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Isolation and Characterization of Bioactive Agents from *Mucuna* pruriens

Piyush Yadav¹, Sanjay B. Bhawar² ¹Research Scholar, Bhagwant University, Rajasthan, India ²Research Guide, Bhagwant University, Rajasthan, India

Correspondence Email: wick07jhon@gmail.com

ABSTRACT

Mucuna pruriens (MP) has long been used in Indian traditional medicine as support in the treatment of Pharmacological properties such as Antiparkinson Effect, Aphrodisiac Effect, Antioxidant Effect, Antitumor Effect and Antibacterial effect. World is endowed with a rich wealth of medicinal plants. Medicinal plants are the local heritage with global importance. In India herbs have always been principle form of medicine and presently they became popular throughout the developed world, as people strive to stay healthy in the face of chronic stress and pollution, and to treat illness with medicines that work to increase the body's own defense. Present studies have demonstrated that the major constituent of the seeds. Besides this, fatty acids and their mono-glyceride derivatives are the important constituents which may contribute towards the remarkable activity of the plant. The activity results of the study will further be utilized for development and identification of bio-markers to give a reliable solution for wellness of humans. The rich Indian plant wealth has made a good contribution to the development of ancient Indian materia medica. In India, during the past one century there has been a rapid extension of the allopathic system of medical treatment. It generated commercial demand for pharmacopoeial drugs and their products. The Ayurvedic medicinal system claims Mucuna pruriens to possess pro-male fertility, aphrodisiac and adaptogenic properties. In a recent study, Mucuna pruriens efficiently recovers the spermatogenic loss induced due to ethinyl estradiol administration. Thus, the seeds of this plant were undertaken for phytochemical investigation to isolate the active secondary metabolites responsible for these pharmacological properties.

Keywords: Mucuna pruriens (MP), Ayurvedic medicinal, Aphrodisiac, velvet beans

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INTRODUCTION

Herbal medications often comprise a variety of pharmacologically active substances; in other cases, the exact elements that are necessary for the therapeutic action are unknown. Many herbalists feel that separated components have lesser therapeutic effects than entire plant extracts, although this is a claim that would need to be proven in each situation. Herbal medications' multi-ingredient nature can make effectiveness testing more difficult than with synthetic pharmaceuticals. One method is to consider the entire herbal extract to be the active ingredient. Extracts must be well defined to improve the repeatability of such research. This is frequently tried by standardising the extract according to a major element (e.g. a pharmacologically active ingredient or, if such an ingredient is not known, a marker suitable substance).[1]

Many of today's synthetic pharmaceuticals have their origins in the plant kingdom, and herbal remedies dominated our pharmacopoeia only approximately 200 years ago. When pharmacology established itself as a dominant area of therapeutics, medical herbalism (i.e. the medicinal use of medicines that include purely plant material) saw a quick fall. Herbalism vanished off the therapeutic map in much of the English-speaking world around a century ago.[2] Many developing countries, on the other hand, never abandoned medical herbalism (for example, Ayurvedic medicine in India, Kampo medicine in Japan, and Chinese herbalism in China), and medical herbalism coexisted with modern pharmacology in other countries, such as Germany and France, albeit at a lower level [3,4]. This scenario has begun to shift again in recent years. According to a more recent US survey, 16.4% of all patients visiting an internal medicine clinic were now using herbal medications.

The plant is an annual climbing shrub with long vines that can reach over 15 metres (50 ft) in length. When the plant is young, it is almost completely covered with fuzzy hairs, but when older, it is almost completely free of hairs. The leaves are tripinnate, ovate, reverse ovate, rhombus-shaped or widely ovate. The sides of the leaves are often heavily grooved and the tips are pointy. In young M. pruriens plants, both sides of the leaves have hairs. The stems of the leaflets are two to three millimeters long (approximately one tenth of an inch). Additional adjacent leaves are present and are about 5 millimetres (0.2 in) long. The isolated and purified compounds, in contrast, may lose their biological activity due to structural change or fail to behave in the same way as in the complex matrix that the original item of food represents. In many parts of the world *Mucuna pruriens* is used as an important forage, fallow and green manure crop.[5]

MATERIAL AND METHOD

Collection of plant material

All the medicinal plant materials were collected around, Uttar Pradesh. Fresh, healthy seeds of *Mucuna pruriens* which were free of diseases were collected from local market, garden and seeds of Mucuna were purchased from local market.

