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PROTECTIVE EFFECT OF ARJUNOLIC ACID (A NATURAL TRITERPENOID SAPONIN OF TERMINALIA ARJUNA (TA)ON ASPIRIN-INDUCED GASTRIC INJURY IN RATS: APOSSIBLE INVOLVEMENT OF INTERCELLULAR ADHESION MOLECULE-1 (ICAM-1) AND VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

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ABSTRACT

The aim of this study is to investigate the effect of arjunolic acid in aspirin-induced gastric ulcer in rats.Twenty four male Sprague- Dawely rats were randomly allocated to four groups; the normal control, aspirin-induced ulceration, ranitidine, and arjunolic acid groups. All groups except the control and aspirin groups received pretreatment drugs for 14 consecutive days. On the 13th and 14th days, aspirin was administered orally to all groups except for control group. The gastroprotective effect of arjunolic acid was assessed by gastric mucosal determination of malondialdehyde, reduced glutathione, nitric oxide and expressions of cyclooxygenase-2, vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1). The changes in these parameters that are affected by aspirin-induced ulcer were corrected by arjunolic acid. Arjunolic acid has a protective effect against aspirin-induced gastric mucosal injury through attenuation of oxidative stress and upregulation of gastric VEGF expression with downregulation of ICAM-1expression.

KEYWORDS: Arjunolic acid, gastric ulcer, intercellular adhesion molecule-1 (ICAM-1) and vascular endothelial growth factor (VEGF)

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INTRODUCTION

Peptic ulcer is a major health hazard in terms of both morbidity and mortality [1]. Peptic ulcer can ensue due to an imbalance between offensive (acid-pepsin secretion, H. pylori, bile) versus defensive factors (mucus, bicarbonates secretion, prostaglandins, blood flow and the process of restitution and regeneration after cellular injury). In addition, it has been reported that gastric ulcer is mainly caused by oxidative stress [2]. Aspirin is a potent nonsteroidal anti-inflammatory drug (NSAID) that is used for the treatment of rheumatoid arthritis and related diseases as well as the prevention of cardiovascular thrombotic diseases[3]. However, NSAIDs produce a broad range of toxic effects, frequently causing gastrointestinal toxicity that result in ulceration, bleeding and perforation of stomach[4]. Even at low-doses, commonly prescribed for the prevention of thrombotic events in high-risk patients, aspirin leads to upper gastrointestinal complications in 0.6–1.2% patients per year [5]. Despite this, routine co-prescription of gastro-protective agents with aspirin or other NSAIDs is usually not advocated [5]. The high degree of efficacy and safety with natural drugs make them more acceptable when compared to other therapeutic intervention. Thus, a widespread search has been launched to identify new anti-ulcer therapies from natural sources to replace currently used drugs of doubtful efficacy and safety [6]. One such plant is Terminaliaarjuna (TA). One of its active constituents is triterpenoid saponins (arjunolic acid, arjunic acid, arjungenin, arjun glycosides) [7].Prolonged administration of TAdid not show any adverse effect on renal, hepatic and hematological parameters [8]. Arjunolic acid is well known for various biological functions, including antidiabetic[9], anti-fungal [10], anti-bacterial [11], antitumor [12] and wound healing [13]. Thus, the aim of this study is to investigate the effects of arjunolic acid on aspirin-induced gastric ulcer in rats and the mechanisms of its possible protective effect.

MATERIALS AND METHODS

Animals

Twenty four male Sprague Dawely rats weighing 180-200 g were used throughout this study. They were obtained from the animal house of Nile Center for Experimental Research in Mansoura. Animals were handled following the Guide for Care and Use of Laboratory Animals as adopted by the International Accreditation Organization and approval from Animal Ethic Committee of Nile Center for Experimental Research (Egypt). All animals in the study were kept at 25° C with 12-h light and dark cycles. The animals were fasted for 24 h before initiating the experiment. They only had free access to water to avoid food-induced enhanced secretion of acid and its effect on gastric lesions. All work is completed under the regulation of Ethical Conduct in the care and use of Nonhuman Animals in Research.

