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ANTIOXIDANT, ANTI-HYPERGLYCEMIC AND ANTI-INFLAMMATORY EFFECTS OF *VIBURNUM LUTESCENS* BLUME

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Summary

Antioxidant, Anti-Hyperglycemic and Anti-Inflammatory Effects of *Viburnum lutescens* Blume

Viburnum lutescens Blume, a Caprifoliaceae family member epidemic to Vietnam is a scarce medication used in traditional medicine to treat ailments such as rheumatism, injuries, measles, and ovarian obstruction. However, there are not many research papers on the properties and chemical components of this plant. Hence, our study is the first to assess the pharmacological capabilities of *Viburnum lutescens* including: anti-inflammation, anti-hyperglycemia and antioxidant that could be used to prevent some chronic illnesses. In our study, 3 extracts of this plants including methanol (MeOH), ethylacetate (EtOAc), and dichloromethane (CH_2Cl_2) were evaluated. The results show that the EtOAc extract performed distinguished properties. We also found that 6α-hydroxybetulinic (VL2), a triterpene acid isolated from *V. lutescens* exhibited a high anti-inflammatory activity against the inhibition of NO production in LPS-activated RAW264.7 cells with IC_{50} value of 56.09 ± 4.76 μM.

Keywords: *Viburnum lutescens, Bioactivity, Antioxidant, Antihyperglycemic, Anti-inflammatory.*

1. Introduction

Inflammation is a pivotal physiological response that aids in the defense of the body against dangerous external agents such as physical or chemical agents, pathogens, and viruses. This kind of response is classified into two main stages: acute and chronic inflammation [1]. The visible manifestations of inflammation are redness, heat, swelling and pain. These symptoms result from the dilation of blood vessels that increases blood flow to the damaged site as well as the massive recruitment of immune cells [1]. Nitric oxide (NO) is an omnipresent signaling molecule in the body and is synthesized by a wide range of NO synthase (NOS) from endothelial cells, and neurons through the breakdown of arginine to citrulline and NO [2],[3]. Moreover, NO is considered an anti-inflammatory agent in the normal condition. On the other hand, when its production is impaired, NO acts as a pro-inflammatory mediator that triggers inflammatory [4].

Many diseases were proved as the repercussion of inflammation including diabetic complications. Type 2 diabetes mellitus (T2DM) is the most common endocrine disease in the world and in Vietnam. T2DM is associated with low levels of peripheral glucose uptake and increased endogenous glucose production as the result of insulin resistance, which leads to numerous complications [5].

In recent time, there is a huge effort to find the active compounds that relent the pathogenesis of inflammation and diabetes mellitus. According to a research, the majority of world's population employs traditional medicine, in another word, herbal extracts to treat acute or chronic ailments [5],[6]. Vietnam is a tropical country with many untapped medicinal resources. *Viburnum lutescens* (Vót vàng nhạt or Giáng cua), a Caprifoliaceae family member endemic to Vietnam has been traditionally used to treat rheumatism, injuries, measles, and ovarian obstruction [7]. Based on our knowledge, there is no study about the anti-inflammatory and anti-hyperglycemic activities of this plant. Hence, this work aims to provide a general view of these properties via investigating antioxidant, anti-hyperglycemic assessment, and anti-inflammatory effects of this plant.

2. Materials and methods

2.1. Sample collection and preparation

Viburnum lutescens Blume leaves were collected from Me Linh Station for diversity, Vinh Phuc, Vietnam in May, 2019, and identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, VAST. A voucher specimen (ML12) was deposited at the University of Science and Technology of Hanoi, VAST. The extraction and isolation was described in our previous study [7]. Six compounds were isolated and identified as ursolic acid (VL1)[8]; 6α-hydroxybetulinic acid (VL2) [7]; (1S,3S,9R)-megastigman-5-en-3,9,11-triol 11-*O*-*β*-D-glucopyranoside (VL3); dihydrovomifoliol-*O*-*β*-D-glucopyranoside (VL4) [9]; (7S,8S)-dihydrodehydrodiconiferyl alcohol 9-*O*-*β*-D-glucopyranoside (VL5)[10]; (7S,8S)-dihydrodehydrodiconiferyl alcohol 9′-*O*-*β*-D-glucopyranoside (VL6) [10] (Fig.2).

