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Establishing Enzyme Inhibition and membrane stability studies in methanolic extracts of *Evolvulus alsinoides*

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A B S T R A C T

Shankhpushpi is botanically termed as *Evolvulus alsinoides*; the extracts have exhibited antioxidant, anti-ulcer, and immunomodulatory activities. In addition, recently scientific research has disclosed adaptogenic and anti-amnesic properties. The main objective of the present work is to investigate the *invitro* therapeutic potential activities of methanolic extract of whole plant of *Evolvulus alsinoides* on albumin denaturation, membrane stabilization test and Proteinase, xanthine oxidase, lipoxygenase inhibitory activities Establishing Anti inflammatory properties, Inhibition of albumin denaturation showed Albumin denaturation in methanolic plant extract (500µg/ml) 82.32 ± 0.50 against standard Aspirin (100µg/ml) 68.40 ± 0.40. Membrane stabilization plant extracts: 65.70 ± 0.60 against standard Aspirin: 84.20±0.40. Xanthine oxidase IC_{50} (μM) ± SEM, methanolic extract: 0.42 ±1.40 against standard Allopurinol: 0.20 ± 0.40. IC_{50} of Anti-lipoxygenase Activity (500µg/ml): 52.60 against standard Indomethacin (50µg/ml): 46.50%. The extract fractions serve as free radical inhibitors or scavenger or acting possibly as primary oxidants and inhibited the heat induced albumin denaturation, proteinase, lipoxygenase and xanthine oxidase (XO), which are agents that directly inhibit the synthesis of uric acid *in vivo*. Certain active constituents present in *Evolvulus alsinoides* plant extracts like flavonoids and polyphenolic compounds possess potent Anti inflammatory therapeutic responses.

Introduction

Extensive research within last three decades has confirmed the molecular basis for most chronic diseases and for the associated inflammation.

The transcription factor, Nuclear Factor-kappaB (NF-κB) that controls over 500 different gene products, has emerged as major mediator of inflammation. Thus

agents that can inhibit NF-κB and diminish chronic inflammation have potential to prevent or delay the onset of the chronic diseases and further even treat them. In an attempt to identify novel anti-inflammatory agents which are safe and effective (Bharat B. Aggarwal, *et al* 2011),

Evolvulus alsinoides (shankhpushpi which is considered as Medhya Rasayana) is an Ayurvedic drug used for its action on the central nervous system, especially for boosting memory and improving intellect. In the Ayurvedic system of medicine, the whole herb of 'Shankhpushpi' has been employed clinically for centuries for its memory potentiating, anxiolytic and tranquilizing properties. The crude extracts of *E. alsinoides* showed a marked reduction in inflammation and edema in adjuvant induced arthritic rat model (Ganju L, *et al.*, 2003).

Evolvulus alsinoides (L) is a perennial herb belonging to the family Convolvulaceae with a small woody and branched root stock (Austin, 2008). This plant is used in traditional medicine in East Asia, India, Africa and Philippines to cure fever, cough, cold, venereal diseases, azoospermia, adenitis and dementia. It has a known nootropic and anti-inflammatory activity (Singh, 2008). Its use in the treatment of neurodegenerative diseases, asthma and amnesia (Goyal & Singh, 2005). Pre-clinical research has justified its ancient claim as brain tonic (Singh, 2008). Several other uses reported for this plant include its ability to boost memory and improve intellect (Sethiya *et al*., 2009), immunomodulatory, adaptogenic as well as anti-oxidant properties (Siripurapu *et al*., 2005). Singh (2008) reported that *Evolvulus alsinoides* is used in the Philippines to cure certain bowel irregularities and as a vermifuge and febrifuge. Infusion of roots, stalks and leaves are all used in Nigeria as a stomaachic.

The preliminary phytochemical screening showed that *E. alsinoides* contains some secondary metabolites such as glycosides, alkaloids, poly phenols, carbohydrates, amino acids and proteins, saponins, volatile oil, flavonoids and tannins (Omogbai BA, *et al* 2011).The plant contains alkaloids such as betaine, shankhapushpine, B-sitosterol and evolvine. Fresh plant contains volatile oil. These compounds help brain stimulation and increase the ability to concentrate. Early phytochemical studies of this species resulted in the isolation and identification of chemical constituents such as triacontane, pentatriacontane. Four unidentified alkaloids A, B, C and evolvine have also been described.

