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Phytochemical Analysis and Hypoglycemic Activity from Ethanolic Extract of *Oroxylum indicum* (L.) Leaves

Nguyen Ngoc Hong, Bui Van Chung and Hoang Khai Le*
HCMC University of Technology (HUTECH) – 475A Dien Bien Phu Street,
Binh Thanh District, HCMC
*Corresponding author: (e-mail: lehoangkhai2016@gmail.com)

Oroxylum indicum (L.) is an important medicinal plant used in traditional medicine. The objective of this study is to evaluate the phytochemical compounds and the anti-hyperglycemic potential of ethanolic extract of *O. indicum* leaves in oral glucose tolerance tests carried out with glucose-loaded mice. Preliminary findings revealed that *O. indicum* showed the presence of carotenoid, essential oil, triterpenoids in free and glycosid forms, tannins, flavonoids and anthraquinones. The composition of ethanolic extract of *O. indicum* leaves was investigated by using an HPLC/MS fingerprinting technique coupled with chemometric analysis. According to the exact mass measurements and MS/MS data, the results showed the identification of four flavonoids such as 5, 7 dihydro - 3 methoxyflavone **(1)**; pinobanksin **(2)**; baicalein **(3)**; and chrysin **(4)**. The triterpenoid betulinic acid **(5)** was identified as constituents of *O. indicum* for the first time.

The effects of three different doses (50 mg/kg, 70 mg/kg, and 100 mg/kg body weight) of ethanolic extract were orally tolerated to body mice before the oral glucose tolerance. The result indicated that percentage of inhibition of ethanolic extract of *O. indicum* leaves at the dose of 100 mg/kg body weight in glucose-induced hyperglycemic mice was effectively similar to standard drug glibenclamide.

Key words: HPLC/MS; hypoglycemic; phytochemical analysis; phenolic compounds; *Oroxylum indicum* (L.)

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Oroxylum indicum (L) Vent. (Family: Bignoniaceae), is a tree well known in ethnic communities of South Asia including Vietnam for treatment of dysentery and rheumatism. In Vietnam traditional medicine, the roots as well as stem bark of *O. indicum* commonly known as 'núc nác' or 'hoàng bá nam' that have been used for centuries for the treatment of various gastric disorders [1]. Its leaves and young stems are used as a food supplement but phytochemical investigation and biological activities from leaves remain little known. Our previous study found that leaves of *O. indicum* are potential sources of natural antioxidants with its ethanolic extract has stronger antioxidant activities than that of aqueous ones [2]. Plants contain a valuable source of natural antioxidants including vitamins, terpenoids, nitrogen, phenolic compounds and some other endogenous metabolites. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from ageing-related diseases has intrigued scientists for a long time. This study aims to examine phytochemical compounds, and potential anti-hyperglycemic activity of ethanolic extract of *O. indicum* leaves in oral glucose tolerance tests carried out with glucose-loaded mice.

MATERIAL AND METHODS

Plant Material and Extraction

Leaves of *O.indicum* were collected from Nhon Trach district, Dong Nai province in May 2014. The sample was dried in the oven at not higher than 60°C and ground into coarse powder. The powdered plant material was macerated with ethanol 90% for 24 h. After filtration, the solvent was removed under reduced pressure to obtain a liquid extract.

Animals

Male Mus *musculus var. albino* mice weighing 22–25 g (6–8 weeks old), were provided by Nha Trang Pasteur Institute (Nha trang, Vietnam). The animals were divided into different groups and fed for one week in standard conditions (70%–80% relative humidity and 12 h photoperiod) for acclimatizing to experimentation condition.

Phytochemical Analysis

Preliminary phytochemical investigation. Identification of phytochemical groups in the sample was carried out using the process described by Ciulei [3] and modified by the Department of Pharmacognosy, Faculty of Pharmacy, University of Medicine and Pharmacy Ho Chi Minh City [4].

Test for carbohydrates (Fehling's test).

An equal volume of Fehling A and Fehling B reagents were mixed together, and 2 ml of it was added to extract and gently boiled. A brick red precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

Iodine test.

The extract was mixed with 2 ml of iodine solution. A dark blue or purple colouration indicated the presence of the carbohydrate.

Test for phenols and tannins.

The extract was mixed with 2 ml of $FeCl_3$ 2%. A blue-green or black colouration indicated the presence of phenols and tannins.

