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Research Article

In vitro antifungal activity of marijuana extract collected from a rural region of Paraguay (Caaguazú)

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Abstract

Cannabis sativa L., commonly known as Marijuana, is a plant traditionally used to satisfy ritual, medicinal and textile needs. Modern medicine has used drugs derived from *Cannabis* for the treatment of diverse symptomatologies that affect human beings such as spasticity produced by multiple sclerosis and convulsions, among others. Despite its medicinal background, its recreational use is one of the determining factors that lead to the criminalization of its cultivation, in addition to the growing market for its illegal trafficking. In view of the need to open the debate and scientific research on the plant, the Paraguayan Congress passed the law that creates a national program for the study and medical and scientific research on the medicinal use of *C. sativa* and its derivatives. The objective of the present work was to evaluate the extraction yields and in vitro antifungal activity against mycelia of *Alternaria solani*, the pathogen causing early blight in tomato crops. The treatment prepared from the ethanolic extract of C. sativa with a final concentration of 10000 ppm produced a 35% inhibition of the mycelium of *A. solani* while the positive control, consisting of a commercial antifungal commonly used against the fungus, produced 100% fungal inhibition. Although the result obtained with the plant extract could be considered as a first study of this as a raw material for the creation of biocidal substances for application in the agricultural field, it is necessary to develop more studies on the target pathogen considering different concentrations of the plant extract until reaching a value that can approximate the results exhibited by the commercial fungicide.

1. Introduction

Cannabis sativa L. (Fig. 1a), commonly known as Marijuana, is a vegetable used since different times by man as a source to satisfy his ritual, textile and medicinal needs [1]. It is a cosmopolitan plant, cultivated for certain purposes although it can also be found wild, its seed possesses nutritional qualities as it contains fats, fibers and proteins [2]. In recent decades, modern medicine has used medicines derived from the plant for the treatment of various symptomatologies that affect humans such as spasticity produced by multiple sclerosis, control of nausea and vomiting derived from chemotherapeutic treatments and convulsions, among others [3]. In Paraguay, the controversy surrounding the plant is generated because its cultivation is illicit [4].

Cannabis is a plant with potential biological activity on certain test organisms, this quality could be due to the complex interaction between its constituent metabolites [5-6]. Tetrahydrocannabinol (Fig. 1b), known as THC, is one of the predominant metabolites

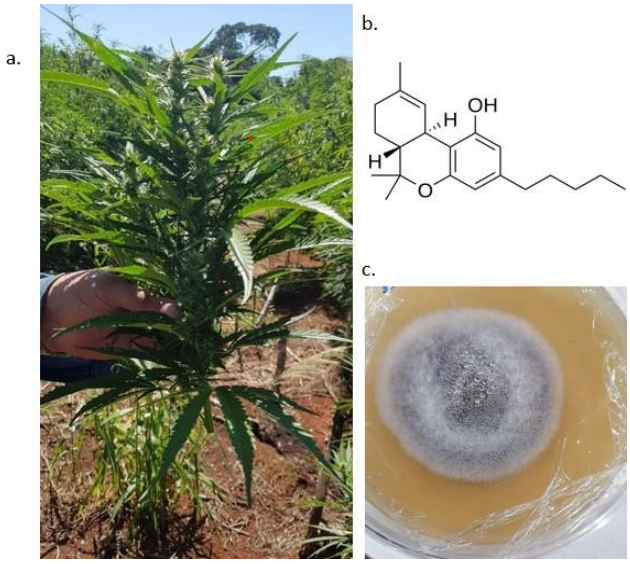

Figure 1. (a) Picture of *Cannabis sativa,* (b) Structure of tetrahydrocannabinol (THC) , (c) Picture of culture of *Alternaria solani*

found in the *Cannabis* plant being responsible for generating psychotropic effects in the consumer, altering the central nervous system [2-3].

Promising biological activities of crude resin obtained from South American varieties of cannabis as well as isolates of its key components have been reported on bacterial and fungal strains [7], results that stimulate the evaluation of the behavior of marijuana-derived substances cultivated in these latitudes of the world.

Despite the historical background on various applications of *Cannabis*, its recreational use is one of the determining factors leading to the criminalization of its cultivation and related relationships, another triggering phenomenon is the growing illegal trafficking market of the plant, Paraguay being one of the largest producers in South America [8]. However, in recent years, through the debate between key actors in the field of illicit drugs and pioneering state initiatives in certain countries around the world, the legal bases have been established on issues related to the regulation of the supply, cultivation, possession and domestic consumption of the plant, as well as controls by *ad hoc* entities and scientific research based on it [9-10].

