

Investigating the Effect of the Ethanolic Extract of Ginger on the Blood Serum Iron and the Growth of Breast Cancer Tumor in BALB / C Female Mice

Abstract

Objective: Breast cancer is one of the most common cancers in women since a combination of factors are involved in the initiation and progression of breast tumors such as iron that is one of the most essential elements in the body. Given its direct contact with cancer and due to the side effects of the chemotherapy, attention has been paid to the medicinal herbs. Ginger is widely consumed in the worldwide diet as a spice. This plant has anti-oxidant, anti-inflammatory, anti-tumor properties and inhibits free radicals because of its polyphenolic compounds. The aim of this study is to investigate the effect of the ginger on the blood serum iron and the growth of breast cancer tumor in BALB / C female mouse.

Methods: In this study, 25 BALB / C female mice of 6 to 8 weeks, weighing approximately 16-23g were randomly divided into 5 groups in the same condition. The first group was healthy and received extraction solvent (normal saline) and the second cancerous group received normal saline and three experimental cancer groups received doses of 20, 50, 100 mg / kg Ethanolic extract of ginger. The tumors were separated from a mouse with spontaneous breast cancer under sterile conditions and they divided into pieces of approximately five millimeters. Then, the mice were anesthetized and surgically tumor parts were implanted under the skin of the right flank and it was sutured with special clips. All of the groups were treated intraperitoneally for 21 days and once a day. After this period, mice were anesthetized and blood was taken from their heart and the plasma iron levels were measured by Ferrozine method. Then the tumor is removed and its size and weight were also assessed. After recording all data, statistical analysis was performed using one-way ANOVA and Tukey's test by SPSS software and the significance level was $p < 0.05$.

The results showed that there is no significant difference between healthy and cancerous group receiving saline in the serum iron level. The cancerous group receiving dose of 20mg/kg compared to the cancerous group receiving saline showed a significant decrease in the serum iron level. The size and weight of tumor in cancerous group receiving 50mg/kg dose of Ethanolic extract of ginger showed a significant decrease compared to the cancerous group receiving saline.

Conclusion: The results suggest that ginger Ethanolic extract has lowering effects on the growth of the tumor and blood serum iron in the female mice with the breast cancer tumor.

Keywords: Breast Cancer, Ginger Ethanolic Extract, Serum Iron, Tumor Growth, BALB / CFemale Mice.

Introduction

Breast cancer is the most common cancer in women and the main cause of death is malignancy in 40 to 44 years old women. This cancer is responsible for 33 percent of all cancers in women and 20 percent of deaths from cancer. (1) For the initiation and progression of breast tumors, a combination of internal and external factors are involved such as elements in nature. (2.3) With respect to the nutritional role of rare elements such as zinc, copper, selenium and especially the essential element of iron and its direct relationship with cancer, many studies are conducted on the how to create or restrict tumor by these elements. (2.4) Iron is the most important element in nature and in a living organism. It is also a necessary mineral that people cannot live without it. (4) Iron construction activity has been proved in the enzymes that have antioxidant action such as Glutathione Peroxidase (GPX), Catalase and Super Oxide Dismutase (SOD). (5) Iron is the most abundant transition metal in the human body because of its role in binding and transferring the oxygen and electron.

Iron ion in healthy cells would not be found in the non-coverage state and it binds to its special proteins such as transferrin and ferritin and this binding prevents the entering of iron to the redox cycle. (6) Although this element is a necessary nutrient for cellular activities such as gene regulation, but it can also acts as a double-edged sword (7) and this will happen if the amount of iron in the blood has been increased. In the low values and dependent to the protein, it is an essential element in the metabolism and cell growth but it will be toxic when it is not adhered. (8) Increase the presence of iron through oxidative stress is known as a harmful factor (9), ferritin serum levels are increased in many cancers including solid and prominent tumors especially in times of metastasis (10,11,12) The importance of iron in tumor growth is clear from the fact that the reduction in serum levels of iron reduces malignant tumor progression (13,14,15)

Today, common cancer treatments are includes surgery, chemotherapy and radiotherapy. Although major problem in cancer treatment failure is metastasis of cancer cells. Unfortunately, it should be said that surgery and radiotherapy are only applied in the treatment of topical cancers and they do not have any use in the cancer metastases. In such cases, the treatment is often based on the chemotherapy. The efficiency of this method is practically limited due to its toxic side effects with high doses. However, it is well known that this type of treatment are weakened and destroyed the body immune system even more than the cancer.

