Current Botany 2024, 15: 76-80
doi: 10.25081/cb.2024.v15.8594
https://updatepublishing.com/journal/index.php/cb

Research Article

ISSN: 2220-4822

Detection and Analysis of melanin pigment in the edible seed coat of some plants

Anitha Rajasekaran*, Geetha Kamalakannan

PG and Research Department of Botany, Bharathi Women's College, Prakasam salai, Broadway, Chennai-600108, Tamil Nadu, India

ABSTRACT

Melanin is a dark phenolic substance present in plants, animals, and microorganisms. The role of melanin in plants is to offer protection against biotic and abiotic conditions. The other possible functions in plants are unknown. Studying the occurrence of melanin in plants is essential to understand the taxonomic evolution of plants and its application in different fields. The deposition and distribution of Phytomelanin in the seed coat was studied using a light microscopy The seed coats of *Phaselous vulgaris* (Leguminaceae), *Vigna mungo* (Fabaceae), *Linum usitatissimum* (Linaceae), *Cicer arietinum* (Fabaceae), *Vigna unguiculata* (Fabaceae), *Sesamum indicum* (padaliaceae) and *Macrotylama uniflorum* (Fabaceae) showed the presence of melanin. Among the plant studied, *S. indicum* and *V. mungo* had a higher yield of melanin per 5 g of seeds. Melanin was extracted, purified and a confirmatory test was also conducted. UV and FT-IR studies were carried out with purified melanin. *V. mungo* and *P. vulgaris* showed no absorbance in the UV spectrum. The Maximum absorbance was found at 276 nm for *S. indicum*, *M. uniflorum* at 283 nm, *L. usitatissimum* at 276.5 nm, and *V. unguiculata* at 286 nm. It is evident from the study that phytomelanin is present in higher concentrations in *S. indicum* followed by *V. mungo*.

KEYWORDS: Phytomelanin, Fabaceae, Leguminaceae, Isolation, *Sesamum indicum*, *Vigna mungo*

Received: September 08, 2023
Revised: May 28, 2024
Accepted: June 14, 2024
Published: June 30, 2024

***Corresponding author:**
Anitha Rajasekaran
Email: anitha.rajasekaran023@gmail.com

INTRODUCTION

Phytomelanin is a dark phenolic pigment present in plants, animals and microorganisms. This pigment has been neglected over the years and recently it has drawn attention due to the enormous application that it possesses. It is reported to contain a broad spectrum of biological activities such as antioxidant (Keles & Özdemir, 2018), antitumor (El-Naggar & El-Ewasy, 2017), antiviral, antimicrobial (Laxmi *et al.*, 2016), chemoprotection, anti- inflammatory, immune stimulant, thermoregulatory and hypoglycemic (Al-Tayib *et al.*, 2014). Hence it has applications in the fields of pharmacology, cosmetics (Kalka *et al.*, 2000), the food industry (Roy & Rhim, 2022) and agriculture. In agriculture, it can be used as a plant protector since it offers resistance to plant pathogens and insects. It has been reported that dark-pigmented seeds are more resistant to soil- borne pathogens and it offers protection against insect pest as well (Cardinale *et al.*, 2021). However, non-melanized seeds were vulnerable to pathogens and insects. In plants, they also offer protection against abiotic stress (Ceccarelli *et al.*, 1987). So far phytomelanins have been isolated from a few dicotyledonous families i.e., Campanulaceae, Convolvulaceae, Umbelliferae, Compositae, Agavaceae, Aloaceae, Amaryllidaceae, and Hyacinthaceae. In monocotyledon, it is found in Asperagales, Zingiberales, Haemodoraceae (Glagoleva *et al.*, 2020) and Poaceae (Bazhenov *et al.*, 2023).

Phytomelanins not only have biological significance but also taxonomic relevance. Studies on the distribution and taxonomic relationship of Phytomelanins would help in determining the phylogenetic relationship of the different taxon. Phytomelanin discovery in certain tribes of Asteraceae has opened new perspectives in evolution of Asteraceae members (Marques *et al.*, 2021). Melanin formation in seed tissue was not studied earlier. But, recently in barley it is reported to be synthesized in chloroplast derived melanoplasts and accumulates in the pericarp of the seed (Shoeva *et al.*, 2020). Since phytomelanins are natural, it has the potential to be utilized in various fields. Occurrence and distribution of melanin in plant tissue have significant implications mainly in seed protection against microbes and Insect attacks. It seems to repel the insects from feeding on the seeds. Hence a search for new phytomelanin sources can lead to new perspectives and applications.

