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Research Article

Dosage of phenolic compounds and evaluation of anti-inflammatory activity *in vivo* of *Rubia tinctorum* extract

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Abstract: This study is part of the search for new biologically active molecules of plant origin. The aim of this study is the *in vivo* evaluation of the anti-inflammatory activity of the hydro-methanolic extract of the underground part (roots) of *Rubia tinctorum* from the Wilaya of Chlef (Algeria). The quantitative estimation of polyphenols, flavonoids, and tannins by the colorimetric method showed that the extract studied is rich in these compounds, with contents equal to 199.17 ± 0.074 mg GAE/g, 53.65 ± 0.042 mg QE/g, and 45.74 ± 0.033 mg CE/g, respectively. The anti-inflammatory activity was evaluated using the model of paw edema induced by the intraperitoneal (IP) route in mice (Morini strain) by carrageenan. The results obtained were compared to those of the reference treatment (diclofenac sodium). The evaluation of the percentage of inhibition of the hydro-methanolic extract of the root of *Rubia tinctorum* at doses of 25, 50, and 100 mg/kg significantly prevented paw edema (p<0.05) after 2 hours (25.07 ± 0.054%, 30.05 ± 0.06%, and 36.31 ± 0.014%, respectively) and after 3 hours (30.50 ± 0.08%, 34.25 ± 0.003%, and 37.8 ± 0.012%, respectively). The anti-edematous effect of the dry extract is due to its richness in tannins and phenolic compounds, which would have an inhibitory action on the mediators of inflammation. These compounds would therefore have interesting anti-inflammatory properties, suggesting a therapeutic application to prevent the inflammatory process.

1. INTRODUCTION

The treatment of inflammation involves the use of steroidal and non-steroidal anti-inflammatory drugs. These molecules, although effective, often present undesirable effects (Bindu *et al*., 2020). Therefore, there is considerable global interest in the search for new sources of natural plant substances with anti-inflammatory activities to improve human health while avoiding the undesirable effects of synthetic molecules (Niki, 2012).

Rubia tinctorum is among the plants having several biological activities (Eltamany *et al*., 2020). Moreover, *Rubia tinctorum* raw extract has been utilized as an anti-bacterial, anti-fungal, and antiinflammatory agent in folk medicines (Shilpa *et al*., 2012). The phytochemical analysis of *R. tinctorum* has stated richness in the chemical compounds. The most examined are alkaloids, phenol, flavonoids, anthraquinones, cardiac glycosides, tannins, coumarins, vitamins, and

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minerals (Aboud, 2010). It is well known that these molecules have many biological activities. Indeed, it has been previously reported as having excellent effectiveness as an anti-inflammatory (Kadam *et al*., 2018).

In this context, the main objective of this stuyis to identify the active compounds from hydro-methanolic extracts of *Rubia tinctorum* with anti-inflammatory activity.

2. MATERIAL and METHODS

2.1. Materials

2.1.1. *Plant material*

The part used in this study is the root of the species *Rubia tinctorum* (Figure 1), which was collected from its natural habitat in the Bissa Forest, north of the commune of Zabouja, Wilaya of Chlef (Figure 2).

Figure 1. The roots of the species *Rubia tinctorum.*

Figure 2. The geographical location of the collection site of the species *Rubia tinctorum* (URL: https://goo.gl/maps/fePAKQen4kCSMQ1QA).

Rubia tinctorum species was collected from the Bissa Forest, Chlef, Algeria, at coordinates 36°27’00" N, 12°29’00" E, and an altitude of 1125 m.a.s.l. The collection occurred in June 2023. Post-harvest, the plant material was shade-dried, powdered, and stored for analysis.

Figure 3. *Rubia tinctorum* roots ground into powder.

2.1.2. *Animal material*

The experimental part (*in vivo*) was carried out on male albino mice of the NMRI (Naval Medical Research Institute) strain, weighing between 20 and 25 g, obtained from the Pasteur Institute of Algiers. The mice were placed in cages for a one-week adaptation period with free access to food and water before being used in the various experiments. The animals were kept in an animal house, fasting for 18 hours, at room temperature.

2.2. Methods

2.2.1. *Extraction process of plant material*

The hydro-methanolic extraction of *Rubia tinctorum* roots was carried out by maceration according to the method of Budic-Letoč *et al*. (2005). The root powder of this plant was macerated in a methanol/water mixture (8:2 V/V) at a ratio of 10 g/100 mL under gentle agitation for 72 hours at room temperature and away from light. This maceration was repeated three times successively with solvent renewal every 24 hours. The three filtered fractions were combined and subjected to a double filtration on filter paper. The filtrates were evaporated to dryness under reduced pressure in a Buchi R-210 type rotary evaporator at 45°C. The dry extract was stored at 4°C until use.

