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A NOVEL EXPERIMENT ON PREVENTION OF BREAST TUMOR FORMATION THROUGH PROPHYLACTIC ADMINISTRATION OF A MULTIHERBAL COMPOUND AGAINST THE CARCINOGENIC CHEMICAL DMBA (7, 12-DIMETHYLBENZANTHRACENE) IN EXPERIMENTAL VIRGIN FEMALE WISTAR RATS- AN INCONCLUSIVE TRIAL

Medical Science

M.V. Ravishankar* JSS Medical College, JSS Academy of Higher Education and Research, Mysuru, Karnataka, India PIN; 570015 *(Corresponding author)

Mohd Suhaimi Wahab School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia

Sunil S Jalalpure Dept. of Pharmacognosy and Phytochemistry, KLE College of Pharmacy, Belgaum, Karnataka.

Sadanand B Patil Dept. of Biochemistry USM-KLE, IMP, Belagaum, Karnataka, India PIN-590010

Dhanashree Patil Dr Prabhakar Kore BSRC, KLE Academy of Higher Education and Research, Belgaum, Karnataka

ABSTRACT

Introduction-Presently existing modern therapies for the breast cancer treatment is having limitations. The ancient Indian medical science has stressed much on the concept of disease prevention to enhance the longevity by facilitating disease free healthy life. It is the time to revisit the application of immune modulation therapy to reduce the growing incidence of malignant diseases.

Objective- Study the breast cancer prevention effect of natural herbal supplements in chemically inducible cancer

Methodology: The healthy rats which have started receiving herbal supplements from the 25th day of post-natal period. They were administered on daily basis for 30 days with the drug Immunocin at a dose of 100mg/kg body weight at different time interval in the form of extract in different age matched experimental group of rats. These different groups of rats were interfered with oral administration of 15mg single dose DMBA (7, 12-Dimethylbenzanthracene) at different time interval to induce the breast tumor. At the end of treatment schedule animals were sacrificed. Blood sample was collected for complete blood count (CBC) analysis. The liver and breast tissue was collected for histopathological examination. Cell viability assay was done by using the drug Immunocin on MCF (Michigan Cancer Foundation) cancer cell lines.

Conclusions: It is uncertain to induce any tumor by using the chemical DMBA. Hence it is uncertain to correlate the tumor prevention activity through prophylactic administration of the drug Immunocin. Through the cell assay, the anticancerous property of our drug on cancer cell lines was found ineffective. Hence our results were negative and inconclusive.

KEYWORDS

Antioxidants, Malignancy, Rejuvenation

Introduction

The cancer is assuming a catastrophic proportions worldwide. Over a period of time our understanding about this disease has improved considerably. This has helped to achieve effective treatment by the clinicians with reducing side effects of its treatment. Current therapies are well established to tame this deadly disease soon after its diagnosis during the early phase itself. However modern therapies are showing much limitation with respect to prevention of cancer and also its complications [1]. Advancing age is one of the factor showing gradual inclination towards malignant changes in susceptible individuals. Where large numbers of malignant diseases were diagnosed during the late adult life. Among the different medical systems evolved worldwide, Ayurveda is considered to be the most ancient one. Based on its literature, number of herbal products are formulated and sold in the Indian market, which are widely used by large Indian community for the maintenance of their good health. But most of such commercially available products still require many more basic, scientific and rational experimental proofs. To study the prophylactic effect of any intervention, the chemical induction of cancer is one of the best alternate. The chemical mode of induction of cancer is widely practiced by using experimental animal models[2]. Cancer ailment is drawing much attention, there will be drastic rise in number of malignant cases from 9, 79,789 in the year 2010 to 2,14,8757 by the year 2020.The total female cancer cases are likely to go up from 75,289 in the year 2010 to 93,563 by 2020. The gynaecological cancers are expected to go from 153850 in the year 2010 to 1, 82,602 by the year 2020. Particularly the breast cancer alone in males and females are expected to go above 10, 00 00 by the year twenty twenty[3].A small observational hospital based study data of cancer patients in Manipal has shown that there is higher relative risk ratio among the followers of non-vegetarian diet when compared with the vegetarians this may suggest the importance of vegetarian diet on healthy life [4]. Ayurvedic herbs are well known for their antioxidant activity. Reactive Oxygen Species (ROS) formation plays an important role on the biological process of carcinogenesis. There must be a balance between free radical formation and its scavenging activity through antioxidant defence mechanism; which can prevent the occurrence of the disease in an individual. This imbalance can makes our body prone for different range of ailments. In the developing countries female breast cancer is the second most deadly disease affecting the large population next to uterine cervical cancer[5].

