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Anti-inflammatory and analgesic activity of various fractions of *Vetiveria zezanioides* (V.Z.) in rodents

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ABSTRACT

Vetiveria zizaniodes (Vetiver) is commonly known for its effectiveness in soil and sediment erosion control. It can tolerate to extreme soil conditions and produce a high biomass even growing in contaminated areas. Vetiver oil has been also used in the treatment of several diseases, including mouth ulcers, fever, headache, inflammation and gastritis. The anti-inflammatory activity was evaluated by carrageenan induced paw oedema and cotton pellet induced granuloma in rats. The analgesic activity was carried out by using acetic acid induced writhing reaction in mice and tail immersion method. Four fractions (n-hexane, chloroform, ethyl acetate and butanol) of V.Z. at a dose 200 mg/kg, p.o. Were tested. In carrageenan induced paw oedema and cotton pellet induced granuloma animal model, the ethyl acetate and chloroform fraction of V.Z. were found to be more significant ($p< 0.01$) and also in acetic acid induced writhing reaction in mice. The n-hexane fraction along with ethyl acetate and chloroform fraction showed significant ($p< 0.01$) increase in reaction time in tail immersion method. The results were further substantiated by biochemical findings, suppressing free radicals, GSH and catalase levels in blood. The V.Z. significantly showed anti-inflammatory and analgesic effect through its anti-oxidant potential

Key words: *Vetiveria zizaniodes*, Anti-inflammatory, Analgesic, Granuloma, Oedema.

INTRODUCTION

Plans used in traditional systems as a medicine since time immemorial but very few species have been thoroughly investigated for their medicinal properties [1,2]. As like *Vetiveria zizanioides* L. Nash commonly known as *Khas Khas*, *Khas* or *Khus* grass in India, is the common source of essential oil used in medicine and perfumery [3,4].

V.Z. a fast-growing perennial tussock grass, it is well known as an ecofriendly plant that prevents soil erosion and its usefulness in the rehabilitation of metalliferous polluted land because of its tolerance to elevated levels of heavy metals [5]. *V.Z.* is also the major source of Vetiver oil, which is used in medicine and perfumery. Vetiver oil has been identified as a national permissible natural food additive in China (China Number System for food, N102) and which is an expensive edible oil in the market [6]. It is a commonly used traditional medicine in Thailand [7,8]. Vetiver oil has been also used in the treatment of several diseases, including mouth ulcers, fever, headache, inflammation and gastritis [9,10]. Moreover, essential oil extracted from V.Z. has been frequently used as a functional ingredient and fragrance in foods, aromatic products and cosmetics. Therefore, the interest in this grass has increased in recent years; however, few articles described the biological activities of this plant [10,11]. Thus, research on the biological properties of V.Z. holds significance for many applications. The anti-inflammatory mechanism of V.Z. Essential Oil in lipopolysaccharide (LPS) -induced murine macrophage cells were investigated.

The main objective of this work was to fractionate the chemical composition in plant and evaluate the antioxidant and anti-inflammatory potential of V.Z. root.

Inflammation is a protective host response to a foreign antigen challenge or tissue injury, which if left untreated, can lead to the loss of the tissue structure as well as function. During the development of inflammation, the concerted actions of molecular signalling determine whether inflammatory cells undergo migration, activation, proliferation, differentiation, or clearance. Many inflammatory processes are self-limiting and self-resolving, which suggest that the existence of endogenous anti-inflammatory and pro resolution mediators during the course of inflammation [12,13,14].

In Ayurvedic literature this plant used in various ailments such as communicative stomachic, and such other gastric disorder. [15]. Earlier studies the plant has the potential, like In-vitro antifungal activity, [16] In-vitro antioxidant activity, [17] Anti-arthritic activity, [18] Antispasmodic & Antihypertensive activity [13]. The aim of the present study was to find out which fraction is responsible for the anti-inflammatory and analgesic activity in V. Z. , So here we performed the fractionation of V.Z. with various solvent according to their polarity, for the achievement of better result related to same .

