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Antioxidant Flavone C-biosides from the aerial parts of *Alternanthera pungens*

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From the 2-butanone fraction derived out of the aqueous alcoholic extract of the fresh aerial parts of *Alternanthera pungens*, three C-glycoflavones, vitexin (1), isovitexin (2) and orientin (3) and an yellow solid composed of their 2"-O-β-D-glucopyranosides (4-6) were isolated and characterised. Fully assigned sets of proton and carbon resonances, based on detailed 1D and 2D NMR measurements, including ^{1}H COSY, HMQC and HMBC experiments, enabled the deduction of definitive evidence for the C- and the O-glycosidic linkages of the three biosides. Antioxidant potential of these six compounds was evaluated using two different assays, namely, the Trolox equivalent antioxidant capacity and the coupled oxidation of β-carotene and linoleic acid (autoxidation) assays. Compounds 3 and 6 were fairly active while the others were mildly active in both the assays.

Alternanthera pungens H.B.et K.[1,2] (Amaranthaceae) [syn.: *Achyranthes repens* L., *Alternanthera repens* (L.) Link; *Alternanthera achyranthra* R.Br] is a folk medicine of Argentina, commonly known as *Yerba del pollo* and recorded in the Pharmacopea National Argentina (1978). A native of American continent is now widely spread as a weed in the tropics and subtropics of the old world and naturalized in several parts of South India. The whole plant is reported to be used in gastric, hepatic and intestinal disturbances, such as dyspepsia, secretory and motor symptoms and the aerial part as diuretic and emollient[3]. The aqueous extract has been found to exhibit spasmogenic properties[4] while the ethanol extract has been evaluated to possess diuretic activity equivalent to furosemide[5]. The aqueous and the ethanol fractions also presented antidiarrhoeal effects[6]. Saponins,alkaloids,steroids,triterpenoids, leucoanthocyanidins[7,8], β-spinasterol[9], a saponinic heteroside of oleanolic acid[10] and choline[11] have been previously described form this plant. The present article deals with the isolation of three C-glycoflavones, vitexin (1), isovitexin (2) and orientin (3) and an yellow solid composed of a mixture of their O-glucopyranosides, 2"-O-β-D-glucopyranosylvitexin (4), 2"-O-β-D-glucopyranosylisovitexin (5) and 2"-O-β-D-glucopyranosylorientin (6), (fig.1), hitherto not reported from this herb, and the unambiguous assignment of the site of glycosylation in the three biosides, based on 1D and 2D NMR data, including ^{1}H COSY, HMQC and HMBC experiments. It is now widely accepted that reactive oxygen species (ROS) play different roles *in vivo*. Some are beneficial and are related to their involvement in energy production, phagocytosis, regulation of cell growth and intercellular signalling, and synthesis of biologically important compounds, while others may be very damaging as they can attack lipids in cell membranes, proteins in tissues or enzymes, carbohydrates and DNA, inducing oxidations and, thus, cause membrane damage, protein modification and DNA damage[12]. Antioxidants tend to reduce free radical formation and scavenge free radicals. Despite the fact that humans have evolved with antioxidant systems to protect against free radicals, which may be endogenous or exogenous, some ROS still escape in quantities sufficient enough to cause damage. Therefore, exogenous antioxidants that scavenge free radicals, especially, those from the relatively harm-

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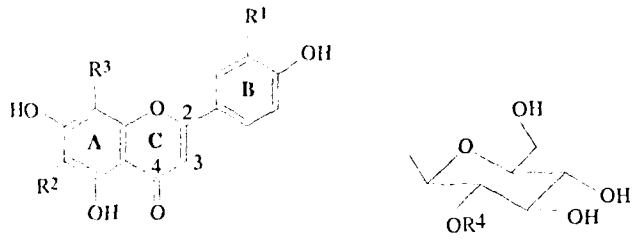

