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Structure activity relationship of flavonoids isolated from angelica shikokiana makino and other analogues as acetylcholine esterase inhibitors

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ABSTRACT: Five flavonoids, namely, kaempferol, luteolin, quercetin, kaempferol-3-*O*-rutinoside, and kaempferol-3-*O*-glucoside were isolated from the aerial part of *Angelica shikokiana* and were examined for acetylcholine esterase inhibition using the Ellman's method. To understand the structure activity relationship, other 17 flavonoids from different classes were also tested. The results showed that 3, 3', and 4' -OH groups are essential for revealing potent activity. Increasing number of OH gp. at ring B increases the activity. Position of hydroxylation at ring B significantly affects the activity. Replacement of hydroxyl gp. at 4' of flavones by OCH_3 results in insignificant decrease in activity. While in flavonols, replacement of hydroxyl gp. at 3' with OCH_3 abolishes the activity. On the other hand, 3' -OH and 4' –OCH_3 gps. in flavane class increase the activity significantly. The double bond at C-2, C-3 is essential for the activity. The β-linkage of ring B to ring Cat C-2 is more active than α-linkage. Attachment of ring B at C-2 is essential for activity. Glycosylation causes significant increase in activity comparing with the activity of their aglycons. The activity of biflavonoid class depends on the site of the second flavonoid attachment.

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1. INTRODUCTION

The main risk factor of neurological diseases is age. For example, it was reported in the USA that people aged 90 years or older accounted for 79.5% of Alzheimer's disease cases compared to 46.7% for those between 71 and 79 years old (Brookmeyer et al., 2011). Meanwhile, there are many data showing strong links with vascular risk factors and other chronic disorders such as hypertension, diabetes, and obesity (Qiu et al., 2009). Strategies that reduce the risks of developing chronic diseases are therefore considered useful in preventing or delaying the degeneration of neurons. Nutritional strategies have been investigated. Some include nutrients like omega-3 fatty acids, folate, vitamin B12, and antioxidants (vitamins A, E, and C) and some include plant secondary metabolites (e.g., polyphenols) (Mohajeri et al., 2015).

AD is a progressive, age-related neurodegenerative disorder that gradually deteriorates over time and is responsible for approximately 60%–80% of dementia cases. The disease is marked by worsening cognitive decline, memory impairment, and behavioral disturbances (Talesa, 2001).

AD is a chronic and progressive neurological disorder that gradually impairs memory and cognitive abilities, leading to irreversible brain deterioration over time. Pathology of the disease is associated with increased oxidative stress or decreased cholinergic response or aggregation of Amyloid β-peptide (Kihara, & Shimohama, 2004).

Inhibition of acetylcholinesterase (AChE), the enzyme responsible for the breakdown of acetylcholine (ACh), increases ACh levels and subsequently helps alleviate the severity of the disease (Racchi et al., 2004).

Flavonoids are a major class of phenolic compounds known for their antioxidant, antitumor, anti-inflammatory, and neuroprotective properties. These neuroprotective activities may be utilized in the development of therapeutic approaches for neurodegenerative disorders, including AD and Parkinson's disease (Parasram, 2017).

Flavonoids have emerged as promising natural candidates for the treatment of AD. Currently, the most effective symptomatic therapies for AD are acetylcholinesterase inhibitors (AChEIs). Owing to their AChE inhibitory potential along with their well-established antioxidant properties, flavonoids may serve as novel multifunctional agents for the management of AD (Uriarte-Pueyo, & Calvo, 2011).

A. shikokiana is a perennial herb belonging to the Apiaceae family and is commonly known in Japan as Inutouki or Yamaninjin. The plant has traditionally been used as an alternative to ginseng roots in herbal medicine (Kimura, & Okuda, 1989).

A previous study (Mira, et al., 2013) demonstrated that the ethanol extract obtained from the aerial parts (stems and leaves) of *A. shikokiana* exhibited neuroprotective activity against H_2O_2-induced neurotoxicity in Neuro-2A cells, in addition to showing AChE inhibitory effects. Neuroprotective properties have also been reported in other Angelica species, including *Angelica sinensis* (Park et al., 2012) and *Angelica dahurica* (Moon et al., 2012).

A. shikokiana is extensively marketed in Japan as a dietary supplement. In a study focusing on neurodegenerative disorders such as AD, 15 compounds were isolated from the aerial parts of the plant, including α-glutinol, β-amyrin, kaempferol, luteolin, quercetin, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, methyl chlorogenate, chlorogenic acid, hyuganin E, 5-(hydroxymethyl)-2-furaldehyde, β-sitosterol-3-O-glucoside, adenosine, isoepoxypteryxin, and isopteryxin.

The isolated compounds were investigated for their in vitro neuroprotective potential through several assays, including acetylcholinesterase inhibition, protection against hydrogen peroxide, amyloid β peptide (Aβ25–35)-induced neurotoxicity in Neuro-2A cells, hydroxyl radical and intracellular reactive oxygen species scavenging, and thioflavin T assays. Among the tested compounds, quercetin demonstrated the strongest AChE inhibitory activity with an IC_{50} value of 35.5 μM, mediated through interactions with His-440 and Tyr-70 residues at the catalytic and anionic binding sites of the enzyme, respectively.

