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Microscopic examination of the larynx (voice box) of Swiss Mice exposed to N-Hexane fraction of *Cannabis sativa* leaf extract

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Abstract

Cannabis sativa (Cannabaceae) is the most widely used illicit drug in the world and has been cultivated for centuries in different parts of the world for its psychoactive properties. However, despite the pharmacological potential and economic prospects, there is a dearth of information on the toxicity profile of Nigerian Cannabis as well as rarely reported cannabis-administered animal models. Therefore, this research examined the microscopic details of the larynx (voice box) of the n-hexane fraction of Cannabis sativa leaves (nHCS) in mice. Dried leaves (2000 g) were extracted with methanol using a Soxhlet apparatus, then partitioned into an n-hexane fraction (nHCS). Forty adult female mice (20- 25 g) were divided into four groups (n=10): Group 1 control (Vehicle (olive oil)-treated group, 10 µL/kg), and groups II-IV received nHCS (200, 400, and 800 mg/kg), orally for 28 days. The animals were then euthanized and sacrificed by cervical dislocation, and the larynx was dissected out and preserved in 10% formalin for histological assessment. The results showed normal histological architecture of the vocal fold epithelium in the control mice compared with epithelial microtrauma, ulceration, denudation, and degeneration in the treated groups. Cannabis sativa administration altered the configuration of the stratified squamous epithelial lining of the mice's vocal fold.

Keywords: *Cannabis sativa*, Injury, Larynx, Microscopy, Vocal fold

1. Introduction

The herbaceous plant Cannabis sativa L. belongs to the Cannabaceae family and is known by many colloquial names, including hemp and marijuana (McPartland et al., 2019). Since ancient times, humans have utilized this plant, and numerous historians have documented its various applications globally (Clarke, 2016; Rupasinghe et al., 2020; Gomez et al., 2021). It has been utilized for recreational and religious purposes, as well as a source

of food, oil, and fiber, according to historical documents. Over the ages, a number of additional applications have also been discovered therefrom, including skin and hair care and cattle feed (Fleming et al., 1998). Although the cannabis plant is the source of all three- cannabis, marijuana, and hashish but differences abound.

Marijuana, in particular, can be called the dried, budded-out flower of the cannabis plant, whereas cannabis is the general name for the plant and all of its derivatives. In contrast, hashish is a more poisonous cannabis derivative from the plant's resinous portions. It is more powerful and has greater effects than marijuana since it has larger concentrations of psychoactive compounds and is typically delivered as a paste (Caputo et al., 2021).

There are various ways cannabis is used and abused. These include inhaling the smoke from its lit dried leaves (smoking), inhaling the aerosol containing its active ingredients using a powered device (vaping), and taking it in the form of food. The smoking of this drug is often done in beer parlour joints and other hidden or open places (Streck et al., 2019). Cannabis may be baked or fried into dishes to create edible marijuana, and an oil extract can be taken as oil droplets or made into capsules. Various forms of cannabis usage are linked to various demographic settings. Additionally, there has been a palpable rise in marijuana usage among young adults in recent years. It becomes important to examine its health impact and the association between cannabis intake and phonation at the histological or microscopic level, considering its rate of abuse.

2. Literature review

An estimated 550 or more bioactive chemicals are present in cannabis (Lowe et al., 2021). Cannabinoids, terpenoids, stilbenoids, lignanamides, carotenoids, flavonoids, and alkaloids are the classes to which these substances belong (Andre et al., 2016). From the cannabis sativa plant, hundreds of chemicals have been identified and characterized. The two most prominent components of cannabis are cannabidiol (CBD) and delta-9-tetrahydrocannabinol (THC). THC is primarily responsible for the psychoactive effects of cannabis. CBD is the most often used illegal substance worldwide, and it is not psychoactive; however, it has drawn increased attention in a number of sectors due to its medicinal potential in a variety of medical ailments (Alves et al., 2020).

For example, recent changes in state legislation in the United States have made cannabis more generally recognized due to the growing focus on medical and recreational usage (Valentino & McKinnon, 2019). The most well-known psychotropic component of cannabis, tetrahydrocannabinol (THC), contains about 500 additional compounds, including over 100 cannabinoids that are chemically similar to THC but whose characteristics are yet unknown. Cannabis strains with higher potencies have been linked to a higher chance of developing cannabis use disorders, such as addiction and psychosis (Blanco et al., 2016). Cannabis has been linked to both reduced vascular endothelial function in animals (Papatheodorou et al., 2016; Wang et al., 2016) and vasodilatory effects on tiny blood arteries (Pacher et al., 2005).

