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IN-VIVO SCREENING OF PET ETHER FRACTION OF LEAVES OF WRITIA TINCTORIA AGINST PSORIASIS VIA SEMI SOLID DOSAGE FORM

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Keywords:

W.tinctoria, ointment preparation, *in-vitro* diffusion study, *In-vivo* study

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ABSTRACT

Wrightia tinctoria R. Br. belongs to family Apocynaceae commonly called as Sweet Indrajao, Pala Indigo Plant, Dyer's Oleander. "Jaundice curative tree"in south India. The object of the present study was to formulte and evaluate the ointment of leaves of *w.tinctoria* ginst psorisis.in the present study physiochemical , phytochemical, TLC,HPTLC, formulation and in-vitro release were investigated successfully. The methanolic extract of leaves of *w.tinctoria* were fractionated with organic solvents based on polarity in that the pet ether fraction was taken for further studies. The physiochemical study showed satisfactory result. The phytochemical screening of leaves of *w.tinctoria* were showed presence of alkaloids, flavanoids, tannins and steroids. TLC and HPTLC showed good spot and peak area for steroids .The quantitative estimation of flavanoids was found to be 2.68mg%w/w. The pet ether fraction rich in steroid were taken for ointment preparation .All the formulation were showed satisfactory result in consistency, pH, melting point, viscosity .The pet ether fraction of wrightia tinctoria leaves showed higher percentage of inhibition against psoriasis .the histopathological studies of pet ether fraction showed the increase in the numbers of the keratinocytes layers including the basal layer.

INTRODUCTION

The genus of *Wrightia* is named after a Scottish physician and botanist William Wright (1740 - 1827). The leaves of this tree yield a blue dye called pala indigo.*W. tinctoria* belongs to family Apocynaceae. It is known by common name as "indrajav"[1]. It has got very important place traditional healing and also is widely recognized medicinal plant. Leaves of this plant showed the presence of flavonoids, glycoflavones-iso-orientin and phenolic acids. Petioles 5mm long. Flowers are usually seen at the tip of branches with 6 cm long cymes, white with fragrance. Calyx and corolla with 5 lobes. Anthers are satiate, ovary bilocular and stigma bifid[2]. Fruits are long follicles up to 50 cm with adhered tips. Seeds are many, linear 1-2 cm long, pointed at the apex .The seeds are released as fruit dehisces. Flowering and fruiting is seen between March to November. It is widely distributed in India and Burma. In deciduous forests, especially in Rajasthan, Madhya Pradesh and peninsular India. Ascending to an altitude of 1300m. *Wrightia tinctoria* R. Br. is considered to be therapeutically very effective jaundice plant in Indian indigenous system of medicine[3]. The juice of the tender leaves is used efficaciously in jaundice. The crushed fresh leaves when filled in the cavity of decayed tooth relieve toothache. In Siddha system of medicine, it is known to be used for psoriasis and other skin diseases[4]. In order to make ensure the use of only genuine and uniform material in preparation of herbal formulation, standardization is still being carried out. Morphological and anatomical aspects as well as differential micro-chemical response have been worked out to identify the characteristic features of the leaf. Physical constant values involving moisture content, ash and extractives as well as qualitative and quantitative estimation of various phytochemicals have been extensively studied. *Wrightia tinctoria* R. Br has shown the presence of lipid, saponin, tannin, alkaloid, phenol, steroid, flavonoid, and some other chemical constituents[5,6]. Oil prepared out of the fresh leaves of the plant has been assigned to analgesic, anti-inflammatory and antipyretic activities and to be effective in the treatment of psoriasis[7].

Collection and authentication: The plants specimen (*Wirghtia tinctoria)*for the proposed study was collected in Chennai, Tamilnadu. It was identified and authenticated by Dr. P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), Tambaram(Chennai). A vocher specimen No. PARC/2010/605

Extraction of steroid from the leaves of *Wrightia tinctoria*

The leaf of *Wrightia tinctoria* R.Br. was shade dried and coarsely powdered. About 500 gms of powder was extracted with methanol (70% v/v) by cold maceration method.

