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Research Article

Performance of Different Solvents and Extraction Methods on Therapeutic Potential of *Mucuna pruriens* (L.) DC. and Chemical Profiling of Screened Extract with Chromatography-Mass Spectrometry Approach

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Abstract

For millennia, people have used natural herbal remedies to cure an extensive variety of illnesses. The present study evaluates the effects of different solvents and extraction methods on antioxidant and cytotoxic potentials of the medicinal plant *Mucuna pruriens* (L.) DC. and the screened extract underwent further chemical profiling. The total phenolic content of the six improved varieties of *M. pruriens* seeds was determined; the variety Arka Dhanvantri scored highly and was subsequently subjected to four different extraction methods using methanol (M) and water (W). The extraction methods adopted include methanol extraction with shaker (M-shaker), methanol extraction with soxhlet (M-soxhlet), water extraction with shaker (W-shaker) and water extraction through boiling (W-boiled). The water extract that was made with the shaker method (W-shaker) had the highest extract recovery and *in vitro* antioxidant activity (IC_{50}=5.9 µg/mL for DPPH scavenging ability, 1.884 M AAE/g extract for the phosphomolybdenum method, 1.73 M AAE/g extract for ferric reducing power, and 158.8 mg EDTA/g extract for ferrous chelating ability). The antioxidant-protective action of the W-shaker extract was further validated in HEK 293T cells using MTT and flow cytometry analyses after inducing oxidative stress in the cells with H_2O_2. It demonstrated a potential cytoprotective effect, peaking at 100 µg/mL concentration. W-shaker extract was also profound for its cytotoxic effect on MCF-7 cells that was dose- and time- dependent. The W-shaker extract comprised phytochemicals from the amino acid, carbohydrate, terpenoid, phenolics, vitamin and nucleoside categories, as per LC-QTOF-MS/MS analysis. Consequently, it is suggested that the water extraction of *M. pruriens* using the shaker extraction method is a viable option for a promising source of natural antioxidants and anticancer phytochemicals. This study further suggested that this extract may be a useful and affordable substitute for synthetic antioxidants. The profound *in vitro* cytotoxicity of water extract against breast cancer cells raises the possibility that its phytochemicals could be used to create a natural anticancer medication. This study is even more pertinent because screened water extract is both economical and environmentally beneficial.

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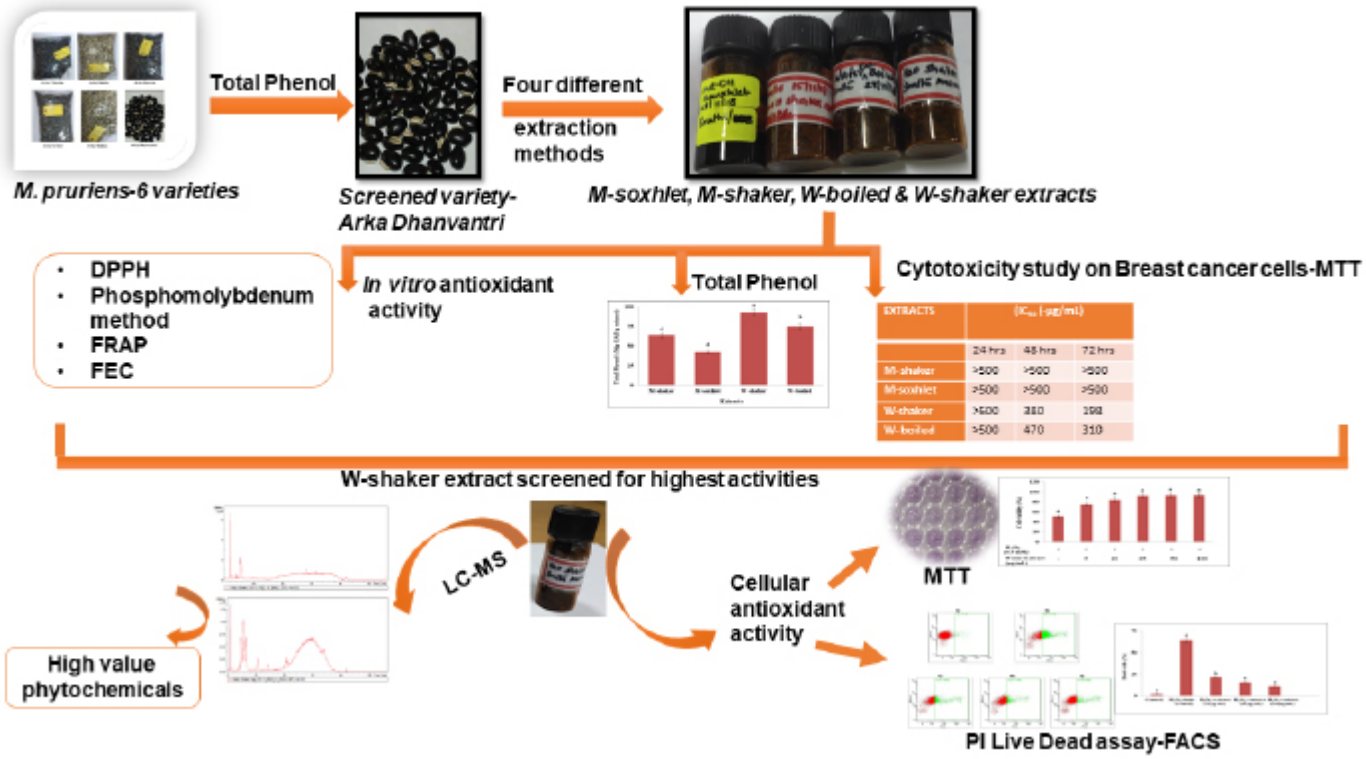
Keywords: *Mucuna pruriens* (L.) DC.; Extraction methods; High-value phytochemicals; Oxidative stress; LC-QTOF-MS/MS; Breast cancer.



Highlights

- Extraction methods and solvents influenced the antioxidant and cytotoxic efficacies of *Mucuna pruriens* (L.) DC.
- W-shaker extract was profound for its antioxidant activity and total phenol content
- W-shaker extract was the most effective against breast cancer cells at 72 h.
- W-shaker extract's chemical profiling affirmed the presence of different metabolites.
- Many of the identified compounds are being reported from the chosen crop for the first time.

Introduction

Phytochemicals, the chemical constituents of plants, are remarkably involved in the prevention and control of degenerative diseases [1]. Recent studies have highlighted the significance of plant secondary metabolites and their pharmacological applications. In order to collect bioactive compounds from plants, extraction is a necessary step. Solvents are important when extracting phytochemicals from various plant sources. Variations in phyto-constituents, antioxidant efficacy and other pharmacological activities are directly correlated with the nature of solvents and methods of extraction being exercised. Low polar solvents like hexane and chloroform are preferred to extract the lipophilic compounds and pigments, whereas mid-polar and highly polar solvents including ethyl acetate, methanol and water are used to extract compounds like polyphenols [2,3]. This emphasises the significance of selecting an appropriate solvent and method for extracting phytochemicals with maximum beneficial effects.

