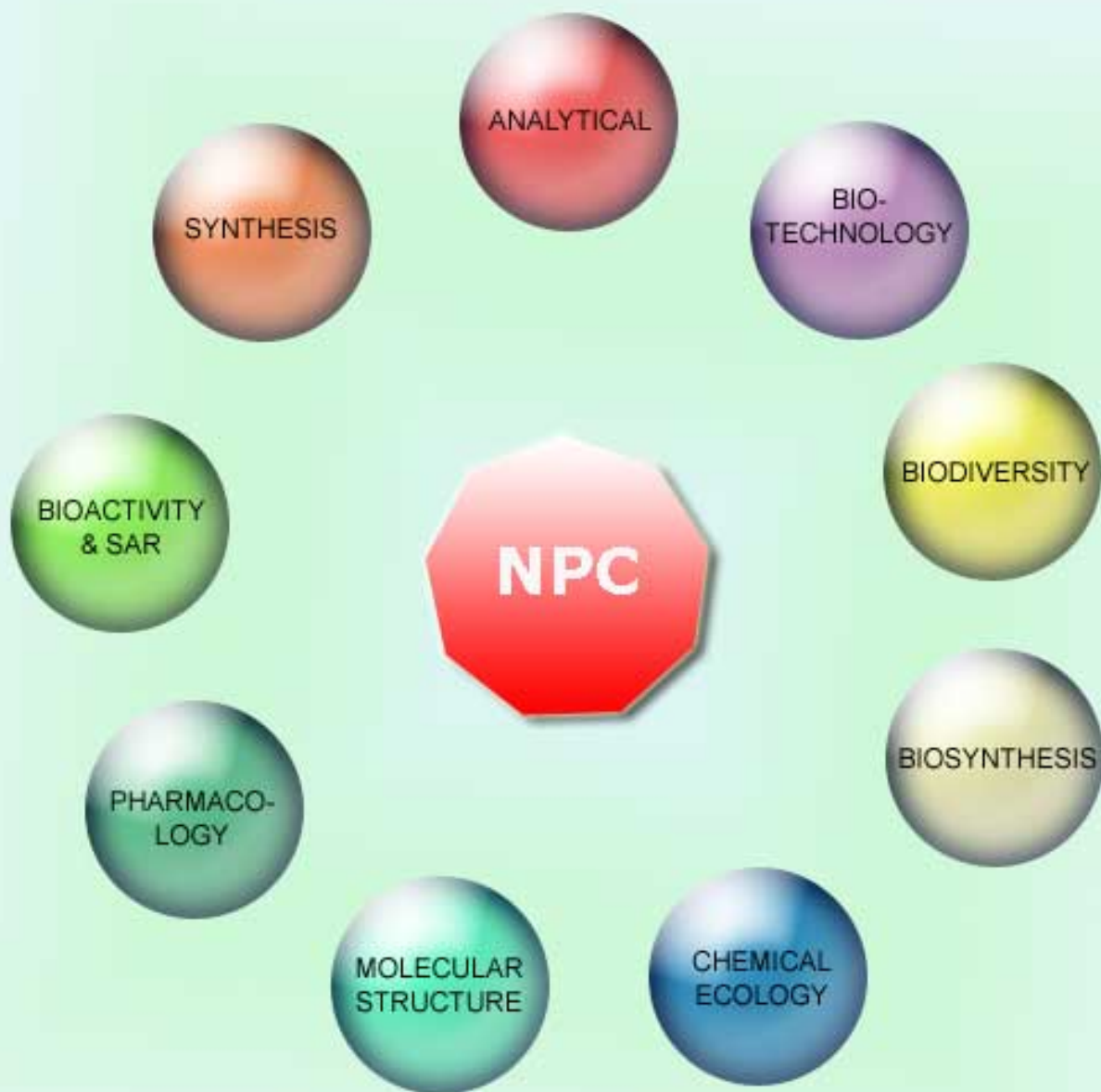


NATURAL PRODUCT COMMUNICATIONS

An International Journal for Communications and Reviews Covering all
Aspects of Natural Products Research



Volume 2. Issue 8. Pages 795-882. 2007
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

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Inhibitory Effects of *Cissus quadrangularis* L. Derived Components on Lipase, Amylase and α -Glucosidase Activity *in vitro*

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Received: May 9th, 2007; Accepted: June 21st, 2007

