

## Chemopreventive and Therapeutic Role of Nutraceuticals in Mice Loaded with Solid Tumor

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**Abstract:** The present study focused on evaluating the effect of different nutraceutical combinations including primarose, omega 3 fatty acids, silymarin, coenzyme Q<sub>10</sub>, antioxidants on tumor growth as well as the metabolic impact-induced by Ehrlich ascites carcinoma (EAC) inoculation in female mice. After 60 days of oral administration of the selected nutraceutical combinations, the tumor size was recorded. Also, serum and liver samples were obtained for different biochemical analyses. These analyses included the determination of serum arginase, L-fucosidase, lipid peroxidation, nitric oxide levels and total antioxidant capacity. Moreover, hepatic Na<sup>+</sup>-K<sup>+</sup>/ATPase activity, sodium and potassium content of hepatic tissue were also assayed. The present results revealed that EAC inoculation (0.2ml of EAC which contains 2.5×10<sup>6</sup> cells) causes significant increase in serum arginase, L-fucosidase, lipid peroxidation, nitric oxide levels and hepatic potassium concentrations, while, total antioxidant capacity, hepatic Na<sup>+</sup>/K<sup>+</sup>-ATPase activity and sodium concentrations were significantly decreased in EAC inoculated mice when compared to control one. On the other hand, the selected nutraceutical combinations significantly reduced tumor size and markedly improved most of the biochemical parameters associated with the inoculation of EAC. This study highlighted the potential effectiveness of the selected nutraceutical combinations in ameliorating the growth of implanted EAC as well as modulating the biochemical parameters that are involved during tumor development in mice.

**Key words:** Primarose, Omega-3 fatty acid, Silymarin, EAC, Rutin, CoQ<sub>10</sub>, Garlic, Solid tumor.

### INTRODUCTION

Chemoprevention is the administration of one or more chemical entities, either as individual drugs or as dietary supplements, to prevent the initiation of premalignant lesions or their progression to cancer or cancer recurrence. Most chemoprevention studies have been conducted with individual compounds, including various nutrients and non-nutrient phytochemicals (Gary 2009). Chemoprevention is based on the concepts of multifocal field carcinogenesis and multistep carcinogenesis. Carcinogenesis is a multistep process in which an accumulation of genetic events within a single cell line leads to a progressively dysplastic cellular appearance, deregulated cell growth, and finally, carcinoma (Anne *et al.* 2004).

Animal experimentation revealed that low activity of antioxidant system was found in experimental carcinoma. In addition, free radicals, particularly oxygen radical, play an important role in the complex course of multistep carcinogenesis (Sun 1990).

Dietary antioxidants are considered beneficial because of their potential protective role in the pathogenesis of multiple diseases associated with oxidative stress such as cancer (Alia *et al.* 2006).

Primrose (PE) is extracted from *Oenothera biennis* L., one species evening primroses, which has been shown to have several pharmacological effects. It was found that PE – induced apoptosis in Ehrlich ascites tumor cells (Arimura *et al.* 2004). Increasing interest in the role of omega 3 ( $\omega^3$ ) fatty acids has arisen in the latest years since evidence of their implication in the cardioprotection has been demonstrated. Recent study demonstrated that oils rich in  $\omega^3$  fatty acids decrease the tumor weight and metastasis number (Espada *et al.* 2007). Silymarin, the standardized extract of *Silybum marianum*, is used as a hepatoprotector in man. Silymarin can protect liver from the damaging effect through its antioxidant properties as it acts as a free radical scavenger (Kidd, 2009). N-Acetylcysteine (NAC), a thiol reducing agent that has antioxidant, antiangiogenic, and anticarcinogenic properties. NAC also down-regulated the production of proinflammatory cytokines (Tosetti *et al.* 2002). Garlic is a popular spice added to several edible preparations. Epidemiological as well as laboratory studies have shown that garlic consumption reduces certain cancer in the stomach and colon. Garlic

has been thought to bring about its anticarcinogenic effect through a number of mechanisms (Agarwal *et al.* 2007). Coenzyme Q<sub>10</sub> is essential component of ATP generation in the oxidative phosphorylation process and it is an antioxidant that preventing lipid peroxidation and scavenging superoxide (Choi *et al.* 2005). Rutin is a type of herbal flavonoids which could suppress rat cancer model via the induction of apoptosis. The effect of rutin on tumor cells *in vivo* showed marked antiproliferative activity (Martinez *et al.* 2002).

The main purposes of the current study was to explore the major complications occurred due to Ehrlich ascites carcinoma cells implantation in female mice, and to explore the potential role of certain nutraceutical combinations for prevention and intervention of the disturbances in the metabolic profile and in the antioxidant status induced by these tumor cells.

## MATERIALS AND METHODS

### **Materials:**

#### ***Ehrlich Ascites Carcinoma Cell Line:***

A line of Ehrlich Ascites Carcinoma (EAC) was supplied from the National Cancer Institute, Cairo, Egypt.

### **Nutraceuticals:**

Primrose: Evening Primrose soft gel capsule was obtained from EMA Pharm Pharmaceutical Egypt and (181.25mg) dissolved in 2.75 ml corn oil. Omega 3: Omega 3 ( $\omega^3$ ) soft gel capsule was purchased from SEDICO Pharmaceutical Co. Egypt, and (4.25mg) was dissolved in 117.64 ml corn oil. Silymarin plus N acetyl cysteine (NAC): Silymarin plus NAC soft gel capsule was purchased from SEDICO Pharmaceutical Co. Egypt and (0.5mg) dissolved in 280 ml corn oil. Garlic: Garlic tablet purchased from ATOS Pharma. Egypt and (3.125mg) was dissolved in 32 ml water. Coenzyme Q<sub>10</sub>: Coenzyme Q<sub>10</sub> capsule provided from Arab Co. for Pharmaceuticals and Medicinal Plants (MEPACO) Egypt and (5mg) was dissolved in 3 ml water. Rutin: Rutin tablet provided by KAHIRA Pharmaceutical and Chemical Industry Company, Egypt and (4.375mg) was dissolved in 6.85 ml water.

