Trop J Nat Prod Res, July 2023; 7(7):3411-3420 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Alkaloids from *Marrubium vulgare L.*: Antioxidant and Anti-Inflammatory Activities as a Function of Extraction Methods

Taha Ouriagli[1,2]*, Abdellatif Amnay[1,2], Sidi M. Raoui[2,4], Faouzi Errachidi[1,2], Fouad O. Chahdi[3], Rachida Chabir[1]

[1]*Human Pathology, Biomedicine and Environment Laboratory, Faculty of Medicine and Pharmacy, Sidi Mohamed Ben Abdallah University, Fez, Morocco*
[2]*Laboratory of Functional Ecology and Engineering Environment, Faculty of Sciences and Technologies, Sidi Mohamed Ben Abdellah University, Fez, Morocco*
[3]*Laboratory of Applied Organic Chemistry, Faculty of Sciences and Technologies, Sidi Mohammed Ben Abdallah University, Fez, Morocco*
[4]*Higher Institute of Nursing professions and Health Techniques of Fez-Meknes, Morocco*

ARTICLE INFO

Article history:
Received 01 June 2023
Revised 17 June 2023
Accepted 10 July 2023
Published online 01 August 2023

ABSTRACT

Marrubium vulgare L, commonly known as *white horehound*, is a medicinal plant widely recognized for its therapeutic properties. It has been extensively studied for its bioactive compounds, including alkaloids, which possess significant antioxidant and anti-inflammatory activities. This study investigates the impact of four extraction methods (soxhlet, refluxing, maceration A, and maceration B) on the antioxidant and anti-inflammatory activities of alkaloids from *Marrubium vulgare L*. The qualitative analysis reveals Emetine and Cephaeline as the predominant alkaloid compounds in the plant leaf extracts. Among the methods, soxhlet and maceration B demonstrate the highest extraction yields (9.81% and 9.57% respectively). Notably, maceration B exhibits exceptional antioxidant activity as ascorbic acid equivalent per gram of dry matter (2370.53 AAE/1gDM), while soxhlet exhibits remarkable anti-inflammatory activity (78.51% inhibition at 500 mg/kg, 180 minutes post-formol injection). These findings underscore the importance of maceration B and soxhlet methods for enhancing alkaloid extraction yield and bioactivity.

Keywords: medicinal plants, *Marrubium vulgare L*, extraction methods, alkaloids, column chromatography, antioxidant activity, anti-inflammatory activity. .

Introduction

Marrubium vulgare L, a medicinal plant of the Lamiaceae family, is widely distributed in the Mediterranean basin, including Morocco.[1] It is valued in the pharmaceutical and food industries and extensively used by Moroccans to treat various ailments.[2] Traditionally, it has been employed for respiratory infections, gastrointestinal disorders, diabetes, anorexia, coughs, and colds.[3] Furthermore, *Marrubium vulgare L* possesses notable hypoglycemic, analgesic, antispasmodic, anti-inflammatory, and antioxidant properties.[4,5]

Despite the potential of herbal medicines in combating oxidative stress and inflammation, the influence of extraction methods on biological evaluations has received limited attention.[6,7] Alternative antioxidants and anti-inflammatory compounds derived from medicinal plants have gained interest in recent years.[8,9] Alkaloids have documented antioxidant and anti-inflammatory properties and potential in preventing cardiovascular diseases.[10-12] However, their clinical utility is hindered by issues like poor bioavailability and rapid metabolism, despite promising results in numerous studies.[13-15]

This study provides a comparative analysis of four extraction methods: soxhlet, refluxing, maceration A, and maceration B. These methods were selected based on their wide usage in phytochemical extraction and potential for yielding valuable alkaloid compounds.[58-61]

*Corresponding author. E mail: *ouriagli.taha@usmba.ac.ma*
Tel: +00212651364577

Citation: Ouriagli T, Amnay A, Raoui SM, Errachidi F, Chahdi FO, Chabir R. Alkaloids from *Marrubium vulgare L.*: Antioxidant and Anti-Inflammatory Activities as a Function of Extraction Methods. Trop J Nat Prod Res. 2023; 7(7):3411-3420 http://www.doi.org/10.26538/tjnpr/v7i7.20

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

The main objective is to determine the most suitable extraction technique for maximizing alkaloid yield and unlocking their potent biological activities. Extraction parameters, including yield, antioxidant activity, and anti-inflammatory activity, will be meticulously assessed. This study's findings will contribute to understanding the alkaloid profile of *Marrubium vulgare L* leaves and facilitate the development of effective extraction techniques for obtaining high-quality alkaloid-rich extracts.

Material and Method

Chemicals and plant material

The glassware used was purchased from Pyrex. Solvents (methanol, ethanol, hexane, chloroform, ethyl acetate, and diethyl ether) were purchased from Honeywell Fluka. Chemicals and reagents (Mayer's reagent, iron chloride, sodium hydroxide, ammonia, hydrochloric acid, sulfuric acid, sodium bicarbonate, sodium phosphate, ammonium molybdate, ammonium hydroxide, potassium ferricyanide, trichloroacetic acid, ascorbic acid, lithium chloride, Gel filtration for chromatography Sephadex G-50, aspirin, and formol) were purchased from Sigma-Aldrich (Casablanca, Morocco). Extracts concentration was done using a rotary evaporator (BUCHI, Rotavapor R-100, Germany), and optical density measurements were performed on Ultraviolet/Visible spectrophotometer (Shimadzu UV-1600 PC UV, Japan). Molecular structures and physicochemical data were obtained from PubChem and SwissADME programs (https://pubchem.ncbi.nlm.nih.gov/, http://www.swissadme.ch/).

Plant material used consists of *Marrubium vulgare L* leaves, which were collected during February and March 2021 in the Fez-Meknes region and were identified by Dr. Chaimae Rais in The National Agency for Medicinal and Aromatic Plants, Taounate, Morocco (Document no. 075/2021). The study was conducted at Human Pathology, Biomedicine and Environment Laboratory, in the Faculty of Medicine and Pharmacy (34.00612274141898, -4.964672169974484).

Alkaloids detection

To assess the presence of alkaloids, a 5 g sample of dried material was subjected to reflux heating with 200 mL of individual solvents (distilled water, ethanol, and diethyl ether). Following this, 0.2 mL of each extract was mixed with 5 mL of 1% aqueous hydrochloric acid solution. Gradual dilutions up to 60% were performed to observe alkaloid precipitation at varying concentrations. Mayer's reagent (three drops) was then added to 1 mL of the filtrate (Table 1). Positive reactions were indicated by turbidity (+), flocculation (++), or the formation of a whitish precipitate (+++).[16]

Extraction methods

For the extraction, 4 experiments were carried out. The first method was performed using the soxhlet apparatus as described by Yubin (2014). A total of 20 g of powder were extracted with 300 mL of absolute ethanol. The extract was evaporated under reduced pressure at 60 °C and the dry residue was dissolved in 40 mL of chloroform and acidified with 5% hydrochloric acid until pH = 3. After 30 minutes, the acidic aqueous phase was extracted with 40 mL chloroform and alkalized by 5% sodium bicarbonate to reach pH 9. The chloroform phase was evaporated at 40 °C and the final residue represents total alkaloids.[17]

The second method was performed using a reflux set-up as described by Koné (2009). The extraction lasted 12 h, and crude extract was obtained after filtration and concentration in rotary evaporator. Next, 40 mL Chloroform was added, and solution was extracted 4 times by 5% H_2SO_4 (v/v). The acidic phase was alkalinized using 50% NH_3 and extracted with chloroform. A dry residue was obtained after concentration in rotary evaporator under reduced pressure.[18]

The last method is maceration. Two experimental protocols were conducted; extracts were named maceration A and maceration B. Maceration A was conducted according to the Togola method (2019) with slight modifications. In brief, 10 g of dry matter was macerated at room temperature by 100 mL of 80% methanol for 48h, then the mixture was filtered using Whatman paper No. 3. Other impurities were eliminated with hexane and methanolic phase was evaporated in rotary evaporator under reduced pressure until dryness to obtain the extract.[19]

