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In-vitro evaluation of *Mucuna pruriens* seeds by nutritional and phytochemical analysis, assessment of antioxidant property and estimation of L-DOPA content by RP-HPLC

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Abstract
In-vitro analysis of *Mucuna prurien* seed was done to assess the nutraceutical potential of raw *Mucuna pruriens* seeds. Proximate analysis of *Mucuna pruriens* seed revealed presence of 93.60± 0.07 dry matter; 88.41± 0.06% organic matter; 5.80 ± 0.02% total ash; 25.08 ± 0.09% crude proteins; 48.48± 0.12% nitrogen free extract, 46.12 ± 0.09% soluble carbohydrate, 13.91± 0.12% neutral detergent fiber, 6.45± 0.1% acid detergent fiber & 2.65± 0.08% acid detergent lignin and 312.00 ± 1.36 kcal energy value. Qualitative phytochemical analysis in aqueous and methanolic extract revealed the presence of alkaloids, flavonoids, saponins, tannins, and phenols in both extracts. Phytosterols and terpenoids were detected in methanolic extract only. Absence of glycosides in both extract. *Mucuna pruriens* seeds were found to possess 0.29±0.21% tannins, 3.9±0.32 mg/100g saponins, 1.78±0.61 mg quercetin equivalent flavonoids, and 104.23±0.19 gallic acid equivalent polyphenols content. RP-HPLC analysis revealed 3.62+0.31% of L-DOPA levels in seeds. *In-vitro* antioxidant assay in isolated goat lymphocyte revealed concentration-dependent increase in % inhibition of radical formation by *Mucuna pruriens* extract thus decrease in H_2O_2 induced oxidative stress in goat lymphocyte suspension. These results of the study indicate that raw *Mucuna pruriens* seeds exhibit both nutritional and bioactive components thus possessing nutraceutical contents which may be of health benefits and suitable for animal utilization.

Keywords: proximate phytochemical, antioxidant , L-DOPA, *Mucuna pruriens*

Introduction
M. pruriens is a leguminous plant native to tropical countries from Africa and Asia, including India, Bangladesh, Srilanka & china (Fung *et al*., 2011) [10]. *M. pruriens* seeds have been considered & reported as wonder plant or magic velvet bean in several published reviews. (Lampariello *et al* 2012, Kavitha and thangamani, 2014, Suryawanshi *et al*., 2020, Natarajan *et al* 2012) [14, 12, 33, 22]. Mostly processed seeds of *M. pruriens* are used as unconventional feedstuff for animal utilization. *M. pruriens* seeds contain high levels of protein, essential fatty acids, carbohydrates, dietary fiber, starch content, energy value, and most of the macro and microelements (Loyra-Tzab *et al*., 2013) [16]. The presence of bioactive substances limits the use of raw *M. pruriens* seeds in animal feeding to meet the nutritional requirement of animals. Seeds are generally processed by boiling, dehulling, and trying to reduce their phytochemical contents (Mugendi *et al*., 2010) [20]. This processing makes *M. pruriens* seeds suitable for animal feeding to meet the nutritional requirement but also destroys bioactive substances that have been demonstrated in several studies to possess health-promoting beneficial properties. Thus raw *M. prurien* seeds can be used as health supplements in animals at scientifically determined levels to provide both nutritional and health benefits, thus overcoming the adverse effect of high levels of *M. pruriens* seed feeding in animals. To assess the nutraceutical potential of raw *M. pruriens* seeds for animal feeding, *in vitro* analysis of *M pruriens* seed powder was carried out to determine nutrient and phytochemical content and *in vitro* antioxidant property evaluation.

Material and Method
A raw black variety of *M. Pruriens* seeds used in this study was obtained from Herbal hills (Isha agro developers, Pune Maharashtra), and the identity was confirmed from department Ayush GADVASU Ludhiana.

These seeds were pulverized to a fine powder in the grinder and used for the study.

