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Antimicrobial and Immunostimulatory Effects of *Senna occidentalis* Ethanolic Extract against *Aspergillus flavus*-Induced Mycotic Keratitis: A Promising Therapeutic Approach

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ABSTRACT

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Background: Mycotic keratitis (MK) poses a significant risk as a severe and difficult-to-treat corneal infection with potentially life-threatening consequences. Fungi are commonly implicated as causative agents. Additionally, improper care of contact lenses increases the susceptibility to MK. **Aim:** This study aimed to investigate the phytochemical composition and therapeutic potential of *Senna occidentalis* ethanolic leaf extract in the treatment of *Aspergillus flavus*-induced mycotic keratitis. **Methodology:** The selected plant extract was tested for antimicrobial activity, analysed for active ingredients and evaluated in vivo for physiological (Kidney and liver functions), histological (corneal tissue healing rates) and immunological responses (IgM levels). **Results:** The GC/MS analysis revealed high levels of some active components, including o-methyl glucose, tetramethyl hexadecenol, hexadecenoic acid, and octadecatrienoic acid. The ethanolic extract of *S. occidentalis* demonstrated potent antimicrobial activity against *A. flavus* in an albino rabbit model. Treatment with the extract at a concentration of 12.5 mg/ml resulted in a significant healing rate without any side effects, as evidenced by reduced levels of blood urea, creatinine, AST, and ALT in infected rabbits. Moreover, *S. occidentalis* extract effectively stimulated the immune system, as indicated by increased levels of IgM in both infected and non-infected rabbits. Histopathological analysis of the eye cornea further confirmed the complete disappearance of inflammation and restoration of normal tissue appearance and layer arrangement. **Conclusions:** These findings highlight the potential of *S. occidentalis* extract as a safe, affordable, and easily accessible treatment for *A. flavus*-induced mycotic keratitis, owing to its rich content of safe and bioactive phytochemicals.

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Keywords: Aspergillus flavus, Senna occidentalis, mycotic keratitis, physiological parameters, corneal histology.

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1. INTRODUCTION

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Mycotic keratitis (MK) is the inflammation of corneal stroma due to the invasion of fungal elements, which is an exogenous infection, entering through injuries of the corneal epithelium [1]. Fungal keratitis is highly invasive into the layers of corneal stroma and antifungal agents are mostly static, leading to prolonged treatment and even surgical intervention [2,79,80]. The occurrence and type of filamentous fungal keratitis are significantly impacted by several environmental conditions, including humidity, rainfall, and wind, as

23 well as seasonal fluctuations [3]. Less common predisposing variables that can contribute to mycotic
24 keratitis include the use of antibiotics or corticosteroids, allergic conjunctivitis, immunological
25 incompetence, and the use of hydrophilic contact lenses [4]. Furthermore, fungal keratitis is typically
26 associated with pre-existing ocular conditions such as insufficient tear secretion or defective eyelid closure,
27 as well as systemic conditions like diabetes mellitus or immunosuppression. It can also occur as a
28 secondary infection on abrasions caused by contaminated contact lenses or on pre-existing epithelial
29 defects resulting from herpes keratitis [5].

30 In two randomized controlled studies, it was found that many cases of mycotic keratitis were resistant to
31 chlorhexidine when administered as a weak substitute for natamycin [6]. *Candida*, a prevalent yeast in
32 ocular infections among immunosuppressed patients, was often managed using topical amphotericin B.
33 However, this treatment had poor efficacy and resulted in calcification of the corneal tissues. Echinocandins
34 (such as caspofungin and micafungin) and fluoroquinolones can be included as combination therapies to
35 provide comprehensive treatment [6]. Corticosteroids are highly undesirable because they promote the
36 growth of fungi by suppressing the immune mechanisms in the eye. This includes inhibiting the movement
37 of immune cells toward the infection site, preventing the ingestion of pathogens by immune cells, reducing
38 the number of immune cells, and blocking the release of certain substances. Therefore, it is not
39 recommended to use topical steroids in the treatment of fungal keratitis [7]. Topical cyclosporine A was
40 proposed as a complementary agent to antifungal treatment due to its ability to hinder the growth of
41 filamentous fungi. Although therapeutic penetrating keratoplasty in certain cases demonstrated sustained
42 graft survival, there is no clinical evidence suggesting that graft survival acts as a limiting factor for fungal
43 keratitis [7].

44 Throughout human history, plants have been utilized as the foundation for numerous traditional remedies
45 and continue to be utilized as supplies for various contemporary pharmaceuticals. According to the World
46 Health Organisation (WHO), approximately 60% of populations in regions with limited resources are unable
47 to acquire or purchase conventional medications. Consequently, almost three-quarters of these populations
48 depend upon medicinal plants to meet their primary healthcare demands [8]. Due to their availability,
49 affordability, safety, potential efficacy, and environmental friendliness, traditional herbal medicines have
50 recently attracted increased attention [9]. The development of Egyptian herbal medicine has a long and
51 complex history, influenced by several cultures. The ancient pharaonic and more contemporary Arabic
52 Unani medicine are the primary and most important origins of all formulations in today's herbal market [10].
53 Phytocompounds found in nature have been shown to have potential antifungal properties due to their
54 chemical composition, which allows them to exhibit various biological activities [11]. These phytochemicals
55 encompass a wide array of components such as phenols, flavonoids, tannins, alkaloids, essential oils,
56 polysaccharides, terpenoids, lignans, glycosides, and more [9].

57 In their study, Essien et al. [12] reported that the leaf oil of *S. occidentalis* demonstrated antimicrobial
58 effects against a range of bacteria including *P. aeruginosa*, *B. cereus*, *E. coli*, and *S. aureus*. However,
59 studies on the antifungal activity of *S. occidentalis* are extremely rare. Generally speaking, the aerial
60 portions of *S. occidentalis* were the only focus of the few research that was conducted on its antifungal
61 activities [13]. Before contemplating the examination of natural plant extracts as viable antifungal
62 alternatives for the management of fungal keratitis, deeper investigations must be performed to assess the

safety of host tissues and functions. These studies are critical to making well-informed decisions about the acceptability and effectiveness of these extracts in the treatment of eye diseases. According to our knowledge and a deep literature survey, there have been no reported studies on the use of *S. occidentalis* leaf extract for treating mycotic keratitis. Accordingly, the primary aims of this work was to report on the chemical composition and antifungal characteristics of leaf extracts from *S. occidentalis*, with the aim of investigating their possible use in the treatment of fungal keratitis.

2. MATERIALS AND METHODS

2.1. Clinical diagnosis of different corneal ulcers

The study conducted a survey on patients diagnosed with different types of corneal ulcers, specifically focusing on mycotic keratitis. The patients were regularly visited at the Ocular Microbiology lab in the Ophthalmology Hospital of Tanta University twice a week from January to December 2022. The symptoms associated with corneal fungal infections include initial foreign body sensation, gradual increase in pain, the presence of a thick area of keratitis with a sticky hypopyon, stromal infiltrates with feathery edges and epithelial defects, as well as coagulative necrosis resulting from the secretion of various fungal enzymes. These enzymes lead to the destruction of keratocytes and collagen lamellae, causing puncture of Descemet's membrane and the passage of fungi into the anterior chamber. Additionally, the study observed various complications accompanying active mycotic keratitis, such as endophthalmitis, perforation, ring abscess, corneal melting, guttering, facet formation, thinning, and corneal opacity. Photographs and clinical descriptions were used to summarize the clinical complications associated with fungal keratitis in all infected eyes.

2.2. Collection, purification, and identification of keratitis fungi

Samples were collected from patients diagnosed with mycotic keratitis using appropriate tools and precautions to avoid perforation and contamination. A sterile and controlled environment was maintained in the hospital laboratory. Corneal scrapings were streaked on Petri dishes containing Sabouraud's dextrose agar (SDA) medium supplemented with chloramphenicol to prevent bacterial contamination. The plates were incubated at 27°C for up to 21 days. Purified fungal colonies were identified and photographed using established references. Identification of fungal genera and species was based on the works of Houbraken et al. [14]. The research protocol was approved by the Research Ethics Committee of Tanta University with an approval code of **34895/9/21**.

