ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Protective and curative role of allyl isothiocyanate against bladder carcinogenesis in rats

Hamdy Abdelhamed IBRAHIM[1], Mahmoud Mohamed GABR[2], Wafaa Mohamed EL-KHOLY[1], Wafaa Sami ABD-ELKHALEK[3], Mahmoud Mohamed ZAKARIA[4]

1. Professor of Animal physiology, Zoology Department, Faculty of Science, Mansoura University, Mansoura, Egypt.
2. Consultant of Cytology & Histology, Department of Biotechnology, Urology and Nephrology Center, Mansoura 35516, Egypt.
3. Biotechnologist, Department of Biotechnology, Urology and Nephrology Center, Mansoura 35516, Egypt.
4. Molecular Biologist, PhD, Department of Biotechnology, Urology and Nephrology Center, Mansoura 35516, Egypt.

Manuscript Info

Manuscript History:

Received: 15 July 2015
Final Accepted: 22 August 2015
Published Online: September 2015

Key words:

allyl isothiocyanate, bladder cancer, rat experimental model, real-time PCR, oxidative stress, antioxidant biomarkers

Corresponding Author

Mahmoud Mohamed ZAKARIA

Abstract

Study's background and aim: Allyl isothiocyanate (AITC) occurs in many cruciferous vegetables such as broccoli, cabbage, cauliflower. They showed chemopreventive activity against different tumors. Our objective is determination of protective effect and cure effect of AITC against bladder cancer (BC) in rats.

Materials and methods: Sixty male Fischer rats were used for this study. Rats were randomized into six groups. At the end of the study period, blood samples were collected for biochemical analysis and the bladder tissues were removed for biochemical measurement and P53 gene expression.

Results: There was no incidence of bladder cancer in normal controls. Meanwhile, all rats in bladder cancer group developed cancer with percentage 100%. In the protective group (AITC+ BC), bladder cancer lesions were seen in 60% of animals. In the curative group (BC+AITC), ten weeks of treatment with AITC 80% of rats were developed bladder cancer lesions. The size of bladder tumor was decreased and only small carcinomas are found.

Conclusion: AITC has an efficient anticancer activity. It could protecting 40% of animals and suppressed all stages of the neoplastic processes.

INTRODUCTION

Bladder malignant tumors are regarded as the most popular widespread malignancies around the world, in addition to it considers superior popularity in developed countries. Bladder malignant tumors are the second most traditional tumor of the genitourinary region, as well as the second most widespread reason for mortality in sufferers with genitourinary region malignancies (1, 2). Carcinogenesis includes 3 recognizable degrees including, initiation, promotion, and progression. Chemoprevention is the usage of factors to prevent, reverse, or suppress tumorigensis at the initiation degree itself (3). There is a great need for agents capable of inhibiting bladder cancer development and recurrence (4).

N-butyl-n-(4-hydxybutyl) nitrosamine (BBN) has a high propensity to induce mutations affecting the expression of genes such as p53 (5). It is reasonable to expect a close correlation between mutagenesis and carcinogenesis, particularly with respect to initiation, before selection of precancerous clones may indicate progression (6).

Allyl isothiocyanate (AITC) occurs in many cruciferous vegetables, is widely and often frequently consumed by humans. Besides antimicrobial activity against a wide spectrum of pathogens, it showed anticancer

activity in both cultured cancer cells and animal models, although the underlining mechanisms remain largely undefined (7). AITC is a component of numerous prevalent cruciferous greens. AITC revealed a noticeable preventing impact on bladder cancers progression and/nontoxic to normal human bladder epithelial cells (4). It is selectively delivered to bladder tumor tissue via urinary excretion and potently prevents bladder cancer development and invasion (4). Outcomes of epidemiologic scientific tests have shown that dietetic isothiocyanates and cruciferous greens consumption are oppositely linked with bladder cancer danger in people (8). There has been great interest in the potential therapeutic effects of these compounds that isothiocyanates (ITCs) inhibit tumor development in many experimental models and are being investigated as possible chemopreventive agents for specific human cancers (9). Available data indicate that urinary concentrations of AITC equivalent are at least 10 times higher than in plasma and tissue levels of AITC equivalent in the urinary bladder 14 - 79 times higher than in other organs after oral AITC administration to rats. These findings indicate that AITC may be effective in the bladder as a cancer chemopreventive compound (7). There has been great interest in the potential therapeutic effects of these compounds that ITCs inhibit tumor development in many experimental models and are being investigated as possible chemopreventive agents for specific human cancers (9).

2. Materials and methods:

2.1. Experimental animals and study groups:

Rat models: Healthy animals including 60 male Fischer rats (140 - 150 g) were obtained from the animal house department of Urology and Nephrology Center, Mansoura University .They maintained under a 12 hour light dark cycle air conditioned at 24 ± 2 ℃ and 50 - 70% humidity. Throughout the study, rats were provided with standard diet and water *ad libitum*. All protocols were approved by the institution's animal welfare regulatory committee. Rats were divided into 6 groups, each contains 10 rats: the 1st group was normal control received standard diet and tap water for 20 weeks, 2nd group (AITC group) received 17 µl AITC dissolved in 2ml soy oil for 20 weeks with stomach tube, 3rd group (soy oil group) received 2ml soy oil (dissolving agent) for 20 weeks with stomach tube, 4th group bladder cancer (BC group) received 0.5 ml of N-butyl-N-(4-hydroxy butyl) nitrosamine (BBN) in 3% NaHCO3 in their drinking water for 10 weeks, then housed another 10 weeks without any treatment (i.e. drink tap water only). 5th group (AITC + BC) Simultaneously group received 0.5 ml of BBN in 3% NaHCO3 in their drinking water simultaneously with AITC for 10 weeks with stomach tube then housed another 10 weeks without any treatment and 6th group (BC + AITC) group received 0.5 ml BBN in 3% NaHCO3 in their drinking water for 10 weeks and thereafter the rats were treated with AITC for additional 10 weeks.