The solvent was filtered and distilled off. Final traces of solvent were removed under vacuum. The total extract thus obtained was fractionated successively with the solvents of increasing polarity viz, petroleum ether, chloroform, ethyl acetate. The process was repeated until the complete extraction of the solvent. The solvent was filtered and distilled off. Final traces of solvent were removed under vacuum. The yield was noted and its percentage was calculated and given in the Table 4.

EVALUATION STUDY

Physicochemical analysis[8]: The physiochemical analysis such as ash value, extractive value, swelling index and loss on drying were carried out as per the procedure given in the standard books. The result was shown in Table 1

Phytochemical studies: The phytochemical screening such as alkaloids, flavanoids, tannins, tri terpenoids, steroids, glycosides, saponin etc were analysed as per the procedure given in the standards. The result was shown in Table 3.

Thin Layer Chromatography: Total methanol extract and its fractions such as petroleum ether fractions, chloroform and ethyl acetate of *Wrightia tinctoria R.Br* were subjected to thin layer chromatographic studies to support preliminary chemical analysis[9]. A number of developing solvent systems were tried, but the satisfactory resolution was obtained in the solvent system for flavonoid was Toluene: Ethyl acetate: Formic acid (5:4:1).The result was shown in Figure 1.

HPTLC FOR STEROIDS

Materials

Standard β-sitosterol was (Merck, India), TLC aluminum plates pre-coated with silica gel 60 F_{254} (10x 10 cm, 0.2 mm thick).

Standard preparation: A stock solution of β-sitosterol (1000 µg mL-1) was prepared by dissolving 10.0 mg of accurately weighed β-sitosterol in Methanol and diluting it to 10.0 ml with Methanol. Aliquots (0.05 ml to 0.5 ml) of this stock solution were transferred to 10 ml standard volumetric flasks and the volume of each was adjusted to 10 ml with Methanol[10]. From this 2, 4,6, 8, and 10 µl of this solution were applied using LINOMAT 5 applicator with the band

width of 8 mm., which gave different concentration 200, 400, 600, 800, and 1000 µg/spot respectively on HPTLC plate (10x 10 cm, 0.2 mm thick) silica gel 60 F 254 .

Preparation of Sample solution: 200 mg of Petroleum ether fraction were taken, dissolved in methanol and transferred to a 10 ml volumetric flask. The volume was made up to the mark with methanol. This solution was further used for HPTLC estimation. From this 5and 10µl of the solution was applied to the plate with the band width of 8 mm

Development : Chromatography was performed on HPTLC silica gel 60 F254 pre-coated plates. Samples were applied on the plates as bands of 10 mm width with the help of a Camag Linomat IV sample applicator at the distance of 15mm from the edge of the plates. The mobile phase constituted of Toluene- Ethyl Acetate- Methanol 7.0+1.0+0.5 (*v/v/v*). The plates were developed up to a distance of 85 mm in a Camag twin trough chamber previously equilibrated with mobile phase for 30 min. The chromatographic conditions had previously been optimized to achieve the best resolution and peak shape.

Detection and Quantification : Following the development the TLC plates were dried in a current of air with the help of an air dryer in a wooden chamber with adequate ventilation. After drying, plates the plates were immediately scanned and quantified at 360 nm. Data of peak area of each band were recorded. Spectra of samples and standard β- sitosterol were matched. The results were shown in Figure 2.

Formulation of ointment

Formulation of Ointment: The required amount of hard paraffin and cetostearyl alcohol was melted on water bath. To this wool fat and white soft paraffin was incorporated, stirred until all the ingredients were melted. The mixture was stirred thoroughly until cold. The required quantity of ointment base was weighed and incorporated the appropriate medicaments in the different ratios; as per the requirements of the different formulations[11,12]. Triturate the semi-solid medicaments with a small amount of the base; on an ointment slab, with the help of a stainless steel ointment spatula until a homogeneous product is formed. The remaining quantities of the base were added until the appropriate medicaments and uniformly mixed with it. The result was shown in table no 1.

Table 1: Composition of the ointment

S. No	F1	F2	F3	F4	F7	F8
Steroid	0.250	0.125	0.125g	0.250g	.0250g	.0125g
Wool fat	1.243g	1.237g	1.243g	1.237g	1.205g	1.218g
Cetosteryl alcohol	1.243g	1.237g	1.243g	1.237g	1.205g	1.218g
Hard paraffin	1.243g	1.237g	1.243g	1.237g	1.205g	1.218g
White soft paraffin	20.521g	21.039g	20.521g	21.039g	20.498g	20.709g

PHARMACOLOGICAL SCREENING

Experimental protocol was approved by the Institutional Animal ethics Committee IAEC. Ref.No: XII/VELS/PCOG/04/2000/CPCSEA/IAEC/22.2.11.

