Journal of Clinical and Experimental Oncology

Research Article A SCITECHNOL JOURNAL

Anticarcinogenic Effect of Epigallocatechin-3-Gallate (EGCG) in Renal Carcinoma Induced by Aristolochic Acid in Male Rats

Sohail Hussain*, Mohammed Ashafaq, Rahimullah Siddiqui, Khaled Hussain Khabani, Ahmad Suliman Alfaifi and Saeed Alshahrani

Department of Pharmacy, University of Jazan, Jizan, Saudi Arabia

***Corresponding author:** Sohail Hussain, Department of Pharmacy, University of Jazan, Jizan, Saudi Arabia, Tel: 966581939262; E-mail: shussainamu@gmail.com

Received: 16 October, 2019, Manuscript No. JCEOG-21-3588;

Editor assigned: 21 October, 2019, PreQC No. JCEOG-21-3588 (PQ);

Reviewed: 04 November, 2019, QC No. JCEOG-21-3588;

Revised: 25 August, 2022, QI No. JCEOG-21-3588 (QI); Manuscript No. JCEOG-21-3588 (R);

Published: 22 September, 2022, DOI: 10.4172/2324-9110.1000315

Abstract

Introduction: To elucidate the chemopreventive effect of Epigallocatechin-3-Gallate (EGCG) a polyphenol found in green tea having antioxidative, anticancer, anti-inflammatory, anticollagenase, and antifibrosis properties property against carcinogenic effect of Aristolochic Acids (AA) extract from a Chinese herb *Aristolochia* species (*Aristolochia and Asarum*) in wister male rats.

Material and Method: The consequences of EGCG a dose (20 mg/kg b.w.) for 30 days was assessed against renal carcinoma induced by AA after a dose of (20 mg/kg b.w.) for 90 days. The biochemical markers (Urea, Uric acid and Creatinine), imbalance in oxidative parameters (MDA, GSH, Catalase and SOD), caspases 3,9 expression and histopathological studies were determined for quantifying the carcinogenic effect of AA.

Results: Treatment of AA results significant increase in free radicals, serum markers, caspases 3 and 9 activity and histopathological changes. After co-treatment with EGCG markedly reverse all the parameters. Furthermore, it also restores the activity of antioxidative enzymes.

Conclusion: Our study shows, that carcinogenic property of AA is restore by treating with EGCG in renal tissue.

Keywords: Aristolochic acid; Caspases; Chemoprevention; Renal carcinoma

Introduction

All around the world approximately 273,000 new cases of renal cancer are added every year. Which is nearly about two percent of all carcinoma [1]. According to report, the most incidence is occurring in Western countries [2]. The new data suggest that there are about 102,000 new cases per year in European countries, while approximately 45,000 people in Europe are likely to die from kidney cancer each year [3]. In KSA, kidney cancer is the third common genitourinary cancer. The estimate 3.4% and 2.0% of all male and female cancers respectively. In 2010, a total of 167 cases were diagnosed in males and 117 cases in females [4]. Oxidative stress has played a significant role in aging and pathogeneses of diseases. Reactive Oxygen Species (ROS) are free radical species, it can modify and make the protein, nucleic acid and lipids dysfunctional [5].

Aristolochia species (*Aristolochia and Asarum*) are used in the Chinese as a traditional medicine (such as fangchi and mutong) and ethnobotanicals. Aristolochic Acid (AA) from various Aristolochiaceae has a possible function cell growth [6]. Pioneered finding shows that in 1961, AA a product of nitrophenanthrene might exert an anti-inflammatory, anti-neoplastic and analgesics. However, because of its role in carcinogenesis was well established as result the product containing AA were called back from market in the early 1980s, in Asian subcontinents still these plants are in use to treat snake bites, arthritis, gout, and coronary artery diseases. In Europe, nephrotocixity due to AA was first reported in 1991, referring to females from Belgium. Today, AA is mainly known to be as geno and nephrotoxic inducing agent in Balkan endemic nephropathy in people living in the Balkan Region [7].

