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Protective effects of *Achillea millefolium* on 5-fluorouracil-induced intestinal mucositis in a Wistar rat model

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Abstract

Introduction: Cancer remains a major global health concern, with chemotherapy being a cornerstone of treatment. However, chemotherapy drugs, such as 5-fluorouracil (5-FU), are associated with severe side effects, including intestinal mucositis. *Achillea millefolium*, a medicinal plant with established anti-inflammatory and antioxidant properties, has been suggested as a potential protective agent against chemotherapy-induced toxicities.

Objectives: This study aims to investigate the ameliorative effects of *A. millefolium* on 5-FU-induced intestinal mucositis.

Materials and Methods: A total of 28 male Wistar rats were randomly assigned to four groups (n = 7/group). Group 1 (control) received normal saline by gavage for nine days. Group 2 received a single intraperitoneal injection of 5-FU (150 mg/kg) on day five. Group 3 was administered *A. millefolium* extract (250 mg/kg, gavage) for nine consecutive days. Group 4 received both *A. millefolium* extract (250 mg/kg, gavage) and a single intraperitoneal injection of 5-FU. The villus-to-crypt ratio, superoxide dismutase (SOD) activity, and catalase (CAT) levels were evaluated.

Results: Our study showed, 5-FU administration led to significant oxidative stress and severe intestinal mucosal damage. Body weight loss was pronounced in the 5-FU/saline group. Co-administration of *A. millefolium* extract with 5-FU significantly reduced oxidative stress, enhanced SOD activity, and improved the villus-to-crypt ratio compared to the 5-FU group.

Conclusion: The findings indicated that *A. millefolium* possesses protective effects against 5-FU-induced intestinal mucositis. These results suggest its potential role in mitigating chemotherapy-related side effects and enhancing patient outcomes.

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Introduction

Cancer is a leading cause of mortality, with an estimated 10 million cancer-related deaths recorded in 2023 (1). Chemotherapy remains an essential treatment modality; however, its adverse effects on healthy tissues limit its therapeutic efficacy (2). Fluoropyrimidine-based chemotherapeutic agent like 5-fluorouracil (5-FU), which a is widely administered in the treatment of various malignancies, including breast, bladder and colorectal cancers (3). A major complication of 5-FU therapy is intestinal mucositis, characterized by enterocyte proliferation impairment, villous atrophy, and increased oxidative stress (4-6). The pathophysiology of intestinal mucositis is multifactorial, involving inflammatory cytokines, oxidative stress, increasing reactive oxygen species (ROS) and epithelial destruction (7, 8). Elevated Malondialdehyde (MDA), nitric oxide (NO), and tumor necrosis factor-alpha (TNF-α) levels contribute to mucosal injury (9). Clinical manifestations include nausea, vomiting, diarrhea, and severe abdominal pain (10). Current treatments focus on symptom management, utilizing anti-inflammatory agents, systemic antibiotics, and analgesics (11). In modern pharmacotherapy, the use of medicinal plants with multiple properties is

Key point

Chemotherapy, especially with drugs like 5-fluorouracil (5-FU), can cause harmful side effects such as intestinal mucositis. This study explored whether *Achillea millefolium*, a plant with antioxidant and anti-inflammatory properties, could reduce this damage. Rats treated with both 5-FU and *A. millefolium* showed less oxidative stress, less tissue damage, and better enzyme activity compared to those given 5-FU alone. The plant extract also helped prevent weight loss and preserved intestinal structure. Overall, *A. millefolium* showed promise in protecting against chemotherapy-induced intestinal damage.

highly regarded. Therefore, finding natural products in this field that could prevent patients from experiencing side effects of chemotherapy drugs is of clinical importance. Antioxidants are substances that can protect the body against various types of oxidative damage caused by ROS (12). Yarrow plant (*Achillea millefolium*) belongs to the family Asteraceae and grows in different parts of Iran and the world. It contains essential oils, flavonoids, alkaloids, and tannins, and has various medicinal properties. The antioxidant properties of this plant, mainly attributed to its phenolic compounds (phenolic acids and flavonoids), are considered its most important benefits. Additionally, the plant has been observed to have antitumor, antimicrobial and liver-protective activity (13). *A. millefolium* is used in treating disorders associated with the gastrointestinal tract, including digestive problems, dyspepsia, abdominal pain, diarrhea, and stomachache (14). Previous studies suggest that *A. millefolium* have ameliorative effect on ulcerative colitis in mice model (15). Furthermore, *A. millefolium* have protective effects against cisplatin-induced ocular toxicity (16). Based on previous research highlighting this plant's effectiveness in treating digestive disorders and its protective properties against various chemotherapy agents, along with the diverse array of antioxidant compounds present in its extract, we aimed to evaluate its potential protective effects against 5-FU-induced intestinal mucositis.

