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Research Article

Acute oral toxicity of *Cannabis sativa* L. co-products in mice

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Abstract

Cannabis sativa L. commonly known as 'Kif', is a medicinal plant belonging to the Cannabaceae family (*Cannabis* genus). It is used in traditional folk medicine to treat several diseases. The recent legalization of cannabis use for medicinal, cosmetic, and industrial purpose in Morocco (law 13.21), has enabled us to assess the potential acute toxicity of extract from its female plant co-products in Swiss mice. In this experimentation, 3 different extraction methods (maceration, decoction, and sonication) were used to determine the efficacy of cannabis extracts and investigate their acute toxicity in mice over a period of 14 days. Decoction showed the highest efficacy (19.7%), followed by maceration (16.2%), and finally sonication (5.4%). Regarding the toxicity results of the oral gavage administration dose at 2,000 mg kg^{-1}, there were no recorded deaths and no changes in the internal organs (kidneys and liver) between treated and control groups of both male and female mice. We also found that body weight of female mice treated by decoction and maceration extracts decreased significantly compared to those treated with sonication extract throughout the experimental period, when treated and untreated male mice show no significant changes (p-value < 0.05). Our findings suggest that the LD_{50} may be greater than 2.000 mg kg^{-1}. Nevertheless, the extracts showed signs of low-level toxicity such as drowsiness, hypoactivity, and tachycardia. Further studies to determine the phytochemical composition of the three extracts of *C. sativa* and assess the chronic and sub-chronic toxicity should be carried out.

Keywords: acute toxicity; *Cannabis sativa* L.; co-products; efficacy; extraction methods

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Introduction

Cannabis sativa L. is a dicotyledon plant that belongs to the Cannabaceae family, cannabis genus, and sativa species (Vastolo *et al*., 2021). *C. sativa* species are commonly known as cannabis or hemp and are among the first domesticated plants. They probably originated in Central Asia around 12,000 years ago and subsequently spread over the world (Crocq, 2022). The cannabis plant has long been cultivated for its fibers, seeds, and psychotropic properties. It has also been used for its numerous qualities that allow a wide range of applications, particularly in agriculture, cosmetics, textiles, food, papermaking, biofuels, biocomposites, and building (Crini *et al*., 2020; Adesina *et al*., 2020; Babiker *et al*., 2021; Rehman *et al*., 2021; Visković *et al*., 2023).

During the last decades, special attention has been paid to cannabis plant since it contains a variety of bioactive compounds with therapeutic effects (Gugliandolo *et al*., 2018). It is also rich in natural antioxidants and other bioactive substances including peptides, phenolic compounds, tocopherols, carotenoids, and phytosterols (Farinon *et al*., 2020). For instance, cannabidiol, a non-psychotropic compound derived from *Cannabis sativa* L., possesses antioxidant and anti-inflammatory properties, as well as neuroprotective, anxiolytic, and anticonvulsant characteristics (Izzo *et al*., 2009; Pellati *et al*., 2018). Potential applications of cannabidiol (CBD) in inflammation, diabetes, cancer, emotional and neurodegenerative illnesses have recently emerged, and its useful actions in the treatment of epilepsy and obesity are promising (Izzo *et al*., 2009). However, cannabidiol may cause adverse effects and toxicity due to its potency, route of administration (vaporized, transdermal or oral), concurrent use of licit and illegal drugs, and potential drug-drug interactions (Huestis *et al*., 2019).

Furthermore, other secondary bioactive compounds derived from cannabis such as polyphenols, terpenes and flavonoids exert beneficial functions in living organisms (Cavalli and Dutra, 2021). Tetrahydrocannabinol (THC), the principal psychoactive component in the cannabis plant, has also several medical properties, such as easing nausea and vomiting, stimulating appetite, as well as reducing pain (EMCDDA, 2019).

