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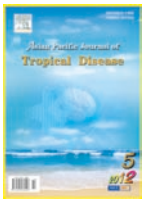

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In ova angiogenesis analgesic and anti inflammatory potency of *Aerva monsoniae* (Amaranthaceae)

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ABSTRACT

Objective: To evaluate the wound healing potency of aqueous extract of *Aerva monsoniae (A. monsoniae)* by *in vitro* method using fertilized eggs, *in vivo* analgesic and anti inflammatory activity in rodents and the anti bacterial activity on the bacterial strains that infect the wound. **Methods:** The whole plant of *A. monsoniae* was extracted with water and then subjected to preliminary chemical screening. It was then evaluated for in ova angiogenesis on fertilized white leg horn eggs using the concentrations of 200-600 μg/mL. The analgesic activity was evaluated in mice using the dose 100 and 250 mg/kg. The anti inflammatory activity was evaluated in rats using the dose 250 mg/kg and 500 mg/kg. In both the parameters water was used as the control and diclofenac was used the standard. The anti bacterial activity on *Staphylococcus aureus* and *Pseudomonas aerugenosa* was performed. **Results:** The phytochemical screening revealed the presence of tannins, flavonoids and saponins. The in ova angiogenesis revealed a dose dependent activity which proves the wound healing claim of the plant as more number of blood capillaries were formed at the site of the drug. The plant proved to be a potent analgesic and anti inflammatory agent at doses 100 mg/kg and 250 mg/kg. The anti bacterial activity was present but at higher doses. **Conclusions:** The parameters studied in the present investigation proved that the plant is a potent wound healer. Further *in vivo* wound healing studies on animal model is desired. As the extract showed potent analgesic, anti inflammatory and anti bacterial properties, it can be considered that when formulated into suitable formulation, and it can reduce the pain, inflammation and infections related to wound very well.

1. Introduction

Plants have the immense potential for the management and treatment of wounds. A large number of plants are used by tribal and folklore in many countries for the treatment of wounds and burns[1]. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms. These phytomedicine are not only cheap and affordable but also safe. The presence of various life-sustaining constituents in plants has urged scientist to examine these plants with a view to determine potential wound healing properties[2]. Many phytopharmaceutical laboratories are now concentrating their efforts to identify the active constituents and modes of action of various medicinal plants[3]. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body. These constituents include various chemical families like alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, and phenolic compounds[4]. Tribal people of Andhra Pradesh are endowed with a deep knowledge concerning the use of wild plants as sources of food and medicine Tribe's constitute an average of 6% of total population of Andhra Pradesh[5]. Andhra Pradesh is the home land of 33 tribes generally distributed in hilly and interior forest areas. Major primitive tribes are Chenchu, Kond, Kutia Khond and Kolam. The Sugalis and Lambadies are the largest tribes found in Andhra Pradesh[6]. It was observed that these tribes use the plant *Aerva monsoniae*, for curing wounds, diabetes, sore throat, urinary colitis and pain. This plant is a perennial herb, 5-50 cm tall. Stem branched from base; branches ascending or stoloniferous, white lanose. Leaves opposite or nearly

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whorled, sessile, grayish green, subulate, linear abaxially white lanose, adaxially glabrous, base attenuate, sometimes vaginate. Spikes terminal, narrowly ovate or terete, white lanose; rachis very short or absent indigenous to many parts of the peninsular in India, Burma and Bangladesh[6]. As no work was carried out on the pharmacological aspects, we undertook this research to evaluate the *in vitro* wound healing property on fertilized chick embryo model. This model was selected to avoid torturing of animals. As pain and inflammation are linked with wound, we evaluated the same by *in vivo* methods on rodents. The antibacterial activity on the bacterial strains that infect wound was also performed.

