

Chemical and antioxidant studies on *Crataegus pentagyna* leaves and flowers

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Abstract

The aim of this study was to investigate the antioxidant potential of *Crataegus pentagyna* leaves and flowers in relation to the phenolic content. The total phenolic, flavonoid and proanthocyanidin contents were determined using Folin-Ciocalteu, aluminium chloride and acid butanol assays, respectively. The radical scavenging activity was evaluated by several in vitro assays: Trolox equivalent antioxidant capacity assay, superoxide anion and hydroxyl radicals scavenging assays. In addition, ferrous ion chelating activity and 15-lipoxygenase inhibition were also investigated. Both leaf and flower extracts had high total phenolic (206.94±4.86 and 184.62±1.71 mg/g, respectively), flavonoid (57.08±0.21 and 67.04±0.52 mg/g, respectively) and proanthocyanidin contents (68.92±1.81 and 97.70±3.81 mg/g, respectively), scavenged ABTS (TEAC=0.64±0.01 and 0.65±0.01 µM Trolox equivalent to 1 µg/mL extract, respectively), superoxide (EC₅₀=0.9±0.0 mg/mL) and hydroxyl radicals (EC₅₀=0.9±0.0 and 0.86±0.05 mg/mL, respectively), chelated ferrous ions (EC₅₀=1.3±0.0 and 1.9±0.0 mg/mL, respectively) and inhibited 15-lipoxygenase (EC₅₀=129.63±0.75 and 151.76±1.65 µg/mL, respectively). It is noteworthy that both extracts showed a higher superoxide anion scavenger activity than catechin (EC₅₀=2.2±0.0 mg/mL). The results of the study indicate that *Crataegus pentagyna* leaves and flowers are a promising source of natural antioxidants.

Keywords: *Crataegus pentagyna* Willd. ssp. *pentagyna*, polyphenols, reactive oxygen species, scavenging activity, chelating activity, 15-lipoxygenase, cardiovascular disease

1. Introduction

The genus *Crataegus* (Rosaceae, hawthorn) consists of more than 280 species that are widespread throughout the world. *Crataegus monogyna* Jacq. (Lindm.) and *Crataegus laevigata* (Poir.) D.C. are found and used in Europe, *Crataegus pinnatifida* Bunge and *Crataegus cuneata* Siebold & Zucc. in China, *Crataegus sinaica* Boiss. in Egypt and *Crataegus aronia* syn. *azarolus* (L.) in Israel (LJUBUNCIC & al. [1], REFAAT & al. [2], KWOK & al. [3], JURIKOVA & al. [4]).

In Europe extracts of hawthorn leaves, flowers and fruits are used as dietary supplements and herbal medicines in the treatment of cardiovascular disorders due to their coronarodilating, positive inotropic, hypotensive and antiarrhythmic effects. Polyphenols, namely flavonoids and oligomeric proanthocyanidins, have been identified as active constituents in these extracts (VERMA & al. [5]).

Both *in vitro* and *in vivo* studies showed the complexity and unique profile of the activity exhibited by hawthorn extracts on the cardiovascular system. The most studied extracts were those isolated from *Crataegus laevigata*. Hawthorn extracts showed positive inotropic effects due to the inhibition of the myocardial Na^+/K^+ -ATPase. Hawthorn extracts decreased blood pressure by inhibition of angiotensin converting enzyme, phosphodiesterase-3 and endothelin-1 release from endothelium thus causing vasorelaxation. In addition, hawthorn extracts activated endothelial nitric oxide synthase with a subsequent release of high levels of nitric oxide, a vasodilating agent. It is worthy to note that hawthorn extracts showed the ability to regulate not only high, but also low blood pressure. The mechanism of this dual activity has not been elucidated yet. Hawthorn extracts enhanced coronary flow, increased the ability of cardiomyocytes to consume oxygen, protected cardiomyocytes from ischemia-reperfusion injury and prevented reperfusion-induced arrhythmia (JURIKOVA & al. [4]), VERMA & al. [5]). The protective activity on cardiomyocytes was associated with a significant decrease in oxidative stress (decrease of xanthine oxidase and NADPH oxidase expressions), upregulation of anti-apoptotic proteins (Bcl-2, Hsp 70), downregulation of pro-apoptotic proteins (cytochrome c, caspase-3), decrease in lactate dehydrogenase and creatine kinase activity (SWAMINATHAN & al. [6]). Apart from cardiac glycosides, hawthorn extracts showed negative chronotropic effects without causing arrhythmia. On the contrary, they exhibited antiarrhythmic effects. Recent studies elucidated several mechanisms with possible implications in the antiarrhythmic activity: prolongation of action potential duration, blockade of potassium currents, decrease in the number of ventricular ectopic beats (MÜLLER & al. [7], [8], GARJANI & al. [9]).

