make final concentration of [50 µg/mL and 100 µg/mL (w/v)], dimethyl sulfoxide (DMSO) at final concentration (0.3%, v/v), and this and media were added to the cells and further incubated for 72h at 37°C, 5% CO_2. The controls included in the analysis were DMSO (0.3%)+ cells and media + cells. Resazurin (Sigma-Aldrich) solution (0.01% w/v) was added to each well at volume 20 µL, the plates were then incubated for 4h. Absorbance was measured using Infinite M2000 Pro TM plate reader (Tecan, Germany) with 544 nm (excitation) and 590 nm (emission). The assay was independently performed three times with six replicates according to a reported protocol [21].

3. Glucose uptake assay

3.1. Experimental design

The maintained HepG2 cell was divided into 3 groups:
Group I: cells of control were cultured with the maintenance media (DMEM, glucose-free, serum-free).
Group II: cells were cultured with metformin at a concentration (100 µM/mL) reconstituted in glucose-free, serum-free media.
Group III: cells were cultured with TOAF at a final concentration (50 µg/mL, w/v) reconstituted in glucose-free, serum-free media.

3.2. 2-NBDG uptake

Differentiated groups of cells were examined for the fluorescent D-glucose derivative [2-deoxyglucose, 2-(N-(7-nitrobenz-2-oxa-1,3- diazol-4-yl)-amino)-2-deoxy-d-glucose] (2-NDBG) uptake [22]. The seeded cells in a 96-well plate with differentiated groups were washed out after incubation of 24h, using phosphate buffer saline (PBS) (200 µL). Subsequently, the cells were incubated with 2-NDBG (for a final concentration of 100 µM reconstituted in glucose-free, serum-free media) for an additional 2h. After treatment, the free 2-NDBG was aspirated and the cells were washed with 200 µL PBS twice, and 200 µL of dimethyl sulfoxide (DMSO) was added to each well. Fluorescence was measured using an Infinite M2000 Proplate reader (Tecan, Germany) with 544 nm (excitation) and 590 nm (emission). The glucose uptake of the control group was set at 100 (2-NBDG) uptake, and the 2-NBDG of the sample treated groups was calculated according to the following equation:

$$\text{2-NBDG uptake (\%)} = (A_{sample}/A_{control}) \times 100$$

where *A* is absorbance.

PHYTOCHEMICAL STUDY

1. Total phenolic and total flavonoid determination

The total phenolic content (TPC) of antidiabetic extract TOAF was estimated using Folin-Ciocalteu reagent to be equivalent to pyrocatechol [23] by applying the equation ($y = 0.0347x + 0.0384$, and the determination coefficient (R^2) = 0.9916) acquired from the pyrocatechol calibration curve (0-4 µg/mL). TPC is expressed as the mean values (± SEM) of pyrocatechol (µg) per 1 g extract. Total flavonoid content (TFC) was estimated using the aluminum nitrate method [24], with the equation acquired from the calibration curve of quercetin ($y = 0.0445x + 0.1352$, $R^2 = 0.9962$) expressed as mean values (± SEM) of µg quercetin per 1 g extract.

2. LC-MS/MS

Shimadzu Nexera model ultra-high performance liquid chromatography (UHPLC) connected with a Shimadzu LCMS 8040 model triple quadrupole mass spectrometer (Shimadzu, Japan) was used to evaluate the antidiabetic extract (TOAF), using a method adapted from Akdeniz, 2018 [25].

The liquid chromatography components were an LC-30 AD gradient pump, a SIL-30AC auto-sampler, a DGU-20A3R degasser and a CTO-10ASvp column oven. The chromatographic separation was performed on an Inertsil ODS-4 model C18 (100 mm × 2.1 mm, 2 µm) column. The column temperature was maintained at 35°C during the analysis. The mobile phase A consisted of water, with the addition of 10 mM ammonium formate (Chem-Lab, Belgium) – 0.1% formic acid (Chem-Lab, Belgium) to the water phase to facilitate chromatographic separation and ionization. The mobile phase B was methanol (Chem-Lab, Belgium). The applied gradient profile was optimized as 5 – 20% B (0-10 min), 20% B (10-22 min), 20 – 50% B (22-36 min), 95% B (36-40), 5% B (40-50 min). The flow rate of the mobile phase was 0.25 mL/min and the injection volume was 4 µL.

