Trop J Nat Prod Res, September 2024; 8(9): 8504-8509 **ISSN 2616-0684 (Print)**
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

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

Original Research Article

Identification of Anti-Nutritional Factors and Trypsin Inhibitory Activity of Three Spontaneous Plants: *Malva sylvestris* L, *Cynara cardunculus* L, and *Spinacia oleracea* L

Nabahat Benmansour[1], Sihem Belkhiter[1,2]*, Mustapha Mounir Bouhenna[3], Houcine Boutoumi[1,4]

[1]*University Saad Dahleb Blida1, BP270, Soumaa, Blida. Algeria*
[2]*Laboratory of Phytopathology and Molecular Biology, National Higher School of Agronomy, ENSA, Algiers 16004, Algeria*
[3]*Scientific and Technical Center of Research in Physical and Chemical Analysis (CRAPC), Bou-Ismail 42004, Tipaza, Algeria*
[4]*Chemical Engineering Laboratory, Department of Process Engineering, BP270, Soumaa, Blida Algeria*

ARTICLE INFO

Article history:
Received 31 July 2024
Revised 13 August 2024
Accepted 14 September 2024
Published online 01 October 2024

ABSTRACT

Malva sylvestris L., *Cynara cardunculus* L., and *Spinacia oleracea* L. are spontaneous plants often consumed by humans and animals. Although, these plants possess nutritional and therapeutic value, they may also contain anti-nutritional factors in their carbohydrate fraction and trypsin inhibitors that interfere with protein digestion. This study aims to identify the anti-nutritional factors, and determine the trypsin inhibitory activity of *Malva sylvestris* L., *Cynara cardunculus* L., and *Spinacia oleracea* L. The sugar contents of the plants' ethanol extracts were determined using the Anthrone and Ortho-Toluidine colorimetric methods. Specific monosaccharides and oligosaccharides were identified by thin layer chromatography (TLC). The polysaccharides (starch and cellulose) contents were also assessed. The trypsin inhibitory activity was evaluated according to standard procedures. The results showed that the sugar contents of the plants ranged from 0.14 to 0.34 g/100 g dw. TLC analysis revealed the presence of sucrose in all the three plant extracts, while two oligosaccharides; raffinose and stachyose were identified in *M. sylvestris* and *S. oleracea*. The extracts exhibited significant trypsin inhibitory activity with *C. cardunculus* demonstrating the highest inhibitory effect with IC_{50} value of 4.92 mg/mL, while the IC_{50} values for *M. sylvestris* and *S. oleracea* where 9.26 mg/mL and 12.24 mg/mL, respectively. The presence of these anti-nutritional factors may interfere with digestion in both humans and animals, hence the need for caution in the use of these plants for medicinal purposes.

Keywords: *Malva sylvestris* L., *Cynara cardunculus* L., *Spinacia oleracea* L, Anti-nutritional factors, Trypsin inhibitory activity.

Introduction

Malva sylvestris L., commonly known as common mallow, is a spontaneous plant widespread in Europe, Western Asia, and North Africa.[1-2] This plant is traditionally used for its medicinal virtues which is attributed to its rich phytochemical composition. The plant is rich in biomolecules such as terpenoids, coumarins, flavonoids, polyphenols, vitamins, and tannins. These constituents contribute to its antioxidant, anti-inflammatory, anticancer, hepatoprotective andantimicrobial effects.[3]

Cynara cardunculus L. is native to the Mediterranean basin. This plant, which belongs to the Asteraceae family, is cultivated for a variety of reasons, including as a bioenergy crop, for its rich bioactive molecules and its nutritional value.[4]This plant exhibits promising pharmacological properties, particularly anti-inflammatory effect, which is beneficial in conditions like inflammatory bowel disease.[5] However, there are also certain subspecies, such as artichoke and cardoon, which are cultivated for the production of food, biofuels and pharmaceutical compounds.[5]

*Corresponding author. Email: belkhiter_sihem@univ-blida.dz
Tel: + 213 770 82 91 36

Citation: Benmansour N, Belkhiter S, Bouhenna MM, Boutoumi H Identification of Anti-Nutritional Factors and Trypsin Inhibitory Activity of Three Spontaneous Plants: *Malva sylvestris* L., *Cynara cardunculus* L., and *Spinacia oleracea* L. Trop J Nat Prod Res. 2024; 8(9): 8504-8509 https://doi.org/10.26538/tjnpr/v8i9.37

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

As a result, this plant, which is much appreciated by the people of the Mediterranean basin, represents a valuable resource for a variety of uses.

