

Phytochemical Screening and Antioxidant Activity of *Uncaria tomentosa* Extract: *In Vitro* and *In Vivo* Studies

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Abstract

Background: *Uncaria tomentosa* is a traditional medicinal herb with antiviral, antioxidant, immunostimulating, anti-inflammatory, and anticancer effects. **Objective:** The present study was conducted to evaluate the antioxidant capacity *in vitro* and *in vivo* and the phytochemical analysis of *Uncaria tomentosa*. **Materials and Methods:** The plant extract was screened for phytochemical compounds and antioxidant capacity *in vitro* using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method and *in vivo* using acetic acid-induced colitis. Colitis was induced in rats by transrectal administration (5 mL/kg) of 4% (v/v) acetic acid. Forty adult albino rats were divided into four groups: control group, acetic acid group, acetic acid + sulfasalazine (100 mg/kg/day) group, and acetic acid + *Uncaria tomentosa* extract (250 mg/kg/day) group. After inducing colitis, sulfasalazine and *Uncaria tomentosa* extract were given orally for 10 days. Data were statistically analyzed, and $P < 0.05$ was considered statistically significant throughout the study. **Results:** Preliminary phytochemical study showed that *Uncaria tomentosa* extract contains flavonoids, phenols alkaloids, saponin, and terpenoids. In the DPPH assay, the extract exhibited considerable antioxidant capacity in a dose-dependent manner. Also, *Uncaria tomentosa* extract dramatically decreased oxidative stress parameters, such as myeloperoxidase enzyme activity and malondialdehyde in colonic tissue. Moreover, *Uncaria tomentosa* treatment attenuated macroscopic colonic scores and histopathological changes induced by acetic acid. **Conclusion:** The findings of this study show that *Uncaria tomentosa* extract could be a source of natural antioxidants and may have a therapeutic effect on ulcerative colitis.

Keywords: Antioxidant, colitis, DPPH, extract, polyphenols, *Uncaria tomentosa*

INTRODUCTION

Herbal remedies have been used to treat diseases for centuries and are considered the primary source of most found medicines.^[1] Medicinal herbs mainly contain several phytochemicals, such as glycosides, alkaloids, triterpenes, saponins, and polyphenols, which are assumed to work synergistically and can be used to treat different diseases.^[2] *Uncaria tomentosa* (UT) (Willd.) DC., widely known as cat's claw, is a member of the Rubiaceae family. It is a creeper vine that grows mainly in the rainy tropical forests of Central and South America. It has long been used as a medicine because of its medicinal characteristics such as anti-inflammatory, antiviral, antioxidant, immunostimulating, and antimutagen agent.^[3] UT contains various chemical components, including oxindole alkaloids, triterpenes, tannins, sterols, flavonoids,

glycosides, and various polyphenols.^[4] Most studies have shown UT's anti-inflammatory, and antioxidant effects arise from oxindole alkaloids. Recently, it was found that the pharmacological activity of this species derives from the synergistic effect of several compounds, especially polyphenolic compounds.^[5] Therefore, we have chosen UT to screen its phytochemical compounds and to investigate its antioxidant properties *in vitro* and *in vivo* against colitis.

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is a practical, efficient, and inexpensive method for assessing *in vitro* antioxidant activity.^[6] DPPH is a stable,

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deep-purple free radical with a notable absorption band at 515–520 nm. It can receive a hydrogen atom or an electron from an antioxidant molecule to become more stable. Antioxidant activity can be determined spectrophotometrically. The less DPPH purple color in the sample, the more free radicals were eliminated.^[7] The data are given as an inhibitory concentration (IC₅₀). IC₅₀ is the sample concentration required to lower the starting DPPH concentration by 50%.^[8]

For *in vivo* estimation of the antioxidant activity of UT, we have chosen acetic acid (AA)-induced colitis as a model for colitis because it is similar to human inflammatory bowel disease in its cause, histopathology, and proinflammatory cytokine profile.^[9] Ulcerative colitis (UC) is an inflammatory bowel disease characterized by mucosal inflammation restricted to the colon and rectum.^[10] The pathophysiology of UC remains unclear. In general, it is widely thought that this disorder is closely related to the high generation of reactive oxygen species and the overexpression of inflammatory cytokines such as tumor necrosis factor- α , interleukin-6, and interleukin-1 β .^[11,12] The proinflammatory nature of oxidative stress byproducts promotes the deterioration of the colonic mucosa and the entry of pathogens, which triggers an immune response and results in cell death and necrosis.^[13,14] Therefore, controlling inflammation and oxidative stress is a critical therapeutic goal for UC.^[15] Thus, the current study directed to explore the phytochemical compound of UT and its antioxidant effect *in vitro* and *in vivo*.

