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LC-ESI-MS/MS analysis, toxicity and anti-anaemic activity of *Rubia tinctorum L.* aqueous extract

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Abstract

The present study investigated the chemical profile, toxicity, and anti-anaemic activity of *Rubia tinctorum* root aqueous extract against phenylhydrazine induced hemolytic anaemia. Phenolic compounds were analyzed by LC-ESI-MS/MS; acute toxicity test was evaluated by administering a single dose of 2,000 mg.kg^{-1} of the extract; anaemia was induced by administration of 40 mg phenylhydrazine by intraperitoneal injection for 2 days. Moreover, the anti-anaemic activity was evaluated by measuring the haematological parameters of rats treated with iron and aqueous extract for 15 days. The LC-ESI-MS/MS analysis results revealed the presence of 31 phytochemical compounds, among them, citric acid was found as the most abundant. No signs of toxicity or death were recorded, indicating that the LD50 of *R. tinctorum* root extract is higher than 2,000 mg.kg^{-1}. Furthermore, the aqueous extract increased red blood cell levels by 69.82 and 71.67 % in the groups treated with 200 and 400 mg.kg^{-1} of the extract, respectively. Besides, a significant increase in hemoglobin from 12.05 ± 0.15 to 12.9 ± 0.52 g.dL^{-1} was noted in rats treated with 400 mg.kg^{-1} of extract. Thus, the data indicate that the root extract could be considered a natural source for the treatment of anaemia.

Introduction

Nowadays, many people suffer from anaemia. It is a global public health phenomenon affecting developed and developing countries via severe consequences for human health and economic and social development (WHO 2008). The most exposed are infants, children in intensive growth, older adults, and pregnant women. It is thought to be responsible for 3.7 % to 12.8 % of maternal deaths during pregnancy and childbirth in Africa and South Asia (Khan *et al.* 2006). Several factors are responsible for the spread of anaemia: malnutrition, attacks by blood parasites such as trypanosomes, plasmodium, and helminths. It has also been reported that the high demand for the developing fetus during pregnancy is a factor in anaemia.

There are many types of anaemia. One of them is hemolytic anaemia, the most frequent sort defined as the destruction or elimination of red blood cells (RBCs) from the circulation before their average life span of 120 days (Dhaliwal *et al.* 2004). It is caused by the abnormality of the constituents of RBC (hemoglobin, an enzyme of energy metabolism of RBC or constitutive protein of the membrane-extrinsic factor). Also by infectious agents, mechanical factors, toxins, etc. (Loustau *et al.* 2011).

Since ancient times, medicinal plants have been considered essential products for the health of communities and individuals. They contain bioactive substances used in traditional medicine and as precursors in the synthesis of valuable drugs at the industrial level.

Interestingly, many plants including *Phyllanthus emblica* L., *Moringa oleifera* L., *Boerhavia diffusa* L., *Hemidesmus indicus* L., *Centella asiatica* L., *Ageratum conyzoides* L., *Hemidesmus indicus* L., *Momordica charantia* L., *Phyllunthus amarus* L., *Punica granatum* L., *Ocimum tenuiflorum* L., *Solanum americanum* L., *Ichnocarpus frutescens* L. are used in the treatment of anaemia, as they are sources of iron and minerals (Silja *et al.* 2008).

Rubia tinctorum belongs to the Rubiaceae family, comprising 6,000 species (Sharifzadeh *et al.* 2014). It is also known as "El foua"(Ezzaki *et al.* 2021), growing in the northern regions of Africa, the Mediterranean regions of Spain, and some areas of Asia, and it is cultivated in the central and western regions of Iran (Sharifzadeh *et al.* 2014). *Rubia tinctorum* has been widely used as a source of red color with its variants for textile dyeing for thousands of years (Degano *et al.* 2009). In addition, several ethnobotanical studies have reported its use in the treatment of various diseases, such as diarrhea (El Haouari and Rosado 2016), rheumatism, cardiovascular diseases (Jouad *et al.* 2001), and kidney stones (Agarwal and Varma 2015). Moreover, it is really appreciated by Moroccan women as a purgative after childbirth (Ezzaki *et al.* 2021). Besides, there are numerous biological studies on *R. tinctorum* that have demonstrated its therapeutic potential as anti-platelet agent, antitumor, hepatoprotective, antimicrobial, diuretic, and stones inhibitory activities (Sharifzadeh *et al.* 2014; Marhoume *et al.* 2019; Eltamany *et al.* 2020; Hoseinzadeh *et al.* 2020).

To the best of our knowledge, no studies have been performed on the anti-anaemic activity of *R. tinctorum* extract so far. Thus, this study aims to evaluate the anti-anaemic potential of *R. tinctorum* aqueous extract on an experimental model of phenylhydrazine-induced hemolytic anaemia and to identify its phytochemical compounds.

Experimental

Chemicals

Distilled water, ammonium formate, acetonitrile, formic acid, sulfuric acid (98 %), chloric acid, hydrochloric acid, ammonium thiocyanate, sodium chloride (9 %), phenylhydrazine, oroofer plus (Bottle of 100 mL as a drinkable solution, iron III poly maltose hydroxide, 20 mg. mL^{-1} of iron).

