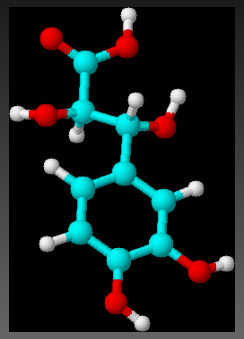


Novel Biologically Active Caffeic Acid-Derived Biopolymer from Different Species of Boraginaceae Family with Potential Therapeutic Effect

Dr. Vakhtang Barbakadze
Head of the Laboratory of Plant Biopolymers

Tbilisi State Medical University
I.Kutateladze Institute of Pharmacochemistry
Tbilisi, Georgia







"Actual isolation and purification of natural products must not be stereotyped; it requires critical spirit, creativity and originality.

In this sense, isolation is an "art" in natural product chemistry"

Y. Tsuda "Isolation of Natural Products", 2004, p.1, Printed by Japan Analytical Industry Co., Ltd.

Symphytum asperum (prickly or rough comfrey)





Symphytum caucasicum (Caucasian comfrey)





Symphytum officinale





Anchusa italica (Italian bugloss)





Introduction

***Symphytum* L. (Comfrey) is a herb already mentioned in ancient literature for its wound-healing properties. Through the ages, *Symphytum* extracts have been used in folk medicine for treatment of different kinds of disorders and wounds due to analgesic, antimicrobial and anti-inflammatory effects.**

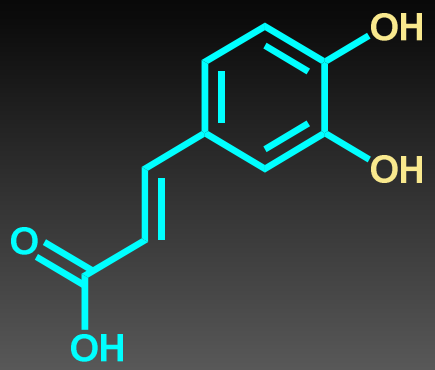
***Anchusa* (Bugloss) extracts also have been used in folk medicine due to anti-ulcer, wound healing and anticancer properties.**

The first representative of a new class of natural phenolic polyethers, namely regular caffeic acid-derived polymer - has been detected in the species of Comfrey - *Symphytum asperum* (SA) *S. caucasicum* (SC), *S. officinale* (SO) and Bugloss (*Anchusa*) – *Anchusa italica* (AI).

Caffeic acid and its derivatives of natural and synthetic origin have antioxidant, anti-inflammatory, hepatoprotective, antimutagenic, anticancer, immunomodulatory, pro-apoptotic activity and inhibitory effect on angiogenesis, tumor invasion, and metastasis. Their radical-scavenging and antioxidative activities are mainly due to the presence of two phenolic hydroxygroups at ortho positions.

It is suggested that antitumor activity of caffeic acid and its derivatives is related to the immunomodulatory properties of the compounds, particularly their capacity to induce apoptosis and necrosis.

The results concerning the structure elucidation of this caffeic acid-derived polymer are presented below.

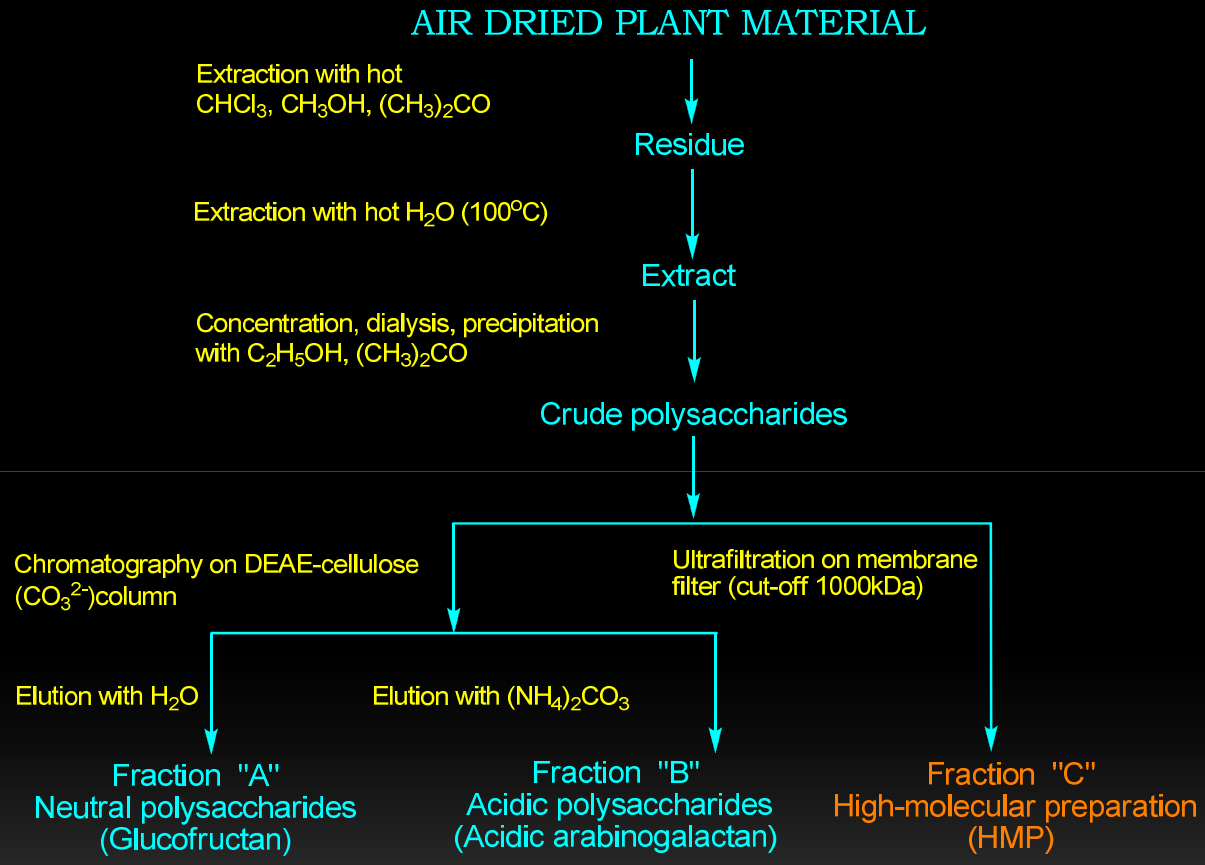


Caffeic acid

History of detection of caffeic acid-derived polymer

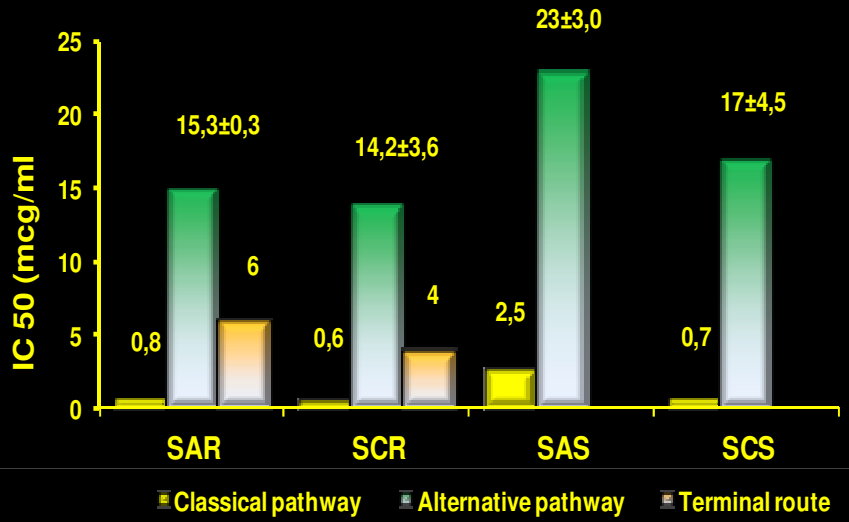
There is interesting history of detection of novel regular caffeic acid-derived polymer in comfrey (*Symphytum*). It is necessary to emphasize that subject matter of our research for a long time was isolation, structure elucidation and investigation of biological activity of polysaccharides from medicinal plants widespread in the Caucasus, in Georgia. According to literary data some polysaccharides show anti-cancer activity due to their immonomodulatory properties. This phenomenon served as the basis for our aim to search immonomodulatory polysaccharides among medicinal plants. The immunomodulatory activities of plant polysaccharide preparations were assessed by testing their effect on functional parameters of humoral and cellular branches of the innate immune system. For the humoral part human complement and for the cellular part human polymorphonuclear leukocytes (PMNs) were selected as relevant immune parameters. Studying the polysaccharide composition of a number of Caucasus flora plants used in folk medicine showed that, unlike the polysaccharides from other plants, the crude polysaccharide preparations from prickly comfrey *S. asperum* and Caucasus comfrey *S. caucasicum* possess a high anti-complementary activity and effectively catch free radicals. However, pure polysaccharides of *S. asperum* and *S. caucasicum* - glucofructan and acidic arabinogalactan had not any anti-complementary and antioxidant activities.

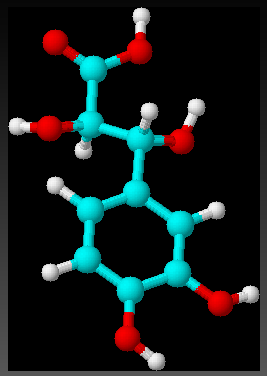
Extraction and fractionation of SA, SC, SO and AI polysaccharides

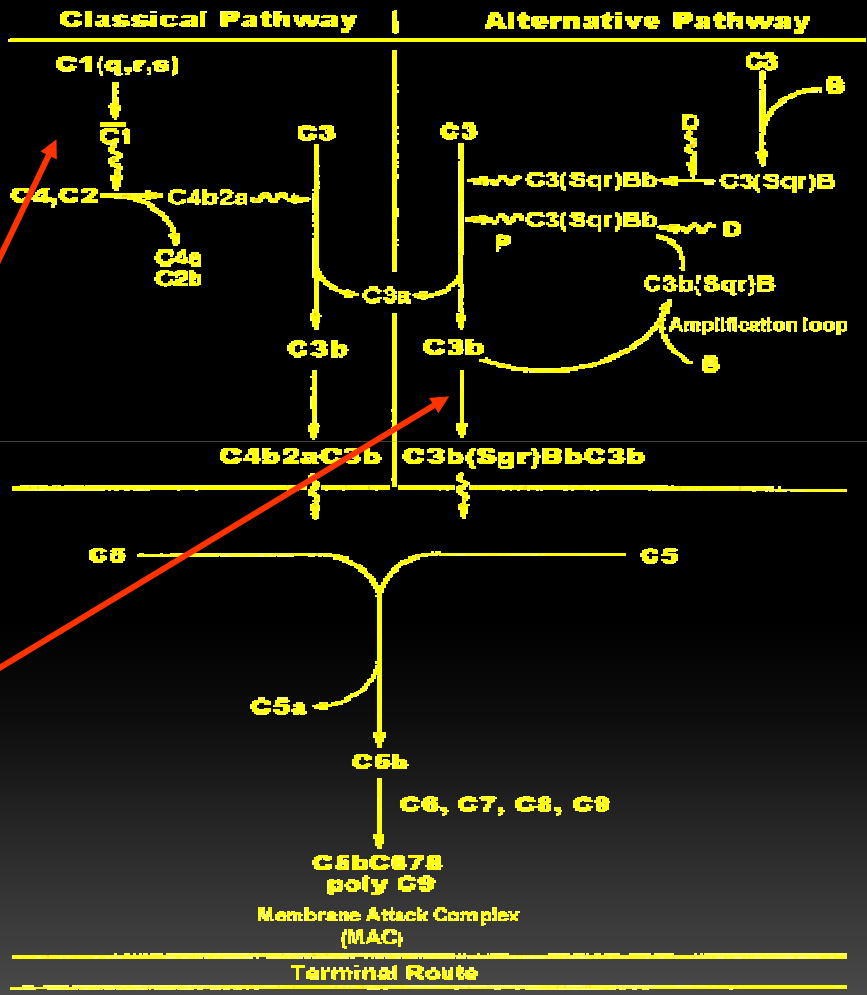


In order to determine the chemical nature of active components, crude polysaccharide preparations were fractionated by ultrafiltration on membrane filters with cut-off values of 10 kDa, 100 kDa and 1000 kDa, which resulted in the retaining of the main anti-complementary activity in the fractions with molecular masses exceeding 1 MDa. The fractionation procedure by ultrafiltration allows to remove most ballast polysaccharides and to obtain water-soluble high-molecular (>1000 kDa) preparations (HMP).

