J Herbmed Pharmacol. 2025; 14(1): 29-42.

http://www.herbmedpharmacol.com doi: 10.34172/jhp.2025.51481

Journal of Herbmed Pharmacology

Chemical profile and bioactive properties of *Cannabis sativa* threshing residue: Vasorelaxant, antioxidant, immunomodulatory, and antibacterial activities

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ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 27 February 2024
Accepted: 21 July 2024
Epublished: 25 December 2024

Keywords:
Cannabis sativa threshing residue, Inflammation, Antioxidant activity, Vasorelaxant activity, Antibacterial activity, Immunosuppressive activity

ABSTRACT

Introduction: *Cannabis sativa* threshing residue is the plant material left after extracting resinous trichomes from the dried female plant. This study examines the phytochemical properties of *C. sativa* threshing residue and screens its vasorelaxant, antimicrobial, and immunomodulatory activities.

Methods: The residue was extracted sequentially using hexane, dichloromethane, ethyl acetate, and ethanol. The four fractions were analyzed by gas chromatography-mass spectrometry. Phenolic and flavonoid contents were estimated using Folin-Ciocalteu reagent and aluminum chloride colorimetric methods. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay determined in vitro antioxidant activity. The vasorelaxant effect was explored using rat mesenteric arterial beds (MABs). Antimicrobial studies on *Salmonella enterica*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* were conducted using microdilution tests. Immunomodulatory activity was determined using cell proliferation, antibody production, and complement tests.

Results: Phytochemical analysis of the four fractions revealed many volatile bioactive compounds, including terpenes like phytol and squalene and cannabinoids like delta-9-tetrahydrocannabinol and cannabidiol (CBD). The fractions had significant polyphenol and flavonoid content. They dose-dependently dilated the MAB and demonstrated varying DPPH free radical scavenging activity. Immunomodulatory activity showed an immunosuppressive effect on cell proliferation and biological functions. The four bacterial strains studied were sensitive to the fractions but with different degrees.

Conclusion: *Cannabis sativa* threshing residue demonstrated diverse pharmacological potential, suggesting its suitability for developing cannabis-based medications targeting hypertension-related damage, free radicals, and bacteria, expanding its potential applications beyond inflammation-related conditions

Implication for health policy/practice/research/medical education:
This work highlights the promising potential of threshing residue from the cannabis cultivar 'Khardala' as a source of bioactive ingredients with notable antioxidant, antibacterial, vasorelaxant, and immunomodulatory activities. This suggests that cannabis by-products might offer a promising approach for developing novel therapeutics.

Please cite this paper as: Mahou Y, Marrakchi Ben Jaafar D, Al Moudani N, Jeddi M, Chda A, Fettoukh N, et al. Chemical profile and bioactive properties of *Cannabis sativa* threshing residue: Vasorelaxant, antioxidant, immunomodulatory, and antibacterial activities. J Herbmed Pharmacol. 2025;14(1):29-42. doi: 10.34172/jhp.2025.51481.

Introduction

Cannabis sativa L., commonly known as "hemp" or "weed," has a rich history spanning over 5000 years as a vital source of food and fiber (1). Despite its psychoactive properties, cannabis stands out as a versatile plant with a diverse array of therapeutic applications. These applications

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encompass glaucoma, pain management, depression, cancer, liver disease, cardiovascular health, inflammation reduction, and addressing metabolic syndrome (1). The impressive versatility of cannabis stems from its myriad phytoconstituents. To date, researchers have isolated and identified a staggering 566 secondary metabolites. These include cannabinoids, terpenes, flavonoids, and various other compounds (2).

Cannabinoids are the most prominent secondary metabolites of *C. sativa*. Over seventy different cannabinoids have been identified, with tetrahydrocannabinol (THC) being the most abundant and known for its psychotropic effects. Cannabidiol (CBD) and cannabigerol (CBG) are also abundant but are non-psychotropic (3).

Terpenes are also found in cannabis; more than 200 terpenoids have been identified (4). These aromatic substances are known for their therapeutic potential (4). Moreover, flavonoids, a large class of secondary metabolites with possible health advantages, are commonly found in cannabis. They are a broad category of secondary metabolites known for their potential health benefits (2). More than 26 flavonoids have been found in cannabis, including conjugated O-glycosides, methylated and prenylated aglycones, and C-glycosides of apigenin, vitexin, isovitexin, quercetin, luteolin, and kaempferol (5).

Cannabinoids and terpenes have predominantly resided in the resinous glandular trichomes of the plant (6). While flavonoids are present in flowers, leaves, and pollen (7,8), they remain undetected in trichomes (9) and seeds (10).

Since 2021, Morocco has embarked on a pioneering journey into the realm of medical cannabis, recently legalizing its cultivation for therapeutic purposes. This bold move positions Morocco as a potential leader in the emerging global medical cannabis market. The cultivation and regulation of medical cannabis in Morocco are subjects of keen interest, with the government working to establish a robust regulatory framework to ensure safe and standardized productions.

In Morocco, the production process of cannabis resin involves beating the dried leaves and mature inflorescences of female plants against a mesh screen, forcing the trichomes to separate and fall off. Following this threshing-sieving process, the residual plant material is discarded as a low-value by-product. Depending on the chemovar of cannabis, it takes between 35 and 70 kg of plant material to produce 1 kg of cannabis resin (11).

While significant attention has been dedicated to the major phytocannabinoids in *C. sativa*, recent research has underscored the presence of a plethora of non-cannabinoid bioactive constituents, including flavonoids, spiroindans, dihyrostilbenes, dihydrophenanthrenes, lignan-amides, steroids, and alkaloids (2).

To optimize the therapeutic potential of Moroccan *C. sativa*, our study aimed to provide the chemical composition of the residue of female plant and to explore its potential vasorelaxant, antioxidant, immunomodulatory and antibacterial effects. The rationale for investigating these pharmacological activities stems from their potential relevance in the context of inflammation-related disorders. Vasodilation, for instance, plays a crucial role in inflammation by promoting increased blood flow to inflamed tissues, facilitating the delivery of immune cells and nutrients necessary for tissue repair. Similarly, antioxidant activity is vital for mitigating oxidative stress, a key driver of inflammation and tissue damage. Furthermore, the inhibition of immune cells may help regulate excessive immune responses that contribute to chronic inflammation. Additionally, antibiotic resistance in microorganisms may exacerbate certain diseases, prolong hospitalization, and increase treatment costs. It is estimated to cause 700 000 deaths annually and is projected to become one of the leading causes of death worldwide by 2050 (12). Therefore, our study aims to assess the potential of *C. sativa* threshing residue against clinically relevant bacteria. By focusing on bacteria such as *Salmonella enterica* (causing foodborne illnesses), *Escherichia coli* (associated with gastrointestinal infections), *Staphylococcus aureus* (known for causing skin infections, food poisoning, and sometimes serious conditions like pneumonia and bloodstream infections), and *Bacillus cereus* (responsible for food poisoning), we aimed to better understand the therapeutic potential of this plant in combating infectious diseases and inflammation-related disorders. These strains were selected due to their significant implications for human health disorders and the prevalence of related infections.