Plant material

The *Rubia tinctorum* plant was gathered in June 2018 in Sidi Bel Abbes (North-West Algeria). It was identified by Dr. Righi K, Department of Botany, University of Mustapha Stambouli, Mascara, Algeria. The plant material was shade-dried in a room for 15 days and pulverized using an electrical grinder. A voucher specimen plant has been deposited at the herbarium of the Sciences Faculty, Mustapha Stambouli University (HAM 02 74).

Preparation of aqueous root extract

The aqueous extract of *Rubia tinctorum* L. was prepared by maceration, using 10 g of powders of the root soaked in 100 mL of distilled water for 2 days with constant stirring. The mixture was filtered through Whatman filter paper No. 1. thereafter the extract was freeze-dried. The obtained residue was maintained at 4 °C (Bruneton 1999).

LC-ESI-MS/MS analysis

The aqueous root extract of *Rubia tinctorum* was subjected to LC-ESI-MS/MS analysis. The compounds were separated on an Agilent Poroshell 120 EC-C18 column in reverse phase (100 mm × 3.0 mm, 2.7 µm). The column temperature was fixed at 25 °C and the volume of injection was 5 µL. The mobile phase consisted of a linear gradient of acetonitrile -20 mM ammonium formate (pH 3.0) (15/100; v/v). The injection rate was 1 $mL.min^{-1}$.
The elution gradient was eluent A (water + 5mM ammonium formate) and eluent B (acetonitrile + 0.1 % formic acid), the solvent flow rate was set to 0.250 $mL.min^{-1}$and the gradient was as follows: 1 min 40 % A – 60 % B; 2 min 70 % A – 30 % B; 3 min 70 % A – 30 % B; 4 min 40 % A – 60 % B; 5 min 10 % A – 90 % B. The Agilent 6460 triple quadrupole mass spectrometer system model tandem mass spectrometer and the electrospray ionization source (ESI) operating in negative and positive ionization modes were used. The conditions of the LC-ESI-MS/ MS analysis were as follows: capillary temperature 350 °C, nebulizer N2 gas flow rate 15 $L.min^{-1}$, fragmentor voltage - 4,400 V. The collision energies (CE) were optimized to generate optimal phytochemical fragmentation and maximum transmission of the desired product ions.

LC-MS/MS method validation analysis

To quantify 37 phytochemicals (17 flavonoids, 15 phenolic acids, 3 non-phenolic organic acids, 1 phenolic aldehyde, and 1 benzopyrene) in *Rubia tinctorum* extract, a comprehensive LC-MS/MS method was optimised and validated (Yilmaz *et al.* 2018). Spike and non-spike standard solutions were used to determine the performance characteristics of the method.
The developed method has been validated in terms of linearity, inter-day and intra-day precision (repeatability), accuracy (recovery), limits of detection and quantification (LOD/LOQ), and relative standard uncertainty (U % at 95 % confidence level (k = 2)). The parameters are listed in Table 1.

Determination of Na, Ca, K

In a glass flask, 3 g of plant powder (roots) was mixed with 8 mL of concentrated H_2SO_4 (98 %) and 2 mL of $HClO_3$ (60 %) for 24 h, all placed at a temperature of 80 °C using a sand bath until the digestion material became a white powder. After that, 8 mL of distilled water was added to the powder. Finally, the mineral elements were measured by a flame atomic absorption spectrophotometer (NV202 spectrophotometer) (Aboud 2010).

Determination of iron (Fe)

The iron was quantified spectrophotometrically by the thiocyanate method. For this, the sample was prepared using 5 g of the root powder, placed in a muffle furnace at 600 °C for 3 h until completely transformed into ashes. Ashes were mixed with 1 M hydrochloric acid and 5 mL of distilled water and then filtered.
Thiocyanate solution was prepared using 38 g of ammonium thiocyanate (NH_4SCN), completed with distilled water to a final volume of 500 mL. For the standard curve, optical density was determined at 490 nm for five standard iron solutions (iron concentrations: 2, 4, 6, 8, 10 × 10 – 5 M) mixed by vortexing with 10 mL of thiocyanate solution. Iron content in the root sample was determined using 10 mL of the sample placed in a dry test tube, then mixed with 10 mL of the prepared ammonium thiocyanate solution. The optical density of the mixture was read at 490 nm. Iron content was deduced from the standard curve. The results were expressed in mg iron.100 g^{-1} of the sample (Bhuvaneswari *et al.* 2015).

Experimental animals

Healthy male rats (aged 8 weeks, weighing 162 – 233 g) were used for acute and anti-anaemic studies. They were obtained from the animal house unit of the University of Mustapha Stambouli, Mascara (Algeria), and kept in the experiment room under normal environmental conditions with a temperature of 22 ± 3 °C, relative humidity of 30 – 70 %, and 12 h light/dark cycle, with free access to a regular diet and water.

The experimental protocols were approved by the Animal Research Ethics Committee of the Mascarian University, Mustapha Stambouli (ARECM) according to the Adelaide University Animal Ethics Committee (Ethics number M/76/98).

