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Research Article

CHEMOPREVENTIVE POTENTIAL OF DIOSGENIN ON MODULATING GLYCOPROTEINS, TCA CYCLE ENZYMES, CARBOHYDRATE METABOLISING ENZYMES AND BIOTRANSFORMATION ENZYMES AGAINST *N*-METHYL-*N*-NITROSOUREA INDUCED MAMMARY CARCINOGENESIS

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ABSTRACT

Objective: Chemically induced carcinogenesis models in the rat widely used for studying the biology of cancer development and evaluating cancer prevetion strategies. The aim of our present investigation attempts to evaluate antitumourogenic efficacy of steroidal sapogenin, diosgenin against NMU induced mammary carcinoma in Sprague Dawley rats.

Methods: Mammary carcinogenesis was induced by administring a single dose of (50mg/kg bodyweight) of NMU intraperitoneally.

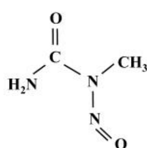
Results: Mammary cancer bearing Group II bearing experimental animals showed enzymatic alterations in the levels of ($P<0.05$) glycoproteins, TCA cycle enzymes, carbohydrate metabolising enzymes Kreb cycle enzymes and biotransformation enzymes.

Conclusion: These biochemical modulations were reverted close to normal levels upon management of steroidal sapogenin in group III animals. From the result of our present analysis, we concluded that diosgenin have anticarcinogenic activity against NMU induced breast cancer.

Keywords: Breast cancer, Mammary cancer, Glycoproteins, TCA cycle enzymes, Biotransformation enzymes, Carbohydrate metabolising enzymes.

INTRODUCTION

Cancer is a foremost health problem globally and the World Health Organization predicts that, by 2030, an estimated 21.4 million fresh cases of cancer and 13.2 million cancer related deaths will occur once a year [1,2]. Breast carcinoma is a variety of cancer instigate from breast tissue [3]. Breast cancer is one of the most common cancers in the world and it remains the second leading cause of cancer deaths [4,5]. It accounts for 10.9% of all cancers, with a reported incidence of about 1.38 million cases in 2008 [6]. Despite the fact that the overwhelming majority of human cases occur in women, few male breast cancer cases also reported [7]. Breast cancer is not a single disease, instead it is a collection of breast diseases that have diverse histopathologies, genomic variations, and clinical outcomes [8]. Mammary cancer etiology is a multifactorial and the risk factors comprise early menarche, late menopause, postmenopausal obesity, hormone replacement therapy, previous and increased lifetime exposure to endogenous or exogenous estrogens [9]. It has been reported that a number of genes including (BRCA1 and BRCA2) have been coupled in the event of breast cancer vulnerability and expansion [10]. Additionally, chemical carcinogens know for extreme formation of oxygen free radicals can cause oxidative damage to biomolecules resulting in lipid peroxidation (LPO), mutagenesis and carcinogenesis. In this correlation, oxygen free radicals induced LPO has been implicated in neoplastic transformation [11].

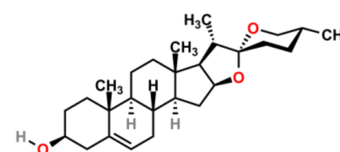


Chemical structure of N-methyl-N-nitrosourea

N-methyl-*N*-nitrosourea (NMU) is the oldest and simplest member of a group of compounds belongs to the alkylnitrosoureas that have the ability to alkylate DNA [12]. The NMU is formed *in vivo* by the interaction of the nitrosonium ion from ingested food preservative sodium nitrite with endogenous *N*-methyl urea. NMU is a highly consistent chemical carcinogen, mutagen, and teratogen [13]. NMU is an alkylating agent and exhibits its toxicity by transferring its methyl group to nucleobases in nucleic acids [14]. NMU is an effectual carcinogen for the induction of breast carcinoma in experimental rats and a very good model for human mammary carcinomas [15].

Considering current treatments reported to produce various side effects and also restriction in the advanced treatment show the way to innovative new approachs for managing breast cancer [16]. Plant derived biological compounds have the prospective to fall down the biochemical disproportion stimulated by various toxins connected with free radicals [17]. Natural chemopreventive agents have been identified to endow with safeguard devoid of causing any side effects and as a good defender against the free radicals [18]. Natural products, especially plant based products, have been frequently examined as anticancer agents [19]. Chemoprevention has been successfully achieved in numerous *in vitro* and as well as *in vivo* studies and has been validated in several human intervention trials [20].

