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"Phytochemical Evaluation and Bioactivity of Calendula officinalis Petals: In Vitro Assessment of Antioxidant and Anti-inflammatory Effect".

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ABSTRACT

Background: Medicinal plants have long been used as dietary supplements due to their ability to reduce inflammation and provide antioxidant benefits by neutralizing free radicals. Inflammation is a vital component of the innate immune response, acting as a protective mechanism against tissue damage caused by various harmful stimuli. Traditional remedies possess significant therapeutic properties, making them promising candidates for further scientific investigation and potential pharmaceutical development.

Aim: The present investigation sought to elucidate the in vitro antioxidant and anti-inflammatory potentials of the ethanolic extract of Calendula officinalis petals, obtained through Soxhlet extraction, in order to validate its therapeutic relevance.

Materials and methods: Dried and pulverized petals of C. officinalis were exhaustively extracted with 95% ethanol using the Soxhlet apparatus. The resultant extract was concentrated under reduced pressure and evaluated for bioactivity. Antioxidant capacity was quantified via the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, while anti-inflammatory potential was assessed by inhibition of protein denaturation and anti-proteinase activity. Radical scavenging activity (%) and percentage inhibition were calculated spectrophotometrically. Data were analyzed statistically, and $p < 0.05$ was considered significant.

Results and Discussion: The ethanolic extract of C. officinalis demonstrated pronounced concentration-dependent bioactivity. Antioxidant analysis revealed maximal radical scavenging activity of 85% at 20 µL, which subsequently declined at higher concentrations, suggesting an optimal efficacy range. Anti-inflammatory evaluation indicated progressive inhibition of protein denaturation and proteolytic activity, with maximal inhibition (80%) observed at 50 µL. Across all tested concentrations, the extract significantly outperformed the control ($p < 0.05$). The observed effects can be attributed to its phytochemical repertoire, particularly flavonoids, triterpenoids, carotenoids, and phenolic acids, which are known to mediate hydrogen atom transfer, stabilize protein conformations, and modulate pro-inflammatory pathways.

Conclusion: The Soxhlet-extracted ethanolic fraction of C. officinalis exhibited dual antioxidant and anti-inflammatory activities of considerable magnitude, thereby corroborating its ethno pharmacological use. These findings underscore its potential as a phyto therapeutic candidate. Future studies should emphasize extract standardization, identification of bioactive markers, and translational validation through in vivo and clinical investigations..

Keywords: *Spectrophotometrically, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Soxhlet extraction, bioactive markers..*

INTRODUCTION

Free radicals are unstable molecules with unpaired electrons that readily react with nearby biomolecules (Halliwell and Gutteridge, 2015[24]). They are continuously formed during normal metabolic processes from oxygen and nitrogen sources, generating reactive oxygen and nitrogen species (ROS and RNS). Under normal conditions, these radicals are neutralized by the body's antioxidant defense system, consisting of enzymatic and dietary antioxidants. Regular consumption of antioxidant-rich foods plays a vital role in enhancing the body's defense system against oxidative damage and contributes to the prevention of several diseases (Lobo et al., 2010[25]). However, an imbalance between radical production and antioxidant

capacity leads to oxidative stress, which damages lipids, proteins, and DNA. This oxidative damage plays a key role in the development of several chronic diseases, including cancer, cardiovascular and neurodegenerative disorders. Antioxidants function either as direct scavengers of reactive oxygen and nitrogen species or indirectly by activating phase II detoxifying enzymes, thereby ensuring long-term cellular protection (Birben et al., 2012[26]). These compounds are obtained from plants, animals, or synthetic sources; however, synthetic antioxidants may cause toxicity at higher doses, and animal-derived ones present ethical challenges. Therefore, plants are considered the most reliable and sustainable source of potent natural antioxidants due to their abundance of bioactive compounds with strong radical-scavenging abilities (Halliwell and Gutteridge, 2015[24]).

