Bot. Bull. Academia Sinica (1991) **32:** 113-119

Effects of tissue-cultured *Anoectochilus formosanus* Hay. extracts on the arachidonate metabolism

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(*Received October 26, 1990; Accepted January 14, 1991*)

Abstract. Tissue-cultured *Anoectochilus formosanus* Hay., a precious Chinese herb used for the treatment of cardiovascular diseases, was examined for its physiological activity on arachidonic acid (AA) metabolism. Extracts of the herb were found to inhibit thromboxane A_2 (TXA_2) production in platelets and to activate prostacyclin (PGI_2) production in aortic endothelial tissues. All the inhibitions and activations found were concentration-dependent. These effects were similar to those obtained previously when wildtype *A. formosanus* plants were used. In addition, the substances inhibiting TXA_2 production in platelets were found to occur in the stems plus roots whereas substances activating PGI_2 production in endothelial tissues were found to occur in the leaves of the tissue-cultured *A. formosanus* plants. By using different precursors, AA and prostaglandin H_2 (PGH_2), it was found that lower production of TXA_2 in platelets and higher production of PGI_2 in endothelial tissues were caused by the inhibition of thromboxane synthase and activation of cyclooxygenase, respectively. These results suggest that the tissue-cultured *A. formosanus* contains at least two different bioactive components affecting TXA_2 and PGI_2 production, which could play important physiological and pharmacological roles in the treatment of cardiovascular diseases.

Key words: *Anoectochilus formosanus*; Arachidonic acid; Endothelium; Platelet; Prostacyclin; Thromboxane A_2; Tissue culture.

Introduction

Anoectochilus formosanus Hayata is an orchid and can be found in the forests below 1500 m throughout Taiwan island (Li *et al.*, 1978). The leaves of the herb are dark green with white reticulated venation on the upper surface and pale purple on the lower surface. The whole plant body of *A. formosanus* has been found to be an effective Chinese herb for the tretment of hypertension, tuberculosis, impotence and underdeveloped children. Normal dosage is about 4-40 g of *A. formosanus*, which is extracted with hot water for about an hour (Kan, 1986). It has been found that the wildtype *A. formosanus* contains substances affecting arachidonic acid metabolism which is involved in the function of the cardiovascular system (Mak *et al.*, 1990). Since this herb is very precious in the Chinese herb market, its existence in the field has been seriously reduced by unlimited collecting. In addition, clonal propagation by conventional methods are slow. Tissue culture methods offer a much better opportunity to multiply the orchid. However, the metabolic pathways of a plant tissue culture may differ from that of the original plant (Nettleship and Slaytor, 1974). In this study, the vegetative propagation of *A. formosanus* by tissue culture has been achieved. The physiological effect on arachidonate metabolism of the bioactive substances extracted from these seedlings has been examined. A comparison was then made between the results of extracts from seedlings derived from tissue cultures and extracts obtained from the wildtype *A.*

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formosanus.

Materials and Methods

Materials

All solvents, laboratory chemicals and thin layer chromatography plates were supplied by Merck Chemicals (Darmstadt, FRG). Radioactive compounds were purchased from Amersham (Amersham, England, UK). Non-radioactive prostaglandins were obtained from the Sigma Chemical Company (St. Louis, MO, USA). Sheep vesicular microsome was purchased from Hilran Biochemical Ltd (Tel-Aviv, Israel). Rabbits (12 month-old) were obtained from the veterans General Hospital (Taipei, Taiwan, ROC).

Vegetative Propagation of ***A. formosanus*** *Hay*.

