Journal of Applied Pharmaceutical Science Vol. 7 (05), pp. 174-183, May, 2017
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2017.70530
ISSN 2231-3354

Phenolic constituents of *Trifolium resupinatum* var. *minus*: Protection against rosiglitazone induced osteoporosis in type 2 diabetic male rats

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ARTICLE INFO

Article history:
Received on: 26/12/2016
Accepted on: 09/03/2017
Available online: 30/05/2017

Key words:
Persian clover, Thiazolidinediones, diabetes, serum bone markers, bone mineral density, osteogenic genes, phytoestrogens.

ABSTRACT

Thiazolidinediones, an antidiabetic drug, promote bone loss and provoke fractures, suggesting a protective role of phytoestrogens rich herbal supplement along with rosiglitazone. The aqueous methanol extract (AME) of *Trifolium resupinatum* L. var. *minus* Boiss. (Fabaceae) and defatted AME (DAME) protective effects were assessed against rosiglitazone (Ros) inducing osteoporosis in type 2 diabetic male rats. Eighty male Albino rats (10/group) were used. OPG, RANKL and β2- microglobulin serum levels, femur BMD (Dexa) and OC, COL and ACP5 osteogenic genes mRNA (qRT-PCR) were evaluated in Ros-rats + AME or DAME (24, 12, 6 mg/kg bw/d, each). Ros-rats + DAME (24 mg/kgbw) revealed the most significant effects on bone biomarkers. It modulated OPG (ng mL^{-1}), RANKL (pg mL^{-1}) and β2- microglobulin (µg mL^{-1}) serum levels. It also improved BMD (mg cm^{-2}) of proximal, distal and total femur bones compared to Ros-rats and up-regulated OC and COL and down-regulated ACP5 expressions. The phenolics of bioactive extract (DAME) were evaluated. Twelve known phenolics were isolated and characterized by chromatographic, chemical and spectral techniques. Their HPLC profiling was also analyzed and showed major phenolics; chlorogenic acid > formononetin> quercetin> pseudobaptigenin. DAME in combination therapy with rosiglitazone provides protective effect on bone, possibly due to its phenolics.

INTRODUCTION

Natural products, particularly plant extracts containing phenolics, are being explored for their protective effects against a variety of disease and disorders, including osteoporosis. Among the various phenolics, flavonoids have been reported to improve bone mineral density (BMD) and bone formation (Ma *et al*., 2008; Weaver, 2012). Isoflavones are a group of flavonoids called phytoestrogens that structurally resemble endogenous estrogens. It has been found to apply prospective health benefits in hormone dependent and age-related diseases, including cancer, cardiovascular disease, menopausal symptoms and osteoporosis (Wu *et al*., 2003). Phytoestrogens plays an important role in bone remodeling (Setchell and Lydeking-Olsen, 2003) and also up-regulate bone morphogenetic protein BMP2 gene transcription (Zhou *et al*. 2003). Isoflavones are found in a number of plant species, predominately in the members of legumes (Stahl *et al*., 1998). The genus *Trifolium* L. belongs to subfamily Faboideae (=Papilinoideae) of family Fabaceae (=Leguminosae), including about 300 species, widely distributed in subtropical and temperate regions of both hemispheres (Sabudak and Guler, 2009).

Some of *Trifolium* species are source of phytoestrogens, among them, the well-known one; *Trifolium pratense* L. (red clover) which is used for the manufacture of herbal drugs and as an alternative to the conventional hormonal replacement therapy (Kolodziejczyk-Czepas, 2012). The major constituents reported from the genus *Trifolium* are flavonoids, mainly isoflavones (Kazakov *et al*., 1973; Oleszek *et al*., 2007; Janda *et al*., 2009; Sabudak and Guler, 2009; Kicel and Wolbiś, 2012; Dabkevičienė *et al*., 2012), in addition to limited amount of fatty acids, clovamids, triterpenes, cyanogenic glucosides and phenolic acids

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(Oleszek *et al*., 2002; Sabudak *et al*., 2007; 2008a, b; 2009; Kolodziejczyk-Czepas, 2012). *Trifolium* species have been used in many countries as forage plants and for treatment of numerous diseases, such as eczema, psoriasis, fever, pneumonia, skin problems, sore throat, lung illnesses, stomachache, meningitis, diarrhea and swelling (Kolodziejczyk-Czepas, 2012). They have been also administered as analgesic, antiseptic, expectorant, and sedative (Sabudak *et al*., 2008b). An anti-diabetic activity has been reported for the seeds of *T. alexandrinum* L. growing in Egypt (Khaled *et al*., 2000). There are few available data for the biological action and phytoestrogen contents of some *Trifolium* species, among them the Persian clover; *T. resupinatum* growing wild in Egypt. The alcohol extract of the aerial parts of *T. resupinatum* L. var**.** *microcephalum* Zoh. showed anti-inflammatory and antioxidant activities in arthritic rats (Sabudak *et al***,.** 2008a), while the hexane extract of *T. resupinatum* L. var. *resupinatum* L. seeds revealed antioxidant action (Sabudak *et al***,.** 2009). Recently, several interesting finding on pharmacological effects and ethnomedicinal value of *T. resupinatum* have been reported; decoction of whole plant is used for whooping cough, skin sores and ulcer and digestive disorders (Renda *et al*., 2013; Sharma and Rana, 2014). Flowers are also used as sedative and to treat coughs (Gulshan *et al*., 2012; Kizilarslan and Özhatay, 2012).

