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Original Article

High Performance Liquid Chromatography Standardisation of the Hydroalcoholic Extract of *Phaseolus radiatus* Beans Based on Vitexin Content

Leena R Dhoble*, Prakash R Itankar

Department of Pharmaceutical Sciences, R.T.M. Nagpur University, Nagpur, Maharashtra, INDIA.

ABSTRACT

Aim: Our research work deals with chromatographic standardisation of the 'smart crop' mungbean or *Phaseolus radiatus* L. Wilczek using high performance liquid chromatography (HPLC) with respect to vitexin, in addition to pharmacognostic and phytochemical analysis. **Materials and Methods:** Pharmacognostic standardisation of the plant included macroscopy, powder microscopy, physicochemical parameters, preliminary phytochemical screening, heavy metal testing and microbial contamination studies. Further, '70% *v/v* hydro-ethanolic mother extract of *Phaseolus radiatus*' (PR) was obtained by maceration. Quantitative estimations and antioxidant assay of PR was performed followed by qualitative thin layer chromatography (TLC). Lastly, PR was quantified by HPLC using vitexin as the marker compound. **Results:** The percent yield obtained for PR was found to be 45.6 % w/w. IC_{50} value of PR was found to be 38.96 µg/ml. The R_f value of PR obtained by TLC was calculated to be 0.78, complementary to the reported R_f of vitexin. Further, HPLC studies helped in the confirmation and quantification of vitexin. **Conclusion:** The study helped in successful development of quality testing parameters and achieve phytochemical standardisation of *Phaseolus radiatus* plant with respect to vitexin, a principle constituent of mungbean. From the results obtained by antioxidant assay, TLC and HPLC, vitexin may be attributed to be one of the phytoconstituents responsible for the significant antioxidant potential of mungbean which can become a marker for deciding the quality of mungbean to be used for preparation of Ayurvedic medicines.

Key words: HPLC, Mungbean, *Phaseolus radiatus*, Quantification, Standardisation, Vitexin.

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INTRODUCTION

Vigna radiata (L.) Wilczek, synonym *Phaseolus radiatus* (Figures 1 and 2) belonging to the Fabaceae family, is a leguminous plant species fondly consumed worldwide as traditional food for more than 3500 years. Commonly known as mungbean, moong, green gram and golden gram, it is indigenous to India and widely cultivated in tropical and sub-tropical countries. The plant is a short-duration (75-90 days) annual creeping crop which grows up to 90 cm in height in warm climates (35°C). Leaves are pale green in colour, alternate and trifoliate in shape characteristics, and flowers are greenish yellow to pale yellow. Fruits contain 10-15 cm ellipsoidal green, yellow or black mottled pods upto 12 cm, and are pendant, glabrous and linear-cylindric.[1,2] The chemical constituents found in this plant include catechin, epicatechin, *p*-hydroxybenzoic acid, *p*-coumaric acid, ferulic acid, protocatechuic acid, sinapic acid, syringic acid, gallic acid, quercetin, rutin, kaempferol, isoquercetin, robinin and kaempferol-7-*O*-rhamnoside. The polyphenols vitexin and isovitexin (Figure 3 and 4) are major phytoconstituents found in the seed coats of mungbean.[3] Ayurveda considers mungbean as daily recommended food possessing a distinctive property due to its nourishing, strength promoting and tissue regenerating benefits. Mungbean soup is used post-*panchakarma* to strengthen the digestive fire. Ayurvedic texts classify it as *drishta prasadana* i.e. good for the eyes, *jwaragna* meaning it relieves fever, *varnya* which improves skin complexion, *pushti bala prada* i.e. nourishes and increases physical strength.[4] Mung beans are proven to possess antioxidant, anti-diabetic, antimicrobial, anti-hyperlipidemic, antihypertensive, anti-inflammatory and anticancer properties. Known adulterants include nutritional substitution of mungbean sprouts with sunflower or soybean sprouts.[1]

Legumes, as human food crops are an excellent source of proteins, minerals, bioactives and vitamins as compared to cereals. Hence, they are also known as 'poor man's meat' addressing the significance of legumes in terms of food security.[3] Mungbeans are a rich, easy to digest and cost-effective source of protein, making it an indispensable pulse used as ingredient in majority Indian households. India produces 30% of the global output of mungbeans.[4] Mung is a nitrogen-fixating legume that works in symbiosis with *Rhizobium* bacteria, ensuring enough quantity of nitrogen in soil, essential for the healthy growth of succeeding plants. Additionally, being a smother crop, it is often intercropped with others to suppress weed growth, consequently minimizing the cost of production.[6] Taken together, it is quite evident that this indigenous 'smart food' has tremendous commercial potential along with major health benefits, food security and sustainable crop production.