The Congress of Paraguay enacted law number 6007 that "*Creates the national program for the study and medical and scientific research of the medicinal use of the Cannabis plant and its derivatives*" [11] and its regulatory decrees for the controlled production of the plant as well as the one that establishes the general conditions for the production and industrialization of *Cannabis*, granting powers of application, control and supervision to governmental entities competent in the matter [12]. The current regulation is a great step forward in the incipient path to research with *Cannabis* focused on different areas of knowledge such as agriculture or medicine. Following this initiatives, we focused our attention on the application of *Cannabis* as a biopesticide against *Alternaria solani* Sorauer (Figure 1c). This genus of fungi presents a pathogenic potential that affects the agricultural sector causing early blight disease [13] in addition to presenting a high incidence in certain crops compared to other fungi and producing certain metabolites that are toxic to humans [14-15]. In addition, the emergence of pests resistant to synthetic biopesticides used in modern agricultural practices generates the need to explore novel and environmentally friendly alternatives in the fight against phytopathogens, a scenario that favors research on extracts of natural origin with pesticidal potential [16]. Current legislation in Paraguay opens the way for scientific research with the Cannabis plant and led this research with the objective of evaluating the extraction yield and *in vitro* antifungal activity of Marijuana extract on mycelia of *Alternaria solani*, in order to have background information that will allow the generation of new knowledge based on *Cannabis*, in addition to generating an approach that promotes critical thinking about the plant or its potential derivatives, trying to banish the idea that the plant can only be used for illicit purposes.

2. Materials and methods

2.1 The plant materials

The plant studied was collected in the city of Caaguazú (Paraguay) as part of an operation carried out by the National Anti-Drug Secretariat - SENAD "Cutting and destruction of marijuana plantation". The sample was collected in April 2020. To ensure the identity of the collected plant species, taxonomic identification was carried out under the guidance of a botanist (Ms. Fátima Piris da Motta) from the Botany area of the Faculty of Exact and Natural Sciences of the National University of Asunción - FACEN UNA.

2.2 Extractive works

The processes of drying, grinding and maceration of the sample were carried out in the SENAD forensic

laboratory, excluding stems and lower leaves, leaving the buds and upper leaves of the plant for the preparation of the extract. The plant was ground with the help of an electric mill until a fine powder was obtained. Once the powder was obtained, it was macerated with absolute ethanol (EtOH). The maceration was carried out during one week, making intermediate agitations on the preparation so that the effect of the solvent was as homogeneous as possible. Once the liquid extract was obtained, it was filtered by gravity to separate the solid particles from the solution, after which the solvent was evaporated with a rotary evaporator.

2.3 Antifungal activity

The isolates of *Alternaria solani* used in this experiment were provided by the Phytomycology laboratory of the Plant Protection area of the Faculty of Agricultural Sciences of the National University of Asuncion - FCA UNA.

The antifungal activity assays were established using a completely randomized design. Sterilized potato-dextrose-agar (PDA) and medium technique poisoned with the treatment to be tested [17] were used as culture medium. 0.1 mL of antibiotic composed of Oxytetracycline was added to the PDA medium in order to inhibit undesirable bacterial growth during the development of the experiment, then 100 mL of the medium was mixed with the plant extract to obtain a final concentration of 10000 ppm. From this stock concentration, solutions of concentrations of 5000 ppm, 2500 ppm and 1250 ppm respectively were prepared, the culture medium being the solvent. A commercial reference antifungal Acronis® (thiophanate-methyl) was used as a negative control and the positive control was the pathogen in an untreated culture medium. The solutions of different concentrations, as well as the controls were added in triplicate in Petri dishes and allowed to stand for twenty-four hours at room temperature of 35 °C to check the sterility of the experimental units, after confirming the viability of the treatments, the pathogen was seeded, extracting a disc of purified Alternaria mycelium of 0.5 mm in diameter with the help of a sterile metal punch and placed in the center of each plate. After seeding was completed in the test treatments, the plates were kept at a temperature of 35°C. Measurement of Alternaria growth diameter began forty-eight hours after sowing, measuring the halo every twenty-four hours and ending once the positive control completely covered the Petri dish containing it, for a total period of one hundred and sixty-eight hours. The fungal growth, recorded at 168 h from the start of incubation, in the presence of treatments at different concentrations of *Cannabis sativa* shown in Table 1. The efficacy of each concentration of the Cannabis extract-based treatment on mycelia was evaluated according to Abbot's formula and classified according to the scale established by the International Organization for Biological Control OIBL [18].

(i) Percentage of inhibition (%) = Control colony growth - colony growth_concentration_x 100

Table 1. Means of fungal growth, recorded at 168 h from the start of incubation, in the presence of treatments at different concentrations of *Cannabis sativa.*

Treatment	n	*Means of fungal growth* (cm)	SD
10000ppm	3	0.4833	0.0058
5000ppm	3	0.5183	0.0115
2500ppm	3	0.5500	0.0050
1250ppm	3	0.5567	0.0029
Control	3	0.6000	0.0050
Acronis®	3	0.0000	0.0000

Control colony growth: Measurements of *Alternaria* spp. growth diameters were evaluated using INFOSTAT® software by applying the ANOVA test with a 5% error and then Tukey's test to identify statistically different groups.

