

EVALUATION OF TOTAL PHENOLS, TOTAL FLAVONOIDS, ANTIOXIDANT, AND ANTICANCER ACTIVITY OF *MUCUNA PRURIENS* SEED EXTRACT

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ABSTRACT

Objective: In recent years, herbal plants have been got more attention due to their diverse presence of phytochemicals and its biological properties. Hepatocellular carcinoma is one of the major worldwide problems primarily caused by hepatitis B and C virus infection. End-stage liver cancer treatment options are limited, thus requiring expensive liver transplantation which is not available in many countries.

Methods: In the present study, the *Mucuna pruriens* seed extract was analyzed for phytochemicals, antioxidant activity, total phenols, and total flavonoids content. The seed extract was further analyzed for its anticancer activity by culturing liver cancer cell line. The above protocols were done by standard methods.

Results: The seed extract of *M. pruriens* revealed more number of phytochemicals in different organic solvents. 1,1- Diphenyl- 2- picrylhydrozyl scavenging activity of plant extract was more in ethanol extract (98.051±0.547) among all other solvents. The total phenols and flavonoids content in ethanol extract were 46.442±0.353 mg gallic acid equivalent/g and 2.254±0.647 mg - quercetin equivalent /g, respectively. IC₅₀ value of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) analysis of Hep-2 cell line was 150 (µg/ml).

Conclusion: The present study revealed about the phytochemical contents and antioxidant potential of *M. pruriens* seeds. Further, the MTT analysis proved that the seed extract was effective against cancer cells and also used to treat many diseases.

Keywords: *Mucuna pruriens*, Antioxidant, Anticancer.

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INTRODUCTION

Cancer is an abnormal growth of cells that grow continually multiplying with the inability to be controlled or inhibited [1]. Cancer has been a constant conflict globally with a lot of development in treatment and preventative remedies. According to Chavan *et al.* [2], the number of people diagnosed with cancer is estimated globally about 11 million people; the victim number will rise to 16 million by 2020. Liver diseases have become one of the major causes of morbidity and mortality in human and animals all over globe and hepatotoxicity due to drugs appear to be the most common contributing factor [3].

Reactive oxygen species are class of highly reactive molecules, resulting by the metabolism of oxygen. These free radicals occur in the body due to lack of antioxidants. Oxidative damage of biomolecules occurs by rapid production of free radicals will lead to degenerative disorders such as aging, diabetes, cancer, and neural disorders [4,5].

Ayurveda, a traditional Indian medical practice using herbal plants drugs has been successful from ancient time [6]. Many medicinal plants contain a large amount of antioxidants such as polyphenols, Vitamin E, and Vitamin C. Natural antioxidants improve the antioxidant capacity of the plasma and reduce the risk of cancer and cardiovascular diseases [7]. There are many synthetic antioxidants available in the market, but they have many side effects [8]. Hence, there is a need for more potent and less toxic antioxidants. It has been found that medicinal plants having polyphenolic compounds such as flavonoids possess antioxidant activity [9]. Polyphenolic compounds such as flavonoids, curcumin, tannins, alkaloid [10], resveratrol, gallic acid [11], and xanthenes [12] are all considered being anticancer compounds. Polyphenols have antioxidant properties which are due to their high reactivity as a hydrogen donor or electron donor which stabilize and delocalize the unpaired electron [13,14].

Mucuna pruriens Linn. belongs to the family *Fabaceae* is the most popular drug in Ayurvedic system of medicine [15-18]. It is commonly known as velvet bean or cowitch or cowhage or Alkushi [19]. All the parts of *M. pruriens* plant possess medicinal value. Seeds of the plants have antidiabetics activity [20], antimicrobial activity [21], and antivenom activity [22]. The phytochemicals present in the seeds of the *M. pruriens* increase the fertility in men [23]. Levodopa synthesis from seeds regulates central nervous system and also used to treat Parkinson's diseases [24-27].

METHODS**Collection and process of plant sample**

The seeds of *M. pruriens* were collected from Namakkal district, Tamil Nadu, during the month of October–November and authenticated by Dr. P. Jayaraman, Botanist, Plant Anatomy Research Centre, Chennai-45. The specimen number is PARC/2015/2214.

