Amol University of Special Modern Technologies

Caspian Journal of Veterinary Science

doi: 10.22034/cjvs.2024.198853

Journal homepage: https://cjvs.ausmt.ac.ir/

Histopathological and biochemical evaluation of protective effects of lemongrass (*Cymbopogon citratus*) essential oil on 5-Fluorouracil-induced intestinal damage in murine model

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Article Info

Article history:

Received: 14 April 2024

Accepted: 3 June 2024

Keywords:

Essence

5-Fluorouracil

Histopathology

Lemongrass

Small intestine

Abstract

5-Fluoraracil (5-FU) is a common chemotherapy drug, but its toxicity is considered a big obstacle to successful cancer chemotherapy. Oxidative stress plays an important role in 5-FU toxicity. Plant compounds reduce the damage caused by oxidative stress. This study aimed to explore the potential protective effects of lemongrass essential oil (LG) on intestinal tissue damage caused by 5-FU. Forty-eight mice were divided into six groups including control, 5-FU (200 mg/kg, IP), 5-FU (200 mg/kg, IP along with LG (125, 250, and 500 mg/kg, PO), and LG alone (500 mg/kg, PO) for six days. On the seventh day, serum and intestinal tissue samples were collected. The histopathological analysis of the intestine tissues revealed that a single dose of 5-FU led to degenerative damage, destruction of the epithelium, shortened villi length, and edema in the small intestine. It also caused a significant increase in serum (malondialdehyde) MDA levels, and a notable reduction in superoxide dismutase (SOD) and catalase serum levels. Oral administration of LG, particularly at doses of 250 and 500 mg/kg, significantly mitigated the severity of these lesions and serum marker alterations. Importantly, the study observed that administering a high dose of LG did not induce any pathological effects in the intestine tissue or serum. LG oil offers protective effects against 5-FU-induced damage in the mice intestine and serum biochemical changes, which can help reduce oxidative damage caused by free radicals generated by 5-FU.

Introduction

Currently, cancer is one of the main causes of death in the world with a high prevalence. Chemotherapy is one common treatment, during which different chemicals are prescribed to patients and each drug works on different phases of the cell cycle, which is why it leads to severe side effects (Hanahan and Weinberg, 2011). Cancer treatment includes a series of strategies to destroy, control, or remove early or advanced cancerous tissue. The diversity and extent of cancer over the past years has led to the development of several treatment methods that are used to fight cancer, depending on the type, location, rate of progress, extent of the disease, and the patient's condition.

Cancer is initially treated with 3 main methods: surgery, radiation therapy, and systemic therapy (including targeted drugs, hormone therapy, and the use of immunomodulatory drugs) (Telloni, 2017). Fluorouracil with the chemical formula C4H3FN2O2 is a prodrug that is converted into ribosyl and deoxyribosyl nucleotide metabolites under biotransformation. The cytotoxicity of 5-fluorouracil is caused by its effects on both DNA and RNA. 5-Fluorouracil (5-FU) is usually taken intravenously and has a half-life of 10 to 15 minutes. This drug is not prescribed orally, because due to the high levels of the dihydropyrimidine dehydrogenase breaking enzyme in

the digestive mucosa, up to 80-85% of the dose is catabolized. There is a pharmacogenetic syndrome due to partial or complete deficiency of DPD enzyme in 5% of all cancer patients, which causes severe toxicity in the form of bone marrow suppression, diarrhea, nausea, vomiting, and neurotoxicity (Parker and Cheng, 1990; Iacopetta *et al*., 2008). 5-FU is considered the most widely used drug in colorectal cancer both as an adjuvant treatment and for advanced treatment. Also, this drug is active against a wide range of solid tumors such as breast, stomach, pancreas, esophagus, liver, head and neck, and anal cancers. The main toxicities are bone marrow suppression, digestive toxicity in the form of mucositis and diarrhea, skin toxicity in the form of hand-foot syndrome, and neurotoxicity. Also, as can be seen from the above, 5-FU affects the synthesis phase of the cell cycle (Longley *et al*., 2003).

