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ANTINOCICEPTIVE, ANTI-INFLAMMATORY, AND ANTIOXIDANT STUDIES ON *WITHANIA SOMNIFERA* (L.) DUNAL

NAVID BAGHALPOUR[1], JAVAD MAHROO BAKHTIYARI[1], ZAINAB M. ALMARHOON[2], DEEPAK CHANDRAN[3], AFNAN KHAN SHINWARI[6], MANOJ KUMAR[4], JAVAD SHARIFI-RAD[5*] AND ZABTA KHAN SHINWARI[6,7*]

[1]*Department of Pharmacology and Toxicology, School of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran: navid.bp1994@gmail.com; javad.bakhtiari1372@gmail.com*
[2]*Department of Chemistry, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia; zalmarhoon@ksu.edu.sa*
[3]*Department of Veterinary Sciences and Animal Husbandry, Amrita School of Agricultural Sciences, Amrita Vishwa Vidyapeetham University, Coimbatore 642109, India; c_deepak@cb.amrita.edu*
[4]*Chemical and Biochemical Processing Division, ICAR—Central Institute for Research on Cotton Technology, Mumbai 400019, India; manojkumarpuniya114@gmail.com*
[5]*Facultad de Medicina, Universidad del Azuay, Cuenca, Ecuador*
[6]*Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan*
[7]*Pakistan Academy of Sciences, Islamabad- Pakistan*
[*]*Corresponding authors: javad.sharifirad@gmail.com; shinwari2008@gmail.com*

Abstract

The goal of this study is to evaluate the antinociceptive and anti-inflammatory activities of various leaf extracts (methanolic extract, chloroform fraction (1), chloroform fraction (2), ethyl acetate, butanol, and hydroalcoholic fractions) of *Withania somnifera* (L.) Dunal plant using high-performance thin-layer chromatography (HPTLC). Total tannins content (TTC), total flavonoid content (TFC), and total phenolic content (TPC) were measured to estimate the polyphenol content. Four different approaches were used to assess the antioxidant potential of the plant. The carrageenan and formalin tests were used to assess anti-inflammatory and antinociceptive properties in rats respectively. The methanolic extract (100 mg/kg and 300 mg/kg), all fractions (100 mg/kg and 300 mg/kg), Diclofenac sodium (25 mg/kg), and normal saline were given to 156 Wistar rats via oral gavage. Also, the acute toxicity of the methanolic extract was analyzed on twenty rats. In comparison to the control group, treatment with the methanolic extract at doses of 100 and 300 mg/kg, both doses of chloroform fraction (1) (30 mg/kg and 100 mg/kg), and butanol fraction 100 mg/kg significantly reduced the pain score in the formalin test as well as the carrageenan paw edema. At the tested dose, the acute toxicity test revealed that it was non-toxic. Furthermore, in the assessments, there was moderate amounts of flavonoid content in these extracts. In conclusion, the present study revealed the phytochemicals present in *Withania somnifera* (L.) Dunal whole plant has anti-inflammatory, antinociceptive, and anti-oxidant effects. Further studies are needed to recognize the exact bioactive compounds that possess these properties and the mechanism of these potential effects in the plant.

Key words: *Withania somnifera* (L.) Dunal, Anti-inflammatory, Analgesic, Antioxidant.

Introduction

Pain is a critical healthcare concern associated with prospective or actual tissue damage; and besides, it is characterized as an unpleasant feeling that drives people to seek pain reduction measures, such as using natural products and their derivatives as an anti-inflammatory agent (Al-Snafi, 2018; Baghalpour *et al.*, 2021; Taheri *et al.*, 2021, Ahmad *et al*., 2022). In recent years, non-steroidal anti-inflammatory (NSAID) drugs and opioids have been essential medications for pain relief; so far, many descriptions have represented the side effects of these drugs containing gastrointestinal upset, cardiovascular effects, renal function abnormalities and risks of dependency (Calixto, 2005; Shinwari *et al.*, 2013 & 2018; Mahmood *et al.*, 2013). As a result, the scientists have been investigating a safer method for pain relief. Inflammation is a state that manifests itself in a variety of ways. Inflammation can weaken the immune system, lead to infections, and delay illness detection and treatment (Hameed *et al*., 2021 and Zahoor *et al*., 2021). Due to the release of chemical mediators during inflammation, there is a clear link between inflammation and pain; this can lead to the development of impulsion and nociceptors, as well as sensors and afferent fibres (Abdulkhaleq *et al.*, 2018; Matsuka *et al.*, 2020).

Natural products with a wide range of biologically active substances can be used to manage many diseases (Ayatollahi *et al*., 2019; Shinwari *et al*., 2020). Many pre-clinical and clinical studies have been undertaken on animals and humans to investigate the efficacy of natural substances (Yuan *et al.*, 2016; Salehi *et al.*, 2019a; Salehi *et al.*, 2019b; Sharifi-Rad *et al*., 2020). In fact, the efficacy of medicinal plants in the treatment of pathological conditions dates from time immemorial (Ruhsam & Hollingsworth, 2018) , with about a third of all Food and Drug Administration (FDA) approved drugs developed in the last two decades deriving from plant sources (Thomford *et al.*, 2018; Newman & Cragg, 2020). This indicates that the search for biologically active molecules from natural products for drug discovery is a wise strategy for novel drug development with the ultimate goal of solving global health challenges such as pain and inflammation.

Herbs have been used for their anti-inflammatory and analgesic effects after old times. *Withania somnifera* (L.)