Herbs containing AA effects 33% of Taiwan population [8-11]. A previous work demonstrated that half of patients exposed with AA have a higher risk of developing cancer. Some finding shows half of the patients with bladder carcinoma or upper Urinary Tract Urothelial Carcinoma (UTUC) are having history of AA exposure. UTUC are common in females and lung cancer associated with AA, pancreatic cancer, many studies in animal model demonstrated earlier the formation of carcinoma at the site of injection such as in fore-stomach, urinary tract and fibrohistiocytic sarcomas [12-14]. Adenocarcinoma of the renal tissue and hypertrophy of the pancreas, hepatocarcinogenesis, ovarian or testicular carcinoma were also reported. The AA also inhibit the anti-apoptotic markers such as bcl-2, ERK1/2 and over expression of pro-apoptotic markers such as PARP and caspase-3,9 [14].

Green tea, which contains polyphenols having antioxidative property is commonly used beverages in the world. Phenolic acids and catechins are the major constituent found in green tea [15]. Many beneficial effects of Polyphenolshas been proved in pathological disease such as cancer, inflammation, diabetes, and cardiovascular diseases [16]. EGCG is having antioxidant, anticancer, anti-inflammatory, anticollagenase, and antifibrosis properties [17,18]. The activities to trap electron and scavengers of free radicals is mainly due phenol ring in its structure reduce the ROS formation and oxidative stress [19].

EGCG induce tumor cell death *via* several mechanisms including caspase-dependent apoptosis, caspase-independent apoptosis, lysosomal membrane permeabilization-mediated cell death, and autophagy and by ameliorating the activities of antioxidive enzymes (GSH, Catalase and SOD) and reduction of lipid peroxidation [20].

Many researchers reported anticarcinogenic effect of EGCG on animal model but none of the studies have been reported till date in animal model taking together EGCG and Aristolochic Acid (AA) [21].

Materials and Methods

In this experiment, male Wistar rats of 225 g to 250 g were used. Rats were maintained according to standard laboratory conditioned.

Recommendation of Institutional Animal Ethics Committee was adopted for all experimental procedure [22].

Male Wistar rats were divided into four experimental groups (each group having six rats).

- The first group served as control and received vehicle saline only (10 ml/kg B.W orally).
- Second group received EGCG (20 mg/kg B.W, orally) only for 90 days.
- Third group was treated with AA (20 mg/kg B.W, orally) in saline 90 days.
- In fourth group AA (20 mg/kg B.W, orally 90 days before) and EGCG (20 mg/kg B.W for last 30 days orally) were given.

Biochemical evaluation

After completion of treatment regime all rats were anesthetized with ether and blood sample was collected by ocular puncture. Blood serum were obtained by centrifuging sample at 3000 rpm for 15 min and keep at -80°C for further analysis of urea, uric acid and creatinine by using commercially available test kits. Thereafter, animals were sacrificed, and kidney tissue was collected out sample prepared in buffer for the biochemical analysis to check the relevance from serum results. LPO and GSH were assayed as biomarkers of oxidative stress along with the antioxidant enzymes (SOD and Catalase). Caspase 3,9 activity was done by calorimetric kit purchased from abcam (Figure 1) [23].

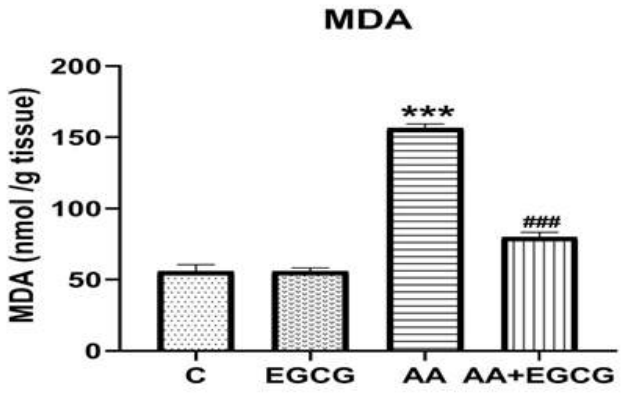

Figure 1: Effect of EGCG on kidney tissue levels of LPO in renal carcinoma induced by AA. Data presented as Mean ± SEM (n=6). **Note:** P<0.001 designates significant difference between only AA with control group, ###P<0.001 and #P<0.001 shows significant difference from AA+EGCG treated group. AA: Aristolochic Acid, EGCG: Epigallocatechin-3-Gallate.

