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PHARMACOLOGICAL EVALUATION AND ACUTE TOXICITY STUDIES OF FERMENTED EXTRACTS OF *ASPERGILLUS FLAVUS AND ASPERGILLUS ORYZAE*

UMAR FAROOQ GOHAR[1], AAMIR MUSHTAQ[2], AMJED HUSSAIN[3], FATIMA RIAZ[1], MUHAMMAD TAYYAB AKHTAR[1] AND HAMID MUKHTAR[1*]

[1]*Institute of Industrial Biotechnology, Government College University Lahore, Lahore, Pakistan*
[2]*Department of Pharmaceutical Sciences, Government College University Lahore, Lahore, Pakistan*
[3]*Department of Zoology University of Kotli, Azad Jammu &Kashmir Pakistan*
Corresponding author's email: hamidmukhtar@gcu.edu.pk

Abstract

The objective of the current study was to investigate the pharmacological advantages of fermented extracts derived from soil fungi commonly encountered in the environment. Twenty soil samples were collected from Lahore, Gujranwala, and Wah Cant for isolation of fungal strains. Among the isolated strains, *Aspergillus flavus* and *Aspergillus oryzae* were chosen. Subsequently, ethyl acetate extracts were obtained from the fermentation broths of these selected strains. Both the fermented extracts i.e. ethyl acetate extract of *A. flavus* (EAe.*A.f*) and ethyl acetate extract of *A. oryzae* (EAe.*A.o*) were analyzed for DPPH free radical scavenging activity. Both EAe.*A.f* and EAe.*A.o* showed free radical scavenging activity as 72.82 % and 36.47 %, respectively. The extracts were then analyzed for antimicrobial activity by using disc diffusion assay and it was found that both the extracts exhibited antibacterial activity against *Staphylococcus aureus, Bacillus subtilis* and *Escherichia coli* while no antifungal action was observed against tested fungal strains. The hind paw edema model was used to assess anti-inflammatory activity of EAe.*A.f* and EAe.*A.o.* It was observed that both the extracts reduced edema up to 43.5±0.7 and 42.9±1.3 %, respectively. The antidiabetic activity was found against streptozotocin induced hyperglycemic albino mice. EAe.*A.f* (400 mg/kg) significantly reduced glycemic level from 438.33±19.15 to 359.11±18.16 mg/dL in 21 days while EAe.*A.o* (400 mg/kg) reduced the same from 422.39±25.28 to 255.33±17.21 mg/dL. The outcomes of acute toxicity study suggested the LD_{50} values of both the extracts were found to be 1300 and 1100 mg/Kg, respectively for EAe.*A.f* and EAe.*A.o.* The findings thus suggested that both *A. flavus* and *A. oryzae* are pharmacologically active as antioxidant, anti-inflammatory, antibacterial and antidiabetic agents. However, the mechanisms underlying these pharmacological activities were not fully elucidated in this study suggesting the necessity for comprehensive investigations to provide detailed insights.

Key words: Fermentation, Soil fungi, *Aspergillus*, Antioxidant, Antimicrobial, Anti-inflammatory & antidiabetic activity.

Introduction

Natural products have been used from centuries for the treatment and prevention of several diseases but the more advanced scientific work on natural product has been contributed since last century. The Fungi Kingdom represents a reservoir of numerous valuable fungal species, many of which inhabit soil ecosystems. These fungi hold significant promise for clinical applications in the prevention and treatment of infectious and metabolic diseases (Delgado *et al.*, 2021). Different active and metabolic products have been isolated from the fungi which are responsible for the biological activities. It has been investigated that *Trichoderma* species are responsible for the synthesis of several secondary metabolites which are proved toxic or lethal for bacterial and fungal pathogens (Peñuelas *et al.*, 2014).

Ascomycetes fungi including *Aspergillus* and *Penicillium* and some *Basidiomycetes* fungi are much famous for the pharmaceutical development in health care setting (Bala *et al.*, 2011). A variety of fungal metabolites have been obtained which can be used for therapeutic purposes and out of which about 30% of fungal metabolites have been isolated from *Aspergillus* and *Penicillium* species.