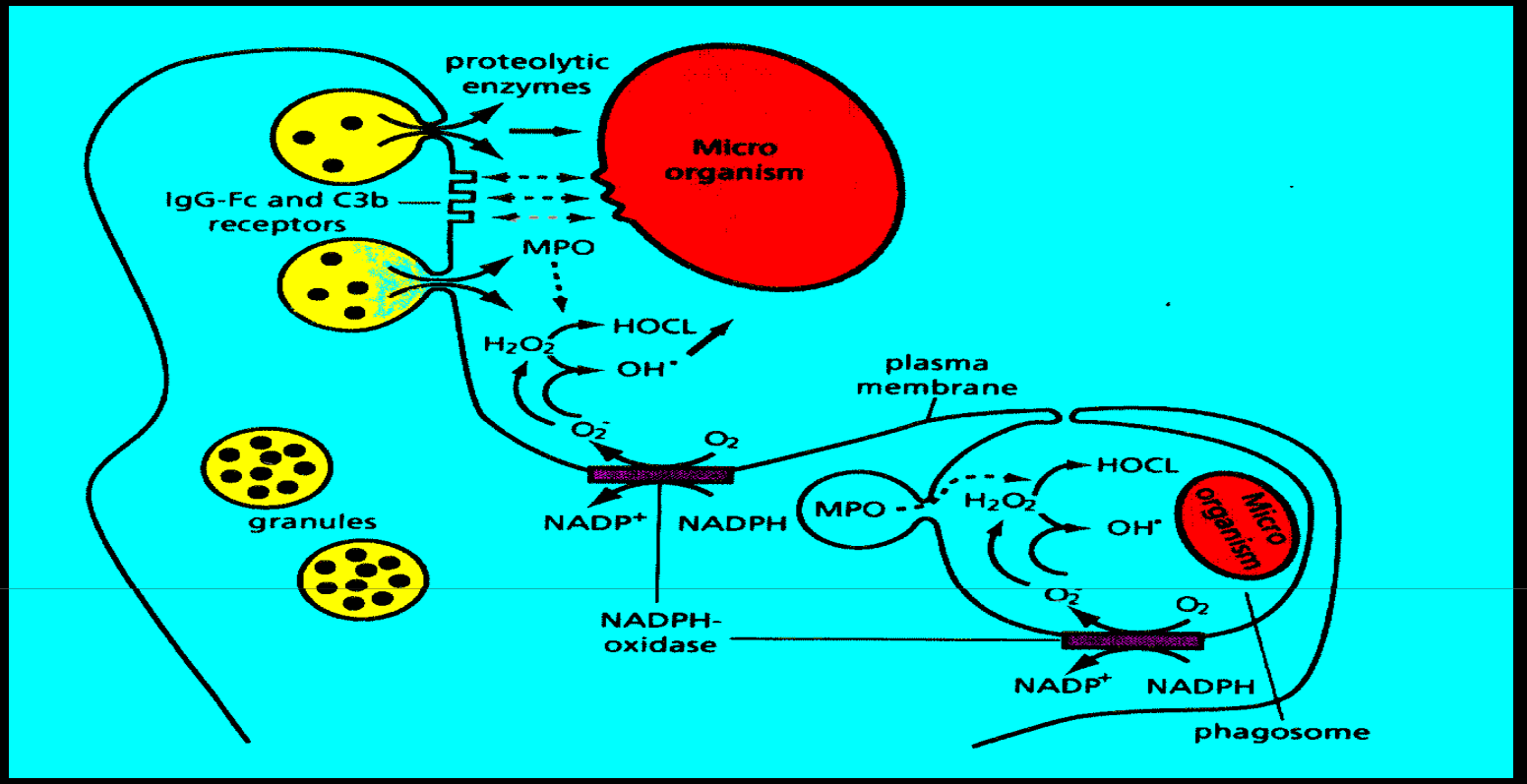
Anticomplementary activity of *Symphytum* polymer





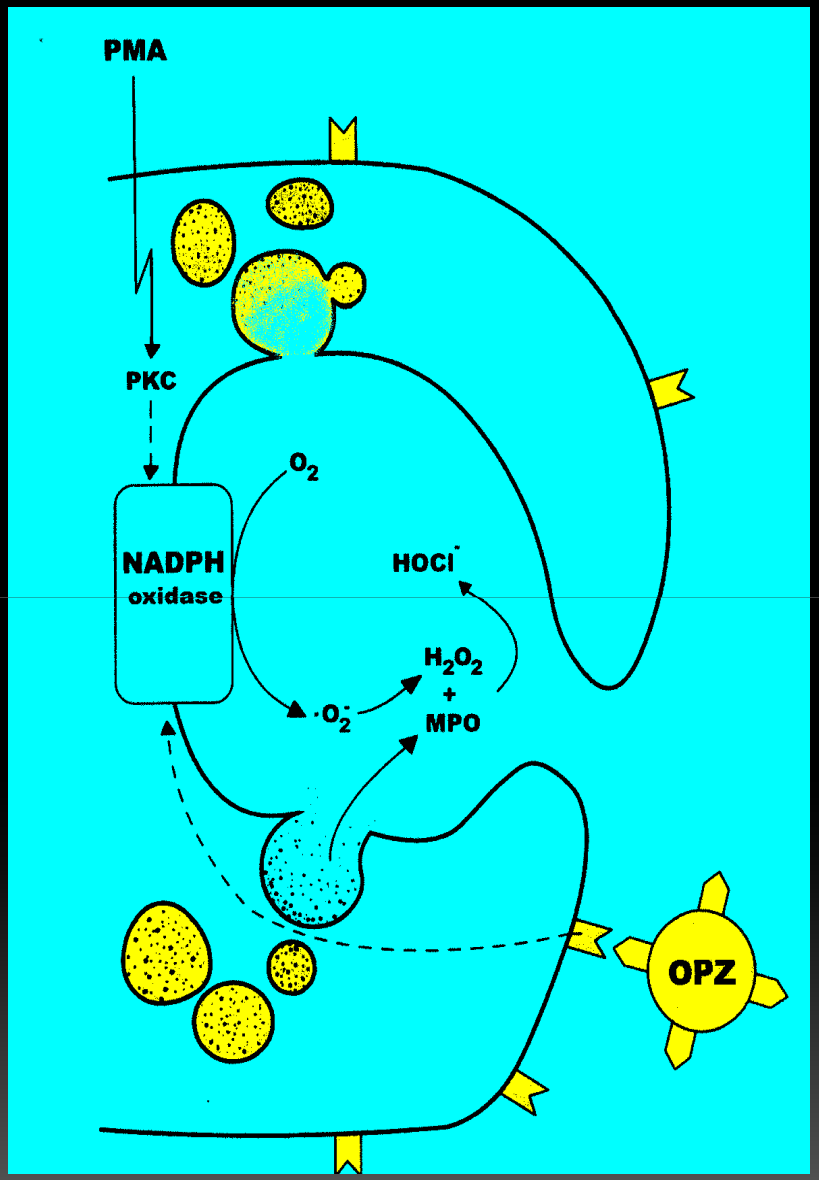


ROS Production in PMNs upon Phagocytosis



Upon phagocytosis, stimulated polymorphonuclear neutrophils (PMNs) produce reactive oxygen species (ROS): superoxide anions which formation is catalyzed by NADPH oxidase ($\cdot O^{2-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot OH$), and hypochlorous acid (HOCI). ROS, produced by stimulated PMNs, play an important role in host defence against invading microorganisms. Upon triggering, PMNs start to consume a large amount of oxygen which is known as the respiratory or oxidative burst. Production of ROS occurs within the cell (phagosome), but also extracellularly, thus causing damage of surrounding tissue. Natural compounds which exhibit anticomplementary activity and/or interfere with ROS production may be useful tools to prevent tissue destruction.

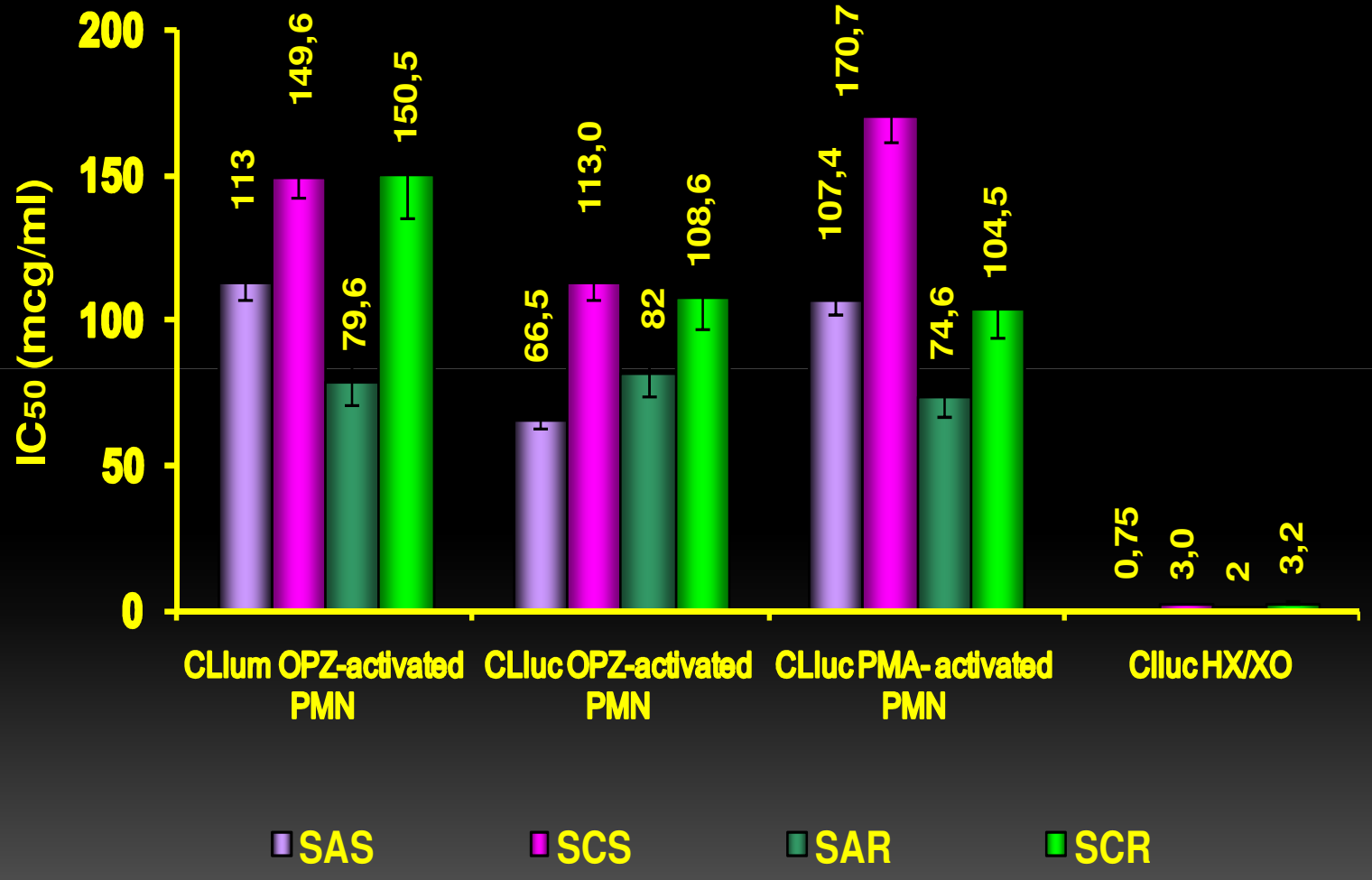
Oxidative Burst of Neutrophils Caused by OPZ or PMA Stimulation



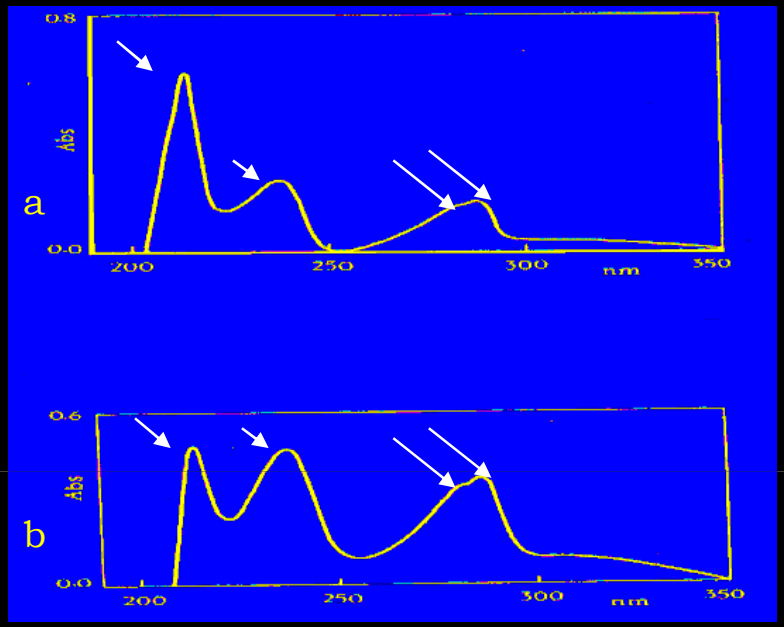
Specificity of the enhancers			
Luminol		Lucigenin	
$\cdot O_2^-$	+ +	$\cdot O_2^-$	+ + +
H_2O_2	+ +	H_2O_2	+ +
$HOCl^-$	+ + +		