Evaluation of immunomodulatory activity

Carrageenin induced pleurisy in mice for leucocyte migration assay*:* Pleurisy is induced in rats by paw edema of 0.5 ml of 1% (w/v) suspension of carrageenin in sterile normal saline. steroid rich fraction from *Wrightia tinctoria* were administered at a dose 500 mg/kg orally 1 hr before and 6 hr after the carrageenan injection[13]. Blood sample was collected 24 hr after carrageenan injection from each rat. Volume is measured and total and differential leucocyte counts were determined. Saline treated group serve as positive control. The results were shown in **Table 6.**

Evaluation for anti-psoriatic activity

Rat ultraviolet ray B photodermatitis model for psoriasis : Male Wistar rats weighing around 300 g are used. Hair on the dorsal skin is clipped and carefully shaved. An area (1.5 × 2.5 cm) on one side of the flank is irradiated for 15 min (1.5 J/cm2) at a vertical distance of 20 cm with UV - B lamps. A biphasic erythema is observed. Immediately after irradiation, initial faint erythema appears, disappearing within 30 min[14]. The second phase of erythema starts 6 h after the irradiation and gradually increases, peaking between 24 and 48 h. The color is brownish-red, and the reaction is confined to the exposed area with a sharp boundary. By 48-72 h after irradiation, dark brown scale is formed on the erythematous lesion.

Method of screening: The formulated ointment were applied topically, once daily, 5 times a week, for 2 weeks. Drugs are applied topically, once daily, 5 times in a week, for 2 weeks. Two hours after the last treatment animals were sacrificed.

The irradiated rats are sacrificed after various time intervals by decapitation under ether anesthesia. Skin biopsies are taken immediately, fixed in 10% formalin and embedded in paraffin. Tissue sections (4 µm thick) are stained with hematoxylin and eosin. Level of orthokeratotic region was measured. The result of each fraction against psoriasis was shown in **Fig. 3.**

Treatment regimen

Standard group: Retino- A 0.05% (Tretinoin cream U.S.P.)**, Control group:** Saline solution

Group 1: Steroid rich fraction 0.5%) (Petroleum ether fraction of *Wrightia tinctoria*), **Group 2:** Steroid rich fraction 1% (Petroleum ether fraction of *Wrightia tinctoria*)

RESULT AND DISCUSSION

Physiochemical analysis: Ash value, Extractive value and Loss on drying of the coarsely air dried powder of the leaves of *Wrightia tinctoria* was carried out and the results were reported in **Table 2.** This data may helpful for identifying and ascertaining the quality of the collected crude drug.

Table 2: Physico-chemical standards of *Wrightia tictoria* R.Br

S. No	Parameters	Wrightia tinctoria (%w/w)
1.	Ash Values	
	Total ash	9±0.010
	Acid insoluble ash	2.4±0.034
	Water soluble ash	5.10±0.157
2.	Extractive Values	
	Alcohol soluble extractive value	15.64±0.507
	Water soluble extractive value	16.9±1.145
	Chloroform soluble extractive	12.10±0.324
3	Loss on drying	7.20±.020

Extraction : The successive fractionation of leaves of *Wrightia tinctoria* R.Br was done in the order of increasing polarity i.e. petroleum ether, Chloroform, Ethyl acetate and petroleum ether, respectively. Compared to other fractions the ethyl acetate fraction of *w.tinctoria* showed more yield and semi solid so these fraction was taken for further studies. The yield was reported in **Table 3**

Table 3: Percentage yield of total extract and its fractions of the leaves of *Wrightia tinctoria* R.Br.

Extract/Fraction	**Percentage Yield (% w/w)**	**Colour**	**Consistency**
Methanol extract	13.24	Dark brown	Greasy liquid
Petroleum ether fraction	2.68	Green	Greasy liquid
Chloroform fraction	2.26	Green	Greasy liquid
Ethyl acetate fraction	2.48	Dark brown	Semi solid

Phytochemical studies: The photochemical screening were carried out successfully

The methanolic extract of leaves of *w.tinctoria* showed presence of alkaloids, tannins, flavanoids, saponin, phenolic compounds, and steroids.