Aspergillus is one of the huge genus of Fungi which is comprising almost 180 filamented fungal species all over the World (Lubertozzi & Keasling, 2009). Numerous species within the Aspergillus genus demonstrate rapid growth across diverse environmental conditions and frequently exhibit resistance to varying salt concentrations. They are readily obtainable from soil, aquatic environments, and endophytic fungi (Kusari *et al.*, 2009), The metabolites isolated form *Aspergilus* fungi have great diversity in their chemical structures which render them towards different pharmacological and therapeutic activities (Cai *et al.*, 2011; Wang *et al.*, 2017). They are also involved in production of secondary metabolites which are responsible for antimicrobial activities (Al-Fakih & Almaqtri, 2019). Aflatoxin, fumagillin, asp-hemolysin, gliotoxin and helvolic acid are very famous and mycotoxins produced from *Aspergilus* fungi (Bhetariya *et al.*, 2011) causing mycotoxicosis in human. Several other compounds have also been isolated from *Aspergilus* like 2-hydroxycircumdatin C, which is an anti-oxidant peptide and 4-2-methoxyphenyl)-1-piperazinyl an important anti-cancer compound (He *et al.*, 2012). Others include stigmast4-ene-3-one (an anti-protozoal agent), iso-aspulvinone (anti-diabetic), probiotics (Lee *et al.*, 2006), lipases and statins (Barrios-González & Miranda, 2010), which have been obtained from *Aspergillus* species (Al-Fakih & Almaqtri, 2019).

In the current era, fermentation methodologies are extensively employed for a multitude of botanicals and natural substances. It is hypothesized that fermented extracts exhibit heightened efficacy compared to their non-fermented counterparts (Wang *et al.*, 2011; Wang *et al.*, 2017). The success of the fermentation depends upon the selection of right strain of microorganism along with appropriate methodology (Kim *et al.*, 2017). The selection

of accurate microbial organism for fermentation promotes the enzymatic metabolism of the extract which ultimately leads to increase in biological action of that substance. It has been reported that the *Aspergillus* species have proved very successful for the purpose of fermentation. The benefit of fermented extracts lies in their potential physiological advantages without concurrent cytotoxic effects. Consequently, these extracts may be utilized without the necessity of purifying bioactive constituents, owing to the potential synergistic interplay among phytochemical components within the fermented extract (He *et al.*, 2011; Wang *et al.*, 2019).

By knowing the utilization of fermentation techniques, different pharmaceutical companies and large research groups in different universities are very much enthusiastic to discover the fungal strains for manufacturing of antibiotics (Larsen *et al.*, 2005) and anticancer medicines (Zhang *et al.*, 2006). Different secondary metabolites have been isolated from *Aspergillus oryzae* which show marked antibiotic activity against a variety of bacteria. They also exhibit 100 % cytotoxicity against the brine shrimp (Shaaban *et al.*, 2014).

Antibiotic resistance has become a global challenge for the researchers and health care professionals to discover the alternate products which may be beneficial against emerging resistant strains of pathogenic bacteria. In response to this challenge, the present study is formulated to employ fermentation extraction techniques, aiming to evaluate the antimicrobial activity of two fungal strains, namely *Aspergillus oryzae* and *Aspergillus flavus*. Additionally, the investigation encompasses the assessment of their anti-inflammatory, antidiabetic, and antioxidant properties.

Material and Methods

Fungal isolation: Soil sampling was done from different places of Gujranwala, Lahore and Wah Cant. Twenty samples were collected by taking soil from 5-10 cm deep from the ground surface, serial dilutions (10^{-3} to 10^{-9}) were made, and 0.1 mL from each serial dilution was poured onto PDA plates which were then incubated at 30°C for 72 hrs. The colonies formed were picked up and streaked onto fresh PDA plates to get pure fungal cultures. The isolated fungal strains underwent sub-culturing every two weeks to sustain their viability. Microbial identification was performed based on morphological attributes including colony size, shape, texture, elevation, pigmentation, and overall appearance. The selected strains of *Aspergillus* species were transferred to PDA slants where they were incubated for at 30°C for four days. The grown colonies of *Aspergillus* species were microscopically examined and were identified by experts from Institute of Botany, University of the Punjab Lahore, Pakistan. Out of these isolates, two species viz., *Aspergillus oryzae* and *Apsergillus flavus* were selected for the studies.

Fermentation & extraction: First of all, spore suspensions of selected strains of the fungus were prepared by adding 5 mL of sterile distilled water in fully grown slants. Sterilized potato dextrose broth, contained in 250 mL Erlenmeyer flasks was inoculated with 1 mL of spore suspension. It was then incubated for fifteen days at 30°C under static conditions. After fifteen days, the culture broth was filtered by using muslin cloth and the supernatant so obtained was used for the extraction of fungal metabolites. The broth culture was mixed with equal amount of ethyl acetate and was mixed properly with vigorous shaking. It was shifted in separating funnel and allowed to stand for 15 min. Subsequently, the upper organic layer was separated, and the solvent was removed via rotary evaporation. The resulting semi-solid extract was then preserved for further investigation of its pharmacological properties.

