Production of Red Pigment from the Root of *Morinda angustifolia* Roxb. var. *scabridula* Craib. by Root Cell Culture

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ABSTRACT

Antraquinone is a group of natural red dye found in the root of *Morinda* sp. which is available in the upper north of Thailand and has been widely used on cotton dyeing. Recently, interest in natural dyes has increased and there is a need to find suitable alternative sources of natural dyes. We have studied one alternative to increase the production of anthraquinone dye by root cell culture of *Morinda angustifolia* Roxb. var. *scabridula* Craib. The major components of the red pigment extracted from the root of this plant was purified and analyzed for the chemical structure and was found to be an anthraquinone pigment morindone. Uncontaminated root cells were obtained by growing the *Morinda* seed on Murashige and Skoog agar medium(MS).The roots were cut into 2.5 mm. pieces and grown in fresh MS medium to get callus. The callus fully proliferation on a modified Gamborg's B_5 medium supplemented with 40 g/l succinic acid, 0.1 mg/l kinetin, 0.2 mg/l auxin and 20 g/l sucrose cultured in shake flask 100 rpm at 25°C for 3 months gave root cells only 0.14 g dry weight whereas the callus cultured on B_5 agar at the same condition gave 1.22 g dry cells within one month. The cultivated callus contained red and yellow pigments. Extraction of the pigment from the cultured cells and separated by Thin Layer Chromatography with the same solvent system as what had been used to separate pigment in the plant's root extract and gave two major components with R_f values comparable to the red pigment extracted from *Morinda* plant's root. The production of anthraquinone dye from root cells cultured for 5 months was 1.4 times of the cells cultured for 3 months and could produce 0.6 times of the dye from the root of 2-3 years old plant.

Key words: Red pigment, *Morinda* sp. Extraction, Root cell culture

INTRODUCTION

Plants are the origin of most natural dyes. Some common plants containing dyes include wood, weld, madder and goldenrod. Alum, bichromate of potash, copper, ferrous sulfate, stannous chloride and tannic acid are common mordants (Lemmens and Wulijirni-Soetjipto,1992). The natural red dyes including the anthraquinones, naphthoquinones and benzoquinones could be also found in varieties of plants (Koyama et al.,2001). Anthraquinones are important naturally occuring pigments that are widely distributed in nature. They are particularly prominent in fungi (Turner and Aldridge , 1983; Gill and Steglich, 1987; Gill, 1994), higher plants

(Thomsom,1987), and lichens (Huneck, 1973, 1984, 1991; Huneck et al.,1991,1994; Elix et al.,1984). Biosynthesis of anthraquinone from plant is derived from isochorismate which is formed by isomerization of chorismate catalyzed by the enzyme isochorismate synthase (EC 5.4.99.6) with Mg^{2+} as cofactor (Polsen et al.,1991). The first aromatic intermediate in the biosynthesis of anthraquinone in higher plants is o-succinylbenzoic acid. It is formed from isochorismate and α-ketoglutaric acid in the presence of thaimine pyrophosphate (Simantiras et al.,1991). This reaction links the Shikimate-Mevalonate pathway to the anthraquinone in plants (Leistner, 1973). *Rubia tinctorum* L., the source of a natural dye, produces anthraquinone pigments in its root . Hairy roots of *R. tinctorum* cultured in liquid medium in the presence of 5 μM IAA showed the maximal growth rate and the highest anthraquinone production while kinetin had no effect. Higher concentrations of sucrose (6-18%) inhibited growth in the presence of phytohormones (5 μM IAA or 0.5 μM NAA). In contrast, in phytohormone-free medium, 12% sucrose resulted in maximal growth and anthraquinone production (Sato, 1991). Cell suspensions of *Morinda citrifolia* are able to produce large amounts of anthraquinones when they are cultivated on B_5 medium containing 1 mg l^{-1} naphtylacetic acid (NAA) (Van der Plas et al.,1995). Twenty five anthraquinones from *Cinchona ledgeriana* callus and suspension cultures and 16 anthraquinones from callus cultures of *Cinchona pubescens* have been reported (Mulder-Krieger et al.,1982; Wijnsma et al., 1984; Robins et al., 1986; Wijnsma et al.,1986). A suspension culture of *C. robusta* produced eight new anthraquinones, robustaquinones A-H, in addition to two known anthraquinones, 1,3,8-trihydroxy-2-methoxyanthraquinone and copareolation 6-methyl ether (Schripsema et al., 1999). The nonionic surfactant Pluronic F-68 has adventageous effects as a growth-stimulating supplement in *M. citrifolia* suspensions at 2% (w/v). It also improved the release of intracellular secondary metabolites (Bassetti and Tramper, 1995). By supplementing the growth medium with 2% (w/v) of Pluronic F-68 for four repeated batch cycles, cells released anthraquinones to a concentration 170-times of the value of the control cells. An average of 55% of the intercellularly stored anthraquinones was recovered from the Pluronic-treated cells against 0.74% of the control (Bassetti et al.,1995).

