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Short Communication

Antityrosinase Inhibitory Activity of Phytochemicals from *Alpinia aquatica* Roscoe

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Abstract

Background: Genus *Alpinia* are commonly used as spices and ingredients in traditional medicines. In the present study, we attempted to isolate the phytochemicals from *Alpinia aquatica* and evaluate their tyrosinase inhibitory activity.

Methods: Phytochemical constituents of the extract were investigated using various chromatographic and spectroscopic methods. The chemical structures of the isolated phytochemicals were established by analysis of their spectroscopic data, as compared to that of reported data. Tyrosinase inhibitory activity was also tested on the extracts and selected compounds using mushroom tyrosinase as the enzyme.

Results: Fractionation and purification of the extracts of *Alpinia aquatica* afforded seven known compounds which are 5-hydroxy-3,7,4'-trimethoxyflavone (**1**), 4',5-dihydroxy-3,7-dimethoxy-flavone (**2**), 2-methoxy-8-(2',4',5'-trimethoxyphenyl)-1,4-naphthaquinone (**3**), *cis*-3*S*-(2',4',5'-tri-methoxyphenyl)-4*S*-[(*E*)-2''',4''',5'''-trimethoxystyryl]cyclohexene (**4**), 2,4,5-trimethoxybenz-aldehyde (**5**), stigmasterol (**6**) and β-sitosterol (**7**). The ethyl acetate extract of pseudostems possessed the highest tyrosinase inhibition of 31.0% among the extracts, while compound (**1**) gave tyrosinase inhibition of 48.0%.

Conclusion: Compounds (**3**) and (**4**) were isolated for the first time from *A. aquatica* and *Alpinia* genus. These phytochemical results suggest that the extracts could assist as a potential source of bioactive compounds. Further research is needed in which the extract could possibly be exploited for pharmaceutical use.

Introduction

The Zingiberaceae or ginger family is one of the largest families of the monocotyledons of Malaysia and one of the most important herbaceous components of the ground cover in many types of tropical forests. This family comprises about 1500 species with 52 genera in the world.[1] They are predominantly found in the tropical region particularly in Southeast Asia. Several members of the Zingiberaceae family are used for some purposes. In Malaysia, the Zingiberaceae species are frequently used as spices, food preservatives, coloring agents and cooking ingredients. Various ginger species provide health-promoting effects and traditionally used for treating nausea, motion sickness, stomachache, asthma, diarrhea, digestive disorder, vomiting, rheumatism, swelling, common cold, and cough.[2] *Alpinia* is the largest, most widespread, and most taxonomically complex genus in the Zingiberaceae with 230 species. It is occurring throughout tropical and subtropical Asia and distributed from Sri Lanka and the Western Ghats of India to China, Japan, all of Southeast Asia, the Pacific as far as Fiji, Samoa, and the Caroline Islands, and Australia as far as southern and northern New South Wales. The *Alpinia* plants have fragrant rootstocks resembling the scent of ginger.[3] *Alpinia aquatica* is widely distributed in West Malaysia, Borneo and Sumatra. It cultivates up to 10 feet high in low to mid-elevation forests and forms clumps with stems from 1-3 meter.[3] Previously, phytochemical studies have shown the presence of phenylbutenoids.[4] Meanwhile, the essential oil compositions of the rhizomes, leaf, and pseudostem of *A. aquatica* had previously been reported.[5,6] In this article, we aim to report detailed phytochemical study and tyrosinase inhibitory activity of *A. aquatica* collected from Sarawak, Malaysia.

Materials and Methods

Plant materials

Alpinia aquatica was collected from Kuching, Sarawak in 2011 and identified by Zaini Assim. The voucher

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specimen (ZA2011) was deposited at the Herbarium of Resource Science and Technology, Universiti Malaysia Sarawak.

General experimental procedures

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Avance 400 Spectrometer. Chemical shifts were reported in ppm relative to tetramethylsilane (TMS) in deuterated chloroform ($CDCl_3$) as the solvent. Melting points were measured by using melting point apparatus equipped with a microscope, Leica Gallen III and were uncorrected. The infrared spectra were taken with a Perkin-Elmer series 1600 spectrophotometer as KBr pellet for solid samples. The Ultraviolet (UV) spectra were recorded on Shimadzu UV 1601PC spectrophotometer. Mass spectral data were obtained from National University of Singapore. Vacuum Liquid Chromatography (VLC) and gravity column chromatography were carried out by using Merck silica gel (230-400 Mesh) and Merck silica gel (70-230 Mesh), respectively. Absorbance for the samples was measured using EPOCH Microplate Spectrophotometer (BioTek Reader).