Materials and Methods

Reagents and chemicals

The Folin-Ciocalteu phenol reagent and DPPH were purchased from Sigma-Aldrich Co. (Steinheim, Germany). Gallic acid, quercetin, sodium carbonate (Na_2CO_3), $AlCl_3$, hexane, dichloromethane, ethyl acetate, and ethanol used in the present study were bought from Merck Company (Darmstadt, Germany).

Plant material

The threshing residue of *Cannabis's* variety known as Khardala was received from farmers in the Tafrante region in Morocco between May and June 2021. An earlier threshing-sieving procedure was performed to remove the resin rich in THC in the glandular trichomes. Seeds were removed from the threshing residue, and only the crushed leaves and inflorescences were used. The plant was identified by Prof. Derraz Khalid (Department of Biology, FST, USMBA, Fez, Morocco) and registered with the specimen number CSFSTF 18 at the FST (Fez, Morocco).

Extraction

Fifty grams of the threshing residue of *C. sativa* underwent sequential extraction using a soxhlet extractor (ISOLAB GERMANY 250 mL 45/40+29/32) for 24 hours each. The

extraction process utilized 300 mL of hexane, followed by dichloromethane, ethyl acetate, and ethanol fractionation. The obtained sequential fractions were dried and stored at 4 °C until further analyses were conducted.

The yields of the sequential fractions: hexane (HFCS), dichloromethane (DFCS), ethyl acetate (EAFCS), and ethanol (EFCS) fractions from the *C. sativa* threshing residue were determined to be 6.2%, 3.4%, 4.2%, and 3%, respectively.

Phytochemical composition

Using gas chromatography/mass spectrometry (GC-MS) on an Agilent 8890GC equipment connected to an Agilent MSD 5977B mass spectrometry detector, the profiles of volatile phytochemicals were determined (13). A volume of 1 µL of sample fraction was injected using a 7693A auto-sampler. A split mode injection was used with a split ratio of 50:1. Helium was used as carrier gas with a flow rate of 1 mL/min, and the injector temperature was set at 250 °C. Compounds were separated on an HP-5MS (phase: 5% Diphenyl- 95% dimethylpolysiloxane) capillary column (30 m × 0.25 mm × 25 µm). The temperature program for the column oven began at 60 °C and ramped up to 295 °C over 15 minutes at a rate of 15 °C/min.

Total phenolic and flavonoid contents

The Folin-Ciocalteu spectrophotometric technique was used to quantify the total phenol content (TPC) (14). The absorbance was measured at 750 nm using a spectrophotometer; gallic acid was employed as a positive control. In terms of milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g dw), the total amount of phenols was calculated. After three replications of each experiment, the results were shown as means ± standard error of the mean (SEM).

The Harborne aluminum chloride spectrophotometric method was used to measure the total flavonoid content (TFC) (15). The sample's absorption was followed at 510 nm. The total amount of flavonoids was determined using a standard quercetin solution's calibration curve. Flavonoids in each gram of dry weight were measured as milligrams of quercetin equivalent (mg QE/g dw). After three replications of each experiment, the results were presented as means ± SEM.

Antioxidant activity

To assay the antioxidant capacity of the four fractions, the free radical-scavenging effect on DPPH radical was evaluated based on the procedure described by Brand-Williams et al (16). In a nutshell, 25 µL of the fraction and 1 mL of an ethanol solution of DPPH were added. After an hour of incubation at room temperature, the absorbance was measured at 515 nm. Three repetitions of each measurement were taken, and the means ± SEM was used to express the findings.

The free radical inhibitory percentage was calculated, indicating the potential to neutralize free radicals and mitigate oxidative stress using the formula below:

$$\%\,inhibition = \left[\left(\frac{Abs\,control - Abs\,sample}{Abs\,control}\right)\times 100\right]$$

Animals

Both sexes of Wistar rats (250–350 g; Charles River) and rabbits (1.8-2.5 kg) were employed for vasomotricity and immunomodulatory experiments. The animals were housed in conditions maintaining a constant room temperature of 20 ± 5 °C. They were provided free access to food and water and handled in compliance with the guidelines outlined in the US National Institutes of Health's standard for the care and use of laboratory animals (National Research Council, 2011. Guide for the Care and Use of Laboratory Animals. Washington, D.C.: The National Academies Press; 2011). The experimental procedures were approved by the local Ethics Committee under reference number 11/2021/CEFST on March 9, 2021.

Vasorelaxant activity

To investigate the vascular effect, the prepared four fractions were injected in doses ranging from 0.01 mg to 0.5 mg into the mesenteric arterial bed (MAB) precontracted with phenylephrine (10 µM). The percentage decrease in perfusion pressure (PP) following exposure to bolus injections was measured, providing insights into potential cardiovascular benefits, including improved blood flow and reduced blood pressure. MAB was isolated as described by Bencheikh et al (17). Urethane (1.5 g/kg) was administered intraperitoneally to anesthetize the rats. Following an abdominal midline incision to open their abdominal cavities, the superior mesenteric artery was quickly localized and cannulated at its origin from the abdominal aorta. A peristaltic pump was used to perfuse the preparation with a warmed Krebs-Henseleit solution (37 °C) at a consistent flow rate of 2 mL/min. By carefully cutting close to the intestinal wall, the entire cannulated MAB was freed from the intestine. The Krebs-Henseleit solution, which contained 118 mmol/L sodium chloride, 15 mmol/L sodium bicarbonate, 4.7 mmol/ L potassium chloride, 1.2 mmol/ L magnesium chloride, 2.5 mmol/ L calcium chloride, 1.2 mmol/ L potassium phosphate, and 11.0 mmol/L glucose, was aerated with carbogen (5% CO_2 in O_2; final pH= 7). After stabilizing for 30 minutes, an oscillograph (50-8622, Harvard Apparatus Limited) and a pressure transducer (Capto SP844) were used to continuously measure and record the MAB's PP. Phenylephrine (10 µM) was continuously injected into the MAB using a syringe pump (M365, Orion) to induce pre-contraction until a PP between 80 and 100 mm Hg was achieved. Once the contraction reached a plateau, 5.5 nmol of acetylcholine was injected via a sidearm into the MAB to assess endothelial integrity. Subsequently,

MABs were exposed to bolus injections to generate dose-response curves. Vasorelaxation was reported as a percentage decline in PP, with 100% relaxation indicating a return to baseline values.

Immunomodulatory activity

Cell proliferation, complement system activity, and immunoglobulin production were evaluated providing insights into the ability of *C. sativa* threshing residue to modulate immune responses and their potential utility in immune-related disorders.

Cell culture

Cell suspensions used in this study were prepared from rabbits. Before any procedure, chloroform was administered to the animal to ensure its comfort and reduce potential distress. Once the animal was anesthetized, euthanasia by intravenous injection, in compliance with ethical and regulatory standards, was used to ensure a rapid and painless death.

