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EXTRACTION, PHYTOCHEMICAL INVESTIGATION AND ANTI ACNE ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *WRIGHTIA TINCTORIA*

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ABSTRACT

Acne vulgaris is a common skin disorder affecting more than 85% of teenagers in the United States. While it is not a lethal or debilitating disorder, it can be painful and disfiguring, causing significant physiological distress, and heavy economic burden. Acne is associated with increases in anxiety, depression, and suicidal ideation. *Wrightia tinctoria* R.Br. (Apocyanaceae) is known as a biologically effective plant for the treatment of jaundice in the Indian traditional system of medicine. It is a wild medicinal tree possessing anti-inflammatory, antidiabetic, antinociceptive, hepatoprotective, antibacterial, antifungal, antiviral, antipsoriatic, anticancerous, anthelmintic, aphrodisiac, analgesic, and antipyretic activities. So aim of present work to evaluate anti-acne activity of *Wrightia tinctoria* using suitable animal model. The acne like inflammatory activity was carried out by measuring the ear thickness. Hydroalcoholic extract of *Wrightia tinctoria* (HEWT) showed significant reduction in the ear thickness. It seems that the increased ear thickness and inflammation caused due to various biochemical, viz. various kinins, histamine and 5- HT is significantly reduced.

Keywords: *Wrightia tinctoria*, Hydroalcoholic extract, Anti acne, Hydroalcoholic extact.

INTRODUCTION

Acne is an inflammatory process that extends into the dermis, hence scarring occurs early. In the non inflammatory phase, when there are only comedones, there is no scarring. Once the non inflammatory lesion evolves into an inflammatory lesion, the mechanisms of wound healing are activated. If the inflammation is extensive and deep, extending into the deep dermis and continues untreated, scarring results. The different types of scars in acne are a result of the extent, depth and degree of inflammation that is followed by repair. A study compared the histopathological and immunohistochemical features of acne lesions that were prone to scarring versus those that did not scar [1].

This results in a plug of keratinous material which distends the sebaceous follicle, forming a comedone. Comedogenesis is thus the accumulation of corneocytes in the pilosebaceous duct [2]. This could be due to either hyperproliferation of ductal keratinocytes, inadequate separation of the ductal corneocytes

(increased stickiness) or a combination of both factors. Androgens also play a role in comedogenesis. The enzyme 5 alpha-reductase (type 1) is present in the infrainfundibulum part of the duct and the sebaceous gland. Antiandrogen therapy results in decreased comedogenesis [3].

The depth and degree of inflammation determines the amount, type and depth of scarring in acne. Acne lesions are unusual because the inflammation is initiated beneath the epidermis in the infrainfundibular region of the pilosebaceous structure. The enzymatic activity and inflammatory mediators destroy the dermal structures, hence scarring involves the deeper structures first. Contraction leads to an atrophic scar [4].

Acne vulgaris is a common skin disorder affecting more than 85% of teenagers in the United States. While it is not a lethal or debilitating disorder, it can be painful and disfiguring, causing significant physiological distress, and heavy economic burden. Acne is associated with increases in anxiety, depression, and suicidal ideation. In 2001, a report estimated that over $1 billion is spent each year in the US on acne related health care visits and acne therapies. However, these therapies are not cures, but rather ways of managing this follicular disorder [5].

Current treatments fall into either two categories: topical or oral. Common topical treatments include benzoyl peroxide, retinoids, and antibiotics (i.e., erythromycin or clindamycin), and common oral treatments include retinoids and antibiotics (i.e., tetracycline and macrolides). In cases of severe acne, combinational treatments are used, usually employing benzoyl peroxide, retinoids, and/or antibiotics together. Antibiotics have been used for over 50 years to treat acne and today one course of topical or systemic treatment typically lasts 3–6 months. Antibiotics are thought to inhibit inflammation indicative of acne when used topically and systemically, as well as target *P. acnes* when used topically[6].

