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Modulatory effect of thymoquinone on the activities of lysosomal and carbohydrate metabolizing enzymes studied in DMBA induced breast cancer rats

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ABSTRACT

Recently we have evaluated the anticancer efficacy of thymoquinone in DMBA induced breast cancer. In the present study an attempt has been made to evaluate the modulatory effect of thymoquinone on the levels of total protein, carbohydrate metabolizing enzymes, status of glycoprotein components, lysosomal enzymes in experimental groups of rats. A single dose of DMBA (20 mg/kg/rat) diluted in olive oil was given orally to induce breast cancer. After 13 weeks of experimentation all animals were sacrificed. The rats were orally administered with 25 mg/kg body weight of thymoquinone. The levels of total protein was determined. The serum and tissue levels of glycoproteins, lipid profile, lysosomal enzymes and carbohydrate metabolizing enzymes were assayed. Upon treatment with thymoquinone, the levels of the above enzymes were significantly normalized. The reduced level of protein was improved upon treatment with thymoquinone. Treatment with thymoquinone altered the levels of glycoprotein components and modulated the activities of lysosomal enzymes indicating the cytostabilising property. Oral treatment with thymoquinone altered the activities of carbohydrate metabolizing enzymes. Also, the altered status of lipid profile in plasma and liver tissues were normalized in thymoquinone treated rats indicating the beneficial effect of thymoquinone in maintaining glucose and lipid homeostasis.

INTRODUCTION

Non-communicable diseases including cancer are raising as a chief public health problems. These diseases are lifestyle related, have a long latent period, and needs specialized infrastructure and human resource for treatment. Interest in cancer research has grown rapidly during the past century as infectious diseases have increasingly been controlled as the result of improved sanitation, vaccination and antibiotics. Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. According to recent statistics, cancer is the second most common cause of death after heart disease. Among various cancers, Breast cancer is a major health problem in women in both developing and developed countries. One in 10 of all new cancers diagnosed worldwide each year is a cancer of the female breast. Also, it is the principal cause of death from cancer among women globally [1]. Breast cancer development and metastasis is a multistep process, a result of the dysfunction of several regulatory features that keep the cells in check. Though many drugs of cancer, none is found to be ideal to sidefffects. Hence, phytotherapy continues to play a prominent role in the treatment of cancer. Plants used for phytotherapy should have potential to increase immunity, reduce the pain and other indirect effects such as nausea, vomiting, fatigue and infections induced by cancer.

Nigella sativa L., commonly known as black seed, belongs to the botanical family Ranunculaceae. Most of the biological properties of the black seeds have been attributed mainly to the quinone constituents of N. sativa, of which thymoquinone is the main active ingredient of the volatile oil isolated from the black seeds [2]. Thymoquinone (TQ) is the primary active constituent of *Nigella sativa* seeds which has been reported for wide array of biological activities such as anti-inflammatory, antioxidant, and anti-neoplastic effects both in vitro and in vivo [3-4]. Recently we have reported the anticancer and liver protective role of thymoquinone in DMBA induced breast cancer rats [5-6]. In the present study an attempt has been made to evaluate the modulatory effect of thymoquinone on the levels of biochemical indices, tumour marker enzymes, carbohydrate metabolizing enzymes, status of glycoprotein components, lysosomal enzymes in control and experimental groups of rats

MATERIALS AND METHODS

Healthy female Sprague dawley wistar rats, at the age group of 45-48 days were used for present investigation. Rats were housed spaciously in individual cages and maintained under standard experimental conditions: temperature 25 ± 1°C, relative humidity 60 ± 5% and 12 ± 1h (light/dark cycle) in Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai-600113. The animals were fed with commercially available balanced pellet diet (Amrut laboratory Animal Feed, Bangalore, India) and water ad libitum. The animals were acclimatized for one week prior to the initiation of experiments. The experimental design was performed in accordance with the current ethical norms approved by the Ministry of Social Justice and Empowerment, Government of India and Institutional Animal Ethics Committee Guidelines (IAEC.No:01/02/2013).

Induction of breast cancer

A single dose of DMBA (20 mg/kg/rat) diluted in olive oil was given orally. Animals were monitored periodically. After 13 weeks of experimentation all animals were sacrificed.

Experimental Design

The rats were divided into four groups each comprising of six rats as detailed below:

- Group I: Control rats
- Group II: DMBA induced rats.
- Group III: DMBA+TQ (25mg/kg bw)
- Group IV: TQ alone

All animals were fasted overnight and sacrificed by sodium pentothal anesthesia followed by cervical decapitation. Blood was collected with and without anticoagulant and the serum was centrifuged at 5000 rpm for 15 min to obtain a clear supernatant and stored at -70°C until its use for further biochemical analysis. Breast tissues from control and experimental groups of rats were immediately excised, washed in ice-cold PBS to remove the blood stains, blotted, weighed and homogenized in Tris-HCL buffer (0.1M, pH 7.4) using a Teflon homogenizer to prepare 10% (w/v) tissue homogenate. This homogenate was centrifuged at 12,000g for 30 min at 4°C to obtain a clear supernatant. This supernatant was pooled and used for further analysis.

