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EXTRACTION AND STANDARDIZATION OF CALENDULA OFFICINALIS LEAVES AND ITS ANTI-INFLAMMATORY ACTIVITY

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ABSTRACT

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. Inflammation was characterized two thousand years ago by Celsius by four Latin words: Rubor, calor, tumor and dolor. It is a protective effort by the organism to remove the damaging stimuli and to start the healing process. Without inflammation, wounds and infections would never heal. On the similar way, progressive destroying of the tissue would compromise the survival of the organism. However, chronic inflammation can also lead to a host of diseases, such as hay fever, atherosclerosis, rheumatoid, and even cancer (e.g., gallbladder carcinoma). It is for that reason as inflammation is normally closely regulated by the body. Inflammation is not a alternative for infection, even in cases where inflammation is caused by infection. Although infection can be caused by a microorganism, inflammation is one of the responses of the organism to the pathogen. Infection with pathogenic microbes often outcomes in a substantial inflammatory response and infections may sometimes leads to an acute inflammatory disease, typically demonstrating as a self-limiting infection of the gastrointestinal tract and the mesenteric lymph nodes, resulting in gastroenteritis and lymphadenitis. The herb *C. officinalis* is traditionally used to treat dysmenorrhea, gastrointestinal ulcers, and internal organ inflammation. It is also used as a diuretic and a diaphoretic in convulsion patients.

KEYWORDS: *C. officinalis,* Anti-inflammatory activity, Analgesic.

1.1. INTRODUCTION

There are over 6,000 herbal plants in India that have been used earlier as herbal medicines in ancient times. Recently, only a handful are generally used in common practice.[1] *Calendula officinalis* (*C. officinalis*), also known as pot marigold, was most frequently used as a medicinal plant in ancient India. *Calendula* is known as "gold" in Old English and *C. officinalis* is an annual herb.[2] Tea is widely consumed worldwide and it is well known that in addition to alkaloids, amino acids, polyphenols, carbohydrates, and aromatics, herbal tea also possesses vitamins and minerals. Consumption of herbal tea might prove beneficial in preventing certain medical conditions, such as heart disease, Parkinson's disease, and various malignancies.[3] *Calendula* is a sanctification and detoxifying herb and the infusion is used in the treatment of long-standing infections.[4] *C. officinalis* belongs to the *Asteraceae*/*Compositae* family, a native to Central Europe and Mediterranean countries. *C. officinalis* grows widely in sunny areas and in a variety of soils.[5]

The herb *C. officinalis* is traditionally used to treat dysmenorrhea, gastrointestinal ulcers, and internal organ inflammation. It is also used as a diuretic and a diaphoretic in convulsion patients.[6] In addition, it is used to treat burns, wounds, and inflammation of the pharyngeal and oral mucosa.[6] *Calendula* also helps the body to detoxify.[7] It has been discovered in the past that dried flower petals have antipyretic, anti-tumor, and cicatrizing properties.[8] The infusion is applied topically as an antifungal and antiseptic medication for treating wounds, scars, freckles, and conjunctivitis.[9] Eyewashes and gargles can also be made from *Calendula* tea.[10] Skin inflammations and rashes in children are additional illnesses that have been treated using marigold flower tinctures. In homeopathy, the tincture of *C. officinalis* is used in the treatment of mental tension and insomnia-related disorders.[11] The genus *Calendula* (Asteraceae) contains approximately 25 herbaceous annual or perennial species, commonest being common marigold Linn., (*Calendula arvensis Linn*., *Calendula suffruticosa Vahl*., *Calendula stellata Cav Calendula alata Rech Calendula tripterocarpa Rupr).*

Synonyms: *Calendula officinalis* is also known as pot marigold, ruddles or common marigold.

Morphological Features *C. officinalis Linn.*, is an annual or biennial plant. It attains a height of 30-60 cm. Its stem is angular, hairy and solid; leaves are lower spatulate, 10-20 cm long and 1-4 cm wide; higher oblong and mucronate, 4-7 cm long; anomocytic stomata within the apical region of outer epidermis, covering and glandular trichomes, elongated sclerenchyma cells, marginal flower heads are bright yellow to orange in color; corolla oblong spatulate, 15- 25 mm long and about three mm wide; corolla of disc flowers rounded, at the very best tridentate, 1.5-2.5 cm long and 4-7 mm in diameter with 5 mm long tubular florets. The powdered *C. officinalis* is raw sienna with a characteristic, aromatic odor and a quite bitter taste.

