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Investigation on the gastrointestinal properties of ethanolic extract of *Cannabis sativa* through *in vivo* and *in vitro* approaches

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ABSTRACT

Introduction: For thousands of years, *Cannabis sativa* has been used for its medical and recreational benefits. Nowadays, there is an increasing interest in the use of *C. sativa* and its non-psychoactive products/effects to treat certain diseases. This study was conducted to examine the gastrointestinal effects of a sequential ethanolic fraction of *C. sativa* threshing residues (EFCS).

Methods: *In vivo* and *in vitro* approaches were used to investigate the gastrointestinal properties of EFCS. Antidiarrheal, antibacterial, spasmodic, laxative, and antiulcer effects were tested respectively against castor oil induced diarrhea, disc diffusion method, isolated tissue preparations, charcoal transit time, and ethanol induced ulcer model.

Results: EFCS induced a spasmodic effect on an isolated rabbit jejunum, which was inhibited by atropine and verapamil. Moreover, under free calcium conditions, the effect of EFCS was significantly reduced. The acute toxicity assay in female and male mice showed that EFCS was a safe product. Additionally, EFCS like loperamide presented antidiarrheal effect in mice and inhibited intestinal fluid secretions. Unlike the standard drug (loperamide), EFCS increased the motility of the intestinal transit. Furthermore, EFCS showed a protective effect against gastric ulcers induced by ethanol in mice and exhibited antimicrobial properties against *Bacillus cereus* strains.

Conclusion: The results of this study reveal that EFCS possesses anti-diarrheal, anti-bacterial, spasmodic, laxative, and anti-ulcer activities. EFCS has potential therapeutic use against gastrointestinal diseases. Also, our work values the non-psychoactive products of *C. sativa*.

Implication for health policy/practice/research/medical education:
Cannabis sativa extract pharmacologically targets muscarinic receptors and induces intestinal spasmodic effect. This study confirms that cannabis may be a potential candidate to manage gastrointestinal disorders.

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Introduction

The scientific perspective on Cannabis medicinal use and its health benefits has shifted in recent years. Our scientific knowledge about the complex endocannabinoid system in our body and about the chemical constituents of *Cannabis sativa* supports the rapid increase in the use of *Cannabis* extracts and *Cannabis* ingredients for medical purposes (1). The World Health Organization recommended that *Cannabis* should be removed from the list of the most "dangerous drugs", thus unlocking the therapeutic benefits of *C. sativa*. Following this recommendation, several countries have chosen to legalize the therapeutic use of *C. sativa* (2).

Cannabis sativa is characterized by a rich chemical

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composition, including terpenes, carbohydrates, fatty acids, phytosterols, phenolic compounds, and the unique compounds of this plant, cannabinoids (3). The latter compounds are meroterpenoids, which are mostly produced in trichomes, the glandular resin in the female inflorescences (4). Typically, Δ9-tetrahydrocannabinol (THC) is a cannabinoid that is most prevalent and well characterized component in *C. sativa* plants. However, the characterization of phytochemical compounds of *C. sativa* starts showing evidence of their effectiveness according to the criteria of modern science. The benefits of phytochemical compounds are numerous, ranging from better management of cancer symptoms to the relief of chronic diseases (5,6).

At present, minor cannabinoids, such as cannabinol, and cannabidiol (CBD), have broad therapeutic potentials against rheumatoid arthritis, neurological disorders, cancer, and cardiovascular diseases (7–9). Terpenoids of *C. sativa* may directly elicit pharmacological effects or act in synergy with cannabinoid molecules (10). Recent studies have shown that Cannabis flavonoids are responsible of a wide range of biological effects such as neuroprotective and antitumorigenic properties (5,11). Plant sterols may reduce plasma cholesterol levels (12). The combination of different secondary metabolites of *C. sativa* is thought to increase its therapeutic properties known as the "synergic effect" (10). The latter is recognized by the fact that the extracts of *C. sativa* may have better pharmacological effects than individual compounds. It has been suggested that CBD-rich extracts may have a better therapeutic profile than purified CBD for treating epilepsy in humans (13). Further works previously reported that anti-inflammatory effect of Cannabis extract was more potent to that of CBD alone (14), and that its antitumor responses *in vitro* was greater to that observed with THC alone (15).

The therapeutic use of *C. sativa* products is however known to be limited by its content of THC that acts on CB1 receptors leading to the psychotropic effects. Literature reviews on the efficacy of medical *C. sativa* are cautionary about its effectiveness and side effects. Since the non-psychoactive compounds of *C. sativa* have low affinity for CB1 receptors (1), they represent an opportunity to discover new medicine.

The remaining residue part after artisanal extraction of resin (rich of THC, which is a psychoactive compound) from *C. sativa* is an unexplored by-product with no commercial value. However, it can be a source of non-psychoactive molecules that can be exploited for their pharmacological properties. Despite increasing evidence that *C. sativa* might potentially be used for gastrointestinal disorders (16), few experimental data are available demonstrating the effect of *C. sativa* on the gastrointestinal tract. As far as we know, no studies have described the bioactive potential of the threshing residue extract of *C. sativa* on gastrointestinal tract. Therefore, this study was conducted to explore the effect of the sequential ethanolic fraction of the threshing residue of *C. sativa* (EFCS) *in vitro* and *in vivo*. The functional chemical groups were characterized by Fourier transform infrared spectroscopy. EFCS's anti-diarrheic, antibacterial, spasmodic, laxative, and potential antiulcer effects were investigated.

Materials and Methods

Chemicals

All solutions were prepared daily and kept on ice until used. Acetylcholine chloride, loperamide hydrochloride, omeprazole, and atropine were dissolved in distilled water; verapamil was dissolved in DMSO (0.05%). All other drugs and solvents used in the present work were purchased from Sigma-Aldrich. All reagents used in this study were of the highest available analytic grade (purity ≥ 98%).

