



Analgesic and Anti-inflammatory Activity of *Withania somnifera* Root Extract

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

The anti-inflammatory and analgesic properties of the 85 % methanolic extract of *Withania somnifera* (WS) root was investigated and the anti-inflammatory effect was compared with the standard drug indomethacin. Analgesic activity was carried out by hot plate and tail flick method. Anti-inflammatory activity was carried out by carrageenan induced paw edema and Freund's adjuvant induced arthritis. The results are related with evaluation of the analgesic activity in hot plate and tail flick method, they reveal that methanolic extract has exhibited significant activity ($P<0.05$) at 150 mg/kg b.wt, itself, whereas, the percentage inhibition exhibited by WS (350 mg/kg b.wt.) in carrageenan induced paw edema is found to be nearer to that of standard drug (10 mg/kg b.wt.). In Freund's adjuvant induced arthritis, WS is seen to decrease the paw volume significantly ($P<0.05$). Significant ($P<0.05$) protection is also observed by elevating antioxidant enzymes. WS does not exhibit toxic effect which is observed in standard drug treatment. The 85 % crude methanolic extract does not present toxic effect as observed in indomethacin treatment. However, the extract has exhibited analgesic, anti-inflammatory and antioxidant activities.

Keywords: Hot plate; tail immersion; carrageenan; complete freund's adjuvant; oxidative stress.

1. INTRODUCTION

Withania somnifera Dunal (ashwagandha) is widely used in Ayurvedic medicine, a traditional

medical system of India. It forms an ingredient in many formulations prescribed for a variety of musculoskeletal conditions (e.g., arthritis, rheumatism), and as a general tonic to increase

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energy, improve overall health and longevity. It is used to prevent disease among athletes and the elderly. Many pharmacological studies have been conducted to investigate the properties of ashwagandha in an attempt to authenticate its use as a multi-purpose medicinal agent [1] anti-inflammatory properties [2], and anti-stress agent [3].

Anbalagan [4] has evaluated the anti-inflammatory effect of dried root powder of *Withania somnifera* in Freund's adjuvant induced inflammation. They have compared the effect of *Withania somnifera* with the standard drug of phenylbutazone. Moreover, they have confirmed that dried powder of *Withania somnifera* root (WS) is active against inflammation. Begum [5] has reported the anti-inflammatory activity of WS powder in carrageenan induced acute inflammation. Likewise, Begum [6] has evaluated the anti-inflammatory activity of WS powder in Freund's adjuvant induced arthritis. In both the models, they have used dried powder of WS. In our study, we have evaluated the anti-inflammatory activity of 85 % methanolic extract of WS in both carrageenan and Freund's adjuvant induced arthritis at minimal dose and compared their efficacy with the standard drug Indomethacin. In the present study, we have also evaluated the analgesic effect of 85 % Methanolic extract of WS by hot plate and tail immersion method.

2. MATERIALS AND METHODS

2.1 Plant Material

The roots of WS were collected from Madurai region, Tamilnadu, India. The plant was identified and authenticated at Centre for Advanced Research in Indian System of Medicine (CARISM), SASTRA University, Thanjavur, Tamilnadu, India and the Specimen of the same is maintained (Voucher number is 0064).

2.2 Extraction

The material was dried under shade. One kg of crushed root of the plant was soaked with seven liters of different solvents like hexane, chloroform, ethyl acetate and 85 % methanol for 7 days. The extract was filtered and concentrated by distillation. The final traces of solvent were dried in a vacuum oven at 50°C. The yield of extract was as follows: In hexane - 0.76 %, in chloroform – 1.34 %, in ethyl acetate – 1.32 %, in 85 % methanol – 4.62 %.

2.3 Qualitative Analysis of WS Extracted with Different Solvents

Different extracts of WS were tested for the presence of various phytoconstituents like 1) alkaloids using dragendorff, wagner and hager's reagent. 2) Flavonoids using aqueous sodium hydroxide solution. 3) Polyphenolics using folin phenol reagent. 4) Phytosterol by Libermann Burchard test which was carried out by mixing extract with acetic acid, acetic anhydride and sulphuric acid, 5) Saponins by testing the extract for the formation of foam by agitating with distilled water. 6) Carbohydrate by Molisch test which was carried out by mixing extract with alcoholic α -naphthol solution and concentrated sulphuric acid solution. 7) Amino acids and proteins using ninhydrin, Molisch and biuret reagent.

