

Bioactive Phenolic Compounds from the Egyptian Red Sea Seagrass *Thalassodendron ciliatum*

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Five flavonoids (rutin, asebotin, 3-hydroxyasebotin, quercetin-3-*O*- β -xylopyranoside, and a racemic mixture of catechin) and caffeic acid were isolated and identified for the first time from seagrass, *Thalassodendron ciliatum*, collected from the Hurghada region in Egypt. The crude extract and the isolated pure compounds were evaluated for their cytotoxic activities against HCT-116, HEPG, MCF-7, and HeLa human cancer cell lines, for their antiviral activity against Herpes Simplex and hepatitis A viruses, and for their antioxidant activity.

Key words: Flavonoids, Red Sea, *Thalassodendron ciliatum*

Introduction

Seagrass is part of a critical, albeit fragile, ecosystem, widely distributed along temperate and tropical coastlines of the world. It is the only marine flowering plant which often lives entirely submerged and completes its life cycle in seawater (Short *et al.*, 2007). It has ecological importance (Hemminga and Duarte, 2000) and is used as a traditional medicine (Torre-Castro and Rönnbäck, 2004).

Interest in marine organisms as sources of new substances is growing. With marine species comprising approximately half of the total global biodiversity, the sea offers an enormous resource of novel compounds (De Vries and Beart, 1995), and it has been classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity (Gerwick and Bernart, 1993). Most studies have focused on the importance of coral reefs and mangroves, limited studies are found on seagrass although it has been reported that this plant is a rich source of secondary metabolites, particularly phenolic compounds (McMillan

et al., 1980) which are interesting for their biological activities.

Few reports exist on the biological activity of either crude extracts or isolated compounds from seagrass (Achamlale *et al.*, 2009; Kontiza *et al.*, 2005, 2008; Rowley *et al.*, 2002; Carbone *et al.*, 2008). *Thalassodendron ciliatum* (Forssk.) Hartog is one of the most common and longest seagrasses along the Egyptian Red Sea. It is characterized by many ‘tannin cells’ in its leaves, more than in any other seagrass (Lipkin, 1988), which means a high phenolic content. There are no previous studies in the literature on the chemical constituents and biological activities of this species. Thus, for the best of our knowledge, this is the first report on the chemistry and biological activities of *T. ciliatum*.

Results and Discussion

As mentioned above, *T. ciliatum* is considered a rich source of phenolic compounds. In this study, five flavonoids and a phenolic acid have been isolated for the first time from the methanolic extract of *T. ciliatum* collected from the shores of

the Red Sea: rutin (quercetin-3-*O*-rutinoside, **1**) (Chaurasia and Wichtl, 1987), asebotin (2',4,6'-trihydroxy-4'-methoxydihydrochalcone 2'-*O*- β -D-glucopyranoside, **2**; Fig. 1) (Fuendjie *et al.*, 2002), 3-hydroxyasebotin (**3**; Fig. 1) (Yao *et al.*, 2005), quercetin-3-*O*- β -D-xylopyranoside (**4**) (Debella *et al.*, 2000), and *trans*-caffeic acid (**6**), in addition to a racemic mixture of (+)-catechin and (–)-catechin (**5**) (Davis *et al.*, 1996).

Asebotin (**2**) and 3-hydroxyasebotin (**3**) are the first dihydrochalcones isolated from a marine organism. In addition, this is the second report for **3** as a naturally occurring compound, because it has previously been found only in the leaves of *Pieris japonica* by Yao *et al.* (2005).

Antioxidant activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity of the *T. ciliatum* total methanolic extract was found to be (71 $\pm$ 1.09)% at 1 mg/mL. In comparison, rutin (**1**) showed higher activity with an EC₅₀ value of 0.99 mM than quercetin-3-*O*- β -D-xylopyranoside (**4**) (1.63 mM), 3-hydroxyasebotin (**3**) (1.62 mM), caffeic acid (**6**) (3.50 mM), and the racemic mixture of catechins (**5**) (3.82 mM), while asebotin (**2**) was inactive. The difference in antioxidant activity is related to the number and positions of hydroxy groups and the nature of any other substitutions on the aromatic rings (Nagendran *et al.*, 2006; Heijnen *et al.*, 2001); the hydroxy groups of the sugar moiety do not contribute to the antioxidant activity (Rezk *et al.*, 2002). Concerning the dihydrochalcones Kozłowski and co-workers (2007) have shown the importance of the double bond between C8 and

C9 for the redox capacity. Its absence decreases the antioxidant activity, and it was found that antioxidant activity depends on the presence of a hydroxy substitution at the C2, C6, and C4' positions (Bentes *et al.*, 2011). The active dihydrochalcone has a conformation in which the aromatic A ring is orthogonal to the carbonyl group, while the inactive dihydrochalcone has a strongly hydrogen-bound phenolic hydroxy group, suggestive of a coplanar conformation. A hydroxy group at the 2' position of the dihydrochalcone A ring is an essential pharmacophore for radical scavenging potential (Nakamura *et al.*, 2003).

