Antiangiogenic Effects of the Apigenin and/or Selenium on Ehrlish Bearing Mice

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Abstract

Apigenin, a plant flavenoid compound has been claimed for its antioxidant, anti inflammatory and recently antiangiogenic property. In this paper, the antiangiogenic activity of apigenin and selenium was tested in vitro as well as in vivo via systemic intramuscular injection targeting the improvement of cancer therapeutic protocols. The angiogenic regulators Matrix metalloproteinase 2 and 9(MMP2 and MMP9), the expression of tumor necrosis factor- α (TNF-α; Key cytokine) as well as the antioxidant markers catalase, superoxide dismutase and glutathione peroxidase (CAT, SOD and GPX) and DNA fragmentation were estimated to monitor efficacy of Apigenin and selenium in cancer treatment strategy. All parameters were determined as a time course on days 16 and 22 after tumor volume reached $1mm^3$. The using of MTT assay on Ehrlich ascites carcinoma (EAC) cells in vitro showed that apigenin and /or selenium inhibit EAC cells proliferation. In vivo, Administration of apigenin and /or selenium to mice bearing tumor reduced significantly the TNF-α expression, MMP 2 and 9 levels and nitric oxide (NO) concentration while, increased the liver antioxidant enzymes (CAT, SOD and GPX) activities. The DNA fragmentation and the expression of antiangiogenic factors TIMP-1 in spleenocytes were significantly increased when compared with their values in mice bearing tumor. From the results obtained, it could be postulated that the synergistic administration of apigenin and selenium might improve the condition of cancer therapy via decreasing the tumor metastasis possibility in addition to increase cancer cells apoptotic transforming factors and conservation of normal cells antioxidant parameters.

Introduction:

Cancer can be viewed as the result of a succession of genetic changes during which a normal cell is transformed into a malignant one while evasion of cell death is one of the essential changes in a cell that cause this malignant transformation **(Hanahan and Weinberg, 2000)**.Apoptosis had linked to the elimination of potentially malignant cells, hyperplasia and tumor progression **(Fulda, 2009)**.New growth in the vascular network is important since the proliferation, as well as metastatic spread, of cancer cells depends on an adequate supply of oxygen and nutrients and the removal of waste products. New blood and lymphatic vessels form through processes called angiogenesis and lymph angiogenesis, respectively. Angiogenesis is regulated by both activator and inhibitor molecules **(Mehmet *et al*., 2012)** . Angiogenesis is a fundamental process in reproduction and wound healing. Under these conditions, neovascularization is tightly regulated. Unregulated angiogenesis may lead to several angiogenic diseases and is thought to be indispensable for solid tumor growth and metastasis. The construction of a vascular network requires different sequential steps including the release of proteases from "activated" endothelial cells with subsequent degradation of the basement membrane surrounding the existing vessel, migration of endothelial cells into the interstitial space, endothelial cell proliferation, and differentiation into mature blood vessels. These processes are mediated by a wide range of angiogenic inducers, including growth factors, chemokines, angiogenic enzymes, endothelial specific receptors, and adhesion molecules. Finally, when sufficient neovascularization has occurred, angiogenic factors are down-regulated or the local concentration of inhibitors increases. As a result, the endothelial cells become quiescent, and the vessels remain or regress if no longer needed. Thus, angiogenesis requires many interactions that must be tightly regulated in a spatial and temporal manner. Each of these processes presents possible targets for therapeutic intervention. Many different proteins have been identified as angiogenic activators (TNF-α, MMP2, MMP9) in addition to NO and other acting as inhibitors (TIMP1). Levels of expression of angiogenic factors reflect the aggressiveness of tumor cells. The discovery of angiogenic inhibitors should help to reduce both morbidity and mortality from carcinomas **(Mehmet *et al*., 2012)**. Targeting inhibition of angiogenesis represents a potential approach in the treatment of solid tumors and such antiangiogenic strategies inhibiting the growth of endothelial cells may be more advantageous than targeting cancer cells. **(Hanahan and Folkman 1996)**. The plant foods that are rich in antioxidants, including carotenoids and flavonoids are considered to induce cardiovascular protection, improve endothelial function, inhibit angiogenesis, and inhibit cell migration and proliferation in blood vessels (**Yamagata et al., 2011).** Apigenin; a flavenoid found mainly in orange, tea, chamomile, onion, and wheat sprouts, has received much attention, due to its strong anticancer effect in various cancer cells, including breast cancer, colon cancer, lung cancer, neuroblastoma, liver cancer, prostate cancer, pancreas cancer, and oral cancer cells **(Begum *and* Prasad, 2012).** Selenium is an essential trace element existing in organic and inorganic

chemical forms which have been shown to play an important role in maintenance of an optimal physiological state of mammalian cells. It has recognized a chemopreventive potential against various forms of environmental stress as well as against tumor development (**Kralova et al., 2012)**.

The present study was to evaluate the influence of the angiogenic regulators modification on the tumor growth targeting the improvement of cancer therapeutic protocols. Thus, the action of apigenin and/or selenium was examined in vitro on Ehrlich ascites carcinoma cells and in vivo in mice bearing Ehrlich cells (a model of solid carcinoma tumor).

Material and Methods:

Experimental animals

All animal procedures and experimental protocols were approved by the Research Ethics Committee and were carried out in accordance with the guide for the care and use of laboratory animals. Swiss albino mice weighing 20 to 25 g were obtained from the Egyptian Organization for Biological Products and Vaccines (Vacsera, Egypt) and housed under controlled conditioning (25±1 °C constant temperature, 55% relative humidity, 12 h dark/ light cycles). Food and water were allowed *ad libitum* during the study period.