Diosgenin, a steroid sapogenin, belonging to the group of triterpenes found in a number of plants including Dioscorea species (yams), fenugreek and Costus speciosus [21]. Steroidal sapogenins are secondary metabolites whose biosynthetic precursors are sterols, especially cholesterol and it is an imperative compound in the pharmaceutical industry as a natural source of steroidal hormones [22,23]. Diosgenin, structurally analogous to cholesterol and other steroids establish a range of biological and pharmalogical activities including antimicrobial [24], antiviral [25], anti-inflammatory activities, anti-diabetes [26], gastrointestinal ailments [27] and also plays an significant responsibility in the control of cholesterol metabolism [28]. Furthermore steroidal saponins (aglycone) also exert anticarcinogeneic properties against a wide variety of tumor cells [29-31]. The role of environmental contaminants such as NMU in enhancing breast cancer risk, the present investigation attempts to prove the anticancer consequence of diosgenin against NMU induced experimental breast cancer.



Chemical structure of Diosgenin

MATERIALS AND METHODS

Animals

Healthy female Sprague Dawley rats at the age group of 45-48 days were used for this present investigation. They were obtained from the central animal house facility, Dr. ALMPGIBMS, University of Madras, Taramani, Chennai, Tamilnadu, India. The animals were kept in large spacious polypropylene cages and received standardized rat pellet and water *ad libitum*. The animal room was well ventilated and a 12 h day and light rhythm was maintained throughout the experimental period. During the course of the study, the temperature was maintained between 27ºC to 37ºC. The maintenance and breeding of experimental animals were followed as defined by the Ministry of Social Justice and Empowerment of India, 1998 (IAEC No. 01/024/2010).

Chemicals

1-Methyl-1-Nitrosourea (NMU**)** and Diosgenin were purchased from Sigma Chemical Company, St Louis, MO, USA. All other chemicals including solvents used were of high purity and of analytical grade marketed by Glaxo Laboratories, Mumbai, and Sisco Research Laboratories Pvt. Ltd, Mumbai, India

Dose fixation

The range of dosage of Diosgenin was selected from the previous report. Preliminary biochemical studies were conducted at the concentration of 20 mg/kg body weight and remarkable response was observed. Hence, this concentration was selected for the present investigation.

Tumor induction

1-Methyl-1-Nitrosourea (NMU**)** was used as a carcinogen for the present investigation. Mammary cancer was induced by a single dose of 50 mg/kg body weight of NMU was dissolved in 0.9% saline adjusted with acetic acid (pH 4.0) and then administered intraperitoneally. After thirteen weeks of experimental period, all the animals were sacrificed.

Experimental Design

The rats were divided into four groups with six animals in each group and were given dose regimen as given below.

Group IControl animals were given normal Saline (0.9%).

Group IIAnimals received a single dose of (50mg/kg bodyweight) of NMU diluted in 0.9% saline adjusted with acetic acid at pH 4.0 and then administered intraperitoneally.

Group IIIAnimals received a single dose of(50mg/kg bodyweight) of NMU diluted in 0.9% saline adjusted with acetic acid at pH 4.0 and then administered intraperitoneally and from the seventh week (i.e.45days after NMU administration) followed by Diosgenin at the concentration of (20mg/kg bodyweight) dissolved in 1% gum acacia (1ml) for 45 days Orally.

Group IVAnimals received Diosgenin at the concentration of (20mg/kg body weight) dissolved in 1% gum acacia (1 ml) for 45 days orally.

Collection of blood and Tissues

At the end of the experimental period, all the animals were anesthetized with diethyl ether, and they were sacrificed by decapitation. Animals were starved overnight before sacrifice. Blood was collected and the serum was separated by centrifugation. The breast and liver tissues were dissected out and washed 2 to 3 times with saline, and known weight of breast and liver were homogenized in 0.1 M Tris-HCl buffer (pH 7.4). The homogenate was subjected to differential centrifugation. The cell organelle such as mitochondria, microsomes and cytosolic fractions were isolated. Total homogenate and subcellular fractions were used for the assay of the following parameters in serum, plasma, breast and liver samples.