Fig. 1: C-glycoflavones

1. R^1=R^2=H; R^3=ß-D-glucopyranosyl (vitexin), 2. R^1=R^3=H; R^2=ß-D-glucopyranosyl (isovitexin), 3. R^1=OH; R^2=H; R^3=ß-D-glucopyranosyl (orientin), 4. R^1=R^2=H; R^3=R^4=ß-D-glucopyranosyl (2"-O-ß-D- glucopyranosylv-itexin, 5. R^1=R^3=H; R^2=R^4=ß-D-glucopyranosyl (2"-O-ß-D-glucopyranosylisovitexin), 6. R^1=OH; R^2=H; R^3= R^4=ß-D-glucopyranosyl (2"-O-ß-D-glucopyranosylorie- ntin)

less natural sources play an important role in cardiovascular disease, aging, cancer and inflammatory disorders as well as in ameliorating drug-induced toxicity. This has accelerated the search for potential antioxidants from traditional medicinal plants.

MATERIALS AND METHODS

Gel chromatographic separation of the chemical constituents was carried out in open column using Sephadex LH-20, Pharmacia and TLC using microcrystalline cellulose, Merck. Trolox, 2,6-di-*tert*-butyl-4-methylphenol and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt from Aldrich Chemical Co. and BHT from Loba Chemie. All other reagents/solvents are of analytical or laboratory grades from standard companies. UV spectra were recorded on Shimadzu UV-160 Spectrophotometer. The FAB mass spectrum was recorded on a JEOL SX 102/DA-6000 Mass Spectrometer/Data System using Argon/Xenon (6 kV, 10 mA) as the FAB gas and m-nitrobenzyl alcohol as the matrix at room temperature. The NMR experiments were performed on Bruker DRX-500 spectrometer, operating at 500 and 125 MHz for ^1H and ^{13}C, respectively, with DMSO-d_6 solutions.

Extraction and isolation:

Fresh aerial parts of *A. pungens* (2.9 kg), collected from Pondicherry and duly authenticated at the Department of Botany, were extracted with boiling ethanol (3×8 l) and concentrated *in vacuo* to yield the aqueous concentrate. This was then partitioned with benzene, diethyl ether and 2-butanone successively, again concentrated and then checked for the availability of phenolic constituents using paper chromatography (PC) (Whatman No.1, 1-butanol:acetic acid:water {4:1:5}, ascending, 28°, 8 h). The 2-butanone fraction (720 mg) that answered positively for flavonoids was passed through a column of Sephadex LH-20 (100 g) in nine portions of 80 mg each and eluted every time using a solvent mixture of methanol:1, 2-dichloroethane (7:3). The eluates of each portion were analysed by PC and combined appropriately and evaporated to afford 5 fractions (I-V). After rejecting fraction I, fractions II-V were again subjected individually to gel chromatography (Sephadex LH-20) and the eluates analysed as before and combined. This again resulted in the collection of five more fractions (1-5). Again after rejecting 1 and leaving aside 2, as it was homogeneous in PC, fractions 3-5 were rechromatographed individually in the same column and processed as before to yield three fractions F1, F2 and F3. F1 had PC behaviour similar to 2 and hence were combined and preserved. As F2 was a mixture of 1-6, it was not processed further. F3 was subjected to preparatory PC (Whatman No. 3, 5% acetic acid, descending, 28°, 6 h) to separate 1 (R_f 0.15), 2 (R_f 0.24) and 3 (R_f 0.08) and each of them was finally purified by chromatography on Sephadex LH 20 using methanol. The combined fractions 2 and F1 upon concentration yielded a pale yellow solid (582 mg). A portion of the solid was then subjected to TLC (microcrystalline cellulose, 1-butanol:acetic acid:water {4:1:5}, ascending, 28°, 10 h), to isolate 4 (R_f 0.42), 5 (R_f 0.50) and 6 (R_f 0.38), which were also purified by passing through Sephadex column.