Fig. 1. *Viburnum lutescens* Blume collected in Melinh, Vinh Phuc

2.2. Antioxidant activity evaluation

DPPH radical scavenging assay

This colorimetric reaction was carried out in accordance with the previous study's protocol with slight modifications [11]. DPPH working solution (0.1 mM) was prepared by dissolving 2,2-diphenyl-1-picrylhydrazyl (DPPH) powder (Sigma-Aldrich, USA) in methanol (MeOH). This working solution was left to stand in the dark for 24 h before being used. Briefly, 30 µL of ascorbic acid or plant extracts was incubated with 570 µL methanolic DPPH working solution. The same volume of MeOH was used as a blank. The react mixtures were left for 20 minutes at room temperature. Then, they were measured their absorbance at 517 nm by the microplate reader SpectraMax® iD5 (Molecular Devices, USA). The experiment was triplicated.

ABTS+ radical scavenging assay

This assay followed the previous study with minor modifications [11]. The ABTS stock was prepared by mixing 7.0 mM aqueous ABTS (Sigma-Aldrich, USA) with 2.45 mM aqueous potassium persulfate $K_2S_2O_8$ (Scharlau, Spain) in ratio 1:1. The stock solution was kept out of light for 24h for complete reaction. The ABTS working solution was prepared by diluting stock with water until the absorbance reached about 0.7. Basically, 30 µL of Trolox (National Institute of Drug Quality Control) or plant extracts was incubated with 570 µL ABTS working solution. The same volume of ethanol was used as a blank. Reaction was left for 10 minutes before being read the absorbance by microplate reader SpectraMax® iD5 at the wavelength of 734 nm. The experiment was repeated three times.

2.3. α-Glucosidase inhibitory activity

The experiment was conducted based on a method of Ranilla et *al* [12] with slight modifications. α-glucosidase was dissolved by the phosphate buffer KPP 0.1M, pH 6.8 to the desired concentration of 0.5 U/mL. The substate p-NPG was also formulated in buffer KPP 0.1M, pH 6.8 to the final concentration of 5 mM. Briefly, 100 µL plant extract was diluted into various concentrations, then was incubated with 200 µL α-glucosidase solution (0.5 U/mL). This mixture was left to stand for 10 minutes at room temperature before the substrate p-NPG (5 mM) was added. The reaction took place for 10 minutes at room temperature. To stop the reaction, 100 µL sodium carbonate 0.2 M was supplemented. The whole mixture was centrifuged 14000 rpm for 5 minutes. The absorbance of the supernatant was measured using the microplate reader SpectraMax® iD5 at 405 nm.

2.4. Cell line and cell culture

Murine macrophage RAW 264.7 cell line is kindly provided by Prof. Jeong Hyung Lee, Kangwon National University, Korea. The cells were cultured in DMEM supplemented with 10% FBS, penicillin and streptomycin (100 µg/mL) and were kept in incubator 37°C, 5% CO_2. The medium was changed in 1 - 2 days to maintain the cell.

2.5. Cell viability assay

Cell proliferation and cytotoxic effect of the extracts (25 µg/mL) were assessed using 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) - based assay. MTT solution (0.5 mg/mL) was added to each well and incubated for 3 h. Then, the culture supernatants were removed, and formazan crystals were dissolved in DMSO. Absorbance was measured at 540 nm using a microplate reader (Molecular Devices, USA).

2.6. Nitric oxide (NO) production measurement

The cells were seeded in 96-well plates and treated with a series of concentration of samples. Lipopolysaccharide (LPS) (0.1 µg/mL) was added in each well except the negative control. The quantity of nitrite was measured using Griess reagent (ratio of cell culture medium/Griess reagent is 1:1). After incubation under room temperature, the absorbance was measured at the wavelength of 540 nm. $NaNO_2$ standard curve was employed to calculate the nitrite concentration.

2.7. Statistical analysis

Statistical analysis was performed on GraphPad Prism 8 software. The experiments were repeated three times and the ANOVA one-way program was run to check the significance.

