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DIETARY PHYTOCHEMICALS AS CHEMOPREVENTIVE FOR LIVER CANCER

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تم تقييم الاحماض الفينولية الموجودة فى درنات البطاطس المعرضة لطرق طهومختلفة وكذا فى لب درنات البطاطس الطازجة والقشر باستخدام كروماتوجرافيا السائل ذات الضغط العالى. اسفرت النتائج عن وجود تلك الاحماض بتركيزات عالية فى درنات البطاطس المطهية بطريقة الشى وكذا فى قشور الدرنات الطازجة. كما تم دراسة تأثير المحلول المائى المحضر من الخلاصة الكحولية الجافة للاحماض الفينولية المحضرة من درنات البطاطس على السمه الخلوية الناتجة من مركب البنزوبيرين وذلك باضافتها قبل ، مع وبعد اضافة المادة السامه على خلايا كبد سمك البلطى المنزرعة فى وسط RPMI عند ضغط اسموزى قدره ٣٣٠ مل اسموزى فى وجود ٥% ثانى اكسيد الكربون عند درجة حرارة ٢٧°. اظهرت النتائج على ان الاحماض الفينولية لها تأثير مثبط للسمية الخلوية واعلى النتائج ظهرت عند استخدامها كمادة متلازمة مع المادة السامة ويليها عند استخدامها كمادة معالجة ثم كمادة وقائية.

Phenolic acids content of potato tubers, subjected to different types of cooking as well as in the peels and peeled fresh tubers, were determined by HPLC. The higher value of phenolic acids was noticed in the tubers cooked by grilling and in the peels of the fresh tubers.

The cytoxicity of the aqueous solution of the prepared dried ethanolic extracts was tested by the use of isolated liver cells of Bolti fish in RPMI 1640 medium at 330 mOsm/kg in the presence of 5% CO_2 and incubated at 27°. Benzo(a) pyrene, B(a) P, was used as toxicating agent. After the incubation period the media were removed, stained with neutral red and incubated for 3 hours. The dye of the stained cells was extracted with 1% acetic acid: 50% ethanol mixture and the absorbance of the extracts was measured at 490 nm. The result of the assay indicated that phenolic acids decrease the cytotoxicity of B (a) P in all treatments. The more effect was noticed for phenolic acid extracts in commitment with B(a) P followed by post then pre-treated ones.

INTRODUCTION

Cancer is a major disease at a worldwide level, accounting for more than 7 million deaths per year.[1] With the increasing of humon activities in modern environment, the chemical agents may represent the major cause of cancer. Amangest of these activities are processing and cooking of foods.[2,3]

Chemoprevention has received growing consideration as a mean of cancer control. Chemopervention is that compounds which have been demonstrated to prevent the occurrence of cancer. Phytochemicals are the most important type of chemopervention. They have nutrational value and pharmacologically active as anti-oxidant, anti-mutagenic and anti-carcinogenic agents.[4-7]

Phytochemicals include allyle sulfides, indolse, phenolic compounds, saponins and trepens. Phenolic compounds commonly occur in food are phenols acid, coumorins, flavanoids.

Phenolic acids occur in nature in the form of esters, ether or in free form. They are structurally related to two main groups of acids as benzoic and cinnamic acids.[8] They are commonly present in some diets as vegetables, (potato, lettuce, cabbage, spinach and wheat bran) and fruits (apples strawberries, pear,

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green and black grape, pineapple, cherry and peech).[9-12]

Phenolic acids are used as anti-oxidant, ani-careogenic, anti-inflammatory, anti-microbial, anti-viral, in the treatment of asthma, various allergic conditions and platelet aggregation.[4,5,7,13-18] It was reported that potato tubers have several kinds of phenolic acids and concentrated in the outer part.[19]

For the wide range activities of phenolic acid which present in high concentration in potato tubers, *Solanum tuberosum* L. Solanaeae family, encouraged us to evaluate phenolic acids in potato tuber either fresh or cooked and to study the influence of their extracts as cancer chemopreventive agents.

MATERIALS AND METHODS

Materials

1- Potato tubers

Potato tubers, *Solanum tuberosum* L. variety king Edward, three weeks old after harvesting were collected from the market and thoughrally cleaned with water.

2- Standards

Phenolic acids standards (gallic, protocatechic, p. cumaric, p.hydroxybenzoic, vanillic, chlorogeonic, caffeic and ferulic acids) were obtained from Sigma Chemical CO.