Thus, either in their natural or synthetic states, cannabinoids are included in pharmaceutical formulations of several drugs, including Cesamet, Marinol, Sativex, and Epidiolex (EMCDDA, 2019). However, THC use may lead to adverse effects and toxicity (Schep *et al*., 2020). The severity and duration of symptoms are proportional to the blood THC levels. After acute use, THC remains in the blood for many hours before being converted to carboxylic acid, which is distributed to fat and then detected in urine for several weeks after consumption. Acute intoxication treatment consists primarily of symptom-based supportive care. Cannabis toxicity can cause convulsions and unconsciousness in children who may require supportive care for this potential symptom (Schep *et al*., 2020).

As in many countries around the world (Manthey *et al*., 2021; Hall, 2015), the cannabis-attributable burden of disease is one of the public health concerns in Morocco. The existing literature shows the causal relationship between nonmedical cannabis use and/or dependence and the onset and/or worsening mental disorders among adults in Morocco (WHO, 2016; Ouanouche *et al*., 2022) and elsewhere (WHO, 2018). Moreover, the Moroccan Poison Control and Pharmacovigilance Center received 616 cannabis-related reports in 2015. Cannabinoid toxicity was recorded in 119 (19.3%) of these cases, including 35 cases of acute toxicity requiring hospitalization and 3 cases of unintentional toxicity in children aged 18 months to 3 years. The remaining cases were linked to long-term cannabis use (Centre Anti Poison et de Pharmacovigilance du Maroc, 2015). Thus, the toxicity and adverse effects of cannabis-derived phytochemicals should be further investigated to have enough data for a rational clinical application.

In Morocco, the extraction of resin, commonly called 'Hashish' which is rich in THC (UNODC, 2022), has been traditionally performed using the process of beating the plant's aerial parts, mainly leaves and female flowering tops. This process generates residues that can be used in a variety of applications. For example, the produced biomass, known as 'Kif' is usually consumed for its psychoactive effects through smoking. Other uses

of this biomass in human agri-food industry (Brownies, cookies, candies, etc) have also been reported (Wang *et al*., 2020; Bailoni *et al*., 2021; Valizadehderakhshan *et al*., 2021; Vastolo *et al*., 2021). However, the short and long-term toxicity of *C. sativa* has been largely sighted by numerous authors. It is mainly caused by the ingestion or inhalation of cannabis-based products (Noble *et al*., 2019).

To our knowledge, the toxicity of 'Kif' used in smoking or in animal or human feeding has not been sufficiently documented. Its use depends on its quality and the good Kif is derived from the female flower tops and their bracts, which are then dried and ground. For its psychoactive use through smoking, its quality also depends on the length and material of the Sebsi (traditional Moroccan pipe) and the material of which it is made. These two factors are important in reducing the harmfulness of the smoke (Bellakhdar, 1997; Merzouki *et al*., 2001; Afsahi, 2017). The purpose of this research was to assess the efficacy of different extracts from *Cannabis sativa* L. co-products, and their acute toxicity in Swiss mice.

Materials and Methods

Sampling and plant material

The plant's botanical identification was completed at the Laboratory of Botany, Faculty of Sciences in Rabat. The voucher number assigned to the tested plant by the Scientific Institute of Rabat is RAB 112735.

The staff of the National Agency for Medicinal and Aromatic Plants in Taounate collected the samples of female cannabis plants co-products from Douar Rkaiba in Taounate region, Morocco, 34°43'55.6N and 4°52'01.8W. These samples were dried for 72 h at 40 °C before being processed into a coarse powder (250 µm) with a suitable grinder. The powder obtained has been stored in an airtight container and kept in a cold, dark, and dry place until the analysis.

Preparation of extracts and extraction efficacy

Maceration: 10 g of plant powder was macerated in 100 ml of ethanol for 24 h at room temperature with electric stirring. After vacuum filtration, the extracted materials were recovered. The solvent was subsequently removed by evaporation in a rotary evaporator at low pressure, and the resulting extract was freeze-dried. Before administration, the extract was dissolved in distilled water.