2.2.2. *Extraction yield determination*

The weight of the crude extracts was determined by the difference between the weight of the full flask (after solvent evaporation) and the empty flask weight, using the formula (Muniyandi, 2018):

$$Yield\ (\%) = \frac{M}{M_0} \times 100$$

Where M is the mass of the dry extract and M0 is the mass of the plant material.

2.2.3. *Polyphenol assay*

The polyphenol assay was carried out according to the method described by Adusei *et al*. (2019), using the Folin-Ciocalteu reagent, which was described as early as 1965 by Singleton and Rossi. An aliquot of 250 µL of the extract of *Rubia tinctorum* roots (1 mg/mL) and gallic acid (standard) was introduced into test tubes and then mixed with 1.25 mL of freshly prepared Folin-Ciocalteu reagent (1/10) and 1 mL of NaCl at 7.5%. After agitation, the different solutions were incubated at 40°C for 30 minutes. The absorbance was measured at 765 nm using a UV-Visible spectrophotometer (Shimadzu-UV-2401PC). A standard curve was prepared using solutions of gallic acid in water at 0.0312, 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL. The results were expressed in milligrams equivalent of gallic acid per gram of dry plant material (mg GAE/g) according to the following formula:

$$C = \frac{C1.V}{m}$$

- C: Total phenolic content in mg of GAE/g
- C1: Concentration of gallic acid established from the calibration curve in mg/mL
- V: Volume of the extract in mL

2.2.4. *Flavonoid assay*

The total flavonoid assay was carried out by the Aluminum trichloride (AlCl3) method described by Ghafar *et al*. (2017). A volume of 500 µL of hydro-methanolic extract of *Rubia tinctorum* roots (1 mg/mL) was added to 150 µL of sodium nitrite (NaNO2) at 5% and 2 mL of distilled water. The mixture was vortexed and left to rest for 5 minutes. Then, a volume of 150 µL of aluminum chloride (10%) was added to the solution and left to rest for an additional 5 minutes, and then 1 mL of sodium hydroxide (NaOH) at 1 M was added to the mixture. With distilled water, the solution was brought to 5 mL, and the absorbance was measured at 510 nm using a spectrophotometer with a blank control solution (all components without extract). A standard catechin solution was prepared at variable concentrations (0.031-1 mg/mL) using the same procedure. The contents were expressed in milligrams equivalent of catechin per gram of dry plant material (mg CAE/g Ext) according to the following formula:

$$C = \frac{C1.V}{m}$$

- C: Total phenolic content in mg of CAE/g
- C1: Concentration of gallic acid established from the calibration curve in mg/mL
- V: Volume of the extract in mL

2.2.5. *Detection of tannin*

The condensed tannin assay was carried out according to the vanillin method described by Julkunen-Titto (1985). A 50 µL aliquot of the hydro-methanolic extract of *Rubia tinctorum* roots was added to 3 mL of 4% vanillin and 750 µL of concentrated hydrochloric acid (HCl). The absorbance of this preparation was measured after 20 min of incubation at 550 nm by a UV-Visible spectrophotometer (Schimadzu-UV-2401PC). The calibration curve was performed in parallel under the same operating conditions using catechin as a positive control at concentrations ranging from 0.031 to 1 mg/mL. The condensed tannin rate was expressed in microgram equivalent of catechin per milligram of extract (mg EC/g) according to the following formula:

$$C = \frac{C1.V}{m}$$

- C: Total phenolic content in mg of EC/g
- C1: Concentration of gallic acid established from the calibration curve in mg/mL
- V: Volume of the extract in mL

2.2.6. *Evaluation of anti-inflammatory activity*

The induction of inflammation was carried out via intraperitoneal (IP) injection of carrageenan under the plantar region of the hind paw, leading to edema in the metatarsal area (Winter *et al*., 1962). To evaluate the effect of *Rubia tinctorum* root extract on acute inflammation, mice were fasted for 12 hours prior to the experiment and divided into three groups:

Negative Control Group: Received an IP injection of distilled water one hour before the injection of 1% aqueous carrageenan solution under the plantar region of the hind paw (Figure 4).

Positive control group (reference lot): Received an IP injection of sodium diclofenac one hour before the injection of 1% aqueous carrageenan solution under the plantar region of the hind paw (Figure 4).

Treated group: Received IP injections of *Rubia tinctorum* extract at doses of 25, 50 mg/kg and 100 mg/kg one hour before the injection of 1% aqueous carrageenan solution under the plantar region of the hind paw (Figure 5).