Dunal, known as ashwagandha, Indian ginseng, poison gooseberry, or winter cherry, is a plant in the nightshade or Solanaceae family. Morphologically, some other species of the genus *Withania* are the same (Zhang *et al.,* 2014; Uritu *et al.,* 2018; Salehi *et al.,* 2019c) Even though thought to be helpful as a medicinal herb in Ayurveda and sold in many countries as a supplement, there is inadequate scientific evidence that it is safe or efficient for treating any disease. In Latin, the type name *W. somnifera* means sleep-inducing. 'Ashwagandha' is a mixture of the Sanskrit words, 'ashwa' meaning 'horse' and 'gandha' meaning 'smell, meaning that the plant's root has a powerful horse-like odor (Bhattacharya *et al.,* 2000a; Kushwaha *et al.,* 2012; Bhadra, 2020). *W. somnifera* is cultivated in some of the drier places of India. It can also be found in Nepal, Srilanka, China, and Iran. *Withania* roots are bitter, astringent, acrid, thermogenic, soporific, aphrodisiac, stimulant, diuretic, and tonic (Visweswari *et al.,* 2013; Gaurav *et al.,* 2015). The plant's leaves include antitumoral, antibiotic, and antihepatotoxic effects (Alam *et al.,* 2012; Palliyaguru *et al.,* 2016). The plant's seed includes hypnotic, milk coagulating, and diuretic effects (Umadevi *et al.,* 2012). *W. somnifera* has been used as an antioxidant, antibacterial, aphrodisiac, liver tonic, adaptogen, and anti-inflammatory agent (Bhattacharya *et al.,* 2000a; Singh *et al.,* 2010). The doses of ashwagandha for humans are usually 4-6 g per day and are expected to be non-toxic and safe (Visweswari *et al.,* 2013). Ashwagandha handles a potent anti-stressor activity and has evidence indicating a reduction of stress-induced changes and has cardioprotective activity in ischemic rats like the effects ascribed to the adaptogens, for example, Panax ginseng. Adaptogens are chemicals that promote the "state of non-specific resistance" in stress, a physiological condition that is associated with a variety of neuroendocrine-immune system problems, due to their anxiolytic, neuroprotective, anti-fatigue, anti-depressive, nootropic, and CNS-stimulating effects (Bhattacharya *et al.,* 2000b; Umadevi *et al.,* 2012; Dutta *et al.,* 2019; Ng *et al.,* 2020). *W. somnifera* consists of more than 35 chemical compounds including flavonoids, alkaloids, steroidal lactones, and saponins, which are all biologically active. Among them, flavonoids, steroidal lactones, alkaloids, and saponins have biological activity (Panossian & Wikman, 2010; Logie & Berghe, 2020). A highly potent compound with various biological activities called Withaferin has been found in this plant, isolated mostly from its root and it has biological activity in many important biochemical reactions in inflammation, including the inflammatory response pathway, regulation of the heat shock protein, modulation of the kinase activity, and the cellular redox balance activity via *Nrf2* regulation (Logie & Berghe, 2020). *W. somnifera* has previously revealed anticancer activity against HepG-2 and MCF-7 cell lines (Sharifi-Rad *et al.,* 2021a). Moreover, *W. somnifera* root extracts is reported to regulate cytokines levels, which shows its therapeutic potential against many important health conditions including the inflammation, pain and cancer (Naidoo *et al.,* 2018). Furthermore, with the popularization, the use of this plant as a food supplement in the market is also increasing. Indeed, both extracts and compounds isolated from *Withania* species exhibit excellent biological activities, including antioxidant, antimicrobial, anti-inflammatory, and chemopreventive abilities, as assessed by both *in vitro* and *in vivo* studies. *W. somnifera* is a member of the *Solanaceae* or the nightshade family, a native of Afghanistan, Sistan and Baluchestan Province of Iran, Pakistan, and India (Mirjalili *et al.,* 2009; Pandit *et al.,* 2013). The genus *Withania*, *W. coagulans* (*Ashutosh booti*), and *W.somnifera* (Ashwagandha) are economically significant and are cultivated in some places. The *Solanaceae* plant family includes 2700 species and consists of 80 genera. *W. somnifera*, a little green shrub usually known as 'Ashwagandha ', is one of the main components of Ayurvedic preparations prescribed for processing properties containing anti-inflammatory, diabetes, antitumor, and antioxidant. It has also been used to treat pain (Sharma *et al.,* 2021; Singh *et al.,* 2021).

Free radicals are molecules that have one or more unpaired electrons produced in many natural biochemical reactions in the cell. Each free radical can turn a non-free radical into a radical, resulting a chain of reactions destroying any cell and tissue. Many diseases are influenced by free radical reactions, such as cancer and cardiovascular disease. Antioxidants are molecules that can reduce the free radical reactions and the damage that it causes to the cells. Any plant with high antioxidant content can have many health benefits as a dietary health promoter (Alam *et al.*, 2012). Many *In-vitro* and *In-vivo* studies have shown significant antioxidant activity from different extracts of *W. somnifera*. Sumathi and Padma (2008) demonstrate high antioxidant compounds content in leaves and tubers in *W. somnifera*. In addition, another study carried out by Alam *et al*., (2011) suggested that *W. somnifera* has an excellent antioxidant activity due to catechin.

W. somnifera extract and fraction might be used to alleviate pain and inflammation, as well as provide other health advantages due to its antioxidant activity. Hence, the objective of this study was to look into and measure the antioxidant activity of *W. somnifera* extract, as well as assess its anti-inflammatory and antinociceptive properties. The findings of this study might be utilized to identify the advantages of dietary inclusion of *W. somnifera* and its potential for the treatment of inflammation and pain.

Materials and Methods

***W. somnifera* collection, preparation, and identification:** **The** areal parts of *W. somnifera* (4 kg) were harvested from Zabol County (Sistan) and Baluchestan Province (Iran), in the flowering season (June 2020) and dried at room temperature under shade. The identity of the control herbarium specimen was confirmed at the laboratory of Phytochemistry Research Center, Tehran, Iran.

Preparation of total extract and fractions of *W. somnifera*: The plant material was grounded after drying. Using the maceration process, powdered material (500 g) was repeatedly extracted with % methanol. The powder was macerated for 72 hours, filtered, and the solvent was replaced

every 24 hours. Afterward, the solvent was evaporated using a rotary evaporator. After the complete concentration of the extraction, doses 100 mg/mL and 300 mg/mL were prepared for the following steps. Also, three fractions were prepared with chloroform, ethyl acetate, butanol, and water solvents, following the liquid-liquid extraction method, as indicated in Figure 1 (Amin *et al.*, 2017).

Phytochemical screening tests: Phytochemical analysis was carried out by using standard methods described by Evans (2009), Yadav & Agarwala (2011) and Banu & Cathrine (2015). Table 1 indicates the mobile and stationary phases.

1. Test for alkaloids: The crude extract was combined with 2mL of 1% HCl and gently heated. The reagents of Mayer and Wagner were then added to the mixture. The presence of alkaloids was determined by the turbidity of the resultant precipitate.

2. Test for terpenoids: The crude extract was dissolved in 2 mL chloroform and then evaporated to dryness. 2ml concentrated H_2SO_4 was added to this and boiled for roughly 2 minutes. The presence of terpenoids was indicated by a grey colour.

3. Test for saponins: Crude extract was mixed with 5ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

4. Test for flavonoids: Concentrated HCl was added drop by drop to a mixture of crude extract and a few shards of magnesium ribbon. After a few minutes, a pink scarlet colour developed, indicating the presence of flavonoids.