Acute toxicity test

To assess the acute oral toxicity of *Rubia tinctorum* aqueous extract, the protocol of the Organization for Economic Cooperation and Development (OECD 2002; Kifayatullah *et al.* 2015) was used with slight modifications. The procedures were carried out in two stages. For each stage 3 Wistar rats were used. Twelve male rats weighing 170 ± 7.5 and 232 ± 7.02 g were randomly divided into two groups of 6 each. A single dose of aqueous extract was dissolved in distilled water at 2,000 $mg.kg^{-1}$ body weight, and it was administered to the first group by oral gavage while the control group received only NaCl 9 %. All animals were submitted to overnight fasting. The animals were under surveillance for the first 4 h after administration, and no food was given during this time. After that, they were observed for 14 days once a day for physical and behavioural changes.

Relative organ weight

To determine the relative weight, on the 15th day of the experiment, the animals were sacrificed (anesthesia), the weights of the following organs were recorded in grams: liver, lungs, kidneys, heart, and spleen. Relative organ weight was calculated as follow (Eq. 1; Kifayatullah *et al.* 2015):

(Organ weight/body weight of the rat (on the day of sacrifice)) × 100 % (1)

The percentage change in body weight

The percentage change in body weight of the experimental animals was calculated (Eq. 2; Desai and Singh 2009):

(final body weight-initial body weight / final body weight) × 100 (2)

Analysis of hematological parameter for toxicity tests

On the 15th day, all the animals were sacrificed by anesthesia. The blood was collected from each rat into an EDTA tube by an abdominal puncture. Blood parameters were assessed using an automatic counter (Abacus 380).

Analysis of biochemical parameter

Biochemical analyses were performed on their serum after the centrifugation of the blood. The biochemical parameters, including aspartate transaminase (AST), alanine transaminase (ALT), glycemia, urea, and creatinine were determined for the control groups and treated groups. All analyses were performed using a clinical chemistry analyzer (Mindray BA-88A).

Anaemia test

It was carried out according to the approach used by (Pandey *et al.* 2016)**,** with some modifications. Anaemia was induced in rats by intraperitoneal injection of phenylhydrazine (PHZ) at 40 $mg.kg^{-1}$ for 2 days. Rats that presented anaemia with a hemoglobin concentration of less than 13 $g.L^{-1}$ were used in the study (Diallo *et al.* 2008).

The anaemic rats were divided into five groups (6 animals per group) and treated daily for 15 days. All four groups of animals were treated with phenylhydrazine (PHZ) except Group (I). The first group (control group) received (NaCl (0.9); 10 $mL.kg^{-1}$ body body weight). Group II (negative group) received (PHZ i.p. 40 $mg.kg^{-1}$ body weight) once daily for 2 consecutive days + (Nacl (0.9); 10 $mL.kg^{-1}$ body weight). Group III (positive control) received (PHZ i.p. 40 $mg.kg^{-1}$ body weight) once daily for 2 consecutive days + Standard treatment (Orofer plus). Group IV received (PHZ i.p. 40 $mg.kg^{-1}$ body weight) once daily for 2 consecutive days + Extract (200 $mg.kg^{-1}$). Group V received (PHZ i.p. 40 $mg.kg^{-1}$ body weight) once daily for 2 consecutive days + Extract (400 $mg.kg^{-1}$).

All tested drugs were administered orally.

Analysis of hematological parameters for antianaemic activity tests

The blood was collected from each rat into an EDTA tube by ocular puncture before the induction of anaemia (day 1); during the test (day 3); and at the end of the test (day 15), were evaluated for blood parameters using an automatic counter (Abacus 380) (Pandey *et al.* 2016).

Statistical analysis

Data analysis was carried out using SPSS IBM 23 statistical package. Analysis of variance (ANOVA) with Fisher's LSD tests and *t*-test were used. The data were presented as mean ± standard error, P<0.05 was considered statistically significant.

Results and Discussion

LC-ESI-MS/MS analysis

In the present study, aqueous extracts of *Rubia tinctorum* roots were assessed for phytochemical composition, acute toxicity, and antianaemic activity thereof. The percentage extraction yield of the extracts was recorded as 18.8 %.

The phytochemical profile of *R. tinctorum* root aqueous extracts investigated by LC-ESI-MS/MS equipment report Fig. 1 and Table 1.

LC-ESI-MS/MS analysis of *Rubia tinctorum aquenos* aqueous extract revealed the presence of many phytochemical compounds. The highest amount was attributed to citric acid (165.80 $\mu g.mg^{-1}$) followed by ascorbic acid (64.99 $\mu g.mg^{-1}$), vanillic acid (52.88 $\mu g.mg^{-1}$), epicatechin (50.78 $\mu g.mg^{-1}$), and 4-hydroxybenzoic acid (49.93 $\mu g.mg^{-1}$), and eleven other compounds with concentrations varying between 1.028 – 47.42 $\mu g.mg^{-1}$.

