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RESEARCH ARTICLE - MEDICAL TECHNIQUES

Expression Profiling of Transglutaminase 2 and Inflammatory Host Response in Aspergillus Fumigatus After Treatment with Curcuma Extracts

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Abstract

Aspergillus fumigatus, is considered the most common human pathogen causing Aspergillosis, and is a tough opportunistic pathogen. This pathogen is very capable of forming biofilms via developing hyphal walls radially. The current therapies are known for their limitations where they couldn't target the proteins involved in undoing host response. Here we aimed to screen the possible antifungal role of Curcumin and its effect on the Transglutaminase 2 and Reactive Oxygen species response. We also screened the levels of cytokines with and without the treatment to confirm the activity. We could neutralize the elevated levels of TG2 by using the treatment (methanol extract). Even the cytokine levels could be normalized with the extract treatment which is usually highly elevated with the fungal infection. We found that TG2 and IL 1β and IL 18 got elevated significantly in control in addition, as well as our extract could confirm the possible role of ROS and its effect on TG2 which is said to play a major role in apoptosis.

Keywords: Aspergillus fumigatus; Transglutaminase 2; ROS; Interleukins

1. Introduction

Aspergillus is known for its ubiquitous nature and saprophytic behaviour, which aids in the recycling of Nitrogen and carbon. Their main job is within the soil to decay the vegetation, these sometimes form spores called conidia, where they disperse and contaminate the air. The genus Aspergillus includes more than one species, impacting public health to a large extent in being beneficial and destructive [1]. Among the human pathogens of Aspergillus, A. fumigatus stands as the first, and then A. flavus, A. terreus, followed by A. niger [2]. It causes a wide range of human infections that overly opportunistically depending on the host immune response [3].

In humans, who are compromised with lung functions like asthma and cystic fibrosis, these spores could lead to allergic bronchopulmonary aspergillosis (ABPA). There are some non-invasive aspergillomas which also may form conidia to stay for longer among the alveoli. Invasive aspergillosis (IA) on the other hand, being more devastating targets the immunocompromised patients killing them. Even people with blood disorders like leukaemia, hematopoietic cell transplant patients, people on corticosteroids, and those with chronic granulomatous disease (CGD) are all more prone to these spores, making the rate of mortality very high [4].

Reactive Oxygen species (ROS) are generated within the body due to the malfunctioning of metabolic enzymes or due to infections. These infections either bacterial, viral, or fungal and whether, they are chronic or acute do leads to the development and production of ROS species. Within, the host, the ROS from the mitochondria also serve as markers for many of the metabolic ailments and apoptosis [5]. Phagocytosis and cytokines secretion, do activate the host enzymes like NADPH Oxidase and Nitric Oxide Synthases, aiding in neutralizing the invading fungal spores. At such times, the host oxidative response responds adequately to the fungal spores and that again depends on the susceptibility of the patients as discussed earlier [6].

Nomenclature			
ABPA	Allergic Bronchopulmonary Aspergillosis	IA	Invasive Aspergillosis
CGD	Chronic Granulomatous Disease	SOD	Superoxide Dismutase
TG2	Transglutaminase 2	DMEM	Dulbecco's Modified Eagle's Medium
YPD	Yeast Peptone Dextrose Agar	PBS	Phosphate-Buffered Saline
FITC	Fluorescein Isothiocyanate		

In response to such oxidative stress, these pathogens try to mitigate transcriptional, post-translation, and many enzymes like Superoxide Dismutase (SOD) and Catalases, enabling them to facilitate or adapt by using diverse strategies ranging from evasion of uptake to intracellular parasitism that permit fungus to survive and replicate within macrophages [7]. Transglutaminase 2 (TG2, EC 2.3.2.13) is said to be the most commonly expressed enzyme with its characteristic cross-linking nature aiding in growth, differentiation, and apoptosis of the cells [8]. TG2 is said to be the major reason for cellular activity with the liver cells when induced by alcoholic and non-alcoholic conditions. This TG2, in turn, inactivates the transcription factor Sp1, which slows down the growth factor receptors expression, killing the cells [9]. Even reactive oxygen species (ROS) response, is also said to activate TG2 within different cell types [10] [11] exhibiting or destructing functions like mortality and morbidity in line with the dosage of the signal. This enhanced TG2, translocates Bax to the mitochondria, releasing apoptosis-inducing genes activating caspase pathways and then finally apoptosis [12].

