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PHYTOCHEMICAL AND IMMUNOMODULATORY SCREENING OF PHYSALIN B, ISOLATED FROM METHANOLIC EXTRACT OF UNRIPE FRUITS OF *PHYSALIS MINIMA*

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ABSTRACT
Phytochemicals are chemicals of plant origin. Phytochemicals are chemicals produced by plants through primary or secondary metabolism. Phytochemicals generally are regarded as research compounds rather than essential nutrients because proof of their possible health effects has not been established yet. Immunology is one of the most rapidly developing areas of medical biotechnology research. All drugs which modify immune response generally categorized as immunomodulators. In the present study, Phytochemical screening included extraction, isolation and identification bioactive compounds of methanolic extract of unripe fruits of *Physalis minima* by different analytical methods. The isolated compound was found to be Physalin B in comparison to the previous published data and it was screened for immunomodulatory activity. So it has been concluded that the methanolic extract of unripe fruits of *Physalis minima* possess a steroidal alkaloid, an active constituent Physalin B, which may be used to treat different ailments.

KEYWORDS: Phytochemical, *Physalis minima*, Physalin B, Immunomodulator.

INTRODUCTION
Phytochemicals are chemicals of plant origin. Phytochemicals (from Greek *phyto*, meaning "plant") are chemicals produced by plants through primary or secondary metabolism. They generally have biological activity in the plant host and play a role in plant growth or defense against competitors, pathogens, or predators. Phytochemicals generally are regarded as research compounds rather than essential nutrients because proof of their possible health effects has not been established yet.

All drugs which modify immune response generally categorized as immunomodulators.[1] These can either function as immunostimulants or immunosuppressants.

Physalis minima is a perennial herb belonging to the family Solanaceae, commonly known as pygmy ground cherry, wild cape gooseberry, native gooseberry. It is pantropical annual herb possess cream to yellowish flower followed by edible yellowish fruit encapsulated in papery cover which turns straw brown on maturity.[4, 5] The results of the preliminary phytochemical analyses in the chloroform, diethyl ether, ethanol, ethyl acetate and methanol extracts of stem, leaves and unripe fruits showed presence of Alkaloids, flavonoids, cardiac glycosides, phenols, saponins, steroids, tannins and terpenoids. Reducing sugars were unable to be separated in all the solvent extracts of P. minima. Amount of phenols eluted by the organic solvents was very low in all the plant parts.[6]

The past studies reported that the plant possess diuretic activity, anti-inflammatory, analgesic, antipyretic, antibacterial, antidiabetic activities.[7-10]

The literature survey revealed that there is no detailed study of chemical constituents using analytical methods such as HPLC, I.R., and NMR. So, the present work was aimed to study the detailed chemistry of active principle present in the methanolic extract of unripe fruits of *Physalis minima* and screening of isolated component for immunomodulatory activity.

MATERIALS AND METHODS
Collection of Plant Material
The unripe fruits of *Physalis minima* were collected from the surroundings of Ilanthakunta, Jammikunta, Karimnagar, Telangana, India. The plant parts were authenticated and deposited at the herbarium of University College of Pharmaceutical Sciences, Satavahana University, Karimnagar, Telangana, India.

Preparation of the extract
The unripe fruits of *Physalis minima* (2.0kg) were kept for maceration with methanol for seven days. The extracts were concentrated in desiccators.[11]

Chemicals
All the chemicals used for the investigation were of analytical grade.

Detection of phytoconstituents
The extract was tested for phytoconstituents by preliminary tests, isolated the compound by Column chromatography and identified pure component through HPLC, identified functional groups by I.R. and structure with the help of NMR data.

Screening of immunomodulatory activity
Methods
- ✓ Carbon clearance test
- ✓ Humoral antibody titre
- ✓ Delayed type hypersensitivity

METHOD OF EVALUATION
Detection of Phytoconstituents
The extract was tested for the presence of Carbohydrates, Tannins, Flavonoids, Alkaloids, Anthocyanin and Betacyanin, Glycosides, Proteins, Steroids and Phytosterols, Phenols.