Study background: Prevention of disease is always a challenging task due to individuals varied pattern of life style including their dietary patterns, genetic profile, exposure to threats, immune competency, psychosomatic stress etc. Ayurvedic literature elaborately describes the special chapters on "Rejuvenation Therapy" popularly known as Rasayana Chikithsa. It is a special prevention strategy which modifies our immune system to fight or prevent the threats with the advancing age. Classical rejuvenation therapy mentions usage of number of herbs along with Ayurvedic principles of drug processing with administration[6]. In the present day, the preventive treatment for first degree relatives of patients suffering from carcinoma of breast is very radical i.e. surgeries like removal of total breast (bilateral mastectomy). Based on this an attempt was made experimentally to test the chemical cancer prevention effect in healthy individuals. The drug Immunocin is a combination of extracts of herbs like Withenia somnifera, Ocimum sanctum, Azdirachta indica, Curcuma longa, Tinospora cordifolia, Boerhaavia diffusa, Piper longum. To test the chemical DMBA effect on mammary gland tumor formation in rats which were already (prophylactic) administered with the trial drug Immunocin were studied to assess their disease acquaintances.

Materials and Method

Type of Study: Animal experimental study Rats[7]

Animals used: Twenty five days old female rats

Drugs used: Tablet Immunocin (GUFIC Biosciences Limited, Belagavi, Karnataka State, India, Manufacture Date Nov.2014 Lic no: L-AUS-109)

Ethical clearance: Resolution No. KLECOP/CPCSEA-Reg.No.221/Res.23-2/09/2016

Form of administration: Chemical DMBA administered by dissolving it in sunflower oil and the Immunocin extract was administered by dissolving it in drinking water

Route of administration: Cancer inducing agent and therapeutic drug, both were administered through oral route
Induction process of experimental breast cancer: by the administration of 15mg single dose of 7, 12 – Dimethyl benzanthracen [8].
Experimental groups: each group contains 6 rats
In all these age matched different group of experimental rats the intervention of the experimental drug (Immunocin) and standard carcinogenic agent (DMBA) was done to test the levels of disease acquaintances.

Treatment control group I: In this group rats were administered with the Immunocin extract alone from the 25th day till 54th day, and later on the 55th day they were administered with the single dose of DMBA, and Immunocin administration was continued for the next 30 days till the age 85th day, and they were sacrificed by the end of 155th day.

Treatment control group II: In this group, on the 55th day the rats were administered with the single dose of DMBA+ single dose of Immunocin. They were observed and sacrificed on the 155th day.

Treatment control group III: In this group these rats were administered with the Immunocin extract from the 25th day till 54th day, and later on the 55th day they were administered with the single dose of DMBA alone and the later were scarified on the 155th day.

Standard control group IV: In this group on the 55th day, rats were administered with DMBA alone as a standard drug for the induction of the malignant mammary tumor, and they were observed and sacrificed on the 155th day.

