Chemoprotective Effects of Geraniin against Azoxymethane Induced Colorectal Cancer by Reduction of Inflammatory Reaction

Ruize Zhou¹, Jia Lei², Yubo Wei¹, and Hongbin Zhang^{1*}

¹ Department of Oncology First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, 650032, P.R. CHINA

² Clinical Technology Experimental Center, Kunming Medical University Haiyuan College, Kunming, Yunnan, 650106, P.R. CHINA

Abstract: The leading cause of cancer-related death is colorectal cancer, and inflammatory bowel disease is a risk factor for this disease. Azoxymethane (AOM) is a potent cancer inducer widely used in rats for colon cancer. The current study was scrutinizing the chemo-protective effect of geraniin against AOM induced colorectal cancer via alteration of oxidative stress and inflammatory cytokines. The rats were divided into different groups such as Group I: normal control, Group II geraniin (20 mg/kg), Group III: received AOM, Group IV-VI: AOM + geraniin (5, 10 and 20 mg/kg), respectively. All group of rats were received treatment for 16 weeks. At the end of the experimental study, the hepatic, biochemical, phase II antioxidant, antioxidant enzymes, cytokines, apoptosis and inflammatory mediators were estimated. Geraniin treatment significantly reduced tumor weight and enhanced body weight. Geraniin administration also altered the level of antioxidant parameters-superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR); phase I enzymes - cytochrome B₅, cytochrome P₄₅₀; phase II enzymes - Glutathione-S-Transferase (GST), UDP-Glucuronyl transferase (UDP-GT) respectively. Obtained results also demonstrate that geraniin treatment reduced the level of pro-inflammatory cytokines such as IL-2, IL-1 α , IL-10, IL-1 β , IL-4, IL-6, IL-12, IL-17A, IFN- γ , tumor necrosis factor- α , G-CSF, and GM-CSF. Geraniin also reduced the expression of IL-1 α , IL-1 β , IL-6, IFN- γ , G-CSF, and GM-CSF. On the basis of result we can conclude that geraniin reduced the colorectal cancer via inflammatory pathway.

Key words: colorectal cancer, geraniin, inflammation, antioxidant

1 Introduction

Recently, colorectal cancer is on the stage of alarming incidence resulted in a highly demand for new pharmacological approaches to overcome the limitations of conventional treatment. Its management includes surgery, radiation, and chemotherapy, depending at which the cancer stage exists¹⁾. Given the widespread use of chemotherapeutic agents i.e. fluorouracil, oxaliplatin, or irinotecan, they have many drawbacks, such as extreme toxicity associated with serious adverse reactions, drug resistance growth, and deficient active site in targeting tumor cells only^{2,3)}.

Studies mentioning the beneficial effects of the administration of natural compounds for a broad range of ailment related to the various complementary and traditional plants available for isolating compound have opened up a growing curiosity in the usage of natural compounds as novel phar-

macological compounds for treating CRC⁴⁾. CRC treatment uses natural compounds can be used throughout the CRC management process. A good plan of diet may prevent the chances of diseases based on high vegetable, fruit, and fiber consumption. It has been revealed that after the onset of disease, natural components can aim to tumor cells and protect recurrence of tumor. In addition, the most essential characteristic of natural compounds having the ability to sensitivity to tumor cells for chemotherapeutic agents after drug resistance has been developed⁴⁻⁶⁾.

It has been documented that edible components such as quercetin and curcumin have managed to conquer the multidrug resistance in various cell lines, which are malignant in nature. This potential for chemosensitivity of bioactive constituents supports the use of these compounds as adjuvant therapy in locally used protocols, but a different mechanism of action against tumor cells can also be based

*Correspondence to: Hongbin Zhang, Department of Oncology, First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan, 650032, P.R. CHINA

E-mail: zhbwydz@163.com

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on exclusively natural compound treatment^{7,8)}. Plant based compounds can therefore show anti-tumor action because of their antioxidant power, their capability of cell growth inhibition and activate apoptosis of tumor cells or via the metastatic cascade modulation^{9,10)}.

