Chemical Analysis and Antitumor Activity of Natural and Semi-Natural Carotenoids of Saffron

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Keywords: *Crocus sativus* L., FT-IR, FT-Raman and ^{1}H NMR, human leukemic cells, LC-MS, UV-Vis, X-ray

Abstract

The qualitative analysis and determination of saffron components using chromatographic and spectroscopic methods is described. Combination of LC-MS data with UV-Vis, FT-IR, FT-Raman and ^{1}H NMR and crystal structure analysis data prove useful information in identifying of saffron compounds. In addition, the effects of saffron carotenoids on cell proliferation and differentiation of K562 and HL-60 cells cancer human cell lines in vitro are demonstrated.

INTRODUCTION

Saffron, in filaments, is the dried, dark red in colour, stigmata of *Crocus sativus* L. flowers. It is used both as a spice for flavouring and colouring food preparation and as a drug in traditional medicine. There are variety of foods that have been spiced with saffron, including cream or cottage cheese, bouillabaisse, chicken, rice, paella, etc. One stigma of saffron weighed about 2 mg, each flower has three stigmata. 150,000 of flowers must be carefully picked one by one in order to produce 1 Kg spice.

The colouring components of saffron are crocins (CRCs), which are unusual water-soluble carotenoids (*cis* and *trans* glucosyl esters of crocetin). The major component is a digentiobiosyl ester of crocetin. Picrocrocin, a monoterpene glycoside, is the principal bitter tasting substance (Tarantilis et al., 1994, 1995). One of the main components of the essential oil of saffron responsible for its aroma is safranal (Tarantilis and Polissiou, 1997). It is a monoterpene aldehyde, formed in saffron during drying and storage by hydrolysis of the picrocrocin.

Retinoids (vitamin A and its natural and synthetic analogs) and carotenoids have been shown to inhibit the growth of certain types of tumour cell lines, including leukemic, neuroblastoma, breast and colon adenocarcinoma. Thus, retinoids, carotenoids and their synthetic analogs have been proposed for experimental chemoprevention and even for the treatment of cancer. However, their toxicity remains an important limiting factor for using this class of compounds at high therapeutical doses.

Many compounds from natural products have shown tumour reducing activity thus useful in the treatment of cancer. Extracts of some spices were found to inhibit the growth of transplanted tumours in mice as well as being cytotoxic to cells in tissue culture.

In this lecture, novel chromatographic and spectroscopic methods for qualitative analysis and determination of saffron substances are present. In addition, the effects of saffron carotenoids on cell proliferation and differentiation of K562 and HL-60 cells cancer human cell lines in vitro are demonstrated.

MATERIALS AND METHODS

Materials

Stigmata of pure red Greek saffron were kindly supplied by the Cooperative of Saffron, Krokos Kozanis.

For determination of saffron components, 5 mL methanol and 5 mL water were added to 100 intact stigmata of saffron (200 mg), and allowed it to undergo maceration for about 2 h in the dark at ambient temperature with occasional stirring. The crude methanolic extract was analysed by LC-MS using both TS and ES LC-MS interfaces.

Crocins (CRCs) are prepared from the red dried stigmata of *Crocus sativus* L.

(Saffron). Saffron was exhaustively and successively extracted by a) petroleum ether 40-60 °C, b) diethyl ether and c) methanol 80%. The methanol extract, after evaporation under vacuum at room temperature, provides a dark red powder residue. This residue was a mixture of crocins having as major component the digentiobiosyl ester derivative of crocetin. Additional purification of mixture of carotenoids was performed by washing with tetrahydrofurane (THF), containing 0.025% of butylatehydroxytoluene (BHT) as preservative.

Dimethylcrocetin (DMCRT), was synthesized by alkaline hydrolysis from CRCs in methanol and purified by extraction with dichloromethane (DCM) and recrystallisation solvent system of DCM-Et_2O 50:50.

Crocetin (CRT) was prepared from CRCs by alkaline hydrolysis and acidification in water and purified by successive extraction with benzene. Additional purification was obtained when benzene was evaporated completely and the powder (dark red colour) was dissolved in pyridine which in turn, eliminated by a flow of nitrogen gas.

All-trans β-carotene and all-*trans* retinoic acid were purchased from Aldrich Chemical Company and Sigma respectively and used without further purification.

The nuclear retinoic acid receptor c (RAR-c) was generously provided by D. Bonnier, H. Gronemeyer and P. Chambon at a concentration of 0.17 mg mL^{-1} in 10 mM Tris buffer (pH 8)-5 mM dithiothreitol-500 mM NaCl-0.15 mM C12M.

Liquid Chromatography-Mass Spectrometry

The LC system consisted of a Hewlett-Packard (Palo Alto, Ca, USA) model HP-1090 Series liquid chromatograph with DR5 binary solvent delivery system and variable volume auto injector. A UV/Vis diode array detector monitored the elution profile at multiple wavelengths. A stainless-steel column Hewlett-Packard ODS Hypersil 799160D-552, 5μm, 100x2.1mm id was used. The mobile phase was linear gradient from 10% to 100% methanol/water solution containing 1% acetic, in 60 min. The solvent flow rate was 0.5 mL/min and the sample injection volume 5μL. Detection was performed simultaneously at 190-600 nm.

The mass spectrometer was a Hewlett-Packard model 5989A, equipped with a hyperbolic quadrupole mass analyser, a high mass option, high energy dynode (HED) detector multiplier detector and a differentially pumped vacuum system with diffusion pumps. The voltage applied to the electron multiplier was 2300 V and to the HED 9000 V. The quadrupole temperature was held at 100°C. The ionization mode was positive. The mass spectrometer scanned from m/z 130 to 1200. A HP mass spectra DOS ChemStation system (G1023B) was used as analytical workstation.

The HP 5989 A mass spectrometer was equipped with an HP thermospray interface. The thermospray probe temperature was set at 100°C and time programmed to 90°C in 60 min, following the column linear gradient elution profile. The source was maintained at 280°C and the aerosol temperature varied from 260-280°C (beginning -end of gradient). The measurements were performed in the filament ON, acquisition mode. To avoid any alteration of chromatographic conditions a post column addition of a 0.2 M ammonium acetate was effected with 1050 HP isocratic pump, giving a final concentration of about 0.1 M buffer after dilution with the column effluent; this provided the volatile buffer for ion evaporation ionization process. The TS-MS spectra look like chemical ionization spectra and fragmentation is related to the vaporization temperature.

