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Investigating the Biological Activities of Moroccan *Cannabis Sativa L* Seed Extracts: Antimicrobial, Anti-inflammatory, and Antioxidant Effects with Molecular Docking Analysis

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Abstract: The present study focuses on assessing the bioactive potential of *Cannabis sativa* L. seed extracts, specifically the organic and aqueous extracts derived from Ketama hemp seeds harvested in Morocco. We tested the antioxidant capacities of the extracts, specifically their ability to scavenge DPPH free radicals. The ethanolic *Cannabis sativa* L. seed extract was particularly effective, demonstrating the lowest IC_{50} value of 0.36mg/mL±0.01, outperforming the reference compound ascorbic acid, which had an IC_{50} value of 0.68mg/mL±0.02. This suggests a significantly high antioxidant potential of the ethanolic extract against the DPPH radical. The β-carotene bleaching test further supported these findings, with the ethanolic and hexanolic extracts showing substantial antioxidant activity, inhibiting the coupled oxidation of linoleic acid and β-carotene by 36%±0.12 and 33%±0.14, respectively. We also assessed the antimicrobial potency of the seed extracts using the Muller-Hinton Agar well diffusion method. The ethanolic extract demonstrated the highest inhibition zone diameter (IZ = 23 mm) against Penicillium sp., whereas the hexane extract had the smallest IZ (07 mm) against Listeria monocytogenes. Microdilution method in a 96-well microplate was employed to determine the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) of the extracts. All extracts showed inhibitory effects against all tested bacterial and fungal strains, with MIC, MBC, and MFC values ranging from 0.03 to 1.25 mg/mL. Lastly, the anti-inflammatory activity of the extracts was tested using the RBC method. The findings indicated that all extracts significantly inhibited heat/hypotonia-induced erythrocyte haemolysis, further exploring their potential therapeutic value.

1. Introduction

Plant-derived compounds have served as the foundation for a vast array of pharmaceuticals and therapeutics throughout human history (Aoussar *et al.,* 2021, Chakraborty *et al.,* 2021, Rajput *et al.,* 2021). Investigating the diverse biological properties of plant extracts provides valuable insights into their potential applications in treating various diseases and promoting human health. These bioactive compounds, such as phenolic compounds, flavonoids, and alkaloids, often exhibit potent antioxidant, antimicrobial, and anti-inflammatory activities, among other beneficial effects (Benkirane *et al.,* 2022; Khadraoui *et al.,* 2022, Nischitha and Shivanna 2022). Furthermore, the exploration of plant-derived bioactive compounds may offer alternative solutions to the challenges posed by antibiotic resistance and the adverse effects associated with synthetic drugs. Cannabis has been known and cultivated by humans since ancient times. C. Sativa L. is an annual plant of the Cannabaceae family (Babiker *et al.,* 2021), the fruit, commonly called "hemp seed" is a smooth, light gray, oval achene, 2.5 to 3.5 mm long and 2.5 to 3 mm in diameter (Burnside *et al.,* 1994). Over the past 30 years, there has been a growing awareness of the versatility and utility of hemp in products ranging from fibers and textiles to food and health products (Byrd 2019). Hemp seeds have been used since ancient times as a base for pharmaceuticals, foodstuffs, and filaments (Yan *et al.,* 2015, Rodriguez-Martin *et al.,* 2019, Martinez *et al.,* 2020). Compared to other medicinal plants, hemp seeds enhance several virtues of the food industry. The ingredient itself can also be used as a food additive for preserving as well as improving the presentation of foods (Rupasinghe *et al.,* 2020, Claro-Cala *et al.,* 2022). Over the past decades, companies, research institutes, and institutions have been interested in C. sativa L. because of its virtues (Andre *et al.,* 2016). The secondary metabolism of cannabis produces interesting molecules with different biological properties: for example, cannabinoids, phenolics, and terpenes are found (Flores-Sanchez and Verpoorte 2008, Pegoraro *et al.,* 2021). Hemp seeds are used both for their edible qualities and for their therapeutic properties. (Bruneau 2016). Cannabis is used as a companion crop to kill many insects, fungi, and weeds (Satyal and Setzer 2014), and its extracts can kill insects, mites, and microbes, and can be used as allelochemicals (McPartland 1997). Thus, essential oil of cannabis seed of Nepalese origin has been evaluated for its cytotoxic, larvicidal, insecticidal (Satyal and Setzer 2014), and antimicrobial activity (Nissen *et al.,* 2010). *Cannabis* leaves also have great potential against pests (McPARTLAND and Cubeta 1997). *Cannabis* is a potential source of phytochemicals and antioxidants responsible for insecticidal activity (Ahmed *et al.,* 2019). Hemp extracts grown in different parts of the world have been shown to have antibacterial (Wasim *et al.,* 1995, Novak *et al.,* 2001, Nissen *et al.,* 2010) and antioxidant activities (Abuzaytoun and Shahidi 2006) and the literature search is rich in studies concerning the antibacterial activity of compounds extracted from high-THC cannabis, which known to contain potent antibacterial agents (Appendino *et al.,* 2008). However, surprisingly few studies have focused on antibacterial activity. The components of hemp seeds include tocopherols, terpenes, and phytosterols which have significant antioxidant activity and may be useful in regulating inflammatory responses (Farinon *et al.,* 2020, Izzo *et al.,* 2020). Therefore, the compounds associated with hemp seeds are anti-inflammatory and immunomodulatory (Farinon *et al.,* 2020).

Morocco, one of the world's leading *cannabis* producers, adopted on Thursday (11 March 2021) a draft law authorizing the medical, cosmetic, and industrial use of cannabis, to convert environmentally damaging prohibited crops into sustainable legal activities that create value and jobs. The bill provides for the establishment of a regulatory company in charge of "developing the agricultural and industrial cycle and controlling the entire production chain, from the import of seeds to marketing, with regulatory scope, approved agricultural cooperatives, certified plants, and control of THC levels, the main psychoactive molecule of *Cannabis*" (Elhamdaoui *et al.,* 2019) .

The primary objective of this study is to examine the antioxidant and antimicrobial properties of organic and aqueous extracts from Cannabis sativa L. seeds harvested in Morocco, through in vitro tests. The ultimate aim is to underscore their potential medicinal and nutritional value, thereby endorsing their broader use. Our research strategy involves several steps. We will assess the antioxidant capacity of the extracts utilizing the DPPH system and the linoleic acid-β-carotene system. We will then evaluate their antibacterial and antifungal effectiveness using the well diffusion method in an MHA medium and the minimum inhibitory concentration. Through these steps, we hope to provide comprehensive insight into the bioactive potential of these seed extracts.

2 Materials et methods

2.1 Plant Materials

In December 2021, hemp seeds were harvested in the regions of Ketama and Chefchaouen in northern Morocco (province of Tangier-Tetouan-Al Hoceima), the main cannabis-producing city in Morocco. And they were taken to the Applied Chemistry and Environment laboratory of the Department of chemistry, University Mohammed 1st, Oujda, Morocco, to complete their environmental drying at room temperature (25°) for one week. The C. Sativa L. seeds were first ground into a fine powder and soaked in hexane. Afterward, a series of solvents including dichloromethane, ethanol, and water were sequentially used to extract the desired substances. The resulting extracts were filtered and concentrated under reduced pressure. To preserve their quality, the extracts were stored in opaque containers at a temperature of 4°C until they were ready to be utilized (Haddou et al., 2022).

2.2 Flavonoid assay

The quantification of total flavonoids was conducted following the method outlined by Kim et al. (Kim et al., 1997), with slight modifications. In brief, 200 µL of an ethanolic extract obtained from cannabis seeds was combined with 1000 µL of distilled water and 50 µL of NaNO2 solution (5%). The mixture was thoroughly mixed and allowed to react for 6 minutes. Subsequently, 120 µL of $AlCl_3$ solution (10%) was added, followed by an additional 5-minute incubation period. Then, 400 µL of NaOH solution (1M) and 230 µL of distilled water were introduced (Kim et al., 1997). To establish a calibration curve, various concentrations (0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 µg/mL) of a quercetin standard solution were prepared. The absorbance of the reaction mixture was measured at 510 nm, using methanol as the blank. All analyses were performed in triplicate. The concentration of flavonoids was expressed as quercetin equivalents (QE) per milligram of dry matter in the extracted sample. The same experimental procedure was followed for both the dichloromethane extract and the aqueous extract.

