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5-*O*-Demethylnobiletin, a polymethoxylated flavonoid, from *Citrus depressa* Hayata peel prevents protein glycation

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ABSTRACT

Advanced glycation end products (AGEs) have been implicated in many age-related chronic diseases and the search for AGEs has intensified. In the present study, eight Okinawan edible or medicinal plants were screened for the antiglycation activity. A polymethoxylated flavonoid, 5-*O*-demethyl nobiletin (DN), was isolated from the chloroform fraction of *Citrus depressa* Hayata (Rutaceae) and was identified for the first time as an AGEs inhibitor. DN prevented protein glycation significantly better than aminoguanidine (AG) with the respective IC_{50} values of 64.2 and 484.3 μM (p = 0.01). Further studies revealed that DN prevented AGEs formation predominantly by inhibiting fructosamine adduct formation while only partly by preventing dicarbonyl generation. Together, these results suggest that further exploration on the identification of AGEs inhibitors from Okinawan plants and in-depth investigations on the AGEs inhibitions by polymethoxylated flavonoids are required.

Keywords: Antioxidant; Advanced glycation endproducts (AGEs); Polymethoxlated flavonoids; 5-*O*-Demethyl nobiletin; Okinawa

1. Introduction

Advanced glycation end products (AGEs) are a complex and heterogeneous group of compounds that have been implicated in many age-related chronic diseases and in protein ageing. These products are associated with diabetic complications, neurodegenerative diseases, cancer and the normal ageing process (Heijst, Niessen, Hoekman, & Schalkwijk, 2006; Negre-Salvayre et al., 2010). The formation of AGEs begins with the autoxidation of glucose and further interactions with proteins will generate several intermediates including Schiff's base, Amadori products, hydroperoxides, and carbonyl compounds.

The inhabitants of Okinawa have traditionally utilized plants as medicinal herbs or in preparing characteristic Okinawan foods. Some researchers have indicated that the intake of typical Okinawan medicinal and edible plants contributes to the longevity of the local people since these plants are rich in phytochemicals with a broad range of bioactivities (Elzaawely, Xuan, & Tawata, 2007; Upadhyay, Chompoo, Kishimoto, Makise, & Tawata, 2011; Upadhyay, Chompoo, Taira, Fukuta, Gima, & Tawata, 2011; Upadhyay, Chompoo, Taira, Fukuta, & Tawata, 2013).

Although antioxidant activities of these plants are well investigated, very few studies on the prevention of AGEs formation have been conducted. A study on *Alpinia zerumbet* has identified kawain and labdadiene as AGEs inhibitors (Chompoo, Upadhyay, Kishimoto, Makise, & Tawata, 2011). Other studies focused on the antihyperglycemic properties; the extracts of *M. charantia* have been extensively studied for anti-diabetic properties (Miura et al., 2001; Virdi, Sivakami, Shahani, Suthar, Banaalikar, & Biyani, 2003). Besides one antiglycation study and a few other antihyperglycemic studies, no previous reports related to the antiglycation activity of the Okinawan plants could be found. Therefore, in this study, the AGEs inhibitory activity of several edible and medicinal plants from Okinawa was investigated. The plants examined in this study are typically present in the Okinawan cuisine,

particularly, *Citrus depressa*, *Caulerpa lentillifera*, *Cladosiphon okamuranus*, and *Momordica charantia*. Others like *A. zerumbet* leaves and *Garcinia subelliptica* fruits are either used in traditional preparations or consumed locally. Finally, a polymethoxylated flavonoid was isolated from one of the fractions and was identified for the first time as an AGEs inhibitor.

2. Material and methods

2.1 Chemicals and reagents

Glucose and bovine serum albumin (BSA) were purchased from Merck, Germany, whereas all other chemicals were bought from Sigma-Aldrich. All the solvents used were of HPLC grade.