Chromatography
a. Thin-layer chromatography (TLC)
Thin layer chromatography (TLC) is a chromatography method which is employed to separate mixtures.[11] The analytes rise in the TLC plate at different rates, finally the mixture is separated.[12]

b. Column chromatography
Every compound in a mixture will have a specific solubility in the solvent and a specific affinity to be adsorbed by the solid adsorbent. No two compounds typically behave precisely alike in these respects. This principle is used in column chromatography.[13, 14]

Preparation of Column
Materials Used
Column of size 90 cm X 4.0 cm
Silica gel 100-200 mesh as the adsorbent

Silica gel 100-200 mesh was poured into the column by tapping to avoid air space between the particles (dry column method). The bottom of the column was plugged with little cotton to prevent the adsorbent pass out.

The methanolic extract of the unripe fruits of *Physalis minima* was subjected to column chromatography over silica gel (100-200 mesh). The column was eluted with solvents of increasing polarity. They are.

1. The solvent system used-starting with Toluene 100%, then Toluene 90% & Ethyl acetate 10% up to Toluene 50% & Ethyl acetate 50% but no spots were observed for these fractions on TLC.[15]
2. Finally, Toluene: Ethyl acetate: Diethyl amine were used in the ratio of 7:2:1, two spots were observed on TLC (Fig 1)
3. Again same solvent system in the ratio of 7:2:2 was used, single spot was observed (Fig 2)

All chromatograms were visualized under ultra violet light.

Sprayed by using Dragendroff's reagent got the orange bands for alkaloids

Chemical Test
The test is positive for Dragendroff's reagent test and Libermann-Buchard test, showing the presence of steroidal alkaloids.

c. Preparative high performance liquid chromatography
This technique was used to identify the specific constituent present in the sample, which was isolated by column chromatography.[16]

HPLC conditions
Column: Hypersil BDS-C18 (150X4.6mm, 5µ)
Mobilephase: A: 0.1% TFA in Water (50%)
B: 0.1% TFA in ACN (50%)
Flowrate: 1.0 ml/min
Column temp: 35°C
Run time: 40min
Programme (Isocratic)
Diluent: MeOH
Sample Preparation: 1.0 mg/mL in diluent
Vail: 96
Injection Volume: 10 uL

Characterization of Isolated Plant Constituent
Isolated Compound
Spectroscopic Methods
The chemical constituents present in the drug possess characteristic features because of which its characterization becomes possible. At every stage of structure determination from isolation and purification of constituents to its final comparison with an authentic sample, the spectral data facilitates the description of structure. Interpretation of molecular spectra is generally based on empirical correlations of spectral data with reasonable assurance to a particular group or arrangement of atoms in the molecule.

Infra red (IR) spectrum is generally complicated and out of many peaks relatively a few can be interpreted with assurance. Proton ('HNMR) spectra provide information about the number, nature and environment of the protons and carbon skeleton in the molecule, respectively.[17- 18]

Infrared Spectrum

The constantly vibrating molecules stretch and bend their bonds with respect to one another, by absorbing infrared light. IR spectrum is highly characteristic to establish the identity of compounds. The region 1430 – 910 cm-1 is called 'fingerprint' region where many more bending vibrations of the molecules are found. The identities of two samples that have identical spectra in the finger print region give conclusive identification of compounds. The crystals obtained from methanolic extract of the unripe fruits of *Physalis minima* was subjected to Infrared Spectroscopy and the spectrum is shown in Fig 4.[19]

FT-IR conditions used

Model Name FT/IR-4100typeA
Light source- Standard
Detector- TGS
Accumulation- 16
Resolution- 4cm^{-1}
Apodization- Cosine
Scanning speed- auto (2mm/sec)
Filter- auto(30000 Hz)

