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Original Article

Potent *in vitro* Antioxidant, Antimicrobial, and Antidiabetic Effects of a Polyherbal Mixture: Evidence from 3T3-L1, β-TC6, and C2C12 Cell Line Studies

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ABSTRACT

Aim and Objectives: The traditional plants *Plumeria pudica, Podranea ricasoliana*, and *Leea macrophylla* have been used for their medicinal properties since ancient times. Our main objective is to identify the preliminary chemicals of these plant leaves to prepare a polyherbal mixture and apply it for *in vitro* DPPH, H_2O_2, and NO scavenging free radical antioxidants test with antibacterial study and evaluated for its *in vitro* antidiabetic potential using 3T3-L1, β-TC6, and C2C12 cell lines. **Materials and Methods:** The macroscopic study as well as the preliminary phytochemical screening of these plants, possess many numbers of metabolites that are responsible for anti-inflammatory and anti-oxidant properties. The plant extract's hydrogen peroxide scavenging activity was assessed by measuring absorbance at 230 nm using a UV-vis spectrophotometer. The disc plate technique uses antibiotics to decrease the various gram-positive and negative bacterial properties. Ethanol-based extract of Polyherbal Mixture (PHM) was subjected to phytochemical analysis. The increase of glucose uptake in 3T3L1 cells (a) and C2C12 myoblast cells over the treatment of PHM extract at varying concentrations ranging from 10 to 500 µg/mL. Metformin and Insulin-stimulated glucose uptake were considered positive controls. Experiments were conducted in triplicate**. Results:** The IC_{50} values of the DPPH, NO, and H_2O_2 scavenging assays revealed that the test compounds were less potent than the standard drugs, yet they still exhibited satisfactory free radical scavenging activity. The ethanolic extract of the Polyherbal Mixture (PHM) showed antibacterial activity that was slightly less potent than the standard antibiotic ciprofloxacin, yet still produced satisfactory results with comparable inhibition zones. Furthermore, the IC_{50} values of the test drug PHM (16.71, 12.0, and 20.8 µg/mL) and the standard drug ascorbic acid (7.07, 6.07, and 8.0 µg/mL) indicate that PHM possesses antioxidant scavenging activity. The zone of inhibition of PHM was satisfactory as compared to the standard drug during the antimicrobial study. Specifically, in 3T3-L1 cells, PHM-500 (2.9±0.25), metformin (2.8±0.41), and insulin (5.9±0.43) increased glucose uptake compared to control. Similarly, in C2C12 myoblasts, PHM-500 (4.37±0.28), metformin (4.91±0.34), and insulin (6.77±0.32) showed increased glucose uptake. **Conclusion:** The poly-herbal mixture is expected to exhibit beneficial anti-microbial, anti-oxidant, and anti-diabetic activities, making it a unique and unpublished formulation.

Keywords: Antibacterial, Antioxidant, Phytochemical Screening, Antidiabetic Potential Using 3t3-L1, B-Tc6, and C2c12 Cell Lines.

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INTRODUCTION

Inflammation is the major cause of the increase in the number of complications day by day in many diseases.[1] Diabetes mellitus is now a global challenge for many developing countries, as per the report of the International Diabetes Federation, more than 500 million people are affected with diabetes worldwide.[2] The rate of complications in diabetes mellitus is high like neuropathy, retinopathy, nephropathy, and angiopathy.[3] The process of angiogenesis and epithelization may be affected by the different free radicals and now the rapid use of modern medicine may increase the rate of complications in wounds formed during diabetes.[4] The healing of wounds becomes slower due to complications of various chemical and synthetic remedies.[5] Our main focus is that naturopathy may be the replacement of modern therapy, which can reduce the rate of complications. Traditionally, many plants were used for the treatment of many diseases.[6]

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Nowadays, 60 percent of the population uses traditional drugs as home remedies for their primary health care.[7] In the traditional system of medicine, the plant *Podranea ricasoliana* is used as an anti-inflammatory drug,[8] as well as the *Plumeria pudica* locally called naga Champa in India, and the plant *Leea macrophylla* also locally called Hatikana plants in India.[9] All three plants have potent antiseptic and anti-inflammatory activities, as shown by different research studies. The combination of plant extracts may increase the rate of wound healing as compared to single-use.[10] Until now there is not much data on *Plumeria* and *Leea macrophyla* for wound healing and anti-diabetic activities. The mixture formulation may be useful of increasing the rate of wound complication during diabetes.[11]

The plant Naga Champa, or *Plumeria pudica*, which belongs to the family Apocyanacee, is used as a traditional medicine in India for its purgative, antidiarrheal activities, the milk juice extract is also useful in rheumatoid arthritis; it possesses anti-inflammatory properties due to the presence of flavonoid and phenolic compounds.[12] The ethanolic leaf extract of *Plumeria pudica* contains several bioactive compounds that make the plants a bactericidal herbal remedy.[13] The ethanol extract produced the inhibition of *Escherichia coli* during the antimicrobial study. The plant *Podrenea ricasoliana*, commonly called a garden plant, Tanfani in India, has not been reported for antidiabetic activity and anti-inflammatory properties till now, but the presence of phenol, flavonoid, and saponin indicates that the flower and leaves may be useful for anti-inflammatory and antioxidant properties.[14] The plant *Leea macrophylla*, the root extract, has the potential for free radical scavenging activity, such as NO and DPPH study. The presence of oleanolic acid and its derivatives, along with stigma sterol, makes the plant suitable for antioxidant drugs. The ethanolic extract of *Leea macrophylla* has also been reported for its wound healing activity by comparison with ointments that produce faster, as well as it would be safer in traditional wound healing as an herbal drug .[15] From different studies, the extract of *Leea macrophylla* has the potential anti-microbial activity against several microorganisms like Blastomyces dermatitis, Candida albicans, and *Aspergillus niger*, Trichophyton sp, *Cryptococcus neoformans*.[16] It is indicated that the ethanolic extract of *Leea macrophyla* has both anti-bacterial and antifungal properties.[17]

