CHAPTER IV

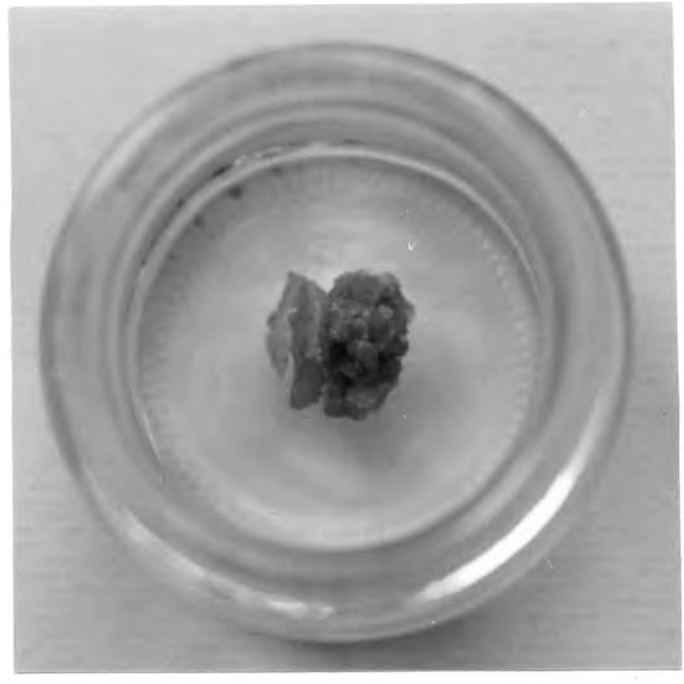
RESULTS

1. EFFECT OF HORMONAL FACTORS ON CALLUS FORMATION

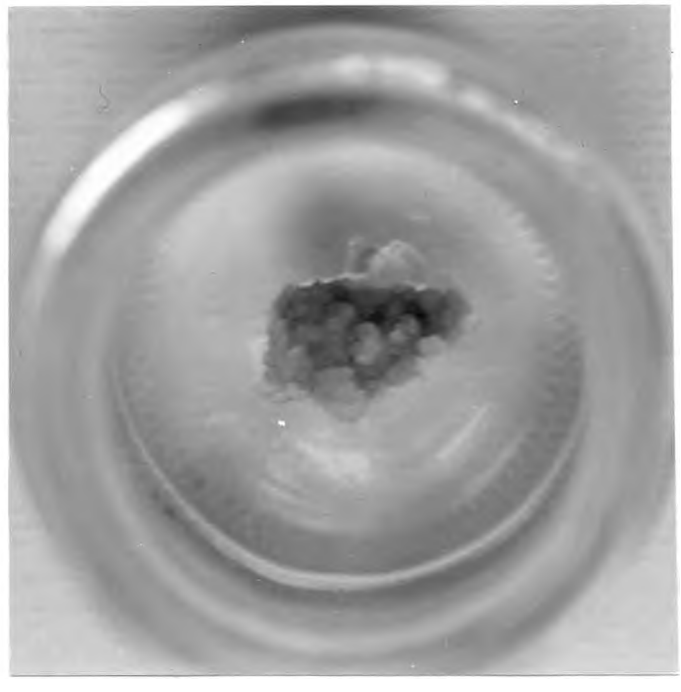
The ability of young leaf explants of *I. balsamina* to form callus was investigated by changing the type and concentration of growth regulators in standard B5 and MS media. From 36 different recipes of the tested media, it was found that B5 medium supplemented with 0.1 mg/l NAA and 1.0 mg/l BA could initiate callus formation from the leaf explants. The callus appeared to be dense with green color (Fig. 8A). This callus could be maintained by a regular four-week subculturing. In addition, 0.1 mg/l 2,4-D and 1.0 mg/l kinetin in B5 medium also effectively stimulated the formation of callus from the explants. The callus formed from this medium was, on the other hand, friable with pale yellow color (Fig. 8B). This callus cultures could be maintained by using the same medium with regular subculturing in a peroid of every three to four weeks.

2. ESTABLISHMENT OF CELL SUSPENSION CULTURES AND ROOT CULTURES

Although the green callus of *I. balsamina* could be maintained by regular subculturing, it was difficult to be used for initiating cell suspension cultures. This was due to the callus consisted of hard compact tissue with small closely packed cells and were not dispersed while shaken in B5 liquid medium. Cell suspension cultures, therefore, could not be established using this hormonal composition. On the other hand, it was found that the yellowish and friable callus was more suitable for initiating cell suspension cultures. The callus was separated into small aggregates upon transfer of some of the callus to B5 liquid medium containing the same hormonal factor composition. It was found, however, that the shaked suspension cultures had a slow growth rate. The cultured cells under these conditions also appeared to be fragile. Cell debris were found under



A



B

Fig. 8 Two forms of *I. balsamina* callus cultures established in B5 media containing different hormonal factors. A) dense and green callus cultures in B5 medium supplemented with 0.1 mg/l NAA and 1.0 mg/l BA and B) friable and yellowish callus cultures in B5 medium supplemented with 0.1 mg/l 2,4-D and 1.0 mg/l kinetin.

microscope in the liquid medium and most of them were accumulated on the interior surface of the glass vessel above the medium. Attempts were then made to improve cell integrity of *I. balsamina* suspension cultures. This was, again, done by changing the types of plant growth regulators in the liquid medium. It was found that B5 medium supplemented with 0.1 mg/l NAA, 0.1 mg/l kinetin and 1.0 mg/l BA stimulated the cultured cells to form root cultures (Fig. 9A). On the other hand, B5 containing 0.1 mg/l 2,4-D and 1.0 mg/l BA resulted in the formation of small aggregates of suspension cultures (Fig. 9B). However, while the root cultures could be maintained by a regular three-week subculturing, the cell suspension cultures were still fragile. Subsequent study found that the increase of calcium in B5 as much as two times of the normal concentration could greatly reduced the cell damage. The basal B5 medium used for maintaining *I. basalmina* cell cultures was therefore modified to contain double concentration of calcium chloride and used for all subsequent subculturing. For the effect of sucrose concentration on cell growth of suspension culture we found that when we reduced the sucrose concentration from 3 to 1%(w/v), the cell suspension cultures had more degree of cell dispersion than the concentration of 3% (w/v). On the other hand, when the sucrose concentration was increased to 5 or 7% (w/v), the degree of cell dispersion was decreased and the culture was slowed growth .

3. DETECTION OF LAWSONE AND 2-METHOXY-1,4-NAPHTHOQUINONE IN VARIOUS IN VITRO CULTURES OF I. BALSAMINA

After a few passages, the callus, cell supension and root cultures of *I. balsamina* were examined for their ability to produce lawsone and 2-methoxy-1,4-naphthoquinone. Crude extracts of each type of *I. balsamina* cultures were prepared and its chemical constituents were separated by thin layer chromatography. In this experiment, crude extracts prepared from various parts of mature *I. balsamina* plant were also examined in order to compare the chemical patterns obtained between the *in vitro* cultures and the whole

