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Antinociceptive activity shown by *Aerva javanica* flowering top extract and its mechanistic evaluation

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ABSTRACT: A grey perennial woolly tomentose shrub of *Aerva javanica* Burm. f. Juss. ex Schult. (Amaranthaceae), based on its folklore and traditionally usage, is needed to investigate for pharmacological potential. Therefore, as investigation of anti-nociceptive potential of the same was carried out and possible mechanism responsible for suppression of nociception was explored. Extract with hydro-alcoholic solvent (AJCE) and its ethyl acetate fraction (AJEAF) were prepared from flowering tops of *A. javanica*. Formalin induced edema model was used to explore involvement of opioid receptors with agonist morphine and antagonist naloxone. Bradykinin induced nociception was used to investigate involvement of Kinin-B_2 receptors with standard aspirin. Xylene-induced ear edema helped in evaluation of involvement of phospholipase-A_2 receptor using prednisolone as standard. Involvement of ATP-sensitive potassium channels was evaluated in formalin induced ear edema model with glibenclamide as antagonist. Involvement of peripheral receptors was studied in extract-induced antinociception using acetic acid induced writhing as peripheral pain model with receptor blockers and agonists- caffeine, atropine, haloperidol, pindolol, yohimbine, prazosin, phenylephrine or clonidine.

Findings of mechanistic investigation showed that analgesic responses involve interaction with opioid receptors ($p<0.001$) in neurogenic (acute) phase while in inflammatory (chronic) phase, no significant interaction with receptor is involved. Involvement of peripheral mechanism was also resulted as indicated by bradykinin inhibition ($p<0.001$). Antiphlogistic mechanism involves inhibition of phospholipase-A_2. Anti-inflammatory mechanism involves interaction of extract with β-adrenergic, cholinergic receptors ($p<0.05$), dopaminergic ($p<0.05$) and adenosinergic ($p<0.05$) receptors. Involvement of α-adrenoceptors and ATP-sensitive potassium channels was ruled out completely. Further, there is still a need to work on more specific fractions and receptors to explore their involvement in anti-nociceptive mechanism. © 2020 iGlobal Research and Publishing Foundation. All rights reserved.

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INTRODUCTION

Folklore usage of herbs in various ailments motivates research of traditional drugs in modern system. Indigenous medical system is much more explored to develop drugs from plants.(1) Traditional use of *Aerva javanica* flower tops in is the basis of present study. Chopra (1956) reported its traditional use as demulcent, diuretic, anthelmintic and also in headache. Swellings were reported to be removed by administration of plant decoction. Thereby, it may be suggested that plant is having antioxidant potential which is responsible for its traditional activity.(2,3)

A. javanica Linn. (Amaranthaceae) also known as '*Patharphori',* is a grey colored woolly, perennial, suffruticose, hoary-tomentose, erect to scandent dioecious conspicuous under shrub, 0.6- 1 m tall. It belongs to family Amaranthaceae. It is native to the region from North Africa to South West Asia (Willis1966; Gupta 1992). It is found almost throughout plains of India.

It has erect branching which is pale stiff and height is up to 1.6 m. Leaves are pale green, alternate, oblong ovate, lanceolate. They have grayish upper surface with matted covering hairs, raceme inflorescence with white sessile woody dense pikes. Flowers are very small and creamy-white in color and appear in January to May. Male flowers are smaller than female. Fruits are globular and immersed in silky white fleece with single black small seed.(4,5) The present study was aimed to evaluate establish anti-nociceptive potential of *A. javanica* and to explore possible mechanism involved behind it.

MATERIAL AND METHODS

Aspirin, Morphine sulfate, diclofenac, glibenclamide, naloxone, haloperidol, pindolol, yohimbine, prazosin, phenylephrine, clonidine (Oasis labs, Jaipur), formalin, carrageenan, xylene, glacial acetic acid, caffeine, atropine, bradykinin (Sigma Aldrich), sodium carboxy methyl cellulose (Na-CMC) (SD fine-chem, Mumbai), prednisolone, surgicals (SMS Hospital Pharmacy, Jaipur).

Collection of plant material and preparation of extracts

A. javanica flowering tops were collected from forests of Jhalana in periphery of Jaipur, Rajasthan and authentication was done at "Department of Botany, University of Rajasthan, Jaipur" vide voucher specimen no. RUBL211644 and authentication certificate *no.Bot/2017/5424*.