Cannabis may therefore theoretically put patients at risk for vocal fold bleeding or damage, according to a research survey. The survey ascertained the prior laryngeal history, marijuana use history, and belief system of participants. There were reported voice symptoms attributable to marijuana use, such as hoarseness, breathiness, and weakness, which the study suggested could lead to immediate or long-term dysfunction (Balouch et al., 2023). According to Meehan-Atrash et al. (2019), metabolites of marijuana smoking may potentially cause dysphonia by altering the vocal folds' viscoelastic characteristics. Therefore, it is likely that the manner in which marijuana is administered has a significant influence on the type and extent of airway injury, leading to varying voice consequences (Meehan-Atrash et al., 2019).

A prior study discovered evidence that marijuana promotes coughing, congestion, and inflammation in a dose-dependent way (Meehan-Atrash et al., 2019). In a similar vein, Balouch et al. (2023) proposed that cannabis consumption may result in phonational hoarseness both instantly and over time. Nevertheless, there

have been few reports on the microscopic details of the larynx of animal models exposed to cannabis. Hence, this study is to investigate the relationship between cannabis use and voice production.

3. Research method

3.1. Plant materials

Cannabis sativa leaves were obtained from the National Drug Law Enforcement Agency, Kwara State Command, Nigeria. Evidence specialists at this State Command verified the plant's identity, characteristics, and validation.

3.2. Extract preparation

The leaves of cannabis sativa were air-dried and milled, and thereafter macerated in 70% methanol for 72 hours. A rotary evaporator operating at a lower pressure and around 400ºC was used to concentrate the extract and remove the solvent. Methanol extract of the plant was dissolved in a mixture of distilled methanol and water, and the fractions were then extracted using n-hexane. The extracts obtained were then stored in the refrigerator at 40ºC until needed for the experiment.

3.3. Animals

Healthy adult female mice (20-25 g) were used for the study and were fed standard mice cubes, and water was provided *ad libitum*. The handling of the experimental animals followed the ethical guidelines for using animals as stated in the National Academy of Sciences (National Academy of Sciences, 2011).

3.4. Experimental design and grouping of animals

Forty adult female mice (20- 25 g) were divided into four groups (n=10). Group I mice were administered olive oil (vehicle), orally and served as the control, while groups II-IV mice were treated with the n-hexane fraction of cannabis extract for 28 days, as shown below;

Group 1 (n=10) received 10 µL/g body weight of Olive oil.

