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In-vitro and *in-vivo* anti-inflammatory activity of *Andrographis serpyllifolia (Rottl. Ex Vahl.) Wt.*

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ABSTRACT

The study was aimed to evaluate the analgesic and anti-inflammatory activity (by both in-vitro and in-vivo) of both chloroform and methanol root extracts of *Andrographis serpyllifolia (Rottl. Ex Vahl.) Wt.* Methods used for the studies were In-vitro 5-Lipoxygenase inhibition assay and In-vivo measurement of rat paw edema and ear edema in rats, acetic acid induced writhing response and hot plate method in albino mice. Chloroform and methanolic extracts of *A. serpyllifolia* root have shown moderate potency in inhibiting 5-LOX and shown significant anti-inflammatory activity. Despite the IC_{50} values are little higher, anti-inflammatory efficacy of these extracts possibly due to other mechanisms apart of 5-LOX inhibition. However, *In-vivo* anti-inflammatory studies revealed that *A. serpyllifolia* methanolic extract has shown higher degree of efficacy when compared to the chloroform extract. In terms of analgesic activity in writhing test, methanolic extract has shown more efficacy than chloroform extract. Hence, it is important to isolate the active principles for further testing the anti-inflammatory efficacy.

Key Words: *Andrographis serpyllifolia*, anti-inflammatory, analgesic, 5-LOX, *In-vivo* animal models.

INTRODUCTION

Tirumala Hills (Rayalaseema region, Andhra Pradesh, India), which lie geographically in the South Eastern Ghats are well-known for the rich heritage of flora. *Andrographis serpyllifolia (Rottl. Ex Vahl.) Wt.* (Acanthaceae) is a trailing and rooting procumbent herb widely distributed throughout South India. All the parts of *A. serpyllifolia* are important in the traditional system of medicine in India which has been extensively used for snake bites, antipyretics, cancer and inflammation (Sekhar *et al.*, 2011). The root extract of the plant is used to cure fever (Ramaswamy *et al.*, 1973). The plant extract is used in treating wounds and also effective in the treatment of Jaundice (Manjunatha *et al.*, 2004). But still there is no specific study on *A. serpyllifolia* for its analgesic and anti-inflammatory activity. Hence, in the present study, chloroform and methanol extracts of roots of *A. Serpyllifolia* was investigated for analgesic and anti-inflammatory activity *in vitro* and *in vivo* in animal models.

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MATERIALS AND METHODS

Collection of plant material

Roots of *Andrographis serpyllifolia (Rottl. Ex Vahl.) Wt.* were collected from surrounding areas of Tirupati and Tirumala hills, was identified and authenticated by the botanist Dr. K. Madhava Chetty, Assistant Professor, Department of Botany, S. V. University, Tirupati. A voucher specimen (Herbarium Accession No. 136) was deposited in the herbarium, Department of Botany, S.V. University, Tirupati.

Table 1: Experimental designs for various *in-vivo* animal models of inflammation.

Groups	Experimental design (Rat paw edema)	Experimental design (Writhing test/Hot plate)	Experimental design (Ear edema test in mice)
Group 1	Control Group	Control Group	Control Group (0.5 mg/ear)
Group 2	Diclofenac 40 mg/kg, p.o.	Diclofenac 40 mg/kg, p.o.	ASC-0.5 mg/ear
Group 3	ASC-100 mg/kg,p.o.	ASC-100 mg/kg,p.o.	ASM-0.5 mg/ear
Group 4	ASC-200 mg/kg,p.o.	ASC-200 mg/kg,p.o.	Diclofenac Sodium (0.5 mg/ear)
Group 5	ASM-100 mg/kg, p.o.	ASM-100 mg/kg, p.o.	
Group 6	ASM-200 mg/kg,p.o.	ASM-200 mg/kg,p.o.	

ASC = *A. serpyllifolia* cholroform extract; ASM = *A. serpyllifolia* methanolic extract

Preparation of Plant Extracts and Phytochemical Screening

The fresh roots (2Kg) of *Andrographis serpyllifolia* subjected for air dried and powdered. The root powder was extracted successively with petroleum ether, chloroform and methanol using soxhlet apparatus. All the extracts were filtered using cotton plug followed by Whatman filter paper. The extracts were concentrated using rotary vaccum evaporator (Buchi USA) and then dried in lyophilizer (Labconco USA) under reduced pressure. The dried extracts were stored in airtight container and placed in refrigerator. The root extracts of *A. serpyllifolia* were analyzed for the presence of flavonoids, alkaloids, glycosides, steroids, phenols, saponins, terpenoid, cardiac glycosides and tannins according to the standard methods (Harborne *et al.*, 1973; Mondal and Suresh, 2012).

