Anti-influenza a virus activity of a new dihydrochalcone diglycosides isolated from the Egyptian seagrass *Thalassodendron ciliatum* (Forsk.) den Hartog

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Abstract

One new dihydrochalcone diglycosides has been isolated from the EtOAc fraction of the Egyptian seagrass *Thalassodendrin ciliatum* (Forsk.) den Hartog, and identified as 6′-*O*-rhamnosyl-(1′′′→6′′)-glucopyranosyl asebogenin for which a trivial name Thalassodendrone was established. Furthermore, five known phenolics were isolated and identified as asebotin, quercetin 3,7-diglucoside, protocatechuic acid, ferulic acid and *p*-hydroxybenzoic acid. The strustures of all isolated compounds were established based on 1D and 2D NMR spectroscopy and HR-Mass spectrometer. The anti-influenza A virus activity of the isolated new compound and asebotin were evaluated, and the obtained results revealed an inhibition dose concentration of asebotin more than Thalassodendrone with IC_{50} = 2.00 & 1.96 μg/mL respectively, and with cytotoxic concentration (CC_{50}) of 3.36 & 3.14 μg/mL respectively.

Keywords: *Thalassodendron ciliatum* (Forsk.) den Hartog; Cymodoceaceae; Dihydrochalcones; Phenolics; Antiviral; SAR studies

1. Introduction

Marine natural products have attracted the attention of biologists and chemists all over the world for the last five decades. Several of the compounds isolated from marine sources exhibited significant biological activity, which is accounted for the ocean to be a source of potential drugs (Bhakuni and Rawat, 2005).

In the tropics, shallow coastal areas are characterised by the existence of extensive seagrass meadows. The seagrasses are found in different habitats such as lagoons behind coral reefs that fringe the coast, mangrove bays or estuaries. The seagrass beds provide food and shelter for a variety of other organisms, including commercially important fish species, and thus constitute a valuable component of the nearshore ecosystem (Howard et al., 1989).

Thalassodendron ciliatum (Forsk.) den Hartog is commonly known as ''Majani kumbi'', it is a very common seagrass species in the Red-Sea, the Western Indian Ocean and the tropical part of the Indo-Pacific region (Den Hartog, 1970).

T. ciliatum is used as ''mafusho'' and as a treatment to relieve smallpox. A mixture of ''short seagrasses'', such as *Thalassia* and *Cymodocea*, is effective against fever and skin diseases. Traditional uses, besides pure medicinal use, people in Chwaka (East Coast of Zanzibar) believe in the power of seagrasses to solve different human problems. Traditional doctors reported seagrasses as an important ingredient for different kinds of magic potions. Moreover, many respondents (45%) reported the use of seagrass beach cast as fertilizer in the cultivated family plots ''shambas''. People reported that seagrasses are especially good for the growth of coconut trees (De La Torre-Castro and Rönnbäck, 2004). Seagrasses have showed great ability to accumulate several elements, and are increasingly used as a biological indicator of environmental quality (Pergent-Martini and Pergent, 2000; Pergent-Martini et al., 2005).

The aims of the presented study were to review the phytochemical constituents of the Egyptian seagrass *T. ciliatum*. searching for the presence of dihydrochalcones and other phenolics, accompanied by an evaluation of the antiviral activity of the new isolates, and then highlights their potentials as candidates for new drugs that may be of value in the treatment and prevention of human and livestock diseases. Extensive chromatographic separation and purification resulted in the isolation of one new dihydrochalcone diglycosides identified as 6′-*O*-rhamnosyl-(1′′′→6′′)-glucopyranosyl asebogenin (compound **1**), together with asebotin, quercetin 3,7-diglucoside, protocatechuic acid, ferulic acid and *p*-hydroxybenzoic acid.

The anti-influenza A virus activity of the isolated asebotin and the new compound (**1**) was evaluated and resulted with IC_{50} = 2.00 & 1.96 μg/mL, respectively.

2. Results and Discussion

The phytochemical investigation of the EtOAc fraction of the MeOH extract of *T. ciliatum* resulted in the isolation of one new compound (**1**), together with asebotin (Guang-Min et al., 2005), quercetin 3,7-diglucoside (Mabry et al., 1970), protocatechuic acid (Achenbach et al., 1988), ferulic acid (Zhilan et al., 2006) and *p*-hydroxybenzoic acid (Takeo et al., 2004), which have been structurally elucidated using 1D, 2D-NMR, HR-MS and by comparison with the literature.