2.3. Detection of pathogenicity tools of isolated fungi

The isolated fungi from corneal ulcers were tested for collagenase and protease activities, the main contributors to corneal ulcers. For the qualitative collagenase assay, fungal isolates were grown on a collagen-dependent liquid medium for one week. The supernatants from the cultures were tested for collagenase activity using a native bovine collagen substrate. The formation of a purple color indicated the presence of collagenase activity [15]. To assess protease activity, a modified method using casein as a substrate was employed. Agar plates containing casein were inoculated with fungal isolates and incubated.

101 The formation of cleared zones around the colonies indicated proteolysis, which was quantified as the
102 percentage ratio of the diameter of the cleared zone to the diameter of the colony [16].

103 For the quantitative estimation of collagenase activity, a liquid medium containing gelatin, glucose, yeast
104 extract, and native bovine collagen was prepared. Fungal isolates were incubated in the medium, and the
105 supernatants were collected. The concentration of L-leucine produced from collagen degradation was
106 measured, and the concentration of total proteins in the supernatants was determined. The collagenase
107 activity was calculated by dividing the L-leucine concentration by the total protein concentration [17].

108 **2.4. Collection, identification, and extraction of wild plants**

109 A total of eight wild plants (*Cotula sericea* [L.f.], *Senna alexandrina* [Mill.], *Senna occidentalis* [L.], *Senna*
110 *italica* [Mill.], *Heliotropium europaeum* [L.], *Pulicaria undulata* [L.], *Aerva javanica* [Burm.f.], and *Cleome*
111 *droserifolia* [Forssk.]) were collected from Halayeb and Shalateen reserved region, Red sea cost, Egypt in
112 the Spring season of 2022. The plants underwent meticulous air drying and were subsequently dried for
113 further investigation. To prepare the different extracts from the dried plants, the method outlined by Kasim
114 et al. [18] was followed.

115 **2.5. Assessment of the anti-fungal characteristics of the plant extracts**

116 A spore suspension of the collected fungi was mixed with sterile Sabouraud's dextrose medium and poured
117 onto sterile Petri dishes. Wells were created in the agar plates, and each well was filled with 20 mg of the
118 tested extract. Three replicates were prepared for each test, and all plates were incubated at 27°C for 3
119 days. The average diameters of the inhibition zones were then measured in millimeters and compared
120 across all plates [19]. The MIC of the investigated plant extracts was performed, and the findings
121 demonstrated that the ethanolic extract of *S. occidentalis* was the most potent plant extract against the
122 predominant fungal keratitis-causing agent *A. flavus*, with MIC of 12.5 mg/ml.

123 **2.6. Molecular Identification of *A. flavus* strain**

124 The predominant fungal keratitis-causing agent *A. flavus* isolate was cultivated on Sabouraud's dextrose
125 medium and subjected to incubation at 28°C for 5 days, as described by [20]. DNA extraction was carried
126 out utilizing the Patho-gene-spin DNA/RNA extraction kit manufactured by Intron Biotechnology, Korea.
127 Polymerase chain reaction (PCR) and sequencing procedures were conducted in collaboration with
128 SolGent, Daejeon, South Korea. The amplification of the ITS regions of the rRNA gene was accomplished
129 using the universal primers ITS1 (forward) and ITS4 (reverse), which were incorporated into the reaction
130 mixture. The primer compositions are as follows: ITS1 (5' - TCCGTAGGTGAACCTGCGG - 3') and ITS4 (5'-
131 TCCTCCGCTTATTGATATGC -3'). Subsequently, the purified PCR product was subjected to sequencing
132 using the same primers along with the incorporation of ddNTPs in the reaction mixture, following the
133 approach outlined by [21]. The obtained sequences were then subjected to analysis using the Basic Local
134 Alignment Search Tool (BLAST) available on the National Centre of Biotechnology Information (NCBI)
135 website. Finally, the analysis of the sequences and the construction of phylogenetic trees were performed
136 using MegAlign software version 5.05, developed by DNA Star.

137 The DNA sequence was aligned in the international GenBank database (http://www.ncbi.nlm.nih.gov/)
138 using BLAST. Evolutionary history was inferred by the Maximum Likelihood method based on the Tamura-

139 Nei model [22]. Evolutionary analysis was conducted by MEGA7 according to Kumar et al. [23] and the
140 nucleotide sequences were deposited in the GenBank.

141 **2.7. Phytochemical investigation of the ethanol extract of *S. occidentalis* using GC/MS**

142 The phytochemical composition of *S. occidentalis* ethanolic extract was performed using Clarus 580/560S
143 gas chromatography/mass spectrometry (GC/MS) (Perkin Elmer Inc., Waltham, MA, USA). An Elite-5 MS
144 column (30 mm x 0.25 mm x 0.25 µm film thickness) was utilized. The oven temperature was programmed
145 to start at 80°C for 7 min., followed by an increase of 10°C/min. until 140°C with a 1 min. hold, then held at
146 200°C for 1 mi., and finally held at 280°C for 10 min. with a rate of 5°C per min. The input and transfer lines
147 were maintained at 250°C. Helium gas was used as the carrier gas at a constant flow rate of 1 ml/min. A
148 sample of 1 µL was automatically injected after a solvent delay of 5 min. by autosampler AS3000 and GC in
149 split mode (1:20) at an ionization energy of 70 eV, EI mass spectra were obtained in full scan mode over
150 the range of m/z 40–650. The ionization chamber temperature was set at 200°C. The identified
151 components of the analyzed extract were determined by comparing their retention times and mass spectra
152 to those of WILEY-09 and NIST 11 mass spectral databases.

153 **2.8. Treatment of experimental animals with *S. occidentalis* ethanolic extract**

154 As a safety confirmative test for the usage of *S. occidentalis* ethanolic extract to be applied as an
155 antifungal agent in the treatment of corneal fungal infection of the experimental animals, the experimental
156 design for the present investigation was performed according to El-Badry [24]. The research protocol,
157 involving sampling, patient and animal model handling, was approved by the Research Ethics Committee
158 of Tanta University (approval code: 34895/9/21). Twins male albino rabbits of the same weight (2 kg) were
159 adapted to the lab conditions for 3 days before the start of work and divided into groups for different
160 treatments as follows: albino rabbits were maintained under a 12 h. light-dark cycle at a temperature of
161 22±2°C and fed with standard diet (Soy protein, 18%; Vitamins mixture, 3.5%; Choline chloride, 0.2%;
162 Soybean oil, 5.6%; Cellulose, 3.4%; L-lysine, 0.3% and Starch, 68.7%) and water. Each group of rabbits,
163 with 3 individuals, was housed separately in special cages. The groups were as follows:

164 *Group A:* Healthy rabbits with no infection and no treatment, ordinary follow-up for normal growth, and
165 normal incubation conditions.

166 *Group B:* The corneal surface of the right eyes of rabbits was infected with a spore suspension of *A. flavus*
167 (10^6/ml) by scratching and swabbing with the spore suspension once daily for a week until noticeable
168 fungal growth was established. The left eyes were left untreated to monitor the progression of the infection.

169 *Group C:* The right eyes of a healthy rabbit were treated twice daily for a week with a water suspension of
170 S. occidentalis ethanolic extract that was redissolved in water.

171 *Group D:* Healthy rabbit right eyes were dropped with the standard concentration of the commercial
172 antifungal drug twice daily for a week.

173 *Group E: A. flavus* infection of albino rabbits' right eyes, treated with the standard concentration of the
174 selected commercial antifungal drug by dropping onto the infected eyes with one drop every 2 h till the end
175 of treatment.

176 *Group F: A. flavus* infection of albino rabbits' right eyes, treated with a combination of the standard
177 concentration of the selected commercial antifungal drug and the MIC of *S. occidentalis* extract by dropping
178 onto the infected eyes every 2 h till the end of treatment.

179 *Group G: A. flavus* infection of albino rabbits' right eyes, treated with the MIC of *S. occidentalis* extract by
180 dropping onto the infected eyes every 2 h till the end of treatment.

181 *Group H: A. flavus* infection of albino rabbits' right eyes, treated with the double MIC of *S. occidentalis*
182 extract by dropping onto the infected eyes every 2 h till the end of treatment.

183 Following the completion of each treatment, rabbit eyes were photographed, and then rabbits were
184 sacrificed after 2 h of the last dose (without using anesthesia). Blood samples were collected from each
185 rabbit separately in sterile test tubes and kept for further assays and corneal samples were excised for
186 further histological examinations.

