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SECONDARY METABOLITES, *IN-VITRO* ANTIOXIDANT AND ANTIULCER POTENTIAL OF THE PHYLLOCLADE EXTRACT OF *MUEHLENBECKIA PLATYCLADA* (F. MUELL) MEISN

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Keywords:

Muehlenbeckia platyclada, cladode, *In-vitro* antiulcer potential, *In-vitro* antioxidant activity, Secondary metabolites, Methanolic extract

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ABSTRACT: The present study aimed to determine secondary metabolites, *in-vitro* antioxidant, and antiulcer activities using various solvent-based *Muehlenbeckia platyclada* extracts. Solvents based phylloclade extraction was used to determine various secondary metabolites such as phenols, flavonoids, terpenoids and tannins and to assess *in-vitro* antioxidant activities such as DPPH, $ABTS^{\cdot+}$, superoxide, hydrogen peroxide, nitric oxide, FRAP, Phosphomolybdenum, metal chelating and reducing power activities. Further, *in-vitro* antiulcer activities such as α-chymotrypsin enzyme, urease enzyme inhibitory activity, H^+ K^+ ATPase activity and acid neutralization capacity (ANC) were performed, and the resultant was analyzed using response surface methodology (RSM) to develop an adequate functional relationship between a response of interest (ME) with independent variables (Time, HCL consumed and NaOH consumed). A correlation coefficient was made between secondary metabolites and *in-vitro* antiulcer activities. Methanolic extract (ME) showed higher levels of secondary metabolites, *in-vitro* antioxidant activities and was subsequently selected to evaluate its antiulcer potential. A significant inhibition for alpha chymotrypsin (88.84%), urease (68.52%), and H^+K^+ ATPase (91.75%) was compared with respective standards. RSM results of ME for ANC indicated that 10 min, 22.66 mL HCl and 30.7 mL NaOH are adequate to get maximum ANC *i.e.* 46 mEq/ml. A significant correlation ($P<0.05$) was found between phenol and ACE (R^2=0.948), between phenol and HKA (R^2=0.941) and phenol and UE (R^2=0.952). These results suggest that ME of *M. platyclada* possesses potential antiulcer activities due to higher levels of secondary metabolites and *in-vitro* antioxidant activities.

INTRODUCTION: Phytocompounds from plants have a variety of therapeutic benefits, and they have been employed in managing several human illnesses. Due to numerous side effects and the high cost of chemical pharmaceuticals, there is a renewed interest in natural medicines in the form of standardized plant extracts [1].

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M. platyclada (F. Muel.) Meisn, a member of the Polygonaceae family that is used as a common natural remedy by many ethnic groups [2], is endemic to the Southern Hemisphere.

The plant is commonly known as centipede, tapeworm, or ribbon bush with several medicinal properties like anti-inflammatory, analgesic, anti-tumor, anti-HIV, anti-diarrheal, anti-fungal, anti-hepatic, anti-lipolytic, antioxidant, immuno-stimulant and antiulcer [2, 3]. According to several studies, phylloclade is a good source of flavonoids [4]. Secondary metabolites in plants combine with nutrients and fibres to form an integrated defense mechanism that protects against illness and stress [5].

Under any type of stress, living system produce more reactive oxygen species (ROS) than enzyme and non-enzyme-based antioxidants, such as superoxide anion radicals, hydroxyl radicals and hydrogen peroxide. Cell damage is brought by an imbalance between free radicals, antioxidants [6-9] and various diseases [10, 11]. Supplementing the diet with antioxidants from natural plant sources is the way to address this issue [12]. Peptic ulcers and ulcerative colitis are the most common gastrointestinal tract lesions. Peptic ulcer disease, which affects many individuals worldwide, occurs when some aggressive and gastroprotective factors are in an unbalanced state. Antacids, weak bases, work to lessen gastric acidity by interacting with it to produce salt and water. They have been shown to have good antiulcer properties; extended use can cause several adverse effects, including diarrhea, renal failure, and constipation [13].

The H. pylori bacteria release the urease enzyme to defend themselves from the stomach's acidic environment. This enzyme converts urea into ammonia, which protects bacteria from the stomach's acidic environment and facilitates their survival [14]. One effective strategy for eliminating stomach bacteria is to inhibit urease activity [15]. Due to their toxicity and less stable chemical and physical features in the natural environment, synthetic medicines cannot be used to modulate urease function [16]. Plant-derived urease inhibitors have great efficiency against microorganisms while less harming human cells [14].

Therefore, the finest antiulcer medications could be derived from plants with urease inhibitory capability [17-23]. One of the proteolytic enzymes chymotrypsin, is released into the duodenum by the pancreas. The most potent inducer for the secretion of chymotrypsin and the release of gastrointestinal hormones occurs when food enters the duodenum in the form of partially digested chyme [24]. When compared to the healthy control groups, illness groups like those with duodenal and stomach ulcers showed a considerable increase in chymotrypsin activity [25].

Hyperchlorhydria is a stomach situation characterized by uncontrolled hypersecretion of hydrochloric acid from the parietal cells of gastric mucosa through the proton pump [26].

It is known that the proton pump enzyme H^+ K^+-ATPase transports H^+ against a concentration gradient in stomach parietal cells, where it is in charge of producing acid. Various natural products of plant origin *i.e. Garcinia kola* [27], *Cissus quadrangularis* [28], *Acalypha wilkesiana* [29], *Azadirachta indica* [30] and *Hypericum perforatum* [31] were found to have the inhibitory potential for H^+/K^+ ATPase. Acidity is a common gastrointestinal problem due to extreme secretion of gastric acid or stomach acid which worsens the stomach lining mucosa and produces ulceration [32]. Antacids are general drugs that reduce or neutralize gastric acid, thereby decreasing gastric pH [33]. Acid neutralization capacity (ANC) is one of the mechanisms for controlling acidity of the stomach [34] and it is defined as the number of moles of acid neutralized by given antacid [35]. ANC screening using various plant extracts has been reported earlier [36-40].

This assay comprises three independent variables, such as consumed volume of HCl, NaOH and Time which are used to determine the end points of the titration during an experiment concerning ANC of a given antacid. Response surface methodology (RSM) is a valid statistical tool to standardize the experimental procedure by applying and studying the effect of different experimental variables and their interactions on the outcome of the result by decreasing the number of experiments [41].

Many studies have proved that RSM is an efficient technique for the optimization of various experimental protocols including standardization of plant extraction process for various *in-vitro* antioxidants (*Vitis vinifera* [42] and *Origanum vulgare* [43] optimized condition for enhanced production of alpha galactosidase enzyme (*Fusarium moniliforme*) [44], normalization of ultrasonic assisted extraction of flavonoids (*Crinum asiaticum*) [45] and selection of specific plant tissue culture media (*Berberis vulgaris*) [46]. The present study was designed to investigate secondary metabolites, *in-vitro* antioxidants and anti–ulcer potential of different solvent-based phylloclade extracts of *M. platyclada*.