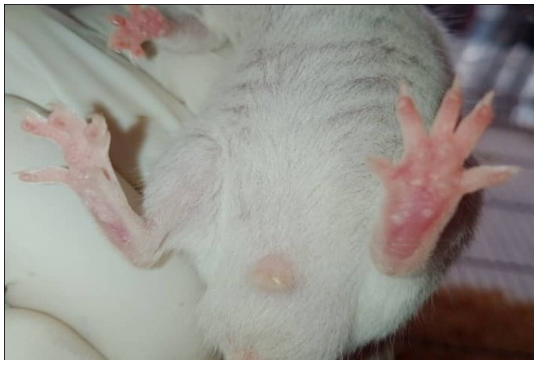

Figure 4. Injection of 1% carrageenan under the plantar of the mouse's hind pawto provokes edema.

Figure 5. IP injection with the extract of *Rubia tinctorum*.

The metatarsal diameter, ankle, and paw circumference at the metatarsal level were measured using a caliper. The average values for the treated groups were compared to the control group and analyzed statistically. The percentage of edema inhibition (% INH) was calculated using the following formula:

$$\%INH = \frac{\%\ AUG\ control\ -\ \%, AUG\ treated}{\%AUG\ control} X100$$

The percentage increase in edema of the mouse hind paw was calculated using the formula (Sango *et al*., 2006):

$$\%AUG = \frac{Vt - Vo}{Vo} X100$$

Where:

- Vo: represents the initial volume of the paw at T=0 (before carrageenan injection)
- Vt: represents the volume of the paw at any time T.

2.2.7. *Statistical analyses*

All experiments were performed in triplicate, and the results are expressed as mean ± standard deviation. Statistical significance was determined using analysis of variance (ANOVA). When significant differences were observed, the analysis was followed by the Newman–Keuls test at a significance level of $p < 0.05$.

3. RESULTS

3.1. Extraction Yield

The extraction yield of the hydro-methanolic extract of *Rubia tinctorum* roots by maceration was 46.97 ± 0.022% on a dry weight basis, calculated as the average of three trials ± standard deviation.

3.2. Total Polyphenol Content

The total polyphenol content of each extract was calculated from the calibration curve and expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g). Optical density was measured at 765 nm. Statistical analysis showed that the phenolic compound content in *Rubia tinctorum* roots was 199.17 ± 0.074 mg GAE/g dry weights.

3.3. Flavonoid Content

The flavonoid content of the hydro-methanolic extract of *Rubia tinctorum* roots was calculated from the calibration curve and expressed as milligrams of catechin equivalents per gram of

extract (mg CE/g). Optical density was measured at 420 nm, yielding a flavonoid content of 53.65 ± 0.042 mg CE/g dry matter.

3.4. Condensed Tannin

The condensed tannin content of the hydro-methanolic extract of *Rubia tinctorum* roots was calculated from the calibration curve and expressed as milligrams of catechin equivalents per gram of extract (mg CE/g). Optical density was measured at 550 nm, with a condensed tannin content of 45.74 ± 0.033 mg CE/g dry weight.

3.5. Anti-Inflammatory

Intraperitoneal administration of carrageenan resulted in an increase in mouse paw volume after 30 minutes. Paw edema progression was measured using a caliper over a period of 0 to 180 minutes at 30-minute intervals (Figure 6).

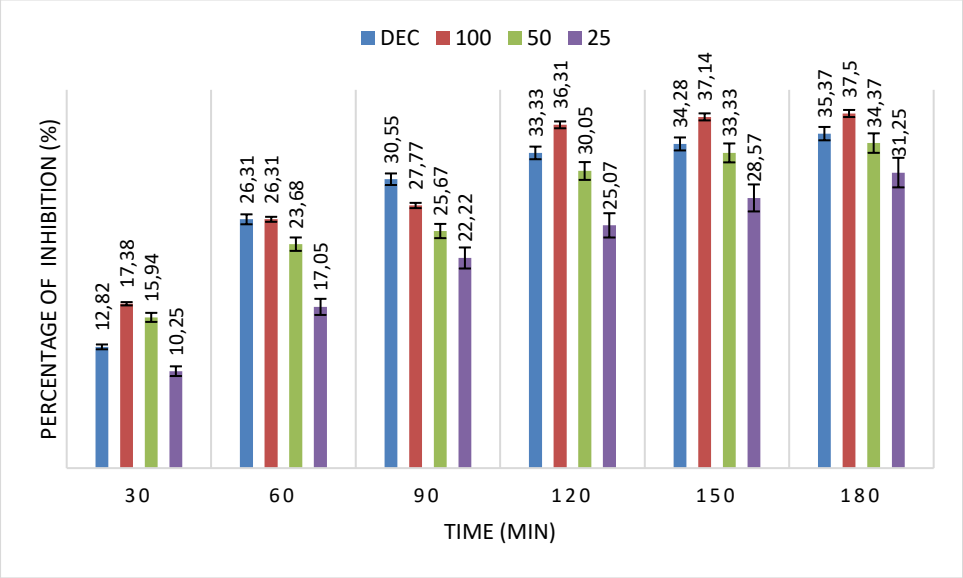

Figure 6. Evolution of the percentage of edema volume inhibition over time.