Test for flavonoids.

Shinoda test.

The extract was mixed with few fragments of magnesium ribbon and concentrated HCl was added dropwise. Pink scarlet colour appeared after few minutes which indicated the presence of flavonoids.

Alkaline reagent test.

The extract was mixed with 2 ml of 2% solution of NaOH. An intense yellow colour was formed which turned colourless on the addition of few drops of diluted acid which indicated the presence of flavonoids.

Test for saponins.

The extract was mixed with 5 ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication of the presence of saponins.

Test for steroid.

The extract was mixed with 2 ml of chloroform and concentrated H_2SO_4 was added sidewise. A red colour produced in the lower chloroform layer indicated the presence of steroids.

Another test was performed by mixing the crude extract with 2 ml of chloroform. Then 2 ml of each of concentrated H_2SO_4 and acetic acid was poured into the mixture. The development of a greenish colouration indicated the presence of steroids.

Test for terpenoids.

The extract was dissolved in 2 ml of chloroform and evaporated to dryness. Then 2 ml of concentrated H_2SO_4 was added and heated for about 2 min. A grayish colour indicated the presence of terpenoids.

Test for alkaloids.

The extract was mixed with 2 ml of 1% HCl and heated gently. Mayer's and Wagner's reagents were then added to the mixture. The turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids.

Test for anthraquinones.

The extract was mixed with 20 ml of chloroform and heated gently for 5 min. The extract was filtered

while hot and allowed to cool. To the filtrate was added with an equal volume of 10% ammonia solution. This was shaken and the upper aqueous layer was observed for bright pink colouration as indicative of the presence of anthraquinones.

Quantification of total phenolic content. The total phenolic content of the ethanolic extract leaves from O. indicum was determined spectrophotometrically using the Folin-Ciocalteu colorimetric method [5].

Quantification of total flavonoid content. The total phenolic content was determined using the method proposed by Park *et al.* [6].

Quantification of tannin content. Tannin content was determined according to the method described by Sun *et al.* [7].

HPLC – MS Conditions

The high-performance liquid chromatography (HPLC) analysis of the extract of *O.indicum* leaves. Chromatographic separation was carried out on a C18 analytical column (ACE 3 C18, 4.6 × 150 mm, 3.5 μm) supplied by Weber Consulting Ltd., Hungary. The mobile phase consisted of water–formic acid (A; 100:0.1, v/v) –methanol- formic acid (B; 100:0.1, v/v). A: B was as follows: 0 min; 90:10; 35 min, 0:100; 60 min, 0:100 the flow-rate was 0.5 ml/min. The column temperature maintained at 40°C. The MS conditions were as follows: Source type: ESI; positive ion negative; collision cell RF: 250.0 Vpp; drying gas: 9.0 l/min; drying heater: 200C; set divert valve: source; pressure of nebulizer: 1.2 bar; set capillary: 4500 V; set end Plate Offset: –500 V; scan begin: 50 m/z; scan end: 2500 m/z; smart parameter setting: not active. Data acquisition was performed using a Chemstation software (Agilent Corporation, MA, USA).

Antihyperglydemic Activity

Albino mice of either sex were randomly divided into five groups of six animals each. Group I: served as control, received distilled water. Group II: hyperglycemic mice received standard drug glibenclamide, 10 $mg.kg^{-1}$ weight body. Group II, IV and V: hyperglycemic mice were treated with leaf 90% ethanolic extract of *O. indicum* at doses of 50 mg/kg, 70 mg/kg and 100 mg/kg body weight, respectively. All of the treatments were given orally. After one hour, all mice were orally treated with $2g.kg^{-1}$ of glucose. The blood samples were collected 2 h after glucose administration. Serum was separated, and blood glucose levels were measured immediately by glucose oxidase method [8].

Statistical Analysis

Results were expressed as mean ± standard deviations. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Student's t-test using statistical software Stagtgraphics Centurion., a p-value less than 0.05 was considered to be statistically significant when compared to control.

RESULTS AND DISCUSSION

Phytochemical Analysis

The identification of phytochemical groups in the plant sample revealed that *O. indicum* leaves contained carotenoid, essential oil, triterpenoids in free and glycosid forms, tannins, polyphenols, flavonoids, and anthraquinones.