Tumor cell line:

Ehrlich ascites carcinoma (EAC) cell line was purchased from the tumor Biology Department, National Cancer Institute, Cairo University. EAC is a murine spontaneous breast cancer that served as the original tumor from which an ascites variant was obtained. Intraperitoneal inoculation resulted in the production of ascites rich in tumor cells. The tumor cell line was maintained in our laboratory by serial intraperitoneal (i.p.) passage in female Swiss albino mice at 7–10 day interval. The EAC cells were prepared under aseptic conditions. EAC cells were tested for viability and contamination using Trypan blue dye exclusion technique (Lazarus et al., 1966). EAC cells were suspended in normal saline so that each 0.2 ml contains 2.5×10^6 EAC cells. Cells were counted under the microscope using Neubauer hemocytometer.

***In vitro* study**

MTT cell proliferation and viability method was used for the measurement of cell proliferation or when metabolic events lead to apoptosis. It is based on the transformation and colorimetric quantification of MTT (3- (4, 5 – dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide). All electron transport systems reduce MTT and other tetrazolium salts and thereby form non-water-soluble violet formazan crystals within the cell. The amount of these crystals can be determined spectro photochemically and serves as an estimation for the mitochondrial activity and hence the number of living cells in the sample **(Freimoser *et al.*, 1999)**.

***In vivo* study**

Induction of solid tumors: Solid tumors were produced by intramuscular inoculation in the right thigh of the lower limb of each mouse with 0.2 ml of EAC cells, which contained 2.5 x 10^6 viable EAC cells. Mice with a palpable solid tumor mass (100 mm3) that developed within 14 days after inoculation were used in the study. Tumor volume (TV) was measured at different time intervals during the experimental period (days 6, 13, 16, and 22) using a Vernier caliper and calculated (**Papadopoulos *et al.*, 1989)**.

Treatments (Apigenin and/or selenium): Apigenin was dissolved in dimethyl sulphoxide (DIMSO) and given to mice by intraperitoneal (i.p.) injection of the maximal tolerated dose (50 mg/kg body weight) as described previously by **Singh et al., 2004**, once every day for 14 days. Selenium was dissolved in 0.9% saline and given to mice by intraperitoneal (i.p.) injection of the maximal tolerated dose (5µg/mice) (**Poirier et al ., 1983)**, once every day for 14 days.

Work design: Forty eight mice were randomly assigned to 8 main groups, 6mice each, and were treated as follows. The first group received vehicle injection (DIMSO in PBS) and served as control. Group (2) : mice wrere treated by i.p. injection of apigenin (50 mg/kg b.wt/day) for 14 consecutive days. Group (3) mice were treated by i.p. injection of selenium (5µg/mice) for 14 consecutive days. Group (4) : mice were treated by i.p. injection of amixture of apigenin (50 mg/kg b.wt/day) and selenium (5µg/mice) for 14 consecutive days .Group (5) 20 mice bearing solid Ehrlich tumor . Group (6) mice bearing solid Ehrlich tumor were received 14 successive apigenin dose starting from the 15^{th} day after EAC inoculation. Group (7) mice bearing solid Ehrlich tumor were received 14 successive selenium dose starting from the 15^{th} day after EAC inoculation. Group (8) mice bearing solid Ehrlich tumor were received 14 successive apigenin and selenium dose starting from the 15^{th} day after EAC inoculation.

***Sample collections*:** Animals were fasted for 16 hours before each sampling. Samples were collected after 16 and 22 days post the tumor volume reached $1mm^3$ Animals were sacrificed and the blood was collected from heart puncher and left for coagulation. The blood was centrifuged and the upper layer (serum) was taken. MMPs were measured in serum of each group. Portion of liver was dissected, weighed and homogenized in physiological saline (9g Na Cl /1000 ml distilled water)for antioxidant and nitric oxide detection . Portion of spleen was removed from animal after dissection and immediately putted at liquid nitrogen, then stored at – 80°C for RNA extraction and conventional PCR analysis, the remaining portion of spleen were used for splenocyte isolation and DNA fragmentation .

Molecular examinations:

The total cellular RNA was extracted from spleen tissue using **RNeasy** mini kit from **Qiagen** according to the manufacturer's protocol. The improvement of RNA extraction and the increase of RNA purity were

carried out by using Qia shredder column. Total RNA was reverse transcribed for 1 h at 37^0C in a reaction mixture containing 5 U RNase inhibitor , 0.5 mM dNTP, oligo dT primer, 1x RT buffer and 5 U MuMLV reverse transcriptase (Qiagen) in a 50 µl reaction. The mixture was placed at room temperature for 10 min, 42^0C for 45 min, and 90^0C for 3 min, and then rapidly cooled on ice. cDNA (1 µl) was PCR amplified. For PCR reaction of TNF-α, and GAPDH, the following conditions were used: one cycle at 95^0C for10 min followed by 94^0C for 50 Sec, 55^0C for 50 Sec, and 72^0C for 50 Sec, for 35 cycles, and one cycle at 72^0C for 10 min. Conditions of TIMP PCR reaction were , one cycle at 95^0C for5 min followed by 94^0C for 60 Sec, 50^0C for 45 Sec, and 72^0C for 60 Sec, for 30 cycles, and one cycle at 72^0C for 10 min.

PCR primers are as below:

- For mRNA of *GAPDH* gene (housekeeping gene) **(Qiu *et al* .,2007):**

Forward primer: 5' - GACCCCTTCATTGACCTCAAC-3'
Reverse primer: 5' - CTTCTCCATGGTGGTGAAGA-3'
Expected product size: 502 bp

- For mRNA of TNF-α gene **(Qiu *et al* .,2007):**

Forward primer: 5'- ACGGCATGG ATCTCAAAGAC-3'
Reverse primer: 5'- CGGACTCCGCAAAGTCTAAG-3'
Expected product size: 324 bp

- For mRNA of TIMP-1gene (**Furukawa *et al* ., 1998)**:

Forward primer: 5'- CCT TTG CAT CTC TGG CAT CTG GCA-3'
Reverse primer: 5'- CCAACA GCC AGC ACT ATA GGT GTT-3'
Expected product size: 437 bp

