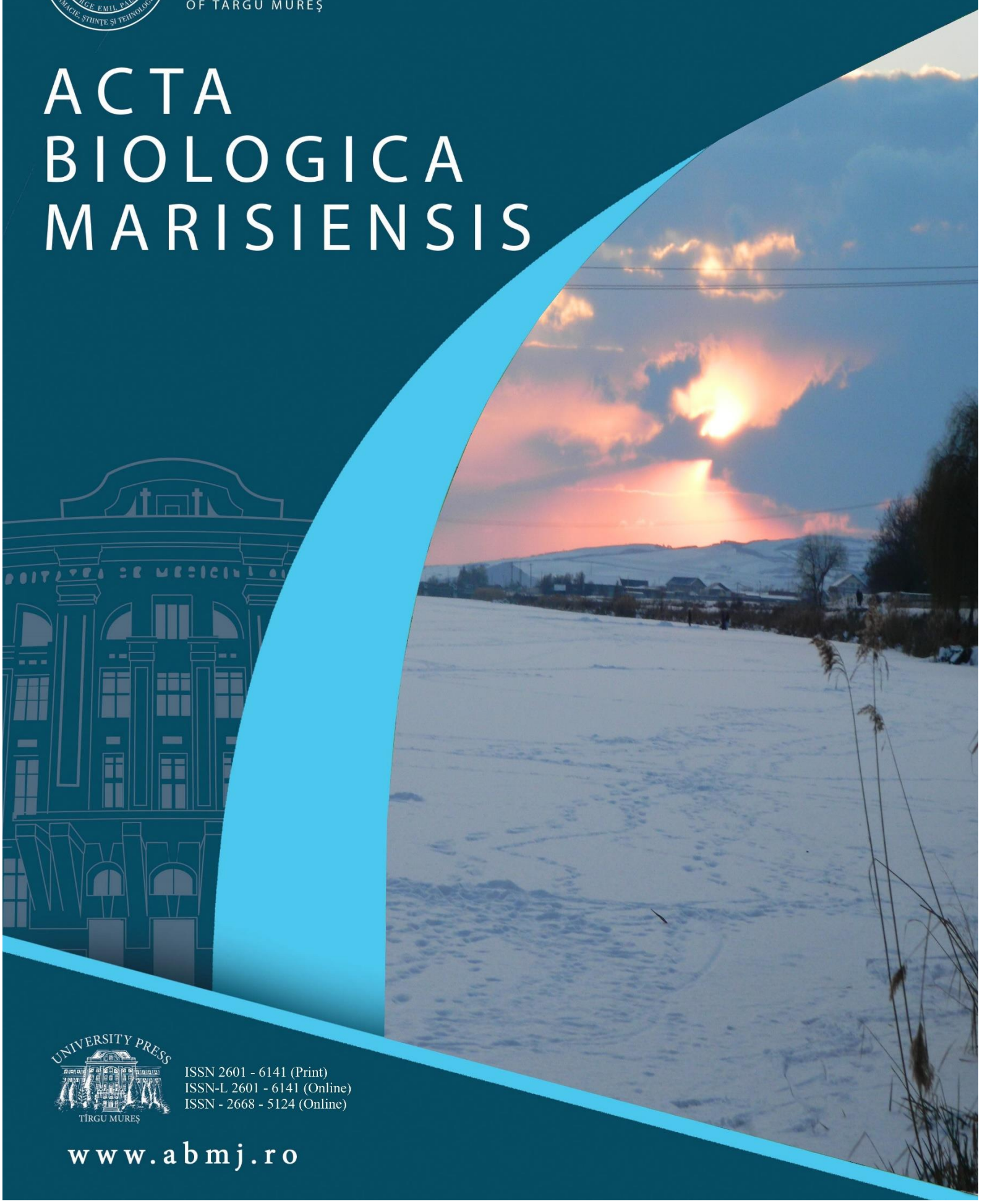
Volume 7 • Issue 1-2 December 2024

"GEORGE EMIL PALADE"
UNIVERSITY OF MEDICINE,
PHARMACY, SCIENCE AND TECHNOLOGY
OF TÂRGU MUREŞ

ACTA BIOLOGICA MARISIENSIS



UNIVERSITY PRESS
TÎRGU MUREŞ

ISSN 2601 - 6141 (Print)
ISSN-L 2601 - 6141 (Online)
ISSN - 2668 - 5124 (Online)

www.abmj.ro

2024 | Volume 7 | Issue 1-2

ACTA BIOLOGICA MARISIENSIS

Official Journal of the George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Târgu Mureș

Acta Biologica Marisiensis
ISSN: 2601 – 6141 (Print)
ISSN-L: 2601 – 6141
ISSN: 2668 – 5124 (Online)
Published by University Press Târgu Mureș in cooperation with CrossRef

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INDIGENOUS *CANNABIS SATIVA* - THE PHARMACOGNOSTICAL ANALYSIS OF A WEED-TYPE VARIETY

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Received: 16 November 2023; **Accepted:** 8 January 2024; **Published:** 30 June 2024

Abstract: *Cannabis sativa* is a well-known plant that has attracted a lot of attention because of its remarkable therapeutic potential. However, a variety of this species is growing invasive in some cultures in Romania. This variety has a different composition compared to the cultivated variety and a notably low Δ9-tetrahydrocannabinol (THC) content, making the occurrence of psychoactive effects highly improbable. This research aimed to evaluate from a pharmacognostical point of view the herbal drug collected from Romania. A preliminary microscopic analysis was performed to confirm the identity of the herbal drug. A qualitative analysis by thin-layer chromatography was performed to identify cannabinoids and flavonoids. Quantitative determinations included the evaluation of total polyphenolic content, total flavonoid content, and hydroxycinnamic acid content, as well as ion chromatographic analysis for anion quantification. Antioxidant and alpha-amylase activities were performed as well. The results indicated that the herbal drug contains only moderate concentrations of polyphenolic compounds, but has a good antioxidant activity, likely attributable to the presence of non-polyphenolic compounds. Moreover, the extract had a moderate alpha-amylase inhibitory activity, suggesting a low potential in nutritional and therapeutic contexts.

Keywords: *Cannabis sativa*, invasive species, cannabidiol, polyphenols, antioxidant activity

1. Introduction

Cannabis sativa L. is cultivated in Romania for its fibers that are used in the textile industry. The cultivated species (encoded NC 5302 10 00) must have a Δ9-tetrahydrocannabinol (Δ9-THC) level below 0.3%, and the seeds must be accompanied by documents that attest the level of THC, according to the Regulation (EU) no 1308/2013

of the European Parliament and of the Council of 17 December 2013. In Romania, *Cannabis sativa* is included in the list of plants dangerous for human consumption (*ORDIN 244 22/04/2005 - Privind Prelucrarea, Procesarea Şi Comercializarea Plantelor Medicinale Şi Aromatice Utilizate ca Atare, Parţial Procesate Sau Procesate Sub Formă de Suplimente Alimentare Predozate*, n.d.). According to Law no. 339/2005 regarding the legal regime of narcotic and psychotropic plants, substances, and preparations, tetrahydrocannabinol is included in the list of substances without recognized interest in medicine. *Cannabis*, *cannabis* resin, extracts, and tinctures from this plant are included in the category of 'Other plants, substances, and preparations containing narcotic and psychotropic substances of medical interest, subjected to strict control', according to the same law (*LEGE 339 29/11/2005 - Privind Regimul Juridic al Plantelor, Substanţelor Şi Preparatelor Stupefiante Şi Psihotrope*, n.d.).

Over time there have been many disputes regarding the nomenclature of *Cannabis* species. A two step classification system has been used in the twenty years, which was initially proposed by Cronquist and Small. The first step is represented by the THC content (a value of 0.3% is the dividing point). Based on the THC content there are two varieties of *Cannabis sativa: Cannabis sativa* subsp. *sativa* (low THC content), and *Cannabis sativa* subsp. *indica* (high THC content). The second step proposed by the two authors is related with the domestication characteristics of the subspecies: *Cannabis sativa* subsp. *sativa* var. *sativa* having domestication characteristics, *Cannabis sativa* subsp. *sativa* var. *spontanea* having the characteristics of a wild population, *Cannabis sativa* subsp. *indica* var. *indica* having the characteristics of a cultured variety, and C*annabis sativa* subsp. *indica* var. *kafiristanica* having the characteristics of a wild population. They also have declared that it is quite difficult to distinguish wild populations from the cultivated ones, due to the hybridization between these populations. Height is usually a less important characteristic in the identification process of the species, because large variations were seen due to the geographical conditions. The morphology of the fruit can offer information about the origin of the *Cannabis* variety. The perianth is present in the wild populations, while in the cultivated populations, the perianth is often missing at maturity. However since the early 1970's and until now, the classification of *Cannabis species* is still on debate (McPartland, 2018; McPartland and Small, 2020; Small and Cronquist, 1976).

Cannabinoids are benzopyranic derivatives, terpenophenolic compounds having 21 carbon atoms in the molecule. In the freshly harvested material, they are found mostly in the form of acids. The percentage of cannabinoids in the herbal drug can vary from 2 to 20%, depending on the variety and the growing conditions (Evans, 2009; Mechoulam and Hanuš, 2000). The herbal drug also contains a percentage of up to 0.01% of spermidine alkaloids (Mechoulam, 1989; Radwan et al., 2021). At the same time, there is also a percentage of up to 0.5% of essential oil, consisting of over 120 constituents. The volatile compounds are mainly monoterpenes and sesquiterpenes, such as: α- and β-pinene, myrcene, limonene, α-bergamotene, α-caryophyllene and β-caryophyllene (Aizpurua-Olaizola et al., 2016, Vasudevan and Sthal, 2020). Also, other classes of substances, such as flavonoids, have been identified in the *Cannabis sativa* species. The most common flavonoids in hemp are flavones (mainly apigenin and luteolin) and flavonols (quercetin) derivatives, while cannflavins A, B, and C are mostly specific to this plant. Based on their

chemical structures, cannflavins A and B can be derived from luteolin (Bautista et al., 2021).

The primary objective of this study was to conduct a phytochemical analysis of an invasive weed-type variety of *Cannabis sativa*, focusing on qualitative and quantitative determinations. The literature is limited regarding the phytochemical composition and potential applications of this invasive species, therefore, we aimed to assess the potential utility of this invasive plant, with an emphasis on the relevance of its practical utilization.

2. Materials and methods

Chemicals

Folin-Ciocâlteu reagent was purchased from Carl Roth GmbH (Karlsruhe, Germany), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, and Trolox were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). ABTS tablets [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid)], 50 mg/tablet were purchased from Roche Diagnostics GmbH (Germany). All other solvents used in this research were of analytical grade.

Plant material and extract preparation

The herbal drug (aerial parts) was collected from a sunflower (*Helianthus annuus*) culture from Bărăganului plain, Romania, in August 2022. The plant material was dried in the shade and deposited in laboratory conditions until analysis. A voucher specimen (SF-CS-22) was deposited at the Department of Pharmacognosy and Phytotherapy, Faculty of Pharmacy, George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureș, Romania. The sample collected presents the morphological characteristics of the *Cannabis sativa* subsp. *sativa* var. *spontanea*.

An aliquot of the herbal drug was subjected to artificial drying in an oven, at 121 °C, for an hour. Hexane and methanol extractions were performed from the artificial dried herbal drug, giving the following extracts CS-DH (hexane) and CS-DM (methanol). Also, methanolic extracts (1%) were prepared using the herbal drug dried in laboratory conditions, in an ultrasonic water bath at 40 °C, for 30 minutes giving the extract abbreviated CS-M.

Micro-anatomical characterization of the herbal drug

Leaf samples were sectioned in the elder stem, and an 80% chloral hydrate solution was used for the clarification of the cross sections. The sections were mounted in a gelatine-glycerin mixture and analyzed with a Micros-Austria microscope.

Identification of cannabinoids by thin-layer chromatography (TLC)

CS-DM, CS-DM, and CS-M extracts were applied to the TLC plates. A 0.01% CBD solution was used as standard, and aliquots from commercial CBD oils were used as control.

The solutions were applied as bands (1 cm) on silicagel 60 F_{254} plates (Merck). Plates were developed in the ascending mode in a saturated chamber using a mixture of hexane: diethyl ether (80:20, *v*/*v*) as the mobile phase. Following development, the dried plates were sprayed with a 0.5% vanillin solution of methanol-hydrochloric acid. The plates were analyzed in visible and at UV_{254} (Galand et al., 2004; Liu et al., 2020; Sherma and Rabel, 2019).

Identification of flavonoids by thin-layer chromatography (TLC)

For the identification of flavonoids, the methanolic extract was used (CS-M), as well as 0.05% methanolic solutions of chlorogenic acid, rutoside, and caffeic acid. The solutions were applied as bands (1 cm) on silicagel 60 F_{254} plates (Merck). Plates were developed in the ascending mode in a saturated chamber using a mixture of ethyl acetate: formic acid: acetic acid: water; 100:11:11:27 (*v/v/v/v*) as mobile phase. Following development, the dried plates were sprayed with Natural substance-polyethylene glycol reagent (NST/PEG). The plates were analyzed in visible and at UV_{365} (Sherma and Rabel, 2019).

Total flavonoid content (TFC)

Total flavonoid content was determined after the method described in the Romanian Pharmacopoeia 10th Edition (*Farmacopeea Română*, 1993). Briefly, 10 mL of the methanolic extract (CS-M) was diluted at 25 mL with methanol and filtered. An aliquot of this solution was mixed with 5 mL of 10% sodium acetate solution, 3 mL of 2.5% aluminum chloride solution, and methanol. The absorbance was measured at 430 nm after 15 minutes against a blank solution. The test was carried out in triplicate. A calibration curve was prepared using a standard solution of quercetin in the concentration range of 0.004 - 0.012 mg/mL.

Total flavonoid content was estimated from the calibration curve of quercetin (R^2 = 0.9948), and the results were expressed as mg quercetin equivalents per gram dry weight (mg QE/ g DW).

Total polyphenolic content (TPC)

The total polyphenolic content was determined after a modified method described in the European Pharmacopoeia 10th edition. Briefly, 40 µL from the methanolic extract (CS-M) were mixed with 3160 µL water, 200 µL Folin-Ciocâlteu reagent, and 600 µL of 9% sodium carbonate solution. The mixture was allowed to stand in the dark at room temperature for 30 minutes. The absorbance was read at 765 nm against a suitable blank. Total polyphenolic content was estimated from the calibration curve of gallic acid (0.002 - 0.025 mg/ mL, R^2 = 0.9649). The results were expressed as mg gallic acid equivalents per gram dry weight (mg GAE/ g DW).

Total hydroxycinnamic derivatives content (HDC)

HDC was determined according to the method described in the European Pharmacopoeia 10th edition (*European Pharmacopoeia (Ph. Eur.)*, 2010), using the CS-M extract. The results were expressed as mg rosmarinic acid equivalents per gram of dry weight (mg RAE/ g DW), and was calculated using equation 1, taking the specific absorbance of rosmarinic acid to be 400:

$$HDC = \frac{A \times 2.5}{m} \times 10 \quad (1)$$

where: A - absorbance of the test solution at 505 nm

m - mass of the substance to be examined, in grams

Ion chromatography analysis

Inorganic anion composition was determined using a Dionex ICS-3000 Ion Chromatography System and a Dionex IonPac® AS23 analytical column under suppressed conditions. The eluent used was sodium carbonate 4.5 mM/sodium bicarbonate 0.8 mM, its flow rate was 1.2 mL/min at 30 °C. The detection was conductometric and the analysis time was 20 minutes. Combined Seven Anion Standard II (Thermo Scientific) was used for the identification and quantification of anions in Cs-M extract.