Lemongrass (LG) with the scientific name *Cymbopogon citratus* (*C. citrates*) belongs to the Poaceae family, which is widely distributed in tropical and subtropical regions of the world. LG is native to India and Sri Lanka (Esmort *et al*., 1998). This perennial plant grows in dense clumps; its height is 1.8 meters and its diameter is about 1.2 meters. It has long stems and rough, thin, and long leaves with a width of 1.3-2.5 cm and a length of 90 cm, which emits a lemon-like smell when broken (Yazdani *et al*., 2004). *C. citrates* species is widely cultivated in several Asian and African countries due to the high amount of citral (70-80%) in its essential oil (Robbing, 1983). This plant is an important source of essential oil used in the food and health industries as a flavoring and perfumer. For this reason, it is also of interest to the pharmaceutical industry (Adeleke *et al*., 2001). Currently, the annual production of LG essential oil in the world is about 1300 tons (Tizianna *et al*., 1998). This plant has different chemical compounds that briefly include phenolics, steroids and fatty alcohols, tannins, flavonoids, and terpenoids (Ross, 2005). This plant has many uses such as food, medicinal and ornamental uses. Traditionally, this plant is used to treat cough, flu, headache, arthritis, epilepsy, malaria, and inflammatory diseases. The pharmacological activities of this plant include anti-diabetic, anti-mutagenic, anti-carcinogenic, anti-inflammatory, antioxidant, insecticidal, anti-AIDS, anti-blood pressure, anti-malarial, anti-skin poisoning, anti-kidney poisoning, and antimicrobial activities (Manvitha and Bidya, 2014).

In this study we aimed to investigate the possible protective effect of LG oil on intestinal tissue damage caused by 5-FU.

Materials and Methods

Preparation of LG essential oil: The aerial parts of young LG leaves were obtained from Mirnia Plant Farm in Babol city, Mazandaran province, Iran. Species confirmation was conducted by Sari Faculty of Agriculture and Natural Resources, Mazandaran province. Essential oil extraction was performed using the hydrodistillation method with a Clevenger apparatus. Gas chromatography analysis was then carried out to identify the chemical components present in the essential oil. For the chemical analysis of LG essential oil constituents, an Agilent 6890 gas chromatography-mass spectrometer was utilized. The system consisted of a column measuring 0.25 mm × 30 m × 0.25 µm and an Agilent technologies 5973 detector with inert MS. The oven temperature was programmed as follows: an initial temperature of 50°C, which was then increased at a rate of 15°C/minute until reaching 240°C, and held for 30 minutes. The samples were dissolved in hexane, and helium was used as the carrier gas. The injector volume was set at 1 µl, with an injector temperature of 300°C and a split ratio of 20:1. Both the injector and detector temperature were maintained at 280°C. To identify the compounds obtained, their retention times and mass spectra were compared with standards or their retention indices (RI) from published data. Additionally, their mass spectra were compared with the National Institute of Standards and Technology (NIST) library.

Preparation and maintenance of the experimental animals: 48 male albino Swiss mice, aged two weeks and weighing an average of 25-35 g were provided from the Pasteur Institute in the northern region of the country. The mice were then randomly allocated into six groups of eight. It is important to mention that all the experimental groups were housed in the same room, each in separate cages, and subjected to uniform rearing conditions, including ventilation and lighting.

The animals in the study were divided into different groups as follows: 1) Control group was given distilled water intraperitoneally and solvent of essential oil orally. 2) 5-FU group was given a single dose of 200 mg/kg of 5-FU intraventricularly to induce intestinal toxicity. 3) 5FU+LDLG group received a low dose (125 mg/kg) of LG essential oil in addition to 5-FU for six days. 4) 5FU+MDLG group received a medium dose (250 mg/kg) of LG essential oil in addition to 5-FU for 6 days. 5) 5FU+HDLG group received a high dose (500 mg/kg) of LG essential oil in addition to 5-FU for six days. 6) HDLG group received only a high dose (500 mg/kg) of essential oil for six days. On the seventh day, blood samples were taken from the animals, and they were euthanized using a carbon dioxide tank to collect

tissue samples for further biochemical and pathological investigations.

