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FUNGICIDAL ACTIVITY OF ROSMARINIC ACID AGAINST *ASPERGILLUS FUMIGATUS AND ASPERGILLUS BREVIPES* ISOLATED FROM RAW COW MILK IN WESTERN SAUDI ARABIA IN SIGHT OF *INVITRO* AND *INSILCO* STUDIES

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Abstract

The potential relationship between the presence of gliotoxinogenic *Aspergillus* species and the surrounding environment of lactating cows. *Aspergillus fumigatus* is the main etiologic agent causing invasive aspergillosis. Also, the *A. fumigatus* complex may be a cause of invasive aspergillosis. In this study, *A.fumigatus* strains were isolated from lactating cow's milk in Taif, Saudi Arabia, and identified using morphological criteria via cultivating *Aspergillus* species inoculations on Czapek agar, and malt extract agar (MEA) plates, followed by examining the activity of the Rosmarinic acid against *Aspergillus* species using diffusion disk and MIC assays. Also interesting was the molecular docking of Rosmarinic acid against 14-alpha sterol demethylase Cyp51B enzyme which considers a target site for fungal infection. The result has shown that morphologic identification of the strains isolated from lactate cow milk displayed two classes of *Aspergillus* species: *Aspergillus fumigatus* which has a colony blue-green in front and converts into colorless in reverse with a diameter is 2-3 cm and a Vesicle diameter 23 µm, the second class was *Aspergillus brevipes* which has a colony color in front is yellow and convert into brown in reverse with diameter is 2 cm and Vesicle diameter 14 µm. The inhibition zone of *A. fumigatus and A. brevipes* were 17 and 15 nm respectively after the strains were investigated with RA. The MIC value of RA against *Aspergillus fumigatus and Aspergillus brevipes* isolated from cow milk was 321 and 326 mg, respectively. The 14-alpha sterol demethylase Cyp51B enzyme was inhibited by RA via the formation of two hydrogen bonds with Ser511 and Arg510 respectively.

Keywords: *Aspergillus Fumigatus, Aspergillus Brevipes*, Raw Cow Milk, Rosmarinic Acid

1. INTRODUCTION

A.fumigatus, is a major etiological agent of human and animal aspergillosis. It is present in contaminated animal environments [1]. Also, it can be isolated from sorghum and maize silages and other contaminated animal feeds [2]. *A.fumigatus* is a pathogenic filamentous fungus that causes a wide range of diseases, including chronic pulmonary aspergillosis and invasive aspergillosis [3]. *A.fumigatus* is capable of producing tremorgenic compounds such as gliotoxin that induce neurological syndromes in farmers who have contact with animals [4]. It is able to produce spores that spread in the air, causing a high risk to both animals and humans. *A.fumigatus* caused infectious diseases in animals, including respiratory infections in chicks, ducks, and horses [5]. The identification of *A. fumigatus* occurred by examining microscopic features. The variations in its phenotype are determined by several factors, such as vesicle size and shape and stipe length. *Aspergillus brevipes* isolated from Australian soil. It is characterized by the production of intensely pigmented mycelium, and of many sessile conidial heads [6]. Conventional

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antifungals are the only treatment for fungal infections such as fluconazole, voriconazole, itraconazole, and ketoconazole. However, there are adverse effects of the use of antifungals, such as diarrhea, abdominal pain, redness of the skin, and liver damage [7]. Rosmarinic acid (RA) is a natural polyphenol found in the Labiatae family. It is present in various herbs, such as Rosmarinus officinalis and Salvia miltiorrhiza, and in Zostera marina seagrass beds. RA has many biological properties, such as antioxidant, anti-inflammatory, anticancer, anti-infectious, antinociceptive, and neuroprotective activities [8]. Previous work showed that RA is hydrolyzed by different esterases and metabolized throughout the gut microbiota before its absorption, which influences the function of this polyphenol [9]. Inside the fungal membrane, the sterol 14a demethylase enzyme (CYP51) is working to catalyze the lanosterol 14a methylation to produce ergosterol, which can regulate the integrity, fluidity, and permeability of the cell membrane [10–13]. Hence, influencing the growth and replication of fungi by inhibiting CYP51 has become a strategy for the development of antifungal agents. In this study, we isolated *Aspergillus fumigatus* and *Aspergillus brevipes* from cow milk, identified them using microscopic methods, and then applied RA against them. Then, study the interaction of RA with the sterol 14α-demethylase enzyme (CYP51) in silico.