Crataegus pentagyna Willd. ssp. *pentagyna* is spread in the southern and south-eastern regions of Romania (PARVU & al. [10]). A literature survey revealed only few biological investigations on the leaves and flowers of *Crataegus pentagyna* growing in Turkey (ÖZYÜREK & al. [11]). In this respect, the aim of this work was to evaluate the antioxidant potential of the leaves and flowers of *Crataegus pentagyna* growing in Romania in relation to the phenolic content. The present work is part of a larger study aiming to explore the possibility of using *Crataegus pentagyna* leaf and flower extracts as ingredients in dietary supplements for cardiovascular diseases and other pathological conditions associated with oxidative stress.

2. Materials and methods

Plant material

Crataegus pentagyna leaves and flowers were collected in Tulcea district in June 2010 and were authenticated in the Department of General Plant and Animal Biology, Faculty of Pharmacy, Ovidius University of Constanta. Before extraction, the leaves and flowers were air-dried in dark at ambient temperature (25°C). Herbarium voucher samples (P-l-2010, P-f-2010) are deposited in the Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy Grigore T. Popa-Iasi.

Chemicals

Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid and ferrous chloride were purchased from Merck (Darmstadt, Germany). 2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ethylenediaminetetraacetic acid (EDTA), pyrogallol, Folin-Ciocalteu's phenol reagent, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4''-disulfonic acid monosodium salt (ferrozine), linoleic acid, lipoxylase from soybean were from Fluka (Steinheim, Germany). (+)-Catechin hydrate, gallic acid, sodium carbonate, iron (II) sulfate

heptahydrate, hydrogen peroxide, sodium salicylate, (R)-(+)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Sigma-Aldrich (Steinheim, Germany). Ammonium iron (III) sulfate dodecahydrate, potassium persulfate and sodium nitrite were obtained from Riedel-de Haën (Seelze, Germany). All other chemicals were of analytical grade.

Extraction

50 g of dried and powdered plant material (leaves and flowers, respectively) were extracted with 500 mL methanol:water (7:3, v/v) for 6 h at room temperature under continuous stirring. The extracts were filtered under vacuum and the residues were re-extracted twice in the same conditions. The extracts were combined, evaporated under reduced pressure at 40°C and freeze-dried. Both extracts were kept in sealed glass tubes at -20°C until studied.

Total phenolic content

The total phenolic content was estimated using Folin-Ciocalteu method as described previously (WANGENSTEEN & al. [12], SINGLETON & ROSSI [13]). Briefly, each extract (0.04 mL) was mixed with ultrapure water (3.16 mL) and Folin-Ciocalteu's phenol reagent (0.2 mL). After 5 min, 20% sodium carbonate (0.6 mL) was added followed by vigorous mixing. The reaction mixture was incubated for 2 h at room temperature before the absorbance was measured at 765 nm. The total phenolic content was expressed in terms of mg gallic acid equivalents (GAE)/g of extract.

Flavonoid content

The total flavonoid content was determined by aluminium chloride assay (OZSOY & al. [14]). Each extract (0.25 mL) was mixed with ultrapure water (1.25 mL) and 5% sodium nitrite (0.075 mL). After 6 min 10% aluminium chloride (0.15 mL) was added. The mixture was incubated for another 5 min followed by the addition of 1 M sodium hydroxide (0.5 mL) and ultrapure water (0.275 mL). After vigorous shaking the absorbance was measured immediately at 510 nm. The total flavonoid content was expressed in terms of mg (+) catechin equivalents/g of extract.

