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In vivo Anti-inflammatory and Analgesic Activities of Ethanol Leaf Extract of *Stevia rebaudiana* in Albino Rats

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Abstract: The present study was undertaken to investigate the anti-inflammatory and analgesic activities of ethanolic extract of *Stevia rebaudiana* leaf in experimental rats. Swiss albino rats were administered with oral dose of ethanolic leaf extract of *S. rebaudiana* at the doses of 200, 300, 400 and 500 mg/kg body weight, and studied for its anti-inflammatory and analgesic activities by using carrageenan-induced hind paw edema, acetic acid-induced writhing, hot plate reaction and tail immersion test in albino rats. Inhibition in paw volume was measured plethysmometrically at different time intervals after carrageenan (1% w/v) injection. As a result, the ethanolic leaf extract of *S. rebaudiana* showed anti-inflammatory and analgesic effects in dose dependent manner when compared with the control and standard drug, aspirin (100 mg/kg, p.o). The paw volumes, pyrexia and writhes in experimental rats were reduced significantly ($p < 0.05$) as compared to that of control, and hot plate test showed significant licking effect in rats. Phenylbutazone, aspirin, morphine and diclofenac sodium were employed as reference drugs for analgesic and anti-inflammatory studies. In the present study, high dosages of the ethanolic leaf extract of *S. rebaudiana* (300, 400 and 500 mg/kg body weight) demonstrated significant anti-inflammatory and analgesic activities in the tested models. Findings of this investigation suggested a potential benefit of ethanolic leaf extract of *S. rebaudiana* in treating conditions associated with pain and inflammation.

Key words: *Stevia rebaudiana*, ethanolic leaf extract, anti-inflammatory activity, analgesic activity.

Introduction

Plant medicines are far and away safer, gentler and better for human health than synthetic drugs. This is so because human beings have co-evolved with plants over the past few million years. We eat plants, drink their juices, ferment and distill libations from them, and consume them in a thousand forms. The World Health Organization (WHO) estimates that 4 billion people or up to 80% of people still rely mainly on traditional remedies such as herbs for their medicines [29]. Herbal medicine is a major component in all indigenous peoples' traditional medicine. Among the various medicinal and culinary herbs, some endemic species are of particular interest because they may be used for the production of raw materials or preparations containing phyto-chemicals with significant antioxidant capacities and health benefits [8]. Crude extracts of fruits, herbs, vegetables, cereals and other plant materials rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food [17].

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Although the plant material has been long used to treat pain and inflammation, no scientific work has been carried out to ascertain the claimed properties. Inflammation is the response of living tissues to injury. It involves a complex array of enzyme activation, mediator release and extravasations of fluid, cell migration, tissue breakdown and repair [24]. The synthetic drugs used to treat inflammation are toxic and not commonly available to the rural folks that constitute the major community of the world [5]. Steroidal and non-steroidal anti-inflammatory drugs are still widely employed as analgesics and to relief inflammatory conditions [22]. The adverse effects of these drugs pose some problems and have placed limitations in their use. Hence, there is a need to continuous search for natural, effective and safer alternatives. Medicinal plants are important sources of new chemical substances with potential therapeutic effects, and literature has also been documented plants with various analgesic and anti-inflammatory activities [14].

Stevia rebaudiana (Bert.), Bertoni is an herbaceous perennial plant of the Asteraceae family. It is native of Paraguay, where it grows wild in sandy soils [13]. Stevia leaf extracts are used in Japan, Korea and certain countries of South America to sweeten soft drinks, soju, soy sauce, yogurt and other foods, whereas in the United States it is used as dietary supplement. The leaf extract of *S. rebaudiana* has been used traditionally in the treatment of diabetes. The main sweet component in the leaves of *S. reba*udiana is stevioside [11]. Stevia sweetener extractives have been suggested to exert beneficial effects on human health, including anti-hypertensive [3], antihyperglycemic non-cariogenic, antihuman rota virus activities, glucose metabolism [27] and renal function [18]. Also the leaves of *S. rebaudiana* can be commercialized as a processed sweet green tea.