5. Test for tannins: Crude extract was mixed with 2ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

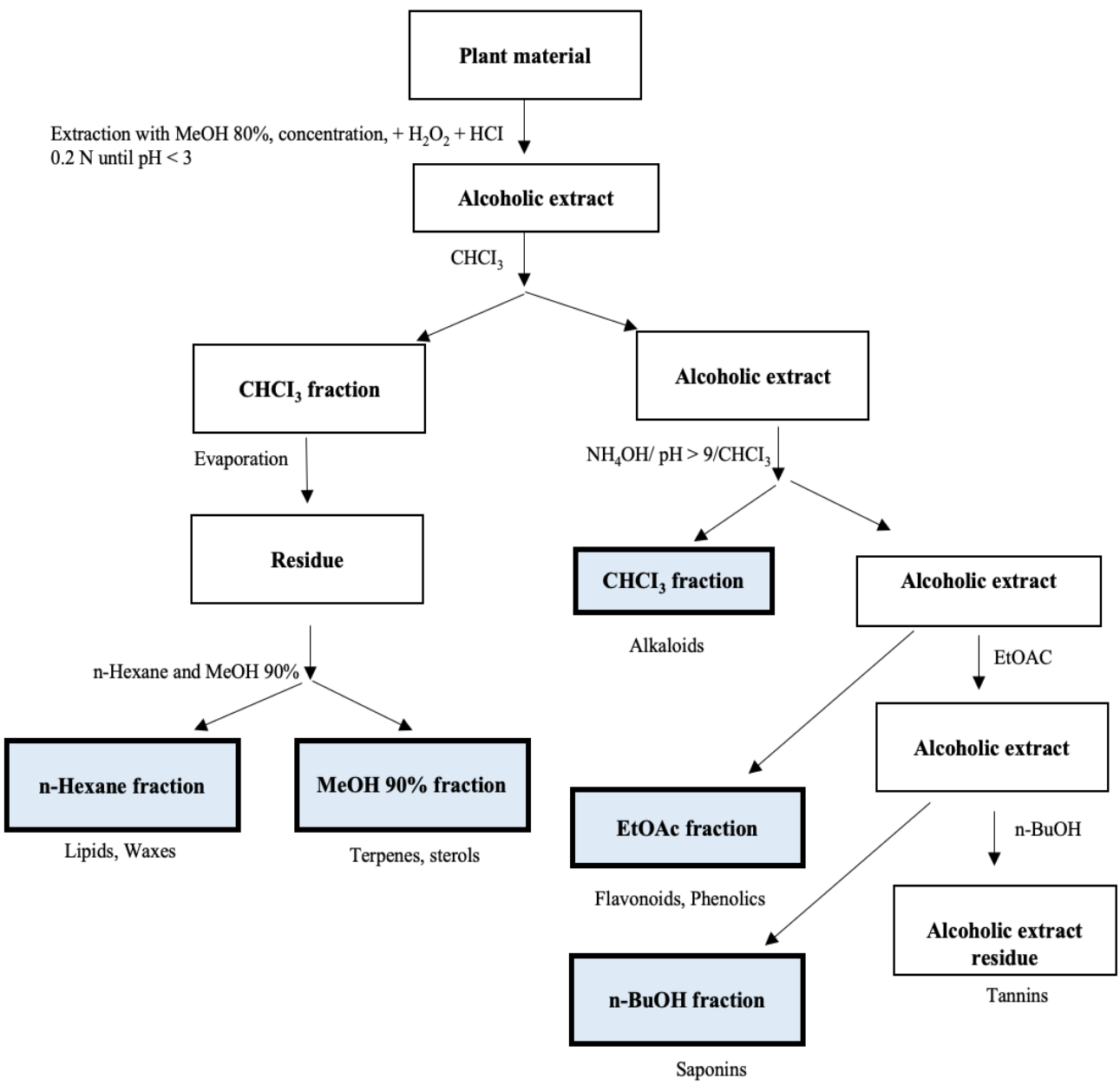

Fig. 1. Flowchart of extraction and fractionation.

Photochemical component	Mobile Phase	Stationary Phase	Derivatization reagent
Terpenoids	toluene-chloroform-ethanol (4:4:1)	Sillica geL 60 F 254 HPTLC Plate 5*10	**AnisAldehyde-sulfuric Acid**
Flavanoids	chloroform-Ethyl acetate-acetone - Formic acid (4:3:2:1)	Sillica geL 60 F 254 HPTLC Plate 5*10	**Natural products**

Table 1. The mobile phase, stationary phase, and derivatization reagent for the phytochemical screening tests.

Photochemical component	Mobile phase	Stationary phase	Derivatization reagent
Terpenoids	Toluene-chloroform-ethanol (4:4:1)	Sillica geL 60 F 254 HPTLC Plate 5*10	AnisAldehyde-sulfuric acid
Flavanoids	Chloroform-Ethyl acetate-acetone -Formic acid (4:3:2:1)	Sillica geL 60 F 254 HPTLC Plate 5*10	Natural products

Total phenolic content (TPC) assay: The Folin-Ciocalteu method is utilized for the estimation of the phenolic content of *W. somnifera* extract. This assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/ phosphotungstic acid complexes, which are determined spectroscopically at 765 nm (Sánchez-Rangel *et al.,* 2013; Shirazi *et al.,* 2014; Sharifi-Rad *et al.,* 2021b).

Total tannins content (TTC) assay: The total tannin content was determined (Tzekiat & Chiang, 2013; Shirazi *et al.,* 2014; Haile & Kang, 2019; Sharifi-Rad *et al.,* 2021b), for which the absorbance was reported at a wavelength of 500 nm, and the calibration curve of catechin at 0-1000 µg/mL was used for calculation. The data resulting from the assessment were reported as mg of catechin equivalents (mg CE) per weight of the sample.

Total antioxidant capacity (TAC) assay: The total antioxidant capacity was studied ((Tzekiat & Chiang, 2013; Shirazi *et al.,* 2014; Rubio *et al.,* 2016; Haile & Kang, 2019; Sharifi-Rad *et al.,* 2021b) and the absorbance of the sample was measured at 695 nm. The results were extracted from a calibration curve of ascorbic acid at the range of 0-200 µg/mL. Finally, the result was described as a mass of ascorbic acid equivalents (mg AAE) per weight of the sample.