Sonication: 10 g of plant powder was mixed with 100 ml of ethanol and placed in a sonicator set to 35 Hz at room temperature for 45 min. The solvent was subsequently removed by evaporation in a rotary evaporator at low pressure, and the resulting extract was freeze-dried. Before administration, the extract was dissolved in distilled water.

Decoction: 10 g of plant powder was mixed with 100 ml of distilled water and boiled for 15 min then filtered through a Whatman paper. The filtrate was collected, and the solvent was evaporated using a rotary evaporator set to 40 °C to obtain an extract which was then freeze-dried. Before administration, the extract was dissolved in distilled water.

All extracts were stored in an airtight container and kept in a cool, dark, and dry place until analysis.

The extraction efficacy of *C. sativa* co-products obtained by the three extraction methods (maceration, decoction, and sonication) is determined by the following formula:

$$\text{Percentage efficacy} = \frac{\text{Extract weight obtained} \times 100}{\text{Total plant matter weight}}$$

This parameter is important for estimating the raw-material amount required for the components' extraction and selecting the most effective extraction method.

Animals

Different *C. sativa* co-product extracts were tested for acute toxicity in adult male and female Swiss mice (28-34 g, 1.5 to 2 months of age) bred in the animal house of the Faculty of Sciences, Mohammed V University in Rabat, Morocco. The animals were kept in cages at room temperature with a light/dark cycle of 12:12 h. They were given a pelleted foods and tap water *ad libitum*. All tests were carried out with strict adherence to the ethical guidelines for investigation of experimental pain in conscious animals (Zimmerman, 1983).

Acute toxicity

The acute toxicity test to determine the cause-effect relationship, the nature of any acute effects, and the lethal dose (LD_{50}) was performed using a single dose of each extract. Our protocol was based on the chemical testing guidelines of the Organization for Economic Cooperation and Development, which were adopted on October 3, 2008, and updated on June 30, 2022 (OECD, 2022). For the acute toxicity assessment, a dose of 2,000 mg kg^{-1} of the extract is indicated in these guidelines.

Before oral gavage administration of the extracts, the animals were fasted for 3 to 4 h. The mice were weighed, marked on the tail, and divided into the 4 groups based on weight homogeneity (1 untreated control group (NT) and 3 treated groups (T)):

- Group NT (control): 10 mice (5 males and 5 females) were given simply distilled water.
- Group T (Decoction): 10 mice (5 males and 5 females) were given the decoction extract.
- Group T (Maceration): 10 mice (5 males and 5 females) were given the maceration extract.
- Group T (Sonication): 10 mice (5 males and 5 females) were given the sonication extract.

After each extract administration, the feeding was stopped for 1.5 h and mice were observed continuously for the first 4 h daily over 14 days. During this period, any unusual sign that occurred was noted, including changes in coat, motility, tremors, grooming, respiration, stool appearance, mobility, body weight changes, and death.

Body weight of untreated (NT) and treated (T) mice of both sexes was measured every two days for 14 days (D0, D2, D4, D6, D8, D10, D12, D14).

After the testing period, the mice were sacrificed, and their organs (liver and kidneys) were removed, weighed, and macroscopically examined.

Statistical analysis

GraphPad Prism 8th Version software was used to analyze the data. Our data were expressed as mean ± standard error of mean (SEM) for continuous variables and as percentage for categorical variables. The One-Way ANOVA Repeated Measures test was performed to compare value of various groups. A P-value < 0.05 indicates that the difference is statistically significant.

Results

Extraction efficacy

The extraction efficacy obtained using different extraction methods is shown in Figure 1. The decoction extract (19.7%) has the best efficacy, followed by maceration (16.2%) and sonication (5.4%).