MATERIALS AND METHODS

Chemical and reagent:
The V.Z. root powder was purchased from Endeavour exports, Tamil Nadu, Carragenan (Himedia Laboratories Pvt Ltd). All other chemicals used for biochemical estimations were of analytical grade.

Animals:
Male Wistar albino rats (250–300 g) were used. Animals were housed under standard conditions (i.e. at 22 ± 2^0C, humidity: 50–55% and 12 hr natural light/dark cycle) and feed with standard pellet diet (Neutrivet Life Sciences, Saswad, Pune.) And water *ad libitum*. Each of these treatment groups consists six animals/group. The protocol of the study was approved by the Institutional Animal Ethics Committee (IAEC) Laboratory animal handling and experimental procedures were performed in accordance with CPCSEA guidelines (Approval number: 198/99).

Preparation of ethanolic extract:
The root powder of *V.Z.* was pulverized to make a coarse powder. This course powder was defeated with petroleum ether and then extracted with 95% ethanol using soxhlet apparatus to obtain the ethanolic extract of *V.Z.* . The extract was dried using a rotary vacuum evaporator [19].

Preparation of fractions:
Ethanolic extract was dissolved in 400 ml of (9:1) ratio of methanol : water and this solution were then transferred to a separating funnel for further extraction by using 50 ml of n-hexane. The mixture was thoroughly shaken for 15 minutes. The n-hexane layer was separated and the remaining methanolic phase was evaporated to dryness .The methanolic extract was diluted with 400 ml water and extracted successively and exhaustively with chloroform, ethyl acetate and butanol in the order of increasing polarity. The fractions were concentrated in a rotary evaporator at reduced pressure. The percentage yield of fractions was 35.18% w/w, 19.42% w/w, 18.67% w/w and 17.53% w/w respectively [20].

Acute toxicity studies:
The acute toxicity of ethanolic extract and the various fractions of V.Z. root extract was determined as per the OECD guideline no. 423 (Acute toxic class method) [21]. It was observed that the ethanolic extract and the fractions were not mortal at 2000 mg/kg dose. Hence, 1/10^{th} (200 mg/kg) of this dose was selected for this study [21].

Dose regimen:
In Carrageenan induced paw oedema, cotton pellets induced granuloma and acetic acid induced writhing models, animal were divided in 6 groups (n=6) where, group-I (Control) received an aqueous suspension of 1% w/v Sodium CMC (10 ml/kg; p.o.). The group-II (Standard) received Diclofenac sodium (10 mg/kg p.o.) And groups III-VI received /kg dose of n-hexane, chloroform, ethyl acetate and butanol fractions of V. Z. Respectively. In tail immersion method all groups of animals received the same dose except group II , where group II received Pentazocin (30 mg/kg p.o.).

PHARMACOLOGICAL STUDIES:

Carrageenan induced paw edema in rats:

Anti-inflammatory activity of V.Z. was tested using the carrageenan-induced rat paw edema model [22]. Experimental animals (Wistar rats) were randomly divided into six groups with six animals in each group. Group I (Control) received vehicle (1% CMC). Group II (Standard group) received Diclofenac sodium at dose 10 mg/kg. From group-III-VI received n-Hexane, chloroform, ethyl acetate and butanol fractions of V.Z. at dose 200 mg/kg respectively. The drugs were administered orally 1hr prior to the injection of 0.1 ml of freshly prepared suspension of carrageenan into the left hind paw of each rat. The paw volume was measured using a plethysmometer (Ugo Basile 7140, Italy) at the time interval of 0.5 hours, 1hr, 2hr, 3hr, 4hr, 5hr and 24hr after administration of Carrageenan. Results were expressed as,

Oedema volume = Vt-Vc

V_t = Paw volume in ml, at time t, after carrageenan administration.
V_c = Paw volume in ml, before carrageenan administration.

Inhibition rate (%) = Ec-Et/Ec×100

EC = oedema volume of control group.
E_t = oedema volume of treated group.

