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RESEARCH ARTICLE

PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITIES OF SEED AND CALLUS EXTRACTS OF MUCUNA PRURIENS (L) DC

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ABSTRACT

The aim of study was to examine the phytochemical screening, total phenols, total flavonoids and antioxidant activity fromseed and callus extracts of *Mucuna pruriens.* The phytochemical analysis revealed the presence of active ingredients such as Tannins, saponins, steroids, cardiac glycosides, phenols, flavonoids, terpenoids, alkaloids and coumarins in the callus extract of *Mucuna pruriens* followed by seed extract. Gallic acid (GA), Quercetin (Q) and Butylated Hydroxy Toluene (BHT) were taken as standard in case of total phenol and Flavonoid and antioxidant activity respectively. Total phenol and flavonoid contents were quantitatively estimated which recorded maximum in callus extract of *Mucuna pruriens*17.25±45 mg Gallic Acid Equivalents (GAE)/g and 8.26 ± 0.55 mg and QuercetinEquivalents (QE)//g). The seed and callus extracts were evaluated for antioxidant activities by DPPH (1, 1– Diphenyl -2- picryl - hydrazyl) radical scavenging assay. Among five different extracts used, maximum antioxidant activity was found in the ethanolic callus extract (91.34 ± 1.0%) of *Mucuna pruriens* followed by seed extract (90.5 ±30%). The powerful antioxidant activity is attributed to the greater amount of total phenols and flavonoids compound in the ethanolic callus extracts of *Mucuna pruriens*.

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INTRODUCTION

Plants are the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer *et al.,* 1999). Most of the people in rural and urban areas of the world are dependent on the medicinal plants for the treatment of infectious diseases. Plant derived medicines are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment. Dietary phytochemicals are considered as an effective tool to cure body disorder. They play important roles as therapeutic agents in prevention of many diseases (Kareem *et al.,* 2010). Therapeutic benefits can be traced to specific plant compounds; many herbs contain dozens of active constituents that, together, combine to give the plant its therapeutic value (Babu *et al.,* 2015). A growing body of evidence indicates that secondary plant metabolites play important roles in human health and may be nutritionally important (Jeeva *et al.,* 2012). Phytochemical screening of various plants has been reported by many workers (Mojab *et al.,* 2003; Parekh and Chanda, 2008). These studies have revealed the presence of numerous chemicals including alkaloids, flavonoids, steroids, phenols, glycosides and saponins. The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites (Hagerman *et al.,* 2008). Free radicals or highly reactive oxygen species are formed by exogenous chemicals or endogenous metabolic processes in the human body. These are capable of oxidizing bio-molecules *viz* nucleic acids, proteins, lipids and DNA and can initiate different degenerative diseases like neurological disorders, cancer, emphysema, cirrhosis, atherosclerosis, arthritis etc (Sathisha *et al.,* 2011). Antioxidants are the compounds which terminate the attack of free radicals and thus reduce the risk of these disorders (Rice-Evans *et al.,* 1996). Presently, much attention has been focused on the use of

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natural antioxidants to protect the human body especially brain tissues from the oxidative damage caused by free radicals. In last two decades, several medicinal plants have shown such effectiveness through the traditional methods of psychoneuro-pharmacology (Meena *et al.,* 2012).The crude extracts of herbs, spices and other plant materials, rich in phenolics and flavonoids are of increasing interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food (Chu *et al.,* 2000). In addition, phenolic compounds and flavonoids are also widely distributed in plants which have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic etc. (Miller, 1996). *Mucuna pruriens* (L). Dc. is a plant of the Fabaceae family, typically found in tropical regions and used for various purposes in traditional medicine in several countries. In India, It is used as a uterine stimulant and aphrodisiac (Longhi *et al.,* 2011) and also used against snake bites (Chikagwa-Malunga *et al.,* 2009). Traditionally, the seeds of *M. pruriens* are used as a tonic and aphrodisiac for male virility. Seeds contain L-DOPA (3,4-dihydroxyphenyl alanine) content, which is a precursor of the neurotransmitter has found to be used in the treatment of Parkinson's disease and mental disorder (Ali *et al.,* 2005). The present study aims to investigate the phytochemical screening, antioxidant activity, total phenol and total flavonoid content from seed and callus extracts of *Mucuna pruriens.*

MATERIALS AND METHODS

Collection of material: The healthy wild *Mucuna pruriens* seeds were collected during the middle of February 2015 from Kolli Hills - Namakkal District, Tamil Nadu, India and seeds were cleaned and stored.