### **Experimental Animals:**

One hundred and twenty Swiss albino female mice (2-3 months old and 20-25g weight) obtained from the Animal House Colony of the National Research Center, Cairo, Egypt, were used in the present study. The animals were acclimated in specific pathogen free plastic cages of dimensions of 42L x 26W x 22H centimeters. The animals were maintained under controlled conditions of humidity (55%), temperature (25±1 °C), and diurnal environment of light and dark (12 h light/dark cycle) at National cancer Institute; Animal Facility Breeding Colony. The mice were freely fed on standard laboratory diet consisting of casein 10%, salts mixture 4%, vitamins 1%, corn oil 10% and cellulose 5% completed to 100g with corn starch (A.O.A.C 1995). Also, tap water was offered liberally. Animals received humane care in compliance with the guidelines of the Ethical Committee of Medical Research of National cancer Institute, Cairo, Egypt.

The animals were randomly assigned into eight experimental groups (15 mice/ group) which were classified as follows: Group (1) the mice in this group were orally administered with 1ml corn oil/mouse daily and served as control group for two months. Group (2): Each mouse in this group was injected intramuscularly (i.m.) in the right thigh with 0.2ml of EAC which contains  $2.5 \times 10^6$  cells for solid tumor induction (El-Gawish 2003). The animals were left without any treatment for two months. Group (3): The mice in this group were orally treated with primrose [7.25 g/kg b.wt.] (Taweechaisupapong *et al.* 2005), plus omega 3 [170 mg/kg b.wt.] (Desager *et al.* 1989) daily for two month, then each mouse was injected with EAC for tumor induction. Group (4): Each mouse in this group was injected with EAC for tumor induction. Then, mice were orally treated with primrose plus omega 3 daily, for two months. Group (5): The mice in this group were orally treated with silymarin plus N-acetylcysteine (NAC) [20 mg/kg b.wt.] (Bhattacharya *et al.* 2000) daily for two months, then each mouse was injected with EAC for tumor induction. Group (6): Each mouse in this group was injected with EAC for tumor induction. Then, mice were orally treated with silymarin plus NAC daily for two months. Group (7): The mice in this group were orally treated with garlic [125 mg/kg b.wt.] (Bhuvanewari 2004), plus coenzyme Q<sub>10</sub> [200 mg/kg b.wt.] (Andeassen 1999), plus rutin [175 mg/kg b.wt.] (Maurya *et al.* 2004), daily for two months, then each mouse was injected with EAC for tumor induction. Group (8): Each mouse in this group was injected with EAC for tumor induction. Then, mice were orally treated with garlic plus rutin plus coenzyme Q<sub>10</sub> daily for two months.

#### **Blood Sampling:**

At the end of the experimental period, fasting blood samples were collected from retro-orbital venous plexus under diethyl ether anesthesia.

Blood samples were collected in dry clean centrifuge tubes and then centrifuged at 3000 rpm for 15 min at 4 °C. Serum samples were collected, stored at -80 °C in clean plastic Eppendorf tubes till analysis.

#### **Liver Tissue Sampling:**

Preparation of liver homogenate: After blood collection, the mice were sacrificed and the whole livers were rapidly dissected, thoroughly washed with isotonic saline and dried on filter paper and then weighed. The individual liver of each animal was divided into two portions. The first portion was homogenized immediately to give 10% (w/v) homogenate in ice-cold medium containing 50 mM Tris-HCl and 300 mM sucrose (Tsakiris *et al.* 2004). The homogenate was centrifuged at 3000 rpm for 10 min in cooling centrifuge at 4 °C. The supernatant (10%) was used for the determination of Na<sup>+</sup>/K<sup>+</sup> ATPase activity.

Digestion of liver tissue: The second portion of each liver was digested in mixture of nitric acid (65 %) and concentrated sulfuric acid (1:1 v/v) at high temperature, then, the digested liver solution was centrifuged at 3000 rpm for 15 min. (I.A.E.A 1980). The supernatant was taken and stored under cooling condition for analysis of sodium and potassium.

#### **Methods:**

##### **Measurement of Tumor Size:**

The size of solid tumor was measured using Vernier caliper, starting from the tenth day-post EAC implantation. The tumor size was calculated according to Papadopoulos *et al.* (1989) using the following formula:

$$\text{tumor size (mm}^3\text{)} = 4/3 \pi (A/2)^2 (B/2)^2;$$

where A and B are the minor and major axis, respectively.

##### **Biochemical Analysis:**

Serum samples of all animals in the experimental groups were subjected to the following determinations. The activity of arginase activity and  $\alpha$ -L-fucosidase (AFU) was estimated by colorimetric method according to the method described by Forsell and Palva (1961) and Zielke *et al.* (1972) respectively.

Malondialdehyde (MDA) as a product of lipid peroxidation was assayed in the supernatant of deproteinized serum by the thiobarbituric acid (TBA) method (Esterbauer and Cheeseman 1990). The nitrite assay kit was used for determination of serum nitric oxide level according to the method of Berkels *et al.* (2004). Total antioxidant capacity in serum was estimated by colorimetric method according to the method of Koracevic *et al.* (2001). Liver samples of all animals in the experimental groups were subjected to the following determinations: Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in the liver homogenate was estimated according to the method of Tsakiris *et al.* (2004). Hepatic sodium and potassium concentrations were estimated by colorimetric method according to the method described by Trinder (1951) and Sunderman and Sundeman (1958) respectively.