Group 2 (n=10) received 200 mg/kg Cannabis sativa n-hexane leaves extract

Group 3 (n=10) received 400 mg/kg Cannabis sativa n-hexane leaves extract

Group 4 (n=10) received 800 mg/kg Cannabis sativa n-hexane leaves extract

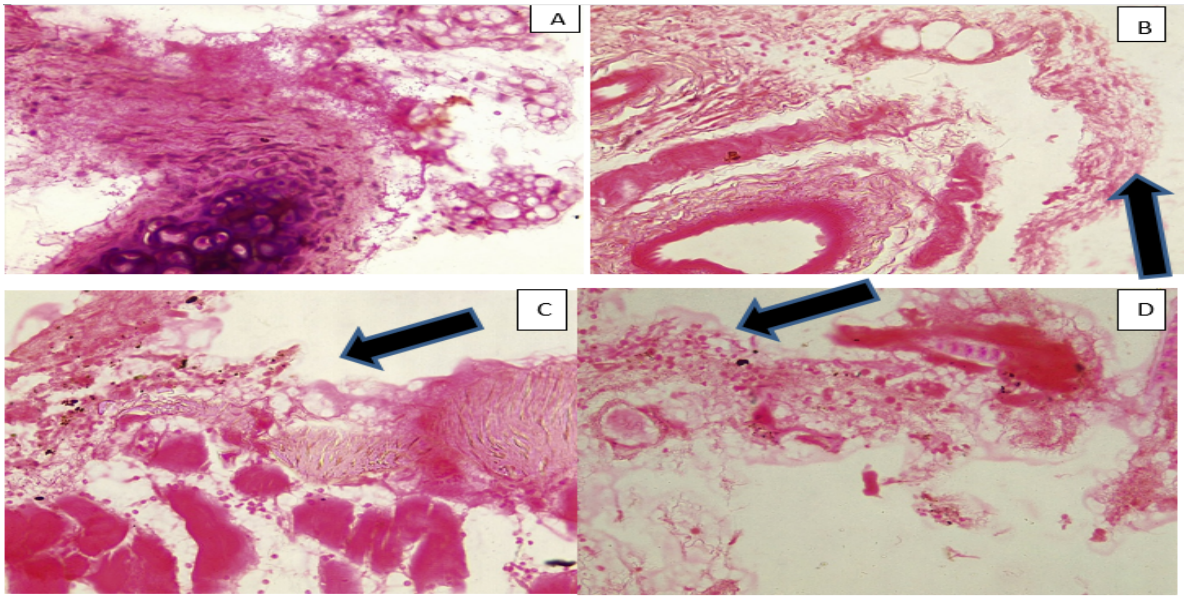
On the 29th day after the last administration, the mice were euthanized and sacrificed by cervical dislocation. The larynx was removed and fixed in 10% formal saline, processed through the steps of dehydration, clearing, infiltration, and embedding, and then sectioned at 6 µm thickness using a Rotary Microtome (Leica RM2125 RTS, Germany). The ribbons were stained with Haematoxylin and Eosin (H & E) for microscopic assessment, using a 500 pixel Leica digital binocular microscope.

4. Data analysis

4.1. Result

The result of this study showed normal histological architecture of the vocal fold epithelium in A. There were mild damage or microtrauma on the epithelial layers in B. Epithelial ulceration on the vocal fold surface epithelium in C. There was loss of the epithelial layer or degeneration, which exposed other underlying tissues in the vocal fold in D.

Figure 1: Photomicrograph showing the microscopic anatomy of the larynx of mice labelled A, B, C, and D



H&E X400

5. Findings and discussion

The vocal folds of the larynx are delicate structures composed of a stratified, non-keratinized squamous epithelium, whereas the ventricular folds and trachea next to the vocal folds are composed of a ciliated columnar epithelium. These microstructures are always in synergy and in comparable rhythmic air pulses in the glottis that cause them to resound. This oscillation is necessary for the production of voice for singing and communication, two of its main purposes. The length and tension of the vocal folds influence the tone of the sound produced. Physical injury from phonation and environmental irritants can induce constant damage to the mucosa of the vocal fold (Levendoski et al., 2014). Cannabis leaves can constitute these irritants.

In this study, the oral administration of cannabis sativa leaf was seen to have altered the normal histological architecture of the vocal fold in a dose-dependent manner, with the 800mg/kg having the highest severity. This is corroborated by other studies. According to a study, oral consumption of cannabis was shown to elicit bouts of anxiety, notably panic and hallucinations, in roughly 50% to 60% of cases compared to smoking marijuana. As a result, the oral method was recommended over inhalation due to dose delivery. According to their findings, animals given cannabis exhibited heightened hostility, aggressiveness, and restlessness between 30 and 60 minutes following drug use. This impact decreased to noticeable slowness and sluggishness, as well as extreme tiredness in the last week of the trial, and it was closely correlated with the cannabis dosage. This may indicate the beginning of the most contentious motivational condition.

According to additional research, long-term cannabis sativa usage may result in the buildup of tar particles in the mouth, which may induce lesions including hyperkeratosis, leukoplakia, and cannabis stomatitis with leukoedema (Harpreet et al., 2019). Regular recreational use of hashish and marijuana was associated with a higher frequency of severe periodontitis, including deeper probing depths and clinical attachment loss (Shariff et al., 2017). Gingival enlargement can also occur after marijuana use, much as phenytoin gingival overgrowth. Newmann et al. (2019) investigated the effects of long-term marijuana use on the microbiota of the oral pharynx and lateral border of the tongue, two common sites for oral cancer, using 16S rDNA sequencing. They discovered that the bacterial populations in both regions were changed by marijuana consumption. However, the changes were only consistent with malignancy at the pharyngeal level. Another study found that tongue squamous cell carcinoma is associated with dysregulation of the endocannabinoid system signaling pathway (Lim et al., 2019). All these studies corroborated the harmful effects of C*annabis sativa* abuse.