A. formosanus seedlings were originally collected from the field and generously supplied by Taiwan Agricultural Research Institute. It was sterilized in 70% alcohol for 15 sec and washed in tap water for 20 min. The herb was sterilized again in 1% sodium hypochlorite by sonication for a few minutes. The disinfectant was decanted and the *A. formosanus* was washed 4-5 times in sterile distilled water. The leaves and roots of the seedlings were removed, and the stem segments with nodes were layered on the surface of autoclaved 1/2 strength MS medium (Murashige and Skoog, 1962) containing 0.3 ppm NAA (naphthaleneacetic acid) and 3 ppm BA (benzyladenine). After the stem segments have developed 3-4 leaves, they were transferred to a MS medium containing 1 ppm NAA and 0.3 ppm kinetin to induce root formation. The cultures were incubated at 25°C in a growth chamber with a day length of 16 h illuminated by Sylvania cool-white fluorescent tubes yielding 27 μmol photons $\cdot m^{-2} \cdot s^{-1}$ (400-700 nm) at plant height. Seedlings with good root systems were transferred to pots containing moss peat, vermiculite and crushed tree fern before being used for physiological experiments.

Extraction of Whole ***A. formosanus*** *Plants Derived from Tissue Cultures*

Oven-dried *A. formosanus* (37.5 g) was extracted with boiling water (1 L) for 48 h and then concentrated to about 200 ml. The concentrate was filtered through Whatman No. 1 paper, and half of the filtrate (about 100 ml) was extracted with two volumes of ethyl acetate. The organic phase was removed and the aqueous phase was re-extracted with another two volumes of ethyl acetate. The two organic phases were combined and evaporated to dryness at room temperature under a stream of nitrogen gas. The residue was dissolved in water (3 ml) and stored at 4°C. The aqueous phase as well as the second half of the filtrate (both about 100 ml) were freeze-dried, and the residues dissolved in 3 ml water.

Preparation of Rabbit Blood Platelets

Rabbit platelets were prepared according to the modified method described by Gerrard (1982). Rabbit blood (4.5 ml) was mixed with a citrate solution (0.5 ml containing 93 mM sodium citrate, 7 mM citric acid and 0.14 M dextrose at pH 6.5). The mixture was centrifuged at 200 x g for 10 min to remove erythrocytes and leucocytes. The supernatant was centrifuged at 1,000 x g for 10 min and the pellet resuspended in 10 mM Tris-HCl buffer, pH 8.0. This suspension was used in the experiment.

Preparation of Bovine Aortic Tissues

Bovine aortic tissues were prepared as described by Salmon and Flower (1982).

Preparation of PGH_2

Preparation and purification of the precursor ^{14}C-PGH_2 were carried out as described by Graff (1982) using sheep vesicular microsome as the enzyme source and ^{14}C-arachidonic acid (^{14}C-AA, 5 μCi) as precursor.

Platelet Incubation

Incubation of rabbit blood platelets with extracts of the tissue-cultured Chinese herbs was carried out as described previously (Mak *et al.*, 1990). Aliquots of 1-ml platelet solution were incubated with 12.5, 25 and 50 μl (approximately 50, 100 and 200 mg dry weight) of *A. formosanus* extract and ^{14}C-AA or ^{14}C-PGH_2 (0.2 μCi, specific activity 59.6 Ci/mol) was added to each of the mixtures for incubation.

Incubation of Bovine Aortic Tissues

Incubation of bovine aortic tissues with extracts of the tissue-cultured Chinese herbs was carried out according to the method described by Mak *et al.* (1990). Aliquots of aortic tissue preparation (2 ml)

were incubated with 12.5, 25 or 50 μl (approximately 50, 100 and 200 mg dry weight) of *A. formosanus* extract and ^{14}C-AA or ^{14}C-PGH_2 (0.2 μCi, specific activity 59.6 Ci/mol) was added to each of the mixtures for incubation.

Results

Vegetative Propagation of ***A. formosanus*** *Hay.*

Fig. 1 show the whole plants that were derived from stem tissue cultures of *A. formosanus*.

