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Second Metabolite Composition, Antioxidative, Tyrosinase Inhibitory, Antibacterial and Anticancer Activity of *Balanophora laxiflora* Extract

Tran Thi Hang[1], Tran Thi Quyen[1], Nguyen Quang Huy[2], Le Thi Phuong Hoa[1,*]

[1]*Faculty of Biology, Hanoi National University of Education, 136 Xuan Thuy, Cau Giay, Hanoi, Vietnam*
[2]*Faculty of Biology, VNU University of Science, 334 Nguyen Trai, Hanoi, Vietnam*

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Abstract: *Balanophora laxiflora* extract contains various compounds including terpenoids, phenolics, and flavonoids. Ethyl acetate fraction of *B. laxiflora* has high content of phenolic compounds (608.21 mg GAE/g), highly correlated with its antioxidant activity including 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging capacity (IC_{50} value of 22.81 µg/mL) and reducing power (2.33), which is comparable to that of ascorbic acid and quecertin. This fraction shows strong tyrosinase inhibitory activity with IC_{50} value of 7.9 µg/mL and mild inhibitory activity against Gram-positive and Gram-negative strains at the concentration of 20 mg/mL, stronger than those of other fractions. Ethyl acetate fraction also exhibited cytotoxicity against human lung carcinoma (LU-1) cell line. n-Hexane fraction shows stronger activity on epidermal carcinoma (KB) cell lines (IC_{50} = 3.45 µg/mL). Median lethal dose (LD_{50}) of *B. laxiflora* methanolic crude extract on experimental mice is 10.64 g/kg body mass. The results suggest new pharmacological use of *B. laxiflora* especially in depigmentation, cancer treatment and further characterization of bioactive constituents and biological activities of ethyl acetate and n-hexane fraction.

Keywords: Balanophora laxiflora, antioxidative activity, tyrosinase inhibition, antibacterial activity, anticancer activity

1. Introduction

In recent years, the search for natural sources of bioactivities has been rising with the global concern for preventive and therapeutic healthcare. Vietnamese medicinal plants are a good source of bioactive compounds as they have traditionally been used to treat ailments. *Balanophora laxiflora* Hemsl. is a dioeciously parasitic plant, mainly distributed in the forests in Hoa Binh, Lao Cai, Yen Bai provinces. The whole plant of *B. laxiflora* has been used as a tonic for blood circulation improvement, recovery, appetite stimulation, and in traditional remedies for muscular pain, diarrhea…[1].

Recent researches have discovered various compounds and bioactivities of *B. laxiflora*. As other species in genus *Balanophora*, *B. laxiflora* possesses hydrolyzable tannins with a phenylacrylic acid derivative such as caffeoyl,

*Corresponding author. Tel.: 84-975399160
Email: lephhoa@yahoo.com

coumaroyl, linked to C-1 of a glucosyl unit by *O*-glycosidic bond [2,3,4]. She et al., (2008) has purified 19 phenolic compounds from 80% acetone extract of *B. laxiflora* collected from China, among which 9 hydrolyzable tannins, 1 phenylpropanoid and 1 phenolic acid showed stronger or similar DPPH scavenging capacity as compared to ascorbic acid (SC_{50}, concentration required for 50% reduction of DPPH radical, ranging from 4.2 – 10.7 μM) [3]. Various extracts from *B. laxiflora* male flowers especially ethyl acetate fraction were reported to have good inhibitory activity against DPPH radical as well as strong superoxide radical scavenging activity and high reducing power. Accordingly, 5 phenolic compounds were isolated from ethyl acetate fraction with 2 compounds showed stronger DPPH and superoxide radical scavenging activity than catechin, a wellknown antioxidant [4]. Anti-inflammatory activity was also espressed in *B. laxiflora*. Among eighteen compounds including phenolics, triterpenoids and phytosterols isolated from tuberous rhizomes of *B. laxiflora* collected in Taiwan, two compounds, isolariciresinol and ethyl caffeate, showed strong inhibitory activity on LPS-stimulated NO production in RAW 264.7 macrophages with IC_{50} (half maximal inhibitory concentration) values of 0.81 and 7.29 μM, respectively. Isolariciresinol had a potent effect on TNF-α production and inhibitory effect on nuclear factor-κB (NF-κB) activation [5]. Latest research on *B. laxiflora* demonstrated strong xanthine oxidase inhibitory activity of ethyl acetate fraction (IC_{50} 14.2 μg/mL) from male flowers. This fraction and two derived hydrolyzable tannins also exhibited *in vivo* hypouricemic effect in hyperuricemic mice, suggested to be potential candidates as new hypouricemic agents [6]. In Vietnam, there is only one report on androgenic activity of B. laxiflora water extract in both intact and orchidectomized rats with the increase in the relative weight of the testis and serum testosterone, glans penis in intact rat and the increase in the relative weight of seminal vesicle, Cowper's glands in orchidectomized rats [7].