To the best of our knowledge, no reports are available in the literature on the anti-inflammatory and analgesic activities of *S. rebaudiana* leaves, therefore, the present work was undertaken to evaluate the analgesic and anti-inflammatory effects of the ethanolic leaf extract of *S. rebaudiana* (ELES) through various *in vivo* assays in experimental rats.

Materials and methods

Plant material

The leaves of *S. rebaudiana* were collected in March 2006 from Jeevan Agro farms, Sagar, MP, India. A herbarium of the source was made and identified, voucher specimen no. Bot/H/3352 was submitted at the herbarium of Department of Botany, Dr. H.S. Gour University, Sagar, MP, India.

Preparation of the ethanolic extract

The air-dried leaves of *S. rebaudiana* (50 g) were powdered and then extracted with 500 ml of ethanol by using Soxhlet apparatus. The crude extract was filtered and evaporated under reduced pressure to give a viscous dark mass with a percentage yield of 4.5% (w/w).

Experimental animals

Animal use protocol was approved by the Dr. Hari Singh Gour University, Sagar, MP, India (Animal Eths Comm/IE/98/Reg No 379/01/ab/CPCSEA) and was in accordance with international standard on the care and use of experimental animals (CCAC, 1993). Swiss albino rats of either sex weighing between 100-125 g were used for the study. Animals were housed under standard conditions of temperature (25°C), 12 h/12 h light/dark cycles and fed with standard pellet diet and tap water.

Toxicity study

Toxicity study was performed with ethanolic leaf extract of *S. rebaudiana*. Extract was dissolved in water and administered orally to different groups of rats in graded ranging from 100-1000 mg/kg for the LD_{50} study using the modified method (Ghosh, 1971). There was no lethality in any of the groups after 7 days of treatment.

Determination of *in vivo* anti-inflammatory activity

Carrageenan-induced paw edema assay

Acute inflammation was produced by injecting 0.1 ml of (1%) carrageenan into plantar surface of rat hind paw [32]. The test samples of with ELES

(200, 300, 400 and 500 mg/kg, orally), and phenylbutazone (100 mg/kg, orally) as reference agent were administered 60 min before carrageenan injection. The paw volume was measured at 0, 1, 2, 3 and 4 h, using a thread to determine the diameter of edema formation size. Differences in diameter between the left and right hind paws were taken as a measurement of oedema.

Determination of in vivo analgesic activity

Acetic acid-induced writhing assay

For the analgesic activity assay, acetic acid solution (15 mg/ml) at the dose of 300 mg/kg body weight was injected (i.p.) and the number of writhes during the following 30 min period was observed [30]. A significant reduction in the number of writhes by the treatment of ELES (200, 300, 400 and 500 mg/kg, orally) as compared to vehicle treated animals which was considered as a positive analgesic response. The percentage inhibition of writhing was then calculated. Aspirin (100 mg/kg, i.p.) was used as standard drug.

Hot plate reaction time assay

Preliminary study of the phenomenon of adaptation of nociceptors to repetitive application of stimulus was undertaken to determine the onset and time span of the adaptation. This knowledge was required to administer the extract and measure the pain threshold at a time when there was no adaptation, i.e., to determine the appropriate dosing interval. The adaptation was studied by examining the relationship between responses to thermal pain and varying frequency of the stimulus application. The paws of mice and rats are very sensitive to heat at temperatures which are not damaging the skin. The responses are jumping, withdrawal of the paws and licking of the paws.

Briefly, Rats were screened by placing them on a hot plate maintained at 55 ± 1°C and the reaction time in seconds for hind paw licking or jumping was recorded [30]. The rats were orally administered the test drug ELES (200, 300, 400 and 500 mg/kg). Only rats which reacted within 15 seconds and did not show large variation when tested on four separated occasions, each 15 min apart, were used in this study. Morphine (5 mg/kg, i.p.) was used as standard. The time for hind paw licking or jumping on the heated plate of analgesiometer was taken as the reaction time.