Considering the serious health issues, there is growing focus on using natural foods and bioactive chemicals on a global scale. The world prefers medicinal herbs since they have a variety of health benefits and are a source of various useful substances. *M. pruriens* (L.) DC. (velvet bean) is one of the most productive and multipurpose underused legumes, with both culinary and medicinal uses

[4]. It is a member of the Fabaceae family and grows worldwide in tropical and sub-tropical regions [4]. Many Asian and African nations, including Brazil, Malawi, Nigeria, Ghana, India, and the Philippines, eat it as food. The leaves and immature pods serve as vegetables in some parts of Asia, and the roasted seeds are also eaten [4]. The plant is a key component in Ayurveda medicine and has a wide range of medicinal potential, most notably anti-action Parkinson's due to L-DOPA [5]. Although this plant has been found to contain valuable phytochemicals like L-DOPA that have a significant impact on human health, the crop deserves much more attention with regard to its phyto-constituents and their contribution to its nutraceutical and pharmacological potentials.

Free radicals mainly reactive oxygen and nitrogen species (ROS and RNS) can intensify oxidative stress and cause cellular damage, which can eventually result in serious diseases like cancer [1]. The pharmaceutical industry currently focuses on finding natural antioxidants that can stop free radicals from being produced and spreading, as well as a treatment for cancer, one of the most dreaded diseases [6]. Considerable scope for improvement exists despite amazing advancements in the diagnosis, treatment, and management of cancer. Even though plenty of chemical compounds derived from plants have recently demonstrated potential as anticancer medications, a plethora of plants and its phyto-constituents have not yet been assessed for anticancer potential.

The current study attempts to evaluate the effectiveness of various extraction methods and solvents on the antioxidant and cytotoxic properties of *M. pruriens*, as well as the chemo-profiling of the potent extract using chromatography mass spectrometry.

Materials and Methods

Chemicals

The following items were purchased from Sigma-Aldrich in the USA: DMSO (dimethyl sulfoxide), DMEM (Dulbecco's modified Eagle's medium), MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide), BHA (butylated hydroxyanisole), DPPH (1,1-diphenyl-2-picryl-hydrazyl), PI (propidium iodide), and gallic acid. FBS, the foetal bovine serum, was acquired from Gibco in Brazil. We purchased methanol, ascorbic acid, hexane, hydrogen peroxide, and chloroform from Merk, India. Sisco Research Laboratories Pvt. Ltd. in India provided the Folin-Ciocalteu reagent, ammonium molybdate, potassium ferricyanide, trichloroacetic acid, ferric chloride, ferrozine, and ethylenediaminetetraacetic acid (EDTA).

Collection of study materials

The study employed matured and sun-dried seeds of six improved varieties of *M. pruriens* (L.) DC. var. utilis (Wall. ex Wight) Baker ex Burck. Arka Aswini, Arka Daksha, Arka Shukla, Arka Charaka, Arka Shubra and Arka Dhanvantri were the six improved varieties used (Figure 1). The seeds were obtained from ICAR- Indian Institute of Horticultural Research (IIHR), Bengaluru, India. The improved varieties used in this study are those developed for commercial cultivation by ICAR-IIHR, Bengaluru, India, through selection and recombination breeding for high seed yield and high L-DOPA content.

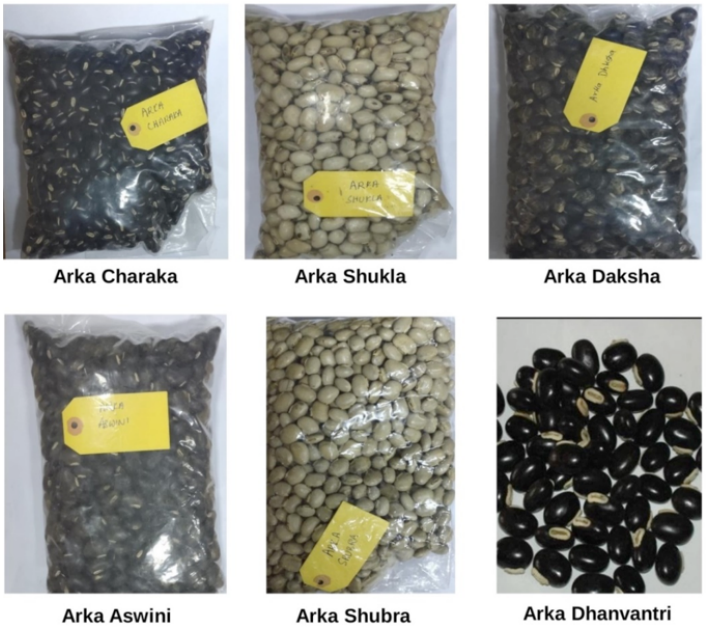


Figure 1: Improved varieties of *M. pruriens* used for the study. Six improved varieties viz. Arka Charaka, Arka Shukla, Arka Daksha, Arka Aswini, Arka Shubra and Arka Dhanvantri were used.

Upkeep of cell lines

For the study, human cell lines HEK 293T (human embryonic kidney cells) and MCF-7 (breast cancer cells) were used. These cells were grown at 37°C in a humidified 5% CO_2 environment in 90% DMEM supplemented with 10% FBS and antibiotics to form the adherent monolayers.

Estimation of total phenol from *M. pruriens*

As phenolic content is considered as the most important contributor for antioxidant activity, the selected *M. pruriens* improved varieties were screened for further studies based on their total phenol content. The phenolic contents were extracted from *M. pruriens* seeds with 80% ethanol and estimated by the Folin-Ciocalteu method [7]. In short, the phenolic contents in ethanolic extract were allowed to react with 20% Na_2CO_3 and Folin-Ciocalteu reagent before being boiled for one minute. Using a UV-VIS spectrophotometer (BioSpectrometer, Eppendorf, India), the absorbance of the resulting molybdenum blue was measured at 650 nm. The calibration curve was prepared using gallic acid

(20-100 µg/mL) as the standard, and the results were displayed as gram gallic acid equivalents /100 gram of tissue (%).

Preparation of extracts

Four alternative methods of extraction were used on the seeds of the *M. pruriens* improved variety that had the highest total phenol content.

Methanol extraction with shaker: The crushed sample (100 g) was dissolved in 200 mL methanol and kept in a shaker at 30°C for 30 min. The supernatant was collected and repeated the process until the residue became colorless (Total time taken for complete extraction-10h; 20 cycles). The supernatant was pooled, filtered and concentrated using an IKA rotary evaporator. The dried extract was labeled as 'M-shaker' and after tracking the extract recovery (the quantity of extract obtained after extraction and complete solvent evaporation), it was kept at 4°C. The extraction was performed using the modified procedure of Saranya et al. [8].

Methanol extraction with Soxhlet apparatus: The crushed sample (100 g) was dissolved in 300 mL methanol and subjected to soxhlet extraction until it became colorless (Total time taken for complete extraction -30h). The extract was filtered and concentrated with an IKA rotary evaporator. The concentrated extract was labeled as 'M-soxhlet' and after noting the extract recovery, it was preserved at 4°C [9].

Water extraction with shaker: The crushed sample (100 g) was dissolved in 200 mL distilled water and kept in a shaker at 80°Cfor 15 min. The supernatant was collected and repeated the process until the residue became colorless (Total time taken for complete extraction -6h; 24 cycles). The pooled supernatant was further centrifuged and concentrated with an IKA rotary evaporator. The dried extract was labeled as 'W-shaker' and kept at 4°C after noting the extract recovery. The extraction was performed using the modified procedure of Saranya et al. [8].

Water extraction through boiling: The crushed sample (100 g) was added to an RB flask containing 200 mL distilled water and boiled for 15 min. The supernatant was filtered and repeated the process until the residue became colorless (Total time taken for complete extraction -5h; 20 cycles). The extract was centrifuged and the supernatant was evaporated to dryness using an IKA rotary evaporator. The concentrated extract was labeled as 'W-boiled' and preserved at 4°C after the extract recovery was recorded [10].

