Rubia tinctorum root extracts: chemical profile and management of type II diabetes mellitus

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The chemical and biological profiling of the root extracts of Rubia tinctorum was performed. The activities of different extracts were determined considering the antidiabetic effect against type II diabetes mellitus together with anti-obesity and hepatoprotective effects and lipid profile. The methanolic extract of Rubia tinctorum exhibited significant results in decreasing body weight, improving lipid profile, normalizing hyperglycaemia, insulin resistance, hyperinsulinemia. Additionally, it showed enhancement of liver tissue structure and function. The methanolic extract, being the most significant one, was subjected to LC-HRMS analysis to determine its chemical constituents. Finally, the chemical constituents were evaluated by molecular docking study that was carried out to identify the interaction of a panel of 45 compounds in silico and to correlate the structures to their anti-diabetic activity. Among the tested compounds, 1-hydroxy-2-hydroxymethyl anthraquinone and naringenin-7-O-glucoside showed the most potent activity as α-amylase inhibitors.

1. Introduction

Diabetes mellitus and obesity are important health problems worldwide. They contribute to the development of various pathological processes such as hypertension, cardiovascular diseases, hyperlipidaemia, certain types of cancer and even Alzheimer’s. There is a strong correlation between diabetes and obesity, where adipose tissue has an important role in diabetes, a disease characterized by hyperglycaemia, insulin hyposcretion, and insulin resistance. The exploitation of phyto-medicine as a therapy for diabetes as well as obesity is a crucial issue for the development of safer alternatives to pharmaceuticals which not only decrease blood glucose but also enhance the antioxidant system. The genus Rubia belongs to the family Rubiaceae and comprises about 70 species. Previous research on Rubia species yielded the isolation and chemical identification of about 250 compounds with different chemical classes which possess diverse pharmacological activities including anti-bacteria, antithrombic, anticancer, anti-inflammatory and anti-oxidant.

Rubia tinctorum L. (Madder root) was used as a source of red dyes in ancient Egypt. In addition, several ethnobotanical surveys have reported its use for treatment of various ailments, such as cardiovascular disease, liver pain, diarrhea, rheumatism and kidney stones. Moreover numerous biological studies have been extensively conducted on R. tinctorum and proved its therapeutic potential as antiplatelet aggregation, antitumor, hepatoprotective, vasoconstriction and protective effect on aorta. The current work was designed to evaluate the antiobesity, antidiabetic and hepatoprotective efficacy of different extracts of R. tinctorum along with profiling of potent bioactive components responsible for the antidiabetic activity. In our strategy, we used LC-HRMS technique to identify the phytochemicals in the root extract of R. tinctorum then to determine the binding mode of the tested compounds with 1HX0 as α-amylase inhibitor by using molecular docking tool.

2. Materials and methods

2.1. In vitro determination of hypoglycemic activity, liver enzymes and lipid profile

2.1.1. Plant material. The roots of Rubia tinctorum were obtained from the Egyptian market and the identity was confirmed in the Faculty of Science, Suez Canal University. A specimen was deposited at Pharmacognosy Department, Faculty of Pharmacy, Suez Canal University, with a code number 2019-RT. Two kilograms of the roots were dried, powdered and extracted with methanol. The extract was dried under vacuum using rotary
evaporator to give 250 g of brownish-red methanolic extract (RM). A
weight of 160 g of the methanol extract were withdrawn and
suspended in 200 mL of distilled water then fractionated with 3 L
of each of hexane, chloroform and ethyl acetate successively. Each
of the three extracts was dried under vacuum using rotary eva-
rator to yield 30, 60 and 40 g of hexane (RH), chloroform (RC) and
ethyl acetate (RE) extracts respectively.

2.1.2. Experimental animals. Thirty-five male Wistar rats
were used in the current study. The base line body weight was in the
range of 118–152 g. They were kept in clean cages with temperature
equals 21 ± 6 °C and normal light-dark cycle. They had free
permission to water and regular diet or HFD. Study protocol was
approved by the Committee of Research Ethics at Faculty of Phar-
macy in Suez Canal University (license number 202004R2).

2.1.3. Experimental design. Wistar male rats were distrib-
uted into seven groups; with 5 rats in every group. First group of
rats was assigned as the normal group and received normal
standard diet, 10% lard fat, 30% glucose) for 7 weeks followed
by small dose of streptozotocin (STZ 1% g L
–1) acetic acid; 30 mg
g kg
–1, S. C). After further five days, blood glucose levels are
determined to insure the incidence of diabetes mellitus. After
that, rats in Group II (diabetic control group) were given
distilled water (1 mL per kg per day, p. o.) till the end of
experiment. Rats in group III were given pioglitazone (10 mg per
kg per day, p. o.) while rats in group IV, V, VI and VII were given
the following extracts RM (200 mg kg
–1), RH (200 mg kg
–1), RC
(200 mg kg
–1) and RE (200 mg kg
–1), respectively for further
four weeks. After completion of the treatment regimens, final
body weight was recorded. The change in body weight was
calculated from the following equation: Δbody weight = (the
final body weight – the initial body weight)/initial body weight
× 100. Similarly, obesity index was calculated by: obesity index
= weight of total adipose tissue/final body weight × 100.

