Comparative Study On The Analgesic, Anti-Inflammatory, And Acute Oral Toxicity Profile Of Vernonia Anthelmintica Seeds Extracted With Different Polarity Solvents

Vishal S. Adak*[1], and Mahesh K. Gupta[1]

[1]School of Pharmacy, Career Point University, Alaniya, Kota, Rajasthan- 325003
*Corresponding author:
School of Pharmacy, Career Point University, Alaniya, Kota, Rajasthan- 325003
E-mail ID: vishaladak83@gmail.com

ABSTRACT

The study investigated the anti-inflammatory, analgesic, and toxicity profiles of different polarity solvent extracts of Vernonia anthelmintica (L.) Willd. (family: Asteraceae) in albino rats and mice. The petroleum ether (VAPE), chloroform (VACE), and hydroalcoholic (VAHA) extracts were evaluated using Carrageenan-induced paw edema (CIPE) and cotton pellet-induced granuloma methods for anti-inflammatory properties, while central and peripheral pain relief activities were assessed using the hot plate and acetic acid-induced writhing test. The study also included toxicological profiling using the Up-and-Down Procedure method. Results showed that VAPE, VACE, and VAHA at a single oral dose of 2000 mg/kg were safe. These extracts demonstrated dose-dependent anti-inflammatory effects, significantly reducing inflammation in CIPE and cotton pellet-induced granuloma models. They also exhibited positive effects on serum and liver tissue biochemical parameters. Moreover, the extracts displayed central and peripheral analgesic effects, as evidenced by increased latency time in the hot plate test and reduced number of writhes induced by acetic acid in a dose-dependent manner. In conclusion, the study highlights the significant dose-dependent anti-inflammatory and analgesic activities of different polarity solvent extracts of V. anthelmintica, with VAHA showing more pronounced effects. Further research is needed to understand the underlying mechanisms of action.

Keywords: Acute oral toxicity, Analgesic, Anti-inflammatory, Dexamethasone, Indomethacin, Tramadol, Vernonia anthelmintica,

INTRODUCTION

Vernonia anthelmintica (L.) Willd., formerly known as Conyza anthelmintica, is indeed a plant belonging to the Asteraceae family.

Commonly known by various names such as kalijiri, somaraaji, black cumin, or purple fleabane, this herbaceous plant is recognized for its potential medicinal properties. The genus Vernonia comprises over a thousand species, widely distributed in subtropical and tropical regions across Asia and Africa. These species find extensive use in both food and medicine [1]. Traditional medicine has long used V. anthelmintica to treat a variety of conditions pertaining to the CNS, kidney, gynecology, GI tract, skin, metabolism, and overall health. It has been documented to exhibit diverse pharmacological activities, including antimicrobial, anticancer, antidiabetic, anti-inflammatory, analgesic, antipyretic, diuretic, and larvicidal properties [2]. A comprehensive examination of the phytochemical composition of V. anthelmintica has revealed the presence of flavones, caffeoylquinic acid derivatives, sesquiterpene, triterpenoids, elemanolide sesquiterpenoids, and guaianolide sesquiterpenoids [3].

Traditional medicines, including herbal and herbo-mineral preparations, continue to hold significance in rural areas, despite advancements in synthetic and semi-synthetic drugs for treating various ailments [4]. Herbal medicines present notable advantages, including efficacy and safety with minimal or no adverse effects, even with prolonged usage. However, these medicines may require an extended duration to manifest therapeutic effects, necessitating long-term studies to establish scientific evidence regarding their safety and efficacy [5]. Furthermore, determining the toxicological profiling of herbal medicine is essential before investigating their efficacy, especially when these plants are traditionally indicated for potential therapeutic benefits. As per World Health Organization reports, over 80% of individuals in developing countries rely on traditional medicines as their primary source of healthcare [6]. Therefore, it is mandatory to evaluate the toxicological profile of plant-based products before its efficacy investigation.

Inflammation functions as an immunological response initiated as a defense mechanism against various triggers, including infections, cell, toxins, microbes, and tissue injury. Physiologically, controlled and self-regulated inflammatory reactions are essential for cellular functions and overall survival [7]. However, sustained, and excessive concentrations can lead to severe damage to vital organs such as the lungs, liver, brain, heart, pancreas, kidneys, and different body organ systems [8]. Uncontrolled inflammatory or immunological responses are linked to the development of lifestyle diseases such as rheumatoid arthritis, diabetes, hypertension, obesity, and more [9]. While allopathic medicines like non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs are effective in treating inflammatory disorders, prolonged use of these medications can result in severe side effects like ulcers, kidney injury, and gastrointestinal bleeding [10]. Additionally, glucocorticoids may exhibit side effects such as infection susceptibility, immunosuppression, and bone damage [11].

The majority of drugs employed to alleviate inflammation, including those derived from the opium poppy plant, diminish the perception of pain in both humans and animals [12,13]. Nevertheless, these agents exhibit addictive tendencies and severe side effects such as respiratory depression, drowsiness, decreased gastrointestinal motility, nausea, endocrine disruption, and overactivation of the autonomic nervous system [14]. Therefore, it is essential to seek safer remedies, predominantly from natural sources, for the treatment of inflammatory disorders and pain.

A few scientific reports on V. anthelmintica (L.) have discussed its potential for anti-inflammatory and analgesic studies. However, to date, there has been a lack of research specifically comparing different polarity solvents extracts for assessing anti-inflammatory, analgesic, and toxicological profiles. The extraction of pharmacologically active phytonutrients is influenced by solvents of different polarity, ultimately impacting biological activity. Therefore, in the present investigation, petroleum, chloroform and hydroalcoholic extracts comparatively evaluated for anti-inflammatory, analgesic and acute oral toxicity using experimental animals. Anti-inflammatory activity was studied using CIPE and cotton pellets induced granuloma pouch model. The central and peripheral analgesic activity was done by the hot plate and acetic acid (AA) induced writhing tests respectively. Acute Oral Toxicity: Up-and-Down Procedure (Test No. 425) was used to assess toxicological profiling.

MATERIALS AND METHODS

Chemicals and reagents

λ-Carrageenan and indomethacin were procured from Sigma Aldrich, St. Louis, MO, USA. Normal saline (sodium chloride injection IP 0.9 %; w/v), indomethacin (Microcid®) capsules, dexamethasone (Dexonal®) tablets and Tramadol (Sifadol®) injection were procured from local market. The biochemical analysis and histopathology work utilized chemicals and reagents of the highest commercial quality.

Collection and authentication of plant material

The seeds of V. anthelmintica plant was procured from local market. The sample was submitted to the Agarkar Research Institute (An autonomous body under the Department of Science and Technology, Govt. of India), Pune for the identification. A voucher specimen (AUTH 22-157) was deposited in the Herbarium of Agarkar Research Institute for future reference.

