Effect of different solvents extraction on recovery of pigments in *Xylocarpus granatum*, endangered medicinal plant

Z. Hasni*[1], J. S. Yaacob[1], A. I. M. Yusoff[1], R. M. Taha[1], R. Yahya[2], A. Bakrudeen Ali Ahmed[1] and K. Ramesh[3]

Xylocarpus granatum is locally known as Pokok Nyireh Bunga. This endangered mangrove species has economical importance. Pigments of *X. granatum* were investigated in the mature leaves of seedlings collected from Carey Island, Selangor, Malaysia. The pigments were solvent extracted using 80% methanol, 80% acetone, 80% *N,N*-dimethylformamide and 100% hexane. As recommended by Bertrand and Schoefs,[1] all the extraction steps were performed under weak light intensity to avoid photosynthetic pigment degradation. The pigments were detected by ultraviolet–visible spectroscopy and thin layer chromatography. Aqueous acetone was the best solvent for pigment extraction compared to methanol, *N,N*-dimethylformamide and hexane.

Keywords: Mangrove, Chlorophyll, UV–Vis spectroscopy, Thin layer chromatography

Introduction

Xylocarpus granatum, commonly known as 'Pokok Nyireh Bung' in Malaysia, is native to the tropical mangrove forests of Africa, Australia, Malaysia and India. *X. granatum* is an endangered mangrove plant and is economically important for wood carvings, furniture and interior construction. It has been helpful for the natural ecosystem and is exploited by both internal and external agents. The internal forces, such as utilisation of the mangroves by fishermen for timber, fuel, fodder and medicine, have been in practice for a long time, even before the systematic identification of these taxa. In Carey Island, this is an essential wood and has importance for the Mah Meri indigenous people, whose evocative carvings are one of the major aspects of their culture.[2]

X. granatum is a traditional medicinal plant, with reported use as astringent, antiparasitic and antidiarrhoeal preparation.[3] Recent studies have shown that stem bark extracts are effective in the treatment of diarrhoea in mice.[4] The stem bark extracts of this evergreen mangrove have been found to contain high amounts of procyanidins and catechins and have been observed to have effect against Gram positive bacteria.[5–7] In China, one novel tetranortripenoid derivative (xylocarponoid A) was isolated from the seeds of *X. granatum*.[8] Moreover, the *X. granatum* fruit constituents, gedunin and photogedunin, have been shown to possess a significant antisecretory effect on peptic ulcers.[9]

Pigments are classified into different groups, such as tetrapyrroles (e.g. chlorophylls), carotenoids (e.g. β-carotene), polyphenolics (e.g. anthocyanin) and alkaloids (e.g. betalains). Chlorophylls and carotenoids are hydrophobic compounds and can be extracted from single or mixed organic solvents.[10] Natural colourants have become increasingly popular with consumers because synthetic colourants are frequently perceived as undesirable or harmful.[11,12]

In this study, the pigments of *X. granatum* were extracted by different organic solvents. Major compounds like chlorophylls and carotenoids were very commonly present in the plant. These pigments have been shown to play crucial roles in photosynthesis. Additionally, other compounds, such as carotenes and xanthophylls, provide a protective mechanism for plant growth under saline stress conditions.[13]

Experimental

Plant collection

X. granatum seeds and propagules (Fig. 1) were collected from the mangrove forest of Carey Island, Selangor, Malaysia. The seedlings were maintained in the garden of the Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia. Six-month-old to 1-year-old seedlings were used in this experiment.

