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Article

Investigation on the *in vitro* antioxidant capacity of methanol extract, fractions and flavones from *Oroxylum indicum* Linn bark

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Antioxidants from natural sources hold high values regarding their indispensible roles in the development of nutraceuticals, pharmaceuticals and cosmetic products. *Oroxylum indicum* L. is a common medicinal plant with a wide range of therapeutic properties, including a notable antioxidant potency that was reported, yet has not been subjected to more detailed studies. The present study evaluated the potency of *Oroxylum indicum* methanol stem bark extract, along with its hexane, ethyl acetate, methanol fractions, three flavones including baicalein, oroxylin A and chrysin using DPPH assay. In terms of IC_{50} values, the crude extract (65,48 µg/mL) exhibited moderate inhibitory activity which was as half potent as that of its ethyl acetate fraction (32,94 µg/mL). This fraction was also superior to the methanol and hexane fractions, as their IC_{50} were 57,19 and 137,95 µg/mL respectively. Remarkably, a yellow powdery sub-fraction consisted of isolated compounds showed powerful activity (32,89 µg/mL) compared to those of its components, revealing the intriguing effect of synergism while giving evidence for the theory of structure-activity relationship between some flavones and their antioxidant capability. Perpetual search for new radical scavenging agents in *Oroxylum indicum* is emboldened considering its partially exploited potential in this study.

Keywords: *Oroxylum indicum*. Flavones. Methanol/extract. DPPH assay. Antioxidants. Inhibitory Concentration 50. Bignoniaceae.

INTRODUCTION

Oxidation is a critical metabolic process thanks to which a body can perform living functions properly. The oxidative metabolism however produces reactive oxygen species (ROS) that in case of highly accumulated, would create oxidative stress that damages cell structure, contributing to pathogenesis of most inflammatory diseases, cardiovascular diseases, neurodegenerative diseases and cancers by attacking biologically relevant molecules (Hensley *et al.*, 2000). Although a body has its own systems to balance the ratio between oxidants and antioxidants, they can get overwhelmed sometimes like every other machinery. Therefore, the supplement of antioxidants is undoubtedly of great importance, as searches for agents with free radicals scavenging activity have been carried out continually. (Brand-Williams, Cuvelier, Berset, 1995; Koleva *et al.*, 2002)

Oroxylum indicum L. (*Bignoniceae*) is a commonly known medicinal plant that distributes throughout the territory of Vietnam, particularly in the Southern and the Central Highlands of Vietnam. Each part of the plant can be utilized as remedies for various diseases. The root bark is an astringent and bitter tonic that can alleviate bronchitis, diarrhea, diaphoretism and rheumatism (Khandhar *et al.*, 2008; Zaveri, Jain, 2009). *O. indicum* seeds are used in treating stomach disorders, pneumonia, and wounds (Dev, Anurag, Rajiv, 2010; Joshi, Alok, Tapan, 2014). The bark can reduce severity of diarrhea, dysentery, coilitis and skin conditions such as ulcer and eczema (Khandhar *et al.*, 2008; Vikrant, Arya 2011). Fruits of *O. indicum* are useful for treating haemarrhoids, cholera and leukoderma (Joshi, Alok, Tapan, 2014). A large number of pharmacological reports on *O. indicum* L. have been conducted. The root bark was shown to have various bioactivities such as antimicrobial, antiulcer, immunomodulatory, antihyperlipidemic, antimicrobial, cytotoxic, and apoptotic effects. Other parts of the plant were also proven to have valuable bioactivities, including the leaves with hepatoprotective effect, and fruits with