Effects on Thromboxane A_2 Production

Fig. 2 and 3 show the radioactivity of the product, TXB_2 (the natural stable product of TXA_2 in blood plasma) scraped from the relevant sites of the TLC plates. Each point is the mean of duplicates, and the standard deviation at all points was less than 5% of the results presented in this communication. Both the whole crude water extract of *A. formosanus* and its ethyl acetate-soluble fraction inhibited TXA_2 production in blood platelets when supplied either with ^{14}C-AA or ^{14}C-PGH_2 as precursors. On the other hand, the aqueous remainder of the crude water extract of the herb stimulated TXA_2 production. All these effects were concentration-dependent. Fig. 4 shows the inhibitory effects of extracts prepared from stems plus roots of tissue-cultured *A. formosanus* plants on TXA_2

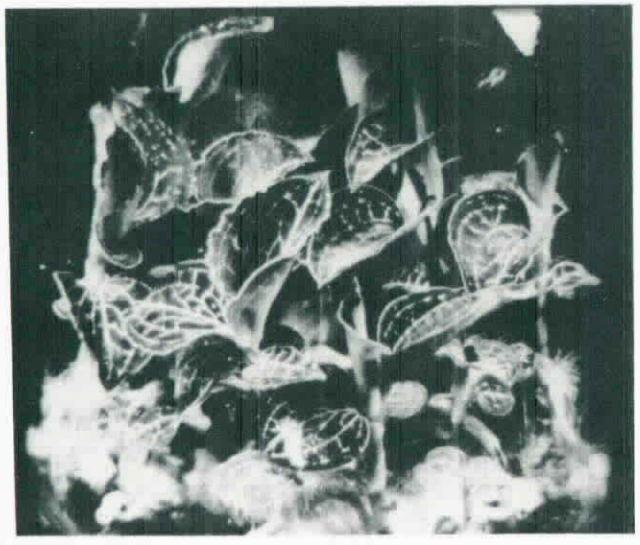

Fig. 1. Picture of the whole plant derived from tissue cultures of *A. formosanus*.

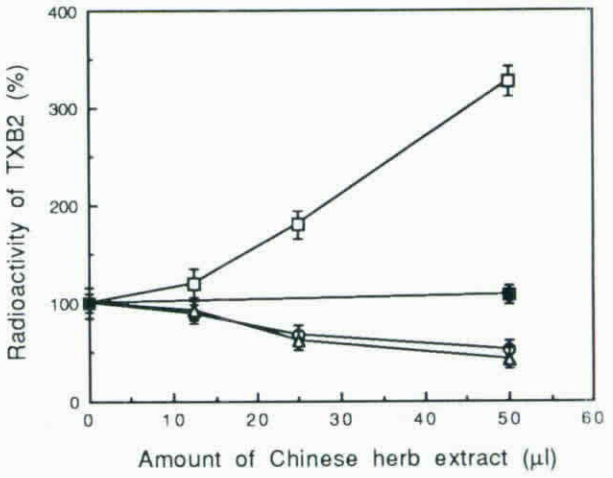

Fig. 2. Effect of extracts from tissue-cultured *A. formosanus* on TXB_2 production by rabbit blood platelets supplied with ^{14}C-AA. ○, crude water extract; △, ethyl acetate-soluble fraction of crude water extract; □, aqueous remainder of crude water extract; and ■, aqueous remainder of crude water extract supplemented with aspirin (250 μg/ml). The TXB_2 was separated by silica gel TLC using the organic phase of ethyl acetate: 2,2,4-trimethylpentane: acetic acid: water (110:50:20:10, v/v) as solvent.

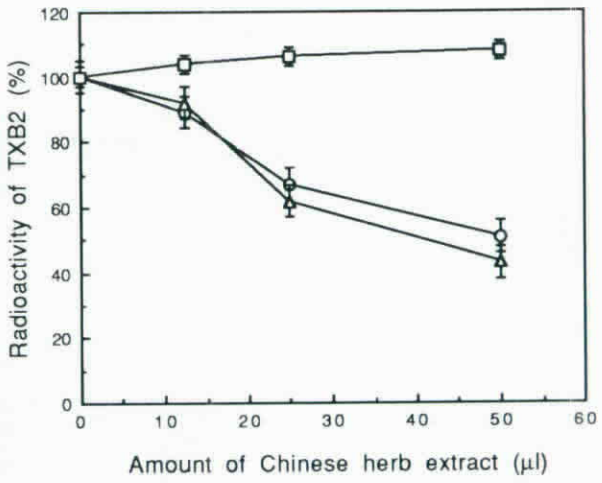

Fig. 3. Effect of extracts from tissue-cultured *A. formosanus* on TXB_2 production by rabbit blood platelets supplied with ^{14}C-PGH_2. ○, crude water extract; △, ethyl acetate-soluble fraction of crude water extract; and □, aqueous remainder of crude water extract. The TXB_2 was separated by silica gel TLC using the solvent system described in Fig. 2.