MATERIALS AND METHODS:

Plant Material Collection, Authentication and Preparation of Extracts: *M. platyclada* was

collected from Kerala and authenticated by the Botanical Survey of India, Coimbatore (BSI/SRC/5/23/2019/Tech. 3431). Phylloclade was cleaned, dried under shade, and ground into fine powder. For the cold plant extraction process, the powder was treated with various solvents (petroleum ether, benzene, chloroform, acetone, methanol, and water) successively based on their polarity in 1:10 ratio for 24 h on an orbital shaker (180 rpm) at room temperature.

The filtrate was concentrated through evaporation at room temperature and dissolved in their respective solvents to get the working solution of 10 mg/ 10 mL (w/v).

Quantitative Determination of Secondary Metabolites: All the various solvent-based phylloclade extracts of different concentrations (0.1-0.5 mg/mL) were subjected to quantitative analyses of different secondary metabolites such as total phenols, flavonoids, tannins and terpenoids.

Determination of Total Phenols: For the determination of total phenolic content, different concentration of extracts were placed in test tubes and made up to a volume of 1 mL with distilled water. Then 0.5 mL of Folin–Ciocalteu reagent (1:1 with water) was added.

After five minutes of incubation, 2.5 mL of sodium carbonate solution (20%) was added, the reaction mixture was vortexed and incubated in the dark for 40 min, and the absorbance was recorded at 760 nm. The amount of total phenols was calculated as Gallic acid equivalents from the calibration curve [47].

Determination of Total Flavonoids: Different concentrations of all the solvent extracts were taken in test tubes and made up to a volume of 1 mL with distilled water. The sample solution was mixed with 0.15 mL of 5% $NaNO_2$ solution. After 6 min, 0.15 mL of 10% $AlCl_3$ solution was added and allowed to stand for 6 min and then 2 mL of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume up to 5 mL and then the mixture was thoroughly mixed and kept for 15 min. The absorbance of the mixture was determined at 510 nm using water as a blank. The analysis was performed in triplicate, and the results were expressed in rutin-equivalent flavonoids per gram extract [48].

Determination of Total Tannins: Tannin content of the samples were determined using insoluble polyvinyl-polypirrolidone (PVP), which binds tannins [49]. Different extracts were placed in test tubes, made up to a volume of 1mL with distilled water and mixed with 100 mg PVP, and incubated at 4ºC for 15 min. Then the mixture was centrifuged for 5 min at 8000 rpm. The non-tannin phenols were determined as mentioned for total phenol content using the clear supernatant. Tannin content was calculated by taking the difference between total and non-tannin phenolic contents.

Determination of Total Terpenoids: Solvent extracts with different concentrations were taken and made into 1mL using distilled water. Then, it was mixed with 3 mL of chloroform and allowed to stand for 3 min after adding 0.2 mL of conc. H_2SO_4, it was incubated for 2 h under dark conditions. Then, it was centrifuged and the supernatant was collected. To this, 3 mL of 95% methanol was added and vortexed. The absorbance of the mixture was determined at 538 nm against water as blank. The total terpenoid amount was calculated as linalool equivalents from the calibration curve [50].

***In-vitro* Antioxidant Assays:** During secondary metabolites analysis, 100µg/ml concentration showed high secondary metabolites content among various phylloclade extracts. Therefore, all the extracts in this concentration were subjected to different *in vitro* antioxidant assays such as DPPH, ABTS•+, Superoxide ($O2^-$), Hydrogen Peroxide (H_2O_2), Nitric Oxide ($NO^·$), FRAP, Phosphomolybdenum, Metal chelating (MC) and Reducing power (RP) activities.

DPPH Radical Scavenging Activity: A solution of 0.135 mM DPPH in methanol was prepared, and 1.0 mL of this was mixed with different concentrations of phylloclade extracts. The reaction mixture was vortexed thoroughly and left in the dark at room temperature 27°C for 30 min and the absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid was used as a standard for reference. The inhibitory concentration (IC_{50}) of several extracts required to

decrease the initial concentration of DPPH by 50% was calculated [51].

ABTS•+ Radical Scavenging Assay: To phylloclade extracts and trolox standards, 1 mL of 7mM ABTS solution was added and allowed to react for 30 min, and the absorbance was taken at 734 nm. The inhibitory concentration (IC_{50}) of several extracts required to decrease the initial concentration of ABTS by 50% was calculated [52].

Ferric Reducing Antioxidant Potential (FRAP): The freshly prepared FRAP reagent (900 µL) was mixed with water (90 µL) and phylloclade extracts. The reaction mixture was incubated for 30 min under 37°C, and the optical density was read at 593nm. The antioxidant power was expressed in ferric – TPTZ reducing power corresponding to that of 1mM ferrous sulfate heptahydrate [53].

Determination of Total Antioxidant Capacity by Phosphomolybdenum Assay: The assay is based on the reduction of Mo (VI) – Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 100 µg/mL phylloclade extracts were combined with 1mL of reagent solution (0.6M sulfuric acid, 2.28mM sodium phosphate, and 4mM ammonium molybdate) and incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695nm, and the antioxidant activity was expressed as the number of ascorbic acid equivalents [54].

Assay of Superoxide Radical ($O2^-$) Scavenging Activity: The assay was based on the capacity of the solvent extracts to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin light–NBT system [55]. Briefly, phylloclade extracts containing 50mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA and 0.1 mg NBT were allowed for 90 sec illumination and then absorbance was measured at 590 nm. The percentage of inhibition and IC_{50} values were determined.

Scavenging of Hydrogen Peroxide (H_2O_2): A solution of hydrogen peroxide (2mM) was prepared in phosphate buffer (pH 7.4) and 100µg/mL solvent-based phylloclade extracts were added to 0.6 mL of H_2O_2. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without H_2O_2 [56].

Assay of Nitric Oxide Scavenging Activity (NO): Sodium nitroprusside in an aqueous solution at physiological pH spontaneously generates nitric oxide, and it interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Nitric oxide scavengers compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM) in phosphate-buffered saline was mixed with different concentrations of solvent extracts and incubated at room temperature for 150 min. After the incubation, 0.5 mL of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylene diamine dihydrochloride was added. The absorbance of the chromophore formed was read at 546 nm. The inhibitory concentration (IC_{50}) of several extracts required to decrease the initial concentration of NO by 50% was calculated [57].

Metal Chelating Activity: Initially, 0.1 mM $FeSO_4$ and 0.25 mM of ferrozine combined and produce a Fe^{2+}-ferrozine complex. To this 0.2 mL of the phylloclade extract was added and incubated for 10 min at room temperature. The absorbance of the mixture was recorded at 562 nm and EDTA was used as a positive control [58].

Fe^{3+} Reducing Power Activity: 100 µg/mL plant extracts in 1mL of methanol were mixed with phosphate buffer (2.5 mL,0.2 mol/L, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture and then centrifuged for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicates increased reducing power[59].