Spinacia oleracea L., commonly known as spinach, is a green leafy vegetable native to Central and South-West Asia. This plant was introduced into North Africa and is much appreciated by the local populations for its flavour and nutritional value.[6]*S. oleracea* also has numerous therapeutic benefits, due to its antioxidant, anti-diabetic, anti-inflammatory and hepatoprotective properties.[7] Its rich bioactive compounds such as quercetin, gallic acid, and caffeic acid contribute to its nutritional and therapeutic values.[8]

Although, these plants have important nutritional values, they may also contain other anti-nutritional factors. These are compounds that can interfere with the assimilation of nutrients which is detrimental to the health of humans and animals by reducing the absorption of amino acids, and minerals.[9] Antinutrients such as tannins, saponins, phytates, oxalates and lectins are abundant in plant foods and can have adverse effects on human health, leading to symptoms such as nausea, bloating and nutritional deficiencies.[10]

Anti-nutritional factors in plants are compounds that interfere with nutrient absorption, such as trypsin inhibitors that inhibit the activity of enzymes involved in protein digestion. Identifying and characterising these factors in plants is crucial to understanding their impact on human health and nutrition.[11]

The aim of this study is to identify anti-nutritional factors in the glucoside fraction of *Malva sylvestris* L., *Cynara cardunculus* L., and *Spinacia oleracea* L., and to assess their trypsin inhibitory activity.

Materials and Methods

Plants collection and identification

Malva sylvestris L., *Cynara cardunculus* L., and *Spinacia olracea* L. were collected in April 2021 in the Honaine district, Tlemcen, north-western Algeria, which is located within the geographic coordinates 35°10'35" North and 1°39'18" West. The Honaine region is bounded by the Mediterranean Sea to the north, the Maghnia plain and the Sbaa Chiyoukh mountains to the south, Beni Khaled and Beni Ouarsous to the east and Dar Yaghmoracene to the west (Figure 1).The plants were identified and authenticated at the herbarium of the Department of botany, National School of Agronomy (ENSA), Algiers.

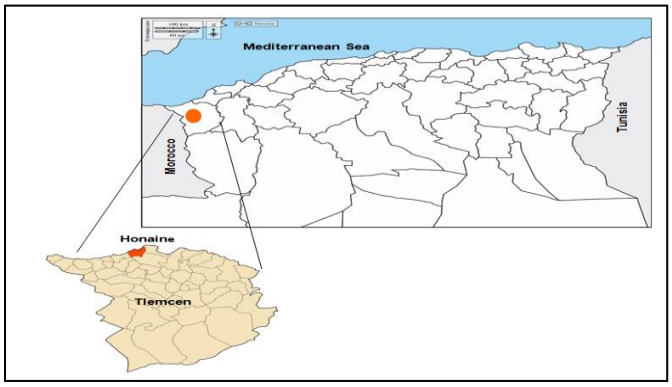

Figure 1: Geographical location of the Honaine region

Determination of total sugar content

The sample solution was prepared by dissolving 2.5 g each of the plants powder in 80% ethanol in separate test tubes, and allowed to stand at room temperature for 30 min.The solutions were filtered, and the filtrates were used for analysis of total sugars using the Anthrone method as described by Cerning-Beroard (1975).[12]

Firstly, a stock solution of glucose (0.06 g/L) was prepared in distilled water. The stock solution (5 mL) was diluted with distilled water to a final volume of 50 mL. Volumes of 0.5, 1.0, 1.5, and 2 mL, corresponding to 30, 60, 90, and 120 µg/10 mL of glucose, respectively were used to preparea calibration curve ofglucose standards.

Additionally, a solution of 2% anthrone and 1.84 g/mL sulphuric acid was prepared. The test solution (2 mL) was mixed with 0.5 mL of the anthrone solution in tubes placed in an ice water bath. Then, 5 mL of sulphuric acid was slowly added to the mixture.The tubes were shaken and immediately immersed in a boiling water bath for 12 min. The reaction was stopped by placing the tubes in an ice water bath. The optical density of the resulting coloured solution was measured at 625 nmagainst a blank solution consisting of water, anthrone and sulphuric acid, using a spectrophotometer (Shimadzu UV-1800 UV/Visible, Japan). The ethanol-soluble sugar content was calculated from the glucose calibration curve.