In many inflammatory disorders there is excessive activation of phagocytes, production of 0_2-, OH radicals as well as non free radicals species (H_2O_2) (Gilham *et al*., 1997), which can harm severely tissues either by powerful direct oxidizing action or indirect with hydrogen peroxide and –OH radical formed from O2- which initiates lipid peroxidation resulting in membrane destruction. Tissue damage then provokes inflammatory response by production of mediators and chemotactic factors (Lewis, 1989).

The reactive oxygen species are also known to activate matrix metello proteinase damage seen in various arthritic tissues (Cotran *et al*., 1994).

Free radicals which have one or more un-paired electrons (superoxide, hydroxyl, peroxyl) are produced in normal or pathological cell metabolism and the compounds that can scavenge free radicals have great potential in ameliorating the

diseases and pathological cells (Halliwell, 1995; Squadriato and Peyor, 1998; Gulcin *et al*., 2004).

The increase in prevalence of multiple drug resistance has showed down the development of new synthetic anti-inflammatory drugs and the new drug is necessary to search for new anti-inflammatory from alternative sources. Phytochemicals from medicinal plants showing anti-inflammatory activities have the potential of filling this need because of structures are different from those of the more studied and their those of the more action may too very likely differ (Fabricant and Fanworth, 2001). Phytochemical analysis of methanolic extract of *Evolvulus alsinoides* proved in total Phenolic content and total Flavonoid and Anti oxidant assay (Zuhaib Zafar, Muralidhar. S. Talkad, *et al*., 2011).

The present study planned on Methanolic extract of whole plant of *Evolvulus alsinoides* for albumin denaturation, membrane stabilization test and Proteinase, xanthine oxidase, lipoxygenase inhibitory activities *invitro* to establish potential therapeutic response of the plant.

Materials and Methods

Plant Materials and Extraction

The plant material was collected from the local rural area of Tumkur district in Karnataka state, India. The plant materials were identified and authenticated. The Plant material were air-dried and pulverized in course powder and was loaded in the Soxhlet apparatus for defating with petroleum ether and followed by extraction with methanol. The methanolic extract of *Evolvulus alsinoides* was subjected to fractionation and dry concentrated extract were used for the study.

Phytochemical Analysis

Phytochemical analysis was carried out for saponins, flavonoids, steroids, phenol, anthroquinone, alkaloids (Obdoni and Ochuko, 2001) and tannins (Kaur and Arora, 2009) were performed.

In Vitro Anti-inflammatory Activity

Inhibition of Albumin Denaturation

Methods of Mizushima and Kobayashi (1968) and Sakat *et al*. (2010) followed with minor
modifications. The reaction mixture was consisting of test extracts and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using HCl, and incubated at 37^0C for 20 min, then heated to 51^0C for 20 min, measured turbidity after cooling spectrophotometrically at 660nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows:

% inhibition= [{Abs control- Abs sample}/Abs control] x 100,

Where Abs control is the absorbance without sample, Abs sample is the absorbance of sample
Extract/standard

Membrane Stabilization Test

Preparation of Red Blood Cells (RBCs) Suspension

Fresh whole human blood (10ml) was collected and centrifuged at 3000 rpm for 10min and were washed three times with equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline (Sadique *et al*., 1989; Saket *et al*., 2010)

Heat Induced Hemolytic Assay

The reaction mixture (2ml) consisted of 1 ml of test sample solution and 1 ml of 10% RBCs suspension, control sample consists only saline. Aspirin was used as a standard drug. All the centrifuge tubes with reaction mixture were incubated in water bath at 56^0C for 30min, cooled and centrifuged at 2500 rpm for 5 min; the absorbance of the supernatants was measured at 560 nm. The experiment was performed in triplicates for all the test samples. Percent membrane stabilization activity was calculated by the formula mentioned above (Shinde *et al*., 1999; Saket *et al*., 2010)

Proteinase Inhibitory Action

The test was performed according to the modified method of (Oyedepo *et al*, 1995 and Sakat *et al*, 2010). The reaction mixture (2ml) was containing 0.06mg trypsin, 1ml of 20mM Tris HCl buffer (pH: 7.4) and 1ml test sample of different concentrations of methanolic extracts. The reaction mixture was incubated at 37^0C for 5min and then 1ml of 0.8% (W/V) casein was added. The mixture was inhibited for 20min with 2ml of 70% perchloric acid, was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was measured at 210nm against buffer as blank. The experiment was performed in triplicate. The percentage of inhibition of proteinase inhibitory activity was calculated.

