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Research Article

In vitro investigation of antifungal efficacy of diclofenac sodium salt against *Aspergillus* spp. strains causing food spoilage: a drug repurposing approach

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Abstract

Fungal species especially Aspergillus spp. causes contamination in food causing food spoilage. *Aspergillus flavus* is the most common fungal pathogen which secrets highly poisionous secondary metabolite aflatoxin which is toxic to mammals. So, to identify a compound which can control contamination of *A. flavus* of food is necessary and challenging. Drug repurposing is an alternative which is less time consuming and economical. Current study aimed to investigate potential of NSAID drug-diclofenac sodium for its antifungal potency. Isolation of Aspergillus species causing food contamination were carried out by the suspension-dilution technique and inoculation on agar medium from peanuts. Antifungal efficacy of diclofenac was conducted using the agar plate and liquid dilution methods. The inhibitory effect of diclofenac sodium was found significantly on *A. flavus and A. niger* in concentration dependent manner. The mycelial was significantly reduced at concentration of 300 µg/ml diclofenac for both *A. flavus and A. niger* strains. The MIC_{50} was achieved at 300 and 1200 µg/ml for *A. flavus* and *A. niger* respectively in liquid medium. Our findings suggest diclofenac can be used effectively to control food spoilage caused by *Aspergillus* species.

Keywords: Diclofenac sodium, *Aspergillus* spp, Drug repurposing, Mycelium inhibition

INTRODUCTION

Plant pathogens and pests are the major reasons for reduced yield and quality of crops[1,2]. Plant diseases are affecting economic development and food security[3]. More than 19,000 species of fungi are known to cause plant diseases. Some of these fungal species may live in a dormant state until conditions change to become proliferative[4]. The average annual loss due to phytopathogens is more than $200 billion[5]. Filamentous fungi have a special ability to grow on very simple substrates whose components are used as food. During their growth and development, they also synthesize different secondary metabolites[6].

Secondary metabolism of fungi is a complex process and different compounds are produced in response to different environmental signals. Some of them are pharmaceutical and industrial interests. Even though, fungal species can also produce a toxic metabolite known as mycotoxins[7,8,9]. Filamentous fungi, especially *Aspergillus flavus*, are a major contaminant in different foods such as fruits, vegetables, and cereals. It is the most common species associated with food spoilage. It also secretes highly poisonous secondary metabolites like aflatoxin. Among all mycotoxins, aflatoxin is the most studied and strictly regulated because of its toxic effect on health and economic loss. It is a common natural carcinogen and is associated with the development of liver cancer. It is classified under group 1 of molecules that show carcinogenicity to humans and animals by the International Agency for Research on Cancer (IARC)[10]. Aflatoxin is also well-known for its toxicity and teratogenic, hepatotoxic, mutagenic, and immunosuppressive properties. It can also target various aspects of the metabolic system. So, it is necessary to develop a safe and efficient way to control the spoilage of *A. flavus* in food[11]. Therefore, it is necessary to develop an approach to prevent the contamination of this fungal species and their toxic mycotoxins to food and to restrict their harmful effect [12].

Pesticide and fungicidal agents are commonly used to prevent the contamination of fungal agents. However, as they are associated with their own toxic effects, their use to fruits and vegetables has to be restricted[13]. Biological control and detoxification processes are other options for controlling the toxicity of contaminants to food. However, production of biopesticides is slow and difficult to be applied to food[14]. So, chemical agents are still the preferable option[15]. Even though, large numbers of these weeds are not efficiently controlled by a number of agrochemicals. Additionally, antibiotic resistance was also observed in these species[16].So, it is necessary to develop a novel approach to control these phytopathogens. Development of drugs is a complicated, long and costly process [17]. It takes nearly 20 years to develop a new drug and it can cost up to $2 billion for the whole process. Similarly, chemical agent discovery is also an expensive and time-consuming process[18]. So, drug repurposing for already known drugs is one approach to the development and discovery of drugs as the pharmacokinetics and biological activities of these drugs are already known. Many of the drugs are already known for successfully repositioning[19]. The antifungal activity of diclofenac sodium has been demonstrated in various studies, showing its effectiveness against *Candida albicans* [20] and *Aspergillus fumigatus* [21]. Various studies are reported on antifungal potency of diclofenac sodium on human fungal pathogens its potency against phytopathogens such as *Aspergillus flavus* are not known. Additionally, *Aspergillus flavus* is an opportunistic human pathogen and can cause aspergillosis disease. On this basis, diclofenac sodium , a non-steroidal anti-inflammatory drug was tested against *Aspergillus flavus and Aspergillus niger* which can serve as the theoretical basis for the development of fungicides.

