

In Vivo Anti-Inflammatory, Anti-Bacterial and Anti-Diarrhoeal Activity of *Ziziphus Jujuba* Fruit Extract

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Abstract

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BACKGROUND: *Ziziphus jujuba* belongs to family Rhamnaceae widely distributed in subtropical and tropical countries. It is used traditionally for several pharmacological purposes including anti-inflammation, antidiarrhoeal and antibacterial, as well as tonic and sometimes as hypnotic (sedative).

AIM: To determine the in vivo antidiarrhoeal, antibacterial and anti-inflammatory activities of *Z. jujuba* fruit ethanolic extract.

METHOD: The fruit was macerated and extracted by 95% (v/v) ethanol. The antidiarrhoeal activity was evaluated using castor oil and *Escherichia coli* induced diarrhoea mouse model. The antidiarrhoeal and antibacterial activity was investigated at graded doses (400-1200 mg/kg). The anti-inflammatory effects were tested using the carrageenan-induced paw oedema in female Wistar rats. Rat's treatment groups received tragacanth, 100 mg/kg diclofenac sodium, 800 mg/kg, 1200 mg/kg or 1600 mg/kg of an ethanolic extract of *Z. jujuba* (EEZJ). All treatment groups were fed with the compounds one hour before carrageenan injection at of rat's paw. Also, the EEZJ was further analysed by HPLC-PDA system for identification of the presence of betulinic acid and quercetin.

RESULTS: EEZJ different doses did not show inhibitory activity against castor oil induced diarrhoea except for the higher (1200 mg/kg) dose. However, the frequency of defecation of stools and watery stool were reduced significantly when compared to control group ($P \leq 0.05$ and $P \leq 0.01$ respectively), resulted in overall 67% inhibition of diarrhoea. Our anti-inflammatory results demonstrated that EEZJ was able to inhibit the carrageenan-induced paw oedema in rats to a significant degree ($p \leq 0.05$) and the paw volume and thickness of both left and right paw were affected compared to the negative control group.

CONCLUSION: EEZJ possesses antidiarrhoeal and antibacterial activity in a dose depending manner and may provide a pharmacological basis for its clinical use in diarrheal diseases. The activity may partially be due to the presence of betulinic acid and quercetin.

Introduction

Ziziphus jujuba Mill. (family: Rhamnaceae), is a thorny tree of medium height, whose fruit resembles buckthorn or olive fruit and had been widely consumed as both fruit and remedy for a long time all around the world. It has been listed among the first most valuable fruits in ancient Chinese medicine books. Moreover, in the Chinese herbal medicine, it is considered as one of the superior herbal medicines and thought to possess the effect of prolonging lifespan by purifying and nourishing blood, treating insomnia and help in digestion. Nowadays, *Z. jujuba*

fruit is believed to be one of the nutritious foods as it contains plenty of nutrients which consists of amino acids, carbohydrates, minerals and vitamins [1] [2].

Regarding the geographical distribution, *Z. jujuba* is widely found in the tropical and subtropical regions of Asia and America as well as in the Mediterranean regions [3]. It is well known in the Arabian Peninsula since ancient time. However, it is believed to be originated in the Algerian town of Annaba, due to which it has been named Annab. Furthermore, the mature fruit of *Z. jujuba* is red to purplish black, resembling small dates. Therefore in China are known as Chinese red date or Chinese

jujuba. The dried pulp of *Z. jujuba* is a source of essential unsaturated fatty acids. The main fatty acids in the jujube are oleic, linoleic (omega-6), palmitic, and palmitoleic acids. Jujube fruits contain various types of amino acids and proteins. The protein and free asparagines content are influenced and accumulated potentially during the ripening and harvesting stage [4].

Dietary fibre and fructose contents of the jujube fruit play a role in the regulation of blood sugar levels by slowing digestion [5]. The major sugars found in the jujube fruit are glucose, fructose, sucrose, rhamnose and sorbitol. The fruit is also abundant in vitamin C, which is one of the water-soluble antioxidants [6]. The postharvest sorting process is important for increasing the economic benefits and dietary values of the jujube fruit, especially vitamin C content protection during storage and marketing [7]. Moreover, the jujube is enriched, nevertheless to a lesser extent, with other vitamins including thiamin, riboflavin, niacin, vitamin B₆, and vitamin A. Jujube fruit is also considered a good source of minerals such as magnesium, phosphorus, potassium, sodium, and zinc [6]. Various studies have shown that the jujube fruit contains many bioactive compounds, including triterpenic acids, flavonoids, cerebrosides, phenolic acids, α -tocopherol, β -carotene, and polysaccharides. Each constituent of the jujube presents some health benefits, thus making it a healthy food choice [8]. The total phenolic compounds in jujube fruit which is accounted for the antioxidant activities are higher compared to other common fruits, such as cherry, apple, persimmon, or red grape [9]. Flavonoids, phenolic acids, tannins, stilbenes, and lignans are derivatives of phenolic compounds [10] [11] [12].