Compound (**1**) was isolated as a yellow amorphous solid, with the molecular formula $C_{28}H_{36}O_{14}$ as determined by a combination of high resolution MALDI-MS (S6, +ve) (*m/z* 598.22561 for $[M + 2H]^+$ calcd 598.22616 for $C_{28}H_{38}O_{14}$) together with the ^{13}C-NMR and HSQC spectra (Experimental Section, S2 & S5) which confirmed the presence of 2 methyl, 3 methylene, 16 methine, and 7 quaternary carbons. The ^{1}H-NMR and ^{1}H-^{1}H COSY spectra (S1 & S3) of compound (**1**) suggested the presence of asebogenin (Harborne & Baxter, 1999) and/or asebotin (Nkengfack et al., 2001) derivative, with the characteristic signals as follow; two triplet signals correlate through a cross-peak and appeared at δ_H 2.88 (2H, *t*, *J* = 7.5 Hz, CH_2-β) and at δ_H 3.46 (2H, *t*, *J* = 7.5 Hz, CH_2-α), which are characteristic for the dihydrochalcones (Hufford and Oguntimein, 1982), two ortho-coupled doublets correlated together and appeared at δ_H 7.06 (2H, *d*, *J* = 8 Hz, H-2 & H-6) and at δ_H 6.68 (2H, *d*, *J* = 8 Hz, H-3 & H-5) which are characteristic for the (AA´BB´) system of the B-ring and indicating an oxygen substitution at the 4-position, another correlated meta-coupled doublets appeared at δ_H 6.29 (1H, *d*, *J* = 2 Hz, H-3´) and at δ_H 6.31 (1H, *d*, *J* = 2 Hz, H-5´). Furthermore, a sharp singlet signal typical for an aromatic methoxyl group appeared at δ_H 3.83 (3H, *s*, H_3CO-4´) & at δ_C 56.24 as confirmed from ^{13}C-NMR and HSQC spectra (S2 & S5). Moreover, a doublet signal appeared at δ_H 5.02 (1H, *d*, *J* = 7 Hz, H-1´´) characteristic for the anomeric proton of a hexose moiety identified to be glucose as shown in ^{13}C-NMR spectrum (S2), and another doublet signal appeared at δ_H 4.68 (1H, *d*, *J* = 1 Hz, H-1´´´) characteristic for the presence of rhamnose moiety, which confirmed by the presence of a doublet methyl appeared at δ_H 1.20 (3H, *d*, *J* = 6 Hz, H_3C-6´´´) and at δ_C 17.92. A number of multiplets appeared between δ_H 3.31–4.03 (10H, *m*) and correlated through a number of contiguous cross-peaks in COSY (S3), which are corresponding to the remaining sugars protons. The HMBC experiment (S4, S8) was conducted to get the attachment positions as

well as the inter-linkage of the methoxy group, glucose and rhamnose moieties through the long rang correlations (^2J & ^3J), the obtained results revealed the presence of three distinguishable cross peaks; the methoxy group at δ_H 3.83 with C-4′ of A-ring at δ_C 167.66, the anomeric proton of glucose at δ_H 5.02 with C-6′ of A-ring at δ_C 161.70, and the anomeric proton of rhamnose at δ_H 4.68 with the methylene hydroxy (CH_2OH) of glucose at δ_C 67.84, confirming (1′′′→6′′) glucorhamnopyranoside. Thus, the structure of compound (**1**) was elucidated as depicted in (Figure **1**) and assigned to 6′-*O*-rhamnosyl-(1′′′→6′′)-glucopyranosyl asebogenin trivially named as Thalassodendrone.

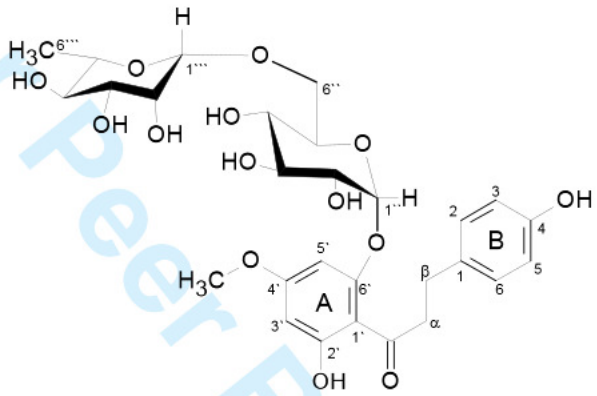

Figure **1.** 6′-*O*-rhamnosyl-(1′′′→6′′)-glucopyranosyl asebogenin (Thalassodendrone)