Assessment of antioxidant activity *in vitro*

Using the DPPH free radical scavenging activity, phosphomolybdenum method, ferric reducing power method, and ferrous chelating ability, the extracts' *in vitro* antioxidant activity was evaluated.

DPPH (1, 1-Diphenyl -2-picryl hydrazyl) free radical scavenging activity: DPPH free radicals were used to test the extracts' capacity to donate hydrogen or scavenge radicals [11]. To summarize, 1.0 mL of extract (concentration range: 1.0-500 µg/mL) was allowed to react with 1.0 mL of 0.004% DPPH in methanol and incubated in the dark for half an hour. Using a UV-VIS spectrophotometer (BioSpectrometer, Eppendorf, India), the absorbance was measured at 517 nm. As a positive control, synthetic antioxidant BHA was used. The extracts' capacity to scavenge 50% of the DPPH free radicals was measured and expressed as IC_{50} value, and its effectiveness was compared to that of synthetic antioxidant BHA.

Phosphomolybdenum method to measure the total antioxidant capacity: The extract underwent incubation for 90 min at 95°C with phosphomolybdenum reagent (28 mM disodium hydrogen phosphate, 0.6 M sulphuric acid, 4.0 mM ammonium molybdate). Further, the absorbances (at 695 nm) were recorded with UV-Vis spectrophotometer (BioSpectrometer, Eppendorf, India) and the results were presented as molar ascorbic acid equivalents per gram of extract (M AAE/ g of extract) [12]. Synthetic antioxidant BHA served as the method's positive control.

The FRP (Ferric reducing power) method: Using the ferric reducing power method, the extracts' reductive capacity was determined [13]. The extract was incubated at 50°C for 30 min after being allowed to react with 0.2 M phosphate buffer at pH 6.6 and then with 1.0% potassium ferricyanide. Trichloroacetic acid (10%) was added to stop the reaction, and the resulting mixture was centrifuged for 20 min at 3000 rpm. A UV-Vis spectrophotometer (BioSpectrometer, Eppendorf, India) was used to measure the absorbance at 700 nm after the supernatant was mixed with distilled water and 0.1% $FeCl_3$. As a positive control, synthetic antioxidant BHA was maintained. Molar ascorbic acid equivalents per gram of extract (M AAE/g of extract) were used to express the result.

Finding the ferrous chelating capacity: This test, which gauged the intensity of the ferrous-ferrozine complex, was used to assess the extracts' capacity to chelate ferrous ions. In summary, the extract was left to react with 2.0 mM $FeCl_2$. After 5.0 min incubation, 5.0 mM ferrozine was added. Using a UV-VIS spectrophotometer (BioSpectrometer, Eppendorf, India), the absorbance was measured at 562 nm after 10 min of room temperature incubation. The milligram of EDTA per gram of extract (mg EDTA/g extract), was used to express the ferrous chelating ability [14].

Determining the extracts' total phenol content

The extracts' total phenolic content was determined using the Folin-Ciocalteu method, which is already covered in previous section. Gallic acid equivalents in milligrams per gram of extract (mg GAE/g extract) were used to express the results.

Assessment of screened extract's cellular antioxidant activity

The extract exhibiting the highest *in vitro* antioxidant activity was subjected to cellular antioxidant efficacy studies on the HEK 293T cells after oxidative stress was induced using H_2O_2. The parameters checked in this section include the extract's cytotoxicity assessment on normal HEK 293T cells, the optimization of H_2O_2 dose, and the extract's impact on HEK 293T cells' oxidative stress caused by H_2O_2.

Assessment of the screened extract's cytotoxicity on normal HEK 293T cells: The HEK 293T cell monolayer culture in a 96-well culture plate was treated to varying concentrations of the screened extract, and incubated at 37°C with 5% CO_2. The MTT assay was used to assess the cell viability after 24 h. In a nutshell, the cells (5×10^3cells/well) were seeded in 96-well micro titer plates and kept overnight at 37°C in an incubator with 5% CO_2 humidity (NuAire, USA). The screened extract was added to the cell at varying concentrations (5.0 - 100 µg/mL), and it was incubated for 24 h. After allowing the cells to react with MTT (5 mg/mL in PBS) for an additional 2.0 h, the formazan crystals produced were dissolved in DMSO. Using a plate reader (Infinite M200 PRO, Tecan, Germany), the absorbance of coloured formazan crystals was measured at 595 nm. By comparing the viability of the treated cells with that of the control, the inhibitory effect in percentage was computed [15].

The optimization of H_2O_2 dose: A 96-well culture plate containing a monolayer culture of HEK 293T cells was incubated for 24 h at 37°C and 5% CO_2 with various concentrations of H_2O_2 (0.1–1.0 mM). The MTT assay was used to assess the cell viability, as previously mentioned. For further studies on oxidative stress, the H_2O_2 dose needed to induce around 50% cell inhibition was taken [15].

The impact of screened extract on oxidative stress caused by H_2O_2 in HEK 293T cells: Oxidative stress was induced by adding the previously optimized H_2O_2 dose to the HEK 293T cell monolayer cultured in a 96-well plate. The screened extract was added to the wells at varying concentrations (5.0 - 100 µg/mL) after 1.0 h, and the mixture was incubated for an additional 24 h at 37°C and 5% CO_2 [15]. The effectiveness of the extract on H_2O_2-induced cellular antioxidant activity on HEK 293T cells was then assessed by subjecting the treated cells to the MTT and PI Live Dead assay.

MTT: In order to comprehend the safeguarding impact of the screened extract against H_2O_2-induced oxidative stress in HEK 293T cells, the cells' monolayer culture was subjected to varying concentrations (5.0 - 100 µg/mL) of the screened extract in the presence of H_2O_2. The most protective dosage was determined using the MTT assay, as previously mentioned.

Flow cytometry assay for Live Dead Cells Employing PI Staining: By assessing the amount of cell death, the screened extract's protective effect against H_2O_2-induced oxidative stress in HEK 293T cells was further observed. Propidium iodide staining the cells allowed for this to be accomplished [16]. In summary, the cells (5×10^4 cells/mL) were seeded in a 24-well culture plate and exposed to an optimal dose of H_2O_2 to induce oxidative stress. The cells were exposed to different concentrations of extract (5.0 - 100 µg/mL) after 1.0 h and allowed to incubate for 24 h. The cells were trypsinized, washed with PBS, and then allowed to react with PI (1.0 µg/mL). Then, a flow cytometer (CytoFLEX S, Beckman Coulter) was used to measure the live and dead populations. CytExpert 2.0 software was used to analyse the collected data.

Evaluation of extracts' *in vitro* cytotoxicity on breast cancer cells

Using the MTT assay, which was explained in the previous section, the cytotoxic impact of four distinct extracts was evaluated on the breast cancer cells (MCF-7) for 24, 48, and 72 h at varying concentrations (5-500 µg/mL).

