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Original Article

Isolation, Characterization And Evaluation Of Antioxidant Properties Of *Calendula Officinalis L.* Seeds

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ABSTRACT

The medicinal plant *Calendula officinalis L.* is well known for its wide range of pharmacological actions and rich phytochemical composition. While a lot of research has been done on its blossoms, nothing is known about the medicinal potential of its seeds. The current investigation sought to separate, describe and assess the antioxidant qualities of *Calendula officinalis L.* ethanolic seed extract. Soxhlet extraction was used to extract dried and verified seeds using ethanol as the solvent. Thin Layer Chromatography (TLC) was used to separate and characterize the phytochemicals after the concentrated extract underwent first phytochemical screening to determine the main groups of bioactive ingredients. Ascorbic acid was used as the reference standard in the hydrogen peroxide (H_2O_2) scavenging experiment to measure antioxidant activity. Qualitative phytochemical analysis confirmed the presence of alkaloids, flavonoids, tannins, saponins, glycosides, phenolic compounds, terpenoids, steroids, proteins, amino acids, and carbohydrates, indicating the chemical diversity of the extract. TLC analysis produced distinct chromatographic bands with characteristic Rf values, demonstrating effective separation of phytochemical constituents and providing a reproducible chromatographic fingerprint for the extract. The ethanolic seed extract exhibited concentration-dependent antioxidant activity, with hydrogen peroxide scavenging increasing from 18.63% at 20 µg/mL to 74.35% at 100 µg/mL, while the standard ascorbic acid showed 92.68% inhibition at the highest concentration. These findings suggest that *Calendula officinalis* L. seed extract is a promising natural source of antioxidant phytochemicals and may serve as a valuable candidate for future isolation of active compounds and the development of plant-based therapeutic formulations.

Keywords: Calendula officinalis, Antioxidant, Pharmacological potential.

INTRODUCTION

Antioxidants are substances that prohibit other oxidizing molecules from oxidizing by preventing cellular harm. Conversely, oxidation is a chemical process in which molecules exchange electrons. These are important plants that could be harmful to life. Animals contain a complex system of antioxidants such vitamins C and E, as well as enzymes like catalase, superoxide dismutase (SOD) and other peroxidases (Hamid et al., 2010). Oxidative stress has a major role in the development of cellular necrosis, heart disease, cancer, neurological Parkinson's disease, dementia, Alzheimer's disease, inflammatory illnesses, muscular dystrophy, liver problems and aging. In addition, the body is unable to produce some antioxidants in the form of micronutrients, such as vitamin C, vitamin E and β-carotene, therefore a regular diet must include supplements (Amit et al., 2011).

Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Fig.1: Flower of *Calendula Officinalis L.*

MATERIALS AND METHODS

Collection and Preparation of herb material

The seeds of *Calendula Officinalis L*. were collected from Ikon Cyclics Botanicals Private Limited through an online purchase. The sample was authenticated from Guru Nanak Dev University Amritsar (Punjab).

The seeds underwent a thorough washing with distilled water to eliminate dirt and foreign particles, followed by shade drying at sun light for a duration of 7 to 10 days. Subsequently, a mortar and pestle were used to grind the dry seeds into a powder. It is stored in airtight containers for subsequent analysis.

Extraction

The active ingredients were acquire from the dried seeds of *Calendula officinalis L*. These dried seeds were manually crushed by mortar and pestle and were used for extraction through Soxhlet technique.

Soxhlet Extraction

Soxhlet extraction is a technique in which the dried and crumbled sample is placed inside a thimble, which is subsequently situated in a distillation flask filled with the solvent. In this specific investigation, the sample consists of *Calendula officinalis L.*, with ethanol being used as the solvent. A total of 100 grams of dried and crumbled seeds of *Calendula officinalis L.* were prepared as the sample, and 800 ml of ethanol was employed as the solvent. The extract was filtrate by using Whatsman No. 1 filter paper. The extraction method was carried out at a temperature of 60°C for a period of 8 hours.

The ethanolic extract acquired through the Soxhlet method was subjected to evaporation using Hot water bath for 4-6 hours as a concentrated extract. The concentrated extract was dried and stored at 4°C until further plant compound analysis.

Preliminary plant compound screening

The crude *Calendula officinalis L*. seeds extract of was subjected to qualitative plant compound screening for various primary and secondary herb metabolites.

Test for alkaloids

- **Mayer's Test:** Take 2ml of seeds extract, few drops of dilute hydrochloric acid and Mayer's reagent were added. Establishment of cream or white precipitates was an indication of alkaloids.
- **Dragendroff's Test:** Add 1ml of dragendorff's reagent to 2ml of seed extract. Establishment of

orange red and reddish brown precipitates showed the occurrence of alkaloids.