2.1.4. Fasting blood glucose determination. Rats were
subjected to overnight fasting. Blood specimens were gathered
from each rat’s tail tip, and fasting glucose was recorded by the use
of an automated blood glucometer (Super Glucocard, Japan).

2.1.5. Liver processing. The rats were sacrificed under
anaesthesia. Each rat’s liver was quickly dissected and washed
out of blood with cold saline solution. The weight of livers was
measured and the following formula was used for determining
the liver index: (liver weight/body weight × 100).

Portion of liver tissue was removed from the biggest hepatic
lobe, fixed in formaldehyde and finally stained with hematox-
ylin and eosin (H&E).

2.1.6. Measurement of serum biochemical parameters

2.1.6.1. Liver enzymes. Spectrophotometrically method was
done with marketable kits (Biocon Diagnostic, Germany) to
evaluate serum activity of liver enzymes; alanine transaminase
enzyme (ALT) (EC 2.6.1.2), aspartate transaminase enzyme
(AST) (EC 2.6.1) in accordance with the protocol reported by the
manufacturer.26

2.1.6.2. Lipid profile. A spectrophotometric assay kits
(Spinreact, Spain) were used to measure serum total cholesterol
(TC)(CHOD-POD), triglycerides (TGs)(GPO-POD, Líquido), high-
density lipoprotein (HDL) (HDLC-P) and low-density lipoprotein
(LDL)(LDLC-D) according to the manufacturer’s protocol.27,28

2.1.6.3. Insulin & leptin ELISA kits. The level of serum insulin
and leptin were determined by a rat insulin and leptin ELISA
kits (PELOBIOTECH GmbH-Am Klopferspitz 19-82152
planning-Germany) following the manufacturer’s protocol.
Insulin resistance was determined using the homeostasis
model assessment index for insulin resistance (HOMA-IR)
index.29

2.1.7. Statistical analysis for the data. Results obtained
from the current study were expressed as mean ± S. E. M and
analysed with the version 16 of SPSS program. A one-way anal-
ysis of variance (ANOVA) was used to analyse quantitative vari-
ables, followed by the multiple comparison test of Bonferroni.
Significant variations were measured at p ≤ 0.05.

2.2. Preparation of the sample and LC-HRMS analysis

The mobile phase working solution (MP-WS) was prepared from
Di-water : methanol : acetonitrile (50 : 25 : 25). One mL of MP-
WS was added to 50 mg weighted dry methanolic extract, vortex
for 2 min. This step was followed by ultra-sonication for 10
min then centrifugation for 10 min at 10 000 rpm. 20 mL
stock (50/1000 µL) was diluted with 1000 µL reconstitution
solvent. Finally, the injected concentration was 1 µg µL
–1 where
10 µLs were injected on positive mode. Also, 10 µL MP-WS were
injected as a blank sample. The used mobile phase consisted of
(A): 5 mM ammonium formate buffer pH 3 containing 1%
methanol and (B): 100% acetonitrile. The flow rate was 0.3
mL min
–1. The used pre-column was in-line filter disks (Phe-
nomenex, 0.5 µm × 3.0 mm) and the column was X select HSS
T3 (Waters, 2.5 µm, 2.1 × 150 mm). Data processing was via
MS-DIAL3.52. Master view was used for feature (peaks) extraction
from total ion chromatogram based on the following criteria:
features should have signal-to-noise greater than 5 (non-
targeted analysis) and features intensities of the sample-to-
blank should be greater than 5.

2.3. Molecular docking

Molecular modelling study was carried out to study the inter-
action of a panel of 28 anthraquinone and 17 flavonoids in silico
and to correlate the structures to their anti-diabetic activity.
Molecular docking study was conducted on a computational
software basis using the Molecular Operating Environment
(MOE 2014.09 Chemical Computing Group, Canada). The three-
dimensional structures of 1HX0 completed with AC1 as alpha-
amylase inhibitor was freely accessible from the protein data
bank (https://www.rcsb.org/structure/1HX0).29 The active sites were
defined using grid boxes of appropriate sizes around the co-crystallized
ligands. These compounds were docked into the receptor active
site, each ligand-receptor complex was tested for binding energy
using MOE and interaction analysis using Chimera as a visualiz-
ing software.
3. Results and discussion

3.1. Effect of different *R. tinctorum* extracts and pioglitazone (10 mg kg⁻¹) on percent change in body weight and obesity index on type II diabetic rats

Treatment with high fat diet followed with STZ (30 mg kg⁻¹) in diabetic group resulted in a significant increase in final body weight (352 ± 7.5%), % change in body weight (152 ± 14.1) and obesity index (5.9 ± 0.5) versus normal group (210 ± 10) (42.5 ± 9) and (0.83 ± 0.07) respectively at p ≤ 0.05 (Table 1). Treatment with pioglitazone (10 mg kg⁻¹) for four weeks after induction of diabetes induced significantly decrease in final body weight, % change in body weight and obesity index when compared with diabetic group at p ≤ 0.05. Pioglitazone is one member from thiazolidines, as it is PPAR-gamma agonist in particular is known to favorably influence the majority of the components of

