Restoring of aspirin disrupted intestinal mucosa barrier by *Alhagi graecorum* alcoholic extract

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Abstract

The current study aims to examine the effect of *Alhagi graecorum* ethanolic extract on intestinal function and histopathological changes in male rabbits. The experiment employed twenty male rabbits. The animals were equally separated into four groups and given treatment for 42 days as follows: control group (C): Rabbits were allowed free supply to drinking water, T1 group: Animals received (10 mg /kg b.w) of aspirin orally, T2 group: were administered 400 mg/ kg b.w ethanolic extract of *Alhagi graecorum* and 10 mg/kg of aspirin orally , T3 group: animals were given 400 mg/ kg b.w alcoholic extract of *Alhagi graecorum* orally. blood samples collected at the end of the experiment from fasting animals to assess serum tumor necrosis factor alpha (TNF-α) and Citrulline levels. Immediately after animals were scarified, small piece from the duodenum was collected for assessment of Occludin protein (ocln gene) expression, small intestine (duodenum) was removed for histopathological examination. The results shown that oral administration of Aspirin for 42 days caused intestinal damage manifested by a substantial decline ($P<0.05$) in ocln gene and serum level of citrulline in the T1group, whereas the animals given *Alhagi graecorum* showed a considerable elevation ($P<0.05$) in the levels of ocln gene and citrulline. While serum level of TNF-α significantly ($P<0.05$) elevated in T1 group comparison with other groups. The histopathological examination revealed pathological changes in aspirin group while giving *alhagi graecorum* alcoholic extract with and without aspirin was effective in modified these changes into semi normal. The alcoholic extract of *Alhagi graecorum* improved intestinal functions and protected intestinal tissue from damage induced by Aspirin.

Keywords: Aspirin, *Alhagi graecorum*, Occludin protein, TNF-α, citrulline, Histopathology of intestinal muosa.

Introduction

Alhagi graecorum Boiss is a herb grows in Iraq and belongs to the family Fabaceae (Elsaied *et al*. 2018). It's an evergreen plant that have thorny twigs (Salama *et al*. 2022). *Alhagi graecorum* is also called camel thorn (El-Hak *et al*. 2019), and In Iraq

is known as Al-Aqoul (Hashim *et al*. 2022). Flavonoids of various types have been isolated and recognized from *Alhagi graecorum* (Ahmad *et al*. 2015). Additionally, there are a number of other polyphenolic compounds, including phenols, alkaloids, resins, terpenoids, as well as other secondary metabolites. *Alhagi graecorum* has diverse bioactive effects, such as antioxidant activity, antimicrobial, cytotoxic and anti-proliferative effects. Therefore, it is used in a variety of pharmaceuticals applications (Salama *et al*. 2021; Sumaiya *et al*. 2022; Saleh and Madany 2014). Besides to its anti-nociceptive, anti-inflammatory, diuretic, muscle relaxant, antipyretic, gastrointestinal, hepatroprotective, antidiarrheal, antiulcerogenic, anti-cancer properties and anti-fungal (Hamad *et al*. 2021; Abd El-hak *et al*. 2019; Tavassoli *et al*. 2020). Furthermore it is used for chronic migraines as an analgesic (Al-Edany 2021). Aspirin has various medicinal values in both veterinary medicine and human. a wide range of anti-inflammatory, antipyretic, analgesic and antiplatelet therapeutic activities (Najwan and Shatha 2009 ;Warner and Mitchell, 2002; Simon *et al*. 2020). It is also beneficial for urinary tract and liver infections and rheumatism, also it is utilize as a schistosomiasis repellent and a laxative (Muhammad *et al*. 2015). The antiplatelet effects of aspirin exerted by suppressing cyclooxygenase-1 action and inhibiting prostaglandin synthesis. However, the decline in prostaglandins induce whole intestinal mucosal damage as well as gastric ulcer (Al-Shaha and Mohammed 2017;Hara *et al*. 2018). Despite the large volume of research on aspirin's effect on the mucosal layer of the stomach (Al-Timimi, 2020 **;** Arul *et al*. 2023), its effect on the lining mucosa of the intestine has not been made clear. Research indicates that the absorption of aspirin occurs primarily in the acidity of the stomach, but it has not been observed to have an effect on the lining of the intestine. Consequently, the current study aimed to identify the effect of aspirin on the structure, function, as well as permeability of the intestinal lining, and on the other hand, to study the efficiency of the plant in reducing these effects.