Objectives

The present study was carried out with two main objectives; i) to isolate *Aspergillus* spp. strains which cause the biodeterioration of peanuts (*Arachis hypogea* L.) ii) to determine the MIC of diclofenac sodium against *Aspergillus* spp. strains in liquid medium.

MATERIAL AND METHODS

Chemicals and culture media

All chemicals and culture media were purchased from Himedia Chemicals Ltd, Mumbai, India.

Sampling

Contaminated peanut (*Arachis hypogea* L.) samples (with envelope, without envelope) were collected from different markets in the Chh. Sambhajinagar, India. Peanut samples were well mixed and kept in plastic bags.

Mycobiota Analysis

The fungi present in the samples were extracted using the suspension-dilution method. A mixture of 10 grams of contaminated crushed peanuts was combined with 90 ml of distilled water and subsequently mixed with tween 80. This mixture was homogenized by stirring for 15 minutes. Serial dilutions were performed starting from the stock solution. From each dilution, 100 microliters were subcultured onto Potato Dextrose Agar (PDA). The plates were incubated at 28 °C for a period of 5 to 7 days. Representative isolates from each sample were purified and preserved on Potato Dextrose Agar (PDA). The identification of the main fungal species was carried out on specific medium and on the basis of morphological characteristics. Purified fungal strains of *Aspergillus flavus* (RAN5) and *Aspergillus niger* (ARN7) were isolated and stored on PDA at 4 ° C.

Effect Of Diclofenac On Mycelial Growth of *Aspergillus* spp. strains

The antifungal properties of diclofenac were examined on the mycelial growth of *A. flavus* strain (RAN5) and *A. niger* (ARN7). To assess the impact of diclofenac on mycelium growth, aliquots of the drug were separately dissolved in distilled water and aseptically added to PDA medium at a temperature ranging from 45 to 50°C. Solid potato dextrose agar plates were prepared with different concentrations of diclofenac, varying from 75 to 2400 µg/mL. Control plates were maintained without test substances (diclofenac sodium). Agar discs (9 mm in diameter) containing the edge of a 5-day-old culture of *A. flavus* (RAN 5) and *A. niger* (ARN7) were placed at the center of each Petri dish. Subsequently, the plates were sealed with parafilm. The incubation of the plates was carried out at 28 ± 2 °C. The growth rate of the colony was assessed by measuring the diameter of the expanding colony. The effect of diclofenac was evaluated after 5 days by calculating the average of two perpendicular diameters of each colony. The antifungal impact of diclofenac on the mycelial growth, in comparison with the control, was determined on the 5th day using the following formula:

Percentage of mycelial inhibition = $[dc - dt/dc] \times 100$

where, dc (cm) is the mean radius of the colony in the control plates and dt (cm) is the mean colony radius value of colonies grown in PDA media containing diclofenac [22].

Determination of Minimum Inhibitory Concentration (MIC) in liquid medium

The minimum inhibitory concentration (MIC) of diclofenac was assessed using the CLSI broth microdilution technique described by Borman *et al*. (2017) with minor adjustments. A spore suspension of *A. flavus* (RAN5) and *A. niger* (ARN7) was introduced into potato dextrose liquid medium to achieve a final concentration of 1×10^4 CFU/ml, and this medium was distributed into the wells of a 96-well plate. Diclofenac sodium was subsequently added to the wells at final concentrations ranging from 75 to 2400 µg/mL. Control tubes containing potato dextrose medium were inoculated solely with the fungal suspension and were incubated at 35^0C for 48 hours. The plate was incubated at 35°C for 48 hours, after which the growth of *A. flavus* (RAN5) and *A. niger* (ARN7) in each well

was evaluated, and the MIC was established. The IC_{50} represents the concentration of the drug necessary to eliminate or inhibit 50 % of the growth of the isolates[23].

Statistical analysis

Values presented are the means with standard deviations, obtained from three different observations. Values in the control and treatment groups for various concentrations were compared using the student's t-test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Mycological analysis

In the examined peanut samples, the pH levels were noted to be between 6 and 7. Two critical factors influencing the growth and spread of mold in stored food samples are moisture content and pH. Our investigation revealed that pH in the peanut samples was suitable for fungal development. The mycological assessment of the peanut samples indicated a prevalence of *Aspergillus* species. *A. flavus* (RAN-5), isolated from peanut samples, was identified and was chosen as the test fungus and *A. niger* (ARN7) was also evaluated.