Trolox equivalent antioxidant capacity test:

This method relies on the generation of a long-lived specific 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) [$ABTS^{\cdot+}$] radical cationic chromophore in phosphate-buffered saline pH 7.4 and its quenching by an antioxidant, which is measured spectrophotometrically at 734 nm[13,14]. It determines the relative ability of the compounds to scavenge $ABTS^{\cdot+}$ as compared to a standard amount of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), the water-soluble vitamin E analogue, expressed as TEAC values. TEAC value is defined as the concentration of standard Trolox with the same antioxidant activity as a 1 mM concentration of the compound under investigation and is taken as an index to evaluate antioxidative activity of an antioxidant. Quercetin, isolated from *Taxillus bracteatus*[15] is used as the reference compound.

Autoxidation of β-carotene:

Oxidation of linoleic acid was measured by the method

described by Pratt[16]. Linoleic acid (20 mg) and Tween 20 (200 mg) were taken in a flask and a solution of β-carotene (2 mg in 10 ml of chloroform) was added. After removal of chloroform 50 ml of distilled water saturated with oxygen was added. Aliquots (200 µl) of the ethanol solution (15 µg/ml) of each compound were added to each flask with shaking. Samples without test compounds were used as blanks, and a sample with 2,6-di-*tert*-butyl-4-methylphenol (BHT) was used as a control substance. After incubation of the samples at 50° for 3 h to effect autoxidation, the absorbance was read at 470 nm at regular intervals. The antioxidant activity was calculated using the relation, antioxidant activity=$[1-(A_0-A_t)/(A_{00}-A_{0t})]\times100$, where, A_0=absorbance at the beginning of the incubation, with test compound; A_t=absorbance at time t, with test compound; A_{00}=absorbance at the beginning of the incubation without test compound; A_{0t}=absorbance at time t, without test compound. Compounds are considered active when their antioxidant activity is close to that of the control substance, BHT.

RESULTS AND DISCUSSION

Systematic chemical examination of the aerial parts of *A. pungens* has resulted in the isolation of three C-glycoflavones, vitexin (1), isovitexin (2) and orientin (3) and an yellow solid composed of their 2"-O-β-D-glucopyranosides (4-6) from the 2-butanone soluble fraction. All the six compounds are reported from this taxon for the first time. The identity of the three familiar flavone C-glycosides (1-3) has been established by comparing their physical, chemical and spectral data with those available in literature[17,18] and further confirmed by mmp. and co-chromatography with authentic compounds[18-20]. Compounds 4-6 exhibited mp., hydrolytic behaviour and spectral (UV, ^{1}H-NMR and Mass Spectral) characteristics as reported earlier for compounds 2"-O-β-D-glucopyranosylvitexin,[18] 2"-O-β-D-glucopyranosylisovitexin[21] and 2"-O-β-D-glucopyranosylorientin[18]. Their ^{13}C-NMR spectra contained 27 carbon resonances confirming the diglycoside nature of the three compounds. The absence of proton resonances correlating to C-8 of compound 4, C-6 of compound 5 and C-8 of compound 6 in HMQC experiments (Table 1 and figs. 2-4) constituted an evidence for the glycosyl substitution at these carbons. The fact that the ^{1}H-NMR spectra of the three compounds contained the characteristic β-linked anomeric doublets (J~7 Hz) at δ=4.8 ppm and at δ=4.2 ppm prompted one to infer the bioside nature of compounds 4-6. Their structures have been unequivocally established by tracing the connectivities of the C-glucosyl and the O-glucosyl moi-