The Indian jujuba, *Ziziphus mauritiana* Lam. and *Z. jujuba* Mill. Are the two main domesticated jujubes. The pantropical genus *Ziziphus* Mill. Includes approximately 170 species with a few species occurring in temperate regions. Recent studies on the biological activities of this fruit have supported the health benefits of jujube as both food and medicinal herb. Different parts of *Z. jujuba* are used traditionally for curing many kinds of illness including diabetes, diarrhoea, liver complaints, urinary disorders, obesity, skin infections, respiratory infections, anaemia, insomnia, cancer, and also for blood purification and modification of the gastrointestinal tract [6] [13] [14]. *Z. jujuba* is used traditionally as a tonic and sometimes as hypnotic-sedative. Additionally, there are studies that had been carried out to test its anxiolytic, anticancer, antimicrobial, anti-inflammatory, anti-allergy, cognitive, anti-nephritic, antioxidant and wound healing properties [8] [15].

The anxiolytic effect *in vivo* had been reported in a polyherbal substance which consists of seed extract of *Z. jujuba* [16]. The possibility of improving cytotoxic activity was suggested to be due to the

presence of coumaroyl moiety at the carbon-3 position of the lupanetype triterpene, extracted from *Z. jujuba* [17]. Selective toxicity against cultured human melanoma cells is also performed by the triterpenes acid and betulinic acid extracted from *Z. jujuba* and *Ziziphus mauritiana* [18]. Notable inhibitory activity performed by ethanolic extract of *Z. jujuba* root on fungi *Candida albicans*, *C. tropicalis*, *Aspergillus flavus* and *A. niger* showed convincing antifungal activity [19]. Additionally, extract of root bark of *Z. jujuba* exhibited antibacterial activity against 20 bacteria [20].

A traditional Chinese prescription, Huangqin Tang with fruit content from *Z. jujuba* had shown remarkable anti-inflammatory and antispasmodic effect [21]. Additionally, *Ziziphus mauritiana* leaf extracts were also found to possess significant anti-inflammatory activity against carrageenan induced rat paw oedema [22]. The anti-allergic activity of the aqueous extracts of leaves of *Z. jujuba* was studied by measuring its inhibitory effect on hyaluronidase or bovine testes activation *in vitro*. *Z. jujuba* was shown to have strong antiallergic and anti-anaphylactic activity [23] [24]. A component of *Z. jujuba* extract, oleamide poses possible potential against Alzheimer's disease as it could be a useful chemopreventative agent [25] [26]. Methanolic extract of *Z. jujuba* was found to show activation effect on choline acetyltransferase *in vitro* as high as 34.1%.

Moreover, *Z. jujuba* also exhibits possibly anti-nephritic effect by increasing renal blood flow and thus reducing inflammation of kidney [27]. Two reports studied 70 antioxidant Korean medicinal plants listed the *in vitro* antioxidant effect of *Z. jujuba* [28] [29]. The wounding healing effect had been reported on the extract of *Z. jujuba* root [30]. Furthermore, a rat model which uses an ointment formula at a dose 0.5% and 1% on topical application proved the wound healing activity of the extract of *Z. jujuba* root [31]. The main aim of the current studies was to further explore the anti-inflammatory and anti-diarrhoeal activities of ethanolic extract of *Ziziphus jujube* fruit.

Materials and Methods

The 1 ml and 10 ml disposable syringes, amber bottles, oral gavage and 27G needle, were purchased from Terumo Tokyo-Japan. Digital Plethysmometer (model 7140, Ugo Basile, Italy), weighing balance (A & D, Tokyo, Japan), freezer (Action International, Kuala Lumpur, Malaysia), refrigerator (Sharp Malaysia), cell culture incubator (CL-170B-8, ESCO, Singapore), rotary evaporator (Butchi Rotavapour model R-114, Büchi Labortechnik AG, Flawil Switzerland). The plastic cages, water bottles, white cloth, tissue paper, masks, gloves,

vacuum pump and vortex, were from Autovortex SA6, Stuart Scientific, UK. HPLC machine (Waters 2695; WATERS CORPORATION, Milford, MA 01757 USA), Ultra-purified water machine (ELGA Labwater Purification System, High Wycombe HP14 3BY, UK).

The dried granules of fruits (2744.58 g) were extracted by cold maceration for 72 hours with 95% of ethanol. The solutions were filtered using vacuum filtration. The filtrates were then concentrated in a rotary evaporator to eliminate ethanol and yielded semi-solid extract. Immersion, filtration and rotary evaporation process were repeated for three times. The extract of *Z. jujuba* was preserved at 10°C until further use [24].

Different concentration of EEZJ, loperamide, and diclofenac sodium salt were freshly prepared in tragacanth compound powder suspension prior administration. Carrageenan powder was added into normal saline to create 1% w/v carrageenan solution at 4°C and stirred immediately. Any remaining lumps were dispersed by using vortex. The solution was warmed to 50°C with stirring. After that, the solution was incubated at 40°C for less than 24 hours before use [32].

Sample (EEZJ) and standards (betulinic acid and quercetin) were weighed and transferred into three 2 ml vials, respectively. After dissolved in 100% methanol, solutions were filtered through 0.45 µm membrane filters before HPLC analysis, Guo et al. [33].

The bacterial inoculum suspension of *E. coli* (ATCC 25927) was prepared by direct transfer of bacteria from stock culture into nutrient broth contained in the universal bottle. Few loops of stock culture were transferred into the sterile nutrient broth and incubated for 24 hours at 37°C prior administration. *E. coli* suspension was then compared with McFarland standards to obtain the desired turbidity of bacterial suspension. The minimum effective dose obtained from this test will determine the doses of choice in the *in vivo* anti-diarrhoeal experiment and *in vivo* antibacterial experiment [33].

