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Polyphenols from *Cydonia oblonga* Miller leaves: Extraction, characterization, and biological activities

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Abstract

We extracted the leaves of *Cydonia oblonga* Miller and qualitative reactions specific to total polyphenols were performed on the leaves with certain reagents. An aqueous extract of the plant leaf turned a solution of FeCl₃ green, a qualitative reaction to an aromatic ring. We observed a color change from orange to light red when we experimented with a 1% vanillin solution in hydrochloric acid. This reaction is characteristic of condensed polyphenols; namely, anthocyanidins (catechins). Total polyphenols were isolated from *C. oblonga* leaves using extraction methods and organic solvents (acetone, chloroform, ethyl acetate), known in the literature. Studies were conducted to investigate the medicinal properties of an extract isolated from the leaves of *C. oblonga* on the mitochondrial membrane status of mouse cells. An extract isolated from the leaves of *C. oblonga* showed antioxidant and membrane-active properties and had a corrective effect on the state of the mitochondrial membrane. It can be concluded that the substances extracted from *C. oblonga* leaves are highly biologically active substances.

1. Introduction

Polyphenols are a diverse group of secondary metabolites derived from plants. They have a crucial therapeutic potential, because of their antioxidant, membrane-stabilizing and anti-inflammatory properties (Sameemabegum *et al.*, 2022). These compounds are synthesized in response to environmental threats such as microbial infections, UV radiation and herbivory, due to their important role in plant defense mechanism (Vijayalakshmi *et al.*, 2023). Their comprehensive structure, characterized by aromatic rings and hydroxyl groups, enables polyphenols to effectively neutralize reactive oxygen species (ROS) and prevent oxidative damage, both within plants and in medicinal applications (Wenting Zhou *et al.*, 2014; Prichko *et al.*, 2017; Ali Esmail Al-Snafi, 2016; Karen Marlene Herrera *et al.*, 2022; Persicmartina *et al.*, 2019; Luminita Dimitriu *et al.*, 2023; Bohn *et al.*, 2014; Liu *et al.*, 2014).

Quince (*Cydonia oblonga* Miller-COM) stands out among other polyphenol-rich plants because of its high phenolic content and traditional use in medicine. COM is native to regions such as Azerbaijan, Turkey and Iran; moreover, it is a deciduous tree and member of Rosaceae family. The leaves of this plant harbor a many important

quantities of bioactive compounds, especially condensed polyphenols like anthocyanidins and flavonoids, which are related with remarkable antioxidant and anti-inflammatory properties, furthermore known for it is fragrant (Sibi *et al.*, 2024; Ashraf *et al.*, 2016; Amsa *et al.*, 2022; Herrera-Rocha *et al.*, 2016; Pereira *et al.*, 2023; Bender *et al.*, 2020).

Oxidative stress is a major contributing factor to the development of various chronic diseases, including diabetes, certain types of cancer, cardiovascular disorders, and neurodegenerative diseases. By neutralizing free radicals and stabilizing plasma membranes, polyphenols have shown potential in reducing these effects. Previous research has shown that polyphenol-rich extracts of COM have antimicrobial, antidiabetic and cardioprotective outcomes, highlighting their importance in traditional and modern medicine. Moreover, they are the good candidates for further pharmacological studies, because of their ability to regulate lipid peroxidation (LOP) and improve mitochondrial function (Alston *et al.*, 2017; Chinnery *et al.*, 2015; Maulana *et al.*, 2021).

Phenolic acids, flavonoids, tannins, and stilbenes are key subgroups of polyphenols, which are plant-derived secondary metabolites. Each subgroup contributes uniquely to the therapeutic properties of polyphenols. The biosynthesis of these compounds is intricately linked to plant growth and environmental interactions, underscoring the importance of seasonality and harvesting practices in obtaining

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extracts with optimal bioactivity. Existence of anthocyanidins in COM leaves proves it is usage as a recourse for isolating biologically active substances with targeted actions (Jonilda *et al.*, 2022; Sri Bhuvanewari *et al.*, 2021; Hajeyah *et al.*, 2020; Diana Melo Ferreira *et al.*, 2022; Novikov *et al.*, 2014).