From the phytochemical point of view; four flavonoids were reported from *T. resupinatum* var. *microcephalum* (Isik *et al*., 2007). They are kaempferol 3-O-(6"-acetyl)-β-D-galactopyranoside, genistein 7-O-β-D-glucopyranoside (please justify the distances). formononetin and formononetin 7-*O*-β- D - glucopyranoside. More recently, Janda *et al*. (2009) isolated nine phenolics from the aerial parts of *T. resupinatum* L. var. *majus* Boiss. They are represented as, two chlorogenic acids; 3-caffeoylquinic acid and 4-caffeoylquinic acid, four quercetin glycosides; quercetin 3-*O*-β-D-glucopyranoside, quercetin 3-*O*-(2"-β- D.glucopyranosyl)-β-D-galactopyranoside), quercetin 3-*O*-(3"-β-D-glucopyranosyl) (6"-α-L-rhamnopyranosyl)-β-D-galactopyranoside and quercetin 3-*O*-(6"-malonyl)-β-D-glucopyranoside), two kaempferol glycosides; kaempferol 3-*O*-(2"-β-D-glucopyranosyl)-β-D-galactopyranoside and kaempferol 3-*O*-(3"-β-D-glucopyranosyl) (6"-α-L-rhamnopyranosyl)-β- D-galactopyranoside, and one isoflavone glycoside; formononetin 7-*O*-β- D-glucopyranoside. An alkenol; 4,15-dimethyl-2-(1,2-dihydroxyethyl)-hexadecane, and an alkyl glucoside; 1-undecene-1-O-β-2',3',4',6'-tetraacetyl gluco-pyranoside, have been isolated from *T. resupinatum var*. *microcephalum* (Sabudak *et al***.,** 2008b). In addition, few fatty acids were reported for *T. resupinatum* var. *resupinatum* (Sabudak *et al*., 2009). Recent studies revealed significant decrease in total BMD in diabetic rats treated with rosiglitazone (Gupta *et al*., 2010; Zgórka, 2011). It was found that there is a strong correlation between the decrease in BMD, and therapy duration by glitazones (Ha *et al*., 2010; Schwartz *et al*., 2006). Thus, there is wide variety of newer therapeutic agents/strategies being examined for the treatment of type 2 diabetes management (T2DM), most of all currently under preclinical and early clinical stages of drug development. In view of the phytochemical and pharmacological properties of *Trifolium* species, this study was designed to assess the protective effect of the extracts from Egyptian *T. resupinatum* aerial parts against rosiglitazone induced osteoporosis in male rats. In addition, the phenolic constituents of the bioactive extract were investigated as well as the localization of isolated phenolics was determined in the HPLC profile.

MATERIAL AND METHODS

General

Streptozotocin was purchased from Sigma- Aldrich Company. Rosiglitazone was provided as a gift from APEX Company (Egypt). It was suspended in 1% Tween 80. Rosiglitazone was orally administered in a dose of 10 mg/kg (Elmegeed *et al*., 2011; Pickavance *et al*., 1999). NMR experiments were recorded on Jeol EX-500 spectrometer. EIMS was measured on Finnigan-Mat SSQ 7000 spectrometer, while ESIMS on LCQ Advantage Thermo Finnigan spectrometer. UV spectra were recorded on Shimadzu model-2401 CP spectrophotometer. CC was carried out on Polyamide 6S (Riedel-De-Haen AG, Seelze Haen AG, D-30926 Seelze Hanver, Germany) and Sephadex LH-20 (Pharmazia, Uppsala, Sweden). PPC (descending) Whatman No. 1 and 3 MM papers, using solvent systems (1) H_2O, (2) 15% AcOH (AcOH: H_2O, 15:85), (3) 30% AcOH (AcOH: H_2O, 30:70) (4) BAW (n-BuOH: AcOH: H_2O 4:1:5, upper layer), and (5) BBPW (C_6H_6: n-BuOH: pyridine: H_2O, 1:5:3:3, upper layer). HPLC profile was recorded on Agilent HPLC 1200 series equipped with diode array detector (Agilent Technologies, Waldbronn, Germany).

Plant Material and extraction

T. resupinatum var. *minus* was freshly collected and identified by Dr. Ibrahim El Garf in April 2009 from Cairo University garden. A voucher specimen (No.446) was deposited in the herbarium of National Research Centre (CAIRC), Egypt. The air-dried powdered (3 Kg) was extracted with 80% MeOH (4 x 10L). The solvent was evaporated under reduced pressure at 60ºC afforded 320 g aqueous methanol extract (AME). AME was defatted with petroleum ether (4 x 1L) yielded 260 g of defatted aqueous methanol extract (DAME).

Acid hydrolysis

Acid hydrolysis of DAME was carried by refluxing at 80°C for 2h in 1.2 M HCl in 50% aqueous methanol (Häkkinen *et al*., 1999). After hydrolysis, the mixture was extracted three times with EtOAc. The EtOAc extract was concentrated in vacuum to yield the aglycones mixture.

Animals

Male albino adult rats (100-120g) used in the experiments were purchased from the Animal House Colony, Giza, Egypt. They were maintained on standard laboratory diet (protein, 16.04%; fat, 3.63%; fiber, 4.1%; and metabolic energy,

0.012 MJ) and water *ad libitum* at the Animal House Laboratory, NRC, Egypt. After an acclimation period of 1 week, animals were grouped and housed individually in filter-top polycarbonate cages, temperature-controlled (23±1°C) and artificially illuminated (12 h dark/light cycle) room free from any source of chemical contamination. All animals received human care in compliance with the guidelines of the Animal Care and Use Committee of NRC, Egypt (09085).