Geraniin, a polyphenol by hydrolysis derived from *Nephelium lappaceum* L. Fruit rind has substantial antioxidant activity *in vitro*, and has previously been considered in metabolic syndrome for its pharmacological property^{11, 12)}. *Nephelium lappaceum* L., belongs to the family of Sapindaceae and indigenous region Southeast Asia. The main phenolic bioactive components present in the rind of *N. Lapacée* L. were isolated as corilagin, elaeocarpusin and geraniin^{12, 13)}. Palanisamy *et al.* suggested that the highest yields of geraniin (35 percent) obtained from *N. lappaceum* dried fruit rind¹⁴⁾. Geraniin is in huge quantity obtained from plant-based medicinal products and foods from Chinese, Unani, and Ayurvedic herbs such as *Phyllanthus niruri*, *Geranium thunbergii* and *G. Sibirica*^{11, 12, 15, 16)}. Literature documented about the distribution, plenty, and therapeutic of geraniin^{11, 12, 15)}. It is also found in various articles that geraniin is highly antioxidant and that cellular OS shows cytoprotective effect evidenced by *in vitro* studies^{16, 17)}. Currently, we have observed in literature that geraniin have substantially significant defense against metabolic dysfunctions caused by HFD in rodents and is not toxic up to 50 mg/kg bw dose level. The protective nature of geraniin in HFD-fed rodents can be associated because of its antioxidant property. On the basis of benefits of geraniin and based on the various facts related to develop novel drugs from traditional plant extraction and isolation. The purpose of current study was to assess the chemo-preventive action of geraniin in the experimental model of rats induced by AOM, carcinogenic agents with colorectal cancer.

2 Materials and Methods

2.1 Chemical

Standard geraniin and azoxymethane were purchased from Sigma Aldrich (St. Louis, USA). HPLC grade *n*-butanol, ethanol, dimethylsulfoxide, and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA, USA). Multi-Analyte ELISA kits for inflammatory mediators and pro-inflammatory cytokines were obtained from Qiagen (Germantown, MD, USA). All the reagents used in the experimental protocol were analytical grade and 95% pure.

2.2 Experimental rodent

Swiss Albino Wistar (70 ± 14 g, gender both) was used for the current experimental study. The rats were kept under polyethylene cage in the standard laboratory conditions such as temperature 20 ± 3°C, 70% relative humidity and

12 h day and night light cycle. The rats were received standard mouse diet and water *ad libitum*. The rats were allowed to acclimate to laboratory conditions for at least 1 week before the experimental study. This research was approved by animal ethical committee of The First Affiliated Hospital of Kunming Medical University, Approved No.2019KMU311.

2.3 AOM injection

Intraperitoneal injection of AOM was used for induction of colorectal cancer. briefly, AOM soluble in physiological saline (0.9%). The 1% suspension of carboxyl methylcellulose (CMC) was used to dissolve the geraniin⁴⁾.

2.4 Treatment group

2.4.1 Experimental procedure

Fifty Swiss Wistar rats were divided into 5 groups and each group contain the 10 rats (aged 6 weeks, average body weight of 20 ± 3 g. The groups of rats were as follow-

- Group I : fed with the basal diet and received the saline
- Group II : fed with the basal diet and received AOM
- Group III: fed with the basal diet and received AOM + geraniin (5 mg/kg)
- Group IV: fed with the basal diet and received AOM + geraniin (10 mg/kg)
- Group V: fed with the basal diet and received AOM + geraniin (20 mg/kg)

The dose selection of geraniin based on the previous published work. All group rats were continuously received *ad libitum*. The water intake, body weight and food intake were monitored at regular time intervals (16 weeks). At the end of the experimental protocol, blood was collected from the group of rats by puncturing the retro-orbital plexus. The rats were scarified after the end of the experimental study and removed the colon tissue for subsequently analysis such as macroscopically analysis, microscopical changes and biochemical analysis in the colorectal tissue.

2.5 Lipid peroxidation

The commercial (thiobarbituric acid reactive substances (TBARS) Assay Kit from Cayman Chemical Item Number 10009055) was used to estimate lipid peroxidation in colorectal tissue.