Preparation of Extract

Seeds of *Mucuna pruriens* was procured locally, authenticated by Professor, Department of Botany, by the studies include organoleptic tests, macroscopic and microscopic observations. They were washed twice using tap water and then washed again in distilled water to remove the dust. The seeds were dried in the shade for 7–12 days at room temperature, until they were free from the moisture and then pulverized into coarse powder. The powdered seeds was added separately in soxhlet tube, methanol was also added to both soxhlet tube and round bottom flask, it was subjected to continuous hot extraction (soxhlet) with methanol at 50-60° Celsius for 16 to 20hrs. Ethanol for ethanolic extract, water for aqueous extract and water and alcohol in a ratio of 50:50 for hydroalcoholic extract respectively. The complete extraction was confirmed by taking about 5 ml solvent from the thimble and evaporated to check for the absence of residue and solvent in siphon was colorless. The extracts were concentrated using Rotary evaporator under reduced pressure below 40°C to get reddish-brown semi solid extract. The obtained mass for each crude drug was weighed, subjected for lyophilized to get free from methanolic solvent and kept in vacuum desiccators. Procedure was repeated to get sufficient amount of extract. Weight of round bottom flask with extract was measured every time and noted for calculation of percentage of yield. Later it was transferred into a container and the filtrate was vacuum concentrated to remove the moisture content. Finally the extract was stored at -4°C [6].

In the laboratory, a fat extractor (Soxhlet extractor) is used for extraction. The fat extractor uses the solvent reflux and siphon principle to continuously extract the solid matter by pure solvent, which saves the solvent extraction efficiency and high efficiency. The solid material is ground prior to extraction to increase the area of solid-liquid contact. The solid material is then placed in a filter paper holder and placed in an extractor. The bottom end of the extractor is connected to a round bottom flask containing a solvent, and is connected to a reflux condenser. The bottom flask is heated to boil the solvent, the vapor rises through the branch pipe of the extractor, is condensed and drops into the extractor, and the solvent is contacted with the solid for extraction. When the solvent surface exceeds the highest point of the material, is repeated such that the solid material is continuously taken as a pure solvent and the extracted material is concentrated in the flask. [7, 8, 9]

Determination of Extractive Values

5 g seed powder of all the study samples were extracted separately with 100 ml solvent (Methanol, Petroleun Ether, Chloroform, water separately) in a closed flask for 24 hours. The flask was intermittently shaken during the first 6 hours and allowed to settle without any movement / disturbance for 18 hours. The extract was filtered with precaution avoiding solvent loss and 25 ml of the filtrate was evaporated to dryness in a dried, weighed, tared flat bottom evaporating dish and dried at 105°C to a constant weight.[10] From the weight of the residue obtained, the percentage of alcohol soluble extractive was calculated in accordance to the air-dried drug and then percentage of alcohol soluble extract was calculated using the following formula:

Percentage yield of extract = (Weight of residue x100x100/ weight of sample x25) % w/w.

Standardization parameters

The different standardization/physicochemical methods were carried out in dried powder of different Extracts of *Mucuna pruriens*.

Foreign Matter Weigh 100gm of the powder drug to be examined or the minimum quantity prescribed in the monograph and spread it out in a thin layer. With the help of eye or by the use of lens (6xs) we can detect the foreign matter. Separate and weigh it and calculate the percentage present.

> 100 g of sample was weighed.

> The sample was spread on a white tile or glass plate uniformly without overlapping.

> Inspected the sample with naked eyes and by means of a lens (5x or above).

