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Anti-nociceptive Activity of *Hygrophila spinosa*, Cow Urine Distillate and their Combination in Balb-C Mice

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ABSTRACT

Medicinal plants are worthy constituents of traditional medicine including therapy for the relief of pain. Evaluation of Anti-nociceptive activity of *Hygrophila spinosa* hydro-alcoholic extract, cow urine distillate and their combination was done by observing latency in reaction time of mice to raise or lick the fore limb or jumping using Eddy's hot plate analgesiometer. The reaction time recorded on Eddy's hot plate analgesiometer for various treatment groups except control was highest at 60 min followed by 120 min, then 30 min and was least at 0 min. At 60 min, the reaction time showed by rats of Group II (Aspirin), Group III (0.5 ml CUD), Group IV (HSE: 400 mg/kg) and Group V (CUD: 0.5 ml + HSE: 400 mg/kg) were 14.833 ± 1.352, 10.833 ± 1.276, 13.000 ± 1.528 and 14.167 ± 0.946 seconds, respectively, which were significantly ($p \leq 0.05$) higher than the reaction time shown by control group rats (3.167 ± 0.477 seconds). The results endorsed the utility of *Hygrophila spinosa* in pain relief treatment as per Ayurvedic texts. The study has also proved the anti-nociceptive activity of cow urine distillate and its combination with *H. spinosa* hydro-alcoholic extract in mice.

HIGHLIGHTS

- Ash of *Hygrophila* is used with cow urine or water in inflammation.
- Pain inhibition by HSE in hot plate latency assay was comparable to Reference drug. CUD potentiated the analgesic action of HSE.

Keywords: Anti-nociceptive, analgesiometer, *Hygrophila spinosa,* cow urine distillate, reaction time

Traditional medicine is blooming worldwide again fetching deep faith of people in healthcare. Most peoples are attracting towards medicinal plants and alternatives based on their folklore utilization. *Hygrophila spinosa* plant is widely distributed and used as a folk medicine in tropical Africa, India, China, Nepal and Malasia (Almeida and Almeida, 2020). In India it is commonly known as Kokilaksha or Gokulakanta. In Chhattisgarh it is called as Maukhala, Kulekhra or Talmakhana. Whole plant, its roots as well as seeds are known for medicinal value in Ayurveda. In India it is used for various medicinal purposes including anti-inflammatory. Talmakhana ash is taken along with cow urine or water and is useful in inflammation (Tripathi, 2019). In north Ethiopia, leaves of *H. schulli* are macerated in alcohol overnight and used for the management of headache and pain secondary to physical damage (Tekulu *et al*., 2020). Cow urine is regarded as an indispensable part of Indian heritages and rituals. In Ayurvedic texts like *Sushruta Samhita* and *Ashtanga Sangraha* cow urine was described to have a variety of pharmacological activities (Nautiyal and Dubey, 2021). There is paucity of literature available

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on pharmacological activities of cow urine distillate with special consideration of its analgesic activity. Bioenhancer is the agent that enhances the potency or efficacy of a substance with which it is administered. It often improves the solubility or adsorption of medicine, or inhibits the action of drug-metabolizing enzymes (Javed *et al*., 2016). Cow urine distillate increases the effectiveness of antimicrobial drugs like refampicin (Randhawa, 2015). Bioenhancing property is attributed to the biochemical role of the chemical constituents. This study aimed to evaluate the analgesic property of *H. spinosa*, indigenous Cow urine (Kosli cow, a recognized breed from Chhattisgarh) distillate and their combination in mice. Aspirin is one of the most frequently used and cheapest drugs in medicine. It belongs to the non-steroidal anti-inflammatory drugs with a wide range of pharmacological activities, including analgesic, antipyretic, and antiplatelet properties. In this study, analgesic activity of *H. spinosa*, indigenous Cow urine distillate and their combination is compared with that of Aspirin.

MATERIALS AND METHODS

Collection of cow urine and distillate preparation

Natural voiding, morning, mid stream urine from Kosli cows of 2-3 year age was collected in a sterile container. Cow urine distillate (CUD) was prepared by condensing the cow urine using a glass distillation apparatus. The preparation was stored at 4°C and used for further study.

Preparation of *Hygrophila spinosa* extract

Leaves of *Hygrophila spinosa* from paddy fields of district Rajnandgaon, (C.G.) were collected. These were shade dried, powdered and stored in air tight container. Extraction was done in Soxhlet apparatus for 18 - 24 hours. Extraction thimbles were prepared using fifty gm of powdered leaves and placed in the extraction chamber. Solvent for extraction consisted of 30% distilled water and 70% methanol to prepare hydro-alcoholic extract (30:70). The extract thus obtained was transferred to the evaporating dishes for evaporation of solvent. Thick extract of pasty consistency was transferred to air tight container and stored in refrigerator for further use and referred as *Hygrophila spinosa* extract (HSE)**.**

Experimental animals

Thirty adult healthy BALB/c mice of either sex weighing 25 ± 3 g were used for evaluation of analgesic activity**.** The animals were housed in polypropylene cages and maintained under standard laboratory conditions (27 ± 2° temperature and 12/12 hr light/dark cycle). The mice were offered *ad libitum* standard commercial feed and clean drinking water. All the animals were observed regularly during the experimental period. The study was carried out with prior approval by the Institutional Animal Ethical Committee (IAEC), College of Veterinary Science and Animal Husbandry, Anjora, Durg, C.G. India and followed the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of` Forests and Environment, Government of India.

