

## ANTIOXIDANT AND CHEMOTHERAPEUTIC POTENTIAL OF *CURCUMA AMADA* RHIZOME EXTRACT ON BENZO(A)PYRENE INDUCED CERVICAL CARCINOMA IN SPRAGUE DAWLEY RATS

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### ABSTRACT

**Objective:** To evaluate the antioxidant and chemotherapeutic potential of *Curcuma amada* Rhizome extract on benzo(a)pyrene (BaP) induced cervical carcinoma in Sprague Dawley rats.

**Methods:** A total of 30 female Sprague Dawley rats were selected to establish cervical cancer model and then divided into 5 groups at random with six mice in each group. Group 1 control, Group 2 BaP (oral), Group 3 BaP for 8 weeks and post-treated with cisplatin (intravenous administration), Group 4 BaP for 8 weeks and post-treated with 250 mg of ethanol extract of *C. amada* (oral), Group 5 BaP for 8 weeks and post-treated with 500 mg of ethanol extract of *C. amada* (oral). 4 weeks after the treatment, the animals were sacrificed, serum separated, and cervical tissues were dissected. Antioxidants and the markers carcinoembryonic antigen (CEA), cancer antigens (CAs) 125, gamma glutamyltransferase (GTT) were assayed in serum and the tissue was used for analyzing tumor burden and sectioned for histopathological assays. 10% tissue homogenate was estimated for antioxidants and membrane-bound enzymes.

**Results:** BaP treated group showed significant ( $p < 0.001$ ) incidence of tumor burden, decreased activities of antioxidants, elevated lipid peroxidation,  $\text{Na}^+/\text{K}^+$  adenosine triphosphatase ( $\text{Na}^+\text{K}^+\text{ATPase}$ ), Calcium adenosine triphosphatase ( $\text{Ca}^{2+}\text{ATPase}$ ), Magnesium adenosine triphosphatase ( $\text{Mg}^{2+}\text{ATPase}$ ), CEA, CA 125, GTT. Treatment with *C. amada* rhizome extract and standard drug cisplatin reverted the antioxidants, serum markers and tissue enzymes.

**Conclusion:** From the results, it can be concluded that *C. amada* Rhizome extract ameliorated BaP induced oxidative stress in the cervical carcinogenicity of rats.

**Keywords:** *Curcuma amada*, *In vivo* antioxidant, Chemotherapy, Benzo(a)pyrene, Cervical carcinoma, Tumor markers.

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### INTRODUCTION

Cervical cancer is the rapid, uncontrolled growth of severely abnormal cells in the cervix. Although infection with human papillomavirus is the major known risk factor for the development of cervical cancer, other etiologic factors associated with this disease include cigarette smoking, deficiencies in micronutrients, lower socioeconomic status, and use of oral contraceptives. Epidemiological studies indicated that cigarette smoking is one of the cofactors that double the risk of cervical cancer [1] since it possesses polyaromatic hydrocarbons, genotoxic and carcinogenic constituents such as tar, nicotine, aromatic amines, nitrosamines, alkenes, and metals. The components of tobacco smoke exert their biological effects through interaction of reactive intermediates with DNA to form DNA adducts and further induce DNA damage [2].

Benzo(a)pyrene (BaP), a potent inducer of carcinogenesis and an important component of tobacco smoke is a representative compound of polycyclic aromatic hydrocarbons (PAHs) which shows cytotoxicity and/or genotoxicity in lung, stomach, and skin of the body. It is catalyzed by cytochromes P450 including CYP 1A1 and CYP 1B1 to form BaP-7, 8-diol which is activated to catechol, the auto-oxidation of which can generate reactive oxygen species (ROS) that cause oxidative DNA damage [3]. The major mechanisms for metabolic activation of BaP include formation of BaP-quinones (BPQs) that are important redox-cycle compounds, produced by a one-electron oxidation whose toxicity may be mediated to ROS such as superoxide anion,  $\text{H}_2\text{O}_2$  and hydroxyl radical ( $\bullet\text{OH}$ ) that chemically attack nucleotides, proteins, carbohydrates, and fatty acids.

These are neutralized by the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and xenobiotic reactivity decreased through the conjugation of reduced glutathione (GSH), catalyzed by GSH S-transferase. When the rate of elimination by antioxidative systems is slower than the rate of ROS production, lipid peroxidation (LPO) and DNA damage may occur leading to oxidative stress and subsequent canceration [4]. BaP has been detected in the cervical mucus of women [5] whose mechanism of oxidative damage on cervical tissue is rarely reported. Measurements of oxidative stress may provide further insight into cervix impairment induced by BaP *in vivo*.

An antioxidant is a molecule that delays the oxidation of proteins, carbohydrates, lipids, DNA and can be classified as the first line defense antioxidants which include SOD, CAT, GSH reductase, selenium, copper and zinc, the second line defense antioxidants which include GSH, vitamin C, albumin, vitamin E, carotenoids, flavonoids, and the third line defense antioxidants include a complex group of enzymes such as lipase, protease, DNA repair enzymes, transferases, methionine sulphoxide reductase for repair of damaged DNA, proteins, oxidized lipids, and peroxides. An effective antioxidant complex has various types of radical catching antioxidant sites that seek and destroy free radicals at many cellular sites thus helping in overall anti-oxidative capacity of cells and towards maintaining the health of biological tissues [6].