Antifungal Effect of Diclofenac sodium on mycelial growth and determination of MIC

The inhibitory effects of various concentrations of Dic on the colony growth of *A. flavus* and *A. niger in vitro* are illustrated in Figures 01 and 02 . We observed that different concentration of Dic led to a decrease in fungal growth, and the reduction in mycelial growth was correlated with the higher concentrations of Dic. The Dic resulted in a notable decrease in mycelium growth for *A. flavus* (RAN 5) at concentrations of 300, 600, 1200, and 2400 µg/mL, with reduction percentages of 82, 87, 90, and 97 respectively. Likewise, for *A. niger* (ARN7), Dic demonstrated a significant decline in mycelial growth at 300, 600, 1200, and 2400 µg/mL concentrations, with inhibition percentages of 89,90,92, and 94 respectively.

The minimum inhibitory concentration (MIC) of Dic was evaluated against foodborne fungi using the dilution method in a liquid environment. Conducting the MIC study is essential for identifying the lowest concentration needed to inhibit fungal growth. At a concentration of 1200 µg/mL, Dic effectively reduced growth of *A. flav*us (RAN-5) by 72%. Additionally, Dic was found to inhibit *A. niger* (ARN-7) at 300 µg/mL, resulting in a 64% reduction. The IC_{50} values were determined to be 300 µg/mL for *A. flavus* (RAN-5) and 1200 µg/mL for *A. niger* (ARN-7) respectively (Fig 03). Here, we found MIC value of Dic is higher in *A. niger* than *A. flavus*.

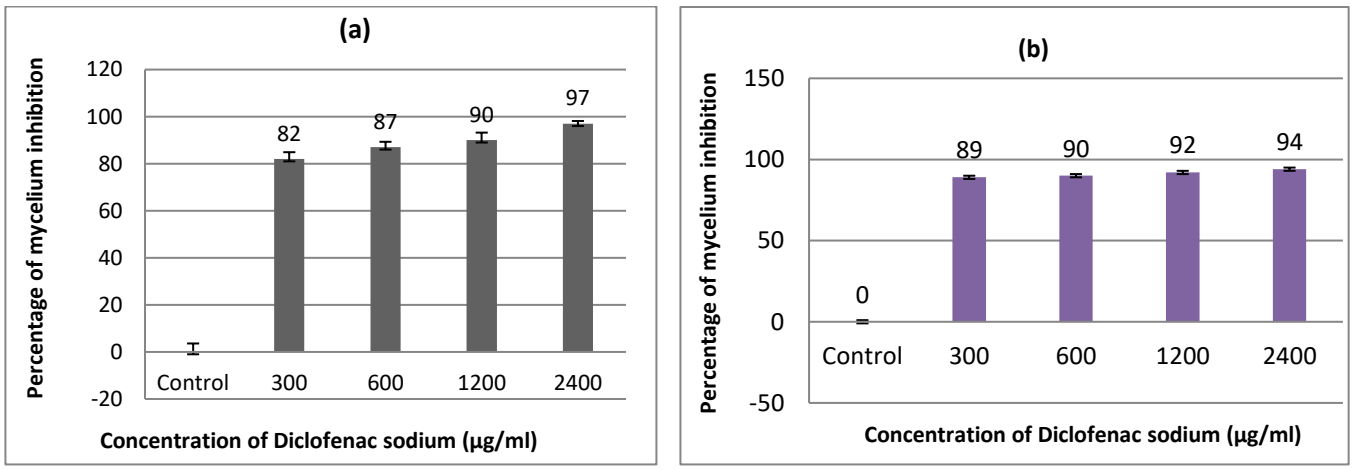
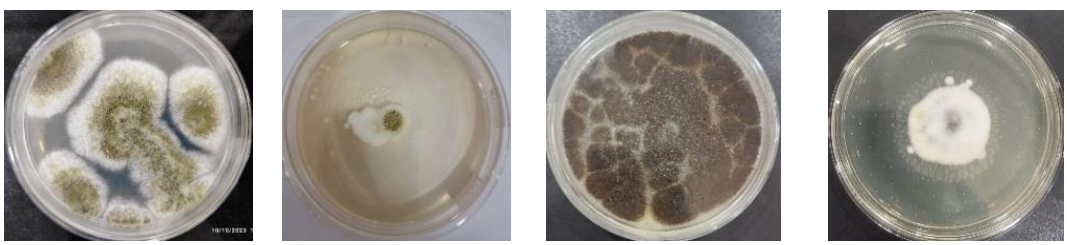


Figure 1: Effect of different concentrations of diclofenac sodium on mycelial colony growth of (a) *A. flavus* (b) *A. niger*. The plates were incubated at a temperature of 28 ± 2^{0}C for 5 days. Values are means (n = 3) ± standard deviations.



(2 A) (2B) (2a) (2b)

Figure 2: Effect of diclofenac sodium on mycelial colony growth of *A. flavus*; control(2A), 300 µg/ml (2B) and *A. niger*; control (2a), 300 µg/ml. The plates were incubated at a temperature of 28 ± 2^{0}C for 5 days.