Identification of oses and oligosides

Separation and identification of sugars and oligosaccharides was performed by thin-layer chromatography. A 30 µg quantity of ethanol-soluble carbohydrates (obtained previously) was spoted on a silica gel TLC plate and separated using a mixture of chloroform, acetic acid and water (44:47:3) as the elution solvent.Three consecutive elutions were performed to achieve optimal separation. The TLC plate was sprayed with diphenylamine-aniline-orthophosphoric acid solution (5:5:1).[13] This resulted in distinct coloration for each sugar or oligosaccharide, as shown in Table 1. This method allowed for the separation and qualitative identification of the different oses and oligosides present in the samples.

Determination of glucose content

Glucose concentration was determined using the Ortho-toluidine colorimetric method.[14] Firstly, Ortho-Toluidine reagent was prepared by dissolving 1.5 g of Thiourea in acetic acid (940 mL, 85%) followed by the addition of 60 mL of Ortho-Toluidine. Next, a colour reagent was prepared by mixing 0.5 mL of the test sample and 4.5 mL of Ortho-Toluidine reagent in a test tube. Then, a reagent black containing 0.5 mL distilled water and 4.5 mL of Ortho-Toluidine reagent was prepared. Standard glucose solutions withconcentrations of 1, 2 and 5 g/L were also prepared. The test tubes were tightly sealed, vortexed, and placed in a boiling water bath for 8 min. Thereafter, the tubes were cooled by placing in a coldwater bath. Optical density of the solutions were measured at 625 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800 UV/Visible, Japan). The concentration of glucose in the sample was estimated from a previously prepared calibration curve of standard glucose solutions (Table 2).

Table 1: Staining of oses and oligosides with diphenylamine aniline reagent

Oses and Oligosides	Colour
Fructose	Brown
Glucose	Dark blue
Sucrose	Green grey
Raffinose	Grey
Stachyose	Grey
Maltose	Green blue

Estimation of starch content

The starch content was determined using the Anthrone colorimetric method as described by Hodge and Hofreiter's (1962).[15] This method involved the hydrolysis of starch into glucose by treating with10% hydrochloric acid (HCl) for 5 h at a temperature of 110°C. The glucose released is then measured using the Anthrone method described earlier. The residue obtained after extraction of the sugars was placed in a flask, to which 50 mL of 10% HCl was added. The mixture was then refluxed for 5 h. Hydrolysis was confirmed to be complete by an iodine test. The reaction mixture was neutralised using a 10% sodium hydroxide (NaOH) solution. It was then filtered into a 100 mL volumetric flask and diluted with distilled water. The glucose content was first determined using the Anthrone colorimetric method as described above. The starch content was determined by multiplying the glucose content by a factor of 0.9 as shown in the following equation.

$$Starch\ content = Glucose\ content \times 0.9$$

Determination of cellulose content

The finely ground sample (2 - 3 g) was placed in a 600 mL beaker, 150 mL of distilled water was added, and the volume was made up to 200 mL with 1.25% sulphuric acid (H_2SO_4). The solution was boiled for 30 min, while maintaining a constant volume of 200 mL by adjusting with distilled water.

The solution was allowed to stand, and filtered using suction. The residue was washed with hot distilled water until it the pH became neutral. The procedure was repeated using 1.25% potassium hydroxide (KOH) solution in place of sulphuric acid. The residues collected on the filter papers were weighed, then placed in a tare vessel and heated in an oven at 103°C until constant weight. The cellulose content was determined using the formula below.

$$\%cellulose = \frac{P2 - P1}{100 - \%H} \times 100$$

Where; P_1 is the mass of the dried filter paper, P_2 is the mass of the dried filter + cellulose, and %H is the water content of the sample. This method eliminated non-cellulosic components and facilitated the quantification of cellulose.

