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IN VITRO PRODUCTION OF SECONDARY METABOLITES FROM GYNURA PSEUDOCHINA (Lour.) D.C.

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Abstract : *Gynura pseudochina* (Lour.) D.C., or commonly called "Daun Dewa" in Indonesian language, is a local medicinal plant believed to be able to treat cancer and some other diseases. Many researchers have been studying its secondary metabolites and some compounds have been identified. From the production point of view, it is also interesting to consider whether or not its *in vitro* culture will give the same spectrum of secondary metabolite. Growth curve of *in vitro Gynura pseudochina* (Lour.) D.C. showed that exponential phase was achieved after three weeks while stationer phase has been started right four weeks (MS media + BA 3%) after inoculation. Samples were, therefore, taken after three and four weeks, respectively, to study the profile of their secondary metabolite. Extraction was carried over some degree of solvent polarities and separated compounds were visualized using UV light at 254 and 365 nm. Additionally, qualitative analysis of the compounds was performed using specific spray reagents for flavonoids, terpenoids and alkaloids, respectively. While terpenoid content seemed to be very similar between that produced by *in vitro* and external plant, there were some evidence on differences of flavonoids and alkaloids content between *in vitro* plant and external one. In general, *in vitro* culture has been observed to produce higher amount and variety of secondary metabolites both in three week- and four week-samples.

Keywords : *in vitro* Gynura pseudochina (Lour.) D.C., secondary metabolite.

INTRODUCTION

Higher plants contain a variety of substances which are useful medicines, food additives, perfumes, etc. However, decreased plant resources, increases in labour cost and other problems in obtaining these high-value added substances from natural plants have pointed toward the use of plant cell culture for production of the products. Because *in vitro* plant cell culture is not affected by changes in such environmental conditions such as climate or natural depredation, improved production may be available in any place or season. Therefore, studies on the production of useful metabolite by plant cell culture have been carried out on an increasing scale since the end of the 1950's. Plant cell culture is viewed as a potential means of producing useful plant products such that conventional agriculture, with all its attendant problems and variables, can be circumvented. These problems include: environmental factors (drought, floods, etc.), disease, political and labour instabilities in the producing countries, uncontrollable variations in the crop quality, inability of authorities to prevent crop adulteration, losses in storage and handling. Thus, the production of useful and valuable secondary metabolites in large bioreactors located in the consuming country is very attractive. Additional advantages of such processes include: controlled production according to demand and a reduced and requirement. However, this technology is still being developed and despite the advantages outlined above, it should be verified, whether or not the culture will produce the same metabolites as the external plant. *Gynura pseudochina* (Lour.) D.C., or commonly called "Daun Dewa" in Indonesian language, is a local medicinal plant believed to be able to treat cancer and some other diseases. Many researchers have been studying its secondary metabolites and some compounds have been identified [15-22].

From the production point of view, it is also interesting to consider whether or not its *in vitro* culture will give the same spectrum of secondary metabolite. Since no research has been reported to answer that issue on *Gynura pseudochina* (Lour.) D.C., we tried to start revealing important facts by comparing the TLC profile of extracts from the plant and that from the *in vitro* culture.

MATERIALS AND METHODS

Plant and materials

Sterile eksplan and plant used in this experiment originated from Mojokerto, East and had been previously identified as *Gynura pseudochina* (Lour.) D.C. by Pusat Informasi dan Pengembangan Obat Tradisional (PIPOT) Universitas Surabaya. Thin Layer Chromatography (TLC) was done using silica gel plate 60 F_{254} with aluminum support. All reagents, solvent and consumables used for the extraction and fractionation were purchased from Merck. Anisaldehyde-Sulfuric acid reagent was prepared by dissolving 1 ml of sulfuric acid and 0,5 ml of anisaldehide in acetic acid to 50 ml of final volume. Dragendorff reagent was prepared by mixing 1.7 g of bismuth sub-nitrate, 20 grams of tartaric acid and 16 grams of KI in 120 ml of water.

In vitro Subculture of *Gynura pseudochina* (Lour.) D.C.