In addition, chlorogenic acid, methyl chlorogenate, quercetin, and luteolin significantly protected Neuro-2A cells from H_2O_2-induced neurotoxicity and effectively scavenged hydroxyl radicals and intracellular reactive oxygen species. Furthermore, kaempferol-3-O-rutinoside, hyuganin E, and isoepoxypteryxin markedly reduced Aβ25–35-induced neurotoxicity and thioflavin T fluorescence (Mira et al., 2015).

2. RESULTS AND DISCUSSION

The results showed that 3, 3', and 4' -OH groups are essential for revealing potent activity (e.g.; quercetin: IC_{50}=35.5 μM) while lacking 3-OH gp. (luteolin; 25% inhibition) or 3'-OH gp. (kaempferol; 30% inhibition) or 3',4' -OH gps. (galangin; 25% inhibition) or 3, 3'-OH gps. (apigenin; 15% inhibition) or 3,3', 4'-gps. (baicalein and chrysin; 10 and 13%, respectively) that decreases the activity significantly. So, flavonoles are more active than flavones. The presence of C-3 OH increase the activity. The presence of 3' or 4' hydroxyl group is essential for the activity, and their absence leads to inactivity of the compound (chrysin) or decreases the activity (galangin).

Increasing number of OH gps. at ring B increases the activity (luteolin and quercetin are more active than apigenin

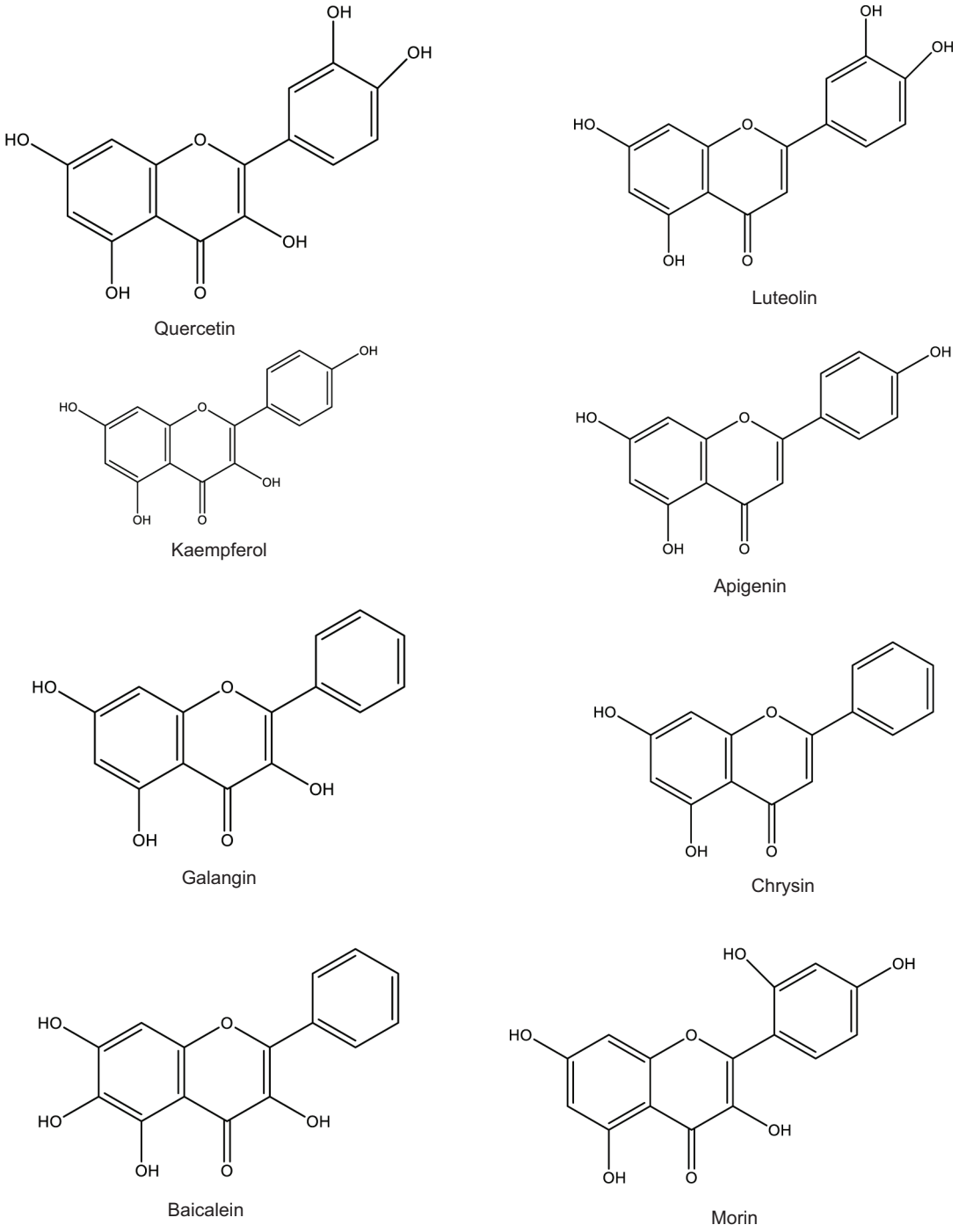
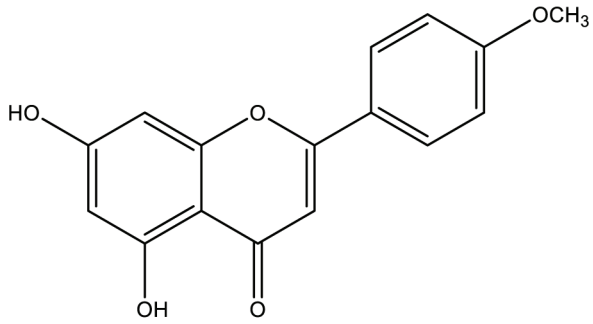


Figure 1. Effect of ring B hydroxylation and C-3 OH.

