PHYTOCHEMICAL SCREENING, ANTIBACTERIAL, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF *ACMELLA OLERACEA* FLOWERS

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ABSTRACT

Acmella oleracea flowers are traditionally used to treat mouth ulcers in the rural areas of Nashik, Maharashtra, India. This research project is an effort to understand the scientific basis for the utility of *A. oleracea* flowers and validate it for potential antimicrobial, anti-inflammatory and antioxidant activity, some of the main causes of mouth ulcers. The aim of this project was to study the phytochemistry of the ethanol extract of *A. oleracea* flowers and evaluate antibacterial anti-inflammatory and antioxidant potential. The phytochemical screening ethanol extract indicates the presence of phenolics, flavonoids, glycosides, alkaloids and carbohydrates. The total phenolic and flavonoid content in the ethanol extract determined by Folin-Ciocalteu reagent and aluminum chloride method were found to be 29.992 mg GAE g^{-1} and 93.2 mg QE g^{-1}, respectively. The antibacterial activity of the ethanol extract was evaluated against *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis and Staphylococcus aureus,* using streptomycin as the reference standard. The best activity was observed against *P. aeruginosa.* The antioxidant activity of the was determined by 2,2-diphenyl-1-picrylhydrazyl, hydrogen peroxide, nitric oxide scavenging and ferric reducing antioxidant power assays. The IC_{50} in the DPPH, hydrogen peroxide and nitric oxide scavenging assays were found to be 89.589 µg mL^{-1}, 89.178 µg mL^{-1} and 50.86 µg mL^{-1} respectively. The anti-inflammatory activity was evaluated by *in vitro* albumin denaturation assay using naproxen as the reference standard. The ethanol extract demonstrated significant antibacterial, anti-inflammatory and antioxidant activity, these combined effects could be responsible for its effectiveness in mouth ulcers and potentially justify the traditional use of *A. oleracea* flowers in mouth ulcers.

Keywords: *Acmella oleracea* flowers, ethanol extract, mouth ulcer, antioxidant, antibacterial, anti-inflammatory, phenolics, flavonoids

INTRODUCTION

Acmella oleracea is a flowering herb, commonly known as toothache plant that has a unique golden yellow color flowers with a red tip, majorly found in the subtropical and tropical regions of the world. It is a rich source of bioactive constituents[1]. It is commonly referred to as akarkara, akmella, ting flowers, eye ball plant, buzz buttons, and sichuan buttons[2]. Alkamides are the most abundant phytochemicals reported in *A. oleracea* whole herb with spilanthol being the major reported alkylamide. Spilanthol is *N*-isobutylamide found in various parts of these herb sometimes used to reduce pain associated toothaches and induce saliva secretion. The traditional uses of this herb include rheumatism, mouth ulcers, sialagogue for stammering, tongue paralysis, antipyretic, sore throat and gum infection[3-5], the leaves are reportedly used in sore throat, rheumatism and gum infection. This herb is well-known as a folk treatment for toothaches and throat and gum infections, particularly in the Irula tribe of Hasanur hills in Erode district of Tamil Nadu, where it is locally known as "Mandal Poo Chedi" [6]. The root paste of this herb is used in throat problems in Betul and Chindwara districts of Madhya Pradesh and for articular rheumatism and snakebite in Cameroon[7]. In Bangladesh, the flowers are reported to be used in leucorrhoea in females. The whole plant pest is used as poisonous sting in Chittagong hills of Bangladesh where this plant is commonly known as Jhummosak[8]. The extract of leaves and roots of plant from this genus demonstrate a wide range of pharmacological activity like anesthetic, antipyretic, insecticidal, antioxidant, antimicrobial, obesity and immune stimulation[9]. No reports are apparently published of *A. oleracea* flower being used in mouth ulcers. This research is an effort to understand the phytochemistry in the ethanol extract of *A. oleracea* flowers. Also reported here are the *in vitro* antibacterial,

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antioxidant and anti-inflammatory activity of the ethanol extract of *A. oleracea* flowers. We try to explore scientific evidence for traditional utility of the flowers to treat mouth ulcers.

MATERIALS AND METHODS

Collection and authentication of plant material

The flowers of *A. oleracea* were collected from the Chas area of Nashik district, Maharashtra, India. Between August – November months and authenticated at Research Laboratory, Department of Botany, Mithibai College, Mumbai (authentication no. MIT0160).