During the study of Ayurvedic literature, the preparation of polyherbal formulations was mentioned in "*Sarangdhar Samhita*" with their dosage and formulation.[18] The combination plant formulation therapeutic activity is rather than a single use of the herb.[19] Some combination formulations in Ayurveda that the combination of neem and ginger shows positive effects, The black pepper, asafoetida, and cumin are utilized for improving the slow digestion process.[20] So there are several formulations found in Ayurveda for polyherbal formulations. The PHF is effective in several diseases due to its composition of different active constituents, and also the effects or side effects are less as compared to the modern medicine system. Some combinations for formulation, like Diyar, are useful for anti-diabetic effects.[21] PHM can target multiple pathways involved in diabetes, such as insulin sensitivity, glucose metabolism, and antioxidant defenses.3T3-L1 adipocytes are the mouse embryonic fibroblast cell line that differentiates into adipocytes, exhibiting insulin resistance.[22] The BT6 cell line is a pancreatic beta-cell line derived from the insulinoma of the rat.[23] It is a commonly used cell line in diabetes research, particularly for studying pancreatic beta-cell function, insulin secretion, and glucose metabolism. The 3T3-L1 cell line is a preadipocyte cell line derived from the mouse embryo.[24] It is a widely used model for studying adipocyte biology, obesity, and insulin resistance.

MATERIALS AND METHODS

2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino, Krebs-Ringers bicarbonate buffer.[25] The suppliers of phosphate-Buffered saline and 2-Deoxyglucose (2-NBDG)[26] were Sigma Aldrich Co. in St. Louis, USA. 1% L-glutamine, glipalamide, and 1% penicillin-streptomycin,[24] ascorbic acid,[27] ethanol, and 1,1-diphenyl-2-picrylhydrazyl are obtained from Sigma and Aldrich Co., S.D. Fine Chemicals, Mumbai, and are of analytical grade. Fatal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), Insulin, glucose, 1% L-glutamine, 1% penicillin-streptomycin, Krebs-Ringers bicarbonate buffer (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino), metformin, Glibenclamide, insulin ELISA kit, ascorbic acid, ethanol and 1,1-diphenyl-2-picrylhydrazyl, Histamine and serotonin and acetic acid are obtained from Sigma and Aldrich Co., S.D. Fine Chemicals, Mumbai, and are of analytical grade.

Collection and authentication of the plant

L. Rasingam, the Scientist In-Charge, Botanical Survey of India, Hyderabad, Telangana, identified and verified the *Plumeria pudica, Podranea ricasoliana*, and *Leea macrophylla* plants that were collected from the Bolangir Hill range in Odisha, India. In the herbarium of the Botanical Survey of India, Hyderabad, a voucher specimen (BSI/DRC/2021-22/Tech/434) was placed (Figure 1).

Plant material and extract preparation

The fresh greenish leaves of *Plumeria pudica, Podranea ricasoliana,* and *Leea macrophylla* were collected during September from the Hill range and villages of Bolangir, Odisha. All the plant's leaves were placed in the shade and dried at room temperature for 15-20 days.[28] After properly drying all the leaves, they are prepared for coarse powder with the help of a mortar and pestle. They were sieved with 100 mesh particle sizes.[29] In order to find out, each powdered plant material was subjected separately to Soxhlet extraction using ethanol, chloroform, and hexane solvents with different polarities.[30] They then contrasted their extractive values. Next, 1.5 kg of powdered plant material underwent a final

extraction by cold maceration for 48-72 hr, with ethanol serving as the solvent.[31] At 50°C, the extract was dried using a rotary evaporator (model R-1001-VN, ZGSIT, China). To find out which phytoconstituents were present, a preliminary screening was done on several extracts.

Preparation of Poly Herbal Mixture (PHM)

The polyherbal mixture was prepared by the ratio of 1:1:1 of the plants *Podranea ricasoliana, Plumeria pudica,* and *Leea macrophylla* of each 15 g of drugs, 5 g was mixed with 120 mL of distilled water and macerated 24 hr at a temperature of 24 hr.[32] After 24 hr, the macerated drugs were boiled for 45 min and then filtered through muslin.

Morphological Evaluation

We have studied the morphological characteristics of *Plumeria pudica, Leea macrophylla*, and *Podrenea ricasoliana.* In this study, characteristics like color, taste, shape, size, and texture (Table 1).

Phytochemical Analysis

The EEPP, EEPS, and EELM are subjected to phytochemical analysis to check for the presence of mucilage, alkaloids, sugars, tannins, phenols, triterpenes, glycosides, and steroids, proteins and amino acids. The solvents used were like N-hexane, Petroleum ether, ethanol and methanol. All the procedures for phytochemical screening were carried out under the reference procedure (Table 2).[33]

In vitro Antioxidant Study

DPPH Free Radical Scavenging Activity

Procurement of Chemicals

1, 1 -diphenyl-2-picrylhydrazyl**,** ethanol was purchased from S.D Fine Chemicals, Mumbai.