Anti-Lipoxygenase Activity

Anti-Lipoxygenase activity was carried out using linoleic acid as substrate and lipoxidase as enzyme (Shinde *et al*., 1999). Test samples were dissolved in 0.25ml of 2M borate buffer with pH 9.0 and added 0.25ml of lipoxidase enzyme solution (20,000U/ml) and incubated for 5 min at 25°C followed by, 1.0ml of lenoleic acid solution (0.6 mM) was added, mixed well and absorbance was measured at 234 nm. Indomethcin was used as reference standard; all tests were carried out in triplicate. A dose response curve was plotted to determine the IC_{50} values. The percent inhibition was calculated from the following equation,

% inhibition= [{Abs control- Abs sample}/Abs control] x 100

Xanthine Oxidase Assay

Xanthine oxidase activity was assayed (Yamamoto *et al*, 1993). Briefly, the reaction mixture consisting of 500 µl of solution A (0.1M phosphate buffer containing 0.4mM xanthine and 0.24 mM NBT), 500 µl of solution B (0.1 M phosphate buffer containing 0.0449 units/ml xanthine oxidase) and 50 µl of a 10% of methanolic plant extracts were incubated in a cuvette at 37^0C for 20 min. Allopurinol were used as a Standard, the enzyme activity was expressed as the increment in absorption at 300 nm spectrophotometrically per unit time.

Statistical Analysis

Analysis of variance (ANOVA) was used to determine the significance of difference between treatment groups ($p<0.05$). Means between treatment groups were compared for significance using Duncan's new Multiple Range post test.

Results and Discussion

Phytochemical Analysis

The phytochemical screening showed that the methanolic extracts of *Evolvulus alsinoides*, the tannin, flavonoids, phenol, alkaloids, steroids, anthraquinones and saponins were present.

Anti Inflammatory Properties

Inhibition of Albumin Denaturation

Protein denaturation is a well documented cause in the mechanism of the anti inflammation activity, ability of different concentration of methanolic extracts of plant on protein denaturation was studied. It was effective in inhibiting heat induced albumin denaturation at 500µg/ml (Table 1). Maximum inhibition 86.32 was observed from plant methanolic extract 82.32, against std, Aspirin: a standard anti-inflammation drug showed the maximum inhibition 68.40 % at the concentration of 100µg/ml

The experiments carried out in triplicates, According to Duncan's Multiple Range Test (DMRT), values followed by different subscripts are significantly different at $P<0.05$, mean ± SEM

Membrane Stabilization Test

Stabilization of RBCs membrane was establishes the mechanism of anti-inflammatory action of different concentrations of methanolic extracts of *Evolvulus alsinoides*, the extract were effectively inhibiting the heat induced hemolysis, these results provide evidence for membrane stabilization and their anti-inflammatory mechanism. This effect may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. The extracts inhibited the heat induced hemolysis of RBCs to varying degree (Table 1). The maximum inhibition was recorded 65.70 ± 0.60 at 500µg/ml in methanolic extract of plant, the aspirin as a standard drug showed the maximum inhibition 84.20%.

Proteinase Inhibitory Activity

The methanolic extracts of *Evolvulus alsinoides* at different concentration exhibited significant antiproteinase activity. The maximum inhibition was observed from methanol extract (74.80) at 500µg/ml, when compared to the standard drug aspirin the maximum proteinase inhibitor activity is 86.70 (Table 1).

Xanthine Oxidase Assay

The maximum inhibition of xanthine oxidase was observed from methanolic plant extract IC_{50} μM (42.00). Maximum inhibition was noticed when compared to the Standard: Allopurinol was 0.20 and Flavonoids: Luteolin + epigallocatechin (1: 1) were 0.62. (Table: 2)

Effect of Different Solvent Extracts on Anti-lipoxygenase Activity

All the extracts significantly inhibited the lipoxygenase activity; it is ranged from 42.50 to 52.60. The methanolic extracts has showed highest anti-lipoxygenase activity at 500µg/ml is 52.60 (Table: 2), the standard indomethacin showed a 46.50% inhibition at a concentration of 50 µg/ml.

