Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
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ORIGINAL ARTICLE

Taxifolin Possesses Anti-Cancer Activity on the 7,12-Dimethylbenz(a)anthracene-Induced Breast Cancer in the Sprague Dawley Rats by Remodeling Nuclear Factor Erythroid 2- Kelch-Like ECH-Associated Protein 1-Heme Oxygenase 1 and Anti-Oxidant Pathways

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Submitted: 19-12-2017 Revised: 28-02-2018 Published: 28-06-2018

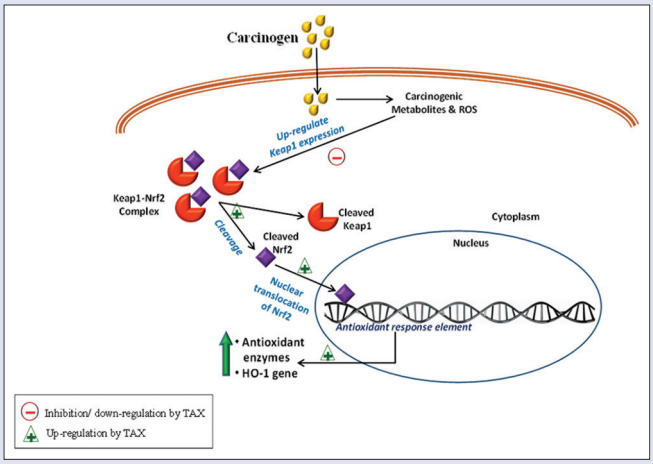
ABSTRACT

Background: In mammary cancer, alterations in various gene expressions and signaling pathways occurs due to the secondary effects of oxidative stress that facilitates cancer by causing genomic instability and mutagenic alterations. Several phenolic compounds are active against various malignancies. Taxifolin (TAX) exhibits diverse bioactivity profile that also contributes toward its anticancer efficacy. **Objective:** The present study has been designed for estimation of the anticancer potential of TAX on 7,12-dimethylbenz(a)anthracene (DMBA)-induced breast cancer in Sprague Dawley (SD) rats. **Materials and Methods**: Molecular docking analysis of Kelch-like ECH-associated protein 1 (Keap-1) and heme oxygenase-1 (HO-1) was carried out using Maestro tool to rationalize the activity of TAX based on their binding potential. This was followed by DMBA administration in air pouch to induce mammary cancer in female SD rats (50–55 days old). After 90 days of cancer induction, the chemotherapeutic potential of TAX was evaluated by the administration of TAX at doses 10, 20, and 40 mg/kg/day. Besides this, the effect of TAX on Keap-1-nuclear factor erythroid-2 (Nrf-2) pathway associated with HO-1 and NADPH:quinoneoxidoreductase 1 (NQO1) expressions and their effect on the anti-oxidative and anti-proliferative activity was also evaluated through immunofluorescence analysis, real-time quantitative polymerase chain reaction, and biochemical estimations. **Results**: TAX revealed protective effect against lipid peroxidation, enzymatic (superoxide dismutase [SOD], manganese-containing SOD, copper- and zinc-containing SOD, catalase, and glutathione peroxidase), and nonenzymatic (reduced glutathione, α-tocopherol, and ascorbic acid) anti-oxidative markers in serum, liver, kidney, and breast tissue of both control and experimental groups. The study revealed upregulation of protective Nrf-2, HO-1, and NQO1 expressions with consequent suppression in Keap-1 mRNA expression. **Conclusion**: This study revealed the potency of TAX in the inhibition of mammary carcinogenesis through Nrf-2-Keap-1-HO-1 and antioxidant pathway.

Key words: 7,12-dimethylbenz(a)anthracene, antioxidant, breast cancer, Kelch-like ECH-associated protein 1, nuclear factor erythroid 2, taxifolin

SUMMARY

- Mechanism of Action of Taxifolin for it's anti-cancer activity on Keap-1-Nrf2-HO-1 Axis.



Abbreviations used: RT-qPCR: Real-time quantitative polymerase chain reaction; ODC: Ornithin decarboxylase; HO-1; Heme oxygenase-1; PAHs: Polyaromatic hydrocarbons; TBARS: Thiobarbituric acid reactive species; NQO1: NADPH:quinoneoxidoreductase 1; Keap-1: Kelch-like ECH associated protein 1; Nrf2: Nuclear factor erythroid 2; SD: Sprague Dawley.

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DOI: 10.4103/pm.pm_601_17

Access this article online
Website: www.phcog.com
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INTRODUCTION

Cancer chemotherapeutics, utilizing naturally occurring components is thrust in cancer research worldwide. Diversity in molecular mechanisms involved in cancer progression leads to continuous and progressive discoveries of newer approaches in cancer therapy. Polycyclic aromatic hydrocarbons (PAH) are a family of structurally related chemicals that comprises major class of environmental carcinogens. These carcinogens along with certain related halogenated compounds result in mammary cancer by epidemiological and laboratory studies. 7, 12 dimethylbenz(a)

Cite this article as: Haque MW, Mohd Siddique MU, Bose P, Pattanayak SP. Taxifolin possesses anti-cancer activity on the 7,12-Dimethylbenz(a)anthracene-Induced breast cancer in the sprague dawley rats by remodeling nuclear factor Erythroid 2- Kelch-Like ECH-Associated Protein 1-Heme Oxygenase 1 and anti-oxidant pathways. Phcog Mag 2018;14:S110-7.

anthracene (DMBA) which is a prototypical PAH is utilized to promote tumors in laboratory animals.[1] It is also involved in the development of cancers with progressive stages, in various parts of the body mainly breast,[2,3] skin,[4] cervical, renal, etc.

