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Evaluation of Antioxidant, Analgesic and Anti-Inflammatory Activity of Hydro - Alcoholic Extract and Ethyl Acetate Fraction of Withania Coagulans Dunal

KEYWORDS: Withania coagulans, Analgesic, Anti-inflamatory, Antioxidant

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ABSTRACT *Background: Present study is designed to evaluate antioxidant, analgesic and anti inflammatory potential of Withania coagulans Dunal in rat and mice which may be the basis for treating various challenging disorder.*

Objective: Evaluating antioxidant, analgesic and anti-inflammatory activity of Withania coagulans Dunal .

Material and Method: Hydroalcoholic fraction and ethyl acetate fraction were extracted from fruits of Withania coagulans Dunal. Anti-inflammatory and analgesic activity was evaluated by carrageenan induced right hind paw edema and acetic acid induced writhing respectively. Antioxidant assay was performed by DPPH scavenging activity.

Result: Hydro alcoholic fraction was found to be effective in treating inflammation (80.43 % inhibition as compared to diclofenac, 88.45 %±0.35) and pain (72.78 % protection as compared to diclofenc, 87.00 %). Ethyl acetate fraction was also effective for treating inflammation (88.19% inhibition) and pain (86.32 % Protection). Both fraction acts as antioxidant (scavange DPPH).

Conclusion: The anti-inflammatory and analgesic activity of ethyl extract of Withania coagulans are close to standard diclofenac with less ADR. Antioxidant potential can give additional benefit to the patients in free radical associated disorder.

1. Introduction

Withania coagulans Dunal is a well-known solanaceous shrub. It is traditionally used as digestant, anti flatulent, sedative, emetic and diuretic (Maurya et al., 2010). In some part of the sub-continent, the berries are used as blood purifier (Maryam et al., 2012). It is well known in the indigenous system of medicine for the treatment of ulcer, rheumatism, edema, and senile debility (Jain et al., 2012). The twigs are chewed is cleaning teeth and the smoke of the plant is inhaled for relief in toothache (Kiritika & Basu, 1981; Atta-ur-Rahman et al., 2003). Withania coagulans is also used traditionally for the treatment of gout, and lumbago and local people used it as an anti-asthmatic (Said, 1970; Iqbal et al., 2010). Withania coagulans is known as Tukhm-e-hyat in Unani system of medicine, cheese maker in english and Akri in hindi. This plant is indigenous to Pakistan and Afganistan.It is also found in some western part of Rajasthan and Punjab and used there as a folk medicine (Iqbal et al., 2010). Fruits of the plant have milk coagulating properties (Iqbal et al., 2010; Atta-ur-Rahman et al., 2003). Previously various type of anolides and other phytoconstituents were isolated from W. coagulans like withanferin A, (Jain et al., 2012), withacoagin (Neogi et al., 1988), Coagulin B,F,G, ergostane type steroidal lactone (Maurya et al., 2012), 5, 20 [alpha](R)-dihydroxy-6 [alpha], 7 [alpha]-epoxy-1-oxo-(5 [alpha]) witha-2, 24-dienolide, a new steroidal lactone (Subramanian et al., 1971), withanolides B (Atta-ur-Rahman et al., 1993), Coagulansin A and B (Subaraju et al., 2006, maurya et al., 2012).This chemical constituent show broad spectrum of activities in different cadre in different forms. Some major therapeutic utility are mentioned as follows - antidiabetic activity (Kirtikar et al., 2012; Hemlatha et al ., 2004; Hoda et al., 2010), antibacterial activity (Arora et al., 2004; Owais et al., 2005), antifungal activity (Dasgupta et al., 1970), anticytotoxic activity (Gabriel et al., 2010, Widodo et al., 2007), myocardial depressant (Budhiraja et al., 1983), withdrawl syndrome, hepatoprotective (Budhiraja et al., 1986), hypolipidemic activity (Hemalatha et al., 2006), wound healing activity (Prasad et al., 2010). This research article mainly gives the information about potent analgesic and anti inflammatory activities of the extract of Withania coagulans in wistar albino rat.