Following Sahoo et al. work [34] the preliminary *in vivo* assay using mice, six mice were separated randomly into two groups of three animals each. The turbidity of *E. coli* inoculum suspensions was adjusted to McFarland Standard No. 1 (3.0×10^8 CFU/ml), No. 2 (6.0×10^8 CFU/ml) prior administration to mice. Group 1 received 1 ml of *E. coli* inoculum suspension of McFarland Standard No.1 while group 2 received 1 ml of *E. coli* inoculum suspension of McFarland Standard No.2. Following treatment of *E. coli* suspension, mice were placed and observed for subsequent eight hours in the separated beaker which consists of white filter paper. The filter paper was changed hourly. Several parameters such as the weight of stools, the frequency of total stools and watery stools were taken for measuring purpose. After

eight hours of observation, mice were sacrificed, and the intestines were removed. Any abnormalities such as ulceration, perforation and redness were observed and recorded. The minimum concentration of *E. coli* that induced abnormalities in intestines of the mice was used in the antibacterial activity experiment.

Base on experiment used by Ayalate and his colleagues [36], thirty mice with a weight of 20-35 g were separated into six groups of five animals each. All five groups of animals were fed orally with 1 ml of *E. coli* suspension using gavage needle with turbidity similar to McFarland Standard No. 1 three hours prior administration of suspension. Group 1 received 0.2 ml of tragacanth 2% orally and served as the control group while group 2 received antibiotic (Amoxicillin 260 mg/kg) and served as positive control. Group 3, 4 and 5 received 400 mg/kg, 800 mg/kg and 1200 mg/kg of EEZJ, respectively. Following treatment with drug and extraction suspension, mice were separated and placed individually into a different beaker containing a white filter paper and observed for the subsequent four hours. The white filter papers were changed hourly.

RA was calculated according to the following formula:

$$\text{Relative area} = \frac{\text{Total mucosal area}}{\text{Total ulcerated area}}$$

Meanwhile, approximately 2000 mg of stools of each mouse were collected and suspended into 5 ml of sterile phosphate-buffered saline (PBS) to produce suspension A, which underwent vigorous shaking by a vortex mixer for at least 15 minutes. Then, 100 µl of the suspension A was diluted into 10 ml of sterile PBS to form suspension B. Afterwards, 100 µL of the suspension B was spread on to the MacConkey agar plates and incubated at 37°C for 24 hours. Later the growth of bacterial colony was observed and counted by using colony counter.

A preliminary study was carried out to determine the dose of carrageenan to induce paw oedema in rats. Moreover, the effective doses of plant extract that exert an observable anti-diarrhoeal effect on castor oil induced diarrhoea, as well as anti-inflammatory effects on carrageenan-induced paw oedema were determined in the current study.

Following Wang et al. method [33], 30 mice were selected and divided randomly into six groups of five animals each. The weight of all mice was in the range of 20-35 g. Group 1 and group 2 were administered with 0.2 ml of 2% tragacanth and 4.2 mg/kg loperamide HCl respectively. Group 3, 4 and 5 received 400 mg/kg, 800 mg/kg and 1200 mg/kg of EEZJ, respectively. Mice acute diarrhoea was induced with 0.2 ml of castor oil one hour after administration of oral suspension. After the supply of castor oil, animals were placed in the separated beakers which consist of white filter paper. The filter papers were

changed every hour for subsequent four hours. Mice were under direct observation for the onset of diarrhoea, weight of stools, and frequency of total stools and watery stools during the four hours. The proportion of the weight of watery stools was calculated based on the formula that developed by Wang et al., [33].

$$\text{Proportion of weight of watery stool} = \frac{\text{Weight of watery stool}}{\text{Total weight of stool}} \times 100$$

The percentage of inhibition of defaecation and diarrhoea were calculated according to the formula that developed by Lumpu et al., [37].

$$\text{Percentage inhibition of defaecation} = \frac{P_c - P_s}{P_c} \times 100$$

Where P_c is the average number of defaecation of control group while P_s is the average number of defaecation of the test group.

$$\text{Percentage inhibition of diarrhoea} = \frac{D_c - D_s}{D_c} \times 100$$

Where D_c is the average number of diarrhoea of control group, while D_s is the average number of diarrhoea of test group.

Acute inflammation was induced by subplantar injection of 0.1 ml carrageenan solution (1% w/v in normal saline) in the hind paw of each rat. Before injection, the rat paw was held still and steady to ensure the injection site was cleared and observable. Different groups of rats were administered either solution of *Z. jujuba* ethanolic extract (800 mg/kg, 1200 mg/kg or 1600 mg/kg), diclofenac sodium (100 mg/kg) or negative control solution (2% tragacanth) orally, one hour before carrageenan injection. The paw volume and thickness were measured by plethysmometer and digital calliper respectively immediately before carrageenan injection (0 h) and the consecutive six hours with the one-hour interval between two readings [38].

Paw volume was measured by dipping the rat paw into the tube until marked level, and the pedal switch was pressed to get the data value after the figure stabilised. The rat feet were kept still and steady during measurement process to improve accuracy and consistency. Every time after data value was taken, the meter was zeroed before next measurement. The thickness of the paw was taken at the marked line to ensure the consistency and accuracy of the data value by using a digital calliper.

