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# Phytochemical and Biological Studies *on Verbascum sinaiticum* Growing in Egypt

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**Abstract –** The aerial parts of *Verbascum sinaiticum* Benth. (Scrophulariaceae) yielded two iridoids that were idenified as ajugol **3** and aucubin **4**. Also, investigation of the flavonoid constituents revealed the isolation and identification of luteolin **1** and chrysoeriol-7-glucoside **2**. All the isolated compounds were identifid by spectroscopicmethods (UV, MS, $^{1}$H-NMR and $^{13}$C-NMR) and in comparison with the literature data. Both alcohol and methylene chloride/methanol extracts (1 : 1) exhibited a hepatoprotective effect by $CCl_4$challenge test.
**Keywords –** Flavonoids, Hepatoprotection, Iridoides, Verbascum sinaiticum

## Introduction

The family Scrophulariaceae comprises about 200 genera and 3000 species. The genus *Verbascum* comprises about 360 species spread chiefly in the Mediterranean region (Boulos, 2002). It is represented in Egypt by six species viz. *V. sinuatum* L., *V. sinaiticum* Benth., *V. schimperianum* Boiss, *V. letourneuxii* Asch., *V. eremobium* Murb. *and V. fruticulosum* Post. (Täekholm, 1974).

Several classes of compounds were detected in *Verbascum* species like iridoid glycosides, flavonoids, phenolic acids, sterols, triterpenes, saponins, polysaccharides and alkaloids (Seifert *et al*., 1989; Vesper and Seifert 1994; Klimek, 1996).

Many iridoids have been isolated from *Verbascum*. Among them was aucubin; which was frist isolated from *Aucuba japonica* Thunb; exhibited marked liver protective activity against $CCl_4$ (Chang *et al*., 1983). *Verbascum* species are used in folk medicine as diuretic, antirheumatic, for wound healing, for opthalmic diseases and against cold and chest diseases (Boulos, 20002). In this study we report on the flavonoid and iridoid compounds and the hepatoprotective effect of the different extracts.

## Experimental

**General** – FAB-MS spectral analyses in negative or positive mode were performed on a VG 70-SEQ Hybrid Mass Spectrometer. All NMR spectra were run on a Bruker DRX-400 instrument. The chemical shifts were reported in δ values (ppm). UV spectra were measured as diluted samples in MeOH. $^{1}$H- and $^{13}$C-NMR spectra were recorded in $D_2O$. Thin layer chromatography for flavonoids was performed on Merck precoated polyamide plates and the solvent system was benzene-methyl ethyl ketone-methanol (40 : 30 : 30). UV detection at 254 and 366 nm was used. Column chromatography was carried out using Merck Silica gel 60 (70 - 230 mesh) as adsorbent. TLC for iridoids was performed on Merck precoated Silica gel 60 F254 plates. Solvent system for TLC was methylene chloride-methanol-water (80 : 20 : 2) and methylene chloride-methanol (17 : 3).

**Plant material** – The plantmaterial was collected in the flowering stage from Wadi El-Arbaien, close to the Monastery of St. Katherine, Sinai, Egypt on October, 2004. The plant was identified by Prof. Dr. K. H. El-Batanouny, Faculty of Science, Cairo University. The voucher specimen has been deposited at the Herbarium of the National Research Centre, Cairo, Egypt. The leaves and flower heads were dried seperately in an oven at 40 °C, then ground into fine powder and kept in tightly closed dark containers.

**Extraction and isolation**

**Flavonoids** – The dried and powdered plant material (2 kg) was exhaustively extracted with MeOH. The extract was concentrated under reduced pressure and the residue was dissolved in water, filtered and partitioned with $CHCl_3$, EtOAc and n-BuOH. The EtOAc soluble fraction was subjected to column chromatography on polyamide and eluted with $H_2O$-MeOH gradient of decreasing polarity (95 : 5, 90 : 10, 85 : 15, etc., 250 ml each). Fractions were combined according to their TLC pattern on polyamide

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plates using the solvent system mentioned above. Fraction eluted with $H_2O$-MeOH (30 : 70) (400 mg) was subjected to repeated column chromatography on Sephadex LH-20 using MeOH as eluant yielding a subfration (80 mg) showed to be a mixture. Crystallization from MeOH yielded **1** which was identified as luteolin. Another portion of EtOAc soluble fracion was subjected to preparative paper chromatography using 30% aqueous acetic acid as solvent system to give **2** which was identified as chrysoeriol-7-O-glucoside.

**Iridoids** – The dried aerial parts (2 kg) was exhaustively extracted with MeOH-$H_2O$ (80 : 20). The extract was concentrated under reduced pressure and the residue (366 gm) was mixed with Celite and packed in a glass column. Elution was done with petroleum ether, $CH_2Cl_2$, $CH_2Cl_2$-MeOH (1 : 1) and MeOH. Only the fraction eluted with $CH_2Cl_2$-MeOH (1 : 1) gave positive test for iridoids (Trim and Hill reagent) (Trim and Hill, 1952).