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antimutagenic effect (Dev, Anurag, Rajiv, 2010; Bisht *et al.*, 2011). Such a wide range of bioactivities may be attributed to *O. indicum*'s phytochemical composition with a high amount of tannins, glycosides, phenolics, phenolic acids, and flavonoids. The leaves of *O. indicum* were reported to contain four flavonoids: chrysin, hispidulin, baicalein and oroxylin A by Hương *et al.* (2013), and quercetin-3-o-α- L-arabinopyranoside, 1-(2-hydroxyethyl) cyclohexane-1, 4-diol and apigenin by Yuan *et al.* (2008). From the seeds of the plants, chrysin, baicalein, baicalein-7-*O*-glucoside (Oroxin A) and baicalein-7-*O*-diglucoside (Oroxylin B) were isolated (Chen, Games, Jones, 2003). Ellagic acid and biochanin-A were also found in the root bark of *O. indicum* (Zaveri, Khandhar, Jain, 2008). The fruits were reported to contain ursolic acid and aloe-emodin besides its main phytocomponents (Yuan *et al.*, 2008). Thanks to this variety of bioactive compounds in the plant composition, *O. indicum* is highly appreciated as a valuable herbal medicine in Vietnam.

Plant is the primary source of antioxidants that are affordable and easily accessible especially in Vietnam, a country endowed with extremely rich flora abundance. Containing high content of phenolics and flavonoids, *O. indicum* therefore has high potential as a strong antioxidant agent. The screening for antioxidant activities of the plant extract from different parts using various solvents is, however, still preliminary and needs to be furthered. It is definitely crucial to conduct this study, considering our long-term national developing schemes with serious focuses on the fostering and conservation of phytomedicines in particular regions, such as the Mekong Delta or the Central Highlands of Vietnam. Indeed, the reevaluation on this specific plant's bioactivity is indispensable for numerous sustainable forest and agricultural management programs in this mission as it certainly will add highlights to the true values and potentials of this medicinal plant. The present study, accordingly, aims to extend the knowledge on the antioxidant activity of *O. indicum* bark by investigating the total methanolic extract, its hexane, ethyl acetate and methanol fractions, 3 pure compounds isolated from the extract and the combination of these by DPPH free radical scavenging assay.

MATERIAL AND METHODS

Chemicals and reagents

Methanol, ethanol, hexane, ethyl acetate, and chloroform were purchased from Fischer Scientific. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) and ascorbic acid for DPPH assay were supplied by from Sigma-Aldrich. HPLC grade methanol, dimethyl sulfoxide (DMSO) and silica gel F_{254} aluminium sheets for TLC was obtained from Merck. Silica gel KG 60 (0.040-0.063 mm) (Scharlau) was used for column chromatography.

Collection and preparation of plant materials

O. indicum stem bark was obtained in Dak Lak, the high mountain central of Vietnam, 2016, and immediately washed with tap water to eliminate possible contaminants. The plant was authenticated by Associate Professor Tran Van Minh of the Institute of Tropical Biology, Vietnam and a voucher specimen (No. HBBIO-06-08-1) has been deposited in the herbarium of Applied Biochemistry Laboratory, Department of Applied Biochemistry, School of Biotechnology, International University, Vietnam National University – Ho Chi Minh City, Vietnam. The preparation of plant extracts, isolation of compounds and assessment of DPPH scavenging activity were carried out in the Institute of Chemical Technology – Vietnam Academy of Science and Technology, Ho Chi Minh City, Vietnam. The bark was dried in the oven at 65°C for 3 days and finely ground before being exhaustively macerated with 10 liters of methanol. This crude extract (CE) was subjected to filtration using Whatmann filter paper No. 42 (125 mm) and concentrated using rotary evaporator.

Partition of the total extract

The crude extract (165 g) was later successively partitioned with 5 L of n-hexane which was added in small portions. Within each time, the mixture was vigorously agitated for 5 minutes using a sonicator. The hexane layer was collected and condensed as hexane fraction (HF). In a similar manner, the partition of the remaining extract with 10 L of ethyl acetate was carried out exhaustively. The extract collected in this step was identified as ethyl acetate fraction (EAF). The remained was identified as methanol fraction (MF). All extracts were concentrated under high pressure and stored at 4 °C for further separation and analysis.

