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Antitumor potential of herbomineral formulation against breast cancer: Involvement of inflammation and oxidative stress

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Breast cancer, the most frequently diagnosed cancer is a leading cause of death in females worldwide. Here, we evaluated the effect of herbomineral formulation [Cruel capsule (CC), an ayurvedic proprietary medicine] on DMBA-induced mammary carcinogenesis in rat. Fifty days postpartum female Sprague-Dawley rats were grouped into control, control treated with herbomineral formulation (30 mg/kg/day, p.o.), disease control, disease treated with herbomineral formulation (30mg/kg/day,p.o.). Breast cancer was induced by dimethylbenz[a]anthracene (DMBA) (60 mg/kg, p.o). Animals were palpated twice weekly for the incidence and tumor size. After 48 h of last dose, blood was collected for estimations of LDH, GGT, C-RP & ESR. Animals were sacrificed and tumors isolated for histopathological examinations and antioxidant studies. Treatment with formulation showed significantly decreased LDH, GGT, CRP & ESR compared to disease control group. Histopathological examinations showed hyperplastic and well demarcated tubular inflammation which was improved by treatment with the formulation. Treatment also showed significant decrease in MDA and increase in GSH and SOD levels in breast homogenate as compared to the disease control group. From the present investigations, we can conclude that the herbomineral formulation (CC) inhibits the tumor progression and the mechanism for the chemopreventive potential of formulation may be due to its anti-inflammatory, antiangiogenic and antioxidant potential.

Keywords: *Adhatoda vasica*, Antiangiogenic, Anticancer activity, Anti-inflammatory, Antioxidant, Ayurvedic,, *Boerhavia diffusa*, Cruel capsule, Dimethylbenz[a]anthracene, *Glycyrrhiza glabra*, Mammary carcinogenesis, *Moringa pterygosperma*, *Piper nigrum*, *Syzygium aromaticum*, *Tinospora cordifolia*, Tumor

Breast cancer, defined as tumors having the expression of estrogen, progesterone and HER-2 receptors, is the second leading cause of cancer mortality in women[1]. Human breast cancers represent a heterogeneous group of tumors that are diverse in behaviour, outcome and response to therapy[1,2].

Several drugs are available *viz*., aromatase inhibitor (anastrozole) and chemotherapeutic agent (paclitaxel, doxorubicin, carboplatin) for the treatment of breast cancer. However, side effects of such drugs are often deleterious, which includes immunosuppression, alopecia, gastrointestinal disturbances, fertility issues, etc. Use of phytochemicals as chemopreventives has acceptance, because of their low toxicity and high tolerability[3]. The current focus of anticancer research is on phytoconstituents capable of not only inhibiting carcinogenic progression but also protect against the side effects associated with chemotherapy.

In this study, we evaluated the effect of oral treatment with the herbomineral formulation named Cruel capsule (CC), an ayurvedic proprietary medicine, prepared from parts of seven different medicinal plants (*Piper nigrum*, *Boerhavia diffusa*, *Glycyrrhiza glabra*, *Adhatoda vasica*, *Syzygium aromaticum*, *Moringa pterygosperma*, *Tinospora cordifolia)* that are used in traditional medicine as anticancer agent in animal model of mammary gland cancer[4] to ascertain the use of ayurvedic proprietary medicine in the management of breast cancer in folklore medicine.

Materials and Methods

Materials

All the plants used in the formulation were authenticated and the formulation was prepared by VIRGO UAP Pharma Pvt. Ltd. (Ahmedabad, India) on the basis of an ayurvedic formulary (Table 1). Dimethylbenz[a]anthracene (DMBA) was procured

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Table 1 — Composition of Cruel Capsule

Ingredients	Botanical name	Content (mg)
Sarveswar Parpati	-	14.4
Suvarna Bhasma	-	3.6
Rasa Kapoor	-	14.4
Swet Mirch	*Piper nigrum*	42.0
Abhrak Bhasma	-	1.2
Ext. Punarnava	*Boerhavia diffusa*	25.0
Ext. Yastimadhu	*Glycyrrhiza glabra*	25.0
Ext. Vasaka	*Adhatoda vasica*	25.0
Hira Bhasma	-	0.3
Rasasindur	-	14.4
Tamra Bhasma	-	27.6
Lavang	*Syzygium aromaticum*	21.6
Panna Bhasma	-	0.5
Ext. Saragava	*Moringa pterygosperma*	25.0
Ext. Rohitak	*Tecomma undulate*	25.0
Ext. Guduchi	*Tinospora cordifolia*	25.0
Excipients	-	q.s.

from Sigma Chemicals (St.Louis, USA). Diagnostic kits were purchased from Accucare Diagnostics Pvt. Ltd (Vadodara, India). The chemicals used in the present study were of analytical grade.