DPPH• assay

Antioxidant activity with DPPH• was determined according to the method published by Laczko-Zöld et al (Laczkó-Zöld et al., 2018). Briefly, 150 µL of extract (CS-M) in different concentrations were mixed with 200 µL DPPH• 0.1 mM solution in a 96 multiwell plate. The mixtures were allowed to stand for 30 minutes, at room temperature, and the absorbance was determined at 517 nm using an Epoch multiplate reader (BioTek Instruments Inc., USA). The inhibition capacity was calculated using Equation 2:

$$IC\% = \frac{A_0 - A_1}{A_0} \, x \, 100 \quad (2)$$

Where: A_0 - absorbance of DPPH• solution, A_1 - the absorbance of the sample solution

The concentration that inhibits 50% of the DPPH• activity (IC_{50}) was calculated by plotting the absorbance of the solution against the logarithm of the concentrations.

Ascorbic acid was used as positive control.

$ABTS^{+\bullet}$ radical scavenging assay

Antioxidant activity with $ABTS^{+\bullet}$ was determined according to a previously described method, with slight modifications (Ştefănescu et al., 2017). The absorbance was measured at 734 nm using an Epoch multiplate reader (BioTek Instruments Inc., USA). The inhibition capacity and IC_{50} were calculated as described in the DPPH• assay. Trolox was used as a positive control.

α-Amylase inhibition capacity

The inhibition capacity of the methanolic extract (CS-M) on *α*-amylase was determined according to a previously published method (Ercan and El, 2016). The DNS reagent was prepared by mixing 8 mL of 5.3 M sodium potassium tartrate tetrahydrate solution in 2 M NaOH, 20 mL of 96 mM 3, 5- dinitrosalicylic acid solution, and 12 mL of water. 0.5 mL of different concentrations of the extract was incubated for 30 minutes with the α-amylase solution (1 U/mL) before the starch solution was added. Further, the mixture was incubated at 37 °C, for 10 minutes. The reaction was stopped by adding the DNS reagent, and the samples were introduced in a boiling water bath for 10 minutes. The absorbance of the solution was measured at 540 nm. One negative control (absence of inhibitor) was set up to obtain 100% enzyme activity. Acarbose was used as a positive control. The inhibition capacity was calculated using the following formula (Equation 3):

$$IC\% = 100 \, x \, (1 - \frac{A \, sample}{A \, control}) \quad (3)$$

The results were expressed as IC_{50} in mg/ mL.

Statistical analysis

All experiments were done in triplicate (n = 3) and results were expressed as means ± standard deviation (SD). The statistical analyses were performed by using GraphPad Prism 9.0 software (San Diego, CA). Statistical differences in the antioxidant activities were determined through analysis of variance (ANOVA). Student *t*-test was used to compare the IC_{50} of the extract and acarbose. A value of *p* lower than 0.05 was considered to be statistically significant.

3. Results and discussions

Micro-anatomical characterization of the herbal drug

The analysis of the cross-section through the leaf revealed a heterogeneous-asymmetric (bifacial) structure; the upper epidermis and the

lower epidermis are made up of a single layer of cells, which delimits the mesophyll made up of palisade tissue and lacunar tissue. Numerous cystolithic trichomes are observed on the upper epidermis.

There are three types of glandular trichomes characteristic of *Cannabis sativa*: bulbous glandular trichome, capitate-stalked glandular trichome, and capitate-sessile glandular trichome. Cannabinoids are synthesized and stored in these trichomes (Raman et al., 2017).

There are also two types of non-glandular, unicellular trichomes: cystolithic trichomes (cystoliths of calcium carbonate) and long and slender trichomes (Raman et al., 2017).

The microscopic analysis of the herbal drug confirmed the identity of the species. As it can be seen in **Figure 1**, numerous trichomes can be found in the herbal drug. The glandular trichomes are responsible for the secretion of the oleoresin (Gurav and Gurav, 2014).

Identification of cannabinoids by thin-layer chromatography (TLC)

The TLC (**Fig. 2.**) analysis revealed that CBD is found in a reduced concentration in the sample dried in laboratory conditions, while the sample dried in the oven at 121 °C for 1 hour, contains higher concentrations of CBD. This conclusion could be drawn based on the correlation between the intensity and dimensions of the CBD - CBD-corresponding zone. This higher concentration of CBD was probably generated through the decarboxylation of its corresponding acid (cannabidiolic acid - CBDA) (**Fig. 3.**).

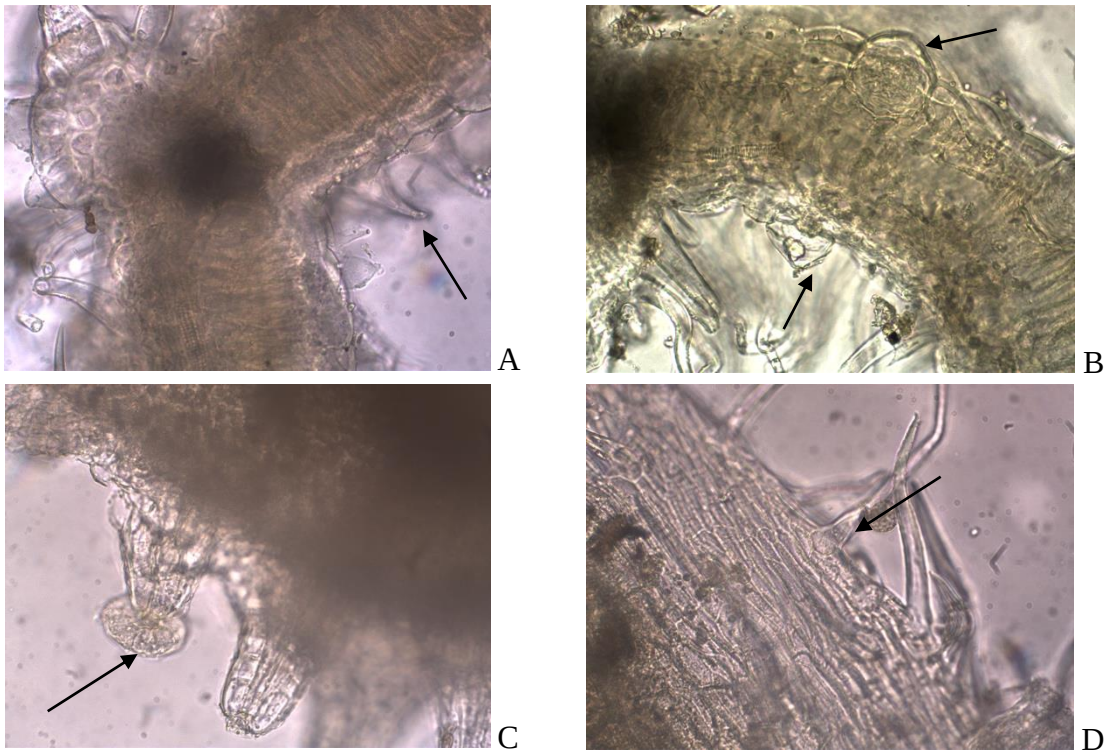

Fig. 1. Microscopic characteristics of the herbal drug: A - non-glandular cystolith-containing conical trichomes; B - Capitate-sessile glandular trichome; C- Capitate-stalked glandular trichome, D - non-glandular longer and slender trichomes

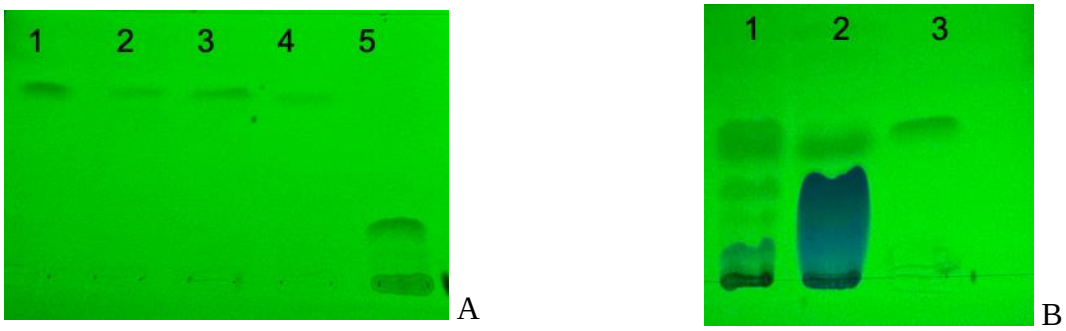

Fig. 2. A: 1 - CBD 0.01% solution, 2 - CBD oil 5%, 3 - CBD oil 10%, 4 - CBD extract 5%, 5 - CS-M; B: 1 - CS-DH, 2 - CS-DM, 3 - CBD 0.01% solution

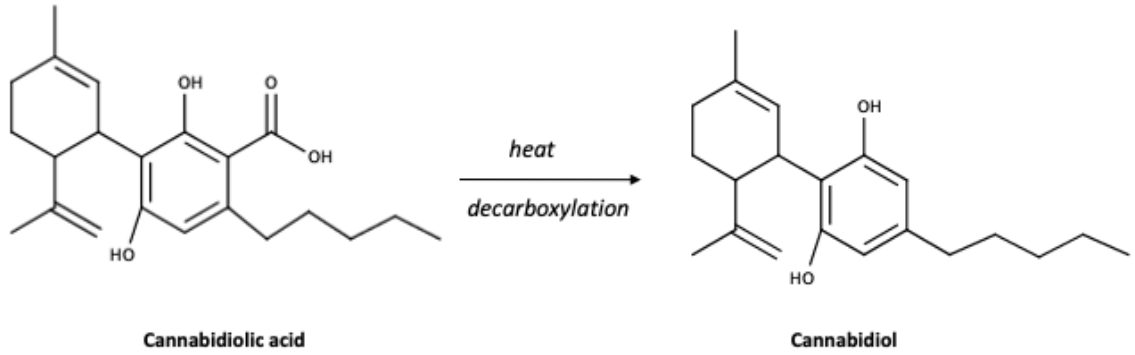

Fig. 3. Decarboxylation reaction of CBDA to CBD

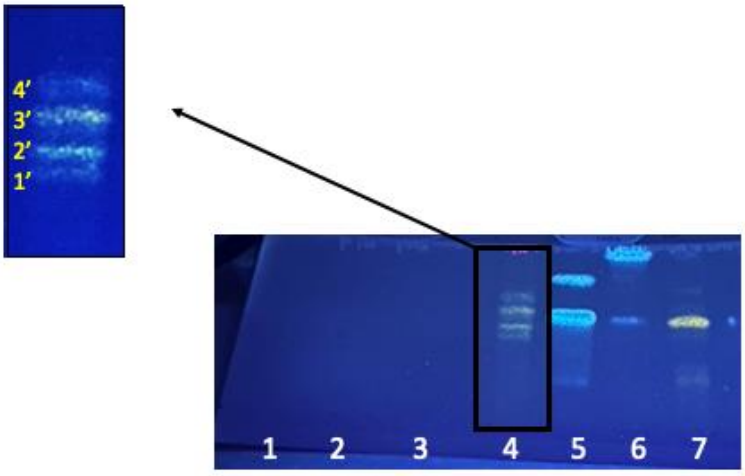

Fig. 4. TLC analysis of flavonoids from *Cannabis sativa*; 1 - 5% CBD oil, 10% CBD oil, CBD extract, 4 - sample, 5 - chlorogenic acid, 6 - caffeic acid, 7 - rutoside

Identification of flavonoids by thin-layer chromatography (TLC)

Separation of flavonoids by TLC revealed the presence of 4 compounds in the sample (**Fig. 4.**). The compounds had different retention factors compared with the standard solutions used. Based on their fluorescence in UV_{365} we can presume that the compounds could be the characteristic *Cannabis* prenilated flavones, cannflavins, but as well, these compounds could be flavons or flavonols derivatives. An HPLC analysis is needed in

order to identify the flavonoids extracted from this herbal drug.

TPC, TFC, HDC

Cannabis sativa is used for its cannabinoid content. However, only a few research papers have described the polyphenolic composition of the herbal drug.

The results obtained in this study (**Table 1.**) showed that the concentration of polyphenolic compounds in the herbal drug is low. Compared to other published data, our samples contained lower concentrations of TPC and flavonoids. However, Hacke *et al*. have used supercritical fluid extraction for extract preparation, which could have led to a better yield (Hacke et al., 2019). It is well known that geographical variability is mainly responsible for the different synthesis of secondary metabolites.

With regard to the flavonoid content, the results obtained in our study are similar to the results obtained by Hacke et al (Hacke et al., 2019).

IC analysis

Ion chromatographic analysis showed the presence of traces of fluoride, chloride, nitrate, phosphate, and sulfate ions (**Table 2.**). Four unidentified anions, possibly low molecular weight organic acids, were also present (**Fig. 5.**).

DPPH and ABTS assay

Antioxidant radical scavenging activity was measured through DPPH and ABTS assays. These methods are frequently used for screening possible antioxidant activities of natural compounds (Gulcin, 2020).

Interestingly, although the total phenolic, flavonoid, and hydroxycinnamic acid composition is quite low, compared to other herbal drugs, the methanolic extract has a powerful antioxidant activity determined with the DPPH assay (**Fig. 6.**). This suggests that other compounds from the extract are contributing to this activity. The potential of the active compounds from the extracts to scavenge the ABTS radical was lower compared with the DPPH assay. This result is probably linked with the ATBS assay being more suitable for exploring the radical scavenging activity of hydrophilic compounds, and with the lipophilicity of cannabinoids and terpenes, it was predictable that the antioxidant activity would be reduced in this assay. The results are in accordance with the results published by Zengin et al, who have tested the antioxidant activity of the essential oil distilled from the aerial parts of *Cannabis sativa*. They have reported that the essential oil had a good radical scavenging activity against the DPPH radical but was inactive against the $ABTS^{\bullet+}$ radical (Zengin et al., 2018).

This method showed that the extract has very good antioxidant activity, with IC_{50} = 0.068 ± 0.004 mg/mL, comparable to that of ascorbic acid used as a standard (IC_{50} = 0.053 ± 0.003 mg/mL).