Total Protein was estimated according to the method of Lowry et al.[7], The levels of glycoprotein components namely hexose, hexosamine and sialic acid in plasma, liver and mammary gland were estimated by the method of Niebes (1972), Wagner (1979), and Warren (1959) [8-10] respectively.

The lysosomal enzymes such as Acid Phosphatase, β-glucuronidase and β-galactosidase, Cathepsin D and Cathepsin B were determined [11-14]. Carbohydrate metabolizing enzymes such as Hexokinase, Phosphogluco isomerase, Aldolase and Glucose-6-phosphatase were assayed [15-18].

The lipids were extracted from liver tissues by the method of Folch et al. (1957) [19]. Cholesterol content, triglycerides, and free fatty acids in plasma and liver tissues were estimated[20-22] . Phospholipid concentration was estimated by the method of Bartlett (1959) [23] by digestion with perchloric acid and the phosphate liberated was estimated by the method of Fiske and Subbarow (1925) [24].

Statistical analysis

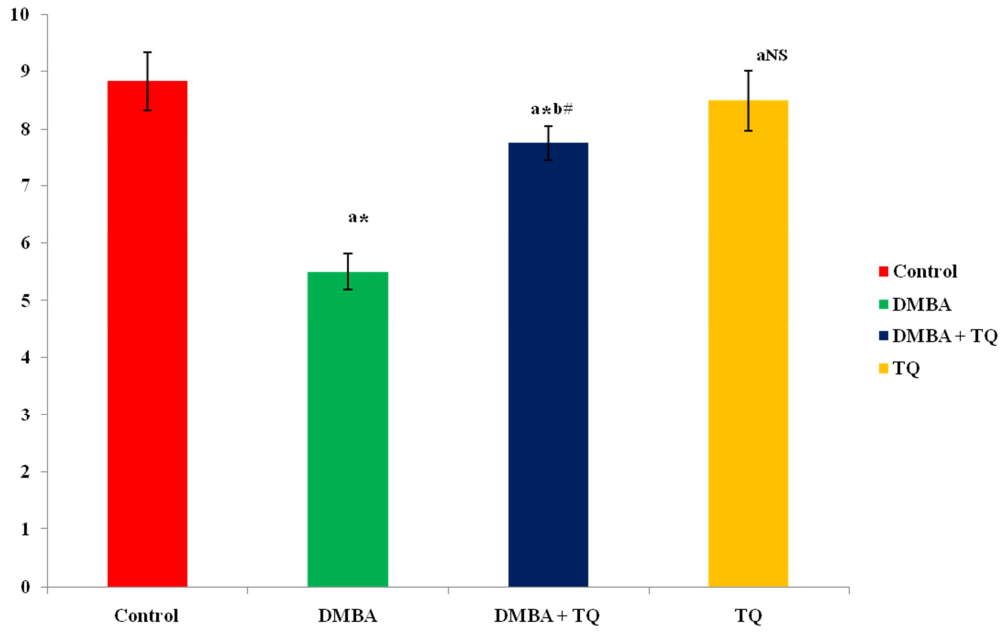
Statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago) statistical package. The results were expressed as mean ± S.E.M One-way ANOVA followed by post hoc test LSD was used to correlate the difference between the variables. Values were considered statistically significant if *p<0.001; @p<0.05; #p<0.01.

RESULTS

Effect of thymoquinone on the levels of total proteins

Figure 1 the levels of total proteins in serum of control and experimental groups of rats. The level of total protein was found to be decreased in DMBA induced rats. Upon treatment with Thymoquinone, the levels were found to be increased. Protein synthesis is an important phenomenon in normal as well as in cancerous condition. Skeletal muscle which embodies the major protein mass of an organism is greatly affected in cancer cachexia. Protein waste implies the underlying metabolic nitrogen imbalance which is being expressed by an elevation in the apparent protein catabolism rate with no changes in apparent synthesis and thus, the host responds to the increased tumor lead by increased tissue protein breakdown [25]. Upon treatment with thymoquinone to DMBA induced rats prevents the protein degradation rate and increases the total protein content by modulating protein biosynthesis.