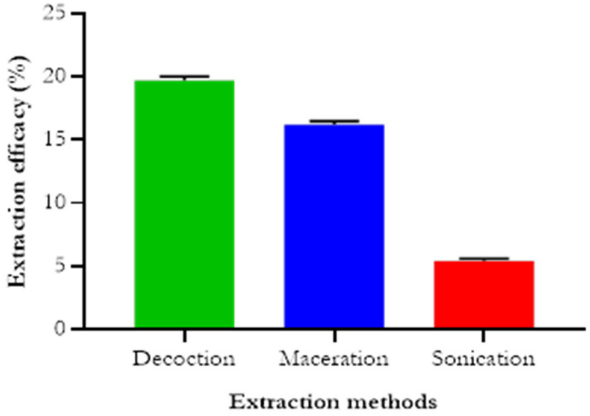

Figure 1. Extraction efficacy obtained by the decoction, maceration, and sonication methods

Body weight

As change in weight may be a sign of toxic effects, a 14-day follow-up was used to determine body weight after extracts' administration. Figure 2 shows that the mean body weight of treated female mice in the T(Decoction) and T(Maceration) groups decreased significantly ($p < 0.05$) compared to that of treated mice with the sonication extract. Figure 3 shows that change in body weight in male mice was non-significant throughout the experimental period in both untreated and treated groups.

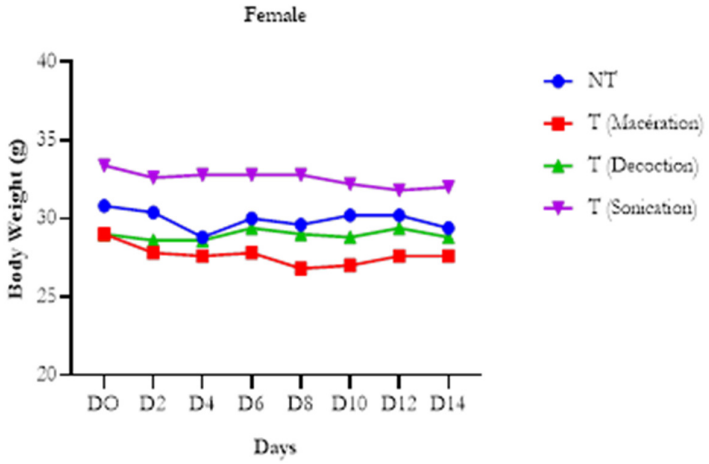

Figure 2. Changes in body weight of untreated and treated female mice throughout the experimental period

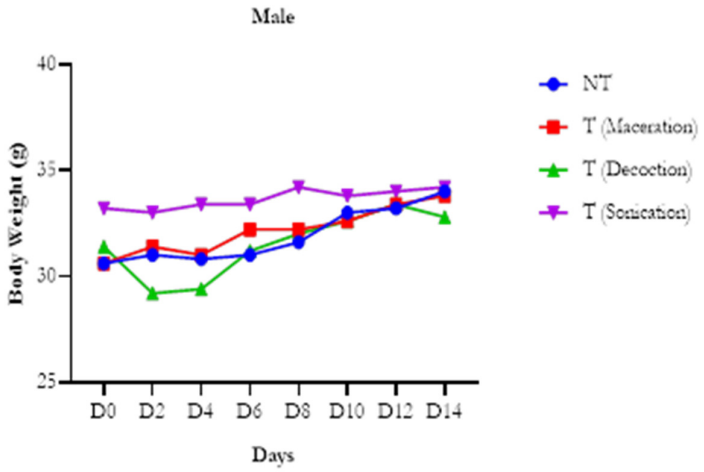

Figure 3. Changes in body weight of untreated and treated male mice throughout the experimental period

Compared to the baseline body weight, our results showed a significant weight loss in female mice of the untreated (NT), T(Maceration) and T(Sonication) batches. with a decrease of 4.5 ± 1.6 %, 4.8 ± 2.3 % and 4.2 ± 0.7 %, respectively (Figures 4 and 6). However, in male mice, a significant body weight gain ($p < 0.05$) was observed in both NT and T(Maceration) groups. with an increase of 10.9 ± 2.8 % and 10.3 ± 3.1 %, respectively (Figures 5 and 6).

