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Evaluation of Antioxidant, Anti-Inflammatory, and Anti-Ulcer Activities of Yellow Apple (*Malus domestica* Borkh) Ethanol Extract from Algeria

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ABSTRACT

The yellow apples (*Malus domestica* Borkh.) belong to the Rosaceae family and are one of the most popular fruits in Algeria. This study evaluated the phenolic content, antioxidant, anti-inflammatory, and anti-ulcer effects of the ethanol extract of yellow apples. The ethanol extract contains a significant amount of phenolic compounds, flavonoids, and tannins with values of 85.06±3.05 mg GAE/g, 2.15±0.07 mg QE/g and 47.76 ± 0. 30 mg TAE/g, respectively. Antioxidant activity was studied using five assays, including DPPH, ABTS, hydroxyl radicals scavenging tests, reducing power, and iron chelation tests. The ethanol extract showed strong antioxidant activity with IC_{50} = 0.22 ± 0.01 mg/mL, 0.34 ± 0.02 mg/mL, 13.24 ± 0.61 mg/mL, 2.36 ± 0.13 mg/mL, and 2.14 ± 0.46 mg/mL, respectively. The anti-inflammatory property was estimated with the inhibition of protein denaturation and showed a percentage inhibition of 56.43% at 1 mg/kg. The ethanol extract administered at 200 and 600 mg/kg doses showed a gastro-protective effect on ethanol-induced ulcers in rats with percentages of protection of 89.49 ± 4.31% and 91.86 ± 1.58% for the 200 and 600 mg/kg doses, respectively. According to this study, consuming yellow apples may have potential benefits for reducing inflammation and preventing stomach ulcers.

Keywords: Apple, Polyphenols, Tannins, Antioxidant, Anti-inflammatory activity, Ulcer.

Introduction

Reactive oxygen species (ROS) are generated during regular cellular functions, primarily in cellular respiration and defence against external agents. They are composed of reactive chemical molecules, including hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-), and superoxide anion (O_2^-).[1]These species have an unpaired electron in their outer orbital, which makes them highly reactive. Excessive production of ROS causes a state of oxidative stress and has been linked to gastrointestinal diseases, neurodegenerative diseases, cardiovascular disease, diabetes, and many other diseases related to oxidative stress.[2]Gastric ulcer disease is a common gastrointestinal disorder that causes damage to the mucosal lining due to the production of pepsin and acid.[3] The leading causes of ulcers are believed to be *Helicobacter pylori* infection and chronic use of non-steroidal anti-inflammatory drugs (NSAID).[4]Additionally, NSAID use increases the risk of complications of ulcers such as upper gastrointestinal bleeding or perforation.[5]Free radicals, like superoxide anion, hydroxyl radical, and hydrogen peroxide, have been shown to play a role in the pathogenesis of ischaemic in the gastrointestinal mucosa, as well as other models of mucosal damage by ethanol, non-steroidal anti-inflammatory drugs, and *H. pylori*.[6] Natural antioxidants found in food, particularly in fruits and vegetables, are crucial for preventing diseases by the scavenging effect of free radicals.[7]

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Phenolic compounds are among the most common types of molecules in the plant kingdom. They can act as antioxidants through various mechanisms, including directly neutralising free radicals, metal chelation, and inhibiting lipid peroxidation. The yellow apples (*M. domestica* borkh.) belong to the Rosaceae family and are one of Algeria's most commonly consumed fruits. They contain a broad class of phytochemicals such as quercetin, catechin, phloridzin, andchlorogenic acid, which are essential antioxidants. The chemical content of apples varies during the maturation period and between varieties of apples.[8] Therefore, this study evaluated the phenolic content and antioxidant, anti-inflammatory, and gastro-protective activities of ethanol extract of *M. domestica* fruit grown in Algeria.

Materials and Methods

Plant material and extract preparation

The yellow apples were collected from Babour, Setif Province, in November 2022. They were identified by Professor Hocine Laouar of the Institute of Agronomy, Setif (Algeria),and a voucher specimen with authentication number MD 22/25 was deposited in the herbarium of the Faculty of Nature and Life Sciences at University Ferhat, Abbas, Setif 1, Algeria. The fruits were washed, the seeds were taken out, and then 1 kg of small fruit pulp was crushed. Phenolic compounds were extracted using ethanol following Markham's method[9] with some modifications. 100 g of yellow apple pieces were soaked in 500 mL of ethanol (80: 20 v/v) for 5 days. This solution was used to extract the most compounds. After filtration and evaporation, it was labelled and stored as an ethanol extract.