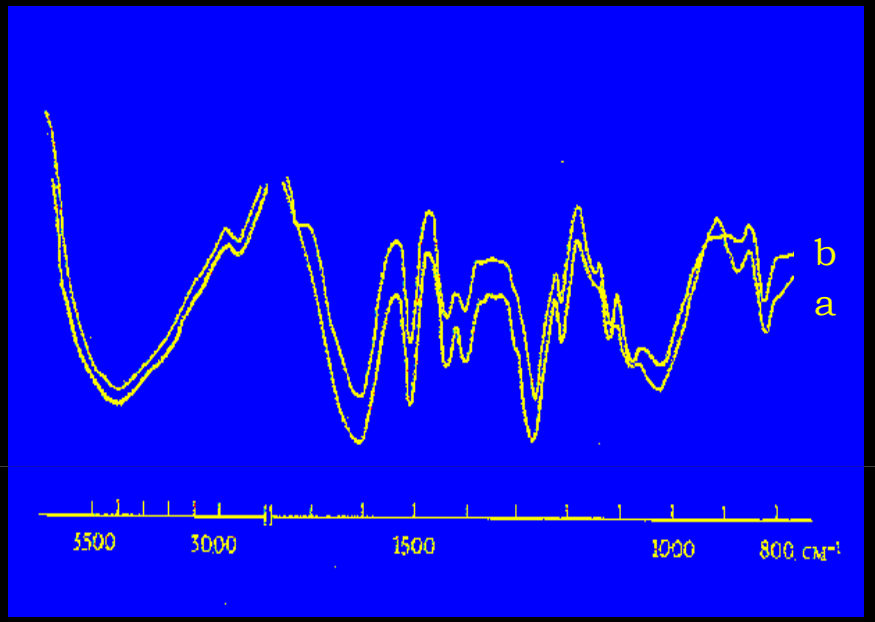
To quantitate the inhibitory effects of the compounds on the generation of ROS after stimulation of PMNs, we used two stimuli which represent different PMN-activation pathways. Opsonized zymosan (OPZ), was used as a model system for opsonized microorganisms. OPZ consists of cell walls of baker's yeast coated with IgG, mannose-binding lectin, and C3b complement fragments. C3b is opsonizing agent. Phagocytes have receptors for C3b. Therefore, covering of microorganisms with C3b will facilitate their recognition and uptake by phagocytes, the most pronounced function of complement activation. Phorbol myristate acetate (PMA) is a soluble agent activating PMNs directly at the level of protein kinase C (PKC) which also leads to the activation of the respiratory burst. Althoough OPZ and PMA both stimulate the superoxide anions-generating NADPH-oxidase, their transductional mechanisms within the neutrophil are quite different. The ability of SAR, SCR, SAS and SCS to inhibit ROS production by human PMNs (mediated either by receptor-dependent OPZ or by receptor-independent PMA) was studied by monitoring the intensity of chemiluminescence enhanced by luminol (CLlum) or lucigenin(CLluc). The use of luminol reveals predominantly hypochlorous acid, while lucigenin is more selective with respect to superoxide anions. Luminol can detect both intra- and extracell ROS production, whereas lucigenin can not penetrate into PMNs and, hence, probes only the extracell space. In order to separate the ROS production and scavenging processes, we performed a control experiment, in which superoxide anions were generated in a cell-free HX/XO system, and measured the corresponding CLluc level.

Antioxidant activities of *Symphytum* polymer



UV (I) and IR (II) spectra of ultrafiltration fractions SA (a) and AI (b) (>1000 kDa)



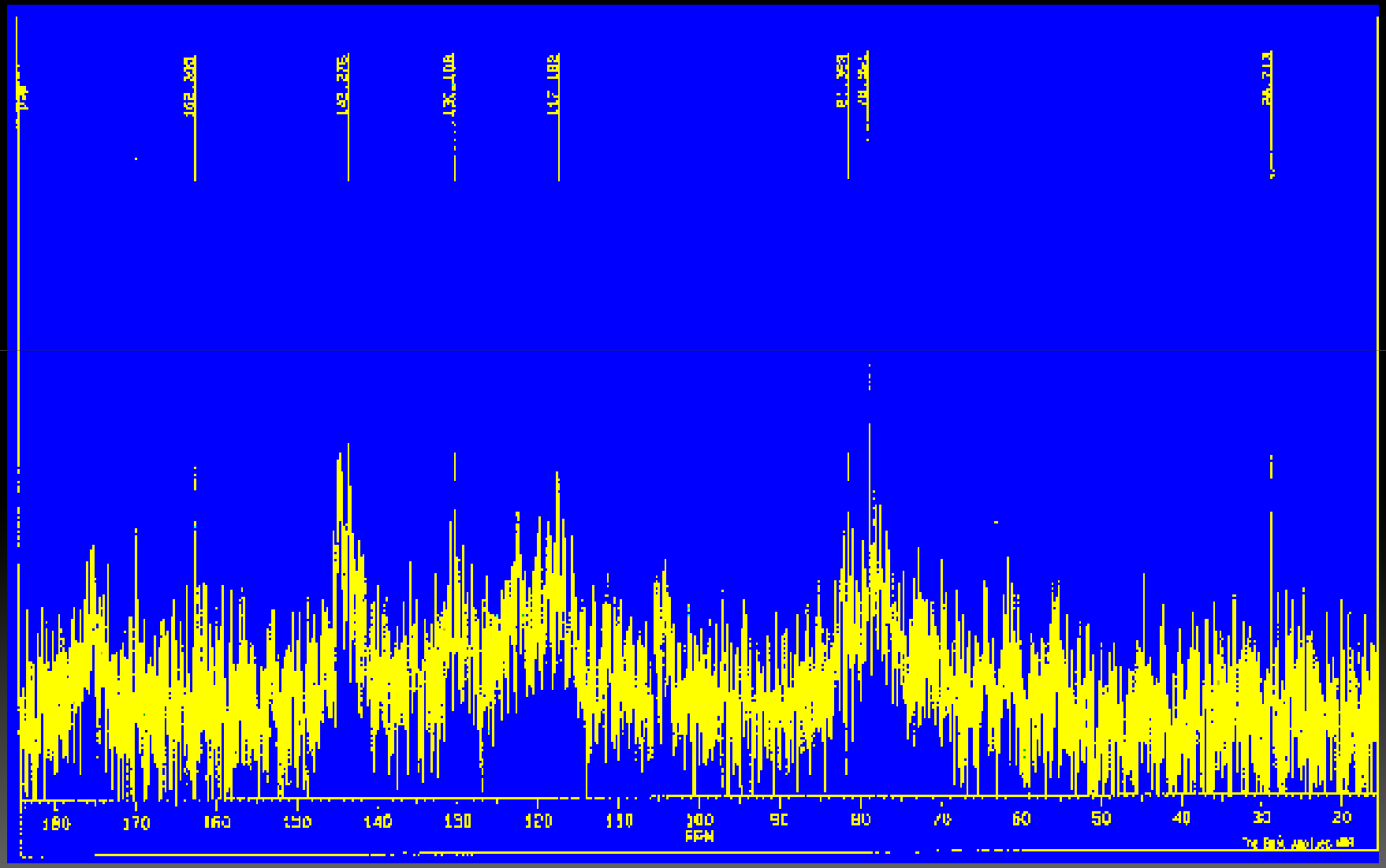


I. The absorption maxima at 213, 237, 282 (shoulder) and 286 nm were observed in the UV spectra of preparations (water) of S.A. (a) and A.I. (b), which could be attributable to substituted phenols.

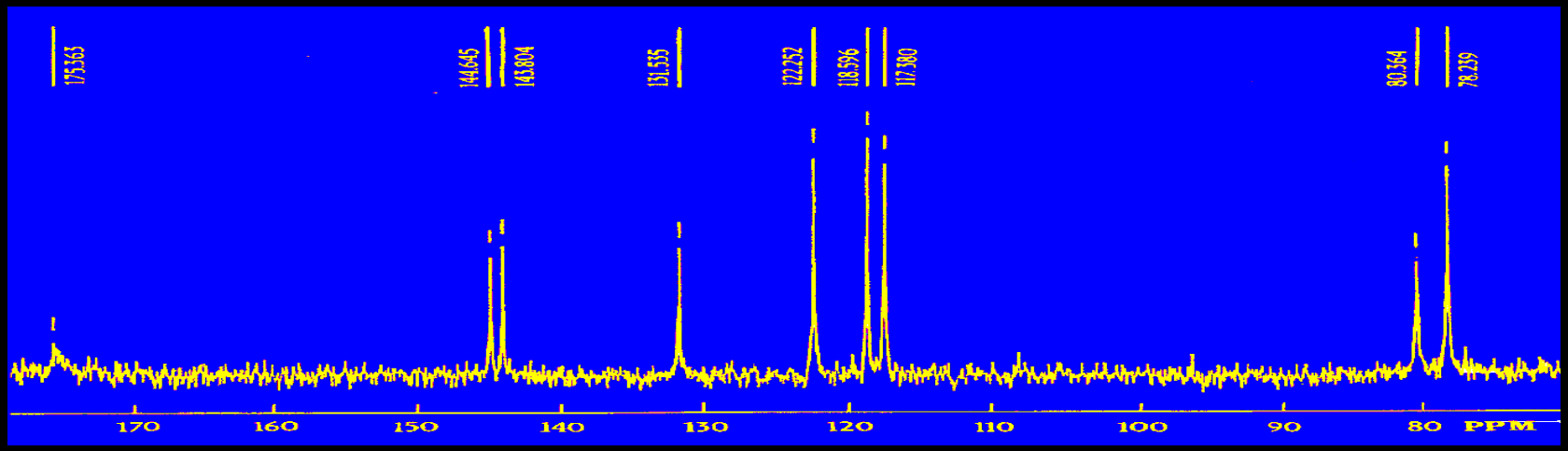
II. The IR spectra of preparations contain absorption bands characteristic of phenol-carboxylic acids: 3400 (OH); 2930 (CH); 1620 (ionized carboxyl) and 1736 cm^{-1} for its ester form (AI); 1600, 1510, and 1450 (aromatic C=C); 1410 and 1220 (phenols); 1270, 1130, 1075 and 1030 (R-O-R'); 880 (C-H in the aromatic ring with one isolated hydrogen atom); and 830 cm-1 (C-H in the aromatic ring with two neighboring hydrogen atoms).

V.Barbakadze et al. Molecules, 2005, V. 10, N 9, P. 1135-1144; V.Barbakadze et al. Chem. Nat. Compds. 2009, V. 45, N 1, P. 6-10.

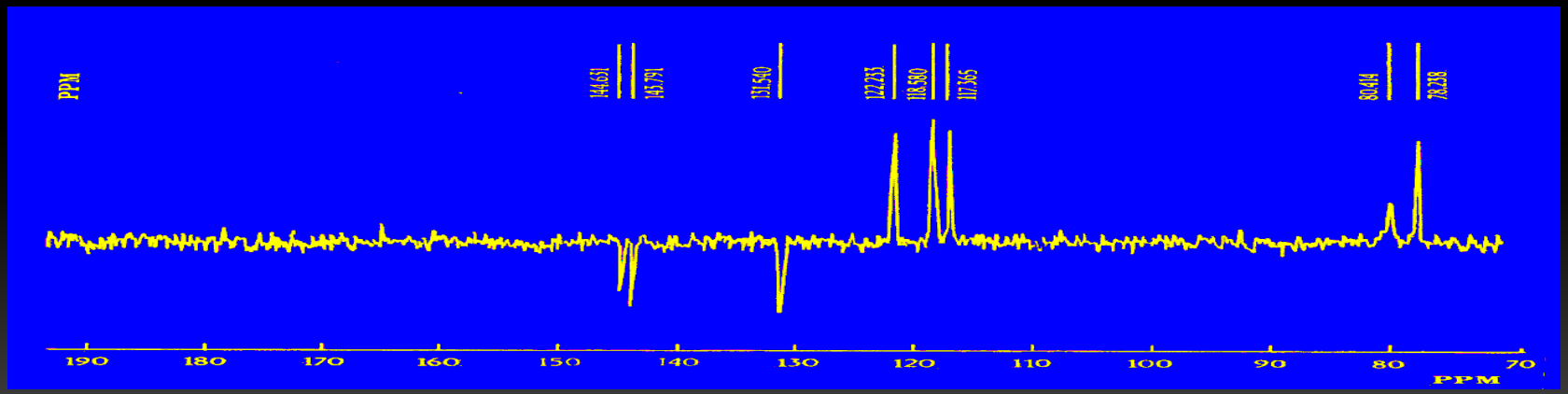
The ^{13}C NMR Initial Spectrum of HMP from SA (at room temperature)



The ^{13}C NMR (a) and APT (b) spectra of HMP from SA, SC, SO (at 80°C)