2.6 Glutathione (GSH/GSSG) assay

The colorectal supernatant was divided into 2 aliquots for estimating GSSG and GSH. The fresh tube containing 25 mmol/L monochlorobimane and glutathione-S-transferase (2 µL) as provided by commercial kits (Biovision, Mountain View, CA, USA, Catalog # K251) and incubated for 30 min at 37°C. Finally estimate the absorbance at 380/460 nm. GSSG and GSH level was estimated by comparison with values from standard curve using freshly prepared GSSG and GSH, which was standardized with protein level in the

colonic mucosa tissue homogenates and the result was expressed as ratio of GSH/GSSG.

2.7 Myeloperoxidase activity assay

The level of myeloperoxidase activity was estimated in the colon supernatant. Briefly, colon sample was mixed into the medium containing 50 mM (pH-6.0) phosphate buffer (PBS) and 1.5 mM N, N, N₀, N₀-tetramethylbenzidine. The reaction was initiated by adding 0.01% hydrogen peroxide and estimated the absorbance at 655 nm at 37°C.

Assay of Alkaline Phosphatase and Lactate Dehydrogenase

2.8 Estimation of lactate dehydrogenase and alkaline phosphatase

Previous reported method was used for estimating the lactate dehydrogenase and alkaline phosphatase with minor modification^{7,8)}.

2.9 Pro-inflammatory cytokines

The pro-inflammatory cytokines includes IL-4, IL-1 α , IL-6, IL-2, IL-10, IL-12, IL-17A, IL-1 β , IFN- γ , G-CSF, GM-CSF and tumor necrosis factor- α were estimated using the ELISA kits following the manufacture instruction.

2.10 RNA Isolation and PCR

TRIzol reagent was used for isolating cellular RNA from the colonic tissues. cDNA was isolated from the total RNA (2 μ g) using the MMuLV reverse transcriptase. The primers are presented in Table 1.

2.11 Statistical analysis

All data were statistically analyzed with GraphPad Prism 5 software. One way analysis of variance followed via Dunnett's test. All the data presented as SD $\pm$ SEM. * p < 0.05 considered as significant.

3 Results

3.1 Effect of geraniin on antioxidant activities

After the supplementation of geraniin, the levels of SOD, CAT, GSH and LPO in all groups rats are displayed in Fig. 1. Owing to the activity of geraniin alone, a significant reduction was observed in the activity of SOD in AOM induced colorectal cancer rats. Treatment with geraniin at high doses improved the level of SOD as caused by AOM and enhanced the level of the enzyme near normal control rats. In comparative with normal control rats, the level of catalase was significantly reduced in the AOM induced the cancer group and reversed by the geraniin as relative to the community of cancer (Group II).

The GSH level was significantly exhausted and LPO level was significantly increased in the AOM cancer group as contrast to the normal control rats. Treatment with geraniin at both dose levels significantly enhanced amount of GSH and induced a substantial (p < 0.05) dose-dependent reduction in LPO level toward the normal range compared with AOM induced colorectal cancer rats, as illustrated in the Fig. 1.

3.2 Effect of geraniin on ACF formation

Figure 2 summarizes the effect of geraniin on the AOM-induced formation of ACF in carcinogenic rats at all doses tested. AOM was administered to all rats in the cancer group and showed a 100% incidence of ACF formation, while no ACF was found in the normal group and rats received geraniin. The number of ACFs created when a comparison was made between the geraniin-treated group of 20 mg/kg bw and the AOM-treated group was also significantly reduced. A higher number of ACF was observed in the distal part of the colon in the AOM treated group and the geraniin-treated groups. Compared to the cancer community, the decrease in the percentage of ACF formation recorded by the geraniin-treated group (AOM).

3.3 Effect of geraniin on body weight

The study-wide change in body weight is presented in

Table 1 list of primers for quantitative real time polymerase chain reaction analysis of inflammatory cytokines.