Proanthocyanidin content

The total proanthocyanidin content was estimated by acid butanol assay (PORTER & al. [15]). Briefly, the reaction mixture consisted of extract (0.5 mL), *n*-butanol-hydrochloric acid reagent (95:5, v/v, 3.0 mL) and ferric reagent (2% ferric ammonium sulfate dodecahydrate in 2 N hydrochloric acid, 0.1 mL). The mixture was shaken vigorously and kept in the water bath at 95°C. After 40 min the mixture was cooled and the absorbance was recorded at 550 nm. The total proanthocyanidin content (mg cyanidin/g extract) was calculated using the molar extinction coefficient of cyanidin ($\epsilon = 17,360 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$) (QA'DAN & al. [16]).

Trolox equivalent antioxidant capacity assay

The assay was used to evaluate the Trolox equivalent antioxidant capacity (TEAC) of *Crataegus pentagyna* extracts. In brief, different concentrations of extracts or Trolox (0.02 mL) were mixed with ABTS radical cation ethanol solution (1.98 mL; $A_{734 \text{ nm}}=0.70\pm 0.02$). The decrease in absorbance at 734 nm was measured after 6 min reaction time at 30°C. (+)-Catechin hydrate was used as positive control. For calculating TEAC values, the percentage of absorbance decrease as a function of concentration was plotted for each extract, Trolox and catechin. TEAC values were calculated as follows: slope of dose-response curve of extracts or catechin/slope of dose-response curve of Trolox (RE & al. [17]).

Superoxide anion radical scavenging assay

Superoxide anion radical scavenging activity was determined by self-oxidation of 1,2,3-phentriol (pyrogallol) assay (WANG & al. [18]). Different concentrations of each extract (0.1

mL) were mixed with Tris-HCl buffer (0.05 M, pH 8.0) containing 1 mM EDTA (2.8 mL) and pyrogallol (6 mM, 0.2 mL). The mixture was vortexed and the absorbance at 325 nm was recorded every 30 s over a period of 4 min. (+)-Catechin hydrate was used as positive control. The inhibitory activity on the self-oxidation of 1,2,3-phentriol was calculated from the absorbance vs. time curves using the following formula: $(1 - \text{slope of extract or catechin} / \text{slope of control}) \times 100$.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was evaluated according to a described procedure with minor changes (JEONG & al. [19]). Different concentrations of each extract (0.225 mL) were mixed with 20 mM sodium salicylate (0.9 mL), 1.5 mM FeSO₄ (0.75 mL) and 6 mM H₂O₂ (0.525 mL). After 30 min incubation at 37°C, the absorbance was measured at 562 nm. (+)-Catechin hydrate was used as positive control. Hydroxyl radical scavenging activity (%) was calculated using the formula: $(1 - \text{absorbance in the presence of extracts or catechin} / \text{absorbance of control}) \times 100$.

Ferrous ion chelating assay

The ability to chelate ferrous ions was measured by ferrozine assay (DINIS & al. [20], TUNG & al. [21]). Various concentrations of each extract (0.4 mL) were mixed with methanol (1.48 mL), 2 mM ferrous chloride (0.04 mL) and 5 mM ferrozine (0.08 mL). After vigorous shaking, the mixture was allowed to stand 10 min at room temperature. The absorbance was measured at 562 nm. EDTA was used as positive control. The ferrous ion chelating activity (%) was calculated using the equation: $(1 - \text{absorbance in the presence of extracts or EDTA} / \text{absorbance of control}) \times 100$.

15-Lipoxygenase inhibition assay

Briefly, different concentrations of extracts (0.05 mL) were mixed with 0.2 M borate buffer at pH 9.0 containing 10,000 U/mL of 15-lipoxygenase (0.95 mL). After 10 min incubation at 25°C, linoleic acid in borate buffer was added (2 mL). The increase in absorbance at 234 nm was recorded for 90 s. (+)-Catechin hydrate was used as positive control. 15-Lipoxygenase inhibition (%) was determined on the basis of the absorbances of the control and samples after 30 and 90 s reaction time using the formula: $100 \times [(A_{\text{control},90} - A_{\text{control},30}) - (A_{\text{sample},90} - A_{\text{sample},30})] / (A_{\text{control},90} - A_{\text{control},30})$ (WANGENSTEEN & al. [12], CRETU & al. [22]).