Proximate analysis of *M. pruriens* seed

The proximate analysis of *M. pruriens* seed powder was done in triplicate following the method on AOAC (2012) [1]. 50 g of *M. pruriens* seed powder was taken in a pre-weighed dry aluminum tray & was dried at 100°C for 24 hours in hot air over. Moisture % was calculated by 100- DM %. The dried samples were used for further analysis proximate principles. Crude protein percent in the sample was calculated from the Nitrogen content of the sample, which is determined by Macro-Kjeldahl by multiplying by 6.25 conversion factor. Crude fat was estimated by SOCS plus apparatus. Total ash was determined by charring sample on a hot plate & igniting it at 650 ^{0}C in a Muffle furnace for 3hr. Organic matter was calculated by subtracting total ash % from dry matter% of the sample. Neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin, and cellulose were estimated according to Van soest *et al*. (1991) [35]. Soluble carbohydrate % was calculated by subtracting crude protein, fat, fiber, and total ash from 100. Energy value Calculated using the formula of Osborne and Voogt (1978) [24]. Energy value (kcal/100g) = (crude protein % × 4) + (crude lipid % ×9) + (Soluble carbohydrates % × 4).

Qualitative phytochemical analysis of *M. pruriens* seed extract

For phytochemical screening first aqueous and methanolic extract of *M. pruriens* seeds was prepared. Qualitative detection of alkaloids, flavonoids, glycosides, saponins, steroids, tannin, terpenoid sand phenols was performed by following standard methods of Parekh and Chanda (2007) [25]. To 1.0ml of the extract, a few drops of dragendorff reagent were added. An orange color developed, indicating the presence of an alkaloid. The presence of flavonoids was identified by adding 5 ml of dilute ammonia solution to a portion of aqueous filtrate of seed extract, followed by the addition of concentrated H2SO4. A yellow coloration absorbed in extract indicated the presence of flavonoids. Glycoside detection was done by keller-kiliani test 1ml of the extract was dissolved in 2 ml chloroform. To that, a few drops of dilute H2SO4 were added to form a layer. A brown color indicated the presence of glycoside. Saponin detection was done by a frothing test. 1 ml extract added to 1 ml alcohol & diluted with 20 ml distilled water and shook well about 15 minutes. The formation of foam in the tube indicated the presence of saponin. Phytosterol was detected by lieberman burchard test. 2 ml acetic anhydride was added to 0.5 g methanolic extract of seed with 2 ml H_2SO_4. The formation of green color indicates the presence of steroids. Tannins were detected by the lead acetate test. A few drops of 1% lead acetate were added to 2 ml of extract. A yellowish coloration indicated the presence of tannins. Terpenoids were detected by salkowiski test 5 ml extract was mixed in 2 ml of chloroform, and 3 ml concentrated H_2SO_4 was added carefully to form a layer. A reddish-brown coloration of the interface was created indicated the presence of terpenoids. Phenols were detected by ferric chloride test. To 5 ml extract few drops of 1% FeCl3 were added. The formation of violet color indicated the presence of Phenol.

Quantitative phytochemical analysis of *M. pruriens* seed

Total phenols were estimated by Folin Ciocalteu reagent (FCR) method (Bray and Thorpe, 1954) [4]. Phenols react with phosphomolybdate in Folin Ciocalteu reagent under alkaline conditions to form a blue-colored complex of molybdenum blue, which was measured at 660 nm calorimetrically. The amount of tannin in the methanolic extract was determined by the Folin Ciocalteu reagent method with some modifications. The Spectrophotornetric method described by Brunner (1984) [5] was used for saponin analysis. Estimation of flavonoids content in plant extract was carried out using the method of Ordon *et al*. (2006) [23].

