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Anti-nociceptive activity of a polyherbal formulation RO7D, in experimental models: Possible mechanisms

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ABSTRACT

The selected herbal preparation RO7D contained the following ingredients. *Centella asiatica, Cassia auriculata, Cynodon dactylon, Rosa damascena, Myristica fragrans, Nelumbo nucifera, Hibiscus Rosa sinensis, Hemidesmus indicus, Glycyrrhiza glabra, Eclipta alba.* The aqueous extract of RO7D was prepared and freshly prepared solution was used for experiments. The anti-nociceptive activity of RO7D was investigated employing acetic acid induced abdominal constriction, hot plate and tail clip assay procedures in swiss albino male mice. The involvement of opioid mechanism is examined using naloxone. The aqueous extract of RO7D exhibited a significant and dose related anti-nociceptive response in all the three assay procedure employed. Therefore, it can be concluded that RO7D possess anti-nociceptive activity. The observations that the pretreatment with naloxone attenuated the anti-nociceptive activity of RO7D strongly suggest a role for opioid pathway in this action.

KEY WORDS: Anti-nociception, Polyherbal formulation, RO7D and Naloxone

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INTRODUCTION

Reverse pharmacology is gaining importance to treat most of the common ailments since the available remedies in modern medicine are known to cause side effects. Around mid 1980s exhaustive studies on plant products were carried out, however, on plant principles. World Health organization (WHO), then insisted on the identification of active principles followed by investigations on pharmacological actions. Unfortunately, significant outcome was not reported. One of the contributing factors for this may be the loss of activity during isolation of active principles with various polar/ non-polar solvents. In order to overcome this hurdle, WHO recommended that scientific support for the herbal products for use in humans could be provided only by using the recommended formulations in the ancient medicine and allowed its use in human being. A significant momentum gained after this created a renewed interest in herbal research.

One of the most common ailment for which humans seek remedy to alleviate is pain. Many over the counter drugs such as paracetomol, is used frequently besides, the use of non steroidal anti-inflammatory drugs are also used. Unfortunately, all the available analgesic and anti-inflammatory agents induce side effects. Therefore it is essential to identify a suitable safe analgesic drug from the herbal source having minimal side effects.

A large number of plants have been shown to exhibit anti-nociceptive activity in experimental models. Among them *Solanum xanthocarpium, Myristica fragrans, Nelumbo nucifera, Glycyrrhiza glabra, Phyllanthus nuri* have been shown to exhibit analgesic activity based folkloric claims [1]. Additionally flavonoids have been investigated to greater extent for their analgesic and anti-inflammatory activity. The hydroxyl ethyl rutoside exhibited anti-nociceptive activity in experimental models [2]. This study was extended further to other flavonoids, gossypin [3] and showed that gossypin can be considered as a potential candidate for clinical trial since it exhibited anti-nociceptive, anti-inflammatory and surprisingly a beneficial anti-ulcer activity.

Currently many herbal food supplements are available in the market for use in situations of pain. Rumi herbals, Chennai has formulated a food supplement for its known analgesic use. This formulation which they coded RO7D includes the following herbs *Centella asiatica, Cassia auriculata, Cynodon dactylon, Rosa damascena, Myristica fragrans, Nelumbo nucifera, Hibiscus rosa sinensis, Hemidesmus indicus, Glycyrrhiza glabra, Eclipta alba, Phyllanthus niruri.* As per the ancient system of medicine the aqueous extract of these plants are used in the management of pain. Hence, in the present study, the aqueous extract of RO7D was investigated for its anti-nociceptive activity employing standard experimental procedures.

MATERIALS AND METHODS

Preparation of Extract

RO7D was soaked in distilled water for 8 hrs and extracted using a Soxhlet extractor for 8 hrs. The collected hot aqueous extract was dried under reduced pressure using a rotatory flask evaporator. The aqueous extract was used for further experiments. A fresh infusion was prepared prior to each experiment.