The triple quadrupole mass spectrometer was equipped with an ESI (electrospray ionization) source that operates in a negative mode. LC-ESI-MS / MS data were collected and processed by the software registered in LabSolutions (Shimadzu, Kyoto, Japan).

In the MRM (multiple reaction monitoring) mode applied for the quantitative determination of analytes studied the molecular (parent) ions and the product ions, the other parameters optimized in the spectrometer were: 350°C interface temperature, 250°C desolvation line (DL) temperature, 400°C heat block temperature, while 3 L/min and 15 L/min were nebulizer and drying gas (N2) flow rates, respectively. The authenticated standards Protocatechuic acid, chlorogenic acid, and luteolin-7-glycoside were purchased from Sigma-Aldrich, Germany.

3. Validation parameters

The method performance was evaluated using standard spiked and non-spiked samples. For method validation, linearity, precision (repeatability and reproducibility), limits of detection (LODs) and quantification (LOQs), trueness (recovery), and relative standard uncertainty (U% at 95% confidence level (k) = 2) are provided in Table 1 and Figure 1.

Table 1. Analytical parameters of LC-MS/MS study

No	Analytes	RT[a]	Precursor ion (m/z)[b]	Fragment ions	Ionization mode	Equation	[c]R²	Linearity (µg/L)	LOD/LOQ (µg/L)[e]	(% RSD[d])		Recovery (%)		U[f]
										Intraday	Interdays	Intraday	Interdays	
1	Protocatechuic acid	7.00	153.4	109.1-108.0	Neg	y=590.460x + 120226	0.9909	100-3200	4.26/5.32	0.0060	0.0060	1.0096	0.9988	0.0215
2	Chlorogenic acid (5-CQA)	8.03	353.3	191.2-85.0	Neg	y=697.935x + 87418.5	0.9910	75-2400	2.44/3.36	0.0074	0.0055	0.9941	0.9999	0.0299
3	Luteolin-7-glucoside	13.20	447.0	285.1-284.1	Neg	y=215.412x + 36852.1	0.9939	75-2400	2.30/3.02	0.0052	0.0037	1.0014	1.0072	0.0086

[a] RT: Retention time, [b] Precursor ion(*m/z*): Molecular ions of standard compounds (m/z oranı), [c] R^2: Correlation coefficient, [d] RSD: Relative standard deviation, [e] LOD/LOQ (µg/L): Limit of determination/Limit of quantification, [f] U (%): Percent relative uncertainty at 95% confidence level (k=2)

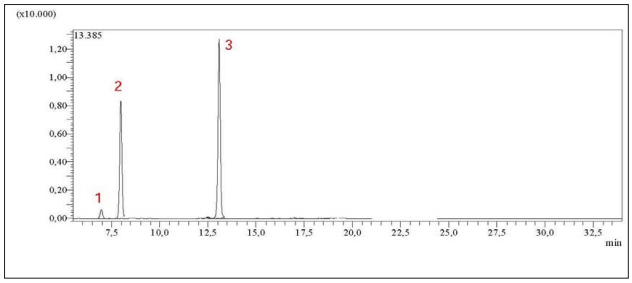


Figure 1. Standard LC-MS/MS chromatogram for: 1 - Protocatechuic acid, 2 - chlorogenic acid and 3 - luteolin-7-glycoside

RESULTS

IN VIVO ANTIDIABETIC STUDY

Acute toxicity test

The oral administration of high dose (up to 2000 mg/kg BW) *T. officinale* root extracts did not exert significant changes in the general behaviour and physical activity of the mice. Observation was estimated according to the OECD 2011 parameters [18], including alertness, motor activity, breathing, restlessness, coma, convulsion, diarrhea and general appearance of the animal. There was no recorded mortality within the 24h observation period after oral administration of the plant extracts. After the entire observation period of 7 days, the mice looked normal regarding physical activity, which demonstrates that the doses of plant extracts used did not exert any adverse effect on the animals tested.

