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ASSESSMENT OF ANTI-OXIDANT, ANTI-BACTERIAL AND ANTI-ULCER ACTIVITIES OF *CALENDULA ARVENSIS* LEAVES

Shailaja K, Kailash Venkatesh K, Meghana K, Soumya M, Vasudha Bakshi, Narender Boggula, Nerella Mounika*

School of Pharmacy, Anurag University, Venkatapur, Ghatkesar, Medchal, Hyderabad, Telangana, INDIA – 500 088.

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Abstract:

Medicinal plants which act as therapeutic agents are also a good source of information for a wide variety of phytochemical constituents which can be developed as drugs with precise and good selectivity. Calendula arvensis is an interesting medicinal plant and it is also known as "Aljemrah" in Morocco. It is a species of flowering plant in the daisy family known by the common name 'field marigold'. It is the important medicinal plant shows various bioactivities. The aim of the present research work is to screen the anti-oxidant, anti-bacterial and anti-ulcer and phytochemical activities of an ethanolic extract of Calendula arvensis leaves. The extract was screened for anti-oxidant activity using reducing power assay, total phenolic and total flavonoid content. The total phenolic and total flavonoid contents were found to be 47.01±1.5 and 62.16±9.7 respectively. In the reducing power assay [RPA] the extract showed significantly reduce in the absorbance to 0.892 at concentration of 250mg as compared to 1.996 with standard Gallic acid. Anti-bacterial activity of Calendula arvensis ethanolic extract were investigated against B. cereus, S. aureus, E. coli, P. aeruginosa using agar disc diffusion technique. The ethanolic extract of Calendula arvensis were potentially effective with variable efficiency against the tested bacterial strains at concentration of 10 mg with standard gentamycin. In acid neutralizing capacity the extract significantly reduced the acid neutralizing capacity to 5.3 at concentration of 1000mg is compared to 35.7 with standard aluminium hydroxide + magnesium hydroxide (500mg). The study reveals that ethanolic extract of Calendula arvensis possess considerable number of phenols, flavonoids, bacterial and antacid capacity. Further, this investigation will be helpful to identify the plant and also provide valuable information to the researchers to establish the pharmacological activities supported with possible mode of action.

Key words: *Calendula arvensis, anti-oxidant activity, anti-bacterial activity, anti-ulcer activity.*

Corresponding author:

Nerella Mounika,
Assistant Professor,
Department of Pharmacology,
School of Pharmacy, Anurag University,
Venkatapur, Ghatkesar, Medchal,
Hyderabad, Telangana, INDIA – 500 088.E-Mail: nerellamounika16@gmail.com
Mobile: +91 98492 57928

QR code

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INTRODUCTION:

Ayurveda is a traditional system of medicine in which herbal therapies were used systematically. Plants have been used for medicinal purposes long before prehistoric period. Ayurveda, the traditional system of medicine continues to be widely practiced on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several synthetic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant material as a source of medicines for a wide variety of human ailments.

India has an ancient heritage of traditional medicine. The material medica provides a great deal of information on the folklore practices and traditional aspects of therapeutically important natural products. Indian traditional medicine is based on various systems including Ayurveda, Siddha, Unani and Homoeopathy. The evaluation of all these drugs is based on phytochemical and pharmacological approaches which lead to drug discovery often is referred to as "natural product screening". Any part of the plant may contain active components like bark, leaves, flowers, roots, fruits, seeds, etc. Plants have been used for thousands of years to flavour and conserve food, to treat health disorders and to prevent diseases including epidemics. The knowledge of their healing properties has been transmitted over the centuries within and among human communities. Active compounds produced during secondary vegetal metabolism are usually responsible for the biological properties of some plant species used throughout the globe for various purposes, including treatment of infectious diseases.