Figure 1 : The levels of protein in control and experimental groups of rats

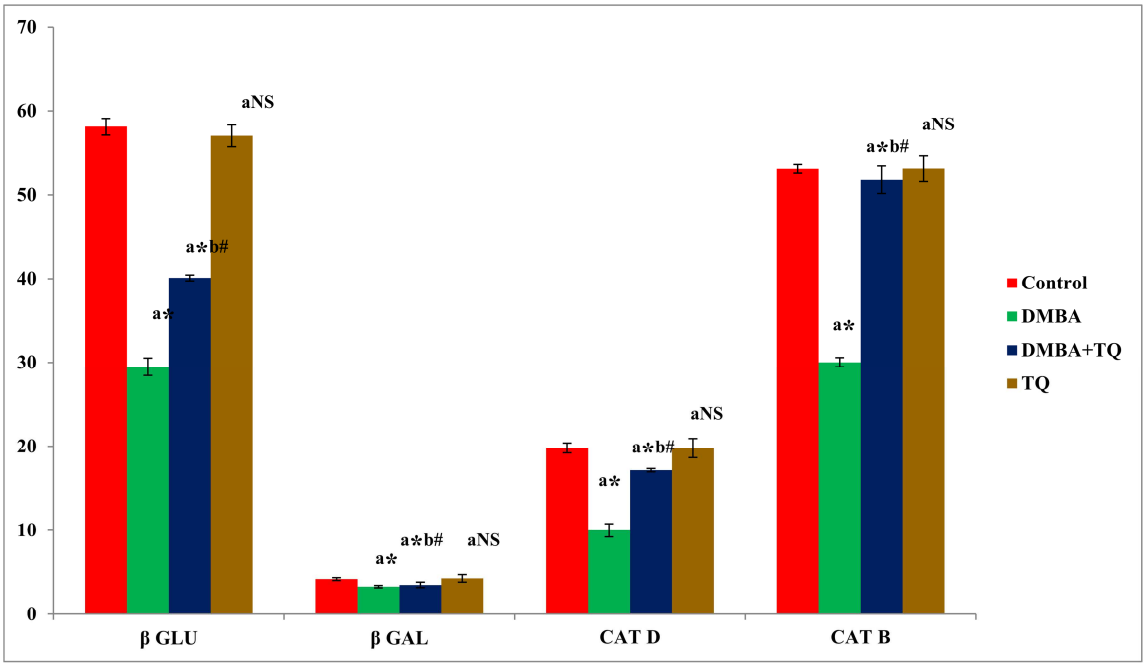


Values are given as mean ± S.D for groups of six rats in each. Statistical significance was compared within the group as follows: a-compared with control rats
b- compared with DMBA induced rats
***p<0.001; @p<0.05; #p<0.01; NS-Not significant**

Effect of thymoquinone on the activities of lysosomal marker enzymes

Figure 2-5 depicts the activities of lysosomal enzymes in serum, mammary, hepatic and renal tissues of control and experimental groups of rats. The activities of lysosomal enzymes were significantly deprived in serum of DMBA induced rats whereas in tissues their levels were increased when compared to control rats. On the contrary, the lysosomal enzyme levels were significantly brought towards normal in thymoquinone treated rats. There was no significant alterations observed in thymoquinone alone treated rats.

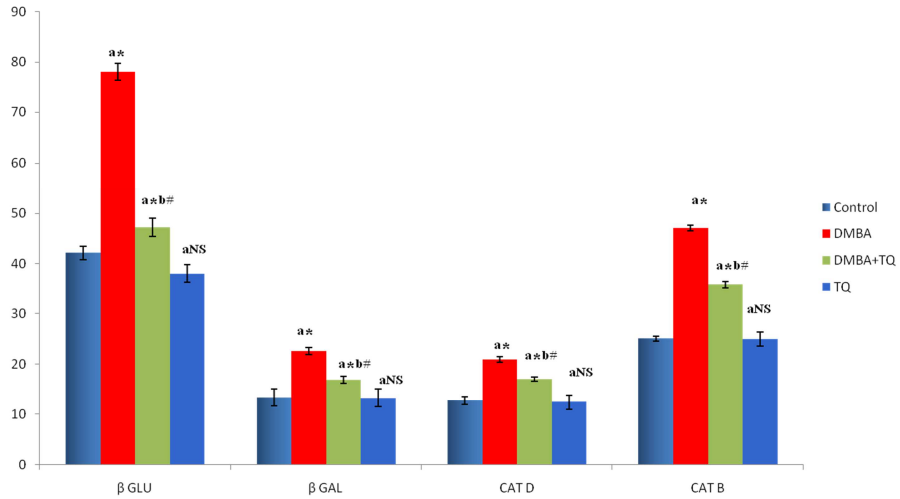
Figure 2: Effect of Thymoquinone on the activities of lysosomal marker enzymes in serum of control and experimental animals



Units : β-Glucuronidase, β-Gal actosidase- µmoles of p-nitrophenol formed/min/L
Cathepsin-D - µmol of tyrosine liberated/min/L
CAT-B- µmol of p-nitroaniline liberated/min/L
Values are given as mean ± S.D for groups of six rats in each. Statistical significance was compared within the group as follows: a-compared with control rats
b- compared with DMBA induced rats
***p<0.001; @p<0.05; #p<0.01; NS-Not significant**

Lysosomes are a group of cytoplasmic organelles present in numerous animal tissues, characterized by their content of acid hydrolases. These enzymes are implicated in tissue remodeling, which occurs during the physiological involution of the uterus, prostate gland, and the mammary gland [26, 27]. Lysosomes contain digestive enzymes capable of degrading all macromolecules such as proteins, nucleic acids, lipids and carbohydrates. These enzymes can trigger apoptosis in human breast carcinoma cells as well as in rat mammary gland cells.