Solvent extraction

Healthy, mature leaves were collected for pigment extraction. Two grams of leaves (the midrib and large veins of the leaves were discarded) was solvent extracted

[1]Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia
[2]Department of Chemistry, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia
[3]Centre for Ionics, Faculty of Science, University Malaya, Kuala Lumpur 50603, Malaysia

*Corresponding author, email zulianahasni@hotmail.com

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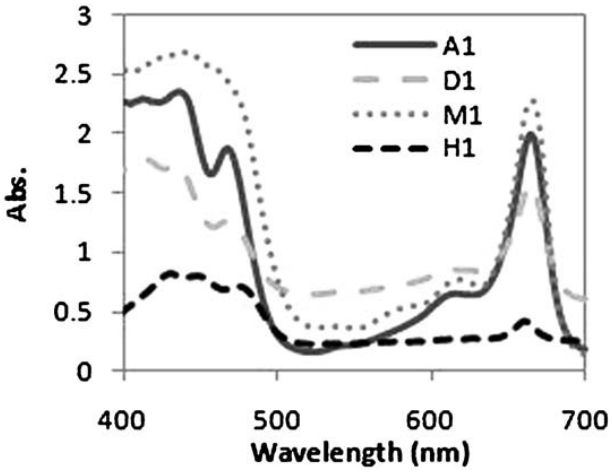
a fruit; *b* flower; *c* trunk; *d* germinated seedling; *e* leaves

1 ***Xylocarpus granatum***



2 **Absorbance spectra of freshly prepared *Xylocarpus granatum* pigments which were isolated from 80% methanol (M1), 80% acetone (A1), 80%DMF (D1) and 100% hexane (H1)**

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separately in each of the four different solvents, namely, acetone, methanol, *N*,*N*-dimethylformamide (DMF) and hexane. A small amount of $MgCO_3$ and sand were added to the solvent. Then, the mixtures were homogenised with a mortar and pestle. The homogenates were centrifuged at 5500 rev min^{-1} for 10 min (4°C). The supernatants were collected and evaporated to a small volume in rotavapour at 25°C. Absorption spectroscopy was performed using a Shimadzu UV-1650 PC spectrophotometer. The remaining supernatants were wrapped in aluminium foil and immediately stored in the dark at 4°C before chromatographic analysis. All the experiments were carried out in dim light.

Thin layer chromatographic (TLC) analysis

Thin layer chromatography of chlorophylls and carotenoids was carried out on commercial TLC aluminium Silica Gel 60 F254 (Merck). The solvent extracts were transferred in the standard manner, and the plates were eluted in a closed chamber. The developing solvent system used to separate the pigments was toluene/ethyl acetate/acetone (80 : 20 : 10). The TLC plates were placed under UV light (360 nm) for visualisation of the separated pigments.

Results and discussion

Chlorophylls are unstable in the presence of acid and light. Magnesium carbonate was added to the mixture to neutralise the acidity of the solvents since trace amounts of acid will promote the conversion of chlorophylls to pheophytins.[14] The addition of sand in the mixture helps to disintegrate the leaf substances and to lyse the cells and acts as an inert material, which improves the extraction process.[15] Pigments could also degrade in the rotavapour. This technique can be improved by adding the inert material $MgSO_4$ as a drying agent[15] instead of using a rotavapour that takes a long time to vapourise the water in the sample.

In addition to chlorophylls and carotenoids, the leaves of mangroves contain many extractable pigments. Spectral analysis of these pigments may provide information on their adaptive features.[13] Figure 2 depicts the absorption spectra of pigments extracted from *X. granatum* in four different solvents. Spectral analysis of the pigments extracted from the leaves showed typical absorption bands of the chlorophyll region broadly from 635 to 680 nm. Spectral analysis exhibited chlorophyll at the red absorption peaks at 663 nm (A1, D1 and H1) and at 666 nm (M1). M1 showed a high intensity of pigments, followed by A1, D1 and H1. The intensity of the pigments in four different solvents at 666 nm for M1 was 2·28 followed by A1 (1·97), D1 (1·55) and H1 (0·41) at 663 nm.

Chlorophyll a and chlorophyll b together with carotenoid absorbed broadly in the blue 'soret' region at 400–500 nm. Since purification of the pigments was not performed in this experiment, spectral analysis of the blue region exhibited overlapping chlorophyll a, chlorophyll b and carotenoid peaks. The intensity of M1 was also higher than A1, D1 and H1 in the blue region.