Like many other bacteria, *P. acnes* is also subject to emerging antibiotic resistance and novel therapies are in high demand worldwide. *Wrightia tinctoria* R.Br. (Apocyanaceae) is known as a biologically effective plant for the treatment of jaundice in the Indian traditional system of medicine[7]. It is a wild medicinal tree possessing anti-inflammatory, antidiabetic, antinociceptive, hepatoprotective, antibacterial, antifungal, antiviral, antipsoriatic, anticancerous, anthelmintic, aphrodisiac, analgesic, and antipyretic activities. Its constituents are of utmost interest to pharmaceutical industries owing to their many actions and biological activities. Till now antiacne activity not reported for selected plant *Wrightia tinctoria*. So aim of present work to evaluate anti-acne activity of *Wrightia tinctoria* using suitable animal model.

Extraction by maceration method

Collected plant drugs namely *Wrightia tinctoria* leaves were cleaned properly and washed with distilled water to remove any kind of dust particles. Cleaned and dried plant drugs were converted into moderately coarse powder in hand grinder. Powdered plant drugs were weighed (300 gm) and packed in (1 liter) air tight glass Bottle. The plant drug was defatted with petroleum ether for about 12 hrs. The defatted plant drugs were subjected to extraction by ethanol and Water (ethanol: water; 70:30) as solvent for about 24 hrs. The liquid extracts were collected in a tarred conical flask. The solvent removed from the extract by evaporation method using hot plate. The extracts obtained with each solvent were weighed to a constant weight and percentage w/w basis was calculated.

Phytochemical Analysis

Preliminary phytochemical screening means to investigate the plant material in terms of its active constituents. In order to detect the various constituents present in the different extracts of leaves of *Wrightia tinctoria*, were subjected to the phytochemical tests as per standard methods. Phytochemical screening was revealed for the presence of alkaloids, glycosides, carbohydrates, tannins, resins, flavonoids, steroids, proteins and amino acids [7-8].

Determination of Alkaloids

Table 1: Identification tests for alkaloids

S. No.	Identification test	Procedure	Observation
1	Mayer's Test	Test solution + Mayer reagent (Potassium mercuric iodide solution)	White or yellow precipitate
2	Dragendorff's Test	Test solution + Dragendorff's reagent (Potassium iodide + bismuth nitrate)	Showed orange red precipitate
3	Wagner's Test	Test solution + Wagner's reagent (iodine solution)	Brown or reddish brown precipitate
4	Hager's Test	Test solution + Hager's reagent (saturated solution of picric acid)	Gives characteristic crystalline ppt.

Determination of Glycosides and Carbohydrates

Table 2: Identification tests for glycosides

S. N.	Identification test	Procedure	Observation
1	Raymond's Test	Test solution + 1 ml of 50% ethanol + 0.1% solution of dinitrobenzene in ethanol + 23 drops of 20% sodium hydroxide solution	Appearance of violet color, which changes into violet.
2	Killer Killani Test	2 ml of extract + glacial acetic acid + one drop of 5% $FeCl_3$ + conc. H_2SO_4.	Reddish brown color appeared at the junction of the two liquid layers and upper layer appeared bluish green.
3	Legal Test	Test solution dissolved in few drops of pyridine + a drop of 2% sodium nitroprusside + a drop of 20% sodium hydroxide solution.	Deep red color produced.

Table 3: Identification tests for carbohydrates

S.N.	Identification test	Procedure	Observation
1	Molisch's Test	2-3 ml. extract +few drops of α-naphthol solution (20% in ethyl alcohol) + 1 ml. conc. H2SO4 added along the side of the test tubes.	Violet ring was formed at the junction of two liquids.
2	Fehling's Test	Extract heated with dil. HCL +NaOH + Fehling's solution A & B	Brick red precipitate was formed
3	Benedict's Test	Extract +equal volume of Benedict's reagent. Heat for 5 min.	Solution appears Green, Yellow or Red

Determination of Tannins, Flavonoids, and Resins

Table 4: Identification tests for tannins

S.N.	Identification test	Procedure	Observation
1	Vanillin- HCl Test	Extract+ vanillin-HCl reagent (1 g vanillin + 10 ml. alcohol + 10 ml. conc. HCl)	Formation of pink or red color.
2	Gelatin Test	Extract solution + aqueous solution of gelatin	White buff color precipitate was formed.

