Arq Ajeeb prevents chemically induced visceral pain in mice

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Abstract

Visceral pain is a characteristic complaint of gastrointestinal disorders. *Arq Ajeeb* (AA), is an Unani pharmacopeial formulation used to treat gastrointestinal pain. The present study aimed to evaluate the efficacy of AA in visceral pain models. An acute toxicity study of AA was done as per OECD guidelines 423. For analgesic activity, Capsaicin-induced visceral and Indomethacin-induced gastric ulcer pain models were used. Adult male Swiss mice were divided into five groups of six each, viz. negative, positive, standard, test A, and B groups. AA administered orally (0.05 and 0.1 ml/kg). In the first test, the pain was induced by intracolonic administration of capsaicin (200 microliters); spontaneous behavior was observed and referred hyperalgesia tested by Von Frey filament. A gastric ulcer was induced with indomethacin (30mg/kg). The standard control animals were treated with omeprazole (20mg/kg) and test groups received AA as in the previous test. Referred hyperalgesia was quantified and the mucosal surface was examined for ulceration. AA was taken to GCMS analysis as well. The result of AA was safe at 300 mg/kg. In the capsaicin model, mice treated with tramadol and AA at both doses showed a significant reduction in pain behavior ($p<0.01$; $p<0.001$; $p<0.05$) respectively. In the indomethacin model, the pain threshold in standard, test groups A and B increased ($p<0.01$; $p<0.001$; $p<0.001$) at 4 hr, and after 24 hours ($p<0.05$; $p<0.05$; $p<0.05$) with significant ulcer reduction ($p<0.01$; $p<0.01$; $p<0.01$) respectively. The study concluded that AA has profound analgesic and anti-ulcerogenic activities in mice, validating the Unani claims that AA is effective in the treatment of gastrointestinal pain.

Keywords: *Arq Ajeeb*; Capsaicin; Indomethacin; Referred hyperalgesia; Visceral Pain;

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1. Introduction

Pain is an unpleasant sensation that is specific to a certain area of the body and frequently defined as a penetrating or tissue-destructive process, as well as a physical or emotional reaction (Fields 2005). Describing pain as an experience separates pain from nociception. Nociceptive pain is the pain that arises from an identifiable lesion causing tissue damage, accompanied by stimulation of nociceptors in somatic or visceral structures (Steeds 2016). Almost every human being experience pain in their lifetime, and in some instances, it may require medical attention and long-term therapeutic management (Shilpi and Jamaluddin 2020).

Previously, visceral organs were thought to be pain-insensitive, but it is now evident that the societal impact of visceral pain is far more significant than that of somatic pain. The activation of nociceptors in the thoracic, pelvic, or abdominal visceral organs leads to pain in the viscera induced by distension, inflammation, or ischemia (Kansal and Hughes 2019). An ironical characteristic of visceral pain is that it is commonly “referred” to as somatic sites (Johnson and Meerveld 2016). Visceral pain is a common complaint in colitis, gastric ulcer, and dyspepsia (Cash et al. 2016). Gastric ulcers are a serious medical problem with approximately 5-10% of the population worldwide suffering from them. Despite continued improvement in pain management in recent years, the pain remains a common medical problem around the world. In developing countries, pain is often undertreated or ignored aspect of treatment (Dureja et al. 2017).

One of the most significant gaps in current therapeutics is the lack of safe and specific medications for the treatment of the pain associated with these disorders (Quibria et al. 2014). In conventional medicine, visceral pain is treated with NSAIDs, opioids, and adjuvant analgesics like tricyclic anti-depressants, etc. which have adverse effects like gastric lesion, hepatic and renal dysfunction; tolerance, dependence, increased incidence of relapses, and drug interactions respectively, the use of these drugs is not successful in all the cases (Quibria et al. 2014). Therefore, the lack of specific treatments for visceral pain conditions and the high prevalence rates of many forms of pain from internal organs have stimulated a search for new analgesic targets aimed explicitly at treating visceral pain (Cervero and Laird 2004).

Plant essential oils have been used for a very long time in many conventional treatments as analgesics. The analgesic activity of the essential oils is based on some of their components including menthol, eugenol, linalool, carvacrol, p-cymene, thymol, and camphor (Lenardao et al. 2016). *Arq Ajeeb* (AA) also known as Jauhar Shifa is a Pharmacopeial formulation of the Unani system of medicine containing *Kafūr* (*Cinnamomum camphora*), *Jawhar Pudina* (*Mentha arvensis*), and *Jawhar Ajwain* (*Trachyspermum ammi*). It has analgesic and carminative properties and is used in the treatment of gastralgia, gastro-oesophageal reflux disease, colitis, nausea, flatulence, cholera, diarrhea, coryza, catarrh (Anonymous 2006), headaches, joint pain, toothache, and otalgia (Kabeeruddin 2014).

The majority of the ingredients in AA are composed of components from essential oils, which are just barely reported to have analgesic efficacy against various pain models (Chahal et al. 2017; Biswas and Ali 2014; Chauhan et al. 2012; Singh and Jawaid 2012). AA has been studied for its antidiarrheal activity in an animal model (Khan et al. 2004) and a clinical trial for headaches (Ahmad et al. 2019). However, AA neither as a whole nor its ingredients has been studied for anti-nociceptive effect in visceral pain models associated explicitly with gastrointestinal disorders. Therefore, in the present study, *Arq Ajeeb* is selected to evaluate its pharmacological efficacy in a diverse range of visceral pain models.