Plant extraction

The *C. sativa* plant was harvested in 2021, in the region of Tafrante in Morocco (37°38′38.67396″N, 5°5′20.92272″W). The plant was identified (Prof. Derraz Khalid, Department of Biology, FST, USMBA, Fez, Morocco) and registered with the specimen number CS-FSTF 18 at the Faculty of Science and Technique (Fez, Morocco). We used only the leaves and bracts of the female *C. sativa* plant without trichomes. The trichomes were eliminated with a threshing method as described previously (17). The obtained threshing residues (leaves and bracts) were grounded into a fine powder; 40 grams of this powder were sequentially extracted with 300 mL of hexane, dichloromethane, ethyl acetate, ethanol, and water using a soxhlet extractor. The sequential extraction yields were 3%, 3%, 3.22%, 7.19%, and 16.2% for hexane (HFCS), dichloromethane (DFCS), ethyl acetate (EAFCS), ethanol (EFCS), and water sequential extracts (AqFCS), respectively. These sequential fractions were dried, stored at 4°C, and protected from light. In the present study, only EFCS was chosen to evaluate its gastrointestinal properties.

Animals

Both sex mice (35–50 g, 2 months) and rabbits (2 kg-2.5 kg, 3 months) from Pasteur Institute (Casablanca, Morocco) were used in this study in strict accordance with the guidelines for the care and use of laboratory animals as described by the National Research Council's Guide for the Care and Use of Laboratory Animals. The present experimental protocol received the local Ethics Committee CEFST approval under reference number No 13 /2021/CEFST.

Tissue preparation

Rabbits were anesthetized, the abdominal cavity was then opened, fragments of jejunum were dissected and cut into

a length of about 1 cm, and then the piece of jejunum was fixed vertically in an isolated organ bath (10 mL volume) filled with Krebs-Henseleit buffer (KHS). Its lower end was attached to a tissue holder in the organ bath and the upper end was connected to an isometric force transducer (UF1 Isometric Transducer). connecting to a polygraph, it was maintained under a tension of 1 g. The KHS was maintained at 37°C and continuously oxygenated with a carbogen mixture (95% O2 + 5% CO2). The tissue preparations were left under tension for at least 30 minutes until stabilization (e.g., until jejunum contractions became rhythmic). After stabilization, segments with weak contractions were removed from the protocol. For each test, we used a new segment of the jejunum.

Involvement of muscarinic receptors and voltage-dependent calcium channels in the spasmogenic effects of EFCS

To assess the intestinal effect of EFCS on muscarinic receptors, a non-selective muscarinic antagonist (Atropine, 1μM) was incubated with the preparations, then EFCS was added cumulatively (0.001-0.3 mg/mL). To verify the involvement of voltage-gated calcium channels (VDCCs) in EFCS responses, similar experiments were performed in the presence of verapamil (0.1 and 1 μM), a voltage-gated calcium channel inhibitor.

Involvement of the release of Ca^{2+} from intracellular stores in the spasmogenic effect of EFCS

To investigate whether the spasmogenic effect of EFCS could also be due to the mobilization of Ca^{2+} channels from intracellular stores, the effect of EFCS induced contractions in a Ca^{2+}-free medium was examined as described by (18).

Rabbit jejunums were contracted for 8–9 minutes in high K+ (100 mM), a modified KHS buffer to reload internal stores with external Ca^{2+}. Then, the preparations were relaxed for 5 to 6 minutes in a Ca^{2+}-free KHS containing 2mM of EDTA, and finally transient contractions were obtained by a bolus injection of submaximal concentration of EFCS (0.1 mg/mL).

Evaluation of the acute toxicity of EFCS in mice

The acute toxicity assay was carried out in accordance with (OECD, 2002) (23). Twenty mice (27/37 g) were divided into four experimental groups (n = 5). After fasting overnight, EFCS was administered orally to each treated group (except the negative group) in single doses of 500, 1000, and 2000 mg/kg, respectively.

During the first 4 hours following treatment, any symptoms of toxicity and or changes in general behavior (hyporeactivity, tremor, abdominal contractions, bleeding, and nausea) were evaluated. The LD50 and the weight evolution were also followed during the 14 days following the treatment.

Evaluation of EFCS effect on gastrointestinal motility

After fasting for 18 hours, mice of both sexes were divided into 4 groups (n=5 per group). Group 1 served as a negative control and received distilled water orally (0.3 mL). Group 2 received loperamide (5 mg/kg), a reference drug for transit delaying (positive control). The treated group 3 received orally a 500 mg/kg of EFCS and the treated group 4 received 500 mg/kg of the EFCS and 5 mg/kg of loperamide.

Thirty minutes after treat animals received 0.5 mL of 10% charcoal suspension via oral gavage. One hour later, animals were sacrificed and the small intestine was removed. Thereafter, the total length of the small intestine and the distance traveled by the activated charcoal were measured with a graduated ruler. The effect of EFCS on gastrointestinal motility was determined by calculating the peristalsis index according to the formula below:

Peristalsis index in percentage:

$$\frac{\text{The distance covred by the activated carbon}}{\text{The total lenght of the intestine}} \times 100$$

Investigation of antidiarrheal properties of EFCS

Mice of both sexes were divided into three groups (n = 6 per group) after fasting for 18 hours. The first group (negative control) received distilled water (the vehicle in which EFCS and loperamide were dissolved). The second group received loperamide (5 mg/kg), which was used as a reference antidiarrheal drug. The treated group received a dose of 500 mg/kg EFCS orally.

