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ANTIMICROBIAL AND ANTIOXIDANT SYNERGY OF *PSORALEA CORYLIFOLIA* LINN. AND *PLUMBAGO ZEYLANICA* LINN.

P. Suman [1], P.V. Smitha [2], K.Y. Ramkumar [1], A. Siva [1], Ch. Murali Mohan [2] and S. Hara Sreeramulu*[1]

Department of Biotechnology, Dr. V.S. Krishna Govt. PG College [1], Visakhapatnam-530013, Andhra Pradesh, India
Department of Biotechnology, GITAM Institute of Technology, GITAM University [2], Visakhapatnam – 530045, Andhra Pradesh, India

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Correspondence to Author:

Dr. S. Hara Sreeramulu

Head of the Department, Department of Biotechnology, Dr. V.S. Krishna Govt. PG College, Andhra Pradesh, India

E-mail: drharasreeramulu@gmail.com

ABSTRACT

Objectives: *Psoralea* corylifolia L. (seeds) and *Plumbago zeylanica* L. (bark) were combined in the ratio of 8: 2 and named as “Swithranasini” and used for the treatment of skin diseases in ayurvedic medicine. Scientific studies validating the therapeutic properties for this indigenous medicine are lacking. This study focused on determining antimicrobial efficacy of methanol and butter milk extracts along with antioxidant efficacies of these plant parts independently and in combination.

Materials and Methods: Antimicrobial activity was tested against seven pathogenic bacteria and six pathogenic fungi by measuring the zone of inhibition. *In vitro* antioxidant activities were assayed by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method and Ferric reducing antioxidant power (FRAP) method.

Results: Individual extracts of the two plants showed better zone of inhibition (12- 40 mm) when applied alone but the activity was more when applied in combination (18-50 mm) against the tested pathogens. DPPH activity of methanol extracts showed higher radical scavenging activity when compared with the positive control, ascorbic acid. The IC_{50} values are 442.080, 457.825, 432.040 and 394.563 ug/ml for A, B, A+B and ascorbic acid respectively. The FRAP method also showed excellent antioxidant potential in combination (85.454 mg of Gallic acid equivalents/g) when compared with individual extracts A (78.571 mg GAE/g) and B (75.472 mg GAE/g).

Conclusion: The results validate the potent use of “Swithranasini” against skin infections and results are an evidence for the existence of synergism among the compounds.

INTRODUCTION: Ayurvedic medicine is a system of traditional medicine native to India and a form of alternative medicine. One of the most significant contributions offered by ayurvedic medicines are the science of herbal combinations. In India, medicinal preparations from plants have been used over a long period for the treatment of ailments called as traditional herbal medicines. This is because orthodox medicine is not available in some places due to a wide range of reasons, among which includes that the first line drugs which are cheap and affordable have become ineffective because of resistance and side

effects. Now however, these plant preparations are becoming more widely used by people all over the world as they understand the gentle strength in them and the fact that most of them can be used safely without the known side effect of drugs [1]. Now, researchers in the field of plant medicines, regard higher plants as living chemical factories that provide a vast number of unusual chemical substances that display a variety of biological actions [2].

Plants are able to produce compounds which though have no apparent function in the primary metabolism of the plant [3] have good activity against bacterial and fungal pathogens when they are able to find their way to accumulate in them. These compounds have had an extensive history of use as therapeutic agents [4].

Psoralea Corylifolia plant is an important herbal medicine as a tonic remedy and is used to improve general vitality. It is also of value in the treatment of skin disorders, including vitiligo. The seed is anthelmintic, antibacterial, aphrodisiac, astringent, cardiac, cytotoxic, deobstruent, diaphoretic, diuretic, stimulant, stomachic and tonic. It is used in the treatment of febrile diseases, premature ejaculation, impotence, lower back pains, frequent urination, incontinence, bed wetting etc. The seed and fruit contain psoralen. The plant yields a useful medicinal oleoresin; it treats kidney disorders, impotence, and lumbago. It is also used externally to treat various skin ailments including leprosy, leucoderma and hair loss.

Plumbago zeylanica root, bark and seed are used for variety of medicinal treatments. The roots of the plant increase the digestion and promote appetite, and small doses stimulate central nervous system, and is made into a paste and applied to the skin to treat abscesses, and other skin diseases including ulcers and scabies.