2.2 Plant materials extraction and isolation of bioactive principle

The plant materials (Table 1) was either collected or bought in local markets of Okinawa, Japan and were verified by one of the co-authors (AU). The methanolic extract of the dried plant parts were partitioned successively with *n*-hexane, chloroform and ethyl acetate. The most active compound was isolated from the chloroform fraction of *C. depressa* peel. The compound identified was 5-*O*-Demethyl nobiletin (DN) *m/z* 389.1 $[M + H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) : δ 12.72 s (1H, 5-OH); δ 7.72 dd (1H, *J* = 8.86, 2.2 Hz, H-6'); δ 7.59 d (1H, *J* = 2.1 Hz, H-2'); δ 7.19 d (1H, *J* = 8.7Hz, H-5'); δ 7.09 s (1H, H-3); δ 4.02, 3.93, 3.88, 3.86, 3.82 s (15H, -OCH_3).

2.3 Inhibition of protein glycation

The inhibition of protein glycation was measured as described by Chompoo et al. (2011), with slight modifications. Briefly, the reaction mixture (500 μL) containing 400 μg BSA, 200

mM glucose and test compounds, in DMSO, at different final concentrations (10 – 500 μM) were incubated at 37^{o}C for one week. Sodium azide (0.02%) was added to prevent bacterial growth. The change in fluorescence intensity (excitation 360 nm, and emission 450 nm) based on AGEs formation was monitored using a spectrofluorometer (Tecan Infinite M200). In order to reduce the interference in the fluorescence signal by the test compounds, parallel incubation at 4^{o}C was performed for all the samples (Upadhyay et al., 2014). The AGEs inhibition was calculated as

% AGEs inhibition = [1-(S-Sb)/(C-Cb)] x 100

where S and C represent relative fluorescence units (RFU) for test samples (in DMSO) and control (test mixtures containing only DMSO) incubated at 37^{o}C, and where Sb and Cb are RFU for samples incubated at 4^{o}C. The concentration required for 50% inhibition (IC_{50}) was determined graphically.

2.4 Measurement of fructosamine adduct and α-dicarbonyl compounds formation

The fructosamine adduct was determined by using the NBT assay and the α-dicarbonyl compounds formation was measured using Girard-T reagent, as described previously (Chompoo et al., 2011).

2.5 Antioxidant activity (DPPH scavenging) and total phenolic content

The DPPH radical scavenging activity and the total phenolic content of the plant extracts and fractions were determined as reported previously (Elzaawely et al., 2007).

2.6 Statistical analysis

The data were analysed by one-way ANOVA. Upon significant difference, means were separated using Tukey HSD range test at $p = 0.01$ with three replications. . Correlations were

determined using Pearson correlation coefficient. All statistical analyses were performed using SPSS Version 20.0 for Windows Vista (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 AGEs inhibition by crude extracts and different fractions

The AGEs inhibition assay revealed that *C. depressa* had significantly better inhibitory activity than other plants and the reference product aminoguanidine (Table 2; $p = 0.01$). Other plants with significant activity were *G. subelliptica* and *L. leucocephala*. On carrying out the inhibition with different fractions, it was found that the chloroform fraction of *C. depressa* had the highest inhibitory activity (63.0 %) followed by ethyl acetate fraction of *G. subelliptica* (45.5 %) (Table 2), and were significantly more active than aminoguanidine, used as a positive control, which had an inhibition of 18.1% ($p = 0.01$). Other plant fractions with considerable AGEs inhibitory activities were *L. leucocephala*, *Miscanthus sinensis*, and *A. zerumbet*.

3.2 AGEs inhibition by 5-O-demethyl nobiletin

The most active fraction obtained from the AGEs inhibitory assay, the chloroform fraction of *C. depressa* was further investigated and 5-*O*-demethylnobiletin (DN) was isolated and identified. It was found that DN had significantly higher AGEs inhibitory activity (IC_{50} = 64.2 ± 3.6 μM) than aminoguanidine (AG; IC_{50} = 484.3 ± 7.3 μM; $p = 0.01$) (Table 3). At a concentration of 100 μM, DN inhibited AGEs formation for more than 60% and at 200 μM the inhibition was 71%. To achieve the same level of inhibition, 900 μM of AG was required (Fig. 1A). Furthermore, 200 μM of DN was significantly superior to 450 μM of AG (at $p = 0.01$), and 100 μM of DN had better inhibitory effect than AG at $p = 0.05$ (Fig. 1A).