Objectives

This study aimed to evaluate strategies for mitigating the adverse effects of chemotherapeutic agents on intestinal inflammation. Accordingly, comprehensive histological, biochemical, and serological assessments were performed in accordance with established protocols and previously published methodologies.

Materials and Methods

Plant material and extract preparation

Achillea millefolium was collected from southwestern Iran during the flowering season. Botanical identification was confirmed at the herbarium unit of Ahvaz Jundishapur University of Medical Sciences. The plant material was air-dried at room temperature, powdered, and subjected to ethanolic extraction (70% ethanol) over three days with continuous agitation. The extract was concentrated using a rotary evaporator and stored at 40°C until use. Phytochemical characterization was performed using a UV-Vis spectrophotometer. Total phenolic content was determined by the Folin-Ciocalteu method (17), ascorbic acid levels were measured using oxalic acid assays (18), and flavonoid content was quantified using $AlCl_3$ colorimetric methods (19).

Study design and animal model

Our experimental research was conducted using Twenty-eight male Wistar rats (230±30 g, 2-3 months old) were procured from Ahvaz Jundishapur university of medical sciences. Following acclimatization (one week, 12-hour light/dark cycle, temperature 22 ± °C), the rats were randomly assigned to four groups:

- Group 1 (control): Normal saline (gavage) for 9 days
- Group 2 (5-FU/saline, injury group): Normal saline (gavage) for 9 days+ single 5-FU injection (150 mg/kg, intraperitoneal, day 5)
- Group 3 (AM, extract group): *A. millefolium* extract (250 mg/kg, gavage) for 9 days
- Group 4 (5-FU/AM, treatment): *A. millefolium* extract (250 mg/kg, gavage) for 9 days+ single 5-FU injection (150 mg/kg, intraperitoneal, day 5)

Achillea millefolium extract was administered orally by gavage at a daily dose of 250 mg/kg, a safe dose that in accordance with dosing protocols established in previous studies (20,21). Intestinal mucositis was induced by intraperitoneal administration of 5-FU (Alembic Pharmaceuticals, India). Based on previous studies a single high dose of 5-FU (150 mg/kg) was injected on day 5 (22). In the control groups, an equal amount of normal saline was injected intraperitoneally. Previous studies have shown that the most significant effects of 5-FU occur three days after injection (23-25). On day 9 of the experiment, we collected blood via cardiac puncture after ensuring that the animal was under deep anesthesia with ketamine (60 mg/kg Sigma, Germany) and xylazine (15 mg/kg Sigma, Germany) intraperitoneal. The rats were then sacrificed, and a midline laparotomy was performed to access the abdominal cavity. Subsequently, the small intestine was removed for macroscopic and microscopic examination.

Macroscopic examination of small intestine

We made a longitudinal incision to open the small intestine. We then assessed congestion, edema, ulcerations, and hemorrhage (26).

Histopathologic examination of tissue

We fixed the intestines in 10% buffered formalin, embedded them in paraffin, cut them into 4-µm slices, and stained them with hematoxylin and eosin. Measurements of villus heights (from the villus tip to the villus-crypt junction) and crypt depths (defined as the depth of invagination between adjacent villi) were performed using a calibrated micrometer under light microscopy. It

is known that mucositis causes a decrease in villus height and an increase in crypt depth (27).

Stool assessment and diarrhea score

The severity of diarrhea was quantified using the Bowen scoring system, which classifies stool consistency as follows: 0 = normal stool; 1 = slightly wet and soft stool, indicative of mild diarrhea; 2 = wet and unformed stool, indicative of moderate diarrhea; and 3 = watery stool, indicative of severe diarrhea (28,29).

Examination of tissue inflammatory cytokine

The small intestine was dissected, and a supernatant of tissue was created. As we TNF-α is a cytokine that is measured for inflammatory responses (30). TNF-α concentrations were assessed using the ELISA (enzyme-linked immunosorbent assay) technique (Zell Bio kits, Germany), following the manufacturer's instructions.

Examination of antioxidant enzymes and oxidative stress

In line with similar studies, we investigated antioxidant enzyme activity and oxidative stress markers. The small intestine was dissected, and tissue homogenates were prepared to obtain supernatants. The concentrations of catalase (CAT), superoxide dismutase (SOD), MDA, and NO were measured using ELISA kits (ZellBio, Germany), following the manufacturer's instructions (31-34).