*Curcuma amada*, commonly known as Mango ginger, is a unique spice having morphological resemblance with ginger (*Zingiber officinale*) but imparts raw mango (*Mangifera indica*) flavor and is reported to contain

ocimene, dihydro-ocimene,  $\alpha$ -pinene,  $\alpha$ -curcumene,  $\beta$ -curcumene, linalool, cuminyl alcohol, keto-alcohol, camphor, turmerone, linalyl acetate, safrole, curcumin, myristic acid, car-3-ene, myrcene, 1,8-cineol, limonene and perillene. The rhizome of the plant has been used for centuries in traditional medicine and is known to have cancer preventive or therapeutic capabilities by suppressing multiple signaling pathways and inhibit cell proliferation, invasion, metastasis and angiogenesis [7]. Its safety combined with its low cost and multiple targeting potential makes *C. amada* an ideal agent to be explored for prevention and treatment of various cancers and fits very well as a candidate for chemoprevention by edible phytochemicals [8]. Hence, this study is focused to evaluate the antioxidant and chemotherapeutic potential of ethanol extract of *C. amada* rhizome by assessing the antioxidants, serum markers, membrane-bound enzymes, and histopathological analysis in Sprague Dawley rats.

## METHODS

### Chemicals

BaP was obtained from Sigma Chemical Co. (St. Louis, USA). Corn oil used as vehicle for BaP and all other chemicals utilized were obtained from reputed local firms (India) and were of highest purity grade.

### Collection of plant sample

Fresh rhizomes of *C. amada* were collected from local market of Coimbatore and were authenticated by G.V.S. Murthy, Scientist "F" and Head, Center of Botanical Survey of India - Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India where the herbarium voucher (BSI/SRC/5/23/2013-14/Tech/544) have been kept identifying the plant species.

### Extraction of plant material

Freshly collected rhizomes were cleaned to remove adhering dust and were cut into pieces, shade dried at room temperature and subjected to size reduction to a coarse powder using Laboratory Willey Mill (Secor Make). Ethanol extract of rhizome was prepared using Soxhlet apparatus, dried in rotary evaporator and used for the study.

### Selection of animals

Adult female Sprague Dawley rats (150-200 g) purchased from KMCH College of Pharmacy, Coimbatore was used for the study. All animals were housed in polypropylene cages containing paddy husk as bedding under standard conditions of humidity (35-60%), temperature ( $25 \pm 2^\circ\text{C}$ ), and 12 hrs light-dark cycle. They were allowed free access to standard pelleted diet throughout the experiment. The study was conducted in accordance with the guidelines of the Institutional Animal Ethics Committee at KMCH College of Pharmacy, Coimbatore, Tamil Nadu, India (Approval No: KMCRET/PhD/10/2013-2014).

### Experimental design

BaP was dissolved in corn oil shortly before oral gavage. The animals were randomly divided into 5 groups (containing 6 animals each) namely control group, BaP group, BaP+cisplatin group, BaP+*C. amada* (low dose) group, and BaP+*C. amada* (high dose) group. All rats except control group were given BaP for induction of cervical cancer as described by Garry *et al.* [9], with minor modifications.

Briefly, each rat was orally administered with 10 mg BaP/kg in 0.2 ml corn oil, twice a week for 8 weeks (a total of 16 BaP instillations per rat). The animals of BaP+cisplatin group were post-treated with cisplatin (10 mg/kg b.wt.) for 4 weeks (daily) by intravenous administration. Rats of BaP+*C. amada* (low dose) group and BaP+*C. amada* (high dose) group were post-treated with 250 mg/kg b.wt. of ethanol extract of *C. amada* (orally) for 4 weeks (daily) and 500 mg/kg b.wt. of ethanol extract of *C. amada* (orally) for 4 weeks (daily), respectively.

### Collection of blood and tissue samples

At the end of treatment period, the animals were anesthetized, blood collected from retro-orbital sinus, serum separated and used for antioxidant and marker assays. Cervical tissues were dissected out immediately and transferred to ice-cold saline for the tissue analysis.

### Preparation of homogenate

About 10% tissue homogenate was prepared using 0.1 M Tris-buffer (pH 7.4) and used for estimation of antioxidants and membrane-bound enzymes.

### Determination of tumor burden

All the animals except in control group were treated with BaP for 8 weeks (twice a week). At the end of the experimental regime, the rats were anesthetized using chloroform; cervical tissues were dissected out, split longitudinally and carefully observed under dissecting microscope for the presence of tumors. The number of nodules were counted and expressed as number of polyps.

### Estimation of SOD

SOD activity was determined by Das *et al.* [10]. Pipetted 1.4 ml aliquot of the reaction mixture in a test tube containing 1.1 ml phosphate buffer, 75  $\mu\text{l}$  methionine, 40  $\mu\text{l}$  triton X-100, 75  $\mu\text{l}$  hydroxylamine hydrochloride, and 100  $\mu\text{l}$  ethylenediamine tetraacetic acid (EDTA). 100  $\mu\text{l}$  of the sample was added followed by preincubation at  $37^\circ\text{C}$  for 5 minutes. 80  $\mu\text{l}$  of riboflavin was added, and the tubes were exposed for 10 minutes to 200 W Philips fluorescent lamps. The control tube contained an equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time, 1.0 ml of Greiss reagent was added to each tube, and the absorbance of the color formed was measured at 543 nm. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition.

### Estimation of CAT

The activity of CAT was measured by Sinha [11]. To 0.9 ml of phosphate buffer, 0.1 ml of serum/tissue homogenate, 0.4 ml of hydrogen peroxide were added. At time 0 s and at 60 s after the addition of the serum/homogenate, 2.0 ml of dichromate-acetic acid mixture was added. The tubes were kept in boiling water bath for 10 minutes and the color developed was read at 620 nm. Standards in the range of 1.2-6.0  $\mu\text{mol}$  were taken and processed as test and blank containing reagent alone. The activity of CAT was expressed as  $\mu\text{mole}$  of  $\text{H}_2\text{O}_2$  decomposed/minutes/mg protein.