Extraction and isolation

The dried rhizomes (250 g), pseudostems (250 g) and leaves (271 g) of *A. aquatica* were extracted successively with *n*-hexane (4.0 L), ethyl acetate (4.0 L) and methanol (4.0 L) using a Soxhlet extractor for 8 h. The extracts were concentrated using a rotary evaporator to give the crude extracts. The ethyl acetate pseudostems extract extract (AASE) (2.1 g) was fractionated by vacuum liquid chromatography (VLC) and eluted with nhexane:EtOAc:MeOH to give six fractions (AASE1-6). Fractions AASE5-6 were subjected to column chromatography (CC) over silica gel (800 g, 12×100 cm) eluted with *n*-hexane:$CHCl_3$ (90:10, 80:20, 75:25, 70:30, 60:40, 50:50, 45:55, 40:60, 35:65, 30:70) to afford 100 fractions. Fraction 20-40 was recrystallized from cold *n*-hexane to give 5-hydroxy-3,7,4'-trimethoxyflavone (**1**). The *n*-hexane pseudostems extract (AAEH) (1.8 g) was fractionated by VLC and eluted with *n*-hexane:EtOAc:MeOH to give five fractions (AAEH1-5). Fractions AAEH3-5 were subjected to CC over silica gel (600 g, 5×60 cm) eluted with *n*-hexane:$CHCl_3$ (90:10, 80:20, 70:30, 60:40, 50:50) to afford 150 fraction. Fraction 35-45 was subjected to CC over silica gel (300 g, 5×60 cm) eluted with *n*-hexane:EtOAc to afford 80 fractions. Fraction 50-70 recrystallized from cold n-hexane to yield *cis*-3*S*-(2',4',5'-trimethoxyphenyl)-4*S*-[(*E*)-2''',4''',5'''-trimethoxystyryl] cyclohexene (**4**). The The MeOH leaves extract (AALM) (1.8 g) was fractionated by VLC and eluted with *n*-hexane:EtOAc:MeOH to give five fractions (AALM1-5). Fractions AALM3-5 was subjected to CC over silica gel (20 g, 5×60 cm) eluted with n-hexane:$CHCl_3$ (90:10, 80:20, 75:25, 70:30, 60:40, 50:50) to afford 100 fractions. Fraction 65-80 were combined and followed by preparative TLC (*n*-hexane:EtOAc, 1:1) to yield 4',5-dihydroxy-3,7-dimethoxyflavone (**2**).

Purification of the methanol rhizomes extract (AARM) (2.2 g) was subjected to vacuum liquid chromatography (VLC) on SiO_2 60 (230-400 mesh) using *n*-hexane and $CHCl_3$ in 5% increasing polarity to give 8 fractions (AARM1–8). The combined fractions of AARM1-3 were purified by column chromatography on silica gel 70-230 mesh to afford 2-methoxy-8-(2',4',5'-trimethoxyphenyl)-1,4-naphthaquinone (**3**). The rhizomes ethyl acetate extract (AARE) (2.0 g) was fractionated by VLC on SiO2 70–230 mesh, using nhexane and EtOAc in 10% increasing polarity to give 15 fractions (AARE1–10). The combined fractions AARE2-4 were purified and recrystallized from hexane:$CHCl_3$ to yield 2,4,5-trimethoxybenzaldehyde (**5**). The combined fractions AARE5-6 and AARE7-8 were purified and recrystallized from hexane:$CHCl_3$ to yield stigmasterol (**6**), and β-sitosterol (**7**).

Tyrosinase inhibitory assay

Tyrosinase inhibitory assay was performed according to the previous study with slight modifications.[7,8] Analyses were run in triplicate and the result was expressed as means ±SD of triplicate.