2.3 High-performance liquid chromatography analyses

The chromatographic analysis was carried out following the protocol of Loukili *et al.,* 2022 with some changes. The chromatographic test was performed with a (waters allianceTM e2695 XC HPLC system) High-performance liquid chromatography (HPLC) equipped with a P 680 Socratic pump. The separation was performed by an Alliance ew2695 C18 column (250x4mm, 5 µm) in the normal phase according to the method described by Loukili *et al.,* 2022, using a gradient solvent system of A (water/ 0.5% acetic acid) and B (methanol) and with a flow rate of 1 ml/min. The detector used is a UV-visible diode detector (PDA Waters 2996). The chosen wavelength depends on the extract's nature and the targeted substances. The reading was at 254, 280, 320 and 340 nm and the identification of compounds was performed at 280 nm. The contents of phenolic compounds are determined from

the calibration curves for each compound. All calibration curves showed high linearity ($r^2 > 0.99$). The identification and quantification of the compounds are performed by comparison of their relative retention time with those of the standards. The results were presented in mg/100 g of per extract.

2.4 Biological Activities

2.4.1 Antioxidant activity

The antioxidant activities of *C. sativa* L. seeds extracts were evaluated using in vitro DPPH and Beta-carotene essays as described elsewhere (Laaroussi *et al.*, 2022).

2,2-Diphenyl-1-Picrylhydrazyl Radical Scavenging Capacity: The capacity for neutralizing free radicals in cannabis seed extract (using Hexane, Dichloromethane, ethanol, and water) was assessed in triplicate utilizing the method described by Miri et al., albeit with minor alterations. In a nutshell, 0.2ml of each varying concentration (0.032, 0.062, 0.125, 0.250, 0.500, 1, and 2mg/ml) of the individual extracts dissolved in methanol was incorporated into 1.8ml of a methanolic solution of DDPH (equivalent to 2 mg of DDPH in a 100 mL methanol solution). The amalgamation was thoroughly vortexed for 60 seconds, and subsequently incubated in a dimly lit room at ambient temperature for a 20-minute duration. Absorbance was recorded at 517 nm using an Ultrospec 7000 UV-visible spectrophotometer. Ascorbic acid was formulated as a reference standard (acting as a positive control) under identical conditions (Miri and Djenane 2019). Each experiment was carried out in triplicate. The DPPH radical neutralizing potential of the extracts was then determined based on the following:

$$\text{Inhibition \%} = ((\text{A control} - \text{A test})/\text{A control}) * 100 \qquad (1)$$

The level of DPPH that was inhibited by the inhibitory activity of the extracts was given as a percentage of concentration corresponding to 50% inhibition (IC_{50}).

Beta-carotene test: The antioxidant power of cannabis seed extracts was evaluated using the β-carotene/linoleic acid bleach assay using the protocol of Kartal 2007 & Ouahabi et al. 2023. A β-carotene/linoleic acid emulsion was synthesized by dissolving 6 mg of β-carotene in 1.5 mL of chloroform, followed by the addition of 30 µL of linoleic acid and 250 mg of Tween 80. The chloroform was then removed using a rotary evaporator, and 100 mL of oxygen-saturated distilled water containing 30% H_2O_2 was subsequently introduced. To achieve homogeneity, the emulsion was vortexed. Thereafter, 175 µL of either the extract or a control antioxidant solution (BHA) with a concentration of 2mg/mL was introduced to 1.25 mL of the pre-existing emulsion. The decoloration kinetics of the emulsion, in the case of the negative control and the mixtures containing either the extracts or BHA, were assessed at 490 nm for a period of 120 minutes at consistent time intervals. The inhibition percentages of β-carotene bleaching by the Cannabis seed extracts were computed using the subsequent equation:

$$\text{inhibition \%} = ([OD_{(E120)} - OD_{(C120)}]/(OD_{(C0)} - OD_{C(120)})*100$$

where:

$OD_{(E120)}$ represents the absorbance in the presence of the extract (antioxidants) at 120 min, $OD_{(C120)}$ is the control absorbance at 120 min and $OD_{C(120)}$ indicates the absorbance of the control at 0 min.

2.4.2 Antimicrobial activity

Inoculum preparation: The antimicrobial properties of plant extracts were assessed on five pure strains sourced from the Laboratory of Microbial Biotechnology at the Faculty of Science in Oujda, Morocco. The tested strains were composed of one Gram-negative bacterium, Escherichia coli (ATCC

1051.4.2. 36), one Gram-positive bacterium, Staphylococcus aureus (ATCC 6538), and three fungal strains, namely Aspergillus niger, Penicillium sp, and Candida Albicans. Bacterial growth was facilitated on Muller-Hinton Agar (MHA) medium, and the samples were incubated at 37 °C for a period of 24 hours (Celiktas et al., 2007). The bacterial load was subsequently adjusted to a concentration of 106 cells/mL. Candida albicans was cultivated on Yeast Extract Peptone Dextrose (YPD) and allowed to incubate at 30 °C for 48 hours. Post incubation, the cell density was regulated to 106 cells/mL. Aspergillus niger and Penicillium sp were cultivated on Potato Dextrose Agar (PDA) at a temperature of 25 °C for a duration of 7 days. Following incubation, the spore concentration was adjusted to 105 spores/mL (Pundir et al., 2013).

Agar Well-Diffusion Method: The experimental protocol involved the introduction of a prepared inoculum to Petri dishes filled with MHA medium for bacterial strains, YPD for C. albicans, and PDA for Aspergillus niger and Penicillium sp. Wells with a 6 mm diameter were aseptically created using a Pasteur pipette and were then filled with 80 µL of the plant extract. Finally, the agar plates were incubated for 24 hours at 37°C for bacterial strains, 48 hours at 30 °C for C. albicans, and 7 days at 25 °C for Aspergillus niger and Penicillium sp. DMSO was used as negative control, Gentamicin and Cycloheximide were used as positive control. After incubation, the inhibition zone of the target strain around the well was measured in mm by with a sliding caliper (Frassinetti et al., 2020).

2.4.3 Microdilution testing

Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC): The determination of MIC, MBC and MFC is important to measure the antimicrobial potential of plant extracts. The MIC was carried out in 96 well-microplate using the microdilution method previously described by Wiegand et al. (Wiegand et al., 2008) with some modifications. The *C. sativa L* extracts were suspended in 2% DMSO (v/v) to yield a concentration of 10 mg/mL. Aliquots of 50 µL of microbial inoculum adjusted to 106 cells/mL for bacteria and yeast and 106 spores/mL for fungal strains, were added to each well containing serial dilutions of plant extracts. The positive control (11th well) was filled with 50 µL of the medium and 50 µL of the microbial suspension while the negative control (12th well) contained the culture broth with 2% DMSO and 50 µL of the microbial suspension. The incubation at 37 °C for 20 h for bacteria, 30 °C for 48 h for yeasts and 25 °C for 48 h for fungi. The microbial growth for bacterial and yeast strains was revealed by adding 15 µL of 0.015% resazurin into each well followed by a further incubation at 37 °C for 2 hours to observe the color changes. For the studied fungal strains, the MIC was determined as the lowest concentration without visible growth.

The MBC and MFC were the determined by sub-culturing 5 µL from each well showing higher concentrations than MIC values on culture medium plates followed by an incubation at 37 °C for 24 h for bacteria, at 30 °C for 48h for yeast strains and at 25°C for 48h for fungal strains.

2.4.4 Anti-inflammatory activity

Bovin Serum Albumin (BSA) Denaturation Assay: The Bovin Serum Albumin (BSA) Denaturation Assay was done according to (Kandikattu et al., 2013) with some modifications. 0.2% (w/v) BSA solution was prepared in tris-saline buffer solution. The test group consists of preparing a series of different concentrations C. *sativa* L. seeds using ethanol as solvent, from these stock solutions, 0.05ml of each concentration was transferred into test tubes containing 5ml 0.2% BSA solution. The standard group consists of preparing a series of concentrations ranging from 100 to 250µg/ml of Ibuprofen, to which is added a volume of 5ml of 0.2% BSA solution. The test tubes were subjected to

thermal stress (75°C/ 5min) and then left to cool. The UV-visible spectrophotometer was used for the determination of the absorbance of solutions at 660 nm. The experiments were carried out in triplets and the average values were calculated. The percentage % of inhibition of BSA protein denaturation was determined using the following formula:

$$\%\text{Inhibition of BSA denaturation} = \frac{\text{DO control} - \text{DO test}}{\text{DO control}} \text{ x } 100$$

DO control corresponds to the measurement of the optical density of the heated solution. DO test is the measurement of the optical density of the heated test solution.