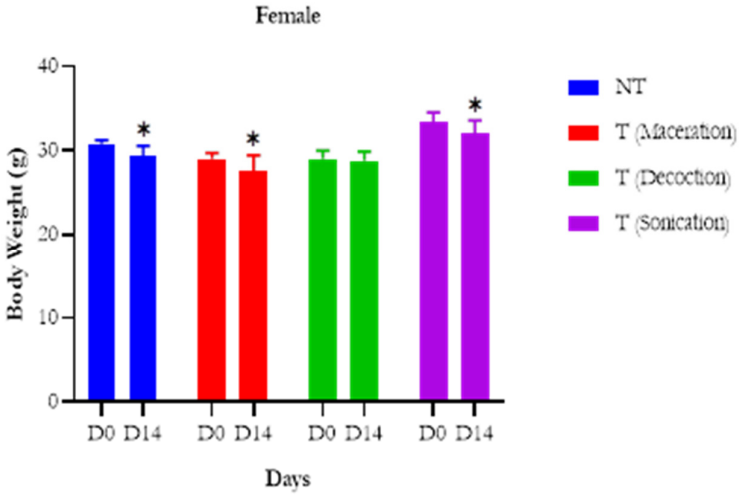

Figure 4. The average body weight variation of untreated and treated female mice throughout the experimental period

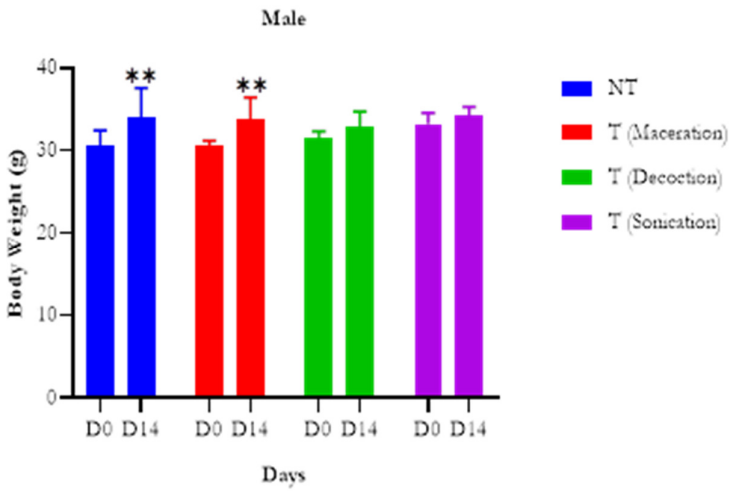

Figure 5. The average body weight variation of untreated and treated male mice throughout the experimental period

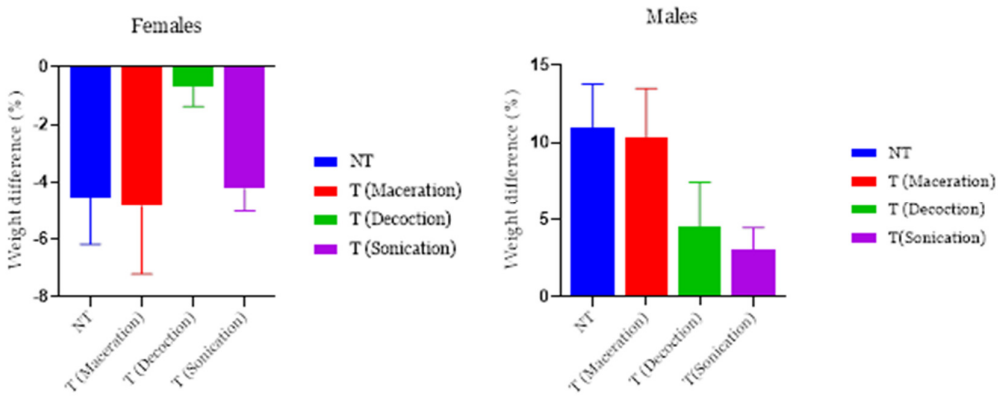

Figure 6. Difference in mean body weight of treated and untreated mice of both sexes between the first day and the 14th of the experiment (in %)