The seeds were air-dried and powered using ball mill machine. The powered sample was weighed and preserved in the closely tight clean jar before the extraction.

Preparation of plant extraction

Plant extracts were prepared by standard methods [28,29]. About 5 g of dried seed fine powder of *M. pruriens* was extracted with 20 ml water, ethanol, acetone, chloroform, and petroleum ether for 1 min using ultra Turrax Mixer (13,000 rpm) and soaked overnight at room temperature. The sample was then filtered through Whatman No 1 filter paper in Buchner funnel. The filtered solution was evaporated under vacuum in a Rotaevator at 40°C to a constant weight and then dissolved in the respective solvent. The concentrated extracts were stored in airtight container in the refrigerator below 10°C.

Preliminary phytochemical analysis of seed extracts

The preliminary analysis of phytochemicals screening was done by standard methods [30,31].

Estimation of total phenolic content

The total phenol content in seed extract of *M. pruriens* was determined by standard method [32]. 100 µl of ethanol extract was mixed with 0.5 ml of Folin-Ciocalteu reagent (1/10 dilution) and 1.5 ml of sodium carbonate (2% w/v). The blends were incubated in the dark at room temperature for 15 min. The absorbance of blue-colored solution of sample was measured at 765 nm using ultraviolet (UV)-spectrophotometer. Gallic acid used to make standard curve. Total phenolic contents were expressed as mg of gallic acid equivalent (GAE) per gram dry weight of plant powder.

Estimation of total flavonoids content

Total flavonoid content in seed extract of *M. pruriens* was determined by aluminum chloride colorimetric method [33]. 0.5 ml of seed extract of *M. pruriens* and the volume was made up to 3 ml by adding methanol. Then, 0.1 ml of 10% aluminum chloride (10%), 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water were added sequentially. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer. A standard generated using known concentration of quercetin. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging activity of *M. pruriens* seeds

Qualitative assay of antioxidant activity

Antioxidant assay of *M. pruriens* was estimated for its free radical scavenging activity using DPPH free radicals.

Principle

DPPH is a stable free radical with purple color (absorbed at 517 nm). If free radicals have been scavenged, DPPH will degenerate to yellow color. The character of this assay shows free radical scavenging activity.

Screening method

Qualitative analysis of antioxidant potential of *M. pruriens* seed was determined by standard method [34]. 50 µl of seed extracts of *M. pruriens* were taken in five separate wells of microtiter plate. 100 µl of 0.1 % methanolic DPPH was added to the different samples and incubated for 30 min in dark condition. The samples were then taken, observed for discoloration from purple to yellow or pale pink which were considered as strong and weak positive response of free scavenging activity, respectively. The antioxidant-positive samples were subjected for further quantitative analysis.

Quantitative assay of antioxidant activity

100 µl of different extracts of seed samples were mixed with 2.7 ml of methanol and incubated for 30 min in dark condition. Subsequently, at every 5 min interval, the absorption maxima of the solution were measured using UV double-beam spectra scan (Chemito, India) at 517 nm. The antioxidant activity of the sample was compared with known synthetic standard of 0.16 % of butylated hydroxytoluene [35].

In vitro anticancer activity

Cell line and culture

Hep-2 (liver) cell lines were obtained from National Center for Cell Sciences, Pune. The cells were maintained in minimal essential media supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO₂ at 37°C.

Reagents

Minimum essential medium was purchased from Hi-Media Laboratories, FBS was purchased from Cistron laboratories, and trypsin, methylthiazolyl diphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from (Sisco research laboratory chemicals Mumbai). All of other chemicals and reagents were obtained from Sigma-Aldrich, Mumbai.

