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# Investigation of Free Radical Scavenging Activity by ESR for Coumarins Isolated from *Tecoma radicans*

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Chemical investigation of the aerial parts of *Tecoma radicans*, *F. bignoniaceae* indicates the presence of four coumarins, 2`,3`-epoxide alloimperatorin (8-methoxy furanocoumarin), pabulenone (5-alkoxy-furanocoumarin), pereflorin B and 17-methylbothrioclinin. One chromone was also isolated peucenin-7-methyl ether (7,5 dioxygenated chromone). Biological screening of this fraction showed no antitumoral effects but antioxidant activity. The method of the assay of the free radical is modified to make combination between the stable DPPH radical and its determination by ESR instead of determining it by colourimetric method. When the coumarin fraction and isolated compounds were examined for free radical scavenging activity, using the stable DPPH free radical and recorded by ESR, it was found that the whole coumarin fraction was the most active (89.06% inhibition of DPPH free radical) and the isolated components are 0, 25.9, 8.45, 13.85 and 50%, respectively.

**Key words:** *Tecoma radicans*, *Bignoniaceae*, antioxidants, Coumarins and chromone

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## INTRODUCTION

*Tecoma radicans* (*Campsis radicans*), *F. bignoniaceae* is a species that belongs to a tropical family but has been introduced in many countries as ornamental. Experimental studies of the biological properties of *Tecoma radicans* are lacking. The aim of this research is to study the phytochemical and biological activity of the fraction containing coumarins from leaves of *T. radicans* which were collected in Egypt.

Two aglycone moieties of the isocoumarin glycosides were isolated from the bark of *Tabebuia impetiginosa*, four iridoid glycosides, two lignan glycosides, three phenyl ethanoid glycosides and eight phenolic glycosides (Warashina *et al.*, 2004). A preliminary phytochemical analysis of the extracts of *Catalpa bignonioides* showed no antimicrobial or antitumoral effects, but prominent anti-inflammatory and antinociceptive actions of the extracts (Mingarro *et al.*, 2003). The common anthocyanin, cyanidin 3-rutinoside were found in *Campsis radicans* petals. 6-Hydroxyluteolin was identified in fresh leaves of *Tecoma australis* (Harborne, 1967). Hortensin which is a methoxylated flavone possessing anticancer properties was isolated from the flower of *Millingtonia hortensis* (Hase *et al.*, 1995). Tissue cultures of *Tecoma sambucifolium* showed accumulation of phenylpropanoid glycosides, the main components were identified as verbascoside, orobanchoside, isoverbascoside and rhodioloside (Pletsch *et al.*, 1993).

## MATERIALS AND METHODS

**Finnigan MS (USA) Spectrophotometer at 70 eV in methanol:** $^1$H-NMR spectra were recorded in $CDCl_3$ solvent on a JEOL EX-300 MHZ Spectrometer. Chemical shifts are given in the δ (ppm) with tetramethylsilane (TMS) as an internal standard. UV spectra measured on Beckman UV 640 spectrometer. ESR was recorded in methanol on Bruker (German).

**Plant material:** The aerial parts (leaves) of *Tecoma radicans* were collected from Orman Garden (The Botanical Garden of Egypt), in March 2004 and identified by Treaze labib, specialist in identification of plants.

**Extraction and isolation:** The dried powdered aerial parts of *Tecoma radicans* (500 g) was extracted with 80% ethanol in a Soxhlet apparatus. The extract was concentrated and treated with an equal volume of 10% KOH solution at room temperature for one hour. The alkaline alcohol extract was diluted with water and extracted with ether. The aqueous layer was acidified with dilute HCl, refluxed for 1.5 h, cooled and extracted with ether, whereby the ethereal extract was evaporated to dryness (coumarins).

The coumarin fraction was dissolved in ethanol and subjected to TLC using silica gel G60, F254 precoated plates, developed with benzene: ethyl acetate (8:2) and sprayed with $I_2$/KI reagent. Six compounds were detected, they were isolated by preparative layer chromatography several times using the above solvent system. These compounds were identified by measuring their mp., UV, $^1$H-NMR, EIMS and compared with the published data, (Murray *et al.*, 1982).

The first four compounds gave violet colour with alcoholic ferric chloride after alkalinization with amm. solution but the last one (chromone) showed violet colour directly with ferric chloride.