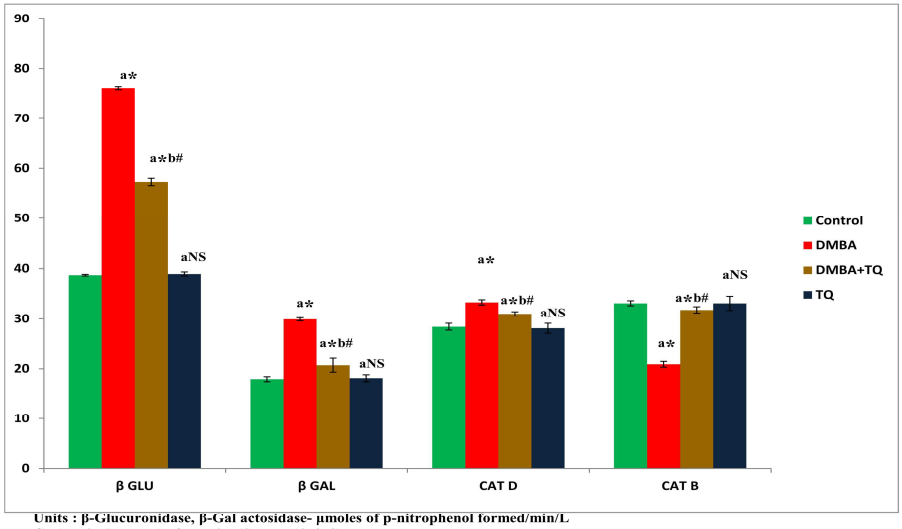
Figure 3: Effect of Thymoquinone on the activities of lysosomal marker enzymes in mammary tissues of control and experimental animals



Units : β-Glucuronidase, β-Gal actosidase- µmoles of p-nitrophenol formed/min/L
Cathepsin-D - µmol of tyrosine liberated/min/L
CAT-B- µmol of p-nitroaniline liberated/min/L
Values are given as mean ± S.D for groups of six rats in each. Statistical significance was compared within the group as follows: a-compared with control rats
b- compared with DMBA induced rats *p<0.001; @p<0.05; #p<0.01; NS-Not significant

β- glucuronidase is shown to be a sensitive marker of lysosomal integrity. The levels of glycan moieties and the activities of glycosidases can be used as diagnostic markers to assess the stage of cancer and can be used as prognostic markers during therapy. During the cancer conditions β-D-glucuronidase and β- D-galactosidase were observed in breast cancer patients [28].

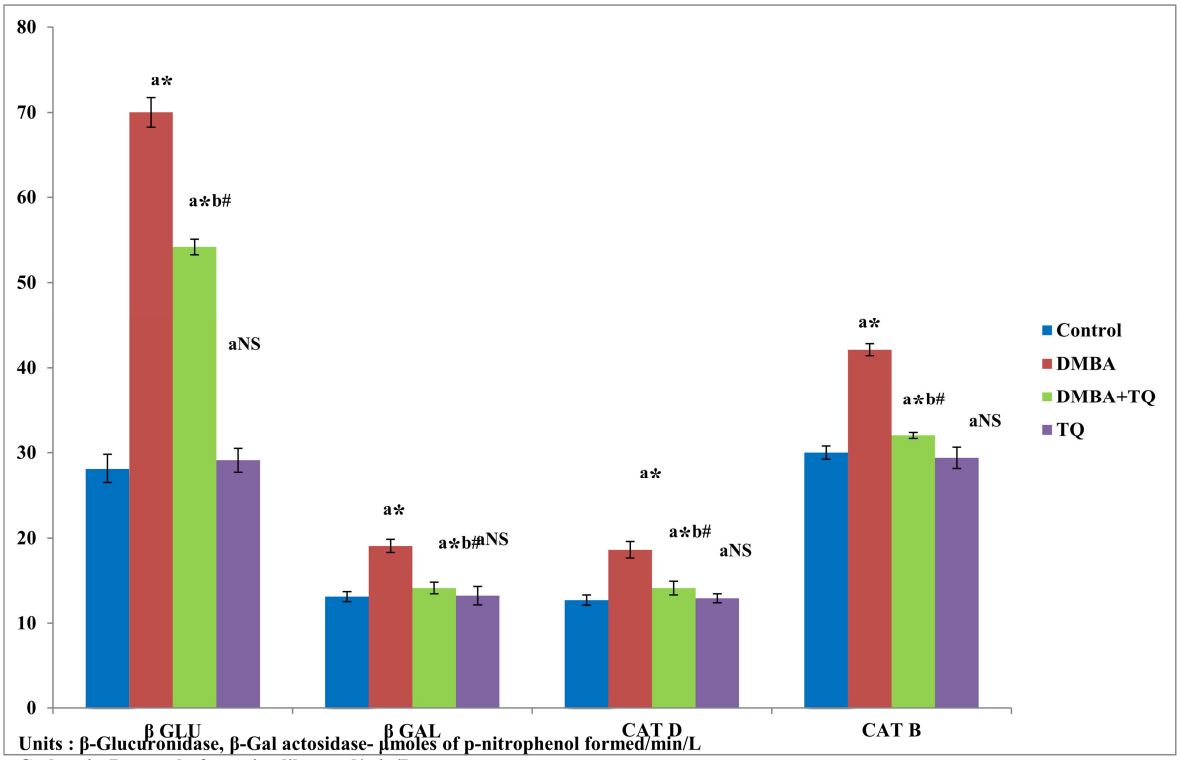
Figure 4: Effect of Thymoquinone on the activities of lysosomal marker enzymes in hepatic tissues of control and experimental animals