Figure 1. a. Mother plant of *Mucuna pruriens*, b) Seeds of *Mucuna pruriens*

Explant preparation

Seed explants (1-year-old mature plants) were surface sterilized by cleaning thoroughly under running tap water for 20 minutes, washed with a solution of labolene (2-3 drops in 100 ml of water) for 5 minutes, and again washed with sterile distilled water. The cleaned explants were treated with 70% ethanol for 1 minute followed by 0.1% mercuric chloride (HgCl2) treatment for 5 minutes under aseptic conditions and washed six times with sterile distilled water to remove traces of HgCl2.

Germination and plant development

After surface sterilization *M. pruriens* seed explants were inoculated onto Murashige and Skoog (MS) [Meena et al., 2012] basal medium supplemented with different concentrations of 2-isopentenyl adenine (2iP) (2.46–12.3 µM) and Gibberellic acid (GA3) (1.44 µM) for germination of plants from the seeds.

Initiation of callus

The *in vitro* cotyledonary leaf and hypocotyl explants were cultured on MS basal media containing various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (1.13, 2.26, 4.52, 6.78, and 9.04 µM); naphthalene acetic acid (NAA) (1.34, 2.68, 5.37, 8.05, and 10.74 µM), 6-Benzylaminopurine BA (1.11, 2.22, 4.44, 6.66, and 8.88 µM), and 2iP (1.23, 2.46, 4.92, 7.32, and 9.84 µM) for callus induction. Primary callus was established from cotyledonary leaf explants. For secondary callus production, a small portion of primary callus was excised using sterile knife holder and was sub-cultured periodically once in 3 weeks. The secondary callus was used for all the experimental studies. A standard approach of Latin square method [Chu et al., 2000] was followed for screening of media to establish optimum culturing of callus by manipulating the concentration of auxins (2,4-D, Indole-3-acetic acid [IAA] and NAA) and cytokinins (BA and 2iP) individually and in combinations. A range of seven concentrations of auxins and cytokinins (0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10 mg/L) were used in this study.

Callus growth

The growth measurement of callus was determined by standard method [Miller, 1996]. The growth of the callus and its biomass was measured in terms of fresh (FW g/L) and dry weight (DW g/L). FW of callus was measured after removing the excess moisture and agar adhering to the callus surface using blotting paper. DW of callus was determined by drying the callus in hot air oven at 60°C for 24 hr and was expressed in g/L DW culture.

Preparation of the seed and callus extracts of *Mucunapruriens*

Preparation of the extracts was done according to a combination of the methods used by Pizzale *et al.,* (2002) and Lu and Foo (2001). About 15g of dried seed fine powder of *Mucuna pruriens* plant material was extracted with 150 mL acetone, ethanol (75%), chloroform, petroleum ether and aqueous extract for 1 min using an Ultra Turax mixer (13,000 rpm) and soaked for overnight at room temperature. The sample was then filtered through Whatman No.1paper in a Buchner funnel. The filtered solution was evaporated under vacuum in a rota-evator at 40° C to a constant weight and then dissolved in respective solvents. The concentrated extracts were stored in airtight container in refrigerator below 10º C.

Phytochemical screening of seed and callus extracts of *Mucunapruriens*

The phytochemical screening of seed and callus extracts were assessed by standard method as described by Savithramma *et al.,* (2011). Phytochemical screening was carried out on the seed extracts using different solvents to identify the major natural chemical groups such as tannins, saponins, flavonoids, phenols, terpenoids, alkaloids, glycosides, cardiac glycosides, coumarins and steroids. General reactions in these analyses revealed the presence or absence of these compounds in the seed extracts tested.

Estimation of total phenol content in seed and callus extracts of *Mucunapruriens*

Total phenolic content in the seed extracts was determined by the Folin-Ciocalteau colorimetric method (Slinkard and

Singleton, 1984). For the analysis, 0.5 ml of dry powdered acetone extracts were added to 0.1 ml of Folin- Ciocalteau reagent (0.5N) and the contents of the flask were mixed thoroughly. Later 2.5 ml of sodium carbonate (Na2CO3) was added and the mixture was allowed to stand for 30 min after mixing. The absorbance was measured at 760 nm in a UVVisible Spectrophotometer. The total phenolic contents were expressed as mg gallic acid equivalents (GAE)/g extract.