Statistical analysis

All experiments were performed in triplicate and all data were presented as mean values \pm standard deviations. The EC₅₀ values were calculated by linear interpolation between values above and below 50% activity.

3. Results and discussions

Contents of total phenols, flavonoids and proanthocyanidins

Previous studies have shown that polyphenols, mainly flavonoids and proanthocyanidins, are the major bioactive compounds in hawthorn species. They are responsible for many of the pharmacological effects of hawthorn extracts (antioxidant, cardiovascular, anti-inflammatory effects) (REFAAT & al. [2], VERMA & al. [5]). Therefore, the total phenolic, flavonoid and proanthocyanidin contents in *Crataegus pentagyna* extracts were estimated according to the methods described by SINGLETON & ROSSI [13], OZSOY & al. [14] and PORTER & al. [15], respectively. These three methods are simple, rapid and inexpensive. Even if these methods have a limited specificity, they are routinely used for the quantitative estimation of the above mentioned phytochemicals and give accurate results (SINGLETON & ROSSI [13], OZSOY & al. [14], PORTER & al. [15], KELM & al. [23], BLAINSKI & al. [24]). The total

phenolic content was higher in leaf extract (206.94±4.86 mg/g) in comparison to flower extract (184.62±1.71 mg/g). Flower extract had a higher flavonoid content than leaf extract (67.04±0.52 and 57.08±0.21 mg/g, respectively). Likewise, proanthocyanidin content, calculated as cyanidin equivalents, was higher in flower extract (97.70±3.81 mg/g) in comparison to leaf extract (68.92±1.81 mg/g) (Table 1).

Table 1. Total phenolic, flavonoid and proanthocyanidin contents in *Crataegus pentagyna* extracts.

Extract	Total phenolic content (mg gallic acid/g extract)	Flavonoid content (mg (+)-catechin/g extract)	Proanthocyanidin content (mg cyanidin/g extract)
Leaf extract	206.94 ± 4.86	57.08 ± 0.21	68.92 ± 1.81
Flower extract	184.62 ± 1.71	67.04 ± 0.52	97.70 ± 3.81

Several studies have reported total phenolic contents ranging from 108.65 to 343.54 mg/g in different extracts from *Crataegus monogyna* leaves with flowers (ÖZTÜRK & al. [25]), total phenolic and flavonoid contents of 38.25-396.04 and 2.12-32.62 mg/g, respectively in *Crataegus azarolus* leaf extracts (AMEL & al. [26]). In view of these literature data, it may be concluded that both *Crataegus pentagyna* extracts contain significant amounts of phenolic compounds and therefore they are expected to have strong antioxidant activity.

Free radical scavenging activity

Free radical scavenging ability of *Crataegus pentagyna* extracts was initially evaluated by TEAC assay. Flower and leaf extracts showed similar TEAC values (0.65±0.01 and 0.64±0.01 µM Trolox equivalent to 1 µg/mL extract, respectively). TEAC value of catechin was higher (5.46±0.16 µM Trolox equivalent to 1 µg/mL catechin) suggesting a stronger antioxidant capacity (Table 2). Free radical scavenging effects of *Crataegus pentagyna* extracts were further evaluated against superoxide anion and hydroxyl radicals, reactive oxygen species involved in the pathogenesis of many cardiovascular disorders.

Oxidative stress plays an important role in the development and progression of many cardiovascular diseases (ischemia, atherosclerosis, cardiomyopathy, cardiac hypertrophy, congestive heart failure, hypertension) and cardiovascular complications of diabetes mellitus (peripheral and coronary artery diseases, diabetic retinopathy) (PHAM-HUY & al. [27], BADESCU & al. [28]).