Antidiabetic test

The mean values (± SEM) of blood glucose levels in the normal mice models are shown in Table 2. Fasting followed by treatment with various *T. officinale* extracts showed no activity.

Diabetic mice blood glucose levels (mean ± SEM) and percentage of decrement from fasting blood glucose levels are listed in Table 3. Blood glucose levels decreased after the administration of the different extracts of *T. officinale*. Petroleum ether and chloroform extracts 400 mg/kg BW showed a reduction percentage of 28.51% at the fifth hour after extracts administration. The highest activity was recorded for the aqueous extract (400 mg/kg BW, 62.33%) at the fifth hour maintained to an approximately similar amount of decrement (60.5%) at a seventh hour after extract administration, which reached significantly higher values than the positive control metformin (58.93%, $p \leq 0.05$).

Subcutaneous glucose tolerance test

We recorded no apparent antidiabetic activity for the various extracts in comparison to metformin within the used range of drug concentration in both tested animal models ($p > 0.05$) in SGTT (Fig. 2-5).

In vitro antidiabetic study

As an attempt to illustrate the mechanism of action of *T. officinale* aqueous extract antidiabetic extract, glucose uptake assay was applied on HepG2 cells, using fluorescent glucose derivative (2-NDBG) as a detector.

Resazurin assay

Antidiabetic extract TOAF, which was evaluated for its cytotoxic effects on HepG2 cells using a resazurin assay, showed that a 50 µg/mL concentration is safe for use in a glucose uptake assay due to its higher percentage of viability (72.51%) compared to 100 µg/mL at (68.15%).

Table 2. Antidiabetic test of *T. officinale* root extracts in normal mice (n=*6*)

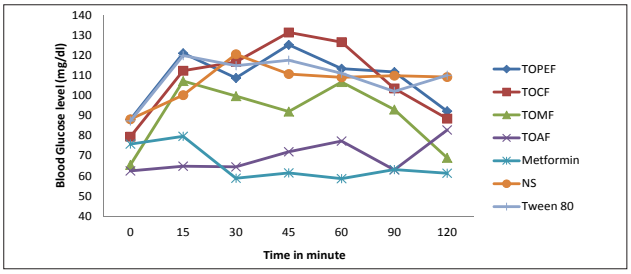
Treatment groups [1]	Blood glucose level mg/dL [2]					
	FBG	1 h	2 h	3 h	5 h	7 h
TOPEF (200 mg)	97.83±5.49	145±1.98	132±1.57	101±1.81	109.67±1.51	121±2.25
TOPEF (400 mg)	98.67±1.11	128.83±4.33	118.16±1.38	114±3.22	104±1.99	93.33±0.9
TOCF (200 mg)	81.5±1.96	94.167±4.26	80.167±2.33	86±2.53	131.83±3.65	122.16±2.26
TOCF (400 mg)	106.5±1.98	129.5±1.77	129.16±2.69	130.83±1.82	124.67±2.54	112.83±2.04
TOMF (200 mg)	80.5±1.13	114.67±1.33	107.167±6.99	103.167±2.79	128.5±3.67	98.17±0.55
TOMF(400 mg)	95.33±1.05	125.833±1.14	118.67±1.21	119.67±0.83	107.33±2.07	93.167±1.38
TOAF (200 mg)	86.67±1.44	134.167±2.82	133.67±2.36	120.5±1.17	132.5±4.56	98.83±0.82
TOAF (400 mg)	81.5±1.96	94.167±4.27	80.167±2.35	86±2.54	131.83±3.66	122.167±2.26
Control I (NS)	99.167±1.27	119.167±1.83	119.67±0.79	109.67±1.86	115.167±2.02	98.5±0.98
Control II (Tween 80)	102.67±1.12	120.167±2.51	118.83±3.07	115±2.24	104±3.68	101.67±3.51
Metformin	95±1.28	93.83±3.09	61.667±2.19	60.167±1.44 (36.667%) [3]	71.167±7.467 (25.087%)	74.333±7.062 (21.754%)