Study Parameters:
1. Histopathology of mammary gland and liver.
2. Complete Blood Count (CBC)
3. MTT (Microculture tetrazolium) assay

Procedure:
The tablet Immunocin is a commercial product contains multiherbal drug combination was purchased from the market. It was made to dissolve in the rectified spirit and later supernatant was filtered, dried, weighed and later was used for its administration by dissolving it in drinking water. The dissolved drug was administered through orally to intragastric by using baby feeding tube. First we have completed the toxicity study as per Organisation for Economic Co-operation and Development (OECD) [9]; it is found that our drug is safe to use with 100mg single dose orally. In our novel experimental study we have started with prophylactic administration of Immunocin drug dose 100mg to all the experimental groups of (normal) rats. In the initial stage of our experimental trial a separate isolated group of rats were given with 20mg single oral dose of DMBA by using the sunflower oil. On its administration all the rats in this group were died within 11days after its administration; but these died rats presented with no signs of breast tumor formation during this period. With the hope of their effective survival we started with the lower single dose of 15mg DMBA administration in all the experimental groups, and the rats were observed for the next 16 weeks. Simultaneously carcinogenic drug (DMBA) administration was interfered with Immunocin drug administration in different groups of rats at different time intervals as stated above. Weekly observation of rats for breast tumor formation along with liver and lymph node enlargement was under regular vigilance. We have regularly checked for the breast, liver and lymph node enlargement as physical signs, but couldn't notice any. To support the possible overt expression of subclinical pathogenesis, subsequently at the end of 16th week we have given an additional 5mg dose of the DMBA in the similar pattern as said above. In spite of an additional dose administration we couldn't notice any tumor formation in the next 4 more weeks i.e. by the end of 20th week of study period. At the end of 5 months all the rats were physically examined thoroughly for the tumor formation and later they were sacrificed through the cervical dislocation. Breast, Liver tissue were collected and sent for histopathological examination and blood sample was sent for complete blood count (CBC).

To test the in vitro Cytotoxicity of our multiherbal compound Immunocin, we have used MTT assay by procuring MCF (Michigan Cancer Foundation) cell lines form NCSS Pune[11,12]. Initially on these cell line culture we tried with the regular dose i.e.1000mg/microliter. Later the cytotoxicity assay was done at time intervals of 24 hours and 48 hours, but we were unable to record IC50 value. Later the same trail we have repeated with 2000mg/micro liter. But in both these cases we are unable to notice the activity of cell viability suppression (cell death). In the present cell assay viability of cells were checked through the finale absorbance calculated for the percentage of cell viability through OD vales (Optical Density OD) at 492nm by using ELISA (Lisa plus, India) reader [12].

Results and Discussions
Food and nutrition plays an important role to maintain the health and fitness of our body. The right intake of quality food and nutrition can boost our immune system. Generally the plant source food items are including vegetables, grains, cereals, fruits, mushrooms etc. Herbs which are considered as drugs will differ from food items with respect to their potency. To support this view there are number of food supplements freely available in the Indian market; irrespective of their health status which are consumed by large Indian population. Often its usage is recommended by the dietitians, nutritionists, Ayurveda or modern physicians. Hence combination of many such herbal products are generally used as food supplements to fulfill some dietary insufficiency. It is believed that these supplements will work in complimentary with the regular food. Sometimes they were used by healthy individuals or by the patients after recovering from a prolonged illness or by the person who is having general debility. The herbal supplement tablet Immunocin may stand as one among them. There are number of herbal preparations were used by the people with hope that such supplements may boost their immune system so that they might get protection against the exogenous or endogenous threats by preventing the occurrence of diseases.

Cancer is one such ailments creating panic in the society for no definite cause. Though the genetic concept and interference of inorganic chemicals for acquiring the cancer is a most widely discussed theory in the present era; their influence on the biological system is having variable duration of latency period. Such similar experimental models were tried under research to study their prevention as well as cure.