2.2. Blood and tissue samples collection:

At the end of the study period, overnight fasted rats were sacrificed under ether anesthesia. Blood samples were collected in clean dry centrifuge tubes. Sera were separated by centrifugation at 2500 rpm for 10 minutes and then quickly frozen at -20 ℃ for further biochemical analysis. Immediately after collecting blood, all animals were dissected and the bladders were removed, cleaned and divided into 2 portions for biochemical measurement and gene expression investigation respectively. Serum and bladder tissues from each group after collecting were frozen until examination.

2.3. Determination of oxidative stress:

Malondialdehyde (MDA) level, nitric oxide (NO) level and hydrogen peroxide (H_2O_2) concentrations were measured using kits purchased from Biodiagnostic Co., Giza, Egypt. Determination of the biomarkers were done according to manual instructions of these kits.

2.4. Determination of antioxidant biomarkers:

Glutathione (GSH) content, superoxide dismutase (SOD) activity, catalase (CAT) activity and Glutathione-S-transferase (GST) activity were measured using Antioxidant Biomarkers (Biodiagnostic Co., Giza, Egypt). Determination of the biomarkers were done according to manual instructions.

2.5. Real time PCR for P53 gene (qPCR)

Total RNA was extracted from rat urinary bladder for each group by using ABIO pure total RNA kit (Aliance Bio, CA.USA) according to manufacturer′s instruction RNA was isolated. Briefly, 1 µg of total RNA which measured by Nano drop spectrophotometer (Nano drop 2000, thermo scientific, USA) was converted to cDNA by high capacity archival kit (Applied Biosystem, California, USA). Run was done using a total volume 25 µl of mixture for each sample in which included 12.5 µl 2X Syber Green Master Mix (Quantitect, Qiagen, USA), 25 pmole of each primers, 2 µl of cDNA template and completed the volume by sterile distilled water . Then the reaction mixture was subjected to Real Time- PCR system (CFX96 Real Time System, Bio Rad, USA). The cycling

parameter, were as follows: initial denaturation at 95 °C for 2 minutes, followed by 40 cycles of 94 °C for 15 second, annealing and elongation at 60 °C for 60 second. Primer sequences were designed at National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov/). P53 forward primer TAGCGACTACTACAGTTAGGGGGT and reverse primer GCTCGATGCTCATATCCGACT. Glyceraldehyde-3- phosphate dehydrogenase was included as reference gene (GAPDH) with forward primer TTGTGCAGTGCCAGCCTCGT and reverse primer TGCCGTTGAACTTGCCGTGG. The estimated PCR product of P53 was 125 bp and GAPDH was 201 base pair. The resulting products were electrophoresed in a 1.5% agarose gel to detect the accuracy and evaluation of qPCR. Photographed by Canon digital camera on UV transilluminator in dark hood system. The mathematical model introduced by M. Pfaffl (10) was achieved for the relative quantification of target gene. In this study, gene expression was expressed relative to that of healthy negative control (normal control group).

2.6. Statistical analysis:

All data are represented as means ± SE. One-way analysis of variance (One-way ANOVA) followed by DUNCAN test was used to determine differences among means of investigated groups. The differences were considered to be statistically significant at $P \leq 0.05$ (11).

3. Results:

Table (1): Bladder cancer incidence in control and different treated rat groups.

Group	Treatment	No. of rats free of tumor	No. of rats developed tumor	Tumor type
1	**Normal control**	**10/10 (100%)**	**0/10 (0%)**	**-**
2	**AITC**	**10/10 (100%)**	**0/10 (0%)**	**-**
3	**Soy oil**	**10/10 (100%)**	**0/10 (0%)**	**-**
4	**BC**	**0/10(0%)**	**10/10 (100%)**	**Transitional**
5	**AITC+BC**	**4/10(40%)**	**6/10 (60%)**	**Transitional**
6	**BC+AITC**	**2/10(20%)**	**8/10(80%)**	**Transitional**

AITC: allyl isothiocyanate (dose =17µl AITC dissolved in 2ml soy oil daily).

BC: Bladder cancer group was given (N- butyl-N-(4-hydxybutyl) nitrosamine) (BBN) (dose = 0.5ml/litre in 3% NaHCO3 in drinking water.

AITC+BC: AITC was given concomitant to BBN.

BC+AITC: AITC was given after the end of BBN.