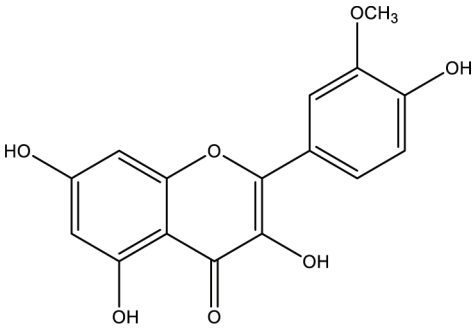
and kaempferol, respectively). Presence of 3' and 4' hydroxyl groups in ring B (luteolin and quercetin) is more active than 4' alone (apigenin and kaempferol). Position of hydroxylation at ring B significantly affects the activity; 3',4' -OH gps. (quercetin) are nearly 100 times more active than OH gps. at C-2', C-4' (Morin, IC_{50}=325 µM). So, the position of ring B hydroxylation significantly affects the activity (Figure 1).

Replacement of hydroxyl gp. at 4' of flavones (apigenin, 15% inhibition) by OCH_3 results in insignificant decrease in activity (acacetin, 13% inhibition) while in flavonols, replacement of hydroxyl gp. at 3' with OCH_3 abolishes the activity (isorhamnetin is inactive compared with quercetin). (Figure 2)

On the other hand, 3'-OH and 4'–OCH_3 gps. in the flavane class increase the activity significantly (hesperitin,

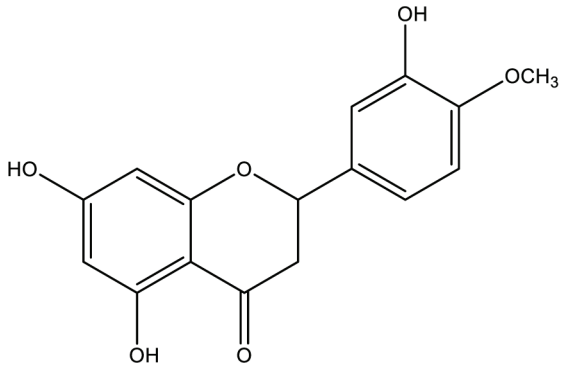


Acacetin

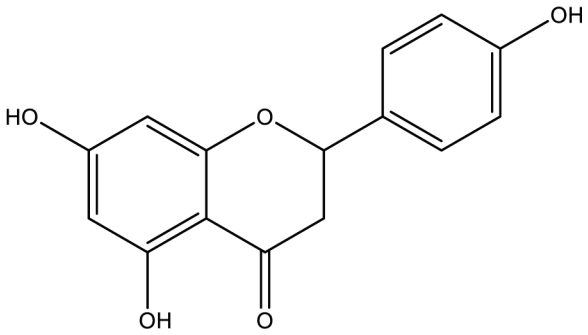


Isorhamnetin

Figure 2. Effect of replacing OH by OCH_3 group at 3' or 4' in flavones and flavonols.



Hesperetin



Naringenin

Figure 3. Effect of OH at 3' and OCH_3 group at 4' in flavanes.

IC_{50} = 66.9 μM while naringenin is inactive) (Figure 3). Comparing the activity of apigenin (flavone) with naringenin (flavanone), it is found that double bond at C-2, C-3 is essential for the activity (Figure 4). The β-linkage of ring B to ring C at C-2 (e.g.; epicatechin, 34% inhibition) is more active than α-linkage (catechin, 7% inhibition). Attachment of ring B at C-2 is essential for activity, and its attachment at C-3 results in abolishing the activity (genistein, inactive). (Figure 5).

Glycosylation, whatever the position, of all flavonoid groups causes significant increase in activity comparing with the activity of their aglycons (e.g., naringin; glycosylation at C-7; showed 25% inhibition while aglycon naringenin is inactive, robinin, kaempferol-3-O-glucoside, and kaempferol-3-O-rutinoside; glycosylation at C-3; with IC_{50} =70, 80.3, and 50.4 μM, respectively, are more active than aglycone kaepmferol and also puerarin; glycosylation at C-8; shows 30 % inhibition while its aglycon genistein is inactive) (Figure 6).

Also, the activity of kaempferol increases by glycosylation at C-3 either by glucose (Kaempferol-3-O-glucoside) or gluco-rhamnose (Kaempferol-3-O-rutinoside) or at C-3 by robinose and C-7 by rhamnose (robinin) (Figure 7).

The activity of the biflavonoid class depends on the site of the second flavonoid attachment. The C-C attachment of the second flavonoid moiety at C-3' (amentoflavone, IC_{50} = 90.2 μM) gave higher activity than its attachment at C-4' OH gp. (hinokiflavone, 32% inhibition) (Figure 8).