Table 1. Total polyphenolic content, total flavonoid content, and total hydroxycinnamic derivatives content

TPC (mg GAE/ g)	**TFC (mg QE/ g)**	**HDC (mg RAE/ g)**
13.6 ± 1.3	7.25 ± 0.8	3.1 ± 0.3

* results are expressed as mean ± standard deviation (n = 3)

Table 2. Anion composition in the extract

No.	Retention time (min)	Name	Concentration ppm
1	3.33	Fluoride	1.895
2	3.52	n.a.	n.a.
3	3.68	n.a.	n.a.
4	4.21	n.a.	n.a.
5	5.55	Chloride	11.13
-	n.d.	Nitrite	n.d.
-	n.d.	Bromide	n.d.
6	10.12	Nitrate	3.919
7	12.95	Phosphate	4.788
8	15.00	Sulfate	2.087
9	17.30	n.a.	n.a.

n.a. - not analyzed, n.d. - not detected

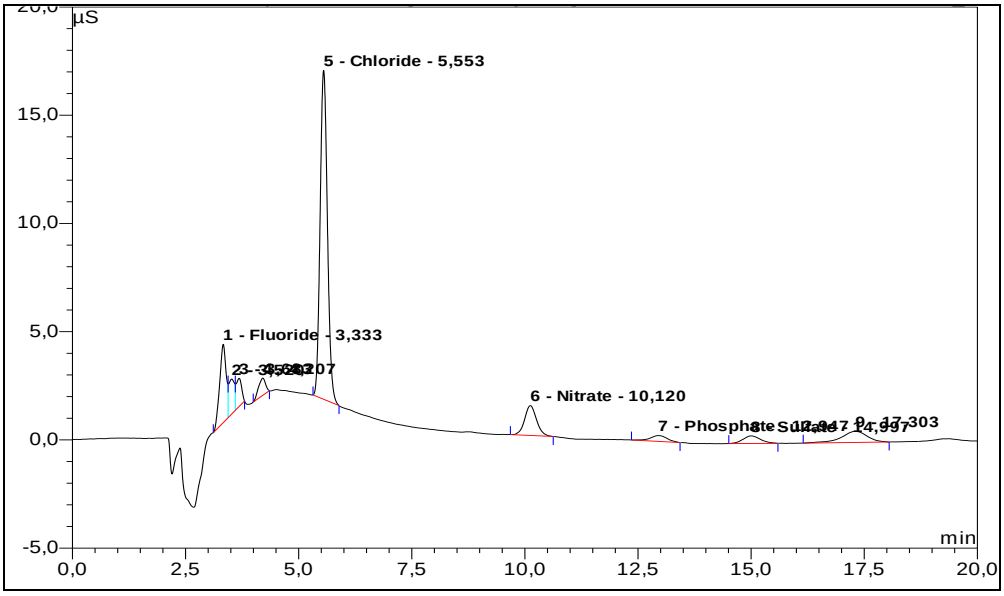

Fig. 5. IC analysis of *Cannabis sativa* extract

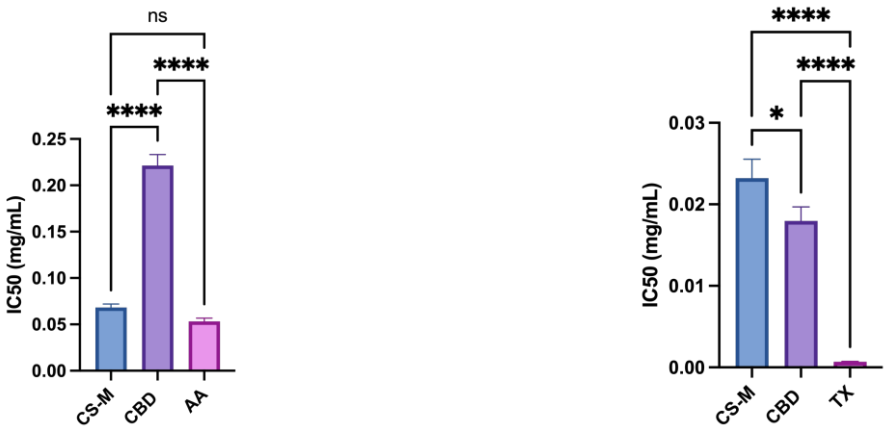

Fig. 6. DPPH and ABTS results; CS - M - *Cannabis sativa* methanolic extract, CBD - cannabidiol, AA - Ascorbic acid, TX - Trolox; *statistically significant difference at $p < 0.05$; ****statistically significant difference at $p < 0.0001$; ns - no significant difference at $p < 0.05$

α-Amylase inhibition capacity

α-Amylase is an enzyme that catalyzes the hydrolysis of starch to maltose and is used as a target for delaying postprandial hyperglycemia in diabetic patients. Usually, the action of these enzyme inhibitors is dose-dependent, and the treatment can reduce the glycated hemoglobin (HbA1c) (Agarwal amd Gupta, 2016; Rosak and Mertes, 2012). An impressive number of natural compounds were found to have the ability to inhibit one of the two enzymes involved in carbohydrate metabolism (Sales et al., 2012). There are only a few studies that have evaluated the α-amylase inhibitory capacity of *C. sativa* extracts, and the results are inconclusive (Hourfane et al., 2023; Shah et al., 2020).

For this assay, the concentration that inhibits 50% of the enzyme's activity (IC_{50}) was calculated from the dose-response curve by plotting the IC% against the logarithm of the concentration. The extract had an IC_{50} of 1.058 ± 0.072 mg/mL, while acarbose had a lower statistically significant IC_{50} of 0.063 ± 0.002 mg/mL at $p < 0.05$. The herbal drug proved to have a minor-moderate effect on α-amylase which excludes a possible application in the prevention of carbohydrate absorption.

Conclusions

In conclusion, the results of the present investigation infer that the *Cannabis sativa* extract possesses potent antioxidant activity, although the concentration of polyphenols, flavonoids, and cinnamic acids is low. Although our obtained results indicate the possibility of utilization of this product as a source of natural antioxidants, further studies are needed to evaluate the safety of this herbal drug.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgement

This work was supported by the George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureș, Research Grant number 163 /7/ 10.01.2023.

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Acta Biologica Marisiensis

VALORIZATION OF THE MEDICINAL PLANT *VACHELLIA HORRIDA (L.)* KYAL. & BOATWR. THROUGH CHEMICAL AND BIOLOGICAL EVALUATION OF ITS EXTRACTS

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Received: 1 August 2023; **Accepted:** 20 May 2024; **Published:** 30 June 2024

Abstract: This study aims to enhance the value of the medicinal plant *Acacia horrida* (synonym of *Vachellia horrida*) by evaluating its chemical and biological properties, specifically focusing on its antioxidant and enzymatic activities. Three extraction methods, namely ultrasonication, maceration, and microwave-assisted extraction, were employed, utilizing various solvent systems to extract bioactive molecules, quantify total polyphenols and flavonoids, and measure antioxidant, anti-inflammatory, antidiabetic, and antimicrobial activities. The antioxidant activity was assessed using several tests, including DPPH and ABTS radical scavenging assays, as well as the CUPRAC and FRAP methods for measuring reducing power. The radical scavenging tests proved to be the most effective. Phytochemical screening of the extracts revealed an abundance of secondary metabolites, particularly total polyphenols and flavonoids, in the ethyl acetate (AE) extract, which likely accounts for its potent antioxidant activity. The study confirms the efficacy and high yield of the ultrasonication method compared to maceration and microwave-assisted extraction, as mentioned earlier. On the other hand, the extracts from this plant exhibited no significant antidiabetic or anti-inflammatory activities. However, they did demonstrate antibacterial activity, with the dichloromethane (DC) and butanolic (BU) extracts displaying notable effects.

Keywords: *Acacia horrida*, antioxidant activity, biological activities, total polyphenols, flavonoids, extraction

1. Introduction

Woody plants, especially trees, captivate our interest with their stems, trunks, canopies, and branches, collectively encompassing their aerial parts. Their majestic beauty and ornamental richness often leave a profound impression on humans, drawing immediate attention to their overall appearance (Zhao et al., 2015; Heywood, 1996; Spichiger et al., 2002). Shrubs, being woody plants with entirely lignified stems branching from the base, manifest in various forms, including single-stemmed, multi-stemmed, or bushy types (Belot, 2004). When a woody plant fails to reach a height of at least 3 meters due to multiple stems or small size, it is classified as a shrub (El Amin, 1990). Epidemiological, *in*

vitro, *in vivo*, and clinical studies have demonstrated that a plant-rich diet can reduce the risk of certain degenerative diseases, such as diabetes, obesity, cardiovascular complications, and cancer (Bruneton, 1999; Gaston, 2016; Afonso et al., 2007). For instance, research indicates that approximately 20 to 50% of all cancer cases can be prevented through plant-based diets (Glade, 1999).

One such widespread shrub across the globe is *Acacia* genus, comprising approximately 1200 species within the Fabaceae family, belonging to the tribe Acacieae. *Acacia* species hold significance in nutrition, ethnopharmacology, and offer potential therapeutic uses (Halliwell, 1999; Subhan et al., 2018; Habellah et al., 2016). Notably, 152 chemical constituents have been identified from Acacia species, primarily concentrated in the leaves, pods, and stem bark, with flavonoids, terpenoids, and phenolic acids being the major isolated compounds. These bioactive compounds exhibit diverse pharmacological activities, such as antibacterial, antifungal, antioxidant, and anticancer effects (Amoussa et al., 2020; Ghedira, 2005).

Studies have revealed that *Acacia* plants foliage presents highly nutritious fodder, particularly rich in proteins. In Algeria, five species belonging to the Fabaceae family, *A. nilotica* (synonym of *Vachellia nilotica*), *A. horrida* (synonym of *Vachellia horrida*), *A. saligna*, *Faidherbia albida*, and *Albizia julibrissin* collected from arid and semi-arid zones demonstrated high protein contents (157-252 g/kg dry matter), with *A. horrida* exhibiting the highest content (551 g/kg dry matter) (Kadi and Zirmi-Zembri, 2016). Consequently, the leaves of these forage shrubs present a promising alternative feed source for ruminant animals, considering the forage deficits in Algeria and the rising costs of raw materials used in concentrated feeds (Mebirouk-Boudechiche et al., 2014). Furthermore, the *Acacia* genus is renowned for its abundance of secondary metabolites. Studies on ten *Acacia* species reported the presence of seventy-six (1-76) molecules, including triterpenes, saponins, flavonoids, polyphenols, tannins, among others (Hussain, 2019).

The objective of this study is to valorize the species *A. horrida*, for which limited data are available concerning its biological properties. Indeed, the research aims to extract bioactive molecules using innovative methods, such as the ultrasonication and microwave-assisted extraction techniques, rather than the conventional approach. Additionally, this study intends to develop biological activity tests, including antioxidant activities, anti-inflammatory activity, antibacterial activity and antidiabetic activity. By pursuing these objectives, we aim to shed light on the potential medicinal properties of *A. horrida* and contribute valuable insights to its biological profile.

2. Materials and methods

The study was conducted at the Biotechnology Research Center (CRBT), in Constantine province, Algeria

2.1. Plant material preparation

In this study, the aerial part, particularly the leaves, of *A. horrida* was used. The plant was collected on March 2023 from Constantine Province of Algeria. The species identification was performed at the Laboratory of Molecule Synthesis and Characterization, CRBT Constantine. The aerial part was cleaned with tap water to remove dust and other contaminants then it was oven dried at 37°C for twenty-four hours. After drying, it was ground into a fine powder using an electric grinder (E8130 Waring blender) **(Fig. 1.)** and stored in small glass jars in dark at 4°C until further use. The obtained powder was subjected to different extraction methods.

Fig. 1. Preparation of *Acacia horrida*

Fig. 2. Different Erlenmeyer flasks prepared for each extraction method

2.2. Extraction Methods

Different extraction methods were used for the extraction of plant essences, namely: solid-liquid extraction by maceration, extraction by ultrasonication and microwave-assisted extraction. For each method, a mass of approximately 50g was immersed in a hydoalcoholic solution (ethanol-water), and then placed in Erlenmeyer flasks **(Fig. 2.)**.

2.2.1. Solid-liquid extraction by maceration

Maceration is a process that involves letting a solid substance soak in a cold liquid to extract soluble compounds or to absorb the liquid to obtain its fragrance or flavor, preserve it, or allow it to decompose. Ethanol is often used to extract phenolic compounds.

A mass of 50g ground plant material were subjected to maceration in 300 ml of a hydroalcoholic solution (ethanol + water). The mixture was mechanically stirred for 24 hours.

After vacuum filtration, two additional extractions on the residue were performed. Subsequently, the ethanolic extract was evaporated under vacuum using a rotary evaporator (BUCHI) at a temperature of 38°C. The mixture was then dissolved in an ultrasonic bath (Elmasonic S70 H) , and the crude extract was collected in a petri dish and dried in an oven (ETUVE MEMMERT UF160 - 161L) at 37°C until all the methanol was evaporated.

2.2.2. Extraction by ultrasonication

Ultrasonic extraction is a rapid, cost-effective, simple, and efficient alternative method that offers numerous advantages in terms of yield, selectivity, time, and energy.

A quantity of 50g of ground plant material was placed in an Erlenmeyer flask and

macerated in 200 ml of a hydroalcoholic solution.

The mixture was allowed to sit for 24 hours. The Erlenmeyer flask was then placed in an ultrasonic extractor and subjected to extraction for 30 minutes. The extract was subsequently evaporated under vacuum at 38°C until the solvent was completely evaporated. Finally, the extract was recovered and dried at 37°C in an oven.

2.2.3. Microwave-assisted extraction

In this method, a microwave oven was used, and the following procedure was adopted: In a beaker, an equal-volume hydroalcoholic mixture (320 ml ethanol + 80 ml H_2O) was added to 70 g of the sample. The experimental setup was then placed in a microwave oven (Samsung ME73A) and subjected to microwave irradiation for 90 seconds at a pressure of 600 mbar.The mixture was recovered, followed by vacuum filtration. Subsequently, the extract underwent evaporation under vacuum at 38°C until the solvent was completely evaporated. Finally, the extract was collected and dried at 37°C in an oven.

2.3. Estimation of total phenols and flavonoids content

2.3.1. Estimation of total phenols content (TPC)

The determination of polyphenols content was carried out according to the method described by (Muller et al., 2012), on a96-well microplate, a volume of 20 µl of extract (1 mg of extract dissolved in 1 ml of methanol) is added to 100 µl of diluted FCR. Then, 75 µl of sodium carbonate (7.5%) is added, and the mixture is kept in the dark for 2 hours at room temperature. The absorbance is measured at 765 nm. A blank is prepared in the same manner by replacing the extract with the solvent used (Methanol). Gallic acid is used as a positive control, and a calibration curve is established based on it.

2.3.2. Estimation of total flavonoid content (TFC)

The test was conducted by using a 96-well microplate according to the method described by (Khlifi et al., 2013), a volume of 1 ml of each extract was mixed with an equal volume of aluminum trichloride (AlCl3) in methanol (2%). (The volumes were scaled down by a factor of 10.) The mixture was kept in the dark for 15 minutes at room temperature. The absorbance was read at 415 nm. A blank was prepared in the same manner by replacing the extract with the solvent used (Methanol). Quercetin was used as a positive control, and a calibration curve was established based on it.

2.4. *In vitro* biological activities assessment of *Acacia horrida* extracts

Evaluation of biological activities was conducted using a PerkinElmer 96-well microplate reader with EnSpire software.

2.4.1. Antioxidant activity

The antioxidant activity of extracts of the species *Acacia horrida* was carried out using various methods: DPPH free radical scavenging, ABTS radical scavenging, Ferric Reducing Antioxidant Power (FRAP), and copper-neocuproine complex (CUPRAC).

2.4.1.1. Free radical scavenging test of DPPH

The test was carried out according to the method described by (Bloi, 1958). On 96-well microplate, a solution of 160 µl of DPPH was mixed with 40 µl of the extract at different concentrations. The mixture was kept at room temperature for 20 minutes, and the absorbance was measured at 517 nm. Ascorbic acid and trolox were used as standards.

2.4.1.2. Test of ABTS radical scavenging

The test was carried out according to the method described by (Re et al., 1999), a concentrations, the absorbance of the reaction medium was measured at 734 nm after 10 minutes of incubation. Ascorbic acid and trolox were used as standards, and their absorbance was measured under the same conditions as the samples.