In upper north of Thailand, natural red dyes extracted from the root of *Morinda* sp. have been widely used on cotton in combination with some kind of mordants. In this region, at least four *Morinda* sp. have been found and the most abundant is *Morinda angustifolia* Roxb. var. *scabridula* Craib. We have studied one alternative to increase the production of natural dye by root cell culture of this plant. The major pigment extracted from root cell culture were purified and identified by chemical analysis. The red pigment from the extract of root cell culture and the major component from the extract of the plant's root were compared.

MATERIALS AND METHODS

Plant Materials Roots and seeds of two or three years old plant of *Morinda angustifolia* Roxb.var. *scabridula* Craib. were used.

Culturing of the Root Cells Sterilized root cells were obtained by growing the *Morinda* seeds on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The roots were cut into 2.5 mm pieces and grown in fresh MS medium to get callus which was fully proliferated on a modified Gamborg's B_5 at 25°C (Gamborg et al.,1968) (Fig.1) in the liquid and solid medium. The amount of anthraquinone dye produced in the cell culture for 3 and 5 months were compared.

Extraction and Characterization of the Major Pigments from Root Cell Culture The root cell culture of *Morinda* sp. was grown on B_5 medium for 1, 3 and 5 months. At each cultured period the produced pigments were extracted from 59 g wet cells with 1.4 litres of chloroform. The crude extract from the root cell culture were evaporated under reduced pressure by rotatory evaporator to obtain the concentrated dyestuff. The crude extract from the root cell cultured for 3 and 5 months were separated by thin layer chromatography on silica gel 60 with chloroform:methanol (9:1 v/v) as eluent. The amount of the dye production from the root cell culture were obtaines by comparing the density of the major band separated on TLC chromatogram on densitometer using the purified morindone from the plant's root as reference.

