

**ROLE OF DIFFERENT POLYPHENOLIC COMPOUNDS IN
MODULATION OF DNA PATTERN, NO LEVEL AND IL-12
LEVEL ON MICE – BEARING SOLID TUMOR**

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ABSTRACT

Polyphenolic compounds are potent antioxidants found in high concentration in a wide variety of plants, associated with numerous activities against chronic diseases. They have anti-cancer and anti-inflammatory effects, but the precise mechanism of protection remains unclear. The aim of this study was to investigate the effect of polyphenols, catechin (4mg/100gm), epicatechin (1mg/100gm), and tannic acid (1mg/100mg) on tumor – bearing (TUB) Swiss albino mice. The tumor was induced by implanting Ehrlich carcinoma (EAC) with subcutaneous injection of 3×10^5 tumor cells from abdominal cavity of syngeneic mice. After 9 days, subcutaneous tumor of approximately 3 mm³ diameter was found. Treatment provided intratumourally for 3 weeks. The results suggested that upon injection of polyphenols, tumor growth was reduced significantly and survival rate was increased by 49-54% with health benefits for animal as compared to control group bearing tumor. Furthermore, pro-inflammatory cytokine IL-12 was elevated in treated mice serum and contributed to preservation of the integrity of DNA pattern. Moreover, nitric oxide production reduced in liver homogenate for treated mice. In conclusion, the present data is promising for the efficacy of polyphenols to use as potent anti-tumor and survival advantage. With shedding for more detail analysis through molecular action in normal cells, it will become possible to be applied as medical treatment in human clinical trials.

Keywords: Polyphenols; DNA integrity; Tumor – bearing mice; IL12 and Nitric oxide.

INTRODUCTION

It is well known that cancer chemoprevention has a significant progress in prevention and/or inhibition of carcinogenesis by administration vast and variable drugs with chemical or natural entities depending on their antimutagenic properties. It has been demonstrated that natural polyphenols possess cancer chemopreventive effects in a wide range of target tumor in rodent, (Gupta *et al.*, 2001), that including lung (Yang *et al.*, 1997), skin (Stangl *et al.*, 2007), liver (Dreosti *et al.*, 1997) and colon (Bermudez-Soto *et al.*, 2007). However, besides capability to decrease frequency of cancer development, they reduce morbidity rate and supporting inhibition of cancer cell survival that give evidences on their protective role (Kuriyama *et al.*, 2006).

In the past decade, clinical data provided a convincing data that polyphenols rich in fruits, apple, green and red tea that consumed by human daily had antioxidant activity (Bhattacharyya *et al.*, 2003). At the same time, these compounds may affect number of Key enzymes including mitogen – activated protein kinase which increased or decreased protein/mRNA (Yu *et al.*, 1997). Recent work demonstrates some of polyphenols that reduce angiogenesis and dihydrofolate reductase activity (Beltz *et al.*, 2006) which would affect nucleic acid. Therefore, in the present study, we examined the effect of various polyphenolic compounds (+) catechin, (-) epicatechin and tannic acid on the growth of solid Ehrlich tumor transplanted *in vivo* syngeneic mice to evaluate the selective antitumor efficacy of these three compounds and to investigate the effect of the administration of these compounds on the antioxidant capacity of mice liver homogenate with significant measurement of immune system regulators "signal 3" interleukin 12 in particular, which known associated with suppression of tumor growth. Also more details and analysis on molecular level DNA integrity is necessary for management the host before safe clinical use of polyphenols for treatment of cancer diseases.