187 **2.9. Biochemical parameters**

188 **2.9.1. Determination of Serum AST and ALT levels**

189 The enzymatic activity of both aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was
190 determined using the method of Reitman and Frankel [25], employing the Diamond Diagnostic kit provided
191 by Diamond Co. Egypt. The procedure involved adding 500 µl of 100 mM phosphate buffer with a pH of
192 7.2, together with 80 mM L-aspartate as a substrate for AST or 80 mM D-L-alanine as a substrate for ALT,
193 and 4 mM α-ketoglutarate to 100 µl of rabbit's serum. The mixture was subjected to incubation at a
194 temperature of 37˚C for 30 min. Subsequently, the mixture was combined with 500 µl of the color
195 developing reagent (4 mM 2, 4-dinitrophenylhydrazine), and then incubated at room temperature for 20
196 min. After that, 5 ml of 0.4 N NaOH was added, followed by thorough mixing, and then incubation at room
197 temperature for 5 min. The absorbance was measured spectrophotometrically at 546 nm against the blank
198 (assay combination without enzyme source). The AST and ALT activities were quantified as U/L AST and
199 U/L ALT, respectively.

200 **2.9.2. Determination of serum creatinine and urea levels**

201 The quantification of creatinine level in the rabbit serum samples was performed following the
202 manufacturer's instructions outlined in the Randox reagent kit manual (Crumlin, County Antrim, UK). The
203 development of a yellow-orange colored complex with alkaline picrate, which could be measured at 492
204 nm, was the basis of creatinine determination [26]. The serum urea concentration was quantitatively
205 determined using the Biolab Kit (Maizy, France) employing enzymatic reaction. The measurement was
206 conducted at 578 nm, following the methodology established by Smith et al. [27].

207 **2.9.3. Determination of serum IgM level**

208 Serum immunoglobulin M (IgM) levels were quantitatively determined using an enzyme-linked 209 immunosorbent assay (ELISA) method based on the protocol described by Takahashi et al. [28]. Various 210 solutions and reagents were prepared, including coating buffer, wash solution, blocking solution, and 211 sample/conjugate diluent. The step-by-step procedure involved coating the wells with a capture antibody, 212 followed by the addition of an HRP detection antibody. The wells were then washed, and an enzyme 213 substrate solution was added, allowing for a reaction to occur. The reaction was stopped using 2 M H_2SO_4, 214 and the absorbance of the plate was measured at 450 nm. The results were calculated by determining the 215 mean value of duplicate readings for each standard, control, and sample, and subtracting the blank 216 reading. The concentration of IgM was calculated using a previously prepared standard curve.

217 **2.10. Histological examination of corneal tissues**

218 The excision of rabbit right eye corneas in the experimental groups was performed according to the 219 protocol outlined by Fischer et al. [29]. The corneas were fixed using alcoholic Bouin fixative, which 220 involved injecting 0.1 ml of the fixative into the anterior chamber of the eye and dropping another 0.1 ml 221 onto the eye surface. After excision, the corneal samples were immediately immersed in the fixative for 24 222 h. Subsequently, the samples underwent a series of steps, including washing with distilled water, 223 dehydration in ethanol, soaking in a solution containing eosin dye, clarification in xylene, embedding in 224 molten paraffin, and sectioning into 5 μm thickness using a microtome. The sections were then stained with 225 hematoxylin and eosin, dehydrated, cleared with xylene, and mounted using a Hoyer mounting medium. 226 The resulting slides were examined under a digital microscope, and images were captured at a 227 magnification of 400X (Celestron Pentaview, USA).

228 **3. RESULTS**
229
230 **3.1. Diagnostic assessment of corneal ulcers**

231 The survey conducted at the Ocular Microbiology lab, Ophthalmology Hospital, Tanta University, from 232 January 2022 to December 2022, revealed that patients diagnosed with various corneal ulcers resulting 233 from corneal fungal infections experienced initial sensations of a foreign object and subsequently 234 experienced escalating pain. Observations revealed a dense region of keratitis accompanied by a viscous 235 hypopyon, stromal infiltrates characterized by delicate borders, and an area of epithelial defect. The fungal 236 enzymes, including phospholipase, collagenase, and proteases, induced coagulative necrosis, resulting in 237 the depletion of keratocytes and the breakdown of collagen lamellae. Consequently, Descemet's 238 membrane was punctured, enabling the fungus to infiltrate the anterior chamber. Mycotic keratitis that is 239 active is frequently associated with complications such as endophthalmitis, perforation, ring abscess, 240 corneal melting, gutter formation, facet formation, thinning, and corneal opacity.

241 **3.2. Characterization of keratitis-causing fungi**

242 According to our earlier investigation [30], we found that the fungal species responsible for mycotic ulcers 243 ranked in the following order: *Aspergillus flavus* > *Aspergillus niger* > *Candida albicans* > *Mucor fuscus*. We

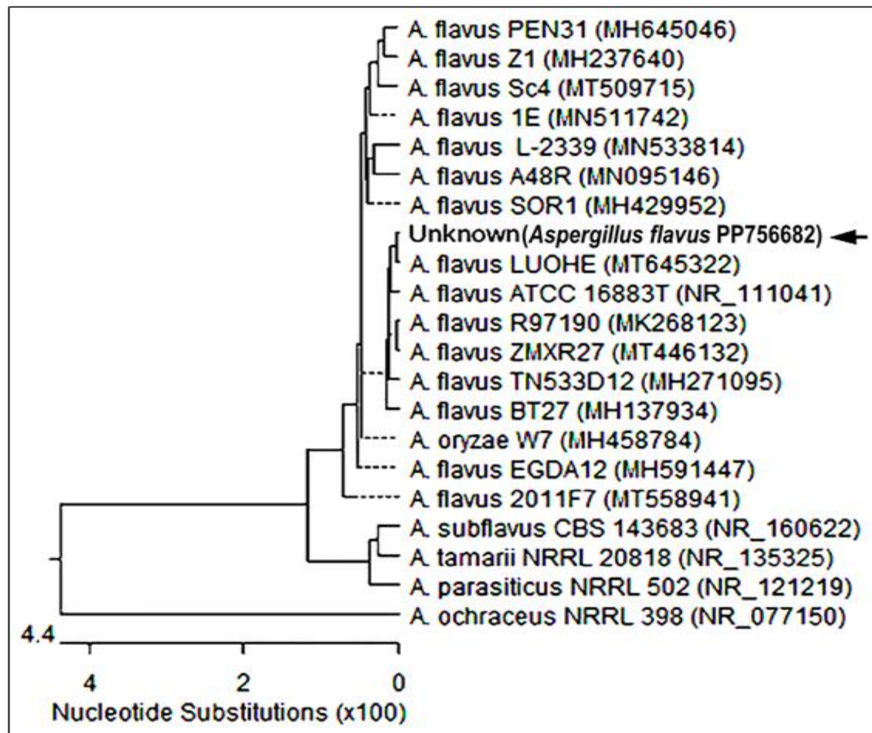
244 assessed the hydrolytic activity of the obtained isolates by conducting casienase and collagenase assays,
245 as these fungi have aggressive capabilities that contribute to their spread in corneal diseases. Our previous
246 investigation revealed significant levels of activity in both casienase and collagenase enzymes in
247 *Aspergillus flavus*, suggesting its prominent function as a corneal invader.

248 **3.3. The anti-fungal characteristics of the investigated plant extracts**

249 Our earlier study demonstrated that the ethanolic extract derived from *Senna occidentalis* exhibited the
250 highest efficacy as an antifungal agent compared to other examined plants. Specifically, it displayed
251 significant effectiveness against *Aspergillus flavus*, which is the most prevalent causal agent of fungal
252 keratitis. Therefore, the ethanolic extract of *S. occidentalis* was selected to investigate its active
253 components to determine its potential as a natural and safe treatment for fungal keratitis.

254 **3.4. Molecular identification of *A. flavus***

255 The utilization of 5.8S-ITS DNA sequences has been documented as a reliable method for the identification
256 and classification of *A. flavus* [31]. Through phylogenetic analysis, it was verified that the *A. flavus* isolate
257 PP756682 represents a mycelial fungus belonging to the Aspergillaceae family and Eurotiales order,
258 characterized by the production of closed fruiting bodies (Figure 1 and Table 1). The 5.8S-ITS DNA
259 sequence of *A. flavus* PP756682 was deposited in the GenBank database with the accession number
260 PP756682.1.