### Estimation of GPx

GPx was measured by the method of Ellman [12]. To 0.4 ml of buffer, 0.2 ml of EDTA, 0.1 ml of sodium azide and 0.2 ml of reduced GSH, 0.1 ml of  $\text{H}_2\text{O}_2$  were added to two test tubes labeled as test (T) and control (C). To the test added 0.2 ml of sample and to the control added 0.2 ml of water. The contents were mixed well and incubated at  $37^\circ\text{C}$  for 10 minutes. The reaction was arrested with the addition of 0.5 ml of 10% trichloroacetic acid (TCA). To determine the GSH content, 1.0 ml of supernatant was removed by centrifugation. To that 3.0 ml of buffer and 0.5 ml of Ellman's reagent, the color developed was read at 412 nm. The activity was expressed in term of  $\mu\text{g}$  of GSH consumed/minutes/mg protein.

### Estimation of total reduced GSH

GSH activity was measured by Moron *et al.* [13] method. A known weight of tissue was homogenized in phosphate buffer. From this 0.5 ml was pipetted out and precipitated with 2.0 ml of 5% TCA. 1.0 ml of supernatant/serum was taken after centrifugation and added 0.5 ml of Ellman's reagent and 3.0 ml of phosphate buffer. The yellow color developed was read at 412 nm. Pipetted out 0.2-1.0 ml standard solution corresponding to a  $\mu\text{g}$  of 40-200. The volume, in all the tubes, was made up to 1.0 ml with distilled water. Then, added 0.5 ml of Ellman's reagent and 3.0 ml of phosphate buffer to all the tubes. The absorbance was read at 412 nm within 2 minutes against the reagent blank. The amount of GSH was expressed as  $\mu\text{g/g}$  tissue.

### Estimation of ascorbic acid

Ascorbic acid was measured by Omay *et al.* [14] method. 1.0 ml of 10% homogenate was precipitated with 5% ice-cold TCA and centrifuged for 20 minutes at 3500 g. 1.0 ml of the supernatant/serum was mixed with

0.2 ml of dinitrophenylhydrazine-thiourea-copper sulfate reagent and incubated for 3 hrs at 37°C. Then 1.5 ml of ice-cold 65% sulfuric acid was added. Mixed well and the solutions were allowed to stand at room temperature for an additional 30 minutes. Absorbance was determined at 520 nm. The results were expressed as  $\mu\text{g}/\text{mg}$  protein.

#### Estimation of lipid peroxidation

Lipid peroxidation was determined by the method of Bishayee and Balasubramaniam [15]. Pipetted out 1.0 ml of tissue homogenate/serum into a test tube labeled test and 1.0 ml of water in test tube labeled "blank." Added 2.0 ml of the thiobarbituric acid-TCA-HCl reagent mixture to all test tubes. Mixed well and placed in boiling water bath for 5 minutes, cooled. Standards in the range of 20-100  $\mu\text{g}$  were treated similarly. The absorbance of clear supernatant was measured against reference blank at 535 nm.

#### Na<sup>+</sup>K<sup>+</sup> adenosine triphosphatase (ATPase)

Bonting [16] method of measuring the activity of Na<sup>+</sup>K<sup>+</sup> ATPase is based on the transport of Na<sup>+</sup>K<sup>+</sup> against concentration gradient at the cost of adenosine triphosphate ATP molecule liberating inorganic phosphate (Pi) that is estimated. To 1.0 ml of Tris buffer, 0.2 ml of each of 184 mM Tris-HCl buffer (pH 7.5), 50 mM MgSO<sub>4</sub>, 50 mM KCl, 600 mM NaCl, 1 mM EDTA and 40 mM ATP were mixed together. The final assay mixture is a final volume of 2.0 ml, containing 92 mM Tris-buffer, 5 mM MgSO<sub>4</sub>, 60 mM NaCl, 1 mM EDTA and 4 mM ATP. After 10 minutes of equilibration at 37°C in an incubator, reaction was started by the addition of 0.1 ml of tissue homogenate. The tubes were incubated for 15 minutes, and the reaction was arrested by the addition of 1.0 ml of 10% TCA. The phosphorus content in the supernatant was estimated by Fiske and Subbarow method. The enzyme activity is expressed as micromoles of Pi liberated/minutes/mg protein.

#### Ca<sup>+</sup> ATPase

Ca<sup>+</sup> ATPase activity was estimated as described by Hjertén and Pan method [17] that measures the amount of inorganic phosphorus liberated. 1 ml of each of 375 mM Tris-HCl buffer (pH 7.6), 25 mM CaCl<sub>2</sub>, 10 mM ATP were mixed well, 0.1 ml of homogenate and 0.1 ml of water were added, and was incubated for 15 minutes at 37°C. The reactions were arrested by the addition of 1.0 ml of 10% TCA. The control tubes received enzyme after the addition of 10% TCA. The phosphorous content in the supernatant was measured by the method of Fiske and Subbarow. The enzyme activity is expressed as micromoles of Pi liberated/minute/mg protein.

#### Mg<sup>+</sup> ATPase

The activity was estimated by the method of Ohnishi *et al.* [18] that involves the estimation of liberated inorganic phosphorus. The enzyme activity was estimated by the addition of 0.1 ml of homogenate to an incubation medium containing 0.1 ml of water and 0.1 ml of each of 375 mM Tris-HCl buffer (pH 7.6), 25 mM MgCl<sub>2</sub> and 10 mM ATP. The final concentration of tris buffer, MgCl<sub>2</sub> and ATP were 75 mM, 5 mM and 2 mM, respectively, in the total incubation volume of 0.5 ml. The reaction was terminated after 15 minutes by the addition of 1.0 ml of 10% TCA. The liberated Pi was estimated by the method of Fiske and Subbarow and enzyme activity is expressed as micromoles of Pi liberated/minutes/mg protein.