The powder may be added to bath teas for acne prone skin or for those suffering from chicken pox. Its bark is used to stop bleeding, cure baldness, and treat diarrhea. In Ethiopia powdered bark, root or leaves are used to treat gonorrhoea, syphilis, tuberculosis, rheumatic pain, swellings and wounds.

There is a continuous & urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and reemerging infectious diseases and skin diseases like athlete's foot, eczema, ringworm, and hair, nails damage due to their ability to obtain nutrients from keratinized material. A single drug may have several pharmacological actions, but it is only those that occur in concentrations reached by standard doses that are considered relevant. Although plants may contain the appropriate constituents, they may be in insufficient amounts to account for the observed effects.

Thus, some herbal medications may produce a more favorable response when an extract is taken in combinations. Traditional system of medicine generally assumes that a synergy of all ingredients of the plants will bring about the maximum of therapeutic efficiency [5].

Many phytomedicines sold in market today are as whole extracts and it is believed that the synergistic interactions between the constituents are responsible for the therapeutic efficiency [6]. It would surely be useful to examine the concept of synergy in an attempt to reduce side effects and improve therapeutic success. The current work is undertaken to validate the role of synergism.

Despite the accumulated knowledge of their ingredients, the mechanical and clinical pharmacology is only known for a small percentage of herbal products [7]. Some herbal combinations are more effective than the constituent herb used alone.[8] Synergy occurs if two or more herbal ingredients mutually enhance each other's effect more significantly than the simple sum of these ingredients [9].

Evidence to support the occurrence of synergy within phytomedicines i.e. within total extract of a single herb, as well as between different herbs in formulation is studied here basing on their antimicrobial and antioxidant activity. It will probably involve a thoroughly new approach, for isolating ingredients individually and in combination, as has been described by Wagner [10].

The plants selected for the study are based on their frequent usage in several formulations and the combinatorial study was also done based on the traditional medicine used for treating skin diseases.

MATERIALS AND METHODS:

Collection of Plants and Identification: In the present investigation, the parts of two plants namely *Psoralea corylifolia* (Pappilionaceae) (seeds) and *Plumbago zeylanica* L. (Plumbaginaceae) (bark) were collected from local market. The identification was confirmed at Department of Botany, Andhra University and Department of Botany, Dr.V.S.Krishna Govt P.G College, Visakhapatnam. The plant selection and testing of synergy was based on the advice of the traditional healers and on traditional usage.

Preparation of Crude Extracts of Plant Parts: The crude extracts of the two plant material were prepared separately by methanol as solvent as described below.

Solvent Extraction: The plant material was brought to laboratory and washed under running tap water and blotted with filter paper then shade dried on laboratory benches by putting news papers. After complete drying the plant material was then ground into powder by using hand mill. The powdered plant material (25 g) was placed in a Soxhlet extractor and was exhaustively extracted using 250 ml of methanol (60 – 80°C). The crude extracts were respectively concentrated *in vacuo* at 40°C using a *rotavapor*. The crude extracts thus obtained were preserved in freezer at -20°C until use. The successive extractive values were 5.6 g and 5.3 g for *P. corylifolia* (seeds) and *P. zeylanica* (bark), respectively. The crude plant extracts were labeled as *Psoralea corylifolia* (seeds) (A) and *Plumbago zeylanica* (bark) (B).

Test Organisms: Bacterial and fungal isolates used in the current work include clinical and pure isolates of seven bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Klebsiella pneumonia*, *Enterobacter aerogenosa* and *Enterococci species*; and six fungal isolates such as *Candida albicans*, *Candida tropicalis*, *Candida bombii*, *Candida utilis*, *Candida nonalbicans* and *Trichophyton rubrum* were obtained from the Department of Microbiology, Andhra Medical College, Visakhapatnam. Nutrient agar (NA) and Potato dextrose agar (PDA) were used for the growth of bacteria and fungi respectively. Pure cultures obtained were kept on respective agar slants at 4 °C until needed. They were sub-cultured once in every fifteen days.

Each inoculum was prepared by inoculating the stock culture into freshly prepared media. All the bacterial strains were incubated at 37 °C for 24 h and fungi at 27°C for 48 h. The test organisms were grown overnight in respective broth media.