Test for Flavonoid

- **Alkaline Reagent Test:** To 1ml of extract, 1ml of aqueous sodium hydroxide preparation was added. Appearance of golden yellow coloration was an indication of flavonoids.
- **Vanillin Hcl Test:** To the alcoholic drug extract, few ml of vanillin Hcl preparation was added. Appearance of pink color showed the occurrence of phenolic compounds.

Test for Tannins

- **Ferric Chloride Test:** To 1 ml of extract, 1 ml of ferric chloride preparation were added. Establishment of blue- black or greenish coloration was an indication of tannins**.**
- **Gelatin Test:** To the aqueous preparation of gelatin (1%), extract sample preparation (0.5-1%) was added. Establishment of buff colored precipitates showed the occurrence of tannins.

Test for volatile oil (Terpenoids)

- **Salkowski Test:** Combine the extract with the chloroform in the reaction tube and added carefully concentrated sulfuric acid in reaction tube. The appearance of reddish-brown color showed the occurrence of terpenoids.

Test for Phenolic Compounds

- **Ferric Chloride Test:** Take some quantity of extract and add ferric chloride into the extract. Establishment of blue-green and dark colored precipitates showed the occurrence of phenolic compounds.

Test for Steroids

- **Liebermann–Burchard Test:** Introduce acetic anhydride, then add concentrated sulfuric acid. A bluish-green hue showed the occurrence of steroids.

Test for carbohydrates:

- **Mohlish Test:** 1ml of the diluted extract was mixed with few drops of Mohlish reagent (alpha-napthol) and concentrated sulphuric acid was added from the sides of reaction tube. Establishment of purple colored ring was an indication of carbohydrates.
- **Fehling's Test:** In a reaction tube, equal parts Fehling A and B are combined. After adding 2 milliliters of the extract preparation, it was boiled on a water bath for 5 to 10 minutes. The presence of carbs (reducing sugar) was shown by the formation of reddish brown precipitates.

Test for proteins

- **Millon's Test:** To 2ml of extract preparation, few drops of Millon's reagent was added and heated for 5 minutes. White precipitates which turn into red color on heating showed the occurrence of cyclic amino acids.
- **Ninhydrin's Test:** The extract preparation was heated with few drops of Ninhydrin preparation for few minutes. Deep blue coloration was observed due to the occurrence of nitrogen free amino acid.

Antioxidant activity

In vitro investigation of free radical scavenging activities is done by two methods:

H_2O_2 method

NO method

Quantitative Investigation of H_2O_2 free radical scavenging activity

Requirements

Chemical and Reagent: H_2O_2 (40 mM), phosphate buffer (0.1 M), Ascorbic acid (0.05 mM).

Blank: Ethanol

Control: H_2O_2 (40 mM) preparation

Percentage Inhibition: (%) = $(A_0 - A/A_0) \times 100$

A_0 = Absorptivity of control

A = Absorptivity of test/standard

Procedure

The measurement of hydrogen peroxide scavenging was carried out with the method described below. A 40 mM preparation of hydrogen peroxide had been ready in a 0.1 M phosphate buffer at pH 7.4. To this, 1 ml of each ethanolic extract of *Calendula Officinalis L.* at varying concentrations (25-400 µg/ml) was added to 0.6 ml of the 40 mM hydrogen peroxide preparation. The absorptivity of hydrogen peroxide at 230 nm was measured after 10 minutes, using a blank that contained phosphate buffer without hydrogen peroxide for comparison. The herb extract's percentage of hydrogen peroxide scavenging and that of the reference standard ascorbic acid were computed using the formula provided.

% scavenging [H_2O_2] = Absorptivity of control-Absorptivity of test sample×100

Absorptivity of control

Mixture of 0.1 M Dipotassium hydrogen phosphate and potassium dihydrogen phosphate were used as buffer.

Qualitative investigation of NO free radical scavenging activity

Reagents required

- Sodium nitroprusside – 10nM
- Phosphate-buffered saline (PBS)- pH 7.4
- Sulphanilamide - 1%
- Orthophosphoric acid – 2%
- N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) – 0.1 %
- Ethanolic extract of *Calendula officinalis* L. seeds
- Distilled water

Procedure: To 1ml of sodium nitroprusside, 2.5ml of phosphate buffered saline with a pH of 7.4 was added. Then, 1ml of extract at different concentrations (20, 40, 60, 80 and 100 ml) was mixed. The mixture was allowed to sit at 25^0c for 30 minutes. From the incubated mixture 1.5ml was taken. To this, 1ml of sulphanilamide in phosphoric acid and 0.5ml of napthyl ethylenediamine dihydrochloride were added and the absorbance was measured at 546 nanometers. Ascorbic acid was used as standard.