So, because of the high side effects of the chemical drugs and low side effects in the medical plants, researchers' tendency to these plants and use their active combinations in the cancer treatment have been increased. In recent years, many plants were studied in the treatment of cancer and antioxidant effects of these plant combinations have been documented. Ginger plant is used as medicine, spice and food. (16) The combination of ginger contains monoterpenes, aldehydes, Citral and Sesquiterpenes and metabolites of smell and aroma and oxygenated derivatives which includes Zingiberene, AR-Curcumene, B-Sesquiphellandrene and b-bisabolene,

Pyrogalloles, gingerols and shogaols, Paradol, Zynjron and volatile oils that constitute 3% of the weight of fresh ginger.(17,18)

Other compounds derived from ginger root are organic germanium (Ge-132) or 2-carboxy ethyl germanium oxide and Quercetin that play an important role in the pharmacological effects of the plants. (19) Ginger has strong antioxidant and anti-inflammatory effect and it also has anti-bacterial, analgesic, antipyretic and lowering blood cholesterol properties (16:20:21). The other effects of ginger on the body are antitumor properties (22.23), remove free radicals (23:24), stimulation of menstruation and relieve menstrual irregularity (25.26), influencing on spermatogenesis (26) and increased sexual desire (25). Gingerol in ginger caused the loss of free radicals, anti-serotonergic, inhibited the production of prostaglandin and sesquiterpens also inhibits free radical production (27).

The aim of this study is to investigate the effect of Ethanolic extract of ginger on the blood serum iron and the growth of breast cancer tumors in BALB / c female mouse.

Materials and Methods

Laboratory Animals: A total of 20 BALB / c female mice with the age of approximately 6 to 8 weeks and weighing 16 -23 grams of Pasteur Institute of Tehran were purchased and they were stored under the standard conditions for laboratory animals (12/12 light / dark, standard pellet food, temperature of 24 ° C and humidity 52 percent).

Then the mice were transferred to the animal house of Azad University for one week in order to adapt to the new circumstances and they were studied. In practical stage of study, mice were divided randomly into 5 groups. They are received extraction solvent (normal saline) in the healthy group, and they are received normal saline in the cancerous group and three cancerous groups are received 20,50 and 100 mg/kg dose of Ethanolic extract of ginger (n=5).

Extraction: 500 g fresh rhizome of ginger was prepared from herbalists and after washing, a specific amount of it was peeled, then it cut in to thin slices, after that it was kept in a proper environment without light for one day and it was powdered after drying. Then it was soxhlet by its solvent, i.e., ethanol for 6 hours and finally honey and dark Ethanolic extract of ginger was prepared after putting it in the rotary with round 50 with 45-50 min at 45 ° C. Then it is transferred to a container with aluminum cover and placed in a desiccator with vacuum pump for 24 hours in order to evaporating the extract solvent and the resulting semi-solid extract was stored at -20 ° C in order to prevent contamination and to continue the experiment.

The action of tumor in the mice: After confirming the mouse model of spontaneous breast cancer by Dr. Zahir Mohammed Hassan (the originator of this method and Professor of Immunology at the School of Medicine in TarbiatModarres University), the cancerous mouse spinals out and the tumor removed from the body and it is divided into pieces of about 5 cubic millimeter in the sterile normal saline with scalpel and surgical blade, then each of the mice were anesthetized by intraperitoneal injection of ketamine / xylazine (at a dose of 10 / 40mg / kg) and divided parts of the tumor are surgically implanted under the skin of the right flank and surgical site was sutured with special clips. About a week after the transplantation, tumor growth was

visible with eye. Injection of different doses of ginger (20 mg/kg, 50 mg/kg and 100mg/kg) was administered a week after the tumor action with ethical principles for each group for 21 days and once a day via intraperitoneal syringe. The control group received saline during this period.

It should be noted that death dose of this extract is 1000 mg/kg orally and it is 458 mg/ml by injection. Finally, after the mice were anesthetized by ether, Heparin dose was absorbed into the syringe in order to prevent blood clots and then blood was taken from their heart and blood samples were emptied into the microfibrers and they were kept in the container of ice water and in dark surroundings. The iron in blood plasma was measured by Ferrozine test. First, the 35 landaof serum sample was taken and after getting it for 5 minutes in the incubation, free iron in serum was readable by a device with HITACHI-917 model versus 240 Landa of Blank spillage with power filtration (OD) = 750nm.