Material and Methods

Preparation of plant extracts

Alhagi graecorum plant was collected in March 2022 from the river area southern Baghdad city, Iraq. The plant samples were cleaned, shade-dried, crushed and for each 50 gm mixed with aqueous ethanol 70% kept in shaker at (35c˚) for (72 hr.) and then thoroughly filtered via different size filters, then placed in incubator for about 3 days The filtrate was concentrated (approximately 10 g / 100 g crushed plant) and used to make a diluent by normal saline. (Marashdah and AL-Hazimi 2010).

Experimental design

Twenty healthy adult male rabbits, weighted (1500- 1750 g) were divided into four equal groups at random.: control group (C): The rabbits in this group were given unlimited access to drinking water., T1 group: Animals received (10 mg /kg b.w) of aspirin orally , T2 group: were administered 400 mg/ kg b.w alcoholic extract of *Alhagi graecorum* and 10 mg/kg of aspirin orally , T3 group: Orally administered 400 mg/kg b.w. alcoholic extract of *Alhagi graecorum*. After 42 days of experiment blood samples collected from the heart for measurement of TNF-α and citrulline. Rabbits were anesthetized, sacrificed via drag the blood from the heart, Immediately after animals were scarified, small piece (100 mg) from the duodenum was collected

for assessment of Occludin protein (ocln gene) expression , after that small intestine was excised, opened longitudinally, washed with normal saline and conserved in 10% neutral buffered formalin, then prepared for paraffin embedding technique and partitioned at 6 µm with rotary microtome, after that stained with hematoxylin and eosin stain (Bancroft and Marilyn, 2008).

Estimation of Serum Citrulline

Serum Citrulline was measured by using ELISA kit from Sunlong Biotech Co., Ltd.

Estimation of serum tumor necrosis factor Alpha (TNFa).

TNF-α concentrations was evaluated following the procedure of a specific rabbits ELISA kit (ELK Biotechnology, china).

PCR detection of occluding gene expression

The integrity of intestinal mucosa of the present experimental animal determined by evaluates the gene expression of the *Ocln.* Protein which is the dominant tight junction protein. Total RNA was extracted from duodenum samples (100mg) using Easy-spin™ (DNA free) total RNA extraction Kit, iNtRON biotechnology, South Korea. RNA isolated according to the manufacturer's protocol. The extracted RNA samples were quaintified (ng/µL), and qualified by using a nanodrop spectrophotometer (Thermo.USA) to measure absorbance at 260 and 280 nm. Total RNA samples were used in the cDNA synthesis step with the AccuPower ® RocktScript RT PreMix Kit from Bioneer, Korea. We used Protocol of GoTaq® 1-Step RT-qPCR System for Real-Time qPCR (Gene expression assay). The relative expression of target genes in duodenum tissue was calculated (2^-ΔΔCT). That dependent on normalization of RT-qPCR (CT values) of target genes relatively to housekeeping gene (GAPDH) as reference gene in control and different treatment groups. The primers for duodenum occludin and the house keeping genes (GAPDH) are listed in table 1.

Table 1: The Primers for Ocln target gene and GAPDH housekeeping gene.

Gene	Primer name	Sequence 5'-3'	Product Size/bp	Accession numbers
Occludin	F	GAACCTACGGAACTGGCTTAC	121	XM_008262319.2
	R	GTGTAGCCTCCATAGCCATAAC		
GAPDH	F	TGGTGAAGGTCGGAGTGAAC	121	NM_001082253.1
	R	ATGTAGTGGAGGTCAATGAATGG		

Statistical analysis

Data is displayed as the Mean± SE. Within the SPSS program, data was analyzed using two-way analysis. LSD was used to test the means at a probability level of ($p<0.05$).