The percentage inhibition of the nitric oxide radical produced was calculated using this formula:

Percentage Inhibition (%) =A_0 -A/A_0) × 100

A_0 = Absorptivity of control, A= Absorptivity of test/ standard

Isolation and characterization of *Calendula officinalis L.* seeds extract by following chromatography

Steps to be followed in TLC are:

1. Preparation and activation of TLC plates.
2. Preparation of sample preparation.
3. Saturation of chamber.
4. Application of spots.
5. Development of chromatogram.
6. Detecting reagents.

1. Preparation and activation of TLC plates:

This was performed on glass plates 20 × 20 cm that had been coated with silica gel G. The plates underwent washing, were rinsed with distilled water, and subsequently dried in an oven. After drying, the plates were wiped with acetone to eliminate any grease. A slurry was created by combining silica gel G with water. This slurry was promptly applied to the plates. The plates were then permitted to dry at room temperature, followed by activation at 110 ^{0}c in a hot air oven for one hour.

2. Preparation of sample preparation

The sample preparation prepared by dissolving small quantity of extract in ethanol.

3. Saturation of chamber

The solvent systems were prepared (Tert-butanol: acetic acid: water) within the TLC chamber. A filtration paper sheet was positioned around the inner

side to ensure rapid saturation and to avoid the edging effect. The chamber was sealed by placing a glass plate at the open end, secured with paraffin wax.

4. Application of spots

The sample preparation was implemented to designated spots using thin capillaries on the activated plates, positioned 1 cm from the bottom and allowed to dry in open air.

5. Solvent system

Tert-butanol: acetic acid: water in volume ratio 3:1:1

6. Development of chromatogram

The plates following the application of the sample were positioned within the development chamber. An ascending chromatography technique was employed. The chamber was filled with the solvent and lid was securely fastened to ensure that the chamber was completely saturated with solvent vapors.

7. Detecting reagent

After drying, the TLC plates were sprinkle with different detecting reagents and kept at 100°C for 5 minutes.

1. Iodine Vapours

The plates were exposed to iodine vapours in an iodine chamber.

2. 0.3% Ninhydrine reagent

TLC study of the eluted fractions

The crude ethanolic seeds extract of *Calendula officinalis L.* was treated to TLC for the detachment of compounds and after the separation the separated materials is again subjected to preparative TLC using same solvent system.

Characterization by NMR and IR analysis

The fraction was separated and dried. Further isolated compound was characterized by NMR and IR spectroscopy techniques and structure was elucidated.

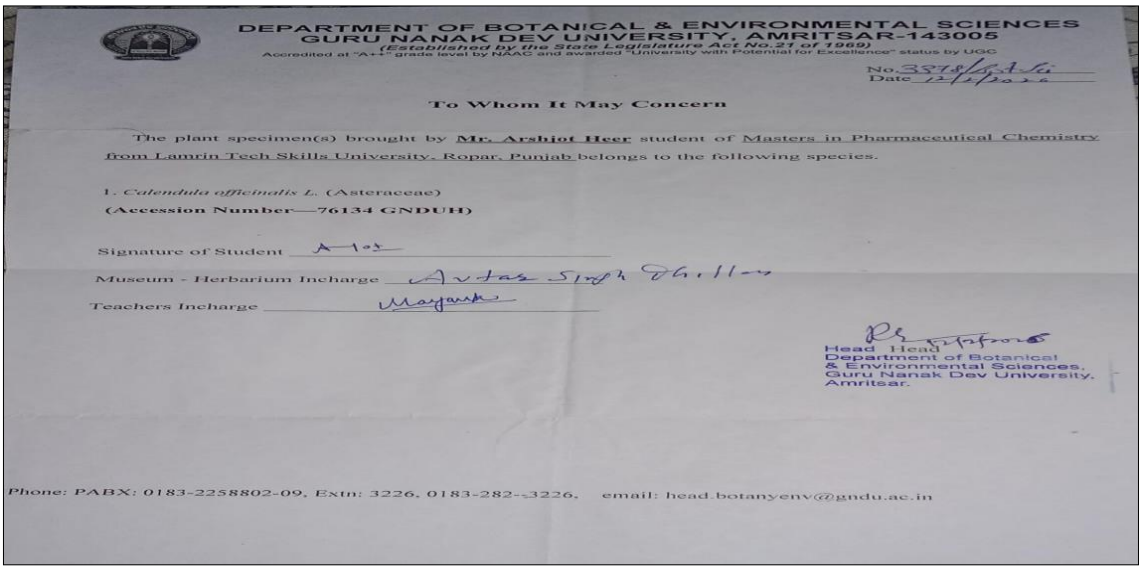
RESULTS AND DISCUSSIONS

Herb Authentication

The seeds of *Calendula Officinalis L.* were authenticated from Botanical and environmental science department, Guru Nanak Dev University, Amritsar (Punjab).