![Table 1](image1)

| Group                | Base line body weight (g) | Final body weight (g) | % change in body weight | Obesity index |
|----------------------|---------------------------|-----------------------|-------------------------|---------------|
| Normal               | 147.5 ± 2.5               | 210 ± 10              | 42.5 ± 9                | 0.83 ± 0.07   |
| Diabetic             | 140 ± 5                   | 352 ± 7.5             | 152 ± 14.1a             | 5.9 ± 0.5     |
| Diabetic + pioglitazone (10 mg kg⁻¹) | 143.5 ± 1.5               | 227.5 ± 17.6b         | 58 ± 13.6b              | 2 ± 0.36b     |
| Diabetic + RM (200 mg kg⁻¹) | 167.5 ± 7.5               | 308 ± 8.8bc           | 84 ± 3.4ab              | 2.6 ± 0.39ab  |
| Diabetic + RH (200 mg kg⁻¹) | 140 ± 2                   | 333.5 ± 16.6cde       | 138 ± 8.2axe            | 5.1 ± 0.17axe |
| Diabetic + RC (200 mg kg⁻¹) | 141 ± 4                   | 291.5 ± 18.6e         | 106 ± 7.1c              | 3.7 ± 0.1c    |
| Diabetic + RE (200 mg kg⁻¹) | 145 ± 10                  | 297.3 ± 3.4c          | 106 ± 15.9c             | 5.3 ± 0.03cde |

* Results are expressed as mean ± S. E. M. and analysed using one-way ANOVA followed by Bonferroni’s test for multiple comparisons. *p ≤ 0.05 versus normal group. *p ≤ 0.05 versus diabetic group. *p ≤ 0.05 versus diabetic + pioglitazone (10 mg kg⁻¹) group. *P ≤ 0.05 diabetic + RM (200 mg kg⁻¹) group. *P ≤ 0.05 diabetic + RH (200 mg kg⁻¹) group. n = 5.

![Table 2](image2)

| Group                | Blood glucose level (mM L⁻¹) | Serum insulin (ng L⁻¹) | HOMA-IR | Serum leptin (ng L⁻¹) |
|----------------------|-----------------------------|------------------------|---------|----------------------|
| Normal               | 97 ± 3.9                    | 2.4 ± 0.05             | 14.3 ± 0.87 | 3.8 ± 0.3 |
| Diabetic             | 172 ± 24a                   | 7.6 ± 0.29a            | 74.79 ± 16.7a | 14 ± 1a   |
| Diabetic + pioglitazone (10 mg kg⁻¹) | 110 ± 5.6b                  | 3.9 ± 0.02ab           | 26.5 ± 1.5b  | 4.9 ± 0.3b |
| Diabetic + RM (200 mg kg⁻¹) | 99 ± 4a                    | 4 ± 0.06b              | 29.8 ± 0.99b | 5.6 ± 0.1b |
| Diabetic + RH (200 mg kg⁻¹) | 125 ± 7.6b                  | 4.1 ± 0.1b             | 34.6 ± 2.8b  | 10.5 ± 0.5b |
| Diabetic + RC (200 mg kg⁻¹) | 111 ± 2.0b                  | 4.2 ± 0.16ab           | 30.14 ± 1.6b | 8.8 ± 0.7b |
| Diabetic + RE (200 mg kg⁻¹) | 102 ± 0.66b                 | 4 ± 0.6b               | 25.5 ± 0.58b | 8.8 ± 0.15b |

* Results are expressed as mean ± S. E. M. and analysed using one-way ANOVA followed by Bonferroni’s test for multiple comparisons. *p ≤ 0.05 versus normal group. *p ≤ 0.05 versus diabetic group. *p ≤ 0.05 versus diabetic + pioglitazone (10 mg kg⁻¹) group. *P ≤ 0.05 diabetic + RM (200 mg kg⁻¹) group. n = 5.

![Table 3](image3)

| Group                | Liver index | AST (U L⁻¹) | ALT (U L⁻¹) |
|----------------------|-------------|-------------|-------------|
| Normal               | 2.3 ± 0.1   | 39 ± 1      | 34.5 ± 1.5  |
| Diabetic             | 3.5 ± 0.2a  | 92.5 ± 0.5a | 78 ± 2.0a   |
| Diabetic + pioglitazone (10 mg kg⁻¹) | 2.5 ± 0.3b   | 50.5 ± 1.5ab | 26.5 ± 2.5b |
| Diabetic + RM (200 mg kg⁻¹) | 2.5 ± 0.02b  | 35 ± 1.01bc | 30 ± 1.01b  |
| Diabetic + RH (200 mg kg⁻¹) | 3.9 ± 0.07cde | 72 ± 1.01d  | 55 ± 1.01ad |
| Diabetic + RC (200 mg kg⁻¹) | 3.07 ± 0.04cde | 65.5 ± 2.5a | 46.5 ± 1.5ae |
| Diabetic + RE (200 mg kg⁻¹) | 3.35 ± 0.02cde | 66 ± 1.01ae | 46 ± 1.01ae |

