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Original Research Article

Antioxidant and Anti-Inflammatory Activities of Saponins Extracted from *Marrubium vulgare L.* Collected in Fez-Meknes Region in Morocco

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ABSTRACT

This study concentrates on the importance of saponins present in *Marrubium vulgare L.* which is a medicinal plant used mainly in Morocco for neurosedative and anti-inflammatory treatment. To prove its importance, in a phytochemical study, antioxidant and anti-inflammatory activities were determined. The phytochemical analysis allowed isolating saponins compounds from the plant leaves, with foam index equal to 550. Saponins compounds extraction was conducted by three different methods. Results showed a yield of 2.87±0.21% by maceration after fat elimination extraction (D), 6.28±0.28% by maceration extraction (E) and 9.86±0.14% by soxhlet extraction(S). Antioxidant activity was done with FRAP and TAC methods. At 0.5 mg/ml, the three extracts exhibited high antioxidant activity, namely ethanolic extract (E), which presented the significant highest value with 9033.58 AAE/1gDM by TAC assay, and 2361.57 AAE/1gDM by FRAP method. In the anti-inflammatory activity, the (E) extract showed significant inhibition at 500 mg/kg and 180 min after formol-injected 96.64% compared to Aspirin which showed an inhibition of 59.27% at 150 mg/Kg. The results have shown the significance of the medicinal plant *Marrubium vulgare L.* and its remarkable biological activities, which are related especially to saponins.

Keywords: Medicinal plants, *Marrubium vulgare L.*, saponins, antioxidant activity, anti-inflammatory activity

Introduction

Marrubium vulgare L. is a perennial herbaceous plant that belongs to the Lamiaceae family.[1] It is native to Central and Western Asia, Southern Europe, and North Africa.[2] This plant is used in traditional medicine against diarrhea, diabetes, rheumatism.[3] Various plant extracts of *Marrubium vulgare L.* showed a high potential of hepatoprotective,[4] antioxidant activities,[5,6] antidiabetic and antihyperlipidemic activities,[7] anti-inflammatory effects,[8] anti-plasmodial activities, [(9)] antiviral, insecticidal, and molluscicidal activities.[10] In addition to some hemolytic, expectorant, and immune-stimulant proprieties.[11]

The aerial part of the *Marrubium vulgare L.* contains several secondary metabolites, the most interesting ones arepolyphenols, flavonoides, tannins, terpenoids,[5] marrubiin, phenolic acids, phenylpropanoid glycoside,[6] and saponins.[12,13] Saponins are bioactive compounds with potential health benefits. Besides their role in plant defense, they are considered to have anti-cancer, anti-inflammatory, antibacterial and antifungal activities,[14] and the antioxidant activity,[15,16] protection against allergies, platelet aggregation, hepatotoxins and microbes.[17]

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The main objective of this study was to demonstrate the importance of saponins from *Marrubium vulgare L.*in terms of antioxidant and anti-inflammatory activities.

Materials and Methods

Biological Material

Marrubium vulgare L. leaves were harvested manually in March 2021 in the Fez-Meknes region (Morocco), dried for a few days at room temperature, and then dried at 40°C in an oven (Binder drying oven FD) in the absence of light and humidity. A fine powder was obtained by crushing the sample in an electric mill. Storage was at room-temperature without light, and humidity preserved it for subsequent extractions.

Characterization of Saponins by Phytochemical Screening

Decoctions of different concentrations were prepared in 100ml boiling water and the suspension was filtered. Ten dilution test tubes of 1/10, 2/10…, 10/10 filtrate were prepared with distilled water. The contents of each tube were shaken for 15 seconds. The foam height was measured 15 minutes after shaking. Foam index (Im) was calculated by formula (1) :

$$Im = \frac{h \times 5}{x} \times 1000 \quad (1)$$

Where: h = height of the foam (cm)
x = number of the Xth tube.