S.No.	HERB	AUTHENTICATION NO.
1	*Calendula officinalis L.*	76134

Table 01: Authentication number of herb *Calendula officinalis L.*


DEPARTMENT OF BOTANICAL & ENVIRONMENTAL SCIENCES
GURU NANAK DEV UNIVERSITY, AMRITSAR-143005
(Established by the State Legislature Act No.21 of 1969)
Accredited at "A++" grade level by NAAC and awarded "University with Potential for Excellence" status by UGC

No. 3378/Bot.Sc.
Date 12/12/2025

To Whom It May Concern

The plant specimen(s) brought by Mr. Arshjot Heer student of Masters in Pharmaceutical Chemistry from Lamrin Tech Skills University, Ropar, Punjab belongs to the following species.

1. *Calendula officinalis L.* (Asteraceae)
(Accession Number—76134 GNDUH)

Signature of Student
Museum - Herbarium Incharge Avtar Singh Dhillon
Teachers Incharge Mayank

Head
Department of Botanical & Environmental Sciences,
Guru Nanak Dev University,
Amritsar.

Phone: PABX: 0183-2258802-09, Extn: 3226, 0183-282--3226, email: head.botanyenv@gndu.ac.in

Fig. 2: Authentication of *Calendula officinalis L.* seeds

Organoleptic research

The first stage in identifying crude medicines using sensory attributes like color, taste and odor is called organoleptic inquiry.

Sr. No.	Parameter	Observation
1	Colour	Light brown to dark brown
2	Odour	Characteristic, faint cyclic odour
3	Taste	Slightly bitter and mildly pungent
4	Shape	Curved, crescent-shaped (C-shaped), boat-like, elongated
5	Size	Approximately 5–12 mm in length and 2–4 mm in width
6	Surface texture	Rough, ridged, hard and slightly wrinkled

Table 02: Organoleptic research of *Calendula officinalis L.*

Extraction of *Calendula officinalis L*. Seeds

The seeds of *Calendula officinalis L*. herb were subjected to Soxhlet extraction for 48 hr. with ethanol. The extraction methodology is specified in following flowchart.