Preparation of its subculture was done using Murashige & Skoog (MS) medium added with BA (Benzil Adenin) hormone of 1.5, 2.5 and 3.0 ppm concentration. Growth index was determined from the last condition since it showed the highest growth rate. Samples were taken after three and four weeks, respectively. Propagation was carried out until 10 grams dry weight was obtained. Drying was performed by neon lamp in air conditioned room.

Extraction and Fractionation

All procedures were performed following the modified Markham method [11] for *Gynura pseudochina* (Lour.) D.C. external plant, *in vitro* subcultures harvested at 3 and 4 week age, respectively. 10 grams of dried plants was macerated by 100 ml mixture of methanol:H_2O (9:1) at room temperature for 12 hours and filtered. The residue was further macerated by 100 ml of methanol:H_2O (1:1) for another 12 hours and followed by filtration. Combined filtrates were concentrated to one fifth volume using vacuum pump at 40°C and extracted by hexane. Organic phase was separated and dried using Na_2SO_4 anhydrate. Ammonium hydroxide was added to the water phase to make it alkaline (pH 9-10) followed by the second extraction using 50 ml of chloroform. Again, the organic phase was separated and dried using Na_2SO_4 anhydrate. The water phase was than acidified by adding acetic acid (pH 5) and finally extracted using 50 ml of ethyl acetate.

Thin Layer Chromatography

The three organic phases were concentrated to 10 ml volume for each and applied on the surface of TLC plates which were than eluted using Hexane: Ethyl acetate (4:1), Methanol:Chloro-form (3:2) and Methanol:Acetonitrile (3:1), respectively for extract in hexane, chloroform and ethyl acetate. After elution, the plates were dried at room temperature and visualized under UV at 254nm and 465nm. Further visualization was also done by spraying specific reagent for identification of terpene, alkaloid and flavonoid using anisaldehyde-sulfuric acid reagent, dragendorff reagent and ammonia vapor, respectively. These reagents were freshly prepared. For revealing with anisaldehyde-sulfuric acid reagent, TLC plates were dried in the oven at 100°C for 25 minutes before analyzed.

RESULTS AND DISCUSSION

Orientation of BA concentration

Murrashige-Skoog (MS) medium was chosen since it was well known as the most effective one for dikotil dan monokotils. Culture using 3 ppm has shown faster growth with the best shoot and leaf formation. This culture was, therefore, used in further work steps described below.

In vitro Subculture of *Gynura pseudochina* (Lour.) D.C.

Maximum growth was achieved for MS medium + BA 3 ppm at the week-4 with the growth index of 2,966. After 4 weeks the plant began to undergo stationary phase where the secondary metabolites normally formed.

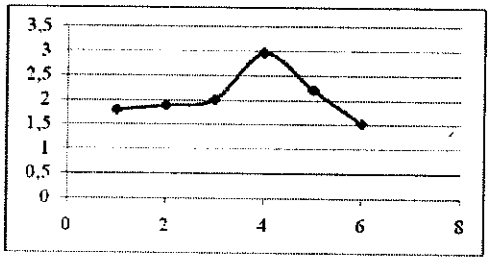
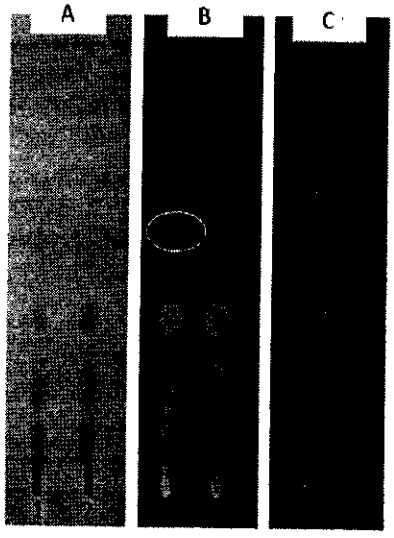


Figure-1. The growth curve of *Gynura pseudochina* (Lour.) D.C. *in vitro* culture, growth index vs time (weeks).