2.4.1.3. Reducing power activity (FRAP)

The reducing power of iron (Fe^{3+}) was determined using the method described by (Oyaizu, 1986). This method is based on the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) by antioxidants, resulting in a blue color formation. In this assay, a volume of 10 µl of the extract at different concentrations was mixed with 40 µl of a phosphate buffer solution (pH 6.6) and 50 µl of a potassium ferricyanide solution (1%). The mixture was incubated at 50°C for 20 minutes. After incubation, 50 µl of 10% trichloroacetic acid (TCA) was added to stop the reaction. Finally, 40 µl of distilled water and 10 µl of a ferric chloride solution (0.1%) were added.

The absorbance of the reaction medium was measured at 700 nm. Ascorbic acid and trolox were used as standards, and their absorbance was measured under the same conditions as the samples.

2.4.1.4. Copper-neocuproine reducing power test (CUPRAC)

The CUPRAC method is based on the reduction of the Copper-Neocuproine complex in the presence of an antioxidant. It is determined according to the method of (Apak et al., 2014).In a 96-well microplate, 40 µl of the sample was added to a mixture containing 60 µl of ammonium acetate buffer, 50 µl of Neocuproine, and 50 µl of copper chloride ($CuCl_2$). After one hour of incubation, the absorbance was measured at 450 nm. Ascorbic acid and trolox were used as standards, and their absorbance was measured under the same conditions as the samples.

volume of 160 µl of ABTS was added to 40 µl of the extract diluted in methanol at various

2.5. Antidiabetic activity (alpha-amylase inhibition)

The inhibitory activity of alpha-amylase was determined using the method of (Zengin et al., 2014) with some modifications. In a 96-well microplate, a volume of 25 µl of the extract at different concentrations was mixed with 50 µl of alpha-amylase solution (1U/ml), then incubated for 10 minutes at 37°C. Subsequently, 50 µl of starch solution (0.1%) was added to the mixture, which was incubated again for an additional 10 minutes at 37°C.

After incubation, 25 µl of hypochlorous acid (1M) and 100 µl of potassium iodide solution were added. The absorbance was measured at 630 nm. Acarbose was used as a standard reference for comparison.

2.6. Anti-inflammatory activity

The anti-inflammatory activity was determined by thermal denaturation of BSA (Kandikattu et al., 2013) with some modifications. A volume of 100 µl of each extract was mixed with 100 µl of Bovine Serum Albumin solution (0.2%) prepared in Tris-HCl buffer (50 mM, pH 6.6). The mixture was allowed to stand for 15 minutes at 37 °C then heated in a water bath at 72 °C for 5 minutes. Absorbance was recorded at 660 nm after cooling to room temperature. Sodium diclofenac was used as a standard.

2.7. Antibacterial activity

The antibacterial activity was determined using the disc diffusion method, known as the aromatogram technique. This involves placing discs soaked in the extract on a solid medium

inoculated with bacterial strains through swabbing, to assess the sensitivity or resistance of the strains to these compounds. The five tested extracts were: methanolic extract, dichloromethane, ethyl acetate, butanolic, and sonication-derived extract.

2.7.1. Pathogenic microbial strains

The antibacterial activity of *Acacia horrida* extracts was assessed against: *Escherichia coli ATCC* 25922 and *Staphylococcus aureus ATCC* 25923.

2.7.2. Application of discs and incubation

Discs of 6 mm diameter made from Wattman paper soaked with the extracts were placed on the surface of Mueller Hinton agar inoculated with the tested bacteria using sterile forceps. Similarly, discs soaked with methanol-water solvent (negative control) and discs of the antibiotic cefotaxime 30 µg (positive control) were placed. Petri dishes are left for 1 hour at room temperature for pre-diffusion, then incubated at 37°C for 24 hours.

2.7.3. Reading and interpretation

Results are interpreted by measuring the zones of inhibition after 24 hours of incubation, represented by a clear halo formed around each disc. The results are expressed according to four levels of activity **(Table 1.)**.

2.8. Statistical analyses

The *in vitro* test results were expressed as the mean ± standard deviation of three trials. The IC50 (inhibition concentration at 50%) and A0.50 (concentration indicating 0.50 of absorbance) values were calculated using linear regression. Multiple comparisons and significance rates were determined by the Tukey's honestly significant difference (HSD) test and univariate ANOVA. Differences were considered statistically significant at a threshold of 0.05.

3. Results

3.1. Total phenols and flavonoids content

The values of total phenols and flavonoids content expressed in (µg/mg extract) of the six extracts of *Acacia horrida* plant: EC: classical extract, ES: sonication extract, EM: microwave extract, DC: dichloromethane extract, AE: ethyl acetate extract, BU: butanol extract, are represented in **Table 2**.

The total phenolic compouns content **(Fig. 3.)**, demonstrate that the highest total phenolic content is recorded for the ethyl acetate extract (435.99±0.76 µg/mg), followed by the sonication extract (SE) (240.63±0.57), and microwave extract (ME) (223.96±1.07). The classical extract (CE) displayed a relatively low value of phenolic compounds (129.54±3.8), while the butanolic extract (Bu) showed the lowest content of phenolic compounds.

The results of flavonoid contents for the various extracts illustrated in table 2 show average values. The highest value is recorded for ES (70.68±3.43) and the lowest for EC **(Fig. 4.)**. Both BU and DC extracts do not contain flavonoids.

3.2. Assesment of biological activities

3.2.1. Antioxidant activity

3.2.1.1. DPPH Test

The antioxidant effect of the studied extracts, represented by their IC_{50} values, shows that the EA has a strong antioxidant power with a value of (5.44±1.28 µg/ml), close to that of the tested standard, Trolox. followed by SE (16.9±0.70 µg/ml), EC (25.96±3.57µg/ml), ME (30.57±0.27 µg/ml), and BU (69.48±1.82µg/ml). The DC extract recorded the lowest antioxidant power (>800 µg/ml) **(Fig. 5.)**.

Table 1. Sensitivity of microbial strains based on inhibition zones (Pouce et al., 2003)

Sensitivity	**Inhibition Zone**
Not sensitive or resistant (-)	Diameter < 8 mm
Sensitive (+)	Diameter between 9 and 14 mm
Very sensitive (++)	Diameter between 15 and 19 mm
Extremely sensitive (+++)	Diameter > 20 mm

Table 2. Total polyphenol and flavonoid contents of the 6 extracts of *Acacia horrida*

Extract	**Phenolic compounds content (µg EAG/ mg extract)**	**Flavonoids compounds content (µg EQ/ mg extract)**
Classic Extract (CE)	129.54±3.8	28.86±2.62
Sonication extract (SE)	240.63±0.57	70.68±3.43
Microwave extract (ME)	223.96±1.07	59.39±3.41
Dichloromethane extract (DC)	-	-
Ethyl acetate extract (EA)	435.99±0.76	47.04±3.15
Butanol extract (BU)	58.02±3.41	-

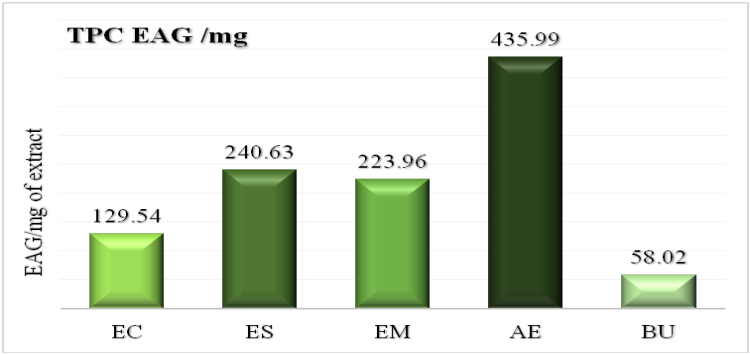

Fig. 3. Total phenolic compound content

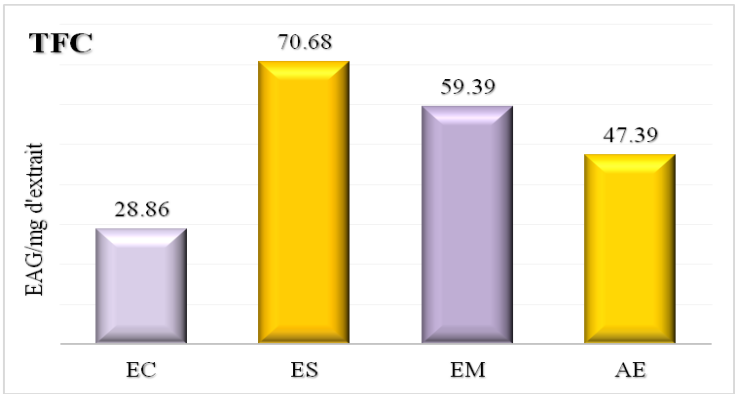

Fig. 4. Total Flavonoid Content

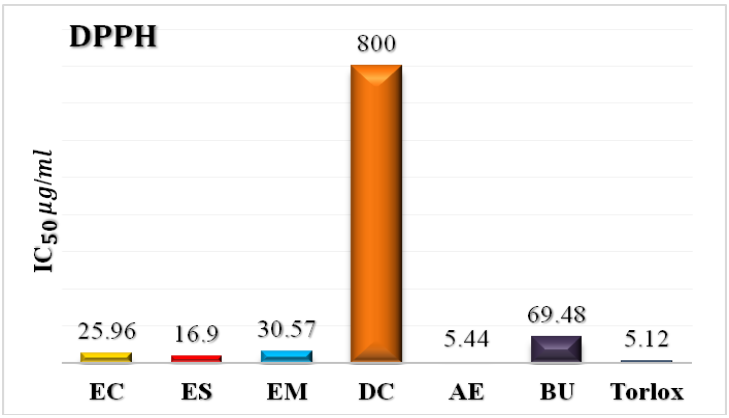

Fig. 5. IC_{50} Values of the Five Extracts of *Acacia horrida* and Trolox for the DPPH Test

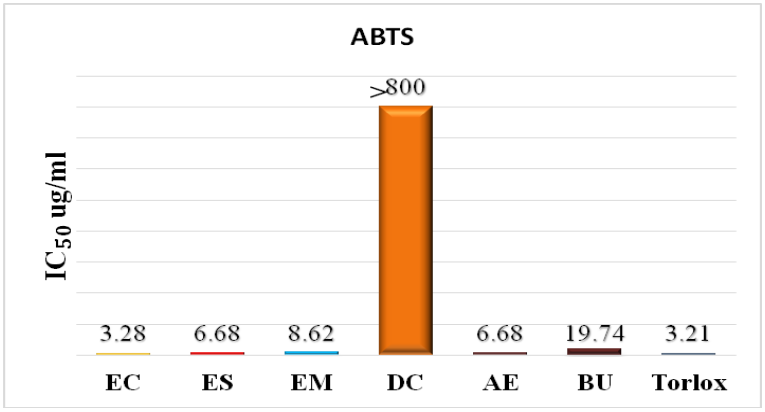

Fig. 6. IC_{50} values of the five extracts of *Acacia horrida* and Trolox for the ABTS test

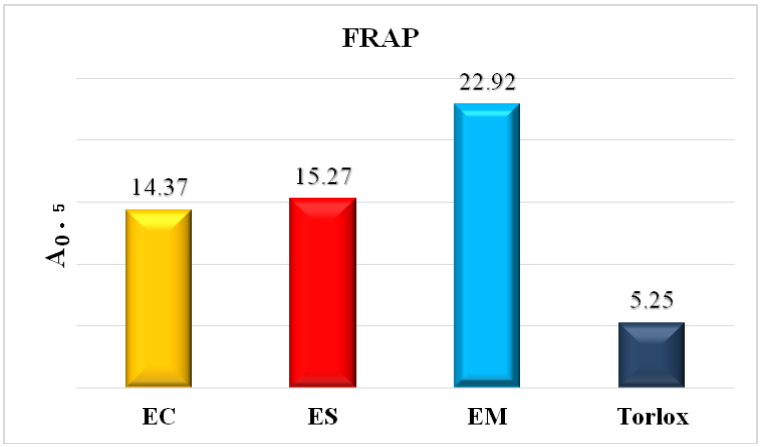

Fig. 7. $A_{0.5}$ values of the extracts compared to the standard for the FRAP test

3.2.1.2. The ABTS radical scavenging test

The results of the antioxidant power by ABTS radical scavenging of the studied extracts, represented by their IC_{50} values **(Fig. 6.)**, revealed that the CE, SE, EA, and ME extracts have a powerful antiradical effect close to that of Trolox.The BU extract shows a relatively weak effect compared to the other extracts, while the DC extract exhibits the lowest activity (> 800 µg/ml).

3.2.1.3. Ferric Reducing Power Test

The results expressed in terms of $A_{0.5}$ (µg/ml) are represented in **Figure 7**.

The three extracts SE,CE,ME show values close to each other but slightly lower compared to Torlox. The extract closest to Torlox is EC.

3.2.1.4. Copper Reduction Antioxidant Capacity (CUPRAC) Test

The $A_{0.5}$ values are of the studied extracts are illustrated in **Figure 8**.

The three tested extracts exhibited weak ferric reducing activities lower than that obtained by Torlox.

3.2.2. Antidiabetic activity

Alpha-amylase is a digestive enzyme that catalyzes the hydrolysis of α-D-glucose polymers at α (1-4) linkages. This enzyme is present in the pancreas and plays an essential role in the digestion of starch present in the food bolus. According to the comparative histogram **(Fig. 9.)**, all tested extracts of *Acacia horrida* show no antidiabetic effect compared to the standard Acarbose.

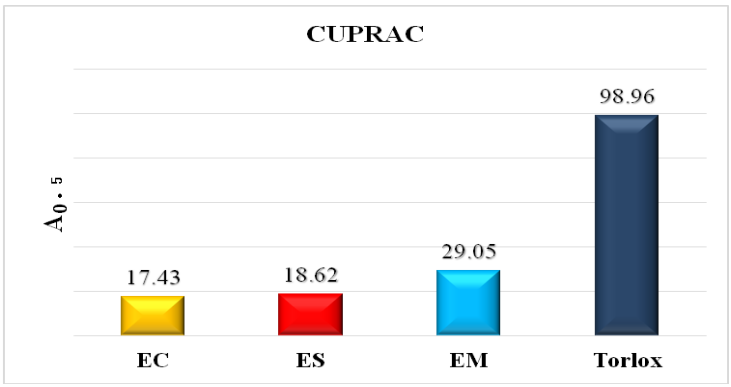

Fig. 8. $A_{0.5}$ values of the extracts compared to the standard for the CUPRAC test

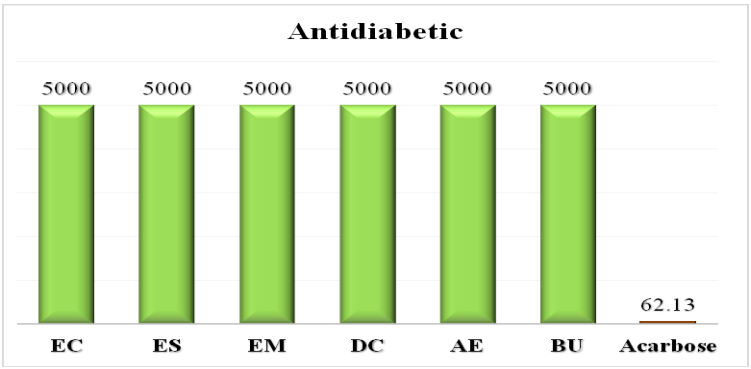

Fig. 9. Comparative histogram of IC_{50} values of the six extracts of *Acacia horrida* with the standard Acarbose

3.2.3. Anti-inflammatory activity

The anti-inflammatory activity was measured relative to the anti-inflammatory standard DICLOFENAC **(Fig. 10)**. According to the results, all extracts of *Acacia horrida* did not exhibit anti-inflammatory activity; the values of the extracts were significantly different from those of the standard Diclofenac.