MATERIALS AND METHODS

Tumors and Animals:

8-week –old , female Swiss albino mice weighing from 20 to 22gm each kept in Animal Center, Genetic Institute Minufiya University –Egypt, under constant conditions (12h light : dark regimen , oriental chow pellet food and water *Ad Libitum*). Ehrlich ascitis tumor cells were maintained in the abdominal cavity of albino mice by intraperitoneal (i.p) injection of 1×10^6 . After the incubated tumor cells reached the exponential growth phase, 3×10^5

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cells per 0.02 ml were subcutaneously transplanted into the dorsal side of the thigh region of the right legs of the mice. When tumor volume reached approximately 3mm³, mice were used for experiments. Only water was given to the mice during the 12 h before polyphenols administration. Tumor volume was calculated by means of the formula $V=0.4 \times a \times (b^2)$, where (a) and (b) are the longer and shorter diameters of the tumor respectively, as measured with calipers.

Doses and Experimental Design:

The doses of (+) catechin, (-) epicatechin and tannic acid were 4mg /100gm, 1mg /100gm and 1mg /100gm respectively. These doses were evaluated by calculating the dose and time effect for each polyphenol separately. Tumor-bearing mice were randomly divided into four groups. Group 1 was the positive control group and the mice received no treatment of any type. Group 2, 3 and 4 received selective doses of (+) catechin, (-) epicatechin and tannic acid respectively, with keeping a negative control mice not inoculated with tumor and received only saline up to the end of experiment. Each experimental group contained 10 mice. Tumor size was measured twice weekly. The mice were sacrificed under anesthesia after 30 days of experiment, in accordance with the ethical standards. The samples were then stored at -20°C until analysis.

Enzyme-linked Immunosorbent Assay:

Enzyme-linked immunosorbent assay (ELISA) systems for IL-12 were detected in serum mice purchased from (Biosource International, INC., Immunoassay Mouse IL-12 p 70 kit, Belgium). Other reagents necessary for ELISA were purchased from Sigma Chemical (ST Louis, MO). The assays were carried out according to the manufacturer's manuals. OD values at 450 nm were measured by a microplate reader system (Bio-rad, Hercules, CA), and the concentrations were calculated using computer program (Bio-rad).

Assessment of Total Genomic DNA Damage:

The DNA damage was evaluated by electrophoresis of DNA extracted from both Kidney and liver removed from dissected mice at the end of experiments. Briefly, specimens from control group and treated mice groups were homogenized and lysed with Nicoletti lysis buffer (10mM tris-HCL, pH :7.5), 100 mM NaCl, 1mM EDTA, 1% SDS and 50 µg/ml proteinase K at 43 °C overnight. The DNA was extracted with an equal volume of phenol following chloroform: isoamyl alcohol wash. The DNA was precipitated with 0.5M NaCl and 95% ethanol at -20 °C overnight. The pellets were resuspended in 50µl at TE buffer and concentration of DNA detected from the absorbance at 260 nm.

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Each DNA sample (10 μ g) was electrophoresed at 50 volts for 14 hours through 1.5% of agarose gel. DNA bands were visualized under UV light staining with ethidium bromide, 100 base-pair DNA ladder (Amersham Biosciences) was applied as a reference marker (Kyprianou and Isaacs, 1988).

Nitrite Assay:

The nitrite concentration in liver homogenate was measured as an indicator of NO production according to the Griess reaction (Kim *et al.*, 1995). Three hundred microliters of absolute ethanol was added to 150 of 30% liver homogenate (PBS and EDTA), centrifuged under cooling condition at 5000 r.p.m for 5 minutes. One hundred of supernatant was mixed with the same volume of vanadium chloride (VCL3) and Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride in water); absorbance of the mixture at 540 nm was determined using a double-beam spectrophotometer (Shimadzu uv- pc 1601, Japan) (Miranda *et al.*, 2001).

Statistical Analysis:

The results of tumor size, nitric oxide and interleukin-12 were presented as Mean \pm SD; statistical analysis was performed using ANOVA test followed by multiple comparisons post-hoc analysis (Tucky), with a P value of less than 0.05 considered significant.

