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ISOMERANZIN AGAINST HERPES SIMPLEX VIRUS IN VITRO FROM *CLAUSENA HEPTAPHYLLA* (ROXB.) W. & ARN.: ISOLATION, STRUCTURE AND BIOLOGICAL ASSAY

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LUU DUC HUY[1], R. CAPLE[2], C. KAMPERDICK[3], NGUYEN THI DIEP[1], R. KARIM[2]

[1]*Institute of Chemistry, Vietnamese Academy of Science and Technology*
[2]*University of Minnesota, Duluth, USA*
[3]*Institute of Plant Biochemistry, Halle, Germany*

SUMMARY

The isomeranzin, a coumarin was isolated from the leaves of Clausena heptaphylla. The structure was elucidated by IR, MS, ^{1}H and ^{13}C-NMR. For the first time, the anti-Herpes simplex virus type 1 & 2 in vitro activity of the compound was reported and discussed.

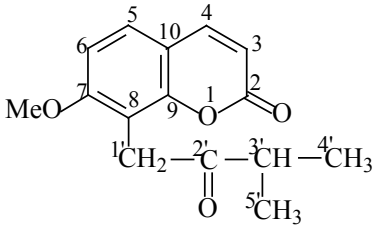
I - INTRODUCTION

Clausena heptaphylla (Roxb.) W. & Arn. (Rutaceae) (Vietnamese name: Giổi 7 lá) is a tropical tree [1]. This species is also widely used in traditional medicine. In Asia (China, India, Vietnam, etc.), it is used for fever. Several groups have reported the isolation of the isomeranzin from *Skimmia japonica* [2], *Triphasia trifloliata* [3], *Clausena anisata* [4]. In this paper we reporte on the isolation of the coumarin from the leaves of C*lausena heptaphylla* growing in Northern Vietnam, the structure elucidation and the anti-Herpes simplex virus type 1 and 2 *in vitro* biological assay of the compound.

II - RESULTS AND DISCUSSION

Specimens of *Clausena heptaphylla* were collected many times, in November 1999, May 2000, October 2001, January 2002, from Ba Vi forest of Northern Vietnam. The same coumarin, isomeranzin was isolated simply in high yield (0.7 ÷ 0.9%).

The structure of obtained coumarin was determined by interpretation of its spectral data (IR, MS, ^{1}H and ^{13}C-NMR) as well as by comparison with spectral data of an authentic sample [2, 5].



Thus, there are IR absorptions at 1608 cm^{-1} (C=O ketone), at 1719 cm^{-1} (C=O lactone).

MS data gave a molecular peak at m/z 260 accompanied by ion peak $[M+1]^+$ at m/z 261 and by fragment at m/z 190 corresponding to the sequential loss of a $COCH(CH_3)_2$ of side-chain, $[M+1- COCH(CH_3)_2]^+$.

The NMR spectrum showed signals characteristic of a coumarin; spin-spin decoupling led to the assignment of doublets at 7.36 and 6.84 ppm (o-aromatic protons) and at 7.62 and 6.19 ppm

(α-pyrone ring protons). The presence of a 3'-methyl-2'-oxobutyl side - chain was indicated by the appearance of a two-protons singlet at 4.00 ppm, a one-proton septet at 2.83 ppm and doublet signals at 1.21 ppm [-CH(C<u>H</u>$_3$)$_2$]. Signal at 3.85 ppm (s, 3H) was assigned for 7 - OMe protons.