**Identification of isolated compounds**

**Compound I:** Isolated at $R_f$ 0.53 with dark bluish violet colour when sprayed with $I_2$/KI and mp 103-105°C. UV : $\lambda_{max}^{methanol}$ 272, 290, 346, Na OH; 272, 300, 376. $^1$H-NMR (300 MHZ, $CDCl_3$), δ ppm. 1.244, 1.332 (6H, s,-$CH_3$), 6.49-6.465 (1H, d, J = 7.5, H-3), 7.699-7.710, 7.718-7.729 (1H, dd, J = 3.3, H-4), 7.519-7.550 (1H, m, H-6), 6.524-6.536 (1H, d, J = 3.6, H-7), 2.349 (2H,m, $CH_2$-1\`), 3.073 (1H, br, CH-2\`). 4.147 (3H,s,-$OCH_3$). EIMS:(m/z, rel. int.) $C_{17}H_{16}O_5$, 300 $M^+$ (50%), 279 (7%), 257 (12%), 229 (9%), 214 (7%), 189 (10%), 187 (9%), 177 (14%) 159 (7%), 150 (17%), 149 (44%), 131(10%), 128 (25%), 107 (24%), 101(82%), 81 (36%), 71 (48%), 57 (57%), 55 (100%).

**Compound II:** It was produced at $R_f$ 0.84 as blue spot with melting point 118-120°C. UV $\lambda_{max}^{methanol}$:268, 291, 335, NaOH 271, 300, 335. $^1$H-NMR (300 MHZ, $CDCl_3$) δ ppm 1.9 (3H, s,-$CH_3$), 2.813 (2H,s,-$CH_2$), 5.304 (2H, s, = $CH_2$) 6.3 (1H, m, H-3), 6.949-6.975 (1H, d, J = 7.8, H-3\`) 7.522-7.541, 7.552-7.571 (1H,d.d. J = 5.7, 3.3, H-2\`), 7.618 (1H, s, H-8), 7.796-7. 825 (1H, d.J = 8.7, H-4), 9.882 s. EIMS (m/z, rel. int.) ($C_{16}H_{12}O_5$) $M^{+1}$ 285 (4%), $M^+$ 284 (19%), 270 (15%), 269(100%), 253 (6%), 241 (8%), 222(7%), 213 (14%), 207 (32%), 149 (9%), 115 (8%), 91 (16%), 81 (15%), 77 (12%), 69 (14%), 57 (62%), 55 (41%).

**Compound III:** It was isolated at $R_f$ 0.18 as pale yellow needles with mp. 198-200. UV $\lambda_{max}^{methanol}$ :240, 276, 307, 418, NaOH 240, 276, 305, 413. $^1$H-NMR (300 MHZ, $CDCl_3$), δ ppm 3.969 (3H, s, $OCH_3$ -8), 3.913, 3. 952 (6H, s, $OCH_3$-3,4). 6.748-6.781 (1H, d., J = 9.9, H-7).

**EIMS:** (m/z, rel. int.), 280 $M^+$ (100%) for $C_{13}H_{12}O_7$, 279 (14%), 259 (10%), 251 (5%), 208 (5%), 203 (5%), 184 (5%), 181 (10%), 167 (22%), 156 (25%), 149 (63%), 128 (26%), 111 (31%), 99 (59%), 97 (69%), 96 (59%), 86 (48%), 84 (59%), 83 (65%), 71 (54%), 57 (82%), 56 (70%), 55 (75%),

**Compound IV:** This compound appeared as yellow spot under UV at $R_f$ 0.6 and mp 98-100°C. UV $\lambda_{max}^{methanol}$ : 276, 350, 386, NaOH: 274, 300, 400. $^1$H-NMR (300 MHZ, $CDCl_3$), δ ppm 1.236, 1.259, 1.271 (3H, t, $CH_3$-14), 1.601 (6H, s, gem dimethyl, $CH_3$-11,12), 2.4 (m, $CH_2$-13), 5.5 (1H,d. J = 2.7, H-10), 6.7 (1H, d., J = 7.2, H-9), 6.921 s, 7.02(1H, d., J = 4.5, H-6), 7.18 (1H, br., H-8), 7.4 (1H, m, H-7), 7.61 s, 7.73 m.

**EIMS:** (m/z, rel. int.), $M^{+1}$ 257 (3%), $M^+$ 256 (12%) due to $C_{16}H_{16}O_3$ 241(7%), 227 (5%), 233 (3%), 213 (7%), 186 (7%), 157 (6%), 149 (7%), 135 (8%), 129 (18%), 128(24%), 101 (100%), 83 (31%), 73 (47%), 60 (30%), 57 (54%), 55 (77%).