According to previous research (18), the animals' spleen and thymus were aseptically removed, and the organs were pushed through a fine wire mesh to create cell suspensions. These cells were cleaned with RPMI, and 154 mM ammonium chloride was used to lyse the red blood cells. The trypan blue 0.1% exclusion test was used to assess the number of viable cells under the microscope. Two mM glutamine, 10% serum, antibiotics (ampicillin 100 U/mL and streptomycin 100 mg/mL), antifungal (fluconazole 2 mg/mL), and 10% serum were added to the RPMI culture medium.

Cell proliferation assay

The MTT assay was used to measure cell growth, according to Mosmann (19). Cell suspension equivalent to 2.105 cells/well was incubated with cannabis threshing fractions prepared at varying concentrations, dissolved in DMSO with a concentration not exceeding 1%, in 96-well plates. The incubation period was 72 hours at 37 °C in a humidified chamber with a 95% air and 5% CO2 atmosphere. Ten microliters of MTT solution (5 mg/mL in PBS) was added. Following a three-hour incubation period, each well received 100 µL of DMSO to dissolve the formazan that the cells had produced. At 570 nm, the optical density was then measured using a spectrophotometer.

Complement test

The study evaluated the effect of *C. sativa* threshing residue on the complement system using a complement test, as previously described (18). The test was conducted to assess the hemolysis of rat red blood cells (RRBCs) in the presence of anti-RRBC antibodies obtained from immunized rabbits.

Firstly, RRBCs were diluted in 0.9% NaCl at a ratio of 1:5 to prepare a suspension after cell washing. The RRBC suspension was then added to serum containing anti-RRBC antibodies, and incubated in the presence or absence of the tested fractions at 37 °C for 4 hours. After the incubation period, the microtubes were centrifuged at 2000 rpm for 3 minutes, and the hemoglobin released was measured by determining the absorbance at 540 nm of the recovered supernatants.

Evaluation of total IgG production by ELISA assay

We used spleen cells to investigate the impact of *C. sativa* threshing residue on the humoral response. With or without the extracted fractions, about 3106 cells were cultured. After that, the mixture was incubated at 37 °C for 72 hours. To determine the amount of IgG antibody, the tubes were centrifuged for five minutes at 2500 rpm. The supernatants were then used. Total IgG was determined using the ELISA enzyme-linked immunosorbent assay. To do this, the plate wells were first sensitized with 100 µL of the supernatants obtained from the antibody production test (containing IgG), diluted ½ in a solution of BBS (100 mM boric acid, 24.3 mM sodium borate, 147.5 mM NaCl, pH 8.4), containing 1 mL/L of Tween-20. The plate was incubated for 1 hour at 37 °C and washed twice with BBS-Tween 20 (0.1%). Then, the wells are blocked with 200 µL of BBS-Tween 20 (2.5%). The plate was incubated at 37 °C for 15 minutes and washed twice with wash buffer. Next, a peroxidase-conjugated secondary antibody (diluted 1/1000 in BBS-Tween 20 (0.1%)) was dispensed at 100 µL per well. The plate was incubated for 2 hours at 37 °C and washed three times with wash buffer. Orthophenylenediamine hydrochloride substrate solution at 0.5 mg/mL was dispensed into all wells in 100 µL aliquots. The plate was then incubated in the dark at 37 °C for 30 minutes, after which 50 µL of HCl (3N) was added to each well to stop the reaction. Absorbance was then measured at 490 nm using an ELISA reader (BioTeK).

Antibacterial activity

Bacterial strain

The antibacterial effects of *C. sativa* threshing residue were evaluated against *S. enterica*, *E. coli, S. aureus,* and *B. cereus*. Before use, the bacterial strains were revived by subculturing in Luria-Bertani (LB) plates at 37 °C for 24 hours. Then, 2 to 3 colonies were aseptically suspended by a metal loop into sterile physiological saline. Turbidity was adjusted, using a spectrophotometer (625 nm), to the 0.5 McFarland scale to obtain a final concentration of 10^8 CFU/mL (20).

Preparation of the inoculum

Bacterial revival was accomplished by subculturing the top of LB agar made in Petri dishes and incubating at 37 °C for 18 to 24 hours. Utilizing the direct colony suspension technique, microbial inocula were obtained from fresh colonies. Thus, to produce a standardized inoculum (10^8 CFU/mL), 2-3 colonies were suspended in sterile saline

(0.9% NaCl) and tested to a 0.5 McFarland standard.

Determination of the minimum inhibitory concentration (MIC) against the tested strains

The microdilution test was used in 96-well microplates to determine minimum inhibitory concentrations (21,22). Each microplate well received a 50 µL addition of the culture medium. The extract was poured into the first column of the wells in a volume of 100 µL. Then, microdilutions were carried out by moving 50 µL from the first column's well to the second, and so on. The 12th well, which included the culture media and strain, was used as a growth control. After that, 50 µL of previously made, 0.5 McFarland-adjusted bacterial inoculum was added to each well to get the concentration up to 10^6 CFU/mL. After incubation of the microplate at 37 °C for 24 hours, 10 µL of resazurin was added to each well as a bacterial growth indicator. A color turn from purple to pink after an additional two hours of incubation at 37 °C indicated the presence of bacteria. To reduce experimental error, trials were run in triplicate.

Determination of the minimum bactericidal concentration (MBC)

To calculate the MBC, 3 µL of each negative well was plated onto LB agar plates and then incubated for 24 hours at 37 °C (23). Three duplicates of each test were executed. The MBC value was considered the lowest concentration at which no discernible growth was seen.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism 8.00 software. Results are presented as mean ± SEM. EC_{50} or IC_{50} values were calculated using linear regression analysis. Comparisons between multiple groups to the control group were performed by analysis of variance test for repeated measurements (one-way analysis of variance [ANOVA]), followed by Dunnett's multiple comparisons tests. Differences were considered statistically significant at $P < 0.05$. Pearson's correlation test was used to determine the correlation between the antioxidant activities (DPPH) and TPC or TFC.

Results

Chemical composition

The GC–MS analysis of the four fractions showed the presence of many bioactive compounds belonging to different phytochemical categories suggesting that a multi-valorization can be carried out on this plant (Tables 1-4).

In the hexane fraction of *C. sativa* threshing residue (HFCS), the major compounds present were delta-9-tetrahydrocannabinol (Δ-9-THC, 48.18%), CBD (8.09%), cannabinol (6.85%). Terpenes such as caryophyllene and some phytosterols such as Sitosterol were also detected in low abundance (Table 1).

In the dichloromethane fraction of *C. sativa* threshing residue (DFCS), the major compounds detected were Δ-9-THC (51.59%). However, terpenes like Neophytadiene and fatty acids like Linoleic acid and Phytosterols were detected in low abundance (Table 2).

In the ethyl acetate fraction of *C. sativa* threshing residue (EAFCS), the major compounds were glycerol monoacetate (26.87%) 2-deoxygalactose, (13.51%), palmitic acid (12.36%), palmitic acid amide (10.742%), octadecanamide (10.40%), phytol (6.98%), and Δ-9-THC (6.96%) (Table 3).

