

Phytochemical screening, metallic ion content and from its impact on Anti psoriasis activity of *Calendula officinalis* and *Phlebodium decumanum* aqueous leaf extracts in animal experiment model

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Abstract

Objective

The aim of this study is to evaluate the influence of metal ions present in soil as well as in the leaves samples of *Calendula officinalis* and *Phlebodium decumanum* for the treatment of psoriasis.

Methods

Looking at the objective, soils and leaves samples were estimated for metal ions by atomic absorption spectrophotometer to determine the influence in anti-psoriatic activity. Thereafter Imiquimod-induced dermatitis lesions were created in grouped mouse. Two plant extracts (aqueous) separately as well as in combinations and standard Retino-A (0.05%) were used. Psoriasis severity index (PSI) was evaluated by phenotypic (redness, erythema, and scales) and histological features (epidermal thickness). Further content of phytochemicals in terms of extract was correlated with the effect of psoriasis activity.

Results:

We observed redness, erythema and scales and the histological features and found a progressive reduction ($P < 0.05$) in the severity of psoriatic lesions (redness, erythema, and scales) from day 7 to 21st day and decreased epidermal thickness in animals treated with combined extracts at the dose of 200 mg/kg b.w. Furthermore plant samples procured from Nandi Hill's, Bangalore, showed better uptake of metals with respect to Fe (2.05 mg/kg), Cu (0.78 mg/kg) and Zn (1.12 mg/kg) which showed positive impact on procurement of maximum amount of extracts that further correlated with the activity, indicated significant reduction of psoriatic lesions.

Conclusion

Results revealed that significant dose dependent antipsoriasis activity of combined aqueous extracts of *Calendula officinalis* and *Phlebodium decumanum* as well as metal ions are also had impact on the procurement of extracts and said activity.

Key words: Epidermal thickness, correlation, *Calendula officinalis*, *Phlebodium decumanum*, PSI, Psoriasis.

Introduction

One of the common immune based chronic autosomal diseases is psoriasis which may develop in any age people. It is also known as genetically influenced inflammatory disease in skin which is identified as salmon colored plaque covered by loosely adherent scales and looks like silver white in color. This disease is spread in whole body in a couple of days and cause total body erythema with scaling known as erythroderma. The disease most frequently affects in to the joints like in the skin of the elbow, knees, scalp, lumbosacral areas, intergluteal cleft and glans penis¹. People are neglecting this dermatitis but it may sometimes associate with arthritis, myopathy, enteropathy, spondylitic heart disease, diffuse cutaneous and mucosal pustules and electrolyte disturbances². Hence proper treatment is required for cure psoriasis in root level. A vast number of allopathic drugs are available for the treatment of psoriasis. Some drugs viz. lithium, β - blockers and chloroquine are provocative factors³ and many drugs are associated with various side effects. Therefore currently emphasizes the importance of using natural herbs for the treatment of skin diseases like psoriasis either in combination or solely in different forms. Whole parts of natural plants such as the root, bark, stem, seed, flowers, or leaves are effective against versatile therapeutic activities. In the present study *Calendula officinalis* and *Phlebodium decumanum* leaves were selected based on the traditional knowledge. *Calendula officinalis* (CO) (Family: Asteraceae), commonly known as pot marigold is abundantly available throughout India and is cultivated in most of soils during sunny climate. The leaves contain carotenoids such as lutein (80%), zeaxanthin (5%) and beta carotene^{4,5}. Apart from that the leaves also content polyphenols, alkaloids, steroids, tannins and flavonoids⁶. Many applications are revealed with the flowers whereas traditionally the leaves are claimed for wound healing activity, treatment