#### Serum cancer markers

Specific cancer markers, carcinoembryonic antigen (CEA), gamma-glutamyltransferase (GGT), and cancer antigen (CA) 125 were done using an automate analyzer Dx C 860i (Beckman Coulter).

#### Histopathological examination

Formalin-fixed tissues were processed for Ehrlich's hematoxylin staining using conventional Laboratory procedure. Briefly, the tissues were dehydrated through ascending grades of Isopropyl alcohol, cleared in xylene, and embedded in low melting point paraffin blocks. Sections of 3 micron thickness were cut, placed serially on clean glass slides and then deparaffinized by immersing in xylene. Three

contiguous sections were made from each cervical tissue and stained with Ehrlich's hematoxylin for evaluation under Labomed binocular light microscope with ultra scope 9.1. Cervical cell injury and other aspects were observed under high power dry objective ( $\times 40$ ).

#### Statistical analysis

The results were expressed as mean  $\pm$  standard error of the mean and analyzed by using one-way ANOVA followed by Dunnett's multiple comparison tests. Data was computed for statistical analysis by using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California, USA, and compared with the vehicle control group. p values ( $p < 0.05$ ) were considered statistically significant.

## RESULTS

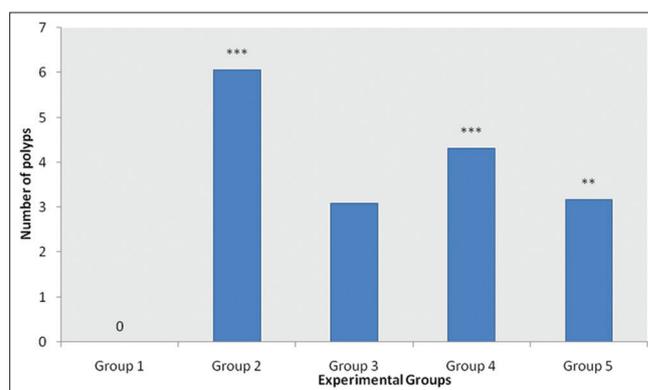
#### Tumor burden

Tumor burden was significantly ( $p < 0.001$ ) increased in BaP-induced rats that are indicated as number of polyps when compared to control animals that lack the polyps. Cisplatin treatment decreased the number of polyps compared to induced group. Administration of 250 mg/kg b.wt. of *C. amada* rhizome decreased the number of polyps at  $p < 0.001$  level while administration of 500 mg/kg b.wt. of rhizome decreased the number of polyps at  $p < 0.01$  compared to control group (Fig. 1).

#### Antioxidants in serum and tissue

Overproduction of ROS can cause severe impairment of cellular functions and necrotic lesions in the cervical tissue of animals. Antioxidants may protect against this toxicity by the preventing their formation [19]. Fig. 2 represents the significant decrease ( $p < 0.001$ ) of serum antioxidants in BaP induced rats. Treatment with standard drug cisplatin increased the antioxidants activities compared to induced group. Administration of rhizome extract (250 mg/kg b.wt.) reverted SOD, CAT activities at  $p < 0.001$  level and GPx at  $p < 0.01$  level compared to control. 500 mg/kg b.wt. of rhizome extract reversed SOD and CAT at  $p < 0.001$  level and non-significantly reverted GPx, GSH, and vitamin C level compared to control group.

Activities of cervical tissue antioxidants were found to be decreased significantly at  $p < 0.001$  level in BaP induced rats (Fig. 3). Treatment with cisplatin increased the activities of antioxidants compared to induced group. 250 mg/kg b.wt. of rhizome extract significantly ( $p < 0.001$ ) reverted SOD and vitamin C activities and non-significantly increased the activities of CAT, GPx, and GSH compared to control. Administration with 500 mg/kg b.wt. of rhizome extract reversed the activities of SOD activity at  $p < 0.01$ , CAT, GSH at  $p < 0.001$  level and non-significantly reverted GPx, and vitamin C compared to control group.



**Fig. 1: Effect of ethanol extract of *Curcuma amada* rhizome on Tumor burden of Experimental animals. Values are Mean  $\pm$  SEM (n=6). \*\*\* $p < 0.001$ : Extremely Significant, \*\* $p < 0.01$ : Highly Significant**

**Lipid peroxidation**

BaP significantly ( $p < 0.001$ ) increased LPO in serum and tissue of rats while cisplatin treatment non-significantly decreased LPO compared to control group. Treatment with *C. amada* rhizome nonsignificantly reduced LPO compared to normal (Fig. 4).

**Membrane-bound enzymes**

The disturbance in ionic homeostasis in cervical cancer patients may result from elevated LPO resulting in increased membrane permeability [20]. The elevation of membrane-bound enzymes activities (Fig. 5) in tissues of BaP-induced rats indicate the deteriorating effect of BaP on the animals. Cisplatin treatment significantly reduced  $\text{Na}^+\text{K}^+$  ATPase and  $\text{Ca}^{2+}$  ATPase activity at  $p < 0.01$  and  $\text{Mg}^{2+}$  ATPase activity at  $p < 0.001$  level compared to control. Treatment with 250 mg/kg b.wt. of *C. amada* rhizome extract significantly reverted  $\text{Na}^+\text{K}^+$  ATPase activity at  $p < 0.05$  and  $\text{Mg}^{2+}$  ATPase activity at  $p < 0.001$  level 0.500 mg/kg b.wt. of *C. amada* rhizome extract non-significantly reverted the enzyme activities to near normal.