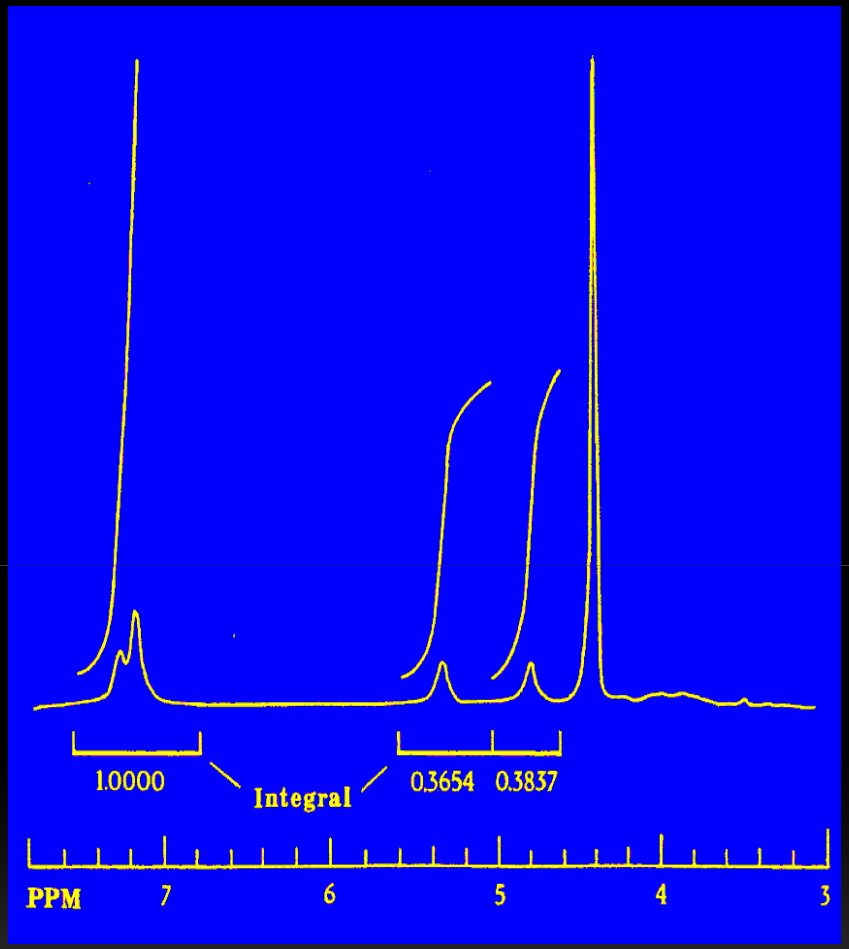
a) Interestingly, the signals of the carbohydrate components are practically unobservable in the spectra of these preparations probably due to their variegated monosaccharide composition. Nine distinct signals corresponding to the carbon atoms of the substituted phenylpropionic acid fragment are observed. A good resolution and the narrow shape of the ^{13}C NMR signals indicate that the compounds under study are regular polymers.

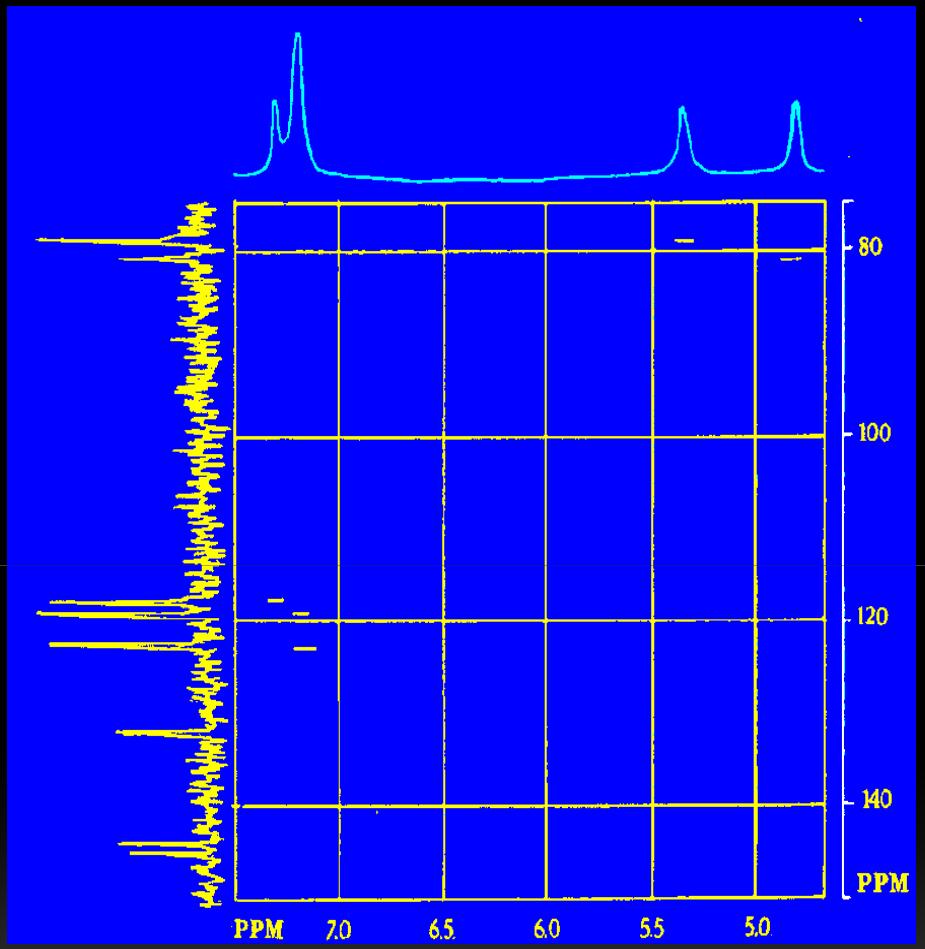


b) From signals observed five should be assigned to CH groups and four signals to the nonprotonated carbon atoms. The two signals with chemical shifts of 78.2 and 80.4 ppm obviously belong to oxygen-bound protonated aliphatic carbon atoms. Six signals were assigned to aromatic carbon atoms (protonated atoms at 117.4, 118.6, and 122.3 ppm and nonprotonated atoms at 131.5, 143.8, and 144.6 ppm). The broadened signal at 175.4 ppm was assigned to the carboxyl group in the compound.

V.Barbakadze et al. Molecules, 2005, V. 10, N 9, P. 1135-1144; V.Barbakadze et al. Chem. Nat. Compds. 2009, V. 45, N 1, P. 6-10.

The ^{1}H NMR (a) and HSQC (b) spectra of HMP- SA, SC, SO





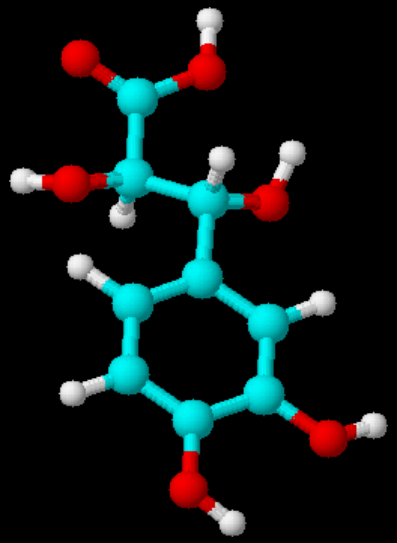
a) The ^{1}H NMR spectrum contains four signals at 4.88, 5.33, 7.13, and 7.24 ppm, one of them (7.13 ppm) with doubled intensity. These signals are broadened, and, therefore, the coupling constants cannot be determined.

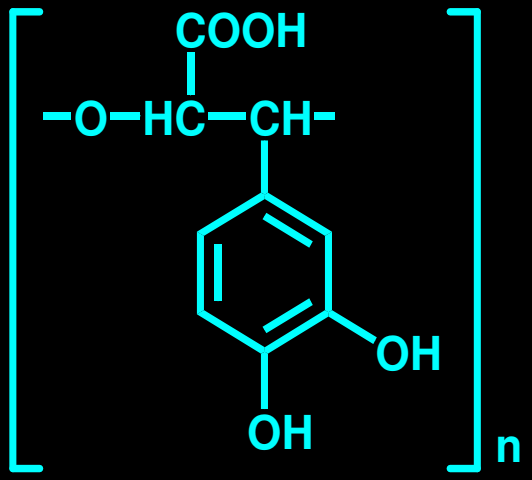
b) The 2D heteronuclear ^{1}H/^{13}C HSQC spectrum exhibits the following correlations between protons and carbon atoms: 4.88/80.4, 5.33/78.2, 7.13/118.6, 7.13/122.3, and 7.24/117.4 ppm.

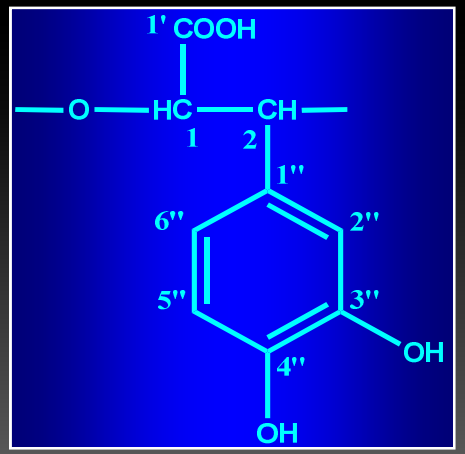
V.Barbakadze et al. Molecules, 2005, V. 10, N 9, P. 1135-1144; V.Barbakadze et al. Chem. Nat. Compds. 2009, V. 45, N 1, P. 6-10.

Assignments of signals in the ^{13}C and ^{1}H NMR spectra of HMP from SA, SC and SO

Atom No	Chemical shift ^{13}C, δ ppm	Chemical shift ^{1}H, δ ppm
1′	175.4	
1	78.2	5.33
2	80.4	4.88
1″	131.5	
2″	117.4	7.24
3″	144.7	
4″	143.8	
5″	118.6	7.13
6″	122.3	7.13





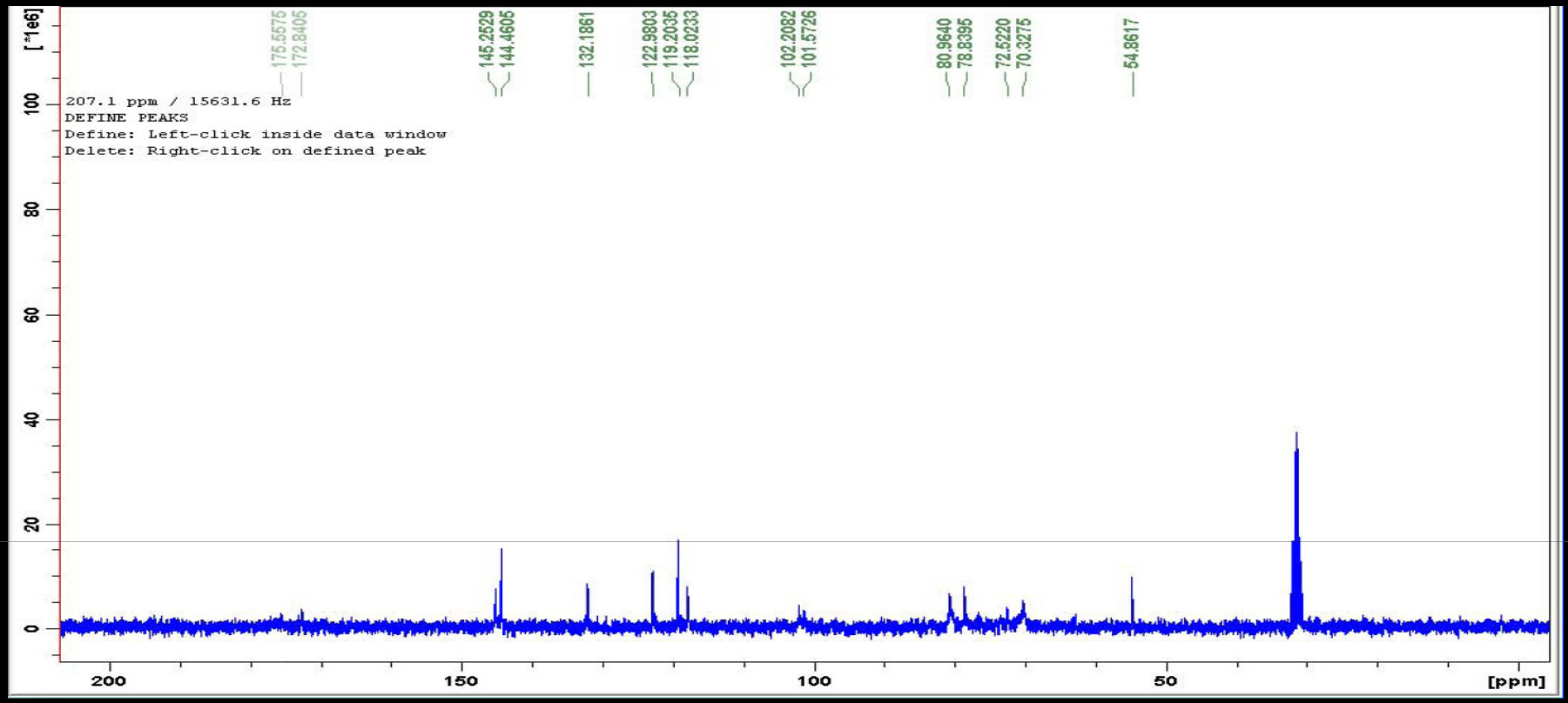


Thus, according to different techniques of NMR spectroscopy the polyoxyethylene chain is the backbone of the polymer molecule. 3,4-Dihydroxyphenyl and carboxyl groups are regular substituents at two carbon atoms in the chain. The repeating unit of this regular polymer is 3-(3,4-dihydroxyphenyl)glyceric acid residue. This compound is a representative of a new class of natural polyethers. Such biopolymer has not been known and has been identified for the first time.