Tail immersion method

In the present study, analgesia was assessed according to the method described by Aydin et al. (1999) [1]. Rats divided in the groups of five each, were held in position in a suitable restrainer with the tail extending out. A 3-4 cm area of the tail was marked and immersed in the water bath thermo-statistically maintained at 55 ± 0.5°C. Within a few minutes, the rats reacted by withdrawing the tail. The reaction time was recorded with a stopwatch. The animals were treated by ELES (200, 300, 400 and 500 mg/kg), or water (vehicle) or standard drug (diclofenac sodium, 50 mg/kg), 30 min before the immersion of the tail. The time reaction was taken at 30, 60, 90, and 120 min after administration of different preparations.

Statistical analysis

Data were expressed as the mean standard deviation (S.D.), and statistical analysis was carried out employing one way ANOVA and Student's t-test. Differences between the data were considered significant at $P< 0.05$.

Results

Anti-inflammatory activity

Carrageenan-induced paw oedema

In the carrageenan-induced oedema test, the results of paw volumes and average oedema formation by ELES and standard drugs are shown in Table 1. There was a gradual increase (dose dependent) in oedema paw volume of rats in the control (carrageenan treated group). However, in the test groups, the ELES treated group (300, 400 and 500 mg/kg body weight) showed a significant reduction in the oedema paw volume. As indicated in Table 1, a dose-related inhibition of hind paws oedema between 2 and 4 h was observed. Phenylbutazone as reference drug (100 mg/kg, orally) produced a significant inhibitory effect comparable to tested ELES.

Table 1. Anti-inflammatory activity of ethanolic leaf extract of *S. rebaudiana* on carrageenan induced rat paw oedema in the right hind-limb paw of rats

Treatment	Dose (mg/kg)	0	Time (h) 1	2	3	4	Average paw edema formation
ELES[a]	200	-	0.36±0.02	0.42±0.01	0.35±0.02	0.32±0.01	0.36±0.02
	300	-	0.33±0.01	0.32±0.04*	0.28±0.00*	0.25±0.05*	0.29±0.01*
	400	-	0.30±0.11	0.27±0.01*	0.25±0.03*	0.21±0.00*	0.25±0.04*
	500	-	0.30±0.01	0.26±0.04*	0.21±0.02*	0.15±0.03*	0.23±0.01*
Phenylbutazone	100	-	0.21±0.01	0.22±0.06*	0.22±0.01*	0.17±0.09*	0.21±0.04*
Control (carrageenan treated)	-	-	0.44±0.11	0.75±0.01	0.88±0.21	0.93±0.10	0.74±0.11*

Values are mean ± SEM (n=6)
*p<0.05 of the difference between the left and right hind paws
[a]Ethanolic leaf extract of *S. rebaudiana*

Analgesic activity

Acetic acid-induced writhing in rats

The ELES at the doses of 200, 300, 400 and 500 mg/kg significantly and dependently reduced the number of abdominal constriction induced in rats by a solution of acetic acid. The results presented in Table 2 showed that the ELES at the doses of 200, 300, 400 and 500 mg/kg exhibited significant ($p < 0.05$) inhibition of the control writhes at the rate of 9.65%, 11.69%, 45.99% and 54.80%, respectively when compared to that of aspirin (100 mg/kg) (65.97%) and control groups (Table 2).

Hot plate reaction time in rats

The hot plate and tail immersion tests are useful in the elucidating centrally mediated antinociceptive responses, which focus mainly on changes above the spinal cord level. All the test and standard drugs significantly ($p<0.01$) reduced the pain as compared to the control group (Table 3). As shown in Table 3, the ELES produced significant ($p < 0.01$) analgesic activity at all the tested doses when compared to that of control. Additionally, ELES at different doses (200, 300, 400 and 500 mg/kg kg) potentiated the analgesic activity (reaction time) 8.00, 12.16, 13.62, 16.14 and 19.17 min, respectively, when compared to morphine (5 mg/kg) as 19.17 min. The higher dosages such as 400 and 500 mg/kg showed more potent activity than lower dosages of ELES.