Drugs investigated

- **Aspirin** 500 mg/kg[14] was supplied by Bayer Company in the form of Aspirin protect 100 mg tablet.
- **Ranitidine** 100 mg/kg [15]was supplied by Adco Company in the form of Ranitidine 150 mg tablet.
- **Arjunolic acid** 20 mg/kg [16] was supplied by Sigma-Aldrich, St. Louis, MO in powder form.

Experimental design

Male Sprague-Dawley rats were randomly divided into four groups (each one containing 6 rats):

Group 1: Normal control group.

Group 2: Aspirin –induced gastric ulceration group; single dose of 500mg/kg aspirin was administered orally for two consecutive days [14].

Group 3: As group 2 but with previous treatment with 150 mg/kg ranitidine, as a standard drug, daily for 14 days (Aspirin on 13th and 14th day) [15].

Group 4: As group 3 but with previous treatment with 20mg/kg arjunolic acid daily for 14 days (Aspirin on 13th and 14th day)[16].

After 6 hours following the last doses of drugs administration[17], the animals were anesthetized. The stomach was removed after the esophageal end had been tied [17, 18] .Then, it was cut along the greater curvature. The contents were collected in tubes and centrifuged at 1000 rpm for 10 minutes. The

resultant supernatant was used for the determination of gastric pH. The stomach was then washed with warm saline, and the inner surface was photographed to allow the assessment of gross gastric lesions by gross lesion score (GLS). Next, the gastric mucosal tissues were scrapped with a glass slide and piece of it was immediately frozen in liquid nitrogen and stored at –80˚C until used for real time-PCR technique. Another piece of the mucosa was homogenized in a 10-fold volume of ice-cold sodium, potassium phosphate buffer (0.01 M, pH 7.4) containing 1.15 % KCl. The homogenates were centrifuged at 600g at 4°C for 10 minutes and stored at -80°C until used for estimation of oxidative stress parameters.

A) Biochemical analysis

A.1. Determination of gastric pH

The 1ml of supernatant was diluted with 9ml of distilled water and the pH was measured with the pH meter.

A.2. Assessment of gastric tissue oxidative stress parameters

- Malondialdehyde(MDA), was estimated by colorimetric method as described by Ohkawa et al. [19].
- Reduced glutathione(GSH) and nitric oxide activity(NO) were estimated by colorimetric method as described by Beutler[20]; and MontgomeryandDymock[21] ,respectively, using commercially available kits provided by Bio-Diagnostic Company, Giza, Egypt.

B) Detection of gastric cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF) and intercellular adhesion molecule-1 (ICAM-1) gene expressions using real time- polymerase chain reaction (PCR)

- **B.1. RNA extraction**
  Total RNA was isolated from gastric tissue homogenates using RNeasy Purification Reagent (Qiagen, Valencia, CA) according to manufacturer's instruction. Concentration of the RNA was assessed using spectrophotometer and the integrity of the RNA was studied by gel electrophoresis on a 1% agarose gel.
- **B.2. cDNA synthesis**
  First-strand cDNA synthesis was performed with the SuperScript Choice System (Life Technologies, Breda, the Netherlands) by mixing 2 µg total RNA with 0.5 µg of oligo(dT)12-18 primer in a total volume of 12µL. After the mixture was heated at 70°C for 10 minutes, a solution containing 50mmol/L TrisHCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl2, 10 mmol/L DTT, 0.5 mmol/L dNTPs, 0.5 µLRNase inhibitor, and 200 U Superscript Reverse Transcriptase was added, resulting in a total volume of 20.5 µL. This mixture was incubated at 42°C for 1 hour.
- **B.3. Real-time PCR**
  Real-time quantitative PCR was done by using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 ng of each primer, which shown in Table 1. The PCR reactions consisting of 95°C for 10 minutes (1 cycle), 94°C for 15 seconds, and 60°C for 1 minute (40 cycles), were performed on step one plus Real Time PCR system (Applied Biosystems). Data were analyzed using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the beta actin genes [22].

C) Determination of gross lesion score (GLS)

The stomach was dissected and opened along the greater curvature.Gastric lesions was studied by macroscopic examination and were classified according to Esplugues and Whittle [23] as following:

0= No lesion.

0.5= One punctiform ulceration< 1mm in diameter.

1= More than one punctiform ulceration.

2= One or more than one ulceration> 1mm in diameter.

3= More than one punctiform ulceration and more than one ulceration> = 1mm in diameter.