CAFFEIC ACID-DERIVED POLYMER;
POLY[3-(3,4-DIHYDROXYPHENYL)GLYCERIC ACID]
(p-DGA);
POLY[OXY-1-CARBOXY-2-(3,4-DIHYDROXYPHENYL)ETHYLENE]

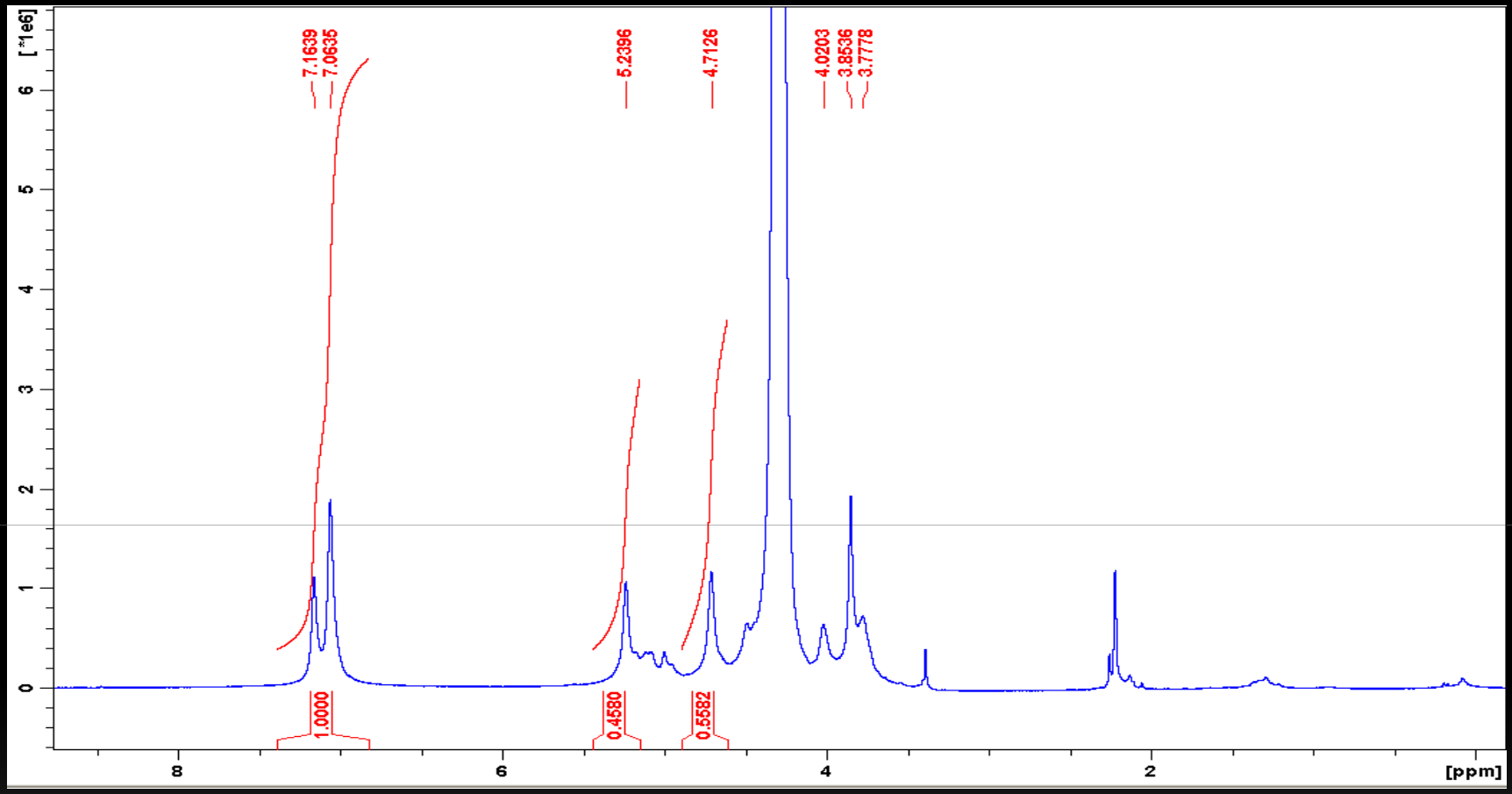
V.Barbakadze et al. Molecules, 2005, V. 10, N 9, P. 1135-1144; V.Barbakadze et al. Chem. Nat. Compds. 2009, V. 45, N 1, P. 6-10.

The ^{13}C NMR spectrum of HMP-AI



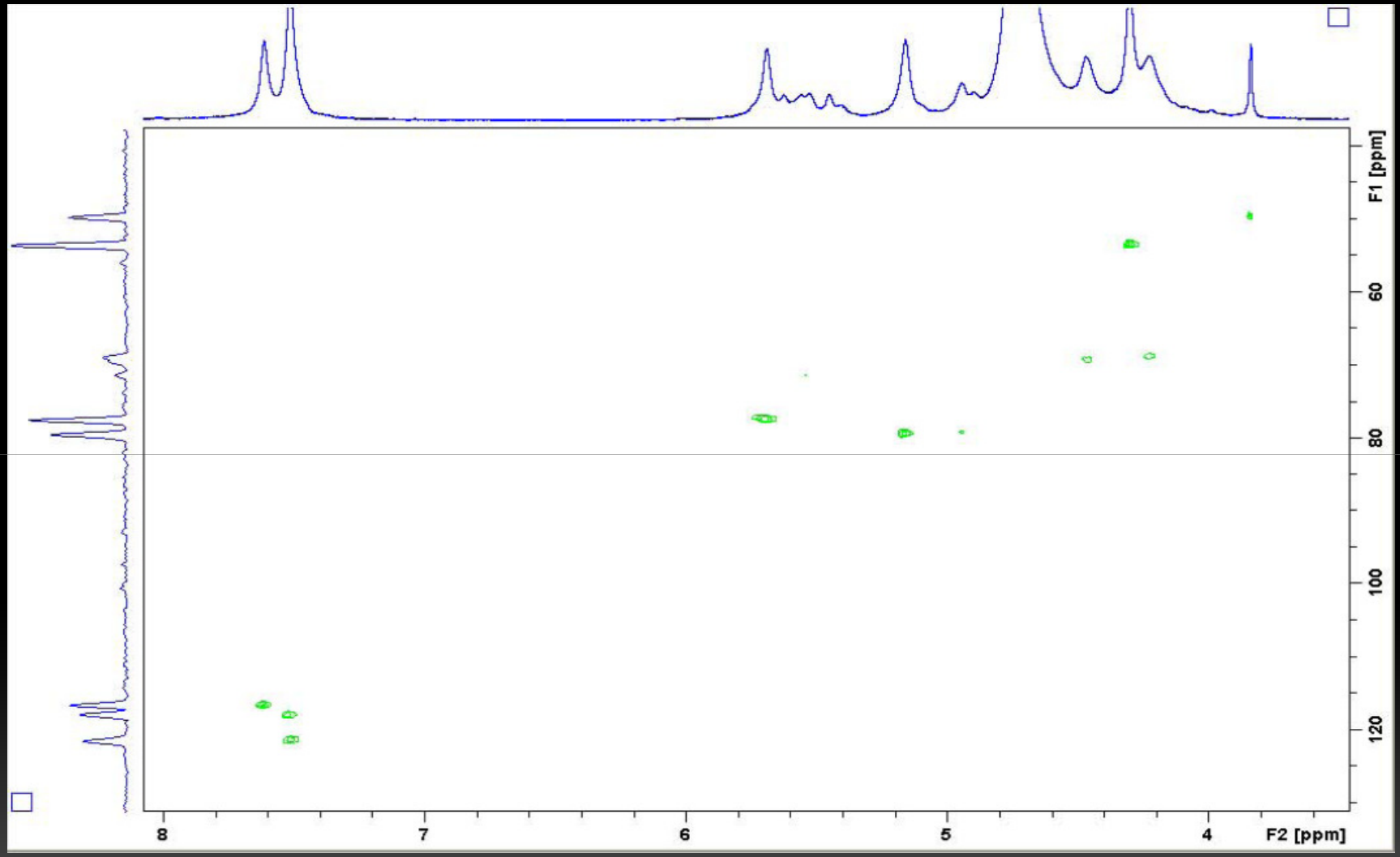
The two signals with chemical shifts of 78.84 and 80.96 ppm obviously belong to oxygen-bound protonated aliphatic carbon atoms. Six signals were assigned to aromatic carbon atoms (protonated atoms 118.02, 119.20 and 122.98 ppm and nonprotonated atoms at 132.19, 144.46, and 145.25 ppm). Then, two non-sharp signals (172.84 and 175.56 ppm) were thought to be due to two carboxyl groups. A resonance in the ^{13}C NMR spectrum at 54.86 ppm, which correlated with the ^{1}H resonance at 3.85 ppm, suggested the presence of methoxy groups in carboxylic acid methyl esters. With this, the signal at 175.56 ppm was attributed to a carboxylic acid group and the signal at 172.84 ppm was assigned to carboxyl groups in methyl ester-form (upfield shifted). About 70 % of the present carboxyl groups were methyl esterified (MeO: ^{13}C, 54.86 ppm; ^{1}H, 3.85 ppm).

The ^{1}H NMR spectrum of HMP-AI



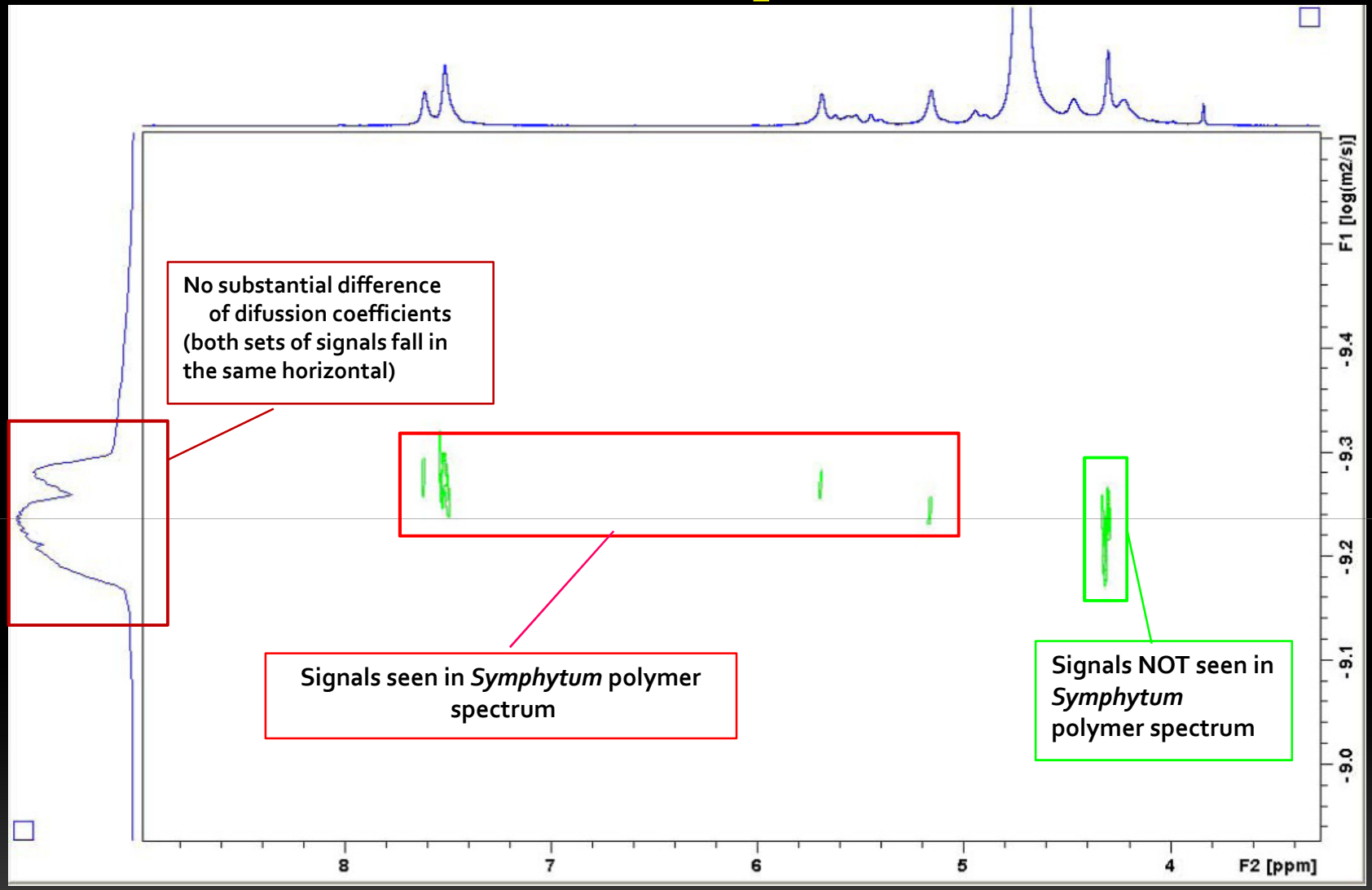
The ^{1}H NMR spectrum of HMP-AI contains five signals at 3.85, 4.71, 5.24, 7.06, and 7.16 ppm, one of them (7.06 ppm) with doubled intensity. These signals are broadened, and, therefore, the coupling constants cannot be determined.