The earliest reference of breast tumour induction with DMBA chemical administration is about 13 weeks[13]. DMBA being a well-known chemical which is widely used as carcinogenic agent in the experiments to induce variety of cancers. DMBA administration causing altered gene expression resulting in immune modulation; which is considered to be one of the important factor play its role in inducing cancer like conditions.There are number of ways through which experimental trails studied the role of DMBA in cancer tumour induction. In this connection they have studied the different molecular cell signalling pathways, where 1 mg dose of DMBA was given on weekly basis till the 6th week. Later they observed that there is an elevated expression of Aryl hydrocarbon Receptor (AhR), C-myc, CyclinD and hyperphosporilation. Which has triggered up regulation of wnt signalling pathway, NF kappa B pathway when compared with the normal mammary gland formation in control groups. Probably these biological factors are leading to mammary tumourogenesis with DMBA[14].The drug Immunocin was used in prophylaxis therapy with the intention of its suppressing action on such oncogenic expressions against the carcinogenic agent DMBA. Alteration and deterioration in immune response can make our body prone for the life threatening diseases like malignancy. Present novel experimental trial is also based on our observations where the incidence of the cancer is often seen among the same family members or among the siblings. It was our hypothetical thinking that, can regular intake of such herbal/food supplements can help the individual who is prone for life threatening diseases like cancer..?. In this regard the Ayurveda has elaborately described chapters under the name of "Rasayana Chikithsa" (Rejuvenation Therapy) which is exclusively meant to achieve the purpose of disease free living in healthy individuals, and to achieve the cure in diseased[6]. With this background we used carcinogenic agent DMBA as a chemical agent which bring cancer like changes by suppressing/influencing the activity of proto-oncogenes in our biological system. Present study is targeted and limited to induce and test the mammary gland tumor formation among the rats by using DMBA which are prior under the influence of multiherbal commercial drug. These two different drugs with diversified activity were

administrate in animals at different timings or time gap; and later were kept under observation. Weekly physical examination of formation of tumor was carried out till the end of our study. The animal was physically examined for any findings; but we couldn't notice formation of any tumors till the end of our experiment. Histopathology of mammary gland and liver both were studied under H&E (Haematoxilin & Eosin) stain; its findings were within normal limits. To see overall changes in the blood, the biochemical parameter i.e. complete blood count (CBC) was used. The end results of CBC was found to be within normal limits. CBC has not shown any changes supporting/indicative of malignancy or inflammation. The ratio of other TC (total count) and DC (differential count) were within normal limits. Among them the neutrophil count, which is one of the marker of inflammatory changes was also found within normal limits. Particularly we have focused on the Neutrophil count which play an important role in body immune response. Under blood investigations the hemoglobin (blood iron) content was referred as an important indicator of possible malignant changes in the body. Where accumulation of large quantity of iron storage protein called Ferritin raise is seen significantly among cancer patients. The body antioxidant enzymes are significantly raised among cancer patients[15].In the present study hemoglobin percentage was showing no indication of anemia in any of the experimental groups. Where reduced hemoglobin percentage (anemia) could be used as one of the tentative sign of malignancy, or it can be also used as an indicator of prognosis in malignant diseases. With respect to Hb%, the results were found within normal limits in all the experimental groups. The findings under CBC were correlated with the histological features of liver and mammary gland, but they were found normal.