DMBA is known to produce DNA adducts.[5,6] In rodents, breast tumors can be induced by DMBA administration which upregulates cellular cytosolic receptor for DMBA.[7] In the initial stage of tumorigenesis, cytochrome P450 enzymes are upregulated, which are responsible for metabolizing DMBA into an epoxide intermediate that readily forms DNA adducts. These adducts play a key role in DNA mutations and malignant transformation which is considered to be associated with PAH-mediated carcinogenesis.[8,9]

Molecular oxygen and free radicals give rise to highly proactive molecules called reactive oxygen species (ROS).[10] Prooxidants and antioxidants imbalance results in oxidative stress. Endogenous oxidative stress may be triggered due to impairment of cellular antioxidant defense system.[11] Oxidative stress-induced by ROS plays a critical role in the pathogenesis of cancer.[12] Nuclear factor-erythroid 2 (Nrf2) is a key transcription factor that stimulates cytoprotective genes transcription in response to oxidative stress, which in turn is controlled by Kelch-like ECH associated protein 1 (Keap-1) (adaptor protein).[13] As a consequence of stress conditions, there is disruption of Keap-1 and Nrf2 interaction leading to Nrf2 accumulation in the cell nucleus. This is followed by Nrf2 binding to the antioxidant response element (ARE) in the promoter region of some phase II enzyme genes leading to expression of enzymes downstream to Nrf2.[14] These enzymes including heme oxygenase-1 (HO-1) and NADPH: quinoneoxidoreductase 1 (NQO-1) provide protection against oxidation. Normally, controlled oxidative stress of body shifts to a progressive neoplasm after activation of certain carcinogenic pathways. Antioxidant molecular components such as Nrf-2, HO-1, hypoxia-inducible factor 1α, and NQO-1 fails to overcome the intracellular free radical loads. DMBA initiate progressive oxidative stress causing failure of antioxidant mechanisms. These changes further activate (upregulate and downregulate) specialized molecular genes/pathways such as nuclear factor-kappa B (NF-κB) and Keap-1 expression causing neoplastic changes in individual or group of cells.

One of the primary markers of carcinogenesis, the endogenous antioxidants perform a pivotal role defense against ROS-induced oxidative damage.[15] Free radicals play an important role in promoting tumors either by direct chemical reaction or by alterating cellular metabolic process,[16] and their scavengers (superoxide dismutase [SOD], catalase [CAT], glutathione peroxidase [GPx]) represent inhibitors at different stages of carcinogenesis.[17] The enzymes located in mitochondrial and cytosolic functions are mainly involved in the biotransformation and detoxification of carcinogens.[18] Superoxide radical is sequentially converted into hydrogen peroxide by SOD and CAT and subsequently to molecular oxygen and water, respectively.[19] Manganese-containing SOD (MnSOD) and copper- and zinc-containing SOD (CuZnSOD) that comprise total SOD content of cell are predominantly situated in the cytosol and mitochondrial matrix, respectively. Elevation of superoxide radical level is associated with a reduction in cellular SOD and CAT antioxidants activity.[20] Carcinogenesis which results in an excessive increase in cellular superoxide and peroxide levels, also elevates SOD and CAT utilization further depleting enzyme levels. Stimulation of oxidative stress due to increase in the cellular level of free radical brings enhances oxidative stress leading to critical changes of cellular antioxidant enzymes.[19]

Epidemiologically, naturally occurring polyphenolic components are evident to possess outstanding antioxidant, anticancer, anti-inflammatory, anti-angiogenic, and apoptotic potentials. These potentials attract different researchers for their uses against various progressive disorders such as neoplasm. Among all, flavonoids are a group of natural products including flavones, flavanones, and isoflavones, and several beneficial biological activities of flavonoids including antioxidant, antitumor, and anti-inflammation properties have been identified in several previous studies.[21-25] Flavonoids have proven their anticancer potentials against different cancer cell lines and experimental models. Taxifolin (TAX) is a flavone derivative having antiproliferative, anti-inflammatory, antidiabetic, and antioxidant activities. TAX has anticancer activity against colon cancer by modulating various molecular pathways such as NF-κB, β-catenin, and tumor necrosis factor-α. We performed *in silico* docking studies by taking few antioxidant and other proteins such as Keap-1 and HO-1. Based on the *in silico* results and previous literature, the present study was designed to elucidate the anticancer potential of TAX against DMBA-induced mammary carcinogenesis in SD rats and elucidating the molecular mechanism involved.

MATERIALS AND METHODS

Materials

DMBA and TAX were procured from Sigma Aldrich (78666-100MG-F) (St. Louis, MO, USA). All other reagents used were of high purity analytical grade.

