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Growth stages modulate phytochemical content and antioxidant property in methanolic extract of *Vigna radiata* (L.) Wilczek leaves.

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ABSTRACT

Plant phytochemicals have extensive applications in the food, pharmaceutical and cosmetic industries. *Vigna radiata* (L.) Wilczek (mung bean) seeds and sprouts are consumed by humans as functional foods worldwide. In this study, we analyzed the phytochemical constituents of *Vigna radiata* (L.) Wilczek leaves by qualitative and quantitative methods at three different developmental stages. Phytochemical analysis of these three stages, i.e., young (7 days), mature (20 days), and old (35 days), showed variability in the amount of phytochemicals and antioxidative properties indicating that phytochemical composition and production alters with the growing stages of plants. Qualitative analysis of phytochemicals showed a higher content of metabolites in younger leaves than in mature and old leaves, except for alkaloids, which were found to be higher in mature leaves. Similarly, the quantified value of phytochemicals also matched the qualitative estimation. Furthermore, the antioxidant potential of young, mature and old leaves was also evaluated and found to vary with leaf age. Such studies can provide information on suitable plant stage for consumption or efficient production and extraction of phytochemicals for medicinal and commercial applications.

1. Introduction

Mung beans and their sprouts are rich sources of both stock and bioactive nutrients in many countries. The stock nutrients include carbohydrates and proteins, which constitute essential requirements for energy exchange and primary metabolic activities. Bioactive nutrients are functionally active phytochemicals such as vitamins, phenolics, flavonoids, tannins, saponins, free amino acids, minerals and fibres (Yang *et al*., 2020). These molecules not only scavenge free radicals to alleviate oxidative stress but also modulate certain enzymatic activities, receptors possessing antidiabetic, hyperlipidaemic and anti-inflammatory properties along with defence and other medicinal uses (Ganesan & Xu, 2018). However, functionally active components show differential level of abundance in different parts of the grain, cotyledons, hulls and sprouts. It is also known that high level of bioactive components like total phenolics in mung bean seeds also responsible for high free radical scavenging activity (Yao *et al*., 2012). The hulls of mung bean are evidenced to contain highest concentrations of total phenolics, flavonoids, condensed tannin, saponin, vitexin and isovitexin, than any other parts of the grain (Luo *et al*., 2016). A comparative assessment of functional substances in germinated and non-germinated mung beans has also been reported indicating the germination have positive effect on the production of metabolites (Huang *et al*., 2014). Increased amount of vitamin C, phenolic and flavonoid contents and antioxidant activity have been reported in mung bean sprouts during 9 days germination (Gan *et al*., 2016). There is also a study regarding the variation in phytochemical components during the germination of *Vigna radiata* (L.) Wilczek in two different seasons. The results of this analysis of phytocomponents showed that the biochemical behaviour of germinating seedlings varied in seed lots collected during the summer and rainy seasons (Vijaylaxmi, 2013). It is imperative that the production of bioactive compounds is controlled by several factors such as the site of cultivation, time of cultivation,

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and light and dark periods. Moreover, different parts of the grain and sprout also account for the differential source of bioactive compounds. Despite different existing research involving variation in phytochemical constituents with respect to different parameters, there is scope to determine the effect of growing stages of cotyledon leaves on the availability of phytochemical compounds. The synthesis and availability of different phytochemicals and antioxidant properties in plants are strongly affected by the growing phases. The variation pattern in metabolite profiles during the germination and growth phases is accompanied by dynamic regulation at the transcription level (Tang *et al*., 2014). Because the role of these phytochemical compounds is recently reported in the control of the genetic and epigenetic makeup of the cells during different growth stages transitioning from vegetative and reproductive stages, their availability might change according to the differential expression pattern specific to site or time. The results of different molecular profiling studies stimulated several research projects comprising multiple developmental stages and environmental conditions in different legume crops, including mung beans. Moreover, transcriptomic and metabolomic analyzes of mung bean sprouts have revealed the regulation and nutritional changes during germination (Wang *et al*., 2020). The comprehensive transcriptional patterns of mung bean seedlings after germination remain poorly understood, which may restrict insights into the molecular events triggering metabolism regulation during the transition from vegetative to reproductive phases. Consequently, rationalization awaits further study. Keeping this school of thought in mind, an investigation of different phytochemical components was conducted during different growth phases of first cotyledon leaves after germination to evaluate the changes in the pattern of secondary metabolite production. This study is a primary step toward unraveling the mechanism of the development and commercialization of more qualitatively nutritious food. The present work deals with the preliminary investigation of the methanol extract of *Vigna radiata* to identify major group of phytochemicals that impart medicinal properties to the plant during developing stages. The free radical scavenging activity of these plant leaf extracts is identified using the 2, 2-diphenyl-l-picrylhydrazyl (DPPH) assay, and the hydrogen peroxide scavenging activity was also evaluated.