The stems of *Cissus quadrangularis* L (Vitaceae) have been consumed for centuries throughout Asia and Africa as a culinary vegetable. The aqueous extract of *C. quadrangularis* stems and leaves contains flavonoids and stilbenes, which inhibit lipase, amylase and α -glucosidase and, therefore, might be, at least in part, related to the claimed anti-obesity activity of the plant. The enzyme inhibition activity is outlined of a novel flavonoid (3-*O*- α -L-rhamnopyranosylkaempferol) (**2**) and stilbene (3-(4-hydroxybenzylidene)-2-(2,5-dihydroxyphenyl)-1-(4-hydroxyphenyl)indane-4,6-diol) (**7**), four known structurally related flavonoids, and one stilbene isolated from the extract.

Keywords: *Cissus quadrangularis* L., obesity, weight control, enzyme inhibition.

Cissus quadrangularis L. (Vitaceae) is an ancient medicinal plant native to the hotter parts of Ceylon and India. The plant has been used in India for promoting the fracture healing process [1]. It has been prescribed in Ayurveda as an anthelmintic, dyspeptic, digestive tonic, analgesic for eye and ear diseases, and in the treatment of irregular menstruation and asthma. In Cameroon, the whole plant is used in oral rehydration, while the leaf, stem and root extracts of this species are important in the management of various ailments. There are several reports on the use of *C. quadrangularis* in the management of obesity and complications associated with metabolic syndrome [2], and on its antioxidant and free radical scavenging activity *in vitro* [3,4]. Various formulations now contain extracts of *C. quadrangularis* in combination with other compounds, used for the purpose of weight management and complications resulting from these conditions.

Phytochemical analyses of *C. quadrangularis* have revealed high contents of ascorbic acid, carotene,

anabolic steroidal substances and calcium. Previous reports indicate the presence of triterpenoids [5-8], steroids [5,6,9-11], stilbenes [11,12], flavonoids [10], lipids [8], and iridoids [11]. We now report two new components of *C. quadrangularis* and the inhibition of enzymes by these and other components that might be related to the therapeutic claims for the plant.

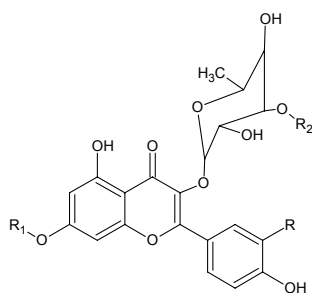
Dried *C. quadrangularis* was extracted and fractionated in order to isolate and identify compounds that might contribute to the anti-obesity activity of either the plant or extracts of it, by inhibition of lipase, α -amylase and α -glucosidase [13] activities. The 50% aqueous ethanol extract of the plant was passed through a column of cation exchange resin (Dowex 50 H⁺ form) from which the inhibitors were eluted. The extract was applied to HP20 resin and three active fractions obtained: unbound, 25% methanol eluate, and 10% acetone in methanol eluate. All showed enzyme inhibition (Table 1) and similar chemical profiles, but the 10% acetone in methanol fraction was more suitable for compound purification.

Table 1: Enzyme inhibition activity (% inhibition).

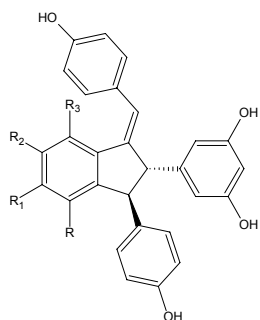
Assay	Roche "Xenical" orlistat	Bayer "Glucobay" acarbose	unbound	25% methanol	10% acetone in methanol
	Lipase (human pancreatic)	53 ± 5	-	64 ± 3	59 ± 6
α-amylase (porcine)	-	100 ± 7	98 ± 3	98 ± 6	92 ± 9
α-glucosidase (<i>S. cerevisiae</i>)	48 ± 15	27 ± 3	91 ± 1	87 ± 0.2	73 ± 4

Samples tested at 2 mg/mL (20 μg/well) with 0.15 mg/mL (lipase), 0.12 mg/mL (amylase) and 0.28 mg/mL (glucosidase), respectively. Results are mean values (n=3) and values represent mean ± S.D

Further chromatography of this fraction by polar flash chromatography on silica, and preparatory and semi-preparatory HPLC on C18 gave seven compounds (Figure 1). Five were identified by comparison with spectroscopic data previously reported in the literature [*Dictionary of Natural Products* (DNP) available on CDROM] as quercitrin (**1**), rhamnitrin (**3**), 3-*O*-α-*L*-rhamnopyranosyl-rhamnocitrin (**4**), quercitrin-3-*O*''-acetate (**5**) and parthenocissin A (**6**).