Biochemical analysis

Hexose was estimated [32], Hexosamine was estimated [33], Sialic acid was estimated [34]. Hexokinase [35], Phosphoglucoisomerase [36], Aldolase [37], Glucose-6-phosphatase and Fructose-1,6-diphosphatase was assayed [38]. The inorganic phosphate was estimated [39], Erythrocyte membrane was isolated [40], with a change in buffer [41], Na^+/K^+-ATPase [42], The activity of Ca^{2+}-ATPase [43] and the activity of Mg^{2+}- ATPase was assayed [44]. Isocitrate dehydrogenase [45], Succinate dehydrogenase [46], Malate dehydrogenase [47] and α-Ketoglutarate dehydrogenase was estimated [48]. The liver microsomes were separated according [49] with slight modification [50]. Cytochrome P_{450} was estimated and the amount of cytochrome b_5 was measured [51], the activity of NADPH-cytochrome P_{450} reductase was assayed [52], Glutathione-S-transferase was assayed [53], UDP-Glucuronyltransferase was estimated [54] as modified [55].

Statistical analysis

Data are presented as the mean ± Standard Deviation (SD). One way analysis of variance (ANOVA) followed by Turkey's multiple comparison method was used to compare the mean of different groups by using SPSS 10.0 student versions. Comparisons were made between group II with III and group IV with I for animal studies.

RESULT

The levels of hexose, hexosamine and sialic acid of plasma, breast and liver of control and experimental animals were presented in Figure 1, 2 and 3 respectively. The levels of hexose, hexosamine and sialic acid were increased significantly in group II cancer bearing animals ($p<0.05$). A concomitant decrease of these glycoproteins levels were observed in group III drug treated animals ($p<0.05$). However, there was no change in group IV drug control animals when compared with group I animals.

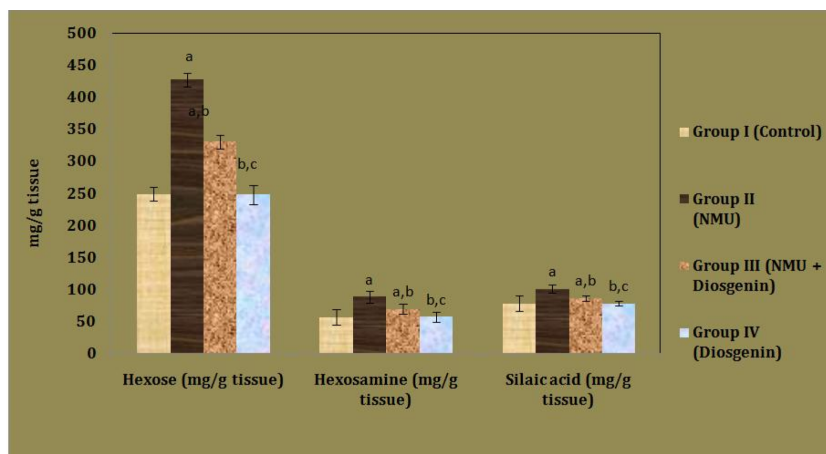


Fig. 1: Effect of diosgenin on the levels of glycoproteins in plasma of control and experimental animals

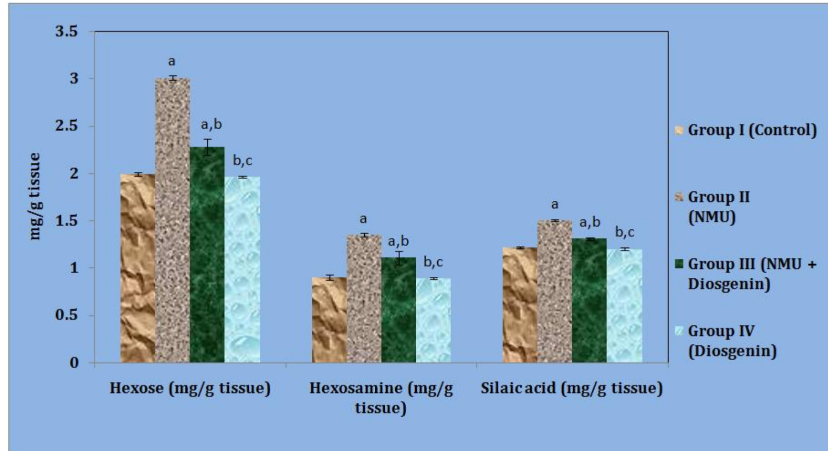


Fig. 2: Effect of diosgenin on the levels of glycoproteins in breast of control and experimental animals