Air dried coarsely powdered plant materials were extracted by maceration using hydro-alcoholic (50:50) solvent for seven days with intermittent shaking. To prevent microbial growth 5 ml of chloroform was added to each vessel. The solvent was removed by vacuum evaporation to obtain syrupy consistency of extract (AJCE). Ethyl acetate fraction (AJEAF) was prepared by solvent fractionation. (6–8)

Laboratory animals

Male wistar rats (180-220 gm) were kept in well ventilated animal house maintained at standard environmental conditions (temperature 25±2^{0}C relative humidity: 55-65% and 12 h light/dark cycle) at Department of Pharmacy, Banasthali University, Rajasthan. The present protocol was approved by *Institutional Animal Ethical Committee (IAEC)* of Banasthali University, Rajasthan, India wide approval no. *BV/3632/2017-2018*. All the animal handling, maintenance and procedures were carried out in accordance to CPCSEA guidelines. (9–11)

Toxicity studies and dose determination

Acute toxicity of extract was determined in overnight fasted wistar rats (n=6 per group), in accordance to OECD-423 guidelines with graded doses (50 -5000 mg/kg, *p.o.*) of extracts. After administration, rats were kept for observations for any toxic symptoms and behavioral changes like hyperactivity, sedation, writhing, diarrheal, restlessness, piloerection etc.) for 2 h for acute toxicity and 14 days for signs of delayed toxicity(9,10).Divisions of 6 animals per group were prepared. A dosing of 1/10 of maximum tolerated dose (MTD) was selected for complete extract (AJCE) and 1/20 MTD dose was selected for fraction (AJEAF).

Evaluation of mechanism

Formalin induced edema and involvement of opioid receptors

Overnight fasted healthy wistar rats were divided into groups of six in each.0.5% w/v sodium CMC (control), extract- AJCE (500 mg/kg) and selected fraction- AJEAF (250mg/kg) in the doses mentioned in experimental protocol *p.o.*, 5 mg/kg *s.c.* morphine (standard), 5 mg/kg *s.c.* naloxone (receptor blocker) or 5 mg/kg of morphine and naloxone (both *s.c.*) were given to animals in respective group. After sixty min, injection of 50 µl of 5% v/v formalin was done into the dorsal surface of the right hind paw of each rat. Time spent in licking or biting of injected paw (defined as the nociceptive response) was observed for the early phase response (periods of 0-5 min post formalin injection) and late phase response (period of 15-40 min post formalin injection). Inhibition of licking (%) time was calculated. Early phase responses indicates inhibition neurogenic phase while late phase responses indicates inhibition inflammatory phase.(12–14)

Role of opioid system- Naloxone, an opioid antagonist, was employed to investigate the role of opioid system in counter-nociceptive response of extracts as assessed by formalin test. Naloxone was administered 50 min after extract/morphine administration or 10 min. prior to formalin injection. The antagonist dose was decided to be effective in a manner to block the *in vivo* effects induced by particular receptor agonist in rats.(15,16)

Bradykinin-induced nociception (involvement of kinin B_2 receptors)

Antinociceptive effect of extract on Bradykinin induced nociception was studied in accordance to the method earlier described by Ferreira *et al*. 10 ml / kg of control (0.5%w/v CMC), Aspirin (100mg/kg) and extracts were administered to animals. After 60 min, 50 µl of Bradykinin (10 nmol conc.) was injected into the plantar ventral surface of the right hind paw. Nociception was induced in rats observed as licking and biting response. Time spent in licking or biting of injected paw

(defined as the nociceptive response) was observed for 10 min. Inhibition of licking (%) time was calculated.(17,18)

Xylene-induced ear edema (involvement of phospholipase A_2 receptor)

Xylene induced ear edema model was studied to report any possible involvement of phospholipase A_2 receptor. 10 ml / kg of control (0.5%w/v CMC), Aspirin (100mg/kg) and extracts were administered *p.o.*to animals as per protocol. After 30 minutes, xylene (30 µl) was instilled to the inner surface of the right ear. Then after 15 minutes, rats were euthanasia was done under ether anesthesia. Both ears were separated off but cutting precisely from the killed rats, sized, and weighed. Difference in weight was observed between right and left ear and in order to determine the effect of extracts on nociception, % inhibition was calculated.(19–21)

Formalin induced paw edema

To investigate the possible involvement of potassium channels (ATP- sensitive) in the anti-nociceptive potential of extracts, rats were pretreated with glibenclamide (10 mg/kg, *i.p.*). The antagonist dose was decided to be effective in a manner to block the in vivo effects induced by particular receptor agonist in rats. Glibenclamide was used as a channel inhibitor. After 15 min, animals were treated with AJCE extract (500 mg/kg, *p.o.*). After 1 hour of extract administration, injected 1% v/v formalin (20 µl in saline) into the right hind paw. Nociception was induced in rats observed as licking and biting response. Time spent in licking or biting of injected paw (defined as the nociceptive response) was observed for 0-5 min and for 15–30 min respectively as early phase and late phase responses. Inhibition of licking (%) time was calculated. Early phase responses indicates inhibition neurogenic phase while late phase responses indicates inhibition inflammatory phase.(15,16,21–23)

Acetic acid induced writhing: peripheral pain model

Possible involvement of some receptor system was elucidated by using their respective antagonists in different rats before extract/standard. Acetic acid (0.6%v/v) was be used to induce abdominal writhing as described by Sani *et al.* After 5 min of acetic acid injection, number of writhes was recorded for 25 min, comparing with control and standard.