In the ethanol fraction of *C. sativa* threshing residue (EFCS), the major compounds were squalene (9.91%), heptacosane (6.11%) palmitic acid methyl ester (5.96%).

Table 1. Phytochemical composition of the investigated hexane fraction of *Cannabis sativa* threshing residue determined by GC-MS analysis

Peak number	Compound	Retention time (min)	Relative abundance (%)
1	m-Xylene	4.655	0.563
2	Eucalyptol	6.612	0.022
3	Dodecane	6.915	0.035
4	L-borneol	8.077	0.021
5	Caryophyllene	10.5	0.114
6	α-Bergamotene	10.601	0.046
7	α-Guaiene	10.639	0.076
8	β-Farnesene	10.753	0.018
9	Humulene	10.791	0.019
10	α-Himachalene	10.968	0.124
11	α-Selinene	11.144	0.043
12	β-Guaiene	11.346	0.042
13	3,7(11)-Selinadiene	11.536	0.122
14	Nerolidol	11.624	0.057
15	Guaiol	11.952	0.181
16	γ-Eudesmol	12.154	0.221
17	α-Eudesmol	12.407	0.467
18	Bulnesol	12.495	0.203
19	α-Bisabolol	12.583	0.216
20	Neophytadiene	13.619	0.258
21	Clovanediol	13.997	0.124
22	Phytol	15.437	0.676
23	Delta-8-THC	16.484	0.509
24	Delta-9-THCV	16.686	0.754
25	Cannabichromene	16.971	0.358
26	**Cannabidiol**	17.343	**8.099**
27	**Delta-9-THC**	17.949	**48.183**
28	Cannabigerol	18.252	1.152
29	**Cannabinol**	18.391	**6.853**
30	Vitamin E	21.433	0.281
31	Campesterol	22.38	0.298
32	Stigmasterol	22.969	0.028
33	γ-Sitosterol	23.415	0.853
34	β-Amyrin	23.819	0.757
35	α-Amytrin	24.413	0.413

Table 2. Phytochemical composition of the investigated dichloromethane fraction of *Cannabis sativa* threshing residue, determined by GC-MS analysis

Peak number	Compound	Retention time (min)	Relative abundance (%)
1	Neophytadiene	13.631	1.164
2	Phytol	15.411	0.966
3	Linoleic acid	15.639	1.378
4	Octadecanoic acid	15.803	0.863
5	Delta-8-THC	16.485	0.601
6	Delta-9-THCV	16.687	0.36
7	Cannabidiol	17.343	0.585
8	**Delta-9-THC**	17.873	**51.59**
9	Cannabigerol	18.126	1.049
10	Cannabinol	18.176	1.672
11	γ-Tocopherol	20.726	1.118
12	Vitamin E	21.383	1.295
13	Campesterol	22.342	1.347
14	Stigmasterol	22.658	1.25
15	γ-Titosterol	23.327	1.093
16	β-Amyrin	23.756	0.978
17	α-Amyrin	24.299	0.469

neophytadiene and β-sitosterol were present but had lower abundance (Table 4).

The chemical compositions determined by GC-MS also highlighted the presence of cannabinoids principally within the less polar fractions with decreasing abundance (HFCS, DFCS, and EAFCS) and their total absence from the most polar fraction (EFCS) (Figure 1).

Vasorelaxant activity on pre-contracted MAB

To investigate whether *C. sativa* threshing residue affects the vascular tone, it was injected in doses ranging from 0.01 mg to 0.5 mg to MABs isolated from rats and continuously pre-contracted with phenylephrine to produce a plateau value of PP between 80 to 100 mm Hg.

Figure 2 shows a fall in PP in a dose-dependent related manner of pre-contracted MAB when challenged by the Soxhlet's fractions of *C. sativa* threshing residue reflecting an endothelium-dependent vasorelaxation. Of the four fractions tested, EFCS was the more powerful, since it caused the most pronounced effect (EC50: 0.161 ± 0.012 mg; maximum effect [Emax]: 74.16 ± 1.44%; n = 3), followed by that caused by HFCE (EC50: 0.280 ± 0.022 mg; Emax: 68.33 ± 2.88%; n = 3), DFCS (EC50: 0.417 ± 0.023 mg; Emax: 51.66 ± 2.88%; n = 3), and EAFCS (EC50: 0.455 ± 0.076 mg; Emax: 48.33 ± 7.6%; n = 3).

Table 4. Phytochemical composition of the investigated ethanol fraction of *Cannabis sativa* threshing residue, determined by GC-MS analysis

Peak number	Compound	Retention time (min)	Relative abundance (%)
1	Undecane	6.86	0.57
3	Neophytadiene	13.25	1.38
4	**Palmitic acid methyl ester**	13.84	**5.96**
5	Digitoxin	14.53	1.20
7	Methyl stearate	15.13	4.43
10	17-Pentatriacontene	16.89	2.55
13	**Heptacosane**	17.75	**6.11**
17	**Squalene**	19.16	**9.91**
21	β-Sitosterol	24.65	1.78

Total phenolic and flavonoid contents

In the present work, TPC ranged from 2.48 ± 0.07 to 8.02 ± 0.45 mg GAE/g dw, while TFC varied from 0.92

Table 3. Phytochemical composition of the investigated ethyl acetate fraction of *Cannabis sativa* threshing residue, determined by GC-MS analysis

Peak number	Compound	Retention time	Relative abundance (%)
1	**2-Deoxygalactose**	5.880	**13.516**
2	**Glycerol monoacetate**	7.016	**26.873**
3	1,4-Diacetyl-3-acetoxymethyl-2,5-methylene-l-rhamnitol	8.430	1.870
4	D-Mannose	9.945	0.704
5	β-D-Glucopyranose.4-o-β-D-galactopyranosyl	11523	1.431
6	Ethyliso-allocholate	11.536	0.741
7	Desulphosinigrin	12.041	1.451
8	3,7-Dimethyl-6-nonen-1-olacetate	12.268	1.826
9	Cyclopropanetetradecanoic acid,2 octymethyl ester	12.571	1.259
10	**Palmitic acid**	13.126	**12.369**
11	**Phytol**	14.061	**6.985**
12	**Palmitic acid amide**	14.490	**10.742**
13	**Octadecanamide**	15.677	**10.404**
14	Cannabidiol	15.904	1.130
15	**Delta-9-tetrahydrocannabinol**	16.421	**6.964**
16	Cannabinol	16.788	1.736

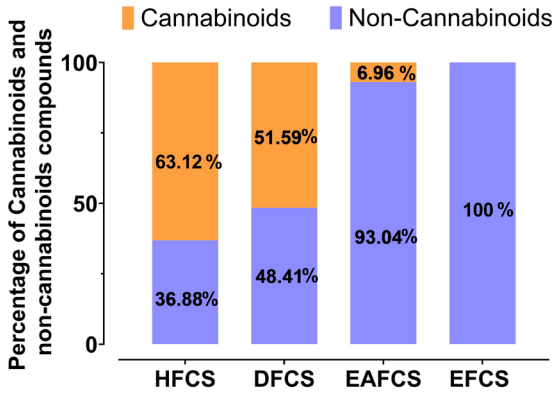

Figure 1. Distribution of cannabinoids and non-cannabinoid components in *Cannabis sativa* threshing residue. HFCS: Hexane fraction of *Cannabis sativa* threshing residue, DFCS: Dichloromethane fraction of *Cannabis sativa* threshing residue, EAFCS: Ethyl Acetate fraction of *Cannabis sativa* threshing residue, EFCS: Ethanol fraction of *Cannabis sativa* threshing residue (EFCS).