Estimation of lipid peroxidation

LPO was estimated by method with some modification was adopted for lipid peroxidation determination [24]. The mixture consisted of 10% trichloroacetic acid and 0.67% thiobarbituric acid. Supernatant was obtained by mixture centrifuged at 3000 g for 15 min. After separation of supernatant, samples were kept in a boiling water bath for 10 min. The sample reading was recorded at 535 nm after cooling. The content of LPO was determined as n moles of Thiobarbituric Acid Reactive Substances (TBARS) formed/h/mg protein by a molar extinction coefficient of 1.56 M^{-1} cm^{-1} × 10^5 M^{-1} cm^{-1} (Figure 2).

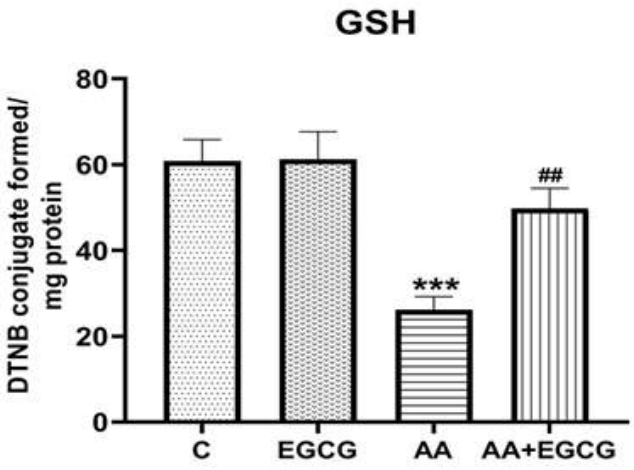

Figure 2: Effect of EGCG on kidney tissue levels of GSH in renal carcinoma induced by AA. Data presented as Mean ± SEM (n=6). **Note:** ***P<0.001 designates significant difference between only AA with control group, ###P<0.001 and #P<0.01 shows significant difference from AA+EGCG treated group. AA: Aristolochic Acid, EGCG: Epigallocatechin-3-Gallate.

Estimation of reduced glutathione

GSH was assessed using the method of 4% SSA was used to precipitate sample PMS in a 1:1 ratio. The mixture was kept for 1 hour at 4°C and then centrifuged at 3000 rpm for 15 minutes at 4°C to separate supernatant. 3 ml assay sample includes 2.2 ml of 0.1 m sodium phosphate buffer (pH 7.4), 0.4 ml of supernatant and 0.4 ml of DTNB. Reading of resultant sample was taken instantly at 412 nm. 1.36 × 10^4 M^{-1} cm^{-1} molar extinction coefficient was used to determine GSH content in µ moles per milligram of protein (Figure 3).

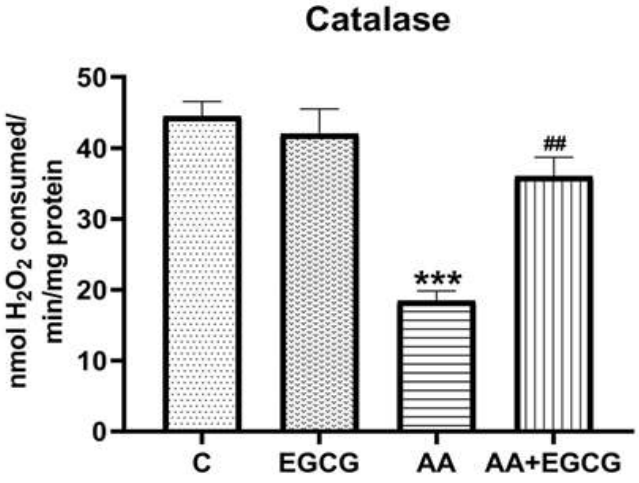

Figure 3: Effect of EGCG on kidney tissue levels of catalase in renal carcinoma induced by AA. Data presented as Mean ± SEM (n=6). **Note:** ***P<0.01 designates significant difference between only AA with control group, ###P<0.001 and #P<0.01 shows significant difference from AA+EGCG treated group. AA: Aristolochic Acid, EGCG: Epigallocatechin-3-Gallate.