Molecular docking of taxifolin on heme oxygenase 1 and Kelch-like ECH-associated protein 1

The co-crystal structures of proteins HO-1 (PDB ID: 3HOK); Keap-1 (PDB ID: 5FNQ); were downloaded from www.rcsb.org. Maestro-9.2, Protein preparation wizard, Ligprep, and Glide module of Schrodinger LLC suite was used for protein preparation, ligand preparation, receptor grid generation, and molecular docking running on the RHEL5 operating system installed on DELL Precision T3400 machine (n-series, Intel Core 2 Quad processor, 8 GB RAM, 500 GB. The software validation was done by extracting internal ligand and redocked into the active site. Protein preparation was carried out using protein preparation wizard and grid was generated through grid preparation wizard picking ligand to specify the binding site. Docking was done using GLIDE 5.0 with XP protocol. The docked conformers were analyzed through XP visualizer. Default parameters were employed during all the computational studies.

Experimental animals

Female Sprague Dawley (SD) rats (50–55 days old) were used for this study and were issued from Central Animal Facility, Birla Institute of Technology, Mesra, Ranchi, Jharkhand, India (Register No. 1968/PO/Re/S/17/CPCSEA). All the studies on animals were performed after approval no. 1972/PH/BIT/17/17/IAEC from the Institutional Animal Ethics Committee. All the experimental rats were maintained with standard laboratory conditions (relative humidity 60% ± 5%, temperature 25°C ± 1°C, and12 h dark and light cycle) throughout the experimental period. These animals were supplied with pellet diet and were provided water *ad libitum*. All the experimental animals were subjected to acclimatization for a week before starting the experiment.

In vivo analysis

Five experimental groups consisting of 8 SD rats (50–55 days old) in each group were used for the study. The total treatment protocol is of 118 days (90 days of tumor promotional stage and 28 days of treatment phase). Cancer was induced using DMBA suspended uniformly in olive oil (20 mg in 0.5 ml) and administered once on day 1 by air-pouch technique.[26-29] The normal control group (Group I) animals were administered with 0.5 ml olive oil in air pouch, the induced control group (Group II) animals were administered with DMBA only,

TAX treatment groups (Group III, IV, and V) were treated with 10, 20 and 40 mg/kg b.w.(i.p.) doses, respectively[30,31] after the promotional stage (90 days after DMBA induction). Tumor location was checked and monitored by palpation[2,29,32,33] each week after the induction process. After 28 days of treatment with TAX, blood samples were collected from all groups of experimental animals by retro-orbital puncture and serum/plasma was separated for biochemical assessments. Later, animals were sacrificed by cervical decapitation[15,28,33] and perfused. Liver, kidney, and mammary tissues were isolated and homogenized in phosphate buffer saline at 4°C and used for the biochemical estimations. Fractions of breast tissues were kept after washing into the liquid nitrogen for protein and mRNA preparations using standard protocols. A slice (2–4 mm) of the breast tissue was also processed for the immunofluorescence assay.

Biochemical analysis for lipid peroxidation, enzymatic and nonenzymatic antioxidants in all treatment groups

To estimate the effect of DMBA and TAX treatment on lipid peroxidation (LPO), enzymatic and nonenzymatic antioxidants biochemical analysis was carried out using previously defined well-established procedures. LPO was estimated on the principle of simple thiobarbituric acid reactive species assay.[34] The sample aliquots were treated with KCN to deactivate CuZnSOD to estimate MnSOD.[35] Total SOD and MnSOD activities were determined on the principle of reduction of nitro blue tetrazolium.[36] CuZnSOD was estimated by calculating the difference between total SOD and MnSOD. Based on the principle that H_2O_2 reduces dichromate in acetic acid to chromic acetate, CAT activity in the test samples was determined.[37] GPx determination in the samples was carried out based on the method described by Rotruck *et al*.[38] Protein content was estimated by the method of Lowry.[39] Reduced glutathione (GSH) was estimated by the principle and method described by Ellman.[40]

Immunofluorescence analysis of nuclear factor-erythroid 2 and Kelch-like ECH-associated protein 1 on mammary tissue[27]

To evaluate the effect of TAX treatment on the expressions of Nrf-2 and Keap-1, immunofluorescence analysis was carried out using standard protocols. In brief, mammary tissues (2–4 mm) after isolation were embedded into ornithine decarboxylase media and kept in liquid nitrogen for few minutes. Cryosectioning was carried out (8–10 μ), and tissue sections (5 μm) were then treated with 4% buffered formalin (cold; 10 min) and OPTIMAX (×1, 1 min). The sections were then stained with primary antibodies (Santa Cruz Biotech, USA), for Nrf-2 and Keap-1 at a concentration of 1 mg/ml incubated for 1 h at room temperature. After washing the slides twice with OPTIMAX (×1) for 1 min, the sections were then incubated with the respective secondary antibodies (Bangalore Genei, India) and incubated for 30 min on ice in the dark. Sections were then washed twice with OPTIMAX (×1, 1 min). Cleaned coverslips were applied over the tissue sections using one drop of flow mount and the slides were analyzed under a confocal microscope (FLOWVIEW, Olympus).