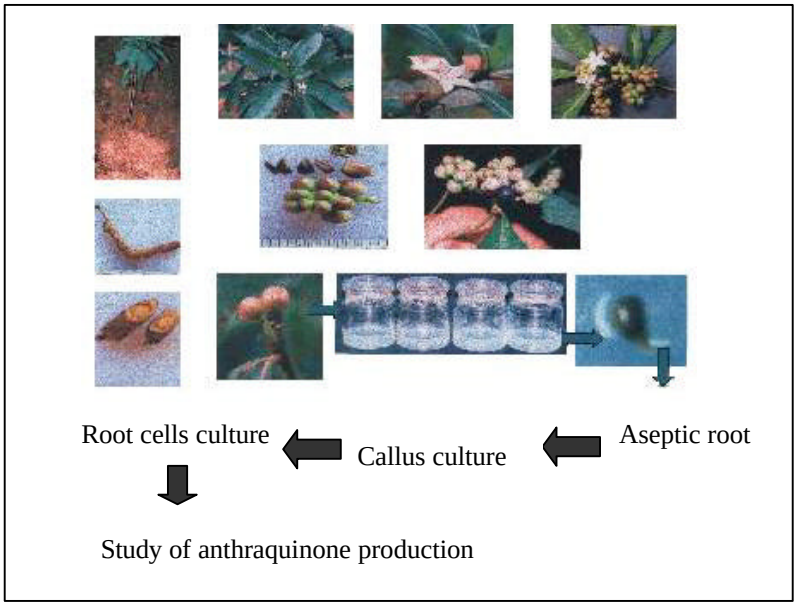


Figure 1. Preparation and culturing of root cells from *Morinda angustifolia* Roxb.var. *scabridula* Craib.

Extraction of Red Pigment from the Plant's Root The root of *Morinda angustifolia* Roxb. var. *scabridula* Craib. were washed, cut into small pieces, dried and ground into powder. One and a half kilograms of the root powder were extracted with 6 litres of 95% methanol in Soxhlet's apparatus until the methanol in the soxhlet was colourless. The methanol in the crude extract was then evaporated under reduced pressure to obtain the concentrated dyestuff.

Analysis of the Red Pigments Classification of the red pigment in the crude extract was performed using chemical tests according to its main structural component (Fransworth, 1966). For the test of anthraquinone dye, the crude extract in methanol was boiled with 0.5 N KOH then dilute H_2O_2 was added and filtered. The filtrate was then acidified with CH_3COOH and extracted with benzene. The anthraquinone dye would show a yellow colour in benzene phase which was separated and shook with dilute ammonium hydroxide, the pink or red colour would appear in ammonium hydroxide phase.

Separation of Major Component of the Red Pigment Major components of the red pigment from the crude extract were separated by column chromatography. The

elution solvent system was found by using thin layer chromatography with silica gel 40 as an adsorbent and characterized the chromatogram by spraying 0.5 M KOH and varied elution solvent system. The most suitable one was used to separate the major component by column chromatography. A glass column 45x3 cm containing 60 g of silica gel 40 was used to separate 3 g of crude extract and eluted with chloroform followed by chloroform : methanol (9:1 v/v) and chloroform : methanol (1:1), respectively. The eluted fractions were dried by evaporation of the eluted solvents.

Purification of the Major Component The major component obtained from column chromatography eluted with chloroform : methanol (9:1) was rechromatographed on silica gel 40 column of 20x1.5 cm saturated with hexane. Fifty milligrams of the major component was passed through the column and eluted with hexane : chloroform (9:1 v/v) by isocratic elution. The eluted fractions were combined and evaporated to dryness and recrystallized in the mixture of chloroform and methanol.

Chemical Structural Analysis of the Purified Pigment The chemical structure of the purified pigment was analyzed by UV-Visible spectroscopy, Mass spectrometry and C^{13} nuclear magnetic resonance spectroscopy.

RESULTS AND DISCUSSION

Production of the red pigment from the root of *Morinda* sp. by root cell culture was firstly done by the extraction of red pigment from the root of *Morinda angustifolia* Roxb. var. *scabridula* Craib. (Fig. 2) followed by purification of the major component and chemical structure analysis. They were found to be in the anthraquinone group by chemical test which were the same as the red pigment produced from 2-3 years old plants of *M. cereia* Ham., *M. citrifolia* Linn. or *M. lucida*. (Perry,1980).The purified component was used as reference to follow the production of anthraquinones from root cell culture.

Figure 2. *Morinda angustifolia* Roxb.var. *scabridula* Craib.

Extraction and Characterization of the Red Pigment from the Root of *Morinda angustifolia* Roxb. var. *scabridula* Craib.