261

262 **Fig.1:** Molecular phylogenetic analysis of the selected *Aspergillus flavus* PP756682 isolate.

263 **Table 1:** Partial sequencing of 18s rRNA of *Aspergillus flavus* PP756682.

No.	Sequence	No.
1	ctgcggaagg atcattaccg agtgtagggt tcctagcgag cccaacctcc cacccgtgtt	60

61	tactgtacct tagttgcttc ggcgggcccg ccattcatgg ccgccggggg ctctcagccc	120
121	cgggcccgcg cccgccggag acaccacgaa ctctgtctga tctagtgaag tctgagttga	180
181	ttgtatcgca atcagttaaa actttcaaca atggatctct tggttccggc atcgatgaag	240
241	aacgcagcga aatgcgataa ctagtgtgaa ttgcagaatt ccgtgaatca tcgagtcttt	300
301	gaacgcacat tgcgcccccct ggtattccgg ggggcatgcc tgtccgagcg tcattgctgc	360
361	ccatcaagca cggcttgtgt gttgggtcgt cgtcccctct ccggggggga cgggccccaa	420
421	aggcagcggc ggcaccgcgt ccgatcctcg agcgtatggg gctttgtcac ccgctctgta	480
481	ggcccggccg gcgcttgccg aacgcaaatc aatctttttc caggttgacc tcggatcagg	540
541	tagggatacc cgctgaactt aagcatatca ataagcggag ga	582

3.5. Phytochemical composition of *S. occidentalis* ethanolic extract using GC/MS

The data reported in Figure 2 and Table 2 depict the phytochemical constituents and the anticipated biological efficacy of the ethanolic extract of *S. occidentalis*, as assessed by the GC/MS technique. The data indicated the identification of 35 phytochemical components with varying prevalence ratios (expressed as area %) with their biological activity, based on the literature survey. The predominant constituents are o-methyl glucose (24.58%), tetramethyl hexadecenol (10.41%), hexadecenoic acid (10.15%), octadecatrienoic acid (5.38%), and tricosane (3.64%). The data additionally demonstrated the presence of various active phytochemicals in the ethanolic extract of S. *occidentalis*. These include d-mannitol, 1-decylsulfonyl, 9-octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis, l-gala-l-ido-octose, myristic acid, 2-hexadecene, 2,6,10,14-tetramethyl, 1-heptadecyne, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, 2-tridecen-1-ol (E), 2-chloroethyl linoleate, stearic acid, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, and octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester. The components 1-monolinoleoylglycerol trimethylsilyl ether, 12-methyl-E,E-2,13-octadecadien-1-ol, and 9-octadecenoic acid, (2-phenyl-1,3-dioxolan-4- yl)methyl ester, cis were determined to be the least abundant in the ethanolic extract of *S. occidentalis*. The identified compounds exhibit many biological activities, including antioxidant, anti-inflammatory, anticancer, and antibacterial activities. Additionally, some of these have long-lasting analgesic properties, antigenotoxic effects, and the capacity to promote the human immune system, as detailed in Table 2.

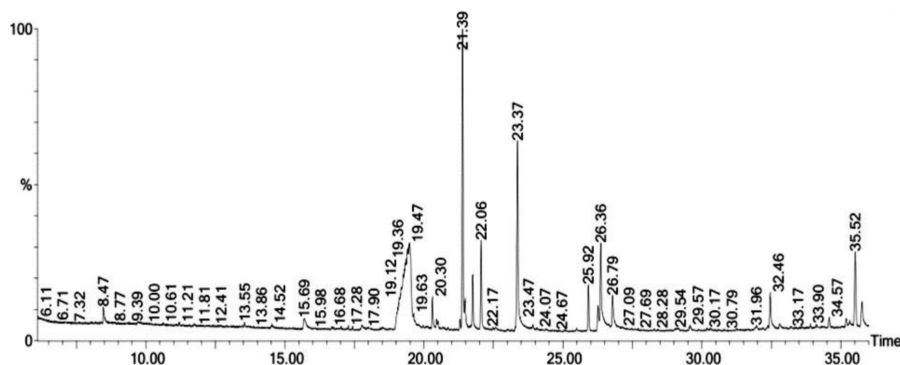


Figure 2: GC/MS chromatogram of of *Senna occidentalis* ethanolic extract analysis.

286 **Table 2:** Chemical constituents and the biological activity of *Senna occidentalis* ethanolic extract.

Peak No.	RT (min.)	Area (%)	Compound name	Chemical formula	Activity	Reference
1	8.469	0.768	9,12,15-Octadecatrienoic acid, 2- [(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)	$C_{27}H_{52}O_4Si_2$	Antimicrobial	[32]
2	8.534	0.378	Androstane-11,17-dione, 3-[(trimethylsilyl)oxy]-,17-[O-(phenylmethyl)oxime], (3à,5à)	$C_{29}H_{43}NO_3Si$	Antimicrobial and antidiabetic	[33]
3	9.705	0.736	1-Monolinoleoylglycerol trimethylsilyl ether	$C_{27}H_{56}O_4Si_2$	Antimicrobial	[34]
4	13.551	0.407	9,12,15-Octadecatrienoic acid, 2- [(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)	$C_{27}H_{52}O_4Si_2$	Antimicrobial	[32]
5	15.692	1.090	d-Mannitol, 1-decylsulfonyl	$C_{16}H_{34}O_7S$	Antimicrobial and anti-cancer	[35]
6	17.763	0.523	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis	$C_{28}H_{44}O_4$	Antimicrobial and antiperspirant	[36]
7	19.484	24.581	3-O-Methyl-d-glucose	$C_7H_{14}O_6$	Antimicrobial, immune activator, and signal regulator	[37]
8	19.634	0.981	l-Gala-l-ido-octose	$C_8H_{16}O_8$	Antibacterial, antiviral, antioxidant, and anti-coagulant	[38]
9	20.304	0.985	Myristic acid	$C_{14}H_{28}O_2$	Antifungal, antiviral, and anticancer	[39]
10	20.454	0.282	4-Cyclopentene-1,3-dione, 4,5-dimethyl-2-phenyl	$C_{13}H_{12}O_2$	Antimicrobial and disinfectant	[40]
11	20.509	0.235	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis	$C_{28}H_{46}O_4$	Antimicrobial, and antiperspirant	[36]
12	21.299	0.314	1-Dodecanol, 3,7,11-trimethyl	$C_{15}H_{32}O$	Antimicrobial, antioxidant, antitumor, antidiabetic, and antihypertensive	[41]
13	21.389	10.410	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	Antimicrobial, antioxidant, anticancer, and hepatoprotective	[42]
14	21.479	1.361	2-Hexadecene, 2,6,10,14-tetramethyl	$C_{20}H_{40}$	Antimicrobial	[43]
15	21.760	2.190	1-Heptadecyne	$C_{17}H_{32}$	Antibacterial, anticancer, antioxidant, antidiabetic, antiemetic, hypoglycemic, antihypertensive, and hypolipidemic	[44]
16	22.060	3.397	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	Antimicrobial, antioxidant, anticancer, and hepatoprotective	[42]
17	22.495	0.137	1-Monolinoleoylglycerol	$C_{27}H_{56}O_4Si_2$	Antimicrobial	[34]