3.2.4. Antimicrobial activity

Five extracts were tested of their antimicrobial power: CE, DC, EA, BU, SE. Only the extracts DC and BU exhibited clear halos of 9 and 7 mm respectively formed around the discs **(Fig. 11)**.

BU exhibited antimicrobial activity against the bacterium *Staphylococcus aureus ATCC* 25923 and DC showed antimicrobial activity against bacterium *Escherichia coli ATCC* 25922.

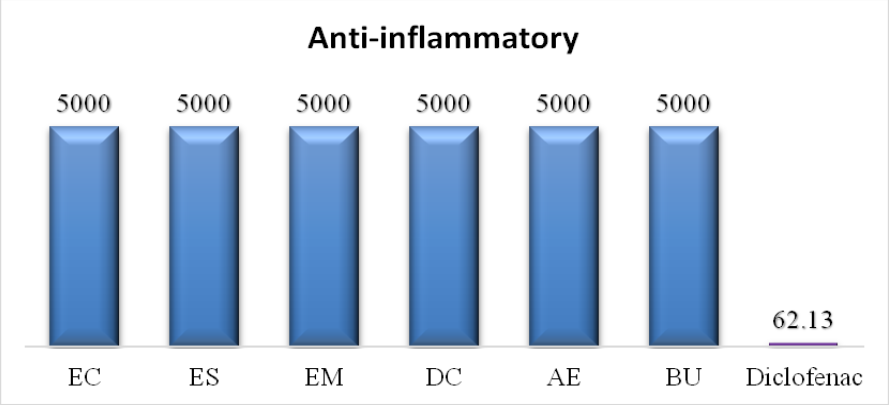

Fig. 10. Comparative histogram of IC50 values of the six extracts of *Acacia horrida* with the standard DICLOFENAC

A. B.

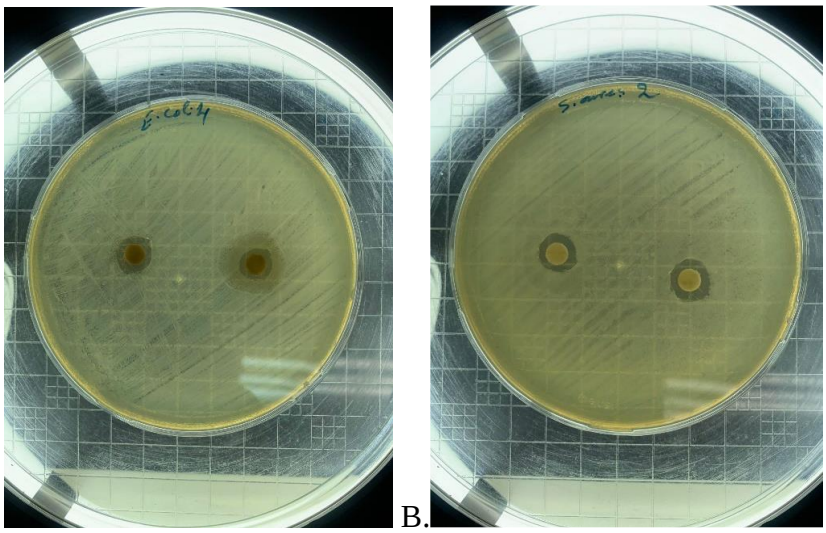

Fig. 11. Antimicrobial activity against of Dichloromethane extract against *Escherichia coli* ATCC 25922 **(A)**; antimicrobial activity of Butanol extract against *Staphylococcus aureus* ATCC 25923 **(B)**

4. Discussion

The results obtained from the study on the antioxidant activity of *A. horrida* extracts provide valuable insights into the potential health benefits of this plant. The study utilized various antioxidant assays, including DPPH, ABTS, FRAP, and CUPRAC, to evaluate the ability of the extracts to scavenge free radicals and exhibit antioxidant properties. The DPPH test revealed that ethyl acetate (EA) extract demonstrated the highest radical scavenging activity, with an IC_{50} value of 5.44 µg/ml, comparable to the standard antioxidant Torlox. This suggests that AE extract has a strong ability to neutralize free radicals, making it a promising candidate for further research as a natural source of antioxidants. The antioxidant effect observed for AE can be attributed to its high concentration of phenolic compounds, known for their antioxidant properties.

On the other hand, the BU and DC extracts have low phenols content, showed relatively low antioxidant activity, confirming that phenolic compounds are responsible for the observed antioxidant effects. Similarly, in the ABTS assay, the ethyl acetate (AE) extract demonstrated significant antioxidant capacity, with an IC_{50} value of 6.68 µg/ml. This confirms the potential of the AE extract as a potent scavenger of ABTS radicals. The EC, also exhibited a strong antioxidant activity, closely approaching the IC_{50} value of Torlox. SE and ME showed also a significative activity. The variation in antioxidant effectiveness among extracts may arise not only from variations in concentration but also from differences in the composition of phenolic compounds obtained through distinct extraction techniques (Rayan and Elfadil, 2019).

Moving on to the FRAP assay, the EC extract stood out with a notable reducing power, with an $A_{0.5}$ value of 3.28 µg/ml, which was almost equivalent to that of the standard Torlox (3.21 µg/ml). This suggests that the EC extract contains potent electron-donating compounds that contribute to its strong reducing capacity. On the other hand, the BU extract exhibited weaker reducing power, indicating a lower concentration of electron-donating compounds in this extract.

The CUPRAC assay also provided insights into the antioxidant potential of the extracts. The EC extract demonstrated the highest antioxidant activity, closely approaching that of the standard. This reinforces the idea that the EC extract is rich in compounds capable of copper reduction. The other extracts, particularly AE and ES, also showed antioxidant capacity, albeit at slightly lower levels compared to EC. However, all the tested extracts exhibited reducing abilities distinct from that of Torlox, indicating that they might contain a different profile of antioxidant compounds.

Regarding the antidiabetic and anti-inflammatory activities, the results were less promising, as all the extracts showed minimal effects compared to the respective standards (acarbose and diclofenac). This suggests that the tested *A. horrida* extracts might not possess significant antidiabetic or anti-inflammatory properties under the study conditions. Further investigations are warranted to explore the potential of these extracts in other experimental models or in vivo studies.

The antimicrobial activity of the extracts was assessed against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Among the extracts, DC and BU demonstrated antimicrobial potential, with clear zones of inhibition around the discs. This indicates that these extracts possess compounds with antibacterial properties against the tested strains, making them potential candidates for further exploration as natural antimicrobial agents.

In conclusion, the results of this study highlight the promising antioxidant and antimicrobial activities of *A. horrida* extracts, particularly the ethyl acetate (AE) and ethyl chloride (EC) extracts. These extracts exhibited significant radical-scavenging and reducing abilities, which could be attributed to their high polyphenolic content. However, further investigations are required to fully understand the chemical composition and potential health benefits of these extracts. Additionally, the limited antidiabetic and anti-inflammatory effects suggest that the plant's therapeutic potential in these areas may be limited or influenced by other factors, which necessitates more in-depth research. Overall, this study contributes to the growing body of knowledge on the potential applications of *A. horrida* extracts in the field of natural antioxidants and antimicrobials.

Conclusions

Acacia horrida, a plant belonging to the Fabaceae family of leguminous shrubs found in arid or semi-arid regions of Algeria, has garnered significant research interest.

In this study, six extracts were prepared using different extraction methods and solvent systems to evaluate various biological activities: EC: classical extract, ES: sonication extract, EM: microwave extract, DC: dichloromethane extract, AE: ethyl acetate extract, BU: butanol extract. Quantitative analysis of total polyphenols using the Folin-Ciocalteu method revealed that the AE extract exhibited the highest polyphenol content, followed by ES, EM, EC, and BU extracts. Similarly, the quantification of total flavonoids using the aluminum chloride method demonstrated that the ES extract had the highest flavonoid content, followed by EM, EC, and AE extracts.

The results indicate that the majority of polyphenols and flavonoids in *A. horrida* are moderately polar and soluble in ethyl ether. Among the extraction methods used, ultrasonication proved to be the most efficient compared to maceration and microwave-assisted extraction. Regarding antioxidant activity, the AE extract displayed a robust antioxidant capacity, likely attributed to its rich polyphenol content. Among the tests employed, the DPPH and ABTS radical scavenging assays were the most effective, with the extract values approaching those of the standard. Concerning biological activities, no significant anti-inflammatory or antidiabetic effects were observed in any of the extracts. However, both DC and BU extracts demonstrated antimicrobial (specifically antibacterial) activity against the pathogenic bacteria *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, respectively. The strength of this antibacterial activity should be further specified in subsequent studies.

Based on these results, the plant extract, could serve as a valuable natural source of antioxidants and antibacterial agents. For future research prospects, the following suggestions are proposed:

- Conduct *in vivo* preclinical studies to validate the in vitro findings.
- Explore the potential use of the studied extracts as antioxidants in agri-food and cosmetic formulations.
- Investigate other parts of the plant, such as flowers and seeds, for potential bioactive compounds.

In conclusion, this study contributes to the understanding of *A. horrida* plant’s medicinal potential and opens avenues for further research on its applications in health and industry.

Acknowledgement

We express our heartfelt gratitude to the Biotechnology Research Center (CRBT) in Constantine province, Algeria, for their invaluable support and collaboration throughout this research. Their expertise and assistance in conducting the experiments were instrumental in the successful completion of this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Acta Biologica Marisiensis

EPIPACTIS TALLOSII A. MOLNÁR & ROBATSCH. (ORCHIDACEAE) IN TRANSCARPATHIA (UKRAINE): NEW DATA ON DISTRIBUTION AND ECOLOGY

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Received: 27 October 2024; **Accepted:** 24 November 2024; **Published:** 30 December 2024

Abstract: In this publication, generalized information on the geographical distribution and ecological affiliation of *Epipactis tallosii* A. Molnár & Robatsch. (Orchidaceae) in Ukraine, and the state of its populations is analysed. In 2016, the species was first observed in Transcarpathia, in the vicinity of the village Nove Selo. Later, in 2020-2021 we found three new localities in the old floodplain willow-poplar forest (association *Salici-Populetum* (Tx. 1931) Meijer-Drees 1936) on the banks of the Borzhava river near villages of Bene and Kvasovo (Beregovo district). The article describes the ecology and habitat of the species in the study region.

Keywords: *Epipactis tallosii*, Flora of Ukraine, Orchidaceae, Transcarpathia

1. Introduction

Woody *Epipactis tallosii* A. Molnár & Robatsch (Orchidaceae) is an autogamous (self-pollinated) and mixotrophic species with rhizomes, which is found in a variety of habitat types. In Hungary, for example, it was common in gallery forests, lowland oak and oak-hornbeam forests and in the more hilly regions, as well as in poplar plantations. (Molnár 2009; Süveges et al., 2019). *E. tallosii* A. Molnár & Robatsch is one of the latest flowering species of its genus and recently, generative individuals have been identified even in October (Molnár 2011). The species was originally described from Hungary (Molnár & Robatsch 1997), then it was confirmed from the Czech Republic and the South of Slovakia where the species was reported to be known from the mid 1990-s (Vlčko 1997; Mereďa 2002; Kolnik & Kucera 2002; Molnár 2011). However, it was not identified until 1997. During 2012-2016, new localities of the plant were found in Ukraine, Romania, Serbia, and Croatia (Süveges et al. 2019). Thus, the species is currently known in seven countries: Hungary, the Czech Republic, Slovakia Serbia, Croatia, Romania and Ukraine.

In the most recent IUCN Red List, *E. tallosii* A. Molnár & Robatsch. is listed as one of the most threatened orchid species in Europe, and is classified as Endangered (EN) at European level (Fay 2011). *E. tallosii* A. Molnár & Robatsch. has been classified by the IUCN categories as Critically threatened (C1) in the Czech Republic (Holub & Procházka 2000; Grulich 2012), as near threatened (NT) in the Carpathian part of Slovakia (Eliáš et al., 2015) and in Hungary (Király 2007). It has been already listed as Endangered (EN) in the Red Data Book of Ukraine Therefore, we suggest that the new habitat of the species is considered in the new edition of the Red Data Book of Ukraine (find data on https://zakon.rada.gov.ua/laws/show/z0370-21#Text).

In the most recent years several species of *Epipactis* Zinn. were added to the Ukrainian Flora. As of 2008, only five species of the genus were known in Ukraine: *E. atrorubens* Hoffm.ex Besser, *E. helleborine* (L.) Crantz, *E. microphylla* (Ehrh.) Sw., *E. palustris* (L.) Crantz, and *E. purpurata* Sm. (Protopopova 1987; Vakhrameva et al. 2008), but in the 2009 edition of the Red Data Book of Ukraine a new species was added to the flora: *E. albensis* Nováková & Rydlo (Didukh 2009). First, *E. albensis* Nováková & Rydlo was discovered in 1997 in Transcarpathia (Drescher et al., 2003). Later, in 2012, systematic studies conducted in the Transcarpathian Lowland confirmed the presence of the species *E. albensis* Nováková & Rydlo at three new localities in Ukraine, namely close to the village of Chetfalva, Koson', Fanchykovo and Drotyntsi of the Beregovo district within the floodplain of the river Tisza (Ljubka et al., 2014). Afterwards, the species was also found in the vicinity of Kyiv district (Tymchenko & Ljubka 2019).

By 2020, *Epipactis* genus is represented by the following species in Ukraine (in alphabetical order): *E. albensis* Nováková & Rydlo, *E. atrorubens* Hoffm.ex Besser, *E. distans* Arv.-Touv., *E. helleborine* subsp. *helleborine*, *E. helleborine* subsp. *tremolsii* (Pau) E. Klein., *E. krymmontana* Kreutz, Fateryga et Efimov., E. *leptochila* (Godfery) Godfery., *E. muelleri* Godfery., *E. microphylla* (Ehrh.) Sw., *E. palustris* (L.) Crantz, *E. persica* (Soó) Hausskn. ex Nannf., *E. purpurata* Sm., and *E. tallosii* A. Molnár & Robatsch (Didukh 2009; Efimov 2008; Kreutz & Fateryga 2012; Fateryga & Kreutz 2012; Fateryga et al. 2013; Fateryga et al.2014; Fateryga et al. 2019; Ljubka et al. 2014; Ljubka 2018; Protopopova et al. 2017; Süveges et al. 2019).

2. Materials and methods

Studies of the species were conducted during 2020-2021 in the Beregovo district of the Transcarpathian Lowlands. Herbarium materials of the M.G. Kholodny Institute of Botany NAS of Ukraine (*KW*) and the herbarium of Ferenc Rakóczi II Transcarpathian College of Higher Education (*KMF*) were analysed. Maps of species distribution were compiled with the help of Quantum-GIS software.

In studies of the species, data about associated plant species, habitat type and the size of the population were gathered.

For documentation, the species were collected of the two flowering specimens (without rhizome), which are stored in the herbarium of KMF. The identification was accomplished by comparing the plants to the description keys in the works of Molnár (2011).

The floral characteristics were measured on a single mounted flower per individual using a vernier calliper measurement tool.

The soil-sampling and the measurement of soil acidity was conducted by means of the ISO 10390:1994 IDT (Soil quality-Determination of pH) methods. We collected soil samples from

three new localities of the species in Ukraine in order to carry out soil reaction characterisation. Soil sampling and soil acidity measurements were undertaken using the methods described by Csoma (2009).

High resolution images of each morphological details were taken with DSLR camera equipped with a macro lens.