Preparation of formulation

All the required ingredients were sieved from 60 # mesh sieve to obtain fine powder. Excipients were sieved separately and mixed in cone blend mixer to obtain homogenous mixture and samples were sent to quality control department for in-process analysis. Empty capsule shells were loaded on semi-automatic capsule loader and filled with specified weight of powder by capsule filling machine. Dust and other particles were removed by hand polishing method and final capsules were sent again to quality control department.

Experimental animals

Seven to eight weeks old female SD rats weighing 170-200 g were obtained from the animal facility of Zydus Research Center, Ahmedabad and were housed in a pathogen-free environment at the animal house of Institute of pharmacy, Nirma University. Animals were maintained under well controlled temperature 22±8°C & humidity 55±5% with 12/12 h light/dark cycle. They had free access to food and water *ad libitum.* All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of Institute of Pharmacy, Nirma University, Ahmedabad (IPS/PCOL/CONS11-12/1003).

Experimental protocol

Female SD rats (28) were randomly grouped into normal control, control treated with herbomineral formulation (30 mg/kg/day, p.o.), disease control and disease treated with herbomineral formulation (30mg/kg/day, p.o.). Breast cancer was induced by oral administration of 60 mg/kg DMBA dissolved in sesame oil[5]. Animals were palpated twice a week after DMBA administration in order to record the presence, location, size, and incidence of tumors. After 5 wk, general parameters like body weight were evaluated. Blood samples were collected after 5 wk for biochemical estimations of lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT), C-reactive protein (CRP) levels using diagnostic kits. Erythrocyte sedimentation rate (ESR) was measured in blood samples. Animals were sacrificed and tumors were separated for Hb% estimation, antioxidant study and histopathological examinations. Statistical analysis results are represented as mean ± SEM. Statistical analysis was performed using Graph pad prism 5 statistical software. Statistical differences between the means of various groups were evaluated using one way analysis of variance (ANOVA) followed by turkey's test. Data were considered statistically significant at $P < 0.05$.

Acute toxicity study

Toxicity of CC was assessed for single dose acute toxicity by employing OECD guidelines 425 using AOT software. Protocol was approved from IAEC *vide* protocol number IAEC-12/11-SDMCRA SP/03. The rats were observed for 14 days, for general appearance, cage side behaviour including increased or decreasing motor activity, convulsions, Straub's reaction, catatonia, muscle spasm, spasticity, ophisthotonus, hyperesthesia, muscle relaxation, anaesthesia, arching and rolling, lacrimation, salivation, diarrheal, writhing movement, mode of respiration and changes in skin colour.

Results

Effect of herbomineral formulation on body weight

A significant ($P < 0.05$) decrease in body weight was observed in disease control group as compared to normal control group. At the same time, diseased rats treated with herbomineral formulation showed increased body weight as compared to disease control animals (Fig. 1).

Effect of herbomineral formulation on tumor size an incidence

Administration of DMBA showed first incidence of tumors at 2 wk after induction and reached to

approximately 131–283 mm^3 size in 30 days. Treatment of rat bearing mammary carcinoma with formulation significantly reduced tumor growth (Fig. 2).

Effect of herbomineral formulation on serum biomarkers

Lactate dehydrogenase

There was a significant (P <0.05) increase in the LDH level in the disease control group as compared to the normal control group. Diseased, but treated with formulation showed statistically significant reduction in the levels of LDH as compared to the disease control group (Fig. 3A).

Gamma glutamyl transferase

A significant (P <0.05) increase in the GGT activity was ascertained in the disease control group as compared to normal control group which was significantly reduced by treatment with formulation (Fig. 3B).

Effect of herbomineral formulation on inflammatory biomarkers

Serum C-reactive protein level (CRP)

Similarly, significant (P <0.05) increase in the CRP level was seen in the disease control group as compared to the normal control group whereas in the disease treated with herbomineral formulation group, CRP levels were found to be significantly reduced as compared to the disease control group (Table 2).