TG2 is also said to elevate the levels of apoptosis-inducing genes within hepatic cells [13] and inactivate the Sp1 transcription factor. This in turn reduces the expression of epidermal growth factor receptors (EGFR) both of which are responsible of cell survival. Once these molecules get reduced in expression, they lead to cellular apoptosis [14]. Suppression of TG2 was also found to reduce cell death [15]. It is also said to enhance the levels of cytokines like interleukins, enhancing the chances of more apoptosis [16].

Curcuma longa (C. longa), is one of the best and potent ethnobotanical herb used globally by people. It belongs to the Zingiberaceae family and is known to contain loads of complex compounds which are used in food, spices, flavouring, and seasoning. It is best used in the cosmetics and pharmaceutical industries [17]. Its invaluable therapeutic properties make it insecticidal [18], antimicrobial [19], antifungal [20], antimalarial [21], antiviral, and a rich antioxidant. It is said to have a toxic effect on many of the fungal species, which destroys not only agriculture but also human health [22, 23].

Here, in the present, we tried to investigate the cellular activity of Curcumin against the human pathogen Aspergillus fumigatus. We tried to deduce a possible cooperative role of TG2 and interleukins in eliciting an apoptotic signal among the host tissues. We thus aim to neutralize such an action of the fungus with methanolic extracts of Curcuma and could block the apoptotic signals.

2. Materials and Methods

2.1. Cell lines maintenance

The Mouse 3T3 fibroblasts from the Wild type mice, were donated by Stellixar Laboratories, India. The cell lines of 3T3 mouse fibroblasts with passage number 12 (P12), were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich), supplemented with L-Glutamine, and FCS. The cells were checked for confluency and frequently trypsinized to maintain the uniform growth of the cells. The cells were checked for optimal growth, daily by maintaining the physical conditions and the growth medium, controlled temperature, incubator that maintains correct pH and osmolality.For the extraction of RNA content, the cells were usually grown to about 80% confluency, and then the total RNA was extracted using the basic protocol given along with the RNeasy mini kit.

2.2. Fungal strain

Aspergillus fumigatus, was cultured on yeast peptone dextrose agar (YPD), and co-incubated with 3T3 and in vitro infection, these strains were initially cultured in YPD broth for 8-16hr at 30°C with continuous shaking at 180 Rcf (xg), These cultures were then rinsed in phosphate-buffered saline (PBS) until a cell growth of 0.8-0.9 by measuring the OD at 600nm using a visible spectrophotometer (Genetix, India).

2.3. Plant extract

Curcumin longa corms were sun-dried and pulverized thoroughly in a blender. About 50gm of powder was extracted with 500ml of methanol, ethanol, and diethyl ether using soxhlet extraction. Following extraction, the crude extract was freeze-dried in a rotary evaporator and stored at -200C until further use.

2.4. Curcumin Antifungal Activity

Several reports suggest that Curcumin is efficient in displaying antifungal activity. From our results, we found that methanol extract alone showed significant results, hence only those results of methanol were shown in figure 1 (The percentage of fluorescent mortal cells relative to the total cells was shown in the images). Here we used the method as described by Bonifácio et al [24] . In brief, the fungal cells were plated into 24 well plates with YPD medium. The cells were then exposed to about 50µg/mL and 100µg/mL of methanol extract for 12hr in an incubator at 300C. Amphotericin B (50µg/mL) was used as a positive control in the study. Following incubation, cells were stained with SYTOX and observed under confocal microscopy. SYTOX is said to penetrate only dead cells and as such differentiation between live and dead cells could be screened effectively [24].

2.5. Determination of ROS in vitro

3T3 cells were seeded onto a 24 well plate and incubated in a CO2 incubator (5%) at 37 °C. The following day, the culture was co-incubated with Aspergillus strain (4 × 106cells) for about 8hr. The ROS production was then analysed based on the incorporation of 5–6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate CM-H2DCFDA (5µM) (Sigma Alrich, USA) for 15min at 37°C. Following incubation, the cells were observed for their fluorescein isothiocyanate (FITC) signals using confocal microscopy (Leica DMRBE; Germany) using the appropriate filters for the FITC assay. The confocal settings (Gain PMT1- 647 V, Gain PMT- 872 V, offset PMT1-30%, and offset PMT2-41%) were kept constant, for all the slides. Image analysis was done, to assess the levels of ROS.