Infectious viral diseases remain a worldwide problem. Viruses have been resistant to therapy or propliylaxis longer than any other form of life due to their nature because they totally depend on the cells they infect for their multiplication and survival. Currently, there are only few drugs available for the cure of viral diseases including acyclovir, the known antiherpetic drug which is modeled on a natural product parent. In order to combat viruses which have devastating effects on humans, animals, insects, crop plants, fungi and bacteria, many research efforts have been devoted for the discovery of new antiviral natural products. So, as part of our ongoing collaborative effort to discover potential anti-viral agents from natural sources, the isolated new dihydrochalcones were tested for their anti-virus activity against influenza A virus using MTT method, and revealed an inhibition dose concentration of asebotin more than compound (**1**) with IC_{50} = 2.00 & 1.96 μg/mL respectively, and with cytotoxic concentration (CC_{50}) of 3.36 & 3.14 μg/mL respectively.

Quantitative structure-activity relationship (QSAR) models are useful in providing a biochemical understanding of the biological activity of natural and synthetic chemicals based solely on molecular structure. Both of the aromatic substituents on both A- & B-rings and the keto-enol functionality of the phenolics can serve as targets for future structure activity

relationship (SAR) studies (Wu et al., 2003). The substituents at the 4-position of the phenyl ring B should have electron-donating properties and most probably this part of the phenolic molecule interacts with the catalytic domain of the enzyme through hydrogen bonds. However, larger substituents in position-4 other than OH are not favourable (Alenka et al., 2002).

It is concluded that the anti-viral inhibitory properties of chalcones and flavonoids are mainly the outcome of electronic interactions between atomic charges within these compounds in both A and B rings and possible receptor-like structures in the cells and prevent the virus attach and penetration to the cell. These agonist-receptor interactions are enhanced by hydrogen bonding contributions and by specific geometrical arrangements associated with each compound.

3. Experimental

3.1. *Plant material*

Seagrass samples of *Thalassodendron ciliatum* (Forssk.) den 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. A voucher specimen (SAA-41) was deposited in the herbarium section of Pharmacognosy Department, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt.

3.2. *Extraction and isolation*

Fresh *T. ciliatum* (800 g) was blended in an electric blender with MeOH, the process was repeated until complete extraction. The resulted extracts were combined, filtered, and the solvent was evaporated under reduced pressure at 45 °C to afford a crude MeOH ext., which is then partitioned between EtOAc and H_2O several times, to afford ethyl acetate fraction (10.61 g). Then the EtOAc fraction was chromatographed on a Sephadex LH-20 column (600 mm) with step gradient elution starting from 30% ethanol in H_2O to 100% ethanol. Fractions of 250 mL each were collected and those exhibiting similar TLC profiles were combined together. Five sub-fractions (**I**–**V**) were obtained and subsequently fractionated on Sephadex LH-20 column; fraction **I** was eluted with sat. BuOH to afford five sub-fractions ($\mathbf{I}_{1\text{-}5}$) of which sub-fraction ($\mathbf{I}_2$) purified on preparative TLC (MeOH-$CHCl_3$ 20:80, v/v) gave compound-**1** (2 mg). Fraction **II** eluted with 100% EtOH gave two sub-fractions ($\mathbf{II}_{1,2}$) on purification on Sephadex LH-20 with 2% EtOH in H_2O resulted in the isolation of asebotin (6 mg). Fraction **III** eluted with 100% EtOH revealed the isolation of quercetin 3,7-diglucoside (4 mg) and protocatechuic acid (2 mg). Fraction **IV** eluted with 100% MeOH resulted in the

isolation of ferulic acid (17.5 mg). Fraction **V** eluted with sat. BuOH to afford *p*-hydroxybenzoic acid (4 mg).

