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Research Article

Anti-Inflammatory, and Anti-Nociceptive Properties of Leaf and Root Extracts of *Amaranthus spinosus*

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Abstract

Inflammation is the body's protective retaliation to obnoxious stimuli, damage and infection. Despite eliciting repairing function, inflammation over prolonged periods of time can lead to the development of severe conditions such as cancer, and numerous cardiovascular, metabolic, neurological, and renal disorders. In this experiment, the ethanolic extracts of *Amaranthus spinosus* L. leaves and roots were evaluated for anti-inflammatory and antinociceptive potential using the acetic acid induced writhing, tail immersion, and hot plate test with mice. Results showed that the extracts of both leaves and roots had reduced inflammation and nociceptive pain. The 500 mg/kg doses of both plant parts showed the most significant results. Both plant part extracts were observed to elicit pain relief effects comparable to a diclofenac sodium, and morphine. The mechanism of this effect may be through the non-selective inhibition of cyclooxygenase-2 (COX-2) enzyme, and activation of μ opioid receptor. The 500mg/kg root extract showed highest anti-inflammatory and antinociceptive action.

Keywords: *Amaranthus spinosus; Inflammation; Analgesia; Pain; Antinociception; Anti-Inflammatory*

Abbreviations

ASP: *Amaranthus spinosus*; LE250 or LE500: Leaf Extract (250 mg/kg or 500 mg/kg) group, N.C: Negative Control Group; RE250 or RE500: Root Extract (250 mg/kg or 500 mg/kg) Group; COX-2: Cyclooxygenase-2; IACUC: Institutional Animal Care and Use Committee; DMSO: Dimethyl Sulfoxide; MPH: Morphine

Introduction

Inflammation and the associated pain is our body's protective response to noxious stimuli, injury, microbial infection, and trauma, helping in the recovery from offending factors and restoration of integrity [1,2]. The entire process of inflammation consists of several cellular events, namely inflammatory mediator release, cell migration, activation of several enzymes, tissue breakdown and repair, and fluid extravasation [3]. Endogenous chemicals known as prostaglandins play a major role in the mediation of acute inflammation as noted by the significant increase in their biosynthesis during this event [4]. Though inflammation acts as a protective mechanism to keep the body safe from harm and to initiate repair, prolonged inflammation can lead to the development of serious conditions like cancer, as the process involves the release of a wide variety of biomolecules in the system [5,6]. Therefore, agents that can block the pathway for inflammation and pain prove to be critical in the management of conditions associated with inflammation.

Amaranthus spinosus Linn. (Bengali name: Kantanotey; Family: Amaranthaceae) is an erect, glabrous, branched herbaceous weed found and cultivated all over Asia, America, Europe, Africa, and Australia. The plant has dense or interrupted spikes borne in the axils of the leaves, can grow up to 120 cm, and has rich protein, carbohydrate, dietary fiber and mineral content [7]. In certain parts of India, *Amaranthus spinosus* (ASP) is used to prevent stomach swelling, and the leaves have been known to treat both rheumatic and stomach pain, inflammation, burns, wounds, eczema, and gonorrhea [8]. It is also reported to be effective in the treatment of internal bleeding, and excessive menstruation, while a decoction of the root of ASP has been found to contain profound antipyretic properties [9]. Studies have also concluded the plant to possess antimalarial, hepatoprotective, antioxidant, antidiabetic, and antihyperlipidemic properties [10-12]. A study suggests that leaf extracts of ASP inhibits 73% of prostaglandin biosynthesis in inflammation [13].

Objective of the Study

The objective of the current study was to determine which part of the plant possessed more anti-inflammatory, analgesic, and antinociceptive properties, and how each part compared with standard drugs.

Materials and Methods

Plant materials

Suitable quantities of the whole plant were freshly collected from Shariatpur, Bangladesh. The plant samples were authenticated and identified taxonomically by the National Herbarium of Bangladesh. A voucher specimen (Accession No. 32781) of the collected plant was deposited at the herbarium for future references. The leaves and roots of the plant were separated, washed, and dried keeping away from direct sunlight, followed by crushing into smaller sizes. About 200g of crushed leaves and roots were kept in 1250 mL of ethanol, in separate conical flasks, and shaken via electric shaker (72 hours) for the extraction of active compounds. The mixture was filtered, and the filtrate was introduced to a rotary evaporator to obtain dry powder of the leaf and root extract. The residue was mixed with ethanol again for further extraction and the process was repeated again.