- 1:** R = OH, R₁ = H, R₂ = H
2: R = H, R₁ = H, R₂ = H
3: R = OH, R₁ = CH₃, R₂ = H
4: R = H, R₁ = CH₃, R₂ = H
5: R = OH, R₁ = H, R₂ = COCH₃



- 6:** R = OH, R₁ = H, R₂ = OH, R₃ = H
7: R = H, R₁ = OH, R₂ = H, R₃ = OH

Figure 1: Structures of compounds isolated from *C. quadrangularis*.

Compound **2** had similar UV, ¹H and ¹³C NMR spectroscopic features to **1**, **3**, **4**, and **5** and was, therefore, deduced to be a flavonoid glycoside. Proton attachments to individual carbon atoms were assigned from pendant and HMQC spectra. The presence of a 1,4-disubstituted benzene ring was

recognized from the distinctive carbon and hydrogen signals, COSY and HMBC correlations given by the characteristic *meta*-split doublets at δ 6.84 and 7.67 ppm in the ¹H NMR spectrum. The ¹³C NMR spectroscopic data showed the presence of single methyl (δ 18.0) and five methine (δ 72.3, 72.4, 72.6, 73.6 and 103.9) carbon atoms; the connectivity was defined from COSY and HMBC spectroscopic data and was consistent with a rhamnose sugar moiety. Doublets in the ¹H NMR spectrum at δ 6.10 and 6.27 were assigned to the flavonoid core and the coupling of 2 Hz to each other was consistent with *meta*-substitution. The ¹H NMR spectrum was the same as for **4** only differing in a singlet at δ 3.76 in the case of **4**, which is due to the methoxy substituent on the flavonoid ring. Because of this, the stereochemistry was assigned to be the same as for the known compound **4**, i.e. α-*L*-sugar linkage. The structural data are consistent with the structure 3-*O*-α-*L*-rhamnopyranosylkaempferol (**2**).

Compound **7** showed similar UV, ¹H and ¹³C NMR spectroscopic features to **6** and was, therefore, deduced to be a stilbene. Proton attachments to individual carbon atoms were assigned from pendant and HMQC spectra. The presence of two 1,4-disubstituted benzene rings was recognized from the distinctive carbon and hydrogen signals, and from the COSY and HMBC correlations given by the characteristic *meta*-split doublets at δ 6.81 and 7.07 and δ 6.81 and 7.29 in the ¹H NMR spectrum. HMBC correlations to quaternary carbons at δ 156.9 and δ 156.5, respectively, indicate a *para*-hydroxyl group connected to the benzene rings. COSY correlations between the triplet at δ 6.27 and the doublet at δ 6.41 in the ¹H NMR spectrum, together with the small coupling constant of 2 Hz, indicate a symmetrical trisubstituted benzene ring. HMBC correlations to the quaternary carbon at δ 160.1 indicate 3,5-dihydroxyl substitution on the ring. COSY correlations between the protons at δ 4.21 and δ 4.36 in the ¹H NMR spectrum indicate

them to be linked and from comparison with **6** are assigned to the five membered ring of the stilbene. The signal at δ 4.21 in the ^1H NMR spectrum shows HMBC correlations to the doublet at δ 6.41 and the aromatic singlet at δ 7.16. This is consistent with a 3,5-dihydroxyl substituted benzene ring being attached to it and it being three bonds away from a substituted double bond. An HMBC correlation between the aromatic singlet at δ 7.16 and the *meta*-split doublet at δ 7.29 in the ^1H NMR spectrum is consistent with a *para*-substituted benzene ring being attached at this point. The signal at δ 4.36 in the ^1H NMR spectrum shows HMBC correlations to the double doublet at δ 7.07 and the quaternary carbons at δ 130.7, 139.0 and 143.8. This is consistent with a *para*-hydroxylsubstituted benzene ring attached at this point and confirms the presence of a benzene ring attached to the carbon with the signal at δ 4.21 and with a substituted double bond being two carbons away. This accounts for the attachment of the *para*-hydroxylphenyl and 3,5-dihydroxylphenyl rings as indicated in Figure 1. HMBC correlations between the aromatic doublets at δ 6.35 and 6.88 in the ^1H NMR spectrum to each other, along with the coupling constant of 2 Hz, indicate them to be *meta* to each other, and both show HMBC correlations to aromatic quaternary carbons, indicating dihydroxyl substitution of the ring. Comparison with **6** indicates them to be part of the stilbene core. An HMBC correlation between the signals at δ 6.88 and 4.36 in the ^1H NMR spectrum places the aromatic methine three bonds from the methine bearing the *para*-hydroxylphenyl ring. The ^1H NMR spectrum for the protons of the five membered ring was the same as for **6** and, therefore, the same stereochemistry was assumed. The structural data are consistent with the structure 3-(4-hydroxybenzylidene)-2-(2,5-dihydroxyphenyl)-1-(4-hydroxyphenyl)indane-4,6-diol (**7**).