[1] TOPEF – petroleum ether extract, TOCF – chloroform extract, TOMF – methanol extract, TOAF – aqueous extract, and NS – normal saline
[2] Values expressed as mean ± SEM
[3] Value in the parenthesis is the percentage of blood glucose decreasing from fasting blood glucose level

Table 3. Antidiabetic test of *T. officinale* root extracts in alloxan-induced diabetic mice (n=6)

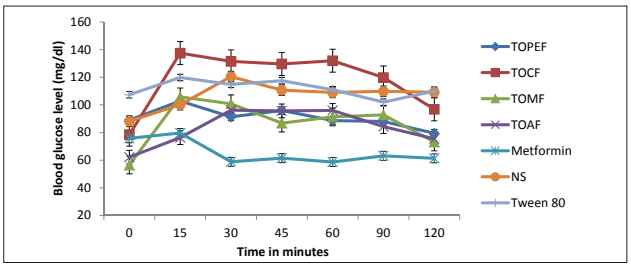
Treatment groups [1]	Blood glucose level mg/dL [2]					
	FBG	1 h	2 h	3 h	5 h	7 h
TOPEF (200 mg)	287.67±3.13	339.83±0.95	376.83±1.71	229.17±4.13 (20.33%)	229.17±4.66 (20.33%) 3	248±4.54 (13.78%)
TOPEF (400 mg)	274.17±2.89	323±0.97	358.83±1.56	196±3.92 (20.51%)	196±0.85 (28.51%)	227.33±0.74 (17.08%)
TOCF (200 mg)	301.17±1.19	367.17±5.48	459.5±2.23	358.83±3.92	212.67±0.68 (29.38%)	210.67±0.65 (30.05%)
TOCF (400 mg)	273.67±1.53	326.67±2.6	388.167±5.03	339.33±5.03	188.17±4.16 (31.2%)	170.33±5.6 (37.7%)
TOMF (200 mg)	364.5±3.03	361.67±0.68	396.67±0.74	465.5±0.95	371±0.9	321.83±5.94
TOMF(400 mg)	348.5±2.5	339±0.63	349.33±5.3	423±0.82	299.83±1.13 (13.965%)	269±5.96 (22.8%)
TOAF (200 mg)	317.5±0.81	300±0.62 (5.511%)	307.17±0.71 (3.254%)	270.17±1.49 (14.908%)	189.33±4.51 (40.36%)	207.33±3.46 (34.69%)
TOAF (400 mg)	309.67±0.74	287.5±0.82 (7.158%)	262.83±0.84 (15.123%)	242.5±5.55 (21.689%)	116.67±3.96 (62.32%) [3]	122.33±2.85 (60.5%)
Control I (NS)	444.67±1.28	427.17±4.53	430.5±0.67	421±5.96	461±3.38	439.33±0.6
Control II (Tween 80)	382.5±1.12	389.33±0.78	393.83±0.61	393±0.95	377.33±1.62	368.83±1.19
Metformin	423.67±1.3	364.5±5.4	307±3.786 (27.54%)	280.833±5.58 (33.71%)	250.33±2.3 (40.91%)	174.±1.32 (58.93%)