Estimation of activity of Superoxide Dismutase (SOD)

SOD activity was estimated by observing the auto-oxidation of (-)-epinephrine. The sample mixture contained glycine buffer (50 mM, pH 10.4) and 0.2 mL of PMS. 4.02 × 10^3 M^{-1} cm^{-1} molar extinction coefficient used to assess SOD activity.

Estimation of catalase activity

Catalase activity was assessed by the method of 3 ml volume of sample consists of 50 µl of PMS, 1.95 ml of phosphate buffer and 1 ml of H_2O_2. The reading of assay sample was measured at 240 nm in kinetic method. 43.6 × 10^3 M^{-1} cm^{-1} molar extinction coefficient used to determine activity of catalase enzyme in nmol H_2O_2 consumed/min/mg protein (Figure 4) [25].

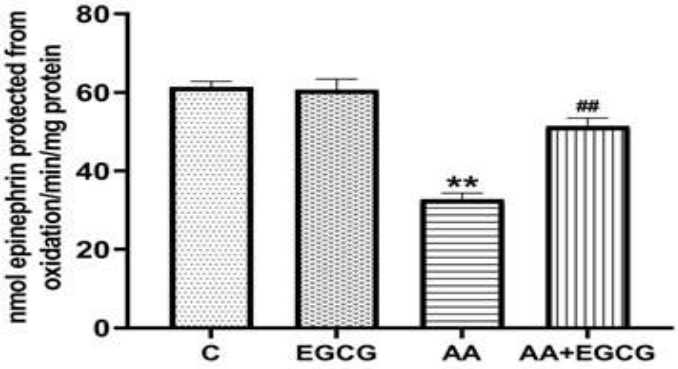

Figure 4: Effect of EGCG on kidney tissue levels of SOD in renal carcinoma induced by AA. Data presented as Mean ± SEM (n=6). **Note:** ***P<0.01 designates significant difference between only AA with control group, ###P<0.01 and #P<0.01 shows significant difference from AA+EGCG treated group. AA: Aristolochic Acid, EGCG: Epigallocatechin-3-Gallate

Estimation of protein

The method of estimation was followed to estimate protein using BSA as standard.

Statistical analysis

Statistical analysis of the data was done by applying the analysis of variance (ANOVA), followed by Tukey-Kramer's test for all experimental parameters. Results of analysis were expressed as mean± SEM of six rats. The p<0.05 was considered statistically significant [26].

Results

Serum markers

Effect of EGCG on AA-induced renal carcinoma administration significantly increased the urea (26.18 ± 3.45 mg/dl to 62.19 ± 4.94 mg/dl), uric acid (4.67 ± 1.07 mg/dl to 13.37 ± 1.43 mg/dl) and creatinine increased the (0.79 ± 0.08 mg/dl to 3.41 ± 0.37 mg/dl) levels when compared to vehicle control groups (p<0.001). EGCG treatment at the doses of 20 mg/dl along with AA significantly attenuated the increase in urea, creatinine and uric acid levels when compared to AA alonetreated group (p<0.01, p<0.001) (Table 1).

Parameters	Control	EGCG	AA	AA+EGCG
Urea	26.18 ± 3.45	23.79 ± 2.31	62.19 ± 4.94	38.27 ± 3.18
Uric acid	4.67 ± 1.07	3.94 ± 0.27	13.37 ± 1.43	7.18 ± 1.13
Creatinine	0.79 ± 0.08	0.81 ± 0.018	3.41 ± 0.37	1.94 ± 0.29

Table1: Biochemical effects of EGCG in serum of AA induced renal carcinoma in rats. **Note:** AA: Aristolochic Acid, EGCG: Epigallocatechin-3-Gallate.