Determination of Antimicrobial Activity: Antimicrobial activity of organic extracts of the plant samples were evaluated by the paper disc diffusion method. [11] For determination of antibacterial activity, overnight grown bacterial cultures were adjusted to 0.5 McFarland turbidity standards. For the determination of antifungal activity, all the fungal isolates were first adjusted to the concentration of 10^6 cfu/ml. The bacterial and the fungal broth cultures of 100 µl each were inoculated onto Nutrient Agar and Potato dextrose Agar plates respectively through spread plate method.

Firstly, stock solutions (100 mg/ml) of each individual plant extract were prepared separately and mixed in the following combinations A+B (8:2) in methanol as a solvent labeled (C) and same A+B (8:2) in butter milk as a solvent labeled (D). Sterile filter paper (Whatman filter paper No.1) discs of diameter 6mm were prepared and 10µl of each extract dilutions alone and in combinations were impregnated onto the discs and carefully placed at the centre of the previously seeded plates with 0.5 McFarland and 10^6 cfu/ml cultures of bacteria and fungi respectively, with sterile forceps. Disc with solvent alone was served as control. Bacterial cultures and *Candida albicans* plates were then incubated at 37°C for 24 h while the other fungal cultures are incubated at (25±2°C) for 48 h.

Antimicrobial activity was determined by measurement of zone of inhibition of growth around each paper disc (mm) . For each extract (test) three replicate trials were conducted against each organism. Each zone of inhibition was measured with a ruler and compared with standard [12].

Determination of *In vitro* Antioxidant Activity Assay:

***In vitro* Antioxidant Activity by DPPH Assay:** DPPH method is based on the reduction of alcoholic DPPH solution (dark blue in colour) in the presence of a hydrogen donating antioxidant converted to the non radical form of yellow colored diphenyl-picryl hydrazine.

4 mg of DPPH was dissolved in 100 ml of ethanol and kept it overnight in dark place for the generation of DPPH radical. The scavenging activity for DPPH free radicals was measured according to the procedure described by Braca *et al*., 2003.[13] An aliquot of 3ml of 0.004% DPPH solution in ethanol and 0.1ml of plant extract at various concentrations were mixed. The mixture was shaken vigorously and allowed to reach a steady state at room temperature for 30 min. Decolorization of DPPH was determined by measuring the absorbance at 517nm. A control was prepared using 0.1ml of respective vehicle (DMSO) in the place of plant extract or ascorbic acid.

***In vitro* antioxidant activity by FRAP Assay:** The ferric reducing antioxidant power (FRAP) [14] property of the extract was determined by taking 1 ml of different dilutions of standard solutions of gallic acid (10-100 µg/ml) or methanolic extract that had been adjusted to come under the linearity range (500 µg/ml), placed in 10-ml volumetric flasks and mixed with 2.5 ml of potassium buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50°C for 20 min and 2.5 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction. To the 2.5 ml of the above solution, 2.5 ml of distilled water was added and then 0.5 ml of 0.1% $FeCl_3$ was added and allowed to stand for 30 min before measuring the absorbance at 593 nm. The absorbance obtained was converted to gallic acid equivalents in mg/g of dry material (GAE/g) using a gallic acid standard curve [15].

Phytochemical Analysis: The plant extracts were subjected to standard phytochemical analyses to test the presence of alkaloids, saponins, glycosides, [10] reducing sugars, phenolics, [14] terpenoids, tannins, flavonoids, [16, 1] steroids and anthraquinone [17, 1].

RESULTS: In the current study two commonly available medicinal plants used by traditional medical practitioners in South India were tested against pathogenic bacteria and fungal strains. The results of phytochemical screening showed the presence of a number of secondary metabolites including reducing sugars, glycosides, phenolics, terpenoids, tannins, carbonyls, flavonoids (**Table 1**).