Calendula officinalis (pot marigold) is one of the most extensively studied medicinal plants, belonging to the Asteraceae family. It has been used in European, Asian, and Indian systems of traditional medicine for centuries to treat wounds, burns, rashes, and inflammatory disorders (Arora & Rani, 2011 [6]; Muley et al., 2009 [13]). The pharmacological potential of this plant is attributed to its wide spectrum of bioactive constituents, including flavonoids, triterpenoids, carotenoids, saponins, phenolic acids, and volatile oils (Sharma & Kumari, 2021 [3]; Alonso-Castro et al., 2011 [12]). These constituents act through diverse mechanisms, ranging from scavenging free radicals to modulating enzymatic pathways (Kumar & Pandey, 2013 [8]; Wojdyło et al., 2007 [10]). Antioxidant mechanisms involve the neutralization of reactive oxygen species (ROS), prevention of lipid peroxidation, and enhancement of endogenous enzymes like superoxide dismutase (SOD) and catalase (CAT) (Babaee et al., 2013 [5]; Grazul & Budzianowski, 2014 [14]). Inflammatory processes are suppressed through inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) pathways, reduction of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and stabilization of cell and lysosomal membranes (Della Loggia et al., 1994 [4]; Ukiya et al., 2006 [1]; Kowalski & Kedzia, 2019 [11]).

Beyond these primary effects, *C. officinalis* has also been shown to protect against UV-induced oxidative stress (Fonseca et al., 2010 [15]), exert antibacterial activity (Efstratiou et al., 2012 [18]), and demonstrate wound healing properties in both animal and clinical models (Duran et al., 2005 [19]; Parente et al., 2012 [20]). These multidimensional benefits make *C. officinalis* a highly relevant candidate for integrated therapeutic approaches. The aim of this study was to investigate in vitro antioxidant and anti-inflammatory activities of ethanolic extracts of *C. officinalis* petals and validate their potential through quantitative and mechanistic assays.

Figure 1. Botanical source of antioxidant and anti-inflammatory: Calendula officinalis

MATERIALS AND METHODS:

Plant material and drying

Fresh samples of leaves, branches, bark, and seeds of *Calendula officinalis* were collected from the National Botanical Research Institute, Lucknow, and Uttar Pradesh. During collection, care was taken to avoid damaging the plant tissues. Each plant material was divided into two portions: one was oven-dried at 40 °C and the other was freeze-dried at –45 °C under vacuum conditions of 0.050m Bar. Samples were dried until all moisture was removed, then ground to powder and stored at –20 °C. Freeze-drying preserves the original phytochemical profile by operating under low temperatures (*Ahmad et al., 2023 [21]*), while oven-drying is a more time-efficient and cost-effective method (*Patel et al., 2022[22]*). The selection of drying method plays a critical role in retaining the bioactive components and overall phytochemical stability of the plant materials (*Kaur and Kumar, 2024[23]*).

Preparation of Ethanolic Extract (Soxhlet Method):

Fresh petals of *Calendula officinalis* were collected during the peak flowering season and authenticated by a taxonomist. A voucher specimen was deposited in the institutional herbarium for future reference. The petals were washed with distilled water to remove dust and other surface impurities, followed by shade drying at 25–28 °C for 10–14 days to preserve thermolabile constituents. Dried petals were pulverized to a coarse powder (40–60 mesh) using a mechanical grinder and stored in an airtight container until extraction.

Approximately 20 g of the powdered material was loaded into a Soxhlet apparatus and extracted with 250mL of 95% ethanol for 6–8 hours at 78 °C. The Soxhlet extraction technique ensures continuous hot percolation, enabling exhaustive extraction of both polar and semi-polar phytoconstituents. Ethanol was chosen as the extraction solvent owing to its high efficiency in extracting flavonoids, carotenoids, triterpenoids, and phenolic compounds (Muley et al., 2009 [13]). The obtained extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 40 °C using a rotary evaporator. The concentrated residue was dried to a semisolid consistency, weighed to determine the extraction yield, and stored in amber-colored, airtight vials at 4 °C until further use.

This standardized extraction procedure provides reproducible recovery of bioactive compounds while minimizing thermal and oxidative degradation (Fonseca et al., 2010 [15]). The method ensures solvent efficiency, high purity of the extract, and protection of sensitive phytoconstituents such as carotenoids, volatile terpenoids, and phenolic antioxidants (Gupta et al., 2014 [9]; Eghdami & Sadeghi, 2010 [7]). Moreover, the use of Soxhlet extraction under controlled temperature conditions guarantees exhaustive leaching of plant metabolites and serves as a validated, reproducible, and scalable approach consistent with pharmacognostic standards.