Method of De Souza et al (2009) was followed to select the doses of drugs administered and to elucidate the possible involvement of receptor systems followed by pain (abdominal writhing) induction using acetic acid.(24,25)

Rats were divided into groups of animals and pre-treated with selected receptor agonist or antagonist such as prazosin (0.15 mg/kg, *i.p.*; α_1-adrenergic antagonist), yohimbine (0.15 mg/kg, *i.p.*; α_2-adrenergic antagonist), phenylephrine (10 mg/kg, *i.p.*; α_1-adrenergic receptor agonist), clonidine (0.15 mg/kg, *i.p.*; central α_2-adrenergic agonist), pindolol (1 mg/kg. *i.p.*; β-adrenergic antagonist), atropine (10 mg/kg, *i.p.*; cholinergic receptor antagonist), caffeine (3 mg/kg, *i.p.*; Adenosine A_1, A_{2A}, A_{2B}, A_3 blocker), haloperidol (20 mg/kg, *i.p.*; Dopamine receptor antagonist), or 15 minutes before the administration of extract fraction (AJCE- 500 mg/kg, *p.o.*).

Statistical analysis

Results obtained as mean± SEM were analyzed using one-way or two way (wherever required) analysis of variance (ANOVA) method followed by *post hoc Tukey's* multiple-comparison test. Results were considered significant at various levels of significance, as little significant at $p<0.05$, significant at $p<0.01$ and highly significant at $p<0.001$.

RESULTS AND DISCUSSION

Toxicity studies revealed that there was no evidence of observable reactions upto 5000 mg/kg (MTD). Dose selected for further investigation was limited to 500 mg/kg (1/10 of MTD) for complete extract (AJCE) and 250 mg/kg dosing (1/20 of MTD) for fraction (AJEAF). Dose of control and standard treatments was defined as per activity. To evaluate the mechanism involved in activity of various extracts, animals were treated with selective receptor blocker and further the effect of drug was studied with and without receptor blocker in different groups.

To evaluate the possible involvement of opioid receptors in anti-nociceptive effect of extracts, formalin induced ear edema model was used with naloxone as non-selective blocker of opioid receptor. In acute phase nociception, very significant difference ($p<0.001$) was observed between effect of morphine and extract fractions. AJCE didn't showed any prominent action while AJEAF showed upto 35% inhibition of edema which was very significantly ($p<0.001$) reversed by naloxone treatment indicating that the action may be mediated through opioid mechanism in acute phase (**Table 1, Figure 1**). In chronic phase studies neither AJCE, not AJEAF reversed the naloxone inhibition, which suggests that there is no involvement of opioid receptor in chronic phase or it may be nullified with some other component of extract. Early phase responses indicates inhibition neurogenic phase while late phase responses indicates inhibition inflammatory phase. Overall, partial early phase correction indicated anti-nociceptive action through opioid receptors in neurogenic phase. Finally, involvement of subtype of opioid receptor needed to be evaluated by further investigation.

Bradykinin acts through G-protein coupled receptor, on dorsal root ganglion (DRG) sensory neurons showing heavy increase in Ca^{2+}.(26,27) This leads to release and synthesis of other second messengers, including prostaglandins, nitric oxide and neurokinins peripherally.(26,28,29)

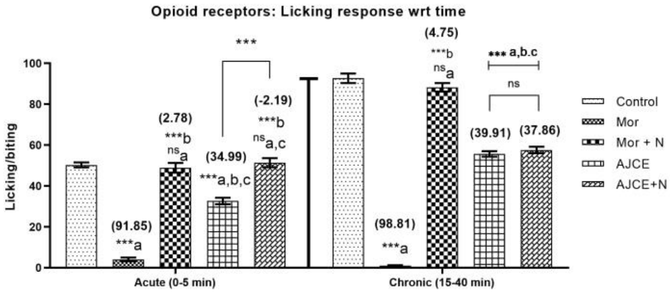

Figure 1. Inhibition of licking responses by extract in formalin induced edema and involvement of opioid receptor. Analyzed statistically using 2-way ANOVA followed by *post hoc Tukey's* multi-comparisons test. Results were compared with control (a), standard- morphine (b) morphine + naloxone (c) with all groups and extracts both time ranges. Significant level represented as * (p<0.05), ** (p<0.01), *** (p<0.001). ns- no significant difference; Mor- morphine, N- naloxone, AJ- *Aerva javanica* extracts suffixed with CE-complete extract. Percentage inhibition (%) is denoted over each group bar in bracket.