After 1 hour of treatment, all animals were given 3 mL/kg of castor oil orally to induce diarrhea, and then they were placed individually in cages with covered bottom with a non-wetting transparent paper that had already been weighted before treatment.

During a 4-hour observation period, the time of the onset of diarrhea, the number of wet stools, the total number of stools, and the total weight of feces (wet and dry diarrheal drops excreted by the mice) were measured and compared to the control group.

The antidiarrheal effect was determined by calculating the % of diarrhea inhibition, the % of total defecation inhibition, and fecal output percentage (FOP) according to the formulas below:

% of diarrhea inhibition:

$$\frac{\text{number of wet stools in the control} - \text{number of wet stools in the treated group}}{\text{number of wet stools in the control}} \times 100$$

% inhibition of total defecation:

$$\frac{\text{total number of stools in the control} - \text{total number of stools in the treated group}}{\text{total number of stools in the control}} \times 100$$

Fecal output percentage (FOP)

$$\frac{\text{Mean faecal weight of each treatment group}}{\text{Mean faecal weight of control group}} \times 100$$

Antibacterial activity of EFCS

Bacillus cereus B1167 and *Salmonella enterica* B801 (from CCMM, hosted in the National Centre for Scientific and Technical Research, CNRST, Rabat, Morocco), were used as standard indicator strains for antimicrobial activity determination. All pathogenic bacteria were cultivated routinely on brain heart infusion broth (BHI, Biokar Diagnostics, France) at 37°C and stored at 4°C on BHI-agar slants. Briefly, 8 mL of molten soft brain heart infusion agar (containing 1.5% BHI and 0.8% agar) was tempered at 45°C and inoculated with pathogenic strains at approximately 108 CFU/mL as described by (19). After solidification, the towers were removed and 100 µL of EFCS was deposited. Then, the plates were incubated at 37°C for 48 hours. Finally, the zones of inhibition surrounding wells were examined.

For minimum inhibitory concentration (MIC) determination, different concentrations of EFCS (0.2, 2 and 20 mg/mL) were used against these pathogenic indicator strains.

Evaluation of gastro-protective/anti-ulcer effects of EFCS

To evaluate the gastro-protective effect of EFCS, we used the ethanol-induced ulcer model *in vivo*. Mice were fasted for 18 hours, then randomized into 4 groups (n = 5 per group), which were divided into two control groups and two test groups. Groups 1 (untreated) and 2 (ethanol ulcerated mice) served as control groups and received just distilled water (vehicle). Groups 3 and 4 were the test groups; group 3 served as the ethanol-ulcerated mice pretreated with 500 mg/kg of EFCS extract and group 4 served as the ulcerated mice pretreated with a reference anti-inflammatory drug, omeprazole (20 mg/kg). Pretreatments were given orally 30 minutes before ulcer induction.

The histological material was fixed, embedded, cut, and stained with hematoxylin, saffron and eosin. Histological changes were observed under a light microscope.

Chemical characterization of EFCS

Fourier transform infrared spectroscopy (FTIR) analysis was performed on a VERTEX 70 - BRUKER spectrometer coupled to a Hyperion microscope, operated in ATR mode (Attenuated Total Reflection) and equipped with a diamond crystal as a single reflecting element. The IR spectrum of EFCS was recorded at room temperature in the range of 4000-400 cm^{-1} with a resolution of 4 cm^{-1} and the number of scan of 124.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM) obtained from n separate experiments. Differences between two groups were analyzed using the student t-test, whereas differences in significance within and between groups were assessed by analysis of variance test for repeated measurements followed by the multiple comparison Bonferroni's test. Differences were considered statistically significant at $P<0.05$.

Results

Fourier transform infrared spectroscopy results

The FTIR spectrum of EFCS is shown in Figure 1 and the results are summarized in Table 1.

Effect of EFCS on the basal tone of rabbit jejunum

The results obtained show the spasmodic effect of the EFCS on the spontaneous contractions of the jejunum. As seen on the original recording, the injection of the extract at the concentrations studied of 0.001 to 0.3 mg/mL contracted rabbit jejunum in a concentration-dependent manner (Figure 2) with a half maximum effective concentration (EC50) value of 0.016 mg/mL (95% CI= 0.014 to 0.018, n=5).

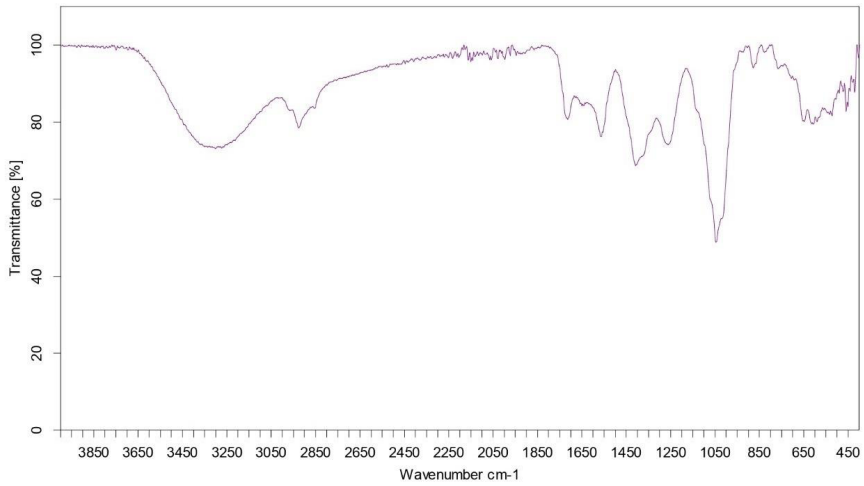

Figure 1. Fourier transform infrared spectroscopy spectrum of sequential ethanolic fraction of *Cannabis sativa* threshing residues.

