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XANTHONES FROM CELL CULTURES OF *HYPERICUM GNIDIOIDES* SEEM.

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تم فى هذا البحث فصل أوكسيجينات وبرينيلات الزانثون من خلايا أنسجة نبات الهيبيريكم اجنيديوايدس سيم. النامية فى وسط بى 5 فى الظلام. باستخدام طرق التحاليل المختلفة تم التعرف على المركبات المفصولة وهى 1،7-ثنائى هيدروكسى زانثون (إيوزانثون)، 1،3،7-ثلاثى هيدروكسى زانثون، 1،3،5،6- رباعى هيدروكسى زانثون ، 1،3،6،7- رباعى هيدروكسى-8-(3-ميثيل بيوت-2-إينيل) زانثون و1،3،6،7- رباعى هيدروكسى-2،8-ثنائى(3-ميثيل بيوت-2-إينيل) زانثون (جاما مانجوستين). وجود كل من 1،7- ثنائى هيدروكسى زانثون و1،3،7- ثلاثى هيدروكسى زانثون والمفصول لأول مرة من خلايا أنسجة سلالات الهيبيريكم يعتبر إشارة الى إشراك إنزيم الإختزال المسئول عن نزع مجموعة 3- هيدروكسيل بالإضافة الى تأكيد طريقة التخليق الحيوى لمركبات الزانثون فى سلالات نبات الهيبريكم.

Oxygenated and prenylated xanthones were isolated from the cell cultures of Hypericum gnidioides Seem. when grown in modified B5 medium in the dark. Based on the spectral methods, the structure of the isolated compounds were elucidated as 1,7-dihydroxyxanthone (euxanthone), 1,3,7-trihydroxyxanthone, 1,3,5,6-tetrahydroxyxanthone, 1,3,6,7-tetrahydroxy-8-(3-methylbut-2-enyl) xanthone and 1,3,6,7-tetrahydroxy-2,8-di(3-methylbut-2-enyl) xanthone (γ-mangostin). The occurrence of both 1,7-dihydroxyxanthone and 1,3,7- trihydroxyxanthone which is recorded for the first time in cell cultures of Hypericum species indicate the presence of a reductase activity responsible for eliminating the 3-hydroxy group and confirm the biosynthetic pathway of xanthones in Hypericum species respectively.

INTRODUCTION

Xanthone derivatives of various structural types have been isolated from several *Hypericum* species.[1-3] Ethanolic extracts from *Hypericum* species (Hypericaceae) have been shown to exert marked effects on the central nervous system.[4,5,6] Best one is that obtained from *Hypericum perforatum* which is widely used as an antidepressant drug.[4] Interestingly, several xanthones from higher plants showed anti-fungal and broad spectrum antibacterial activity viz., mangostin and gartanin from *Garcinia mangostana* L. (Guttiferae).[7] Similar properties have been reported for jacareubin and 6-deoxyjacareubin, two chromen-oxanthones isolated from *Callophyllum inophyllum* L. (Guttiferae).[8,9] Mangostin also exhibited significant anti-inflammatory properties.[10] 1,7-Dihydroxyxanthone, 1,3,7-trihydroxyxanthone and 1,3,5,6-tetrahydroxy-xanthone have anti-inflammatory effect through the suppression of chemical mediators released from mast cell and neutrophil de-granulation.[11]

Xanthones from *Garcinia subelliptica* were found to have anti-oxidative properties.[12] A number of xanthones from *Cansacora decussata* and *Garcinia mangostana* exhibit significant tuberculostatic and anti-viral activities respectively.[13,14] The dihydrofuran-oxanthone epoxide psorospermin exhibit pronounced cytotoxicity and anti-tumour activity against lymphocytic leukemia and mammary and colon tumour systems.[15]

In the present work, the callus induction from the young shoots of *Hypericum gnidioides* Seem. and the isolation and structural elucidation of the xanthones known to be constituents present in Hypericaceae were carried out.

EXPERIMENTAL

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Apparatus

Melting points were determined on Koffler's heating stage microscope. UV spectra were carried out in Unicam SP-1750 Ultraviolet Spectrometer. IR spectra were determined in Unicam SP-1025 Infra Red Spectrometer. EI-MS spectra were performed on Hitachi M-80 and on MAT 311A, 70 ev. Spectrometer. ^{1}H-NMR (500 MHz) spectra were performed in DMSO-d_6 using TMS as internal standard. The TLC was performed on silica gel 60 F_{254}-coated aluminium sheets (Merck, Darmstadt, Germany). The preparative TLC was carried out on silica gel 60 F_{254}-coated glass sheets.