against burn, infections which are mainly due to presence of essential phytoconstituents in leaves. Scientifically leaves are also claimed as having antimicrobial activity⁷, hepatoprotective activity⁸ and wound healing activity⁹. Thereafter *Phlebodium decumanum* (PD) (family Polypodiaceae), commonly known as ornamental fern, is abundantly available in damp region of many places in India¹⁰. The leaves contains many chemicals viz. alkaloids, various fatty acids like oleic acid, linoleic acids, linolenic acids, arachidonic acid, eicosapentaenoic acid, elaidic acid, arabinopyranosides, ecdysone, ecdysterone, juglanin, kaempferols and melilotoside¹¹. Thereafter literature survey revealed the presence of anti-inflammatory activity¹², antioxidant¹³, wound healing, improve immune system¹⁴, antimicrobial, anthelmintic¹⁰ etc. Henceforth therapeutic activities of plant constituents are greatly depend on the soil fertility, climatic conditions and content of metal ions in the accumulated plant parts¹⁵⁻¹⁹. Many literatures reported various activities based on the effectiveness of either extracts in combinations or with isolated compounds but very scanty reports on impact of soil fertility and content of soil metal ions and their uptakes by the plant foliage on therapeutic efficacy. No such literatures are available on relation with metal ion content and activity of the selected plants in this investigation. Looking at that, in the present study *Calendula officinalis* and *Phlebodium decumanum* leaves are selected from West Bengal and Karnataka zones of India for establishment of effective treatment as well as impact of foliage metal ions against psoriasis.

Materials and Methods

Selection of experimental zones

In the present investigation, hilly region of Darjeeling, West Bengal and Nandi Hills, Bangalore, Karnataka zones were selected for collection of leaves samples of said plants because of the soil nature and natural habitat of plant species. Hilly region soil is highly acidic where as soil of Nandi hills slightly basic but both are hill areas (Figure-1). The Terai region lies between latitude 26°30'30" to 27°8'45" N 88° and 88°56' 15" E longitude whereas Bangalore lies between latitude: 12°58'38 N and longitude: 77°35'14 E in which Nandi Hills is located in latitude 13.3667⁰ N and longitude 77.6833⁰ E. Average annual rain fall of Darjeeling and Bangalore are about 2547 mm and 870 mm respectively.

Authentication and preparation of plant samples

The leaves of said plants were taxonomically identified and authenticated by Dr. Rajasekharan P E, Principal Scientist, Department of plant Biotechnology, Indian Institute of Horticultural Research, Bangalore. The voucher specimens of both leaves collected from West Bengal and Karnataka (KCP/34/WB-PD/2016-17; KCP/35/WB-CO/2016-17; KCP/36/KAR-PD/2016-17 and KCP/37/KAR-CO/2016-17) have been deposited in the herbarium section of the Pharmacognosy Department of Krupanidhi College of Pharmacy, Bangalore for further reference.

Leaves were collected during June month of the year 2016 from both the places and transported in sealed plastic cover to the laboratory processing. Leaves are cleaned with running tap water and oven dried at 60⁰ C for 2 -3 hours. Shade dry was not recommended because during rainy season moisture content in environment was high and there were more possibility for microbial growth rather drying. After oven dried the leaves were blended in mixer grinder into coarse powder and separately kept in air tight sealed plastic cover, labeled properly for further investigation.

Analysis of soil sample for metal ion content

Total metals and DTPA extractable metals (iron: Fe; copper: Cu; zinc: Zn; lead: Pb; cadmium: Cd; nickel: Ni; Arsenic: As and chromium: Cr) were determined with the help of Atomic Absorption Spectrophotometer (AAS, Perkin Elmer model: AAnalyst 100; Australia) by acid digestion method. 10 g of soil sample was taken in a conical flask and 20 ml of 0.005 M DTPA (0.005 M DTPA; 0.1 M Triethanol amine and 0.01 M CaCl₂, 2 H₂O) was added to it. Then it was shaken for 2 hours on a mechanical shaker and it was filtered with Whatman No. 42 filter paper. Then the filtrate was determined for various metal contents in different soils. Blank samples were also prepared for corrections. All the samples were checked by carrying out triplicate analyses for the reproducibility of the method used.