2.4 Experimental Animals

In this study, 102 adult Wistar albino rats weighing 180–210 g, obtained from Centre for Advanced Research in Indian System of Medicine (CARISM), Animal house, SASTRA University, Tamilnadu, India were used. The rats were fed with standard laboratory chow and sterile water before the experiment. The animal laboratory was equipped with automatic temperature ($22 \pm 1^{\circ}\text{C}$) and lighting controls (12 h light/12 h dark).

2.5 Experimental Protocol for Analgesic Activity

In the Hotplate test [7] rats were divided into different groups as follows. Each group consists of six animals. Group I - Control animals – 5% Tween 80 in water, Group II- Treatment I- 150 mg/kg b.wt. extract, Group III- Treatment II- 250 mg/kg b.wt. Extract, Group IV- Treatment III - 350 mg/kg b.wt. Extract. Four experimental groups were used. Rats treated with saline solution were employed as controls. The experimental groups were treated with WS (150, 250 and 350 mg/kg b.wt, p.o.). In this test, animals were individually placed on a hot plate maintained at a constant temperature ($55 \pm 0.3^{\circ}\text{C}$). The latency to first sign of hind paw licking or jump response to avoid heat nociception was taken as an index of nociceptive threshold with cut off time of 15 sec. The nociceptive threshold was observed every 60 min up to 4 hours after the drug administration.

2.6 Experimental Protocol for Analgesic Activity

In the Tail immersion method [8] rats were divided in to different groups as follows. Each group consists of six animals. Group I - Control animals – 5 % Tween 80 in water), Group II - Treatment I- 150 mg/kg b.wt. extract, Group III - Treatment II - 250 mg/kg b.wt.extract, Group IV- Treatment III- 350 mg/kg b.wt. extract. The antinociceptive effect was determined in rats using the tail-flick test by analgesiometer. The responses were elicited every 15 min up to 60 min after treatment, with WS under study and the vehicle. Four experimental groups were used. Rats treated with saline solution were employed as controls. The experimental groups were treated with WS (150,250 and 350 mg/kg p.o.)

2.7 Experimental protocol for Carrageenan induced inflammation

Anti-inflammatory effects of 85 % methanolic extract of WS was investigated which was induced by carrageenan [9,10]. The animals were divided in to following groups as follows. Group I- Normal - 5% Tween 80 in water, Group II- Standard - 10 mg/kg b.wt. indomethacin, Group III - Treatment I- 150 mg/kg b.wt. extract, Group IV- Treatment II - 250 mg/kg b.wt. extract, Group V- Treatment III - 350 mg/kg b.wt. extract. The anti-edematogenic properties of the WS extract was studied using the carrageenan-induced edema model. Groups of six rats were used. Animals were given a saline solution, indomethacin (10 mg/kg b.wt, p.o) and a dose of a selected WS extract, 1 h before administration of an intradermal injection of carrageenan (0.1 ml of a 1% solution in 0.9% saline solution) into the plantar region of the right hind paw. The contralateral paw was injected with 0.1 ml saline solution. The group treated with the WS extract was injected with 150, 250 and 350 mg/kg b.wt, p.o. The paw volume was measured before the injection and each hour after for a period of 4 h by means of volume displacement methods. The difference between the left paw and the right paw volumes indicated the degree of inflammation. The average increase in paw volume of each group was calculated and compared with the control (saline) and the indomethacin groups.

2.8 Experimental Protocol for Freund's Adjuvant Induced Arthritis

Animals were divided in to four groups as follows. Each group consists of six animals.

Group I - Normal -5 % Tween 80 in water, Group II – Control - Complete Freund's adjuvant (CFA), Groups III – Treatment - 250 mg/kg b.wt. extract + CFA, Group IV – Treatment - 10 mg/kg b.wt. Indomethacin + CFA. The right foot pad of each rat was injected subcutaneously with 0.05 ml of Complete-Freund's Adjuvant agent [11]. The animals were treated with extract and standard drug for 45 days. On 46th day all the animals were sacrificed by cervical dislocation under ether anesthesia. Liver and kidney was excised and washed with saline. 10 % tissue homogenate was prepared in Tris buffer and various biochemical parameters like Thio barbituric acid Reactive Substances (TBARS) [12] Reduced Glutathione (GSH) [13], Glutathione peroxidase (GPx) [14] and catalase [15] were estimated.