Results

The histopathological examination of the control groups (fig.4&5) revealed normal intestinal villi, normal cytoarchetecture of the goblet cells, enterocytes and intestinal glands. While the histopathological examination of duodenum in T1 group represented in showed sever enteritis which characterize by severe damage of most intestinal, sever epithelial sloughing, luminal tissue debris and submucosal vascular congestion (fig.6). The magnified figures revealed sever damage of villus with marked epithelial sloughing, thickness of villus associated with infiltration of mono nuclear leukocytes mainly lymphocytes and macrophages (fig.7).). In T2 group The histopathological figures of duodenum showed Normal appearance of duodenal characterized mild apical epithelial hyperplasia, marked increased in submucosal Brunner's glands and increase number of goblet cells (fig. 8 & 9). While in T3 group the histopathological figures of duodenum showed normal appearance of epithelium of villus which revealed significant increase in height and thickness that associated with hyperplasia of lamina propria and increased in width of the lacteal vessels. The depth of intestinal glands (epithelial crypts) also revealed significant increased with marked increase in population of goblet cells. The submucosal layer also revealed marked increased in thickness of Brunner's glands (fig.10&11). The magnification figure revealed normal appearance of enterocytes that showed mild hyperplasia (fig.12).

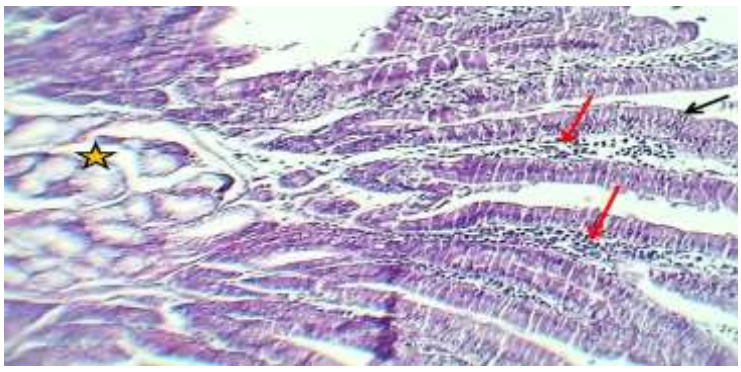

Figure 4: section of duodenum (Control) shows: Normal appearance of epithelium of villus (Black arrow), lamina propria (Red arrows) & Brunner's glands (Asterisk). (H&E stain.100x)

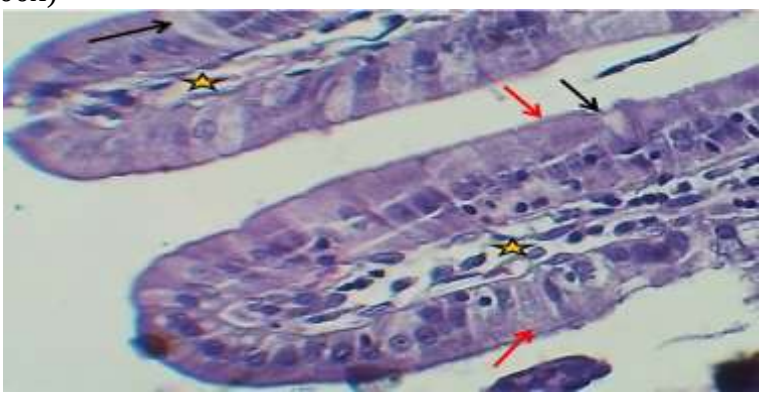

Figure 5: section of duodenum (Control) shows: Normal appearance of goblet cells (Black arrow), enterocytes (Red arrows) & cellular lamina propria (asterisks). (H&E stain.400x)