2. MATERIALS AND METHODS

2.1. In vitro studies

2.1.1. Strains isolation

Aspergillus strains isolated from lactate cow milk, the cows were selected randomly and we identified the fungal species using morphological characterization. The *Aspergillus* species strains came from a sampling carried out on 10 dairy establishments located in Taif, in the western region of Saudi Arabia. Each of them had between 20 and 50 milking cows, with daily average milk production ranging from 10 to 20 liters per cow. A total of 200 individual milking cows were sampled following the random systemic according to the Thrusfield method (2007). The udders were disinfected with 70% ethanol, dried with individual paper towels, and the first jet of milk was discarded. Fungal isolations were performed on Petri plates containing dichloro rose bengal chloramphenicol agar (DRBC) and dichloro 18% glycerol agar (DG18) (Pitt and Hocking, 1997). Colonies that resembled *A. fumigatus* were taken and subcultured in Malt Extract Agar (MEA), which was subsequently incubated at 25 °C for 7 days in darkness [15].

2.2. Morphological identification

A.fumigatus and *A. brevipes* strains were identified using morphological criteria. Briefly, the strains were grown for 7 days as 3-point inoculations on Czapek agar, and malt extract agar (MEA) plates at 25 °C, and on CYA at 37 °C. Morphological data were analyzed through analysis of variance (ANOVA) was used to determine the significant differences between means [6].

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2.3. The anti-fungal activity of Rosmarinic acid against *A. fumigatus* isolates

The inhibition zone assay was carried out to investigate the fungicidal activity of RA. The inoculum suspension was prepared from colonies grown overnight on an agar plate and inoculated into malt broth. A sterile swab was immersed in the suspension and used to inoculate fungi using malt agar plates. RA was dissolved in dimethyl sulfoxide (DMSO) with serial dilution starting with a concentration of 10 mg/ml to determine the MIC value, while the inhibition zone was measured around each well after 48 h at 28 ^{o}C [15].

2.4. Insilco studies

2.4.1. Preparation of Molecular Systems

The three-dimensional structure of the 14-alpha sterol demethylase Cyp51B enzyme was obtained from the Protein Data Bank (PDB code: 6CR2). Rosmarinic acid with a chemical structure of $C_{18}H_{16}O_8$ was downloaded from the Drug bank database [13].

2.4.2. Molecular Docking

Autodock Vina was used to generate all protein structures for docking and used to explore the molecular interaction of ligands and proteins the exhaustiveness parameter was left at its default value and the grid size was set to 20 20 20 with a spacing of 1. The binding site was created using the ligand's core from the PDB structure. Additionally, 3D binding interactions were shown using the Pymol and 2D structures were generated using Ligplot.

3. RESULTS

3.1. Morphological characterization

The morphologic characterization of the strains isolated from raw cow milk displayed two classes of *Aspergillus* species, *Aspergillus fumigatus* as shown in figures 1, and 2 and summarized in table 1, the colony color in front is blue-green while turn on colorless in reverse as shown in figure 1 (a &b). The colony diameter is 2-3 cm with a Vesicle diameter of 23 µm. The second class of *Aspergillus* species that was found was *Aspergillus brevipes* as shown in figures 3, and 4 and summarized in table 1, the colony color in front is yellow while turns brown in reverse as shown in figure 1 (a &b). The colony diameter is 2 cm with a Vesicle diameter of 14 µm.

Table 1: The morphologic characterization of some *Aspergillus fumigatus* and *Aspergillus brevipes* strains isolated from raw cow milk

Strains name	Colony diameter (media ± SD) Cm	Condition on MEA	Colony color on MEA		Microscopic features (media ± SD)		
	Czapek's agar		Front	Reverse	Stipe (µm)		Vesicle diameter (µm)
					Length	Wide	
Aspergillus fumigatus	2-3 ± 1.2	Abundant	blue -green	colorless	6.0	2.2	23
Aspergillus brevipes	2± 0.9	Abundant	yellow	brown	7.9	3.0	14

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Figures (1a) and (1b): are showing *Aspergillus fumigatus var ellipticus* on Czapek's agar