An index of foam higher than 100 indicates saponins are present in the plant.[18]

Saponins Extraction via Maceration after fat elimination

A total of 20 g of plant powder was prepared with 300ml of pure hexane for 2 hours under reflux. Following the removal of the organic phase,

the residue was macerated in 150 ml absolute ethanol for 24 hours. After the filtration, the ethanol phase was evaporated at 60°C using a rotavapor (BUCHI, Rotavapor R-100, Germany) after it had been filtered. An extraction of the dry residue was performed with 200 ml of H_2O/petroleum ether v/v. After 30 minutes of heating at 50 °C, the whole was cooled. 40 ml of n-butanol was used for aqueous phase extraction. After decantation, the organic phase was evaporated.[19]

Extraction of Saponins by Soxhlet Apparatus

An amount of 20 g of *Marrubium vulgare L*. powder was placed in a cellulose cotton cartridge, and 300 ml of methanol (99% analytical grade) was added. A Wittman's filter paper with a porosity of 0.22 mm was used to filter the recovered solution. After evaporating the extractive solution using a rotaevaporator (BUCHI, Rotavapor R-100, Germany), we obtained the dry extract.[20] To extract the organic phase, 100 ml of n-butanol/H_2O v/v solution was used for 30 min,[19] then concentrated in an evaporator at 60 °C under 800 mbar pressure and 120 rte/min.[20]

Extraction of saponins by maceration

A quantity of 40 g of *Marrubium vulgare L*. powder was macerated for 48 hours in 600 ml of absolute ethanol. Filtration was performed using Wattman's filter paper, which had a porosity of 0.22 mm. Dry extract were obtained by evaporating extractive solutions. After decantation with 80 ml of H_2O/n-BuOH v/v, organic phase extraction was carried out in a bath at 60 °C under 800 mbar pressure with 120 rte/min. Extracts were stored at 4°C until further use.[21]

Antioxidant Activity by FRAP

The ferric reducing antioxidant power (FRAP) was determined according to the method proposed.[22] A 0.5 ml saponin extract was mixed with 1.25 ml of phosphate buffer and 1.25 ml of diluted potassium ferricyanide was used to measure the FRAP. In a water bath, the mixture was kept at 50 °C for 20 minutes. The reaction was stopped by adding 1.25 ml of 10% trichloroacetic acid after cooling. Following centrifugation at 3000 rpm for 10 minutes, the top layer solution of 1.25 ml was mixed with 1.25 ml of distilled water and 0.25 ml of ferric chloride solution 0.1%. The standard was ascorbic acid. And absorbance was measured at 700 nm.

Antioxidant Activity by TAC

TAC test was conducted according to Mouffouk*et al* (2019). An amount of 0.5 ml of extract was combined with 4.5 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Incubation was performed at 95 °C for 90 minutes in tubes containing reaction solution. As samples cooled to room temperature, absorbance was measured at 695 nm. Total antioxidant capacity was expressed as ascorbic acid equivalent per gram of dried matter (AAE/1gDM). 900 µl of the reagent mentioned above is mixed with 100 µl of solvent for the blank.[23]

S*ephadex gel chromatography*

To separate the extracts, 400 ml of each extract at a concentration of 20 g of Sephadex G50 Gel, was added to a chromatography column (3 x 60 cm), previously balanced with 100 ml (NaOH 0,5 M and LiCl 0.025 M). The absorbance was measured using a Shimadzu UV-1600 PC UV spectrophotometer.[24]

Animals

During all experimental procedures, animals were cared for and used according to regulations of the College of Animal Science and Techniques of Sidi Mohammed Ben Abdellah University, under the ethical approval number 08/2021.

A total of 12 one-day-old male Ross-308 chicks (average body weight 42.38 ± 1.87 g), obtained from animal house of the Faculty of Science and Techniques of Fez, Sidi Mohammed Ben Abdellah University, were randomly divided into 4 treatment groups containing one cage of 3 chicks, of which a cage is set for positive control (Aspirin) and another one as negative (physiological solution). The chicks were housed in a temperature-controlled room with a continuous light source and provided them with feed and distilled water daily. Table 1 shows chick's mass. During the test, the temperature was maintained between 28°C and 31°C and the relative humidity was set to 50%. A 13-day feeding experiment was conducted at the Faculty of Science and Techniques of Fez, Sidi Mohamed Ben Abdellah University, with 500 grams in the first three days and 700 grams in the last three.[25]