This study concentrating on extraction, identification and biological assessing of polyphenols from leaves of COM, with an emphasis on their effects on mitochondrial lipid peroxidation and membrane stability. The formalized processes of extraction and *in vitro* assessments provide valuable insights into the concentration-dependent activities of these compounds. By elucidating the antioxidant and membrane-active properties of COM polyphenols, the aim of this study is to provide a basis for their application in the development of new therapeutic agents for the treatment of oxidative stress-related diseases.

2. Materials and Methods

2.1 Collection and preparation of plant material

COM leaves were collected from mature plants during the autumn. The collected leaves were thoroughly cleaned with distilled water, air-dried at room temperature, and ground into a fine powder using a laboratory grinder. The powdered leaves were stored in airtight containers under dry condition for future analysis. Fresh leaves of *Cydonia oblonga* Miller (quince) were collected in September 2023 from their natural habitat in the Khorezm region, Gurlan district, Uzbekistan (41.8222°N, 60.3701°E). The plant (Figure 1) was authenticated by Professor A. D. Matchanov, at the Institute of Bioorganic Chemistry and a Voucher Specimen has been deposited in the Herbarium of the Institute of Bioorganic Chemistry, Academy of Sciences of Uzbekistan, for future reference (No: IBOCH 1557).



Figure 1: Quince fruit and leaves on a tree in its natural growth environment.

2.2 Extraction of polyphenols

By using multi-step solvent extraction process, polyphenols were extracted. First, 100 g of crushed leaf material is pre-treated with pure chloroform to remove lipophilic substances. Each extraction taking 2 h until the solvent became colorless and each repeated 3 times. The residue was then extracted with 70% aqueous acetone (1:6 w/v) at 55-60°C for 2 h. To optimize polyphenol yield, the process was repeated three times. The acetone extract was filtered,

and the solvent was evaporated using a rotary evaporator at 50-55°C under reduced pressure. The aqueous phase was further extracted with ethyl acetate (1:4 v/v) using a separatory funnel. The ethyl acetate fraction was dried over anhydrous sodium sulfate and concentrated under vacuum to obtain a polyphenol-rich fraction, designated as PF-4. This fraction was stored at -20°C for subsequent analyses.

2.3 Qualitative analysis of polyphenols

Firstly, the color of dark green ring was observed upon treating an aliquot (3 ml) of aqueous extract with a drop of 1% FeCl₃ solution, indicating the presence of hydroxy aromatic rings. Also, the reaction of the extract with 1% vanillin in concentrated HCl turned pink color, showing the presence of anthocyanidins.

2.4 Antioxidant activity

The antioxidant activity of PF-4 was determined by analyzing its effect on lipid peroxidation (LPO) in rat liver mitochondria. Mitochondria were separated from male Wistar rats (200-250 g) by differential centrifugation. The mitochondrial suspension (0.5 mg/ml protein) was incubated in a medium containing 125 mM KCl, 10 mM Tris-HCl (pH 7.4), 20 μM FeSO₄, and 400 μM ascorbate. LPO was commenced by the addition of Fe²⁺/ascorbate, and the formation of thiobarbituric acid-reactive substances (TBARS) was determined by spectrophotometrically at 532 nm. The blocking of LPO by PF-4 was studied by adding different concentrations (3, 10, 20, and 30 μg/ml) of the extract to the incubation medium. Standard experiments were completed under the same condition without PF-4. The experiments on rat liver mitochondrion, performed according to European Directive 2010/63/EU on the protection of animals used for scientific purposes, September 22, 2010, official Journal of the European Union, L 276/33- L276/79. The experiments on animals were carried out in accordance with the International Declaration of Helsinki, the Council of International Organizations of Medical Sciences (CIOMS), and the "Regulations on the Bioethics of the Use of Laboratory Animals in Scientific Research". National University of Uzbekistan, Department of Biophysics and Biochemistry (Report dated 22.02.2019). Rats were anesthetized in ether and decapitated.

2.5 Statistical analysis

All experiments were conducted in triplicate, and the results were expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way ANOVA, followed by Tukey's post hoc test, with a significance level set at *p*<0.05.