3- Equipments

1- High pressure liquid chromatography, Alltech USA.
2- Freeze drier, Birchover Ltd., Letchworth herts.
3- Wily mill, Tecato, Boulder, CO, USA.
4- Rotary evaporator, Staffordshire.
5- Hemocytometer, Boxter Healthcare Corp Mcgaw Pork .
6- Nikon microscope, Melville, Ny.
7- C18 sep-pak cartridge, phenomenex Co., Torrance, CA, USA.
8- MR-5000 microtiter plate reader.
9- Flat pottom well plates.

Methods

I- Phenolic acid determination

Several HPLC methods for phenolic acid determination were reported.[12,20-22] In our work we aimed to find a new HPLC method for simultaneous separation and determination of eight phenolic acids (gallic, protocatechuic, p-hydroxybenzoic, vanillic, caffeic, chlorogenic, p. coumaric and ferulic) using UV detector. So some mobile phases were tried as ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:27 v/v) and ammonium acetate: methanol in different ratios 92:8, 90:10, 88:12 and 86:14. Also different flow rates, 0.8, 1.0 and 12 ml/mn were tested. The results are listed in Table 1.

II- Phenolic acid extraction and determination

Potato tubers washed with water dried and divided into two groups. Group I whole tubers, group 2 peeled to obtain, peels and peeled tubers

Group I was subjected to the following cooking methods.

Cooking method	Temperature°	Time (min)
Boiling (in water)	100	30
Deep frying (in corn oil)	185	8
Microwaving	218	30
Grilling (electric oven)	121	45

The samples of fresh and cooked potato (group 1 and 2) were cut into small cubes, frozen in liquid nitrogen, then lyophilized for 72 hours and grind. The obtained powders were stored at –20° in air tied bags until using. According to Onyeneho and Hattiarachchy method (1993)[20] the deffated lyophilized powders were exhausted three times with 95% ethanol. The solvent was distilled off and the aqueous extract was freeze-dried under nitrogen and the residues were dissolved in water (10 mg/ml) filtrated and passed through an ultra sep pak C_{18} extraction cartridge. The adsorbed polyphenals were desrobed with methanol. 20 µl were injected and eluted with ammonium acetate buffer and methanol (88:12 v/v pH 5.4), at flow rate 1 m/min and UV detected at 265 nm at room temperature. The results are presented in Tables 2-4.

In order to estimate the respective recaveries of the standard phenolic acids they were treated in the same way as the potato tubers then chromatographed singly or in mixtures. Identification and quantitative

determination were calculated from their R_t and peak areas as well as from the preparing standard curves for each acid which showed linearity range from 25-2000 ug/ml. The results are listed in Table 5.

III- Preparation of liver cell culture

Liver was removed from the anesthetized. Bolti fish *Tilapia nilotica* according to Elhassaneen method,[23] washed with Hank`s balanced salt solution (HBSS), then minced and suspended in trypsin 0.25% and EDTA 0.2% mixture, stirr will then strained through three layer of sterile cheese cloth. The cell solution was mixed with culture media, buffer as well as some nutrients, then centrifuged. The supernatant was discarded and the cell pellet was washed twice with HBSS then suspended with 10% media which stained with 0.4% trypan blue before counting. The stained cells were counted using a hemocytometer.

For the formation of monolayer liver cells several culture samples were prepared and examined daily with Nikon inverted microscope for 9 days. The results are showed in Table 6.

IV- Determination of optimum condition of culture

Three media (RPMI 1640, Minimum essential and Leibovitz 15),[23] three osmolalities concentration 310,330 and 350 mOsm/kg and incubation temperatures at 37, 27 and 20° were studied. To determine the importance of CO_2 pressure the cultures were incubated in the absence or presence of 5 and 10% of CO_2. The results are presented in Figure 1.

Effect of enzyme on the liver cell yield

Two experiments were carried out on the liver cells one of them the liver cells were washed with HBSS buffer and the other not. The two tests were digested separately in 0.25% enzyme, either trypsin or chemotrypsin, in the presence of 0.02% EDTA at different period 10, 20 & 30 min. The cells were determined using homocytometer after staining with trypan blue. The results are illustrated in Figure 2.

Cytotoxicity assays

Isolated liver cells of Bolti fish were seeded with 100 µl of RPMI-1640 growth medium in cell well. Eight fold dilutions of benzo(a) pyrene were prepared and 100 µl of different dilutes were added to each well and incubated at 27° for 24 hours in the presence of 5% CO_2 tension.

