Chemical Composition and biological activities from the *Cleistocalyx operculatus*

Dang Thi Thuy My [1], Xuan Luong Ngo [2*]
[1] Faculty of Natural Sciences and Technology, Tay Nguyen University, 567 Le Duan, Ea Kao, Daklak 630000, Vietnam
[2] Hong Duc University, 565 Quang Trung Str, Hac Thanh, Thanh Hoa 40000, Vietnam

* Corresponding Author: **Xuan Luong Ngo**

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Abstract
The methanol extract from *Cleistocalyx operculatus* collected in Vietnam, was partitioned into hexane, ethyl acetate, and aqueous extract. A compounds was identified, including 2',6'-dihydroxy-4'-methoxychalcone (**1**), and guided by anti-inflammatory activity. Their chemical structures were elucidated by interpreting nuclear magnetic resonance (NMR) spectral data and comparing them with existing literature. The anti-inflammatory potential of isolated compounds was assessed by measuring their ability to inhibit nitric oxide (NO) production. Notably, 2',6'-dihydroxy-4'-methoxychalcone (**1**) significant NO production inhibition with an IC_{50} value of 47.58 ± 2.3 µM, comparable to the reference compound Positive control Cardamonin (3.02 µM) displayed little to no significant inhibitory activity and inhibited α-glucosidase, with an IC_{50} value of 161.3 μg/mL (the positive control acarbose, with an IC_{50} value of 214.1 μg/mL). These findings suggest that 2',6'-dihydroxy-4'-methoxychalcone from *Cleistocalyx operculatus* possess considerable potential as an inflammatory inhibitor and α-glucosidase further investigation and development.

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Introduction

Vietnam is situated in a tropical monsoon and sub-equatorial climate zone, characterized by abundant rainfall and high humidity, which fosters a highly diverse range of flora. Although comprehensive and large-scale botanical studies remain limited, synthesized data from various sources indicate that Vietnam is home to over 7,000 species of higher plants [1]. Among these, more than 2,000 species have been utilized by the local population as sources of food, timber, essential oils, and traditional medicine. Within Vietnam's flora, the group of aromatic plants (essential oil-bearing plants) is particularly rich and diverse. To date, approximately 657 species belonging to 357 genera and 114 families have been documented, accounting for 6.3% of the total species, 15.8% of the genera, and 37.8% of the families [2-3]. While researchers have identified numerous compounds with therapeutic properties, many of these substances exhibit certain limitations, such as undesirable side effects. One of the major sources of raw materials for pharmaceutical production is derived from natural products. Therefore, considerable attention has been directed toward the investigation of bioactive compounds isolated from natural sources. Among the plant species of interest is *Cleistocalyx operculatus* Roxb. (commonly known as "vối") [4].

In Vietnam, this species is widely distributed, particularly in the Central region, where it is traditionally used to prepare a decoction consumed as a beverage to stimulate digestion [5]. The leaves and flower buds are also used in traditional medicine for the treatment of acne, ulcers, and scabies. Recently, the Institute of Traditional Medicine has explored the application of *C. operculatus* in the treatment of gastrointestinal disorders, pharyngitis, and dermatological diseases. Notably, recent studies have indicated that the extract of the flower buds constitutes a component of cardiotonic formulations [6].

The *Cleistocalyx operculatus* Roxb. is a medium-sized woody tree that can reach a height of approximately 12–15 m. The bark is dark brown to nearly black and longitudinally fissured. Branches are cylindrical or occasionally quadrangular and smooth [7]. The leaves are obovate to elliptic or broadly ovate, attenuate at the base with a short acuminate apex. Both surfaces are pale green with brownish dots; the lamina is thick, leathery, and firm. Mature leaves exhibit black dots on the abaxial surface. The petiole is relatively short, approximately 1–1.5 cm in length.The flowers are small, greenish-white, and arranged in clusters of 3–5 in the leaf axils. The flower buds are elongated, with four petals and numerous stamens. The inflorescences are pyramidal and spread along the axils of fallen leaves [8].