Plant material

Hypericum gnidioides seeds were kindly identified and provided by Prof. Dr. L. Beerhues and Dr. Wagner Barillas, Institute for Pharmaceutical Biology, University of Bonn, Germany.

Authentic reference xanthones

The reference xanthones were obtained from Prof. Dr. L. Beerhues, Institute for Pharmaceutical Biology, Technical University-Braunschweig, Germany. To whom the author is greatly indebted.

Cell cultures

Callus cultures of *Hypericum gnidioides* Seem. were established from the young shoots of sterile germinated seeds. After surface sterilization, stem and leaf segments were placed on B5 solid medium[16] (Table 1) supplemented with 2 g/l NZ-Amine, 2.0 mg/l 1-naphthylacetic acid, 2.0 mg/l indole-3-acetic acid and 0.5 mg/l 6-(furfurylamino)-purine. The resulting callus tissue was transferred to the modified liquid B5 medium (without agar) and grown in the dark. Cultures (50ml) were shaken in 300-ml Erlenmeyer flasks at 100 r.p.m. and 25°.

Table 1: Composition of B5 medium.[16]

Ingredients	Amount used (mg/l medium)
NaH_2Po_4. H_2O	150
$CaCl_2$. $2H_2O$	150
$(NH_4)_2SO_4$	134
$MgSO_4$. $7H_2O$	250
KNO_3	2500
$FeSO_4$. $7H_2O$	25.6
Na_2EDTA. $2H_2O$	34.27
KJ	0.75
$MnSO_4$. H_2O	10
H_3BO_3	3
$ZnSO_4$. $7H_2O$	3
Na_2MoO_4. $2H_2O$	0.25
$CuSO_4$. $5H_2O$	0.025
$CoCl_2$. $6H_2O$	0.025
Nicotinic acid	1
Thiamine dichloride	10
Pyridoxal hydrochloride	1
myo-Inositol	100
Hormone	
2,4-Dichlorophenoxyacetic acid	1
♣ Preparation: mix ingredients with about 600 ml distilled water in a 1 liter glass beaker, add and dissolve 20 g/l sucrose. Bring the solution up to 1 liter by adding distilled water, then adjust the pH to 5.5 with a concentrated KOH solution. Add 7 g/l agar, place the beaker on the hot plate and frequently stir the solution until the agar is dissolved, divide the solution in 300-ml Erlenmeyer flasks each one with 50 ml, keep the cap close and sterilize at 120° for 20 min in autoclave.	

Extraction and isolation of xanthones

Extraction of constituents

Xanthones were extracted according to a published method.[17] Cells (100 g) were ground in 700 ml acetone and the homogenate was filtered. The residue was extracted twice with 500 ml acetone. The acetone phases were combined and evaporated to dryness. The residue (9 g) was re-dissolved in 100 ml methanol and subjected to TLC and HPLC analyses.

Isolation of xanthones

The methanolic residue was subjected to thin layer chromatography (TLC) on silica gel 60 F_{254}-coated aluminium sheets (Merck,

Darmstadt, Germany) using cyclohexane – dichloromethane – ethylformate – formic acid (35: 30: 30: 1 by vol.) (system I) as a mobile phase revealed seven spots, five of them are majors, attained yellow colours with 1% w/v $AlCl_3$ and were separated by preparative TLC on silica gel 60 F_{254}-coated glass sheets (Merck, Darmstadt, Germany) using the solvent system mentioned above to compounds **1**-**5**. Further purification of compounds **1**-**5** was achieved by HPLC (L-6200 A intelligent pump, L-4000 UV detector, Merck Hitachi, Japan). This was performed on a reversed phase-(RP)-8 column (Nucleosile® 100-5; 25 cm long, 0.4 cm i.d.; Macherey-Nagel, Düren, Germany) using water (A) and methanol (B) as the solvents. The following gradient was employed: 35% B for 2 min, 35-70% B within 20 min, then isocratic elution at 70% B. The flow rate was 1 ml/min and the detection wavelength set to 254 nm.