Oral toxicity of *T. resupinatum*

Acute oral toxicity of AME of *T. resupinatum* var. *minus* was investigated. Two groups of six rats in each, were administrated orally the AME (1.2 and 120 mg/kg bw, respectively). A group of animals treated with vehicle served as control. There were no visible signs of toxicity or mortality in the two groups during and after 7 d of treatment. Accordingly, it was promising to go on the study.

Experimental design

Eighty rats were divided into eight groups with ten rats in each group. The groups were as follows: G1; healthy rats treated orally with saline (Control), G2-G8 are diabetic rats received orally rosiglitazone (10 mg/kg bw) (Ros-rats). Experimental diabetes was induced by a single-dose of (45 mg/kg bw, intraperitoneally, ip) streptozotocin (STZ) injection according to the method described by Patil *et al*. 2011. G2; Ros-rats, G3-G5; Ros-rats orally treated with AME (6, 12 and 24 mg/kg bw) and G6-G8; Ros-rats orally treated with DAME (6, 12 and 24 mg/kg bw). Rosiglitazone, AME and DAME were suspended in 1% tween and given each as single doses orally to the rats once per day for 30 d (Gupta *et al*., 2010).

Sample Collections

At the end of the experimental period, blood samples from fasting rats were withdrawn from retro-orbital venous plexus under diethyl ether anesthesia in dry clean centrifuge tubes. Blood samples were centrifuged at 3000 rpm for 15 min at 4°C where the clear sera were separated and immediately stored at –20 ºC in a clean plastic eppendorf until analyses. The animals were then rapidly sacrificed and the right femurs were harvested. Each right femur bone was carefully cleaned; length and weight were recorded and then stored in formalin buffer 10% for dual energy X-ray absorptiometry (DEXA). BMD of each right femur were measured by DEXA using Norland XR46, version 3.9.6/2.3.1 instrument (Norland X-R-46 version 3.9.6, Peachtree City, GA, USA) equipped with dedicated software for small animal measurements. This technique provided an integrated measure of right femur proximal, distal and total areas.

Analytical Determinations

Serum osteoprotegerin (OPG) and receptor activator of nuclear factor-κB Ligand (RANKL) levels were determined by enzyme linked immunosorbent assay (ELISA) technique using R and D Elisa (Sorin Biomedica, Eti-System, Denlay Instruments Ltd, England) kit (O'Brien *et al*., 2001; Teng *et al*., 2002).While serum β2-microglobulin level was assayed by ELISA procedure using International Immuno-Diagnostics kit (Orgentec Diagnostika GmbH, Mainz, Germany) (Crisp *et al*., 1983).

Expression of osteogenic genes

Isolation of total RNA

Total RNA was extracted from the bone samples obtained from the intertrochanteric region of the proximal femur of male rats by the standard TRIzol® Reagent extraction method (Invitrogen, Germany). Briefly, bone samples were pulverized under liquid nitrogen and homogenized in 1 mL of TRIzol® Reagent. Afterwards, the homogenized sample was incubated for 15 min at room temperature. A volume of 0.2 mL of chloroform per 1 mL of TRIzol® Reagent was added. Then the samples were vortexed vigorously for 15 sec and incubated at room temperature for 3 min. The samples were centrifuged for no more than 12,000 x *g* for 15 min at 4ºC. After centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA was remained exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube.

The RNA which was often invisible before centrifugation was precipitated forming a gel-like pellet from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 mL of isopropyl alcohol was added per 1 mL of TRIzol® Reagent used for the initial homogenization. Afterwards, the samples were incubated at 15 to 30 ºC for 10 min and centrifuged at not more than 12,000 x *g* for 10 min at 4ºC. The RNA was precipitated which was often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely. The RNA pellet was washed once with 1 mL of 75% ethanol.

The samples were mixed by vortexing and centrifuged at no more than 7,500 x *g* for 5 min at 4ºC. The supernatant was removed and RNA pellet was air-dried for 10 minutes. RNA was dissolved in diethylpyrocarbonate (DEPC) treated with water by passing solution a few times through a pipette tip. Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1).

Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

Reverse transcription (RT) reaction

Complete Poly(A)$^+$ RNA isolated from male rat bone samples was reverse transcribed into cDNA in a total volume of 20 µL using Revert Aid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany) based on previously published methods (Khalil and Booles, 2011; Kassem *et al*., 2016).

Table 1: Sequences of primers of the osteogenic genes.

Genes	Sequence 5´ to 3		Accession no.
	Forward primer	Reverse primer	
OC	CCAGCGGTGCAGAGTCCAGC	GACACCCTAGACCGGGCCGT	NM_199173.3
COL1A1	AGCGGACGCTAACCCCCTCC	CAGACGGGACAGCACTCGCC	NM_000088.3
ACP5	GATCTCCAAGCGCTGGAACT	TGGTCTGTGGGATCTTGAAGTG	Logar *et al*., 2007
TUB-β	GAGGGCGAGGACGAGGCTTA	TCTAACAGAGGCAAAACTGAGCACC	NM_001069.2
β-actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTGCTG	Deng *et al*., 2008

OC; Osteocalcin, COL; Collagen, ACP5; Acid phosphatase, TUB-β; Tubulin β.

qRT-PCR and calculation of gene expression

PCR reactions and calculation of gene expression were analysed according to the method of Kassem *et al*., (2016). The qRT-PCR of osteogenic genes; osteocalcin (OC), collagen (COL) and acid phosphatase 5 (ACP5) were normalized on the bases of tubulin β (TUB-β) and ß-actin expression (Table 1).