The ethanol extract of *Evolvulus alsinoides* had in vitro broad spectrum antimicrobial activity. Thus extracts from the plant can be used to control infections caused by Salmonella typhi, Escherichia coli, Klebsiella pneumoniae and Staphylococcus aureus (Omogbai, BA. and Eze, FA, 2011).

Results of our findings confirmed the use of *Evolvulus alsinoides* as a traditional medicine. We found strong anti inflammatory activities specifically in the Methanolic extracts of *Evolvulus alsinoides*. High TPC values found in methanol extract imply the role of phenolic compounds in contributing these activities. Plant phenolic compounds have been found to possess potent anti-inflammatory activity (Sakat *et*

al., 2010; Roy *et al*., 2010; Garg *et al*., 2010). These flavonoids have been found to possess anti-inflammatory properties in various studies (Lin *et al*., 2008; Lopez-Lazaro, 2009; Yoshida *et al*., 2009; Amarlal *et al*., 2009).

Strong presence of tannins in all the solvent extracts may explain its potent bioactivities are known to possess potent a anti-inflammatory properties (Souza *et al*., 2007; Fawole *et al*., 2010). The Saponins have already shown as anti-inflammatory activity (Gepdireman *et al*., 2005), the presence of terpenoids has shown anti-inflammatory properties (Neichi *et al*., 1983).

Denaturation of proteins is a well documented causative factor for inflammation. The inflammatory drugs (salicylic acid, phenyl butazone etc) have shown dose dependent ability to thermally induced protein denaturation (Mizushima and Kobayashi, 1968).

Similar results were observed from many reports from plant extract (Sakat *et al*., 2010). The extracts may possibly inhibit the release of lysosomal content of neutrophils at the site of inflammation. These neutraphils lysosomal constituents include bactericidal enzymes and proteinases, which upon extracellular release cause further tissue inflammation and damage (Chou, 1997). The precise mechanism of this membrane stabilization is yet to be elucidated; it is possible that the methanolic extract of *Evolvulus alsinoides* produced this effect surface area/volume ratio of the cells, which could be brought about by an expansion of membrane or the shrinkage of cells and an interaction with membrane proteins (Shinde *et al*., 1999).

Proteinases have been implicated in arthritic reactions. Neutrophils are known to be a source of proteinase which carries in their lysosomal granules many serine proteinases. It was previously reported that leukocytes proteinase play important role in the development of tissue damage during in inflammatory reactions and significant level of protection was provided by proteinase inhibitors (Das and Chatterjee, 1995). Recent studies have shown that many flavonoids and related polyphenols contributed significantly to the anti-inflammatory activities of many plants (Luo *et al*., 2002; Okoli and Akah, 2004).

The mode of action of the extracts and standard anti-inflammatory drugs could be connected with binding to the erythrocyte membranes with subsequent alteration of the surface charges of the cells (Hess SM *et al* 1972). It has been reported that certain saponins and flavanoid exerted stabilizing effect on lysosomal membrane both *invivo* and *invitro* while tannins and saponins possess ability to bind cations, thereby stabilizing erythrocyte membranes and other biological macromolecules (Van-Cangeghen P, 1972).

Hence, the presence of bioactive compounds in the methanol and ethanol extracts of ethanol extract of *Evolvulus alsinoides* may contribute to its, antimicrobial, antioxidant and anti inflammatory activity. The present investigation has shown that the different solvent extracts of methanolic extract of *Evolvulus alsinoides* have shown the presence of active phytochemicals which are showed significantly anti-inflammatory properties were confirmed.

These activities may be due to strong occurrence of polyphenolic compounds such as alkaloids, flavonoids, tannins, steroids, terpenoids, phenols and Saponins. The anti-inflammatory activity was comparable with standard aspirin.

This medicinal plant by *in vitro* results appear to be establishing scientific evidence to support traditional medicinal uses and indicate a promising potential for the development of an anti-inflammatory agent from methanolic extract of *Evolvulus alsinoides* plant.

LOXs are sensitive to antioxidants and the most of their action may consist in inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipid peroxy-radical formed in course of enzyme peroxidation. This can limit the availability of lipid hydroperoxide substrate necessary for the catalytic cycle of LOX. The results obtained from our studies on ethanol extract of *Evolvulus alsinoides* has shown as potential anti-inflammatory activity.