Table 1. The main Fourier Transform Infrared Spectroscopy bands of sequential ethanolic fraction of *Cannabis sativa* threshing residues

Wavenumber (cm^{-1})	Band assignments
3630-3000	OH (stretching vibration) of alcohol and phenol (associated hydrogen bonds)
3565-3400	OH: intra- and intermolecular hydrogen bond of phenol
3000-2850	CH_2 (methylene) + CH_3 (methyl) groups of alkyl chain, and CH2 (methylene) + CH (methylidene) groups of terpene cyclic chain
2960	$v_{as}CH_3$: asymmetric stretching vibration of methyl group
2923	v_sCH_3 and $v_{as}CH_2$: symmetric stretching vibration of methyl group and asymmetric stretching vibration of methylene group
2853	v_sCH_2: symmetric stretching vibration of methylene group
1730	C=O (stretching vibration) of associated acidic group: CO_2H
1720	C=O (stretching vibration) of carbonyl group: normal ketone
1640	δOH of adsorbed water overlapped with vC=C (stretching vibration) of alkene group
1580	C_{ar}=C_{ar} skeletal vibration of phenol lied to aromatic ring with C-H_{ar} out of plane bending mode at 870 and 700 cm^{-1}
1425	δCH_2 + $\delta_{as}CH_3$: in pane bending mode of alkyl and terpene cyclic chains
1375	δ_sCH_3: in pane deformation mode of alkyl chain
1265	C_{ar}-O (stretching vibration) of branched aromatic ring (RO-Ar) of phenolic compounds
1050	C-O (stretching vibration) of polysaccharides
900, 820	C-H_{ar} (out of plane bending): two C-H_{ar} adjacent as well as isolated C-H_{ar} in trisubstituted aromatic ring (1, 3, 5-position in phenol compound)
870, 700	Out of plane deformation of isolated C-H_{ar} in the case of 1, 2, 3, 5-tetrasubstituted aromatic ring
725	$r(CH_2)_n$): rocking mode provides information on the length of the hydrocarbon chain which is less than or equal to 4 carbon atoms
700-450	δ_{oop}O-H: out of plane bending mode of OH phenolic.

Cholinergic-spasmogenic response of EFCS

The cholinomimetic effect of EFCS was examined on the segments of jejunum pretreated with atropine. Figure 3 shows an attenuation of the response of acetylcholine used as a positive control on atropine-pretreated preparations. On these same preparations, the spasmodic effects of EFCS were drastically inhibited by the use of atropine, confirming the implication of muscarinic receptor activation by EFCS.

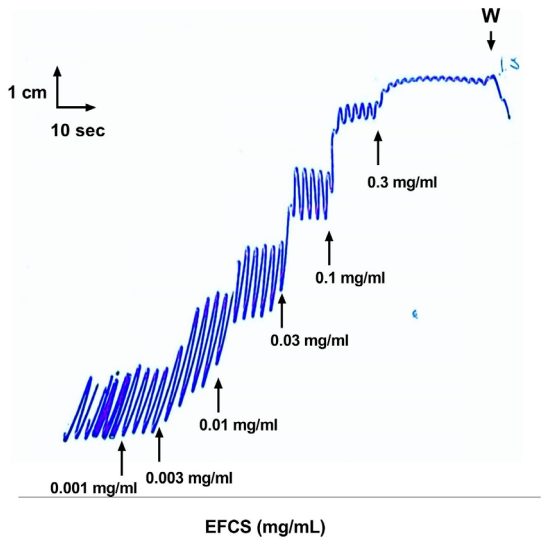

Figure 2. Original recording showing the spasmodic effect of sequential ethanolic fraction of *Cannabis sativa* threshing residues (EFCS) on spontaneous jejunum contractions. The cumulative administration of EFCS at 0.001, 0.03, 0.01, 0.03, and 0.3 mg/mL contracted rabbit jejunum in a concentration-dependent manner. W: wash).

Involvement of extracellular calcium in EFCS induced contraction

In order to evaluate whether the effect of EFCS involves a stimulation of voltage-gated Ca^{2+} channels (VGCC), the experiments were carried out in the absence and presence of verapamil, a VGCC blocker. As shown in Figure 4, verapamil (0.1 to 1 µM) inhibited EFCS responses (0.001-0.3 mg/mL), and shifted its contraction responses curves to the right. The Emax was reduced from 100% (control) to 38.6 ± 3.34 %; n = 4 ($P < 0.0001$) and to 10.44 ± 2.57 %; n=4 ($P < 0.0001$) in the presence of 0.1 µM and 1 µM of verapamil, respectively.

Involvement of intracellular calcium in EFCS induced contraction

Under Ca^{2+}-free conditions, contraction induced by the extract should result only from Ca^{2+} released from the sarcoplasmic reticulum via IP3 activation. Our results showed that the phasic contraction induced by EFCS under Ca^{2+}-free medium was about 46 % of that obtained

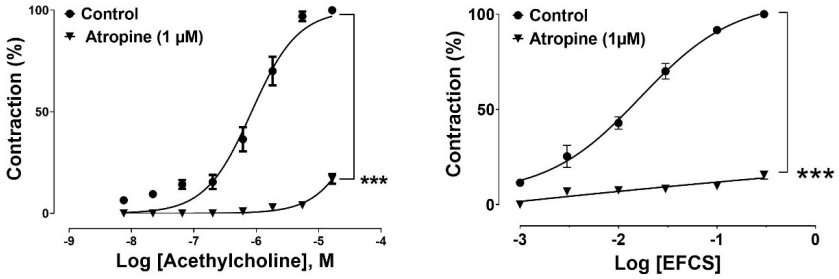

Figure 3. Cumulative concentration–response curves to acetylcholine (left panel) or sequential ethanolic fraction of *Cannabis sativa* threshing residues (EFCS) (right panel) in the absence (control) and in the presence of 1μM of atropine on the rabbit jejunum. ***$P < 0.001$ significantly different from the respective control.

