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Ginkgetin or Isoginkgetin: The Dimethylamentoflavone of *Dioon edule* Lindl. Leaves

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Authors' contributions

This work was carried out in collaboration between both authors. Author AM designed the study, performed the spectroscopic analysis, wrote the protocol and wrote the first draft of the manuscript. Author DEA achieved the literature searches. Both authors achieved the chromatographic isolation, structure elucidation, read and approved the final manuscript.

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ABSTRACT

Aims: Phytochemical study of the biflavonoid content of *Dioon edule* Lindl. leaves in addition to quantification of the phenolic content and evaluation of their antioxidant potential.
Methodology: Chromatographic isolation of the total alcohol extract of the leaves followed by the spectroscopic identification of the isolated compounds was performed adopting 1D, 2D NMR techniques. The total phenolic content (TPC) was determined using the Folin-Ciocalteu method and the total flavonoids content (TFC) was measured by complexation with aluminum chloride and the antioxidant activity was evaluated with DPPH (2.2-diphenyl-1-picrylhydrazyl) assay.
Results: Phytochemical investigation of *Dioon edule* Lindl. leaves afforded the isolation of 7,4′,7″,4‴-tetramethylamentoflavone (1), 4′,4‴-dimethylamentoflavone (isoginkgetin) (2) biolbetin (3) and amentoflavone (4) in addition to β-sitosterol (5). A more accurate assignment of ^{13}C-NMR data of the methylated amentoflavone derivatives is performed using 2D NMR (HMQC and HMBC). Compounds 2 and 5 are reported for the first time in *Dioon edule* Lindl. leaves. Previous report on

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D. edule Lindl. leaves afforded the isolation of ginkgetin (6) as a dimethylamentoflavone but our investigation proved that it is isoginkgetin. The TPC was 0.27±0.01 gallic acid equivalents in mg/g plant material. The TFC was 1.8473±0.077 rutin equivalents in mg/g plant material. The plant exhibited a good antioxidant property as DPPH scavenging activity was 3.29±0.251 mg ascorbic acid equivalent antioxidant capacity in 1 g plant material.

Conclusion: The phytochemical study of *D. edule* Lindl. leaves afforded isoginkgetin instead of the previously reported ginkgetin. The use of NMR provides the most powerful tool for a more accurate assignment of biflavonoids.

Keywords: Dioon edule; isoginkgetin; amentoflavone; antioxidant activity; total phenolic; total flavonoids.