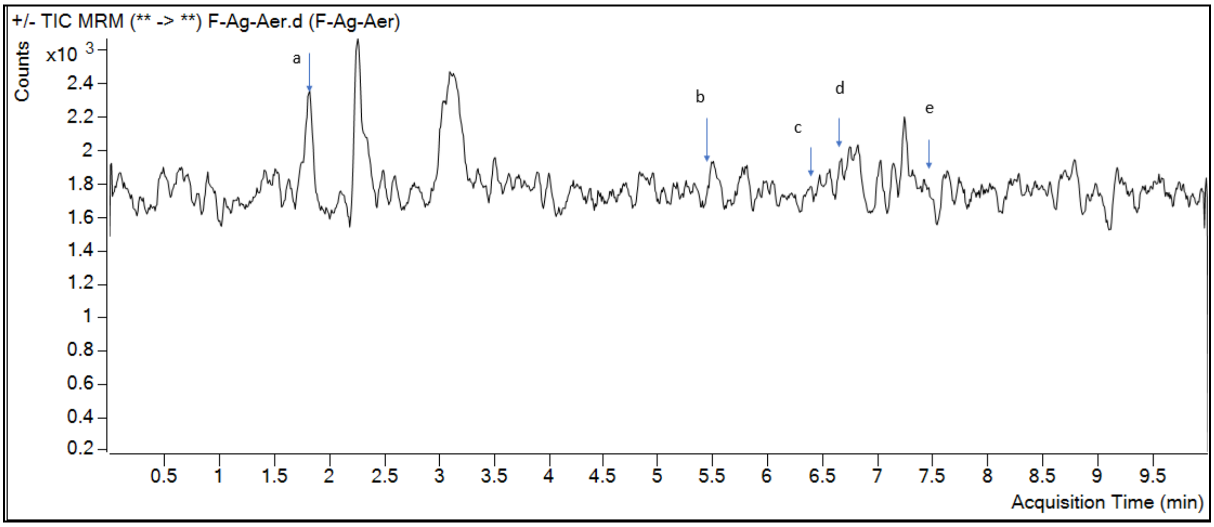

Fig. 1. LC-ESI-MS /MS chromatogram of *Rubia tinctorum* aqueous extract. a – citric acid; b – 5-O-caffeoylquinic acid; c – 4-hydroxyibenzoic acid; d – vanillic acid; e – trans-ferulic acid.

Although *Rubia tinctorum* roots are widely used in traditional medicine (Manojlovic *et al.* 2005), a few studies were found in the literature on the phytochemicals composition of aqueous extract thereof. According to the previous findings, a large variety of phenolic compounds were identified in the *Rubia tinctorum* root. A study by Aboud (2010) revealed the presence of anthocyanidins, chalcone, and kaempferol. Similarly, a previous investigation using LC-ESI-MS/MS reported the presence of further compounds that are munjistin, pseudopurpurin, rubiadin, ruberythric acid, pseudopurpurin, lucidin, primeveroside, and nor-damnacanthal (Lajkó *et al.* 2015). Thus, comparing these results, the richness of *R. tinctorum* root in terms of phenolic compounds' diversity can be confirmed.

Mineral composition of aqueous extract

Table 2. shows the mineral content of *Rubia tinctorum* roots. The results revealed the macro mineral elements (sodium (Na), potassium (K), calcium (Ca), and micro mineral elements (iron (Fe)) at varying concentrations. These results are consistent with those of Aboud (2010), who indicated that the concentrations of sodium (Na), potassium (K), and iron (Fe) in the root of *Rubia tinctorum* were higher, whereas calcium (Ca) was found to be at the lowest concentration.

Table 2. Mineral composition of *Rubia tinctorum* root.

Mineral component	Na	K	Ca	Fe
Content. ppm	0.115 ± 0.01	1.68 ± 0.01	0.043 ± 0.02	3.32 ± 0.07

The data present the mean value of three replicates ± SD. ppm – parts per million; Na – sodium; K – potassium; Ca – calcium and Fe – iron.

Study of acute toxicity

In the present study, the acute toxicity of the extract was investigated using a single dose of 2000 mg/kg/body weight of the extract on 12 rats. The mortality or morbidity was determined after 14 days. Besides morbidity, behavioral changes such as respiration, temperature, convulsions, diarrhea, shaking, and itching were examined during the period of the test, as reported in Table 3. The administration of a single dose of aqueous extract did not cause any sign of toxicity (morbidity or mortality) in all animals. Similarly, no behavioral changes were observed in rats.

On the other hand, our data showed no significant change (P>0.05) in the liver, kidney, heart, and pancreas weights in either treated or untreated rats after 15 days (Table 4).

Besides, macroscopic observations of the organs displayed neither morphological alterations nor changes in their color texture.

The effect of the aqueous extract of *Rubia tinctorum* on haematological parameters is summarized in Table 5. We observed an increase in the platelet count (PLT), mean corpuscular hemoglobin concentration (MCH), mean corpuscular volume (MCV) in treated rats (P>0.05). However, white blood cells (WBC), red blood cells (RBC), hemoglobin (HB), and lymphocytes (LMY) were significantly decreased. Regarding the biochemical parameter levels, the results are shown in Table 6. Compared to the control group, no significant changes (P>0.05) in creatinine (Crea), glutamate-pyruvate transaminase (TGP), triglycerides (Triglyc) and cholesterol levels were observed. Moreover, a significant decrease in urea and cholesterol was detected (P<0.05).