##### **Statistical Analysis:**

The data were statistically analyzed according to Steel and Torrie (1980) using SPSS computer Program. The results were presented as mean  $\pm$  SD. The differences between mean values were determined by analysis of variance (ANOVA test), followed by Duncan's multiple rank test (1955) using MSTAT-C computer program. Statistical significance of the relationships between variables was calculated by linear regression analysis. Difference was considered significant when P value was  $\pm$  0.05.

##### **Results:**

The present results showed that administration of different nutraceutical combinations [(primrose +  $\omega^3$ FA), (silymarin + N-acetylcysteine), or (garlic + CQ10+ rutin)], either prior to or after EAC inoculation resulted a significant decrease ( $P < 0.01$ ) in tumor size as compared to untreated EAC group (Table1).

The data of the current study represented in Table (2) revealed that EAC implantation causes significant increase ( $P < 0.01$ ) in serum arginase and L-fucosidase activities as compared to that of the normal control group. Administration of the different nutraceutical combinations, either prior to or after EAC inoculation produced significant reduction ( $P < 0.01$ ) in serum arginase and L-fucosidase activities as compared to that of EAC control group.

Our results in Table (3) showed that EAC induces significant increase ( $P<0.01$ ) in serum malondialdehyde (MDA) and nitric oxide (NO) levels as compared to that of the normal control group. Administration with the different nutraceutical combinations either prior to or after EAC inoculation produced significant depletion ( $P<0.01$ ) in serum MDA and NO levels as compared to that of EAC control group. On the other hand, data in Table 3 showed that EAC induced significant decrease ( $P<0.01$ ) in serum total antioxidant capacity as compared to that of the normal control group. Significant increase ( $P<0.01$ ) in serum total antioxidant capacity was demonstrated in the groups pre- or post-treated with either one of the different nutraceutical combinations as compared to EAC-bearing group.

Current results in Table 4 revealed that EAC induces significant inhibition ( $P<0.01$ ) in hepatic  $\text{Na}^+/\text{K}^+$ -ATPase activity as compared to control group. Administration with the nutraceutical combinations either prior to or after EAC inoculation resulted in significant increase ( $P<0.01$ ) in hepatic  $\text{Na}^+/\text{K}^+$ -ATPase activity as compared to untreated EAC-bearing mice. Data depicted in Table (4) also showed that EAC induced significant reduction ( $P<0.01$ ) in hepatic sodium level as compared to that of the normal control group and the administration of the different nutraceutical combinations either prior to or after EAC inoculation produced significant elevation ( $P<0.01$ ) in hepatic sodium concentration as compared to untreated EAC-bearing mice. In contrast, EAC induced significant increase ( $P<0.01$ ) in hepatic potassium concentration as compared to that of the normal control group and the administration of different nutraceutical combinations either prior to or after EAC inoculation induced significant decrease ( $P<0.01$ ) in hepatic potassium level when compared to untreated EAC-bearing mice.

**Table 1:** Protective and therapeutic effects of different nutraceutical combinations on tumor size in mice bearing EAC.

Groups	Tumor size (mm <sup>3</sup> )
Control	0.000
Mice bearing EAC	3.42 ± 0.09** <sup>a</sup>
Primrose + ω <sup>3</sup> FA (P)	1.00 ± 0.05** <sup>b</sup>
Silymarin + NAC (P)	1.25 ± 0.09** <sup>b</sup>
Garlic + CoQ <sub>10</sub> + Rutin (P)	1.33 ± 0.07** <sup>b</sup>
Primrose + ω <sup>3</sup> FA (T)	1.40 ± 0.06** <sup>b</sup>
Silymarin + NAC (T)	1.64 ± 0.09** <sup>b</sup>
Garlic + CoQ <sub>10</sub> + Rutin (T)	1.41 ± 0.07** <sup>b</sup>

<sup>a</sup>Differences in relation to the control group, <sup>b</sup>Differences in relation to EAC group.

\*\* Significant change at  $p<0.01$ .

(P) protective effect, (T) therapeutic effect

**Table 2:** Protective and therapeutic effects of different nutraceutical combinations on serum arginase and l-fucosidase activities in mice bearing EAC.

Groups	Arginase (U/L)	L-fucosidase (U/L)
Control	23.35 ± 0.86	2.13 ± 0.26
Mice bearing EAC	64.02 ± 2.00** <sup>a</sup>	9.02 ± 0.41** <sup>a</sup>
Primrose + ω <sup>3</sup> FA (P)	33.27 ± 0.94** <sup>b</sup>	3.80 ± 0.30** <sup>b</sup>
Silymarin+ NAC (P)	35.96 ± 1.12** <sup>b</sup>	5.40 ± 0.38** <sup>b</sup>
Garlic + CoQ <sub>10</sub> + Rutin (P)	37.87 ± 1.11** <sup>b</sup>	5.66 ± 0.27** <sup>b</sup>
Primrose + ω <sup>3</sup> FA (T)	36.02 ± 1.02** <sup>b</sup>	5.65 ± 0.42** <sup>b</sup>
Silymarin+ NAC (T)	36.60 ± 1.52** <sup>b</sup>	5.85 ± 0.34** <sup>b</sup>
Garlic + CoQ <sub>10</sub> + Rutin (T)	39.43 ± 1.16** <sup>b</sup>	6.15 ± 0.41** <sup>ba</sup>

<sup>a</sup>Differences in relation to the control group, <sup>b</sup>Differences in relation to EAC group.

\*\* Significant change at  $p<0.01$ .

(P) protective effect, (T) therapeutic effect

**Table 3:** Protective and therapeutic effect of different nutraceutical combinations on serum malondialdehyde, nitric oxide and total antioxidant capacity in mice bearing EAC.