Plant profile

Asteraceae, also known as Compositae, is the family with the large number of species among the Dicotyledons. *Calendula* belongs to this family and includes several species namely *Calendula arvensis Linn, Calendula officinalis Linn, Calendula suffruticosa* Vahl, *Calendula stellate* Cav, *Calendulla alata* Rech, *Calendula tripterocarpa* Rupr, which are commonly used as ornamental and medicinal plants. According to the ancient records, the *Calendula* flowers were used as a symbol of remembrance and believed to give great forces of warmth and benign compassion to the human soul, especially helping to balance the active and receptive modes of communication. Besides, the *Calendula* essential oil has been reported to be used in care of the elderly. *Calendula* is used in Ayurveda for the treatment of fever and cancer. It is used for its various biological activities like anti-inflammatory, anti-mutagenic, diuretic, anti-spasmodic activities. AIDS, cancer, dengue, fever, and so many diseases are the serious threatening to the present world. The aerial parts of the *Calendula* are traditionally used in the treatment of kidney stones and gall stone.

The aim of the present study is to assess the phytochemical screening, anti-oxidant, anti-bacterial and anti-ulcer activities of ethanolic extract of *Calendula arvensis leaves.*

Figure 1: *Calendula arvensis* plant

MATERIALS AND METHODS:

Ethical approval

This experiment was approved by the Institutional Animal Ethical Committee (IAEC) (I/IAEC/AGI/001/2022 WR ♀♂), School of Pharmacy, Anurag University, Venkatapur, Ghatkesar, Medchal, Hyderabad, Telangana.

Study area

The investigation was conducted at Pharmacology Laboratory, Department of Pharmacology, School of Pharmacy, Anurag University, Venkatapur, Ghatkesar, Medchal, Telangana.

Collection of plant material

The bulk quantity of freshly *Calendula arvensis* leaves were collected from local area of Uppal, Hyderabad, Telangana, India.

Figure 2: *Calendula arvensis* leaves powder

Preparation of extract

The collected powder is then subjected to the extraction process by a Soxhlet extraction method using 90% ethanol as a solvent at room temperature for 20-24 h. The ethanolic extract was then concentrated by simple distillation to dry. The collected extract was stored in desiccators and used for further pharmacological study.

Experimental design

Preliminary phytochemical studies:

Preliminary phytochemical tests of leaves of *Calendula arvensis* ethanolic extract (CAEE) was performed by using standard protocols.

Pharmacological Screening

Anti-oxidant activity

Total phenolic determination:

The Total phenolic content was determined using the Folin-Ciocalteu reagent. The 0.1 mL of Calendula arvensis ethanolic extract was diluted with 1 mL of distilled water. The mixture is added to a solution of 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL of 20% sodium carbonate solution. The reaction mixture was incubated for 2 hours, and, finally, the volume was levelled to a final volume of 10 mL, and the absorbance was recorded at 765 nm using UV-VIS spectrophotometer. Gallic acid (0–200 µg/mL) was used for calibration of standard curve. The total phenolic content was expressed as milligram gallic acid equivalent (mg GAE)/g dry weight of plant material.

Total flavonoid determination:

The method given by Zhishen et al. which is used for analyzing the total flavonoid content (TFC) employing rutin as a standard. To 1mL of Calendula Arvensis ethanolic extract, a solution of distilled water, 5% $NaNO_2$, and 10% $AlCl_3$ were added. The reaction mixture was incubated for 5 min. To the above reaction mixture, 1M NaOH and 2.4 mL distil water were added to get the final volume of 10 mL. The absorbance of samples was read at 510 nm by using UV-VIS spectrophotometer. The total flavonoid content was expressed as mg rutin equivalents per gram (mg RE/g) through the calibration curve.

Reducing power assay:

The 1 mL of Calendula Arvensis ethanolic extract was added to a 2.5 mL of phosphate buffer and 2.5 mL of 1% potassium ferricyanide. Then the reaction mixture was incubated for 20 minutes at 50 °C. To this reaction mixture add 2.5 mL of 10% TCA and centrifuged. The supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$. The absorbance of sample was recorded at 700 nm by using UV-VIS spectrophotometer. The assay was carried out in triplicate, and the results are expressed as mean ± standard error (SE). As the absorbance of the sample increases with concentration indicates high reducing potential of the samples.

Anti-bacterial activity
Disk-diffusion method:
Agar disk-diffusion method was developed in 1940. This method is widely used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. Nowadays, many accepted and approved standards are published by the Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeasts testing. Although not all fastidious bacteria can be tested accurately by this method, the standardization has been made to test certain fastidious bacterial pathogens like *Streptococci*, *Haemophilus influenzae*, *Haemophilus parainfluenza*, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, using specific culture media, various incubation conditions and interpretive criteria for inhibition zones.