Table 1: Changes in the chicks' masses as a function of time

Chicks Weight (G)	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	ADFI (G/BIRD)	ADG (G/BIRD)	FCR (G/G)
Chick 1	41.56	48.82	52.93	83.01	123.32	199.66	264.63	33.45	17.16	1.95
Chick 2	44.50	45.25	58.25	78.75	121.96	194.95	279.08	33.48	18.04	1.86
Chick 3	41.60	50.50	59.12	78.13	131.06	212.68	270.73	34.37	17.62	1.95
Chick 4	42.39	46.91	56.99	81.55	123.98	199.85	265.16	33.66	17.14	1.96
Chick 5	38.81	54.76	55.65	78.92	127.59	214.50	272.35	34.35	17.97	1.91
Chick 6	42.25	50.21	58.93	83.05	129.50	208.03	292.40	39.18	19.24	2.04
Chick 7	39.69	47.89	55.22	88.61	135.04	205.71	275.41	33.07	18.13	1.82
Chick 8	44.43	50.50	52.30	87.30	129.69	198.49	273.12	38.68	17.59	2.20
Chick 9	43.73	51.16	60.06	80.32	128.47	208.25	280.04	33.02	18.18	1.82
Chick 10	43.39	47.63	60.54	78.21	136.28	197.68	277.23	39.91	17.99	2.22
Chick 11	41.65	50.94	55.79	83.32	123.08	202.45	268.00	33.88	17.41	1.95
Chick 12	44.60	47.22	61.39	79.04	121.71	197.67	282.63	33.92	18.31	1.85
Average	42.38	49.32	57.26	81.69	127.64	203.33	275.06	35.08	17.90	1.96
Standard dev	1.87	2.54	2.94	3.51	4.95	6.38	7.92	2.57	0.58	0.13

Average Daily Feed Intake (ADFI) use an 'average' value from feed analysis we've taken.

Average Daily Gain (ADG) can be defined as the average amount of weight gain each day during the feeding period. It can be calculated by taking the amount of weight an animal has gained since the last weight and dividing the weight by the number of days since that last weight.

Feed Conversion Rate (FCR) is calculated by dividing the kg of feed eaten daily, by the kg of live weight gained daily. Which is ADFI/ADG.

Anti-inflammatory activity

On days 14, 15 and 16 of the experiment, randomly chosen chicks from each treatment group were weighed after a 12 h feed withdrawal. The average daily feed intake (ADFI), the average daily weight gain (ADG), and the feed conversion ratio (FCR) were calculated using the body weight and feed intake recorded by cages during different periods.[26]

In each chick, the initial volume (V_0) of the left leg was measured by an electronic caliper before treatments were administered. Various treatments were given by oral gavage, as shown below by the different cages:

NC: Physiological solution at 10 ml/kg (Negative Control)

CSM3: Aqueous saponin extract solution by maceration method at 300 mg/Kg

CSM5: Aqueous saponin extract solution by maceration method at 500 mg/Kg

AA: Aqueous solution of Aspirin at 150 mg/Kg

30 minutes after oral administration, each chick was injected with 50 µl of 2.5% formol solution into the left leg. After injection, the volume of injected legs was measured 60 and 180 minutes later. Based on the formula (2), we determined the extent of the process by calculating the Percentage Increase (%PI) in chick paw volume:

$$\%PI = \frac{Vt - V0}{V0} * 100 \quad (2)$$

Where;

V_t: volume of the leg at time t

V_0: initial volume of the leg

Subsequently, the anti-inflammatory activity was assessed by calculating the percentage of inhibition (%INH) based on the formula (3):

$$\%INH = \frac{\%PIc - \%PIe}{\%PIe} * 100 \quad (3)$$

Where:

$\%PI_c$: percentage increase in negative control

$\%PI_e$: percentage increase in extract/ Aspirin

Statistical analyses

All groups were evaluated three times in parallel, expressing the results as means ± standard deviations (SDs).

Results and discussion

Saponins characterization

Results in Figure 1 confirm the presence of saponins in *Marrubium vulgare L.* leaves. Interestingly, the foam height remained constant at 1.1 cm across all decoction concentrations (1%, 2%, 3%, and 4%) in test tube 10, regardless of the amount of plant material used.