The foreign matter (mentioned above) was separated manually.

After complete separation, the foreign matter was weighed and determined percent weight by weight (% w/w) present in the sample.

> Percentage of foreign matter was calculated using the following formula. For which gross weight of the sample was noted.

Where,

W= Weight of the sample.

W1= amount of Foreign Matter.

W2 = amount of drug taken.

Total Ash value

Ash was calculated by weighing 2 gm of a dried crude drug sample in a crucible. It was then heated using a hot plate. The residue of carbon was obtained as a result of ignition when placed in a muffle furnace at 500°C till the residue disappears. The sample was allowed to cool and weighed. The total ash was determined by the following equation. This is used to determine the quality and purity of the crude drug. Ash contains inorganic materials like Phosphates, carbonates, and silicates of sodium, potassium, magnesium, calcium etc. 2-3 g of powder drug was taken in crucible and kept it in the furnace at a temperature not exceeding 450°C until it got free from carbon. The crucible was then cooled in a desiccator, and the ash weight was noted. Percentage of total ash was calculated with reference to the air dried sample of the crude drug.[11]

%Total Ash Value = (W1-W2)/Wx100

Where,

W= Weight of the sample.

W1= Weight of ash + Crucible.

W2 = Weight of empty crucible.

Water soluble Ash value

The obtained ash was dissolved in 25 ml distilled water and kept aside for 5 min. The insoluble material was obtained by filtration using with Whatman paper grade (589/3) and then washed with hot water. The crucible was burnt for about 15-20 min maintained at 450°C; which indicates the absence of carbon. Insoluble matter was calculated by weighing the ash.[12] Water soluble ash was calculated in term of percent by using the following formula.

% Water soluble Ash value = ((W1-W2)/W3)x100

Where,

W1= Weight of total ash

W2= Weight of water insoluble ash

W3= Weight of crude drug taken.

Acid insoluble Ash value

This represents the residue obtained after boiling the total ash with 25 ml (2M) Hydrochloric acid for 10 minutes. The matter insoluble was collected on Whatman filter paper grade (589/3) and clean with hot water burn in the crucible for about 15-20 min for the maintain the temperature not exceeding 450°C. Acid insoluble matter was calculated in terms of percentage by using the following formula.

The ash obtained from the total ash content, was boiled for 5 minutes with 25 ml of dilute hydrochloric acid, the insoluble matter obtained was collected on an ashless filter paper, which was washed with hot water and ignited the crucible in the flame. Then crucible is cooled, and weighed. The filter paper and residue was put together into the crucible, heated gently until vapours stops and heated further till carbon disappears.[13] The crucible was cooled in a desiccator. The percentage of acid-insoluble ash was calculated using the formula

% Acid insoluble Ash value = (W1/W2) x100

Where,

W1= Weight of acid insoluble ash W2= Weight of crude drug taken

Loss on drying (Moisture content)

5 g of the powdered drug was accurately weighed in pre-weighed Petri dish and dried under hot air oven for 120 minutes maintained at 1100C after that it was kept in desiccators at room temperature. The result was calculated by using the following formula [14].

% Loss on drying =
$$(W1/W2) \times 100$$

Where, W1= Loss in weight of sample

W2= Weight of sample

Water soluble extractive value

5 gm sample (crude drug) was macerated in 100 ml distilled water in a closed container for 6 hours with continuous stirring in orbital shaker assembly after that it was allowed to stand for 18 hr. Evaporate 25 ml filtrate till dryness and dried at 110°C. Weigh the sample without delay and calculate the extractive value [15].

Alcohol soluble extractive value

This was estimated using the same procedure as in the case of water soluble and using alcohol in place of water [15].

Determination of pH Values

10 g seed powder was weighed, 100 ml distilled water was added to it so as to get 10% solutions. It was then mixed continuously with the help of clean and dry glass rod for about 45 minutes till it became a homogenous mixture. It was then filtered through filter paper so as to remove the insoluble components. The pH meter was calibrated using standard buffer solution of pH 7 and pH 9 and then pH value was measured on pH meter [16].