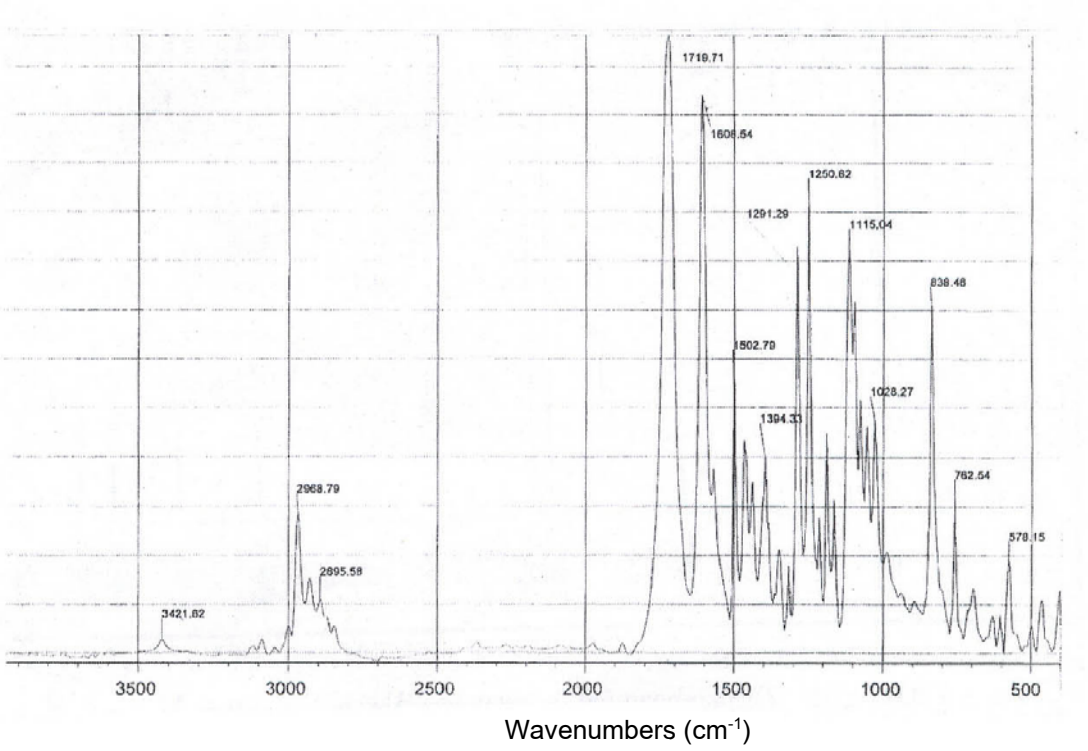


Figure 1: IR spectrum of isomeranzin (KBr)