Foam index equal 550, in accordance with,[18] our results indicate that Moroccan *Marrubium vulgare L.* contains saponins in its leaves. Several studies have reported the presence of saponins in *Marrubium vulgar L.*[13,27]

Extraction of Saponins

Three different methods were used to extract saponins. Each extract yield is calculated from dry weight of *Marrubium vulgare L.* leaves.

According to Figure 2, the results showed that there is a remarkable difference in the extraction yield of saponins, the best yield was observed in the Soxhlet method (S) with a yield of 9.86±0.14%, while the other methods showed a lower yield namely, 6.28±0.28% for maceration (E) and 2.87±0.21% for maceration after dilapidation (D).

Previous studies have conducted the extraction for 8 h using Soxhlet apparatus, they obtained 7.7% as extraction yield.[28] The yield of 9.86% found in this study can be attributed to the extraction time. A 12 h extraction may be responsible for our obtained yield. Additionally, the yield of tea saponins for an extraction lasted 6 h in other studies conducted by Zhao (2020) was 7.28%. This shows the importance of extraction time as a crucial parameter in extraction yield.[29] Also, other factors can affect the extraction results than time; Lahcen (2020) extracted saponins from *Cistus Creticus* plant leaves using the maceration method after removing fat molecules through dilapidation, and the results were totally different, they obtained 1.6% while we found 2.87%.[19]

Antioxidant Activity

Total antioxidant capacity

In Figure 3, the three extracts possess higher antioxidant power than ascorbic acid at all concentration. Evaluation of the antioxidant capacity was performed by measuring the concentration equivalent to ascorbic acid for each gram of dry matter (AAE/1gDM) as follows:

Figure 4 illustrates the antioxidant capacity of the three extracts. As can be seen, all extracts possess a remarkable antioxidant capacity at all concentration, especially the (E) extract with a value of 9033.58 AAE/1gDM. However, the slightly lower values obtained by (D) and (S) extracts can be explained by the difference in extraction conditions for each method. As we know that (S) and (D) extracts are obtained with heating, the active principle causing this important antioxidant activity may be exposed to degradation when the temperature attained high degrees. This also explain the lowest value obtained by S extract, of which the heating lasted for 12 h.

Ferric Reducing Antioxidant Power

The ferric reducing antioxidant power of *Marrubium vulgare L.* extracts was evaluated using the FRAP method, as it shown in Figures 5 and 6. The antioxidant activity measure ascorbic acid equivalent concentrations for one gram of dry matter. Results are summarized in Figure 7.

The (E) extract exhibited the highest antioxidant activity when compared to ascorbic acid, while the (D) extract showed a similar ferric reducing power as ascorbic acid. The (S) extract was totally bellow ascorbic acid. However, it was found that the ferric reducing antioxidant power increases with concentration for all three extracts, which means that all extracts can reduce ferric ions to ferrous ions.

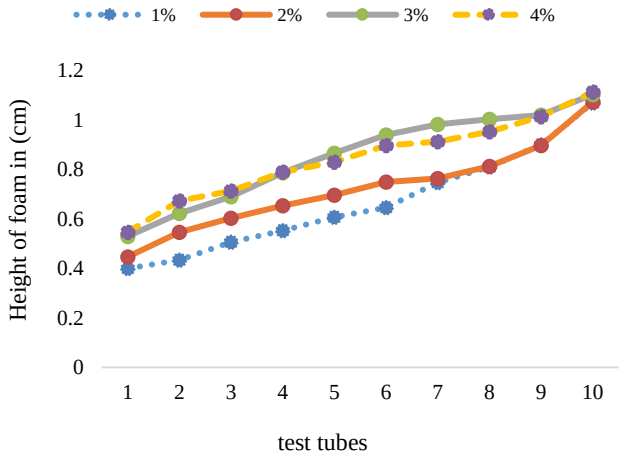

Figure 1: Foam height as a function of dilution of different decocts.