2. Materials and methods

2.1. Collection and authentication

The plants were collected in the month of November and December from the Medicinal Plant Garden, Nalanda College of Pharmacy, Nalgonda district, Andhra Pradesh, India. The plants were identified and authenticated as *Aerva monsoniae* L.; Family-Amaranthaceae; Voucher No.: 824 (Flora of the presidency of Madras- Vol.II; Reprinted Edition-1957), by Prof. Dr. B. Bhadraiah M.Sc., Ph.D, H.O.D, Department of Botany, Osmania University, Hyderabad. A Herbarium was prepared and deposited in the Department of Botany, Osmania University, Hyderabad, Andhra Pradesh, India. The plants species were shade dried and stored for further experimentation.

2.2. Collection of fertilized chick embryos

The fertilized white leg horn eggs were provided by Tirumala breeders (P) Ltd., Hanmakonda, Warangal District, Andhra Pradesh, India and collected at Tirumala breeders (P) Ltd., Vadaigudem, Yadagirigutta, Nalgonda Dist., Andhra Pradesh, India.

2.3. Procurement of rodents

Wistar albino rats (150-200 g) and Swiss albino mice (25-30 g) of either sex were procured from National Institute of Nutrition, Hyderabad, A.P, India. The experimental protocol was approved from the Institutes animal ethics committee under the reference no. NCOP/IAEC/approval/39/2011 and then experimental studies were undergone according to their rules and regulations. The animals were housed under standard environmental conditions and had free access to standard pellet diet and water *ad libitum*.

2.4. Procurement of microbial cultures

The bacterial strains *Pseudomonas aerugenosa (P. aerugenosa)* MTCC 741 and *Staphylococcus aureus (S. aureus)* MTCC 96 were procured as freeze dried form from microbial type culture collection, Chandigarh, India.

2.5. Preparation of extract

About 500 g of the dried powdered whole plant was taken, defatted with the petroleum ether and extracted with distilled water for 18 h at 45 ℃. The thick mass obtained was evaporated with help of vacuum rotary evaporator and then subjected to preliminary chemical tests[8,9].

2.6. In ova angiogenesis

Chorioallantoic membrane (CAM) model was used for the *in vitro* wound healing model. In this method embryonated chicken eggs (9 days old) were selected and a small window (1 cm^2) was made on the shell. Through the window, a sterile disc of cellulose treated with 200, 400 and 600 μg/mL of the aqueous extract of *A. monsoniae* were placed inside triplicate sets of eggs at the junction of two blood vessels. The windows were resealed and the eggs were incubated at 37 ℃ in a well humidified chamber for 72 h. The windows were then opened and the growth of new capillary blood vessels were observed and finally compared with the control eggs containing sterile discs without the extract[10].

2.7. Safety evaluation

The preferred rodent species were female mice, although other rodent species may be used. Females mice selected were nulliparous and non-pregnant. Each animal, at the commencement of its dosing, were between 8 and 12 weeks old and their weights were between 25-30 g. All the animals were fasted prior to dosing. Twelve animals were taken and divided into four groups each comprising of three animals. Group I animals received 5 mg/kg, Group II received 50 mg/kg, Group III received 300 mg/kg and Group IV received 2 000 mg/kg. The test substance was administered in a single dose by gavages using a stomach tube[11].

2.8. Analgesic activity by Eddy's hot plate method

Swiss albino mice of either sex weighing about 20-25 g were used for this experiment. All the animals were fasted for 18 h. Animals were divided into four groups, each group with 6 animals. Group I was denoted as positive control which were applied with diclofenac sodium gel (100 mg/mL), Group II was treated as the negative control which were applied with water, Group III and Group IV were the test

groups which were applied with aqueous extracts at dose levels of 100 mg/kg and 250 mg/kg body weight. All the animals were applied with the respective treatments onto the plantar surface of the hind paw by gently rubbing 50 times with the index finger. The animals were placed on the hot plate and the time until either licking or jumping occurred was recorded by a stop watch. The latency was recorded before and after 5, 10, 15, 30, 60, 90, 120, 240, 360, 720, 1 440 minutes respectively. The prolongation of latency times was compared and was calculated[12,13].