Phytochemical investigation of DAME

Isolation and identification of phenolics

The DAME was subjected to Polyamide CC (5.5x125cm) eluted with H_2O and followed by MeOH/H_2O mixtures of decreasing polarity. A total of 49 fractions were collected, each 250 mL; these were controlled by PC to give four main fractions [FA (20-40% MeOH/H_2O), FB (50-60% MeOH/H_2O), FC (70-80% MeOH/H_2O) and FD (90%MeOH/H_2O-100%MeOH)]. FA was chromatographed on Sephadex CC (2.5x85cm) using H_2O:MeOH (1:1) as eluent yielding four main subfractions, each was subjected to PPC using 15% AcOH, H_2O and BAW, followed by Sephadex CC using 100% MeOH to yield compounds **4** and **8-11**. PPC using BAW and 15% AcOH (double solvent) of FB afforded compounds **6** and **7**. Repeating Sephadex CC (2.5x85cm) of FC on gradient elution with MeOH/H_2O then 100% MeOH yielded compounds **2**, **3** and **12**. FD was also chromatographed on PPC eluted with 50% AcOH afforded compounds **1** and **5**.

***HPLC profile of DAME*.**

One gram of DAME (before and after hydrolysis) were dissolved in 10 mL methanol of HPLC grade and analyzed by an Agilent HPLC 1200 series equipped with diode array detector. Chromatographic separations were performed using a Waters column C18. The binary mobile phase consisted of (A) acetonitrile and (B) 0.1% acidified water with formic acid. The elution profile was: 0-1 min 100% B (isocratic), 1-30 min 100-70% B (linear gradient), 30-35 min 70-20% B (linear gradient). The flow rate was 1 mL/min and the injection volume was 5 µL. Chromatograms were recorded at 280 nm.

This analysis enabled the characterization of flavonoids on the basis of their retention time and UV spectra. The isolated compounds were used as reference standards (0.1mg/10mL MeOH) to determine them in DAME and to detect their localization in HPLC profile. Separated phenolic peaks were initially identified by direct comparison of their retention times with those of isolated compounds. Standard solution was then added to the sample and peaks were identified by the observed increase in their intensity (El Souda *et al*., 2014).

Statistical analysis

All data were analyzed using the General Liner Models (GLM) procedure of Statistical Analysis System (Aronow *et al*., 1990) followed by Scheffé-test to assess significant differences between groups. The values are expressed as mean±SEM. The statements of significance were based on probability of $P < 0.01$ and $P < 0.05$.

RESULTS

Serum OPG, RANKL and β2-microglobulin levels

The effect of AME and DAME supplementation on serum OPG, RANKL and β2- microglobulin levels in rosiglitazone-induced osteoporosis in male rats are represented in Table 2. Administration of rosiglitazone to male rats caused significant increase in serum RANKL and β2-microglobulin levels associated with significant decrease in serum OPG level in comparison with the healthy control rats. However, Ros-rats + AME showed a significant increase in the serum OPG level at the highest dose only (24 mg/kg bw) and a slight changes in the levels of the other two parameters as compared with Ros-rats. Furthermore, Ros-rats + DAME with all doses revealed highly significant increase in serum OPG level with a concomitant significant decrease in serum RANKL and β2-microglobulin levels as compared with the Ros-rats in a dose dependant manner (24 >12 > 6 mg/kg bw).

Table 2: Levels of serum OPG, RANKL and β2-microglobulin Ros-rats alone or combined with AME and DAME.

Groups treatment mg/kg bw/d		OPG (ng mL-1)	RANKL (pg mL-1)	β2-microglobulin (µg mL-1)
Control		3.42 ± 0.32^{a}	58.220 ± 07.04^{d}	0.21 ± 0.04^{b}
Ros-rats		1.62 ± 0.26^{c}	162.42 ± 11.34^{a}	0.46 ± 0.05^{a}
Ros-rats + AME	6	1.64 ± 0.22^{c}	158.26 ± 12.08^{a}	0.38 ± 0.06^{a}
	12	1.84 ± 0.28^{b}	152.12 ± 08.14^{a}	0.34 ± 0.04^{ab}
	24	1.92 ± 0.32^{b}	132.24 ± 09.16^{b}	0.31 ± 0.03^{ab}
Ros-rats + DAME	6	1.90 ± 0.42^{b}	138.18 ± 06.42^{b}	0.34 ± 0.02^{ab}
	12	2.14 ± 0.46^{ab}	112.36 ± 08.22^{c}	0.26 ± 0.05^{b}
	24	2.46 ± 0.44^{ab}	96.640 ± 06.12^{c}	0.22 ± 0.04^{b}

^{a,b,c} Mean values within column with unlike superscript letters were significantly different ($^{a:}$ $P<0.01$, $^{b, c:}$ $P<0.05$). Ros-rats: Diabetic male rats treated by Roziglitazones (10mg/kg bw/d).