RESULTS

Effect of aspirin-induced gastric ulceration on tested parameters

As shown in Table 2,there was a significant decrease in gastric GSH levels as well as pH measurements in aspirin induced gastric ulceration group when compared to control

group ($p<0.05$). In addition, there was a significant increase in gastric MDA and NO activities in aspirin induced gastric ulceration group as compared to control group. There was a significant elevation of gastric m-RNA gene expression of COX-2 (Figure 1) and ICAM-1 (Figure 3) in aspirin induced gastric ulceration group in comparison with control group. However, there was a significant reduction of gastric m-RNA gene expression of VEGF in aspirin induced gastric ulceration group as compared to control group ($p<0.05$) (Figure 2).

Effect of ranitidine treatment on tested parameters in aspirin-induced gastric ulceration group.

Table 2 showed that, there was a significant increase in gastric GSH activities as well as gastric pH in ranitidine treated aspirin-induced gastric ulceration group when compared to non-treated aspirin-induced gastric ulceration group($p<0.05$). In addition, there was a significant decrease of gastric MDA and NO in ranitidine treated aspirin-induced gastric ulceration group as compared to non-treated aspirin-induced gastric ulceration group. In addition, ranitidine treatment in aspirin-induced gastric ulceration rats produced a significant reduction of gastric gene expressions of COX-2 (Figure 1) and ICAM-1 (Figure 3) with a significant elevation of gastric gene expression of VEGF (Figure 2) as compared to non-treated aspirin-induced gastric ulceration rats($p<0.05$).

Effect of arjunolic acid treatment on tested parameters in aspirin-induced gastric ulceration group.

As illustrated in Table 2, arjunolic acid treated aspirin-induced gastric ulceration group showed a significant increase in gastric GSH and gastric pH as compared to non-treated aspirin-induced gastric ulceration group($p<0.05$). In addition, there was a significant decrease of gastric MDA and NO levels in arjunolic acid treated aspirin-induced gastric ulceration group as compared to non-treated aspirin-induced gastric ulceration group. Furthermore, comparing its effect on GSH with that of ranitidine, arjunolic acid was significantly better than ranitidine. Arjunolic acid treated aspirin-induced gastric ulceration group produced a significant reduction of gastric gene expressions of COX-2 (Figure 1) and ICAM-1(Figure 3)while, a significant elevation of gastric gene expression of VEGF (Figure 2)was determined when compared to non-treatedaspirin-induced gastric ulceration group($p<0.05$).

Pathological results (Gross lesion score)

Table 3showed that aspirin produced gross gastric lesions in all rats varying from score 1-3 with a mean score of2 ± .89 (Fig. 4A). Ranitidine produced gross gastric lesions in only three rats ranging from 0.5-1 with a mean score of 0.42±0.49 (Fig. 4B). While, arjunolic acid produced gross gastric lesions in one rat of score 0.5 only, with a mean score of 0.08 ±0.2 (Fig. 4C). Both mean scores of ranitidine and arjunolic acid are significantly lower as compared to aspirin group.

Statistical analysis

All the data are expressed as mean ± SD.Differences between groups were assessed by one-way analysis of variance (ANOVA). Statistical computations were done on a personal computer using the computer software SPSS version 13 (Chicago, IL, USA). Statistical significance was considered when $P \leq 0.05$.

Table 1
Primer Sequences Used For Real Time-PCR

Primer	Sequence
COX-2	Forward: 5′- GCA TTC TTT GCC CAG CAC TTC ACT -3′ Reverse: 5′- TTT AAG TCC ACT CCA TGG CCC AGT -3′
ICAM-1	Forward: 5’- GCCGCTCATTTACACCTATTA-3′ Reverse: 5′- TTCCTTTTCTTCTCTTGCTTG-3′
VEGF	Forward:5′-TTCGTCCAACTTCTGGGCTCTT -3′ Reverse: 5′-CTCCTCTTCCTTCTCTTTCTCCCC-3′
Beta actin	Forward:5′- TCT GGC ACC ACA CCT TCT ACA ATG 3′ Reverse: 5′- AGC ACA GCC TGG ATA GCA ACG 3′

Table 2

Effect Of Aspirin Alone And Its Combination With Either Ranitidine Or Arjunolic Acid Administration On Gastric pH, Reduced Glutathione (GSH), Malondialdehyde (MDA), And Nitric Oxide (NO).(Mean±SD).