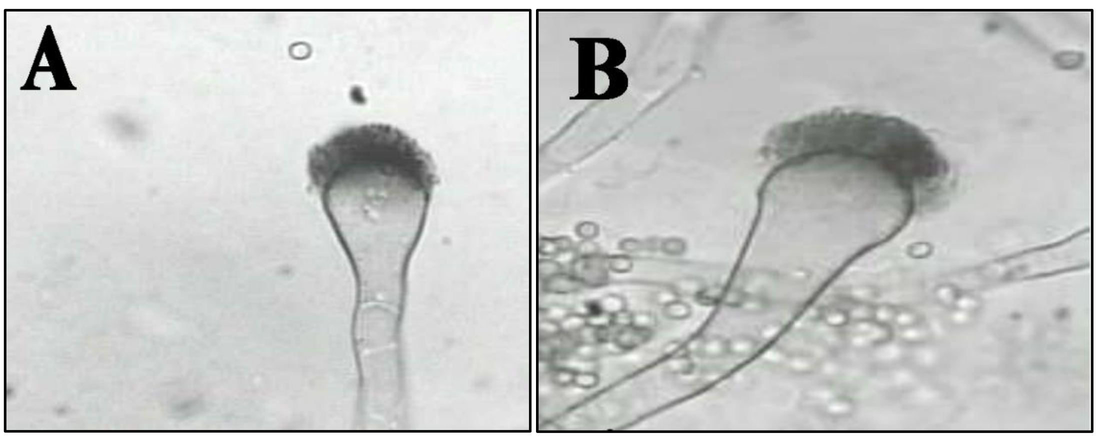

Figure (2a) is showing *Aspergillus fumigatus* Stalked conidial heads. From slide culture on Czapek agar. x 1000 while figure (2b) is exhibiting *Aspergillus fumigatus* Sessile conidial heads. From culture on malt agar. x 1000.

Table 2: The number of *Aspergillus* strains isolated from from raw cow milk.

Total number of sample	*Aspergillus fumigatus*	*Aspergillus brevipes*
100	**12**	**7**

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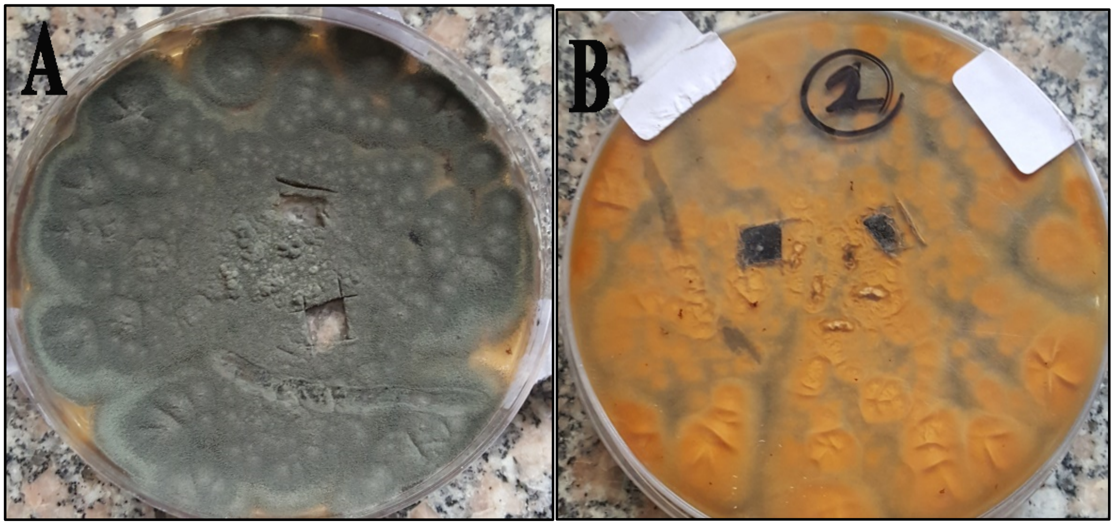