Estimation of L-DOPA content in *M. pruriens* seed by RP-HPLC

Quantitative estimation of L-DOPA was done by Agilent high-performance liquid chromatography (HPLC) as per protocol by Shivananda *et al.* (2003) [28] with some modifications. 100 mg of L-DOPA (Himedia) was weighed and dissolved in 100 ml of 0. 1M H_3P0_4. The column used was Agilent C-18 column(2mm X 150mm size), PDA detector set at 280 nm was used for analysis. The total run time was 10 min. Isocratic mobile phase of Acetonitrile: Methanol:0.1 M H3PO4 in the ratio of 10:10:80 was used. The flow rate used was 1 ml/ min. The injection volume used was 5 µl with autosampler. The observed retention time of L-DOPA was in the range of 8.87 to 8.90 min. Standards at 20,50 100,150 & 200 ppm were run under the specified conditions to get the standard curve of concentration against peak areas. L-DOPA content in the sample was calculated from the peak areas from the standard curve. The percentage of L-DOPA in the seed sample was calculated using the following formula

$$\frac{\text{Area of sample x standard wt(mg) x sample dilution x purity of standard x 100}}{\text{Area standard x standard dilution x sample wt (mg) x 100}}$$

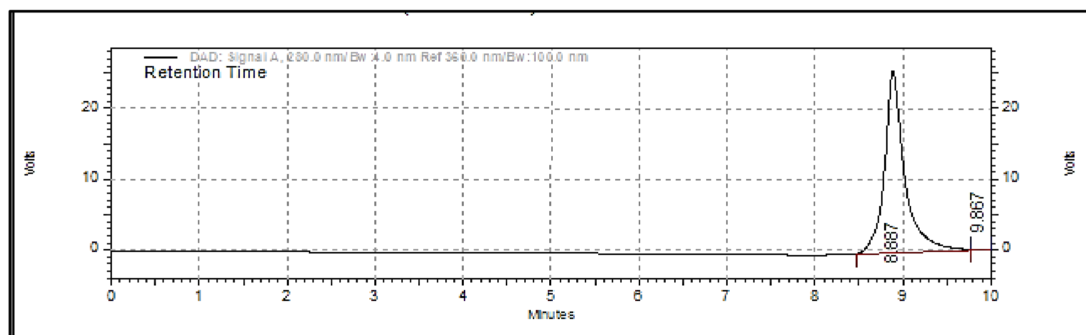

Fig 1: L-DOPA peak and retention time in RP-HPLC

Assessment of in-vitro antioxidant properties of *M. pruriens* extract in isolated goat lymphocyte homogenate-

The in-vitro antioxidant property of various concentrations (0, 50, 100, 200, and 300 µg/ml) of methanolic extract of powdered *M. pruriens* seed against various concentrations of the ascorbic acid standard was measured for H2O2 induced lipid peroxidation level in isolated goat lymphocytes by TBRS assay. Lymphocytes from blood samples were isolated using Histopaque- 1077(Sigma). Cells were counted in a hemocytometer. To observe the antioxidant potential of *M. pruriens* seed extract and lymphocyte suspension (2 x 10^6 cells/ml) were divided into four treatment groups. Treatment 1 consists of lymphocyte suspension only; treatment 2 contains lymphocyte plus 100 µM H2O2, treatment 3 contains lymphocyte suspension with 100 µM H2O2 plus the varying concentration of the ascorbic acid standard (50, 100 200 to 300µg/ml), and treatment 4 contains lymphocyte suspension with 100 µM H2O2 plus the varying concentration of *M. pruriens* seed extract (50, 100 200 to 300 µg/ml). Cells suspension were incubated for 1 hr at 37 ^{0}C, and then lymphocytes suspension was homogenized and used to determine lipid peroxidation level by MDA (malondialdehyde) assay as per Fraga *et al*. (1988) [9]. The assay evaluated the formation of a colored adduct after the stoichiometric reaction between thiobarbituric acid (TBA) and several lipid-derived aldehydes, including malondialdehyde (MDA). MDA level was determined by using a molar extinction coefficient of 1.52X10^5 /M/cm at 532 nm. Antioxidant activity was expressed as % inhibition of radical formation by using the following formula.