The effective extraction of the red pigment from the plant's root could be done by continueously extraction of the dried root powder with methanol. About fifty percents of water lost upon drying of the root before grinding. The amounts of the red pigments found in the extract were from dried root powder. The crude extract of the red pigment obtained from 1.5 kg of the root powder was 267.5 g which was about 14% of root dry weight which was the amount of pigments generally found in the plants. At least three components have been separated from the crude extract by column chromatography on silica gel 40 eluted with chloroform and chloroform : methanol solvent system (Table 1). The major component was obtained when eluted by chloroform : methanol (9:1) which was 8.33% of the crude extract or 1.5% of the dried root powder. Among chemical tests for various groups of dyes, the extracted red pigments fell in the group of anthraquinone dye that gave yellow colour in acidic solution (pH 1.5-6), orange in basic side (pH 6.5-9) and red colour at high pH value (>pH 10).

Table 1. Separation of major components of red pigments from the crude extract of *Morinda angustifolia* Roxb.var. *scabridula* Craib. root by column chromatography on silica gel 40

Solvent system	Separated components	Total amount (g)	% of root dry weight
Chloroform	Component I	0.04	0.0
$CHCl_3$:CH_3OH (9:1)	Component II	0.25	1.5
$CHCl_3$:CH_3OH (1:1)	Component III plus Other compounds Separated component III	2.259 very low	- very low

(-) not calculated

Purification and Structural Analysis of the Major Component

The major component II has rechromatographed on the same column and eluted with hexane : chloroform (1:1). The colour fraction was evaporated to dryness and recrystallized in hot methanol which was acidified with 10%HCl solution and left overnight at 5°C. The orange needle shape crystal of the major component was found to be very pure when checked with thin layer chromatography technique using various developing solvents.

The UV-Visible spectrum of the purified major component had maximum absorption at 446 nm in absolute ethanol (Fig.3). The mass spectrum of the purified dye in Fig.4 showed molecular ion (M-H) at m/e equal to 269 but the resulted mass spectrum was measured by using FAB-(negative mode), therefore the molecular mass of this compound should be 270. Fig.5 showed C^{13}-NMR spectrum of the compound which was exactly the same as the C^{13}-NMR spectrum of a reference compound morindone. The assignment of C^{13}-NMR spectra of major component was shown in Table 2. It could be concluded from these analysis results that the major component of the red pigment from the root of *Morinda angustifolia* Roxb. var. *scabridula* Craib.

was a compound with M.W. of 270, melting point at 282°C had molecular formular $C_{15}H_{10}O_5$ and had chemical structure as morindone (Fig.6). Morindone was also obtained from the bark of *Coprosma australis* after exhaustively extracted with ethanol and purified (Roberts et al., 1977).