2. Materials and Method

2.1 Plant material

The dried fruits of Withania coagulans have been purchased from crude drug market, Dariyagunj, Delhi and identified by Dr M.P. Sharma, Taxonomist, Department of Botany, Jamia Hamdard, New Delhi. A voucher specimen (Voucher N.O- PRL/RMH/2010/01) is submitted in the Phytochemical Research Laboratory, Faculty of Pharmacy, Jamia Hamdard, New Delhi.

2.2 Extraction

The dried fruits (3 kg) were coarsely powdered and extracted with methanol-water (90:10) with the help of soxhlet apparatus(brand: Cole-Parmer,Mumbai,India). The extracts were concentrated on a steam-bath and dried under reduced pressure to get 432gm (14.4% yields) of dark brown mass. Conc hydro-alcoholic extract with small volume of 2N hydrochloric acid to the p^H upto 3.5 was used.The extract after hydrolysis for 3-4 hrs was put on the crushed ice and cooled for 1 hr, then the volume of the total content was measured. Then equal volume of hydrolyzed extract and ethyl acetate was taken in a partioning funnel for the fractionation of ethyl acetate fraction from hydrolysed hydro-alcoholic extract . The process is repeated thrice and organic layer pulled was evaporated to dryness and measured 96 gm (3.2% yields). The fractioned ethylacetate fraction was dissolved in a little quantity of methanol and adsorbed on silica gel (60-120 mesh) for preparation of slurry. It was dried in air and chromatographed over silica gel column packed in dichloromethane. The column was eluted with dichloromethane, ethyl acetate and methanol successively in the order to increase polarity to isolate the various compounds.

2.2.1 TLC Finger printing

Solvent systems were developed for establishing the TLC

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patterns for the ethyl acetate fraction of the Withania coagulans. Various visualization techniques were used to come up with the best TLC fingerprint, like UV 254, UV 366. The developed plates were dried in air, visualized in UV at wavelengths 254 and 366 nm and photographed. (Table 1)

2.2.2 HPTLC Scanning
The developed plates were taken to the CAMAG HPTLC scanner IV for the densitometric scanning. The plates were scanned with UV 254 nm and UV 366 nm. (Figure 1-1.6)

2.2.3 Homogeneity of the fractions
The fractions collected were subjected to TLC to check homogeneity of various fractions. Chromatographically identical fractions were combined and pooled and kept for crystallization.

2.3 Evaluation Procedure
Melting point was determined on Perfit melting point apparatus. Spectra were obtained with following apparatuses: FTIR: Jasco FT/IR-5000; UV: Lambda Bio 20 Spectrophotometer, MeOH; 1H-NMR (400 MHz): Advance DRY 400, Bruker Spectrospin, CDCl 3; 13 CNMR (75 MHz): Advance DRY 100, Bruker Spectrospin, CDCl3 with TMS as an internal standard; MS: TOF-ES-ionization, JEOL-JMS-DX 303. Column chromatograpy: Silica gel (Qualigens), 60-120 mesh; TLC: Silica gel G (Qualigens). Spots were visualized by exposure to iodine vapors, UV radiation and by spraying reagents.

2.4 Antioxidant Assay
2.4.1 DPPH scavenging activity
This assay determines the antioxidant activity of the test extract towards stable free radicals. The free radical scavenging property of plant extracts were determined using DPPH (2,2 –Diphenyl-1-Picrylhydrazyl) radical. The test samples were treated with different concentration (10µg/ml to 200µg/ml) at a ratio of 3:1, (3 ml extract solution is mixed with 1ml of 0.1 mM solution of DPPH in ethanol). After 30 min at room temperature, the absorbance values were measured at 517 nm and converted into percentage of antioxidant activity. Ascorbic acid was used as a standard control. Each assay was repeated thrice and recorded as mean of the triplicate. Capacity to scavenge DPPH radical was calculated by using following equation (Baheti et al., 2005; Viturro et al., 1999). % scavenging Effect = [1- Abs. (s) /Abs. (c)] x100 Where, Abs. (s) = Absorbance of sample, Abs. (c) =Absorbance of control. (Fig no 2.)

2.5 Animals
Wistar albino rats of weight 150–180 g & age 7-8 weeks and mice weight 25-35 gm were taken from central animal house facility, Jamia Hamdard. The animals were maintained as per Committee for the Purpose of Control and Supervision of Experiments on Animals (Registration no. 173/CPCSEA) guidelines at a temperature (25 ± 20 °C) and relative humidity (30-70%) with a 12:12 light-dark cycle, in the animal house facility of the department under ambient condition. The animals were kept on purified diet and water ad libitum. The project was approved by the Institutional Animal Ethics Committee (IAEC), Jamia Hamdard.