Chemical profiling of screened extract with LC-QTOF-MS/MS

To find potential metabolites that may have contributed to the extract's high activities, LC-QTOF-MS/MS analysis was performed on the sample that was screened for the highest levels of antioxidant activity and cytotoxicity. The sample was dissolved in HPLC grade water (10 mg/mL) and subjected to analysis using Thermo Fisher Scientific Ultimate 3000 UHPLC system in conjunction with Brucker's impact HD Q-TOF MS/MS (Quadrupole Time-of-Flight-mass spectrometer) equipped with an ESI ion source. LC specifications: Thermo Scientific Syncronis C18 column,150 mm x 4.6 mm, with a particle size of 5.0 µm; Mobile system A- water containing 0.1% acetic acid and B- acetonitrile containing 0.1% formic acid. Gradient programme: B at 11% (0 - 5.0 min), 21% (5.0 - 8.0 min), 33% (8.0–15 min), 70% (15–19 min), 82% (19–35 min), 100% (35–40 min), and 11% (40–56 min). At 280 nm, a UV-visible detector was employed to develop the chromatogram. Fragment voltage: 50 V; capillary voltage: 4.5 KV; dry gas: high purity N_2 (12.0 L/min); nebulizer pressure: 3.0 bar; dry temperature: 180°C were the source parameters. In both positive and negative modes, MS/MS was executed. Mass scan range: 20–1500 m/z. Compass data analysis software was used to analyse the MS and MS/MS data. Using the KEGG database and Metfrag software, the compounds were identified.

Statistical analysis

ANOVA was carried out using MSTATC software (version 1.41). Using the 'RANGE' procedure of MSTATC, the DMRT

(Duncan's multiple range) test was used to check for significant differences ($p \leq 0.05$). According to DMRT, the different letters indicate the significant difference ($p \leq 0.05$). The means of the three replicates were used to express the values. The correlation coefficient (r), which is used to explain the relationship between total phenol and antioxidant activity of extracts, was measured using the MSTATC "CORR" procedure.

Results and Discussion

Total phenol content from *M. pruriens* varieties

The total phenol content of Arka Dhanvantri was found to be very high (3.02%), statistically significant, and formed a separate cluster, whereas Arka Daksha showed the lowest total phenol content (1.46%). Total phenol content estimated from other samples was amidst these two varieties (Figure 2). Based on this result, Arka Dhanvantri was chosen for further investigations. Variability in total phenol content from *M. pruriens* seeds was reported by researchers. Siddhuraju and Becker [17] reported 5.65g% total phenol in *M. pruriens* seed.

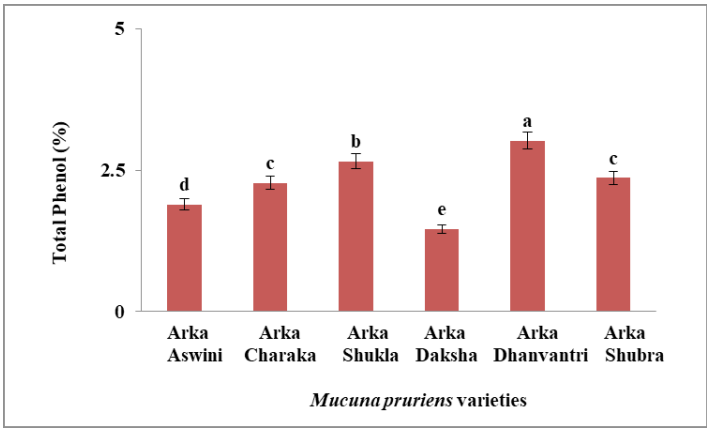


Figure 2: Total phenol content of improved varieties of *M. pruriens* selected for the study. Arka Dhanvantri showed highest total phenol content; the values are the averages of three separate experiments; significant statistical difference is shown by different letters at $p \leq 0.05$.

Extract recovery

The *M. pruriens* extracts were evaporated to dryness using a rotary evaporator and the recovery of solvent-free extracts was noted. It was demonstrated that the recovery of W-shaker extract was high (29.3%), followed by W- boiled extract whereas M-shaker and M-soxhlet recovery were found to be low. The details of extract recovery are displayed in Figure 3. The difference in extract yield might be attributed to differences in extraction methods, the polarity of different solvents used, and the varying extractability of solvents for different classes of phytochemicals [18].

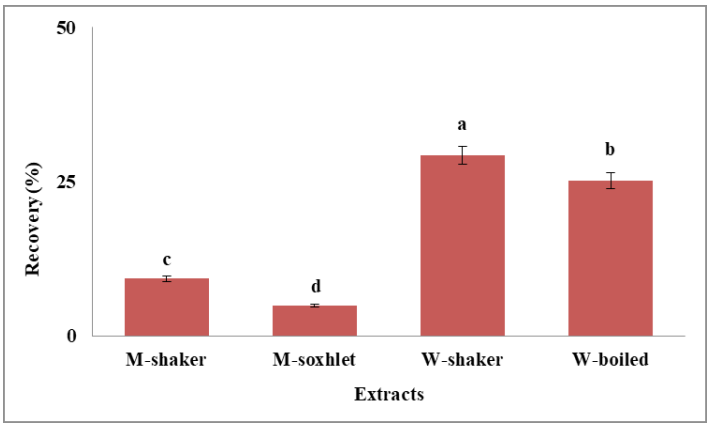


Figure 3: Recovery of different extracts of *M. pruriens* (%). W-shaker extract was profound for the extract recovery; The values are the means of three separate experiments; Significant statistical difference is shown by different letters at $p \leq 0.05$; M-shaker- Methanol extraction with shaker; M-soxhlet- Methanol extraction with soxhlet; W-shaker- Water extraction with shaker; W-boiled- Water extraction through boiling.

Antioxidant activity *in vitro* of *M. pruriens* extracts

The root cause of approximately 200 human diseases is oxidative stress, which is caused by free radicals. Antioxidants can protect against oxidative stress and delay the onset of many chronic diseases. Plants are one of the most plentiful natural sources for active antioxidants [6]. Although the antioxidant ability of various *M. pruriens* extracts has been reported, the effect of solvents and extraction methods on its antioxidant potential, as well as the contribution of phytochemicals to its pharmacological values, have received little attention. The current section focused on these aspects to assess the potency of extracts obtained through different methods for scavenging strongly reactive free radicals using various mechanisms.

DPPH free radical scavenging activity exploits the antioxidant compounds' proton donating ability. Table 1 displays the inhibitory concentration (IC_{50} in µg/mL) of each extract needed to scavenge 50% of DPPH free radicals. The extracts' capacities to scavenge DPPH free radicals varied greatly from one another. Though all of the extracts demonstrated remarkable ability to scavenge DPPH, water extract obtained using the shaker (W-shaker) had a significantly lower IC_{50} value (5.9 µg/mL) and was statistically comparable to W-boiled. This low IC_{50} value indicates that W-shaker extract has a high potential of hydrogen/

electron donating ability to stabilize the free radicals, with an effect comparable to that of BHA-the synthetic antioxidant (IC_{50} - 2.7 µg/mL). Proton donor groups like SO_3H and -COOH may be accountable for the W-shaker extract's capacity to scavenge DPPH. Additionally, various studies have documented the ability of various *M. pruriens* extracts to scavenge DPPH free radicals [19,20].

To ascertain the molybdenum reduction ability/total antioxidant capacity, the phosphomolybdenum assay was employed. The sample's capacity to convert Mo (VI) into Mo (V) is the basis for the assay, and the ensuing shift in absorbance was determined. According to the data (Table 1), water extract obtained from shaker (W-shaker) had a very high total antioxidant activity (1.884 M AAE/g extract) when compared to the other three extracts. M-shaker and W-boiled extracts demonstrated moderate activity (0.774 and 0.863 M AAE/g extract, respectively) and were statistically on par, whereas M-soxhlet extract showed the lowest activity (0.244 M AAE/g extract). Previous reports have also shown that *M. pruriens* can reduce molybdenum [21].