2,2'-Diphenyl-1-picrylhydrazyl (DPPH) assay: The DPPH assay was performed and the absorbance was determined at λ max 517 nm against a blank sample (equal amount of the DPPH solution and methanol). A calibration curve of the ascorbic acid aqueous solution was conducted at the range of 0-50 µg/mL. Finally, the results were reported as mg AAE per weight of the sample (Phatak & Hendre, 2014; Xie & Schaich. 2014; Sharifi-Rad *et al.,* 2021b). The amount of the DPPH scavenging was reported as a percentage calculated via the following equation:

$$\text{Percent scavenging of DPPH}\bullet = [(A^0 - A^1)/A^0] \times 100$$

A^0=The control absorbance, A^1=The extract absorbance

Ferric reducing antioxidant power (FRAP) assay: The FRAP assay was done (Firuzi *et al.,* 2005; Henderson *et al.,* 2015; Sharifi-Rad *et al.,* 2021b), for which the absorbance was reported at λmax 593 nm, and a calibration curve was illustrated using the ascorbic acid solution at the range of 0-50 µg/ mL. The results were described as mg AAE per weight of the sample. The increased absorbance of the mixture showed a more significant reduction power.

2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay: The ABTS assay was carried out (Pinto *et al.,* 2005; Ozgen *et al.,* 2006; Sharifi-Rad *et al.,* 2021b), and the absorbance was calculated at 734 nm. The standard curve was illustrated by 0-150 µg/mL concentration of ascorbic acid, and the results were reported as mg AAE per weight of the sample.

Preparation of Methanolic extract and the fractions doses: A heterogeneous suspension of the concentrated methanolic extract and the fractions of *W. somnifera* were made with distilled water and tween 80. Different doses (100 mg and 300 mg) of the methanolic and 30 mg and 100 mg doses from the fractions were prepared. With a 25 mg/kg dose, Diclofenac was injected by the Intraperitoneal (IP) route as standard material for positive control. Oral gavage of NaCl solution was done for the negative control group. The extraction with the dose of 1 mL/kg was administered by oral gavage route.

Study groups: A total number of 156 Wistar rats weighing 100–120 g were selected from the animal house facility at Pharmacy School of Shahid Beheshti University of Medical Sciences, Tehran, Iran. Formalin test was performed on 62 rats, and paw edoema was assessed in another 62 rats. Humidity, light, temperature, food, and water were all kept at the same levels for the rats (Zimmermann, 1983).

Formalin test: Half an hour before running the test, the rats were treated with normal saline (negative control group), the methanolic extract at doses of 100 mg/kg, and 300 mg/kg, the fractions at doses of 100 mg/kg and 30 mg/kg by oral gavage route. For the positive control group, Diclofenac 25 mg/kg was injected by the IP route. 40 µL Formalin (5%, in 0.9% normal saline) was used to induce paw edema in the sub-planar tissue of paws. Behaviour of the rat was scored and recorded for 60 minutes after the injection. The pain score system used was the one described by Dubuisson & Dennis (1977) and Tjølsen *et al*., (1992) as "0 = normal weight-bearing on the injected paw; 1 = limping during locomotion or resting the paw lightly on the floor; 2 = elevation of the injected paw so that at most the nail touches the floor; and 3 = licking, biting or grooming the injected paw". The score was recorded every five minutes.

Carrageenan-induced paw edema test: Saline, Diclofenac, and the extract were given to the rats 30 minutes before carrageenan injection. After 30 minutes, the carrageenan was injected into sub-plantar tissues of the left hind paw of each rat. The thickness of paws was determined using an open-top cylinder filled with mercury before and three hours after carrageenan injection, using a digital balance. The digital balance measures this force. After that, the volume can be calculated by the density of mercury using the following equation (Taheri *et al.*, 2021).

$$V=m/\rho$$

In this equation, V is the volume of the displaced liquid ρ in the density of the liquid (for mercury ρ=13.534 g/cm^3), and m is the weight of the displaced liquid (Morris, 2003). The anti-inflammatory potential was obtained after measuring the paw edema before and after carrageenan injection and treating it with extract of *W. somnifera.*

Statistical analysis: Results are analyzed as mean ± standard deviation (SD). For analyzing the behavioural changes as a result of pain from the formalin test, the area under the curve (AUC) of the pain score-time graph was calculated. The results were then analyzed by one-way analysis of variance (ANOVA) and Bonferroni's post-test, considering $p<0.05$ as significant. For the carrageenan test, the data were also analyzed by one-way analysis of variance (ANOVA), and then a Tukey's post-test was performed, which $p<0.05$ was considered as significant. The statistical analysis was carried out by GraphPad Prism® 8.0.

Results

Chloroform fractions (1), (2), ethyl acetate, butanol, and hydroalcoholic fractions were tested for phytochemical assessments and are described in Table 2, Figs. 2 and 3.

Phytochemical screening: Phytochemical analysis showed that terpenes, saponins and flavonoids were identified as the major phytochemicals in the fractions, while tannins and alkaloids were found in minor concentrations only. The final results of the phytochemical screening tests of the fractions are according to Table 2.

Table 2. Phytochemical screening results.

Photochemical component	Status
Terpenes	****
Flavonoids	***
Alkaloids	*
Saponins	****
Tannins	**

To determine the flavonoids, the natural product reagent was sprayed on the HPTLC plate, wherein which the light-yellow spots at the visible spectrum and the sharp blue lines in fluorescent light reveal flavonoids (Fig. 2). A significant part of flavonoids was found in fraction 4 (butanol) and fraction 5 (hydro alcoholic).

For the identification of terpenoids, the HPTLC plate was sprayed with the Anisaldehyde sulfuric acid reagent and theterpenoids appeared as purple-blue lines under the visible light (Fig. 3). The majority of terpenes were detected in fraction 1 (chloroform fraction (1)).

Polyphenol estimation: The phenolic content of *W. somnifera* extract was measured by assessments of TPC, TFC, and TTC. The results were derived from the calibration curve of gallic acid and were expressed as gallic acid equivalents (GAE), quercetin equivalent (QE), and catechin equivalent (CE), respectively. As shown in Table 3, the results from TPC, TFC, and TTC of *W. somnifera* extract was 13.47 ± 1.45 mg GAE/g, 0.18 ± 0.01 mg QE/g, 1.95 ± 0.29 mg CE/g, respectively.

Table 3. Polyphenols in *W. somnifera* extract.

TPC (mg GAE/g)	TFC (mg QE/g)	TTC (mg CE/g)
13.47 ± 1.45	0.18 ± 0.01	1.95 ± 0.29

All data are measured on a dry weight basis and presented as mg/g mean ± standard deviation (n = 3). TPC: Total phenolic content; GAE: gallic acid equivalents; TFC: total flavonoid content; QE: quercetin equivalents; TTC: total tannins content; CE: catechin equivalents.

