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Research Article

COMPARATIVE PHYTOCHEMICAL EVALUATION AND ANTIBACTERIAL ACTIVITY OF TWO DIFFERENT GERMPLASM OF *MUCUNA*

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ABSTRACT

Two different germplasm of *Mucuna* collected from agro geographical regions was evaluated for its antioxidant potential, phytochemical compounds and antibacterial activities. Between two, black-coloured germplasm was registered for higher levels of phytochemical compounds viz, total free phenolics (4.281g 100^{-1}), tannins (0.356 g $100g^{-1}$), flavanoids (0.381g 100g-1), alkaloids (1.540 g 100g-1) and saponins (0.525 g 100g-1). Appreciable levels of nitric oxide scavenging (46.16%) and iron chelating activity (51.16%) was observed in both the germplasm. While, increasing the concentration of solvent extract together with increasing in the antioxidant activities was noticed. In general, solvent extracts were effective against microbes such as *E.coli*, *B.subtilis*, P. *aeruginosa* etc, as shown in inhibitory zones. High possibility for the existence of carboxylic acids, aromatic compounds in the studies seeds extracts as evidenced by FTIR spectra. To sum up, the antibacterial, antioxidant potential of the extracts this might be responsible by such compounds. However, further mechanistic study is needed to validate such specified isolated compounds in *in vivo.*

Keywords: Germplasm**;** *Mucuna*; Phytochemical compounds; Antioxidant activities; FTIR

INTRODUCTION

Mucuna pruriens (*Fabaceae*), velvet bean, is found in Asia, America, Mexico and Eastern Nigeria. It has been used as green manure cover crop (GMCC); improving the soil quality and in food the seeds are used as soup thickeners. In Ayurveda, the decoction of the seeds is known to be used for remedying tuberculosis, diabetes and cancer. *Mucuna pruriens* is also used to prepare various formulations, which are used as medicines or alleviating certain diseases[1] .The phytochemical research based on ethno-pharmacological information is generally considered as an effective approach in the discovery of new anti-infective agents from higher plants[2]. Chemical constituents may be therapeutically active or inactive. The chemical constituents may be used for the various purposes besides antimicrobial agents. Antibiotics are also known to disturb the natural intestinal microflora[3], thus, depriving the benefits of these microbes to human body. Medicinal plants exhibit antibacterial activity[4], since they contain innumerable biologically active chemical constituents. The extract also exhibits anti-inflammatory activity, as it is known to inhibit carrageenin-induced edema[5]. Seeds of this wild legume are widely used for treating male sexual dysfunction in Unani Medicine[6]. Over the past two decades, intensive efforts have been made to discover clinically useful antibacterial/antifungal drugs[7,8,9]. The present study is aimed to evaluate the antibacterial activity and to validate the chemical compounds which are responsible for the bioactivity in two different accessions of *Mucuna*.

MATERIALS AND METHODS

Collection of Seeds

The *Mucuna* seed germplasm (white- coloured and black -colored seed coat), were collected from Tamil Nadu, Western Ghats, South India, After drying thoroughly in sunlight for 2-3 days, the parts were thrashed to remove mature seeds; the seeds after thorough cleaning were stored in plastic containers at room temperature (25°C) until further use.

Preparation of Seed samples

Dry mature seeds of different accessions (10 g each) were powdered in a Wiley Mill to 60-mesh size with suitable precaution to avoid contamination of samples. The powders were stored in plastic containers at room temperature (25°C) until further use.

Solvent Extraction

Solvent systems used for the extractions were acetone, ethanol, chloroform, petroleum ether, hexane, methanol and water. Soxhlet and flask extraction procedures were adapted for extraction. 10g of each powered samples were packed in muslin cloth and used for extraction by soxhlet apparatus at a temperature below the boiling temperature of each solvent. A portion of the powdered plant samples was soaked in the conical flask containing solvent, wrapped with aluminum foil and placed in shaker for 48 hours at 120-130 rpm.

After 48 hours, the extracts were filtered using Whatman filter paper No: 1. the solvent was evaporated and the residue was dissolved in sterile dimethyl sulfoxide (DMSO-9:1) in 50 mg/ml concentration. The extract was filtered using 0.22 micro filters (Type GV- Millipore) and stored at 4°C for further antibacterial activity study.