2. Material and methods

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2.1. Experimental animals

The study involved male Swiss mice weighing 20-30 g obtained from CPSCEA registered breeder; acclimatized before the experiment for one week, maintained under standard laboratory conditions throughout the experiment; provided with standard mice diet and water *ad libitum* except during behavioral testing; housed in clean polypropylene cages at room temperature 25±2 °C, humidity at 55–65% with 12-h light/ 12-h dark cycle, cared as per the guidelines of CPCSEA. The study was started after obtaining ethical clearance from the Institutional Animal Ethics Committee (IAEC) of the National Institute of Unani Medicine (NIUM), Bangalore (Reg. no: IAEC/VI/18/ IA/06).

2.2. Chemicals, Standard drugs, and Instruments

The chemical utilized in this study was of analytical grade and obtained from a trusted supplier. Capsaicin from Sigma-Aldrich (USA); Indomethacin from Micro lab Ltd.; Omeprazole from Dr. Reddy's Laboratory; Tramadol hydrochloride from Zydus Healthcare Ltd., India. Raised wire mesh (6×6mm apertures) floor and clear plastic ventilated box (18×13×15cm) were assembled at the local market of Bengaluru. The Homemade Von Frey filament (HmVFF) was handcrafted according to the method (De Sousa et al. 2014) with the use of nylon monofilament fishing line (0.35 mm diameter, DIY Crafts® enterprises, India), crafts sticks (0.9 cm × 11.2 cm × 0.2 cm), Loctite® 495 liquid adhesive. The forces exerted by HmVFF were calibrated with a weighing scale (PERSICA Gravimetrix AG-XB 120A, Swiss-made) accordingly.

2.3. Plant materials and preparation of Arq Ajeeb

All the ingredients of AA were procured from NIUM pharmacy. The ingredients were identified as *Jawhar kafūr* (*Cinnamomum camphora* (L.) PRESL.), *Jawhar Ajwain* (*Trachyspermum ammi* (L.) Sprague.), and *Jawhar Pudina* (*Mentha arvensis* L.) which were chemically verified by Dr. Mohammad Abdul Rasheed Naikodi, Research Associate (Chemistry) at Drug Standardization Research Unit of National Research Institute of Unani Medicine for Skin Disorder, Hyderabad, Telangana, India with the reference no. 19-10/2020-NRIUMSD/Tech/642 dated 31 December 2020. A voucher specimen (Ref. no. 100/IA/Res/2021) was deposited in the drug museum, Department of Ilmul Advia (Pharmacology), NIUM, Bengaluru, for future reference. *Arq Ajeeb* (AA) was prepared according to the method mentioned in the National Formulary of Unani Medicine Part-I (Anonymous 2006). All three ingredients were taken in a 2:2:1 ratio (*Kafūr: Jawhar Pudina*: *Jawhar Ajwain*) and mixed in a moisture-free glass container until they were liquefied. The transparent homogenous liquid thus obtained was filled in an airtight bottle and stored under hygienic conditions in a cool and dry place.

2.3.1 Doses of the drug

The human therapeutic dose of AA mentioned in Unani literature is 3-5 drops (Anonymous 2006). The dose for the mice was calculated by taking the higher dose (5 drops) based on the body surface area of the animal (Nair and Jacob 2016) and was found to be 42.64 mg/kg or

0.05ml/kg for the low dose and 85.28mg/kg or 0.1ml/kg for high dose. At the time of experiment, the doses were freshly prepared in 5% Tween 80 (vehicle) and administered orally by gastric cannula.

2.4. GC-MS analysis

The analysis of the volatile constituent was run on an Agilent 19091 S-433 GC-MS system coupled to mass spectrometry in the lab of Merieux Nutri Sciences, Bangalore Private Limited, under the following analytical conditions: A DB-1 (5% phenyl Methyl siloxane) fused silica capillary column (30 × 0.25 mm × 0.25 µm) was used with helium as carrier gas. The temperature was programmed from 70°C (1 min) to 290°C (at 15°C/min) and then held for 15 min and programmed maximum to 320°C. The injector port (split ratio, 20:1) was at 225°C. The recording was performed at 70 eV, mass range 50- 550 amu. To identify the essential oil constituents, the mass spectra library (NIST Mass Spectrometry Data Centre) was searched, and by comparing with literature data the relative amounts of individual components were calculated based on GC peak areas (Facundo et al. 2005).

2.5. Acute toxicity study

Arq Ajeeb was evaluated for acute oral toxicity as per OECD guidelines 423 (OECD 2011) in the animal house facility of NIUM, Bangalore, Karnataka, India. The drug was tested in a stepwise procedure with three rats in each step, the animals fasted for 12 hours with free access to water. In the morning the animal's weight was recorded and AA in a single dose i.e., 300 and 2000 mg/kg dissolved in 1 ml of 5% Tween80 was administered by oral route with a gastric cannula. After administration of the AA, animals were placed individually in a cage and observed for any sign of acute toxicity and behavioral changes as well as mortality at 0, 30, 120, 240 min, and periodically during the first 24 hours, and daily thereafter for a total period of 14 days. The parameters observed include piloerection, grooming, trembling, diarrhea, breathing difficulty, constantly changing position, immobility, anorexia, ataxia, urination, and coma.