Preparation of plant extracts

The dried powdered seeds of V. anthelmintica plant was extracted with petroleum ether (60–80°C) and successively extracted with chloroform and 70% ethanol (ethanol: water; 7:3 ratio) in Soxhlet extractor. The extracts, so obtained, were concentrated by solvent recovery, and evaporated to dryness at 50°C in hot air oven. Extractive

values were determined for all extracts, correctly labeled, and stored in sealed containers.

Experimental animals

Male Sprague Dawley Rats and Swiss albino mice were obtained from the Global Bioresearch Solution Pvt Ltd. Bhor, Pune. These animals were housed in polypropylene cages within a controlled environment featuring a room temperature of 22±1°C, a relative humidity, 60%-70% and a 12:12-hour light and dark cycle, all maintained in an animal facility (615/PO/Re/S/2002/CPCSEA; dated on 11th June 2002). The study procedures adhered strictly to the guidelines outlined by the Committee for Control and Supervision of Experiments on Animals (CCSEA), Govt. of India. The animal experimental study protocols (RDCOP/Pcol-11/IAEC/2022-23/11) approval was received from the Institutional Animal Ethical Committee (IAEC) at Rajgad Dnyanpeeth's College of Pharmacy, Bhor, Dist. Pune – 412 206, India, prior to the initiation of the experiment.

Phytochemical analysis

The presence of various phytoconstituents in the seeds of V. anthelmintica was examined using the petroleum ether, chloroform, and hydroalcoholic extracts, following the procedure outlined by Khandelwal [15].

Acute oral toxicity

Acute oral toxicity study was performed using the method of Test No. 425: Acute Oral Toxicity: Up-and-Down procedure [16]. Healthy female albino mice weighing 30-35 g were selected and acclimatized for one week under standard conditions. A limit test was conducted at 2000 mg/kg p.o. as a single dose, with mice fasting for overnight before dosing, although they had access to water ad libitum. The dose of vehicle or respective test compound was administered to a single mouse from each group. Observations were made closely during the initial 0.5 hour, followed by continuous monitoring for 4 hours. Feed was provided 2 hours post-dosing. Upon the survival of the treated mouse, all other animals were administered the same dose. A parallel procedure was followed for a vehicle-treated control group (0.25% Na-CMC). The various animal groups underwent careful observation for potential toxic effects within the initial 6 hours and subsequently at regular intervals throughout a 14-day period. Mice that survived were constantly observed for the occurrence of any toxic reactions, and their body weights were consistently monitored. At end of the experiment, blood samples were collected by retro-orbital plexus method and hematology analysis was performed. Blood serum was separated for biochemical evaluations. Vital organs were excised following euthanasia via cervical dislocation, washed with normal saline, and weighed.

Carrageenan induced rat paw edema model

The assessment of anti-inflammatory activity utilized the carrageenan-induced rat paw edema assay [17, 18]. Edema was induced by injecting 100 µL of a 1% freshly prepared carrageenan solution in distilled water into the right-hind paws of all groups. Prior to carrageenan injection, animals treated with a single dose of vehicle or, indomethacin (10 mg/kg) VAPE (100, 200 & 400 mg/kg), VACE (100, 200 & 400 mg/kg) or VEHA (100, 200 & 400 mg/kg), administered 60 minutes in advance. Paw thickness was measured at 0 (before carrageenan injection) and 1, 2, 3, 4, and 5 hours post-injection. The paw edema was calculated as the difference between the paw volume at "0 hour" and respective hours. The experiment was performed in two sets (day-1 and day-2) with 3-3 animals in each group. The readouts of two experiments were clubbed for the final calculations.

Cotton pellet induced granuloma pouch model

The cotton pellet-induced granuloma pouch model was conducted following the modified methods outlined by Ashok P. et al [19] and Panthong A. et al [20]. Wistar rats were organized into separate groups, each consisting of six rats. Pellets, made from absorbent cotton wool and weighing 20±1 mg, were sterilized at 120ºC for 2 hours in hot air oven. The abdominal area was carefully shaved, cleaned with 70% ethanol. In the anaesthetised rats, two cotton pellets were subcutaneously implanted in the abdomen region. Vehicle or dexamethasone or test compounds were treated orally throughout the 7-day experimental period. On the 8th day post-implantation, animals were anesthetized, and the pellets were dissected, dried at 50ºC for 22 hours, and weighed. All the group of animal's cotton pellet weight (wet and dry) were recorded. Next, the exudate weight, granuloma weight, and biochemical parameters were calculated.

Biochemical analysis

Blood was withdrawn from animal by retro-orbital plexus, centrifuged and serum was collected. The serum GOT, GPT and Total protein levels were estimated. Animals were humanely sacrificed, liver tissue was excised, washed with normal saline, homogenised using phosphate buffer saline and GOT, GPT, Total protein and alkaline phosphatase levels were analysed.

Hot plate test

The hot plate test was employed to estimate thermal hyperalgesia, following the procedure outlined by Balkrishna A. et al [17] with slight adjustments. Mice were placed within the perspex cylinder of the hot plate apparatus (Ugo Basile, Italy) maintained at 55.0 ± 0.5°C. The time taken for a discomfort behaviour (such as paw licking or jumping) was recorded as the response latency at 0 (before drug treatment), 30, 60, 90, and 120 min. This test was conducted one hour after the administration of vehicle or tramadol (40 mg/kg; i.p.)

or VAPE (100, 200 & 400 mg/kg) or VACE (100, 200 & 400 mg/kg) or VEHA (100, 200 & 400 mg/kg). To prevent potential accidental paw damage, a maximum cut-off time of 18 seconds was set. This test was analysed in a blinded manner by a researcher unaware of the treatment conditions.

Acetic acid (AA) induced writhing test

To assess the peripheral analgesic properties of different extracts derived from V. anthelmintica seeds, the AA-induced writhing test was employed, according to the protocol outlined by Uddin M.M.N. et al [21] and Bhuiyan M.M.R. et al [22] with slight modifications. All the animals were allocated into different groups and treated orally with vehicle or, indomethacin (10 mg/kg) VAPE (100, 200 & 400 mg/kg), VACE (100, 200 & 400 mg/kg) or VEHA (100, 200 & 400 mg/kg). Pain induction was achieved by intraperitoneally administering 1% AA (0.1 mL/10 g body weight), after 40 minutes of drug administration. After the injection of AA, the cumulative count of abdominal writhing responses was observed for a duration of 10 minutes, starting five minutes post-injection. The percentage inhibition of writhing, representing analgesic activity, was calculated using the following formula:

Inhibition (%) = (No. of writhes in control animals – No. of writhes in drug treated animals) X 100
(No. of writhes in control animals)

Statistical analysis

The data are presented as mean ± standard deviation (SD; n=6, except n=5 for acute oral toxicity) for each group. Statistical analysis was performed using GraphPad Prism software 5.0. A One-way or Two-way analysis of variance (ANOVA) was conducted, followed by Dunnett's multiple comparison t-test or Bonferroni's multiple comparison t-test, respectively, to determine statistical differences. Significance levels were denoted as *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to the diabetic control group, and #$p<0.05$ compared to the normal control.