**Serum markers**

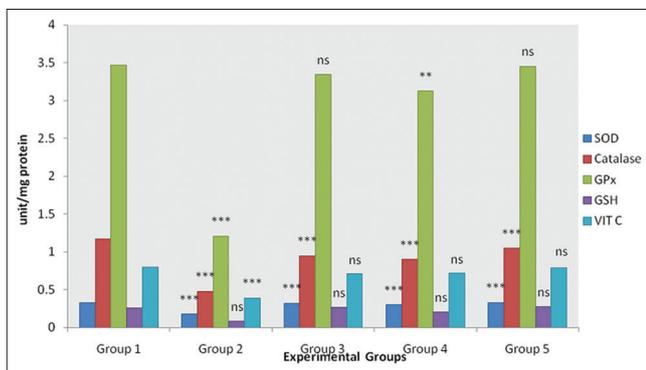
Enzymes that are present in much higher concentration inside the cell are released into the systemic circulation as the result of tumor necrosis or a change in the membrane permeability of the cells that signal the presence of malignancy. Fig. 6 shows the significant increase ( $p < 0.001$ ) of tumor cancer markers in BaP-induced group. Treatment with cisplatin reduced the level of markers compared to control 0.250 mg/kg b.wt. of rhizome extract significantly reduced the markers at 0.01% while 500 mg/kg b.wt. of rhizome extract non-significantly reduced the serum markers compared to control rats.

**Histopathological evaluation**

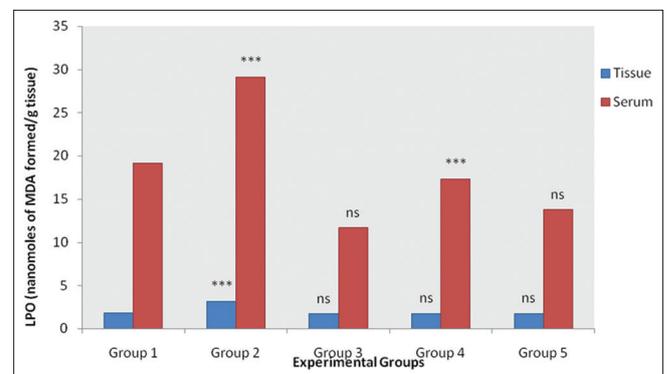
The effect of BaP induced cervical carcinogenesis was also apparent by histological examination. Fig. 1 shows Ehrlich's hematoxylin stained cervical epithelial tissue. In control group of rats, cervical epithelium had normal architecture (Fig. 7a). In BaP induced rats the cervical tissue showed confluent necrosis leading to cervical dysplasia and Stage-I cervical intraepithelial neoplasia (CIN) with individual cells round to polygonal with moderate eosinophilic cytoplasm and round to vesicular nuclei with some showing nucleoli (Fig. 7b). Administration of cisplatin reduced necrosis in cervical tissue of experimental animals (Fig. 7c). Rats treated with 250 mg/kg b.wt. of *C. amada* showed minimal changes in architecture imparting the treatment effect on features of chronic cervicitis (Fig. 7d). Treatment with 500 mg/kg b.wt. of *C. amada* reverted the normal architecture of cervical tissue that showed the anticancer potential of phytochemicals present in the extract (Fig. 7e).

**DISCUSSION**

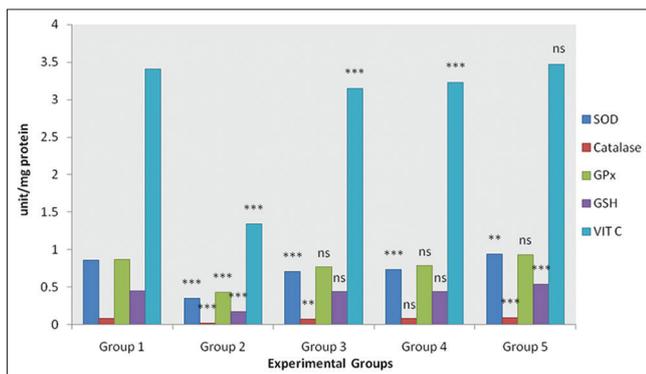
Cervix carcinoma is one of the leading causes of mortality and important public health problem worldwide, particularly in the developing countries and accounts for 80-85% of all the female genital tract malignancies [21]. Epidemiological studies have implicated that cigarette smoking is an independent risk factor for the development of cervical cancer. since it contains a complex mixture of nicotine, cotinine, BaP and 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone, the potent inducers of carcinogenesis [22]. The present study has been carried out to investigate the antioxidant and chemotherapeutic potential of *C. amada* rhizome extraction BaP induced cervical carcinoma in female SD rats.



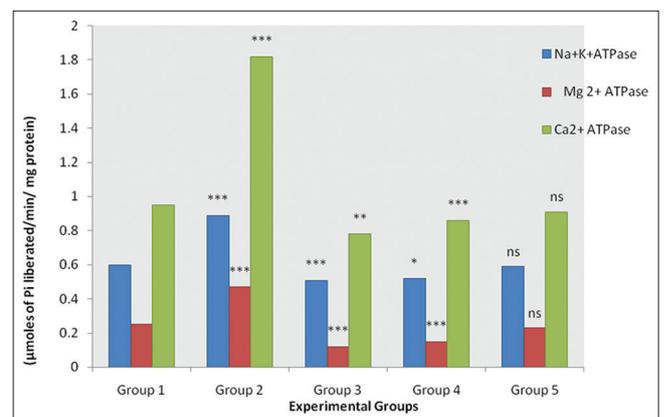
**Fig. 2: Effect of ethanol extract of *Curcuma amada* rhizome on Antioxidants in serum of Experimental animals. Values are Mean  $\pm$  SEM (n=6). \*\*\* $p < 0.001$ : Extremely Significant, \*\* $p < 0.01$ : Highly Significant. ns: non-significant**



**Fig. 4: Effect of ethanol extract of *Curcuma amada* rhizome on Lipid Peroxidation of Experimental animals. Values are Mean  $\pm$  SEM (n=6). \*\*\* $p < 0.001$ : Extremely Significant, ns: non-significant**