Cotton pellets induced granuloma in rats:

The Wistar albino rats (125-150 GM) of either sex were divided into six groups (n=6) fasted overnight and allowed free access to water *ad libitum*. The animals were anaesthetized with light ether anaesthesia and 20 ± 1 mg of the sterile cotton pellet was inserted one in each axle and groin of rats by making a small subcutaneous incision. All groups received drug treatment for 7 days consecutively [16]. On 8^{th} day animals were again anaesthetized and blood was collected for analysis of biochemical parameters (SGOT, SGPT & ALP levels) and antioxidant parameters (SOD, CAT, GSH and LPO). The animals were then sacrificed and cotton pellets along with granuloma mass (wet weight) were weighted and dried at 60^oC and again reweighted (dry weight). The percentage inhibition pellet was calculated by

Percentage of inhibition = WC - wed/WC X 100

Where W_c=difference in pellet weight (GM) of the control group and
W_d=difference in pellet weight (GM) of the test group and drug group.

Acetic acid induced writhing in mice:

The writhing syndrome was elicited by IP injection of acetic acid and numbers of writhes displayed were recorded. After 1 hour of drug treatment animals from all groups challenged with I. p. Injection of acetic acid (300 mg/kg) and the number of writhes were observed for 30 minutes at a time interval of 10 minutes [23].

Tail immersion Test:

Prior to analgesic experiments, the animals were screened for the sensitivity test by immersing the tail of the rats gently in hot water maintained at 55^0C. The animal immersing the tail from hot water within 5 seconds was selected for the study. The selected rats were divided into six groups (n=6). After drug treatment, the reaction time was measured at 0, 15, 30, 45 and 60 minutes by immersing the tail in hot water maintained at 55^0C [24].

Statistical analysis:

The results have been indicated in terms of mean ± SEM, Difference between the groups was statistically determined by One way ANOVA with Dunnett's test. The level of significance was set at $^{**}p < 0.01$, $^{*}p < 0.05$.

RESULTS

Phytochemical investigation:
Preliminary phytochemical investigation showed the presence of the different phytochemical analysis present in different fractions of V. Z. root [Table 1].

Effect of fractions of V. Zezanioides on carrageenan induced paw edema in rats:
Diclofenac sodium treated group showed significant inhibition ($p < 0.01$) of paw volume from 1st year to the 24th hour compared to control group. Groups treated with n-hexane fraction at 4th ,5th and 24th hour; chloroform fraction from 2nd to 24th hour; ethyl acetate fraction from 1st to 24th hour and butanol fraction from 4th to the 24th hour produced a significant decrease ($p< 0.01$) art in paw oedema volume [Table 2].

At 3rd hour n-hexane, chloroform, ethyl acetate and butanol fraction of *V. zezanioides* produced 23.42% ,34.23%, 42.79% and 6.75% inhibition respectively when compared to a standard which showed 49.09% inhibition ($p < 0.001$). At 5th hour, showed 13.38%, 36.4%, 44.76% and 10.87% inhibition respectively when compared to standard [55.23 %] and at the 24th hour showed 25.96%, 42.30%, 52.88% and 14.42% inhibition respectively when compared to Standard [66.82%] ($p < 0.001$) [Figure 1].

To increase in inflammation, there is a marked increase in the ESR count Total WBC, Lymphocytes, Neutrophils and RBC count in the control group. There was no effect of haemoglobin in all groups. Standard, ethyl acetate and chloroform fraction showed significant ($p < 0.01$) decreased in all hematological parameters except haemoglobin [Table 3].

Effect of fractions of V. Zezanioides on cotton pellet induced granuloma in rats:
The group treated with Diclofenac sodium, ethyl acetate and chloroform showed a significant decrease ($p <0.01$) in granuloma weight (wet weight and dry weight) as compared to control group. Orally administered dose of 200 mg/kg of ethyl acetate, chloroform, n-hexane and butanol fraction of *V.Z.* produced 38.12% 24.78%, 11.94% and 3.00% granuloma inhibition rate respectively when compared to standard [52.48%] [Table 4].