Units : β-Glucuronidase, β-Gal actosidase- µmoles of p-nitrophenol formed/min/L
Cathepsin-D - µmol of tyrosine liberated/min/L
CAT-B- µmol of p-nitroaniline liberated/min/L
Values are given as mean ± S.D for groups of six rats in each. Statistical significance was compared within the group as follows: a-compared with control rats
b- compared with DMBA induced rats *p<0.001; @p<0.05; #p<0.01; NS-Not significant

Cathepsin D and Cathepsin B is an aspartic endoprotease which is ubiquitously expressed in lysosomes of all tissues and catalytically active at acidic pH values that vary according to substrates, but is mostly found in intracellular vesicles, lysosomes, phagosomes and late endosomes. Cathepsin D and B plays a major role in the digestion of extracellular matrix (ECM) components and is implicated in tumor invasion and metastasis. It plays a proteolytic role in the digestion of the ECM components and plays a crucial role in tumour metastasis. In most breast tumours and it is over expressed from 2-50 fold compared to its concentration in other cell types such as fibroblasts or normal mammary glands [29]. In the present investigation, increased level of cathepsin-D and B was observed in the DMBA-induced rats. Recuperation of lysosomal enzymes upon thymoquinone treatment to DMBA induced rats may be due to the membrane stabilizing property of thymoquinone on lysosomal membranes, which protects the rapid leakage of enzymes and obstruct the rise in the enzymatic activity.

Figure5: Effect of Thymoquinone on the activities of lysosomal marker enzymes in renal tissues of control and experimental animals

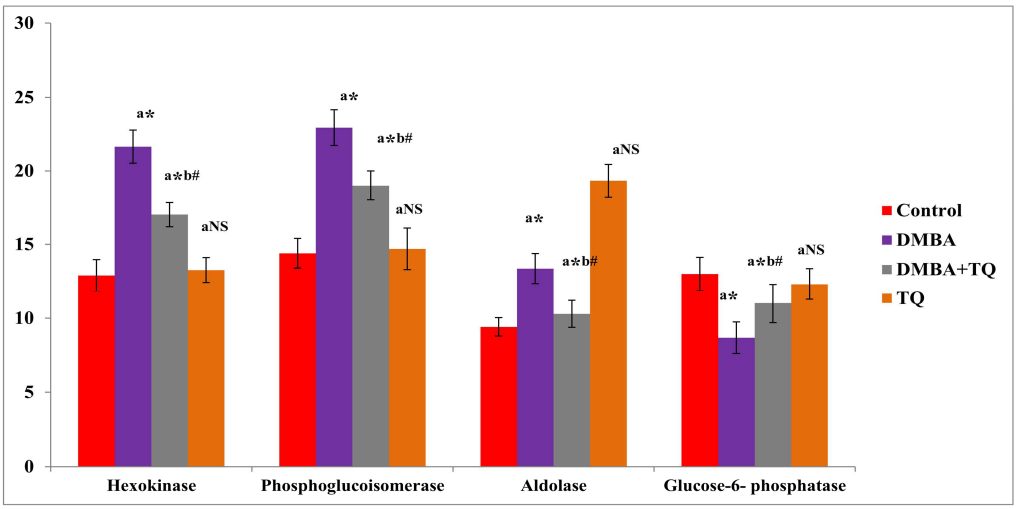


Units : β-Glucuronidase, β-Gal actosidase- µmoles of p-nitrophenol formed/min/L
Cathepsin-D - µmol of tyrosine liberated/min/L
CAT-B- µmol of p-nitroaniline liberated/min/L
Values are given as mean ± S.D for groups of six rats in each. Statistical significance was compared within the group as follows: a-compared with control rats
b- compared with DMBA induced rats *p<0.001; @p<0.05; #p<0.01; NS-Not significant