Antioxidant potential: The antioxidant activity determined in all methods resulted from 0.1 mg/mL concentration from the extract. To facilitate the comparison between data, ascorbic acid was used as a pattern. Figure 4 indicates the results of antioxidant activity quantified by the DPPH, FRAP, ABTS, and TAC methods for the plant extract.

Effects of *W. somnifera* on behavioral changes of animal models in the formalin test: The administration of *W. somnifera* extracts significantly reduced pain score relative to the control group in the formalin test. Effects of *W. somnifera* methanolic extract on behavioural changes of animal models in the formalin test compared with the positive and negative control groups are illustrated in Fig. 5. When compared to treatment with 100 mg/kg methanolic extract, therapy with 300 mg/kg methanolic extract resulted in the highest reduction in pain score, demonstrating that the effect of the extract is dependent upon the dose.

Behavioural studies in the formalin model showed decreased pain scores in the animal models treated with *W. somnifera* extracts compared to the control group. The area under the curve of Figure 5 was calculated, and the results are shown in Fig. 6. The analysis shows a significant difference between the test groups and the negative control group. *W. somnifera* methanolic extract and Diclofenac showed a considerable effect on the pain score. Furthermore, there is no significant difference between the group treated with 300 mg/kg of *W. somnifera* methanolic extract and the positive control group treated with Diclofenac. The antinociceptive effect revealed at a dose of 300 mg/kg was found similar to the standard drug Diclofenac according to Fig. 6.

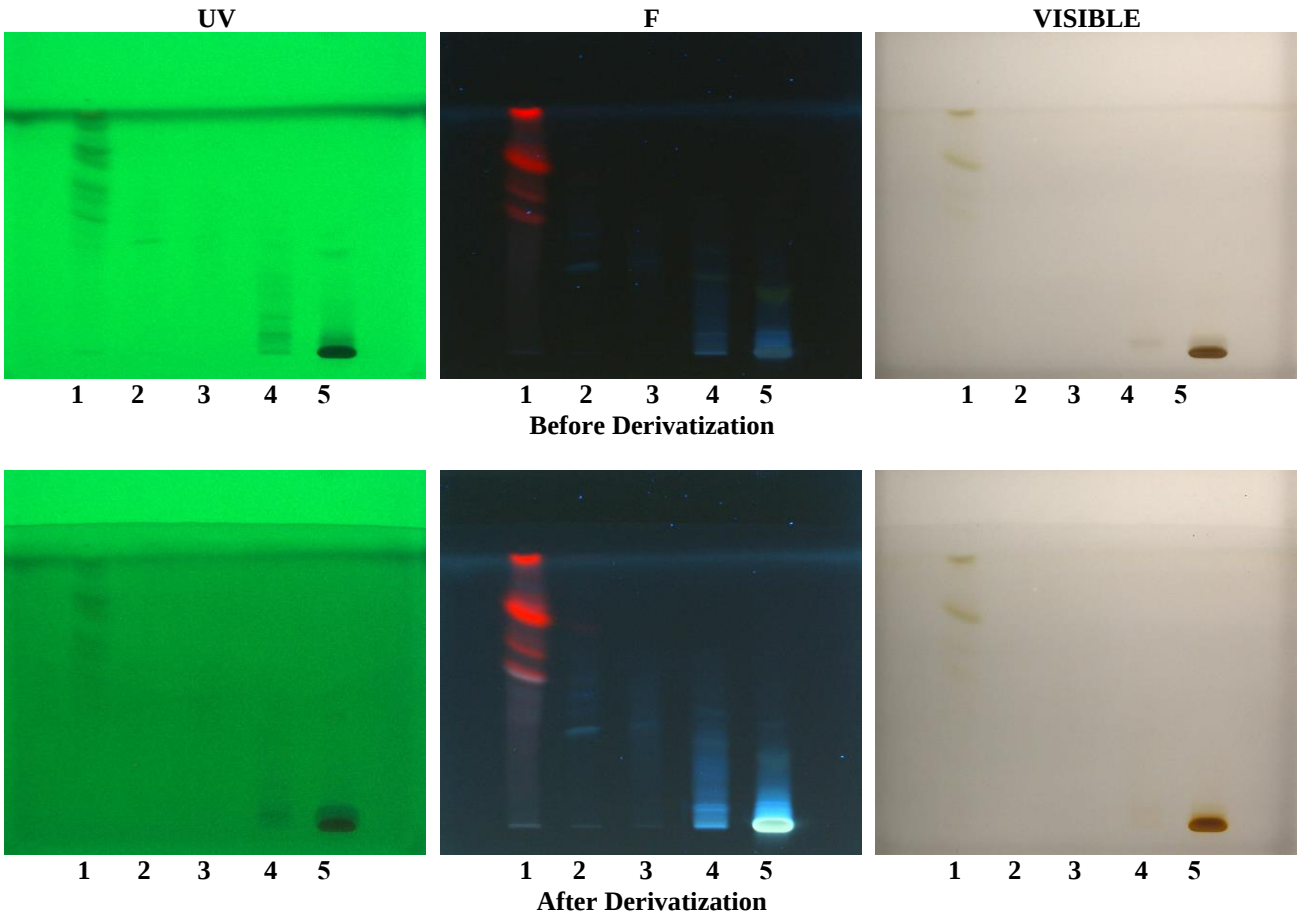

Fig. 2. Flavonoids identified in *W. somnifera* by HPTLC. 1: Chloroform (1) 2: Chloroform (2) 3: Ethyl acetate 4: Butanol 5: Hydroalcoholic