Statistical analysis

Statistical analysis was conducted using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Body weight, villus height, crypt depth, and other data were analyzed using one-way ANOVA with Tukey post hoc test for multiple comparisons. All data were expressed as mean± SEM except for disease severity scores which were expressed as medians and range. Meanwhile, values of $P < 0.05$ were considered significant

Results

Phytochemical analysis of Achillea millefolium extract

The qualitative and quantitative phytochemical composition of *A. millefolium* extract is presented in Table 1.

Table 1. Quantitative phytochemical analysis content of the analysis of *Achillea millefolium*

Phytochemical analysis	mg/g
Total phenol	13.5±0.6
Total flavonoid	12.8±0.4
Ascorbic acid	11.4±0.9

Table 2. Stool examination scores in rats assessed according to Bowen's diarrhea scoring system

Experimental groups		Diarrhea score		*P* value*
		Mean	SD	
G1 (n = 7)		0.0	0.0	0.05
G2 (n = 7)		2.643	0.3780	
G3 (n = 7)		0.143	0.3780	
G4 (n = 7)		2.143	0.6268	
Experimental groups		**Mean difference**	**Std. Error**	***P* value****
G1	G2	-2.6429*	0.2202	<0.001
	G3	-0.1429	0.2202	0.915
	G4	-2.1429*	0.2202	<0.001
G2	G1	2.6429*	0.2202	<0.001
	G3	2.5*	0.2202	<0.001
	G4	0.5	0.2202	0.133
G3	G1	0.1429	0.2202	0.915
	G2	-2.5*	0.2202	<0.001
	G4	-2.0*	0.2202	<0.001
G4	G1	2.1429*	0.2202	<0.001
	G2	-0.5	0.2202	0.133
	G3	2.0*	0.2202	<0.001

G1: Control group, G2: 5-FU group, G3: AM extract, G4: 5-FU/AM extract
SD: Standard deviation. * One-way ANOVA, ** Post hoc Tukey test.

Diarrhea score

According to Bowen's diarrhea score system, the diarrhea score of the rats was recorded. The extract group (G3) group did not suffer from diarrhea compared to control group ($P = 0.915$, Table 2). After 5-FU administration, 5-FU/AM and 5-FU group showed moderate or more diarrhea on day 7 and improved diarrhea on days 8 and 9. There was a statistically significant difference between the 5-FU and the 5-FU/AM group ($P = 0.133$, Figure 1).

Macroscopic examination of intestinal tissue

Figure 2 illustrates the macroscopic evaluation of the small intestine, focusing on vascular congestion, edema, and mucosal ulceration. The 5-FU group exhibited severe vascular congestion, extensive edema, and notable ulcer formation in the intestinal mucosa. In contrast, the 5-FU/AM-treated group demonstrated a significant reduction in vascular congestion and edema, suggesting a protective effect of *A. millefolium* against 5-FU-induced damage.

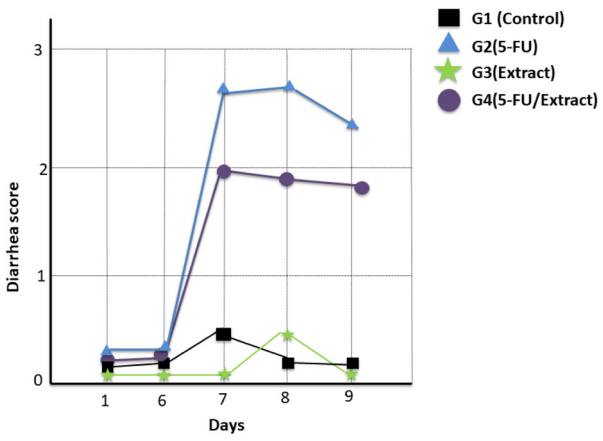

Figure 1. Diarrhea scoring in groups. The values are expressed as mean ± SEM (n=7). G1: control; G2: 150 mg/kg of 5-FU; G3: 250 mg/kg of AM (8 days); G4 250 mg/kg of AM (8 days) then 150 mg/kg of 5-FU.