According to our results, DL50 is superior to 2,000 mg.kg^{-1}. A single dose of 2,000 mg.kg^{-1} did not produce any visible signs or symptoms of toxicity in all treated animals. In addition, we observed no changes in the rat's behavior, no toxic symptoms, no changes in hematological parameters, and no deaths. Therefore, based on the OCDE method of acute toxicity, the *Rubia tinctorum* aqueous extract can be considered a non-toxic substrate (OECD 2002).

Table 3. Clinical signs in acute oral toxicity study of *Rubia tinctorum* aqueous extract in Wistar rats exposed for a dose 2,000 mg.kg^{-1}.

Observation	Control group	Treated group
Temperature	NR	NR
Food intake	NR	NR
Rate of respiration	NR	NR
Change in skin	NO	NO
Eye color	NR	NR
Diarrhea	NP	NP
General physique	NR	NR
Coma	NO	NO
Death	NO	NO
Drowsiness	NP	NP

Notes: NR – normal; NO – not observed; NP – not present.

Table 1. *Rubia tinctorum L.* roots aqueous extracts LC-ESI-MS/MS analysis.

Compound	Rt	Ion transitions [m/z]	Ion. mode	R2	DW [μg.mg^{-1}]	Linearity range [μg. L^{-1}]	LOD [μg.L^{-1}]	LOQ [μg.L^{-1}]	Recovery [%]
Tartaric acid	1.696	149-87	Negative	0.999	ND	62.5-2,000	12.309	37.3	100.7
Citric acid	1.793	191.1-111	Negative	0.999	165.8091	125-2,000	6.237	18.9	100.55
Ascorbic acid	1.802	175.1-114.9	Negative	0.999	64.9996	62.5-2,000	7.75	23.5	99.6
Fumaric acid	1.821	115-71.1	Negative	0.999	16.0948	31.25-2,000	6.43	19.5	100.77
Maleic acid	1.821	115-71.2	Negative	0.999	ND	62.5-2,000	6	18.2	99.8
Chicoric acid	1.989	472.8-310.5	Negative	0.999	31.7746	250-2,000	50.16	152	89.1
Gallic acid	2.605	169-125	Negative	0.998	ND	62.5-2,000	9	54.6	98.9
5-O-caffeoylquinic acid	5.526	353-191	Negative	0.999	47.4291	250-2,000	64.68	196	86.2
4-Hydroxyibenzoic acid	6.531	137-93.1	Negative	0.999	49.9302	62.5-2,000	2.376	7.2	100.7
Catechin	6.660	288.9-245.1	Negative	0.999	31.4229	62.5-2,000	2.57	7.8	100
Epicatechin	6.666	353-191	Positive	0.998	50.7805	62.5-2,000	2.9	8.8	100.6
Hesperidin	6.674	611.3-357	Positive	0.999	ND	125-2,000	32.67	99	99.5
Rutin	6.675	608.9-299.4	Negative	0.997	ND	125-2,000	28.5	85	97.8
Vanillic acid	6.687	167-151.8	Negative	0.998	52.8816	62.5-2,000	2.54	7.7	100
Syringic acid	6.703	197.1-181.8	Negative	0.999	ND	62.5-2,000	4.22	12.8	100.5
Caffeic acid	6.703	178.9-135.1	Negative	0.999	1.0283	125-2,000	25.74	78	99.7
Luteolin -7-glucoside	6.740	449-286.9	Positive	0.997	ND	62.5-1,000	16.5	50	100.6
Apigenin-7-O-glucoside	6.808	430.8-267.4	Negative	0.998	ND	125-1,000	18.24	55.3	100.8
Quercetin-3-glucoside	6.816	432.7-299.5	Negative	0.995	ND	62.5-2,000	9.87	29.9	100.1
Oleuropein	6.849	539.1-275.1	Negative	0.999	ND	62.5-2,000	17.35	52.6	101.9
Rosmarinic acid	6.875	358.9-160.7	Negative	0.998	27.9655	62.5-2,000	15.9	48.2	100.6
P-coumaric acid	6.919	163-119	Negative	0.999	10.2367	62.5-2,000	3	9.1	100.3
4- Hydroxybenzaldehyd	6.929	121-92	Negative	0.999	10.79	31.25-2,000	1.91	5.7	99.9
Trans-ferulic acid	6.968	193.1-133.9	Negative	0.998	51.0571	31.25-2,000	7.26	22.3	100.3
Gentisic acid	7.243	153-109	Negative	0.999	14.1623	250-2,000	44.55	135	99.9
Protocatechuic acid	7.243	152.9-108.9	Negative	0.999	30.2216	62.5-2,000	15.44	46.8	100.4
Quercetin	7.306	300.7-150.9	Negative	0.997	ND	62.5-1,000	14.85	45	98.8
Apigenin	7.555	269-117	Negative	0.999	ND	125-2,000	17.82	54	101.2
Naringenin	7.588	270.9-119.1	Negative	0.999	ND	125-2,000	24.37	73.8	101.2
Trans-cinnamic acid	7.591	148.8-104.8	Negative	0.999	26.1761	62.5-2,000	13.59	41.2	102
Kaempferol	7.613	284.9-116.9	Negative	0.998	ND	62.5-2,000	12.39	37.5	101.1

Rt – retention time, FC – final concentration, ND – not detected, R2 – coefficient of determination, RSD –relative standard deviation, LOD/LOQ (μg.L^{-1}) – limit of detection/quantification, DW – dry weight.