Determination of trypsin inhibitory activity

The trypsin inhibitory activity was determined according to the method described by Kakade *et al*..[16] First, the following solutions were prepared: 0.005N Tris buffer pH 8.2, BAPNA (Benzoyl-DL-Arginine-p-Nitroanilide) substrate solution (40 mg/100 mL in Tris buffer), and trypsin solution (4 mg/200 mL in 0.001N HCl). An extract solution was obtained by adding 1 g of sample into 50 mL of 0.01N NaOH, and the mixture was agitated for 1 to 2 h at while maintaining the pH at 8-10. Aliquots (0.6, 1, 1.4, and 1.8 mL) of the extract were incubated with 2 mL of trypsin in 5 mL BAPNA for 10 min at 37°C (Table 3). The reaction was stopped with 1 mL of 30% acetic acid. The reaction mixture was filtered, and the absorbance of the filtrate was measured at 410 nm. A blank control tube without extract and another control tube without trypsin were used to estimate the trypsin inhibitory activityby calculating the percentage of inhibition of the hydrolysis of BAPNA. BAPNA is a chromogenic substrate commonly employed for assessing trypsin activity.

The percentagetrypsin inhibitory activity was calculated using the following equation: $Inhibition\ (\%) = \frac{[(Ac-As)]}{Ac} \times 100$

Where; Ac = absorbance of the control; As = absorbance of the sample.

The IC_{50} values, which indicate the concentration at which 50% inhibition occurs, were obtained by performing non-linear regression analysis on a plot of percentage trypsin inhibition against sample concentrations for the three plants.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). The difference was considered significant at $P \leq 0.05$.

Results and Discussion

Sugar content of the plants

The presence of free sugars, including sucrose, in plant cells is vital for overall development and plays a significant role in various physiological processes.[17]

The ethanol-soluble sugar contents of the plants were estimated from the glucose calibration curve. The results showed no significant difference ($P \geq 0.05$) among the three species (Table 3). The sugar content was slightly higher in *C. cardunculus* (0.34 g/100 g dw) compared to *M. sylvestris* and *S. oleracea* which gave values of 0.14 g/100 g dw and 0.2 g/100 g dw, respectively.

The sugar contents obtained in this study are significantly lower compared to that recorded by Mandim et *al*.,[18] who found that soluble sugar content varied according to the stage of maturity of *C. cardunculus* with values ranging from 1.03 g/100 g dw to 7.49 g/100 g dw. The authors explained that the composition of free sugars changes according to the stage of maturation of the plant. On the other hand, for *M. sylvestris*, Petkova *et al*.[19] found out that the soluble sugar content was 42.9 g/100 g of fresh matter. This shows that the soluble sugar content fluctuate depending on the quality of the plant material (fresh or dry). For *S. oleracea*, it was found out that the carbohydrate content varied among the different genotypes of *S. oleracea* species, with values ranging from 8.66 g/100 g dw to 5.68 g/100 g dw.[20]

Table 2: Composition of standard solution for preparation of the calibration curve

Tube Number	N°0	N°1	N°2	N°3	N°4	N°5
Orthotoluidine (mL)	4.5	4.5	4.5	4.5	4.5	4.5
Distilled water (mL)	0.5	0.5	0.5	0.5	0.5	0.5
Standard solution (g/L)	0	2 g/L at 1/100	5 g/L at 1/100	1 g/L at 1/10	2 g/L at 1/10	5 g/L at 1/10
Glucose pg/tube	0	10	25	50	100	250

pg: picogram

Table 3: Experimental protocol for the determination of trypsin inhibitory activity

Tubes	Reagent blank	Control	N°1	N°2	N°3	N°4	N°5
Extract (mL)	0	2	0	0.6	1	1.4	1.8
Distilled water (mL)	2	0	2	1.4	1	0.6	0.2
Trypsin (mL)	2	0	2	2	2	2	2
BAPNA (mL)	5	5	5	5	5	5	5
Acetic acid (mL)	1	1	1	1	1	1	1

Sugars identified in the plants

Following the TLC analysis of the ethanol-soluble carbohydrate fraction, sucrose was detected in the three plants (Figure 2). On the other hand, stachyose was identified exclusively in *M. sylevestris*, while Raffinose was identified in *M. sylvestris* and *S. oleracea*. The galactosides raffinose and stachyose are considered to be anti-nutritional factors in plants, especially legumes such as groundnuts and soya.[21] Their presence in *M. sylvestris* and *S. oleracea* shows that these two plants contain anti-nutritional factors in their glucoside fractions.