$$\% \text{ Inhibition of radical formation in test} = \frac{\text{MDA level in test}}{\text{MDA level in Control}} \times 100$$

Results & Discussion

Proximate Analysis of *M. pruriens* seed

Results of proximate analysis have been shown in table no 1. The present result indicates that *M. pruriens* seeds contain a moderate level of crude protein comparable to other leguminous seeds like chickpeas, peanuts, black beans green peas. Less percentage of ADF indicates high digestible content in the *M. pruriens* seeds. The energy value obtained in raw *M. pruriens* seeds was 304.48 kcal/100 g, which was equivalent to 12.67 MJ/kg of DM. Loyra Tzab *et al*. (2013) [16] reported a metabolizable energy value for *M. pruriens* fresh whole pod of 9.7 MJ/kg DM value. The energy value of *M. pruriens* seeds compares favorably with many green crops such as rape, maize, sugarcane, and Lucerne (Mcdonald *et al*., 2002) [18].

In a similar result, Vadivel and Pugalenthi (2007) [34] found a crude protein of 27.30%±0.02. Siddhuraju *et al*. (2000) [32] reported that lower levels of crude lipids (2.8-4.9%) among the investigated accession of *M. pruriens*. Ezeagu *et al*. (2003) [8] reported the presence of total carbohydrates in the range of 59.20-64.88 g/100 g in the study of 12 accessions of *M. pruriens*. Ashidi *et al*. (2019) [2] result reported crude protein 28.62, crude fat 1.87%, crude fiber 5.16, total ash 4.23, moisture 11.18%, nitrogen-free extract 60.13% in dehulled air-dried *M. pruriens* seeds.

Table 1: Proximate composition of *M. pruriens* seeds powder-

Sr. No	Parameters	Percentage
1)	Moisture	6.84± 0.04
2)	Dry matter	93.60± 0.07
3)	Ash	5.80 ± 0.02
4)	Oragnic Matter	88.41± 0.06
6)	Crude Protein	25.08 ± 0.09
7)	Crude Lipid	2.26 ± 0.13
8)	Soluble Carbohydrate	46.12 ± 0.09
9)	Nitrogen free extract (NFE)	48.48± 0.1
10)	Energy Value (kcal/100 gm)	312.00 ± 1.36
11)	NDF	13.91± 0.12
12)	ADF	6.45± 0.1
13)	Hemicellulose (NDF-ADF)	7.50± 0.09
14)	Cellulose(ADF-ADL)	3.9 ± 0.18
15)	ADL (Lignin)	2.65± 0.08

Qualitative and quantitative phytochemical analysis of *M. pruriens* seed extract

M. pruriens seed constitutes one of the richest sources of proteins suitable for human or animal diets, but their utilization is limited due to the presence of antinutritional components. Phytochemicals are often considered to be nutritionally undesirable. While these phytochemicals also confer some beneficial effects to animals if fed at smaller levels (Daromala *et al*. 2015) [7]. Hence it is important to estimate the major phytochemicals for utilizing them for food or feed purpose. Results of qualitative phytochemical screening of aqueous and methanolic extract of *M. pruriens* seeds has been shown in table no 2. Quantitative determination of phytochemicals in methanolic extract of *M. prurien* seeds has been presented in table no 3. Qualitative phytochemical analysis in aqueous and methanolic extract revealed the presence of alkaloids, flavonoids, saponins tannins in both extracts, while also phytosterols and terpenoids were detected in methanolic extract. Glycosides were not detected in both extracts.

Most of the values of estimated phytochemicals in *M. pruriens* seeds were in consonance with previous cited work by different researchers. The total phenolic content of velvet bean varied from 3.1 to 4.9%, and Tannins of *Mucuna* seeds generally range between 0.03 and 0.06% (Mohan and Janardhanan, 1995: Vadivel and Janardhanan, 2000) [19, 11]. Siddhuraju and Becker 2001b [29, 30] reported Saponins % in *Mucuna* seeds ranged between 1.2 and 1.3 while in our study we found comparatively lower saponin content of 0.77±0.32% .