***In-vitro* Antiulcer Assays:** Based on higher levels of secondary metabolites and potential *in-vitro* antioxidant activities, plant methanolic extract was selected for various *in-vitro* antiulcer assays.

α – Chymotrypsin Assay: A total volume of 100 µL assay mixture contained 60 µL Tris-HCl buffer (50 mM pH 7.6), 10 µL methanolic extract, and 15 µL (0.9 units) purified chymotrypsin enzyme. The contents were mixed and incubated for 20 min at 37°C and pre-read at 410 nm. The reaction was

initiated by the addition of 15 μL (1.3 mM) substrate (N-succinyl phenyl-alanine-P nitroanilide). The change in absorbance was observed after 30 min at 410 nm. All reactions were performed in triplicates. Chymostatin (0.5 mM) was used as a 'positive control [60].

$$\text{Percentage of inhibition} = [Activity\ (control) - Activity\ (sample)] \times 100$$

Where activity (control) is the control absorbance and activity (sample) is the sample absorbance

Urease Inhibition Assay: Nessler's reagent 61 determined the urease inhibition assay. A solution consisting of 0.25 mL urease (0.1 mg/mL) from jack bean, 2 mL of Tris – HCl buffer (pH 8.0) and 0.25 mL urea (60 mM) were incubated with 100 μL of the methanolic extract at 30°C for 20 min. After incubation, 1 mL of 10% trichloroacetic acid and 0.5 mL Nessler's reagent were added to stop the reaction. The absorbance was measured at 436 nm and thiourea was used as the standard inhibitor.

The percentage inhibition was calculated from the formula:

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

Where IC (%) is the inhibitory activity of UE. T is the absorbance of the test sample and C is the absorbance of the control sample

***H+ K+ ATPase* Inhibition Assay:**

Preparation of H^+ K^+-ATPase Enzyme: To prepare H^+ K^+-ATPase enzyme, fresh goat stomach was obtained from a local slaughterhouse at Coimbatore, Tamil Nadu, India (IAEC No: KMCRET/ReRc/Ph. D/19). The stomach was cut open, the gastric fundus mucosa was cut off, and the inner layer was scraped out for parietal cells [61]. Thus, obtained cells were homogenized in 16 mM Tris buffer (pH 7.4) containing 10% Triton X-100 and centrifuged at 6000 rpm for 10 min. The supernatant (enzyme extract) was used to determine the H^+, K^+- ATPase inhibition.

Assessment of H^+, K^+- ATPase Inhibition Assay: The reaction mixture containing 0.1mL of enzyme extract and methanolic cladode extract (100μL) was pre-incubated for 60 min at 37°C. The reaction was initiated by adding substrate 2 mM ATP (200 μL). 2 mM $MgCl_2$ (200 μL) and 10 mM KCl (200 μL) were added and incubated for 30 min at 37°C. Then the reaction was stopped by the addition of assay mixture containing 4.5% ammonium molybdate and 60% perchloric acid followed by centrifugation at 2000 rpm for 10 min and inorganic phosphate released was measured spectrophotometrically at 660nm [62]. Omeprazole (0.5mM) was used as a positive control.

Percentage of enzyme inhibition was calculated by using the formula;

$$\text{Percentage of inhibition} = [\text{Activity (control)} - \text{Activity (sample)}] \times 100$$

Where activity (control) is the control absorbance and activity (sample) is the sample absorbance.

Application of RSM to Optimize Different Parameters of ANC: Results obtained for ANC was analyzed using RSM to develop an adequate functional relationship between a response of interest (ME) and some independent variables (Time, HCl consumed and NaOH consumed for ANC).

Experimental design and Optimization: The RSM with CCD (Central Composite Design) was selected to optimize the process variables for enhanced ANC. The experimental design comprised 20 experimental runs with 14 factorial and 3 axial points (α), respectively at a distance of ±2cm from the center and 3 replicates of central points. The number of experiments was calculated from the following equation:

$$N = 2k \text{ (factorial points)} + 2k \text{ (axial points)} + n_0 \text{ (central points)}$$

N is the total number of experiments, k is the independent variable number, and n0 is the replicate number of the central points, resulting in an experimental design of 20 points. A second order polynomial regression model was used to correlate the relationship between independent variables and dependent variable responses i.e., Time, HCL consumed and NaOH consumed for ANC.

The second order polynomial regression model was as follows:

$$Y = \beta 0 + \sum \beta i X i 3 i = 1 + \sum \beta i i X i 2 + \sum\sum \beta i j X i X j + \varepsilon 3 j = i + 12 i = 13 i = 1$$

Where Y is the dependent variable responses for ANC (Y_1), β_0 is the model constant, β_i, β_{ii}, and β_{ij} are model coefficients, X_i and X_j are the coded value of independent variables, and ε is an error. Subsequently, additional experiments were carried out to verify the error in the process variables statistically.

Acid Neutralizing Capacity: The acid neutralizing capacity (ANC) of *M. platyclada* methanolic extract (100µl) was compared with the standard antacid Gelucil (10 mg/mL). Water was added and mixed to the 5 mL plant extract to make the total volume of 70 mL and then 30 mL of 1N HCl was added. It was stirred for 15 minutes and added 2-3 drops of phenolphthalein solution. The excess HCl was immediately titrated with 0.5 N sodium hydroxide solution drop wise until a pink color is appeared [63].

The moles of acid neutralized is calculated by,

$$\text{Moles of acid neutralized} = (Volume\ of\ HCl \times Normality\ of\ HCl) - (Volume\ of\ NaOH \times Normality\ of\ NaOH)$$

$$\text{Acid neutralizing capacity (ANC) per gram of antacid} = Moles\ of\ HCl\ neutralized\ /\ Grams\ of\ antacid/extrac$$

Statistical Data Analysis: Statistical analysis was carried out by one-way analysis of variance (ANOVA) test and Pearson's Correlation Coefficient (R^2) using a statistical package program (SPSS 10.0). The mean comparisons were considered statistically significant when $p \leq 0.05$. The data obtained from ANC was evaluated through the Minitab software (version 21.1.1, LLC, Inc., 2021 State College, Pennsylvania, USA), and the regression model fit values were assessed based on the R^2 (coefficients of determination).