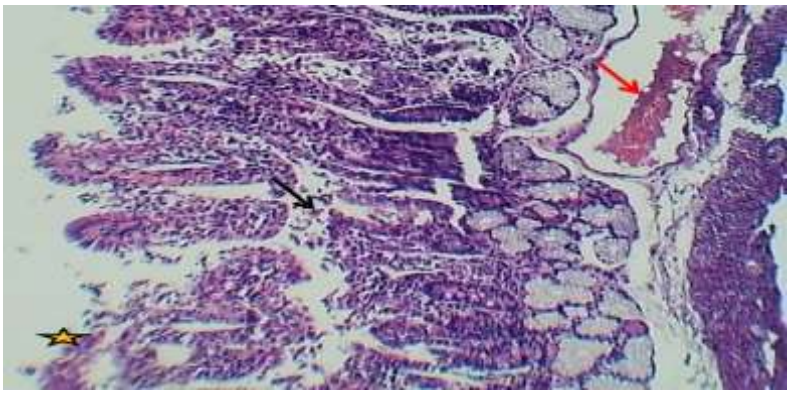

Figure 6: section of duodenum (T1) shows: sever enteritis characterize by severe damage of intestinal villi and infiltration of inflammatory cells in lamina properia (Black arrow) epithelial sloughing, tissue debris (Asterisk), and submucosal congestion (Red arrow). (H&E stain 100x)

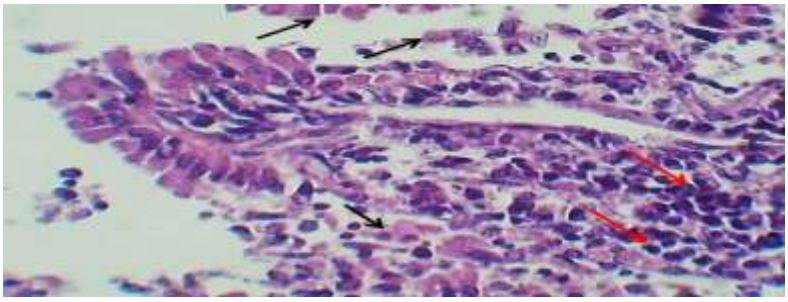

Figure 7: section of duodenum (T1) shows sever damage of villus with marked epithelial sloughing, (Black arrows) infiltration of mono nuclear leukocytes mainly lymphocytes and Macrophages (red arrows). (H&E stain 400x)

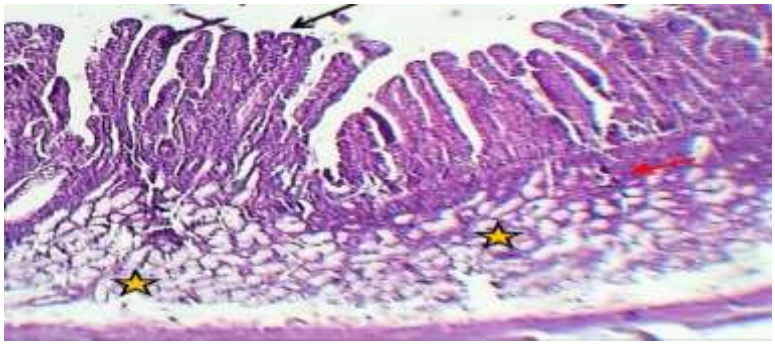

Figure 8: section of duodenum (T2 group) shows: Normal appearance of villus (Black arrows), with marked increased in Brunner's glands (Asterisks) & epithelial crypts (Red arrow). (H&E stain 400x)