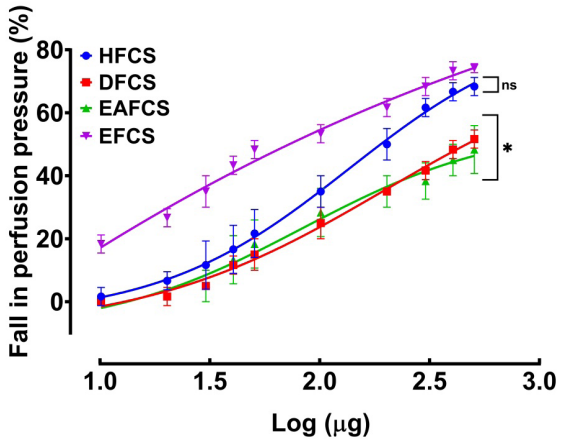

Figure 2. The fall in perfusion pressure (PP; mm Hg) of pre-contracted mesenteric arterial bed (MAB) with phenylephrine (10 µM), challenged with the four investigated fractions of *Cannabis sativa* threshing residue. Dose-response curves were generated for the Hexane (HFCS), Dichloromethane (DFCS), Ethyl Acetate (EAFCS), and Ethanol (EFCS) fractions of *Cannabis sativa* threshing residue. Results are presented as mean ± SEM (n = 3, independent experiments). ns: non-significantly different (HFCS vs EFCS). *P < 0.05 Significantly different from the EFCS (EFCS vs EAFCS or DFCS); One-way analysis of variance (ANOVA) followed by post hoc multi-comparisons Dunnett's test.

± 0.02 to 3.44 ± 0.61 mg QE/g dw. Both polyphenols and flavonoids were distributed among different fractions in the same decreasing order: EFCS > HFCS > EAFCS > DFCS (Figure 3).

DPPH free radical-scavenging activity

The antioxidant activity in the free radical scavenging process was assessed by the DPPH method. Four fractions showed IC_{50} ranged from 0.27 mg/mL to 0.99 mg/mL. The HFCS and EFCS were the most effective with IC_{50s} of 0.27 ± 0.03 mg/mL and 0.28 ± 0.01 mg/mL, respectively, followed by the DFCS (0.95 ± 0.02 mg/mL) and EAFCS

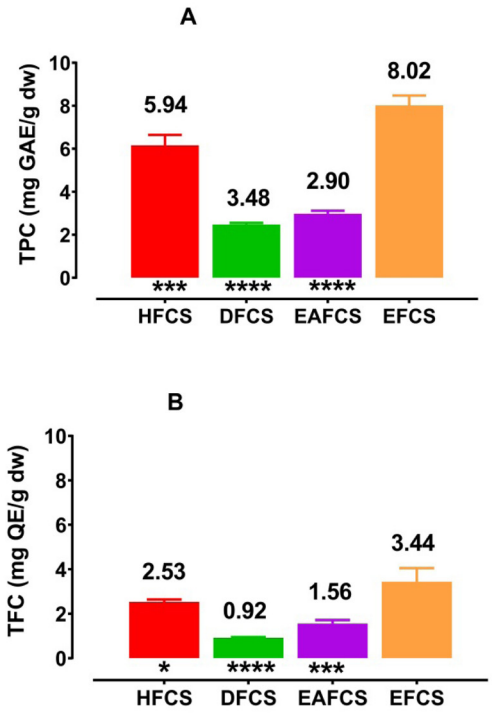

Figure 3. Total phenolic content (TPC) and total flavonoid content (TFC) of the Hexane (HFCS), Dichloromethane (DFCS), Ethyl Acetate (EAFCS), and Ethanol (EFCS) fractions of *Cannabis sativa* threshing residue. The absorbance against the reagent blank was determined using the Folin-Ciocalteu and Harborne aluminum chloride for phenolic (A) and flavonoid (B) compounds, respectively. GAE: Gallic acid equivalents; QE: quercetin equivalents. Results are presented as mean ± SEM (n = 3, independent experiments). ****P < 0.0001, ***P < 0.001, *P < 0.05 compared to the EFCS group; one-way analysis of variance (ANOVA).

(0.99 ± 0.06 mg/mL) (a higher EC_{50} value indicates a lower activity) (Figure 4).

Effect on immune cell proliferation

In this part, we have evaluated the effects of four fractions on the proliferation of splenocytes (composed of B-lymphocytes, T-lymphocytes, and macrophages) and thymocytes (composed of T-lymphocytes and natural killer cells). The proliferation was evaluated by MTT assay (Figure 5). We observed that:

- HFCS did not modify splenocyte proliferation ($P \geq 0.05$ compared to the negative control) but reduced thymocyte proliferation by 16% (from 0.49 ± 0.07 to 0.41 ± 0.11; $P < 0.01$) at 5 mg/mL.
- DFCS did not significantly modify splenocyte proliferation ($P \geq 0.05$ compared to the negative control) but inhibited thymocyte proliferation by 33% (from 0.49±0.07 to 0.34 ± 0.05; $P < 0.01$) at 5 mg/mL.
- EAFCS showed no effect on thymocyte proliferation but inhibited splenocyte proliferation by 38% (from 0.6±0.1 to 0.37 ± 0.06; $P < 0.01$) at 5 mg/mL.
- EFCS induced inhibition of both immunity cells,

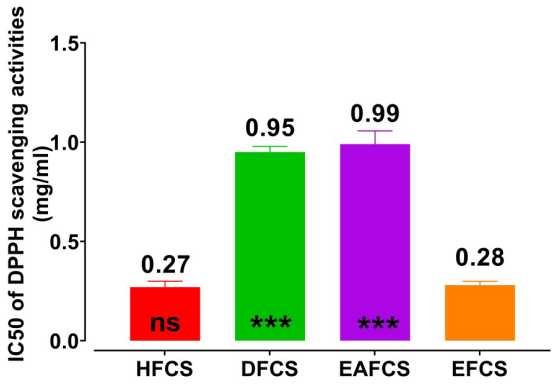

Figure 4. Radical scavenging activity (IC_{50}) of the hexane (HFCS), Dichloromethane (DFCS), ethyl acetate (EAFCS), and ethanol (EFCS) fractions of *Cannabis sativa* threshing residue. Values are means ± SEM from 3 independent experiments. ***P< 0.001 Significantly different from the EFCS group (EFCS vs EAFCS or DFCS); one-way analysis of variance (ANOVA), followed by post hoc multi-comparisons Dunnett's test. ns: non-significantly different from the EFCS group (HFCS vs EFCS).