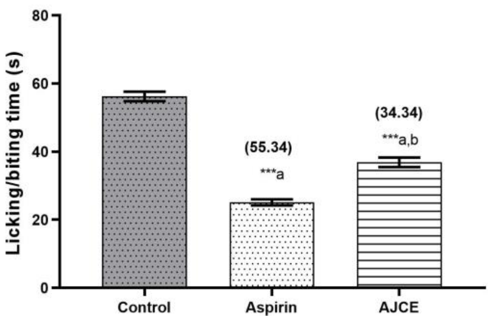

Figure 2. Effect of extract on bradykinin induced nociception. Analyzed statistically using one-way ANOVA followed by *post hoc Tukey's* multi-comparisons test. Results were compared with control (a), and standard- aspirin with all groups. Significant level represented as * (p<0.05), ** (p<0.01), *** (p<0.001). ns- no significant difference. Inhibition (%) is shown in bold and bracket above the respective column.

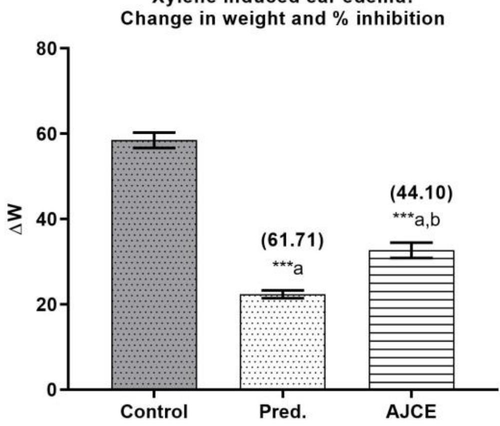

Figure 3. Effect of extract on xylene induced ear edema. Analyzed statistically using one-way ANOVA followed by *post hoc Tukey's* multi-comparisons test. Results are compared with control (a), and standard- prednisolone (pred) with all groups. AJ- *Aerva javanica* extracts suffixed with CE-complete extract. Significant level represented as * (p<0.05), ** (p<0.01), *** (p<0.001). ns- no significant difference. Inhibition (%) is shown in bold and bracket above the respective column.

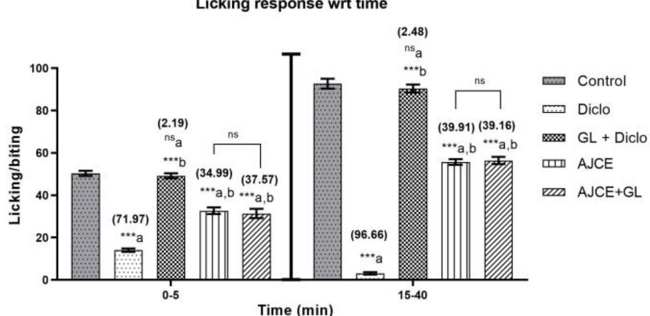

Figure 4. Study of involvement of ATP-sensitive potassium channels in formalin induced edema.
Analyzed statistically using 2-way ANOVA followed by *post hoc Tukey's* multi-comparisons test. Results are compared with control (a), and standard- diclofenac (b) with all groups and extracts in between (with and without GL) separately at both time ranges. Significant level represented as * (p<0.05), ** (p<0.01), *** (p<0.001). ns- no significant difference; GL- Glibenclamide, AJ- Aerva javanica extracts suffixed with CE-complete extract. Percentage inhibition (%) is denoted over each group bar in bracket.

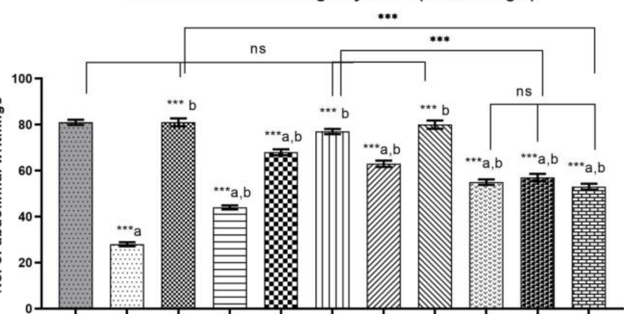

Figure 5. Effect of extracts on abdominal writhes- Involvement of adrenergic system (α-adrenergic).
Analyzed statistically using One way ANOVA followed by *post hoc Tukey's* multi-comparisons test. Results are compared with control (a), and standard-diclofenac(b), with all groups and extracts in between (with and without blocker-YO / PR). Significance level represented as * (p<0.05), ** (p<0.01), *** (p<0.001), ns- no significant difference; PR- Prazosin, PH- Phenylephrine, YO-Yohimbine, CL-Clonidine, AJCE- *Aerva javanica* complete extract.