A Hewlett-Packard 59987A Electrospray interface was used. The needle was grounded and charging occurs by keeping the cylindrical electrode at about -3 kV, the end plate at -3.5 kV and the capillary at -4.5 kV. The potential difference between the skimmer and the end of the capillary was varied between 150 and 300 V. The others lenses were held at potential to obtain maximum signal intensity. Hot high purity nitrogen was used for desolvation. The HPLC flow was splitted 1/150 times resulting flow into the electrospray source was about 3 μl/min.

Spectroscopy

Infrared spectra were recorded on KBr discs of dry substance (2 mg/150 mg KBr) using a Nicolet 750 Magna IR (USA) Fourier transform spectrometer. It is equipped with a DTGS detector. Spectra were acquired using the Omnic (3.1 version) FT-IR software at a resolution of 4 cm^{-1} and with 100 accumulations.

Raman measurements were performed directly on the dry powder with a Bomem DA 3.02 Fourier transform modified IR spectrometer equipped with an Olympus BH-2 (objective ×80) microscope accessory, an Nd^{3+}:YAG laser operating at 1064 nm, an InGaAs detector. Spectra were obtained at a resolution of 4 cm^{-1}, with a laser power 300 mW. For each spectrum 100 scans were averaged. In addition, a Nicolet 750 magna IR with Raman accessory was used. The beamsplitter was CaF2, the source power was 0.5 W, and the wavelength was 1064 nm (near-IR).

Micro-FT-SERS was performed with a Model DA 3.02 FT-IR spectrometer (Bomem) modified to work in the near-infrared region.12 A liquid nitrogen-cooled InGaAs detector was used. The excitation source was 1064 nm radiation from an Nd:YAG laser (CVI, Model C-95) operating at 200 mW. Aqueous silver hydrosol was prepared by reduction of silver nitrate (Prolabo, Paris France) with trisodium citrate (Sigma, St Louis, MO, USA). The hydrosol was activated by the addition of sodium perchlorate solution (Sigma), up to a final concentration of 0.06 M.

Resonance Raman spectra were obtained with a DILOR Omars-89 Raman spectrometer, supplied with a multi-channel (512 photodiodes) detector. A Spectra-Physics Model 2020-03 argon ion laser was used for excitation (457.9 nm). All band intensities were corrected for the monochromator-detector response.

A Varian-Unity 600 was used for NMR experiments: ^{1}H 1D in CD_3OD, proton frequency 600.042 MHz, and (2) ^{1}H-^{1}H 2D COSY.

X-Ray Analysis

A crystal of DMCRT 0.1 x 0.3 x 0.35 mm dimensions was scaled in a Lindermann glass capillary to prevent sublimation. Intensity data were collected on a Syntex $P2_1$ diffractometer, upgrated by Crystal Logic. The refinement, based on F, proceeded by full - matrix least squares, in which $\sum w\Delta F^2$ was minimized. The H-atoms have been located by $\Delta\rho$ map and refined isotropicaly. Seven reflections showing poor agreements were given zero weight during final refinement cycles, and the maximum shift / e.s.d. = 0.080.

Cell Culture, Growth Inhibition and Differentiation

K562 is a human erythroleukia cell line, established from a patient with chronic myelogenous leukaemia in blast transformation. K562 cells grew exponentially from $5x10^4$ to $8x10^4$ at 37 ^{o}C, in humidified 5% CO_2 incubator, in RPMI (Gibco, Cergy Pontoise, France) supplemented with 10% fetal calf serum (Flobio), 2 mM L-Glutamin and antibiotics. Cell growth and viability were determined by Tryptan Blue exclusion staining. Cells were routinely examined for mycoplasma contamination.

Cells in exponential growth phase at $5x10^4$/mL density multiwell dishes (Nunc) were incubated for 3 days at appropriate concentration of CRCs, DMCRT and all-*trans* β-carotene (2.5-80 μM). The percentage of differentiated cells was determined by scoring benzidine positive. K562 were stained using a benzidine-H_2O_2 method which cells giving an unequivocally positive benzidine reaction, seen as intense blue cytoplasmic staining, are know to synthesize haemoglobin. The extent of differentiation was expressed in terms of: (Benzidine Positive Cells/Total Cells)x100.

The percentage of growth inhibition by concentration of CRCs, DMCRT and All-trans β-carotene was calculated from 100% of control cells.

The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, proliferates, in sly in suspension culture and consists predominantly (>90 %) of promyelocytes. These cells can be induced to differentiate to morphologically and functionally mature granulocytes by incubation with a wide variety of compounds. Thus, this cell line provides a unique system for studying growth inhibition in vitro.

Cells, in exponential growth phase at a $5x10^4$ /mL density 25 cm^2 dishes (Nunc), at 37°C, in humidified 5% CO_2 incubator, in RPMI 1640 (Gibco, Cergy Pontoise, France) supplemented with 10% fetal calf serum (fcs, Laboratoire Institut Jacques Boy, Reims, France), 2 mM L-Glutamin and antibiotics, were incubated for 3 or 5 days at the appropriate concentration of retinoids, natural carotenoids and their derivatives of *Crocus sativus* L. Cells were routinely examined for mycoplasma contamination.

The percentage of growth cell of HL-60 cells for each drug concentration and time incubation was determined by the MTT survival test. The MTT test is based on capability of the survival cells to transform the soluble tetrazolium salt [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to a precipitate of formazan. These crystals of formazan were dissolved in dimethylsulfoxyde (DMSO) and the optical density was measured spectrophotometrically (Microplates reader, Series 750, Cambridge Technology INC).

Differentiation activity of ATRA, DMCRT, CRT and Saffron extract (CRCs) has been carried using NBT test (Nitra Blue Tetrazolium). After each culture condition in presence of ATRA and carotenoids of Saffron (3 or 5 days), cells are washed twice with PBS and the pellets are resuspended in 0.2 mL NBT solution containing 0.5 mL of foetal calf serum, 0.5 mL of 1% NBT, 4 mL of RPMI and 1 mL of tetraphorbolacetate (TPA).