Analysis of leaf samples

Leaf samples were pretreated with concentrated nitric acid in digestion flask followed by mixed with acid mixtures. Digestion was carried out at 200⁰C until dense white fumes of H₂SO₄: HClO₄ were evolved and finally obtained white residue. Subsequently the digested samples were diluted with deionized water and volume made up to 50 ml. Final solutions were analyzed for various heavy metal contents (Cd, Cr, Cu, Fe, Ni, Pb and Zn) using atomic absorption

spectrophotometer (AAS; Perkin Elmer model: A Analyst 100; Australia). Air-acetylene is used as the common oxidant/fuel combination gas in AAS and the concentration of the above said elements was determined by using the standard condition. The wavelengths were selected for the analysis based on the concentration ranges of sample and the linear relation between the absorbance (AU) and concentration of the determined element. Blank samples were also prepared for corrections. All the samples were checked by carrying out triplicate analyses for the reproducibility of the method used.

Preparation of plant extract and their phytochemical screening

Stored coarsely powdered samples (250 g) were used for preparation of extract by direct reflux method using distilled water as solvent at 45^o C for 8 hrs. Thereafter extracted liquids were filtered with Whatman No. 1 filter paper and evaporated with the rotary flash evaporator at 45^oC and stored in refrigeration condition (at 4^oC) in glass bottles for further experimentation. The yield of extracts was calculated followed by presence of various phytochemicals were screened qualitatively by various chemical tests for the detection of constituents like alkaloids, flavonoids, steroids, tannins, glycosides, terpenoids and others as methods described by following standard methods^{20, 21}.

Selection of animals

Healthy Albino mice (50-70 g) obtained from Krupanidhi Pharmacy institutional animal housing facilities were used for the present investigation. Animals were housed in polypropylene cages and were left seven days for acclimatization to animal room and was kept under controlled conditions (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 (KCP/PCOL/06/2017).

Acute dermal toxicity

Acute dermal toxicity studies were carried out using Albino mice as per according to the Organization for Economic Cooperation and Development guidelines no. 402²². Albino mice (six animals per group) were divided into two groups. Animal hairs were removed from the dorsum portion of body surface area and applied at a dose of 2000 mg/kg body weight for two different extracts. The animals were observed and recorded for change of redness, erythema, sleep pattern,

behavior pattern and mortality for a period of 14 days. Thereafter skin irritation test also was carried out with the aqueous extracts for 72 hours.

Grouping of animals and experimental method

Based on toxicity study, the following groupings of animals were carried out (Table-1). Group-I is normal (untreated), Group-II is received standard drug, Retino-A 0.05% (Tretinoin cream U.S.P.) - Janssen-Cilag Pharmaceuticals (Trademark of Johnson & Johnson, U.S.A.) in cream form (positive control). The Group III to VIII mouse was administered a daily topical dose of 62.5 mg of 5% imiquimod cream (IMQ, Aldara; 3M Pharmaceuticals, UK) to a 3 cm × 4 cm shaved area on their backs for 7 consecutive days and observed for induced psoriasis.

An objective scoring system was applied based on the clinical psoriasis area and severity index²³. Redness, erythema, and scales were scored independently on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3, marked; and 4, very marked. The cumulative score (sum of redness, erythema, and scaling) served as a measure of the psoriasis severity index (PSI) (scale 0–12)²⁴. After induced psoriasis from the 8th day onwards extract treatment were started once daily, 5 times in a week, for 21 days. At the end of the study, animals were anesthetized using high dose carbon dioxide gas in closed desiccator. The specimen of skin was collected and preserved in glass vials containing 10% formalin solution for histological examination. Longitudinal sections of mice skin specimen (about 5 mm diameter and 5 µm thickness) were prepared by microtomy and stained with hematoxylin and eosin (H and E) dye for histological examination.