Acute toxicity signs (in vivo) of Cannabis sativa L.

The extracts of *C. sativa* administered by gavage at the highest dose of 2.000 mg kg^{-1} caused certain clinical signs of toxicity in treated groups (Table 1). It should be noted that all signs were observed during the first 4 h following extract administration and throughout the experimental period (14 days). They were evaluated in treated groups compared with untreated groups.

During the first two hours following the extracts administration, some clinical toxicity signs were observed in both males and females. We noted somnolence, isolation, and a decrease in spontaneous mobility in almost all treated mice, except males treated with maceration extract. Mice of both sexes treated with maceration extract experienced bradycardia. Additionally, tachycardia and tremors were observed in some mice treated with sonication and decoction extracts.

Polyuria, diarrhea, and a change in feces color were noted within the first three days following the administration of decoction extract, but then these signs disappeared.

Throughout the experiment, no deaths were observed in either males or females treated with the three extracts studied, suggesting that the LD_{50} may be greater than 2.000 mg kg^{-1} body weight.

Table 1. Clinical signs observed in mice after administration of *Cannabis sativa* L. extracts

Symptoms	NT Mice		T Mice					
			$T_{(Decoction)}$		$T_{(Maceration)}$		$T_{(Sonication)}$	
	♂	♀	♂	♀	♂	♀	♂	♀
Hypoactivity	-	-	+	+	+	+	+	+
Drowsiness	-	-	+	+	+	+	+	+
Bradycardia	-	-	-	-	+	+	-	-
Tachycardia	+	+	+	+	-	-	+	+
Tremors	-	-	+	+	-	-	+	+
Isolation	-	-	+	+	-	+	+	+
Grouping	-	-	-	-	+	-	-	-
Diarrhea	-	-	+	+	-	-	-	-
Polyuria	-	-	+	+	-	-	-	-

♂: male mice; ♀: female mice; +: presence of signs; -: absence of signs

Macroscopic observation of organs

Macroscopic observation of the liver and kidneys of mice treated with the three *C. sativa* extracts showed no significant differences compared to those of untreated mice. The liver (L) and kidneys (K) of all groups showed no visible abnormalities under the naked eye (Figure 7).