However, several reports have pointed major issues with mungbean crop like insect adulteration, substitution, mould spoilage, lower weight of beans, decreased nutritional value and low seed germination rates.[7] All these factors reduce the commercial value of mung crop in the

Correspondence: Ms. Leena R Dhoble, Department of Pharmaceutical Sciences, R.T.M. Nagpur University, Nagpur-440 033, Maharashtra, INDIA.
E-mail: leena.dhoble@outlook.com

Figure 1: *Phaseolus radiatus* plants.
(Source: http://www.innerpath.com.au/matmed/herbs/Vigna~radiata.htm)

Figure 2: *Phaseolus radiatus* beans or mungbeans.
(Source: https://en.wikipedia.org/wiki/Mung_bean)

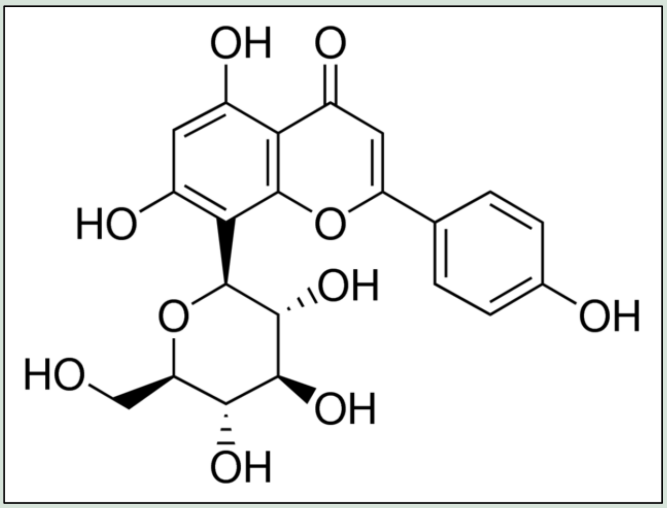

Figure 3: Chemical structure of vitexin.
(Source:https://www.sigmaaldrich.com/IN/en/product/sial/49513?gclid=EAIaIQobChMIyJiWq9Op9gIVDhsrCh3dEgS2EAAYASAAEgIrVPD_BwE)

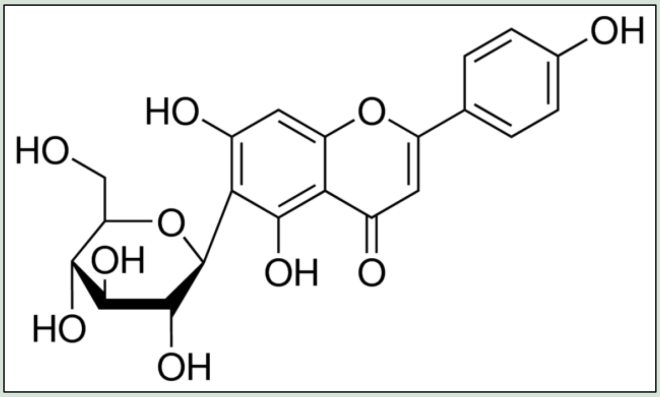

Figure 4: Chemical structure of isovitexin.
(Source:https://www.sigmaaldrich.com/IN/en/product/sigma/17804?gclid=EAIaIQobChMIoam20NOp9gIVkzMrCh09yQu-EAAYASAAEgIhEvD_BwE)

market, necessitating a need for its standardisation moreover, when it is going to be used as per Ayurvedic procedures for cure and management of diseases listed earlier. To fill this knowledge gap our study, for the first time, specifically focused on chromatographic standardisation of mungbean extract using high performance liquid chromatography with respect to vitexin, which is a major phytoconstituent present in seed coat of mungbeans with potential to be used in several inflammatory related conditions.

MATERIALS AND METHODS

Crude plant material

The mungbeans were procured from local market and authenticated (Specimen no. 10339) by Dr. Nitin Dongarwar, Professor from Department of Botany, R.T.M. Nagpur University, Nagpur.