3. MATERIALS AND METHODS

3.1. Reagent and chemicals

AChE derived from electrophorus electricus, with a specific activity of 500 U/mg, along with 17 flavonoids, were obtained from Sigma-Aldrich. Acetylthiocholine iodide (ACTI) was purchased from Tokyo Chemical Industry, while 5,5-dithiobis[2-nitrobenzoic acid] (DTNB) was supplied by FUJIFILM Wako Pure Chemical Corporation. All remaining chemicals were acquired from Merck.

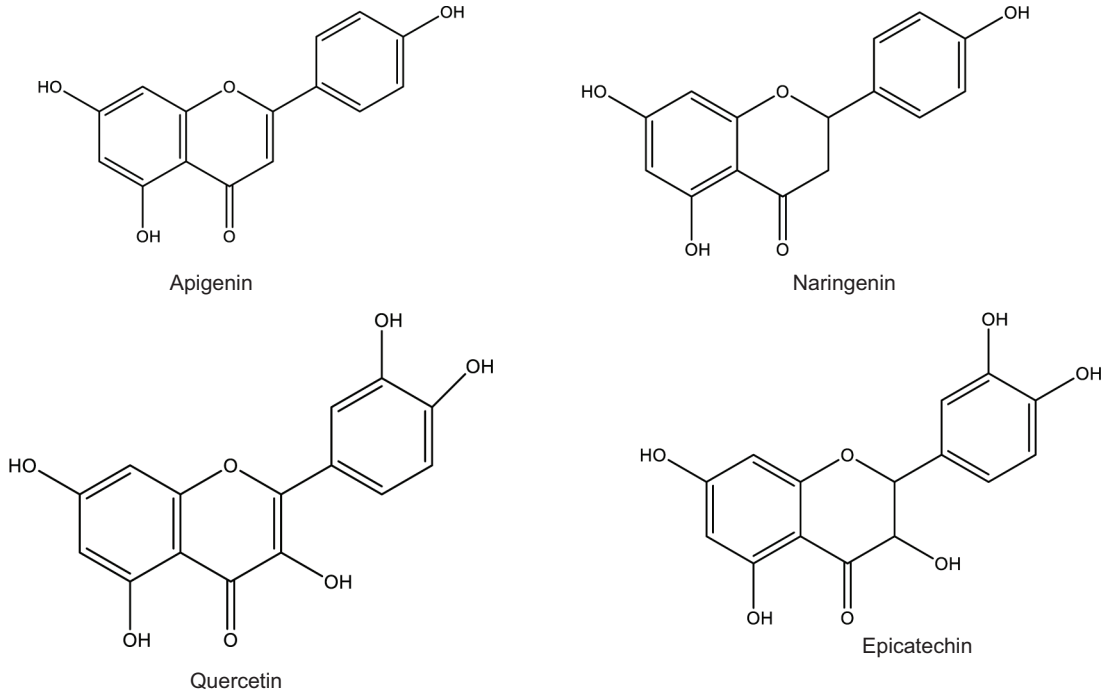


Figure 4. Effect of double bond at C-2, C-3.

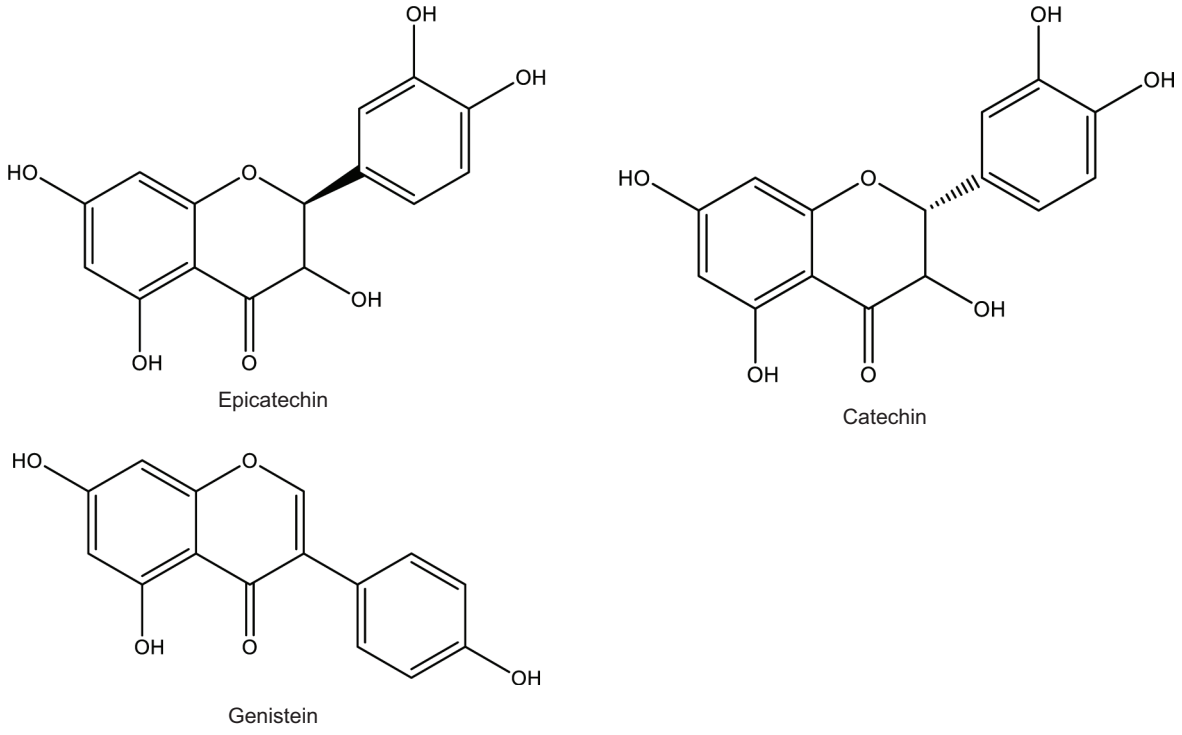


Figure 5. Effect of α and β linkage of ring B to ring C at C-2 and attachment at C-3.