Statistical Analysis

The experimental results were represented as mean ± SD and analyzed using one-way analysis of variance by Tukey–Kramer multiple comparisons test. Statistical calculations were performed using Graph Pad 5 Software Inc, San Diego, CA, USA. P < 0.05 was considered statistically significant in all the groups. Risk assessment code (RAC) of metals in soil was performed following the procedure described by Singh et al. (2005)²⁵, as:

$$\text{RAC (\%)} = \left(\frac{\sum_{n=1}^{n=3} F_n}{\sum_{n=1}^{n=6} F_n} \right)$$

Where “F_n” is the concentration of metal in “nth” fraction.

Result

Analysis of soil sample for metal ion content

Soil samples were collected from both the geographical locations and analyzed for preliminary soil tests likely soil pH, organic carbon, color of soil etc. followed by total metals and DTPA extractable metals were analyzed by Atomic Absorption spectrophotometer. Results revealed very high acidic soil (pH 4.32) of Darjeeling region than that of Nandi hills soil (pH: 5.20). All other results were tabulated in table-2.

Analysis of leaves sample for metal ion content

Collected leaves samples were also analyzed for uptake of metals separately by Atomic Absorption spectrophotometer and results revealed that collected leaves samples from Nandi hills, Bangalore contained more metal ion uptakes by the leaves and the results were tabulated in table-3.

Yield of the extracts and phytochemical screening

Yield of the aqueous extracts from both the collected area was calculated and the results are tabulated in figure-2. Results revealed that leaves samples of PD and CO, collected from Bangalore zone was higher than that of samples collected from Darjeeling, West Bengal zone.

Percentage yield was calculated and found that PD sample was higher (11.48%) than CO sample (9.68 %), collected from Nandi hills region and the same trend was followed for collected sample from Darjeeling. PD sample showed the percentage yield of leaves sample was 10.52% whereas for CO extract showed 8.8 %.

Phytochemical screening with respect to chemical tests were carried out and revealed the presence of various group of phytochemicals in all four leaves aqueous extracts which was depicted in table-4.

Acute dermal toxicity

The study revealed the aqueous leaves extract of both CO and PD are non toxic when tested at maximum dose levels of 2000 mg/kg, body weight. Neither mortality nor any sign of

toxic reactions were found throughout the study period. Furthermore no skin irritation was observed with the applied extracts even after 72 hours of study.

Anti psoriatic activity

Topical application of 62.5 mg of 5% Imiquimod was applied for 7 days resulted in the development of induced psoriasis on each group of mice (Gr-II to Gr-VIII). After 3-4 days, the back skin of the mice started to display signs of erythema, scaling, and thickening (Figure-3).

Various changes such as redness, erythema, and silvery scales on exposed area were marked visually and found an increase up to 7th day and the cumulative score, PSI was significantly ($P < 0.05$) increased as indicated in Table 5 and Figure-4.

After 7th day, 8th day onwards upto 3 weeks extracts were applied topically for Gr-II to Gr-VIII. The severity of psoriatic lesions was evaluated by visual and histological studies. In Group II, topical application of Retino-A cream (0.05%) reduced ($p < 0.05$) the severity of redness, erythema, and scales from day 7 to 21st day. Thereafter drastic reduction ($p < 0.01$) of phenotypic changes like redness erythema, scales observed for Gr-VII and Gr-VIII where combined extracts were applied and the results showed dose dependant manner. Among the responses the plants that procured from Nandi hills, Bangalore, Karnataka state showed more significant results (Table-6, 7, 8 and 9) than sample procured from Darjeeling, West Bengal state (Table-10, 11, 12 and 13). Combined extracts at 200 mg/kg b.w resulted more significant PSI score ($p < 0.01$) in day 14th as well as day 21st than later geographical zone. Interestingly the results were quite better in terms of reduction of redness, erythema, scales and cumulative score in animals which showed the therapeutic efficacy of selected said plant samples on induced psoriasis than applied standard drug.