After 30 min of incubation at 37°C in the dark, cytopsin slides were prepared and stained and 300 cells were scored for the presence of blue-black formazan granules. Percentage of NBT positive cells were estimated in the same way as the number of cells, which contain purple-coloured granulations, over the 300 cells per each smear preparation.

RESULTS AND DISCUSSION

Saffron components have been determined (Tarantilis et al., 1995) in crude plant extract by LC–MS using both TSP and ESP interfacing systems. Figure 1 shows the structures and molecular formula of most abundant compounds identified in saffron extract. Figure 2 demonstrates the reversed-phase HPLC separation chromatogram recorded at three wavelength (250, 308 and 440 nm) and Figure 3 the total ion current chromatogram and selected-ion chromatograms, corresponded well with the LC-UV/Vis trace. The number of absorption bands and the general aspects of their UV spectra allowed the attributions of the picks of the chromatogram in three compounds family: a) picrocrocin and derivatives of picrocrocin, b) flavonoid derivatives and c) crocins.

Three classes of substances are recognized to determine the organoleptic characteristics of saffron; in particular, picrocrocin is the bitter agent of this plant. Concerning the LC–MS analysis of crocins, the mass spectra acquired on-line allowed to differentiate *cis* and *trans* isomers and to establish the sequence of glucoses. TSP-MS was found suitable for the unequivocal molecular mass determination of mono-and diglycosides of crocetin, but it failed in higher glycosides. In contrast, LC–ESP- MS appears to have greater potential than LC–TSP -MS as an identification technique for glycosidic carotenoids bearing up to five glucoses. The two interfacing systems showed similar behaviour for the small molecules (safranal, picrocrocin derivatives and flavonoids), TSP and ESP mass spectra displaying predominantly protonated and sodiated molecular species.

The carotenoids of saffron, crocins (CRCs), and their derivatives, dimethylcrocetin (DMCRT) and crocetin (CRT) were studied using FT-IR, FT-Raman, ^1H NMR spectroscopies and X-ray analysis.

The FT-IR spectra (Figure 4) of Crocins (CRCs), dimethylcrocetin (DMCRT) and crocetin (CRT), in solid state, have characteristic absorbance bands between 1706 and 1664 cm^{-1} ($\nu_{C=O}$) and in the region between 1243 and 1228 cm^{-1} (ν_{C-O}). Two main Raman lines were observed in the FT-Raman spectra (Figure 5) near 1540 and 1166 cm^{-1} which are respectively assigned to ($\nu_{C=C}$) and (ν_{C-C}) stretching modes (Tarantilis et al., 1998).

DMCRT (dimethylcrocetin) redissolved to a 1:1 mixture CH_2Cl_2 and diethyl ether and kept at 4 ^oC to obtain crystals suitable for X-ray analysis in 4 days (Tarantilis et al.,

1994). The stereoview with a numbering scheme of the molecule is given in fig 6.The molecule has the all-*trans* configuration and forms a long planar conjugated system which includes a crystallographic inversion center, located at the midpoint of the C(15) - C(15') bond.

Crocins have been isolated, purified by HPLC and verified as *trans* and 13 *cis* isomers using UV-Vis, Raman and ^{1}H NMR spectroscopy (Figure 7) (Assimiadis et al, 1998). The 13-*cis* compound showed in a UVvis spectrum an additional peak at 330 nm, in FT-Raman spectrum the presence of a peak at 1138 cm $^{-1}$, and in the ^{1}H NMR downfield shifts of protons 12 and 15 and an upfield shift of proton 14, with respect to the all-*trans* isomer, according to differences in their skeletal structures. These features are shown also by the profile of their two-dimensional spectra.

The effects of carotenoids of *C. sativus* on cell proliferation and differentiation of K562 and HL-60 cancer human cell lines have been studied and compared with those of *β*-carotene and all-*trans* retinoic acid (Morjani et al, 1990; Tarantilis et al, 1994).

We have compared the effects of all trans-*β*-carotene, CRCs and DMCRT on the growth inhibition and the induction of the erytroid differentiation of K562 cells. These carotenoids efficiently inhibited the cell growth when K562 cells were cultured during 3 days in presence from 2.5 up to 80 μM. 50% of cellular growth inhibition were obtained with 7, 10 and 18 μM for DMCRT, all trans-*β*-carotene and CRCs respectively. These carotenoids were also found to be inducers at the erythroid differentiation of K562 cells. A significant effect was observed when cells were incubated with 10 μM of carotenoid during 3 days (corresponding to 50% of cell inhibition). From 10 to 80 μM the percentage of benzidine positive cell was increased from 15% to 45% for the MMCRT.

The comparison of carotenoids of *C. sativus* with those of all-trans on cell proliferation and differentiation of HL-60 cells demonstrates that carotenoids of saffron both as crocins mixture (CRCs) or pure derivatives CRT and DMCRT are highly effective in antiproliferation and inducing differentiation of HL-60 leukemic cells.

In fact the concentrations that induced 50% inhibition of cell growth were 1.2 μM for DMCRT, 5 μM for CRT and 6.6 μM for CRCs during 3 days of culture (Figure 8). These values were different, 0.8, 2 and 2 μM for DMCRT, CRT and CRCs respectively, when the time of culture was prolonged for two days more. The minor values correspond always to its derivative DMCRT that seems to be more effective. The same effect was observed for ATRA that was used as reference with 0.5 and 1.2 μM during 3 and 5 days of culture experiments respectively. This result is in agreement with reports from different works indicating that retinoids efficiently inhibit cell growth of both normal and transformed cells. At this time retinoids (vitamin A ; ATRA ; 13-*cis* retinoic acid etc.,) are viewed by many having the greatest potential for immediate application as chemopreventive agents in human, though they have demonstrated a level of toxicity that precludes their use except in subjects at higher risk of cancer.