**Compound V:** This compound showed violet colour by adding alcoholic ferric chloride and blue fluorescent under UV at $R_f$ 0.67 and mp. 100-102°C. UV $\lambda_{max}^{methanol}$ :208, 227, 243, 251, 257, 262-NaOH: 210, 235, 240, 246, 254, 259. $^1$H-NMR (300 MHZ, $CDCl_3$), δ ppm 1.65, 1.70 (6H, s, gem dimethyl), 2.30 (3H, s,-$CH_3$-2), 3.91 (3H, s, -$OCH_3$), 3.35 (2H, d., J = 8.0, Ar-$CH_2$), 5.28 (1H, m, -CH = C), 6.2 (1H, s, H -3), 6.38 (1H, s, H-8).

**EIMS:** (m/z, rel. int.), $M^+$ 274 (23%) due to $C_{16}H_{18}O_4$, 273 (42%), 272 (61%), 271 (86%), 245 (16%), 233 (47%), 203 (12%), 187 (10%), 177 (12%), 161 (12%), 150 (29%), 139 (45%), 135 (64%), 111(33%), 108 (38%), 91 (47%), 85 (31%), 69 (58%), 60 (100%), 57 (77%), 55 (93%).

**Screening for cytotoxic activity:** Successive extracts of *T. radicans*, pet. ether, chloroform, ethyl acetate extracts and the coumarin fraction isolated from the 80% ethanol extract of the aerial parts dried under reduced pressure, then 2 mg of each extract was dissolved in DMSO to give concentration of 100 μg 0.1 $mL^{-1}$. They were screened *in vitro* using a single tumour (*Ehrlich ascites* carcinoma cells). The tumour was maintained in the laboratory by weekly intraperitoneal transplantation in female albino mice from the animal house of Cairo Cancer Institute. A set of sterile test tubes was used for each test solution, where $2.5\times10^6$ tumour cells per mL were suspended in phosphate buffer. Different dilution of each test solution (0.1 mL) was added separately to the suspension, kept at 37°C for two hours (EL-Hossary *et al*., 2000). Trypan blue dye exclusion test was then carried out to calculate the percentage of nonviable cells. Nonviable cells stained blue. Concentrations causing less than 30% nonviable cells in the suspension, are considered inactive while those producing more than 70% nonviable cells are considered active.

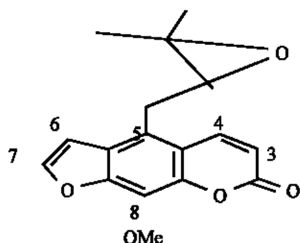
**Determination of the antioxidant activity using DPPH as stable free radical:** (Makhmoor, 2003; Acqua and Innocenti, 2004). Coumarin fraction (10 mg) isolated from 80% ethanol extract of *T. radicans* was dissolved in 1 mL of methanol, DPPH also was prepared in concentration of 10 mg $mL^{-1}$ methanol (as source of stable free radical). Standard solution was prepared from vitamin C as antioxidant (standard), Cid Company (Egypt), with concentration of 10 mg $mL^{-1}$ methanol. Each test solution (10 μL) and standard (10 μL)was added to 190 μL of DPPH solution, the control was prepared from 0.2 mL DPPH, they were all incubated at 37°C for 30 min. Electron Spin Resonance of DPPH was recorded and the percentage inhibitions of the free radical was calculated from the double integration areas. Each of the isolated compounds was tested for radical scavenging activity but in concentration of 3 mg $mL^{-1}$ methanol (because the isolated compounds were very small) and the double integration areas were recorded by ESR after carrying out with the same method as mentioned above.

## RESULTS AND DISCUSSION

Five compounds were isolated from the coumarins fraction (80% ethanol extract of *T.radicans*) and their structures were established on the bases of their spectral data. Four of them are coumarins giving violet colour with alcoholic ferric chloride after alkalinization with amm. and one is chromone giving violet colour with ferric chloride directly.