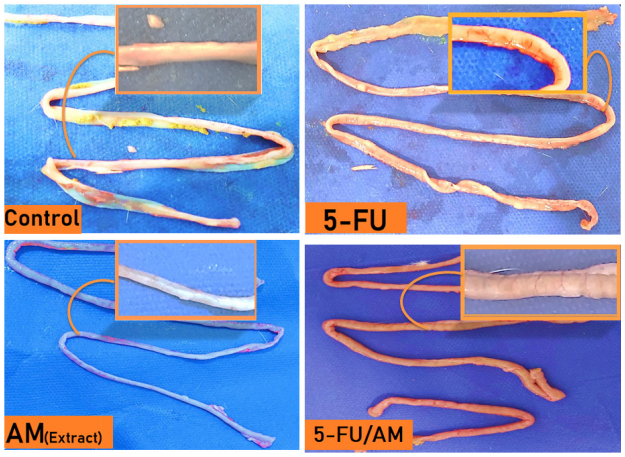

Figure 2. Small intestine in different groups (sections were magnified).

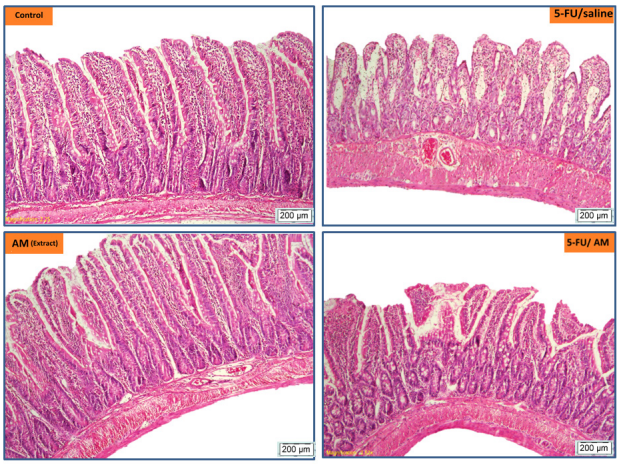

Figure 3. Histological findings of small intestines in rats in different groups (×40).

Microscopic examination of intestinal tissue

Histological analysis was performed to assess the impact of *Achillea millefolium* extract on intestinal architecture. The 5-FU group exhibited a disrupted intestinal mucosa, characterized by epithelial flattening, villous shortening, and thickening of the lamina propria due to inflammation. Crypt depth was significantly increased, indicating cellular proliferation in response to mucosal damage (Figure 3). Figure 4 provides a comparative overview of intestinal histology across experimental groups, with villous height and crypt depth values detailed in Table 3 and Table 4. Meanwhile, Table 3 shows the microscopic measurements and statistical analyses of villus height group by group and across the four study groups. Group 2 (5-FU group) exhibited a significant reduction in villus height compared to other groups ($P<0.05$), confirming the induction of intestinal mucosal injury. Groups 3 (AM group) and 4 (5-FU/AM) showed significant improvements in this parameter compared to group 2 ($P<0.05$), suggesting modulatory effects. With respect to crypt depth, a statistically significant increase in crypt depth was noted only in group 2 (5-FU) compared to other groups ($P<0.05$). There is no significant overall difference was observed between group 4 (5-FU/AM group) and group 1 and group 3 (Table 4). Moreover, Figure 4 provides a graphical representation of these differences.

Inflammatory cytokine levels

TNF-α level, assessed as an inflammatory marker, are presented in Table 5. In both overall and pairwise comparisons, group 2 (5-FU) exhibited a significant

Table 3. Morphometric measurements of villus height in the absorptive epithelium of the small intestine obtained by light microscopy (µm)

Experimental groups		Villus height (µm)		*P* value*
		Mean	SD	
G1 (n = 7)		386.4286	60.09675	0.05
G2 (n = 7)		215.5714	99.23853	
G3 (n = 7)		395.7143	43.41165	
G4 (n = 7)		343.5714	35.61768	
Experimental groups		**Mean difference**	**Std. Error**	***P* value****
G1	G2	170.85714*	34.44778	<0.001
	G3	-9.28571	34.44778	0.790
	G4	42.85714	34.44778	0.225
G2	G1	-170.85714*	34.44778	<0.001
	G3	-180.14286*	34.44778	<0.001
	G4	-128.0 *	34.44778	0.001
G3	G1	9.28571	34.44778	0.790
	G2	180.14286*	34.44778	<0.001
	G4	52.14286	34.44778	0.143
G4	G1	-42.85714	34.44778	0.225
	G2	128.0*	34.44778	0.001
	G3	-52.14286	34.44778	0.143

G1: Control group, G2: 5-FU group, G3: AM extract, G4: 5-FU/AM extract
SD: Standard deviation. * One-way ANOVA, ** Post hoc Tukey test.

