Evaluation of microscopic structure of *Justicia wynaadensis* and the stability of its color extracted by using conventional and microwave extraction method

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Revised: 2 February 2015 / Accepted: 5 February 2015 / Published online: 26 February 2015

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Abstract *Justicia wynaadensis* locally known as Moddu Soppu belongs to the family Acanthaceae. The aqueous bluish purple color extracted from the leaves and stems of *Justicia* is used in the preparation of a sweet dish by the natives of Kodagu district, Karanataka, India, exclusively during the monsoons. This traditional practice is believed to keep the people healthy throughout the year. Owing to its potential to be used as a natural colorant there is a strong need to develop efficient extraction method for maximum yield of colorant and preliminary scientific study of stability. Microscopy was carried out to find out the location of purple color in both leaves and stem and to study the anatomical details. An optimized microwave extraction method for extraction of colorant from *Justicia* has been developed. The microwave oven extraction yield of colorant is 9.41 % (± 0.8598) under the optimized conditions of extraction time 30 min., extraction temperature 150 °C and ratio of liquid to solid 30 ml/g. The study demonstrated that the purple extract of the plant was stable against pH range 6.0–8.0, low temperature (4 °C), high temperature (≥ 50 °C) and sensitive to light. Thus these observations recommend the use of *J. wynaadensis* extract as a food colorant in neutral or slightly alkaline products

(bakery, milk, egg etc.) and could be the potential source for the food colorant market.

Keywords *Justicia wynaadensis* · Microscopy · Microwave extraction · Stability

Introduction

Justicia wynaadensis locally known as Moddu Soppu is a small branched shrub belonging to the family Acanthaceae. The aqueous bluish purple colored extract is used in the preparation of a sweet dish by the natives of Kodagu district, Karanataka, exclusively during the monsoons. This plant is reported to be endemic to the region of Western Ghats and common in forest of Irpu (Ponnamma and Manjunath 2012a, b). The juice from the stem and leaves of this plant is extracted by boiling in water. The deep bluish purple colored extract with a unique flavor is known to possess 18 medicinal principles useful to improve human health. This traditional practice is believed to keep the people healthy throughout the year.

Ethanopharmacological survey in Kodagu district has reported traditional medicinal use of this plant in asthma and immunity (Lingaraju et al. 2013). The plant is used as an external application over rheumatic swellings by the people of Kuruchiar tribes of tirunelli forest, Wayanad district, Kerala. Total phenolics, flavonoids and alkaloids have been quantitatively evaluated in methanolic extract of root, stem and leaf of the *Justicia wynaadensis*. (John et al. 2013; John et al. 2014). The extract of the plant *Justicia* was studied for its catalase as well as peroxidase activity and phenolics and flavonoids were also been identified and estimated (Medapa et al. 2011). The said plant has also been reported to lower cellular cholesterol and cholesteryl ester concentration and

Research highlights

- Present work is the first report of microscopic characterization of *Justicia wynaadensis* for the origin of color
- Microwave oven extraction method has been optimized for maximum yield of colorant i.e. 9.41 % (± 0.8598).
- The study demonstrated that the purple extract of the plant is stable against pH range 6.0–8.0, low temperature (4 °C), high temperature (≥ 50 °C) and sensitive to light.
- The study recommends the use of *J. wynaadensis* extract as a food colorant in neutral or slightly alkaline products (bakery, milk, egg etc.).

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also known to possess novel inhibitory effect on the uptake of ox-LDL by human macrophage cell line (Subbiah and Norman 2002). The volatile phytocomponents present in the methanolic extract of its aerial plants were studied by GC-MS technique to ascertain the medicinal properties of the plant (Ponnamma and Manjunath 2012a). A rapid method for multiple shoot regeneration from the nodal explants of *Justicia* has also been reported (Ponnamma and Manjunath 2012b). It has also been studied for thorough preliminary phytochemical screening of aqueous extract and comparative HPTLC fingerprint profiles of fresh and dry powder aqueous extract. HPTLC fingerprint profile has been developed for the separation of color components for its proposed application as a natural colorant in the field of food industry (Nigudkar et al. 2014).

Natural plant colorants have been in high demand by the food industry for replacing synthetic dyes such as FD& C red 40 and the banned FD& C red 2 for the past decade (Francis 1989; Fossen et al. 1998). Highly stable colorant system may already be present in the nature, however they need to be identified and characterized for their phytochemical compositions and stability attributes. *Justicia* offers high potential as colorant source which can be easily extracted from stem and leaves throughout the year and have a history of folklore use for its edible purpose. It is necessary to develop efficient extraction method for maximum yield of color. It is also essential to study the stability of color for its applications.