S. No	Gene	Primer Sequence	
		Forward	Reverse
1	IL-1 α	CGAAGACTACAGTTCTGCCATT	GACGTTTCAGAGGTTCTCAGAG
2	IL-1 β	GCAACTGTTCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
3	IL-6	TAGTCCTTCCCTACCCCAATTTC	TTGGTCCTTAGCCACTCCTTC
4	IFN- γ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
5	G-CSF	ATGGCTCAACTTTCTGCCCCAG	CTGACAGTGACCAGGGGAAC
6	GM-CSF	GGCCTTGGAAGCATGTAGAGG	GGAGAACTCGTTAGAGACGACTT
7	β -actin	GGCTGTATTCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Fig. 1 showed the effect of geraniin on the antioxidant parameters of azoxymethane induced colorectal cancer. a: LPO, b: GSH, c: CAT and d: SOD. Values are expressed as means $\pm$ SEM. * $p < 0.05$ consider as significant, ** $p < 0.01$ consider as more significant and *** $p < 0.001$ consider as significant.

Fig. 2 showed the effect of geraniin on the incidence of ACF formation of azoxymethane induced colorectal cancer. a: ACF count in proximal colon and b: ACF count in distal colon. Values are expressed as means $\pm$ SEM. * $p < 0.05$ consider as significant, ** $p < 0.01$ consider as more significant and *** $p < 0.001$ consider as significant.

Fig. 3 showed the effect of geraniin on the body weight of azoxymethane induced colorectal cancer. a: ACF count in proximal colon and b: ACF count in distal colon. Values are expressed as means $\pm$ SEM. * $p < 0.05$ consider as significant, ** $p < 0.01$ consider as more significant and *** $p < 0.001$ consider as significant.

Fig. 3. All rats in different groups, except the AOM treated group presented an increase in body weight, which at the end of the experiment revealed a reduction in body weight. No substantial difference ($p > 0.001$) was observed in the relative ratio of organ/body weight among the groups examined (Fig. 4).

3.4 The effect of geraniin on Myeloperoxidase activity assay

Myeloperoxidase is a type of pro-inflammatory mediator and through inflammation, activity of Myeloperoxidase has amplified. The rats supplemented with AOM revealed significant alteration in the response marker of inflammatory.

Fig. 4 showed the effect of geraniin on the organ weight of azoxymethane induced colorectal cancer. a: Liver, b: Lung, c: Spleen, d: Heart, e: Colon and f: Kidney. Values are expressed as means $\pm$ SEM. * $p < 0.05$ consider as significant, ** $p < 0.01$ consider as more significant and *** $p < 0.001$ consider as significant.

Fig. 5 showed the effect of geraniin on the myeloperoxidase activity of azoxymethane induced colorectal cancer. a: ACF count in proximal colon and b: ACF count in distal colon. Values are expressed as means $\pm$ SEM. * $p < 0.05$ consider as significant, ** $p < 0.01$ consider as more significant and *** $p < 0.001$ consider as significant.

i.e. Up-regulation in the activity of myeloperoxidase compared with normal control rats. Administration of geraniin significantly declined the activity of myeloperoxidase induced by AOM in a dose dependent manner (Fig. 5).

3.5 Effect of geraniin on GSH/GSSG ratio

Figure shows that the AOM induced the colorectal cancer group displayed substantial improvements in oxidative markers demonstrated by a reduction in the ratio of GSH/GSSG with respect to the relevant normal control rats. Although, geraniin in a dose dependent manner prevented the rats from colorectal damage caused by AOM. From the result, worth mentioning that geraniin may be a potent drug, which significantly declined the ratio of GSH/GSSG induced by carcinogenic agent (Fig. 6).

3.6 The effect of geraniin on Micronuclei (MN) cell counting and cytotoxicity index

Micronucleus assay was used as a prognostic analysis for finding of carcinogenic-relevant chromosomal alterations/damage. The MN test for rats bone marrow was most commonly used as an *in vivo* assay to classify genotoxic effects

of various carcinogenic agents^{19, 20}. The cytotoxicity index (CI) as the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) was determined. Results are shown in Fig. 7.

