ANTI-INFLAMMATORY ACTIVITIES OF COMPOUNDS ISOLATED FROM *AMANITA CAESAREA* COLLECTED IN LAM DONG PROVINCE

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(Received: July 01, 2022; Accepted: August 10, 2022)

Abstract - Five natural secondary metabolites as cinnamic acid (**1**), (+)-catechin (**2**), (−)-epicatechin (**3**), *p*-coumaric acid (**4**), and ferulic acid (**5**) were isolated from *Amanita caesarea* based on anti-inflammatory activity-guided extraction. Their structures (**1**–**5**) were determined by NMR spectra as well as by comparison with previously reported literature. Compounds **3** and **5** have been isolated from *A. caesarea* for the first time. The anti-inflammatory activity through inhibition of nitric oxide (NO) production of isolates (**1**–**5**) was evaluated. Among them, compounds **2** and **3** exhibited strong inhibitory activity with IC_{50} values of 4.8 and 5.7 μM, respectively. Compounds **4** and **5** with IC_{50} values of 18.4 and 9.6 μM, respectively showed moderate inhibitory activity. The results proposed that *A. caesarea* might exert anti-inflammatory effects due to its mainly NO-inhibitory constituents

Key words - *Amanita caesarea*; flavonoid; NO production; cytotoxic; RAW264.7 cells

1. Introduction

A. caesarea is an edible mushroom commonly known as Caesar's mushroom. This species is a member of the *Amanita* genus. The *Amanita* genus contains about 1000 species and is widely distributed throughout the world [1]. Almost *Amanita* species are either toxic or hallucinogenic [1,2]. Historical evidence suggests that nearly 90% of reported cases of lethal poisonings are caused by the consumption of *Amanita* species [2]. However, several pharmaceutical effects as antioxidant, antiproliferative, immunostimulatory, antibacterial, cytotoxic, pesticidal, larvicidal, anticancer, antitumor, anti-cholinesterase, osteolytic, and antiviral activities were found in *Amanita* species [3]. Especially, *in vitro* studies showed that some *Amanita* mushrooms such as *A. augusta* and *A. muscaria* exhibited potentially anti-inflammatory activity [4, 5]. *A. caesarea* possesses antioxidant and antimicrobial [6, 7, 8], lowering cholesterol [9], and neuroprotective activities [10-12]. Previous studies showed that this mushroom presented phenolics [7, 8, 13-16], sterols [17], alkaloids [9, 18], polysaccharides [11, 19, 20], and fatty acids [7, 21]. Despite the number of studies, there has been no isolation of phenolic compounds and anti-inflammatory activity from *A. caesarea*, especially the species from Vietnam. Our results showed that the dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc) extracts of *A. caesarea* exhibited appreciable inhibitory activity in lipopolysaccharide (LPS)-induced NO production (IC_{50} values of 238.7 ± 12.6 and 146.5 ± 5.8 μg/mL, respectively) (Table 1). Therefore, these extracts were used to isolate compounds and evaluate the inhibitory activity of the isolated compounds against NO production in the RAW 264.7 cells model.

2. Materials and Methods

2.1. Experimental

ECD spectra were recorded on a JASCO J-810 spectropolarimeter. Other spectroscopic measurements and chromatographic techniques are previously described [22-24].

2.2. Materials

The whole mushroom of *A. caesarea* was collected at Langbiang Biosphere Reserve, Lam Dong Province, Vietnam, and this sample was identified by Prof. Dr. Nguyen Phuong Dai Nguyen, Faculty of Science and Technology, Tay Nguyen University. A voucher specimen (LB012) is deposited at the Department of Experimental Biology, Tay Nguyen University.