So, this fraction was subjected to column chromatography on silica gel (Merck) and eluted with $CH_2Cl_2$-MeOH-$H_2O$ (80 : 20 : 2), 200 ml fraction). Fractions were combined according to their TLC pattern on Silica gel plates and using the above mentioned solvents. The spots were detected by spraying the plates with 2N $H_2SO4$ and heating at 110 °C for 5 min. Three fractions were collected. One fraction gave positive iridoid test and was rechromatographed on Silica gel column and eluted with $CHCl_3$-MeOH gradient of increasing polarity (95 : 5, 90 : 10, 80 : 20, etc, 200 ml each). Fractions were combind according to their TLC pattern. Four fractions were collected; the fraction eluted with $CHCl_3$-MeOH (80 : 20) was dissolved in 10% aqueous MeOH and purified on Amberlite X $AD_7$ column. Elution was carried out with 10, 30, 50% aqueous MeOH and finally with 100% MeOH. Fraction eluted with 10% aqueous MeOH gave positive iridoid test and showed to contain two compounds by analytical HPC.

**Preparative HPLC of Iridoid Fraction** – The iridoid fraction was subjected to preparative HPLC (10 µl each injection) using Nucleosil 100 $C_{18}$ column (250 × 8 mm). 30% aqueous MeOH was used as solvent system (isocratic) with flow rate 1 ml/min. Detection was done using UV detector at 190 - 225 nm. Two peaks were observed at retention times 6.72 and 12.63 min. Two iridoids were obtained and identified as ajugol (**3**) and aucubin (**4**).

**Biological Studies on Isolated Rat Hepatocytes** – Monolayers of rat hepatocytes isolated and cultured from rat liver (Seglen, 1976) were used to study the toxicity of both alcoholic and $CH_2Cl_2$-MeOH (1 : 1) extracts. Stock solutions (20 µg extract in 1 µl DMSO) were prepared. The concentration of the extract that kills half of the cells were determined ($LC_{50}$) using Trypan-blue exclusion. Duplicate dishes for each concentration were used.

Both alcohol and $CH_2Cl_2$-MeOH (1 : 1) extracts were tested for their hepatoprotective effect by $CCl_4$ challenge test (Kiso *et al*., 1983). The concentration of the extract that protected the hepatocytes from-the toxic effect of $CCl_4$ is considered the effective biologically active concentration for hepatoprotection (Roberts *et al*., 1994).

## Results and Discussion

**luteolin (1)** – The UV spectum of the compound in MeOH showed band I at 349 nm and band II at 267 nm which indicate it might be a flavone type. A bathochromic shift in band I (57 nm) was oberved by addition of NaOMe indicating the presence of a free OH at position 4'. Also by addition of $AlCl_3$ a bathochromic shift in band I (76 nm) was observed indicating the presence of a free OH at position 5 and/or orthodihydroxyl groups. Hypsochromic shift in band I (36 nm) in $AlCl_3$ spectrum after the addition of HCl indicating the presence of orthodihydroxyl groups. On addition of NaOAc, a bathochromic shift in band II (13 nm) was observed indicating the presence of free OH group at position 7. Addition of NaOAc/$H_3BO_3$ gave a bathochromic shift in band I (23 nm) indicating the presence of orthodihydroxyl groups in B-ring. EI-MS spectrum showed a molecular ion peak at *m/z* 286 which corresponds to the molecular formula $C_{15}H_{10}O_6$. Fragment ions at m/z 285, 258, 124 and at 135 were observed. The fragment ion peak at *m/z* 153 is more intense than the fragment ion peak at 152 indicating that it is a 5,7-dihydroxyflavone.

$^1$H-NMR spectrum of compound **1** in DMSO showed a couple of doublets at δ 6.40, which were attributed to H-6 and H-8 (A-ring), respectively and the typical three-spin system of the 1, 3, 4-trisubstituted B-ring: a meta-coupled doublet at δ 6, 8 (H-5') and ortho and meta coupled doublet of doublets at δ 7.4 (H-6').

$^{13}$C-NMR spectra of compound **1** (Table 1) were very similar to those of standard luteolin therfore compound **1** was identified as luteolin.

**Chrysoeriol-7-*O*-glucoside (2)** – The UV spectrum of the compound in MeOH showed band I at 347 nm and band II at 254 and 267 nm indicating that the substance may be a flavone. A bathochromic shift in band I (55 nm) was observed upon addition of NaOMe indicating the presence of free OH group at position 4'. Also, a bathochromic shift in band I (71 nm) was observed on addition of $AlCl_3$ indicating the presence of free OH group at position 5 and/or orthodihydroxyl groups. No hypsochromic