RESULTS

Polyphenols induce tumor regression

Figure (1), showed changes in the mean tumor volume (Mean \pm SD) in mice bearing EAC before and after treatment as a function of different doses selected from doses administered after tumor inoculation. The selection depends on survivals and toxicity (data not shown). The untreated control tumors (positive control) grew to approximately 2.2 - 2.5 mm³. Where tumor volume regression was recorded after treatment with each polyphenol and the most significantly effective dose was 4 mg, 1 mg and 1mg for catechin, epicatechin and tannic acid respectively.

Over expressing IL-12 confer systemic tumor protection:

The effect of various polyphenols was examined in mice bearing tumor and serum of different mice groups were tested, Table - (1) shows the decrease of IL-12 in serum of positive control mice by 52.5% while treatment of mice with polyphenols induced significant elevation closed to normal control mice (not bearing tumor) and received saline only. At the same time catechin induced a high level of IL-12 as compared to other used polyphenols.

Effect of polyphenols on nuclear damage in kidney and liver of mice:

Figures (2) and (3) showed the DNA electrophoretic mobility extracted from mice kidney and liver compared to that treated with different polyphenols. Smear DNA of tumor bearing mice is appear; not significantly damage, but treatment with polyphenols clearly modulated and repaired the pattern of total genomic DNA. Heart DNA not significantly changed in all tested groups (data not shown).

Effect of polyphenolic compounds on the NO content in liver tissue:

Mice bearing tumor (positive control) showed a significant increase by 49.7 % in the liver NO content compared to control one which received saline only. On the other hand, treatment of tumoural mice with different polyphenols (4mg catechin, 1mg Epicatechin and 1mg tannic acid/ 100gm mouse) for three weeks resulted in an increased levels of NO by 13.9 %, 12.8 % and 8.0 % respectively compared to normal control and in a significant decrease by 23.8 %, 24.6 % and 32.6 % for catechin, epicatechin and tannic acid respectively compared with (Positive control) as shown in (Table; 2).

DISCUSSION

Cancer is one of the most causes of death, despite the advanced of cancer chemotherapy approaches. Overall the years, molecular biology elucidate, provide and develop a novel chemotherapeutic and biological agents that appear to be promising by administration of one or more chemical entities, either as drug or as naturally occurring constituents. In the past decade polyphenolic agents found in a popular drink (tea) which considered to be second consumed by human after water and also some other fruits provided a convincing argument in its antioxidant activity and anti-cancer protection and reduction in a variety of animal bioassay system which involve the lung, liver, breast and colon (Dreosti *et al.*, 1997).

The present study have shown that polyphenols induced reduction in the tumor growth as shown in figure (1), this reduction was beneficial to the host through increasing the life span of EAC-bearing animal, at the same time; prevent growth of EAC cells and their penetration to the peritoneal cavity of Swiss albino mice compared to the untreated group of mice bearing tumor. This finding is one of the characteristic effects of antitumor drugs and moreover, is in agreement with various findings suggesting that tea and its polyphenolic components possess anticancer effect (Mukhtar and Ahmad, 2000).

In parallel, we have also investigated the immunoregulatory cytokine interleukin 12 which known as pro-inflammatory cytokine possessing anti-cancer and antiangiogenic properties (Katiyar *et al.*, 2006 and Granado-

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Serrano *et al.*, 2006). In our study, the polyphenols induced elevation in serum cytokine IL-12 as shown in table (1). This significant elevation followed by a significant reduction in subcutaneous tumor growth in the groups treated with polyphenols compared with untreated group "positive control". We suggest that cytokine elevation induced regulation and production of different cytokines IL-3 , IL-6 , GM-CSF, IFN- and TNF , such cytokines may exert activation of polymorphnuclear leukocytes especially neutrophils which are essential for host defenses and for inflammatory response toward such tumor reduction. At the same time figures (2) and (3) illustrated that the treated mice with polyphenols; exhibit a DNA repair in slightly and partially damage pattern, but this kind of repairing may be due to the reduction of some key enzyme by polyphenols such as dihydrofolate reductase as noticed by Beltz *et al.*, (2006), and may also IL-12 dependent following nucleotide excision repair mechanism.