Maceration B method was performed according to Rojas-Vera (2021) with some adjustments. Briefly, 5g of dry leaves powder was extracted with methanol for 10h at 4 °C, the solution was filtered and concentrated in rotary evaporator under reduced pressure until dryness. The crude extract was acidified to pH 2 with 5% H_2SO_4 and undesired compounds were removed with Et_2O (v/v). Alkaloid extract was obtained by alkalizing aqueous solution to pH 9 using 25% NH_4OH and extracting it with EtOAc (v/v).[20]

Total Alkaloid Yield Determination

The extraction rate, defined by formula (1), is obtained by multiplying the ratio of the mass of crude extracts in a dry state (Ps) to the mass of the plant material used (Pr) by one hundred.

$$Y(\%) = \frac{Ps}{Pr} \times 100 \qquad (1)$$

Antioxidant activity evaluation

The ferric reducing antioxidant power method (FRAP) was performed according to Motto (2021). For this, different concentrations of the extract (0.1/0.2/0.3/0.4/0.5 mg/mL) were combined with 2.5 mL of phosphate buffer and 2.5 mL of 1% potassium ferricyanide. The mixture was then incubated at 50 °C in a water bath for 20 minutes. After cooling, 2.5 mL of 10% trichloroacetic acid was added to stop the reaction. Following a 10-minute centrifugation at 3000 rpm, the upper layer of the solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride solution. Ascorbic acid served as standard and absorbance was measured at 700 nm using a Shimadzu UV-1600 PC UV spectrophotometer.[21] Antioxidant activity was expressed as ascorbic acid equivalent per one gram of dry matter (AAE/gDM).

The total antioxidant capacity test (TAC) was conducted as described by Mouffouk (2019). According to this, 100 µL of extract was treated with 900 µL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate), absorbance was measured at 695 nm against a blank containing 100 µL of methanol combined with 900 µL of the reagent mentioned above.[16] The total antioxidant capacity was expressed as ascorbic acid equivalent per gram of dry matter (AAE/gDM).

Steric Exclusion Chromatography

Steric Exclusion Chromatography technique (SEC) was performed using Sephadex G-50 gel to distinguish the alkaloid molecules in each extract. Extract compounds were separated according to their size and shape. The procedure was conducted in accordance with Rigane (2015), 20 g of Sephadex G-50 gel was added to 100 mL of mobile phase (NaOH 0.05 M and LiCl 0.025 M). The sample then was deposited at 20 mg/mL. The flow rate was set at 5 mL/min and isolated fractions were analyzed by Shimadzu UV-1600 PC UV spectrophotometer at 380 nm, using a fraction volume of 5 mL.[22]

Anti-inflammatory activity

Animal care and management

The protocol was approved by the Institutional Animal Care Committee located in the Faculty of Medicine and Pharmacy of Fez (FMPF) according to French technical specifications for production, under the ethical approval number 08/2021.

The animal care and management procedure were conducted according to Amo-Mensah (2020). A total of 18 one-day-old male Ross-308 chicks (average body weight 42.54 ± 2.16 g), obtained from animal house of the Faculty of Science and Techniques of Fez, Sidi Mohammed Ben Abdellah University, were randomly distributed into 6 treatment groups containing one cage of 3 chicks, of which a cage is serving as the positive control (Aspirin) and another one as negative control (Physiological water). All chicks were housed in a temperature-controlled room with continuous light and had access to feed and distilled water ad libitum. The temperature was maintained at 28 to 31 °C and the relative humidity was set at 50% throughout the trial. The feeding experiment was conducted at the Faculty of Science and Techniques of Fez, Sidi Mohamed Ben Abdellah University, in 13 consecutive days, with 500 g in the first three days and 700 g for the rest of the experiment. The food was withdrawn on the day before the experiment.[23]

Sample Collection and Procedures

On days 14, 15 and 16 of the experiment, randomly chosen chicks from each treatment group were weighed after 12h feed withdrawal. The average daily feed intake (ADFI), average daily gain (ADG), and feed conversion ratio (FCR) were calculated based on cage weights and feed intakes collected during the aging process.[24] The initial volume (V_0) of each chick's left leg was measured using a caliper. Then, different treatments were administered by oral administration, as indicated by the cages below.

NC: Physiological water at 10 mL/kg (Negative Control)
AS3: Aqueous alkaloid extract solution by soxhlet method at 300 mg/kg
AS5: Aqueous alkaloid extract solution by soxhlet method at 500 mg/kg
AM3: Aqueous alkaloid extract solution by maceration B method at 300 mg/kg
AM5: Aqueous alkaloid extract solution by maceration B method at 500 mg/kg
PC: Aqueous extract solution of Aspirin at 150 mg/kg (Positive Control)

Thirty minutes after oral administration, each chick was injected with 50 µL of 2.5% formol solution into the left leg. Injected legs volumes were measured 1 and 3 hours after injection. The extent of the process was assessed by determining the Percentage Increase (%PI) in chick paw volume using equation (2) below:[23]

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

V_t: volume of the leg at time (t)
V_0: initial volume of the leg

Subsequently, the anti-inflammatory activity was obtained by calculating the inhibition percentage (%INH) as identified by formula (3):

$$\%INH = \frac{\%PIc - \%PId}{\%PId} * 100 \qquad (3)$$

$\%PI_c$: percentage increase in physiological water
$\%PI_d$: percentage increase in drug (Extract / Aspirin)

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 1: Dilutions used to detect alkaloids in test tubes

Dilutions (%)	100	90	80	70	60
Extractive solution (mL)	0.20	0.18	0.16	0.14	0.12
Hydrochloric acid 1% (mL)	5	4.50	4	3.50	3
Total Volume (mL)	5.20	4.68	4.16	3.64	3.12
Distilled water required quantity (mL)	0	0.52	1.04	1.56	2.08

Statistical analysis

All results are expressed as the means ± standard error of the mean (SEM). Statistical significance was determined using a two-tailed student's t-test performed with SPSS software. Each experiment was replicated at least three times, and values of ($p < 0.05$) were regarded as significant.

Results and discussion

Alkaloids detection

The three tests yielded similar outcomes, with turbidity observed in tubes with concentrations exceeding 70%, while no signal was observed in tubes at 60%. These specific cases are detailed in Table 2.

It appears from the analysis that the alkaloids test was positive. As a result, *Marrubium vulgare L* leaves hold alkaloids, and by simple turbidity appearance, however, only minimal amount is considered.

Several studies in the literature have investigated the chemical composition of *Marrubium vulgare L* leaves. Rivera (2019) and Haratym (2017) reported the presence of alkaloids, saponins, and various groups of phenolic compounds in *Marrubium vulgare L* leaves.[25, 26] Furthermore, Tripathi (2017) and Njinga (2020) identified alkaloids in different plants belonging to the Lamiaceae family, such as Mentha piperita, Ocimum tenuiflorum, and Ocimum gratissimum Linn.[27, 28]

Total Alkaloid Yield

Extraction results are illustrated in Figure 1.

Figure 1 illustrates that maceration B and soxhlet methods yield higher extraction rates (9.81% and 9.57% respectively) compared to refluxing and maceration A. These methods are known for their efficiency in permeabilizing plant cell walls. Additionally, the choice of extraction solvent is crucial, considering its polarity, when aiming to dissolve target compounds effectively. Previous studies have demonstrated that organic solvents with varying polarities, including ethanol, n-butanol, acetone, ethyl acetate, n-hexane, and petroleum, effectively retain alkaloids.[62-66] As most alkaloids have medium polarity,[29] solvents like ethanol (used in the soxhlet method) and acidic ethyl acetate solution (used in the maceration B method) are ideal for extracting alkaloids, penetrating deeper and releasing more extractable compounds. This explains the higher yields obtained for soxhlet and maceration B methods, which may also explain the low values obtained by maceration A and refluxing methods, since a high polarity organic solvent as methanol was applied.