3. Results and discussions

3.1. Description and diagnostic characteristics

E. tallosii A. Molnár & Robatsch. is (9) 10-12 cm high. The stem is yellowish green; the leaves are 2-3 lanceolate or oval, with slightly undulated margins, inflorescence is near one-sided with (4) 5-8 small flowers, usually wide open but we saw exemplars with slightly opened flowers and pale-green sepals, the junction epichile-hypochile is „U" shaped, but with a very narrow transition: the hypochile nectariferous, coloured from the inside from light green to brown, the epichile is whitish to pinkish in colour, triangular-oval in shape, viscidium is well developed but non-functional. Peak flowering in Ukraine starts at the end of July - through the middle of August (**Fig. 1**).

Fig. 1. *Epipactis tallosii* Á. Molnár & Robatsch: **a.** general view; **b.** flowers (Photo: T. Ljubka, 2019, 08.09. Kvasovo)

3.2. Distribution, habitat, and populations data of *E. tallosii* in Transcarpathia

New localities of *E. tallosii* in Ukraine were found between the villages of Bene and Kvasovo K1, K2, K3 (Beregove district) in 2020-2021 years (**Fig. 2**).

E. tallosii A. Molnár & Robatsch. typically is grown in association *Salici-Populetum* (Tx. 1931) Meijer Drees 1936 (class *Salicetea purpureae* Moor 1958 alliance *Salicion albae*

Soó 1951). The typical habitat of *E. tallosii* A. Molnár & Robatsch. is represented by the association *Salici-Populetum* (Tx. 1931) Meijer Drees 1936 (*Salicetea purpureae* Moor 1958 class, *Salicion albae* Soó 1951 alliance). In the new locations for *E. tallosii*, non-typical species for this association prevails in the habitat. This is due the intensive agriculture activities outside the sites.

In the studied plants communities, the canopy cover is ca. 60%. Co-dominant species are *Populus alba* L. (30%) and *Salix alba* L (30%), with an admixture of *Populus nigra* L. In the shrub-layer the following species were identified: *Cornus sanguinea* L. (40%), *Crataegus monogyna* Jacq., *Rosa canina* L., *Sambucus nigra* L., *Vitis vulpina* L., *Rubus caesius* L., and undergrowth of *Fraxinus angustifolia* Vahl., *Juglans regia* L., *Acer negundo* L., *Acer campestre* L., *Malus domestica* L., *Padus avium* L. The herb layer has a coverage of 30-70% and in its composition we identified herbaceous vascular plants such as *Arctium lappa* L., *Ambrosia artemisiifolia* L., *Bellis perennis* L., *Circaea lutetiana* L., *Bidens tripartita* L., *Echinocystis lobata* (Michx.) Torr. & A. Gray., *Erigeron annuus* (L.) Pers., *Equisetum arvense* L., *Geum urbanum* L., *Hedera helix* L., *Humulus lupulus* L., *Lysimachia nummularia* L., *Plantago major* L., *Prunella vulgaris* L., *Stellaria media* (L.) Vill., *Sanicula europaea* L., *Taraxacum officinale* L., *Trifolium arvense* L., *Viola odorata* L., and *Urtica dioica* L.

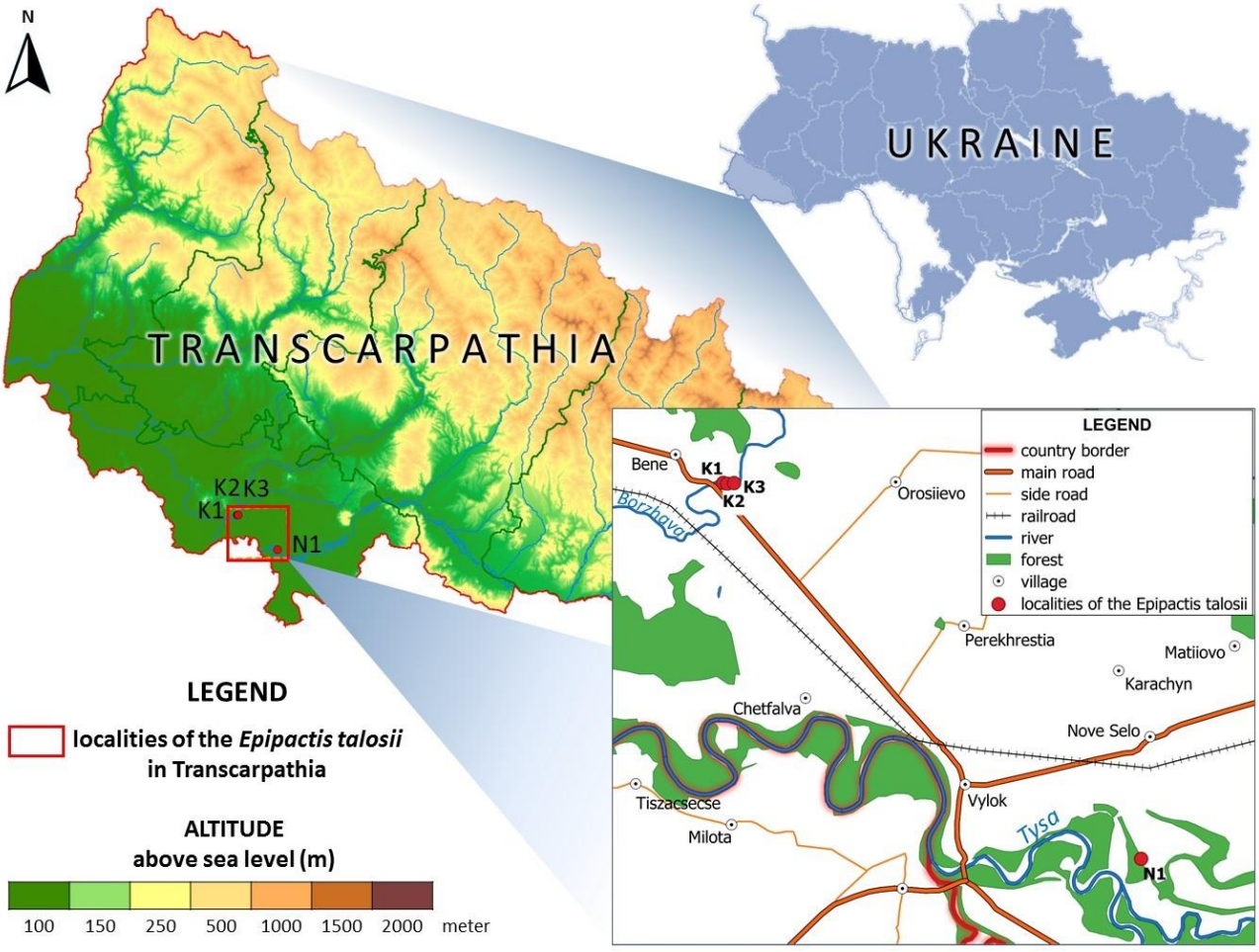

Fig. 2. Map of the distribution of *Epipactis tallosii* Á. Molnár & Robatsch in Transcarpathia

Table. 1. Localities and results of chemical analysis of soil with the presence of *E. tallosii*

Species	Locality	Altitude	Soil reaction pH_{KCl} - pH_{H2O}	GPS coordinate	Number of *E. tallosii* or individuals
E. tallosii	Nove Selo (N1)	117 m	5,5 - 7,9	48.09777, 22.88282	5 - 9
E. tallosii	Kvasovo (K1)	113 m	5,4 - 6,6	48.16299, 22.77457	2 - 5
E. tallosii	Kvasovo (K2)	113 m	6,0 - 7,4	48.16283, 22.77559	3 - 7
E. tallosii	Kvasovo (K3)	114 m	5,7 - 6,8	48.16295, 22.77738	3 - 5

Previous studies on the populations of the species reported that in Croatia the population size varies between 35-45, in Romania – 80-100, Serbia – 18-70 of individuals observed among plantations of *Populus* × *canadensis* Moench (Süveges et al. 2019). The occurrence of this species in Ukraine is restricted to the old floodplain forest of the banks of river Borzhava, and extend to a maximum of 15 m away from the river at approximately 113-117 a.s.l.

At the localities in Transcarpathia we found contrast compared to populations of this species from other countries. The new populations do not have a large number of individuals and range from 2 to 7. In our opinion, the number of individual populations depends on the hydrological regime of rivers and the total amount of average monthly precipitation in the region.

The soil measurements in three localities in Ukraine, are presented in **table 1**. The soil reaction is between pH_{KCl} – 5.4-6.0 and pH_{H2O} – 6.6-7.9. The soil test shows that the pH level of the surveyed area matches the data from Hungary. 5.5 to 8.0 pH_{H2O} (Molnár 2011). In addition, soils of river floodplains are formed during floods, leaving a floodplain alluvium, which is characterized by having acidic environment in humid conditions and neutral or basic environment in dry conditions. This is also confirmed by our results of chemical analysis of the soil.

Conclusions

Currently, 4 localities of *E. tallosii* A. Molnár & Robatsch are known from Ukraine, namely in the Transcarpathian Lowland of the Transcarpathian region. It seems that the number of populations of *E. tallosii* Barbaro A, Kreutz C.A.J (2007) *Epipactis tallosii* A. Molnár & Robatsch subsp. *zaupolensis* Barbaro & Kreutz subsp. nov. (Orchidaceae) in northeastern Italy (Friuli Venezia Giulia) has been similarly documented (Barbaro et Kreutz, 2007).

The study region is small; they occupy small areas and are often represented by single individuals, sometimes by small groups. Populations of the species are more commonly found in acidic to weakly basic soils, this is confirmed by our results. The species occurs up to 120 m above sea level, mainly in the valleys of the rivers Tisza and Borzhava, on alluvial soils. In Ukraine, populations have been found in floodplain forests dominated by species of the genus *Populus*, whose trunk circumference exceeds 80 cm, belonging to the association *Salici-Populetum*, whereas outside Ukraine the species can occur in poplar plantations and, sometimes, on hills with oak and hornbeam. Such ancient floodplain forests in the region are the main habitats of the species which is likely to be the result of favourable environmental conditions for fungi without which the growth of both *E. tallosii* A. Molnár & Robatsch and other mixotrophyc orchid species is impossible. In our opinion, new sites

of the species may be identified, the most likely habitats of the species are poplar plantations and willow poplar floodplain forests. However, further systematic research is needed. The species requires appropriate protection measures in all places where the species is present. Given the nature of the distribution, its status, we have prepared a description of the species in the Red Data Book of Ukraine. At the plant's sites harvesting, (collecting) deforestation, violation of hydrological conditions and drainage of plots is prohibited.

Acknowledgement

We are very grateful to Zsuzsanna Csoma (Ferenc Rákóczi II Transcarpathian Hungarian College of Higher Education, Ukraine) for assistance in measuring chemical analysis of soil.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Acta Biologica Marisiensis

EVALUATION OF *IN-VITRO* ANTHELMINTIC ACTIVITIES OF METHANOL LEAF EXTRACT OF *JATROPHA CURCAS* LINN ON THE EGG AND LARVAE OF *ASCARIS SUUM*

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Received: 27 October 2024; **Accepted:** 29 November 2024; **Published:** 30 December 2024

Abstract: High rates of infections and re-infections of soil transmitted helminthes (STHs) is a public health challenge perhaps due to resistance to anthelmintics in STHs endemic areas. This public health challenge necessitates renewed efforts to discovering newer agents. This study evaluated the ovicidal and larvicidal activities of methanol leaf extract of *Jatropha curcas* against *Ascaris suum.* The plant's material was collected, pulverized and extracted by cold maceration using 70% methanol, and qualitative analyzed for phytochemicals. Graded concentrations (25, 50 and 100 mg/ml) of the extract were tested for *Ascaris suum* egg's hatch inhibition (EHI) and larvae development inhibition (LDI). Distilled water and 1 mg/ml albendazole served as negative and positive controls, respectively. The extract caused a dose-dependent EHIs of 82.57, 89.44 and 92.08% inhibitions at 25, 50 and 100 mg/ml, respectively. In addition, larvae development was inhibited by 91.72, 95.52 and 98.11% at 25, 50 and 100 mg/ml, respectively. The EHI and LDI of the extract at 100 mg/ml was not significantly different (p<0.05) relative to albendazole that produced EHI and LDI of 94.01 and 99.14%, respectively. The phytochemical detected were alkaloids, glycosides, flavonoids, saponins, steroids, and tannins. *Jatropha curcas* extract demonstrated excellent anthelmintic activity justifying its uses in ethno-medicine.

Keywords: Anthelmintic, *Ascaris suum*, *Jatropha curcas*, phytochemicals

1. Introduction

There had been public outcry at the high rate of infections and re-infections of soil transmitted helminthes (STHs) especially in the rural areas with Pre-school age and school age children being the most vulnerable groups (Rahimi et al., 2022). It is estimated that about 1.5 - 2 billion people were infected with STHs globally (WHO, 2022; WHO, 2020). This staggering high figure is not only disturbing; it poses great concern to global health and the economic status of many Nations. Programs to mitigate the burden in children and other vulnerable groups such as adolescent girls and pregnant women have however been put in place. These efforts include improved environmental and personal hygiene, health

education and advocacy for the use of preventive chemotherapy intervention in areas endemic for the transmission at regular interval (Kache et al., 2020). Sequel to these, various Governmental and Non-governmental Organisations embarked on mass deworming exercises of the vulnerable groups in their respective homes and schools, routinely, using any of albendazole, mebendazole, levamisole and pyrantel pamoate or albendazole/mebendazole combination therapy (WHO, 2017; Yarinbab and Darcha, 2019). Notwithstanding the initiatives for preventive chemotherapy intervention initiatives, soil-transmitted helminth (STH) infection is still a major public health problem especially in developing countries; both the prevalence and the intensity of infections remain high, and the intervention has been insufficient in controlling STH morbidity (Yarinbab and Darcha, 2019; Ame et al., 2022). The target population was also not adequately covered which might probably be due to limited availability of effective drugs. Additional factors militating against their successful treatment are the evolution of reduction in cure rate of the available effective drugs and high rate of re-infections, contributing greatly to the development of resistance by the parasites to the drugs (Ghazanfar et al., 2024; Agne et al., 2021; Aurelie et al., 2024). The imperative to investigate alternative treatment options or novel drugs is therefore not optional but a necessity to avert the possible unpleasant consequences of their infestation which are often associated with the disorders such as malnutrition, stunted growth in children coupled with intellectual impairment, cognitive deficits and diminished school attendance, while parasitized pregnant women are anaemic and their newborn have low birth weight (Nisa et al., 2022; Ojo et al., 2023).

Plants have commonly been considered to be possible alternative and important source of new drugs as large number of secondary metabolites that may serve as reservoir for novel drugs and therapeutic agents are continuously being discovered and extracted from them (Kalpesh and Priya, 2020). Apart from this, a good proportion of the population across the globe still adopt traditional methods of treatment, using herbal extracts, which have been claimed to produce beneficial responses especially in the developing nations (Amar and Arun, 2020). These have resulted in renewed interest and efforts toward screening medicinal herbal plants that are used locally as remedies for many diseases including helminthic infections (Hassen et al., 2022).