			trimethylsilyl ether			
18	22.580	0.246	12-Methyl-E,E-2,13-octadecadien-1-ol	$C_{19}H_{36}O$	Antimicrobial, antidiabetic, and cardiovascular-protective	[45]
19	23.370	10.154	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	Antimicrobial, antioxidant, anti-inflammatory, anticancer, and hypocholesterolemic	[46]
20	23.840	0.165	9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis	$C_{28}H_{44}O_4$	Antimicrobial, and antiperspirant	[36]
21	23.925	0.547	9,12,15-Octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)	$C_{27}H_{52}O_4Si_2$	Antimicrobial	[32]
22	25.916	1.715	2-Tridecen-1-ol (E)	$C_{13}H_{26}O$	Anticancer	[47]
23	26.261	1.196	2-Chloroethyl linoleate	$C_{20}H_{35}ClO_2$	Antibacterial, antifungal, and anti-inflammatory	[48]
24	26.361	5.378	9,12,15-Octadecatrienoic acid, (Z,Z,Z)	$C_{19}H_{32}O_2$	Antimicrobial, antioxidant, anti-inflammatory, myorelaxant, anticancer, antigenotoxic, cardiovascular-protective, neuro-protective, and anti-osteoporotic	[49]
25	26.652	0.146	12-Methyl-E,E-2,13-octadecadien-1-ol	$C_{19}H_{36}O$	Antimicrobial, antidiabetic, and cardiovascular-protective	[45]
26	26.787	1.629	Stearic acid	$C_{18}H_{36}O_2$	Anticancer	[50]
27	29.108	0.281	9-Octadecenoic acid (Z)-, phenylmethyl ester	$C_{25}H_{40}O_2$	Antimicrobial	[51]
28	29.578	0.620	3-Trifluoroacetoxypentadecane	$C_{17}H_{31}F_3O_2$	Antidiabetic, anti-inflammatory, antioxidant, anti-ulcerogenic, hepato-protective, and antimicrobial	[52]
29	31.964	0.362	1-Heptatriacotanol	$C_{37}H_{76}O$	Antimicrobial, anti-inflammatory, and antioxidant	[53]
30	32.459	2.230	Hexadecanoic acid, 2-hydroxy-1- (hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	Antimicrobial, and antioxidant	[54]
31	32.799	0.309	Phen-1,4-diol, 2,3-dimethyl-5-trifluoromethyl	$C_9H_9F_3O_2$	Antimicrobial	[55]
32	34.590	0.850	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl	$C_{13}H_{22}OSi_2$	Antimicrobial	[56]
33	35.200	0.353	3,5,6-Trimethyl-p-quinone, 2-(2,5- dioxotetrahydrofuran-3-yl)thio	$C_{13}H_{12}O_5S$	Antimicrobial	[57]
34	35.515	3.648	Tricosane	$C_{23}H_{48}$	Ophthalmic and antimicrobial	[58]
35	35.760	1.673	Octadecanoic acid, 2-hydroxy-1- (hydroxymethyl)ethyl ester	$C_{21}H_{42}O_4$	Antimicrobial, and antioxidant	[59]

287 **3.6. The visual manifestation of rabbit eyes afflicted with *Aspergillus flavus***

288 The histological implications of *Aspergillus flavus*, commercial antifungal agent (fluconazole 150 mg/ml),
289 the ethanolic extract of *S. occidentalis*, and their combined effect on the external appearance of albino
290 rabbit cornea are depicted in Figure 3. Throughout the experimental stages, the eyes of albino rabbits
291 exhibited a healthy and intact corneal structure, with normal eyeball and eyelid appearance in the non-
292 infected, non-treated right eye (Figure 3-a). Conversely, Figure 3-b revealed complete damage to corneal
293 tissues, fungal growth filling the anterior chamber and inner eyeball space, and pronounced inflammation in
294 the *A. flavus*-infected, non-treated right eye of albino rabbits. Interestingly, the right eye of albino rabbits
295 treated with *S. occidentalis* ethanolic extract at its MIC value (12.5 mg/ml) exhibited a normal appearance
296 (Figure 3-c). However, the commercial dose of fluconazole (150 mg/ml) caused observable inflammation
297 and hypersensitivity in the treated right eye of albino rabbits (Figure 3-d).

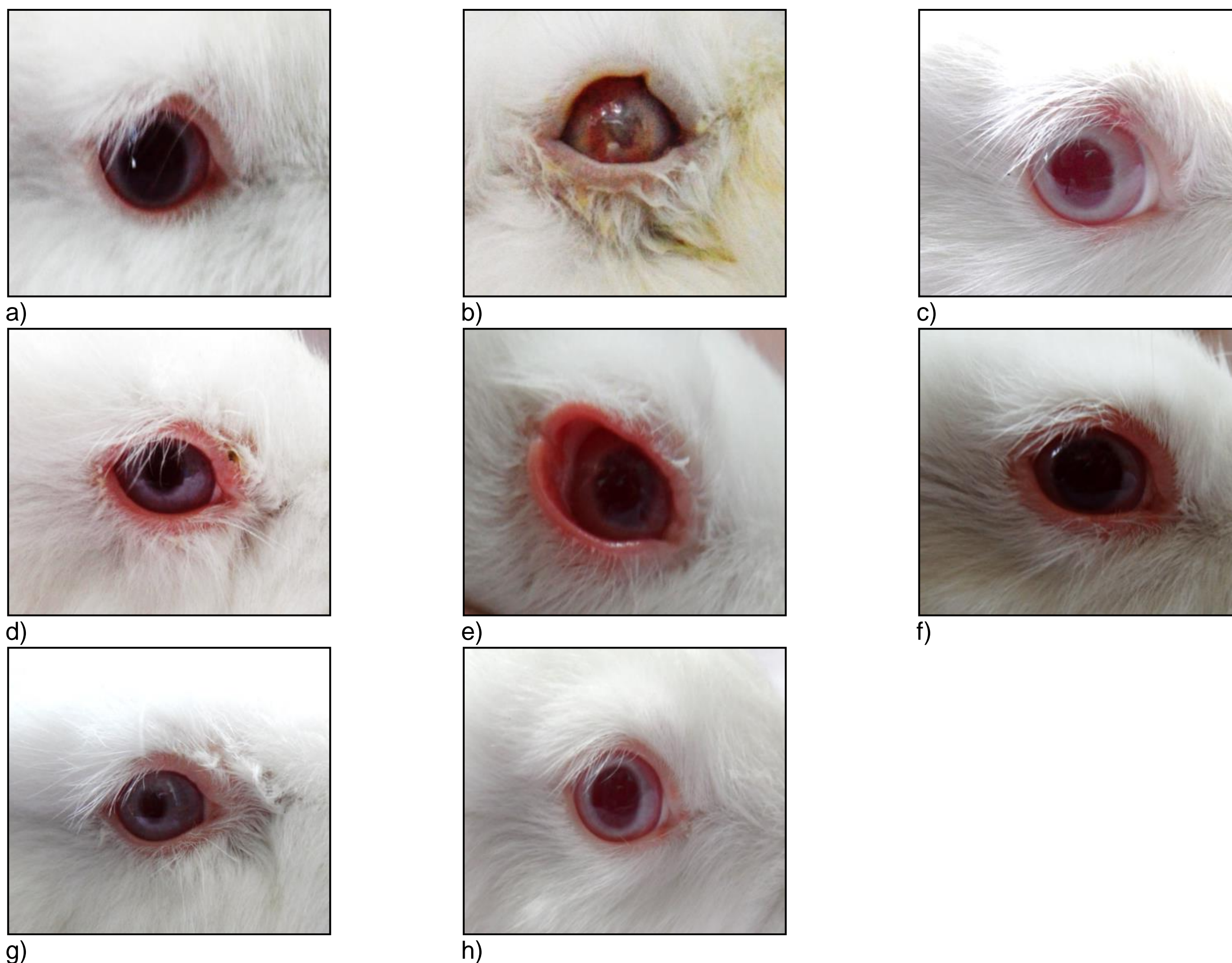
298 Treatment of *A. flavus*-infected eyes of albino rabbits with fluconazole (150 mg/ml) resulted in a slow
299 response and low healing rate. Although fungal growth was inhibited within three weeks, inflammatory
300 effects remained (Figure 3-e). In contrast, the combination of *S. occidentalis* extract with fluconazole
301 accelerated the visual manifestation of the healing rate and decreased inflammation symptoms during
302 fungal infection treatment (Figure 3-f).

303 Figure 3-g illustrates the significant healing rate observed in *A. flavus* infection, with the complete
304 disappearance of inflammation and the restoration of a normal appearance in the infected right eye of
305 albino rabbits following treatment with the MIC dosage of *S. occidentalis* extract within only two weeks.
306 Furthermore, a more definitive demonstration of the effect of *S. occidentalis* on fungal keratitis in vivo was
307 achieved by treating the infected eye with double the MIC concentration (25 mg/ml) of the extract. This
308 resulted in the highest healing rate of corneal tissues, complete inhibition of fungal growth, and the
309 disappearance of inflammation in the treated eye within ten days (Figure 3-h).