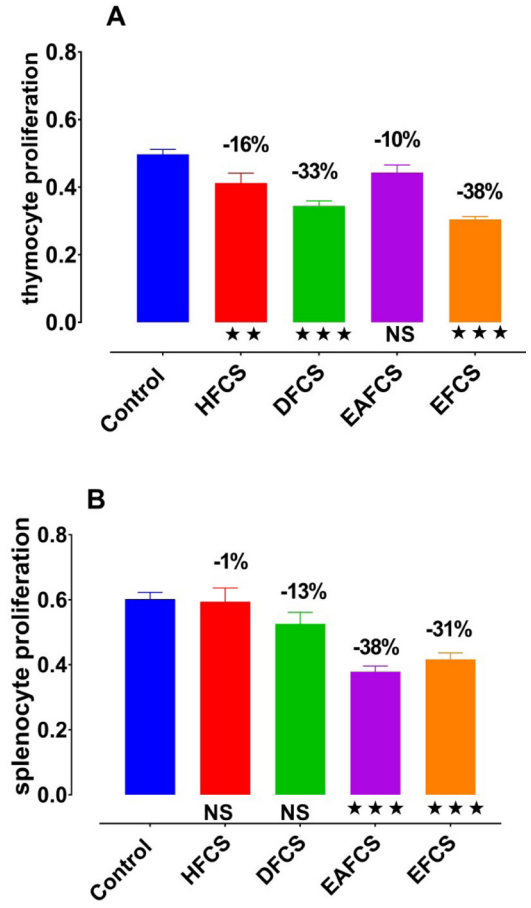

Figure 5. Influence of *Cannabis sativa* threshing residue on thymocyte (**A**) and splenocytes (**B**) cell proliferation. Cells were treated for 72 hours with the hexane (HFCS), dichloromethane (DFCS), ethyl acetate (EAFCS), and ethanol (EFCS) fractions of *Cannabis sativa* threshing residues. Values are means ± SEM from 4 independent experiments. **P < 0.01 and ***P < 0.001 indicate significantly different from the control (solvent) group; one-way analysis of variance (ANOVA), followed by post hoc multi-comparisons Dunnett's test.

with respective inhibition of 38% (from 0.49±0.07 to 0.3 ± 0.03; P<0.01) and 31.8% (from 0.6 ± 0.1 to 0.41 ± 0.07; P<0.01) for thymocytes and splenocytes, at 5 mg/mL.

Effect on antibody production and complement activity

The effect of the *C. sativa* threshing residue on IgG antibodies produced by splenocytes using ELISA assay is depicted in Figure 6. After a 72-hour exposure period, HFCS increased IgG production by 16% (from 0.98 ± 0.04 to 1.14 ± 0.05; n=4; P<0.01), while EFCS reduced it by 5% (from 0.98 ± 0.04 to 0.93 ± 0.05; P<0.01). No significant effect was observed with DFCS and EAFCS (P≥0.05, compared to the negative control).

Effect on complement activity

Regarding complement activity, results indicated that hexane and ethanol fractions inhibited complement activity highly by 57% (from 1 ± 0.22 to 0.43 ± 0.05; P<0.01) and 58% (from 1 ± 0.22 to 0.42 ± 0.03; P<0.01), respectively. However, the other fractions did not significantly modify this activity (Figure 7).

Antibacterial activity

The antibacterial activity was evaluated on four bacterial strains, two gram-negative bacteria (*E. coli* and *S. enterica*) and two gram-positive (*S. aureus* and *B. cereus*), using a microplate microdilution method. All the tested fractions showed significant antibacterial effects.

The results obtained from the antibacterial activity showed that the four strains studied were all sensitive but to different degrees. Therefore, MIC and BMC values

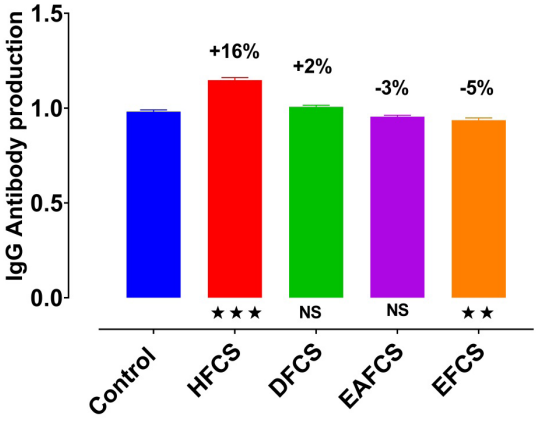

Figure 6. Influence of *Cannabis sativa* threshing residue on total IgG production in isolated splenocytes determined by ELISA. Cells were treated for 72 h with the hexane (HFCS), dichloromethane (DFCS), ethyl acetate (EAFCS), and ethanol (EFCS) fractions of *Cannabis sativa* threshing residues. Values are means ± SEM from 4 independent experiments. **P < 0.01 and ***P < 0.001 depict a significant difference from the respective control (solvent); one-way analysis of variance (ANOVA), followed by post hoc multi-comparisons Dunnett's test. NS: Non-Significantly different from solvent.

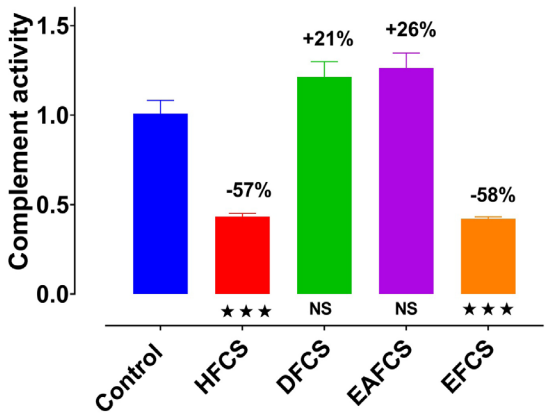

Figure 7. Influence of *Cannabis sativa* threshing residue on complement production in rat red blood cells. Cells were treated for 4 h with the hexane (HFCS), dichloromethane (DFCS), ethyl acetate (EAFCS), and ethanol (EFCS) fractions of *Cannabis sativa* threshing residues. Values are means ± SEM from 4 independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ depict a significant difference from the respective control (solvent); one-way analysis of variance (ANOVA), followed by post hoc multi-comparisons Dunnett's test. NS: Non-significantly different from solvent.

vary according to the fractions and the bacterial strains (Table 5).

The HFCS showed the best inhibitory effects against *S. enterica* and *B. cereus* (MIC of 2.5 mg/mL), followed by *S. aureus* (MIC of 3.125 mg/mL). On the other hand, the HFCS showed a weak inhibition against *E. coli* (MIC of 6.25 mg/mL).

For the DFCS, it took 0.0781 mg/mL to effectively stop *S. enterica* and *B. cereus* from growing, while *E. coli* and *S. aureus* were less sensitive to DFCS (MIC=1.875 mg/mL). Additionally, EAFCS exhibited moderate efficacy against gram-positive *S. aureus* (MIC 3.125 mg/mL) and considerable antibacterial activity against gram-positive *B. cereus* (MIC values of 0.781 mg/mL). On the other hand, EAFCS showed a moderate effect against gram-negative *S. enterica* (1.562 mg/mL) and *E. coli* (6.25 mg/mL).