Animals

Rats weighing 200 to 250 g were used in this study and obtained from the Pasteur Institute in Algiers, Algeria. Rats were housed in a temperature-controlled environment for one week before being used in experiments. They had free access to food and water. The animal experiments were according to the guidelines of the Committee of the Algerian Association of Sciences in Animal Experimentation (http://aasea.asso.dz/articles/) under Law No.8808/1988, associated with veterinary medical activities and protection of animal health (N◦JORA: 004/1988).

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Phytochemicals analysis

Total polyphenol content estimation

The Folin-Ciocalteu method was used to estimate the total polyphenols content in the extract.[10] 100 µL of the sample was mixed with 500 µL of Folin-Ciocalteu reagent. After 4 minutes, 400 µL of sodium carbonate 7.5% was added. The absorbance of the mixture was measured at a wavelength of 765 nm after 1 and 30 mins. The results are expressed as the number of milligrams equivalent of gallic acid per gram of dry extract weight.

Flavonoid content estimation

The flavonoids of the extract were quantified using the $AlCl_3$ method.[11] 1 mL of the chosen dilution solution of the extract was added to 1 mL of $AlCl_3$ (2%). After incubation at laboratory temperature for 10 minutes, the absorbance was measured at a wavelength of 430 nm. Results were expressed as mg quercetin equivalent per g of dry extract weight.

Tannins content estimation

The tannin amount present in the extract was evaluated based on the sedimentation property of haemoglobin, which is characteristic of tannin.[12] Briefly, a blood volume (absorbency = 1.6) was added to the same extract volume (appropriate concentration). After the incubation for 20 minutes at room temperature, the centrifugation process was carried out for 10 minutes at a speed of 4000 rpm, and the supernatant was read at a wavelength of 576 nm. Results were expressed as mg tannic acid equivalent per g of dry extract weight.

In vitro antioxidant activity

DPPH scavenging test

DPPH is commonly used to assess the antioxidant activity of phenolic compounds and plant extracts. This test uses a 2, 2-diphenylpicrylhydrazyl (DPPH) molecule, a stable radical with a violet colour, and by its interaction with the antioxidant compound (AH), it turns yellow.[13]0.5 mL of different concentrations of the plant extracts were added to 0.5 mL of DPPH (0.004%) methanol solution. After incubating at room temperature and in the dark for 30 minutes, the absorption was measured at 517 nm. Free radical inhibition percentage (I%) was calculated as follows:

$$I\% = \frac{A\ control - A\ sample}{A\ control}\ x\ 100$$

A control is the absorbance of the blank solution (containing all reagents except the test compound), and A sample is the absorbance of the test compound. IC_{50} value (the concentration required to scavenge 50% DPPH free radicals) was calculated.

ABTS scavenging test

The ABTS test is one of the most widely used methods for determining the scavenging effect of plant extracts, as described by Re *et al*.[14]The ABTS radical was first prepared by mixing ABTS solution (7 mmol/L) and potassium persulfate solution (2.45 mmol/L) overnight and then diluting this solution with methanol to obtain an absorbance of 0.7 at 734 nm.50 µL of different concentrations of the sample extract were added to 1 mL of the methanol solution of ABTS. The mixture was incubated at room temperature in the dark for 30 min, and the absorbance was measured at 734 nm.ABTS free radical inhibition percentage (I%) was calculated using the same method as DPPH.

Hydroxyl radical scavenging test

The ability of the extract to scavenge hydroxyl radicals was measured using the Smirnoff and Cumbes method.[15] 0.5 mL of $FeSO_4$ (1.5 mmol), 0.350 mL of H_2O_2 (6mmol), and 0.150 mL of sodium salicylate (20 mmol) were added to 0.1 mL of sample test. The absorbance was read at 562 nm after one-hour incubation at 37 C°. The scavenging effect was calculated as follows:

$$\% = \frac{Acontrol - Asample}{Acontrol}\ x100$$

A control is the absorbance of the control (without sample).A sample is the absorbance of the test sample.