Animals

Wistar rats weighing 150-200 g and Albino mice 20-30g of either sex (National Institute of Nutrition (NIN), Hyderabad, India) was used for *in-vivo* pharmacological studies. Animals were maintained under standard laboratory conditions at 25±2°C, 50±15% RH and normal photoperiod (12h dark / 12h light). Commercial pellet diet (NIN) and water were provided *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee of Vaagdevi College of Pharmacy, Hanamkonda, Warangal, Andhra Pradesh, India and by the Animal Regulatory Body of the Government of India (Regd. No. 1047/ac/07/CPCSEA). The experimental designs for various *in-vivo* models of inflammation were tabulated in table 1.

Acute Toxicity

Acute oral toxicity study was performed as per OECD-423 guidelines (acute toxic class method). Albino mice (n =6) of either sex selected by random sampling technique were used for the study. The animals were kept fasting for overnight providing only water, after which the extracts were administered orally at the dose level of 5mg/kg body weight by oral feeding needle and observed for 14 days. Mortality was not observed, the procedure was repeated for further higher dose such as 50, 300, 2000 mg/kg body weight (Ecobichon, 1997).

In-vitro 5-Lipoxygenase Inhibition

The assay mixture contained 80mM linoleic acid and sufficient amount of potato 5-lipooxygenase enzyme in 50mM phosphate buffer (pH-6.3) and incubate for 2 minutes for reaction. The reaction was initiated by the addition of enzyme buffer mixture to substrate (linoleic acid) and the enzyme activity was monitored as in increase in absorbance at 234 nm using UV kinetic mode on (Agilent technologies-Varian Cary-50) UV-visible spectrophotometer. In the inhibition studies, the activities were measured by incubating various concentration of test substance with enzyme buffer mixture for 2 minutes before addition of the substrate. The assay was performed in triplicate and mean values were used for the calculation. Percentage inhibition was calculated by comparing slope or increase in absorbance of the test substance with that of control enzyme activity. The activity of 5-LOX was compared with the standard positive control, Zileutin (Reddenna *et al.*, 1990; Ulusu *et al.*, 2002).

Acetic Acid-induced Writhing Test

Albino mice, weighing 20–30 g, were randomly divided into groups (n=6), acetic acid was administered intraperitonialy to the experimental animals to create pain sensation. The extracts were solubilized in the ratios of (1:1) propylene glycol one drop and starch suspension. The plant extract, *A. serpyllifolia*

Table 2: Phytochemical screening of *A. serpyllifolia* methanolic and chloroform extracts.

Photochemical Constituents	Methanolic extract	Chloroform extract
Flavonoids	+	+
Alkaloids	+	+
Glycosides	+	+
Steroids	+	+
Phenols	+	+
Terpenoid	+	+
Saponins	+	+
Resins	-	-
Tannins	+	+
Cardiac Glycosides	+	+

– =absent, + =present

in two different doses (100 mg/kg and 200 mg/kg, b.w) and vehicle were administered orally 30 minutes prior to intraperitoneal administration of 1% v/v acetic acid solution (0.1mL/10g) but diclofenac sodium was administered 15 minutes prior to acetic acid injection. Then the animals were placed on an observation table. Each mouse from all groups were observed individually for counting the number of writhing they made in 15 minutes commencing just 5 minutes after the intraperitoneal administration of acetic acid solution. Full writhing was not always accomplished by the animal, because sometimes the animals started to give writhing but they did not complete it. This incomplete writhing was considered as half-writhing. Accordingly, two half-writhing were taken as one full writhing. The number of writhes in each treated group was compared to that of a control group while diclofenac sodium (40 mg/kg) was used as a reference substance positive control.

Measurement of Paw Edema

Edema was induced by injecting 0.1mL of 1% solution of carragenan in saline to (sub plantar) right hind paw of rats. The plant extract, *A. serpyllifolia* in two different doses (100mg/kg and 200 mg/kg, b.w) and vehicle were administered orally 60 minutes prior to injection of carragenan. The volume of edema of injected and contra collateral paws were measured at 0.5, 1, 1.5, 2, 3, 4, 5 hours after induction of inflammation using a plethysmograph and the percentage of anti-inflammatory activity was calculated.