Pharmacognostic standardisation

WHO guidelines on quality control methods for herbal materials (2011) were followed for examination of the coarsely powdered crude plant material (CPM). Macroscopy, powder microscopy, ash value, extractive value, loss on drying, swelling index, foaming index, determination of foreign matter, heavy metal testing and microbial contamination of CPM were examined.[8] Lastly, preliminary phytochemical screening was carried out for both CPM and mother extract to infer the presence of various primary and secondary metabolites like sugars, proteins, sterols, alkaloids, saponins, tannins and flavonoids.[9]

Extraction

The CPM (100 g) was macerated with the menstruum (ethanol + water in 70:30 % *v/v* ratio) for a period of 7 days, with occasional stirring. Extract was then filtered using Whatman filter paper. The marc was repeatedly subjected to maceration until complete drug exhaustion was achieved. This '70% *v/v* hydroalcoholic mother extract of *Phaseolus radiatus* beans' (PR) was further evaporated using Rotary evaporator to obtain a concentrated mother extract of semisolid consistency and the yield was calculated as '% *w/w*'.[10] (Table 1)

Table 1: Specifications of PR.

CPM	*Phaseolus radiatus*/ mungbean
Plant part used	Seeds or beans
Form of RPM	Coarsely powdered
Method of extraction	Maceration
Quantity of RPM used	100 g
Quantity of extract obtained	45.6 g
% Yield	45.6 % w/w

Analysis

Quantitative estimations

The quantitative estimations of phenolics,[11] tannins,[12] flavonoids, flavanols,[13] alkaloids,[14] carbohydrates[15] and saponins[16] were carried out to calculate the amount of these primary and secondary metabolites, which may be potentially responsible for various pharmacological activities of the plant.

Antioxidant activity

The evaluation of antioxidant activity of PR was done by using free radical scavenging *in vitro* assay. 1M solution of 1, 1-diphenyl -2-picrylhydrazyl (DPPH) was prepared by dissolving 4.0 mg of DPPH in 100 ml of 99% methanol and stored in cool and dark place. Standard ascorbic acid solution was prepared by dissolving 2.0 mg of the acid in 2.5 ml of water to yield a stock solution of 800 µg; further 6 aliquots corresponding to 12.50 µg, 25 µg, 50 µg, 100 µg, 200 µg and 400 µg were prepared by serial dilution. Sample solution was prepared by dissolving 1.0 mg of mung mother extract in 25 ml of 99% methanol in a conical flask, further subjected to mechanical shaking at room temperature for about 30 min. This was followed by centrifugation at 6000-8000 rpm for 15 min. Afterwards, the tubes were taken out and supernatant liquid was filtered. This solution was dissolved in 10 ml of 99% methanol to yield 400 µg/ml solutions; further 6 aliquots corresponding to 12.50 µg, 25 µg, 50 µg, 100 µg, 200 µg and 400 µg were prepared by serial dilution and absorbance was measured 30 min later at 517 nm. % DPPH scavenged and IC_{50} value of PR was calculated.[17]

Thin Layer Chromatography (TLC)

The test samples were prepared in methanol and applied on pre-coated Merck® aluminium alloy TLC Silica gel 60 F_{254} plate in the form of a band, with the help of fine capillaries.[18,19] Development of plates was done using Camag® chromatographic rectangular glass chamber and were air dried. R_f value was calculated using the formula: R_f value = Distance travelled by sample/ Distance travelled by solvent.

High Performance Liquid Chromatography (HPLC)

Chromatographic conditions: UFLC Shimadzu, SPD-M20A with PDA detector was employed for HPLC quantitative analysis. Analytical column C_{18} (Spinchotech Pvt. Ltd. Enable) was used and temperature was maintained at 40°C. Mobile phase were prepared in closed solvent bottles and sonicated for about 20 min, the injection volume was 20 µl. Data acquisition for vitexin, PR extracts and its fractions was performed at 340 nm (λ_{max} of vitexin) using acetonitrile : formic acid :: 99.5:0.5 v/v as the mobile phase with flow rate of 0.3 ml/min. (Table 2) The retention time, peak intensities and other chromatographic factors of marker compound, mother extract and its fractions were comparatively analyzed using LC solutions software.