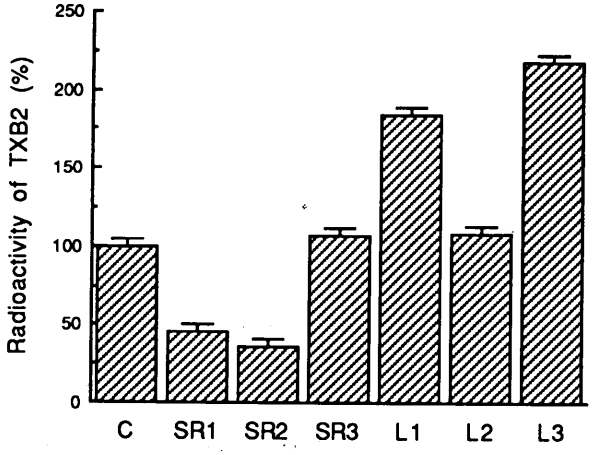

Fig. 4. Effect of extracts from tissue-cultured *A. formosanus* on TXB_2 production by rabbit blood platelets supplied with ^{14}C-AA. C, control; SR1, crude water extract of stems and roots; SR2, ethyl acetate-soluble fraction of crude water extract of stems and roots; SR3, aqueous remainder of crude water extract of stems and roots; L1, crude water extract of leaves; L2, ethyl acetate-soluble fraction of crude water extract of leaves; L3, aqueous remainder of crude water extract of leaves.

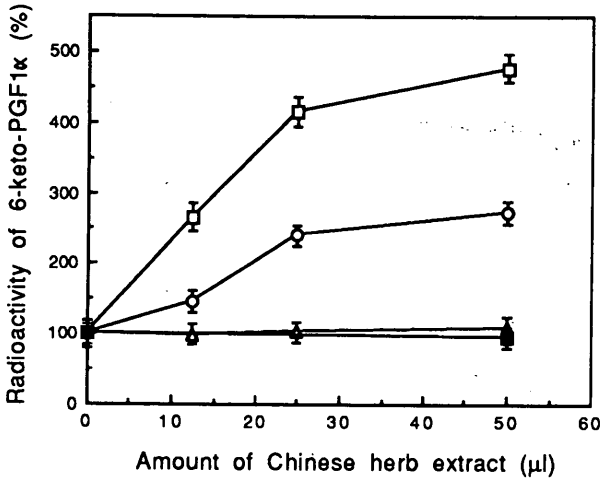

Fig. 5. Effect of extracts from tissue-cultured *A. formosanus* on 6-keto-$PGF_1\alpha$ production by bovine endothelial tissues supplied with ^{14}C-AA. ○, crude water extract; △, ethyl acetate - soluble fraction of crude water extract; □, aqueous remainder of crude water extract; and ■, aqueous remainder of crude water extract supplemented with aspirin (250 μg/2ml). The product, 6-keto-$PGF_1\alpha$, was separated by silica gel TLC using the solvent system described in Fig. 2.