TPA Induced Ear Edema

Each mouse received 2.5µg of TPA (12-*O*-tetradecanoylphorbol-13-acetate) dissolved in 20µL of 70% EtOH. This was applied by an automatic pipette in 20µL volumes to both anterior and posterior surfaces of the right ear. The left ear (control) received the same volume of solvent (EtOH 70%), simultaneously with TPA. Diclofenac sodium (0.5mg/ear) was used as a standard drug. The thickness of each ear was measured 4h after induction of inflammation using a gauge calipers. The edema was expressed as the difference between right and left ears due to TPA application.

Hot Plate Method

Albino mice of either sex were selected, weighed and divided into six groups (n=6). The time of reaction to pain stimulus of the mice placed on the plate, heated at 55°±0.5°C was recorded at 1, 2, 3, 4 and 5 hrs, after the administration of the plant extracts, *A. serpyllifolia* in two different doses (100 mg/kg and 200mg/kg , b.w) and vehicle were administered orally before 60 minutes. The increase in reaction time against control group was calculated.

Statistical Analysis

Values from *in-vivo* anti-inflammatory and analgesic activity shown in tables and figures were Mean ± SD for six animals. Analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc multiple comparison tests (by "GraphPad Prism 5" software) was applied for determining the statistical significance between different groups. The results were significant in the level of *$p < 0.05$, **$p<0.01$ & ***$p< 0.001$.

RESULTS

Phytochemical Screening

In the methanolic and chloroform extracts of *A. serpyllifolia,* flavanoid, alkaloid and phenols were present. Glycosides, saponins, tannins and cardiac glycosides are also present. Resins are absent (Table 2).

Acute Toxilogical Studies

To establish the safety of the extracts (methanolic and chloroform) administered to both male and female mice. We observed no significant toxic signs or death during the 14 days observation period. None of the mice showed clinical toxic signs such as

Table 3: *In-vitro* 5-LOX inhibition of *Andrographis serpyllifolia* extracts.

Plant extract (*A. serpyllifolia*)	IC_{50} (µg/ml)
Methanolic root extract	42.14
Chloroform root extract	107.72
Petroleum ether root extract	180.06
Zileutin	4.36

anorexia, depression, lethargy, jaundice, dermatitis and also, no mortality happened throughout the examination. The plant extracts did not exhibit any mortality up to the dose level of 2000mg/kg. So, the extracts were safe for long term administration. The results were found that there is no testicular toxicity for 1000mg/kg of *Andrographis paniculata* (Burgos *et al.*, 1987), hence the dose of the extracts were up to 1000 mg/kg was safe.

In-vitro 5-LOX inhibition

The IC_{50} values of petroleum ether, chloroform and methanolic extracts of *A. serpyllifolia* and standard drug, zileutin against 5-LOX inhibition were found to be 180.06µg/mL, 107µg/mL, 42.14µg/mL, and 4.36µg/mL respectively (Table 3). The 5-LOX inhibition activity of *A. serpyllifolia* extracts were not reported and published by other workers till date.

Effect Plant Extracts on Rat Paw Edema

Carrageenan-induced edema is a biphasic response. The first phase is mediated through the release of histamine, serotonin and kinins where as, the second phase is related to the release of prostaglandin and slow reacting substances which peak at 3h.

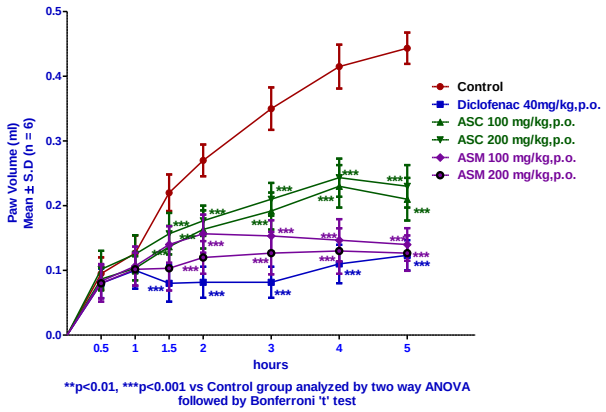

Figure 1: Effect of both *A. serpyllifolia* methanolic root extract (ASM) chloroform root extract (ASC) and on rat paw edema of wistar rats.