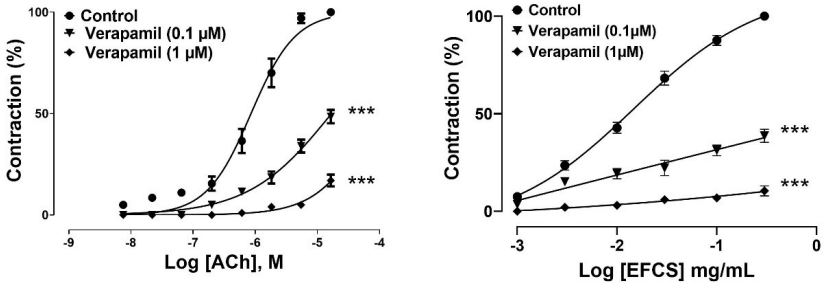

Figure 4. Cumulative concentration response to sequential ethanolic fraction of *Cannabis sativa* threshing residues (EFCS) or acetylcholine (ACh) on rabbit jejunum in the absence and in the presence of 0.1 μM and 1 μM of verapamil, respectively. The values are mean ± SEM (n = 3); *** $P < 0.001$ significantly different from the respective control.

under normal conditions, demonstrating the implication of Ca^{2+} release from intracellular stores in the related spasmogenic effect.

Evaluation of the acute toxicity of EFCS in mice (*in vivo*)

The acute toxicity study of EFCS on mice showed that the three doses tested, namely 500, 1000, and 2000 mg/kg were safe. We did not observe any animal losses; all treated animals survived at the tested doses with no mortality and no signs of toxicity during the examined period. Furthermore, neither the treated nor the control groups showed significant changes in body weight during the 14 days of observation following the treatment.

Effect of EFCS on gastrointestinal motility

In order to evaluate the pharmacological effect of EFCS on gastrointestinal motility *in vivo*, intestinal transit was determined for each group of mice by measuring the distance covered by activated charcoal. The groups were as follows: negative control (mice treated with distilled water the vehicle), loperamide group (mice treated with loperamide at 5 mg/kg), EFCS group (mice treated with EFCS at 500 mg/kg), and loperamide + EFCS group (mice treated with loperamide (5 mg/kg) and EFCS at 500 mg/kg). The results obtained are presented in Figure 5.

In mice given distilled water (control group), the distance was about 46.55 ± 1.83 % of the total length of the small intestine (Figure 5). However, loperamide at 5 $mg.kg^{-1}$, the reference drug for transit delaying, caused a significant inhibition of charcoal transit (31.35 ± 2.62 %), in contrast to the EFCS -treated group, which produced a significant improvement of the intestinal transit (72.00 ±1.38 %).

EFCS (500 mg/kg) induced stimulation of intestinal transit was significantly ($P<0.01$) antagonized by the co-administration of loperamide (5 $mg.kg^{-1}$) (Positive control).

Effect of EFCS on castor oil induced diarrhea

Three mice groups were tested: control group, which received distilled water (the vehicle); EFCS group, which received EFCS at a concentration of 500 mg/kg; and loperamide group, which received loperamide at a concentration of 5 mg/kg. Each group was then treated with castor oil at 3 mL/kg of body weight.

Control mice treated with castor oil expressed diarrhea for the following four hours. Loperamide and EFCS extract markedly reduced the induced diarrhea. Indeed, the oral pre-treatment of mice with the examined dose of EFCS at 500 mg/kg reduced the number of wet stools and the total number of defecations when compared with the control as depicted in Table 2. Furthermore, the extract reduced the

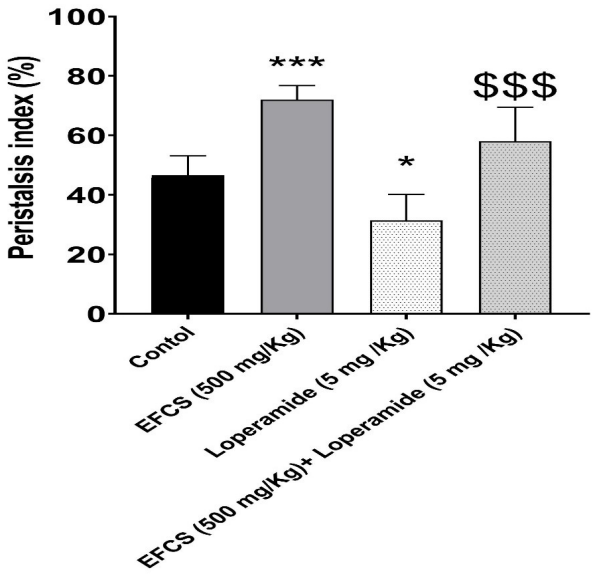

Figure 5. Effects of sequential ethanolic fraction of Cannabis sativa threshing residues (EFCS) on gastrointestinal transit in mice with and without loperamide's induced constipation. Peristalsis index (%) = (distance travelled by the charcoal) / (total length of small intestine) ×100%. ***$P < 0.001$ (EFCS vs control); *$P < 0.05$ (loperamide vs control); $^{\$\$\$}P < 0.001$ (EFCS vs loperamide + EFCS).

percentage of fecal output (Table 2). In comparison with the total percentage inhibition of defecation, the extract was twice as effective against wet stools.

Antibacterial activity

EFCS was subjected to antimicrobial evaluation against *B. cereus* (as gram-positive bacteria) and *S. enterica* (as gram-negative bacteria). Indeed, EFCS was active against *B. cereus* (MIC of 2 mg/mL). However, *S. enterica* was resistant to EFCS (20 mg/mL).