Reducing power test

The reducing power of the extract was assessed using the method developed by Chung *et al*.[16] In brief, 0.1 mL of various sample concentrations was mixed with the same volume of sodium phosphate buffer (0.2 M, pH = 6.6) and 0.1 mL of K_3FeCN_6. After mixture incubation for 20 minutes at 50 °C to reduce ferricyanide to ferrocyanide, 0.25 mL (1%) trichloroacetic acid was added to stop the reaction, followed by centrifugation at 3000 rpm for 10 minutes.0.25 mL of the supernatant was mixed with 0.25 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1%). The absorbance was measured at 700 nm.

Iron chelating activity

The extract's ability to chelate iron (Fe^{+2}) was assessed using the Decker and Welch method.[17] In brief, 50 µL (0.6 mM) $FeCl_2$ and 450 µL methanol were added to 250 µL extract solution. After five minutes, 50 µL (5 mM) ferrozine solution was added to start the reaction. The absorbance at 562 nm was measured after 10 minutes. The following equation was used to calculate ferrozine-Fe^{+2}inhibitions:

$$\% = \frac{Acontrol - Asample}{Acontrol}\ x100$$

A control is the absorbance of the control (without sample). A sample is the absorbance in the presence of the sample. The IC_{50} value, representing the concentration at which 50% inhibition occurs, was calculated. A lower IC_{50} value indicates a stronger chelating effect of the extract.

In vitro anti-inflammatory test

To investigate the anti-inflammatory activity of the extract, the protein denaturation method described by Akkouche *et al.* was adopted[18] with some modifications. Fresh eggs were washed and broken carefully, separating the egg whites from the yolks. The volume of the obtained egg whites was measured and then placed in a solution (20mM pH = 6, 8) Tris-HCL previously prepared to obtain a diluted solution (1/100, v/v). The solution was shaken gently for 10 minutes and then filtered. 2 mL of this solution was placed in test tubes containing different extract concentrations. After the incubation for 30 minutes at a temperature of 74°C, the absorbance was measured at 650 nm. Inhibition of protein degradation was calculated as follows:

$$\% = \frac{Acontrol - Asample}{Acontrol}\ x100$$

A control is the absorbance of the control (without sample). A sample is the absorbance in the presence of the sample.

Ethanol-induced gastric ulcer

Gastric ulcer was induced with ethanol to study the effect of *M. domestica* Borkh L. fruit extract on gastric ulcer according to the method of Abdulla *et al*.[19]Rats weighing between 200 and 250 g were used. The experimental animals were divided into four groups, each group consisting of five rats:

Group 1: the negative control was given distilled water.
Group 2: represented the positive control, which was treated with omeprazole (20 mg/kg) and approved as a standard anti-ulcer.
Group 3: treated with 200 mg/kg ethanol extract of apple fruit.
Group 4: treated with 600 mg/kg ethanol extract of apple fruit.
After 1 hr of treatment, each group received 2.5 mL/kg of ethanol (99%) by oral administration, and after 30 min, rats of all groups were sacrificed by cervical dislocation. The rats were dissected, the stomach was removed, and then it was opened according to the large curvature. It was properly cleaned using 0.9% NaCl solution. Then, it was fixed and photographed to calculate the area of ulcers using the Image J program.

$$Protection\% = \frac{ulceration\ rate\ of\ control - ulceration\ rate\ of\ sample}{ulceration\ rate\ of\ control}\ x100$$

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Statistical analysis

The *in vitro* experimental results were expressed as mean (M) ± the standard deviation (SD), while the *in vivo* results were expressed as mean ± standard error of the mean (SEM). A one-way analysis (ANOVA) followed by Dunnet's test was used to compare the values of the extracts with the control with the availability of the following criteria $p< 0.05$. Statistical results were carried out using Graph Pad Prism.