3. Results and discussion

The response of *Alternaria solani*; in terms of inhibition against the different concentrations of the ethanolic extract of *Cannabis* was associated with the toxicity scale (Table 2).

Table 2. Toxicity level classification of treatments based on *Cannabis sativa* extract against *Alternaria solani*

Concentration of EtOH *Cannabis* extracts	Percentage of inhibition of *Alternaria*	Scale of toxicity*
10000 ppm	35.10	Slightly toxic
5000 ppm	25.35	Innocuous
2500 ppm	15.97	Innocuous
1250 ppm	13.92	Innocuous
CP	100.00	Toxic
CN	0.00	Innocuous

CN: Pathogen in the absence of treatment

CP: Pathogen with commercial fungicide treatment *[18].

For the extraction of Cannabis, we followed the work of Moreno-Sanz et al. [19], they evaluated the biological activities of *Cannabis* on cell lines involved in hypoxia and inflammation processes and found different plant activities using ethanol and hexane as working solvents respectively. In the present work, we decided to work with ethanol extracts and the yield of extraction was 0.4%.

For the antifungal activity, the 10000 ppm concentration of *Cannabis* extract produced the highest percentage inhibition of *Alternaria solani* mycelia. This finding is congruent with that obtained by Bashir et al. [13] where higher concentration treatments prepared with 20% *Cannabis sativa* extracts were tested *in vitro* against *Alternaria solani*.

The greatest challenge of this study was to obtain authorization for the collection of the plant. Since it is an illicit plant, whose cultivation and possession are penalized in the country, they come from crop eradication operations carried out by law enforcement authorities concerning the control of narcotic substances.

As a limitation within the work, the availability of the plant to obtain the extract to be tested. Access to this type of sample is subject to cultivar eradication operations carried out by law enforcement authorities in anti-drug matters. This makes it difficult to obtain homogeneous plant samples in terms of growth stage, climatic and agricultural conditions at the time of collection. In Paraguay, this is the first work carried out with an extract obtained from *C. sativa* of illicit origin, thus building the basis for future research with the plant.

4. Conclusions

The extract obtained from buds and upper leaves of the *Cannabis sativa* plant collected in a rural area of Paraguay showed modest antifungal activity against *Alternaria solani*. This could be a precedent for further research using extracts of the plant in order to design biocidal agents based on it and broaden the spectrum of natural substances for agricultural pest control. Future studies should focus on the highest concentration tested in this experiment as a starting point. It is necessary to have a significant amount of raw material in order to guarantee the obtaining of crude extracts to be tested, or to optimize the extraction process using techniques other than maceration, and the behavior of the marijuana extract should also be evaluated *in vitro* and *in vivo* experiments against other fungal strains and varieties of phytopathogens such as bacteria, viruses and parasites. In spite of the scarce background available on the use of the plant as an antifungal, this work aims to create a precedent to promote the study of marijuana with potential applications in the agricultural field.

List of abbreviations

THC: Tetrahydrocannabinol
SENAD : National Anti-Drug Secretariat
UNA : National University of Asunción
FACEN : Faculty of Exact and Natural Sciences
FCA : Faculty of Agricultural Sciences
OIBL:International Organization for Biological Control
PDA : Potato-dextrose-agar
EtOH : Ethanol
ANOVA : Analysis of variance

Ethical approval

Attached is Fiscal Note No. 285 issued by Attorney Osvaldo Esteban García Giménez, fiscal agent of the Specialized Unit in the Fight against Drug Trafficking -V Department - Caaguazú, authorizing the use of plant samples collected during the Caaguazú-Canindeyú operation carried out in Campos Morombi on April 4, 2020 in the Republic of Paraguay for the purposes set forth in notes dated March 16 and April 12, 2021 respectively sent by Lic. Mario Ricardo Galeano Chena, Director of the Specialized Forensic Directorate - DFE of the National Anti-Drug Secretariat - SENAD, Paraguay. In addition, enclosed is a copy of the legal proceeding act of delivery of samples by the intervening authorities and a favorable opinion issued by the Directorate of Legal and Juridical Affairs of the National Anti-Drug Secretariat - SENAD for the realization of the research project.

Authors' contributions

Drafting and revision of the manuscript, field sampling, execution of experiments, revision of statistical analysis, management for obtaining legal permits for plant use, R.G; Literature review, Drafting and revision of the manuscript, statistical analysis,

execution of experiments, L.C.; Taxonomic identification of the plant, revision of the manuscript, F.P.; Drafting and revision of the manuscript, G.C.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Conflicts of interest

The authors of this work declare that there is no conflict of interest.

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