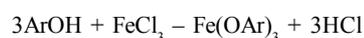
3. Results

3.1 Qualitative reaction for polyphenols

To determine the presence of polyphenolic compounds in the leaves of CoM, several specific color qualitative reactions were carried out.

3.1.1 Qualitative reaction with FeCl₃

One drop of 1% FeCl₃ solution was added to 3 ml of the aqueous plant extract. The color of the solution turned dark green (Figure 2a). This is a qualitative reaction for hydrocarbons in the aromatic ring. The general scheme of this reaction is as follows:



3.1.2 Qualitative reaction with a vanillin solution

Dried quince leaves were poured with boiling water and left for 5 min, after which a 1% vanillin solution in concentrated HCl was

added, and the solution acquired a bright red color, which is a qualitative reaction characteristic of anthocyanins (Figure 2b) (Jonilda *et al.*, 2022; Matmuratov *et al.*, 2024).

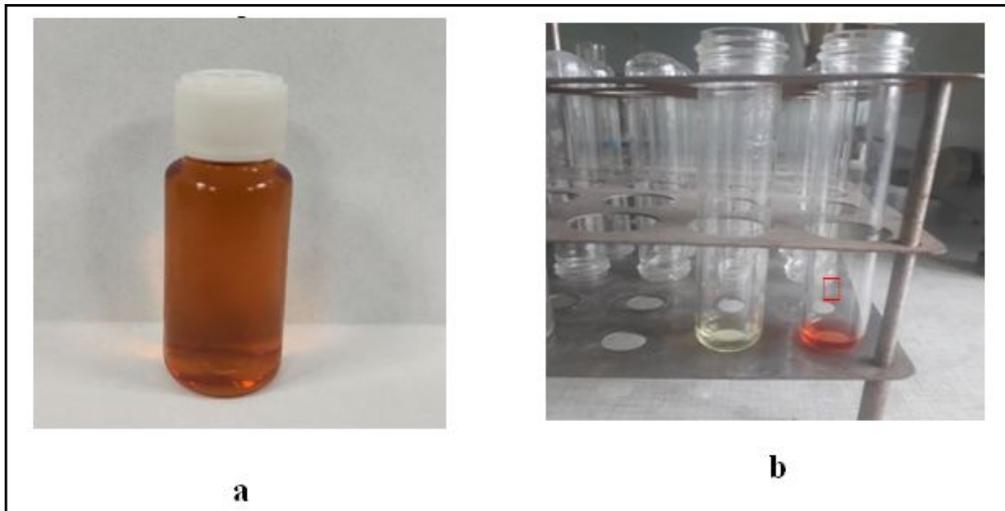


Figure 2: General qualitative reactions to polyphenols.

3.2 Extraction of polyphenols from COM leaves

Qualitative reactions revealed that the quince leaves contain polyphenols. In the next stage of our work, polyphenols were isolated from quince leaves using an extraction method (Figure 3). Quince leaves were collected in September and dried in the shade. According

to previously described methods, 100 g of dried plant leaves were pre-extracted with chloroform (ratio 1:10) and purified from lipophilic compounds. The extraction process was performed three times for 2 h each. The raw materials were dried in a fume hood until the solvent odor disappeared and then extracted with a 70% aqueous acetone solution (ratio 1:6) three times at 55-60°C for 2 h.