1. INTRODUCTION

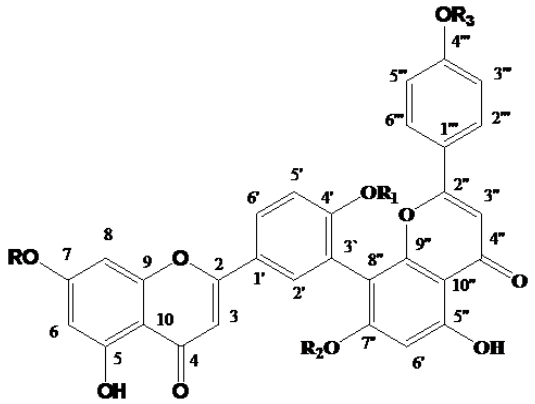
Biflavonoids are flavonoid-flavonoid dimers with varied chemical structures due to the possibility of different flavonoid dimer combinations. For example, flavanone-flavone, flavone-flavone, flavone-flavonol are possible. In addition, the bond connections between the flavonoids may be C-C bond or C-O-C bond. Moreover, a connecting bond may have diverse positions: 3-4''', 4'-4''', 3'-8'',etc. In natural biflavonoids, many hydroxyl/methoxyl groups are substituted at different positions [1]. The major occurrence of biflavonoids is in the gymnosperms and their distribution and patterns of occurrence are very related to plant evolution [2]. The ability to dimerize flavones to form these biflavonoids appears to have arisen early in the development of vascular plants. However, biflavonoids have been detected in some angiosperms and their presence represents the retention of a primitive flavonoid character in these angiosperms and confirms the widely held view that angiosperms arose from gymnosperm-like ancestors [2]. Plants containing biflavonoids as major constituents are not widely distributed, the most famous one is *Ginkgo biloba* leaves [1] which possess antioxidant, anti-ischemic, neuro-protective, cardiovascular, and cerebrovascular properties, and exert beneficial effects against cognitive deficits, including Alzheimer's-type and multi-infarct dementia, as well as peripheral vascular disease [3]. Examples of biflavonoids isolated from *G. biloba* are amentoflavone, ochnaflavone, ginkgetin and isoginkgetin [4]. Bilobetin, sciadopitysin and 7,4',7''4''''-O - methylamentoflavone enhance osteoblast differentiation suggesting their therapeutic potential in osteoporosis [1]. Biflavonoids have a role in inhibition of histamine release from mast cells and inhibition of lymphocyte proliferation, suggesting their anti-inflammatory/antiallergic potential [1]. Biflavonoids isolated from *Podocarpus henkelii leaves* (7,4',7",4"'-tetramethoxy amentoflavone, isoginkgetin and podocarpusflavone–A) showed low antibacterial and antifungal activity with low cytotoxicity [5]. Several amentoflavone derivatives showed antibacterial activity [6,7]. Ginkgetin and isoginkgetin were the first biflavones whose structures were elucidated and confirmed by complete syntheses [8]. Many of the early reported methyl biflavones met correction of their structures since the structure elucidation in the sixties and the seventies were dependent on co-chromatography with isolated authentic - which itself might be misidentified- as in case of sotetsuflavone and bilobetin in *Cycas revoluta* [9,10] and sotetsuflavone and sequioflavone in *Araucaria* [11]. Many early reports included unidentified positional isomers of partial methyl ether derivatives of biflavonoids because of the great difficulty in separating complex mixtures [12], even the reported methylated biflavones were sometimes doubtful due to the lack of ^{13}C-NMR data at that time. There is only one article traced concerning the phytochemical study of *D. edule* leaves which isolated seven biflavonoids; amentoflavone, bilobetin, sequoiaflavone, ginkgetin, sciadoptysin, 7,4',7",4"'-tetra-*O*-methylamentoflavone and diooflavone (hexa-*O*-methylamentoflavone) [13]. The biflavones were identified by direct comparison with authentic samples using m.m.p., co-chromatography in three solvents, and ^{1}H-NMR studies of the acetates. In our study of *D. edule* cultivated in Egypt; we isolated four biflavonoids including isoginkgetin instead of reported ginkgetin and provided complete comparative NMR data of both compounds.

2. MATERIALS AND METHODS

2.1 General Experimental

For the spectrophotometric analysis; vortex mixer, sonication water bath and Shimadzu UV-visible (UV-1650) spectrophotometer were used,

absolute ethanol, $NaNO_2$, NaOH were of analytical grade. $AlCl_3$, Folin Ciocalteu reagent, DPPH, gallic acid and rutin were purchased from Sigma Aldrich Chemicals, Germany. 1D and 2D NMR spectra were recorded on a Bruker Avance III 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR with BBFO Smart Probe and Bruker 400 MHz AEON Nitrogen-Free Magnet (Bruker AG, Switzerland) using $CDCl_3$ residual proton peak at 7.24 (s) ppm and acetone-d_6 residual proton peak at 2.05 ppm as an internal reference standard. Data were analyzed using Topspin 3.1 Software.



	R	R_1	R_2	R_3
1	CH_3	CH_3	CH_3	CH_3
2	H	CH_3	H	CH_3
3	H	CH_3	H	H
4	H	H	H	H
6	CH_3	CH_3	H	H

Fig. 1. Structure of amentoflavone and methylated derivatives

2.2 Plant Material and Sample Preparation

D. edule leaves were collected from Mohamed Ali Palace in Giza Governorate in August 2015 and identified by Dr. Abdelhalim Mohamed (Plant Taxonomy Department, Agricultural Research Institute, Egypt). Voucher specimen were deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Beni-Suef University (voucher NO. BUPD-60). Plants were dried in oven at temperature not exceeding 40℃ and pulverized.