RESULTS:

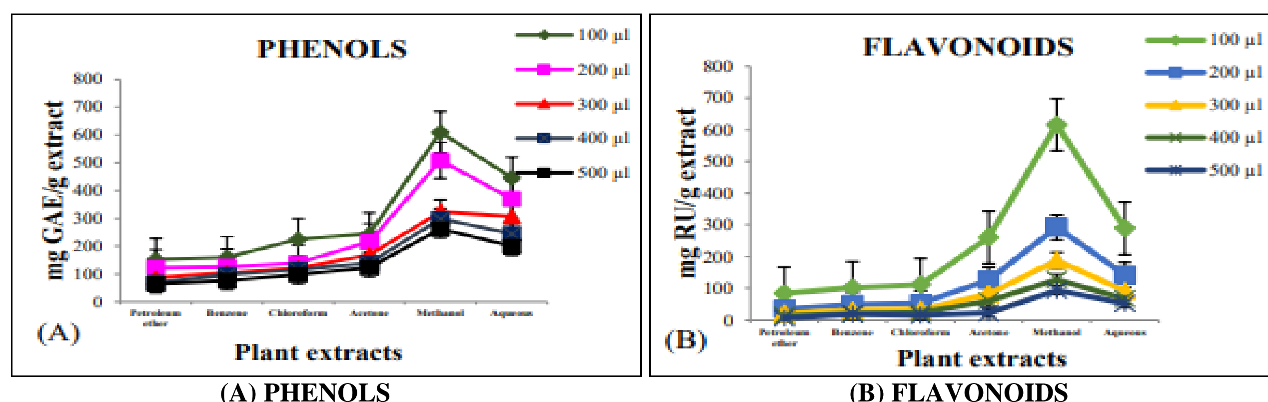
Quantitative Determination of the Secondary Metabolites: The results of total phenols, flavonoids, tannins, and terpenoids are given in **Fig. 1**. For quantification of these secondary metabolites, different concentrations of various solvent based phylloclade extracts of *M. platyclada i.e.* 100, 200, 300, 400 and 500 µg/mL were taken. Of these concentrations, 100µg/mL showed high content of all the secondary metabolites analyzed.

Total Phenol Content: Total phenolic content of various solvent-based phylloclade extracts of *M. platyclada* varied widely and ranged from 65.6 to 610 mg GAE/g extract. Methanolic extract showed higher total phenolic content *i.e.* 610 ± 4.0 mg GAE/g extract than the other solvent extracts **Fig. 1A**.

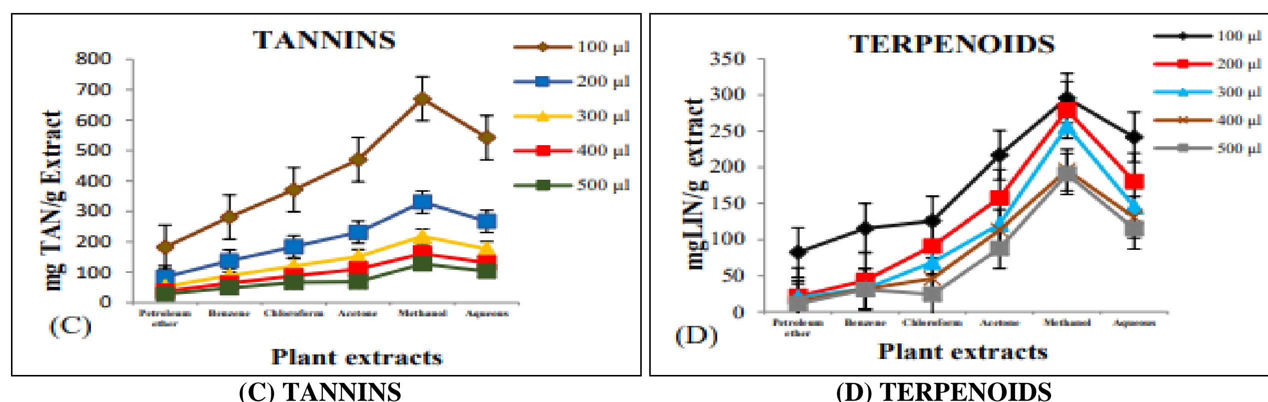
Total Flavonoid Content: Total flavonoid content was found to be high in methanolic extract (615.66 ± 1.52 mg RU/g extract) followed by aqueous and acetone extracts (289.33 ± 2.08 and 260.66 ± 2.51 mg RU/g extract, respectively. Low flavonoid content was observed in petroleum ether extract i.e. 84.0 ± 2.0 mg RU/g extract **Fig. 1B.**

Estimation of Tannin Content: Total tannin content of the various extracts was varied, and the range was found from 27.86 to 670.33 mg TAN/g extract. Among various extracts, methanolic cladode extract registered higher tannins, i.e. 670.33±3.05 mg GAE/g extract **Fig. 1C.**

Estimation of Terpenoid Content: Total terpenoid content of various phylloclade extracts of *M. platyclada* was observed in the range of 11.0 to 295.66 mg LIN/g extract. Of various extracts used, methanolic extract showed maximum terpenoid content *i.e.* 295.66±4.16 mg LIN/g extract **Fig 1D.**



(A) PHENOLS **(B) FLAVONOIDS**



(C) TANNINS (D) TERPENOIDS

FIG. 1: QUANTITATIVE ANALYSIS OF SECONDARY METABOLITES OF *M. PLATYCLADA*

In-vitro Antioxidant Activities:

DPPH Radical Scavenging Activity: The data on DPPH$^•$ radical scavenging activity of the various solvent-based phylloclade extracts of *M. platyclada* along with the best-known natural antioxidant standard, ascorbic acid is presented in **Table 1**. Low mean IC_{50} value was found for methanolic extract (5.22 µg/ mL), indicating its potential to scavenge DPPH free radicals compared to standard ascorbic acid (18.59 µg/ mL). Next to the methanolic extract, aqueous extract (5.70 µg/ mL) showed significant activity. Among all solvent-based extracts, petroleum ether extract had higher IC50 value, i.e. 17.59 µg/ mL, indicating its poor scavenging activity.

Total Antioxidant Activity by ABTS$^{•+}$ Radical Cation Decolorization Assay: Table 1. shows the ABTS radical scavenging ability of various phylloclade extracts. It can be ranked as methanolic extract (IC_{50} 1.34 µg/mL) > aqueous extract (IC_{50}1.38 µg/mL) > acetone extract (IC_{50} 1.37 µg/mL) > chloroform extract (IC_{50} 1.47 µg/mL) > petroleum ether extract (IC_{50} 1.9 µg/mL) > benzene (IC_{50} 3.9 µg/mL).

Nitric Oxide Radical Scavenging Activity: Among various extracts, the methanolic extract showed the highest nitric oxide scavenging activity 1.25 µg/mL IC_{50} than that of the standard rutin (7.98 µg/mL IC_{50}).