310 **3.7. Biochemical parameters of treated rabbits**

311 To assess the safety parameters of synthetic and natural antifungal agents and evaluate the potential of *S.*
312 *occidentalis* as an effective remedy against corneal fungal infection, various physiological and
313 immunological responses were measured in blood samples collected from sacrificed rabbits *in vivo* (Table
314 3). Infection with *A. flavus* was found to significantly elevate the levels of liver function markers, with
315 alanine aminotransferase (ALT) reaching 56 U/L and aspartate aminotransferase (AST) reaching 52 U/L in
316 the infected, non-treated rabbit group (gp. b) compared to the healthy, non-treated rabbit group. However,
317 treatment of infected rabbits with *S. occidentalis* extract demonstrated a positive impact on liver function, as
318 indicated by ALT and AST levels of 39 and 36 U/L, respectively, in the infected and plant extract-treated
319 with 12.5 mg/ml extract rabbit group (gp. g). These values were comparable to the ALT and AST levels of
320 44 and 43 U/L, respectively, in the non-infected, non-treated rabbit group (gp. a), and 38 and 34 U/L,
321 respectively, in the non-infected, plant extract-treated rabbit group (gp. c). These findings were further
322 supported by similar results obtained using double the MIC concentration of the plant extract (gp. h).

323 Conversely, treatment with the commercial fluconazole solution resulted in a noticeable increase in liver
324 function parameters, with ALT and AST reaching 87 and 56 U/L, respectively, in the non-infected,
325 fluconazole-treated rabbit group (gp. d). These levels were even higher in the infected and fluconazole-
326 treated rabbit group (gp. e), reaching 92 U/L for ALT and 71 U/L for AST. However, when fluconazole was
327 combined with *S. occidentalis* extract to treat infected rabbits (gp. f), the levels were significantly reduced to
328 65 U/L for ALT and 53 U/L for AST, indicating a beneficial effect of the combined treatment. These results
329 highlight the potential of *S. occidentalis* extract as a promising and safe alternative for the treatment of
330 corneal fungal infections, as it exhibited positive effects on liver function markers, while the commercial
331 fluconazole solution showed some hepatotoxicity. The combined treatment demonstrated a synergistic
332 effect, effectively reducing liver function levels and suggesting enhanced efficacy in combating fungal
333 infections.



334 **Fig. 3:** Effect of *Senna occidentalis* ethanolic extract on the external appearance of the cornea of albino
335 rabbits' eyes. a) non-infected untreated, b) *Aspergillus flavus*-infected untreated, c) *Senna occidentalis*
336 MIC-treated without infection d) Fluconazole-treated without infection (150 mg/ml), e) *A. flavus*-infected
337 treated with fluconazole, f) *A. flavus*-infected treated with combination of *S. occidentalis* MIC and
338 fluconazole, g) *A. flavus*-infected treated with *S. occidentalis* MIC, and h) *A. flavus*-infected treated with
339 *Senna occidentalis* double MIC

340 **Table 3:** Liver and kidney function parameters and immunoglobulin M (IgM) levels in response to the
341 ethanolic extract of Senna occidentalis used in the treatment of in vivo-induced mycotic keratitis in rabbits.

Rabbit groups	Liver function		Kidney function		Immune response
	ALT (U/L)	AST (U/L)	Urea (mmol/L)	Creatinine (mg/dL)	IgM (mg/dL)
a	44	43	45	1.0	18
b	56	52	58	1.2	24
c	38	34	43	0.6	26
d	87	65	68	1.3	21
e	92	71	73	1.5	26
f	65	53	55	1.0	33
g	39	36	37	0.6	40
h	36	34	33	0.7	47

342 a) non-infected untreated, b) *Aspergillus flavus*-infected untreated, c) *Senna occidentalis* MIC-treated without
343 infection d) Fluconazole-treated without infection (150 mg/ml), e) *A. flavus*-infected treated with fluconazole,
344 f) *A. flavus*-infected treated with combination of *S. occidentalis* MIC and fluconazole, g) *A. flavus*-infected
345 treated with *S. occidentalis* MIC, and h) *A. flavus*-infected treated with *Senna occidentalis* double MIC. ALT =
346 alanine transaminase, AST = aspartate transaminase, and IgM = immunoglobulin M.

347 The impact of experimental treatments on kidney function parameters, urea and creatinine levels, is
348 presented in Table 3. The results revealed that fungal infection significantly elevated the levels of blood
349 urea and creatinine in infected rabbits, reaching 58 mmol/L and 1.2 mg/dL, respectively, compared to the
350 control group levels of 45 mmol/L and 1.0 mg/dL. However, S. *occidentalis* extract effectively reduced blood
351 urea and creatinine levels to equal or even lower than those observed in healthy rabbits (43 mmol/L and
352 0.6 mg/dL, respectively).

353 Conversely, treatment with fluconazole resulted in a noticeable increase in blood urea and creatinine levels
354 in the treated rabbits (68 mmol/L and 1.3 mg/dL, respectively). Moreover, in *A. flavus*-infected rabbits,
355 fluconazole treatment further elevated these levels to 73 mmol/L and 1.5 mg/dL, respectively. However, the
356 combination of *S. occidentalis* extract with fluconazole effectively lowered blood urea and creatinine levels
357 in the infected rabbits (55 mmol/L and 1.0 mg/dL, respectively). Notably, a significant decrease in urea and
358 creatinine levels was observed when the infected rabbits were treated with *S. occidentalis* extract at the
359 MIC dosage, with levels of 37 mmol/L and 0.6 mg/dL, respectively. Furthermore, the enhancement of
360 kidney function parameters in rabbit blood was remarkable when treated with a double MIC concentration
361 of *S. occidentalis* extract, as it yielded levels even lower than those of the healthy control group (33 mmol/L
362 and 0.7 mg/dL, respectively). These findings provide compelling evidence for the potential of *S.*
363 *occidentalis* extract to restore kidney function parameters to normal or improved levels, surpassing the
364 effects of fluconazole treatment. These results hold great promise for the development of novel therapeutic
365 approaches for fungal infections of the cornea, emphasizing the remarkable potential of *S. occidentalis*
366 extract.

367 The determination of serum immunoglobulin M (IgM) levels in the blood samples of experimental rabbits
368 provides insights into the intrinsic immune response elicited by the various experimental treatments (Table
369 3). Notably, the levels of IgM in infected rabbits, fluconazole-treated rabbits, and infected fluconazole-

370 treated rabbits were found to exhibit a compromised immune response, with values of 24, 21, and 26
371 mg/dL, respectively, in comparison to the healthy control group's IgM level (18 mg/dL). In contrast, the
372 administration of *S. occidentalis* extract demonstrated a beneficial effect on stimulating the immune system
373 of both infected and non-infected rabbits. This fortuitous response played a vital role in expediting and
374 facilitating the complete healing of the targeted infection. Notably, the treatment with *S. occidentalis* extract
375 increased the IgM levels of healthy rabbits (26 mg/dL). Furthermore, the combination of *S. occidentalis*
376 extract at the MIC dosage with fluconazole in infected rabbits led to a significant elevation of IgM level (33
377 mg/dL). Similarly, when *S. occidentalis* extract was administered at the MIC dosage in *A. flavus*-infected
378 rabbits, a further increase in IgM levels was observed (40 mg/dL). The highest level of IgM was attained
379 when *S. occidentalis* extract was applied at double the MIC concentration in *A. flavus*-infected rabbits,
380 resulting in a remarkable level of 47 mg/dL.