MATERIALS AND METHODS

Distribution of Phytomelanin in Seeds

Phaselous vulgaris (BWCPV, Leguminaceae), *Macrotylama uniflorum* (BWCMU-Fabaceae), *Vigna mungo* (BWCVM-Fabaceae), *Cicer arietinum* (BWCCA-Fabaceae), *Vigna unguiculata* (BWCVU-Fabaceae), *Sesamum indicum* (BWCSI-Pedaliaceae), *Linum usitatissimum* (BWCLU-Linaceae) are dark pigmented edible seeds rich in protein and antioxidants were selected for screening of phytomelanins. The seeds were procured from the local market of Chennai, Tamil Nadu and a voucher specimen of the above seeds BWCPV, BWCMU, BWCVM, BWCCA, BWCVU, BWCSI and BWCLU respectively was deposited in the Department Herbarium. The seeds were soaked overnight and hand sections were made and observed under light microscopy. The seed coat region of all the seeds were observed and photographed.

Isolation of Phytomelanin

Five grams of the above seeds were soaked in 0.1, 0.5 and 1 M Sodium hydroxide and extracted after 72 hours. The extracts were filtered and diluted 20 times and absorbance was observed in ShimadZu UV- Visible spectrophotometer. The absorption spectra were measured between 200 and 400 nm.

Confirmatory Test for Phytomelanin

The following tests were conducted as confirmatory test to confirm Phytomelanin.

Solubility Test

Phytomelanins has a unique solubility. It is soluble only in alkali solutions and insoluble in water. The obtained melanin was dissolved in alkali such as sodium hydroxide and observed for it solubility.

Decolorization Test

The Melanin solution obtained by dissolving in alkali was treated with 5% hydrogen peroxide and observed for rapid decolourization.

Precipitation Test

To 1mL of the melanin extract few drops of ferric chloride solution is added and observed for precipitation.

Purification of Phytomelanin

After 72 hrs of alkali extraction, the extracts of various seeds were treated with 2 M hydrochloric acid and allowed to precipitate. The contexts were centrifuged at 4000 xg for 5 mins. The pellet was suspended in water twice and centrifuged at 4000 xg for 5 mins. The pellet thus obtained was collected and rinsed in ethylacetate and later ethanol to remove carbohydrates, proteins, and lipids. The residue was collected and dried at 100ºC. The dried powder was scraped and the yield of phytomelanin was estimated. Thus obtained phytomelanin was checked for its purity by dissolving in water and the texture and colour recorded.

UV–VIS Spectroscopic Analysis of Phytomelanin

The purified phytomelans of the various seed coats were dissolved in 0.1 M sodium hydroxide and subjected to UV analysis in a Shimadzu Double beam UV- Visible Spectrophotometer. The samples were scanned through 200 to 300 nm and the maximum absorption was recorded.

FT–IR Spectrosopy

The FT-IR spectrum of phytomelanin was obtained by grinding it with Potassium bromide (1:100). The spectrum was obtained in a Single Beam IR Spirit FT-IR Spectrophotometer, (Shimadzu, Japan) with 190 to1100 nm range. The scan range was between 250 cm^{-1} to 400 cm^{-1}.

Statistical Analysis

The standard deviation was determined by Statistical tool in Excel 2010 version.

RESULTS AND DISCUSSION

Distribution of Phytomelanins in the Seed Coat

The seed coats of *P. vulgaris*, *V. mungo*, *L. usitatissimum*, *C. arietinum*, *V. unguiculata*, *S. indicum* and *M. uniflorum* were studied and found that the seed coat of *P. vulgaris* consists of the palisade, sub epidermal, and parenchyma layers protecting the cotyledons inside. Pigmentation was found in the outer palisade layer and inner parenchyma layer (Figure 1).