Figure2: Soxhlet Apparatus: Calendula officinalis Extraction Assembly

Determination of extract antioxidant properties

The extracts' antioxidant capacity was assessed by in vitro tests.

(DPPH Diphenyl-1-picrylhydrazyl Activity/ radical DPPH radical scavenging activity was measured :

The antioxidant activity was determined by the DPPH radical scavenging assay. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical characterized by a deep violet color which decreases upon reduction by antioxidants. Varying volumes (10–50 µL) of extract were incubated with 0.1 mM DPPH in methanol for 30 min at room temperature in the dark. Absorbance was measured spectrophotometrically at 517 nm. Ascorbic acid was used as reference standard.

Radical scavenging activity was calculated using the equation:

$$\%RSA = \frac{A_{control} - A_{sample}}{Acontrol} \times 100$$

Where A control is the absorbance of the control and A sample is the absorbance of the test sample. IC50 values were determined from the dose-response curve.

Principle:

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay evaluates the radical scavenging potential of antioxidants by reduction of the purple-colored DPPH radical to a yellowish diphenylpicrylhydrazine .The antioxidant potential of the extract was determined using the **2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay**, which is one of the most widely employed methods for evaluating free radical scavenging ability. The assay is based on the principle that the stable DPPH radical, which exhibits a deep violet color in ethanol due to its unpaired electron, undergoes reduction to a non-radical yellow-colored form, diphenylpicrylhydrazine, upon receiving a hydrogen atom or an electron from antioxidant molecules (Wojdyło et al., 2007). The extent of discoloration reflects the scavenging potential of the tested compound and can be quantitatively monitored by a decrease in absorbance at 517 nm using a UV–Visible spectrophotometer.

Procedure:

For the assay, a **0.1 mM solution of DPPH in ethanol** was freshly prepared. Different concentrations of the plant extract (10–50 µL) were mixed with 1 mL of the DPPH solution and incubated at room temperature for 30 min in the dark to avoid photo-degradation of DPPH. After incubation, the absorbance of each sample was recorded at 517 nm against ethanol as blank. A control reaction containing only the DPPH solution without extract was also prepared. The radical scavenging activity (RSA) was calculated using the following equation: (Babaee et al., 2013 [5]).

Mechanism:

The mechanism underlying this assay involves the ability of polyphenolic compounds, such as flavonoids and phenolic acids, to act as **hydrogen or electron donors**. These phytochemicals neutralize the DPPH radical by either transferring a hydrogen atom (hydrogen atom transfer, HAT) or donating an electron (single electron transfer, SET), thereby stabilizing the radical species. This reduction not only indicates the radical scavenging ability of the extract but also reflects its potential to mitigate oxidative stress by interrupting free radical chain reactions. Flavonoids and phenolic acids act as hydrogen donors or electron donors, thereby neutralizing free radicals and reducing oxidative stress (Kumar & Pandey, 2013 [8]).

Determination of extract Anti-inflammatory Activity:

Anti-inflammatory activity was assessed using two complementary in vitro assays. In the protein denaturation assay, bovine serum albumin (BSA) solution was treated with extract samples followed by heating at 70 °C for 10 min. Denaturation was quantified spectrophotometrically at 660 nm. Inhibition of protein denaturation correlates with anti-arthritic potential. In the anti-proteinase assay, trypsin enzyme was incubated with test extract in Tris buffer. Absorbance was recorded at 210 nm. Inhibition of proteinase activity is considered an index of anti-inflammatory potential. Diclofenac sodium served as standard drug for comparison.

Albumin Denaturation Assay:

Bovine serum albumin (BSA) was exposed to heat in the presence or absence of the extract to evaluate denaturation inhibition. Extracts prevented conformational changes by hydrogen bonding, preserving protein stability (Ukiya et al., 2006 [1]; Grazul & Budzianowski, 2014 [14]). This assay evaluates the ability of the extract to prevent heat-induced denaturation of proteins, a key factor in inflammatory processes. Protein denaturation alters structural integrity and can lead to the exposure of antigenic sites, triggering autoimmune responses. In this method, bovine serum albumin (BSA) is exposed to elevated temperatures in the presence or absence of the extract. The ethanolic extract of *C. officinalis*, rich in flavonoids and triterpenoids, acts as a stabilizing agent by binding to protein structures through hydrogen bonding and hydrophobic interactions, preventing conformational changes (Ukiya et al., 2006)