In order to elucidate biochemical and bioactive significance as well as extend the use of *B. laxiflora*, this paper evaluated second metabolite composition as well as antioxidative, tyrosinase inhibitory, antibacterial activity and anticancer activity of *B. laxiflora* from Vietnam.

2. Materials and Methods

Materials

- *B. laxiflora* plants were collected in Lao Cai province.

- Bacteria strains including *Staphylococus aureus, Escherichia coli, Pseudomonas aeruginosa*, *Samonella enterica* and *S. typhimurium* and cell lines of human epidermal carcinoma (KB), lung carcinoma (LU), hepatocellular carcinoma (HepG2) were obtained from Institute of Chemistry, Vietnam Academy of Science and Technology.

- Swiss mice (*Mus musculus*), weighed 18 – 20 g, were purchased from National Institute of Hygiene and Epidemiology.

Methods

Sample extraction and fractionation

The fresh plants were washed with distilled water to remove adhering debris and dust, and then soaked in methanol for 3 days and extracted in an ultrasonic bath for 30 mins at room temperature. The extraction was performed in three replicates. The extracts were mixed and concentrated in a rotary evaporator at 40°C, and then lyophilized. The crude extract was further fractionated sequentially in different solvents including n-hexane, ethyl acetate, butanol and water. The four fractions were concentrated by vacuum evaporation. All of the extracts were stored at -20°C until use.

Thin layer chromatography

Extract solutions were prepared at the concentration of 10 mg/mL in absolute

methanol. Various solvent systems, e.g. n-hexane/ethyl acetate, ethyl acetate/ methanol, chloroform, chloroform/methanol, chloroform/ methanol/ water were used as the mobile phase. The plate was sprayed with 5% sulfuric acid, heat dried, and observed under visible light and UV radiation at 254 nm. Qualitative evaluation of the plate was done by determining the migration behavior of the separated substances given in the form of Rf value.

Determination of total phenolics and flavonoids

Total phenolics content was evaluated according to Waterhouse (2002) [8], using gallic acid as the standard. The result was expressed as mg gallic acid equivalents (GAE) per gram dry weight of extract.

Total flavonoids were determined following the method described by Sapkota et al., (2010) [9], using quercetin as the standard. Flavonoid content of extracts were calculated in mg quercetin equivalents (QE) per gram dry weight of each extract by comparison with the quercetin standard curve.

Antioxidant activity

Antioxidant activity was evaluated by determining free radical scavenging potential using DPPH according to Blois [10]. The reaction mixture contained 20 µL of extract solutions and 180 µL of 0.3 mM DPPH solution. Ascorbic acid was used for comparison with extracts. DPPH scavenging activity was calculated using the following formula:

DPPH scavenging activity (%) = $[(A_{control} - A_{sample})/(A_{control})] \times 100$

where $A_{control}$ represents the absorbance of the control and A_{sample} is the absorbance of the test sample. The IC_{50} value is deduced from the logarithm curve of scavenging capacity vs. sample concentration.