Table 4. Effects of the aqueous extract of *Rubia tinctorum* on the relative weight of organs and relative body weight in rats during acute toxicity study.

Group	Relative organ weight [%]					Relative body weight. [%]
	Heart	Liver	Spleen	Kidney	Lung	
Temoin	0.31 ± 0.02	3.16 ± 0.44	0.32 ± 0.03	0.63 ± 0.04	0.633 ± 0.03	4.88
ER	0.376 ± 0.04	3.606 ± 0.39	0.35 ± 0.06	0.608 ± 0.04	0.733 ± 0.12	13.16

The data present the mean value of three replicates ± *SD*. Results were analysed by standard *t*-test, treated group were compared to the control group, *P*>0.05. ER – group treated with aqueous extract of *Rubia tinctorum*.

These results are in agreement with those of Marhoume *et al.* (2019) who found that the butanolic extract had no toxic effects on treated rats. Moreover, it was consistent with the study of Karim *et al.* (2010), reporting that the tolerated dose of aqueous extract of *Rubia tinctorum* was up to 10 $g.kg^{-1}$ body weight in albino mice, and no mortality and toxic symptoms were observed.

Table 5. Effects of *Rubia tinctorum* aqueous extract on haematological parameter in rats during 14 d of oral acute toxicity study.

Haematological parameters	Control group	Treated group (2,000 mg. mL^{-1})
WBC /10^3 μL^{-1}	12.2466 ± 1.30	9.43 ± 1.09
LYM /10^3 μL^{-1}	7.965 ± 0.47	6.3433 ± 1.62
RBC/ 10^6 μL^{-1}	9.263 ± 0.30	7.26 ± 0.68
HB / $g.dL^{-1}$	17.06 ± 1.19	14.166 ± 1.15
CCMH /g. dL^{-1}	33.9 ± 0.78	33.9 ± 0.78
PLT /10^3 μL^{-1}	625.33 ± 108.7	652 ± 37
TCMH / pg	18.433 ± 0.83	19.5 ± 0.26
PCV / %	54.33 ± 3.05	57.333 ± 0.57

The data presents the mean value of three replicates ± SD. Results were analysed by standard *t*-test, the treated group was compared to the control group, *P*>0.05. RBC – red blood cells; WBC – white blood cells; HB – hemoglobin; LMY – lymphocytes; PLT – platelet count; CCMH – mean corpuscular hemoglobin concentration in red blood cells; TCMH – mean corpuscular hemoglobin content in hematite; PCV – packed cell volume.

Table 6. Effects of *Rubia tinctorum* aqueous extract on biochemical parameter in rats during 14 d of oral acute toxicity study.

Biochemical parameter	Control group	Treated group (2,000 mg. mL^{-1})
Uree	0.56 ± 0.01	0.386 ± 0.03
Crea	4.76 ± 0.30	4.7 ± 0.26
TGO	66.69 ± 20.81	54.58 ± 13.65
TGP	32.3 ± 3.39	32.2 ± 6.89
Cholesterol	0.66 ± 0.05	0.48 ± 0.10
Triglyceride	0.4 ± 0.06	0.423 ± 0.14

The data presents the mean value of three replicates ± SD. Results were analysed by standard *t-test*, treated group were compared to the control group; *P*>0.05. Crea – creatinine; TGP – glutamate-pyruvate transaminase; TGO – aspartate-amino transferase.

Anti-anaemic activity

The effect of acute administration of root aqueous extract on haematological parameters is shown in Tables 7, 8, and 9. The haematological parameters were measured before the treatment (D0), after phenylhydrazine-induced anemia (D2), and up to 15 days.

As stated in Table 8, the treatment with phenylhydrazine decreased RBC, HB, HT, MCH, MCV levels and increased MCHC level compared to the control group.

Table 7. Estimation of haematological parameter before induction of the anaemia.