Figures (3a) and (3b) are displaying *Aspergillus brevipes* on Czapek's agar

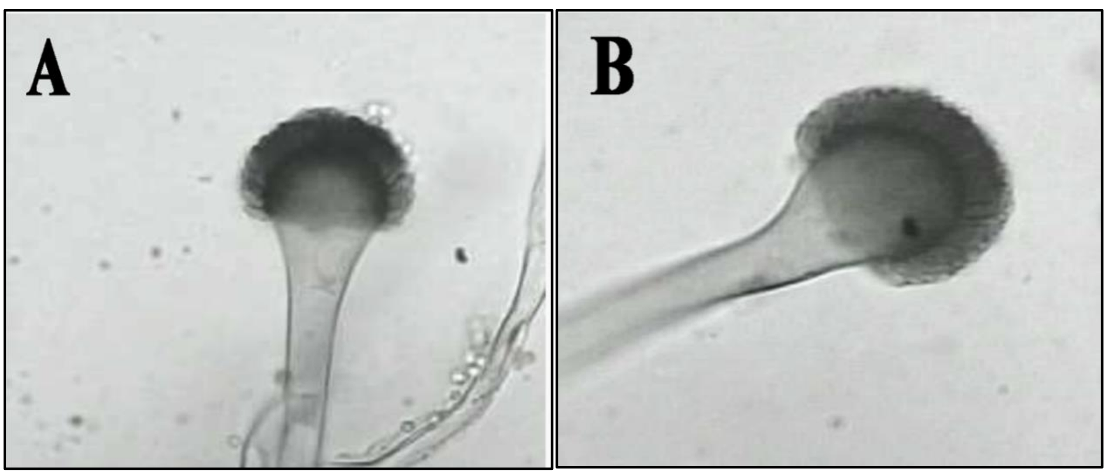

Figure (4a) reveals *Aspergillus brevipes* Stalked conidial heads. From slide culture on Czapek agar. x 1000 while figure (4b) shows *Aspergillus brevipes* Sessile conidial heads. From culture on malt agar. x 1000.

3.2. The susceptibility of *Aspergillus fumigatus* and *Aspergillus brevipes* isolates against Rosmarinic acid

Rosmarinic acid has good anti-fungal activity against many funguses, such as *Candida albicans*, [16]. As table 3 and figures 5 a and b reveal. RA has a strong effect on both isolates of *Aspergillus fumigatus and Aspergillus brevipes* that are isolated from cow milk. The inhibition zone of *A. fumigatus* is 17 nm, while, RA has less effect on *A. brevipes; it is* 15 nm. Similarly, the minimal inhibitory concentration of RA against *Aspergillus fumigatus and Aspergillus brevipes* isolated from cow milk was 321 and 326 mg, respectively.

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Table 3: The inhibition zone of Rosmarinic acid against *A. fumigatus* and *A. brevipes* isolates raw cow milk.

Tested microorganisms	Inhibition Zone (nm)	MIC (mg)
Aspergillus fumigatus	17 nm	321 ±2.3
Aspergillus brevipes	15 nm	326 ± 1.6

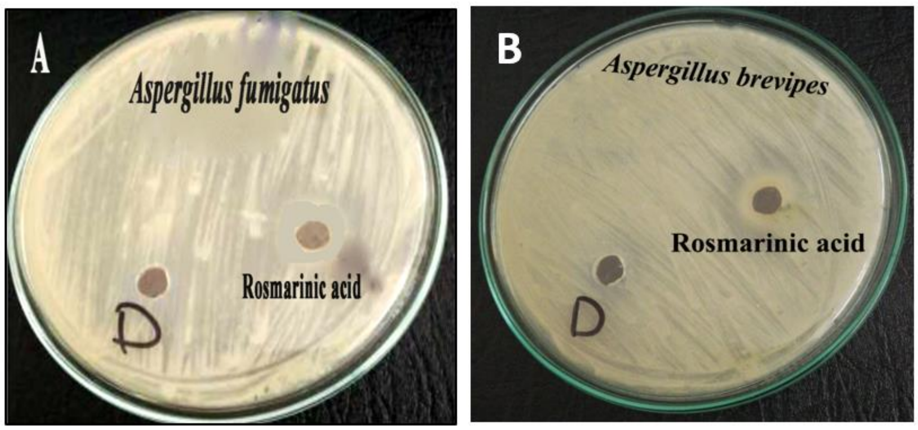

Figure (5a) is displaying inhibition zone diameter produced by rosmarinic acid against *Aspergillus fumigatus* while figure (5b) is showing inhibition zone diameter produced by rosmarinic acid against *Aspergillus brevipes*

3.3. Molecular Docking

The 2D binding conformation rosmarinic acid and sterol 14α-demethylase enzyme (CYP51) as shown in figure 6, RA made 1 hydrogen bond with Ser511 and 1 hydrogen bond with Arg510. As figure 7 showed the result of molecular docking, 3 d interaction revealed that between rosmarinic acid and sterol 14α-demethylase enzyme (CYP51), The binding conformation rosmarinic acid in the active site of sterol 14α-demethylase. The 3d protein position indicated polar hydrogen bond and yellow color indicate the non-polar ones which refer to a red color figure 8 displayed.