Animals

Male Wister rats weighing between 150 – 220 gm were used for this study. The animals were obtained from animal house, IRT Perundurai Medical College, Erode, Tamilnadu, India. On arrival, the animals were placed in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24±2^{o}C and relative humidity of 30 – 70 %. A 12:12 light: day cycle was followed. All animals were allowed to have free access to food (commercial rat chow, M/s. Hindustan Lever Ltd, Mumbai) and water. All the experimental procedures and protocols used in this study were in accordance with the guidelines of the CPCSEA. The study protocol was approved by the animal ethics committee.

Acute Toxicity Study

Acute oral toxicity studies were performed [4] according to OECD-423 guidelines (acute toxic class method). Male Swiss albino mice (*n* = 3/each dose) were selected by random sampling technique were employed in this study. The animals were fasted for 4 h with free access to water only. The aqueous extract of RO7D (suspended with 0.5%, w/v, CMC) was administered orally at a dose of 5 mg/kg, to separate group of mice and mortality was observed for 3 days. If mortality was observed in 2/3 or 3/3 animals, then the dose administered was considered as toxic dose. However, if the mortality was observed in only one mouse out of three animals then the same dose was repeated again to confirm the toxic effect. If mortality was not observed, the procedure was then repeated with higher doses such as 5, 50, 300 and 2000 mg/kg.

Anti-nociceptive Activity

Acetic Acid Induced Writhing

Animals were injected intraperitoneally (0.6%, 10 ml/kg) of freshly prepared acetic acid. The numbers of writhing were observed in each animal for 15 min following acetic acid injection. The mice were treated with the aqueous extract of RO7D (100, 200, 400 and 800 mg/kg) or saline (10 ml/kg., vehicle) or standard drug (Morphine, 1 mg/kg) 30 minutes before administration of acetic acid. Decreases in number of writhing after test drug were observed [5].

Eddy's Hot Plate

The procedure described by Eddy and Leimbach (1953) was adopted for this assay [6]. Animals were placed on a hot plate maintained at 55 ± 0.5ºC. A maximum time of 15 sec was

only allowed to avoid thermal injury to the paws. The animals were placed on the hot plate and time taken by the animal to lick the hind paws or to jump from the hot plate was considered as the reaction time. The mice were first treated with the aqueous extract of RO7D (100, 200, 400 and 800 mg/kg) or saline (10 ml/kg., vehicle) or standard drug (Morphine, 1 mg/kg). After 15 minutes, a significant increase in the reaction time as compared with pretreatment value was considered as anti-nociceptive response.

Tail Clip (Mechanical) Method

This method described by Bianchi and Franschine, 1954 was employed [7]. A mechanical pressure at the base of the tail was applied with the help of a sleeved bull-dog clamp. The time taken by the animal to make attempts to dislodge the clip (biting) was considered as the reaction time. A maximum period of 15 sec was allowed to avoid injury to the tail. The mice were treated with the aqueous extract of RO7D (100, 200, 400 and 800 mg/kg) or saline (10 ml/kg., vehicle) or standard drug (Morphine, 1 mg/kg).

In all the three assay procedures, the role for opioid mechanism in the anti-nociceptive activity of RO7D, if any, was investigated. Opioid antagonist, naloxone was used for this purpose. The anti-nociceptive activity of aqueous extract of RO7D was investigated in all the models were carried out in naloxone 10mg/kg treated (i.p 30 minutes prior to RO7D administration) treated animals.

Statistical Analysis

The results were represented as mean ± SEM. The data's were analyzed by one way analysis of variance (ANOVA) followed by Dunnett's "t" test. *P* value <0.05 was considered as significant.

RESULTS

Acute Toxicity Study

The toxicity study reveals the safety of RO7D in mice. There was no marked change in the general behavior up to 2000 mg/kg., body weight of RO7D. No mortality was observed during the observation period.