To gain additional insights on the mechanism of action of these polyphenols and their antioxidant effect studies were performed on nitric oxide level, in all groups of mice under investigation. It was found that the animal bearing tumor and treated with different polyphenols showed a significant decrease in nitric oxide level compared to positive control (Table 2). This result indicates that polyphenols are responsible for this regression and strongly have anti-inflammatory property. This means that the polyphenols have numerous effects regulating cancer cell growth, inhibiting shedding and invasion by their antioxidant capacity. We suggest that the phenolic rings act as electron traps to scavenge peroxy radicals, superoxide anions and prevent oxidation reactions which may lead to inhibition of nitrate production. The free radical scavenging property of polyphenols is in agreement with (Curin *et al.*, 2006).

We can conclude that polyphenols are uniquely enable through activation of IL-12 to induce inhibitory effect on the Ehrlich tumor and their effects were beneficial for survival of mice, at the same time, they exhibit antioxidant activities and anti-inflammatory effects by depleting NO level in tumor bearing mice, with challenge effect on DNA pattern repair. Another strategy for evaluation of such polyphenols on caner risk should be undertaken in the molecular level, to get more information about their precise mechanism with shedding more analysis on normal cells and to see if it is possible to be used as medical treatment in human clinical trials.

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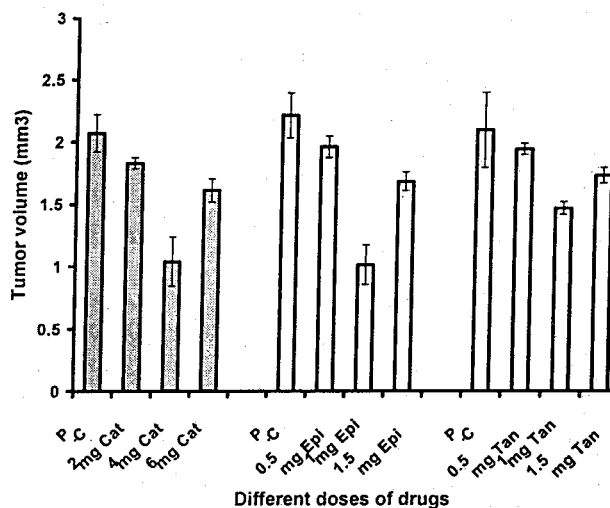
Table (1) : The interleukin-12 (IL-12) concentrations in control mice, mice – bearing solid tumor and others treated with tannic acid, catechin and epicatechin.

Animal groups	Mean ± SD
Control	6.427 ± 1.002
Positive control	3.050 ± 0.5763***
Tannic acid	4.872 ± 0.4784
Catechin	5.712 ± 1.044
Epicatechin	4.962 ± 0.5737

Table (2) : The nitric oxide (NO) concentrations in control mice, mice – bearing solid tumor and others treated with tannic acid, catechin and epicatechin.

Animal groups	Mean ± SD
Control	0.2076 ± 0.01918
Positive control	0.3108 ± 0.01211**
Tannic acid	0.2094 ± 0.03453
Catechin	0.2366 ± 0.04989
Epicatechin	0.2342 ± 0.03255

(**): Highly significant change (p< 0.01) .
 (***) : Very highly significant change (p<0.001).



Figure(1): Volume of solid tumor (mean ± SD) in mice bearing EAC before or after the treatment with different doses of catechin, epicatechin and tannic acid.

(P.C. : Positive Control, Cat. : Catechin; Epi. Epicatechin; Tan. : Tannic acid).