Antibacterial assay: For the antibacterial assay, we opted to utilize five bacterial strains sourced from the culture bank of IIB, GC University Lahore. These included Gram-positive strains such as *Staphylococcus aureus* and *Bacillus subtilis*, as well as Gram-negative strains such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae*. Muller Hinton Agar (MHA) was used for the activation and maintenance of bacterial cultures. Disc diffusion method was used for antibacterial assay of the fungal samples. The 24 hrs old bacterial cultures of *P. aeruginosa, E. coli, K. pneumonia, S. aureus* and *B. subtilus* were evenly streaked on the sterile petri plate. The sterile filter paper discs (6 mm in diameter) were soaked in fungal extracts (100 uL) and then placed in petri plates above the agar surface. The petri plates were covered up after labeling and were incubated overnight at 37°C. After that, the zones of inhibition were measured using mm scale (Zhandabayeva *et al.*, 2021). Ciprofloxacin (5µg) was used as control for each bacterial strain.

Antifungal assay: The antifungal activity of ethyl acetate fungal extracts was assessed against fungal strains including *Candida albicans*, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus oryzae* using the disc diffusion method. Sabouraud Dextrose Agar (SDA) plates were employed for this experimental procedure. Fresh inoculum/spore suspension (10^5 spores/mL) of fungi was prepared from SDA slants. We used sterilized filter paper discs (6 mm in diameter) and dipped them in fungal samples (100 uL) and then put them in petri plates on the surface of agar. They were then incubated at 30°C for three days and then zone of inhibition of fungal growths were measured using mm scale (Zhandabayeva *et al.*, 2021). Vericonzol was used standard antifungal agent for comparison.

Antioxidant activity: The DPPH assay was used after slight modification for the assessment of anti-oxidant activity *A. oryzae* and *A. flavus* ethyl acetate extracts. We freshly prepared 0.004 % solution of DPPH in methanol and mixed 5 mL of it with 0.5 mL of fungal extracts in dark room. The mixture was then incubated for half an hour at room temperature and absorbance was read at 517 nm with the help of spectrophotometer. The test tube marked as control contained only DPPH and butylated hydroxy anisole (BHA) was used as a positive control.

Pharmacological activities: Antidiabetic and anti-inflammatory activity of ethyl acetate extract of *A. flavus* (EAe.*A.f*) and ethyl acetate extract of *A. oryzae* (EAe.*A.o*) was performed. Pharmacological activities were performed on male Swiss albino mice (25 ± 5 g) of 4-5 weeks old only. The animals were put in animal house of Dep. of Zoology, GC University Lahore, Pakistan. The animals were provided standard living conditions i.e. temperature 37°C, 50% humidity and alternative light and dark period of 12 hrs each. The ethical approval for the use of animals was obtained by animal's ethics committee of the Zoology department of GC University vide letter no ZOO-AEC-GC-U-1792-22. The animals were acclimatized in the experimental zone of animal house for one week before the start of experiments and were provided free excess to food and water *ad libitum*.

Anti-inflammatory activity: For assessment of anti-inflammatory activity of ethyl acetate extract of *A. flavus* (EAe.*A.f*) and ethyl acetate extract of *A. oryzae* (EAe.*A.o*), we used hind paw edema model. The animals were divided into four groups; normal control (administered only normal saline), standard control (administered with diclofenac sodium 10 mg/Kg), Test control-1 (administered with 400 mg//Kg EAe.*A.f*) and Test control-1I (administered with 400 mg//Kg EAe.*A.o*). The inflammation was induced in the left hind paw of all the mice of all groups except normal control group by administration of 0.1 ml of carrageenan (1 % w/v) on sub plantar surface with the help of syringe. After administration of carrageenan, it was waited until the induction of inflammation and edema (Tekulu *et al.*, 2020). The inflammation and edema was measured by using Vernier caliper and after induction of inflammation the standard drug and test substances were applied to standard control and test control groups and reduction in inflammation and edema was observed up to 4 hrs.