MATERIALS AND METHODS

Preparation of *Uncaria tomentosa* extract

The plant UT is cultivated and collected from Kirkuk, Iraq. Then it was dried in the shade for several days and was grounded finely. About 500 g of grounded plant powder of UT was macerated with a mixture of ethanol:water (80:20) in the dark at room temperature (22–25°C) for 3 days. The mixture was then filtered using Whatman no. 1 filter paper. The filtrate was then condensed at a temperature not exceeding 40°C using a rotary evaporator (Heidolph) to get greenish gummy exudate (60 g), which was kept at 4°C until employed for phytochemical analysis and in the experiment.^[15]

Phytochemical screening and high-performance liquid chromatography analysis

The crude extract of UT was used for phytochemical screening to identify its chemical compound.^[16] Then, High-performance liquid chromatography (HPLC) analysis by liquid chromatography (Shimadzu, USA) was used to determine the main flavonoids in the plant extract, in which identifications were accomplished by comparing the retention times of examined materials to authentic standards prepared under identical chromatographic conditions.

In vitro antioxidant activity by using 2, 2-diphenyl-1-picrylhydrazyl assay

The antioxidant capacity of the extracts was evaluated using DPPH method.^[17] A stock solution of DPPH (1.3 mg/mL) was produced in ethanol. Mixing 100 μ L of DPPH with 3 mL of ethanol, the absorbance at 517 nm was measured. Ascorbic acid was employed as a reference standard. The different concentrations of the extract and ascorbic acid (50, 75, 100, 150 μ g/mL) were prepared. Every 1 mL sample solution was diluted with 3 mL ethanol, and 100 μ L from DPPH stock solution was added. The test tubes were maintained in the dark at room temperature for 30 min. Then the absorbance was read at 517 nm using a spectrophotometer (Shimadzu, Japan) against ethanol as a blank. The DPPH antioxidant activity was calculated according to this formula:

$$\% \text{Inhibition} = \left[\frac{(A_0 - A_1)}{A_0} \right] \times 100,$$

where A₀ was the absorbance of the control and A₁ was the extract's or standard's absorbance. Then, the IC₅₀ was determined through linear regression analysis of the dose-response curve plotting between % inhibition and concentrations.

Ethical approval

All methods for the current study's experimental protocol and the subject information and consent form were reviewed and approved by a local ethics committee according to document number (20200865) on 8/12/2020. This research was carried out following the Helsinki Declaration's guidelines.

Animals and study design

Forty male albino Wister rats (200 $\pm$ 20 g) were obtained from the College of Science, Babylon University. The animals were housed in standard plastic cages at a temperature 28°C–30°C and 50% humidity, with 12 h of light/dark cycle, had free access to a commercial diet, and were allowed to drink tap water. The rats were separated randomly into four groups (10 in each group): group 1 (control group) received an intrarectal infusion of normal saline (2 mL) on the day of induction, whereas other groups received 4% v/v AA (5 m/kg) transrectally on the day of induction; group 2 (colitis group) received normal saline orally; group 3 received sulfasalazine 100 mg/kg/day orally^[18] (positive control); group 4 received orally UT extracts 250 mg/kg/day.^[19] All treatment started 2 h after the AA installation and continued the treatment for 10 days.^[20] After 24 h from the final oral dose of the treatments, rats were anesthetized with diethyl ether to sacrifice them. The abdomen was dissected, and the colon was excised and cleaned with chilled normal saline. The colon of all rats was assessed macroscopically. Then, the samples were cut into two pieces: one for fixation in 10% neutral formalin for histopathological study. The

second part was kept at -80°C for tissue homogenization until oxidative stress parameters could be measured.