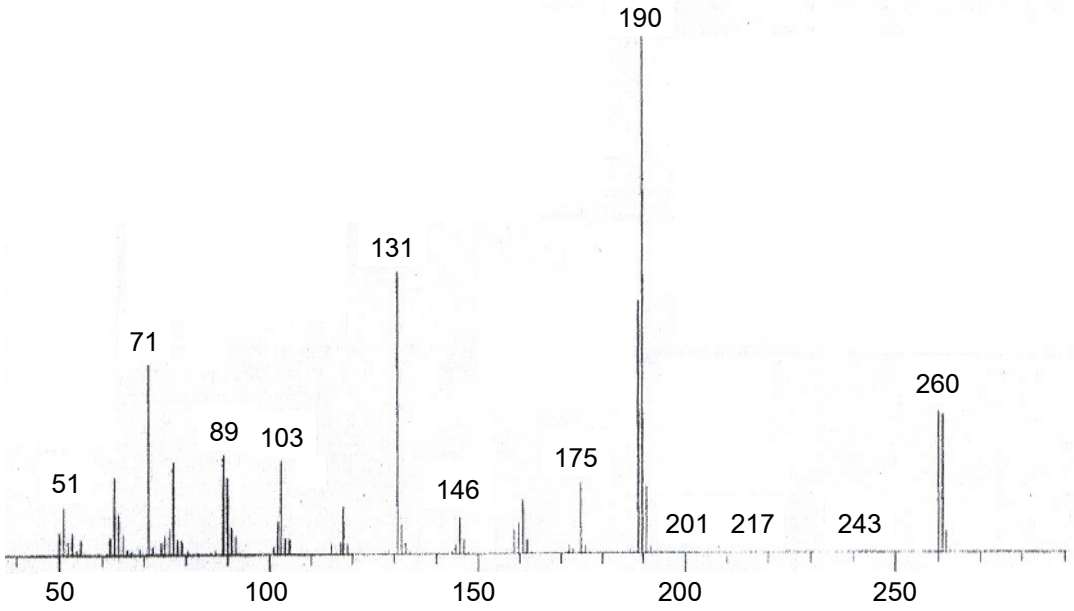


Figure 2: MS spectrum of the product ($CHCl_3$)

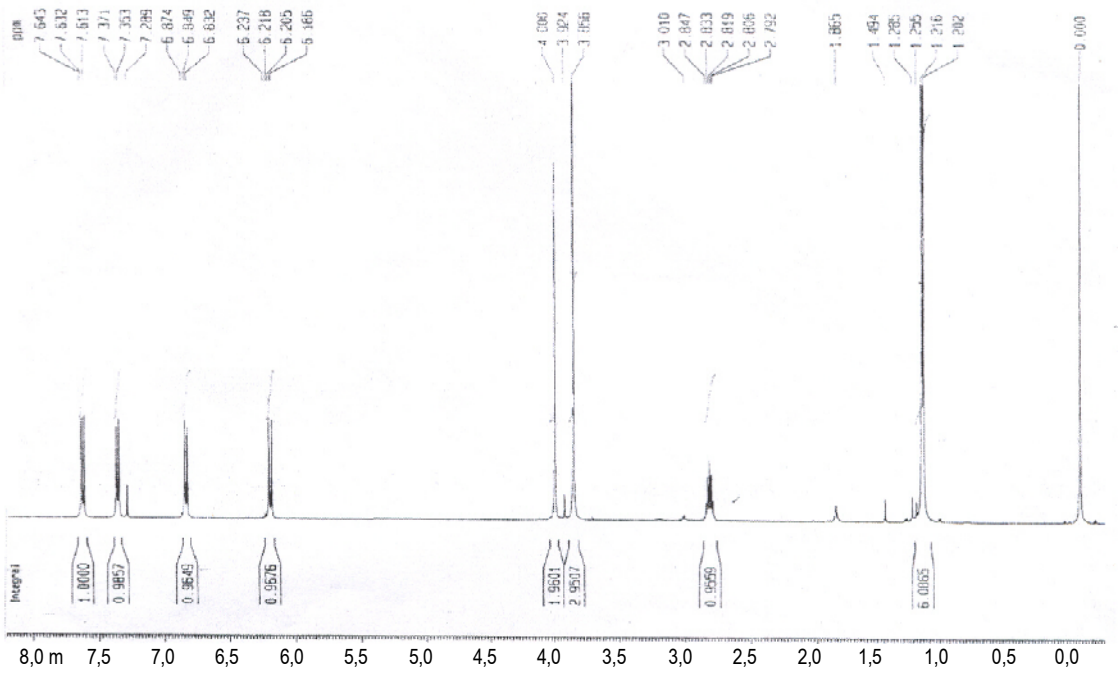


Figure 3: ^{1}H-NMR spectrum of the product (500 MHz, $CDCl_3$)