2.3. Extraction and Isolation

The dried whole mushroom of *A. caesarea* (1.0 kg) was extracted with 96% ethanol (EtOH) using an ultrasonic bath system for 30 mins. The extract was then filtered before being evaporated under reduced pressure to give a crude EtOH extract. The EtOH extract (50 g) was then suspended in hot water and partitioned with dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc) successively to obtain CH_2Cl_2 (15 g), EtOAc (20 g), and water (H_2O) extracts, respectively after removing solvents. The CH_2Cl_2 extract (15 g) was applied on a silica gel chromatography column (CC) and eluted with *n*-hexane-acetone (50:1 to 0:1) to yield nine fractions (Fr.C.1 - Fr.C.9). Fraction C.6 (1.2 g) was subjected to a silica gel CC and eluted with CH_2Cl_2-methanol (10:1 to 3:1), to give four sub-fractions (Fr.C.6.1 - Fr.C.6.4). Compound **2** (415 mg) was isolated from sub-fraction C.6.2 (520 mg) by RP-C18 CC, eluted with acetonitrile-H_2O (1:1 to 2:1). Compound **3** (65 mg) was isolated from sub-fraction C.6.3 (380 mg) by RP-C18 CC, eluted with methanol-H_2O (1:2 to 2:1). The EtOAc soluble fraction (20 g) was also chromatographed on a silica gel chromatography column

(CC) using a stepwise gradient of CH_2Cl_2-acetone (30:1 to 0:1), to yield eight fractions (Fr.E.1 - Fr.E.8) according to their TLC profiles. Fraction E.2 (310 mg) was subjected to a silica gel CC and eluted with CH_2Cl_2-methanol (20:1 to 7:1) to obtain three sub-fractions (Fr.E.2.1 - Fr.E.2.3). Compounds **1** (25 mg) and **5** (12 mg) were obtained from sub-fraction E.2.2 (220 mg) by RP-C18 CC, eluted with methanol-H_2O (1:3 to 1:1). Fraction E.3 (260 mg) was also subjected to a silica gel CC and eluted with CH_2Cl_2-methanol (10:1 to 5:1) to obtain three sub-fractions (Fr.E.3.1 - Fr.E.3.3). Compound **4** (22 mg) was isolated from sub-fraction E.3.3 (85 mg) by RP-C18 CC, eluting with a gradient of methanol-H_2O (1:3 to 1:1).

*Cinnamic acid (**1**)*: White powder; ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H (*ppm*): 7.82 (1H, *d*, *J* = 16.0 Hz, H-7), 7.57 (2H, *m*, H-2/H-6), 7.42 (3H, *m*, H-3/H-4/H-5), 6.48 (1H, *d*, *J* = 16.0 Hz, H-8); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C (*ppm*): 172.7 (C-9), 147.4 (C-7), 134.3 (C-1), 130.9 (C-4), 129.1 (C-2/C-6), 128.6 (C-3/C-5), 117.6 (C-8).

*(+)-Catechin (**2**)*: Colorless solid; $[\alpha]_D^{25}$ +15.4° (*c* 0.1, MeOH); CD (*c* 0.15, MeOH): $\Delta\varepsilon_{228}$ (nm) – 3.22, $\Delta\varepsilon_{280}$ (nm) – 1.85; ^{1}H-NMR (500 MHz, Methanol-d_4) δ_H (*ppm*): 6.84 (1H, *d*, *J* = 2.0 Hz, H-2′), 6.77 (1H, *d*, *J* = 8.0 Hz, H-5′), 6.72 (1H, *dd*, *J* = 8.0, 2.0 Hz, H-6′), 5.93 (1H, *d*, *J* = 2.0 Hz, H-8), 5.86 (1H, *d*, *J* = 2.0 Hz, H-6), 4.57 (1H, *d*, *J* = 8.0 Hz, H-2), 3.98 (1H, *dt*, *J* = 8.0, 5.5 Hz, H-3), 2.85 (1H, *dd*, *J* = 5.5, 16.0 Hz, H-4$_{ax}$), 2.52 (1H, *dd*, *J* = 8.0, 16.0 Hz, H-4$_{eq}$); ^{13}C-NMR (125 MHz, Methanol-d_4) δ_C (*ppm*): 157.9 (C-7), 157.6 (C-5), 157.0 (C-9), 146.3 (C-4′), 146.3 (C-3′), 132.3 (C-1′), 120.1 (C-6′), 116.2 (C-5′), 115.3 (C-2′), 100.9 (C-10), 96.4 (C-6), 95.6 (C-8), 82.9 (C-2), 68.9 (C-3), 28.6 (C-4).