In biochemical parameters, the control group showed a marked increase in SGOT, SGPT, and ALP levels. The group treated with Diclofenac sodium, chloroform and ethyl acetate fraction showed significant ($p < 0.01$) decrease while as n-hexane fraction showed significant ($p < 0.05$) decrease in SGOT, SGPT, and ALP level but a butanol fraction did not show any significant result [Figure 2].

In antioxidant parameters, the control group showed decreases in SOD, CAT and GSH levels and increase in LPO level. The group treated with standard, ethyl acetate and chloroform fraction showed significant ($p <0.01$) increased in SOD, CAT and GSH levels and decreased in LPO level while as n-hexane fraction did not show any effect on SOD and GSH level but showed a significant increase ($p < 0.01$) in CAT level and decrease ($p < 0.05$) in LPO level. Butanol fraction showed a significant increase ($p< 0.05$) in CAT level [Table 5].

Effect of fractions of V.Z. on acetic acid induced writhing in mice:
The control group showed increased in number of writhes. Groups treated with Diclofenac sodium, ethyl acetate and chloroform fraction showed significant ($p < 0.01$) decreased in number of writhes for 30 minutes and n-hexane fraction showed significant ($p < 0.05$) decreased in number of writhes but a butanol fraction did not show significant result [Figure 3].

Effect of fractions of V.Z. on Tail immersion Test:
Groups treated with Diclofenac sodium, n-hexane, chloroform and ethyl acetate fraction showed a significant increase ($p < 0.01$) in reaction time at 15, 30, 45 and 60 minutes as compared to control group and butanol fraction did not show significant decrease in reaction time at 60 min but showed a significant decrease ($p < 0.05$) in reaction time at 15, 30, 45 minutes [figure 4].

Table 1: Phytochemical Tests for various fractions of *V.Z.*:

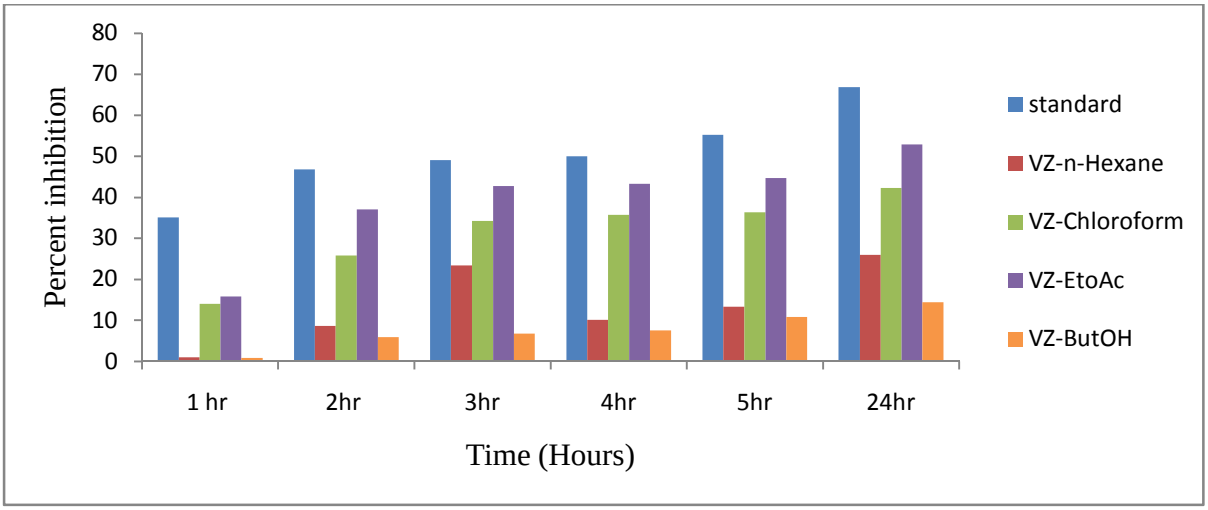
Phytochemicals	N-Hexane fraction	Chloroform fraction	Ethyl acetate fraction	N-Butanol fraction
Alkaloids	+	+	+	-
Glycosides	+	+	+	+
Carbohydrates	-	+	-	-
Steroids and terpenoids	+	+	+	-
	+	+	+	-
Flavanoids	+	+	+	-
Tannins	+	+	-	+
Phenolic Compounds	+	+	+	+
Triterpenes	-	-	-	+
Sugar test	-	+	+	+
Saponins	+	+	+	-
Fixed Oils	-	+	-	-
Protein	-	-	-	+

Table 2: Effect of *V. Z.* Fractions on carrageenan induced hind paw oedema.

