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"Protective Effect of Indian Medicinal Plant against Acute-Reserpine and Chronic-Haloperidol Induced Orofacial Dyskinesia in Rats"

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Abstract: It was determined that the findings of this study have the potential to contribute to the treatment of psoriasis since the only various therapies that are presently available aim solely to lessen the intensity of the symptoms. Therefore, there is a need for treatments that are efficient, affordable, and have fewer side effects that address the cause of the disorder. Both the ethyl acetate and the methanolic extract of herbs are having significant anti-psoriatic activity through the inhibition of keratinocyte proliferation.

Introduction

i. Selection of Indian medicinal plants

The Indian medicinal plants [Cassia sophera (Family: Fabaceae), Mallotus philippinensis (Family: Euphorbiaceae)] traditionally used for treatment of Psoriasis was collected from Tirumala Hills, Tirupati, Andhra Pradesh for the present study. The plant material was identified and authenticated by Botanist.

ii. Collection and Authentication of selected plants:

The Indian medicinal plants [Cassia sophera (Family: Fabaceae), Mallotus philippinensis (Family: Euphorbiaceae)] are collected from Tirupati hills and also from Chittoor, Andhra Pradesh, and authenticated Botanist Dr. P. Jayaraman, Director, Plant Anatomy Research Centre (PARC), Tambaram, Chennai, Tamil Nadu, A voucher specimen (SVCOP- 1-2016) of the plants no: SVCOP 2016/025 and 026 has been deposited at the herbarium unit of the Department of Pharmacognosy, Sri Venkateswara College of Pharmacy, Chittoor.

iii. Preparation of Extracts

All the plants were washed and air dried individually and after drying the plant material is individually powdered coarsely. The Coarsely powdered dried aerial part of Cassia sophera and Mallotus philippinensis were mixed in equal ratio 1:1:1 (5 Kg) were extracted in 50 % aq. ethanol and the extract is fractioned with various solvents like hexane, chloroform, ethyl acetate and methanol to yield the respective fractions. All the fractions were collected in a 5 liter conical flask, filtered, and the solvent was evaporated to dryness under reduced pressure in a Rotary evaporator at 40^{o}-45^{o}C. All the fractions were stored in a well closed air tight container and kept in desiccator and it is used for preliminary phytochemical analysis.

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iv. Preliminary Phytochemical analysis of different extracts of herbs

The preliminary phytochemical group tests of various extracts of Plants were performed by the standard methods **(Kokate. 2005)** to identify the presence of various chemical constituents.

Literature Survey

Based on the complete ethno medical and ethno botanical literature survey,

The plants were identified and collected from Tirupati hills, Andhra Pradesh and authenticated by the botanist for the correct identification of plants. The medicinal value of a crude drug depends on the presence of chemical constituents of physiological importance. The compounds that are responsible for the therapeutic effect are usually the secondary metabolites. All the collected plants are cleaned from debris washed, shade dried and were coarsely powdered and mixed in equal ratio and extracted using different organic solvents like of hexane, chloroform, ethyl acetate and methanol based on polarity. The medicinal value of a crude drug depends on the presence of chemical constituents of physiological importance. The compounds that are responsible for the therapeutic effect are usually the secondary metabolites. So the plant materials are subjected to preliminary screening for the detection of various plant constituents. The solvent is removed from extracts by distillation under reduced pressure. The concentrated extracts were kept in a dessicator and were used for further experiment. Each extract was weighed and its percentage in terms of air-dried weight of plant material was calculated and also the consistency of the extracts was noted. The hexane, chloroform, ethyl acetate and methanol extracts of the plant extracts were subjected to identification of phytoconstituents. Total flavanoid and alkaloids content in Plant extract was estimated by spectrometric method. Thin layer chromatographic studies (TLC) were done. HPTLC technique is useful for identification of plants and their extracts because each plant species produce a distinct chromatogram with unique marker compounds used for plant identification. In cytokine inhibition assay, the alkaloidal and flavonoidal fraction showed remarkable inhibition of IL-17 and TNF- α, key cytokines involved in the pathogenesis of psoriasis at higher concentration. The serum TNF-*α*, IL-1, IL-6, IL-8, IL-12, and IL-17 levels were significantly higher in active psoriatic patients than in controls. Regulation of the inflammatory events initiated or perpetuated by keratinocytes could so represent an important strategy for the treatment of psoriasis and other chronic inflammatory skin diseases. Thus inhibition of TNF-*α*, IL-1, IL-6, IL-8, IL-12, and IL-17 could be employed as criteria for the evaluation of anti-psoriatic activity.