Red Blood Cell (RBCs) preparation: The study and evaluation of the anti-inflammatory activity of *C. sativa L* seeds was carried out using the RBC method according to a protocol described in the literature with a few modifications: anesthesia of Wistar rats which had not received NSAIDs for 2 weeks before the experiment has been carried out. Fresh blood was collected and deposited in glass tubes containing a sterilized anticoagulant (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% sodium chloride). The erythrocyte cell concentrates were recovered after centrifugation at 3000 rpm (15 min), then washed with sterile saline solution (0.9% w/v NaCl, pH 7.2). A 10% suspension was reconstituted using a sterilized solution of 10 mM sodium phosphate buffer containing: 0.2 g NaH2PO4, 1.15 g Na2HPO4, 9 g NaCl for a volume of 1 L of distilled water. The reconstituted erythrocytes were used as such for subsequent tests of hemolysis induced by hypotonicity or heat. The estimate of the percentage of hemolysis was calculated assuming that the hemolysis induced in the control was 100%.

Hypotonia-Induced Hemolysis: C. *sativa L* extracts were prepared at different concentrations using distilled water. 0.1mL was added to the 10% erythrocyte suspension. Diclofenac sodium has been used as the reference anti-inflammatory drug. The mixtures were incubated (1 hour at 37°C), followed by a centrifugation step at 3500 rpm/15 min. hemoglobin content was estimated at 540 nm. The tests were carried out in triplets (Chanda and Juvekar 2019).

Heat-Induced Hemolysis: C. *sativa L* extracts were preprepared at different concentrations using isotonic phosphate buffered solution. 0.1mL was added to the 10% erythrocyte suspension. The vehicle solution was the control containing the isotonic phosphate buffer. Diclofenac sodium (prepared with buffer) was used as the reference anti-inflammatory drug. An incubation was carried out for all the reaction mixtures (60° C. for 30 min), then the tubes are left to cool to ambient temperature. Finally, centrifugation at 3000rpm/10min was performed. hemoglobin content was estimated at 540 nm. The tests were carried out in triplets (Chalini et al., 2017). The determination of the percentage of inhibition of hemolysis induced by Hypotonicity/heat was carried out according to the following formula:

$$\%\text{Inhibition of erythrocyte hypotonicity} - \text{induced hemolysis} = \frac{\text{DO control} - \text{DO test}}{\text{DO control}} \text{ x } 100$$

DO control is the measurement of the optical density of the heated/hypotonia control solution, while DO test is the measurement of the optical density of the heated/hypotonia sample solution.

2.5 Molecular docking studies in silico

The structures of the compounds contained in XX have been downloaded from the Puchem database in SDF format. Molecular docking studies have been carried out through the Pyrx 0.8 virtual screening software, using Autodock Vina and Autodock 1.5.7 as bioinformatics Tools. 15 structures were of interest as the following: Compound 1: Benzoic Acid, Compound 2: Coumarin, Compound 3:

3,3'-Dimethoxybenzidine, Compound 4: Cianidanol, Compound 5 : Hesperidin, Compound 6 : 6-Hydroxyflavone ,Compound 7 : 8-Methoxyflavone ,Compound 8 : Naringin, Compound 9 : Cinnamic acid, Compound 10 : Ferulic acid, Compound 11 : 4-Hydroxycinnamic acid, Compound 12 : Sinapic Acid, Compound 13 : Caffeic Acid, Compound 14 : Chlorogenic Acid and Compound 15 : Rutin. The three -dimensional configurations of crystalline structures of Bovine Serum Albumin BSA and Human Lipoxygenase LXO (PDBID: 3V03 and 4NRE respectively), were downloaded from Protein Data Bank in the PDB format. These protein structures were prepared using autodock, through the addition of polar hydrogen atoms and partial Kollman charges. Thus, the PDBQT files were ready. The energy minimization process of phytochemical compounds was carried out using Pyrx 0.8. Subsequently, the molecular docking of these compounds was carried out towards the two target receptors. The obtained molecular docking poses were viewed and analyzed using Discovery Studio Visualizer. The best conformations were chosen on the basis of the free energies' values obtained.

3 Results and discussions

3.1 Flavonoid assay

The results presented in the histogram (Figure 1) show that the total flavonoid contents vary considerably between the different extracts. The ethanolic extract recorded a maximum of flavonoids (12.82 mg/100g±0.00), followed by the dichloromethane extract which contained lower contents (6.47 mg/100g±0.03) and the aqueous extract by a content of 5.61 mg/100g ±0.00 While the lowest concentration of flavonoids was measured in the hexane extract (4.22 mg/100g±0.00). The amount of flavonoids in cannabis seed extracts depends on the polarity of the solvents used in the preparation of the extracts. Compared to Leonard, *et al.,* raw hemp seeds had a total flavonoid and polyphenol content lower than 2.85±0.05 mg GAE/g (Leonard et al., 2021). Compared to Pojic et al., determined higher levels of flavonoids and polyphenols 4.3-6.3 x 10-4 mg gallic acid/kg (Pojić et al., 2014). Most likely different marijuana strains, solvents, and extraction methods contributed to this difference in total phenols (Li *et al.,* 2020).

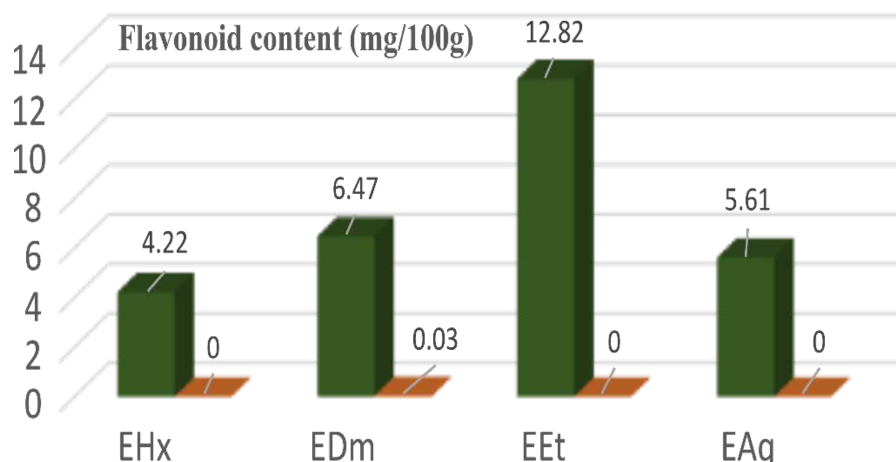

Fig. 1 Histogram of the total flavonoid content (mg/100g)

3.2 HPLC analysis of Cannabis sativa L.

The chemical composition of C. *sativa* L. extracts was performed. The chromatograms were presented at different wavelengths according to the families of secondary metabolites searched (Senior et al., 2012). The results of the analyses have been described in Table 1. The HPLC chromatograms have been interpreted based on the UV absorption bands of the families of secondary metabolites used to classify the metabolites of the different large families. In fact, the percentages of maximal absorption

across all wavelengths were used to attribute the polyphenol family. Table 1 shows eighteen phenolic compounds were identified and quantified in all extracts tested. Including 4-Hydroxybenzoic acid, Vanillic acid, Vanillin and Naringin are the major compounds found in the analyzed extracts. Naringin, 4-Hydroxybenzoic acid and cinnamic acid are the main compounds detected in an ethanolic extract with a content of 741.02, 421.8 and 88.86 mg/100g, respectively. Concerning the aqueous extract, the compounds 4-Hydroxybenzoic acid, Vanillic acid and Vanillin are the major compounds, and they presented the following values 494.15, 300.24, 652.38 mg/100g, respectively. Finally, the dichloromethane extract has modest polyphenol content compared to the other extracts. Vanillin and Quercetin 3-O-β-D-glucoside have great values (60.15 and 58.60 mg/100g, respectively). The primary antioxidants are thought to be phenolic compounds. These phenolic chemicals are also in charge of a number of the essential sensory characteristics of extracts, such as their bitter flavor and pungent sensation (Vuolo *et al.,* 2019). Babiker *et al.,* 2021 & Pojić *et al.* 2014) identified the presence of gallic acid, 3,4-dihydroxybenzoic acid, (+)-catechin, 1,2-dihydroxybenzene, and syringic acid as the major phenolic compounds in the extract of *Cannabis sativa* seeds, while other phenolic compounds were detected at lower levels (<0.74 mg/100 g). Kalinowska *et al.* have identified the primary phenolic compounds found in this plant seed, which include vanillic acid, ferulic acid, p-coumaric acid, (-) epicatechin, catechin, kaempferol, and procyanidin B2 (Kalinowska *et al.,* 2022). According to Flores-Sanchez and Verpoorte (Flores-Sanchez and Verpoorte 2008), approximately 20 flavonoids have been identified in cannabis, with the majority falling under the flavone and flavonol subclasses. These flavonoids include apigenin, luteolin, kaempferol, and quercetin in their O-glycoside versions, as well as unique methylated isoprenoid flavones called cannflavin A and cannflavin B, which are exclusive to cannabis (Ross et al., 2005).