Erythrocyte Sedimentation Rate (ESR)

The ESR levels also showed significant increase in the disease control group as compared to normal control group. However, in the disease treated with herbomineral formulation group ESR was found to be significantly reduced as compared to the disease control group (Table 2).

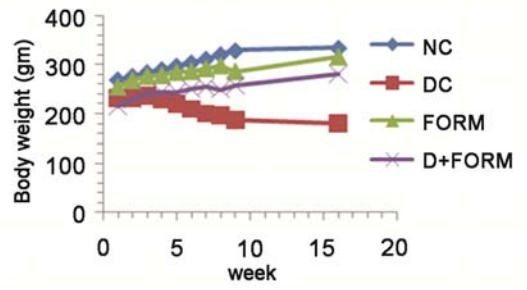

Fig. 1 — Effect of herbomineral formulation on body weight. [Values expressed as Mean±SEM of 6 animals each, * Significantly different from normal control (P <0.05), # Significantly different from disease control group (P <0.05). NC, Normal control; DC, Disease control; FORM, Normal animals treated with formulation (30 mg/kg); and D+FORM, Diseased animal treated with formulation]

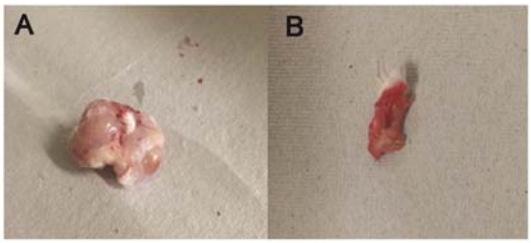

Fig. 2 — Effect of herbomineral formulation on tumor size an incidence. [Values expressed as Mean±SEM of 6 animals each, *Significantly different from normal control (P <0.05), # Significantly different from disease control group (P <0.05). NC, Normal control; DC, Disease control; FORM, Normal animals treated with formulation (30 mg/kg); and D+FORM, Diseased animal treated with formulation]

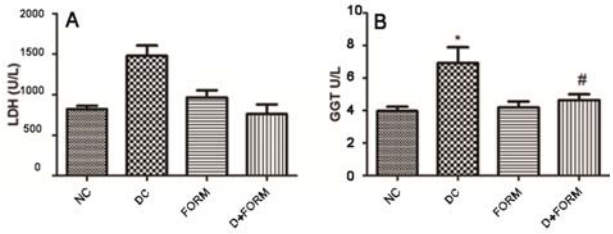

Fig. 3 — Effect of herbomineral formulation on serum biomarkers. (A) Lactate Dehydrogenase; and (B) Gamma Glutamyl Transferase. [Values expressed as Mean±SEM of 6 animals each, *Significantly different from normal control (P <0.05), # Significantly different from disease control group (P <0.05). NC, Normal control; DC, Disease control; FORM, Normal animals treated with formulation (30 mg/kg); and D+FORM, Diseased animal treated with formulation]

Table 2 — Effect of formulation on inflammatory and antioxidant parameters

Group	CRP level (mg/l)	ESR (mm/h)	MDA level (nmol/mg protein)	GSH (µg/mg protein)	SOD (U/mg protein)
NC	199.2± 20.94	2.24 ± 0.06	1.41 ± 0.07	7.28 ± 0.69	2.49 ± 0.16
DC	300.9 ± 10.22*	3.47 ± 0.27*	2.56 ± 0.09*	2.68 ± 0.46*	1.61 ± 0.10*
FORM	218.3 ± 5.90	2.42 ± 0.05	1.51 ± 0.07	6.52 ± 0.70	2.55 ± 0.07
D + FORM	219.5± 9.02#	2.51 ± 0.13#	2.00 ± 0.15#	5.10 ± 0.28#	2.32 ± 0.06#

[Values expressed as Mean±SEM of 6 animals each, * Significantly different from normal control (P <0.05), # Significantly different from disease control group (P <0.05). NC, Normal control; DC, Disease control; FORM, Normal animals treated with formulation (30 mg/kg); and D+FORM, Diseased animal treated with formulation]