Effect of RO7D on Acetic Acid Induced Writhing in Mice

The number of abdominal constriction after i.p. injection of 0.6% acetic acid 33.17±1.05 was reduced to 6.50 ± 0.99 by 1mg/kg of morphine. The aqueous extract RO7D at 100mg/kg did not significantly antagonize the acetic acid induced abdominal constriction (33.67±0.76). In fact, more inhibitory activity was recorded. This may be due to the expression of minimal anti-nociceptive activity of naloxone in lower doses reported earlier [8]. However, in higher doses it produced a significant dose related reduction in the number of abdominal constrictions to

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26.33 ± 0.40, 17.17 ± 0.91 and 10.17 ± 1.65 at 200, 400 and 800 mg/kg respectively. In naloxone pretreated animals, the aqueous extract of RO7D produced a significantly reduced anti-nociceptive effect when compared with that observed in RO7D alone treated animals (Table 1).

Table 1. Effect of morphine or aqueous extract of RO7D on the acetic acid induced abdominal constrictions in saline and naloxone pretreated mice.

Drug Treatment	Dose (mg/kg)	Number of abdominal constriction	
		Absence of Naloxone	Presence of Naloxone
Saline	10ml/kg	33.17±1.05	34.0±0.9
Morphine	10	6.50±0.99**	33.0±0.2€
Aqueous Extract of RO7D	100	31.67±0.67†	29.0±0.75
Aqueous Extract of RO7D	200	26.33±0.42**†@	29.1±0.6
Aqueous Extract of RO7D	400	17.17±0.91**†@	24.7±0.5€
Aqueous Extract of RO7D	800	10.17±0.65**†@	19.4±0.3€

Each value represents the mean ± SEM of six animals.
F value 191.85 (ANOVA),*P<0.005 and **P<0.001 compared with saline value.
@ P<0.005 when compared with 100mg /kg value. € ,P < 0.01 when compared with those obtained in the absence of naloxone. † P < 0.01 when compared to morphine value

Effect of RO7D on Eddy's Hot Plate in Mice

The reaction time after thermal stimulus 1.20 ± 0.14 in saline animals was raised to 12.55 ± 0.35 seconds in morphine 10mg/kg treated animals. The aqueous extract of RO7D at 100mg/kg significantly increased the reaction time to 3.32 ± 0.22. Increase of the dose of RO7D to 200mg did not produce any further significant elevation when compared with 100/kg dose. However, at 400mg/kg, the reaction time was increased to 10.57 ± 0.22 and at 800mg/kg to 12.38 ± 0.26. Further, it was observed that RO7D produced a significantly reduced anti-nociceptive response in naloxone pretreated animals when compared with the animals which received RO7D alone (Table 2).

Effect of RO7D on Tail Clip in Mice

The reaction time after mechanical stimulus 0.97 ± 0.08 seconds in saline treated animal was raised to 13.83 ± 0.46 in morphine (10mg/kg) treated animals. The aqueous extract of RO7D at 100 mg/kg significantly increased the reaction time to 4.32±0.44. Increment of the dose of RO7D to 200mg / kg did not produce any further significant increase when compared with 100mg / kg. However, in higher doses of 400 or 800mg/kg RO7D produced a significant dose related increase in relation time 13.82 ± 0.29 and 14.30 ± 0.17 respectively. Like in other

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assay procedures, naloxone pretreatment significantly attenuated the anti-nociceptive activity of RO7D in mechanical procedure (Table 3).

Table 2. Effect of morphine or aqueous extract of RO7D on the reaction time in the hot plate method in saline and naloxone pre-treated mice.

Drug Treatment	Dose (mg/kg)	Reaction Time (sec)	
		Absence of Naloxone	Presence of Naloxone
Saline	10ml/kg	1.20±0.14	1.8±0.1
Morphine	10	12.55±0.35**	2.4±0.1€
Aqueous Extract of RO7D	100	3.32±0.22*	3.5±0.2
Aqueous Extract of RO7D	200	5.52±0.20*	3.8±0.1€
Aqueous Extract of RO7D	400	10.57±0.22**†	7.1±0.3€
Aqueous Extract of RO7D	800	12.38±0.26**†	8.2±0.2€

Each value represents the mean ± SEM of six animals.
F value 191.85 (ANOVA).*P<0.005 and **P<0.001 compared with saline value.
€ ,P < 0.01 when compared with those obtained in the absence of naloxone.
†<0.005 when compared with 100mg /kg value.