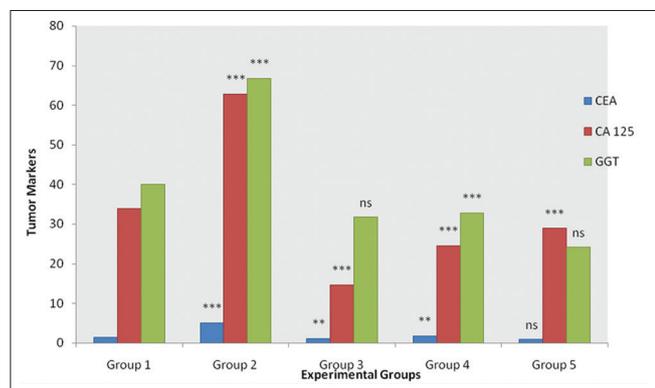
**Fig. 3: Effect of ethanol extract of *Curcuma amada* rhizome on Antioxidants in Cervical Tissue of Experimental animals. Values are Mean  $\pm$  SEM (n=6). \*\*\* $p < 0.001$ : Extremely Significant, \*\* $p < 0.01$ : Highly Significant. ns: non-significant**



**Fig. 5: Effect of ethanol extract of *Curcuma amada* rhizome on Membrane bound enzymes of Experimental animals. Values are Mean  $\pm$  SEM (n=6). \*\*\* $p < 0.001$ : Extremely Significant, \*\* $p < 0.01$ : Highly Significant, ns: non-significant**

BaP, an extremely potent pro-carcinogen, is metabolized by cytochrome P-450 based mono-oxygenase system, the major phase I biotransformation enzyme to a variety of carcinogenic metabolites that are responsible for initiating tumor genesis. BaP-metabolites may directly form ROS through redox cycling or disrupt mitochondrial electron transport leading to formation of more ROS including superoxide anions, hydroxyl radicals and hydrogen peroxide that play a vital role in initiating and progressing oxidative stress that cause peroxidation damage to lipids resulting in damage of organelle and cellular membrane. An increased rate of ROS production occurs in highly proliferative cancer cells, owing to the presence of oncogenic mutations that promote aberrant metabolism. Increased oxidative stress is well documented in transformed cells [23] and growing evidence suggests that ROS act as second messengers in intracellular signaling cascades which induce and maintain the oncogenic phenotype of cancer cells [24].

Since BaP is an environmental pollutant and is believed to be a risk factor for human chemical carcinogenesis, it is important to identify the naturally occurring agents that could modulate BaP-induced tumorigenesis. Most of the synthetic drugs available today are cytotoxic, immunosuppressant and may cause a variety of side effects in the normal organs of the body [25]. Since natural sources play a vital role in cancer prevention and treatment, the antitumor agents currently used in the medicine are of natural origin [26].



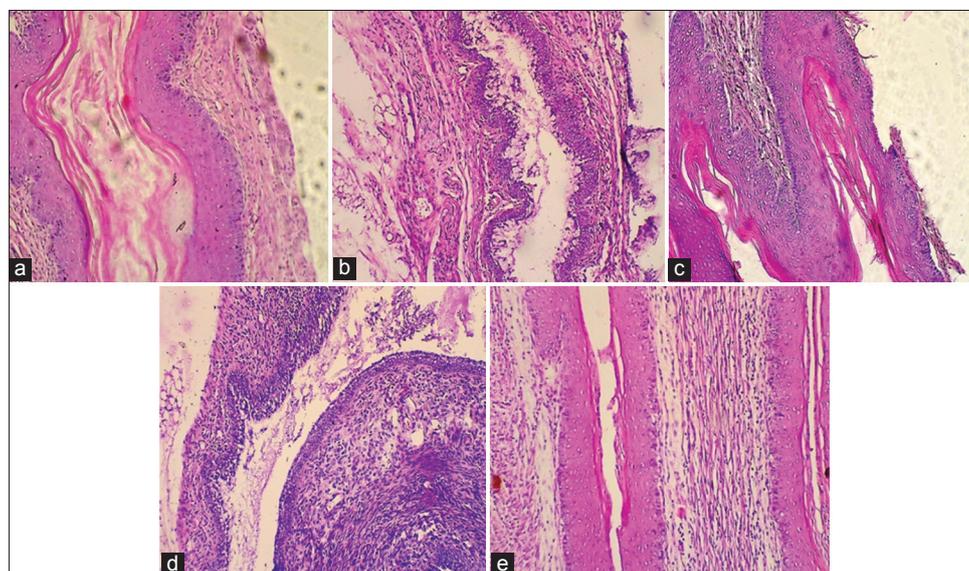
**Fig. 6: Effect of ethanol extract of *Curcuma amada* rhizome on Tumor Markers of Experimental animals. Values are Mean  $\pm$  SEM (n=6). \*\*\*p<0.001: Extremely Significant, \*\*p<0.01: Highly Significant, ns: non-significant**

Recent progress in molecular biology and pharmacology enhances natural phytochemicals derived from medicinal plants that have gained significant recognition in the potential management of several human clinical conditions, including cancer. Secondary metabolites like polyphenols, terpenoids like limonene [27] and flavonoids have been reported to possess antioxidant, antimutagenic and anticancer properties in many studies because of their unique pharmacophores and medicinal properties. They may act as antioxidants, nutrient protectors and also responsible for the prevention and treatment of radical-mediated disorders. The preventive mechanisms of tumor promotion by these phytochemicals range from inhibition of genotoxic effects, increased antioxidants and anti-inflammatory activity, inhibition of proteases and cell proliferation, protection of intracellular communications to modulate apoptosis and signal transduction pathways [28].