The inner layer of pigment may be protoanthocynidin. The seed coats of fabaceae members (*V. mungo*, *C. arietinum* and *M. uniflorum*) generally contained sclerieds, especially macrosclerieds and osteosclerieds. *C. arietinum* seed coat contained a distinct outer palisade, inner palisade, hourglass cells of hypodermis, parenchyma layer, and an inner cuticle (Pandey *et al.*, 1989). Macrosclereids were found extending across the outer and inner palisade layers. The outer and inner palisade layers and parenchyma layers were pigmented (Figure 1). In the case of *V. mungo*, the seed coat is a single layer with a characteristic single row of osteosclerieds. The outer layer was densely pigmented. *V. unguiculata* had three layers outer and inner palisade layers and a single row of osteosclereids. The inner palisade layer was intensely pigmented. *M. uniflorum* the seed coat was not differentiated into layers. A single prominent layer of parenchymatous seed was observed. The seed coat was thick and pigmented. High levels of phenolic content reported in the seed coat of Leguminaceae members play an important role in the nutritional quality of the seeds. The antifungal property of these seeds could be due to tannins present in the seed coat. Active polymerization is due to the action of peroxidases and

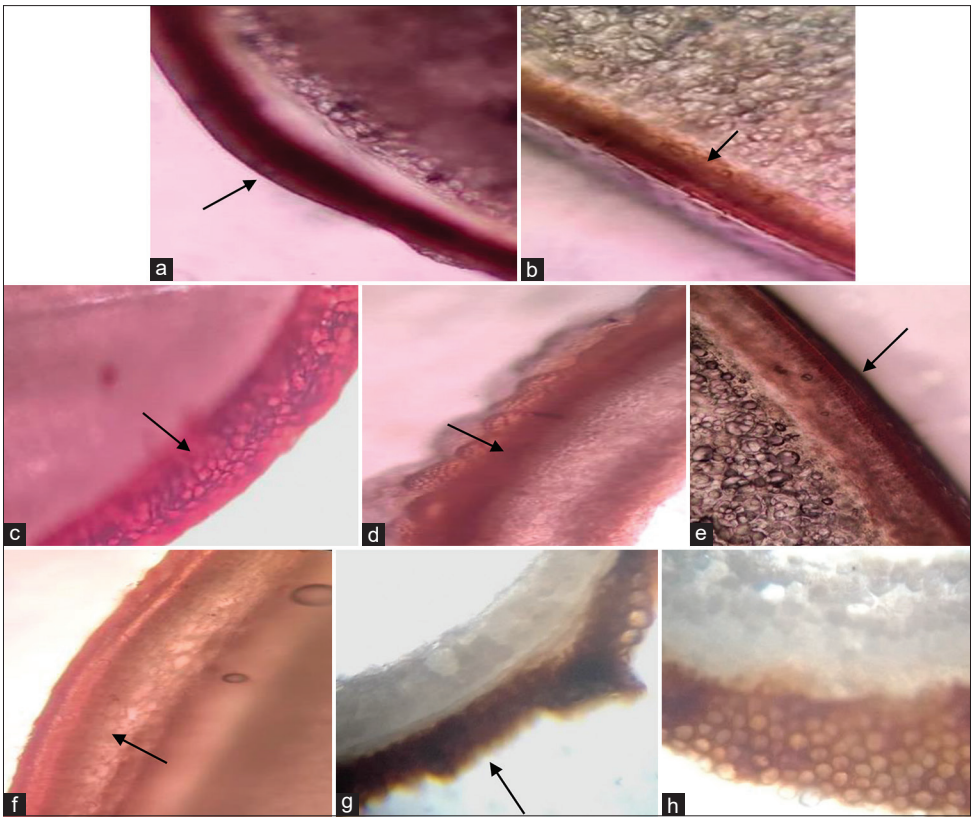

Figure 1: Light Microscopy observation of phytomelanin distribution in seed coat. a) *Vigna mungo* (Fabaceae), b) *Macrotyloma uniflorum* (Fabaceae), c) *Linum usitatissimum* (Linaceae), d) *Cicer arietinum* (Fabaceae), e) *Vigna unguiculata* (Fabaceae), f) *Phaselous vulgaris* (Leguminaceae), g and h) *Sesamum indicum* (Pedaliaceae). Arrows indicate phytomelanin deposition

catechol oxidases that are found in the epidermis layer (Gillikin & Graham, 1991). *S. indicum* seed coat had a layer of sclereids which had deposits of melanin.