In contrast, the natural carotenoids, crocins (CRCs) and their derivative's dimethylcrocetin (DMCRT) and crocetin (CRT), are not pro-vitamin A precursors and could be less toxic at high doses cancer chemotherapy. This hypothesis is supported by in vivo experiments where a delay in the onset of tumour formation with an increase in the life span of treated mice (200 mg/Kg body weight) as compared to the untreated controlled was observed. Furthermore, haematological and biochemical studies on the same animals have shown a complete absence of sever toxicological manifestations at the liver, kidney or bladder, since most of the biochemical limits were the normal range. Finally the lethal-dose (LD_{50}) required to kill 50% of the above animals receiving saffron extract was very high > 600 mg/Kg body weight.

The most important part of this study is that the carotenoids of saffron and especially its derivative DMCRT induced a functional terminal differentiation of the human promyelocytic leukemia cell line HL-60. Maximal differentiation (approximately 70%) occurs at a concentration 5 μM of DMCRT that is almost equally effective to ATRA (85%) and superior to CRT (50%) and CRCs (48%). In the same experiment with 2 μM concentration of ATRA, DMCRT, CRT and CRCs the percentages of

differentiation were 70%, 45%, 20% and 15% respectively (Figure 9). Thus, although HL-60 cells are leukemic origin with a pronounced differentiative response to ATRA the differentiation induction observed by the carotenoids of saffron seems to be significant with a potential advantage on the toxicity.

The anticarcinogenic action of these carotenoids may be explained in two ways: a) there is a widely accepted hypothesis that these compounds function as traps of free radicals, the radical-trapping potential of carotenoids might be derived from a 90° rotated configuration twisting the 13, 14, (or 13',14') double bond, b) there is also an other plausible hypothesis concerning their inhibiting interaction with biological molecules such as topoisomerase II involved in DNA synthesis.

FT-Raman and FT-SERS can be used as powerful probes for investigating the effect of differentiating agents (retinoids and carotenoids) in cancer cells related to RAR expression (Beljebbar et al, 1997). By comparing the Resonance Raman (a) and FT-SERS (b) spectra, we can see that the structure of the DMCRT was not perturbed on adsorption on a colloidal silver surface(Figure 10).

The Raman intensity in the spectrum of DMCRT with RAR-γ is less intense than that in the free DMCRT. This decrease in relative and global intensity is the result of the interaction of DMCRT with the Retinoid Acid Receptor. The intensity ratio I_{1541}/I_{1165} was the same in both cases, 1.58. However, the ratio I_{1541}/I_{1210} decreased on complexation (2.5 for free DMCRT and 1.9 for the DMCRT-RAR-γ complex) (Figure 11). These results demonstrate that DMCRT can interact specifically with RAR-γ. Based on these findings, it is most probable that this molecule can interact indirectly with DNA through the nuclear receptor RAR- γ.

Since the HL60 cell line expresses the nuclear RAR, the observed spectral changes (Raman intensity) may correspond to the interaction of free DMCRT with RAR (DMCRT-RAR complex). In fact the Raman intensity of DMCRT spectrum in the HL60 (b) is less intense than in K562 (c) cells. This decrease in intensity is the result of the interaction of DMCRT with the Retinoid Acid Receptor at different intracellular accumulation in HL60 cells. The similarity between the signal of DMCRT in K562 (b) and that of free DMCRT (a) can be explained either by the absence of RAR or by the absence of any interaction between the drug and receptor (Figure 12).

CONCLUSIONS

LC-MS is suitable for the separation and determination of crocetin glycosides, safranal and picrocrocin (glycoside of safranal) in crude saffron extract. The UV-visible and the mass spectra recorded on-line provide information about the identification of *trans* and *cis* isomers, molecular masses and the sequence of glucoses.

This type of natural carotenoids of *C. sativus* and specially their derivatives having comparable biological activities to those of the ATRA and they could be used for the prevention of human leukaemia and as alternative antitumour agents in cancer chemotherapy, alone or in combination with other anticancer drugs in order to increase their effectiveness and minimise their toxicity.

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Figures

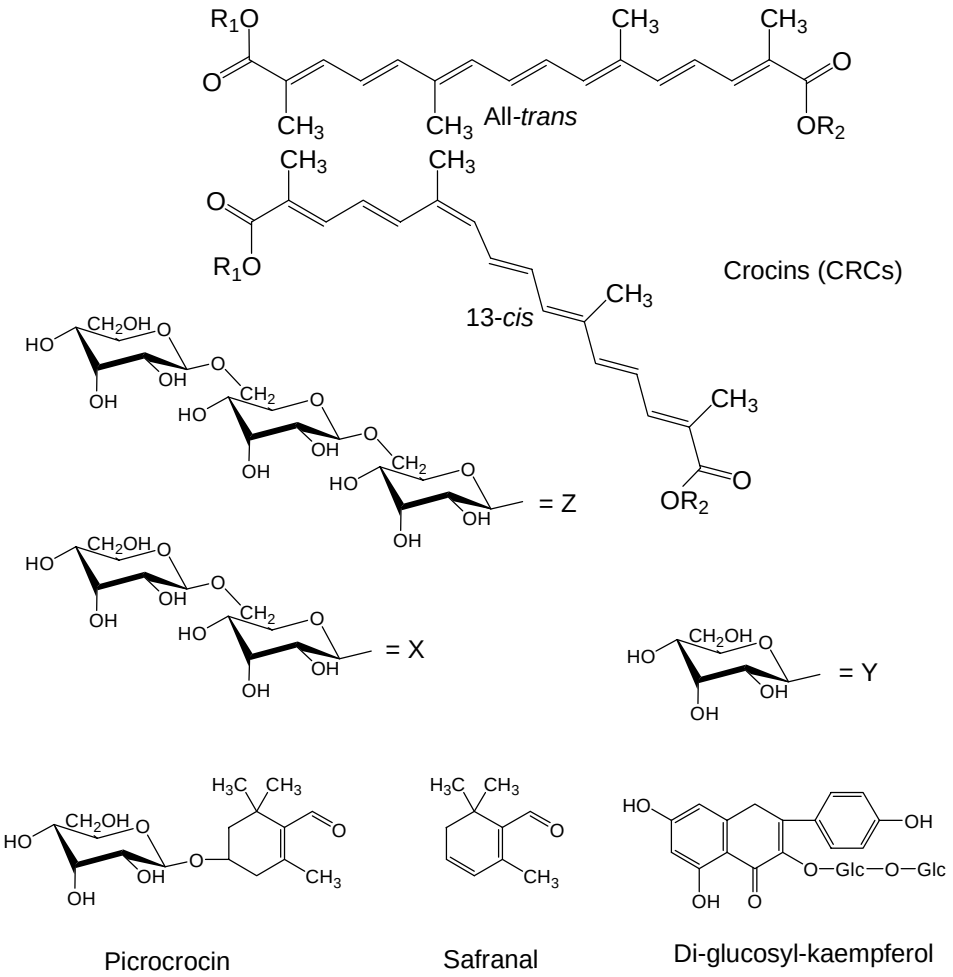


Fig. 1. Structure of saffron components.