3.3 Inhibitory effect of DN on fructosamine adduct and glyoxal formation

The results of the inhibitory effect of DN on fructosamine adduct showed that DN (IC_{50} = 264.3 ± 11.3 μM) was significantly stronger than aminoguanidine (IC_{50} > 1000 μM) (Fig. 1B; p = 0.01). However, DN did not have better activity in preventing dicarbonyl formation than aminoguanidine (Fig. 1C).

4. Discussion

In the present study, the anti-glycation activities of methanolic extracts and ethyl acetate and chloroform fractions of eight Okinawan plants were studied. The rank order of anti-AGEs potency of the crude extracts was: *C. depressa* > *G. subelliptica* > *L. leucocephala* > *A. zerumbet* > *C. lentillifera* > C. *okamuranus* > *M. charantia*. Among different fractions, the chloroform fraction of *C. depressa* and the ethyl acetate fraction extract of *G. subelliptica* showed the best results. Therefore, the chloroform fraction of *C. depressa* peel was further investigated for identification of bioactive constituents.

The role of radical scavenging species (ROS) is extremely important during the autoxidation of sugar to form AGEs, and most of the antioxidants, at least *in vitro*, have profound AGEs inhibitory activities. Hence, the aim was to investigate if this is also true for the Okinawan plant extracts which contain a wide range of different classes of compounds. We explored the DPPH scavenging activity of different plant fractions and quantified the total phenolic content (Fig. 2). The results showed that ethyl acetate fractions with higher phenolic content naturally had better scavenging activity. However, with poor total phenolic content, the chloroform fraction did not reveal superior scavenging activity. Therefore, to determine the possible linkage between ROS and AGEs inhibition in different fractions, a correlation study between these between these two activities were performed. With a high Pearson

coefficient of 0.805 at p = 0.01 (Fig. 3A), ethyl acetate fraction revealed strong positive correlation whereas no correlation could be established in the chloroform fraction (Pearson coefficient of 0.021 at p = 0.01; Fig. 3B). These data suggested that the AGEs inhibitory properties of a plant depend not only on the antioxidant phenolic metabolites, but also on certain compounds that do not have any significant ROS inhibiting activity. Furthermore, it could be observed that some of the data points in chloroform fractions do not comply with the correlation statistics (top left corner in Fig 2B). These points specify that although they have poor DPPH scavenging activity, they have a very high anti-AGEs activity. These sets of data belong to the chloroform fraction of *C. depressa*, and therefore, attention was focused on the identification of the major compound present in this fraction.

5-*O*-Demethylnobiletin (DN), a polymethoxylated flavone, was isolated and identified as the major compound present in the chloroform fraction (Fig. 1D). It was seven times more active in inhibiting AGEs formation than aminoguanidine. Similarly, DN inhibited the formation of fructosamine adducts by more than thirteen times that of aminoguanidine. Fructosamine is an Amadori product formed by the glycation of amino acid via Schiff's base (Singh, Barden, Mori, & Beilin, 2001). It appears that aminoguanidine had better activity than DN in preventing the formation of dicarbonyls. This could be explained by the fact that aminoguanidine reacts with dicarbonyls and forms adduct (Thornalley, Langborg, & Minhas, 1999). Therefore, the relative amount of reactive dicarbonyl left for the formation of Girard adduct is less compared than with the test system containing DN. Although the amount of glyoxal formed in samples treated with DN was higher than those treated with aminoguanidine, the glyoxal content in the former was significantly lower than non-treated samples (Fig. 1C). These results suggested that DN inhibited AGEs formation predominantly by inhibiting fructosamine adduct formation while only partly by preventing dicarbonyl generation.