Table 4. Morphometric measurements of crypt depth in the absorptive epithelium of the small intestine obtained by light microscopy (µm)

Experimental groups		Crypt depth (µm)		*P* value*
		Mean	SD	
G1 (n = 7)		190.0	11.44552	0.05
G2 (n = 7)		220.5714	14.86447	
G3 (n = 7)		187.5714	7.99702	
G4 (n = 7)		195.2857	7.31925	
Experimental groups		**Mean difference**	**Std. Error**	***P* value****
G1	G2	-30.57143*	5.79086	<0.001
	G3	2.42857	5.79086	0.975
	G4	-5.28571	5.79086	0.798
G2	G1	30.57143*	5.79086	<0.001
	G3	33.00000*	5.79086	<0.001
	G4	25.28571*	5.79086	0.001
G3	G1	-2.42857	5.79086	0.975
	G2	-33.00000*	5.79086	<0.001
	G4	-7.71429	5.79086	0.552
G4	G1	5.28571	5.79086	0.798
	G2	-25.28571*	5.79086	0.001
	G3	7.71429	5.79086	0.552

G1: Control group, G2: 5-FU group, G3: AM extract, G4: 5-FU/AM extract
SD: Standard deviation. * One-way ANOVA, ** Post hoc Tukey test.

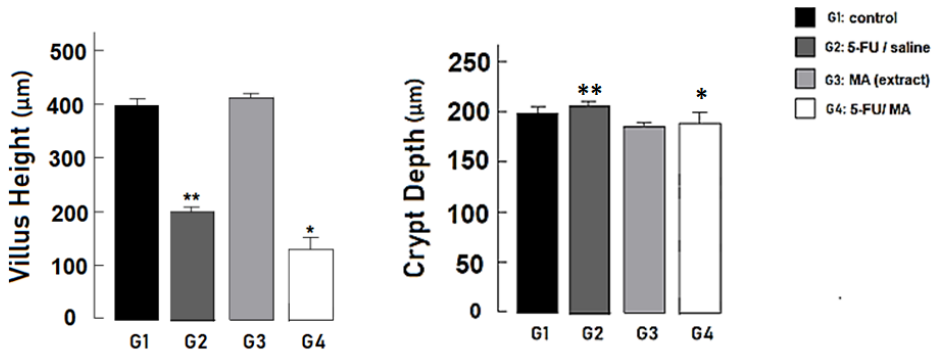

Figure 4. Quantitative value of histologic findings. Villus height, Crypt depth; The values are expressed as mean ± SEM (n=7), **Significant difference from the control group (G1) ($P < 0.05$), *Significant difference from the G2 group ($P < 0.05$).

increase in TNF-α expression compared to all other groups, particularly the control group ($P<0.05$). Group 4 (5-FU/AM group) showed a statistically significant reduction in TNF-α levels relative to group 2, indicating an anti-inflammatory response. Group 3 exhibited a significant decrease in this parameter compared to group 1 ($P<0.05$), suggesting the anti-inflammatory potential of the extract. Figure 5 provides a graphical representation of these differences.

Antioxidant activity

The concentrations of MDA, NO, SOD, and CAT in intestinal tissue are presented in the tables below**.** A significant increase in MDA and NO levels was observed in group 2 compared to the other groups ($P < 0.05$), indicating elevated oxidative stress (Tables 6 and 7). In contrast, group 4 showed a significant reduction in MDA and NO concentrations relative to group 2 ($P < 0.05$). Additionally, group 3 exhibited a significant decrease in these parameters compared to group 1 ($P < 0.05$), suggesting the antioxidant potential of the extract. Conversely, SOD and CAT levels were significantly decreased in group 2 compared to other groups ($P<0.05$). Treatment with the extract in group 4 led to a significant increase in both SOD and CAT levels compared to group 2 ($P<0.05$; Tables 8 and 9). Similarly, group 3 demonstrated significantly higher SOD and CAT activities compared to group 1 ($P<0.05$). Collectively, these findings indicate that the extract possesses notable antioxidant properties. A visual summary of these changes is provided in Figure 6.