* Results are expressed as mean ± S. E. M. and analyzed using one-way ANOVA followed by Bonferroni’s test for multiple comparisons. *p ≤ 0.05 versus normal group. *p ≤ 0.05 versus diabetic group. *p ≤ 0.05 versus diabetic + pioglitazone (10 mg kg⁻¹) group. *P ≤ 0.05 diabetic + RM (200 mg kg⁻¹) group. *P ≤ 0.05 diabetic + RH (200 mg kg⁻¹) group. n = 5.
Insulin resistance characteristic of type 2 diabetes mellitus including adiposity, dyslipidaemia, hyperglycaemia and changes in liver and ovaries. However, its effect in weight gain was previously discussed. On the other hand, its role in decreasing weight gain, enhancing lipid profile and stimulation of lipid mobilization from visceral part to the lower part of body was also reported. The current results are in agreement with these articles, as pioglitazone treatment reduced total body weight and decreased liver-fat resulting in elevation of insulin sensitivity in these tissues. Additionally, the effect of pioglitazone is related to the correct

![Fig. 1](histopathological picture for hepatic specimens stained with hematoxylin and eosin with magnification power 40×. (A) Histopathology images for liver sections from normal group uniform hepatocytes arranged in plates radiating from central vein (black arrows) (H&E, 40×). (B) Diabetic group in which hepatocytes show evidence of injury, hydropic degeneration (black arrows) and steatosis (red arrows) (H&E, 40×). (C) Diabetic + pioglitazone (10 mg kg⁻¹) shows mild histopathological changes (H&E, 40×). (D) Diabetic + RM (200 mg kg⁻¹) group which shows mild histopathological changes. (E) Diabetic + RH (200 mg kg⁻¹) group which shows mild hydropic degeneration of hepatocytes (black arrows) and many hepatocytes show uniform morphology (red arrows) (H&E, 40×). (F) Diabetic + RC (200 mg kg⁻¹) which shows moderate degeneration in hepatocytes (black arrows) (H&E, 40×). (G) Diabetic + RE (200 mg kg⁻¹) which shows moderate hydropic degeneration of hepatocytes (black arrows). Congested sinusoids are seen (red arrows) (H&E, 40×). (H) Effect of different extracts of *Rubia tinctorum* and pioglitazone (10 mg kg⁻¹) on percent of liver steatosis. (A) Normal group. (B) Diabetic group. (C) Diabetic + pioglitazone (10 mg kg⁻¹). (D) Diabetic + RM (200 mg kg⁻¹). (E) Diabetic + RH (200 mg kg⁻¹). (F) Diabetic + RC (200 mg kg⁻¹). (G) Diabetic + RE (200 mg kg⁻¹). Results are expressed as mean ± S. E. M. and analyzed using one-way ANOVA followed by Bonferroni’s test for multiple comparisons. aP ≤ 0.05 versus normal group. bP ≤ 0.05 versus diabetic group. n = 5.)
choice of its dose as lower and higher doses of pioglitazone may exert no or adverse action like sodium water retention and weight gain. So, the dose of pioglitazone should be monitored and well selected. In the current study the selected dose was (10 mg kg⁻¹) which is considered to be medium dose and has significant effect in all measured parameters.

Similarly, diabetic rats treated with RM, RC or RE (each 200 mg kg⁻¹) extracts showed a significant decrease in final body weight and % change in body weight, however, only diabetic rats treated with RM (200 mg kg⁻¹) and RC (200 mg kg⁻¹) showed a significant improvement in obesity index compared with diabetic group. On the other hand, the group treated with the extract RH (200 mg kg⁻¹) couldn’t show any significant enhancement in final body weight, % change in body weight or obesity index in comparison with diabetic group at p ≤ 0.05. Moreover, the results achieved by the treatment with the extract RM (200 mg kg⁻¹) were the best in improving the decrease % change in body weight and obesity index (Table 1).

3.2. Effect of different *R. tinctorum* extracts and pioglitazone (10 mg kg⁻¹) on blood glucose level, serum insulin, insulin resistance and serum leptin level on type II diabetic rats

The current results showed significant increases in blood glucose level (mM L⁻¹), serum insulin level (ng L⁻¹), HOMA-IR and serum leptin level (ng L⁻¹) in diabetic group in comparison with normal group at p ≤ 0.05 (Table 2). However, treatment with pioglitazone (10 mg kg⁻¹) significantly decreased blood glucose level (mM L⁻¹), serum insulin level (ng L⁻¹), HOMA-IR and serum leptin level (ng L⁻¹) when compared to diabetic group at p ≤ 0.05. Additionally, treatment with RM, RH, RC and RE (each of 200 mg kg⁻¹) significantly induced a decrease in blood glucose level (mM L⁻¹), serum insulin level (ng L⁻¹), HOMA-IR and serum leptin level (ng L⁻¹) when compared to diabetic group at p ≤ 0.05. However, the most significant results were obtained from the group treated with RM (200 mg kg⁻¹) concerning normalization of the level of serum leptin (ng L⁻¹) at p ≤ 0.05 (Table 2).