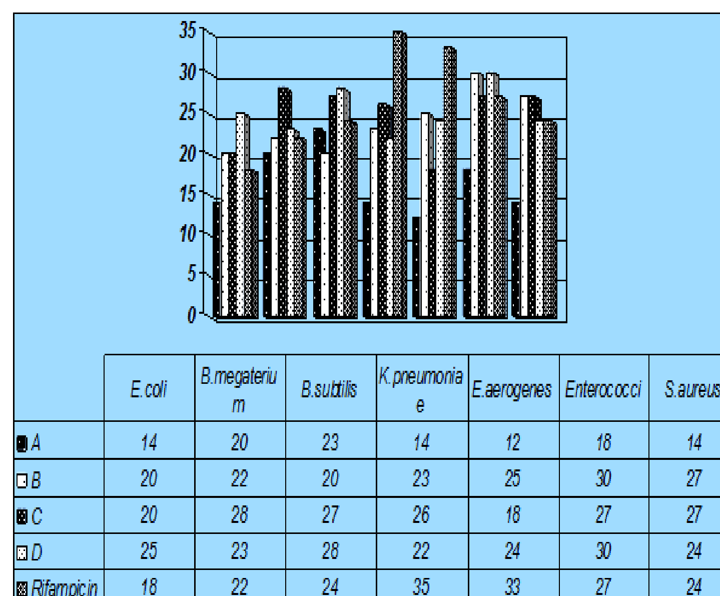
TABLE 1: PHYTOCHEMICAL ANALYSIS OF *PSORALEA CORYLIFOLIA* AND *PLUMBAGO ZEYLANICA*

Phytoconstituents	*Psoralea corylifolia*	*Plumbago zeylanica*
Reducing Sugar	-	+
Tannins	+	+
Glycosides	+	+
Saponins	+	+
Flavonoids	+	+
Steroids	+	+
Anthraquinone	-	-
Phenolics	+	+
Terpenoids	-	-

a) +: Positive, b) −: Negative

The presence of different secondary metabolites especially those known to be responsible for antimicrobial and antioxidant activity was confirmed. The presence of these metabolites in plants has been linked to the antimicrobial and antioxidant activities of the plants [18, 13].

The antimicrobial activity of the plant extracts of individuals and combinatorial zones of growth inhibitions are shown in **Fig. 1 & 2**.



	E.coli	B.megaterium	B.subtilis	K.pneumoniae	E.aerogenes	Enterococci	S.aureus
A	14	20	23	14	12	18	14
B	20	22	20	23	25	30	27
C	20	28	27	26	18	27	27
D	25	23	28	22	24	30	24
Rifampicin	18	22	24	35	33	27	24

FIGURE 1: BAR CHART SHOWING RESULTS OF ANTIBACTERIAL SUSCEPTIBILITY OF TEST ORGANISMS TO METHANOL EXTRACTS OF *PSORALEA CORYLIFOLIA* (A), AND *PLUMBAGO ZYLANICA* (B), ALONE AND IN COMBINATION IN METHANOL SOLVENT (C), AND BUTTER MILK (D), RIFAMPICIN.

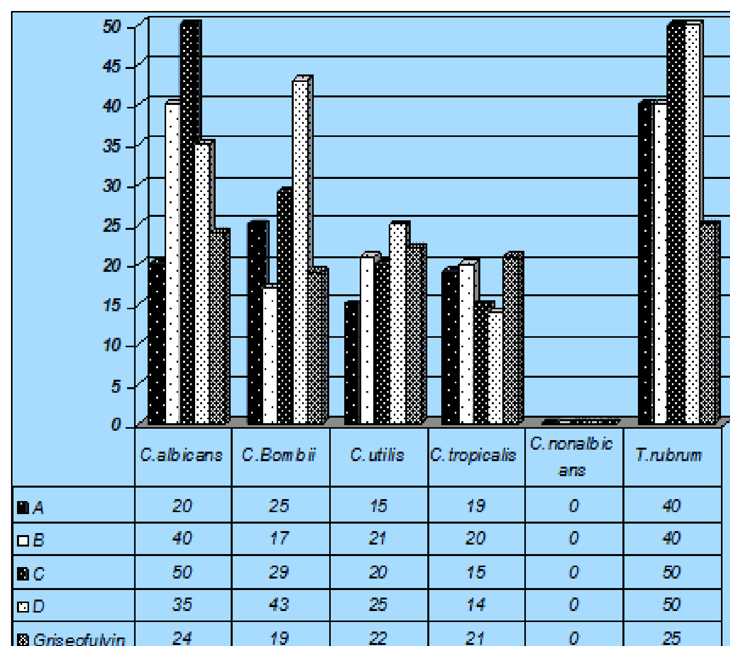


FIGURE 2: BAR CHART SHOWING RESULTS OF ANTIFUNGAL SUSCEPTIBILITY OF TEST ORGANISM TO METHANOL EXTRACTS OF *PSORALEA CORYLIFOLIA* (A), AND *PLUMBAGO ZYLANICA* (B), ALONE AND IN COMBINATION IN METHANOL SOLVENT (C), AND BUTTER MILK (D), GRISEOFULVIN.