Results and Discussion

The medicinal values possessed by the plant of *Alpinia* genus have led to phytochemical studies by many researchers. It has been reported that hundreds of secondary metabolites have been successfully isolated. In this study, phytochemical investigation on the leaves of *A. aquatica* species has been investigated. Seven compounds have been isolated from *A. aquatica* which are 5-hydroxy-3,7,4'-trimethoxyflavone (**1**), 4',5-dihydroxy-3,7-dimethoxyflavone (**2**), 2-methoxy-8-(2',4',5'-trimethoxyphenyl)-1,4-naphthaquinone (**3**), *cis*-3*S*-(2',4',5'-trimethoxyphenyl)-4*S*-[(*E*)-2''',4''',5'''-trimethoxystyryl]cyclohexene (**4**), 2,4,5-trimethoxybenzaldehyde (**5**), stigmasterol (**6**) and β-sitosterol (**7**). The identification of all compounds was achieved by physical properties: UV, IR, 1D/2D NMR, and MS. These data were also confirmed by comparison with previously reported spectral data. The chemical structures of the isolated compounds as in Figure 1. Many of these constituents were previously isolated from Zingiberaceae family mainly *Alpinia* genus. Compound (**1**) has been isolated previously from *A. flabellata*,[9] while compound (**2**) from *Alpinia purpurata*.[10] Besides, compounds (**3**) and (**4**) have been isolated previously from *Zingiber cassumunar*,[11] while compound (**5**) from *A. flabellata*.[12] In addition, compounds (**6**) and (**7**) have been reported previously from *A. globosa*.[13] Compounds (**3**) and (**4**) were isolated for the first time in the genus of *Alpinia*.

Spectral data

5-Hydroxy-3,7,4'-trimethoxyflavone (**1**) - Yellow crystalline needles (0.03 g, 0.14%); m.p. 130-132°C; IR (KBr): 3445, 1658, 1599, 1442, 1258, 1175 cm^{-1}; UV/Vis λ_{max} ($AlCl_3$/HCl) nm: 211, 276, 302, 344, 401; ^{1}H NMR (400 MHz, $CDCl_3$): 3.88 (3H, s, 3-OCH_3), 3.90 (3H, s, 7-OCH_3),

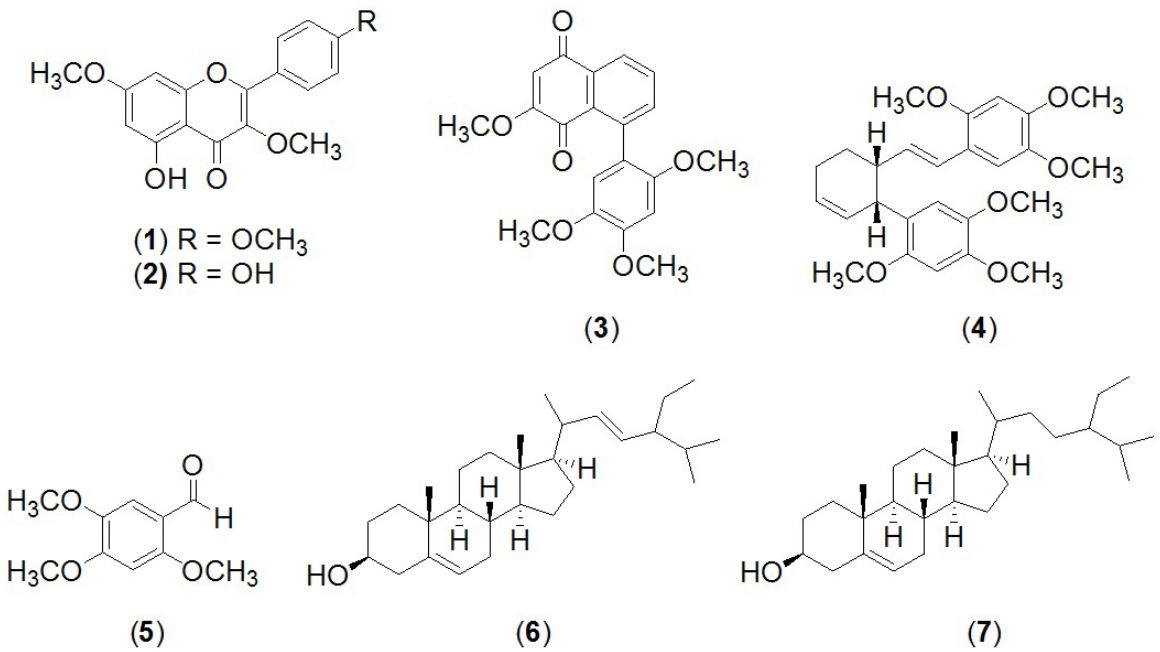

Figure 1. Chemical structures of isolated compounds from *A. aquatica*.