Reperfusion of the ischemic myocardial regions is the only way to restore normal blood flow and prevent myocardial infarction. The increase in blood flow is associated with an increase of oxygen level in the myocardial tissue, generation of reactive oxygen species and oxidative stress-induced damage of myocardial tissue (ischemia-reperfusion injury) (MIMIĆ-OKA & al. [29]). Generation of superoxide anion radical is a key event in ischemia-reperfusion injury. It is mainly produced by xanthine oxidase, impaired mitochondrial activity and neutrophil activity. Superoxide anion radical and other reactive oxygen species (hydrogen peroxide, hydroxyl radical) produce oxidative damage to cardiomyocytes and endothelial cells with an increase in vascular permeability (GALVEZ & al. [30]).

Superoxide anion radical is indirectly involved in the development of atherosclerosis; it reduces ferric ions to ferrous ions, the latter generating hydroxyl radical *via* Fenton reaction. Hydroxyl radical oxidizes polyunsaturated fatty acids in low-density lipoproteins, a process which initiates the formation of atherosclerotic plaques. In addition, the final products of lipid peroxidation (4-hydroxy-nonanal) are cytotoxic leading to tissue injury (GALVEZ & al. [30]).

In hypertension, circulating leukocytes produce high levels of superoxide anion radical and hydrogen peroxide. Superoxide anion radical converts the vasodilating nitric oxide into the highly cytotoxic peroxynitrite anion. The decrease in nitric oxide levels causes arteriolar vasoconstriction. Lipid peroxidation is enhanced leading to final products with vasoconstrictor effects such as F2-isoprostanes. Reactive oxygen species promote other events associated with hypertension (proliferation of vascular smooth muscle cells, deposition of collagen on arterial wall) (MIMIĆ-OKA & al. [29], GROSSMAN [31]).

Reactive oxygen species induce myocardial damage resulting in cardiomyopathy. Besides, reactive oxygen species activate enzymes (mitogen-activated protein kinases) and transcription factors (nuclear factor-κB) which stimulate not only cardiac myocytes to undergo hypertrophy, but also interstitial and perivascular fibrosis. Both cardiomyopathy and cardiac hypertrophy increase significantly the risk of heart failure (MAULIK & al. [32]).

All these data clearly indicate that a reduction of oxidative stress might be beneficial in the management of many cardiovascular diseases.

In superoxide anion radical scavenging assay, both extracts showed similar effects ($EC_{50}=0.9\pm 0.0$ mg/mL) being more active than the positive control, catechin ($EC_{50}=2.2\pm 0.0$ mg/mL). *Crataegus pentagyna* extracts scavenged hydroxyl radical with comparable EC_{50} values (0.86 ± 0.05 mg/mL for flower extract; 0.9 ± 0.0 mg/mL for leaf extract); according to the EC_{50} values, both extracts were only three times less active than catechin ($EC_{50}=0.27\pm 0.02$ mg/mL) (Table 2). As catechin has been reported to be an efficient scavenger of superoxide and hydroxyl radicals (KASHIMA, [33]), it is obvious that *Crataegus pentagyna* extracts exhibited significant scavenging effects against both radicals.

Ferrous ion chelating activity

In living organisms iron exists as ferrous and ferric ions. The ferric ions are relatively biologically inactive while ferrous ions are involved in the generation of hydroxyl radical through Fenton reaction (VALKO & al. [34]). Therefore, ferrous ions chelation is one of the main mechanisms of reduction of oxidative stress. Flower and leaf extracts chelated ferrous ions with EC_{50} values of 1.9 ± 0.0 and 1.3 ± 0.0 mg/mL, respectively; EDTA, the positive control, showed a significantly higher chelating capacity ($EC_{50}=6.46\pm 0.25$ μg/mL) (Table 2).

15-Lipoxygenase inhibition

15-Lipoxygenase plays a key role in the initiation and development of early atherosclerotic lesions. The enzyme oxidizes polyunsaturated fatty acids in low density lipoproteins and induces the expression of vascular cellular adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) thus promoting monocyte chemotaxis and adhesion to endothelial cells (BOLIK & al. [35]). According to the EC_{50} values, leaf extract showed a higher inhibitory activity towards 15-lipoxygenase than flower extract (129.63 ± 0.75 and 151.76 ± 1.65 μg/mL, respectively). Both extracts were less active than the positive control, catechin ($EC_{50}=27.66\pm 0.57$ μg/mL) (Table 2).

Table 2. Antioxidant activity of *Crataegus pentagyna* extracts.