Preparation of standard: 1.0 mg of vitexin was dissolved in 10 ml methanol and sonicated for about 20 min and filtered using minipore filters (0.22 mm) to yield stock sample solution of 100 µg/ml, subsequently diluted in HPLC grade methanol to produce 10 µg/ml (10 ppm) standard solution for analysis purpose.

Preparation of sample: Samples of PR and its fractions were prepared in the concentration of 10 ppm, similarly as the standard.

Procedure: The chromatographic conditions were set as per the given parameters and mobile phase was allowed to equilibrate with stationary phase; indicated by a steady baseline. The standard solution and prepared samples were injected using Hamilton syringe and the chromatograms were recorded for standard and samples.[20-22]

Calculations: Post-data acquisition, the concentration (µg/ml) of standard present in the sample solutions was calculated using the formula:

$$\text{Concentration}_{sample} = \text{Peak area}_{standard} / \text{Peak area}_{sample} * 100$$

Table 2: Chromatographic specifications used for quantitative analysis of vitexin and PR using high performance liquid chromatography.

Make	UFLC Shimadzu, SPD-M20A with PDA detector
Column	C_{18} by Spinchotech Pvt. Ltd. Enable
Column temperature	40°C
Injection volume	20 µl
Flow rate	0.3 ml/min
Wavelength (λ_{max})	340 nm
Separation	Isocratic
Mobile phase (v/v)	Acetonitrile: formic acid :: 99.5:0.5
Software	LC solutions

Table 3: Macroscopic characteristics of mungbeans.

Colour	Odour	Taste	Shape	Size	Surface characteristics
Dark green	Not distinct	Slightly sweet	Oblong	1-3 mm	Smooth, shiny

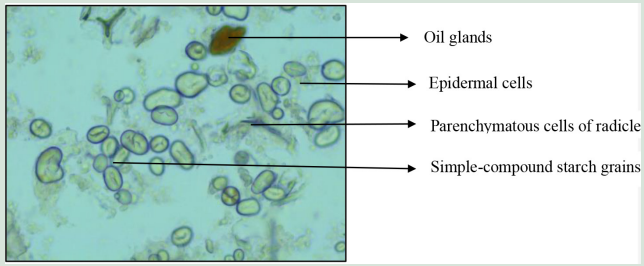

Figure 5: 20x Powder microscopy of mungbeans.
Powder microscopy was performed using Leica® Microsystems DM 2000 microscope and toluidine blue was used as the general staining agent. The general characteristics of grains/ beans can be seen above.

RESULTS

Parameters like macroscopy (Table 3), powder microscopy (Figure 5), physicochemical examination (Table 4) and microbial contamination studies (Figure 6) were found to be within standard API limits.[23] Both, CPM and PR were found to be rich in sterols, alkaloids, saponins, tannins,

Table 4: Various physicochemical properties of mungbeans.

Sr. no.	Parameter	Value
1.	Total ash value	3.65 % w/w
	Acid insoluble ash	0.52 % w/w
	Water soluble ash	1.25 % w/w
2.	Water-soluble	13.7 % w/w
	Alcohol-soluble	5.77 % w/w
	Hydro-alcoholic (70% ethanol:30% water)	7.22 % w/w
3.	Loss on Drying	6.35 % w/w
4.	Swelling index	0.43
5.	Foaming index	100
6.	Foreign matter	Nil
7.	Heavy metal testing	
	Arsenic (As)	Below detection limit
	Lead (Pb)	0.153 mg/kg
	Mercury (Hg)	Below detection limit

All the parameters were found to be within API limits.

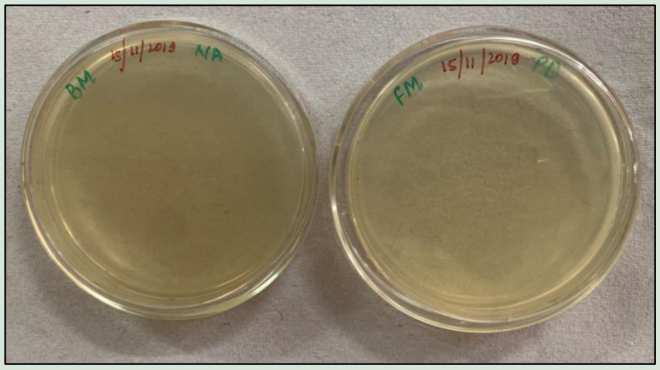

Figure 6: Microbial contamination study of PR in (left)Nutrient Agar and (right)Potato Dextrose media.
There was 'no microbial contamination' in PR as observed above.