Hematological parameter	GR(I)	GR(II)	GR(III)	GR (IV)	GR(V)
RBC /10^3 μL^{-1}	8.26 ± 0.3**	7 ± 0.03***	8.76 ± 0.25***	8.23 ± 0.078***	7.84 ± 0.03***
HB / $g.dL^{-1}$	17.6 ± 1.19**	15.7 ± 0.43***	17.9 ± 0.31	16.3 ± 0.11***	16.3 ± 0.06***
HT / %	45.83 ± 2.30***	43.28 ± 0.22***	50.96 ± 0.14***	47.99 ± 0.35***	46.38 ± 0.03
CCMH /g. dL^{-1}	34.3 ± 0.72***	33.8 ± 0.02***	35.2 ± 0.32***	33.8 ± 0. 27***	35.1 ± 2.23 ***
MCH /fl	17.7 ± 0.64***	19.5 ± 0.02	35.2 ± 0.32***	33.8 ± 0. 27***	35.1 ± 2.23 ***
PCV / %	40.59 ± 0.12***	43.28 ± 0.03***	50.96 ± 0.71	47.99 ± 0.001***	46.38 ± 0.04***

**P*<0.05; ***P*<0.01; ****P*<0.001, significantly different from the control group. Results were analyzed by ANOVA *test*. Values expressed are means ± SD (n = 3). RBC – red blood cells; HB – hemoglobin; PCV– packed cell volume; HT – haematocrit; CCMH – mean corpuscular hemoglobin concentration; MCH – mean corpuscular hemoglobin.

Table 8. Estimation of haematological parameter after induction of anaemia.

Hematological parameter	GR(I)	GR(II)	GR(III)	GR(IV)	GR(V)
RBC /10^3 µL^{-1}	8.32 ± 0.51*	4.25 ± 0.035***	4.66 ± 0.04***	4.72 ± 0.21***	4.63 ± 0.02***
Hb g.dL ^{1}	17.78 ± 0.26*	11.35 ± 0.35***	11.1 ± 0.21	12.05 ± 0.15***	12.85 ± 0.07***
Ht / %	45.88 ± 0.33***	35.98 ± 2.26***	38.01 ± 0.33***	36.58 ± 0.35	38.12 ± 0.02
CCMH /g. dL ^{1}	33.9 ± 0.47***	39.85 ± 0.49	40 ± 0.21***	52.8 ± 3.45***	37.35 ± 3.04***
MCH /fl	18.81 ± 0.51***	17.8 ± 1.41***	18.6 ± 0.49	17.4 ± 0.3***	18.15 ± 0.07
PCV / %	41.33 ± 2.4***	33.48 ± 5.79***	39.25 ± 2.16	32.03 ± 5.86***	42.62 ± 3.55

$^*P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001, significantly different from the control group. Results were analyzed by ANOVA *test*. Values expressed are means ± SD (n = 3). RBC – red blood cells; HB – hemoglobin; PCV– packed cell volume; HT – haematocrit; CCMH – mean corpuscular hemoglobin concentration; MCH – mean corpuscular hemoglobin.

However, iron and root aqueous extract administration significantly raised the levels of RBC, HT, HB, MCH, and PCV (P<0.001) (Table 9). Indeed, iron treatment exhibited strong improvement in the RBC levels (from 4.66 ± 0.04 10^3 µL^{-1} to 6.96 ± 1.25 10^3 µL^{-1}). Similarly, the aqueous extract enhanced RBC levels in a dose-dependent manner, by 69.82 and 71.67 % for GR IV and GR V, respectively. Moreover, an increase of hemoglobin (HB) and hematocrit (Ht) in treated groups (III, IV, and V) was noted as compared to the anemic rat group (GR II).

Our results revealed that MCHC increased considerably in all groups after treatment with phenylhydrazine. However, aqueous extract and iron resulted in a decrease of MCHC level from 38.01 ± 0.33, 32.09 ± 1.88, 38.12 ± 0.02 g. dL^{-1} to 30.55 ± 2.89, 31.3 ± 0.92, 32.86 ± 0.65 g.dL^{-1} in GR III, GR IV and GR V, respectively.

Our results are similar to those of Pandey *et al.* (2016) who reported a significant reduction in haematological parameters in PHZ-injected rats compared to a control group. This result is related to the toxicity of phenylhydrazine. Indeed, the administration of phenylhydrazine into the bloodstream causes oxidative stress in erythrocytes, membrane disruption, and embrittlement as well as hemolysis which triggers events such as premature ageing of erythrocytes, resulting in the lack of hemoglobin and circulating erythrocyte (Ogbe *et al.* 2010; Marhoume *et al.* 2019).

Table 9. Estimation of haematological parameter after treatment.

Haematological parameter	GR(I)	GR(II)	GR(III)	GR (IV)	GR(V)
RBC /10^3 µL^{-1}	8.66 ± 0.03*	6.07 ± 0.30***	6.96 ±1.25***	6. 76 ± 0.35***	6.46 ± 0.17***
HB / g.dL^{-1}	17.89 ± 0.41**	11.95 ± 0.21***	13.25 ± 0.77*	12.66 ± 0.69***	12.9 ± 0.52***
HT / %	46.02 ± 1.45**	35.28 ± 0.86***	39.6 ± 1.27*	32.09 ± 1.88***	38.13 ± 1.70***
CCMH g.dL^{-1}	33.97 ± 0.85***	32.2 ± 0.28***	30.55 ± 2.89***	31.3 ± 0.92**	32.86 ± 0.65
MCH / fl^{-1}	18.98 ± 0.10***	19.15 ± 0.07*	18.35 ± 1.90**	18.43 ± 0.61***	20.63 ± 2.08**
PCV/ %	41.56 ± 0.78***	33.28 ± 0.86***	42.6 ± 1.27	34.09 ± 2.30***	43.13 ± 1.70**

$^*P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001, significantly different from the control group. Results were analyzed by ANOVA *test*. Values expressed are means ± SD (n = 3). RBC – red blood cells; HB – hemoglobin; PCV– packed cell volume; HT – haematocrit; CCMH – mean corpuscular hemoglobin concentration; MCH – mean corpuscular hemoglobin.