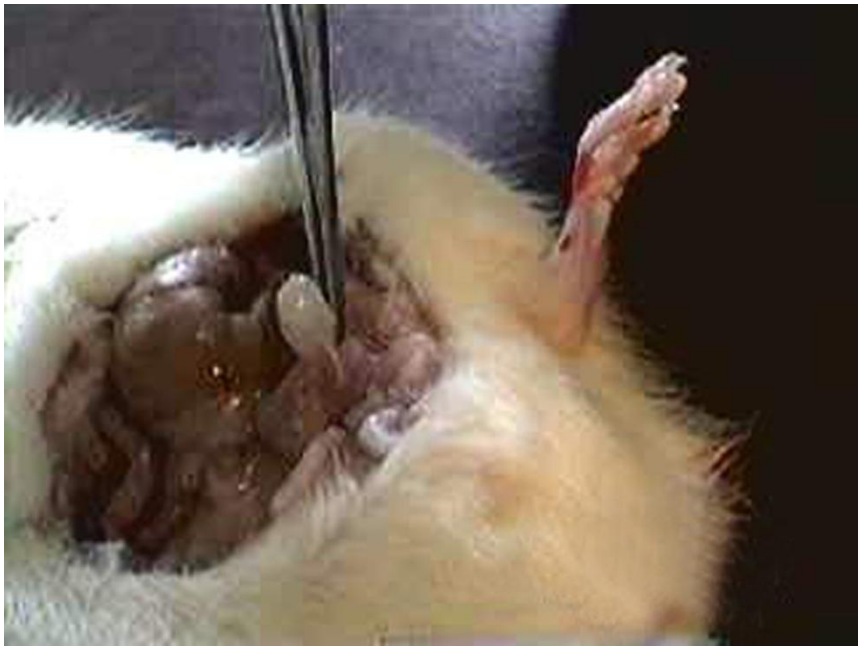

Figure (1): light photograph showing normal bladder of rat from a group of normal control and hasn't any changes in appearance .

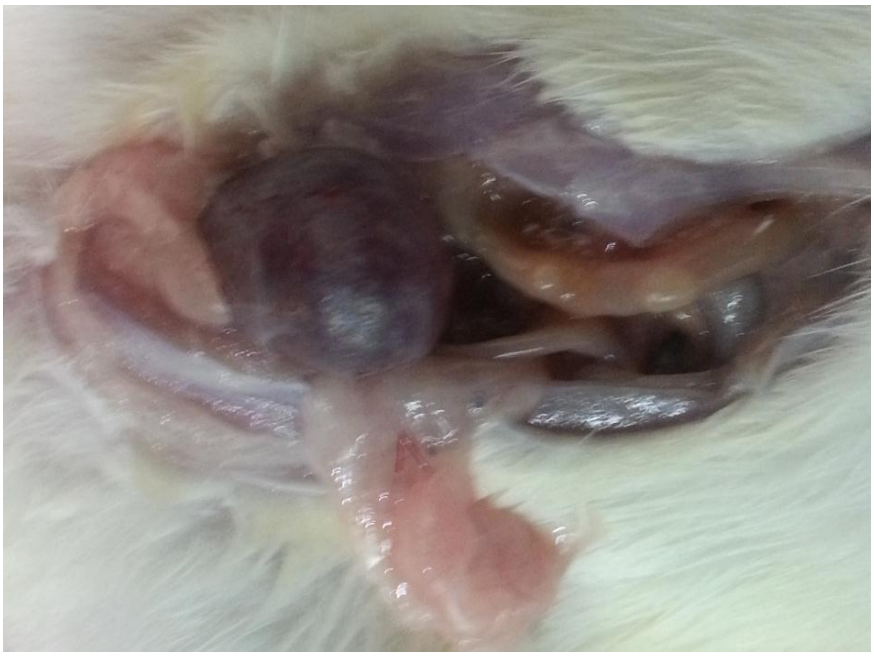

Figure (2): Light photograph showing the bladder of a rat after 20 weeks of initiation of bladder tumor, the tumor with large size in the bladder.

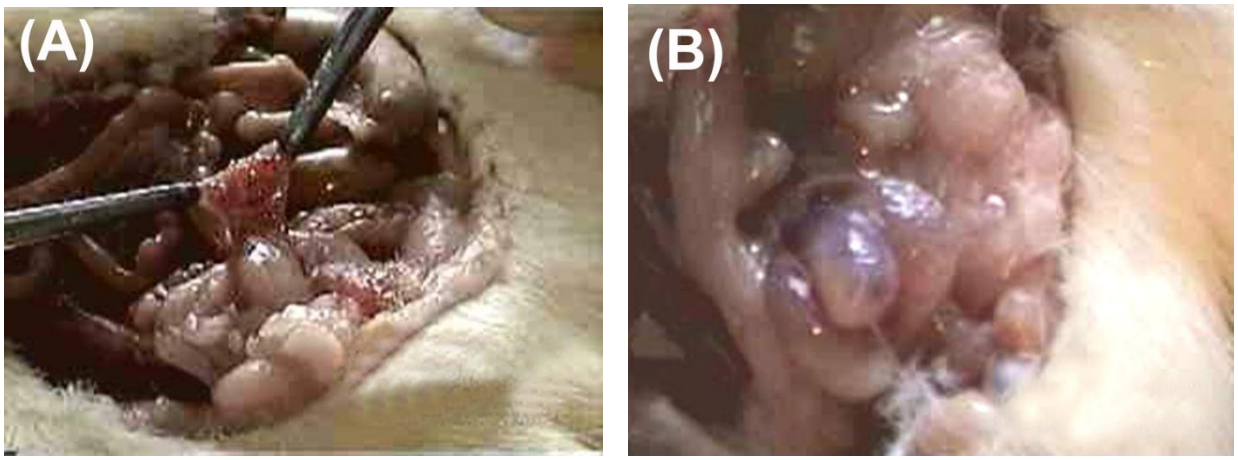

Figure (3): A) Light photograph of the rat bladder from group AITC+BC with significantly decreased size of tumor compared bladder cancer. B) The bladder of a rat from group BC+AITC with significantly decreased size of the tumor.