Crocins:	Glucosyl esters of crocetin	Formula	MM*
Crocin-5:	R_1=Three *β*-D-glucosyl (Z), R_2=*β*-D-gentiobiosyl (X)	$C_{50}H_{74}O_{29}$	1138
Crocin-4:	R_1=R_2=*β*-D-gentiobiosyl (X)	$C_{44}H_{64}O_{24}$	976
Crocin-3:	R_1=*β*-D-gentiobiosyl (X) R_2=*β*-D-glucosyl (Y)	$C_{38}H_{54}O_{19}$	814
Crocin-2:	R_1= *β*-D-gentiobiosyl (X) R_2=H	$C_{32}H_{44}O_{14}$	652
Crocin-2':	R_1=R_2= *β*-D-glucosyl (Y)	$C_{32}H_{44}O_{14}$	652
Crocin-1;	R_1=*β*-D-glucosyl (Y), R_2= H	$C_{26}H_{34}O_9$	490
Crocetin:	R_1=R_2= H	$C_{20}H_{24}O_4$	328
Dimethylcrocetin	R_1= R_2= CH_3	$C_{22}H_{28}O_4$	356
Picrocrocin:	glycoside of safranal	$C_{16}H_{26}O_7$	330
Safranal:		$C_{10}H_{14}O$	150
Di-glucosyl-kaempferol:		$C_{27}H_{30}O_{16}$	610

*Molecular Mass

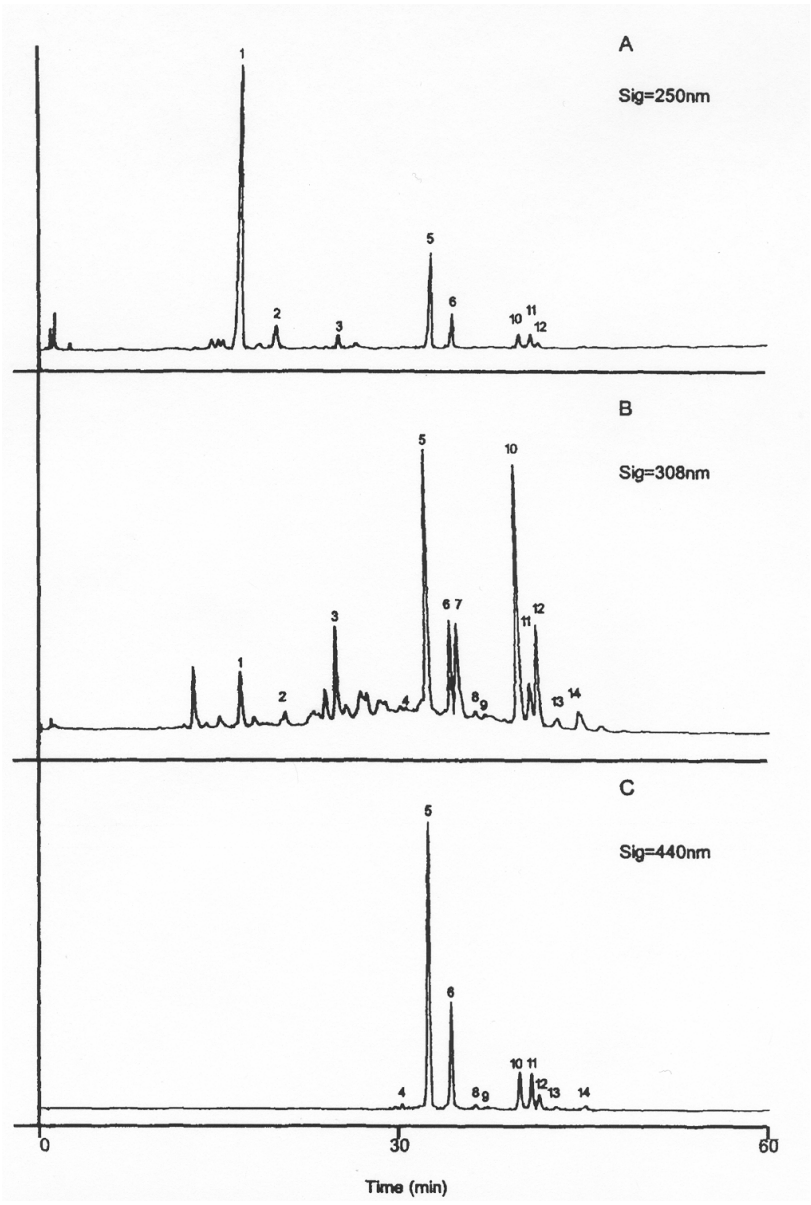


Fig. 2. Chromatographic signals of a methanol-water (50:50) extract of saffron recorded at A) 250 nm, B) 308 nm and C) 440 nm. Peaks 1 = picrocrocin, 2 = picrocrocin acid form; 3 = kaempferol diglycoside; 4 = *trans*-crocin-5 and 9 = *cis*-crocin-5; 5 = *trans*-crocin-4, and 10 = *cis*-crocin-4; 6 = *trans*-crocin-3 and 12 = *cis*-crocin-3; 8 = *trans*-crocin-2' and 14 = *cis*-crocin-2'; 11 = *trans*-crocin-2 (*trans* gentiobiosyl ester of crocetin); 13 = *cis*-crocin-1 and 7 = safranal.