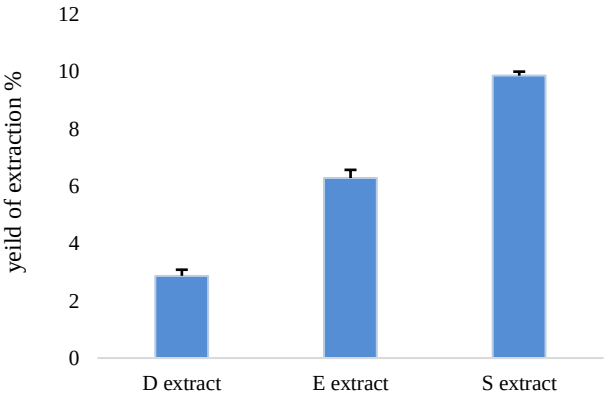

Figure 2: yield of three different methods.

The reason behind lower value obtained for the (S) extracts may be due to the heating step used during the extraction. This process can relatively affect active principles, namely if they are thermolabile.
Previous studies have reported the remarkable antioxidant power of *Marrubium vulgare L.*,[30] indicating that methanolic and hydro-alcoholic extracts from this plant displayed high antioxidant activity. Other studies confirmed the importance of *Marrubium vulgare L.* as antioxidant using the PCL method, methanol and acetone extracts showed an interesting antioxidant power (261.41 and 272.90 µmol TE/g respectively).[6] This was similar to the study conducted by Boulila (2015),[31] they showed that ethyl acetate and ethanolic extracts of *Marrubium vulgare* were significantly effective in terms of DPPH scavenging activity. this later reduces the stable DPPH radical to a non-radical form (DPPH-H) resulting in a decrease in absorbance at 517 nm. The extent of this decrease indicates the strength of the antioxidant.[32] Consequently, the EtOAc fraction had the strongest antioxidant capacity, exhibiting IC_{50} values between 68.98 µg/ml and 118.15 µg/ml. The reason behind all these significant results on total extracts can be attributed to saponins, as they provide powerful activities in terms of iron and molybdate reducing power, which is asserted by Baguia-Broune (2018),[33] reporting that saponin extracts showed a higher antioxidant activity, as measured by spectrophotometry, against the free radical DPPH when compared with (E). *senegalensis* leaves (EC_{50} = 1.0333 mg/ml) and *S. longepedunculataroots* (EC_{50} = 1.3900 mg/ml). Phenolic compounds of *Marrubium vulgare L.* are also provide important antioxidant activity as found by Hayat J et *al* (2020).[5]

Chromatography Sephadex G-50
To estimate the molecular weight of the compounds contained in each extract, we performed steric exclusion chromatography on Sephadex G-50 gel. Graphs for each sample are shown Figures 9 and 10.:

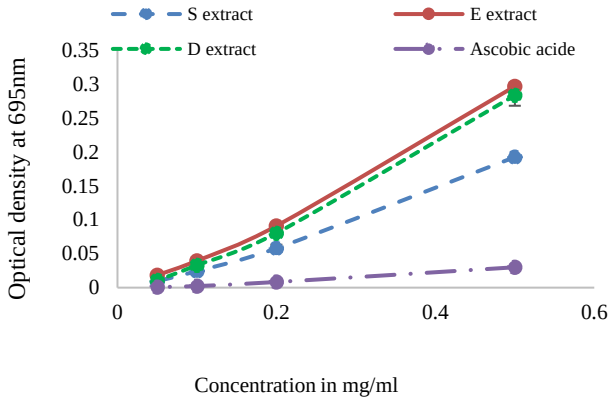

Figure 3: Antioxidant capacity of saponins extracts from *Marrubium vulgare L.*

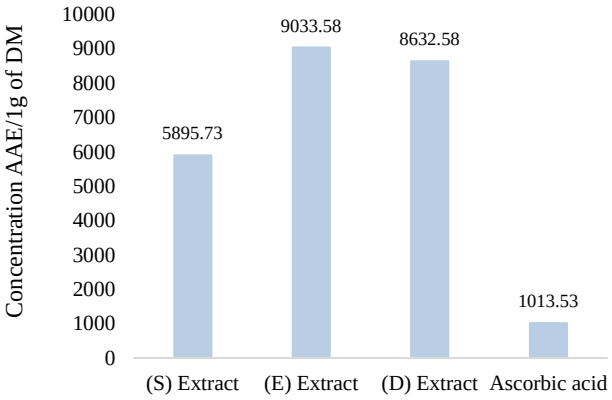

Figure 4: Antioxidant capacity of three extracts at 0.5mg/ml.