Despite the fact that cannabidiol, one of the active components in Cannabis sativa, may be toxic and have negative effects due to its potency, the way it is administered (orally, transdermally, or vaporized), concurrent use of both legal and illegal substances, as well as potential drug interactions could exacerbate the symptoms (Huestis et al., 2019). The primary psychoactive ingredient in cannabis, tetrahydrocannabinol (THC), offers several therapeutic benefits, such as lowering pain, increasing appetite, and alleviating nausea and vomiting (Gonçalves et al., 2019). According to reports, cannabis-derived secondary bioactive chemicals such as flavonoids, terpenes, and polyphenols have positive effects on living things (Cavalli & Dutra, 2021).

Cannabinoids are therefore included in pharmaceutical formulations of a number of medications, such as Cesamet, Marinol, Sativex, and Epidiolex, either in their natural or synthetic forms (Gonçalves et al., 2019). However, THC consumption can be harmful and have negative consequences, as blood THC levels are directly correlated with the intensity and duration of symptoms (Schep et al., 2020). THC stays in the blood for many hours after acute use until it is transformed into carboxylic acid, which is subsequently transported in the form of fat and found in urine for a few weeks. The mainstay of therapy for acute intoxication is symptom-based supportive care. Children who are exposed to cannabis poisoning may experience convulsions and coma, which may necessitate supportive treatment (Schep et al., 2020).

It's important to note that various plant compounds have distinct pharmacological effects on people. According to Rajput and Kumar (2018), these effects have the potential to significantly impact the body's regular physiological processes. Respiratory difficulties, changed perception, altered heart function, and compromised renal functioning are examples of such changes. Additionally, there are other psychological impacts linked to cannabis usage. Additionally, mouth dryness was noted in those who smoked tobacco, methaqualone, and marijuana. A study's histological examinations revealed dysplastic alterations in the buccal mucosa epithelium, such as aberrant mitotic activity, anucleated squamous cells, immature cell morphologies, and enhanced nuclear pleomorphism (Caruntu et al., 2021).

Cannabis use has been associated with a number of changes, including lung conditions in humans, such as symptoms of chronic bronchitis, an increase in acute bronchitis episodes, changes to DNA, and abnormalities in the structure and function of alveolar macrophages (Tashkin et al., 1997). Deep inhalation and holding one's breath while smoking marijuana have been linked to subcutaneous emphysema, pneumothorax, and pneumomediastinum. Additionally, a clear correlation was found with the incidence of squamous cell malignancies of the upper and lower respiratory tracts.

6. Implications of the study

Most recently, cannabis is the most often grown, transported, and used illegal substance. Cannabis usage has increased significantly over the past several decades, particularly in industrialized nations where legalization has led to a rise in the global cannabis user population. Cannabis has been legalized in those nations mostly due to its medical advantages, but its negative consequences cannot be disregarded. Its global popularity due to its psychotropic effects has contributed to its global expansion (Bonini et al., 2018). It is a major social issue since the availability and cultural acceptance of its use, in addition to its pharmacological characteristics, impact how it affects an individual's health.

Evaluation of the effects of cannabis is made possible by social permissiveness and the acceptability of cannabis use in certain nations. Its medicinal benefits are obvious, but it's crucial to understand the hazards associated with it. Worsening of chronic rhinosinusitis, tinnitus, changes to the vocal cords, and head and neck malignancies are the most frequent issues associated with its usage (Luísa, 2022).

7. Recommendation

Since inhalation is the most common mode of administration, issues with the upper respiratory tract are also anticipated to increase. Hence, more research is encouraged in this area due to the rising indiscriminate abuse of *Cannabis sativa*.

8. Conclusion

The vocal cord epithelium forms a structural framework of tensile strength and barrier integrity with pliability structure suitable for vibration. Cannabis sativa administration altered the configuration of the stratified squamous epithelial lining of the mice vocal fold, affecting the viscoelastic properties of the lamina propria of the vocal fold mucosa, which determines its vibratory behavior.

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