Measurement of the tumor size and weight: after the mice were anesthetized with ether, the tumor was removed from the body and the length and width of tumor were measured with calipers. The tumor size is obtained by the following formula:

$$tumorsize = (length) \times (width)^2 \times 0.5$$

Also, the tumor weight was determined with precision scales.



Figure 1: Measurement of tumor growth

Statistical analysis of data was conducted using one-way ANOVA (ony-wayANOVA) and Tukey test and significance level of $p < 0.05$ was considered.

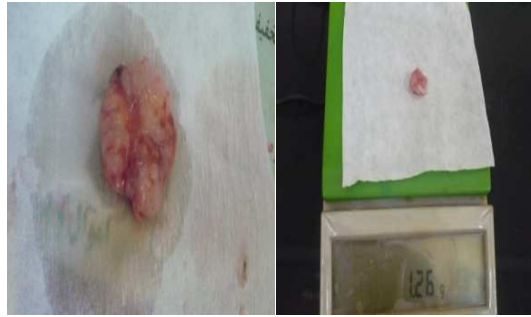


Figure 2: Measurement of tumor weight

Results

The results showed that there is no significant difference in the serum iron level of cancerous group receiving saline compared to the healthy group receiving saline. The cancerous group receiving dose of 20mg/kg ginger showed a significant decrease in the serum iron level compared to the cancerous group receiving solvent extract (normal saline) ($p < 0.05$).

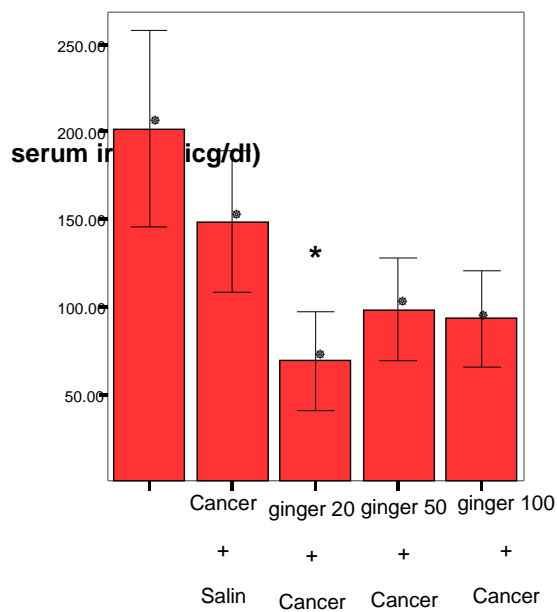


Diagram 1: The effect of the Ethanolic extract of ginger on the iron serum level in the female mice with breast cancer tumor

BALB / C female mice with breast cancer tumor in the flank are received saline and different doses of Ethanolic extract of ginger (20, 50 and 100 mg/kg) intraperitoneally for 21 days. In the end, mice were anesthetized with ether and bloodletting was done from heart and the plasma iron levels were determined by Ferrozine test. The diagrams indicate mean \pm standard deviation (Mean \pm SEM).

(n = 5) (P < 0/05)

The results imply that the size and weight of tumors showed a significant decrease in cancerous group receiving the 50mg/kg dose of Ethanolic extract of ginger compared to the cancerous group receiving saline. ($P < 0.05$).

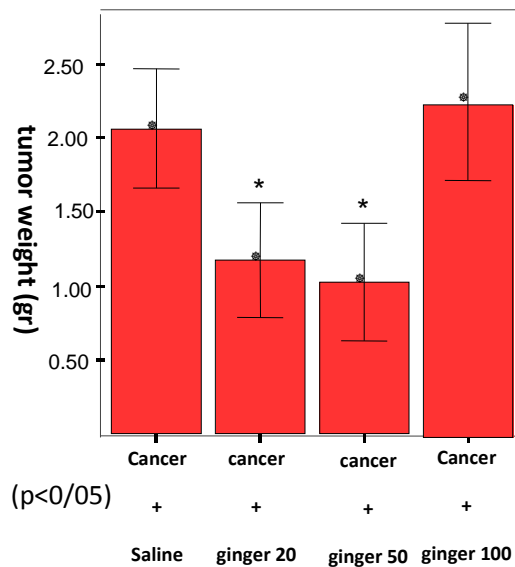


Diagram 2: The effect of the Ethanolic extract of the ginger on the size and weight of the breast cancer tumor in female mice