The ability of antioxidants to reduce ferric (III) ion to ferrous (II) ion was also measured by the ferric reducing power method. A compound's reducing power, which is connected to its electron-transfer capacity, is a crucial measure of its antioxidant activity. Table 1 presents a noteworthy variation in their ferric reducing capacity based on extract. The activity ranged from 0.875 to 1.73 M AAE/g extract. W-shaker extract had the highest activity (1.73 M AAE/g extract), while M-soxhlet extract had the lowest. Researchers have also reported the ferric-reducing ability of various *M. pruriens* extracts [22].

The potential of extracts to chelate ferrous ions was also assessed, and it ranged from 147.7 to 158.8 mg EDTA/g extract (Table 1). W-shaker extract showed a high ability to chelate ferrous ions (158.8 mg EDTA/g extract), whereas M-soxhlet extract showed a lower ferrous chelating ability. M-shaker and W-boiled extracts also demonstrated moderate activity. Secondary metabolites usually lessen the pro-oxidant activity of metal ions by chelating them [1]. Dhanasekaran [19] reported that *M. pruriens* aqueous seed extract exhibited dose-dependent metal chelating ability.

In conclusion, it was discovered that the W-shaker extract was highly successful in scavenging DPPH free radicals. The W-shaker extract further exhibited noteworthy ferrous chelating ability, reducing power, and total antioxidant activity. Its other biological effects, like anticancer activity, may be greatly influenced by this antioxidant capacity. Hence, the W-shaker extract was screened for further studies.

Extract	Antioxidant activity			
	DPPH scavenging capacity (IC_{50} -µg/mL)	Total antioxidant activity (Phosphomolybdenum Method; M AAE/g extract)	Ferric Reducing Power (M AAE/g extract)	Ferrous chelation ability (Mg EDTA/g extract)
M-shaker	16.75^{b}	0.774^{b}	1.417^{b}	152.6^{b}
M- soxhlet	20.5^{a}	0.244^{c}	0.875^{c}	147.7^{c}
W-shaker	5.9^{c}	1.884^{a}	1.730^{a}	158.8^{a}
W- boiled	9.0^{c}	0.863^{b}	1.299^{b}	150.6^{bc}
BHA	2.7	4.6	3.5	-

M AAE – Molar Ascorbic acid equivalents.

Mg EDTA– Milligram EDTA equivalents

M-shaker- Methanol extraction with shaker; M-soxhlet- Methanol extraction with soxhlet; W-shaker- Water extraction with shaker; W-boiled- Water extraction through boiling.

Values are means of three replicates (n=3).

Significant statistical difference is shown by the superscript letters at $p \leq 0.05$.

Table 1: Antioxidant activity *in vitro* of *M. pruriens* extracts.

Total phenol content of *M. pruriens* extracts

Plant phenolics, the electron rich molecules, are various derivatives of hydroxybenzene and are one of the major contributors to the medicinal utility mainly antioxidant activity of plants [23]. Phenolics exhibit antioxidant activity through a variety of mechanisms. Because of this diverse mechanism of antioxidant activity, phenolics have emerged as an intriguing class of compounds for researchers interested in natural health beneficial phytochemicals [1]. Total phenol estimation from selected extracts is thus critical. The total phenol content of four *M. pruriens* extracts varied significantly, ranging from 42 to 92.96 Mg GAE/g extract (Figure 4). W-shaker extract had the highest amount of total phenol, followed by W-boiled extract, and M-soxhlet extract had the lowest total phenol content. Kavitha [20] reported the total phenolic content of *M. pruriens* ethanol extract as 46.442±0.353 mg gallic acid equivalent/g.

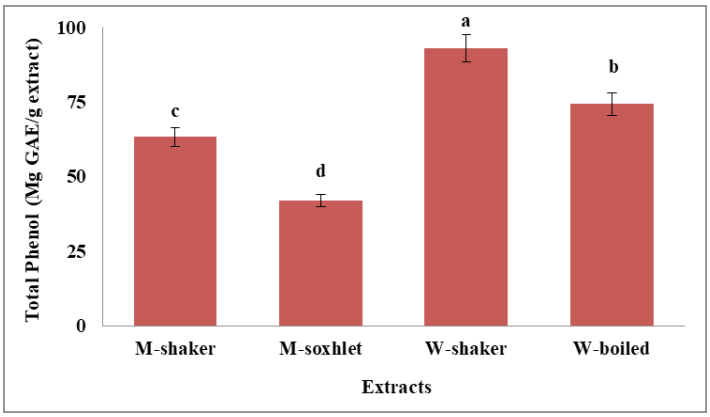


Figure 4: Total phenol content of different extracts of *Mucuna pruriens*. W-shaker extract had the highest amount of total phenol; Mg GAE – Milligram Gallic acid equivalents; M-shaker- Methanol extraction with shaker; M-soxhlet- Methanol extraction with soxhlet; W-shaker- Water extraction with shaker; W-boiled- Water extraction through boiling; The values are the averages of three independent experiments; Significant statistical difference is shown by different letters at $p \leq 0.05$.

Impact of total phenolic content on antioxidant activity through correlation analysis

Researchers investigated the relationship between plant antioxidant potential and total phenolic content [1]. The antioxidant activity of the extracts and their overall phenolic content were correlated in this study. Total phenolic content and the IC_{50} value for each extract to scavenge DPPH free radicals have a high negative correlation (r = -0.96), which suggests that total phenol is a contributing factor to the extracts' high radical scavenging ability. Additionally, these extracts' total phenolic content had correlation coefficients (r) of +0.96, +0.94, and +0.90 with their antioxidant activity as determined by the phosphomolybdenum method, ferrous chelating activity, and FRP, respectively. This positive correlation indicated that an extract's antioxidant activity increased with its total phenol content.

Screened extract's antioxidant capacity to attenuate oxidative stress in HEK 293T cells

A compound's *in vitro* antioxidant activity can be correlated to its cellular antioxidant effects. The current section aims to establish the potential of the W-shaker extract to prevent oxidative stress within the cells, as it dominated the *in vitro* antioxidant section of the present study. The cellular antioxidant activity of the W-shaker extract has been investigated in normal HEK 293T cells, which are immortalized human embryonic kidney cells. The oxidative stress status they display is more realistic because their metabolic conditions are closer to those of normal human cells [24]. One free radical chain reaction propagator that enters cells easily and damages different types of cells through oxidative damage is hydrogen peroxide. So it is commonly employed to trigger oxidative stress in investigations of the antioxidant activity of cells [25]. In the current investigation also, HEK 293T cells were exposed to H_2O_2 to cause oxidative damage.

Initially, the toxic impact of various concentrations (5-100 µg/mL) of W-shaker extract on HEK 293T cells was assessed with MTT assay. It is considered safe to proceed with the taken concentrations since the cells' viability was greater than 90% at all concentrations (93.51 % even at the highest concentration-100 µg/mL). (Figure 5).

Further, the H_2O_2 dose was optimized in order to establish a model of oxidative injury induced by hydrogen peroxide. It came to light that cell viability was decreased in a dose-dependent manner by H_2O_2 concentrations ranging from 0.1 to 1.0 mM (Figure 6). At 0.4 mM, the growth of cells was 50% (50.12%) inhibited by H_2O_2. Therefore, in the subsequent experiments, the ideal injury concentration of 0.4 mM H_2O_2 was selected in order to evaluate the protective effect of screened W-shaker extract against oxidative stress. Our H_2O_2 dose optimisation is supported by Liu et al.'s [24] report of a 45% reduction in HEK 293T cell viability when 0.4 mM H_2O_2 was used.

