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Original article

Comparative *in vitro* Anti-Inflammatory Potential of Fresh and Stored Methanolic Leaf Extracts of *Myrtus communis* L.: An Egg Albumin Denaturation Study

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Keywords.
Myrtus communis, Anti-inflammatory, Egg Albumin Denaturation Models, New and old extract.

ABSTRACT

Myrtus communis L. has been documented to have pharmacological characteristics, including the ability to reduce inflammation. However, little is known about how long-term storage affects the biological activity of the leaf extracts. This study aimed to use an egg albumin denaturation model to compare the in vitro protein-stabilizing and initial anti-inflammatory potential of freshly prepared and stored methanolic M. communis leaf extracts. The anti-inflammatory potential of M. communis methanolic leaf extracts was assessed at doses of 0.125–2 mg/ml by use of the egg albumin denaturation assay. The reference medications utilized were ibuprofen and diclofenac. IC50 values were estimated from the concentration–response data; absorbance was measured at 660 nm, and percentage inhibition was computed. Every experiment was carried out three times. Although the fresh extract exhibited significantly more activity than the stored extract, both extracts showed protein-stabilizing action. The fresh extract showed an estimated IC50 of 0.158 mg/ml and inhibition of 40.3–89.5% across the tested doses. With an estimated IC50 of 0.656 mg/ml, the old extract demonstrated 0–76.5% inhibition. As a result, the efficacy of the stored extract was around four times lower than that of the fresh preparation. With estimated IC50 values less than 0.125 mg/ml, diclofenac and ibuprofen showed greater efficacy than both plant extracts. The protein-stabilizing activity of fresh methanolic M. communis leaf extract was higher than that of the stored one, indicating that the biological activity of the extract may be influenced by storage conditions. However, because comparable chemical profiling was not done, the observed difference cannot be directly linked to phytochemical breakdown. These results encourage additional phytochemical, cellular, and in vivo studies and emphasize the significance of storage and extract stability in the repeatability of plant-derived preparations.

Introduction

Inflammation is a complex protective response that plays an essential role in maintaining tissue integrity following infection, injury, or other harmful stimuli [1,2,3]. Although the inflammatory response is initially protective, excessive or persistent inflammation can contribute to tissue damage and the development or progression of several pathological conditions [4,5,6]. Consequently, the search for effective anti-inflammatory agents remains an important area of pharmacological research. Non-steroidal anti-inflammatory drugs (NSAIDs), including diclofenac and ibuprofen, are widely used to control inflammatory conditions [7,8]. However, their use may be associated with adverse effects, particularly following prolonged administration [9,10]. This has encouraged continued investigation of medicinal plants as potential sources of biologically active compounds with anti-inflammatory properties.

Myrtus communis L. (Myrtaceae), commonly known as myrtle, is an aromatic medicinal plant containing a variety of secondary metabolites, including phenolic compounds and flavonoids [11,12,13]. These phytochemicals have been associated with several biological activities and may contribute to the pharmacological properties of the plant [14,15,16].

One of the commonly employed preliminary *in vitro* approaches for investigating anti-inflammatory activity is the inhibition of protein denaturation. Protein denaturation has been associated with inflammatory processes [17,18], and substances capable of preventing heat-induced protein denaturation may therefore demonstrate potential anti-inflammatory activity. Egg albumin is frequently used as a protein substrate in this type of screening assay [19].

Despite substantial research on the plant's inherent anti-inflammatory effect, the main research gap is the scant evidence regarding whether extended storage modifies the protein-stabilizing activity of methanolic M. communis leaf extracts. The present study was designed to investigate the anti-inflammatory potential of the methanolic leaf extract of the Libyan M. communis using the egg albumin denaturation method. Particular attention was given to the stability of the plant bioactive constituents during a long storage period by the comparison between a fresh extract and an old extract (collected in the same season and stored for one year at 4°C). The activity of the extracts was also compared with selected conventional anti-inflammatory drugs.

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Research Questions

This study aimed to answer many questions (1) Can heat-induced denaturation of egg albumin be quantitatively inhibited by freshly obtained methanolic *M. communis* leaf extract?**, (2)** Does the efficacy of methanolic M. communis leaf extract to prevent heat-induced denaturation of egg albumin change over time?, (3) Do fresh and long-stored methanolic *M. communis* leaf extracts require different concentrations to reach 50% inhibition (IC50)?, and (4) Do methanolic *M. communis* leaf extracts, both fresh and old, show concentration-dependent suppression of egg albumin denaturation?.