Groups	Malondialdehyde (nmol/ml)	Nitric Oxide( mM/L )	Total Antioxidant Capacity ( μmol/L)
Control	1.08 ± 0.06	24.60 ± 1.65	1.71 ± 0.01
Mice bearing EAC	2.71 ± 0.12** <sup>a</sup>	55.21 ± 1.07** <sup>a</sup>	0.80 ± 0.03** <sup>a</sup>
Primrose+ω <sup>3</sup> FA (P)	1.26 ± 0.08** <sup>b</sup>	26.08 ± 1.11** <sup>b</sup>	1.21 ± 0.02** <sup>b</sup>
Silymarin+NAC (P)	1.30 ± 0.10** <sup>b</sup>	27.23 ± 0.89** <sup>b</sup>	1.10 ± 0.02** <sup>b</sup>
Garlic+CoQ <sub>10</sub> + Rutin (P)	1.31 ± 0.09** <sup>b</sup>	29.31 ± 0.91** <sup>b</sup>	1.06 ± 0.02** <sup>b</sup>
Primrose+ω <sup>3</sup> FA (T)	1.32 ± 0.07** <sup>b</sup>	30.73 ± 1.31** <sup>b</sup>	1.14 ± 0.02** <sup>b</sup>
Silymarin+NAC (T)	1.63 ± 0.04** <sup>b</sup>	31.15 ± 1.51** <sup>b</sup>	1.09 ± 0.01** <sup>b</sup>
Garlic+CoQ <sub>10</sub> +Rutin (T)	1.65 ± 0.06** <sup>b</sup>	32.00 ± 1.14** <sup>b</sup>	1.07 ± 0.02** <sup>b</sup>

<sup>a</sup> Differences in relation to the control group.

<sup>b</sup> Differences in relation to EAC group.

\*\* Significant change at  $p<0.01$ . (P) protective effect, (T): therapeutic effect

**Table 4:** Protective and therapeutic effect of different nutraceutical combinations on hepatic Na<sup>+</sup>/K<sup>+</sup>-ATPase activity, sodium and potassium concentrations in EAC bearing mice.

Groups	Na <sup>+</sup> /K <sup>+</sup> -ATPase (μmol pi/h/mg protein)	Sodium (mmol/g)	Potassium (mmol/g)
Control	2.52 ± 0.08	690.58 ± 8.90	647.90 ± 2.80
Mice bearing EAC	0.61 ± 0.03** <sup>a</sup>	417.83 ± 9.47** <sup>a</sup>	759.38 ± 7.33** <sup>a</sup>
Primrose + ω <sup>3</sup> FA (P)	2.04 ± 0.04** <sup>b</sup>	665.00 ± 9.69** <sup>b</sup>	652.60 ± 2.11** <sup>b</sup>
Silymarin + NAC (P)	1.92 ± 0.03** <sup>b</sup>	622.90 ± 8.93** <sup>b</sup>	658.10 ± 4.60** <sup>b</sup>
Garlic + CoQ <sub>10</sub> + Rutin (P)	1.85 ± 0.05** <sup>b</sup>	615.40 ± 15.00** <sup>b</sup>	658.00 ± 5.40** <sup>b</sup>
Primrose + ω <sup>3</sup> FA (T)	2.04 ± 0.09** <sup>b</sup>	629.26 ± 8.67** <sup>b</sup>	663.40 ± 2.80** <sup>b</sup>
Silymarin + NAC (T)	1.68 ± 0.03** <sup>b</sup>	619.71 ± 12.38** <sup>b</sup>	672.70 ± 3.60** <sup>b</sup>
Garlic + CoQ <sub>10</sub> + Rutin (T)	1.67 ± 0.05** <sup>b</sup>	613.29 ± 6.24** <sup>b</sup>	679.40 ± 5.10** <sup>ba</sup>

<sup>a</sup> Differences in relation to the control group.<sup>b</sup> Differences in relation to EAC group.\*\* Significant change at  $p < 0.01$  (P) protective effect, (T): therapeutic effect

### Discussion:

Chemoprevention provides a practical approach to identify potential inhibitors of cancer development as well as affords the appropriate strategy to study the mechanism of carcinogenesis. Dietary constituents seem to hold considerable promise as potential chemopreventive agents through modulating the metabolic fat of protoxicants through competing pathways of detoxication system enzymes. In the present work we studied the most important complications occurred due to Ehrlich ascities carcinoma cells (EACc) in mice as well as role of some selected nutraceutical combinations in the regression of tumor growth and in counteracting its metabolic complications. Nutraceutical combinations were given prior to tumor inoculation "as protective agents" or after tumor inoculation "as Therapeutic agents".

The data obtained from the present study revealed that concurrent administration with primrose and omega-3 (ω<sup>3</sup>) produced significant reduction in tumor size in EAC bearing mice. Primrose, containing about 75% of linoleic acid (LNA) and 9% of gamma-linolenic acid (GLA), was reported to inhibit carcinogenesis in the rat and mouse (Arimura *et al.* 2004). The suggested mechanism for evening primrose extract (EPE) to inhibit tumor cell growth includes the rapid increase in the intracellular peroxides levels, loss of mitochondrial membrane potential and the release of cytochrome c from mitochondria to cytosol which induces apoptosis. Moreover, EPE elicits an accumulation of cells in the G<sub>1</sub> phase and inhibits DNA synthesis (Arimura *et al.* 2003). Horia and Watkins (2005) stated that supplementation of LNA elevates the concentration of eicosapentanoic acid (EPA) – mediated tumor reductions, attenuated new tumor formation and reduced tumor growth. In addition, GLA supplementation was reported to inhibit tumor growth and development *in vitro* as well as *in vivo* through suppression of cyclooxygenase-2 (Cox-2) over expression/PGE2 biosynthesis.

Regarding the effect of omega-3 on the regression of tumor growth, Espada *et al.* (2007) reported that oils rich in ω<sup>3</sup> decrease the tumor weight and metastasis number via complex multiple pathways. One of which is the influencing on the gene expression involved in the control of cell growth, differentiation, angiogenesis and metastasis. Moreover, oils rich in ω<sup>3</sup> significantly reduced the tumor weight due to a reduction in mitosis of tumor tissues (Ikeguchi *et al.* 2004).