The extent of inflammation was expressed as percent (%) oedema and calculated as below [39]:

$$\% \text{ oedema} = \frac{\alpha - \beta}{\beta} \times 100$$

Where α and β are the paw volume/ thickness after carrageenan injection and paw volume/ thickness before carrageenan injection respectively. The anti-inflammatory effects were deduced from the

extent of inflammation. The greater the extent of inflammation, the less anti-inflammatory effects exerted by that treatment group.

HPLC analysis was performed on a Waters 2695 Alliance HPLC system (Waters Corporation, United States of America), equipped with a quaternary pump solvent management system, an auto-sampler, and an on-line degasser. The separation was carried out with an XBridge™ C18 column (4.6 mm x 250 mm) while raw data were detected by Waters 2998 PDA and processed with Empower™ Software. The column temperature was 25°C.

The mobile phase for betulinic acid HPLC qualification was composed of A (acetonitrile) and B (0.1 % acetic acid) at the flow rate of 1.0 ml/min. The elution concentration of mobile phase were: 0-5 min (A: 20%; B: 80%), 6-25 min (A: 70%; B: 30%), 26-45 min (A: 90%; B: 10%) and detected at the wavelength of 205nm. Re-equilibration duration was 15 minutes between individual runs [40].

HPLC analysis for qualification of quercetin was carried out by gradient elution beginning with a mobile phase A (acetonitrile) and B (0.2% of acetic acid) at the flow rate of 0.5 ml/min. The elution concentration of mobile phase were: 0-12 min (A: 30%; B: 70%), 12-13 min (A: 33%; B: 67%), 13-31 min (A: 48%; B: 52%), 31-35 min (A: 63%; B: 37%), 35-80 min (A: 100%; B: 0%) and detected at the wavelength of 205nm [41]. Re-equilibration duration was 15 minutes between individual runs.

The chromatographic peaks from EEZJ were identified by comparing the retention time to the reference standard compounds which underwent the same condition of elution. Each sample and reference standard were analysed for three times for the precision of the analysis.

Data obtained were analysed statistically using GraphPad PRISM. All data are presented as means \pm standard error of the mean (S.E.M). Data were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. The level of statistical significance was set at $P \leq 0.001$ (***) or $P \leq 0.01$ (**) or $P \leq 0.05$ (*) when compared to control group.

Result

The yield of a crude ethanolic extract of *Z. jujuba* was 1620.68 g, from 2744.58 g. The yield percentage was 59.05%. 90% of the extract was subjected to *in vivo* test while another 10% was subjected to qualification analysis.

Two parameters were employed in this test, which is the number of colonies growing in the petri dish and the ulcer index. Positive control greatly reduces the number of colonies growing on petri dish; nevertheless, the results were not sufficient to be statistically significant. Test groups postulate a decreasing trend in reducing the number of colonies in a dose depending manner. However, the results are not statistically significant.

However, Table 1 shows that the EEZJ inhibited the growth of bacteria *in vivo* in a dose depending manner, although there were no ulcers observed in our experiment. Orally administered *E. coli* demonstrated some redness like inflammation and ulcerative presentation at the intestinal tissue during post-mortem session.

Table 1: Antibacterial activity

Compound	Dosage (mg/kg)	Number of Colonies	Ulcer Index
Control	-	162.83 ± 49.57	0.82 ± 0.02
Amoxicillin	260	60.67 ± 18.11 ^{ns}	0.80 ± 0.00 ^{ns}
EEZJ	400	140.83 ± 48.14 ^{ns}	0.82 ± 0.04 ^{ns}
EEZJ	800	122.83 ± 52.64 ^{ns}	0.77 ± 0.05 ^{ns}
EEZJ	1200	117.00 ± 38.26 ^{ns}	0.88 ± 0.02 ^{ns}

(means ± S.E.M; n = 6); ns P ≥ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); *P ≤ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); **P ≤ 0.01 compared to control (One-way ANOVA followed by Dunnett's Test).

Based on Table 2, the extractions produced no significant results in parameters such as the proportion of watery stools, and the onset of diarrhoea when compared statistically to the control group. However, there is a trend of reducing the proportion of the weight of watery stool in mice treated with different concentration of EEZJ. Not only that, but mice treated with EEZJ also delayed the onset of diarrhoea compared to control group. However, the results obtained were not statistically significant. Among three concentrations of EEZJ, only EEZJ 1200 mg/kg produced a significant result (P ≤ 0.01) in reducing a number of watery stools. This significant level was comparable with positive control as it also produced a significant result (P ≤ 0.01). Furthermore, EEZJ 1200 mg/kg was the only test group that produced a significant result (P ≤ 0.05) when compared to control group in reducing a total number of the stool. From Table 3, it shows increasing inhibition of both defaecation and diarrhoea frequency with increasing concentration of EEZJ. Positive control produced the similar significance level when compared to negative control (P ≤ 0.05) regarding reducing a total number of stool. Loperamide was the only group that showed the significant result in all parameters when compared to control group.

Table 2: Preliminary antidiarrhoeal test

Compound	Dosage (mg/kg)	The proportion of Weight of Watery Stool (%)	The onset of Diarrhoea (hour)	Total Number of Defecation	Number of Watery Stool
Control	-	92.73 ± 6.70	1.00 ± 0.00	12.67 ± 1.20	11.67 ± 1.76
EEZJ	100	96.19 ± 3.62 ^{ns}	1.33 ± 0.33 ^{ns}	12.67 ± 1.76 ^{ns}	11.67 ± 2.33 ^{ns}
EEZJ	400	75.47 ± 8.81 ^{ns}	2.67 ± 0.33**	13.67 ± 1.76 ^{ns}	9.00 ± 1.00 ^{ns}

(means ± S.E.M; n = 3); ns P ≥ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); *P ≤ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); **P ≤ 0.01 compared to control (One-way ANOVA followed by Dunnett's Test).