2.9. Anti inflammatory activity by carrageenin induced rat paw oedema

Wistar albino rats weighing 150-200 g were taken and divided into three groups with six animals in each group. Group I was denoted as positive control and were applied with diclofenac sodium gel (100 mg/mL), Group II was treated as the negative control which were applied with water, Group III and Group IV were the test groups which were applied with aqueous extracts at dose levels of 250 mg/kg and 500 mg/kg body weight. All the animals were applied with the respective treatments onto the plantar surface of the hind paw by gently rubbing 50 times with the index finger 60 minutes prior to carrageen injection. Inflammation was induced by a 0.1 mL subcutaneous injection of 1% w/v suspension of carrageenin in saline to the plantar surface of right hind paw. The paw volume was measured plethysmographically immediately after injection and again at 1, 2, 3, 4, 8, 12, 16 hours and eventually 24 h after drug administration[13,14].

2.10. In vitro antibacterial activity

The anti bacterial activity was screened adopting Kirby-Bauer method using the standardized sterile paper disc-agar diffusion method. The bacterial strains used were *S. aureus* and *P. aerugenosa*. All Petri dishes and graduated measuring pipettes were heat sterilized in an autoclave at 120 ℃ for 1 h. All plates were prepared with equal thickness of nutrient agar. Concentrations of 1-8 mg/disc of aqueous extracts of *A. monsoniae* were prepared and 50 μL of each test solution was incorporated into sterile disc and placed on the solidified nutrient agar plates inoculated with the bacterial strains. Gentamycin (10 μg/disc) and water were employed as positive and negative control, respectively. The plates were then incubated at 37 ℃ for 24 h. All the tests were performed in triplicates and the results were expressed as mean of inhibition diameters (mm)[15].

2.11. Statistical analysis

Results were expressed as mean±SD. The difference between experimental groups was compared by One-way Analysis of Variance (ANOVA) followed by Dunnett Multiple comparison test (control vs test) using the soft ware Graph Pad Instat.

3. Results

The preliminary chemical tests performed for the aqueous extract of *A. monsonaie* revealed the presence of carbohydrates, tannins, flavonoids and saponins. The *in vitro* wound healing property by CAM model revealed that the aqueous extract of *A. monsoniae* possessed angiogenesis activity from slight to marked difference which was in dose dependent manner. Increase in the size of blood vessels at a dose of 200 μg/mL was slight as compared to the control on the same day, whereas at a dose of 400 μg/mL caused a marked increase in the size and number of the blood vessels. The 600 μg/mL concentration showed a slight increase in the size and number of blood vessels (Figure 1). Hence the results showed that the plant has powerful wound healing property.

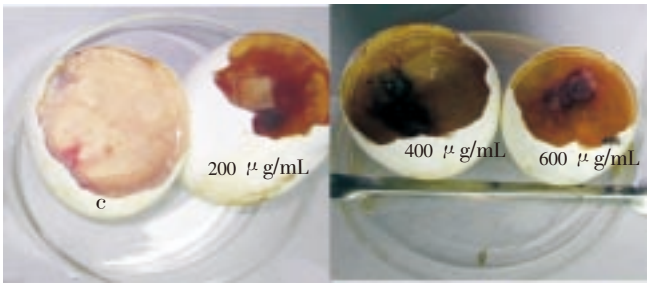

Figure 1. Angiogenesis activity shown by the plant extract on hen egg embryo.