Phytochemical screening of the Seed extract

Solvent extract of seed powder was analyzed for the presence of glycosides, tannins, phenols, flavanoids, alkaloids, saponins, steroids and terpenoids in accordance with the standard methods [10,11].

Estimation of Total Phenolics

Total phenolic contents in the plant extracts were determined with the Folin-ciocalteu reagent according to the method of Wolfe and co workers[12]. The absorbance of seed extracts was measured at 765 nm. Total phenol content was expressed as gallic acid equivalents.

Estimation of Total Tannins

The total tannin content in the two extracts was determined by modified method of Burns[13]. The absorbance was measured at 755 nm .The total tannin in the extract was expressed as equivalent to tannic acid.

Estimation of total Flavanoids

Total flavanoids were estimated by adopting the methods of Ordonez et al[14]. The absorbance was measured at 420 nm. Total flavanoids content was calculated as quercetin equivalents.

Estimation of Total Alkaloids

The total alkaloid contents in the seed powder samples were measured using 1, 10-phenanthroline method[15] with slight modifications. The absorbance of red coloured complex was measured at 510nm against reagent blank. Alkaloid contents were estimated and it was calculated with the help of standard as colchicines. The values were expressed as $g.100g^{-1}$of dry weight.

Estimation of Total Saponins

The total saponins contents in the seed powder extracts were determined with the Folin-ciocalteu reagent according to the method of Sutharsingh et al.[16]

Evaluation of Total antioxidant capacity by Phosphomolybdenum method

The total antioxidant capacity of the plant extracts was assessed by phosphomolybdenum method[17]. An aliquot of 0.3 ml of the sample solution (three replicates) was mixed with 2.7 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The absorbance of the test sample was measured at 695 nm. Gallic acid was used as standard. The antioxidant activity was expressed for the samples as ascorbic acid equivalents (mg/g of ethanol extract).

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was measured by following the method of Garrat[18]. The reaction mixture (3ml) containing 2 ml of sodium nitroprusside (10mM), 0.5 ml of phosphate buffer saline (1M) were incubated at 250ºC for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite was pipetted and mixed with 1 ml of sulphanilic acid reagent (0.33%) and allowed to stand for 5 min for completing diazotization. Then 1 ml of naphthylethylene diamine dihydrochloride (1% NEDA) was added, mixed and allowed to stand for 30 min. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions which can be estimated by the use of Griess Illosvery reaction at 540 nm.

Superoxide radical scavenging activity

Measurement of superoxide anion scavenging activity was performed based on the method described by Winterbourne[19]. The assay mixture contained sample with 0.1ml of Nitro blue tetrazolium (1.5 mM NBT) solution, 0.2 ml of EDTA (0.1M EDTA), 0.05 ml riboflavin (0.12 mM) and 2.55 ml of phosphate buffer (0.067 M phosphate buffer). The control tubes were also set up where in DMSO was added instead of sample. The reaction mixture was illuminated for 30 min and the absorbance at 560 nm was measured against the control samples. Ascorbate was used as the reference compound.

Iron chelating activity

The method of Benzie and Strain was adopted for the assay[20]. The reaction mixture containing 1 ml of 0.05% O-Phenanthroline in methanol, 2 ml ferric chloride (200µM) and 2 ml of various concentrations ranging from 10 to 1000µg was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator. The experiment was performed in triplicates.

Determination of Reducing Power assay

Reducing activity was carried out by following the method of Oyaizu[21]. Different concentration (1000, 500,250,125 µg /ml) of seed extracts (dry ethanol) were prepared with DMSO and taken in test tube as triplicates. To test tubes 2.5 ml of sodium phosphate buffer and 2.5 ml of 1% potassium ferric cyanide solution was added. These contents were mixed well and were incubated at 50^{o} C for 20 minutes. After incubation 2.5ml of 10% TCA was added and were kept for centrifugation at 3000rpm for 10 minutes. After centrifugation 5ml of supernatant were taken and to this 5ml of distilled water was added. To this about 1ml of 1% ferric chlorite was added and was incubated at 35^{0} C for 20 minutes. The O.D (absorbance) was taken at 700nm and the blank was prepared by adding every other solution but without extract and ferric chloride (0.1%) and the control was prepared by adding every other solution but without extract. Increase in absorbance of the reaction mixture indicates increased reducing power. The experiment was conducted in triplicates and values are expressed as equivalents of ascorbic acid in µg / mg of extract.