The leaves and flower buds of the *Cleistocalyx operculatus* plant contain a variety of valuable natural compounds, among which three main groups have been identified: terpenoids (mainly of the oleanane and ursane skeletons), flavonoids (especially C-methylated flavonoids), and phloroglucinols (in the form of polycyclic phloroglucinols). These compounds possess high biological potential, exhibiting antibacterial, antioxidant, anti-inflammatory, and antihyperglycemic activities [9, 10].

. Among these, flavonoids represent the predominant class of compounds in this genus [10]. Biological activity studies have shown that crude extracts and isolated pure compounds from *Cleistocslyx* species exhibit anticancer, analgesic, antiviral, hepatoprotective, antidiabetic, and cardioprotective effects [11]. Among them, *Cleistocslyx Operculatus* has been traditionally used as a tonic for strengthening tendons and bones, and for the treatment of fever, measles rash, pain, and gastritis. Previous investigations have indicated that crude extracts and isolated compounds from this species exhibit diverse biological activities, including anticancer, antioxidant, hepatoprotective, antiviral, anti-inflammatory, and analgesic effects. However, studies specifically addressing the anti-inflammatory activity of this species remain limited to date [12-16]. Therefore, when testing its chemical activity, it is possible to discover different active constituents according to the above factors. This study aims to analyze and characterize the chemical composition and biological properties of *Cleistocalyx operculatus* essential oil collected in Vietnam, in support of its potential exploitation and effective utilization. This study was conducted to explore the therapeutic potential of *Cleistocslyx Operculatus* and to identify bioactive compounds with biological activities, thereby contributing to the development of plant-derived agents for the management of inflammatory and inhibited α-glucosidase disorders.

Materials and Methods

General Experimental Procedures

The NMR spectra (^{1}H and ^{13}C) were measured on a Bruker Avance 500 MHz spectrometer (Bruker Daltonics, Ettlingen, Germany). Chemical shifts were recorded in δ values (ppm). Column chromatography (CC) was performed using RP-C18 (Merck, 17 mesh), silica gel (Merck, 0.040-0.063 mm), and Sephadex LH-20 (Sigma–Aldrich, MO, USA). Thin-layer chromatography (TLC) was conducted using RP-C18 F254s and silica gel F254 plates (Merck). Compounds were initially detected under UV light and later visualized by heating after spraying with 10% H_2SO_4. Column chromatography employed Kieselgel 60 silica gel (particle size: 0.04-0.063 mm, 60-200 µm, Merck, Germany) and RP-18 (particle size: 40-63 µm, Merck, Germany). Thin-layer chromatography used pre-coated Kieselgel 60 F254 plates (Merck, Germany). All solvents used were extra-pure grade (min > 99.5%) without requiring additional purification.

Plant Material

The plant of *Cleistocslyx Operculatus* was collected in Vietnam, in May 2022 and identified by Quoc-Binh Nguyen, Ph.D., Vietnam National Museum of Nature, Vietnam Academy of Science and Technology (VAST). The voucher specimen (TE-01) was carefully stored at the Faculty of Natural Science and Technology, Hong Duc University, Thanh Hoa Province, Vietnam.

Extraction and Isolation

Leaves and small branches (2,165 g) of *Cleistocalyx operculatus* (Roxb.) Merr. & Perry collected in Triệu Sơn were air-dried, pulverized, and macerated with methanol at room temperature for 10 days. The solvent was subsequently removed under reduced pressure to yield a crude methanolic extract (315 g).

The methanolic extract was suspended in water and successively partitioned with n-hexane, ethyl acetate, and n-butanol. Evaporation of the respective solvents under reduced pressure afforded the corresponding fractions: *n*-hexane extract (7.2 g), ethyl acetate extract (5.4 g), and *n*-butanol extract (11.0 g).

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The *n*-hexane and ethyl acetate extracts were subjected to thin-layer chromatography (TLC) on pre-coated silica gel plates (Merck) to determine suitable solvent systems for subsequent column chromatography of the respective fractions.The ethyl acetate extract was fractionated by silica gel column chromatography using chloroform–methanol (19:1 to 4:1, v/v) as the eluent. Six fractions were obtained. Fraction 3 yielded compound 1 (172 mg).