Histopathological study

Histological examination showed an increased epidermal thickness, hyper proliferation of Keratinocytes, granulocyte infiltration, the presence of micro abscess, capillary loop dilatation in IMQ induced mouse skin as compared to normal mouse skin (Figure 5A and B). Thereafter epidermal thickness of extract treated animals was compared with the untreated animals which showed remarkable decrease of thickness than applied standard (Figure-6). It observed that thickness of epidermis cell was significantly lesser (26.18 μM), (** $p < 0.01$) when combined PD and CO extracts were applied at 200 mg/Kg b.w. than that of standard (40.14 μM) in terms of

reduced epidermal thickness, hyper proliferation, granulocyte infiltration, the presence of micro abscess and capillary loop dilatation (Figure-7 A, B and C).

Correlation coefficient

Data were analyzed for correlation study between uptakes of essential metals in leaves with reduction of epidermal thickness in psoriasis treatment. Results revealed progressive high significant. PD and CO leaves samples procured from Nandi Hill's showed better uptake of Fe (2.05 mg/kg and 1.88 mg/kg respectively), Cu (0.78 mg/kg and 0.62 mg/kg respectively) and Zn (1.12 mg/kg and 0.98 mg/kg) than Darjeeling samples. **Extracted plant samples were calculated for percentage of extracts and revealed Nandi hill's samples gave more extract due to higher content of metallic ions (Figure-2).** These metal contents further correlated with the reduction of epidermal thickness which showed significant results. Increased content of metals in PD and CO leaves sample more decreases the epidermal thickness (94.33 μM and 97.30 μM respectively on 21st Day) (Table-14), which was better than sample procured from Darjeeling, West Bengal (98.10 μM and 100.20 μM respectively on 21st Day).

Discussion

Metal ion content in soil and leaf sample

The results (Table-2) show that the pH and organic carbon content in Darjeeling soil was 4.32 and 0.64 % while that of the same in Nandi Hill Soil was 5.18 and 0.32 % suggested that the soil of Darjeeling was more acidic compared to Nandi Hill Soil. As regards to the organic carbon content, it was observed that the amount was much higher (0.64%) in Darjeeling compared to Nandi Hill Soil (0.32%) which might be due to variation climatic conditions especially in temperature. The prevailing temperature in Darjeeling region was much lower compared to Nandi Hill which might be explained by the less loss of organic carbon in the former region resulting from the very little oxidation of organic carbon from the soil than that of the latter Nandi Hill Soil causing greater rate of oxidation of organic carbon.

As regards to the total and available heavy metal content in soils, it was found that the amount of both total and available metal concentration was always higher in Nandi Hill soil compared to Darjeeling soil which might be explained by the variation in the initial higher amount of metals as well as the variation in pedogenic processes of soil formation where

dominant pedogenic process was laterisation in case of Nandi Hill soil resulting an accumulation of sesquioxides and loss of silica and the reverse is the case with Darjeeling soil where podzolisation process is dominant in which accumulation of silica and loss of sesquioxides was occurred²⁶. The amount of DTPA extractable Zn content was in the deficient level (0.51 mg/kg) and marginally deficient (0.61 mg/kg) in Nandi Hill soil based on the critical level as 0.60 mg/kg. However, such decreased availability of Zn and Cu in soils might be explained by their greater fixation and adsorption as well as greater interaction between soil components²⁷. The results also reveal that the amount of DTPA- extractable non-nutrient heavy metals (Cr,Cd,Pb and Ni) were far below the toxic limit based on the test value²⁸. The amount of DTPA – extractable Ni, Cd, Cr and Pb contents were recorded a very low value in both the soils of Darjeeling and Nandi Hill soils which might be due to the higher organic carbon content in the former soil and higher pH in the latter soil resulting from the complexation of those heavy metals with organic matter in Darjeeling soil and higher adsorption of those metals onto sesquioxides in Nandi Hill soil . The availability of Ni associated with organic colloids is highly pH dependent which reduced the rate of dissociation of Ni fulvic acid complexes with increased pH and decreased ionic strength²⁸. The availability of non-nutrient heavy metals such as Cr, Cd, Ni and Pb and also beneficial micronutrients like Fe, Zn and Cu in soil might be attributed to the individual soil characteristics, particularly soil pH²⁹, cation exchange capacity³⁰, different oxides of Fe, Al and Mn³¹ and amount of organic matter content³².