RESULTS & DISCUSSION

Phytochemical analysis

The petroleum ether, chloroform and hydroalcoholic extracts of V. anthelmintica seeds investigated for the presence of various phytoconstituents. The VAHA showed the presence of alkaloids, carbohydrates, glucosides, phytosterols, saponins, phenolic compounds and tannins, proteins and amino acids and flavonoids at moderate to strong levels. The VACE displayed the existence of alkaloids, carbohydrates, glucosides, phytosterols, fixed oils and fats, saponins, phenolic compounds and tannins at mild levels and flavonoids at moderate levels. However, VAPE exhibited moderate levels of fixed oils and fats (Table 1).

Table 1. Phytochemical analysis of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica

Phytochemical tests/reagent(s) used	Pet. ether extract of V. anthelmintica	Chloroform extract of V. anthelmintica	Hydroalcoholic Extract of V. anthelmintica
Alkaloids			
Dragendorff's test	-	+	+++
Hager's test	-	+	+++
Wagner's test	-	+	++
Carbohydrates			
Molisch's test	-	+	++
Barfoed's test	-	+	++
Benedict's test	-	+	++
Glycosides			
Molisch's test after hydrolysis	-	+	+++
Physterols			
Liebermann's Burchard's test	+	+	+++
Fixed oils and fats			
Spot test	++	+	-
Saponification test	++	+	-
Saponins			
Foam test	-	+	+++
Haemolysis test	-	+	++
Phenolic compounds and tannins			
Ferric chloride test	-	+	++
Lead acetate test	-	+	++
Proteins and amino acids			
Biuret test	-	-	++
Ninhydrin test	-	-	++
Flavonoids			
Shinoda test	**-**	**++**	**+++**

Mild: +; Moderate: ++; Strong: +++; - Absent

Acute oral toxicity

Behavioral observations and body weight

The effect of Petroleum ether, chloroform and hydroalcoholic extracts of V. anthelmintica seeds treated at 2000 mg/kg on behavioural patterns and body weight is displayed in Table 2 and 3. No animals were found with convulsions & tremors, coma in treated with VAPE, VACE and VAHA at the dose of 2000 mg/kg. All the animal's eyes, fur and skin, mucous membrane, somato motor activity & behaviour pattern, Sleep, Urination (colour) were found normal. Salivation was slightly increased in VACE treated mice for first 30 min. However, respiration rate was elevated in the animals administered with VAPE and lowered in the VAHA treated mice after 30 min of drug

administration. There was no animal mortality observed in any group till day-14. Animals treated with VAPE, VACE and VAHA displayed no itching behaviour throughout the experimental period. VACE and VAHA showed no significant ($p>0.05$) effect on body weight (absolute and body weight change). However, VAPE treated animals displayed significant increase in body weight change in comparison to control animals.

Table 2. Effect of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica on behavioural responses (acute oral toxicity)

Parameters	Observations																							
	30 Min				4 h				24 h				48 h				Day-7				Day-14			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Convulsions & tremors	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Coma	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Consistency of faeces	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr
Eye	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr
Fur and Skin	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr
Itching	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Mucous membrane	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr
Mortality	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF
Respiration rate	Nr	E	Nr	L	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr
Salivation	Nr	Nr	E	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr
Somatomotor activity & behaviour	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr
Urination (colour)	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr
Sleep	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr	Nr

A: Normal control; B: VAPE; C: VACE; D: VAHA; Nr: Normal; NF: Not Found; E: Elevated; L: Lowered

Organ weight

Animal's kidney weight (relative) was significantly elevated in VACE ($p<0.01$) treated groups in comparison to the normal mice. However, data displayed no significant ($p>0.05$) variations in the mice's heart and liver weight ($p>0.05$) treated with VAPE, VACE and VEHA (Table 3).

Table 3. Effect of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica on animal body weight change and relative organ weight (acute oral toxicity)

Groups	Body Weight Change (%)			Relative Organ Weight (Per 10g)		
	Day-1	Day-7	Day-14	Heart	Kidney	Liver

NC	1.77±0.91	0.94±1.01	2.80±2.10	0.072±0.00	0.140±0.01	0.644±0.01
VAPE-2000	1.13±0.64	2.17±2.19	6.21±2.41*	0.074±0.00	0.155±0.02	0.673±0.03
VACE-2000	2.61±1.17	1.67±1.81	4.15±3.50	0.076±0.00	0.186±0.02**	0.678±0.05
VAHA-2000	2.69±0.75	3.20±2.23	4.59±3.00	0.076±0.01	0.169±0.01	0.626±0.03

Values in the results are expressed as mean ± SD (n=5). *p<0.05, **p<0.01, ***p<0.001, significantly different in comparison to normal control at respective time points. (Body weight change- A Two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison t-test; Relative organ weight- A One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison t-test)

Renal and liver function tests

The effect of VAPE, VACE and VAHA on renal and liver function test is displayed in Table 4. Serum creatinine and urea level were not significantly (p>0.05) altered in the treatment groups as compared to normal mice. Furthermore, in liver functions tests, SGOT, SGPT, total proteins, total bilirubin, alkaline phosphatase, albumin, and globulin level were found to be normal (p>0.05) in VAPE, VACE and VAHA treated animals.

Table 4. Effect of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica on renal and liver function tests (acute oral toxicity)

Groups	Renal Function Test		Liver Function Test						
	Creatinine (mg/dL)	Urea (mg/dL)	SGOT (U/L)	SGPT (U/L)	Alkaline Phosphatase (U/L)	Total Bilirubin (mg/dL)	Total Protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)
NC	0.69±0.14	57.47±6.38	90.22±11.09	55.40±7.56	158.61±8.95	0.26±0.03	7.28±1.45	3.55±0.41	3.33±0.17
VAPE-2000	0.65±0.15	58.66±5.39	90.52±7.05	59.28±6.57	157.84±7.91	0.28±0.03	7.18±0.92	3.65±0.42	3.48±0.31
VACE-2000	0.70±0.14	57.73±7.72	83.74±7.83	56.54±6.97	160.46±7.42	0.26±0.03	8.00±1.18	3.29±0.40	3.31±0.27
VAHA-2000	0.75±0.17	55.16±4.47	91.50±10.52	55.84±4.47	155.70±4.85	0.25±0.04	7.17±1.63	3.74±0.21	3.34±0.27

Values in the results are expressed as mean ± SD (n=5). *p<0.05, **p<0.01, ***p<0.001, significantly different in comparison to normal control. (A One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison t-test)

Lipid profile

Total cholesterol, triglycerides, HDL-c and LDL-c levels showed no significant ($p>0.05$) alterations in the VAPE, VACE and VAHA treated mice compared to normal animals (Table 5).