2.9 Statistical Analysis

The values have been expressed as Mean \pm S.D. Significant difference have been observed using One Way Analysis of variance (ANOVA) by SPSS software version 12.0. $P < 0.05$, was considered as significant difference. Groups having different alphabets are differ significantly at $P < 0.05$

3. RESULTS

3.1 Qualitative Analysis of WS Extracted with Different Solvents

Qualitative analysis showed the presence of alkaloids in chloroform extract, phenols in Ethylacetate and 85 % methanolic extract and phytosterol, saponin and carbohydrates in 85 % methanolic extract can be seen Table 1.

3.2 Analgesic Activity (Hot Plate Method and Tail Immersion Method)

In our present study maximum anti-nociceptive effect of methanolic extract in hot plate method was observed at 3rd hour by the dose of 250 mg/kg b.wt. ($P < 0.05$, Table 2). Moreover, significant difference has been observed at 150 mg/kg b.wt. of extract against 0 hour in hot plate test and also in tail immersion method in Table 2 and 3. Significant difference has not been observed by increasing the dose of the extract against lower dose treated animals.

3.3 Carrageenan Induced Inflammation

85 % Methanolic extract treatment is found to decrease the carrageenan induced edematous

paw volume significantly against Group I animals. The decrement is seen to be dose dependent. Nevertheless, the percentage protection exhibited by extract at the dose of 350 mg/kg b.wt is nearer to that of 10 mg/kg b.wt. of standard drug treatment. The results of all groups of rats can be seen in Table 4.

3.4 Freund's Adjuvant Induced Arthritis

The paw volume of Freund's adjuvant administered animals is observed to be decreased by treating animals with extract. Animals treated with extract at the dose of 250

mg/kg b.wt. for 45 days exhibit similar activity as that of Indomethacin at the dose of 10 mg/kg b.wt in Table 5. TBARS level in liver tissue of Freund's adjuvant administered rats is noticed to be increased in diseased rats ($P < 0.05$, Table 6). Treatment with extract for 45 days is seen to return back the TBARS level to normal level. Indomethacin administration has elevated the level of TBARS in kidney tissue in Fig 1. The antioxidants like GSH, GPx and catalase of treated rats have indicated the increase against Freund's adjuvant administered animals can be seen in Table 6 ($P < 0.05$).

Table 1. Qualitative analysis of *Withania somnifera* extracted with different solvents

Phyto-constituents	Hexane	Chloroform	Ethylacetate	85% Methanol
Alkaloids	-	+	-	-
Flavonoids	-	-	+	++
Polyphenolics	-	+	+	++
Phytosterol	-	-	-	+
Saponins	-	-	-	+
Carbohydrates	-	-	-	+
Amino acids and proteins	-	-	-	-

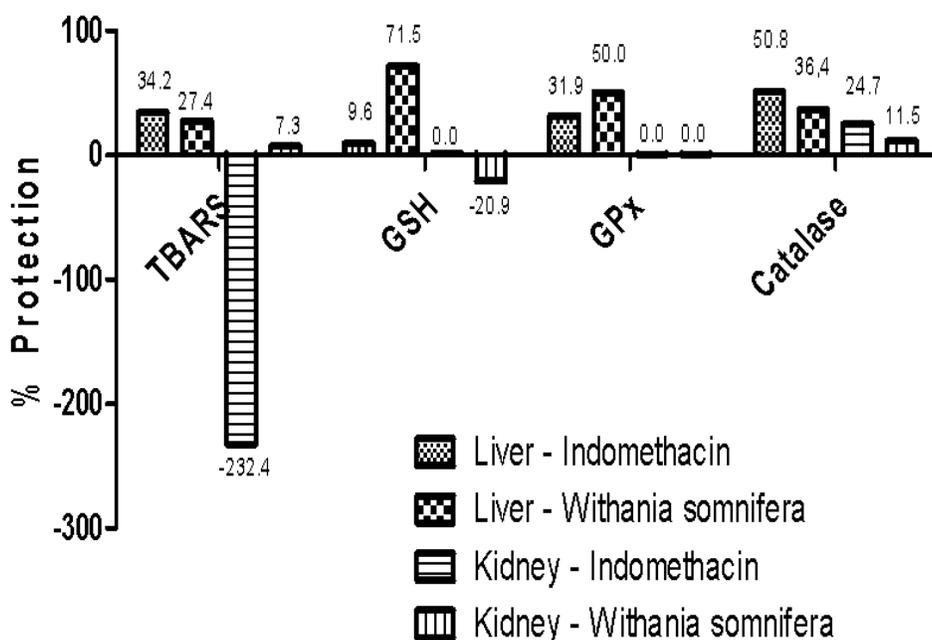


Fig. 1. %Effect of *Withania somnifera* and Indomethacin in Freund's adjuvant induced oxidative stress