Chemicals and equipment

All chemicals used were of analytical grade, and both chemicals and equipment were provided by the Department of Pharmaceutical Sciences, North South University, Dhaka, Bangladesh.

Animals and experimental design

102 male Swiss Albino mice, 3 - 4 weeks of age and weighing between 20 - 25g, were collected from the Animal Research Branch, International Center for Diarrheal Disease and Research, Bangladesh (ICDDR, B). For the acetic acid induced writhing test, 42 animals were divided into 7 groups (6 animals in each) in the following order: negative control group (N.C), control group, diclofenac sodium-treated (10 mg/kg, IP) group, leaf extract-treated (250 or 500 mg/kg, po) groups (LE250 and LE500) and root extract-treated (250 or 500 mg/kg, po) groups (RE250 or RE500). Animals in both control groups were given 0.5% dimethyl sulfoxide (DMSO) in saline orally. All groups were given 0.7% v/v acetic acid injection (0.1 mg/10g) intraperitoneally, except N.C which received physiological saline (IP). For both the tail immersion, and hot plate test, animals were divided into 6 groups (5 animals in each) for each test in the order: control group, morphine-treated (5 mg/kg, po) group (MPH) and the plant extract groups as mentioned above. 0.5% DMSO was used in the feeding of the plant extracts to aid solubilization of the plant powder. All animals were kept under standard environmental conditions (temperature: 24.0 ± 1.0°C, relative humidity: 55 - 65% and 12hr light/12 hr dark cycle) and had free access to feed and water *ad libitum*. All animals were handled following the standard guidelines of the Animal Care and Use Policy by the Institutional Animal Care and Use Committee (IACUC), North South University [14].

Acetic acid induced writhing test

The analgesic activity of the samples was evaluated using acetic acid induced writhing test in mice [15]. In this method, acetic acid was administered intra-peritoneally to the experimental animals to create a sensation of pain. In the present study diclofenac sodium (10 mg/kg, IP) was used to serve the purpose of comparing the analgesic activity of the extract to that of a standard NSAID treatment. The plant extract and 0.5% DMSO in saline were administered orally 30 minutes prior to intraperitoneal administration of 0.7% v/v acetic acid solution (0.1 ml/10g) but diclofenac sodium was administered 15 minutes prior to acetic acid injection. The animals were allowed to rest for 5 minutes and then were placed on an observation table. Each animal was observed individually for the number of writhing they made in the next 15 minutes. Full writhing was not always accomplished by the animal, as sometimes the animals started to give writhing but did not complete it. Hence, this incomplete writhing was considered as half-writhing. Accordingly, two half-writhing were taken as one full writhing. The number of writhing in each treated group was compared to the control group while diclofenac sodium was used as a reference substance.

Tail immersion test

The tail immersion test was performed to evaluate the central mechanism of analgesic activity as reported by Aydin., *et al* [16]. Painful reaction produced by thermal stimuli through dipping the tail tips into hot water and its inhibition by agents proposes presence of antinociceptive activity. An area of the tail was marked and immersed in the water bath thermostatically maintained at 52.5 ± 0.2°C. The withdrawal time of the tail from hot water (in seconds) was noted as the reaction time. Antinociceptive activity of the extract under study would be confirmed if it increased tail withdrawing time.

Hot plate test

This test was performed according to the method previously reported by Lanhers., *et al.* and Adzu., *et al* [17,18]. A transparent glass cylinder (10 cm in height, and 20 cm in diameter) was used to keep the animal on the heated surface of a hot plate. The test was carried out at a fixed temperature of 55 ± 0.5°C. Response was defined as licking or biting of a paw or jumping (where all four paws leave the plate). The time in seconds between the start and reaction time was recorded as the response time. The mice exhibiting response time greater than 30s (seconds) or less than 5s were excluded. The response time was determined at 0, 30, 60, 90 and 120 minutes after initiation of treatment. A response time of 60s was defined as complete analgesia.

Statistical analysis

All data are presented as mean ± standard error of the means (SEM), analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 8 software (USA). A probability level (*p* value) lower than 0.05 was considered as statistically significant.

Results

Acetic acid induced writhing test

The ethanolic extracts of both leaves and roots of ASP showed remarkable inhibition of pain in animal groups (Table 1). All plant extract groups showed decreased number of writhing compared to the control. The LE500 and RE500 groups demonstrated highest percent of pain inhibition (51.24 and 61.57%) compared to control. This data suggests a dose dependent analgesic response of the extract; higher doses were seen to reduce pain to greater extents. Diclofenac sodium, a standard NSAID (Non-Steroidal Anti-Inflammatory Drug) taken for comparison, showed a percent of inhibition of 66.97%. The values of the negative control (N.C) was deducted from all experimental groups.