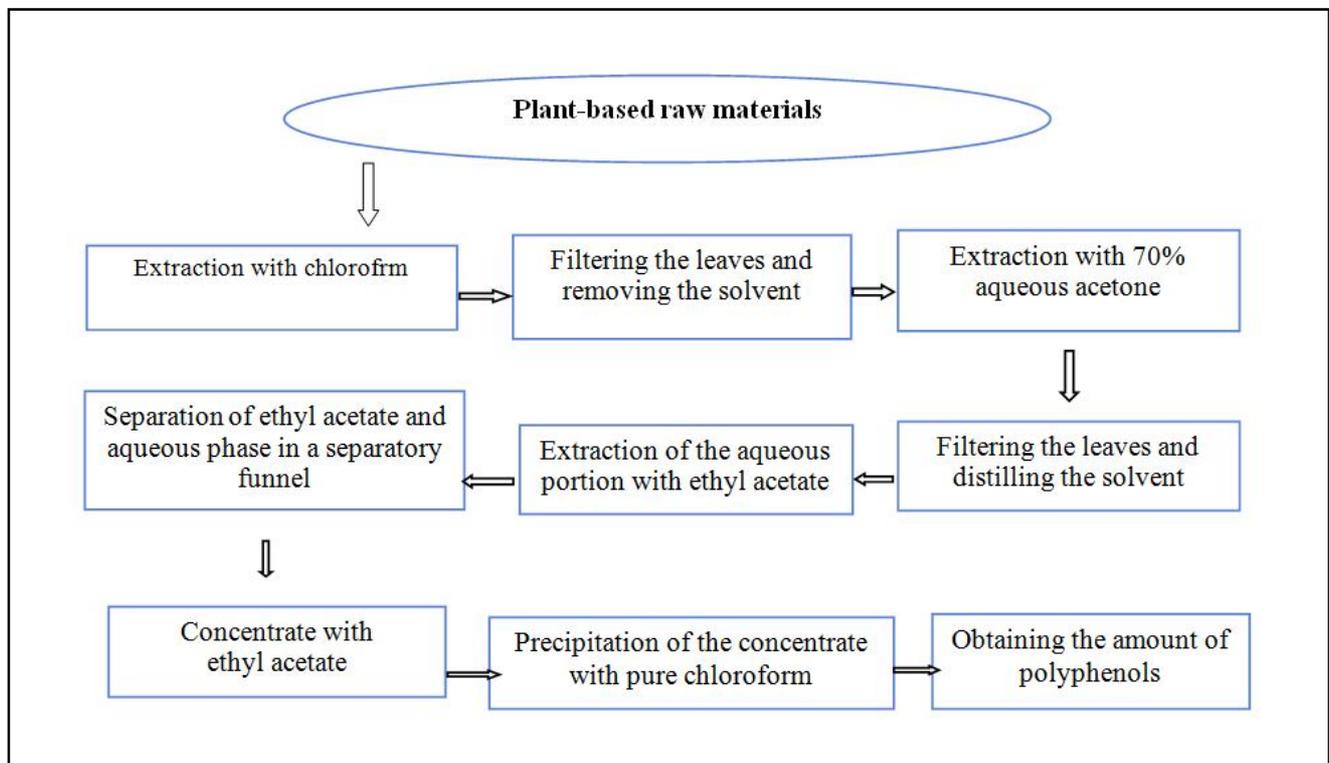


Figure 3: Extraction of polyphenols from plants.

3.3 Study of the influence of biologically active substances on the process of lipid peroxidation in mitochondria

The acetone extracts were collected and filtered, and acetone was removed using a rotary evaporator. The solvent was left in a fume hood until evaporation was complete. After extraction of the aqueous portion with ethyl acetate (1:4 ratio), 1500 ml of the ethyl acetate fraction was separated using a separatory funnel. The extract was dried over anhydrous Na_2SO_4 and concentrated in vacuo (50-55°C) to obtain ethyl acetate concentrate. The ethyl acetate concentrate (800 ml) was precipitated with pure chloroform (1:4), and the precipitate was filtered through a No. 4 Schott funnel and washed with petroleum ether. The mixture was then dried in a vacuum drying cabinet at room temperature. Polyphenols (1.5%) were obtained from 100 g of the plant, 1.5% of polyphenols were obtained (Maulana *et al.*, 2021; Jonilda *et al.*, 2022).

4. Discussion

The study of the medicinal properties of biologically active plant substances *in vitro* makes it possible to determine and evaluate the mechanisms of antioxidant action based on the study of the LPO

process in the liver mitochondria of laboratory animals. Thus, the corrective effect of the polyphenol extract (PF-4) isolated from the leaves of COM on the process of lipid peroxidation was observed as a result of Fe^{2+} /ascorbate induction of mitochondrial membranes. Fe^{2+} /ascorbate is a convenient inductor system for *in vitro* studies. This allows direct determination of the effectiveness of lipophilic antioxidants by inducing the LPO process under the influence of the system. At the same time, scientific literature indicates a violation of the antioxidant system in membranes (Alston *et al.*, 2017; Chinnery *et al.*, 2017).