3.93 (3H, s, 4'-OCH_3), 6.48 (1H, d, *J* = 2.0 Hz, H-8), 6.41 (1H, d, *J* = 2.0 Hz, H-6), 7.06 (2H, d, *J* = 8.8 Hz, H-3', H-5'), 8.12 (2H, d, *J* = 8.8 Hz, H-2'/H-6'), 12.69 (1H, s, 5-OH); EIMS *m/z* 328 [M^+, $C_{18}H_{16}O_6$].[9]

4',5-Dihydroxy-3,7-dimethoxyflavone (**2**) - Yellow crystals (0.005 g, 0.02%); m.p. 247-248°C; IR (KBr): 3424, 1657 cm^{-1}; UV/Vis λ_{max} ($AlCl_3$/HCl) nm: 211, 276, 303, 349, 398; ^1H NMR (400 MHz, $CDCl_3$): 3.89 (3H, s, 3-OMe), 3.94 (3H, s, 7-OMe), 6.69 (1H, d, *J* = 2.4 Hz, H-8), 6.34 (1H, d, *J* = 2.4 Hz, H-6), 7.05 (2H, d, *J* = 8.8 Hz, H-3'/H-5'), 8.08 (2H, d, *J* = 8.8 Hz, H-2'/H-6'), 9.53 (1H, s, 4'-OH), 12.78 (1H, s, 5-OH); EIMS *m/z* 314 [M^+, $C_{17}H_{14}O_6$].[10]

2-Methoxy-8-(2',4',5'-trimethoxyphenyl)-1,4-naphthaquinone (**3**) - Orange crystals (0.012 g, 0.35%); m.p. 162-163°C; IR (KBr): 1685, 1644, 1614 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): 3.67 (3H, s, 2'-OCH_3), 3.86 (3H, s, 5'-OCH_3), 3.87 (3H, s, 2-OCH_3), 3.96 (3H, s, 4'-OCH_3), 6.16 (1H, s, H-3), 6.59 (1H, s, H-3'), 6.72 (1H, s, H-6'), 7.55 (1H, dd, *J* = 7.6, 1.6 Hz, H-7), 7.74 (1H, t, *J* = 7.6 Hz, H-6), 8.17 (1H, dd, *J* = 7.6, 1.6 Hz, H-5); EIMS *m/z* 354 [M^+, $C_{20}H_{18}O_6$].[11]

cis-3*S*-(2',4',5'-trimethoxyphenyl)-4*S*-[(*E*)-2''',4''',5'''-trimethoxystyryl]cyclohexene (**4**) - Colourless crystalline needles (0.04 g, 1.19%); m.p. 133-134°C; IR (KBr): 2933, 1607, 1519, 1206, 1036, 871 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): 1.78 (2H, m, H-5α/β), 2.23 (2H, m, H-6α/β), 2.82 (1H, m, H-4), 3.88 (6H, s, 5'''-OCH_3/5'-OCH_3), 3.81 (6H, s, 4'''-OCH_3/4'-OCH_3), 3.75 (3H, s, 2'''-OCH_3), 3.71 (3H, s, 2'-OCH_3), 4.13 (1H, m, H-3), 5.74 (1H, d, *J* = 9.6 Hz, H-2), 5.83 (1H, dd, *J* = 16.0, 8.4 Hz, H-1''), 6.01 (1H, d, *J* = 9.6 Hz, H-1), 6.46 (1H, s, H-6'''), 6.48 (1H, s, H-6'), 6.49 (1H, d, *J* = 16.0 Hz, H-2''), 6.73 (1H, s, H-3'''), 6.79 (1H, s, H-3'); EIMS *m/z* 440 [M+, $C_{26}H_{32}O_6$].[11]

2,4,5-Trimethoxybenzaldehyde (**5**) - Colourless crystals (0.005 g, 0.15%); m.p. 112-113°C; IR (KBr): 2924, 1613, 1457 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): 3.91 (3H, s, 2-OCH_3), 3.95 (3H, s, 5-OCH_3), 4.00 (3H, s, 4-OCH_3), 6.52 (1H, s, H-6), 7.36 (1H, s, H-3),10.35 (1H, s, CHO); EIMS *m/z* 196 [M^+, $C_{10}H_{12}O_4$].[12]

Stigmasterol (**6**) - White solids (0.02 g, 0.59%); m.p. 158-159°C; IR (KBr): 3439, 2932, 1639 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): 5.38 (2H, d, *J* = 5.2 Hz, H-6'), 5.17 (1H, dd, *J* = 15.2, 8.4 Hz, H-22'), 5.03 (1H, dd, *J* = 15.2, 8.4 Hz, H-23'), 3.55 (2H, m, H-3'), 0.95 (3H, d, *J* = 7.6 Hz, H-26'), 0.94 (6H, d, *J* = 6.8 Hz, H-21'), 0.89 (6H, s, H-18'), 0.88 (6H, t, *J* =7.2 Hz, H-29'); GC-MS *m/z* 412 [M^+, $C_{29}H_{48}O$].[13]