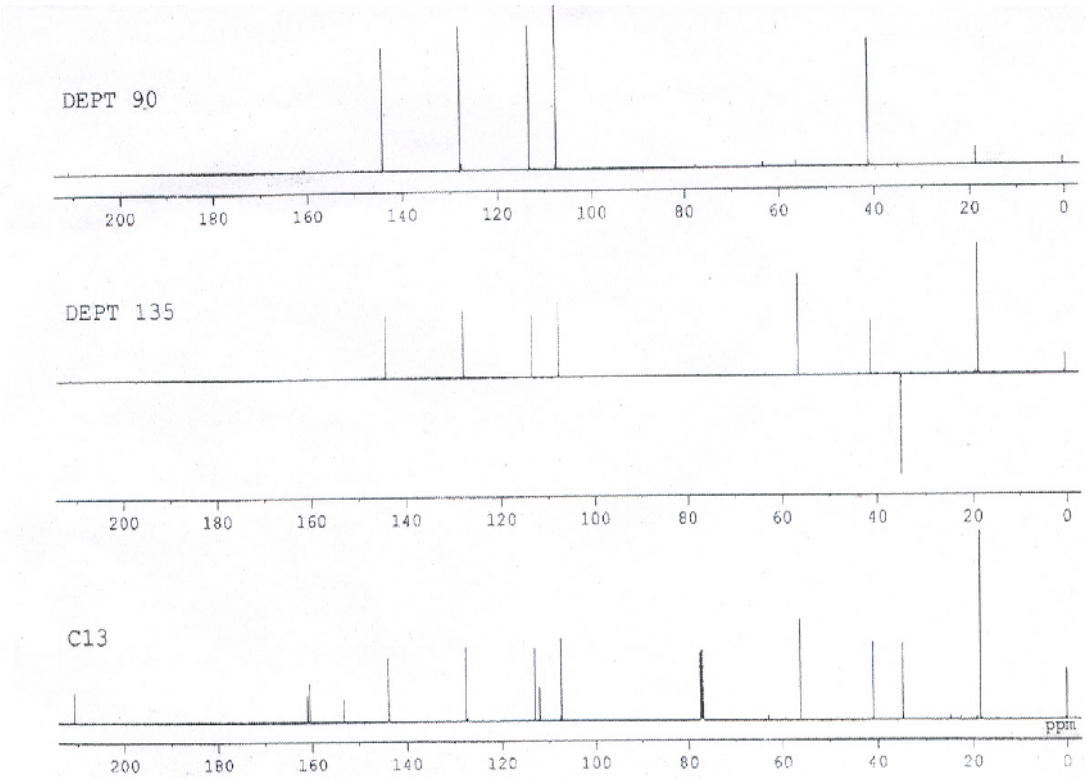


Figure 4: ^{13}C-NMR spectrum of the product (500 MHz, $CDCl_3$)

The ^{13}C-NMR spectrum also gave support to this assignment. Thus, 15 carbon atoms of obtained compound were indicated by the appearance of 15 signals. The DEPT 135 spectrum confirmed that the coumarin possesses 01 CH_2, 08 CH and CH_3; the DEPT 90 spectrum - 05 CH. So, there are 06 quaternary C in this compound.

The effectiveness and cytotoxicity of the compound in different solvents is summarized in Table 1. As can be observed from the table, this compound is cytotoxic to Vero cells. Although some of the solvents used were cytotoxic at the concentrations used, synergistic cytotoxic effects were observed when the compound was combined with the solvent, especially in the case of ethanol. Within 24 hours the monolayer of cells was completely destroyed in the 10% ethanol plus 1 mg/ml compound, while in the 10% ethanol, the cells were about 40% destroyed. At this point in time viral growth had only been observed in cells treated with DMSO and infected with HSV-1. However, in other solvents with 1mg/ml compound, the monolayer was 40% or less destroyed after 24 hours. Long term exposure of the cells to the solvent/ compound mixture at the maximum concentration of each destroyed the cells.