Table 5: Identification tests for flavonoids

S.N.	Identification test	Procedure	Observation
1.	Lead acetate test	Filter paper strip was dipped in the alcoholic solution of extract. Ammoniated with ammonia solution	Color changed from white to orange.
2.	Shinoda Test	Extract + 5 ml. 95% alcohol + few drops of conc. HCl + 0.5 g magnesium turning.	Pink color observed

Table 6.7: Identification tests for resins

S.N.	Identification test	Procedure	Observation
1.	Color detection with ferric chloride	Extract + alcohol + few drops of FeCl3 solution.	Green color appears
2	Turbidity Test	Extract solution (2 g of drug in methanol) +5 ml distilled water.	Turbidity appears

Determination of Steroids, Proteins and Amino-acids

Table 6: Identification tests for steroids

S.N.	Identification test	Procedure	Observation
1.	Liebermann- Burchard Test	2 ml. extract + Chloroform + 1- 2ml. acetic acid + 2 drops H_2SO_4 from the side of the test tube	First red, then blue and finally green color appeared.
2.	Salkowski Reaction	2 ml. of extract +2 ml. chloroform + 2 ml. conc. H_2SO_4. Shake well.	Chloroform layer appeared red color and acid layer shows greenish fluorescence.

Table 7: Identification tests for proteins and amino-acids

S.N.	Identification test	Procedure	Observation
1	Biuret's Test	3 ml. of extract + 4% NaOH + 2-3 drops of 1% copper sulphate solution.	Presence of red/violet coloration
2	Precipitation test	Mix with absolute alcohol White ppt. 3. Ninhydrin's Test Extract + Ninhydrin's reagent in boiling water bath for 10 min.	Violet color appeared.

Estimation of total phenolic content

Estimation of total phenolic content Total phenolic content of all the extracts was evaluated with Folin-Ciocalteu method. Samples containing polyphenols are reduced by the Folin-Ciocalteu reagent there by producing blue colored complex. The phenolic concentration of extracts was evaluated from a gallic acid calibration curve. To prepare a calibration curve, 0.5mL aliquots of 12.5, 25, 50, 100, 200, and 400 µg/mL methanolic gallic acid solutions were mixed with 2.5 mL Folin– Ciocalteu reagent (diluted ten-fold) and 2.5 mL (75 g/L) sodium carbonate. After incubation at 25°C for 30 min, the quantative phenolic estimation was performed at 765 nm against reagent blank by UV Spectrophotometer 1650 Shimadzu, Japan. The calibration curve was constructed by putting the value of absorbance vs. concentration. A similar procedure was adopted for the extracts as above described in the preparation of calibration curve.

All determinations were performed in triplicate. Total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per g of extract.

Estimation of total flavonoids content

The aluminum chloride colorimetric method was modified from the procedure reported by Woisky and Salatin. Quercetin was used to make the calibration curve. Ten milligrams of quercetin was dissolved in 80% ethanol and then diluted to 0.3, to 6 µg/mL. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Shimadzu spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Similarly, 0.5 mL of Hydroalcoholic extracts and Flavonoid standard solutions (100 ppm) were reacted with aluminum chloride for determination of Flavonoid content as described.

Pharmacological activity

Animals

Wistar rats (180-200 g) and Swiss albino mice (males; 20–25 g) were used in the present study. The animals were procured from College of Veterinary Science and Animal Husbandry Mhow, Indore (M.P), India. They were provided normal diet and tap water ad labium and were exposed to 12-h light and 12-h dark cycle. The animals were acclimatized to the laboratory conditions before experiments. Experimental protocol was approved by Institutional Animal Ethics Committee. Care of the animals was taken as per guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. Experiment protocol was approved by Institutional Animal Ethics Committee.