Examination of clinical symptoms and necropsy: During the experimental period, the clinical symptoms of the mice were carefully observed. On the seventh day, the mice were humanely euthanized, and blood samples were collected from their hearts. Subsequently, histopathological samples of the intestinal tissue were taken.

Preparation of tissue sections and histopathological Examinations of intestinal tissue: The tissue samples were placed in a 10% buffered formalin fixative solution for fixation. After the fixation process, the samples underwent various tissue processing steps, and thin slices, 5 µ thick, were prepared and stained with hematoxylin and eosin for examination under a light microscope. The severity of tissue damage was qualitatively assessed and the following scores were given to the severity of histopathological lesions: 0: None, 1: Mild, 2: Moderate, and 3: Severe (Mirzakhani *et al*., 2020). Imaging was conducted using a microscope camera for comparison between experimental groups.

Serum biochemical analyses: To determine the levels of malondialdehyde in the serum of the experimental groups, a commercial kit for malondialdehyde analysis was used. Tissue homogenate or standard samples were mixed with a prepared working solution and incubated in a bain-marie at 95°C. After centrifugation, the optical absorption of the solution was measured at a wavelength of 532 nm. The levels of superoxide dismutase (SOD) and catalase enzymes were measured following the instructions of the Navand Salamat kit (Iran).

Statistical analyses: The results of the pathological analyses were interpreted using Graph Pad Prism version 8.01. Because of the semi-quantitative nature of data obtained from histopathological changes, Kruskal-Wallis and post hoc Dunn's multiple comparison tests were performed. The values of histological changes were presented as quartiles minimum value, first quartile, median, third quartiles, and maximum value. Biochemical results were analyzed using SPSS software version 24, with results reported as mean and standard deviation. One-way ANOVA and Tukey's post hoc test were used to assess differences between experimental groups.

Results

Gas chromatography-spectroscopy: The gas chromatography-mass spectrometry results of LG essential oil were presented in Table 1. It is evident that geranial and neral, two isomers of citral, constitute 49.38% and 35.54% of essential oils, respectively. These two compounds were the predominant components of the essential oil, with a combined total of approximately 85%. This indicated that citral is a major constituent of LG essential oil.

Histopathological results: Transverse sections of intestinal tissue were utilized to assess the protective impact of LG essential oil on damages induced by the chemotherapy drug 5-FU in mice.

Table 1. Chemical components of lemongrass (LG) essential oil.

No	Component[a]	Calculated RI[b]	Abundance (%) [c]
1	Myrcene	991	10.64
2	(Z)-β-Ocimene	1037	0.52
3	(Z)-β-Ocimene	1056	0.26
4	Linalool	1099	0.92
5	Citronellal	1149	0.34
6	Citronellol	1225	0.61
7	Neral d	1238	35.54
8	Geranial d	1266	49.38
9	Geranyl acetate	1379	0.34
	Total identified (%)	-	98.55

[a] Compounds were listed in order of their elution from a HP-5MS column. [b] Linear retention index on HP-5MS column, experimentally determined using homologous series of C_8-C_{30} alkanes. [c] Relative percentage values were means of three determinations with a RSD% in all cases below 10%. [d] [a]Neral and geranial were isomers of citral hence when combined approximately 85 % in this study

Microscopic images were depicted in Fig. 1, showcasing the severity of lesions presented as scores (Fig. 2). Histopathological analysis of intestinal tissue slides from various treatment groups revealed that a single dose of 5-FU led to degenerative damage, epithelial destruction, villi shortening, and edema in the small intestine (Fig. 1B and Fig. 2). Oral administration of LG essential oil at 125 mg/kg did not significantly affect the severity of intestinal tissue damage (Fig. 1C and Fig. 2). In contrast, doses of 250 and 500 mg/kg resulted in a notable reduction of these damages. The highest protective effect was observed with the highest dose (500 mg/kg) (Fig. 1D, Fig. 1E, and Fig. 2). Furthermore, it was noted that

administering LG essential oil at 500 mg/kg did not induce any notable pathological effects in the intestine (Fig. 1F and Fig. 2).