Reducing power assay

The reducing power of the extracts was determined by the method of Sapkota et al., [9]. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid and quercetin was used as standard.

Tyrosinase inhibitory activity of fractions was evaluated according to Yagi et al., [11] using mushroom tyrosinase and L-DOPA (dihydroxyphenylalanine) as the substrate. Kojic acid was used for comparison. The percent inhibition of tyrosinase activity was calculated as:

Tyrosinase inhibitory capacity (%) = $[(A - B)/A] \times 100$

where A stands for the absorbance at 475 nm without the test sample and B is the absorbance at 475 nm with the test sample. IC_{50} values were calculated based on the logarithm curve.

Antibacterial activity assay

The antibacterial activity was tested against *S. aureus*, *E. coli*, *P. aeruginosa, S. enterica* and *S. typhimurium* by using the agar well diffusion method [12]. Fractions were dissolved in methanol at a concentration of 20 mg/mL. Methanol served as a negative control and kanamycin as the positive control. Antibacterial activity was determined by measuring the diameter of the inhibition zone formed around the well.

Anticancer activity assay

B. laxiflora extracts were tested against human cancer cell lines including epidermal carcinoma (KB), lung carcinoma (LU-1), and hepatocellular carcinoma (HepG2) according to the method described by Scudiero et al [13] at the Laboratory of Applied Biochemistry, Institute of Chemistry.

Acute toxicity

Mice were housed in plastic cages and acclimatized for one week at experimental room condition with provided access to water and food. They were assigned to 2 groups of 10 individuals. Dried powder of *B. laxiflora* crude extract was administered by oral gavage in a single dose at a volume 0.2 - 0.4 mL/10 g body weight after the mice were fasted for 8 hrs. The control animals received a vehicle solution. All mice were monitored daily within three days for

any additional behavioral or clinical signs of toxicity before receiving a new dose.

Statistical analysis

Data were analyzed using Microsoft Excell software and Student's t-test. Results were expressed as means ± standard deviation. A level of p value less than 0.05 was considered to be significant.

3. Results and Discussion

Thin layer chromatography

The four fractions of *B. laxiflora* plants were subjected to thin layer chromatographic analysis to investigate second metabolite profile using different solvent systems. As a result, n-hexane/ethyl acetate 4:1, chloroform and chloroform/methanol /water 4:2:0.1 provided best separation and detection of compounds for n-hexane fraction, ethyl acetate fraction, for butanol and for water fraction, respectively.

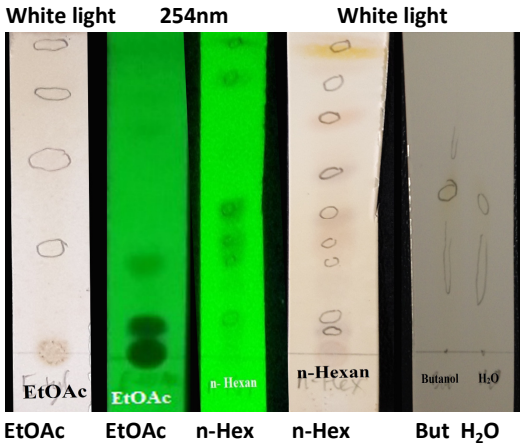

Fig.1. Thin layer chromatogram of *B. laxiflora* extracts. EtOAc: ethyl acetate fraction, n-Hex: n-hexane fraction, But: butanol fraction and H_2O: water fraction.

Ethyl acetate fraction and n-hexane fraction gave more coloured bands than butanol and water fractions (Fig. 1). They possessed terpenoids (with purple colour), flavonoids (yellow, orange) and phenolics (blue), among which terpenoids are predominant. Water fraction had the fewest bands.