2',6'-dihydroxy-4'-methoxychalcone (1): were obtained as white amorphous powders, with a melting point of 221-222^{0}C; $[\alpha]^{25}_D$ = + 57.0 (c = 0.14, MeOH); ^{1}H-NMR ($CDCl_3$, 500 MSHz) δ (ppm): 3.88 (3H, *s*, 2'-OCH_3); 5.94 (1H, *d*, *J* = 2.40 Hz, H3'); 5.95 (1H, *d*, *J* = 2.40 Hz, H-5'); 7.35 (3H, *m*, *J* = 17.4 Hz, H-3, H-4, H-5); 7.55 (2H, *dd*, *J* = 2.40; 7.32 Hz, H-2, H-6); 7.65 (1H, *d*, *J*=15.60, H-9); 7.85 (1H, *d*, *J* = 15.6 Hz, H-7); ^{13}C-NMR ($CDCl_3$) δ (ppm): 55.4 (-C4'); 91.4 (C-5'); 96.0 (C-3'); 105.1 (C-1'); 127.7 (C-8); 128.2 (C-2,6); 128.9 (C-3,4,5); 135.5 (C-1); 141.9 (C-7); 163.4 (C-1'); 165.3 (C-4'); 167.3 (C-2'); 192.8 (C-9).

Cell Culture

The RAW264.7 cells (Manassas, VA, USA) were cultured at 37°C in a humidified atmosphere with 5% CO_2 and maintained in Dulbecco's Modified Eagle Medium (DMEM)

supplemented with 10% heat-inactivated FBS (fetal bovine serum) (Cambrex, Charles City, IA, USA), penicillin (100 units/mL), and streptomycin (100 µg/mL). The RAW264.7 cells were counted by hemocytometer. The trypan blue dye exclusion method was used to assess the viability of the RAW264.7 cells.

NO Production

To measure the nitrite content in cell culture supernatants, the level of NO production was assessed following established protocols (To *et al*. 2024a, 2024b, 2024c; Hoang *et al*. 2024). The RAW 264.7 cells were seeded (1×10^5 cells/well) in 24-well plates and incubated at 37°C, 5% CO_2 for 12 hours. Subsequently, the medium was aspirated from each well, and fresh FBS-free DMEM was added. The isolated compounds (at different concentrations of 1, 3, 10, and 30 µM) were prepared (in FBS-free DMEM). After treatment of 1 hour, the RAW 264.7 cells were stimulated with or without 1 µg/mL of lipopolysaccharide (LPS) for 24 hours. Nitrite was measured by Griess reagent (1% sulfanilamide +0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid). Specifically, 100 µL of cell culture medium was mixed with 100 µL of Griess reagent and then incubated for 10 minutes at room temperature. The absorbance was measured at 540 nm by a microplate reader (Biotek, Winooski, VT, USA). Fresh culture medium was used as a blank, and a sodium nitrite ($NaNO_2$) standard curve was used to determine the nitrite quantity. L-NMMA was employed as a positive control in this experiment.

Inhibition of α-glucosidase assay

The *α*-glucosidase inhibitory protocol was carried out according to previously assay with slight modification (to Trang *et al*. 2012). Extracts and compounds were dissolved in DMSO and diluted with phosphate buffer (pH 6.8) to various concentrations of 250, 100, 50, 25 and 10 *µ*g/ml. In a 96-well plate, a reaction mixture containing 100 *µ*L of phosphate buffer (100 mM, pH 6.8), 40 *µ*L of extracts or compounds, 20 *µ*L *α*-glucosidase (0.4 U/mL, CAS No 9001-42-7, Sigma) were pre-incubated for 15 min at 37 °C. Then, 40 *µ*L substrate *p*-nitrophenyl-*α*-D-glucopyranoside (pNPG, 2.5 mM, CAS No 3767-28-0, Sigma) were added to the mixture. The reaction was stopped by 100 *µ*L of Na_2CO_3 (0.1 M) for each well after 20 min incubation. Without test compound was set up as a blank control and acarbose was used as a positive control. Experiments were done in triplicates. The absorbance of the released *p*-nitrophenol was measured at 405 nm with microplate reader (BioTek, USA). The results expressed as percentage inhibition, were calculated using the following equation:

Inhibition (%) = [(OD control – OD sample) / OD control] x 100

The IC_{50} value showed the concentration of compound inhibiting 50% of *α*-glucosidase activity. The IC_{50} value was calculated using TableCurve software.