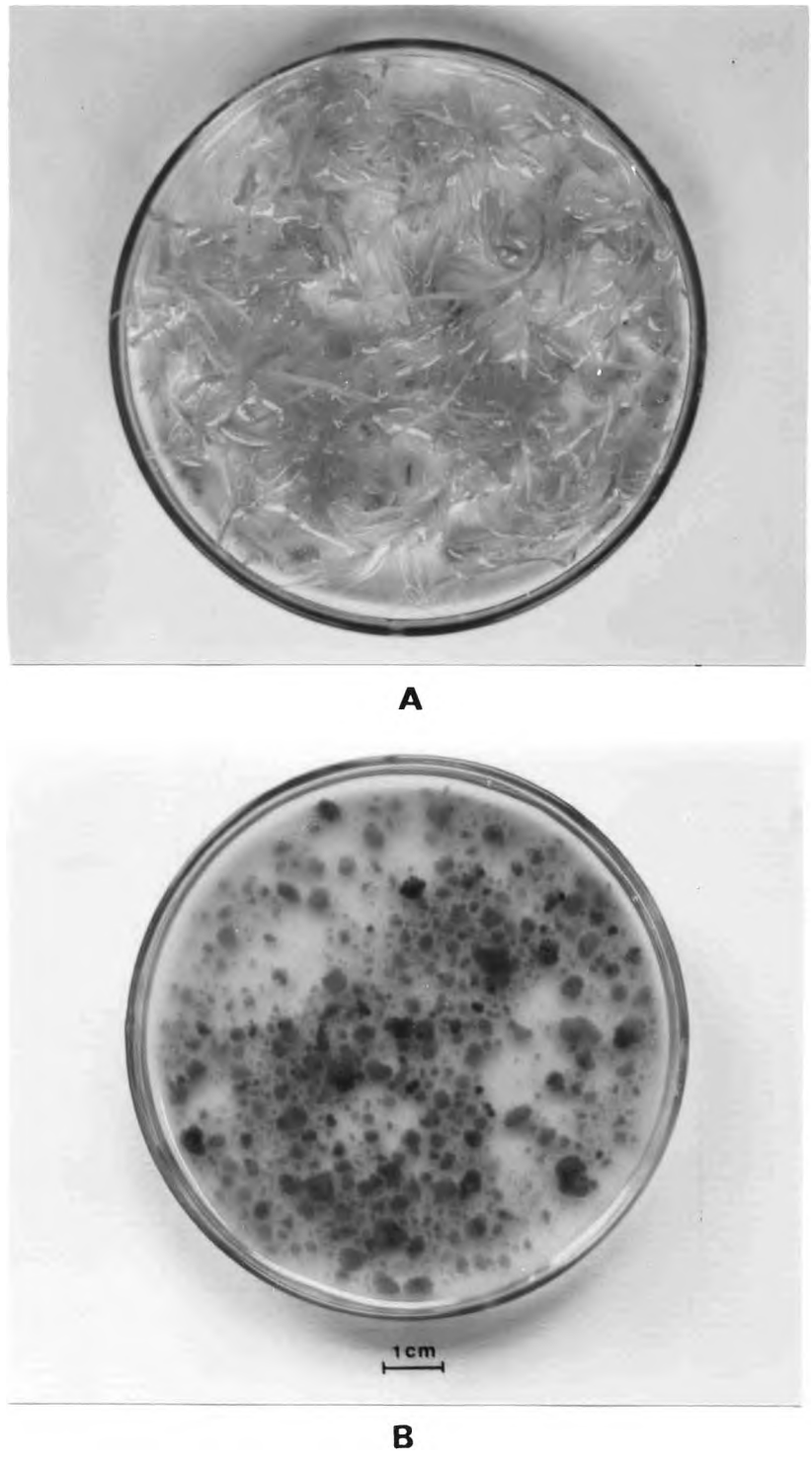


Fig. 9 Two types of *I. balsamina* cultures maintained in B5 liquid media containing different hormonal factors: A) root culture in B5 medium supplemented with 0.1 mg/l NAA, 0.1 mg/l kinetin and 1.0 mg/l BA and B) cell suspension culture in B5 medium (2 x calcium concentration) supplemented with 0.1 mg/l 2,4-D, 1.0 mg/l BA and 1 % (w/v) sucrose.

plants. The crucial part of this TLC analysis was to find an effective solvent system that allows the separation of lawsone and 2-methoxy-1,4-naphthoquinone from other constituents in the crude extracts. Careful study of various solvent systems showed that the optimum resolution of the two naphthoquinones from the other components could be achieved by using two solvent systems run consecutively in one dimension of the TLC plate (silica gel). The first and second solvent systems were chloroform : petroleum ether (80:20) and benzene : acetic acid (98:2), respectively. Under these conditions, the hRf values of lawsone was found to be 55 and of 2-methoxy-1,4-naphthoquinone was 77. Fig. 10 shows various chemical patterns of the crude extracts obtained from callus, cell suspension, root cutures and various parts of the mature *I. balsamina* plants. It can be seen that the callus, cell suspension and root cultures had different abilities in synthesizing lawsone and 2-methoxy-1,4-naphthoquinone. While both naphthoquinones could not be detected in the cell suspension cultures (Fig. 10C), lawsone was found to be present in the root cultures (Fig. 10B) and 2-methoxy-1,4-naphthoquinone in the cullus cultures. (Fig. 10A). The identity of lawsone and 2-methoxy-1,4-naphthoquinone produced by the *in vitro* cultures was confirmed by their UV-absorption spectra which were identical to the spectra of the authentic compounds (Fig. 11,12). It should be noted that the root cultures could also produce a compound which showed very close hRf to 2-methoxy-1,4-naphthoquinone (Fig. 11B). This unknown compound, however, showed different UV-absorption spectrum from that of 2-methoxy-1,4-naphthoquinone (Fig.13). Its structure elucidation is underway.

In the whole plant, the TLC patterns also showed different distribution of lawsone and 2-methoxy-1,4-naphthoquinone in various parts (Fig. 10 D-F). While the leaves (Fig. 10E) and roots (Fig. 10G) apparently contained lawsone and 2-methoxy-1,4-naphthoquinone, the stem seemed to have only 2-methoxy-1,4-naphthoquinone (Fig. 10F).

4. DETECTION OF SCOPOLETIN IN I. BALSAMINA CULTURES

In addition to lawsone and 2-methoxy-1,4-naphthoquinone, the TLC chromatogram obtained from all the *in vitro* cultures of *I. balsamina* also showed a spot corresponding to the hRf value of 32 (Fig.10). The spot showed a characteristic of blue

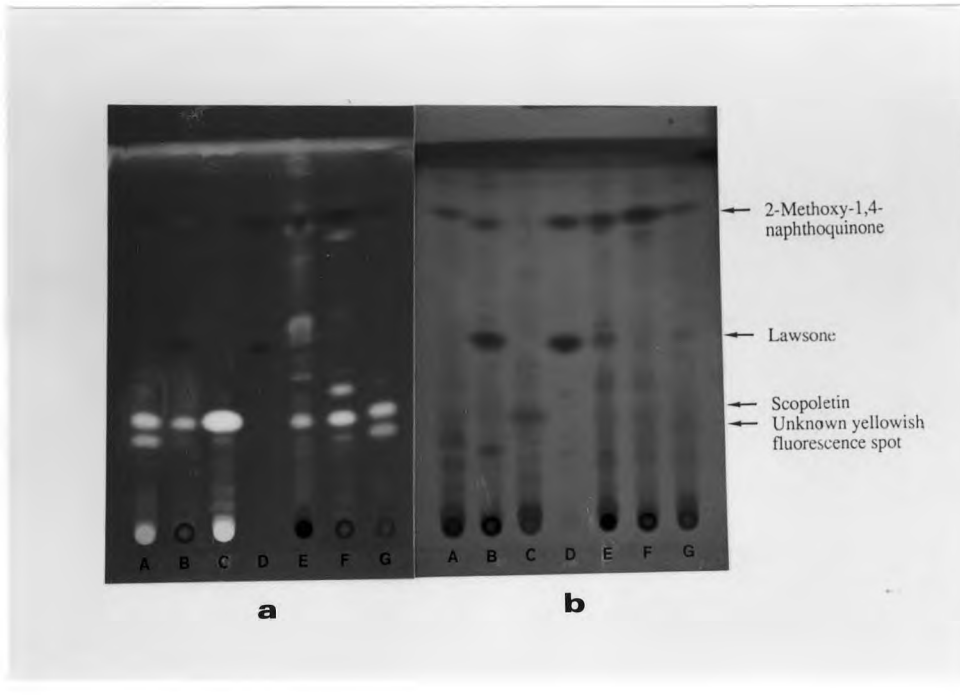


Fig. 10 TLC patterns of crude extracts obtained from various *in vitro* cultures and various plant parts of *I. balsamina* : A) callus culture B) root culture C) cell suspension culture D) standard lawsone and 2-methoxy-1,4-naphthoquinone E) leaves F) stems G) root a) under UV light at 365 nm and b) under UV light at 254 nm