Principle of assay

This is a colorimetric assay that measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilized with an organic solvent (e.g., isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

In vitro assay for cytotoxicity activity (MTT assay)

The cytotoxicity of sample (*Mucuna* seed extract) on Hep-2 (liver) cell line was determined by the MTT assay [36]. Cells (1×10⁵/well) were plated in 1 ml of medium/well in 24-well plates (Costar Corning, Rochester, NY). After 24 h incubation, the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 24 h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200 µl/well (5 mg/ml) of 0.5% MTT phosphate-buffered saline solution was added. After 4 h incubation, 0.04 M HCl/isopropanol was added. Viable cells were determined by the absorbance at 570 nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC₅₀) was determined graphically. The absorbance at 570 nm was measured with a UV-spectrophotometer using wells without sample containing cells as blanks. The effect of the samples on the proliferation of Hep-2 (liver cancer cell line) was expressed as the % cell viability, using the following formula:

Table 1: Preliminary phytochemical analysis of *M. pruriens* seed extracts

Phytochemicals	Aqueous	Ethanol	Acetone	Chloroform	Petroleum Ether
Tannin	++	+++	-	-	-
Saponin	++	+++	-	-	-
Flavonoids	++	++	+	-	-
Quinones	+	+	+	-	-
Glycosides	-	-	-	-	-
Cardio glycosides	-	-	-	-	-
Terpenoids	+	+	+	-	-
Phenol	+	++	+	-	-
Coumarin	+	++	+	-	-
Steroids	+	+	+	-	-
Alkaloids	+	++	+	-	-
Anthocyanin	-	-	-	-	-
B-cyanin	+	+	-	-	-

++: High amount, +: Present, -: Absent

% cell viability=Absorbance of treated cells/A570 of control cells×100%.

RESULTS AND DISCUSSIONS

Many plants display their characters for anticancer, anti-inflammatory, antibacterial, and anti-allergic nature [37] and could be useful in therapeutic roles [38,39]. The phytochemical constituents of *M. pruriens* seeds were shown in Fig. 1 and Table 1. Ethanol extract of *M. pruriens* revealed more phytocontents than other extracts.

Tannins have general antimicrobial and antioxidant activities [40]. Current reports show that tannins may have potential value such as cytotoxic and antineoplastic agent [41]. Saponins have antifungal properties [42,43]. Saponins are used in hypercholesterolemia, hyperglycemia, antioxidant, anticancer, anti-inflammatory, and weight loss [44,45]. Alkaloids tend to be organic and natural ingredients that have nitrogen and are also physiologically active together with sedative and analgesic roles. Several workers have reported the analgesic [46,47], antispasmodic, and antibacterial properties of alkaloids [46,47]. They also reduce stress and depression symptoms [48]. Steroids have been reported to have antibacterial properties [49], and they are very important compounds, especially due to their relationship with compounds such as sex hormones [50].

Qualification assay of *M. pruriens* seed results were shown in Fig. 2 and Table 2. The results revealed that the aqueous and ethanol extractions were shown highly positive responses. Quantitative free scavenging activity of *M. pruriens* seeds were shown in Table 3, which indicates that the ethanol extract was shown more percentage of free radical scavenging activity among other extractions.

Total phenols content and total flavonoids content of the ethanol extract of seed were shown in Table 4. Several studies have described the antioxidant potential of medicinal plants which are rich in phenolic compounds [51,52]. They also possess other biological properties such as antiaging, anti-apoptosis, anti-carcinogen, anti-atherosclerosis,

anti-inflammation, cardiovascular protection, and improvement of endothelial function [53].

Flavonoids have been reported to exert a wide range of biological activities such as antioxidant, anti-inflammatory, antimicrobial, anti-allergic [9,54,55], cytotoxic anti-tumor, treatment of neurodegenerative diseases, and vasodilatory action [56-58]. In the present study, the significant amount of phenols and flavonoids were observed which might responsible for free radical scavenging activity.

The MTT assay is a rapid and highly accurate colorimetric approach that widely used to determine cell growth and cell cytotoxicity, particularly in the development of new drug. It measures cell membrane integrity by determining mitochondrial activity through enzymatic reaction on the reduction of MTT to formazan [59-61].