Finally, the protective impact of W-shaker extract towards H_2O_2-induced oxidative damage in HEK 293T cells was evaluated using MTT and PI live dead cell assay. Treatment with varying concentrations of W-shaker extract mitigated the 0.4 mM H_2O_2-induced reduction in the percentage of viable cells in the MTT assay (Figure 7). The extract at 50 µg/mL significantly boosted HEK 293T cell viability to 92.64%, and its protective effect is statistically similar to that of concentrations of 25 and 100 µg/mL. These three concentrations were therefore picked for additional cytotoxicity study using flow cytometry (PI live dead cell assay).

Using a PI live dead cell assay and FACS, the mechanistic aspects of cytotoxicity as well as the protective effect of the W-shaker extract on H_2O_2-induced oxidative damage in HEK 293T cells were further investigated. Propidium iodide is a nucleic acid stain that only becomes internalized in a cell when the membrane is broken [16]. To identify the live and dead populations, the H_2O_2-induced HEK 293T cells underwent treatment with the indicated concentrations (25, 50, and 100 µg/mL), screened based on the MTT result, stained with PI, and then subjected to flow cytometry analysis (Figure 8). The percentage of dead cells in H_2O_2 treated cells was observed to be higher (63.97%) as a result of oxidative stress. The percentage of dead cells decreased to 21.55, 15.73, and 10.8%, respectively, when W-shaker extract was added to these cells at concentrations of 25, 50, and 100 µg/mL (Figure 9). This result showed that W-shaker extract had a concentration-dependent protective effect on oxidative stress-induced HEK 293T cells, with a high level of protection at 100 µg/mL. In our recent publication [9], we have observed a similar effect for sequentially extracted water extract from the seeds of *M. pruriens* to attenuate oxidative stress and alleviate cell death in HEK 293T cells.

The findings of this section demonstrate that W-shaker extract significantly decreased cell death and successfully maintained cell viability during oxidative stress. The inhibition of oxidative stress and improved viability in HEK 293T cells may be due to the activation of endogenous antioxidant enzymes and the glutathione system, as well as the active ingredients of this extract's capacity to reduce intracellular ROS.

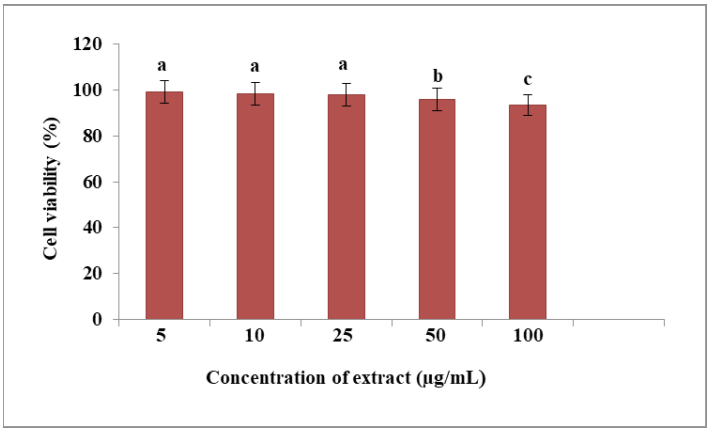


Figure 5: Assessment of the screened W-shaker extract's cytotoxicity on normal HEK 293T cells. The tested extract concentrations were non-toxic to the cells as the cell viability was > 90% at all the concentrations; Values are expressed as mean of three replicates; significant statistical difference is indicated by different letters at $p \leq 0.05$.

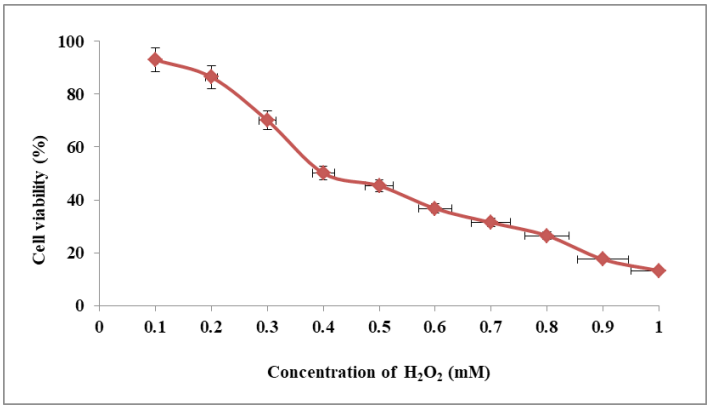


Figure 6: The optimization of H_2O_2 dose. In subsequent experiments, the ideal injury concentration of 0.4 mM H_2O_2 was determined because it produced a 50% inhibition of cell growth. The mean of three replicates is used to express values.

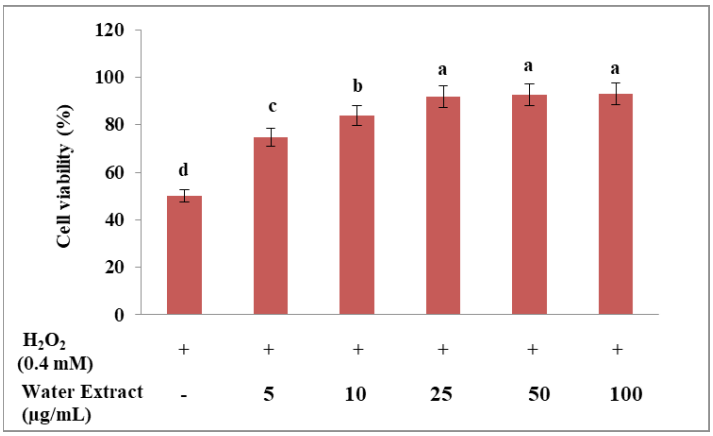


Figure 7: The impact of screened extract on oxidative stress caused by H_2O_2 in HEK 293T cells-MTT Assay. The viability of HEK 293T cells treated with H_2O_2 was significantly enhanced by extract at concentrations of 25, 50, and 100 µg/mL. The different letters indicate a significant statistical difference at $p \leq 0.05$. The values are the averages of three replicates.