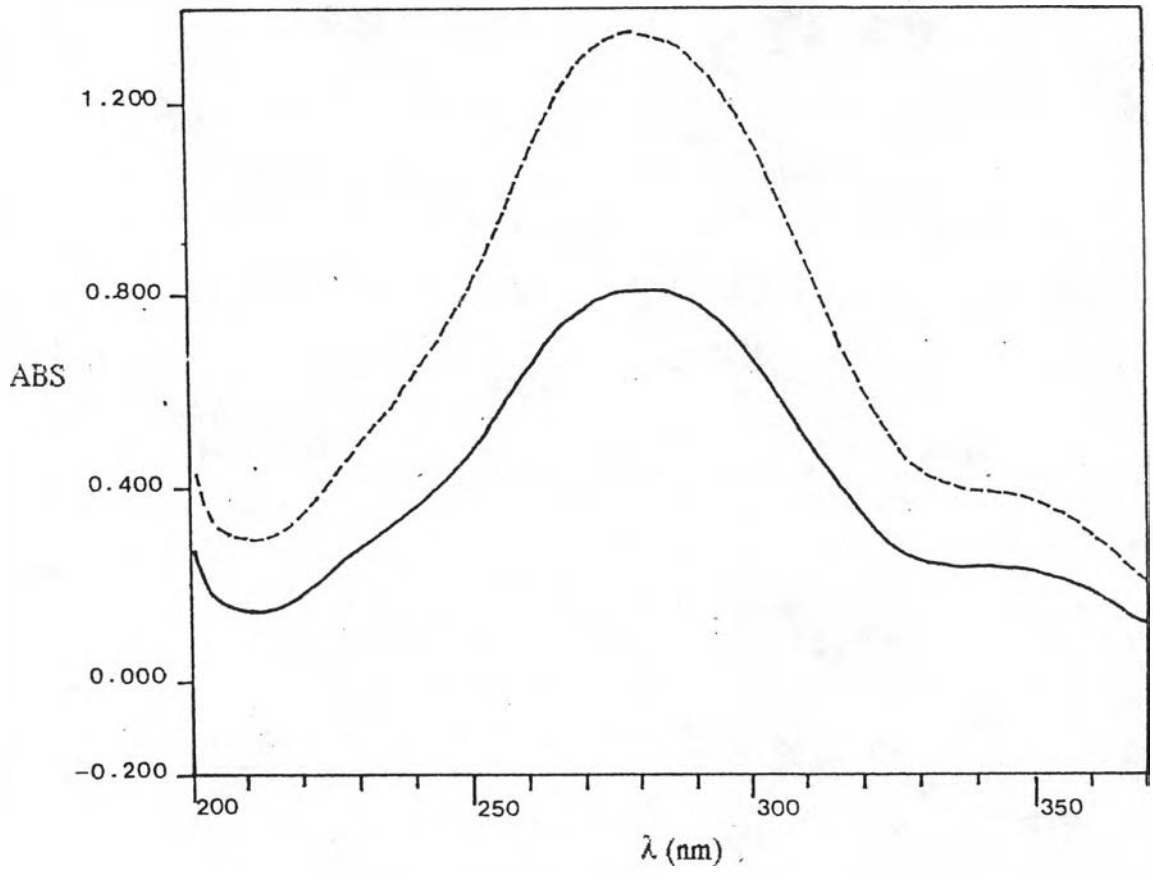


Fig. 11 UV-absorption spectra of authentic lawsone (----------) and the compound of similar hRf value obtained from *I. balsamina* rooot culture (———).

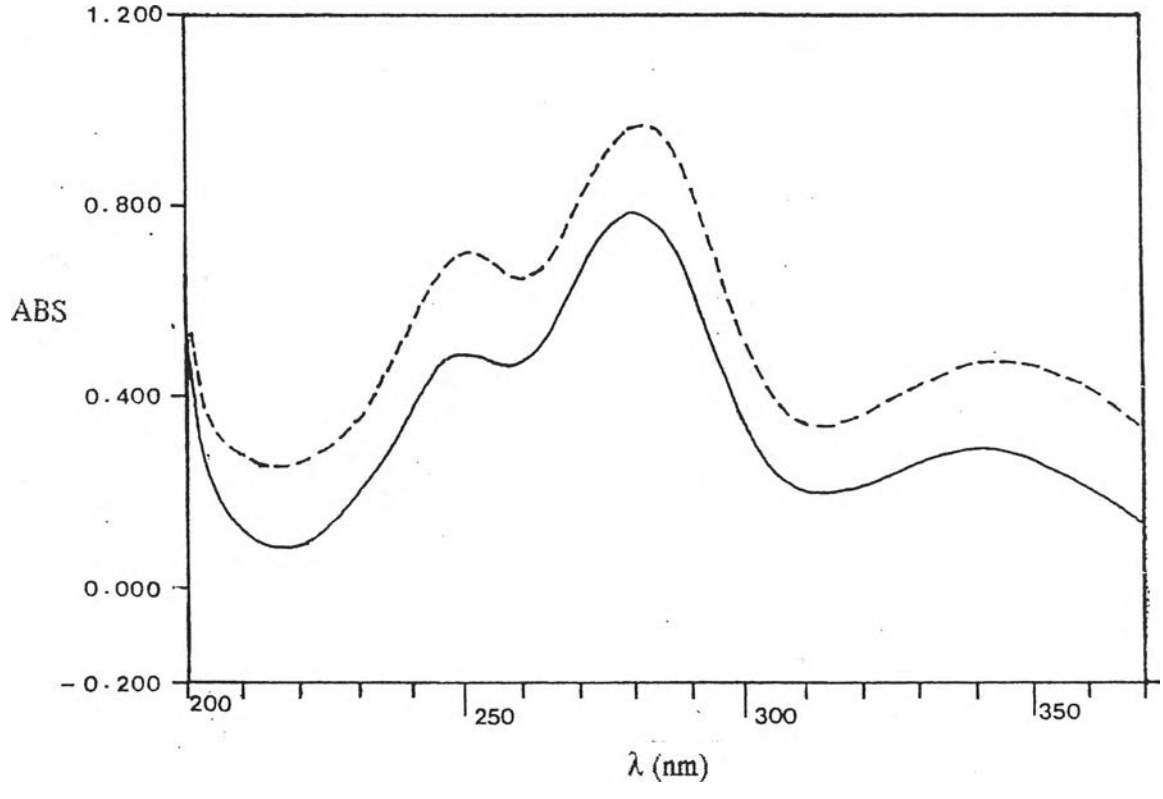


Fig. 12 UV-absorption spectra of authentic 2-methoxy-1,4-naphthoquinone (--------) and the compound of similar hRf value obtained from *I. balsamina* callus cultures (———).

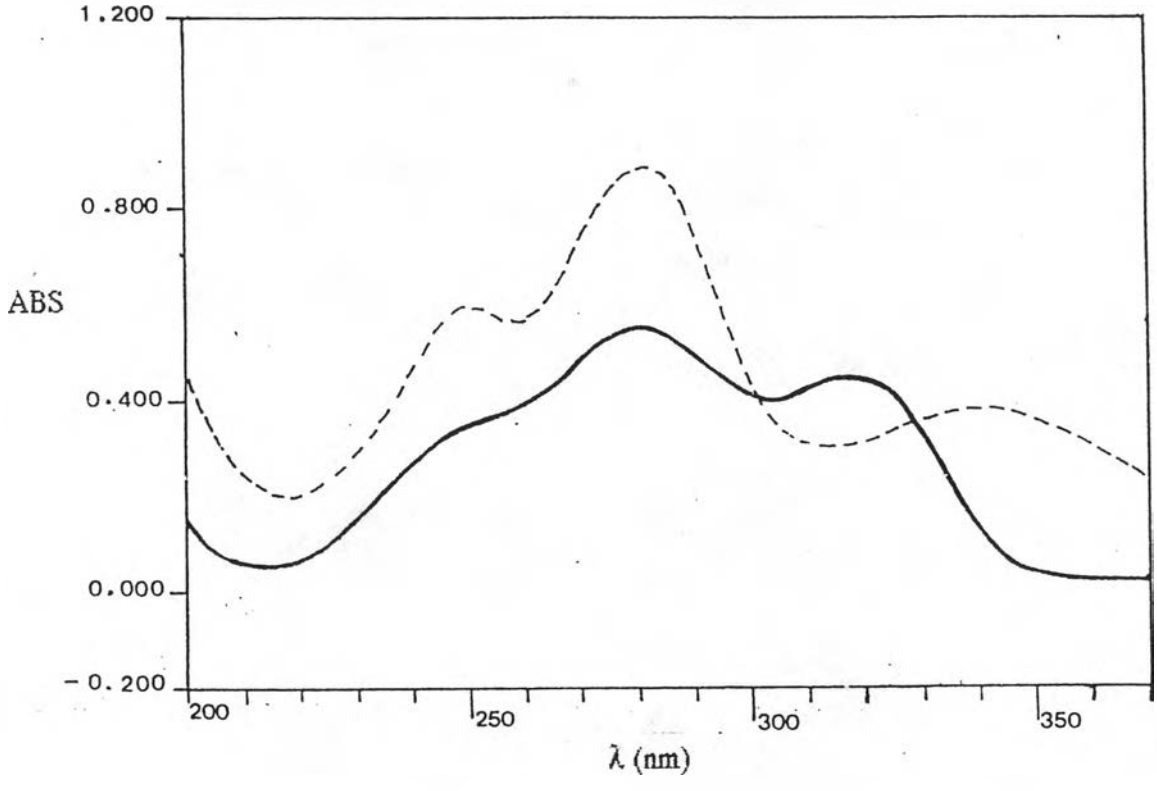
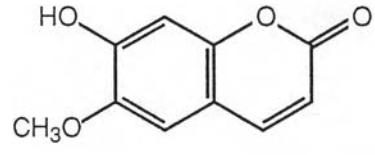