*3.2.1. 6′-O-rhamnosyl-(1′′′→6′′)-glucopyranosyl asebogenin (**1**)*

Yellow amorphous solid, ^{1}H-NMR (CD_3OD, 500 MHz) δ_H 6.29 (1H, *d*, *J* = 2 Hz, H-3′), δ_H 6.31 (1H, *d*, *J* = 2 Hz, H-5′), δ_H 7.06 (2H, *d*, *J* = 8 Hz, H-2 & H-6), δ_H 6.68 (2H, *d*, *J* = 8 Hz, H-3 & H-5), δ_H 2.88 (2H, *t*, *J* = 7.5 Hz, CH_2-*β*), δ_H 3.46 (2H, *t*, *J* = 7.5 Hz, CH_2-*α*), δ_H 3.83 (3H, *s*, H_3CO-4′), δ_H 5.02 (1H, *d*, *J* = 7 Hz, H-1′′), δ_H 3.57 & δ_H 4.03 (2H, br*d*, *J* = 10 Hz, CH_2-6′′), δ_H 1.20 (3H, *d*, *J* = 6 Hz, H_3C-6′′′), δ_H 3.31–3.68 (8H, *m*, CH-2′′, 3′′, 4′′, 5′′, 2′′′, 3′′′, 4′′′, 5′′′); ^{13}C-NMR δ_C 107.55 (C-1′, *C*), δ_C 167.32 (C-2′, *C*), δ_C 95.95 (C-3′, *CH*), δ_C 167.66 (C-4′, *C*), δ_C 69.13 (C-5′, *CH*), δ_C 161.70 (C-6′, *C*), δ_C 133.75 (C-1, *C*), δ_C 130.41 (C-2 & -6, *CH*), δ_C 116.10 (C-3 & -5, *CH*), δ_C 156.40 (C-4, *C*), δ_C 29.40 (CH_2-*β*), δ_C 47.10 (CH_2-*α*), δ_C 206.98 (C=O), δ_C 56.24 (H_3CO-4′), δ_C 102.27 (C-1′′, *CH*), δ_C 74.77 (C-2′′, *CH*), δ_C 77.26 (C-3′′, *CH*), δ_C 71.43 (C-4′′, *CH*), δ_C 78.59 (C-5′′, *CH*), δ_C 67.84 (CH_2-6′′), δ_C 102.19 (C-1′′′, *CH*), δ_C 72.40 (C-2′′′, *CH*), δ_C 73.52 (C-3′′′, *CH*), δ_C 74.11 (C-4′′′, *CH*), δ_C 69.83 (C-5′′′, *CH*), δ_C 17.92 (H_3C-6′′′). HMBC and COSY correlations (S8), MALDI-MS (*m/z* 598.22561 for $[M + 2H]^+$ calcd 598.22616 for $C_{28}H_{38}O_{14}$).

3.3. *General experimental procedures*

NMR: 1D-spectra were obtained using a pulse sequence supplied from *JEOL JNM-AL*-400 MHz NMR spectrometer for (^{1}H, ^{13}C-NMR, DEPT-45, -90, -135 and ^{1}H–^{1}H COSY) in DMSO-d_6. 2D-spectras (HSQC and HMBC) were obtained using a pulse sequence supplied from Varian Gemini VNMR-500 MHz NMR spectrometer. Chemical shifts were given in values (ppm) relative to trimethylsilane (TMS) as an internal reference. High-resolution MALDI-MS: High resolution mass spectra were obtained on JEOL JMS-700N for electron ionization or on JEOL JMS-T100 TD for electrospray ionization, using *α*-Cyano-4-hydroxycinnamic acid (*CHCA*) as a matrix (*m*/z 189.17).

3.4. *Assay for Antiviral* Activity

3.4.1. *Cells and viruses*

Mardin-Darby canine kidney cells (MDCK) were grown in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (unless otherwise stated) at 37°C in a 5% CO_2 incubator. Original virus solution: influenza virus A/WSN/33 (3.72×10^7 $TCID_{50}$/mL), with 100TCID50/well infection.

3.4.2. *Cytopathic effect inhibition assay*

MDCK cells were seeded (100 μL/well = 3.0×10^4 cells/well) in 96-well plates and cultured in MEM/10% FBS for 2 days at 37ºC to >90% confluence. 40µL of the test sample (1mg sample dissolved in 1mL of DMSO) (4% solution, 2 fold dilutions) were added to 960 μL of MEM (-), and then from the mixture 120 μL/well were added to the each well of 96-wells. Cells were then washed with PBS and infected with approximately 50 plaque forming units (PFU) of influenza virus (1000$TCID_{50}$/mL). 100 μL aliquot of the cell suspension was added to each well of a 96-well flat-bottomed microtitre tray containing 100 μL of various concentrations of the test sample. After 3-days incubation at 37°C in 5% CO_2, the number of viable cells was determined by the MTT method (Pauwels et al., 1988). The cytotoxicity of the each compound was evaluated in parallel with the antiviral activity, which was based on the viability of mock-infected cells, as monitored by the MTT method (See supplementary material S7). The 50% antiviral effective dose (EC_{50}) and the 50% cytotoxic dose (CC_{50}) of the sample were determined. The absorbances were determined with Tecan Infinite® 200 PRO Modular Microplate Readers at a test wavelength of 560 nm.