Preparation of ethanol extract of *A. oleracea* flowers

The flowers of *A. oleracea* were dried at room temperature for 6-7 days and powdered using a mechanical grinder, the powder was extracted by maceration in ethanol (100 g powder in 1000mL ethanol for 3-4 days and filtration using a Buchner funnel)[10-11].The ethanol was evaporated on a rotary vacuum evaporator at 60-65 °C to obtain the dried extract that was stored in a refrigerator at 4°C for further use[12].

Microscopic characteristics of flowers of *A. oleracea*

The microscopic characteristics of *A. oleracea* powder were assessed as per the Ayurvedic Pharmacopeia of India[13]. The dried powder of flowers was cleaned with NaOH and mounted in glycerin medium after staining. Different staining reagents (phloroglucinol + conc. hydrochloric acid, picric acid, iodine solution) were used to examine the powder characteristics[14].

Phytochemical screening of ethanol extract of *A. oleracea* flowers

The ethanol extract of *A. oleracea* flowers was evaluated to identify the presence of phytoconstituents like phenolic, tannins, flavonoids, saponins, glycosides and alkaloids using reported methods[14].

Estimation of total phenolic content in the ethanol extract of *A. oleracea* flowers

The total phenolic content in the ethanol extract was determined by using the Folin-Ciocalteu reagent using gallic acid as the reference standard[15-16]. 1 mL extract was oxidized with dilute Folin-Ciocalteu reagent (5 mL), after 5 min the mixture was neutralized with 4 mL sodium carbonate (7.5% w/V) and incubated for 120 mins at 37 ºC, the absorbance of test solution was measured at 765 nm using UV/Visible spectrophotometer (UV Shimadzu 1900), The total phenolic content is expressed as mg GA g^{-1} equivalent of extract[17-18].

Estimation of total flavonoid content in the ethanol extract of *A. oleracea* flowers

The total flavonoid content in the ethanol extract was determined by the aluminum chloride colorimetric method described by Kumar et al , 1 mL of extract, 1.5 mL of methanol and 0.3 mL 5% w/V sodium nitrite ($NaNO_2$) were mixed in a test tube, after 5 min, 0.5 mL of 2%w/V aluminum chloride ($AlCl_3$) was added, the solution was allowed to stand for 6 min, then 0.5 mL of 1M NaOH was added, giving a red colored complex. The absorbance of this solution was determined at 510 nm using quercetin as the reference standard. The total flavonoid content is expressed as mg quercetin equivalent per gram of extract (mg g^{-1})[19-20].

Antibacterial study of ethanol extract of *A. oleracea* flowers

Agar well diffusion method was used to determine the antibacterial activity of the ethanol extract. 0.1 mL of the fresh bacterial suspension of *E. coli, P. aeruginosa, B. subtilis and S. aureus* was spread on Muller Hinton agar plates[21-22]. Using a sterile cork borer 6 mm diameter four wells were prepared, 100 µL of extract was added into the well. Streptomycin (10 µL of 10 ppm solution) was used as reference standard and ethanol as negative control. The agar plates were incubated at 37 ˚C for 24 h and zone of inhibition was measured[23-24].

Determination of antioxidant activity

The antioxidant activity of the ethanol extract was evaluated using the DPPH, FRAP and nitric oxide radical scavenging assays as per reported protocols[25].

DPPH free radical scavenging assay

The free radical scavenging activity of the ethanol extract was evaluated using the DPPH assay as per reported protocol[26-29]. 1 mL of ethanol extract, and 2 mL of 10 mM DPPH solution was added in a test tube. The reaction mixture was mixed, incubated for 30 min in the dark and the absorbance was determined at 517 nm, using ascorbic acid (AA) as the reference standard and IC_{50} was calculated. The percent inhibition of DPPH radical was calculated using the formula

$$\text{Percent inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 = absorbance of control and A_1= absorbance of test solution