2.6. Pharmacological study

2.6.1. Evaluation of Arq Ajeeb on Capsaicin induced visceral pain model

This test was carried out by the method (Laird et al., 2001) with some modifications in the treatment schedule and observation method. Healthy, adult Swiss male mice were categorized into five groups of 6 mice each, namely negative control, positive control, standard control, test group A, and test group B. On the day of the experiment, the animals were housed in an individual transparent plastic box on an elevated platform (dimension: 18x13x15cm) with a wire mesh floor. The negative control group was treated orally with 1ml /100gm of 5% Tween80 throughout the experiment. The standard group was administered tramadol (10 mg/kg). The remaining two groups received AA at the doses of 0.05 ml/kg & 0.1ml/kg (diluted in 5% Tween 80), respectively. All the drugs were administered orally by gastric cannula. After a one-hour habituation period and administration of vehicle, standard, and AA in respective groups; animals were removed from the compartments, and the pain was induced by capsaicin solution (200 microliters of 0.1%, dissolved in 10% ethanol, 10% Tween 80 and 80% Saline), administered into the colon 4 cm proximally from the ano-cutaneous line via the anus with a transparent 1.0 mm diameter cannula in all groups except negative control. Before intracolonial capsaicin injection, petroleum jelly was applied to the perianal area to avoid direct contact with the irritant. The animals of the negative control group were administered with normal saline instead of capsaicin. Immediately after intracolonic administration, the animals were returned to the transparent cage for a 20 min observation period, where spontaneous behavior was observed and counted. Visceral pain behavior was considered licking, contraction of the abdomen, stretching, humpbacked position, and hunching. After 20 minutes, referred mechanical hyperalgesia was tested by using a series of HmVFF (De Sousa et al. 2014) (forces of 0.16g, 0.4g, 0.8g, 1.6g, 2.0g, and 3.26g) in ascending order, to the lower and mid abdominal area except for perianal and genital area. Repeated stimulation of the same area was avoided to prevent sensitization. The lowest filament that elicited a withdrawal response was considered the threshold stimulus; each filament was applied thrice in a slightly slanting position for 2 seconds and with an inter-stimulus interval of 5 to 10 seconds. The response to the filament was considered positive if immediate licking and scratching of the application site, sharp retraction of the abdomen, or jumping was observed.

2.6.2. Evaluation of Arq Ajeeb on Indomethacin-induced gastric ulcer pain

The study was carried out by the method (Hummel et al. 2017) with some modifications in the assessment of referred hyperalgesia. Mice were fasted for 12h, then dosed orally with 30mg/kg indomethacin to induce gastric ulcer. The animals of the negative control group were treated with 1ml /100gm of 5% Tween 80, orally throughout the experiment while the animals of standard control were treated with omeprazole (20 mg/kg body weight) orally 2h post-indomethacin administration. The remaining two groups received AA at the doses of 0.05 ml/kg & 0.1ml/kg, respectively. Referred hyperalgesia was quantified by measuring the threshold to withdrawal from the application of a tactile stimulus to the upper abdominal area. Tactile hypersensitivity was measured 4 and 24 hours following indomethacin administration using HmVFF (bending force 0.008g, 0.02g, 0.4g, 0.8g, 1.4g, 2g, 3g, 3.58g). To evaluate the anti-ulcerogenic effect of AA, after completion of the experiment, all the animals of each group including negative control were sacrificed under Isoflurane anesthesia. The mucosal surface was examined for ulceration under a magnifying lens (10x magnification) and scored by the method (Brzozowski et al. 1998) with some modifications in the scoring scale.

Determination of the degree of ulceration: Normal stomach (0); Red colouration (0.1-0.5); Spot ulcer (1); Haemorrhagic streak (1.1-1.5); Ulcer (2); Perforation (3).

The average degree of single ulceration (ADU) for each group was determined by adding the degree of single ulceration (DSU) and dividing it by the number of animals. Based on mice with ulceration (%MU), the ulcer index was calculated by the following formula (Srimal 1984).

$$\text{Ulcer index} = \frac{(\text{ADU})(\%\text{MU})}{100}$$

ADU – Average degree of single ulceration

% MU – Percentage of mice with ulceration

The percentage of ulcer protection was determined by the following formula.

$$\%\ \text{Protection} = \frac{\text{Control means ulcer index} - \text{Test mean ulcer index}}{\text{Control means ulcer index}} \times 100$$

2.7. *Statistical analysis* All the data were analyzed on GraphPad Prism 8 (Version 9.1.1, USA) using ANOVA followed by post hoc Tukey's Kramer multiple comparison test. Data are presented as mean ± SD. $P<0.05$ was considered statistically significant.