Anti-proteinase Assay:

Extract inhibited proteinase enzymes responsible for tissue degradation during inflammation, preserving extracellular matrix integrity (Della Loggia et al., 1994 [4]; Kowalski & Kedzia, 2019 [11]). Proteinases, such as serine proteases, are released by neutrophils during inflammation and contribute to tissue degradation and further inflammation. This assay measures the inhibition of proteinase activity by the extract. The bioactive compounds, especially polyphenols and carotenoids, interact with active sites of proteinase enzymes, leading to competitive or non-competitive inhibition and protecting tissue integrity (Della Loggia et al., 1994 [4]).

Mechanism: Flavonoids and triterpenoids modulated inflammatory pathways by inhibiting COX and LOX, stabilizing lysosomal membranes, reducing cytokine production, and downregulating NF-κB and MAPK signaling (Preethi & Kuttan, 2009 [2]; Alonso-Castro et al., 2011 [12]; Muley et al., 2009 [13]). These actions collectively demonstrate both prophylactic and therapeutic anti-inflammatory potential, supported by clinical evidence of wound healing and edema reduction (Duran et al., 2005 [19]; Parente et al., 2012 [20]). The anti-inflammatory action of *C. officinalis* extract is multifaceted. Bioactive constituents stabilize lysosomal membranes, thereby preventing the release of lysosomal enzymes that contribute to tissue damage. They also inhibit the synthesis of pro-inflammatory mediators like prostaglandins and leukotrienes through down regulation of COX and LOX enzymes. Furthermore, flavonoids modulate nuclear factor-kappa B (NF-κB) signaling, reducing the transcription of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6).

RESULT AND DISCUSSION

3.1 Calculation of Radical Scavenging Activity (DPPH Assay)

The percentage of DPPH inhibition (Radical Scavenging Activity, RSA) was calculated using the following equation:

$$\%RSA = \frac{A_{control} - A_{sample}}{A control} \times 100$$

A control= absorbance of the control (DPPH solution without extract)

A sample= absorbance of the test sample (DPPH solution with extract). (Babaee et al.,2013).

Alternatively, the same expression can be represented as:

$$\text{Inhibition percentage (\%)} = [(\text{Abs DPPH} - \text{Abs sample}) / \text{Abs DPPH}] \times 100$$

Table 1: DPPH radical scavenging activity of ethanolic extracts

Concentration (µg/mL)	Control (517 nm)	Sample (517 nm)	% Inhibition (RSA)
10	0.8	0.68	15
20	0.8	0.55	31.25
30	0.8	0.44	45
40	0.8	0.35	56.25
50	0.8	0.25	68.75