The qualitative phytochemical estimation indicated that *O. indicum* leaf extract contained a significant amount of phenolic, flavonoid and tannin content which confirmed its antioxidant property. Phenolic contents are very essential plant constituents because they can act as reducing agents, hydrogen donors, and a metal chelator. They also act as radical scavenger due to their hydroxyl groups. Flavonoids show their antioxidant action through scavenging or chelating process. The phytochemical analysis showed that ethanolic extract of *O. indicum* possessed the high total phenolic content — 77.81 ± 0.04 mg gallic acid equivalent/g dry weight, flavonoid content — 49.74 ± 0.06 mg rutin equivalent/g and tannin — 21.15 ± 0.01 mg catechin equivalent/g. The results acquired in this study thus suggested that the identified phytochemical compounds might be the bioactive constituents and this plant was proving to be a valuable reservoir of bioactive compounds of substantial medicinal merit.

HPLC — MS Conditions

The identity compounds flavonoids enriched in the extracts of *O. indicum* investigated by HPLC-ESI/MS. Representative HPLC–MS chromatograms of the leaves of *O. indicum* presented in the chromatogram (Figure 1). HPLC chromatograms of extracts from representative samples showed main flavonoid group of the compound according to their retention time and mass spectrum.

Table 1. Chromatographic and spectrometric data compounds found in the leaves of *O. indicum*.

Peak	R_t (min)	Formula	MS theory (m/z)	MS applied (m/z)	Identification
1	11.20	$C_{16}H_{12}O_5$	285.075	285.075	5,7 dihydro- 3 methoxyflanone
2	14.80	$C_{15}H_{12}O_5$	271.060	271.068	Pinobanksin
3	17.40	$C_{15}H_{10}O_5$	269.044	269.051	Baicalein
4	15.50	–	–	257.086	–
5	18.90	$C_{15}H_{10}O_4$	253.056	253.051	Chrysin
6	24.10	–	–	293.216	–
7	32.60	$C_{30}H_{48}O_3$	455.351	455.352	Betulinic acid
8	35.20	–	–	279.237	–

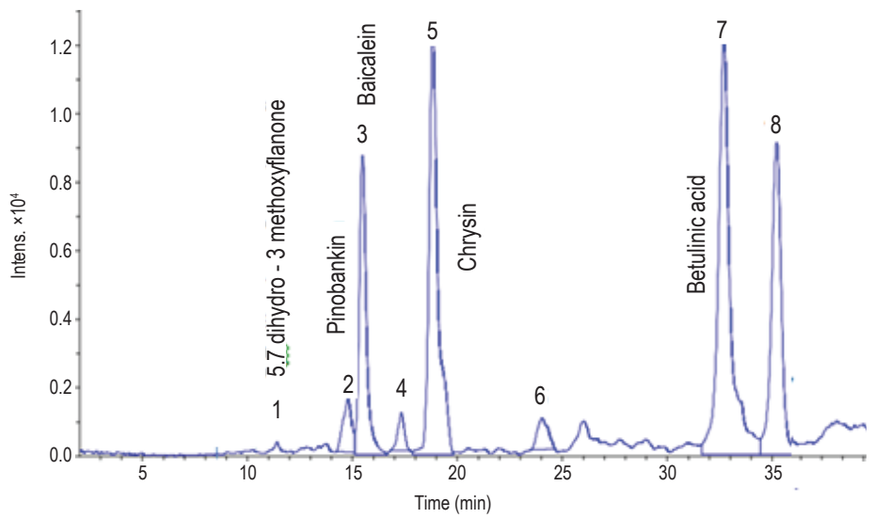

Figure 1. HPLC-ESI/MS chromatograms of ethanolic extract from leaves *O. indicum*.

The identified compounds in the samples are shown in Table 1. Additionally, most of the compounds peaks observed in the fingerprint of the *O. indicum* leaves extract were identical with the compound found in the study from their seed and body plants. These compounds were found in Rt between 11.2 and 35.2 min, include 5,7 dihydro- 3 methoxyflanone [9], pinobanksin [10, 11], baicalein [11,12] , chrysin [11–13], and betulinic acid [14]. The peak 5 showed at Rt of 18.9 min with $[M-H]^-$ ion at m/z 253.05 agrees with that chrysin was found in the samples. The peak 7 showed at Rt of 32.7 min with [M-H]- ion at m/z 456.35 agrees with that betulinic acid was found in the samples. The presence in *O. indicum* of acid betulinic was not previously reported. In addition, the peak 7 and peak 5 in the result of the study was the major compounds in the samples known. The peaks at Rt of 15.50 min, 24.10 min, and 35.20 min were found in the samples, but data MS of peaks were unknown. The groups of constituents eluted at Rt of 15.50 min (peak 4), 24.10 min (peak 6) and 35.20 min (peak 8) were identified unknown. These compounds are currently under investigation in our laboratory.