Cytotoxic activity

The crude extract exhibited cytotoxic activity against HCT-116, HEPG, MCF-7, and HeLa human cancer cell lines with IC₅₀ values of (4.2 $\pm$ 0.51), (8.12 $\pm$ 0.34), (4.12 $\pm$ 0.77), and (9.8 $\pm$ 0.25) μ g/mL, respectively. Quercetin-3-*O*- β -D-xylopyranoside (**4**) showed higher activities against HEPG cells [(7.25 $\pm$ 0.39) μ M] and HCT-116 cells [(11.17 $\pm$ 0.39) μ M], 3-hydroxyasebotin (**3**) was found to be active against HCT-116 cells [(9.77 $\pm$ 0.52) μ M], while asebotin (**2**) was active against HEPG cells [(8.55 $\pm$ 0.42) μ M] (Table I). 3-Hydroxyasebotin and the racemic mixture of catechin exhibited cytotoxic activity only against the HCT-116 cell line. None of the isolated compounds showed activities against HeLa and MCF-7 cancer cell lines.

In vitro cytotoxic activities of plant polyphenols may be related to their prooxidant activity. Bhat *et al.* (2007) have proposed that such prooxidant action could be a common mechanism for anti-

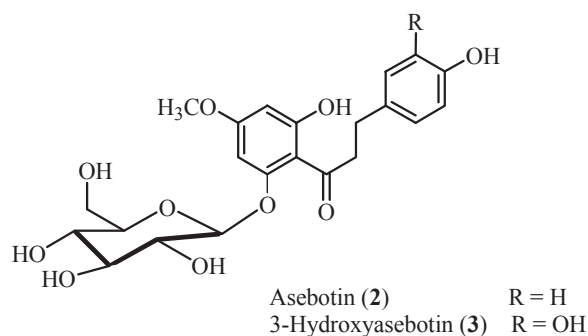


Fig. 1. Chemical structures of asebotin and 3-hydroxyasebotin isolated from *T. ciliatum*.

Table I. Cytotoxic activity of compounds isolated from *T. ciliatum* against HCT-116 and HEPG human cancer cell lines.

Compound	IC ₅₀ [μ M]	
	HCT-116	HEPG
Rutin (1)	20.0 $\pm$ 0.81	32.76 $\pm$ 1.6
Asebotin (2)	14.32 $\pm$ 0.88	8.55 $\pm$ 0.42
3-Hydroxyasebotin (3)	9.77 $\pm$ 0.52	Inactive
Quercetin-3- <i>O</i> - β -D-xylopyranoside (4)	11.17 $\pm$ 0.39	7.25 $\pm$ 0.39
Racemic mixture of (+)-catechins (5)	49.95 $\pm$ 0.96	Inactive
<i>trans</i> -Caffeic acid (6)	23.03 $\pm$ 0.72	17.48 $\pm$ 0.38
Doxorubicin (positive control)	2.33 $\pm$ 0.14	2.02 $\pm$ 0.07

cancer and chemopreventive properties of plant polyphenols. Indeed, a common mechanism would better explain the anticancer effects of polyphenols with diverse chemical structures as well as the preferential cytotoxicity towards cancer cells. The authors hypothesized that the anticancer mechanism of plant polyphenols involves mobilization of endogenous copper, possibly chromatin-bound copper, and the consequent prooxidant action. It is well established that plant polyphenols of various classes behave both as prooxidants and antioxidants.

Catechin, epicatechin, and rutin were reported to exhibit potent anticancer activity (Chen *et al.*, 2009). Individual catechin and epicatechin shows free radical scavenging activities against DPPH radicals (Thiraviam *et al.*, 2004). Epicatechin has low cytotoxicity to human breast cancer cells (MCF-7) with an IC_{50} value of $102 \mu\text{g/mL}$ (Le Baila *et al.*, 1998), and it is inactive against lung PC-6 and HEPG2 human cancer cell lines (Takashi *et al.*, 2008). While epicatechin shows activity against HCT-116 cells (Uesato *et al.*, 2001), catechin was found to be effective against pancreatic cancer (McMillan *et al.*, 2007). There is no previous report on the activity of a mixture of catechin and epicatechin.