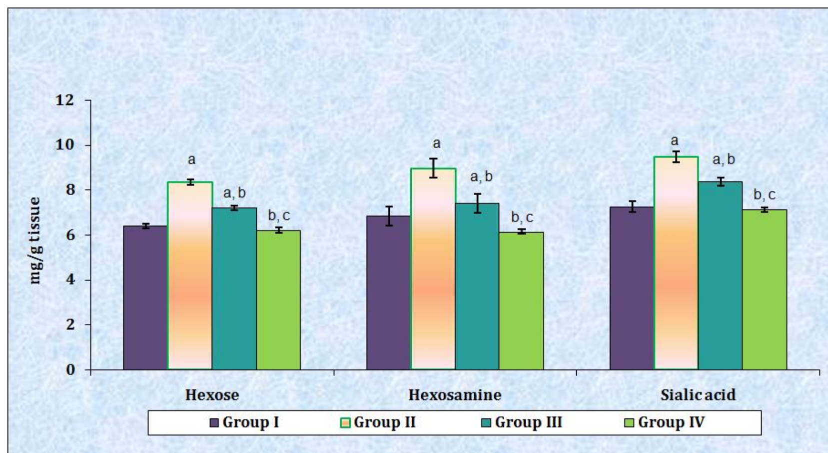


Fig. 3: Effect of diosgenin on the levels of glycoproteins in liver of control and experimental animals

Each bar expressed as mean + SD for six animals in each group

a - Group I Vs Group II, III and IV, b - Group II Vs Group III and IV, c - Group III Vs Group IV

The significance at the level of $p<0.05$

The efficacy of diosgenin on the levels of carbohydrate metabolizing enzymes in liver of control and experimental animals are presented in Figure 4. In breast the activity of the carbohydrate metabolizing enzymes such as hexokinase, phosphoglucoisomerase and aldolase were found to be significantly elevated ($p<0.05$) and the glucose-6-phosphatase and fructose-1,6-diphosphatase ($p<0.05$), were significantly decreased in group II animals when compared with group I control animals. All the carbohydrate metabolizing enzymes were significantly altered with the treatment of diosgenin in group III animals ($p<0.05$) when compared with group II NMU induced animals. There was no remarkable changes were observed in group IV drug alone treated animals when compared to group I animals.

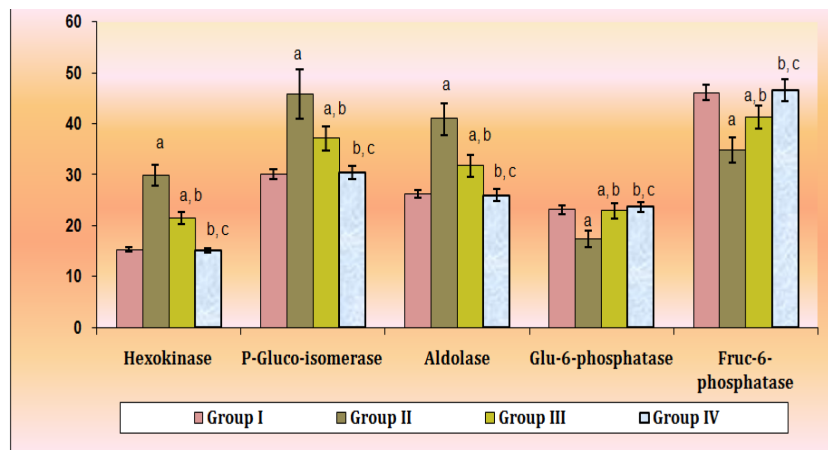


Fig. 4: Effect of diosgenin on the levels of carbohydrate metabolizing enzymes in liver of control and experimental animals

Units are expressed as; Hexokinase – n moles of glucose-6- phosphate liberated/mg protein/min; Phosphogluco-isomerase (n moles of fructose liberated/mg protein/min); Aldolase (n moles of glyceraldehydes liberated/mg protein/min); Glucose-6-phosphatase (n moles of inorganic phosphate liberated/mg protein/min); Fructose-1,6-diphosphatase (n moles of inorganic phosphate liberated/mg protein/min)

Each bar expressed as mean + SD for six animals in each group

a - Group I Vs Group II, III and IV, b - Group II Vs Group III and IV, c - Group III Vs Group IV

The significance at the level of $p<0.05$

Figure 5 and 6 depicts the activities of Na^+/K^+, Ca^{2+} and Mg^{2+} ATPases in erythrocyte membrane and liver of control and experimental animals respectively. NMU induced group II breast cancer bearing animals shows a significant decline in the levels of Na^+/K^+, Ca^{2+} and Mg^{2+} ATPases ($p<0.05$) when compared with control animals. These levels were found to be significantly increased on diosgenin treatment ($p<0.05$) in group III animals when compared to group II animals. On the other hand, there was no significant variations in group IV diosgenin alone treated animals when compared with group I control animals.