Phytochemical of medicinal plant extracts

Preliminary phytochemical screening of the extracts has shown the presence of alkaloids, tannins, saponins, steroids and flavonoids [17, 18].

Tests for Carbohydrate

Preparation of stock solution: Extract was dissolve in ethanol followed by hydrolyzed with 2N HCl stock solution was prepared to proceed with chemical parameters.

(a) **Molish's test (General test):** 3 ml stock solution, and add a few drops of α - naphthol solution in alcohol with proper shaking is done. Add 2 ml of conc. H2SO4 slowly from the side of the test tube. The result show purplish-violet ring at the junction of two liquids.

(b) **Barfoed's test**: Barfoed indicator and stock solution mix equally heated for 2- 3 min, cooled them bricks color is observed.

(c) **Benedict's test**: mixed with few drops of Benedict's indicator in stock solution and boiled in a water bath for 4 min, observation shows yellow, green and red color depends on the amount of reducing sugar present in stock solution.

(d) **Fehling's Test**: Filtrates obtained from Benedict's test was mixed with dilute hydrochloric acid to neutralize the alkali. Fehling's solutions A & B was added and then heated in a water bath. The presence of reducing sugar was confirmed by the appearance of a reddish precipitate.

Tests for Proteins

(a) Stock solution preparation: Extract was dissolve in ethanol to prepare stock solution was prepared to precede the chemical parameters.

(b) Biuret Test (General test): 3 ml of stock solution and mix 4% sodium hydroxide solution, add few drops of one percent copper sulphate observe the inference of pink or violet color.

(c) Million's test: 5 ml million's reagent was added to 3 ml stock solution. This was heated in a water bath; the white precipitate was obtained. Further results show brick red color precipitate. It shows the presence of protein.

(d) Xanthoprotein test: 1 ml conc. sulphuric acid was added to 3 ml stock solution. The creamishwhite precipitate was obtained. Further, the precipitate was boiled and then yellow color was observed; then adds ammonia hydroxide the precipitate turned into orange color

Tests for Steroids

Preparation of test extracts solution

The extracts were refluxed separately with an ethanolic solution of potassium hydroxide till complete saponification. The saponified extract was diluted with ethanol and the unsaponifiable matter was extracted with diethyl ether. The ethereal extract was evaporated and the residue (unsaponifiable matter) was subjected to the following test by dissolving the residue in the chloroform.

(a) Salkowski reaction: To 2 ml of extract, add 2 ml chloroform and 2 ml. conc.H2SO4. Shake well. Chloroform layer appears red and acid layer shows greenish yellow fluorescence.

(b) Liebermann Burchard test: Crude extract was mixed with few drops of acetic anhydride, boiled and cooled. Concentrated sulphuric acid was then added from the sides of the test tube and observed for

the formation of a brown ring at the junction of two layers. Green coloration of the upper layer and the formation of deep red color in the lower layer would indicate a positive test for steroids and triterpenoids respectively.

(c) Liebermann's test: Mixed 3 ml extract with 3 ml of acetic anhydride. Heated and cooled. Added few drops concentrated sulphuric acid observed for blue color.

Test for Amino Acids

(a) Ninhydrin test (General test): Ninhydrin test was conducted for amino acids in general and presence of cysteine was checked by adding 40% NaOH and 10% lead acetate solution to sample solution. Appearance of black lead sulphate precipitate after boiling confirmed the test (Sadasivam and Manickam, 1996).

(b) Test for Cysteine: To 5 ml of sample solution few drops of 40% NaOH and 10% lead acetate solution was added and boiled. Appearance of black precipitate of lead sulphate was formed confirming the presence of Cysteine. It showed that amino acid is present in different extract.

Test for Glycosides

Stock solution: It can prepare by dissolving different plant extract in the ethanol.