Table 1
Effect of aqueous extract on mice by hot plate method

Group	Reaction time (seconds)											
	5	10	15	30	45	60	90	120	240	360	720	1440
I	1.167±0.017	1.233±0.110	1.233±0.018	1.580±0.085	1.483±0.035	1.577±0.040	1.560±1.620	1.617± 0.015	1.05±0.03	1.045±0.035	0.800±0.020	0.673±0.025
II	5.567±0.029**	4.907±1.729**	7.007±0.129**	8.647±1.124**	6.78±0.02**	5.650±0.062**	4.200±0.035**	4.003±0.400**	2.41±0.01*	1.967±0.030**	0.983±0.015**	0.953±0.056**
III	2.537±09.07**	1.570±0.102**	2.950±0.061**	3.480±0.435**	4.873±0.020**	5.840±0.052**	6.003±0.025**	7.083±0.076**	7.120±0.130**	5.463±0.228**	1.500±0.050**	1.480±0.028**
IV	1.943±0.047*	1.100±0.043*	1.783±0.170*	0.920±0.052**	2.327±0.075**	0.870±0.147**	1.120±0.105**	1.757±0.040*	1.867±0.060**	0.980±0.020**	1.023±0.077**	0.953±0.050**

Group I: Control; Group II: standard treated with diclofenac sodium; Group III: test treated with 100 mg/kg extract of *A. momsoniae*; Group IV: test treated with 250 mg/kg extract of *A. momsoniae*. Each value expresses mean±SD ** significantly different from control $P<0.01$.

Table 2

Effect of aqueous extract of whole plant on carrageenin- induced paw oedema in rats

Group	Paw volume (mL)							
	1	2	3	4	8	12	18	24
I	3.7620±0.1597	3.5210±0.0049	3.8390±0.5800	3.3850±0.1750	3.2310±0.0036	3.3600±0.1100	3.8520±0.0040	3.7930±0.0600
II	8.9580±0.0560**	8.3750±0.0290**	7.8750±0.0150**	4.5000±0.0047**	4.2000±0.0030**	3.9800±0.0520**	3.8700±0.0030**	3.7880±0.0030**
III	6.7830±0.0030**	6.5460±0.0120**	6.2990±0.0100**	5.8020±0.0073**	5.2510±0.0030**	4.2950±0.0060**	4.0120±0.0050**	3.7190±0.0030*
VI	8.8910±0.0100**	8.2530±0.0075**	7.6870±0.0035**	7.1910±0.0180**	6.8966±0.0032**	6.6520±0.0030**	6.5140±0.1560**	6.0260±0.0160**

Group I: control; Group II: standard treated with diclofenac sodium; Group III: test treated with 250 mg/kg extract of *A. momsoniae*; Group IV: test treated with 500 mg/kg extract of *A. momsoniae*. Each value expresses mean±SD ** significantly different from control $P<0.01$

The acute toxicity studies showed that the plant extract did not produce any kind of toxic symptoms and it was safe to use up to a dose of 2 000 mg/kg body weight.

The *in vivo* analgesic activity by hot plate method revealed that the plant extract was an effective analgesic agent at lower dose. It was observed that the animals treated with the standard diclofenac showed potent activity up to 30 minutes, but later the reaction time was observed to be reduced. Whereas in the case of the animals treated with 100 mg/kg, it was observed that the reaction time increased after initial 30 minutes of treatment and the time was found to be maximum at 240 minutes. The animals treated with 250 mg/kg exhibited poor reaction time. The control treated animals was devoid of analgesic activity (Table 1).

The anti inflammatory activity performed by carrageenin induced paw edema revealed that the plant extract possessed a potent activity at 250 mg/kg. It was observed that the animals treated with this dose had shown a better reduction in paw volume when compared to standard and 500 mg/kg treated animals. At third hour of treatment, it was noticed that the group I, II and IV animals showed the same paw volume. Later by 4th to 8th hour the group II animals showed drastic reduction in paw volume, whereas the group I and IV showed no change in paw volume. The maximum anti inflammatory activity of the group II animals was found at 18th hour, later by 24th hour the paw volume started to increase, which revealed that the anti inflammatory activity started reducing (Table 2).

Table 3

Zones of inhibition in antibacterial studies (mm).

Bacterial strains	Control	Standard	Test (aqueous extract of *A. monsonaie*)							
		10 μg	1 mg	2 mg	3 mg	4 mg	5 mg	6 mg	7 mg	8 mg
S. aureus	6	13	8	8.5	8.5	9	10	10.5	11	11
P. aerugenosa	6	12	8	8.5	8	9	9.5	9.5	10	11

Including disc diameter 6 mm.