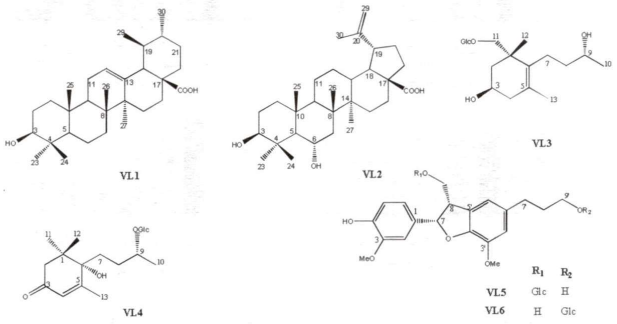

Figure 2. Chemical structure of compounds isolated from *V. lutescens*

3. Results and Discussion

3.1. Antioxidant property assessments

We observed that EtOAc extract had distinguished scavenging potential among three samples. Even at lower concentration of 12.5 µg/mL, it neutralized more than 60% of DPPH free radical (Fig. 3A). According to Fig. 3B, EtOAc extraction neutralized DPPH radical in dose-dependent manner and it has the IC50 of 8.459 ± 0.058 µg/mL, being two times higher than that of the positive control, ascorbic acid (4.567 ± 0.017 µg/mL).

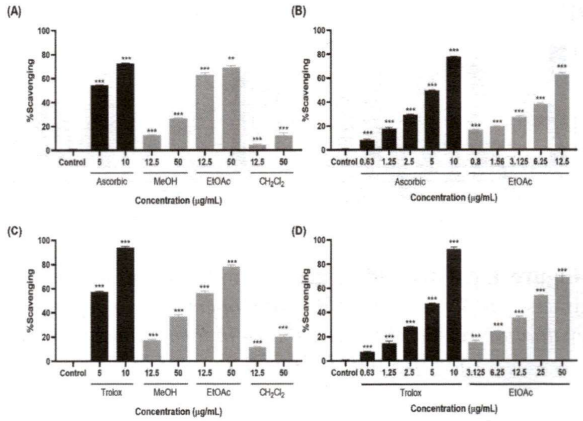

Figure 3. Evaluation of the free radical-neutralizing ability of the extracts. ***(A); (C):*** *Screening result of DPPH and ABTS assay, respectively.* ***(B); (D):*** *IC_{50} value of EtOAc extract on DPPH and ABTS assay, respectively. Ascorbic acid and Trolox was used as a positive control. The data was shown in form of mean ± SD (***$p < 0.001$; **$p < 0.002$; n=3).*

EtOAc extract showed a strong ABTS free radical scavenging capability compared to other extracts since it inhibited approximately 60% and 80% respectively at concentrations of 12.5 µg/mL and 50 µg/mL (Fig. 3C). Thus, EtOAc extract was chosen for the further step. ABTS free radical scavenging capability of EtOAc extract is dose-dependent (Fig. 3D). While the concentration of Trolox to inhibit 50% of ABTS free radical has been recorded to be 4.601 ± 1.21 µg/mL, the concentration of EtOAc extract to achieve the same effect was 21.740 ± 0.13 µg/mL.

The antioxidant property of *Viburnum lutescens* Blume aligned with that of other members of genus *Viburnum*. The genus *Viburnum* has been proven to be rich in essential chemical compositions such as flavonoids and phenols, which also contribute significantly to the free radical scavenging ability [13]. Recently, a new study indicated that flavonol glycoside compounds isolated from the leaves of *V. lutescens* strengthen the antioxidant result of this experiment [14]. Especially, antioxidant compounds such as phenols and flavonoids found in *V. lutescens* can also support reducing inflammation-related diseases by reducing the oxidative stress and reactive oxygen species which are critical factors contributing to the inflammatory process [15].

3.2. α-Glucosidase inhibitory assay

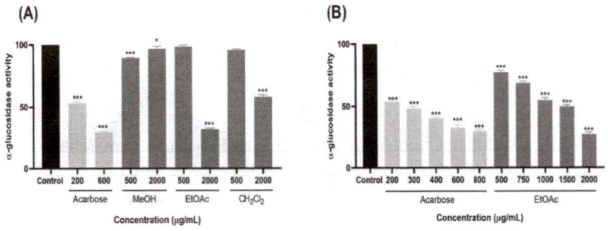

Figure 4. α-Glucosidase activity under the influence of extracts. ***(A)*** *Screening result.* ***(B)*** *Concentration dependent evaluation result. Data was shown in form of mean ± SD (***p <0.001; *p < 0.05; n=3).*

The goal of T2DM treatment is to keep patients' blood sugar levels as close as possible to normal. The hydrolysis of starch, glycogen, and numerous other oligosaccharides by the enzymes glucosidase results in an increase in postprandial blood glucose. The inhibition of α-glucosidase was expected to keep blood sugar levels close to normal [16]. In this study, extracts from *V. lutescens* were studied for the inhibition of this enzyme.