One remaining question pertains to the significant difference in extraction yields between the two maceration methods, despite their similar principles. The disparity can be attributed to the absence of acidification in maceration A, resulting in the non-recovery of alkaloid salts throughout the experiment. This limitation may explain the lower yield value of 2.04% obtained and serves as the sole disadvantage of this method.

Special consideration is given to Rahman (2018) who conducted a phytochemical study on P. quadrifolius, a plant from the Lamiaceae family. The study revealed a significant alkaloid content of approximately 13.92%. However, it is important to note that a direct comparison of the obtained yields is not feasible due to the different plant species under investigation. Therefore, variations in yields are expected under these experimental conditions.

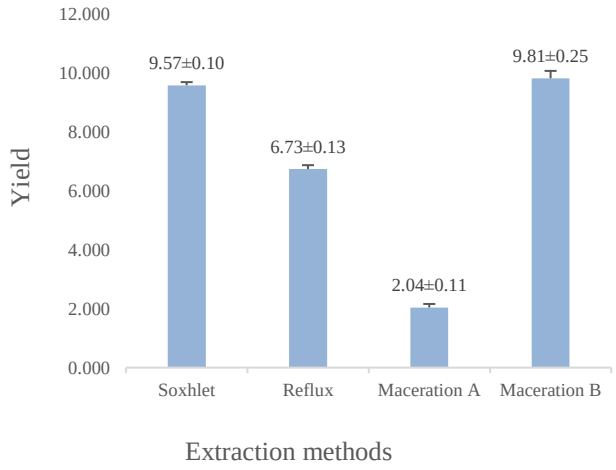


Figure 1: Extraction yields obtained by different methods

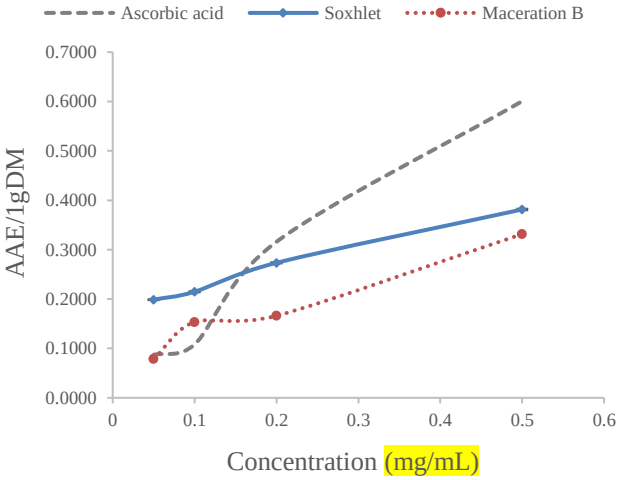


Figure 2a: Antioxidant activity of the methanolic extract by maceration A and refluxing compared to ascorbic acid

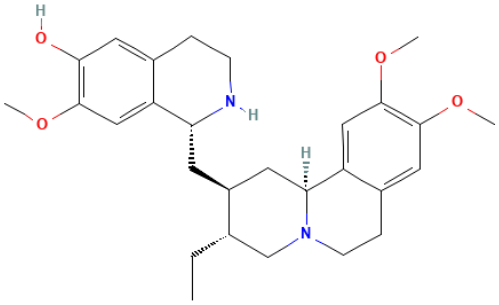
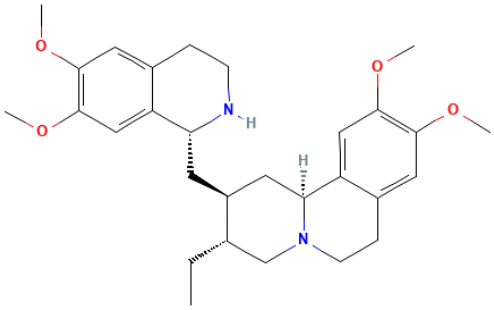
Table 2: Alkaloids detection results from different concentrations.

	Dilutions (%)				
Extract solvents	**100**	**90**	**80**	**70**	**60**
Distilled water	+	+	+	+	-
Diethyl ether	+	-	-	-	-
Ethanol	+	+	+	-	-

Key: - = absence, + = turbidity, ++ = flocculation, +++ = whitish precipitate.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 3: Molecular structure and physicochemical proprieties of the two alkaloids (PubChem).

	Cephaeline	Emetine
Molecular structure		
IUPAC Nomenclature	(1R)-1-[[(2S,3R,11bS)-3-ethyl-9,10-dimethoxy-2,3,4,6,7,11b-hexahydro-1H-benzo[a]quinolizin-2-yl]methyl]-7-methoxy-1,2,3,4-tetrahydroisoquinolin-6-ol	(2S,3R,11bS)-2-[[(1R)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl]methyl]-3-ethyl-9,10-dimethoxy-2,3,4,6,7,11b-hexahydro-1H-benzo[a]quinolizine
Molecular formula	$C_{28}H_{38}N_2O_4$	$C_{29}H_{40}N_2O_4$
Molecular weight	466.622 g·mol−1	480.6389 ± 0.0276 g/mol
Melting point	115 °C	74 °C

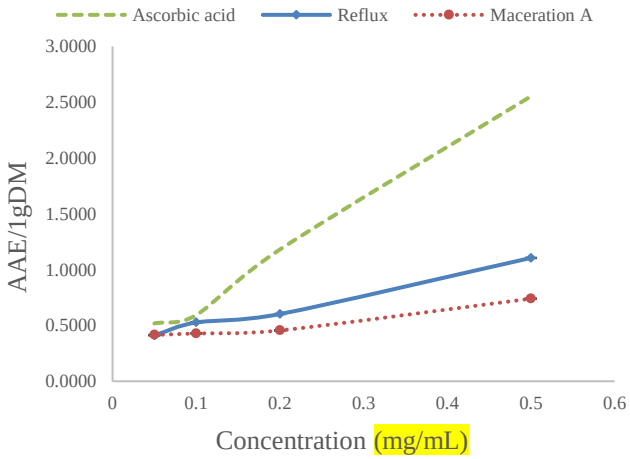


Figure 2b: Antioxidant activity of the aqueous extract by maceration B and soxhlet compared to ascorbic acid

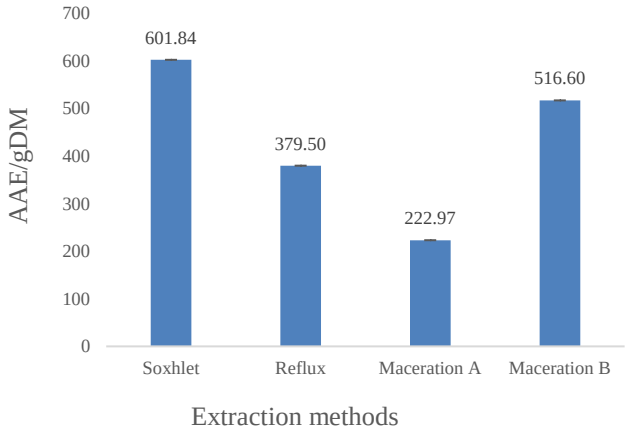


Figure 3: Antioxidant activity of the extracts at 0.5mg/mL

Antioxidant activity evaluation

Ferric Reducing Antioxidant Power

Due to the close relationship between antioxidant activity and reducing capacity,[31] the FRAP assay can provide reliable information about the antioxidant properties of a substance.[32] Curves expressing antioxidant activity by FRAP are displayed in figures (2a) and (2b) as optical density versus concentration.

Figures (2a) and (2b) clearly indicate that all extracts, except at concentrations less than or equal to 0.1 mg/mL, exhibit significantly lower antioxidant activity compared to ascorbic acid. However, there is a positive correlation between the activity of the extracts and their concentrations, suggesting that the alkaloid extracts act as reductones by reducing ferric (III) ions to ferrous (II) ions.

Below is the reducing power antioxidant activity evaluated at 0.5 mg/mL by measuring equivalent concentration of ascorbic acid per gram of dry matter (Figure 3).