Table 1. Total phenolic and flavonoid content of *B. laxiflora* fractions

Fraction	Total phenolic content (mg GAE/g fraction)	Total flavonoid content (mg QE/g fraction)
n-Hexane	203.34 ± 3.61[a]	22.75 ± 1.81[a]
Ethyl acetate	608.21 ± 5.84[b]	71.26 ± 4.73[b]
Butanol	271.00 ± 5.70[c]	29.36 ± 4.80[c]
Water	24.53 ± 7.68[d]	3.24 ± 0.81[d]

GAE: gallic acid equivalents, QE: quercetin equivalents, [a, b, c, d]: significant difference among fractions $p<0.05$

Previous report showed similar results on *B. abbreviata* Bl. Terpenoids were dominant in all fractions including ethyl acetate fraction [14]. Only a few terpenoids were isolated from *B. laxiflora* tuberous rhizomes such as triterpenes lupeol, lupa-12,20(29)-dien-3β-ol [5]. More terpenoids are suggested be isolated and characterized from *B. laxiflora*.

Phenolics and flavonoids contents

Phenolic compounds are commonly found in various parts of all sorts of plants. They have been widely investigated in many medicinal plants and plant foods for they are responsible for multiple biological effects [4,15]. Total phenolic and flavonoid content of *B. laxiflora* fractions was examined.

The result showed that *B. laxiflora* fractions had large amount of phenolics except water fraction but small amount of flavonoids, which agreed with the thin layer chromatography analysis. The total content of phenolics and flavonoids in the ethyl acetate fraction was approximately three times as much as those in the n-hexane and butanol fraction. The water fraction had very low amount of phenolics and flavonoids. The level of phenolic compounds in fractions from *B. laxiflora* plant was much higher than that in fractions from male flowers as compared to the previous report. Ethyl acetate fraction from male flowers contained 460.0 mg GAE/g [4].

Various phenolic compounds were isolated from *B. laxiflora* male flowers and rhizomes. Some of them were indicated to have strong antioxidant activity, anti-flammatory activity and hypouricemic effect [3-6]. Phenolics exhibit a wide variety of beneficial biological activities including antiviral, antibacterial, antihypertensive, antilipoperoxidant, hepatoprotective, and anti-carcinogenic actions [15]. Some compounds from other *Balanophora* species were reported with HIV inhibitory effect, hypoglycemic effect [2]. With high content of phenolics, *B. laxiflora* fractions especially the ethyl acetate fraction are suggested to be further characterized since its biological effects could be attributed to the presence of these valuable constituents.

DPPH scavenging activity

Antioxidants are believed to be highly effective in the management of tissue impairment caused by reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals [15]. DPPH free radical scavenging assay is an easy, rapid and sensitive method, which is widely used for antioxidant screening of plant extracts. In the presence of an antioxidant, the DPPH radical obtain one more electron, decolorized, and the absorbance decreases as a result. Table 2 shows IC_{50} values for DPPH scavenging activity of *B. laxiflora* fractions.

Table 2. DPPH scavenging activities of *B. laxiflora* fractions

Sample	IC_{50} (μg/mL)
n-Hexane fraction	75.89 ± 1.34^{a}
Ethyl acetate fraction	22.81 ± 2.03^{b}
Butanol fraction	60.71 ± 2.34^{c}
Water fraction	2037.4 ± 238.18
Ascorbic acid	18.53 ± 1.62

$^{a, b, c, d}$: significant difference among fractions $p<0.05$

It was observed that *B. laxiflora* fractions had a dose-dependent DPPH scavenging potential except water fraction. The ethyl acetate fraction exhibited much stronger activity than n-hexane and butanol fractions. Although half-maximal DPPH inhbitory concentrations of those fractions were not low as those from *B. laxiflora* male flowers [4], the capacity of ethyl acetate fraction was significantly comparable to that of ascorbic acid, with IC_{50} values of 22.81 ± 2.03 μg/mL and 18.53 ± 1.62 μg/mL, respectively. The result also suggested major antioxidative compound more likely concentrate in flowers.