Table 5. Effect of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica on lipid profile (acute oral toxicity)

Groups	Lipid Profile			
	Total Cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL-c (mg/dL)	LDL-c (mg/dL)
NC	90.56±7.24	98.20±8.79	36.44±5.60	34.22±4.36
VAPE-2000	94.89±7.15	96.11±8.49	38.40±6.69	33.32±4.74
VACE-2000	86.69±6.58	95.82±9.50	35.64±6.66	34.15±4.59
VAHA-2000	88.94±7.17	93.55±9.73	36.52±6.19	34.70±3.77

Values in the results are expressed as mean ± SD (n=5). *p<0.05, **p<0.01, ***p<0.001, significantly different in comparison to normal control. (A One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison t-test)

Hematology parameters

Table 6 illustrates the effect of VAPE, VACE and VAHA on haematology parameters tested at 2000 mg/kg. Blood parameters Haemoglobin, RBCs, WBCs, platelets, differential WBCs viz. monocytes, neutrophils lymphocytes, eosinophiles and basophiles were found no considerable ($p>0.05$) variations by the treatment of test compounds. However, monocytes levels we prominently ($p<0.01$) decreased by the VAHA treatment.

Table 6. Effect of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica on haematology parameters (acute oral toxicity)

Groups	Haematology Parameters								
	Hb (g/dL)	RBCs (10^6/µL)	WBCs (10^3/µL)	Platelets (10^3/µL)	Monocytes (%)	Neutrophiles (%)	Lymphocytes (%)	Eosinophiles (%)	Basophiles (%)
NC	15.55±0.63	5.90±0.29	5.59±0.43	763.80±39.33	1.82±0.46	32.40±5.59	60.40±11.93	2.00±1.00	0.40±0.55
VAPE-2000	15.75±0.80	5.68±0.49	5.67±0.88	792.40±25.00	1.60±0.55	30.20±3.03	61.80±9.98	1.80±0.84	0.20±0.45
VACE-2000	15.54±0.51	5.54±0.60	5.57±0.32	778.20±29.18	1.60±0.55	28.60±5.73	63.00±10.37	2.20±0.84	0.20±0.45
VAHA-2000	15.23±1.18	5.88±1.11	5.10±0.32	762.20±63.76	0.60±0.55**	31.00±5.00	56.40±9.02	2.00±1.00	0.40±0.55

Values in the results are expressed as mean ± SD (n=5). *p<0.05, **p<0.01, ***p<0.001, significantly different in comparison to normal control. (A One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison t-test)

Carrageenan induced rat paw edema model

Table 7 represents the paw volume differences at different time points (1 h, 2 h, 3 h, 4 h, 5 h) for various treatment groups with different doses. The control group exhibited increasing paw volume differences over time, while treatment groups such as VAPE at 200 and 400 mg/kg demonstrated notable reductions in paw volume at 3 ($p<0.05$), 4 hr ($p<0.05$) and 2 ($p<0.01$) to 5 ($p<0.01$) hr respectively. The dose-dependent responses observed in several treatment groups highlight the significance of dosage in influencing the outcomes. However, VACE at 100, 200 and 400 mg/kg displayed prominent reduction in paw edema at 4hr ($p<0.05$), 3-4hr ($p<0.01$) and 2-5 hr ($p<0.01$) respectively. Similarly, VEHA at 100, 200 and 400 mg/kg exhibited prominent anti-inflammatory effect by lowering paw edema at 4-5hr ($p<0.05$), 2-5hr ($p<0.01$) and 2-5 hr ($p<0.001$) respectively. The anti-inflammatory effect shown by the test compounds, VAPE, VACE and VEHA was found to be in dose-dependent manner. The standard drug, indomethacin (10 mg/kg) displayed significant ($p<0.001$) reduction in paw edema at all time points.

Table 7. Effect of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica on carrageenan induced paw edema

Groups	Paw Edema (mL) at Different Time Points (% Anti-inflammatory Activity)				
	1 h	**2 h**	**3 h**	**4 h**	**5 h**
NC	0.22±0.08 (0.00±0.00)	0.88±0.17 (0.00±0.00)	1.09±0.09 (0.00±0.00)	1.03±0.06 (0.00±0.00)	0.90±0.13 (0.00±0.00)
INDO-10	0.11±0.06* (51.91±26.67)	0.20±0.06*** (77.17±7.35)	0.35±0.06*** (67.90±5.36)	0.50±0.16*** (50.97±15.62)	0.43±0.08*** (51.96±8.77)
VAPE-100	0.19±0.04 (15.39±13.95)	0.88±0.05 (2.14±2.85)	1.04±0.07 (5.30±5.66)	0.95±0.04 (7.95±4.17)	0.82±0.08 (8.01±8.38)
VAPE-200	0.14±0.06 (36.01±25.20)	0.81±0.06 (8.87±6.67)	0.98±0.05* (9.37±5.00)	0.92±0.03* (10.71±2.66)	0.80±0.03 (10.61±2.91)
VAPE-400	0.14±0.05 (37.40±21.22)	0.75±0.05** (15.09±6.08)	0.93±0.04*** (13.98±3.38)	0.90±0.03** (12.18±2.92)	0.77±0.03** (14.53±3.85)
VACE-100	0.16±0.04 (26.84±16.13)	0.83±0.10 (8.74±8.53)	0.99±0.09 (9.60±7.91)	0.91±0.05* (11.85±5.29)	0.81±0.06 (10.06±6.58)
VACE-200	0.15±0.03 (29.77±14.09)	0.79±0.04 (10.75±4.93)	0.88±0.04*** (18.74±3.93)	0.88±0.05** (13.96±4.80)	0.80±0.07 (11.17±8.05)
VACE-400	0.13±0.05 (39.69±23.43)	0.73±0.05** (16.98±5.53)	0.78±0.06*** (28.11±5.11)	0.81±0.04*** (21.59±3.73)	0.73±0.08*** (18.81±8.73)
VAHA-100	0.17±0.04 (21.37±16.75)	0.82±0.12 (9.87±10.49)	0.99±0.10 (8.83±8.27)	0.91±0.05* (11.36±4.73)	0.77±0.07** (14.15±8.13)
VAHA-200	0.16±0.03 (25.95±13.41)	0.74±0.04** (16.23±4.17)	0.84±0.04*** (22.27±3.85)	0.81±0.07*** (20.94±6.62)	0.76±0.07** (15.64±7.67)
VAHA-400	0.14±0.04 (35.88±20.07)	0.52±0.07*** (41.13±7.78)	0.64±0.05*** (41.47±4.39)	0.65±0.13*** (36.69±12.49)	0.68±0.10*** (23.65±10.89)