Enzymatic activity

The values of TBARS used to measures of lipid peroxidation was significantly (p<0.001) rise in AA treated rats as compared to control. Co-treatment with EGCG significantly (p<0.001) reduces the TBARS as compared to AA alone treated rats. The levels of non-enzymatic antioxidants and activities of enzymes were significantly (GSH, catalase) (p<0.001) and SOD (p<0.01) reduces in renal tissues of only AA administered rats. EGCG co-treatment at 20 mg/dldose with AA significantly GSH, catalase and SOD (p<0.01) ameliorates the activities of antioxidants [27].

Caspases estimation

To determine the apoptosis of renal cell due to AA, we quantify the apoptosis marker such as caspase-3 and 9 in kidney tissues. AA significantly enhanced the expressions of cleaved caspase-3,9 were significantly (p<0.001) increased in AA alone administered rats as compared to the control group. EGCG co-treatment at dose of 20 mg/kg along with AA significantly attenuated the expression of cleaved caspase-3 and 9, when compared to AA alone group (p<0.05) (p<0.01) (Figures 5 and 6).

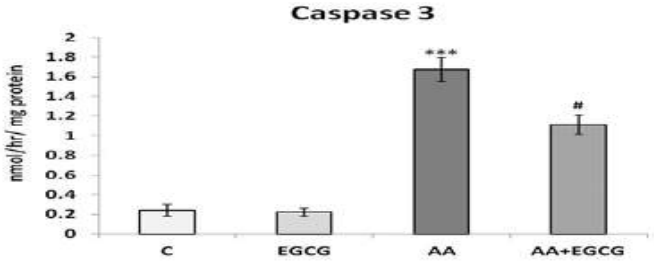

Figure 5: Effect of EGCG on kidney tissue levels of caspase 3 in renal carcinoma induced by AA. Data presented as Mean ± SEM (n=6). **Note:** ***P<0.001 designates significant difference between only AA with control group and #P< 0.05 shows significant difference from AA+EGCG treated group. AA: Aristolochic Acid, EGCG: Epigallocatechin-3-Gallate.

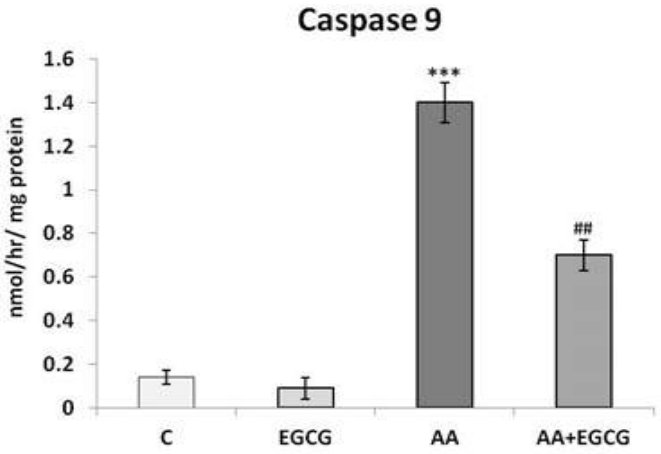

Figure 6: Effect of EGCG on kidney tissue levels of caspase 9 in renal carcinoma induced by AA. Data presented as Mean ± SEM (n=6). **Note:** $^{***}P<0.001$ designates significant difference between only AA with control group and $^{\#}P<0.01$ shows significant difference from AA+EGCG treated group. AA: Aristolochic Acid, EGCG: Epigallocatechin-3-Gallate.

Histopathological studies

Findings of histopathological showed that tissues from control rats (Control) is having intact histo-morphology, however renal tissues from AA group display severely damaged and necrosis in the tubular epithelium, infiltration of inflammatory cells and accumulation of homogenous eosinophilic casts in the lumen of the tubules. Kidney tissue sections from EGCG with AA treated group showed reduces all the morphological changes in tubular epithelial compared to AA alone treated group (Figure 7) [28].