Free radicals contribute to many forms of human illness such as aging, cancer, atherosclerosis, diabetes, Alzheimer's disease and other neurodegenerative disorders. They are chemical species containing one or more unpaired electrons that makes them highly unstable and able to cause damage to other molecules as they extract electrons from them in order to attain stability [15]. The DPPH scavenging capacity of the ethyl acetate fraction of *B. laxiflora* may be due to their donation of hydrogen to a free radical, reducing it to a nonreactive species. The scavenging activity of *B. laxiflora* fractions showed a high correlation to their phenolic content (R_2 = 0.9845), suggesting the contribution of phenolic compounds, which have redox properties, adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [15]. Previous researches have also revealed the highly positive correlation between total phenolic content and antioxidant activity of various

extracts [4,15]. Furthermore, some compounds, mainly hydrolysable tannins, isolated from *B. laxiflora* showed strong scavenging activities, even stronger than ascorbic acid [3,4].

Reducing power

The antioxidant activity of plant extracts can be measured by using reducing power assay. In the reducing power assay, the more antioxidant compounds convert the oxidation form of iron (Fe^{+3}) in ferric chloride to ferrous (Fe^{+2}).

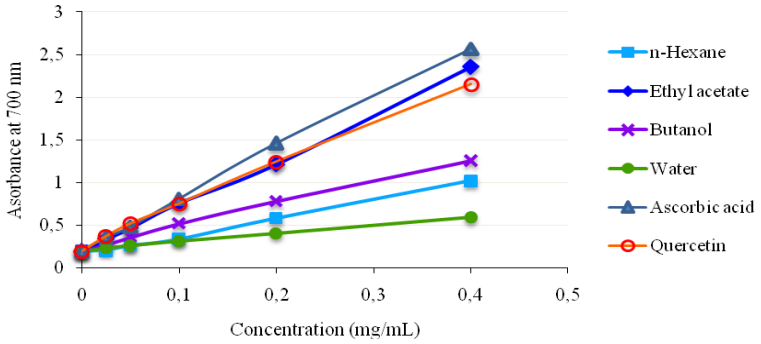

Fig.2. Reducing power of *B. laxiflora* fractions.

Table 3. Concentration of *B. laxiflora* fractions at absorbance 0.5 compared with ascorbic acid and quercetin as standards in reducing power assay

Sample	Concentration mg/mL (Absorbance 0.5)
n-Hexane fraction	0.159 ± 0.0026^{a}
Ethyl acetate fraction	0.056 ± 0.0012^{b}
Butanol fraction	0.105 ± 0.0032^{c}
Water fraction	0.301 ± 0.0021^{d}
Ascorbic acid	0.049 ± 0.0017^{e}
Quercetin	0.052 ± 0.003^{b}

$^{a, b, c, d}$: significant difference among fractions $p<0.05$

The results showed that the reducing power of ethyl acetate fraction from *B. laxiflora* was significantly comparable to ascorbic acid and quercetin (Figure 2, Table 3). The reducing power of ethyl acetate fraction reached higher level (2.33) than quercetin (2.17) at the concentration of 0.4 mg/mL. The reducing power of three other fractions was much lower. Water fraction was at the lowest level. The data demonstrated the high correlation between reducing power and DPPH scavenging activity, supporting previous report [4]. Ferric ion-reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action, and is strongly correlated with other antioxidant properties [4,11].

3.2.1. Tyrosinase inhibitory activity

Tyrosinase is the key enzyme in melanin synthesis and catalyzes the first two reactions converting tyrosine and L-DOPA to dopaquinone, leading to the formation of pigments [11]. Formation and accumulation of melanin is a response to various physical and physiological changes in the body. *B. laxiflora* fractions were subjected to test tyrosinase inhibitor activity to elucidate their biological activities.