The agar plates are inoculated with a standardized inoculum of the test microorganism. Then, filter paper discs of (about 6 mm in diameter), containing the test compound at a desired concentration, are placed on the agar surface. The Petri dishes are incubated under suitable conditions. Generally, antimicrobial agent diffuses into the agar and inhibits germination and growth of the test microorganism and then the diameters of inhibition growth zones are measured. Moreover, the agar disk-diffusion method is not the appropriate method to determine the minimum inhibitory concentration (MIC), as it is impossible to quantify the amount of the antimicrobial agent diffused into the agar medium. Nevertheless, an approximate MIC can be calculated for some microorganisms and antibiotics by comparing the inhibition zones with stored algorithms.

Anti-ulcer activity:
Acid neutralizing capacity:

The acid neutralizing capacity of ethanolic extract of various concentrations (100mg, 500mg, 1000mg, 1500mg) was compared with standard antacid aluminum hydroxide + magnesium hydroxide (500mg). To the 5 ml quantity of test substance (*Calendula arvensis* ethanolic extract) water is added to make up to the final volume 70 ml and mixed for 2 min. Then add 30ml 0f 1.0N HCl is added to the test substance and standard substance and stir for few min. Then add few drops of phenolphthalein indicator and stir. The test substance and the standard substance is titrated with 0.5N sodium hydroxide solution dropwise until pink colour is appeared. The moles of acid neutralizing capacity are calculated by,

Moles of acid neutralized = (Volume of HCl × Normality of HCl) – (Volume of NaOH × Normality of NaOH)

Acid Neutralizing capacity (ANC) per gm of Antacid = Moles of HCl neutralized / Gm of antacid

Statistical analysis

All analyses were run in triplicate and the results were expressed as mean ± standard deviation (SD). The data were analysed by using one - way analysis of variance (ANOVA) followed by Dunnett's 't' test. P values <0.05 were considered as significant.

RESULTS AND DISCUSSION:

The present study was designed to evaluate the anti-oxidant, anti-bacterial and anti-ulcer activities of *Calendula arvensis* leaves extract. Literature review conducted on this plant indicated the presence of various active constituents like glycosides, flavonoids, saponins, terpenoids, carbohydrates, alkaloids etc. since these compounds were soluble in ethanol and was used as solvent for extraction.

Preliminary phytochemical screening:
The preliminary phytochemical analysis of ethanolic extract of *Calendula Arvensis* (CAEE) indicated the presence of various active constituents like steroids, carbohydrates, glycosides, tannins, saponins, triterpenoids, flavonoids and alkaloids. The results are shown in Table 1.

Table 1: Phytochemical screening

Type of phytoconstituent	Ethanolic extract
Alkaloids	
Mayer's test	++
Wagner's test	++
Dragendorff's test	++
Hager's test	++
Flavonoids	
Shinoda test	++
Alkaline reagent	++
Steroids and terpenoids	
Liebermann Burchard test	++
Salkowski test	++
Phenolic compound and tannins	
Fecl3 test	++
Lead acetate test	++
Saponin glycosides	
Foam test	--
Glycosides	
Type of phytoconstituent	**Ethanolic extract**
Alkaloids	
Mayer's test	++
Wagner's test	++
Dragendorff's test	++
Hager's test	++

(++) Indicates presence, (--) indicates absence.

***In vitro* anti-oxidant activity:**

Total phenolic and total flavonoid contents:

The ethanolic extract of *Calendula arvensis leaves* showed that the total phenolic content was found to be 47.01± 1.5 and the total flavonoid content was found to be 62.16 ± 9.7. The results were shown in Table 2.

Table 2: Total phenolic and total flavonoid contents of CAEE

Extract	Phenolic content	Flavonoid content
Ethanolic extract	47.01± 1.5	62.16± 9.7

*Values were expressed as mean±SD. *Significantly different from P<0.05.*

Reducing power assay:

The ethanolic extract of *Calendula arvensis* leaves were taken and were used to study the reducing power assay. The absorbance is taken as 700nm, the absorbance is taken on Y-axis and the concentrations are taken on X-axis. The Gallic acid (GA) is taken as the standard drug. The absorbance increases as the concentrations are increased; the absorbance of the test extract is less than the absorbance of the standard drug. The results for the following have been shown in the below given Table 3 and the graph has been plotted for the same.