2.3 Extraction

Plant sample (0.1 g) was extracted for 2 h with 5 ml of 80% methanol at room temperature on a sonication water bath. The mixture was centrifuged at 1400 x g for 20 min and the supernatant was decanted into a 25 ml measuring flask. The precipitate was re-extracted under identical conditions. Supernatant was combined and used for total antioxidant activity, total phenolic content and total flavonoid content. For the phytochemical study of *D. edule* leaves (930 g powder) was percolated with 80% methanol (6L X 3). All methanol from the extract was evaporated under reduced pressure to yield 105 g crude extract. The crude extract was suspended in water and fractionated using *n*-hexane, EtOAc and *n*-butanol saturated with water to yield 2 g, 55 g and 12 g respectively.

2.4 Phytochemical Study of *Dioon edule* Leaves

Ten grams of the EtOAc fraction was chromatographed over VLC (silica gel, 50 g, 11x4 cm) eluted with 90% DCM in *n*- hexane then 100% DCM then adding MeOH in 1% increments. Fifty ml fractions were collected and screened using TLC to give four fractions (DE-1:4). Fraction DE- 1 (50 mg, eluted with 100% DCM) was purified over Sephadex-LH-20 eluted with 60% MeOH in DCM to yield compound 1 (4 mg).

Fraction DE- 2 (80 mg, eluted with 3% MeOH in DCM) precipitated compound 2 (60 mg) while fraction DE- 3 (600 mg eluted with 8-10% MeOH in DCM) was rechromatographed over silica gel column (90 g, 70x2 cm) eluted isocratically with 5% MeOH in DCM to give compound 3 (11 mg) and compound 4 (120 mg). (DE-4) was chromatographed over Sephadex-LH-20 eluted with MeOH to give compound 4 (15 mg).

The *n*- hexane fraction was chromatographed over silica gel column (30 g, 25x2 cm) eluted with *n*-hexane and increasing polarity by adding 2% increments of EtOAc to get compound 5 (14 mg) in the fraction eluted with 12% EtOAc.

2.5 Determination of Total Phenolic Contents (TPC)

TPC was determined using Folin–Ciocalteu reagent as previously described [14]. Three hundred microliters of extract were mixed with 2.25 ml of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min; 2.25 ml of sodium carbonate (60 g/L) solution was added to the mixture. After 90 min at room temperature,

absorbance was measured at 725 nm using spectrophotometer. Results were expressed as mg gallic acid equivalents in 1 g of dried sample (mg GAE/g).

2.6 Determination of Total Flavonoid Content (TFC)

TFC was determined using colorimetric method described by reference [14]. Half milliliter of the extract was mixed with 2.25 ml of distilled water in a test tube followed by addition of 0.15 ml of 5% $NaNO_2$ solution. After 6 min, 0.3 ml of a 10% $AlCl_3$ solution was added and allowed to stand for another 5 min before 1.0 ml of 1 M NaOH was added. The mixture was mixed well with vortex. The absorbance was measured immediately at 510 nm using spectrophotometer. Results were expressed as mg rutin equivalents in 1 g of dried sample (mg RE/g).

2.7 DPPH Free Radical Scavenging Assay

The scavenging activity of the extracts was estimated by using 1,1-diphenyl-2-pycrylhydrazyl (DPPH) as a free radical model and a method adapted from reference [14]. An aliquot of 300 µL of samples or control (80% methanol) were mixed with 3.0 ml of 500 µM (DPPH) in absolute ethanol. The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark. The mixture was measured spectrophotometrically at 517 nm. The free radical scavenging activity was calculated as follows: Scavenging effect (%) = [1- {absorbance of sample/absorbance of control}] *100. A standard of ascorbic acid was run using several concentrations ranging from 0.05 to 0.25 mg/ml. A standard curve was then prepared by plotting the percentage (%) of free radical scavenging activity of ascorbic acid versus its concentration. The final result was expressed as mg ascorbic acid equivalent antioxidant capacity in 1 g of sample (mg AEAC/g).

2.8 Statistical Analysis

All experiments were carried out in 3 replicates and presented as mean ± standard deviation of (SD) using SPSS version 22.0.