Antidiabetic activity: Antidiabetic activity of ethyl acetate extract of *A. flavus* (EAe.*A.f*) and ethyl acetate extract of *A. oryzae* (EAe.*A.o*), was assessed in streptozotocin induced hyperglycemic albino mice. Fresh solution of streptozotocin was prepared by dissolving streptozotocin in sterile citrate buffer of pH 4.5. The animals were divided into four groups (n = 6); normal control (administered only normal saline), standard control (administered with Metformin 500 mg/Kg//p.o), Test control-1 (administered with 400 mg//Kg EAe.*A.f*) and Test control-1I (administered with 400 mg//Kg EAe.*A.o*). For induction of hyperglycemia the animals of all the groups except normal control group, were administered single intraperitoneal dose of streptozotocin 150 mg/Kg. Hyperglycemia was induced over a period of approximately four days, and blood glucose concentrations were assessed using a glucometer via sampling a drop of blood from the tail of mice. Only those mice exhibiting blood glucose levels exceeding 200 mg/dL were enrolled in the study. The standard drug and test extracts were orally administered to the animals for three weeks after induction of hyperglycemia and blood glucose level was measured on 1st, 7th, 14th and 21st day of treatment to assess the antidiabetic activity (Nguyen *et al.*, 2020).

Acute toxicity study: Acute toxicity study of ethyl acetate extract of *A. flavus* (EAe.*A.f*) and ethyl acetate extract of *A. oryzae* (EAe.*A.o*), was performed on 6-8 week old female albino mice. They were divided into different groups with n = 5. The animals were administered with single oral doses of EAe.*A.f* and EAe.*A.o*, respectively to different groups in dose of multiple of 1000 mg/Kg and dose is increased until the animals died. In this way the median lethal dose LD_{50} of both test extracts were found and the animals were observed for the signs of toxicity after administration of each dose i.e., sedation, hypnosis, piloeraction, hyper stimulation, cyanosis and tremors (Mushtaq *et al.*, 2021).

Statistical analysis: The data were expressed as mean ± S.E and One-way ANOVA followed by Dunnets *t*-test was applied as statistical tool to analyze the data. Graph pad Prism version 07 was used as a software to analyze the data set. The level of significance was marked as $p<0.05$.

Results

Soil sampling: Nine soil samples were collected from which 36 fungal strains were isolated (Table 1), out of which nine fungal isolates were resembling *Aspergillus* species. Those strains were further studied by re-steaking them on PDA plates and the pure colonies were studied for their characteristics first by gross and then by microscopic examination (Table 1). The soil samples collected from Gujranwala and Lahore more abundantly contained *A. oryzae* and *A. flavus* which were then used in the fermentation processes and the biological activities of the fermented extracts were found.

Antibacterial and antifungal activity: Antimicrobial activities as studied by disc diffusion method indicated that ethyl acetate extract of *A. oryzae* (EAe.*A.o*) showed maximum zone of inhibition (10.9±0.02 mm) against *S. aureus.* While zones of inhibition against *B. subtilis* and *E. coli* were recorded as 10.6±0.05 mm and 10.2±0.01 mm, respectively. Similarly, ethyl acetate extract of *A. flavus* (EAe.*A.f*) presented maximum growth (20.1±0.01 mm) against *B. subtilis.* Zone of inhibition by ciprofloxacin has also been shown in the table 2. Neither of the fungal strains showed antibacterial activity against *P. aeruginosa* and *K. pneumonia* (Table 2). The results of antifungal activity indicated that both the fungal extracts (EAe.*A.o* and EAe.*A.f*) did not show inhibition of growth against *A. fumigatus, A. niger, C. albicans, A. oryzae* and *S. cerevisiae* (Table 2).

Antioxidant activity: The results of in-vitro antioxidant study indicated that EAe.*A.f* extract showed maximum free radical scavenging activity 72.82% at a concentration of 150 µg/mL while least activity was recorded as 57.19% at a concentration of 400 µg/mL. It was also observed that maximum free radical scavenging activity was noted as 36.47% by EAe.*A.o* extract at a concentration of 150 µg/mL (Table 3).

Table 1. Number of fungal strains of *Aspergillus* species isolated from different locations along with their general colony characteristics.