By analyzing the activities of the four alkaloid extracts, we noticed that soxhlet and maceration B are the two most efficient extraction methods, with an antioxidant effect of 601.84AAE/gDM and 516.60AAE/1gDM respectively. However, when compared to ascorbic acid, none of the extracts exhibited remarkable ferric reducing activity. This led us to conclude that alkaloids are weak antioxidants for ferric reducing activity.

Figures 4a and 4b illustrate the total antioxidant capacity based on TAC test. They are displayed as optical density versus concentration.

Maceration B and soxhlet extracts exhibit consistently high antioxidant capacities across all concentrations, surpassing even that of ascorbic acid. The refluxing extract also demonstrates notable antioxidant capacity within the concentration range of 0.12 mg/mL to 0.3 mg/mL. However, outside of this range, the antioxidant capacity is comparatively low in comparison to ascorbic acid.

Figure 5 outlines the equivalent concentration of ascorbic acid per gram of dry matter at 0.5 mg/mL:

Once more, the results obtained demonstrate that soxhlet and maceration B are the best two extraction methods, with antioxidant values of 2241.58AAE/gDM and 2370.53AAE/1Gdm respectively, which confirm the proficiency of these methods.

The results are in good agreement with other study conducted by Putra (2022), which has shown that the soxhlet extract produced the highest antioxidant activity level based on FRAP testing, with 36.20 µmol

GAE/g (gallic acid equivalent). The author's attention was focused not only on soxhlet method effectiveness, but also on the ultrasonic assisted extraction (UAE) as it can be an excellent technique for industrial applications thanks to shorter extraction times. However, the study primarily supports previous findings that highlighted the superior antioxidant activity and extraction yields achieved through the soxhlet extraction method.[33] Similar outcomes were discovered by Aguilar-Villalva (2021), revealing that the highest antioxidant capacities were found in soxhlet samples from all tested *Annonaceae* species and extraction solvents.[34] Interesting results were also attributed to the maceration technique, as Eddahhaoui (2022) who indicated that macerated methanol extracts show a remarkable antioxidant activity, with an IC_{50} of 0.99±0.01 µg mL^{-1}, 22.14±0.60 µg mL^{-1}, and 137.55±0.85 µg mL^{-1}, for DPPH, ABTS, and FRAP respectively.[35]
Still, the reason behind the huge differences between the obtained values by the two antioxidant activity methods remains unclear. One of the big advantages for the TAC assay is that it involves reducing Mo (VI) to Mo (V) using antioxidant compounds found in plant extracts. Alkaloid extracts have a significant antioxidant capacity, so they can destroy a free radical by transferring an electron to the phosphomolybdenum complex. When the absorbance during the TAC assay is higher, the TAC values are higher, making them range from 520.99 AAE/1gDM to 2241.58 AAE/1gDM. On the other hand, iron reduction ability of the studied samples from ferric tripyridyltriazine [Fe (III)-TPTZ] complex to ferrous tripyridyltriazine [Fe (II)-TPTZ] complex in FRAP reagent correlates well with their antioxidant power, which is an electron-donating ability indicator.[36] An increase in FRAP value means that the extracts have a higher reducing power and electron transfer ability towards the FRAP reagent,[37] which could not be an important mechanism for alkaloids as antioxidants. Therefore, alkaloids extracts were non-responsive to the FRAP reagent for almost all concentration levels assessed compared to ascorbic acid.[38,39] Another reason to consider is the family type of alkaloids, since different solvents dissolve bioactive molecules depending on polarity, which may also depend on the structural conformation of these alkaloids.[40-43]
An explanation regarding the increase in FRAP values in accordance with the concentration of alkaloid extracts is still required. Bala (2013) and Gutiérrez (2014) reacted to this issue by indicating that total phenolic content in aqueous and methanol extracts is positively correlated with FRAP assay. This means that phenolic compounds were the main responsible for this low positive reducing potential, they break the free radical chain by donating a hydrogen atom.[44] In extracts, these phenolic compounds are incompatible with alkaloids, so the antioxidant properties were exclusively dependent on alkaloids.[45]
Note: For the following experiments, only extracts of maceration B and soxhlet methods were analyzed, as they showed optimal results regarding all previous proceedings.

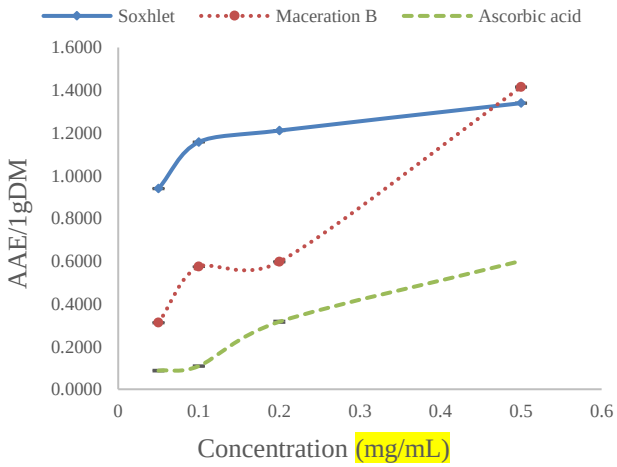


Figure 4a: Antioxidant capacity of aqueous extract by maceration B and soxhlet compared to ascorbic acid

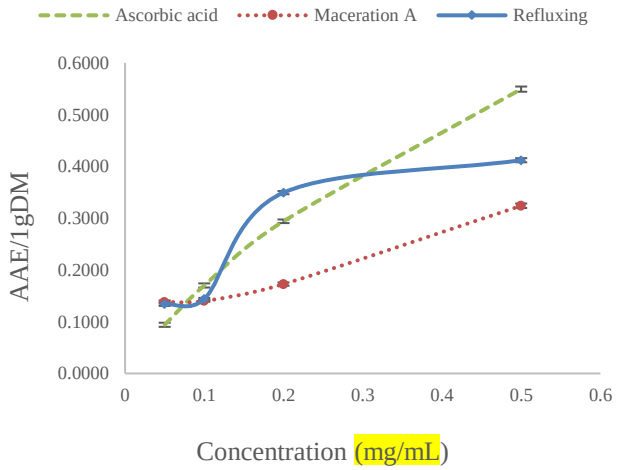


Figure 4b: Antioxidant capacity of Chloroform extract by maceration A and refluxing compared to ascorbic acid

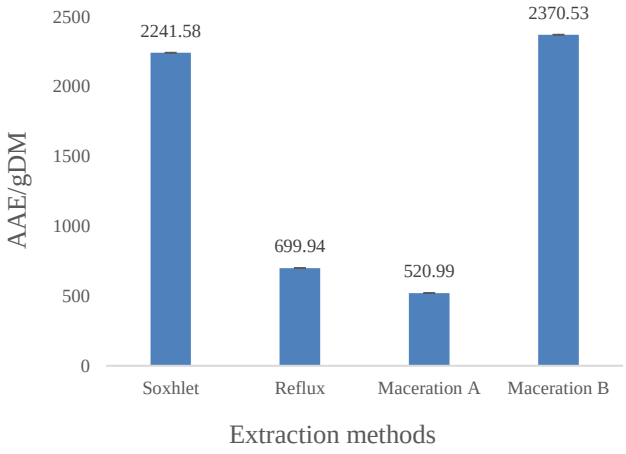


Figure 5: Antioxidant capacities of the extracts at 0.5 mg/mL

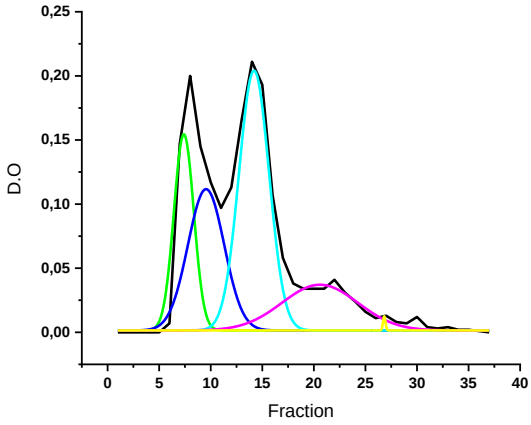


Figure 6: Elution profile of maceration B extract

Steric Exclusion Chromatography
To separate the compounds presented in each sample, gel filtration technique was employed. Results revealed 5 detected signals for the two alkaloid extracts, obtained from Origin 8.5 software. Sephadex G-50 gel could retain small molecular size compounds and let the large molecules crossing along the column first through the mobile phase. For that reason, all the intense signals obtained are representing alkaloid molecules, and they are considered high molecular mass fractions.[46] Our results can be effectively interpreted by utilizing this information.