Objectives as stated in the project proposal:

The following are the broad objectives of this work:

- To separate steroid fraction from Indian medicinal plants
- To separate steroid fraction for phytoconstituents using TLC, HPTLC and HPLC.
- To study the effect of the fractions on the level of cytokine-IL-17, IL-22, TNF-γ, lipooxygenase and keratinocytes.
- To study the anti-psoriatic activity using in-vivo

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Methodology

TEST FOR ALKALOIDS:

- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of plant extracts were separately treated with few drops of dilute hydrochloric acid and filtered. The filtrates were then treated with Dragendroff's reagent (Sodium iodide, basic bismuth carbonate, glacial acetic acid and ethyl acetate). Development of orange brown precipitate in chloroform and methanol fractions indicated the presence of alkaloids.
- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of plant extracts were separately treated with few drops of dilute hydrochloric acid and filtered. The filtrates were treated with Wagner's reagent (Iodine and potassium iodide). Development of reddish brown precipitate in chloroform and methanol extracts suggested the presence of alkaloids.
- Small quantities of hexane, chloroform, ethyl acetate and methanolic extracts of plant extracts were separately treated with few drops of dilute hydrochloric acid and filtered. The filtrates were treated with Hager's reagent (Aqueous solution of picric acid). Formation of yellowish precipitate in chloroform and methanol fractions demonstrated the positive response for alkaloids.

TEST FOR REDUCING SUGAR:

- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of Plant e**xtract** were separately dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume of Benedict's reagent were mixed in a test tube separately and heated for few minutes. Formation of brick red precipitate in methanol and aqueous extracts confirmed the presence of reducing sugars.
- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of Plant extracts were separately dissolved in minimum amount of distilled water and filtered. To the filtrate equal volume Fehling's solutions in a test tube separately heated for few minutes. Development of brick red color in methanolic fraction demonstrated the presence of reducing sugars.

TEST FOR GLYCOSIDES:

Anthraquinone glycosides (Modified Borntrager's test):

- About 10 mg of hexane, chloroform, ethyl acetate and methanolic fractions of plant extracts were boiled with dilute hydrochloric acid for 5 minutes and few drops of ferric chloride solution were added, filtered while hot and cooled. The filtrate was then extracted with benzene and the benzene layer was separated. Equal volume of dilute ammonia solution was added to the benzene extract and shaken well. Rosy pink colour was not observed in the ammonia layer showing the absence of anthraquinone glycosides.

Cardiac glycosides (Keller-Kiliani test):

- About 10 mg of hexane, chloroform, ethyl acetate and methanolic fractions of plant extracts were boiled with 70% alcohol for 3 minutes and filtered. To the filtrate 5 ml of water and 0.5 ml of a strong solution of lead acetate were added, shaken well and filtered. The clear filtrate was treated with equal volume of chloroform and chloroform layer was evaporated. The residue was dissolve in 3 ml of glacial acetic acid and to this two drops of ferric chloride solution were added. The contents were transferred to a test tube

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containing 2 ml of concentrated sulphuric acid. No colour reaction was observed indicating the absence of cardiac glycosides.

Cyanogenetic glycosides (Griard's test):

- Small quantity of hexane, chloroform, ethyl acetate and methanolic fractions of plant extracts were placed in a stoppered conical flask with just sufficient water to cover it. A sodium picrate paper strip was inserted through the stopper so that it was suspended in the flask and it was set-aside for 2 hours in a warm place. No change in the color of the sodium picrate paper was observed indicating the absence of cyanogentic glycosides.

TEST FOR PHYTOSTEROLS:

Libermann-Burchard reaction:

- 10 mg of hexane, chloroform, ethyl acetate and methanolic fractions of Plant extract were separately dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was then added to the mixture followed by 2 ml of concentrated sulphuric acid. A reddish violet ring at the junction of the two layers in hexane and methanolic extracts confirmed the presence of steroids.

Salkowski test:

- When concentrated sulphuric acid and chloroform were added to hexane, chloroform, ethyl Acetate and methanol fractions of plant extract a reddish-blue colour was produced in the chloroform layer and green fluorescence in acid layer, of hexane and methanolic extracts suggesting the presence of steroids.

TESTS FOR SAPONINS:

- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of plant extracts dissolved separately in minimum amount of distilled water and shaken in a graduated cylinder for 15 minutes. Absence of stable foam in all the fractions suggested the absence of saponins

TEST FOR TANNINS:

- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of plant extract dissolved in minimum amount of distilled water and filtered. The filtrate treated with 10% aqueous potassium dichromate solution. yellowish brown precipitate in all the fractions demonstrated the presence of tannins
- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of Plant extract dissolved in minimum amount of distilled water and filtered. The filtrate was allowed to react with 10% aqueous lead acetate solution. Yellow colour precipitate formation in all the fractions indicated the presence test for tannins.
- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of Plant extract dissolved in minimum amount of distilled water and filtered. The filtrates are then allowed to react with 1 ml of 5% ferric chloride solution. Greenish black colour in all the fractions indicates the presence of tannins.