Thin layer chromagraphy was unable to identify glucose and fructose in the ethanol-soluble carbohydrate fraction of the plants. This is not surprising because the Anthrone method used gives an overall measurement of sugars, the presence of certain sugars may be masked by the presence of other molecules that react with Anthrone and are present in large quantities.[22]

In contrast to the results of the present study, Petkova *et al*.,[19] reported the presence of sucrose, glucose and fructose in *M. sylvestris* using a more accurate HPLC technique. Additionally, Sarmiento-Tomalá *et al*.,[23] identified another sugar, Rhamnose, in *M. sylvestris*.

Sucrose, glucose and fructose has been identified in *S. oleracea* using thin layer chromatography and HPLC techniques.[22] However, in *C. cardunculus*, the most abundant sugars are sucrose and glucose.[24] The variation in the quantity and composition of free sugars in plants can be attributed to several factors including geographical location, maturity stage and genotype.[18]

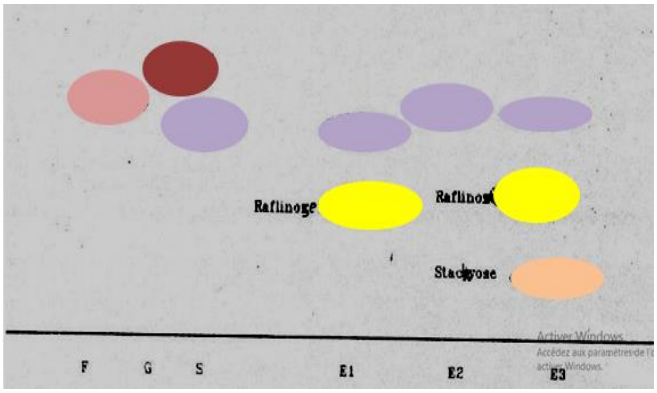

Figure 2: Separation of ethanol-soluble sugars by chromatography on a silica gel TLC plate; F: fructose; G: glucose; S: sucrose; E1: *S. oleracea;* E2: *C. cardunculus;* E3: *M. sylvestris.*

Glucose content of the plants

Glucose plays a crucial role in the diet of humans and animals, serving as a primary source of energy, that is easily assimilated by the body.[25] In the present study, glucose content was estimated using the Ortho-Toluidine colorimetric method, and the results revealed a significant difference ($P \leq 0.05$) in the glucose content among the three plants species (Table 4). The glucose content was highest in *S. oleracea* which gave a value of 0.67 g/100g dw, followed by *C. cardunculus* with glucose content of 0.54 g/100g dw, while *M. sylvestris* had the lowest glucose content of 0.34 g/100g dw (Table 4). These findings are similar to that of Silva *et al.*,[26] who found glucose content ranging from 0.10 to 0.557 g/100g dw in the leaves of *C. cardunculus*, and Petkova *et al*,[19] who found 0.61 g/100 g dw of glucose in *M. sylvestris*. Conversely, in *S. oleracea* , Pereira *et al*,[20] recorded a higher glucose content which ranged from 2.11 g/100 g dw to 2.46 g/100 g dw in *S. oleracea*.

Polysaccharides (Starch and Cellulose) content

Starch is an essential component in plants as it is the main reserve of carbohydrates.[27] As shown in Table 4, the leaves of *S. oleracea* contain a higher amount of starch than *M. sylvestris* and *C. cardunculus* ($P \leq 0.05$). Research has shown that the starch content of *S. oleracea* leaves varies depending on light intensity.[22] Proietti *et al.*,[28] demonstrated that in leaves exposed to low light intensity, starch accounted for 68.5% of the total carbohydrates, whereas in leaves exposed to high light intensity, starch accounted for only 48.2%. However, in *M. sylvestris*, the seeds have the highest starch content.[29] Cellulose was the predominant polysaccharide in all the three plants compared to the other polysaccharides, and it was significantly higher than starch in all the three plants ($P \leq 0.018$). Among the three plants, *C. cardunculus*is had the highest cellulose content of 9.84 g/100 g dw. *M. sylvestris* and *S. oleracea* had cellulose content of 6.42 g/100 g dw and 6.94 g/100 g dw, respectively (Table 4). These values were lower compared to that found by Francaviglia *et al*.,[30] who recorded a cellulose content varying between 29.0 g/100 g dw and 34.3 g/100 g dw in *C. cardunculus* leaves. For *M. sylvestris*, similar values were found by Mousavi et *al*.,[31] who recorded a cellulose content of 5.6 g/100 g dw, equivalent to 56% of the plant's dry weight. Although, cellulose is not specifically mentioned as an anti-nutritional factor, it is possible that it can affect digestion in animals due to its resistance to digestion and its effect on the viscosity of the content of the digestive tract.[32]