Table 5: Preliminary phytochemical screening of CPM and PR.

Sr. no.	Phyto-constituent	Test	Observations		Inference
			CPM	PR	
1.	Sterols	Salkowski test.	++	++	Sterols present
		Lieberman burchard test.	++	++	
2.	Alkaloids	Dragendorff's test.	++	++	Alkaloids present
		Mayer's test.	++	++	
		Wagner's test.	++	++	
		Hager's test.	++	++	
3.	Saponins	Foam test.	++	++	Saponins present
4.	Tannins	Ferric chloride test.	++	++	Tannins present
		Lead acetate test.	++	++	
5.	Flavonoids	Shinoda test.	++	++	Flavonoids present
6.	Sugars	Molisch's test.	++	++	Sugars present
		Barfoed's test.	++	++	
7.	Proteins	Biuret test.	++	++	Proteins present
		Xanthoproteic test.	++	++	
8.	Amino acids	Ninhydrin test.	++	++	Amino acids present

where, ++ indicates 'presence', CPM-crude plant material and PR-70% v/v hydroalcoholic mother extract of *Phaseolus radiatus* beans

Table 6: Quantitative estimation of various primary and secondary metabolites present in PR.

Sr. no.	Parameter	Value
1.	Total Phenolic Content	55.0 mg/g eq. of Gallic acid
2.	Total Tannin Content	33.5 mg/g eq. of Tannic acid
3.	Total Flavonoid Content	62.67 mg/g eq. of Rutin
4.	Total Flavanol Content	5.96 mg/g eq. of Rutin
5.	Total Alkaloid Content	0.5 % w/w
6.	Total Carbohydrate Content	1.81 % w/w
7.	Total Saponin Content	0.36% w/w of Diosgenin

flavonoids, sugars, proteins and amino acids as observed in preliminary phytochemical screening (Table 5). The % yield of mother extract of *P. radiatus* by maceration using 70% v/v hydroalcoholic solvent was calculated to be 45.6% w/w. The quantitative estimations of PR included total tannin content, total phenolic content, total flavonoid content, total flavanol content, total alkaloid content, total carbohydrate content and total saponin content which was calculated to be 33.5 mg/g eq. of tannic acid, 55.0 mg/g eq. of Gallic acid, 62.67 mg/g eq. of Rutin, 5.96 mg/g eq. of Rutin, 0.5% w/w, 1.81 % w/w and 0.36% w/w of Diosgenin respectively (Table 6). Significant antioxidant potential of PR was confirmed by DPPH free radical scavenging assay as evident by the IC_{50} value of 38.96 µg/ml (Figure 7, Tables 7 and 8). The R_f value of PR obtained by TLC was calculated to be 0.78, complementary to the reported R*f* of vitexin (Table 9). Lastly, quantitative HPLC analysis of vitexin and PR was performed successfully using acetonitrile : formic acid :: 99.5 : 0.5 v/v as the mobile phase and the concentration of vitexin in PR was found to be 13.94 µg/ml. (Figures 8 and 9, Table 10). Owing to the knowledge of mechanisms underlying pain and inflammation, and the findings from this study with respect to the significant antioxidant potential, chemical nature of vitexin and its abundance in PR, hence the possible mechanism of action of mungbean may be attributed to the potential suppression of IL-1β-induced production of NO and prostaglandin E2 (PGE_2) possibly causing an anti-inflammatory effect.[24]

DISCUSSION

Pharmacognostic standardisation of *Phaseolus radiatus* or mungbean encompassed physicochemical examination and preliminary phytochemical screening. Sterols, alkaloids, saponins, tannins, flavonoids, sugars, proteins and amino acids were found to be present in both, RPM and PR. '70% v/v hydroalcoholic extract of *Phaseolus radiatus*' (PR) showed highest extractive values and hence this solvent ratio was selected for extraction by maceration. Quantitative estimations confirmed the presence of various primary and secondary metabolites, enlightening the presence of numerous phytoconstituents with therapeutic potential. PR displayed significant antioxidant potential as calculated by the DPPH free radical scavenging assay. TLC confirmed the presence of vitexin, which was further quantified by HPLC.