Table 1. Results of the HPLC analysis of *C. sativa* L seeds extract

Sample (mg/100 g)	Retention time (min)	EDM	EET	EAq
Gallic acid	2.4	0.61	0.91	0.40
Hesperidin acid	4.07	0.45	0.45	26.66
4-Hydroxybenzoic acid	5.89	6.22	421.8	494.15
Caffeic acid	6.4	0.821	0.16	0.21
Syringic acid	6.72	1.29	2.20	1.60
Vanillic acid	6.91	4.1	3.84	300.24
Vanillin	7.11	60.15	57.65	652.38
p-Coumaric acid	8.15	0.24	0.44	0.92
Sinapic acid	8.41	18.61	52.66	0.41
Ferulic acid	8.46	0.09	14.22	0.29
Naringin	9.39	3.02	741.02	46.08
Quercetin 3-O-β-D-glucoside	9.6	58.60	3.45	0.12
Rutin	9.64	0.29	0.24	36.53
Salicylic acid	11.3	0.108	9.33	0.10
Quercetin	12.21	0.36	0.16	0.13
Cinnamic acid	12.4	0.12	88.86	31.59
Kaempferol	13.72	1.11	1.09	51.84
Chalcone	17.39	4.5	4.43	4.35

EDM: Dichloromethane extract, EET: Ethanol extract, EAq: Aqueous extract

Phenolic compounds are a type of plant compound that have antioxidant properties and are known to have a variety of health benefits. The exact chemical composition of *Cannabis sativa* seed extract can vary depending on a number of factors, including the strain of cannabis plant used, the extraction method, and the processing of the extract. also, the environmental factors, such as climate, soil conditions, and harvesting time, can also influence the chemical composition of the plant material. These factors can affect the levels of specific compounds in the plant, leading to variations in the chemical profile. The sample size and the statistical methods used to analyze the data can also play a role in the variation of the results.

3.3 Antioxidant activity by DPPH test

The results of the concentration of the extract that can reduce 50% (IC_{50}) of the DPPH of cannabis seeds are grouped in Figure 2: We can deduct from the results that the 50% reduction of DPPH was achieved by the ethanolic extract (0.36 mg/ml), aqueous extract (0.78 mg/mL), and ascorbic acid was taken as reference. This allows us to note that our extracts have a relatively very important antioxidant capacity. Comparing with the results of a study done by Ahmed et al., , we observe that the best DPPH inhibitions (%) was recorded by the aqueous extract and acetone ranged from 34.20±1.10 to 55.57±1.20 % (Ahmed et al., 2019). According to Pellegrini, et al., results of the evaluation of the antioxidant activity of hemp essential oils by the DDPH and FRAP test showed a very interesting antioxidant potential, with 63.38 ± 0.08 mg TE/g HEO for the DPPH test, and 438.52 ± 6.92 mg TE/g HEO for the FRAP test (Pellegrini et al., 2021). Other authors have shown that the antioxidant potential of essential cannabis oil in the DDPH and FRAP assays is low but higher in the ABTS assay (Benelli et al., 2018, Zengin et al., 2018, Nafis et al., 2019). So, we conclude that the antioxidant activity observed in our *C. sativa* L. seeds extracts is due to the presence of polyphenols in the extracts (ethanolic and aqueous). This is already confirmed by the phytochemical study of the organic extracts by HPLC/UV that we have done.

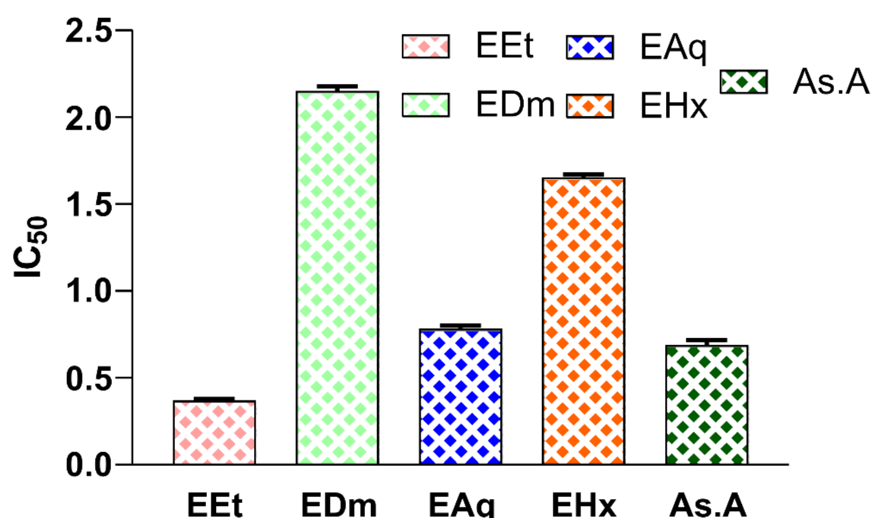

Fig. 2. IC50 of C. Sativa L seeds extracts (EAq: Aqueous extract EEt: Ethanolic extract; EDm: Dichloromethane extract; EHx: Hexane extract)

3.4 Antioxidant activity by the β-carotene decolorization technique

The antioxidant activity of C. *sativa* L. seed extracts was also evaluated by the beta-carotene bleaching assay as represented in Figures 3 and 4. Inhibition is assessed by tracking the decrease in absorption caused by beta-carotene bleaching over time. The results are expressed as a percentage of inhibition compared to the initial uptake. The results show that the best antioxidant activity was

obtained by the ethanolic extract and hexane extract with a percentage of 36% ±0.12 and 33% ±0.14, respectively, followed by the dichloromethane extract with a percentage of 3.52% ±0.19. Finally, the aqueous extract showed the lowest activity (1.73% ±0.20). The lowest activity was obtained by the aqueous extract with a percentage of 1.73% ±0.20.

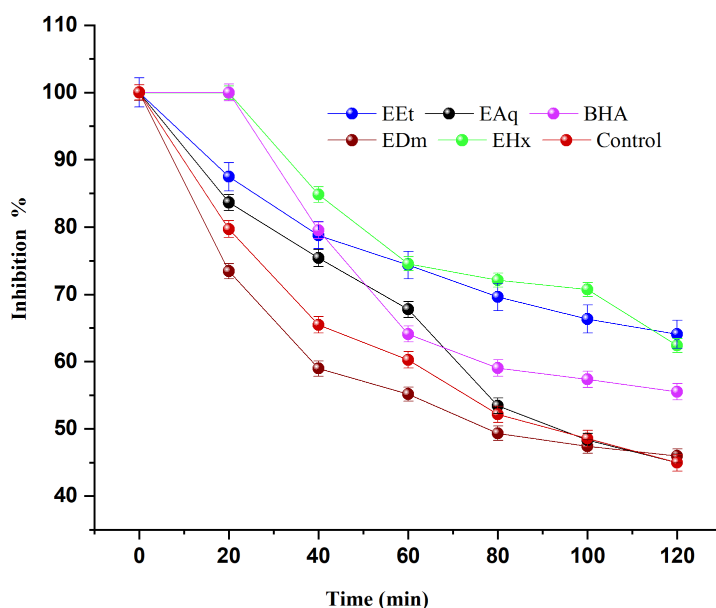

Fig. 3. Antioxidant activity of cannabis seed extracts compared to BHA and negative control