Silymarin in combination with N-acetylcysteine (NAC) significantly reduce tumor size in mice bearing EAC. Various studies indicated that silymarin exhibits cancer preventive and anticarcinogenic effects. The inhibitory effect of silymarin on tumor cell growth and proliferation is associated with a G<sub>1</sub> arrest cell cycle progression in concomitant with the induction of up to 19-fold increase in the protein expression of cyclin-dependent kinase (CDK) inhibitor Cip<sub>1</sub>/P21 (Tyagi *et al.* 2002). Katiyar *et al.* (1997) stated that silymarin exerts high protective effects against carcinogenesis in different mouse tumorigenicity models via inhibition of mRNA expression of an endogenous tumor promoter TNF-α. Silymarin has been shown to be a strong antioxidant capable of scavenging free radicals via protection of the membrane from ROS toxicity, thus inhibiting the initiation as well as promotion stages of carcinogenesis (Sen 1995).

Concerning the cooperative effect of N-acetylcysteine in inhibiting tumor size in EAC bearing mice, Chiao *et al.* (2004) demonstrated the ability of dietary N-acetylcysteine to inhibit tumorigenesis. NAC may prevent initiation of carcinogenesis and modulate the post-initiation phase by targeting cell cycle regulators and apoptosis induction.

Administration of garlic, coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>) and rutin showed significant reduction in tumor size in EAC bearing mice. Many previous studies have demonstrated that garlic possesses anticarcinogenic and immunomodulatory effects, due to its organosulfur compounds. The role of garlic in inhibiting tumor growth is attributed to its antiproliferation activity (Oommen *et al.* 2004). Moreover, the active constituent of garlic inhibits the growth of cancer cell via the induction of apoptosis (Hassan and Ajoene, 2004). Also, it has been shown that garlic oil suppresses ferric nitrilotriacetate (Fe-NTA) (a free radical generating compound) mediated induction of ornithine decarboxylase (ODC) activity and DNA synthesis in cancer cells (Agarwal *et al.* 2007).

Coenzyme Q<sub>10</sub> is an endogenous enzyme cofactor that may provide protective benefits as an antioxidant. CoQ<sub>10</sub> and rutin could affect tumor growth in the present study by inhibiting ROS generation (Alia *et al.* 2006 and Wang *et al.* 2008). CoQ<sub>10</sub> also has the ability to suppress DNA strand breaks in lymphocytes challenged with oxidant stress leading to apoptosis (Allevaa *et al.* 2001).

The results of the present study revealed that EAC inoculation induces significant increase in serum arginase activity as compared to that of the normal control. The elevated activity of arginase has been demonstrated in different carcinomas that indicate its relation to cancer (Erbas *et al.* 2007). Arginase activity has been found to be stimulated significantly by prostaglandin (PGE2) via the induction of arginase I expression (Ochoa *et al.* 2007). Thus, cyclooxygenase-2 (COX-2) enzyme and in turn prostaglandins inhibitors have been shown to suppress the development and metastasis of tumors (Muller and Scherle 2006).

Serum arginase activity in EAC inoculated mice administered with primrose in combination with Omega-3 fatty acids showed significant decrease.  $\omega^3$  fatty acids have been found to decrease pro-inflammatory cytokine and PGE2 production during inflammatory response as they have anti-inflammatory properties. The mechanism of action for this anti-inflammatory activity appears to occur via displacement of arachidonic acid (AA) in the cellular membrane leading to the production of less metabolically active compounds such as PGE2. This means that  $\omega^3$  fatty acids decrease the substrate availability for PGE2 (Horia and Watkins 2005). Additionally,  $\omega^3$  fatty acids have been shown to induce significant alterations in nuclear transcription factor activation, particularly (NF $\kappa$ B) via peroxisome proliferators-activated receptor alpha signaling (Mishra *et al.* 2004), disturbing the pathway of PGE2 generation. Therefore, they could suppress the production of PGE2 and consequently inhibit the activity of arginase.

It has been found that silymarin in combination with NAC could significantly inhibit the activity of serum arginase in EAC bearing mice. Also it has been shown to inhibit the lipopolysaccharide induced production of PGE2 as it could inhibit COX-2 mRNA expression. NAC has been found to decrease COX-2 mRNA gene expression. Thus, this combination acts synergistically to inhibit arginase activity via inhibition of COX-2 mRNA expression (Guo *et al.* 2007).

The current results revealed that the administration of garlic in combination with CoQ<sub>10</sub> and rutin significantly decrease serum arginase activity in EAC-bearing mice. The possible mechanism for this event is the inhibition of PGE2 production by suppressing the expression of COX-2 protein via suppression of NF $\kappa$ B. NF $\kappa$ B targeting to the deterioration of PGE2 pathway (Chang and Chen 2005), or through inhibition of several factors such as interleukin-10 (IL-10) and transforming growth factor- $\beta$  (TGF- $\beta$ ) that participate in PGE2 production *in vivo*. Furthermore Skaltsa *et al.* (2000) stated that flavonoids including garlic and rutin directly modulated AA metabolism and consequently inhibit PGE2 production.

The present results revealed that L-fucosidase activity (AFU) showed significant increase in mice bearing EAC. The increased serum AFU activity in tumor bearing mice in the present work may be attributed to the secretion of tissue-plasminogen activator especially urokinase type from tumor cells. This glycoprotein serine protease contains an unusual a- fucosidase-sensitive O-linked fucose residue on threonin-61 within the epidermal growth factor domain (Grizzi *et al.* 2007). Lysosome and its enzyme complements, AFU have been suggested as potentially important during several stages of carcinogenesis. Therefore, inhibitors of lysosomal enzymes could have useful effect in controlling carcinogenesis (Tappel 2005).