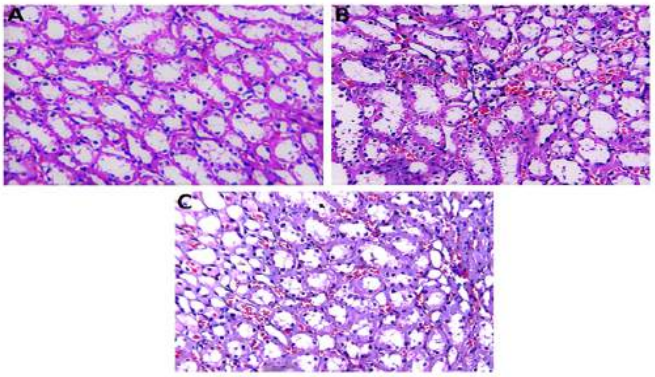

Figure 7: A) Effect of EGCG on morphological changes in AA treatment rat using H and E staining in the kidney of control, AA and single doses of AA+EGCG groups. Kidneys from the control group; B) showed no histological changes. Pathological alteration included tubular necrosis, epithelial degeneration, tubular dilatation, and swelling in AA treated rats; C) Prominent histological changes were observed in kidneys from rats treated with AA and EGCG when compared with kidneys in rats treated with AA only.

Discussion

Chemotherapeutic regimens are commonly used to inhibit the cancer progression, however, these drugs mostly having negative effects. Chemotherapy drugs not only have side effects but tumor cells also develop resistance. Renal carcinoma is a commonly occurring and has attracted considerable attention in the field of cancer research. In the present study, we estimated that EGCG co-treatment with AA largely reduced the renal carcinoma using rat model. The results of study show that EGCG treatment reduced the renal oxidative stress, elevated enzyme activities, decrease expressions of caspases and protected histopathological changes [29]. Reports of shows elevated levels of urea, uric acid and creatinine may be due to strong correlation between renal carcinoma and oxidative stress. Increase in H_2O_2 and O_2 production changes the filtration surface area and modifies the filtration coefficient, so these factors might reduce the glomerular filtration resulting in accumulation of urea, uric acid and creatinine in the blood [30].

According to the previous reports, we showed that AA induces significant increase in urea, uric acid and creatinine (renal function biomarkers) inserum. Furthermore the precise mechanism of inducing renal cancer by AA is still unknown, but previous researchers are agreed on the mechanism such as imbalance in the oxidative enzymes, cell death by apoptosis/necrosis and increase in inflammatory pathways are the principle mode of action of causing renal carcinoma by AA in rats [31]. Researchers also disclosed that the AA increases free radicals production which results oxidative damage and lipid peroxidation in tissues [32]. The produced free radicals such as superoxide and hydroxyl radicals attack and changes cell and cellular component such as nucleic acids, amino acids, lipids and other macromolecules as a result causes cell necrosis [33].

Conclusion

In the present study levels of TBARS were increased and GSH levels decreased in AA treated animals. Whereas EGCG treatment increases GSH level and decreases inhanced TBARS content by its antioxidant property. Previously it was observed that polyphenol having antioxidant protect from oxidative damage by TBARS and increases GSH levels. Antioxidant enzymes activity diminish in AA treated rats. Treatment of EGCG significantly protected enzymes activity (SOD and catalase). Antioxidants enzymes play important role in scavenging free radicals and also inhibit the carcinogenic progression in renal tissue reported earlier model. EGCG treatment also suppresses expression of caspase activity by its antiapoptotic property in AA treated rats. EGCG also protected histological alteration in AA treated rats by its anti-inflammatory, antioxidantive and antiapoptotic property reported in various studies in animal model.

We concluded that AA treatment involves in cancer progression in rat kidney. Whereas EGCG protected histological changes, reduces oxidative stress, enhances anti-oxidative enzymes activity and caspase expression by its potent antioxidant, anti-inflammatory, antiapoptic and anticancer activity.

Acknowledgement

I highly thank full to Jazan University, Jazan, KSA for financial support for this work under the future scientist project number FR6-49.

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