Colitis induction

The colitis was produced using AA in accordance with the method described by Atarbashe and Abu-Ragheb.^[21] Before inducing colitis, rats were fasted for at least 24 h to empty the colon of feces, but the water was interrupted for 2 h before producing colitis. Briefly, under anesthesia with light ether, rats received 5 mL/kg of 4% v/v AA solution as a single transrectally infusion (8 cm away from the anus) using a medical polyurethane tube for enteral feeding. After AA installation, rats were held in a horizontal position for 2 min to prevent leakage of AA. The rats in the control group received the same treatment using 0.9% normal saline instead.

Assessment of macroscopic colonic score

The degree of macroscopic alteration was evaluated by awarding scores.^[22] Briefly, the grading system is as follows: no visible change (grade 0); no ulcer, mucosal hyperemia only (grade 1); hyperemia and mild edema with little erosion, no ulcers (grade 2); one ulcer and inflammation at one site (grade 3); two or more site of inflammation or ulceration (grade 4); severe ulceration extending >1 cm along the colon and tissue necrosis (grade 5); and damage extending >2 cm along the entire colon and tissue necrosis (grade 6).

Measurement of myeloperoxidase activity and malondialdehyde level

Myeloperoxidase (MPO) activity was determined in colon tissue samples using a modified method described by Bradley *et al.* (1982).^[23] 0.1 g of colon tissue was homogenized in 1 mL of potassium phosphate buffer (50 mM, pH = 6). The homogenate was homogenized in an ice bath with 5 mL buffer solution. Then, the homogenate was centrifuged at 4°C for 15 min at 15,000 rpm. The final amount was 3 mL of phosphate buffer (50 mM), 0.167 mg/mL O-dianisidine dihydrochloride, and 0.0005% hydrogen peroxide. Lastly, by measuring absorbance at 460 nm using a spectrophotometer, MPO activity was calculated as U/g (units/gram).

The amount of malondialdehyde (MDA) was determined spectrophotometrically at 535 nm based on the interaction between MDA and thiobarbituric acid according to the method previously described by Buege and Aust (1978).^[24]

Histopathological study

For histological evaluation, the tissues of samples were preserved in 10% neutral formalin and incorporated in paraffin blocks. Then, 5- μm thick sections were sliced and stained with hematoxylin and eosin (H&E). An evaluation of microscopic abnormalities of a colonic lesion according to previously reported grading criteria^[25] was as follows: (1) loss of the architecture of mucosa (0–3), (2) muscle thickening (0–3), (3) leukocyte infiltration (0–3), (4)

formation of crypt abscess (0–1), and (5) loss of goblet cells (0–1); for a scale of 0–3 = absent, mild, moderate, and severe and scores 0–1 = absent or present. A blinded histopathologist examined each slide under a microscope; the maximum score was 11.

Statistical analysis

All data were expressed as mean $\pm$ standard deviation (SD) and analyzed by statistical package for social sciences, SPSS version 23. One-way of variance (analysis of variance) followed by least-significant difference test as a *post hoc* test was used to show significant differences between groups. The Spearman correlation test measured the linearity of the DPPH result. $P < 0.05$ was considered statistically significant throughout the study.^[26]

RESULTS

Phytochemical screening of *Uncaria tomentosa* extract

The qualitative phytochemical tests of the UT extract detected the presence of flavonoids, alkaloids, steroids, saponins, and terpenoids. According to high-performance liquid chromatography analysis, several flavonoids were found in UT hydroethanolic extract such as catechin, rutin, p-Coumaric acid, quercetin, ferulic acid, apigenin, kaempferol, luteolin, and isorhamnetin at different retention time, as shown in Table 1.

In vitro antioxidant activity of *Uncaria tomentosa*

As shown in Figure 1 and Table 2, the DPPH antioxidant capacity of UT was compared to that of ascorbic acid, which provided as a reference. Extract and standard IC₅₀ values were 74.34 g/mL and 33.34 g/mL, respectively. There is a significant direct relationship between extract and standard concentration with inhibition percentage.