RNA isolation, cDNA synthesis and real time quantitative polymerase chain reaction analysis

Total RNA was extracted from cells by TRI reagent® (Sigma-Aldrich) and purified by adding a mixture containing 150 μL DDW, 8 μL glycogen, 20 μL sodium acetate (3M), and 600 μL of absolute ethanol. The RNA mixture was kept in −80°C for 2 h and centrifuged at 13,000 rpm for 15 min at 4°C to obtain the purified RNA for cDNA synthesis. First-stranded cDNA was synthesized by cDNA Synthesis Kit (Bio-Rad) with 1 μg of RNA and oligo dTTP according to the manufacturer's instructions. The equivalent amount of cDNA was subsequently amplified in 20 μl reaction using the 2x SYBR qPCR Master Mix (Kapa-Biosystems) and specific primers. The qPCR of all gene was performed using the following sense and antisense primers: sense, 5′-TGCCCCTGTGGTCAAAGTG-3′ antisense, 5′-GGTTCGGTTACCGTCCTGC-3′ for Keap-1, sense: 5′-TAGATGACCATGAGTCGCTTGC-3′ antisense: 5′-GCCAAACTTGCTCCATGTCC-3′ for Nrf-2 and sense 5′-AAGCCGAGAATGCTGAGTTCA-3′ and antisense 5′-GCCGTGTAGATATGGTACAAGGA-3′ for HO-1, sense 5′-AGAGAGTGCTCGTAGCAGGAT-3′ and antisense 5′-GTGGTGATAGAAAGCAAGGTCTT-3′ for NQO1. The cycling conditions for Keap-1, Nrf2, HO-1, NQO1 were as follows: one cycle at 95°C for 180s; 39 cycles at 95°C for 10s, and 58°C for 30s. The validation of PCR amplification was verified through the presence of a single peak during melting curve analyses (60.0°C–95.0°C, increment 0.5°C, for 0.05). Each RT-qPCR experiment was repeated three times. β-Actin was used as internal control.

Statistical analysis

Statistical comparisons between control and treatment mean values (± standard deviation) were analyzed using one-way ANOVA and Dunnett's *t*-test. The level of significance was considered as $P < 0.05$.

RESULTS

Molecular docking and binding pattern analysis

The results of molecular docking of reference molecule with HO-1 [Figure 1a] and molecular docking of TAX with HO-1 [Figure 1b] revealed that TAX actively fitted at active site of HO-1 (3HOK) in the similar fashion of reference molecule. The molecule was positioned in such a way that C phenyl ring exhibit Π-Π interaction with Hem300. As Hem is the active catalytic part of HO enzyme, if any molecule interacts with heme, probably it could have the potent inhibitory activity. It also displayed the polar H Bonding with GLU32, GLY139, and ASP140 residues. We suppose these interactions determine the potent HO inhibitory effect of TAX. The results of molecular docking of reference molecule with Keap-1 [Figure 1c] and molecular docking of TAX with Keap-1 [Figure 1d] revealed that the TAX molecule get occupied at the active site of Keap-1 (PDB ID: 5FNQ) in the similar fashion of reference molecule. The phenyl C ring of TAX was positioned at the place of 4-chlorophenyl ring of reference molecule and displayed the interaction with ARG415. It also exhibit two polar H bond with LEU365 and VAL463, respectively. These displayed interactions gave the good docking score compared to reference molecule and explained the inhibitory effect of Keap-1 receptor. These overall contributions of ligand amino acid interactions displayed better docking score that the reference molecule. Molecular modeling studies, docking scores (which are more than reference molecules) suggested that these overall interactions with amino acid residues at active site determine the potent inhibitory effect of TAX and could be used against various types of cancer.

Taxifolin restored lipid peroxidation, enzymatic and nonenzymatic antioxidants in all treatment groups

Restorative potential of TAX on antioxidant marker enzymes were evaluated at 10, 20, and 40 mg/kg b.w. doses in DMBA-induced mammary cancer bearing animals. DMBA-induced animals revealed statistically significant ($P < 0.005$) elevation in LPO [Figure 2a] and a subsequent decrease ($P < 0.005$) in total SOD [Figure 2b], MnSOD [Figure 2c], and CuZnSOD [Figure 2d]. The subsequent significant ($P < 0.005$) decrease

