IMMUNOHISTOCHEMICAL AND HISTOPATHOLOGICAL ASSAYS OF WATER-SOLUBLE PEPTIDES (WSPS) BASED CYTOTOXINS IN DMBA-INDUCED BREAST CANCER RAT MODELS

S. Mahmood[1], M. U. Islam[2], T. Tariq[1], F. Tariq[3], A. Parveen[4] M. I. Khan[1], M. N. Faisal[5] and A. Sameen[4*]

[1]National Institute of Food Science and Technology, University of Agriculture, Faisalabad, Pakistan
[2]Department of Human Nutrition and Dietetics, The University of Faisalabad, Pakistan
[3]University of Agriculture Faisalabad, Sub Campus Toba Tek Singh, Pakistan
[4]Department of Food Science and Technology at Government College Women University, Faisalabad (GCWUF), Pakistan
[5]Institute of Physiology & Pharmacology, University of Agriculture, Faisalabad, Pakistan

*Corresponding author's email: dr.ayshasameen@gcwuf.edu.pk

ABSTRACT

Bioactive peptides-based drugs, being a novel therapeutic approach as an alternative to conventional chemotherapy, are the next-generation option. In this milieu, the present study was designed to evaluate the anti-tumoral activities of Water-Soluble Peptides (WSPs) derived from casein, whey, fish bones, and mixed WSPs in 7,12-dimethylbenz (a) anthracene (DMBA)-induced mammary carcinoma. The mammary carcinoma was induced in rats by administering 60mg/kg DMBA by dividing into two doses intra-gastrically through gavage. The rats were divided into 6 groups: G_0 (Negative control), G_1 (Doxorubicin receiving rats), G_2 (Casein WSPs extract), G_3 (Whey WSPs extract), G_4 (Fish bones WSPs extract), and G_5 (Mixed WSPs). Physical parameters and biochemical assays were performed at three intervals (1st, 6th, and 12th week). The WSPs significantly (p<.001) improved the activity of antioxidant enzymes in carcinogenic animals. Furthermore, the fish and mixed WSPs also prevented the increase in the concentration of tumor biomarkers that are carcinoembryonic antigen (CEA), erythrocyte sedimentation rate (ESR), and p53 in experimental rats. Continual increase in liver function enzymes was also observed in G_1 and G_2 as compared to G_4 and G_5. The immunohistochemical analysis was also conducted to assess the expression of proteins related to epigenetic alterations. The results revealed that p53 protein expression decreased in G_1 and G_5, whereas the expression of breast cancer gene-1 (BRCA1) and breast cancer gene-2 (BRCA2) increased in G_1, G_4, and G_5 due to increased immunoreactivity and well-defined nuclear staining. This can be concluded from the findings that G_4 and G_5 exhibited the strong hepatic toxicity mitigation potential as compared to G_1. This study opens the window to consider bioactive peptides for the development of functional foods or new drugs for anticancer therapy.

Keywords: neoplasm, memory tissues, oxidative stress, natural therapies, bioactive peptides, therapeutic potential

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INTRODUCTION

Bioactive peptide-based novel health-based strategies have been approved as effective in the prevention and treatment of cancer. So by focusing on the current scenario, protein amino acid profiling and peptides sequencing have become attractive approaches for determining the breast cancer biomarkers in the blood and breast tissues of a cancer patient (Biancarosa *et al.,* 2017). Cancer-targeting bioactive peptides are highly membrane permeable, block the tumor binding sites, and create perforations in cancer cell membranes, responsible for cell cycle arrest and induction of apoptosis (Guero *et al.,* 2021).

Structurally, bioactive peptides are comprised of a specific sequence of amino acids (2-30 units), which have potential as therapeutic as well as functional food components (Lafarga *et al.,* 2014; Zaky *et al.,* 2022). Milk (casein and whey) and fish bone-derived bioactive peptides composed of essential amino acid sequences, which, when characterized and hand sequenced, cause immuno-modulation, target tumor cell growth inhibition, and decrease treatment toxicity as compared to conventional drugs. Therapeutic peptides possess several physiological benefits potentially due to their small size, targeted tumor membrane penetration, reduced side effects, higher specificity, stability, and absorption (Cicero *et al.,* 2017).

Increased intake of high-quality proteins from animal sources containing all essential amino acids and bioactive peptides is considered a major regulator of healthy cell growth and inhibition of carcinogenic pathways (Dabiri *et al.,* 2016). The protein content of most fish waste is 15-30%, and among these, salmon and tuna have the highest protein content (16-24%), including higher proportions of essential amino acids. Furthermore, proteins obtained from fish muscles consist of 70-80% structural proteins, while the remaining 20-30% have sarcoplasmic proteins (Qiao *et al.,* 2018). Recent advancements in personalized nutrition and proteomics techniques have led to an escalation in the beneficial use of breast cancer biomarkers to ensure patients (Zeweil *et al.*, 2019) receive optimum treatment.

Breast cancer is the second leading cause of cancer-related mortality worldwide. Breast cancer occurs due to a complex aggregation of genetic changes characterized by abnormal cellular proliferation. It is a highly variable and manageable type of cancer, which, if diagnosed at early stages, can reverse the long-term associated side effects. Breast cancer containing lobules or ducts has heterogeneous histology, various signaling growth factors activation, and response to treatment therapies vary according to the stage (Aziz *et al.,* 2016). In many cancers, including breast cancer, the cell signaling molecules, such as pro-apoptotic and anti-apoptotic genes (Bax, Bcl-2), tumor-suppressor protein (p53), and breast cancer genes (BRCA1/2), are mutated and altered (Genovese *et al.,* 2017). Aging, late childbirth, early puberty, family history, gene mutations (p53, BRCA1, and BRCA2 genes), a sedentary lifestyle, poor breastfeeding practices, lack of hygiene, hormone replacement therapy, drugs, and radiation therapies, along with increasing breast density, are some major contributing factors for its onset (Xu *et al.,* 2017).

Various carcinogenic compounds are used to induce breast cancer in rats in experimental settings. The administration of DMBA causes strong immunosuppression and activation of carcinogenic events, leading to mammary carcinoma. (Zeweil *et al.,* 2019). Moreover, DMBA results in the disruption of tissue redox balance by increasing the production of free radicals (Ashraf *et al.,* 2021). Moreover, bioactive peptides demonstrated anti-cancerous effects by promoting apoptosis, regulating the functionality of the immune system, and inhibiting angiogenesis. Several studies have reported the cytotoxic potential of these peptides since biologically active peptides can arrest cell cycle (G_2/M phase), induce cellular apoptosis (caspase 3 activation), and reduce cytotoxicity of CD44 (+) cells (Jang *et al.,* 2008; Yu *et al.,* 2014; Li *et al*., 2020)

By keeping in view, the above-mentioned disease-preventing facts of bioactive peptides, milk and fish bones-based bioactive peptides were analyzed for their therapeutic potential in attenuating and modifying protein expression in breast cancer.

MATERIALS AND METHODS

Chemicals and Raw Materials: To produce water-soluble peptides from dairy sources (whey and casein), buffalo milk was collected from the Dairy Farm of the University of Agriculture, Faisalabad, Pakistan. Tuna Fish bones were purchased from Kataria Fish Farm, Tehsil Summandri, Faisalabad. DMBA for the induction of breast cancer in rat models was procured from TCI (Tokyo Chemical Industry) chemical manufacturer. While all other chemicals used in the accomplishment of research work were purchased from Sigma-Aldrich (St. Louis, MO, USA), Merck, and Fisher Scientific (CHEMTREC®, USA).