Since IC₅₀ value for Hep-2 (liver) cell line (150 µg/ml) *Mucuna* seed extract was found to be effective, the reduction percentage of MTT at 24 h also estimated for Hep-2 (liver) cells (Fig. 3, Table 5 and Graph 1). When incubated with the extract, it induced cytotoxicity in a significant manner which implicates the damage to the member integrity of the cell when contributed with control. The cytotoxicity was minimized in the extract-treated cells and near normal level was attained at various

Table 2: Qualitative free radical scavenging activity of *M. pruriens*

Plant samples	Response
C=Control	-
S=BHT (Standard)	+++
E=Ethanol	+++
Aq=Aqueous	++
A=Acetone	±
C=Chloroform	-
PE=Petroleum ether	-

+++ : High amount, + : Present, ± : Semi-positive, - : Absent. BHT: Butylated hydroxytoluene

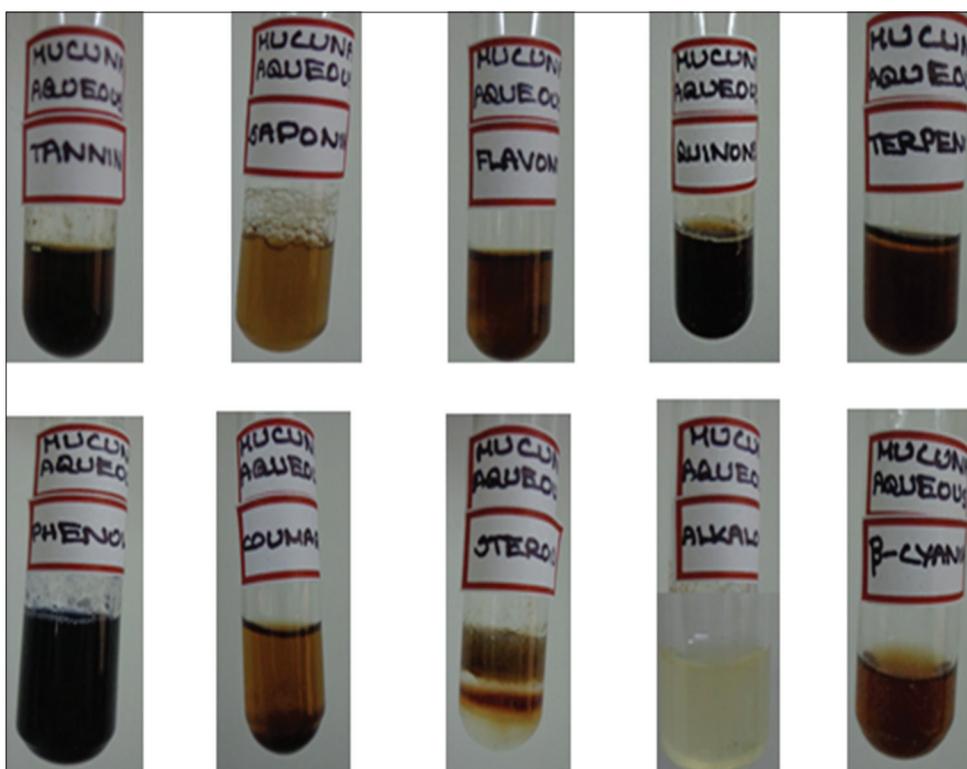


Fig. 1: Preliminary phytochemical analysis of ethanol extract of *Mucuna pruriens* seed extracts

Table 3: DPPH free radical scavenging activity of *M. pruriens* seed extracts

Samples	Time in minutes						
	0	5	10	15	20	25	30
Standard	61.038±0.547	90.909±0.547	97.402±0.547	98.051±0.547	98.701±0.547	98.701±0.547	98.701±0.547
Aqueous	90.909±0.547	92.857±0.547	93.506±0.547	95.454±0.547	96.103±0.547	96.753±0.547	96.753±0.547
Ethanol	94.155±0.547	96.753±0.547	97.402±0.547	97.402±0.547	*98.051±0.547	*98.051±0.547	*98.051±0.547
Acetone	70.129±0.547	79.220±0.547	81.168±0.547	81.818±0.547	82.467±0.547	83.116±0.547	83.766±0.547
Petroleum ether	**53.896±0.547	56.441±0.547	59.090±0.547	59.740±0.547	59.740±0.547	59.740±0.547	60.389±0.547
Chloroform	57.142±0.547	59.090±0.547	59.090±0.547	59.740±0.547	60.389±0.547	60.389±0.547	61.038±0.547

Each value is mean±SD value of 5 samples. Standard versus ethanol *p<0.001, standard versus petroleum **p>0.01. *p level of significance, **p level of insignificance. SD: Standard deviation, DPPH: 1,1- Diphenyl- 2- picrylhydrozyl