Sample No.	No of strains isolated	Fungi	Location	Topography	Texture	Color
LS-1	2	*A. niger*	Lahore	Irregular	Powdery	White with Black spores
LS-2	3	*A. flavus*	Lahore	Irregular	Powdery	Green
LS-3	6	*A. parasiticus*	Lahore	Irregular	Powdery	Green
GS-1	4	*A. versicolor*	Gujranwala	Regular	Powdery	Green
GS-2	9	*A. oryzae*	Gujranwala	Irregular	Powdery	Green
GS-3	5	*A. sulphureus*	Gujranwala	Flat	Velvety	Dirty white with Orange spores
WS-1	1	*A. fumigatus*	Wah Cantt	Irregular	Powdery	Dark Green
WS-2	6	*A. nidulans*	Wah Cantt	Irregular	Powdery	Dirty Green
WS-3	5	*A. terreus*	Wah Cantt	Flat	Velvety	Cinnamon Brown

Table 2. Antibacterial & Antifungal activity of *A. oryzae* and *A. flavus* against different strains of bacteria and fungi.

Organisms		Zone of inhibition (mm) for antibacterial & antifungal Activity		Control (Ciprofloxacin and vericonazol)
		Ethyl acetate extract of *A. oryzae* (EAe.*A.o*)	Ethyl acetate extract of *A. flavus* (EAe.*A.f*)	
Bacterial strains	*E. coli*	10.2 ± 0.01	10.2 ± 0.02	24
	B. subtilis	10.6 ± 0.05	20.1 ± 0.01	20
	S. aureus	10.9 ± 0.02	10.3 ± 0.01	23
	P. aeruginosa	-	-	24
	K. pneumonia	-	-	22
Fungal strains	*A. fumigatus*	-	-	10
	A. niger	-	-	12
	C. albicans	-	-	10
	A. oryzae	-	-	10
	S. cerevisiae	-	-	

Table 3. Percentage inhibition of DPPH by ethyl acetate extracts of *A. oryzae* & *A. flavus*.

No.	Sample concentrations (µg/ml)	Percentage inhibition of DPPH (%)	
		Ethyl acetate extract of *A. oryzae* (EAe.*A.o*)	Ethyl acetate extract of *A. flavus* (EAe.*A.f*)
1.	150	36.47	72.82
2.	200	28.41	62.65
3.	250	27.17	64.64
4.	300	31.51	60.42
5.	400	27.79	57.19

Table 4. Anti-inflammatory activity of ethyl acetate extracts of *A. oryzae* & *A. flavus* in albino mice.

Groups	Treatment	Paw volume (mm)				
		0 hr	1 hr	2 hr	3 hr	4 hr
Normal control	Normal saline 5 mL/kg	0.23 ± 0.02	0.20 ± 0.01	0.22 ± 0.03	0.20 ± 0.03	0.21 ± 0.04
Negative control	Carrageenan (1 % w/v)	0.56 ± 0.05	0.54 ± 0.03	0.53 ± 0.07	0.49 ± 0.04	0.45 ± 0.03
Standard control	Diclofenac sodium 10 mg/Kg	0.55 ± 0.06	0.41 ± 0.05	0.35 ± 0.04	0.31 ± 0.04	0.23 ± 0.05
Test control-I	Ethyl acetate extract of *A. oryzae* (EAe. *A.o*) 400 mg/kg	0.57 ± 0.05	0.42 ± 0.07	0.38 ± 0.05	0.36 ± 0.04	0.24 ± 0.04
Test control-II	Ethyl acetate extract of *A. flavus* (EAe. *A.f*) 400 mg/kg	0.55 ± 0.03	0.36 ± 0.06	0.32 ± 0.07	0.30 ± 0.04	0.22 ± 0.08

Anti-inflammatory activity: The results indicated that the administration of caragenan significantly increased ($p<0.05$) the paw volume in albino mice in comparison to normal control group. After induction of inflammation in the animals the treatment of animals with ethyl acetate extract of *A. flavus* (EAe.*A.f*) 400 mg/kg significantly reduced the paw volume from 0.55±0.03 mm to 0.22±0.08 in hrs. Similarly, the administration of animals with standard drug diclofenac sodium to standard control group and ethyl acetate extract of *A. oryzae* (EAe.*A.o*) 400 mg/kg to test control-I also significantly reduced the paw volume up to 0.23±0.05 mm and 0.24±0.04 mm respectively, in comparison to negative control group in six hours (Table 4). The EAe.*A.f* 400 mg/kg treated mice showed 43.5±0.7% reduction in paw volume after six hrs is comparison of negative control group. Similarly, treatment of animals with EAe.*A.o* 400 mg/kg reduced the inflammation up to 42.9±1.3 % in six hrs (Table 5).