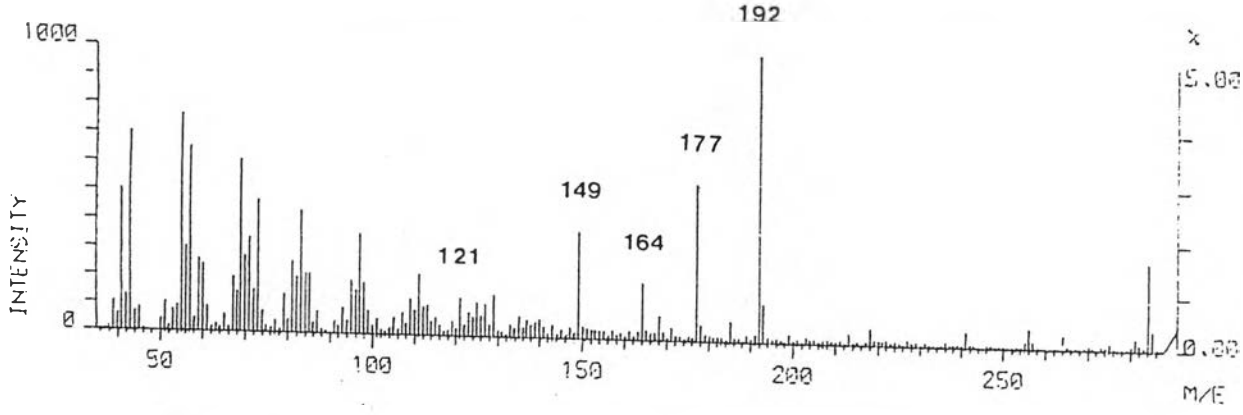
Fig. 13 UV-absorption spectra of authentic 2-methoxy-1,4-naphthoquinone (---------) and the unknown compound of similar hRf value obtained from *I. balsamina* root culture (———).

fluorescence under UV light (365 nm). By preparative TLC, the compound could be purified to the amount that was sufficient for mass spectral analysis. The obtained mass spectrum (Fig. 14) revealed the presence of the molecular ion at m/z 192 (base peak) and also other main peaks at m/z 177, 164,149 and 121. This information and subsequent UV-absorption spectral analysis revealed that the blue fluorescence spot was scopoletin, a coumarin derivative which also found in various parts of *I. balsamina* plant. The structure of scopoletin in shown below.



For detailed interpretation of the mass spectrum of scopoletin(Fig. 15) , the peak at *m/z* 177 corresponds to the emission of a methyl radical to provide the conjugated oxonium ion. The peak at *m/z* 164 is arised by expulsion of carbon monoxide from the lactone carbonyl group. The peak at *m/z* 149 can be described by two pathways. Firstly, it comes from the explusion of carbon monoxide, which is highly stable neutral particle. Secondly, from the loss of a methyl radicle to form another conjugated oxonium ion. The peak at *m/z* 121 corresponds to the loss of another carbon monoxide from the carbonyl group of oxonium ion.

For UV-absorption spectrum of this compound (Fig. 16), the result also showed identical to the spectrum of the authentic compound of scopoletin. This confirmed the identity of scopoletin which was produced by all the *in vitro* cultures of *I. balsamina* . In addition to scopoletin, we also found another unknown spot in which its hRf value (*ca* 23) was a little bit lower than scopoletin and had a yellowish fluorescence characteristic under UV light (365 nm) (Fig.10). This spot had UV-absorption spectrum similar to scopoletin (Fig. 17) Its structure is, however, still unknown.



MASS SPECTRUM : (24 TO 25)
SAMPLE:PHARKPHCOM 18 SEP 91
NOTE :114/2 EI,70V,300UA,CHAMB.TEMP.150
BASE PEAK : M/E 57.2 INT. 631.2

M/E	RAW INT.	R.INT.	SIGMA(%)	
41.0	427.1	676.5	9.65	
43.0	605.9	959.7	13.70	
55.0	485.9	769.7	10.99	
57.0	631.3	1000.0	14.27	
60.0	458.9	719.0	10.26	
69.0	374.0	592.4	8.45	
71.0	371.5	588.5	8.40	
73.0	554.7	878.5	12.54	
83.0	286.6	453.9	6.48	
97.0	230.4	365.0	5.21	END

MASS SPECTRUM : (24 TO 25)
SAMPLE:PHARKPHCOM 18 SEP 91
NOTE :114/2 EI,70V,300UA,CHAMB.TEMP.150
BASE PEAK : M/E 57.2 INT. 631.2

M/E	RAW INT.	R.INT.	SIGMA(%)	
111.0	131.5	208.4	5.44	
112.0	78.0	123.5	2.80	
113.0	78.7	124.6	3.25	
115.0	105.1	165.4	4.30	
121.0	62.1	98.5	2.57	
125.0	74.7	118.3	3.09	
129.0	282.2	447.0	11.68	
143.0	66.3	105.0	2.74	
149.0	396.7	628.3	16.42	
157.0	86.2	136.6	3.57	
164.0	83.5	132.3	3.45	
167.0	93.3	147.9	3.86	
171.0	88.5	140.2	3.66	
177.0	234.8	372.0	9.72	
185.0	124.5	197.3	5.15	
192.0	436.1	690.9	18.05	END

Fig. 14 Mass spectrum of unknown compound obtained from *I. balsamina* root cultures.

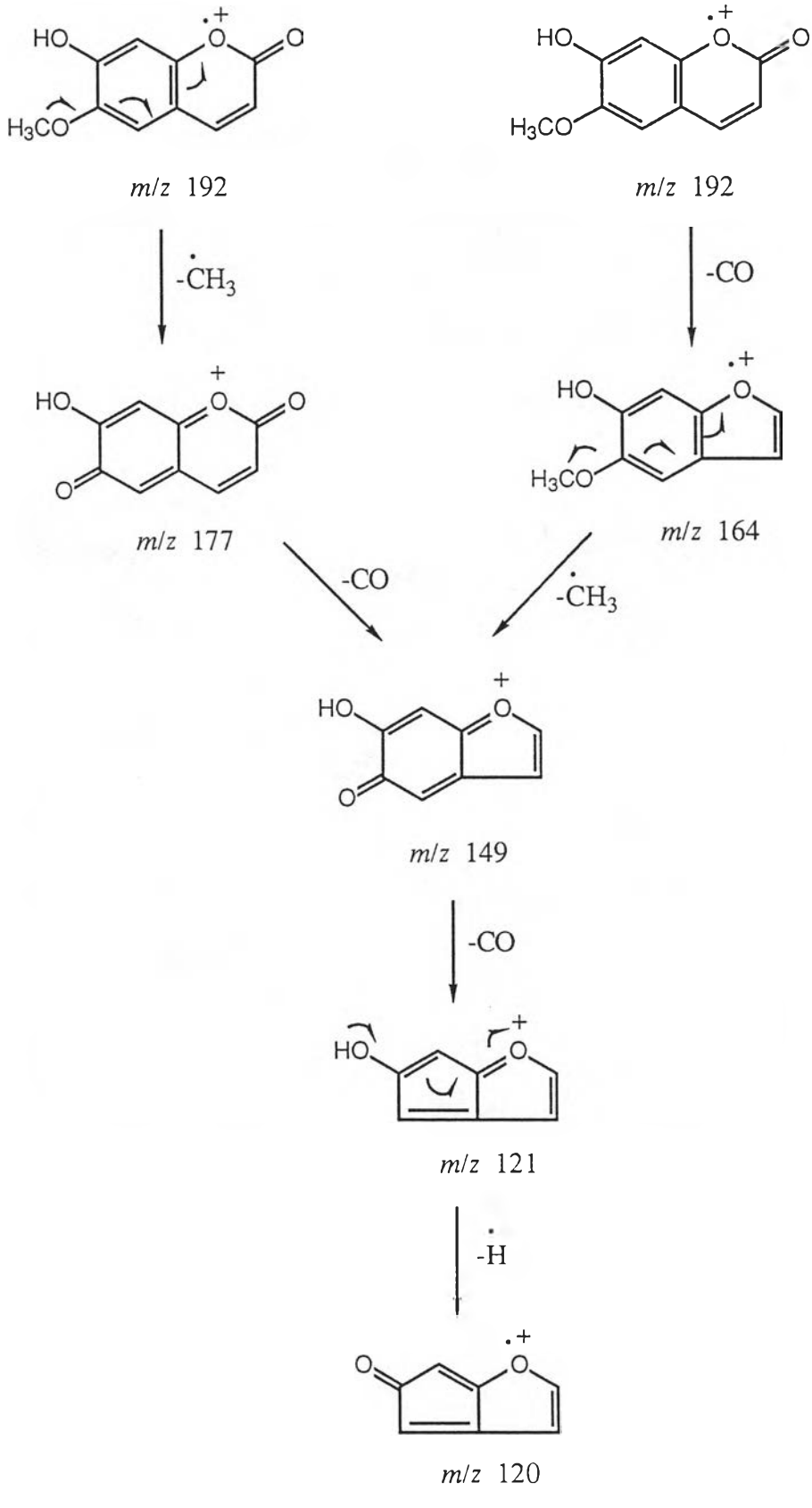


Fig. 15 Fragmentation of scopoletin.