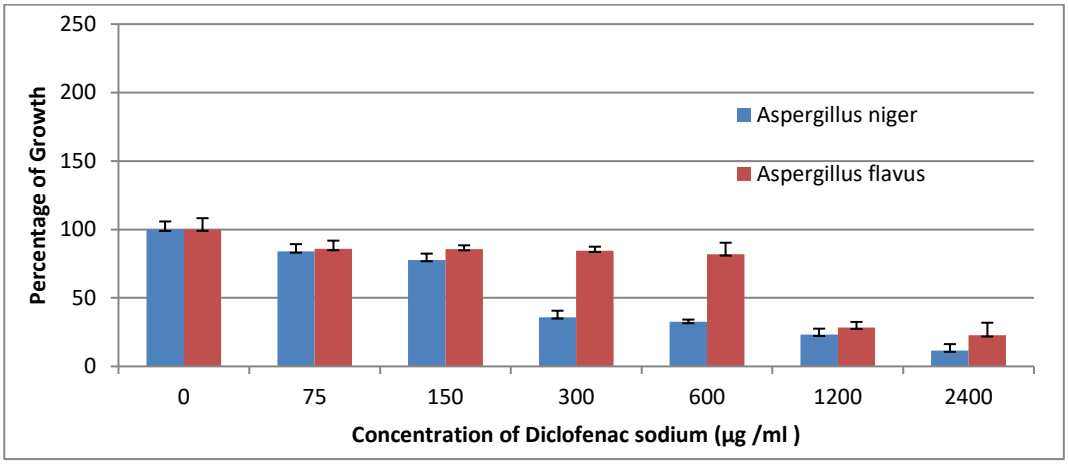


Figure 3: Effect of different concentrations of diclofenac sodium on growth of (a) *A. flavus* (b) *A. niger* in liquid medium. The plates were incubated at a temperature of 35 ^{0}C for 48 hrs. Values are means (n = 3) ± standard deviations.

DISCUSSION

Due to the emergence of drug resistance, repurposing of drugs may be a promising strategy for drug development. It is a promising approach to finding effective drugs and overcoming the problem of the emergence of drug resistance. There are various studies done with a drug repurposing approach to screen a library of drugs with anti-inflammatory, anti-tumour, and antimicrobial efficiency[24]. In the same way, in the agricultural field, the efficacy of halofuginone, kaempferol, honokiol and tavaborole against phyto-pathogens has been reported [24,25]. An *et al* in 2023, screened 600 drugs against pathogenic fungi and 82 drugs were reported effective against *Rhizoctonia solani, Sclerotinia sclerotiorum, Botrytis cinerea, Phytophthora capsici, Fusarium graminearum* and *Fusarium oxysporum*, respectively[24]. In this present work, we have investigated the potential of Dic against aflatoxin producing *Aspergillus flavus* and *Aspergillus niger* isolated from contaminated peanuts. *In vitro* antifungal assay, *Aspergillus* spp. was found sensitive to the different concentrations of diclofenac sodium salt. Our data clearly show a significant reduction in mycelium growth of both fungal strains tested. The diclofenac sodium salt at 300 µg/ml showed significant mycelium inhibition for both *Aspergillus* species. There are a number of findings available suggesting antifungal effects of Dic against human pathogens. Dic at 500 µg/ml showed significant activity in the human fungal pathogen *Aspergillus fumigatus* [21]. Rusu *et al* in 2014 reported the antifungal efficacy of Dic in combination with antifungal agents against *C. albicans* and *C. krusei*[26]. However, there are no studies evaluating the effects of Dic on *A. flavus* and *A niger* isolated from contaminated food. Dic significantly inhibited the growth of *A. flavus* and *A niger* in a concentration-dependent manner. Our results are in agreement with the above findings. These findings suggest the efficiency of Dic as an antifungal in food protection. However, further study is required to find out the toxicity and its effect on the quality of food.

CONCLUSION

Our study reports antifungal efficacy of diclofenac sodium salt against *A. flavus* and *A. niger*. It shows significant inhibitory effects on the growth of *Aspergillus spp.* in a concentration-dependent manner. This observed efficacy of Dic against mycelial growth of *Aspergillus spp*. suggests the repurposing diclofenac as an antifungal agent, particularly for food protection applications. Our findings align with previous findings on the antifungal efficacy of diclofenac and expand its scope to use against foodborne fungal pathogens. However, further investigations are needed, including *in vivo* studies, toxicity, and its effect on food quality. We suggest diclofenac would be a cost-effective and efficient solution for managing fungal contamination and spoilage in food.

Abbreviations

Dic-Diclofenac sodium salt, NSAID-Non steroidal anti-inflammatory drug

Conflict of interest: Authors declare no conflict of interest.

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