	Gastric pH	GSH (mg/g tissue)	MDA (nmol/g tissue)	NO (µmol/L)
Control	4.57 ± .08	128.4± 2.17	263.82± 5.73	23.73± 2.30
Aspirin	2.43 ± .23*	76.42± 1.86*	403.72± 16.17*	53.3± 2.02*
Ranitidine+ Aspirin	6.41 ± .08#	91.89± 4.52#	325.4± 6.95#	33.88±3.71#
Arjunolic Acid+ Aspirin	6.71 ± .08#^	107.68± 9.17#^	292.27± 8.87#^	32.82±4.81#

** Significant difference from control group*

 # Significant difference of arjunolic acid or ranitidine as compared to aspirin-induced gastric ulceration group.

^ Significant difference between arjunolic acid and ranitidine treated gastric ulceration group.

Table 3

Effect of Arjunolic Acid and Ranitidine on Gastric Mucosal Gross Lesion Scores (GLS) in Control and Aspirin Groups (Mean±SD).

Groups	No of animals	No of animals showing different GLS					Mean GLS in each group
		0	0.5	1	2	3	
Control	6	0	0	0	0	0	0
Aspirin	6	0	0	2	2	2	2 ± .89*
Ranitidine+Aspirin	6	3	1	2	0	0	0.42±0.49#
Arjunolic Acid+ Aspirin	6	5	1	0	0	0	0.08 ±0.2#

** Significant difference from control group.*

#Significant difference from aspirin group.

Gross lesion score (GLS):0= No lesion, 0.5= One punctiform ulceration< 1mm in diameter, 1= More than one punctiform ulceration, 2= One or more than oneulceration> 1mm in diameter and3= More than one punctiform ulceration and more than oneulceration> = 1mm in diameter (Esplugues and Whittle, 1990)

DISCUSSION

Peptic ulcer is a major health problem [1]. NSAIDs produce gastro-intestinal toxicity and are one of the causes of gastric ulceration [3]. Arjunolic acid, a natural triterpenoid saponin of TA, is well known for various biological functions including antioxidant and wound healing activities [13]. The purpose of the present study is to determine whether arjunolic acid plays any protective role against aspirin induced gastric ulcer and if so, what mechanism it utilizes for its protective action. In the present study, aspirin produced a significant reduction of gastric pH content.Our results were similar to previously reported studies in which aspirin reduced the gastric pH and increased the volume of gastric juice in rats [24, 25]. Gastric acid has been shown to play an important permissible role in NSAID associated mucosal injury [26]. Suppression of gastric acid by a variety of antiulcer drugs [27] provides effective and rapid healing of ulcer [28]. Our study documented that administration of arjunolic acid for 14 days showed significant increase in gastric pH when compared to aspirin ulcerated rats. This may be explained by a study of Devi et al., 2007 [18] who observed significant increase in gastric pH in TA extract when compared to NSAID induced gastric ulcer rat and attributed its gastroprotective effect to its direct action on acid producing cells. We also found a significant decrease in gastric GSH in rats treated with aspirin when compared to normal control rats. It was reported that GSH is found in high concentration in the gastric mucosa of rats and humans [18]. Glutathione is important for the maintenance of mucosal integrity, and its depletion in the gastric mucosa induces macroscopic mucosal ulceration [29]. Furthermore, our study showed that aspirin administration produced a significant increase in gastric MDA levels in comparison with normal rats. Our results coincided with the results of El-far et al., 2012 [30] who documented that aspirin administration increased the concentration of MDA, an index of lipid peroxidation, in gastric mucosa compared to the normal control. The NSAID

induced ulceration causes accumulation of oxygen free radicals, which cause lipid peroxidation, resulting in reduced membrane integrity of surface epithelial cells, thereby causing gastric ulcers [31].Moreover, this may explain our findings, which documented a significant increase in gastric GLS showed with aspirin group when compared to normal control rats.