**Table 1.** $^{13}$C-NMR of isolated flavonoids and standard luteolin

| Carbon No. | Compound 1 | Compound 2 |
|---|---|---|
| 2 | 164.3 | 164.2 |
| 3 | 103.3 | 103.4 |
| 4 | 182.1 | 182.0 |
| 5 | 161.9 | 161.1 |
| 6 | 99.2 | 99.5 |
| 7 | 164.5 | 163.0 |
| 8 | 94.2 | 95.1 |
| 9 | 157.7 | 156.9 |
| 10 | 104.1 | 105.4 |
| 1' | 121.9 | 121.3 |
| 2' | 113.7 | 110.4 |
| 3' | 146.1 | 56.2 |
| 4' | 150.1 | 148.1 |
| 5' | 116.4 | 115.8 |
| 6' | 119.4 | 120.5 |
| 1" | | 100.1 |
| 2" | | 69.6 |
| 3" | | 73.2 |
| 4" | | 65.7 |
| 5" | | 76.4 |
| 6" | | 60.7 |

shift was observed on addition of HCl to confirm the absence of orthodihydroxyl groups. Also, no bathochromic shift in band II was obscured on addition of NaOAc indicating the absence of free OH group at position 7.

FAB-MS showed a molecular ion peak at *m/z* 287 (M-Glc-$CH_3$), 269 (M-Glc-$OCH_3$), 153 ($A^+$) and at m/z 149 ($B^+$) were observed.

$^1$H-NMR spectrum of the compound showed two singlet signals at δ 6.40 and 6.85 corresponding to H-8 and H-3, respectively. Also, a meta coupled doublet at ä 7.6 (H-2") and an ortho coupled doublet at δ 6.95 (H-5`) were observed. A singlet at δ 3.9 attributed to the methoxy group was observed.

$^{13}$C-NMR chemical shifts are tabulated in Table 1 and were very similar with data reported. So, compound **2** was identified as chrysoeriol-7-O-glucoside.

**Ajugol (3)** – compound **3** was identified as ajugol depending on its $^1$H-NMR spectrum (in $D_2O$) It showed a doublet signal at δ 5.8 corresponding to H-1 with $J$ = 1.95 Hz. Also, a couple doublet of doublets was observed at δ 6.48 ($J$ = 6.15, 1.95 Hz) and δ 5.25 ($J$ = 6.15, 3 Hz) corresponding to H-3 and H-4, respectively. The protons on C-7 were appeared at 2.4 (dd, $J$ = 13.5, 6 Hz) and 2.09 (dd, $J$ = 9.75 Hz). The methyl protons were observed as a singlet at δ 1.52.

The sugar protons were assigned as follows : H-'1 at δ 5.08 (1H, d, $J$ = 7.5 Hz), H-6' at δ 4.18 (dd, $J$ = 12, 2.5 Hz) and δ 4.0 (dd, $J$ = 12, 6 Hz) (Table 2). These data were found to be identical with those reported for ajugol (Agostini *et al*., 1982).

**Table 2.** $^1$H-and $^{13}$C-NMR of the isolated iridoids and ajugal and aucubin

| Carbon No | Compound 3 ($^1$H) | Compound 4 ($^1$H and $^{13}$C NMR) |
|---|---|---|
| 1 | 5.8 | 5.28(98.8) |
| 3 | 6.48 | 6.32(140.0) |
| 4 | 5.25 | 5.14(105.7) |
| 5 | 3.0 | 2.8(42.7) |
| 6 | 4.32 | 4.58(81.0) |
| 7 | 2.4 | 5.88(129.0) |
| 8 | 2.09 | (147.2) |
| 9 | 2.86 | 3.16(46.8) |
| 10-$CH^3$ | 1.52 | 4.3(59.9) |
| 1' | 5.08 | 4.8(95.8) |
| 2' | | (73.2) |
| 3' | | (76.6) |
| 4' | | (70.0) |
| 5' | | (76.1) |
| 6' | 4.18,4.0 | 4.8, 3.9(61.1) |

**Aucubin (4)** – the comound was identified as aucubin depending on its $^1$H- and $^{13}$ C-NMR spectra (in $D_2O$). $^1$H-spectrum showed a doublet signal at δ 5.28 corresponding to H-1 ($J$ = 5.23 Hz). Also, a couple doublet of doublets were observed at δ 6.32 ($J$ = 6, 1.5 Hz) and at δ 5.14 ($J$ = 6.3 Hz) corresponding to H-3 and H-4, respectively. A series of multiplets appeared at δ 2.8, 4.58, 5.88 and 3.16 corresponding to H-5, H-6, H-7 and H-9, respectively. H-10 was observed at δ 4.3. The sugar signals were assigned and were consistent with those of glucose.

$^{13}$C-NMR spectrum was also very similar to that reported for aucubin (Bianco, 1978) (Table 2).

**Biolagical Studies** – The alcohol extract of *V. sinaiticum* exhibited a lethal concentration ($LC_{50}$) of 400 μg/ml on cultured hepatocytes, whereas the $LC_{50}$ for $CH_2Cl_2$/MeOH extract was 600 μg/ml. Both extracts exhibited a hepatoprotective effect against $CCl_4$ induced cytotoxicity (25 μg/ml and 10 μg/ml for alcoholic and $CH_2Cl_2$/MeOH extracts, respectively). This hepatoprotective activity may be attributed to the presence of iridoid compounds (Chang *et al*., 1983) which constitute the major component of $CH_2Cl_2$-MeOH (1 : 1) extract.

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