3. RESULTS

The phytochemical study of *D. edule* plant afforded the isolation of five compounds; 7,4′,7″,4‴-tetramethylamentoflavone [13], isoginkgetin [6,15], Biolbetin [9], amentoflavone [9] and β-sitosterol [16] in which β-sitosterol and isoginkgetin are isolated for the first time. *D. edule* leaves were reported to contain ginkgetin (7,4'-dimethylamentoflavone; 6) [13] but according to our phytochemical investigation of the plant cultivated in Egypt we isolated isoginkgetin (4',4'''-dimethylamentoflavone), there was a complete absence of other dimethylamentoflavones as no mixture was isolated. Spectrophotometric analyses of TPC, TFC and DPPH free radical scavenging ability of *D. edule* leaves (Table 2) showed that the TPC was 0.27±0.01 gallic acid equivalents in mg/g plant material while the TFC was 1.8473±0.077 rutin equivalent in mg/ g plant material and the plant exhibited a good antioxidant property as DPPH scavenging activity was 3.29±0.251 mg ascorbic acid equivalent antioxidant capacity in 1 g plant material.

4. DISCUSSION

The ^{1}H-NMR spectrum of compound 1 in acetone-d_6 showed two D_2O exchangeable 5-OHs at δ_H 13.09 (s, H-5''), 12.77 (s, H-5) ppm indicating the presence of two flavonoid units linked together as biflavonoid. The presence of four methoxyl signals with a spectrum signals similar to amentoflavone [9] suggested the presence of tetramethylamentoflavone in which the methoxy groups are located at the remaining hydroxyl groups at 7,4′,7″,4‴ positions. Thus compound 1 was identified as 7,4′,7″,4‴-tetramethylamentoflavone. This compound was previously synthesized by partial demethylation of the permethylamentoflavone and ^{1}H-NMR data of its acetate was reported [13]. ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H 13.09 (s, H-5''), 12.77 (s, H-5), 7.97 (d, *J* = 8.3, H-6'), 7.87 (s, H-2'), 7.46 (d, *J* = 8.5, H-2''',6'''), 7.16 (d, *J* = 8.6, H-5'), 6.82 (d, *J* = 8.5, H-3''',5'''), 6.62 (s, H-3), 6.59 (s, H-3''), 6.52 (s, H-6''), 6.43 (s, H-6), 6.34 (s, H-8), 3.85* (s,4'''-OCH_3), 3.82 (s, 7-OCH_3), 3.80 (s, 4'-OCH_3), 3.77* (s, 7'-OCH_3). Data with a superscript may be interchanged. There was one report of the ^{13}C-NMR data of 7,4′,7″,4‴-tetramethylamentoflavone to which our data were consistent [15].

Compound 2 showed two aromatic methoxyl signals in the ^{1}H NMR spectrum and a shift in the aromatic signals compared to amentoflavone [9]. This compound was dimethyl ether of amentoflavone. Compared to amentoflavone; ^{13}C NMR data of compound 2 showed upfield shift in the C-5' indicating 4'-*O*-methylation and a slight downfiled shift in C-2''' indicating 4'''-O-methylation [6], [15]. ^{13}C-NMR data was consistent with isoginkgetin [9] but not the

previously reported ginkgetin [15]. Our report of the presence of isoginkgetin in *D. edule* may be due to environmental variation between the previously reported plant and the Egyptian one or incorrect earlier identification of the biflavonoids. We encourage the second assumption as the previous report was at a time in which NMR was starting [13]. The NMR data reported for ginkgetin by Castaneda et al. [17] was inaccurate and incomplete compared to that reported by Markham et al. [15], since ^{13}C NMR assignment of C-2 and C-7 should be interchanged and the ring -B in unit 1 should be revised. Comparative ^{13}C NMR data reported by Markham et al. [15] and Castaneda et al. [17] for ginkgetin and our isolated compound isoginkgetin are compiled in Table 1.

Compound 3: It has similar pattern as compound 2 with only one aromatic methoxyl signal at δ_H 3.89 ppm and slight shift in the aromatic proton signals indicating monomethylated amentoflavone. ^{1}H NMR data of compound 3 was consistent with what is reported about amentoflavone-4'-methyl ether commonly known as bilobetin [9]. ^{1}H and ^{13}C-NMR data of compound 4 was consistent with what is reported about amentoflavone [9] while ^{1}H and ^{13}C-NMR data of compound 5 was consistent with β-sitosterol [16]. This plant shows biflavonoid content close to *G. biloba* leaves.