Table 4. Tyrosinase inhibitory activity of *B. laxiflora* fractions

Sample	IC_{50} (µg/mL)
n- Hexane	31.81 ± 6.78^{a}
Ethyl acetate	7.90 ± 0.58^{b}
Butanol	15.12 ± 4.19^{c}
Water	128.42 ± 26.64^{d}
Kojic acid	2.6 ± 0.57

As shown in Table 4, *B. laxiflora* fractions exerted good inhibitory effect on mushroom tyrosinase activity except water fraction. The activity was concentration dependent. The inhibitory activity increased with the increase in concentration of the extracts (data not shown). The ethyl acetate fraction showed strongest tyrosinase inhibitory activity followed by butanol fraction, n-hexane fraction, and water fraction. Research of Ogi et al (2011) revealed strong tyrosinase inhibitory activity of *B. fungosa* 50% ethanol extract (IC_{50} = 15 µg/mL) and its fractions (chloroform and butanol fraction, IC_{50} 8.2 and 9.6 µg/mL, respectively), from which two phenolic compounds were isolated and exhibited tyrosinase inhibition (IC_{50} 5.7 and 5.9 µg/mL, respectively) as well as depigmentation effect in HMVII human melanoma cells and in three-dimensional human skin model [16]. Papuabalanol B purified from ethyl acetate extract of *B. papuana* also showed inhibition of tyrosinase dose-dependently (IC_{50} 23.3 µM) at lower level than kojic acid [17].

This is the first report demonstrating potential inhibitory effect of *B. laxiflora* on tyrosinase activity. Further study is suggested to implement especially on ethyl acetate fraction to extend the application of *B. laxiflora* in healthcare and skincare.

Antibacterial activity

B. laxiflora fractions showed mild inhibitory activity on both Gram negative and Gram positive bacteria at concentration of 20 mg/mL. Ethyl acetate fraction inhibited all tested bacterial strains. This fraction had stronger activity than the two other fractions (Table 5). Methanol/water fraction of *B. abbreviata* also exerted inhibitory activity on *S. aureus* but at low level, with the minimal inhibitory concentration of 250 mg/L [14].

Anticancer activity

There is still lack of report on anticancer actitivy of the genus *Balanophora* though they possess a number of bioactive phenolic compounds. There is only one report indicating cytotoxicity of four hydrolysable tannins isolated from methanol extract of *B. japonica* ariel parts to HepG2 cancer cell lines with IC_{50} values ranging from 4.22 to 48.2 µM [18]. In order to further characterize biological activity of *B. laxiflora*, its fractions were tested with KB human cancer cell line.

Table 5. Antibacterial activity of *B. laxiflora* fractions

Bacterial strain	Inhibition zone diameter (mm)				
	Positive control	Negative control	Ethyl acetate fraction	Butanol fraction	Water fraction
S. aureus	32.5 ± 0.5	-	16.7 ± 1.0	11.3 ± 0.4	-
E. coli	31.5 ± 1.3	-	13.5 ± 0.7	12.5 ± 0.7	12.5 ± 0.7
P. aeruginosa	28.0 ± 2.8	-	10.5 ± 0.7	-	-
S. enterica	31.6 ± 1.1	-	15.0 ± 0.7	-	-
S. typhimurium	30.2 ± 1.0	-	6.3 ± 0.4	-	-

Negative control: methanol, Positive control: kanamycin, "-": not determined

Table 6. Anticancer activity of *B. laxiflora* fractions

Sample	IC_{50} (µg/mL)
n-Hexane fraction	3.45
Ethyl acetate fraction	>128
Butanol fraction	>128
Water fraction	>128
Ellipticine	0.31

Among *B. laxiflora* fractions, only n-hexane fraction showed inhibitory activity on KB human cancer cell line at high level (IC_{50} = 3.45 µg/mL) (Table 6). Further study on the effect of n-hexane and ethyl acetate fractions on LU-1 and HepG2 cancer cells revealed inhibitory activity of ethyl acetate fraction against LU-1 cells (IC_{50} = 96.65 µg/mL). The result

demonstrated anticancer activity of *B. laxiflora* and suggested further characterization of n-hexane fraction and ethyl acetate fraction from *B. laxiflora*.