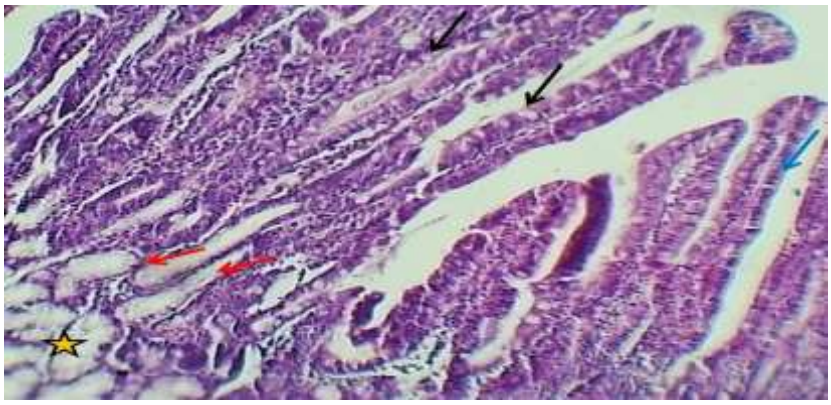

Figure 9: section of duodenum (T2 group) shows: Normal appearance of villus with increase in goblet cells (Black arrows), normal Brunner's glands (Asterisk). (H&E stain 100x) يرفع السهم

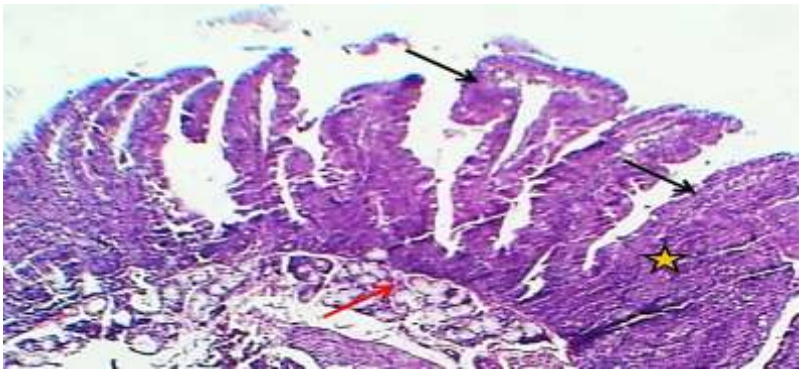

Figure 10:section of duodenum (T3 group) shows: marked increase thickness of villus (Black arrow), associated with hyperplasia of lamina propria (Asterisk), and Brunner's glands (Red arrow). (H&E stain 100x)

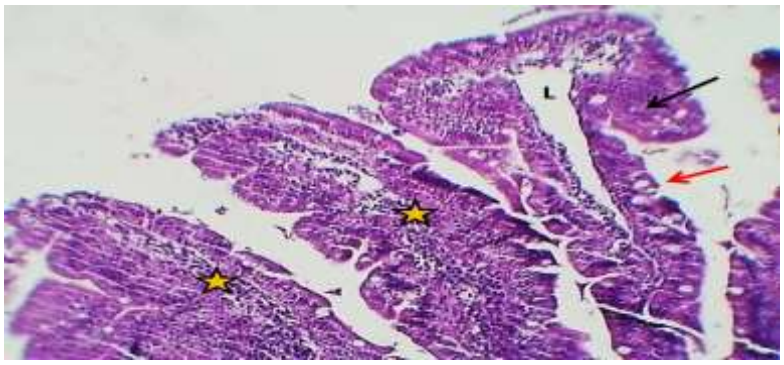

Figure 11: section of duodenum villus (T3 group) shows: Normal appearance of epithelium (Red arrow) with mild cellular hyperplasia of lamina propria (Asterisks) and lacteal vessels (L). (H&E stain 100x)

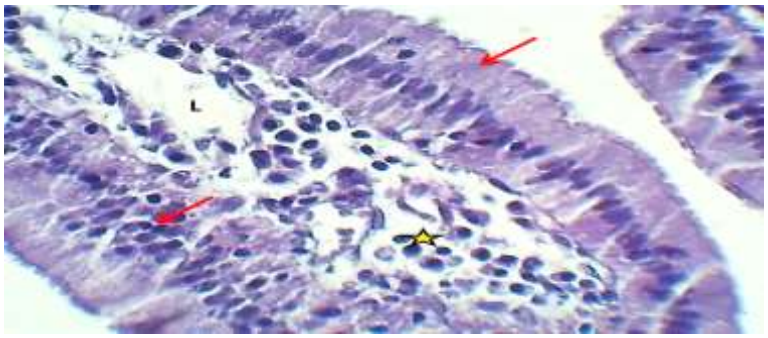

Figure 12: section of duodenum villus (T3 group) shows: Normal appearance of enterocytes with mild hyperplasia (Red arrows) & cellular lamina propria (Asterisks) and lacteal vessels (L). (H&E stain 400x)