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TEST FOR PROTEINS AND FREE AMINO ACIDS:

- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of Plant extract were dissolved in a few ml of distilled water separately and treated with Ninhydrin (Triketohydrindene hydrate) at the pH range of 4 to 8. The purple coloration suggested the presence of amino acids.
- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of Plant extract were dissolved in a few ml of distilled water separately and treated with Millons reagent. The formation of purple coloration in methanol and aqueous extracts suggested the presence of amino acids.
- Small quantities of hexane, chloroform, ethyl acetate methanolic fractions of Plant extract were treated with equal volume of 5% NaOH and 1%$CuSO_4$ solution, and colour change was observed in methanolic extract indicating the presence of proteins and free amino acids.

TEST FOR GUMS AND MUCILAGE:

Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of Plant extract dissolved in minimum amount of distilled water and filtered. The filtrate was treated with equal volume of concentrated sulphuric acid. Then, it was treated with 15% alcoholic solution of α-naphthol (Molish's reagent). Formation of red-violet ring at the junction of sulphuric acid layer and in methanol fraction indicated the positive test for gums (Molish's test).

TEST FOR FLAVANOIDS:

Shinoda Test

To the of hexane, chloroform, ethyl acetate and methanolic fractions of Plant extract, magnesium turnings and few drops of concentrated hydrochloric acid were added and boiled for 5 minutes. Red colour was produced in ethyl acetate, methanolic fractions showing the presence of flavanoids.

Alkali test:

- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of Plant extract were dissolved in ethanol separately and was hydrolyzed with 10% sulphuric acid and cooled. Then, it was extracted with diethyl ether and divided into three portions in three separate test tubes for each extract. 1ml of diluted sodium carbonate, 1ml of 0.1M sodium hydroxide and 1ml of diluted ammonia solution were added to the first, second and third test tubes of both extracts respectively. Development of deep yellow colour produced in ethyl acetate, methanolic fractions showed the presence of flavanoids.

TESTS FOR FIXED OIL AND FATS:

Spot test:

- Small quantities of hexane, chloroform, ethyl acetate and methanolic fractions of Plant extract various extracts were passed separately between the filter paper leaves stain in all the fractions. Indicates the presence of oils and fats

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Saponification test:

- Few drops of 0.5N alcoholic potassium hydroxide were added to all fractions with few drops of phenolphthalein. The mixture was heated on a water bath for 1-2 hours. Characteristic change was observed in all the fractions which indicated the presence of oils and fats.

Thin layer chromatographic studies (TLC) of flavanoids

Pre coated silica gel GF_{254} Plate 15 cm×20 cm (E. Merck, Mumbai, India) was used as the stationary phase. Ethyl acetate fraction was dissolved in ethanol. This fraction was applied by means of a Linomat IV sample applicator to the plates about 1 cm above the edge. The chromatogram was developed up to 10 cm with Toluene: Ethyl acetate: Formic acid as the solvent system in a CAMAG twin trough chamber. The developed TLC plate was observed under UV-light. From the thin layer chromatographic studies, the presence of various flavanoids was observed with Rf values between 0.14 and 0.74.

High performance thin layer chromatography

High performance thin layer chromatography (HPTLC) is modern adaptation of TLC with improved versatility, separation efficiency and detection limit. HPTLC is useful for identification of plants and their extracts because each plant species produce a distinct chromatogram with unique marker compounds used for plant identification. It is used a quality control tool since comparison of chromatograms of different lots can demonstrate the similarities and differences between the test samples and standard chemical markers. HPTLC is a reliable method for quantitation of a nanogram level even when present in complexes formulation. HPTLC finger print analysis is used for rapid identity check, for monitoring purity of drugs, for a detection of adulterants for determining whether a material is derived from defined botanical species also to known whether the constituents are clearly characterized (Sethi, 1996).

Development of HPTLC fingerprint

- **Instrument**

CAMAG TLC Scanner 3 "Scanner 3 – 070408" S/N 070408 (1.41.21) was used for detection and CAMAG Linomat V sample applicator was used for the application of the tracks.

- **Sample**

The fractions used for taking HPTLC finger prints were ethyl acetate and methanol of plant extracts (10 µl).

- **Stationary phase**

Aluminum sheets pre coated with silica gel Merck G $F_{254,}$ 0.2mm layer thickness were used as the stationary phase.