There was no incidence of bladder cancer in normal control rats; (Figure 1) as well as groups treated with AITC alone or with its solvent (soy oil). Meanwhile, in bladder cancer treated group (BC group) all rats developed bladder cancer with percent 100% appeared as small papillary superficial bladder tumor ten weeks after BBN administration. Furthermore, after 20 weeks of carcinogen initiation, the rats showed progressed big size bladder tumor with transitional type as shown in (Figure 2). Rats treated with AITC for 10 weeks simultaneously with the carcinogen then left another 10 weeks without any treatment (group AITC+BC), bladder cancer lesions were seen in 60% of animals (Figure 3A), 4 of 10 rats are free of bladder cancer lesions. Thus, AITC is highly efficient in protecting 40% of animals and suppressed all stages of the neoplastic process.

In (group BC+AITC), the effect of AITC was assessed on already established malignant lesions. The results exhibited that, ten weeks of treatment with AITC in rats with developed bladder tumor decreased 8 of 10 rats developed bladder cancer lesions compared to 100% of incidence of bladder cancer bladder cancer group. The size of bladder tumor was decreased and only small carcinomas are found indicating suppressed stages of the neoplastic bladder cancer process (Figure 3B).

2- Biochemical parameter:

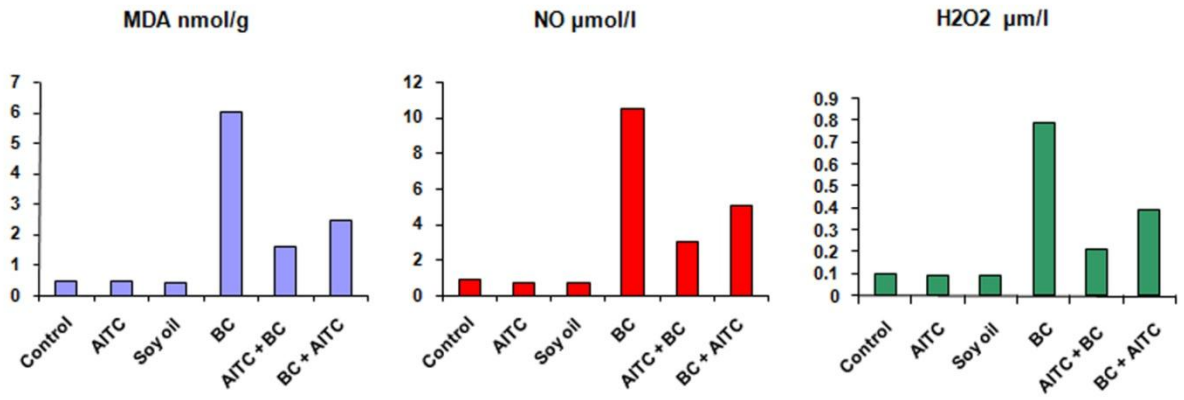

Figure (4): Serum MDA level, NO level and H_2O_2 concentration in control and different treated rat groups.

Figure 4 showed no significant changes in AITC and soy oil of control groups in serum MDA level, NO level and H_2O_2 concentrations when compared with healthy control group. There was an increased serum MDA level, NO level and H_2O_2 concentrations noticed in bladder cancer group when compared with healthy control rats. Diminished bladder tissue MDA level, NO level and H_2O_2 concentrations in AITC+BC and BC+AITC groups were observed when compared with bladder cancer group.