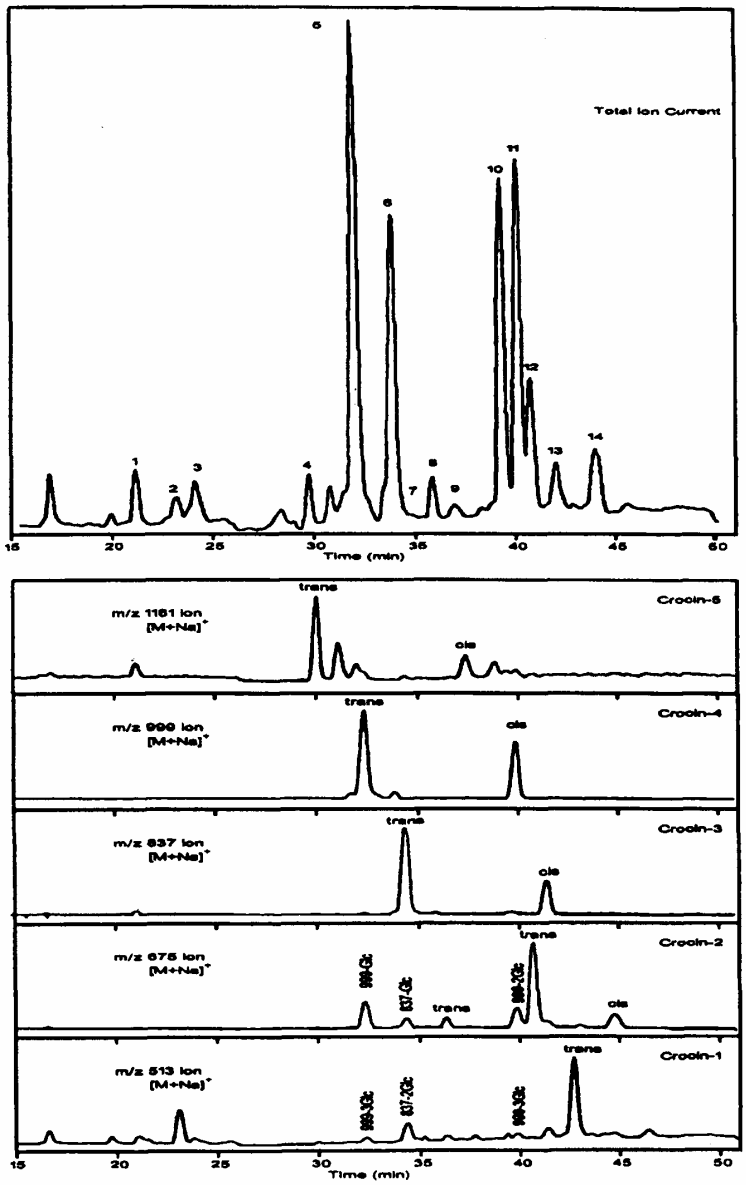


Fig. 3. Total ion chromatogram and selected-ion chromatograms for the HPLC-ES-MS analysis of a methanol-water (50:50) extract of saffron.

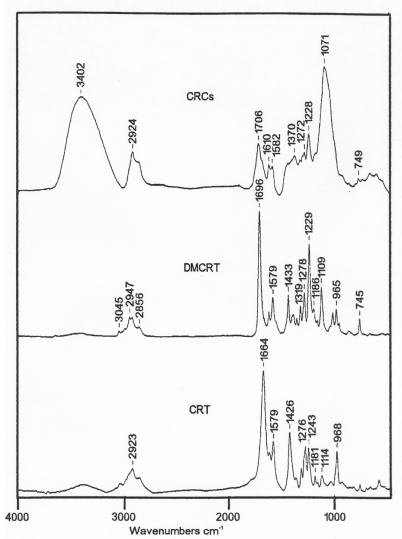


Fig. 4. FT-IR spectra of CRCs, DMCRT and CRT. Spectra are taken in solid state (in KBr pellet).

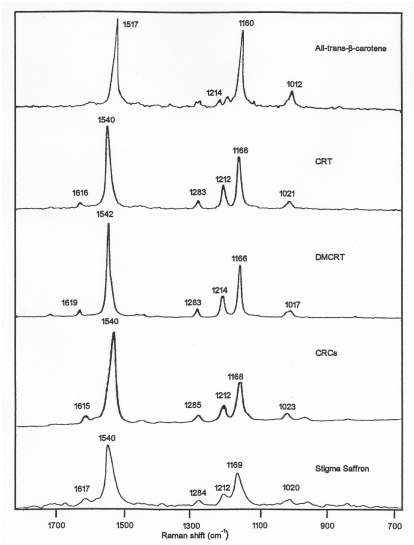


Fig. 5. FT-Raman spectra of all-*trans*-b-carotene, CRT, DMCRT, CRCs and intact stigma of saffron. Spectra are taken in solid state.

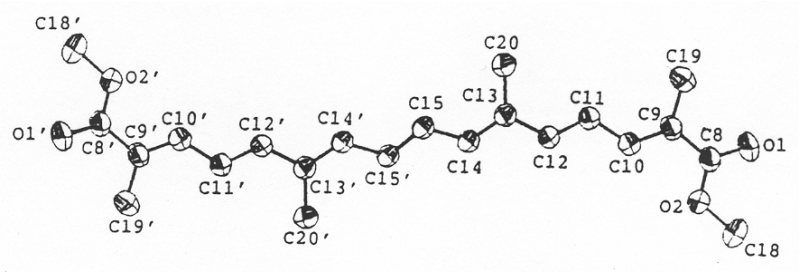
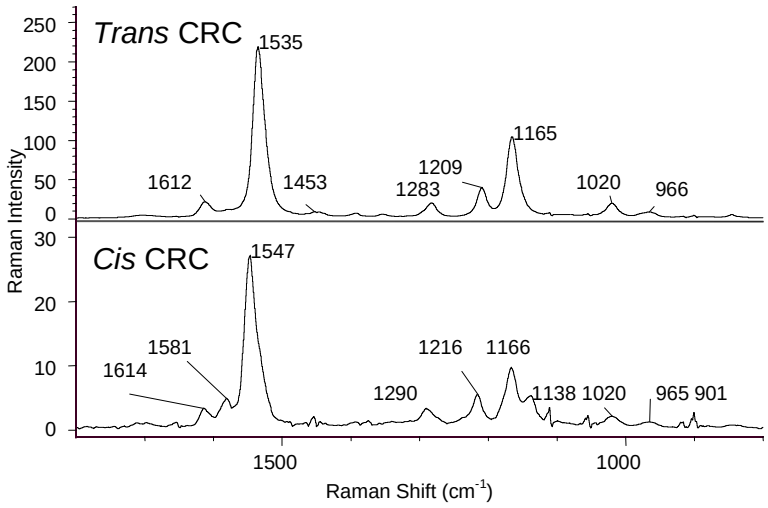


Fig. 6. An ORTEP view of the molecule dimethylcrocetin (DMCRT).