Petroleum ether extract showed a Very low scavenging effect with an IC_{50} value 2.94 µg/mL **Table 1.**

TABLE 1: MINIMAL INHIBITORY ACTIVITY OF DIFFERENT SOLVENT-BASED EXTRACTS OF *M. PLATYCLADA*

	IC_{50}				
Different solvent extracts	**DPPH (µg/ml)**	**ABTS (µg/ml)**	**$NO^·$ (µg/ml)**	**H_2O_2 (µg/ml)**	**O_2^- (µg/ml)**
Petroleum ether	17.59±0.001^d	1.9±0.04^d	2.94±0.002^d	2.33±0.03^d	26.4±0.003^b
Benzene	10.13±0.001^c	3.9±0.03^e	1.43±0.002^c	2.72±0.004^d	50.7±0.004^c
Chloroform	8.2±0.002^c	1.47±0.01^c	1.47±0.004^c	2.13±0.003^b	41.04±0.003^c
Acetone	11.28±0.001^d	1.37±0.005^b	1.62±0.001^c	2.22±0.003^c	56.44±0.003^d
Methanol	5.22±0.001^a	1.34±0.02^a	1.25±0.004^a	2.1±0.003^a	25.14±0.003^a
Aqueous	5.71±0.003^b	1.38±0.01^b	1.79±0.003^b	3.5±0.002^b	28.6±0.002^b
Standard	18.59±0.004	5.04±0.003	7.98±0.03	5.78±0.004	32.36±0.003

Values are expressed as mean± SD of three replicates. Means followed by the different letters. Within the column are significantly different at $P<0.05$ level. Standards: 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) – ascorbic acid, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) – Trolox, Nitric oxide ($NO^·$) – Rutin, Hydrogen Peroxide (H_2O_2) – Rutin, Superoxide radical scavenging activity (O_2^-) – Rutin.

Hydrogen Peroxide Radical Scavenging Activity: Of various extracts analyzed, methanolic extract was capable of scavenging hydrogen peroxide effectively compared to other extracts.

It showed low mean IC_{50} value (2.1 µg/mL) than the standard (5.78 µg/mL IC_{50}). Next to the methanolic extract, the aqueous extract had 3.5 µg/ml IC_{50} value **Table 1.**

Superoxide Radical Scavenging Activity: Among various extracts, the methanolic extract showed better superoxide anion radical scavenging activity *i.e.,* 25.14µg/mL IC_{50}.

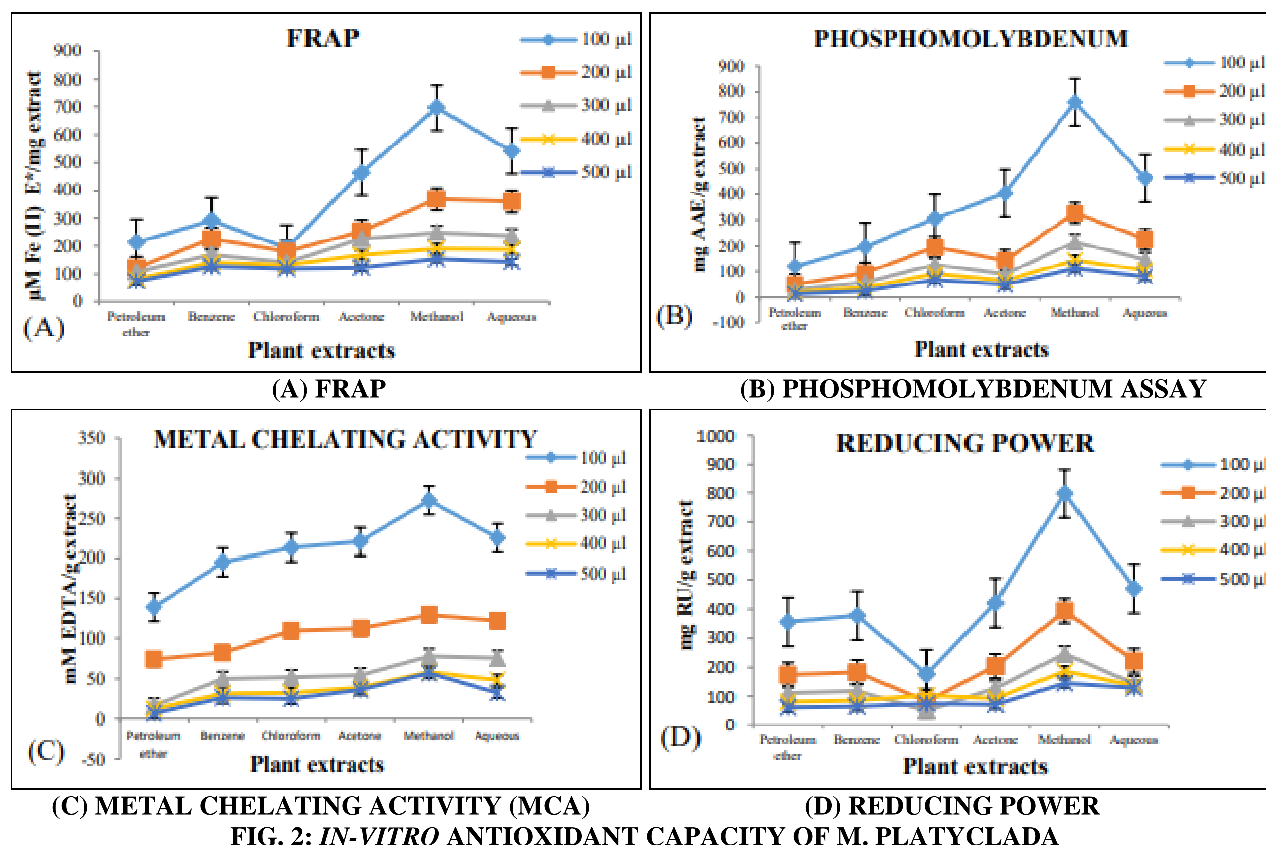
Acetone extract shows less inhibitory effect with IC_{50} value (56.44 µg/mL) for this activity **Table 1.**

Ferric Reducing Antioxidant Potential (FRAP): FRAP assay measures the reducing potential of plant antioxidants reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a coloured ferrous tripyridyltriazine (Fe^{2+}-TPTZ). FRAP values of the different solvent extracts of *M. platyclada* are shown in **Fig 2A.** Of various extracts, the methanolic extract had the highest FRAP value *i.e.* 696.66 µM Fe (II) E*/mg extract, followed by aqueous extract *i.e.* 541.66 µM Fe (II) E*/mg extract. Petroleum ether extract showed the lowest FRAP value *i.e.* 192.66 µM Fe (II) E*/mg extract. Results indicate that the methanolic extract has the stronger ferric-reducing power than other solvent extracts.