Results and Discussion

The ethanol extract's percentage extraction yield of 1kg of yellow apple fruit was 9.72%. The polyphenols, flavonoids, and tannins evaluations showed the values of 85.06±3.05 mg GAE/g, 2.15±0.07 mg QE/g, and 47.76 ± 0. 30 mg TAE/g of dry extract, respectively (Table 1). Phenolic compound constituents are extracted from fresh, frozen, or dried plant samples as a powder to facilitate the entry of the solvents into the plant cells, resulting in a more significant amount of obtained compounds.[20]In general, solvent polarity affects the extract yields and influences the class of compounds obtained and the amount of antioxidant molecules that can be extracted. The extraction time, temperature, ratios of sample to the solvent, and chemical composition of the plant also play an essential role in the extraction process.[21] Our results showed that ethanol with water can increase the extraction yield, as confirmed by the study of Baron and his collaborators[22], who showed that polar solvents, such as methanol, ethanol, and the mixture of methanol or ethanol with water, are among the most preferable solvent systems for the extraction of phenolic compounds. It was also shown to be effective in extracting phenolic compounds from various parts of plants, such as fruits, seeds, and leaves, which have been reported to be potent antioxidants. Polyphenols are present in the majority of fruits and vegetables. They are a diverse group of secondary metabolites that include one or more aromatic rings with several hydroxyl groups linked to the ring. Phenolic acids and flavonoids are the most common dietary polyphenols.[23]In this study, we found that the phenolic content in an ethanol extract from *M. domestica* fruits aligned with the results of Djenidi *et al.*[24], who analysed the total phenolic amount of methanol extract from M. *domustica* fruit grown in Algeria. Furthermore, we discovered that tannins, considered good sources of antioxidants, are abundant in apple fruit ethanol extract.[25]Our findings agreed with the results of Saoudi *et al.*[26], who reported a value of 31.08 mg tannic acid equivalent/g of dry apple extract. The ethanol extract exhibited antioxidant activity by different mechanisms (Table 2). The DPPH assay was used to evaluate the scavenging effect of free radicals by an antioxidant molecule. It is a common and simple colorimetric method for measuring antioxidant properties. [27] *M. domestica* extract had an effective antioxidant activity with IC_{50} = 0.22 ± 0.01 mg/mL compared to BHT used as a positive control (0.01 ± 0.001 mg/mL). Similarly, the extract could inhibit the ABTS radical with an IC_{50}value of 0.34 ± 0.02 mg/mL compared with EDTA; the extract has a lower ability than the control with an IC_{50} value of 0.015 ± 0.00 mg/mL. The OH^- ion produced in the presence of H_2O_2 and $FeSO_4$ oxidises iron (II) to iron (III) ions. By calculating the inhibitory concentration of 50% hydroxyl radicals compared to vitamin C, which was used as a reference drug, it was observed that the apple extract has a scavenging effect with an IC_{50} value of 13.24±0.61 mg/mL. As observed in Table 2, yellow apple extract was found to have a reducing capacity of 2.36 ±0.13 mg/mL compared to BHT, which gave an IC_{50} of 0.518±0.006 mg/ml. Iron chelation activity was measured by inhibiting the formation of a ferrozine-Fe^{2+} complex in the presence of apple extract. Ferrozine can form a complex red coloration with Fe^{2+} (ferrozine-Fe^{2+}). The extract showed the ability to inhibit the formation of ferrozine-Fe^{2+} complex with an IC_{50} value of 2.14 ± 0.46 mg/mL lower than EDTA, with the IC_{50} recorded at 0.003 mg/mL concentration. Antioxidants serve as radical scavengers and prevent lipid peroxidation, among other antioxidant processes. The extract's ability was tested using different methods to determine its antioxidant properties. The DPPH and ABTS tests are commonly used to evaluate the ability of antioxidant substances to scavenge free radicals in plant samples. The tests determine the extract's capacity to donate hydrogen, as indicated by the reduced colour intensity of the radicals' solutions.[28] ABTS radical is less stable than DPPH, which has a blue-green colour when adding the antioxidant compounds. To study its activity, the intensity of the colour decreases until it disappears; this is linked to these compounds' ability to donate hydrogen. In addition, ABTS is widely used in determining the antioxidant effect of food extracts because the ABTS radical has a maximum absorption at a wavelength of 734 nm. In contrast, most foods do not absorb at this wavelength, in addition to being suitable for both hydrophilic and lipophilic systems.[29]

Table 1: Yield extraction, polyphenols, flavonoids, and tannins content in ethanol extract of *M. domestica*

	Yield extraction (%)	Polyphenols content (mg GAE/ g)	Flavonoids content (mg QE /g)	Tannins content (mg TAE /g)
M. domestica ethanol extract	9.72	85.06±3.05	2.51±0.07	47.76±0.30

Results were expressed as mean ± SD.