As shown in Table 4 and Table 5, anti-inflammatory activity of *Z. jujuba* is significant when compared to negative control group (2% tragacanth) all the time. The experimental findings from the carrageenan-induced rat paw oedema model showed that the *Z. jujuba* ethanolic extract reduced the paw volume significantly (P ≤ 0.001) from 1 h to 6 h. Inhibition of paw oedema formation was shown in three doses of *Z. jujuba* ethanolic extract. However, the anti-inflammatory effect exerted by *Z. jujuba* ethanolic extract was less potent than that found with positive control drugs. Furthermore, the onset of anti-inflammatory action of *Z. jujuba* ethanolic extract was found to be comparable with that of control positive drug.

Table 3: In vivo antidiarrhoeal activity

Compound	Dosage (mg/kg)	The proportion of Weight of Watery Stool (%)	The onset of Diarrhoea (hour)	Total Number of Stools	Number of Watery Stool	Inhibition of Defecation (%)	Inhibition of Diarrhoea (%)
Control	-	83.07 ± 5.98	1.83 ± 0.31	16.33 ± 1.50	13.17 ± 2.06	0.0	0.0
Loperamide	4.2	42.49 ± 14.57*	3.67 ± 0.56*	6.83 ± 2.75*	3.83 ± 1.72**	58.16	70.89
EEZJ	400	77.34 ± 3.70 ^{ns}	2.33 ± 0.21 ^{ns}	11.17 ± 1.90 ^{ns}	9.00 ± 0.97 ^{ns}	31.63	31.66
EEZJ	800	73.00 ± 3.90 ^{ns}	2.33 ± 0.33 ^{ns}	10.50 ± 1.38 ^{ns}	8.67 ± 1.01 ^{ns}	35.70	34.17
EEZJ	1200	66.23 ± 12.06 ^{ns}	2.83 ± 0.48 ^{ns}	8.50 ± 1.82*	4.33 ± 1.45**	47.95	67.09

(means ± S.E.M; n = 6); ns P ≥ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); *P ≤ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); **P ≤ 0.01 compared to control (One-way ANOVA followed by Dunnett's Test).

According to Table 4 and 5, both three doses of *Z. jujuba* ethanolic extract successfully controlled the percentage of paw oedema under 40%. 1200 mg/kg *Z. jujuba* ethanolic extract treatment group has a higher percentage of paw oedema inhibition when compared to that of 800 mg/kg and 1600 mg/kg *Z. jujuba* ethanolic extract treatment groups. 800 mg/kg *Z. jujuba* ethanolic extract treatment group has weakest anti-inflammatory effect at the corresponding time point.

Table 4: Percentage of right paw oedema (%) calculated from the change in paw volume after treatment

Group	Treatment	Dose (mg/kg)	Percentage of right paw oedema (%) after treatment (Mean ± SEM)					
			1 h	2 h	3 h	4 h	5 h	6 h
1	EEZJ	800	7.60 ± 1.12***	14.00 ± 1.58***	20.80 ± 1.72***	25.40 ± 1.69***	30.20 ± 2.08***	36.00 ± 2.68***
			8.00 ± 1.27***	13.40 ± 1.20***	18.40 ± 1.50***	24.00 ± 1.79***	30.00 ± 2.43***	32.80 ± 3.41***
2	EEZJ	1200	6.00 ± 0.45***	16.00 ± 1.76***	21.40 ± 1.33***	26.80 ± 1.78***	30.40 ± 1.86***	35.00 ± 2.24***
			3.20 ± 1.07***	8.00 ± 1.30***	13.60 ± 2.34***	18.00 ± 2.67***	21.20 ± 2.56***	23.20 ± 2.35***
3	EEZJ	1600	28.00 ± 4.72	45.80 ± 5.00	68.00 ± 5.06	89.20 ± 6.59	105.20 ± 10.10	114.00 ± 10.07
			28.00 ± 4.72	45.80 ± 5.00	68.00 ± 5.06	89.20 ± 6.59	105.20 ± 10.10	114.00 ± 10.07
4	Diclofenac Sodium	100	3.20 ± 1.07***	8.00 ± 1.30***	13.60 ± 2.34***	18.00 ± 2.67***	21.20 ± 2.56***	23.20 ± 2.35***
5	Control	100	28.00 ± 4.72	45.80 ± 5.00	68.00 ± 5.06	89.20 ± 6.59	105.20 ± 10.10	114.00 ± 10.07

(means ± S.E.M; n = 5); ns P ≥ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); *P ≤ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); **P ≤ 0.01 compared to control (One-way ANOVA followed by Dunnett's Test); ***P ≤ 0.001 compared to control (One-way ANOVA followed by Dunnett's Test).

Apart from that, all three doses of *Z. jujuba* ethanolic extract showed a similar trend of oedema development. Additionally, the *Z. jujuba* ethanolic extract produced the dose-dependent significant anti-inflammatory activity as the lowest dose, 800 mg/kg

showed least anti-inflammatory activity when compared to other two with the higher dose.

