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Antioxidant and Antinociceptive Activity of Methanol Extract of Rhizomes of *Iris Kashmairrania* in Heat and Chemical-Induced Pain

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ABSTRACT

Pain is a sensation which in many cases represents the only symptom for the diagnosis of several disorders. Throughout ages man has used many different remedies for pain relief, among which medicinal herbs are most common due to their efficacy and relative safety due to their natural origin. *Iris kashmiriana* Baker (*I. kashmiriana*) is an important member of the family Iridaceae, locally known as Mazarmund in Kashmir valley. The plant owes its significance because of its endemic nature to the region and its use in alternative medicine as emetic, cathartic, diuretic and expectorant since long time. This study evaluated the antioxidant and antinociceptive effect of the methanolic extract of *I. kashmiriana* rhizomes in mice. Qualitative phytochemical screening of methanolic extract was carried out to identify the phytoconstituents. The *In vitro* antioxidant activity of methanolic extracts of *I. kashmiriana* rhizomes (IKME) was assessed against reducing power assay using standard protocols. The methanolic extract was assessed for antinociceptive activity using chemical and heat induced pain models such as hot plate and acetic acid-induced writhing test models in mice at the doses of 100 and 200 mg/kg (p.o.). Morphine sulphate (5mg/kg, i.p.) was used as reference analgesic drugs. The phytochemical screening results showed that the extract had flavonoids, steroids, saponins, phenolics and terpenoids which have been associated with anti-nociceptive activities. IKME demonstrated potent and dose-dependent antinociceptive activity in the chemical and heat induced mice models ($p < 0.001$). Above findings suggest that *I. kashmiriana* rhizomes extract possesses significant antinociceptive activity in albino mice with reference to positive and negative control groups; however further studies on a large number of animals with clinical trials are required to confirm safe and effective use of this rhizomes extract in humans for pain purpose.

Keywords: *Iris kashmiriana,* Iridaceae, Morphine sulphate, Phytochemical screening, Antinociceptive.

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INTRODUCTION

Herbal drugs are manufactured from fresh or dried plants having interrelated compounds that work together. In comparison pharmaceutical drugs are solitary compounds that are often synthetic in nature. Herbal drug expert treated their patient using herbs, food and lifestyles modifications to cure through addressing the root cause of illness; however modern medicine usually manages only the symptoms of diseases with strong compounds that they contain. They seldom address the actual cause of ailment. It means that herbals treatment follows relatively holistic approach for remedial purposes[1,2]. Ethno-pharmacological research on medicinal plants can promote the development of newer and safer compounds. These studies may help in the discovery of a new drug molecule from the herbal source and hence may endorse the use of traditional drugs in humans. Medicinal plants have strong historical background as remedy for different ailments. Around 25% of our current prescription medicines have been originated from medicinal plants. These products have been used as medication and dietary supplements in both developing and developed countries[3]. In several countries, herbal drugs are still the most common available health source for the majority of the population and its use has been encouraged by the government authorities along with modern medicine[4,5]. Herbal medicines have been investigated under strict control and approved by the health authorities of developed countries worldwide. They are generally treated as dietary supplement and World Health Organization has given separate guidelines to ensure their safety and efficacy[6,7]. However in developing countries, herbal medicines are used for the management of various disorders and their use is generally accepted in the society as alternate system of medicine[8,9]. Herbal drugs therefore have potential for improving general health and lower health care cost[10]. Pain can be defined as a stressful sensation in a specific part of the body. International Association for the Study of Pain defines it as "an unpleasant sensory and emotional experience related with real or probable tissue injury"[11]. Pain encourages the person to