Hydrogen peroxide scavenging (H_2O_2) assay

Hydrogen peroxide (40mM) solution in phosphate buffer (pH 7.4) was prepared. 2 mL of ethanol extract at 20-100ppm concentration was taken, 1.36 mL hydrogen peroxide solution (40mM) was added, the final volume made up 10 mL in phosphate buffer saline (pH 7.4), this solution was allowed to stand for 10 min, and the absorbance measured at 230nm, using phosphate buffer saline as blank. The percent inhibition of peroxide radical was calculated using the formula[30-31]

$$\text{Percent inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 = absorbance of control and A_1= absorbance of test solution

Nitric oxide scavenging assay

10 mg mL^{-1} stock solution of ethanol extract and gallic acid each was prepared. From this solution, different concentrations of ethanol extract and gallic acid each ranging from 10-100ppm were prepared. Griess reagent (0.1% w/V of naphthylethylene diamine dihydrochloride, 1% w/V sulphanilamide and 2.3% w/V phosphoric acid) was prepared freshly. 10 mM solution (1 mL) of sodium nitroprusside was dissolved in phosphate buffer saline (pH 7.4) and 2 mL of test sample was added in a test tube. This solution was incubated at 25˚C for 150 min. After incubation, 3 mL of Griess reagent was added to the mixture, and incubated at 37 ºC for 30 min, absorbance measured at 546 nm. The percent nitric oxide radical inhibition was calculated using the formula[32]

$$\text{Percent inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 = absorbance of control and A_1= absorbance of test solution

Ferric reducing -antioxidant power (FRAP) assay

FRAP assay was conducted as per to the procedure described by Benzie and Strain[33]. The FRAP reagent was made using 5 mL of 10 mM 2,4,6-tris(2-pyridyl)-s- triazine (TPTZ) solution in 40 mM hydrochloric acid, 5 mL of 20 mm ferric chloride in 50 mL of acetate buffer (pH 3.6). 1 mL of ethanol extract and 2 mL of FRAP reagent were taken in a test tube and the reaction mixture vortexed and incubated for 30 min at 37˚C. The absorbance was measured at 593 nm. The standard curve of $FeSO_4$ was plotted and result expressed as mM $FeSO_4$ equivalent per mg of extract[34-35].

Determination of Anti-inflammatory activity

The anti-inflammatory activity of the ethanol extract was assessed by the *in vitro* albumin denaturation assay as described by Chandra, Dey et al[36] with some modifications. A stock solution of 10 mg mL^{-1} of the ethanol extract was prepared, from this a series of solutions ranging from 25, 50, 75 and 100 ppm were prepared in ethanol. 2 mL of 5% egg albumin, 2 mL of phosphate buffer saline and 3 mL of extract solution were added in the test tube and allowed to stand for 15 min at 37 ˚C. The solution was mixed thoroughly and heated at 70 ˚C for 5 min. The absorbance was measured at 660 nm[37] using phosphate buffer saline (PBS) as blank. The percent protein denaturation was calculated by the formula:

$$\text{Percent inhibition} = \frac{(A_0 - A_1)}{A_0} \times 100$$

where A_0 = absorbance of control and A_1= absorbance of test solution

RESULTS AND DISCUSSION

Microscopic characteristics of flowers of *A. oleracea*

The powder characteristics of *A. oleracea* flowers are shown in Fig. 1 and the physicochemical parameters of the powder are given in Table I. The percent yield of the ethanol extract was found to be 3.95±0.15 % w/w. The results of phytochemical screening indicated the presence of phenolics, flavonoids, glycosides, alkaloids and carbohydrates (Table II).

Table I: Physicochemical parameters of ethanol extract of *A. oleracea* flowers

Physicochemical parameter	Percent w/w ± SD
Total ash	13.4±0.061
Acid insoluble ash	5±0.08
Acid soluble ash	8±0.052
Water soluble ash	10±0.025
Loss on drying	0.15±0.015
Ethanol extract	3.95±0.097

The results are an average of three estimates

Table II: Phytochemical screening of ethanol extract of *A. oleracea* flowers

Sr. No	Constituent	Test	Inference
1	Alkaloids	Mayer test	+
		Dragendorff test	+
		Wagner test	+
2	Glycosides	Keller Kiliani test	+
		Legal test	+
3	Flavonoids	Lead acetate test	+
		Aluminium chloride test	+
		Shinoda test	+
4	Tannins	Gelatin test	+
5	Phenolics	Ferric chloride test	+
6	Carbohydrates	Molisch test	+
7	Saponins	Foam test	-
8	Proteins and amino acids	Xanthoproteic test	-
9	Fats and fixed oils	Filter paper press test	+