- **Mobile phase**

$CHCl_3$: MEOH: FA: GAA (7: 2: 1: 1) was used as the mobile phase for developing the chromatogram for ethyl acetate fraction. Ethyl acetate: Hexane (4:6) for methanolic fraction. The mobile phase was taken in a CAMAG twin trough glass chamber.

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- **Detection wavelength**

The developed plates were examined at wavelength 254 and 366 nm.

Chromatographic condition

Sample : Ethyl Acetate fraction
Stationary phase : Silica gel GF_{254}
Mobile phase : $CHCl_3$: MEOH: FA: GAA (7: 2: 1: 1)
Scanning wavelength : 340nm
Applied volume : 5, 10, 15µl
Development mode : Ascending

The percentage recovery was calculated for each extracts analysis by comparing the values with standard.

HPTLC PROFILE of Methanolic extract :

Instrument used : CAMAG make HPTLC.
Software : win CATS 1.4.3
Sample Applicator : Linomat 5.
Detection : @254nm, @366nm in Densitometry TLC Scanner 3
Sample preparation: The sample was prepared in corresponding solvents (50mg in 1 ml)
Stationary Phase : HPTLC plates silica gel 60 F 254.
Mobile Phase : n-Butanol: Acetic Acid: Water (4:1:1)
Derivatization : 5% Ferric chloride reagent, Dragendorff reagent
Sample Solution : 4µl sample is applied as 8mm band for each spot

ESTIMATION OF PHYTOCONSTITUENTS

Estimation of Steroids

1ml of test sample of steroid solution was transferred into 10 ml volumetric flasks. Sulphuric acid (4N, 2ml) and iron (III) chloride (0.5% w/v, 2 ml), were added, followed by potassium hexacyanoferrate (III) solution (0.5% w/v, 0.5 ml). The mixture was heated in a water-bath maintained at 70±20° C for 30 minutes with occasional shaking and diluted to the mark with distilled water. The absorbance was measured at 780 nm against the reagent blank.

Determination of Alkaloid

The plant extract (1mg) was dissolved in dimethyl sulphoxide (DMSO), added 1ml of 2 N HCl and filtered. This solution was transferred to a separating funnel, 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added. The mixture was shaken with 1, 2, 3 and 4 ml chloroform by vigorous shaking and collected in a 10-ml volumetric flask and diluted to the volume with chloroform. A set of reference standard solutions of atropine (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. The absorbance for test and standard solutions were determined against the reagent blank at 470 nm with an UV/Visible spectrophotometer. The total alkaloid content was expressed as mg of AE/g of extract.[17-18].

Determination of total Flavonoid content

The total flavonoids determined by complementary colorimetric methods, aluminum chloride method and the values obtained were summed up to give the final value. The method was

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performed for ethyl acetate and methanol fraction of Cassia sopheraand Mallotus philippinensisI) Aluminum chloride colorimetric method Quercetin was used to make the calibration curve.

1 mg of Quercetin was dissolved in 100 ml ethanol to produce (10 µg/ml). From this solution 0.1, 0.2, 0.3, 0.5, 0.8, 1 ml taken and diluted up to 10 ml ethanol to produce 1, 2, 3, 5, 8, 10µg/ml concentrations respectively. The standard solution was separately mixed with 1 ml of ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of reaction mixture was measured at 415 nm with Shimadzu 1800 spectrophotometer. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. About 1.0 ml of extract solution containing 4 mg extracts was reacted with Aluminum chloride for determination of flavonoids content as described in above procedure; the percentage of total flavonoids was calculated from calibration curve (Maurya& Singh, 2010).

IN VITRO ANTIPSORIATIC ACTIVITY - HACAT KERATINOCYTES CELL INHIBITION ASSAY

The activity was carried out in HaCaT human keratinocyte cell line. Human HaCaT keratinocytes were obtained from NCCS, Pune, India. The cells were seeded at a concentration of 1.0×105 cells/ml in a 96 well microtitre plate and grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (BioWest). After 24 h, the supernatant was decanted and the monolayer was washed once. Then 100 µl of test drug dilution, flavonoidal and steroidal fraction of plant extract of a alkaloidal prepared with above media was added per well in microtitre plates. The plates were then incubated at 37oC for 3 days in 5% CO2 atmosphere. Antiproliferant activity was assessed by performing the Sulphorhodamine B (SRB) assay. Cells were fixed by adding 25 µl of ice-cold 50% trichloroacetic acid on top of the growth medium and the plates were incubated at 4oC for 1 h, after which plates were washed to remove traces of medium, drug and serum. SRB stain (50 µl; 0.4% in 1% acetic acid) (Sigma) was added to each well and left in contact with the cells for 30 min after which they were washed with 1% acetic acid, rinsing 4 times until only dye adhering to the cells was left. The plates were then dried and 100 µl of 10 mM Tris buffer (Sigma) added to each well to solubilise the dye. The plates were shaken gently for 5 min and absorbance read at 550 nm using a micro plate reader (Biorad, USA). Data obtained at different concentrations were used for IC50 calculations.