2.6. RNA extraction

The cells (P12) were treated and co-infected as in the previous section. Cells were treated with curcumin and positive control at 50µg/ml. The over confluent cell cultures, which optimally contain about, 4 to 10 million cells, were used for the extraction process. The basic protocol using the RNeasy spin columns, provided with the RNeasy kit, was followed for the process. The confluent cultures were initially washed with PBS (pH-7) and were treated with the Trypsin EDTA solution. The cell suspension obtained was later used for the extraction process (the optimal number being about 4 – 106 cells). The cells were later digested using the RLT buffer supplied along with the kit. The digested cell suspension was then added onto the RNeasy spin columns and was allowed to go through the chain of extraction treatments, like treating with washing buffer, centrifuged at high speeds, and washing them with the RPE buffer. The RNA obtained was then re-suspended into RNase-free water.
The total RNA content obtained, was quantified and was reverse-transcribed by using Superscript II Reverse transcriptase (Sigma), by using random primers. About 2µg of the RNA sample and about 1.25mM of dNTPs (Roche Applied Biosciences) were used for the production of the complementary DNA from the RNA content. All the RNA samples thus obtained, were cross-checked for genomic DNA contamination, by running the RNA samples on the 1.5% agarose gels.

2.7. Real-time PCR

Primers used for the traditional PCR were used for the real-time PCR also. The real-time PCR assay was then performed according to Salam Abbas et al [25] using the iQTM SYBR Green Supermix (HiMedia). The primers (600nM) and 1µl of the RT products were used with a total volume of 12.5µl. The assay was done in duplicates along with Housekeeping gene GAPDH.

2.8. Expression of TG2 members

Real-time PCR was carried on the samples in the Corbett Research cycler (Bio-Rad). The panDerm_F primers FW 5' AGTATGAGCATGGGCAACGA 3' and RV 5' ATACAGGGGATCGGAAAGTG 3' of about 600nM was used in this assay (product length 408bp). 1.3µl of the RNA was used for about 40 cycles at 93°C for 55s, 62°C for 45s, and with an elongation at 71°C for 1min. GAPDH primers (FW: 5' TGGAAAGCTGTGGCGTGAT 3'; RV: 5' CCCTGTTGCTGTAGCCGTAT 3'; Product length 395bp] was used [Ciesielska, , 2018] was also amplified for a comparative analysis of the gene. The comparative analysis was done by the ΔΔCt method.

2.9. Cytokine assay

The cells (P12) were treated and co-infected as in the previous section. Cells were treated with curcumin and positive control at 50µg/ml. The cytokines like IL 1β and IL 18 which are supposed to be elevated by ROS reaction, were measured using the ELISA kit (Everone Biosciences). The protocol was followed according to the manufacturers' instructions. The supernatant of cells cultured (control and treatment) was pinned down at 8000rpm, 10min, and stored at −20°C until further use. The cells without treatment were used as control. The day before the assay, the 96well plate was impregnated with the capture antibody against the specific molecules. The samples were added to their respective wells and done in triplicates. The plate was incubated for about 2hours and the following washing with secondary antibody conjugated with HRP. H2O2-tetramethylbenzidine was sued as the substrate and after the incubation, the reaction was stopped using 2N H2SO4 and the absorbance was read at 405nm in a plate reader (Genetix). The concentration of the study proteins released into the media was estimated by colorimetric Assay using the standard graph. The minimal detection for IL 1β and IL 18 was adjusted to 20pg/ml. All assays were performed in duplicate.

3. Results and Discussion

3.1. Antifungal activity

From the scan reports, it was evident that there was more deaths observed (as visible by green-stained cells) in the case of Positive control and Curcumin also. Curcumin was also found to show significant antifungal activity when compared to positive control. Our results follow the findings of Praditya (2019). [26] Praditya stated the Curcuma is said to have toxic effects that stops the growth of mycelia. Their findings are related to the plant pathogens, but still, we could conclude their study that Curcuma could be an effective antifungal.

3.2. ROS activity

From the confocal scan images, it was evident that (as viewed from the green stain) there is a lot of secretion out of the cells which could be due to some soluble factors (Not confirm ROS). Since many reports state that most of the fungal infections do cause ROS response and is evident by the green signal secreting out of the cell cytoplasm Bonifácio et al [24] (Ficociello,) [27] From the very base, we could hypothesize

that our findings might be due to ROS response. The level of ROS is found to be quite significant when compared to the negative control (cells alone without infection by the fungus). Such high levels of ROS production could make the strain pathogenic.