Water-soluble peptides preparation: To obtain the water-soluble fractions of casein, whey, and fish bones, the method elucidated by Rafiq *et al. (*2016) was followed. WSPs were extracted through homogenization of sample in D. water through proteolytic activity and centrifugation for separation of soluble fraction The water-soluble fractions obtained were filtered and freeze-dried by employing a lyophilizer (CHRIST®, ALPHA 1-4 *LD* plus*,* Germany). The synergistic effect of WSPs and mix fraction containing an equal ratio (1:1:1) of casein, whey, and fish WSPs was also investigated.

Peptides characterization: The water-soluble peptides (WSPs) in casein, whey, fish bones, and mix were characterized and purified following the method illustrated by Rafiq *et al*. (2016). An RP-HPLC system (Agilent, Series 1100, San Diego, California, USA) was used. The freeze-dried water-soluble peptide extracts of casein, whey, and fish (50 mg) mixed with solvent A (1 mL) consisting of trifluoroacetic acid (TFA) 0.1% were subjected to centrifugation for 10 min at 500 rpm and then filtered using a filter paper 0.45 µm. After this, a sample at a concentration rate of 50µL was injected into the column to measure the separation. The chromatographic dissociation was carried out at room temperature at a flow rate of 1.0 mL/min. Absorbance was checked at 215 nm wavelength by using a UV-spectrophotometer.

In vivo study: Contemporary study was conducted in National Institute of Food Science and Technology and Animal House, University of Agriculture Faisalabad. Permission was taken from the Institutional Bioethical Committee of the University of Agriculture, Faisalabad. All animals received human care according to the recommendations provided by the Committee. Rats were provided with standard diet and *ad libitum* tap water. They were kept in a temperature regulated room with 22°C temperature and a 12 hours light cycle.

Preparation of therapeutic dose: The therapeutic dose was finalized by conducting the acute toxicity test according to the method described by Akshatha *et al.* (2018). Female Wister rats were selected for the acute toxicity assay and administered with water-soluble peptides (casein, whey, fish bones, and mix) extracts at a dose level of 2000 mg/kg of body weight through intraperitoneal injections. Rats receiving the dose were monitored for 24 hours and up to 14 days for the development of any clinical symptoms, toxicity, or mortality. It was evaluated that all water-soluble peptide extracts have no lethal effect on experimental animals even at a dose level of 2000 mg/kg of body weight. By considering this, a 600 mg dose was chosen as a safe therapeutic dose to use in a further efficacy model for evaluation of the anticancer ability of bioactive peptides in mammary carcinoma.

Animals and cancer induction: To evaluate the effect of peptide extracts in a dose-dependent manner, sixty female Wistar rats were collected from the National Institute of Health (NIH), Islamabad, and placed in the animal room of NIFSAT, UAF, Pakistan. For one week, rats were fed a normal diet and water for acclimatization and housed under optimal conditions of light (12/12 light and dark cycle), temperature (27±2°C), relative humidity (60±10%), and pathogen-free surroundings.

Breast cancer in experimental rats was induced according to the method elucidated by Kinoshita *et al.* (2020). DMBA at a dose level of 60 mg/kg of body weight was dissolved in corn oil. It was then administered to female Wister rats on alternate days by dividing into two doses injected intragastrically through gavage at the beginning of the trial (Karnam *et al*., 2017). Rats were examined twice a week by touching, rubbing, and palpating to check tumor induction.

Study design: All animals were randomly divided into 6 groups: G_0 (Negative control), G_1 (Doxorubicin receiving rats), G_2 (Casein WSPs, 600mg/kg b.w.), G_3 (Whey WSPs, 600mg/kg b.w.), G_4 (Fish bones WSPs, 600mg/kg b.w.), and G_5 (Mixed WSPs, 600mg/kg b.w.). At the age of 8th weeks, all the rats were provided with freshly prepared normal feed pellets and cancer-inducing chemicals. Breast cancer was induced after 3 weeks of DMBA administration, and then standard drug doxorubicin and WSPs were given to all experimental groups except the negative control. Standard chemotherapy drug doxorubicin at a dose level (0.54 mg kg−1 b.w.) was administered to G_1 intravenously through injections once a week for 4 weeks, starting from the 6th week of the study. Physical and biochemical parameters were assessed throughout the study at three phases (1st, 6^{th,} and 12th). Afterwards, at the age of 20 weeks, all the animals were decapitated under controlled environmental conditions.

Serum and tissue antioxidant levels: At the age of 20th weeks, all the animals were anesthetized in a vaporized permeable CO_2 chamber and then decapitated under controlled environmental conditions. Mammary tissues of rats were excised, washed, and homogenized in phosphate buffer (50mM) at a pH of 7.4, centrifuged for 20 min at 5000 rpm, and then cooled at 4°C. The obtained supernatant after centrifugation was added to Eppendorf tubes and stored at -40°C. The level of antioxidant enzymes was determined according to the work of Kamisli *et al.* (2015). The activity of catalase (CAT) in serum and breast tissues was determined by following the protocols mentioned by Krishnamoorthy and Sankaran (2016). The superoxide dismutase (SOD) enzyme activity was evaluated spectrophotometrically according to the method expounded by Zargar and Masood (2022).

Tumor Biomarkers, Liver Function Tests (LFTs), and Hematology: The levels of carcinoembryonic antigen (CEA) and erythrocyte sedimentation rate (ESR) were estimated by the method elaborated by Linjawi *et al.* (2015). The activity of liver enzyme aspartate aminotransferase was determined according to the method of Huang *et al.* (2006). Alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities were assessed using commercial reagent kits through a spectrophotometer, following the method of Huang *et al.* (2006). Hematology parameters, including red blood cells (RBCs) and white blood cells (WBCs) levels, were measured by the method described by Vitak *et al.* (2017), while the platelet count was inspected by using the techniques implicated by Linjawi *et al.* (2015) et Ugbogu *et al.* (2019).

Histopathological analysis: Histopathological assessment of rat breast tissues was carried out by following the protocols of the pathology diagnosis of human breast tissues. After decapitation, mammary tissues were excised from the mammary glands of rats, followed by fixation of all rat organs in 10% neutral buffered formalin. After 10 days, the tissues were dehydrated (in graded ethanol) and embedded in paraffin wax (Denkert *et al.,* 2016). These tissue sections were mounted on glass slides and stained to observe under a light microscope (BX51, Olympus Company, Japanese). The photographs and digital images of slides were obtained by using a light microscope according to the method suggested by Feng *et al.* (2015).

Immunohistochemical Assay: Immunohistochemical (IHC) analysis for the evaluation of p53, BRCA1/2 (Breast cancer antigen gene 1/2) protein expression was conducted according to the method described by Hedau *et al.* (2015; Al-Dhaheri *et al.,* 2018). The avidin-biotin-peroxidase complex immunohistochemistry was carried out to assess the protein expressions in tumor-transplant tissues. The breast tissues embedded in paraffin were cut into 4 μm thickness and placed on positively charged slides for analysis. The slides were treated with primary monoclonal antibodies for p53 and

BRCA1/2, washed with phosphate buffer solution (PBS, wash buffer) three times for 3 min each, and stored overnight at 4˚C in a humidified box. The DAB (3,3'Diaminobenzidine) substrate chromogen agent was used to observe the reaction in the tissues, and excess substrate solution was removed by washing with tap water after incubation of tissues at room temperature for 5-10 min. Each slide obtained was subjected to the digital scanner for scanning digital photographs.

Statistical analysis: Quantitative data obtained after the completion of the bio-efficacy trial were analyzed using factorials under the two-way analysis of variance (ANOVA), followed by Tukey HSD (post-hoc test) for observing the variations among groups and multiple mean comparisons. Results are presented as mean ± SEM. A P value of < 0.05 was set to be considered significant in all experimental groups. The statistical software package SPSS v. 23 was used for data analysis.