Administration of primrose in combination with  $\omega^3$  FA produced significant decrease in serum AFU activity in EAC-bearing mice. Primrose plays an important curative role in cancer treatment through inhibiting tumorigenesis and cell proliferation. It could inhibit urokinase type plasminogen activator and in turn AFU activity. It is well known that the major circulating physiologic inhibitor of urokinase-1 type plasminogen activators *in vivo* is plasminogen activator inhibitor type 1 (PAI-1). It has been demonstrated that primrose could increase PAI-1 production and consequently inhibits urokinase-type plasminogen activator activity (Dichtl *et al.* 2002).  $\omega^3$  fatty acids has been reported to increase PAI-1 mRNA levels which are the primary source of circulating PAI-1 production *in vivo*. This is the proposed mechanism by which  $\omega^3$  FA could inhibit AFU activity in the current study.

In the present study, the inhibitory effect of silymarin plus NAC on serum AFU activity in EAC-bearing mice could be attributed to their direct suppressing action on the expression of urokinase plasminogen activator-1 mainly on the transcriptional level (Chu *et al.* 2004).

Co-administration of garlic and CoQ<sub>10</sub> together with rutin resulted in significant inhibition of serum AFU activity in EAC bearing mice. Garlic, CoQ<sub>10</sub> and rutin could exhibit this effect through their anti-lipid peroxidation and antioxidant properties particularly for garlic (Koseoglu *et al.* 2010). The inhibition of lipid peroxidation means the reduction of free radicals induced release of lysosomal enzymes, including AFU. The suggested mechanism for such antioxidant activity has been related to the localization of CoQ<sub>10</sub> within the lipid



bilayer, where it can prevent autocatalytic free radical reaction by stacking among phospholipids molecules and keeping the quinone ring in nonpolar phase. CoQ<sub>10</sub> inhibits lipid peroxidation by either scavenging free radicals directly or reducing  $\alpha$ -tocopheroxyl radicals (Kwong *et al.* 2002). Rutin inhibits the increase in the level of thiobarbituric acid reactive substances. Also, it has been reported that rutin could inhibit superoxide ions that participate in the initiation of microsomal lipid peroxidation (Saravanan and Prakash 2004). Therefore, the combination of garlic, CoQ<sub>10</sub> and garlic succeeded to inhibit AFU activity in serum through restricting lipid peroxidation reactions and products, and in turn preserving lysosomal enzymes (Kwong *et al.* 2002).

The results of the current study revealed that serum malondealdehyde (MDA) level showed significant increase in EAC bearing mice. This finding is greatly supported by Balasubashini *et al.* (2006). It has been reported that free radicals particularly oxygen radicals plays an important role in the complex course of multistep carcinogenesis. The enhanced lipid peroxidation product in the present study may be attributed to over production of ROS. High levels of ROS have been reported to damage many biomolecules and exert diverse cellular and molecular effects including mutagenicity and changes in gene expression that lead to initiation and promotion of carcinogenesis. In addition, ROS have been found to modulate signaling events in the cell and play a functional role in the pathogenesis of malignancy (Behrend *et al.* 2003).

Supplementation with all nutraceutical combinations lead to significant decrease in serum MDA level in EAC-bearing mice. It has been demonstrated that primrose and  $\omega^3$ FA, reduce lipid peroxidation products via the inhibition of oxidative stress as an expression of the effect of free radicals on cell membranes and cellular defense. Also this supplementation enhances resistance to free radical attack and reduces lipid peroxidation as well as this type of fatty acids could increase total antioxidant capacity (Oarada *et al.* 2008). Silymarin plus NAC protects against oxidative damage via their ability to inhibit lipid peroxidation (Khazanov and Vengerovsky 2007 and Al-Tanbolry *et al.* 2009). Garlic supplementation offers significant protection against oxidative stress by inhibiting lipid preoxidation and activating antioxidant defense enzyme in the system (Saravanan and Prakash 2004). This effect of garlic could be attributed to its highly antioxidant constituents such as allicin, vitamins and phenolic compounds. The cytoprotective properties of CoQ<sub>10</sub> are related to a reduction of ROS generation and oxidized/reduced glutathione ratio in cultured hepatocytes (Gonzalez *et al.* 2009).

Rutin has been found exert a protective effect against lipid peroxidation reaction *in vitro* as free radical scavenger and metal chelating agent. The inhibitory effect of rutin on lipid peroxidation and production of MDA depend mainly on its superoxide dismutation activity since it has been suggested that superoxide ion participates in the initiation of lipid peroxidation reaction and production of MDA (Afanas'ev *et al.* 1998).

The present study revealed that serum NO level significantly increased in EAC bearing mice. Overproduction of NO has been implicated in the tissue damage caused by inflammation, contributing to tumor promotion. It has been reported that elevated level of lipid peroxidation and its products stimulate host cells, mainly monocytes/ macrophages to produce and release NO by the induction of inducible nitric oxide synthase (iNOS) activity resulting in tissue as well as DNA damage (Raso *et al.* 2001).

Supplementation with primrose in combination with  $\omega^3$ FA showed significant decrease in serum NO level in EAC bearing mice. Primrose through its active constituent LA has been found to decrease the production of proinflammatory products such as NO and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). LA acts as anti-inflammatory agent and it could decrease the expression of iNOS mRNA and iNOS promoter activity and consequently NO production LA suppressed iNOS expression at least in part through inactivation of NF- $\kappa$ B as the activation of NF- $\kappa$ B involved in the transcriptional expression of iNOS gene (Sheu *et al.* 2006).  $\omega^3$  FA could reduce serum NO level via its effect on NO synthesis through the inhibition of iNOS protein expression (Aldrige *et al.* 2008).