EFCS showed low antibacterial activity compared to other fractions, especially against *S. enterica* (MIC = 25 mg/mL), *E. coli,* and *B. cereus* (MIC = 12.5 mg/mL). The MBC values were globally higher than the obtained MIC values (Table 5). The MBC/MIC values were small (less than 6): for DFCS and EFCS against *S. aureus*, for all fractions except DFCS against *B. cereus*; and for EFCS against *S. enterica*.

Discussion

According to GC-MS analysis, several bioactive chemicals were found in various fractions; Cannabinoids were detected in the less-polar fractions. Delta-9-THC was found in high abundance in the hexane fraction (48.18%) and dichloromethane fraction (51.59%). This could be explained by the fact that the used cultivar is known for its high THC content. It should be noted that even though we used only the threshing residue, cannabinoids were still detected in the less polar fractions, and following the successive extractions, their abundance decreased among the polar fractions until becoming absent in the most polar fraction, namely EFCS. Many volatile terpenes, such as caryophyllene, humulene, and squalene were present in the Soxhlet fractions. Previous work on cannabis composition has highlighted the presence of caryophyllene and humulene as the most abundant sesquiterpenes found in cannabis (24). Squalene has been previously reported in some cannabis species (25).

Interestingly in the non-polar fractions and specifically in the hexane fraction (HFCS), 22 terpenes were detected but only at trace levels; in fact, their relative abundance was less than 1% for each terpene. In contrast, squalene's relative abundance reached 9.91% in EFCS. Phytosterols were found at trace levels in the HFCS (γ-sitosterol), DFCS (γ-sitosterol), and EFCS (β-sitosterol). These components have also been reported in previous investigations of the phytochemical analysis of cannabis (26,27). Fatty acids, such as linoleic acid and palmitic acid, were also detected. However, in the literature, these fatty acids have only been detected in the cannabis seed (28,29). Since we did not use the seeds, we suggest that the presence of such a compound is possibly due to accidental leakages of seeds during the sieving process.

All the investigated Soxhlet fractions had fairly high TPC and TFC levels. Both polyphenols and flavonoids were distributed among the different fractions in the same decreasing order; the maximum phenolic concentration was found in the ethanol fraction, which

Table 5. Minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations of *Cannabis sativa* threshing residue against bacterial strains

Fractions	Gram-positive bacteria				Gram-negative bacteria			
	Staphylococcus aureus		*Bacillus cereus*		*Escherichia coli*		*Salmonella enterica*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
HFCS (mg/mL)	3.12	25	2.50	10	6.25	ND	2.50	ND
DFCS (mg/mL)	1.87	3.75	0.07	ND	1.87	ND	0.07	10
EAFCS (mg/mL)	3.12	25	0.78	1.56	6.25	ND	1.56	ND
EFCS (mg/mL)	6.25	2.5	12.5	25	12.5	ND	25	25

HFCS: Hexane fraction of *Cannabis sativa* threshing residue; DFCS: Dichloromethane fraction of *Cannabis sativa* threshing residue; EAFCS: Ethyl Acetate fraction of *Cannabis sativa* threshing residue; EFCS: Ethanol fraction of *Cannabis sativa* threshing residue; ND: Not determined

was followed by the hexane fraction, while ethyl acetate and dichloromethane are ranked third and fourth, respectively. Our findings are in agreement with a previous study (30), where the TPC of seven different extracts of cannabis threshing residue ranged from 1.90 to 19.07 mg GAE/g dw; the ethanol fraction had ten times more TPC than ethyl acetate. Another study by Pavlovic et al (31) reported that certain flavonoids, such as cannflavin C, were present in significant amounts in hexane extracts of *C. sativa* flowers. Indeed, there are many flavonoids present in cannabis, and the most significant ones are apigenin-7-oglucoside, vitexin, luteolin-7-O-glucoside, and quercetin-7-O-glucoside (32). The leftover threshing residue from *C. sativa* should therefore be regarded as an abundant source of flavonoids and polyphenols.

Polyphenols are thought to be responsible for the antioxidant effect in many plants (33,34). Indeed, polyphenols are able to protect cells from oxidative damage brought on by reactive oxygen species (ROS). ROS are bioproducts of physiological oxygen metabolism and are important regulators of several processes. However, because these ROS cause permanent DNA damage, lipid peroxidation, and enzyme inactivation, their persistent presence can cause tissue damage and cell death (16).

The IC_{50} values for DPPH scavenging activity of the investigated fractions ranged from 0.24 mg/mL to 0.94 mg/mL, in the following order: EFCS, HFCS, EAFCS, and DFCS. EFCS, which exhibited the strongest antioxidant activity, also had the highest total phenolic and flavonoid contents; a similar association was seen with the HFCS, which comes in second place. The results obtained in our study are consistent with those of a previous study (35), demonstrating the strong antioxidant effects of the hexane extract of *C. sativa,* rich in CBD and other phytocannabinoids. Similarly, our investigation found high amounts of phyto-cannabinoids in the hexane fraction of *C. sativa* threshing residue (HFCS). Furthermore, cannabinoids can enhance the production of endogenous antioxidant systems, such as glutathione peroxidase and superoxide dismutase (36). Therefore, the presence of phytocannabinoids might contribute to the antioxidant activity of non-polar cannabis fractions (HFCS).

The presence of terpenes, even in small quantities, may contribute to the antioxidant effect of cannabis fractions (37). Phytols and α/β-amyrin exhibited promising antioxidant capacities by scavenging DPPH free radicals (38,39). β-caryophyllene γ/α-eudesmol and α-bisabolol showed only poor to moderate antioxidant activity when evaluated by DPPH scavenging assay (40-42). Squalene was shown to be unable to scavenge DPPH (43). Furthermore, it has been reported that γ-tocopherol and β-sitosterol are effective DPPH scavengers and antioxidants (44,45).

Vascular oxidative stress is a significant contributor to endothelial dysfunction, primarily by reducing the levels of the endothelium-derived relaxing factor, specifically nitric oxide. This alteration disrupts the vasodilator response to physiological or neuro-humoral stimuli, leading to functional impairments in vascular health (46). Antioxidants are essential for maintaining the integrity and function of the endothelium by shielding it from oxidative damage. This helps to strengthen the vascular system. Vasorelaxation is indeed a primary treatment approach for various cardiovascular disorders such as hypertension and atherosclerosis (46). Therefore, interpreting the studies involving both antioxidant and vasorelaxant assays can provide a more comprehensive understanding of the potential cardiovascular benefits of natural products.