**Table 4** Effect of different *R. tinctorum* extracts and pioglitazone (10 mg kg⁻¹) on lipid profile, serum triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) in the experimental groups of type II diabetes in rats

| Group | Serum TG (mg dL⁻¹) | Serum TC (mg dL⁻¹) | HDL (mg dL⁻¹) | LDL (mg dL⁻¹) |
|-------|-------------------|-------------------|---------------|---------------|
| Normal | 54.5 ± 3.5 | 63 ± 3.03 | 45 ± 11 | 24.5 ± 0.5 |
| Diabetic | 161 ± 16.1 | 116.5 ± 3.5 | 20 ± 2.02 | 55.5 ± 4.5 |
| Diabetic + pioglitazone (10 mg kg⁻¹) | 42.5 ± 2.5 | 88.5 ± 1.6 | 40.5 ± 3.5 | 30 ± 2.02 |
| Diabetic + RM (200 mg kg⁻¹) | 68 ± 1.01 | 72 ± 1.01 | 36 ± 0.5 | 23 ± 1.01 |
| Diabetic + RH (200 mg kg⁻¹) | 98.5 ± 0.5 | 89.5 ± 0.5 | 30.5 ± 0.5 | 46.5 ± 0.5 |
| Diabetic + RC (200 mg kg⁻¹) | 85 ± 2.02 | 82.5 ± 0.5 | 26 ± 1.01 | 45.5 ± 3.5 |
| Diabetic + RE (200 mg kg⁻¹) | 80.5 ± 0.5 | 76.5 ± 0.5 | 26.5 ± 0.5 | 39.5 ± 0.5 |

*Results are expressed as mean ± S. E. M. and analyzed using one-way ANOVA followed by Bonferroni’s test for multiple comparisons. *p* ≤ 0.05 versus normal group. *p* ≤ 0.05 versus diabetic group. *p* ≤ 0.05 versus diabetic + pioglitazone (10 mg kg⁻¹) group. *p* ≤ 0.05 diabetic + RM (200 mg kg⁻¹) group. *p* ≤ 0.05 diabetic + RH (200 mg kg⁻¹) group. n = 5.

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![Chromatogram of methanolic root extract of *Rubia tinctorum* in positive mode ion.](http://example.com/chromatogram.png)
3.3. Effect of different *Rubia tinctorum* extracts and pioglitazone (10 mg kg\(^{-1}\)) on liver index, serum liver enzymes level, hepatic tissue histopathological changes and percent of steatosis on type II diabetic rats

In the current study, diabetic group showed a significant increase in liver index with a value (3.5 ± 0.2) versus (2.3 ± 0.1) in normal group at \(p \leq 0.05\) (Table 3). Diabetic group showed elevation in serum liver enzymes AST and ALT in comparison with normal group at \(p \leq 0.05\). Furthermore, treatment of diabetic rats with only pioglitazone (10 mg kg\(^{-1}\)) or the extract RM (200 mg kg\(^{-1}\)) for four weeks could significantly normalize the liver index in comparison with diabetic group at \(p \leq 0.05\). Additionally, treatment with pioglitazone (10 mg kg\(^{-1}\)), RM, RH, RC and RE (each 200 mg kg\(^{-1}\)) significantly induced a decrease in both two serum liver enzymes ALT and AST in comparison with diabetic group at \(p \leq 0.05\).

Finally, diabetic group showed evidence of injury; hydropic degeneration (black arrows) and a significant increase in percent of steatosis (red arrows) (H&E, 40×) when compared to normal group at \(p \leq 0.05\) (Fig. 1). Treatment with either pioglitazone (10 mg kg\(^{-1}\)) or any of the *Rubia* extracts showed enhancement in liver architecture and a significant decrease in percent of steatosis with a significant reduction in hydropic degeneration of hepatocytes (black arrows) and many hepatocytes show uniform morphology (red arrows) (H&E, 40×) in comparison with diabetic group at \(p \leq 0.05\) (Fig. 1). However, the results obtained by treatment with the extract RM were the best and closer to the normal group (Table 3).

Table 5 Anthraquinones previously isolated from *Rubia tinctorum* root and detected in the extract by LC-HRMS analysis (positive mode)