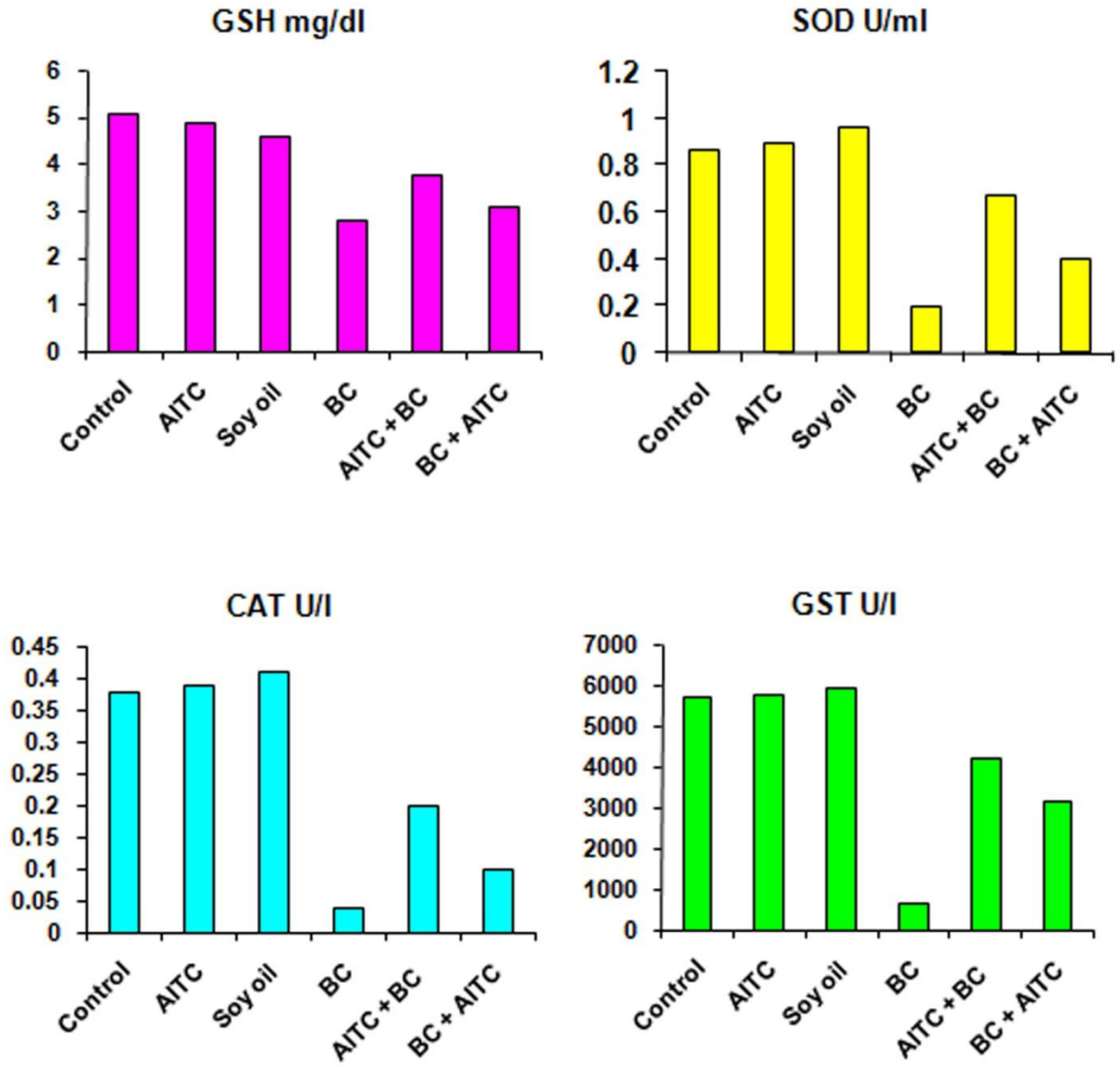

Figure (5): Serum GSH concentration, SOD activity, CAT activity and GST activity in control and different treated rat groups.

No significant changes in serum GSH content, SOD activity, CAT activity, GST activity in AITC and soy oil control groups when compared with healthy control group. There was a decreased serum GSH content, SOD activity, CAT activity, GST activity were noticed in bladder cancer group when compared with healthy control rats. Elevated serum GSH content, SOD activity, CAT activity, GST activity in AITC+BC and BC+AITC groups were observed when compared with bladder cancer group.

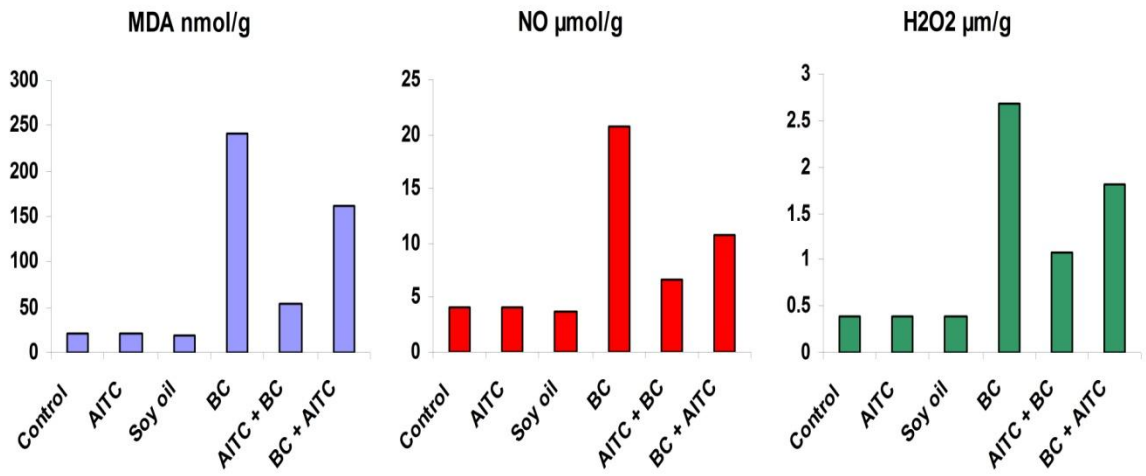

Figure (6): Bladder tissues MDA level, NO level and H_2O_2 concentration in control and different treated rat groups.

Figure 6 showed no significant changes in AITC and soy oil of control groups in bladder tissue MDA level, NO level and H_2O_2 concentrations when compared with healthy control group. Figure 6 showed an increased bladder tissue MDA level, NO level and H_2O_2 concentrations were noticed in bladder cancer group when compared with healthy control rats. Diminished bladder tissues MDA level, NO level and H_2O_2 concentrations in AITC + BC group and BC + AITC were observed when compared with bladder cancer group.