According to Table 6 and 7, the three doses of *Z. jujuba* ethanolic extract successfully controlled the thickness of paw that due to oedema when compared with that of the negative control group. The 1200 mg/kg *Z. jujuba* ethanolic extract treatment group has a higher percentage of paw oedema inhibition when compared to the 800 mg/kg and 1600 mg/kg *Z. jujuba* ethanolic extract treatment groups. However, all the three doses of *Z. jujuba* ethanolic extract exhibited lower anti-inflammatory activity than the positive control group.

Table 5: Percentage of left paw oedema (%) calculated from the change in paw volume after treatment

Group	Treatment	Dose (mg/kg)	Percentage of left paw oedema (%) after treatment (Mean ± SEM)					
			1 h	2 h	3 h	4 h	5 h	6 h
1	EEZJ	800	6.80 ± 1.16***	13.40 ± 2.54***	19.80 ± 3.09***	25.80 ± 3.80***	31.00 ± 4.38***	37.00 ± 4.45***
2	EEZJ	1200	6.00 ± 0.63***	12.60 ± 1.75***	17.40 ± 2.11***	24.40 ± 4.13***	29.40 ± 4.68***	32.20 ± 4.12***
3	EEZJ	1600	5.80 ± 0.37***	12.80 ± 1.36***	17.40 ± 1.17***	25.00 ± 1.82***	29.00 ± 1.92***	34.20 ± 2.48***
4	Diclofenac Sodium	100	2.20 ± 0.37***	6.40 ± 0.81***	10.20 ± 2.60***	14.00 ± 2.59***	17.60 ± 2.46***	19.80 ± 2.29***
5.	Control	100	23.60 ± 4.78	41.80 ± 4.60	60.00 ± 4.37	78.00 ± 6.72	93.20 ± 6.58	102.60 ± 6.81

Means ± S.E.M; n = 5); ns P ≥ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); *P ≤ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); **P ≤ 0.01 compared to control (One-way ANOVA followed by Dunnett's Test); ***P ≤ 0.001 compared to control (One-way ANOVA followed by Dunnett's Test).

The HPLC chromatograms of the reference standards and EEZJ are shown in the Fig. 1A, 1B, 1C and 1D, respectively. UV absorption, retention time and identification results of peaks are presented in Table 8.

Table 6: Percentage of right paw oedema (%) calculated from the change in paw thickness after treatment

Group	Treatment	Dose (mg/kg)	Percentage of right paw oedema (%) after treatment (Mean ± SEM)					
			1 h	2 h	3 h	4 h	5 h	6 h
1	EEZJ	800	14.00 ± 4.93 ^{ns}	19.33 ± 4.41 ^{ns}	21.67 ± 3.38**	25.33 ± 2.91*	20.33 ± 9.33**	26.67 ± 7.13**
2	EEZJ	1200	6.33 ± 1.45*	14.33 ± 2.33*	20.67 ± 1.76**	22.67 ± 3.48**	23.00 ± 5.20*	21.00 ± 6.81***
3	EEZJ	1600	10.33 ± 0.88 ^{ns}	20.00 ± 1.53 ^{ns}	25.33 ± 0.88*	24.67 ± 6.57*	26.33 ± 5.78*	24.67 ± 1.20**
4	Diclofenac Sodium	100	3.00 ± 0.58**	8.00 ± 1.00**	10.00 ± 1.53***	8.66 ± 3.28***	10.00 ± 4.50**	13.33 ± 3.18***
5.	Control	100	19.33 ± 3.53	31.00 ± 4.73	39.00 ± 4.36	46.00 ± 2.52	56.00 ± 4.51	62.33 ± 3.71

Means ± S.E.M; n = 5); ns P ≥ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); *P ≤ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); **P ≤ 0.01 compared to control (One-way ANOVA followed by Dunnett's Test); ***P ≤ 0.001 compared to control (One-way ANOVA followed by Dunnett's Test).

Peak 1 represented the present of betulinic acid at the retention time of 42.169 minutes from reference compound. EEZJ showed a peak at the same retention time. Hence, EEZJ consists of betulinic acid as the peak was comparable to reference compound (Fig. 1).

Peak 2 appeared at 16.821 minutes in the reference compound and represented as quercetin. EEZJ showed a peak at the same retention time

which indicates the presence of quercetin in the extract tested (Fig. 1).

Discussion

People in different communities commonly use plant or plant derivatives to cure illnesses without scientific evidence. The current studies aimed to evaluate anti-diarrhoeal as well as anti-inflammatory activity potential of *Z. jujuba* fruit. Castor oil was given to mice to imitate the diarrhoea symptom while carrageenan was given to rats to induce inflammation at the paw of the rats.