The results of TNF-α are represented in figure 1. Which revealed that the oral intubation of aspirin (T1 group) caused significant rise ($P<0.05$) in TNF- α (43.44±5.00) in compared to control, T2, T3 groups (10.49±1.14), (14.57±1.54), (10.58±1.59). Meanwhile oral administration of ethanolic extract of *alhagi graecorum* cause significant reduction ($P<0.05$) in TNF- α compared to T1 group. The results of Citrulline are illustrated in figure 2. That showed substantial ($P<0.05$) reduction of citrulline in T1 group (3.34±0.48) as compared to control, T2,T3 groups (8.70±0.31), (7.95±0.48), (9.66±0.89), which showed statistical ($P<0.05$) elevation in serum citrullin levels. Gene expression of Occludin represent in figure 3. In which T1 group showed substantial reduction ($P<0.05$) in gene expression (0.29±0.01) in compared to other experimental groups. While the gene expression significantly elevated ($P<0.05$) in T2 and T3 groups (1.02±0.10), (4.24±0.24) compared to T1 group.

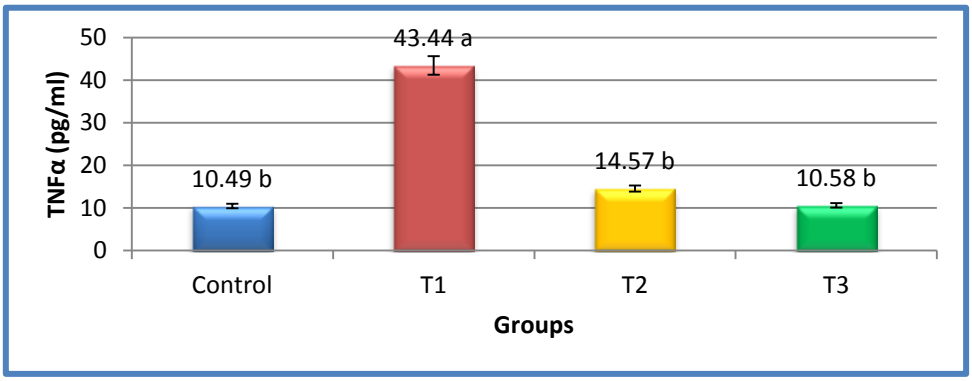

Figure 1: Effect of oral administration of Aspirin and *Alhagi graecorum* alcoholic extract on the TNF (pgl/ml). LSD=8.37

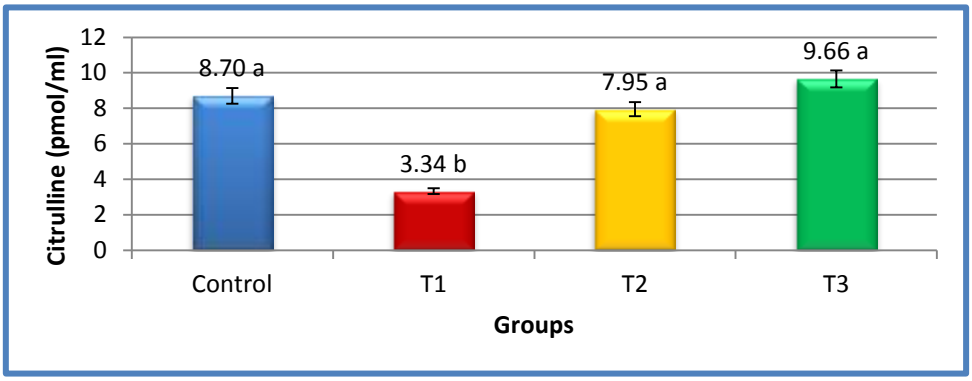

Figure 2: Effect of oral administration of Aspirin and *Alhagi graecorum* alcoholic extract on the citrulline (pmol/ml). LSD=1.75