3. Results

3.1. Capsaicin-induced visceral pain test

The intracolonic instillation of capsaicin (0.1%, 200µl/mice) evoked a significantly ($p<0.001$) higher number of pain-related behaviors, which subsided significantly ($p<0.001$) by AA at a low dose while a little less but significant reduction in pain behavior was observed with AA at high dose ($p<0.05$) and tramadol ($p<0.01$) in comparison to positive control. However, AA manifested more strong effect at a low dose. The animals in the positive control showed mixed behavioral responses like licking, stretching, and squashing, while the animals in standard and test groups showed only licking behavior. The pain threshold for mechanical stimulation was also found to increase significantly by 2.70±0.86 gm ($p<0.05$) at a low dose of AA; standard control showed a pain threshold of 2.02±0.89gm ($p<0.01$) and AA at a high dose showed 2.15±0.86gm though the pain threshold was more than the positive control, statistically non-significant.

3.2. Indomethacin-induced gastric ulcer pain test

Indomethacin-treated mice (positive control) demonstrated referred abdominal hypersensitivity significantly ($p<0.001$) in comparison to negative control which was reduced significantly ($p<0.001$; $p<0.001$; $p<0.01$) in test groups A, B, and standard control respectively in comparison to positive control at 4h post indomethacin dosing. At 24 hr, positive control again showed a significantly low pain threshold ($p<0.05$) in comparison to negative control; and both the test and standard group showed significant ($p<0.05$) exhibition in referred abdominal hypersensitivity, though it was less than the pain threshold observed after 4 hours in the same animals. Pain threshold was compared between 4h and 24h. The mean pain threshold in the negative control was observed at 3.48±0.24 gm while in the positive control group, it was reduced by 2.19±1.34gm ($p=0.04$). The animals of standard control showed the same level of pain threshold as found in negative control i.e., 3.48±0.24gm; almost similar results were observed in test group A i.e., 3.12±0.24gm ($p=0.21$) and test group B 3.39±0.30. The result showed that hyperalgesia caused by indomethacin-induced ulcers subsided within and/or after 24h. The effect of AA on gastric ulcers induced by indomethacin was also analyzed. Ulcer index was found to be significantly increased to 1.12 ($p<0.001$) in comparison to the negative control of 0.003. In the standard drug (omeprazole), test drug AA in low and

high doses showed a significant reduction ($p<0.001$) in ulcer index i.e., 0.04, 0.08, 0.04, and the percentage of ulcer reduction was calculated as 96.42%, 92.85%, and 96.42%, respectively.

3.3. Gas chromatography-mass spectrometry analysis

GCMS analysis of AA was carried out and 5 different compounds were identified with a retention time range of 5.81- 8.39 min. The active principle, area of peak, molecular formula, and retention time (RT) are presented in Table 3 and figure 5. The predominant volatile compounds found in GC-MS are (-)-Camphor, Levomenthol, and Thymol, these related compounds have also been reported by other researchers viz. D-Camphor (Guo et al. 2016), Levomenthol (Chagas et al. 2020), and thymol (Chahal et al. 2017).

3.4.Acute toxicity study

According to OECD 423, the study of AA's acute toxicity was conducted at 300 and 2000 mg/kg. AA was found to be safe at 300mg/kg but a mild toxic effect was observed at 2000mg/kg in some animals.

4. Discussion

This study aims to determine the anti-nociceptive effect of AA on abdominal pain. A technically simple and solely visceral mouse model of chemical stimulation of the colon by capsaicin has been established (Laird et al. 2001). The intracolonic capsaicin administration in mice represents an appropriate translational model of visceral pain since the application of capsaicin to the human gut causes intense suffering and is referred mechanical hyperalgesia; hence, it is a well-validated human model of visceral pain (Gonzalez-Cano et al. 2013). Therefore, in the present study “Capsaicin induced visceral pain model” was selected to evaluate the antinociceptive effect of AA; two parameters, spontaneous pain behavior, and referred mechanical hyperalgesia were analyzed to assess the effect of AA in mice.

This study aims to determine the anti-nociceptive effect of AA on abdominal pain. A technically simple and solely visceral mouse model of chemical stimulation of the colon by capsaicin has been established by mice pre-treated with a low and high dose of AA and showed a substantial reduction in pain behaviors ($p<0.001$, $p<0.05$), respectively. AA at a low dose showed a pronounced effect than at a high dose and in tramadol-treated animals ($p<0.01$).

Intracolonic capsaicin administration produced referred mechanical hyperalgesia in the abdomen, which was assessed by HmVFF. The mean pain threshold in the mice pre-treated with both the doses of AA and tramadol increased compared to the positive control, but a significant result ($p<0.05$) was observed in the animals of test group A only treated with a low dose of AA. The results demonstrated that AA is more effective at a human therapeutic dose as no dose-dependent effect was recognized, instead a decreased reduction of pain was observed at a high dose and in tramadol-treated animals. Based on the results, it can be concluded that the low dose of AA is more potent than the high dose. A very similar result in which the alpha and beta-amyrin mixture at smaller doses showed greater efficacy and concluded that the amyrin mixture might have a dual action on capsaicin response. The mechanism of action of AA might be the same as observed in amyrin (Oliveira et al. 2005).