1,7-Dihydroxyxanthone (euxanthone, compound **1**, Fig. 1). 9 mg (0.09 mg/g callus fresh weight), yellowish fine needles (CH_3OH), mp 225-227°, R_f = 0.84 (system I). It showed an orange-yellow fluorescence under UV light and a positive $FeCl_3$ test. UV: $\lambda_{max,}$ (CH_3OH, nm) 236, 258, 287, 311 sh, 384; λ_{max} (CH_3OH + NaOMe nm) 238, 262, 291, 342, 422; λ_{max} (CH_3OH + NaOAc, nm) 235, 258, 287, 310, 384; λ_{max}, (CH_3OH + NaOAc/H_3BO_3, nm) 235, 258, 287, 310, 384; λ_{max} (CH_3OH + $AlCl_3$, nm) 234, 273, 285, 348, 433; λ_{max} (CH_3OH + $AlCl_3$/HCl, nm) 235, 272, 284, 346, 433. IR ν_{max} (KBr) cm $^{-1}$: 3290 (OH), 1640 (C=O), 1590 (C=C), 1300 and 1090 (C-O-C stretching vibrations). EI-MS (*m/z*): 228 [M^+]. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 12.53 (1H, s, OH-C1), 7.70 (1H, d, J= 2.2 Hz, H-8), 7.58 (1H, t, J= 8.1 Hz, H-3), 7.45 (1H, d, J= 8.6 Hz, H-5), 7.40 (1H, dd, J= 8.1 and 1.8 Hz, H-2), 7.30 (1H, dd, J= 8.6 and 2.2 Hz, H-6), 6.94 (1H, dd, J= 8.1 and 1.8 Hz, H-4).

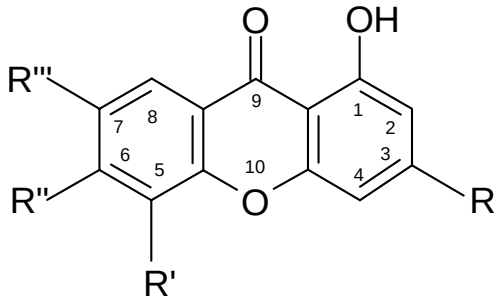
1,3,7-Trihydroxyxanthone (compound **2**, Fig. 1). 4 mg (0.04 mg/g callus fresh weight), yellow needles (CH_3OH), mp 214-216°, R_f = 0.62 (system I). This compound gave an orange-yellow fluorescence under UV light and a positive $FeCl_3$ test. UV: λ_{max} (CH_3OH, nm) 240, 262, 310, 375; λ_{max} (CH_3OH + NaOMe, nm) 245, 266, 340, 410; λ_{max} (CH_3OH + NaOAc, nm) 241, 264, 370, 422; λ_{max} (CH_3OH + NaOAc/H_3BO_3, nm) 240, 263, 371, 422; λ_{max} (CH_3OH + $AlCl_3$, nm) 241, 270, 356, 435; λ_{max} (CH_3OH + $AlCl_3$/HCl, nm) 239, 268, 354, 435. IR ν_{max} (KBr) cm^{-1}: 3310 (OH), 1640 (C=O), 1600 (C=C), 1270 and 1130 (C-O-C stretching vibrations). EI-MS (*m/z*): 244 [M^+]. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 12.71 (1H, s, OH-C1), 7.48 (1H, d, J= 8.3 Hz, H-5), 7.41 (1H, d, J= 2.3 Hz, H-8), 7.22 (1H, dd, J= 8.3 and 2.3 Hz, H-6), 6.41 (1H, d, J= 2.1 Hz, H-4), 6.21 (1H, d, J= 2.1 Hz, H-2).

1,3,5,6-Tetrahydroxyxanthone (compound **3**, Fig. 1). 7 mg (0.07 mg/g callus fresh weight), yellow needles (CH_3OH), mp 206-208°, R_f = 0.37 (system I). It gave a positive $FeCl_3$ test and a yellow fluorescence under UV light. UV: λ_{max} (CH_3OH, nm) 250, 283 sh, 326; λ_{max} (CH_3OH + NaOMe, nm) 265, 290, 374; λ_{max} (CH_3OH + NaOAc, nm) 270, 315, 385; λ_{max} (CH_3OH + NaOAc/H_3BO_3, nm) 272, 316, 385; λ_{max} (CH_3OH + $AlCl_3$, nm) 252, 295, 372; λ_{max} (CH_3OH + $AlCl_3$/HCl, nm) 251, 294, 370. IR ν_{max} (KBr) cm $^{-1}$: 3440 (OH), 1642 (C=O), 1600 (C=C), 1255 and 1100 (C-O-C stretching vibrations). EI-MS (*m/z*) 260 [M^+]. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 13.34 (1H, s, OH-C1), 7.46 (1H, d, J= 8.1 Hz, H-8), 7.1 (1H, d, J= 8.1 Hz, H-7), 6.48 (d, J= 1.4 Hz, H-4), 6.3 (d, J= 1.4 Hz, H-2).