Biochemical Results: Biochemical analysis results of the serum from experimental groups were presented in Table 2. The analysis revealed an increase in malondialdehyde (MDA) levels, a byproduct of lipid peroxidation, in the plasma of mice treated with 5-FU.

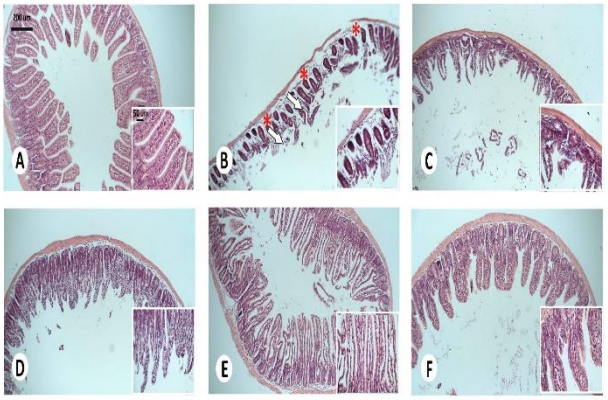
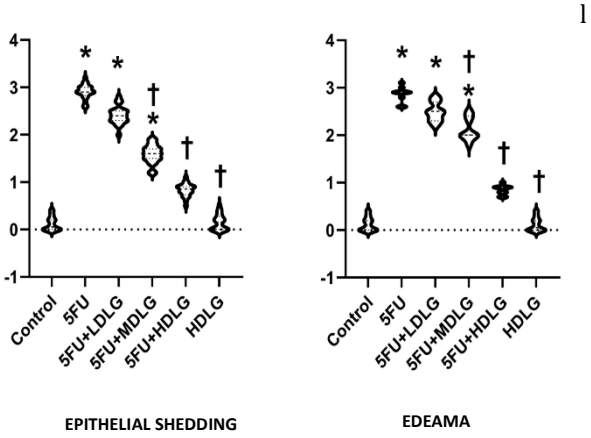


Fig. 1. Micrographs of intestinal tissue of different experimental groups. A- Cross-section of the intestinal tissue of the control group B- Cross-section of the intestinal tissue treated with a single injection dose 5- Fluorouracil (5-FU): shedding of mucus and shortening of the intestinal villi (arrowhead) with severe edema in the intestinal mucosa (*) C, D, and E - Cross-sections of intestinal tissue in the groups receiving lemongrass (LG) essential oil with low, medium and high doses, respectively, along with a single injection dose. F: Cross section of intestinal tissue in the group receiving LG essential oil alone: normal structure of intestinal tissue (H&E, 100 & 400x)



oil in the 5-FU+HDLG group resulted in a significant reduction of MDA in the serum ($p < 0.05$). The SOD enzyme levels in the 5-FU and 5-FU + LDLG groups were notably lower compared to the control group, with statistical significance ($p < 0.001$) and ($p < 0.01$) respectively. However, the inclusion of LG essential oil in the 5-FU + MDLG and 5-FU + HDLG groups brought the SOD levels closer to those observed in the control group. The HDLG group exhibited significantly higher SOD levels than the 5-FU group. The catalase enzyme levels in the 5-FU and 5-FU + LDLG groups showed a significant decrease compared to the control group, with statistical significance ($p < 0.001$) and ($p < 0.05$) respectively. Conversely, the catalase levels in the 5-FU + MDLG, 5-FU + HDLG, and HDLG groups demonstrated a significant increase compared to the 5-FU group, with statistical significance ($p < 0.05$) and ($p < 0.01$) respectively.

Table 2. Effects of lemongrass essential oil in different doses on induced serum level changes of MDA, SOD, and catalase in mice.