Acute toxicity in mice

The results of acute toxicity of *B. laxiflora* crude extract in mice were obtained from three replicates and illustrated in Table 7.

Table 7. Acute toxicity of *B. laxiflora* crude extract in mice

Dosage (g/kg body mass)	Percent mortality
5.0	0
7.5	3.3
10.0	33.3
12.5	76.7
15.0	100

Data in Table 7 was used to plot a logarithmic curve and LD_{50} value was calculated as 10.64 g/kg body mass. Consequently, safe dose was calculated as 1.06 g/kg body mass. This is the first report on acute toxicity and safe dose of *B. laxiflora* in mice. It is a significant reference for future application of *B. laxiflora* as dietary supplement.

4. Conclusions

B. laxiflora extract possessed high content of phenolics which mainly concentrated in ethyl acetate fraction, in high correlation with strong antioxidative activity including DPPH scavenging capacity and reducing power which was comparable to potent antioxidants as ascorbic acid and quercetin. Ethyl acetate fraction also exerted strong inhibitory activity on tyrosinase reaction in melanin synthesis, moderate activity against Gram positive and negative bacteria and LU-1 cancer cell line. The results suggest new application of *B. laxiflora* extract in healthcare and skincare. With further characterization of ethyl acetate fraction for other biological activities and bioactive compounds together with acute toxicity data on mice, *B. laxiflora* extract will be a potent source for production of nutraceuticals.

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Thành phần hợp chất thứ cấp, hoạt tính chống oxy hoá và ức chế tyrosinase, kháng khuẩn và kháng ung thư của dịch chiết *Balanophora laxiflora*

Trần Thị Hằng[1], Trần Thị Quyên[1], Nguyễn Quang Huy[2], Lê Thị Phương Hoa[1]

[1]*Khoa Sinh học, Đại học Sư phạm Hà Nội, 136 Xuân Thủy, Cầu Giấy, Hà Nội, Việt Nam*

[2]*Khoa Sinh học, Trường Đại học Khoa học Tự nhiên, ĐHQGHN, 334 Nguyễn Trãi, Hà Nội, Việt Nam*

Tóm tắt: Dịch chiết *Balanophora laxiflora* chứa các hợp chất terpenoid, phenol, flavonoid. Cao phân đoạn ethyl acetate của *B. laxiflora* chứa hàm lượng cao các hợp chất phenol (608,21 mg GAE/g), tương quan chặt chẽ với hoạt tính chống oxy hoá kể cả hoạt tính quét gốc tự do 1,1-diphenyl-2-picrylhydrazyl (DPPH) (IC_{50} = 22,81 µg/mL) và lực khử (2,33), gần tương đương với axit ascorbic và quecertin. Cao phân đoạn này cũng thể hiện hoạt tính ức chế mạnh tyrosinase với giá trị IC_{50} 7,9 µg/mL, hoạt tính kháng các chủng vi khuẩn Gram dương và Gram âm ở nồng độ 20 mg/mL, mạnh hơn các cao phân đoạn khác. Cao phân đoạn ethyl acetate cũng ức chế dòng tế bào ung thư phổi của người (LU-1). Cao phân đoạn n-hexane thể hiện hoạt tính mạnh hơn ở dòng tế bào ung thư biểu mô (KB) (IC_{50} = 3,45 µg/mL). Liều độc (LD_{50}) của cao tổng số methanol của *B. laxiflora* trên chuột thí nghiệm là 10,64 g/kg thể trọng. Kết quả nghiên cứu gợi ý tác dụng dược lý mới của *B. laxiflora* đặc biệt trong việc làm sáng da, điều trị ung thư cũng như nghiên cứu thêm về các hợp chất có hoạt tính sinh học và hoạt tính của cao phân đoạn ethyl acetate và n-hexane.

Từ khoá: Balanophora laxiflora, chống oxy hoá, ức chế tyrosinase, kháng khuẩn, kháng ung thư.