Inflammation (Latin, *inflammare*, to set on fire) is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants.[12]

Inflammation was characterized two thousand years ago by Celsius by four Latin words: Rubor, calor, tumor and dolor. It is a protective effort by the organism to remove the damaging stimuli and to start the healing process. Without inflammation, wounds and infections would never heal. On the similar way, progressive destroying of the tissue would compromise the survival of the organism. However, chronic inflammation can also lead to a host of diseases, such as hay fever, atherosclerosis, rheumatoid, and even cancer (e.g., gallbladder carcinoma). It is for that reason as inflammation is normally closely regulated by the body. Inflammation is not a alternative for infection, even in cases where inflammation is caused by infection. Although infection can be caused by a microorganism, inflammation is one of the responses of the organism to the pathogen. Infection with pathogenic microbes often outcomes in a substantial inflammatory response.[13]

and infections may sometimes leads to an acute inflammatory disease, typically demonstrating as a self-limiting infection of the gastrointestinal tract and the mesenteric lymph nodes, resulting in gastroenteritis and lymphadenitis.

1.2 Types of Inflammation

Inflammation can be classified as either *acute* or *chronic*.

Acute transient inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. It is mediated by autacoids and characterized by increased vascular permeability, local vasodilatation & exudation of fluid & plasma proteins (edema). Poly morph nuclear neutrophils as inflammatory cells. A flow of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue.

Subacute, This phase is characterized by emigration of leukocytis, predominantly neutrophills & phagocytic cell.

Chronic inflammation, also known as prolonged inflammation, occur either after the causative agent of acute inflammation persist for a long time, or the stimulus is such that it include chronic inflammation from beginning & associated with presence of lymphocytes & macrophages, prolifiration of blood vassels, fibrosis & tissue necrosis. It leads to a progressive move in the type of cells present at the site of inflammation and is characterized by destruction and healing of the tissue from the inflammatory process simultaneous.

MATERIAL AND METHODS

3.1. MATERIAL

3.1.1. EXTRACTION

Leaves of *Calendula officinalis* were shade dried and crushed into powder using crushing machine. Powder were extracted using Soxhlet extraction using different polarity solvents. About 1000 mg of dried Leaves of. *Calendula officinalis* powder was weighed into a round bottom flask. 10 mL of methanol was added to the flask and the mixture was then sonicated for about 30 min with intermediate shaking. The extract was then filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India). The same procedure was performed twice and filtrate obtained was evaporated to dryness. Final volume was adjusted to 5.0 mL with methanol in volumetric flask. The same procedure was performed twice and filtrate obtained was evaporated to dryness. Final volume was adjusted to 5.0 mL with methanol in volumetric flask.

Using above procedure ethanolic, water and chloroform extract were prepared for *Calendula officinalis* plant sample.

3.2.2. STANDARDISATION

3.1.2.1. Macroscopic Characters

(a) Size: Length and width was measured in millimetre by a graduated ruler. the thickness was measured by screw gauge.

(b) Colour: Diffuse Daylight was used to identify the exact colour.

(c) Surface texture and characteristics: Hardness, softness, smoothness, roughness were all identified by touching the powdered material.

(d) Odour: The powdered material was smelt the strength of odour of the powdered material was determined whether (distinct, none, weak, strong, etc)

(e) Taste: The powder was tasted in small amount to know the taste.(WHO guidelines)

Determination of ash value

Total ash value

The ash remaining after ignition of medicinal plant materials is determined by three different methods which

measure total ash, water soluble ash, and acid soluble ash.

Procedure: 2 g of the ground air dried material was accurately weighed and placed in a previously ignited and tarred crucible. The material was spread in an even layer and was ignited by gradually increasing the heat to 500-600 C until it was white, indicating the presence of carbon. It was cooled in desiccator for 30 min and weight without delay. The content of total ash calculated in mg/g of the air dried materials. The calculations and answer are written in results and discussion.

Determination of acid insoluble ash

Procedure: To the crucible containing the total ash, 25ml of HCl was added and covered with a watch glass and boiled gently for 5 min watch glass was rinsed with 5 ml of hot water and liquid was added to the crucible. The insoluble matter was collected on ash less filter paper and washed with hot water until the filtrate was neutral. After that the filter paper containing the insoluble matter was transferred to the original crucible, fried on a hot plate and ignited to constant weight. The residue was allowed to cooled in desiccator for 30 min and weighed without delay. The content of acid insoluble ash calculated in mg of air dried material. the results are tabulated in next portion. (Khandelwal 2010).