RESULTS

HPLC-Based Characterization of Peptides: The HPLC peaks representing characterization of casein, whey, fish bones, and mixed water-soluble peptides are in Figure 1 (A-D). Each peak in the Figure showed the area proportional to the peptide concentration. The results revealed that peptides were eluted at retention times between 9 and 30 min among all types of WSPs. The maximum peptide concentration was determined in mixed WSPs from peak 3 (9.22 mg/mL) at RT between 18-20 min, followed by peak 4 (7.23 mg/mL) at RT between 20-22 min, respectively. Figure 1 (A) also depicts that the maximum number of peaks was also eluted in mixed WSPs. Figure 1 (D) portrayed the minimum number of peaks for peptides and the lowest peptide concentration (2.41 mg/mL at RT between 14-16 min) from peak 2 was observed in WSPs.

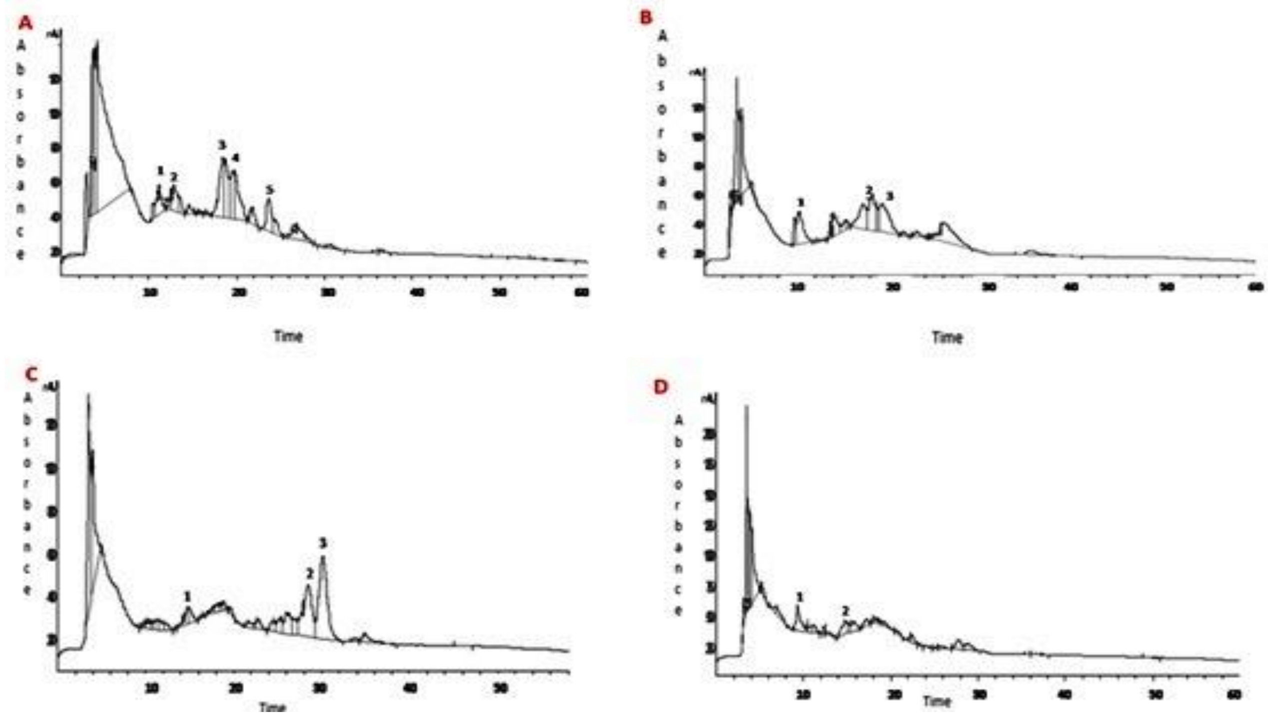


Figure 1 A: RP-HPLC profiling of Mix WSPs, B: RP-HPLC profiling of fish WSPs, C: RP-HPLC profiling of casein WSPs, D: RP-HPLC profiling of whey WSPs

Effect of Treatment on Physical Parameters: The feed intake, water consumption, and weight of all rats indicated highly significant differences ($P<0.01$) as shown in Figure 2 (A-C). The feed intake of the negative control group (G_0) portrayed the highest level as compared to other groups. Whilst, in the G_1 (doxorubicin receiving rats) demonstrated the lowest feed intake. However, the feed intake and water consumption increased significantly in G_4 and G_5, which was more than in G_2 and G_3 ($P<0.05$). Furthermore, treatment with mixed peptides significantly raised the body weight of rats as compared to G_1, exhibiting markedly low body weight.

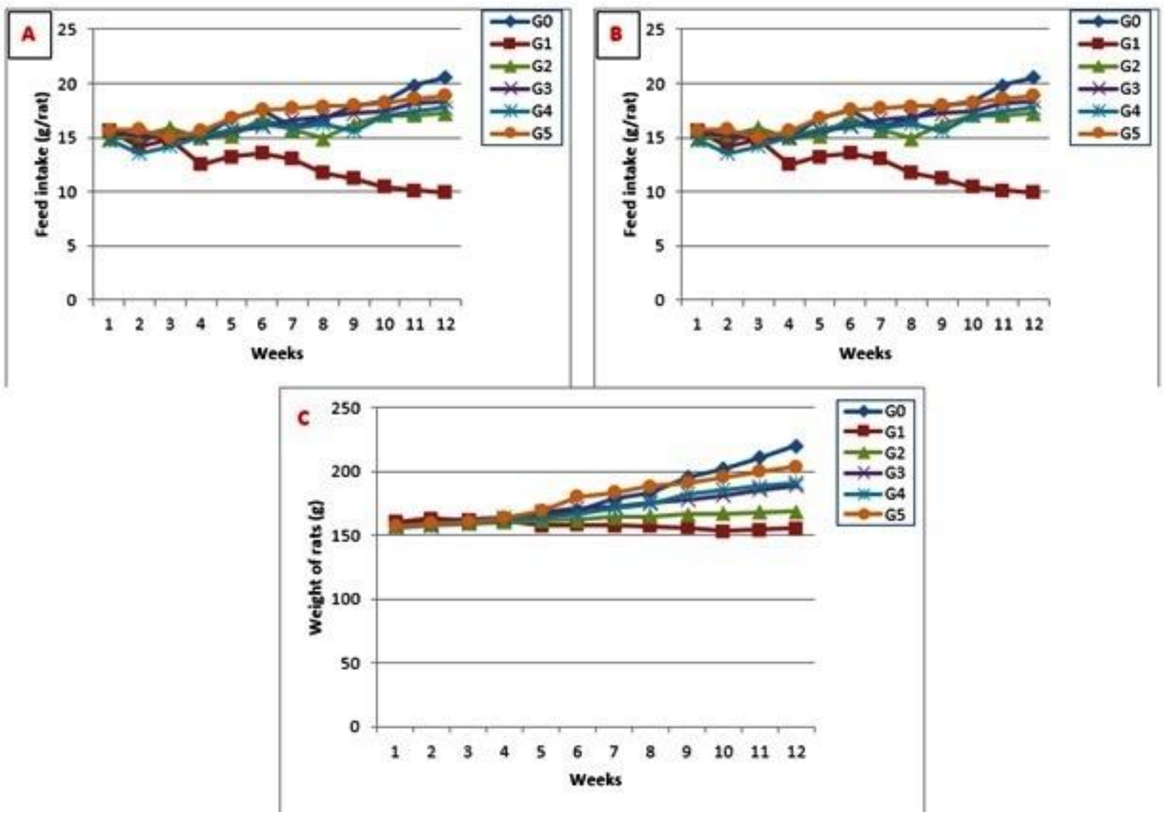


Figure 2 (A-C) Physical parameters and weight of breast cancer-bearing rats (A) feed intake, (B) drinking water consumption, and (C) weight of rats

Effect of Treatment on Serum and Tissue Antioxidants: The analysis of serum and mammary tissues antioxidant enzymes (SOD and CAT) depicted a significant ($P<0.05$) effect of WSPs. The results presented in Figure 3 (A-B) revealed that serum SOD activity was highest in G_1 followed by G_5. In mammary tissues, a rise in SOD levels was seen as $G_1>G_5>G_4$. Moreover, Figure 3 (C-D) explicit significant alterations in serum and mammary tissue CAT levels in response to different treatments. The highest level of serum CAT was assessed in G_1 and G_5. The mix WSPs significantly maintained the serum and mammary tissues SOD and CAT levels of rats.