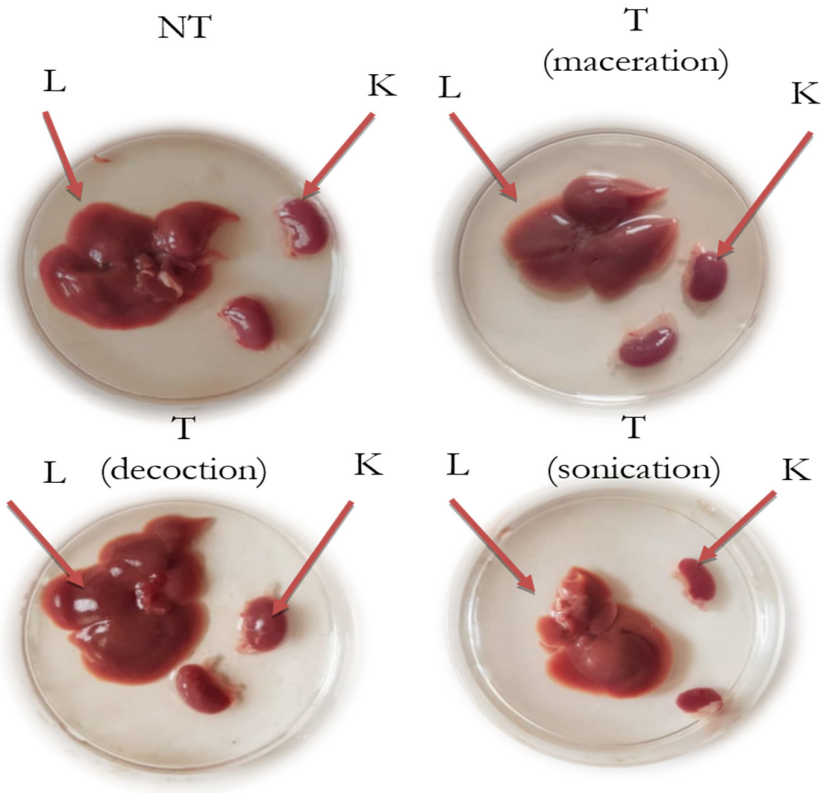

Figure 7. Macroscopic appearance of the organs of untreated and treated mice by co-product *Cannabis sativa* L. extract

Discussion

Hemp contains more than 500 chemical components, including cannabinoids, flavonoids, terpenoids, alcohols, aldehydes, and alkaloids (Liktor-Busa *et al*., 2021). In adequate doses, all parts of the cannabis plant have therapeutic effects against several illnesses, such as pain, inflammation, and mental illnesses (Hourfane *et al*., 2023).

Different kinds of secondary metabolites are found in various amount throughout the plant. The inflorescences and leaves contain the most cannabinoids, while the stem, roots and seeds have less of them (Odieka *et al*., 2022). Therefore, when harvesting only the flowering tops are collected (Jin *et al*., 2020).

Regarding the evaluation of extraction yield, our results showed that the highest efficacy was observed in the extract obtained by decoction. Thus, the extraction of total principles using this method would be more reasonable provided they exhibit better biological activity and the lowest toxicity effects (Brunetti *et al*., 2020).

The available information on the yield of chemical components extracted from plants is very limited and does not allow us to make comparisons with the findings of the current study. However, calculating the extraction yield is of major importance in estimating the quantity of raw material to be harvested to extract the target principles, while preserving biodiversity as well as environmental balance (Hassikou *et al.*, 2014).

Our results showed variable level of extraction efficacy, with the decocted product resulting in the highest efficacy. The literature on *C. sativa* extract yields is still very limited and specific to cannabinoids. Thus, the available information is insufficient for an objective comparison of our findings with those of previous studies. Nevertheless, Rožanc *et al*. (2021) reported that cannabis flower extraction via the Soxhlet method showed a high efficacy (20.50%) followed by the maceration method (16.30%). Similarly, the yield via aqueous extraction for *C. sativa* leaves was significantly high (11.68 g) (Ahmed *et al*., 2019).

Furthermore, the plant toxicity was found to be relatively low during long-term human use. It was evaluated by toxicity tests that allow researchers to determine possible adverse effects and to provide evidence of the exposure-response relationship (Saleem *et al*., 2017).

Update have been made regarding the toxic effects of the inflorescence, resin, and cannabinoids (THC, CBD). According to recent reports, all cannabis-related products are poisons, and the dose is of crucial importance in determining whether the product is a remedy or a poison (Saleem *et al*., 2017; World Health Organization et WHO Expert Committee on Drug Dependence, 2018).

In the present study, the administration of extract derived from decoction, maceration and sonication methods at the dose of 2.000 mg kg^{-1} showed the toxicity signs with variable intensities. The main clinical signs observed, particularly during the 2 hours following gavage, were somnolence, hypoactivity and tachycardia. The tachycardia may be due to the stress caused by restraint and gavage, since this sign was observed also in untreated mice, while hypoactivity and somnolence may be a result of the anxiolytic effect of cannabis extracts (Gamelin *et al*., 2021).