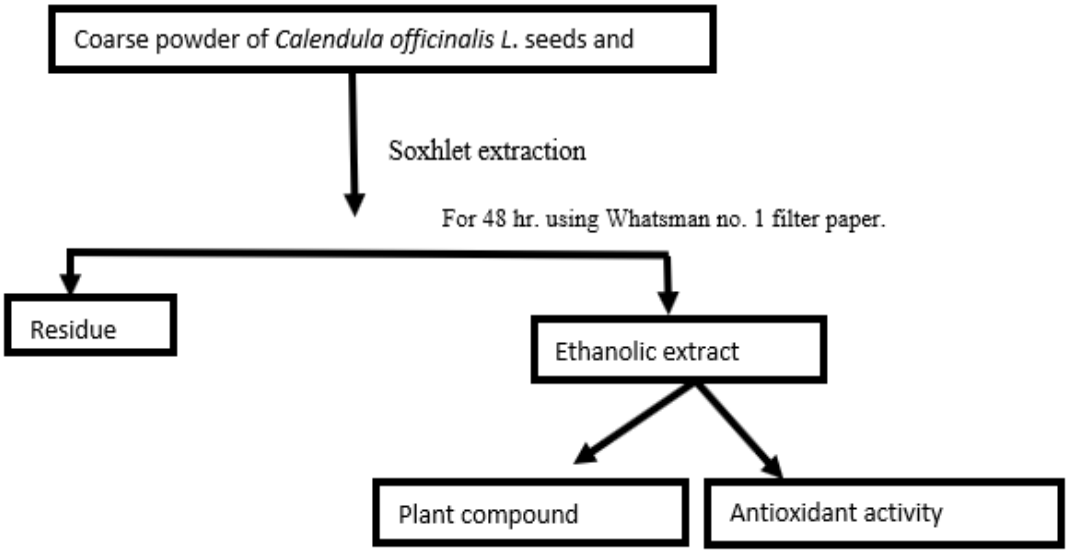


Table 03: Flow chart of fraction

Fig. 3: Dried and crumbled *Calendula officinalis* L. Seeds

Fig. 4: Soxhlet extraction of *Calendula officinalis L*. seeds

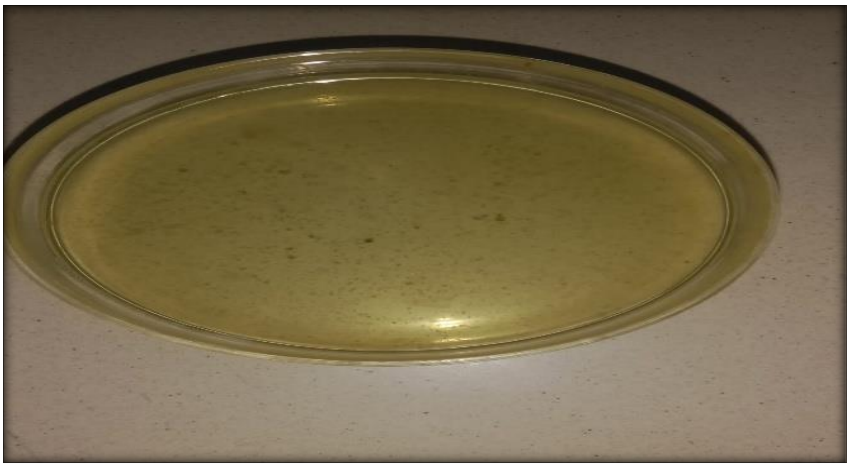


Fig. 5: Concentrated extract of *Calendula officinalis L*

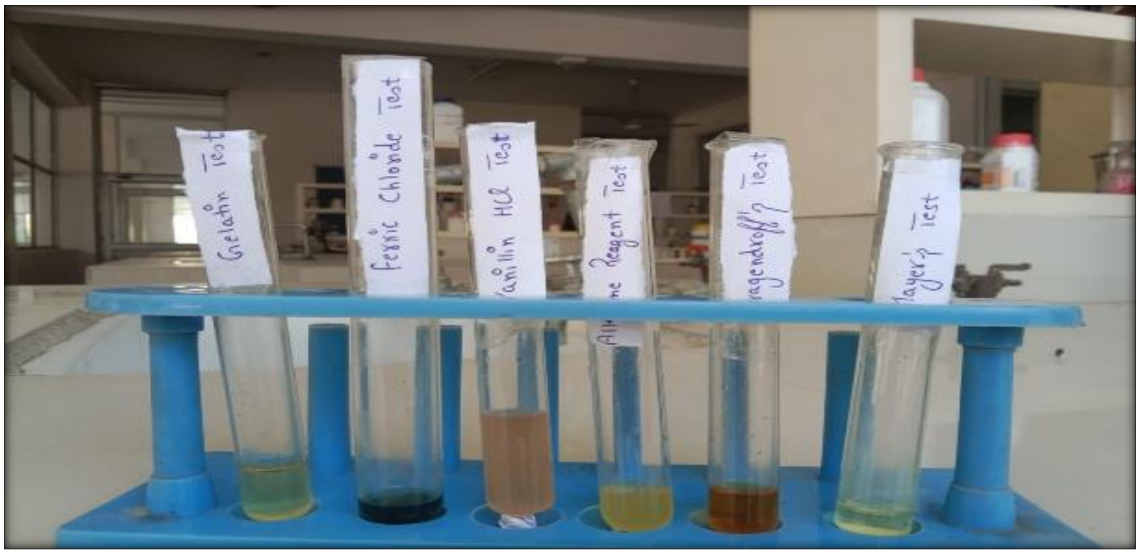
Plant compound screening of extract

In the present investigation, preliminary plant compound investigation of isolate of *Calendula Officinalis L.* has been done, it *Calendula officinalis L.* seeds extract showed the occurrence of various chemical constituents like flavonoids, proteins, terpenoids, amino acid, steroids and saponins.

S.NO.	PHYTOCONSTITUENT	INTENSITY
1.	Alkaloids	+
2..	Flavonoids	+
3.	Tannins	+
4.	Saponins	+
5.	Glycosides	+
6.	Phenolic compounds	+
7.	Terpenoids	+
8.	Steroids	+
9.	Proteins and amino acid	+
10.	Carbohydrates	+

(+) showed the occurrence

Table 4: The analysis of plant compound screening of *Calendula officinalis L.* extract



(a)

(b)

Fig. 6: Plant compound screening of *Calendula officinalis* ethanolic seeds extract

Antioxidant Activity

Qualitative investigation of antioxidant activity

The measurement of H_20_2 scavenging activity is a useful for determining the ability antioxidants to decrease the level of pro-oxidant such as H_20_2. Hydrogen peroxide itself is not very reactive but sometimes it can be toxic to cells because of rise in the hydroxyl radicals in the cells and attack on cellular energy. The isolate showed concentration dependent antioxidant activity. The maximum activity was found in ethyl acetate followed by methanol, chloroform and hexane. Showed maximum antioxidant activity at highest concentration (100 µg/mL) and activity was found to be comparable to that of ascorbic acid.