Estimation of Total Flavonoid Content in seed and callus extracts *Mucunapruriens*

Total flavonoids content in the ethanolic leaf extracts was determined by the aluminium chloride colorimetric method (Mervat *et al.,* 2009). 0.5 ml of leaf extracts of *Mucuna pruriens* at a concentration of 1mg/ ml were taken and the volume was made up to 3ml with methanol. Then 0.1ml AlCl3 (10%), 0.1ml of potassium acetate and 2.8 ml distilled water were added sequentially. The test solution was vigorously shaken. Absorbance was recorded at 415 nm after 30 minutes of incubation. A standard calibration plot was generated at 415nm using known concentrations of quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent /g of sample.

Qualitative analysis of Antioxidant activity of *Mucuna pruriens*

The antioxidant activity of the seed and callus extracts of *Mucuna pruriens*was determined by following the method as described by George *et al.,* (1996); Susanti *et al.,* (2007). 50µL of seed and callus extracts of *Mucuna pruriens*were taken in the microtiter plate. 100µL of 0.1% methanolic DPPH was added with the samples and incubated for 30 minutes in dark condition. The samples were then observed for discoloration; from purple to yellow and pale pink were considered as strong and weak positive respectively. The antioxidant positive samples were subjected for further quantitative analysis.

Quantitative analysis of free radical scavenging activity of *Mucunapruriens*

The antioxidant activities were determined using DPPH (Sigma-Aldrich) as a free radical. Seed and callus extracts of 100µl were mixed with 2.7ml of methanol and then 200µl of 0.1 % methanolic DPPH was added. The suspension was incubated for 30 minutes in dark condition. Initially, absorption of blank sample containing the same amount of methanol and DPPH solution was prepared and measured as a control (Lee *et al.,* 2005). Subsequently, at every 5 min interval, the absorption maxima of the solutions were measured using a UV double beam spectra scan (Chemito, India) at 517nm. The antioxidant activity of the sample was compared with known synthetic standard of 0.16% Butylated Hydroxy Toluene (BHT). The experiment was carried out in triplicates. Free radical scavenging activity was calculated by the following formula: % DPPH radical scavenging = [(Absorbance of control - Absorbance of test Sample) / (Absorbance of control)] x 100

RESULTSS AND DISCUSSION

The surface sterilized seed explants were inoculated on MS medium containing 2iP (2.46-12.3 µM) with GA3 (1.44 µM). Seed germination in most of the treatments was recorded within 15 days of culture (Fig. 1a, b). The prominent seedling developed on MS basal medium supplemented with 4.92 µM 2iP+1.44 µMGA3 showed significant growth response of 61.6±2.8% germination with an average shoot length of 5.43±0.05 cm and an average root length of 3.76±0.25 cm and healthy seedlings were developed after 40 days of culture (Fig. 1c). The *in vitro* seedlings were used for further experimental studies.The growth of callus development varied from cotyledonary leaf and hypocotyl explants. Explants inoculated on MS medium supplemented with an individual concentration of 2,4-D, NAA, BA, and 2iP. Cotyledonary leaf explants inoculated on MS medium containing 4.92 µM 2iP was noticed to be significantly higher than hypocotyl explants (Fig 1f). A total of 53 combinations of auxin and cytokinins were tried for optimum callus biomass production. The hormone combination for optimum callus biomass production was standardized, and the callus biomass was 186.22 g/L FW and 15.54 g/L DW in MS solid medium supplemented with 4.52 µM - 2,4-D, 2.22 µM – BA, and 4.92 µM - 2iP after 24 days of culture (Fig.2a). This standardised callus has been used for further experimental studies. In the present study, phytochemical screening was performed with ethanol, chloroform, petroleum ether, acetone and aqueous seed and callus extracts of *Mucuna pruriens*. The ethanolic seed and callus extracts of *Mucuna pruriens*were rich in terpenoids, quinones, saponins, Cardiac glycosides, steroids, phenols, flavonoids, coumarins, tannins and alkaloids (Table 1 & 2). Phytochemical constituents such as tannins, phenols, alkaloids and several other aromatic compounds or secondary metabolites of plants serve as defense mechanism against predation by many micro-organisms, insects and herbivores (Britto and Sebastian, 2011).

Preliminary screening of phytochemicals may be useful in the detection of the bioactive principles and subsequently may lead to the drug discovery and development (Doss, 2009). Phenolics are the most widespread secondary metabolites in plant kingdom. These diverse groups of compounds have received much attention as potential natural antioxidant in terms of their ability to act as radical scavengers. Phenolic compounds are a class of antioxidant agents which act as free radical terminators (Shahidi and Wanasundara, 1992). In our study, total phenol content (TPC) of *Mucuna pruriens*seed and callus extracts was estimated by using Folin- Ciocalteau colorimetric method and represented in terms of gallic acid equivalent (GAE). The results of the present study showed that the phenol contents of the ethanoliccallus extracts in terms of Gallic acid equivalent were obtained17.25±45mg GAE/ g followed by seed extract 14.54±0.5mg GAE /g.