Treatment (mg/kg, p.o.)	Volume of paw (ml)						
	0 h	1 h	2 h	3 h	4 h	5 h	24 h
Control	0.65± 0.03	1.14± 0.06	1.86± 0.05	2.22± 0.07	2.38± 0.04	2.39± 0.10	2.08± 0.03
Standard	0.68± 0.04	0.74± 0.04**	0.99± 0.06**	1.13± 0.05**	1.19± 0.03**	1.07± 0.02**	0.69± 0.03**
VZ- n-Hexane	0.70± 0.02	1.12± 0.01	1.70± 0.02*	2.01± 0.03*	2.14± 0.01**	2.07± 0.02**	1.54± 0.01**
VZ -Chloroform	0.67 ±0.02	0.98± 0.01*	1.39± 0.01**	1.46± 0.02**	1.53± 0.02**	1.52± 0.02**	1.25± 0.02**
VZ- EtoAc	0.65 ±0.03	0.96± 0.01**	1.17± 0.01**	1.27± 0.01**	1.35± 0.01**	1.32± 0.02**	0.98± 0.01**
VZ -But OH	0.69 ±0.03	1.13± 0.01	1.75± 0.02	2.07± 0.04	2.20± 0.02**	2.13± 0.02**	1.78± 0.03**

Where VZ- Vetiveria zezanioides, EtoAc- Ethyl acetate, But OH- Butanol.

Figure 1: Effect of *V. zezanioides* fractions on percentage inhibition of carrageenan induced hind paw oedema.



Where VZ- Vetiveria zezanioides, EtoAc- Ethyl acetate, But OH- Butanol.

Table 3: Effect of *V. Z.* Fractions on various hematological parameters at 5th hour.

Hematological Parameters	Treatment and dose (mg/kg, p.o.)					
	Control	Standard	VZ- n-Hexane	VZ -Chloroform	VZ- EtoAc	VZ -But OH
ESR (Mm/h)	04.95 ± 0.55	2.13 ± 0.20**	3.61 ± 0.15*	2.93 ± 0.26**	2.41 ± 0.15**	4.11 ± 0.25
Hb %	12.28 ± 0.62	12.35± 0.84	11.71 ± 0.52	12.28 ± 0.68	11.75 ± 0.40	11.91 ± 0.63
Total WBC count (cu.mm)	8950 ± 329	4468 ± 214**	6132 ± 174**	5415 ± 190**	4904 ± 214**	6709 ± 134**
Lymphocytes (%)	79.16 ± 2.50	53.33± 2.47**	71.50 ± 1.20*	68.00 ± 1.52**	60.66 ± 1.68**	75.66 ± 1.49
Neutrophils (%)	40.33 ± 1.38	26.83± 2.21**	34.16 ± 0.47*	32.50 ± 1.45**	31.33 ± 1.40**	34.59 ± 1.34*
RBC (Millions/Cu, mm)	06.62 ± 0.28	2.92 ±0.28**	4.81 ± 0.16**	3.86 ± 0.15**	3.24 ± 0.15**	5.71 ± 0.12*