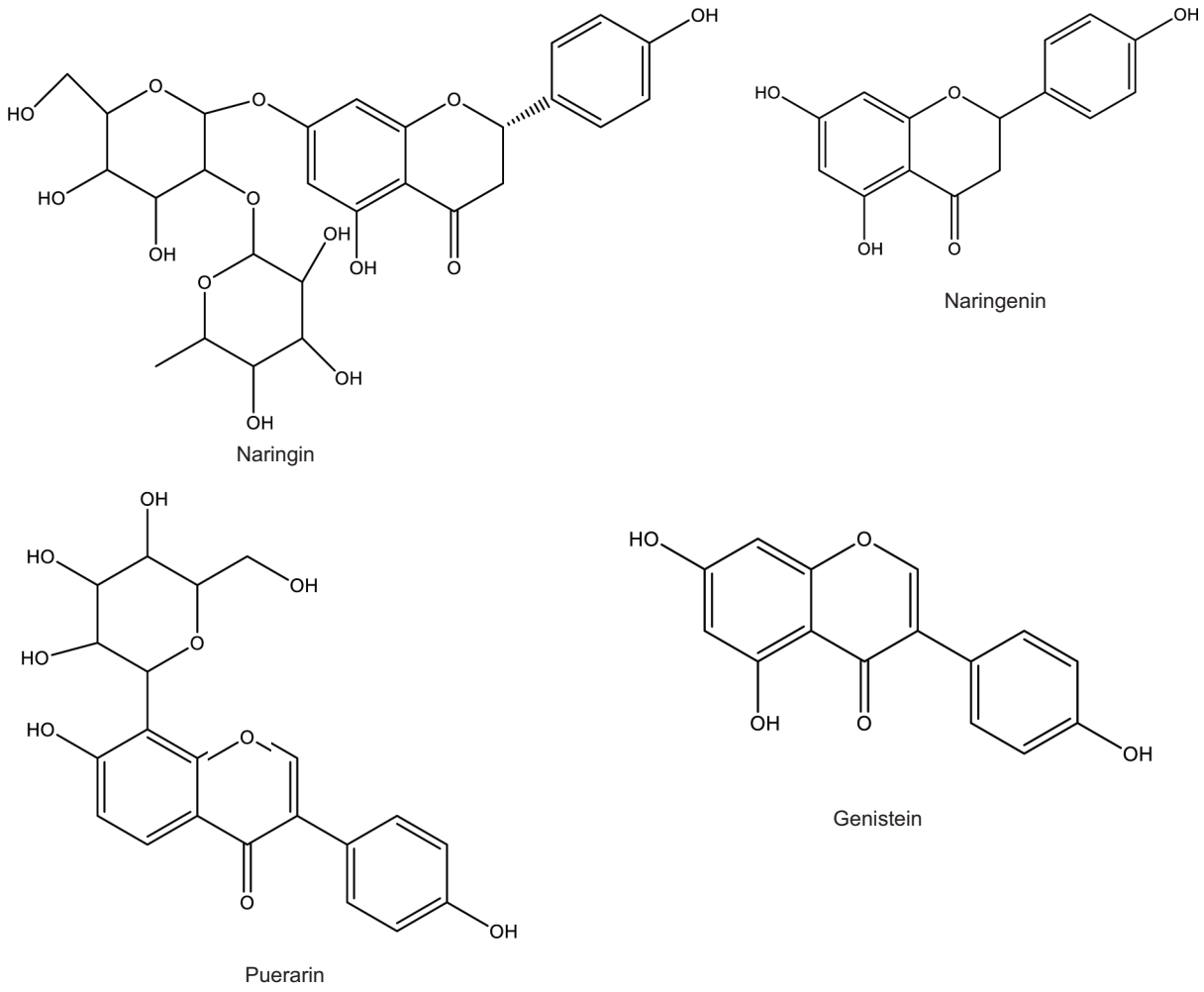


Figure 6. Effect of glycosylation on activity.

The aerial parts of *A. shikokiana* were extracted, and bioassay-guided fractionation led to the isolation of 15compounds, namely, α-glutinol, β-amyrin, kaempferol, luteolin, quercetin, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, methyl chlorogenate, chlorogenic acid, hyuganin E, 5-(hydroxymethyl)-2-furaldehyde, β-sitosterol-3-O-glucoside, adenosine, isoepoxypteryxin, and isopteryxin. Notably, adenosine was isolated from *A. shikokiana* for the first time (Mira, et al., 2015).