Inhibition of enzyme activity: All but one of the flavonoids showed good α -amylase inhibition (Table 2). Quercitrin (**1**) differs from the novel 3-*O*-rhamnopyranosylkaempferol (**2**) only by one hydroxyl substituent, but this results in a decrease in α -amylase inhibition. However, methylation of a ring hydroxyl, as in the case of rhamnitrin (**3**), or acetylation of the sugar, as in the case of quercitrin-3-*O*''-acetate (**5**) causes the inhibition to be restored. All the flavonoids show similar responses to lipase inhibition, but again the inhibition of α -glucosidase shows variation for the various structural features.

The two stilbenes, **6** and **7** display significantly different inhibition profiles. Parthenocissin A (**6**) shows good inhibition of α -amylase, whereas the novel 3-(4-hydroxybenzylidene)-2-(2,5-dihydroxyphenyl)-1-(4-hydroxyphenyl)indane-4,6-diol (**7**) is fairly specific as an inhibitor of the α -glucosidase. It is interesting to note that **1** and **5** promoted activity of the glucosidase.

We have not determined the K_i values of the more potent of the individual inhibitors since we were more interested in indicating the individual effects of each component of the extract. It is important in such studies, however, to look for some specificity of inhibition to prove that the inhibition is not a therapeutically unattractive, non-specific interaction with proteins in general. We have shown here that the compounds show specific inhibition of different enzymes. The inhibition of lipase did not go above 60%, even with use of the commercial inhibitor Orlistat® at the same top concentration. The lipase and amylase seemed much more prone to inhibition by the *C. quadrangularis* components in general than the α -glucosidase.

The presence of several components exhibiting inhibition of amylase, lipase and glucosidase suggests that the plant could well have an effect on the ability of the gastrointestinal tract to utilize the plant and other sources of nutrients efficiently. Inhibitors of lipase such as Orlistat® and glucosidases, such as Glyset® and Acarbose®, are used as drugs to treat obesity and diabetes type 2. These drugs, however, often cause side-effects and it may well be that the combination of several components in *C. quadrangularis* working on different enzymes has a better tolerated effect. Although the combined effect of the aqueous extract was inhibition of the glucosidase activity, it is of interest to note that **1** and **5** actually promoted the glucosidase activity. Such promotion of enzyme activity can be due to binding to allosteric sites on the enzyme. While the effect of increased glucosidase activity by some components on either weight control or diabetes is difficult to understand, there are many α -glucosidases present in the gastrointestinal tract and within cells, and inhibition of some may be beneficial to the control of either weight or diabetes, whereas increased activity of certain glucosidases may be promoting health in other ways. We have only used one α -glucosidase here and the compounds may vary in effects and activity on other α -glucosidases. The ability of the individual components identified to reach the sites of

Table 2: Enzyme inhibition activity (% inhibition).

Assay	Roche	Bayer	1	2	3	4	5	6	7
	"Xenical" orlistat	"Glucobay" acarbose							
Lipase (human pancreatic)	53 ± 5	-	51 ± 3	55 ± 6	47 ± 4	52 ± 5	60 ± 6	41 ± 2	35 ± 4
α-amylase (porcine)	-	100 ± 7	50 ± 3	88 ± 6	94 ± 9	95 ± 7	88 ± 6	94 ± 4	51 ± 8
α-glucosidase (<i>S. cerevisiae</i>)	48 ± 15	27 ± 3	-28 ± 1	-7 ± 0.2	54 ± 4	50 ± 4	-74 ± 4	67 ± 4	89 ± 9

Samples tested at 2mg/mL (20µg/well) with 0.15mg/mL (lipase), 0.12mg/mL (amylase) and 0.28mg/mL (glucosidase) respectively. Results are mean values (n=3) and values represent mean ± S.D.

enzyme activity will also vary and will be influenced by individual differences and no doubt also by variations in other food consumed with *C. quadrangularis* and formulation of products containing it.