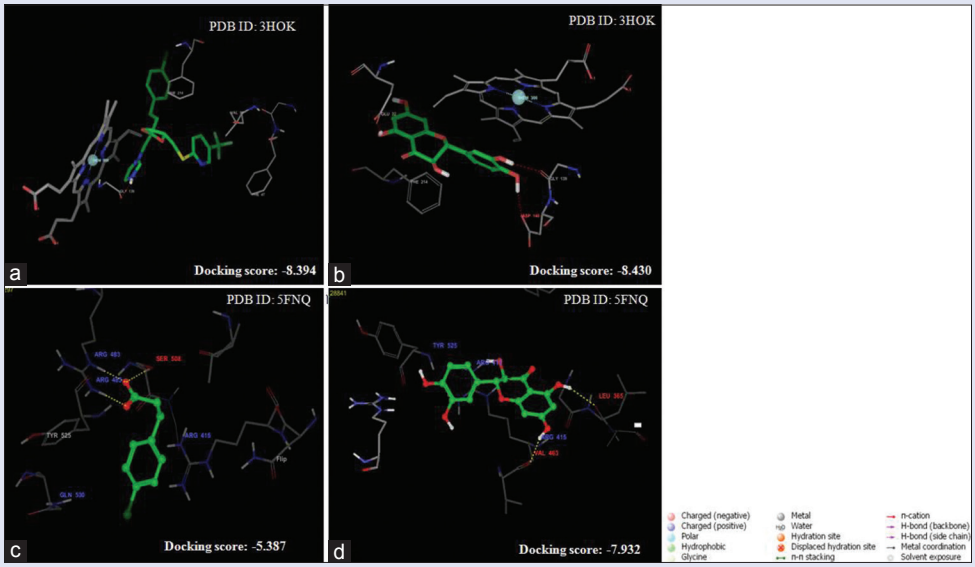

Figure 1: Molecular docking analysis of reference molecule and TAX, Where (a) overlay of reference molecule at the active pocket of HO-1; (b) overlay of TAX at the active pocket of HO-1; (c) overlay of reference molecule at the active pocket of Keap-1; (d) overlay of TAX at the active pocket of Keap-1. TAX: Taxifolin; HO-1: Heme oxygenase 1; Keap-1: Kelch-like ECH-associated protein 1

in enzymatic CAT [Figure 2e], GPx [Figure 2f] and nonenzymatic GSH [Figure 2g], ascorbic acid [Figure 2h], α-tocopherol [Figure 2i] antioxidant markers in serum, kidney, liver, and mammary tissues was recorded as compared to control group of animals. TAX treatment restored these enzyme levels significantly ($P < 0.05$) in a dose-dependent manner as compared to induced cancer-bearing animals. Hence, TAX potentially prevents peroxidation of lipids and improved antioxidant profile in animals with mammary cancer.

Taxifolin altered the expressions of nuclear factor erythroid 2 and Kelch-like ECH-associated protein 1 during immunofluorescence analysis of mammary carcinoma tissue

To determine the TAX mediated changes in cytoplasmic expression of Keap-1 and nuclear translocation of Nrf-2, Immunofluorescence assay was carried out. The results corroborate with earlier studies showing up-regulation of Keap-1 in cancer-bearing untreated animals which on treatment with TAX was down-regulated in a dose-dependent manner [Figure 3a]. Further, Nrf2 upregulation and increased nuclear translocation was observed after TAX treatment which is represented in Figure 3b.These results reveal that TAX causes alteration in the expression of Keap-1 and it was involved in the nuclear translocation of Nrf-2 at the tested dose (40 mg/kg b.w).

Taxifolin restored the mRNA expressions of Kelch-like ECH-associated protein 1, nuclear factor erythroid 2, heme oxygenase-1, and NADPH: quinoneoxidoreductase 1 in mammary carcinogenesis

To define the effect of TAX on Keap-1, Nrf-2, HO-1, and NQO1, mRNA analysis was carried out [Figure 4]. DMBA-induced group animals revealed mRNA over expression of Keap-1 signaling proteins ($P < 00.5$), while the expression of Nrf-2, HO-1 and NQO1 was significantly reduced as compared to control group. TAX treatment caused dose-dependent restoration of the mRNA levels. Group III (TAX 10 mg/kg/day) revealed significant ($P < 0.05$) decrease while group IV (TAX 20 mg/kg/day) and group V (TAX 40 mg/kg/day) showed highly significant ($P < 0.005$) reduction in Keap-1 mRNA expression in comparison to the induced control group. On the other hand, treatment with TAX significantly ($P < 0.005$) increased the RNA expressions of Nrf-2, HO-1 and NQO1 with respect to the DMBA induced cancer group of animals.

DISCUSSION

We used DMBA-induced rat mammary carcinogenesis for the investigation of the underlying mechanisms of various compounds behind their antioxidant and chemotherapeutic potentials because of its high human relevance. Many reactive intermediates (O_2^-, H_2O_2, OH) are produced by DMBA after its metabolism primarily binds to nucleophilic sites of cellular macromolecules potentiating oxidative stress leading to human breast carcinoma.[41] The pro-oxidant-antioxidant balance is disrupted which impedes protective mechanisms of endogenous antioxidant causing pathophysiological and biochemical changes leading to loss of cellular integrity.[42] This study was performed to evaluate the underlying molecular mechanism of Nrf-2-Keap-1-HO-1 and antioxidant pathways.

Endogenous metabolism of DMBA produces oxidative radicals causing serious damage to cellular components that triggers cancer-promoting signals.[41] Primarily cellular and extracellular antioxidant enzymes prevent free radical-mediated mutagenesis or tumorigenesis. Increased production of reactive free radicals causes failure of available antioxidant defense leading to carcinogenesis.[27] This could be correlated with progressive carcinogenesis as previous.[27] Overproduction of free radical formation accounts for excessive peroxidation leading to increased formation MDA.[43] This can be associated with proliferation and metastasis of breast cancer cells.[15] Reduction of cellular SOD and CAT antioxidant activities is associated with increased level of superoxide radical.[20] The clearance of xenobiotics, carcinogens, cellular peroxides is mainly mediated by GPx, and its depletion causes accumulation of intracellular peroxide in body potentiating polyunsaturated fatty acid degradation, cross-linking of lipids, proteins, and nucleic acid.[28] Depression of GSH level in DMBA-induced animals might