The effect of diosgenin on the mitochondrial TCA cycle enzymes such as ICDH, SDH, MDH and α-KGDH in the breast of control and experimental animals are showed in Figure 7. A significant decrease in the levels of TCA cycle enzyme were observed in group II animals when compared to group I control animals ($p<0.05$). In this connection, the levels of TCA cycle enzymes are significantly increased in group III diosgenin treated animals ($p<0.05$) when compared to group II cancer bearing animals. They were observed no changes in group IV drug administered animals when compared with group I control animals.

In the present investigation the effect of diosgenin on the activities of Phase I and Phase II drug metabolising enzymes in liver microsomes of control and experimental animals are presented in Figure 8 and 9. In group II breast carcinoma bearing animals, the levels of phase I enzymes such as cytochrome P_{450}, cytochrome b_5, and NADPH cytochrome C reductase, were found to be decreased considerably ($p<0.05$) when compared with group I animals. Interestingly phase II biotransformation enzymes such as UDP glucronyl tranferase were significantly increased. However, Glutathione-S-transferase was significantly decreased in group II ($p<0.05$) cancer bearing animals when compared with control group I. These altered xenobiotic enzymes were brought back to near standard level in diosgenin treated group III animals ($p<0.05$) comparable to that of group II animals. No difference was noted in group IV drug control animals when compared to group I control animals.

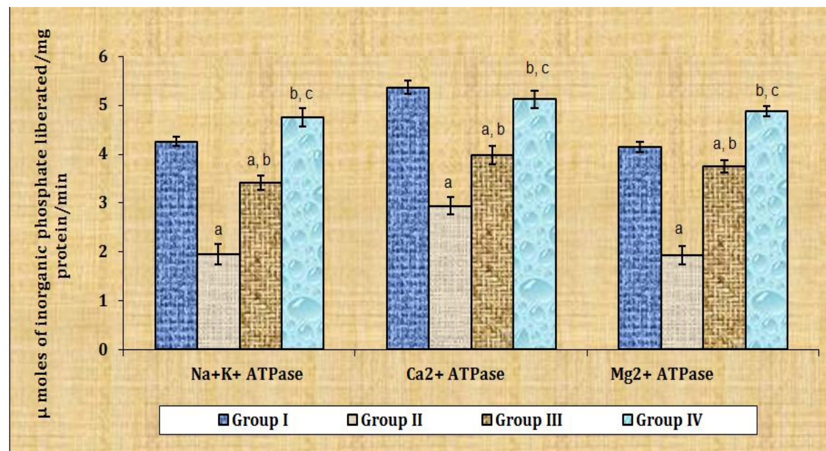


Fig. 5: Effect of diosgenin on ATPases in erythrocyte membrane of control and experimental animals

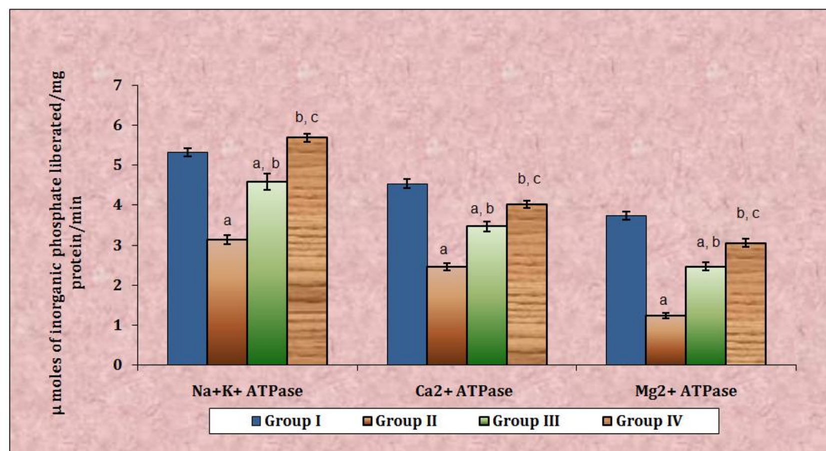


Fig. 6: Effect of diosgenin on ATPases in liver of control and experimental animals

Each bar expressed as mean + SD for six animals in each group

a - Group I Vs Group II, III and IV, b - Group II Vs Group III and IV, c - Group III Vs Group IV