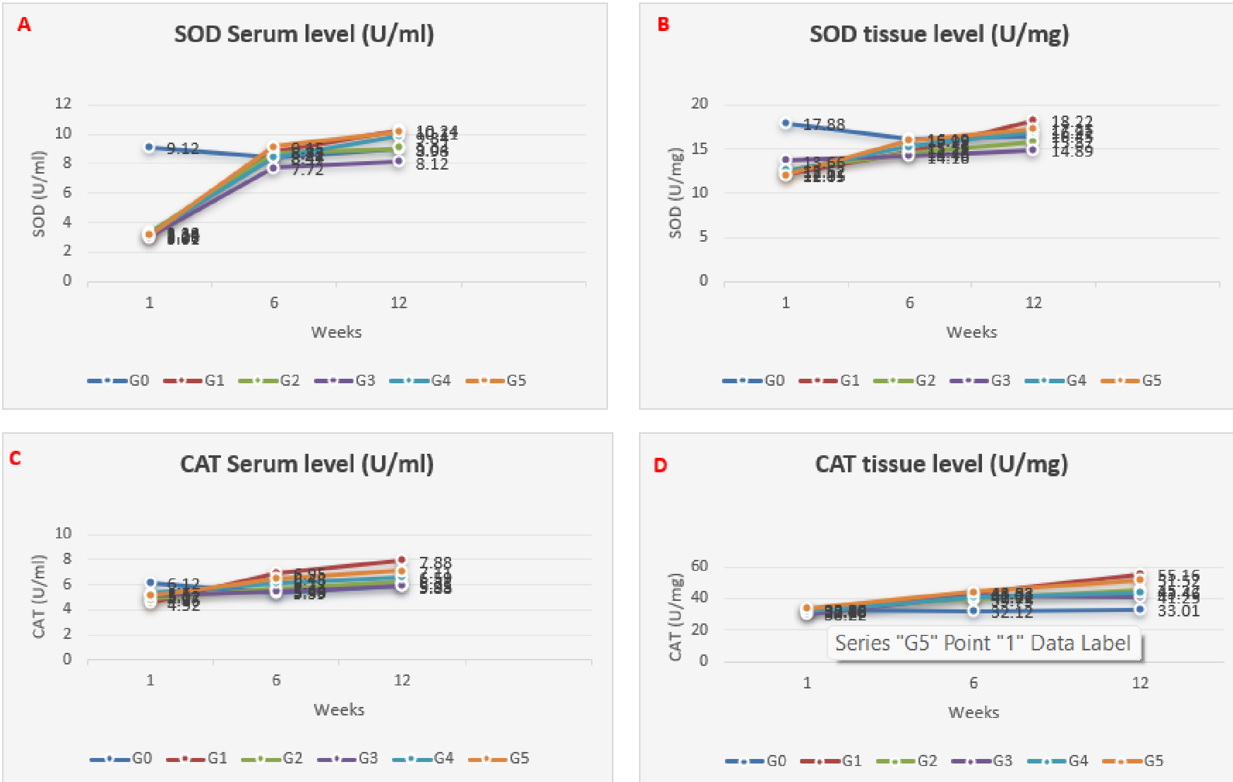


Figure 3 (A-D) Antioxidant enzymes status

Effect of Treatment on Tumor Biomarkers and Liver Enzymes: The anti-tumoral effect of WSPs was also determined on the most used tumor markers CEA and ESR. The administration of DMBA caused a considerable increase in serum tumor biomarker levels. However, the exposure to WSPs significantly ($p < 0.05$) controlled the rise in CEA (Figure 4 A) and ESR levels (Figure 4 B) in breast cancer rats. The introduction of the standard drug doxorubicin significantly prevented the elevation of tumor biomarkers in experimental animals, as observed in the mixed WSPs group.

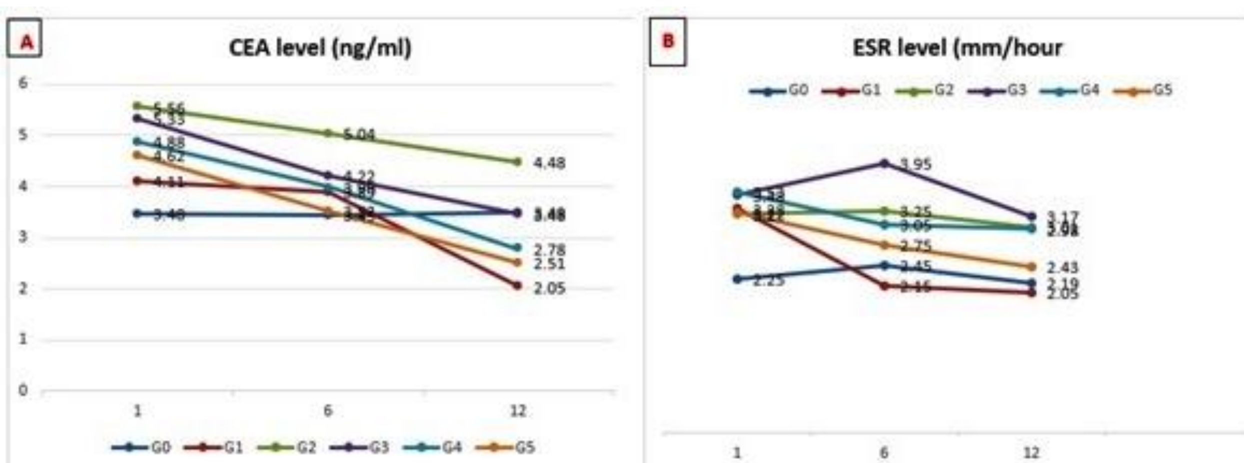


Figure 4 (A-B) Serum tumor biomarkers

The effect of WSPs exposure on liver toxicity in DMBA-induced breast cancer rats was assessed through liver function enzymes. Figure 5 (A, B, and C) depicts the significantly higher ($p < 0.05$) level of alanine transaminase (ALT) and alkaline phosphatase (ALP) in doxorubicin-administered rats (G_1). However, the activities of AST and ALT increased; the maximum activities of AST and ALT were highest in rats treated with doxorubicin (G_1). At the same time, a non-significant difference in ALP was measured in G_1, as compared to their level in G_4 and G_5. Furthermore, the rats treated with mixed WSPs indicated significantly lower (p<0.05) activity of ALT, AST, and ALP compared with G_1 and G_4.