The inhibition of α-glucosidases has been shown to be potentially therapeutically useful in diseases other than diabetes type 2 and weight control [14]. In particular such inhibitors have been studied as potential anti-viral and anti-cancer agents and it may well be that inhibition of α-glucosidases by *C. quadrangularis* explains the wide traditional medicinal use of the species. Promotion of specific glycosidase activities can also be therapeutic where those enzymes are deficient [14].

Experimental

Structure elucidation: Structure elucidation was carried out using NMR spectroscopy (Bruker DRX500) for full ¹H and ¹³C assignments. The assignment of proton and carbon NMR signals was determined from extensive 2D NMR spectroscopic experiments, including ¹H-¹H COSY, HMQC, and HMBC. LCMS (Waters Integrity) gave EIMS data for molecular weight and/or fragmentation assignment.

Literature searches were conducted using the *Dictionary of Natural Products* (DNP) available on CDROM.

Plant material: *Cissus quadrangularis* was supplied dry from the Cameroon. The reference for the voucher specimen for is No. 18668/SRF/Cam, identified by the Cameroon National Herbarium, Yaounde, Cameroon.

Extraction and isolation: The dried *Cissus quadrangularis* (2.3 kg) was extracted with 50% aqueous ethanol (10 L) overnight. The filtered extract

was loaded onto two cation exchange columns (Dowex 50 H⁺ form, 700 cm³) on which the inhibitors were not retained (Table 1). The extract (8 L) was applied to an HP20 cartridge (6 x 75cm) pre-equilibrated with 50% aqueous methanol (5 L) and eluted with 25% aqueous methanol (3 L), followed by 10% acetone in methanol (6 L) to give three active fractions: unbound (37 g), 25% methanol eluate (27 g) and 10% acetone in methanol eluate (14 g). All showed enzyme inhibition and similar chemical profiles but the 10% acetone in methanol fraction was more suitable for compound purification. The 10% acetone in methanol extract was bound onto silica and applied to a KP-SilTM silica Flash 75S cartridge (7.5 x 9.0 cm) pre-equilibrated with heptane (5 L) and eluted with 75% heptane in ethyl acetate (1 L), 50% heptane in ethyl acetate (1 L), 25% heptane in ethyl acetate (1 L), 100% ethyl acetate (1 L) and 95% ethyl acetate in methanol (1 L) to give 5 fractions.

Preparatory HPLC of fraction 4, conducted on a Water's Nova-Pak^R HR C18 column (2 x (40 x 100 mm) in series, 6 µm, 60A⁰) at a flow rate of 55 mL/min and a monitoring wavelength of 225 nm with an acetonitrile/water gradient (80% water : 20% acetonitrile containing 0.1% TFA, to 65% water : 35% acetonitrile containing 0.1% TFA over 15 mins) gave rise to parthenocissin A (**6**) (25 mg). Further purification of this fraction on a Water's Nova-Pak^R HR C18 column (25 x 100 mm, 6 µm, 60A⁰) at a flow rate of 15 mL/min and monitoring wavelength of 225 nm with a methanol/water gradient (55% water : 45% methanol, containing 0.1% TFA, to 30% water : 70% methanol, containing 0.1% TFA over 15 mins) gave rise to quercitrin-3-*O*'-acetate (**5**) (12 mg) and 3-(4-hydroxybenzylidene)-2-(2,5-dihydroxyphenyl)-1-(4-hydroxyphenyl)indane-4,6-diol (**7**) (19 mg). Preparative HPLC of fraction 5 on a Water's Nova-Pak^R HR C18 columns [2 x(40 x 100 mm) in series, 6 µm, 60A⁰] at a flow rate of

55 mL/min and a monitoring wavelength of 210 nm with an acetonitrile/water gradient (80% water : 20% acetonitrile, containing 0.1% TFA, to 60% water : 40% acetonitrile, containing 0.1% TFA over 20 mins) gave rise to quercitrin (**1**) (35 mg), 3-*O*-rhamnospyranosylkaempferol (**2**) (13 mg), rhamnitrin (**3**) (10 mg) and 3-*O*- α -L-rhamnopyranosylrhamnitrin (**4**) (13 mg)

Kits and reagents used for bioassays: The purified compounds and extracts were made up 2 mg/mL solutions in water using initially a drop of DMSO, if required, for solubility.