Tail immersion test in rats

As presented in Table 4, ELES at the doses of 500 mg/kg ($P < 0.01$) body weight showed a significant elongation of reaction time, 30 min after oral administration of the extract. After 60 min, the ELES at the doses of 400 and 500 mg/kg ($P < 0.05$) body weight showed a significant elongation. After 90 min, ELES at the doses of 300, 400 and 500 mg/kg body weight showed a significant ($P < 0.05$) elongation of reaction time. However, after 120 min, ELES at the doses of 200, 300, 400 and 500 mg/kg body weight showed no significant elongation of reaction time.

Discussion

In the present study, anti-inflammatory and analgesic activities of ethanolic leaf extract of *S.*

Table 2. Effect of ethanolic leaf extract of *S. rebaudiana* on writhing induced by acetic acid in experimental rats

Treatment	Dose (mg/kg)	Number of writhes (per 30 min)	Inhibition (%)
ELES[a]	200	29.21±3.71	9.65
	300*	28.55±2.81*	11.69*
	400*	17.46±1.76*	45.99*
	500*	14.61±2.51*	54.80*
Aspirin	100*	11.00±1.40*	65.97*
Control (saline)	5 ml/kg	32.33±2.71	-

Values are mean ± SEM (n=6)
*p<0.05 as compared to control
[a]Ethanolic leaf extract of *S. rebaudiana*

Table 3. Effect of ethanolic leaf extract of *S. rebaudiana* on hot plate reaction time in experimental rats

Treatment	Dose (mg/kg)	Mean latent time	
		Initial	After 30 min
ELES[a]	200	8.58±0.21	8.00±0.11
	300	8.21±0.43	12.16±1.22*
	400	9.42±1.14	13.62±1.32*
	500	9.10±1.21	16.14±1.11*
Morphine	5	9.74±0.87	19.17±1.33*
Control (saline)	5 ml/kg	8.74±0.07	8.14±0.56

Values are mean ± SEM (n=6)
*p<0.05 as compared to control
[a]Ethanolic leaf extract of *S. rebaudiana*

Table 4. Protective effects of ethanolic leaf extract of *S. rebaudiana* on tail withdrawal reflex by tail immersion in experimental rats

Treatment	Dose (mg/kg)	Reaction time (min) Mean ± SEM			
		30 min	60 min	90 min	120 min
ELES[a]	200	1.3±0.3	1.5±0.02	2.0±0.1	2.7±0.2
	300	1.9±0.1	2.2±0.01	2.8±0.01	3.5±0.2*
	400	2.9±0.5	3.3±0.3*	4.6±0.1*	6.3±0.3*
	500	3.4±0.2	4.0±0.01*	5.3±0.3*	6.9±0.01*
Diclofenac sodium	50 mg/kg	6.2±0.3*	8.8±0.4*	9.1±0.1*	9.4±0.1*
Control (saline)	5 ml/kg	1.4±0.01	1.7±0.04	2.6±0.1	2.9±0.2

Values are mean ± SEM (n=6)
*p<0.05 as compared to control
[a]Ethanolic leaf extract of *S. rebaudiana*

rebaudiana (ELES) were investigated using animal experimental models those could determine the effectiveness of the extract on inflammatory-mediated nociception (abdominal writhing test), non-inflammatory-mediated nociception (hot plate test and tail flick test) or both types of nociception (the carrageenan-induced paw oedema test) and provides some evidence on the mechanism implicated in this effect.

Most of the anti-inflammatory drugs now available are potential inhibitors of cyclooxygenase (COX) pathway of arachidonic acid metabolism which produces prostaglandins. Prostaglandins are hyperalgesic, potent vasodilators and also contribute to erythema, edema, and pain. The inhibition of carrageenan induced inflammation in rats is an established model which has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic [19]. The first phase occurs within one hour of carrageenan inflammation and is attributed to the release of cytoplasmic enzymes, histamine and serotonin, from the mast cells. The second phase (>1.0 h) is mediated by an increased release of prostaglandins in the inflammatory area and continuity between the two phases is provided by kinins. Since the ELES significantly inhibited paw edema induced by carrageenan in the second phase, this finding suggests a possible inhibition of cyclooxygenase synthesis by ELES, because the carrageenan inflammatory model basically reflects the actions of prostaglandins [9]. This effect is similar to that produced by non-steroidal anti-inflammatory drugs such as indomethacin, whose mechanism of action is inhibition of the cyclooxygenase enzyme, which catalyzes the synthesis of cyclic endoperoxides important in the formation of prostaglandins [16]. Based on the results obtained from this study, it can be suggested that anti-oedematogenic effects of the ELES on carrageenan-induced oedema might be related to inhibition of inflammation mediator formation.