1,3,6,7-tetrahydroxy-8-(3-methylbut-2-enyl) xanthone (compound **4**, Fig. 1). 8 mg (0.08 mg/g callus fresh weight), yellow needles (CH_3OH), mp 200-202°, R_f = 0.44 (system I). This compound showing an orange fluorescence under UV light was positive to $FeCl_3$ test. UV: λ_{max} (CH_3OH, nm) 242, 258, 313, 365; λ_{max} (CH_3OH + NaOMe, nm) 243, 269, 321, 400; λ_{max} (CH_3OH + NaOAc, nm) 243, 267, 319, 385; λ_{max} (CH_3OH + NaOAc/H_3BO_3, nm) 242, 267, 320, 386; λ_{max} (CH_3OH + $AlCl_3$, nm) 238, 272, 353, 415; λ_{max} (CH_3OH + $AlCl_3$/HCl, nm) 238, 271, 352, 413. IR ν_{max} (KBr) cm^{-1}: 3470 (OH), 1640 (C=O), 1590 (C=C), 1235 and 1140 (C-O-C stretching vibrations). EI-MS (*m/z*): 328 [M^+]. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 13.61 (1H, s, OH-C1), 6.45 (s, H-5), 6.19 (d, J= 1.4 Hz, H-4), 6.12 (d, J= 1.4 Hz, H-2), 5.25 (t, J= 6.3 Hz, = CH -), 4.15 (d, J= 6.3 Hz, -CH_2-), 1.74 and 1.65 (6H, s, CH_3-14, 15).

1,3,6,7-tetrahydroxy-2,8-di(3-methylbut-2-enyl) xanthone (γ-mangostin) (compound **5**,

Fig. 1). 4 mg (0.04 mg/g callus fresh weight), yellow amorphous powder (CH_3OH), mp 199-200°, R_f = 0.51 (system I). It gave an orange fluorescence under UV light and a positive $FeCl_3$ test. UV: $\lambda_{max,}$ (CH_3OH, nm) 243, 260, 318, 366; λ_{max} (CH_3OH + NaOMe, nm) 244, 278, 344, 398; λ_{max} (CH_3OH + NaOAc, nm) 242, 269, 324, 377; λ_{max} (CH_3OH + $NaOAc/H_3BO_3$, nm) 243, 270, 324, 377; λ_{max} (CH_3OH + $AlCl_3$, nm) 241, 272, 359, 420; λ_{max} (CH_3OH + $AlCl_3$/HCl, nm) 241, 273, 358, 418. IR ν_{max} (KBr) cm^{-1}: 3475 (OH), 1641 (C=O), 1610 (C=C), 1285 and 1100 (C-O-C stretching vibrations). EI-MS (*m/z*): 396 [M^+]. ^{1}H-NMR (500 MHz, DMSO-d_6): δ 13.92 (1H, s, OH-C1), 6.79 (1H, s, H-5), 6.36 (1H, s, H-4), 5.30 (1H, t, J= 7.4 Hz, H-12), 5.27 (1H, t, J= 6.9 Hz, H-17), 4.20 (2H, d, J= 6.9 Hz, H-16), 3.34 (2H, d, J= 7.4 Hz, H-11), 1.83 (3H, s, CH_3-19), 1.78 (3H, s, CH_3-20), 1.64 (6H, s, CH_3-14, 15).