Values in the results are expressed as mean ± SD (n=6). *p<0.05, **p<0.01, ***p<0.001, significantly different in comparison to normal control at respective time points. (A Two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison t-test)

Cotton pellet induced granuloma pouch model

The results represented in Table 8 reveal substantial variations in inflammatory parameters across different treatment groups studied by cotton pellet induced granuloma pouch model. Animals treated with VAPE at 100 (p>0.05), 200 (p<0.05) and 400 mg/kg (p<0.001) demonstrated considerable reduction in granuloma wet weight. However, VAPE showed marginal effect (p>0.05) on inhibition of dry weight, exudate, and granuloma weight at treated doses levels. VACE treated animals at 200 (p<0.01) and 400 mg/kg (p<0.001) exhibited substantial decline in wet weight granuloma. However, exudate weight (45.78±11.7% Inhibition) was prominently (p<0.01) reduced by the VACE only at high dose, 400 mg/kg. Moreover, animals administered with VEHA at 200 mg/kg displayed considerable reduction in wet weight (p<0.001), dry weight (p<0.001) and granuloma weight (p<0.01). A high dose of VEHA (400 mg/kg) was found to be effective in lowering wet weight (p<0.001), dry weight (p<0.001), exudate (p<0.01) and granuloma weight (p<0.001) significantly. The inhibition of exudative and granuloma weight was displayed as 48.51±12.3 and 41.54±19.4% respectively. The standard drug, dexamethasone exhibited significant inhibition (p<0.001) in wet, dry weight granuloma, exudate, and granuloma weight at 5 mg/kg dose.

In the biochemical analysis, serum GOT, GPT and total protein levels were significantly (p<0.001) elevated in disease control animals compared to normal. Animals treated with VAPE at 400 mg/kg displayed significant reduction in SGOT (p<0.001) and SGPT levels (p<0.01). However, there were no significant changes observed in total protein levels. Similarly, treatment of VACE at 400 mg/kg was effective in considerable decline in elevated SGOT (p<0.001), SGPT (p<0.001) and total protein levels(p<0.01). VACE at 200 mg/kg dose showed significant (p<0.01) inhibitory effect in GOT enzymes. Furthermore, serum GOT (p<0.001), GPT (p<0.001) and total proteins (p<0.05) levels were significantly lowered by VEHA at 400 mg/kg. However, 200 mg/kg dose of VEHA displayed substantial reduction in SGOT (p<0.001) and SGPT (p<0.05) levels (Figure 1, A-C). Similarly, elevated levels of liver tissue GOT was significantly lowered by VACE (200; p<0.05 & 400 mg/kg; p<0.001) and VAHA at 400 mg/kg (p<0.01). GPT level was prominently (p<0.001) reduced by VAPE, VACE and VAHA at 200 and 400 mg/k except VAPE only at 400 mg/kg. Total proteins levels were significantly declined by VAPE (p<0.05), VACE (p<0.001) and VAHA at 400 mg/kg (p<0.001). However, VAHA displayed additional effect at 200 mg/kg (p<0.05). Elevated level of ALP was considerable decreased by VACE (400 mg/kg; p<0.05) and

VAHA at 200 ($p<0.05$) and 400 mg/kg ($p<0.01$). The dexamethasone, standard drug was found effective ($p<0.001$) in lowering elevated levels of all the liver tissues biochemical parameters (Figure 2, A-D).

Table 8: Effect of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica on cotton pellet induced granuloma test

Groups	Wet weight (mg)	Dry weight (mg)	Exudate weight (mg)	Exudative Inhibition (%)	Granuloma weight (mg)	Granuloma Inhibition (%)
NC	211.27±10.7	86.83±8.2	124.44±16.9	0.00±0.00	66.83±8.2	0.00±.00
DEXA-5	130.89±11.9***	44.59±12.8***	86.30±20.3***	55.10±16.3	24.59±12.8***	63.20±.19.2
VAPE-100	201.35±2.8	87.32±4.9	114.03±6.4	32.81±5.2	67.32±4.9	2.69±.4.0
VAPE-200	193.83±8.5*	82.97±9.5	110.86±15.0	35.36±12.0	62.97±9.5	9.21±.8.3
VAPE-400	183.10±14.7***	73.39±7.1	109.71±10.9	36.29±8.8	53.39±7.1	20.11±.10.6
VACE-100	199.82±6.8	82.74±3.5	117.09±9.5	30.35±7.6	62.7±3.5	6.13±.5.3
VACE-200	187.93±7.1**	75.03±6.1	112.90±7.1	33.72±5.7	55.0±6.1	17.65±.9.2
VACE-400	171.89±14.4***	74.01±6.1	97.89±14.6**	45.78±11.7	54.0±6.1	19.19±.9.1
VAHA-100	201.70±4.9	82.84±6.6	118.86±3.7	28.93±3.0	62.8±6.6	7.14±.8.6
VAHA-200	178.14±13.8***	68.56±9.9**	109.58±7.5	36.39±6.1	48.6±9.9**	27.34±.14.8
VAHA-400	153.56±11.1***	59.07±13.0***	94.49±15.3**	48.51±12.3	39.1±13.0***	41.54±.19.4

Values in the results are expressed as mean ± SD (n=6). *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different in comparison to normal control. (A One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison t-test)