Nuclear Magnetic ResonanceSpectroscopy (NMR)

Nuclear Magnetic Resonance Spectroscopy deals with the study of spin changes in the presence of magnetic field, at the nuclear level when radio frequency energy is absorbed. As we are analyzing organic compounds for the nature, type, number and environment of protons (Hydrogen), the solvent used in the NMR spectroscopy should not contain hydrogen atoms. Hence we usesolvents Carbon tetrachloride (CCl4), Deuterated chloroform (CDCl3), Deuterated Water (D2O), Deuterated Methanol (CD3OD), Deuterated acetic acid (CD3COOD), Deuterated dimethyl sulphoxide (DMSO). The crystals obtained from methanolic extract of the unripe fruits of *Physalis minima* was subjected to NMR and the spectrum is shown in Fig 4.[19]

Screening of immunomodulatory activity of isolated component, Physalin B

Carbon clearance test

Swiss albino mice were divided into five groups which were administered drug for 5 days orally. On the last day, mice were injected with 0.1ml Indian ink via the tail vein. Blood samples were withdrawn at 0min and 15min. A 50µL blood sample was mixed with 4ml, 0.1% Sodium carbonate solution and the absorbance of this solution was determined at 660nm. The phagocytic index K was calculated using the following equation:

K= (Log OD1-Log OD2/15

where OD1 and OD2 are the optical densities at 0 and 15min respectively.

Delayed type hypersensitivity

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T-lymphocytes and their products (lymphokines). The cell mediated immune response was assessed by DTH reaction, i.e. Footpad reaction.

Humoral antibody titre

The animals were immunized by injecting 0.1ml of SRBCs suspension, containing 1×10^8 cells, intraperitoneal on day 0. Blood samples were collected in micro centrifuge tubes from individual animal by retro orbital puncture on day 7. Briefly, equal volumes of individual serum samples of each group were pooled. To serial two fold dilutions of pooled serum samples made in 25µl volume of normal saline, in U-bottomed micro titration plates were added 25µl of freshly prepared 1% suspension of SRBCs in saline. After mixing, the plates were incubated at 37ºc for 2h and examined visually for agglutination. The reciprocal of the highest dilution of the test serum causing visible haemagglutination was taken as the antibody titre.[20-23]

RESULTS AND DISCUSSIONS

Phytochemical screening

TLC analysis

TLC of methanolic extracts of the unripe fruits of *Physalis minima* used in this study revealed the presence of alkaloids by using Dragendroff's reagent to reveal characteristic orange bands of alkaloids (Fig 1 & 2)

HPLC analysis

Results of HPLC analysis (Fig 4) of *Physalis minima* methanolic extract of unripe fruits, at 205nm, shows presence of a constituent as evidenced by the chromatogram obtained at retention time 2.967.

IR Report

FTIR spectrum analysis of *Physalis minima* unripe fruits was found the presence of hydroxyl group, aliphatic methyl group, keto group and ether linkage.

NMR spectral data

The 1H NMR spectrum of 1 displayed signals for three methyl groups at δ 1.225, 1.212, 1.197 and five characteristic signals of a –CH2– group at δ 3.613, 3.627, 3.641, 3.654, and 3.669. Therefore, compound 1was identified as Physalin B, in comparison with published data.

Screening of immunomodulatory activity

The isolated compound Physalin B was screened in animals using the haemagglutinating antibody titre to assess humoral immune response and Carbon clearance test to assess scavenging activity. The animals were also evaluated for delayed type hypersensitivity by the difference between the pre and post challenge footpad thickness. They have shown significant immunostimulant properties i.e., immunodulatory activity for all the methods used. The data were analyzed using statistical methods and compared to that of the standard drug, obtained values at a dose of 200mg/kg body weight (Table 2). . The significance in difference was accepted at $P<0.05$.