The results showed that the plant extracts were effective against both tested bacterial and fungal strains. The individual methanol extract of seeds of *P. corylifolia* showed activity against all the seven bacterial strains tested (12-23 mm) and antifungal activity was shown only against four fungal isolates *viz*. *C. albicans*, *C. bombii*, *C. utilis*, *C. tropicalis* and *T. rubrum* (15-40 mm), individual methanol extract of bark of *P. zeylanica* also showed activity against all tested bacterial strains (23-38 mm) and fungal strains (17-40 mm), except for *C. nonalbicans*.

P. corylifolia and *P. zeylanica* were mixed (A+B) in the ratio of (8:2) in methanol as solvent and labeled (C) and same A+B (8:2) were mixed in butter milk as solvent and labeled (D). When these two plant extracts applied alone they showed activity but comparatively less when they applied in combination. The inhibitory zone of methanol combination (C) ranged from 18-28 mm and butter milk combination (D) ranged from 22-30 mm against all the tested bacterial strains.

For fungal strains the inhibitory zone of methanol combination (C) ranged from 23-50 mm and butter milk combination (D) ranged from 24-50 mm, except for *C. nonalbicans* which did not show any zone of inhibition.

The *in vitro* antioxidant activity of the plants was assessed individually and also in combination based on 1, 1- diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and the ferric reducing antioxidant power (FRAP). Methods like FRAP measure only the hydrophilic antioxidants, while others like DPPH detect merely those soluble in organic solvents, particularly alcohols.[19] DPPH antioxidant activity of methanol extracts showed potent radical scavenging activity. The results were compared with the positive control, ascorbic acid (**Table 2**) and in **Figure 3**.

TABLE 2: CONCENTRATION DEPENDENT PERCENT INHIBITION OF DPPH RADICAL BY METHANOL PLANT EXTRACTS ALONE AND IN COMBINATION. A (PLUMBAGO ZEYLANICA), B (PSORALEA CORYLIFOLIA), C (COMBINATION OF A+B).

Conc. (ug/ml)	A	B	C	Ascorbic acid
10.0	1.068	0.427	1.923	1.196
25.0	2.777	1.495	3.952	4.225
50.0	3.380	2.884	14.957	12.362
100.0	15.792	13.141	14.957	24.479
250.0	26.401	25.534	28.525	40.256
500.0	42.502	42.636	45.089	58.842
750.0	64.089	65.803	68.320	64.673
1000.0	73.312	76.525	79.081	80.724

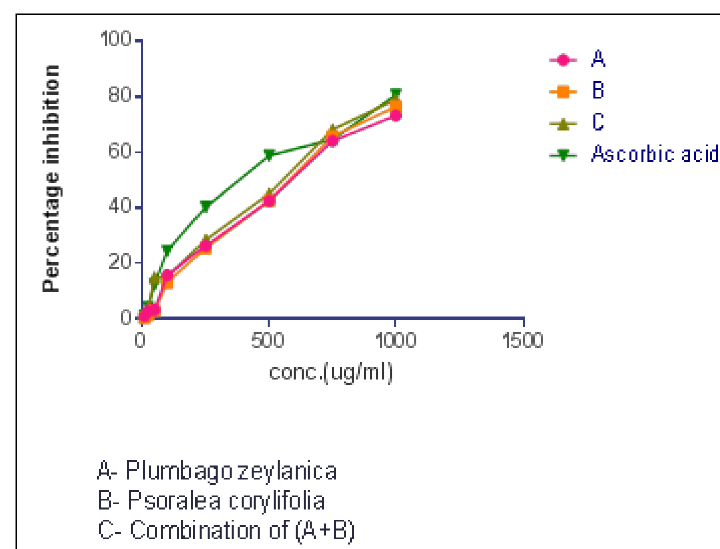


FIGURE 3: CONCENTRATION DEPENDENT PERCENT INHIBITION OF DPPH RADICAL BY METHANO PLANT EXTRACTS ALONE AND IN COMBINATION

The IC_{50} values are 442.080, 457.825, 432.040 and 394.563 ug/ml for A, B, A+B and ascorbic acid, respectively. The FRAP method also showed excellent antioxidant potential in combination (85.454 mg of Gallic acid equivalents/g) when compared with individual extracts A (78.571 mg GAE/g) and B (75.472 mg GAE/g).