Table 2. Anti-nociceptive effect of *Withania somnifera* in hot plate test

Groups	Dose	Reaction time in hour				
		0 hr	1 hr	2 hr	3 hr	4 hr
Response time in seconds						
Group I	-	3.1 ± 0.90	3.1 ± 1.50	3.8 ± 0.96	3.1 ± 0.30	4.8 ± 0.50
Group II	WS 150 mg/kg	3.6 ± 0.90 a	6.1 ± 1.40 c	6.6 ± 1.80 cd	7.3 ± 3.30 d	5.4 ± 0.50 b
Group III	WS 250 mg/kg	3.1 ± 0.50 a	4.5 ± 1.20 ab	5.0 ± 1.50 b	8.5 ± 2.60 c	4.5 ± 0.90 ab
Group IV	WS 350 mg/kg	2.1 ± 0.30 a	4.5 ± 1.30 b	3.9 ± 0.80 b	6.6 ± 3.60 d	5.4 ± 0.40 c

Values are Mean ± SD. One Way ANOVA was carried out using SPSS software version 12.0. Significant difference ($P < 0.05$) has been observed between 0 hour and different hours of the same group. Values not sharing common alphabets are differ significantly at $P < 0.05$

Table 3. Anti-nociceptive effect of *Withania somnifera* in tail immersion test

Drug	Dose	Response time in sec.					
		Reaction time in min.	0 min.	15 min.	30 min.	45 min.	60 min.
Group I	-		4.3 ± 0.50	4.0 ± 0.80	3.8 ± 0.50	5.3 ± 0.96	4.0 ± 0.80
Group II	WS 150 mg/kg		2.0 ± 0.0 a	3.3 ± 0.50 b	3.6 ± 0.80 b	3.4 ± 0.50 b	3.6 ± 0.50 b
Group III	WS 250 mg/kg		2.8 ± 0.30 a	3.6 ± 0.50 b	3.9 ± 0.30 b	3.4 ± 0.30 b	3.0 ± 0.80 b
Group IV	WS 350 mg/kg		2.5 ± 0.60 a	2.9 ± 0.30 ab	3.1 ± 0.30 ab	3.8 ± 0.60 b	3.4 ± 0.50 b

Values are Mean ± SD. One Way ANOVA was carried out using SPSS software version 12.0. Significant difference ($P < 0.05$) has been observed between 0 hour and different hours of the same group. Values not sharing common alphabets are differ significantly at $P < 0.05$

Table 4. Effect of *Withania somnifera* in Carrageenan Induced Rat Paw Edema

Groups	Dose mg/kg b.w	Increase in Paw edema (Mean \pm SD) in mm	% Inhibition of Paw edema
Group I	5 % Tween 80	2.00 \pm 0.14 c	-
Group II	Indomethacin 10	0.99 \pm 0.10 a	50.20
Group III	WS 150	1.42 \pm 0.13 b	29.05
Group IV	WS 250	1.46 \pm 0.06 b	26.93
Group V	WS 350	0.96 \pm 0.05 a	52.13

Values are Mean \pm SD. One Way ANOVA was carried out using SPSS software version 12.0. Significant difference ($P < 0.05$) has been observed between different groups. Values not sharing common alphabets are differ significantly at $P < 0.05$

Table 5. Effect of *Withania somnifera* in Freund's Adjuvant Induced Rat Paw Edema

Days	Freund's adjuvant	Freund's adjuvant + Indomethacin(10 mg/kg b.wt.)	Freund's adjuvant + WS (250 mg/kg b.wt.)		
	Paw volume	Paw volume (mm)	Percentage inhibition	Paw volume (mm)	Percentage inhibition
1	4.27 \pm 0.08	4.32 \pm 0.11 a	-	3.97 \pm 0.14 a	-
8	6.3 \pm 0.43	6.2 \pm 0.33 bc	1.59	6.3 \pm 0.34 c	-
15	6.68 \pm 0.51	6.38 \pm 0.41 c	4.49	5.98 \pm 0.38 c	10.75
22	6.7 \pm 0.87	6.08 \pm 0.25 b	9.25	5.8 \pm 0.24 b	18.31
29	6.7 \pm 0.46	5.85 \pm 0.19 b	12.69	5.03 \pm 0.22 b	22.62
36	6.63 \pm 0.32	4.78 \pm 0.19 a	27.90	4.48 \pm 0.21 a	31.08
43	6.45 \pm 0.39	4.52 \pm 0.11 a	29.92	4.35 \pm 0.05 a	32.03