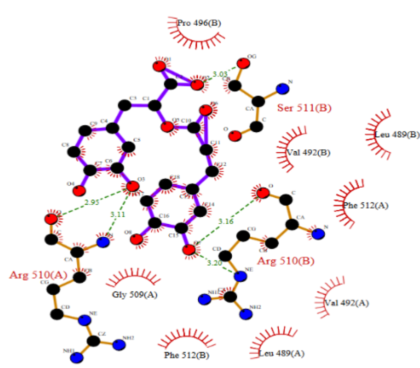

Figure 6: The 2D interaction between rosmarinic acid and sterol 14α-demethylase enzyme (CYP51)

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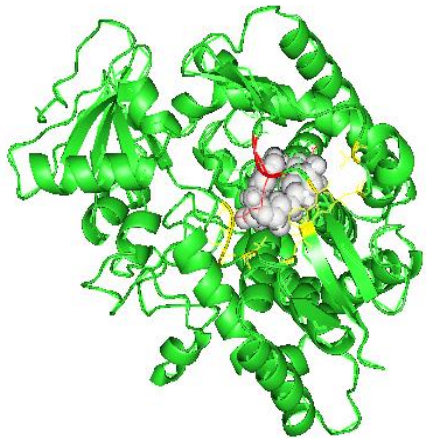

Figure 7: The 3D ineraction between rosmarinic acid and sterol 14α-demethylase enzyme (CYP51)

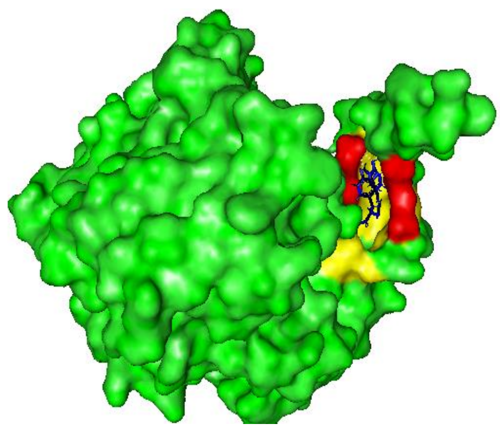

Figure 8: The 3D protein position between rosmarinic acid and sterol 14α-demethylase enzyme (CYP51).

4. DISCUSSION

The *Aspergillus* genus is classified into various sections [16]; *A. fumigatus* belongs to the section Fumigati. According to Pellegrino et al. (2013), the presence of *A. fumigatus* in the surrounding environment is related to a mycological survey of these strains isolated from milk samples. Also, Pellegrino et al. reported that *A. fumigatus* is the second isolated species in milk after A. *flavus*. Furthermore, it's able to associate with high somatic cell counts as well as gliotoxinogenic in milk [17]. A fumigatus is the most common species that causes invasive aspergillosis [18]. Previous studies demonstrated that the main fungus isolated from mycobiota isolated from raw materials and milking cows were Aspergillus and Fusarium fungus. Due to the contamination of farms, fungi frequently exist in the cattle environment special cow, the milk is contaminated by toxinogenic fungi [19]. The contaminations of lactating cow's mammalian glandule with A. fumigatus are easy because of the surrounding environment. Most studies report the role of azoles as an antifungal agent against aspergillosis [20]. Aspergillosis becomes pathogenic to cows as well as the farm handlers'. Silimialry, *Aspergillus brevipes* exist in the soil. Also, *Aspergillus brevipes* is quite unlike any other species of Aspergillus hitherto [21]. RA is present in basil which belongs to the Lamiaceae family [22]. The Rosmarinus (officinalisL.) essential oil (REO) has a fungicide activity against fusarium pathogenic strains [23]. In our work, we isolated the Aspergillus species from raw cow milk in Taif city and isolated the fungal using the Thrusfield method [15]. Then identified and characterized the species using a morphological approach, then investigated the activity of RA against the fungal isolated species, and study the molecular docking using auto-vina docking, the result has been shown that the isolated from raw cow milk were *Aspergillus brevipes* and *Aspergillus fumigatus* and the Fungicide activity of RA is more strongly against *Aspergillus fumigatus* than *Aspergillus brevipes* species. The molecular docking showed the binding of rosmarinic acid with sterol 14α-demethylase enzyme (CYP51) via one hydrogen bond with Ser511 and 1 hydrogen bond with Arg510 as shown in figures (6, 7, and 8). The previous report mentioned that REO has a strong activity to inhibit the mycelial growth of Fusarium spp at a concentration of 1000 µg /ml [23]. Interestingly, the RA inhibition mechanism of *C.Albicans* depends on inhibiting the TTM-RTPase enzyme [7]. Also, the sterol 14a-demethylase enzyme (CYP51) belongs to the cytochrome P450 family that consider a target site for fungal infection. The hydrophobic interaction is the only approach for inhibitors binding to CYP51.