Red peppers contain a strong compound called capsaicin, which is used topically or subcutaneously in both humans and test subjects. It stimulates the vanilloid receptor (Transient Receptor Potential cation channel V1 or TRPV1) situated on polymodal C-fibres and initiates a cascade of events that include neuronal excitation, the release of pro-inflammatory mediators, desensitization of receptors, and neuronal toxicity (Caterina et al. 1997) that causes a burning sensation followed by a refractory state producing analgesic effects against a subsequent nociceptive stimulus (Szallasi and Blumberg 1999). Oliveira et al. (2005) established that capsaicin induces nociceptive pain-related behaviors in rodents following sub plantar (paw licking), or intracolonic (abdominal licking, stretching, squashing of the lower abdomen against the floor, and abdominal retractions) application (Oliveira et al. 2005). Therefore, it was thought-provoking to examine the possible modulation of capsaicin-induced acute pain and visceral nociception by AA in the present study. Numerous reports reveal that naturally occurring compounds, like terpenoids, unsaturated dialdehydes, and phenolic ketones can suppress these behaviors (Dedov et al. 2002; Luo et al. 2012). AA is composed of camphor, thymol, and menthol; the former is one of the natural medicinal components with anti-inflammatory, analgesic, and antibacterial activity. Camphor activates and then desensitizes transient receptor potential vanilloid-1 (TRPV1) (Xu et al 2005) but inhibits the TRPA1 channel, expressed in most nociceptive DRG neurons (Nagata et al. 2005). Camphor-activated TRPV1 currents underwent significant desensitization and tachyphylaxis, which might exhibit analgesic properties together with inhibition of TRPA1. Tramadol is the antinociceptive

reference drug-producing analgesic activity by more than one mechanism of action (Hara et al. 2005). However, it is considered that partial action through opioid receptors is involved in the analgesic effect of Tramadol (Adriana et al. 2012). *Arq Ajeeb* exhibited a pronounced antinociceptive effect in capsaicin models of acute pain and visceral nociception in mice. Although its exact mechanism is unclear, the suppression of C-type fiber activation and interaction with the endogenous opioid system seem likely for the antinociceptive action as seen by tramadol (Oliveira et al. 2005).

According to Unani concept, any sudden change in the temperament or/and breach in continuity affect the physical condition of any organ leading to pain. Corrosive humor, irritant matter, and viscous pneuma are the predisposing factors of abdominal pain (Ibn Sina 2007; Ibn Rushd 2017). Capsaicin causes irritation and inflammation at the site of contact or accruement due to its high caustic and ulcerative qualities. Quantitatively vitiated humor cause tension or stress while qualitatively they produce irritation or ulceration in organs and sometimes together induce pain. The ingredients of AA are resolvent, analgesic, and anesthetic, they may relieve pain by normalizing the temperament and discontinuity and desensitizing the nerve endings (Ibn Sina 2007). *Trachyspermum ammi* and *Mentha arvensis* owing to their demulcent, resolvent, and analgesic properties, disperse the matters which induce pain, while *Cinnamomum camphora* by its sedative and anesthetic actions diminishes the sensation in the concerned part. It is beneficial in desensitizing the local nerve which carries the impulse of pain (Ibn Sina 2007). *Arq Ajeeb* did not only offer respite from the pain behavior but also showed a marked reduction in referred hyperalgesia; the findings suggest that AA produced its analgesic effect by more than one mechanism due to the varied nature of its ingredients.

The indomethacin-induced gastric ulcer pain model reiterates the human condition after oral administration to mice or rats. After administering Indomethacin in fasting animals, mucosal injury, inflammation, and referred visceral hyperalgesia were observed (Dey et al. 2009). Analogous to other gastrointestinal disorders, gastric ulcer pain is diffused. Further, it can be referred to as somatic structures and may present itself atypically given the dichotomization of sensory fibers that innervate visceral tissues (Iannitti et al. 2014). Meanwhile, ulcer pain is noticeable on palpation or mechanical stimulation of the abdomen both in dogs (Vonderhaar 1993) and humans (Ben et al. 2012); it was extrapolated to mice and quantified the referred abdominal hypersensitivity by measuring the number of behavioral responses evoked by Von

Frey filament stimulation (Wantuch et al. 2007; Hummel et al. 2010). Therefore, the indomethacin-induced ulcer pain model, referred abdominal hypersensitivity was quantified by measuring the threshold to withdrawal from the application of HmVFF stimulation on the upper abdominal area at the 4th and 24th-hour post indomethacin administration. Indomethacin-treated mice (positive control) demonstrated a significantly reduced pain threshold ($p<0.001$) compared to negative control at 4 h post indomethacin administration. A very significant elevation in pain threshold was observed in the animals treated with low ($p<0.0001$) and a high dose ($p<0.001$) of AA and omeprazole showed pain threshold ($p<0.01$) at 4h post indomethacin dosing. After 24 hours, the threshold to withdrawal from applying a tactile stimulus to the abdominal area was quantified again on the same animal of all the groups and compared between 4h and 24h; a further increase in the pain threshold was observed but statistically not significant. After intergroup comparison within 24 hours, both the test and the standard groups showed significant ($p<0.05$) elevation of pain threshold compared to the positive control however, it was less than the pain threshold observed after 4 hours in the same animals. On observation of internal mucosa of the stomach no apparent ulcer or perforation was found in the animals, but for a congestion of the mucosa and hemorrhagic streak; ulcer scoring was done. In positive control animals, ulcer score was significantly increased ($p<0.001$) compared to the negative control. Omeprazole, AA in low and high doses exhibited a significant reduction ($p<0.001$) in ulcer score, and the percentage of ulcer reduction was noted at 96%, 92%, and 96%, respectively. Unlike the previous results, AA exhibited a pronounced anti-ulcerogenic effect at both doses, which was more significant than omeprazole. The ingredient of AA viz. *T. ammi* ethanolic extract displayed a significant reduction in ulcer index and ulcer score (Ramaswamy et al. 2010). Hence, the striking analgesic and anti-ulcer effect of AA is the synergistic effect of the total response of its ingredients.