avoid bothersome situations, to care for a wounded body part until healing and to escape related incidences in future. Most pain subsides quickly as the painful stimulus is removed and the body has repaired, but sometimes may remain regardless of elimination of the stimulus and obvious healing of the body part. Occasionally pain begins in the absence of any detectable stimulus, injury or disease. Generally pain has been classified in three classes: nociceptive, inflammatory and pathological pain caused by damage to the nervous system or by alteration in its function e.g. fibromyalgia, irritable bowel syndrome and tension type headache. The pain pathway usually involves transduction, transmission, modulation and perception[12]. Drugs commonly in use for pain management and are classified as opioids analgesics, non-steroidal anti- inflammatory drugs (NSAIDs) and steroidal anti-inflammatory medicines. All these medicines have adverse effects e.g. dependence, constipation and respiratory problems with opioids[13], gastrointestinal risk and renal insufficiency with NSAIDs and immune system depression, increasing chances of infection and decreasing wound healing by steroidal treatment[14,15]. Medicinal plants have been extensively used for a long time to avoid these adverse effects. It is necessary to discover new plants to come up with naturally safe and effective pain killer therapy. Plants exemplify still a large available and unexplored source of new chemicals that can help in the development of novel analgesic drugs[16,17].

The Iris plant belonging to family Iridaceae is worldwide in distribution, the genus comprising of about 300 species known for their ornamental relevance and medicinal value. The species of the genus Iris are very useful for pulmonary asthma, cancer, inflammation, liver and uterus diseases[18]. The intensive phytochemical investigations of various iris species have resulted in the isolation of a variety of compounds including quinones, triterpenoids, flavonoids, isoflavonoids and stilbene glycosides[19]. Flavonoids and isoflavonoids are important plant secondary metabolites with structural diversity and are consumed by human as dietary constituents[20]. The isoflavone rich dietary consumption is reported to reduce risk of cancer particularly breast and prostate cancer[21,22]. The role of isoflavones in cancer[23,24] osteoporosis, cardiovascular diseases and menopausal symptoms in addition to their antioxidant[25] antimicrobial[26] anti-inflammatory and estrogenic activities[23,27] is well documented. *I. kashmiriana* is one of an important member of this family Iridaceae, locally known as Mazarmund in Kashmir. The plant has been widely used in traditional medicine and modern clinical preparations to treat cold, flu, malaria, toothache, cancer, bacterial and viral infections and bruise. The phytochemical analyses of the different extracts of *I. kashmiriana* have revealed the presence of different compounds including flavonoids, isoflavonoids, glycosides and tannins. The medicinal importance of the plant prompted isolation of a variety of pharmacologically active compounds including quinones, triterpenoids, flavonoids, isoflavonoids and stilbene glycosides[28]. The use of *I. kashmiriana* in different painful conditions in folk medicine but lack of scientific study reporting its antinociceptive activity in both chemical- and heat-induced nociception models convinced us to design the present study to evaluate the antinociceptive effect of methanolic extract of the rhizomes of *I. kashmiriana*.

MATERIALS AND METHODS

Plant material

Rhizomes of *I. kashmiriana* were collected from Pinnacle Biomedical Research Institute (PBRI), Near, Bharat Scout and Guides Campus, Shanti Marg, Shyamla Hills Road, Depot Chouraha, Bhopal, Madhya Pradesh 462003, India. The sample was identified by senior Botanist Dr. Zia-Ul-Hassan, Professor and head department of Botany, Safia College of Arts and Science, peer gate Bhopal. A herbarium of plants was submitted to the specimen library of Safia College of Arts and Science, peer gate Bhopal and The specimen voucher no. of *I. kashmiriana* was 112/Bot/Saf/230.

Chemical reagents

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Extraction

Dried pulverized rhizomes of *I. kashmiriana* were placed in thimble of soxhlet apparatus. Soxhlation was performed at 60°C using petroleum ether (40-60ºC) as non-polar solvent at first. Exhausted plant material (marc) was dried and then extracted with methanol. For each solvent, soxhlation was continued till no colour was observed in siphon tube. For confirmation of exhausted plant marc (i.e. completion of extraction), colorless solvent was collected from siphon tube and completion of extraction was confirmed by absence of any residual solvent, The entire extract was concentrated to dryness using rotary flash evaporator under reduced pressure and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts[28].