Among three extracts, EtOAc exhibited the most potent inhibitory activity to α-glucosidase. At higher concentration of 2000 μg/mL, α-glucosidase was antagonized more than 50% (Fig. 4A). The calculated IC_{50} value of acarbose was 228.67 ± 3.40 μg/mL, which was consistent with the previous study [17]. EtOAc extract noticeably prevented α-glucosidase from hydrolyzing substrate p-NPG and its IC_{50} value was 1105.15 ± 3.37 μg/mL, fivefold higher than that of acarbose (Fig. 4B).

By our knowledge, there is no specific research about anti hyperglycemic effects of *Viburnum lutescens* However, there are some reports revealed that *Viburnum* species have been used for the purpose of anti-hyperglycemic, for example, *Viburnum foetidum* and *Viburnum dilatatum* are folk medicines which have been proved to reduce the diabetic stages [18],[19].

3.3. Anti-inflammatory activities

Protecting tissues from damage, antioxidants can prevent unwanted inflammatory responses occur. The hallmark of diabetes, hyperglycemia, can also induce inflammatory programming of macrophages. In the next step of this study, the anti-inflammatory activities of extracts from *V. lutescens* also investigated.

The cytotoxicity and NO production inhibitory effects of the extracts from Viburnum lutecens Blume

To select the non-cytotoxic concentration of these samples on RAW 264.7 cells before going to the bioactivity, MTT assay was performed. As shown in Fig. 5A, the plant extracts MeOH, EtOAc, CH_2Cl_2, showed neglected cytotoxic effect at the concentration up to 100 μg/mL compared to LPS making them suitable to test the inhibitory effect on NO production. The same result was obtained with positive control - cardamonin. All extracts reduced the production of NO at the concentrations range from 30 to 100 μg/mL. The EtOAc extract decreased the NO production more than 50% at the concentration of 100 μg/mL (Fig. 5B). Hence, in the next step, the extract EtOAc was used to investigate the anti-inflammatory in different doses.

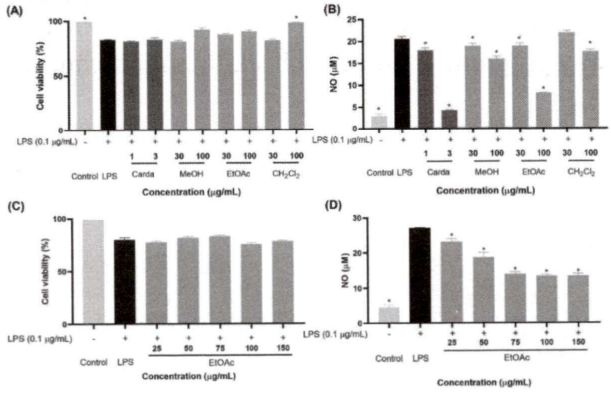

Figure 5. Cytotoxicity of plant extracts in RAW 264.7 cells. ***(A);(C):*** *Cell viability;* ***(B);(D):*** *NO production inhibitory results. The data are expressed as mean ± SD. *p < 0.05, n=3 indicates a significant difference between LPS stimulation value.*

We observed no cytotoxicity at different concentrations of EtOAc extract but the reduction of NO production in dose-dependent manner. IC_{50} value of EtOAc extract was 56.88 ± 1.02 μg/mL (Fig. 5 C and D). The isolates were subsequently investigated for the inhibitory activity of NO production in the LPS-activated RAW 264.7 macrophages. As the result, VL2 significantly reduced NO yield but did not cause cell damage (Table 1).

Table 1. The cytotoxicity and NO production inhibitory effects of the compounds isolated from *Viburnum lutescens* Blume

Compounds	Conc. (μM)	Cell viability	NO (μM) [b]
Control		100 ± 6.03	0.21 ± 0.23**
LPS		87.49 ± 0.56	20.78 ± 1.97
Cardamonin[a]	1	95.65 ± 5.97	15.13 ± 0.46**
	3	97.53 ± 4.21	5.45 ± 0.23
VL1	25	34.85 ± 1.66**	10.28 ± 0.69**
	50	12.90 ± 1.27****	2.02 ± 0.31**
VL2	**25**	**107.28 ± 1.57**	**13.81 ± 0.32***
	50	**109.88 ± 3.46**	**5.92 ± 0.01****
VL3	25	81.38 ± 4.96	21.14 ± 0.31
	50	80.61 ± 2.77	20.19 ± 1.03
VL4	25	87.64 ± 7.26	16.88 ± 1.55
	50	86.80 ± 16.01	14.82 ± 0.31
VL5	25	88.23 ± 0.99	15.93 ± 3.71
	50	82.61 ± 1.13	16.35 ± 0.71
VL6	25	81.69 ± 2.74	25.25 ± 0.83
	50	86.23 ± 2.74	15.59 ± 1.07

[a]Positive control; [b] Experiment was done in triplicate;
Data represent mean ± SD (*, **, $p < 0.05$, 0.01 respectively; n = 3).