Four species of nematodes are collectively referred to as Soil transmitted Helminthic (STH): *Ascaris lumbricoides* (round worm), *Trichuris trichiura* (whipworm), and *Necator americanus* or *Ancylostoma duodenale* (hookworms) (Sumo et al., 2021). Amongst the STH infections ascariasis caused by *A. lumbriciodes* is the most prevalence (Addisu and Mebrate et al., 2020), infecting an estimated one sixth of human population globally, with the highest rate of infection occurring in Sub-Saharan Africa, America, China and East Asia (WHO, 2022). Gonzalez Quiroz et al. (2020) in their study to determine the prevalence and intensity of STH infections, nutritional status, and anemia in children, using multistage stratified probability sampling also reported that *A. lumbricoides* and *T. trichiura* were responsible for most of the infections.

A. lumbricoides and *A. suum* which infect human and pigs respectively are two parasitic nematodes which are closely related and bear significant morphological similarities. In a molecular and morphological study of *A. suum* in a human - pig contact scenario in north-eastern Brazil, concomitant infection has been demonstrated in human and pig, suggesting zoonotic transmission cycles (Bacelar et al., 2023). The analysis of research carried out on

pig farmers in Bali province, Indonesia using a molecular testing showed that ascariasis in human was also caused by *A. suum* indicating that *A. suum* was a zoonosis (Augustina et al., 2023). A comparison of the protein profile of their body wall and reproductive organs manifested considerable similarities in banding pattern which reflect their close genetic relationship (Alba et al., 2009). Additional experimental studies suggested the possibility of cross-infection, cross-breeding and hybridization between them (Silva et al., 2021; Easton et al., 2020). An *A. suum* was removed from a 75 years old Patient with a small enterotomy and identified based on morphological and molecular analysis, suggesting that *A. suum* can function as a relevant agent of human zoonosis. The pig ascaris was therefore suggested to be important source of human ascariasis in endemic area where both human and pig live closely (Romano et al., 2021). It is therefore imperative that any effort toward the total control and eradication of ascariasis in human must not ignore other factors such as the presence of pigs in such environments. The observed evidences of strong relationship and similarities have thus strengthened the justification of the routine usage of *A. suum* as experimental research surrogate for human form, *A. lumbricoides* (Deslyper et al., 2021).

Jatropha curcas (**Fig. 1**), commonly called physic nut or purging nut, belonging to the family of Euphorbaceae is a small branched tree that is widely. It is characterised by features such as succulent smooth grey bark which when cut or its leaf plucked, exudes whitish watery latex which causes brown stains that are difficult to remove. The latex dries on hard surface to form sticky paste. It is a drought resistant perennial plant. However, it sheds most of its leaves during dry season. When fully grown, it usually attains a height of 3 to 5 meters but may exceed this under optimal soil and climatic conditions (Kamal et al., 2011; Namrata et al., 2023). Although its natural habitat is reported to be South and Central America, South-east Asia, Africa, and India, the plant is currently distributed widely across the tropical regions (Riayatsyah et al., 2021). In Nigeria, it is cultivated in all regions, with greater prevalence in the central area of the country. It is called *Binidazugu* in Hausa, *Lapalapa funfun* in Yoruba, (Adebusuyi et al., 2021) *Olulu idu* in Ibo, *Gyedan* in Tiv, it is termed *Ochigbede* in Idoma and known as *Omangba* in Igede. The root, stem-bark, leaves, seeds and fruits of the plant have been widely used in traditional folk medicine in many parts of West Africa and the world for the treatment of diverse diseases (Igbinosa et al., 2011).

Fig. 1. *Jatropha curcas* in its natural habitat taken at Area C staff residential Quarters of the Samaru campus, Ahmadu Bello University Zaria, Nigeria

The plant had been screened for its anti-inflammatory and antimicrobial activities. The anthelmintic activity of the plant was documented by Iwu (1993) and Sarabia et al. (2022). These folkloric claims of the plant's uses, to the best of our knowledge had not been validated scientifically. Therefore, the aim of this study was to evaluate the *in-vitro* anthelmintic properties of the methanol leaf extract of *J. curcas* on the eggs and larvae of *A. suum*.

2. Materials and methods

2.1. Chemicals

Studies Methanol, (Sigma Aldrich, USA), Potassium hydroxide, sulphuric acid, and albendazole used were of analytical grade. The extract and reagents were freshly prepared prior to each test by dissolving a known weight of each of them in a measured volume of distilled water. The required concentration was prepared by serial dilution.

2.2. Collection and authentication of plant materials

Jatropha curcas plant was collected from Area C staff residential Quarters of the Samaru campus, Ahmadu Bello University Zaria, Nigeria located on latitudes 11°15′ N -11°3′ N and longitudes 7°30′ E -7°45′ E and authenticated at Herbarium unit of Department of Botany, Ahmadu Bello University Zaria where the voucher number 1911 was assigned

2.3. Extraction of plants materials

The plant leaves were collected, cleaned, air-dried to constant weight and pulverized to a coarse powder using mortar and pestle. Two hundred and fifty grams (250 g) of powdered leaf was macerated in 2 litres of 70% methanol for 72 hours. The mixture was filtered using Whatman's No. 1 filter paper. The filtrate was concentrated to dryness by heating over a water bath at 50℃ to give solvent free extract used for the study.

2.4. Phytochemical analysis of the extracts

Phytochemical screening was carried out using standard tests of Trease and Evans (2002) for qualitative phytochemical constituents including alkaloids, cyanogenic glycosides, flavonoids, saponins and steroids.

2.5. Collection and authentication of the worms *Ascaris suum*

Live adult female *A. suum* was collected from the intestine of local pig which was slaughtered in piggery slaughter slab in Sabon Gari Zaria. The intestine containing the worms was collected in a beaker and taken to Helminthology laboratory of the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria, where it was opened and *A. suum* isolated. The worm was authenticated.

2.6. Removal and preparation of *Ascaris suum* eggs

The extraction of *A. suum* eggs from the worm for the investigation was conducted with the method outlined by Coles et al. (1992) and Suleiman et al. (2014). The worm collected was gently crushed in mortar, transferred into a beaker containing 0.5 M potassium hydroxide (KOH) solution and filtered after 30 minutes to obtain the eggs. The filtrate was centrifuged at 1,500 rpm for 3 minutes and supernatant decanted to recover the eggs. Distilled water was added to the egg sample, mixed, centrifuged and the water at the surface

decanted. The procedure was repeated three times. The eggs sample was further washed by centrifuging with embryonating fluid (0.1 M sulphuric acid) for another three times. A 0.1 ml of the egg sample was smeared on microscopic slide and observed under light microscope at x 40 magnification to obtain the egg counts. The number of eggs in 0.1 ml sample was used to estimate the eggs in the remaining egg sample and diluted with distilled water such that 0.2 ml of the sample contained 100 eggs.

2.7. *In vitro* egg hatch inhibition (EHI) assay

The protocols established by the World Association for the Advancement of Veterinary Parasitology (WAAVP) as documented by Coles et al. (1992) and Iqbal et al. (2004) was adopted for the *in vitro* egg hatch inhibition (EHI) assay. Hundred (100) freshly collected *A. suum* eggs contained in 0.2 ml of the egg sample were distributed in each of a flat-bottomed micro titration well and designated as groups 1, 2, 3, 4 and 5. The same volume, 0.2 ml of each concentration of the extract (25, 50 and 100 mg/ml) was added to mix with the eggs in well group 2, 3 and 4 respectively, while 0.2 ml each of the negative control, distilled water (DW) and 1 mg/ml albendazole (Alb), the positive control were added to eggs in well group 1 and group 5, respectively. The plates were incubated at 27 ℃ for 18 days for egg hatching. The embryonating fluid was intermittently dropped on to the culture to avoid desiccation. On the 19th day, a drop of 10% lugol's iodine solution was added to the culture to stop eggs from further hatching. All the unhatched eggs and the first stage larvae within the egg shell were counted in all the cultured well of the different treatment to assess the hatch inhibitory effects. The percentage inhibition of egg hatch was calculated for each concentration using the formulae (1) described by Suteky and Dwatmadji (2011) and Suleiman et al. (2014) as:

$$\% \text{ inhibition} = 100 \left(1 - \frac{X1}{X2}\right) \quad (1)$$

Where X1 = number of hatched eggs in extract and positive control micro titration well, X2 = number of hatched eggs in negative control well. The experiment was replicated 6 times.

2.8. *In vitro* larval development inhibition (LDI) assay

The technique described by Assis et al., (2003) was employed. Larvae obtained after 18 days of egg incubation were washed three times with distilled water. Subsequently, each well of a flat-bottom microtitration plate, labelled 1 through 5, received 0.2 ml of a distilled water sample containing 100 larvae. One hundred (100) µl of lyophilized penicillin-streptomycin was added to each well to control fungal growth (Okoli et al., 2016). Twenty (20) µl of nutritive medium of Earle's balance salt solution was added into each well (Igbal et al., 2004) after 48 hours of incubation at 27℃ to provide nutrients to the larvae. A volume of 0.2 ml of each of the graded concentrations (25, 50 and 100 mg/ml) of the extract were added to larvae in wells 2, 3 and 4 respectively, 24 hours after the addition of the nutrient medium. The negative control treatment (well 1) and positive control (well 5) were similarly mixed with 0.2 ml of distilled water and 1 mg/ml albendazole respectively. After seven days of incubation, one drop of 10% Lugol's iodine solution was added to each well. The larvae were categorised into distinct developmental stages (L1, L2, and L3), and the L3 larvae were enumerated. Data was presented as percentage inhibition of L3 using the formula (2) of Cavier (1973) as:

Percentage Inhibition = $\frac{N-n}{N}$ x 100% (2)

Where N = number of L3 in negative control well, n = number of L3 in extract and positive wells. The experiment was replicated 6 times.

2.9. Data analysis

Data were expressed as Mean ± standard Error of Mean and percentages where applicable. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* test for multiple comparisons. The value $p< 0.05$ were considered significant.

3. Results

3.1. Phytochemical screening of the methanol leaf extract

Methanol extract of *J. curcas* tested positive for the presence of alkaloids, carbohydrates, cyanogenic glycosides, flavonoids, saponins, steroids, and tannins after a qualitative phytochemical screening (**Table 1**). Micrograph pictures representative of observations made in a larvae development inhibition assay carried out with or without methanol extract of *J. curcas* at 50 mg/ml or 100 mg/ml are presented in **figure 2**. The development of *A. suum* larvae, **figure 2 B and C**, show inhibited larvae or un-larvated egg with abnormal or distorted content with more significant inhibition at 100 mg/ml. However, a full larva emerges from untreated control as shown in **figure 2 D**.

3.2. *In vitro* egg hatch inhibition of methanol leaf extract of *Jatropha curcas* on *Ascaris suum*

The result showed that the extract exhibited concentration dependent egg hatch inhibition (EHI) of 82.57 and 89.44% at the doses of 25 and 50 mg/ml, respectively. Albendazole, the standard drug used as positive control 100 mg/ml of the extract showed percentage EHI of 94.01 and 92.08%, respectively (**Table 2**).

3.3. Percentage inhibition of larvae development by methanol leaf extract of *Jatropha curcas* against *Ascaris suum*

The result showed that the extract exhibited concentration dependent larvae development inhibition (LDI) of 91.72 and 95.52% at 25 and 50 mg/ml, respectively. At 100 mg/ml, the LDI of the extract was 98.11% and statistically similar to 99.14% produced by albendazole, the standard drug used as positive control as shown in **table 3**.

Table 1. Preliminary phytochemical screening of the methanol leaf extract of *Jatropha curcas*

Phytochemical group	Observation
Alkaloids	+
Carbohydrates	+
Cyanogenic glycosides	+
Flavonoids	+
Saponins	+
Steroids	+
Tannins	+

+ = present

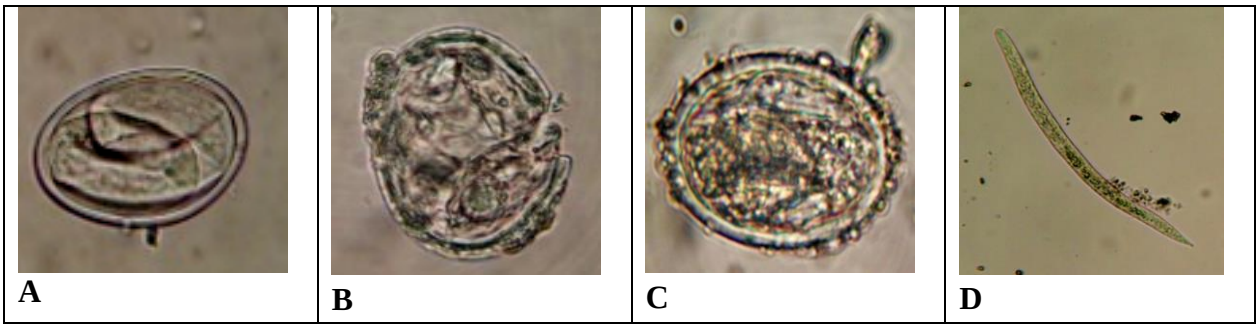

Fig. 2. Microphotograph of larvae development inhibition assay of *Ascaris suum* larvae (Magnification = *100): **A.** Freely developing larval stage of *A. suum* egg after 18 days incubation; **B.** Inhibited *A. suum* egg that failed to larvate after treatment with 50 mg/ml methanol leaf extract of *Jatropha curcas*; **C.** Inhibited *A. suum* egg that failed to larvate after treatment with 50 mg/ml methanol leaf extract of *J. curcas*; **D.** Fully larvated *A. suum* egg in the control treated with distilled water.

Table 2. *In vitro* egg hatch percentage inhibition of methanol leaf extract of *Jatropha curcas* on *Ascaris suum*

Concentration (mg/ml)	Means ± SEM	Percentage Inhibition (%)
DW(0.2 ml)	94.67 ± 0.62	0
25	16.50 ± 1.06[a,b]	82.57
50	10.00 ± 0.58[a,b]	89.44
100	7.50 ± 0.43[a]	92.08
Alb	5.67± 0.67[a]	94.01

Data presented as mean ± SEM; analysed using one way analysis of variance (ANOVA) followed by Bonferroni *post hoc test*; n = 6; [a] and [b] = significantly difference at $p < 0.05$ from DW and Alb. Respectively; DW = distilled water; Alb. = albendazole

Table 3. *In vitro* larvae development inhibition of methanol leaf extract of *Jatropha curcas* on *Ascaris suum* larvae

Concentration (mg/ml)	Means ± SEM	Percentage Inhibition (%)
DW	96.67 ± 0.42	0
25	8.00 ± 0.45[a,b]	91.72
50	4.33 ± 0.43[a,b]	95.52
100	1.83 ± 0.54[a]	98.11
Alb	0.83± 0.31[a]	99.14

Data presented as mean ± SEM; Statistical analysis was carried out using one way analysis of variance (ANOVA), followed by Bonferroni post *hoc test*; n = 6; [a] and [b] = significantly difference at $p< 0.05$ from DW and Alb. Respectively; DW = distilled water; Alb. = albendazole

4. Discussions

The selection of the plant for the study was based on the reported folkloric use of the plant as an anthelmintic (Trease and Evans, 2002; Suleiman et al., 2014). The challenges posed by limited availability of synthetic anthemintic agents, the decrease in cure rate, the emerging resistance of helminthes to treatment and high cost of synthetic anthelmintic agents have necessitated the desires to research into alternate means, including the herbal plants to

supplement the exclusive use of available synthetic anthelmintic agents (Ahmed et al., 2023).

The study investigated *in vitro* anthelmintic effects of *J. curcas* against *A. suum* using the conventional drug, albendazole as standard. The *in vitro* screening of potential anthelmintic agents prior to their *in-vivo* testing has been shown to be rational and cost effective strategy since this minimizes the number of experimental animals necessary for development of new therapeutic agents (Belga et al., 2024).