The significance at the level of $p<0.05$

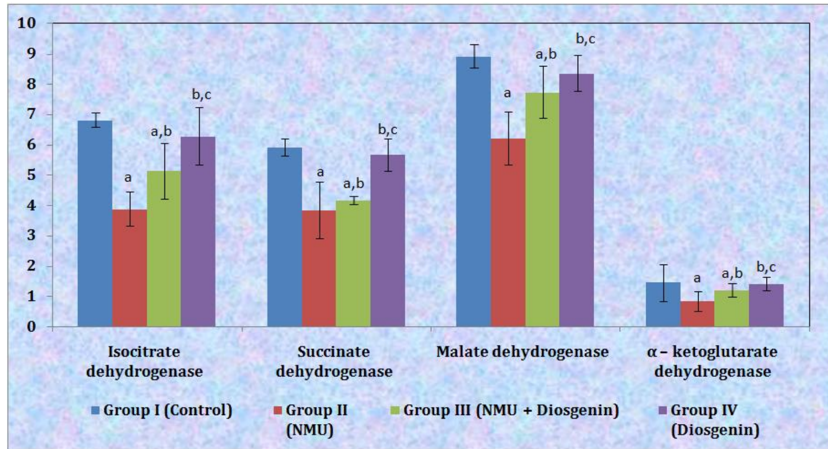


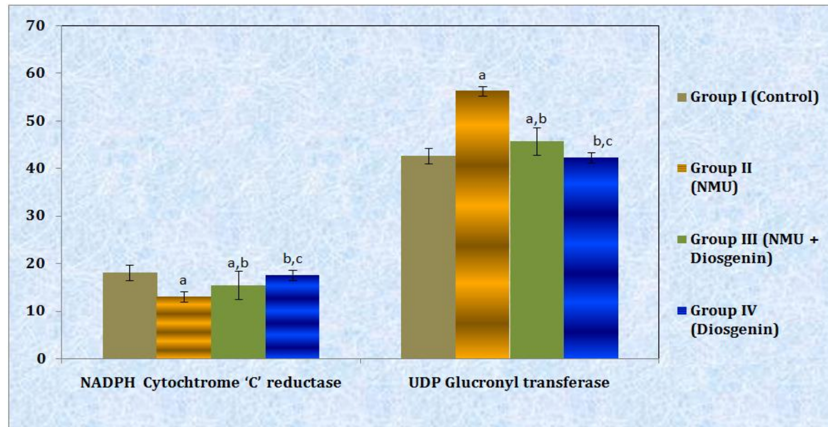
Fig. 7: Effect of diosgenin on the levels of TCA cycle enzymes in liver of control and experimental animals

Units: ICDH - n mol of α – ketoglutarate formed/mg protein/min; SDH - µ mol of succinate oxidized/mg protein/min; MDH - µ mol of NADH oxidized/ mg protein/min; αKGDH - µ mol of potassium ferrocynade liberated/mg protein/min.

Each bar expressed as mean + SD for six animals in each group

a - Group I Vs Group II, III and IV, b - Group II Vs Group III and IV, c - Group III Vs Group IV

The significance at the level of $p<0.05$



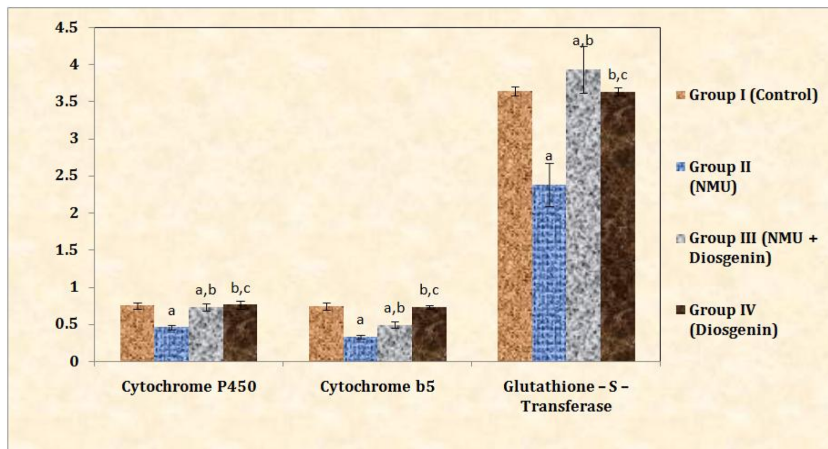


Fig. 8 and 9: Activities of phase I and phase II biotransformation enzymes in liver of control and experimental animals

Units: Cytochrome P450 - n moles/mg microsomal protein/min; Cytochrome b_5 - n moles/mg microsomal protein/min; NADPH Cytochtrome 'C' reductase - n moles/mg microsomal protein/min; Glutathione–S– Transferase - µ mole of CDNB conjugated/mg microsomal protein/min; UDP Glucronyl transferase - units/min/mg microsomal protein.