Evaluation of anti-nociceptive activity

The study was conducted to assess the analgesic effect of Kosli cow urine distillate, HSE and their combination using hot plate latency assay (Eddy and Leimbach, 1953). Screening of mice was done prior to the experiment and thirty mice showing reaction time of within 5 sec to thermal stimulus of 55 ± 5 °C were selected and divided into five groups of 6 animals each. Group I mice acted as control group and administered with normal saline only. Animals of Group II as standard group were administered Aspirin (@ 100 mg/kg, p.o.) as reference drug. Group III and IV mice received HSE @ 400 mg/kg and Kosli cow urine distillate (0.5 ml) orally, respectively. Group V was orally treated with the combination of HSE (400 mg/kg) and Kosli cow urine distillate (0.5 ml). The reaction time of all the five group mice was recorded after 1h of administration of test drug. At 0 min, 30 min, 60 min and 120 min, animals were lowered onto the surface of hot plate (55 ± 5°C) (Eddy's analgesiometer, Techno) enclosed with cylindrical glass and the time elapsed between placing of mice onto the hot plate and appearance of the signs of discomfort i.e., to raise or lick the fore limb or jumping in an attempt to escape was noted as the reaction time (RT). The design of experiment is depicted in Table 1.

STATISTICAL ANALYSIS

Data obtained was analysed statistically by using SPSS

version 25 and presented as mean ± standard error of the mean (M ± SEM). Comparison was made by Duncan's Multiple Range Test (Snedecor and Cochran, 1994).

Table 1: Experimental design for evaluation of Analgesic activity of CUD, *H. spinosa* hydro-alcoholic extract and their combination in mice

Group	No. of animals	Treatment
I	6	Control: Normal saline orally
II	6	Reference Drug: Aspirin @ 100 mg/kg, p.o.
III	6	HSE @ 400 mg/kg orally
IV	6	Cow urine distillate of Kosli cow @ 0.5 ml orally
V	6	HSE @ 400 mg/kg orally +Cow urine distillate of Kosli cow @ 0.5 ml orally

RESULTS AND DISCUSSION

The reaction time of mice to raise or lick the fore limb or jumping in Eddy's Hot plate analgesiometer at various time intervals is tabulated in Table 2 and depicted in Fig. 1. Latency in reaction time was the delay in showing the reaction by the animals. The reaction time of mice of Group I, II, III, IV and V was 2.333 ± 0.211, 2.167 ± 0.401, 1.667 ± 0.211, 2.333 ± 0.422 and 1.500 ± 0.224 seconds, respectively. There was no significant ($p \leq 0.05$) difference in reaction time at 0 minute.

The latency in reaction time at 30 min for Group I, II, III, IV and V was -1.000 ± 0.258, 6.000 ± 0.931, 3.833 ± 0.703, 4.667 ± 0.615 and 8.667 ± 1.820 seconds, respectively. At 60 min, it was 0.833 ± 0.543, 12.667 ± 1.145, 11.333 ± 1.498, 8.500 ± 1.360 and 12.667 ± 0.989 seconds for Group I, II, III, IV and V, respectively. At 120 min it was 0.000 ± 0.258, 12.500 ± 1.455, 9.333 ± 0.843, 7.167 ± 0.833 and 11.333 ± 0.667 seconds Group I, II, III, IV and V, respectively. It was significantly ($p \leq 0.05$) increased in all treatment groups as compared to control groups. Maximum effect was observed in the combination treatment group (Group V) and was maximum at 30 min and 60 min. The effective dose of HSE producing 50 percent pain inhibition percent (ED_{50}) in mice may be less than 400 mg/kg, whereas for combination of HSE and CUD, the approximate oral dose is supposed to be less than the administered dose.

Hot plate test is most sensitive to centrally acting analgesics. This method illustrates centrally mediated antinociceptive responses which focus generally on supraspinal integrated changes. Centrally acting analgesics act by raising the threshold for pain and altering the physiological response to pain also. Thermal induced pain/nociception indicates narcotic involvement (Besra *et al*., 1996) and Thermal nociceptive tests are more sensitive to opioid μ receptors (Abbott and Young, 1988).