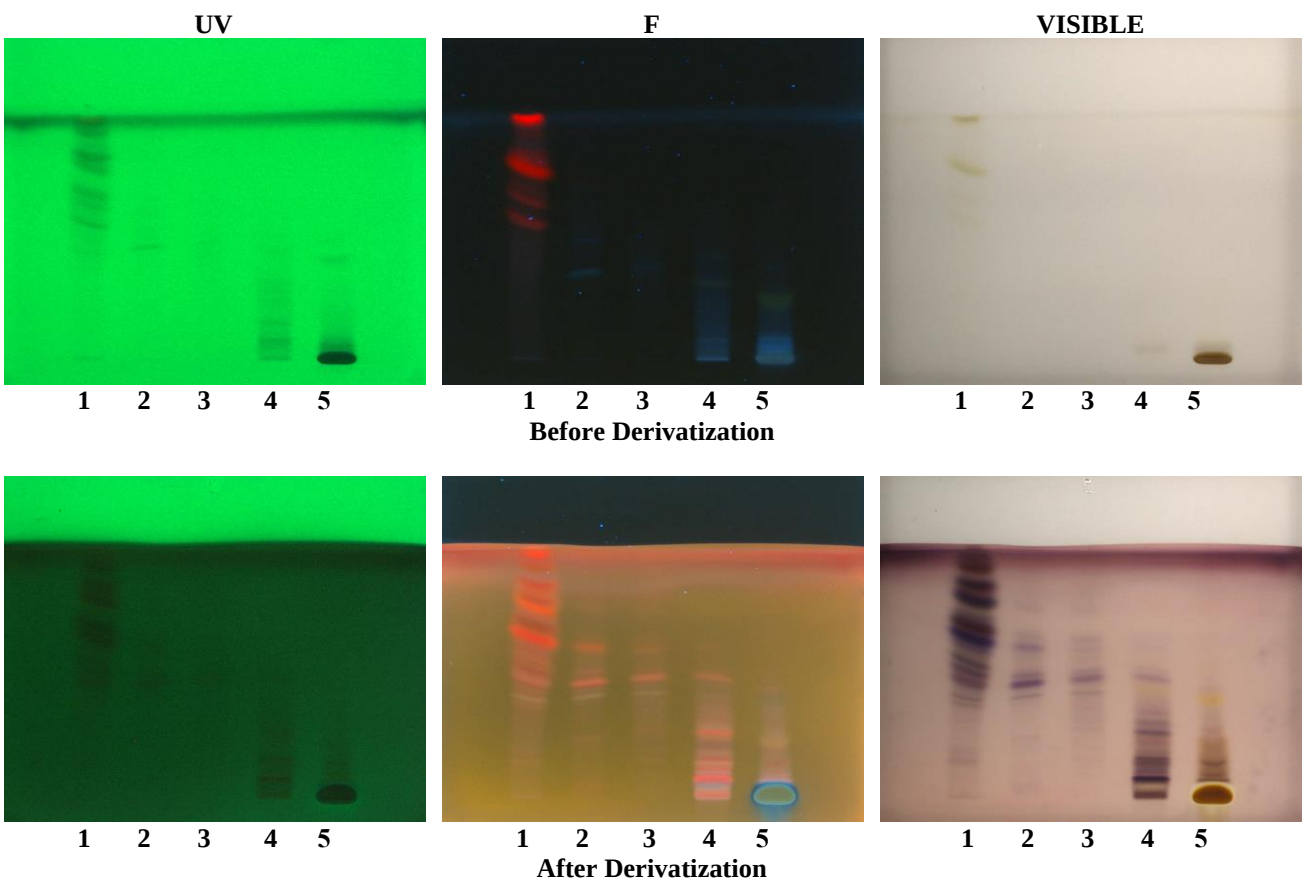

Fig. 3. Terpenoids identified in *W. somnifera* by HPTLC. Where, 1: Chloroform (1) 2: Chloroform (2) 3: Ethyl acetate 4: Butanol 5: Hydroalcoholic.

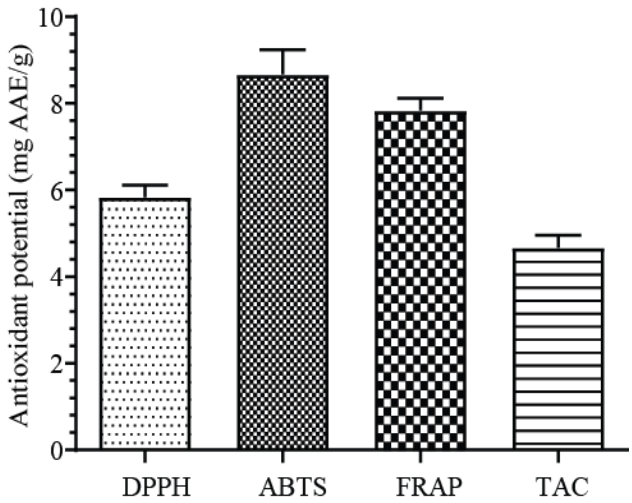

Fig. 4. Antioxidant potential of *W. somnifera* extract based on DPPH, ABTS, FRAP, and TAC tests.

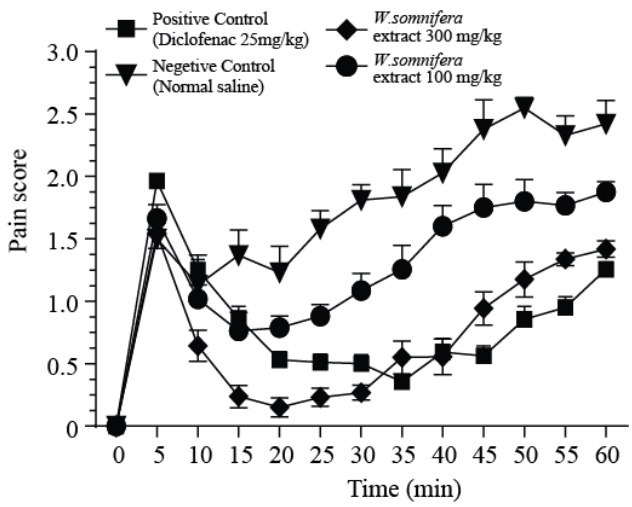

Fig. 5. Effects of *W. somnifera* methanolic extract on behavioural changes of animal models in the formalin test compared with the positive and negative control groups.

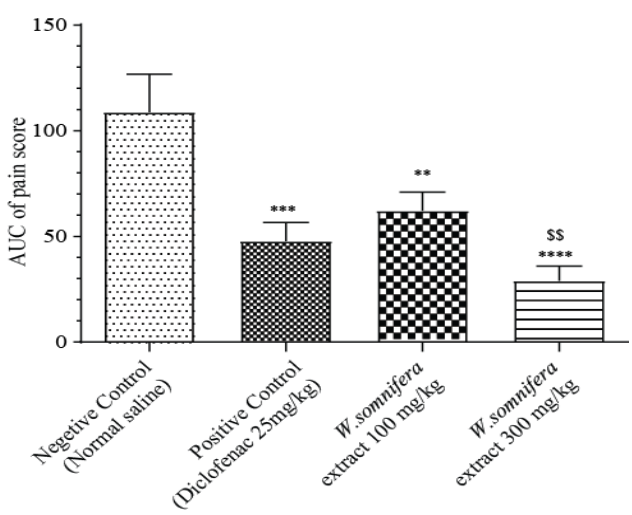

Fig. 6. The area under the curve of pain score from Figure 5. Results are expressed as mean ± SD. The significant difference compared with the negative control group (normal saline) $p<0.0001$****. Significant difference with positive control group (diclofenac) $p<0.05$ $, $p<0.01$$, $p<0.001$$$, $p<0.0001$.