Caffeic acid exhibits antioxidant activity *in vitro* and has been shown to have multiple pharmacological properties including inhibition of HIV replication (Kashiwada *et al.*, 1995) and induction of apoptosis in cancer cell lines, and causes tumour growth inhibition and regression in animals (Chung *et al.*, 2004; Orsolic *et al.*, 2004). Caffeic acid shows anticancer activity against HeLa cells at a concentration of $6.25 \mu\text{g/mL}$ and against subcutaneous tumour growth either given before tumour cell inoculation or after stabilization of tumour growth because of higher intracellular copper levels in cancer cells (Bhat *et al.*, 2007).

Antiviral activity

The crude extract showed 100% inhibition of hepatitis A (HAV) and Herpes Simplex (HSV-1) viruses at lowest concentration tested ($20 \mu\text{g/mL}$). The antiviral activity of the crude extract against HAV was lost by fractionation, which could be explained by the synergistic action of several compounds in the crude extract.

Asebotin (**2**), quercetin-3-*O*- β -D-xylopyranoside (**4**), and *trans*-caffeic acid (**6**) showed viral per-

centage reduction [$(96.6 \pm 0.91)\%$, $(70 \pm 0.98)\%$, $(53 \pm 0.77)\%$, respectively] of plaque formation of HSV-1 at 1 mg/mL and were not cytotoxic.

It has been reported that rutin has no antiviral activity, while sulfated rutin demonstrated some anti-HSV-1 activity with an IC_{50} value of $(88.3 \pm 0.1) \mu\text{M}$ (Orhan *et al.*, 2010; Tao *et al.*, 2007). Caffeic acid exhibits strong activity against HSV-1, HSV-2, and ADV-3, catechin also strongly inhibits replication of acyclovir-resistant HSV-1-ACVr. Compounds with anti-HSV activity have a broad range of structures: polyphenols, polysaccharides, tannins, flavonoids, etc. (Thompson, 2006). The presence of *ortho*-trihydroxy groups in the B-ring is important for compounds exhibiting anti-HSV, complement classical pathway inhibition, and radical scavenging effects (De Bruyne *et al.*, 1999).

Seagrasses are no link between marine algae and land-living higher plants but are derived from land plants which have secondarily recolonized marine habitats. Therefore, seagrasses share most features of their primary and secondary metabolism with their relatives from the Alismatales order which live in land and freshwater habitats (Heglmeier and Zidon, 2010). A previous study showed that the sterol compositions of some seagrasses are typical of higher plants (Gilan *et al.*, 1984).

This is the first report on the chemical composition and biological activity of *T. ciliatum*. The isolation of dihydrochalcones from a marine organism is quite interesting from the chemotaxonomical point of view because the chemical composition of the seagrasses is not fully covered and the characterization of this type of marine aquatic plants is highly recommended based on the chemical profile. We also report the biological activity of the two dihydrochalcones as well as of the other pure compounds. Further studies are required on the toxicity of *T. ciliatum* to humans when consumed as food supplement and/or on its formulation as a therapeutic drug against HSV-1 and HAV and as an anticancer agent.

Material and Methods

General experimental procedures

Optical rotations were measured on a Perkin-Elmer model 343 Plus polarimeter (Überlingen, Germany) using a sodium lamp at 25°C . NMR spectra were recorded on a Bruker AMX 500

instrument (Karlsruhe, Germany) operating at 500 MHz for ^1H NMR and at 125 MHz for ^{13}C NMR. Two-dimensional NMR spectra were obtained with the standard Bruker software. Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was used for column chromatography. For thin layer chromatography (TLC) aluminum sheet silica gel 60 F₂₅₄ pre-coated plates (Merck, Darmstadt, Germany) were used.

Plant material

Seagrass samples of *Thalassodendron ciliatum* (Forssk.) Hartog were collected from Magawish city near Hurghada, Egypt in October 2008, and were identified by Prof. Dr. Monir Abd-El Ghaney, Botany Department, Herbarium, Faculty of Science, Cairo University, Cairo, Egypt.

Extraction and isolation

Fresh *T. ciliatum* (800 g) was blended in an electric blender with methanol; the process was repeated until exhaustion. The combined extracts were filtered, and the solvent was evaporated under reduced pressure at 45 °C. The crude extract was partitioned between ethyl acetate (EtOAc) and H₂O several times.