Table 5. Morphometric measurements of crypt depth in the absorptive epithelium of the small intestine obtained by light microscopy (µm)

Experimental groups		TNF-α level (ng/g)		*P* value*
		Mean	SD	
G1 (n = 7)		47.1400	0.51643	0.05
G2 (n = 7)		105.3757	0.71561	
G3 (n = 7)		39.8743	0.86240	
G4 (n = 7)		54.0757	0.41243	
Experimental groups		**Mean difference**	**Std. Error**	***P* value****
G1	G2	-58.23571*	0.34771	<0.001
	G3	7.26571*	0.34771	0.001
	G4	-6.93571*	0.34771	<0.001
G2	G1	58.23571*	0.34771	<0.001
	G3	65.50143*	0.34771	<0.001
	G4	51.30000*	0.34771	<0.001
G3	G1	-7.26571*	0.34771	<0.001
	G2	-65.50143*	0.34771	<0.001
	G4	-14.20143*	0.34771	<0.001
G4	G1	6.93571*	0.34771	<0.001
	G2	-51.30000*	0.34771	<0.001
	G3	14.20143*	0.34771	<0.001

TNF-α: Tumor necrosis factor-α, G1: Control group, G2: 5-FU group, G3: AM extract, G4: 5-FU/AM extract SD: Standard deviation. * One-way ANOVA, ** Post hoc Tukey test.

Discussion

Chemotherapeutic agents, particularly 5-FU, remain essential in the treatment of various malignancies (2). However, their clinical use is often limited by severe adverse effects, most notably gastrointestinal toxicity, which significantly impairs patients' quality of life and treatment adherence (4,5). Damage to the small intestinal mucosa is largely attributed to increased oxidative stress and a concomitant decrease in antioxidant defenses within

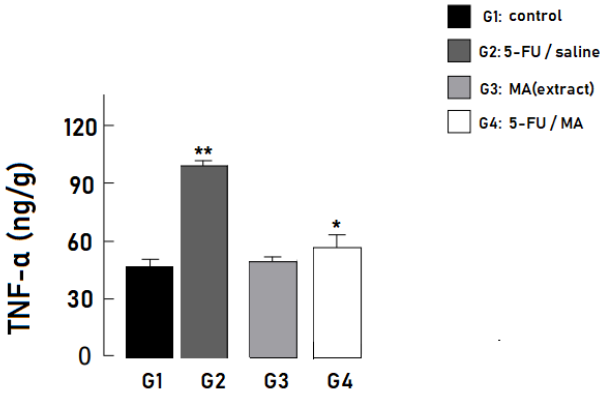

Figure 5. Concentration of tumor necrosis factor-alpha (TNF-α) in the intestine. The values are expressed as mean ± SEM (n=7), **Significant difference from the control group (G1) ($P < 0.05$). *Significant difference from the G2 group ($P < 0.05$).

Table 6. Malondialdehyde (MDA) concentration in small intestinal tissue determined by ELISA

Experimental groups		MDA (nmol/mL)		*P* value*
		Mean	SD	
G1 (n = 7)		0.9314	0.009	0.05
G2 (n = 7)		3.2171	0.10242	
G3 (n = 7)		0.8014	0.01676	
G4 (n = 7)		1.2357	0.25585	
Experimental groups		**Mean difference**	**Std. Error**	***P* value****
G1	G2	-2.28571*	0.07451	<0.001
	G3	0.13	0.07451	0.324
	G4	-0.30429*	0.07451	0.002
G2	G1	2.28571*	0.07451	<0.001
	G3	2.41571*	0.07451	<0.001
	G4	1.98143*	0.07451	<0.001
G3	G1	-0.13	0.07451	0.324
	G2	-2.41571*	0.07451	<0.001
	G4	-0.43429*	0.07451	<0.001
G4	G1	-0.30429*	0.07451	<0.001
	G2	-1.98143*	0.07451	<0.001
	G3	-0.43429*	0.07451	<0.001

G1: Control group, G2: 5-FU group, G3: AM extract, G4: 5-FU/AM extract SD: Standard deviation. * One-way ANOVA, ** Post hoc Tukey test.

Table 7. Nitric oxide (NO) concentration in small intestinal tissue determined by ELISA

Experimental groups		NO (µmol/g)		*P* value*
		Mean	SD	
G1 (n = 7)		5.4714	0.20432	0.05
G2 (n = 7)		25.2914	0.17418	
G3 (n = 7)		4.2271	0.14874	
G4 (n = 7)		6.1143	0.7231	
Experimental groups		**Mean difference**	**Std. Error**	***P* value****
G1	G2	-19.82000*	0.08487	<0.001
	G3	1.24429*	0.08487	<0.001
	G4	-0.64286*	0.08487	<0.001
G2	G1	19.82000*	0.08487	<0.001
	G3	21.06429*	0.08487	<0.001
	G4	19.17714*	0.08487	<0.001
G3	G1	-1.24429*	0.08487	<0.001
	G2	-21.06429*	0.08487	<0.001
	G4	-1.88714*	0.08487	<0.001
G4	G1	.64286*0	0.08487	<0.001
	G2	-19.17714*	0.08487	<0.001
	G3	1.88714*	0.08487	<0.001

G1: Control group, G2: 5-FU group, G3: AM extract, G4: 5-FU/AM extract SD: Standard deviation. * One-way ANOVA, ** Post hoc Tukey test.