3.7 Effect of geraniin on Alkaline phosphatase and Lactate Dehydrogenase

Figure 8 displays the ALP and LDH levels in rats belonging to the group of control and experimental. AOM administration encourages an elevation in ALP and LDH levels. Treatment with different doses of GER greatly decreased levels of ALP and LDH as comparative to normal control rats and AOM-induced rats (Fig. 8). No such alteration was found in the control normal rats and plane GER levels of the ALP and LDH enzymes.

3.8 The effect of geraniin on pro-inflammatory cytokines activation

Earlier studies have revealed that blocking inflammatory cytokines reduce colitis severity significantly. By the method of ELISA array, we examined the levels of pro-inflammatory cytokines in the colon tissue to explore the

Fig. 6 showed the effect of geraniin on the GSH/GSSG of azoxymethane induced colorectal cancer. **a**: ACF count in proximal colon and **b**: ACF count in distal colon. Values are expressed as means $\pm$ SEM. * $p < 0.05$ consider as significant, ** $p < 0.01$ consider as more significant and *** $p < 0.001$ consider as significant.

Fig. 7 showed the effect of geraniin on the Micronuclei (MN) cell counting and cytotoxicity index of azoxymethane induced colorectal cancer. **a**: Micronuclei (MN) cell counting and **b**: cytotoxicity index. Values are expressed as means $\pm$ SEM. * $p < 0.05$ consider as significant, ** $p < 0.01$ consider as more significant and *** $p < 0.001$ consider as significant.

Fig. 8 showed the effect of geraniin on the ALP and LDH level of azoxymethane induced colorectal cancer. **a**: ALP and **b**: LDH. Values are expressed as means $\pm$ SEM. * $p < 0.05$ consider as significant, ** $p < 0.01$ consider as more significant and *** $p < 0.001$ consider as significant.

mechanisms of inhibition of AOM induced carcinogenesis via GER treatment. AOM induced colorectal cancer, levels pro-inflammatory cytokines in colon such as IL-6, IL-1 α , IL-10, IL-1 β , IL-2, IL-4, IL-12, IL-17A, IFN- γ , tumor necrosis factor- α , G-CSF and GM-CSF was significantly elevated (Figs. 9 and 10). Administration of GER in a dose dependent manner significantly improved pro-inflammatory cytokines levels. This finding indicated that GER substantially decreased proinflammatory cytokine levels in the tissue of colon, which were mediated by AOM in rats.

3.9 Effect of geraniin on inflammatory cytokine gene expression

We looked at the effect of GER on the inflammatory cytokine genes expression to authenticate the cytokine value found in the ELISA analysis. RT-PCR was used to determine the impact of GER at the target genes present in colon tissue. As illustrated in Fig. 11. Inflammatory cytokines such as IL-6, IL-1 α , IFN- γ , G-CSF, IL-1 β and GM-CSF in the AOM treated group were significantly bigger than normal control rats (Fig. 11). Treatment with GER significantly reduced the significantly measured inflammatory

Fig. 9 showed the effect of geraniin on the pro-inflammatory cytokines of azoxymethane induced colorectal cancer. **a:** IL-2, **b:** IL-4, **c:** IL-1 α , **d:** IL-1 β , **e:** IL-6 and **f:** IL-10. Values are expressed as means $\pm$ SEM. * p < 0.05 consider as significant, ** p < 0.01 consider as more significant and *** p < 0.001 consider as significant.

Fig. 10 showed the effect of geraniin on the pro-inflammatory cytokines of azoxymethane induced colorectal cancer. **a:** INF- γ , **b:** TNF- α , **c:** IL-12, **d:** IL-17A, **e:** G-CSF and **f:** GM-CSF. Values are expressed as means $\pm$ SEM. * p < 0.05 consider as significant, ** p < 0.01 consider as more significant and *** p < 0.001 consider as significant.

Fig. 11 showed the effect of geraniin on the pro-inflammatory cytokines expression of azoxymethane induced colorectal cancer. **a:** IL-6, **b:** INF- γ , **c:** IL-1 α , **d:** IL-1 β , **e:** G-CSF and **f:** GM-CSF. Values are expressed as means $\pm$ SEM. * p < 0.05 consider as significant, ** p < 0.01 consider as more significant and *** p < 0.001 consider as significant.

cytokines expression relative to the AOM treated group. This study suggests that supplementation of GER in a dose dependent manner transcriptionally suppressed the inflammatory cytokines in colorectal tissue.