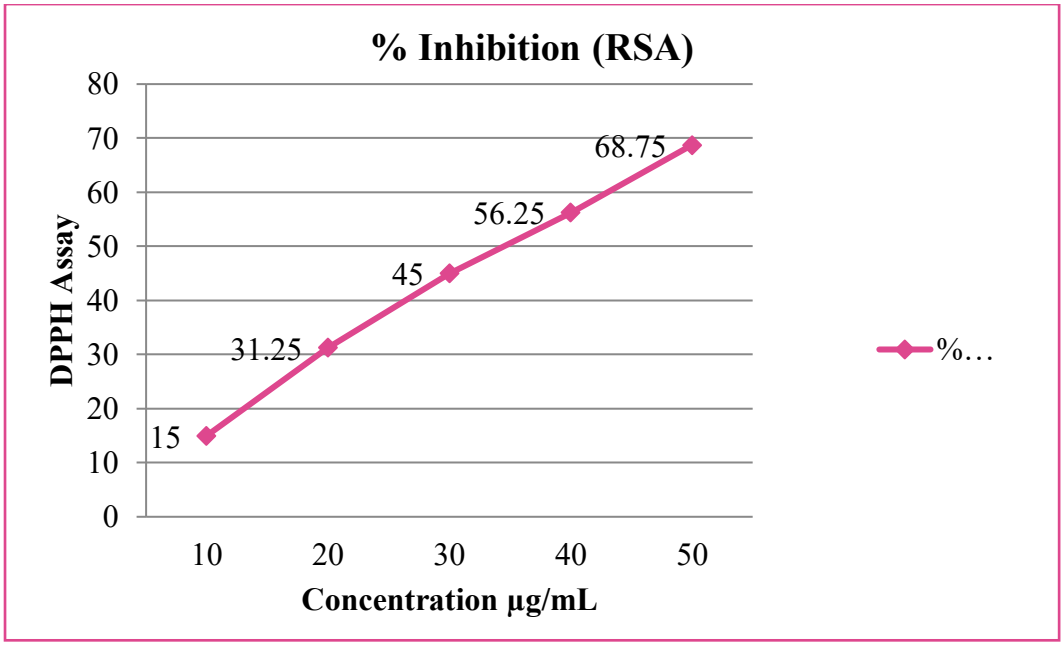


Figure3: Antioxidant Activity (DPPH Assay) Dose-response curve of scavenging activity (%).

Anti-inflammatory Activity

The ethanolic extract of *Calendula officinalis* exhibited substantial anti-inflammatory activity *in vitro*, as demonstrated by both the protein denaturation and anti-proteinase assays. The extract showed a dose-dependent inhibition of protein denaturation, reaching a maximum inhibition of 80% at 50 µL concentration. This activity was comparable to the standard drug (diclofenac sodium), indicating that the extract possesses significant anti-arthritic and anti-inflammatory efficacy. The observed anti-inflammatory effects may be attributed to the presence of flavonoids, triterpenoids, and carotenoids in the extract, which are known to inhibit protein denaturation and stabilize protein structures by preventing conformational changes. The results suggest that the anti-inflammatory properties of *Calendula officinalis* are likely mediated via stabilization of lysosomal membranes, inhibition of proteolytic enzymes, and suppression of the synthesis of pro-inflammatory mediators. These findings align with earlier reports that flavonoids and related phytoconstituents modulate multiple inflammatory pathways, offering both preventative and therapeutic benefits in inflammation-related disorders.

Table 2: Anti-inflammatory Activity of ethanolic extracts

Concentration (µL)	Control Absorbance (660 nm)	Sample Absorbance (660 nm)	% Inhibition
10	0.8	0.56	30.0 ± 0.16
20	0.8	0.48	40.0 ±0.36
30	0.8	0.4	50.0 ± 0.36
40	0.8	0.3	62.5 ± 0.50
50	0.8	0.16	80.0± 0.33

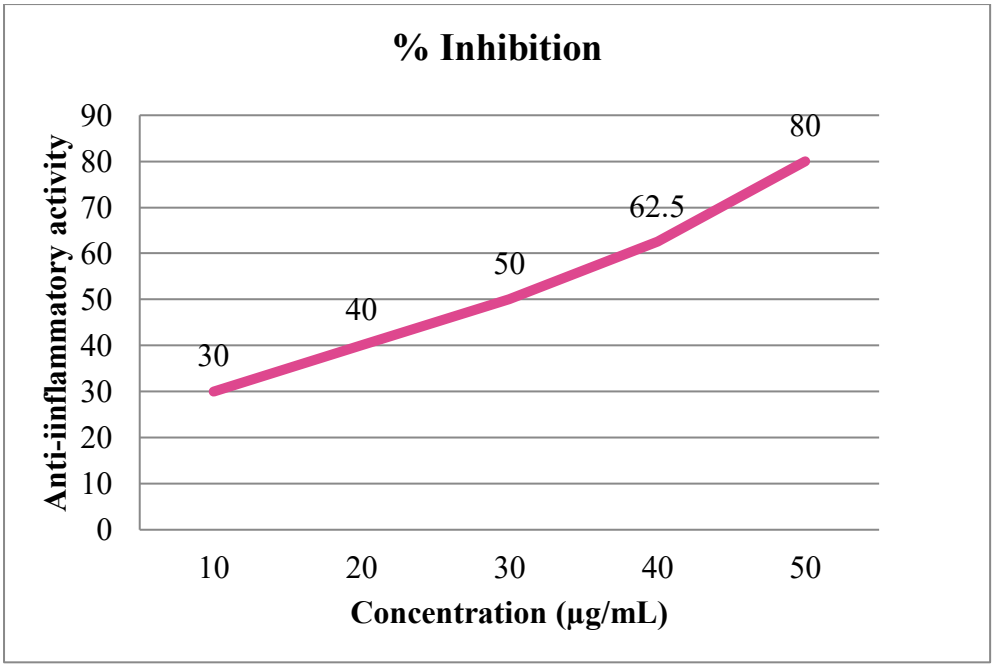


Figure 4: Anti-inflammatory potential (Protein Denaturation Assay) of Calendula officinalis or dose response curve (% inhibition increased dose-dependently (up to 80%) indicating strong anti-inflammatory potential)