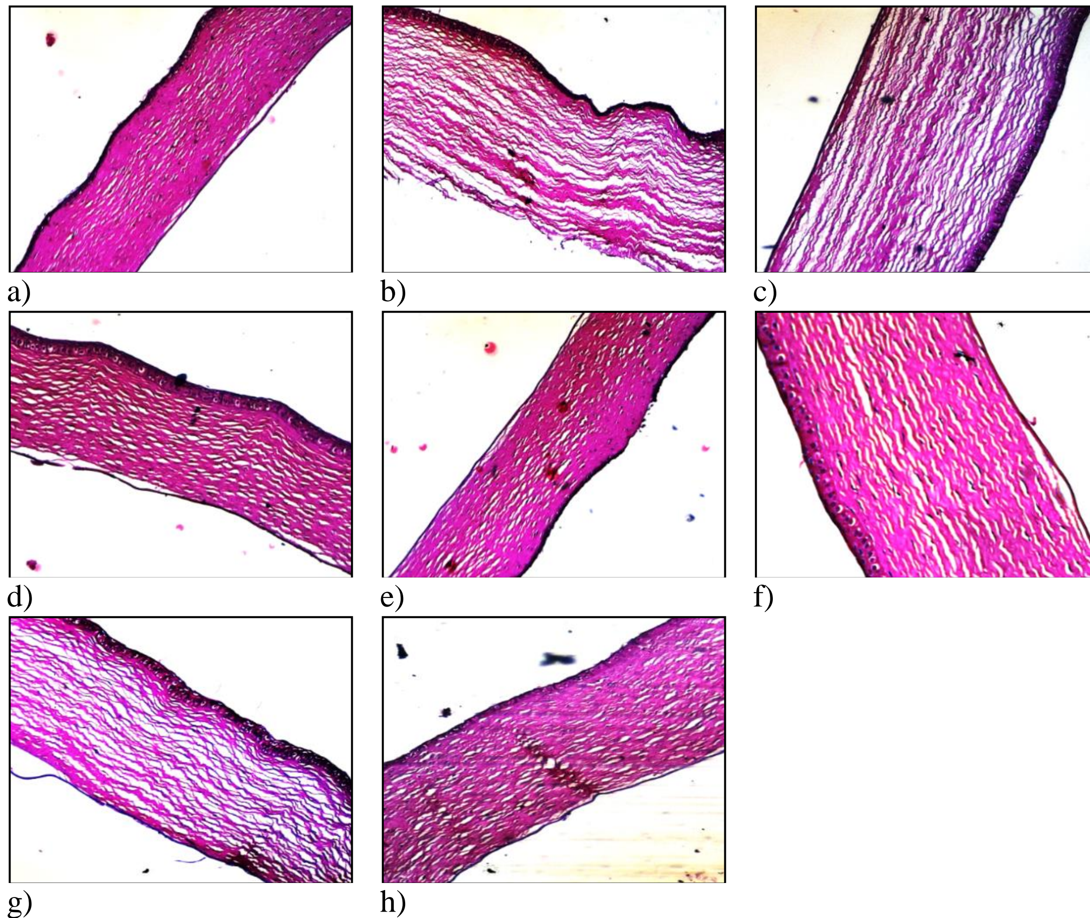
381 **3.8. Histopathological effects of *Senna occidentalis* extract on the corneal tissues**

382 Following the *in vivo* investigation of the antifungal and histological effects of *S. occidentalis* extract on
383 albino rabbit corneas, the corneas were excised meticulously and preserved in a 10% formalin solution.
384 Subsequently, transverse sections of the corneas were stained, allowing for examination under a light
385 microscope. Photographic documentation was conducted for all animal groups involved in the study (Figure
386 4). Figure 4-a illustrates the cornea of healthy rabbits, exhibiting an intact and regular layer with normal
387 thickening and smooth epithelium and endothelium. In contrast, Figure 4-b displays the cornea of *A. flavus*-
388 infected rabbits, showing significant damage and distortion across all tissue layers.

389 The application of *S. occidentalis* extract to non-infected rabbit corneas, as depicted in Figure 4-c,
390 preserved the regular arrangement and thickness of the corneal layers. Conversely, treatment with a
391 commercial fluconazole solution on non-infected rabbit corneas (Figure 4-d) resulted in noticeable
392 inflammation of the stroma, disintegration of the endothelium, and distortion of the epithelium.

393 Microscopic examination of infected rabbit corneas after three weeks of fluconazole treatment, as depicted
394 in Figure 4-e, revealed poor healing signs and persistent side effects of the drug. The corneal tissues
395 exhibited stromal infiltration, thickening, incomplete endothelium regeneration, and irregular epithelium
396 appearance. These complications manifested despite the inhibition of fungal growth. However, the
397 combination of *S. occidentalis* extract at MIC dosage with fluconazole (Figure 4-f) increased the likelihood
398 of avoiding the side effects associated with the commercial drug. The corneas displayed an intact
399 endothelium and relatively regular epithelium. Moreover, the use of the minimum inhibitory concentration
400 (MIC) of *S. occidentalis* extract resulted in faster inhibition of fungal growth and stimulated the healing of
401 corneal tissues after controlling the infection. This treatment led to complete regeneration of the
402 endothelium, regular arrangement of the epithelium, and reduced stromal infiltration (Figure 4-g). The
403 fastest and most complete inhibition of fungal growth within corneal tissues was achieved by using double
404 the MIC of *S. occidentalis* extract (Figure 4-h). This treatment resulted in a healed cornea with a normal
405 appearance, exhibiting regular arrangement and thickness of the epithelium, stroma, and endothelium.

406



407

408 **Fig. 4:** Histopathological effects of *Senna occidentalis* ethanolic extract on the corneal tissues of albino
409 rabbits. a) non-infected untreated, b) *Aspergillus flavus*-infected untreated, c) *Senna occidentalis* MIC-
410 treated without infection d) Fluconazole-treated without infection (150 mg/ml), e) *A. flavus*-infected treated
411 with fluconazole, f) *A. flavus*-infected treated with combination of *S. occidentalis* MIC and fluconazole, g) *A.*
412 *flavus*-infected treated with *S. occidentalis* MIC, and h) *A. flavus*-infected treated with *Senna occidentalis*
413 double MIC

414 4. DISCUSSION

415 Fungal keratitis poses a significant health concern in developing countries. The prevalence of this condition
416 is notably higher in regions with limited access to quality healthcare, inadequate hygiene practices, and
417 suboptimal agricultural and environmental conditions. Factors such as warm and humid climates,
418 agricultural occupations, improper contact lens use, and ocular trauma contribute to the increased
419 incidence of fungal keratitis in these settings [60]. Moreover, limited awareness about the disease, delayed
420 diagnosis, and challenges in accessing appropriate antifungal medications further exacerbate the burden of
421 fungal keratitis in developing countries. The lack of resources and infrastructure to support effective
422 management and treatment strategies adds to the complexity of addressing this issue. Therefore,
423 implementing comprehensive public health programs, improving healthcare infrastructure, promoting
424 hygiene education, and ensuring the availability of affordable antifungal medications are crucial steps
425 toward mitigating the impact of fungal keratitis in developing countries [61].

426 Despite chemical antifungal treatments being proven effective against fungal keratitis, their utilization
427 involves potential dangers to patient well-being and health consequences. More precisely, these artificial
428 pharmaceutical substances might lead to localized negative consequences such as irritation and allergic
429 inflammation of eye tissues, as well as causing discomfort to the patient [62]. Administration of the drug
430 throughout the body may also result in issues related to toxicity and possible interactions with other drugs,
431 posing a risk to the health and safety of the patient [63]. Furthermore, an increasing issue in long-term
432 treatment is the emergence of drug resistance among fungal infections.

433 In order to address these issues, the use of natural product extracts derived from plants has emerged as a
434 possible alternative therapeutic strategy. In the current study, ethanolic extract of *S. occidentalis* was
435 explored as a potential natural antifungal agent against *A. flavus* that causes fungal keratitis. The present
436 work investigated the potential of the ethanolic extract derived from *S. occidentalis* as a natural antifungal
437 agent against *A. flavus*, the causative agent of fungal keratitis. The phytochemical analysis using GC/MS
438 approach revealed the existence of several phytocompounds with a broad spectrum of biological activity.
439 The prevalent compounds were o-methyl glucose, tetramethyl hexadecenol, hexadecenoic acid,
440 octadecatrienoic acid, and tricosane. In addition, the ethanolic extract of *S. occidentalis* possessed other
441 active phytochemicals, such as d-mannitol, 1-decylsulfonyl, 9-octadecenoic acid, l-gala-l-ido-octose,
442 myristic acid, 2-chloroethyl linoleate, stearic acid, hexadecanoic acid, octadecanoic acid, and others. These
443 compounds that were identified have been reported to provide health benefits to the human body, such as
444 antioxidant, anti-inflammatory, anticancer, and antibacterial activity [64]. Additionally, they have long-lasting
445 analgesic effects, antigenotoxic effects, and may stimulate the human immune system (Table 1).