The results confirm the potent use of "Swithranasini" against skin diseases and results are an evidence for the existence of synergism among the compounds.

DISCUSSION: The results confirmed that the combinational use of these two plants exhibit synergistic antimicrobial activity against both bacterial and fungal strains. This is explained by the fact that the secondary metabolites identified in the extracts are known to have combinational antimicrobial properties.

In the present study, individual extracts of *Psoralea corylifolia* showed significant antimicrobial activity and showed the presence of phytoconstituents like tannins, phenols, steroids, saponins, glycosides and flavonoids that are known to have antimicrobial activity [20, 21].

Similarly potent activity was observed in the previous study on hexane extract of *P*. *corylifolia* seed extract where the zone of inhibition was found to be in concentration of 5µg / ml for bacterial strains and 4µg / ml for fungal strains [22]. This was further supported by the potent antimicrobial activity on leaf extract of *P*. *corylifolia* L. which may be due to the presence of alkaloids, flavonoids and phenolic compounds present in the plant as secondary metabolites [23].

The current study on individual extract of *P. zeylanica* showed good antimicrobial activity and showed the presence of tannins, phenols, steroids, saponins, glycosides and Flavonoids. The antimicrobial activity of methanol, chloroform and alcoholic leaf extracts of *P. zeylanica* was reviewed and found to posses' potent activity when tested against various Gram-negative and Gram-positive bacteria. The results of qualitative screening of phytochemical components in leaves of *P. zeylanica* revealed the presence of alkaloids, glycoside, reducing sugars, simple phenolics, tannins, lignin, saponins and flavonoids that might contribute to the antimicrobial activity [24].

In vitro antioxidant studies of *P*. *zeylanica* was carried out previously on ethanol root extract using DPPH free radical scavenging, nitric oxide scavenging and Superoxide scavenging methods at dose of 100–1000 mg Ml^{-1}. The ethanol extract showed good antioxidant activity in these above methods with the maximum activity in DPPH free radical scavenging model [25].

Similarly *in vitro* antioxidant properties of aqueous and solvent extract of seeds of *P*. *corylifolia* L. were evaluated previously employing superoxide scavenging activity, lipid peroxidation, hydroxyl radical scavenging activity, DPPH radical scavenging activity.

The results suggest strong antioxidant potential of alcohol and water (1:1) extract of seeds of *P. corylifolia* that could play an important role in the modulation of oxidative stress [26].

Literature only suggested the individual activities of these plants but according to the present study combinatorial effect of plant extracts (A+B) was found more effective than when used alone. The results further confirmed the good antimicrobial activity of the combination of plant extracts against the test organisms (Figure 1 & 2) as well as good combinatorial antioxidant activity (Figure 3).

The secondary metabolites from plants are good sources for combination therapy. The therapeutic indications of medicinal plant-based extracts are in most cases, empirical, and practitioners of phytotherapy intuitively believe that a total extract acts better than an equivalent dose of an isolated substance [27].

The obtained results might be considered sufficient to further studies for the isolation and identification of the active principles and to evaluate the possible synergism among extract components for their antioxidant and antimicrobial activity.

To the best of our knowledge, synergistic activity of methanol extracts of *Psoralea corylifolia* and *Plumbago zeylanica* has not been reported earlier. Research on synergism is very limited and few studies have been reported [18, 28, 29, 30].

CONCLUSION: To conclude, the study of synergy is an emerging field in all aspects, and there is much to be developed and understood in all areas of knowledge. New technological tools that enable the mechanisms of action of combined natural extracts or synthetic agents to be tracked will enable the creation and corroboration of differentiated medical therapies, increasing the quality of existing health treatments.

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