Table 2: *In vitro* antioxidant activity of the ethanol extract of *M. domestica* using different assays

	DPPH IC_{50}(mg/mL)	ABTS IC_{50}(mg/mL)	OH° IC_{50}(mg/mL)	RP E_{C50}(mg/mL)	Chelating IC_{50} (mg/mL)
M. domestica extract	0.21 ±0.01***	0.34 ± 0.02***	13.24±0.61***	2.36 ±0.13***	2.14 ±0,46***
BHT	0.01±0.001	0.015±0.00034	-	0.051±0.006	-
EDTA	-	-	-	-	0.003±0.005
Vitamin C	-	-	0.084±0.007	-	-

Results were expressed as means + SD, n=3. *** $p< 0.001$, the comparison was realized against the correspondent standard.

Apple extract demonstrated a significant DPPH and ABTS radicals scavenging activity, possibly linked to its high polyphenolic content. The relationship between the radical scavenging and the polyphenol contents compositions has been previously reported by Angeli *et al.*[30],

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who found that apple extract had good antioxidant activity with IC_{50} values of 2.91±0.25, 1.82±0.14 and 1.53±0.12 mg of TEAC/g of fresh weight for three varieties. The hydroxyl radical is one of the most harmful radicals for biological systems. Its scavenging protects the body from many diseases. Numerous studies have examined the antioxidant properties of flavonoids and the link between their structures and radical scavenging activity. Flavonoids' effective radical scavenging needs a 3', 4'-dihydroxy group (catechol structure) in the B ring, which donates electrons to target radicals. Moreover, the 3-OH and 5-OH groups in relation to a 4-carbonyl function and C2-C3 double bond raise radical scavenging activity.[31]

Numerous compounds depend on their antioxidant mechanism in their reducing capacity. The term "reducing power" refers to the extract's capacity to convert Fe^{+3} to Fe^{+2}, demonstrating its antioxidant properties through electron transfer.[32] In the present study, apple extract had a significant reducing power that may be due to the high content of phenolic compounds. Some studies found a good correlation between plant extracts' phenolic amounts and antioxidant capacity.[33, 34]Free metal ions, such as iron and copper, are powerful catalysts in oxidation reactions and the production of free radicals, especially the hydroxyl radical, which is one of the most effective radicals in biological systems. Thus, iron chelating using plant extracts inhibits the Fenton reaction, reducing hydroxyl radical production. The results obtained in the study of Djenidi *et al*.[24]corroborate the capacity of yellow apple fruit extract to chelate the iron ions with an IC_{50} value of 9.18 ± 0.11 mg/mL. However, it is difficult to compare the results because several factors mentioned above affect the extraction of antioxidant compounds.

In the *in vitro* anti-inflammatory activity assay, the protective effect of heat denaturation of albumin protein in the presence of an ethanol extract was measured. The results indicated that the extract could protect proteins at a 1 mg/mL concentration with a 52.43% efficacy, compared to the Aspirin, the positive control, which exhibited 97.74% higher protection ability (Figure 1). Inflammation is the response of tissues to stimuli induced by different factors, including microbial infections, toxic chemical molecules, heat, and injuries. Protein denaturation is associated with the manifestation of various inflammatory diseases. Hence, compounds that protect proteins against denaturation may be considered anti-inflammatory agents**.** [35]As shown in this study, apple extract exhibited a dose-dependent inhibition of protein denaturation.

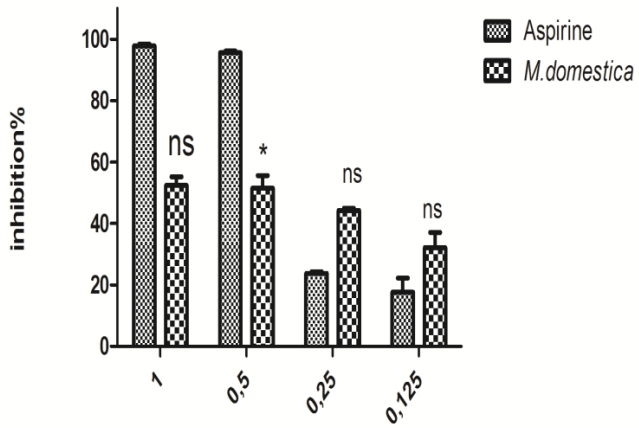

Figure 1: Inhibition of egg albumin protein degradation in the presence of *M. domestica* extract and aspirin. Data were presented as mean ± SD. ns: $p \geq 0.5$, *: $p \leq 0.1$, compared to aspirin.