[1] TOPEF – petroleum ether extract, TOCF – chloroform extract, TOMF – methanol extract, TOAF – aqueous extract, and NS – normal saline
[2] Values expressed as mean ± SEM
[3] Value in the parenthesis is the percentage of blood glucose decreasing from fasting blood glucose level
[4] Significant vs metformin (p≤0.05)



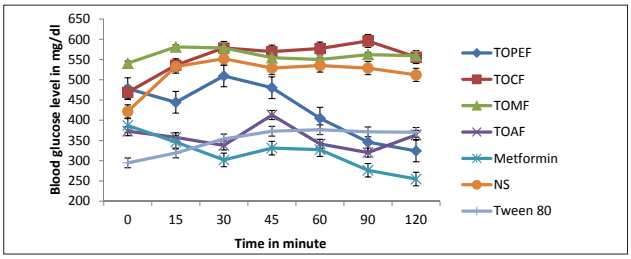
The data were expressed as mean ± SEM for n=6. TOPEF = Petroleum ether extract, TOCF = Chloroform extract, TOMF = Methanol extract, TOAF = Aqueous extract, NS = Normal saline

Figure 2. Antidiabetic of *T. officinale* root extracts (200 mg/kg of BW) measured by the subcutaneous glucose tolerance test in normal mice



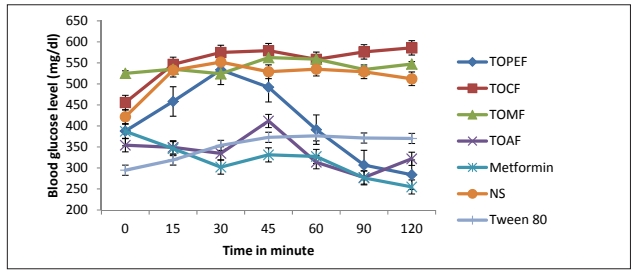
The data were expressed as mean ± SEM for n=6. TOPEF = Petroleum ether extract, TOCF = Chloroform extract, TOMF = Methanol extract, TOAF = Aqueous extract, NS = Normal saline

Figure 3. Antidiabetic effect of *T. officinale* root extracts (400 mg/kg) measured by the subcutaneous glucose tolerance test in normal mice



The data were expressed as mean ± SEM for n=6. TOPEF = Petroleum ether extract, TOCF = Chloroform extract, TOMF = Methanol extract, TOAF = Aqueous extract, NS = Normal saline

Figure 4. Antidiabetic effect of *T. officinale* root extracts (200 mg/kg of BW) measured by the subcutaneous glucose tolerance test in alloxan-induced diabetic mice

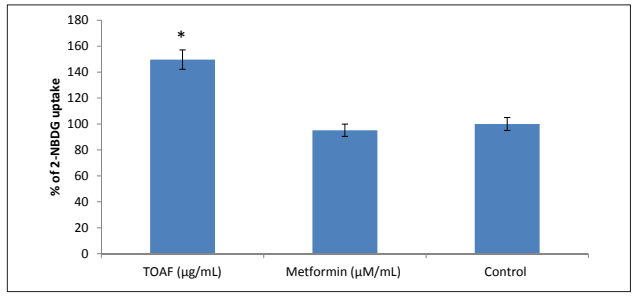


The data were expressed as mean ± SEM for n=6. TOPEF = Petroleum ether extract, TOCF = Chloroform extract, TOMF = Methanol extract, TOAF = Aqueous extract, NS = Normal saline

Figure 5. Antidiabetic effect of *T. officinale* root extracts (400 mg) measured by the subcutaneous glucose tolerance test in alloxan-induced diabetic mice

Glucose uptake assay

A significant elevation in the 2-NDBG uptake ($p<0.05$) was recorded for the HepG2 cells in the presence of the *T. officinale* root aqueous extract (TOAF), in comparison to the negative control and metformin (Fig. 6).