2. Materials and Methods

The seeds of *Vigna radiata* (L.) Wilczek were collected fromthe commercial market of Malgodown area of Cuttack district of Odisha and authenticated at Department of Botany, Ravenshaw University. The plant parts were examined and identified with the help of regional flora, specimen was further confirmed by refereeing to the book Flora of Odisha available at Department of Botany, Ravenshaw University. The seeds were grown in 36 cell trays during mid-March of summer/spring season and provided optimal sunlight and water for normal growth. Healthy plant leaves were chosen at three different developmental stages – young, mature and old having different growth time duration of 7 days, 20 days and 35 days respectively. Leaves are collected for comparative analysis of active components present among them during the developmental stages.

2.1. Preparation of plant extracts

10 grams of *Vigna radiata* (L.) Wilczek leaves were air dried under shade at room temperature and milled to coarse powder. The obtained dried powder was subjected to successive Soxhlet extraction with (250 ml) methanol for 40 cycles. The extract thus obtained was concentrated to dryness in a flash evaporator under reduced pressure and controlled temperature. The obtained residue was yellowish brown to dark brown colour with thick and sticky paste. The extract was filtered through Whatman No. 1 paper and was quantified before being stored in -4^{o}C refrigerator under airtight condition for further uses.

2.2. Phytochemical Screening Test

The presence of phytochemical constituents in the methanolic extracted young, mature, and old leaves of *Vigna radiata* (L.) Wilczek were carried out to identify metabolites such as alkaloids, total soluble carbohydrate, glycosides, amino acids, tannins, flavonoids, steroids, terpenoids and saponins. For all the qualitative phytochemical screening the final concentration was kept at 1gm of plant leaf extract in one ml of methanol (Agidew, 2022; Kumar *et al*., 2020).

3. Quantitative determination of leaf constituents of *Vigna radiata*

The phytoconstituents particularly, tannins, total phenol content, total flavonoid content and alkaloids found in methanolic extract of *Vigna* leaves during the qualitative screening was quantitatively determined by standard procedures. For all the quantitative measurement the final concentration was kept at 1mg of plant leaf extract in one ml of methanol.

3.1. Determination of tannin content

The tannins were determined by Folin-Ciocalteu method. 1mg/ml of the plant leaf extract was added to a volumetric flask (10 ml) containing 7.5 ml of distilled water and 0.5 ml of Folin-Ciocalteu phenol reagent, 1 ml of 35 % Na_2CO_3 solution. The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard

Table

Phytochemical Screening	Test protocol	Result
Alkaloids (Wagner's Test)	1 ml of extract treated with 2 ml of Wagner's reagent	Appearance of Reddish-Brown color
Soluble Carbohydrates (Molisch's Solution Test)	1 ml of extract added with few drops of 10% alcoholic solution of α-naphthol, followed by 1ml of concentrated H_2SO_4 along the side of the test tube.	Purple-violet ring at the junction of two liquids confirms the presence of soluble carbohydrates.
Glycosides (Keller-Kiliani Test)	5 ml of extract added with 2 ml of glacial acetic acid, followed by adding one drop of ferric chloride ($FeCl_3$) and 1 ml of concentrated H_2SO_4 into the tube	Appearance of reddish-brown colour
Amino Acids (Ninhydrin Test)	2 ml of extract added with 0.5 ml of Ninhydrin solution and boiled for 2 minutes followed by cooling	Appearance of blue colour
Tannins (Braymer's test)	1ml of extract mixed with 2ml of distilled water along with few drops of 5% ferric chloride solution	Blue-black or blue-green precipitate indicates the presence of tannins
Flavonoids (Aqueous NaOH test)	1 ml of extract dissolved in 3 ml warm distil water and filtered. To this solution added a few drops of 10% aqueous NaOH in 4 ml of solution	A yellow colour appears and become colourless with adding few drops of HCl
Steroids (Liebermann-Burchard)	To 1ml extract added 10 ml of cold acetic acid followed by addition of conc. H_2SO_4 carefully	Colour appears from violet to blue or bluish green
Terpenoids (Salkowski's Test)	In 5 ml of extract added 2 ml of chloroform followed by addition of 3ml of conc. H_2SO_4 to form a layer	Formation of reddish-brown colour layer
Saponins	1 ml extract diluted to 5 ml by adding methanol and incubated at 50°C for 15 minutes. It is followed by cooling and then adding 3ml of distilled water. This solution shaken vigorously for about 5 minutes	Frothing which persisted on warming was taken as evidence for the presence of saponins