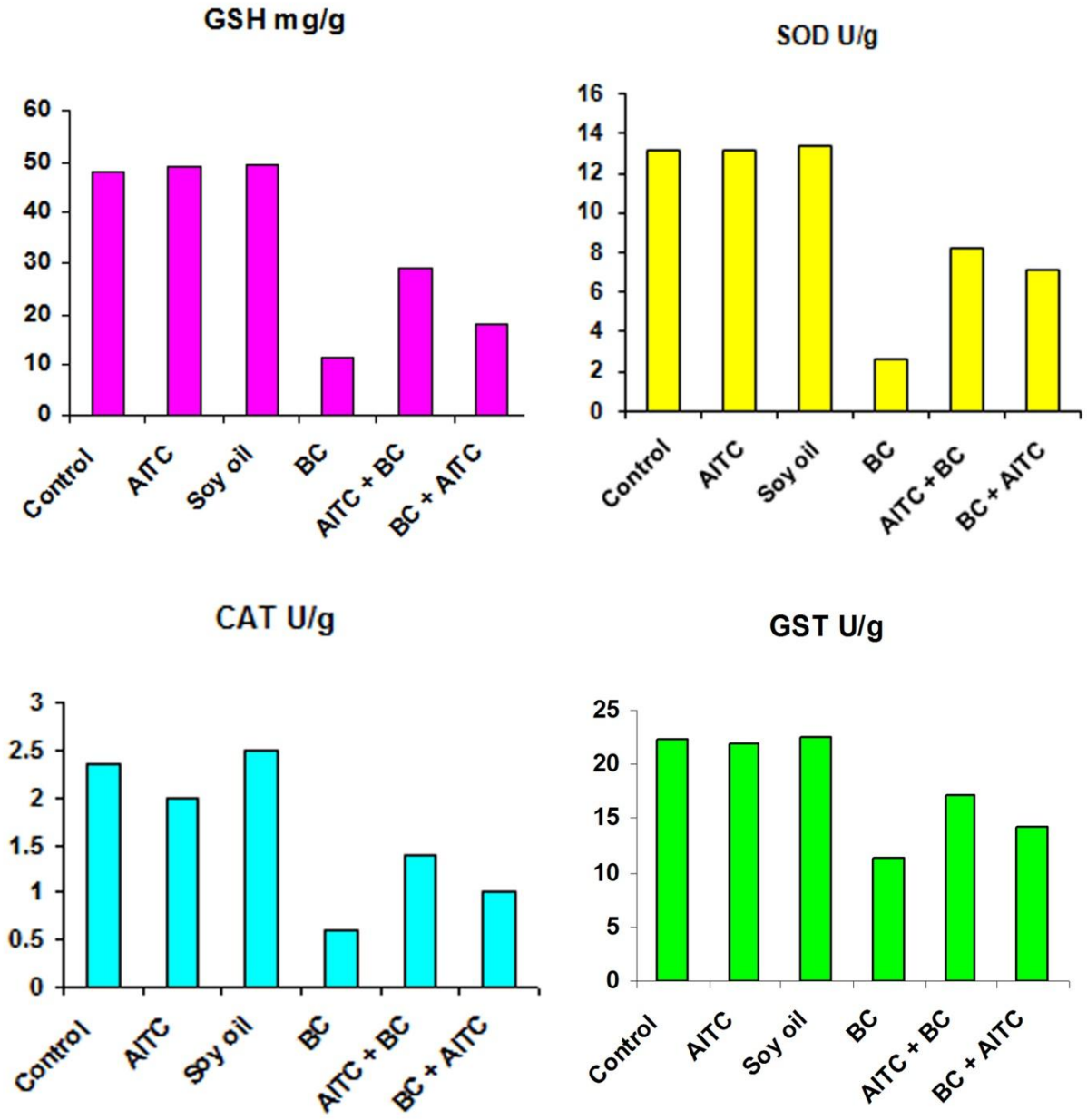

Figure (7): Bladder Tissue GSH concentration, SOD activity, CAT activity and GST activity in control and different treated rat groups.

There is no significant changes in AITC and soy oil of the control group in bladder tissue GSH content, SOD, CAT and GST activities when compare with healthy group (Fig.7). It showed a decrease in bladder tissue GSH content, SOD, CAT, and GST activities in bladder cancer group. An elevation in these enzymes in AITC+BC and BC+AITC groups were observed when compare with the bladder cancer group.

3- Gene expression assay

P53 gene was calculated relative to that of control normal group. Negative control AITC group was slightly changed in fold (non-significant) compared to normal control group. The solvent of the AITC was soy oil, when soy oil group was added to the normal control group there is no fold changed also in p53 expression. In bladder cancer group there was great change occur in fold reached to 25 fold in gene expression. In bladder cancer treated with allyl isothiocyanate (BC+AITC group) the fold decrease than positive control (BC) group reach to 7.8 fold in expression.

In bladder cancer treated with allyl isothiocyanate (BC then AITC) the fold decrease than positive control (BC group) reach to 5.5 fold in expression.