TLC results of alkaloids for methanolic extract of unripe fruits of *Physalis minima*

Fig 1

Fig 2

HPLC fingerprints of isolated compound from methanolic extract of unripe fruits of *Physalis minima*

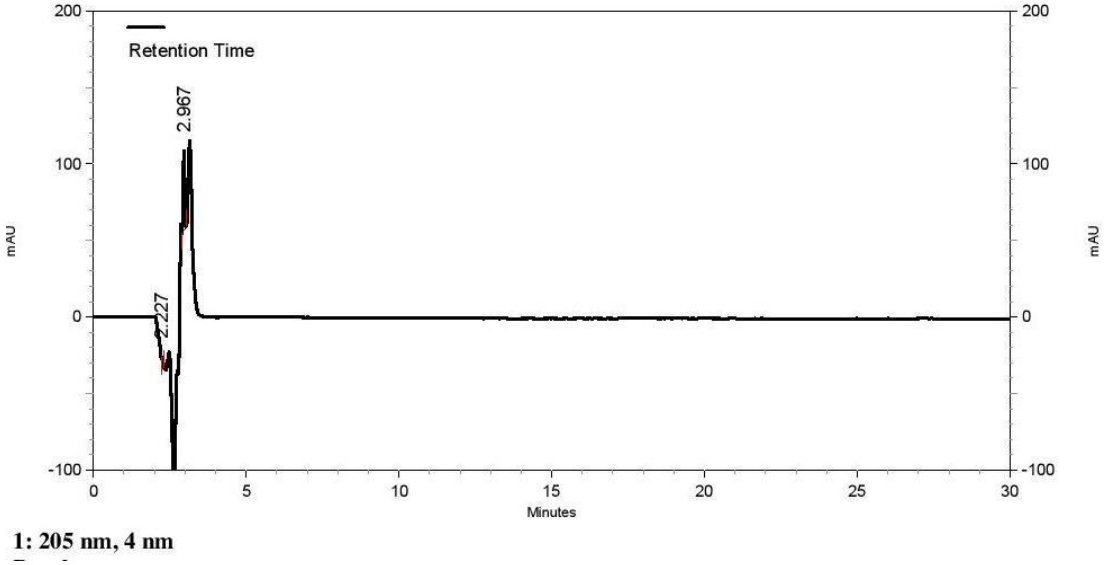

Fig 3

Fourier Transform Infrared Spectroscopy (FTIR) of isolated compound from methanolic extract of unripe fruits of *Physalis minima*