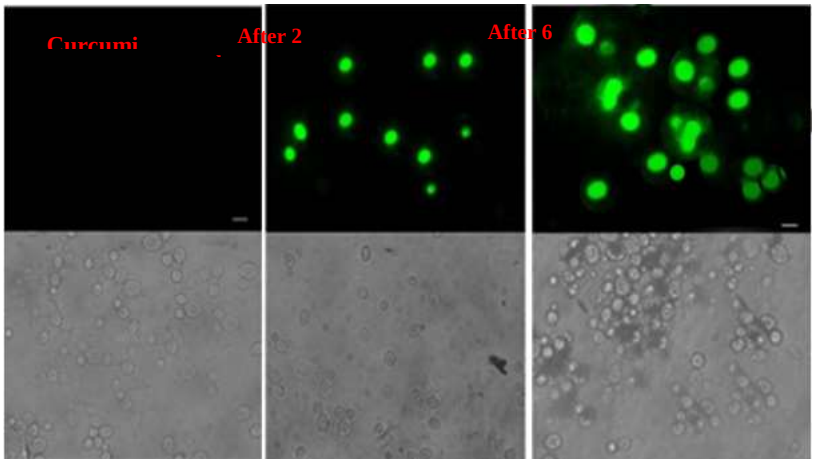

Fig. 1 Cell Viability or growth inhibition of A. fumigatus treated with Curcumin methanol extract along with Amphotericin B (positive control). The percentage of fluorescent mortal cells relative to the total cells was shown in the images. (SYTOX Green staining) Scale bar 5 µm. The bottom images are of a bright field

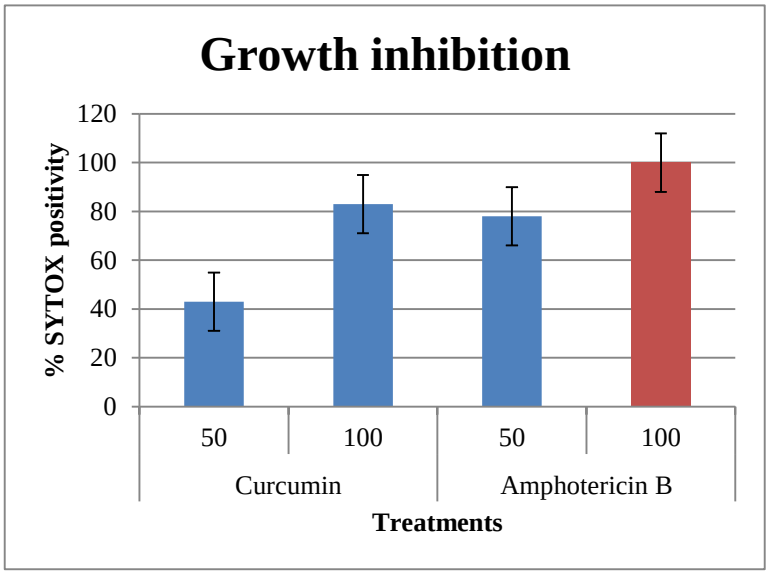

Fig. 2 Cell Viability or growth inhibition of A. fumigatus treated with Curcumin methanol extract along with Amphotericin B (positive control). The percentage of fluorescent mortal cells relative to the total cells was shown in the graph. All the results are mean of triplicates and expressed as percentage ±SD

3.3. Gene expression studies

All the results in the real-time analysis were normalized to GAPDH and the results obtained were the mean values of the duplicates. Each gene was analysed individually, in the samples with its negative control (Sterile Water). The Ct value with the lower $\Delta\Delta^{Ct}$ values was used as a calibrator. The Ct values above, 25 were shown to contain no amplification.

From the melting curves, it was evident that there is an elevation in the expression of TG2 members when compared to the treatment. This might be due to the very reason for the release of ROS into the culture medium. On the other hand, curcumin could lower the expression levels to a great extent. The Ct value was found to be 26 and 30 for Curcumin and positive control respectively. This could confirm the possible antifungal role of Curcumin and its significant effect on the TG2 expression which is solely responsible for apoptosis of the cell lines. The expression of the PC and Curcumin was found to be 24 and 56 times less than that of the control. Control was treated as hundred percent.