β-Sitosterol (**7**) - White solids (0.02g, 0.59%); m.p. 160-161°C; IR (KBr): 3439, 2932, 1639 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): 5.38 (2H, d, *J* = 5.2 Hz, H-6), 3.55 (2H, m, H-3), 0.94 (6H, d, *J* = 6.8 Hz, H-21), 0.89 (6H, s, H-18), 0.88 (6H, t, *J* =7.2 Hz, H-29), 0.71 (3H, d, *J* = 7.2 Hz, H-26); GC-MS *m/z* 414 [M^+, $C_{29}H_{50}O$].[13]

Tyrosinase, a multifunctional copper-containing oxygenase that is widely distributed in nature catalyzes the hydroxylation of a monophenol and the conversion of O-diphenols to the corresponding O-quinones.[14] Tyrosinase inhibitors are also imperative in cosmetic applications for skin whitening effects.[15] Since plants are a rich source of bioactive chemicals, and mostly free of harmful side effects, there is an increasing interest in finding natural tyrosinase inhibitors from them. In the present study, tyrosinase inhibitory activity of extracts and compounds (**1-4**) were investigated using L-DOPA as substrate and mushroom tyrosinase as the enzyme. The positive control used in mushroom tyrosinase assay was kojic acid as it has been understood to act as a potent tyrosinase inhibitor. Figure 2 shows the percentage of inhibitory activity at 1 mg/mL. The percent inhibition of tyrosinase activity expressed as mean of duplicate independent experiments. The ethyl acetate extract of pseudostems possessed the highest

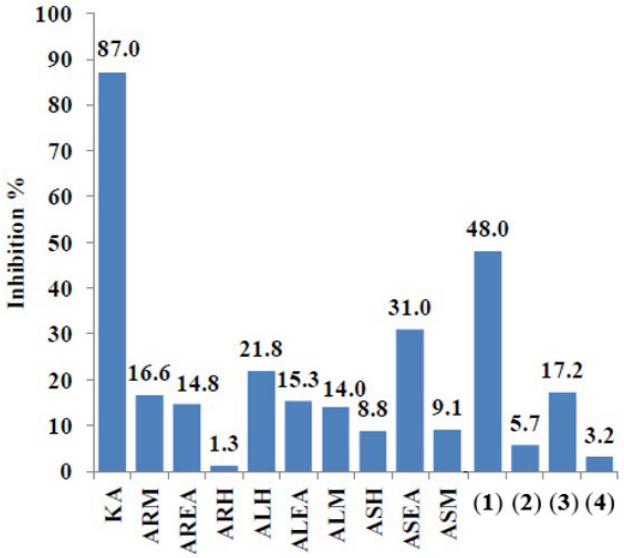

Figure 2. Percentage inhibition of tyrosinase activity of extracts and compounds (**1-4**). (KA = Kojic acid; ASH = A. *aquatica* pseudostem hexane, ASEA = *A. aquatica* pseudostem ethyl acetate, ASM = *A. aquatica* pseudostem methanol, ALH = *A. aquatica* leaves hexane, ALEA = *A. aquatica* leaves ethyl acetate, ALM = *A. aquatica* leaves methanol, ARH = *A. aquatica* rhizomes hexane, AREA = *A. aquatica* rhizomes ethyl acetate, ARM = *A. aquatica* rhizomes methanol).

percent of tyrosinase inhibition of 31.0%. Among the isolated compounds, compound (**1**) possessed the highest percentage of tyrosinase inhibition, 48.0% among the other isolated compounds. Tyrosinase inhibition percentage of the isolated compounds varied from 3.2-48.0% which are lower than that of positive control, kojic acid (87.0%). It has been reported in the previous study that *Alpinia* species can inhibit tyrosinase enzyme and reduce melanogenesis.[16,17] Furthermore, flavonoids are well known to show good inhibition of tyrosinase activity.[18] In this case, flavonoids were the major phytochemicals isolated from *A. aquatica* seems to justify the tyrosinase inhibition.

Conclusion

In conclusion, our study led to the isolation and identification of seven phytochemicals from *A. aquatica*, and compound (**1**) exhibited moderate tyrosinase inhibitory activity. These findings support further research to investigate the mechanism of an isolated compound from the extract for better understanding as a potential inhibitor for tyrosinase and may be of interest to clarify the physiological role of the enzyme.

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Conflict of Interests

The authors claim that there is no conflict of interest.

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