Analysis of the resulting PCR products on 1% agarose gels showed single-band amplification products with expected sizes. Semi quantitation was performed using gel documentation system (BioDO, Analyser, Biometra, Gottingen, Germany). According to the amplification procedure, relative expression of each studied gene (R) was calculated according to the following formula: densitometrical units of each studied gene/densitometrical units of GAPDH. The presence and activity of specific MMP species (MMP -2 and 9) were initially detected in the serum using substrate (gelatin) gel electrophoresis **(Birkedal-Hansen and Taylor 1982)** . A buffer of 4% SDS, 0.15 mol/L Tris (pH 6.8), 20% glycerol and 0.5% (w/v) bromophenol blue was added to the serum sample. Serum samples mixed with buffer were directly added to 10% SDS–acrylamide gel containing 0.1% (w/v) gelatin (sigma) and separated by running on a mini gel apparatus at 15 mA/gel, and then gels were gently rocked in a 2.5% Triton X-100 solution for 30 min at room temperature. Gels were then incubated overnight at 37 °C in substrate buffer containing 50mmol/L Tris–HCl (pH 8), 5 m mol/L CaCl2 and 0.02% NaN3. Gels were subsequently stained for 30 min in 0.5% Coomassie Blue R-250 dissolved in a 1:3:6 solution of acetic acid, isopropyl alcohol and water. The gel was scored for the presence/absence MMP activity by a blinded evaluator and photographed. MMP-2 and MMP-9 could be detected on the SDS gel as transparent bands.

Biochemical assays: Nitric oxide level in the liver was determined as nitrite by Griess reaction (**Miranda *et al.*, 2001**). Briefly 100 µL sample was incubated with equal amount of Griess reagent (one part of 0.1% N(1-naphthyl)-diamine dihydrochloride in distilled water and one part 1% sulfanilamide in 5% concentrated H3PO4) and incubated for 10 min at room temperature and the absorbance of color developed was measured at 540 nm. The glutathione peroxidase (GSH-Px) activity level was assayed by method relies on the following reaction: $2\ GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$. The residual GSH concentration was calculated and the amount of GSH consumed per unit time is a measure of the catalytic activity of GPx **(Gross *et al*., 1967)**. Superoxide dismutase (SOD) activity was estimated by detection of superoxide anions using nitroblue tetrazoluim formazan color development (**Minami and Yoshikawa, 1979)**. Catalase (CAT) activity was assayed in tissue homogenate. The dichromate/acetic acid reagent can be thought of as a stop bath for catalase activity. By addition of acetic acid to reaction medium, catalase activity destroyed and any hydrogen peroxide which hasn't been split by the catalase will react with the dichromate to give a blue precipitate of perchromic acid. This unstable precipitate was then decomposed by heating to give the green solution; this green color was measured photometerically at 570 nm **(Sinha, 1972**).

DNA fragmentation assay was performed according to **Sellins and Cohen (1987)** with modification. DNA extract was stained by 1 µl of ethidium bromide and loaded on each well of a 1.5% agarose gel. The gel was visualized under an UV light and photography was performed with a transilluminator.

Results

In Vitro

The MTT test shows significant alteration in the EAC cells viability when incubated with different concentrations of apigenin and/or selenium. Apigenin and sodium selenite inhibit cells growth in a dose and time dependent manner. The maximal inhibitory concentration of Apigenin and sodium selenite were approximately 80 µg/ml and 5µg respectively, after 24 h of incubation. When cells incubated with 30 µg Apigenin and 5µg sodium selenite, synergistic anti proliferative effect was observed. The sodium selenite and Apigenin combination induced 81% inhibition of cell growth (Fig 1).

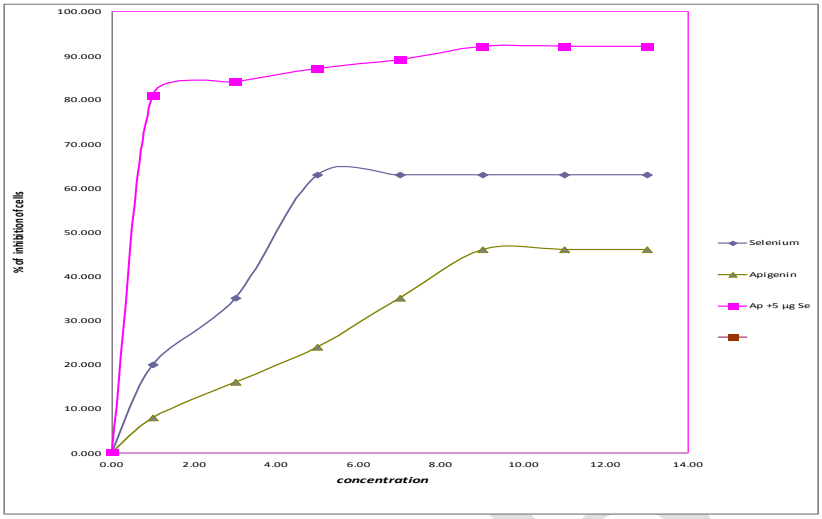

Fig (1): Cell Viability at different Apigenin and/ or Selenium concentrations

In vivo

Tumor volume: A consecutive treatment of the animals with apigenin , selenium or their combination for 14 day starting after the tumor volume reached 1 mm^3 (14 day after tumor inoculation) caused a marked suppression of tumor growth at days 6, 13, 16 and 22 from the day of tumor volume reaching 1 mm^3 . The (E +AP + Se) mice reveal the most reduced tumor volume as compared with other groups.