*(–)-Epicatechin (**3**)*: Colorless solid; $[\alpha]_D^{25}$ –22.1° (*c* 0.1, MeOH); CD (*c* 0.15, MeOH): $\Delta\varepsilon_{238}$ (nm) + 2.08, $\Delta\varepsilon_{272}$ (nm) – 3.12; ^{1}H-NMR (500 MHz, Methanol-d_4) δ_H (*ppm*): 7.01 (1H, *d*, *J* = 2.0 Hz, H-2′), 6.83 (1H, *dd*, *J* = 8.5, 2.0 Hz, H-6′), 6.79 (1H, *d*, *J* = 8.5 Hz, H-5′), 5.97 (1H, *d*, *J* = 2.0 Hz, H-8), 5.95 (1H, *d*, *J* = 2.0 Hz, H-6), 4.84 (1H, *d*, *J* = 3.0 Hz, H-2), 4.20 (1H, *t*, *J* = 3.0 Hz, H-3), 2.89 (1H, *dd*, *J* = 5.0, 12.0 Hz, H-4$_{ax}$), 2.76 (1H, *dd*, *J* = 3.0, 12.0 Hz, H-4$_{eq}$); ^{13}C-NMR (125 MHz, Methanol-d_4) δ_C (*ppm*): 158.1 (C-7), 157.8 (C-5), 157.5 (C-9), 146.0 (C-4′), 145.9 (C-3′), 132.4 (C-1′), 119.5 (C-6′), 116.0 (C-5′), 115.4 (C-2′), 100.2 (C-10), 96.5 (C-6), 96.0 (C-8), 80.0 (C-2), 67.6 (C-3), 29.3 (C-4).

*p-Coumaric acid (**4**)*: White solid; ^{1}H-NMR (500 MHz, Methanol-d_4) δ_H (*ppm*): 7.18 (1H, *d*, *J* = 16.0 Hz, H-7), 6.98 (2H, *d*, *J* = 8.5 Hz, H-2/H-6), 6.38 (2H, *d*, *J* = 8.5 Hz, H-3/H-5), 5.86 (1H, *d*, *J* = 16.0 Hz, H-8); ^{13}C-NMR (125 MHz, Methanol-d_4) δ_C (*ppm*): 171.2 (C-9), 161.0 (C-4), 146.8 (C-7), 131.1 (C-2/C-6), 127.2 (C-1), 116.8 (C-3/C-5), 115.5 (C-8).

*Ferulic acid (**5**)*: Amber-colored solid; ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H (*ppm*): 7.44 (1H, *d*, *J* = 16.0 Hz, H-7), 7.03 (1H, *d*, *J* = 2.0 Hz, H-2), 6.91 (1H, *dd*, *J* = 8.5, 2.0 Hz, H-6), 6.67 (1H, *d*, *J* = 8.5 Hz, H-5), 6.16 (1H, *d*, *J* = 16.0 Hz, H-8), 3.75 (3H, *s*, 3-OCH_3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C (*ppm*): 171.1 (C-9), 150.6 (C-4), 149.4 (C-3), 147.0 (C-7), 127.9 (C-1), 124.1 (C-6), 116.5 (C-5), 116.0 (C-8), 111.7 (C-2), 56.5 (3-OCH_3).

2.4. Biological Assay

Cell culture, cell viability assay and the determination of NO production were performed according to the methods previously described [22-25].

2.5. Statistical Analysis

Inhibitory activity assay was performed in triplicate. The results are presented as the means ± standard error of the mean.