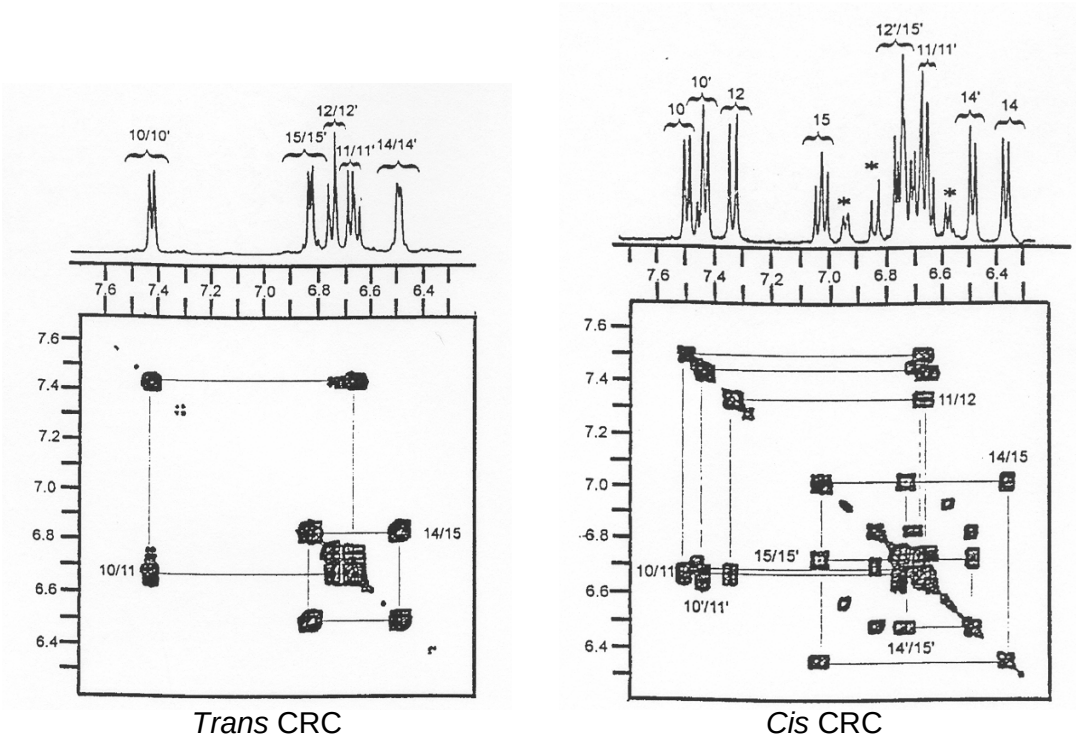


Fig. 7. FT-Raman (top) and 600 MHz ^{1}H 1DNMR and ^{1}H-^{1}H 2D COSY NMR (bottom) spectra of all-*trans* and 13-*cis* digentiobiosyl ester of crocetin.

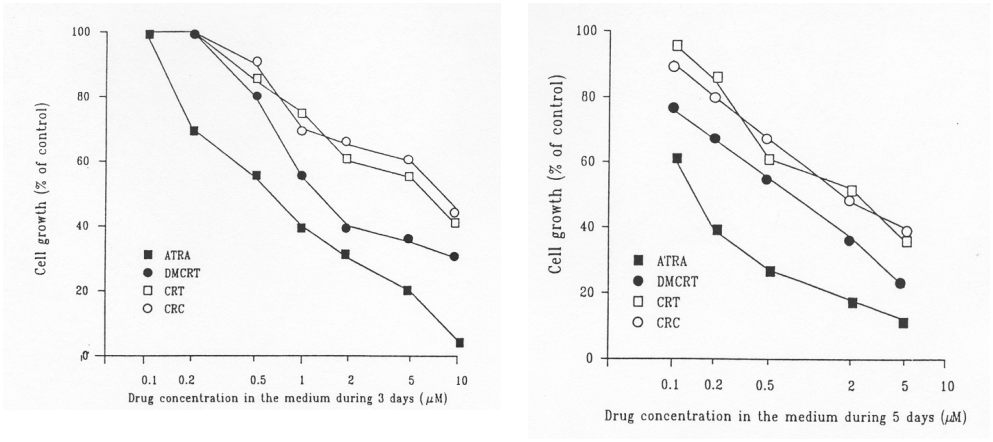


Fig. 8. Differentiation of HL-60 cells by ATRA, DMCRT, CRT and CRCs. Cells are incubated at respectively 2 and 5 μM concentrations of each compounds during 3 (left) and 5 days (right). Percentage of NBT positive cells was estimated in the same way as the number of cells which contain blue-black formazan granules, over 300 cells per each slide preparation. Each point represents the mean of three experiments, and standard deviation represents 10% of each mean.

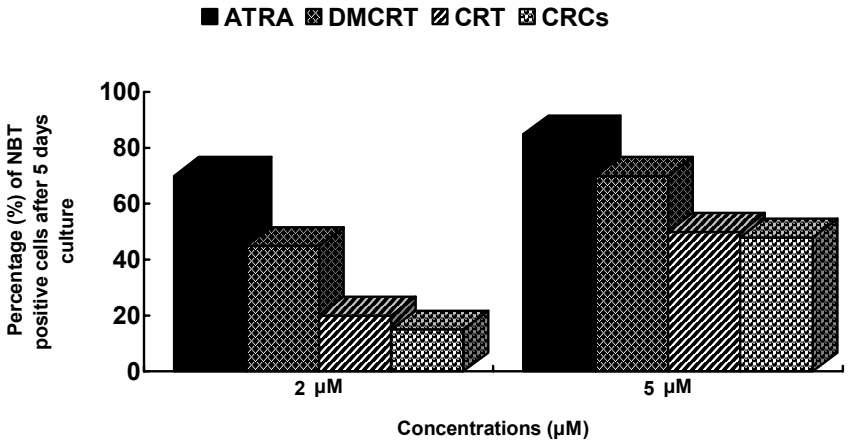


Fig. 9. Differentiation of HL-60 cells by ATRA, DMCRT, CRT and CRCs. Cells are incubated at respectively 2 and 5 μM concentrations of each compounds during 5 days. Percentage of NBT positive cells was estimated in the same way as the number of cells, which contain blue-black formazan granules, over 300 cells per each slide preparation (see materials and methods). Each point represents the mean of three experiments, and standard deviation represents 10% of each mean.