Water insoluble ash

Procedure: To the crucible containing the total ash, 25ml of water was added and boiled for 5 min. The insoluble matter was collected in ash less filter paper and washed with hot water. The crucible was ignited for 15 min at a temperature not exceeding 450 C. The weight of residue was subtracted in mg from the weight of total ash and content of water soluble ash was calculated in mg/g of air dried material. the results are recorded.(WHO guidelines).

Determination of Extractive values

Exhaust the powdered drug into different solvents to obtain extracts of different polarity. Then further treat the small quantities of extract to get the chemical composition of crude drug. Different solvents are used according to their polarities to get the maximum number of phytochemical present in the drug.

Procedure of extractive value

4 g of coarsly powdered air dried material was accurately weighted and placed in glass stoppered conical flask. It was macerated with 100 ml of the solvent specified for the plant material concerned for 6 hours, shaken frequently and then allowed to stand for 18 hrs. Solvent was filtered without loss and 25 ml of filtrate was transferred to the tarred bottom dish and evaporated to dryness on a water bath. The content of extractable matter in mg/g of air dried material was calculated, The result are reported.

Aqueous extractive value

Distilled water was used as a solvent and rest of the procedure is followed as mentioned.

Methanol Extractive Value

Methanol was used as a solvent and rest of the procedure is followed as mentioned above.

Ethanol extractive value

Ethanol is used as a solvent here and the above mentioned procedure was followed.

Chloroform Extractive Value

Chloroform was used as a solvent and the above mentioned procedure was followed.

3.1.2.2. PHYTOCHEMICAL SCREENING

Ethanolic and aqueous extracts of *Calendula officinalis* was treated with different reagents to prove the presence of different phytochemicals (Treasa 2002).[61]

Test for Tannins

(a) Extract + 5% ferric chloride solution: Extract was treated with 5% solution of ferric chloride. The formation of dark blue or greenish black products shows the presence of tannin.

Test for Alkaloids

(a) Dragendorff's Test: The extract was treated with potassium bismuth iodide solution (Dragendroff's Reagent). The formation of orange red precipitate confirms the presence of alkaloids.

b) Mayer's Test: Potassium mercuric iodide solution was added to the extract. The formation of cream coloured or whitish yellow precipitate confirms the presence of alkaloids.

Test for Carbohydrates

(a) Molisch's Test: The extract was first treated with α-napthol and then conc sulphuric acid was poured from the side. Formation of purple or reddish violet colour indicated the carbohydrates presence.

Test for Saponins

(a) Foam test: Small quantity of extract was added to 20 ml of distilled water and shaken for 15 mins in a graduated cylinder. Formation of 1 cm of layer above the liquid shows the presence of saponins.

Test for Flavanoids

(a) Shinoda test: To extract, add magnesium turnings in small quantity, then to this solution drop drops of conc HCl. The colour which appear after sometime will be green to blue, pink scarlet or red.

(b) Alkaline reagent test: Few drops of sodium hydroxide were added to the extracts which results in formation of deep yellow colour. This liquid turns colourless on addition of dil acid which confirm the presence.

(c) Zinc hydrochloride test: Formation of red colour on addition of zinc dust and HCl into the extract confirms presence of flavonoids.

Test for Steroids and Sterols

(a) Salkowski Test: The extract is first treated with chloroform and then with sulfuric acid. Two different layers are form. the choloform layer appears bluish red or cherry and the acid layer appears green fluorescence.

(b) Libermann-Burchard Test: A mixture of extract, glacial acetic acid, chloroform, acetic anhydride was heated and cooled. Then a few drops of conc sulphuric acid was added to obtain bluish green colour.

Test for Amino Acids

(a) Ninhydrin Test: Few drops of 0.2% ninhydrin reagent(0.1% sol in n-butanol) was added the extracts to obtain blue colour which showed the presence of amino acid, protein, peptides.

Test for protein

(a) Biuret Test: A pink colour solution is obtained in addition of 1% CuSO4 and sodium hydroxide which on pouring into extract gives pink or purple violet colour.

(b) Millon's Test: Sulphuric acid was added into the extract and million's reagent was further added. The solution was boiled which shows the formation of yellow precipitate.

3.2.3. ANTI-INFLAMMATORY ACTIVITY

Inflammation in rat paw by carrageenan

1% w/v of carrageenan was used to produce inflammation in rats. The animals were kept on fasting for 16 hours prior to administration of carrageenan. 0.1 ml of carrageenan was injected into the right hind paw.

Experimental Protocol

Animal were divided into 4 groups each containing six animals.