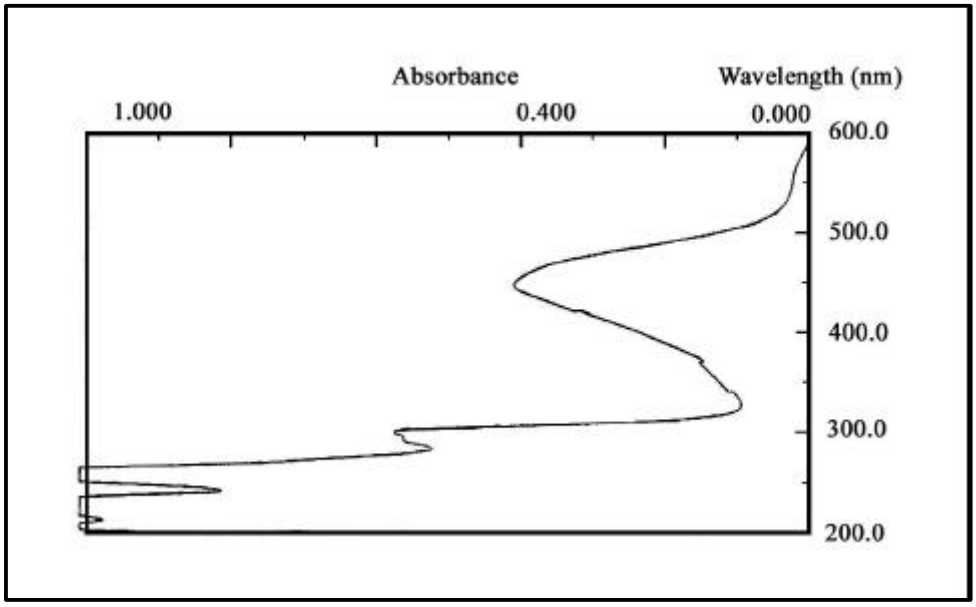


Figure 3. UV-Visible spectrum of the red pigment from the root of *Morinda angustifolia* Roxb. var. *scabridula* Craib.

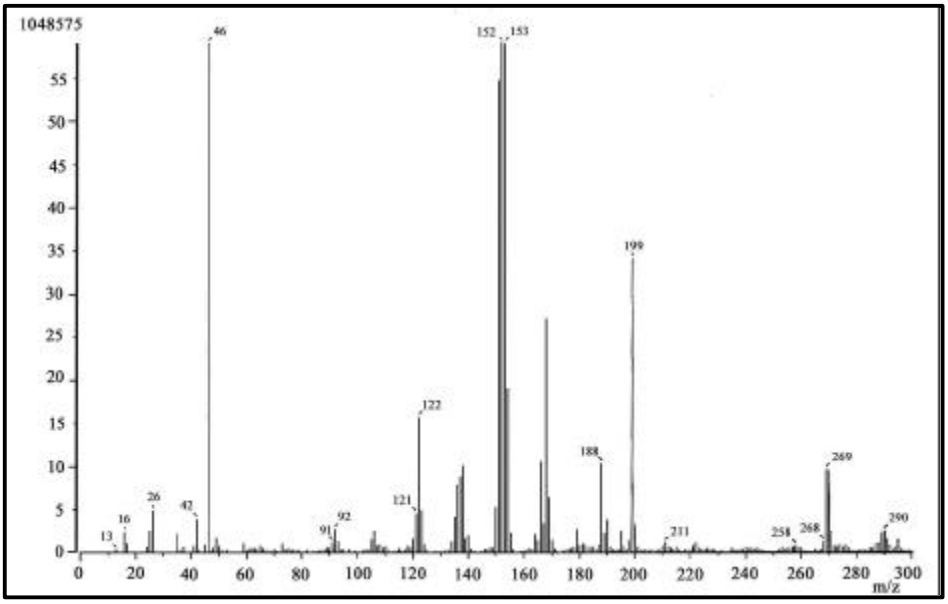


Figure 4. Mass spectrum of the red pigment from the root of *Morinda angustifolia* Roxb. var. *scabridula* Craib.

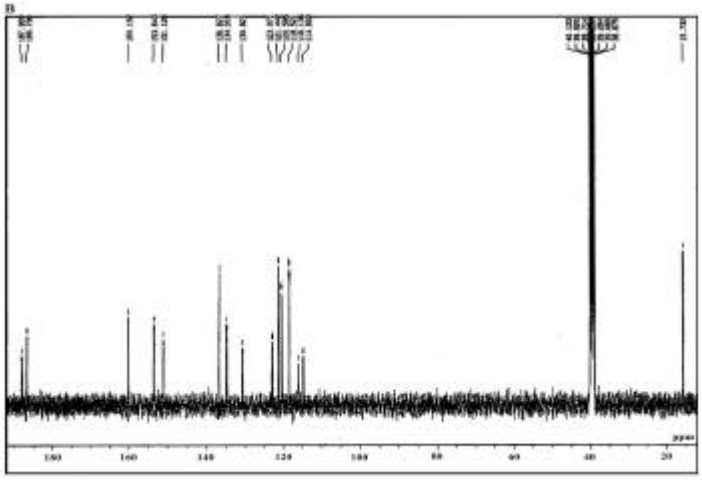


Figure 5. C^{13}-NMR spectrum of the red pigment from the root of *Morinda angustifolia* Roxb. var. *scabridula* Craib.