Statistical Analysis

Data are presented as mean ± SD. Graphs were generated, and statistical analyses were performed using SigmaPlot 6.0 and SigmaStat 3.1 (Systat Software, San Jose, CA, USA). ANOVA followed by Tukey's test was used to compare multiple groups with pre-validated data. To compare the two groups, an unpaired Student's t-test was conducted using Prism (GraphPad Software, San Diego, CA, USA). Non-parametric ANOVA with Tukey's test and Bonferroni post hoc analysis or correlation analysis as appropriate were employed for comparisons involving more than two groups. Statistical significance was indicated by $*p < 0.05$ and $**p < 0.01$.

Results and Discussion

The *Cleistocalyx operculatus* methanolic extract was partitioned into *n*-hexane and ethyl acetate-soluble fractions. Chromatographic purification of the ethyl acetate-soluble fraction led to the isolation of three compound (1).

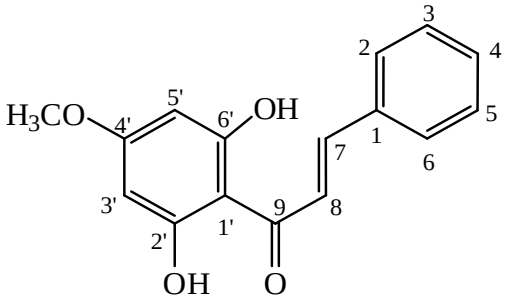

Fig 1: Chemical structures of the compound isolated from *Cleistocalyx operculatus*.

Compound 1 was obtained as pale-yellow needle-shaped crystals with a melting point of 163–165°C. The ^{1}H_NMR spectrum exhibited characteristic signals of two hydroxyl protons involved in intramolecular hydrogen bonding at δ_H 13.7 ppm. The aromatic region indicated a trisubstituted benzene ring (ortho- and para-substitution pattern), with two meta-coupled protons appearing at δ_H 6.02 ppm (J = 2.0 Hz, H-5′) and 5.94 ppm (J = 2.5 Hz, H-3′), confirming meta coupling. Two olefinic protons observed at δ_H 7.82 and 7.65 ppm were assigned to H-7 and H-8, respectively. The ^{13}C_ NMR and DEPT spectra displayed 16 carbon signals, including nine methine carbons and six quaternary carbons, among which a carbonyl carbon resonated at δ_C 191.8 ppm and a methoxy carbon at δ_C 55.0 ppm. The spectra also indicated the absence of two methoxy groups, accounting for the reduced number of carbon signals.Substituent positions were further established by HMBC correlations. The three protons of the methoxy group at δ_H 3.71 ppm showed a 3 correlation with C-4′ (δ_C 164.9 ppm). The singlet signal of the hydroxyl proton at δ_H 13.7 ppm exhibited 2 and 3 correlations with C-1′ (δ_C 105.2 ppm), C-2′ (δ_C 166.1 ppm), and C-3′ (δ_C 95.9 ppm). Comparison of the spectroscopic data of 1 with published literature allowed its identification as a chalcone-type flavonoid, namely 2′,6′-dihydroxy-4′-methoxychalcone [15]. Compound 1 has been reported to possess notable biological activities, including cytotoxic and chemopreventive effects against prostate cancer, carcinoma, and breast cancer, as well as antiprotozoal and antiparasitic activities. Its relatively simple structure may enable it to serve as a promising lead compound for the development of novel antiemetic agents [16].