3.2. Experimental

3.2.1. ACh Esterase inhibitory assay

AChE inhibition activity was determined using Ellman's method [14,15]. In this assay, AChE hydrolyzes the substrate ACTI into acetate and thiocholine. In neutral to alkaline conditions, thiocholine reacts with 5,5-dithiobis[2-nitrobenzoic acid] (DTNB) to produce a yellow-colored 2-nitro-5-thiobenzoate anion, which is quantified spectrophotometrically at 405 nm.

Briefly, in a 96-well microplate, a reaction mixture containing 25 µL of 15 mM ACTI, 125 µL of 3 mM DTNB in buffer B (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.02 M $MgCl_2 \cdot 6H_2O$), 50 µL of buffer A (50 mM Tris-HCl, pH 8.0, 0.1% BSA), and 25 µL of the test sample (dissolved in 25% DMSO) was prepared, and absorbance was recorded at 405 nm every 16 seconds for 10 readings using a microplate reader.

Subsequently, 25 µL of AChE solution (0.25 U/mL in buffer A) was added, and absorbance measurements were again taken 10 times at 16-second intervals. A 25% DMSO solution served as the negative control. Enzyme activity was calculated from the slope of absorbance versus time and expressed as a percentage relative to a control assay without an inhibitor. Flavonoids were evaluated at a concentration of 400 µg/mL. To correct for interference from compound color or spontaneous substrate hydrolysis, the pre-enzyme

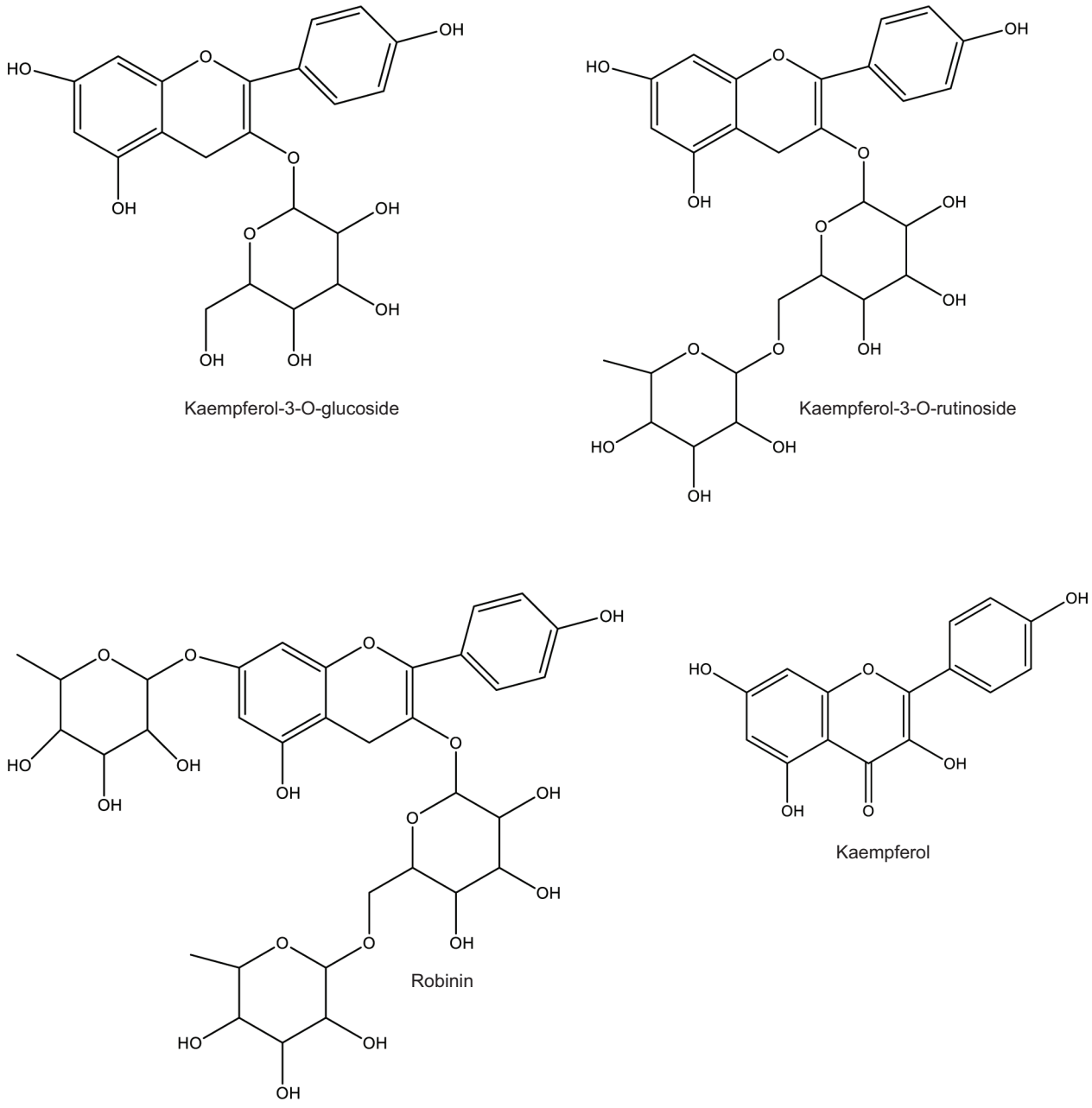


Figure 7. Effect of glycosylation at C-3, C-7.

addition absorbance was subtracted from post-enzyme readings (Figures 9 and 10).