| \(R_t\) (min) | Molecular formula \(m/z\) \([M + H]^+\) | Name of the compound | Substituents \(R_1\) \(R_2\) \(R_3\) \(R_4\) \(R_5\) |
|---------------|---------------------------------|---------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| 7.18 | C₂₆H₂₈O₁₄ | 565.1557 | Lucidin-3-O-primveroside | OH | CH₂OH | O-primverose | H | |
| 8.82 | C₂₅H₂₆O₁₃ | 535.1451 | Ruberythric acid | OH | O-Glucose (6-1)xylose | H | H | |
| 8.92 | C₁₅H₁₀O₇ | 301.0348 | Pseudopurpurin | OH | COOH | OH | OH | H | |
| 9.57 | C₁₅H₁₀O₆ | 285.0399 | Munjistin | OH | COOH | OH | H | |
| 10.08* | C₁₄H₁₀O₅ | 257.045 | Purpurin | OH | OH | H | OH | H | |
| 10.28* | C₁₄H₁₀O₅ | 257.045 | Anthragallol | OH | OH | OH | H | H | |
| 12.29** | C₁₄H₁₀O₄ | 241.0501 | Alizarin | OH | OH | H | H | H | |
| 12.31** | C₁₄H₁₀O₄ | 241.0501 | Xanthopurpurin | OH | H | OH | H | H | |
| 12.64 | C₁₄H₁₀O₃ | 271.0606 | 1,4-Dihydroxy-2-hydroxymethyl anthraquinone | OH | CH₂OH | H | OH | H | |
| 12.64 | C₁₄H₁₀O₃ | 225.0551 | 2-Hydroxy anthraquinone | OH | OH | H | H | H | |
| 13.31 | C₁₄H₁₀O₃ | 255.0657 | Rubiadin | OH | CH₃ | OH | H | H | |
| 13.96 | C₁₄H₁₀O₃ | 299.0919 | 1,4-Dihydroxy-2-ethoxymethyl anthraquinone | OH | CH₂OC₂H₅ | H | OH | H | |
| 13.77*** | C₁₅H₁₀O₅ | 271.0606 | Anthragallol-3-methyl ether | OH | OH | OCH₃ | H | H | |
| 13.83*** | C₁₅H₁₀O₅ | 271.0606 | Lucidin | OH | CH₂OH | OH | H | H | |
| 18.08**** | C₁₅H₁₀O₅ | 239.0708 | 1-Hydroxy-2-methyl AQ | OH | CH₃ | H | H | H | |
| 18.75**** | C₁₅H₁₀O₅ | 239.0708 | 7-Hydroxy-2-methyl AQ | H | CH₃ | H | H | OH | |
| 18.80** | C₁₅H₁₀O₅ | 255.0657 | Xanthopurpurin-3-methyl ether | OH | H | OCH₃ | H | H | |
| 19.02** | C₁₅H₁₀O₅ | 255.0657 | Alizarin-1-methyl ether | OCH₃ | OH | H | H | H | |
| 19.53** | C₁₅H₁₀O₅ | 255.0657 | Alizarin-2-methyl ether | OH | OCH₃ | H | H | H | |
| 19.77** | C₁₅H₁₀O₅ | 255.0657 | Xanthopurpurin-1-methyl ether | OCH₃ | H | OH | H | H | |
| 20.16 | C₁₅H₁₀O₅ | 255.0657 | 1-Hydroxy-2-hydroxymethyl anthraquinone | OH | CH₂OH | H | H | H | |
| 20.5 | C₁₅H₁₀O₅ | 285.0763 | Anthragallol-2,3-dimethyl ether | OH | OCH₃ | OCH₃ | H | H | |
| 20.84 | C₁₅H₁₀O₅ | 239.0708 | 2-Methoxy-anthraquinone | H | OCH₃ | H | H | H | |
| 21.30**** | C₁₅H₁₀O₅ | 269.0814 | Alizarin-dimethyl ether | OCH₃ | OCH₃ | H | H | H | |
| 21.40**** | C₁₅H₁₀O₅ | 269.0814 | Xanthopurpurin dimethyl ether | OCH₃ | H | OCH₃ | H | H | |
| 23.0**** | C₁₅H₁₀O₅ | 253.0864 | 1-Methoxymethyl anthraquinone | CH₂OCH₃ | H | H | H | |
| 24.12**** | C₁₅H₁₀O₅ | 253.0864 | 1-Methoxy-2-methyl anthraquinone | OCH₃ | CH₃ | H | H | H | |
| 25.52 | C₁₅H₁₀O₅ | 266.0943 | 2-Ethoxymethyl anthraquinone | H | CH₂OC₂H₅ | H | H | H | |

\*\*, \***, \****: interchangeable values.
3.4. Effect of different \textit{R. tinctorum} extracts and pioglitazone (10 mg kg\(^{-1}\)) on lipid profile, serum triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) on type II diabetic rats

Treatment with high fat diet followed with STZ (30 mg kg\(^{-1}\)) in diabetic group resulted in a significant increase in serum triglycerides (TG) (mg dL\(^{-1}\)), total cholesterol (TC) (mg dL\(^{-1}\)) and low-density lipoprotein (LDL) (mg dL\(^{-1}\)) and a significant decrease in high-density lipoprotein (HDL) (mg dL\(^{-1}\)) in comparison with normal group at \(p < 0.05\) (Table 4). Treatment with pioglitazone (10 mg kg\(^{-1}\)) resulted in a significant decrease in serum triglycerides (TG) (mg dL\(^{-1}\)), total cholesterol (TC) (mg dL\(^{-1}\)) and low-density lipoprotein (LDL) (mg dL\(^{-1}\)) and a significant increase in high-density lipoprotein (HDL) (mg dL\(^{-1}\)) in comparison with diabetic group at \(p < 0.05\).

Furthermore, all extracts induced a significant decrease in serum triglycerides (TG) (mg dL\(^{-1}\)), total cholesterol (TC) (mg dL\(^{-1}\)) and low-density lipoprotein (LDL) (mg dL\(^{-1}\)) at \(p < 0.05\) (Table 4). Moreover, treatment with RM (200 mg kg\(^{-1}\)) resulted in a significant improvement and normalization in high-density lipoprotein (HDL) serum level (mg dL\(^{-1}\)) in comparison with diabetic group at \(p < 0.05\) (Table 4).