Acute Oral Toxicity [9]

The acute oral toxicity study was carried out as per the guidelines set by Organization for Economic Cooperation and Development (OECD) revised draft guidelines 423 B ("Up and Down" method) received from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Assessments of Anti acne In-vivo Models [10-11]

Based on the pilot screening the following protocol was carried out. In pilot screening 6 rats were taken under study which showed that the granulomatous inflammation remain constant from day 6th day to 10^{th} day.

Table 8: Protocol study for in-vivo anti acne activity on male Wistar rats

S. No	Groups	Induction of Acne	Treatment
1	Normal control	No induction	Vehicle
2	Control (acne induced)	Heat killed P. acnes in PBS	Vehicle
3	Treated with Standard	Heat killed P. acnes in PBS	Clindamycin 200mg/kg b.w.,p.o.
4	Treated with HEWT	Heat killed P.acnes in PBS	HEWT 100mg/kg b.w.,p.o
5	Treated with HEWT	Heat killed P.acnes in PBS	HEWT 150mg/kg b.w.,p.o
6	Treated with HEWT	Heat killed P.acnes in PBS	HEWT 200mg/kg b.w.,p.o

Thus, depending on the protocol given, the animals were divided into 6 groups containing 6 in each and kept in metabolic cages. All animals had free access to regular rat show and drinking water ad libitum during the study[12].

Induction of acne by Propionibacterium acnes

The acne like inflammatory model was produced in the ears of male Wistar rats (180-200g) by subcutaneous injection of heat-killed bacteria (65^0C for 30 min).

Measurement of ear thickness

Ear thickness was measured as an index of inflammatory strength and acne. Thickness was measured by using a vernier calliper. Thickness was measured once every day for the first week of induction, then every other day until 35th day.

Statistical Analysis

The data were expressed as mean ± SEM and tested for significance by one way ANOVA and Dunnett t-test and results were regarded as significant when $p < 0.05$.

RESULTS AND DISCUSSION

Acne vulgaris is a chronic inflammatory disease results in the formation of inflamed and/or noninflamed eruptions *Propionibacterium acnes* are the anaerobes, in the skin which grow in the sebaceous region. Various antibiotics like tetracycline, Clindamycin, and erythromycin etc. and other drugs like benzoyl peroxide are used for acne treatment. The various drawbacks of synthetic drugs are different side effects and resistant developed towards these drugs. Herbal therapy is required to overcome the above drawbacks

and treat the acne. So in the present study *Wrightia tinctoria* was selected for the anti-acne activity. The preliminary phytochemical study was carried out according to standard literature. This revealed that they contain various phytoconstituents which can be responsible for the anti-acne activity.

The screening of phytoconstituents was done subjectively and quantitatively as a preliminary step. Using the maceration method, preliminary screening was carried out using solvents such as Pet ether and Hydroalcoholic (ethanol: water; 70:30v/v). *Wrightia tinctoria* (Leaves) were studied. Phytochemical study revealed the presence of several compounds in variable amounts in the leaves portions. Hydroalcoholic solvent extract produced the best results. The Hydroalcoholic contained substantial levels of alkaloids, flavonoids, and phenols. Other phytochemicals found in *Wrightia tinctoria* include tannins, saponins, quinones, and fats/oils, which are present in moderate levels.

The percentage yield of dry extract of *Wrightia tinctoria* was 13.58% in pet ether and 19.44 % in Hydroalcoholic extract. The total phenolic content in leaves (mg/100mg) in hydroalcoholic extract was found to be 23.11 µg/100mg dried extract respectively. The total flavonoid content in leaves extract of *Wrightia tinctoria*, in hydro alcoholic extract showed the concentration of 22.18 µg/100mg respectively.

Table 9: Preliminary phytochemical screening of *Wrightia tinctoria* leaves.