Results expressed as (mean ± SEM). The bar with (*) symbol indicates a significant elevation of the 2-NBDG uptake ($p<0.05$) in comparison to metformin and control groups using student t-test analysis

Figure 6. 2-NBDG uptake of *T. officinale* root aqueous extract (TOAF) at (50 µg/mL), Metformin at (100 µM/mL), control untreated group using HepG2 cell line

Phytochemical study

The total phenolic and flavonoid contents for the antidiabetic aqueous extract of *T. officinale* root was (46.97±2.88 equivalent to µg pyrocatechol per 1 g dry extract) for the phenolic content and flavonoids contents (22.2±0.22

equivalent to µg quercetin per 1 g dry extract) expressed as mean ± SEM. The LS-MS/MS analysis revealed the presence of three evaluated analytes with the highest quantity of chlorogenic acid (Tab. 4 and Fig. 7).

Table 4. Evaluated analytes quantity in *T. officinale* root aqueous (TOAF) extract using LC-MS/MS technique

Evaluated analyte	Quantity (µg/g of dry extract)
Protocatechuic acid	16.87
Chlorogenic acid (5-CQA)	66.9
Luteolin-7-glucoside	18.39

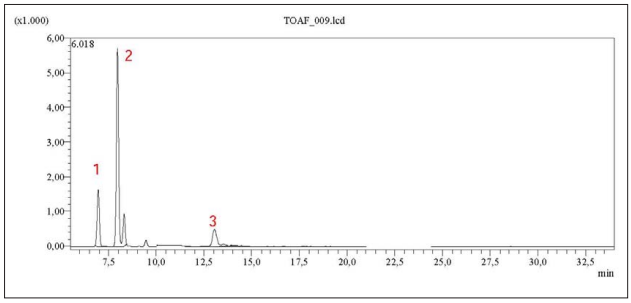


Figure 7. LC-MS/MS chromatogram for *T. offficinale* root aqueous extract (TOAF). [1] Protocatechuic acid, [2] Chlorogenic acid and [3] Luteolin-7-glucoside

DISCUSSION

T. officinale root extracts showed no apparent activity in normoglycemic mice at evaluated concentrations (200 and 400 mg/kg BW). The results indicate the advantageous of the plant extracts over the conventional antidiabetic drugs characterized by hypoglycemia.

The aqueous extract (TOAF) results revealed a strong significant antidiabetic activity at the highest concentration (400 mg/kg BW) in alloxan-induced diabetic mice started at the fifth-hour post-extract administration and maintained until the seventh hour of administration ($p \leq 0.05$). A similar manner of blood glucose-lowering activity was recorded at lower concentration (200 mg/kg) for the same extract (40.36% and 34.69%) at the fifth and seventh hour, respectively, but insignificant in comparison to the positive control metformin ($p > 0.05$).

Other extracts of *T. officinale* root such as chloroform (200 and 400 mg/kg BW) and methanol (400 mg/kg BW) showed more than 25% blood glucose reduction, but at a statistically insignificant potency ($p > 0.05$) compared with metformin at the tested concentrations. Of note, Khan and Shechter (1991) suggested that a 25% reduction in blood glucose levels can be considered a significant hypoglycemic activity [26].

The delayed antidiabetic response expressed by petroleum ether, chloroform and methanol extracts could be due to the presence of the slow absorption of the phytoconstituents in the gastrointestinal tract that required a lag phase to reach a sufficient concentration at the targeted site. A similar delayed hypoglycemic pattern was recorded for plants such as *Phyllanthus debilis* L [27], and *Nycanthus arbor-tristis* L [28].

SGTT was used to evaluate the efficacy of the extracts to diminish the postprandial glucose level. The subcutaneous route of administration was indicated instead of the commonly used oral route to circumvent the possible false-positive results that might result from delayed absorption of glucose due to interaction with sticky/waxy and viscous extracts (especially the petroleum ether and chloroform extracts). Results of SGTT showed insignificant blood glucose reduction in comparison to metformin in both normoglycemic and alloxan-induced diabetic mice. The maximum blood glucose reduction of *T. officinale* root extracts in SGTT was recorded for the petroleum ether extract in diabetic mice at 120 min time interval at 400 mg/kg BW.