2.6 Anti-inflammatory activity
2.6.1 Carrageenan induced rat paw edema
Anti-inflammatory activity was investigated in Wistar albino rats of either sex weighing 150–200 gm using carrageenan induced rat paw edema method (Afzal et al., 2012, Winter et al., 1962). The animals were divided randomly into four groups having six animals in each. The right hind paw edema was induced by sub-planter injection of 100 µL of 1% carrageenan solution in 0.9% saline and volume of paw edema (ml) was determined by digital Plethysmometer before and after 3 & 4 h of carrageenan injection. The standard drugs diclofenac at a dose of 100 mg/kg, hydroalcoholic extract (500 mg/kg) and compound WC-1 (300 mg/kg) were administered p.o 1 h prior to carrageenan injection. The control group received only 0.5% w/v solution of carboxymethyl cellulose (CMC). The percent edema inhibition was calculated according to the following equation:

% edema inhibition ¼ Vc–Vd=Vc - 100 Where, Vc represents the mean increase in paw volume in the absence of test drug (control) and Vd represents the mean increase in paw volume after treatment with test and standard drugs. The anti-inflammatory activity of the aqueous extract and compound 1 relative to that of diclofenac was also determined (Table 2).

2.7 Analgesic Activity
2.7.1 Writhing Test
Animals were divided into four groups having 6 rats in each group. Group 1 served as negative control and was treated orally with vehicle (0.5% w/v CMC solution). The second groups received Diclofenac were served as positive controls. The third and fourth group animals received the hydroalcoholic plant extracts and Ethyl acetate fraction at the doses of 500 mg/kg and 300mg/kg respectively. One hour after oral administration of these substances, each animal was injected intraperitoneally with 0.3% acetic acid, in a volume of 0.1 mL/10 g body weight.

After acetic acid injection, the number of stretchings or writing responses per animal was recorded (Koster et al., 1959) during a subsequent 20 min and the results are expressed as mean± S.E.M in Table 3.

Analgesic activity was measured as percent decrease in writhings (% protection) when compared to control. The percent protection was calculated using the following formula:

% Protection = {1-(number of writhing in test/ writhings in control)}×100.

2.8 Statistical analysis
All the data were expressed as mean±S.E.M., and analysis of variance (ANOVA) was used for the statistical analysis. The values were considered to be significant when the P value < 0.01.

3. Result and Discussion
Result from the pharmacological evaluation of hydroalcoholic extract, ethyl acetate extract of Withania coagulans shows analgesic activity against writhing effects induced by acetic acid (Koster et al., 1959, Afzal et al., 2012). The maximum significant inhibitory action (P< 0.01) exerted by the isolated compound at the dose of 300 mg/kg found to be 80.32%.

Hydro alcoholic extract of the plant also showed significant (P< 0.01) analgesic activity and found to be 72.78% at a dose of 500 mg/kg compared to standard drug diclofenac at a dose of 100 mg/kg which showed 92% protection.

It is very well known that intraperitoneal injection of acetic acid produced pains by stimulating chemosensitive nociceptors (Stai et al.,1995).It also cause pain by irritating the visceral surface leading to liberation of histamine, bradikynin, prostaglandins and serotonin (Schowb and Dubost, 1984; Garcia et al., 2004) through which an opioid agonist, opioid partial agonist and non-steroidal anti-inflammatory agents act. Since, Withania coagulans are traditionally known to treat pain, (Budhiraja et al. 1977) ,the analgesic activity of ethyl acetate fraction and hydroalcoholic extract by writhing test and found that the ethyl acetate extract of Withania coagulans have very potent analgesic efficacy which may act by inhibiting these chemicals responsible for pain and scavenging free radicals.