Guilin and his co-workers [28] reported that ROS-generating Candida species could enhance cellular activity, especially nuclear TG activity within the hepatic cells, which eventually led to apoptosis, even same results were seen in the liver cells of mice infected with Candida species. Similar findings were reported by Shrestha and his co-workers, [29], that co-incubating hepatocyte with Candida species, induces hepatic cell death with elevated TG levels. This is again due to ROS. And when used with TG2 inhibitor, 6-diazo-5-oxo-norleucine tetra peptide (ZDON), apoptosis was greatly reduced. Our findings are in line with the above two findings. On coinfection, we could find ROS response, and on

treatment, the level was found to reduce. The same was seen in the case of elevated levels of TG2 when co-infected with fungus. The possible explanation could be due to ROS response, the TG2 activity was inhibited which was very high in the case of control.

3.4. Cytokine assay

From the table, it was found that treatment has reduced the IL 1β and IL 18 levels when compared to the control values. The control values were found to be 57.89± 0.11pg/ml and 26.24± 0.26pg/ml for IL 1β and IL 18 respectively. Curcumin (5029µg/ml) significantly reduced IL 1β levels to 21.23 ± 0.28pg/ml from 57.89 ± 0.11pg/ml value of the control. (see table 1) Positive control got reduced 5.11± 0.71pg/ml. The same results were observed even for the IL 18 levels. The values of curcumin and positive control for IL 18 were found to be 09.45± 0.45 and 02.56± 0.61 which is significantly lesser than the control (26.24± 0.26pg/ml) $P < 0.005$.

ROS response created by Glucose levels was found to enhance IL 1β and IL 18 levels within adipocytes. Studies were done by (Akira,2019) [30] on C. albicans also reported the same that, when cells are stressed with ROS, they could initiate inflammasome, which eventually triggers the caspase 1 activation and increases the levels of IL 1β and IL 18 within the cells. Similar findings were reported by Manoranjan Sahoo (2011) where they found the cells co-infected with fungal strains triggered caspase 1 and apoptosis via increasing the interleukins levels. Our study is following the above findings, with enhanced levels of IL 1β and IL 18 among the cells without treatment. This could be the possible explanation stating that the fungal infections could initiate ROS response and increase the levels of these cytokines to make the host cells apoptotic.

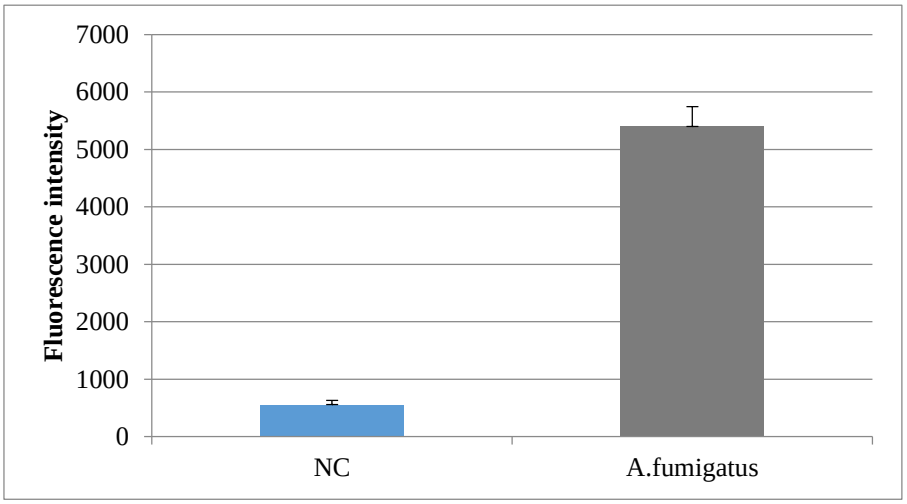

Fig. 3 The Fluorescence intensity of the ROS activity with and without treatment. NC: Negative control. All the values are average of triplicates