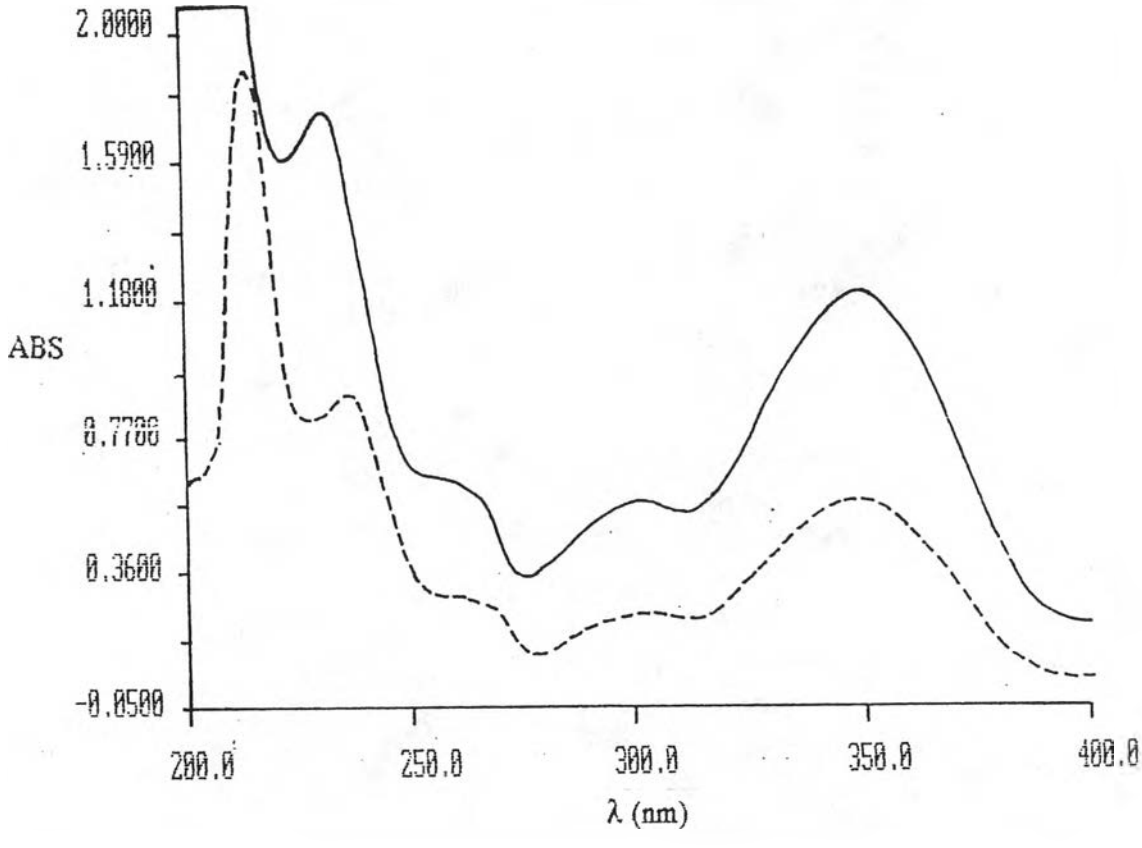


Fig. 16 UV-absorption spectra of authentic scopoletin (---------) and the compound of similar hRf value obtained from *I. balsamina* root cultures (———).

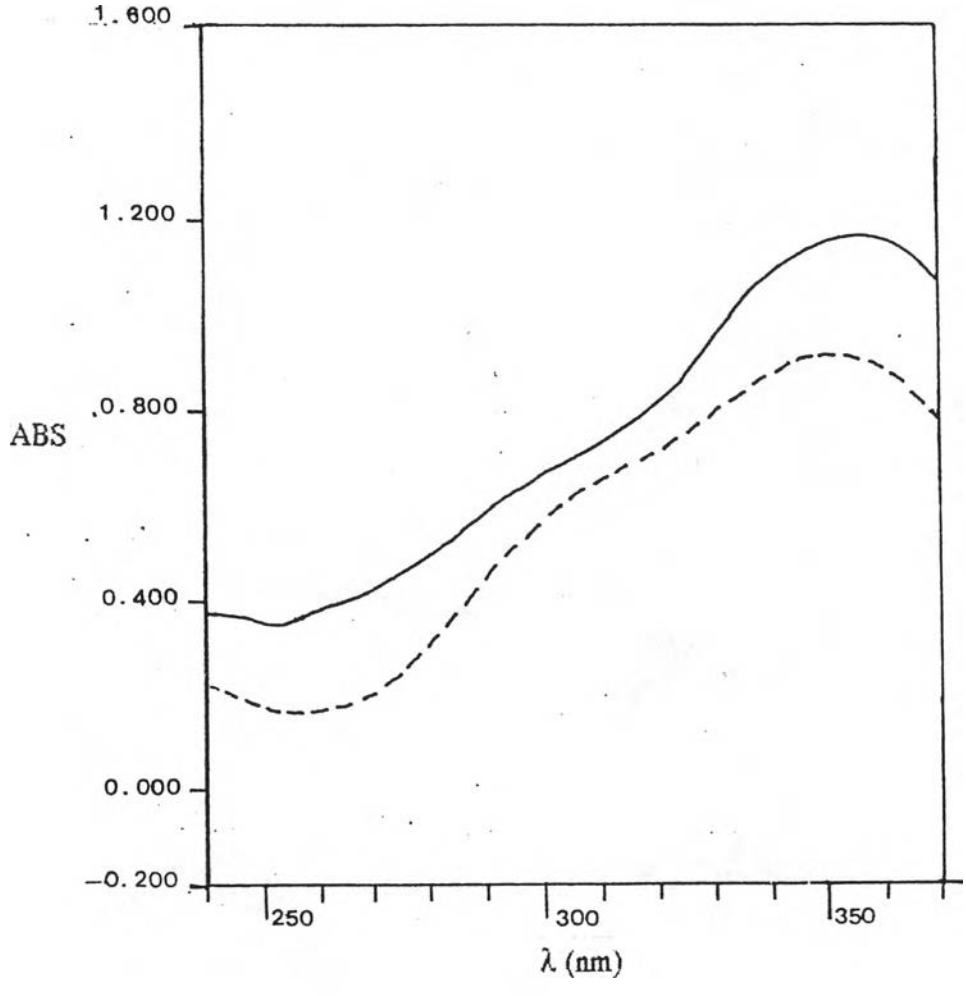


Fig. 17 UV-absorption spectra of authentic scopoletin (----------) and the unknown compound of yellowish fluorescence obtained from *I. balsamina* root cultures (———).

5. QUANTITATIVE ANALYSIS OF LAWSONE AND 2-METHOXY-1,4-NAPHTHOQUINONE

The complete separation of lawsone and 2-methoxy-1,4-naphthoquinone from other components by double-development TLC (Fig. 10) allowed both naphthoquinones be quantitated simultaneously by densitometry. This method is simple, rapid and able to detect the naphthoquinones in the absolute amounts down to *ca.* 0.1 μg. A linear relationship between the amount of lawsone (Fig. 18) or 2-methoxy-1,4-naphthoquinone (Fig. 19) and its corresponding peak integrated areas were obtained in the range between 0.1 and 2.0 μg in each case.