Antidiabetic activity: It was observed that the animals treated with EAe.*A.o* 400 mg/Kg lowered blood glucose level up to 255.33±17.21 mg/dL after 21st day of treatment in comparison to standard control drug metformin which presented the values as 222.72±14.55 mg/dL. The treatment of animals with EAe.*A.f* 400 mg/Kg lowered blood glucose (359.11±18.16 mg/dL) less

significantly in comparison to standard control animals. Hence, the results indicated that EAe.*A.f* 400 mg/Kg possess more antidiabetic potential than that of EAe.*A.f* 400 mg/Kg (Table 6).

Effect on body weight: Induction of diabetes by using streptozotocin significantly reduced the body weigh in mice in comparison to normal control group. Streptozotocin treated mice presented body weight as 28.11±1.66 and 30.08±1.9 g respectively on 14th and 21st day of study in comparison to 31.11±1.8 and 33.31±1.7 g of the normal control group. The animals administered with metformin did not exhibit a significant alteration in body weight when compared to the normal control group. Similarly, the mice treated with either EAe.*A.o* 400 mg/Kg or EAe.*A.f* 400 mg/Kg did not present any significant change in body weight of mice. Their body weights were recorded as 32.27±1.9 and 33.21±2.1 respectively on 21st day of study (Table 7).

Acute toxicity: Animals were administered with single acute doses of test extracts and it was observed that the no animal died up to a dose level of 2000 mg/Kg. The animals started dying at dose level 3000 mg/Kg for both extracts. It was observed that all the animals died when extracts were administered in 4000 mg/Kg. Hence the LD_{50} values were calculated as 1100 and 1300 mg/Kg respectively for EAe.*A.o* and EAe.*A.f* (Table 8). Moreover, no clear signs for sedation, hypnosis, piloeraction, hyper stimulation, cyanosis and tremors were observed after administration of acute toxic doses in animals.

Table 5. Percentage inhibition of edema in mice after treatment with ethyl acetate extracts of *A. oryzae* & *A. flavus*.

Groups	Treatment	Percentage inhibition in paw volume (%)			
		1 hr	2 hr	3 hr	4 hr
Test control-I	Ethyl acetate extract of *A. oryzae* (EAe. *A.o*) 400 mg/kg	29.6 ± 1.6	32.0 ± 1.2	42.8 ± 1.9	42.9 ± 1.3
Test control-II	Ethyl acetate extract of *A. flavus* (EAe. *A.f*) 400 mg/kg	32.0 ± 1.1	37.2 ± 1.9	37.5 ± 0.8	43.5 ± 0.7

Table 6. Antidiabetic activity of ethyl acetate extracts of *A. oryzae* & *A. flavus* in albino mice.

Groups	Treatment	Blood glucose level (mg/dL)			
		1st Day	7th Day	14th Day	21st Day
Normal control	Normal saline 5 mL/kg	88.33 ± 17.18	84.52 ± 14.21	89.76 ± 15.88	91.14 ± 16.08
Negative control	Streptozotocin 150 mg/Kg/ip	449.17 ± 21.14	498.87 ± 17.22	549.12 ± 18.12	559.88 ± 23.11
Standard control	Metformin 500 mg/Kg	402.72 ± 20.19	302.93 ± 16.71	272.12 ± 19.19	222.72 ± 14.55
Test control-I	Ethyl acetate extract of *A. oryzae* (EAe.*A.o*) 400 mg/kg	422.39 ± 25.28	366.54 ± 19.29	288.96 ± 14.88	255.33 ± 17.21
Test control-II	Ethyl acetate extract of *A. flavus* (EAe.*A.f*) 400 mg/kg	438.33 ± 19.15	412.61 ± 14.59	401.09 ± 17.21	359.11 ± 18.16

Table 7. Effect of ethyl acetate extracts of *A. oryzae* & *A. flavus* *A. oryzae* on body weight in albino mice.