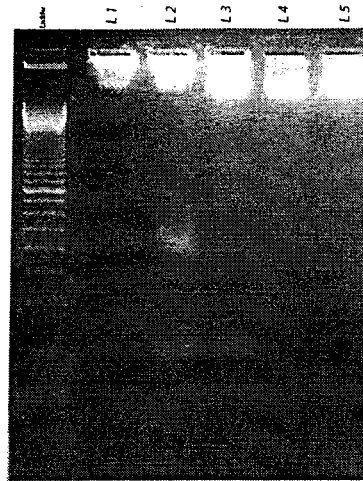
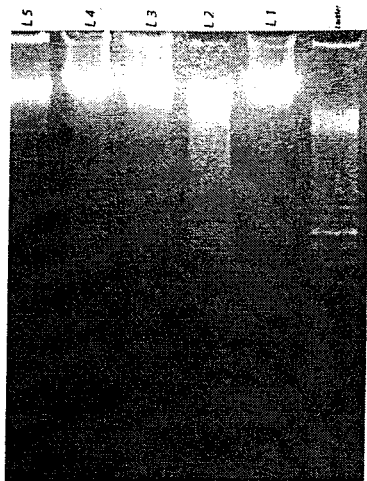


Fig (2): Gel electrophoresis of mice's kidney liver

Fig (3): Gel electrophoresis of mice's liver

Ladder (DNA ladder), *Lane (1)*: control, *Lane (2)*: Ehrlich carcinoma tumor (ECT) DNA without treatment, *Lane (3)*: ECT DNA with tannic acid treatment, *Lane (4)*: ECT DNA with catechin treatment, *Lane (5)*: ECT DNA with epicatechin treatment.

"تأثير المركبات عديدة الفينول في تثبيط انتشار الأورام، تحفيز عوامل الالتهاب وحماية سلامة الحامض النووي DNA في الفئران الحاملة للأورام"

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المركبات الفينولية هي من مضادات الأكسدة الفعالة وتوجد في عديد من النباتات ولها نشاطات ضد عديد من الأمراض المزمنة. لهذه المركبات تأثيرات ضد السرطان وضد الالتهابات ولكن تبقى ميكانيكية الحماية غير معروفة.

تهدف هذه الدراسة الى دراسة تأثير المركبات الفينولية مثل الكاتاشين (٤ مجم/١٠٠ جم) والإبيكاتاشين (١ مجم/١٠٠ جم) وحمض التانيك (١ مجم/١٠٠ جم) على الفئران البيضاء الحاملة للأورام السرطانية الصلبة. تم إحداث الورم السرطاني بزراعة خلايا سرطانية (إرلش) بالحقن تحت الجلد بما يعادل 3×10^5 خلية سرطانية من التجوييف البطنى لفئران معدية حاملة لهذه الخلايا. بعد ٩ أيام، يصل حجم الورم السرطاني المزروع الى حوالي 3 mm^3 ويستمر الحقن بمركبات البوليفينول داخل الورم لمدة ثلاثة أسابيع.

أوضحت النتائج أن حقن المركبات عديدة الفينول في الأورام المحدثه إختزل حجم الورم بصورة معنوية وكذلك إرتفع معدل البقاء بنسبة ٤٩-٥٤% مع تمتع الفئران بحالة صحية جيدة مقارنة بالفئران الحاملة للورم ولم يتم علاجها. علاوة على ذلك ارتفعت معدلات تركيز معامل الالتهاب الإنترليكون ١٢ في مصل الفئران وساعد ذلك في حفظ سلامة صورة الحامض النووي DNA. بالإضافة إلى ما تقدم أختزلت معدلات أكسيد النيتريك في كبد الفئران المعالجة بالمركبات الفينولية. نستخلص من كل ما تقدم أن مركبات البوليفينول لها قدرة عالية ومن الممكن إستخدامها لزيادة معدلات البقاء وعلاج السرطان. بدراسة تفاصيل تحليلية أخرى على التأثير الجزئى لمركبات البوليفينول على الخلايا الطبيعية نستطيع إستخدام هذه المركبات كعلاج طبي على المستوى البشرى.