Evaluation of Anti-Inflammatory Activity via Inhibition of NO Production

The *in vitro* anti-inflammatory activity, assessed through the inhibition of nitric oxide (NO) production in RAW 264.7 cells, was evaluated for the crude methanol extract (CO), *n*-

hexane extract (COH), ethyl acetate extract (COE), and aqueous extract (COW), as well as for the isolated 2',6'-dihydroxy-4'-methoxychalcone (1) obtained from *Cleistocalyx operculatus*. The results are presented in Table 1.

Table 1: Inhibitory effects on NO production in RAW264.7 cells *in vitro from Cleistocalyx operculatus*

Sample	Percentage of NO production inhibition (%)	IC_{50}
Negative control (–)	100.00 ± 0.9	-
Positive control (+) [Cardamonin]	81.54 ± 2.3	3.02 µM
LPS	0.00 ± 2.5	-
CO	58.33 ± 1.1	49.17 µg/ml
COH	56.62 ± 1.9	92.54 µg/ml
COE	67.14 ± 2.1	31.94 µg/ml
COW	32.51± 2.0	-
1	47.58 ± 2.3	-

The results indicated that among the crude extracts of the *Cleistocalyx operculatus*, COE and CO exhibited notable *in vitro* inhibitory activity against NO production, with IC_{50} values of 31.94 and 49.17 µg/mL, respectively. Among the isolated compound 2',6'-dihydroxy-4'-methoxychalcone (1) showed the most potent anti-inflammatory activity, with an IC_{50} value of 47.58 ± 2.3 µg/mL. The remaining extracts and pure compounds displayed little to no significant inhibitory activity.

α-Glucosidase Inhibitory Activity

The total methanol residue, *n*-hexane extract, chloroform extract, ethyl acetate extract and and two isolated compound from *Cleistocalyx operculatus* were evaluated for *α*-glucosidase inhibitory activity. As shown in Table 2, the total residual methanol showed weak inhibitory ability against α-glucosidase while the n-hexane extract showed no inhibitory activity. Both chloroform and ethyl acetate extracts showed good inhibitory activity, however, ethyl acetate extract showed better inhibitory activity, so it was chosen by us to proceed with the isolation of compounds.

Table 2: *α*-glucosidase inhibitory activity of the *Cleistocalyx operculatus*

Sample	Inhibition (I, %)					IC_{50} (*µ*g/mL)
	250 *µ*g/mL	100 *µ*g/mL	50 *µ*g/mL	25 *µ*g/mL	10 *µ*g/mL	
CO	85.4 ± 1.4	28.5 ± 2.5	2.1 ± 2.6	–	–	125.1
COH	78.3± 2.7	19.1 ± 1.3	–	–	–	187.5
COE	93.8 ± 1.4	41.8 ± 2.8	6.5 ± 1.7	–	–	115.4
COW	78.2 ± 2.3	56.0 ± 3.1	26.52 ± 0.66	8.1 ± 1.5	–	98.4
1	87.2 ± 1.3	38.6 ± 1.4	18.6 ± 1.2	-	-	161.3
Acarbose						214.1

*: I > 100%; –: I < 1%

The results showed that both compounds had stronger inhibitory activity against α-glucosidase enzyme than the positive control, acarbose, with IC_{50} values of 2',6'-dihydroxy-4'-methoxychalcone (1) being 161.3 *µ*g/mL much smaller than that of acarbose (IC_{50} = 214.1 *µ*g/mL). Compound 1 showed stronger inhibitory activity against α-glucosidase enzyme, thus having potential use as an antidiabetic drug.

Conclusion

This study successfully isolated and characterized three compounds: including 2',6'-dihydroxy-4'-methoxychalcone (1) from *Cleistocalyx operculatus*, with a particular focus on their inhibitory effects on NO production in RAW 264.7 cells. Among the isolated compound (1), significant anti-inflammatory activity, with an IC_{50} value of 47.58 ± 2.3 µM. Compound 1 showed stronger inhibitory activity against α-glucosidase enzyme with IC_{50} values of 161.3 *µ*g/mL. The results provide substantial evidence for the efficacy of these compounds in modulating inflammatory responses, although further research is needed to explore their interactions with other inflammatory and inhibitory activity against α-glucosidase enzyme.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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