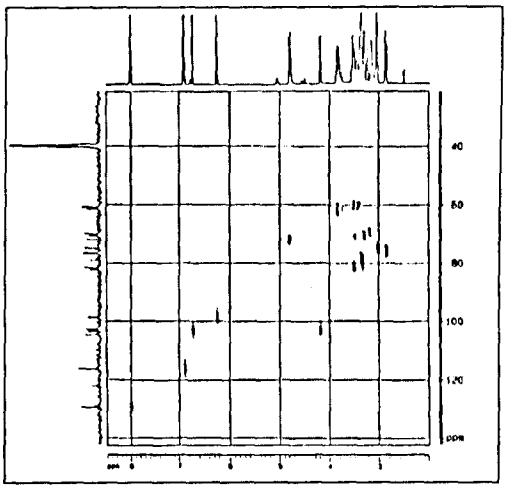

Fig. 2: HMQC spectrum of compound 4

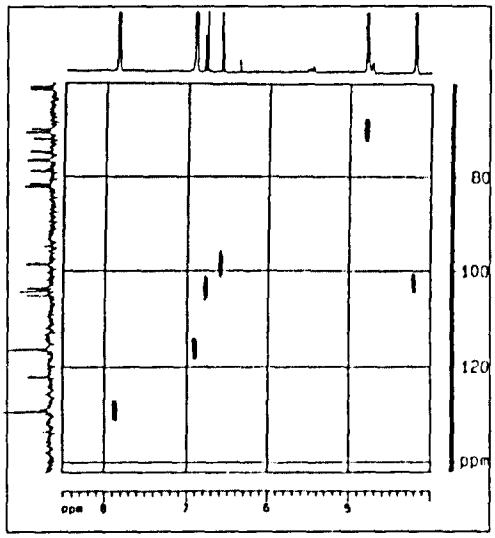

Fig. 3: HMQC spectrum of Compound 5

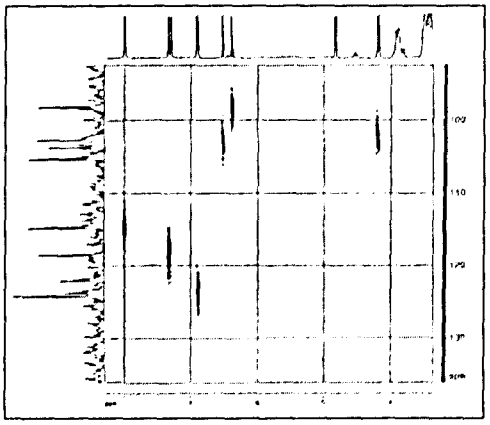

Fig. 4: HMQC spectrum of Compound 6

TABLE 1: NMR DATA (500/125 MHZ $^{1}H/^{13}C$, DMSO-D_6) OF COMPOUNDS 4-6

Position	δ, ppm					
	4		5		6	
	1_H, multiplicity, (J_{H-H}, Hz)	^{13}C	1_H, multiplicity, (J_{H-H}, Hz)	^{13}C	1_H, multiplicity, (J_{H-H}, Hz)	^{13}C
2		164.203		164.030		164.197
3	6.737, s	104.078	6.786, s	105.238	6.504, s	103.624
4		182.425		181.924		182.419
5		161.002		161.121		160.998
6	6.245, s	98.571		106.629	6.392, s	98.564
7		162.978		162.823		162.973
8		105.561	6.601, s	97.994		107.284
9		156.616		156.349		161.486
10		104.295		104.075		104.070
1'		122.137		122.001		122.130
2'	7.999, d (9.0)	129.284	7.879, d (8.7)	129.423	7.997, d (2.0)	115.559
3'	6.901, d (9.0)	116.271	6.923, d (8.7)	116.872		145.142
4'		161.496		160.983		150.615
5'	6.901, d (9.0)	116.271	6.923, d (8.7)	116.872	6.884, d (9.0)	124.526
6'	7.999, d (9.0)	129.284	7.879, d (8.7)	129.423	7.311, dd (9.0, 2.0)	118.461
1"	4.810, d (7.0)	71.933	4.817, d (7.2)	71.982	4.831, d (7.6)	71.929
2"	3.482, m	82.191	3.491, m	82.984	3.403, m	82.184
3"	3.393, m	78.852	3.407, m	78.623	3.512, t (9.2)	78.845
4"	3.320, t (9.1)	70.535	3.328, t (9.7)	70.591	3.497, t (9.2)	70.529
5"	3.405, m	81.598	3.418, m	81.672	3.412, m	81.598
6" α	3.843, dd (2.0, 11.7)	61.387	3.857, dd (2.2, 11.8)	61.420	3.430, dd (12.2, 2.7)	61.384
β	3.616, dd (5.5, 11.7)		3.704, dd (5.7, 11.8)		3.442, dd (12.2, 6,2)	
1"'	4.200, d (7.0)	103.073	4.196, d (7.2)	103.232	4.217, d (7.2)	103.070
2"'	2.985, m	74.780	2.996, m	74.820	3.052, m	74.777
3"'	3.058, t (9.5)	76.649	3.106, t (9.4)	76.538	3.231, m	76.400
4"'	3.198, t (9.5)	69.904	3.204, t (9.4)	69.800	3.403, m	69.903
5"'	2.878, ddd (2.0, 5.5, 11.8)	76.404	2.936, ddd (2.2, 5.6, 11.8)	76.043	3.008, ddd (2.2, 5.8, 12.0)	75.122
6" α	3.855, dd (2.0, 11.8)	60.834	3.933, dd (2.2, 11.8)	60.791	3.882, dd (2.2, 12.0)	60.830
β	3.487, dd (5.5, 11.8)		3.537, dd (5.6, 11.8)		3.605, dd (5.8, 12.0)	