Screening for Antibacterial activity

Bacterial Strains

Microorganisms (*Protease vulgaris, E. coli, B. subtilis, E. faecalis, S. aureus, K.pneumonia, Enterobacter aerogens, Yersinia enterocolitia*) were obtained from the Department of Biotechnology, SRM University, Chennai, India.

The agar well diffusion method as described by Ntiejumokwu and Alemika [22]and Ogueke et al[23] was used to determine the inhibitory effects of the seeds extracts against the isolates. The bacterial isolates were first grown in nutrient broth for 18 h at 37°C, then 0.2 ml of the broth culture of the isolates were aseptically inoculated onto a molten nutrient agar which had been cooled to 45°C, mixed gently and poured into sterile petridishes and allowed to set. The suspension was diluted with sterile distilled water to obtain approximately 10^{-5} CFU/ml.

These were delivered into wells (8 mm diameter) bored unto the surface of the inoculated nutrient agar plates. The extracts were allowed to diffuse into the medium for 30 min. The plates were incubated at 37°C for 24 to 48 h. The zones of inhibition were measured in millimeter diameter using meter rule [24].

Fourier Transformer Infra red Spectrophotometer (FTIR) mediated Identification of functional group

Fourier Transform Infrared (FTIR) spectrophotometer was used to identify the characteristic functional groups in the seed extract. A small quantity (5 mg) of the seed extract was dispersed in dry potassium bromide (KBr). The mixture was thoroughly mixed in a mortar and pressed at pressure of 6 bars within 2 min to form a KBr thin disc. Then the disc was placed in a sample cup of a diffuse reflectance accessory. The IR spectrum was obtained using Perkin Elmer 2000 infrared spectrometer. The sample was scanned from 400 to 4000 cm^{-1} for 16 times to increase the signal to noise ratio.

RESULT AND DISCUSSION

The qualitative phytochemical analysis of ethanol extracts of seed of *M. pruriens* registered the presence of alkaloids, steroids, terpenoids, tannins, flavanoids, phenols, glycosides, and saponins (Table-1).

Table: 1: Phytochemical screening of *Mucuna pruriens* (black and white coloured germplasm)

Phytochemical	Acetone		Chloroform		Ethanol		Methanol		Hexane		Petroleum Ether		Water	
	BS	WS	BS	WS	BS	WS	BS	WS	BS	WS	BS	WS	BS	WS
Alkaloids	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Steroids	+	+	+	+	-	-	+	+	+	+	+	+	-	-
Triterpenoids	+	-	-	-	-	-	-	-	+	+	+	+	-	-
Flavanoids	-	-	+	+	-	-	-	+	-	-	-	-	-	-
Tannins	-	+	-	-	+	+	-	+	-	-	-	-	+	+
Phenols	-	+	-	-	+	+	-	+	-	-	-	-	+	+
Glycosides	-	-	-	-	-	+	-	-	+	+	-	-	-	-
Saponins	+	+	+	+	+	-	-	+	-	-	+	+	+	+

(+) Present; (-) Absent, BS- black colored seed; WS-white colored seed

Between two germplasm, phytochemical compounds viz., phenolics, tannins, flavanoids, alkaloids and saponins were found to be present higher levels in black-coloured seeds i.e., 4.281 GAE g $100g^{-1}$,0.356 TAE $g100g^{-1}$; 0.381 QE g $100g^{-1}$; 1.540 CE g $100g^{-1}$ and 0.525, respectively. (Table-2).