Sephadex G-50 was conducted to separate the compounds contained in each sample. Results revealed 3 intense signals detected for each extract. The stationary phase consists of Sephadex beads, this latter is able to keep molecules with a small volume and let the large molecular weight ones elute along with the mobile phase. Thus, all the intense signals obtained are considered high molecular mass fractions and represented saponins molecules.
The (E) extract graph indicates four polymeric saponins, in accordance with fraction numbers N°8 to N°10, N°11 to N°12, N°15 to N°21, and N°23 to N°26, of which the first and the third ones are the most major compounds with an optical density of 3. The (D) extract three polymeric saponins, appeared according to fraction numbers from N°9 to N°10, N°16 to N°18, and N°19 to N°22, which indicate that these extracts contain three saponin molecules of which the first one is the most present compound in that extract, since it indicates an optical density of 3. The (S) extract shows three polymeric saponins appeared in accordance with fraction numbers of N°8 to N°11, N°15 to N°22, and N°23 to N°33, this means that the extract contains three saponin molecules and the first one is considered as the principal compound in this extract, with an optical density of 3. However, a small difference between these extracts existed, which is the fourth polymeric saponin signal in the (E) extract, and the lack of this fourth signal presence in the other extracts may be due to overheating. The missing molecule is pretty much to be considered as thermolabile.
Prior *Marrubium vulgare L.* composition studies affirmed these findings, demonstrating the plant's possession of three saponin molecules: vulgarcoside A, 3-hydroxyapigenin-4'-O-(6"-O-p-coumaroyl]-beta-D glucopyranoside, and Apigénine-4'-O-(6''-O-p-coumaroyl)-β-D-glucopyranoside.[34]

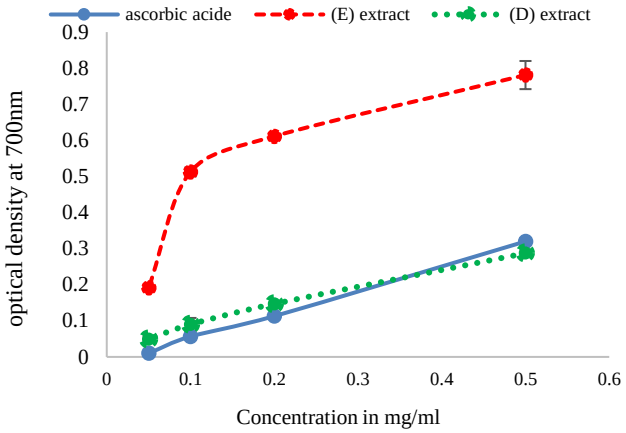

Figure 5: Ferric reducing power assay of E and D extracts of ethanolic solvent

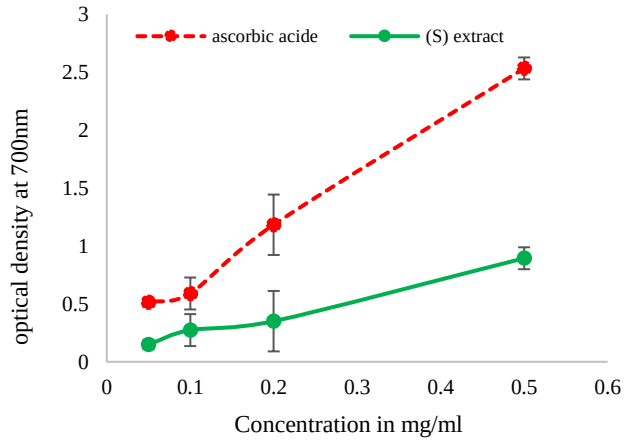

Figure 6: Ferric reducing power assay of S extract of methanolic solvent

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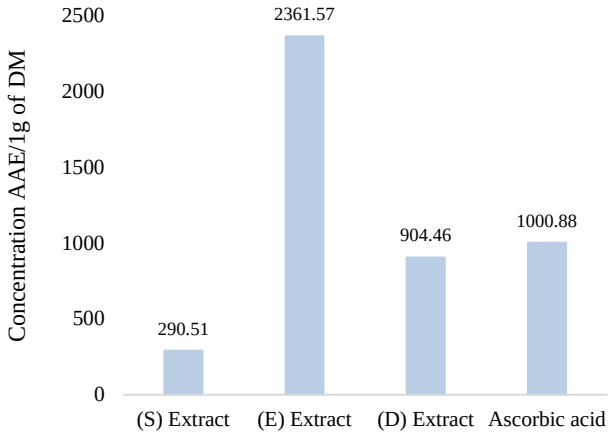

Figure 7: Antioxidant activity FRAP of three extracts at 0.5 mg/ml.