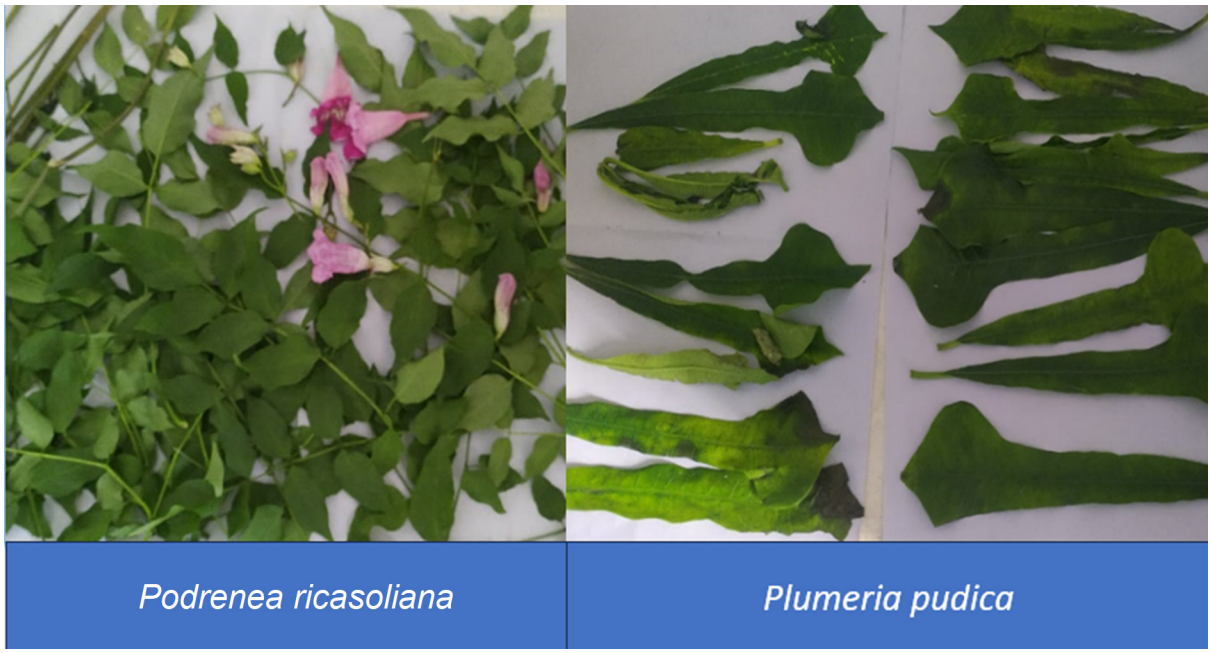

Figure 1: *Podrenea ricasoliana* flowering plants and *Plumeria pudica* leaves.

Table 1: Final Zone of Inhibition.

Name of the organism	Standard (Ciprofloxacin 50 µg/mL)	T_1 (50 mg of EEPP)	T_2 (100 mg of EEPP)	T_3 (150 mg of EEPP)	T_4 (200 mg of EEPP)	Control
B. pumilus (Gram+ve)	6 mm	10 mm	10 mm	9 mm	9 mm	-
B. subtilis (Gram+ve)	6 mm	10 mm	10 mm	9 mm	9 mm	-
E. coli (Gram-ve)	5 mm	8 mm	7 mm	6 mm	6 mm	-
P. aeruginosa (Gram-ve)	5 mm	10 mm	10 mm	9 mm	9 mm	-

AA: Ascorbic Acid, **PHM:** Poly Herbal Mixture.

Preparation of Stock Solutions of Extract and Riboflavin

10 mg of PHM extract and riboflavin were taken and dissolved in 10 mL of water to give a standard stock solution of plant extract and riboflavin, respectively to provide a 1 mg/mL solution. From this, various dilutions of extract such as 50, 100, 150, 200 µg/mL were made with water.[34]

Principle

The reduction of an alcoholic DPPH solution (dark blue in color) in the presence of an antioxidant that donates hydrogen results in the nonradical form of yellow-colored diphenyl-picryl hydrazine, which is the basis of the DPPH assay method.[35]

Reagents

1, 1 -diphenyl-2-picrylhydrazyl (DPPH, 0.004%) solution

To generate DPPH radicals, 100 mL of ethanol were mixed with 4 milligrams of DPPH, and the mixture was left in a dark place overnight.[36]

Procedure

The scavenging activity for DPPH free radicals was measured according to the procedure. 0.1 mL of plant extract at different concentrations and 3 mL of 0.004% DPPH solution in ethanol were combined to create an aliquot.[37] After a vigorous shake, the mixture was left to settle at room temperature for half an hour. By measuring the absorbance at 517 nm, the decolorization of DPPH was ascertained. 0.1 mL of the appropriate vehicle was used in place of the plant extract to create a control.[38]

$$Percentage\ inhibition = \frac{\text{Average control O.D.} - \text{Test sample O.D.}}{\text{Average control}} \times 100$$

Calculation of 50% Inhibition Concentration

A graph was created by plotting the optical density at each concentration of the extracts and Ascorbic acid, with the X- and Y-axes representing concentrations and percentage inhibition, respectively.[39] The graph was extrapolated to find the 50% inhibition concentration (IC_{50}) of the test sample and Ascorbic acid as the standard drug (Figure 2).

IC_{50} calculation: it is the concentration in which 50% of the free radicals are scavenged by the standard drug like Ascorbic acid or test drugs like PHM. It is calculated from the linearity graph with the following formula:

Y=mX + c; Y=% of inhibition (50), X=log (concentration), m= slope, c= intercept,

$$IC_{50} = ((50\text{-}c)/m) \text{ X } 100)$$

Hydrogen Peroxide Scavenging Activity

Chemicals such as hydrogen peroxide, NaH_2PO_4, and Na_2HPO_4 were obtained from Sisco Research Laboratories. A stock solution of plant extract and riboflavin (1 mg/ml) was prepared, with further dilutions made using phosphate buffer. Reagents included a hydrogen peroxide solution (43 mM) and a phosphate buffer (0.1 M, pH 7.4) created by mixing specific amounts of NaH_2PO_4 and Na_2HPO_4.[40]

Procedure

By measuring the concentration of a 40 mM hydrogen peroxide solution at 230 nm using a Shimadzu UV-vis 1700 spectrophotometer against a phosphate buffer pH 7.4, the plant extract's capacity to scavenge hydrogen peroxide was evaluated. After mixing plant extract (10-50 µg/mL) with 0.6 mL of hydrogen peroxide solution (43 mM), the absorbance at 230 nm was measured after 10 min and compared to a blank solution that did not contain hydrogen peroxide.[41]

The percentage of scavenging of hydrogen peroxide of PHM extract and standard compounds was calculated using the following equation:

$$Percentage\ inhibition = \frac{Average\ control\ O.D. - Test\ sample\ O.D.}{Average\ control} \times 100$$

Calculation of 50% Inhibition Concentration

An X-axis representing concentrations and a Y-axis representing percentage inhibition was used to plot the optical density attained at each concentration of the extracts and riboflavin. A 50% inhibition concentration of riboflavin was found by extrapolating the graph to the test sample (Figure 3).