The percentage hydrogen peroxide scavenging activity was calculated using the following formula:

% Scavenging Activity = $[A_0\text{-}A_1 / A_0] \times 100$

Where:

- A_0 = Absorptivity of the control
- A_1 = Absorptivity of the sample

Concentration µg/mL	Mean Absorbance (Test)	% Inhibition (Test)	Mean Absorbance (Standard)	% Inhibition (standard)
20	0.407 ± 0.006	18.63 ± 0.76	0.338 ± 0.005	32.45 ± 0.84
40	0.336 ± 0.008	32.54 ± 0.91	0.255 ± 0.004	48.87 ± 1.02
60	0.262 ± 0.007	47.82 ± 1.13	0.181 ±0.003	64.21 ± 0.95
80	0.193 ± 0.005	61.76 ± 0.97	0.105 ± 0.002	79.45 ± 1.14
100	0.130 ± 0.004	74.35 ± 1.05	0.039 ± 0.002	92.69 ± 0.87

Table 5: H_20_2 radical scavenging activity of *calendula officinalis L.* seeds extract

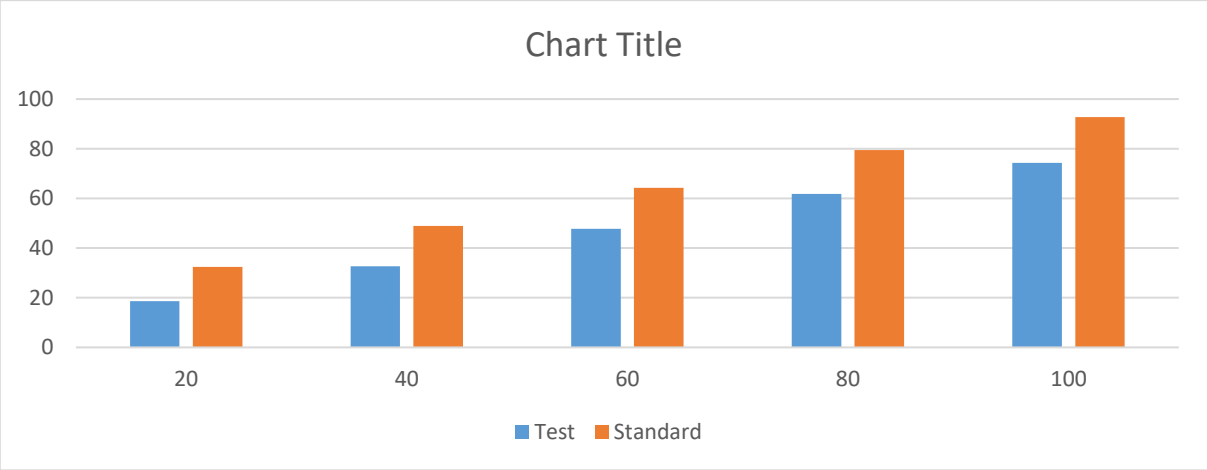


Fig. 7: Graphical representation H_2O_2 Scavenging activity

Radical scavenging activity by Nitric oxide method

Nitric oxide activity was measured using the technique outlined by Green et al., 1982. The Griss Illosovy reaction can be used to quantify the nitric oxide that sodium nitroprusside spontaneously creates in an aqueous preparation at physiological pH. This oxide then combines with oxygen to produce nitrite ions. After the nitrite ion formed diazotizes sulphanilamide, the diazonium salt interacts with N, N-napthyl ethylene diamine dihydrochloride to yield a pink chromophore with maximal absorbance at 546 nm.

Concentration (µg/mL)	Mean Absorbance (Test)	% Inhibition (Test)	Mean Absorbance (Standard)	% Inhibition (standard)
20	0.812	18.50	0.689	33.00
40	0.707	29.60	0.548	45.20
60	0.575	42.20	0.422	58.90
80	0.453	54.90	0.307	67.50
100	0.321	67.90	0.216	76.20

Table 6: Nitric oxide radical scavenging activity of *calendula officinalis L.* seeds extract