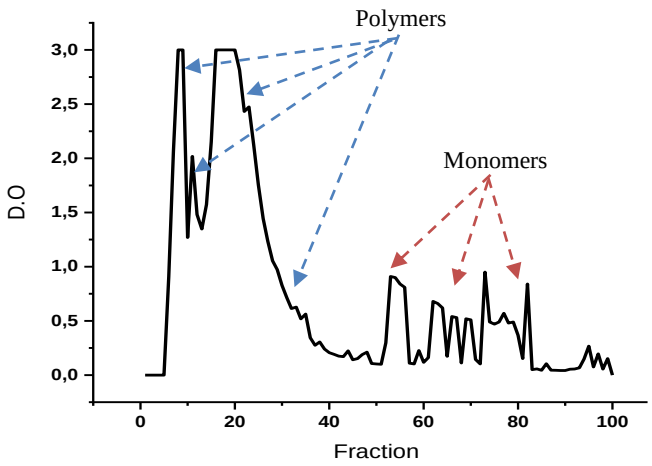

Figure 8: Molecular distributions of (E) extract

Table 2: Effects of extracts and standard drugs on formol-induced left leg of chicks

Extract/Drug	Dose (mg/kg)	60 mins		180 mins	
		Swell. Thick ± SEM	Inhibition (%)	Swell. Thick. ± SEM	Inhibition (%)
NC	-	76.352 ± 0.893	0	95.011 ± 0.593	0
CSM3	300	19.09 ±0.129	74.994	15.776±0.927	83.395
CSM5	500	14.568±0.807	80.912	3.187±0.350	96.645
AA	150	50.262±0.859	34.155	38.695±0.587	59.271

The results are quite similar. However, the only difference to consider is that fourth signal appearing on E extract chromatogram. The reason behind this difference can be related to some potential impurities that were may be analyzed with the other saponin compounds. Another reason to consider are climatic and environmental sampling conditions where the plant has been taken.

Anti-inflammatory activity

The anti-inflammatory activity was investigated using an in vivo model by extract's oral administration to chicks. After administration of formol, an increase in chick's leg volume was measured in 60 min and 180 min.

The (E) extract showed significant inhibition at 60 min, 74.99% and 80.91% at 300 mg/kg and 500 mg/kg respectively, compared to aspirin which showed an inhibition of 34.15% at 150 mg/Kg. Indeed, we observed an increase in inhibition at 180 min with 83.39% and 96.64% at 300 mg/kg and 500 mg/kg respectively, and 59.27% for Aspirin at 150 mg/Kg. These findings showed the importance of saponins extracts of *Marrubium Vulgare L.* on inflammation inhibition. Anti-inflammatory activity results are presented in Table 2.