Table 1: Anti-HSV activity of the product

Sample	Anti-HSV-1	Anti-HSV-2
10% ethanol	+/+	++/+
10% ethanol + 1 mg/ml cmpd	?/+++	?/+++
5% ethanol	+++/-	+++/+
5% ethanol + 0.5 mg/ml cmpd	++/++	?/+++
2.5% ethanol + 0.25 mg/ml cmpd	+++/++	+/+
10% DMSO	?/+++	?/+++
10% DMSO + 1 mg/ml cmpd	?/+++	?/+++
5% DMSO	++/++	++/+
5% DMSO + 0.5 mg/ml cmpd	?/+++	?/+++
2.5% DMSO + 0.25 mg/ml cmpd	+/++	++/++
10% ethyl acetate	+++/-	++/-
10% ethyl acetate + 1 mg/ml cmpd	NT/+++	?/+++
5% ethyl acetate	+++/-	+++/-
5% ethyl acetate + 0.5 mg/ml cmpd	++/+	++/+
2.5% ethyl acetate + 0.25 mg/ml cmpd	+++/-	++/-
0.85% NaCl + 1 mg/ml* cmpd	?/+++	?/+++

*did not fully dissolve, so less than 1 mg/ml
- = no viral growth/cytotoxicity (~ 0%)
+/- = very little viral growth/cytotoxicity (< 5%)
+ = definite viral growth/cytotoxicity (< 25%)
++ = major viral growth/cytotoxicity (< 60%)
+++ =extreme viral growth/cytotoxicity (up to 100%)
? = cannot determine viral growth due to extreme cytotoxic effects
NT = not tested

Although the compound does not demonstrate spectacular effects against either virus in Vero cells, 0.5 mg/ml compound in 5% ethyl acetate or 0.25 mg/ml compound in 2.5% ethyl acetate generally provided about 40% protection from both HSV-1 and HSV-2 with little to no cytotoxic effects. The other combination that proved effective was 0.25 mg/ml compound in 2.5% DMSO. This combination provided about 40% protection against HSV-2, though it was also more cytotoxic than either ethyl acetate mixture. Against HSV-1, it provided about 70% protection, though it was fairly (~ 40%) cytotoxic. Finally, 0.25 mg/ml in 2.5% ethanol provided about 70% protection against HSV-2 with minor cytotoxic effects.

First symbol refers to viral toxicity, second symbol to cytotoxicity. Thus +++/- would show extreme viral growth, but no cytotoxic effects and +/+++ shows some viral growth but extreme cytotoxic effects

Based upon this study, this compound showed some promise as an anti-HSV agent, though it was not as effective as other plant-originated compounds have been. The combinations most effective against HSV-1 (0.25 mg/ml in 2.5% DMSO) or HSV-2 (0.25 mg/ml in 2.5% ethanol) were also cytotoxic. Whether this cytotoxicity is due to trace chemicals remaining from the extraction process, or from the compound itself is uncertain. When the compound was dissolved in ethyl acetate, it was effective against both viruses, albeit to a lesser extent, and not as cytotoxic. This compound was cytotoxic to cells even when it was at its maximum limit of solubility in water (with 0.85% NaCl). Further studies should focus on examining the compound at lower concentrations (0.5 mg/ml down to 0.05 mg/ml or lower), examining the role of solvent at lower concentrations (2.5% ethyl acetate plus virus), examining the effectiveness of a water extract of the plant, or elucidating the mechanism of synergistic cytotoxicity of ethanol and the compound. It is possible that the compound reacts with the ethanol, changing the compound to a more cytotoxic form.

III - EXPERIMENTAL

1. General experimental procedures

Solvents were purified and distilled prior to use.

Optical rotation was measured on ATAGO POLAR- D.

Melting point was determined on Boetius apparatus (D. Germany).

IR spectra were recorded on FT-IR-IMPACT-410 as KBr pellets .

^{1}H-NMR and ^{13}C-NMR spectra were recorded on Bruker AM 500 spectrometer in $CDCl_3$ with TMS as internal reference.

MS spectra were recorded as solutions in $CHCl_3$ on MS-5989B spectrometer.

Analytical thin-layer and column chromatography was performed using silicagel Merck GF_{254}.

Biological assay was performed in the University of Minnesota, Duluth, USA.

2. Plant material

The dried leaves of *Clausena heptaphylla* used in this investigation was collected from the Ba Vi Forerst, Northern Vietnam many times. The plant was determined by Botanist, Dr Tran Ngoc Ninh, Vietnamese Academy of Science and Technology.