Assay for the inhibitory effects on IL-l*α*, IL-1β, IL-6, IL-8, IL-17 and TNF-α biosynthesis Endotoxin (LPS) from Escherichia coli 055:B5 was obtained from Difco (Detroit, MI). Heparin was purchased from Takeda (Osaka, Japan) and ELISA kits from RayBio® (RayBiotech, Inc.). Blood collection About 20 mL of odcollected from healthy manvolunteers after an overnight fast of 10–12 h. containing 20U heparin/ml by venapuncture and 30% solution is prepared by suspending in supplemented RPMI-1640 medium containing 100 U/ml penicillin and 100 mg/ml streptomycin. Procedure lipopolysaccharide stimulated human peripheral mononuclear cells (LPS) (1 µg/ml) was dissolved in the supplemented RPMI-1640 media at a concentration of 3 µg/ml. The test sample was dissolved in DMSO at concentrations of 1, 3, 10, and 30 µg/ml and each of these concentrations was diluted with the supplemented RPMI-1640 media (1:100). Only DMSO was contained in control suspension. Equal volumes from each of three solutions (whole blood, LPS and test sample) were mixed and the mixture was incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air for 18—24 h. The supernatant of culture prepared by

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centrifugation was stored at -20°C until the assay of cytokine. The concentrations of the human cytokines (IL-lα, IL-1β, IL-6, IL-8, IL-17, TNF-α) were assayed using an ELISA kits

The ratio (%) of inhibition of the cytokine release was calculated by the following equation:

$$\text{Inhibition (\%)} = 100\times(1\text{-}T/C)$$

where T represents the concentration of the cytokine in the culture supernatant with the test represents the concentration of the cytokine in the culture supernatant with the solvent control

IN-VIVO ANTIPSORIATIC ACTIVITY

Animals

Healthy male Wistar rats (120-170 g) and Swiss albino mice (25- 30 g) obtained from the institutional animal housing facilities were used for the study. Animals were housed in polypropylene cages and were left seven days for acclimatization to animal room, which was kept under controlled conditions (a 12 h light-dark cycle at 22±2°C) and fed on standard pellet diet and water ad libitum. All animals were taken care of under ethical consideration as per the guidelines of CPCSEA with approval from the Institutional Animal Ethics Committee ([CPCSEA] IAEC/SVCOP /52/2017).

Acute toxicity studies

During the acute toxicity study, th methanolic and ethylacetate extract, was administered orally and animals were observed for mortality and behavioral responses. There was no mortality observed even at 2000 mg/kg for the extract and at 500 mg/kg for the compounds. All the animals were normal and there were no gross behavioral changes till the end of the observation period.

Rat ultraviolet ray B photodermatitis model for psoriasis

Mice (six animals per group) were exposure of the rat's skin to UV radiation using a UV-B bulb (wavelength 280-315 nm) induced proinflammatory reaction in the skin that resembles the one observed in psoriasis. This was evident by the altered skin parameters; the most important of which are the increase in epidermal thickness to almost double the normal size, absence of stratum granulosum and the movement of neutrophils towards the epidermis, all symptoms typical of psoriasis., (Michael et al., 2005).

The hairs of one side of the flank of the rat were clipped with scissors followed by careful shaving, taking precaution to avoid injury to the skin. The animals were then placed on a curved wooden block and their legs tied around it, to avoid contact with the floor. This arrangement prevented the movement of the animal during its subsequent exposure to UV radiation. into seven groups (six animals per group). The control group animals received normal Except for an area of 1.5 × 2.5 cm on the depilated skin, the entire animal was covered with a UV resistant film. The uncovered area of 1.5 × 2.5 cm was then irradiated for 20 min with a UV-B lamp kept at a vertical distance of 20 cm from the skin (Vogel et al., 2002). The animals were divided saline (10 ml/kg, p.o.) and standard group received retinoic acid (0.5 mg/kg, p.o.). The remaining groups were treated orally with the methanolic and ethyl acetate extract of plant herbs (200 and 400 mg/kg)) once daily, five times a week, 12 h after irradiation, for two weeks. Two hours after the last treatment animals were sacrificed; longitudinal sections of the tail skin were made and

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prepared for histological examination with hematoxylin-eosin staining (Nakaguma et al., 1995; Singhal and Kansara 2012). Histopathological examination Sections were examined for the presence of Munro's microabscesss, elongation of rete ridges, and capillary loop dilation by direct microscopy. The vertical epidermal thickness between the dermoepidermal junction and the lowest part of the stratum corneum (n = 3 measurements per scale, n = 3 scales per animal, n = 6) were examined. The percentage relative epidermal thickness of all the groups was calculated in comparison to the positive control group (100%; n = 54 measurements per treatment). It was also examined for Mean thickness of stratum corneum and stratum granulosum. All measurements were made at a magnification of 400× using a digital camera attached to an Olympus microscope, and used software to take measurements.