	Control	5-FU	5-FU+ LDLG	5-FU+M DLG	5-FU+H DLG	HDLG
MDA (nmol/ ml)	73.71 ± 12.77	97.14 ± 13.82 [b]	85.14 ± 17.07[b]	87.61 ± 12.23[b]	70.46 ± 14.85[a]	74.39 ± 9.88 [a]
SOD (u/ml)	216.24 ± 1.87	208.17 ± 1.22 [b]	209.20 ± 4.10 [b]	212.39 ± 1.54 [a]	213.16 ± 2.21[a]	213.58 ± 2.92[a]
Catalase (nmol/ min/ml)	6.69 ± 0.71	5.24 ± 0.53 [b]	5.52 ± 0.40 [b]	6.8 ± 0.41 [a]	6.86 ± 0.60 [a]	6.65 ± 0.56 [a]

MDA: Malondialdehyde; SOD: Superoxide dismutase. a indicated significant differences at p <0.05 in comparison to 5-FU and b indicated significant differences at p <0. 05 in comparison to control.

Fig. 2. The results of histopathological examinations of the severity of lesions in the intestinal tissue in different experimental groups. * Means that it was significant with the control group. † Means that it was significant with 5-FU receiving group ($p < 0.05$).

Discussion

Cancer is a prevalent cause of death worldwide, and chemotherapy is a common treatment method. Chemotherapy drugs target different phases of the cell cycle, leading to various acute and long-term side effects. While some progress has been made in managing these side effects, solutions have not been found for all cases (Launay *et al*., 2007; Rosner and Perazella, 2017; Krishnamurthi and Macaron, 2019). The purpose of a current study was to explore the effects of LG essential oil on the complications caused by the chemotherapy drug 5-FU in the intestines of mice. The study aimed to evaluate whether the essential oil can provide protection when used alongside 5-FU. This study revealed that a single dose of 5-FU led to degenerative damage, epithelial destruction, villi shortening, and edema in the small intestine. These findings were in line with the previous studies (Leocádio *et al*., 2015; Fideles *et al*., 2020; Gan *et al*., 2020). Oral administration of LG essential oil in different doses could reduce the severity of the lesions induced by 5-FU but the most significant reduction was observed when the animals were treated with the highest dose (500 mg/kg), although the medium dose of the LG essential oil was also significantly effective against the toxicity induced by 5-FU on intestinal tissue. Similar findings were reported by researchers about some essential oils or herbal extracts previously (Zheng *et al*., 2019; Lotfi *et al*., 2021). The biochemical analysis of our experiment revealed a significant reduction in the levels of SOD and catalase along with a notable increase of MDA amounts in the serum following the administration of a single dose of 5-FU. The effect of 5-FU on serum markers was consistent with other research findings (Fideles *et al*., 2020; Lotfi *et al*., 2021) indicating that oxidative stress is involved in 5-FU toxicity. 5-FU-induced excessive oxidative stress has been reported repetitively in multiple researches (Numazawa *et al*., 2011; Lamberti *et al*., 2012; Rtibi *et al*., 2018). Our analyses showed that LG essential oil contains geranial and neral, two isomers of citral, constitute 49.38% and 35.54% of essential oils, this indicated that citral is a major constituent of LG essential oil. Citral has been reported to have strong antioxidative properties (Guimarães *et al*., 2011; shi *et al*., 2016). Tissue damage by toxic compounds is due to the phenomenon of oxidative stress related to the disruption of the balance between oxidants such as free radicals and antioxidants. In this research, oxidative damage induced by 5-FU was significantly reduced in the 5-FU + HDLG group compared to the 5-FU group, which indicates the antioxidant property of LG essential oil which has led to inhibiting 5-FU related free radicals' destructive effects. Antioxidant enzymes such as superoxide dismutase and catalase decreased in the 5-FU group, but the levels of superoxide dismutase and catalase increased with the consumption of LG essential oil, which has antioxidant properties. Therefore, it can be concluded that the reduction of the amount of tissue damage in the treatment groups with medium and high doses of LG essential oil is due to the presence of antioxidant compounds in this substance and through the reduction of oxidative damage caused by free radicals resulting from 5-FU.

Acknowledgment

This research has been financially supported by a grant from the Amol University of Special Modern Technologies, Amol, Iran.

Conflict of Interest

The authors do not have any potential conflict of interest to declare.

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