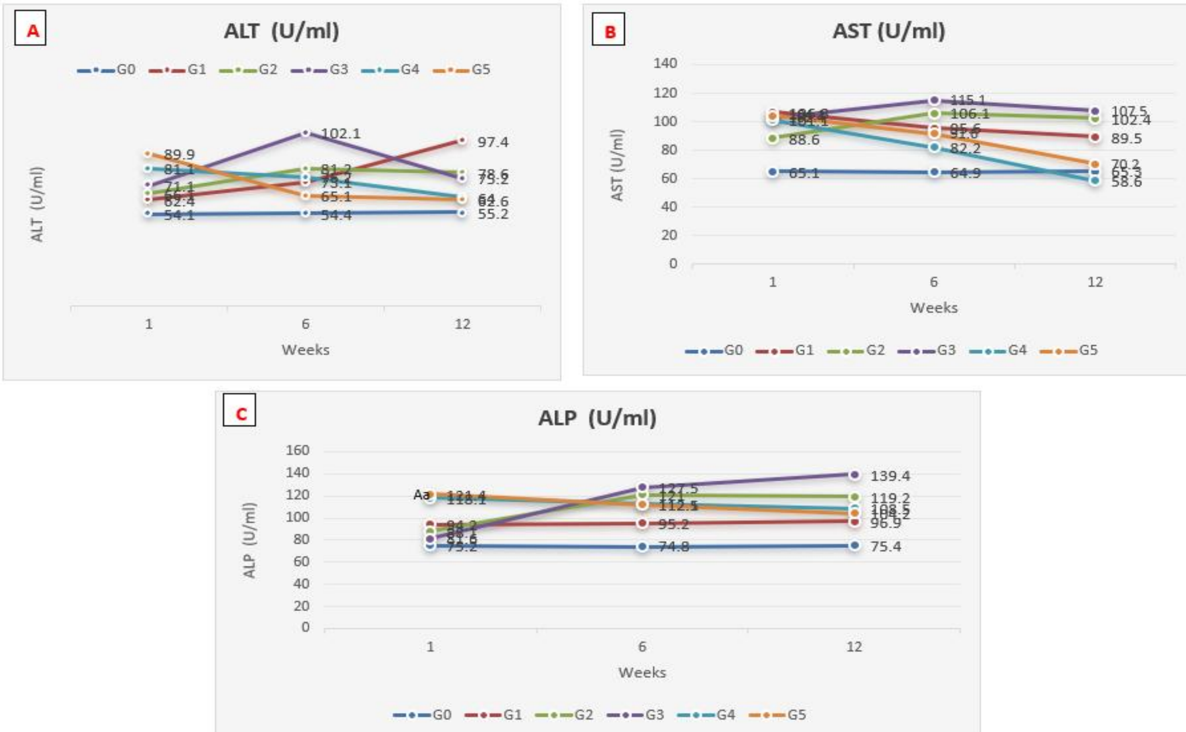


Figure 5 (A-C) Liver function enzymes

Effect of Treatment on Haematological Indices: Haematological indicators were analyzed in this experiment due to high susceptibility to toxic metabolites and chemicals. The findings given in Figure 6 (A-C) portray the statistically significant effect (P<0.05) of all treatments on red blood cells (RBCs) and hemoglobin (Hb) levels, while non-significant (P>0.05) influence white blood cells (WBCs). The maximum decline in RBCs and Hb level was measured in G_1. However, in rats treated with fish and mixed WSPs (G_4 and G_5), the decreasing trend of RBCs and Hb was lower than that of the decline in G_1, G_2, and G_3, respectively. Moreover, the administration of mixed WSPs effectively controlled the level of WBCs.

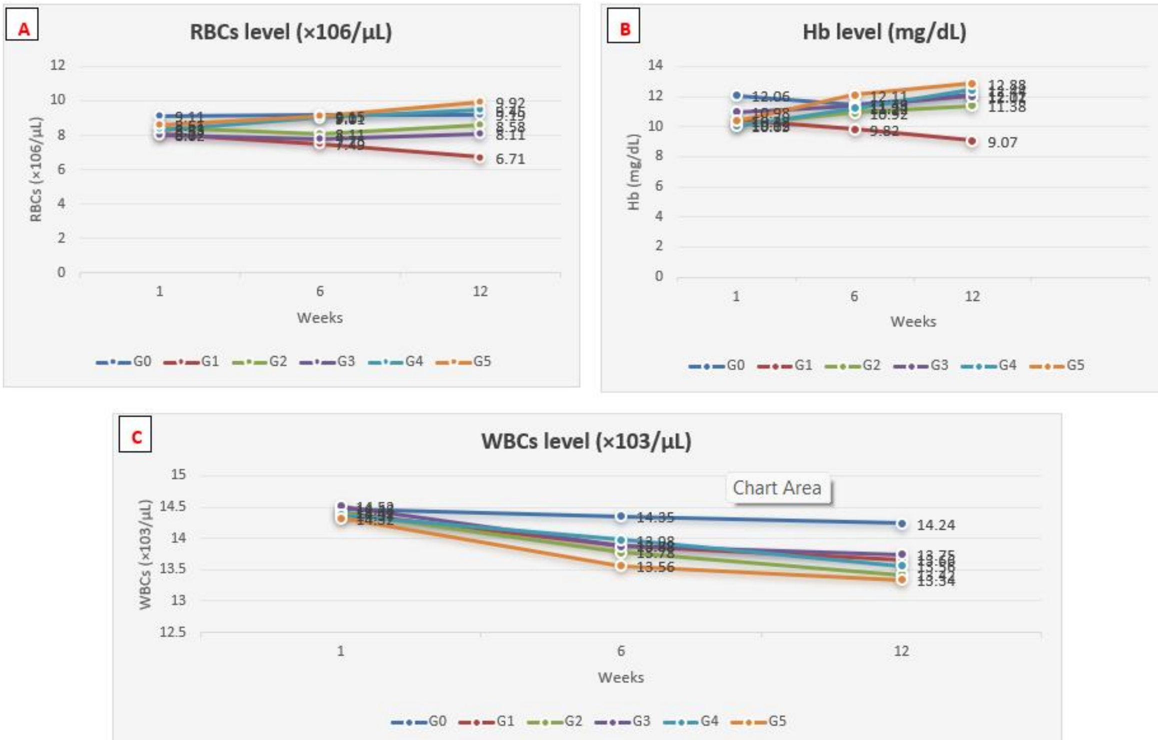
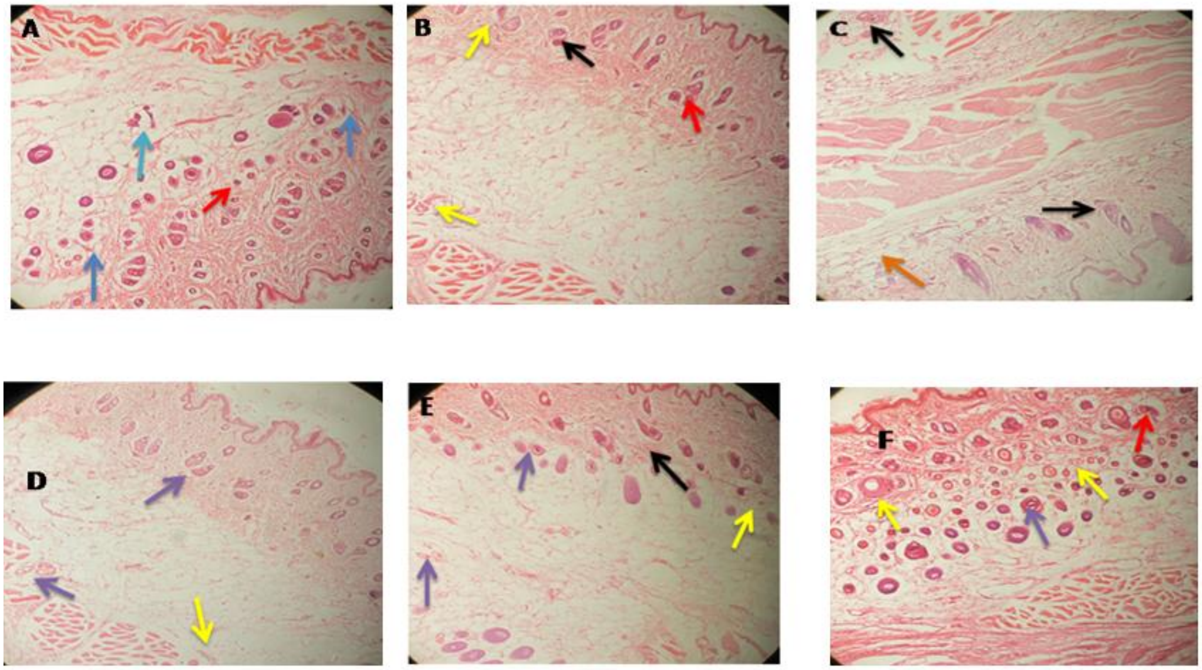


Figure 6 (A-C) Hematological parameters.