BALB / C female mice with breast cancer tumor in the flank were received saline and different doses of Ethanolic extract of ginger (20, 50 and 100 mg/kg) intraperitoneally for 21 days. In the end, mice were anesthetized with ether and tumor was removed and its size and weight was determined. The diagrams indicate mean \pm standard deviation (Mean \pm SEM). ($n = 5$) ($P < 0.05$)

Discussion

Many of the herbs and spices have pharmacological and biochemical properties including antioxidant and anti-inflammatory properties which are involved in the activities of anti-mutagenic and anti-carcinogenic. Given that tumor progression is closely related with inflammation and oxidative stress, the compound which has antioxidant and anti-inflammatory properties can be an anti-carcinogen factor. (28) Investigating the effect of the Ethanolic extract of the ginger on the size and weight of the tumor showed a significant decrease in the cancerous group receiving 50 mg/kg dose of Ethanolic extract of the ginger compared to the cancerous group receiving saline.

In various studies, the impact of the materials of the ginger extract on the prevention of the proliferation of human cancer cells through apoptosis has been demonstrated(28,29). Blessy et al (2009) investigated the Ethanolic extract of the ginger rhizome on the cysts caused by DMBA in the mice and showed that this extract in addition to having lipid peroxidation leads to neoplastic

transformation by its antioxidant property and prevents tumor formation. (25) Afshari et al (1389) investigated the effect of the cytotoxicity alcoholic extract of the fresh ginger on the breast and liver cancer cells and found that fresh rhizome alcoholic extract of the ginger has no toxic effect on the breast and liver normal cells (HPEG2) but it showed lethal effect on the cancerous cells according to the dose of weight material. The morphology changes reflect the induction of apoptotic cell death (apoptosis) in cancer cells which were treated with ginger.

Suzuki (1997) investigates the gingerol resulting from the ginger on the tumor growth and found that this combination has anti-angiogenic effect and inhibits tumor growth and its metastasis. Yoshikawa et al (1994) investigated the anti-oxidant ginger and found that ginger has antioxidant, anti-inflammatory and anti-tumor activities. Choudhari and his colleagues investigated the aqueous extract of ginger on the cancer cells and the results showed that this extract has poly phenolic combinations that affects directly on the microtubules of the cancer cell and shears their structure so it leads to the morphological changes and destroyed the structural and functional characteristics of tubulin and microtubule and causes apoptosis of the cancer cells. The reports obtained from 2006 showed that ginger in the Hep2 cells or liver cancer cells causes the Apoptosis by increasing the production of reactive oxygen (35). It has an important effect on reducing tumor growth and tumor weight.

Researchers found that 6-gingerol of the pungency of ginger inhibits the proliferation of the vascular endothelial cell (VEGF) and FGF by its anti-tumor and anti-bacterial activity and prevents the formation of the ion channels by these cells and halts the cell cycle in G1 phase and prevents the angiogenesis *in vivo* and *in vitro* (36). According to the conducted studies, wayneloyidketones derived from ginger root called 6-gingerol and 6- paradol showed anti destruction powerful activity against skin cancer cells and also reduce tumor cells by inhibiting the production of TNF (tumor necrosis factor) (37).

Ginger has a compound called Curcumin and directly inhibits fibroblast growth hormone and epidermal growth hormone, thereby inhibits angiogenesis in cancer cells (38). So, Ginger likely to be involved in the recovery process of malignant tumors whose research on breast tumors was similar to this research results on reducing growth and weight of tumor by sub-peritoneal injection of the Ethanolic extract of the ginger with different doses on the growth and weight of tumor. This is also due to anti-tumor, anti-angiogenesis of this extract.

In a study conducted in 2010 on the 6-gingerol, the result implies that this combination of ginger extract inhibits the cell adhesion, movement, invasion and activity of MMP_2, MMP_9 proteins that is selectively expressed in endothelial cells and increased the mitosis in the breast cancer cell lines $MDA-MB_{231}$ (39). Also, it was found during the investigations on the 6-Shogaols that this active compound of ginger completely prevents the $MDA-MB_{231}$ cell invasion of breast cancer through stimulating the PMA activity (ferbel 12-myristat and 13-acetat) by inhabitation of NF-KB activity (40).