3. Extraction and isolation

Leaves of *Clausena heptaphylla* were dried at 40 ÷ 50^{0}C and powdered. The powder (500 g) was extracted with 3 litres MeOH 90^0 at 80^{0}C. The extract was filtered and concentrated in vacuo to yield 84 g extract. The concentrated extract was fractionated into Hexan, EtOAc, BuOH, and H_2O-soluble fractions, sequentially. The EtOAc-soluble fractions (28.7 g) was subjected to silicagel column chromatography. Elution with Hexan / EtOAc 10 : 1 ÷ 10 : 2 gave isomeranzin (4 g, 0.8%). Recrystallization from Hexan / EtOAc 10 : 1 gave needles melting at 58 ÷ 60^{0}C (60^{o}C ÷ 62^{0}C (from pentane) [2]), $[\alpha]_D$ 0^0 ($CHCl_3$) (0^0 [2]). IR (γ, cm^{-1}, KBr) : 838, 1028, 1115, 1250, 1291, 1394, 1502, **1608, 1719**, 2968. MS (m/z, $CHCl_3$) : 261 $[M+1]^+$, 260 $[M^+]$,

190 [M+1-COCH$(CH_3)_2$]$^+$. ^{1}H-NMR (δ, ppm, $CDCl_3$): 1.21 [d, 6H, j = 6.93 Hz, 3' - CH($C\underline{H}_3)_2$]; 2.83 (septet, 1H, 3'-H); 3.85 (s, 3H, 7-OCH_3); 4.00 (s, 2H, 1' - CH_2); 6.19 (d, 1H, 4-H, j = 9.46 Hz); 6.84 (d, 1H, 6-H, j = 8.60 Hz); 7.36 (d, 1H, 5-H, j = 8.60 Hz); 7.62 (d, 1H, 3-H, j = 9.46 Hz). ^{13}C-NMR (δ, ppm, $CDCl_3$): 18.42 (4'-C, 5'-C); 34.69 (1'-C); 40.88 (3'-C); 56.12 (7 - OCH_3); 107.26 (6-C); 111.95 (10-C); 112.91 (8-C); 112.97 (3-C); 127.55 (5-C); 143.80 (4-C); 153.20 (9-C); 160.44 (7-C); 160.97 (2-C); 210.76 (2'-C).

4. Methods of biological activity assay [6 -9]

The sample was prepared in four solvents. It was prepared at a concentration of 1 mg/ml in 0.85% NaCl plus a 10% solution of one of the three solvents: dimethyl sulfoxide (DMSO), ethyl acetate or ethanol.

These solutions were assayed against HSV-1 and HSV-2 using a cell protection assay. Immortalized African Green Monkey Kidney (Vero) cells (ATCC) were grown to a monolayer in minimal essential medimum (MEM) (Sigma) supplemented with 10% fetal calf serum (FCS) (Sigma) in 12 well microtiter plates (Falcon). The cells were infected with about 100 virus particles of HSV-1 strain E377 or HSV-2 strain MS per well. After one hour, the cells were treated with 1 ml of the treatment (for 1 mg/ml compound in 10% ethanol) or 1 ml of saline (for virus control and cell control) and given 1 ml 2x MEM. The cells were incubated for 72 hours and observed for cytopathic effects from the virus and cytotoxic effects from the samples. After 72 hours, the cells were stained with 0.05% crystal violet in 35% methanol.

IV - CONCLUSION

1. The isomeranzin was isolated many times and simply from the leaves of the *Clausena heptaphylla* in high yield (0.7 ÷ 0.8%). The structure of the compound was determined by interpretation of its spectral data (IR, MS, ^{1}H and ^{13}C-NMR) and by comparison with spectral data of an authentic sample.

2. The isomeranzin showed some promise as an anti-Herpes simplex virus agent.

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