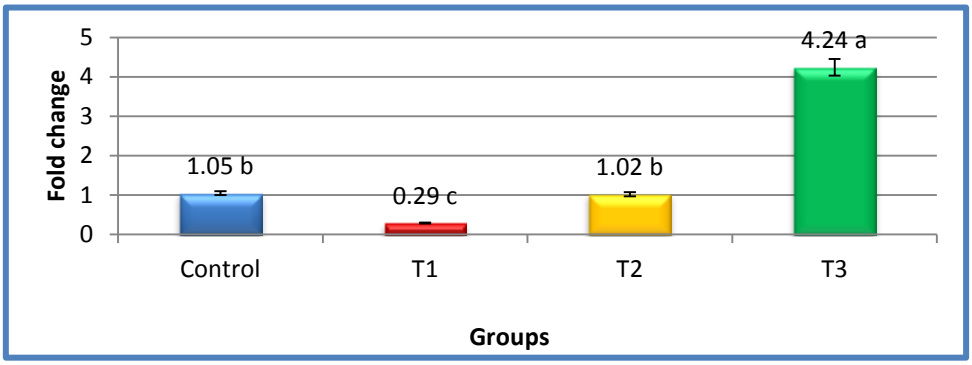

Figure 3: Effect of oral administration of Aspirin and *Alhagi graecorum* alcoholic extract on the gene expression. LSD=0.46

Discussion

The present model for aspirin induced enteritis in rabbits, indicated that this drug's effects extended from the stomach, site of absorption, to the intestine. The histopathological changes of aspirin administered animals may be due to oxidative stress caused by aspirin which result in intestinal mucosal damage (Fukui *et al*. 2012; Takayama *et al*. 2018). The high production of ROS is the main cause for oxidative stress in intestinal mucosa. The mechanism involved in ROS production by aspirin related to cell membrane lipids disorganization and raft caveoli formation which mediate a wide molecular mechanisms and cellular signal pathways alteration (Han *et al*. 2016; An *et al*. 2021). Mitochondrial impairment result from increased reactive oxygen species (ROS) formation and increased in mitochondrial membrane permeability (MMP), can cause protein and mitochondrial DNA (mtDNA) damage resulting in enzymatic chain failure and mutations that weaken mitochondrial function. These processes lead to abnormal cell signaling, premature cell senescence, initiation of inflammation, and apoptosis (Zhunina etal. 2021). Inflamed intestinal mucosa by aspirin in the present model of rabbits, characterized by macrophages and lymphocytes cells infiltation in lamina properia of vili.

While administration of *Alhagi graecorum* modified these changes to seminormal this may because the antioxidant effect and free radical scavenger of the phenolic and flavonoids constituent of *Alhagi graecorum* (Manhal 2017; Al‑Saleem *et al*. 2019). According to studies, that mitochondrial permeability transition (MPT) pore sealing