*C. amada*, popularly known as mango ginger, is a perennial, rhizomatous spice, belonging to Zingiberaceae family. It is found wild as well as in cultivation in various parts of world, used in food industry as basic ingredient in pickles, preserves, candies, sauces, curries, salads and also medically as coolant, astringent and to promote digestion. Its rhizome has been traditionally used for healing of wounds, cuts, itching and used against scabies, lumbago, stomatitis, inflammation in the mouth and ear, ulcers on the male sex organs [29]. It possesses antifungal, anti-inflammatory, analgesic, anticancer, antihyperglyceridemia [30], and carminative properties [31]. The rhizome extracts were observed to be potent antimutagen based on its antioxidative activity [32].

Comparison of tumor burden between control and BaP treated animals revealed that BaP induction caused significant tumor burden leading to tumor genesis. Treatment with *C. amada* rhizome extract significantly reduced tumor burden that indicates its anticancer efficacy. This is in accordance with the statement that the reliable criterion for judging the potency of an anticancer drug is the prolongation of the life span of tumor-bearing animals [33].

Antioxidant status has been suggested as a useful tool in estimating the risk of oxidative damage induced carcinogenesis. The antioxidative defense systems that include antioxidant enzymes like SOD, CAT, GPx simultaneously neutralize chemical reactive intermediates produced by endogenous pathways and/or xenobiotic metabolism [34]. SOD detoxifies superoxide radicals to hydrogen peroxide, CAT decomposes hydrogen peroxide to water and oxygen and GPx converts peroxides and hydroxyl radicals into non-toxic forms [35]. GSH constitutes the first line of defense against free radicals and plays an important role in



**Fig. 7: (a-e) Histological changes of benzo(a)pyrene induced cervical cancer in experimental animals**

the detoxification of electrophilic substances and prevention of cellular oxidative stress [36]. Several studies have shown that many radical scavengers, including naturally occurring compounds such as vitamin C, have been found to possess a strong antioxidant activity related to a strong scavenging capacity [37].

Studies show that serum of patients with CIN and carcinoma of the cervix evidenced changes in impairment of either enzymatic or non-enzymatic antioxidant systems [38]. In the present study, we observed significantly lowered activities of serum and tissue SOD, CAT, GPx, GSH and vitamin C in BaP induced rats when compared with those of the control group that was caused by the neoplastic cells that sequestered essential antioxidants from circulation to supply the demands of growing tumor [39]. Reduced tissue antioxidants were due their leakage from the cytoplasm caused by peroxidative damage of lipids that confirms the severe oxidative stress status of the cervical tissue. Ramakrishnan *et al.* [40] cited decreased SOD activities, Vinodhkumar *et al.*, [41] showed decreased CAT in various carcinogenic conditions that may be due to the increased LPO. Decline in GPx activity may be attributed to the reduction in the levels of reduced GSH and increase in the level of peroxides during carcinogenesis [42].

Treatment with standard drug cisplatin increased activities of antioxidants in serum and tissue of experimental animals. Administration of low and high dose of *C. amada* rhizome extract significantly reverted serum and tissue SOD and CAT activities and non-significantly reverted the activities of GPx, GSH and vitamin C that may be caused by the presence of phytochemicals like phenolic compounds [43] in the extract that act as natural antioxidant due to their redox properties and plays an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [44], flavonoids like gallic acid [45] that inhibit cancer cell growth and plays an important role in the prevention of malignant transformation and cancer development by acting as a free radical scavenger and an inducer of differentiation and apoptosis in leukemia, lung cancer, colon adenocarcinoma cell lines and in normal lymphocyte cells [46], terpenoids like difurocumenonol, amadannulen and amadaldehyde that scavenge the damaging reactive oxygen species such as hydroxyl radicals, peroxyradicals, superoxide anion that are known to be involved in inflammation and carcinogenesis [47] by their radical scavenging activities [48] and curcuminoids like curcumin has been shown to suppress transformation, proliferation, and metastasis of tumors [49]. This is in accordance with [50] who stated that regular consumption of fruits and vegetables is strongly associated with reduced risk of developing chronic diseases such as cancer as the phytochemical extracts from it exhibit strong antioxidant activity. The result is also supported by many studies that were conducted (*in vitro*) in various extracts of the rhizome like aqueous [51], methanol [52], chloroform [53], petroleum ether, ethyl acetate and ethanol [54] that exhibited antioxidant activity. Recently an inverse association between antioxidant nutrients and cervical cancer was shown suggesting protective effect of natural extract against cervical dysplasia [55].

Numerous reports have documented that LPO is an important parameter in evaluating the level of oxidative stress in organisms [56]. BaP is a very effective carcinogen with a capability to induce enormous amounts of free radicals, which in turn reacts with lipids causing LPO, the products of which include malondialdehyde that has been reported to be involved in the formation of tumors [57]. In this study, BaP induction caused generation of excessive free radicals leading to significant increase of LPO level in serum and tissue of rats. Pan *et al.* [58] had reported an increase of LPO after exposure to BaP and the concentration changes of BPQs metabolites in the body. Increase of tissue LPO in BaP treated groups indicated that the cervix suffered from severe and continuous LPO caused by the free radicals that participate in BaP epoxidation [59]. Treatment with *C. amada* rhizome significantly reduced LPO in rats when compared to cisplatin treatment that would be caused by the presence of terpenoids in the extract that exerted LPO inhibitory activity and curcumin that suppressed LPO induced tissue

inflammation [60]. The present result correlates with report stating that non-polar extracts showed good LPO inhibitory activity [61].