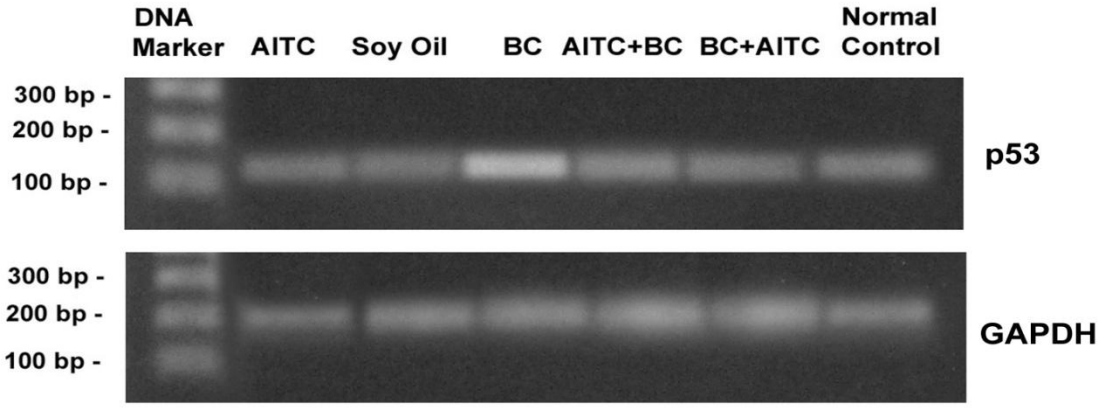

Figure (8): Bladder p53 gene expression in control and different treated rat groups

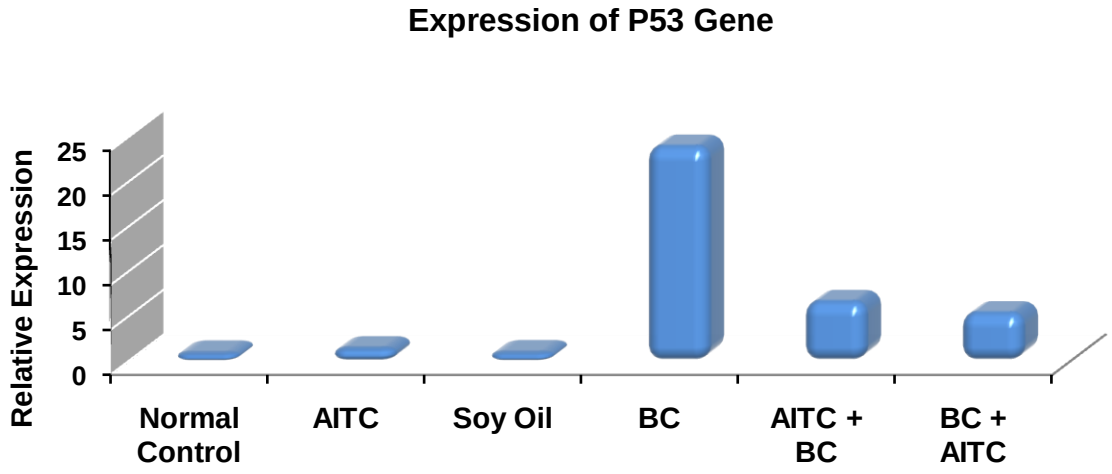

Figure (9): Gene expression was expressed relative to that of healthy negative control group.

4. Discussion:

The present results showed that BBN induced bladder cancer in fischer rats when added to drinking water for 10 weeks and found that bladder tumors were induced in fischer rats at almost 100% incidence (ten of ten rats) , The data exhibited that BBN-induced Fischer rat urinary bladder cancer. In addition, after 20 weeks of initiation of bladder tumor progressed to big sized tumor of transitional type. Meanwhile, rats treated with AITC for 10 weeks simultaneously with the carcinogen then left another 10 weeks without any treatment , bladder cancer lesions were seen in 60% of animals, 4 of 10 rats are free of bladder cancer lesions. Thus, AITC is highly efficient in protecting 40% of animals and suppressed all stages of the neoplastic process. The effect of AITC was assessed on already established malignant lesions and the results exhibited that, ten weeks of treatment with AITC in rats with developed bladder tumor decreased 8 of 10 rats (80%) developed bladder cancer lesions compared to 100% of incidence of

bladder cancer. The size of bladder tumor was decreased and only small carcinomas are found indicating suppressed stages of the neoplastic bladder cancer process. In harmony with the present results, BBN is a specific bladder carcinogen and represents an important class of human carcinogens. Despite the well-recognized phenomenon that orally administered BBN occurs cancer almost exclusively in the urinary bladder with high degree of tissue specificity. BBN, in rodents, was strongly correlates with the tissue specificity of mutagenesis of the carcinogen. Just as BBN induces only bladder tumors upon oral administration, it was mutagenic only in the bladder (6).