Five flavonoids, namely, kaempferol, luteolin, quercetin, kaempferol-3-O-rutinoside, and kaempferol-3-O-glucoside were isolated from the aerial part of *A. shikokiana* and were examined for ACh esterase inhibition using the Ellman's method. (Ellman et al., 1961)

To understand the structure activity relationship, other 17 flavonoids from different classes were also tested. IC_{50} was calculated for compounds with activity > 50% inhibition. The compounds with activity < 50% inhibition were determined as % inhibition at 250 µM (Mukherjee et al., 2007).

3.3. Statistical analysis

Acetylcholinesterase inhibition IC_{50} values of flavonoids were determined using Probit analysis in SPSS software

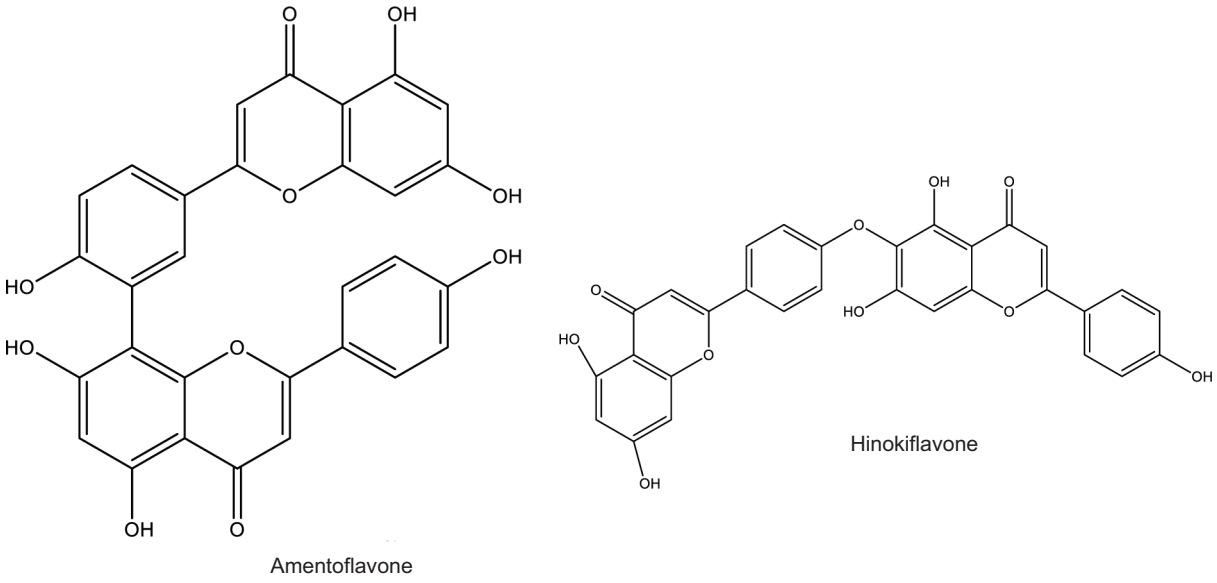


Figure 8. The attachment of bioflavonoids.

Table 1
Results of acetylcholine esterase inhibitor assay.

Compound	IC_{50}
I-Flavones	
1-Luteolin	>500 (30% at 250 µM)
2-Baiclein	>500 (10% at 250 µM)
3-Apigenin	>500 (15% at 250 µM)
4- Acacetin	>500 (13% at 250 µM)
5-Chrysin	Inactive
II-Flavonols	
1-Quercetin	35.5
2-Galangin	>500 (25% at 250 µM)
3-Morin	325
4-Kaempferol	>500 (30% at 250 µM)
5-Isorhamnetin	Inactive
III-Flavanes	
1-Naringenin	Inactive
2-Hesperitin	66.9
3- Epicatechin	>500 (34% at 250 µM)
4-Catechin	>500 (7% at 250 µM)
IV-Isoflavones	
Genistein	Inactive
V-Flavonoid glycosides	
1-Robinin	69.8 (at 125 µM 55%)
2-Kaempferol-3-O-glucoside	50.4 (at 125 µM 60%)
3-Kaempferol-3-O-rutinoside	80.3 (at 125 µM 70%)
4-Naringin	>500 (25% at 250 µM)
5-Puerarin	>500 (30% at 250 µM)
VI-Biflavonoids	
1-Amentoflavone	92.2
2-Hinokiflavone	>500 (32% at 250 µM)

(Version 16.0 for Windows; SPSS Inc., Chicago, IL, USA) based on five different concentration levels. All results were expressed as mean ± standard deviation (SD).

Statistical significance was assessed using one-way ANOVA, followed by Dunnett's post-hoc test, performed with GraphPad Prism software (Version 5.0 for Windows; GraphPad Software Inc., San Diego, CA, USA).

AUTHOR CONTRIBUTIONS

Amira Mira: Research concept and design, Asmaa E. Sherif: Collection and/or assembly of data, Amira Mira and Asmaa E. Sherif: Data analysis and interpretation, Asmaa E. Sherif: Writing the article, Ahmed Ashour: Critical revision of the article, Kuniyoshi Shimizu: Final approval of the article.