Determination of Total Antioxidant Capacity by Phosphomolybdenum Assay: The total antioxidant activity by phosphomolybdenum assay was based on the reduction of Mo (VI) to Mo (V) in the presence of the antioxidant compound and subsequent formation of a green phosphate/ Mo (V) complex at acidic pH and at a higher temperature. The antioxidant capacity was reported as ascorbic acid equivalents. The methanolic extract exhibited the best antioxidant activity (760.66 mg AAE/g extract) followed by the aqueous extract (464.66 mg AAE/g extract). The results indicated that the methanolic extract contains powerful antioxidants as compared to other solvent extracts **Fig. 2B.**

Metal Chelating Activity: The metal chelating ability of the extracts was measured by the ferrous ion ferrozine complex formation. The chelating activity has been mentioned as EDTA equivalents. Each extract interferes with the formation of Ferrous ion (Fe+2) and ferrozine complex, suggesting its metal chelating activity. Among the solvent extracts, methanol shows the highest chelating activity of 272.66 Mm EDTA/g extract, followed by the aqueous extract (225.66 Mm EDTA/g) **Fig. 2C.**

Reducing Power Activity: A compound's reducing capacity may be a significant indicator of its potential antioxidant activity. The results of the reducing power assay show the highest activity for methanolic extract (798.33 mg RUT/g extract) and the lowest activity for petroleum ether (355.6 mg RUT/g extract) **Fig. 2D.**



(A) FRAP **(B) PHOSPHOMOLYBDENUM ASSAY**

(C) METAL CHELATING ACTIVITY (MCA) **(D) REDUCING POWER**

FIG. 2: *IN-VITRO* ANTIOXIDANT CAPACITY OF M. PLATYCLADA

Correlation Coefficient between Secondary Metabolites and *In-vitro* Antiulcer Activites: Correlation coefficient was made between secondary metabolites and *in-vitro* antiulcer activities. Phenol showed significant correlation (P<0.05*) for alpha chymotrypsin (0.948), H^+ K^+ ATPase (0.941) and urease (0.952) while flavonoids and tannin had significant correlation only for urease. Terpenoids exhibited maximum linear correlation for urease and acid neutralization capacity **Table 2.**

TABLE 2: PEARSON'S CORRELATION COEFFICIENT ANALYSIS BETWEEN SECONDARY METABOLITES AND *IN-VITRO* ANTIULCER ACTIVITES

Correlation coefficient (r)	Alpha chymotrypsin	H+ K+ ATPase	Urease	ANC
Phenols	0.948*	0.941*	0.952*	0.977**
Flavonoids	0.842	0.999**	0.899*	0.988**
Terpenoids	0.974**	0.849	0.912*	0.883*
Tannins	0.837	0.999**	0.895*	0.988**

Correlation coefficients (R) with the level of significance **=p<0.01, *=p<0.05.

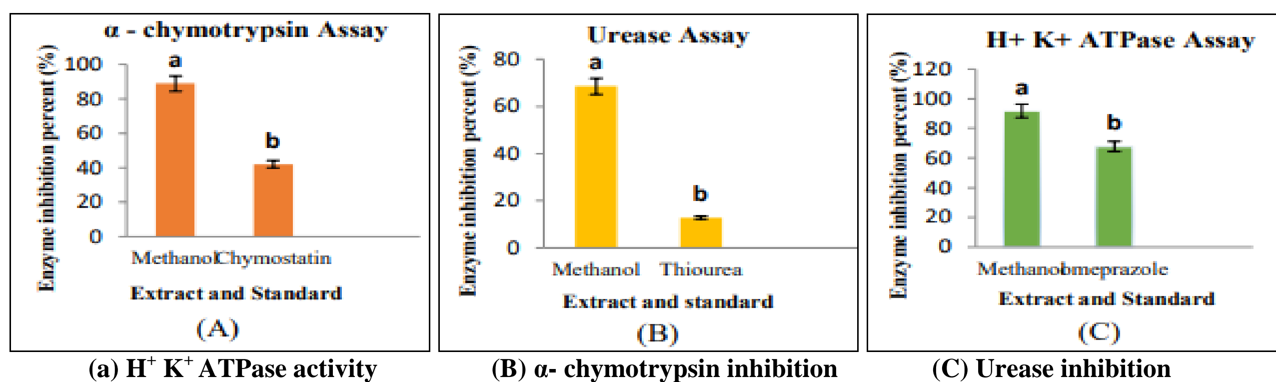
***In-vitro* Antiulcer Activity:** During *in-vitro* antioxidant analysis, the methanolic extract showed better antioxidant and free radical scavenging activity among various solvent-based phylloclade extracts. Therefore, this extract was selected for the *in-vitro* antiulcer activity.

α-chymotrypsin Assay: Methanolic extract of *M. platyclada* was tested against α-chymotrypsin enzyme **Fig. 3B.** The extract showed maximum inhibitory activity (88.84%) for alpha chymotrypsin enzyme compared to the standard chymostatin (42.51%).

Urease Enzyme Inhibition Assay: The urease enzyme inhibition assay was done by utilizing standard thiourea, which showed minimum inhibitory percent (12.84%), whereas methanolic extract showed the maximum percent of inhibition (68.52%) **Fig. 3C.**

H^+ K^+ ATPase Activity Assay: During H^+ K^+ ATPase activity assay, the methanolic extract showed significant (P<0.05) proton pump inhibitory activity in the goat gastric mucosal homogenate **Fig. 3A.** The inhibitory activity was comparable to the standard drug omeprazole.

The methanolic extract of *M. platyclada* potently reduced the hydrolysis of ATP by the goat gastric ATPase (91.75%). Omeprazole (100 µg/ml) was used as a positive control, which reduced the activity (67.68%).



(a) H^+ K^+ ATPase activity (B) α- chymotrypsin inhibition (C) Urease inhibition

FIG. 3: *IN-VITRO* ANTIULCER ACTIVITIES OF *M. PLATYCLADA*

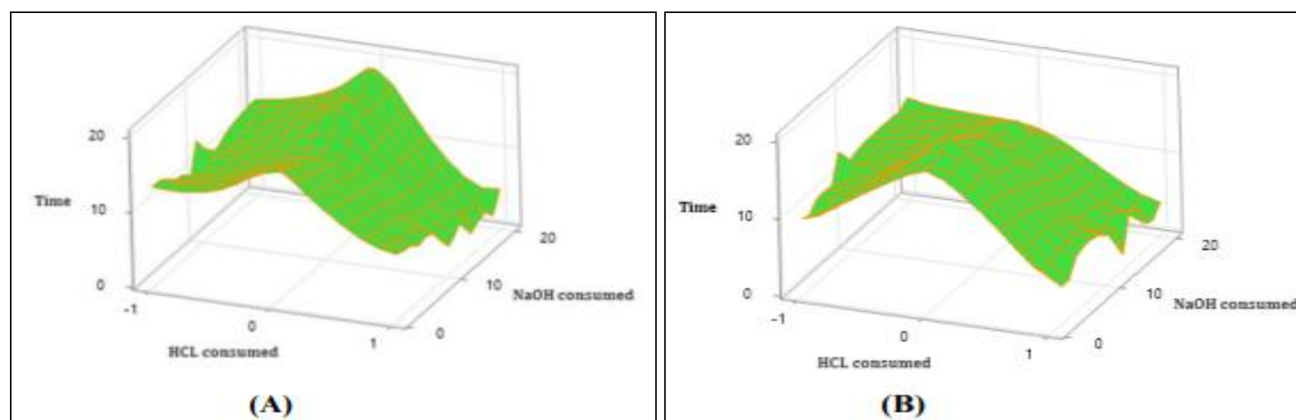
Model Fitting of Response Surface Methodology (RSM): Results obtained for ANC were analyzed using RSM to develop an adequate functional relationship between a response of interest (ME) and some independent variables (Time, HCl consumed, and NaOH consumed for ANC). The model CCD was fixed to investigate the effect of Time, HCl, and NaOH consumed for ANC **Table 3**. These three factors were assessed using the second-order model. The consumed volume of HCl (X_1) and NaOH (X_2) was increased with increasing Time (X_3). When the titration reached its endpoint, the ANC value was found for both the sample and the standard. **Table 3** shows the design matrices of the experiments using CCD.