Based on the elution profiles depicted in Figures 6 and 7, the chromatogram of maceration B extract reveals three prominent signals with significantly higher optical density values (0.21 compared to 0.02 in other monomers). These signals correspond to fractions No. 7, No. 8, and No. 13, indicating the presence of three alkaloid molecules. Also, the reason behind other peaks' appearance is suggested to be due to the presence of other potential monomers. In contrast, the soxhlet extract exhibits only two prominent peaks with optical densities of approximately 0.33 and 0.65, corresponding to fractions No. 7 and No. 8 respectively. This indicates that the soxhlet extract predominantly comprises two major alkaloid molecules.

The attained findings were quite unexpected. It has been found that maceration B extract contains one more alkaloid molecule over the soxhlet extract. The most likely explanation of this negative result is that molecular distributions depend on the genetics and the environment of the samples taken.[47] It is also important to consider various procedures for obtaining these extracts. Additionally, some thermolabile compounds may be overheated when performing the soxhlet method, resulting in their degradation. All these factors may be the reason why these extracts showed significant qualitative and quantitative differences in terms of composition. Therefore, comparing the alkaloids profile in these two extracts is challenging.[47] The mentioned reasons may also be responsible for varied optical values obtained (maximum reached values are 0.21 and 0.65 for maceration B and soxhlet methods respectively), which means that soxhlet alkaloids have a higher polymerization degree than maceration B alkaloids. Thus, the soxhlet method may yield alkaloids with larger molecular structures, indicating possible differences in their chemical properties.

Despite all, there is a good match between the crude extracts' composition, especially for the two alkaloid molecules present in each one. Previous studies supported this result and reported that *Marrubium vulgare L* contains two different alkaloid molecules, named Cephaeline and Emetine.[48] Moreover, Rosales-López (2020) and Asano (2001) also performed qualitative analysis by column chromatography using Silica gel, they obtained Emetine and Cephaeline within retention times around 7.58 min and 8 min, respectively.[49,50] In fact, we applied a 1 fraction/min rate, which means that the two alkaloid molecules were obtained at around 7th and 8th minute for both extracts. As it is obvious, the results are quite similar, and pretty much Emetine and Cephaeline are the two alkaloid molecules included in the extracts.

Nevertheless, the polarity of the two alkaloids detected was of interest to us. We intended then to explore other research highlighting Emetine and Cephaeline polarity index. Consequently, Asano (2002) have developed the metabolism of Cephaeline and Emetine, they assumed that the two alkaloid molecules are of medium polarity compounds indeed, since they can't create hydrogen bonds when in contact with extraction solvents. Except Cephaeline which contains a hydroxy group that allows it to transform into a high polar metabolite by glucuronidation. In fact, the two studied compounds are quite similar, and Emetine can demethylated to get converted to Cephaeline.[51] Additionally, both alkaloids dispose of a topological polar surface area (TPSA) ranging from 20 Å to 130 Å as indicated by SwissADME software analysis. This confirms our approximation in section II, as these alkaloids are considered average polarity compounds. Therefore, the reason we used to explain high and low values of extraction yield is proved. For visual representation of chemical structures and properties, the reader is referred to Figure 8.

Anti-inflammatory activity

To examine the anti-inflammatory activity, we used an in vivo animal model to know oral administration of extracts to chicks. A gradual increase in chick's leg volume was observed and measured 1h and 3h after 50 µL of 2.5% formol injection. Chicks' gained weighs during feeding period are shown in Table 4. Average Daily Feed Intake (ADFI), Average Daily Gain (ADG), and Feed Conversion Ratio (FCR) were calculated after 13 sequential days of the aging period. All is represented in Table 5.

Table 6 summarizes the anti-inflammatory data of maceration B and soxhlet extracts of *Marrubium vulgare L* regarding the in vivo animal model at 300 mg/kg and 500 mg/kg. As revealed below, maceration B and soxhlet extracts showed significant inhibition 1h after formol injection, best results were attributed to the 500 mg/kg dose to know 69.97% and 44.43% respectively, compared to Aspirin which showed an inhibition of 34.15% at 150 mg/kg. More notably, the two extracts significantly inhibited chicks' hind paw edema 3h after formol injection, with 50.32% and 78.51% at 500 mg/kg, respectively, while Aspirin inhibition was 59.27% at 150 mg/kg. On the contrary, no effect was observed at 300 mg/kg.

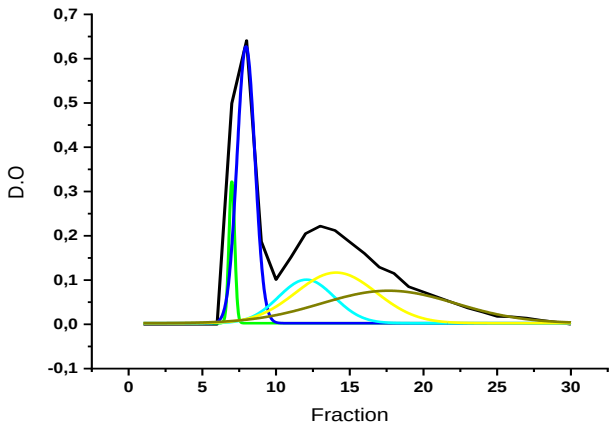
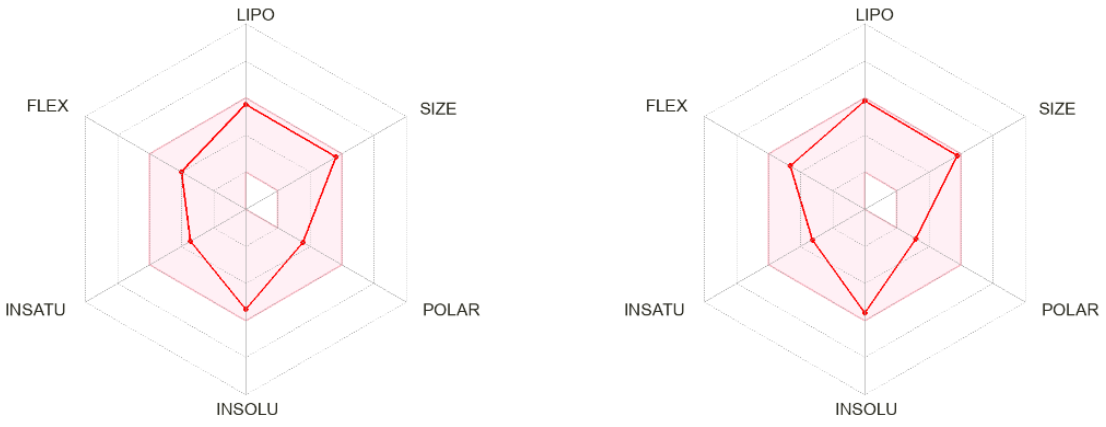


Figure 7: Elution profile of soxhlet extracts



(a) **(b)**

Figure 8: ADME analysis of proposed drugs: Cephaeline (a) and Emetine (b)