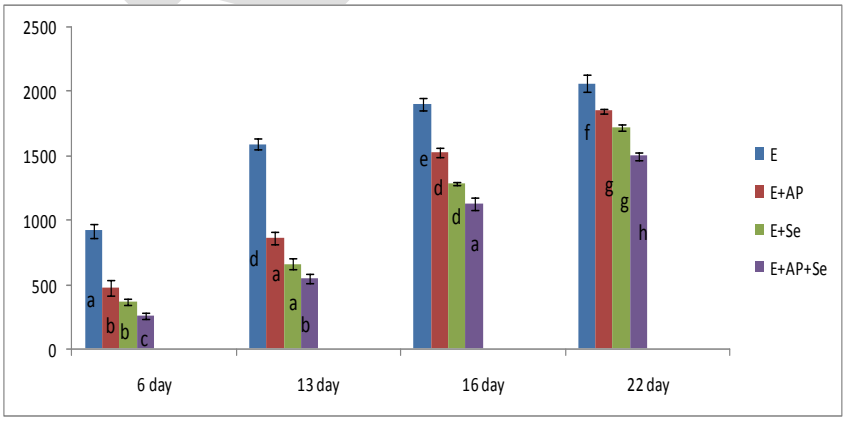

Fig (2): Values of tumor volume in different animal groups

The columns with unlike label letters are considered significantly different at P< 0.05.

TNF-α expression: A significant induction of TNF-α expression (1580%; at P<0.05 on the 1st experimental interval) was observed in samples isolated from the spleen of Ehrlich bearing mice with substantial increases on the 2ed experimental interval (2173 % at P<0.05).The pretreatment with Ap, Se or Ap+ Se of mice bearing tumor induced remarkable modulation in TNF-α gene expression compared to TNF-α gene expression in Ehrlich bearing mice however a lesser effect was recorded on the 2ed experimental interval. Further, the administration of Ap +Se induced more pronounced decrease in TNF-α gene expression than Apigenin or selenium alone (Fig 3).

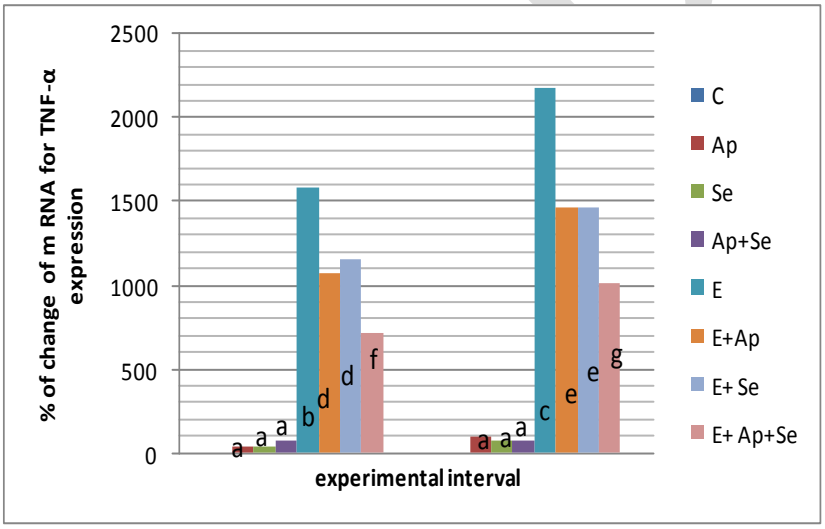

Fig (3): percent of change of TNF-α m-RNA expression in different animal groups

The zero line represents the control values, take label a and Columns represents the percentage of changes from control. The columns with unlike label letters are considered significantly different at P< 0.05.

Serum MMP-2 and MMP9: A significant increases (P<0.05) in MMP2 and MMP9 concentration (131 and 92 % from control values respectively) were observed in samples isolated from Ehrlich bearing mice) on the 1st experimental interval substantial increase (P< 0.05) on the 2ed experimental interval (271 and 139%, respectively). The pretreatment of mice bearing with Ap, Se or Ap+ Se tumor according to the present protocol induced remarkable modulation in MMP2 and MMP9 concentration as compared to their concentration in Ehrlich bearing mice however a lesser effect was recorded on the 2ed experimental interval. The result obtained revealed that the administration of Ap +Se induced more pronounced decrease in MMP2 and MMP9 concentration than Apigenin or selenium alone (Fig 4,5).

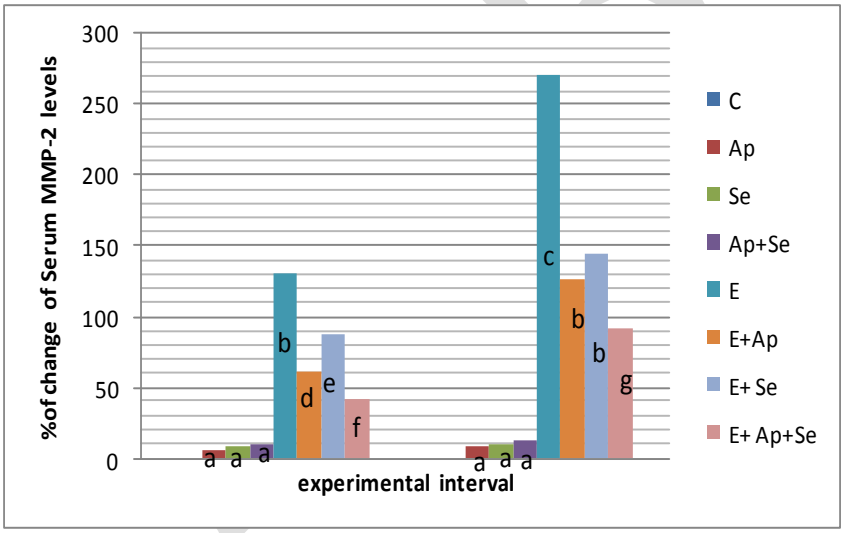

Fig (4): percent of change of MMP-2 levels in different animal groups

The zero line represents the control values , take label a and Columns represents the percentage of changes from control. The columns with unlike label letters are considered significantly different at P< 0.05.