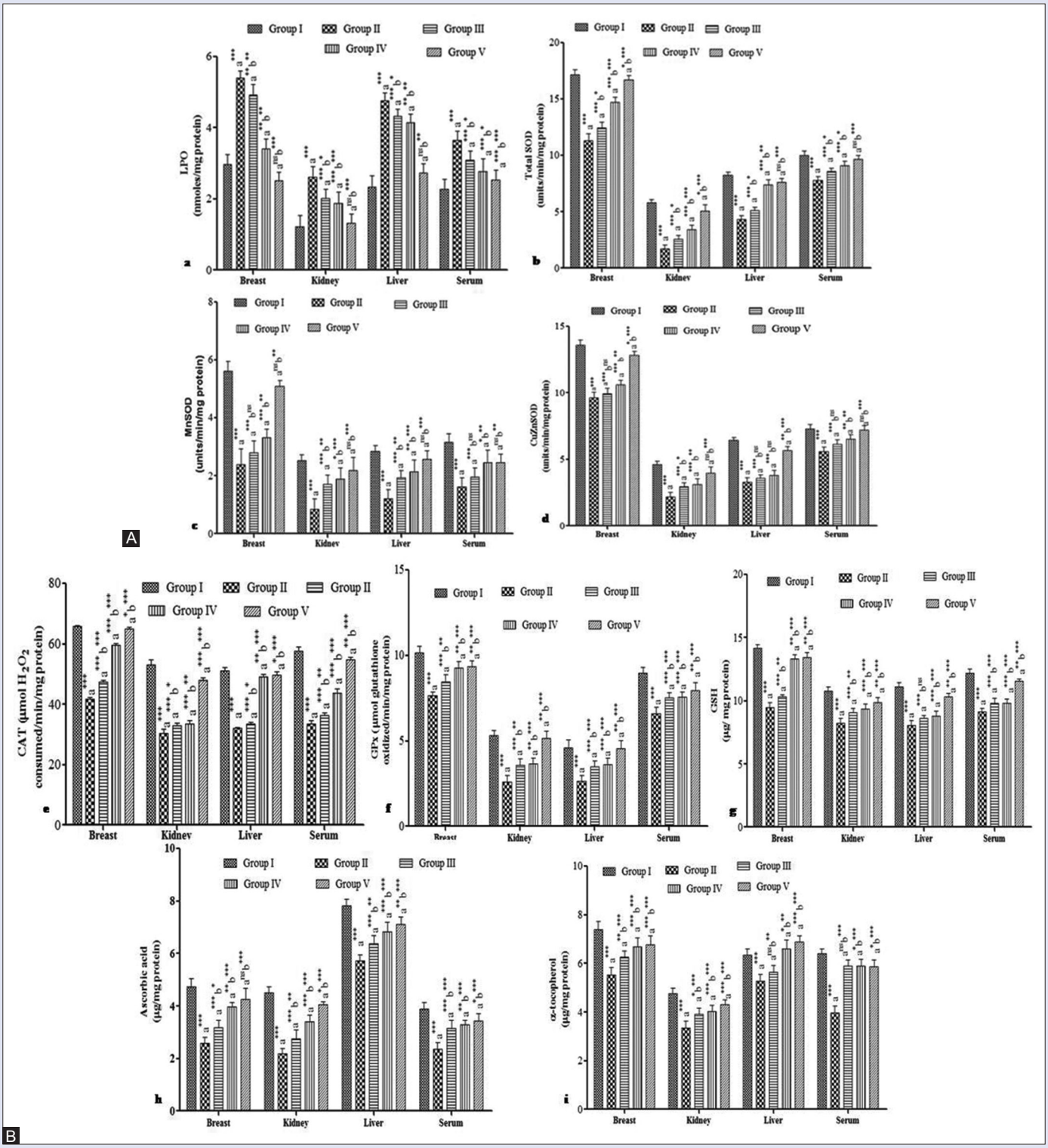

Figure 2: (A) Biochemical estimations of enzymatic and nonenzymatic antioxidants in all treatment groups. Where (a) estimation of LPO, (b) total SOD, (c) MnSOD, (d) CuZnSOD. Where each value is represented as mean ± SEM; *n* = 8 Group I: Normal control; Group II: Induced control group; Group III: TAX (10 mg/kg/day); Group IV: TAX (20 mg/kg/day) and Group V: TAX (40 mg/kg/day). Comparisons: a = Group II, III, IV, V with Group I; b = Group III, IV and V with Group II. Significant: $^{NS}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (B) Biochemical estimations of enzymatic and nonenzymatic antioxidants in all treatment groups. Where (e) CAT, (f) GPx, (g) GSH (h) ascorbic acid and (i) α-tocopherol, Where each value is represented as mean ± SEM; *n* = 8 Group I: Normal control; Group II: Induced control group; Group III: TAX (10 mg/kg/day); Group IV: TAX (20 mg/kg/day) and Group V: TAX (40 mg/kg/day). Comparisons: a = Group II, III, IV, V with Group I; b = Group III, IV and V with Group II. Significant: $^{NS}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. TAX: Taxifolin; LPO: Lipid peroxidation; SOD: Superoxide dismutase; MnSOD: Manganese-containing SOD; CuZnSOD: Copper and zinc containing SOD; SEM: Standard error of mean; CAT: Catalase; GPx: Glutathione peroxidase; GSH: Reduced glutathione