Silymarin in combination with NAC produces significant decrease in serum NO level in EAC bearing mice and this finding could be explained as silymarin suppressed iNOS gene expression as reported by Gu *et al.* (2003) who demonstrated that silymarin causes a strong protective effect against carcinogenesis via down-regulation of inflammatory and angiogenic responses including iNOS, and this effect is mediated through the inhibition of NF- $\kappa$ B/Rel transcription factor which is the critical regulator of iNOS gene expression Silymarin suppresses cytokine-induced NO production. These cytokines include interferon- $\gamma$  (IFN- $\gamma$ ) and/or interleukin-1 $\beta$ -induced iNOS expression. The antioxidant NAC has been shown to reduce NO production via the inhibition of iNOS activity. The inhibition of iNOS activity is accompanied by a decrease in iNOS mRNA level (Araki *et al.* 2007). There is data constitute preliminary evidence that NAC might have cytoprotective actions of potential relevance against oxidative stress, mediated partially through the modulation of NO production (Mansour *et al.* 2008).

A combination of garlic with CoQ<sub>10</sub> and rutin significantly decreased serum NO level in mice bearing EAC. The active compounds present in garlic could suppress NO synthesis and this action is mediated via a reduction of iNOS mRNA expression which leads to decreasing iNOS mRNA stability or reducing transcriptional activity. Chang *et al.* (2005) suggested that the inhibitory effect of garlic on NO level is mainly due to inhibiting the expression of iNOS protein and partly by iNOS enzyme inhibition as well as increasing NO clearance. The antioxidant property of CoQ<sub>10</sub> allows it to inhibit free radicals including NO production. Rutin has been found to inhibit nitrite production by activated macrophage in vitro (Guruvayoorappan and Kuttan 2007).

The results of the current study revealed significant reduction in serum total antioxidant capacity (TAC) in EAC bearing mice. This finding could be explained as the excess production of free radicals can be detoxified by the endogenous antioxidants causing their cellular stores to be depleted (Velmurugan and Nagini 2005). The reduction of SOD activity may be due to loss of Mn<sup>2+</sup>-containing SOD activity in EAC bearing mice and the loss of mitochondria, leading to a decrease in total SOD activity in the liver. Also, an inhibition of catalase (CAT) activity is recorded in EAC-bearing mice. The inhibition of SOD, CAT activities and a reduction in glutathione level as a result of tumor growth were also reported (Gupta *et al.* 2004). This phenomenon could be attributed to the exhaustion of these antioxidants especially glutathione and glutathione containing enzymes in the detoxification of free radicals and peroxides, generated due to tumor inoculation. These free radicals conjugate with GSH ultimately protects the cells and organs from oxidative stress. This action of these antioxidant enzymes results in a depression of overall CAT activity as well as SOD activity. This finding is greatly supported by Raj Kapoor *et al.* (2007). Moreover, Ekambaram *et al.* (2008) attributed the decrease in the total antioxidant level (enzymatic and non enzymatic), due to tumor inoculation, to the increasing of circulating lipid peroxides which reportedly leads to the accumulation of superoxide anions that are capable of traversing membranes causing deleterious effects at sites beyond the tumor.

Primrose in combination with  $\omega^3$  FA produced significant increase in serum TAC in mice bearing EAC. Primrose could reduce oxidative stress not only by inhibiting lipid peroxidation, but also by reinforcing the glutathione-dependent antioxidant defense system.  $\omega^3$  FA has been also reported to improve oxidative stress and stimulate the antioxidant defense system (Bouzidi *et al.* 2010).

The present study showed that silymarin together with NAC could significantly enhance the TAC in EAC bearing mice. Silymarin could increase the activities of the antioxidant enzymes in the animals administered with carcinogen. Silymarin could also protect against oxidative stress via reversing the oxidant-antioxidant imbalance during carcinogenesis (Kiruthiga *et al.* 2007). Animals treated with NAC showed an improvement in the antioxidant activity as indicated by the increase in the total antioxidant status in serum. Also, the administration of NAC to high fat diet fed rats prevented the buildup of oxidative stress by restoring normal activities of the enzymatic antioxidant and normal levels of the non-enzymatic antioxidant in serum (Yang *et al.* 2006).

Administration of garlic and CoQ<sub>10</sub> in combination with rutin could significantly enhance the TAC in EAC bearing mice. This effect has been attributed to modifying the balance between oxygen radicals and antioxidant defense and their strong antioxidant properties (Perumal *et al.* 2005 and Wang *et al.* 2008).

The decreased activity of hepatic Na<sup>+</sup>, K<sup>+</sup>-ATPase in mice bearing EAC in the present study could be attributed to the decreased expression of mRNAs of  $\beta$ -isoform ( $\beta_1$  and/or  $\beta_2$ ) of ATPase enzyme. This down regulation of  $\beta$ -subunits has been suggested to be associated with the loss of tight junctions and epithelial polarity of the liver cells of mice inoculated with cancer cells (Rajasekaran and Rajasekaran 2003). Na<sup>+</sup>, K<sup>+</sup>-ATPase activity is blocked in EAC implanted animals as a result of the production of specific inhibitor protein (IF<sub>1</sub>) from cancer cells. Therefore, the decreased activity of hepatic Na<sup>+</sup>, K<sup>+</sup>-ATPase in mice inoculated with EAC may be resulted from some changes such as the altered molecular structure of the Na<sup>+</sup>, K<sup>+</sup>-ATPase, change in membrane fluidity and/or inhibition of its activity by specific protein. Moreover, it has been demonstrated that there is another growth related protein called translation ally controlled tumor protein (TCTP). This protein is correlated with cell cycle progression and malignant transformation (Tuynder *et al.* 2002). This protein acts as a cytoplasmic suppressor of Na<sup>+</sup>, K<sup>+</sup>-ATPase by interacting with the third cytoplasmic domain of its catalytic  $\alpha$ -subunit. Over expression of TCTP during carcinogenesis and this is responsible for the inhibition of Na<sup>+</sup>, K<sup>+</sup>-ATPase activity (Kim *et al.* 2008).