S.N.	Phytoconstituents	Test Name	Hydroalcoholic Extract
1	Alkaloids	Mayer's Test	**+(ve)**
		Dragendorff's Test	**-(ve)**
2	Glycosides	Raymond's Test	**+(ve)**
		Killer Killani Test	**+(ve)**
3	Carbohydrates	Molisch's Test	**-(ve)**
		Fehling's Test	**-(ve)**
4	Tannins	Vanillin- HCl Test	**+(ve)**
		Gelatin Test	**-(ve)**
5	Flavonoids	Lead acetate	**+(ve)**
		Shinoda Test	**+(ve)**
6	Resins	Color detection with ferric chloride	**-(ve)**
		Turbidity Test	**-(ve)**
7	Steroids	Libermann- Bur chard Test	**+(ve)**
		Salkowski Reaction	**+(ve)**
8		Biuret Test	**+(ve)**

	Proteins & Amino	Precipitation test	**-(ve)**
	acids	Ninhydrin Test	**+(ve)**
9.	Phenols	Ellagic Acid Test	**+(ve)**

Table 10: Total Phenolic Content of Hydroalcoholic extract of *Wrightia tinctoria*

Sample	Total phenolic content GAE mcg/ml
Hydroalcoholic extract 100µg/ml	23.11± 0.001

n=3, values are given in SEM

Table 11: Total Flavonoid content of hydroalcoholic extract *Wrightia tinctoria*

S. N.	Extracts 100µg/ml	Flavonoid content Quercetin equivalent mcg/ml
1	Hydroalcoholic extract (100µg/ml)	22.81 ± 0.001

n=3, values are given in SEM

Table 12: Changes in the animal behavior after administration of Hydroalcoholic extract of *Wrightia tinctoria* 2000 mg /kg dose

	Time after administration (h)					
Gross activity	**2**	**3**	**5**	**7**	**12**	**24**
Respiration	-	-	-	-	-	-
Writhing	-	-	-	-	-	-
Tremors	-	-	-	-	-	-
Convulsion	-	-	-	-	-	-
Salivation	-	-	-	-	-	-
Diarrhoea	-	-	-	-	-	-
Mortality	-	-	-	-	-	-
Skin irritation	+	-	-	-	-	-
Eye irritation	-	-	-	-	-	-
Sedation	+	-	-	-	-	-
CNS Depression	+	-	-	-	-	-

+: Indicates that change was observed; –: Indicates that there was no change

Table 13: Effect of Clindamycin (standard) and Hydroalcoholic extract of *Wrightia tinctoria* (HEWT)

S. No	GROUP	Mean thickness ±SEM					
		Day1	Day3	Day5	Day6	Day7	Day10
1	Control	1.53±0.016	1.36± 0.012	1.27± 0.021	1.26± 0.026	1.26± 0.02	1.26± 0.02
2	Clindamycin	1.45±0.0054****	1.27± 0.0031****	0.19±0.0018***	0.10±0.0048***	0.10±0.004***	0.10±0.0046***
3	HETW (100mg/kg)	1.51±0.0016*	1.33±0.0041*	0.39±0.0021*	0.33±0.005*	0.33±0.0048*	0.33±0.004*
4	HETW (150mg/kg)	1.51±0.0019*	1.33± 0.0042*	0.30±0.0040*	0.29±0.0263*	0.29.02618*	0.29±0.026*
5	HETW (200mg/kg)	1.48±0.0038****	1.30±0.004**	0.26±0.0014**	0.19±0.0027**	0.19±0.0025**	0.19±0.002**

***P<0.05

Table 14: Percentage inhibition of P. acne induced granulomatous inflammation treated with standard and Hydroalcoholic extract of *Wrightia tinctoria* (HEWT).

Sl. No.	Test Material	Percentage Inhibition (%)					
		Day1	Day3	Day5	Day6	Day7	Day10
1	Std	5.5	6.6	85	92	92	92
2	HETW (100mg/kg)	1.30	2.20	69.2	73.8	73.8	73.8
3	HETW (150mg/kg)	1.30	2.20	76.3	76.9	76.9	76.9
4	HETW (200mg/kg)	3.26	4.41	78.4	84.9	84.9	84.9

CONCLUSION

The acne like inflammatory activity was carried out by measuring the ear thickness. Hydroalcoholic extract of *Wrightia tinctoria* (HEWT) showed significant reduction in the ear thickness. It seems that the increased ear thickness and inflammation caused due to various biochemical, viz. various kinins, histamine and 5- HT is significantly reduced.

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