In present work, the macroscopic and microscopic evaluation of stem and leaf was carried out to investigate the location of bluish purple color from *Justicia wynaadensis* as it morphologically does not show presence of color except occasional presence of purple color streaks at nodal region especially during monsoon. The aim of present work is also to optimize efficient extraction method over traditional maceration method to obtain maximum yield of colorant in less time. Our approach involved determining the stability of this colorant for its proposed application in the food industry.

Material and methods

Source: *Justicia wynaadensis* was collected from Coorg district, Karnataka, India. The herbarium specimen is prepared and authenticated from Botanical survey of India, Pune (voucher no. GNKC-7). The fresh stem and leaves were washed thoroughly and used for further studies. All the chemicals and solvents used are analytical grade.

Equipment and apparatus

Multifunctional microwave oven (Sharp carousel) model number R-880B having 0–1200 W range was used for extraction. The emission frequency of the microwave oven was

2450 MHz. The extraction power and extraction temperature cannot be set at the same time in this oven. For filtration, cold centrifuge (Hermle Labortechnik GmbH, Z326K) was used. Stability studies were carried out using UV-visible spectrophotometer (Shimadzu, 1650). Phase contrast microscope A-210 equipped with 3MPx camera was used for microscopic work.

Microscopy

The leaf and stem were studied for its morphological characteristics and a thin free hand section of leaf passing through midrib and Transverse section (T.S.) of stem passing through node and internode without stain was studied for its microscopic characteristics especially to find out the location of bluish purple color pigment and characterization of microscopic details.

Extraction methods of *Justicia* colorant

The colored juice of *Justicia* is extracted by conventional maceration method. However, to obtain maximum yield of colorant in less time, an efficient method of microwave oven extraction has been developed.

1. **Conventional maceration method:** Maceration extraction was performed in a water bath at 100 °C; 5.0 g fresh sample was placed into a 250 ml glass flask with 100 ml water and extracted for 90 min.
2. **Optimization of Microwave oven extraction method (MWE):** There are many parameters that affect the microwave extraction efficiency, such as particle size of sample, liquid to solid ratio, extraction temperature and microwave power. Based on the preliminary experimental results, appropriate ranges of extraction time, extraction temperature and ratio of solid to liquid were selected. The variables for every factor are given in Table 1. The weighed plant material was taken in conical flask and water was added to it. The flask was plugged with cotton and kept in microwave oven. The oven was operated at convection mode where the temperature of the oven can be monitored keeping the microwave power constant. After microwave extraction in oven, the samples were filtered through Whatman paper no. 41 and centrifuged for 15 min. at 10,000 rpm. The percent yield of the extract

Table 1 Variables for Microwave oven extraction

Independent variables	Ranges
Ratio of sample: solvent (g/ml)	10, 20, 30, 40, 50
Temperature (°C)	130, 150, 160, 180, 200
Time (min.)	10, 20, 30, 40, 50

was calculated for every sample. The optimized MWE was repeated thrice and Standard deviation was calculated. Microwave extracted colorant residues had been reconstituted in water and checked for its $\lambda_{\max}$ using UV–visible spectrophotometer.

Measurement of color and stability

The leaves and stems of *J. wynaadensis* were extracted in microwave oven with previously optimized conditions. The colored extract was filtered through whatman filter paper no. 41 and centrifuged at 10,000 rpm for 15 min. A scan from 400 to 800 nm was performed in order to generate the characteristic absorption spectra of the sample using UV–visible spectrophotometer. The sample was prepared initially at pH = 7.4 (original pH of the extract) with absorbance reading of 0.8 at the wavelength of maximum absorption in the visible region. The sample was prepared at six different pH values (2, 4, 6, 8, 10, and 12) using 0.1 N NaOH and 1 N HCl. Additionally 5 % ethanol was added to avoid microbial growth. The effect of pH on colorant stability was performed immediately and between 1 h and 1 month interval at 25 °C with the samples inside capped vials sealed with parafilm. The spectra were recorded between 200–700 nm on UV–visible spectrophotometer.

The sample after extraction was stored under refrigeration condition (4 °C). It was transferred to different glass vials which were then kept at a) temperature 15 °C, 40 °C, 50 °C, 70 °C, 100 °C and 150 °C, b) light condition (day and night cycle) and dark condition. Different time intervals for 1 month were selected. At appropriate time intervals, the samples were taken out to measure the absorbance at its $\lambda_{\max}$ 589nm.