4. DISCUSSION

Maceration is intended to soften and break down the plant cell wall to release soluble phytochemical compounds while preserving thermosensitive molecules. Additionally, extracting at room temperature with continuous agitation can maximize the extraction of bioactive compounds and prevent their degradation. However, excessively high temperatures can inactivate bioactive compounds and reduce extraction yield in the chosen solvent (Lahmar *et al*., 2017). According to Do *et al*. (2014), the combined use of water and organic solvents can facilitate the extraction of chemicals soluble in either water or the organic solvent. Furthermore, the presence of water in the extraction system effectively increases the yield of extracted compounds and maintains their structural quality (Kraza *et al*., 2021).

Our results align with those of Ghafari *et al*. (2018), who reported extraction yields by maceration for *R. tinctorum* roots using two solvents: methanol (49.97%) and water (45.35%). Other studies found lower yields, such as 17.3% for hydro-methanolic extract and 18.8% for aqueous extract of *R. tinctorum* roots (Houari, 2022). Rhazi *et al*. (2015) suggested that prolonged extraction time may decrease the extract yield due to the degradation of natural substances like polyphenols. In summary, the extraction of phenolic compounds from plant material is influenced by several factors, including the extraction method, maceration time, agitation level, temperature, and solvent type (Saidi, 2019).

Comparing the polyphenol content of the hydro-methanolic extract from *R. tinctorum* roots with other studies on the same plant part, this study's extract exhibits a very high content of these compounds. This finding is consistent with Essaidi *et al*. (2017), who reported that the

hydrolyzed extract of *R. tinctorum* root contains 78.26 mg GAE/g dry weight. They also confirmed the phenol content in the methanolic extract (38.84 ± 0.6 mg GAE/g dry weight), which is significantly lower than the results presented here. Similarly, Rovčani *et al*. (2015) found that the total polyphenol content in the ethanolic extract of *R. tinctorum* root was 14.7 mg GAE/g, while the aqueous extract had 18.2 mg GAE/g.

The results obtained by Essaidi *et al*. (2017), who worked on *R. tinctorum* roots, recorded flavonoid values of 25.36 mg GAE/g in the hydrolyzed extract and 13.41 ± 0.34 mg/g in the methanolic extract. Additionally, Rovčanin *et al*. (2015) found that the flavonoid content in *R. tinctorum* root was 14.7 mg CE/g and 1.1 mg CE/g in the hydroethanolic and aqueous extracts, respectively. These values are significantly lower than our results.

This variability can be attributed to several factors, including drying conditions during extraction (method, time, temperature, particle size), solvent choice, the number of extraction steps, and the plant's geographical origin (Gheffour *et al*., 2015). It's evident that the flavonoid levels in our extracts are lower than the total phenol content, suggesting that the extracts contain other phenolic compounds with different chemical structures than flavonoids, possibly non-flavonoid compounds (Kraza *et al*., 2021). A study conducted by Houari (2022) on *R. tinctorum* roots showed that the tannin content in the aqueous extract (131.68 ± 0.00 mg CE/g dry weight) is significantly higher than that recorded in our study. In contrast, Rovčanin *et al*. (2015) determined a content of 6.2 mg GAE/g in the hydroethanolic extract and 7.3 mg CE/g dry weight in the aqueous extract, despite using the same extraction procedure (hydro-alcoholic maceration) as the previous study.

These observed differences in tannin quantity may be attributed to various factors, including plant maturity, growth stages, biochemical and structural processes in plant tissue, altitude, lighting conditions, humidity, and harvest season (Cezarotto *et al*., 2017). The paw edema test induced by carrageenan is commonly used to determine the anti-inflammatory potential of natural products. The development of paw edema in the mouse hind limb following carrageenan injection has been described as a biphasic event (Kim *et al*., 2020).