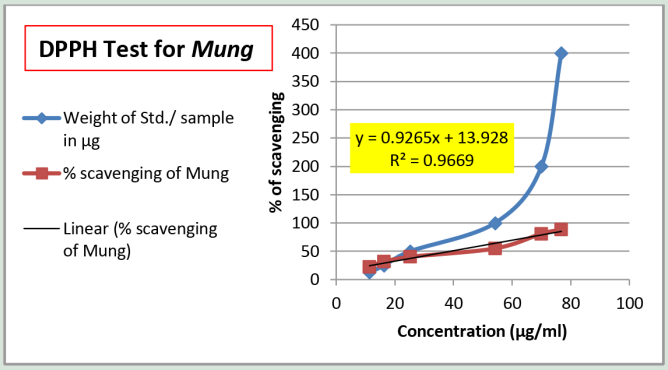

Figure 7: DPPH antioxidant activity of PR.

Table 7: Antioxidant assay of PR; absorbance and % scavenging.

Weight of Std./ sample in µg	Absorbance of Std. Ascorbic acid at 517 nm	Absorbance of PR at 517 nm	% Scavenging of Std. Ascorbic acid	% Scavenging of PR
12.5	0.958	0.456	11.37	22.58
25	0.905	0.402	16.28	31.74
50	0.808	0.352	25.25	40.23
100	0.495	0.265	54.20	55.00
200	0.324	0.112	70.02	80.98
400	0.251	0.069	76.78	88.28
Blank	1.081	0.589	00	00

Table 8: IC_{50} value of PR in comparison with standard ascorbic acid.

Sample	IC_{50} Value
Standard ascorbic acid	24.76 µg/ml
Mung mother extract	38.96 µg/ml

Table 9: Specifications for TLC of PR.

PR	Standard	Solvent system	R_f value
Mung	Vitexin	Ethyl acetate: Acetic acid: Formic acid: Water::100:11:11:27 (v/v)	0.78

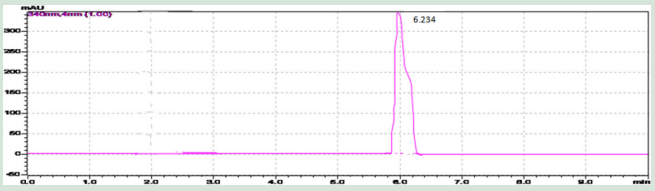

Figure 8: HPLC chromatogram of vitexin; reference standard for PR.

CONCLUSION

Our present work has led to two important conclusions (1) successful development of quality testing parameters and phytochemical standardisation of *Phaseolus radiatus* plant with respect to vitexin, a principle secondary metabolite, and (2) presence of vitexin was confirmed by TLC and quantified by HPLC; the amount of vitexin present in 70% v/v hydroalcoholic extract of *Phaseolus radiatus* was found to be 13.94 µg/ml.

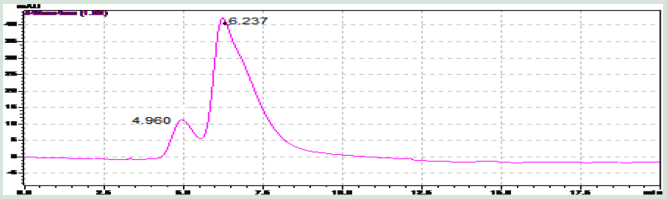

Figure 9: HPLC chromatogram of PR.

Table 10: Retention time, peak area and concentration of PR in reference to standard vitexin.

Sr. no.	Sample	Retention time (minutes)	Peak area	Sample concentration (µg/ml)
1.	PR	6.237	3840163	13.94
2.	Std. Vitexin	6.234	2753105	10.00

ACKNOWLEDGEMENT

The authors acknowledge the facilities provided by the University Department of Pharmaceutical Sciences, RTM Nagpur University, Nagpur for successful completion of the study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

%: Percent; **API:** Ayurvedic Pharmacopoeia of India; **CPM:** crude plant material; **DPPH:** 1, 1-diphenyl -2-picrylhydrazyl; **HPLC:** high performance liquid chromatography; **IC_{50}:** Half-maximal inhibitory concentration; **IL-1β:** Interleukin-1-beta; **M:** molar; **mg:** milligram; **mg/g eq.:** milligram/ gram equivalent; **ml:** millilitre; **mm:** millimetre; **nm:** nanometre; **NO:** nitric oxide; **PGE_2:** prostaglandin E2; **ppm:** parts per million; **PR:** '70% v/v hydro-ethanolic mother extract of *Phaseolus radiatus*'; **R_f:** Retention factor; **rpm:** revolutions per minute; **TLC:** thin layer chromatography; **v/v:** volume/volume; **w/w:** weight/weight; **λ_{max}:** wavelength of maximum absorption; **µg:** microgram; **µg/ml:** microgram/ millilitre; **µl:** micro litre.