The results are an average of three estimates

Determination of phytoconstituents in ethanol extract of *A. oleracea* flowers

Phytochemicals, primarily secondary metabolites, are non- nutritive plant chemicals which have different medical benefits. Medicinal plants continue to be an interesting source of natural compounds such as phenolics and flavonoids. The total phenolic and flavonoid content in the extract is depicted in Table III calculated from the regression equation of the calibration curve (Y=0.084 x +0.1192 and R^2=0.9925) and (Y=0.0072x – 0.047 and R^2 =0.9928), respectively.

Table III: Total phenolic and total flavonoid content ethanol extract of *A. oleracea*

Test Sample	Total phenolic content (mg g^{-1} GAE) ± SD	Total flavonoid content (mg g^{-1} QE) ± SD
Ethanol extract of *A. oleracea* flowers	29.992± 0.43	93.2± 1.03

The results are an average of three estimates

Antibacterial activity of ethanol extract of *A. oleracea* flowers

The antibacterial activity of the extract is expressed in the terms of zone of inhibition against the selected strains of bacteria (*E. coli, P. aeruginosa, B. subtilis, and S. aureus)* and depicted in Table IV. The ethanol extract showed the best activity against *P. aeruginosa*.

Table IV: Antibacterial activity of ethanol extract of *A. oleracea* flowers

Sr. No.	Concentration of ethanol extract of *A. oleracea*	Zone of inhibition (mm) ± SD			
		Microorganism			
		E. coil	*P. aeruginosa*	*B. subtilis*	*S. aureus*
1	5 %, 100 µL	9± 0.57	10.6± 1.5	8± 1.71	6± 1.0
2	10 %, 100 µL	10± 0.57	14± 0.5	9± 1.5	8± 0.57
3	15 %, 100 µL	11± 1.0	14± 1.1	10± 1.0	11 ± 1.1
4	20 %, 100 µL	12± 0.56	15± 1.0	10± 0.15	11± 0.56
5	Streptomycin, 10 ppm, 10µL	20±0.67	21±0.28	22±0.32	20±0.65

The results are an average of three estimates

ANTIOXIDANT ACTIVITY

DPPH free radical scavenging assay

This assay is based on the measurement of the scavenging capacity of the test extract. The hydrogen atom donating ability of the extract is determined by the decolorization of 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH which is reduced to form the hydrazine, that leads to change in colour from violet to yellow. The scavenging ability of both extract and the reference standard as ascorbic acid were found to be concentration dependent (Table V and Fig. 2). With increasing concentration, the scavenging ability of the extract and ascorbic acid was found to increase; the IC_{50} of the extract was found to be 89.589 µg mL^{-1} and for ascorbic acid 101.106 µg mL^{-1}.

Table V: DPPH radical scavenging activity of ethanol extract of *A. oleracea* flowers and ascorbic acid

Concentration (ppm)	DPPH scavenging activity	
	Ethanol extract of *A. oleracea* flowers ± SD	Ascorbic acid ± SD
20	14.64±0.183	18.77±0.042
40	29.18±0.041	43.78±0.44
60	42.06±0.268	65.73±0.612
80	54.63±0.126	84.20±0.135
100	63.32±0.089	99.24±0.056

The results are an average of three estimates; Ascorbic acid was used as positive control