Methods

Plant Material Collection and Extraction

The Research and Consulting Department, Faculty of Pharmacy, Omar Al-Mukhtar University, Al-Bayda, Libya, officially authorized the collection of *M. communis* leaves (Approved: 1-6-20/2020). All methods used to collect plant material complied with national and institutional regulations. Both old and fresh *M. communis* leaves were collected in the summer of 2026 and 2025, respectively, from AL-Hmama, a location in north-eastern Libya that surrounds Al-Bayda city. Ensaf H. Dakeel, a taxonomist at Omar Al-Mukhtar University's Faculty of Sciences in Al-Bayda, Libya, identified and categorized the plant. The leaves were cleaned with tap water, allowed to air-dry, crushed, and then extracted using methanol (1:10 w/v) for 72 hours at room temperature using cold maceration. A rotary evaporator was used to concentrate the filtrate, which was then allowed to air-dry. The old extract was stored at 4°C in a clean, closed glass container**,** while the fresh extract was investigated directly**.** Two extract conditions were investigated and designated as the fresh extract and the old extract.

Reference drugs

Diclofenac (50 mg) and ibuprofen (400 mg) were used as reference anti-inflammatory treatments. All drugs were purchased from local pharmacies.

Egg albumin denaturation assay

The anti-inflammatory activity of the extracts was assessed using the egg albumin denaturation method according to Chandra and his team [20]. The samples were evaluated at different concentrations. Concentrations of 2, 1, 0.5, 0.25, and 0.125 mg/ml were examined. The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin solution (2.5g of Egg albumin powder dissolved in 25ml distilled water), 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of *M. communis* extract. Similar volume of double-distilled water served as control. Then the mixtures were incubated at (37°C) in water bath incubator for 15 min and then heated at 70°C for 5 min. After cooling, their absorbance was measured at 660 nm. Diclofenac sodium and Ibuprofen were used as reference drug and treated similarly. The assay was done in triplicates.

Calculation of inhibition

The percentage inhibition of albumin denaturation was calculated according to the following equation:

"% inhibition = [(An − As)/An] × 100,"

Where, As = absorbance of test sample, An = absorbance of control. The extract/drug concentration for 50% inhibition (IC50) was determined by plotting percentage inhibition with respect to control against treatment concentration.

Statistical analysis

The experiment was repeated in triplicate, and the results were presented as mean ± standard deviation. One-Way ANOVA was used to determine the probability value (P value), which is considered significant if it is ≤ 0.05.

Results

The anti-inflammatory activity of fresh and old methanolic extracts of *M. communis* leaves was evaluated using the egg albumin denaturation method, with diclofenac and ibuprofen used as reference drugs. The results demonstrated a concentration-dependent inhibition of albumin denaturation by both extracts. The fresh extract showed inhibition percentages of 40.3%, 77.6%, 85.5%, 86.2%, and 89.5% at concentrations of 0.125, 0.25, 0.5, 1, and 2 mg/ml, respectively. In comparison, the old extract produced inhibition percentages of 0%, 3.5%, 38.0%, 76.5%, and 57.3% at the same concentrations, respectively as shown in Table 1. The estimated IC_{50} values were 0.158 mg/ml for the fresh extract and 0.656 mg/ml for the old extract, indicating greater anti-inflammatory activity of the fresh extract (Table 2). Among the reference drugs, diclofenac and ibuprofen produced inhibition values ranging from 65.5% to 98.2% and 66.6% to 94.0%, respectively, although 50% inhibition was not reached within the tested concentration range; therefore, their IC_{50} values were estimated to be below 0.125 mg/ml. Overall, the fresh *M. communis* extract demonstrated stronger anti-inflammatory activity than the old extract, as indicated by its substantially lower IC_{50}.

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Table 1. Anti-inflammatory effect of methanol extract of leaves of *Myrtus communis*

Serial dilution mg/ml	Fresh Extract		Old Extract		Diclofenac (50mg) Tab.		Ibuprofen (400mg) Tab.		Control (Dis. H2O) Absorbance 660nm
	A	%	A	%	A	%	A	%	
2	0.076	89.5	0.31	57.3	0.013	98.2	0.043	94	0.726
1	0.1	86.2	0.17	76.5	0.041	94.4	0.135	81.4	
0.5	0.105	85.5	0.45	38	0.192	73.5	0.184	74.6	
0.25	0.162	77.6	0.7	3.5	0.250	65.5	0.242	66.6	
0.125	0.433	40.3%	1.001	Abs. > control	0.262	63.9	0.79	Abs. > control	