Our current study has demonstrated that the four cannabis threshing residue fractions induced a dose-dependent vasorelaxation on rat pre-contracted MAB. Interestingly, over all the screened fractions, EFCS caused the most pronounced effect, followed by that caused by HFCE, DFCS, and EAFCS. The statistical analysis revealed that among the four examined fractions, the vasodilator effect of EFCS was significantly correlated with the phenolic content (R^2 = 0.95). These findings have led us to hypothesize that the phenolic components are active chemicals associated with the observed vasodilation. Moreover, plants high in polyphenols are important for preserving vascular health and averting cardiovascular diseases (47). In our previous study on the EFCS, HPLC analysis revealed the presence of flavonoid-phenolic compounds, notably apigenin and luteolin glycosides (48). Furthermore, we have previously shown that flavonoids can activate G protein-coupled receptors to produce their biological effects. (47). Flavonoids have been shown to induce vasorelaxant effects through endothelial muscarinic receptors (47,48). Additionally, EFCS significantly reduced inflammation *in vivo* (49). These discoveries further underscore the significance of polyphenols in promoting vascular health and highlight the potential therapeutic advantages of EFCS in mitigating cardiovascular disorders and inflammation.

It is also plausible that volatile compounds, such as those identified, may contribute to the observed vasodilation in MAB. This suggestion stems from studies demonstrating the vasorelaxant properties of specific terpenes, such as beta-caryophyllene, alongside other compounds like palmitic acid and selinene, on rat thoracic aorta artery rings (50).

Furthermore, cannabinoids have also demonstrated therapeutic use in the pathological states of cardiac malfunction and vascular abnormalities because they improve the function of both the heart and arteries (51). However, it is unlikely that THC and CBD present in the non-polar fractions are responsible for the observed effect. CBD did not affect vascular tone in the MAB of mice, while THC was rather vasoconstrictive in the rat MAB (52).

In this work, we evaluated the antibacterial activity of the tested cannabis fractions against four bacterial strains.

The fractions had strong inhibitory effects against gram-positive bacteria (*S. aureus* and *B. cereus*) and gram-negative bacteria (*E. coli and S. enterica*). Our results are in concordance with previous reports demonstrating the cannabis extracts activities against gram-positive bacteria (*B. subtilis, Bacillus pumilus, S. aureus,* and *Micrococcus flavus*) and gram-negative bacteria (*Proteus vulgaris and Bordetella bronchiseptica*) (53,54).

Escherichia coli had low sensitivity to the tested fractions. In this regard, Kaur et al showed that *C. sativa* leaf extract had strong antibacterial activity against *B. subtilis* and *S. aureus* and moderate effect against *E. coli* (55). Furthermore, an interesting antibacterial activity of cannabis inflorescences has been reported on *Paenibacillus* larvae, a pathogen of the honey bees larvae, causing the American Foulbrood disease (MIC = 20.80–76.83 µg mL-1) (56). Malikova et al (57) studied ethanolic extracts of cannabis during its development cycle and reported a powerful activity against *S. aureus* (MIC values from 32 to 64 µg/mL).

The antibacterial potential of the plant might be due to the phytochemical composition of the tested cannabis extracts, especially the presence of cannabinoids. Indeed, the most active extracts tested in this study were those having the highest cannabinoid content. Moreover, DFCS extract, which had the highest Δ-9-THC percentage (51.59%) showed the highest antimicrobial activity, followed by HFCS (having 48.18% Δ-9-THC) and EAFCS (<10% Δ-9-THC). While the EFCS extract, whose chemical analysis showed a lower content of bioactive compounds and the absence of cannabinoids, had the lowest antibacterial activity. This finding is in line with the results reported by Skalla et al (58) who showed that ethanolic extracts were less effective against 7 tested bacterial strains and 12 dermatophytes compared to butane and dimethyl ether extracts. It has been reported that THC and THCA induce DNA damage, while CBD causes oxidative and cytotoxic damage (59). This potential could be enhanced by terpenoids, which increase the penetration of cannabinoid compounds into the bacterial cells. An antibacterial power has also been previously reported for other compounds, such as terpenes (Caryophyllene) that have been found in the hexane extract of *C. sativa* (53); poly-sterols, especially β-sitosterol have also been identified as the major compounds of *Parthenium hysterophorus* crude extract, which showed a significant antibacterial activity against two Vibrio species, *B. cereus and Proteus mirabilis* (60).

Regarding immunomodulation activity, the results showed that hexane, dichloromethane, and ethanol fractions had a significant suppressive effect on thymocytes, leading to a reduction in T-lymphocyte proliferation. The ethyl acetate and ethanol fractions also inhibited splenocyte proliferation, which correlated with a 5% inhibition in antibody production. Interestingly, EFCS treatment affected both splenocyte viability and biological activity, resulting in a reduced humoral response. Additionally, treatment with HFCS and EFCS strongly inhibited complement secretion. It can be presumed that the inhibition of thymocytes and splenocyte proliferation is possibly attributable to certain cannabinoids that remained in the threshing residue, in particular in HFCS and DFCS. McKallip et al (61) reported that *in vivo* administration of Δ9-THC to mice resulted in thymic atrophy that correlated with T-lymphocyte apoptosis and inflammatory cytokine suppression. Furthermore, there is evidence suggesting that CBD influences all phases of cellular inflammation, altering various mechanisms to favor its immunosuppressive effect and thereby impacting the functionality of a diverse array of immune cells (62). The cannabinoid system has now been shown to be involved in regulating the immune system through its immunomodulatory properties both *in vivo* and *in vitro* (63). The inhibition observed could also be due to non-cannabinoid compounds, as is the case with EFCS, which contains no cannabinoids.

The literature has emphasized the potential of non-cannabinoid compounds to modulate the immune response and reduce inflammation. Among these compounds are terpenes (64) such as α-pinene, trans-nerolidol, D-limonene, linalool, phytol, β-caryophyllene, and β-lemene, as well as phenolic compounds such as apigenin, and quercetin (65). These compounds have been found to target specific immune cells and their functions involved in the pathological processes underlying inflammation. For example, some terpenes interact with the CB2 receptor of the endocannabinoid system, which is responsible for regulating pain and inflammation (66). Others have been shown to influence the activity of T-lymphocytes, B-lymphocytes, and other immune cells. In addition, phenolic compounds modulate inflammatory pathways by reducing the production of pro-inflammatory cytokines (65).

Conclusion

This work highlights the promising potential of *C. sativa* threshing residue as a source of bioactive ingredients with diverse pharmacological benefits, particularly in the context of inflammation. Our findings demonstrate that the non-cannabinoid fractions, specifically EAFCS and EFCS, exhibit notable antioxidant, antibacterial, vasorelaxant, and immunomodulatory activities. This suggests that leveraging cannabis processing by-products may offer a promising approach for developing novel therapeutics aimed at managing inflammatory conditions. Further investigations in preclinical and clinical studies are warranted to fully elucidate the potential of *C. sativa* threshing residue in combating inflammation and related disorders.

Authors' contribution

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Conflict of interests

The authors state that there is no conflict of interest to declare.

Ethical considerations

The experimental procedures were approved by the local Ethics Committee under reference number 11/2021/CEFST on March 9, 2021.

Funding/Support

This work was supported by ANPMA/USMBA/CNRST, project: VPMA-2019-9.

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