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CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

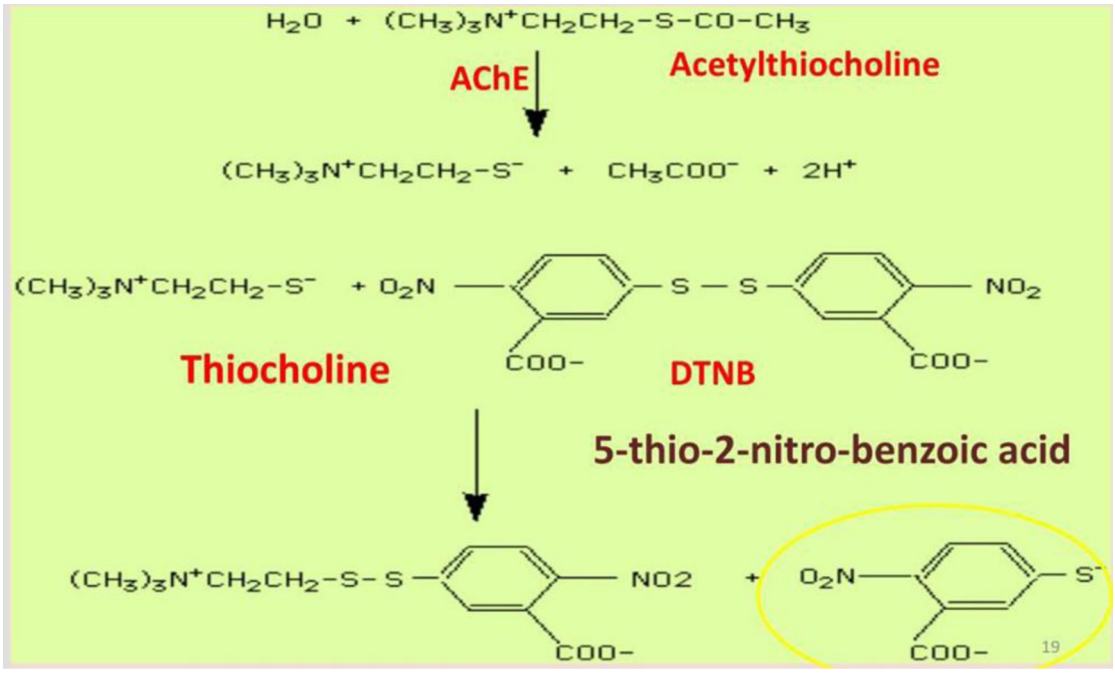

Figure 9. Principle of acetylcholine esterase inhibitory assay.

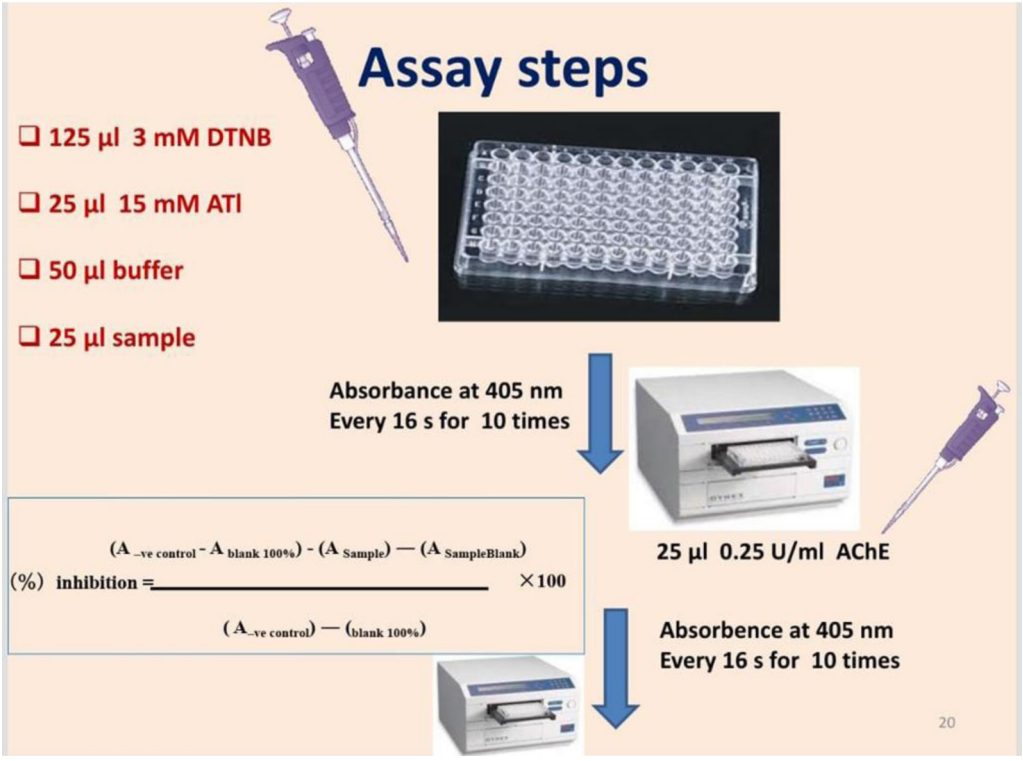

Figure 10. Acetylcholine esterase inhibitory assay method.

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ABBREVIATIONS

AD, Alzheimer's disease; AchE, Acetylcholinesterase; Ach, Acetyl choline; AChEI, Acetylcholinesterase inhibitors; ACTI, Acetylthiocholine; DTNB, 5-thio-2-nitro-benzoic acid; Gp, Group.

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