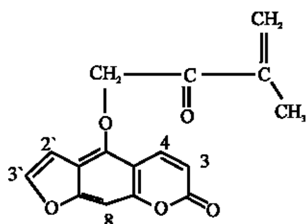
**Compound I:** It was isolated as the major coumarin of *T. radicans* with dark bluish violet with $I_2$/KI. Its chromatographic behavior and UV with spectral data as suggestive for alloimperatorin methyl ether epoxide. Analytical and high resolution mass spectrometric data were in accord with the molecular formula $C_{17}H_{16}O_5$ and molecular wt.300. $^1$H-NMR spectrum of the compound contained signals in the aromatic region in accord with 5,8-distributed furanocoumarin. Doublet of doublet at δ ppm 0 7.699-7.710 and 7.718-7.729 with J value, 3.3 for H-4. At δ ppm 7.519-7.550 for H-6 and at 6.49-6.465 with J = 7.5 for H-3. The last aromatic proton at δ 6.524-6.536 with J = 3.5 for H-7. The presence of m signal at 3.073 for the aliphatic proton of the epoxide CH-2` while at 2.349 for $CH_2$-1`. The presence of singlet signal at 4.147 for $OCH_3$ group at position 8.

This compound was isolated before naturally from the shoots of *Thamnosma*, *montana* F. *Rutaceae* and it is the first time to be isolated from *Bignoniaceae* (Kutney *et al*., 1972, 1969).




2`, 3`-epoxide of alloimperatorin methyl ether

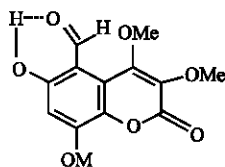
**Compound II:** MS of compound II showed a molecular ion peak m/z at 284 (mol. formula $C_{16}H_{12}O_5$). This compound has 100% absorbance at 269 and showed main fragments of 5-alkoxy-furanocoumarin. The $^1$H-NMR spectra showed all the characteristic signals for pabulenone. The doublet signal at δ ppm 7.796-7.825 with J = 8.7 indicates that it is 5-substituted by oxygen. The presence of singlet signal at 7.618 revealed the presence of proton 8 while the aliphatic protons appeared at δ ppm 2.813 for-$CH_2$ and 5.304 for terminal methylene = $CH_2$.




Pabulenone

This compound was isolated and reported from the root of *Peucedanum ostruthium* (Reisch *et al*., 1975).

**Compound III:** Compound III was suggested to have the molecular formula $C_{13}$ $H_{12}O_7$ based on m/z 280, it was attributed to pereflorin B and confirmed by its UV and $^1$H-NMR spectra. The singlet signals at δ ppm 3.969, 3.913 and 3.952 are for-$OCH_3$ groups at carbons 8, 3 and 4, respectively. While the aromatic proton at δ ppm 6.748-6.781 with J = 9.9 is for the proton at carbon 7.

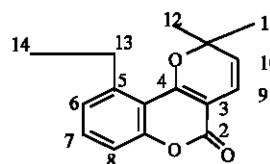



Pereflorin B

Pereflorin B had been isolated before from some families and lastely from the roots of *Perezia multiflora*, *F. Compositae*, (Joseph-Nathan *et al*., 1978), it is the first time to be isolated from *Bignoniaceae*.

**Compound IV:** The molecular formula of compound IV was considered to be $C_{16}H_{16}O_3$ on the bases of mass spectra and molecular wt. 256. The aromatic protons of positions 6, 8 and 7 appeared at δ ppm 7.02, 7.18 and 7.4, respectively. The aliphatic protons of methyl group 14 are at δ ppm 1.236-1.259-1.271 as triple signal, while the 6 protons of the gem dimethyl are present as singlet signal at δ ppm 1.601. The two protons of the side chain showed multiple signal at 2.4.

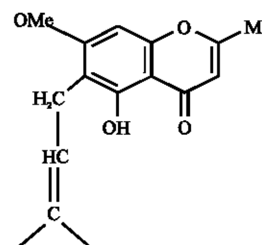
This compound was found to be 17-methylbothrioclinin and it was defined before in *Bothriocline laxa*, *F. Compositae* with other Coumarins and chromones (Bohlmann and Zdero, 1977).




17-methylbothrioclinin

**Compound V:** The UV and mass spectra of this compound are identical with the chromone peucenin-7-methyl ether. The aromatic protons at δ ppm 6.05 and 6.32 are for H-2 and H-4. Doublet at δ 3.3 ppm and m at 5.2 are for $CH_2$- and CH- of the side chain at carbon 6. The gem dimethyls appeared as singlets at δ ppm 1.65, 1.75 while the 3 protons of methyl-3 appeared at δ 2.32 ppm. This chromone peucenin-7-methyl ether was isolated before from *Angelica officinalis* (Harkar *et al*., 1984).