Moreover, our study showed a significant increase in gastric NO when compared to normal control rats. One of the mechanisms by which aspirin damages the gastric mucosa is the increased production of NO due to the overexpression of iNOS[32]. The excessive release of NO from gastric epithelial cells induced by aspirin has been reported to exert detrimental effects [33, 34]. Arjunolic acid treatment for 14 days resulted in significant decrease in gastric MDA, NO and significant increase in gastric GSH in comparison to aspirin induced gastric ulceration. Devi et al., 2007 [18]reported that TA extract showed a significant elevation in gastric GSH when compared to NSAID treated rats. It is expected that arjunolic acid exerts the same effects of TA extract on aspirin treated rats. It has been found that oxygen-derived free radicals are implicated in the mechanism of acute and chronic ulceration in the gastric mucosa and scavenging these free radicals can play an appreciable role in healing the ulcer [35.] Arjunolic acid, the triterpenoidsaponins in TA, is a potent antioxidant as evidenced from its free radical scavenging activity. The reduction of NO levels might be due to the direct scavenging effect of NO by the carboxyl group and the NO radical quenching ability of arjunolic acid [36.] Interestingly, our study demonstrated that aspirin treated rats showed significant higher gastric COX-2 expression than normal control rats. This is inconsistent with previous studies that concluded that COX-2 isoform is constitutively expressed in the gastric mucosa after NSAIDs administration [38, 39]. Increased COX-2 expression can be seen following exposure of the mucosa to an irritant, induction of ischemia [40] or when COX-1 activity is suppressed with aspirin [41, 42].Davies et al., 1997 [42] documented that aspirin administration caused a profound suppression of systemic COX activity and extensive gastric mucosal damage resulted in a rapid induction of COX-2 expression in the stomach. The inhibition of COX-1 upregulates COX-2 expression and COX-2/PGs may counteract the deleterious effects of gastric hypermotility due to COX-1 inhibition[39.] The expression of COX-2 is higher in the ulcerative margins and at sites of inflammation and disappears at regions close to the cicatrisation[43].The cellular origin of increases in COX-2 mRNA and protein has yet to be defined, however, a prominent role of infiltrating lymphocytes and fibroblastic cells of the lamina propria was noticed [39.]

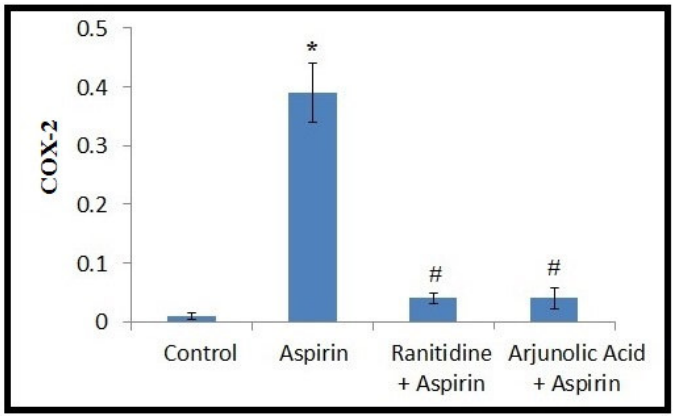
In the present study, we found that treatment with arjunolic acid for 14 days showed a significant decrease in gastric COX-2 expression near normal ranges. It was reported that COX-2 was subsequently found to be expressed at low levels in healthy tissues, including the stomach [44]. Arjunolic, acid treatment affects the arachidonic acid metabolism by cyclooxygenase thus exerting its anti-inflammatory activity [45]. It is known that VEGF is a fundamental angiogenic factor, which stimulates formation of granulation tissue and new micro vessels via angiogenesis that in turn accelerates gastric and duodenal ulcer healing [46]. In our study, there was a significant reduction of gastric VEGF expression in aspirin ulcerated rats in comparison with normal control rats. It was reported that NSAIDs interfere with the ulcer healing by inhibiting epithelial cell proliferation, migration and angiogenesis and by blocking growth factor-triggered signaling pathways like VEGF [47]. To date, we are the first to show the upregulation of VEGF expression, during ulcer healing after arjunolic acid administration. This may explain the ulcer healing effect of arjunolic acid as reported by Chaudhari et al., 2006 [13].To our knowledge, it was reported that COX-2 induces the production of several growth factors, including VEGF, and has an important role in tissue repair. In contrast, our results documented that VEGF did not seem to be dependent on COX-2 expression. Raut et al., 2004 [48] reported that blocking COX-2 production in pancreatic cancer cell did not affect VEGF. These contradictory findings suggest that the relationship between COX-2 and VEGF is complex and more studies are required toestablish the causal relationship between COX-2 and VEGF in gastric ulcer model.