Polymethoxylated flavones are a group of methoxylated phenolic compounds found exclusively in tissues and peels of *Citrus* species (Manthey & Grohmann, 2001). There has been an increasing interest in the health promoting properties of these flavones. The chemistry and bioactivity of a polymethoxylated flavonoid, nobiletin has been reviewed extensively (Li, Wang, Guo, Zhao, & Ho, 2014). Several studies have shown that this group of compounds has antimutagenic, antiproliferative, anti-inflammatory and hypolipidemic properties (Calomme, Pieters, Vlietinck, & Vanden Berghe, 1996; Manthey & Guthrie, 2002; Lee et al., 2013). Recently, it has been reported to have anti-allergic effect by suppressing activation of phosphoinositide 3-kinase (Onishi et al., 2014). Although the anti-diabetic effects of polymethoxylated flavones have been reported (Lee et al., 2010), this is the first report on the antiglycating activity of this group of compounds.

Conclusion

Okinawan plants have been suggested to contain highly potent health benefit constituents and it is believed that consumption of such plants attributes to the healthy longevity of the islanders. This study explored the antiglycation activity of Okinawan plants which were not investigated before for the AGEs inhibitory properties. This result identified 5-*O*-demethylnobilietin as a novel AGEs inhibitor from *Citrus depressa* and hence anti-glycation studies should be performed for other polymethoxylated flavonoids. Since the ethyl acetate fractions of *Garcinia subelliptica* showed a high antiglycation activity, further investigations should be done to identify the potent bioactive compounds. These results may be useful in developing protocols for the effective use of the functional properties of these plants. Finally, this study also partly provides pharmacological explanations of the possible linkage between Okinawan diet and health.

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Table 1 Names and parts of plants investigated in the study

Species	Family name	Common name	Okinawan name	Consumed parts	Investigated parts
Alpinia zerumbet (Pers.) B.L. Burtt and R.M. Smith	Zingiberaceae	Shell ginger	Getto	Leaf	Leaf
Caulerpa lentillifera J. Agardh	Caulerpaceae	Sea grapes	Umi budou	Fruit	Fruit
Citrus depressa Hayata	Rutaceae	Flat lemon	Shikwasha	Fruit	Peel
Cladosiphon okamuranus Tokida	Chordariaceae	-	Mozoku	Leaf	Leaf
Garcinia subelliptica Guttiferae	Clusiaceae	-	Fukugi	Fruit	Leaf
Leucaena leucocephala (Lam.) de Wit	Fabaceae	Leucaena	Ginnem	-	Leaf
Miscanthus sinensis Andersson	Poaceae	Zebra grass	Susuki	-	Ariel
Momordica charantia L.	Cucurbitaceae	Bitter melon	Goya	Fruit	Fruit

Table 2 AGEs inhibition (%) by crude extracts and fractions of different plants.

Plant	Crude extract 250 μg/ml	Chloroform fraction		Ethyl acetate fraction	
		25 μg/ml	250 μg/ml	25 μg/ml	250 μg/ml
A. zerumbet	19.3 ± 1.5^{c}	27.5 ± 1.3^{c}	$37.1 \pm 1.8^{d,e}$	13.4 ± 0.7^{b}	31.9 ± 1.3^{c}
C. lentillifera	$15.7 \pm 0.9^{b,c}$	1.2 ± 0.1^{c}	24.1 ± 1.2^{c}	14.6 ± 0.8^{b}	23.6 ± 1.1^{b}
C. depressa	$32.1 \pm 1.1^{d,e}$	42.3 ± 2.1^{d}	63.0 ± 3.1^{f}	17.8 ± 0.9^{c}	31.9 ± 1.7^{c}
C. okamuranus	$14.6 \pm 1.2^{a,b}$	28.5 ± 2.0^{c}	41.3 ± 2.0^{e}	13.7 ± 0.7^{b}	25.2 ± 0.9^{b}
G. subelleptica	25.8 ± 2.1^{e}	32.7 ± 1.9^{c}	39.8 ± 1.6^{e}	38.7 ± 1.3^{e}	45.5 ± 1.2^{d}
L. leucocephala	29.7 ± 1.5^{d}	28.8 ± 1.4^{e}	40.4 ± 2.0^{d}	24.4 ± 1.2^{d}	32.6 ± 1.8^{c}
M. sinensis	$16.4 \pm 1.2^{b,c}$	38.2 ± 1.7^{d}	$39.4 \pm 1.9^{b,c}$	23.7 ± 1.3^{d}	25.2 ± 1.0^{b}
M. charantia	11.1 ± 0.7^{a}	1.3 ± 0.1^{a}	5.8 ± 0.3^{a}	6.9 ± 0.5^{a}	18.1 ± 0.6^{a}
Aminoguanidine	-	6.9 ± 0.4^{b}	18.1 ± 0.1^{b}	-	-