Phytochemical screening

The crude methanolic extract of *I. kashmiriana* was qualitatively tested for the detection of alkaloids, flavonoids, saponins, tannins, glycosides, carbohydrates, reducing sugars, proteins, glucosides, terpenoids, and steroids following standard procedures[29].

Reducing power assay

A spectrophotometric method was used for the measurement of reducing power. For this 0.5 ml of each of the extracts was mixed with 0.5ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml of 1% potassium ferricyanide (10 mg/ml). The reaction mixture was incubated at 50 ºC for 20 min separately, and then rapidly cooled, mixed with 1.5 ml of 10% trichloroacetic acid and centrifuged at 6500 rpm for 10 min. An aliquot (0.5ml) of the supernatant was diluted with distilled water (0.5ml) and then ferric chloride (0.5ml, 0.1%) was added and allowed to stand for 10 min. the absorbance was read spectrophotometrically at 700 nm. Ascorbic acid (AA) was used as standard for construction of calibration curve[30]. Plot a curve of absorbance versus concentration. Increased absorbance of the reaction mixture indicates increase in reducing power.

$$\text{Reducing Power (\%)} = (As / Ac) \times 100$$

Here, Ac is the absorbance of control and As is the absorbance of samples (extracts) or standards.

Animals

All ethical and handling guidelines were followed as set by Indian Legislation and approved by Institutional Animal Ethics Committee. All animals were procured and housed in animal house maintained under standard hygienic conditions. Animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of Pinnacle Biomedical Research Institute (PBRI) Bhopal (Reg No. 1824/PO/ERe/S/15/CPCSEA). Protocol Approval Reference No. PBRI/IAEC/PN- 1808.

Acute oral toxicity

Acute toxicity study of the prepared rhizomes extracts of *I. kashmiriana* was carried out according to the Organization for Economic Co-Operation and Development (OECD) Guidelines-423[31] the animals were fasted for 4 h, but allowed free access to water throughout. Nulliparous healthy female mice were used for this study. 3 animals per step were selected. Dose selected 5, 50, 300, 2000 mg/kg body weight. Immediately after administration of extract, all of the animals were observed for a total of 14 days based on established criteria, observations of behavior pattern changes in skin and eye, respiration, tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. With special attention given during the first 4 hours, clinical signs or mortality were noted. On day 15, all of the animals were euthanized by cervical dislocation. Acute toxicity was determined as per reported method[32].

Antinociceptive activity test

Acetic acid-induced writhing test

To evaluate the analgesic activity of the plant extract acetic acid writhing model in mice was conducted accordingly the procedure described by Koster et al[33]. The test samples (methanolic extract of *I. kashmiriana* 100 and 200 mg/kg body weight respectively), standard (morphine, 10 mg/kg body weight per orally) and control (1% Tween 80 in distilled water at the dose of 10 ml/kg body weight) were given and after 30 min 0.85% acetic acid was injected intra-peritoneally (i.p.). The writhing (constriction of abdomen, turning of trunk and extension of hind legs) was observed randomly after 15 min of interval and its frequency was counted for up to 25 min in each group of animals. The percent inhibition (% analgesic activity) was calculated by:

$$\%\text{Inhibition} = [(A-B/A)] \times 100$$

Where, A=Average number of writhing of the control group; B =Average number of writhing of the test or standard groups.

Hot plate test

The hot-plate test was performed according to the method described by Eddy and Leimbach (1953)[34] with slight modification. The temperature of the metal surface of Eddy's hotplate was set at 52±2°C. The mice that showed fore paw licking, withdrawal of the paw(s) or jumping response within 15s on hotplate were selected for this study 24h prior to the experiment. Mice were fasted overnight with water given *ad libitum*. The mice were then treated with 0.9% sodium chloride solution as vehicle (0.1 ml/ mice, p.o.), HMCR (50, 100, and 200 mg/kg), and morphine as positive control (5 mg/kg, i.p.). IKME was administered