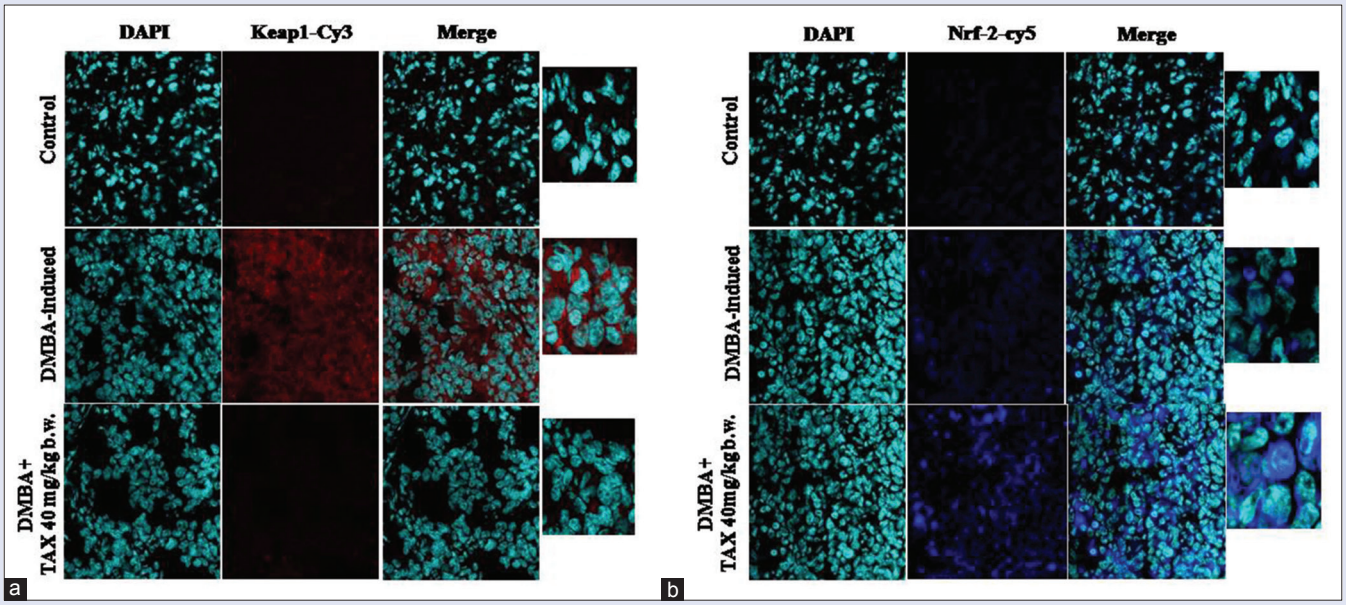

Figure 3: Immunofluorescence analysis of Keap-1 and Nrf-2 in mammary tissue of all experimental animals. *n* = 8. DAPI: florescent-blue (nucleus); Cy3: red; Cy5: dark blue. (a) Degradation of Keap-1 after TAX treatment to DMBA induced mammary cancer tissue. Keap-1 degradation is represented in a closer view (small extended slides). For the analysis immunofluorescence staining (×160) was used. (b) TAX treated experimental groups revealed Keap-1 dependent Nrf2 nuclear translocation (×170). The translocation of Nrf2 from cytosol to nucleus is represented in a closer view (small extended slides). DMBA: 7,12-dimethylbenz (a) anthracene; TAX: Taxifolin; Keap-1: Kelch-like ECH-associated protein 1; Nrf2: Nuclear factor erythroid 2

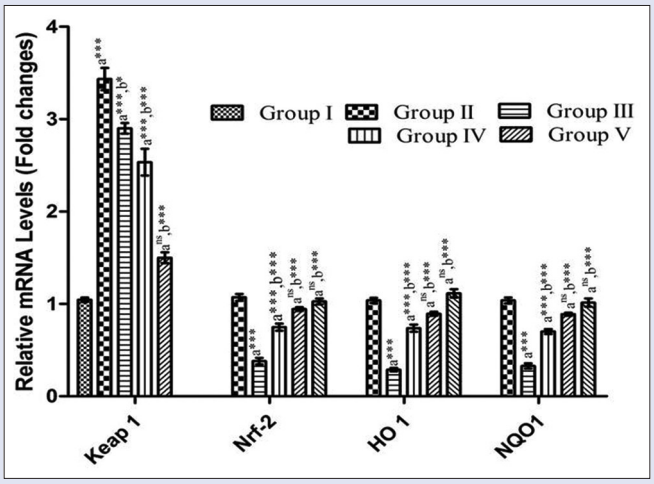

Figure 4: Relative mRNA expression of Keap-1, Nrf-2, HO-1, NQO1. Where each value is represented as mean ± SEM; *n* = 8. Group I: Normal control; Group II: Induced control group; Group III: TAX (10 mg/kg/day); Group IV: TAX (20 mg/kg/day) and Group V: TAX (40 mg/kg/day). Comparisons: a = Group II, III, IV, V with Group I; b = Group III, IV and V with Group II. Significant: $^{NS}P > 0.05$; $^{*}P < 0.05$; $^{***}P < 0.001$. TAX: Taxifolin; Keap-1: Kelch-like ECH-associated protein 1; Nrf2: Nuclear factor erythroid 2; SEM: Standard error of mean; HO-1: Heme oxygenase 1; NQO1: NADPH: quinoneoxidoreductase 1

be associated with free radical-mediated increased utilization of and in protecting "SH" containing proteins from lipid peroxides.[19] In our study also, MDA level was found to increase in DMBA treated animals which decreased on TAX treatment. On the other hand, the induced control groups revealed depression in various enzymatic and anti-enzymatic antioxidant levels which increased dose-dependently on TAX treatment which is also in accordance with other studies.