solutions of gallic acid (20, 40, 60, 80 and 100 µg/ml) were prepared in the same manner as described earlier. Absorbance for the plant leaf extract and standard solutions was measured against the blank at 725 nm with UV/Visible spectrophotometer. The tannin content was expressed in terms of mg of GAE/g (Galvão *et al*., 2018).

3.2. Determination of total phenol content (TPC)

TPC was determined using the spectrophotometric method.1mg/ml of plant leaf extractin triplicate were introduced into test tubes followed by 1.5 ml of Folin-Ciocalteu's reagent (diluted 10 times with water) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were incubated for 30 min and absorption at 765 nm was measured. Total phenolic contents were expressed in gallic acid equivalents (mg per 100-gram dry leaves) (Stanojeviæ *et al*., 2009).

3.3. Total flavonoids content (TFC)

The flavonoids content was determined by aluminium trichloride method using quercetin as reference compound. A volume of 1ml of plant leafextract was added to 0.6 mL of a 5% $NaNO_2$ solution making a total volume of 1.6 ml. The mixture was allowed to stand for 5 minutes and then 1.2 ml of aluminium trichloride (10%) was added and incubated for another 5 min followed by addition of 6mL of NaOH (1M). The final volume of the solution adjusted to 20mL with distilled water. After 15 min of incubation the mixture turned to pink, and the absorbance was measured at 510 nm. The concentration of flavonoid was expressed as mg quercetin equivalent (QE) per gram of extract (Rebaya *et al*., 2015).

3.4. Determination of Alkaloid

The plant leaf extract having concentration of 1mg/ml was oven dried and mixed with 1 ml of dimethylsulphoxide (DMSO) followed by addition of 1ml of 2N HCl and then filtered. This solution was transferred to a separating funnel with the addition of 5 ml of bromocresol green and 5 ml of phosphate buffer (pH 7.4). The mixture was shaken with differential volume of 1, 2, 3 and 4 ml of chloroform by

vigorous shaking. The extract was collected in a 10-ml volumetric flask and diluted to the volume with chloroform in the ratio of 1:1. A set of reference standard solutions of caffeine (20, 40, 60, 80 and 100 µg/ml) were prepared and spectrophotometrically measured. The absorbance for plant leaf extract and standard solutions were determined against the blank at 470 nm. The total alkaloid content was expressed as mg of caffeine per ml of extract (John *et al*., 2014).

4. Antioxidant potential of leaf extract of *Vigna radiata*

In nutraceutical investigations, *in vitro* antioxidant activity assessment methods are often used to screen and confer antioxidant potential to plants or their phytochemicals (Kasote *et al*., 2015). The free radical scavenging potential can vary with the aging or the growing stages of plants. To evaluate this objective, several *in vitro* assays were measured for antioxidant properties of leaves at different age duration were carried out.

4.1. Determination of the radical scavenging ability using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

2, 2-diphenyl-1-picrylhydrazyl (DPPH) was used to determine the free radical scavenging activity of *Vigna radiata* (L.) Wilczek leaf extracts. Briefly, different concentrations (12.5-400µg/ml) of the plant leaf extract of *Vigna* leaves were added with an equal volume of methanolic DPPH solution (0.1mM) and incubated at 37°C for 30 min. The absorbance of the DPPH radical without an antioxidant, i.e., negative control, was also measured. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH Scaveging Effect (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where $A_{o,}$ is the absorbance of the negative control reaction and A_1 is the absorbance in the presence of plant leaf extract.When DPPH reacts with antioxidant, DPPH was reduced, and the colour changed from deep violet to light yellow. This was measured at 517 nm. The experiments were carried out in triplicate and the results expressed as a percentage of the control (Sahu *et al*., 2013).