Bone mineral density (BMD) levels

The results show the effect of AME and DAME administration on BMD-proximal, BMD-distal and BMD-total areas of femur bones in rosiglitazone-induced osteoporosis in male rats. The Ros-rats showed significant decrease in BMD of the

proximal, distal and total measured areas in comparison with the healthy rats. On the other hand, the three measurements of BMD for Ros-rats + AME increased slightly except for the high dose. However, Ros-rats treated with all doses of DAME caused significant increase in BMD (24 >12 > 6 mg/kg bw) compared with the Ros-rats (Table 3).

Table 3: BMD in proximal, distal and total areas of femur bones of Ros-rats alone or combined with AME and DAME after 30 days of daily treatment.

Groups treatment mg/kg bw/d		BMD-proximal (mg cm^{-2})	BMD-distal (mg cm^{-2})	BMD-total (mg cm^{-2})
Control		109.6 ± 14.2^{a}	111.2 ± 08.6^{a}	111.3 ± 06.4^{a}
Ros-rats		82.30 ± 06.4^{b}	81.20 ± 07.2^{b}	82.10 ± 04.8^{b}
Ros-rats + AME	6	83.80 ± 04.6^{b}	84.20 ± 06.8^{b}	84.60 ± 06.2^{b}
	12	88.20 ± 10.4^{ab}	89.40 ± 08.2^{ab}	88.80 ± 11.4^{ab}
	24	92.40 ± 08.2^{ab}	94.60 ± 10.2^{ab}	94.20 ± 10.4^{ab}
Ros-rats + DAME	6	94.80 ± 11.4^{a}	96.20 ± 08.8^{a}	96.60 ± 09.2^{a}
	12	98.40 ± 12.4^{a}	97.60 ± 10.4^{a}	98.60 ± 10.8^{a}
	24	102.8 ± 08.6^{a}	104.6 ± 10.2^{a}	106.0 ± 09.2^{a}

^{a,b,} Mean values within column with unlike superscript letters were significantly different ($^{a:}$ P<0.01, $^{b:}$ P<0.05). Ros-rats: Diabetic male rats treated by Roziglitazones (10mg/kg bw/d).

Expression changes in osteogenic genes

The qRT-PCR of osteogenic genes (OC, COL and ACP5) is summarized in Figure 1. The results revealed that Ros-rats showed significantly lower expression values of OC, COL genes in comparison with the healthy male rats. While, Ros-rats treated with AME at 24 mg/kg bw caused significant increase in OC and COL expression compared with Ros-rats. Moreover, all doses of DAME caused significant increase in OC and COL expression as compared with the Ros-rats, in which the highest expression levels of OC and COL genes were showed in the high dose of DAME. In the term of ACP5 gene, the present work revealed that the Ros-rats showed significantly higher expression values of ACP5 mRNA in comparison with the healthy male rats. However, Ros-rats treated with AME showed that only the high dose of AME decreased significantly ACP5 expression compared with the Ros-rats.

Moreover, all doses of DAME caused significant decrease in ACP5 expression as compared with the Ros-rats, in which the lowest expression levels of ACP5 gene were showed in Ros-rats treated with AME (24 mg/kg bw).

Phytochemical constituents of DAME

Identification of the isolated compounds

Twelve flavonoids were isolated from DAME (Figure 2). They were identified as quercetin (1), quercetin 3-*O*-β-glucopyranoside (2), quercetin 3-*O*-α-rhamnopyranoside (3), rutin (4), Kaempferol (5), daidzein (6), genistein (7), formononetin (8), formononetin7-*O*-β-glucopyranoside (9), pseudobaptigenin (10), pseudobaptigenin 7-*O*-β-glucopyranoside (11) and chlorogenic acid (12).

The structure elucidation were determined by R_f values, colour reactions, acid hydrolysis, MS spectrometry, UV spectrophotometry (Mabry *et al*., 1970; Markham, 1982), 1D-NMR (^{1}H and^{13}C) and 2D-NMR (HMBC and HMQC) spectroscopy. Their spectroscopic data were in agreement with previously reported values (Mabry *et al*., 1970; Agrawal, 1989; Coward *et al*., 1993; Markham and Geiger, 1994; Isik *et al*., 2007; Fedoreyev *et al*., 2008; Janda *et al*. 2009). In this study, all phenolics were isolated for the first time from *T. resupinatum* except compounds **2**, **8** and **9**. To the best of the authors´ knowledge, no previous phytochemical investigations have been reported on the present variety of *T. resupinatum* (*minus*).