Trypsin inibhitory activity

Figure 3 shows the trypsin inhibitory activity of the three plant extracts (*M. sylvestris*, *S. oleracea*, and *C. cardunculus*). The IC_{50} values were 9.26 g/mL, 12.24 mg/mL, and 4.92 mg/mL, respectively. The results showed that *C. cardunculus* had the lowest IC_{50} value (4.92 mg/mL), which indicates that *C. cardunculus* is highly effective in inhibiting trypsin activity. It was observed that, as the concentration of the extracts decreases, the percentage inhibition of trypsin increases, suggesting a diminishing inhibitory effect. At a concentration of 6 mg/mL, the inhibitory effect of the three plants on trypsin activity was very strong compared to the effect at the maximum concentration of 18 mg/mL (Table 5).

In a study by Kunitz (1949),[33] it was observed that increasing the concentration of soya extract led to an increase in trypsin inhibitory activity. However, it was noticed that this relationship was not linear and that the inhibitory effect of the plant extracts reached a maximum and then declined at very high concentrations. This observation may be due to the possibility that at the maximum concentration, all the trypsin molecules might have been fully bound by the inhibitor in the extract, and no unbound trypsin is available for additional inhibition. This results in a decrease in the observed inhibitory effect, despite the increase in extract concentration.

Table 4: Sugar composition (g/100 g dry weight) of *M. sylvestris, S. oleracea and C. cardunculus*

	Ethanol-soluble sugars	Glucose	Starch	Cellulose
M. sylvestris	0.14 ± 0.06	0.34 ± 0.09	0.31 ± 0.07	6.42 ± 0.47
S. oleracea	0.20 ± 0.11	0.67 ± 0.24	0.61 ± 0.22	6.94 ± 0.63
C. cardunculus	0.34 ± 0.32	0.54 ± 0.14	0.49 ± 0.12	9.84 ± 3.70
t, df	t = 3.93, df = 3	t = 5.828, df = 2	t = 5.32, df=2	t = 7.258, df = 2
P-value (two tailed)	0.0509 n.s	0.0340*	0.0336*	0.0185*

Data represent mean ± SD (n = 3). n.s: not significant, *p < 0.05, **p < 0.01, ***p < 0.001.

Table 5: Trypsin inhibitory activity of *M. sylvestris*, *S. oleracea*, and *C. cardunculus*

Percentage Inhibitory activity (%)					IC_{50} (mg/mL)
Plant (extract)	**0.6 mL (6 mg/mL)**	**1 mL (10 mg/mL)**	**1.4 mL (14 mg/mL)**	**1.8mL (18 mg/mL)**	
M. sylvestris	64.67 ±0.1	52.47± 0.45	41.25± 0,07	23.25± 0,27	9.26
S. oleracea	73.28±0.24	60.16 ± 0.27	43.14 ± 0.17	33.21 ± 0.11	12.24
C. cardunculus	63.18 ± 0.59	43.63 ± 0.32	6.3 ± 0.28	3.37 ± 0.39	4.92

Data represent mean ± SD (n = 3).