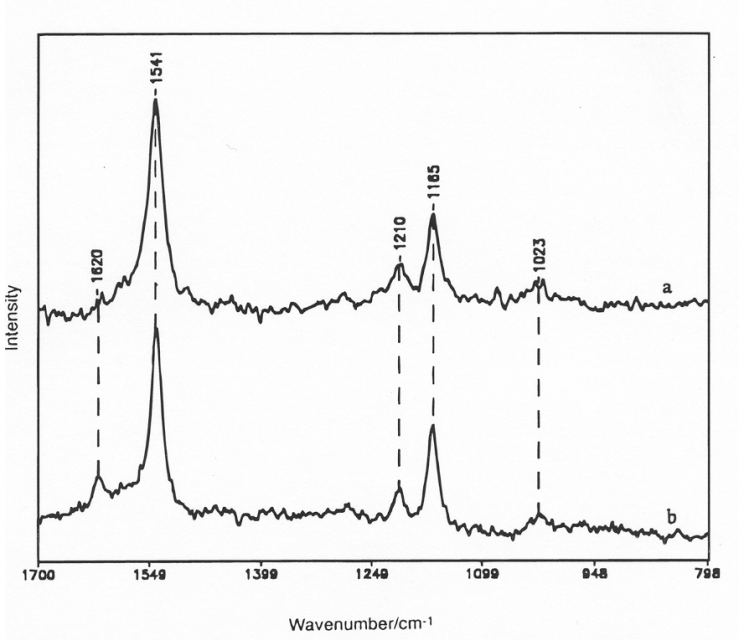


Fig. 10. (a) Resonance Raman and (b) FT-SERS spectra of DMCRT at concentrations of $5x10^{-4}$ and $5x10^{-6}$ M, respectively. Conditions: (a) laser power 200 mW at 457.9 nm, resolution 4 cmÉ1, 100 accumulations and (b) laser power 100 mW at 1064 nm, resolution 4 cm^{-1}, 300 accumulations.

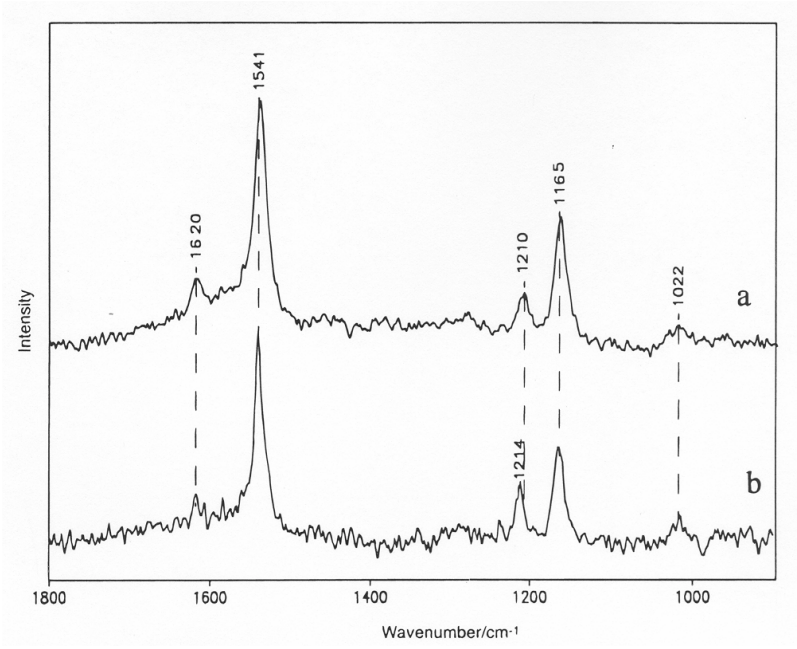


Fig. 11. FT-SERS spectra of (a) free DMCRT and (b) its complex with RAR-c at concentrations of $5x10^{-6}$ M and $1x10^{-5}$ M, respectively. All other conditions were the same as before.

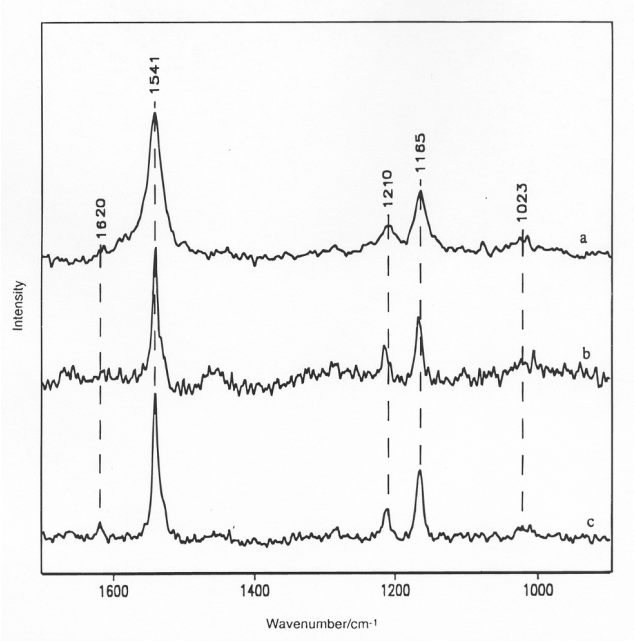


Fig. 12. (a) RR spectrum of DMCRT in solution and FT-Raman spectra of DMCRT in (b) HL60 cells and (c) K562 cells. Experimental conditions: (a) same as before; (b) and (c) laser power 40 mW at 1064 nm, spectral resolution 4 cm^{-1}, 100 accumulations.