Comparison of TLC Profiles

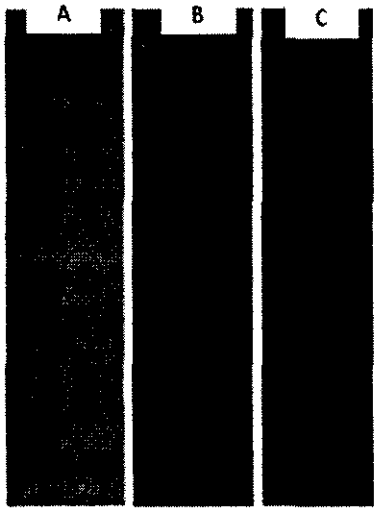
This study was limited to terpenes, alkaloids and flavonoids, which were the most common substances related to anti-cancer agent. Here, terpenes were isolated using hexane as the appropriate solvent, while alkaloids and flavonoids were isolated using chloroform and ethyl acetate, respectively. For revealing the terpene contents, we used anisaldehyde-sulfuric acid spray reagent. The TLC profile was shown in the picture below (Fig.2). There is high similarity between chemical contents of the culture and those of external *Gynura pseudochina* (Lour.) D.C., except for one compound characterized by a blue spot at R_f of 0.59, which is not present in the *in vitro* culture.



A: visualized by UV light 254nm
B: visualized by UV light 365nm
C: visualized by Anisaldehide reagent
1: external plant. 2: cultured plant

Figure-2. Identification of *Gynura pseudochina* (Lour.) D.C. by TLC (silica gel F254, heksane:ethyl acetate 4:1), revealed by anisaldehyde-sulfuric acid reagent.

However, as revealed by Anisaldehide-sulfuric acid reagent, there is a weak spot at the corresponding R_f. Thus, it is still unclear whether the blue fluorescing compound visualized at 365nm UV wavelength is really disappear in the *in vitro* culture, or it just underwent a minor structure modification and lost its fluorescence property.
The work was continued by revealing the alkaloid contents, for which we used Dragendorff spray reagent followed by drying at 100°C in the oven for 25 minutes before analyzed. From the TLC profiles (Fig.3), it is obvious that external plant of *Gynura pseudochina* (Lour.) D.C. does not contain a significant amount of alkaloids, while the *in vitro* culture shows one spot characterizing an alkaloid at 0.47, a contrast case to that regarding terpenes.



A: visualized by UV light 254nm
B: visualized by UV light 365nm
C: visualized by Dragendorf reagent
1: external plant, 2: cultured plant

Figure-3. Identification of *Gynura pseudochina* (Lour.) D.C. by TLC (silica gel F254, methanol:chloroform 3:2), revealed by dragendorff reagent.

This work has shown that cultured plant cells could produce reduced quantities and different profiles of secondary metabolites when compared with the intact. Another researcher argued that the poor product expression could be attributed to a lack of differentiation in cultures [12]. On the other hand, it has been also proved in this study that cultures can over-produce metabolites compared with the external plant. There are reports on a number of other examples of cultured cells producing metabolites not observed in the plant, eg. *Lithospermum erythrorhizon* cultures have been observed to synthesize rosmarinic acid [13].
The last visualization was performed using ammonia vapor to identify the present of flavonoid. There we found almost no significant difference between TLC profile between culture and plant of *Gynura pseudochina* (Lour.) D.C. as depicted by Fig.4. However, the red spot at R_f 0.76 was somehow stronger in the *in vitro* culture.

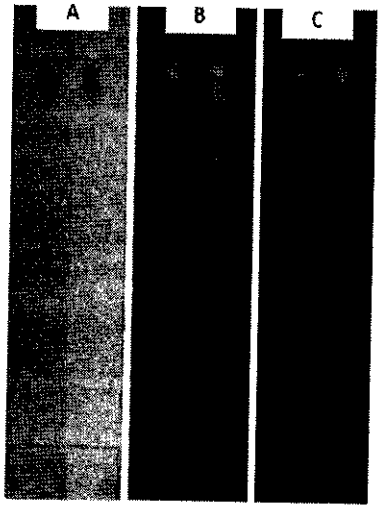


Figure-4. Identification of *Gynura pseudochina* (Lour.) D.C. by TLC (silica gel F254, methanol:acetonitrile 3:1), revealed by ammonia vapor.

Note: we presented only the TLC picture generated from 4 week-age cultures. However, the 3 week-age cultures has been analyzed and concluded as the same result.

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