The stability data were studied by analysis of variance (ANOVA) at $\alpha = 95$ % confidence level.

Result and discussion

Morphological characteristics

Stem: Elongate with distant nodes, slender, terete, striate, glabrous or nearly so, cylindrical, swollen at the nodes with occasional purple colored streaks.

Leaf: leaves opposite decussate, 7.5 to 10 × 2.5 to 4.0 cm, elliptic – lanceolate, acuminate, pubescent on nerves beneath, petiole 1.2 cm long (Fig. 1).

Transverse section (T.S) of stem

T.S of stem passing through node and T.S. of stem passing through internode were studied mainly to compare the presence

of purple color as stem node shows occasional presence of purple color streaks and to study their anatomical details.

Transverse section (T.S) of stem passing through node

Epidermis is wavy in outline, single layered made up of tubular cells, probably with bluish colour. It is followed by hypodermis which is few layered, made up of chlorenchyma cells. Next to this is a region of narrow cortex which is divided into three parts i.e. outer, middle and inner cortex. The outer cortex covers the collenchyma cells which are interrupted by vertical bands of 1–2 layers of cells filled with purple colour. Middle cortex is a narrow zone of spherical cells rich purple in colour. Inner cortex is 5–7 layered made up of chlorenchyma cells extended in continuation of the vertical bands of purple colour cells in the region of outer cortex/collenchyma cells. Vascular cylinder covers the vascular bundles which are arranged in a ring of six. Endodermis is a single layer of barrel shaped cells, wavy in outline. Pericycle is not distinguishable. Phloem is 2–3 layered. Cambium is not very distinguishable. Abundant secondary xylem was observed with rows of xylem sclerenchyma with scattered pores. Primary xylem is projected in the pith below secondary xylem at certain points. Pith is a very broad zone of parenchyma. Many cells of pith are filled with raphides – bundles of needle like crystals of calcium oxalate and also with star shaped crystals i.e. sphaeraphides. Besides this calcium carbonate crystals or cystoliths are also observed in the epidermal region of the stem node (Fig. 2).

T.S of stem passing through internode

Shows same anatomical characters as that of node with the exception that as it is observed in the middle cortex region

Fig. 1 *Justicia wynaadensis* plant

Fig. 2 A-T.S of stem passing through node, B- Enlarged view of T.S stem node showing blue layer on epidermis and middle cortex cells rich in purple color, C-T.S of stem nodal peeling showing cystoliths

Fig. 3 A- T.S of stem passing through internode, B- T.S of stem passing through internode with well developed secondary xylem

instead of purple color, the cells are filled with green color i.e. chlorophyll. (Fig. 3)

T.S of leaf passing through midrib

Transverse section of leaf passing through the midrib showed an upper and lower, single-layered epidermis, occasionally bluish purple colored. Epidermis is covered with two types of epidermal hairs or trichomes i.e. uniseriate multicellular trichomes are present all over the upper and lower epidermis and glandular trichomes are also observed with a unicellular head and stalk. Stomata are observed on both upper and lower epidermis with scattered calcium carbonate crystals or cystoliths. It is followed by 3–4 layers of collenchyma cells in the midrib region along upper and lower epidermis. In the midrib region ground tissue is made up of compactly arranged parenchyma cells and covers the crescent shaped stellar region with vascular bundles. The xylem consisted mostly of vessels and a strip of cambium was present between the xylem and phloem tissues. The chlorenchyma cells which are oval in shape and 3–4 layered are running continuous in the midrib region along the upper epidermis. The lamina is dorsiventral with the mesophyll differentiated into a palisade and spongy tissue. Below the upper epidermis is a single layer of elongated, closely

arranged, palisade parenchyma cells. Spongy parenchyma tissues are almost radially elongated with intercellular spaces. (Fig. 4)

Microwave extraction in oven

The effect of different solvent proportion on the colorant yield is shown in Fig. 5a. It showed that the yield of colorant is greatly influenced by the volume of the water. The yield of color is affected by different extraction temperatures (130 °C, 150 °C, 160 °C, 180 °C, 200 °C respectively) were shown in Fig. 5b. The yield of colorant was greatly influenced by the extraction temperature when extraction temperature reaches at 150 °C, maximum yield was obtained. Further increase in temperature resulted in decrease in the extraction yield. This data suggests that the higher temperature beyond 150°C may lead to the thermal degradation of color. When other two factors (extraction temperature and ratio of solvent to sample) were fixed at 150°C and 30 ml per gram, the result indicated that the yield of total colorant increased with the increase of microwave extraction time in the beginning of extraction. The yield reached its maximum at 9.41 % in 30 min. When extraction time exceeds more than 30 min, the yield of colorant does not increase with the increase of extraction time. Hence, the