Administration of primrose together with  $\omega^3$  FA significantly stimulated hepatic Na<sup>+</sup>, K<sup>+</sup>-ATPase activity in EAC-bearing mice. GLA can activate both Na<sup>+</sup>, K<sup>+</sup>-ATPase and Ca<sup>2+</sup>-ATPase. This action of GLA could be attributed to its ability to correct the lipid profile of the membrane by modulation of steroid-induced protein synthesis and/or by being converted to an eicosanoid with several possible actions and/or by action on phospholipases A and C or G proteins beside its direct effect to activate protein kinases which would result



in phosphorylation of membrane proteins. These mechanisms could all play a role in the modulation of membrane ATPases (Haag *et al.* 2000).  $\omega^3$  FA has been found to enhance  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity via phosphorylation of its  $\alpha$ -subunit with protein kinase C (Haag *et al.* 2003).

The current study demonstrated that the administration of silymarin in combination with NAC causes significant increase in hepatic ATPase activity in EAC-bearing mice. Silymarin has been found to prevent lipoperoxidation and its associated cell damage in experimental models (Soto *et al.* 1998). This property makes silymarin able to participate in preventing the loss of ATPase activity. NAC could restore oxidant/antioxidant balance and maintain cell integrity. Also it offers protection of membrane bound enzymes ( $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and  $\text{Ca}^{+2}$  - ATPase) by restoring the lipid composition along with the activity of these ATPases. These properties of NAC may be responsible for the observed increase in the activity of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, in participation with silymarin in EAC-bearing mice in the current study (Kamboj *et al.* 2006).

Garlic in combination with  $\text{CoQ}_{10}$  and rutin significantly enhanced hepatic ATPase activity in EAC-bearing mice. Garlic extract administration has been shown to stimulate liver ATPase activity. The suggested mechanism is related to the antioxidant activity of garlic since the enhanced susceptibility of membrane to lipid peroxidation leads to loss of ATPase activity and altered cell functions. Antioxidant compounds in garlic could activate ATPase activity via the inhibition of radical generation and lipid peroxidation of the membrane along with the preservation of mitochondrial function (Pari and Murugavel 2007).  $\text{CoQ}_{10}$  is essential component of ATP generation in the oxidative phosphorylation process. In addition  $\text{CoQ}_{10}$  has a potent antioxidant activity and it could prevent lipid peroxidation of the cell membrane (Choi *et al.* 2005). These properties of  $\text{CoQ}_{10}$  may account for its ability to stabilize cell membranes preserving cellular integrity and function, and help in restoration of ATPase activity (Lass and Sohal 2000).

Rutin cooperate with garlic and  $\text{CoQ}_{10}$  to cause an increase in hepatic ATPase activity. We could propose that the antioxidant and free radical scavenging activity of rutin may be responsible for its stimulatory action on ATPase (Jiang *et al.* 2007).

The results of the present study revealed that hepatic sodium concentration shows significant decrease, while hepatic potassium concentration shows significant increase in EAC-bearing mice. These results are in accordance with those of Ibsen and McKee, (1967). These findings could be explained as the net transport of  $\text{Na}^+$  and  $\text{K}^+$  ions by liver cells is almost entirely dependent on the energy made available by oxidative phosphorylation and the energy is lost during carcinogenesis.

Administration with a combination of primrose and  $\omega^3$  reverses the changes in hepatic sodium and potassium concentrations in EAC bearing mice. The effect of this combination could be attributed to the stimulation of sodium channels by n-3 fatty acids via membrane-bound kinase (Mies *et al.* 2007). At the same time, these polyunsaturated fatty acids have been found to inhibit potassium channels (Poling *et al.* 1996). By this action, the combination of primrose and  $\omega^3$  fatty acid could restore the balance between sodium and potassium in hepatic tissue.

Administration of silymarin in combination with NAC could restore hepatic sodium and potassium concentrations in EAC-bearing mice. Herein, we could suggest that the stimulation of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activity due to silymarin and NAC administration may contribute in the regulation of hepatic sodium and potassium concentration in EAC-bearing mice. Moreover, Kuo *et al.* (1993) stated that the treatment with NAC inhibits potassium channel activity in cells exposed to ionizing radiation. The restoration of glutathione content of the cells contributes in the protective effect of NAC on potassium channel (Shartava *et al.* 1999).

Garlic in combination with  $\text{CoQ}_{10}$  and rutin modulated hepatic sodium and potassium concentration in EAC-bearing mice. Our results go hand in hand with Chetty *et al.* (2004) who supported the effect of garlic in modulation of liver trace element contents including sodium and potassium. The possible mechanism of action in this regard could be related to the enhancement of hepatic  $\text{Na-K}^+$ ATPase activity by garlic supplementation. The antioxidant properties of garlic and the inhibition of free radical generation are contributed in this effect.  $\text{CoQ}_{10}$  is essential for the antioxidant defense system. It could also restore ATP synthesis and attenuate oxidative stress (Quinzii *et al.* 2008). This property of  $\text{CoQ}_{10}$  may be responsible for its effect in correcting sodium and potassium concentrations in the liver of EAC-bearing mice. Rutin has a powerful antioxidant activity and membrane-stabilizing action as well as it has the ability to increase ATPase activity (Zyma 1988). These properties of rutin may be involved in its modulating action on hepatic sodium and potassium contents in the current study.

In conclusion, the present study provided obvious evidence on the beneficial effects of the selected nutraceutical combinations in reducing tumor growth and counteracting the metabolic disorders associated with EAC inoculation. These positive effects may be attributed to their anti-inflammatory properties, powerful antioxidant activities. Noteworthy, the protective effect of the selected nutraceutical combinations were more promising than the therapeutic one and the combination of primrose with  $\omega^3$  was the most effective one.

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