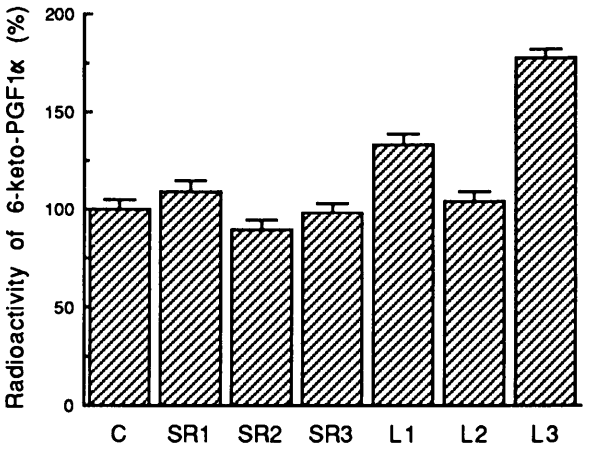

Fig. 6. Effect of extracts from tissue-cultured *A. formosanus* on 6-keto-$PGF_1\alpha$ production by bovine endothelial tissues supplied with ^{14}C-PGH_2. C, control; SR1, crude water extract of stems and roots; SR2, ethyl acetate -soluble fraction of crude water extract of stems and roots; SR3, aqueous remainder of crude water extract of stems and roots; L1, crude water extract of leaves; L2, ethyl acetate-soluble fraction of crude water extract of leaves; L3, aqueous remainder of crude water extract of leaves.

production in platelets. Activatory effects on TXA_2 production were obtained when extracts of the leaves of the Chinese herb were tested.

Effects on Prostacyclin Production

Fig. 5 shows that the whole crude water extract of *A. formosanus* and its ethyl acetate-insoluble aqueous remainder activated 6-keto-$PGF_1\alpha$ (the stable hydrolysis product of PGI_2) production in bovine aortic tissues when incubated with ^{14}C-AA as precursor. The activation of PGI_2 production by these extracts could be completely prevented by the addition of aspirin (250 μg/2 ml). The ethyl acetate-soluble fraction of the crude water extract did not stimulate 6-keto-$PGF_1\alpha$ production. No effect on PGI_2 production was observed in tissues incubated with PGH_2 as precursor. Fig. 6 shows the activatory effects of leaf extracts of tissue -cultured *A. formosanus* on PGI_2 production in endothelial tissues.

Discussion

Prostaglandins help mediate many physiological

functions (Johnson *et al.*, 1983). Prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) are important mediators of most of these physiological functions and have opposite effects on blood coagulation and vascular activity (Moncada *et al.*, 1983; Dusting *et al.*, 1983). PGI_2 is mainly produced in vascular endothelium and has potent platelet antiaggregation and vasodilatory effects. TXA_2 is produced in platelets and is the most potent platelet aggregator and vasoconstrictor known. It is suggested that PGI_2 plays an important role in vascular disease, as a local defense hormone (Steer *et al.*, 1980). Altered production of PGI_2 and TXA_2 can disrupt haemostasis in blood vessels, with effects on platelet aggregation and bleeding time. In this report, the whole plant of tissue-cultured *A. formosanus* was extracted with boiling water, and the aqueous solution was extracted with ethyl acetate in order to study the effects on TXA_2 production in rabbit platelets and PGI_2 production in bovine aortic tissues. Results indicate that the crude water extract and its ethyl acetate-soluble fraction inhibit TXA_2 production in blood platelets incubated with either AA or PGH_2 as precursor, and these inhibitory effects are concentration-dependent. Incubation of platelets with the ethyl acetate-insoluble aqueous remainder of the crude extract activate TXA_2 production only when AA is used as precursor. With the AA precursor, there was activation of PGI_2 production on incubation of aortic tissues with both the crude water extract and the ethyl acetate-insoluble aqueous fraction of the crude extract, but not with the ethyl acetate-soluble fraction of the crude extract. These activating effects were also found to be concentration-dependent. No effect on PGI_2 production was observed on incubation with PGH_2 as precursor. These results suggest that tissue-cultured *A. formosanus* contains at least two bioactive components, one of which affects TXA_2 production in platelets and the other PGI_2 production in aortic endothelium. Furthermore, because of the inhibitions obtained with the ethyl acetate-soluble fractions when using AA and PGH_2 as precursors, suggest that the ethyl acetate-soluble fraction of the crude extract might contain a thromboxane synthase inhibitor. The ethyl acetate-insoluble aqueous phase of the crude extract might contain an activator of cyclooxygenase, as shown by the activation of PGI_2 occurring only with AA as precursor. It is supported by the reduction of the activation by the cyclooxygenase inhibitor, aspirin. These effects were compared to the previous work carried out with extracts of wildtype *A. formosanus* (Tables 1 and 2). The date show that they have similar effects on TXA_2 and PGI_2 production. These results also suggest that, both wildtype and tissue-cultured *A. formosanus* contain similar bioactive substances involved in the regulation of arachidonate metabolism, and the bioactive substances might play important roles in the phar-