(100 and 200mg/kg p.o.) 30 min before the experiment while morphine sulphate was administered (10 mg/kg, i.p.) 15 min before the experiment. The response in the form of fore paw licking, withdrawal of the paw(s) or jumping was recorded at 30, 60, 90, and 120 min following treatment. A cut off period of 20s was maintained to avoid paw tissue damage. The results of the hot plate test are expressed as a percentage of the maximal possible effect (%MPE), which was calculated using following formula:

$$\%\text{MPE} = [(\text{Postdrug latency-predrug latency})/ (\text{Cut off period-predrug latency})] \times 100$$

RESULTS AND DISCUSSION

The crude extracts so obtained after the soxhlation process, extract were further concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extraction. The percentage yield of methanolic extract was found to be 2.95 %. Phytochemical analysis of methanolic extract of *I. kashmiriana* rhizomes showed the presence of carbohydrate, flavonoids, phenolics, tannin, saponins, triterpenoids Table 1.

Table1 Phytochemical analysis of methanolic rhizomes extract of *I. kashmiriana*

S. N.	Constituents	*I. kashmiriana*
1.	**Alkaloids**	
	Mayer's reagent test	-ve
	Wagner's reagent test	-ve
	Hager's reagent test	-ve
2.	**Carbohydrates**	
	Molish's test	+ve
	Fehling's test	+ve
	Benedict's test	+ve
	Barfoed's test	+ve
3.	**Proteins and amino Acids**	
	Biuret test	+ve
4.	**Flavonoids**	
	Alkaline reagent test	+ve
	Lead acetate test	+ve
5.	**Glycoside**	
	Borntrager test	-ve
	Legal's test	-ve
	Killer-Killiani test	-ve
6.	**Tannin and phenolic compounds**	
	Ferric chloride test	+ve
	Lead acetate test	+ve
	Gelatin test	+ve
7.	**Saponin**	
	Foam test	-ve
8.	**Test for triterpenoids and steroids**	
	Salkowski's test	+ve
	Libbermann-Burchard's test	+ve

Antioxidant activity of the samples was calculated through reducing power assay. The reducing ability of the compound usually depends on the reductants, which have been exhibited antioxidative capacity by breaking the free radical chain, donating a hydrogen atom. Reducing power assay was calculated in extracts and the values indicated a better activity Table 2& Fig. 1.

Table 2 Result of reducing power assay

S. No.	Concentration (µg/ml)	Ascorbic acid	Methanolic Extract
1.	20	0.107	0.061
2.	40	0.122	0.088
3.	60	0.139	0.102
4.	80	0.156	0.123
5.	100	0.182	0.145

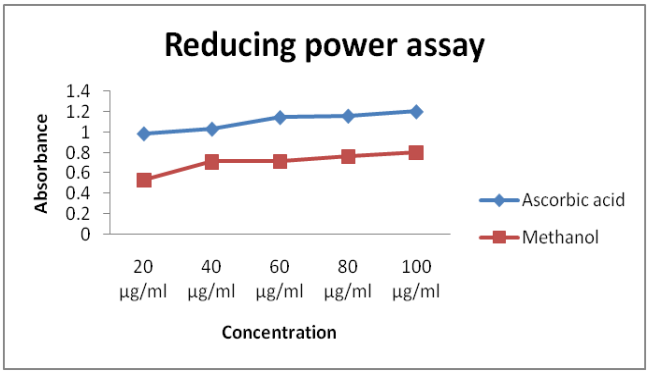

Fig. 1 Reducing power assay

Acute oral toxicity was calculated at four different concentrations 5mg/kg, 50mg/kg, 300 mg/kg and 2000 mg/kg. Observations were performed in groups of three and no mortality was observed Table 3. The acute toxicity results showed that methanolic extracts of *I. kashmiriana* rhizomes was safe up to a dose of 2000 mg/kg body weight. Based on acute toxicity data, two different dosages 100 and 200 mg/kg (p.o.) were selected for in vivo anti-nociceptive activity.