As displayed in Table 9, the anaemia was restored by daily oral administration of *Rubia tinctorum* aqueous extract (200, 400 mg /kg/body weight) and iron for 15 days. To the best of our knowledge, this is the first study revealing the anti-anaemic activity of the *Rubia tinctorum* plant, thus rendering the comparison difficult.

There are several possible explanations for this finding. The first one is that it might be due to its composition of polyphenols. As indicated in Table 2, most of the compounds detected and identified have been previously reported in the literature for their antioxidant and/or anti-inflammatory bioactivity. Ogbe *et al.* (2010) reported that secondary metabolites like flavonoids and alkaloids repair free radical damage to red blood cells and protect them from oxidative stress. Anthocyanins have been used in strengthening kidney function, treating anaemia, promoting blood circulation, and eliminating blood stasis in traditional Chinese medicine (Sari *et al.* 2019). Moreover, Innih *et al.* (2020) reported that the aqueous leaf extract of *Spondias mombin* caused stimulation of the lymphoid follicle at different degrees, from mild to moderate and increased the number of red blood cells in the red pulp. Koriem *et al.* (2018) observed that oral administration of 5-O-caffeoylquinic acid for two-week protects against anaemia and mineral disturbances in 4-tert-octylphenol toxicity by enhancing oxidative stress and apoptosis in rats.

Furthermore, the return of haematological indices in the treated group to normal ranges are not necessarily related to the reported phytochemical constituents (Ohadoma 2016), which brings us to mineral elements. In fact, Musyoka *et al.* (2016) reported that copper and iron have synergetic effects promoting hematopoiesis. Moerever, Sheth *et al.* (2021) pointed out in their study that the anti-anaemic activity of Raktavardhak Kadha can be attributed to its iron content and it may prevent hemolytic anaemia induced by phenylhydrazine. Thus, it is not surprising to find a positive anti-anaemic effect in our study, as iron is the most abundant element in our plant.

Besides, the antianaemic effect of *Rubia tinctorum* could be due to the presence of vitamins such as ascorbic acid or organic acids. Many authors have demonstrated that vitamin B6, vitamin B12, vitamin C, vitamin E, and folic acid play an important role in the erythropoietic mechanism, especially in the presence of iron, copper, and other elements such as cobalt (Musyoka *et al.* 2016). A study performed by Zhang *et al.* (2020) on the effects of malic and citric acids on growth performance, antioxidant capacity, hematology, and immune response of *Carassius auratus gibelio* showed that the appropriate addition of citric acid and malic acid to the diet regulated hematological parameters and the expression of immune-related genes and improved the antioxidant capacity of *Carassius auratus gibelio* fish. Similarly, in a previous study (Salovaara *et al.* 2002), it was suggested that organic acids improve iron absorption. The researchers studied the effect of tartaric, malic, succinic, citric and oxalic, and fumaric acids on Fe (II) and Fe (III) uptake in the human epithelial cell line Caco-2. The results showed that tartaric, malic, succinic, and fumaric acids increased the uptake of Fe (II) and Fe (III), and citric and oxalic acids increased the Fe (III) uptake. Based on these results, it can be stated that the presence of organic acids in our plant may be one of the factors that contributed to the anti-anaemic activity. Furthermore, Tang *et al.* (2013) found that citric acid and L-malic acid have protective effects on myocardial ischemia/reperfusion injury, which may be associated with their anti-inflammatory, antiplatelet aggregation, and direct protective effects on cardiomyocytes.

In summary, these findings showed remarkable antianaemic effects. It constitutes a scientific basis justifying the traditional use of *Rubia tinctorum* in anaemic disease.

On the other hand, in future studies, it will be better to measure also the ferritin and serum vitamin levels which are important markers in anaemia diagnostic.

Conclusion

This is the first report on the antianaemic activity of the aqueous extract obtained from the roots of *Rubia tinctorum*. Based on these results, it can be concluded that the LD50 of the aqueous extract of *Rubia tinctorum* roots was much above 2,000 $mg.kg^{-1}$ and that oral administration of this extract up to 400 $mg.kg^{-1}$ is safe for nutrious and therapeutic uses. Furthermore, it can be suggested that the root of *Rubia tinctorum* L. has an anti-anaemic effect, inhibiting the hemolysis of red blood cells. The antianaemic activity may be due to the composition of the extract contents including polyphenols, iron, and other non-identified molecules. Therefore, *Rubia tinctorum* extract could be a promising treatment for anaemia.

Further studies are required to understand the mechanism involved in the anti-anaemic action of *Rubia tinctorum* and to identify active constituents of the plant.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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