According to the method described by Borenfreund and Puerner[24] the medium was removed gently than 0.2 ml of neutral red (NR) stain (50 µg/ml) were added to each well and incubated for 3 hours, allow stain uptake. The stained media were removed and the cells were washed three times with 1% formaldehyde, calcium chloride mixture. To extract the dye from the stained cells 0.2 ml of 1% acetic acid and 50% ethanol mixture was added to the culture, keep for 10 minutes at room temperature then agitate for 15 second. The absorbance of the extracted dye was measured by using MR-5000 microtiter plate reader at a wavelength of 490 nm.

The same procedure was adopted for the determination of the cytotoxicity of phenolic acid alone and phenolic acid pre, with and post-treated with benzo(a) pyrene. The results are illustrated in the Figures 3 and 4.

RESULTS AND DISCUSSION

The proposed HPLC method Table 1 revealed good separation of the eight tested phenolic acid with well marked retention time by the use of ammonium acetate buffer (pH5.4): methanol (88:12 v/v) as a mobile phase, flow rate 1.0 ml/min and UV detector at 265 nm. Sharp separation for the phenolic acid the mixture peaks was also noticed. The identification of the individual peak was confermed by single spiking of the acids separately.

Table 1: Retention times of phanolic acids detected with ultraviolet and florescence detectors.

Peak	Phenolic acids	Retention time (min)
		Ultraviolet
1	Gallic acid	1.567
2	Protocatechuic acid	1.789
3	p. Hydroxybenzoic acid	2.385
4	Vanillic acid	2.797
5	Caffeic acid	3.645
6	Chlorogenic acid	4.902
7	p. Coumaric acid	5.741
8	Ferulic acid	8.232

Table 2 indicated that the phenolic acid content in potato tubers, peels and peeled tubers were similar in presentation but differ in quantitation. The highest value was noticed in the peels followed by the whole tubers and finally the peeled tubers. Chlorogonic acid recorded high concentration followed by caffeic and protocatechuic with decreasing order. Ferulic acid showed the lowest concentration.

Onyeneho and Hettiarachchy[20] and Kumar *et al.*[25] measured the tested phenolic acids in potato peels but recorded low values. Malmbreg and Theander[26] observed the same phenolic acid with the exception of gallic and p. coumoric acids in the peels and peeled tubers. The higher values in the present study could be attributed to the method of extraction, potato varieties and localities in addition to the method of determination.

The data in Table 3 indicated that all cooking methods reduced the individual and total phenolic acids. Microwaving showed the highest reduction followed by frying, boiling than grilling with increasing order. Also the Table cleared that some acids were sensitive to the cooking methods as gallic, vanillic, p.hydroxybenzoic and protocatechaic while the others were more or less stable.

Table 2: Phenolic acid contents in different parts of potato tubers.

Part / Phenolic acids	Extract (mg/100 g) of		
	Whole potato tubers	Peeled tubers	Peels
	Mean ±SD	Mean ±SD	Mean ±SD
Gallic acid	219.69 ±1.16	224.61 ± 1.01	189.90 ± 5.61
Protocatechuic acid	297.71 ±1.84	269.37 ± 4.39	310.95 ± 0.38
p.Hydroxybenzoic acid	126.84 ± 0.70	112.66 ± 1.70	206.18 ± 4.36
Vanillic acid	43.77 ±0.53	34.12 ± 1.54	100.62 ± 3.93
Caffeic acid	302.04 ± 0.35	300.56 ± 3.04	457.74 ± 17.38
Chlorogenic acid	500.75 ±25.36	373.94 ± 19.60	1045.38 ±71.82
p.Coumaric acid	49.59±0.32	43.53 ± 3.02	82.34 ± 5.86
Ferulic acid	31.43 ± 0.55	28.05 ± 0.03	46.10 ± 4.49
Total	1571.80 ± 30.80	1386.80 ± 34.30	2439.21 ± 113.83

Table 3: The effect of cooking methods on phenolic acid contents of potato tubers.

Method / Phenolic acids	Extract (mg/100 g of tubers) using				
	Control	Grilling	Boiling	Frying	Microwaving
	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD
Gallic acid	224.61 ± 1.01	194.44 ± 2.26	149.59 ± 2.26	173.58 ± 2.28	143.57 ± 1.45
Protocatechuic acid	269.37 ± 4.39	256.39 ± 2.90	134.87 ± 0.43	222.67 ± 11.29	109.08 ± 2.45
p.Hydroxybenzoic acid	112.66 ± 1.70	105.90 ± 0.19	99.64 ± 2.07	74.23 ± 0.35	31.39 ± 1.30
Vanillic acid	34.12 ± 1.54	30.11 ± 0.49	27.14 ± 0.31	26.56 ± 0.43	18.05 ± 0.20
Caffeic acid	300.56 ± 3.04	298.97 ± 0.54	287.60 ± 1.37	293.29 ± 0.16	284.83 ± 2.27
Chlorogenic acid	373.94 ± 19.60	354.96 ± 2.59	340.10 ± 1.09	256.73 ± 15.41	282.03 ± 19.00
p.Coumaric acid	43.53 ± 3.02	41.28 ± 1.16	42.90 ± 0.15	29.37 ± 2.57	25.14 ± 0.27
Ferulic acid	28.05 ± 0.03	27.02 ± 0.04	27.59 ± 0.12	25.55 ± 0.03	23.71 ± 0.18
Total	1386.80 ±34.30	1309.10±10.20	1109.40±7.80	1102.00±32.50	917.80±26.10