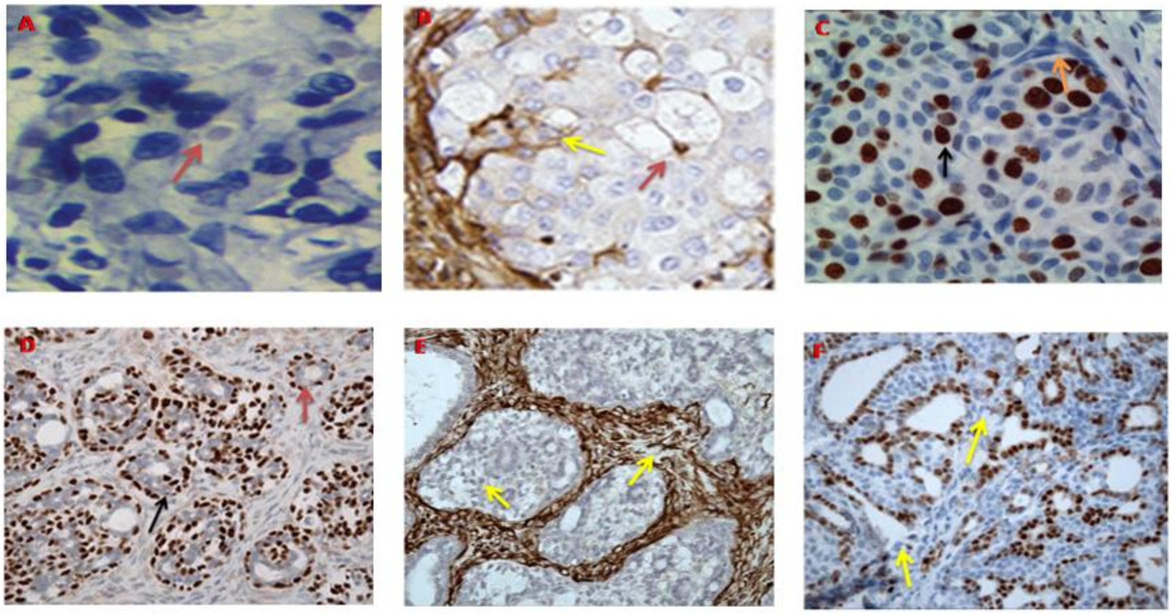
Histopathology: The histopathological analysis of the mammary tissues of all rats from the experimental groups also showed the different histological structures. Figure 7 (B) shows the histopathological picture of G_1 and dense adiposity associated with mild inflammation adjacent to the tumor area is identified. Moreover, the appearance of apoptotic cells and hyperplastic lobules hyperplasia are also found in ductal carcinoma. Figure 7 (C and D) explicitly shows the mammary tissues of G_2 and G_3 in which chronic inflammation, neoplasia of lesions, and different size nucleus are assessed. The atypical acini and ductules proliferate widely in G_2 and G_3. Figure 7 (E) depicts the breast tissues of G_4 and exhibits the improved histological characteristics of breast tissues. Mild to the moderate peritumoral inflammatory response in the tumor area and mild apoptosis were observed in G_4. Figure 7 (F) indicated the mammary tissues of G_5 and unveiled moderate apoptosis and neutrophil infiltration. Furthermore, the signs of hyperplasia of lobules were also reduced in this group.



A= G_0, B= G_1, C=G_2, D=G_3, E=G_4, F=G_5

Figure 7 (A-F) Histopathological characteristics of mammary tissues of all experimental groups A) Red arrow= Neutrophil infilteration; Blue arrow= cellular congestion B) Yellow arrow= Apoptosis induction; Red arrow= Neutrophil infilteration; Black arrow= Breast cancer development C) Black arrow= Breast cancer development in acini and ductules of tissues; Orange arrow= atypical acini and ductules D) Purple arrow= Chronic inflammation and neoplasia of lesions; Yellow arrow= Apoptosis induction E) Purple arrow= moderate peritumoral inflammation and neoplasia of lesions; Yellow arrow= mild apoptosis induction; Black arrow= Breast cancer development in acini and ductules of tissues F)Purple arrow= Chronic inflammation and decline neoplasia of lesions; Yellow arrow= Apoptosis induction; Red arrow= Neutrophill infilterationand reduced signs of hyperplasia.

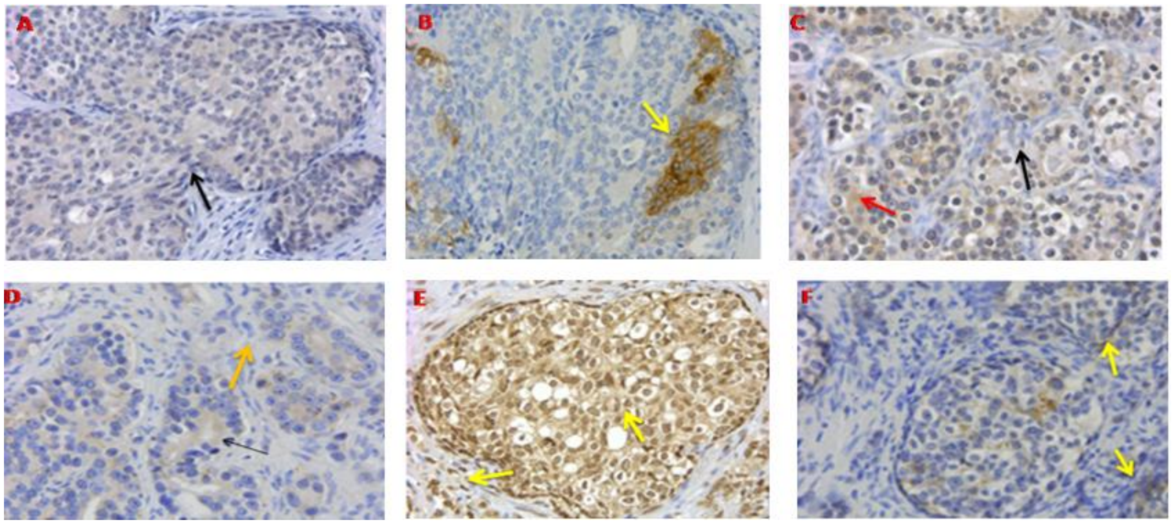
Immuno-histochemistry Assay for p53, BRCA1 and BRCA2: Immunohistochemistry (IHC) staining is the key method to determine the presence and location of proteins associated with tumortumor development and prognosis in tissue sections. Abnormal alteration in p53 expression leads to the development of aggressive tumortumors and stimulation of oncogenic events via inhibition of apoptosis (Li et al., 2020). At the end of the experimental trial, the breast tissues of female Wister rats from all different groups were analyzed through immunohistochemistry analysis. The paraffin-embedded tissue sections were processed to assess the p53 immunoreactivity and expression in breast tissues to understand the extent of apoptosis and tumortumor localization. Figure 8 (A-F) reveals that increased p53 expression occurs in G_2 and G_3 because of sharply defined nuclear and cytoplasmic staining (darker the stain more expression of proteins), whereas decreased expression occurs in G_1, G_4, and G_5 due to lightened nuclear staining and localization.