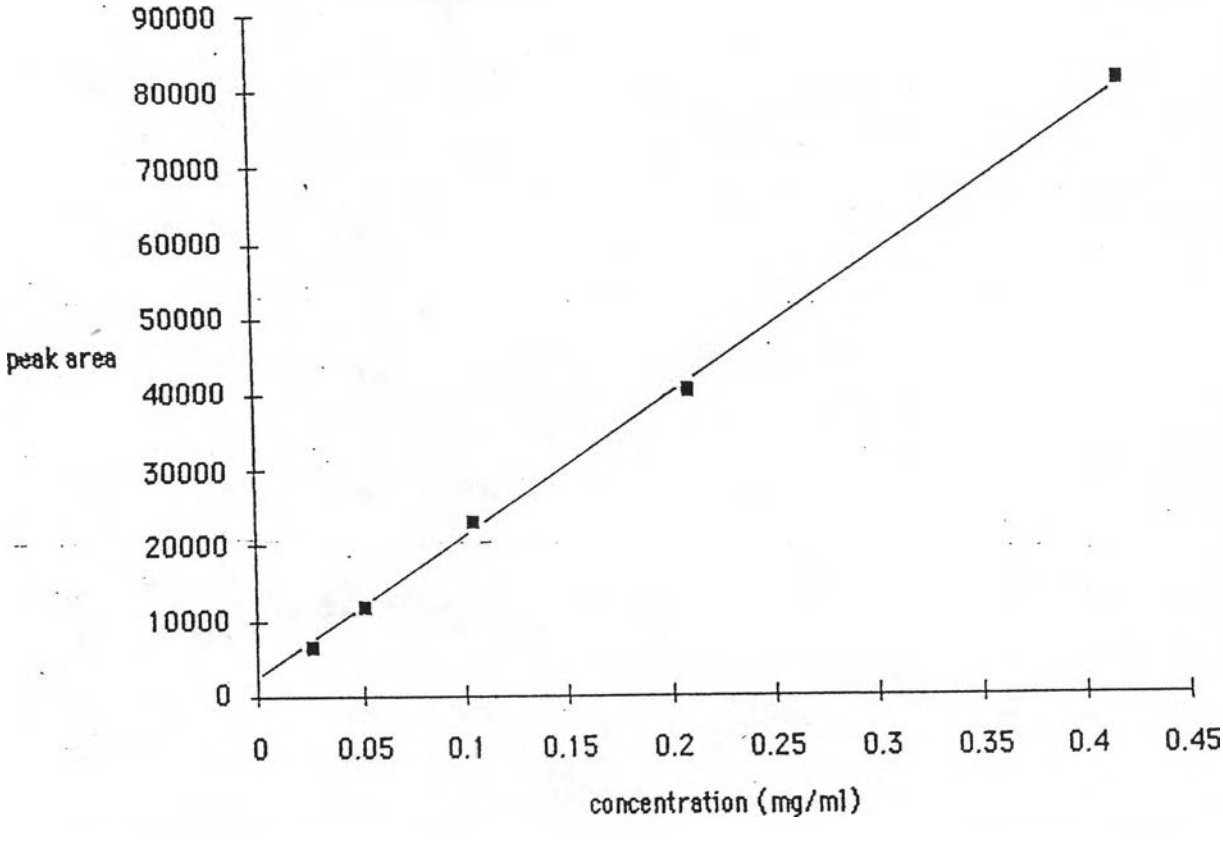


Fig. 18 Standard curve of lawsone established by TLC-densitometric method.

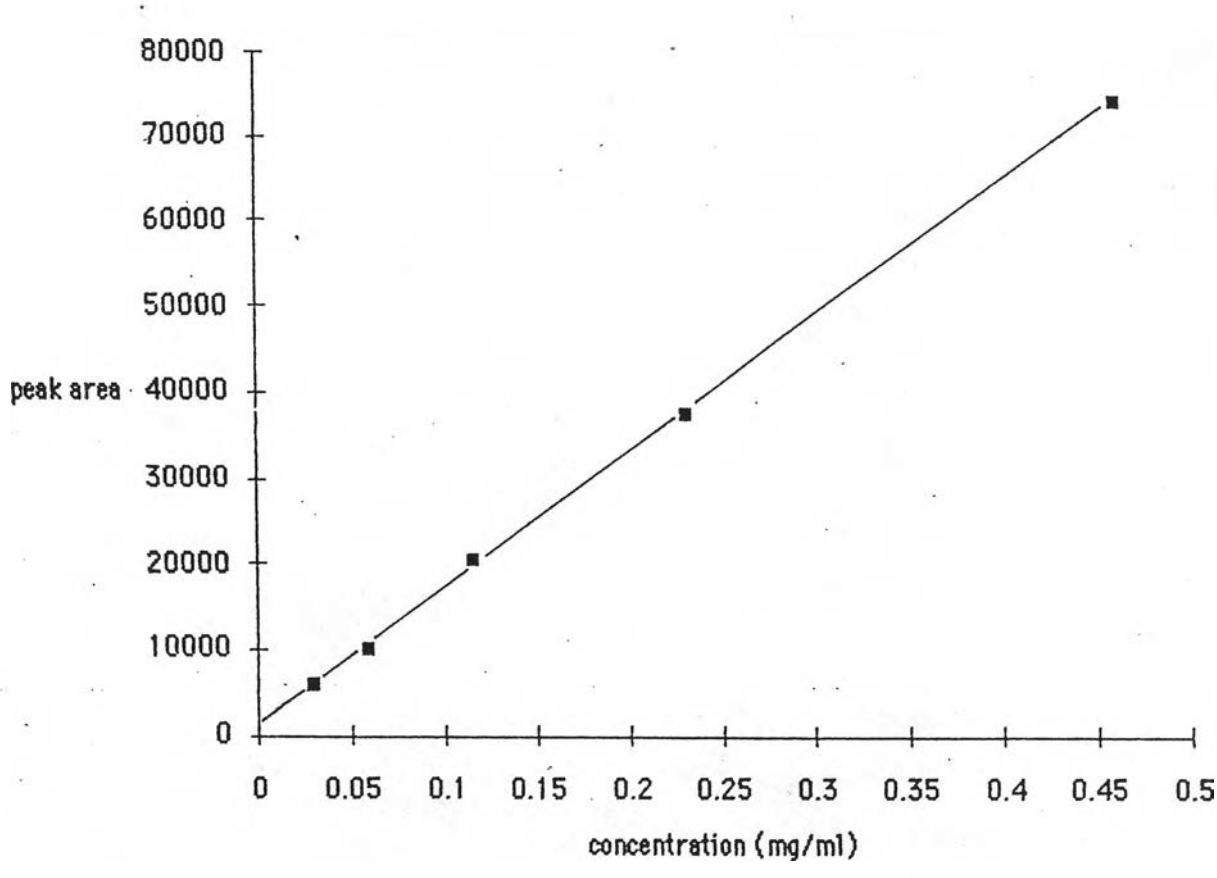


Fig. 19 Standard curve of 2-methoxy-1,4-naphthoquinone established by TLC-densito - metric method.

Based on this densitometric analysis, TLC chromatograms of various crude extracts mentioned above were produced (Fig. 20) and their content of lawsone and 2-methoxy-1,4-naphthoquinone were determined. It was found that the 2-methoxy-1,4-naphthoquinone content in callus cultures was only 0.004 % dry weight compared to that in the leaves, stems and roots which were found to be 0.130, 0.075 and 0.032% dry weight, respectively (Table 9). On the other hand, the root cultures accumulated lawsone up to 0.158% which appeared to be 3.5 times higher than its content found in the leaves (0.045 %).

The accuracy of the TLC-densitometry was confirmed by HPLC method. Under the HPLC conditions, the standard lawsone and 2-methoxy-1,4-naphthoquinone had a good separation from each other as shown in the chromatrogram in Fig. 21. The standard curves of lawsone (Fig. 22) and 2-methoxy-1,4-naphthoquinone (Fig. 23) established by the HPLC method apparently showed good linear relationship. Subsequently, each unknown concentration of standard lawsone and 2-methoxy-1,4-naphthoquinone was determined and compared with that obtained by the TLC-densitometric method. The results showed that the concentrations of the naphthoquinones determined by both methods were very closed from one another (Table 10). This suggested that the TLC-densitometric method was reliable.

Table 9. Lawsone and 2-methoxy-1,4-naphthoquinone content in various form of *I. balsamina* cultures and various parts of the intact plants

Material	% dry weight	
	Lawsone	2-Methoxy-1,4-naphthoquinone
Callus cultures	-	0.004
Cell cultures	-	-
Root cultures	0.158	-
Leaves	0.045	0.13
Stems	< 0.001	0.075
Roots	0.002	0.032