Values are Mean \pm SD. One Way ANOVA was carried out using SPSS software version 12.0. Significant difference ($P < 0.05$) has been observed between different groups. Values not sharing common alphabets are differ significantly at $P < 0.05$

Table 6. Effect of *Withania somnifera* in Freund's Adjuvant Induced Oxidative stress

Parameters	Organs	Groups		
		Normal	Freund's adjuvant	Freund's adjuvant + WS (250 mg/kg b.wt.)
TBARS (nmoles/mg protein)	Liver	1.19 \pm 0.08 a	1.57 \pm 0.18 b	1.14 \pm 0.24 a
	Kidney	1.18 \pm 0.05 a	1.23 \pm 0.13 a	1.14 \pm 0.09 a
GSH (μ g of GSH/mg protein)	Liver	38.03 \pm 7.49 b	23.27 \pm 1.24 a	39.9 \pm 8.72 b
	Kidney	40.99 \pm 3.3 b	35.36 \pm 2.4 b	27.96 \pm 5.52 a
Catalase (mM of H ₂ O ₂ consumed/minute/mg protein)	Liver	19.28 \pm 1.41 b	13.84 \pm 1.42 a	18.88 \pm 1.74 b
	Kidney	19.34 \pm 1.38 a	15.61 \pm 1.04 b	17.41 \pm 1.56 ab
GPx (μ g of GSH utilized/min /mg protein)	Liver	0.153 \pm 0.02 b	0.104 \pm 0.0009 a	0.156 \pm 0.0003 b
	Kidney	0.1091 \pm 0.0011 a	0.1087 \pm 0.0002 a	0.1092 \pm 0.0005 a

Values are Mean \pm SD. One Way ANOVA was carried out using SPSS software version 12.0. Significant difference ($P < 0.05$) has been observed between different groups. Values not sharing common alphabets are differ significantly at $P < 0.05$

4. DISCUSSION

Pain and edema are the outcome of inflammatory reaction. Pain has been described as nature's early sign of morbidity. It is reported

that chemical production of pain usually results in an inflammatory response in biologically active system [16]. In our present study, we have assessed both the analgesic and anti-inflammatory activity of WS.

Different extracts of WS have been taken to account the presence of various phytoconstituents in different extracts so as to choose the particular extract having the presence of maximum phytoconstituents for further study. Since most of the phyto-constituents are eluted out in the 85 % methanolic extract (Table 1), we have used the same for further pharmacological evaluation.

Significant difference has been observed at 150 mg/kg b.wt. ($P<0.05$) of extract against 0-hour response reveals that the extract is more potent at minimum dose itself (Table 2). Significant difference has not been noted down between Group II against Group III and IV. It directs that maximum activity is at minimum dose. However, it should be further evaluated by carrying out the bioavailability and pharmacokinetics study of the extract. Likewise, in tail immersion method maximum effect is observed at 150 mg/kg b.wt. of extract (Table 3) pointing out that the antinociceptive effect of WS is present at 150 mg/kg b.wt. itself. It is reported that stimulation of sympathetic nerves is responsible for production of pain [17] Although the direct mechanism of action is still not established, the antinociceptive effect of WS might be due to the suppression of sympathoimetic amines. Antinociceptive effect of WS might be due to the presence of phenolic compounds. Phenolic compounds are reported to have analgesic activity through inhibition of the enzyme prostaglandin synthetase more specifically the endoperoxidase [18] Carrageenan induced edema model is a better model to assess anti-inflammatory activity of substances [9]. Hence the same method is adopted in our study.

The development of edema in the paw of the rat after the injection of carrageenan is due to release of histamine, serotonin and prostaglandin like substances [19]. WS is able to suppress edema and this effect may be due to the inhibitory effects on the release of histamine, 5 – hydroxyl tryptamine and kinin like substances which are reported to release from mast cell degradation during first hour of carrageenan induced artificial paw edema [20]. This decrement of paw volume might be due to the presence of phenolic compounds in the extract. Compounds like phenolic compounds and flavonoids are reported to produce anti-inflammatory action by decreasing capillary permeability [21].