The ICAM-1, one of the major adhesion molecules, plays a pivotal role in the

inflammatory reaction by increasing leukocyte adhesion to endothelium and promoting trans endothelial migration of leukocytes to inflammatory sites [29].In our study, rats treated with aspirin showed a significant elevation of gastric ICAM-1 m-RNA gene expression when compared to normal rats. Andrews et al., 1994 [49] reported that aspirin ulcerated rats showed a significant increase in ICAM-1 expression in the gastric microcirculation. The NSAIDs are believed to have the effect on nuclear translocation of nuclear factor (NF)-κB, which modulates the expression of several adhesions molecules, including ICAM-1 [50]. Our study showed that rats treated with arjunolic acid for 14 days showed a significant reduction of gastric ICAM-1 m-RNA gene expression when compared to aspirin ulcerated group. It was reported that arjunolic acid regulates the expression of various molecules including chemokines, proinflammatory cytokines andICAM-1 in a rat model of diabetes [51].

Figure1

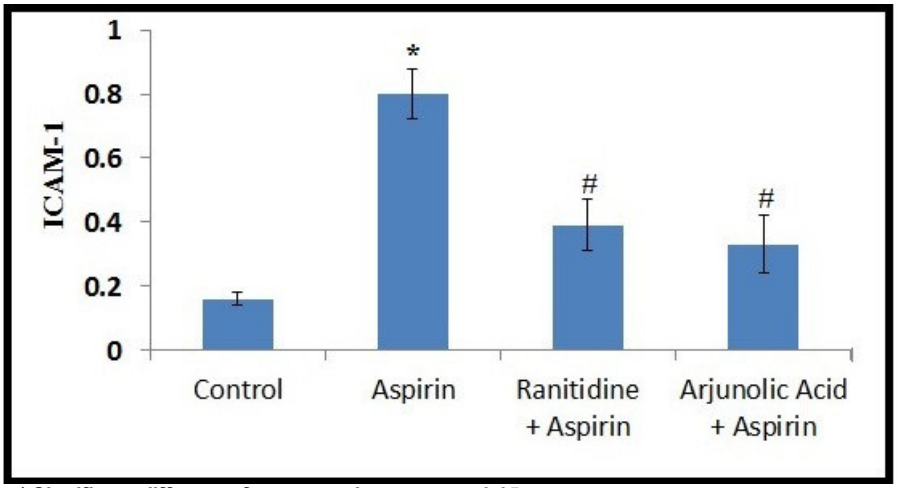
Effect of Aspirin Alone and Its Combination with Either Ranitidine or Arjunolic Acid Administration on Gastric m-RNA Gene Expression of Cyclooxygenase-2 (COX-2).



** Significant difference from control group at p <0.05*
 # Significant difference from aspirin group at p <0.05

Figure 2

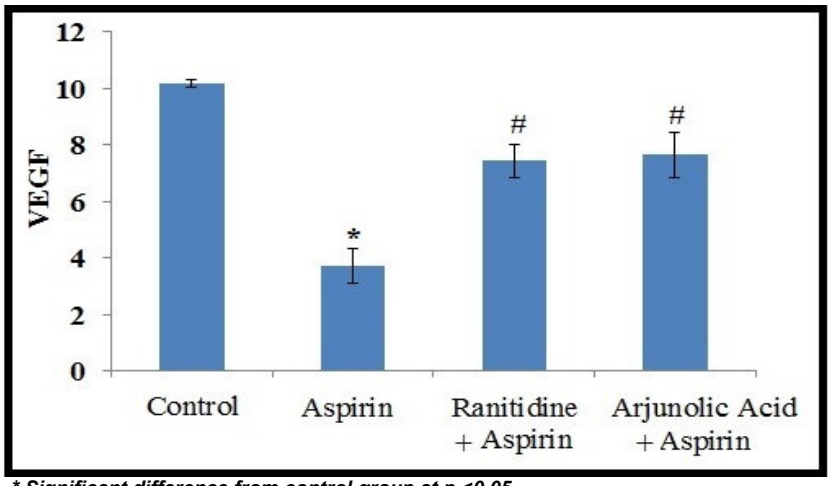
Effect of Aspirin Alone and Its Combination with Either Ranitidine or Arjunolic Acid Administration on Gastric m-RNA Gene Expression of Intercellular Adhesion Molecule-1 (ICAM-1).