Our findings suggests that DMBA has not shown any mammary or liver tumors which is of benign or malignant in nature. Experiments have clearly shown that the single dose oral route administration of DMBA or its multiple intermittent small dose administration has supported the formation of breast tumor in a period between 8-14 weeks. Some experiments also quotes the formation of tumor within the time duration ranging from 13th week to 53rd week on single dose administration. With these references initially we have set our expected tumor formation by the end of 16 weeks duration, which we couldn't achieve. Later we have extended our study for 4 more weeks with the additional drug DMBA administration dose of 5mg. But in spite of it we couldn't notice any physical signs of mammary gland tumor formation. Despite the total 20mg of DMBA administration, the rats were started gaining the weight, showing normal/regular intake of food and water, regular bowel habits with routine physical activities. To test set objectives of our present study, we have proceeded with DMBA administration in rats which are under Immunocin drug therapy at different intervals. Our experimental observations under split dose of (15mg+5mg) DMBA administration was contradicting with the outcome of single dose 20mg DMBA administration, where all the animals were died within 11 days. But our experimental trial observations were continued in all the groups regularly as before. After waiting for some more weeks by considering our experimental limitations we have concluded our experiment by the end of 5th month. Generally the drug which is considered to be anticancerous is showing the reduction (cell death) in number of viable cancer cells. In the present trail under MTT cell assay, the MCF cell viability was found undisturbed with the Immunocin compound. These observations were in contrast with the drugs which are generally considered to be anticancerous, which are showing IC 50 (inhibitory quotient) values which express the cell viability in terms of its percentage. Anticancerous activity was assessed through the final percentage of MCF cell absorbance values; where the absorbance value is inversely proportional to cell viability. In the present MTT assay IC 50 (inhibitory quotient) value assessment of Immunocin compound has shown no signs of its activity; which has failed to check the cell viability of MCF cell line (Photo no. 9).

Though we are unable to induce any type of tumors, our observations under the histopathology of the breast tissue was showing quite interesting pattern. At the end of the study none of these rats in the group were showing proper development of alveoli and duct pattern of the mammary gland which is corresponding with their normal physical age; later in all the experimental groups it was confirmed through physical and histopathological examination (Photos no. 2,4,6 and 8) Microscopic appearance of breast tissue is suggestive of its subnormal growth pattern. Regular development of duct system and acini is called branching morphogenesis. This process is seems to be halted, hence it may suggestive of organ suppression. Where the duct is seen in the form of scattered without glandular (acinar) pattern with limited adipose and connective tissue; hence it is suggestive of under development of the mammary gland which is not matching with the age of the animal. Usually in puberty mammals will show exuberant duct and alveolar growth pattern with intense tissue remodeling in the lining epithelium[16]. The sexual maturity through timely production of sex hormones in these experimental rats might have suppressed. Because of these reasons the rats which regularly (physiological) attain their sexual maturity around 6th week (Age) might have failed[17]. Probably, the lack of estrogens and its strong influence on overall development of reproductive system including ovary (female gonad) might have adversely affected by our drugs like Immunocin and DMBA. The normal age dependent growth pattern of reproductive organ (Mammary gland) maturity in these animals seems to be suppressed (Photo no. 10). Histopathological of mammary gland and liver is showing no tumor formation. The blood and tissue findings may suggest that the carcinogenic chemical DMBA has failed to influence any tumor formation or failed to induce any inflammatory changes in the body; which can be correlated with normal histopathological appearance of liver tissue in all experimental groups (Photos no. 1,3,5, and 7). Our observations/findings at this 15mg single dose do suggests that its effect was within manageable biological safety limits hence showing no changes in blood or histopathological parameters. Hence at this dose the chemical was found biologically ineffective to induce cancer in its gross form in 16-20 week duration of latency period. To influence carcinogenic effects it may require much higher single dose or escalating multiple dose administration. The drug Immunocin being a multi herbal product probably influencing adversely on the physiological events in the development of normal mammary gland alveolar/duct pattern. Suppression of these physiological events could be due to the effect/influence of phytochemicals present in this drug. Hence our overall experimental findings supports the statements of one of the author, where DMBA was considered to be a non-reliable, inadequate, time consuming, and expensive method when compared with direct in situ cell line injection into the breast tissue to induce cancer in rats[18]. Sequential hormone signaling pattern is essential not only for the overall development female reproductive system, but it can even influence the changes in disease condition as as well[19]. Both these drugs might have suppressed the age dependent sexual maturity by adversely influencing the activity of endocrine glands. In the present study the induction of mammary gland tumor has failed. It is obviously contradicting the previous reports; for such unmatched outcome of our experiments, i personally consider it as our own limitation in designing our experiment at all levels. Our findings in the present study is totally inconclusive. Hence present study findings suggest further more basic experiments to conclude any suppressive effect of our drugs on the hypothalamo pituitary adrenal (HPA) axis[20].