446 The investigation conducted by Yadav et al. [65] revealed that the extract of *S. occidentalis* includes
447 substantial quantities of saponins, flavonoids, sterols, triterpenes, and tannins. These phytochemicals are
448 secondary metabolites that have been found to be highly effective against a variety of fungal infections,
449 including *C. albicans*, *A. flavus* [9], *Trichophyton rubrum*, *T. mentagrophytes* [66], *A. niger* and *A. fumigatus*
450 [67]. In addition, Yakubu et al. [68] conducted a GC/MS study on the ethanolic extract of *S. occidentalis*
451 leaves and found a biologically active mixture. The extract exhibited noticeable antifungal activity. This
452 finding was further supported by Abubakar and Umar [69], who validated the antifungal activity of the
453 methanolic extract of *S. occidentalis* against *A. flavus, A. niger*, and *C. albicans*. Furthermore, according to
454 Xu et al. [70], fatty acids, which are abundant in the ethanolic extract of *S. occidentalis*, could potentially
455 contribute to the suggested therapeutic effect of antifungal activity. Therefore, *S. occidentalis* exhibited
456 significant antifungal activity against the causative agent *A. flavus* of mycotic keratitis. The activity of this
457 extract could be ascribed to bioactive secondary metabolites, including fatty acids, flavonoids, polyphenols,
458 and other compounds. Additional investigation is necessary to investigate the therapeutic capacity of this
459 plant in tailoring natural alternatives to commercially produced antifungals.

460 The results of our study revealed that the right eye of the albino rabbit treated with MIC of *S. occidentalis*
461 extract seemed normal, but the commercial dose of fluconazole caused evident inflammation and
462 hypersensitivity in the treated right albino rabbit eye. The extract of *S. occidentalis* maintained the normal
463 architecture and thickness of the uninfected layers of the cornea. In addition, the utilization of MIC dosage
464 of *S. occidentalis* extract resulted in a more rapid inhibition of fungal growth and promoted the recovery of

465 corneal tissues following infection management. This was accompanied by the complete regeneration of
466 the endothelium, the regular organization of the epithelium, and a reduction in the infiltration of the stroma.
467 Our findings align with previous research, highlighting the significance of natural botanical extracts in the
468 treatment of eye disorders. Ruszymah et al. [71] found that the aqueous extract of *Centella asiatica* may
469 have the potential to enhance the healing of corneal epithelial wounds. Furthermore, Agarwal et al. [72]
470 documented that the aqueous extract of *Curcuma longa* effectively inhibited endotoxin-induced uveal
471 inflammations in rats by decreasing TNF-α activity.

472 An increase in liver function indicators, involving ALT and AST values, was found as a result of the *A. flavus*
473 infection of albino rabbits under investigation. In contrast, the administration of *S. occidentalis* extract to
474 infected rabbits had a positive impact on the liver function parameters. Conversely, the use of the
475 commercial fluconazole solution in the treatment of the fungal infection resulted in a noticeable elevation of
476 liver enzyme levels in the infected rabbits treated with fluconazole. The study indicated a promising
477 potential for utilizing *S. occidentalis* extract as an antifungal treatment for mycotic keratitis. Additionally, it
478 was found to effectively reduce the levels of blood urea and creatinine, which serve as indicators of kidney
479 function, to a level that is equal to or lower than that of healthy rabbits. In their study, Roy et al. [73]
480 concluded that oral administration of *Senna* sp. extract did not have any adverse effects on liver functions
481 in animal models used for treatment. Furthermore, Yang et al. [74] validated the safety of *Senna* sp. extract
482 on the various functions and tissues of the liver and kidney in albino mice while treating their dry eyes.
483 Agbodjogbe et al. [75] examined the safety of *Senna* sp. leaf extract in rats by quantifying the toxicity of the
484 extract to rats. The treatment of albino rats with *Senna* sp. leaf extract indicated that the extract can be
485 classified as non-toxic to rats. Nevertheless, the study on sub-acute toxicity revealed that the aqueous
486 extract of *Senna* sp. had a negative impact on several biochemical markers associated with liver, kidney,
487 and muscle function when administered at doses exceeding 2000 mg/kg. In addition, supporting to our
488 data, experiments conducted on in vivo have demonstrated that the extracts obtained from the roots,
489 leaves, and aerial parts of *S. occidentalis* can enhance liver function by reducing the activities of ALT, AST,
490 alkaline phosphatase (ALP), and bilirubin in rats with chemically-induced liver damage [76,77]. The tissue
491 protective action of *S. occidentalis* has been attributed to its distinct composition of secondary bioactive
492 compounds.

493 The successful healing of cornea fungal infection in the treated rabbits depends on the combined
494 effectiveness of the administered antifungal treatment and the internal immune response. The extract of *S.*
495 *occidentalis* demonstrated a significant ability to enhance the immune system of rabbits in both infected
496 and non-infected groups. This resulted in faster and more thorough healing of the targeted infection. The
497 level of IgM, a key indicator of the immune response, reached its peak in infected rabbits treated with
498 double the MIC of *S. occidentalis*. A recent study investigated the impact of *Senna* sp. leaf extract on dry
499 eye in animal models. The study found that the extract promoted the regeneration of corneal tissues and
500 did not negatively affect liver and kidney functions. Additionally, the extract enhanced the antifungal activity
501 of macrophages, indicating an improved immune response against fungal eye infections [74]. Plant
502 extracts, which are natural immunomodulators, enhance a compromised immune system and reduce an
503 over-aggressive immunological response [78]. Therefore, it is crucial to evaluate the immunomodulatory
504 activity, safety, and toxicity of local herbal formulations as they are reported to have these properties.

505 The administration of a novel antimicrobial product *in vivo* necessitates the conduction of safety approval
506 tests on the host tissue and function, alongside the identification of discernible indicators implying the swift
507 and complete healing of the host tissues after the effective inhibition of the microbial infection. Upon
508 scrutinizing the corneal tissue layers of the treated groups, it was observed that the ethanolic extract of *S.*
509 *occidentalis* exhibited a notable inhibition in *A. flavus* mycelial growth. This was accompanied by a positive
510 rate of healing and regeneration of the corneal tissues, characterized by their normal arrangement,
511 thickness, and texture on both the outer and inner surfaces. The positive outcome can be attributed to the
512 potent antimicrobial activity of the phytochemical components present in the ethanolic extract of *S.*
513 *occidentalis*, which effectively eradicated the *A. flavus* infection within the corneal stroma. Moreover, the
514 rapid regeneration of the corneal stroma and collagen-bound layers can be ascribed to the invigorating
515 effects of the *S. occidentalis* phytocomponents on the immune system and the production of IgM. These
516 elements ensure an ample supply of blood and nutrients, expedite cell division in the corneal layers and
517 contribute to the orderly arrangement, thickness, and texture of the regenerated layers. Furthermore, it was
518 determined that the normal growth of corneal tissues relies on the proper functioning of other organs in the
519 treated animal model, as evidenced by the normal levels of liver and kidney function parameters observed
520 in the rabbits treated with the *S. occidentalis* ethanolic leaf extract in this investigation.

521 **5. CONCLUSION**
522
523 The phytochemical composition of *S. occidentalis* has shown potential as a safer alternative with reduced
524 side effects and the ability to address anti-fungal resistance against fungal keratitis in rabbits caused by *A.*
525 *flavus*. These findings highlight the efficacy of *S. occidentalis* extract in enhancing the immune response of
526 rabbits, both in the presence and absence of infection, without adversely affecting liver and kidney function.
527 The observed increase in IgM levels indicates the immunostimulatory properties of *S. occidentalis* extract,
528 which contribute to a more robust and efficient healing process. Histopathological examination also
529 revealed that *S. occidentalis* extract promotes the healing of affected corneal tissues in rabbits. These
530 results suggest that *S. occidentalis* may be a promising adjunct treatment to enhance immune response
531 and facilitate infection resolution. However, further clinical assessment is needed to thoroughly evaluate the
532 effectiveness, toxicity profile, and optimal application of *S. occidentalis* extract for mycotic keratitis. Well-
533 designed research studies are necessary to bridge the remaining knowledge gaps and determine the
534 effectiveness and utility of herbal medications for this specific purpose.

535 Disclaimer (Artificial intelligence)

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544 technology

545

546 Details of the AI usage are given below:

547 1.

548 2.

549 3.

550 **COMPETING INTERESTS**
551
552 All authors certify that they have no affiliations with or involvement in any organization or entity with any
553 financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.
554 Authors are responsible for correctness of the statements provided in the manuscript.
555
556 **AUTHORS' CONTRIBUTIONS**
557
558 A.S.E., E.E.E., M.S.E, S.M.A., and K.M.S designed the research plan and wrote the manuscript. A.E., and
559 EE performs the experimental work. All authors have read and approved the final manuscript.
560
561
562 **ETHICAL APPROVAL**
563
564 This study was approved by the Research Ethics Committee of Tanta University with an approval code of
565 34895/9/21.
566
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