Methanol has been reported to be more efficient than acetone under some circumstances.[4] Methanol removed 20% more pigment than 90% acetone when tissue grinding was performed. In addition, methanol removed three times more pigment than 90% acetone with sonication.[16] Sartory and Grobbelaar[17] found that 90% acetone was an inefficient organic solvent compared to methanol or 95% ethanol. However, it has been shown that the use of methanol as a solvent for

Table 1 **R_f values of freshly isolated *X. granatum* pigments separated on a commercial silica gel plate and toluene/ethyl acetate/acetone (80 : 20 : 10) as the mobile phase***

Pigment/solvent	R_f values			
	A1	M1	D1	H1
β-carotene	0·96	0·92	0·90	0·97
Chlorophyll a	0·50	0·50	0·50	0·50
Chlorophyll b	0·44	0·45	0·42	0·40
Xanthophyll 1	0·26	0·29	0·28	0·28
Xanthophyll 2	0·12	…	…	…
Breakdown product (pheophytin)	…	0·7	…	…

*A1: acetone; M1: methanol; D1: *N,N*-dimethylformamide; H1: hexane.

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extraction resulted in unstable pigments, and the absorption band of pigments was broad and less sharp since methanol enhances the degradation of chlorophylls by opening the isocyclic ring.[18] Although 100% acetone was not found to yield the highest amount of chlorophyll from any particular species, its use as an extracting solvent strongly inhibited the formation of degradation pigments.[19]

Alternatives to aqueous acetone for chlorophyll extraction are DMF, dimethyl sulphoxide (DMSO) and methanol. In DMF and DMSO, as in aqueous 80% acetone, chlorophyll a and b exhibit sharp Qy peaks.[20] Cell disruption for pigment extraction is not required when extracting with DMF, and the pigments remain stable for up to 20 days when stored in the dark at 5°C, according to Schuman *et al.*[21] and Simon and Helliwell.[16] However, DMF and DMSO are more toxic than acetone,[20] which decrease their appeal as efficient solvents.[19]

When observed under UV light (360 nm), fluorescence bands were observed in all the TLC plates. Table 1 depicts the R_f value of the four different solvent extracts. β-carotene, chlorophyll a, chlorophyll b and xanthophyll 1 were observed in all four solvent extracts. However, xanthophyll 2 was only observed in A1 (80% acetone) and H1 (100% hexane). Breakdown products were detected on the M1 TLC plate. In general, the separation illustrated in the TLC plates had four zones, labelled β-carotene (R_f=0·9), chlorophyll a (R_f=0·5), chlorophyll b (R_f=0·4) and xanthophyll (R_f=0·2) from top to bottom of the silica gel layer for all the solvents (Table 1). However, in M1, additional zones for pheophytins (R_f=0·7) were found to be separated at an R_f higher than the original chlorophyll, resulting from the loss of magnesium ions from chlorophyll molecules.[22]

The use of silica gel promoted the degradation of pigments and resulted in multiple chlorophyll zones.[23] Hinesol could have been used as a chlorophyll preserving substance to reduce pigment degradation.[23] The solvent systems reported in the literature often give acceptable resolution between chlorophyll a and chlorophyll b but poor resolution between chlorophyll b and xanthophyll as overlapping bands. This problem can be solved by adding small quantities of alcohol, of which methanol has been found to be superior to ethanol and isopropanol.[15]

Conclusions

In conclusion, it was shown that chlorophyll and carotenoid were detected in all the solvents. In addition, some alternative extractants, such as DMF,[20] were more toxic to plant cells than aqueous acetone. Methanol could extract more pigments compared to other solvents; however, it could cause pigment degradation.[18] In this study, we have confirmed that aqueous acetone is the most suitable solvent for chlorophyll and carotenoid extraction as compared to hexane, methanol and DMF. Polar solvents like hexane are less suitable for pigment extraction. Purification of pigments should be performed to obtain precise results.

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