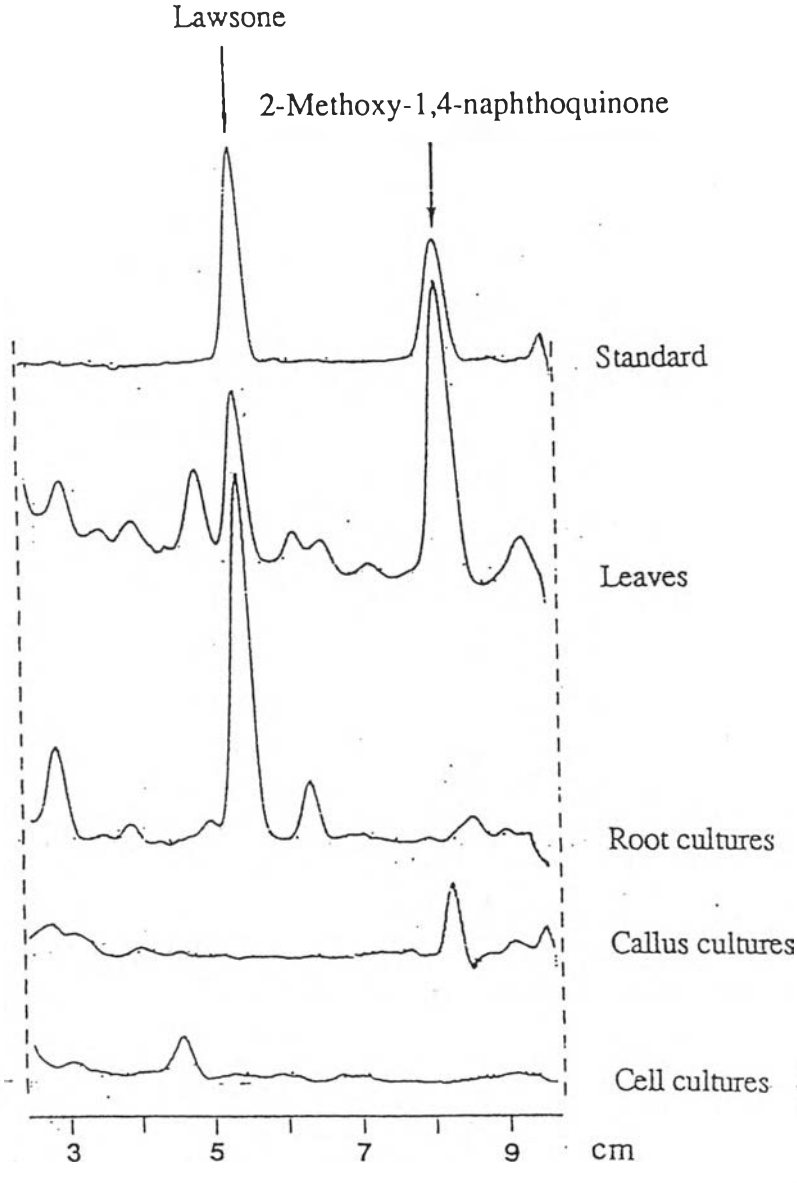


Fig. 20 TLC-densitometric chromatograms of the crude extracts of various *in vitro* cultures of *I. balsamina* as compared to the chromatogram of the leaves. The arrows indicate the position of the standard lawsone and 2-methoxy-1,4-naphthoquinone.

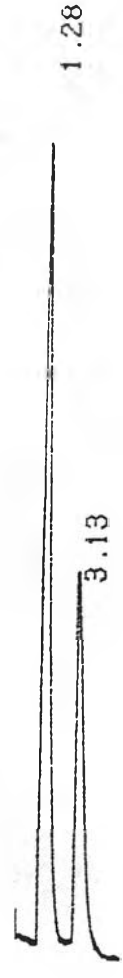


Fig. 21 HPLC-chromatogram of lawsone (RT = 1.28 min) and 2-methoxy-1,4-naphthoquinone (RT = 3.13 min). The HPLC conditions : SP-C18-5 column, 4.0 mm x 15 cm ; methanol water (65:35); 1 ml/min.

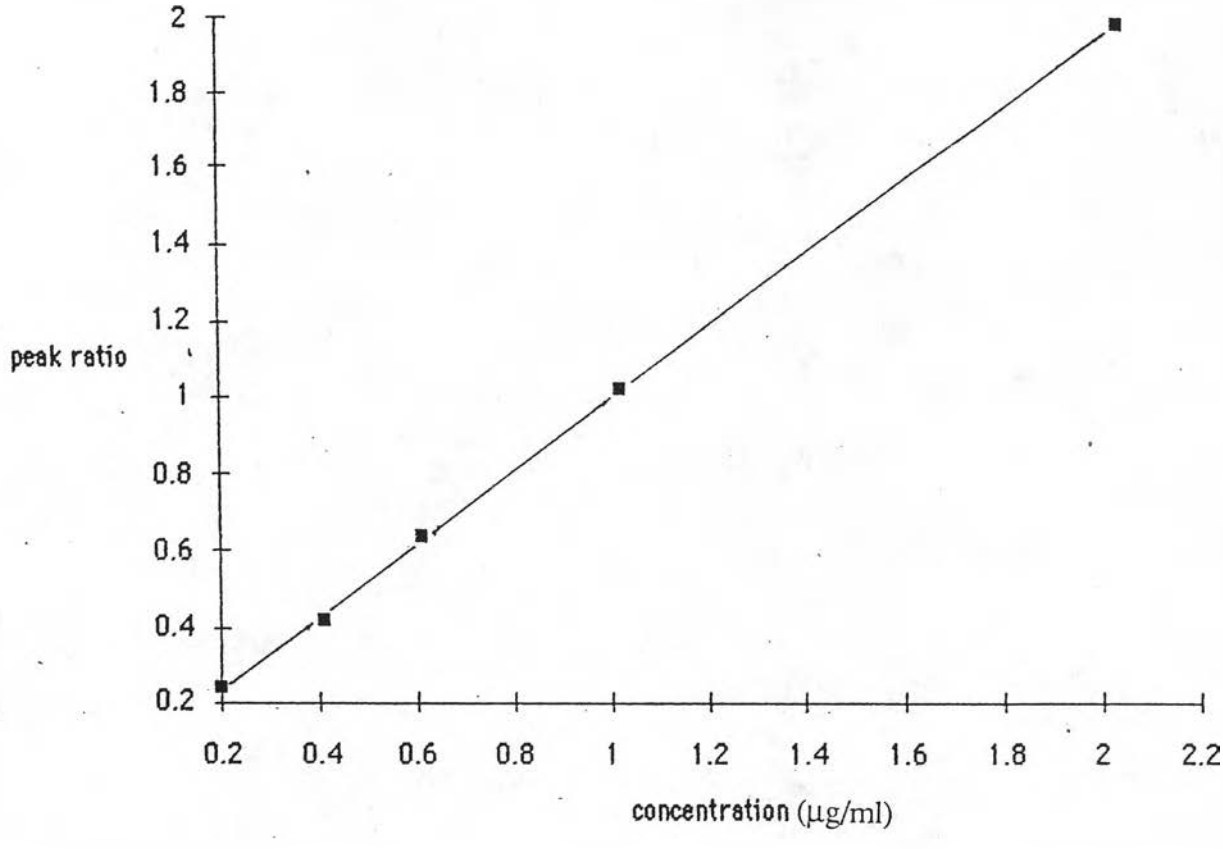


Fig. 22 Standard curve of lawsone established by HPLC method.

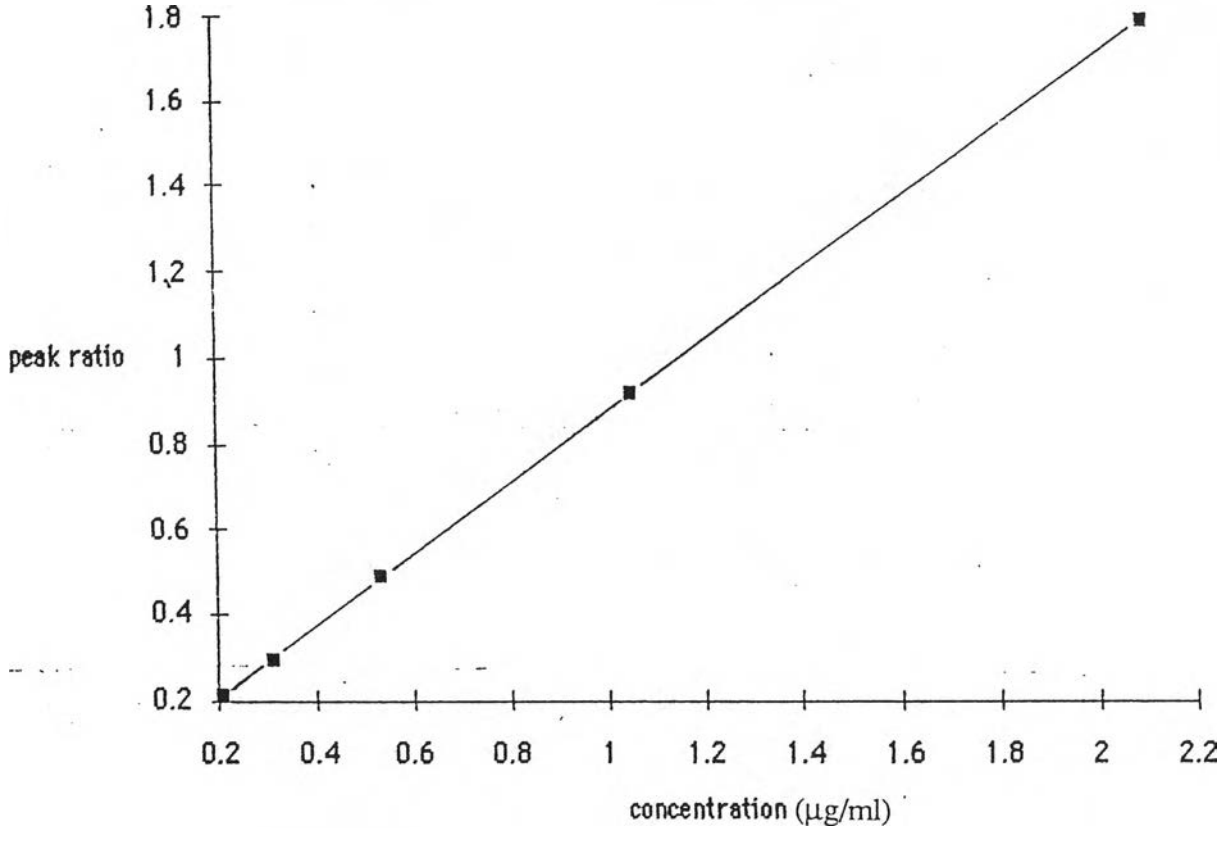


Fig. 23 Standard curve of 2-methoxy-1,4-naphthoquinone established by HPLC method.