3. Results and Discussion

3.1. Determination of Isolated Compounds

The ^{1}H-NMR spectrum of **1**, **4**, and **5** displayed characteristic signals due to aromatic protons of a benzene ring, together with those of hydroxyl and methoxy groups, while their ^{13}C-NMR spectrum revealed the signals of aromatic carbons, oxygen-substituted carbons, and a quaternary carbon (C-1) in a benzene ring (Figure 1). The ^{1}H- and ^{13}C-NMR spectra revealed that the presence of a *trans*-olefinic group [δ_H 7.14-7.82 (1H, *d*, *J* = 16.0 Hz, H-7), 5.86-6.48 (1H, *d*, *J* = 16.0 Hz, H-8)/δ_C 146.8-147.4 (C-7), and δ_C 115.5-117.6 (C-8)] and a carboxyl group δ_C 171.1-172.7 (C-9) indicated **1**, **4**, and **5** to be unsaturated carboxylic acid derivatives [16,29,30]. Compound **1** displayed 5 aromatic protons (H-2/H-3/H-4/H-5 and H-6) while compound **4** possessed 4 aromatic protons (H-2/H-3/H-5 and H-6) but showed one oxygenated carbon at C-4 in the ^{1}H- and ^{13}C-NMR spectra. Compound **5** possessed 3 aromatic protons (H-2/H-5 and H-6) but showed two oxygenated carbons (C-3 and C-4), and a methoxy group [δ_H 3.75 (OC<u>H</u>$_3$)/δ_C 56.5 (O<u>C</u>H$_3$)] at C-3 (Figure 1). Comparison of the ^{1}H- and ^{13}C-NMR data of these compounds with those published in the literature led to the identification structures of **1**, **4**, and **5** to be cinnamic acid [26], *p*-coumaric acid [29], and ferulic acid [30], respectively.

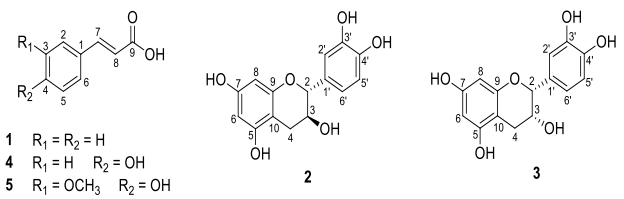

***Figure 1.** Chemical structure of isolated compounds (**1–5**)*

Compounds **2** and **3** were isolated as colorless solids. The optical rotation value of **2** was +15.4, while compound **3** was –22.1. The ^{1}H-NMR spectrum of **2** and **3** displayed five aromatic protons (H-6/H-8/H-2′/H-5′ and H-6′), two oxymethines [δ_H 4.57 (H-2), and δ_H 3.98 (H-3) for **2**, and δ_H 4.84 (H-2) and δ_H 4.20 (H-3) for **3**], and a methylene group (2H-4), while their ^{13}C-NMR spectra revealed the signals of two oxymethine carbons [δ_C 82.9 (C-2) and δ_C 68.9 (C-3) for **2**, and δ_C 80.0 (C-2), δ_C 67.6 (C-3) for **3**], a methylene carbon [δ_C 28.6 (C-4) for **2**, and δ_C 29.3 (C-4) for **3**] (Figure 1). The above observation indicated that these compounds are flavan-3-ol [27,28]. Detailed analysis of the ^{1}H-NMR spectrum of **2** and **3** revealed the 2,3-*trans*-isomer $J_{2,3}$ = 8.0 Hz (compound **2**) and 2,3-*cis*-isomer for $J_{2,3}$ = 3.0 Hz

(compound **3**) [31]. The CD spectrum of **2** showed two negative cotton effects at $\Delta\varepsilon_{228}$ – 3.22 and $\Delta\varepsilon_{280}$ – 1.85, while the CD spectrum of **3** showed a positive cotton effect at $\Delta\varepsilon_{238}$ + 2.08 and a negative cotton effect at $\Delta\varepsilon_{272}$ – 3.12, suggesting 2*R*,3*S* configuration for **2** and 2*R*,3*R* configuration for **3** [32]. Comparison of the ^{1}H- and ^{13}C-NMR data of these compounds with those published in the literature led to the identification structures of **2** and **3** to be (+)-catechin [27] and (–)-epicatechin [28], respectively.

3.2. Cell Viability and NO Production Inhibition of Isolated Compounds

First, cell viability was tested to determine the non-toxic concentration of the isolates (**1**–**5**) and was evaluated by MTS assay [25]. The isolates (**1**–**5**) were toxic to RAW 264.7 cells at the concentration of 100 μM (Figure 2), therefore the chosen concentrations for the next experiment were 1, 3, 10, and 30 μM.