The antidiabetic extract (TOAF) was introduced to the cell-based *in vitro* study to identify the potential mechanism of antidiabetic activity of the extract. The obtained results showed a significant elevation ($p < 0.05$) in the 2-NDBG uptake using HepG2 cells in comparison to metformin. The results could indicate a metformin mimic-like mechanism of action of *T. officinale* antidiabetic activity. Metformin oral hypoglycemic agents belong to the biguanide class, exerting their activity through activation of AMP-activated protein kinase (AMPK) in the liver, consequently leading to a series of pharmacological effects, including, inhibition of glucose production, improved hepatic sensitivity to insulin and lipid synthesis [29,30]. Accordingly, *T. officinale* root aqueous extract may be hypothesized to be linked to the activation of the insulin signaling cascade, resulting in stimulation of glucose transporter 2 (GLUT 2) which facilitates the glucose translocation in the cells.

In vivo and *in vitro* study outcomes suggest a dual activity of the *T. officinale* root as an antihyperglycemic and hypoglycemic prophylaxis remedy. Our findings are supported by a hypothesis made by Iddrisu and coworkers [31] on the *T. officinale* activity on blood glucose concentration. Iddris and coworkers suggested that *T. officinale* enhances glucose uptake by the liver, which means normalizes the glucose levels in the blood, and later the glucose is released gradually when the body is running out of energy/glucose. In fact, it could be able to delay the onset of hypoglycemia, but assists in the prevention of hyperglycemia. Consequently, *T. officinale* lowers blood glucose concentrations, but not to the hypoglycemic state. The dual activity of *T. officinale* is further supported by the findings of both Jinchun and Jie [32], who suggested that *T. officinale* might delay the hypoglycemic onset and improve fatigue in mice, and Nnamdi and coworkers [33] who recorded blood-glucose-lowering efficacy after *T. officinale* root consumption using an animal model.

The antidiabetic extract TOAF was introduced to the phytochemical study to determine the probable phytochemicals responsible for the physiological activity. The study results showed the presence of the high phenolic compound, but low flavonoid concentrations. Phenolics and flavonoids are compounds known for their potential to lower blood glucose levels and antidiabetic potential [34-36]. This might be attributed to the potent antidiabetic activity of the aqueous extract.

LC-MS/MS data revealed the presence of chlorogenic acid (5-CQA), the main constituent of the aqueous extract (66.9 µg/g of extract), which is in an agreement with the Kenny *et al*., [37] data that recorded 0.09-0.51% Dw of chlorogenic acid in the *T. officinale* root. Chlorogenic acid is a known phytoconstituent that contributes to a crucial role in glucose metabolism by various mechanisms [38,39]. Other detected active constituents were protocatechuic acid and luteolin-7-glucoside in lower quantities. Both constituents also contribute to the antihyperglycemic potential of *T. officinale* root aqueous extract (400 mg/kg BW) [40,41]. The detected phytoconstituents are characterized by strong antioxidant activity [42,43] might provide a curial role in the antihyperglycemic activity of *T. officinale* root by amelioration of the oxidative stress – a well-known mechanism affecting glucose homeostasis. Future studies should clarify whether the full activity of the extract is due to a single phytochemical component or a synergistic effect of a mixture of diverse compounds present in each extract.

CONCLUSION

The current study demonstrated the *in vivo* antidiabetic potency of various root extracts of *T. officinale* in both normal and alloxan-induced diabetic mice and cell-based *in vitro* antidiabetic activity. Results revealed a significant antidiabetic potency of the aqueous extract comparable to metformin, with speculation of the activation of the insulin signaling pathways, and slight activity of petroleum ether and chloroform extracts in diabetic-induced animals with non-apparent effects in normoglycemic animals.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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