Table 2. C^{13}-NMR chemical shiff (ppm) of major component

Peak No.	Position of C^{13}	Chemical shiff (ppm)
1	C-10	187.909
2	C-9	186.798
3	C-5	160.192
4	C-2	153.643
5	C-1	151.126
6	C-7	136.827
7	C-8a	134.918
8	C-6	130.821
9	C-4a	123.070
10	C-4	121.442
11	C-9a	120.595
12	C-8	118.521
13	C-10a	116.136
14	C-3	114.902
15	6-CH_3	15.732

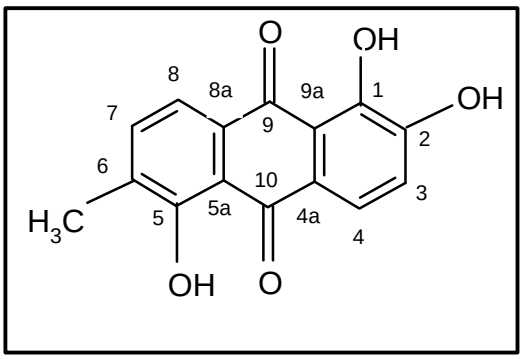


Figure 6. Chemical structure of morindone, the major component of the red pigment extracted from *Morinda angustifolia* Roxb. var. *scabridula* Craib.

Production of the Red Pigments from Root Cell Culture

The root of *Morinda angustifolia* Roxb. var. *scabridula* Craib. was failed to grow on solid medium after washing and soaking in 10% clorox dued to contamination of microorganism from soil. Therefore, we chose another alternative to culture root cells from the seeds of this plant on liquid and solid medium.

Sterillized root cells were obtained by growing the *Morinda* seed on MS medium. The roots were cut into 2.5 mm pieces and grown in fresh MS medium to get callus which was fully proliferated on a modified Gamborgs's B_5 at 25°C in liquid and solid culture. The root cell was cultured in shake flask of 100 rpm for 14 to 30 days. It was found that the root cell grew slowly and gave very low yield that was not enough for detection of the pigment production. Culturing of the root cell on B_5 agar medium for 1 month could produce red and yellow pigments. Table 3 showed the amount of anthraquinone dye produced in the root cell cultured on Gamborgs's B_5 agar medium for 3 and 5 months compared to the amount extracted from the root of 2-3 years old plant. This was done by comparing of the density of the major band from TLC chromatogram using purified morindone as reference on densitometer (Fig. 7)

Table 3. Anthraquinone dye production from *Morinda* root cell culture.

Sample	Cultivation (Months)	Anthraquinone (% of dry material)
Morinda root powder	2 – 3 years	1.5
Root cell culture	5	0.89
Root cell culture	3	0.65

Anthraquinone dye from the plant's root was found to be 1.5 % of dry root powder. The major component from root cell cultured for 5 and 3 months were 0.89 and 0.65 % of dry cells, respectively. It was found that the root cells cultured for 5 months increased 2.6 times of root cell weight and the pigment production was 1.4 times of the root cells cultured for 3 months and 0.6 times of the amount of pigment found in the root powder of 2-3 years old plant.