Effect of thymoquinone on the activities of glycolytic and gluconeogenic enzymes

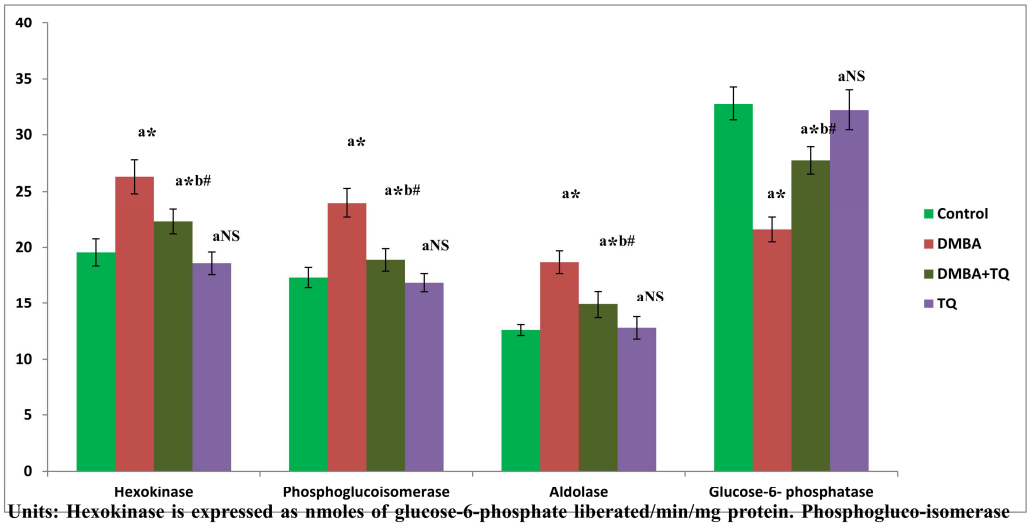
The effect of thymoquinone on the activities of carbohydrate metabolizing enzymes in the mammary gland and liver of control and experimental animals are presented in figures 6 and 7 respectively. The levels of Hexokinase, Phosphogluco-isomerase and Aldolase in mammary and liver tissues of DMBA induced rats were found to be elevated significantly whereas the Glucose-6-phosphatase was found to be decreased when compared to the Control and Thymoquinone control . On the other hand, these enzyme levels were significantly brought back to near normal levels in Thymoquinone treated rats when compared to DMBA induced rats. Whereas, no significant changes were observed in Thymoquinone alone treated rats when compared to control rats.

Figure 6: Effect of thymoquinone on activities of glycolytic and gluconeogenic enzymes in the mammary tissue of control and experimental animals.



Units: Hexokinase is expressed as nmoles of glucose-6-phosphate liberated/min/mg protein. Phosphogluco-isomerase is expressed as nmoles of fructose liberated/min/mg protein. Aldolase is expressed as nmoles of glyceraldehyde liberated/min/mg protein. Glucose-6- phosphatase is expressed as nmoles of Pi liberated/min/mg protein. Values are given as mean ± S.D for groups of six rats in each. Statistical significance was compared within the group as follows:
a-compared with control rats
b- compared with DMBA induced rats *p<0.001; @p<0.05; #p<0.01; NS-Not significant

Figure7: Effect of thymoquinone on activities of glycolytic and gluconeogenic enzymes in the hepatic tissue of control and experimental animals.



Units: Hexokinase is expressed as nmoles of glucose-6-phosphate liberated/min/mg protein. Phosphogluco-isomerase is expressed as nmoles of fructose liberated/min/mg protein. Aldolase is expressed as nmoles of glyceraldehyde liberated/min/mg protein. Glucose-6- phosphatase is expressed as nmoles of Pi liberated/min/mg protein. Values are given as mean ± S.D for groups of six rats in each. Statistical significance was compared within the group as follows:
a-compared with control rats
b- compared with DMBA induced rats *p<0.001; @p<0.05; #p<0.01; NS-Not significant

Hexokinase levels occupy an important place in determining the glycolytic capacity of cancer cells [30]. Hexokinase is an isoenzyme that catalyzes the phosphorylation of glucose to glucose-6-phosphate and plays a pivotal role in the

maintenance of glucose homeostasis [31]. In the present study, a significant increase in the activity of hexokinase was observed in DMBA induced rats. This may be due to the fact that tumors catabolise large amount of glucose because glucose is the preferred substrate for most of the rapidly growing cancer cells.

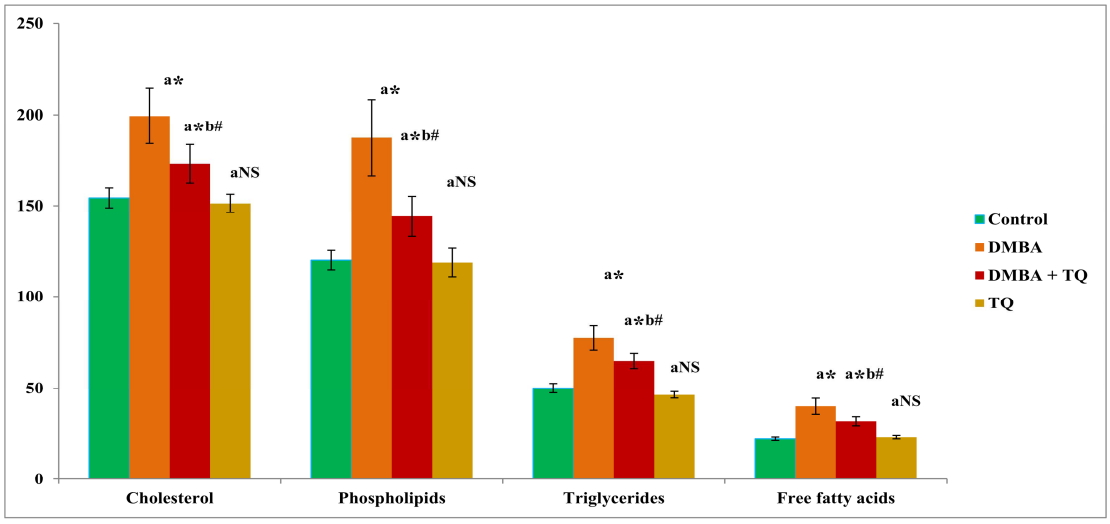
Phosphoglucoisomerase (PGI) catalyzes the conversion of glucose-6-phosphate to fructose-6- phosphate. PGI is an indicator of metastatic growth and is elevated in patients with neoplasm, especially after metastasis. In the present investigation, the increased levels of phosphoglucoisomerase was found in DMBA induced rats, which may be due to the higher glycolytic rate in tissues and further leakage from the destruction of neoplastic tissues. Aldolase is also found to be elevated in the tumor bearing animals. Sibley and Fleisher (1954) have reported that the activity of aldolase was elevated in the breast cancer condition [32].