5. CONCLUSION

In this work, we study the fungicide activity of RA against *Aspergillus fumigatus*, and *Aspergillus brevipes* which were isolated from raw cow milk in Taif city Saudi Arabia by using diffusion disk and MIC methods. The result has shown that RA has a good effect against *Aspergillus fumigatus*, and *Aspergillus brevipes* strains. The inhibition zone of A. *fumigatus* and A. *brevipes* were 17 and 15 nm respectively after the strains were investigated with RA. The MIC value of RA against *Aspergillus fumigatus* and *Aspergillus brevipes* isolated from cow milk was 321 and 326 mg, respectively. The 14-alpha sterol demethylase Cyp51B enzyme was inhibited by RA via the formation of two hydrogen bonds with Ser511 and Arg510 respectively. Finally, RA is a promising agent against fungal species with few adverse effects on both humans and animals.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

Acknowledgments

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Conflict of Interest Statement

According to the authors, there were no financial or commercial ties that would raise the possibility of a conflict of interest while conducting the study.

Reference

1) Keller, L.A.M., Keller, K.M., Monge, M.P., Pereyra, C.M., Alonso, V.A., Cavaglieri, L.R.,et al., 2012. Gliotoxin contamination in and pre- and postfermented corn, sorghum and wet brewer's grains silage in Sao Paulo and Rio de Janeiro State, Brazil. J. Appl.Microbiol. 112, 865–873.

2) Pena, G.A., Monge, M.P., González Pereyra, M.L., Dalcero, A.M., Rosa, C.A., Chiacchiera, S.M., Cavaglieri, L.R., 2015. Gliotoxin production by Aspergillus fumigatus strains from animal environment. Micro-analytical sample treatment combined with a LC-MS/MS method for gliotoxin determination. Mycotoxin Res. 31, 145–150.

3) Latgé J-P, Chamilos G. 2019.Aspergillus fumigatus and aspergillosis in 2019.Clin Microbiol Rev 33:e00140-18. https://doi.org/10.1128/CMR.00140-18

4) Girma, G., Abebaw, M., Zemene, M., Mamuye, Y., Getaneh, G., 2016. A review on aspergillosis in poultry. J. Vet. Sci. Technol. 7, 382. http://dx.doi.org/10.4172/2157-7579.1000382

5) Pena, G. A., Alonso, V., Manini, M. V., Pellegrino, M., & Cavaglieri, L. R. (2018). Molecular characterization of Aspergillus fumigatus isolated from raw cow milk in Argentina: Molecular typing of A. fumigatus from raw cow milk. *International journal of food microbiology*, *275*, 1-7.

6) Samson, R.A., Hong, S., Peterson, S.W., Frisvad, J.C., Varga, J., 2007. Polyphasic taxonomy of Aspergillus section Fumigati and its teleomorph Neosartorya. Stud. Mycol. 59,147–203. http://dx.doi.org/10.3114/sim.2007.59.14

7) Swari, D. A. M. A., Santika, I. W. M., & Aman, I. G. M. (2020). Antifungal Activities of Ethanol Extract Of. *Journal of Pharmaceutical Science and Application*, *2*(1), 28-35.