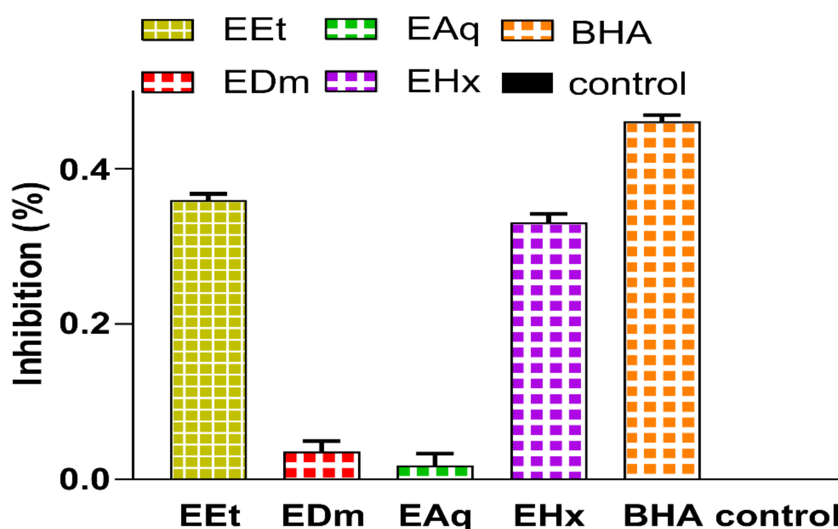

Fig. 4. Percentage of inhibition of the extracts compared to the initial absorption

According to the GC results, we found that the major component in our hexane extract is linoleic acid with a percentage of 42. 92%, and according to a study made by Ha's team on the Inhibition of benzo(a)pyrene-induced neoplasia of mouse stomach by conjugated dienoic derivatives of linoleic acid, showed that linoleic acid is more resistant to oxidation (Ha et al., 1990). so, we can see that linoleic acid in our hexane extract is responsible for this antioxidant activity. The ethanolic extract has already shown its power by the DPPH test, and its last results obtained by Beta-carotene bleaching confirm the previous results, and this antioxidant power present by the ethanolic extract of cannabis seed is due to the presence of an important quantity of total polyphenols (130 mg/100g).

3.5 Evaluation of antibacterial and antifungal activity

We studied the antibacterial activity of *C. sativa* L. seeds by applying the well diffusion method on MHA medium. The antibacterial activity was estimated in terms of the diameter of the inhibition zone around the wells containing the extracts to be tested against the bacterial and fungal strains. The results are presented in Tables 2 and 3.

Table 2. Antibacterial activity of different extracts of *C. sativa* L seed

Extract	***Gram-negative bacteria***		***Gram-positive bacteria***	
	Escherichia coli	***Pseudomonas aeruginosa***	***Staphylocoque aureus***	***Listeria monocytogenes***
EAq	12±0.01	10±0.02	12±0.02	08±0.01
EDm	14±0.01	10.50±0.01	16±0.01	08±0.02
EEt	15±0.02	09.2±0.02	18±0.02	7.90±0.02
EHx	12.50±0.01	11±0.02	12.50±0.01	07±0.02
T- DMSO	00±00	00±00	00±00	00±00
T+ Gentamicin IZ	21±1	25±1.70	25±1.20	25±0.60

EAq: aqueous extract, EAq: dichloromethane extract, EEt: ethanolic extract, EHx: hexane extract

Table 3. Antifungal activity of different extracts of *C. sativa* L seed

Extract	**Inhibition zone (mm)**			
	Penicillium sp	***Candida Albicans***	***Aspergillus niger***	***Rhodotorula sp***
EAq	12±0.02	13±0.01	13.00±0.01	7±0.01
EDm	13±0.01	16±0.02	14.00±0.01	7±0.02
EEt	**23**±0.02	16±0.02	18.50±0.01	9.50±0.01
EHx	10±0.01	12.50±0.01	14.20±0.02	9.50±0.01
T- DMSO	00±00	00±00	00±00	00±00
Cycloheximide	30.00±1.00	24.30±0.03	22.00±1.00	25.00±0.12

EAq: aqueous extract, EAq: dichloromethane extract, EEt: ethanolic extract, EHx: hexane extract

3.5.1 MIC of *Cannabis sativa* seed extracts

The diameters of the inhibition zones were determined using the well diffusion technique, while the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC) of the plant extracts under investigation were ascertained via the microdilution method (as shown in Tables 4 and 5). It has been proposed that plant extracts are deemed active if they present an inhibition zone diameter of ≥ 10 mm (Usman et al., 2009). The tested plant extracts demonstrated antimicrobial properties against all investigated bacterial and fungal strains, with inhibition zone diameters varying between 12 and 23 mm. The ethanol extract yielded the largest inhibition zone diameter (IZ = 23 mm) against Penicillium sp., while the smallest inhibition zone diameter (IZ = 07 mm) was recorded for the hexane extract against L. monocytogenes. According to the data, the microdilution results showed that the four extracts exhibited an inhibitory effect against all the bacterial and fungal tested strains with MIC values ranging from 0.03 to 1.25 mg/mL. Moreover, the results showed that the seed extracts of *C. sativa* L. showed bactericidal and fungicidal potentials with values ranging from 0.03 to 1.25 for both MBC and MFC. The Gram-positive bacterium *S. aureus* was found to be the most sensitive tested strain towards all the tested seed extracts while the fungi *Penicillium sp*. was the most resistant strain. The antimicrobial potential of the studied extracts increased order :

EET>EDm>EHx>EAq.

Table 4. Minimum inhibitory concentrations (MIC) and Minimum bactericidal concentration (MBC) of *C. sativa* L seed extracts against Gram-positive bacteria

Extract	Minimum inhibitory concentration and Minimum bactericidal concentration (mg/mL)			
	Gram-positive bacteria			
	Escherichia coli		*Staphylococcus aureus*	
	MIC	*MBC*	*MIC*	*MBC*
EHx	0.15±0.10	0.31±0.20	0.07±0.30	0.15±0.10
EDm	0.07±0.40	0.15±0.30	0.03±0.20	0.07±0.20
EEt	0.07±0.20	0.15±0.10	0.03±0.30	0.03±0.10
EAq	0.31±0.30	1.25±0.10	0.15±0.10	0.62±0.40

Table 5. Minimum inhibitory concentrations (MICs) and Minimum fungicidal concentration (MFC) of *C. sativa* L seed extracts

Extract	Minimum inhibitory concentration and Minimum fungicidal concentration (mg/mL)					
	Fungi					
	Penicillium sp		*Candida Albicans*		*Aspergilus niger*	
	MIC	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*
EHx	0.31±0.20	0.62±0.10	0.31±0.10	0.31±0.20	0.15±0.10	0.62±0.10
EDm	0.07±0.20	0.15±0.20	0.03±0.50	0.07±0.10	0.07±0.20	0.15±0.30
EEt	0.15±0.10	0.15±0.10	0.07±0.20	0.15±0.20	0.03±0.10	0.07±0.10
EAq	0.62±0.00	1.25±0.10	0.31±0.10	0.62±0.30	0.31±0.00	1.25±0.20

These results are in accordance with several investigations which reported that *Cannabis sativa* seeds extracts have a strong antimicrobial potential against a large range of microbes including multidrug-resistant bacteria and fungal strains (Isahq et al., 2015, Schofs et al., 2021). Moreover, additional studies proved that *C.sativa* extracts possess an inhibitory effect against Methicillin-resistant *S.aureus* (MRSA) and reduced the cell viability as well as the biofilm formation of *S.aureus* (Chakraborty et al., 2018, Frassinetti et al., 2020).Our findings may be explained by the chemical composition of the extracts which showed a high flavonoids content for the ethanolic extract followed by the Dichloromethane extract. Indeed, several studies have linked the antimicrobial activity of plant extracts to flavonoids, these latter are known to possess a remarkable antimicrobial potential by several mechanisms including the inhibition of the cytoplasmic membrane function, the inhibition of nucleic acid synthesis and the inhibition of energy metabolism (Tsuchiya and Iinuma 2000, Cushnie and Lamb 2005) .Furthermore, recent studies reported that among the 565 compounds that constitute the natural constituents of *Cannabis* species, 120 compounds belong to the cannabinoid class (ElSohly et al., 2017). Furthermore, numerous studies investigated the antimicrobial potency of cannabinoids. A recent study reported that the cannabidiol own an inhibitory effect of the release of the bacterial membrane vesicles from Gram-negative and Gram-positive bacteria (Kosgodage et al., 2019, Feldman et al., 2020). Reported that various cannabinoids have remarkable antimicrobial activity against Gram-positive pathogens particularly the MRSA isolates and their biofilms, potentially in synergy with antibiotics especially gentamicin and ampicillin. According to Marika Pellegrini et al., *C. sativa* L. seed oil was effective against *Listeria monocytogenes* strains ATCC 19114 and LM4 with a minimum inhibitory concentration value of 2.50 mL/mL. For *Staphylococcus aureus* strains St 32, St 47 and St 39, the minimum inhibitory concentration values were 5 mL/mL, 1.25mL/mL and 2.50mL/mL

respectively (Pellegrini et al., 2020). Zengin et al., showed that the minimum inhibitory concentration values for Gram-positive strains were generally more sensitive to essential oils than Gram-negative strains (Zengin *et al.,* 2018). Claudet *et al.,* confirmed that this resistance of Gram-negative bacteria to essential oils is common and is attributed to their hydrophilic permeability barrier of lipopolysaccharide in the outer membrane, which provides protection against highly hydrophobic molecules (Claudet *et al.,* 2017).