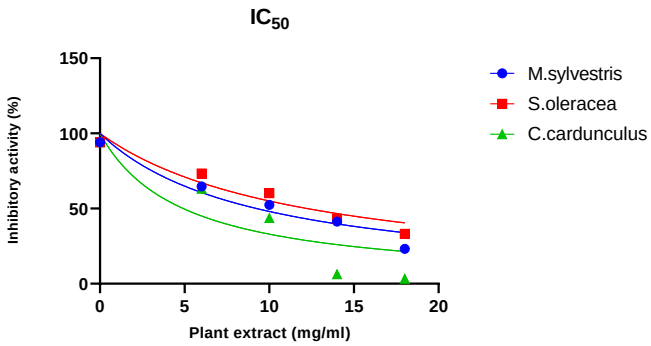

Figure 3: Trypsin inibhitory activity of the ethanol-solubles plants exract

Conclusion

M. sylvestris, *S. oleracea* and *C. cardunculus* are highly versatile plants with a long history of use in traditional medicine. Their nutritional properties, combined with their rich phytochemical composition, make them a valuable addition to human health. Despite their long history of use in traditional medicine, these plants may present potential toxic effects, particularly at high doses or in the event of drug interactions. Findings from this study have shown that these plants contain oligosaccharides such as raffinose and stachyose, and high amount of cellulose. Ethanol extracts of the plants posses significant trypsin inhibitory activity. The presence of these components coupled with their trypsin inhibitory activity may act as anti-nutritional factors that will affect digestion in humans and other monogastric animals. For this reason, there is the need to exercise caution in the use of these plants. Therefore, further studies, including controlled clinical trials should be conducted to fully assess the bioavailability, long-term toxicity, safety, and therapeutic efficacy of these plants.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

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

References

1. Ryu H, Ryu M, Jeon EJ, Park YJ, Kang B, Ono Y, Kim OK, Lee SJ. *Malva sylvestris*, a New Host of *Puccinia modiolae* in Korea. Plant Dis. 2023; 107(10):3285.
2. Panchenko K and Pospielov S. The effect of *Malva sylvestris* L. plant density on its productivity. Grail Sci. 2023; (25):160-162.
3. Batiha GES, Tene ST, Teibo JO, Shaheen HM, Oluwatoba OS, Teibo TKA, Al-kuraishy HM, Al-Garbee AI, Alexiou A, Papadakis M. The phytochemical profiling, pharmacological activities, and safety of *Malva sylvestris*: a review. Naunyn Schmiedebergs Arch Pharmacol. 2023; 396:421-440.
4. Pappalardo HD, Toscano V, Puglia GD, Genovese C, Raccuia SA. *Cynara cardunculus* L. as a multipurpose crop for plant secondary metabolites production in marginal stressed lands. Front Plant Sci. 2020; 11:240.
5. Mateus V, Estarreja J, Silva I, Barracosa P, Teixeira-Lemos E, Pinto R. Effect of *Cynara cardunculus* L. var. altilis (DC) in Inflammatory Bowel Disease. Appl Sci. 2021; 11:1629.
6. Baba Aissa F. Encyclopédie des plantes utiles (Flore d'Algérie et du Maghreb). Substances végétales d'Afrique, d'Orient et d'Occident. (1ère éd.). Ed. Edas; 1999. 178 p.
7. Hussain F, Kayani HUR, Bashir S. Antioxidant, antidiabetic and structural analysis of *Spinacia oleracea* leaf. Pak J Biochem Biotechnol. 2022; 3(1):1-11.
8. Ammarellou A and Mozaffarian V. The first report of iron-rich population of adapted medicinal spinach (*Blitum virgatum* L.) compared with cultivated spinach (*Spinacia oleracea* L.). Sci Rep. 2021; 11(1):22169.
9. Pomar C and Remus A. 242 The Impact of Feed Formulation and Feeding Methods on Pig and Poultry Production on the Environment. J Anim Sci. 2022; 100(3):124.
10. Popova A and Mihaylova D. Antinutrients in Plant-based Foods: A Review. Open Biotechnol J. 2019; 13:68-76.
11. El-Ramady H, Hajdu P, Törős G, Badgar K, Llanaj X, Kiss A, Abdalla N, Alaa Omara AD, Elsakhawy T, Elbasiouny H, Elbehiry F, Amer M, El-Mahrouk MS, Prokisch J. Plant Nutrition for Human Health: A Pictorial Review on Plant Bioactive Compounds for Sustainable Agriculture. Sustainab. 2022; 14(14):8329-8329.
12. Cerning-Beroard J. A note of sugar determination by the Anthrone method. Cereal Chem. 1975; 52:857-860.
13. Ghebregzabher M, Rufini S, Monaldi B, Lato M. Thin-layer chromatography of carbohydrates. J Chromatogr. 1976; 127(2):133-162.
14. Audigié C, Figarella J, Zonszain F. Manipulation d'analyse biochimique. (1st ed). Paris. 1980. 274 p.
15. Hodge JE and Hofreiter BT. Carbohydrates. In: Whistler RL and Miller JNB (eds.). Methods in carbohydrate chemistry. New York: Academic Press; 1962. 17-22 p.
16. Kakade ML, Rackis JJ, McGhee JG, Puski G. Determination of trypsin inhibitor activity of soy products: A collaborative analysis of an improved procedure. Cereal Chem. 1974; 51:376.
17. Lara-Cruz GA and Jaramillo-Botero A. Molecular Level Sucrose Quantification: A Critical Review. Sensors. 2022; 22:9511.
18. Mandim F, Petropoulos SA, Fernandes Â, Santos-Buelga C, Ferreira ICFR, Barros L. Chemical Composition of *Cynara Cardunculus* L. var. *altilis* Heads: The Impact of Harvesting Time. Agron. 2020; 10(8):1088.
19. Petkova N, Popova A, Alexieva I. Antioxidant properties and some phytochemical components of the edible medicinal *Malva sylvestris* L. J Med Plants Stud. 2019; 7(1):96-99.
20. Pereira C, Dias MI, Petropoulos SA, Plexida S, Chrysargyris A, Tzortzakis N, Calhelha RC, Ivanov M, Stojković D, Soković M, Barros L, C F R Ferreira I. The Effects of Biostimulants, Biofertilizers and Water-Stress on Nutritional Value and Chemical Composition of Two Spinach Genotypes (*Spinacia oleracea* L.). Molecules. 2019; 24(24):4494.
21. Sanyal R, Pradhan B, Jawed DM, Ghosh T, Yadav S, Bera, B, Behera TK, Pradhan S. Spatio-temporal expression pattern of raffinose synthase genes determine the levels of raffinose family oligosaccharides in peanut (*Arachis hypogaea* L.)