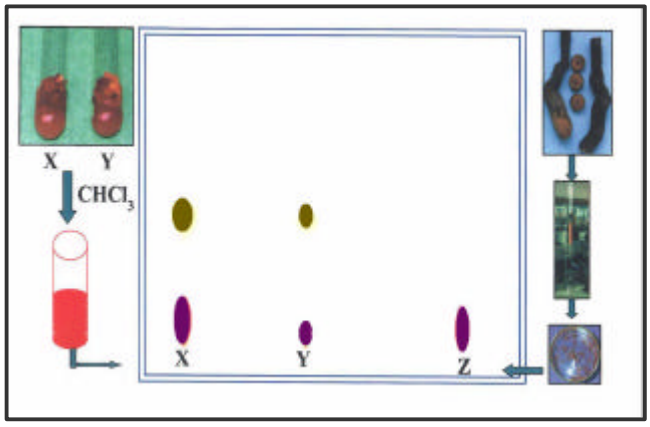


Figure 7. TLC analysis of the anthraquinone produced by of *Morinda angustifolia* Roxb. var. *scabridula* Craib. root cell culture X = crude extract from 5 months old callus, Y = crude extract from 3 months old callus, Z = reference morindone

Extraction, Purification and Analysis of the Red Pigment from the Root Cell Culture

The extraction of the red pigment from root cell culture with $CHCl_3$ gave two major spots on TLC with the R_f values the same as the components extracted with ethanol (data not shown). One component (red) was morindone and other one (yellow) had R_f values the same as the component from alkali extract of the root which has not yet been identified (Fig.8).

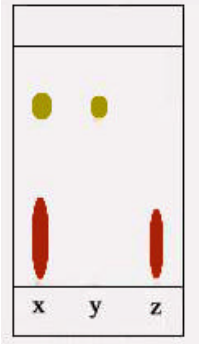


Figure 8. TLC analysis of the anthraquinone from X = crude extract from root cell culture
Y = alkali extract of the plant's root
Z = reference morindone (purified from the ethanol extract of the plant's root)

The UV-Visible and IR spectra of the purified unknown component from root cell culture and the alkali extract of the plant's were the same as anthraflavic acid (2,6-dihydroxyanthraquinone)(Pouchert, 1975.) but gave different spectra from the reference morindone (Fig.9 and 10). Table 4 and 5 showed the functional groups of the purified unknown component from root cell culture and alkali extract from the root of this plant. The red pigment from two sources included the same functional groups as O-H bending, asymmetric stretching of alkanes, symmetric stretching of alkanes, N-H in-plane bending of alkyl and aryl-NH-R and C-H out of plane bending of aromatic and had the difference of the functional groups as C-N stretching of aryl-NH_2 and alkyl-NH_2, C-N stretching of aryl-NH-R and C-O-C asymmetric stretching of aromatic. However, The structure elucidation of the purified unknown pigment will be confirmed by H^1 and C^{13}- NMR and mass spectrometry in the near future.

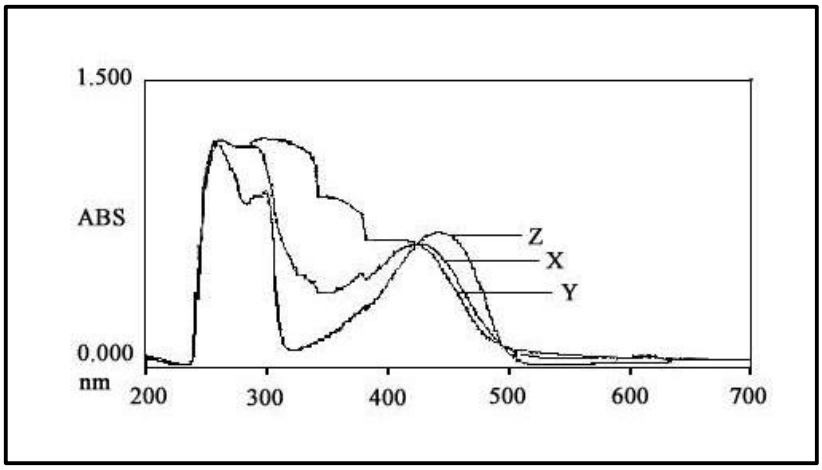


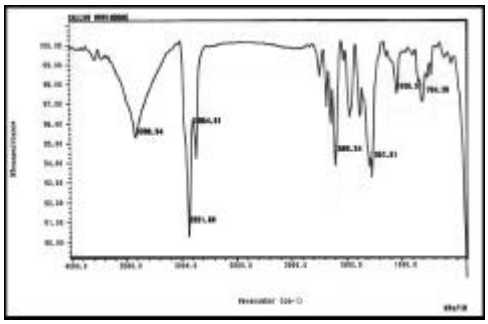
Figure 9. UV-Visible spectra of anthraquinone dye from crude extract of root cell culture (X), alkali extract of the plant's root (Y) and reference morindone (Z).