Table 7: Percentage of left paw oedema (%) calculated from the change in paw thickness after treatment

Group	Treatment	Dose (mg/kg)	Percentage of left paw oedema (%) after treatment (Mean±SEM)					
			1 h	2 h	3 h	4 h	5 h	6 h
1	EEZJ	800	12.67 ± 3.18**	22.00 ± 7.55 ^{ns}	28.67 ± 6.33 ^{ns}	27.33 ± 5.55**	30.33 ± 6.94**	30.67 ± 4.41***
2	EEZJ	1200	7.00 ± 0.58***	15.00 ± 1.53*	19.33 ± 0.88**	19.00 ± 1.16***	22.67 ± 1.67***	19.67 ± 1.76***
3	EEZJ	1600	10.33 ± 0.33**	19.67 ± 2.73 ^{ns}	25.00 ± 3.00*	1.20*	4.36**	27.33 ± 3.53***
4	Diclofenac Sodium	100	3.00 ± 0.58***	6.00 ± 1.00**	8.33 ± 0.67***	8.33 ± 1.33***	6.33 ± 1.33***	12.00 ± 7.69***
5.	Control	100	22.33 ± 2.03	31.33 ± 1.67	40.33 ± 2.33	46.67 ± 2.67	53.33 ± 1.20	56.00 ± 2.08

Means ± S.E.M; n = 5); ns P ≥ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); *P ≤ 0.05 compared to control (One-way ANOVA followed by Dunnett's Test); **P ≤ 0.01 compared to control (One-way ANOVA followed by Dunnett's Test); ***P ≤ 0.001 compared to control (One-way ANOVA followed by Dunnett's Test)

The minimum effective dose of EEZJ that determined in the preliminary test was brought into the experimental test. Moreover, two higher concentration of EEZJ (800 mg/kg and 1200 mg/kg) were added into the test to evaluate the effectiveness in gradual increasing dose.

Table 8: Method employed, retention time, and ultraviolet absorption of betulinic acid, quercetin and ethanolic extract of *Ziziphus jujuba*

Compound	Method	Constituent	Retention Time (min)	UV Absorption (nm)
Standard 1	Zhang et al. 2008	Betulinic Acid	42.169	205
EEZJ	Zhang et al. 2008	Betulinic Acid	42.169	205
Standard 2	Guo et al. 2011	Quercetin	16.821	205
EEZJ	Guo et al. 2011	Quercetin	16.821	205

Loperamide (loperamide hydrochloride) served as a positive control group in the experiment as it acts directly at the opioid receptors on the circular and longitudinal intestinal mucosa. Similar to other μ-receptor agonists, loperamide inhibits peristalsis of the intestine and prolongs the transit time of the digested content. Loperamide also stimulates the reabsorption process and alters the transport of water and electrolytes. Thus, it reduces the faecal volume and decreases the fluid and electrolyte loss. Loperamide not only used in acute diarrhoea patient but also successfully applied in chronic diarrhoea patient for several years without evidence of tolerance [42].

Based on the results obtained and presented in Table 3, mice treated with different concentrations of EEZJ produced a reduced number of watery dropping as well as a total number of faeces.

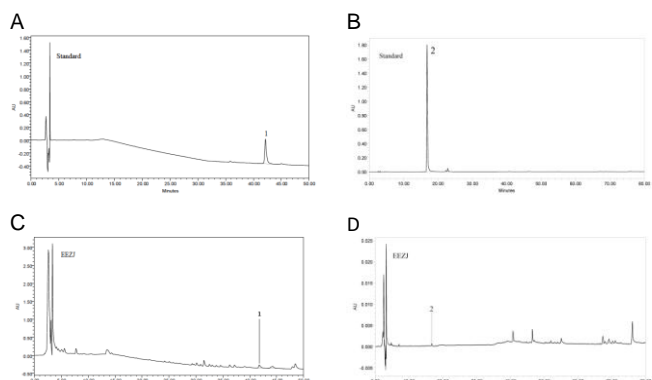


Figure 1: A) Chromatographic profile of standard; 1 = Betulinic acid; B) Chromatographic profile of standard; 2 = Quercetin; C) Chromatographic profile of ethanolic extract of *Ziziphus jujuba*; 1 = Betulinic acid; D) Chromatographic profile of ethanolic extract of *Ziziphus jujuba*; 2 = Quercetin

This result is similar to the studies done by Oh and Ryu [43], where they proposed that Korean oriental medicine which containing *Z. jujuba* fruit, can reduce the number of faeces of mice in five hours of observation. A similar observation was reported by Hu et al. [44] where a Chinese traditional medicine (Wei-Chang-An-Wan extract) containing *Z. jujuba* fruit was able to reduce the total number of faeces significantly and the number of watery dropping.

Stool weight depends on many factors such as water composition, number of bacteria and fibre content in the stool. In addition to this, total stool weight is highly correlated with the frequency of defaecation [45]. In our experiment, EEZJ did not only reduce the number of stool but also induced mice to produce a lesser weight of watery stool. Muller et al. [46] in their work concluded that the gastrointestinal transit time would affect the weight of stool where longer gastrointestinal transit time will produce lighter stool weight. Which was also tested by Rao and Lakshimi [44], where they also displayed the aqueous extract of *Z. jujuba* leaves to increase the gastrointestinal transit time. Hence, it may indicate that *Z. jujuba* fruit in our experiment may reduce stool weight by increasing the gastrointestinal transit time.

EEZJ did not only reduce stool weight and some faeces, but it also delayed the onset of first watery dropping compared to control group. *Z. jujuba* can reduce the intestinal motility, and this has been described by Rao and Lakshimi [47]. Hu et al. [44] demonstrated that *Z. jujuba* containing traditional medicine able to attenuate the gastrointestinal tract motility in a dose depending manner. Based on the same studies, *Z. jujuba* containing traditional medicine also decreased the spontaneous contraction of

isolated jejunum in animals. In our study, the result showed that EEZJ could reduce the number of faeces as well as the weight of faeces in a dose depending manner. Moreover, it significantly attenuated the onset of first watery dropping from the mice. With these characteristics, EEZJ can be considered as possessing antidiarrhoeal activity against castor oil-induced diarrhoea.