Flavonoids are regarded as one of the most widespread groups of natural constituents found in plants. The value of flavonoid content varies in various plants. It has been recognized that flavonoids show antioxidant activity and their effects onhuman nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process (Kessler *et al.,* 2003, Cook and Samman, 1996). The results of the present study showed that the flavonoid contents of the ethanolic callus extracts in terms of quercetin equivalent were found to be maximum (8.26±0.55mg QE/ g) followed by seed extract (7.3±0.6mg QE/ g) (Table 3). The seed and callus extracts of *Mucuna pruriens* were used for antioxidant studies. Analysis on different extraction of acetone, ethanol, petroleum ether, chloroform and aqueous extract showed the presence of antioxidants.

Table 1. Phytochemical screening from seed extracts of *Mucuna pruriens* (Kolli Hills - accession)

Phytochemicals Tested	Seed extracts of *Mucuna pruriens*				
	Aqueous	Ethanol	Chloroform	Petroleumether	Acetone
Tannins	+	+	-	-	+
Saponin	++	++	+	-	+
Quinones	+	++	+	+	++
Terpenoid	+	++	+	+	+
Steroids	+	+	+	+	++
Flavonoids	+	++	-	-	+
Phenol	++	++	+	+	+
Alkaloid	+	+	-	-	-
Coumarins	+	++	-	+	-

Key : + = positive, ++ = strong positive , - = negative

Table 2. Phytochemical screening from Callus extracts of *Mucuna pruriens* (Kolli Hills - accession)

Phytochemicals Tested	Callus extract of *Mucuna pruriens*				
	Aqueous	Ethanol	Chloroform	Petroleumether	Acetone
Tannins	+	+	+	+	+
Saponin	+	+	+	-	-
Quinones	+	++	-	+	++
Terpenod	+	++	-	+	+
Steroids	+	++	-	+	+
Flavonoids	++	+	-	+	-
Phenol	+	++	+	+	+
Alkaloid	+	+	-	-	-
Coumarins	+	++	-	+	+

Key : + = positive, ++ = strong positive , - = negative

Table 3. Estimation of Total phenol and flavonoid contents from seed and callus extracts of *Mucuna pruriens*

S.no.	Plant sample	Total phenol content (mg GAE/g)	Total flavonoid content (mg QE/g)
1	Seed extract	14.54 ± 0.65	*7.30* ± 0.60
2	Callus extract	17.25 ± 0.45	8.26 ± 0.55

Table 4. Qualitative antioxidant activity of seed and callus extracts of *Mucunapruriens*

S.No	Extractions	*Mucunapruriens*	
		Seed	Callus
		-	-
	BHT (standard)	++	++
S1	Aqueous	+	+
S2	Acetone	+	+
S3	Ethanol	++	++
S4	Chloroform	Semi positive	Semi positive
S5	Petroleum ether	Semi positive	Semi positive

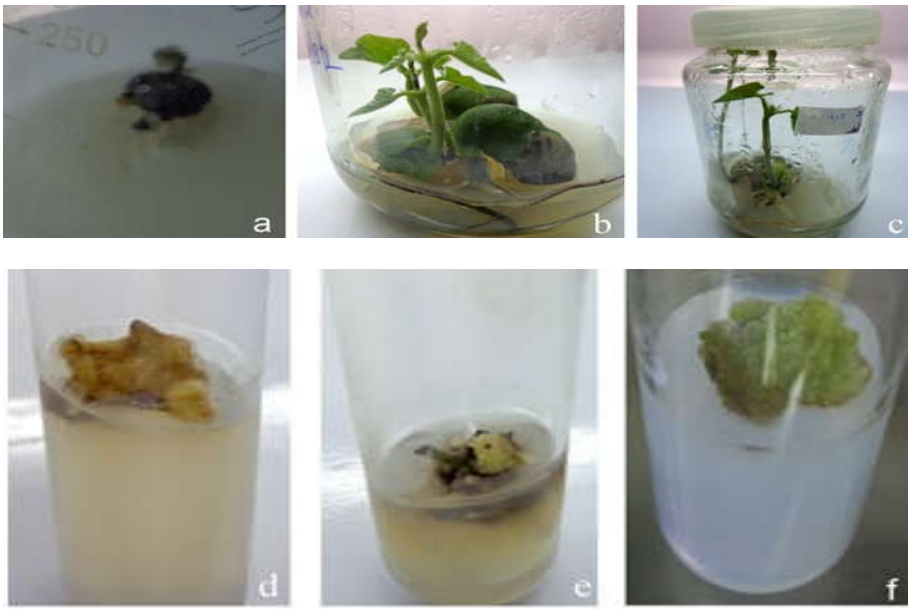
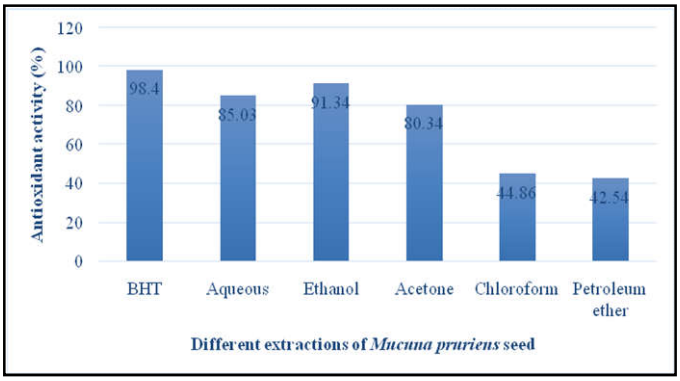