Table 2: Effect of PHM and Glibenclamide on insulin secretion from β-TC6 cell lines.

Conc. in µg/mL	Insulin in ng/mL
Control	1.32±0.27
Control (Glucose)	3.64±0.46
PHM10	3.89±0.61
PHM 50	4.61±0.51
PHM 100	6.31±0.80
PHM 150	8.83±1.18
PHM 250	12.74±1.43
PHM 500	22.51±2.86
GLIB 10 µm	34.59±3.46

DETERMINATION OF NITRIC-OXIDE RADICALS SCAVENGING ACTIVITY

Chemicals used included sodium nitroprusside, sulphanilamide, naphthyl ethylene diamine hydrochloride, and phosphoric acid. A 1 mg/mL stock solution of plant extract and riboflavin was prepared, with dilutions of 50-200 µg/mL. Nitric oxide scavenging activity was evaluated by incubating plant extract with sodium nitroprusside and Griess reagent, and absorbance was measured at 570 nm after 30 min of incubation. Vehicle control was also used.[42]

Procedure

Nitric oxide production was induced by sodium nitroprusside and quantified using the Griess assay.[43] Sodium Nitroprusside (SNP) in aqueous solution at physiological pH (7.4) spontaneously releases Nitric Oxide (NO) radicals (59, 60). The generated NO rapidly interacts with oxygen, forming Nitrite Ions (NO2-). The produced nitrite ions can be quantitatively estimated using the Griess reagent.[44] Nitric oxide scavengers outcompete oxygen for NO molecules, diminishing nitrite ion formation.[45] Poly Herbal Mixture was evaluated for its nitric oxide scavenging activity by incubating sodium nitroprusside (5 mM) with varying concentrations of PHM in phosphate-buffered saline at 25°C for 150 min. At specified intervals, 0.5 mL aliquots of the incubation mixture were withdrawn and mixed with 0.5 mL of Griess reagent. The absorbance of the resulting chromophore, formed through diazotization and coupling reactions, was measured at 546 nm using the Griess reagent, with potassium nitrite standards serving as references.

Calculation of Percentage Inhibition

The percentage inhibited by the extract of nitric oxide production was calculated by using the formula:

$$Percentage\ inhibition = \frac{Average\ control\ O.D. - Test\ sample\ O.D.}{Average\ control} \times 100$$

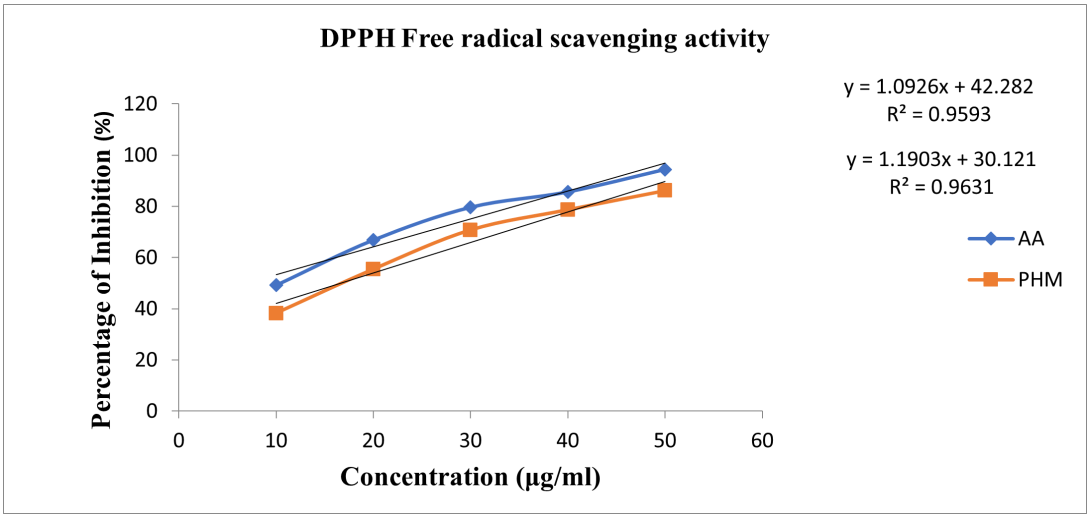

Figure 2: The Percentage (%) of inhibition of ascorbic acid and Test drugs (PHM) on DPPH radical scavenging.

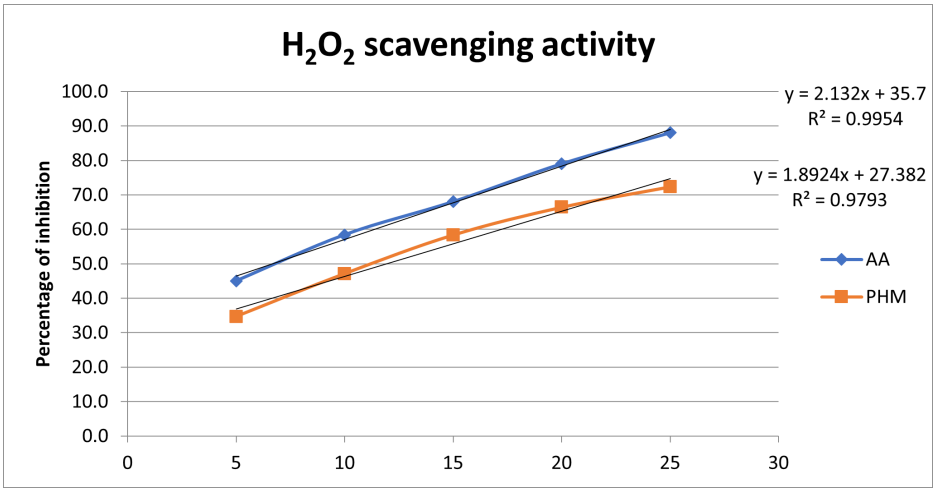

Figure 3: The Percentage (%) of inhibition of ascorbic acid and Test drugs (PHM) during H_2O_2 free radicals scavenging Assay.