Groups	Treatment	Body weight (g)			
		1st Day	7th Day	14th Day	21st Day
Normal control	Normal saline 5 mL/kg	25.29 ± 1.3	28.71 ± 2.1	31.11 ± 1.8	33.31 ± 1.7
Negative control	Streptozotocin 150 mg/Kg/ip	24.42 ± 2.1	26.18 ± 1.7	28.11 ± 1.66	30.08 ± 1.9
Standard control	Metformin 500 mg/Kg	23.91 ± 2.3	28.88 ± 1.6	31.22 ± 1.71	35.66 ± 1.8
Test control-I	Ethyl acetate extract of *A. oryzae* (EAe.*A.o*) 400 mg/Kg	26.91 ± 1.7	28.96 ± 1.9	30.12 ± 1.81	32.27 ± 1.9
Test control-II	Ethyl acetate extract of *A. flavus* (EAe.*A.f*) 400 mg/kg	24.71 ± 1.6	27.51 ± 1.59	29.99 ± 1.21	33.21 ± 2.1

Table 8. Acute toxicity study EAe. *A.o* and EAe. *A.f* along with calculation of LD_{50}.

Groups	Acute toxicity of EAe. *A.o*				Groups	Acute toxicity of EAe. *A.f*			
	Dose difference (X)	Mortality	Mean mortality (Y)	X * Y		Dose difference (X)	Mortality	Mean mortality (Y)	X * Y
Toxic control-IA (1000 mg/Kg)	0	0	0		Toxic control-IB (1000 mg/Kg)	0	0	0	
Toxic control-IIA (2000 mg/Kg)	1000	0	0		Toxic control-IIB (2000 mg/Kg)	1000	0	0	
Toxic control-IIIA (3000 mg/Kg)	1000	3	1.5	1500	Toxic control-IIIB (3000 mg/Kg)	1000	4	2	2000
Toxic control-IVA (4000 mg/Kg)	1000	5	4	4000	Toxic control-IVB (4000 mg/Kg)	1000	5	4.5	4500

Discussion

Pharmaceutical industries generally rely on bioactive molecules which are synthesized for the management of different diseases. Nature is the biggest source of biologically active ingredients which lead to the development of potentially active therapeutic agents (Adeoyo *et al.*, 2019; Havenga *et al.*, 2019). The fungal kingdom represents a significant resource in this regard, comprising nearly one and a half million species. However, only approximately 5% of these fungal species have been thoroughly explored to date. There exists a compelling necessity for further scientific investigation aimed at pharmacologically evaluating the diverse metabolites produced by fungi (Saleem *et al.*, 2007; Khattak *et al.*, 2021). Among all fungal genera, *Aspergillus* genus is famous for the isolation and development of secondary metabolic products which display their anticancer, antifungal and antibacterial activities (Khattak *et al.*, 2018; Zulqarnain *et al.*, 2020). Among *Aspergillus* species, *A. oryazae* is one of the famous biotechnological tool used in fermentation industries (Son *et al.*, 2018) and *A. flavus* finds its application in herbicide industry (Khattak *et al.*, 2018).

By knowing the importance of *Aspergillus* species the study was conducted to evaluate the soil samples of different places to identify the fungal strains. The organic matter and appropriate moisture contents in soil provide the best medium for microbial growth (Delgado *et al.*, 2021). Out of thirty-six samples, the nine were confirmed to contain *Aspergillus* species which were grown on PDA medium for further isolation. Researches have proved that PDA is an excellent medium for growth of mycelia of fungi (Al-Fakih & Almaqtri, 2019). So, we used this media for the rapid growth and isolation of fungal strains. Fungal strains were isolated through the examination of their phenotype, revealing a prevalence of Aspergillus strains in our samples. Prior studies have indicated that soil serves as a prolific reservoir of Aspergillus species. Building upon this understanding, we adopted a soil-centric approach for this study, collecting soil samples to isolate fungal strains.

After isolation of two fungal strains *A. flavus* and *A. oryazae* from soil samples we prepared ethyl acetate extracts of them after fermentation which were named; ethyl acetate extract of *A. flavus* (EAe.*A.f*) and ethyl acetate extract of *A. oryzae* (EAe.*A.o*). Both the extracts, EAe.*A.f* and EAe.*A.o* were first tested for *in-vitro* antioxidant activity and it was found that both of them presented marked antioxidant potential. Previously, a number of compounds belonging to polysaccharides (α-D-glucans), monosaccharides (glucose) and proteins have been isolated from *A. flavus* which are responsible for antioxidant activity (Khattak *et al.*, 2021). Similarly, *A. oryzae* is responsible for production of phenols (gallic acid), flavonoids (gallocatechin) and related metabolites during fermentation process which are responsible for its antioxidant potential (Shaaban *et al.*, 2014). The antioxidant activity is indirectly linked to anti-inflammatory activity as suggested by our findings. Both extracts significantly reduced the edema and inflammation in mice which might be due to the inhibition of toxic free radicals which are induced by carrageenan (Makni *et al.*, 2019). Furthermore, it has also been reported that during fermentation process, *A. oryzae* and *A. flavus* are responsible to secrete out certain enzymes responsible for metabolism of inflammatory proteins and carbohydrates (Son *et al.*, 2018).