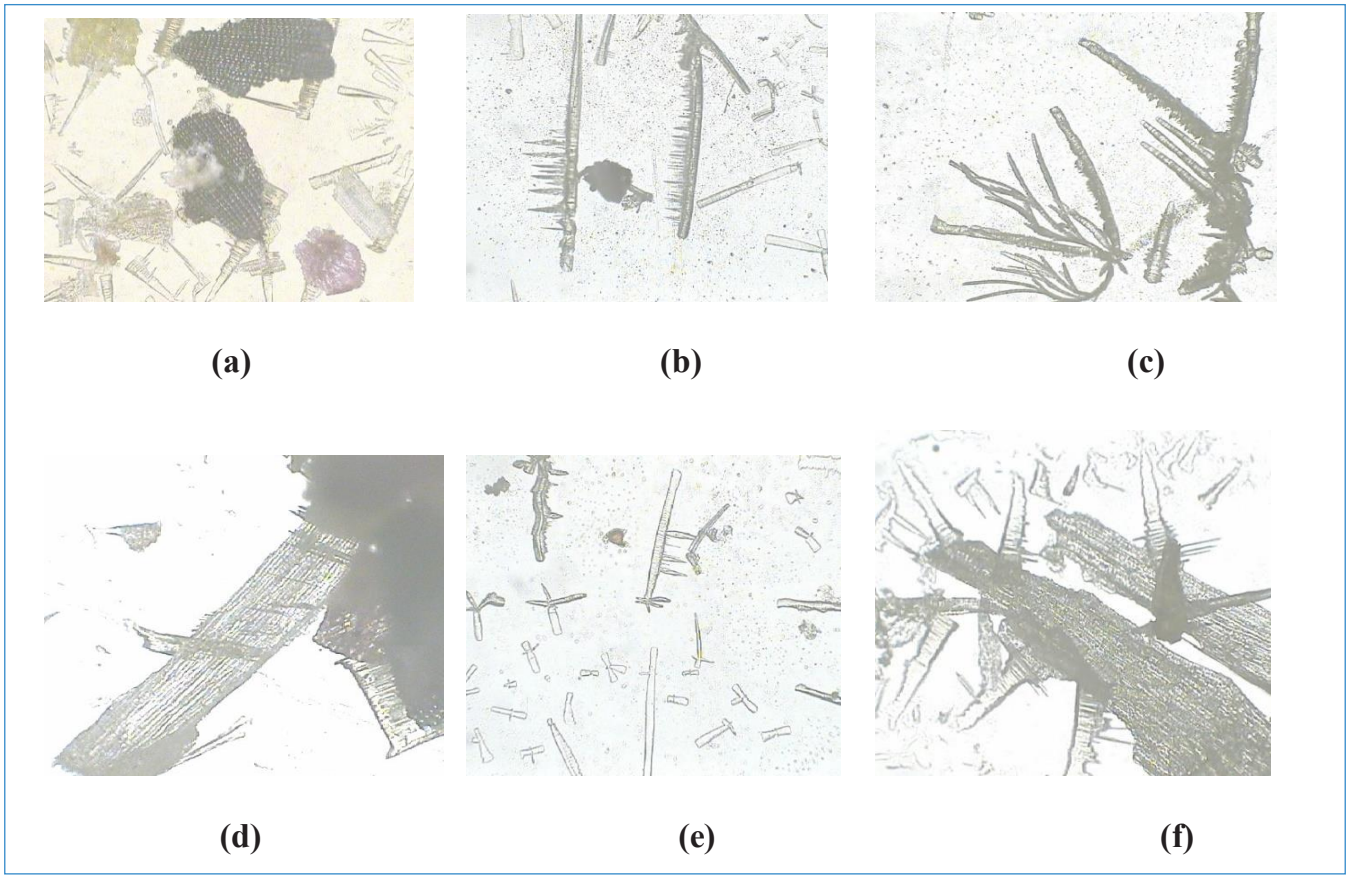

Fig. 1: Powder characteristics of *A. oleracea* flowers consist of (a)- ground tissue, (b)- simple multicellular hair, (c)- simple multicellular thin wall hair, (d)- polygonal rectangular epidermal cells, (e)- thin wall hair and vesicles with no uniform content (f)- vascular system

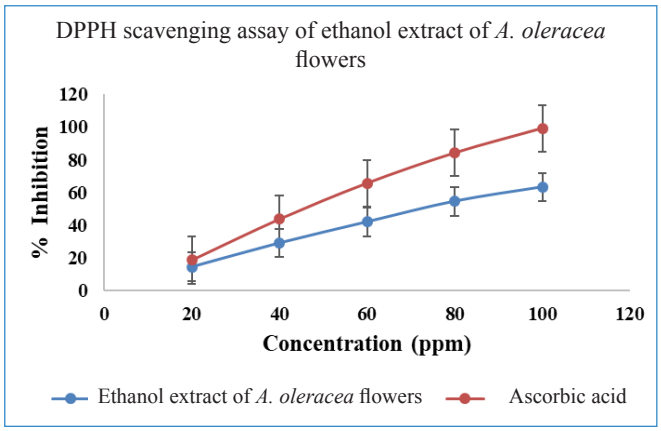

Fig. 2: DPPH radical scavenging activity of ethanol extract of *A. oleracea* flowers and ascorbic acid