In Unani literature, the causes of gastric ulcers have been described to be hot and irritant humor, waste products, intake of hot and spicy foods, excessive use of alcohol, chronic gastritis, and indigestion (Ibn Sina 2007; Khan 2011). AA may neutralize the irritant humor and arrest the secretion of corrosive waste products. Furthermore, AA and its ingredients have been mentioned to possess sedative, hypnotic, resolvent, and demulcent properties (Momin 1855; Khan 2018). These effects may have a role in improving gastric lesions either through the mediation of the nervous system or by producing a local effect of neutralization, healing, and

cytoprotection. Unani scholars have also mentioned it to be helpful in various gastric disorders gastralgia, indigestion, food poisoning, and gastric ulcers; all of these conditions are associated with the weakening of the mucosal defense system (Ibn Sina 2007; Khan 2011). Therefore, these effects must be scientifically proved as done by the authors and found fruitful results.

Unani medicine contains a variety of bioactive components, whether it is taken as a single medication, a compound formulation, or an extract. GC-MS analysis of *Arq Ajeeb* confirmed the presence of (-)-camphor, levomenthol, and thymol, which have already been proven for analgesic activity. HPTLC fingerprinting of a single ingredient of AA also quantified the amount of camphor, menthol, and thymol. The effect of camphor on the acute inflammatory response *in vitro* and *in vivo* demonstrated that camphor reduces the topical ear edema induced by croton oil in mice and reduces leukocytes chemotaxis *in vitro* (Silva-Filho et al. 2014). Some studies performed with extracts and essential oil of plants containing camphor as one of the significant constituents demonstrated a reduction of inflammatory mediators, such as proinflammatory cytokines (IL-1β, IL-6, and TNF) and prostaglandin E2 in macrophages culture (Nikkhah Bodagh et al. 2019). Considering the studies cited in the literature about camphor properties, the analgesic properties could be attributed to this compound. Thymol partly blocks voltage-gated sodium and potassium channels and directly activates GABA receptors for aminobutyric acid; it also reversibly inhibits prostaglandin synthesis, which is probably related to the analgesic effect of thymol in endodontic therapy (Vonapart et al. 2008). Menthol reverses cinnamaldehyde-induced heat hyperalgesia, an effect that may have been a consequence of the blockade of TRPA1 by this compound (Silva-Correa et al. 2021). Thus, it can be said that the significant analgesic effects may be contributed to the synergism of all the three ingredients of AA, especially camphor.

Arq Ajeeb's possible side effects were looked into because Unani physicians commonly use it to treat pain, digestive, and respiratory conditions. The results of the acute toxicity study revealed mild toxic effects in two animals at 2000 mg/kg. Therefore, a detailed toxicity study is recommended in a planned manner, including sub-acute, sub-chronic, and chronic toxicity studies in different species so that its lethal dose can be established and target organ toxicity could be identified.

The present research outcome demonstrated that AA produces a significant antinociceptive effect in different kinds of induced pain in rodents. The findings validate its therapeutic efficacy as claimed by Unani physicians in a diverse nature of pain associated with gastrointestinal disorders. AA exhibiting feeble analgesic effect at a high dose compared to the low dose indicates its efficacy at a human therapeutic dose, which is advantageous given its uncertain safety profile at a high dose in the toxicity study. In all the experimental models, animals pre-treated with a single dose only showed a significant analgesic effect. A more pronounced effect is expected after multiple administrations.

5. Conclusion

The investigation's findings indicate that the *Arq Ajeeb* contains compounds with analgesic and anti-ulcer effects that are pharmacologically active. Thus, we presume that *Arq Ajeeb* can be used as a therapeutic agent to treat the diverse natures of gastrointestinal pain and gastric ulcer. However, to identify the active principle and specific mechanism of action, additional models of abdominal pain and more thorough phytochemical research are needed.

Table 1: Analgesic effect of *Arq Ajeeb* on Capsaicin-induced visceral pain model

Groups	Treatment	No. of Pain behavior (Mean ± SD)	Pain threshold (g force)
Negative control	5% Tween 80 (1ml/100g) orally + 200 µL Normal saline i. col.	6.33±1.63	2.71±0.86
Positive control	5% Tween 80 (1ml/100g) orally + 200 µL of 0.1% Capsaicin i. col.	21.5±3.56 a***	1.00±0.49 a*
Standard control	Tramadol (10mg/kg) orally + 200 µL of 0.1% Capsaicin i. col.	13.50±1.87 b**	2.02±0.89
Test group A	*Arq Ajeeb* (0.05ml/kg) orally + 200 µL of 0.1% Capsaicin i. col.	9.17±4.17 b***	2.70±0.86 b*
Test group B	*Arq Ajeeb* (0.1ml/kg) orally + 200 µL of 0.1% Capsaicin i. col.	15.00±4.19 b*	2.15±0.86