agents and antioxidants can significantly prevent mitochondrial toxicity (Salimi *et al*. 2019). Also this study revealed increase in TNF-α in aspirin administrated group. Due to intestinal mucosal injury, many proiflammatory cytokines will be released to the circulation (Ota *et al*. 2019). The activated macrophages and lymphocytes in lamina properia of villi in aspirin administered rabbits stimulate the release of pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) (Sethi and Hotamisligil 2021). Intestinal mucosal injury correlated with inflammatory cytokines release like TNF and mDNA damamge (Bindi *et al.* 2020). TNF-α is a key regulator that has diverse effects on the inflammatory response (Hameed and Hassan 2022). TNF is released rapidly to enhance the acute-phase inflammatory response via TNFRs which consequently sustains inflammatory conditions (Sanchez-Munoz *et al*. 2008; Gomez-Bris *et al*. 2023). Experimental studies demonstrate that TNFR plays an important role in maintaining intestinal integrity (Roulis *et al*. 2011). TNF-targeting biological agents have improved the therapeutic approach to inflammatory diseases. (Mao *et al*. 2017). TNF fusion protein is effective in mild-to-moderate ulcerative colitis (UC), and not related with immune suppression, while inducing beneficial anti-inflammatory immune modulation (Almon *et al*. 2021). Meanwhile TNF decreased in animals received *Alhagi graecorum*, may be due to the function of Flavonoid content of *Alhagi graecorumvia* in upregulating the gut hormone glucagon-like peptide (GLP)-2 that might improve the function of intestinal barrier (Oteiza *et al*. 2018) or preserving the tight junction barrier and structure of the intestine (Hu *et al*. 2019). Also this study denoted that serum level of citrulline in aspirin administrated group significantly degrease. Plasma citrulline concentration is a sensitive biomarker for the intestinal injury and dysfunction (Wataru *et al*. 2018; Konieczny et al. 2022). Aspirin caused oxidative stress, which destroyed epithelial tight junctions and increased permeability of the intestinal barrier. (Akifumi *et al*. 2012; Takayama *et al*. 2018). Loosing permeability and aspirin induced ROS stressed enterocytes, contribute to decreased citrulline synthesis by the intestinal mucosa. The protective effect of alcoholic extract of *Alhagi graecorum* on intestinal mucosa and maintenance of gut health and barrier function, mediated by free radical scavenging activities (Soni *et al*. 2015;Ghassan 2016; Shakiba *et al*. 2016). The contents of this plant for different antioxidant phytochemicals (Tavassoli *et al*. 2020) make it a good candidate for intestinal oxidative stress relieves. Present results in accordance with (Gargoum *et al*. 2013 Jafri *et al*. 2023) home found that phenolic and flavonoids act as antioxidant against aspirin oxidative stress. On the other hand the expression of occludin gene downregulated in aspirin group. NSAID including aspirin may induce suppression of PG formation results in a severe mucosal injury. This mucosal injury caused via inhibition of COX-1 enzyme pathway leading to inhibition of PG. Inhibition of prostaglandins result in ischemic intestinal mucosa. Due to role of PG in the regulation of small-intestinal blood flow (Akifumi *et al*. 2012; Lavie *et al*. 2017). Following onset of ischemia, tight junction proteins, are complexes that highly disrupted (Ronaldson *et al*. 2012). Particularly occludine is a tight junction protein that affected by ischemia in different tissue such as brian (Abdullahi *et al*. 2018; Sugiyama *et al*. 2023). The down regulation of occluding gene expression in aspirin group suggest a strong transcriptional responses to aspirin due to intestinal ischemia (Slifer *et al*. 2020). Whereas the expression of occluding gene upregulated in *Alhagi graecorum* groups could be explained by different mechanisms attributed to its bioactive compound. This may duo to its protective effect on intestinal mucosa and maintenance of gut health and barrier function as mentioned previously because it's

bioactive compound particularly flavonoids and phenolic compounds exert antioxidant effects on mucosal cell wall against oxidative stress induced by aspirin (Shaker *et al*. 2022). Restoration of blood flow in ischemic intestinal mucosa stimulates the rapid mechanisms of intestinal mucosal repair via increase of tight junction proteins (Slifer *et al*. 2020). The antioxidant and intinflammatory activities exerted by *Alhaji graecorum* extract restore the intestinal injury ischemia down regulated occluding that caused by aspirin oxidative stress (Lui *et al*. 2022). The high contents of *Alhaji graecorum* for phytochemicals bring about a transcriptional role for regulation of occludine protein synthesis. Since natural polyphenol compound derived from plant sources can up regulate expression of tight junction proteins in intestinal mucosa (Altaf *et al*. 2022).

Conclusion

We revealed that *Alhagi graecorum* protected the small intestinal mucosa from Aspirin-induced damage. As a result, this study suggests that *Alhagi graecorum* may be the best option for treating aspirin-induced gastrointestinal damage. These findings should be considered in future recommendations.

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