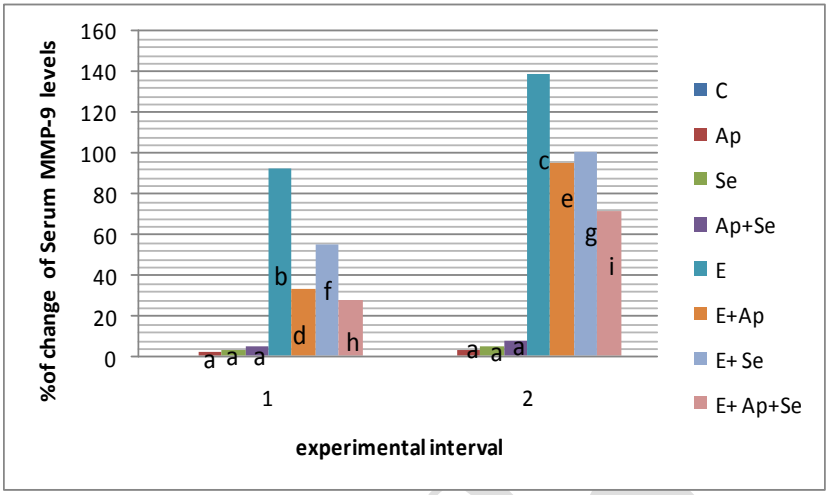

Fig (5): percent of change of MMP-9 levels in different animal groups

The zero line represents the control values , take label a and Columns represents the percentage of changes from control. The columns with unlike label letters are considered significantly different at P< 0.05.

TIMP-1expression: A significant reduction of TIMP-1 expression (63- % ; at P<0.05 on the 1st experimental interval) was observed in samples isolated from the spleen of Ehrlich bearing mice with substantial decreases at the 2ed experimental interval (76-% at P<0.05).The pretreatment with Ap, Se or Ap+ Se to mice bearing tumor induced remarkable increase in TIMP-1 gene expression as compared to TIMP-1 gene expression in Ehrlich bearing mice. However a lesser effect was recorded on the 2ed experimental interval. The administration of Ap + Se to E, R or E+R mice induced more pronounced increases in TIMP-1 expression than apigenin or selenium alone (Fig 6).

The zero line represents the control values, take label a and Columns represents the percentage of changes from control. The columns with unlike label letters are considered significantly different at P< 0.05.

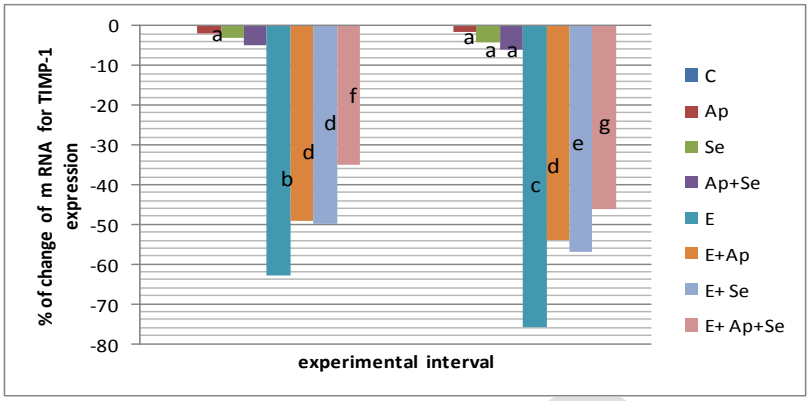

Fig (6): percent of change of TIMP-1 m-RNA expression in different animal groups

Liver NO: A significant increases ($P<0.05$) in NO concentration (65% from control value) was observed on the 1st experimental interval in samples isolated from the liver of Ehrlich bearing mice with substantial increases(95%) on the 2ed experimental interval. The pretreatment of mice bearing tumor with Ap, Se or Ap+ Se according to the present protocol induced remarkable modulation in NO concentration as compared to NO concentration in Ehrlich bearing mice. However a lesser effect was recorded on the 2ed experimental interval. The Ap + Se exert more obvious decrease in liver NO concentration than Apigenin or selenium alone (Fig 7).

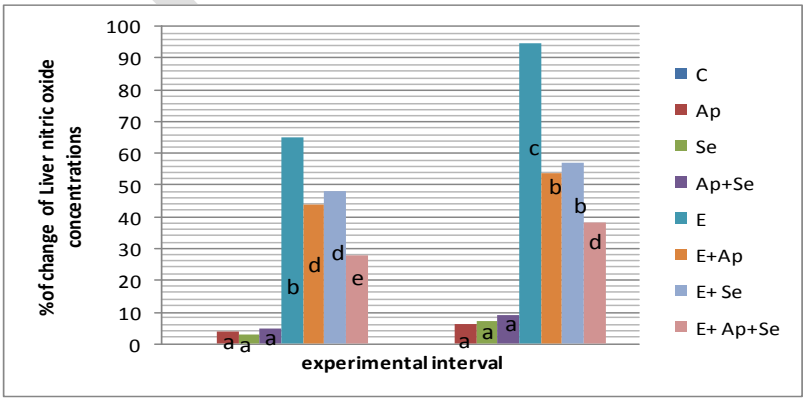

Fig (7): percent of change of NO concentrations in different animal groups

The zero line represents the control values, take label a and Columns represents the percentage of changes from control. The columns with unlike label letters are considered significantly different at P< 0.05.

Liver antioxidant enzymes: The data obtained reveals significant decreases (P<0.05) in GPX activity (-135%),SOD (-45%) and CAT (-19%), respectively. This was observed in samples isolated from the liver of Ehrlich bearing mice when compared to their values in control mice on the 1st experimental interval) with substantial reduction on 2ed experimental interval (-17, -50 and -30%), respectively. The pretreatment of mice bearing tumor with Ap, Se or Ap+ Se according to the present protocol induced remarkable increase in GSH-PX, SOD and CAT activity compared to their activity in Ehrlich bearing mice. However a minor effect was recorded on the 2ed experimental interval. Further, the synergistic administration of Ap and Se induces the most increases in liver antioxidant enzyme activity compared to either apigenin or selenium (Fig 8,9,10).