The Results from anti-inflammatory activity which was evaluated via carrageenan induce edema (Table 2) revealed that hydroalcoholic extract (500 mg/kg, p.o) and ethyl acetate extract (300 mg/kg, p.o) were found to be efficacious against inflammation (% inhibition 80.43 and 83.19 respectively) which was complimentary to the result of standard drug diclofenac having inhibition of 88.45%. Free radicals like superoxide anion [O], hydroxyl radical [OH], and hydrogen peroxide [H

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2O2]. These are controlled and balanced by the antioxidants level which is cellular defense mechanism of the body. It includes SOD, GPx, Catalase, Glutathione (GSH/GST) scavenger system (Shinya et al., 1995). The imbalance leads to oxidative stress that is the main cause of various organ toxicities. Thus free radical scavenging is very essential for preventing organ injury associated with shock, inflammation. (Jain et al. 2010, Hemalatha et al., 2004). Hydroalcoholic extract of Withania coagulans showed remarkable free radical scavenging activity. The results of absorbance and % inhibition showed decrease in the concentration of DPPH radical due to the scavenging property of extract and standard ascorbic acid, as a reference standard. The anti-inflammatory action of the compound and the extract of the plant may have this property due to its antioxidant property as the leaves extract of this plant is known to inhibit inflammation by inhibiting COX-2 responsible in mediating inflammation (Jayaprakasam and Nair, 2003).

Table1: TLC fingerprinting

S.No	Sample	Solvent system	Wavelength (nm)	Rf value	Number of spots
1	Ethyl acetate fraction	Toluene:Chloroform: Formic acid (6:4:1 v/v/v)	254 nm	0.35,0.47, 0.53,0.65, 0.79,0.96	6
2	Hydro-alcoholic extract	Chloroform:Methanol: Formic acid (7:2.5:0.5 v/v/v)	366 nm	0.19,0.32, 0.42,0.48, 0.79	5

Table 2: Anti-inflammatory activity of hydroalcoholic and ethyl acetate extract of Withania coagulans against carrageenan induced paw edema in Wistar albino rats.

Groups	Treatment	Dose	Paw edema volume mean (ml) ± SEM			% inhibition ± SEM[a]		Activity related to Diclofenac
			0 hr	after 3 hr	after 4 hr	after 3 hr	after 4 hr	
I	Normal Control (0.5% w/v CMC solution)	1 ml/kg	0.78± 0.012	1.84± 0.012	1.95± 0.08	---	---	---
II	Standard (Diclofenac)	100 mg/kg	0.75± 0.011	0.82± 0.019	0.85± 0.020	82.63± 0.63	88.45± 0.35	85.54
III	Hydroalcoholic extract of Withania coagulans	500 mg/kg	0.73± 0.018	0.85± 0.045	0.86± 0.076	76.24± 0.12	80.43± 1.13*	78.82
IV	Ethyl acetate fraction of Withania coagulans	300 mg/kg	0.71± 0.015	0.89± 0.056	0.84± 0.034	86.82± 0.09	88.19± 2.43**	87.97

[a] S.E.M. denotes the standard error of the mean; ** P<0.01, * P<0.05 compared to Diclofenac, n=6

Table 3: Effect of hydroalcolic extract and ethyl acetate extract of Withania coagulans on acetic acid induced writhing in mice.

Groups	Treatment	Dose	No. of writhings ± SEM	% Protection
I	Normal Control (0.5% w/v CMC solution)	1 ml/kg	77.30±2.24	--
II	Standard (Diclofenac)	100 mg/kg	8.69 + 0.78**	87.00
III	Hydroalcoholic extract of Withania coagulans	500 mg/kg	19.49±1.02	72.78
IV	Ethyl acetate fraction of Withania coagulans	300 mg/kg	09.56±0.76**	86.32

Data were analyzed by ANOVA followed by Dunnett's multiple comparison test; values are expressed as mean ± SEM. **p<0.01 (n=6).