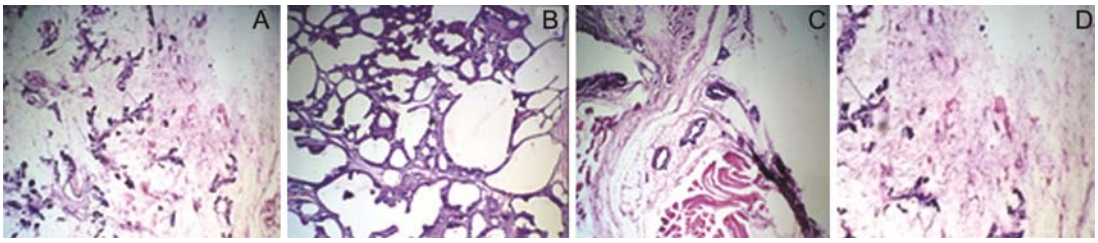

Fig. 4 — Effect of herbomineral formulation on histopathological examinations of mammary gland. (A) normal control; (B) disease control; (C) disease treated with formulation; and (D) normal treated with formulation

Effect of herbomineral formulation on antioxidant parameters

Malondialdehyde Level

In MDA levels, the disease control group showed significant increase (P <0.05) as compared to the normal control group, while the disease treated with herbomineral formulation showed significantly reduced MDA level. However, no significant change in MDA levels was observed in normal treated with formulation as compared to normal control animals (Table 2).

Reduced glutathione levels

The results showed a significant (P <0.05) decrease in the GSH level in the disease control as compared to the normal control group. Whereas in the disease treated with herbomineral formulation group, GSH level was found to be significantly increased as compared to the disease control group (Table 2).

Superoxide dismutase levels

A significant (P <0.05) decrease in the SOD level was observed in the disease control group as compared to the normal control group while the diseased animals treated with formulation showed statistically significant improvement in SOD levels. There was slight increase in the SOD levels in the normal treated with herbomineral formulation when compared to the normal control group showing the presence of antioxidant potential in the formulation (Table 2).

Effect of herbomineral formulation on histopathological examinations of mammary gland

In histopathological examinations of normal control animals, normal ductular and alveolar structure of mammary gland was observed. There was no sign of inflammation or tissue damage, and hyperplasic lesions were observed. While in disease control group, mammary gland carcinoma; showing hyperplastic and well demarcated tubular carcinoma was found. The tubular and alveolar epithelium showed diffused hyperplasia with minimal rim of skeleton muscle. Treatment with herbomineral formulation to diseased animals showed mild to moderate ductular and alveolar structure of mammary gland without sign of inflammation or tissue damage and erosions. Treatment demonstrated protection against hyperplasic and neoplastic lesions. Normal treated animals with herbomineral formulation showed normal ductular and alveolar structure of mammmary gland and no sign of inflammation or tissue damage and erosions (Fig. 4).

Acute toxicity study

The test drug did not produce any mortality up to the dose of 2000 mg/kg p.o. At the dose level studied, the drug also did not produce any observable toxic effect except for mild irritation. Based on the observation made and recorded it can be concluded that the Ayurvedic proprietary medicine Cruel capsule (CC) is safe without any toxic effects.

Discussion

Present chemotherapeutics do not specifically target tumor cells, but interfere with cell division or inhibit enzymes involved in DNA replication or metabolism of normal dividing cells of rapidly regenerating tissues. This limits the therapy of breast cancer with present chemotherapeutics[6]. Considering the side effects associated with synthetic chemotherapeutics and the potential of traditional and folk medicines in the management of breast cancer, we have explored Cruel capsule (CC), an ayurvedic proprietary medicine, in animal model of DMBA-induced mammary cancer .

The 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary cancer models in rats have been employed to identify substances with chemopreventive activity in many herbal drugs and formulations[7]. In the present study, DMBA causes decrease in body weight of rats as compared to the normal control group; and these results are in consistent with the previous reports which showed decreased body

weight with DMBA[8]. Treatment with herbomineral formulation produced an improvement in body weight indicating that formulation reduces toxicities produced by DMBA.

Lactate dehydrogenase (LDH) is an enzyme that, under anaerobic conditions, catalyzes the reversible transformation of pyruvate to lactate. Upregulation of LDH ensures an efficient anaerobic/glycolytic metabolism for tumor cells and reduces dependence on oxygen[9]. In the present study, the increased LDH level in the disease control group may be due to increased hypoxic stress and anaerobic metabolism for cancer cells, which ultimately cause the release of hypoxia inducible factor (HIF) and formation of new blood vessels for the growth of developing tumor. Decreased LDH level observed in herbomineral formulation treated group suggests the reduction in hypoxic stress and anaerobic metabolism. Moreover, LDH plays an important role in inflammation and DNA damage. Thus, decreased LDH in the treated groups proves beneficial role in the prevention of inflammation and ultimately mammary gland cancer.