4.2. Assay of Hydrogen peroxide (H, O,) scavenging activity

Hydrogen peroxide scavenging activity of the plant leaf extract was measured spectrophotometrically. A solution of hydrogen peroxide was prepared with phosphate buffer (pH 7.4) making it to the final concentration of 40mM. Plant leaf extract (100-500µg/ml) were added to 0.6 ml of hydrogen peroxide solution and spectrophotometrically assessed at 230nm. The absorbance was measured after incubating for 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide.

$$\text{Scavanged } H_2O_2 \text{ (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100$$

Where $A_{o,}$ is absorbance of the negative control reaction, and A_1 is the absorbance inthe presence of the plant leaf extract (Meenatchi *et al*., 2017).

5. Statistical Analysis

All the analyses were performed in triplicate and the results were statistically analyzed and expressed asmean (n=3) ± standard deviation (SD). Statistical calculations were performed in Microsoft office Excel 2010.

6. Results and Discussion

6.1. Qualitative phytochemical analysis

Qualitative analysis for different phytochemical constituents were performed, and it was observed that the methanolic plant leaf extract possesses diverse concentration of metabolites during different growth stages. The 7-day young leaves have more amount of bioactive compounds present than the 20- and 35-days old plant leaf extracts. In a recent study by Wang and associates, the 6-day plant cotyledon leaf extract transcriptome profile reveals upregulation of more than 80 genes that takes part in the regulation of hydroxycinnamic acid and phenyl propanoic acid biosynthesis as well as the synthesis of aromatic amino acid that are precursor of poly phenolic compounds like lignans, coumarins, flavonoids (Wang *et al*., 2020). In another study on the coffee plant and *Moringa oleifera*, the age of the leaf and the kind of solvent do affect the composition of phytochemical profile (Chen *et al*., 2018; Nobossé *et al*., 2018). In table 1, the list of phytochemical constituents presents in the methanolic plant leaf extract indicating majority of the compounds decreases with aging of the leaves except for alkaloids and steroids. These observations can be correlated with the findings of Shukla and Singh, in case of *Papaver somniferum,* more alkaloids are found in the young and mature cotyledonary leaves but with growing stages more alkaloids are in the reproducing organs and trace amount in leaves (Shukla and Singh, 2001). Another recent report regarding the influence of developmental stages on secondary metabolites in medicinal plants by Li and his group states that monoterpenes and sesquiterpenes starts synthesizing in the cotyledon stage but synthesis of specific oils is observed in later stages of growth. In addition, the phytochemical content production also depends on the different vegetative and reproducing stages, environmental

Table 1:

Phytochemical screening of methanolic extract of 3 different developmental stages of *V. radiata* leaves.

Chemical Constituents	7 days extract	20 days extract	35 days extract
Alkaloid	+	+++	++
Carbohydrates	+++	+	+
Glycosides	+	-	-
Phenols	+++	+++	++
Tannins	+++	+	+
Amino acids	+++	++	++
Flavonoids	+++	++	+
Terpenoids	+++	++	+
Saponins	++	-	-
Steroids	++	+	+

+++: Strong positive test; ++: Low positive test; +: Weak positive test; –: Negative test.

stress factors, circadian rhythm, and soil microbial community. On the other hand, glycosides, phenols, flavonoids and saponins were detected in high amount in young leaves and very little to no detection in mature and old leaves (Li *et al*., 2020).

Quantitative determination of the pharmacologically important chemical phytoconstituent indicates the presence of phytochemicals in varying amount during the developmental phases of leaves. The estimation was carried on alkaloids, total phenol content, total flavonoid content, tannins and steroids and interestingly, the qualitative indicators as carried out to screen the phytochemicals correlate with the quantitative estimation of the phytochemicals that were measured using spectrophotometer.

Total alkaloid content reported as the caffeine equivalent were derived from standard curve (y=0.0058x+0.0122, R^2=0.9904). The concentration of total phenolic and tannin contents of 7-, 20- and 35-days leaf extract were determined by the gallic acid equivalent per ml of leaf extract from the standard curve (y=0.0041x-0.0165, R^2=0.9946). Total flavonoid content in the leaf extracts of *Vigna* were calculated against mg quercetin equivalent in the leaf extract of 7, 20 and 35 days with the standard equation of (y=0.0033x+0.0112, R^2=0.998). On the other hand, the steroids concentration in the leaves found to be higher in young leaves (7 days) as compared to older days. Quantitatively, the measured amount of steroid were measured in mg of cholesterol equivalent as calculated from the equation (y=0.0018x-0.0411, R^2=0.9605). The measured values are shown in table 2.