As mentioned above, soxhlet and maceration B extracts produced potential anti-inflammatory effects in experimental chicks when assessed by formol. The alkaloid extracts administered orally showed considerable anti-inflammatory activity at 500 mg/kg by reducing the increase caused by formol at the chicks' leg. As the plant exerts an important anti-inflammation as compared with Aspirin, these results are consistent with another study by Kanyonga (2011), who conducted the anti-inflammatory test on methanolic extract of *Marrubium vulgare L* realized by a soxhlet apparatus, using carrageenan-induced hind paw oedema model by oral administration on adult male mice at 200 mg/kg. As of 4.5h after injection, inhibition was 34.0% while reference drug (Indomethacin) was 38.7%. In this regard, Indomethacin exhibited a remarkably similar inhibitory effect to the extract, which shows the high impact of a soxhlet method.[52] However, our inhibition values (78.51% and 69.97% for soxhlet and maceration B extracts respectively) could be explained by the high concentration we used (500 mg/kg) compared to theirs (200 mg/kg), large concentration values can also be attributed to increasing the inflammation inhibition, therefore. Nevertheless, this approximation may not be valid for all cases, as low concentrations of the extract could show significant anti-inflammatory activity. For instance, Jin (2022) studied the anti-inflammatory effects of major alkaloids in many mono terpenoid indole alkaloids (MIAs) obtained from *Kopsia officinalis* by a similar protocol to that of maceration B. In short, the dried and powdered leaves of *K. officinalis* were extracted using 90% methanol through refluxing. The resulting residue was dissolved in water and adjusted to pH 3 using 0.1% aqueous hydrochloric acid. After filtration, the solution was basified to pH 9 using 10% ammonia and then extracted with ethyl acetate to obtain separate alkaloid layers. The anti-inflammatory activity was investigated on male mice (23 – 25 g) by utilizing the carrageenan-induced paw edema models, with Aspirin as the positive control.[53] At only 40 mg/kg, the three major inhibition ratios reached 43.7%, 50.3%, and 46.1%. These results surpassed Aspirin's anti-inflammatory effect, which achieved an inhibition ratio of 38.9% at 200 mg/kg. Clearly, low concentrations of the extract can exert significant anti-inflammatory effects[53]. Similarly, Xie has proved in vivo the efficiency of natural anti-inflammatory steroidal alkaloids, by evaluating the anti-inflammatory activity of methanolic extract from *Veratrum grandiflorum* by refluxing method, using the carrageenan-induced paw edema animal models with dexamethasone as the positive control. The extract significantly inhibited the carrageenan-induced paw edema compared with control, inhibitory ratios of methanolic extract were 36.3% and 47.2% at 0.5 mg/kg and 1.0 mg/kg respectively, approximately equal to dexamethasone (49.5%) at 5.0 mg/kg.[54]

All these in vivo studies have proved the efficiency of natural alkaloids as anti-inflammatory agents by the carrageenan-induced paw edema animal model, which were extracted already by soxhlet, maceration B, and refluxing methods. Apart from that, these significant anti-inflammatory effects are also attributed to bioactive phytochemicals found in *Marrubium vulgare L* and other medicinal plants of the Lamiaceae family.[55-57]

Conclusion

The present study investigated the influence of extraction methods on the biological activities of alkaloids. Four extraction methods were examined, and their effects on antioxidant and anti-inflammatory activities were evaluated using *Marrubium vulgare L* alkaloids. The study commenced with the characterization of alkaloids through precipitation reactions, which confirmed their presence in the leaves of the plant. Among the tested extraction methods, maceration B and soxhlet techniques yielded the highest extraction yields (9.81% and 9.57% respectively). The antioxidant activity assays (FRAP and TAC) revealed that maceration B extracts exhibited the strongest antioxidant activity (2370.53 AAE/1gDM). Qualitative analysis identified Emetine and Cephaeline as the major alkaloids in the extracts. An in vivo animal model study demonstrated the effectiveness of the soxhlet method in reducing inflammation with a percentage inhibition of 78.51%, surpassing the performance of Aspirin as a reference (59.27%). These findings underscore the pivotal role of extraction methods in determining the biological activity of alkaloid extracts. However, the use of different solvents for each extraction method poses a limitation in accurately comparing the results. Therefore, future research should concentrate on exploring alternative extraction methods and employing specific solvents that align with the polarity of the targeted compounds. Such efforts will enhance our understanding of the remarkable potential of phytochemicals extracted from plants in terms of their biological activities

Conflict of Interest

The authors declare no conflict of interest.

Table 4: Average weight per bird during feeding period

Aging period	Average weight (g/bird)
Day 1	42.54 ± 2.16
Day 3	48.45 ± 2.37
Day 5	54.87 ± 2.83
Day 7	80.95 ± 3.70
Day 9	128.76 ± 4.54
Day 11	203.18 ± 6.05
Day 13	273.77 ± 7.79

Table 5: ADFI, ADG, and FCR for 13 days of feeding experiment

ADFI	35.35 ± 2.71
ADG	17.78 ± 0.55
FCR	1.98 ± 0.13

Values are mean ± standard deviation (SD) of triplicate analyses.

Table 6: Anti-inflammatory findings of maceration B and soxhlet extracts by animal model in vivo on chicks

Extract/Drug	Dose (mg/kg)	1h		3h	
		Swell. Thick. ± SEM	Inhibition (%)	Swell. Thick. ± SEM	Inhibition (%)
NC	-	76.35 ± 0.89	0	95.01 ± 0.59	0
AM3	300	68.72 ± 2.34	09.99	75.37 ± 0.54	20.66
AM5	500	37.93 ± 1.06	69.97	28.51 ± 0.53	50.32
AS3	300	64.26 ± 0.84	15.81	48.16 ± 1.45	49.30
AS5	500	42.42 ± 0.51	44.43	20.41 ± 0.92	78.51
PC	150	50.26 ± 0.85	34.15	38.69 ± 0.58	59.27

Values are mean ± standard deviation (SD) of triplicate analyses.

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.

Funding

Financial support from Sidi Mohamed Ben Abdellah University (Laboratory of Functional Ecology and Engineering Environment, and Laboratory of Human Pathology, Biomedicine and Environment) regarding this research is gratefully acknowledged.