The 2D heteronuclear $^{1}H/^{13}C$ HSQC spectrum of HMP-AI



The following correlations between protons and carbon atoms: 3.85/54.86, 4.71/80.4, 5.24/78.84, 7.06/119.2, 7.06/122.98, and 7.16/118.02 ppm are detected.

The 2D DOSY experiment



The similar diffusion coefficient for the methylated and non-methylated signals of HMP-AI is observed. Both sets of signals fell in the same horizontal. This would imply a similar molecular weight for methylated and non-methylated polymers.

V.Barbakadze et al. Nat. Prod. Commun., 2010, V. 5, N 7, P.1091-1095.

Assignments of signals in the ^{13}C and ^{1}H NMR spectra of HMP-AI (δ, ppm)

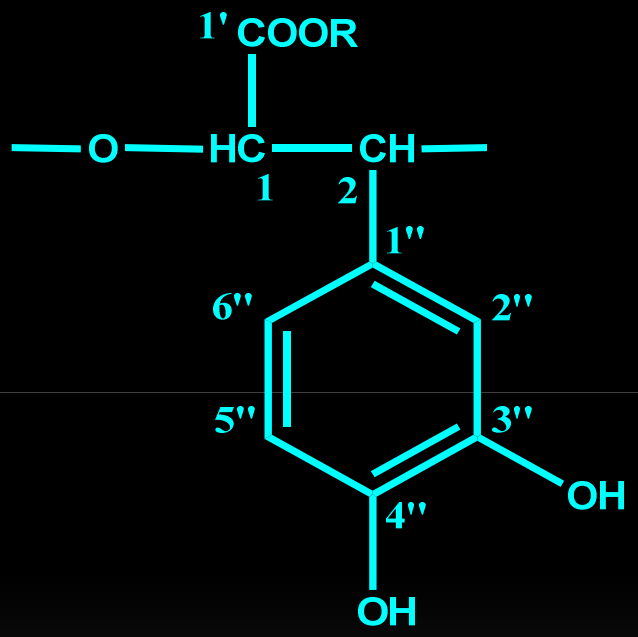
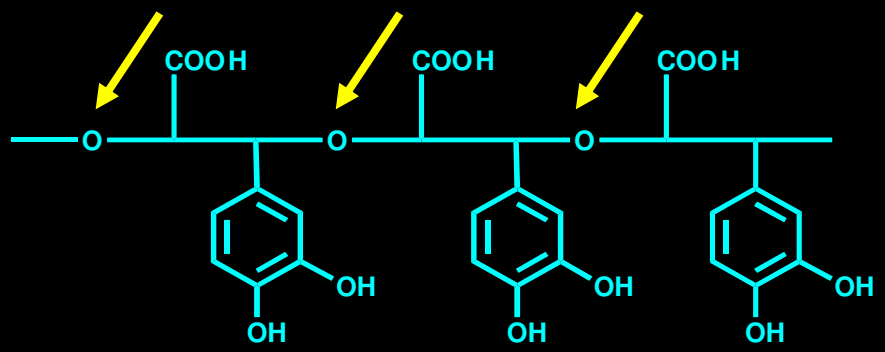


Fig. The repeating unit of HMP-AI; R=H, CH_3.

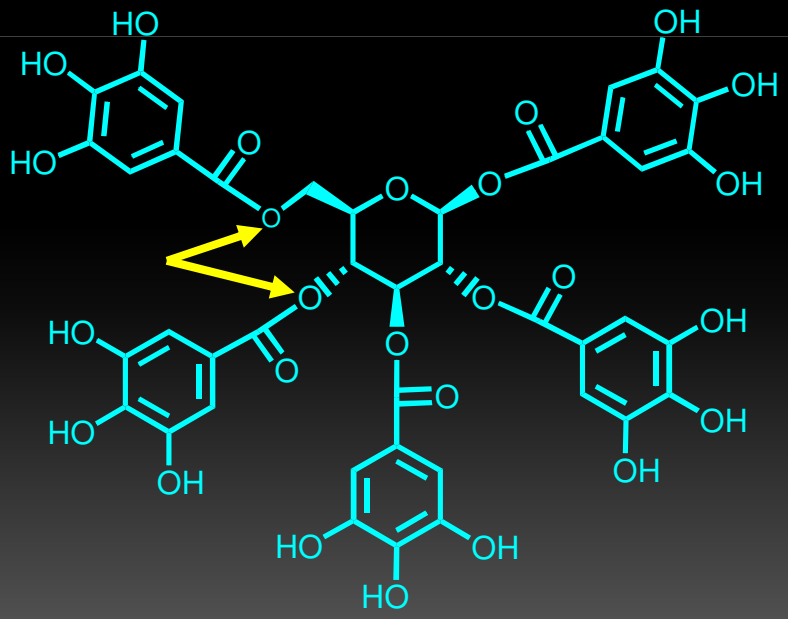
C atom no.	^{13}C chemical shift	^{1}H chemical shift
1'	175.56 (COOH) 172.84 ($COOCH_3$) 54.86 (OCH_3)	 3.85 (OCH_3)
1	78.84	5.24
2	80.96	4.71
1''	132.19	
2''	118.02	7.16
3''	145.25	
4''	144.46	
5''	119.20	7.06
6''	122.98	7.06

Most of the carboxylic groups (70%) of HMP-AI unlike the HMP SA, SC, SO are methylated (MeO: ^{13}C, δ 54.86 ppm; ^{1}H, δ 3.85 ppm). The extent of methyl esterification was calculated by comparing the integral intensity of the methyl ester signal (3.85 ppm, 0.5 H) to that of the aliphatic proton signal at H1 (5.24 ppm, 0.7 H) in a ^{1}H NMR experiment.

V.Barbakadze et al. Nat. Prod. Commun., 2010, V. 5, N 7, P.1091-1095.



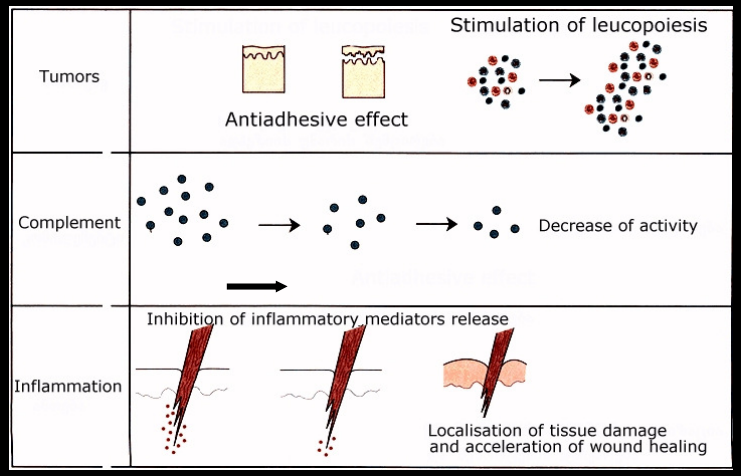
Caffeic acid-derived polyether



Pentagalloyl glucose (constituent of tannic acid)

An advantage of this new polymer is the low susceptibility to hydrolysis and, hence, high stability, which is related to the presence of only ether bonds in the backbone structure thus being a much more stable compound than for example tannic acid that is composed of ester-linked glucose and gallic acid moieties.

Established effects of caffeic acid-derived polymer



- Abrogation of the adhesion of melanoma cells to tumor-conditioned medium- and VEGF-activated endothelial cells.

V.Barbakadze et al. Bull. Georg. Natl. Acad. Sci. 2008, V. 2, N 3, P. 108-112.

- Haematopoietic efficacy of polymer: in mice drug-induced leukopenia the polymer caused significant stimulation of leucopoiesis.

M. Moistsrafishvili, et al. Investigation of Georgian biologically active compounds of plant and mineral origin. Tbilisi, 2010, Issue 2(17) p.91-93.

- Increases spontaneous *in vitro* apoptosis of B-chronic lymphocytic leukaemia cells.

L. Kardava et al. Bull. Georg. Natl. Acad. Sci. 2000, V. 162, N 4, P. 47-50.

- Antioxidant activity and anticomplementary activity due to the inhibition of xantine oxidase and complement convertase, respectively .

V.Barbakadze et al. Pharmaceutical Chemistry J. 2007, V.41, N 1, P. 14-16.

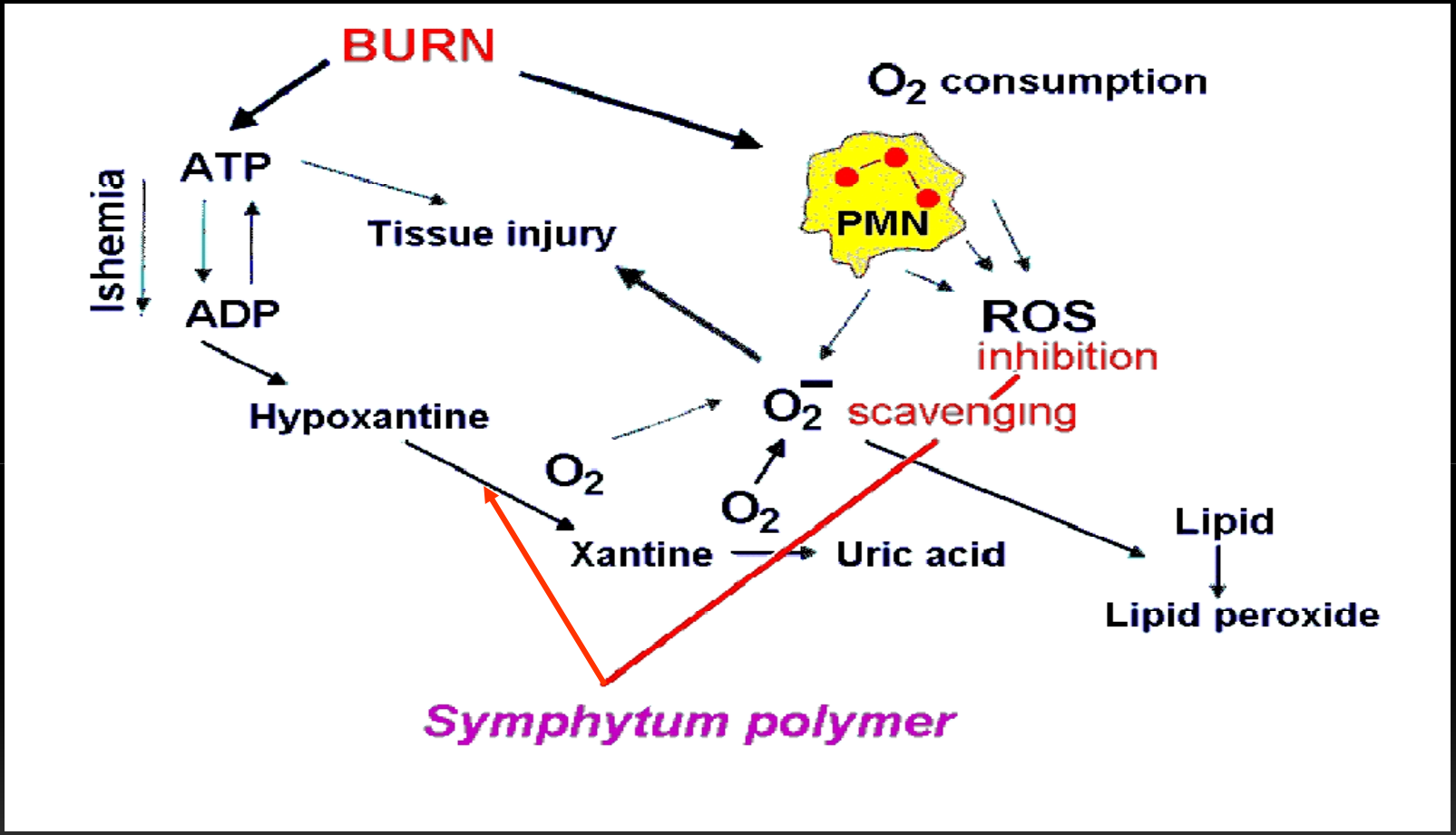
- Burn and wound healing effect due to the shortening of the second phase of wound healing - the inflammatory response.

K.Mulkijanyan et al. Bull. Georg. Natl. Acad. Sci. 2009, V. 3, N 3, P. 114-117.

- The strong efficacy against prostate cancer cells suggesting their high potential in prostate cancer patients.