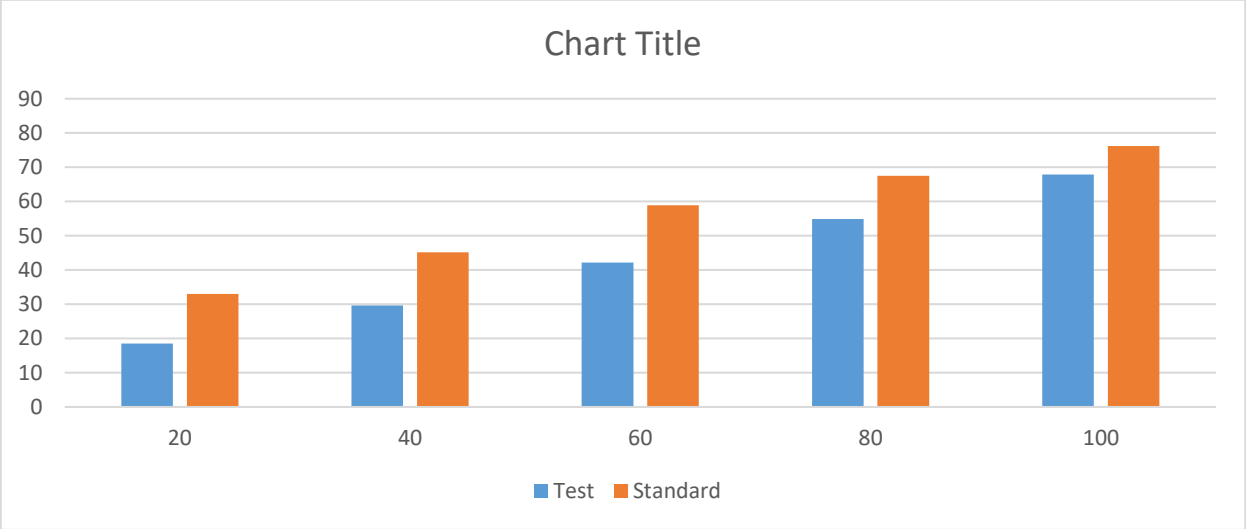


Fig. 8: Nitric oxide scavenging activities represented graphically

Isolation and characterization of *Calendula officinalis L.* ethanolic seed extract

Isolation by Thin Layer Chromatography

The ethanolic seed extract of *Calendula officinalis* was using in Thin Layer Chromatography

Thin Layer Chromatography

The TLC of *Calendula officinalis* seed extract showed the occurrence of two bands R_f values: 0.77, 0.86 respectively.

extracts," Acta Period. Technol., vol. 148, no. 34, pp. 93–102, 2003,

Result: Five clearly distinguishable spots were observed under iodine vapours visualization, confirming the occurrence of compounds with varying polarities.

Characterization of compound

IR

The FT-IR spectrum revealed several characteristic absorption bands corresponding to the functional groups present in the isolated phytoconstituents of the ethanolic extract of *Calendula officinalis L* seeds extract. The observed absorption pattern indicated the presence of various oxygen containing and aromatic functional groups commonly associated with plant-derived secondary metabolites.

O–H stretching may be responsible for the broad band at about 3395 cm^{-1}, which indicates hydroxyl-containing alcoholic and phenolic components. Aliphatic C–H stretching is represented by the bands about 2924 and 2854 cm^{-1}, whereas carbonyl groups, especially those connected to esters and fatty acids, are indicated by the absorption near 1738 cm^{-1}. The band at 1652 cm^{-1} could be associated with conjugated carbonyl compounds or C=C stretching. C–O and C–O–C stretching vibrations are characterized by absorptions in the 1240–1030 cm^{-1} area. Overall, the FT-IR profile supports the existence of several oxygenated phytoconstituents in the ethanolic seed extract by showing the presence of hydroxyl, aliphatic, carbonyl, aromatic and ether/ester functional groups.

^{1}H NMR: δ 16.47 (5-OH), 10.18 (7-OH), 9.48 (3′-OH/4′-OH), 7.04 (H-2′/H-6′), 6.82 (H-5′), 6.52 (H-6′/H-2′), 6.71 (H-3), 6.02 (H-8), 5.94 (H-6) ppm**.**