3.6 Anti-inflammatory activity of C. sativa L. seeds

According to the results obtained (Figures 5 and 6), it turned out that the various extracts of C. *sativa L* exhibited a dose-dependent inhibitory activity against the denaturation of BSA proteins, namely the dichloromethane extract (IC_{50}=107.73 mg/mL), the ethanolic extract (IC_{50}=93.09 mg/mL), the aqueous extract (IC_{50}=139.93 mg/mL), but relatively low in comparison with the reference anti-inflammatory drug (IC_{50}=62.19 µg/mL). The different extracts of C. *sativa L* seeds studied showed a significant inhibitory power against erythrocyte hemolysis induced by heat/hypotonicity. The results obtained from Figures 5 and 6 show a dose-dependent inhibition of the various extracts against the hemolysis of the red blood cells prepared. heat-induced hemolysis was inhibited by the various dichloromethane, ethanolic and aqueous extracts with IC_{50}s of 75.55 mg/mL, 80.81mg/mL and 89.30µg/mL respectively, in comparison with ibuprofen (IC_{50} =59.50). While the erythrocyte hemolysis induced by hypotonicity was inhibited by the different dichloromethane, ethanolic, aqueous extracts presenting IC_{50} of 73.54 mg/mL, 66.26mg/mL and 107.32 µg/mL respectively, in comparison with the 'Ibuprofen (IC_{50}=55.75). The study and evaluation of the anti-inflammatory activity was carried out according to the in vitro method of stabilization of the RBC membrane, whose hemolysis was induced by two agents: heat or Hypotonicity. The different extracts of C. *sativa L* seeds growing in Morocco showed a considerable inhibitory effect against erythrocyte hemolysis induced by both heat and hypotonicity in a dose-dependent manner, probably explained by the ability of these extracts to effectively protect the membrane of rat red blood cells. This activity can be attributed to the presence of bioactive molecules, such as Hesperidin and Naringin which showed the highest affinity values towards the two studied proteins LXO and BSA respectively.

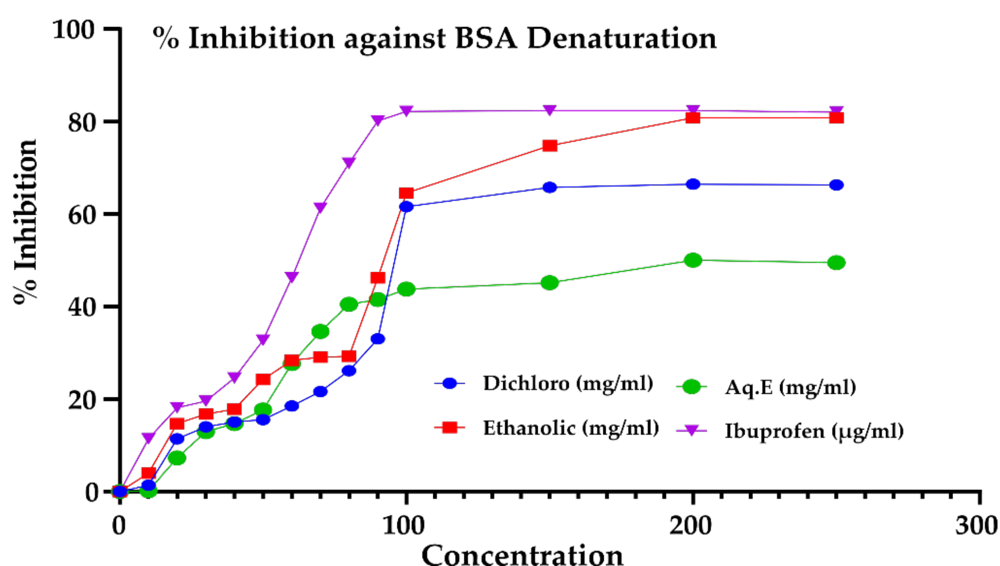

Fig. 5. % Inhibition of *C. sativa* L extracts against BSA protein denaturation in comparison to Ibuprofen

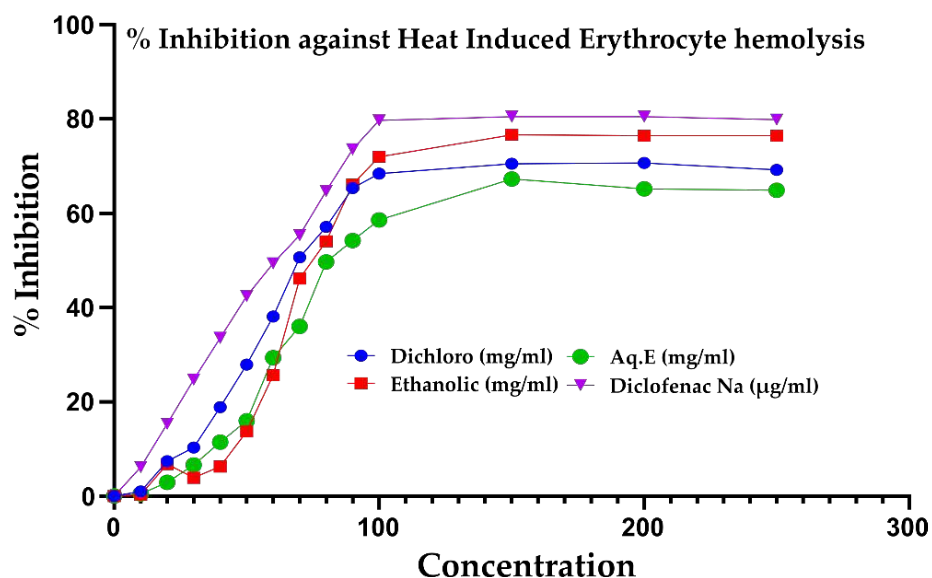

Fig. 6. % Inhibition of *C. sativa* L extracts against Heat induced RBC denaturation

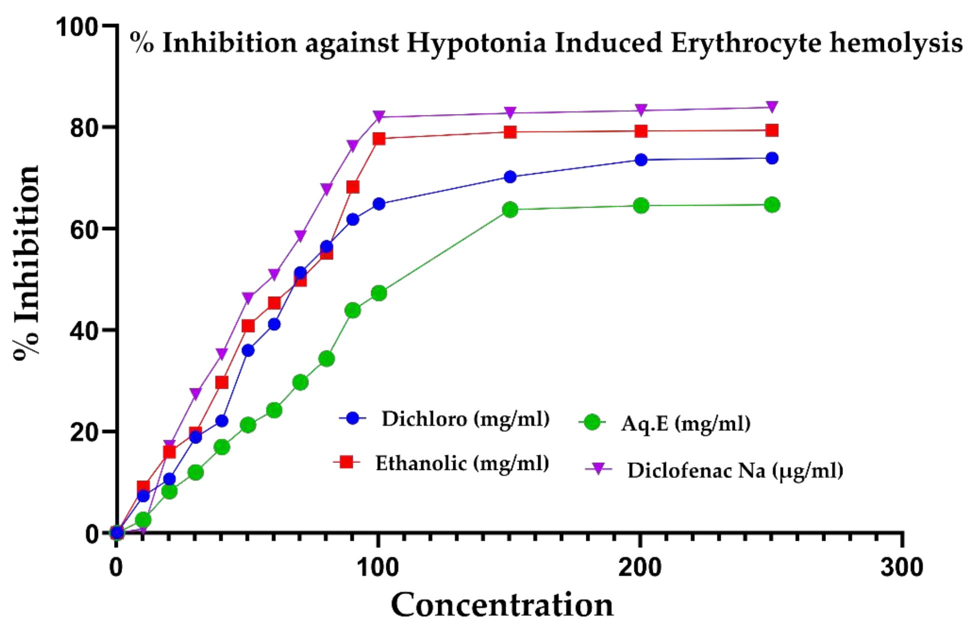

Fig. 7. % Inhibition of *C. sativa* L extracts against Hypotonia induced RBC denaturation