Group	Mean of Total Writhing	Mean ± SEM	Writhing (%)	Inhibition of Writhing (%)
Control	37.08	37.08 ± 7.172	100	-
Diclofenac sodium	12.25	12.25 ± 2.932	33.037	66.963**
LE250	21.58	21.58 ± 3.330	58.207	41.793
LE500	18.08	18.08 ± 4.269	48.768	51.232*
RE250	29.17	29.17 ± 1.726	78.659	21.341
RE500	14.25	14.25 ± 2.257	38.430	61.569**

Table 1: *Effect of leaf and root extracts of Amaranthus spinosus extract on acetic acid induced writhing.*
Values are presented as mean ± SEM, where n = 6.
**: Significantly different (p < 0.05) from control group using one-way ANOVA followed by Tukey's multiple comparison test.*
***: Significantly different (p < 0.01) from control group using one-way ANOVA followed by Tukey's multiple comparison test.*

Tail immersion test

All animals treated with plant extract showed increased response time to thermal stimuli (Table 2). Morphine (MPH), a standard antinociceptive drug, showed significant response in the inhibition of this type of pain at 30 and 60 minutes compared to control. Both LE500 and RE500 groups were shown to have significantly increased response time to stimuli at 60 minutes. No significance in effect was observed between the MPH and LE500 and RE500 groups.

Group	Response Time (in seconds) at Fixed Intervals				
	0 minutes	30 minutes	60 minutes	90 minutes	120 minutes
Control group	2.34 ± 0.135	2.66 ± 0.348	3.17 ± 0.359	2.84 ± 0.188	2.74 ± 0.193
Morphine	3.08 ± 0.382	5.33 ± 0.662**	6.03 ± 0.328***	4.10 ± 0.270	3.47 ± 0.348
LE250	2.67 ± 0.312	3.21 ± 0.374	3.60 ± 0.229	3.32 ± 0.463	2.99 ± 0.297
LE500	2.92 ± 0.254	4.15 ± 0.504	4.94 ± 0.323**	3.78 ± 0.395	3.30 ± 0.312
RE250	2.84 ± 0.375	3.20 ± 0.587	3.75 ± 0.352	3.34 ± 0.392	3.12 ± 0.158
RE500	2.94 ± 0.407	4.44 ± 0.376	4.77 ± 0.135**	4.01 ± 0.111	3.26 ± 0.163

Table 2: *Effect of leaf and root extracts of Amaranthus spinosus extract on tail immersion test.*
Values are presented as mean ± SEM, where n= 5.
**: Significantly different (p < 0.05) from control group using one-way ANOVA followed by Tukey's multiple comparison test.*
***: Significantly different (p < 0.01) from control group using one-way ANOVA followed by Tukey's multiple comparison test.*
****: Significantly different (p < 0.001) from control group using one-way ANOVA followed by Tukey's multiple comparison test.*

Hot plate test

ASP extract treated animals showed increased response latency to heat (Table 3). MPH significantly increased response time compared to control. The LE500 and RE500 groups exhibited most significant results compared to control at 60, 90 and 120 minutes. The RE500 group was seen to have greater significance of antinociceptive effect than LE500 at 120 minutes compared to control. No significance in effect was seen between MPH and the LE500 and RE500 groups.

Group	Response Time (in seconds) at Fixed Intervals				
	0 minutes	**30 minutes**	**60 minutes**	**90 minutes**	**120 minutes**
Control group	5.45 ± 0.407	5.44 ± 0.429	4.78 ± 0.571	4.64 ± 0.435	4.08 ± 0.292
Morphine	6.94 ± 0.462	11.57 ± 0.901	12.83 ± 2.100**	11.81 ± 0.603***	9.17 ± 0.677***
LE250	6.08 ± 1.617	7.95 ± 2.363	8.35 ± 0.459	6.73 ± 0.354	5.28 ± 0.400
LE500	7.19 ± 0.627	11.04 ± 0.685	12.21 ± 0.692**	9.09 ± 0.708***	7.76 ± 0.750**
RE250	6.52 ± 0.722	7.44 ± 1.686	7.85 ± 2.005	6.56 ± 0.516	5.32 ± 0.442
RE500	6.07 ± 1.555	10.68 ± 1.812	12.12 ± 1.317**	9.29 ± 0.920***	8.66 ± 0.354***