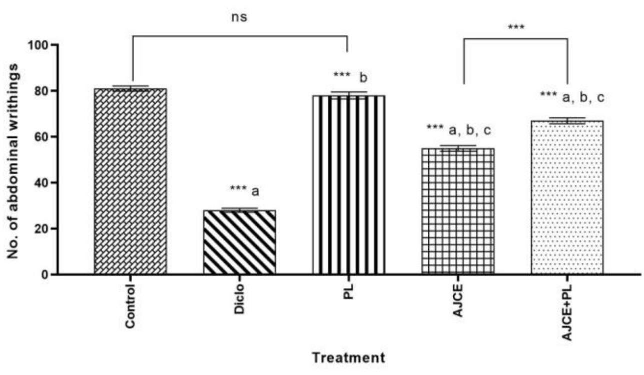

Figure 6. Effect of extracts on abdominal writhes- Involvement of adrenergic system (β-adrenergic).

Analyzed statistically using One way ANOVA followed by *post hoc Tukey's* multicomparisons test. Results were compared with control (a), standard-diclofenac (b) and blocker- pindolol (c) with all groups and extracts in between (with and without blocker-pindolol). Significance level represented as * (p<0.05), ** (p<0.01), *** (p<0.001), ns- no significant difference; PL- Pindolol, AJCE- *Aerva javanica* complete extract.

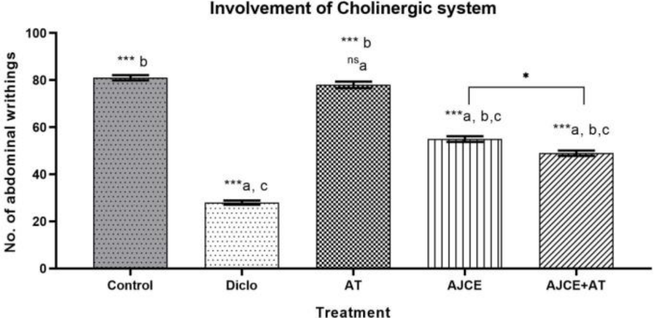

Figure 7. Effect of extracts on abdominal writhes- Involvement of cholinergic system.

Analyzed statistically using One way ANOVA followed by *post hoc Tukey's* multicomparisons test. Results were compared with control (a), standard-diclofenac (b) and antagonist- AT (c), with all groups and extracts in between (with and without blocker-AT). Significance level represented as * (p<0.05), ** (p<0.01), *** (p<0.001). ns- no significant difference; AT- Atropine, AJ- *Aerva javanica* extracts suffixed with CE-complete extract

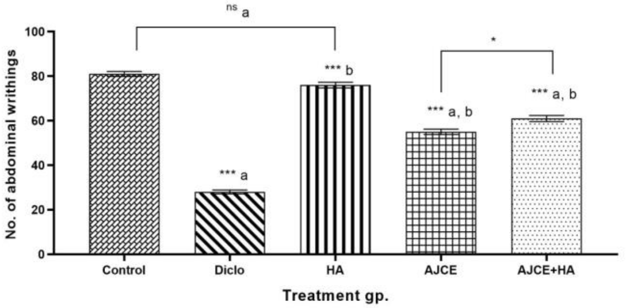

Figure 8. Effect of extracts on abdominal writhes- Involvement of dopaminergic system.

Analyzed statistically using One way ANOVA followed by *post hoc Tukey's* multicomparisons test. Results were compared with control (a), and standard- diclofenac (b) with all groups and extracts in between (with and without haloperidol). Significance level represented as * (p<0.05), ** (p<0.01), *** (p<0.001), ns- no significant difference; HA- Haloperidol, AJCE- *Aerva javanica* complete extract.

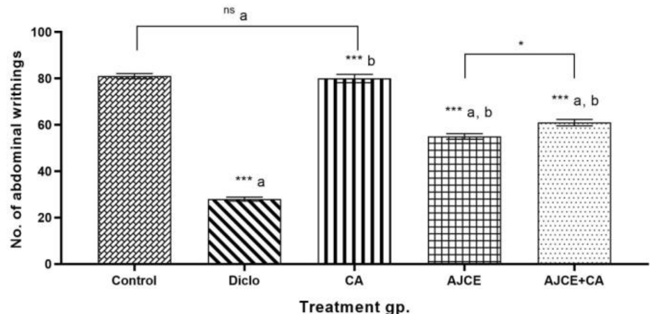

Figure 9. Effect of extracts on abdominal writhes- Involvement of adenosinergic system.

Analyzed statistically using One way ANOVA followed by *post hoc Tukey's* multicomparisons test. Results were compared with control (a), and standard- diclofenac (b) with all groups and extracts in between (with and without caffiene). significance level represented as * (p<0.05), ** (p<0.01), *** (p<0.001). ns- no significant difference; CA- caffeine, AJCE- *Aerva javanica* complete extract.