The data represent mean ± SE of three replicates. Values with the same superscript in one column are not significantly

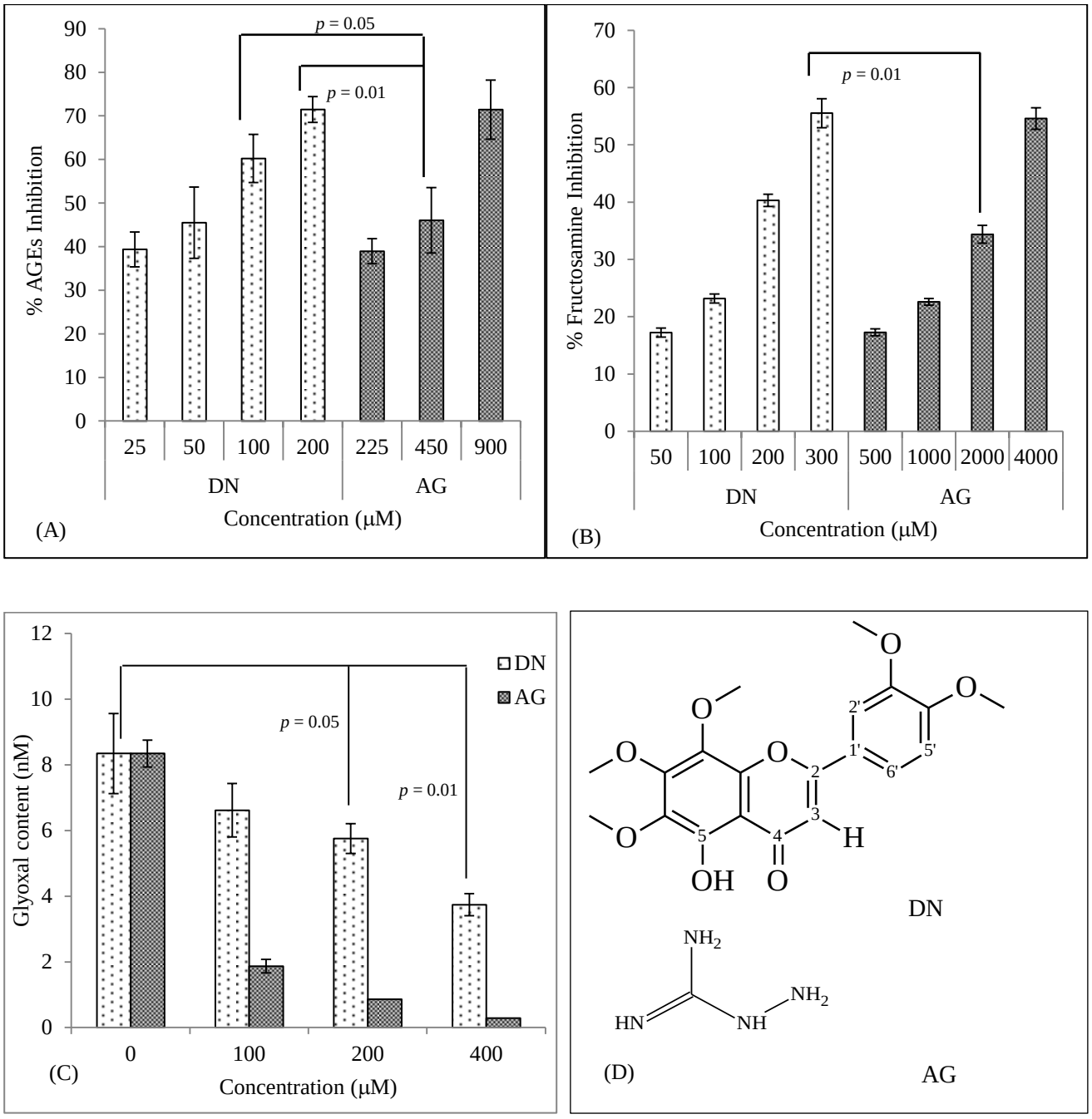
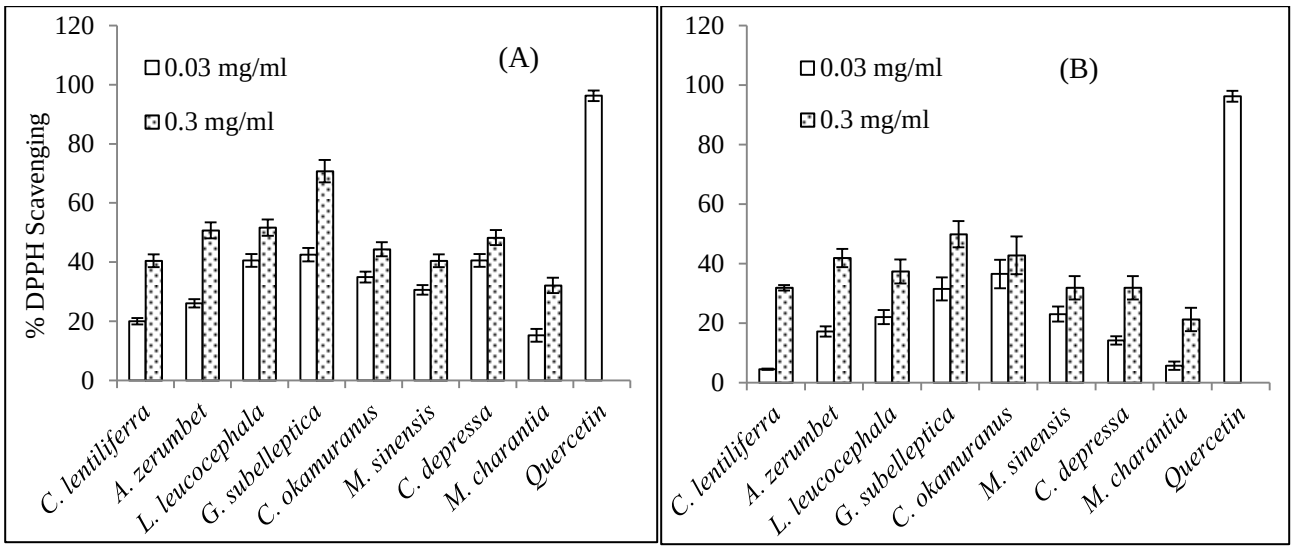