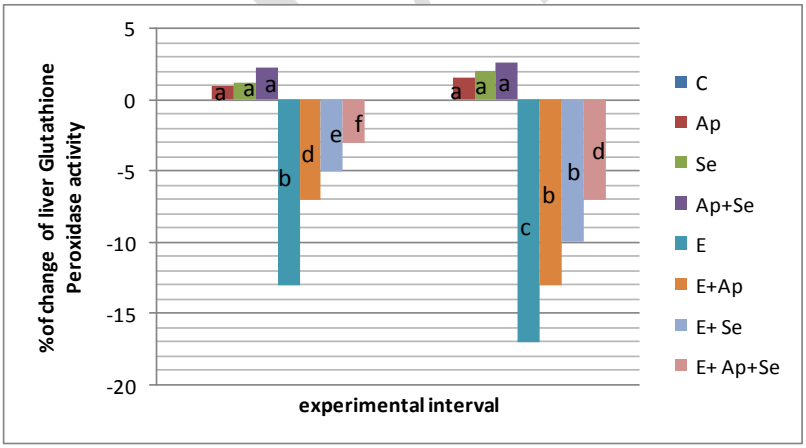

Fig (8): percent of change of GPX activity in different animal groups

The zero line represents the control values, take label a and Columns represents the percentage of changes from control. The columns with unlike label letters are considered significantly different at P< 0.05.

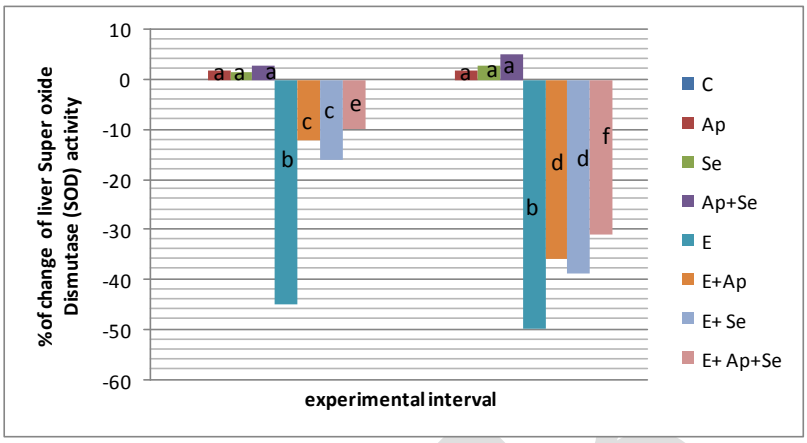

Fig (9): percent of change of SOD activity in different animal groups

The zero line represents the control values, take label a and Columns represents the percentage of changes from control. The columns with unlike label letters are considered significantly different at P< 0.05.

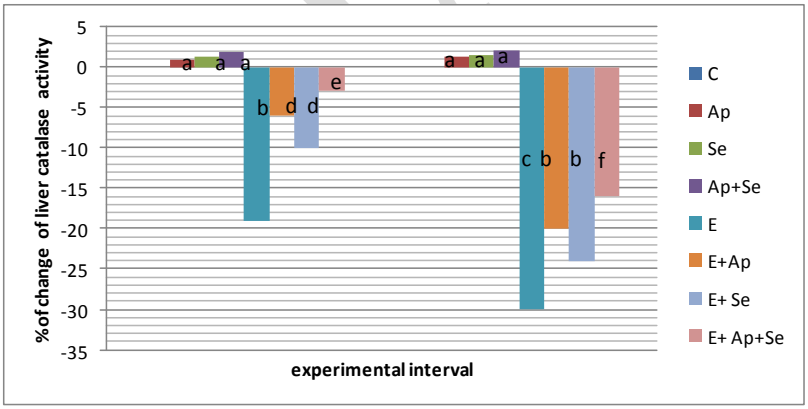

Fig (10): percent of change of CAT activity in different animal groups

The zero line represents the control values, take label a and Columns represents the percentage of changes from control. The columns with unlike label letters are considered significantly different at P< 0.05.

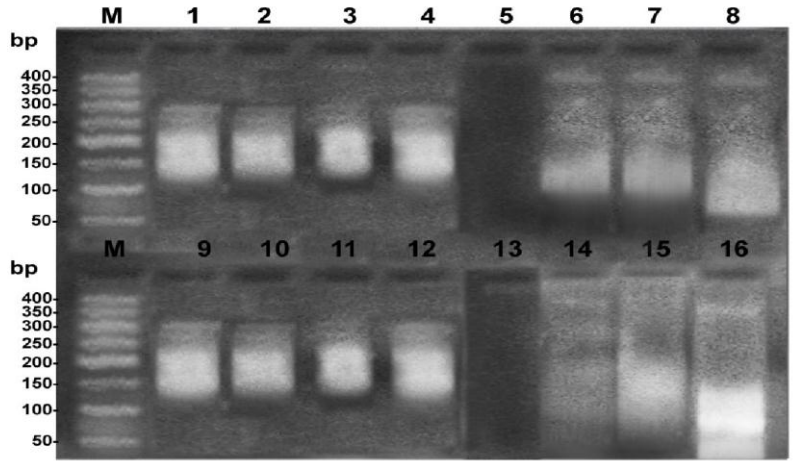

Fig (11): DNA fragmentation electrophoretic pattern

M is the marker lane. Lane: 1, 2, 3, 4 for control, apigenin, selenium and apigenin+selenium respectively. Lane: 5 for EAC bearing mice, lane :6 for E+Ap group ,lane :7 for E+Se group lane 8 for E+Ap+Se group, lane 1-8 for group of the first time interval, lane 9-16 for the same groups at the second experimental time interval .

DNA fragmentation

The electrophoresis pattern of DNA in spleenocyte displayed disappears of fragmentation in mice bearing EAC cells (Lane 5; 1st interval and 13 2nd interval). The administration of Apigenin and /or selenium retrieves the bands which manifest the present of DNA fragmentation (lane 6, 7 and 8; 1st interval and 14, 15, 16; 2nd interval).