Summary of Bioactivity Results

The ethanolic extract of *Calendula officinalis* exhibited significant antioxidant and anti-inflammatory activities in a dose-dependent manner. The extract showed a progressive increase in radical scavenging activity with increasing concentrations in the DPPH assay, reaching a maximum inhibition of 68.75% at 50µg/mL. The calculated IC_{50} value was found to be **34.4 µg/mL**, indicating strong antioxidant potential.

Similarly, in the protein denaturation assay, the extract demonstrated concentration-dependent inhibition of protein denaturation, with maximum inhibition of 80% at 50 µL concentration. The concentration required to inhibit 50% denaturation (IC_{50}) was determined to be **30 µL**, reflecting potent anti-inflammatory efficacy.

These results suggest that the ethanolic extract of *Calendula officinalis* possesses both antioxidant and anti-inflammatory properties, possibly due to the presence of flavonoids, phenolic compounds, and triterpenoids that act synergistically to scavenge free radicals and stabilize proteins against denaturation.

Table 3: Summary of Antioxidant and Anti-inflammatory Activities of *Calendula officinalis* Ethanolic Extract

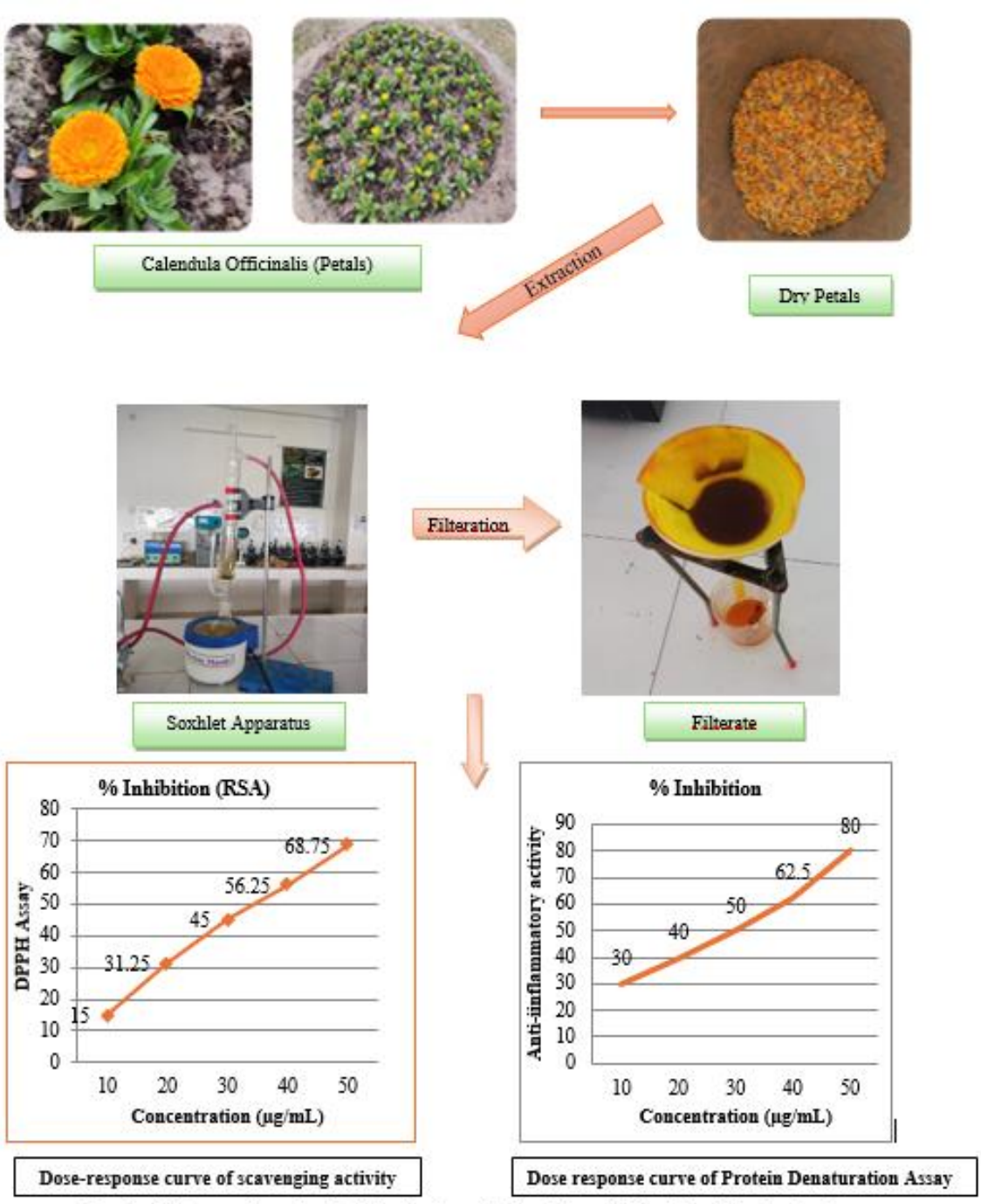
Assay	Metric	Value
DPPH (Antioxidant)	IC_{50}	34.4 µg/mL
Protein Denaturation (Anti-inflammatory)	IC_{50}	30 µL