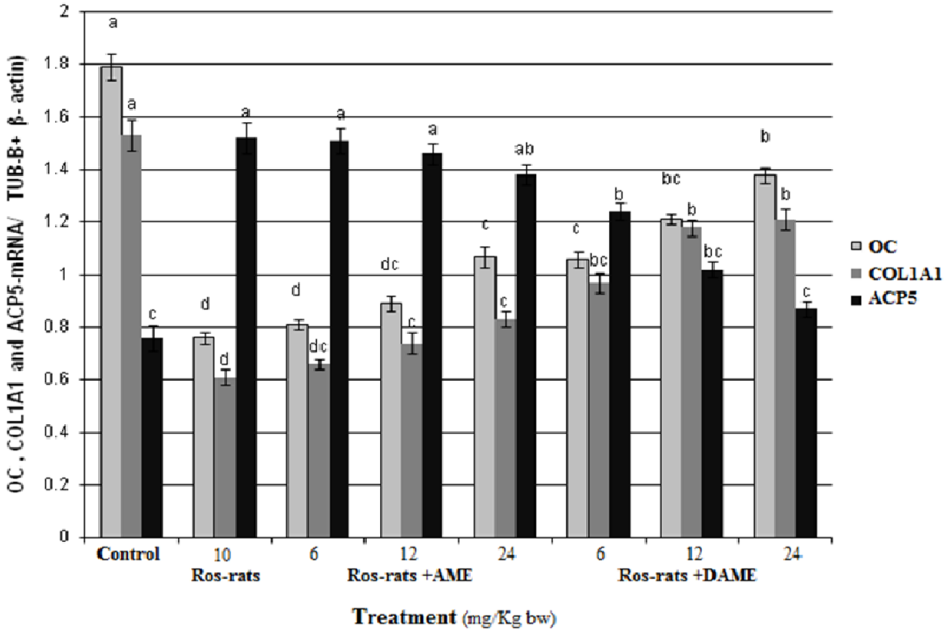

Fig. 1: The alterations of (OC, COL1A1 and ACP5) mRNA in bone tissues isolated from intertrochanteric region of the proximal femur of male rats treated with Rosiglitazone alone (Ros-rats) or combined with *AME* and DAME. Data are presented as mean ± SEM. ^{a,b,c} Mean values within tissue with unlike superscript letters were significantly different (^{a} P<0.01, $^{b, c, d}$ P<0.05).

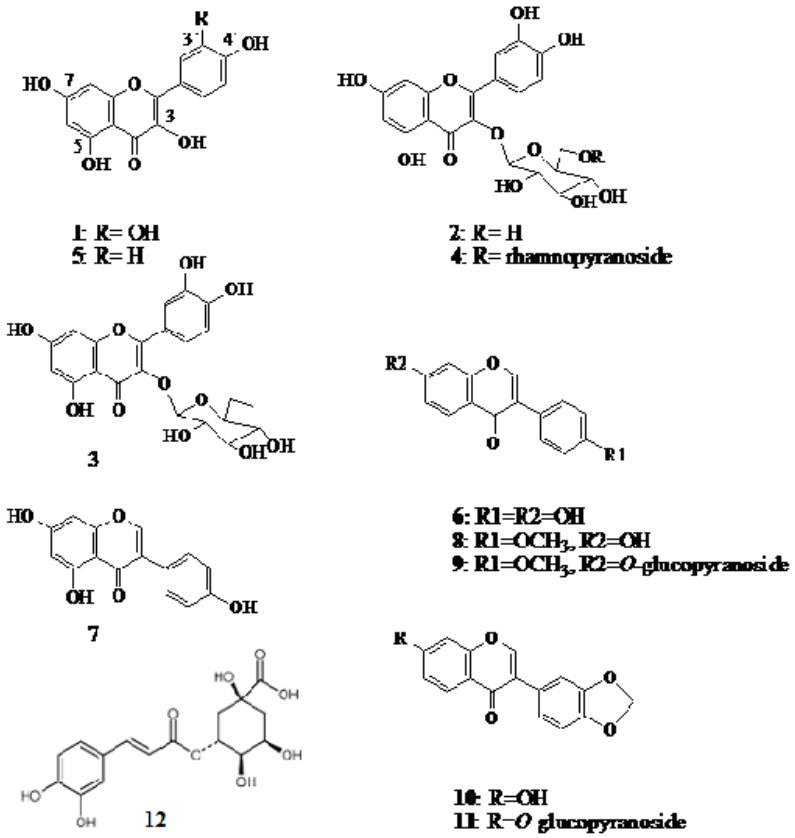

Fig. 2: Chemical structural of compounds 1-12. 1) Quercetin , 2) Quercetin 3-*O*-β-glucopyranoside, 3) Quercetin 3-*O*-α-rhamnopyranoside, 4) Rutin, 5) Kaempferol, 6) Daidzein, 7) Genistein, 8) Formononetin, 9) Formononetin7-*O*-β-glucopyranoside, 10) Pseudobaptigenin, 11) Pseudobaptigenin 7-*O*-β-glucopyranoside, 12) Chlorogenic acid