Bhatia [22] has mentioned that hydrogen sulphide synthesizing enzyme level is noted to

be increased along with the paw volume. The significant decrease of paw volume of WS might be due to their effect on hydrogen sulphide synthesizing enzyme which should be evaluated further. Freund's adjuvant, prepared from certain heat-killed species of Mycobacteria by dispersion in mineral oil alone (i.e. no emulsifier), induces a chronic polyarthritis in 10 days or more after being injected in the footpad or tail or ears of sensitive rat strains [11]. Chatterjee [23] has mentioned that inhibition of adjuvant induced arthritis in rats is one of the most suitable test procedures to screen anti-arthritic agents since it closely resembles WS root to arthritic animals has resulted in the suppression of generalized immunological response to the constituents of the tubercle bacilli which become disseminated after administration in the sub-plantar region.

Freund's adjuvant disease is a complicated one due to the involvement of many organs other than the joints, notably the liver, bone marrow, spleen, etc. This made us to evaluate the effect of extract in Freund's adjuvant affected organs other than joints like liver and kidney [24]. A major systemic event that occurs in the rat following the induction of inflammation is the marked alteration in the cellular defence mechanism. Many cellular defence mechanisms are directed against the toxic effects of these radicals in inflammatory process. TBARS which is formed as an end product for these toxic effects has been quantified in our present study. The decrement of TBARS observed by treatment has been suspected as the effect of antioxidant activity of WS. Anti-lipid peroxidative activity of WS has been reported earlier by Dhuley [25,26,27]

To reconfirm the antioxidant activity various antioxidants like Glutathione, catalase and glutathione peroxidase level are also estimated. GSH level is found to be decreased significantly in liver tissue of diseased rats against normal ($P<0.05$, Table 6). This non-enzymatic antioxidant is observed to be increased by treatment. Effect of WS in increasing the level of GSH has been reported earlier by Singh [3]. In kidney tissue, the treatment is kept to be decreased (Table 6). This might be due to the mobilization of GSH from kidney to protect the animal from damage caused by Freund's adjuvant.

Catalase and GPx levels of both liver and kidney homogenate is found to decrease significantly in diseased rats ($P<0.05$, Table 6). This might be

due to the utilization of enzymes by the diseased animals to protect the organ from damage caused by Freund's adjuvant. Treatment with extract is acted to bring back the level of these enzymes to normal level. This might be due to the antioxidant effect of WS. The increasing catalase and GPx by WS might be due to the presence of glycowithanolides [3].

Fig. 1 reflects the better effect of WS than the standard drug Indomethacin. Administration of Indomethacin for a longer duration (10 mg/kg b.wt. for 45 days) is reported to develop lipid peroxidation of kidney. The toxic effect of indomethacin has been recorded earlier by Hemieda [28,29]. The toxic effect is not seen in WS treatment. This result reflects that WS treatment prevail better protection in anti-arthritis and anti-inflammatory effect without toxic effect. Likewise, the antioxidants like GSH (71.5 %), GPx (50.0 %) levels are found to get increased drastically in the liver homogenate by treating animals with WS. Indomethacin administration is recorded to increase these GSH and GPx level only by 9.6 % and 31.9 %, respectively. These results reveal that WS extract treatment is exhibiting better activity when compared to standard drug treatment.

5. CONCLUSION

In conclusion, 85 % methanolic extract of WS exhibits antinociceptive effect at the lower dose and anti-inflammatory activity in carrageenan induced paw inflammation at the lower dose. Moreover, the anti-inflammatory activity exhibited by extract is nearer to that of standard drug Indomethacin. Treating Freund's adjuvant induced arthritic animals of extract for 45 days is found to decrease the paw volume. The paw volume decrement of extract treatment is looked to be similar to that of Standard drug indomethacin. Further, Indomethacin drug treatment is seen to develop a toxic effect in kidney which is reflected in the level of TBARS. Similar toxic effects have not been observed by extract and the same dose of extract increased the level of antioxidants comparatively better than that of Indomethacin. It reflects that 85 % methanolic extract of WS treatment aid better activity than Indomethacin for chronic treatment.

CONSENT

It is not applicable

ETHICAL APPROVAL

All the animal experiments were performed after getting clearance from Animal ethical clearance (Clearance No. 11/SASTRA/IAEC/RPP) from SASTRA University, Thanjavur, Tamil Nadu, and India.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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