S. Shrotriya et al. Carcinogenesis, 2012, 33(8), 1572-1580.

Generation of ROS in injured tissue and their scavenging by *Symphytum* polymer



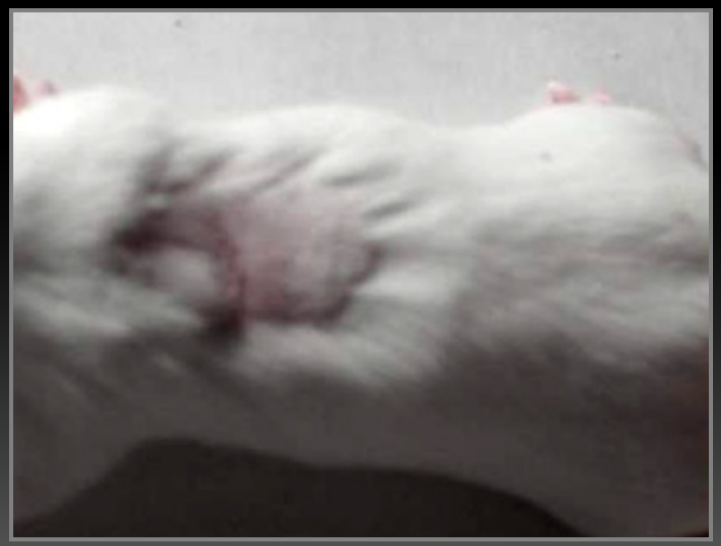
Besides generation of superoxide anions by stimulated PMNs, these radicals may also arise in chronic wounds where ischemic conditions may convert the enzyme xanthine dehydrogenase into xanthine oxidase (XO) which catalyses the conversion of oxygen into superoxide anions causing tissue damage. During this process XO converts hypoxanthine (HX) to xanthine and subsequently to uric acid. So, scavenging of superoxide anions either produced by PMNs or through XO is regarded beneficial for wound healing and in inflammatory process.

Wound healing effect of *Symphytum* polymer



Day 4





Day 10

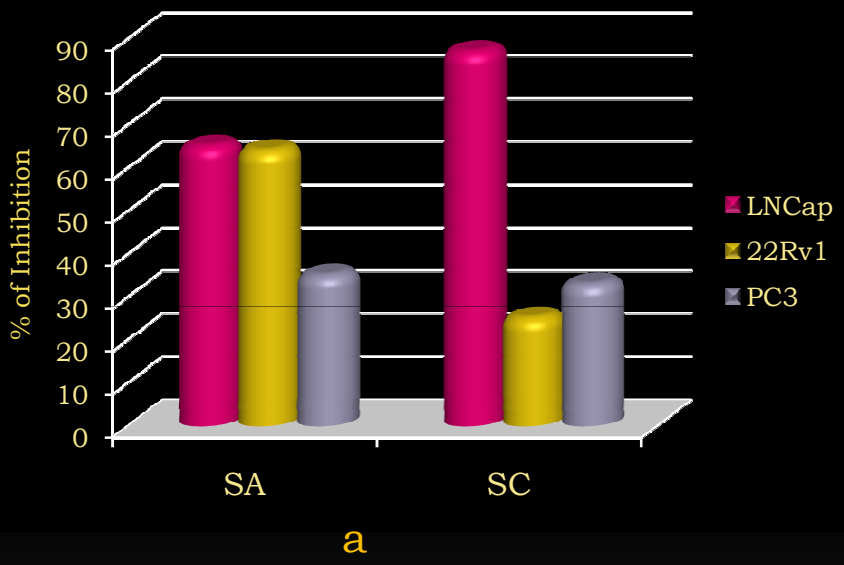


Symphytum Polymer's 1% ointment

Control (vehicle)

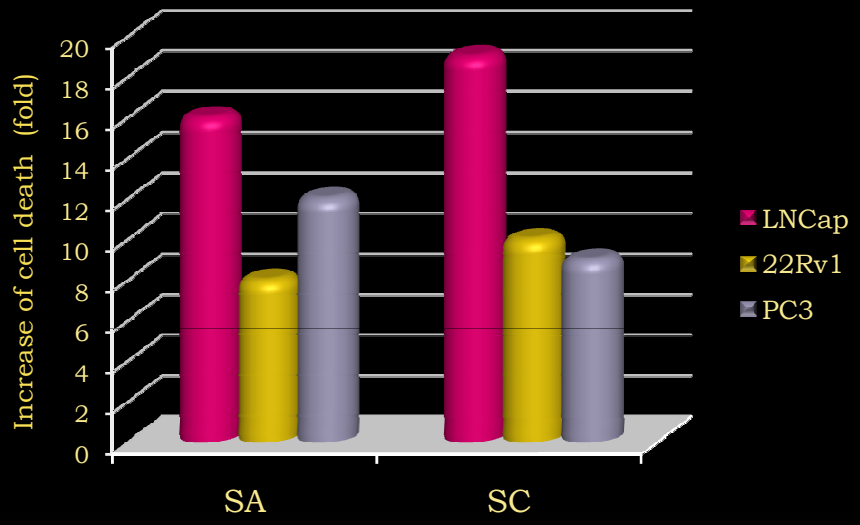
In vitro anti-cancer efficacy of novel phenolic polymers from *Symphytum asperum* (SA) and *S.caucasicum* (SC)

Effects of SA and SC (100 mcg/ml) on PCa cells growth after 48 hours



a

Effects of SA and SC (100 mcg/ml) on PCa cells death after 48 hours



b

In androgen-dependent (LNCaP) and -independent (22Rv1 and PC3) human prostate cancer (PCa) cells SA treatment (100 mcg/ml for 48h) decreases the live cell number by 65, 64 and 35% (a) and increases the cell death by 16, 8 and 12 folds (b) in LNCaP, 22Rv1 and PC3 cells, respectively. Similarly, SC treatment (100 mcg/ml for 48h) decreased the live cell number by 87, 25 and 33% and increased the cell death by 19, 10 and 9 folds in LNCaP, 22Rv1 and PC3 cells, respectively.

S. Shrotriya et al. American Association for Cancer Research 100th Annual Meeting, Denver, Colorado, USA. Abstracts. 2009, N 921.

Fig. 1. Poly[3-(3,4-dihydroxyphenyl)glyceric acid] (p-DGA) and m-DGA selectively inhibit growth and induce death in human prostate cancer (PCA) cells. (A) The chemical structure of p-DGA and m-DGA. (B-D) PCA androgen-independent 22Rv1, androgen-dependent LNCaP cells and immortalized non-neoplastic prostate epithelial PWR-1E cells were treated with vehicle (sterile DI water) or two different concentrations of m-DGA or p-DGA (50 and 100 µg/mL) for 24 and 48 h. Afterwards, cells were collected and total cell number (viable plus dead cells) as well as dead cell population were determined by trypan blue exclusion assay. The data are presented as mean (n=3) ± standard error of mean (SEM) and represent at least three independent experiments. *, P<0.001; $, P<0.05. *S.Shrotriya et al., Carcinogenesis, 2012, 33(8), 1572-1580.*

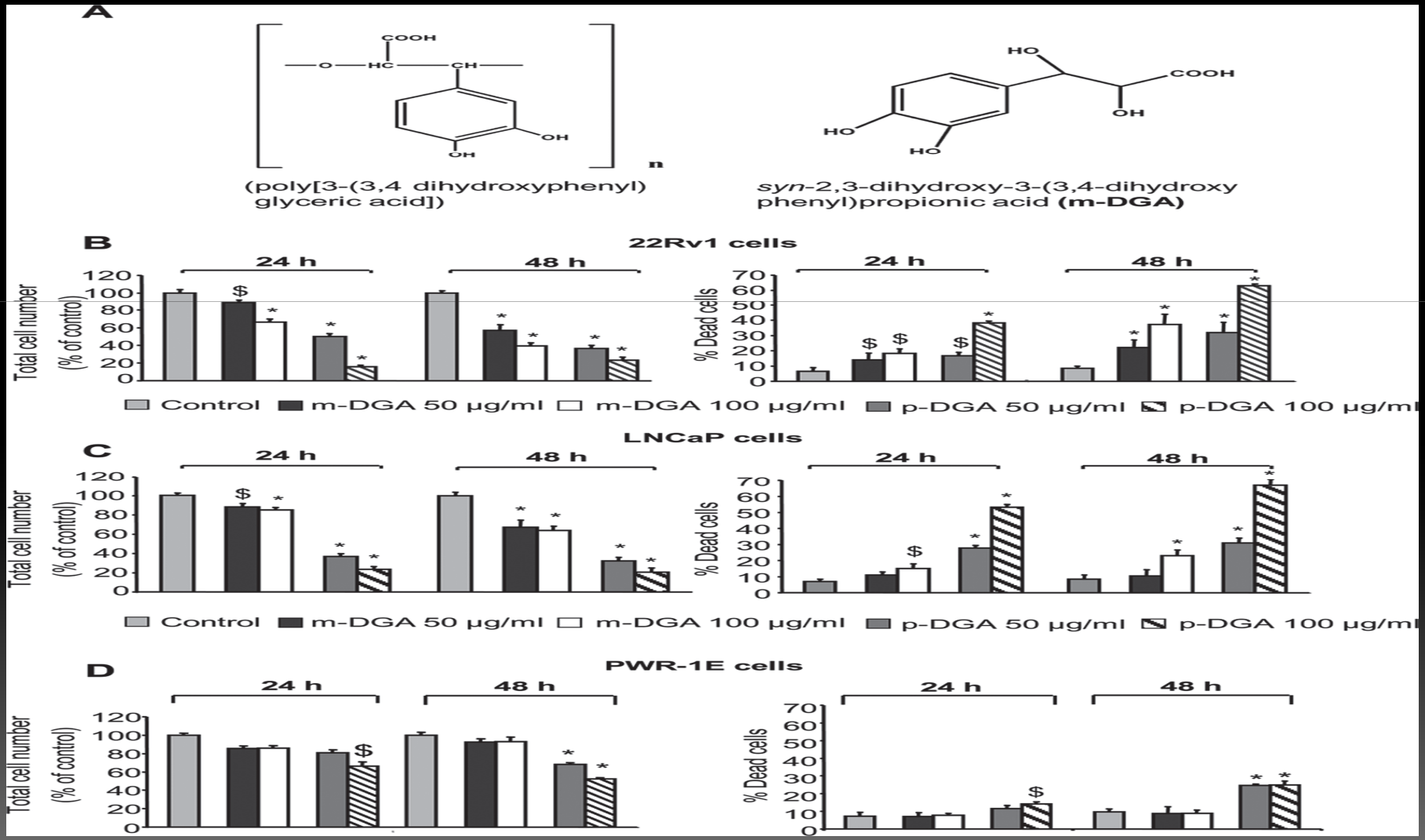
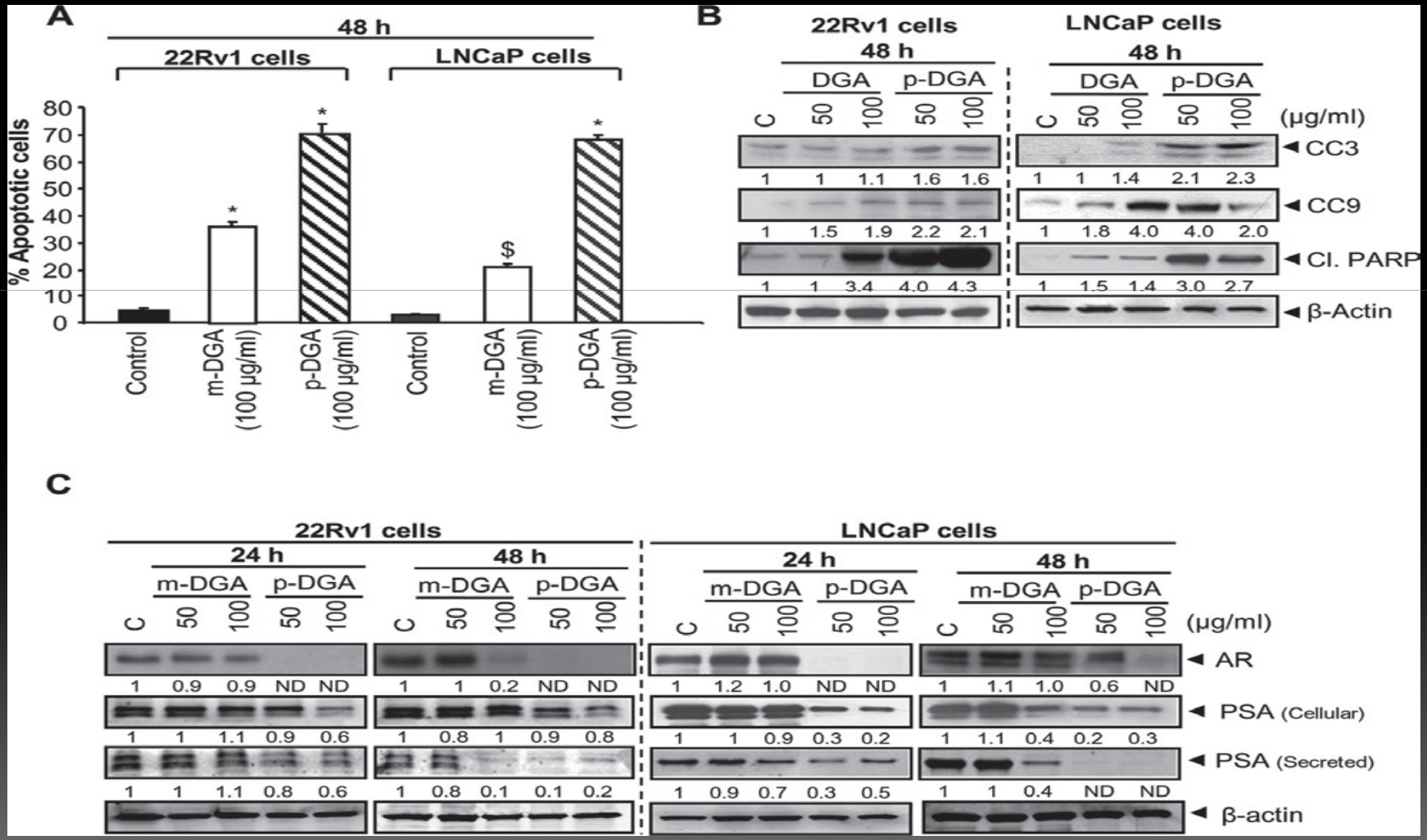
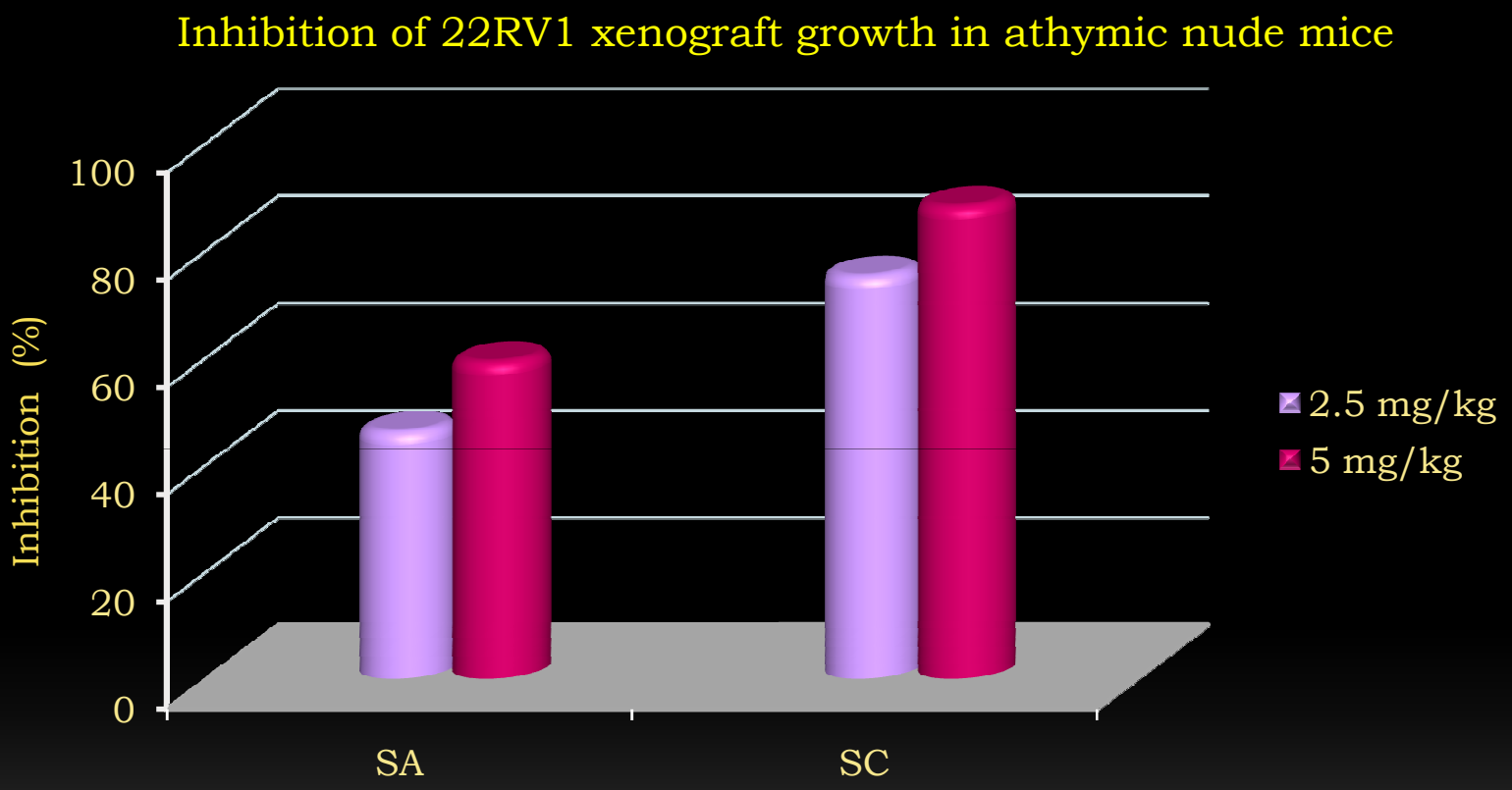