In vivo antioxidant activity of *Uncaria tomentosa*

Effect of Uncaria tomentosa extract on oxidative stress parameters (myeloperoxidase activity and malondialdehyde level)

Rectal installation of AA induced a highly significant increase ($P < 0.001$) in MPO activity (76.29 ± 2.23) and

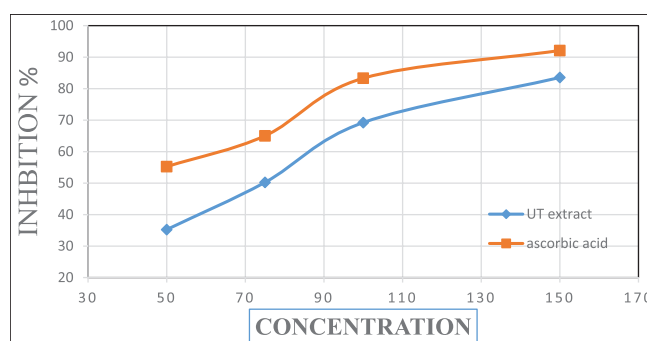


Figure 1: Correlation between the concentration of UT extract and ascorbic acid with inhibition percentage of DPPH

Table 1: High-performance liquid chromatography analysis of the main flavonoids in UT extract shows retention time in minutes and quantity in ppm

Types of flavonoids	R _i (min) of standard	R _i (min) of UT	Quantity of standard	Quantity of UT
Catechin	1.293	1.318	5	4.8
Rutin	1.562	1.565	5	5.13
p-Coumaric acid	1.774	1.780	5	5.5
Quercetin	1.923	1.921	5	5.1
Ferulic acid	2.089	2.09	5	5.1
Apigenin	2.610	2.600	5	5.12
Kaempferol	2.897	2.870	5	5.12
Luteolin	3.556	3.505	5	5.05
Isorhamnetin	3.845	3.790	5	5.02

ppm: part per million, R_i = retention time**Table 2: Antioxidant capacity of UT extract determined by the DPPH test**

Concentration (μg/mL)	% inhibition (DPPH scavenging)	
	UT extract	Standard (ascorbic acid)
50	35.24	55.23
75	50.23	65.01
100	69.22	83.3
150	83.53	92.1
IC50 value	74.34	33.34

MDA level (48.92 ± 1.42) in colonic tissue as compared to the control group (18.29 ± 1.82 ; 10.63 ± 1.23), respectively, as shown in Figure 2a and b.

Also, treatment with UT extract and sulfasalazine significantly ($P < 0.001$) decreased the increment in the MPO activity (44.30 ± 2.06 and 30.27 ± 2.06), respectively, and decreased the MDA level (27.45 ± 1.9 and 18.82 ± 1.66), respectively, upon comparison with colitis group, as shown in Figure 2a and b.

Effect of *Uncaria tomentosa* extract on macroscopic changes score and histopathological changes

The macroscopic feature was assessed with grades ranging from 0 to 6. The control group did not have a score 0.00 ± 0.00 , whereas the colitis group manifested significant damage (5.15 ± 0.26), such as thickening of the colon and massive tissue necrosis and ulceration. Treatment with UT extract and sulfasalazine significantly lowered the extent of the visible damage (2.83 ± 0.41 and 1.96 ± 0.33), respectively ($P > 0.001$; Table 3).

In addition, as shown in Figure 3, specimens from the control group exhibited normal colon histology. Rectal injection of AA caused diffuse damage and necrosis with severe mucosal ulceration and was associated with massive inflammatory cell infiltration. Therefore, the colitis group has a higher histopathological score than the control

group ($P < 0.001$; Table 2). Treatment with UT extract and sulfasalazine resulted in an improved histological pattern with fewer mucosal ulcers, preservation of crypts, and markedly decreased inflammatory cell infiltration. Therefore, AA + UT and AA + sulfasalazine groups had lower microscopic scores than the colitis group ($P < 0.001$; Table 3).

DISCUSSION

The preliminary phytochemical screening of UT detected the presence of alkaloids, flavonoids, polyphenols, steroids, saponins, glycosides, and terpenoids. This result was consistent with a study reported by Laus *et al.*^[27] that revealed the accumulation of oxindole alkaloids in the UT leaves and bark. Also, other studies have demonstrated that a cat's claw contained substances other than oxindole alkaloids, which may be responsible for its medical properties, such as coumarins, flavonoids, quinovic acid glycosides, and triterpenes.^[28]

According to high-performance liquid chromatography analysis in this study, the hydroethanolic extract of UT contains different flavonoids and polyphenols, such as catechin, rutin, p-Coumaric acid, quercetin, ferulic acid, apigenin, kaempferol, and others, in high proportion. Phenolic chemicals are essential plant ingredients known to be potent chain-breaking antioxidants. Phenolic substances may directly contribute to antioxidant activity.^[29] According to some research, human mutagenesis and carcinogenesis may be inhibited by phenolic substances.^[30,31]