Besides typical medicinal properties, several antinutritional compounds of *Mucuna* seeds have several beneficial properties. Polyphenols are important phytochemicals due to their free radical scavenging and *in vivo* biological activities (Bravo 1998) [3]. Tannins were known to have antioxidant and antibacterial properties. Saponins are known to have hypocholesterolemic, hypoglycemic, and anti-inflammatory properties. Verma *et al*. (2014) [36] reported that these compounds are known to possess free radical scavenging action thus have antioxidant properties. Alkaloids are nitrogen-containing compounds that have sedative, antistress, and analgesic roles. Phytosterols are natural sterols that may mimic the role of steroid hormones.

Table 2: Qualitative phytochemical analysis of *M. pruriens* seeds

Sr. No	Phytochemicals	Aqueous Extract	Methanolic Extract
1)	Alkaloids	+	+
2)	Flavonoids	+	+
3)	Glycosides	-	-
4)	Saponins	+	+
5)	Phytosterols	-	+
6)	Tannins	+	+
7)	Terpenoids	-	+
8)	Phenols	+	+

Table 3: Quantitative phytochemical analysis of *M. pruriens* seeds

Sr. No	Phytochemical	Concentration
1	Total phenol content mg GAE/g	64.23±0.39
2	Tannin content mg/100 g	29.17±0.21
3	Saponin content mg/100 g	771.9±0.32
4	Flavonoid content mg QE/g	1.78±0.61
5	DOPA %	3.62+0.31

Estimation of L-DOPA content in *M. pruriens* seed by RP HPLC-

Standard graph generated of peak area against varying concentration of L-DOPA standard has been graphically represented in fig 2. An appreciable amount of L-DOPA of 3.62+0.31% was found in the HPLC analysis of *M. pruriens* seeds. Siddhuraju & Becker (2001) [29, 30] reported L-DOPA concentration of 4.32% in the *M. pruriens* seeds. Prakash and Tiwari (1999) [27], found that L-DOPA varies from 3.6% to 4.2% in three accessions of *M. pruriens* bean collected in India. These results indicate that *M. pruriens* seeds are a good natural source of L-DOPA. The presence of L-DOPA in *Mucuna* seeds is a major impediment to utilizing it as food or feed. L-DOPA is known to possess anti-stress and neuroprotective activity (Pati *et al*., 2010) [26], but can have toxic effect if consumed at higher doses. L-DOPA has shown to have a negative effect on thyroid (Maayan *et al* 1986) [17]. Acute levodopa administration were found to reduce cortisol release in patients with Parkinson's disease (Muller e al 2007) [21].

Ingested L-DOPA is decarboxylated to dopamine by the enzyme L-aromatic amino acid decarboxylase in the intestinal mucosa. Only about 1% of the orally ingested L-DOPA crosses the blood-brain barrier into the central nervous system and the basal ganglia. A study by Malunga *et al*., 2008 Showed that ingested *M. pruriens* L-DOPA was extensively metabolized in the gastrointestinal tract and liver of lambs. Similarly, P Sidharaju & K becker (2001) [29, 30] showed that *M. pruriens* and L-DOPA were ruminally degraded, making it less toxic to the ruminants. Thus the overall presence of nutritional and bioactive components both in *M. pruriens* seeds may contribute to nutraceutical potential for livestock feeding.