22. seed. Sci Rep. 2023; 13(795). https://doi.org/10.1038/s41598-023-27448-2
23. Degli Agosti R and Greppin H. Extraction, identification and assay of sugars in Spinach (*Spinacia oleracea* cv. Nobel) petioles during photoperiodic acclimation. Bot Helv. 1987; 97:329-340.
24. Sarmiento-Tomalá GM, Miranda-Martínez M, Gutiérrez-Gaitén YI, Delgado-Hernández R. Chemical Study, Antioxidant Capacity, and Hypoglycemic Activity of *Malva pseudolavatera* Webb & Berthel and *Malva sylvestris* L. (Malvaceae), Grown in Ecuador. Trop J Nat Prod Res. 2020; 4(12):1064-1071.
25. Petropoulos SA, Pereira C, Ntatsi G, Danalatos N , Barros L, Ferreira ICFR. Nutritional value and chemical composition of Greek artichoke genotypes. Food Chem. 2018; 267:296–302.
26. Bennett KA, Turner LM, Millward S, Moss SEW, Hall AJ. Obtaining accurate glucose measurements from wild animals under field conditions: comparing a hand-held glucometer with a standard laboratory technique in grey seals. Conserv Physiol. 2017; 5(1):cox013.
27. Silva LR, Jacinto TA, Coutinho P. Bioactive Compounds from Cardoon as Health Promoters in Metabolic Disorders. Foods. 2022; 11:336.
28. Cho YG and Kang KK. Functional Analysis of Starch Metabolism in Plants. Plants. 2020; 9(9):1152.
29. Proietti S, Paradiso R, Moscatello S, Saccardo F, Battistelli A. Light Intensity Affects the Assimilation Rate and Carbohydrates Partitionin in Spinach Grown in a Controlled Environment. Plants. 2023; 12:804.
30. Sabri FZ, Belarbi M, Sabri S, Alsayadi MMS. Phytochemical Screening and identification of some compounds from Mallow. J Nat Prod Plant Resour. 2012; 2(4):512-516.
31. Francaviglia R, Bruno A, Falcucci M, Farina R, Renzi G, Russo DE, Sepe L, Neri U. Yields and quality of *Cynara cardunculus* L. wild and cultivated cardoon genotypes. A case study from a marginal land in Central Italy. Eur J Agron. 2016; 72:10–19.
32. Mousavi SM, Hashemi SA, Behbudi G, Mazraedoost S, Gholami A, Omidifar N, Babapoor A, Rumjit NP. A review on health benefits of *Malva sylvestris* L. nutritional compounds for metabolites, antioxidants, and anti-inflammatory, anticancer, and antimicrobial applications. Evid-Based Complement Alternat Med. 2021; 2021: 5548404.
33. Yacout MHM. Anti-nutritional factors & its roles in animal nutrition. J Dairy Vet Anim Res. 2016; 4 (1):237-239.
34. Kunitz M. Crystalline soybean trypsin inhibitor. J Gen Physiol. 1947; 30(4):291-310.