Calculation of 50% Inhibition Concentration

On the graph, where concentrations are plotted on the X-axis, optical density is plotted on the Y-axis, and each concentration of the extracts and riboflavin is plotted, the curve was extrapolated to determine the 50% inhibition concentration of the test sample (Figure 4).

Anti-Microbial Activity of Polyherbal Mixture

The leaves and leaflets of plants *Plumeria pudica, Podranea ricasoliana,* and *Leea macrophylla* were dried and powdered. Then, the extraction process was carried out with an ethanolic solution by using the method of extraction.[46] The Soxhlet apparatus was used for this process. The controlled temperature was maintained between 75°-80°C for all three herbs. The extracted residue was collected and properly dried under reduced pressure using the rotatory evaporator, which kept the temperature at 40-45°C. All the herbs were properly weighed and the extraction efficiency was carried out by using the references of air-dried substance. The airdried polyherbal mixture was dissolved in different solvents with their concentration. Finally, the zone of inhibition was calculated and compared with standard PHM antimicrobial activity drugs. The standard drug was ciprofloxacin against *B. subtilis*, and *Pseudomonas aeruginosa*.[47]

Preparation of Poly Herbal Mixture (PHM)

After the process of solvent extraction, the prepared ethanolic extracts of *Podranea ricasoliana, Plumeria pudica*, and *Leea macrophylla* were stored in a well-closed container. The PHM was prepared in the ratio of 1:1:1 [48] of the plants *Podranea ricasoliana, Plumeria pudica*, and *Leea macrophylla*, respectively. The 1 mg ethanolic extract from *Podranea ricasoliana*, 1 mg from *Plumeria pudica*, and 1 mg from Leea macrophylla. The total 3 mg ethanolic extracts were dissolved in 30 mL of distilled water and prepared as a mixture as followed by the standard procedure.[49] We have taken different concentrations of PHM for antioxidant studies.

Test Organisms

The following strains of *Bacillus subtilis* (*B. subtilis*) (MTCC no 441) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (MTCC no 424) were acquired from MTCC, IMTEC, Chandigarh, Punjab.

Preparation of Nutrient Agar Media

We prepared the nutrient agar media by following the standard procedure.[50] Nutrient Agar by adding 6.2 g of Nutrient Agar in 200 mL of distilled water. The medium was autoclaved for 15 min at 121°C. It was cooled and 25 mL poured on each Petri dish and placed on the refrigerator for solidification.

Procedure

We followed this method of antimicrobial study of EPHM (Ethanolic extracts polyherbal formulation).

Disc Plate Technique

It is the most used technique for determining the susceptibility of microorganisms to plant extracts or chemotherapeutic agents.[51] Small paper (what man filter paper) discs impregnated with known concentrations of extracts or chemotherapeutic agents and kept on the surface of an inoculated agar plate. After incubation, the zones of inhibition were measured around the disc. We prepared four agar plates for testing of standard drugs ciprofloxacin and four plates for testing of EPHM of different concentrations against the microorganisms *Staphylococcus aureus, Bacillus subtilis, Escherichia coli*, and *Pseudomonas aeruginosa*. The culture was placed on the plate by copper loop, and the Zones were prepared. [52] We measured the growth of bacterial culture on the Petri plates from day 1 to 5, which were 0.5 mm,0.7 mm, 0.9 mm,1.2 mm and 1.3 mm, respectively. Then we applied the test and standard drugs day by day. The test drugs were given with different concentrations of 50 mg/mL, 100 mg/mL, 150 mg/mL, and 200 mg/mL over the zone. Finally, we measured the Zone

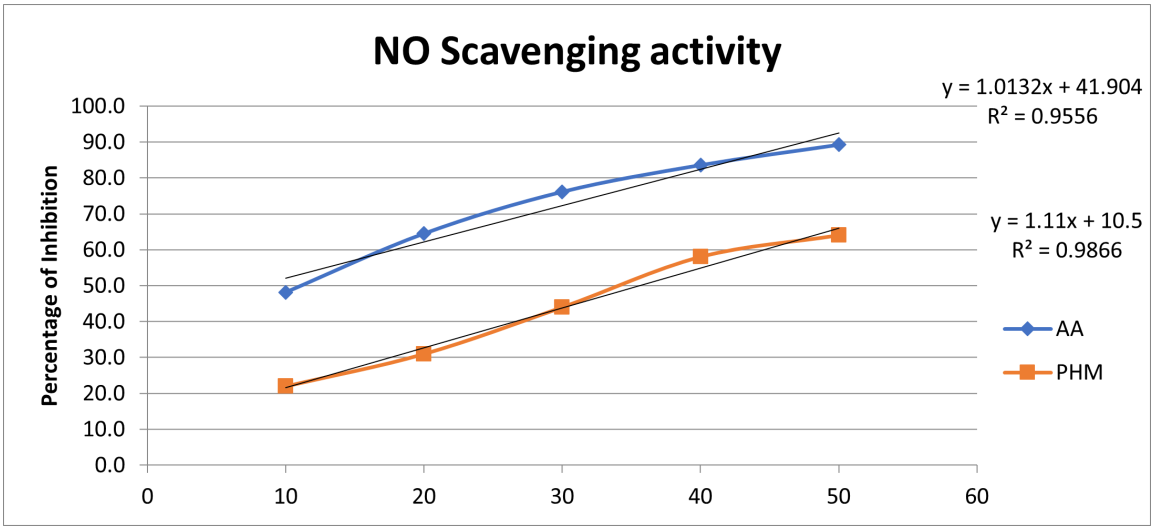

Figure 4: The Percentage (%) of inhibition of ascorbic acid and Poly Herbal Mixture on NO radical scavenging.

of inhibition that the test drugs EPHM inhibited significantly shown in Table 1 and Figure 5.[53]

Method of Glucose Uptake in 3T3L1 Cells (a) and C2C12 Myoblast Cells

Ethanol-based extract of Polyherbal Mixture (PHM) was subjected to phytochemical analysis. The increase of glucose uptake in 3T3L1 cells (a) and C2C12 myoblast cells over the treatment of PHM extract at varying concentrations ranging from 10 to 500 µg/mL. Metformin and Insulin-stimulated glucose uptake were considered positive controls. Experiments were conducted in triplicate was carried out on the rat model.[54]