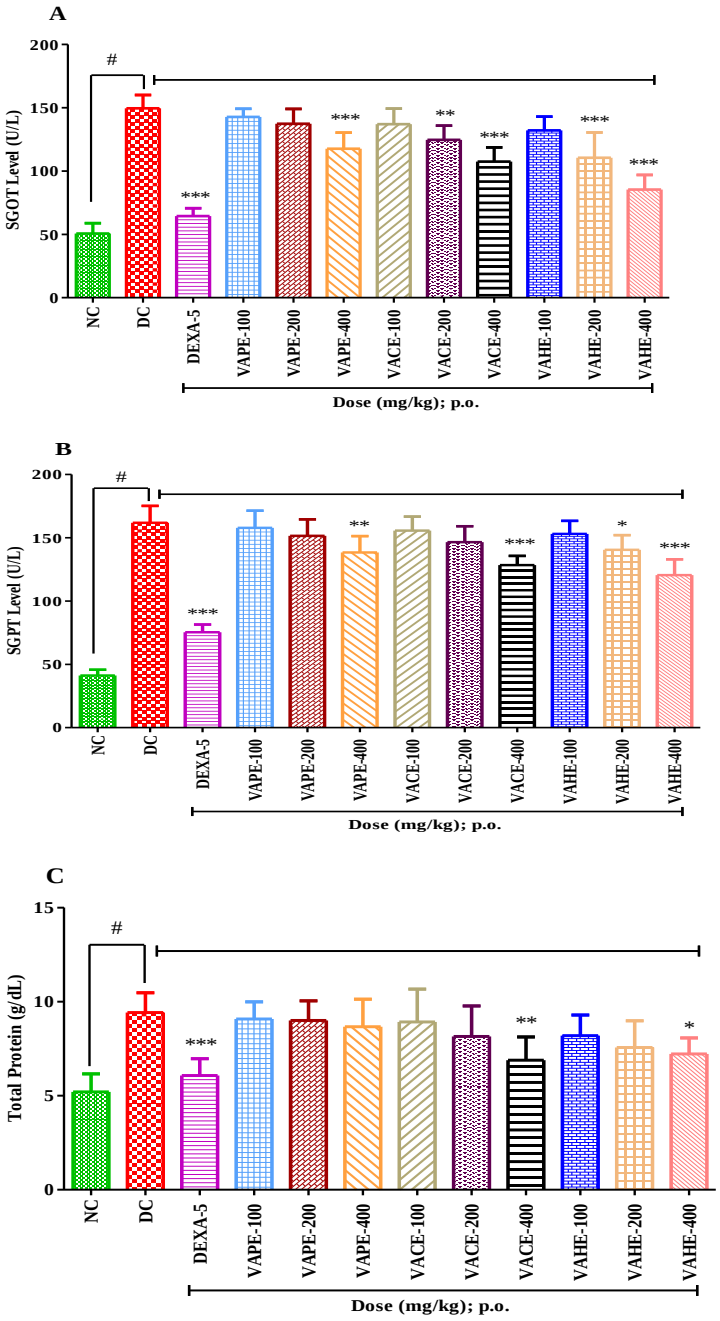

Figure 1: Effect of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica on serum enzymes in rats with cotton pellet-induced granuloma test A) SGOT level B) SGPT level C) Total Protein. Values in the results are expressed as mean ± SD (n=6). *p<0.05, **p<0.01, ***p<0.001, significantly different in comparison to disease control. #p<0.05, significantly different in comparison to normal control. (A One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison t-test) **Abbreviations:** NC: Normal control; DC: Disease control; DEXA 5: Dexamethasone 5 mg/kg; VAPE: Vernonia anthelmintica petroleum ether extract; VACE: Vernonia anthelmintica chloroform extract; VAHE: Vernonia anthelmintica hydroalcoholic extract

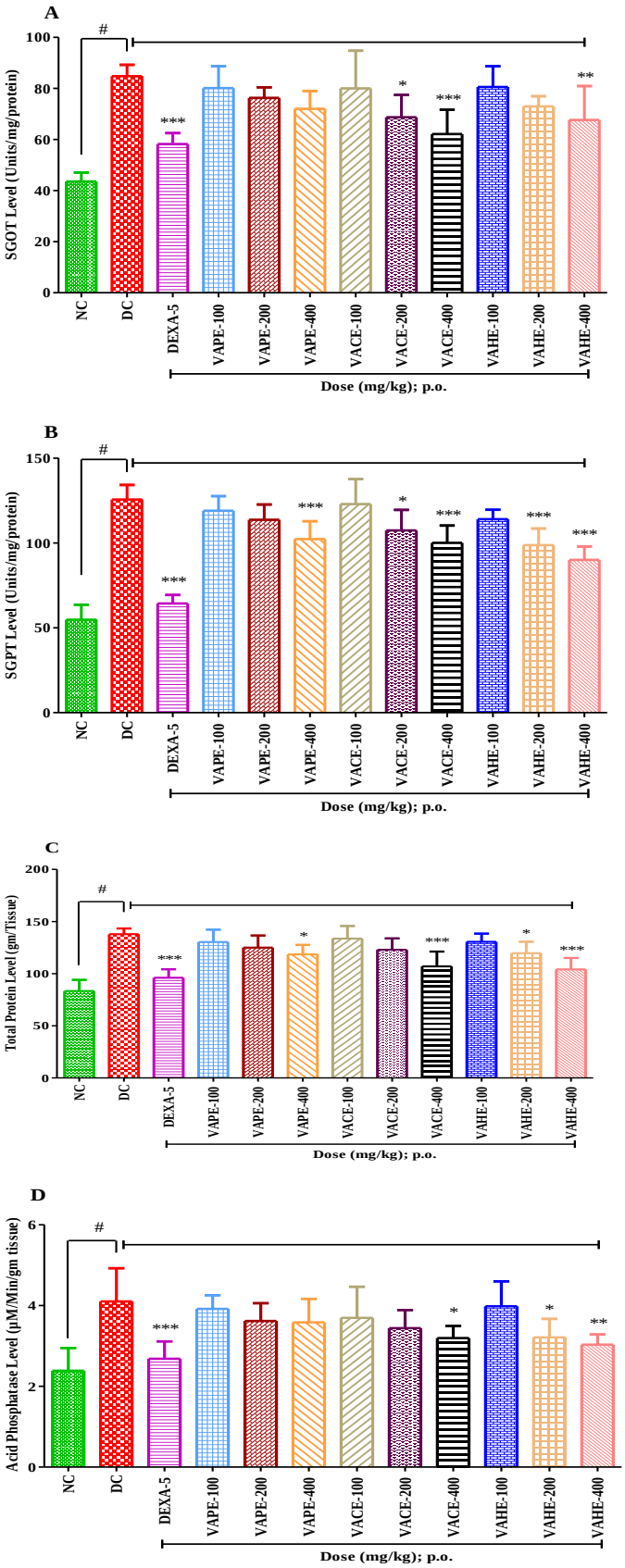

Figure 2: Effect of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica on liver enzymes in rats with cotton pellet-induced granuloma test A) SGOT level B) SGPT level C) Total Protein D) Acid Phosphatase. Values in the results are expressed as mean ±

SD (n=6). *p<0.05, **p<0.01, ***p<0.001, significantly different in comparison to disease control. #p<0.05, significantly different in comparison to normal control. (A One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison t-test) **Abbreviations:** NC: Normal control; DC: Disease control; DEXA 5: Dexamethasone 5 mg/kg; VAPE: Vernonia anthelmintica petroleum ether extract; VACE: Vernonia anthelmintica chloroform extract; VAHE: Vernonia anthelmintica hydroalcoholic extract.