Extract/ Positive control	Trolox equivalent antioxidant capacity (TEAC)	Superoxide anion radical scavenging activity (EC_{50})	Hydroxyl radical scavenging activity (EC_{50})	Ferrous ion chelating activity (EC_{50})	15-Lipoxygenase inhibitory activity (EC_{50})
Leaf extract	$0.64\pm 0.01^*$	$0.9 \pm 0.0^{***}$	$0.9 \pm 0.0^{***}$	$1.3 \pm 0.0^{***}$	$129.63 \pm 0.75^{**}$
Flower extract	$0.65\pm 0.01^*$	$0.9 \pm 0.0^{***}$	$0.86 \pm 0.05^{***}$	$1.9 \pm 0.0^{***}$	$151.76 \pm 1.65^{**}$
(+)-Catechin	$5.46\pm 0.16^*$	$2.2 \pm 0.0^{***}$	$0.27 \pm 0.02^{***}$	-	$27.66 \pm 0.57^{**}$
EDTA	-	-	-	$6.46 \pm 0.25^{**}$	-

* μM Trolox equivalent to 1 μg/mL extract/catechin; ** μg/mL; *** mg/mL

Antioxidant activity has already been reported for other *Crataegus* species (*C. oxyacantha*, *C. orientalis*, *C. aronia* syn. *azarolus*, *C. monogyna*, *C. meyeri*, *C. pontica*). An ethanol extract of *Crataegus orientalis* leaves showed good activity in diphenylpicrylhydrazyl (DPPH) radical scavenging assay ($62.91 \pm 3.40\%$ at 10 mg/mL) and β -carotene bleaching assay (42.37% at 1 mg/mL) (BOR & al. [36]). An aqueous extract of *Crataegus aronia* leaves inhibited the oxidation of β -carotene, 2,2'-azobis(2-amidino-propan) dihydrochloride (AAPH)-induced plasma oxidation, iron-induced lipid peroxidation in rat liver homogenate and increased intracellular glutathione levels; in addition, the extract showed a significant superoxide anion scavenging activity (50% at $42 \pm 6 \mu\text{g/mL}$ in xanthine-xanthine oxidase assay) (LJUBUNCIC & al. [1]). The antioxidant activity of methanol extracts of the leaves and flowers belonging to 14 *Crataegus* species growing in different regions of Turkey was investigated by different assays: CUPRAC (cupric reducing antioxidant capacity), FRAP (ferric reducing antioxidant power), ABTS/persulfate and Folin assays. An extract of *Crataegus pentagyna* leaves proved to be the most active among other *Crataegus* extracts ($\text{TEAC}_{\text{CUPRAC}} = 0.378 \pm 0.004$, $\text{TEAC}_{\text{FRAP}} = 0.132 \pm 0.001$, $\text{TEAC}_{\text{ABTS}} = 0.423 \pm 0.112$ and $\text{TEAC}_{\text{Folin}} = 0.752 \pm 0.201 \text{ mmol TR g}^{-1}$). To the best of our knowledge, this is the single report on the antioxidant activity of *Crataegus pentagyna* leaves and flowers, evaluating only the ability to reduce cupric and ferric ions, ABTS radical cation and Folin reagent (ÖZYÜREK & al. [11]). A comparison of the results in ABTS/persulfate assay with our data is difficult due to different experimental protocols. However, besides ABTS radical cation scavenging activity, our study evaluated the capacity to scavenge reactive oxygen species, chelate ferrous ions and inhibit 15-lipoxygenase. In our study, according to the EC_{50} values, *Crataegus pentagyna* extracts proved to be remarkable superoxide anion radical scavengers but also efficient hydroxyl radical scavengers and inhibitors of 15-lipoxygenase.

4. Conclusions

Our study demonstrated that extracts of *Crataegus pentagyna* leaves and flowers contain high levels of polyphenols and possess marked antioxidant effects. These results indicate a potential health benefit of *Crataegus pentagyna* leaf and flower extracts in oxidative stress-related cardiovascular disorders. The *in vivo* antioxidant and cardioprotective effects of *Crataegus pentagyna* extracts need to be evaluated.

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