eties to the basic flavone skeleton adopting heteronuclear chemical shift correlation techniques. In a polyphenolic glycoside the characteristic anomeric proton doublet is a convenient 'structural reporter resonance' that offers a convenient starting point to identify the individual spin systems of the sugar ring in order to assign the spectrum by coupling-correlation[22]. The interglycosidic linkage at C-2" of the three C-glycoflavones could be inferred from the

TABLE 2: ANTIOXIDANT ACTIVITIES OF COMPOUNDS 1-6 IN THE TEAC AND AUTOXIDATION ASSAYS

Compound	TEAC Assay	Autoxidation Assay	
	mM±SD[a]	t=60 min	t=120 min
1	1.182±0.04	22.6	9.7
2	1.235±0.05	25.2	10.8
3	2.526±0.04	48.5	37.5
4	1.201±0.02	20.9	8.2
5	1.296±0.04	26.4	11.0
6	2.743±0.03	50.6	41.4
Quercetin	4.012±0.05		
BHT[b]		72.8	65.3

[a]n=3; [b]BHT=2,6-di-*tert*-butyl-4-methylphenol, butylated hydroxytoluene, standard control substance.

characteristic anomeric doublets (H 1'"), which correlated to the characteristically downfield shifted 2" carbon resonances around 82 ppm[23-25] (fig.5). The 2-D-NMR experiments to detect directly the connectivities of the sugar moieties in the three 2"-O-glucopyranosyl flavone C-glucopyranosides are being accomplished for the first time. The identification of the glycosidic linkages in the earlier instances has been effected only on the characteristic down field shift of the concerned *ipso* carbon and the concomitant up field shift of the adjacent carbon resonances[23-26]. The structures of the three biosides have now been unambiguously confirmed in the light of detailed 1D and 2D NMR data, including ^{1}H COSY, HMQC and HMBC experiments that trace directly the nuclear connectivities.