Accordingly, the results obtained from treatment of the diabetic rats with the methanolic extract of \textit{Rubia tinctorum} were the best and more close to normal group and pioglitazone treated group either in decreasing body weight, obesity, improving lipid profile, normalization hyperglycaemia, insulin resistance, hyper-insulinemia or in enhancing liver tissue structure and function.

3.5. LC-HRMS analysis

Based on the results of the biological activities of different extracts of \textit{R. tinctorum} which revealed that the best extract was the methanolic one, accordingly, the methanolic extract was subjected to LC-HRMS analysis to identify the flavonoids present in it. The results are presented in Table 6.

| \(R_t\) (min) | Molecular formula | \(m/z\) \([M + H]^+\) | Name of the compound | Substituents | \(R_1\) | \(R_2\) | \(R_3\) | \(R_4\) | \(R_5\) | \(R_6\) |
|-------------|------------------|----------------|----------------------|-------------|-------|-------|-------|-------|-------|-------|
| 7.44        | C\(_{22}\)H\(_{36}\)O\(_{16}\) | 609.1456 | Luteolin-3-7-diglucoside | H | OH | H | O-Glucose | O-Glucose | OH |
| 8.67        | C\(_{22}\)H\(_{30}\)O\(_{13}\) | 593.1507 | Kaempferol-7-neohesperidoside | H | OH | H | O-Neohesperidose | OH | OH |
| 9.41        | C\(_{22}\)H\(_{22}\)O\(_{13}\) | 607.1663 | Hesperetin-7-O-neohesperidoside | H | OH | H | O-Neohesperidose | OH | OCH\(_3\) |
| 10.89       | C\(_{22}\)H\(_{23}\)O\(_{14}\) | 591.1714 | Acacetin-7-rutinoside | H | OH | H | O-Rutinoside | H | OH |
| 11.30       | C\(_{22}\)H\(_{34}\)O\(_{11}\) | 445.0771 | Baicalein-7-O-glucuronide | H | OH | OH | O-Glucuronide | H | H |
| 11.59       | C\(_{22}\)H\(_{26}\)O\(_{11}\) | 447.0928 | Luteolin-7-O-glucosid | H | OH | H | O-Glucose | OH | OH |
| 11.60       | C\(_{22}\)H\(_{27}\)O\(_{10}\) | 433.1135 | Naringenin-7-O-glucoside | H | OH | H | O-Glucose | H | OH |
| 12.30       | C\(_{22}\)H\(_{30}\)O\(_{12}\) | 433.0771 | Quercetin-3-D-xylidoside | H | OH | OH | O-Xylose | OH | OH |
| 12.36       | C\(_{22}\)H\(_{29}\)O\(_{12}\) | 447.0928 | Quercitrin | H | OH | OH | O-Glucose | OH | OH |
| 13.63       | C\(_{22}\)H\(_{29}\)O\(_{10}\) | 431.0978 | Kaempferol-3-O-\(\alpha\)-rhamnoside | H | OH | OH | O-Rhamnoside | OH | OH |
| 13.77       | C\(_{22}\)H\(_{32}\)O\(_{12}\) | 477.1033 | Isorhamnetin-3-O-glucoside | H | OH | OH | O-Glucose | OH | OCH\(_3\) |
| 13.83       | C\(_{22}\)H\(_{30}\)O\(_{8}\) | 285.0399 | Luteolin | H | OH | OH | OH | OH |
| 14.77       | C\(_{22}\)H\(_{30}\)O\(_{8}\) | 271.0607 | Naringenin | H | OH | OH | OH | OH |
| 14.90       | C\(_{22}\)H\(_{30}\)O\(_{3}\) | 269.045 | Apigenin | H | OH | OH | OH | OH |
| 15.84       | C\(_{22}\)H\(_{32}\)O\(_{6}\) | 299.0556 | Hesperetin | H | OH | OH | OH | OCH\(_3\) |
| 15.61       | C\(_{22}\)H\(_{32}\)O\(_{5}\) | 283.0607 | Acacetin | H | OH | OH | OH | OCH\(_3\) |
| 9.82        | C\(_{22}\)H\(_{26}\)O\(_{13}\) | 611.1976 | Neohesperidin dihydrochalcon | | | | | | |

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\textbf{Table 6} Flavonoids of \textit{Rubia tinctorum} root extract identified by LC-HRMS analysis (positive mode)
3.6. Docking study

\( \alpha \)-Amylase has been considered as an important therapeutic target for the management of type 2 diabetes mellitus, hence, we aimed to elucidate the binding mode of the tested compounds with 1HX0 as \( \alpha \)-amylase inhibitor.\(^4\) We performed induced fit molecular docking studies with the compounds under investigation. The docking results with docking scores, and the hydrogen bonded residues are given in Table 7. Additionally, 3D representative images of some of the high binding affinities of both anthraquinone and flavonoids compared to AC1 as the co-crystallized ligand are shown in Fig. 3.