4 Discussion

Looking at the possibility of these medications for a long time, however, as a general guideline, render this type of chemoprevention ineffective. Epidemiological, laboratory

and clinical studies indicate that inflammatory bioactive constituents have special ways of preventing the development and growth of cancer^{3, 18}. The effects of GER have been examined in this study is an effort to explore the various sources from botanical plants against this major medical problem. In laboratory research, AOM, which is a mutagenic agent was frequently used in animal chemoprevention of colorectal cancer.

The current study first noticed on Geraniin's anti-colon carcinogenesis activity against colon cancer induced by AOM in rats model, thus confirming the earlier states of the association of antioxidant and anti-inflammatory properties, which may be helpful in supporting the mechanism behind anticancer¹⁹. Furthermore, this study demonstrated the *in vivo* model of anticolon cancer activity of Geraniin, which supports the previous results correlated with *in vitro* model. Additionally, this study also demonstrated the geraniin's ability to alleviate the enzymatic antioxidant action involving SOD, CAT, GSH and LPO and used in determining the mechanisms behind the activity of GER against AOM induced colorectal cancer.

Additionally feeding of Geraniin has not altered body weight profiles steady with earlier anti-tumor properties using a similar cancer experiment on an animal model²⁰. Because of metabolite reactive nature, methyl diazonium ion, that activates mutagenicity by inducing damage to chromosomes and micronuclei (MN) cell induction, AOM may cause oxidative and damage to DNA following administration, resulting in morphological alterations in colon related to the production of aberrant crypt foci (ACF)²¹. Aberrant crypt foci (ACF) are presumed lesions of neoplastic, which appear to be abnormally big, stained in dark complexion and slenderly increase from normal crypt and may develop into colorectal carcinogenesis. Usually, ACF is observed after the supplementation of the mutagenic agent AOM and develop as soon as 2–4 weeks²². Geraniin's capability to minimize the count of ACF can also be partly due to the high level of antioxidant property of the drug and the capacity to amend the antioxidant endogenous mechanism in the tissue of the colon.

GSH is one of the important intracellular antioxidants used to remove oxidative stress. GSH's antioxidant potential is accomplished by the SH group's direct association with ROS or by acting as a coenzyme to detoxify ROS reaction²³. As mentioned earlier, by depleting the amount of GSH, AOM induces oxidative stress; Geraniin treatment reserves the content of GSH in this research. AOM also depleted the level of CAT and SOD in addition to GSH, which is the inverse and restored by geraniin to normal value. A marked increase in the LPO level was also caused by the administration of AOM, which was decreased by geraniin, suggesting the cytoprotective activity of the extract. One of the strong intracellular antioxidants required to eliminate oxidative stress is GSH. GSH's antioxidant ability is

achieved through the –SH group's direct association with ROS or acts as a coenzyme to detoxify the reaction of reactive oxygen species). As previously mentioned, AOM causes oxidative stress by lowering the GSH content; in the current work, treatment with geraniin reserves the GSH content suggesting the defensive property of geraniin. In addition to GSH, AOM also drained CAT and SOD levels, which were reversed and geraniin restored to normal data¹⁹. AOM administration also provoked a significant elevation in the level of LPO, which geraniin decreased, suggesting its cytoprotective action.

5 Conclusion

In brief, this research reveals that Geraniin boosts the pharmacological property against AOM induced colorectal carcinogenesis by amending various pro and anticancer moieties, which play a vital role in the management of colorectal cancerous cell by regulating damage to DNA, cell development, inflammation and proliferation. The pro-inflammatory cytokines include IL-6, IL-2, TNF-alpha, and Myeloperoxidase activity plays an important role to show the underlying mechanisms. In this context, our outcome shows a therapeutic efficacy by providing the preclinical development of Geraniin in the management of colorectal tumorigenesis in patients. Although, more research work must scrutinize the pharmacological activity and to explore its anticancer properties by inducing colon inflammation and tumorigenesis in people by AOM.

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