Gamma-glutamyl transferase (GGT) is a cellular enzyme with wide tissue distribution primarily in the breast, kidney, pancreas, liver and prostate. Measurement of GGT activity helps in diagnosis and treatment of metastatic tumors. Elevated activities of GGT observed in the DMBA-treated group are indicative of DMBA-induced damage and the subsequent leakage of these enzymes into circulation[10]. GGT acts as a useful marker for hepatic metastasis from breast and colon primaries[11,12]. Treatment with herbomineral formulation to DMBA-treated animals significantly decreased GGT activities. Herbomineral formulation contains medicinal plants, such as *Boerhavia diffusa*, *Glycyrrhiza glabra* and *Moringa pterygosperma*, and according to previously reported study, they have been reported to have cytoprotective effect as well as antimetastatic effect[13]. Punarnavine, an alkaloid from the *Boerrhavia diffusa* has shown to significantly inhibit peritoneal angiogenesis in an Ehrlich ascites carcinoma *in vivo*. The antiangiogenic action has been recently demonstrated using *in vivo* sponge implant angiogenesis model and punarnavine was found to inhibit neovascularization in a dose-dependent manner[14]. Also, studies have shown potent inhibition of drug efflux activity of breast cancer resistance protein (BCRP/ABCG2) by 2 rotenoids boeravinones G and H, indicating a cytoprotective as well as antimetastatic effect on the epithelial cells of mammary gland[15]. The flavonoid, isoliquiritigenin (ISL) from *Glycyrrhiza glabra* has been shown to inhibit human breast cancer metastasis, preventing resistance, migration and invasion by down-regulation of COX-2 and CYP P450 signaling[16]. Also, it inhibits the growth of androgen-dependent and independent prostate cancer cell lines LNCaP and DU145, respectively[16]. Studies have shown that ISL diminishes cell viability, 5-bromo-2'-deoxyuridine incorporation and clonogenic ability in both MCF-7 and MDA-MB-231 cells and also induces apoptosis. Similarly, *Moringa pterygosperma* contains 4-(4'-O-acetyl-a-L-rhamnopyranosyloxy)benzyl isothiocyanate, 4-(a-L-rhamnopyranosyloxy)benzyl isothiocyanate and niazimicin which is known to inhibit tumor promotion[17]. The chloroform extract of *Tecomella undulata* demonstrated antiproliferative effects on chronic myeloid leukemia cells. Induction of apoptosis was mediated by cell cycle arrest.

C-reactive protein (CRP) is a biomarker for inflammation. It is synthesized in hepatocytes and belongs to the family of acute-phase proteins, the concentration of which change in response to neoplasia. These changes are upregulated by cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor (TNF)[18]. In this study, elevated CRP level was found in the disease control group. Whereas, CRP level was found to be reduced in herbomineral formulation treated groups. Similarly, ESR is also a marker of inflammation. Further, high ESR was observed in the disease control group whereas, ESR was found to be reduced in the disease treated with herbomineral formulation. Therefore, it was suggested that herbomineral formulation reduces the inflammation and there by reduces the progression of mammary gland cancer. Herbomineral formulation contains *Piper nigrum*, *Adhatoda vasica* and *Syzygium aromaticum* which have been shown to possess anti-inflammatory activity in various preclinical and clinical studies[19]. *Piper nigrum* is reported to contain the major constituent piperine, which has been found to be responsible for the antitumor action by inhibiting P-gp mediated transport[20]. *Adhatoda vasica* contains vasicinone which is responsible for the anticancer activity. Studies have shown that vasicinone showed concentration dependent inhibition of cell proliferation with downregulation of p110α and p85 subunits of

PI3K[21]. *Syzygium aromaticum* contains eugenol which possesses anticancer activity with apoptotic inducing capacity. Eugenol interferes with various cell signalling pathways, specifically the NFKB[22]. *Tinospora cordifolia* which contains tinocordiside and yangambin has been shown to be active against cancer cell lines[23]. Therefore, it can be suggested that herbomineral formulation interfere with one of the pathways of synthesis of IL-6 or IL-8 or TNF by inhibition of release of biomarkers and thereby neoplasia.