The test employed a one-way Analysis of Variance (ANOVA) with a post hoc Tukey's Kramer multiple comparison test. n=6, a- positive control vs Negative control; b- standard, test group A & B Vs positive control; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, i.col.= intracolonic

Table 2: Effect of *Arq Ajeeb* on Indomethacin-induced gastric ulcer

Groups	**Treatment**	**Referred hyperalgesia Pain threshold (Force in g)**			**Indomethacin-induced gastric ulcer**			
		0 hr	4 hr	24 hr	ADU	%MU	Ulcer index	% Reduction
Negative control	5% Tween80 (1ml/100g)	3.58±0.00	3.38±0.30	3.48±0.24	0.02±0.04	17%	0.003	-
Positive control	Indomethacin (30mg/kg) + 5% Tween 80 (1ml/100g)	3.58±0.00	1.30±0.92 a***	2.19±1.34 a*	1.12±0.53 a***	100%	1.12	-
Standard control	Indomethacin (30mg/kg) + Omeprazole (20mg/kg)	3.58±0.00	2.86±0.94 b**	3.48±0.24 b*	0.08±0.10 b***	50%	0.04	96.42
Test group A	Indomethacin (30mg/kg) + AA(.05ml/kg)	3.58±0.00	3.29±0.32 b***	3.12±0.24 b*	0.13±0.12 b***	66%	0.08	92.85
Test group B	Indomethacin (30mg/kg) + AA(0.1ml/kg)	3.58±0.00	3.12±0.62 b***	3.39±0.30 b*	0.08±0.10 b***	50%	0.04	96.42

The test employed one-way Analysis of Variance (ANOVA) with post hoc Tukey's Kramer multiple comparison test, paired 't-test between 4h and 24h, Values expressed as Mean ± SD, n=6, a- positive control Vs Negative control; b- standard, test group A & B vs positive control; * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Table 3: Identification of compounds presented in the *Arq Ajeeb* by GCMS analysis

Peak	RT	Formula	Molecular weight (g/mol)	Compound name	Synonyms
1.	6.426	$C_{10}H_{16}O$	152	bicyclo[2.2.1]heptan-2-one,1,7,7,-trimethyl-,(1s)-	L-camphor, (-)-Camphor, S-(-)-Camphor
2.	6.803	$C_{10}H_{20}O$	156	Levomenthol	(-)-Menthol, L-Menthol
3.	6.390	$C_{10}H_{14}O$	150	Thymol	p-Cymen-3-ol, Thymic acid, 3-Hydroxy-p-cymene, 6-Isopropyl-m-cresol

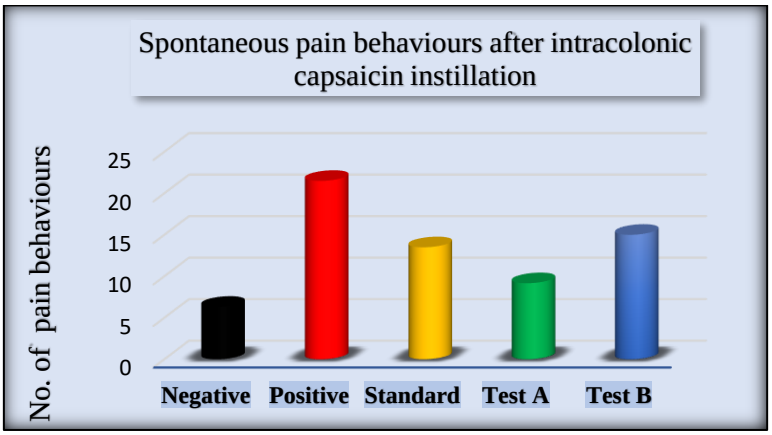

Figure 1. No. of pain behaviours observed in the 20 min post capsaicin administration

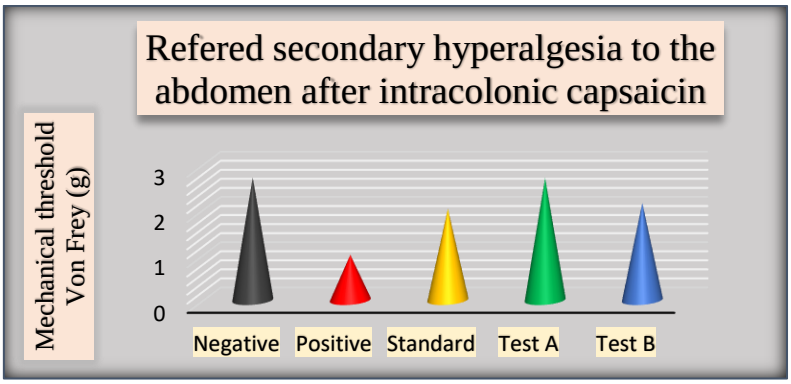

Figure 2. Response to the mechanical stimulation of the abdomen with HmVFF

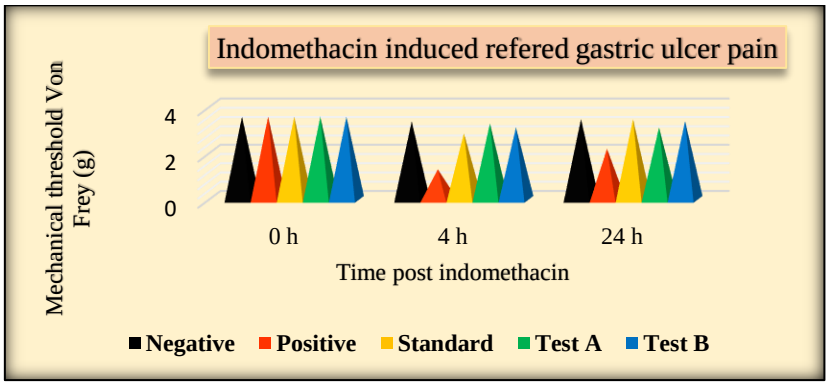

Figure 3. Dose-response and time course for the effect of AA on referred gastric ulcer pain