Each bar expressed as mean + SD for six animals in each group

a - Group I Vs Group II, III and IV, b - Group II Vs Group III and IV, c - Group III Vs Group IV

The significance at the level of $p<0.05$

DISCUSSION

Breast cancer is the third most common malignancy affecting female population, and approximately 19% of breast cancer mortality was reported worldwide [56]. Glycoproteins are common components of cell surfaces and are also commonly found as constituents of lysosomes and among the products secreted/exposed by the cell [57]. The cell surface glycoproteins have been shown to play important roles in tumourogenesis [58]. Elevation of glycoprotein contents are useful indicators of carcinogenic process and these changes alter the rigidity of cell membranes [59]. Abnormal increase in the levels of plasma glycoprotein components have been related to the changes in hepatic cells during neoplastic transformation. Sialic acids are widely distributed in nature as terminal sugars in glycoproteins or glycolipids, impart a net negative charge to cell surface and are reported to be important in cell-to-cell and cell-to-matrix interactions [60]. It was previously demonstrated that neoplastic transformation leads to elevated plasma sialic acid concentration [61] through the shedding or secreting of sialic acid from the tumor cell surfaces [62]. In the present study increased levels of glycoproteins in plasma, liver and breast tissues of cancer bearing animals were observed. The lower level of glycoproteins in limonin-treated animals confirms the antimetastatic activity of the drug. Since diosgenin has already been demonstrated to inhibit tumor growth, the present evidence further supports the anticancer property of diosgenin.

Carbohydrate metabolism plays a central task in cancerous condition and it is one of the most common and profound in malignant tissues, particularly those with the higher growth rates, their capacity to utilize and catabolization of glucose at high rates. The high glycolysis rate is important for rapid proliferating cancers, not only as a major energy source but also to provide such cells with precursors for nucleotide and lipid biosynthesis [63]. The early changes of the carbohydrate metabolism are of particular interest, since anomalies of glycolytic and gluconeogenic pathways are well known from biochemical investigations of cancer conditions [64]. Hexokinase levels occupy an important place in determining the glycolytic capacity of cancer cells [65]. It is a rate-limiting enzyme which catalyses the conversion of glucose to glucose-6-phosphate in the first step of the glycolytic pathway. Phosphoglucoisomerase serves as a good index of cancer condition and which act as a catalyst in the conversion of glucose-6-phosphate to fructose-6-phosphate and it is an indicator of metastatic growth with elevated levels in patients with neoplasms, especially after metastasis [66]. In the present investigation, increased levels of phosphoglucoisomerase were observed in breast cancer bearing animals which may be due to the higher glycolytic rate in hepatic tissues and further leakage from destruction of neoplastic tissues. Glucose-6-phosphatase is a marker enzyme for liver microsomal activity and it is greatly inhibited in cancer bearing animals. Decreased activities of glucose-6-phosphatase and fructose-1-6-diphosphatase in cancerous conditions [67]. In the present investigation, a decrease in the level of glucose-6-phosphatase was observed in cancer bearing animals. Therefore, the observed reduction in activities of these enzymes in cancer bearing animals in the present study may be due to the higher lactate production in neoplastic tissues. It was suggested that the drugs could selectively target the energistic metabolism of cancer cells are of great remuneration to combat the disease [68]. In the present study, treatment with diosgenin significantly altered the levels of these enzymes to near normal, which may be due to inhibition of glycolytic pathway and activation of gluconeogenesis. This cleary showed that diosgenin may interrupt the energy requirement of neoplastic tissues leading to the suppression of cancer progression.

Adenosine Triphosphatases (ATPases) are integral part of the membrane structure regulating ion transport across cellular membrane, cellular volume, osmotic pressure and membrane permeability that are indispensable enzyme for providing metabolic energy to the living processes [69]. ATPase are lipid dependent membrane bound enzymes involved in active transport process and are implicated in the pathogenesis of tissue injury [70]. Changes in membrane lipid leads to change in membrane fluidity, which in turn adjust the ATPase activities and cellular functions [71]. The Na^+/K^+ ATPase have a transmembrane catalytic subunit which regulates the solute concentration inside the cell thereby regulate the osmotic pressure. The activities of Na^+/K^+ ATPase are highly susceptible to the lipid peroxidation, which often is generated in cancerous condition [72]. ATPase activity was inhibited during lipid peroxidation caused by intra and extra cellular generation of activated oxygen and the inhibition of Ca^{2+} ATPase is probably due to LPO and oxidation of membrane [73]. It is reported that alterations in the concentration of cytosolic Mg^{2+} leads to a significant change in cellular functions [74]. In the present investigation, the Na^+/K^+, Ca^{2+} and Mg^{2+} ATPase were significantly decreased in NMU induced group II cancer bearing animals. Administration of diosgenin brought back these membrane bound ATPases to standard level in group III animals which reflects that diosgenin can maintain structural integrity probably by protecting the membrane ATPase from the deleterious effect of lipid peroxidation.