Table 8. Superoxide dismutase (SOD) concentration in small intestinal tissue determined by ELISA

Experimental groups		SOD (U/cg Hb)		*P* value*
		Mean	SD	
G1 (n = 7)		46.7086	0.68548	0.05
G2 (n = 7)		11.8043	3.67504	
G3 (n = 7)		75.3943	0.65169	
G4 (n = 7)		35.5457	1.00123	
Experimental groups		**Mean difference**	**Std. Error**	***P* value****
G1	G2	34.90429*	1.04891	<0.001
	G3	-28.68571*	1.04891	<0.001
	G4	11.16286*	1.04891	<0.001
G2	G1	-34.90429*	1.04891	<0.001
	G3	-63.59000*	1.04891	<0.001
	G4	-23.74143*	1.04891	<0.001
G3	G1	28.68571*	1.04891	<0.001
	G2	63.59000*	1.04891	<0.001
	G4	39.84857*	1.04891	<0.001
G4	G1	-11.16286*	1.04891	<0.001
	G2	23.74143*	1.04891	<0.001
	G3	-39.84857*	1.04891	<0.001

G1: Control group, G2: 5-FU group, G3: AM extract, G4: 5-FU/AM extract SD: Standard deviation. * One-way ANOVA, ** Post hoc Tukey test.

Table 9. Catalase (CAT) concentration in small intestinal tissue determined by ELISA

Experimental groups		CAT (U/mg Hb)		*P* value*
		Mean	SD	
G1 (n = 7)		101.9057	0.95807	0.05
G2 (n = 7)		28.1343	0.53553	
G3 (n = 7)		109.9614	0.6803	
G4 (n = 7)		97.4543	0.308	
Experimental groups		**Mean difference**	**Std. Error**	***P* value****
G1	G2	73.77143*	0.3548	<0.001
	G3	-8.05571*	0.3548	<0.001
	G4	4.45143*	0.3548	<0.001
G2	G1	-73.77143*	0.3548	<0.001
	G3	-81.82714*	0.3548	<0.001
	G4	-69.32*	0.3548	<0.001
G3	G1	8.05571*	0.3548	<0.001
	G2	81.82714*	0.3548	<0.001
	G4	12.50714*	0.3548	<0.001
G4	G1	-4.45143*	0.3548	<0.001
	G2	69.32*	0.3548	<0.001
	G3	-12.50714*	0.3548	<0.001

G1: Control group, G2: 5-FU group, G3: AM extract, G4: 5-FU/AM extract SD: Standard deviation. * One-way ANOVA, ** Post hoc Tukey test.

intestinal tissues (6).

In recent years, natural compounds with strong antioxidant properties have gained attention for their potential to mitigate chemotherapy-induced toxicity without compromising therapeutic efficacy (12). *Achillea millefolium*, rich in bioactive compounds such as caffeic acid, ferulic acid, rosmarinic acid, quercetin, and rutin, has demonstrated protective effects in various oxidative stress models (13,14). The present study evaluated whether *A. millefolium* extract could attenuate 5-FU-induced intestinal mucosal injury.

As expected, histopathological analysis confirmed the destructive effects of 5-FU, including villus disorganization, mucosal thickening, congestion of the intestinal wall, and impaired absorption of water and nutrients (26,27). These alterations were associated with diarrhea, as confirmed by stool assessment and diarrhea scoring (28). Notably, co-administration of *A. millefolium* extract (group 4)