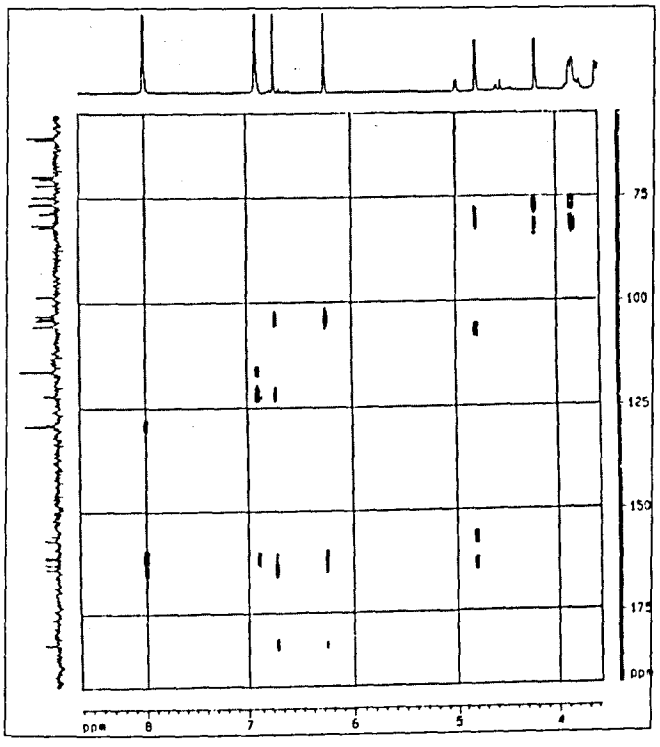

Fig. 5: HMBC spectrum of compound 4

Owing to the incomplete efficiency of our endogenous defence systems and the existence of some pathophysiological situations, ROS are produced in excess and at the wrong time and place. Increased generation of these oxidative free radicals, or impaired antioxidant defence mechanisms have been implicated in the aging process, neurodegenerative conditions including Parkinsonism and Alzheimer's disease, in chronic stress-induced perturbed homeostasis, including immunosupression, inflammation, diabetes mellitus, peptic ulcer and other disease conditions[27]. Exogenous antioxidants are, therefore, needed to ameliorate the cumulative effects of oxidative damage over the life span. It may be inferred from the large number of studies made in the past 25 years that free radical mediated reactions are also responsible for a wide range of chemotherapy-induced side-effects, and that antioxidants are able to protect non-malignant cells and organs against some of the damaging effects of cytostatic agents[28]. Flavonoids are ubiquitous phytochemicals with remarkable *in vitro* antioxidant property[29]. The constituents of black tea, for instant, theaflavin digallate is about 6.2 times and epicatechin gallate is about 4.9 times more active than either vitamin C or E. The major flavonoids of *Silybum marianum* (silymarin) have been reported to exhibit synergistic actions with cisplatin and doxorubicin (adriamycin) in

test tubes[30] apart from offsetting the nephrotoxicity of cisplatin in animals[31]. The ubiquitous phytoflavonol, quercetin, has been used in a study to evaluate the protective capacity of antioxidants against drug-induced, superoxide-mediated cytotoxicity[32]. The activity is mainly due to their redox properties, which can play an important role in neutralising free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The antioxidant activity of the isolated compounds in the pure form has been evaluated by two different assays, namely, the Trolox equivalent antioxidant capacity (TEAC) assay and the coupled oxidation of β-carotene and linoleic acid (autoxidation) assay and the results are summarised in Table 2. Compounds 3 and 6 exhibited 63% and 68% of the activity of quercetin and were about 2.5 times more active than Vitamin C and E in the TEAC assay while compounds 1,2,4 and 5 were relatively of lower activity (32% and less). The antioxidative effect of the six compounds on the autoxidation of linoleic acid was also measured at t=60 and 120 min, employing bleaching of β-carotene as model system (Table 2). Again compounds 3 and 6 exhibited 66% and 70% of the activities of quercetin respectively and the others were of only 40% or less after 60 min.

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. In particular, linoleic acid and arachidonic acid are the target of lipid peroxidation. It is generally thought that the inhibition of lipid peroxidation by antioxidants may be due to their free-radical scavenging activities. Studies have indicated a direct correlation between the radical scavenging activity and the structure of the flavonoid skeleton[13,33,34]. Accordingly, the observed activity of compounds 3 and 6 may be attributed to the presence of the catechol group in ring B and possibly to the 2, 3- double bond conjugated with the 4-oxo group (α-β- unsaturated carbonyl chromophore) of ring C. The absence of the ring B catechol group in the rest of the compounds may be responsible for their lower activity. The significance of the presence of 3-OH in the flavonoid skeleton in enhancing the radical scavenging activity is also obvious from the decreased activity of 3 and 6 as compared to the reference compound, quercetin.

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