Fig. 3. Effect of p-DGA and m-DGA on apoptosis and AR in human PCA cells. Human PCA 22Rv1 and LNCaP cells were treated with vehicle or m-DGA or p-DGA (50 and 100 µg/mL) for 24 and 48 h. (A) After 48h of treatment, both adherent and non-adherent cells were collected, stained with annexin V/PI and analyzed by flow cytometry for the apoptotic cell population. The data are presented as mean (n=3) ± SEM and represents two independent experiments. *, P<0.001; $, P<0.05. (B) Whole cell lysate were prepared after treating 22Rv1 and LNCaP cells with m-DGA or p-DGA for 48 h and used to analyze the protein expression of cleaved caspase 3 (CC3), cleaved caspase 9 (CC9), and cleaved PARP (Cl. PARP) by western blotting. (C) Western blotting was performed for AR and PSA; and membranes were re-probed with β-actin to check equal protein loading. For the secreted PSA expression, media was collected and analyzed for PSA expression by immunoblotting. In each case, the media loading volume was normalized with the respective protein value of the cell lysate. The densitometry data presented below the bands are "fold change" as compared to control after normalization with respective loading control (β-actin). ND: Not detectible.



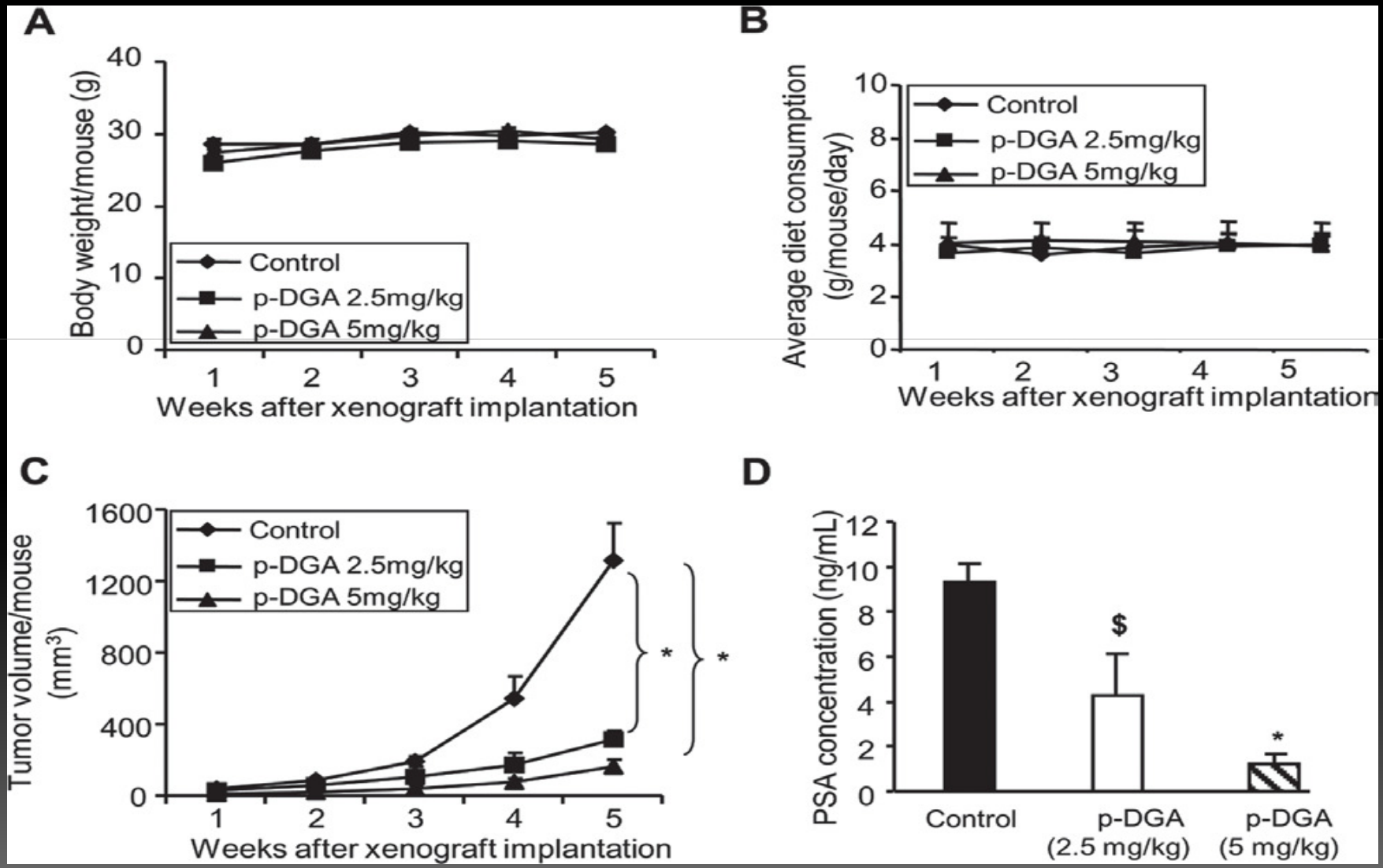
In vivo anti-cancer efficacy of novel phenolic polymers from Symphytum asperum (SA) and S.caucasicum (SC)



Oral gavage feeding of SA (2.5 and 5.0 mg/kg body weight) and SC (2.5 and 5.0 mg/kg body weight) 5 days/week for 5 weeks caused a marked time-dependent inhibition in 22RV1 tumor xenograft growth which accounts for 46% and 59% decrease in SA treated animals and 75% and 88% decrease in SC treated animals, respectively.

S. Shrotriya et al. Carcinogenesis, 2012, 33(8), 1572-1580.

Fig. 4. Effect of p-DGA oral administration on the growth of human PCA 22Rv1 tumors and secreted PSA in athymic nude mice. 22Rv1 cells at the density of 1×10^6 were injected subcutaneously on the right flank of each male athymic nude mouse; and p-DGA (2,5 mg/kg or 5,0 mg/kg body weight) was administered through oral gavage route 5 days/week for 5 weeks. (A) The body weight of the animals was monitored throughout the experiment duration and presented as body weight/mouse in grams (g). (B) The diet consumption of the animals was also monitored throughout the experiment duration and presented as average diet consumption/mouse/day in grams (g). (C) Tumor volume was measured and presented as tumor volume/mouse (mm^3). (D) At the end of the study, blood was collected from mice, plasma was isolated and PSA level was determined by ELISA. Data are presented as mean ± SEM, where n=12 to 15 animals in each group for the data in panels A–C; and 4 animal samples for each group for the data shown in panel D. *, P<0.001; $, P<0.05.



Mechanism of anti-cancer efficacy of caffeic acid-derived polymer

Thus, a novel phytochemical poly[3-(3,4-dihydroxyphenyl)glyceric acid] (p-DGA) suppressed the growth and induced death in prostate cancer (PCA) cells, LNCaP and 22Rv1, with comparatively lesser cytotoxicity towards non-neoplastic human prostate epithelial PWR-1E cells. Molecular studies suggested that p-DGA caused G1 arrest in PCA cells through modulating the expression of cell cycle regulators, especially an increase in Cyclin-dependent kinase inhibitors (CDKIs) (p21 and p27). In addition, p-DGA induced apoptotic death in PCA cells by activating caspases, and also strongly decreased Androgen Receptor (AR) and Prostate-Specific Antigen (PSA) expression. Consistent with *in vitro* results, our *in vivo* study showed that p-DGA feeding strongly inhibited 22Rv1 tumors growth by 76 and 88% at 2.5 and 5 mg/kg body weight doses, respectively, without any toxicity, together with a strong decrease in PSA level in plasma; and a decrease in Proliferating Cell Nuclear Antigen (PCNA), AR, and PSA expression but increase in p21/p27 expression and apoptosis in tumor tissues from p-DGA-fed mice.

Conclusion

- **The first representative of a new class of natural polyethers - regular caffeic acid-derived polymer, namely POLY[3-(3,4-DIHYDROXYPHENYL)GLYCERIC ACID] or POLY[OXY-1-CARBOXY-2-(3,4-DIHYDROXYPHENYL)ETHYLENE] - has been isolated from comfrey species *Symphytum asperum*, *S. Caucasicum*, *S.officinale* and Bugloss (*Anchusa italica).***

- **Most of the carboxylic groups (70%) of *Anchusa* polyether unlike the polymer of *S.asperum*, *S.caucasicum* and *S.officinale* are methylated.**

- **The caffeic acid-derived polymer has wide spectrum of biological activity: anticomplementary, antioxidant, antiinflammatory properties, burn and wound healing effect.**

- **Pre-clinical investigation revealed the strong efficacy of p-DGA against prostate cancer cells and identifies this polymer as a potent agent against PCA without any toxicity, and supports its clinical application suggesting high potential in prostate cancer patients.**

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Photos

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Laboratory of plant biopolymers



THANK YOU FOR

YOUR PATIENCE

AND ATTENTION