The result of the investigation in which the extract produced statistically significant ($p< 0.05$) dose-dependent egg hatch inhibition (EHI) and larva development inhibition (LDI) activities indicated that the plant posses anthelmintic activities. The EHI of 82.57 and 89.44 at 25 and 50 mg/ml respectively indicated that at lower concentrations the plant has moderate activities while the LDI at all concentrations and EHI at 100 mg/ml had satisfies the recommendation of good and effective anthelmintic agents (Igbal et al., 2004; Suteky, 2011). Albendazole which is the standard drug showed higher anthelmintic activity, but not significantly different ($p<0.05$) compared to the extract at the highest test concentration of 100 mg/ml. The difference in activity profiles of albendazole and the extract is suggested to be due to the higher state of purity of albendazole relative to the extract.

The mechanism of anthelmintic activities of *J. curcas* extract is still not fully understood, however, previous studies have suggested that the mechanism of inhibition of egg hatching and larval development of different parasite were related to the inhibition of cell division and/or the formation and development of vital structures of the parasite (Gallardo et al., 1998). One of the mechanisms of uptake of the extract could also be by diffusion through the worm's eggshell or the cuticles of larvae (Hassen et al., 2022).

J. curcas extract has higher LDI compared to EHI and this could probably be due the greater exposure of larvae to the component of the extract than the eggs. The eggs have four protective layers of eggshell which provided it with supplementary mechanical strength (Wharton, 1980) which confers additional resistance on the eggs compared to larvae in which these extra protections were absent. This further suggested that the extract might have acted by diffusion through the egg shell and cuticle of the larvae. The preliminary phytochemical screening of the plant extracts used for this study which revealed the presence of tannins, alkaloids, glycosides and flavonoids supported the findings of Igbinosa et al. (2011). The anthelmintic activity of the extracts might be due to presence of these secondary metabolites, tannins, flavonoids and other bioactive compounds present in the extract (Tchetan et al., 2022; Tulasi et al., 2020).

Tannins were suggested to have interfered with energy generation of worms by uncoupling oxidative phosphorylation or binds to the free protein of the gastrointestinal tract of the worms which lead to their death (Mute, 2009). On the other hand, alkaloids were also suggested to be responsible for the anthelmintic activity of herbal plants (Kalpesh and Priya, 2020; Tirkey, 2019). Consequently, the anthelmintic properties of methanol leaf extract of *J. curcas* may potentially be attributed to the presence of these secondary metabolites, which could have interacted synergistically.

Conclusions

Methanol leaf extract of *Jatropha curcas* demonstrated anthelmintic activity against *Ascaris suum* comparable to albendazole, the standard drug. This work therefore validates the folkloric claims of anthelmintic properties

of the plant as the extract has shown excellent ovicidal and larvicidal activities against *A. suum* which was used as surrogate for human ascaris. The study therefore suggests that ***J. curcas*** could be a potential source of novel anthelmintic agent.

Acknowledgement

The authors gratefully acknowledge Magaji Yusuf of Helminthology laboratory of the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University, Zaria, for his technical assistance, Musa Mohammed and Namadi Sanusi of Botany Department, Ahmadu Bello University, Zaria for identification and authentication of the plant.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Acta Biologica Marisiensis

ANALYSIS OF DIFFERENT TYPES OF HONEY TO EMPHASIZE GENETICALLY MODIFIED SEQUENCES

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Received: 30 November 2024; **Accepted:** 15 December 2024; **Published:** 30 December 2024

Abstract: Food safety and consumer health protection is a particularly important aspect, always in the attention of researchers. The authentication of honey, but also its mislabelling, is one of the current challenges related to quality monitoring. Therefore, a rigorous monitoring of the different types of honey on the market is required, to ascertain whether they are properly labeled and to detect the possible presence of modified genes. The aim of this study was to analyze honey samples, to identify the possible presence of genetically modified genes, using the technique based on DNA analysis, called real-time PCR. For this purpose, ten samples of honey were analyzed. DNA isolation was performed with Quick-DNA™ Plant/Seed Miniprep kit. For identification a potential presence of the genetically modified genes in the honey samples it was used a real-time PCR kit - Xpert qDetect P-35S, T-NOS and P-FMV -DNA amplification kit, that allows detection by real-time PCR of specific DNA sequences from the 35S promoter, NOS terminator and/or FMV promoter present in total DNA previously purified from honey samples. The results showed that two honey samples - M2 and M3 contain genetically modified sequences. It can be concluded that these results illustrate the importance of honey quality monitoring for the effective detection of genetically modified organisms.

Keywords: honey, genetically modified sequences, food quality, screening, real-time PCR

1. Introduction

Honey is a natural product produced by honeybees (*Apis mellifera* L.) from various plant secretions (Bertelli et al., 2010; vanEngelsdorp and Meixner, 2010; Siddiqui et al., 2017). Honey is marketed as a food with scientifically and clinically proven content to have beneficial health properties. Honey has many health benefits arising from the regular consumption of honey, such as the elimination of disorders of the gastrointestinal tract and cardiovascular disorders of the cardiovascular system (vanEngelsdorp and Meixner, 2010; Majtan et al., 2021). Some studies showed an antioxidant activity of honey (Jamróz et al., 2014; Piljac-Zegarac et al., 2009) and showed that there was a direct correlation between the content of phenols, the antioxidant activity and the color intensity of the honey. Due to the presence of phenolic constituents, honey has antibacterial activity (Majtan et al., 2021) anticancer, anti-inflammatory (Samarghandian et al., 2017), antithrombotic, immune, immunomodulatory and analgesic properties. Some studies have also shown that honey decreases cardiovascular risk factors without an

increase in body weight (Yaghoobi et al., 2008; Siddiqui et al., 2017).

Genetically modified organisms (GMOs) are organisms in which the genetic material is modified, by introducing portions of DNA from another organism, to give it resistance or to increase its production. Thus, new crops were obtained, resistant to pests, herbicides and insecticides, and the production of many plants such as: corn, rapeseed, potato, rice, sugar beet was increased. After 1998 there was an exponential increase in genetically modified foods. Among the plants that have had a rise and a special economic importance are: corn, cotton, soybeans, tomatoes, potatoes, rapeseed, flax and many others (Cristea and Denaeyer, 2004).

According to DIN ISO 9000, the term "quality" is defined as ”the totality of characteristics relevant to the ability of a product to fulfill its requirements”. In order to obtain healthy and safe products, without neglecting economic and ecological aspects, the concept of food quality should be much broader. Thus, several aspects must be taken into account: requirements of the producer, consumer, supervision and legislative bodies (Böhme et al., 2016; Müller and Steinhart, 2007).

As defined by European Directive 18/2001/EC, in 2018, the European Court of Justice (ECJ) showed that organisms obtained through mutagenesis are also genetically modified organisms (GMOs) (Broll et al., 2019; Kuntz, 2020; Ryan et al., 2020). Genetically modified (GM) pollen not authorized in the EU cannot be present in honey. The honey must be labeled if it contains more than 0.9% pollen from authorized GM plants in relation to the total pollen content (Zmijewska et al., 2013; Villanueva-Gutierrez et al., 2014).

Food adulteration as well as it’s mislabeling has become a cause of concern for people worldwide. To monitor and control the authenticity of food, but also to guarantee the correct and accurate labeling of food products, the use of analytical methods is necessary. Thus, using these methods, we must ensure that the components included in a food product are of the nature and quality declared by the seller (Cheftel, 2005; Ortea et al., 2016).

Traditional methods such as SDS-PAGE and other classical genetic techniques such as restriction fragment length polymorphism (RFLP), real-time polymerase chain reaction (Real-Time PCR) are still used based on molecular masses. Also, methods such as techniques related to proteomics, metabolomics and genomics are increasingly used, helping to elucidate the limitations of previous methodologies (Böhme et al., 2016; Ortea et al., 2016).

DNA-based techniques are considered routine analyzes for food analysis. They enable the detection of food fraud in complex foods. Many of the classical polymerase chain reaction (PCR)-based techniques (DNA sequencing, RFLP, multiplex-PCR, real-time PCR, microarray, random amplified polymorphic DNA, and microsatellites) have been used to determine species, cultivars, and geographic origin in food products. At the same time, new techniques based on genomics were used to verify the authenticity of food products. Thus, attempts have been made to improve the performance of classical DNA-based techniques in terms of specificity, sensitivity and sample processing capacity (Böhme et al., 2016; Haynes et al., 2019).

The aim of this study was to analyze honey samples, to identify the possible presence of genetically modified genes, using the technique based on DNA analysis, called real-time PCR.

2. Materials and methods

For the DNA-based technique, 10 honey samples from different market were analyzed in the study, coded as follows: M1-M10.

DNA isolation from honey samples was carried out using the Quick-DNATM Plant/Seed Miniprep kit - Zymo Research, which provides a simple and rapid isolation of high-quality DNA for PCR analysis, without inhibitors, from a variety of plant sample sources (Instruction manual - Quick-DNA^{TM} Plant/Seed Miniprep kit).

The amplification of DNA extracted from honey samples was carried out with the help of the Xpert qDetect P-35S, T-NOS and P-FMV kit (Grisp Research Solutions), which allows the detection by Real-Time PCR of specific DNA sequences from the 35S promoter, NOS terminator and/or FMV promoter present in total DNA previously purified from honey samples. The detection limit is 10-100 µg of GMO DNA, which allows detection of only 0.01-0.1% of target DNA in food samples if 100 ng of total DNA is used (Instruction manual - Xpert qDetect P-35S, T-NOS and P-FMV). The parameters of the amplification program are detailed in **Table 1**.

Table 1. The parameters of the amplification program (Instruction manual - Xpert qDetect P-35S, T-NOS and P-FMV)

Steps	**Temperature**	**Time**	**Cycles number**
Enzyme activation	50˚C	2 minutes	1
Initial Denaturation	95˚C	5 minutes	1
Denaturation	95˚C	30 seconds	40
Primer alignment	60˚C	30 seconds + channel data acquisition FAM and ROX	
Extension	72˚C	30 seconds	

3. Results

Since 1994, more than 100 genetically modified plants have been approved for use as food or animal feed. Effective detection of genetically modified organisms in food and feed is essential for the implementation of national legislation. Given the high diversity, an initial generic screening for the presence of the most common genetically modified materials is usually the first step in GMO analysis to reduce the volume of subsequent identification analyses. Since the 35S promoter from cauliflower mosaic virus (CaMV) and the NOS terminator from *Agrobacterium tumefaciens* are the most common elements present in transgenic materials in food and feed, detection of these regulatory sequences by PCR amplification is the most logical choice. However, as they do not cover some important GMOs, such as soybean MON89788, sugar beet H7-1 or rapeseed GT73, to ensure the widest possible detection, the kit used is also suitable for promoter detection from FigWort mosaic virus (P-FMV) by PCR (Instruction manual - Xpert qDetect P-35S, T-NOS and P-FMV).

Specific amplified DNA target sequences could be observed in real-time in the amplifier software. Each amplification curve recorded represents relative fluorescence units for each individual sample compared to a fluorescence threshold value, depending on the fluorophores used in the reaction (FAM and ROX channels). The analyzed samples are considered to be

positive, if a fluorescence value higher than the fluorescence threshold value is recorded. At the same time, the amplifier software automatically records the Ct (Threshold Cycle) values, values that are detected, for each individual sample, at the intersection between the amplification curve and the fluorescence threshold signal value. For each sample, 2 analyzes were performed in repetition, the device automatically calculating the average of the analysis values for the 2 analyses.

According to the specifications of the Xpert qDetect P-35S, T-NOS and P-FMV kit (Grisp Research Solutions), a sample is considered positive if a positive Ct is recorded for both FAM and ROX channels or if the Ct is positive for FAM channel and Ct = N/A on ROX channel. Samples are considered negative if Ct = N/A on the FAM channel and Ct is positive on the ROX channel. If both the GMO channel (FAM channel) and the internal control (ROX channel) have signals below the fluorescence threshold level (Ct = N/A), it means that the PCR reaction has been inhibited. For positive control samples, they must be positive for the FAM channel and positive but not significant for the ROX channel. Negative control samples should record Ct = N/A for both channels.

The results recorded for the FAM channel show whether the sample is positive for the presence of GMOs, and the results recorded for the ROX channel show whether the amplification reaction proceeded under normal conditions without being inhibited. The results showed that the positive control samples recorded positive Ct values for the FAM channel, and for the negative control samples, the Ct values were below the fluorescence threshold level. All amplification reactions showed detectable results.

Honey samples M2 (Ct FAM = 28.22; Ct ROX = 23.33) and M3 (Ct FAM = 35.11; Ct ROX = 23.81) were detected as positive only for the P-FMV sequence, recording positive Ct values for both detection channels. In the case of the other honey samples, the recorded results were negative for all 3 analyzed sequences (P-35S, T-NOS, P-FMV). These results show that honey samples M2 and M3 contain genetically modified sequences (**Table 2**).

Table 2. Amplification results for all samples analyzed

Sample type	**Ct values**					
	P-35S		**T-NOS**		**P-FMV**	
	FAM	**ROX**	**FAM**	**ROX**	**FAM**	**ROX**
M1	N/A	24.66	N/A	23.28	N/A	24.12
M2	N/A	24.20	N/A	23.46	28.22	23.33
M3	N/A	24.55	N/A	24.18	35.11	23.81
M4	N/A	24.26	N/A	24.23	N/A	24.06
M5	N/A	24.25	N/A	24.00	N/A	24.55
M6	N/A	24.05	N/A	24.37	N/A	24.62
M7	N/A	23.85	N/A	24.22	N/A	23.93
M8	N/A	24.11	N/A	24.82	N/A	24.41
M9	N/A	24.87	N/A	24.85	N/A	24.43
M10	N/A	24.76	N/A	24.87	N/A	24.46
Pozitive control	25.23	23.62	24.45	23.50	24.34	23.16
Negative control	N/A	N/A	N/A	N/A	N/A	N/A

In a study that carried out the analysis of honey from the Yucatan peninsula in Mexico, the authors showed that following the PCR analysis, 2 positive samples for genetically modified soy out of a total of 9 samples were confirmed, which demonstrates the importance of monitoring the quality of honey (Villanueva-Gutierrez et al., 2014). Another study describes the possibilities and limits of detection of genetically modified rape in rape honey by real-time PCR and the sensitivity of PCR methods for the detection of genetically modified rape was demonstrated (Waiblinger HU et al., 2005).

Since cotton is one of the main plants used as a source of honey, Chinese researchers became concerned to see if there are genetically modified DNA sequences contained in honey. Researchers have developed a DNA extraction procedure and a PCR protocol for the detection of foreign DNA sequences in bee honey. They used a PCR protocol, whereby genetically modified cotton DNA sequences, 125-550 bp in length, could be specifically amplified and detected (Cheng et al., 2007).

The increasing number and diversity of genetically modified organisms for the food and feed market requires the development of advanced methods for their identification and detection. This problem can be solved by using next generation sequencing (NGS) and DNA barcoding techniques (Saltykova et al., 2022).

Conclusions

The results obtained in this study illustrate the importance of honey quality monitoring for the effective detection of genetically modified organisms, but also the effectiveness of the real-time PCR technique for performing an initial screening to detect the presence of genetically modified sequences. Taking into account the results obtained, a complete analysis is required, to quantify the genetically modified sequences, but also to verify the authenticity of all the ingredients in the honey samples, using other methods (DNA barcoding, next generation sequencing).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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