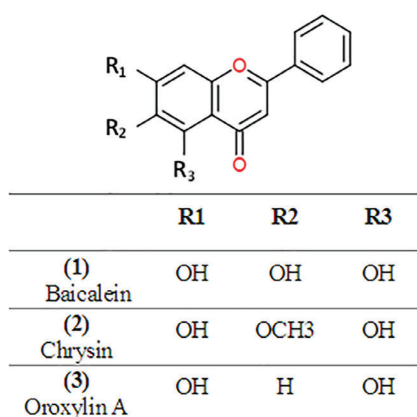
Fractionation of EA extract

For the fractionation, EAF was chromatographed on a glass column (d = 7 cm, l = 45 cm) packed with 370 g silica gel KG 60 (0.040-0.063 mm) (Scharlau). An amount of 20 g of EAF was mixed with silica gel (mass ratio 1:1) and applied into the prepared column that was later eluted by a gradient solvent system including hexane, ethyl acetate and methanol. The collected fractions were examined by TLC so that main components of each were

exposed. After the elution with hexane-ethyl acetate (3:1 and 1:1), a yellow powder, namely EAF-sub, was collected and chosen to be subjected to further separation as its chromatogram revealed only 4 visible bands at relatively different Rf when developed in a saturated TLC chamber containing chloroform.

Isolation and identification of compounds (1), (2) and (3)

EAF-s was dissolved completely in methanol, injected to a semi-prep HP 1050 HPLC system and separated using an Agilent ZORBAX Eclipse XDB-C18 column (21.2 × 150 mm, particle size 5 μm). The solvent system consisted of methanol: water (68:32) was pumped through the column at a flow rate of 8.00 ml/min, while detection wavelength was set to 245 nm. Compound **(1)** was from EAF-s fraction after the process, leaving another sub-fraction as the mixture of compound **(2)** and **(3)**. MPLC was used in the next step for isolation of remained compounds. Specifically, compound **(2)** and **(3)** were subjected to a glass column (d = 2 cm; l = 50 cm) packed with 80g silica gel KG 60 (0.040-0.063 mm) with chloroform (100%) as the eluent at a flow rate of 2.00 mL/minute. All 3 compounds **(1)**, **(2)**, and **(3)** (Figure 1) were identified from ^{13}C- and/or ^{1}H- NMR spectra recorded on a NMR Bruker Avance II spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C), with dimethyl sulfoxide as internal standard, in reference with published literature (Yan *et al.*, 2011; Mouffok *et al.*, 2012; Hương *et al.*, 2013).



	R1	R2	R3
(1) Baicalein	OH	OH	OH
(2) Chrysin	OH	OCH3	OH
(3) Oroxylin A	OH	H	OH

FIGURE 1 - Chemical structures of isolated compounds in the study.

Spectral data

Baicalein (1): brownish yellow powder; M.P. 256 °C; ^{1}H NMR (500 MHz, DMSO) δ 12.64 (s, 1H), 10.63 – 9.23 (m, 1H), 8.06 (dd, J = 8.1, 1.4 Hz, 2H), 7.64 – 7.46 (m, 3H), 6.92 (s, 1H), 6.63 (s, 1H).

Oroxylin A (2): light yellow powder; M.P. 202 °C; ^{1}H NMR (500 MHz, DMSO) δ 12.78 (s, 1H), 8.04 (dd, J = 8.0, 1.3 Hz, 2H), 7.78 – 7.45 (m, 3H), 6.87 (s, 1H), 6.41 (d, J = 1.6 Hz, 1H), 6.11 (d, J = 1.7 Hz, 1H), 3.70 (s, 1H), 3.17 (s, 1H); ^{13}C NMR (126 MHz, DMSO) δ 181.87, 162.83, 152.73, 152.50, 131.85, 130.85, 129.10, 126.29, 104.57, 94.62, 59.77.