Glucose-6-phosphatase, a crucial gluconeogenic enzyme, is mainly found as an integral protein in the lumen of the endoplasmic reticulum of liver tissues that catalyzes the dephosphorylation of glucose-6- phosphate to glucose [33] and it is transported out of the liver to increase blood glucose concentration. Glucose-6-phosphatase is unique among the gluconeogenic enzymes and also a marker enzyme for liver microsomal activity. The activities of gluconeogenic enzymes were significantly inhibited in DMBA induced rats. This may be due to the higher lactic acid production of neoplastic tissues, and it has been proved that the tumor utilizes a large proportion of lactate for glycolysis and protein synthesis [34]. Oral treatment with thymoquinone showed a significant drop in the activities of glycolytic enzymes and a concomitant elevation in the levels of gluconeogenic enzymes. This modulation may be due to the antitumor activity of the drug either by inhibiting the glycolytic enzymes activities or by the suppression of tumor progression.

Effect of thymoquinone on the levels of lipid profile

Figure 8 and 9 depicts the effect of thymoquinone on the levels of lipid profile in the control and experimental group of rats. Elevated levels of total cholesterol, phospholipids, triglycerides and free fatty acids in the plasma and liver tissues were observed in DMBA induced rats when as compared to the control rats. Treatment with thymoquinone decreased the levels of lipid profile indicators to near those of the control rats. Thymoquinone alone treated rats did not show any significant changes on the level of lipid profile indicators when compared to control rats.

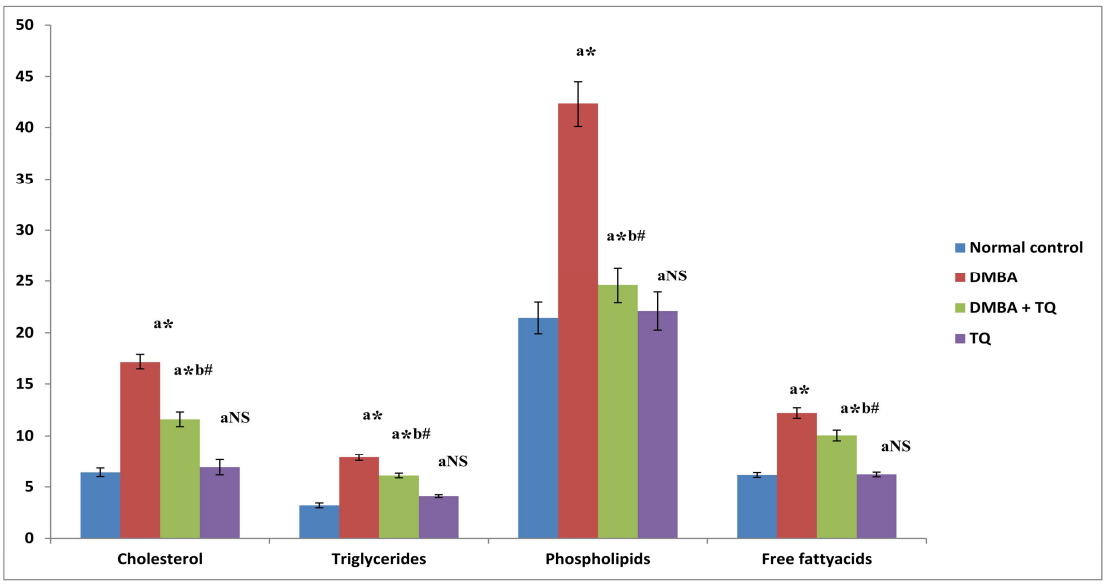
Figure 8: The levels of Lipid profile in plasma of control and experimental groups of rats



Unit: mg/dl
Values are given as mean ± S.D for groups of six rats in each. Statistical significance was compared within the group as follows: a-compared with control rats
b- compared with DMBA induced rats
***p<0.001; @p<0.05; #p<0.01; NS-Not significant**

Cancer is associated with higher lipid metabolizing activity and cholesterol metabolism is regulated differently during tumour growth. Deregulated cholesterogenesis observed in tumours implicated an over production that could result in the enrichment of tumour cell membrane with cholesterol [35]. Abnormal levels of lipid profile and their changes in lipid metabolizing enzymes are proportionate to the disease stage. In the present study, it was observed the elevated levels of Total cholesterol, triglycerides, phospholipids and free fatty acids in DMBA induced cancer bearing rats. The progression and proliferation of the tumour cells depends on the elevation of cholesterol and phospholipids levels which modifies the lipid fluidity of the tumour cell membranes thereby increasing the rate of malignancy in tumours condition [36]. In Thymoquinone treated rats, the lipid profile was reduced to normal levels. It has been shown by maintain the balance between pro-oxidant/antioxidant and stimulate various antioxidant enzymes to suppress the carcinogenic activity and also protects normal cell kinetics from DMBA induced deleterious changes.