Figure 1. *In vitro* germinated seedling developed and callus induction from *Mucuna pruriens*. (a) Seeds inoculated on MS medium supplemented with 2-isopentenyl adenine 4.92 µM and Gibberellic acid 1.44 µM. (b) *In vitro* seedlings developed from *Mucuna pruriens* seeds. (c) Cotyledonary leaf and hypocotyl developed from *Mucuna pruriens* seeds. (d) Callus initiation from cotyledonary leaf explants of *Mucunapruriens*. (e) Callus initiation from hypocotyl explants of *Mucunapruriens*. (f) Secondary callus developed from cotyledonary leaf explants of *Mucuna pruriens*

100µl of seed and callus extracts were estimated for free radical scavenging activity using 1,1-Diphenyl-2-picryl hydrazyl (DPPH) assay. The samples were observed for the colour change from purple to yellow and pale pink were considered as strong positive and weak positive respectively (Table 4).

Figure 2 a. Optimum callus biomass developed on MS medium 2,4-D (4.52 µM), BA (2.22 µM) and 2iP (4.92 µM)

a)



b)

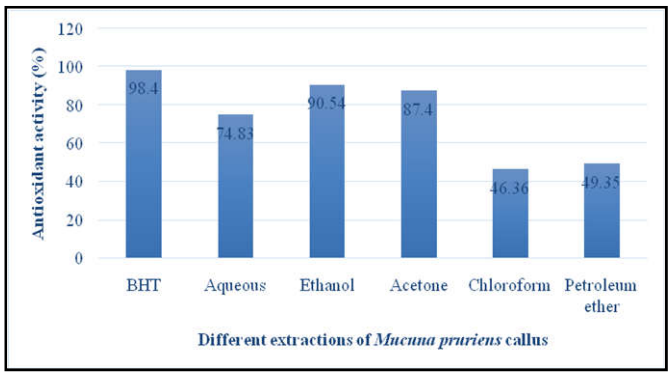


Figure 3. Quantitative analysis of antioxidant activity from seed and callus extracts of *Mucuna pruriens*

Among five different solvent callus extracts of *Mucuna pruriens*, the ethanolic seed and callus extract of *Mucuna pruriens* collected from kolli hills recorded the most effective DPPH radical scavenging activity (91.34 ± 1.05 %) followed by seed extract (90.5 ±30%). (Figure 3a,b). *Mucuna pruriens* value being very close to synthetic antioxidant (BHT) aspositive control (98.36±1.4%). The seed and callus samples of *Mucuna pruriens*, ethanolic callus and seed extracts recorded higher percentage of free radical scavenging activity followed by aqueous, acetone, chloroform and petroleum ether. In conclusion, phytochemical composition, total phenol, flavonoid contents and antioxidant activity of medicinal plants are very important in identifying new sources of therapeutically and industrially important compounds. It is imperative to initiate an urgent step for screening of plants for secondary metabolites. The present communication attempts to assess the status of phytochemicals, total phenol, flavonoid content and antioxidant activity. Thus from our findings, it is concluded that the ethanolic extracts from dry powdered callus of *Mucuna pruriens* has a superior level of antioxidant activity. The powerful antioxidant effect is attributed to the greater amount of phenol and flavonoid compound in the callus extracts of *Mucuna pruriens.*

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