Table 2:

Yield of alkaloids, total phenolics, total flavonoids, tannins, and steroids contents of young, mature, and old leaf extract of *V. radiata.* The value is expressed as mean ± SD from minimum of three independent experiments. TPC and Tannin data was expressed as microgram of gallic acid equivalent (mg GAE) per ml of extract. Steroid data was expressed as microgram of cholesterol equivalent (mg CHO) per ml of extract. Flavonoid data was expressed as microgram of quercetin equivalent (mg QE) per ml of extract. Alkaloid data was expressed as microgram of caffeine equivalent (mg CAF) per ml of extract.

Phytoconstituents	7-days extract	20-days extract	35-days extract
Alkaloids $[\frac{\mu g \text{ of CAF}}{ml \text{ of extract}}]$	34.6 ± 0.529	67.1 ± 1.404	59.5 ±1.7
Total phenolic $[\frac{\mu g \text{ of GAF}}{ml \text{ of extract}}]$	190 ± 4.58	176 ± 4.52	89.2 ± 1.56
Total flavonoids $[\frac{\mu g \text{ of QE}}{ml \text{ of extract}}]$	34.8 ± 3.37	28.5 ± 1.34	15.8 ± 1.34
Tannins $[\frac{\mu g \text{ of GAF}}{ml \text{ of extract}}]$	120.8 ± 4.8	36.9 ± 4.41	37 ± 1.73
Steroids $[\frac{\mu g \text{ of CHO}}{ml \text{ of extract}}]$	66 ± 1.40	46 ± 1	45 ± 0.57

6.3. Antioxidant and free radical scavenging activities of Vigna radiata (L.) Wilczek extract

The DPPH radicals scavenging activity demonstrate the effect of plant leaf extract of different days having antioxidant property through their hydrogen donating ability, which reduces the stable violet DPPH radical to the yellow DPPH. A high percentage of radical scavenging indicating a strong antioxidant activity in the tested sample. The extracts showed concentration dependent antioxidant activity. Furthermore, the extract which contained the considerable amount of total phenolics, and flavonoids effects in reducing DPPH and inhibiting hydrogen peroxide oxidant. The IC_{50} value of scavenging activities on DPPH radical was carried out using an online IC_{50} calculating tool i.e., https://www.aatbio.com/tools/ic 50-calculator (Inc, 2023). The observed values are shown in table 3.

Similarly in case of H_2O_2 scavenging activity, IC_{50} value of scavenging activity of 7-, 20- and 35- days leaves were shown in table 4.

Table 3:

DPPH inhibition percentage and calculated IC_{50} value of *Vigna* leaf extracts of different days.

Concentration (µg/ml)	7-days extract (% Scavenging)	20-days extract (% Scavenging)	35-days extract (% Scavenging)
12.5	46.547	43.544	42.643
25	48.649	46.547	46.246
50	51.351	48.949	48.348
100	52.553	51.652	50.450
200	53.453	52.853	51.952
400	56.156	54.955	52.553
IC_{50}	3.97	5.12	3.96

Table 4:

H_2O_2 scavenging activity and calculated IC50 value of *Vigna* leaf extracts of different days.

Concentration(µg/ml)	7-days extract (% Scavenging)	20-days extract (% Scavenging)	35-days extract (% Scavenging)
100	14.551	13.483	10.787
200	26.854	23.427	16.011
300	38.202	36.966	23.202
400	55.562	54.607	37.579
500	67.416	61.292	44.831
IC_{50}	620.66	330.49	372.45

7. Conclusion

The phytochemical screening in three different stages of leaves - young, mature, and old shown difference in availability of phytochemicals. Qualitative analysis showed that the younger leaves have higher phytochemical content than the older leaves. Quantitative screening indicated that the methanolic plant extract had the highest metabolite content in the young leaf samples, except for alkaloids. Similarly, the DPPH assay and H_2O_2 scavenging activity varied because of the aging process.It is generally accepted that plants undergo series of biochemical and physiological changes during developmental stages viz seedling, vegetative, reproductive and senescence stage. These developmental phases also alter active phytoconstituents and secondary metabolites. Therefore, these changes in the chemical composition of mung beans during germination will also lead to substantial and important changes in their pharmacological properties. These phytoconstituents seem to have the potential to act as a source of useful metabolites and to improve the health status of consumers because of the presence of various compounds that play a vital role towards good health. It also benefits the herbal and phytochemical manufacturing industries to determine the

suitable stage for extraction of plant metabolites for commercial production.

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