Figure 9: The levels of lipid profile in liver tissues of control and experimental groups of rats



Unit:mg/g of wet tissue
Values are given as mean ± S.D for groups of six rats in each. Statistical significance was compared within the group as follows: a-compared with control rats
b- compared with DMBA induced rats
***p<0.001; @p<0.05; #p<0.01; NS-Not significant**

Table 1-3 depicts the levels of glycocomponents of glycoproteins in plasma, liver and mammary tissue of control, DMBA induced and thymoquinone treated rats. Elevated levels of hexose, hexosamine and sialic acid in plasma, liver and mammary tisssue were observed in cancer induced rats when compared to the controls. Upon drug administration, the animals responded better to Thymoquinone and the levels of those biochemical parameters were significantly decreased in a dose dependent manner when compared to control rats.

Table 1: Effect of thymoquinone on the levels of glycoproteins in plasma of control and experimental animals

Groups	Control	DMBA	DMBA+TQ	TQ
Hexose	146.62±8.1	197.68±11.11 ^{a*}	150.75±8.16 ^{a*b*}	148.60±9.60 ^{aNS}
Hexosamine	38.40±3.4	50.34±4.4 ^{a*}	41.80±4.2 ^{a*b**}	41.88±3.7 ^{aNS}
Sialic acid	54.23±4.6	127.67±9.56 ^{a*}	93.50±7.54 ^{a*b*}	91.94±8.28 ^{aNS}

Units: mg/dl

*Values are given as mean ±S.D for groups of six rats in each. Statistical significance was compared within the group as follows: a-compared with control rats b-compared with DMBA induced rats *p<0.001; @p<0.05; #p<0.01; NS-Not significant*

Table 2: Effect of thymoquinone on the levels of glycoproteins in breast tissues of control and experimental animals

Groups	Control	DMBA	DMBA+TQ	TQ
Hexose	1.69±0.74	4.16±0.34 ^{a*}	2.06±0.14 ^{a*b*}	1.96±0.14 ^{aNS}
Hexosamine	0.70±0.03	1.44±0.11 ^{a*}	0.90±0.05 ^{a*b**}	0.87±0.05 ^{aNS}
Sialic acid	0.24±0.02	0.80±0.03 ^{a*}	0.29±0.02 ^{a*b*}	0.32±0.03 ^{aNS}

Units: mg/g of defatted tissue

Values are given as mean ± S.D for groups of six rats in each. Statistical significance was compared within the group as follows: a-compared with control rats b-compared with DMBA induced rats *p<0.001; $^{@}$p<0.05; #p<0.01; NS-Not significant

Table 3: Effect of thymoquinone on the levels of glycoproteins in Liver tissues of control and experimental animals

Groups	Control	DMBA	DMBA+TQ	TQ
Hexose	3.35±0.24	9.65±0.73 ^{a*}	4.10±0.35 ^{a*b*}	4.15±0.39 ^{aNS}
Hexosamine	3.50±0.25	11.20±1.12 ^{a*}	4.50±0.36 ^{a*b**}	4.35±0.42 ^{aNS}
Sialic acid	2.92±0.24	6.30±0.44 ^{a*}	3.66±0.35 ^{a*b*}	3.52±0.30 ^{aNS}

Units: mg/g of defatted tissue

*Values are given as mean ± S.D for groups of six rats in each. Statistical significance was compared within the group as follows: a-compared with control rats b-compared with DMBA induced rats *p<0.001; $^{@}$p<0.05; #p<0.01; NS-Not significant*

The elevated levels of plasma glycoprotein components in cancer condition may be due to the leakage of the disturbed membrane components from either disintegrating or dying neoplastic cells or may be due to the consequent shedding of plasma membrane and due to increased synthesis by sequential addition of monosaccharide units to parent protein molecule catalyzed by multiple glycosyltransferases such as sialyltransferase (NeuAc-T), galactosyltransferase (Gal-T), fucosyltransferases (Fuc-T A and Fuc-T B) [37-39]. An increased levels of glycoprotein components in mammary tissue is observed in the present study. Oral treatment with thymoquinone resulted in the normalization of glycoprotein components levels which indicates the cytostabilising property of thymoquinone.

CONCLUSION

The results of the present study indicate that thymoquinone exerts anticancer effect through its role in modulating the glycoprotein components, lysosomal membrane stability. Also, the altered enzyme activities of carbohydrate metabolism were normalized upon treatment with Thymoquinone. Furthermore, the reason for the observed anticancer activity could be due to the inhibition of glycolytic pathway and activation of gluconeogenesis via antioxidant activity. Thus, it can be concluded that thymoquinone may modulate the energy requirement of neoplastic tissues and resulting in suppression of tumor growth.

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