Table 3: Reducing power assay

	Absorbance at 700nm	
Concentrations (µg/ml)	**Test extract (CAEE)**	**Standard (GA)**
50	0.246	1.22
100	0.453	1.485
150	0.597	1.663
200	0.781	1.742
250	0.842	1.996

*Values were expressed as mean±SD, *Significantly different from P<0.05.*

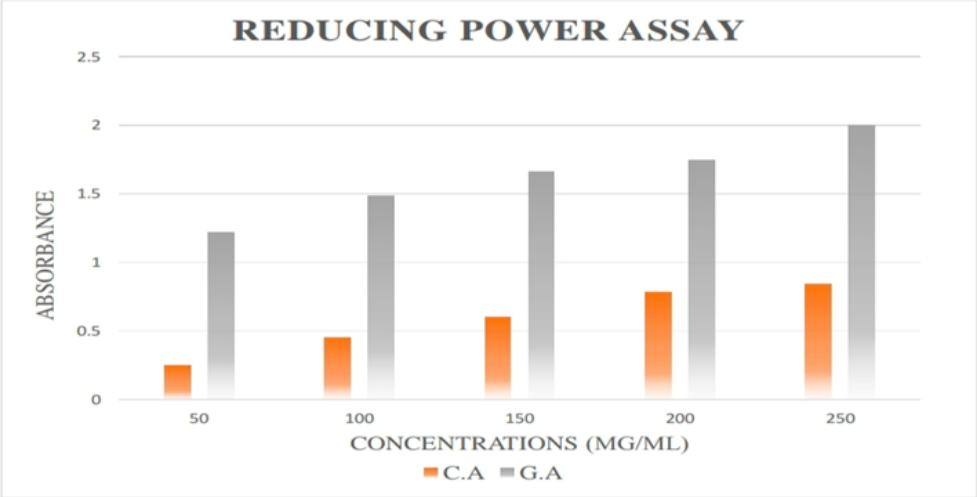

Figure 3: Reducing power assay

Antibacterial activity:

Calendula arvensis ethanolic extract were investigated to evaluate their anti-bacterial activity of Gram-positive bacteria (*B. cereus and S. aureus*) and Gram-negative bacteria (*E. coli and P. aeruginosa*) using disc diffusion method. The results revealed that all plant extracts were potentially effective in suppressing microbial growth of bacteria with variable potency. The inhibitory effect of CAEE started at 2.52mg/ml with inhibition zones of 9.8 and 8.5mm against *S. aureus and P. aeruginosa*. CAEE was found to be effective with concentration of (10mg/ml) against *B. cereus, S. aureus, E. coli* and *P. aeruginosa* suppressing their growth with inhibition zones of 14.8, 17.8, 14.8 and 17.8mm respectively

Table 4: Anti-microbial screening test of ethanolic plant extract (10mg/ml) against some bacterial strains

	Zone of inhibition(mm)			
	Gram +ve		**Gram -ve**	
	B. cereus	*S. aureus*	*E. coli*	*P. Aeruginosa*
CAEE	14.8	17.8	14.8	17.8
Standard	16.9	20.8	15.8	13.6

Table 5: MIC of the most effective plant extract against *S. aureus* and *P. aeruginosa*

Conc. (mg/ml)	Inhibition zones	
	Gram(+ve) bacteria (S. aureus)	Gram(-ve) bacteria (P. aeruginosa)
1.28	0	0
2.52	9.8	8.5
5.2	14.9	13.6
10.2	18.8	16.5
12.8	21.6	18.6
15.2	23.8	22.8