Fig. 1. Effects of 5-*O*-demethyl nobiletin (DN) and aminoguanidine (AG) on AGEs inhibition (A), fructosamine formation (B), and glyoxal content (C). Note the differences in the concentrations of test compounds used in (A) and (B). The figures represent mean ± SE of three replicates, and the significant difference measured at $p = 0.01$ and 0.05. Chemical structures of test compounds (D).



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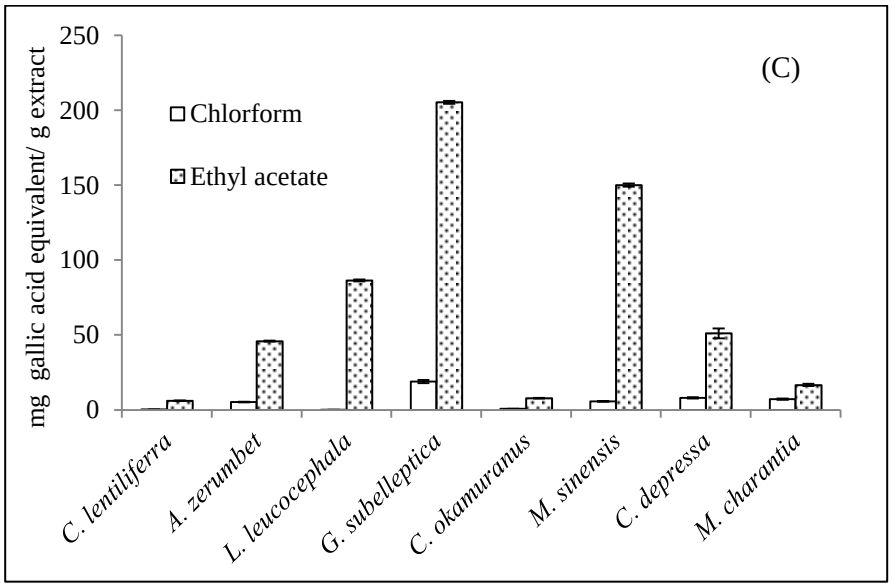

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14 **Fig. 2**. DPPH scavenging activity of ethyl acetate (A) and chloroform (B) fractions and total phenolic

15 content (C) of test samples. The data represent mean ± SE of three replicates.

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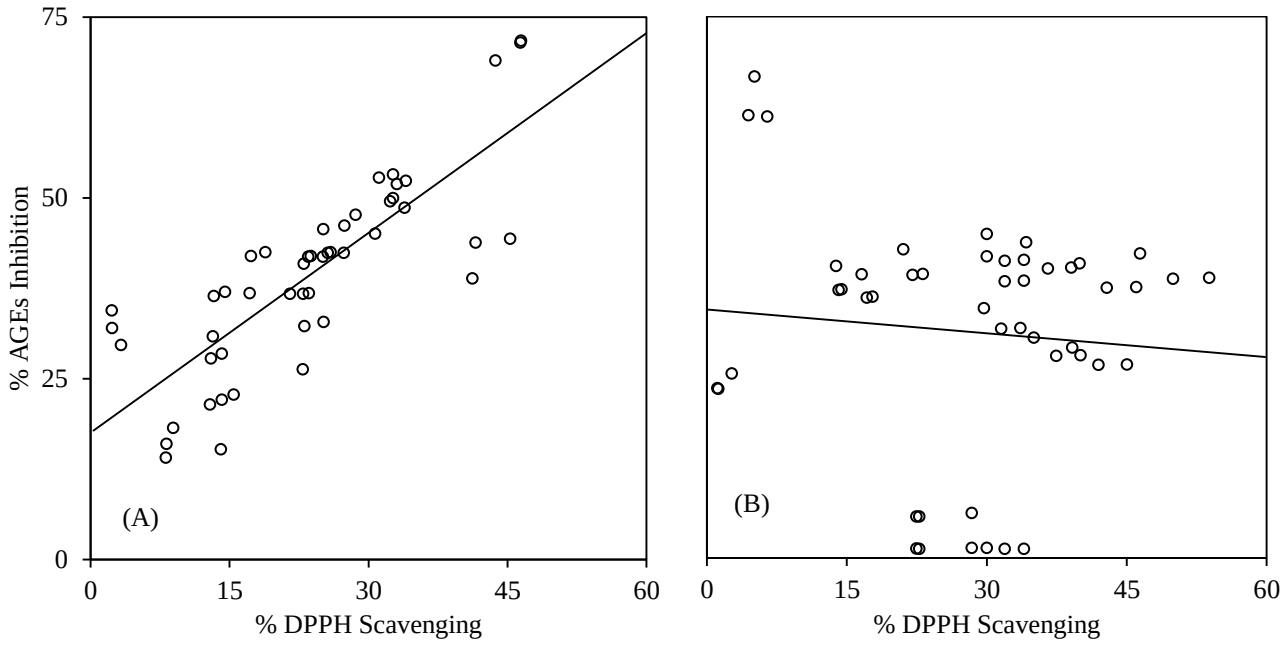

Fig. 3. Correlation between DPPH scavenging and AGEs inhibition of ethyl acetate (A) and chlorofrom (B) fractions of different plant extracts.

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