VL2 suppressed NO production on RAW 264.7 stimulated with LPS

VL2 at all treated concentrations did not cause significant cell death (Fig. 6A). Simultaneously, VL2 suppressed NO production in dose-dependent manner with IC_{50} value of 56.09 ± 4.76 μM (Fig. 6B).

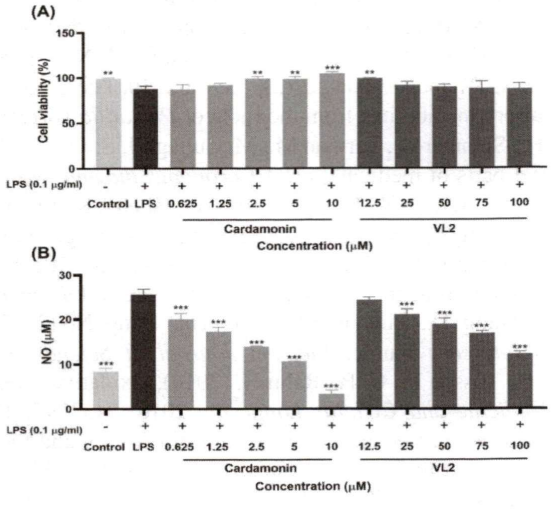

Fig. 6. Evaluation of cell viability and NO production of RAW 264.7 cells when exposed to VL2. *(A) RAW 264.7 cells' survival rate when were treated with VL2.* ***(B)*** *NO production of VL2-treated LPS-induced RAW 264.7 cells. The data are expressed as mean ± SD. ***$p < 0.001$, **$p < 0.002$ indicates a significant difference between LPS stimulation value.*

Human body's defense mechanism cannot work smoothly without the inflammation. After the injured site was approached by immune cells such as macrophages or lymphocytes, NO is one of inflammatory mediators being released [20]. Normally, accepted level of NO will help protect the body from the invasion of microorganism, however, inflammatory factors cause iNOS activated and stimulate the production of NO which contributes significantly to the inflammation process [21]. Therefore, analyzing NO inhibition is a crucial step to evaluate the efficiency of inflammatory treatment.

Viburnum species have been widely applied as folk medicines in Vietnam before, including medicines for treating cough, diarrhea, and especially diseases related to inflammation. Traditionally, *Viburnum lutescens* Blume has been used to treat a common disease which is rheumatoid arthritis. Since rheumatism caused mainly by the immune system and can lead to several dangerous chronic diseases such as diabetes and heart diseases [22], finding a way to reduce inflammation and also hyperglycemic condition could have huge therapeutic implications. In this study, EtOAc extract exhibited the high anti-inflammatory effect with IC50 of 56.88 ± 1.02 μg/mL. We also found that, 6α-hydroxybetulinic acid (VL2) from *V. lutescens* showed inflammation inhibitory activity with IC_{50} value of 56.09 ± 4.76 μM. This is the first report of anti-inflammatory effect of this triterpene acids.

4. Conclusion

In our present study, 03 extracts from *Viburnum lutescens* Blume were screened for several biological properties including antioxidant, anti-hyperglycemia, anti-inflammatory effects. Among them, EtOAc showed the most potential when it had a relatively strong free radical scavenging capability, strong α-glucosidase antagonist, and anti-inflammation. 6α-hydroxybetulinic acid (VL2) - a triterpene acid from *V. lutescens* showed inflammation inhibitory activity with IC_{50} value of 56.09 ± 4.76 μM.

Viburnum lutescens Blume has long been used as a traditional remedy. However, due to limitations, only investigations on the therapeutic characteristics and chemical compositions of this plant have been conducted that so far. We discovered that this plant possesses relatively efficient anti-inflammatory, antioxidant and antihyperglycemic actions, which explains why it is a decent medication. Our research represents a forerunner as well as a foundation for future research on the components of this plant.

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Conflicts of interest: All authors declare that there are not any conflicts of interest.

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