Hot plate test

Figure 3 illustrates the analgesic effect of VAPE, VACE and VAHA using hot plate test. Mice treated with VAPE at 200 (p<0.05) and 400 mg/kg displayed significant rise in latency time at 30, 60, 90 and 60 and 90 min compared to normal animals respectively, confirming its analgesic effect. Further, 200 (p<0.05) and 400 mg/kg (p<0.01) dose of VACE displayed substantial rise in latency time at 30-, 60-, and 90-min. Administration of VAHA at doses of 200 mg/kg and 400 mg/kg resulted in a considerable (p<0.001) rise in latency time at 30, 60, and 90 minutes, supporting its analgesic effects. VAHA and VACE at 400 mg/kg represented pain relieving effect (p<0.01) till 120 min. Notably, the Tramadol-treated group demonstrated a significant (p<.001) increase in latency times compared to the normal control, suggesting a prolonged analgesic effect.

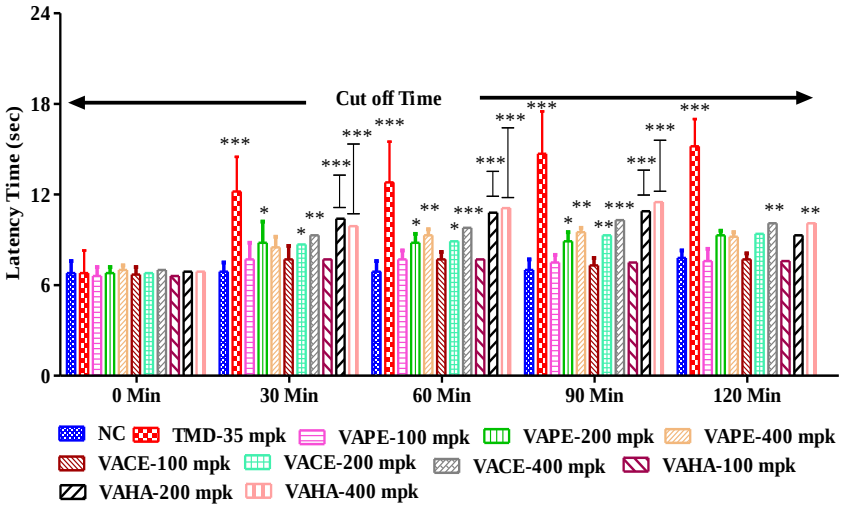

Figure 3: Effect of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica on hot plate test (Latency time). Values in the results are expressed as mean ± SD (n=6). *p<0.05, **p<0.01, ***p<0.001, significantly different in comparison to normal control. (A One-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison t-test) **Abbreviations:** NC: Normal control; TMD-35 mpk: Tramadol-35 mg/kg; VAPE: Vernonia anthelmintica petroleum ether extract; VACE: Vernonia anthelmintica chloroform extract; VAHE: Vernonia anthelmintica hydroalcoholic extract

Acetic acid induced writhing test

The writhing responses and the percentage of inhibition is displayed in Figure 4. Indomethacin displayed a highly significant ($p<0.001$) reduction in writhes, demonstrating a substantial 55.11% inhibition and confirming its potent analgesic effect. VAPE administered mice prominently ($p<0.01$) reduced numbers of writhes particularly at high dose of 400 mg/kg (14.49% inhibition). Additionally, mice treated with VACE at 200 ($p<0.05$) and 400 ($p<0.001$) mg/kg represented noticeable decrease in withing production, providing support for its analgesic effects. Furthermore, VAHA treated animals exhibited substantial ($p<0.001$) decline in writhing count at 200 and 400 mg/kg, with % inhibitory activity being 36.58% & 47.98% respectively. These findings suggest dose-dependent analgesic effects for the investigated compounds, with the VAHA showing a more pronounced effect than the VAPE and VACE.

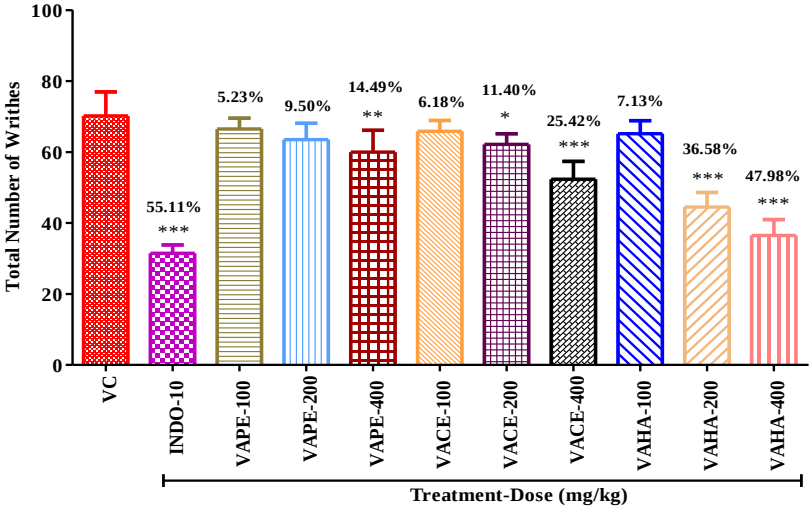

Figure 4: Effect of petroleum ether, chloroform and hydroalcoholic extract of V. anthelmintica on acetic acid induced writing test. Values in the results are expressed as mean ± SD (n=6). *p<0.05, **p<0.01, ***p<0.001, significantly different in comparison to vehicle control. (A One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison t-test) **Abbreviations:** VC: Vehicle control; INDO 10: Indomethacin 10 mg/kg; VAPE: Vernonia anthelmintica petroleum ether extract; VACE: Vernonia anthelmintica chloroform extract; VAHE: Vernonia anthelmintica hydroalcoholic extract.