TABLE 3: DESIGN MATRICES OF TIME (X_1), HCL CONSUMED (X_2) AND NAOH CONSUMED (X_3) FOR THE ACID NEUTRALIZATION CAPACITY OF *M. PLATYCLADA* METHANOLIC EXTRACT AND STANDARD USING CCD DESIGN

			Code level			Response variable (experimental & predicted)		
Run	**Type**	**Blk**	**A**	**B**	**C**			
1	Factorial	1	0	0	0	X_1	X_2	X_2
2	Factorial	1	0	0	-1.68	10.6	22.6	30.7
3	Factorial	1	1	1	1	16.3	22.3	30.6
4	Factorial	1	0	0	0	19.1	22.1	30.5
5	Axial	1	-1	1	1	20.9	21.9	28.6
6	Axial	1	0	-1.68	0	20.7	21.7	28.4
7	Axial	1	0	0	0	20.5	21.5	28.2
8	Factorial	1	-1.68	0	0	20.9	20.9	26.8
9	Factorial	1	0	0	1.68	20.7	20.7	26.6
10	Factorial	1	0	1.68	0	20.5	20.5	26.4
11	Factorial	1	-1	1	-1	19.9	19.9	25.4
12	Factorial	1	-1.68	0	0	19.7	19.7	25.3
13	Center	1	1	-1	1	19.6	19.6	25.2
14	Center	1	0	0	0	18.9	18.9	24.9
15	Center	1	1	-1	-1	18.7	18.7	24.7
16	Factorial	1	0	01	0	18.6	18.6	24.6
17	Factorial	1	1	-1	-1	17.9	17.9	23.9
18	Factorial	1	-1	0	-1	17.7	17.7	23.8
19	Factorial	1	0	1	0	17.4	17.4	23.4
20	Factorial	1	-1	-1	1	16.9	16.9	22.9

BLK: Block, Time (X_1), HCL consumed (X_2) and NaOH consumed (X_3).

The values of the response variables for Time (4.9-10 min), HCl consumed (16.9-22.6 mL), and NaOH consumed (22.9-30.7 mL). After experimental runs, 22.66 mL HCl, 30.7 mL NaOH and 10 min were found to be required to get maximum ANC **Fig. 4.**



(A) THE ASSOCIATION EFFECT OF ANC AND ME WITH TIME (MIN), HCL CONSUMED (ML) AND NAOH CONSUMED (ML), (B) THE ASSOCIATION EFFECT OF ANC AND STANDARD WITH TIME (MIN), HCL CONSUMED (ML) AND NAOH CONSUMED (ML)

FIG. 4: RESPONSE SURFACE PLOTS OF *M. PLATYCLADA* (METHANOL EXTRACT AND STANDARD)

Acid Neutralization Capacity (ANC): The *in-vitro* acid neutralization effect of the methanolic extract of *M. platyclada* was compared with the standard antacid gelucil (10mg/mL). ANC of the standard was found to be 65mEq/extract, whereas it was 46mEq/extract for methanolic extract **Table 4**.

TABLE 4: *IN-VITRO* ACID NEUTRALIZATION CAPACITY OF *M. PLATYCLADA* METHANOLIC EXTRACT AND THE STANDARD (GELUCIL)

Extract	Volume of NaOH Consumed (mL)	mEq of acid consumed
MEMP	30.7mL	46mEq
Standard	16.9mL	65mEq

MEMP: methanolic extract of *M. platyclada*, Standard: Gelucil. The acid-neutralizing capacity of Standard antacid and methanolic extract of *M. platyclada* (100µg/ml) was observed *in-vitro*. The data represent the mean volume of NaOH consumed (mL) and mEq of acid consumed. The methanolic extract shows a maximum ANC (46 mEq /ml) than the standard (65 mEq /ml).

TABLE 5: REGRESSION ANALYSIS AND ANOVA OF MODEL FITTING DESIGN IN ORDER TO EXAMINE THE STATISTICAL SIGNIFICANCE OF ANC

Source	DF[a]	Adj SS[b]	Adj MS[c]	F value[d]	P Value[e]
Regression	2	588	294	64.91	<0.001
Pt type	2	588	294	64.91	<0.001
Error	17	77	4.529		
Total	19	665			

[a]Degrees of freedom, [b]Adjusted sum of squares, [c]Adjusted mean square measures, [d]Test for comparing model variance with residual (error) variance, [e]Probability of seeing the observed F value if the null hypothesis is true.

Table 5 represents the regression analysis and ANOVA of model fitting design to examine ANC's statistical significance. Large F value *i.e*. 64.91 of ANC indicating the CCD model selected for this analysis was found to be significant. P value obtained (<0.001) in the model response was relatively very low, indicating this model's significance. A large F value and small p-value shows that the independent variables significantly impact the respective response variables. The predicted R – square (Pre. R^2) value indicates how well a regression model predicts response values, while the adjusted R – square (Adj. R^2) indicates the descriptive power of the regression model while including a diverse number of variables. In this study, the Adj. R^2 values were found to be 0.845 and 0.889 for ANC of ME and ANC of standard.

DISCUSSION: Ulcerative colitis is a chronic disease that causes the colon to become inflamed regularly. The condition mostly affects the colon and rectum, only damaging the innermost mucosa. The moderate symptoms of the disease include diarrhoea, stomach pain and progressively loose stools. Additionally, when the illness worsens, the patient may develop anemia, weight loss, exhaustion, and loss of appetite. Antacids (which include bismuth for convenience), anticholinergics, H_2-receptor antagonists, and liquorice derivatives are the four categories of peptic ulcer medications. Most commercially available antacids contain magnesium and aluminium but have undesirable side effects, like diarrhoea and constipation. Due to their widespread availability, patient acceptability, efficacy, safety, and low cost, people have used herbal medications for a long time to prevent these issues. Globally, herbal medicine is a well-liked complementary therapy for ulcerative colitis, and hundreds of clinical trials have supported its efficacy. This study aimed to assess the secondary metabolites quantification, *in-vitro* antioxidants and antiulcer potentials of different solvent-based phylloclade extracts of *M. platyclada*. During secondary metabolites analysis, among various solvent-based phylloclade extracts, 100µg/ml extract showed high content of all the secondary metabolites analyzed. Therefore, all the extracts in this concentration were subjected to different free radical scavenging assays such as DPPH, FRAP, ABTS•+, phosphomolybdenum, superoxide radical ($O2^-$), hydrogen peroxide (H_2O_2), nitric oxide ($NO^.$), metal chelating activity (MCA) and reducing power. Among various plant extracts, ME showed a higher level of *in-vitro* antioxidant activity.