Chrysin (3): yellow powder; M.P. 284 °C; ^{1}H NMR (500 MHz, DMSO) δ 12.89 (s, 1H), 8.05 (dd, J = 8.1, 1.4 Hz, 2H), 7.69 – 7.46 (m, 3H), 6.91 (s, 1H), 6.56 (s, 1H), 3.79 – 2.55 (m, 18H), 2.50 (tt, J = 12.0, 6.0 Hz, 8H), 1.99 (s, 1H), 1.62 – 1.34 (m, 1H), 1.26 (d, J = 24.3 Hz, 6H), 1.16 (dd, J = 24.0, 6.7 Hz, 1H), 0.97 – 0.58 (m, 2H); ^{13}C NMR (126 MHz, DMSO) δ 181.25, 167.68, 162.52, 161.33, 157.66, 131.75, 130.94, 129.09, 126.24, 104.94, 102.75, 99.79, 94.54.

In vitro antioxidant activity

In vitro antioxidant activity of isolated compounds was investigated using DPPH free radical scavenging assay with slight modifications (Amin, Norazaidah, Emmy, 2006). Briefly, testing samples of CE, HF, MF, EAF, EAF-sub, compounds **(1), (2)** and **(3)** were prepared in various concentrations 20, 40, 60, 80 and 100 (μg/mL) by dissolution and dilution in DMSO. Blank solution and ascorbic acid were used as negative and positive control, respectively. An aliquot of 50 μL of prepared sample was then loaded into separated well. A volume of 150 μL of ethanolic DPPH was added and mixed well. The mixtures were immediately incubated at room temperature, in the absence of light for 30 minutes before the absorbance at 517 nm were measured. The assay was performed in triplicates. The percentage of inhibition of DPPH free radical was evaluated following the below equation:

$$\% \text{ Inhibition} = (\text{ODc} - \text{ODs}) \times 100\%/\text{ODc}$$

where ODc is the OD value of negative control and ODs is the OD value of testing sample.

IC_{50} value indicating the concentration at which a sample would inhibit free radicals by 50% was also calculated.

Statistical analysis

All results were expressed in terms of Mean

± Standard Error of Mean (SEM). The correlation coefficients between generated data in the determination of antioxidant activity and the concentration of samples were calculated in Graph Pad Prism package 6.

RESULTS AND DISCUSSION

The potent antioxidant activity of *O. indicum* has been recognized through some significant studies. Different parts of the plant were proven to possess scavenging ability of varied potencies in a comparative study by Sannigrahi et al. (2010), in which the bark extract exhibited its superior antioxidant activity. As the search for new antioxidants has never ceased, more research subjecting *O. indicum* ethyl acetate bark extract needs to be conducted for the sake of novel and effective fractions or compounds. In this study, we aimed to confirm and report new insights into the pharmacological activities of *O. indicum* methanol bark extract and its components, particularly antioxidant effect by performing DPPH assay.

The inhibitory activity of all samples was investigated at various concentrations (Figure 2). The crude extract of the plant (CE) has exhibited a quite remarkable activity at 100 µg/mL (63.16%). Out of the three fractions derived from this crude extract, ethyl acetate fraction (EAF) had the greatest abundance of antioxidants compared to hexane and methanol ones (HF and MF) and thus, further separation was carried out on this fraction. EAF was submitted to a silicagel column chromatography and yielded a sub-fraction that was a yellow, powdery solid in large quantity, namely EAF-sub. From this sub-fraction, three pure compounds isolated and identified based on their physical characteristics (Figure 1), TLC chromatographs and NMR spectra in reference to published literature. The compounds were then found to be baicalein, chrysin and oroxylin A. However, the radical inhibitory activity of chrysin was undetectable at all examined concentrations, when that of oroxylin A, detectable only at 20 and 80 µg/mL, indicated a very insignificant level of activity compared to the control and baicalein. The inhibition percentage of baicalein, as found to be 45.64 ± 1.31, was comparatively lower than that of standard ascorbic acid (89.70 ± 0.17) at the concentration of 100 µg/mL. The EAF-sub sample with the presence of all three compounds, nonetheless, showed surprisingly strong activity (82.63 ± 0.16) that reached almost as high as ascorbic acid's.