Table 3. Effect of morphine or aqueous extract of RO7D on the reaction time when tested by tail clip method in normal and naloxone pre-treated mice.

Drug Treatment	Dose (mg/kg)	Reaction Time (sec)	
		Absence of Naloxone	Presence of Naloxone
Saline	10ml/kg	0.97±0.08	0.8±0.09
Morphine	10	13.83±0.46**	2.1±0.6€
Aqueous Extract of RO7D	100	4.32±0.44*	3.9±0.3
Aqueous Extract of RO7D	200	6.68±0.23**†	4.1±0.5
Aqueous Extract of RO7D	400	13.82±0.29**†	8.4±0.5€
Aqueous Extract of RO7D	800	14.30±0.17**†	9.0±0.4€

Each value represents the mean ± SEM of six animals.
F value 191.85. *P<0.005 and **P<0.001 compared with saline value.
€ ,P < 0.01 when compared with those obtained in the absence of naloxone.
†P<0.005 when compared with 100mg /kg value

DISCUSSION

Renewed interest on the use of herbal products especially as food supplements is gaining importance. This is because the World Health Organization has agreed for reverse pharmacology as described in the review of literature. Globally, many herbal products are being

used as food supplements for prophylaxis of common ailments such as hypertension, diabetes etc. The technical expertise available in the field of molecular biology and biotechnology assist in providing scientific evidences to support the folkloric claims of several herbal food supplements.

Prior to the pharmacological investigation of any herbal product it is mandatory to investigate the safety profile of these herbal products. It is documented that if a herbal substance is free from side effects or adverse effects up to 2000 mg/kg, it is considered safe for clinical use. In the present study, the absence of any acute toxicity observed for RO7D is in line with those observed for individual ingredients recorded earlier. [9, 10, 11, 12] thus proving the safety profile of RO7D.

In the folkloric claim, it was recommended that the aqueous extract when administered orally was found to be effective and hence, the aqueous extract was used for investigation.

Morphine, a known opioid analgesic inhibited the acetic acid induced abdominal constrictions in mice validating the assay procedures. Similar observations were also made in thermal and mechanical assay methods. The observation that the aqueous extract of RO7D produced a dose related inhibition of abdominal constriction induced by acetic acid similar to morphine suggests that RO7D exhibit anti-nociceptive activity. This view was confirmed from the results obtained in thermal and mechanical assay procedures where RO7D increased the reaction time to the noxious stimulus such as thermal and mechanical were recorded. Therefore, it can be concluded that RO7D possess anti-nociceptive activity.

The anti-nociception of any substance can be mediated either through opioid pathway or cyclo-oxygenase inhibition. In the present study, the role of opioid mechanism in the anti-nociceptive activity of RO7D was tested using naloxone, an opioid antagonist. The observations that that pretreatment with naloxone attenuated the anti-nociceptive activity of RO7D in all the three assay procedures strongly indicate a role for opioid pathway in this action. Further experiments like ligand binding studies and pA_2 determination will confirm this view. These results provide scientific support to the folkloric claim of the ingredients of RO7D suggesting their use for the management of pain. However, the efficacy of RO7D when compared with morphine was found to be less.

Flavonoid, vitamin P like substances maintain capillary integrity [13, 14] possibly by interfering with the release of prostaglandins (15). RO7D includes herbs which contains flavonoids. (1). Several flavonoids like hydroxyl ethyl rutoside, gossypin, flavones and its hydroxyl derivatives [16, 17] possess anti-nociceptive activity [2, 18, 19] Gossypin has a unique property of possessing anti-nociceptive, anti-inflammatory and especially anti-ulcer property [3]. Based on this, it can be suggested that the ingredients of RO7D could involve cyclooxygenase pathways also to elicit anti-nociception. It is interesting to mention here that RO7D has been shown to exhibit anti-ulcer activity [20]. Therefore, like gossypin, RO7D is yet another potential compound for clinical trial for use in pain and inflammation as it does not produce the common side effect gastritis, rather protects from this problem.

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