A = Absorbance at 660nm % = Percentage of inhibition

Table (2): IC50s of ant-inflammatory inhibition of tested extracts and drugs

Test substance	Concentrations around 50% inhibition	Estimated IC_{50}
Fresh *M. communis* extract	0.125 mg/ml = 40.3%; 0.25 mg/ml = 77.6%	0.158 mg/ml
Old *M. communis* extract	0.5 mg/ml = 38.0%; 1 mg/ml = 76.5%	0.656 mg/ml
Diclofenac	50% inhibition was not reached	<0.125 mg/ml
Ibuprofen	50% inhibition was not reached	<0.125 mg/ml

Discussion

Instead of just highlighting the plant's anti-inflammatory properties, this study offers a comparative evaluation of the impact of extended storage on the in vitro protein-stabilizing activity of methanolic *M. communis* leaf extract. By preventing heat-induced denaturation of egg albumin, the methanolic leaf extract of *M. communis* was shown in this study to have significant *in vitro* anti-inflammatory potential. The fresh extract's significantly higher activity as compared to the old extract was the most notable discovery. The fresh extract caused 40.3% inhibition at 0.125 mg/ml and rose to 89.5% at 2 mg/ml in the egg albumin powder model, while the estimated IC50 values for the fresh and aged extracts were 0.158 and 0.656 mg/mL, respectively. In order to achieve 50% inhibition, the fresh extract needed a concentration that was around four times lower than the old extract, according to the IC50 calculations. This result implies that the age or state of the extract may have a significant impact on the chemicals that protect proteins from denaturation.

A higher concentration or better preservation of thermolabile or oxidation-sensitive phytochemicals may be linked to the fresh extract's increased activity. The various secondary metabolites found in *M. communis* leaves, especially flavonoids and phenolic substances, may contribute to the plant's biological characteristics. The current study's extraction process comprised 72 hours of methanolic maceration, rotary evaporation, and storage of the extracts at -20°C. Depending on the length of time and storage conditions, phenolic compounds may experience oxidation, degradation, polymerization, or other chemical changes. Therefore, it is conceivable that the decreased activity seen for an older extract could be explained by variations in the phytochemical content of the two. However, this explanation should be considered a feasible hypothesis rather than a proven mechanism because the current study did not conduct a comparative phytochemical profile of the fresh and aged extracts.

Further evidence for a true concentration-related effect comes from the fresh extract's concentration-dependent response. Inhibition in the albumin powder assay rose from 40.3% at 0.125 mg/ml to 77.6% at 0.25 mg/ml and then to 89.5% at 2 mg/ml. Such an increase suggests that the extract's ability to prevent or lessen heat-induced protein denaturation was typically improved by increasing extract concentration. Inhibiting the process of protein denaturation, which disrupts proteins' natural structure, is frequently used as a first screening method for compounds that may have anti-inflammatory qualities. However, rather than providing concrete proof of the prevention of inflammation in a biological system, this assay should be viewed as a measure of protein-stabilizing action. Therefore, the observed activity does not prove pharmacological anti-inflammatory efficacy on its own, but it does indicate the extract's anti-inflammatory potential.

The comparison with traditional anti-inflammatory medications is a crucial component of our study. Within the measured concentration range, diclofenac and ibuprofen showed high inhibition values; reported inhibition ranged from 65.5–98.2% for diclofenac and 66.6–94.0% for ibuprofen. Since 50% inhibition was not obtained as a distinct concentration within the studied range, their IC50 values were reported as being < 0.125 mg/ml. Since the reference medications are well-known pharmacological agents with specific molecular targets and optimized pharmacological activity, their greater activity is not surprising. Crucially, it should not be assumed that the discovery that the fresh plant extract approached significant inhibition at comparatively low concentrations indicates that it is equivalent to either ibuprofen or diclofenac. While the reference medications include specific active pharmacological ingredients, the crude extract contains a variety of elements at varying quantities. Therefore, a direct comparison of IC50 values does not prove medicinal equivalency but is helpful in characterizing relative assay performance.

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From a pharmacognostic and pharmacological standpoint, the distinction between the fresh and aged extracts is especially significant. The purpose of the study was to determine whether extract condition affects anti-inflammatory action, and the findings corroborate this theory. With an estimated IC50 of 0.656 mg/ml as opposed to 0.158 mg/ml for the fresh extract, the aged extract showed noticeably less activity.

Because the concentration–response relationship for the old extract was not consistently monotonic, the results obtained with it also need to be interpreted cautiously. At 0.125, 0.25, 0.5, 1, and 2 mg/mL, the reported inhibition values were 0%, 3.5%, 38.0%, 76.5%, and 57.3%, respectively. A straightforward concentration-dependent pharmacological response is not compatible with the decrease in inhibition from 76.5% at 1 mg/ml to 57.3% at 2 mg/ml. This may be due to the precipitation or turbidity of the extract at greater concentrations, interference of extract constituents with absorbance measurements, or instability of constituents at higher concentrations. Therefore, it is necessary to repeat the measurements and the statistical analysis at the higher concentrations to confirm the result.