In our study, it was found that the protective effect of AITC against tumor formation was clearly apparent and more potent when given the carcinogen that four out of ten rats were free of tumor. Also the rest six rats developed tumors smaller than that in cancer untreated rats and also when comparing to its treatment with AITC after bladder cancer induction for 10 weeks. When AITC was administered after 10 weeks initiation of cancer, it had an inhibiting effect on the number and size of the developed tumor where 2 rats of ten were free of tumor. Such results indicated protective effect of AITC which could be related to that AITC causes strong cell cycle arrest and apoptosis in bladder cancer Similarly, it was seen that oral administration of AITC at a low dose level significantly inhibits cancer growth and muscle invasion in a rat model that mimics the development and recurrence of bladder cancer in humans (4). The results were explored that the cancer chemopreventive activity of AITC is efficiently and selectivity to the bladder target tissue, since 17µl AITC induced morphologic changes that had clearly defined as apoptotic death in bladder cancer cells. Inhibition of cell proliferation by AITC may be associated with the cell cycle arrest and/or induction of apoptosis (7). The chemopreventive effects of AITC as evidenced from its activity in lowering MDA level, NO level and H_2O_2 concentrations. The BBN was used to evaluate MDA level, NO level and H_2O_2 concentration and p53 gene folds in BBN-treated rats. Our results showed higher MDA level, NO level and H_2O_2 concentration and p53 gene folds in BBN-initiated bladder carcinogenesis. Also, GSH content, SOD, CAT and GST activities are important cellular protectants against carcinogens and oxidants. Our results are in line with studies reveal that low levels of antioxidants are associated with an increased risk of cancer (12). Antioxidant depletion in the circulation may be due to their consumption in scavenging of lipid peroxidase as well as sequestration by tumor cells (13). Therefore, if these systems are insufficient, severe metabolic malfunctions and oxidative damage to DNA may result, which, experimental studies in animals and *in vitro* have suggested, are an important factor in carcinogenesis (14). Studies have shown that dietary isothiocyanates and cruciferous vegetable intake are oppositely associated with bladder cancer danger in mammals (3). AITC had much less effect on normal human bladder epithelial cells and it failed to elicit significant cell cycle arrest and apoptosis in the normal cells at the concentration that were highly effective against the cancer cells. These results suggested that AITC may selectively target malignant cells versus normal cells in bladder *in vivo* (4). AITC is primarily metabolized through the mercapturic acid pathway *in vivo*. An initial conjugation through its (–N=C=S) group with GSH gives rise to the corresponding conjugate, which then undergoes further enzymatic modifications to finally form N- acetyl cysteine (NAC) conjugate, which excreted in the urine (7).

The P53 tumor suppressor gene encodes for a protein that plays an important role in cell cycle regulation and apoptosis mechanisms (15). In the present study, there was a significant increase in p53 gene in bladder tissue in bladder cancer group than in AITC groups and in comparison with the control group. In harmony with the present results, BBN has a high propensity to induce mutations affecting the expression of genes for example p53 (5). Bladder cancer group denoted great change occur in gene expression. In bladder cancer treated with allyl isothiocyanate (BC+AITC) the fold decreased than in cancer group in gene expression but in bladder cancer treated with allyl isothiocyanate (BC then AITC) the fold decreased more than bladder cancer group. An elevated degree of specificity of BBN in the induction of bladder malignant tumors in rodents strongly correlates with the tissue specificity of mutagenesis of the carcinogen, just like BBN causes only bladder cancers upon oral consumption, it was mutagenic only in the bladder(6). So AITC has an efficient in protecting 40% of animals and suppressed all stages of the neoplastic processes.

5. References:

1.Ahmad A, Biersack B, Li Y, Kong D, Bao B, Schobert R, et al. Targeted regulation of PI3K/Akt/mTOR/NF-κB signaling by indole compounds and their derivatives: mechanistic details and biological implications for cancer therapy. Anti-cancer agents in medicinal chemistry. 2013;13(7):1002.

2.Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians. 2011;61(2):69-90.

3.Prabhu B, Padma R, Alwin D, Pazhanivel N, Balakrishnan D, Sundaresan S. Protective Effect of Diindolylmethane against N-Butyl-N-(4-hydroxybutyl) Nitrosamine-induced Bladder Carcinogenesis. Journal of Experimental & Clinical Medicine. 2014;6(4):132-8.

4.Bhattacharya A, Tang L, Li Y, Geng F, Paonessa JD, Chen SC, et al. Inhibition of bladder cancer development by allyl isothiocyanate. Carcinogenesis. 2010;31(2):281-6.
5.Vasconcelos-Nóbrega C, Colaço A, Lopes C, Oliveira P. BBN as an Urothelial Carcinogen. In Vivo. 2012;26(4):727-39.
6.He Z, Kosinska W, Zhao Z-L, Wu X-R, Guttenplan JB. Tissue-specific mutagenesis by N-butyl-N-(4-hydroxybutyl) nitrosamine as the basis for urothelial carcinogenesis. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2012;742(1):92-5.
7.Zhang Y. Allyl isothiocyanate as a cancer chemopreventive phytochemical. Molecular nutrition & food research. 2010;54(1):127-35.
8.Saw CLL, Cintron M, Wu TY, Guo Y, Huang Y, Jeong WS, et al. Pharmacodynamics of dietary phytochemical indoles I3C and DIM: Induction of Nrf2-mediated phase II drug metabolizing and antioxidant genes and synergism with isothiocyanates. Biopharmaceutics & drug disposition. 2011;32(5):289-300.
9.Xiang J, Alesi GN, Zhou N, Keep RF. Protective effects of isothiocyanates on blood-CSF barrier disruption induced by oxidative stress. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2012;303(1):R1-R7.
10.Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic acids research. 2001;29(9):e45-e.
11.Heiberger RM, Neuwirth E. One-way anova. R Through Excel: Springer; 2009. p. 165-91.
12.Gecit I, Aslan M, Gunes M, Pirincci N, Esen R, Demir H, et al. Serum prolidase activity, oxidative stress, and nitric oxide levels in patients with bladder cancer. Journal of cancer research and clinical oncology. 2012;138(5):739-43.
13.Sharma A, Rajappa M, Saxena A, Sharma M. Antioxidant status in advanced cervical cancer patients undergoing neoadjuvant chemoradiation. British journal of biomedical science. 2007;64(1):23.
14.Marnett LJ. Oxyradicals and DNA damage. Carcinogenesis. 2000;21(3):361-70.
15.Syrigos KN, Karapanagiotou E, Harrington KJ. The clinical significance of molecular markers to bladder cancer. Hybridoma and hybridomics. 2004;23(6):335-42.