The brain and spinal cord play a major role in central pain mechanisms. The dorsal horn of the spinal cord is endowed with several neurotransmitters and receptors including somatostatin, neuropeptides, inhibitory amino acid, nitric oxide, endogenous opioids, and the monoamines which are the major targets for pain and inflammation [21]. The abdominal constriction response induced by acetic acid is a sensitive procedure to evaluate peripherally acting analgesics [10]. This method is not only simple and reliable but also affords rapid evaluation of peripheral type of analgesic action. Flavonoids may increase the amount of endogenous serotonin or may interact with 5-HT2A and 5-HT3 receptors which may be involved in the mechanism of central analgesic activity [20]. In this study, ELES (400 and 500 mg/kg) showed highest analgesic activity which may be due to its high flavonoid contents responsible for free radical scavenging activity, as these free radicals are involved during pain stimulation, and antioxidants showed reduction in such pain. Previously we reported strong free radical scavenging activity of ELES [26].

To evaluate a possible central analgesic effect of the ELES at supraspinal and spinal levels, the hot plate and tail-flick tests were used [33]. Hot plate method was originally described by Woolfe and MacDonald (1994). This test has been found to be suitable for the evaluation of centrally but not of peripherally acting analgesics. The present findings of the study indicate that the ELES may be centrally acting. The tail-flick response is believed to be a spinally mediated reflex and the paw-licking hot plate response is more complex supraspinally organized behavior [4]. The effectiveness of analgesic agents in the tail-flick pain model is highly correlated with relief of human pain perception. The tail immersion test was considered to be selective to examine compounds acting through opioid receptor; the tested ELES increased pain threshold, basal latency, which indicates that it may act *via* centrally mediated analgesic mechanism. Narcotic analgesics inhibit both peripheral and central mechanism of pain, while nonsteroidal anti-inflammatory drugs inhibit only peripheral pain [7]. The ELES inhibited pain with both mechanisms, suggesting that the ethanolic leaf extract of *S. rebaudiana* may act as a narcotic analgesic.

The results of the present study shown that the crude ethanolic leaf extract of *S. rebaudiana* exhibited very high anti-inflammatory and analgesic activities. These activities may be linked

to the presence of polyphenolic compounds present in the ELES. The efficacy of most herbal remedies is attributed to various active constituents in combination.

Since, anti-inflammatory activity of many plants has been attributed to their flavonoids [23], tannins [31], triterpenes [6]. Many plants containing flavonoids have been shown to have diuretic, laxative, antispasmodic, anti-hypertensive, and anti-inflammatory actions 23. Flavonoids and saponins are well known for their ability to inhibit pain perception as well as anti-inflammatory properties due to their inhibitory effects on enzymes involved in the production of the chemical mediator of inflammation [25]. Further, flavonoids are able to inhibit neutrophils degranulation and thereby decrease the release of arachidonic acid 15. Therefore, analgesic and anti-inflammatory activity of ELES, may be due to the presence of phytoconstituents like flavonoids, tannins, saponins, triterpenes and coumarins as reported in phytochemical investigation [28].

Conclusion

Based on the results of the present study, it can be concluded that the ELES has potential anti-inflammatory and analgesic activities in a dose dependent manner against both acute and chronic phases. Hence, this study provides scientific basis for the traditional medicinal uses of these plants for analgesic and anti-inflammatory activities. Further studies are in progress to isolate the active constituents responsible for the observed effects, and to elucidate the possible mechanisms of action responsible for the analgesic and anti-inflammatory activities of *S. rebaudiana*.

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