The area under the curve calculated from Figure 1 shows a significant effect of the methanolic extract of *W. somnifera*. The pain score of the group treated with 25 mg/kg of Diclofenac and the plant extract at the dose of 300 mg/kg was lower than other treatments based on figure 3. In the post-test, chloroform fraction (1), chloroform fraction (2), ethyl acetate, butanol, and hydroalcoholic fractions showed a significant reduction in pain score as compared to the negative control group of rats. Chloroform fraction (1) and butanol fraction decreased the pain score remarkably. Furthermore, these two fractions showed more effect at the dose of 100 mg/kg. This can demonstrate a dose-dependent impact on pain score. Figure 7 displays a comparison between positive control, negative control, and test groups with the treatment of all the fractions.

Fractions show a significant impact on pain scores as compared to the negative control groups. Bar charts show significant effects on pain scores chloroform fraction (1) and butanol fraction Fig. 7.

The effects of *W. somnifera* on behavioral changes of animal models in the paw edema test: The anti-inflammatory potential of the test samples, which were *W. somnifera* methanolic extract, at a dose of 100 and 300 mg/kg, and 30 and 100 mg/kg for the fractions, respectively, against the acute paw edema induced by carrageenan, is depicted in Figure 8 and Figure 9. The crude extract (methanolic) and the fractions induced a remarkable and dose-dependent response.

Figure 9 reveals the results of paw volume after administration of Chloroform fraction (1), chloroform fraction (2), ethyl acetate, butanol, and hydroalcoholic fractions Diclofenac in carrageenan-induced edema. All the fractions reduced the paw edema induced by carrageenan significantly in comparison with the negative control group. A dose-dependent reduction of the paw volume was observed.

As shown in Figure 8, the fractions have a substantial and dose-dependent impact on paw edema reduction in the carrageenan-induced paw edema test.

Discussion

Natural bioactive compounds are a rich source for the spread of analgesic and anti-inflammatory agents. Given the wide range of *Withania* species applications in Ayurvedic medicine for multiple aims, an increasing number of studies have progressively addressed their biological effects. Numerous substances of diverse chemical classes have been discovered by chemical analysis of various *W. somnifera* plant sections. The biologically active chemical constituents of *W. somnifera* are alkaloids (isopellertierine, anferine), steroidal lactones (withanolides, withaferins), saponins containing an additional acyl group (sitoindoside VII and VIII), and withanoloides with a glucose at carbon 27 (sitonidoside XI and X). Among them, withanolides (steroidal lactones) have been used in an increasing number of drug-formulations, given their promissory therapeutic abilities (Gaurav *et al.*, 2015). Furthermore, previous studies have

determined that *W. somnifera* has safety and efficacy in rheumatoid arthritis, type 2 diabetes, memory, and cognitive impairment, hypothyroidism, and chronic stress (Tandon & Yadav, 2020). *W. somnifera* leaves and fruits are rich in terpenoid compounds and polyphenols, containing phenolic acids, flavonoids, and tannins. Polyphenols are compounds including phenolic acids, flavonoids, and tannins. Before, it was defined in studies that phenolic and terpenoid compounds of this plant could have anti-inflammatory and antinociceptive potentials. Various species like *W. somnifera*, *W. coagulans*, *W. riebeckii*, and *W. chevalieri* revealed the highest amount of phenolic and terpenoid compounds that are considered to impart antinociceptive and anti-inflammatory potential (Prakash, 2017; Maleki *et al.,* 2019). Triterpenoids are widely circulated in nature. They have been studied for their biological activities, containing antibacterial, antiviral, antitumor, antioxidation, and anti-aggregation function (Ghosh, 2021). The Withanolides are a group of naturally occurring polyoxygenated steroidal lactones assembled on a C_{28} ergostane skeleton (Misico *et al.,* 2011; White *et al.,* 2016).

The hydroxyl groups in the compounds present in the extract of *W. somnifera,* are the main reason for their free radical scavenging activity. As a basic determination, *W. somnifera* extract phenolic contents were estimated by TFC, TPC, and TTC, and their results were reported as GAE, QE, and CE, respectively. TPC measurement was performed using the Folin-Ciocalteu reagent. The Folin-Ciocalteu reagent has the ability to react with many of the reducing compounds in the plant. The plant extract is a pool of different compounds, including many reducing agents such as non-phenolic and phenolic compounds, including ascorbic acid (Morris, 2003). TFC in the extract of *W. somnifera* was estimated by the primary determination of aluminum chloride colorimetric. The method is based on the carbonyl group of flavonoids. The aluminum chloride reacts with the carbonyl group and create a stable complex. The outcome data were derived from a calibration curve of quercetin (Zhong *et al.,* 2020). TTC value of *W. somnifera* extract was detected using a vanillin solution in methanol. The tannins are divided into hydrolysable tannins and condensed tannins. Both of these two groups can be detected by this method. This study's resulting data clearly shows that *W. somnifera* has significant amounts of phenolic, flavonoid, and tannin content. These assays prove the considerable potential for the food and nutraceutical industry. Depending on the technique of extraction, W. somnifera and other Withania genus plants have varying biological effects, particularly in terms of their antioxidant potential and phytochemical contents. The methanol-chloroform-water (1:1:1) extract of W. somnifera roots, which had the highest content of all phytochemical constituents except tannins, had higher antioxidant and reducing activities when compared to water, acetone, and aqueous methanol (1:1) extracts (i.e. total antioxidant capacity of methanol-chloroform-water (1:1:1) was 83.354 1.828, aqueous methanol (1:1) was 76.978 2.210, and water was 68.439) (Ganguly *et al.,* 2018). In our study, *W. somnifera* extract was assessed for antioxidant activity and presented great antioxidant properties and could be attributed to the considerable amounts of flavonoids, alkaloids, tannins, saponin glycosides, and phenolic compounds in the extract. The flavonoids are important antioxidant compounds with great radical scavenging activity. Many studies have shown a linear correlation between the total phenolic and flavonoids content and the antioxidant potentials (Aryal *et al.,* 2019). To study the potentials of the *W. somnifera* as an antioxidant agent, many antioxidant assays with different mechanisms were performed, including project DPPH, ABTS, FRAP, and TAC. The determination of radical scavenging ability was carried out using ABTS and DPPH, while TAC and FRAP methods were performed to determine the reduction potential of the extracts. The free radical scavenging activity can be measured via the DPPH assay. Based on the TFC assay in this study and its correlation with the value of the DPPH assay, the DPPH assay value might be significantly influenced by the TFC (Tang *et al.,* 2019). The ABTS radical scavenging activity is due to the hydrogen-donating potential. Both DPPH assay and ABTS assay detect the free radicals scavenging abilities of an extract. In this study, the difference between the results of antioxidant activity in ABTS assay and DPPH assay might be due to the phenolic compounds and their complex structure in *W. somnifera,* which caused an underestimation of DPPH scavenging activities. The FRAP assay is a reachable method for assessing the antioxidant or reducing the extract's ability and its compounds. It is based on the rapid reduction of Fe^{3+} and forming Fe^{2+} due to the interaction with an antioxidant. In this study, the high reduction power of Fe^{3+} is reflected in the high value of result data of FRAP assay. This might be due to the TPC value since there is a correlation between the FRAP and the TPC. Similar to FRAP, the mechanism of total antioxidant capacity (TAC) assay is based on electron transfer, and can be concluded that compounds with antioxidant activity and phenolic compounds have the potential to reduce molybdenum (VI) to molybdenum (V). Between the tests used for the antioxidant activity assay, the FRAP test indicated the highest antioxidant activity in the sample containing *W. somnifera* extract (Zhong *et al.,* 2020; Sharifi-Rad *et al.,* 2021b).