After cancer induction, various intermediate components increase ROS causing cancer progression. Nrf2 is a cytosolic, Keap-1 conjugated redox-sensitive transcription factors that evident to initiate ARE-mediated protective responses after oxidative stress initiated Keap-1-Nrf2 complex cleavage and further nuclear translocation of Nrf2. In carcinogenesis, upregulation of Keap-1 and simultaneous downregulation of Nrf-2 expression was evident that enhances the cellular susceptibility toward ROS[44] leading to further carcinogenesis progression.[45] DMBA-treated animals revealed elevated Keap-1 levels inhibiting Nrf2 nuclear translocation and mediated antioxidant protect.[27] The increased nuclear translocation of Nrf-2 after TAX treatment to mammary cancer-bearing animal further increased intracellular antioxidant levels which were reflected in the biochemical analysis of enzymatic and nonenzymatic antioxidants in all treatment groups.

The activation of Keap-1-Nrf2 pathway due to oxidative stress leads to the expression of cytoprotective enzymes, such as NQO1 and HO-1.[46] Similarly, TAX-treated animals revealed increased mRNA expression of Nrf2 which resulted in increased mRNA expression of antioxidant proteins HO-1, NQO1 as represented in Figure 5. This proposes antioxidant promoting activity of TAX.

CONCLUSION

TAX revealed a high binding affinity toward Keap-1, HO-1. Further TAX potentially down-regulate Keap-1 and subsequently up-regulated the expressions of Nrf-2 and associated HO-1 and NQO1 proteins in cancer-bearing animals. Thus, TAX efficiently increased the antioxidant competence in the cancer cells. These molecular changes are well reflected in the biochemical analysis of endogenous antioxidant profile that revealed a significant increase in protective enzymatic and nonenzymatic antioxidants after TAX treatment in cancer bearing animals. In brief, TAX can potentially inhibit mammary cancer growth in DMBA-induced human resembling mammary carcinoma by inducing antioxidant, apoptosis, and inhibiting cell survival and growth.

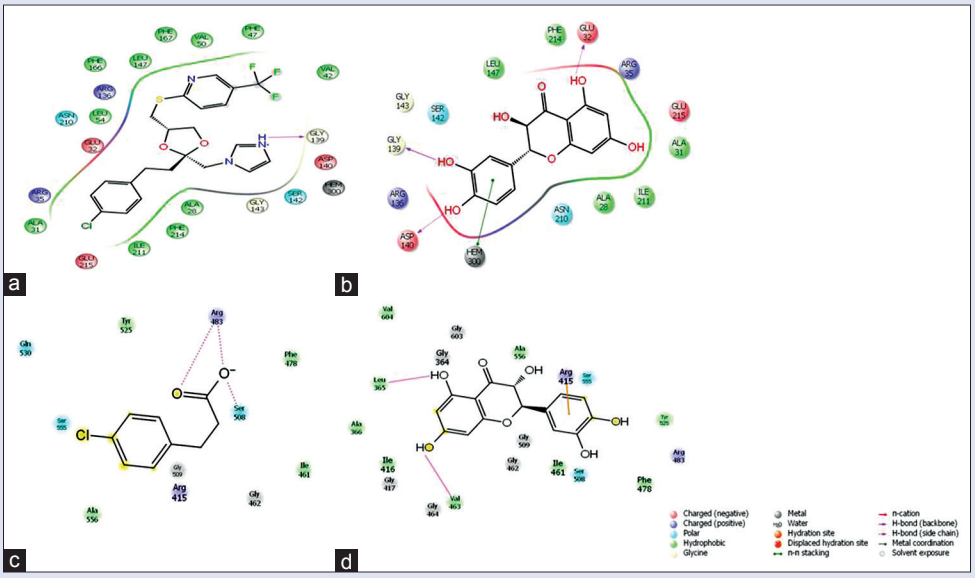

Figure 5: Molecular docking analysis of reference molecule and TAX, Where (a) 2D interactions of reference with HO-1 (b) 2D interactions of TAX with HO-1; (c) 2D interactions of reference molecule with Keap-1 and (d) 2D interactions of TAX with Keap-1 (pose view). TAX: Taxifolin; HO-1: Heme oxygenase 1; Keap-1: Kelch-like ECH-associated protein 1; 2D: Two-dimensional

Acknowledgements

The authors would like to thank the Birla Institute of Technology for providing the necessary facilities for this work.

Financial support and sponsorship

The authors are grateful to the University Grant Commission (Basic Scientific Research [BSR]), Government of India for giving financial support for this work (UGC Grant number: No. F.736/2007 [BSR]).

Conflicts of interest

There are no conflicts of interest.

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