Conclusion

The carcinogenic agent DMBA has failed to induce expected tumors in this setup, hence we are unable to conclude possible malignant tumor prevention activity of the drug Immunocin under prophylactic therapy. The multiherbal drug even in its higher concentrations was not able to show anticancerous effect on MCF cancer cell lines. Hence a small pilot study can give a better practical inputs to continue the study to the next level. Hence it is essential to start with a pilot study in a small setup; certainly it will guide us about the experimental trouble shooters which can save our time, resource and money. In research experiments bias is an unavoidable event or it is the limitation of any experiment in particular. There may be several factors involved in the present experiment which might have influenced its final outcome. Hence further experimental evaluation may be required to suggest the appropriate induction dose, the time duration for the induction of mammary gland tumors by using the chemical DMBA. To test the prophylactic effect of any commercial drug by using a carcinogenic agent, it needs a meticulous experimental evaluation in a stage wise manner. Any supporting reference for such invasive experiments can't be tried at once. They may pose practical difficulty in replicating/reproducing/initiating the experiments in the exact manner in which others have executed; where our experimental outcome is one such evidence which is mismatching with them, hence pilot studies are essential.

Nil effect shown under MTT assay of our drug on the MCF cell line

study may indicate lack of anticancerous property. Even though single experiment can't answer all the questions, it makes us to be skeptical about the efficacy of drugs especially when administered in its compound (multiherb) form. Irrespective of individuals' health status, the probability of downstream/negative/adverse influence or mutual interaction of variety of phyto constituents from different herbs under one biological context can't be ruled out.

Competing Interests: Here all the authors declares that they don't have any competing interests in any manner.

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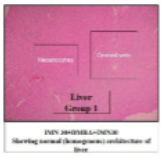
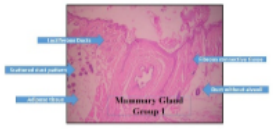
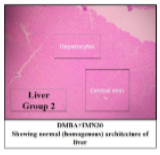
Group I, Liver and Mammary Gland