Xanthone	R	R'	R''	R'''
1	H	H	H	OH
2	OH	H	H	OH
3	OH	OH	OH	H

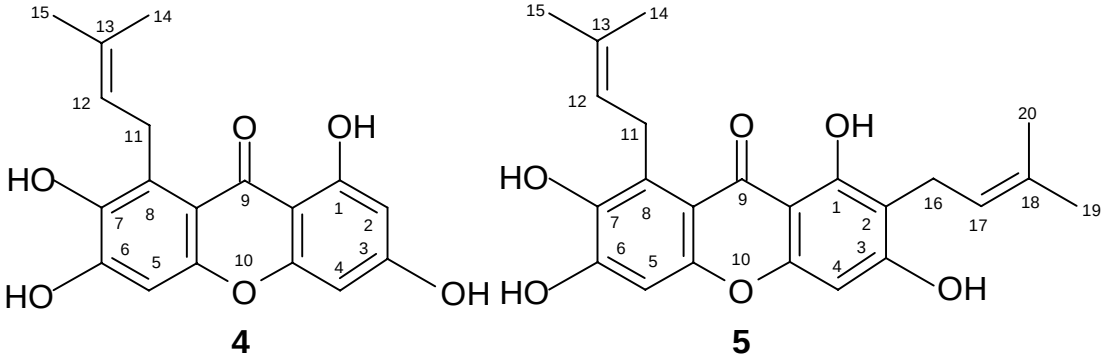


Fig. 1: Xanthones isolated from *Hypericum gnidioides* Seem. cell cultures.

RESULTS AND DISCUSSION

Cell cultures of *H. gnidioides* Seem. grown in modified B5 medium accumulate a remarkable spectrum of secondary metabolites. They are isolated and identified as xanthones (**1-5,** Fig. 1**)** by UV, IR, mass and ^{1}H-NMR spectroscopy, as well as co-chromatography (TLC and HPLC) with samples of reference xanthones.

The UV spectra of polyoxygenated xanthones vary according to their oxygenation pattern.[18] This technique provides helpful information about the location of free hydroxyl groups by running the spectra in the presence of certain additives such as sodium methoxide (NaOMe), sodium acetate (NaOAc), sodium acetate/boric acid (NaOAc/H_3BO_3), aluminium chloride ($AlCl_3$) and aluminium chloride/hydrochloric acid ($AlCl_3$/HCl). Sodium methoxide is a strong base can ionise hydroxyl groups at all positions of xanthone skeleton resulting in a significant alterations of the absorption spectrum. Sodium acetate being a weaker base ionise only the hydroxyl groups of enhanced acidity (C-3/C-6), which are activated by the *para* carbonyl function, causing bathochromic shift. In addition, it can ionise some other hydroxyl groups partially e.g. C-4, resulting only in changes in absorption intensity and having no effect on the hydroxyl group at C-2. Aluminium chloride causes a bathochromic effect on C-1 hydroxyl group, in addition, it can detect *ortho*-hydroxyl system. Sodium acetate/boric acid can also detect *ortho*-hydroxyl system as $AlCl_3$.

The compounds **1-5** were obviously phenolic since they gave a positive ferric chloride test and contained appropriate bands in their IR spectra.

The EI-MS spectrum of compound **1** showed a [M^+] peak at *m/z* 228 corresponded to the molecular formula $C_{13}H_8O_4$. Its UV spectrum revealed bathochromic shifts upon addition of sodium methoxide and aluminium chloride, but not with sodium acetate indicating the absence of hydroxyl groups at C-3 and C-6. The IR spectrum suggested the presence of hydroxyl group(s) (3290 cm^{-1}) and a hydrogen-bonded carbonyl (1640 cm^{-1}). The ^{1}H-NMR spectrum showed signals for *ortho*-coupled protons (δ 7.58, t, J= 8.1 Hz; 7.45, d, J= 8.6 Hz), signal for *meta*-coupled proton (δ 7.7, d, J= 2.2 Hz) and signals for *ortho* and *meta*-coupled protons (δ 7.40 and 6.94, dd, J= 8.1 and 1.8 Hz; 7.30, dd, J= 8.6 and 2.2 Hz). A low field singlet (δ 12.53) represents the 1-hydroxy group.[19] The UV, IR, mass and ^{1}H-NMR spectroscopic data of compound **1** were identical with those reported for 1,7-dihydroxyxanthone.[11,20]

Compound **2** gave a [M^+] peak at *m/z* 244 in EI-mass spectrum consistent with the molecular formula $C_{13}H_8O_5$. The UV spectrum exhibited bathochromic shifts upon addition of sodium acetate, aluminum chloride and sodium methoxide. The IR spectrum revealed a characteristic bands for the hydroxyl group(s) and a hydrogen-bonded carbonyl group at 3310 and 1640 cm^{-1} respectively. The ^{1}H-NMR spectrum showed signal for *ortho*-coupled proton (δ 7.48, d, J= 8.3 Hz), signals for *meta*-coupled protons (δ 7.41, d, J= 2.3; 6.41 and 6.21, d, J= 2.1) and signal for *ortho* and *meta*-coupled proton (δ 7.22, dd, J = 8.3 and 2.3 Hz). A low field singlet (δ 12.71) represents the 1-hydroxy group.[19] The UV, IR, mass and ^{1}H-NMR spectroscopic data of compound **2** were in accordance with those published for 1,3,7-trihydroxyxanthone.[11,21,22]