Table 4: Total phenols and total flavonoids content of ethanol extract of *M. pruriens* seeds

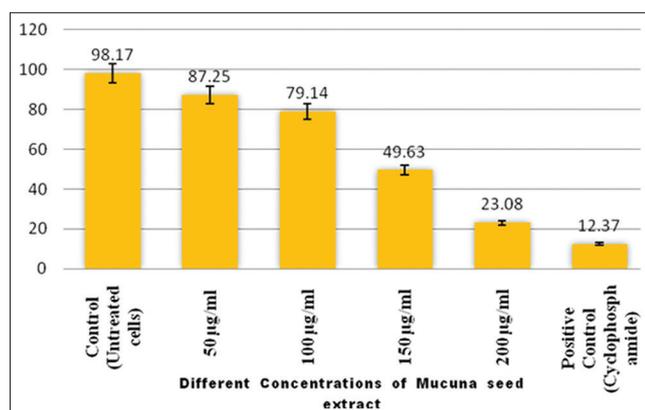
Content	<i>M. pruriens</i>
Total phenols content (mg GAE/g dry sample)	46.442±0.353 mg/g
Total flavonoids content (mg QE/g dry sample)	2.254±0.647 mg/g

Each value is mean±SD value of 3 samples. GAE: Gallic acid equivalent, QE: Quercetin equivalent

Table 5: MTT reduction (%) on Hep-2 cell line

Concentration (µg/ml)	MTT reduction (%)	IC ₅₀
Control	98.17	150 (µg/mL)
50	87.25	
100	79.14	
150	49.63	
200	23.08	
Cyclophosphamide (Positive control)	12.37	

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

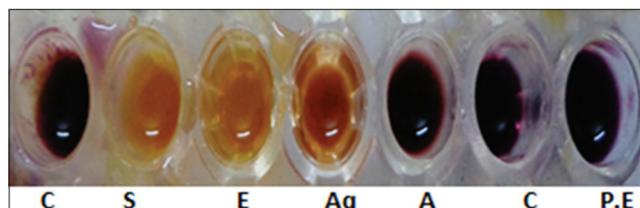
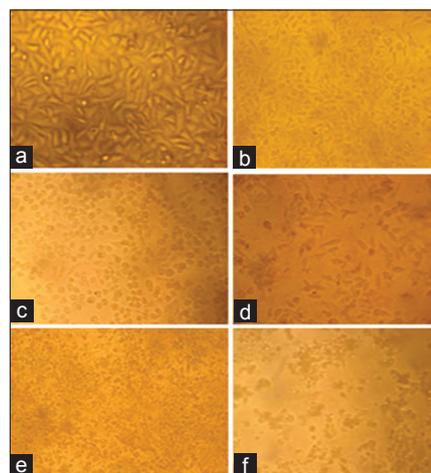


Graph 1: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide reduction (%) on Hep-2 cell line

concentrations (50 µg/ml, 100 µg/ml, 150 µg/ml, and 200 µg/ml), and maximum effect was found when treated at 150 µg/ml. From the above results, it was confirmed that *Mucuna* seed extract at 150 µg/ml seems to offer significant protection and maintain the structural integrity of the hepatocellular membrane and this active concentration was followed for further studies.

CONCLUSION

It can be summarized that the plant selected for the present study having importance in traditional medicine can be considered as a source for

Fig. 2: Qualitative free radical scavenging activity of *Mucuna pruriens* seedsFig. 3: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide reduction (%) on Hep-2 cell line (a) control cells (untreated), b: *Mucuna pruriens* seed extract 50 µg/ml, (c) *M. pruriens* seed extract 100 µg/ml (d) *M. pruriens* seed extract 150 µg/ml (e) *M. pruriens* seed extract 200 µg/ml (f) cyclophosphamide (positive control) 80 µg/ml

the isolation, identification, and development of novel and effective anticancer and antioxidant agents. Hence, the research data of the present findings may serve as a guideline for the standardization and validation of natural drugs containing the selected medicinal plants as ingredients.

AUTHOR CONTRIBUTION

Kavitha K: The research work was designed and carried out.

CONFLICTS OF INTERESTS

The author does not have any conflict of interest.

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