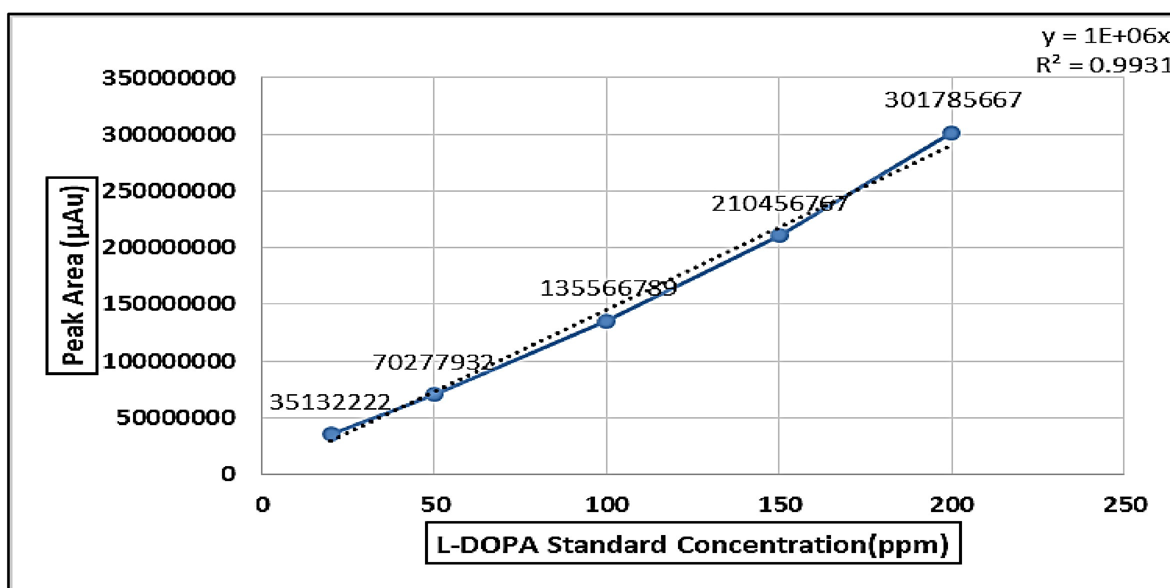

Fig 2: Standard graph of peak area against varying concentration of L-DOPA standard

Results of the in-vitro antioxidant assay on isolated lymphocyte suspension with different concentration of *M. pruriens* seed extract has been shown in table no 4, and % inhibition of radical formation has been graphically represented in fig 3. Concentration-dependent increase % inhibition of radical formation; thus, reduction in H_2O_2 induced oxidative stress in lymphocyte suspension has been found in both ascorbic acid standard and *M. pruriens* seed extract, indicating the presence of antioxidant property in *M. pruriens* seed. But antioxidant activity remains less compared to ascorbic acid at any evaluated concentration. In Similar findings, Longhi *et al.* (2011) [15] demonstrated that acid extract of *M. pruriens* has marked antioxidant activity due to high phenolic content. It was confirmed by comparing its antioxidant activity to that of vitamin C, BHA, Trolox, etc., as measured through the DPPH and ABTS assays. Kumar *et al.* (2010) [13] reported that alcohol extract of *M. pruriens* seeds showed significant antioxidant activity comparable with standard ascorbate and total phenol content. Siddharaju and Becker (2003) [31] said that *in vitro* assays with ethyl acetate

and methanolic extract of *M. pruriens* seeds showed high antioxidant and free radical scavenging activities. This antioxidant property in *M. pruriens* seed is may be due to the presence of polyphenol and flavonoid contents.

Table 4: Results of invitro antioxidant assay of *M. pruriens* seeds extract.

Sr No	Treatment		MDA (TBRS) Concentration (nMol/10^6 Cells)	% Inhibition of radical formation
1)	Lymphocyte		67.31 ± 0.98	-
2)	Lymphocyte + H_2O_2		412.91 ± 1.32	-
3)	Lymphocyte suspension + H_2O_2+ Ascorbic acid standard	50 µg/ml	291.45 ± 1.29	29.42
		100 µg/ml	242.87 ± 1.11	41.19
		200 µg/ml	203.51 ±1.21	49.29
		300 µg/ml	168.56 ± 0.97	59.18
4)	Lymphocyte suspension+ H_2O_2+ *M. pruriens* Seed Extract	50 µg/ml	344.67 ± 1.76	16.53
		100 µg/ml	298.23 ± 1.65	27.78
		200 µg/ml	264.54 ± 1.14	35.94
		300 µg/ml	211.32 ± 0.98	48.83