Fig. 4 a, –T.S of leaf passing through midrib, b- Surface view of leaf peeling showing stomata and cystoliths, c,d – Trichomes. Abbreviations: cyst – cystolith, epi – epidermis, oc -outer cortex, coll – collenchyma, ic - inner cortex, chlo – chlorophyll, mc - middle cortex, vb - vascular bundles,

pth – pith, stom – stomata, uepi -upper epidermis, lepi - lower epidermis, pls - palisade tissue, spts - spongy tissue, gr.ts - ground tissue, tri – trichome, sec. xy - secondary xylem, gtri - glandular trichomes

Fig. 5 **a-** Effect of solvent proportion, **b-** Effect of temperature, **c-** Effect of time on the yield of colorant

extraction time optimized is 30 min. The influence of extraction time on the yield of total colorant is shown in Fig. 5c. The percent yield of colorant was found to be 9.41 (± 0.8598).

Comparison of microwave extraction (MWE) with maceration extraction method

As shown in Table 2, the optimized MWE has produced 9.41 % of color extract which is higher than that of maceration extraction (5.62 %). The MWE has advantage of reducing extraction time, saving energy and increased yield as compared to maceration extraction.

The colorant obtained by both the methods has been separated on TLC according to the method described for *Justicia* extract (Nigudkar et al. 2014) to ensure fingerprint pattern of color bands. TLC fingerprint showed similar pattern for both the methods which further confirms that the color components have not degraded during microwave oven extraction. (Fig. 6)

Table 2 Comparison of microwave extraction and traditional maceration extraction

Extraction method	Maceration extraction	Microwave oven
Temperature (°C)	100	150
Time (min.)	90	30
Ratio solid/liquid (g/ml)	30	30
Yield of color (%)	5.62	9.41

UV–Visible spectroscopy analysis and stability studies

The characteristic purple color manifested by the color extract could be the consequence of the presence of compounds derived from anthocyanin group. Fig.7

Fig. 6 Comparison of Conventional (C) and Microwave oven extraction (M) method

shows the absorption spectra generated during the analysis of the aqueous color extract. The preliminary phytochemical study shows the positive tests for presence of anthocyanins (Nigudkar et al. 2014).

A maximum absorbance peak ($\lambda_{\max}$) was detected at 589 nm. This value is higher than those corresponding to anthocyanins like petunidin ($\lambda_{\max} = 543$ nm), malvidin ($\lambda_{\max} = 542$ nm) or delphinidin ($\lambda_{\max} = 546$ nm). This fact suggests a bathochromic effects probably caused by co-pigmentation mechanisms. It is a consequence of a typical anthocyanins intermolecular association within flavonoids like tannins and catechins or glucocyclic groups which can act like co-pigments (Gris et al. 2007; Eiro and Heinonen, 2002; Boulton 2001; Andersen and Markham 2006).

The co-pigmentation phenomenon was observed already in 1916 by Willstätter and Zollinger (1916), who noticed the color of a grape pigment, oenin (malvidin 3-glucoside), to change its hue to bluer red by the addition of tannin or gallic acid. Copigmentation was first studied with flower colors. Robinson and Robinson (1931) observed the same anthocyanin to produce different color hues in different flower petals, and different anthocyanins to produce the same colors in different parts of plants.

In 1915, Willstates and Mieg related the red-purple color of *Delphinium ajacis*, with delphinidin molecule. It have been observed that when delphinidin suffer co-pigmentation, its $\lambda_{\max}$ is shifted until closer values to that observed for *Justicia* aqueous extract ($\lambda_{\max} = 589$ nm).

They reported that an increase in cafeic acid concentration shifts the $\lambda_{\max}$ to highest value and also increase the possibility of hyperchromic effects in anthocyanins at pH value between 3.0 and 4.0. Other reports indicate that during the formation of cafeic acid/delphinidin complex, $\lambda_{\max}$ value is shifted until 585 nm. On the other hand, Schreiber et al. observed that the complexation of delphinidin with metallic ions like Al^{3+} displaced $\lambda_{\max}$ even 589 nm. (Espinosa-Morales et al. 2012)

At pH 6.0–7.5, the aqueous extract achieved purple color at $\lambda_{\max} = 589$, but further increase in pH induced a bluish shift to reach maximum around pH = 8.0, while after pH = 8.0 up to 12.0, the $\lambda_{\max}$ values decreased. The pH variation also affected color intensity of color extract. A pH decreases from 6.0 to 2.0 resulted shift in color change towards orange giving $\lambda_{\max} = 491$ (pH = 2.0 and 4.0). The maximum color intensity of purple color extract was reached in the alkaline region at pH = 7.5. (Fig. 8a, b).