L. usitatissimum seed coat is reported to possess 5 distinct layers, outer cuticle, epidermis, sclerenchymatous hypodermis, fibrous layer of parenchyma cells, and inner pigment layer. Dense pigmentation was observed in the lignified sclerenchymatous hypodermis layer which was made of 3-4 layers of cells. A prominent cuticle was also evident. A similar observation was reported by Park (2007). Phytomelanin precursor molecules synthesized in the chloroplast are deposited in the epidermis where it is polymerized. It is probably why it is more prominently found in the epidermis and hence offers protection to the seeds.

Extraction of Phytomelanins

The extraction of melanin is often complicated since it is bound to other cellular components. Phytomelanins were extracted with three concentrations of sodium hydroxide. The pigment extraction was directly proportional to the concentration of the alkali. 1 M NaOH extracted more pigments than 0.5 and 0.1 M. A similar result was reported by Selvakumar *et al.* (2008). Since phytomelanins have a very distinct property, identification using conventional phytochemical tests is possible. Phytomelanins are not soluble in organic and in organic solvents, or acids, readily soluble in alkali, sparingly soluble in DMSO, and insoluble in water (Shoeva *et al.*, 2020). It shows decolourization in Potassium permanganate, Potassium dichromate, sodium hypochlorite, and hydrogen peroxide. In the present study, 5% hydrogen peroxide showed rapid decolorization and a positive reaction with ferric chloride. It formed a precipitate immediately which indicated the presence of polyphenols. Phytomelanins are phenolic compounds. Precipitation with Ferric chloride is a vital step that indicates its purity.

UV-VIS Spectroscopy

The extracted pigments were subjected to UV-Vis spectroscopy. It was found that all extracts of the selected seeds had no absorption at the visible spectrum. Maximum absorption was evident in the range of 250-300 nm. Pralea *et al.* (2019) reported that phytomelanins have maximum absorption at 190-300 nm. The high UV light absorption of melanin is apparently due to the complex conjugated molecules in the melanin structure which absorb and scatter photons of UV light (Hou *et al.*, 2019).The isolated phytomelanins from the sunflower seed coat had a maximum absorption at 280 nm. In the present study, *S. indicum* seed coat melanin had absorbance at 276 nm (Figure 2a), *M. uniflorum* at 283 nm (Figure 2b), *L. usitatissimum* 276.5 nm (Figure 2c), *V. unguiculata* at 286 nm (Figure 2d), *V. mungo* and *P. vulgare* showed no absorbance.

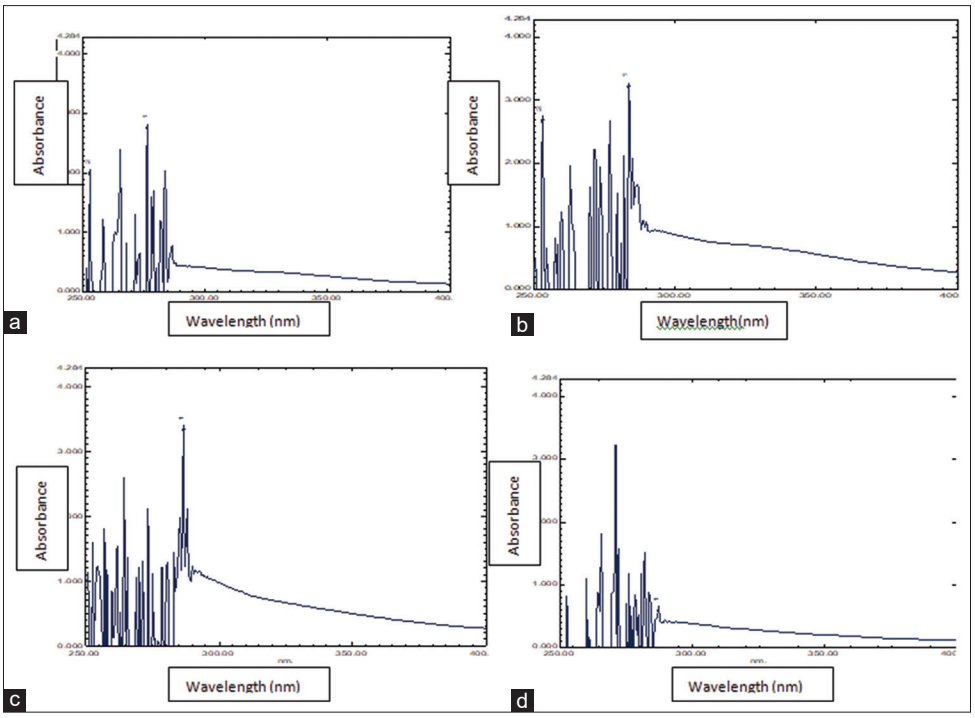

Figure 2: UV-Vis spectrum of the phytomelanin. a) *Sesamum indicum*, b) *Macrotylon uniflorum*, c) *Linnum usitatissimum* and d) *Vigna unguiculata*. Note the maximum absorption at 276, 283,276.5 and 286 nm of the respective phytomelanin