The chloroform and pet.ether fraction of leaves of *w.tinctoria* showed presence of terpenoids and steroids .the ethyl acetate fraction of leaves of *w.tinctoria* showed presence of flavanoids, terpenoids and steroids. The results were shown in table 4.

Table 4: Preliminary phytochemical screening of *Wrightia tinctoria*

Extract/Fractions	**Alkaloid**	**Carbohydrate**	**Glycoside**	**Protein**	**Amino acid**	**Saponin**	**flavonoid**	**Phenolic compound**	**tannin**	**Terpenoid**	**Steroid**
Wrightia tinctoria R.Br											
Total methanolic extract	+	-	-	-	-	+	+	+	+	+	+
Petroleum ether fraction	-	-	-	-	-	-	-	-	-	+	+
Chloroform fraction	-	-	-	-	-	-	-	-	-	+	+
Ethylacetate fraction	-	-	-	-	-	-	+	+	-	-	+

Estimation of steroid content*:* The total amount of steroid in the dried powder, total methanolic extract and ethyl acetate fraction were determined by spectroscopic method at a wave length of 415 nm. The results were shown in **Table 5**.

Table 5: Determination of steroid content

S. No	**Extract/fraction**	**mg %w/w**
1	Powder	0.12
2	Total methanolic extract	0.23
3	Pet.ether fraction	0.21

Thin Layer Chromatography: To support phytochemical screening, total methanolic extract, petroleum ether fraction, chloroform fraction and ethyl acetate fraction were subjected to thin layer chromatography. The total methanolic extract showed 5 well clear spots R_f values 0.87, 0.76, 0.62, 0.38, 0.17 and ethyl acetate fraction shows 2 spot, of which one spot of R_f value 0.69

matches with standard (Rutin) R_f value (0.69) The petroleum ether fraction showed 11 well separated spots with R_f values 0.07, 0.13, 0.15, 0.23, 0.36, 0.43, 0.53, 0.57, 0.72, 0.85, 0.94 and chloroform fraction shows 6 clear spots with R_f values 0.07, 0.13, 0.15, 0.72, 0.85, 0.94 in Toluene: Ethyl acetate: Formic acid (5:4:1) solvent system and iodine was used as detecting agent. The results were reported in **Fig.1**

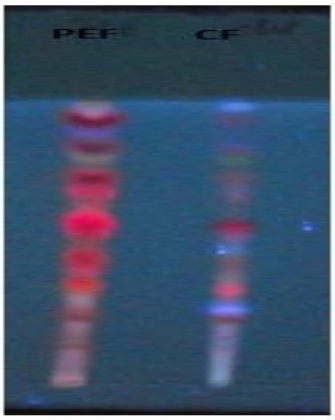


Fig 1: TLC of pet.ether and chloroform fraction

High performance thin layer chromatography for Steroid (*Wrightia tinctoria)***: HPTLC** was done for Petroleum ether fraction extract of *Wrightia tinctoria* against the standard drug Beta sitosterol. The standard concentrations, test sample concentrations were found at different track levels. The *Rf* value of standard Beta sitosterol was found to be 0.12 and peak area 363.40[13]**.The** Petroleum ether fraction extract of *Wrightia tinctoria* showed ten peaks, the second peak *Rf* value (0.13) was slightly coinciding with standard *Rf* value and its peak area was 527.00**.** All the results were shown in HPTLC chromatogram. The peak purity of β- sitosterol was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot. The result was shown in figure 2.