Lipase: Sigma Lipase-PS kit (catalogue number 805-A). Human pancreatic lipase was diluted 1.5:5 prior to use. The assay was carried out at 20°C using 10 μ L test compound, 5 μ L enzyme solution, 90 μ L substrate, and 30 μ L activator reagent. Color formation was measured at 550 nm after 30 minutes.

Amylase: Sigma INFINITY powder reagent (catalogue number 568-20); Sigma α -amylase (A6255, porcine pancreatic amylase as saline suspension) diluted to 35 units/mL. The assay was carried out at 20°C using 10 μ L enzyme solution, 10 μ L test compound, and 150 μ L INFINITY reagent. The rate of color formation was measured over 5 minutes, after which the final absorbance was measured at 405 nm as an endpoint recording.

α -Glucosidase: Sigma α -glucosidase (G5003, from *Saccharomyces cerevisiae*) at 2 units/mL in phosphate buffer pH 6.0. Sigma *p*-nitrophenyl- α -D-glucopyranoside, 5 mM in phosphate buffer pH 6.0. The assay was carried out at 20°C using 10 μ L enzyme solution, 10 μ L test compound, and 50 μ L substrate solution. The reaction was carried out for 7 minutes; color formation was measured at 415 nm

following the addition of 80 μ L 0.4 M glycine solution, pH 10.4, to stop the reaction. Other glycosidase assays were carried out using enzymes from Sigma and appropriate *p*-nitrophenyl substrates [13].

3-*O*- α -L-Rhamnopyranosylkaempferol (**2**)

UV/Vis λ_{\max} (MeOH) nm (log ϵ): 200, 264, 342.

^1H NMR (500 MHz, MeOH): δ 0.79 (3H, d, $J = 6$ Hz), 3.23 (1H, m), 3.59 (1H, m), 3.59 (1H, m), 4.11 (1H, m), 5.27 (1H, d, $J = 2$ Hz), 6.10 (1H, d, $J = 2$ Hz), 6.27 (1H, d, $J = 2$ Hz), 6.84 (2H, dd, $J = 2, 9$ Hz), 7.67 (2H, dd, $J = 2, 9$ Hz).

^{13}C NMR (500 MHz, MeOH): δ 18.0 (CH₃), 72.3 (CH), 72.4 (CH), 72.6 (CH), 73.6 (CH), 95.2 (CH), 100.3 (CH), 103.9 (CH), 106.4 (C), 117.0 (2xCH), 123.1 (C), 132.3 (2xCH), 136.7 (C), 159.0 (C), 159.7 (C), 162.0 (C), 163.7 (C), 166.3 (C), 180.1 (C=O).

HPLC-MS: m/z , 288 (100) (C₂₁H₂₂O₁₀), 257, 120.

3-(4-Hydroxybenzylidene)-2-(2,5-dihydroxyphenyl)-1-(4-hydroxyphenyl)indane-4,6-diol (**7**)

UV/Vis λ_{\max} (MeOH) nm (log ϵ): 200, 323.

^1H NMR (500 MHz, MeOH) δ : 4.21 (1H, broad s), 4.36 (1H, broad s), 6.27 (1H, t, $J = 2$ Hz), 6.35 (1H, d, $J = 2$ Hz), 6.41 (2H, d, $J = 2$ Hz), 6.81 (4H, dd, $J = 2, 9$ Hz), 6.88 (1H, d, $J = 2$ Hz), 7.07 (2H, dd, $J = 2, 9$ Hz), 7.16 (1H, s), 7.29 (2H, d, $J = 9$ Hz).

^{13}C NMR (500 MHz, MeOH): δ 58.5 (CH), 61.6 (CH), 98.9 (CH), 102.0 (CH), 104.3 (CH), 107.1 (2xCH), 116.3 (2xCH), 116.4 (2xCH), 123.6 (CH), 125.9 (C), 129.3 (2xCH), 129.6 (2xCH), 130.7 (C), 131.6 (CH), 139.0 (C), 143.8 (C), 148.0 (C), 148.1 (C), 150.1 (C), 156.5 (C), 156.9 (C), 157.8 (C), 160.1 (2xC).

HPLC-MS: M^+ 454 (C₂₈H₂₂O₆), m/z 360, 347 (100), 331, 239, 227, 226, 215, 199, 180, 149, 107, 95.

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