Table 2: Quantitative analysis of phytochemical constituents *M. pruriens* (black and white coloured germplasm)

Phytochemical compounds	*Mucuna pruriens* (WS)g/100g	*Mucuna pruriens* (BS)g/100g
Phenolics[a]	4.016 ± 0.039	4.281 ± 0.035
Tannins[b]	0.305 ± 0.025	0.356 ± 0.071
Flavanoids[c]	0.343 ± 0.040	0.381 ± 0.058
Alkaloids [d]	1.410 ± 0.184	1.540 ± 0.098
Saponins	0.509 ± 0.094	0.525 ± 0.015

All the values are determine triplicates mean ± Standard Error ;

a- Gallic acid equivalent; b - Tannic acid equivalent ;

c- Quercetin equivalent; d - Colchicines equivalent's BS-black seed ;WS-white seed

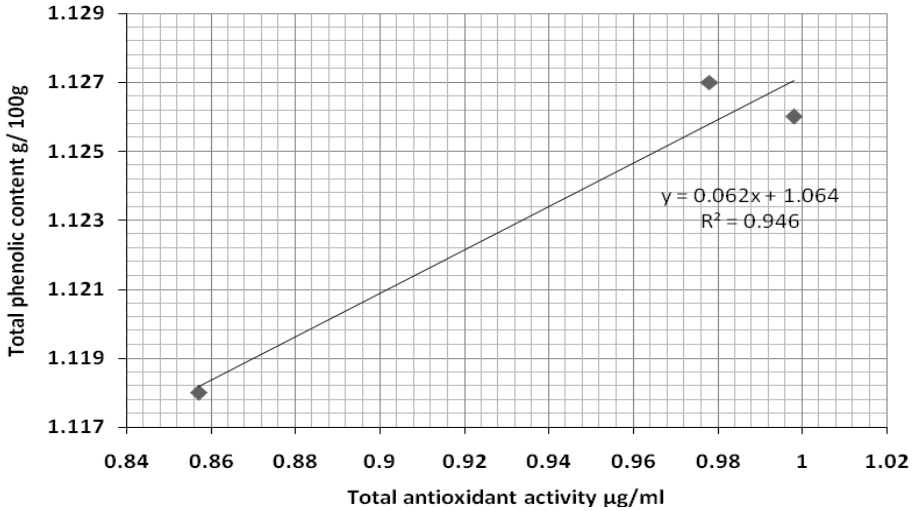

Fig. 1: Scatter plot showing the correlation between Total antioxidant activity (%) and Total phenols (g/100g) in black and white - coloured germplasm.

Table 3: Ethanolic extract of *M.pruriens* black and white coloured germplasm

Concentration in µg/ml	Nitric oxide scavenging activity (%)		Superoxide radical scavenging activity (%)		Iron chelating activity (%)		Reducing power capacity (%)	
	BS	WS	BS	WS	BS	WS	BS	WS
125	32.31±0.01	32.01±0.21	13.31±0.01	12.01±0.02	16.31±0.01	14.01±0.21	1.01±0.02	0.97±0.02
250	37.30±0.10	36.13±0.01	21.30±0.00	19.13±0.31	32.30±0.10	30.13±0.01	4.23±0.02	4.1±0.02
500	42.05±0.21	41.05±0.23	27.05±0.11	25.05±0.22	37.05±0.21	35.05±0.23	7.31±0.02	6.8±0.02
1000	46.16±0.04	46.12±0.02	33.16±0.04	32.16±0.12	51.16±0.04	50.16±0.02	11.2±0.02	10.01 ±0.02

All the value determined triplicates mean ± standard error. BS-black colored seed; WS-white colored seed

Total phenolic content of the seed extract of both black and white coloured germplasm significantly correlated with total antioxidant activity was y=0.062x + 1.064(R^2= 0.946) compared to other studied antioxidants(Fig-1).

These results obtained in the present study are in good consonance with the earlier reports on the other germplasm of *Mucuna* [25,26]. The percentage of iron chelating activity was accounted upto 51.16% (black coloured germplasm) and 50.16% (white coloured germplasm) as ascorbic acid equivalents (Table- 3).