DPPH scavenging capacity has been widely utilized as a rapid and accurate indicator to evaluate plant extracts' general antioxidant activity *in vitro* attributed to phenolic components.^[32] Antioxidants are believed to affect DPPH because of their ability to donate hydrogen.^[33] The results of the DPPH antioxidant capacity of the UT show a direct relationship between UT extract concentration and the percentage of inhibition. Even though the DPPH result of the extract is less than that of ascorbic acid, this finding demonstrated that the extract can donate protons and may act as a free radical inhibitor or scavenger. Our finding is compatible with the previous study reported by Navarro *et al.*^[34] who found that the UT extract exhibited a high level of antioxidant activity, as measured by DPPH capacity, peroxy radical-trapping capacity, and superoxide radical scavenging activity.

This study investigated the potential antioxidant effects of UT extract on AA-induced colitis in rats. The experimental model of UC induced by AA closely resembles the human UC in clinical manifestations such as weight reduction, diarrhea, and macroscopic and microscopic abnormalities.^[35] In the present study, the untreated colitis group demonstrated a significant increase in inflammatory inflow, ulcerative area, and colonic necrosis compared to the control group. All of

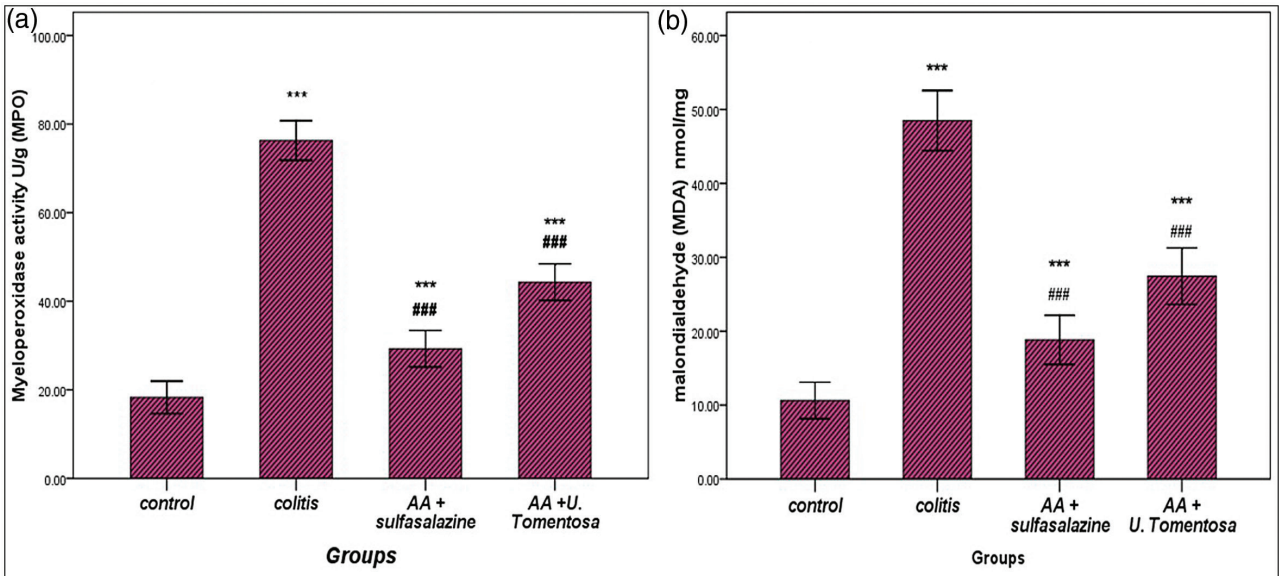


Figure 2: Effect of UT extract and sulfasalazine on (a) MPO and (b) MDA. ****P* < 0.001 vs. control group and ###*P* < 0.001 vs. AA group