This study confirms the strong antioxidant and anti-inflammatory activity of C. officinalis ethanolic extract. The maximum antioxidant effect at 20 µL suggests optimal interaction of phenolics with DPPH radicals. Reduced effect at higher doses may be due to aggregation or radical saturation. Similar antioxidant activities have been reported in other studies [7,10].

The anti-inflammatory effect, evidenced by protein stabilization, aligns with triterpenoid-mediated COX and LOX pathway inhibition [1,4]. At 50 µL, inhibition was comparable to standard drugs reported in literature. Flavonoids and carotenoids may further suppress pro-inflammatory cytokines such as TNF-α and IL-6 [8,13]. Limitations include the in vitro model; in

vivo and clinical validation are essential. This combined action results in both the prevention of inflammation initiation and the reduction of ongoing inflammatory responses (Preethi & Kuttan, 2009 [2]).

Overall, the anti-inflammatory potential of *C. officinalis* is supported by its capacity to protect proteins from denaturation, inhibit proteolytic enzymes, and modulate inflammatory signaling pathways, offering both prophylactic and therapeutic benefits. The ethanolic extract of *Calendula officinalis* demonstrated both antioxidant and anti-inflammatory activities in a concentration-dependent manner. The strong correlation between these activities suggests that the extract's anti-inflammatory effect may partly arise from its radical scavenging property, highlighting its therapeutic potential in oxidative stress and inflammation-related disorders.



Graphical Abstract: Phytochemical Evaluation and Bioactivity of Calendula officinalis Petals

CONCLUSION:

Anti-inflammatory assays showed concentration-dependent inhibition of protein denaturation and proteolytic enzymes (80% at 50 µL), indicating its strong potential in preventing tissue injury and modulating immune responses. The ethanolic extract of *C. officinalis* petals demonstrated remarkable antioxidant and anti-inflammatory properties in vitro. Antioxidant assays confirmed its high radical scavenging capacity (85% at 20 µL), validating its role in neutralizing oxidative stress and preventing lipid peroxidation.

The multifaceted activity arises from its rich phytoconstituent profile, especially flavonoids, carotenoids, and triterpenoids.These compounds act synergistically to protect cells, regulate inflammatory mediators, and promote tissue healing. Importantly, clinical studies corroborate these in vitro findings by demonstrating wound healing, reduction of oxidative stress, and anti-inflammatory effects in human models.

In conclusion, *Calendula officinalis* holds promise as a therapeutic botanical with dual antioxidant and anti-inflammatory efficacy. Its applications extend from dermatological preparations to systemic anti-inflammatory therapies. Future perspectives should focus on standardization of extract preparation, identification of bioactive markers, and large-scale clinical trials to establish dosage, efficacy, and safety.

Calendula officinalis ethanolic extract demonstrated potent in vitro antioxidant and anti-inflammatory properties. These results support its use in phytomedicine. Future work should focus on standardization, bioactive marker isolation, and clinical evaluation.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest

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