Pain induced through the introduction of bradykinin into the right hind paw of the experimental rat was very significantly (*p<0.001*) inhibited by oral administration of 500 mg/kg AJCE with about 34.34% inhibition. Protein kinase C (PKC) sensitize the silent NMDA-glutamate receptors centrally at postsynaptic neuron (30,31). Bradykinin directly activates PKC by kinin B_2 receptor binding(17) and produces noxious or even innocuous stimuli. Our results supported the same by demonstrating the ability of AJCE (500 mg/kg) to suppress the nociception {though significant difference (*p<0.001*) with standard- aspirin} caused by bradykinin.(32,33)(**Table 2, Figure 2**)

Xylene-induced ear edema method was used to assess the effect of extracts on acute edematous inflammation. Method is useful to evaluate effect of steroidal or non-steroidal agents of antiphlogistic nature mechanised through inhibition of phospholipase A_2(34). Accumulation of fluid leading to acute edema is resulted due to intense irritation caused by application of xylene (neurogenic inflammatory response). Drugs that suppress this response are likely to act as anti-inflammatory agent. Results indicated an increase in weight of right ear to about 58.5 mg in control. A significant (*P<0.001*) reduction in ear weight was observed due to AJCE and prednisolone treatment by 32.7 and22.4 mg respectively and inhibition was reported to be 44.10% and 61.71% respectively (**Table 3, Figure 3**). Although, activity of extract was very significant (p<0.001) when compared to control, it did not significantly reduce the inflammatory response as compared to standard Prednisolone. Steroidal inflammation suppressant drugs found to be more active than NSAIDs against edema induced by xylene, hence prednisolone was used as standard.(35) Finally, by xylene induced ear edema model, this may be predicted that extracts in study are anti-inflammatory in nature and may involve some components that are antiphlogistic. Their mechanism may involve inhibition of phospholipase A_2.

In order to study the possible involvement of ATP-sensitive potassium channels in anti-nociceptive effect of extract in formalin induced edema, channel blocker- Glibenclamide (GL) was used. In both acute and chronic phase studies, extract showed very significant (p<0.001) improvement in licking duration in comparison to control, indicating the anti-nociceptive action of extract but effect was not comparable with standard-diclofenac.(**Table 4, Figure 4**) No significant change in antinociceptive potential was reported in both phases, which clearly over-ruled the involvement of ATP-sensitive potassium channels in anti-nociceptive effect of extract in both acute and chronic phases.

Table 1. Study of involvement of opioid receptors in formalin induced edema.

Treatment & dose	Acute phase (0-5 min)		Chronic phase (15-40 min)	
	Duration of paw licking (sec.)	Inhibition %	Duration of paw licking (sec.)	Inhibition %
Control (1 ml / 100 g body weight	50.3 ± 1.2	--	92.7 ± 2.3	--
Morphine* (5 mg / kg body	4.1 ± 0.9	91.85	1.1 ± 0.2	98.81
Morphine + Naloxone (5 mg / kg b.w. of each *s.c.*)	48.9 ± 2.4	2.78	88.3 ± 2.1	4.75
AJCE (500 mg / kg b.w. *p.o.*)	32.7 ± 1.6	34.99	55.7 ± 1.3	39.91
AJCE + Naloxone (500 mg / kg b.w. *p.o.*& 5 mg / kg b.w. *s.c.*)	51.4 ± 2.2	-2.19	57.6 ± 1.6	37.86
AJEAF (250 mg / kg b.w. *p.o.*)	22.6 ± 1.7	55.07	42.5 ± 1.4	54.15
AJEAF + Naloxone (250 mg / kg b.w. *p.o.*& 5 mg / kg b.w. *s.c.*)	49.2 ± 2.4	2.19	40.1 ± 0.9	56.74

*used as standard treatment. Only higher doses of extracts were taken based on earlier results. Naloxone was used as opioid receptor blocker as per experimental protocol. AJCE- *Aerva javanica* complete extract and AJEAF- ethyl acetate fraction of the same. All the values are mean ± SEM of 6 rats. Statistically analyzed by two way ANOVA followed by *post hoc Tukey's* multiple comparison test atP<0.001, P<0.01, P<0.05 using graphpad PRISM ver 8.01

Table 2. Effect of extract on bradykinin induced nociception.