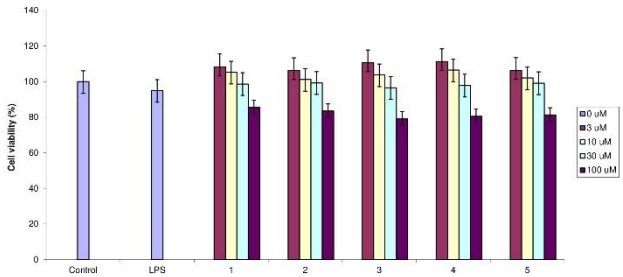

Figure 2. *Effect on cell viability compounds **1**–**5***

To check the NO production inhibitory activity, the RAW 264.7 cells were treated with isolated compounds with several concentrations (1, 3, 10, and 30 μM), and the level of NO production was measured using the Griess reaction [22-24]. Table 1 revealed that compound **2** exhibited the strongest inhibitory on NO production (IC_{50} = 4.8 ± 0.2 μM), followed by compound **3** exhibited inhibitory effects with an IC_{50} value of 5.7 ± 0.5 μM. Compounds **4** and **5** showed moderate inhibitory NO production with IC_{50} values of 18.4 ± 1.2 and 9.6 ± 0.8 μM, respectively, while compound **1** was inactive (IC_{50} > 30 μM).

Table 1. *NO production inhibition of compounds **1**–**5***

Extracts / Compounds	IC_{50} values[a]
EtOH[b]	348.2 ± 10.5
CH_2Cl_2[b]	238.7 ± 12.6
EtOAc[b]	146.5 ± 5.8
1	> 30
2	4.8 ± 0.2
3	5.7 ± 0.5
4	18.4 ± 1.2
5	9.6 ± 0.8
Celastrol[c]	1.0 ± 0.1

[a]IC_{50} of compounds were in μM. [b]IC_{50} of extracts were expressed in μg/mL. [c]Positive control for NO production. Values are mean ± S.D (n = 3).

NO is produced by iNOS in macrophages, hepatocytes, and renal cells, under the stimulation of LPS, tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), or interferon-gamma (IFN-γ) [33]. The overproduction of NO by iNOS has been implicated in the pathology of several inflammatory disorders, including septic shock, tissue damage after inflammation, and rheumatoid arthritis [34-36]. Therefore, inhibiting iNOS activity or downregulation of iNOS expression is one of the ways to reduce inflammation. From our results, the flavonoid (+)-catechin (**2**) strongest inhibited NO production (4.8 ± 0.2 μM), followed by (–)-epicatechin (**3**) exhibited inhibitory effects with an IC_{50} value of 5.7 ± 0.5 μM, presumably because of the presence of the 3,4-hydroxylation(s) in the benzene ring (Figure 1) and this result is similar to those of previously reported [22-24]. Meanwhile, the inhibitory activities on NO production of compounds **4** and **5** were significantly reduced (IC_{50} values of 18.4 ± 1.2 and 9.6 ± 0.8 μM, respectively) and compound **1** was inactive, presumably because of the lack of 3-hydroxylation (compounds **1** and **4**) of the benzene ring or the presence of the methoxy groups (compound **5**) in benzene ring (Figure 1) [22-24,37-39]. These results suggested that flavan-3-ol bearing the 3,4-hydroxylation of the benzene ring could be considered as new lead compounds for the development of agents against NO production.

4. Conclusion

Through biological guide isolation, five compounds, cinnamic acid (**1**), (+)-catechin (**2**), (–)-epicatechin (**3**), *p*-coumaric acid (**4**), and ferulic acid (**5**) were isolated from the dichloromethane and ethyl acetate fraction of *A. caesarea*. Their chemical structures were determined by the interpretation of NMR spectral data and comparison with published data. (–)-epicatechin (**3**) and ferulic acid (**5**) have been isolated from *A. caesarea* for the first time. Compound **2** showed the most potent inhibitory activity against the LPS-induced NO production with IC_{50} values of 4.8 μM, followed by compounds **3**–**5** with IC_{50} values of 5.7, 18.4, and 9.6 μM, respectively. The results proposed that the active constituents from *A. caesarea* can be used for research and development of inflammatory agents and the use of this mushroom may be beneficial in the handling of inflammation.

Acknowledgments: This research is funded by National Foundation for Science and Technology Development (NAFOSTED) under grant number 108.05-2020.06.

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