Hydrogen peroxide (H_2O_2) scavenging assay

The scavenging capacity of the ethanol extract of *A. oleracea* flowers was found to be concentration dependent as depicted in Table VI and Fig. 3, the IC_{50} of the extract and ascorbic acid (used as a reference standard) were found to be 89.178 µg mL^{-1} and 227.5794 µg $mL^{-1,}$ respectively. Ascorbic acid was used as reference standard.

Table VI: Hydrogen peroxide scavenging (H_2O_2) activity of ethanol extract of *A. oleracea* flowers and ascorbic acid

Concentration (ppm)	Hydrogen peroxide scavenging activity	
	Ethanol extract of *A. oleracea* flowers ± SD	Ascorbic acid ± SD
20	13.33±0.159	46.27±0.301
40	29.41±0.085	62.55±0.360
60	39.72±0.056	73.46±0.215
80	56.09±0.301	89.22±0.102
100	64.73±0.165	99.98±0.004

The results are an average of three estimates; Ascorbic acid was used as positive control

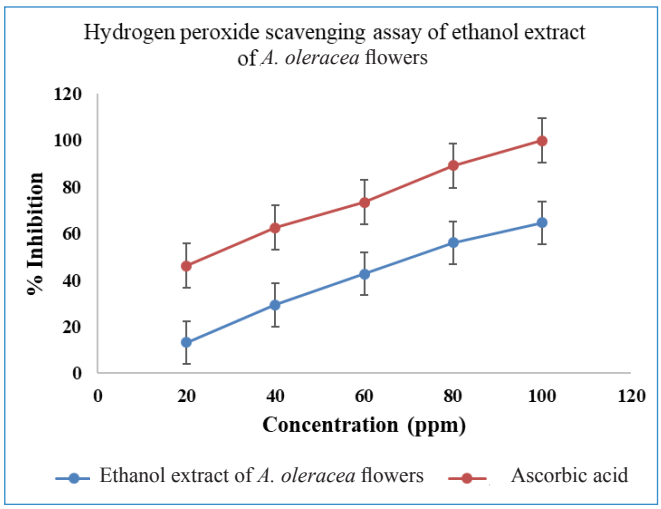

Fig. 3: Hydrogen peroxide scavenging (H_2O_2) activity of ethanol extract of *A. oleracea* flowers and ascorbic acid

Nitric oxide scavenging assay

Nitrite radical scavenging assay is based on formation of colored complex of nitric acid with Griess reagent. Nitrite radical scavenging assay of the extract and ascorbic acid as the reference standard was plotted at different concentrations depicted in Table VII and Fig. 4. The antioxidant activity increased with increase in concentration, the IC_{50} of the extract was found to be 50.86 µg mL^{-1} and for ascorbic acid 248.19 µg mL^{-1}, respectively.

Table VII: Nitric oxide radical scavenging activity of ethanol extract of *A. oleracea* flowers and ascorbic acid

Concentration (ppm)	Nitric oxide radical scavenging activity	
	Ethanol extract of *A. oleracea* flowers ± SD	Ascorbic acid ± SD
20	12.66±0.027	14.35±0.185
40	22.19±0.015	30.12±0.316
60	31.88±0.091	41.95±0.106
80	42.81±0.005	54.27±0.509
100	49.90±0148	65.010±0.387

The results are an average of three estimates; Ascorbic acid was used as positive control

Ferric reducing -antioxidant power (FRAP) assay

The ferric reducing antioxidant power assay determines the antioxidant capacity of the test extract. This assay based on the reduction of ferric form to ferrous in the presence of an antioxidant. The FRAP value obtained from the linear regression of ferrous sulphate standard curve (y=0.703x + 0.1,031 r^2= 0.998).

The FRAP result is expressed as mM $FeSO_4$ equivalent per mg of extract. The FRAP value was found to be 1.96± 0.21 mmol dm^{-3} g^{-1} extract from the calibration curve.