Statistical analysis

Values were represented as mean ± SEM. Data were analyzed using one-way analysis of variance (ANOVA), and group means were compared by the Tukey-Kramer Multiple Comparison test using Instat-V3 software. p values < 0.05 were considered significant.

Results and Discussions

Table 1: Ash values of POLYHREBS

Total ash (%)	Acid Insoluble ash (%)	Water soluble ash (%)
11.23±.115	1.126±0.34	13.340±1.45

Table 2. moisture and fat content of polyherbs

Parameter	%
1. Moisture content	5.4
2. Fat content	2

Table 3. Biochemical composition of polyherbs

Parameter	Values
Carbohydrate mg/g	0.462±0.012mg/g
Protein mg/g	13.29±0.244 mg/g
Amino Acid mg/g	2.224±0.160 mg/g
Total free Phenols (mg/g)	14.3±0.126 mg/g
Tannins (mg/g)	12.1±0.27mg/g

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Total Flavonoids **Total alkaloids**	**91.09± 0.04mg of QEof extract in ethylacetate fraction** **66.08± 0. 33mg/g in methanolic fraction** **66.01± 0.049 mg/g AE/mg of** **91.09± 0.04mg/g**

Table 4: Estimation of metals/Heavy metals in dried material of Polyherbs

S.No	Name of the metal/Heavy metal	Amount in mg
1.	**Sodium**	57.34
2.	**Potassium**	49.32
3.	**Phosphorous**	23.6
4.	**Magnesium**	0.89
5.	**Calcium**	67.7
6.	**Copper**	0.12
7.	**Selenium**	0.6
8.	**Zinc**	0.32
9.	**Lead**	Less than 1 PPM

Table 5: Rf values of standard amino acids and sample of Polyhrebs by THIN LAYER CHROMATOGRAPHY

S.No	Name of the Amino Acid	Rf value	S.No	Name of the Amino Acid	Rf value
1.	L- Proline	0.379	**14.**	L- Histidine	0.102
2.	L-Serine	0.214	**15.**	DL- 2-amino-N-butric acid	0.355
3.	DL-Nor leucine	0.695	**16.**	L.Glycine	0.193
4.	L.Ornithine	0.129	**17.**	L.Arginine	0.163
5.	DL.Threonine	0.238	**18.**	L- Tyrosine	0.373

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6.	L-Cysteine	0.121	**19.**	Phenyl alanine	0.587
7.	L-Leucine	0.677	**20.**	Lysine	0.141
8.	DL-Valine	0.468	**21.**	Tryptophan	0.627
9.	3-3,4-dihydroxyphenyl alanine	0.280	**22.**	Methionine	0.156
10.	L.Hydroxy proline	0.218	**23.**	L- Cysteine	0.419
11.	DL- Isoleucine	0.618	**24.**	L – Aspartic acid	0.349
12.	L. Alanine	0.227	**25.**	SAMPLE	0.162,0.236 0.226,0.158 0.162
13.	L- Glutamic acid	0.160			

Table 6: Percentage yield of total extract of plant material

Parameter	70% Ethanolic extract
Colour of extract	**Dark green**
Consistency	**Semisolid**
Percentage yield (% w/w)	**12.50**

Table 7: Preliminary Phytochemical Analysis of various fractions of Polyherbs (Kokate,1997

Chemical Test	Hexane fraction	Chloroform fraction	Ethyl acetate fraction	Methanol fraction
Alkaloids	-	-	+	+
Carbohydrates	-	-	-	+
Steroids	+	+	+	+
Tannins	-	-	-	+

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Proteins	-	-	+	+
Terpenoids	-	-	-	-
Flavonoids	-	-	+	+
Gums &mucilage	-	-	-	+
Oils &fats	+	+	+	+

It is observed that preliminary phytochemical studies show the presence of flavanoids, alkaloids, terpenoids, tannins, amino acids, carbohydrates and steroids.

The preliminary phytochemical screening of ethyl acetate and methanol fraction of Cassia sophera and Mallotus philippinensiswas performed. The Preliminary phytochemical analysis was made clearly indicated the presence of alkaloids, proteins, glucosides, steroids, tannins and flavonoids.