Table 4 showed the collective results of cooking methods. Griling method was the most suitable method that cleared from the lowest percent of change.

Table 4: The effect of cooking methods on total phenolic acid contents of potato tubers.

Treatment	Total phenolic acids (mg/100g extract) Mean ±SD	Percent of change
Control	1386.8 ± 34.3	0
Grilling	1309.1 ± 10.2	5.6
Boiling	1109.4 ± 7.8	20.0
Frying	1102.0 ± 32.5	20.5
Microwaving	917.8 ± 27.1	33.8

Table 5 showed the percent recovery of the phenolic acids. Most of them was above 95%, this conferm the used extraction procedure was suitable.

Table 5: Precent recovery of phenolic acids.

Phenolic acids	Recovery (%)	S.D. (%)
Gallic acid	97.12	2.79
Protocatechuic acid	96.71	2.08
p. Hydroxybenzoic acid	94.14	3.41
Vanillic acid	98.80	1.07
Caffeic acid	93.23	4.10
Chlorogenic acid	92.59	4.92
p. Coumaric acid	98.22	1.73
Ferulic acid	97.81	2.08

Regarding the optimum condition of liver cell culture, Figure 1, the studies revealed that the optimum condition was performed by the use of RPMI-1640 media at 33o mOsm/kg in the presence of 5% CO_2 incubated at 27°.

Table 6 indicated that the number of adherent cells increased by the time reaching maximum at 9 days of incubation, conversely the number of nonadherent cells showed a slight increase in the first five days of incubation followed by a steady decline. Accordingly all the expermints of this study were performed at 5 days.

Table 6: Growth kinetics of isolated Bolti liver cells.

Time of Incubation /Days	Viable cell count (in 10^4) Non-adherent	Adherent	Viability %
1	11.4 ± 1.1	0.9 ± 0.3	90.1 ± 3.1
3	11.9 ± 2.0	3.8 ± 0.5	87.9 ± 2.8
5	13.2 ± 1.7	12.7 ± 3.2	84.6 ± 3.9
7	11.8 ± 2.2	22.6 ± 3.3	82.3 ± 4.1
9	9.2 ± 1.8	28.4 ± 2.9	78.9 ± 1.8

As regard the enzyme effect on the liver cells, Figure 2, clarified that the rinsed cells gave higher result at 20 min for both enzymes then the non rinsed cells. In addition trypsin showed good result than chemotrypsin.

Figure 3 and 4 indicated that the phenolic acids assay decreased the cytotoxicity of B(a) p in all studied treatments. The more effect was noticed for the extracted phenolic acid in commitment with B(a) p followed by post then pre-treated ones.

The best concentrations that decreased cytotxicity of B(a) p were recorded for 10^{-3}, 10^{-7} and 10^{-4} mg/ml respectively for the tested treatments.