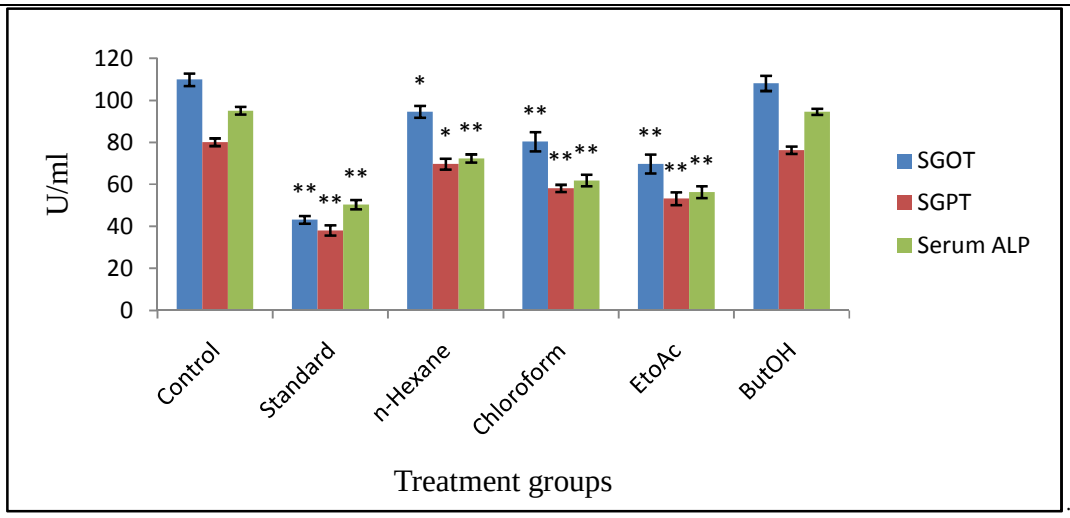
Where VZ- Vetiveria zezanioides, EtoAc- Ethyl acetate, But OH- Butanol.

Table 4: Effect of *V. Z.* Fractions on granuloma weight and the percent inhibition rate in cotton pellet induced granuloma in rats:

Treatment and dose (mg/kg, p.o.)	Granuloma Wt Weight (mg)	Granuloma Dry Weight (mg)	Inhibition Rate (%)
Control	212.50±3.17	131.17±1.24	-
Standard	127.17±2.56**	062.33±1.20**	52.48%
VZ- n-Hexane	198.67±3.55*	115.50±3.84*	11.94%
VZ -Chloroform	161.83±4.56**	098.66±2.88**	24.78%
VZ- EtoAc	145.17±3.08**	081.16±1.80**	38.12%
VZ -But OH	208.67±2.24	126.33±2.23	03.0%

Where VZ- Vetiveria zezanioides, EtoAc- ethyl acetate, But OH- butanol.

Figure 2: Effect of V. Z. Fractions on serum parameters [SGOT, SGPT and Serum ALP levels] in cotton pellet induced granuloma in rats.



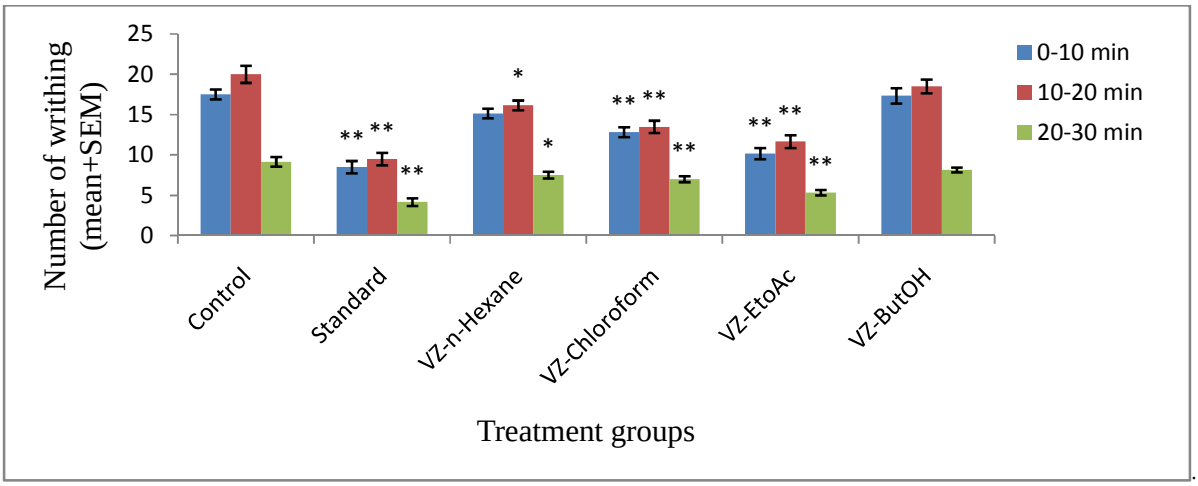
Where VZ- Vetiveria zezanioides, EtoAc- Ethyl acetate, But OH- Butanol.