Effect of EFCS of the gastric mucosa in ethanol-treated mice

The histological study revealed that the ulcerogenic group (Figures 6 B1 and B2) showed severe damage to the gastric mucosa with the presence of vascular congestion, infiltration of inflammatory cells, and destruction of the mucosa in comparison with the normal stomach (Figure 6A).

The group pretreated with omeprazole, a known gastroprotective drug, showed a protection and a reduction of visible gastric lesions demonstrating a more or less normal aspect of the gastric mucosa, but with the presence of inflammatory cell infiltration (Figure 6C). In addition, the administration of EFCS at 500 mg/kg induced a reduction in the injured areas (Figure 6D) and thus showed a cytoprotection comparable to that conferred by omeprazole.

Discussion

It has long been known that Cannabis preparations are used in traditional medicine to manage a variety of health problems, including dysentery and cholera, as well as other gastrointestinal conditions (20). The aims of the present study were to provide scientific evidence of the use of *C. sativa* in the treatment of gastrointestinal disorders. Our study also focused on valuing the residue part of *C. sativa* from artisanal extraction for medical purposes.

In the present study, the gastrointestinal effects of EFCS were investigated *in vitro* and *in vivo*. In *in vitro* study, the effect of EFCS was explored on the spontaneous contraction of an isolated rabbit jejunum. EFCS promoted contraction of the intestines in a concentration-dependent manner, showing a significant increase in the smooth muscle tone, demonstrating a spasmogenic effect, similar to that resulted from acetylcholine. In view of these findings, the mechanism by which EFCS exerted its cholinomimetic effects in rabbit jejunum was investigated.

One of the possible mechanisms that could imitate the action of acetylcholine is the activation of muscarinic receptors, in particular the M1 and M3 subtypes. These receptors are metabotropic and belong to Gq protein-DAG/IP3. The cholinergic effect of acetylcholine on these receptors leads to an increase in cytosolic Ca^{2+} concentration, which may promote smooth muscle contraction (21).

To assess whether the spasmogenic effect of EFCS was mediated by an acetylcholine-like mechanism via the activation of muscarinic receptors, preparations were pre-treated with atropine (1µM), an anti-muscarinic agent. Atropine abolished both acetylcholine and EFCS induced

Table 2. Effect of sequential ethanolic fraction of *Cannabis sativa* threshing residues (EFCS) and loperamide on castor oil induced diarrheal in mice

Treatment	Total number of feces	Number of wet feces	Total weight of feces (g)	% inhibition of defecation	% inhibition of diarrhea	% FOP
Control	7.1 ± 2.93	4.67 ± 1.91	1.40 ± 0.57	-	-	-
Loperamide (5 mg/kg)	3.50 ± 1.43 **	1.33 ± 0.54 **	0.96 ± 0.39 ns	51.16	71.42	68.61
EFCS (500 mg/kg)	3.67 ± 1.50 **	0.67 ± 0.27 ***	1.11 ± 0.45 ns	48.43	85.71	79.47

The number of wet feces, total fecal number, and total fecal weight contents were measured at the same time during the experiment to calculate the percentage inhibition of defecation, the percentage inhibition of diarrhea, and the percentage of fecal output (FOP) respectively. Six mice per group were assayed for fecal parameters analysis. Data represent the means ± SEM. Ns $P \geq 0.05$; **$P < 0.01$ and ***$P < 0.001$ pretreated groups with EFCS or loperamide compared to castor oil group (control group).

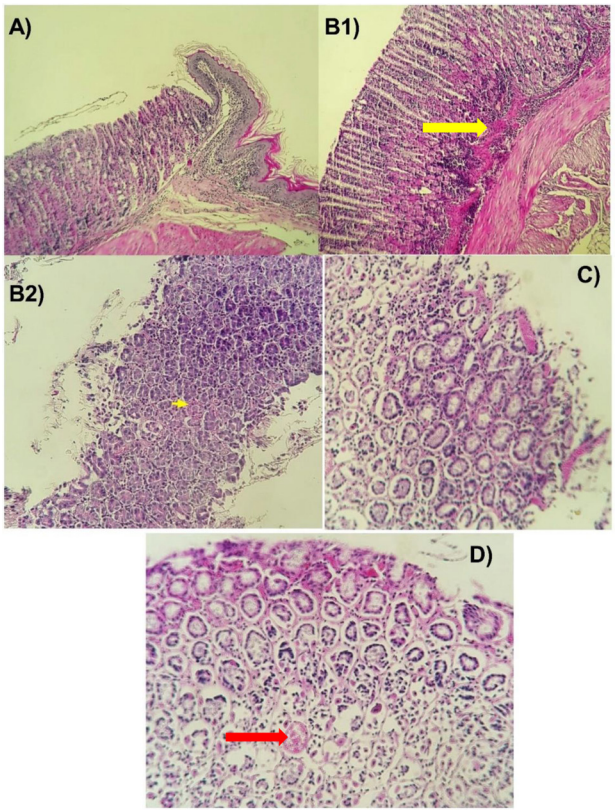

Figure 6. Effect of sequential ethanolic fraction of *Cannabis sativa* threshing residues on the gastric epithelium after ethanol-induced ulcer in mice. Normal control (A). There was severe disruption to the surface epithelium and infiltration of inflammatory cells into the submucosal surface, as well as a focus of vascular congestion (yellow arrow) in the EtOH group (B1 and B2) (H&E stain 10×). Discreet vascular congestion in EtOH + omeprazole (20 mg/kg) is seen (C). Subtle vascular congestion in EtOH + EFCS 500 mg/kg (D) (red arrow) (H&E stain 10×).

contraction. Accordingly, these findings support the hypothesis that EFCS induces contraction by activating muscarinic receptors.