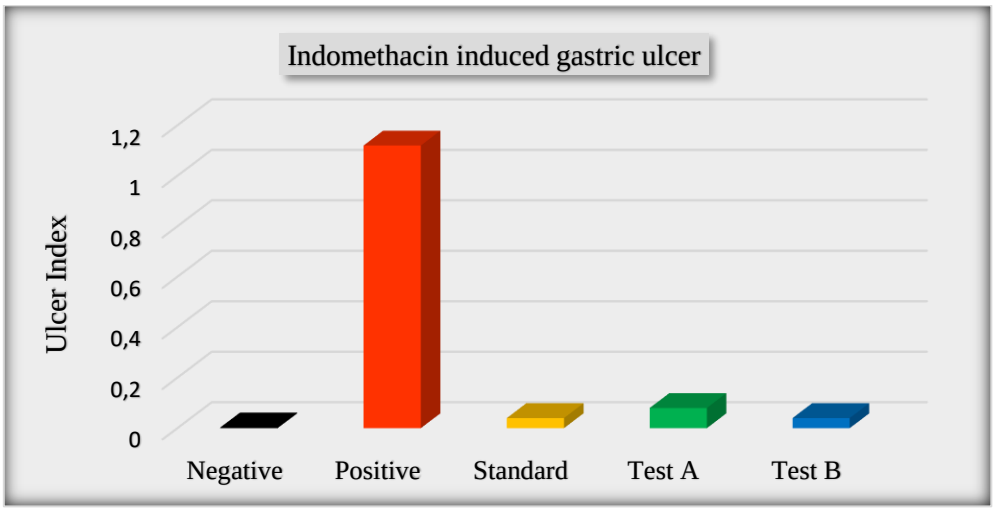

Figure 4. Effect of *Arq Ajeeb* on gastric ulcer lesion

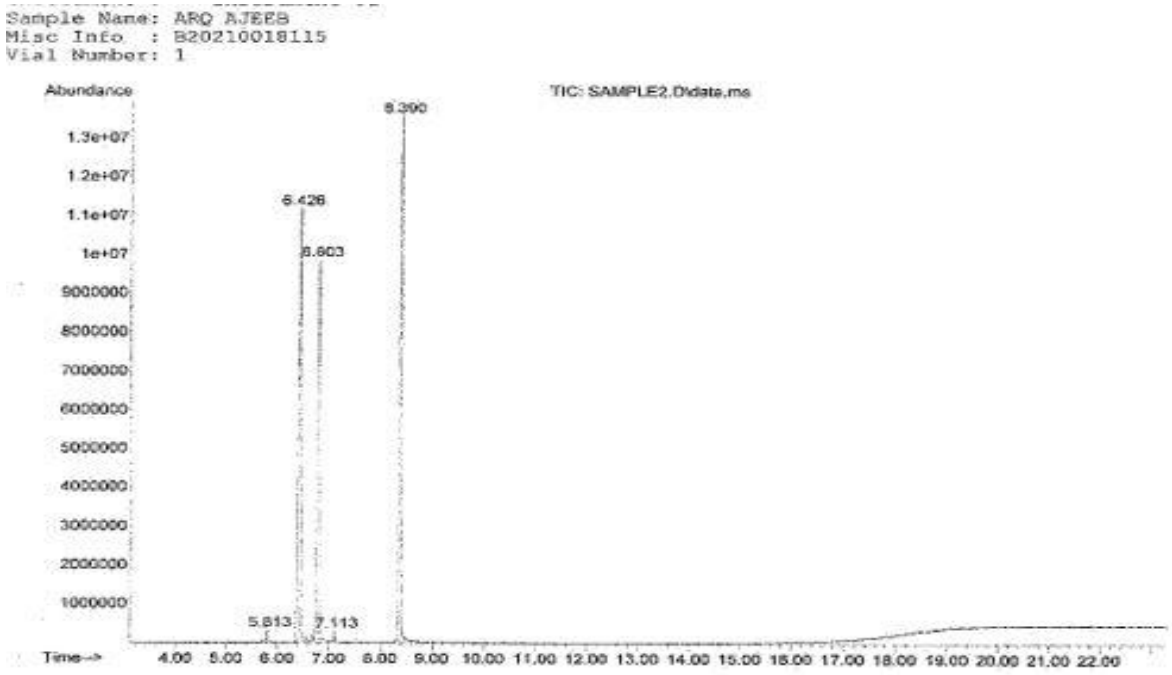

Figure 5. Chromatogram of AA by GC-MS

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