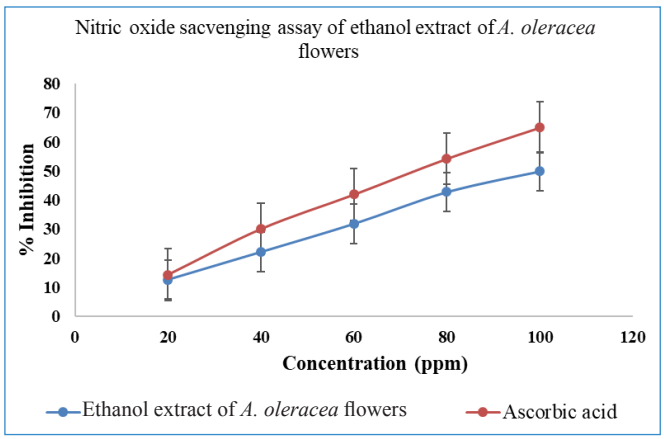

Fig. 4: Nitric oxide radical scavenging activity of ethanol extract of *A. oleracea* flowers and ascorbic acid

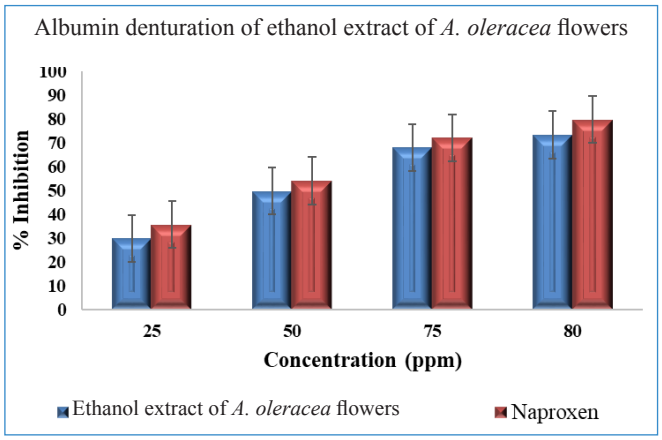

Fig. 5: Albumin denaturation assay of ethanol extract of *A. oleracea* flowers and naproxen

ANTI-INFLAMMATORY ASSAY

Albumin denaturation assay

Inhibition of albumin denaturation indicates the ability of anti-inflammatory agent to prevent denaturation of proteins. The assay denaturation is based on damage associate molecular pattern, also known as DAMP. These DAMPs are associate with scavenging receptors (SRs) which lead to activation of inflammatory mediators like cytokines and further spread the inflammation. Albumin is a common protein of the skin i.e. it is considered for protein denaturation. The extent of inhibition of albumin

denaturation correlates with the anti-inflammatory activity of the test sample. The inhibition of albumin denaturation is concentration dependent for both ethanol extract and naproxen (reference standard), as depicted in Table VIII and Fig. 5. The ethanol extract exhibited maximum percent inhibition of albumin denaturation at 100 ppm.

Table VIII: Albumin denaturation assay of ethanol extract of *A. oleracea* flowers and naproxen

Concentration (ppm)	Percent inhibition (%)	
	Ethanol extract of *A. oleracea* flowers ± SD	Naproxen ± SD
25	29.29±0.164	35.71±0.115
50	49.77±0.078	54.09±0.145
75	68.01±0.04	72.19±0.06
100	73.35±0.053	79.48±0.05

The results are an average of three estimates; Naproxen was used as positive control

CONCLUSION

The flowers of *A. oleracea*, a herb endemic to the Indian peninsula, is traditionally used to treat mouth ulcers in Nashik of Maharashtra, India. This project was an effort to study the phytochemistry of the flowers and potential biological activity that could explain the traditional use in mouth ulcer. The extract was found to be rich in phenolics and flavonoids. It showed good antioxidant activity in DPPH, NO, FRAP assays along with good antibacterial activity. The ethanol extract showed comparable anti-inflammatory activity to that of standard naproxen sodium in the *in vitro* albumin denaturation assay. These combined effects could be responsible for its effectiveness in mouth ulcers and potentially justify the traditional use of *A. oleracea* flowers in mouth ulcers and has the potential to be developed into a topical delivery system for convenient application in treatment of mouth ulcers.

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