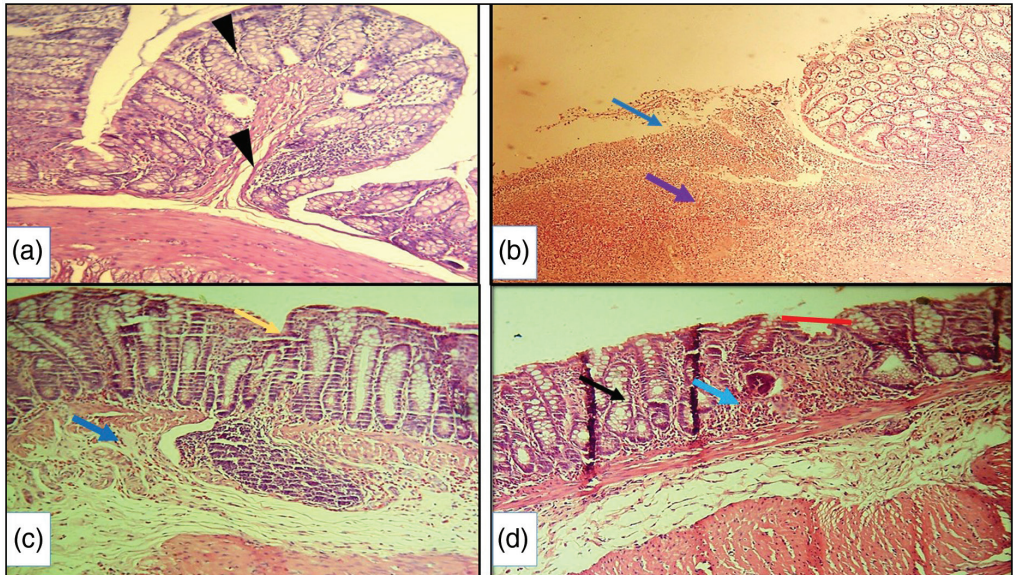


Figure 3: Photomicrographs of colonic tissue sections for (a) control group shows intact colonic mucosa and submucosa (arrowhead); (b) colitis group shows UC features: ulceration (blue arrow) and inflammatory cells infiltration (purple); (c) AA + sulfasalazine and (d) AA + UT extract groups show an improved histological feature with mild erosion mucosa (yellow arrow), less inflammatory cell infiltration (blue), a smaller ulcer (red line), and mucosal regeneration (black arrow); H&E stain, $\times 10$

Table 3: Histopathological changes score and macroscopic changes score of experimental groups				
Parameter	Control group	Colitis group	AA + sulfasalazine group	AA + UT group
Histopathological score	0.0 $\pm$ 0.0	10.48 $\pm$ 0.44***	4.00 $\pm$ 0.32###	5.92 $\pm$ 0.35###
Macroscopic score	0.00 $\pm$ 0.00	5.15 $\pm$ 0.26***	2.83 $\pm$ 0.41###	2.83 $\pm$ 0.41###

SD, standard deviation; Values are expressed as mean $\pm$ SD
****P* < 0.001 vs. control group
###*P* < 0.001 vs. AA group

the previously stated variables are major UC indicators.^[36] The findings revealed that UT extract significantly mitigated AA-induced colitis in rats. Our findings were agreed with a previous study that found the UT aqueous extract protected human erythrocytes from oxidative stress and relieving chronic intestinal inflammation caused by indomethacin.^[2,37]

Oxidative stress is a critical factor contributing to tissue damage in UC. Excessive production of free radicals by activated macrophages and neutrophils in inflamed mucosa leads to the pathophysiology of UC and

damages the intestinal mucous membranes.^[38] Finally, our finding showed that the MDA level was elevated in the colonic tissue of the untreated colitis group, but treatment with UT extract decreased its tissue level. MPO is an indicator of leukocyte infiltration and was upregulated in inflammatory bowel disease.^[39] This study showed elevated MPO activity in the colitis group and dramatically reduced with UT extract treatment. Consequently, it supports UT extract's antioxidant and anti-inflammatory action.

CONCLUSIONS

The antioxidant analysis showed that UT has a higher antioxidant capacity and therapeutic antioxidant effects on experimentally induced colitis. This result may be because it contains flavonoids, polyphenols, and other phytochemicals. This study's findings indicated that UT might be a source of natural antioxidants that could be useful in preventing or slowing the progression of oxidative stress-related degenerative diseases.

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Conflicts of interest

There are no conflicts of interest.

Authors' contribution

All authors contributed to the conception and design of the study, analysis of data, statistical analysis, preparation, editing, and reviewing of the article.

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