Table 3: *Effect of leaf and root extracts of Amaranthus spinosus extract on hot plate test.*
Values are presented as mean ± SEM, where n= 5.
**: Significantly different (p < 0.05) from control group using one-way ANOVA followed by Tukey's multiple comparison test*
***: Significantly different (p < 0.01) from control group using one-way ANOVA followed by Tukey's multiple comparison test*
****: Significantly different (p < 0.001) from control group using one-way ANOVA followed by Tukey's multiple comparison test*

Discussion

Inflammation is our body's protective retaliation against obnoxious stimuli such as injury and infection, which are carried out by the release of a large number of biomolecules [19]. Sustained inflammation has been consequently labeled as a cause behind the development of several cardiovascular, neurological, intestinal, and renal disorders, even cancer [5,20-24]. The present study aimed to answer which part of ASP, the leaves or the roots, exhibited more potent anti-inflammatory and antinociceptive action.

Prostaglandins are key mediators of inflammation and pain in injury, and in the acetic acid writhing test, the writhing in mice is a result of increased biosynthesis of prostaglandins and other endogenous biomolecules [25,26]. Diclofenac sodium, a standard NSAID taken as reference, reduced the number of writhing in mice by non-selective inhibition of the cyclooxygenase-2 (COX-2) enzyme that converts arachidonic acid to prostaglandins in inflammation [27-29]. The extracts of both leaves and roots of ASP were seen to reduce the number of writhing in the animals. The LE500 and RE500 groups (500 mg/kg leaf or root extract) showed the most significant results (Table 1), which are comparable to the anti-inflammatory effect of diclofenac sodium (% of inhibition = 66.96%). Hence, it can be concluded that ASP extracts inhibit inflammation in a similar fashion to the NSAIDs, by the inhibition of COX-2 enzyme. It was also observed that the RE500 group exhibited the highest percentage of inhibition (61.569%) than any other part of the plant extract group. This suggests that the higher anti-inflammatory potential of the roots of ASP.

The tail immersion and hot plate tests are reliable methods used to assess antinociceptive efficacy of compounds inhibiting centrally acting pain [17,30]. Morphine, a standard opioid analgesic, caused significant increase in reflex time (or response time to stimuli) by acting on the μ opioid receptor [31]. All plant extract groups showed increased response times to thermal stimuli; the LE500 and RE500 groups, once again, showed the most significant result. In both tests, both LE500 and RE500 groups exerted similar extent of analgesia and antinociception. In the hot plate test, the RE500 group was observed to have a more significant effect than LE500 group at 120 minutes, compared to the control. Results from the above tests suggest that the active compounds present in the leaves and roots of ASP may exert analgesic effect in a similar fashion to morphine, by acting on the μ opioid receptors on spinal and supraspinal levels.

The exact mechanism through which ASP mediates its anti-inflammatory and antinociceptive effects is still under study. Despite not having a defined mechanism of action, it is postulated by numerous studies that these effects are mediated by non-selective inhibition of COX-2 enzyme and the activation of μ opioid receptor [32-34]. It is noteworthy to mention that ASP's analgesic properties may be attrib-

uted to its rich content of flavonoids, phenolics, and antioxidants, as these categories of compounds are widely known to exert protective effects in our body [35-37]. In our study it was seen that the RE500 group (500 mg/kg root extract) showed the most significant results in the inhibition of pain. This suggests that either the roots of the plant may contain anti-inflammatory, or analgesic compounds absent in the leaves, or the same biomolecules may be present at a higher concentration in the roots.

Conclusion

Our study demonstrates the anti-inflammatory, analgesic, and antinociceptive properties of *Amaranthus spinosus* (ASP) and that the roots of the plant are more potent inhibitors of inflammation than the leaves. The mechanism by which ASP elicit these effects may involve the inhibition of cyclooxygenase-2 (COX-2) enzymes, scavenging of free radicals that participate in inflammation, and the activation of μ opioid receptor. ASP may exert these effects due to the presence of numerous flavonoids, phenolics, and antioxidants. Roots of ASP may be more potent than the leaves in the suppression of inflammation and nociceptive pain. ASP maybe used as an anti-inflammatory agent, however, more studies aimed towards understanding its molecular mechanism of action are required.

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Conflict of Interest

The author declares that there is no conflict of interest associated with this paper and also that there was no funding provided that could have significantly influenced its outcome.

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