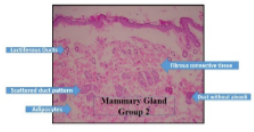




Photos no. 1 & 2

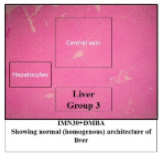
Group 2, Liver and Mammary Gland

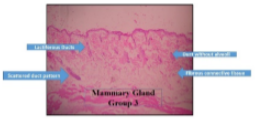




Photos no. 3 & 4

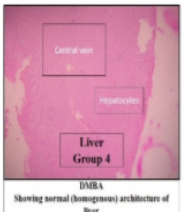
Group 3, Liver and Mammary Gland





Photos no. 5 & 6

Group 4, Liver and Mammary Gland



Photos no. 7 & 8

MCF Cell lines

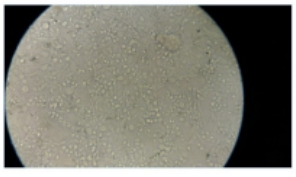


Photo no. 9, MTT Assay

Breast seen in virgin rats
Aged 24 weeks

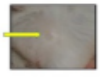


Photo no. 10, showing small breast size in all DMBA administered rats

References

1. Sudhakar A. History of cancer, ancient and modern treatment methods. J. Cancer sci Ther. 1(2):14, doi:10.4172/1948.100000e2.1 (date of access 12-10-2015)
2. Miller E C. Some cancer perspectives on chemical carcinogenesis in humans and experimental animals: Presidential address. Cancer research.1978; 38: 1479-1496.
3. Takiar R, Nadayali D, Nandakumar A. Projections of Number of Cancer Cases in India (2010-2020) by Cancer Groups.Asian Pacific J Cancer Prev. 2010; 11: 1045-1049
4. Kamath R, Mahajan KS, Ashok L, Sanal TS. A study on risk factors of breast cancer among patients attending the tertiary care hospital, in Udupi district. Indian J Community Med.2013; 38(2): 95-99.
5. Semra S. The Role of Antioxidants in Cancer Prevention and Treatment. Indoor and Built Environment. 2003:12(6):401-404.
6. Kumar SA. Rationality of rasayana therapy as adoptogernic, anti-oxidant and ant-inflammatory agent. International research journal pharmacology.2011; 2(12):259-260.
7. Workman P, Aboagye EO, Balkwill F, Balmain A, Burder G, Chaplin DJ. Guidelines for the welfare and use of animals in cancer research. British Journal of Cancer. 2010; 102:1555 – 1577
8. Alfredo Carlos S. D. Induction of experimental mammary carcinogenesis in rats with 7,12 dimethylbenz (a)anthracene. Rev. Hosp. 2004; 59 (5).
9. Organization for economic co-operation and development guideline for testing of chemicals, 1/14-14/14, 423 Adopted 17th December 2001. (Cited on 4-42013) Available from iccvam.niehs.nih. Gov/SuppDocs/FedDocs/OECD/OECD_GL423. pdf
10. MTT cell proliferation Assay groups. molbiosci. northwestern. edu/ morimoto/ research/ Protocols/II.../2.%20MTT.pdf online document Date of Access 4-2-2018.
11. Regina V and Uma Rajan KM. In-vitro assay for Cytotoxicity activity in ethonolic extract of fruit rind of Couropita Guianensis aubl. Int. J.Curr.Microbiol.App.Sci (2014) 3(10) 169-176
12. Valter R. M. Lombardi, Iván Carrera, and Ramón Cacabelos, "In Vitro Screening for Cytotoxic Activity of Herbal Extracts," Evidence-Based Complementary and Alternative Medicine, vol. 2017, Article ID 2675631, 8 pages, 2017. doi:10.1155/2017/2675631
13. Macejova D, Brtko J. Chemically induced carcinogenesis: A comparison of 1-Methyl-1-Nitrosourea, 7,12-Dimethyl benzanthracene, Dimethylnitroso-amine and azoxymethan and Azoxymethan models: (Mini review). Endocrine regulations. 2001: 35; 53-59.
14. Currier N, Soloman SE,Demicco EG,Chang DL,Farago M,Ying H, et al. Oncogenic signaling pathwats activated in DMBA-induced mouse mammary tumours. Toxicol pathol. 2005; 33 (6): 726-37.
15. Mishra S, Sharma D C, Sharma P. Studies of biochemical parameters in breast cancer with and without metastasis. Indian journal of clinical biochemistry. 2004:19 (1); 71-75.
16. Sternlicht M D. Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. Breast Cancer Res. 2006; 8(1):201;36.
17. Susman E J, Houts R M, Steinberg L. et al. Longitudinal development of secondary sexual characteristics in girls and boys between ages 91/2 and 151/2 years. Arch Pediatr Adolesc Med. 2010; 16:166–173.
18. ` Whirledge S and Cidlowski JA. Glucocorticoids, Stress, and Fertility. Minerva Endocrinol. 2010 35(2): 109–125.
19. Brisken C, O'Malley B. Hormone Action in the Mammary Gland. Cold Spring Harb Perspect Biol. 2010; 2(12): a003178.
20. Abbasalipourkabir R, Dehghan A, Salehzadeh A, Shamsabadi F, Abdullah R. Induction of mammary gland tumor in female Sprague Dawley rats with LA7 cells. African Journal of Biotechnology 2010; 9(28), 4491-4498.