Results of present study agreed with the analysis of Shallangwa *et al.* [36], who reported that the extract of *M. domestica* could protect the proteins' denaturation. Flavonoids are one of the most common secondary metabolites in fruits. Their biological activities are usually related to their structure. The C_2 and C_3 double bonds, 3', 4' OH in the B-ring and 5,7 OH in ring A, are important anti-inflammatory sites in flavonoids. Also, the presence of OH groups is crucial for inhibiting lipoxygenase enzymes.[37]Peptic ulceration represents one of the most common digestive diseases.

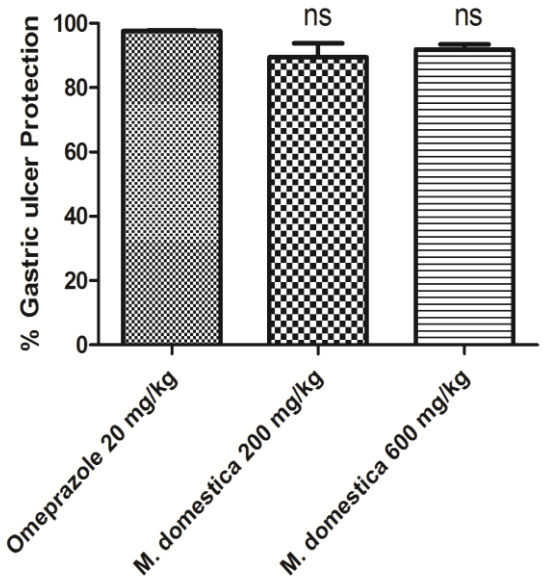

Figure 2: Percentage of protection after the pretreatment with apple ethanol extract and omeprazole. Results are expressed as means ± SEM (n = 5) ;(ns: no significant difference, compared to omeprazole.

Different studies suggest that *Helicobacter pylori* infection and non-steroidal anti-inflammatory drugs (NSAID) are significant causes of the pathogenesis of gastric ulcers. As a result of these factors, immune cells produce ROS.[38] Thus, yellow apple extract, which contains a high content of phenolic compounds, may be employed as an anti-inflammatory agent to avoid the detrimental effects of synthetic drugs. Rats with ethanol-induced gastric mucosa lesions were pre-treated. Oral administration of rats with ethanol extract prepared from *M. domestica* fruits (200 and 600 mg/kg) protected the mucosa from ulceration. Omeprazole (20 mg/kg) was used as a positive control agent. In comparison to the control group with an ulcer index of 31.96±2.41%, the treatment of rats with apple extract (200 and 600 mg/kg) reduced lesion area with ulceration percentages of 7.74±1.533 % and 2.616±0.8354%, respectively. The omeprazole group (20 mg/kg) as positive control showed an important reduction in gastric lesions (1.98±0.63 %). Ethanol extract showed a gastro-protective effect in a dose-dependent manner with a protection percentage of 89.49±4.31 and 91.68± 1.58 % for 200 and 600 mg/kg doses, respectively (Figure 2). The antioxidant mechanism of these polyphenols can significantly contribute to the treatment of ulcers. The gastro-protective effect of apple extract has been confirmed by Hamauzu *et al*.[39], who showed that phenolic compounds are the main functional factor in the anti-ulcer activity of apple juice. Abdelaleem *et al*.[40] indicated the ability of apple seed extract to protect the stomach mucosa of rats in gastric ulceration induced with indomethacin via antioxidant and anti-inflammatory effects.

Conclusion

The findings of this study suggest that the ethanol extract of yellow apple fruit, widely consumed in Algeria, contains a considerable

amount of phenolic compounds that could be beneficial in pharmaceutical applications. The extract also demonstrated strong antioxidant capacity, anti-inflammatory effects, and a high ability to protect the stomach mucosa from ethanol-induced ulcers. However, further studies on apple fruit extract are necessary to comprehend the exact mechanism of its protective effects and its relationship to antioxidants and inflammation.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

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

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