The ethyl acetate fraction (10.61 g) was chromatographed on a Sephadex LH-20 column (600 mm) with step gradient elution starting from 30% ethanol in H₂O to 100% ethanol. Fractions of 250 mL each were collected and those exhibiting similar TLC profiles were combined. Subfractions were subsequently fractionated on Sephadex LH-20 columns with different elution systems to allow the purification and identification of six phenolic compounds; rutin (**1**), asebotin (**2**), 3-hydroxyasebotin (**3**), quercetin-3-*O*- β -D-xylopyranoside (**4**), a racemic mixture of (+)-catechin and (–)-catechin (**5**), and *trans*-caffeic acid (**6**).

Asebotin (2): Fine colourless needles. – UV: λ_{max} = 282 nm (MeOH). – M.p. 140 – 143 °C. – $[\alpha]_{\text{D}}^{22}$ –44.4° (c 0.275, MeOH). – ^1H NMR: δ = 7.06, 6.68 (2H each, d, 8.6 Hz, H-2/H-6 and H-3/H-5), 6.30 and 6.11 (1H each, J = 2.4 Hz, H-3', 5'), 5.07 (d, J = 7.3 Hz, H-1''), 3.47 (2H, t, J = 7.5 Hz, α -H) and 2.88 (2H, t, J = 7.5 Hz, β -H) of C-*a, b* (Fuendjiep *et al.*, 2002). – ^{13}C NMR: δ = 206.9 (qC, C=O), 167.4 (qC, C-6'), 167.2 (qC, C-4'), 161.9 (qC, C-2'), 156.4 (qC, C-4), 133.8 (qC, C-1), 130.4 (2CH, C-2 and C-6), 116.1 (2CH, C-3 and C-5), 107.5 (qC,

C-1'), 102.2 (CH, C-1''), 96.5 (CH, C-5'), 94.8 (CH, C-3'), 78.5 (2CH, C-3'' and C-5''), 74.7 (CH, C-2''), 71.2 (CH, C-4''), 62.5 (CH₂, C-6''), 56.1 (CH₃, OCH₃-4'), 47.1 (CH₂, C- α), 30.7 (CH₂, C- β).

3-Hydroxyasebotin (3): Feathery fine needles. – UV: λ_{max} = 279 nm (MeOH). – M.p. 155 – 157 °C. – $[\alpha]_{\text{D}}^{22}$ –36.0° (c 0.519, MeOH). – ^1H NMR: δ = 6.69 (d, J = 2.0 Hz, H-2), 6.56 (dd, J = 8.1 and 2.0 Hz, H-6), 6.65 (d, J = 8.1 Hz, H-5), 6.30 and 6.11 (1H each, J = 2.4 Hz, H-3', 5'), 5.07 (d, J = 7.6 Hz, H-1''), 3.46 (2H, t, J = 7.6 Hz, α -H) and 2.83 (2H, t, J = 7.6 Hz, β -H) of C-*a, b* (Yao *et al.*, 2005). – ^{13}C NMR: δ = 206.9 (qC, C=O), 167.5 (qC, C-6'), 167.2 (qC, C-4'), 161.9 (qC, C-2'), 146.0 (qC, C-3), 144.3 (qC, C-4), 134.6 (qC, C-1), 120.7 (CH, C-6), 116.6 (CH, C-2), 116.2 (CH, C-5), 107.5 (qC, C-1'), 102.1 (CH, C-1''), 96.5 (CH, C-5'), 94.7 (CH, C-3'), 78.5 (2CH, C-3'' and C-5''), 74.7 (CH, C-2''), 71.2 (CH, C-4''), 62.5 (CH₂, C-6''), 56.1 (CH₃, OCH₃-4'), 47.1 (CH₂, C- α), 31.0 (CH₂, C- β).

Cytotoxic activity

Cytotoxicity was determined using the sulforhodamine B colorimetric assay (SRB) and scored as percentage reduction of cell viability of treated culture versus untreated control culture. Triplicate wells were prepared for each individual concentration (5, 10, 20, and 30 $\mu\text{g}/\text{well}$). The IC₅₀ values of the cell growth were obtained from the drug dose-response curves using the software Origin 6.1 as previously described by De Vries and Beart (1995).

Antioxidant activity

Antioxidant activity was determined by measurement of DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging percentage according to the method described by Chung *et al.* (2002).

Antiviral activity

The total extract as well as the isolated pure compounds were tested against HSV-1 and HAV-MBB. The cytotoxicity of samples was determined by a method previously described by Van den Berghe *et al.* (1978). Tenfold serial dilutions of each sample were made starting from 10⁰ (1 mg/mL) to a 10^{–4} dilution. Each concentration was tested in triplicate. The antiviral activity of the samples was determined at non-cytotoxic concentration by the plaque assay method (Kaul *et al.*, 1938).

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