DISCUSSION

A preliminary toxicological assessment is required to verify the safety of herbal medicines [23]. In the present investigation, the acute oral toxicity profile of the VAPE, VACE and VAHA was studied using OECD guideline 425. Among many other toxicity indicators, clinical signs and symptoms are the primary observations that reveal the toxic effects of drugs on essential body organs [24]. The results indicated, no adverse effects such as convulsions, tremors, or coma were observed. Salivation increased slightly in VACE-treated mice, and

respiration rates varied. However, no mortality or itching behaviour occurred, indicating general safety. VAPE-treated animals exhibited a significant increase in body weight change. Kidney weight showed elevation in VACE group. The heart and liver weights remained unaffected. Further, serum creatinine and urea levels were within normal ranges across all treatment groups, signifying no adverse effects on renal function. Liver function tests, including SGOT, SGPT, total proteins, total bilirubin, alkaline phosphatase, albumin, and globulin levels, were normal, suggesting no hepatotoxicity. The lipid profile, encompassing total cholesterol, triglycerides, HDL-c, and LDL-c levels, exhibited no significant alterations in VAPE, VACE, and VAHA treated mice compared to normal animals, indicating lipid homeostasis. Haematological parameters play a crucial role as sensitive indicators, reflecting physiological changes in animals in response to environmental pollutants or exposure to toxic stressors [25]. These results suggest haemoglobin, RBCs, WBCs, platelets, and differential WBCs, remained unaffected by the treatment, suggesting the absence of hepatotoxicity. Notably, monocyte levels were decreased in the VAHA group. In brief, the study demonstrates the safety profile of VAPE, VACE, and VAHA at 2000 mg/kg, with minimal impact on behavioural parameters. The observed effects on behavioural response, body weight and organ weight indicate potential physiological responses that requires further investigation.

The anti-inflammatory potential of VAPE, VACE and VEHA were investigated using carrageenan induced rat paw edema model. The observed outcomes align with the biphasic nature of the inflammatory response following carrageenan injection. The dose-dependent reduction in paw edema, particularly evident in VAPE at 200 and 400 mg/kg, corresponds to the late accelerating phase (2–6 h after injection of carrageenan). This phase involves increased production of polyphenols (PGs), oxygen-derived free radicals, and cyclooxygenase-2 (COX-2). The anti-inflammatory effects of VACE at 100, 200, and 400 mg/kg, as well as VEHA at 100, 200, and 400 mg/kg, support their potential modulation of these late-phase mediators. Furthermore, the considerable reduction in paw edema at 2 hr for VAPE, VACE and VEHA aligns with the early phase of inflammation (0–2 h after injection of carrageenan). This phase is characterized by the involvement of inflammatory mediators like histamine, 5-hydroxytryptamine, and bradykinin [26, 27]. The results suggest that V. anthelmintica seed extracts may influence both early and late phases of inflammation, confirming as a potential anti-inflammatory agent. The standard drug, indomethacin, serving as a reference, further supports the efficacy of the tested extracts in modulating the inflammatory response at various stages.

The cotton pellet granuloma method is a widely adopted approach for evaluating the transudative, exudative, and proliferative aspects of subacute inflammation [19]. The wet weight of the granuloma is notably influenced by the fluid absorbed by the pellet, whereas the dry weight provides a reliable measure of the

granulomatous tissue formed. The present study findings suggested that, VAPE, VACE and VEHA displayed substantial reduction in wet weight of granuloma, confirm their effect on vascular permeability. To examine the effect of test compounds on proliferative phase of inflammation, dry weight of granuloma was measured. VAPE, VACE and VEHA. Among all these test compounds, only VEHA at 200 and 400 mg/kg displayed positive impact on proliferative phase of inflammation. Most of the studies reported that, NSAIDs like diclofenac exhibits marginal inhibitory effect on granuloma formation. However, the steroidal drug exhibits significant granuloma inhibition. Similarly, dexamethasone at 5 mg/kg noticeably reduced granuloma weight. During the cotton pellet-induced granuloma test, evaluations were conducted on both blood serum and liver homogenate (10% w/v in Tris HCl Buffer) for activities related to Glutamate Oxaloacetate Transferase (GOT), Glutamate Pyruvate Transferase (GPT), Total Proteins, and Acid Phosphatase (ACP). The results indicated VAPE, VACE and VAHA considerable reduced the levels of GPT, GOT, and Total Proteins in both blood serum and liver tissue (except VAPE on serum total proteins and liver tissue GOT). Moreover, a dose-dependent inhibition of acid phosphatase activity in liver tissue was observed by VACE and VAHA. The observed inhibitory effects on GOT and GPT activities by these plant extracts may influence the formation of polypeptides like bradykinin and other kinin-like substances released during the inflammatory process. Additionally, the dose-dependent inhibition of acid phosphatase suggests a potential interference with the synthesis of lysosomal enzymes by anti-inflammatory drugs, thereby stabilizing the lysosomal membrane and impacting the inflammatory process, as suggested by previous studies [27].

The analgesic properties of the test compounds were assessed through the hot plate and acetic acid-induced writhing tests. The hot plate test is employed to investigate central analgesic activity in animal models utilizing thermal stimuli as pain inducers. These approaches emphasize modifications above the spinal cord level, offering a meaningful depiction of centrally mediated anti-nociceptive responses [28, 29]. In the current studies, VAPE, VACE, and VAHA exhibited a noteworthy increase in latency time compared to control animals, confirming a central anti-nociceptive effect. In this method, pain is induced through a supra-spinal reflex that engages μ1, κ3, δ1, and σ2 opioid receptors. From the study outcomes, it can be suggested that the analgesic effect of the plant extracts could be due to alterations in the pain induction via supra-spinal reflex pathways. The acetic acid-induced writhing response method is a commonly employed technique for evaluating the peripheral analgesic activity of plant components, utilizing acetic acid as a primary pain inducer in animal models. This response is believed to be initiated by peritoneal mast cells and prostaglandin pathways. After the intraperitoneal administration of acetic acid, an increased release of inflammatory mediators, including substance P, bradykinin,

serotonin, histamine, and prostaglandins, occurs, and this subsequent release triggers abdominal constrictions and the pain sensation [30, 31]. The study results suggest that the analgesic effect of VAPE, VACE, and VAHA may be attributed to the presence of pharmacologically active compounds in their extracts, which contribute to the inhibition of inflammatory mediators.

CONCLUSION

Based on the study findings, it can be demonstrated that the administration of VAPE, VACE, and VAHA at a single oral dose of 2000 mg/kg was determined to be safe. Additionally, the extracts exhibited significant dose-dependent anti-inflammatory effects, potentially attributed to the presence of pharmacologically active compounds inhibiting various inflammatory mediators such as serotonin, histamine, prostaglandins, substance P, and bradykinin. The plant extracts also showcased both central and peripheral analgesic effects, possibly through the inhibition of supra-spinal reflex pathways and inflammatory mediators, respectively. Notably, VAHA displayed a more pronounced effect on reduction of inflammation and nociceptive pain compared to VAPE and VACE. However, further studies are recommended to elucidate the exact mechanism of action.

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation**.**

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