Treatment & dose	Licking time (sec)	% inhibition
Control (1ml/100g body weight *p.o.*)	56.2 ± 1.4	--
Aspirin* (100mg/kg body weight	25.1 ± 0.9	55.34
AJCE (500mg/kg body weight *p.o.*)	36.9 ± 1.4	34.34

*used as standard treatment. Control- 0.5%w/v sodium CMC. AJCE- *Aerva javanica* complete extract. All the values are mean ± SEM of 6 rats. Statistically analyzed by repeated measures ANOVA followed by Tukey's Multi-comparison test atP<0.001, P<0.01, P<0.05 using graphpad PRISM ver. 8.01

Table 3. Effect of extract on xylene induced ear edema

Treatment & Dose	Change in weight (mg)	% inhibition
Control (1ml/100g body weight *p.o.*)	58.5 ± 1.8	--
Prednisolone* (20mg/kg body weight	22.4 ± 0.9	61.71
AJCE (500mg/kg body weight *p.o.*)	32.7 ± 1.8	44.10

*used as standard treatment. Control- 0.5%w/v sodium CMC. All values are represented as mean ± SEM of 6 rats. AJCE- *Aerva javanica* complete extract. Statistically analyzed by repeated measures ANOVA followed by *post hoc Tukey's* multi-comparison test at P<0.001, P<0.01, P<0.05 using graphpad PRISM ver. 8.01

Table 4. Study of involvement of ATP-sensitive potassium channels in formalin induced edema

Treatment & dose	Acute phase (0-5 min)		Chronic phase (15-40 min)	
	Duration of paw	Inhibition	Duration of paw	Inhibition
Positive control (0.5% w/v Na-CMC 10ml/kg b.w. *p.o.*)	50.3±1.2	--	92.7±2.3	--
Diclofenac* (20 mg / kg body weight *p.o.*)	14.1±0.8	71.97	3.1±0.6	96.66
Diclofenac + Glibenclamide (20 &10 mg / kg body weight	49.2±1.2	2.19	90.4±1.9	2.48
AJCE (500 mg/kg b.w.)	32.7±1.6	34.99	55.7±1.3	39.91
AJCE + Glibenclamide	31.4±2.2	37.57	56.4±1.7	39.16

*used as standard treatment. Only higher doses of extracts were taken based on earlier results. Glibenclamide was used as ATP sensitive potassium channel blocker as per experimental protocol. All the values are mean ± SEM of 6 rats. AJCE- *Aerva javanica* complete extract. Statistically analyzed by repeated measures ANOVA followed by *post hoc Tukey's* multiple comparison test at$P<0.001$, $P<0.01$, $P<0.05$ using graphpad PRISM ver 8.01

Table 5. Effect on abdominal writhes- Involvement of various receptor systems.

Treatment	No. of writhes /	% inhibition
Control (1 ml / 100g body weight *p.o.*)	81 ± 1.1	--
Diclofenac* (20 mg / kg body weight *p.o.*)	28 ± 0.9	65.43
Caffiene (CA) (3 mg / kg body weight *i.p.*)	80 ± 1.8	1.23
Atropine (AT) (10 mg / kg body weight *i.p.*)	78 ± 1.4	3.70
Haloperidol (HA) (20 mg / kg body weight *i.p.*)	76 ± 1.3	6.17
Prazocine (PR) (0.15 mg / kg body weight *i.p.*)	81 ± 1.7	0.00
Phenylephrine (PH) (10 mg / kg body weight *i.p.*)	44 ± 0.9	45.68
PH+PR (10 mg / kg and 0.15 mg/kg body weight *i.p.)*	68 ± 1.3	16.05
Yohimbine (YO) (0.15 mg / kg body weight *i.p.*)	77 ± 1.1	4.94
Clonidine (CL) (0.15 mg / kg body weight *i.p.*)	63 ± 1.4	22.22
YO + CL (0.15 mg / kg body weight *i.p. of both*)	80 ± 1.8	1.23
Pindolol (1 mg / kg body weight *i.p.*)	78 ± 1.5	3.70
AJCE (500 mg /kg body weight *p.o.*)	55 ± 1.2	32.10
AJCE + CA (500 mg / kg b.w. *p.o.*& 3 mg / kg b.w. *i.p.*)	61 ± 1.4	24.69
AJCE + AT (500 mg / kg b.w. *p.o.*& 10 mg / kg b.w. *i.p.*)	49 ± 1.1	39.51
AJCE + HA (500 mg / kg b.w. *p.o.*& 20 mg / kg b.w. *i.p.*)	61 ± 1.4	24.69
AJCE + PL (500 mg / kg b.w. *p.o.*&1 mg / kg b.w. *i.p.*)	67 ± 1.3	17.28
AJCE + YO (500 mg /kg b.w. *p.o.*&0.15 mg / kg b.w. *i.p.*)	57 ± 1.6	29.63
AJCE + PR (500mg / kg b.w. *p.o.*&0.15 mg / kg b.w. *i.p.*)	53 ± 1.3	34.57