Determination of Steroids

The highest concentration of steroids was measured 24.44± 0.024mg of extract in ethyl acetate fraction and 37.29± 0.41mg of AE/g of extract in methanolic fraction.

Determination of total alkaloid contents

The alkaloid contents were examined in plant extracts and expressed in terms of atropine equivalent as mg of AE/g of extract. The highest concentration of alkaloid was measured 66.01± 0.049mg of extract in ethyl acetate fraction and 41.08± 0.33mg of AE/g of extract in methanolic fraction.

Determination of total flavonoid content

The content of flavonoids was expressed in terms of Quercetin equivalent mg of mg Quercetin/g of extract. The concentration of flavonoids in ethyl acetate and methanol fraction of Cassia sophera andMallotusphilippinensis was estimated 91.09± 0.04mg/g and 66.08± 0.33mg/g. High solubility of phenols and flavonoids in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction.

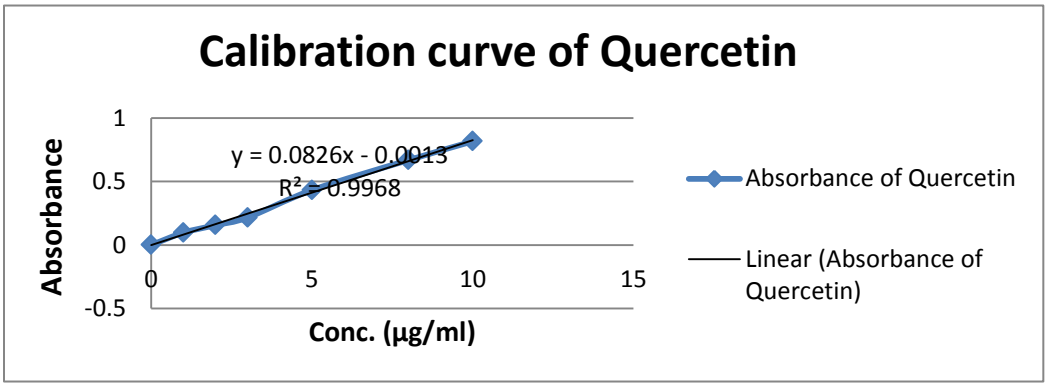

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Figure 1. Calibration curve of Quercetin

Table 8: Protein as Amino Acids was determined by using HPLC System (LACHROM – 700) in extracts

S.No.	Name of the Amino Acid	Amount in mg
1.	Glutamic acid	**0.089**
2.	Asparagine	**0.056**
3.	Glutamine	**0.034**
4.	Arginine	**0.317**
5.	Alanine	**0.125**
6.	Threonine	**0.206**

Table 9 : R_f values of ethyl acetate and methanolic fractions of Polyherb by HPTLC
The fractions used for taking HPTLC finger prints were ethyl acetate and methanol **fractions of plant extracts (10 µl).**

S. No.	Name of the fraction	Solvent system	Detection wavelength	No. of spots	R_f value
1	Ethyl acetate	chloroform: methanol: formic acid: glacial acetic acid (7:2:1: 1)	254 & 366	09	0.07, 0.13, 0.18, 0.31, 0.42, 0.54, 0.64, 0.72, 0.88
2	Methanol	ethyl acetate: hexane (4:6)	254	10	0.04, 0.07, 0.16, 0.24, 0.37, 0.51, 0.57, 0.65, 0.76, 0.82

- **Stationary phase**

Aluminum sheets pre coated with silica gel Merck G $F_{254,}$ 0.2mm layer thickness were used as the stationary phase.

- **Mobile phase**

$CHCl_3$: MEOH: FA: GAA (7: 2: 1: 1) was used as the mobile phase for developing the chromatogram for ethyl acetate fraction. Ethyl acetate: Hexane (4:6) for methanolic fraction. The mobile phase was taken in a CAMAG twin trough glass chamber**.**

- **Detection wavelength**

The developed plates were examined at wavelength **254 and 366 nm.**

- **<u>Chromatographic condition</u>**

Sample : Ethyl Acetate fraction

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Stationary phase : Silica gel GF_{254}
Mobile phase : $CHCl_3$: MEOH: FA: GAA (7: 2: 1: 1)
Scanning wavelength : 340nm
Applied volume : 5, 10, 15µl
Development mode : Ascending

- **Instrument**

CAMAG TLC Scanner 3 "Scanner 3 – 070408" S/N 070408 (1.41.21) was used for detection and CAMAG Linomat V sample applicator was used for the application of the tracks.
The percentage recovery was calculated for each extracts analysis by comparing the values with standard.