Between two germplasm studied, the black- coloured ones was reported to have high level of phytochemical compounds and showed better antioxidant activities. Antioxidants can be defined as the compounds of lipids or other molecules by inhibiting the initiation or propagation of an oxidizing chain reaction and by many other mechanisms and thus prevent disease [27,28]. The involvements of free radicals in the pathogenesis of a large number of diseases are well documented. A potent scavenger of free radicals may serve as a possible preventative intervention for the diseases [29]. The various methods for measuring antioxidant activity differ in their reaction mechanisms, the target substance on which they act, the conditions under which they are carried out and the way in which results are expressed. As a result, polyphenols with a different relationship structure antioxidant activity can come out; in this sense, the relationship between the chemical skeleton of flavonoids and the ability to scavenge radicals has been suggested elsewhere [30,31].

Antibacterial activity of *Mucuna pruriens* seed extracts against *P. vulgaris, E.coli, B. subtilis, E. faecalis, S. aureus, K. pneumonia, E. aerogens and Y. enterocolitia* were represented (Table- 4&5).

Chloromphenicol, standard antibiotic, was used for comparing with the seed extracts. In comparison with other solvents used in the present study, methanol, ethanol and water extracts of seeds were given better efficiency and showed significant antibacterial activity against all the studied pathogens. On overall, the seed extracts are very active against all the tested pathogens. In the case of solutions with a low activity, however, a large concentration or volume is needed. Because of the limited capacity of discs, holes or cylinders are preferably used[32]. It

has been suggested that the antibacterial activity is mainly due to the presence of essential oil, flavonoids and triterperoids & other natural polyphenolic compounds of free hydroxyl groups[33].

Based on the results of above experiments, FTIR spectroscopic studies were carried out to validate the phytochemical compound deciphering for such activities (Table-6).

Table 4: Antibacterial activity of velvet bean (black colored seeds) by well diffusion method

Test Microorganisms	Zone of inhibition(mm diameter)								
	Positive control	Solvent control	chloroform	Methanol	Ethanol	Petroleum ether	Water	Acetone	Hexane
E.coli	20	-	12	11	12	9	11	11	12
P. vulgaris	13	-	11	10	9	10	11	9	9
K.pneumoniae	16	-	9	9	11	9	9	7	9
E.faccalis	19	-	9	10	7	10	9	9	10
E.aerogens	14	-	10	8	9	7	8	8	8
B. subtilis	17	-	12	9	9	9	9	10	7
Y. enterolitica	16	-	11	8	10	11	7	9	8
S.aureus	21	-	9	8	11	7	7	9	7

Concentration of extract- 100µl/well, (-) - No zone of inhibition observed, Positive controls – chloromphenicol (10µg/ml), Solvent control - 10% DMSO.

Table 5: Antibacterial activity of *M.pruriens* (white colored seeds) by well diffusion method

Test Microorganisms	Zone of inhibition(mm diameter)								
	Positive control	Solvent control	chloroform	Methanol	Ethanol	Petroleum ether	Water	Acetone	Hexane
E.coli	20	-	11	12	10	9	11	9	9
P. vulgris	13	-	10	9	9	9	11	9	8
K.pneumoniae	16	-	9	9	11	8	9	8	9
E.faccalis	19	-	9	11	9	11	8	9	11
E.aerogens	14	-	10	10	9	7	6	8	7
B.Subtilis	17	-	12	9	8	8	9	11	9
Y. enterolitica	16	-	11	8	10	11	7	9	7
S.aureus	21	-	9	11	12	10	7	8	7

Concentration of extract- 100µl/well, (-) - No zone of inhibition observed, Positive control– chloromphenicol (10 µg/ml), Solvent control - 10% DMSO.

Table 6: IR Spectroscopic analysis of *M.pruriens,* which showing Characteristic absorption peaks at IR range.

Wave number (cm^{-1})		Type of Bond	Compound type
BS	WS		
3399.44	3298.43	N-H(s), O-H	Amines ,Alcohol
2925.45	2925.72	N-H (s)	Amine salts
-	2364.07	N-H (s), O-H	Amine salt, Amides
1744.78	1744.91	C=0(s), N-H (b)	Amides
1655.98	1654.11	C=0 (s), N-H (b)	Amides
1545.96	1542.09	Asym.(s), Asym.(s) strong	Aliphatic; aromatic, nitro compounds
1407.00	1406.29	P-CH3 (b)	Phosphine
1249.82	1247.61	C-F (S),C-Br (s)	Flurides, Aryl Bromides
-	1157.55	C-Br (s) ,C-I (s)	Bromides, Iodides
1050.16	1052.18	C-Br (s) ,C-I (s)	Bromides, Iodides
860.79	860.67	S-O (s)	Sulfonates
572.33	572.59	C-Br (s) C-I (s)	Bromides, Iodides

(-) – Not observed; BS-Black coloured germplasm, WS- White coloured germplasm

The spectra of seed extracts were recorded in the form of an interferrogram to which results of various functional groups were exhibited between the wave length of 400–4000 cm^{-1}. Eight functional groups were found(Fig -2&3).