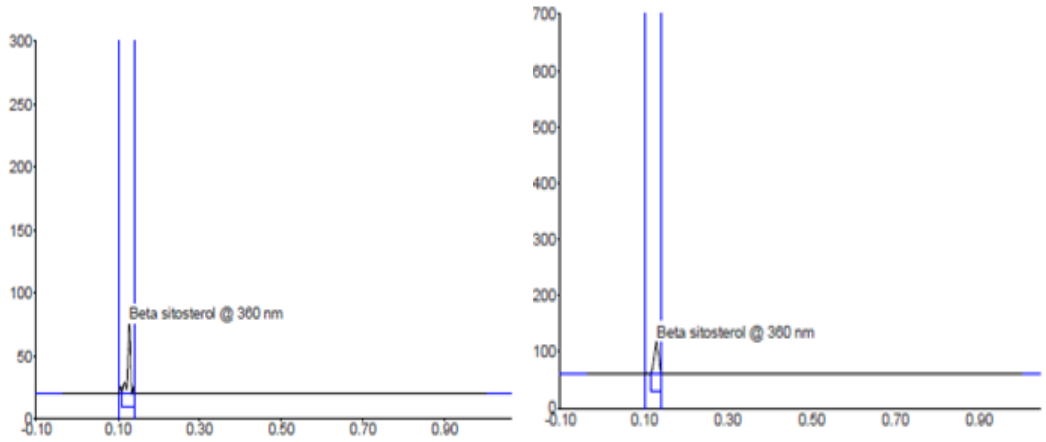


Fig. 2: Chromatogram of β-sitosterol and pet. ether fraction

Pharmacological activity

Carrageenan induced pleurisy in mice for leucocyte migration assay

Effect of steroids on leucocyte migration by the Carrageenan induced pleurisy in mice. The results were shown in **Table 6.** steroids are the potent immunomodulators. Immune system have crucial role in the proliferative stages of psoriasis. So the immune modulator plays an immensive steps in the treatment of psoriasis. steroids have been reported for their immunosuppressive activity. These compounds balance the immune system and will help in the treatment of psoriasis.

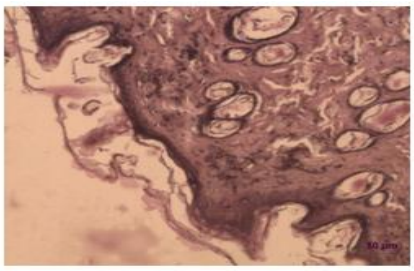
Table 6: Carrageenan induced pleurisy in mice for leucocyte migration assay

S. No	**Control**	**Steroid rich fraction *Wrightia tinctoria***	
		250 mg/kg	500 mg/kg
1	7600	9600	9900
2	7400	9200	9800
3	7100	8500	9900
4	7800	9600	9900
5	7800	9500	9700
6	7400	9700	9800
7	7516± 271.42	9350± 450.56**	9833± 81.65**

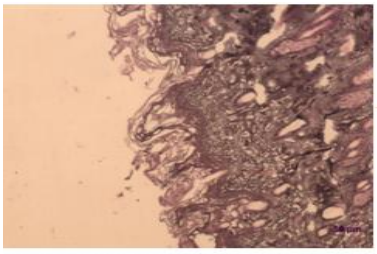
Anti psoriatic activity

Rat ultraviolet ray B photodermatitis model for psoriasis

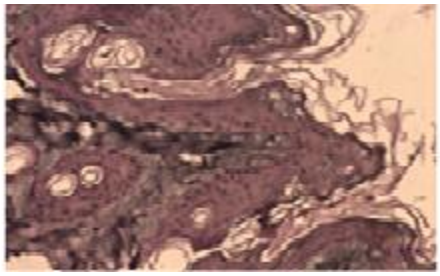
petroleum ether fraction from *Wrightia tinctoria* screened for their possible antipsoriatic activity using ultraviolet ray B photodermatitis model. Formulations were applied topically in the form of a ointment[14]. Drug activity is defined by the increase in the numbers of the keratinocytes layers including the basal layer. Representative examples of the histological specimens underlying the histometrical investigation are shown in **Fig. 3**



Control (Normal rat skin)



Standard (Retino A cream 0.05%. USP)



pet.ether fraction (0.1%)

Fig 3: Longitudinal histological sections through the skin of Ultra Violet-B treated rat totally for 2 weeks, HE staining (original magnification 40×).

CONCLUSION

At present, psoriasis remains pathologies for which no complete cure is available. In the present study, formulation containing steroids from *Wrightia tinctoria* showed markable effect in anti-psoriatic study. On the basis of above observations, it may be concluded that the local application of this formulation as a valuable therapeutic approach to control psoriasis. However, further studies will be needed to elucidate the mechanism(s) involved in anti-psoriatic effect with references to phytoconstituents steroid.

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