Table 4: Effects of both *A. serpyllifolia* methanolic root extract (ASM) and chloroform root extract (ASC) against TPA-induced ear edema in mice as measurement of swelling thickness.

Test Groups	Dose (mg/ear)	Swelling thickness (µm) ± S.E.M
Control Group	0.5	262.34 ± 21.8
A. serpyllifolia methanolic extract (ASM)	0.5	120.25 ± 16.2***
A. serpyllifolia chloroform extract (ASC)	0.5	155.80 ± 13..2***
Diclofenac Sodium	0.5	22.35 ± 0.5***

The chloroform and methanol extracts of *A. serpyllifolia* root at the dose level of 100mg/kg and 200mg/kg decreased the oedema significantly ($p < 0.001$) at 3rd and 4th hour after administration. When compared to the control group, the effect was almost comparable with standard drug diclofenac sodium at 3rd and 4th hour after administration (Figure 1). Methanolic extract of *A. serpyllifolia* root has exhibited more degree of anti-inflammatory activity than chloroform extract.

Effect of Plant Extracts on Mouse Ear Edema

Similar pattern of activity was followed for both the measurements of ear swelling thickness. Both chloroform and methanolic root extracts of *A. serpyllifolia* were shown significant anti-inflammatory effect in mouse ear models. The swelling thickness of chloroform extract (500 µg), methanolic extract (500 µg) and control was shown as 120.25µm, 155.80 µm and 262.34 µm respectively. This result revealed that the methanolic extract

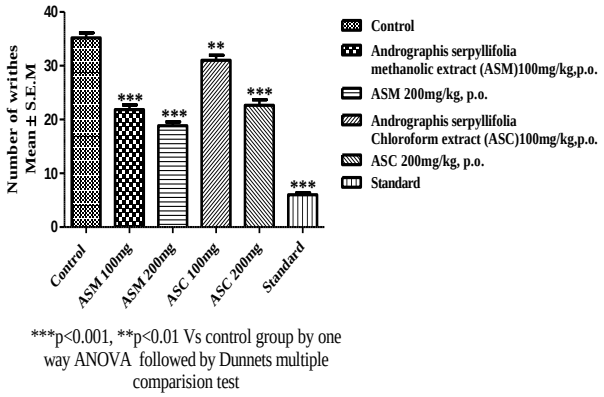

Figure 2: Effect of both *A. serpyllifolia* methanolic root extract (ASM) and chloroform root extract (ASC) on acetic acid induced writhes in albino mice.

exhibited more degree of anti-inflammatory activity than chloroform extract (Table 4).

Effect of plant extracts on acetic acid induced writhes

The number of writhings was more in the chloroform extract with various doses of 100 and 200 mg/kg than methanolic extract with the same doses. This is clearly indicated that both chloroform and methanolic root extracts of *A. serpyllifolia* were shown significant reduction in the number of writhes, but methanolic extract exhibited more degree of anti-inflammatory activity than methanolic extract (Figure 2).

Effect of plant extracts on Hot plate induced algesia

Standard drug, Tramadol 10 mg/kg, p.o. has shown significant analgesic activity at the intervals of 1h (*p<0.05), 2h (***p<0.001) and 3h (***p<0.001) where as chloroform and methanolic extracts of *Andrographis serpyllifolia* have not shown any significant analgesic activity at two dose levels of 100 mg/kg and 200mg/kg in mice, data tabulated in the Table 5.

DISCUSSION

A. serpyllifolia methanolic extract might possess potent active principles which inhibits 5-LOX enzyme. IC_{50} values of methanolic and chloroform root extracts of *A. serpyllifolia* were found to be 42.14 µg/mL and 180.06 µg/mL respectively. It is apparent that these plant extracts are as potent as standard drug zileutin in inhibiting 5-LOX. However, potency of the drug always not necessarily reflects the efficacy of the drug. With this point of view, carried out *in-vivo* anti-inflammatory and analgesic activity of *A. serpyllifolia* root extracts were performed.