(a) Keller Killiani Test: 2-3 ml test solution deal with few drops of 5% FeCl3 and CH3COOH solution and mixed together add H2SO4 and observed for the formation of two layers. Reddish brown layer is observed at the upper side of the test tube and blue - green colour is at the lower side shows an actual test for glycosides.

(b)

В

romine water test: Test solution was dissolved in bromine water and found precipitate yellow formation shows that presence of glycosides.[19]

Tests for Flavonoids

For the test of flavonoids is perform in that pieces of magnesium ribbon and HCl concentrated were mixed with aqueous different crude extract after 5-10 minutes and pink color observe which confirmed that flavonoids are present.

Tests for Vitamins

Equal quantity of 12-16 units in chloroform 1 ml and was mixed 8-10 ml antimony trichloride solution was added a moving blue color is obtained directly which shows the presence of vitamin A.

Test for Ascorbic acid the sample solution (1 ml 2% w/v) was dissolved in 5 ml distilled water. To this solution pour 2-3 drops of sodium nitroprusside (5% w/v) was poured and then NaOH solution (2ml) was added. To the above mixture drop by drop added 0.5- 0.6 ml of HCl and the medium was stirred until the yellow color is converted to blue. Test for Vitamin D Equal quantity of 100 unit of vit. D was dissolved in chloroform and mixed antimony trichloride 10 ml mixture, pinkish-red color is obtained directly which shows the presence of vit. D.

Tests for Tannins and phenolic compounds

(a) For the tannins estimation: 2ml distill water was added in 1 ml ethanolic extract. The green to blue or blue to black coloration was observed by adding 2 to 3 drops of diluted FeCl3 mixture.

(b) Aqueous extract of 2 ml was added to water 2 ml, few drops of dilute ferric chloride solution were then added. Bluish-green color confirmed that tannins were present.

Test for alkaloids

a) Preliminary test: A 100gm of an alcoholic extract was dissolved in dilute hydrochloric acid. Solution was clarified by filtration. Filtrate was tested with Dragendroff's and Mayer's reagents. The treated solution was observed for any precipitation.

b) Confirmatory test: Five grams of the alcoholic extract was treated with 40% calcium hydroxide solution until the extract was distinctly alkaline to litmus paper, and then extracted twice with 10ml portions of chloroform.

RESULT

Organoleptic Properties of Mucuna pruriens

Organoleptic properties are the critical parameter for the rapid identification and consumer acceptance. Sensory evaluation-visual macroscopy, colour, odour, taste, fracture are the common features helped in identification of the crude drug. Organoleptic tests are sometimes conducted to determine if food or pharmaceutical products can transfer tastes or odors to the materials and components they are packaged in. Shelf life studies often use taste, sight, and smell (in addition to food chemistry and toxicology tests) to determine whether a food product is safe to consume. Measurements of pepper spiciness on the Scoville scale depend upon an organoleptic test. The quality of extracts used in phytotherapy is assessed in part using organoleptic tests. Organoleptic qualities are considered part of hurdle technology. Indicators identified organoleptically as part of European Union wine regulations are assessed when qualifying for a

Quality Wine indicator. Organoleptic analyses are, occasionally, still used when the protocol for a certain sample does not have a high enough sample throughput to meet the demand. In this case, organoleptic analyses serve as a primary screen to determine which samples must be analyzed according to the original method protocol, and which samples need no further sensory analysis. The organoleptic properties of *Mucuna pruriens* have been mentioned in Table Below:

Sr.No.	Properties	Outcome	
1.	Colour	Dark bluish	
2.	Shape	Irregular	
3.	Odour	Characteristic Odour	
4. Texture		Rough, plain	
5.	Taste	Sweet And Bitter	

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Extraction of Selected Mucuna pruriens

Medicinal plants are extracted and processed for direct consumption as herbal or traditional medicine or prepared for experimental purposes. The concept of preparation of medicinal plant for experimental purposes involves the proper and timely collection of the plant, authentication by an expert, adequate drying, and grinding. This is followed by extraction, fractionation, and isolation of the bioactive compound where applicable. In addition, it comprises determination of quantity and quality of bioactive compounds.