3.7 Molecular docking studies

The evaluation of the results of the anti-inflammatory activity of the extracts was carried out through bioinformatics outlines allowing the clarification of atomistic actions mechanisms. For this goal, molecular marking has been used against receptors widely described in the literature; Bovine Serum Albumin (BSA) and lipooxygenase (LXO) (Pdbid. 3V03, 4nre respectively). The results of molecular docking of phytochemicals against BSA and LXO are shown in Tables 6 and 7. It is clearly indicated that compound 5: Hesperidin (PCID: 10621), compound 8: Naringin (PCID: 442428) and compound 15: Rutin (PCID: 5280805) showed the highest affinities towards the active sites of BSA and LXO, and even better than the co-crystallized ligands used in this study (-3.3 and -5 Kcal/mol respectively). The binding

energies with the active site of BSA were estimated as -9.1 Kcal/mol for Hesperidin, -9.5 Kcal/mol for Naringin and -8.7 Kcal/mol for Rutin. For LXO, the binding energies of Hesperidin, Naringin and Rutin were estimated by -10.4 Kcal/mol, -9.6 Kcal/mol and -9.3 Kcal/mol respectively (Scheme 1).

Table 6. Proteins selected for molecular docking, presented with free binding energy values with co-crystallized ligands

Protein Name	PDB ID	Grid Box Center	Grid Box Size	Reference Drug Structure	Free Binding Energy ΔG (kcal/mol)
Bovine Serum Albumin	3V03	Center-X=66.284 Center-Y=29.969 Center-Z=40.184	Center-X=40 Center-Y=40 Center-Z=40		ΔG= -3.3
Human Lipooxygenase	4NRE	Center-X=-14.206 Center-Y=-55.964 Center-Z=-19.495	Center-X=40 Center-Y=40 Center-Z=40		ΔG= -5

Table 7. Binding free energy of phytocompounds from *C. sativa* L seeds, values are expressed in (kcal/mol)

Compound	Binding Free Energy ΔG (kcal/mol)	
	Bovine Serum Albumin	**Human Lipooxygenase**
1 (PCID : 243)	-5.6	-5.8
2 (PCID : 323)	-6.9	-6.2
3 (PCID : 8411)	-7.5	-6.4
4 (PCID : 9064)	-7.8	-7.4
5 (PCID : 10621)	-9.1	-10.4
6 (PCID : 72279)	-8.8	-8.3
7 (PCID : 213728)	-8.3	-7.9
8 (PCID : 442428)	-9.5	-9.6
9 (PCID : 444539)	-6.5	-5.8
10 (PCID : 445858)	-6.4	-6.7
11 (PCID : 637542)	-6.4	-6.3
12 (PCID : 637775)	-6	-5.8
13 (PCID : 689043)	-6.4	-6
14 (PCID : 1794427)	-7.3	-7.9
15 (PCID : 5280805)	-8.7	-9.3

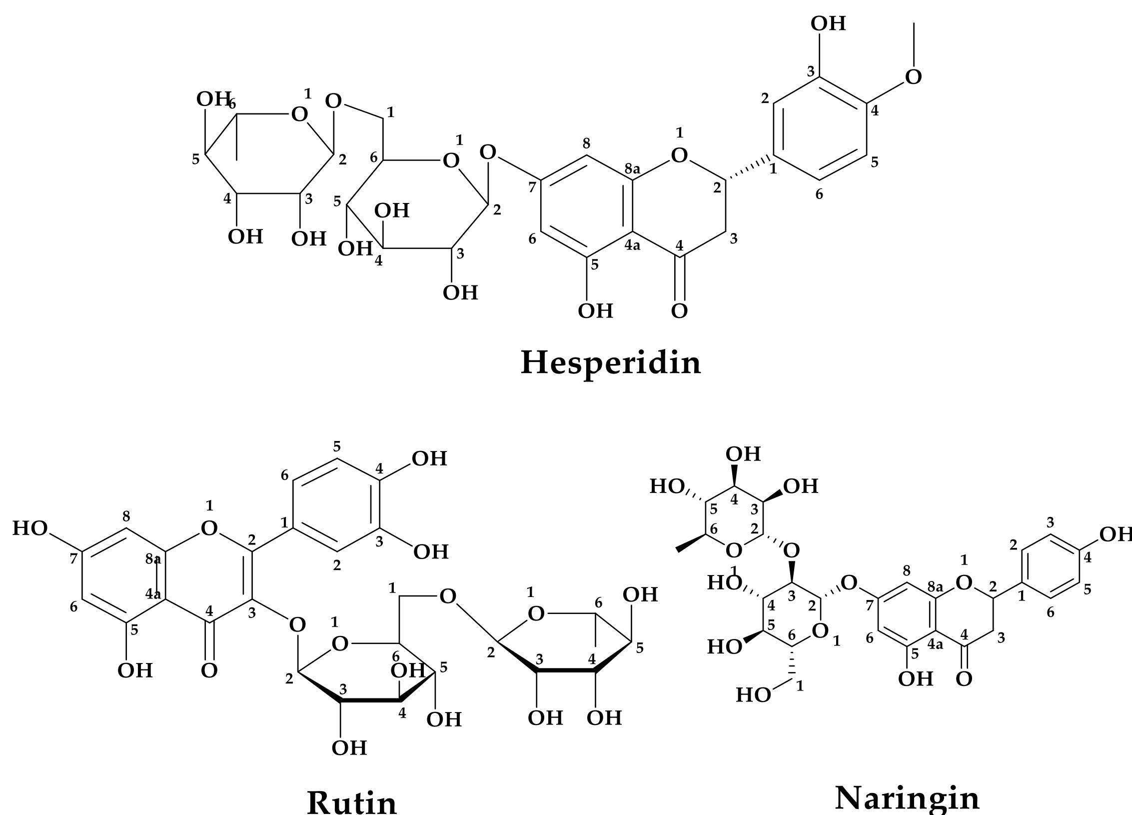

Fig. 8. Molecular structure of the three major compounds

The docking molecular analyzes were carried out with the aim of clarifying the interaction modes of the first three compounds exhibiting the highest affinities towards target receptors, thus explaining the mechanism of action performed by these phytochemical molecules (Tables 9 & 10). Generally, Conventional Hydrogen Bonds, Carbon Hydrogen Bonds, and Alkyl Bonds were the major interactions forming the molecule-protein complexes. Naringin who showed the highest inhibition power towards the active site of the BSA, has recruited several residues of amino acids through solid interactions essentially of the conventional hydrogen bond type (LEU112, LEU115, ARG144, ARG185, LEU189), carbon hydrogen bonds (LYS114, HIS145), Pi-Akyl (PRO110, VAL188), and Pi-Sigma (ILE141). The two compounds Hespiridin and Rutin approximately recruited the same amino acids as those who interact with Naringin such as LEU112, LEU115, ARG144, ARG185, LEU189, LYS114, HIS145 and PRO110. While it was Hespiridin who executed the best affinity towards the active site of LXO, thus forming interactions of the Conventional Hydrogen Bonds type (TRP109, GLU141, ARG145, ASN173, His394, ARG407), Alkyl (Val117), and one an unfavorable Donor-Donor with GLN137. Hespiridin has approximately recruited the same amino acids engaged in the interaction of protein with the co-crustallized ligand such as ASN173, His394 and arg407. Narinjin and Rutin who showed affinity values of -9.6 and -9.3 kcal/mol respectively recruited common amino acids with Hespiridin such as Arg145, ASN173, His394, ARG407.

The molecular mooring of phytochemical compounds extracted from *C. sativa* L. seeds has been made towards Bovine Serum Alumin and Lipooxygenase, which are proteins widely described in scientific literature. The results clearly show the presence of bioactive compounds with significant inhibitory power towards the active sites of the cressing proteins. Hespiridine, Naringin and Rutin were the compounds presenting the values of the most faile liaison energies, thus, the most important inhibitory activities among all the phytochemical compounds studied, which far exceeded the inhibitory activities of co-Crystallized ligands.