Table 4. The functional groups of the purified unknown component from the root cell culture

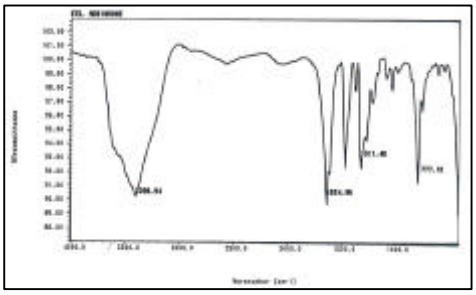
Wavenumber (cm^{-1})	Functional groups
3398.94	O-H bending
2921.60	Asym.stretch of alkanes
2864.61	Sym.stretch of alkanes
1589.34	N-H in plane bending of alkyl and aryl-
1261.61	C-N stretch of aryl-NH_2
1026.51	C-N stretch of alkyl-NH_2
784.28	C-H out of plane bending of aromatic

Table 5. The functional groups of the major component of alkali extract from the root of this plant

Wavenumber (cm^{-1})	Functional groups
3320.57	O-H bending
2921.60	Asym.stretch of alkanes
2850.39	Sym.stretch of alkanes
1560.84	N-H in plane bending of alkyl and aryl-NH-R
1290.11	C-N stretch of aryl-NH_2
1119.13	C-O-C asym.stretch of aromatic
713.03	C-H out of plane bending of aromatic



A



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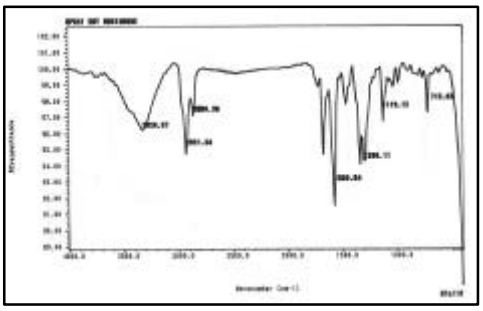

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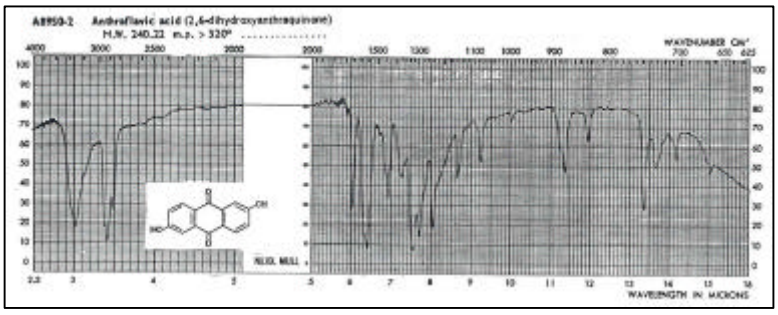

D

Figure 10. IR spectrum of the purified unknown pigment from root cell culture (A), alkali extract of the root plant (B), reference morindone (C) and anthraflavic acid (D) (Pouchert, 1975).

CONCLUSION

Increase production of anthraquinone dye could be obtained on solid culture of *Morinda angustifolia* root cells. Modification of culture by infection with *Agrobacterium rhizogenes* and cultured in Murashige and Skoog liquid medium containing 3% sucrose and in the presence of 5% μM IAA showed the maximal growth rate and highest anthraquinone production (Sato et al., 1991). This could be an alternative to increase the production of natural dye from this plant in the future.

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