Table 10. calculated concentration of lawsone and 2-methoxy-naphthoquinone by TLC densitometric method compared with HPLC method.

Standard	Concentration (mg/ml)	calculated concentration (mg/ml)		% CV	
		HPLC	TLC-demitrometer	HPLC	TLC-demitrometer
Lawsone	0.128	0.126	0.125	1.56	2.34
2-Methoxy-naphthoquinone	0.131	0.128	0.128	2.29	2.29

6. CHEMICAL PATTERNS OF THE CRUDE EXTRACTS OF I. BALSAMINA ROOT CULTURES AND THE WHOLE ROOTS.

To observe whether the secondary metabolites produced by the root cultures and the whole roots showed similar or differant patterns, comparative study of the two sources was carried out. Crude extracts were prepared from both materials and analyzed by the established TLC-densitometry. It was found that the chromagram of the root culture extract was extremely different from that of the whole roots (Fig. 24). The former showed less number of the extractable compounds than the latter. With respect to the naphthoquinones, the root cultures appeared to contain high content of lawsone (with nondetectable 2-methoxy-1,4-naphthoquinone) while the whole roots showed mainly the peak of 2-methoxy-1,4-naphthoquinone with trace amount of lawsone.

7. TIME-COURSE OF GROWTH AND LAWSONE PRODUCTION BY I. BALSAMINA ROOT CULTURES.

The relationship between growth and lawsone production of *I. balsamina* root cultures during a period of 20 days were also examined in this study. the dry weight of the harvested root mass was used as a parameter for expressing the culture growth. The resulted growth cycle (Fig. 25A) showed a very short lag phase. Soon after the inoculation the root cultures appeared to increase in their dry weight . The increase in the biomass was

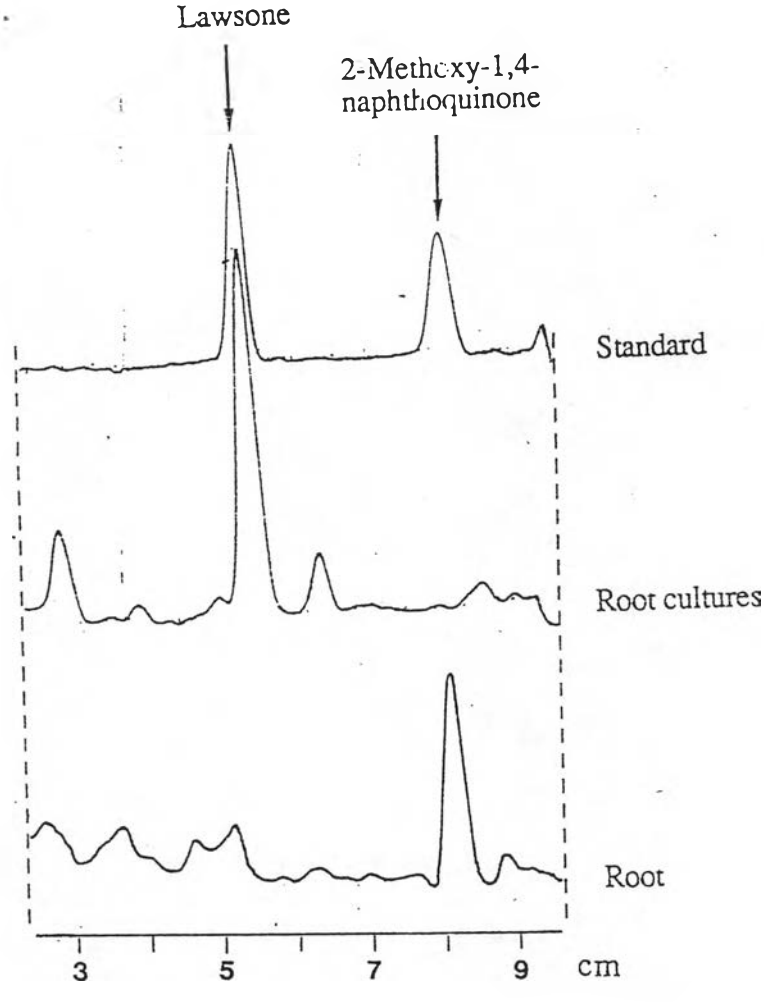


Fig. 24 Comparison TLC-densitometric chromatogram of crude extracts of root cultures and root of *I. balsamina* , standard lawsone and 2-methoxy-1,4-naphthoquinone.

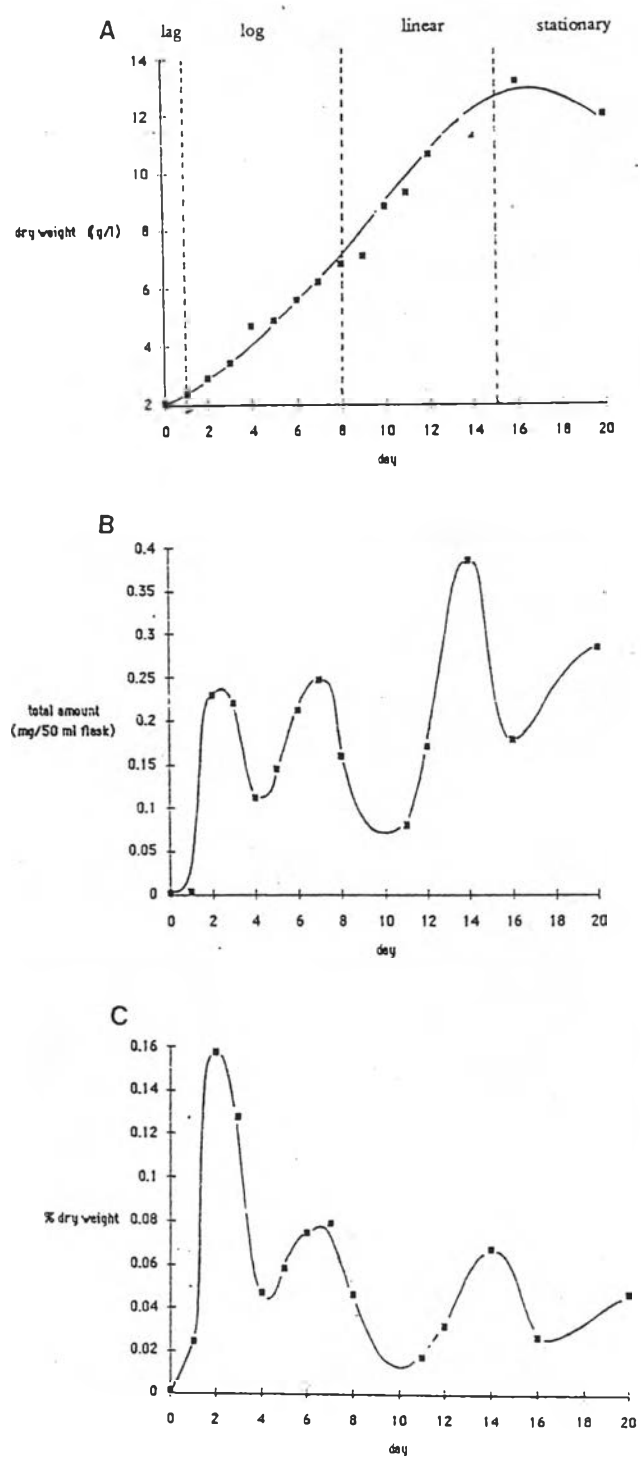


Fig. 25 Time-coruse of dry weight (A), lawsone content in % dry weight (B) and total lawsone yield per 50 ml flask (C) for root cultures of *I. balsamina*.

continued at a relatively constant rate for approximately 12 days before slowing down to a zero-increase rate at day 16. Thereafter, the culture dry weight began to decline. The highest value of the biomass obtained was 0.6 mg/flask (day 16 or 17) equivalent to approximately 6 times of the inoculated root culture mass.

For lawsone production, it appeared that there was a fluctuation of lawsone accumulation during the growth cycle of the root cultures. The production curve expressed using the unit of either total lawsone produced per flask (50-ml medium) (Fig. 25B) or percent lawsone on the dry weight basis (Fig. 25C) showed at least 3 peaks of lawsone accumulation at days 2,7 and 14 . The total content of lawsone accumulated in these peaks was found to be 0.230, 0.250 and 0.390 mg/50 ml flask equivalent to 0.158 , 0.079 and 0.068% dry weight respectively.