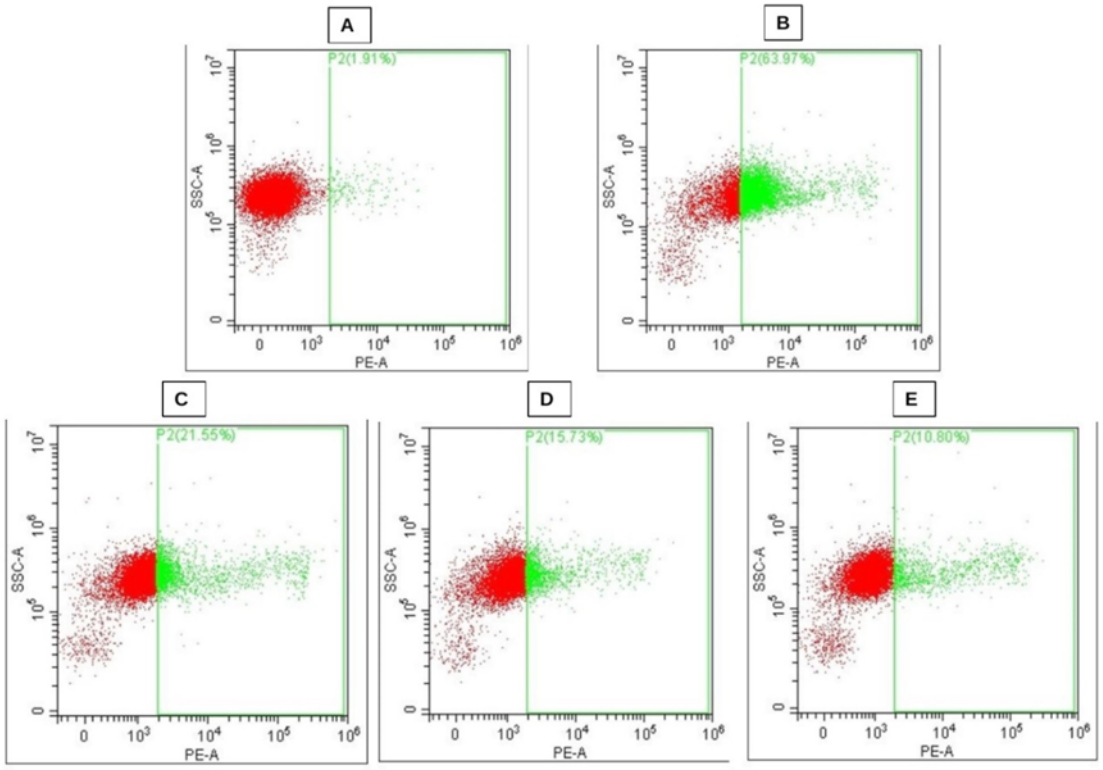


Figure 8: Flow cytometry analysis (PI live dead assay) to establish the protective impact of W-shaker extract on oxidative stress in HEK 293T cells induced by H_2O_2. (A) Control, (B) H_2O_2-0.4mM alone, (C) H_2O_2+extract-25 µg/mL, (D) H_2O_2+extract-50 µg/mL, (E) H_2O_2+extract-100µg/mL. The extract's ability to protect against oxidative stress was demonstrated by the dose-dependent decrease in cell death.

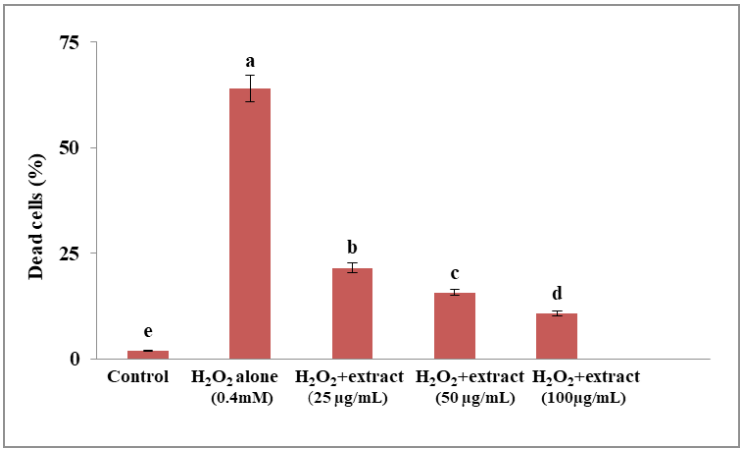


Figure 9: W-shaker extract's protective effect on H_2O_2-induced oxidative stress in HEK 293T cells as determined by the PI Live Dead Cell Assay. The bar diagram displays the percentage of dead cells in each treatment and shows that W-shaker extract has a concentration-dependent protective effect on oxidative stress-induced HEK 293T cells, with the highest effect observed at 100 µg/mL extract concentration; The values are the averages of three replicates; Significant statistical difference is shown by different letters at $p \leq 0.05$.

M. pruriens extracts' cytotoxic potential on breast cancer cells

An extract's or compound's potential antioxidant activity can be connected to its bioactivities, such as anticancer potential. There are numerous findings on the connection between bioactive compounds' antioxidant and anticancer properties [26]. Antioxidants may inhibit the proliferation of cancer as they are exceptionally good at scavenging free radicals, which are one of the main causes of carcinogenesis [6]. Hence, the current section evaluated the cytotoxicity of the four *M. pruriens* extracts on MCF-7 cells with the MTT assay. The MTT assay was conducted on extracts varying in concentration from 5 to 500 µg/mL over three time intervals (24, 48, and 72 h), and the IC_{50} was determined (Table 2). The IC_{50} value for all four extracts was greater than 500 µg /mL for 24 h, indicating moderate cytotoxicity. When the time of exposure increased to 48 and 72 h, the water extracts (W-shaker and W-boiled) showed an improved cytotoxicity. Among these, W-shaker extract was found to be more efficient, with an IC_{50} of 380 and 198 µg/mL for 48 and 72 h, respectively. This suggested that the cytotoxic effect of *M. pruriens* water extract increased in a time- and dose-dependent manner. The slow-releasing cytotoxic phytomolecules in water extract might be the responsible factor for the increase in cytotoxic effect observed with longer exposure times. Sruthi et al. [9] revealed the effectiveness of *M. pruriens* seeds' sequentially extracted water extract on MCF-7 cells and similar trend was observed. The anticancer efficacy of *M. pruriens* seed (crude methanolic extract) towards MCF-7 cells was also reported by Sinha et al. [27].

Extract	Cytotoxic potential in IC_{50}-µg/mL		
	24 h	48 h	72 h
M- shaker	>500	>500	>500
M-soxhlet	>500	>500	>500
W-shaker	>500	380	198
W- boiled	>500	470	310
M-shaker: Methanol extraction with shaker; M-soxhlet: Methanol extraction with soxhlet; W-shaker: Water extraction with shaker; W-boiled: Water extraction through boiling. Values are the averages of three replicates (n=3).			

Table 2: *M. pruriens* extracts' cytotoxic potential on breast cancer cells.

Metabolite profiling of screened W-shaker extract with LC-QTOF-MS/MS analysis

Metabolic profiling is essential for identifying the bioactive compounds that contribute to the high activities of W-shaker extract, and LC-MS is a well-established tool for metabolite identification. Hence, LC-QTOF-MS/MS analysis was performed for the W-shaker extract screened for remarkable antioxidant activity and *in vitro c*ytotoxicity and the possible compounds were identified (Figure 10). The Mass-based search and MS/MS spectra of each peak analysed using Metfrag revealed the presence of thirteen compounds from W-shaker extract, including amino acid and its related compounds, carbohydrate, alkaloid, terpenoid, flavonoid, hydroxycinnamic acid, vitamin and nucleoside categories (Table 3). The amino acids identified from W-shaker extract were L-arginine, phenylalanine, isoleucine, and aspartic acid, which had previously been reported from *M. pruriens* [5]. Various researchers investigated the antioxidant activities of these amino acids [28, 29]. Amino acids are often considered as synergistic antioxidants because they enhance the antioxidant effect of primary antioxidants mainly by chelation of pro-oxidative metal ions and regeneration of oxidised primary antioxidants [30]. Thus, the identified amino acids may have had a synergistic effect on the extremely high antioxidant efficacy of W-shaker extract. The oligosaccharide raffinose and the amino acid compound L-DOPA from W-shaker extract have previously been reported from *M. pruriens* [5]. Researchers discovered that raffinose, in conjunction with ROS-scavenging enzymes and non-enzymatic antioxidants, can act as an antioxidant [31]. L-DOPA is the primary chemical component from *M. pruriens* and is mainly used to treat Parkinson's disease; it has antioxidant property also [8]. Deguelin is a natural rotenoid of the flavonoid family with antioxidant, anticancer, anti-inflammatory, antidiabetic and antimicrobial properties [32]. Riboflavin, the B vitamin, has previously been reported from *M. Pruriens* [5]. The nucleoside guanosine identified from *Mucuna* has previously been reported as a natural antioxidant, with the ability to prevent oxidative