Initially, 10 μM Fe_2SO_4 and 600 μM ascorbate were used to induce LPO in mitochondrial membranes. The addition of a fixed amount of Fe^{2+} /ascorbate to the incubation medium directly induces the formation of mitochondrial membranes and stimulates the LPO process. In this case, the permeability of the mitochondrial membranes increased, the membrane potential decreased, and permeability was observed. The LPO process induced in mitochondria exposed to Fe^{2+} /ascorbate was designated as the control group (100%). The studies examined the effects of the extract isolated from COM leaves (PF-4) on the LPO process in the mitochondrial membranes (Figure 4).

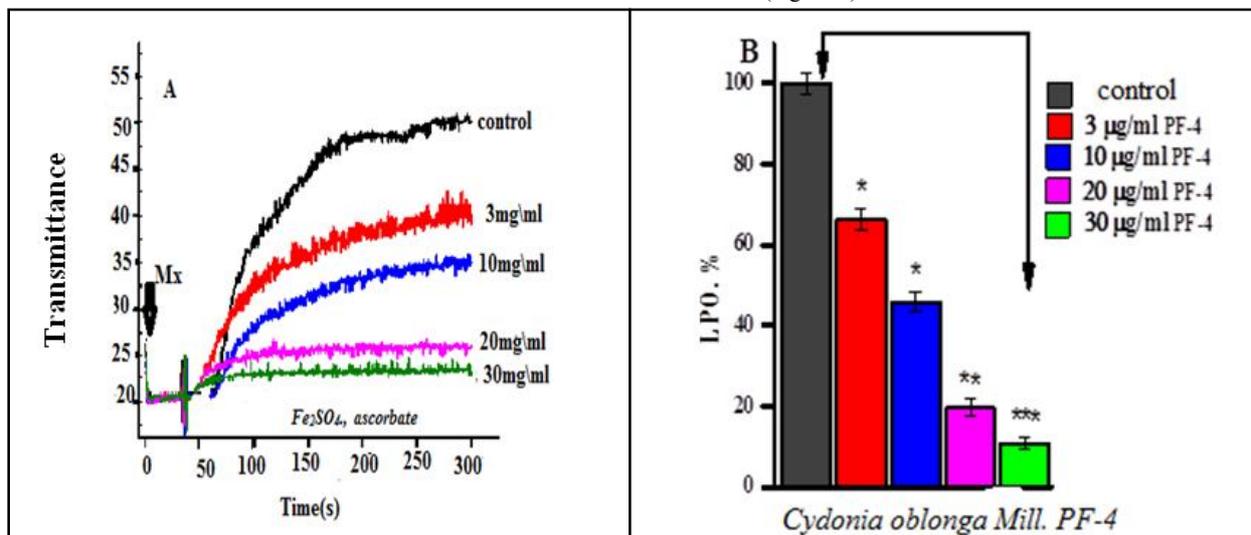


Figure 4: Inhibitory effect of PF-4 extract isolated from *C. oblonga* leaves on the LPO process in mitochondria.

Note: The ordinate axis shows the division of mitochondria, expressed as %, the abscissa axis shows the concentration of the PF-4 extract. IS: KCl-125 mM, Tris-HCl-10 mM, pH 7.4. Concentrations: Fe_2SO_4 -10 μM , ascorbate-600 μM ; mitochondrial protein 0.5 mg/ml. Control - Fe^{2+} /ascorbate. *** $p < 0.001$.