HPTLC Scannings

Figure 1 HPTLC plate of Withania coagulans, ethyl acetate fraction

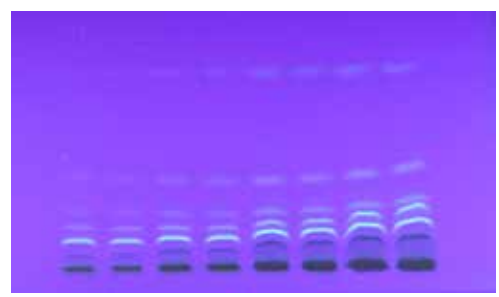

Figure 1.2 Chromatogram of Withania coagulans ethyl acetate fraction

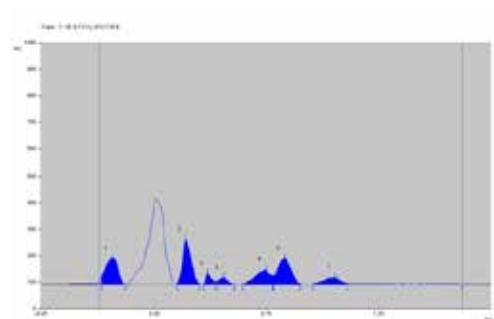

Figure 1.3 Chromatogram of Withania coagulans ethyl acetate fraction

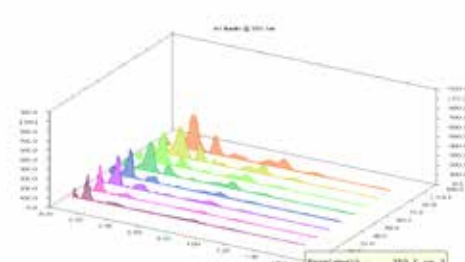

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Figure 1.4 HPTLC plate of Withania coagulans hydroalcoholic extract

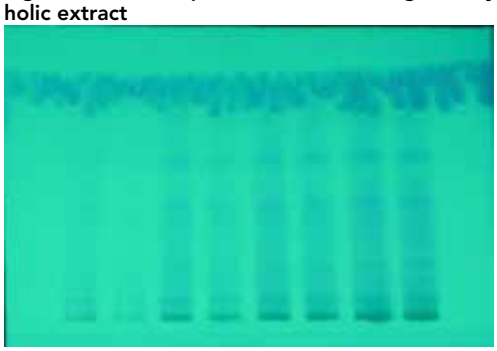


Figure 1.5 Chromatogram of Withania coagulans hydroalcoholic extract

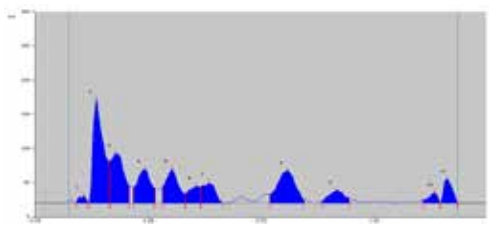


Figure 1.6 3D Chromatogram of Withania coagulans hydroalcoholic extract

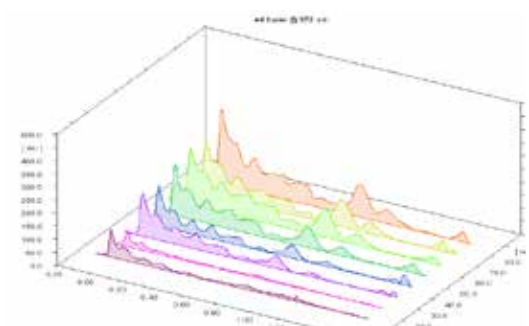
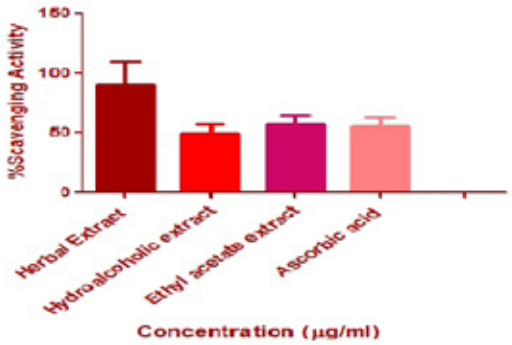


Figure 2



4. Conclusion

Thus on the basis of % inhibition against carraginaan induced rat paw edema and % protection against acetic acid induced writhings it can be concluded that the Withania coagulans Dunal is empowered with plethora of analgesic and anti inflammatory activity. On that basis it can be said that the compound may involve inhibition of agent which is broadly responsible for causing pain and inflammation like cyclo-oxygenase and other inflammatory or pro inflammatory mediators. This may be the pathophysiological basis for the treatment of various challenging disorder associated to pain and inflammation. As the mechanism of action is similar to standard drug diclofenac,so it may be the suitable agent like diclofenac with lesser or no side effect. The antioxidant potential gives additional benefit in older people suffering from inflammatory disease associated with pain like arthritis and disease associated with free radical formation.

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