Ca^{2+} cytosolic is the primary cause of contraction in smooth muscle cells. Smooth muscle may contract phasically with rapid contraction and relaxation, or tonically with steady, continuous contractions; the tonic contraction is prolonged for a while and is especially dependent on extracellular Ca^{2+} (21). The influx of extracellular Ca^{2+} through voltage-gated calcium channels (VGCC) plays a vital role in regulating the peristaltic movements of the intestine, as well as its tonic contraction response (21). Thus, we theorized that EFCS through muscarinic receptors might be acting on VGCC, which allows extracellular calcium to enter the muscle cell and trigger the contraction. The pretreatment of the jejunum preparation with verapamil, a VGCC blocker, shifted to the right the concentration response curve of EFCS and acetylcholine. These findings support the hypothesis that EFCS like acetylcholine activates Ca^{2+} influx through the VGCC to produce their spasmogenic effects.

Increased cytosolic calcium is regulated by multiple mechanisms, including the PLC-phosphatidylinositol 3-kinase pathway (PI3), one of the most ubiquitous pathways. This pathway can be mediated by muscarinic receptors activation, which allows Ca^{2+} to be released from intracellular stores, namely the endoplasmic reticulum (21).

Since the effect of EFCS seems to be muscarinic receptor dependent, the possible interaction of EFCS with Ca^{2+} release from the internal stores was also examined in the present study. To verify this hypothesis, experiments were performed in a Ca^{2+}-free medium. Under these conditions, contractions induced by EFCS should be resulted only from Ca^{2+} released from sarcoplasmic reticulum via PI3 activation. Our findings showed that EFCS in a Ca^{2+}-free medium still induced a phasic contraction that was approximately 46% of the EFCS-induced contraction obtained under normal conditions, indicating that Ca^{2+} release from intracellular stores was involved in the genesis of EFCS contraction.

An evaluation on the acute toxicity of EFCS on mice was conducted. Both genders of mice received an acute orally treatment of single dose of EFCS at 500, 1000, and 2000 mg/kg, and the behavior of the animals was observed for 4 hours and at least once a day for 14 days. We did not observe any significant changes in the behavior of animals, which suggests that the extract does not have immediate toxic side effects. During the 14-day observation, there were no animal deaths and no interferences with weight evolution. As determined by 423/2001 OEDC international guidelines, this result indicates low acute toxicity with an estimated LD50 exceeding 2000 mg/kg.

Given the safety of EFCS, *in vivo* experiments were carried out to investigate its gastro-functionality. To confirm the spasmodic response to EFCS seen *in vitro*, a series of experiments were carried out *in vivo* using the charcoal method as described above, so we decided to investigate if a single dose of EFCS (the minimal dose tested in the acute toxicity, 500 mg/kg) might alter the intestinal motility in mice *in vivo.*

As expected, when mice were treated with 500 mg/kg of EFCS, intestinal distance traveled by the charcoal marker was significantly increased when compared to controls, thus confirming that oral administration of the extract promotes a spasmogenic effect. In addition, the constipation produced by loperamide was significantly antagonized by the co-administration of EFCS. Based on these results and corroborating data found *in vitro*, EFCS may promote laxative effects. Our findings support previous studies that found acetylcholine receptors to be involved in the laxative effect of extracts and natural compounds (22). The EFCS was found to be the only fraction from *C. sativa* that induced intestinal stimulant activity, whereas non-polar fractions of *C. sativa* threshing residues (hexane (HFCS), dichloromethane (DFCS), ethyl

acetate (EAFCS)) demonstrated no excitatory activity (data not shown), indicating that spasmogenic components possess polar properties. It is also reported that flavonoids (aglycones/glycosides), mangiferin (xanthone glycoside), and cardiac glycosides could interfere with cholinergic receptors (23-27) and are more likely to be soluble in polar solvent.

It has been reported by previous studies that *C. sativa* extract could be useful against diarrhea. Moreover, the implication of endocannabinoid system against diarrhea caused by toxin of cholera was demonstrated before by (28). Thus, we decided in the present study to examine the effect of a single dose of EFCS on diarrhea caused by castor oil on mice. Our hypothesis was that EFCS might have an anti-secretory mechanism to stop the manifestations related to diarrhea rather than an inhibition of intestinal motility which could not be the case of EFCS as demonstrated above.

Castor oil has been reported to induce diarrhea by increasing the hydric volume of intestinal content and by preventing the re-absorption of sodium, chloride, and water, namely due to the release of ricinoleic acid (29,30). The results show that EFCS can act as an anti-diarrheal agent, as it was shown that EFCS (500 mg/kg) inhibited castor oil-induced diarrhea at least by inhibiting liquid stools. These results suggest that the anti-diarrheal action of EFCS is probably due to its ability to inhibit intestinal secretions, to improve the hydro-electrolytic flow from the intestinal lumen to the plasma pole, causing a reduction in the accumulation of intraluminal fluid induced by castor oil/ ricinoleic acid.

Various bacteria are responsible for enteric infections, which may lead to gastric ulcer (31). In the present study, EFCS was subjected to antimicrobial evaluation against *Bacillus cereus* (as gram-positive bacteria) and *S. enterica* (as gram-negative bacteria). EFCS was active against *Bacillus cereus* (MIC of 2 mg/mL). This toxin-producing bacterium, responsible for intestinal illnesses, has a thick peptidoglycan layer and lacks an outer membrane. However, *S. enterica*, involved in salmonellosis outbreaks and considered the most common foodborne pathogen, was resistant to EFCS, probably due to the presence of the outer membrane specific of gram-negative bacteria (32).