The mass spectrum was compatible with that of data using the software. Their information compounds were established based on extensive spectroscopic MS data analysis and by the comparison with spectroscopic data reported in the literature. The structural of compounds flavonoids from leaves of *O.indicum* are currently being in our continuing studies on the quantitative analysis of flavonoids by HPLC with the purpose of assessing the chemical quality of phytomedicines.

Antihyperglydemic Activity

Some flavonoids have hypoglycemic properties because they improve altered glucose and oxidative metabolisms of diabetic states [15]. Hypoglycemic activity of leaf extract of *O. indicum* was studied in hyperglycemic mice for comparison level compatible with previous reports. The results obtained from this study indicated that the ethanolic extract of *O. indicum* leaves lowered serum glucose levels significantly when compared to the control group at nearly all doses examined in a dose-dependent manner. The only exception, where there was not a significant reduction of serum glucose levels compared to control animals, was in the case at a dose of 50 mg/kg body weight. The anti-hyperglycemic activity was informed at a dose of 100 mg/kg than the other two doses (62.68% inhibition), while the standard drug glibenclamide produced 65.29% inhibitory activity at 10 mg/kg dose (Table 2). The result of the group received standard drug and the group which received extract with at dose of 100 mg/kg did not undergo different statistical analysis under the experimental conditions of the present study ($p < 0.05$).

Table 2. Effects of ethanolic extract of *O.indicum* leaves on serum.

Treatment	Dose	Serum glucose (mmol/l)	**%** Inhibition
Group I (Glucose)	2 g/kg	14.55 ± 2.34^{a}	–
Group II (Glibenclamide)	10 mg/kg	5.05 ± 1.13^{c}	65.29
Group III (*O. indicum*)	50 mg/kg	12.5 ± 0.46^{ab}	14.08
Group IV (*O. indicum*)	75 mg/kg	10.68 ± 0.56^{b}	26.60
Group V (*O. indicum*)	100 mg/kg	5.43 ± 0.62^{c}	62.68

Data were expressed as mean ± S.D., n = 6. In each column different letters mean significant differences ($p < 0.05$).

A comparative analysis of the results obtained indicated that the ethanolic extract of *O. indicum* leaves at doses of 100 mg/kg had the highest antihyperglycemic effect, followed by doses of 75 mg/kg, 50 mg/kg body weight ($p>0.05$). It was noted that leaves of *O. indicum* contained antihyperglycemic flavonoids compounds, which could account for the antihyperglycemic effects obtained with the leaves of this plant in the present study and antihyperglycemic constituents were yet to be reported in the leaves of the plants studied. Nevertheless, the results indicated the presence of such constituents. Reduction of serum glucose levels by a plant extract can stem from several factors. The extract may influence in a positive manner the pancreatic secretion of insulin, or the extract may increase the glucose uptake [16,17]. The extract possibly may inhibit glucose absorption in gut, thus reducing the presence of glucose in serum [18]. Flavonoids represent another beneficial group of naturally occurring compounds with hypoglycemic potentials. These are widely distributed in the plant kingdom and exhibit distinctive pharmacological properties. The flavonoids can be widely classified into different categories such as flavanols, flavones, catechins, and flavanones. Some flavonoids have hypoglycemic properties because they improve altered glucose and oxidative metabolisms of diabetic states [15]. The exact mechanisms through which the extracts lowered the serum glucose levels in hyperglycemic mice in the present study, as well as the identification of phytochemical constituents responsible for the anti-hyperglycemic effects, is currently under investigation in our laboratory.

CONCLUSIONS

The results from this study have confirmed the hypoglycemic activities of ethanolic extracts of O. indicum that supported the folkloric uses of the medicinal herbs in the control or management of diabetes mellitus. This promising result encourages further investigations including bioassay of the fractionated extract might lead to the isolation of compounds that were responsible for the hypoglycemic effects and which could be further developed to modern anti-diabetic drugs.

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