References

1. Rodríguez Villanueva J, Martín Esteban J, Rodríguez Villanueva L. A reassessment of the *Marrubium vulgare L*. herb's potential role in diabetes mellitus type 2: first results guide the investigation toward new horizons. Medicines. 2017; 4(3): 57.
2. Rodriguez Villanueva J, Martin Esteban J. An insight into a blockbuster phytomedicine; *Marrubium vulgare L*. herb more of a myth than a reality? Phytother. Res. 2016; 30: 1551–1558.
3. Bahammou Y, Tagnamas Z, Lamharrar A, Idlimam A. Thin-layer solar drying characteristics of Moroccan horehound leaves (*Marrubium vulgare L.*) under natural and forced convection solar drying. Solar Energy. 2019; 188: 958–969.
4. Elberry AA, Harraz FM, Ghareib SA, Gabr SA, Nagy AA, Abdel-Sattar E. Methanolic extract of *Marrubium vulgare* ameliorates hyperglycemia and dyslipidemia in streptozotocin-induced diabetic rats. Int J Diabetes Mellit. 2015; 3(1): 37-44.
5. Kadri A, Zarai Z, Békir A, Gharsallah N, Damak M, Gdoura R. Chemical composition and antioxidant activity of *Marrubium vulgare L*. essential oil from Tunisia. Afr J Biotechnol. 2011; 10(19): 3908-3914.
6. Alimpić A, Oaldje M, Matevski V, Marin PD, Duletić-Laušević S. Antioxidant activity and total phenolic and flavonoid contents of *Salvia amplexicaulis Lam*. extracts. Arch Biol Sci. 2014; 66(1): 307-316.
7. Rubió L, Motilva MJ, Romero MP. Recent advances in biologically active compounds in herbs and spices: a review of the most effective antioxidant and anti-inflammatory active principles. Crit Rev Food Sci Nutr. 2013; 53(9): 943-953.
8. Ng ZX, Koick YTT, Yong PH. Comparative analyses on radical scavenging and cytotoxic activity of phenolic and flavonoid content from selected medicinal plants. Nat Prod Res. 2021; 35(23): 5271-5276.
9. Bouterfas K, Mehdadi Z, Elaoufi MM, Latreche A, Benchiha W. Antioxidant activity and total phenolic and flavonoids content variations of leaves extracts of white Horehound (*Marrubium vulgare Linné*) from three geographical origins. Ann Pharm Fr. 2016; 74(6): 453–462.
10. Azam S, Hadi N, Khan NU, Hadi SM. Antioxidant and prooxidant properties of caffeine, theobromine, and xanthine. Med Sci Monit. 2003; 9(9): 325-330.
11. Hu S, Kim BY, Baik MY. Physicochemical properties and antioxidant capacity of raw, roasted and puffed *cacao* beans. Food Chem. 2016; 194: 1089-1094.
12. Mudenuti NV de R, de Camargo, AC, de Alencar SM, Danielski R, Shahidi F, Madeira TB, Grossmann MVE. Phenolics and alkaloids of raw *cocoa* nibs and husk: The role of soluble and insoluble-bound antioxidants. Food Biosci. 2021; 42: 101085.
13. Teng H, Chen L. Polyphenols and bioavailability: An update. Crit Rev Food Sci Nut. 2019; 59(13): 2040-2051.
14. Muller AG, Sarker SD, Saleem IY, Hutcheon GA. Delivery of natural phenolic compounds for the potential treatment of lung cancer. Daru. 2019; 27(1): 433-449.
15. Šalamúnová P, Cupalová L, Majerská M, Treml J, Ruphuy G, Šmejkal K, Ruphuy G, Šmejkal K, Štěpánek F, Hanuš J, Hošek J. Incorporating natural anti-inflammatory compounds into yeast glucan particles increases their bioactivity *in vitro*. Int J Bio Macromol. 2021; 169: 443-451.
16. Mouffouk S, Gómez-Ruiz S, Benkhaled M, Carralero S, Haba H. Phytochemical Composition, Antioxidant and Antibacterial Activities of Crude Extracts from the Species *Euphorbia Atlantica Coss*. Pharm Chem J. 2019; 53(9): 831-837.
17. Yubin JI, Miao Y, Bing W, Yao Z. The extraction, separation, and purification of alkaloids in the natural medicine. J Chem Pharm Res. 2014; 6(1): 338-345.
18. Koné D. Enquête ethnobotanique de six plantes médicinales maliennes: extraction, identification d'alcaloïdes - caractérisation, quantification de polyphénols : étude de leur activité antioxydante. [Thèse de doctorat. Metz, Français: Université Paul Verlaine]. 2009. Available from: https://hal.univ-lorraine.fr/tel-01752627.
19. Togola I, Konaré MA, Diakité M, Diarra N, Tounkara F, Sanogo R, Dembélé D. Evaluation of total alkaloid content at different stages of development of *datura innoxiamill*. A plant used in traditional medicine in Mali. Am. J. Innov. Res. Appl. Sci. 2019; 9(2): 200-207.
20. Rojas-Vera J, Buitrago-Díaz A, Possamai L, Timmers L, Tallini L, Bastida J. Alkaloid profile and cholinesterase inhibition activity of five species of Amaryllidaceae family collected from Mérida state-Venezuela. S Afr J Bot. 2021; 136: 126-136.
21. Motto AE, Lawson-Evi P, Bakoma B, Eklu-Gadegbeku K, Aklikokou K. Antihyperlipidemic and antioxidant properties of hydro-alcoholic extracts from *Anogeissusleiocarpus* (Combretaceae). Heliyon. 2021; 7(4): e06648.
22. Rigane H, Chtourou M, Ben Mahmoud I, Medhioub K, Ammar E. Polyphenolic compounds progress during olive mill wastewater sludge and poultry manure co-composting, and humic substances building (Southeastern Tunisia). Waste Manag Res. 2015; 33(1): 73-80.
23. Amo-Mensah J, Darko G, Borquaye L. Anti-inflammatory and antioxidant activities of the root and bark extracts of *Vitex grandifolia* (Verbanaceae). Sci Afr. 2020; 10. e00586.
24. Yang L, Liu G, Zhu X, Luo Y, Shang Y, Gu X. The anti-inflammatory and antioxidant effects of leonurine hydrochloride after lipopolysaccharide challenge in broiler chicks. Poult Sci. 2019; 98(4): 1648-1657.
25. Rivera PB, Rebaza GF, Tuesta AT, Gutierrez RR, Melo DC, Fiestas GV, Herrera LM. Determinación estructural de flavonaspresentes en el extractometanólico de hojas de *Marrubium vulgare L*. "Cordon". Rev. Peru Med. Integr. 2019; 4(3): 90-95.
26. Haratym W, Weryszko-Chmielewska E. Ultrastructural and histochemical analysis of glandular trichomes of *Marrubium vulgare L*. (lamiaceae). Flora. 2017; 231: 11-20.
27. Tripathi IP, Tripathi R, Tiwari A. Investigation of biologically active phytoconstituents present in selected plants material of Verbenaceae, lamiaceae and Fabaceae family. Int. J. Multidiscip. Curr. Res. 2017; 5: 31-37.
28. Njinga NS, Bakare-Odunola MT, Sa'ad TA, Abdulmajeed FF, Bello RH, Saadu RA, Shittu AO. Effects of ultra-violet, red and sun light on the stability of phytochemicals, antiradical and antimicrobial activity of *ocimumgratissimum linn*. (lamiaceae). The East and Central Afr J Pharm Sci. 2020; 23(3): 121-133.
29. Mukherjee PK. Bioactive phytocomponents and their analysis. In: Quality control and evaluation of herbal drugs; 2019. 237-328 p. Doi: https://doi.org/10.1016/B978-0-12-813374-3.00007-7.
30. Rahman MM, Devi R, Megha PU. Phytochemical screening and *in-vitro* antimicrobial activity of *Pogostemon quadrifolius* (benth) of lamiaceae. Int J Pharmaceutical Sci Res. 2018; 9(6): 2438-2445.