The results of antimicrobial testing on EAe.*A.f* and EAe.*A.o* extracts revealed significant antibacterial activity against *E. coli, S. aureus,* and *B. subtilis*. However, these extracts did not demonstrate antibacterial efficacy against *P. aeruginosa* and *K. pneumoniae*. Similarly, no antifungal effects were observed against *C. albicans, A. oryzae, A. niger, S. cerevisiae,* and *A. fumigatus* with both extracts. It is noteworthy that Aspergillus species are known to produce bioactive secondary metabolites capable of inhibiting the growth of various bacterial strains (Wang *et al.*, 2017; Al-Fakih & Almaqtri, 2019). Coumarins, unsaturated fatty acids, coumarins, pentahydroxy anthraquinone, benzopyrone compounds, dihydroxydodecanoic acid and hexylitaconic acids are potent antibacterial secondary metabolites have been reported to be produced by *A. oryzae* after fermentation process (Son *et al.*, 2018).

Previous studies have also reported that the *A. flavus* is responsible for production of potentially active antimicrobial metabolites. Seurotin-A is one of the most active metabolite is involved in inhibition of microbial growth against various bacterial strains (Khalid, 2020). Thus the antibacterial activity shown by EAe.*A.o* and EAe.*A.f* against the aforementioned bacterial strains might be due to the presence of their actively secreted metabolic products. Both of our extracts remained fail to show antifungal activity. The possible reasoning of this fact is that the fungal strains against which antifungal potential was noted might be due to development of resistant against EAe.*A.f* and EAe.*A.o.* The previous studies have shown that the different fungi have evolved the resistance mechanisms against antifungal test substance. For example, *A. flavus* show resistance against conventional antifungal substance due to the presence of its high β-D-glucan contents (Lima *et al.*, 2019).

The findings suggest that both the fungal extracts; EAe.*A.f* and EAe.*A.o* show strong antidiabetic activity against streptozotocin induced hyperglycemia in mice. The fermentation process is responsible for the production of several secondary metabolic products by the fungal strains. These metabolic products are responsible for reducing the oxidant load in the pancreas of mice (García-Mora *et al.*, 2017). It has been investigated that *A. flavus* and *A. oryzae* produce certain phenolic compounds, bioactive peptides and precursor proteins which present marked antidiabetic activity (Nguyen *et al.*, 2020). The results indicated that the treatment of animals with EAe.A.*f* and EAe.*A.o.* for consecutive 21 days significantly reduced the body weights as compared to normal control group. This has been proposed that the treatment of animals with fungal extracts is responsible for cellular intake of glucose (El-Gharbawy *et al.*, 2016), thus less plasma glucose was available to be converted into fats in liver. Our findings of acute toxicity studies suggested LD_{50} values as 1300 and 1100 mg/Kg, respectively for EAe.A.*f* and EAe.*A.o*. Previously, various toxic substances like gliocladic acid,

fellatunine, meleagrin, heptelidic acid, cordycepin and kojic acid have been reported to be produced by *A. flavus* (Kovač *et al.*, 2020). Theses toxins in higher concentrations may be proved lethal for living organisms. However, our findings suggested that the both of fungal extracts are considerably safe and did not produce cyanosis, hypersecretions, hyper salivations, excitability, sedation or hypnosis in mice.

Conclusion

The outcomes of our investigation reveal that both the fermented ethanolic extracts derived from *A. oryzae* and *A. flavus* exhibit robust antibacterial activity against *B. subtilis, S. aureus,* and *E. coli.* Furthermore, they demonstrate potent antioxidant properties and display strong and rapid anti-inflammatory effects, in addition to exhibiting antidiabetic activity. Acute toxicity studies indicate that both extracts are reasonably safe; however, comprehensive toxicity assessments are warranted to elucidate potential chronic toxic effects. Similarly, further research is needed to elucidate the mechanisms underlying their antibacterial, anti-inflammatory, and antidiabetic activities.

Declaration of competing interest: It is hereby declared that there is no any conflict of interest. The ethical approval for the use of animals was obtained by animal's ethics committee of the Zoology department of GC University, Lahore vide letter no ZOO-AEC-GC-U-1792-22.

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