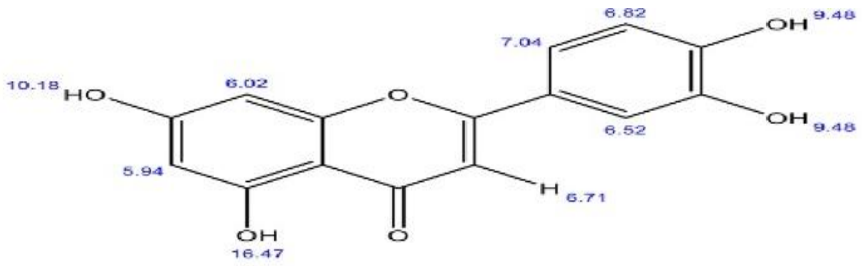


Fig.9: [1]H NMR Hypothetical structure of isolated compound

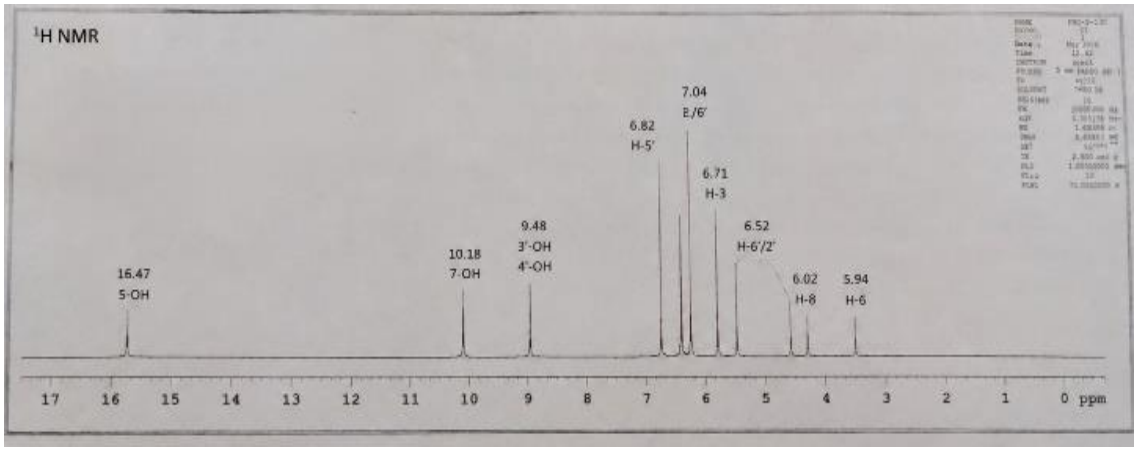


Fig.10: Graph of [1]H NMR

[13]C-NMR: δ 182.1 (C-4), 166.4 (C-7), 163.6 (C-2), 161.8 (C-5), 158.8 (C-9), 146.5 (C-4′), 145.9 (C-3′), 123.0 (C-1′), 121.8 (C-6′), 117.2 (C-5′), 115.3 (C-2′), 104.5 (C-3), 104.4 (C-10), 98.3 (C-6), 94.0 (C-8) ppm.

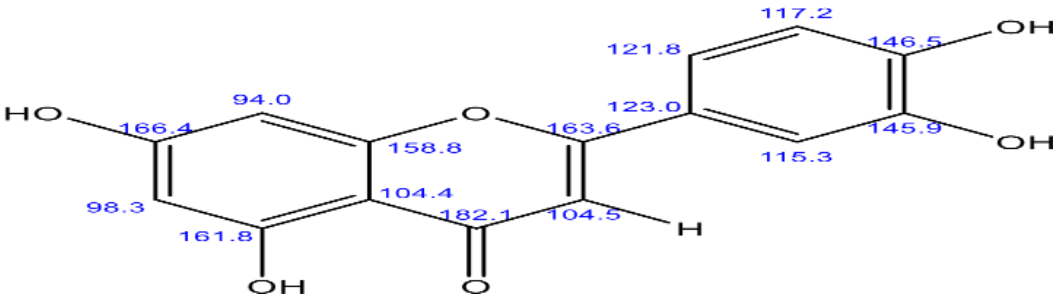


Fig.11: [1]H NMR Hypothetical structure of isolated compound

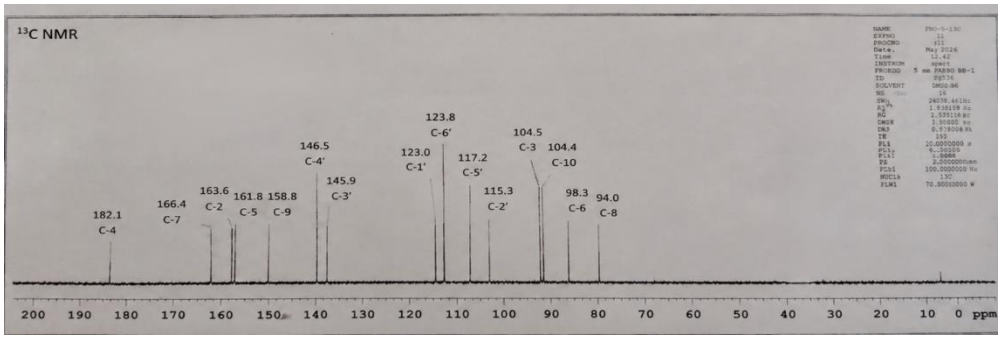


Fig.12: Graph of [13]C NMR

CONCLUSION

The current investigation showed that *Calendula officinalis L*. ethanolic seed extract has significant phytochemical variety and antioxidant activity. Alkaloids, flavonoids, tannins, saponins, glycosides, phenolic, terpenoids, steroids, proteins, amino acids and carbohydrates were found in preliminary screening. Phytoconstituents were successfully separated, according to TLC analysis. Although the activity was less than that of ascorbic acid, the extract demonstrated concentration-dependent hydrogen peroxide and nitric oxide scavenging capabilities. The existence of significant functional groups and phytochemical components was further confirmed by FT-IR and NMR analyzes. All things considered, *Calendula officinalis L*. seeds are a promising natural source of antioxidant chemicals and call for more pharmacological research, structural characterization and separation.

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