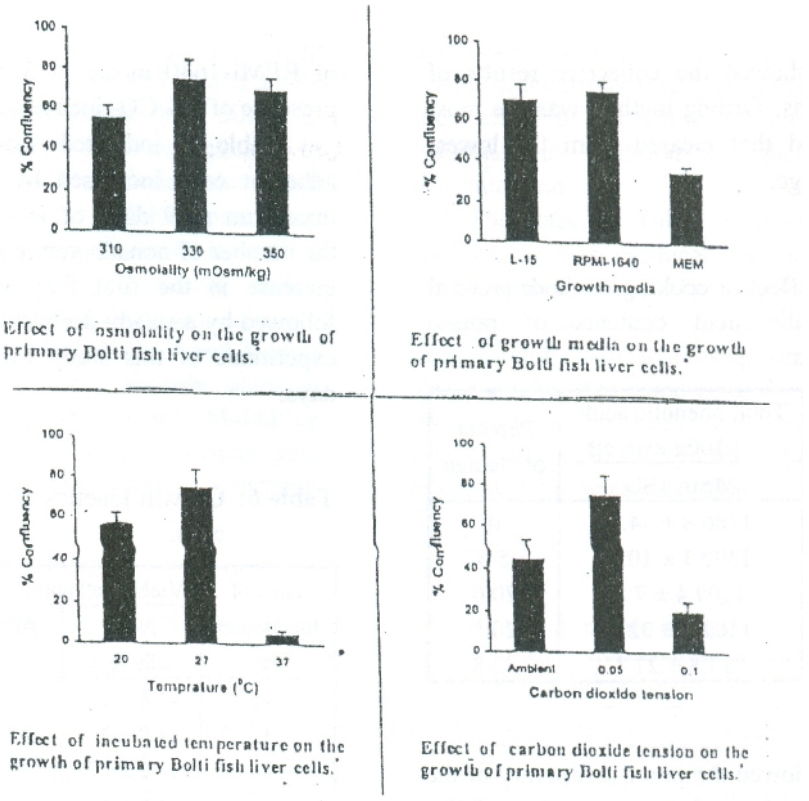
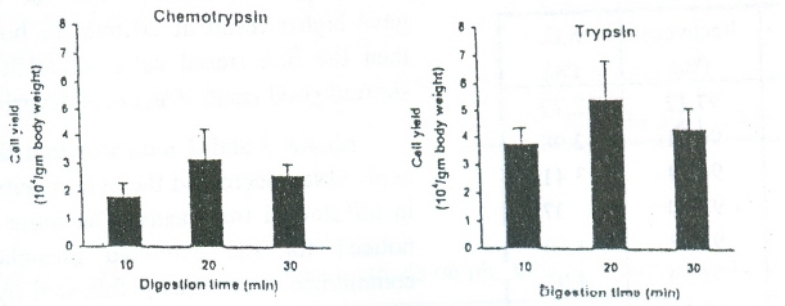


Fig. 1: Culture characteristics of isolated Bolti fish liver cells.

a- Non-rinsed cells



b- Rinsed cells

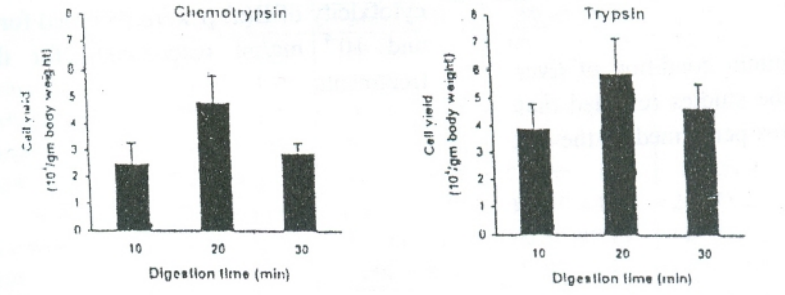


Fig. 2: Effect of digestion enzymes, with or without predigestion rinse with HBSS buffer, on cell yield of isolated Bolti fish liver cells.

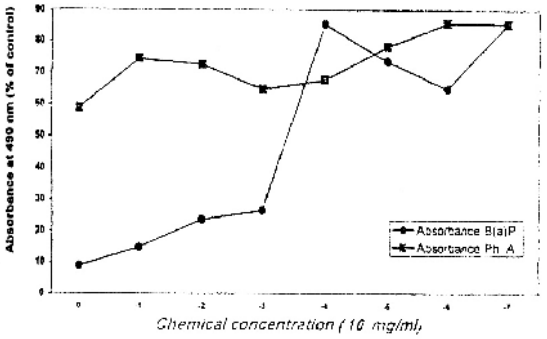


Fig. 3: Cytotoxicity of benzo(a) pyrene and phenolic acids as determined by neutral red (NR) assay.

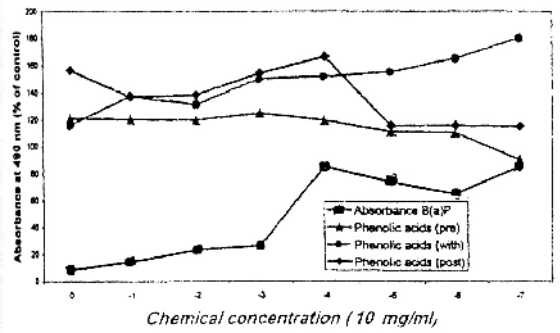


Fig 4: The influence of extracted phenolic acid of potato peels "pre, with and post-treated" on the cytotoxicity of benzo(a) pyrene as determined by neutral red (NR) assay.

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