According to the conducted research on the (DEG) 6- Dehydrogingerdione compound made from ginger, researchers found the inhibition effect of this substance on the MCF-7 and

MDA-MB₂₃₁ in breast cancer (41). Based on the invitro studies on one of the compounds resulting from ginger called 1-stoksi cavicle acetate (ACA), the results showed that ACA clearly reduces the viability of cancer cells by inhibiting the reactive oxygen in two cell lines of *MDA-MB₂₃₁* and MCF-7 within 24 hours and prevents its invasion (42). During the research conducted on the Zerumbone that is another compound of ginger, the results showed that this compound is the reduction factor in the expression of the CXCR4 protein. This leads to the suppression of its inducing performance i.e. tyrosine kinase and finally inhibiting the NF-KB activity in the MCF7 / HER-2 cells of breast cancer (43). In the wake of another study on the antioxidant activity (33) and antioxidant of ginger extract based on the inhibition of hydroxyl radicals, it is one of the Carcinogenic factors in different temperatures and concentrations. (44) Ginger have vitamin C and other antioxidants so it can remove ROS which is one of the species of reactive oxygen and is a free radical and it can prevent the peroxidation of the plasma membrane lipids (45). Ginger consumption significantly increases the peroxidase glutathione enzyme as an antioxidant (46). Existing gingerols in ginger causing the disappearance of free radicals, anti-serotonergic, inhibiting the production of prostaglandins (47) and anti-inflammatory effect (48).

Sesquiterpens are also inhibited the production of free radicals. (47) Shugaol and similar structural components in ginger have anti-inflammatory properties and prevent this process through inhibition of Cyclooxygenase and 5-lipoxygenase pathways from the biosynthesis leucotrienosand prostaglandins which are inflammatory compounds (49). This is consistent with Felipe et al research that they specified the antioxidant activity and inhibition of the peroxidation lipid by aqueous and alcoholic extracts of ginger. Also, they found that this plant acts as a dual inhibition of Cyclooxygenase and 5-lipoxygenase that inhibits the prostaglandin (E2) and it takes the advantage of better treatment and fewer side effects compared to the non-steroidal anti-inflammatory drugs (50). It also inhibits the Arachidonic acid that causes platelet aggregation and the formation of thromboxane B2 and prostaglandin D2, which are frequently carcinogenic(49, 51).

The 6-gingerol combination of ginger decreased levels of intracellular peroxides particularly due to the high density in concentration and through its antioxidant activity. (52)

Since ginger oil can prevent oxidative damage and also, it has inhibition effect on the DNA damage induced by H_2O_2 , so it acts as a collector of oxygen radicals which implies Ginger oil is antioxidant. (53)

In this study, there is significant decrease in the serum levels of iron in the cancer group receiving 20 mg/kg dose of Ethanolic extracts of ginger compared to the cancerous group receiving saline. Since there has not been conducted any specific research based on the direct effect of ginger on the reducing iron levels in the blood serum of breast cancer, so this study is new and important in this respect.

According to the various studies, the levels of iron in women with bladder cancer was significantly lower than the control group (54,55). The results of the study also showed a reduction of iron serum levels in mice with breast cancer receiving normal saline compared to

the healthy group but the mean difference is not significant. According to other studies, iron serum level is increased significantly in other cancers such as liver cancer, stomach cancer and kidney cancer and colon cancer compared to the normal individuals (56). Therefore, coordination between this study and the results of the present research cannot be observed. Because, there is a significant decrease in the cancer group receiving 20mg/kg dose of Ethanolic extract of ginger compared to the cancerous group receiving saline. According to the conducted research, additional iron of cell with production of ROS (a form of reactive oxygen) and free radicals such as superoxide anion and hydroxyl radical damages the proteins, lipids and DNA. It is toxic and also produce the final products of the mutagenesis and peroxide genotoxic and reduce the mitochondrial respiration and relates to the various human diseases, including cancer (57).