A= G_0, B= G_1, C=G_2, D=G_3, E=G_4, F=G_5

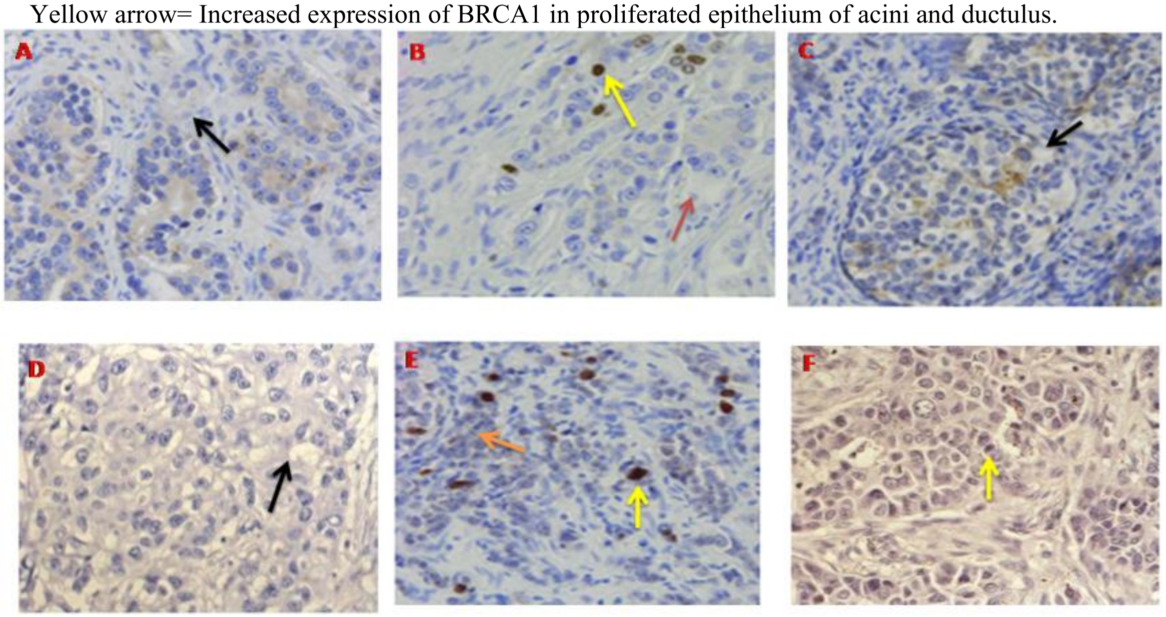
Figure 8 (A-F) Immunohistochemical analysis of p53 expression A) Red arrow=No p53 immunoreactivity observed in negative control group, lightening of nuclear staining in ductal and acini of breast tissues B) Yellow arrow= Decreased immunoreactivity of p53 Red arrow= lightening of staining in ductal and acini C) Black arrow= p53 immunoreactivity increased and sharply defined nuclear staining, Orange arrow= cytoplasmic staining revealed the diffuse pattern D) Black arrow= sharply defined nuclear staining of p53 as expression of p53 increased, Red arrow= lightening of staining in ductal and acini of tumor area is shown by brown color E) Yellow arrow= More larger and irregular tumor cell nuclei are indicated by deep invaginations from surfaces and p53 Immunohistochemical staining was decreased as lighter color of cytoplasm and negatively stained nuclei. F) Yellow arrow= p53 immunoreactivity was decreased and less sharply defined nuclear staining due to less expression of p53 and nuclear localization.

Breast cancer genes (BRCA1/BRCA2) are responsible for the activation of tumor suppressor genes (TSGs), thus reversing the abnormal proliferation of cancer cells. The results representing immunohistochemical staining of BRCA1/BRCA2 expression in all experimental rats are presented in Figures 9 **(**A-F) and 10 (A-F). It is speculated from the results that all treatments significantly affected the BRCA1/BRCA2expression. The protein expression of BRCA1/BRCA2 was increased significantly in G_1, G_4, and G_5 as assessed by sharply defined nuclear staining in acini and ductules of proliferated mammary tissues. The results of immunohistochemistry portrayed the reduction in cancer cell proliferation from fish and mixed WSPs as the same as the standard drug.



A= G_0, B= G_1, C=G_2, D=G_3, E=G_4, F=G_5

Figure 9: Immunohistochemical expression of BRCA1 in breast cancer experimental rat tissues. The photomicrographs show (A) Black arrow= No BRCA1 immunoreactivity (B)Yellow arrow= Increased expression of BRCA1 (C)Red arrow= decreased expression of BRCA1 in proliferated epithelium of acini and ductulus; Black arrow= decreased BRCA1 immunoreactivity (D) Black arrow= decreased BRCA1 immunoreactivity; Orange arrow= less sharply defined cytoplasmic staining, larger and irregular nuceli (E)Yellow arrow= Increased expression of BRCA1 in in proliferated epithelium of acini and ductulus (F) Yellow arrow= Increased expression of BRCA1 in proliferated epithelium of acini and ductulus.



A= G_0, B= G_1, C=G_2, D=G_3, E=G_4, F=G_5

Figure 10 Immunohistochemical expression of BRCA2 in breast cancer experimental rat tissues. The photomicrographs show (A) Black arrow= No BRCA2 immunoreactivity and expression (B)Yellow arrow= Increased expression of BRCA2; Red arrow= BRCA2 localized inside nucleous (C)Black arrow= decreased expression of BRCA1 in proliferated epithlium of acini and ductules, less cytoplasmic staining and irregular nuclei (D) Black arrow= decreased BRCA2 immunoreactivity and less nuclear staining (E)Yellow arrow= Increased expression of BRCA2 in in proliferated epithlium of acini and ductules; well defined cytoplasmic and nuclear staining, Orange arrow= well defined cytoplasmic and nuclear staining (F) Yellow arrow= Increased expression of BRCA2 in proliferated epithlium of acini and ductules.

DISCUSSION

Owing to the significant role of bioactive peptides in regulating certain aspects of the functionality and communication of cells, the researchers' interest in bioactive peptides has increased in the past few decades (Zaky *et al.,* 2022; Chakrabarti *et al.,* 2018; Colletti, *et al.,* 2022). Moreover, the development of encapsulated peptides through nanoparticle technology may improve membrane permeability and penetration of peptides to reduce cancer cell resistance to drugs. It has been speculated by various researchers that larger molecular weight and complicated structure of intact proteins make their digestion and absorption more difficult than peptides (Skripnikov, *et al.,* 2011; Qiao, *et al.,* 2018).

Based on the research hypothesis that casein, whey, fish bones, and mixed WSPs may reduce breast cancer progression, the present study was planned to investigate the anti-cancer potential of water-soluble peptides (Offret *et al.,* 2019), reporting anti-inflammatory peptides from Atlantic fish (Mackerel) by using the RP-HPLC characterization followed by two steps. The first fraction elution was measured between 15-20 minutes, while the second elution was noticed at 19 minutes of retention time. The second step elevated the activity of the anti-inflammatory peptides , as it was 20.833 times higher than the initial peak. (Wang, *et al.,* 2013) characterized the casein hydrolysates synthesized through enzymatic hydrolysis. The chromatographic quantification (HPLC) of the generated peptides during the enzymatic hydrolysis was performed. Furthermore, (Yi *et al). (*2017; Iosageanu *et al.,* 2021) determined the antioxidant activity of bioactive peptides from fish. The findings speculated that peaks at retention time 2-5 min contain hydrophilic small-sized peptides with high permeability. The peptides synthesized in the current experiment were slightly different from those of another researcher, which could be due to variations in protein sources (casein, whey, fish bones, and mix), composition, and different processing conditions (time and temperature).