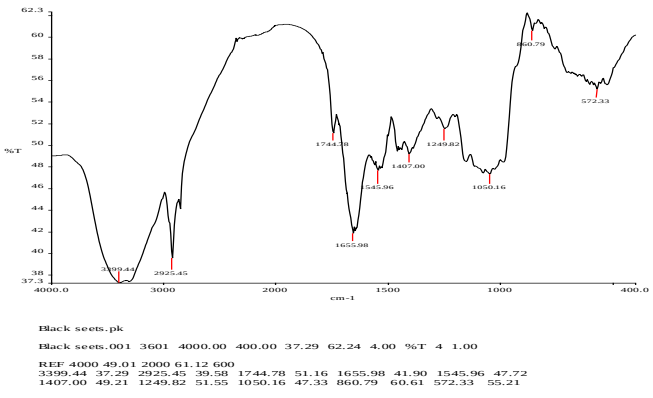

Fig. 2: IR Spectroscopic analysis *M.pruriens*(black - coloured germplasm)

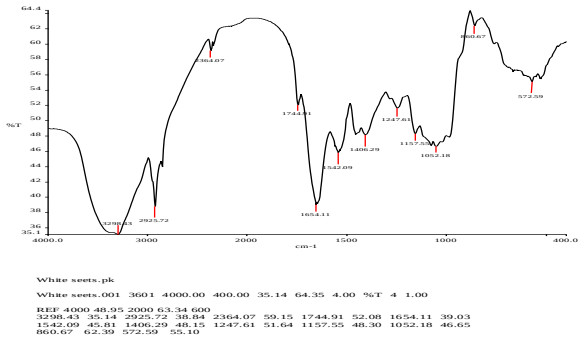

Fig. 3: IR Spectroscopic analysis *M.pruriens*(white coloured germplasm)

The spectrum denoted a broad range at 3399.44 and 3298.43cm^{-1} for black and white –coloured accessions, respectively. Which has been assigned to NH(s), O-H stretching vibrations which in turn conforms amines and alcohols present in the extract. Strong to medium intensities of broad band was observed at 1744.78 - 1744.91 cm^{-1} and 1655.98-1654.11cm^{-1} in both the seed extracts. This confirms the presence of carboxylic acid groups. Other bands are observed to be appearing at 1050.16-1052cm^{-1} and 860.79-860.67 cm^{-1} are attributed to the existence 1545.96-1542.09 cm^{-1}aromatic groups and also bromides and sulfonates groups respectively. The antibacterial activity of the seed extracts might be attributed by the presence of these studied functional compounds such as carboxylic acid, aromatic acids and bromides. In other cases, Jabeen et al[34] had mentioned that the elimination of fungal pathogens by the seed extract of *Moringa oleifera* can be attributed to the presence of carboxylic acids. The results of the present study is highly correlated with other reports obtained by Shittu and co workers[35], who had earlier reported on the seeds of *Sesame radiatum* wherein the carboxylic acids being responsible for the antimicrobial activity.

CONCLUSION

Two different germplasm of *Mucuna* was collected and evaluated for its phytochemical quality and biological activities. Between two germplasm, black coloured germplasm was registered for higher levels of phytochemical compounds and expressed significant antioxidant potential. Ethanol and methanolic solvents extracts of seed seemed to be brought better extraction of phytochemical compounds studied in the present report. Antioxidative potential of seed extract is highly comparable with standards. FTIR spectroscopic study revealed the presence of amines, carboxylic acid, and other aromatic compounds, which might be responsible for such activities. Further mechanistic study is needed to isolate the specified compounds to assess the bioactivities in vivo.

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