The purple color extract initially showed attractive colors at all pH values, however the high color intensity in the alkaline region (pH = 7.0–12.0) decreases significantly after 72 hrs at room temperature (25°C). The color of these unstable solutions increasingly shifted towards brown and yellow with time. After 1 month period, alkaline region showed browning and yellowing of solutions. However, pH = 6.0 solution had shown purple hue. (Fig. 8c).

The color extracted from *Justicia* is particularly unstable in acidic media and can only be extracted if neutral solvents are employed. This type of behavior of

Fig. 7 Visible spectra of *Justicia wynaadensis* aqueous extract

Fig. 8 **a**- Spectrum charts at different pH, **b** and **c**- Pictures of colorants at all pH after 1 h and 30 days respectively

anthocyanin is reported in Advances in flavonoid research (Harborne and Williams 2000).

Temperature stability

The thermal stability of purple color extract was studied at the storage temperatures, 4 °C to 50 °C as well as at high temperatures of 70 °C to 150 °C which are the common food processing temperatures for example cooking, pasteurizing, frying, blanching etc. (Lee et al. 2011). It was found that the purple extract showed gradual degradation to about 94.73 % residual color in about 30 days at 4 °C. It was more interesting to observe that the purple extract of *Justicia* is relatively more stable at 50 °C, a temperature above 45 °C which is for testing the stability of cosmetic products, compared with those at room temperature (25 °C) and 40 °C as typified by a longer stabilization period and greater retention of its color (90.78 %) even after 30 days. (Fig. 9b)

As a light would cause photodegradation of anthocyanin, the effect of light on the stability of purple extract was conducted at room temperature (25 °C for 14 days). The color was retained better when kept in the dark. Fig. 9a shows that the stability of the sample kept in the dark was 65.22 % of purple color remained whereas 52.02 % of residual color remained for the samples kept in the light after 12 days of storage.

Fig. 9c shows the degradation profiles of *Justicia* at high temperatures. The $t_{1/2}$ values of purple anthocyanin were longer than the period required for high processing temperatures of some food and cosmetics products.

Results for ANOVA performed for determining the relation between temperature and residual color percent revealed that there is significant difference between these two parameters at $p = 0.05$. The residual color percentage decreases with the increase in temperature and hence, the residual color percentage significantly depends upon the temperature.

Conclusion

Present work is the first report of microscopic characterization of *Justicia wynaadensis* especially for the location of color source. Microscopy was carried out and it is found that nodal region of stem shows presence of purple pigment in the middle cortex region and layer of blue pigment is found along upper and lower epidermis of the leaf as well as stem. However, purple color was not observed in the middle cortex of internodal region of the stem as the cells are filled with chlorophyll pigment.

The microwave extraction method was optimized for maximum yield of extraction. The optimized microwave extraction can save time, energy, improve extraction yield of total colorant.

Fig. 9 a-Effect of light and dark conditions, b-Effect of temperatures, and c-Effect of Higher temperatures

The stability of color was studied using spectrophotometer at different pH, temperature and light and dark conditions. *Justicia* colorant at pH 6.0–7.5, the aqueous extract achieved purple color at $\lambda_{\max} = 589$ whereas at around pH = 8.0, it has its most bluish color. These observations recommend the use of *Justicia* extract as food colorants in neutral or slightly alkaline products (bakery, milk, egg etc.)

The study demonstrated that the purple *Justicia* extract has great thermal stability and its capability to withstand high temperature for duration longer than the normal processing temperature for certain food and cosmetic products.

Thus these results suggest that colorant extract from *Justicia wynaadensis* represent inexpensive plant with high pigment yield and it can be propagated easily throughout the year that could be a source of anthocyanins for the food colorant market.

To have a better understanding of the mechanism and kinetics of degradation of the purple anthocyanins, further investigation using purified anthocyanins is warranted.

Acknowledgments The authors wish to express sincere thanks to G. N. Khalsa College and GNIRD department for providing facilities and support. The authors are thankful to Dr. Hema Sane for giving her valuable inputs in understanding microscopic details and Ms. Gauri Shende for her valuable help in statistical analysis.

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