Compound **3** was also a xanthone. Its ^{1}H-NMR spectrum contained signals for two pairs of aromatic protons, one *ortho*-coupled (δ 7.46 and 7.1, d, J= 8.1 Hz) and the other *meta*-coupled (δ 6.48 and 6.3, d, J= 1.4 Hz). A low field singlet (δ 13.34) represents the 1-hydroxyl group.[19] Its UV spectrum revealed a bathochromic shifts upon addition of NaOMe, NaOAc, NaOAc/H_3BO_3, $AlCl_3$ and $AlCl_3$/HCl. The IR spectrum showed a characteristic bands for the hydroxyl group(s) and a hydrogen-bonded carbonyl group at 3440 and 1642 cm^{-1} respectively. The mass spectrum showed a molecular ion peak at *m/z* 260 which corresponded to the molecular formula $C_{13}H_8O_6$, revealing the presence of four hydroxyl groups. Comparison of the UV, IR, mass, ^{1}H-NMR spectroscopic data with literature values established the identity of compound **3** as 1,3,5,6-tetrahydroxy-xantone.[23,24]

Classification of compound **4** as a xanthone on the basis of its UV spectrum implies that a C_5-unit and four oxygen functions be added to the nucleus to reach a molecular weight of 328 [M^+] consistent with the molecular formula $C_{18}H_{16}O_6$. This is supported by bathochromic shifts upon addition

of NaOAc, NaOAc/H_3BO_3, $AlCl_3$ and $AlCl_3$/HCl. The IR spectrum revealed two characteristic bands for the hydroxyl group(s) and a hydrogen-bonded carbonyl group at 3470 and 1640 cm^{-1} respectively. The ^{1}H-NMR spectrum of compound **4** showed a low field singlet (δ 13.61) represents the 1-hydroxyl group[19] and signals for *meta* coupled protons (δ 6.19 and 6.12, d, J= 1.4 Hz). The C_5-unit is a prenyl (3-methylbut-2-enyl) group showing two high-field three-proton signals and a vinylic proton signal at expected chemical shift values while the methylene group signal is at rather low field (δ 4.11). This indicates that it is situated at C-8 of the xanthone nucleus *i.e.*, in the *peri* position and, thus, subject to an anisotropic effect from the carbonyl group. The solitary aromatic proton in this ring also resonates at a δ-value in agreement with its positioning at C-5 rather than at C-8.[25] The UV, IR, mass and ^{1}H-NMR spectroscopic data of compound **4** were identical with those reported for 1,3,6,7-tetrahydroxy-8-(3-methylbut-2-enyl) xanthone.[26,27]

Compound **5** was also a xanthone. Its ^{1}H-NMR spectrum showed a low field singlet (δ 13.92) characteristic for the 1-hydroxyl group[19] and two aromatic protons one on each ring resonate at a δ-values in agreement with their positioning at C-4 and C-5. The UV spectrum implies that two prenyl and four oxygen functions be added to the nucleus to reach a molecular weight of 396 [M^+] consistent with the molecular formula $C_{23}H_{24}O_6$. The IR spectrum showed two characteristic bands for the hydroxyl group(s) and a hydrogen-bonded carbonyl group at 3475 and 1641 cm^{-1} respectively. Matching the ^{1}H-NMR and mass spectra of compounds 4 and 5 concluded that compound 5 has two prenyl groups. The UV, IR, mass and ^{1}H-NMR spectroscopic data of compound **5** were identical with those reported for (γ-mangostin).[28,29]

It is interesting to note that compounds **1**-**5** were found to have biological and pharmacological activities as mentioned before in the introduction part, consequently, this experiment from economic point of view provides a good conditions for large scale production of xanthones.

The occurrence of 1,7-dihydroxyxanthone in cell cultures of *Hypericum gnidioides* confirms its occurrence in cell cultures of *Hypericum androsaemum* which points to the biosynthetic involvement of a reductase eliminating the 3-hydroxy group.[30]

1,3,7-Trihydroxyxanthone is isolated for the first time from cell cultures of *Hypericum* species; its occurrence confirms the biosynthetic pathway of xanthones in *Hypericum* species.[21,31]

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