Pain inhibition by HSE in this study was comparable to Reference drug, aspirin. CUD potentiated the analgesic

Table 2: Effect of *H. spinosa* hydro- alcoholic extract,CUD and their combination on Anti-nociceptive activity in mice

Group	Treatment	Reaction time (seconds)				Latency in Reaction time		
		0 min	30 min	60 min	120 min	30 min	60 min	120 min
I	Normal saline orally	2.333 ± 0.211	1.333 ± 0.211^{c}	3.167 ± 0.477^{c}	2.333 ± 0.211^{d}	-1.000 ± 0.258^{c}	0.833 ± 0.543^{c}	0.000 ± 0.258^{d}
II	Aspirin 100 mg/kg, p.o.	2.167 ± 0.401	8.167 ± 0.703^{ab}	14.833 ± 1.352^{a}	14.667 ± 1.229^{a}	6.000 ± 0.931^{ab}	12.667 ± 1.145^{a}	12.500 ± 1.455^{a}
III	HSE: 400 mg/kg orally	1.667 ± 0.211	5.500 ± 0.885^{b}	13.000 ± 1.528^{ab}	11.000 ± 0.730^{bc}	3.833 ± 0.703^{b}	11.333 ± 1.498^{ab}	9.333 ± 0.843^{bc}
IV	CUD: 0.5 ml orally	2.333 ± 0.422	7.000 ± 0.931^{ab}	10.833 ± 1.276^{b}	9.500 ± 1.088^{c}	4.667 ± 0.615^{b}	8.500 ± 1.360^{b}	7.167 ± 0.833^{c}
V	HSE 400 mg/kg + CUD: 0.5 ml orally	1.500 ± 0.224	10.167 ± 1.851^{a}	14.167 ± 0.946^{ab}	12.833 ± 0.654^{ab}	8.667 ± 1.820^{a}	12.667 ± 0.989^{a}	11.333 ± 0.667^{ab}
Level of Significance		NS	*	*	*	*	*	*

Values indicate Mean ± SEM of n=6 animals; Values with different superscript differs significantly ($P \leq 0.05$)* within a column; NS: Non Significant.

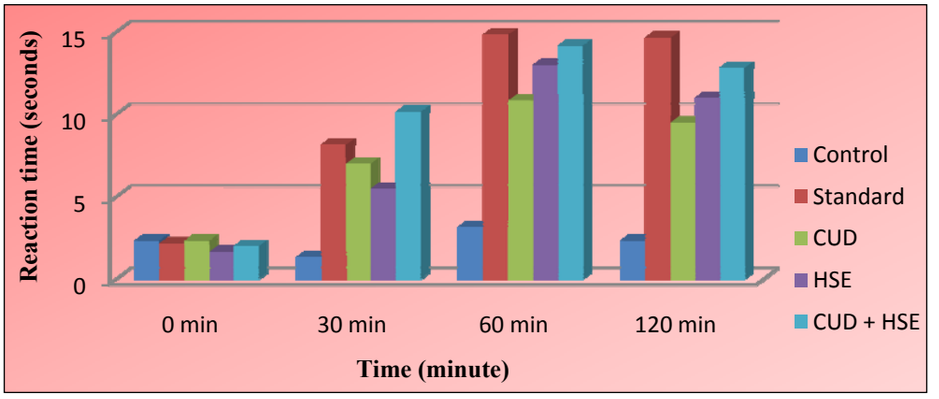

Fig. 1: Effect of *H. spinosa* hydro-alcoholic extract, CUD and their combination on Analgesic activity in mice

action of HSE in combination form. This action is supposed to be mainly by influencing prostaglandins synthesis. Most of drugs with anti-inflammatory and analgesic action possess antipyretic action also. Sterols, terpenoids and flavonoids in *H. spinosa* may contribute to this action. Presence of uric acid and polyphenols in CUD may be involved in exhibition of this activity.

The results of this study endorsed the utility of *H. spinosa* and cow urine distillate in pain relief treatment as per *Ayurvedic* texts. Significantly ($p \leq 0.05$) increased reaction time in CUD, HSE and their combination treated mice indicated activity at supraspinal level suggesting their central analgesic activity. Shanmugasundaram and Venkataraman (2005) found that pain inhibition percent by Hot plate test for *H. auriculata* aerial part extract was 36.5% and 67.3% at dose of 200 mg/kg and 400 mg/kg b.wt., respectively in mice. Bellah *et al*. (2017) found anti-nociceptive action of *H. spinosa* by acetic acid induced writhing test in dose-dependent manner where the highest dose (500 mg/kg) achieved the maximum percentages of pain inhibition (58.8%). Tekulu *et al*. (2020) found that leaf extract of *H. schulli* possessed significant inhibition of acetic acid-induced writhes in mice in a dose-dependent manner. The oral dose of the ethanolic extract at doses of 200 and 400 mg/kg reduced the acetic acid-induced abdominal writhes by 48.76 ± 12.39% and 57.89 ± 10.92%, respectively. Wate *et al*. (2012) revealed notable analgesic activity of both cow urine and its distillate. The CUD showed significant analgesic activity after 90 minutes of the administration (RT was 9.34 ± 0.09 min). Whereas, Jagadeesh (2007) found no significant analgesic activity in cow urine treated male and female rats at dose of 0.1 ml, 0.2 ml and 0.3 ml/100 gm. The analgesic action of *H. spinosa* in this study may be attributed to the bioactive molecules i.e., secondary metabolites present in it.

CONCLUSION

The study suggests analgesic activity of HSE. CUD has bioenhanced the analgesic activity of HSE.

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