Also the chromone derivative peucenin was isolated with pabulenone coumarin from *Peucedanum ostruthium* (Reisch *et al*., 1975).




peucenin-7-methyl ether

**Cytotoxicity assay:** The results obtained in cytotoxicity assay against *Ehrlich ascites* carcinoma cells were very low, so this plant is ineffective as a cytotoxic agent.

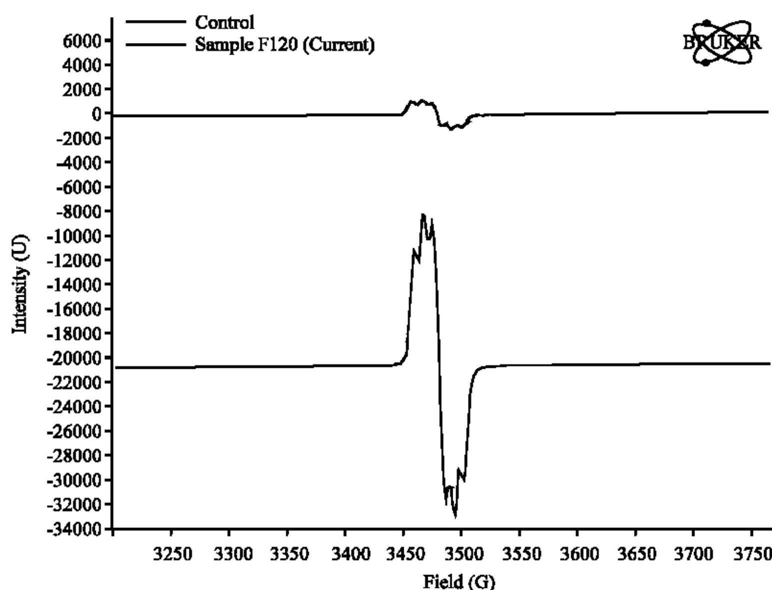



Fig. 1: ESR of control (DPPH) and coumarin

**Free radical scavenging activity:** Testing the antioxidant activity of the whole Coumarin fraction and the purified isolated compounds by the inhibition of the stable free radical of DPPH. According to the ESR of each test solution, the double integration areas of DPPH free radical were calculated after the addition of the inhibitor. The total coumarin extract showed higher activity as free radical scavenger as shown in Fig. 1 (89.06%. Inhibition), 50% for peucenin-7-methyl ether, 25.9% for pabulenone, 13.85% for 17-methylbothrioclinin and 8.45% for pereflorin B as shown in Table 1.

Alloimperatorin methyl ether oxide has no any activity as free radical scavenger. Vitamin C showed 95.45% inhibition). We find here that the whole coumarin fraction was the most inhibitor for DPPH free radical, so the isolated compounds may be more potent by increasing their concentrations.

This method of DPPH radical scavenging assay was modified from the method of Makhmoor (2003) and the methods reported in 2004 for Acqua and Innocenti they measured the tested extracts or compounds colourimetricaly, but it is more accurate to record the free radical by its ESR and no interference from colours of the tested compounds. The higher activity of the isolated chromone peucenin-7-methyl ether is attributed to the conjugation of double bonds and the methoxy group in the molecule, it can possibly contribute to the activity of this compound as a scavenger for DPPH free radical. This is confirmed by (Hashem, 2000-2001) isolated the chromone 3-[2-(3,5-dimethoxy phenyl) ethenyl]-2-methyl from *Erucaria microcarpa* and it was proved to be active as a singlet oxygen scavenger.

Table 1: Inhibition of DPPH radical by coumarins isolated from *T. radicans*

| Compounds | Double integration area | Percentage inhibition |
|---|---|---|
| DPPH | 638.0 | |
| Vitamin C | 29.0 | 95.45 |
| Coumarin ext. | 69.0 | 89.20 |
| Compound I | 638.0 | - |
| Compound II | 472.7 | 25.90 |
| Compound III | 584.0 | 8.45 |
| Compound IV | 549.6 | 13.85 |
| Compound V | 319.0 | 50.00 |

The most common natural antioxidants are flavonoids and phenolic acids (Larson, 1988), these are not only the defensive molecules in prevention of different pathological disorders but are commonly used in industry for the prevention of oxidative degradation of polymers and synthetic and natural pigments. Sequential extracts of *Chaerophyllum hirsutum* were found to have antioxidant activity when they were tested also using DPPH by Acqua and Innocenti (2004). Free radicals have been proposed to induce cellular damage which may play a role in heart diseases, rheumatoid arthritis, cancer, inflammatory disorders as well as in aging processes (Heilmann *et al*., 1995).

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