31. Guinda A, Rada M, Delgado T, Castellano JM. Pentacyclic triterpenic acids from *Argania spinosa*. Eur J Lipid Sci Technol. 2011; 113(2): 231-237.
32. Firuzi O, Lacanna A, Petrucci R, Marrosu G, Saso L. Evaluation of the antioxidant activity of flavonoids by "ferric reducing antioxidant power" assay and cyclic voltammetry. Biochim Biophys Acta Gen Subj. 2005; 1721(1-3): 174-184.
33. Putra L, Sukweenadhi J, Nathania C, Wibowo E, Buschle-Diller G, Marianti Purwanto M. Comparative study of polyphenolic compound extraction from empty palm fruit bunches and sugarcane pulp. Heliyon. 2022; 8(2): e08951.
34. Aguilar-Villalva R, Molina G, España-Sánchez B, Díaz-Peña L, Elizalde-Mata A, Valerio E, Azanza-Ricardo C, Estevez M. Antioxidant capacity and antibacterial activity from *Annona cherimola* phytochemicals by ultrasound-assisted extraction and its comparison to conventional methods. Arab J Chem. 2021; 14(7): 103239.
35. Eddahhaoui F, Boudalia M, Harhar H, Chahboun N, Tabyaoui M, Guenbour A, Zarrouk A, Bellaouchou A. Effect of the extraction technique on the bioactive compounds and the antioxidant capacity of the *Chamaerops humilis L.* fruit (pulp and seeds). Chemical Data Collections. 2022; 40: 100882.
36. Li L, Chiroma S, Hashim T, Adam S, Mohd Moklas M, Yusuf Z, Rahman S. Antioxidant and anti-inflammatory properties of *Erythroxylum cuneatum* alkaloid leaf extract. Heliyon. 2020; 6(6): e04141.
37. Amaeze OU, Ayoola GA, Sofidiya MO, Adepoju-Bello AA, Adegoke AO, Coker HAB. Evaluation of antioxidant activity of *Tetracarpidium conophorum (Müll. Arg) Hutch & Dalziel* leaves. Oxid Med Cell Longev. 2011; 2011: 1-7.
38. Suktham T, Jones A, Soliven A, Dennis G, Shalliker R. A comparison of the performance of the cupric reducing antioxidant potential assay and the ferric reducing antioxidant power assay for the analysis of antioxidants using reaction flow chromatography. Microchem J. 2019; 149: 104046.
39. Purewal S, Kaur P, Sandhu K. Bioactive profile and antioxidant properties of *Kinnow* seeds: A report broadening its potential. Appl Food Res. 2022; 2(2): 100135.
40. Shimada K, Fujikawa K, Yahara K, Nakamura T. Antioxidative properties of xanthan on the autoxidation of *soybean* oil in cyclodextrin emulsion. J. Agric. Food Chem. 1992; 40(6): 945-948.
41. Fukumoto LR, Mazza G. Assessing antioxidant and prooxidant activities of phenolic compounds. J Agric Food Chem. 2000; 48(8): 3597-3604.
42. Aksoy L, Kolay E, Ağılönü Y, Aslan Z, Kargıoğlu M. Free radical scavenging activity, total phenolic content, total antioxidant status, and total oxidant status of endemic *Thermopsis turcica*. Saudi J Biol Sci. 2013; 20(3): 235-239.
43. Lfitat A, Zejli H, Bousraf F, Bousselham A, El Atki Y, Gourch A, Lyoussi B, Abdellaoui A. Comparative assessment of total phenolics content and *in vitro* antioxidant capacity variations of macerated leaf extracts of *Olea europaea L.* and *Argania spinosa (L.)* Skeels. Mater Today Proc. 2021; 45: 7271-7277.
44. Bala A, Haldar PK. Free radical biology in cellular inflammation related to rheumatoid arthritis. OA Arthritis. 2013; 1(2): 15.
45. Gutiérrez DMA, Bah M, Garduño MLR, Mendoza SOM, Serrano VC. Anti-inflammatory and antioxidant activities of methanol extracts and alkaloid fractions of four Mexican medicinal plants of Solanaceae. Afr J Tradit Complement Altern Med, Complementary and Alternative Medicines. 2014; 11(3): 259-267.
46. El moujahed S, Chahdi F, Rodi Y, El ghadraoui L, Lemjallad L, Errachidi F. The Moroccan *Pomegranate*: An Underrated Source of Tannins Extracts and Natural Antimicrobials from Juice Processing Byproducts. Waste Biomass Valorization. 2021; 12(10): 5383-5399.
47. Saad H, Charrier-El Bouhtoury F, Pizzi A, Rode K, Charrier B, Ayed N. Characterization of *pomegranate peels* tannin extractives. Ind Crops Prod. 2012; 40: 239-246.
48. Al-Snafi AE, Al-Saedy HA, Talab TA, Majid WJ, El-Saber Batiha G, Abolfazl JS. The bioactive ingredients and therapeutic effects of *Marrubium vulgare*- A review. Int J Biol Pharm Sci Arch. 2021; 1(2): 9-21.
49. Rosales-López C, Muñoz-Arrieta R, Abdelnour-Esquivel A. Emetine and cephaeline content in plants of *Psychotria ipecacuanha* in Costa Rica. Rev. Colomb. Quim. 2020; 49(2): 18-22.
50. Asano T, Kushida H, Sadakane C, Ishihara K, Wakui Y, Yanagisawa T, Kimura M, Kamei H, Yoshida, T. Metabolism of *ipecac* alkaloids cephaeline and emetine by human hepatic microsomal cytochrome P450s, and their inhibitory effects on P450 enzyme activities. Biol Pharm Bull. 2001; 24(6): 678-682.
51. Asano T, Watanabe J, Sadakane C, Ishihara K, Hirakura K, Wakui Y, Yamashita M. Biotransformation of the *ipecac* alkaloids cephaeline and emetine from *ipecac* syrup in rats. Eur J Drug Metab Pharmacokinet. 2002; 27(1): 29-35.
52. Kanyonga PM, Faouzi MA, Meddah B, Mpona M, Essassi EM, Cherrah Y. Assessment of methanolic extract of *Marrubium vulgare* for anti-inflammatory, analgesic and anti-microbiologic activities. J Chem Pharm Res. 2011; 3(1): 199-204.
53. Jin Q, Zhao Y, Liu Y, Zhang R, Zhu P, Zhao L, Qin X, Luo X. Anti-inflammatory and analgesic monoterpenoid indole alkaloids of *Kopsia officinalis*. J Ethnopharmacol. 2022; 285: 114848.
54. Xie T, Zhao Y, Wang H, Chen Y, Wei X, Wang Z, He Y, Zhao L, Luo X. New steroidal alkaloids with anti-inflammatory and analgesic effects from *Veratrum grandiflorum*. J Ethnopharmacol. 2022; 293: 115290.
55. Tang Y, Deng J, Li L, Yan J, Lin L, Li Y, Lin Y, Xia B. Essential oil from *Prunella vulgaris L.* as a valuable source of bioactive constituents: *In vitro* anti-bacterial, anti-viral, immunoregulatory, anti-inflammatory, and chemical profiles. S Afr J Bot. 2022; 151: 614-627.
56. Aitfella Lahlou R, Bounechada M, Mohammedi A, Silva L, Alves G. Dietary use of *Rosmarinus officinalis* and *Thymus vulgaris* as anticoccidial alternatives in poultry. Anim Feed Sci Technol. 2021; 273: 114826.
57. Silva A, Tewari D, Sureda A, Suntar I, Belwal T, Battino M. Nabavi SM, Nabavi SF. The evidence of health benefits and food applications of *Thymus vulgaris L.* Trends Food Sci Technol. 2021; 117: 218-227.
58. Rosales PF, Bordin GS, Gower AE, Moura S. Indole alkaloids: 2012 until now, highlighting the new chemical structures and biological activities. Fitoterapia. 2020; 143: 104558.
59. Klein-Júnior LC, Vander Heyden Y, Henriques AT. Enlarging the bottleneck in the analysis of alkaloids: A review on sample preparation in herbal matrices. Trends Analyt Chem. 2016; 80: 66-82.
60. Dey P, Kundu A, Kumar A, Gupta M, Lee BM, Bhakta T, Dash S, Kim HS. Analysis of alkaloids (indole alkaloids, isoquinoline alkaloids, tropane alkaloids). In: Recent advances in natural products analysis. Elsevier; 2020. 505-567 p.
61. Sinan KI, Saftić L, Peršurić Ž, Pavelić SK, Etienne OK, Picot-Allain MCN, Mahomoodally MF, Zengin G. A comparative study of the chemical composition, biological and multivariate analysis of *Crotalaria retusa L.* stem barks, fruits, and flowers obtained via different extraction protocols. S Afr J Bot. 2020; 128: 101-108.
62. Onakurhefe P, Achuba FI, George BO. Phytochemical analysis and chemical characterization of extracts and blended mixture of *palm oil* leaf. Trop J Nat Prod Res. 2019; 3(9): 282-297.

63. Olorukooba B, Maiha BB, Chindo AB, Ejiofor JI, Nasir HA, Sani BM, Balogun SM. Antimalarial activity of the n-Butanol fraction of Uapaca togoensis (Pax) stem bark in mice. Trop J Nat Prod Res. 2018; 2(1): 29-33.
64. Adamu K, Musa H, Musa AO, Mikailu AS. Antifungal activity of Ethyl-acetate Leaf Extract and Terpenoid-Rich Fraction from *Hyptis spicigera Lam*. Trop J Nat Prod Res. 2020; 4(3): 63–68.
65. Alaribe CS, Lawal M, Momoh R, Idaholo U, Mudabai P, Adejare AA. Phytochemical and Toxicological Properties of Sclerotium from the Edible Fungus-Pleurotus tuber-regium. Trop J Nat Prod Res. 2018; 2(5): 235-239.
66. Sani NA, Kawu MU, Bako IG, Sulaiman MH. Mammogenic and lactogenic effects of leaf extract and fractions of *Launaea taraxacifolia* and resveratrol in lactating Wistar rats. Trop J Nat Prod Res. 2020; 4: 8-13.