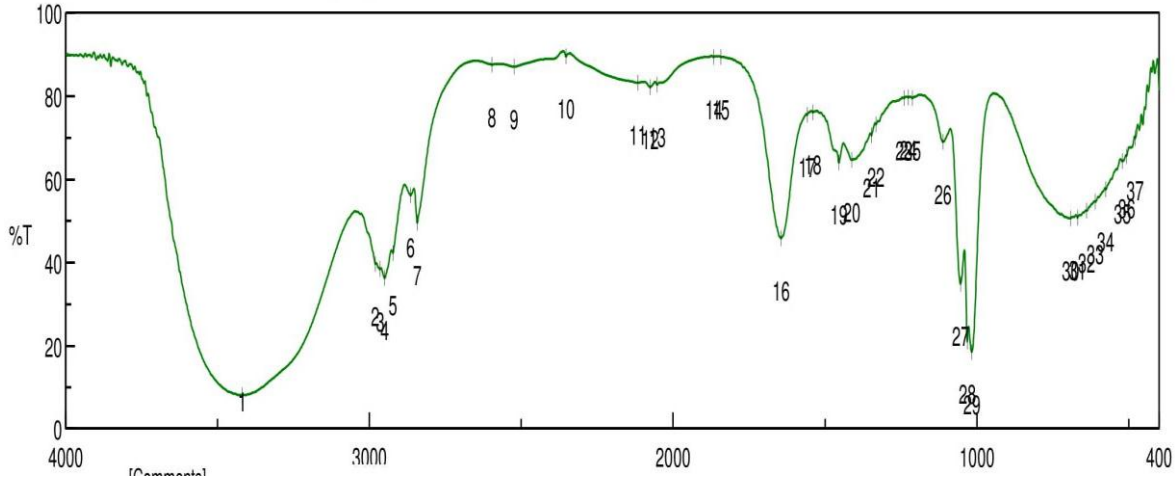

Fig 4

FTIR spectral data interpretation

Table 1: The FTIR spectrum of isolated compound from methanolic extract of unripe fruits of *Physalis minima* are given in Fig 4.

Extract prepared in	Peak number	Peak value	Functional group
Methanol	1	3400	RR'N-H (Tertiary amines)
	4	2950	Aliphatic –CH
	16	1650	C=O (Keto group)

NMR spectral data of isolated compound from methanolic extract of unripe fruits of *Physalis minima*

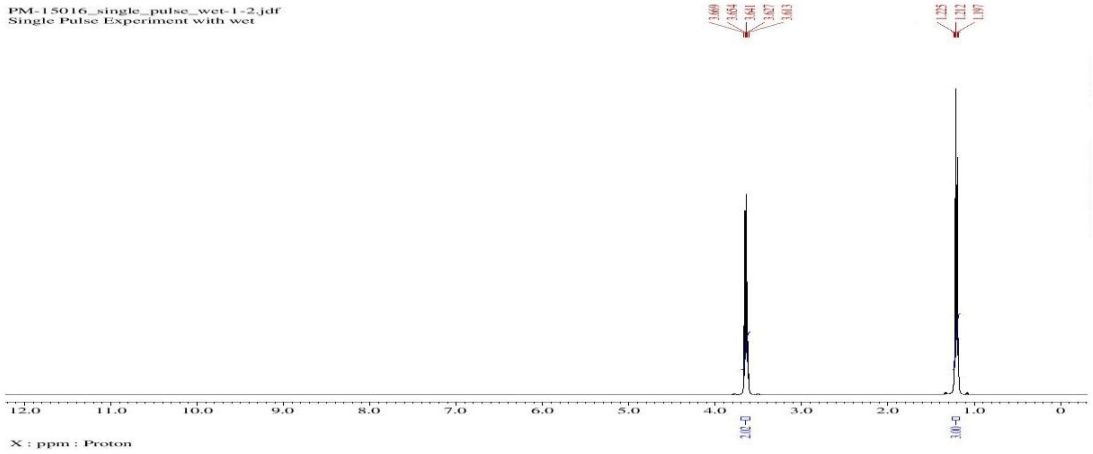

Fig 5

Effect of methanolic extract of unripe fruits of *Physalis minima* by Carbon Clearance test, Humoral Antibody (HA) Titre and Delayed Type Hypersensitivity (DTH) response

Treatment dose	Carbon Clearance test	DTH response	HA Titre
Control	0.065±0.2	09.00±0.011	3.20±0.51
Std (Levamisole)	0.069±0.1	09.84±0.013	3.36±0.54
PME (200mg)	0.073±0.1	12.09±0.020	5.05±0.22
PME (400mg)	0.075±0.2	12.88±0.032	7.38±0.45
PME (600mg)	0.079±0.2	14.86±0.036	10.90±0.51

Values are mean ± SEM; n=6 in each group; P<0.05 in comparison with control

CONCLUSION

In the present study, the reports of HPLC reveal the presence of the compound with retention time 2.967, I.R. reveals the the presence of hydroxyl group, aliphatic methyl group, keto group and ether linkage. The 1H NMR spectrum of 1 displayed signals for three methyl groups at δ 1.225, 1.212, 1.197 and five characteristic signals of a –CH2– group at δ 3.613, 3.627, 3.641, 3.654, and 3.669. Therefore, compound 1was identified as Physalin B, in comparison with published data and the isolated compound screened for immunomodulatory properties was found to be significant.