The ethanol extract of *Evolvulus alsinoides* inhibited the lipoxygenase enzyme activity.

This indicates that plant ethanolic extract of *Evolvulus alsinoides* is more useful in studies of inflammation and in various related physiological studies and diseases such as aging, cancer etc. activity and stabilized the Red Blood Cells membrane. The solvent fractions exhibited a moderate XO inhibitory activity and therefore may due to presence of bioactive constituents and these can useful in the treatment of XO induced diseases.

Many studies have suggested that flavonoids exhibit biological activities, including antiallergenic, antiviral, anti-inflammatory, and vasodilating actions. These pharmacological effects are linked to the antioxidant properties of flavonoids. Protective effects of flavonoids are ascribed to their capacity to suppress ROS formation by inhibiting some enzymes or chelating trace elements involved in free radical production, scavenge radical species and more specially the ROS, and improve regulation antioxidant defense (Ferrali, *et al* 1997, Elliott, *et al* 1992, Hirano, R. *et al* 2001, Cotelle, *et al* 1996).

Table.1 Effect of methanolic extract of evolvulus alsinoides on albumin denaturation, membrane stabilization and proteinase inhibitory activity percentage inhibition

Test sample	Albumin denaturation	Membrane stabilization	Proteinase inhibition
Methanolic extract (500µg/ml)	82.32±0.50a	65.70 ±0.60b	74.80±0.80b
Aspirin (100µg/ml)	68.40±0.40c	84.20±0.40a	86.70±0.50a

Table.2 Effect of methanolic extract of Evolvulus alsinoides on Xanthine oxidase, Anti-lipoxygenase inhibitory activity percentage inhibition

Test sample	Xanthine oxidase IC_{50} (μM) ±SEM	Anti-lipoxygenase Activity (IC_{50} µg/ml)
Methanolic extract (500µg/ml)	0.42 ±1.40a	52.60
Std : Allopurinol	0.20 ± 0.40	-------
Luteolin + epigallocatechin (1: 1)	0.62 ± 0.20	-------
Std : Indomethacin (50µg/ml).	----------	46.50%

Xanthine oxidase inhibitors (XOI) are typically used in the treatment of nephropathy and renal stone diseases linked to hyperuricemia. There has been recent interest in the potential benefit of XOI in the prevention of vascular disease, because of emerging evidence suggesting a role for serum uric acid in the development of cardiovascular disease; the enzyme is an important source of oxidative stress in the vasculature (Higgins, P *et al* 2009). XOI are agents that directly inhibit the synthesis of uric acid *in vivo*. Certain active constituents present in crude plant extracts like flavonoids and polyphenolic compounds have been reported to possess XOI (Chang, W. S. *et al* 1993, Costantino, L. *et al* 1992). These findings have opened the possibility of isolation of new natural compounds, which can be possible inhibitors of XO, and led to the growing interest in the investigation of medicinal plants. The activity of flavonoids as inhibitors of xanthine oxidase *in vitro* has been reported. The absence of a hydroxyl group at C-3 enhances slightly the inhibition effect on XO (Cos P. L, *et al* 1998, Van Hoorn, D. E. C. *et al* 2002, Arimboor, R. *et al* 2011).

These extracts also exhibited antioxidant and anti-inflammatory properties strongly. These extracts reduced the activity of lipoxygenase, xanthine oxidase activities. Purification of each bioactive compound can be needed and purified compound can also increase their activity. This report proposing its potential application as a lead compounds for designing potent anti-inflammatory activity and they can be used for treatment of various diseases (Cancer, neurological disorder, aging and inflammatory).

In conclusion, the present study results indicated that the methanolic extract of *Evolvulus alsinoides* possess antioxidant and anti-inflammatory properties. The extract fractions serve as free radical inhibitors or scavenger or acting possibly as primary oxidants and inhibited the heat induced albumin denaturation, proteinase, lipoxygenase and xanthine oxidase (XO), which may serve as therapeutic agents for hyperuricemia and/or gout. Xanthine oxidase inhibitors are agents that directly inhibit the synthesis of uric acid *in vivo*. Certain active constituents present in crude plant extracts like flavonoids and polyphenolic compounds have been reported to possess XOI.

These therapeutic responses were due to the strong occurrence of polyphenolic compounds in *Evolvulus alsinoides* plant extracts

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