The researchers reported that Quercetin is one of the ginger polyphenolic compounds which prevents the peroxidation of the lipid dependent on the iron ion by binding the free iron ions (58). Acetoxychavicol (ASA) as one of the ginger polyphenol compounds can neutralize the free iron ion of blood by giving the electron to the free radical (oxidant) (59,60). 6-gingerol is one of the most important compounds of the ginger on the oxidase xanthine system which is responsible for the production of reactive oxygen species (Ros), such as superoxide anions that has inhibitory and antioxidant activity (61,62)

Probably the ethanol extract of ginger used in the present study with the mechanism of destruction of free radicals and its relationship with free iron and the subsequent inhibition of xanthine oxidase activity reduces the serum levels of iron in the three groups treated with ginger compared to the intact and Cancer group. So, various research results are consistent with the present research results.

According to the conducted studies concerning the effects of carcinogenic metals such as iron for the activation of transcription factors sensitive to redox, it requires the AP-1, NF-KB and p53 and also catalyzes the production of ROS in Fenton reaction to target the genes. Zerombon as one of the effective and active compounds in the ginger could leads to the breakdown of message transcripts in carcinogenesis, such as COX_2 , TNF- α , IKB, INOS and finally inhibits NF-KB (that has a significant role in the development of cancer) by ceasing the production of free radicals such as superoxide anion and reducing the oxidative stress which is one of the factors for the emergence of free iron (63, 64).

Thus, according to the results of research and the above studies, the important role of ginger in reducing the excess iron levels in mice with breast cancer will be proved once again.

According to the studies, it was found that Curcumin is one of the other compounds of the ginger extract which has affinity with metal ions in aqueous and ethanol solution under the controlled PH conditions (65). It was found during further investigation that curcumin creates a strong bind with the iron in the aquatic environment (66, 67). So, it is proved that curcumin is a strong iron binder under neutral to slightly acidic environment although its affinity to other metals is less known (68). That is, curcumin prevents the adverse effects of this element in confronting with free radicals generated by iron through giving a hydrogen electron from the keto-enol or B- Dai

ketone group or through giving hydrogen from its phenolic group to the free radicals and by formation of B-Dai keton radical or phenol radical (69,70, and 23).

It is conducted that these observations are consistent with the results if this study based on the significant reduction in serum iron in the cancerous mice treated with 20 mg/kg dose of ethanol extract of ginger compared to the cancerous group receiving saline because of the synthetic iron. The OH produced by Fenton reaction can increase the breast cancer risk by lipid peroxide and activation of tumorigenesis and prevention of tumor suppressor factors (71). According to the results of previous research based on the increased iron with production of ROS which is damaged the proteins, fats and DNA, Ethanolic extract of ginger decreases the DNA damage by cleaning mechanism of free radicals such as ROS, superoxide anion by decreasing oxidative stress (57).

According to the research on the lipid peroxidation, it was found that factors that can cause lipid peroxidation at low levels are included ferritin (as a source of iron) and xanthine oxidase.

It is noteworthy that combination of both of them would be a major lipid peroxidation (15) and are involved in the development of certain cancers. (72, 73)

Free iron leaves the membranes with disruption and cell death after activity because of the following reasons:

transition metal ions have high ability exposure to oxidation and react with free radicals, and since enzymes such as dismutase superoxide that play an important role in free radicals have three different transition metals such as copper, manganese and iron in their active site and iron plays an important role in binding and transferring with oxygen and it is the most abundant transition metal in the human body and due to the destructive role of free iron in the cell based on the redox activity which causes lipid peroxidation, the lipid peroxidation occurs when the chain reaction between free radicals and unsaturated acyl fatty groups is in the cell membranes and molecular oxygen(6)

According to the other studies, the excess iron consumption increases the incidence of mammary tumors in mice (74). According to the studies on the Ethanolic extract of the ginger rhizome and the ability of per-oxidative anti-lipid, this plant is an agent for preventing tumor growth. (25)

Since in healthy cells, the iron ions are never found in the non-coverage state and always are binding to their proteins such as transferrin and ferritin, so healthy cells prevent from entering the iron ion to the redox cycle (6). According to various studies, researchers found that iron deficiency reduces tumor progression (75, 76)

Since neoplasm cells have high reaction to the iron coupler factors, and reduce the transferrin receptors which are the growth factors influencing on the cancer cells, so tumor death occurs without harmful effects to the host body by regulating free radical (77).

According to the studies and results, reduction in serum levels of iron in the blood were significantly reduced tumor growth and weight tumor in mice with breast cancer treated with Ethanolic extracts of ginger.

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