Many plants of the gymnosperms contain biflavonoids without tannins while other closer species contain tannins in the form of flavan-3-ols [6]. *D. edule* leaves don't contain tannins; the TPC was 0.27±0.01 while the TFC was higher (1.8473±0.077 due its content of biflavonoids). The plant exhibited a good antioxidant property as DPPH scavenging activity was 3.29±0.251 mg ascorbic acid equivalent antioxidant capacity in 1 g plant material (Table 2).

Table 1. ^{13}C NMR data of isoginkgetin (2) and the reported ginkgetin (6)

Position	2	6[§]	6[£]
2 (2″)	163.8[a] (163.5[a])	163.5 (163.6)	165.01(163.09)
3 (3″)	103.6 (104.0)	103.5 (102.5)	103.09(103.09)
4 (4″)	182.5 [b] (182.2[b])	181.9 (182.0)	181.83(182.04)
5 (5″)	160.9 (161.9)	161.5 (160.4)	161.03(160.48)
6 (6″)	99.1 (99.4)	98.5 (98.6)	98.67(98.01)
7 (7″)	164.7 (162.6)	165.1 (161.7)	163.96(162.12)
8 (8″)	94.6 (104.2)	92.6 (103.8)	92.61(103.6)
9 (9″)	157.9 (154.8)	157.3 (154.3)	157.21(154.64)
10 (10″)	104.1 (104.2)	104.7 (103.5)	103.19 (NR)
1′ (1‴)	123.0 (123.3)	122.3 (121.2)	119.99(120.78)
2′ (2‴)	131.3 (128.2)	128.2 (128.0)	NR (127.9)
3′ (3‴)	122.1 (114.9)	121.7 (115.8)	122.89(114.43)
4′ (4‴)	161.1 (162.6)	160.6 (161.0)	159.66(161.03)
5′ (5‴)	112.1 (114.9)	111.7 (115.8)	116.17(131.34)
6′(6‴)	128.7 (128.2)	130.7 (128.0)	114.43(127.9)
7- *O*CH_3	--------	NR	55.49[d]
4′-*O*CH_3	56.3[c]	NR	56.01[d]
4‴-*O*CH_3	55.9 [c]	----------	----------

[§]Data from reference (15); NR: not reported
[£] Data from reference [17]
Data with the same superscript in the same column may be interchanged

Table 2. Total phenolic, total flavonoid and DPPH scavenging ability of *Dioon edule* Lindl. leaves

Plant material	TPC [a]	TFC [b]	DPPH assay [c]
Dioon edule leaves	0.27±0.01	1.8473±0.077	3.29±0.251

Values are presented in mean ± SD (n = 3)
[a]Total phenolic was expressed as mg gallic acid equivalent in 1 g of dry sample. (mg GE/g)
[b]Total flavonoid was expressed as mg rutin equivalent in 1 g of dry sample. (mg RE/g)
[c] DPPH free radical scavenging activity was expressed as mg ascorbic acid equivalent antioxidant capacity in 1 g of dry sample (mg AE/g)

The high antioxidant activity of phenolic substances is often attributed to the presence of acidic OH groups, which are potent H donors to free radicals and the resulting phenoxy radicals are easily stabilized by electron delocalization across the conjugated aromatic system efficiently. Also intramolecular hydrogen bonding between neighboring–OH and C=O groups increase aromatic-ring conjugation and raise the H donation power of the molecule, because the resulting radicals are more delocalized [18]. *D. edule* leaves exhibit powerful antioxidant activities because of its biflavonoid content which possess all of these structural features.

5. CONCLUSION

Our investigation of the biflavonoid content of *D. edule* Lindl. leaves proved that the dimethylamentoflavone present is isoginkgetin and not the previously reported ginkgetin. The use of NMR provides the most powerful tool for a more accurate assignment of biflavonoids. The biflavonoid content of *D. edule* is similar to *G. biloba* which have several medicinal uses so *D. edule* leaves may be a good candidate for incorporation in herbal medicine formula.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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