Furthermore, the water used as a solvent is quite polar and does not completely dissolve the cannabinoids, monoterpenes, and sesquiterpenes, which may have a link with the apparition of certain symptoms such as diarrhea and polyuria. Ethanol and methanol, however, have a strong affinity with these compounds which allows their quick dissolution (Liktor-Busa *et al*., 2021; Odieka *et al*., 2022).

Our findings are consistent with those reported by Yassa *et al*. (2010) who observed hypoactivity as a result of oral administration of an ethanolic extract of *C. sativa* leaves in male albino rats.

Moreover, we noted no deaths during the entire experimental period, which suggest that the lethal dose 50 (LD_{50}) of the three extracts studied may be higher than 2,000 mg kg^{-1}. However, Yassa *et al*. (2010) reported a LD_{50} of 1,729.6 mg kg^{-1} for an ethanolic extract of *C. sativa* leaves. This difference could be related to the solvent or the extraction method used. According to the Hodge and Sterner scale, our extracts are classified as slightly toxic substance (Hodge and Sturner, 2005).

Another study assessed toxic effects of a crude extract of cannabis on Fischer rats and revealed that its LD_{50} value, after oral administration, was 1,380 mg kg^{-1} for females and 3,300 mg kg^{-1} for males (Thompson *et al*., 1973). This study also showed that the acute toxicity of THC was low compared to the crude extract, and that its LD_{50} values for Wistar-Lewis rats were lower than those observed in Fischer rats, with values of 1,160 mg kg^{-1} vs. 1,910 mg kg^{-1} for males and 860 mg kg^{-1} vs. 1,040 mg kg^{-1} for females (Thompson *et al*., 1973; Saleem *et al*., 2017).

On the other hand, an ex-vivo study conducted by Sazmand *et al*. (2018) showed that treating stem cells from rat bone marrow with a low dose (1,000 ng ml^{-1}) of hydroalcoholic cannabis extract increased their growth while a dose of 10,000 ng ml^{-1}, caused cells death through apoptosis.

The change in mean body weight (BW) showed no statistically significant difference in the three groups of treated mice (T(Decoction), T(Maceration), and T(Sonication)) compared to the NT mice of male gender throughout the experimental period. The same result was reported by Bailey *et al*. (2022) in a study of the effect of subacute exposure of five groups of male mice at doses of 0.04, 0.1, 0.4, 1 and 4 mg kg^{-1} of a water-soluble cannabinol compound. Similarly, Maqbool *et al*. (2023) also found that the BW of mice was not affected by the addition of ethyl acetate and n-hexane extracts of *C. sativa* leaves to their diet in a comparative study of the effect of these two extracts on the restoration of muscle function after peripheral nerve injury.

In our experiment, macroscopic observation of the liver and kidneys of the treated mice showed no abnormalities in color, size, or external appearance compared to the organs of the untreated control group.

Conclusions

In the present study, the obtained results showed that extraction by the decoction method provided the highest yield. Acute toxicity evaluation of the three cannabis extracts at a dose of 2,000 mg kg^{-1} in both sexes of mice revealed some signs of toxicity but no deaths were recorded, and significant changes in BW were observed for female mice treated by decoction and maceration extracts compared to those treated with sonication, when treated and untreated male mice show no significant changes. In addition, macroscopic observation of the liver and kidneys of T mice showed no difference compared to those of NT mice.

These preliminary results revealed that using cannabis residue presents no notable risk at the studied dose. Thus, residual biomass generated by hemp can be used in human and animal feeding. Further studies are needed to determine the phytochemical composition of the plant in order to identify its active component(s) responsible for the observed signs of toxicity, and to assess the chronic and sub-chronic toxicity of *Cannabis sativa* L.

Authors' Contributions

All authors have contributed to the study design, data collection, and manuscript write-up. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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