As shown in Table 7, most of the tested derivative were docked and bound to amino acids of the receptor binding site with high and mild binding affinities (energies), while other derivatives couldn't be docked. In reference to the co-crystallized ligand (AC1) forms two major interactions with the Val 163 and Gly 106 as the key amino acids residues, we found two of the anthraquinones with high binding affinity (\(-13.92-21.03 \text{ kcal mol}^{-1}\)), and four flavonoids (\(-16.16-23.56 \text{ kcal mol}^{-1}\)) towards \( \alpha \)-amylase inhibition by forming the same key interactions. Nine of the anthraquinones, and twelve of flavonoids showed mild binding affinities (\(-9.05-15.45 \text{ kcal mol}^{-1}\)) and (\(-16.59-27.45 \text{ kcal mol}^{-1}\)), respectively, as they form only one hydrogen bond with either Val 163 or Gly 106.

As shown in Fig. 3, three-dimensional representation of two highly docked compounds as two active leads relative to the AC1 with moieties of ligand and receptor involved in the interaction, interaction-type, bond-length for each docking procedure. Among anthraquinone, 1-hydroxy-2-hydroxymethyl anthraquinone forms two hydrogen bonds through the hydroxyl groups as

| Group    | Binding affinity | Identified compound | Binding energy (kcal mol\(^{-1}\)) | Ligand-receptor interactions with |
|----------|------------------|---------------------|------------------------------------|----------------------------------|
| Anthraquinone* | High          | 1-Hydroxy-2-hydroxymethyl AQ\(^{\text{\#}}\) | \(-13.92\)                            | 2 HB with Gly 106 and Val 163    |
|          | Mild             | 1,4-Dihydroxy-2-ethoxyethyl anthraquinone | \(-9.05\)                            | 1 HB with either Val 163 or Gly 106 |
|          |                  | 2-Hydroxyanthraquinone | \(-9.36\)                            |
|          |                  | 2-Methoxy-anthraquinone | \(-10.43\)                           |
|          |                  | Lucidin-3-O-primeroside | \(-24.9\)                            |
|          |                  | Lucidin               | \(-11.9\)                            |
|          |                  | Purpurin              | \(-14.78\)                           |
|          |                  | Xanthopurpurin dimethylether | \(-11.34\)                         |
|          |                  | Xanthopurpurin       | \(-11.68\)                           |
|          |                  | Rubiadin              | \(-15.45\)                           |
| Flavonoids* | High          | Baicalein-7-O-glucuronide | \(-19.66\)                        | 2 HB with Val 163                |
|          |                  | Kaempferol-3-O-L-rhamnoside | \(-20.95\)                         |
|          |                  | Naringenin-7-O-glucoside\(^{\*}\) | \(-16.16\)                         |
|          |                  | Neohesperidin dihydrochalcon | \(-23.36\)                         |
|          | Mild             | Acacetin 7-rutinoside | \(-21.21\)                           |
|          |                  | Apigenin              | \(-16.59\)                           |
|          |                  | Hesperetin            | \(-20.98\)                           |
|          |                  | Hesperetin-7-O-neohesperidoside | \(-25.20\)                         |
|          |                  | Isorhamnetin-3-O-glucoside | \(-22.21\)                         |
|          |                  | Kaempferol-7-neohesperidoside | \(-17.04\)                         |
|          |                  | Luteolin              | \(-18.08\)                           |
|          |                  | Luteolin-3-7-diglucoside | \(-18.38\)                           |
|          |                  | Luteolin-7-O-glucoside | \(-27.45\)                           |
|          |                  | Naringenin            | \(-17.67\)                           |
|          |                  | Quercetin-3-D-xylloside | \(-19.95\)                           |
|          |                  | Quercetin             | \(-20.89\)                           |

\(^{\#}\)Highly-bonded interactive docked compounds in the same way like AC1. \(^*\)The rest of compounds of both groups weren't able to bind with the receptor pocket.
H-donor with Val 163 with bonds length 2.05 Å, and H-acceptor with Gly 106 with bonds length 1.74 Å. Among flavonoids, naringenin-7-O-glucoside forms two hydrogen bonds through the hydroxyl groups as H-donors with Val 163 and Gly 106 with bonds length 1.59 and 1.53 Å.

From docking study, we conclude the good affinity of compounds under investigation through their hydroxyl and carbonyl active groups that bind inside the tested target (α-amylase inhibition compared to AC1), which is correlated to anti-diabetic activity.

In addition to α-amylase inhibition activity, most of the compounds detected in the extract proved to possess antioxidant and anti-inflammatory activities. It is well known that both activities play an important role in treatment of diabetes mellitus. This also can justify the activity of the methanolic extract of Rubia tinctorum which accumulate a great number of compounds showing antioxidant, anti-inflammatory and α-amylase inhibition activities.

### 4. Conclusions

The current study proved that the methanolic extract of Rubia tinctorum showed significant results in decreasing body weight, improving lipid profile, normalizing hyperglycaemia, insulin resistance, hyperinsulinemia in addition to enhancing liver tissue structure and function. There activities could be attributed to its chemical constituents that exert antioxidant, anti-inflammatory and α-amylase inhibition activities. As indicated by the docking study, 1-hydroxy-2-hydroxymethyl anthraquinone and naringenin-7-O-glucoside were the most potent as α-amylase inhibitors.

### Conflicts of interest

There are no conflicts to declare.
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