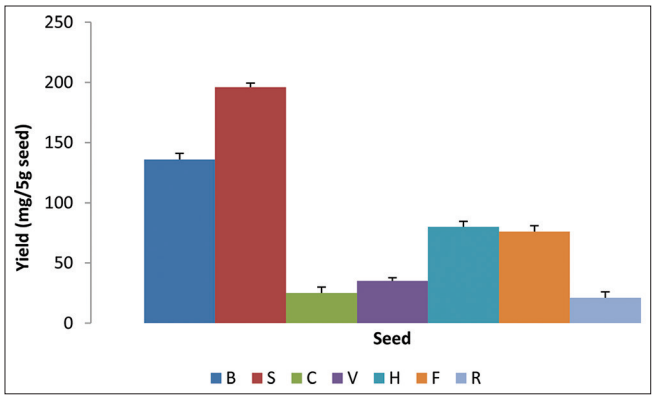

Figure 3: Yield of Phytomelanin/5 g of seed. B-*Vigna mungo*, S-*Sesamum indicum*, C-*Cicer arietinum*, V-*Vigna unguiculata*, H-*Macrotyloma uniflorum*, F-*Linum usitatissimum* and R-*Phaselous vulgaris*. All values in triplicate ± SD

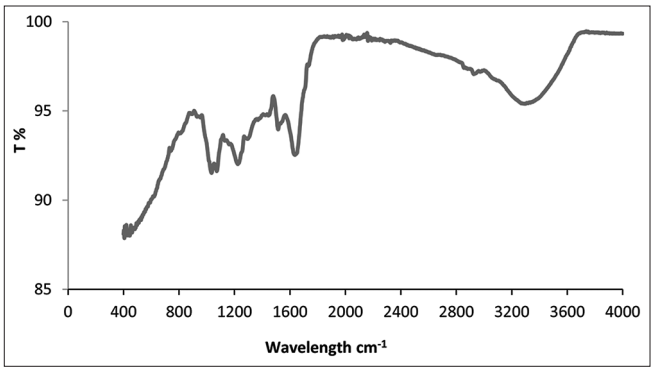

Figure 4: FT-IR Spectrum of phytomelanin isolated from *Sesamum indicum*

Yield of Melanin

The yield of the purified phytomelanins obtained per 5 g of seed was estimated. A higher yield of melanins was obtained in *S. indicum* (196 mg/5 g), followed by *V. mungo* (136 mg/5 g) and *M. uniflorum* (80 mg/5 g) and a lower yield were recorded in *P. vulgare* (21 mg/5 g) (Figure 3). The phytomelanin obtained from *M. uniflorum* seed coat was amorphous, flakey powder in *S. indicum* seed coat and was crystalline in *P. vulgaris*, *V. mungo* and *V. unguiculata* and resinous in *L. usitatissimum* seed coat extracts. The purified phytomelanins also showed no solubility in water and dissolved readily in NaOH.

Fourier Transform Infra-Red Spectroscopy

In the present study all the purified samples of *S. indicum*, *V. unguiculata* and *M. uniflorum* exhibited absorption between 3200-3500 indicating N-H and OH stretching, C=C bonding between 1600-1500 cm^{-1} and 1320-1210 cm^{-1} indicating –C-O stretching and band stretching around 1024 indicating a pyrone C-O-C structure, band between 1230-1020 shows C-N Bonding (Qi *et al.*, 2020) (Figures 4, 5 & 6).