4. Conclusions

In the present study, the Egyptian seagrass *T. ciliatum* was phytochemically investigated for its secondary metabolites, which resulted with the isolation of one new dihydrochalcone diglycosides together with asebotin, flavonoid diglycosides, and three phenolic acids. The anti-influenza A virus activity was evaluated for the newly isolated dihydrochalcone diglycosides along with its mono glycoside derivative, which resulted with virus inhibition with IC_{50} = 2.00 & 1.96 μg/mL respectively, and with cytotoxic concentration (CC_{50}) of 3.36 & 3.14 μg/mL respectively.

Supplementary material

Original NMR data (S1 – S5), high resolution MALDI/MS (S6) of compound (**1**), detailed protocol for anti-influenza A virus (S7), and some selected 2D correlations (S8) are available online.

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References

Achenbach, H., Stöcker, M., & Constenla, M.A. (1988). Flavonoid and other constituents of *Bauhinia manca*. *Phytochemistry, 27,* 1835–1841.

Alenka, S.-P., Ales, K., & Tom, S. (2002). QSAR of flavonoids: 4. Differential inhibition of aldose reductase and $p56^{lck}$ protein tyrosine kinase. *Croatica Chemica Acta, 75*, 517–529.

Bhakuni, D.S., & Rawat, D.S. (2005). Bioactive Marine Natural Products. India, Anamaya Publishers.

De La Torre-Castro, M., & Rönnbäck, P. (2004). Links between humans and seagrasses–an example from tropical East Africa. *Ocean & Coastal Management, 47*, 361–387.

Den Hartog, C. (1970). The Seagrasses of the World. pp. 275. Amsterdam, North Holland Publishers.

Guang-Min, Y., Yu, Ding., Jian-Ping, Zuo., Hong-Bing, Wang., Yu-Bo, Wang., Bing-Yang, Ding., Pauline, Chiu., & Guo-Wei, Qin. (2005). Dihydrochalcones from the Leaves of *Pieris japonica*. *Journal of Natural Products*, *68,* 392–396.

Harborne, J.B., & Baxter, H. (1999). The Handbook of Natural Flavonoids, Vol. (2), p. 175. Chichester, John Wiley & Sons.

Howard, R.K., Edgar, G.J., & Hutchings P.A. (1989). Faunal Assemblages of Seagrass Beds. In: Larkum, A.W.D., McComb, A.J., & Shepherd, S.A. (Eds.), Biology of Seagrasses. pp. 539–564. Amsterdam, Elsevier.

Hufford, C.D., & Oguntimein, B.O. (1982). New dihydrochalcones and flavanones from *uvaria angolensis*. *Journal of Natural Product, 45,* 337–342.

Mabry, T.J., Markham, K.R., & Thomas, M.B. (1970). The Systematic Identification of Flavonoids. pp. 354. Berlin, Springer-Verlag.

Nkengfack, A.E., Van Heerden, F.R., Fuendjiep, V., & Fomum, Z.T. (2001). Asebotin, a dihydrochalcone glucoside from *Guibourtia tessmannii*. *Fitoterapia, 72,* 834–836.

Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J., & De Clercq, E. (1988). Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *Journal of Virological Methods, 20,* 309–321.

Pergent-martini, C., & Pergent, G. (2000). Marine phanerogams as a tool in the evaluation of marine trace-metal concentration: an example from the Mediterranean. *International Journal of Environment and Pollution, 13,* 126–147.

Pergent-martini, C., Leoni, V., Pasqualini, V., Ardizzone, G.D., Balestri, E., Bedini, R., et al.

(2005). Discriptors of posidonia oceanica meadows: use and application. *Ecological Indicatiors, 5,* 213–230.

Takeo, Y., Tomohisa, I., Shozo, F., & Yasuo, K. (2004). Phenolic compounds and flavonoids as plant growth regulators from fruit and leaf of *Vitex rotundifolia*. *Zeitschrift für Naturforschung*, *59c,* 509–514.

Wu, J.H., Wang, X.H., Yi, Y.H., & Lee, K.H. (2003). Anti-AIDS agents 54. A potent anti-HIV chalcone and flavonoids from genus *Desmos*. *Bioorganic and Medicinal Chemistry Letters, 13,* 1813–1815.

Zhilan, L., Juan, W., Pingniang, S., Chengyun, W., & Yongjia, S. (2006). Microwave-assisted extraction and high-speed counter-current chromatography purification of ferulic acid from *Radix Angelicae sinensis*. *Separation and Purification Technology, 52,* 18–21.