Table 5: Effect of V.Z. fractions on antioxidant parameters

Treatment (Mg/kg, p.o.)	Antioxidant parameter			
	GSH (µg of GSH/g of tissue)	LPO (NM of MDA/g of tissue)	SOD (Units/mg of tissue)	CAT (µM of H_2O_2/g of tissue/min)
Control	30.18±0.68	16.74±0.63	71.01±0.68	12.05±0.60
Standard	38.01±0.92**	12.75±0.49**	79.89±0.85**	18.63±0.61**
VZ- n-Hexane	31.91±0.51	14.78±0.19*	73.19±0.44	14.67±0.31**
VZ -Chloroform	34.31±0.17**	13.76±0.41**	75.71±0.43**	16.51±0.37**
VZ- EtoAc	36.78±0.33**	13.01±0.31**	77.83±1.03**	17.82±0.65**
VZ -But OH	30.56±0.73	16.42±0.35	71.57±0.47	14.36±0.27*

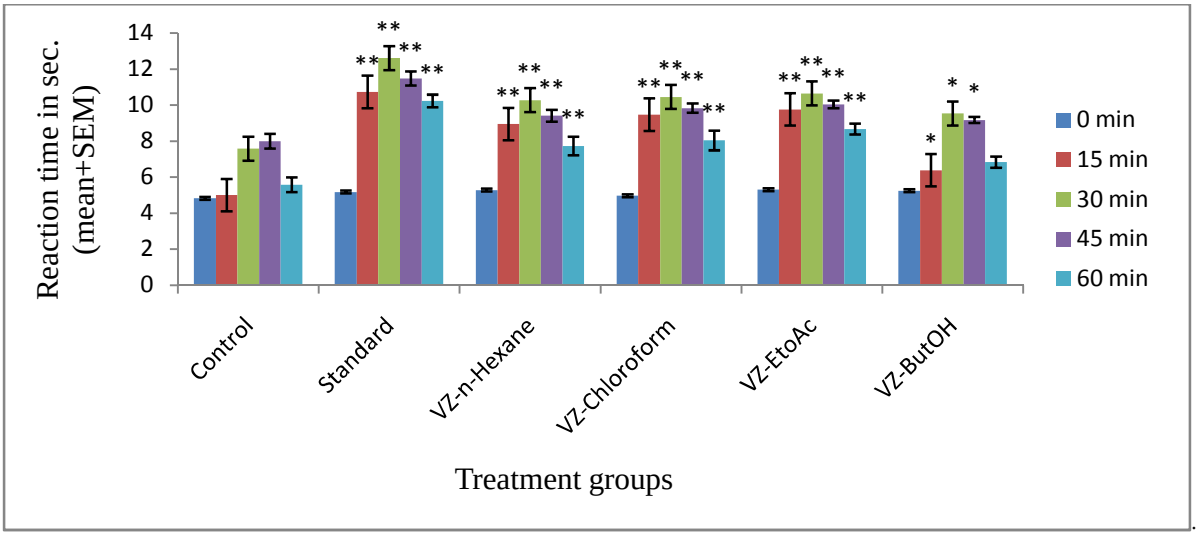
Where VZ- Vetiveria zezanioides, EtoAc- Ethyl acetate, But OH- Butanol.

Figure 3: Effect of *V. Z.* Fractions on Acetic acid induced writhing in mice:



Where VZ- Vetiveria zezanioides, EtoAc- Ethyl acetate, But OH- Butanol.