Cell Lines and Culture Media Preparation

The mouse preadipocyte cell line 3T3-L1, mouse myoblast cells (C2C12 cells), and mouse pancreatic cells (β-TC6 cells) were cultivated using the protocol described in. To prepare Dulbecco's Modified Eagle's Medium (DMEM), β-TC6 cells by heating them to 37°C and supplemented with 15% 1% L-glutamine, 1% penicillin-streptomycin, and Fetal Bovine Serum (FBS).[55] The β-TC6 cells were promptly frozen in a 37°C water bath before being carefully placed into a vial and placed into a 15 mL centrifuge tube with 9 mL of previously heated medium. After 5 min of centrifugation at 1200 rpm, the supernatant was removed, and the cell pellet was resuspended in 10 mL of new medium. After that, the cell suspension was transferred to a 25 cm^2 flask and incubated at 37°C in a humidified environment containing 5% CO_2. Every two to three days, the medium was switched until convergence was reached.[56] For the 3T3-L1 preadipocytes, DMEM supplemented with 10% FBS and 1% penicillin-streptomycin was used. After being frozen, 3T3-L1 cells were similarly moved to β-TC6 cells. After centrifuging the cells, removing the supernatant, and reconstituting them, they were then put into a 75 cm^2 culture flask. Next, the cells were incubated under the same conditions with regular changes to the media until confluence was achieved. C2C12 myoblasts were cultured in DMEM supplemented with 1% penicillin-streptomycin and 20% FBS. After the C2C12 cells were defrosted and centrifuged, the cell pellet was reconstituted in a new medium and plated in a flask measuring 25 cm by 25 cm.[57] The cells were cultured at 37°C in 5% CO_2 once they achieved around 70-80% confluence. To obtain the cells for subculturing, the media was changed every two to three days. The day after confluence, the cells were cultured in DMEM supplemented with 20% FBS(v/v), antibiotics, 1% penicillin-streptomycin, and 5 µg/mL insulin to encourage adipocyte differentiation.[58] The culture media was switched to DMEM supplemented with 20% FBS (v/v), antibiotics, and 5 µg/mL insulin for four days to induce differentiation. The media was changed to DMEM with 20% (v/v) FBS and antibiotics on the sixth day following differentiation induction.[59] These processes involve thorough adherence to sterilization protocols to protect cell viability and prevent contamination.

Test Solutions Preparation

The stock solution for the cell line study was made at a concentration of 1 mg/mL. After being weighed, *Plumeria pudica* was dissolved in distilled Dimethyl Sulfoxide (DMSO). 10% FBS and 11.1 mM glucose were added to the DMEM to expand the solution's volume.[60] The solution was then filtered to make sure it was sterile. Three distinct cell lines-C2C12 myoblast cells, 3T3-L1 adipocytes, and β-TC6 pancreatic beta cells-were serially diluted twice.

Determination of Cell Viability by MTT Assay

Insulin Secretion Assay

Measurement of Insulin Secretion from β-TC6 Cells

To maintain β-TC6 pancreatic beta cells under standard circumstances, the volume of Dulbecco's Modified Eagle Medium is modified with 100 U/mL streptomycin, 100 U/mL penicillin,

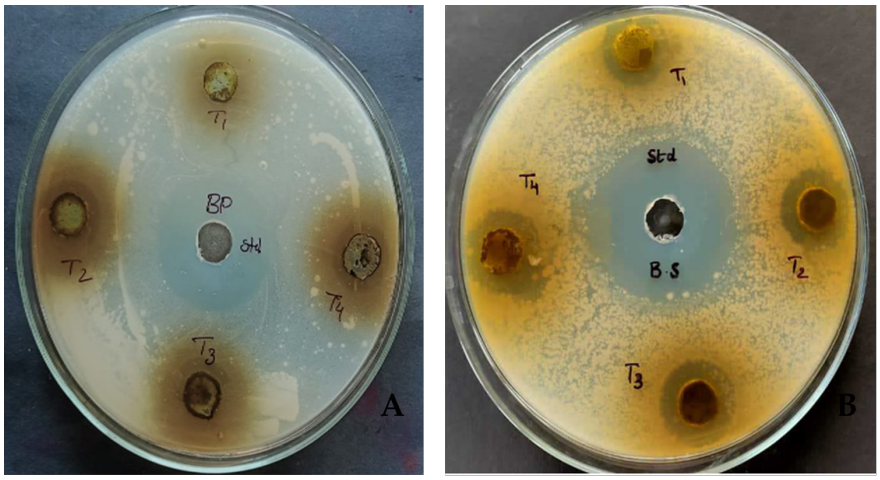

Figure 5: The final day of the Zone of inhibition by Test drugs (PHM) on culture media as compared to standard drug Ciprofloxacin.

and 10% fetal bovine serum to promote optimal cell development and viability.[61] Because the cells were subcultured every five to six days under these circumstances, the density was consistently at the ideal level for the population's general health. The PHM was administered to the cells at various dosages to measure the dose-dependent amount of insulin secretion after a good culture of β-TC6 was formed. The cells were seeded in 24-well plates.[62] Subsequently, ensuring that the seed is homogeneous would yield a high cell density and, hence, consistent exposure to the therapy. Following treatment, Krebs-Ringers bicarbonate buffer was used for incubation at 37°C.[63] The pH of the buffer was adjusted to 7.4. To allow for the natural release of insulin in reaction to the extract, if any, the cells' environment was stabilized back to an equilibrium state.[64] To assess the insulin concentration in later testing, the post-incubation supernatants were collected and kept in a freezer at -20°C after additional centrifugation. Using Glibenclamide as a positive control, the amount of insulin secreted by the β-TC6 cells was measured.[65] A mouse insulin Enzyme-Linked Immunosorbent Assay (ELISA) kit is used to detect insulin in the supernatant. Insulin from the samples binds with insulin-specific antibodies coated onto the plate wells, followed by a secondary antibody conjugated to an enzyme, in this sandwich ELISA experiment.[66] A spectrophotometer was used to assess the color of the enzymatic reaction, and the precise quantification of insulin levels was made possible by the fact that the change in optical density was proportional to the concentration of insulin.[67]