The antioxidant effect of PF-4 was observed when increasing concentrations of Fe^{2+} /ascorbate (3, 10, 20, and 30 $\mu\text{g/ml}$) were added to the medium. In studies of 3 $\mu\text{g/ml}$ PF-4 induced by Fe^{2+} /ascorbate, the permeability of mitochondrial membranes was 66.3 ± 1.75 compared to the control, and the LPO process was inhibited by 33.6%. This indicated the obvious antioxidant properties of PF-4. An increase in the concentration of PF-4 in the incubation medium also increased its ability to activate the LPO process. That is, the addition of 10 $\mu\text{g/ml}$ PF-4 to the incubation medium led to a mitochondrial permeability of 45.8 ± 1.40 , and the LPO process was inhibited by 54.12%. In addition, the permeability of the mitochondrial membrane was 19.6 ± 0.83 under the influence of PF-4 at a dose of 10 $\mu\text{g/ml}$ and 10.7 ± 0.89 under the influence of 30 $\mu\text{g/ml}$, while the LPO process in the mitochondria was 80.4% and was inactivated by

80.2%. These data prove that the PF-4 extract isolated from COM leaves has pronounced antioxidant properties.

According to the scientific literature, the structure and function of mPTP play an important role in the regulation of cellular metabolic processes (Hajeyah *et al.*, 2020; Diana Melo Ferreira *et al.*, 2022; Novikov *et al.*, 2014). According to Szweczyk and a number of other scientists, it is possible to regulate mPTP dysfunction in various pathologies using modulators and pharmacological drugs (Raghad, 2023; Truong, 2019). Therefore, experimental pharmacology and medicine consider mPTP a potential biomarker in the search for promising bioactive substances in plants (Al-Snafi, 2016). Several pharmacological agents and modulators of the mPTP have been identified. Among them, classical inducers (doxorubicin, progesterone,

dehydroepiandrosterone, estradiol, Ca^{2+} , or GnRH) strongly affect the mPTP structure and cause pore opening in experimental studies. Conversely, some modulators (cyclosporin A, bongrokrekat, buterol, ubiquinone, and sodium hydrosulfide) have an ingressive effect on mPTP. They cause a violation of membrane potential, directly affecting the mitochondrial membrane. In the experimental experiments, their inducing effect on mPTP was observed in the concentration range (of 10⁻⁸ to 10⁻⁴ M). It has also been reported that mPTP inducers simultaneously stimulate apoptosis in cancer (Mohammed, 2022). The antioxidant and membrane-stabilizing properties of PF-4 extracts align with earlier findings on the role of bioactive compounds in environmental management. For instance, (Tilyabaev *et al.*, 2017a; Togaev *et al.*, 2024) explored termite control through chemical applications, and their work complements studies

on insect pheromones as integral components in pest control strategies (Tilyabaev *et al.*, 2017b).

In our experiments, Ca^{2+} ions were chosen for mPTP induction, and we used a Ca^{2+} concentration of 30 μM . In the initial screening experiments, the addition of Ca^{2+} to the incubation medium increased the mPTP permeability (t -300 s) compared to that of the control, and this value was taken as 100%. In *in vitro* studies, the PF-4 extract isolated from COM leaves exerted an inhibitory effect on mPTP induced by Ca^{2+} ions at concentrations (2, 5, 10, and 15 $\mu\text{g/ml}$) (Figure 5). The inhibitory activity of PF-4 towards mPTP opening was evident from the first concentration of 2 $\mu\text{g/ml}$ added to the incubation medium (34.6%). The infective activity of the PF-4 extracts in relation to mPTP, depending on the concentrations added to the incubation medium, was as follows: 5 $\mu\text{g/ml}$ (45.1 ± 1.12), 54.8%; 10 $\mu\text{g/ml}$ (35.4 ± 1.27), 64.5%; and 15 $\mu\text{g/ml}$ (17.6 ± 0.81), 82.3%.