*Values were expressed as mean±SD, *Significantly different from P<0.05.*

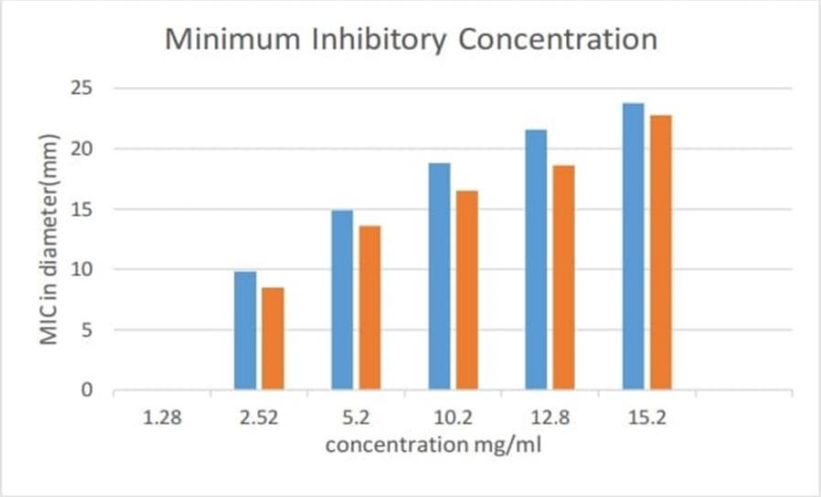

Figure 4: Minimum inhibitory concentration

Anti-ulcer activity:

Acid neutralizing capacity (ANC): The neutralizing effect of ethanolic extract was studied for three concentrations (100mg, 500mg, 1000mg) and standard aluminium hydroxide +magnesium hydroxide [$Al(OH)_3$ + $Mg(OH)_2$] (500mg). The results obtained envisage that the extract at concentration 100mg, 500mg, and 1000mg, showed a significant reduction in acid neutralizing capacity (ANC) i.e.,7.7, 16.8, and 5.3 respectively as compared to standard $Al(OH)_3$ + $Mg(OH)_2$ (500mg) which is 35.7. The extract at a concentration 1000mg has been found to neutralize acid more significantly as compared to standard. The results have tabulated in table and Graph.

Table 6: Effect of *Calendula Arvensis* ethanolic extract of an acid neutralizing capacity

S. NO.	Concentration (mg)	Volume of NaOH consumed (ml)	mEq of Acid consumed	ANC per gram of Antacid
1	100	44.6	7.7	77
2	500	43.2	8.4	16.8
3	1000	49.4	5.3	5.3
4	500 $Al(OH)_3$ + $Mg(OH)_3$	24.3	17.85	35.7

*Values were expressed as mean±SD, *Significantly different from P<0.05.*

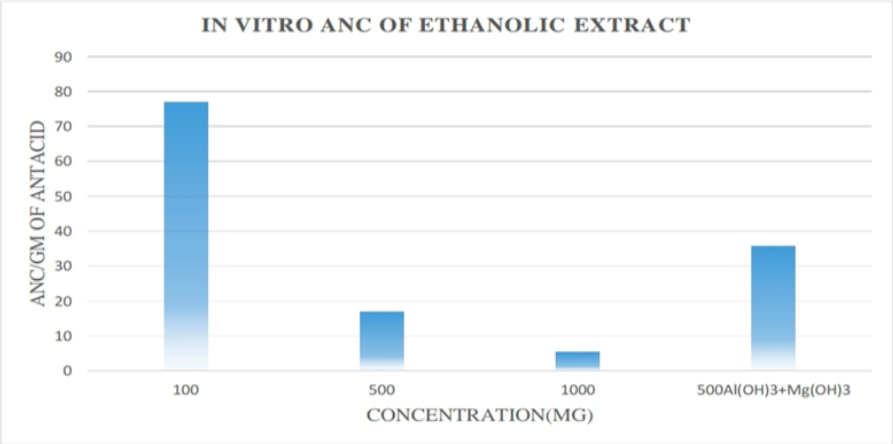

Figure 5: Acid neutralizing capacity

CONCLUSION:

The ethanolic leaf extract of *Calendula arvensis* exhibits anti-oxidant, anti-bacterial and anti-ulcer activities. It shows good anti-oxidant, anti-bacterial and anti-ulcer activity effects produced by *Calendula arvensis* leaves may be due to high content of phenolic compounds and tannins, sterols, flavonoids, alkaloids and carbohydrates.

Declarations

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Author contributions

All authors contributed to experimental work, data collection, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Competing interest statement

All authors declare that there is no conflict of interests regarding publication of this paper.

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