Control- 0.5%w/v sodium CMC, * standard treatment. Results were compared with control, standard-diclofenac and respective receptor blocker, with all groups and extracts in between (with and without blocker). AJCE- *Aerva javanica* complete extract. PR- prazosin, PH-phenylephrine, YO-yohimbine, CL-clonidine, HA- haloperidol, AT- atropine, PL- pindolol, CA- caffeine. Statistically analyzed by repeated measures ANOVA followed by Tukey's multi-comparisons test at $P<0.001$, $P<0.01$, $P<0.05$ using graphpad PRISM ver 8.01

Probable involvement of adrenergic, cholinergic, adenosinergic and dopaminergic receptors, on extract-induced antinociception studied on acetic acid induced nociceptive (abdominal writhes) rats, was investigated using their respective receptor antagonist. (**Table 5, Figure 5-9**)

On blocking the adrenergic receptors with yohimbine and prazosin, no significant change in anti-nociceptive action of drug was observed after adrenergic receptor blockage which counters involvement of adrenergic system. To study the possible involvement of adrenergic system, various adrenergic blockers (yohimbine, prazosin and pindolol) with or without agonist (phenylephrine, clonidine) and AJCE was used in various groups. AJCE showed very significant ($p<0.001$) reduction in abdominal writhing as compared to control group. But effect was not comparable with standard diclofenac. There found no significant blockage of anti-nociceptive response of extract when treated with yohimbine (α_2) and prazosin (α_1), the receptor blockers of α-adrenoceptor. Significant difference ($p<0.001$) in depression of writhes were seen when extract group was compared with extract and pindolol (a non-selective β- blocker) group. This over-ruled the assumption of involvement of any subtype of α-adrenoceptor receptor but supports the probable involvement of β-adrenoceptor(36). Selectivity in β-adrenoceptor involvement needs to be investigated.

Possible involvement of cholinergic system was studied by using atropine (AT) - a nonselective cholinergic antagonist. Very little significant ($p<0.05$) increase in writhing were observed with atropinization (blockage by atropine) which suggest that there may be few compounds present in extracts, but in very less quantity, that might be showing partial cholinergic potential. Inhibition of writhing was seen even after blockage which suggested that there might be only partial involvement of cholinergic receptors and further some compounds may be showing adrenergic and cholinergic

involvement. This may be due to the presence of broad range of components in crude extract.

Possible involvement of dopaminergic system was studies by using haloperidol (HA) as receptor antagonist. (Table 5, Figure 8) When HA was used alone, no effect (no significant difference) was seen on HA treated group when compared with control. There was little significant difference ($p<0.05$) found in response of AJCE and AJCE+HA group. This indicates that action of AJCE may be blocked with HA which indicates the possible dopaminergic involvement in response of AJCE against abdominal writhes.

To study the possible involvement of adenosinergic receptor system in extract-induced antinociception, Caffeine, a non-selective adenosinergic receptor antagonist, was used. It blocked the anti-nociceptive action of *AJCE* with little significance ($p<0.05$). Results indicate possible involvement of adenosinergic receptors in mechanism of *A. javanica* extract. Pharmacologically, caffeine acts by low affinity blocking of adenosine A_1, A_{2A}, A_{2B} and A_3 receptors(37,38). Out of these in particular A_1 is important in producing antinociception, with reduction in PGE2 (39,40) and triggering of NO/cGMP/PKG/K related acute pain pathway, and increasing pain threshold (41) as well as inhibiting release of glutamate (42) responsible in chronic pain.

CONCLUSION

In conclusion, presented study evolved the anti-nociceptive action of flowering top extract of *A. javanica* and its fractions. Findings of mechanistic investigation showed that in analgesic interaction involves interaction with opioid receptors (in neurogenic phase). Involvement of peripheral mechanism was also resulted as indicated by bradykinin inhibition. Anti-inflammatory mechanism involves interaction of extract with β-adrenergic, cholinergic receptors, dopaminergic and adenosinergic receptors. Antiphlogistic mechanism also involved inhibition of phospholipase A_2. Involvement of ATP-sensitive potassium channels was ruled out completely.

FUTURE PROSPECTIVE

Involvement of subtype of opioid receptor needed to be evaluated by further investigation. Only partial involvement of cholinergic receptors was obtained with flowering top extract of *A.javanica*. Therefore, there is still a demand to work on more receptors to explore their involvement in anti-nociceptive action. There is a need to further fractionate the extract and to move on isolation of components to explore the specific mechanism involved.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY

Not declared.

ETHICAL APPROVAL

"All applicable international, national, and/or institutional guidelines for the care and use of animals were followed."

"All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted."

"This article does not contain any studies with human participants performed by any of the authors."

The present protocol was approved by *Institutional Animal Ethical Committee (IAEC)* of Banasthali University, Rajasthan, India vide approval no. *BV/3632/2017-2018*.

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