Discussion:

The metastatic spread of tumor cells is responsible for the majority of cancer deaths, and with few exceptions, all cancers can metastasize. Clinical findings have long suggested that by providing a pathway for tumor cell dissemination so tumor associated neovascularization is a key component of metastatic spread. Angiogenesis, the growth of new blood vessels from an existing vasculature, is a critical process in the formation of solid tumor growth beyond 1–2 mm in diameter. The Angiogenic process is a balance between stimulatory and inhibitory switch' allowing the tumor to induce the

formation of microvessels from the surrounding host vasculature **(Mehmet *et al.*, 2012)**. The experimental data reveals that the significant increases ($P<0.05$) in TNF-α , MMP2 and MMP9; the angiogenic activators acting as stimulatory switch for the new vascular formation was associated with decreases in TIMP1 concentration an inhibitory switch in mice bearing tumors when compared with their values in normal control mice (Fig 3,4,5,6).The imbalance between angiogenic activators and inhibitors was observed on the 1st experimental interval with substantial alteration on the 2nd interval. The increase in TNF-α gene expression could be interpreted in the view of its role in neovascularization process. TNFα is a pleiotropic, proinflammatory cytokine produced by activated monocytes and macrophages **(Kim *et al.*, 2009).**

Altered levels of proinflammatory and proangiogenic factors are observed in various forms of cancer, TNF α expression was related to differentiation, invasiveness, and angiogenesis of various tumor **(Feng *et al* ., 2011)**. Interaction of cancer cells with endothelial cells is a pivotal step in both tumor angiogenesis and metastatic dissemination. Recently, **Mortensen *et al.*** have observed spontaneous fusion between cancer cells and endothelial cells in vitro and in vivo, which provide a novel type of tumor–endothelial cell interaction and may strongly modulate the biological behavior of tumors **(Mortensen *et al*.,2004)** . TNFα is a major inflammatory mediator that induces multiple changes in Endothelial cell (EC) gene expression including induction of adhesion molecules, integrins, and matrix metalloproteinases (MMPs**) (Song *et al* ., 2012)**.It acts as autocrine growth factors for tumor angiogenesis. These cytokines could be prometastatic or proangiogenic and their deregulated expression directly correlates with the metastatic potential of several human carcinomas **(Yhee *et al* ., 2012)**.The matrix metalloproteinases (MMPs) are a family of enzymes involved in many physiological processes involving matrix remodeling, and appear to be essential in angiogenesis, tumor cell invasion and metastasis. MMPs are considered to be primarily responsible for extracellular matrix (ECM) degradation. These enzymes are zinc-dependent and secreted in inactive

proenzymatic forms that require activation. MMP-2 and 9 (gelatinases A and B) were found to be over-expressed in many invasive tumor cells. Several experiments have confirmed the key role of these enzymes in angiogenesis **(kumar and Kuttan, 2011)**. Our results demonstrates similar pattern in MMP2 and MMP9 changes as in TNF-α (Fig 4,5,3). The significant increase in MMP2 and MMP9 activities in EAC bearing mice could be attributed to TNF- α over expression. TNF-α stimulates MMP-2 and MMP-9 activities in human corneal epithelial cells via the activation of focal adhesion kinase (FAK)/ extracellular regulated protein kinase (ERK) signaling **(Yang *et al.*, 2012)**. The increase in MMP-2 and MMP-9 was associated with significant decrease inTIMP-1 expression . MMP activation generally inhibited by natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs). TIMP-1 decreases migration of endothelial cells and also inhibits capillary tubule formation in vitro and angiogenesis in vivo **(Nahid *et al.*, 2010).** In addition, The experimental data reveals a significant increase ($P<0.05$) in liver NO concentration of EAC bearing mice during the 2 time points of the experiment (Fig 7). The increase in NO concentration might be due to TNF-α over expression. It has been demonstrated that TNF-α is a mediator of NO synthesis. The earliest stages of angiogenesis are defined by vasodilatation mediated by nitric oxide (NO) and an increased vascular permeability of pre-existing capillaries or post-capillary venules in response to VEGF which over expressed by TNF α **(kumar and Kuttan ,2011)**. NO is produced from L-arginine by a family of NO synthase (NOS) isoenzymes. The inducible form of nitric oxide synthase was expressed mainly through TNF- α activated pathways **(kumar and Kuttan, 2011)**. It is not expressed in resting cells and produces NO only when induced by certain cytokines and microbial products or lipopolysaccharide.

Our data shows that the increases in angiogenic activators are accompanied with increases in tumor volume (Fig 2). The neovasculaturization enhances the ability of the tumor to grow as well as increases its invasiveness and metastatic ability **(Xu *et al.*, 2002)**. Moreover, the increases in tumor

volume in mice bearing EAC is associated with significant depletion in antioxidant parameters (GPx , SOD and CAT) in addition to the acceleration in NO synthesis (Fig8,9,10,7). A reduction in GPx along with the decrease in SOD and CAT activity was observed in the liver of tumor-bearing mice **(Manoharan *et al*., 2006)**. Moreover, the tumor growth can cause antioxidant disturbances and acceleration in nitric oxide synthesis in vital organs of the tumor host **(Gonenc *et al*., 2001).** CAT, most abundant in the liver is responsible for the decomposition of H_2O_2 to oxygen and water. SOD reduce superoxide anion ($O_2^{\bullet -}$) into H_2O_2. The glutathione peroxidase (GPx) removes H_2O_2 by coupling its reduction with the oxidation of GSH. Glutathione peroxidase (GPx) can also neutralize other peroxides, such as fatty acid hydroperoxides **(Badr El-Din, 2004).** The decrease in GPx which plays an important role in metabolizing lipid peroxides in the liver is potentially ascribable to inactivation by the increase in ROS or lipid peroxide formations. The loss of mitochondria in tumor host could be responsible for the decrease in total SOD activity in liver tissues of the tumor host **(Sahu *et al* ., 1977).** when the oxidative damage is extreme as a result of tumor growth ROS scavenging enzymes such as SOD,GPX and catalase are degraded which in turn lead to increase in free radical which cause oxidative stress . Free radical and oxidative stress in turn increase the expression of TNF - α which responsible for the successive steps in the angiogenesis process leading to continuous tumor growth. Further, the electrophoresis pattern of DNA isolated from mice bearing EAC cells (+ve control) displayed absence of fragmentation (Fig11) and loss of apoptosis which expressed as increases of tumor volume. Some types of cancers are characterized by defects in apoptosis leading to immortal clones of cells **(Ghobrial *et al*., 2005).** The present study interested in control of angiogenic process as a promising approach in overwhelming cancer which could be occurs through many points in the angiogenic pathway. The antiangiogenic capacity of apigenin supported by selenium was examined. The administration of apigenin and /or selenium to mice bearing tumor induced remarkable improvement in the level of angiogenic activators