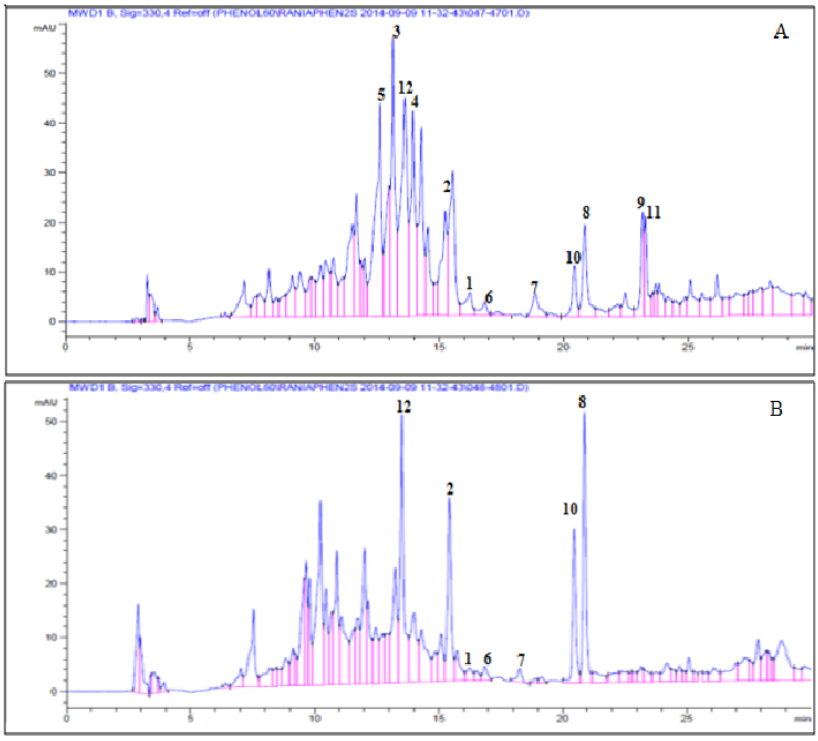

Fig. 3: HPLC at 280 nm profile of phenolics from *T. resipunatum* extract DAME (A) before hydrolysis, (B) after hydrolysis. 1) Quercetin , 2) Quercetin 3-*O*-β-glucopyranoside, 3) Quercetin 3-*O*-α-rhamnopyranoside, 4) Quercetin 3-*O*-β-rutinoside, 5) Kaempferol, 6) Daidzein, 7) Genistein, 8) Formononetin, 9) Formononetin7-*O*-β-glucopyranoside, 10) Pseudobaptigenin, 11) Pseudobaptigenin 7-*O*-β-glucopyranoside, 12) Chlorogenic acid.

Qualitative HPLC profile of DAME

The DAME (before and after hydrolysis) was subjected to HPLC analysis (Table 4, Figure 3) using the isolated compounds as standards (each, 0.1mg/10mL MeOH, purity approximately 98%). After hydrolysis, it was found that chlorogenic acid > formononetin > quercetin > pseudobaptigenin are the major phenolic skeletons at retention times 13.64, 20.86, 15.44 and 20.44 with % area 5.91, 5.57, 4.61 and 3.36, respectively. Moreover, the other skeletons (kaempferol, daidzein and genistein) are represented at retention times 16.19, 16.8 and 18.8 with % area 0.47, 0.42 and 0.38, respectively.

Table 4: HPLC analysis of DAME before and after acid hydrolysis.

Flavonoids	Rt	Area %	
		Before	After
Quercetin (1)	15.44	1.95	4.61
Quercetin 3-*O*-β-glucopyranoside (2)	13.16	5.17	-
Quercetin 3-*O*-α-rhamnopyranoside (3)	13.99	4.73	-
Rutin (4)	12.64	3.41	-
Kaempferol (5)	16.19	1.04	0.47
Diadazein (6)	16.8	0.51	0.42
Genestien (7)	18.8	0.48	0.38
Formononetin (8)	20. 86	2.54	5.57
Formononetin 7-*O*-β-glucopyranoside (9)	23.18	1.87	-
Pseudobaptigenin (10)	20. 44	1.31	3.36
Pseudobaptigenin 7-*O*-β-glucopyranoside (11)	23.29	1.47	-
Chlorogenic acid (12)	13.64	2.73	5.91
Total		27.21	20.72

DISCUSSION

Certain medical treatments may cause series of bone disorders leading to osteoporosis (Abd Jalil *et al*., 2012). Glitazones (thiazolidinediones) are relatively new antidiabetic drugs used in type 2 diabetes treatments. Rosiglitazone is a high affinity ligand and activator of the peroxisome proliferators-activated receptor-γ (PPAR-γ). It acts by increasing sensitivity to endogenous insulin and consequently inducing a hypoglycemic effect. Most of the rosiglitazone effects are mediated via this transcription factor (Lecka-Czemik *et al*., 1999; Lee *et al*., 2003). It triggers PPAR-2 functions as a principal negative regulator of osteoblast differentiation (Sabudak and Guler, 2009).

Fujioka *et al*. (2007) suggested that the phytoestrogen daidzein has a specific, sexually dimorphic effect on bone formation and BMD during growth period in mice.

Moreover, Benvenuti *et al*. (2012) reported that estrogens and androgens in addition to the phytoestrogens genistein and quercetin are able to counteract the negative effects of rosiglitazone on bone. They suggested the possibility of using steroid receptor modulators, such as plant-derived phytoestrogens, which lack evident adverse effects.

In the present study, *T. resipunatum* aerial parts extracts (AME and DAME) were assayed for their protective effects against bone loss induced by roziglitazone in diabetic male rats and phytochemical investigation of phenolic constituents of the bioactive extract was carried out. Ros administration revealed significant bone loss in experimental animals, possibly through its interaction with PPAR-γ2 isoforms, which is critical for the regulation of osteoblast and adiposities differentiation (Rzonca *et al*., 2004). Treatment of Ros-rats with *T. resipunatum* extracts especially DAME showed a dose dependant- significant increase in micro-hardness of bones when compared to Ros-rats. This could be attributed to the resorption/desorption of calcium, phosphorous caused by Ros through supplemented *T. resipunatum* extracts DAME. The treatment also resulted in a significant reduction in serum OPG level. This finding was supported by that of Lazarenko *et al*. (2007), who reported that Ros affect the level of OPG mRNA and reduce production of OPG from osteoblasts and/or marrow cells of mice. Meanwhile, the DAME administration produced a significant increase in the serum OPG level in Ros-rats. *Trifolium* species rich in isoflavones has direct effect on the osteoblast cells stimulating OPG expression (Cho *et al*., 2012; Shu-Jem *et al*., 2013).

Moreover, treatment with Ros in the current study caused a significant elevation in the serum RANKL and β2-microglobulin levels. These findings agreed with that of Cho *et al*. (2012), who reported that rosiglitazone promotes osteoclastogenesis via increasing RANKL, β2-microglobulin and decreasing the OPG expression.