** Significant difference from control group at p <0.05.*
 # Significant difference from aspirin group at p <0.05.

Figure 3

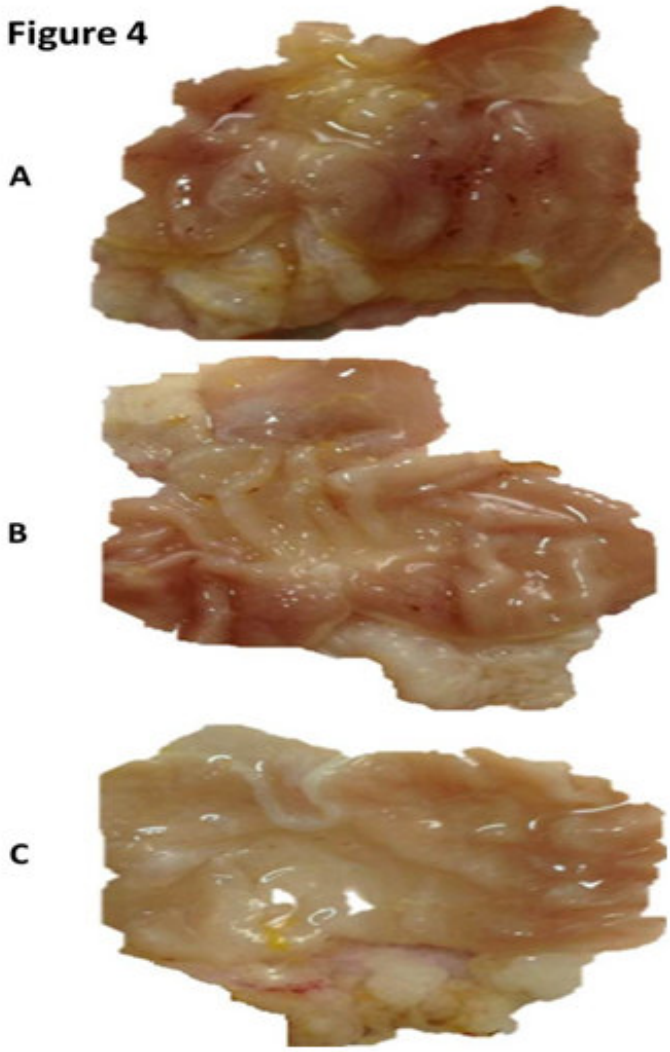
Effect of Aspirin Alone and Its Combination with Either Ranitidine or Arjunolic Acid Administration on Gastric m-RNA Gene Expression of Vascular Endothelial Growth Factor (VEGF).



** Significant difference from control group at p <0.05.*
 # Significant difference from aspirin group at p <0.05

Figure 4

Photograph of The Gastric Mucosa Showing Gross Lesion Score (GLS) In A) Aspirin Induced Gastric Ulceration Group, B) Ranitidine Treated Gastric Ulceration Group And C) Arjunolic Acid Treated Gastric Ulceration Group.



CONCLUSION

The results of this study concluded that arjunolic acid has a gastroprotective effect that was evident from significant reduction in the GLS in arjunolic acid treated rats against ulcerated rats. Arjunolic acid can exhibit antioxidant properties by several mechanisms including decreasing gastric MDA, NOand increasing gastric GSH. It also has an additional protective mechanism through its effect on the elevated level of the vascular inflammation markers as ICAM-1 as well as its inducing effect on VEGF. Thus, this study provides a new mechanism for the gastroprotective effect of arjunolic acid. Furthermore, comparing its effect with that of ranitidine, the traditionally used anti-ulcer drugs, arjunolic acid has a similar effect. Finally, preliminary clinical trials have to be undertaken to evaluate the therapeutic efficacy of arjunolic acid.

Conflict of interest

Conflict of interest declared none.

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