The results (Table 3) reveal that the amount of non-nutrient heavy metal concentrations (Cr,Cd,Ni and Pb) in both PD and CO have been varied between soil types and kind of medicinal plants, being slightly higher in PD compared to CO plant in both the soils. Such low content of those metals in plants might be due to very low content of those metals in both the soils resulting from the variation of soil reaction as well as amount of organic carbon content in soils²⁸. Karak *et.al* (2005)³³ reported that the concentration of Zn and other non-nutrient heavy metals in soil solution and their availability to crops is controlled by sorption-desorption reactions at the surfaces of soil colloidal materials. The results of the present investigation are similar to that of the result reported by earlier investigators^{34, 35}.

However, the overall results reveal that the amount of available trace heavy metals including beneficial and non-nutrient metals depends on the nature and properties of soils, pedogenic processes of soil formation etc. In case of Darjeeling soil exchangeable Al is mainly

responsible for the development soil acidity while that of the same in Nandi Hill soil, extensive leaching and at the same time accumulation of sesquioxide are responsible for acidity. Since all those metal concentrations as their available forms in both soils are very low and the absorption in and uptake of those metals by plants are also reportedly low. Based on the results of the present investigation, the cultivation of medicinal plants in both soils are suitable without affecting their medicinal value.

Yield of the extracts

Yield of extracts were determined w/w and tabulated in result. The yield of extract showed slight increased amount that procured from Nandi Hill, Bangalore. This may be due to higher accumulation of Fe, Zn and Cu and lesser content of non essential heavy metals in leaf samples of CO and PD. Earlier report also is the said evident of the same¹⁹.

Antipsoriasis activity

In recent year many plant extracts in combinations or in sole are applicable for psoriasis treatments. But the main concern is to discovery of new drugs from the plant extracts which is more potent than the extracts against any kind of human health hazards. The main reason behind the selection of these two plants leaf because both the plants enhanced the immunity and act strongly against any infections due to their high antioxidant activities^{36, 10}. It was revealed that selected plant extracts showed significant antipsoriasis activity due to the presence of important secondary metabolites (discussed earlier in introduction part).

It was scientifically proved that Prostaglandin E₂ produced by the cyclo-oxygenase pathway results psoriasis by dilating skin capillaries which increases leukocyte infiltration and stimulates keratinocyte cell growth³⁷. During induction of psoriasis, 5% imiquimod cream was used which showed redness, erythema and scales within 7 days in the skin of mice. Histologically, psoriatic skin contains thickened epidermis with large number of inflammatory cells and absence of granular layer. In this study fully developed psoriatic lesions were treated with combined herbal aqueous extracts of PD and CO and compared with marketed standard drug along with untreated animal. Results revealed significant reduction of skin epidermal thickness with the treated aqueous extracts.

Previous studies have established that antioxidants could play an effective role in the psoriasis treatment³⁸. Plant secondary metabolites such as flavonoids, triterpenoids and

polyphenolic compounds are well known for antioxidant activity and for their anti-inflammatory, antiproliferative, Immunomodulatory activities^{39, 40}. These characteristics of polyphenolic phytoconstituents are beneficial for the treatment of psoriasis which is present in huge quantities in PD and CO leaves. Preliminary phytochemical screening through chemical tests revealed the presence of these constituents which showed antipsoriasis activities.

Conclusion

The results of this present study demonstrate that the combined extracts of PD and CO provide significant antipsoriasis activity and the effect was dose dependent. Selected said plants showed remarkable activity than that of marketed standard Retino drug. Hence further isolation of newer chemicals and clinical trial is needed for establishment of effective herbal drug formulation against psoriasis with new drug discovery.

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Conflict of Interest

None reported