The anti bacterial activity of the plant extract revealed that *S. aureus* and *P. aerugenosa* were highly sensitive at higher doses (Table 3). It was observed both the bacteria were equally sensitive to the plant extract.

4. Discussion

Flavonoids are potent inhibitors of many bacterial strains, inhibit important viral enzymes, such as reverse transcriptase and protease, and destroy some pathogenic protozoans. Yet, their toxicity to animal cells is low. Flavonoids are major functional components of many herbal and insect preparations for medical use. Modern authorized physicians are increasing their use of pure flavonoids to treat many important common diseases, due to their proven ability to inhibit specific enzymes, to simulate some hormones and neurotransmitters, and to scavenge free radicals[2]. Wound healing is a complicated process, which involves a series of stages like inflammation, granulation, wound contraction and epithelisation followed by tissue remodeling. Any extract that is used for its wound healing activity may exhibit one or more of the biological activities which could be the basis for it to be used traditionally as a medicine; some of these activities may be analgesic, anti inflammatory, anti microbial, antioxidant, *etc*[16].

The whole plant was extracted with the distilled water and phytochemical tests performed reveals that the presence of phytochemicals like carbohydrates, phenolic compounds (tannins), flavonoids and saponins. Literature survey has revealed that tannins promote wound healing activity through several mechanisms that include chelation of free radicals; antioxidant, antimicrobial and astringent property.

The present investigation on in ova angiogenesis revealed that abundant new blood capillaries were formed at the site of extract which indirectly confirms the wound healing potentiality of the plant. Overall comparison shows the plant showed a significant increase in the size and number of blood vessels formation showing that in the proliferative phase and remodeling phase of the wound healing activity plays a key role by forming the new blood capillaries and improving the circulation in the remodeled muscle. It was observed that the plant extract exhibited potent anti bacterial activity when screened for the two pathogenic bacterial species that infect the wound. The phytochemicals responsible for this may be the flavonoids.

Phenolic acids have been reported to posses anti inflammatory, analgesic, antioxidant and wound healing properties[17]. Following injury or injection of carrageenin,

it takes 2 h for prostaglandin to be released, a time period equivalent to the time lapse between the administrations of carrageenin and the extract. Thus the time lapse or dosing interval used in the paw-pressure method was adequate to permit the release of prostaglandin. It was observed that the aqueous extract of the plant had produced a potent anti inflammatory activity which had started increasing from the third hour of treatment[14]. However, prostaglandin could not be implicated in the mediation of pain in the hot-plate method since responses of mice to thermal stimulus occurred within seconds, a time too short to permit the release of prostaglandin. This suggests that chemical mediators other than prostaglandin may be involved in the mediation of pain. Mediators of inflammation are released sequentially with histamine and serotonin being the first to be released within the first 30 min, followed by kinins which are released about 1 h and prostaglandins, 2 h after induction of inflammation[14]. Because the analgesic property of the extract was also demonstrated in the hot-plate method, it could mean that the extract in addition to inhibiting prostaglandin may also interfere with the metabolism of other analgesic agents or block the receptor sites of these agents. The development of the paw edema in the rats after the injection of carrageenin has been described as a biphasic event, the first phase is due to release of histamine and serotonin (5-HT) (1 h), first plateau phase is maintained by kinin like substance (2 h) and second accelerating phase of swelling is attributed to prostaglandin release (3h)[18,19]. In the present research investigation the *in vitro* wound healing property was performed the results of which were found to be very promising[20]. We evaluated the other parameters like analgesic, anti inflammatory and anti bacterial activities as these are linked with wound. The results obtained were found to be satisfactory, as the extract was proved to be potent analgesic, anti inflammatory and anti bacterial agent. Since all the parameters linked to wound healing were proved, further studies in *in vivo* wound healing parameters are yet to be performed for final confirmation. Further research is envisaged on the identification, isolation and characterization of the phytochemical responsible for the potent pharmacological action.

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