Based on the results of all the tests, there was significant antioxidant activity in the *W. somnifera* extract. This is due mainly to the high phenolic content and the flavonoid content in the *W. somnifera* extract. The formalin test as a representation of nociception treats pain differently in two phases, divided by time: the first phase (the first 0-5 minutes), called the neurogenic phase, is generated peripherally it happens through the direct stimulation of nociceptive neurons. The second phase (20-25 minutes) occurs between the activation of central neurons, exceptionally the neurons of the dorsal horns in the spinal cord. The second phase is thought to be inflammation-induced pain due to the activity of cytokines (for example, prostaglandins, serotonin, histamine, and bradykinin). These facts can be used to define the antinociceptive mechanism.

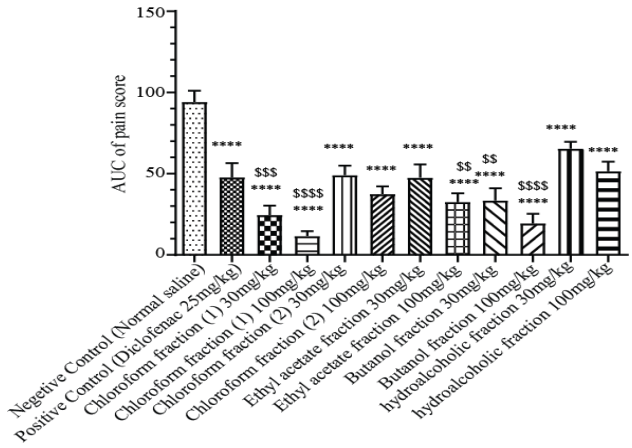

Fig. 7. Effects of *W. somnifera* Chloroform fraction (1), Chloroform fraction (2), ethyl acetate, butanol, and hydroalcoholic fractions on behavioural changes of animal models compared to positive and negative control groups. Results are expressed as mean ± SD. The significant difference compared with the negative control group (Normal saline) p<0.0001****. Significant difference with positive control group (diclofenac) p<0.05$, p<0.01$$, p<0.001$$$, p<0.0001$$$$.

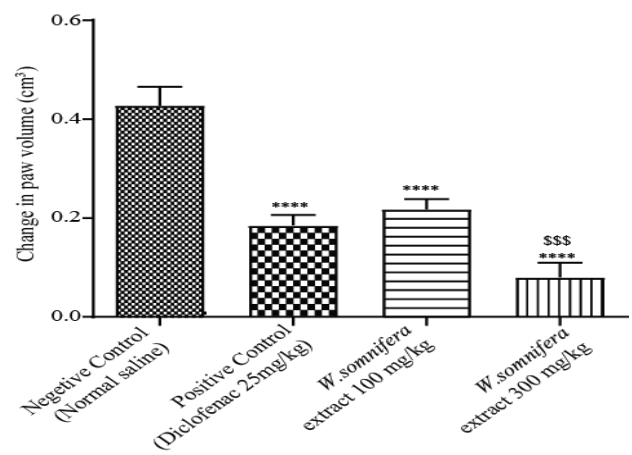

Fig. 8. Effects of *W.somnifera* methanolic extract on carrageenan-induced paw edema. Results are represented as mean ± SD. Significant difference compared with negative control group (Normal saline) p<0.0001****. Significant difference with positive control group (diclofenac) p<0.05$, p<0.01$$, p<0.001$$$, p<0.0001$$$$.

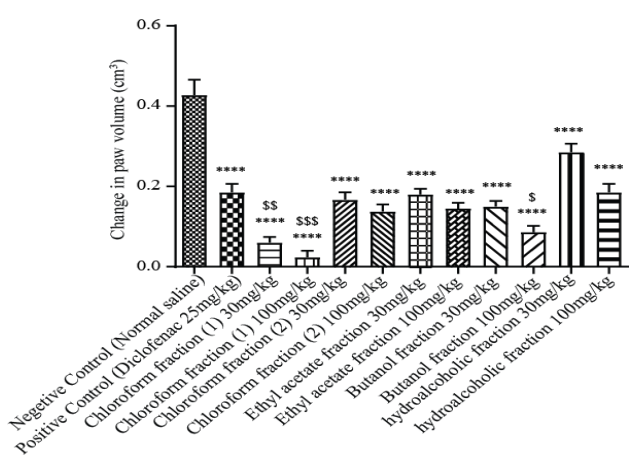

Fig. 9. Effects of *W. somnifera* of Chloroform fraction (1), Chloroform fraction (2), ethyl acetate, butanol, hydroalcoholic fractions and standard drug Diclofenac on carrageenan-induced paw edema. Results are presented as mean ± SD. The significant difference compared with the negative control group (Normal saline) p<0.0001****. Significant difference with positive control group (diclofenac) p<0.05$, p<0.01$$, p<0.001$$$, p<0.0001$$$$.

Conclusion

Our outcome reveals that the extracts and fractions of *W. somnifera* had antinociception activity against both the inflammatory and neurogenic phases of the formalin test. Our carrageenan-induced paw edema test results show that the methanolic extract, chloroform fraction (1), and butanol fraction have anti-inflammatory activity. The present study revealed that *W. somnifera* shows the activities under examination, including anti-inflammatory, antinociceptive, and antioxidant effects. *W. somnifera* and its components can be utliized in current formulations for analgesic, anti-inflammatory, sedative/anxiolytic, and antioxidant properties. Further studies are needed to recognize the exact compounds with these effects and the mechanism of these potential effects in the plant.

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