The moderately coarse powder of the seeds of *Mucuna pruriens* was subjected to extraction with water as solvent in order to find the active constituents. The obtained aqueous extract of seeds of *Mucuna pruriens* was dried and weighed. The percentage yield of plant drug was calculated as per standard method. The weighed extract of plant drug was stored in desiccators for further use. The yield was found to be (9.21 % w/w of crude drug) of aqueous extract with semisolid mass of Brownish-Black colour when extracted with water for 18 hrs. Obtained results were recorded in table: Table 2. Extractive values obtained from Mucuna pruriens seeds

Table 2: Extractive values obtained if on Mucunu pruriens seeds						
Sr.No	Solvent	Color of extract	% yield			
1.	Water	Dark Bluish	9.21%			
2	Pet other	Blackish	5 42%			

1.	Water	Dark Bluish	9.21%
2.	Pet ether	Blackish	5.42%
3.	Chloroform	Dark Bluish	8.20%
5.	Methanol	Dark Bluish	19.05%

Table 3: Phytochemical constituents of Mucuna pruriens (Methanol Seed Extract)

Sr. no.	Constituents	Methanol seed extract
1.	Carbohydrates	+
2.	Tannins and Phenolics	-
3.	Amino acids	+
4.	Flavonoids	+
5.	Saponins	-
6.	Fixed oils and Fats	-
7.	Alkaloids	+
8.	Glycosides	-
9.	Phytosterols	-
10.	Starch	+
11.	Proteins	+
12.	Steroids	+
13.	Reducing Sugar	+
14.	Resins	+

"+" shows present "-" shows absent

Physicochemical Standardization of Mucuna pruriens

Standardization of drugs means confirmation of its identity and determination of its quality and purity. The quality control standards of various medicinal plants, used in indigenous system of medicine, are significant nowadays in view of commercialization of formulations based on medicinal plants. The quality of herbal drugs is the sum of all factors, which contribute directly or indirectly to the safety, effectiveness, and acceptability of the product.

Lack of quality control can affect the efficacy and safety of drugs that may lead to health problems in the consumers. Standardization of drugs is needed to overcome the problems of adulteration and is most developing field of research now. Therefore, there is an urgent need of standardized drugs having consistent quality.

Sr.No	Parameters % w/w	Ethanol Extract	Pet. Ether extract	Chloroform extract	Methanol extract
1.	Foreign Matter	2.05	1.86	1.45	1.20
2.	Loss on drying (Moisture content)	5.01	4.45	3.75	4.55
3.	Alcohol soluble ash	7.86	6.28	5.85	8.35
4.	Determination of pH Values	6.14	6.12	6.32	6.41

Table 4: Standardization pai	rameters of Mucuna pruriens
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Foreign matter

Foreign matter is defined as any kind of outside contaminant introduced to a drug product at any point in its production or distribution. It was found highest in ethanol extract and lowest in methanol extract.

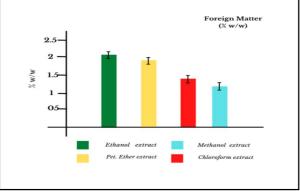


Figure 1: Graph showing foreign matter in different solvents

Moisture content

Moisture content can be expressed on wet or dry basis. Moisture content on dry basis is the mass of water to the mass of dry solid. It was found highest in water extract and lowest in chloroform extract.