The antioxidant property of various extracts, fractions and constituents of *O. indicum* bark was as well presented by their IC_{50} values (Figure 3). The value of crude extract was lower than the one reported by Sannigrahi et al. (2010) (65.48 µg/mL compared to 149.59 µg/mL), regarding the same employed plant parts and solvents of extraction. Out of the three fractions derived from CE, EAF fraction showed the strongest antioxidant activity with its significantly smaller IC50 values in comparison with HF and MF, being 32.94 µg/mL, 137.95 µg/mL and 57.19 µg/mL, respectively. The sub-fraction EAF-sub exhibited a quite as low IC50 as EAF (32.89 µg/mL to 32.94 µg/mL) and showed a surprisingly superior scavenging capability to those of its components, including baicalein, chrysin and oroxylin A. These naturally occurring flavones, often introduced as remarkable neuroenhancing and neuroprotecting substances (Cheng *et al.*, 2008; Lee *et al.*, 2010; Nabavi *et al.*, 2015), did not exerted significant antioxidant activity singly in the present study and thus, should be utilized

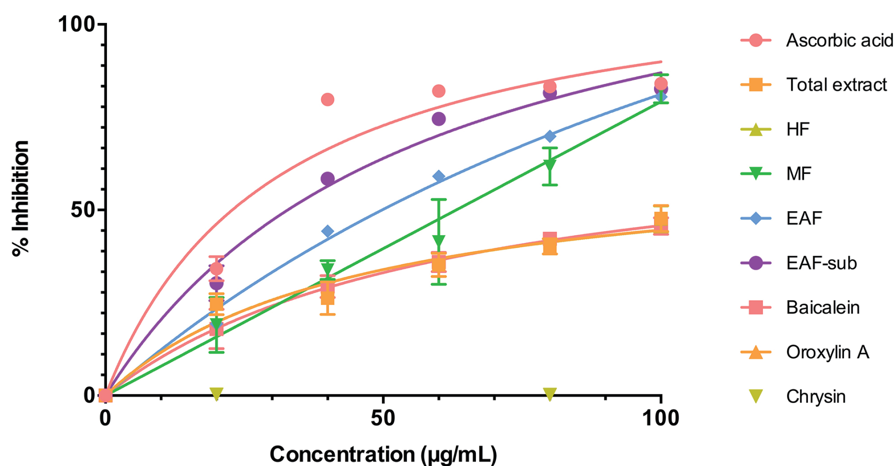


FIGURE 2 - DPPH free radical inhibiting activity (%) at various concentration of the total extract, hexane (HF), methanol (MF) and ethyl acetate (EAF) fractions, sub-fraction of ethyl acetate fraction (EAF-sub), baicalein, oroxylin A, chrysin and ascorbic acid. - *not determined*. Each point represents the Mean ± SEM.

when combined as a mixture. This also encourages the search for novel antioxidants with outstanding potency in other components of *O. indicum*, especially the ethyl acetate and methanol fractions, as their identities might have not yet been unveiled in the present study.