REFERENCES

1. Ghosh MN; Fundamentals of Experimental Pharmacology; 2nd Edition; Scientific Book Agency; Kolkata, 1984; 156-157.
2. Kulkarni SK; Hand Book of Experimental Pharmacology; 3rdEdition; Vallabh Prakasan, 1999; 117-171.
3. John A Parrotta; Healing Plants of Peninsular India; CABI publishing, 675.
4. https://en.m.wikipedia.org/wiki/Physalis_minima.
5. Harborne JB, Methods of extraction and isolation; In: Phytochemical methods; Chapman and Hall, London, 1988; 60-66.
6. Wagner, H. Plant Drug Analysis. A thin Layer chromatography Atlas, Heidelberg, Springer-Verlag Belgium, 2nd ed, 2002; 227, 305.
7. A. Marston, M. Maillard and K. Hostettmann, The role of TLC in the investigations of medicinal plants of Africa, South America and other tropical regions, GIT Laboratory Journal, 1997; 1: 36-39.
8. K. Hostettmann, J-L. Wolfender and S. Rodriguez, Rapid detection and subsequent isolation of bioactive constituents of crude plant extracts, Planta Medica, 1997; 63(1): 2-10.
9. Kurt Hostettmann, Strategy for the Biological and Chemical Evaluation of Plant Extracts Invited lecture presented at the International Conference on Biodiversity and Bioresources: Conservation and Utilization, Phuket, Thailand, November 1997; 23–27.
10. JI Yubin, The extraction, separation and purification of alkaloids in the natural medicine; Journal of Chemical and Pharmaceutical Research, 2014; 6(1): 338-345.
11. N Hosoda, Agricultural and Biological Chernistry, 1979; 43(4): 821-825.
12. K. Hostettmann *et al.*, Rapid detection and subsequent isolation of bioactive constituents of crude plant extracts, Planta Medica, 1997; 63(1): 2-10.
13. Jayathirtha MG, Preliminary immunomodulatory activities of methanol extract of *Ielipta alba* and *Centella asitica*, Phytomedicine, 2004; 11: 361-365.
14. Popova IE, A determination of lignans in flaxseed using liquid chromatography with time-of-flight mass spectrometry, Journal of Chromatography A, 2009; 1216: 217–229.
15. Zhang ZY, Role of peroxidase in anthocyanin degradation in litchi fruit pericarp, Food Chemistry, 2005; 90: 47–52.

16. Heftmann F, Chromatography: Fundamentals and Application of Chromatographic and Electrophoretic Techniques. 1992, 5th edn., Elsevier, Amsterdam, The Netherlands.
17. A. Marston, The role of TLC in the investigations of medicinal plants of Africa, South America and other tropical regions, GIT Laboratory Journal, 1997; 1: 36-39.
18. Feuske M, Schonbeiter H, Thin-layer chromatography on silica coated aluminium sheet as an adjunct to radioimmunoassay of steroids. Journal of Chromatography, 1991; 563: 178-183.
19. K Hostettmann, Rapid detection and subsequent isolation of bioactive constituents of crude plant extracts, Planta Medica, 1997; 63(1): 2-10.
20. Hancock WS, High Performance Liquid Chromatography in Biotechnology. Wiley-Interscience, New Jersey, USA, 1990.
21. Eberhardt TL, Chinese Tallow Tree (Sapium Sebiferum) utilization: Characterization of extractives and cell-wall chemistry. Wood Fiber Sci, 2007; 39: 319-324.
22. Hazra KM, Isolation of antibacterial pentahydroxy flavones from the seeds of *Mimusops elengi* Linn. African Journal of Biotechnology, 2007; 6(12): 1446-1449.
23. Makare N, Immunomodulatory activity of alcoholic extract of *Mangifera indica* L. in mice, Journal of Ethnopharmacology, 2001; 78: 133-137.