Figure 4: Effect of *V. Z.* Fractions *on Tail immersion Test:*



Where VZ- Vetiveria zezanioides, EtoAc- Ethyl acetate, But OH- Butanol.

DISCUSSION

Inflammation, the body defence mechanism. The acute inflammation is characterized by vasodilation, exudation of plasma release of various inflammatory mediators, cytokines, growth factor and emigration of leukocytes .While the featured of chronic inflammation include infiltration of mononuclear cells, proliferation of fibroblast, blood vessel and increased connective tissue.

Carrageenan-induced oedema in rats has a biphasic inflammatory response. The first phase (0–2.5 h) is associated with the release of several mediators such as histamine, serotonin and kinins, as well as TNF-α, IL-1, IL-2 IFN-α release on vascular permeability. These mediators together stimulate the molecular events, as well as inflammation process and nociception [25,26]. The second phase (4 h) is correlated with the enhancement of prostaglandins oxygen derived free radicals and inducible cyclooxygenase release [27]. Since the second phase (oedema) of inflammation induced by carrageenan is sensitive to most clinically effective anti-inflammatory drugs, this assay are

useful for studying the anti-edematous effect of natural products [28]. It is presumed that the antioxidant potential of V.Z. is responsible for reducing the oxidative stress and leads to anti-inflammatory potential. When fractionation of V.Z. we observed that the ethyl acetate fraction gives the best result as compare to the other faction. In case of the antioxidant parameters, rats treated with the aqueous extracts of V.Z. in the dose of 200 mg/kg body weight showed significant increase in the activity of SOD, CAT and GSH with a decrease in MDA level in granulation tissue compared with controls. These enzymes are known to quench the superoxide radical and thus prevent the damage of cells caused by free radicals [29].

The cotton pellet implanted into the subcutaneous tissues of the rats formed a granuloma with Distinct borders from the surrounding tissues, Cotton pellet induced chronic inflammatory response characterized by granuloma formation, fluid infiltration & undifferentiated connective tissue was measured by weighing the dried pellets after implantation & treatment. Nonsteroidal anti-inflammatory drugs decrease the size of granuloma which results from the cellular reaction by inhibiting granulocyte infiltration, preventing the generation of collagen fibres and suppressing mucopolysaccharides. Hence V.Z. fractions of ethyl acetate and chloroform effectively lower the infiltration at the site of granulation.

In the acetic acid-induced writhing test, the drugs that affect motor activity may give false-positive or negative results. Therefore, to assess whether the effects of drugs in the acetic

Acid-induced writhing test is because of nonspecific actions in motor activity, we performed a hole-board test, a simple method for measuring the several aspects of behaviour of mice in the open field [30,31]. The results confirmed that none of the treatment with drugs used in the acetic acid-induce writhing test produces a drastic decrease in motor activity that could possibly impair writhing behaviour. Accordingly, it has been shown that antidepressants increased the pain threshold without modifying open-field behaviours [32]. Therefore, the inhibition of writhing behaviour observed in the present study is based on changes in the pain threshold rather than on some impairment of motor activity.

The present study clearly demonstrates that the method used to restrain the animal greatly influences the results of the tail immersion test. Both the intensity and duration of the analgesic effect of a test drug could be estimated as low or high, depending on the method used to restrain the animal. Furthermore, the temperature of the water bath appears to be an important factor affecting the outcome of the test. Being simple and technically less demanding, the tail immersion test has been used by many investigators. This test has been utilized as a tool to investigate the pharmacology of the opioid system during ontogeny [33,34]. In these studies, while the tail flick latency is measured, It is possible that V.Z. extract exerts an analgesic effect probably by inhibiting the synthesis of prostaglandins.

In our study on the effect of ethyl acetate and chloroform fraction of V.Z. on carragenan induce paw oedema (200 mg/kg body weight), we found that ethyl acetate and chloroform fraction possesses a better effect on the dose of 200 mg/kg body weight. Since V.Z. is ubiquitous and abundantly grown, it could be a fairly economical therapeutic agent for inflammation management as a prohealer, as well as to control analgesic effect.

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