- **Chromatographic condition**

Sample : Methanolic fraction
Stationary phase : Silica gel GF_{254}
Mobile phase : Ethyl acetate: Hexane (4:6)
Scanning wavele : 340nm
Applie : 5, 10, 15µl
Development mode : Ascending

Table: 10 HPTLC profile of ethyl acetate fraction

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.05	2.4	0.07	16.0	1.88	0.08	0.2	170.3	0.46
2	0.11	0.1	0.13	22.9	2.69	0.17	11.7	558.5	1.49
3	0.17	11.8	0.18	12.6	1.49	0.21	1.0	201.3	0.54
4	0.23	1.0	0.31	69.6	8.18	0.35	32.5	2415.9	6.46
5	0.35	32.6	0.42	243.2	28.59	0.47	60.1	10991.9	29.40
6	0.47	60.1	0.54	238.7	28.06	0.60	57.7	12944.2	34.62
7	0.60	57.8	0.64	166.4	19.56	0.70	39.2	7007.2	18.74
8	0.70	39.3	0.72	46.7	5.49	0.83	0.0	2306.6	6.17
9	0.84	0.5	0.88	34.6	4.07	0.90	2.0	795.9	2.13

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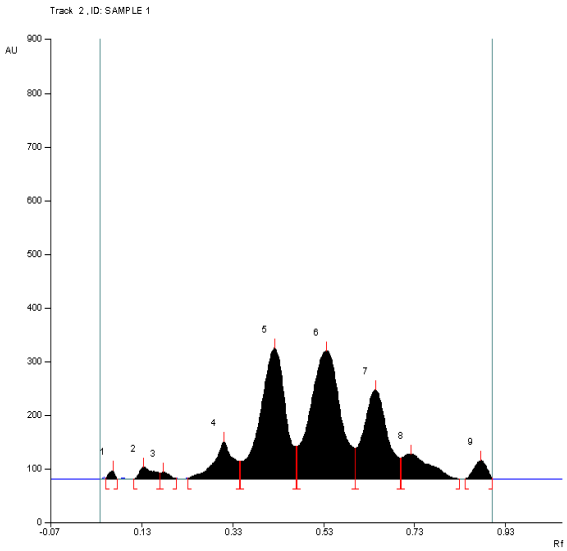
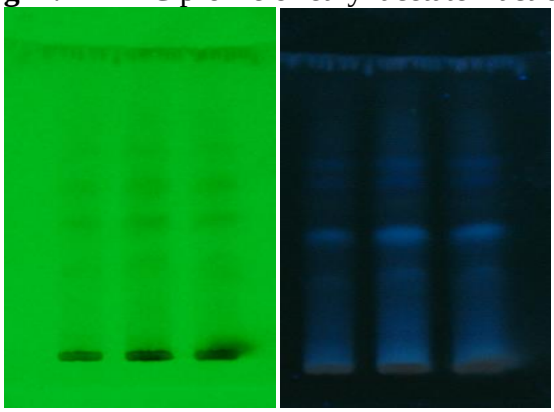


Fig 2 : HPTLC profile of ethyl acetate fraction



254 nm **366 nm**

Fig .3: TLC Profile of ethyl acetate fraction

HaCaT keratinocytes cell inhibition assay

The cytotoxic effect of ethyl acetate and methanol fraction of Cassia sophera and Mallotusphilippinensis were evaluated using HaCaT cells, a rapidly multiplying human keratinocyte cell line, as a model of epidermal hyper proliferation in psoriasis. The tested extract showed appreciable antiproliferant activity in HaCaT cell line due to the presence of flavonoids. The results were validated using asiaticoside as positive control. Ethyl acetate fraction of Cassia sophera and Mallotus philippinensis showed antiproliferant activity significantly (24.27±4.94µg/ml) in HaCaT cell line. Methanol fraction of Cassia sophera and Mallotusphilippinens is showed appreciable antiproliferant activity (29.27±5.67µg/ml) in HaCaT cell line. Asiatic side showed a potent activity with IC50 value of 33.69 µg/ml.

Conclusion

It was concluded that this research can be contributed to treat the psoriasis because only multiple treatments are currently available only to reduce the severity of the symptoms. So there is a need for effective ,affordable therapies with fewer side effects that address the cause of

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disorder and the ethyl acetate and methanolic extract of herbs are having significant anti psoriatic activity through the inhibition of keratinocyte proliferation.

The following research works are to be carried out in future (future work)

- Scaling up of methodology to industrial scale by making creams, ointments and capsules
- To conduct clinical trials in order to prove the quality, safety and efficacy of dosage form.
- To identify which constituents responsible for anti psoriatic activity

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