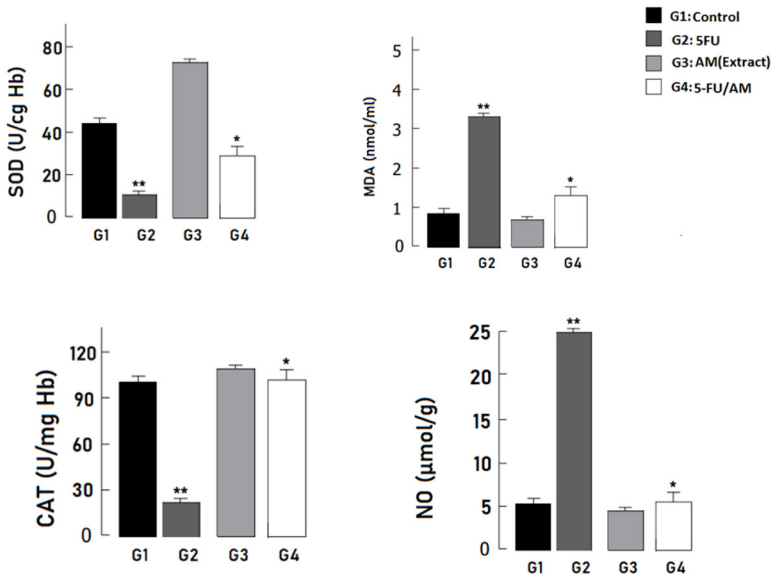

Figure 6. Concentration of superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT), and nitric oxide (NO) in the intestine. The values are expressed as mean ± SEM (n=7), **Significant difference from the control group (G1) ($P < 0.05$). *Significant difference from the G2 group ($P < 0.05$).

markedly reduced diarrhea severity compared to the 5-FU-only group, suggesting improved absorptive function.

Histomorphometric evaluation of villus height and crypt depth remains a reliable method for assessing intestinal mucosal injury and regeneration in experimental chemotherapy models (29). In contrast, co-treatment with *A. millefolium* extract significantly preserved villus height and normalized crypt depth, indicating protection of mucosal architecture. Animals receiving the extract alone (group 3) showed no histopathological abnormalities relative to controls, confirming its tolerability at the administered dose.

Inflammatory responses were evaluated via TNF-α, a key pro-inflammatory cytokine implicated in chemotherapy-induced intestinal injury (30). TNF-α expression was significantly elevated in the 5-FU group, reflecting robust inflammation and epithelial damage, but markedly reduced in the co-administration of *A. millefolium* extract (group 4). Group 3 also showed reduced TNF-α compared to control, supporting the anti-inflammatory activity of the extract.

Oxidative stress analysis revealed a significant decrease in CAT (31) and SOD (32) activities in the 5-FU group, consistent with antioxidant depletion. Both group 3 and group 4 exhibited significantly higher CAT and SOD activities, with partial restoration in the co-treatment group despite ongoing 5-FU exposure. Lipid peroxidation marker MDA (33,34) and nitrosative stress marker NO (35-38) were elevated in the 5-FU group, whereas co-treatment significantly reduced both. Group 3 also exhibited lower MDA and NO levels compared to controls, reinforcing the extract's antioxidant potential.

Collectively, these results suggest that *A. millefolium* extract protects against 5-FU-induced intestinal injury through enhancement of endogenous antioxidant defenses, suppression of oxidative and nitrosative stress, and attenuation of pro-inflammatory signaling. These findings are consistent with previous studies showing that 5-FU-induced intestinal injury is driven by oxidative stress and inflammation, ultimately leading to apoptosis and mucosal damage.

While the translational potential of *A. millefolium* is promising, it should be emphasized that these findings are based on an animal model. Further studies are warranted to elucidate the precise molecular pathways involved, evaluate dose–response relationships, and confirm safety and efficacy in clinical settings. Pending successful translation to human models, *A. millefolium* could be developed into pharmaceutical formulations such as herbal tablets, teas, or other delivery systems to support gastrointestinal health during chemotherapy.

In summary, the present study demonstrates that *A. millefolium* extract attenuates 5-FU-induced intestinal mucosal injury by enhancing antioxidant enzyme activity, reducing oxidative and nitrosative stress, and suppressing pro-inflammatory cytokine production. Functional improvements, including reduced diarrhea severity and preservation of villus architecture, further support its protective role. These findings highlight *A. millefolium* as a promising candidate for adjunctive therapy during chemotherapy, although confirmation in human clinical trials is essential before clinical application.

Conclusion

This study provides novel evidence of *A. millefolium's* protective effects against 5-FU-induced intestinal mucositis. By attenuating oxidative stress and inflammatory responses, this medicinal plant offers potential as an

adjunctive therapy to reduce chemotherapy-associated toxicities. Further clinical investigations are warranted to explore its therapeutic applications in cancer patients.

Authors' contribution

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Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

Ethical approval for this study was obtained from the Animal Care and Ethics Committee (ACEC) of the Abadan University of Medical Sciences (Ethical code#IR.ABADANUMS.REC.1399.134). All experimental protocols were conducted in compliance with the regulations of the Research Ethics Committee of this University and Iranian Ethical Guidelines for the use of animals in research. Additionally, all animal experiments were conducted in accordance with protocols approved by the United States National Institutes of Health (NIH, 1978). Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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