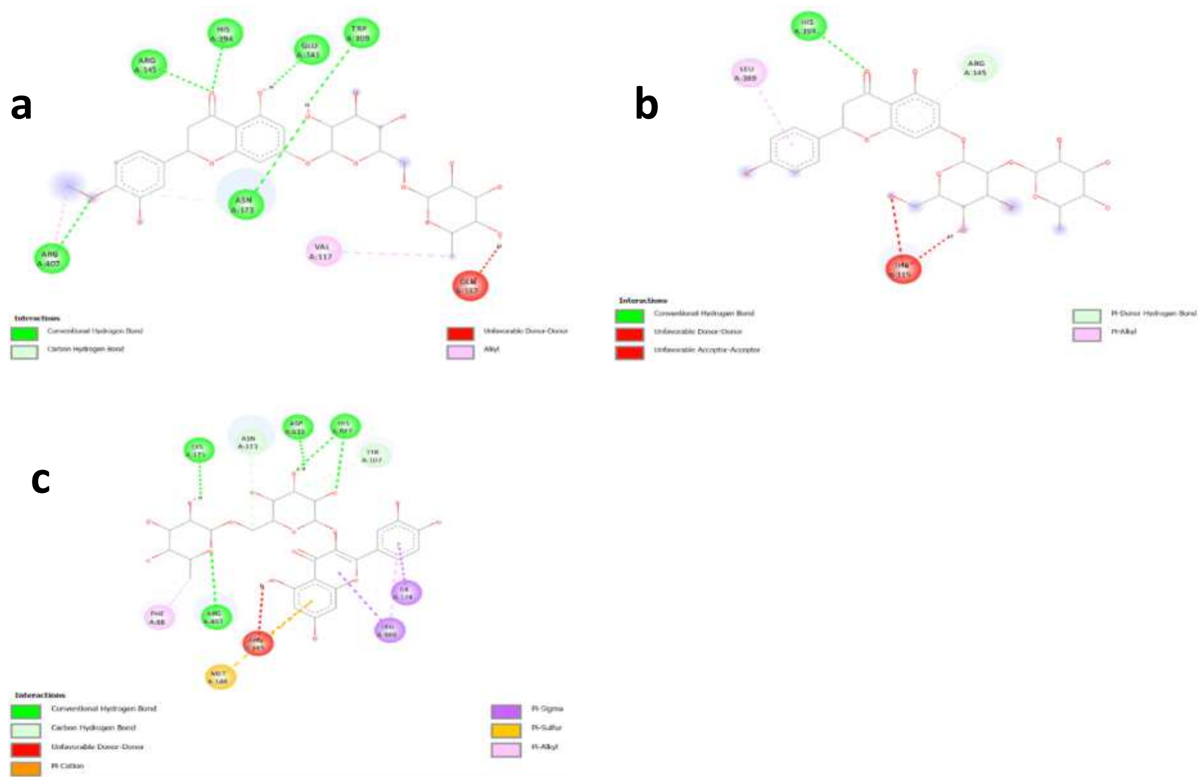

Fig. 9 2D-representations of the chemical bonding modes of the complexes formed between A. Hesperedin, B. Naringin, & C. Rutin compounds and the catalytic site residues of human lipooxygenase (PDBID: 4NRE)

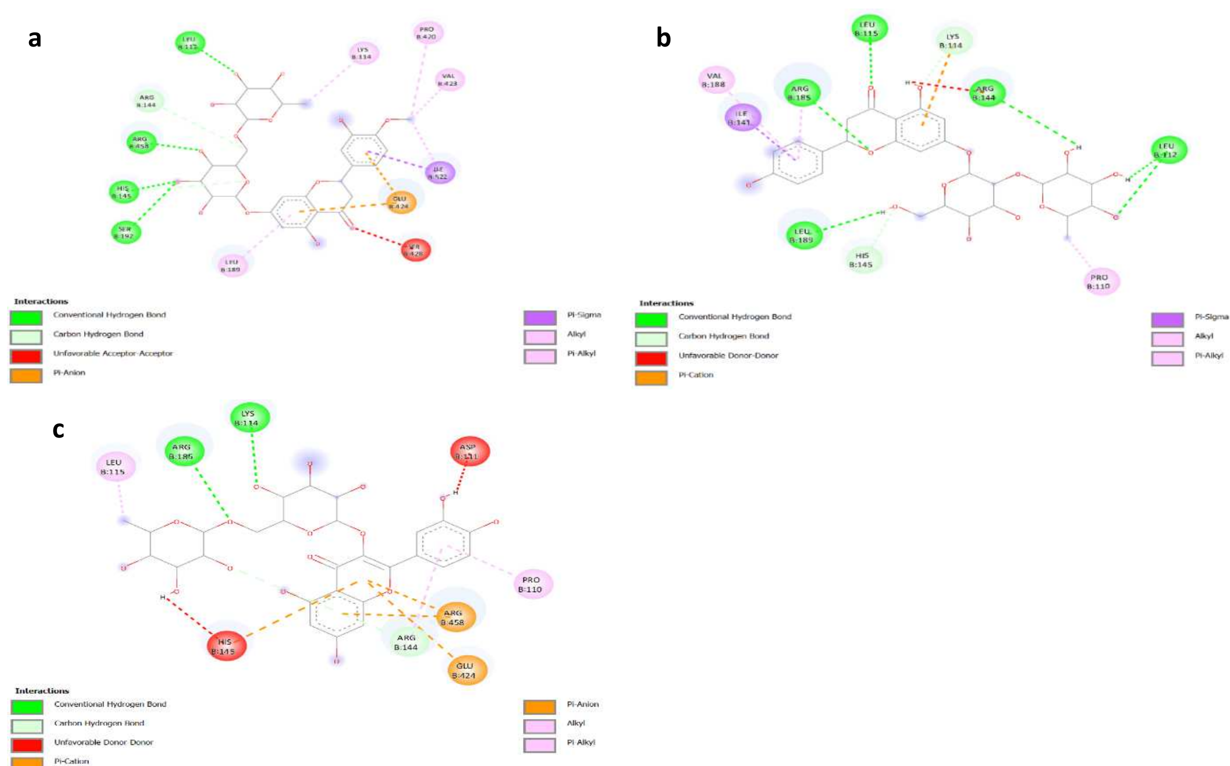

Fig. 10. 2D-representations of the chemical bonding modes of the complexes formed between A. Hesperedin, B. Naringin, and C. Rutin compounds and the catalytic site residues of Crystal structure of Bovine Serum Albumin (PDBID: 3V03)

Hydrogen, hydrophobic, and electrostatic links were the main interactions described in the formation of Ligand-Protein complexes with these three phytocomposed. This could explain the great stability of the trained complexes by comparing them to the Co-Crystallized Ligands references. Based on these results, we can conclude the coherence between the results in silico and those in vitro of the study of anti-inflammatory activity. In addition, they demonstrate the presence of natural origins promoters' compounds, capable of providing effective inhibition of BSA and LXO.

Conclusion

This research aimed to investigate the antioxidant, antimicrobial, and anti-inflammatory properties of various extracts derived from *Cannabis sativa* L. seeds. The investigation revealed that a variety of extracts obtained from *cannabis* seeds possess the capacity to effectively neutralize the 2,2-diphenyl-1-picrylhydrazyl radical and exhibit antimicrobial characteristics against a spectrum of bacterial and fungal strains. These extracts showcased significant bactericidal and fungicidal effects, with BMC and FMC values spanning from 0.03 to 1.25. The research findings, in conjunction with the evaluation of the anti-inflammatory activity of *Cannabis sativa* L. seeds, demonstrated that the diverse extracts under examination exhibited considerable inhibitory properties against heat/hypotonia-induced erythrocyte hemolysis. The results of this study may serve as a foundation for further investigations into *Cannabis sativa* L. seeds and their potential applications, given their demonstrated strong antioxidant capacities and promising antimicrobial potential. This potential is attributed to the presence of polyphenols and flavonoids, as revealed in the phytochemical analysis. The noteworthy antioxidant, antimicrobial, and anti-inflammatory properties of *Cannabis sativa* L. seeds propose their potential incorporation in food processing, pharmaceutical, and cosmetic industries, potentially providing added value.

Declarations

"Ethical approval Not applicable."

"**Competing interests** "The authors of this work declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper."

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"**Availability of data and materials** All data used to support the finding of our study are available from the corresponding author upon request."

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