It was examined whether EFCS could heal ulcer *in vivo* using an ethanol-induced gastric model, which stimulates ulcer formation through various mechanisms, including mucus exhaustion, mucosal damage, hemorrhaging, and inflammation. Our findings showed that EFCS significantly reduced hemorrhagic lesions and inflammation when compared to the control animals given ethanol alone as an ulcer agent. Based on these findings, the EFCS extract demonstrated a significant cytoprotective activity.

FTIR analysis of EFCS revealed the presence of some organic chemical constituents, including several types of polar groups, such as alcohol, phenol, and acidic functions, as well as an aromatic fraction (phenolic). The presence of both alcohol and phenol groups was proved by the presence of strong and wide signal (*hydrogen-bonded*) in the range of 3630-3000 cm^{-1} connected to *stretching* vibrations of νO-H (33,34). The intense peak centred at 1050 cm^{-1} is assigned to νC-O (*stretching mode*) of carbohydrates molecules reflecting the higher polar character of the extract (35), while the broadening signal at 1265 cm^{-1} is assigned to νCar-O (*stretching* mode) of substituted aromatic ring by polar groups, such as hydroxyl or methoxyl (33,35).

The band at 1730 cm^{-1} is correlated to the carbonyl acidic group (-CO2H) justified by the presence of an inclination in the spectrum profile between 2450 and 3200 cm^{-1} (O-H acidic) (36,37) contributing to the enhancement of the polar character of the extract.

Phenolic compounds are confirmed by the presence of the following characteristic signals at 1580 and 1500 cm^{-1} related to *stretching* vibrations of aromatic ring (νCar=Car) (34,38), as well as the range between 3565-3400 cm−1, which is connected to the *intramolecular hydrogen* bond of O-H phenolic (35). The polar character of the phenolic ring is clearly manifested by the broadening in the Car=Car signal (33). The branched aromatic nuclei by alkoxy (R-O-) and/or hydroxyl (O-H) groups (substituted phenol compound) is revealed by the presence of the following bands at 1265 cm^{-1} corresponding to *stretching* mode of νCar-O (35). The degree of substitution of phenolic compounds is justified by the presence of aromatic fingerprint bands (*out of plane bending* mode) at 870 cm^{-1} (strong) and 700 cm^{-1} (weak) associated to isolated Car-H *i.e.* 1,2,3,5-tetrasubstituted phenol (38). Other aromatic fingerprint bands at 900 cm^{-1} (medium) and 820 cm^{-1} (less strong) indicate the presence of both two adjacent Car-H and isolated Car-H *i.e.* 1,3,5-trisubstituted phenol (35,37).

Regarding the carbonyl group, its presence is indicated by its frequency at 1720 cm^{-1}, which corresponds to νC=O (stretching vibration) of normal ketone (33,39). The broad band at 1640 cm^{-1} could be correlated to the plane bending mode (δOH) of adsorbed water overlapping with νC=C (stretching vibration) of the alkene structure.

Stretching vibrations of alkyl (CH2 + CH3) and cyclic chains (CH2) are detected between 3000 and 2850 cm^{-1} (asCH3 = 2960 cm^{-1}, sCH3 + asCH2 = 2923 cm^{-1}, sCH2 = 2853 cm^{-1}), and confirmed by the in-plane deformation mode at 1425 cm^{-1} (asCH2) and 1375 cm^{-1} (sCH3) (34,36,38). The weak rocking peak (r(CH2)n) at 725 cm^{-1} provides information on the length of the alkyl chain linked to the aromatic ring, which is 'n' is less than or equal to 4 carbon atoms (37).

The gastro properties of EFCS could be related to several compounds. Indeed, various secondary metabolites have been identified in the ethanolic fractions of *C. sativa*, the most relevant being the cannabinoids, flavonoids, phytosterols, and terpenes (31,40,41). Previous studies

have shown that flavonoids contribute to gastrointestinal protection against ulcers caused by Helicobacter pylori (42), and that cannabinoids protect the gastric mucosa against ethanol and stress-induced damage (43).

The contribution of other chemical entity has also been reported in ethanolic fraction of *C. sativa*, in particular phytosterols and terpens. In fact, according to previous phytochemical data, the amount of phytosterols, with representative of β-sitosterol in *C. sativa* ethanolic fractions (40), has been reported to improve experimental colitis in mice with a target against pathogenic bacteria (44). This effect occurred via the inhibition of the expression of antimicrobial peptide and the regulation of the cytokine profiles in mice. The gastrointestinal protective effects of β-sitosterol could also be attributed to the induction of antioxidant enzymes and the improvement of immune function in intestinal tissues (44). In addition, numerous *in vitro*, animal, and clinical trials have shown the medicinal properties of Cannabis terpenes/terpenoids, including anti-inflammatory, antioxidant, and gastroprotective properties (45). Therefore, these compounds reported present in the ethanolic fraction of *C. sativa* might be the principal candidate behind the EFCS observed effect, which need further investigation.

Conclusion

This study showed that EFCS had a spasmogenic effect, possibly at least through the activation of muscarinic receptors and modulation of both extracellular and intracellular calcium. Furthermore, EFCS had an antidiarrheal, antiulcer, and laxative properties. Because of its potential benefits on the gastrointestinal tract, EFCS could be a valuable source of beneficial bioactive compounds in the pharmaceutical field.

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Authors' contribution

CA and BR conceived the original idea and were in charge of overall direction and planning. CA, ZY, AS, and MY carried out the experiments. EAK contributed with statistical analysis. BA contributed to the chemical characterization. EFH contributed to the histological analysis. CA and BR wrote the manuscript with the support from EFH, BA, AS and EAK.

Conflict of interest

The authors declare no conflict of interest.

Ethical considerations

The present experimental protocol has received the local Ethics Committee CEFST approval under reference number 13 /2021/CEFST.

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