(TNF-α, MMP2, MMP9, NO) when compared with their correspondence values in EAC mice (Fig 3, 4,5,7). The pre-treatment with apigenin significantly mitigated serum TNF- α levels in EAC mice. The modulation of the TNF-α triggered VEGF signaling pathway by targeting specific signaling molecules **(Kim *et al* ., 2009).** Apigenin blocked the secretion of IL-8, inhibit COX-2 and iNOS expression in stimulated mouse macrophages and suppresses inflammatory responses through inactivation of NF-kappa B. It attenuates neutrophil and lymphocyte adhesion to endothelial cells and adhesion of monocytes to human umbilical vein endothelial cells HUVECs, by regulating the expression of ICAM and VCAM which leads suppression of angiogenesis **(Bisht *et al* ., 2010).** Moreover, apigenin was observed to have some inhibitory effects on iNOS enzyme activity. It strongly inhibited expression of iNOS responsible for NO production **(Sang *et al* ., 2008)**.

Selenium could inhibit the angiogenesis of hepato- carcinoma, by down regulating the expression of tumor necrosis factor alpha, VEGF and interactions with the factors VEGF, TNF α , IGF-II, NO and T-NOS to delay the development of hepatocarcinoma in rats **(Liu *et al*., 2010)**. Also, sodium selenite lead to inhibition of tumor necrosis factor alpha in Human umbilical vein endothelial cells (HUVECs) and in turn lead to suppression of MMP2 &MMP9 activity with dramatic increase in TIMP expression **(Zhang *et al*., 2002)**. Selenium compounds have inhibitory effect on iNOS, which is the sole, NO generating synthase in macrophages (**Mandy *et al*., 2012)**.

Further, significant regression in tumor volume accompanied with noticeable increase in TIMP1 (angiogenic inhibitor) as well as appearance of fragmented DNA pattern were observed (Fig 6, 11). These results could be attributed to the antiangiogenic and proapoptotic action of apigenin and selenium. Apigenin inhibited the tumor growth and induced apoptotic machinery through suppression of VEGF induced angiogenesis (**Silvan and Manoharan., 2012)**. It exhibited anti-proliferative and pro-apoptotic effects on pancreatic adeno carcinoma cells in vitro and in mice xenograft in vivo **(Jonathan *et al*., 2012)**. In addition, it inhibits hepatoma cell growth both

in vitro and in vivo, which is coupled with induction of G2/M arrest and causes significant apoptosis (**Caia *et al.*, 2011)**. Sodium selenite and other different selenium forms could be able to inhibit cancer metastasis and primary tumor growth in multiple types of cancer in animals (**Liping *et al.* 2011)**.

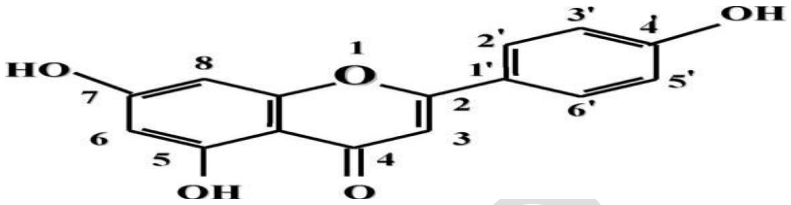

Fig (12): Molecular structure of apigenin **(Mariappan et al., 2012)**.

The experimental data also, shows significant amelioration in the antioxidant parameters (CAT, SOD, GPx) in EAC mice pretreated with apigenin, and /or selenium when compared with their equivalents values in EAC mice (Fig 8,9,10). The increase in the level of these antioxidants after the administration of apigenin may be due to the direct reaction of apigenin with ROS. The free radical scavenging property of apigenin might be attributed to the number of OH groups possessed in 4′, 5 and 7th position of its structure. The antioxidant potency may be attributed to the double bond between carbon atoms two and three of the C ring (Fig 12). The 4 and 5 OH groups with 4 oxo function in A and C rings are giving maximum radical scavenging potency to apigenin **(Noorizi *et al*., 1998).** Se was found to be a component of antioxidant defenses either as agent able to scavenge free radicals, or as a component of a family selenoenzymes. Se incorporates into the first identified selenoprotein; glutathione peroxidase (GPx)**.** Members of the GPx family are effective in catalyzing the reduction of hydrogen peroxide (H_2O_2) and lipid peroxides. The Se-dependent GPx provided a plausible mechanism for cancer prevention. The GPx activity was maximized in tissues of animals fed normal amounts of Se and was not elevated as dietary Se was increased 10-fold at higher levels necessary to achieve chemoprevention. The Se supplements exerted their chemopreventive effect in a manner unrelated to the saturated levels of GPx

(Jayaprakash and Marshall , 2011). Sodium selenite causes cytotoxicity and apoptotic mediated cell death through DNA and mitochondrial membrane potential damage. The cell death (both apoptosis and necrosis) is positively associated with the production of ROS, which can be generated within the mitochondria and can damage mitochondrial components. Mitochondrial membrane potential damage is a nearly sign and also a very sensitive marker of cytotoxicity **(Selvaraj *et al.*, 2013)**. Moreover, Se suppresses cancer cell growth and induces mitochondrial apoptosis via calcium and calcium dependent generation of reactive oxygen species (ROSs) **(Wang *et al.*, 2005).**

It could be postulated that, Apigenin and/or selenium could inhibit cancer growth through controlling the angiogenic process via amendment of the balance between angiogenic activators and inhibitor, maintenance of antioxidant system as well as induction of tumor intercellular ROS.

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