Weight loss is the most common symptom during advanced cancer metastasis, affecting the quality of life, survival, and potential outcomes of anticancer drug therapy (Mariadoss *et al.,* 2019). The increasing demands of cancerous cells for more and more energy result in depletion of energy reserves and lead to considerable weight loss. Although the exact mechanism of weight loss among cancer patients is still not clear, it has been speculated that altered nutrient metabolism might be the reason (Fearon *et al.,* 2013). In the present study, remarkable weight loss was observed in rats after exposure to DMBA, as this chemical significantly reduced food consumption and decreased energy intake. These findings are consistent with *in vivo* studies of Mariadoss *et al.* (2019) and Rajendran *et al.* (2019). This loss of body weight in rats might be attributed to altered energy metabolism after administration of DMBA (Rajendran, *et al.,* 2019). Moreover, in the current experiment, the chemotherapy drug doxorubicin resulted in a severe reductio in the body weight of rats, which is further supported by several *in vivo* studies (Patel *et al.,* 2013; Arroyo-Acevedo *et al.,* 2015; Rocha *et al.,* 2019). The drastic body weight losses after administration of chemotherapeutic agents were due to altered metabolism, loss of appetite, increased energy expenditure, and high catabolism (Hiensch *et al.,* 2020). Consequently, weight gain was lowered in rats administered with DMBA and doxorubicin, while it improved significantly upon exposure to ,WSPs' bioactive peptides responsible for increasing appetite and improved feed intake.

It has been reported that DMBA exposure results in the production of peroxide as well as superoxide free radicals and depletes antioxidant enzymes, thus causing oxidative stress (Zeweil *et al.,* 2019). It has been observed that reactive oxygen species (ROS) accumulation may cause serious complications through the onset of cellular damage and inflammatory processes (Ibrahim, *et al.,* 2018). The oral supplementation of fish and mixed WSPs significantly reversed the changes to near normal. Thus, it was concluded that WSPs improved the antioxidant enzyme status in DMBA-induced breast cancer rats. It is reported that peptides may exert antioxidant effects through the donation of a hydrogen atom and metal chelating potential (Xing et al., 2019). . The findings of research studies (Zeweil, *et al.,* 2019; Kasapovic *et al*., 2010; Yi *et al.,* 2020; Krishnamoorthy and Sankaran, 2016; Henry *et al.,* 2020) further support current outcomes.

Serum tumor biomarkers play a remarkable role in cancer progression, particularly in the management and selection of suitable systemic therapy Duffy *et al.,* 2017). The continual increase in biomarkers is a sign of negative treatment response or recurrence of cancer (Kabel, 2017). A rise in CEA level was associated with high tumor incidence, increased age, and non-triple negative breast cancer (Li *et al*., 2018). In current research, the tumor biomarkers CEA and ESR were significantly increased in rats administered with DMBA, while exposure to WSPs and doxorubicin significantly controlled the level of these biomarkers. These findings are closely related to investigations of (N'guessan *et al.,* 2021) in which *Nymphaea lotus*-derived polyphenol extract caused a decline in ESR levels as compared to a positive control group. Similarly, zuegbuna *et al*(2022) and Antonilli *et al* (2016) reported the higher ESR rate and CEA in breast cancer patients. Furthermore, Pandi et al). (2011) assessed the anti-tumor characteristics of Taxol, synthesized from an endophytic fungus on DMBA-induced mammary tumor rats, and reported a significant reduction in CEA levels of taxol group as well as doxorubicin treated group.

The DMBA metabolizes into DMBA-3-4 epoxide and free radicals in the presence of cytochrome P450 enzymes, resulting in liver toxicity and injury (Hosny *et al.,* 2021). To analyze liver toxicity, the liver function enzymes

ALT, AST, and ALP is most commonly assessed (Chane, *et al.,* 2023). The increase in liver enzymes is associated with DMBA-induced toxicity and the release of enzymes into the blood circulation subsequently (Oleshchuk et al., 2019). In the current study, the exposure to DMBA caused an elevation in liver enzymes in the serum of rats. Conclusively, the WSPs caused effective control of liver enzymes in rats with DMBA-induced breast carcinoma. The findings are in line with the results of (Dakrory *et al.,* 2015; Hamad *et al.,* 2011; Anber, 2018; Oluboyo *et al.,* 2015; Rocha *et al.,* 2019). It was unveiled that peptides comprising aromatic amino acids, histidine, tryptophan, and cysteine are involved in the suppression of oxidative stress via lipid peroxyl radical trapping, donating electrons to electron-deficient moieties, and binding to prooxidant metal ions, which may further support the therapeutic potential of WSPs exhibited in current research (Ajibola *et al.,* 2011; Esfandi *et al.,* 2019; Toldra *et al.,* 2018).

The histopathological analysis is performed to identify all the alterations in tissues related to disease and responses to systemic therapy (Rocha *et al.,* 2019). Apart from physical parameters and biochemical markers, the histopathological investigation in the present study showed mild to moderate peritumoral inflammatory response, fewer lobules of hyperplasia, and moderate apoptosis in the G_1, G_4, and G_5 groups.

To assess the possible underlying protective mechanism of WSPs, immunohistochemistry of the breast tissues was performed. In current research, WSPs significantly reduced the p53 expression. Loss of p53 functioning can stimulate the oncogenic events via loss of apoptosis. As a result of DNA mutations, the level of p53 increases, which in turn elevates the p21 transcription of p53 and thus modulates the cell cycle arrest at the G_1 phase. This process helps the cells to survive until the abnormal changes or damage are removed (Li *et al.,* 2020). Yang *et al*. (2018) and Dia and Krishnan (2016) investigated the effect of peptides on cancer cell lines and reported that increasing the dose of peptides and incubation period resulted in down-regulation of cell cycle-associated gene expression (Cdk4), and up-regulation of pro-apoptotic gene Bax.

BRCA2 stimulates apoptosis in cancer cells via the TRAIL receptor signalling pathway and activation of caspase 8, in addition to the DNA repair mechanism (Sadeghi *et al.,* 2020). Yan *et al.* (2019) investigated the BRCA1 modulated apoptosis in serum-depleted breast cancer cell lines (MCF-7). The results revealed that BRCA1 stimulated the programmed cell death via induction of phosphorylation of the JNK pathway, Fas-ligand interaction, and activation of caspases (8 and 9). Moreover, peptides stimulated the cell cycle arrest and inhibited cancer cell proliferation in breast and cervical cancer cell lines (Marcela *et al.,* 2016). The results obtained from the present study are in correspondence to these findings. On the basis of serum, histopathological, and immunohistochemical analysis, it is concluded that water-soluble peptides can reduce the risk of oxidative stress, inflammation, and cancer.

Conclusion: The WSPs derived from milk proteins (casein, whey), fish bones, and mixed sources showed a promising next-generation treatment option for breast cancer due to their small size, immune system regulation, cancer cell targeting, and less toxicity to healthy cells. Further clinical and efficacy trials are needed to understand the detailed structural composition and deep cancer cell penetration ability of bioactive peptides. In the current research work, WSPs proved effective for suppressing the tumor biomarkers via regulating the antioxidant enzymes as well as increasing the expression of breast cancer proteins (BRCA1 and BRCA2). Concisely, the value-added products containing bioactive peptides can curtail the alterations associated with breast cancer.

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