Glucose Uptake assay

The glucose absorption was measured in both 3T3-L1 adipocytes and C2C12 myoblast cells using techniques outlined in the literature, with minor adjustments made for appropriate adaptation based on experimental requirements.[68] Adipocytes were treated with different PHM test solutions at concentrations ranging from 10 to 500µg/mL for 24 hr on the ninth day of differentiation. Medium alone and 1X PBS were used as negative controls, metformin was used as a positive control, and 10 µM was utilized as a positive control. Furthermore, 3T3-L1 cells undergo insulin-stimulated GLUT-4 translocation when exposed to 10 µM insulin; this served as a supplemental positive control in this study to examine insulin-mediated glucose uptake pathways.[69]

To remove any remaining test solution, the cells were washed with 1X PBS following seeding and a 24-hr incubation period. To stop the fluorescent probe from photodegrading, the cells were then given DMEM supplemented with 80 µM of the fluorogenic glucose analog 2-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG) for 60 min at 37°C in the dark.[70] This was carried out in order to ascertain the initial glucose consumption during the presence of the extract. Insulin was administered to the cells to measure the insulin-mediated absorption of glucose following an incubation with 2-NBDG.[71] Unincorporated 2-NBDG was eliminated, and the uptake response was successfully halted by washing the cells with prechilled 1X PBS. A fluorescence microplate reader was used to quantify intracellular fluorescence, a marker of glucose absorption, with the excitation wavelength set to 485 nm and the readout wavelength set to 535 nm.[72] To improve the specificity of the glucose absorption signal, measurements made in each trial without the addition of 2-NBDG were subtracted.[73]

The quantification of basal and insulin-stimulated glucose uptake was aided by this rigorous and precise methodological approach, which also yielded important insights into the cellular mechanisms governing glucose metabolism in either adipocytes or myoblasts as well as the modulating effects that the tested extracts may have.[74] The cells were first cultured at 37°C in DMEM media with high glucose, 20% fetal bovine serum, 1% penicillin-streptomycin, and 5% CO_2.[75] After the cells had expanded to fill the culture dish's surface area and achieved 80-100% confluence, the differentiation media was added to the fluid to enable differentiation.[76] To limit growth factors and induce a cell cycle exit, this medium swap used high-glucose DMEM supplemented with a lower serum concentration of 2% horse serum in place of FBS.[77] After the cells were kept in this differentiation medium, the media was changed often every 48 hr for 4-7 days. Successful differentiation is characterized by the elongation, alignment, and fusion of myoblasts into multinucleated myotubes during this time.[78] Under a light microscope, the developed myotubes were examined for morphological alterations and confirmed by muscle-specific markers.[79] The myotubes might then be used for any additional metabolic research that is biologically related to the metabolism and activities of skeletal muscles, such as insulin-stimulated glucose uptake assays.

RESULTS

The PHF has a significant presence of alkaloids, glycosides, and flavonoids with protein tannin and carbohydrates in the phytochemical screening of the PHM. In the DPPH, Hydrogen Peroxide, and NO scavenging, the method must be defined activity test the percentage of inhibition was significantly found in comparison with standard drugs ascorbic acids. The IC_{50} values in DPPH were 21.17, 33.16, and 38.16, respectively, for the test drugs. The IC_{50} values in H2O2 were 72.3 for test drugs PHM and 88.0 for standard drugs ascorbic acid. The IC_{50} values in NO were 64.0 for test drugs PHM and 89.2 for standard drugs ascorbic acid. The PHM also inhibited the growth of bacterial culture on Petri plates, proving that this mixture has good antioxidants, anti-inflammatory, and anti-bacterial properties as compared to the standard drugs ciprofloxacin. A dose-dependent increase in glucose uptake was observed in both 3T3-L1 and C2C12 myoblast cells, with PHM-500, metformin, and insulin exhibiting significant effects. Specifically, in 3T3-L1 cells, PHM-500 (2.9±0.25), metformin (2.8±0.41), and insulin (5.9±0.43) increased glucose uptake compared to control.

Table 3: Effect of EEPP and Metformin on glucose uptake in C2C12 and 3T3-L1 Cell Lines.

Conc. in µg/mL	Insulin in ng/mL	
	C2C12 cell lines	3T3L1 cell lines
Control	1.01±0.03	1.01±0.08
PHM10	1.06±0.11	1.1±0.16
PHM 50	1.22±0.11	1.3±0.19
PHM100	1.29±0.12	1.6±0.13
PHM 150	1.42±0.21	1.9±0.18
PHM 250	1.71±0.25	2.2±0.23
PHM 500	2.32±0.22	3.2±0.23
Metformin 10 µM	3.87±0.31	3.7±0.32

Similarly, in C2C12 myoblasts, EEPP-500 (4.37±0.28), metformin (4.91±0.34), and insulin (6.77±0.32) showed increased glucose uptake (Table 3).

Antimicrobial Activity

The final day of the Zone of inhibition by PHM was on culture media as compared to standard drug ciprofloxacin. The final day of the antibacterial assay revealed a significant zone of inhibition against *Bacillus subtilis* (B. subtilis), and *Pseudomonas aeruginosa* (P. aeruginosa) by PHM. The final zone of inhibition measured of test drug PHM was 19.7mm, 18.6mm,21.2mm, and 18.7mm respectively as compared to the standard ciprofloxacin was 21.6 mm,22.1 mm, and 25.2 mm,21.5 mm. (Mentioned the Result data on Table 1).