Table 3 Acute oral toxicity

S. No.	Groups	Observations/ Mortality
1.	5 mg/kg Bodyweight	0/3
2.	50 mg/kg Bodyweight	0/3
3.	300 mg/kg Bodyweight	0/3
4.	2000 mg/kg Bodyweight	0/3

Table 4 shows the antinociceptive effect of IKME and standard drug (Morphine) assessed using the hot plate test. IKME, at the doses of 100 and 200 mg/kg, exhibited significant ($p < 0.001$) ability to prolong the latency of response to thermal-induced nociception throughout the whole experimental period. The effect was dose-dependent and IKME showed stronger effect at 100 and 200 mg/kg doses. The hot-plate test is a specific test carried out to verify involvement of central mechanism with compounds/drugs showing antinociceptive activity[25].In this work, HMCR showed a marked inhibition on thermal induced hyperalgesia as it showed significant increase in latency ($p < 0.001$) compared to control. Morphine (10 mg/kg i.p.) was used as a standard drug which demonstrated a stronger analgesic effect than IKME. The effect was evident from the elongation of the latency time till the 4th observation (120min). The effect of compounds or plant extract in such mechanism by increasing the latency are suggested to act like centrally mediated drugs[35] by activating the periaqueductal gray matter (PAG) to release endogenous peptides (i.e., endorphin or enkephalin). These endogenous peptides descend the spinal cord and function as inhibitors of the pain impulse transmission at the synapse in the dorsal horn[36]. The acetic acid induced writhing test is widely used for antinociceptive screening and involves local peritoneal receptors (cholinergic and histamine receptor) as well as the mediators of acetylcholine and histamine[37]. In the abdominal tissues acetic acid injection produces peritoneal inflammation, which triggers a response characterized by writhing. Such types of responses are induced by the release of endogenous mediators of pain such as prostaglandins, bradykinine and cytokines (TNF-α, IL-1β and IL-8) that stimulate the nociceptive neurons, which are sensitive to non steroidal anti-inflammatory drugs (NSAIDs) and opioids[38]. In our study, IKME inhibitied the acetic acid induced writhing in a dose dependent manner (Table 5). These responses can be described as a typical model of inflammatory pain in which the sensory neurons are depolarized by directly activating a non-selective cationic channel of cutaneous, visceral and other types of peripheral afferent C fibers[39]. Thus, the significant reduction in the number of acetic acid-induced writings by IKME indicates the antinociceptive potential of this plant and confirms its traditional use for the relief of inflammatory pain.

Table 4 Antinociceptive effect of *I. kasmiriana* extract and morphine in hot plate test

Groups	Mean latency ± SD			
	30	**60**	**90**	**120**
Control	6.00± 0.816	6.66±0.471	7.00± 0.816	7.66± 0.471
Morphine	9.00 ± 0.816	10.0±2.94	11.33 ± 1.24	15.33 ± 1.24
IKME 100	8.00 ± 0.816	8.66±1.24	9.33 ± 0.942	10.66± 0.471
IKME 200	8.33± 0.417	9.66 ± 1.24	11.00± 0.816	13.33 ± 1.69

Table 5 Antinociceptive effect of methanolic extract of *I. kashmiriana* on acetic acid writhing test

Groups	Number of writhes Mean ± SD
Control	37.66± 2.05
Morphine	11.00 ± 1.63
IKME 100	21.66± 2.05
IKME 200	28.33 ± 2.86

CONCLUSION

The results of the present study indicate that IKME exhibited significant antinociceptive activity at all the tested doses in mice. The effect is dose dependent and statistically significant particularly at 100 and 200 mg/kg doses. Taking these findings into account, it seems quite possible that IKME contains constituents with promising antinociceptive activity. The traditional use of the plant in the treatment of painful conditions can be affirmed by this study. However, further studies are required to isolate the bioactive compounds and elucidate the precise mechanisms responsible for the antinociceptive activity.

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