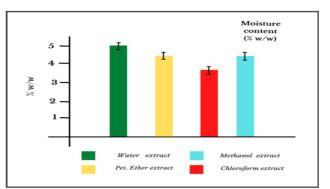


Figure 2: Graph showing moisture content in different solvents

Alcohol soluble ash

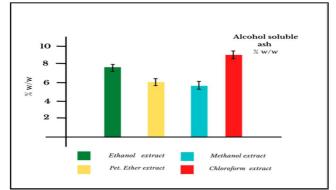


Figure 3: Graph showing Alcohol soluble ash in different solvents

Sr.No	Parameters % w/w	Ethanol extract	Pet. Ether drug extract	Chloroform drug extract	Methanol drug extract
1.	Ash value	4.02	5.31	4.46	3.88
2.	Swelling index	0.14	0.13	0.16	0.15
3.	Water soluble ash	2.98	2.44	3.13	3.51
4.	Acid insoluble ash	2.86	2.45	1.84	3.95

Table 5: Physicochemical Standardization of Mucuna pruriens

Ash value

Ash values help determine the quality and purity of the crude drug, especially in powdered form. The object of ashing a vegetable drug is to remove all traces of organic matter which may otherwise interfere in analytical determination.

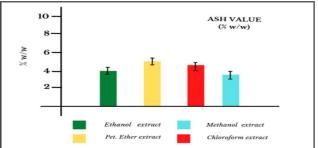


Figure 4: Graph showing ash value in different solvents

Swelling index

Swelling study indicates the capacity of the polymer to imbibe water for swelling, thereby producing mucosal adherence and ultimately controlling the release of drugs over time.

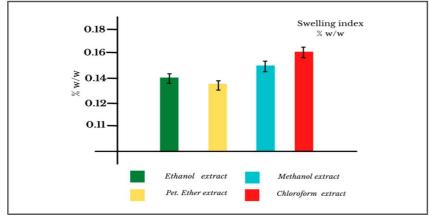


Figure 5: Graph showing swelling index

Water soluble ash

Water soluble ash value in different solvents was found highest in chloroform and lowest in pet ether extract.

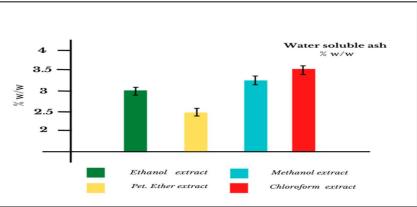


Figure 6: Graph showing water soluble ash in different solvents

Acid insoluble ash

Acid insoluble ash indicates the amount of silica present. Acid insoluble ash value in different solvents Was found highest in methanol extract and lowest in pet ether extract.

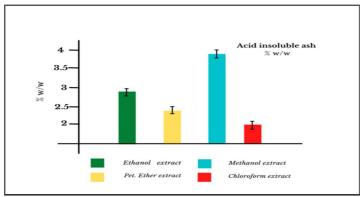


Figure 7: Graph showing acid insoluble ash in different solvents

DISCUSSION AND CONCLUSION

The results arrived and inferences drawn, conclusively validates the phytochemical effect of mucuna as follows. The *Mucuna pruriens* was identified collected and shade dried. The various extract obtained after the extraction were subjected to phytochemical screening for determination of various class of pharmaceutical constituents present in *Mucuna pruriens*. Carbohydrates, Amino acids, Flavonoids, Alkaloids, Steroids, Proteins were found positive in Methanol seed extract. The shade dried plant material was converted into moderately coarse powder. Ash content value of *Mucuna pruriens* was found 4.02% w/w, 5.31% w/w, 4.46% w/w, 3.88% w/w were of Ethanol drug ext, Pet. Ether drug ext, Chloroform drug ext, Methanol drug ext respectively. Whereas Water soluble ash was found to be 2.98%, 2.44%, 3.13%, 3.51% were of Ethanol drug ext, Pet. Ether drug ext, Methanol drug ext respectively. Acid insoluble ash was found to be 2.86%, 2.45%, 1.84%, 3.95% were of Ethanol drug ext, Pet. Ether drug ext, Chloroform drug ext, Pet. Ether drug ext, Chloroform drug ext, with the exception of a few medications, the *Mucuna pruriens* plant material is not already in use, and that it should be introduced for the benefit of the community at a national level.

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Conflict of Interest

The authors confirm no conflict of interest.

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