Inflammation process causes an alteration in human body's physiological responses and ultimately resulting in pain, heat, redness, swelling and loss of function. Carrageenan-induced paw oedema is an example of acute inflammation which results in swelling. It had been widely used as an experimental animal model to discover and evaluation of anti-inflammatory potential [48].

Carrageenan is a complex group of polysaccharides made up of repeating galactose-related monomers. The inflammatory response is usually quantified by an increase in paw size which is observed for around five hours after carrageenan injection [32]. Inhibition of carrageenan-induced inflammation has been shown to be highly predictive of anti-inflammatory activity in human inflammatory disease and doses of nonsteroidal anti-inflammatory drugs (NSAIDs) in this model correlate well with effective dose in patients [49]. Using antagonists of various mediators of inflammation showed that the inflammatory response to carrageenan consisted of three phases [50]. The primary phase is mediated by both histamine and 5-hydroxytryptamine. The second phase starts after one hour and persists for six hours after carrageenan injection. This phase is kinin-mediated.

The final phase is attributed to the local production of prostaglandins, which is derived from arachidonic acid by the action of cyclooxygenase (COX) enzymes. Inhibition of these enzymes is the basis of action of the NSAIDs of major clinical importance in the treatment of pain and inflammation [51]. Cyclooxygenase 2 (COX-2) is a pro-inflammatory enzyme which is in charge of making prostaglandins (PG), and it is also the site of action targeted by non-steroidal anti-inflammatory drugs such as diclofenac and COX-2 inhibitors. However, COX-2 is normally produced within the second hour after carrageenan is induced to cause inflammation. This means PG is not involved in oedema formation in the very first hour but instead contribute to oedema within second and the subsequent hours.

According to Di Lorenzo et al. [52], carrageenan injection evokes neutrophil infiltration immediately after injection and persists for six hours. O_2^- , OH and H_2O_2 which are derived from neutrophils, are suggested to cause a sustained increase in permeability through damaging the endothelial cells [53]. Thus, carrageenan-induced paw oedema has been an important tool in the development of NSAIDs.

A role for neutrophil-derived reactive oxygen species, nitric oxide, and peroxynitrite in carrageenan-induced inflammation has also been identified, and some specific inhibitors have been identified which have potential clinical use.

Diclofenac sodium is superior to the other clinically established non-steroidal anti-inflammatory agents in carrageenan-induced paw oedema [54]. It exerts their effect by blocking prostaglandin synthetase system. In the present study, diclofenac sodium significantly expressed its anti-inflammatory activity in Table 4-7. The result of the present study showed and solidified that diclofenac sodium has significant anti-inflammatory activity.

According to the anti-inflammatory experiments that were carried out, *Z. jujuba* ethanolic extract successfully controlled the inflammation of the paw. At 1200 mg/kg dose of *Z. jujuba* ethanolic extract displayed the highest paw oedema inhibition. The mechanisms of action of *Z. jujuba* ethanolic extract may be similar to that diclofenac sodium whereby the anti-inflammatory effect observed might be due to the inhibition of expression and activity of COX-2.

Based on the chemical analysis done, it was found that *Z. jujuba* fruit contains both betulinic acid and quercetin in it. Earlier studies showed that most of the medical plants could counter dysentery and diarrhoeal incident. Most of the medical plants contain alkaloids, saponins, flavonoids, sterols and triterpenoid [55] and they are responsible for the mechanism of antidiarrhoeal activity. Betulinic acid belongs to the pentacyclic triterpenoid group [56], whereas quercetin belongs to flavonoids group [13]. From the earlier statement, we understand that both triterpenoid and flavonoids capable of possessing antidiarrhoeal activity. This is further support by Ezekwesili et al. [57] studies, where he described that quercetin relaxes the intestinal smooth muscle and inhibits bowel contraction which probably due to inhibition of intracellular release of calcium ions from the sarcoplasmic reticulum. Other than that, Begum et al. [58] claimed that triterpenoid also had been shown to possess antispasmodic activity in a dose depending manner.

From previous studies, betulinic acid is considered to have a potent anti-inflammatory activity where betulinic acid can reduce the production of TNF- α as well as nitric oxide in mice. Additionally, betulinic acid also found to promote the concentration of IL-10 upon LPS stimulation [59][60], and quercetin was able to regulate down the inflammatory response of *in vitro* bone marrow-derived macrophages. They further proof that quercetin to inhibit the release of cytokine and induce the nitric oxide synthase via inhibition of NF- κ B pathway without modification of c-Jun N-terminal kinase activity.

Besides that, different kinds of methods were tried during the chemical analysis for better resolution

and separation of target peak. All of it was run at the maximum wavelength of 205 nm as the study compound from *Z. jujuba* has wavelength maximum at 205 nm. According to Guo et al. [33], suggested acetonitrile to be used as a mobile phase because acetonitrile has low wavelength maximum compared to methanol where acetonitrile avoids blank interference during the analysis. Therefore, low peaks of EEZJ can be observed due to less blank interference. Guo et al. group reported the mobile phase with a buffered acid to produce a better separation of peaks. This is particularly in case of betulinic acid analysis as buffer acid improved the peak shape and eliminated the tailing of the target peak [61].

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