Consistent with previous evidence of anti-denaturation activity, a study done by Belahcene [21], who used the BSA denaturation assay, which is closely linked to the egg-albumin model used in this study, and they assessed a methanolic extract of *M. communis* leaves and report an IC_{50} of 23.86 ± 0.31 µg/mL for BSA denaturation, with 58.27 ± 0.19% inhibition at 200 µg/mL. Diclofenac exhibited more potent action, with an IC50 of 9.82 ± 3.14 µg/ml. More significantly, the interpretation is reinforced by the *in vivo* literature. Myrtucommulone, a component extracted from myrtle leaves, was shown by Rossi to reduce carrageenan-induced paw edema in a dose-dependent manner and to lower a number of inflammatory indices, such as TNF-α, IL-1β, leukocyte infiltration, MPO activity, and LTB_4 [22,23]. Therefore, proof that *M. communis* ingredients can affect real inflammatory pathways [24,25] supports the action seen in this study assay. In addition, Rasool [26] highlighted the significance of extraction solvent and phytochemical profile in influencing biological activity by demonstrating the anti-inflammatory efficacy of various *M. communis* leaf extracts *in vivo* and further investigating their phenolic content. The current observation that *M. communis* leaf extract can suppress protein denaturation *in vitro* is supported by these findings, but direct quantitative comparison of the data is not possible due to variations in experimental conditions.

All things considered, the current results show that methanolic *M. communis* leaf extract, especially the fresh preparation, can prevent heat-induced albumin denaturation in vitro. The IC50 values of the fresh and old extracts differed by about four times, suggesting that the age or condition of the extract may be a significant factor in determining the activity that was assessed. However, rather than being evidence of clinical anti-inflammatory activity, the results should be viewed as preliminary pharmacological screening. For further phytochemical, mechanistic, cellular, and in vivo research, the study offers a helpful basis. However, the current results supported the methanolic leaf extract of *M. communis's* initial *in vitro* anti-inflammatory potential by showing that it had detectable protein-stabilizing effect in the egg albumin denaturation model. The fresh extract demonstrated a concentration-related rise in albumin denaturation inhibition With an estimated IC50 of 0.656 mg/m, while the old extract showed significantly (Pv <0.05) less activity, suggesting a roughly fourfold difference in potency between the two extract conditions. These results imply that a decrease in the assessed protein-stabilizing activity of *M. communis* is linked to extract storage or aging. Thus, by showing a significant difference in activity between freshly prepared and preserved extracts, the study answers its main research issue. The results also suggest that when assessing and standardizing the biological activity of plant extracts, their state and storage history may be significant factors. However, the observed difference cannot be directly linked to the degradation of specific bioactive ingredients because the preserved extract did not exhibit a consistently concentration-dependent response and the study did not the phytochemical content of the two extract conditions.

Under experimental conditions, diclofenac and ibuprofen demonstrated greater efficacy than moringa extracts, suggesting that crude plant extracts do not match the efficacy of known anti-inflammatory drugs but regarding to the drugs side effects, and overall, the results show that the methanolic extract of fresh *M. communis* leaves exhibits greater protein-stabilizing capacity compared to the long stored one. These findings encourage further research to elucidate the mechanisms underlying this marked difference and its pharmacological significance using comparative phytochemical analysis, established cell models, and *in vivo* studies.

Conclusion

The current work supported the preliminary in vitro anti-inflammatory potential of Myrtus communis leaf extract by showing protein-stabilizing action in the egg albumin denaturation model. With IC^2_0 values of 0.158 and 0.656 mg/mL, respectively, the fresh extract demonstrated more activity than the preserved extract, suggesting a roughly four-fold change in potency. These results imply that the extract's biological activity may be influenced by its history or storage conditions. However, since no comparison phytochemical research was done, the difference cannot be directly linked to the breakdown of individual phytochemicals. In the examined settings, diclofenac and ibuprofen were more active than the plant extracts. Overall, the results justify additional phytochemical, cellular, and in vivo research and emphasize the significance of extract preservation in preserving biological activity.

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Recommendation

To understand the chemical foundation of the observed variation in activity, more research should compare the phytochemical profiles of fresh and old *Myrtus communis* extracts. To find the best conditions for maintaining extract activity, standardized studies of storage time and temperature are advised. Cellular and *in vivo* models should be used in addition to the protein-denaturation experiment to verify the findings' anti-inflammatory significance, and in order to determine which chemicals are responsible for the observed protein-stabilizing action, individual bioactive ingredients should be examined.

Conflict of interest. Nil

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