Group 1 (Carrageenan control group): Animals were treated with 1% carrageenan dissolved in distilled water injected before the study.

Group 2 (standard group): Animals were given 10mg/kg diclofenac sodium dissolved in DMSO(dimethyl sulfoxide). After one hour edema is produced in the animals by injecting carrageenan.

Group 3 (treatment group): Animals were treated 250 mg/kg of methanolic extract of *Dioscorea deltoidea*. After one hour edema is produced in the animal by injecting carrageenan.

Group 4 (treatment group): Animals were treated with 200mg/kg of aqueous extract of *Dioscorea Deltoidea*. After one hour edema is produced by injecting carrageenan.

The change in the paw volume was noted and further calculated to obtain a degree of inflammation. The dose of drugs and extracts for rats and mice were calculated by the below mentioned formula: Dose = (Std. /1000) x b. w Where, Std. is the standard dose of the drug and b.w. is the body weight of the animals.

4.4. ANTI-INFLAMMATORY ACTIVITY

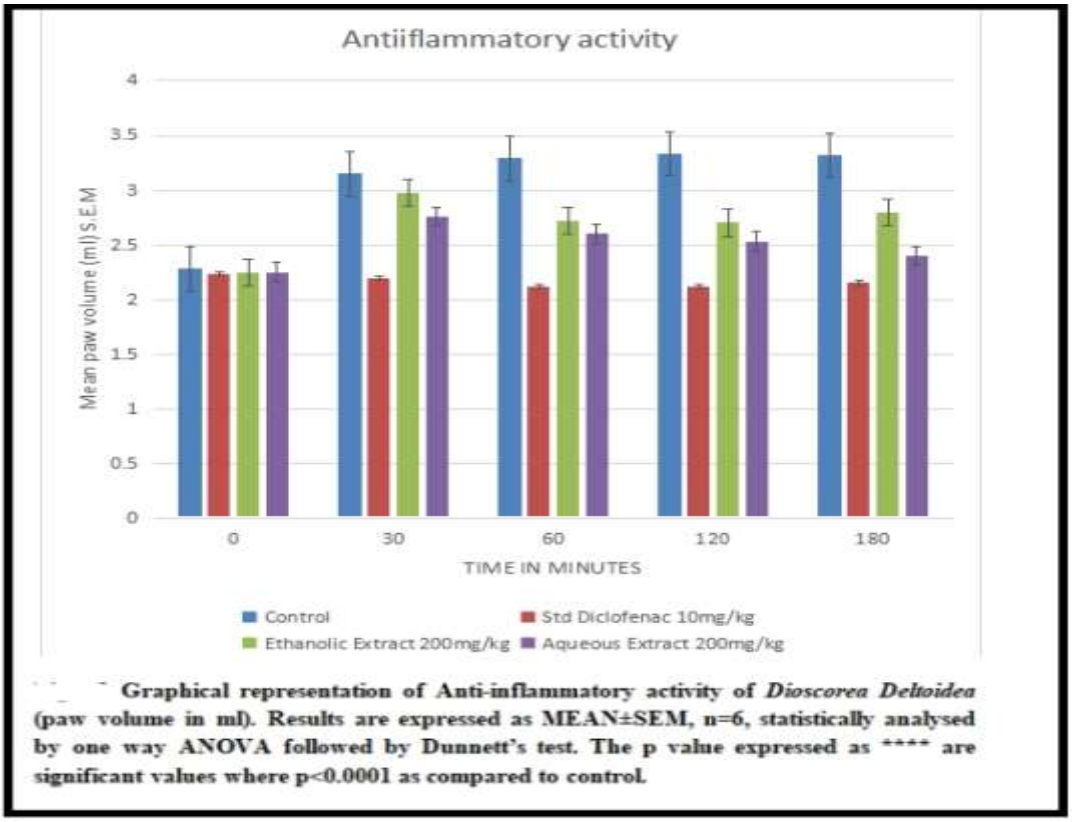
4.4.1. Effect of *C. officinalis* Leaves on inflammation

Measurement of paw volume by plethysmometer.

Table 1: Effect of extract of *C. officinalis* (ml) on rat paw.