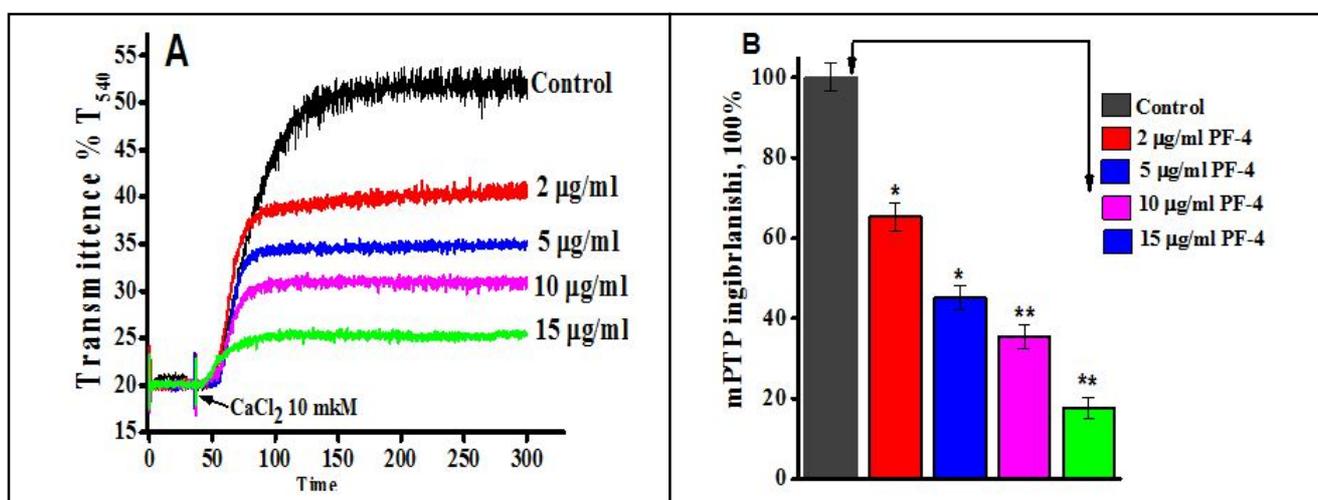


Figure 6: Effect of PF-4 extract isolated from COM leaves on the state of mPTP.

Note: the ordinate axis shows the division of mitochondria, expressed in %, the abscissa axis shows the concentration of the PF-4 extract. IS: 200 mM sucrose, 20 μM EDTA, 5 μM succinate, 2 μM rotenone, 20 mM Tris, 20 mM HEPES and 1 mM KH_2PO_4 , pH 7.4. Control – 10 μM Ca^{2+} . * $p < 0.05$; *** $p < 0.001$.

Lower concentrations (from 1 to 30 $\mu\text{g/ml}$) of PF-4 extract from COM leaves *in vitro* had a corrective effect on mitochondrial swelling induced by Fe^{2+} -ascorbate and Ca^{2+} . The observed effective effect of PF-4 extract on membranes was dependent on the concentration added to the incubation medium. The obtained results indicate that the PF-4 extract isolated from COM leaves is a bioactive substance with membrane-active properties and that this extract can be used in the future to create new drugs with antioxidant and antiradical properties.

5. Conclusion

This study successfully demonstrated the potent antioxidant and membrane-stabilizing properties of polyphenols extracted from COM leaves. The qualitative and quantitative analyses confirmed the presence of bioactive compounds, particularly anthocyanidins that contribute to the extract's ability to inhibit lipid peroxidation and stabilize mitochondrial membranes *in vitro*. The dose-dependent activity of the PF-4 extract highlights its therapeutic potential, paving the way for its application in managing oxidative stress-related conditions.

The findings emphasize the importance of COM as a valuable source of bioactive polyphenols and its potential integration into

pharmaceutical and nutraceutical formulations. Future research should focus on *in vivo* studies to further validate these results and explore the molecular mechanisms underlying the observed biological effects. Additionally, advanced analytical techniques could be employed to identify and isolate specific active compounds, enhancing the scope for targeted therapeutic applications.

Furthermore, understanding the seasonal and geographical variations in polyphenol content could provide insights into optimizing the extraction process for maximum efficacy. The application of standardized protocols for polyphenol extraction and analysis will ensure reproducibility and facilitate the development of commercial formulations. The antioxidant properties observed in this study highlight the relevance of polyphenols not only in therapeutic interventions but also as functional ingredients in dietary supplements and cosmetics.

Overall, this research establishes a strong foundation for the utilization of COM polyphenols in addressing oxidative stress-related disorders, contributing to the broader field of plant-based therapeutics. The integration of traditional medicinal knowledge with advanced scientific methodologies offers promising avenues for the development of innovative solutions to contemporary health challenges.

Conflict of interest

The author declares no conflicts of interest relevant to this article.

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