In a study conducted by Sharifzadeh *et al*. (2014), the inhibition percentages of paw edema volume for the hydro-ethanolic extract of *Rubia tinctorum* roots at concentrations of 600 mg/kg and 1000 mg/kg were (35.12±%) and (44.21%), respectively, after 1 hour, and (38.6%) and (43.06%), respectively, after 3 hours. However, our study's extract showed inhibition percentages at concentrations of 25 mg/kg and 100 mg/kg as (21.05±0.06%) and (26.31±0.014%), respectively, after 1 hour, and (31.25±0.003%) and (37.5±0.012%), respectively, after 3 hours. This indicates that our extract more effectively reduced mouse paw edema compared to the other study.

The biological activity of a plant extract is linked to its chemical composition, the functional groups of major compounds, and their synergistic effects (Tasneem *et al*., 2019). Indeed, certain flavonoids possess potent inhibitory activity against various enzymes, such as protein kinase C, tyrosine kinase, and phospholipase A2. The latter is known to be responsible for the formation of inflammatory mediators such as prostaglandins and leukotrienes (Habouche *et al*., 2019). Considering that our hydro-methanolic extract of *Rubia tinctorum* is rich in flavonoids, this explains its anti-inflammatory activity. Thus, the anti-inflammatory activity of plants relies on secondary metabolites such as polyphenols, saponins, alkaloids, and tannins. Notably, flavonoids may exert inhibitory effects on inflammation (Banerjee *et al*., 2014; Sani *et al*., 2014). These active substances can act at various stages of the inflammatory response through kinases like protein kinase C and mitogen-activated protein kinase (Azab *et al*., 2016).

Numerous studies have demonstrated that polyphenols and their metabolites can mitigate inflammatory responses through various pathways. For instance, they inhibit the expression of proinflammatory genes (Karasawa *et al*., 2011), reduce the production of inflammatory mediators, and suppress the activity of enzymes involved in inflammatory processes, such as cyclooxygenase, lipoxygenase, MAPK (mitogen-activated protein kinase), and IKK (κ kinase

inhibitor) (Leiherer *et al*., 2013). Therefore, polyphenols play a crucial role in anti-inflammatory activities (Sahu and Saxena, 2013). The anti-inflammatory effect of extracts is likely due to their inhibitory action on the activation of NF-kappa B and the genetic transcription factor that activates TNF-α and IL-1β in the synovial tissue lining the joint (Taheri *et al*., 2022). Polyphenols also modulate MAPK signaling and arachidonic acid signaling to reduce the inflammatory response at various levels, including blocking the release of TNF-α (Yahfoufi *et al*., 2018).

The anti-inflammatory mechanisms of polyphenols make them a promising therapeutic agent for treating and preventing inflammatory diseases. However, the anti-inflammatory effect of polyphenols may differ depending on various factors, including their chemical structure, dose, mode of administration, and individual differences (Dinarello, 2010)

5. CONCLUSION

In conclusion, we can say that the secondary metabolites are presented as complex mixtures due to the structural and functional diversity of their constituents, despite being derived from the same basic entities. Our study highlights the valorization of *Rubia tinctorum*, demonstrating the beneficial effects of polyphenols in inflammation. The hydro-methanolic extraction by maceration of the underground part (roots) of *Rubia tinctorum* provided a yield of 46.97 ± 0.022%. The total polyphenol content, estimated by the Folin–Ciocalteu method, was 199.17 ± 0.074 mg GAE/g of dry extract. The flavonoid content, determined by the $AlCl_3$ method, was 53.65 ± 0.042 mg QE/g of dry matter. The condensed tannin content, measured by the vanillin method, was 45.74 ± 0.033 mg CE/g of dry matter. These results highlight the richness of the hydro-methanolic extract of *R. tinctorum* roots in phenolic compounds.

The *in vivo* study of the anti-inflammatory activity of *Rubia tinctorum* extract, administered intraperitoneally after inducing paw edema in mice using carrageenan, showed an inhibition percentage of (26.31 ± 0.014%) after 1 hour at a dose of 100 mg/kg, with complete reduction of edema after 2 hours. This anti-inflammatory action may be attributed to the extract's chemical composition, including polyphenols, flavonoids, and tannins, or their synergistic effects. However, this topic warrants further research due to the encouraging results, demonstrating the potential use of *Rubia tinctorum* as a remedy for various pathological conditions.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors. **Ethics Committee Number**: ANDRS-NRP-EC- No: 1049/2024. National Agency for the Development of Health Research (ANDRS), National Research Project (NRP).

Authorship Contribution Statement

Amine Bengag: Concept and design, analysis and interpretation, data collection, overall responsibility. **Rouam Djawed**: Writing the article, statistical analysis. **Meziane Ahmed Malika**: Critical revision of the article, final approval of the article.

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