According to our findings, (E) extracts of *Marrubium Vulgare L.* produced potential anti-inflammatory effects in experimental chicks when assessed by formol. The saponins extracts administered orally showed significant anti-inflammatory activity at 300 mg/Kg and 500 mg/Kg ($p < 0.05$) by reducing the increase caused by formol at the chicks' leg. Suggesting that the plant exerts an important anti-inflammation as compared with reference (Aspirin), these results are in good agreement with another study by Kanyonga (2011),[35] who conducted the anti-inflammatory test on methanolic extract of *Marrubium Vulgare L.* using carrageenan-induced hind paw oedema model by oral administration on adult male mice at 100 and 200 mg/kg. The anti-inflammatory activity value for (E) extracts was 83.39% and 96.64% respectively at 300 mg/Kg and 500 mg/Kg may be explained by the high concentration used (300 and 500 mg/Kg) compared to their case (200 mg/Kg), the extract's concentration also has an essential role in increasing the inflammation inhibition, therefore. Similarly, many studies have proved in vivo the efficiency of natural anti-inflammatory saponins,[36] who conducted the carrageenan-induced paw edema test on clean-grade mice (18–22 g) of both sexes after revealing two triterpenoid saponins (SP-R1 & SP-R2) from the medicinal plant *Panax stipuleanatus* by Maceration method, with sterile 0.9% saline as control and dexamethasone 10 mg/kg as reference. The two saponins were orally administered for seven days, and each mouse was given a subcutaneous intraplantar injection with 1% carrageenan suspended in sterile 0.9% saline into the right hind paw. At 10 mg/kg, SP-R1 and SP-R2 showed inhibition rates of 25.70% and 29.76% at the 1st h, respectively, which increased to 33.76% and 55.40% at the 4th h. However, the anti-inflammatory activity value was 74.99% and 80.91% at the 1st h, respectively for 300 and 500 mg/Kg, may be explained by the high concentration we used (300 and 500 mg/Kg) as compared to theirs (10 mg/Kg), the extract's concentration also has an essential role in increasing the inflammation inhibition,. I It cannot be confirmed that saponins are poor anti-inflammatory agents, as we were able to obtain optimal values only at higher concentrations. In contrast, the carrageenan-induced paw edema animal model has proven the effectiveness of natural saponins as anti-inflammatory agents, these significant anti-inflammatory effects are also attributed to secondary metabolites of *Marrubium Vulgare L.* and other medicinal plants belonging to Lamiaceae family.

Several investigations have demonstrated that *Marrubium Vulgare*'s bioactive phytochemicals are responsible for these significant anti-inflammatory activities (extract of *Marrubium Vulgare L.*).[37,38,39]

Conclusion

This study focused on saponins from *Marrubium vulgare L.* and their efficacy as an antioxidant and anti-inflammatory. Compared to ascorbic acid, the three extracts showed strong antioxidant activity. The anti-inflammatory activity of the (E) extract showed a particularly important activity compared to Aspirin. As a result, saponins from *Marrubium vulgare L.* are high antioxidant and anti-inflammatory compounds, which make them a potential new source of bioactive natural products. Further in vivo studies are needed to better understand how these bioactive molecules work, their therapeutic dose, and their cellular sites of action.

Monomers

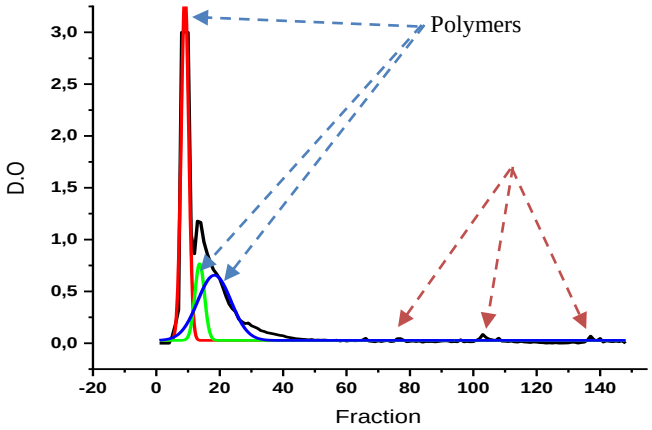

Figure 9: Molecular distributions of (D) extract

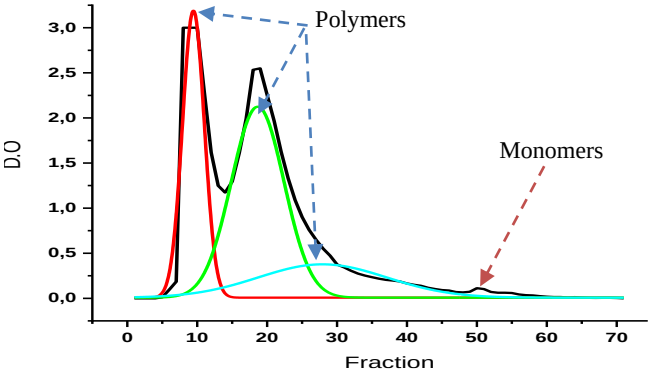

Figure 10: Molecular distributions of (S) extract

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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