The DAME supplementation caused a significant decrease in serum RANKL and β2-microglobulin levels in the Ros-rats. It has been reported that significant reduction in serum RANKL level resulted in rats treated with *T. medium* L. and *T. pratense* extracts as compared to that in untreated OVX rats (Cegieła *et al*., 2012).

Furthermore, our study demonstrates that male Ros-rats showed significantly low levels of OC, COL expression and higher expression of ACP5 mRNA in bone samples compared with healthy rats. This finding is consistent with previous studies by (Cho *et al*., 2012) that reported rosiglitazone treatment reduced collagen and osteocalcin expression levels in bone marrow cells. However, supplementation of DAME to Ros-rats reversed the effect of Ros on OC, COL and ACP5 expression. Our data demonstrates that DAME supplementation to male Ros-rats was more effective than AME supplementation. It exerts a significant dose dependant protection and intervention against the negative effect of Ros on bones of diabetic animals. These results prompt us to chemically investigate the *T. resupinatum* DAME bioactive extract.

Phytochemical investigation of DAME and its phenolic profile using HPLC analysis (before and after hydrolysis) demonstrated that the phenolic acid; chlorogenic acid, the isoflavones; formononetin and pseudobaptigenin and the flavonol; quercetin are the major constituents (as free aglycons). The effectiveness of DAME supplementation might be attributed to a higher carbohydrate and phenolic contents including phytoestrogens, than in AME. Referring to Setchell and Lydeking-Olsen (2003) and Yuan *et al*. (2007), higher carbohydrate content may contribute to the creation of better environment that stimulates the growth of intestinal microflora. This effect is

responsible for the metabolism of polyphenols including the conversion of glycosides to aglycones (bioactive molecules) and their further conjugation in blood. Examples of these, the isoflavone glycosides formononetin and daidzin are converted to their aglycones (formononetin and daidzein), then to the active equol; daidzein metabolite (Cegieła *et al*., 2012).

Chlorogenic acid (CGA) is a major constituent of DAME, characterized by its anti-inflammatory and anti-oxidant activities. It protects bone structure from deterioration by exerting positive effects on bone mass, bone micro architecture and bone strength (Hooshmand *et al*., 2011). CGA inhibits osteoclast differentiation and bone resorption by down-regulation of receptor activator of Nuclear Factor Kappa-B Ligand-Induced Nuclear Factor of Activated T Cells c1Expression (Kwak *et al*., 2013). CGA dose-dependently inhibited RANKL-mediated osteoclast differentiation in bone marrow macrophages (BMMs) without any evidence of cytotoxicity.

The phytoestrogen quercetin is considered as one of the bioactive anabolic molecules for bone. Wattel *et al*. (2004) showed that quercetin exert a beneficial effect in bone remodeling not only via inhibiting osteoclast activity, but also via a protective effect on osteoblasts through its antioxidant activity. Furthermore, recent findings reported that at low concentrations, quercetin enhanced osteogenic differentiation *via* a mechanism independent from estrogen receptor activation (Kim *et al*., 2006).

Marini *et al*., 2007 reported that the metabolic balance of bone formation and resorption can be restored by high isoflavone intake. Isoflavones not only suppress bone breakdown but at the same time enhance the new bone formation. Taken together Bhargavan *et al*. (2009) and Ishimi *et al*. (2002) studies, they indicated that isoflavones (formononetin and pseudobaptigenin) may positively enhance osteoblast function and bone anabolism probably not only through estrogen-like action but also *via* interaction with other signaling pathways such as NF-kB or MEK-Erk pathways depending on the isoflavone metabolite, and this is shown in pre-clinical studies. The present data revealed that formononetin is a main phytoestrogen in DAME, it has structural similarity to 17β-estradiol and can bind to estrogen receptors in bone tissue. Vandenput and Ohlsson (2009) stated that estradiol levels are more strongly associated with BMD than testosterone levels in adult men.

Our data, yet preliminary, suggest the possibility to counteract the negative effects of TZD on bone using isoflavones rich plant extract such as *T. resipunatum* aerial parts extract, which lack adverse effects.

CONCLUSION

Therapeutic advantages may be realized through combination regimes. In the present study, administration of DAME to Ros-rats resulted in marked modulation of bone biomarkers RANKL, β2-microglobulin and OPG, and improvement of femoral BMD and content, as well as it reversed the effect of Ros on the osteogenic genes OC, COL and ACP5 expression. Twelve phenolic compounds were isolated from the bioactive extract DAME; ten of them were isolated for the first time from *T. resupinatum*. A qualitative HPLC profile elucidates its major phenolics fingerprint (chlorogenic acid, formononetin, quercetin and pseudobaptigenin), which possess potential antioxidant activity and anti-osteoporosis properties in restoring the metabolic balance of bone formation and resorption. The increased risk of bone loss and fractures in TZD users indicated by clinical evidence necessitate more research to elucidate possible therapeutic strategies and improve bone safety.

ACKNOWLEDGMENT

Financial support and sponsorship: The authors acknowledge the financial support of Science and Technology Development fund (STDF) project no. 5024 and the National Research Centre (NRC) project no. 10010002.

Conflict of Interests: There are no conflicts of interest.

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How to cite this article:

Kassem MES, Marzouk MM, Mostafa AA, Khalil WKB, Booles H. Phenolic constituents of *Trifolium resupinatum* var. *minus*: Protection against rosiglitazone induced osteoporosis in type 2 diabetic male rats. J App Pharm Sci, 2017; 7 (05): 174-183.