8) Marinho, S.; Illanes, M.;Ávila-Román, J.; Motilva, V.; Talero,E. Anti-Inflammatory Effects of Rosmarinic Acid-Loaded Nanovesicles in Acute Colitis through Modulation of NLRP3 Inflammasome. *Biomolecules* **2021**, *11*, 162. https://doi.org/10.3390/biom11020162

9) Hitl, M.; Kladar, N.; Gavarić, N.; Božin, B. Rosmarinic Acid-Human Pharmacokinetics and Health Benefits. Planta Med. 2020, doi: 10.1055/a-1301-8648.

10) Shi N, Zheng Q and Zhang H (2020) Molecular Dynamics Investigations of Binding Mechanism for Triazoles Inhibitors to CYP51. Front. Mol. Biosci. 7:586540.doi: 10.3389/fmolb.2020.586540.

11) Lee, Y., Puumala, E., Robbins, N., and Cowen, L. E. (2020). Antifungal drug resistance: molecular mechanisms in Candida albicans and beyond. Chem. Rev.[Epub ahead of print]. doi: 10.1021/acs.chemrev.0c00199

12) Choi, J. Y., and Roush, W. R. (2017). Structure based design of CYP51 inhibitors.Curr. Top. Med. Chem. 17, 30–39. doi: 10.2174/1568026616666160719164933

13) Hargrove, T. Y., Friggeri, L., Wawrzak, Z., Sivakumaran, S., Yazlovitskaya, E. M.,Hiebert, S. W., et al. (2016). Human sterol 14 alpha-demethylase as a target for anticancer chemotherapy: towards structure-aided drug design. J. Lipid Res. 57, 1552–1563. doi: 10.1194/jlr.M069229.

14) Thrusfield, M., 2007. Veterinary Epidemiology, 3rd Edn. Blackwell Science, Oxford, USA,pp. 1–593.

15) Hindler, J.A.;Howard,B.J. and Keiser, J.F.(1994): Antimicrobial agents and Susceptibility testing. In: Howard BJ (Editor), Clinical and pathogenic Microbiology. Mosby-Year Book Inc., St. Louis, MO, USA.

16) Peterson,S.W.(2008).Phylogenetic analysis of *Aspergillus* species using DNA sequences from four loci. Mycologia 100, 205–226.doi: 10.3852/mycologia.100.2.205.

17) Pellegrino, M., Alonso, V., Vissio, C., Larriestra, A., Chiacchiera, S.M., Bogni, C., Cavaglieri, L., 2013. Gliotoxinogenic *Aspergillus fumigatus* in the dairy herd environment. Mycotoxin Res. 29, 71–78.

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18) Zhao, J., Kong, F., Li, R., Wang, X., Wan, Z., & Wang, D. (2001). Identification of *Aspergillus* fumigatus and related species by nested PCR targeting ribosomal DNA internal transcribed spacer regions. *Journal of clinical microbiology*, *39*(6), 2261-2266.

19) Alonso, V.A., Monge, M.P., Dalcero, A.M., Keller, K.M., Rosa, C.A. da R., Cavaglieri, L.R., Chiacchiera, S.M., 2009. Contribution of raw materials on dairy cattle feedstuff aflatoxin contamination in Central Argentina. Rev. Bras. Med. Vet. 31, 92–99.

20) Verweij, P.E., Chowdhary, A., Melchers, W.J.G., Meis, J.F., 2016. Azole resistance in *Aspergillus fumigatus*: can we retain the clinical use of mold-active antifungal azoles? Clin. Infect. Dis. 62, 362–368. http://dx.doi.org/10.1093/cid/civ885.

21) Smith, G. (1952). *Aspergillus brevipes* n. sp. *Transactions of the British Mycological Society*, *35*(4), 241-IN1.

22) Petersen, M. and Simmonds, M.S.J. (2003) Rosmarinic Acid. Phytochemistry , 62, 121-125. https://doi.org/10.1016/S0031-9422(02)00513-7

23) da Silva Bomfim, N., Nakassugi, L. P., Oliveira, J. F. P., Kohiyama, C. Y., Mossini, S. A. G., Grespan, R., Nerilo, S. B., Mallmann, C. A., Abreu Filho, B. A. and Machinski, M. (2015b). Antifungal activity and inhibition of fumonisin production by Rosmarinus officinalis L. essential oil in Fusarium verticillioides (Sacc.) Nirenberg. Food Chemistry, 166, 330-336.