Contribution details

Concept, design and definition of intellectual content: Dhoble LR and Itankar PR

Literature search and experimental studies: Dhoble LR

Data acquisition and data analysis: Dhoble LR

Manuscript preparation: Dhoble LR

Manuscript editing and manuscript review: Dhoble LR and Itankar PR

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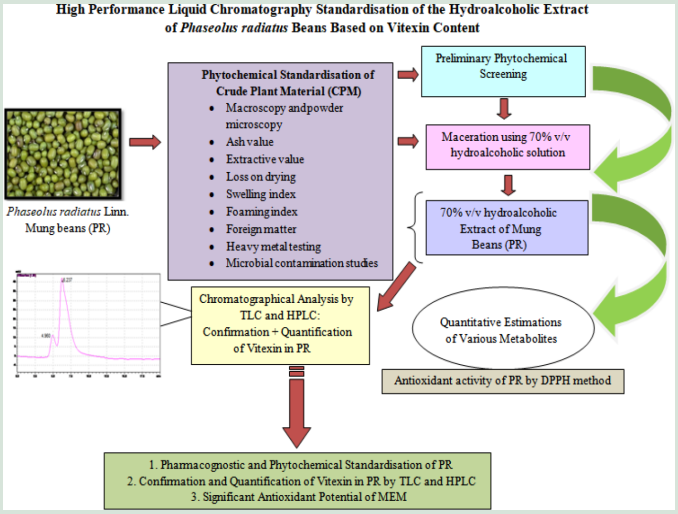
GRAPHICAL ABSTRACT



SUMMARY

The present work deals with the chromatographic standardisation of *Phaseolus radiatus* L. Wilczek beans using high performance liquid chromatography (HPLC) with respect to vitexin, in addition to pharmacognostic and phytochemical analysis. Experimental work encompassed pharmacognostic standardisation, extraction, quantitative estimations of various primary and secondary metabolites like phenolics, tannins, flavonoids, flavanols, alkaloids, carbohydrates and saponins. Analytical experimentation involved examination of antioxidant activity, qualitative thin layer chromatography (TLC) and HPLC of extract. Quantitative estimations confirmed the presence of various primary and secondary metabolites, enlightening the presence of numerous phytoconstituents with therapeutic potential. '70% v/v hydroalcoholic extract of *Phaseolus radiatus*' displayed significant antioxidant potential as calculated by the DPPH free radical scavenging assay. TLC confirmed the presence of vitexin, which was further quantified by HPLC. Based on these results, it can be concluded that (1) quality testing parameters and phytochemical standardisation of *Phaseolus radiatus* plant with respect to vitexin, a principle secondary metabolite were successfully developed, and (2) presence of vitexin was confirmed by TLC and quantified by HPLC; the amount of vitexin present in 70% v/v hydroalcoholic extract of *Phaseolus radiatus* was found to be 13.94 µg/ml. From the results obtained by our research work, vitexin may be attributed to be one of the phytoconstituents responsible for the significant antioxidant potential of mungbean which can become a marker for deciding the quality of mungbean to be used for preparation of Ayurvedic medicines.

ABOUT AUTHORS

Dr. Prakash R. Itankar is a highly experienced Professor in Pharmacognosy and Phytochemistry at University Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra. His area of expertise involve validation of medicinal plants, ethnopharmacology, validation of Panchagavya Ayurvedic Chikitsa, patents and publications. He has successfully received project grants in collaboration with MODROB, AICTE, and NMPB.

Leena Raju Dhoble is a Ph.D. research scholar at Dr. Prakash Itankar Lab in University Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur, Maharashtra. She has completed her Master's degree with specialisation in pharmacognosy and phytochemistry, as well as Bachelor's degree in Pharmacy from University Department of Pharmaceutical Sciences, Nagpur. Her doctoral research focuses on 'standardisation and validation of novel ethnopharmacological combination of herbs for management of osteoarthritis'. Her fields of interests include ethnopharmacology, natural products chemistry, traditional medicine and chromatography.

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