The most widely used primary test to screen new anti-inflammatory agent's measure the ability of a compound to reduce local edema induced in the rat paw by injection of an irritant agent (Bonnie *et al.*, 2005). Carrageenan induced oedema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1-2 h) of the carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages (Winter *et al.*, 1962). The similar trend was shown by methanolic extract of *Euphorbia heyneana* Spreng. (Ganga Rao *et al.*, 2011).

A. serpyllifolia methanolic root extract has shown more significant anti-infalmmatory effect in two inflammatory models (carragenan induced paw edema and TPA induced ear edema) than chloroform root extract. Most important finding in this study is strong correlation between *in-vitro* and *in-vivo* anti-inflammatory data. Though potency of 5-LOX inhibitory activity of this *A. serpyllifolia* root extracts was found to be low and anti-inflammatory efficacy of these plant extracts was found to be high and moreover, effect was dose dependent. This evidence heightened the importance of this plant extract for further evaluations to isolate the active principles that are contributing to the observed anti-inflammatory activity.

Studies are under progress in our laboratory to isolate the compounds and to study the biological activities of the same. However, it is speculated that the probable mechanism of anti-inflammatory action of *A. serpyllifolia may* be due to its influence

Table 5: Effects of both *A. serpyllifolia* methanolic root extract (ASM) and chloroform root extract (ASC) on analgesia produced by hot plate method in mice.

Name of the Group	Reaction time (Sec)				
	1 hr	2 hr	3 hr	4hr	5hr
Control group	16.3 ± 0.64	17.2 ± 0.98	17.6 ± 1.56	16.2 ± 1.22	18.1 ± 1.46
Diclofenac Sodium (40 mg/kg)	19.54 ± 0.5*	23.36 ± 0.84***	24.5 ± 0.96***	22.5 ± 0.96***	20..5 ± 1.26
ASC-100 mg/kg, p.o.	16.16 ± 1.71	18.50 ± 0.80	17.12 ± 1.24	18.42 ± 1.24	18.63 ± 0.85
ASC-200 mg/kg,p.o.	17.91 ± 1.02	17.08 ± 1.35	18.25 ± 1.85	17.32 ± 0.74	18.74 ± 1.57
ASM-100 mg/kg, p.o.	17.85 ± 1.52	18.45 ± 1.28	19.12 ± 2.84	18.12 ± 1.35	19.82 ± 0.95
ASM-200 mg/kg,p.o.	18.84 ± 1.34	18.50 ± 0.86	17.07 ± 1.29	18.76 ± 1.55	17.41 ± 1.37

ASC = *A. serpyllifolia* cholroform extract; ASM = *A. serpyllifolia* methanolic extract

Each value is Mean ± S.E.M (n=6), *Denotes significance difference when compared to control values at *p<0.05, ***p<0.001.

on the cycloxygenase pathway (George *et al.*, 2010 a, 2010 b) since it is interfering with prostaglandins biosynthesis as evidenced by the maximum anti-inflammatory activity at the end of the third hour after the challenge with carageenan. *A. serpyllifolia* methanolic extract has shown more degree of analgesic activity than exhibited potent analgesic activity than chloroform extract at the dose levels of 100, 200mg/kg in acetic acid induced writhing test. The same results were reported on *Trigonella foenum-graecum* Linn. (Moli *et al.*, 2011). But both extracts did not exhibit the analgesic activity in hot plate method. This clearly indicated that the plant extracts which are possibly act through the peripheral mechanisms (Richardson *et al.*, 1998) but not via the central mechanisms. Extracts have demonstrated to have analgesic effect in acetic acid induced writhes. This result provides evidence that plant extracts might possess active principles which produce peripheral analgesic effect without mediating spinal and supra-spinal actions. At present, there are no reports on investigation to identify the active components present in methanolic extract of *A. serpyllifolia*.

CONCLUSION

Andrographis serpyllifolia methanolic root extract has shown moderate potency in the inhibition of 5-LOX. Methanolic root extract of *A. serpyllifolia* exhibited more degree of anti-inflammatory activity than chloroform extract in *in-vitro* studies. Methanolic extract of *A. serpyllifolia* exhibited more degree of analgesic activity than chloroform extract in *in-vivo* studies. Based on the correlation between both *in-vitro* data and *in-vivo* data, it was concluded that *A. serpyllifolia* methanolic root extract possibly consists of more anti-inflammatory active principles than chloroform extract. Further investigations are anticipated to identify the active components and lead to their further clinical use.

DECLARATIONS

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