Polyphenols are one of the abundant plant secondary metabolites and are ubiquitously present in various parts of the plants. Polyphenols reported in *M. platyclada* are morin-3-O – α rhamnopyranoside, kaempferol – 3 – O – β - gluco-pyranoside, quercetin 3-0-α-rhamnopyranoside and (+) – catechin. The phytocompound quercetin has an effective antiulcer activity that protects and prevents biochemical and morphological changes caused due to the induction of ulcer with ethanol and indomethacin. Kaempferol is a common flavonoid with antioxidant and anti-inflammatory properties with gastric antiulcer effect. Tannins are polyphenols used in the medicinal industry due to their astringent properties. During gastric ulcers, tannins react with the stomach protein and precipitate to form a protective cover, promoting resistance to proteolytic enzymes responsible for ulcer formation. Monoterpenoids are believed to be capable of changing the harmful effect of stress and

serve as non-steroidal anti-inflammatory drugs. Lupeol, a pentacyclic terpenoid, possesses gastroprotective activity against ethanol's ulcerogenic effects in the experimental mice. These studies indicate the potential activity of terpenoids against ulcers. The total antioxidant activity determinations were carried out using all the plant extracts of *M. platyclada* of various extracts, ME showed much antioxidant properties compared to other extracts.

In general, antioxidants act as reducing agents for completely eliminating various free radical intermediates with further inhibition of oxidation. $DPPH^{\bullet}$ is a stable free radical and it can accept an electron or hydrogen radical to become a stable diamagnetic molecule. In the present study, ME exhibited the highest $DPPH^{\bullet}$ radical scavenging activity than the other solvent extracts, which was likely due to their higher flavonoid content, the most important phytocompounds for scavenging activity. This trend of $DPPH^{\bullet}$ free radical scavenging activity had already been well documented in the literature for various plant species.

M. platyclada phylloclade extracts were found to have effective antioxidants, which was confirmed through various *in vitro* assays. In FRAP assays, ferric tripyridyltriazine (Fe (III)-TPTZ) becomes ferrous tripyridyltriazine (Fe (II)-TPTZ) through reaction with a reductant under low pH. (Fe (II)-TPTZ exhibits an intense blue colour and can be measured at 593 nm. An increase in absorbance is directly related to the amount of antioxidant present in the reaction mixture. A FRAP value of ME calculated was similar to the reports compared to the standard. $ABTS^{\cdot +}$ assay is an excellent technique for determining hydrogen-donating and chain-breaking antioxidants. In the present study, the efficacy of ME showed higher $ABTS^{\cdot +}$ cation radical scavenging activity compared to other extracts. Since ME contains high molecular weight, it can scavenge ABTS more easily$^{\cdot +}$ radicals. In the present investigation, among various extracts, ME significantly showed higher scavenging activity of $O_2^{\cdot -}$ which is produced in biological systems during cellular respiration and causes damage to biomolecules. Therefore, the removal or neutralization of $O_2^{\cdot -}$ is important to protect the cells from their deleterious effects. $NO^{\cdot}$ becomes toxic after reaction with oxygen or $O_2^{\cdot -}$ radicals. Various phenols and flavonoids have been found to neutralize $NO^{\cdot}$ radicals in previous studies. Several plant extracts have been reported to manifest antioxidant activity by exhibiting high FRAP values *in-vitro* (*Croton caudatos* [64], *Oroxylum indicum* [65], *Garcinia atrovirdis,* and *Cynometra cauliflora* [66].

Based on the data, it was understood that MCA was found higher in ME than other extracts. It was evident from the study that ME of *M. platyclada* possesses a better MCA, which might play a protective note against metal-catalyzed oxidative damage. The reducing power assay evaluates the ability of an antioxidant to donate an electron. In this assay, the ability of extracts to reduce Fe^{3+} to Fe^{2+} was determined. The reducing activities of various extracts of *M. platyclada* was observed in comparison with the standard. Among all the extracts, ME showed the highest reducing ability.

Herbal medicines play a significant role in treating gastro-duodenal diseases because they have less side effects and are more affordable than synthetic drugs. α-chymotrypsin enables the cleavage of peptide bonds by hydrolysis reaction. In this study, ME of *M. platyclada* showed more inhibitory activity against α-chymotrypsin than standard chymostatin. Maximum chymotrypsin inhibitory activity was reported in various plants such as *Acacia concinna*, *Caesalpinia bonducella*, *Lathyrus sativus*, *Mucura pruriens*, *psoralea corylifolia* and *Sapindus mucoross* [67]. Urease (UE) is responsible for the hydrolysis of urea to ammonia and carbamate, the final step of nitrogen metabolism in living organisms. The present study showed profound inhibition of urease by ME compared to standard. H^+ K^+ ATPase is a key enzyme in inducing acidity. This investigation studied the potential of ME to inhibit H+ K+ ATPase in-vitro isolated from goat stomachs. The result showed that ME had more significant proton inhibitory activity than standard. In ANC, ME showed a significant reduction (46mEq/extract) compared to standard (65mEq/extract). RSM is an effective statistical tool for optimizing various complex processes simply by saving experimental periods. It is the first report in which RSM tool was utilized to optimize the parameters for the enforced activity of ANC. This statistical tool was applied for the

optimization of plant extraction methods for *Cynomonium songaricum* for prediction of various secondary metabolites biosynthesis from cell suspension culture using yeast extract as elicitor; for optimization of ultrasonic-assisted extraction of flavonoids from *Crinum asiaticum* [68] and optimization of the best conditions such as pressure, temperature and CO_2 flow rate for extraction of triterpenes and sterols from jackfruit seed using supercritical CO_2 extractor. The correlation coefficient analysis between secondary metabolites and the *in-vitro* antiulcer activities gives significantly correlated values. Many research reports are available about the gastroprotective effects associated with several plant extracts rich in antioxidants [69]. Several authors have stated the positive correlation between phenolic content and antioxidant potential of various plant extracts.

CONCLUSION: An examination of the phytochemical composition, antioxidant effectiveness, and antiulcer activity of *M. platyclada* was conducted in this study. Results showed that secondary metabolites such as phenols, flavonoids, tannins, and terpenoids were present in high ME levels, which were further found to have significant *in-vitro* antioxidant and antiulcer properties. These results suggest that ME of *M. platyclada* possesses potential antiulcer activities due to higher levels. These experimental data validate the use of this plant in ethnic groups.

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