Assay of membrane-associated enzyme activities indicate the alterations of the membrane under diseased status. ATPases are intimately associated with plasma membrane and participates in energy requiring translocation of sodium, potassium, calcium, and magnesium. Higher level of LPO process ultimately impairs the membrane function by decreasing its fluidity and altering the activities of membrane-bound enzymes and receptors [62]. Na<sup>+</sup>K<sup>+</sup> ATPase is a ubiquitously expressed integral membrane protein that carries out the extrusion and uptake of Na<sup>+</sup> and K<sup>+</sup> ions across the plasma membranes of cells and is critical in maintaining physiological electrochemical gradient that is essential for cell survival and activities. Alterations in Na<sup>+</sup>/K<sup>+</sup> - ATPase alters the Na<sup>+</sup>/Ca<sup>2+</sup> exchange mechanism which may play a vital role in regulating the cellular calcium levels. Ca<sup>2+</sup> ATPase, the best characterized pumps that have been linked to cancer are important regulators of intracellular calcium concentration that enable muscle contraction, relaxation and control calcium signaling pathways [63]. Mg<sup>2+</sup> ATPase is believed to be responsible for control of membrane permeability and maintain intracellular Mg<sup>2+</sup>, changes of which can control protein synthesis and cell growth [64]. This study shows significant elevation of these enzyme activities in tissues that may be due to peroxidation of membrane lipids that alters the lipid milieu, leading to alterations in membrane fluidity and cell deformability [65] that indicate the persistent deteriorating effect of BaP in cancer-bearing animals. Kolanjiappan *et al.* [66] had reported elevated Na<sup>+</sup>K<sup>+</sup> ATPase activity in erythrocytes of cervical cancer patients and [67] reported elevated activity of Ca<sup>2+</sup> ATPase and Mg<sup>2+</sup> ATPase in BaP induced toxicity of experimental mice. Treatment of experimental rats with cisplatin significantly reduced the activities of these enzymes. Post-treatment of low dose rhizome extract significantly decreased the enzyme activities by protecting the "SH" groups of enzymes from the oxidative damage through the inhibition of peroxidation of membrane lipids [68]. Minimizing the alterations in the activities of these enzymes indirectly indicates the membrane stabilizing action of *C. amada* rhizome extract in BaP induced cervical carcinogenesis of experimental rats.

Analysis of tumor marker enzymes serves as an indicator of cancer response to environment. Distribution of many biochemical, immunological and molecular properties of the host have been observed in BaP mediated cancer conditions [69]. With this concept, the cancer specific markers CEA, CA 125 and GGT were evaluated in the present study. CEA is a glycoprotein, the elevated levels of which are found in smokers [70] and cervical cancer patients [71]. The enhanced marker level in BaP induced animals indicates the correlation that exists between CEA and tumor burden. This elevation was significantly inhibited by low dose of rhizome extract that may be caused by curcumin that inhibited tumor initiation by decreasing cytochrome P450 and aryl hydrocarbon hydroxylase activity with a concomitant decrease in BaP-DNA adduct in cells treated with BaP [72], as that of standard drug cisplatin.

CA 125, a membrane glycoprotein is the potential tumor marker of cervical cancer. Raised CA 125, associated with the stage of cervical disease is of some prognostic significance and has been detected in patients with newly diagnosed carcinoma of the cervix, squamous cell cervical cancer, cervical adenocarcinoma and are associated with advanced tumor stage, large tumor size, high histological grade, lymph-vascular space involvement, deep cervical stromal invasion and lymph node involvement [73]. The elevation of this marker correlates with the intraepithelial neoplastic lesions that were observed in histology of rats treated with BaP. A significant decrease of CA 125 was observed in rats treated with low dose of rhizome extract when compared to that of cisplatin treatment which may be due to the presence of terpenoids that act at various stages of tumor development, inhibit initiation and promotion of carcinogenesis, induce tumor cell differentiation and apoptosis and suppress tumor angiogenesis, invasion and metastasis through regulation of various transcription and growth factors as well as intracellular signaling mechanisms [74].

GGT, the marker of pathologic oxidative stress in cancer patients is a key enzyme that catalyzes the transfer of the glutamyl moiety linked through glutamate gamma carboxylic acid to cysteine favoring the recovery of constituent amino acids for subsequent intracellular GSH resynthesis and has been traditionally regarded as a component of the cell protection system against oxidative stress whose dysregulated expression has been detected in several tumor types [75]. In the present study, BaP induction elevated the GGT level in Group II rats that reflected the malignant cell transformation and tumor burden in carcinoma of the cervix [76]. Treatment with low dose of rhizome extract significantly reverted the marker level to near normal level when compared to control group that would be due to the presence of curcuminoids in the extract that induced repair of DNA oxidative damage and chromosomal aberrations [77] caused by BaP metabolism.

In the histological examination, a series of pre-cancerous lesions were observed in cervixes of BaP-treated group, showing that BaP can cause different injuries to the cervical tissue epithelium [78]. As the crude therapeutic preparations are more effective and less toxic than the isolated active components or their respective synthetic counterparts because they contain the total family of medicinal compounds just as they are found in their natural source and hence offer less risk of side effects the treatment with rhizome extract rectified the cervical lesions induced by BaP in the experimental rats.

## CONCLUSION

BaP, a prototypical PAHs is a widespread environmental pollutant and thought to be one of the etiological factors in human chemical carcinogenesis. Since experimental carcinogenesis and modulatory action on antioxidant enzymes help to evaluate the chemotherapeutic potential of natural products, in this study, we screened the potential of ethanol extract of *C. amada* rhizome on BaP-induced cervical carcinogenesis in female Sprague Dawley rats. The extract significantly ( $p < 0.001$ ) increased serum and cervical tissue antioxidant activity, inhibited LPO in serum and tissue, decreased membrane-bound enzymes and tumor markers that may be caused by the presence of active components in the crude extract that might have facilitated the detoxification and excretion of oxidized metabolites of BaP by acting as synergists for the therapeutic effects. The findings of the present investigation support this extract to be an effective anticancer agent, therefore triggering for preclinical studies and pharmacokinetic profiling to determine its mechanism of action and clinical doses for human population.

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