The present study has shown the presence of phytomelan in Fabaceae, Lianaceae and Pedaliacae. Phytomelanin has antioxidant properties and new sources of phytomelanin can be ultilized for pharmacological purposes and can also contribute to the nutritive values of theses seeds. However, only limitation is the solubility, dosage and toxicity if any need to be studied

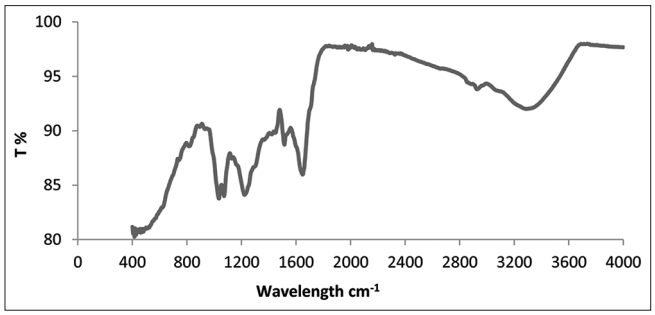

Figure 5: FT-IR Spectrum of phytomelanin isolated from *Vigna unguiculata*

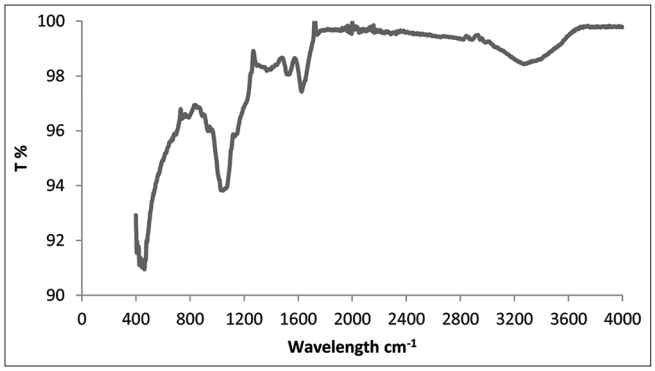

Figure 6: FT-IR Spectrum of phytomelanin isolated from *Macrotyloma uniflorum*

in an animal model before being used for the pharmacological purposes.

CONCLUSION

Studies on phytomelanin distribution are few, the significance of the work is the discovery of phytomelanin in Fabaceae, Linaceae, and Pedaliacaeae genus which is not reported earlier. The isolated phytomelanin can be formulated as a plant protector either as foliar spray or seed dressing since it is reported to possess antimicrobial properties. Hence it will have its application in agriculture for the control of plant diseases. Further work on the antimicrobial properties of phytomelanin will justify the above statement.

ACKNOWLEDGMENT

The Author wishes to thank the Head of the PG Research Department of Botany and the Principal, of Bharathi Women's College for providing the laboratory facility to carry out this work. The authors also wish to thank Marina Labs, Chennai for their help during the course of the work.

REFERENCES

Al-Tayib, O. A., El Tahir, K. E., Idriss, M. H., Eram, K. E., & Hassib, A. M. (2014). *Nigella sativa* L seeds melanin: A new hypoglycemic agent. Comparison with insulin in alloxan-diabetic rats. *Scholars Academic Journal of Pharmacy, 3*(4), 332-335.

Bazhenov, M. S., Litvinov, D. Y., Divashuk, M. G. (2023). Melanin found in wheat spike husks. Preprint BioRxiv. https://doi.org/10.1101/2023.05.30.542842

Cardinale, M., Viola, M., Miceli, E., Faddetta, T., Puglia, A. M., Maggini, V., Tani, C., Firenzuoli, F., Schiff, S., Bogani, P., Fani, R., & Papini, A. (2021). The Cypsela (Achene) of Echinacea Purpurea as a Diffusion Unit of a Community of Microorganisms. *Applied Microbiology and Biotechnology, 105*, 2951-2965. https://doi.org/10.1007/s00253-021-11212-2

Ceccarelli, S., Grando, S., & Van Leur, J. A. G. (1987). Genetic diversity in barley landraces from Syria and Jordan. *Euphytica, 36*, 389-405. https://doi.org/10.1007/BF00041482

El-Naggar, N. E.-A., & El-Ewasy, S. M. (2017). Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment produced by newly isolated microbial cell factories *Streptomyces glaucescens* NEAE-H. *Scientific Reports, 7*, 42129. https://doi.org/10.1038/srep42129

Gillikin, J. W., & Graham, J. S. (1991). Purification and developmental analysis of the major anionic peroxidases from the seed coat of *Glycine max*. *Plant Physiology, 96*, 214-220. https://doi.org/10.1104/pp.96.1.214