damage to DNA, reduce ROS production, and protect mice from gamma-radiation-induced death [33]. Hederagenin 3-O-arabinoside is a triterpenoid saponin with anticancer efficacy [34] and Apigenin 7-O-[beta-D-apiosyl-(1->2)-beta-D-glucoside] is a flavonoid glycoside. Flavonoid glycosides have been shown in studies to prevent cancer, atherosclerosis, and chronic inflammation in humans by slowing oxidative degradation [35]. Caffeic acid 3-glucoside is a hydroxycinnamic acid derivative. Nwaji et al. [36] reported caffeic acid from *M. pruriens* and Gulcin [37] demonstrated its antioxidant activity. To the best of our knowledge, deguelin, hederagenin 3-O-arabinoside, apigenin 7-O-[beta-D-apiosyl-(1->2)-beta-D-glucoside] and caffeic acid 3-glucoside were reported for the first time from the plant studied. We have already reported the compounds L-DOPA, proline, raffinose, trigonelline , 2-Hydroxy-3-(4-hydroxyphenyl) propenoate, umbelliferone, 3,4-Dihydroxybenzaldehyde, parasorbic acid, beta-maltose, aspidodasycarpine, histidine, and coniferyl acetate from the sequentially extracted water extract of *M. pruriens* seed by LC-QTOF-MS/MS [9]. These highly valued constituents identified from W-shaker extract using LC-MS alone or in combination are thought to contribute to its remarkably high antioxidant activity and cytotoxicity on breast cancer cells. Unknown metabolites were also found in this extract, which may have contributed to its high activity. As a result, bioassay guided isolation, purification, and identification, followed by experimental validation, are required to identify the molecule/s exhibiting excellent antioxidant and cytotoxic activity in order to develop a natural product drug.

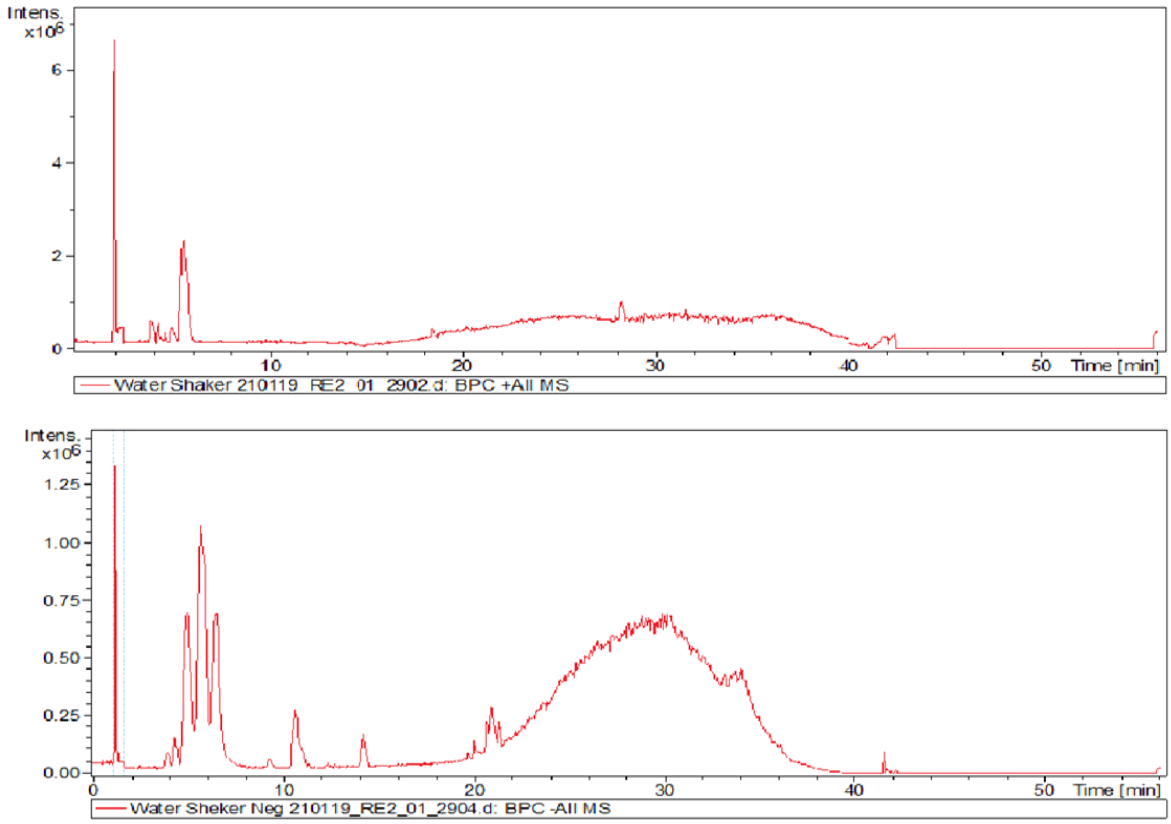


Figure 10: Base peak chromatogram for metabolite profiling of screened W-shaker extract by LC-QTOF-MS/MS at Positive mode and Negative mode.

S. No.	Retention time (RT)	Phytochemicals Identified	Detected adducts (Da)	Monoisotopic mass (Da)
	Positive mode			
1	4.2	Arginine	175.122	174.112

2	4.8	Raffinose	505.183	504.169
3	5.5	L-Dopa (3,4-Dihydroxy-L-phenylalanine)	198.08	197.069
4	5.6	Deguelin	395.15	394.142
5	6.7	Guanosine	284.103	283.092
6	6.8	L-Isoleucine	132.104	131.095
7	7.4	Riboflavin	377.139	376.138
8	8.6	Phenylalanine	166.089	165.079
9	12.6	D-Aspartate	134.047	133.038
10	13.2	Apigenin 7-O-[beta-D-apiosyl-(1->2)-beta-D-glucoside]	565.159	564.148
11	24.8	Hederagenin 3-O-arabinoside	605.413	604.398
	Negative mode			
12	4.8	Caffeic acid 3-glucoside	341.082	342.095
13	12	Scandoside methyl ester	403.129	404.132

Table 3: The phytochemicals identified from the screened W-shaker extract by LC-QTOF-MS/MS.

Conclusion

The present work compared the antioxidant efficacy of extracts obtained through different solvents and extraction methods. The study demonstrated that using water, an environmentally sustainable solvent, with an easy extraction method of shaking, it is possible to prepare effective extract from *M. pruriens*, which can be used as natural antioxidants and can replace synthetic ones in many food and cosmetic applications. The W-shaker extract/its metabolites can also be considered for further mechanistic studies on breast cancer prevention and its efficacy in developing as a natural anticancer drug. Even though the current study chemically profiled the active phytochemicals of screened extract through LC-MS, we could not structurally elucidate and confirm these effective compounds. Hence, further research is warranted to confirm the bioactive metabolites identified with LC-MS, which contributed to the remarkable medicinal potential addressed in this study, through structural elucidation.

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Ethics Approval and Consent of Participate: There is no need for ethical approval and informed consent in this study.

Human and Animal Rights: This study involved no human or animal participation.

Conflict of interest: The authors declare no conflict of interest.

CRediT authorship contribution statement:

DS: Conceptualization, Formal analysis, Data Curation, Methodology, Investigation, Writing-original draft, Writing-review & editing. CJ: Supervision, Resource**s**, Writing-review & editing.

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