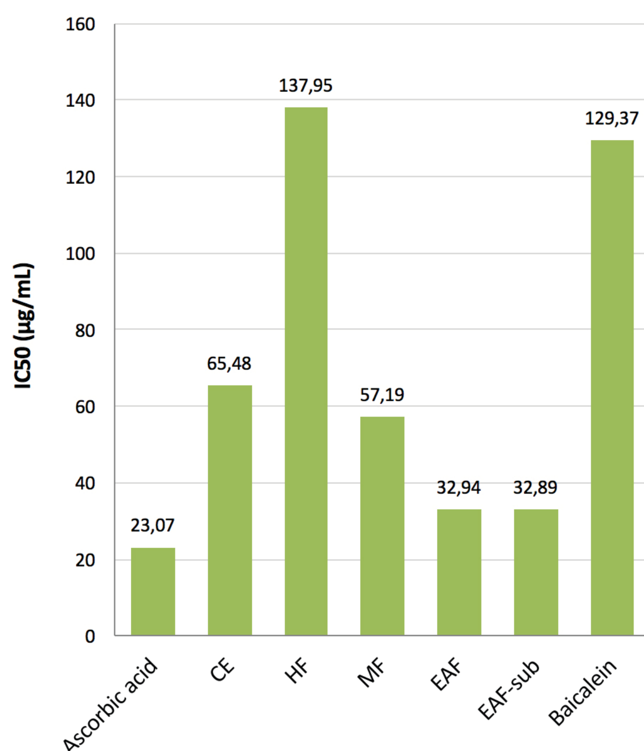


FIGURE 3 - DPPH IC50 (µg/ml) of the crude extract, hexane, methanol and ethyl acetate fractions, subfraction of ethyl acetate fraction, compound (1) and ascorbic acid.

The structure-activity relationship of antioxidants fell into the category of flavonoids has been in discussion for long as a tool of revelation and explanation for the capability of a substance (Rice-Evans *et al.*, 1996). While all isolated compounds in the present study share similar features as being flavones, they are only different in the functional groups attached on 6-C, illustrating how an A-ring substitution can impact an antioxidant's potency. The superior effect of baicalein to the other two compounds might be ascribed to its 6-OH group when methoxy group at this same position might have diminished the effect of oroxylin A (Pietta, 2000; Heim, Tagliaferro, Bobilya, 2002), possibly due to an increase in hydrophobicity of the molecule as a result of *O*-methylation (Heim, Tagliaferro, Bobilya, 2002). Indeed, more studies focusing on the conformation of investigated compounds in relation with their pharmacological effects using different assays would be helpful to reach a valid conclusion.

A central metabolic process enabling the living and all functionalities of every organism as it is, oxidation unfavorably increases the amount of ROS in a condition termed "oxidative stress", and is responsible for the deterioration of many biomolecules. So as to protect the components of cells made up of proteins, lipids and nucleic acids, antioxidants come into play by forming several lines of defense against ROS and restore the balance disturbed by the stress (Hensley *et al.*, 2000; Irshad, Chaudhuri, 2002). Many methods for detection and assessment of antioxidant activity are constructed considering the complicated nature of oxidative and antioxidative systems inside the body. The DPPH assay used in this investigation reveals whether a sample possesses scavenging action on active radicals by donating hydrogen atoms to DPPH molecules (Brand-Williams, Cuvelier, Berset, 1995). Because of its cost-saving and simple practice, together with the ability to generate data with high correlate coefficient while constructing standard curve for determination of IC_{50} values (Table I), DPPH assay is widely used for the assessment of drugs and foodstuffs with high antioxidant potential. It is also recommended that investigated extract and components of *O. indicum* in this present study should be tested by various assays with different types of active radicals, both *in vivo* and *in vitro*, so that their mechanisms and targets of action can be elucidated and exploited properly to the fullest.

CONCLUSION

The antioxidant property of *O. indicum* extract, its fractions and isolated flavones has been demonstrated through DPPH assay in our study. Significant differences in the scavenging ability of three fractions derived from the partition with solvents of distintive polarity, while disclosing strong antioxidants in the extract mostly are moderately or highly polar, are evidence for later studies to narrow their research down to the ethyl acetate and methanol fractions when the antioxidant profile of *O. indicum* is brought into analysis. Comparably

TABLE I - Correlation coefficient (R^2) of DPPH assay

Ascorbic acid	CE	HF	MF	EAF	EAF-sub	Compound (1)
0.9872	0.9992	0.8626	0.9735	0.9985	0.9980	0.9980

higher activity of a subfraction to those of its flavones components individually – a telltale of synergetism in free radical scavenging, and minor structural differences between such flavones that suggest a closer look to the structure-activity relationship both pave the path for more extensive research on plant-derived antioxidants.

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AUTHORS' CONTRIBUTION

This work was carried out in collaboration between all authors. Author DHTT performed the experiment and wrote the manuscript. Authors PVT designed the study and supervised experimental process. Author HLS contributed to the conceptualization and supervision of the study, and finally to the revision of the manuscript. All authors have read and approved the final manuscript.

CONSENT

Not applicable.

COMPETING INTERESTS

Not applicable.

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