Mitochondria are the most important target of reactive oxygen species (ROS) and it is essential cell organelle plays a significant role in cell metabolism and hence it is the source of energy during oxidative phosphorylation. Enhanced production of ROS and free radicals in mitochondria resulting in mitochondrial DNA mutations which indirectly impair glucose sensing by reducing intracellular concentrations of ATP [75]. Most cancers probably start with an interruption of the Krebs cycle that arrests aerobic metabolism and force the cells to revert back to anaerobic metabolism. Mammary carcinoma induced rats showed a significant reduction in the activities of the Krebs cycle enzymes which proves the defect in the aerobic oxidation of pyruvate that might cause the low production of ATP molecules [76]. In the present investigation, the decreased activities of mitochondrial TCA cycle enzymes were observed in NMU induced breast cancer bearing animals may be due to the mitochondrial damage caused by NMU induced oxidative stress. Administration of diosgenin increased the activities of the mitochondrial enzymes activitiy that render protection against NMU induced breast cancer which inevitably suggested that diosgenin is very efficient in maintaining the mitochondrial membrane integrity. This could be due to the protective role of diosgenin by acting an antioxidant and ROS-scavenging potential.

Biotransformation enzymes participate not only in the metabolism of naturally occurring chemicals but also participate in metabolism of various artificial chemicals and drug. The metabolism of xenobiotics are performed by the biotransformation enzymes such as Phase I and Phase II enzymes which perform an important function by converting biologically inactive compound into active or toxic metabolites [77]. Phase I enzyme catalyzes functional group of xenobiotic into hydrophilic substrate. Phase II enzymes make the molecule less reactive by conjugation of the functional group with glutathione, sulphate or glucuronic acids. These reactions generally make the substrate into water soluble and the conjugated endogenous compound further facilitates the excretion of the product. Induction of Phase II enzymes is an important mechanism of chemoprevention [78]. It was reported that Cyto P_{450} levels were increased in liver tissues of NMU induced breast cancer bearing animals [79]. In the present study, increased level of Cyto $P_{450,}$ NADPH-cytochrome reductase and Cyto b_5 reductase was observed in NMU induced mammary cancer bearing animals may be due to the utilization of this enzyme to excrete the NMU metabolites.

Glutathione -S- transferases (GST) are inducible enzymes important in the detoxication of many different xenobiotics in mammals. The GST achieve detoxication by catalyzing the conjugation of reduced glutathione to various electrophilic substrates [80] serves as a marker for hepatotoxicity in rodent system, and also plays an important role in carcinogen detoxification [81]. Consequently, inhibition of GST activity and depletion of GSH levels might potentiate the deleterious effects of many environmental toxicants and carcinogens. GSTs are also engaged in the intracellular transport of variety of hormones, endogenous metabolites, and drugs, by virtue of their capacity to bind these substances [82]. The decreased activity of GST in group II cancer bearing animals may be due to the excessive utilization of this enzyme in conjugation process and also may be due to the enhancement of the covalent binding of NMU

metabolites to cellular DNA and results in an increase in the degree of cell damage leading to neoplastic growth. UDPGT catalyze the transfer of UDPGA to a wide variety of acceptor substrate to form O-, N-, S- and C-glucronides and the majority being O-glucronides [83]. In the present study, decreased level of UDPGT was observed in liver of cancer bearing animals may be due to the peroxidative damage to the microsomal lipids in cancer conditions [84]. There was a concomitant decrease in breast cancer bearing animals in the levels of phase II enzymes. Diosgenin treated animals, due to its anticarcinogenic activity it has been shown to prevent NMU induced cancer presumably by the regulating Phase I and Phase II metabolizing enzymes and through its strong antioxidant activity.

CONCLUSION

The diosgenin notably ameliorates the changes on glycoproteins, carbohydrate metabolizing enzymes, ATPases, mitochondrial TCA cycle enzymes and xenobiotic enzymes in drug treated cancer bearing animals. Results of our present research, we put forward that steroidal sapogenin diosgenin show evidence of its antitumorogenic potential through standardizing the status of various biochemical enzymes in NMU induced breast cancer in Sprague Dawley rats.

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