Glagoleva, A. Y., Shoeva, O. Y., & Khlestkina, E. K. (2020). Melanin Pigment in Plants: Current Knowledge and Future Perspectives. *Frontiers in Plant Science, 11*, 770. https://doi.org/10.3389/fpls.2020.00770

Hou, R., Liu, X., Xiang, K., Chen, L., Wu, X., Lin, W., Zheng, M., & Fu, J. (2019). Characterization of the physicochemical properties and extraction optimization of natural melanin from *Inonotus hispidus* mushroom. *Food Chemistry, 277*, 533-542. https://doi.org/10.1016/j.foodchem.2018.11.002

Kalka, K., Mukhtar, H., Turowski-Wanke, A., & Merk, H. (2000). Biomelanin Antioxidants in Cosmetics: Assessment Based on Inhibition of Lipid Peroxidation. *Skin Pharmacology and Applied Skin Physiology, 13*(3-4), 143-149.

Keles, Y., & Özdemir, Ö. (2018). Extraction, purification, antioxidant properties, and stability conditions of phytomelanin pigment on the sunflower seeds. *International Journal of Secondary Metabolite, 5*, 140-148. https://doi.org/10.21448/ijsm.377470

Laxmi, M., Kurian, N. K., Smitha, S., & Bhat, S. G. (2016). Melanin and bacteriocin from marine bacteria inhibit biofilms of foodborne pathogens. *Indian Journal of Biotechnology, 15*(3), 392-399.

Marques, D., Marzinek, J., & De-Paula, O. C. (2021). A new report of phytomelanin in cypselae of Vernonieae: the case of the type species of *Lychnophora* Mart. *Annals of the Brazilian Academy of Sciences, 93*(4), e20210204. https://doi.org/10.1590/0001-3765202120210204

Pandey, A. K., Wilcox, L. W., Sack, F. D., & Stuessy, F. (1989). Development of the phytomelanin layer in fruits of *Ageratum conyzoides* (Compositae). *American Journal of Botany, 76*(5), 739-746. https://doi.org/10.1002/j.1537-2197.1989.tb11368.x

Park, K.-I., Ishikawa, N., Morita, Y. Choi, J.-D., Hoshino, A., & Iida, S. (2007). A bHLH regulatory gene in the common morning glory, *Ipomoea purpurea*, controls anthocyanin biosynthesis in flowers, proanthocyanidin and phytomelanin pigmentation in seeds, and seed trichome formation. *Plant Journal, 49*(4), 641-654. https://doi.org/10.1111/j.1365-313X.2006.02988.x

Pralea, I.-E., Moldovan, R.-C., Petrache, A.-M., Ilieș, M., Hegheș, S.-C., Ielciu, I., Nicoară, R., Moldovan, M., Ene, M., Radu, M., Uifălean, A., & Iuga, C.-A. (2019). From extraction to advanced analytical methods: the challenges of melanin analysis. *International Journal of Molecular Sciences, 20*(16), 3943. https://doi.org/10.3390/ijms20163943

Qi, Y., Liu, J., Liu, Y., Yan, D., Wu, H., & Li, R. (2020). Polyphenol oxidase plays a critical role in melanin formation in the fruit skin of persimmon (*Diospyros kaki* cv. 'Heishi') *Food Chemistry, 330*, 127253. https://doi.org/10.1016/j.foodchem.2020.127253

Roy, S., & Rhim, J. W. (2022). New Insight into Melanin for Food Packaging and Biotechnology Applications. *Critical Reviews in Food Science and Nutrition, 62*(17), 4629-4655, https://doi.org/10.1080/10408398.2021.1878097

Selvakumar, P., Rajasekar, S., Periasamy, K., & Raaman, N. (2008). Isolation and characterization of melanin pigment from *Pleurotus cystidiosus* (telomorph of *Antromycopsis macrocarpa*). *World Journal of Microbiology and Biotechnology, 24*, 2125-2131. https://doi.org/10.1007/s11274-008-9718-2

Shoeva, O. Y., Mursalimov, S. R., Gracheva, N. V., Glagoleva, A. Y., Börner, A., & Khlestkina, E. K. (2020). Melanin formation in barley grain occurs within plastids of pericarp and husk cells. *Scientific Reports, 10*, 179. https://doi.org/10.1038/s41598-019-56982-y