Table 1. *Comparison of the effects of tissue-cultured and wildtype* ***A. formosanus*** *extracts on TXA_2 production in rabbit blood platelets*

Amounts of Chinese herb used were 50 μl. Precursors used were ^{14}C-AA (A) and ^{14}C-PGH_2 (B). N represents no significant change of TXA_2 production.

Chinese herb extract		Effects on TXA_2 production (%)	
		Tissue-cultured	Wildtype*
Whole crude water extract	A	52±2	40±2
	B	48±2	38±2
Ethyl acetate-soluble fraction of crude water extract	A	45±2	25±1
	B	42±2	28±1
Ethyl acetate-insoluble fraction of crude water extract	A	327±12	320±15
	B	N	N

*The data reported by Mak *et al.* (1990).

Table 2. *Comparison of the effects of tissue-cultured and wildtype* ***A. formosanus*** *on PGI_2 production in bovine aortic tissues*

Amounts of Chinese herb used were 50 μl. Precursor used was ^{14}C-AA. N represents no significant change on PGI_2 production.

Chinese herb extract	Effects on PGI_2 production (%)	
	Tissue-cultured	Wildtype*
Whole crude water extract	272±10	485±15
Ethyl acetate-soluble fraction of crude water extract	N	N
Ethyl acetate-insoluble fraction of crude water extract	477±16	435±15

*The data reported by Mak *et al.* (1990).

macological and physiological effects of *A. formosanus* (Mak *et al.*, 1990). Furthermore, the inhibitory substance on TXA_2 production and activatory substance on PGI_2 production were shown to be distributed in stems and roots, and leaves respectively of the tissue-cultured *A. formosanus*. It is interesting to know that, as described previously (Krikorian and Steward, 1969; Staba, 1969), plant tissue cultures often produce extremely low concentrations of the compounds of interest. Results shown here do however suggest that the wildtype and tissue-cultured *A. formosanus* contain similar amounts of the bioactive substances affecting prostaglandin metabolism. The similarities indicate that the tissue-cultured type could be a suitable substitute for the wildtype *A. formosanus* in the treatment of cardiovascular disease. However, the bioactive substances of these two different types of *A. formosanus* are at present unknown, and remain to be studied. As described in the Introduction, the wildtype is very precious and is difficult to obtain. Through the large scale tissue culture of *A. formosanus*, sufficient materials for the isolation and characterization of the bioactive substances of *A. formosanus* can however readily be obtained.

Acknowledgements. This work was supported by the National Science Council, Republic of China (Grants NSC77-0606-B006-12 and NSC78-0420-B006-05).

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組織培養金線蓮之萃取液對花生四烯酸新陳代謝的影響

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金線蓮是一種治療心臟血管疾病的高貴中藥材，本研究乃探討組織培養培育的金線蓮對花生四烯酸代謝的生理活性。由組織之萃取物中，發現含有抑制血小板中前列凝素合成和促進動脈內皮組織中前列環素產生之效果，所有抑制和促進作用都與濃度成正相關。此結果與先前用野生金線蓮所得之結果類似。此外，抑制血小板中前列環素產生的物質是存在於金線蓮的根莖部位，促進內皮組織中前列凝素合成的物質則在葉部。利用不同的先驅物質反應得知，造成血小板中前列凝素產量降低及內皮組織中前列環素產量提高的原因，乃是分別抑制前列凝素合成酵素及活化環化加氧酵素(cyclooxygenase)所造成的結果。這些數據顯示，經由組織培養所得之金線蓮中，含有至少兩種不同之生物活性物質，它們可以影響前列凝素及前列環素的生成，其在治療心臟血管疾病之生理及藥理方面擔當了極重要的角色。