DISCUSSION

Native plants have long been employed as a traditional source of raw materials for the production of pharmaceuticals.[80] Before they are accepted and used globally, two interrelated issues, efficacy and safety must be addressed to modernize the age-old practice of herbal medicine passed down through the generations.[81] Many research articles have proved that a mixture of herbs possesses more therapeutic effects as compared to the use of a single herb.[82] The mixture of DIHAR is useful for the treatment of diabetes as compared to the lone herb. Ayurveda and another traditional system of medicine also proved that the mixture produced a potent therapeutic.[83] The aforementioned parameters were significantly enhanced by the polyherbal mixture in a dose-dependent manner. Histopathological findings corroborated the biochemical results. In summary, this research has yielded new knowledge regarding the effectiveness of polyherbal blends in treating hyperglycaemia and its associated problems in individuals with diabetes mellitus.[84] There are several research articles published that the PHM of different herbs have anti-oxidants, anti-microbial and anti-inflammatory properties.[85] Traditionally, *Plumeria pudica* has been used to treat various illnesses, such as neurological disorders, helminthic infections, inflammation, and nociceptive disorders[86] Alkaloids, terpenoids, vitamins, carbohydrates, flavonoids, glycosides, saponins, fixed oils, and a few more unidentified substances are the components that have been isolated.[87] Additionally, the plant has a variety of pharmacological properties, including anti-inflammatory, anti-diarrheal, nociceptive, anthelmintic, and activity against gynaecological illnesses, ulcerative colitis, and neurological conditions including Alzheimer's disease and leishmaniasis. The leaves of the plant *Leea macrophylla* may have antibacterial, antioxidant, anti-inflammatory, analgesic, neuropharmacological, and anti-diabetic properties, according to several research.[88]

The plant parts were employed by tribal societies as dietary supplements and as a cure for various illnesses. Well-established *in vitro* and *in vitro* investigations were carried out to gather proof of the plant components' pharmacological actions.[89] The leaves of *Podranea ricasoliana* have both antifungal and antibacterial activities in pharmacological research.[90] Our objective was all these three plants individually possess significant antioxidant, antimicrobial, and anti-inflammatory activity so that their EPHM or ethanolic polyherbal mixture will be beneficial for anti-microbial and anti-oxidant activity and this mixture is unique and unpublished. A statistically significant ($p<0.05$) conclusion was drawn from this study when we discovered that the PHM test drug sample in Beta-TC6 cells outperformed glibenclamide (10 µM) at high glucose concentrations of 11.1 mM. Nicotinic receptors may play a role in this calcium-dependent insulinotropic effect.[91] Increased insulin secretion and glucose absorption through upregulating the levels of GLUT-2 and GLUT-4 in these cells. In a similar vein, 3T3L1 cell lines showed enhanced insulin absorption when given varying concentrations of PHM.[92] GLUT1 and GLUT4 receptor expression promotes glucose absorption in 3T3-L1 cells. Similarly, in 3T3 cells, Peroxisome Proliferation-Activated Receptor γ (PPARγ) and Cytosine-Cytosine-Adenosine-Adenosine-Thymidine/Enhancer-binding protein α (CCAAT/Enhancer-binding protein α, also called C/EBPα) are responsible for adipogenesis. In both 3T3L1 and C2CL2 myoblast cells, the mean±SD of glucose absorption was higher than the basal level, according to the results.[93] Since these cell lines also express the GLUT-4 protein, which increases glucose uptake by activating the insulin signaling pathway, the glucose uptake is statistically significant, with a p-value of less than 0.05. Every trial result shows that PHM has antidiabetic potential.

CONCLUSION

As compared to the standard drug ascorbic acid in both H_2O_2 and NO scavenging antioxidant assay, the study demonstrates that the antibacterial and antioxidant properties of the Polyherbal Mixture (PHM) leaves of *Plumeria pudica, Podranea ricasoliana*, and *Leea macrophylla* have a significant rate of IC_{50} value.

Staphylococcus aureus, Bacillus subtilis, Escherichia coli, and *Pseudomonas aeruginosa* are just a few of the bacteria cultures that respond well to the inhibition of bacterial culture in plate method. Consequently, our study indicates that PHM from *Podranea ricasoliana, Leea macrophylla*, and *Plumeria pudica* may be a useful natural antioxidant source and that it may be used as a rejuvenating drug with a variety of therapeutic benefits. The PHM was also shown to have a promising anti-diabetic impact from the cell line research. PPAR γ activation and glucose transporter expression are the primary causes of the insulinotropic action of PHM seen in these cell lines. *In silico, in vivo*, and confirmatory studies of active principles can all help to validate this further.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

IC_{50}: Half Maximal Inhibitory Concentration; **PHF:** Poly Herbal formulation; **NBDG-2:** (N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **EPHM:** Ethanolic Extract of Polyherbal formulation**; mm:** Millimetre; **mg:** Milligram; **NaH_2PO_4:** Sodium dihydrogen phosphate; **H_2O_2:** Hydrogen peroxide; **NO:** Nitric Oxide; **1X PBS:** Phosphate buffered saline; **2-NBDG:** 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose; **DM:** Diabetes mellitus; **DMEM:** Dulbecco's Modified Eagle Medium; **DPPH-2:** 2-Diphenyl-1-picrylhydrazyl; **EEPP:** Ethanolic extract of *Plumeria pudica*; **ELISA:** Enzyme-Linked Immunosorbent Assay; **FBS:** Fatal Bovine Serum; **FFAs:** Free Fatty Acids; **GLUT-4:** Glucose transporter type 4; **IC_{50}:** 50% concentration of inhibitor; **MTT:** Metabolic activity-based cell viability assay; **T1DM:** Type 1 diabetes mellitus; **T2DM:** Type 2 diabetes mellitus; **v/v:** Volume/volume; **DMSO**- Dimethyl sulfoxide.

SUMMARY

This study investigates the antioxidant, antimicrobial, and antidiabetic properties of a polyherbal mixture using various cell lines (3T3-L1, β-TC6, and C2C12). The results demonstrate the mixture's potent effects in the antioxidant activity, which neutralizes free radicals on its antimicrobial activity, inhibiting microbial growth and antidiabetic activity: potential therapeutic benefits for diabetes management. The findings suggest the polyherbal mixture's potential as a multifaceted therapeutic agent, warranting further research for its applications in healthcare.

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