There is an increase in nutrients demand in cancer cells for their growth, thereby tumor tissue needs increase in blood supply which is triggered by cancerous tissue angiogenesis. Increase in blood vessels needs increase in Hb for oxygen supply[24]. A significant increase in Hb% level was observed in formulation treated group, whereas in disease treated with herbomineral formulation Hb% level was found to be reduced. The formulation contains minerals, such as *Sarveswar Parpati*, *Suvarna Bhasma*, *Rasa Kapoor*, *Abhrak Bhasma*, *Hira Bhasma*, *Rasasindur*, *Tamra Bhasma and Panna Bhasma*. These active principles of *Sarveswar Parpati*, *Rasa Kapoor*, *Abhrak Bhasma* and *Rasasindur* reported to strengthen the tissue, heal ulcers and decreases the tumor growth by inhibition of angiogenesis. Parpati is the form which is obtained in flakes, gets disintegrated in the body at the level of Grahani. *Sarweswar parpati* has been found to be a Raskalpa used as anticancer drug in Ayurveda[25]. *Swarna Bhasma* (Gold ash) has shown to reduce tumor in several solid tumors i.e. lung, liver, pancreas, gall bladder and rectum cancer[26]. *Hira bhasma* is also known as 'vajra bhasma' since it is prepared from purified diamond. It provides a balance to all ailments including various chronic diseases such as cancers, tuberculosis, diabetes, obesity and chronic anemia. According to the basic principles of Ayurveda, human body consists of seven different tissues i.e. Rasa, Rakta, Mansa, Meda, Asthi, Majja and Shukra. The tissues affected due to cancer can be directly treated with heerak bhasma[27]. *Rasa Kapoor*, *Abhrak Bhasma* and *Rasasindur* have also been shown to have beneficial role in side effects associated with anticancer treatment in Ayurveda[28,29].

The assessment of reactive oxygen species (ROS)-induced lipid peroxidation and the status of antioxidants are widely used in the detection and evaluation not only of environmental carcinogens, but also of presumptive anti-mutagens and anti-carcinogens[30]. ROS generation is a major factor involved in all steps of carcinogenesis, *viz*. initiation, promotion and progression. The use of antioxidants during cancer treatment enhances therapy by reducing the free radicals generation. Tamra bhasma has been shown to inhibit lipid peroxidation preventing rate of aerial oxidation of reduced glutathione and induction of SOD in rat liver homogenate. The results suggested that it is a strong antioxidant with no reported adverse effects at the therapeutic dose. Natural drugs, which are used in Ayurveda, have also been proved to have antioxidant properties[31,32]. It is evident from the results that increased level of MDA was found in cancer bearing animals when compared to the control group. On the contrary, reduced level of MDA was observed in disease treated with herbomineral formulation indicating that it is a potent free radical scavenger. The medicinal plants present in the formulation are reported to have antioxidant activities[19]. During the process of neutralizing toxic reactants, herbomineral formulation protects proteins, lipids, mitochondrial DNA and nuclear DNA from oxidative damage. The present study reveals the protective activity of formulation in the cancer-bearing animals, having altered antioxidant status caused by carcinogenesis.

The non-enzymatic antioxidant systems are the second line of defense against free radical damage. GSH is involved in scavenging the electrophilic moieties involve in the cancer initiation. In the present study, herbomineral formulation showed increased level of GSH clearly suggesting their antioxidant property. All these *Bhasmas* which are used in herbomineral formulation also have the ability to reduce the three doshas or imbalances related to *vaata*, *pitta* and *kapha* (fundamental bodily bio-elements of Air, Fire and Earth).

The histopathological evaluation showed that hyperplastic and well demarcated tubular carcinoma, the tubular and alveolar epithelium shows diffuse hyperplasia in disease control group, whereas in the disease treated with herbomineral formulation there is no sign of inflammation or tissue damage, observed. The results show that the herbomineral formulation produced antitumor activity by inhibition of hyperplastic or neoplastic lesions thereby progress of the disease and metastasis.

Conclusion

From the present investigations, we can conclude that the herbomineral formulation inhibits the tumor

progression in animal model of breast cancer by cytoptotective, antimatastatic, anti-inflammatory, antiangiogenic and antioxidant activity of various phytoconstituents and minerals present in formulation. Therefore the use of herbomineral formulation as a neo adjuvant or adjuvant therapy may be beneficial in treatment of breast cancer.

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Conflicts of interest

The authors state that there are no conflicts of interest pertaining to this manuscript.

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