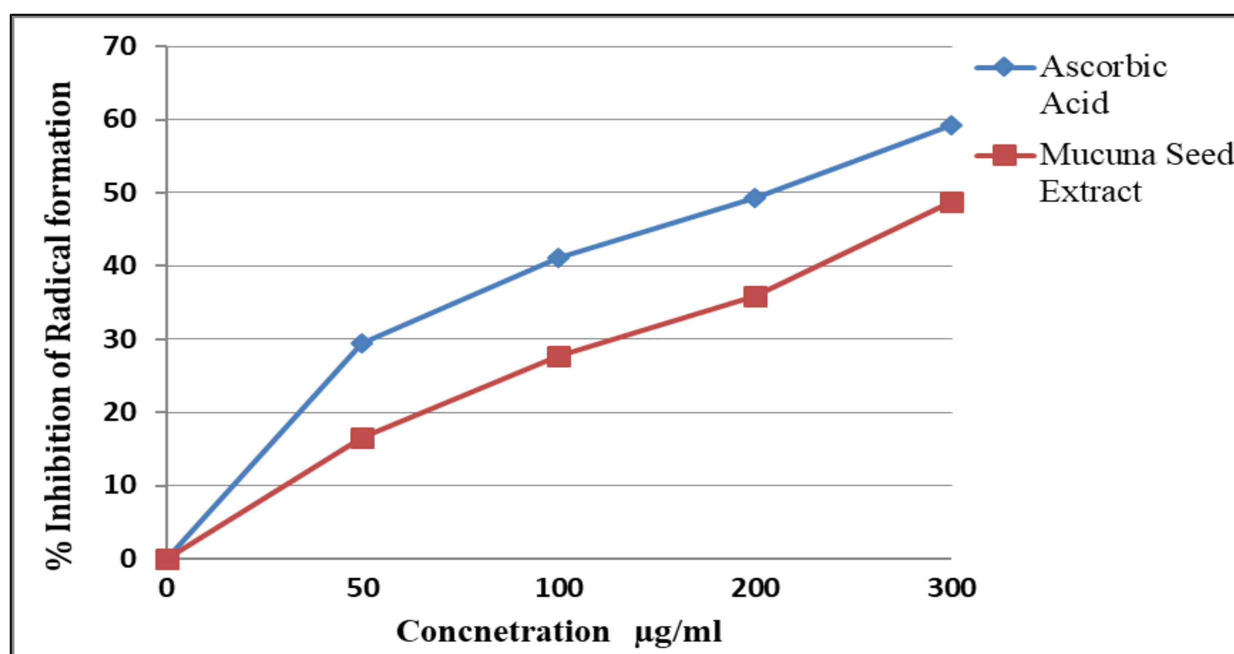

Fig 3: % Inhibition of radical formation by *M. pruriens* seed extract compared to ascorbic acid standard

Conclusion

Thus it may be concluded that raw *M .pruriens* seeds are a rich source of nutrients equivalent to other leguminous plants. Several phytochemical components identified in *M. pruriens* seeds may provide additional health benefits to the animals if raw seeds are used at scientifically determined levels. As revealed from RP-HPLC analysis, *M. pruriens* seeds are a good natural source of antistress molecule L-DOPA. *M. pruriens* seed extracts served as a significant source of natural antioxidants, which might be helpful in preventing the progress of various oxidative stress-related conditions. Though presence of bioactive substance limits use of raw *M. pruriens* seeds at higher level. These raw seeds can be better utilized at lower level in order to provide both nutritional and health beneficial bioactive effects to the animals. Extensive *in vivo* studies in animals can be carried out to determine scientifically defined levels of raw seeds as nutraceutical suitable for animal feeding.

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