S. No	Treatment	Dose (mg/kg)	Mean paw volume (ml) S.E.M				
			0 min	30 min	60 min	120 min	180 min
1.	Control (Carageenan 1%)	Normal saline (NIL)	2.28± 0.20	3.15± 0.04	3.29 ± 0.20	3.33± 0.20	3.32± 0.08
2.	Standard Diclofenac	10 mg/kg	2.23± 0.10	2.19± 0.13****	2.12 ± 0.12***	2.12± 0.10****	2.15 ± 0.17
3.	ethanolic Extract	200 mg/kg	2.24± 0.09	2.98± 0.09	2.72 ± 0.12	2.70± 0.15*	2.80 ± 0.09
4.	Aqueous Extract	200 mg/kg	2.25± 0.06	2.76± 0.06*	2.60± 0.08*	2.53± 0.08**	2.40± 0.09

The resultant activity is show by MEAN± SEM and further calculated by ANOVA and multiple comparison done with tukey's test. The comparison of groups done against control group led to p values. ***p≤0.001, **p≤0.01, *p≤0.05

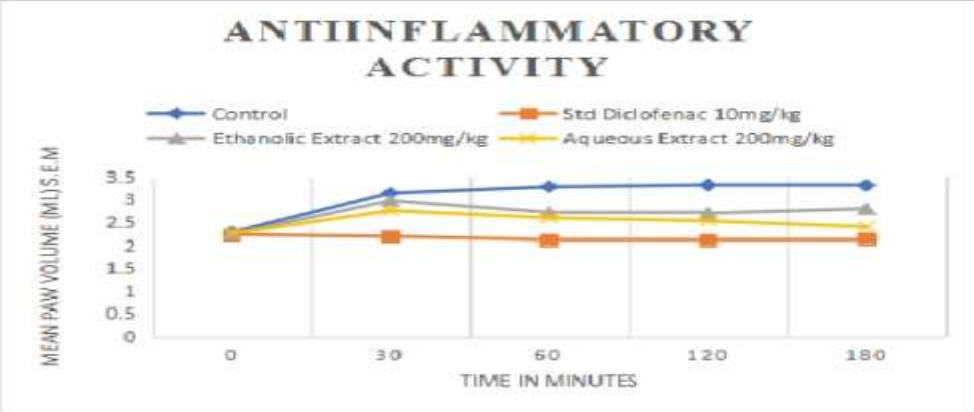


Graphical representation of Anti-inflammatory activity of *Dioscorea Deltoidea* (paw volume in ml). Results are expressed as MEAN±SEM, n=6, statistically analysed by one way ANOVA followed by Dunnett's test. The p value expressed as ** are significant values where p<0.0001 as compared to control.**

Fig 1.

Table 4.4.2: Effect of *C. officinalis* ethanolic and aqueous extract on inhibition of joint inflammation (paw volume in ml)

Treatment	After 0 min	After 30 min	After 60 min	After 120 min	After 180 min
Control	-	-	-	-	-
Standard	3.58%	28.48%	36.56%	37.33%	42.24%
ethanolic Extract	2.21%	5.39%	17.32%	21.92%	21.78%
Aqueous Extract	2.65%	12.38%	20.97%	27.02%	27.98%



: Graphical representation of % Inhibition of joint inflammation (paw volume in ml). Results are expressed as MEAN±SEM, n=6, statistically analysed by one way ANOVA followed by Dunnett's test. The p value expressed as ** are significant values where p<0.0001 as compared to control.**

Fig 2

CONCLUSION

Medicinal plants have been in use since ancient times according to their therapeutic activities. Large number of herbs are used to treat pain in different parts of the body according to their safety. *Calendula officinalis* is one of those species of *Asteraceae* family which has flowers and leaves of medicinal as well as nutritional value. Different solutions are made to treat metabolic disorders, orthopaedic disorders, autoimmune disorder, CNS disorder, heart disorders, digestive disorders, sore throat, diarrhoea, abdominal pain, irritability, wounds, burns, anaemia.

The pharmacological effects of various chemical constituents of *Calendula officinalis* makes it a very useful drug. In this study, we have carried out the extraction and standardisation of the leaves of the plant *Calendula officinalis*. Moreover, the phytochemical screening was also performed for the analysis of chemical constituents. Based on the phytochemical screening, which was carried out in this study, we concluded the presence of these valuable constituents which are previously reported for anti-inflammatory activities. Further, we carried out pharmacological activities anti-inflammatory) on two extracts: aqueous and ethanolic extract of *Calendula officinalis* by rat paw edema produced by carrageenan and method respectively. The values represented in the table are mean ± SEM, One-way ANOVA followed by Dunette's test, $p<0.001$, $p<0.01$, $p<0.05$. The aqueous extract of *Calendula officinalis* was better in inhibiting the inflammation by 27.98% as compared to ethanolic extract 21.78%. Same were the results with analgesic activity.

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