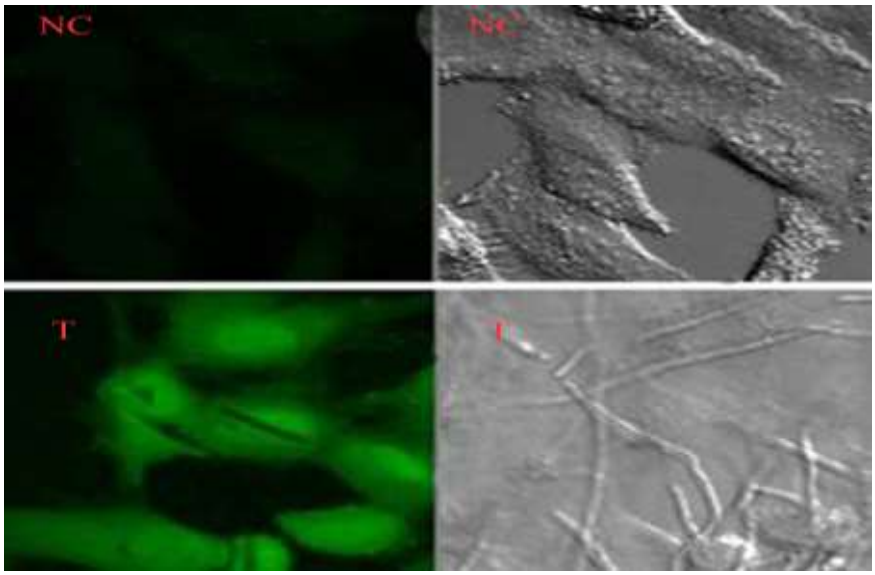

Fig. 4 Confocal scan images showing the cells co-infected with fungal strain with 5µM CM-H2DCFDA. (Scale bar=20µm). NC: Negative control; T: Treatment

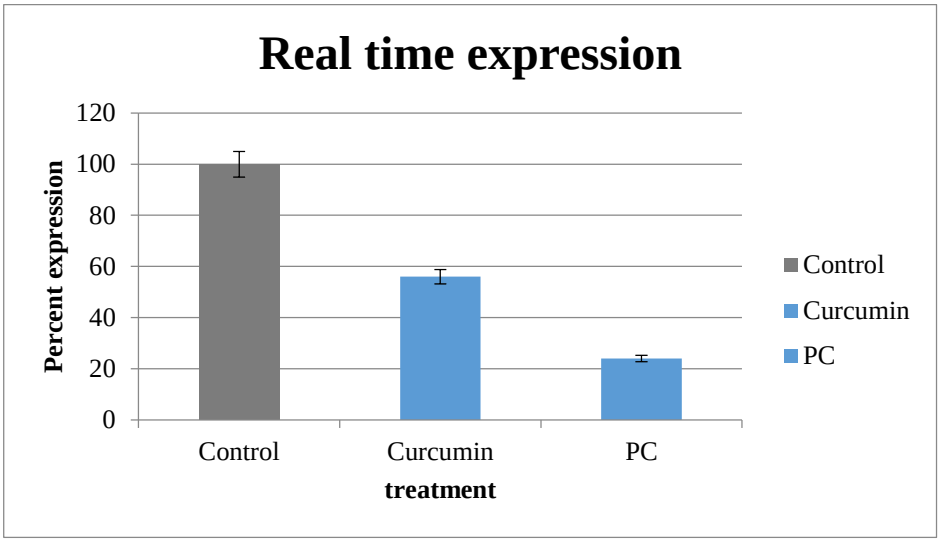

Fig. 6 Graph showing the $2-\Delta\Delta^{Ct}$ values for the gene member TG2 for both the control and treatments. PC: positive control (Amphotericin B) All the experiments were average of duplicates. GAPDH is used as a calibrator

Table 1 Table showing the concentration of IL 1β and IL 18 in pg/ml. All the values are average of triplicates and expressed as value ± SD

	IL-1β	**IL-18**
Control	57.89± 0.11	26.24± 0.26
Curcumin	21.23± 0.28	09.45± 0.45
PC	5.11± 0.71	02.56± 0.61

5. Conclusion

There exists urgency to screen new antifungal compounds against Aspergillus species especially human pathogen. Aspergillus fumigatus infections have been increasing due to the limitations among the existing therapies. Mycelia and biofilm formation are pathogenic initiators which get enhanced with elevated levels of ROS and cytokine levels within the host. In the present context, we confirmed the antifungal nature of the Curcuma longa by expression studies of cytokines concerning TG2. We found that TG2 and cytokines like IL 1β and IL 18 got elevated significantly in the control and that on treatment we could neutralize their expression. In addition, our extract could confirm the possible role of ROS and its effect on TG2 which is said to play a major role in apoptosis.

References

[1] akoma, D.O., Department of Biological Sciences (Microbiology Option) Faculty of Science Benson Idahosa University Benin City. 2017.

[2] Hassan, A.S., et al., In-vitro assessment of first-line antifungal drugs against Aspergillus spp. caused human keratomycoses. Journal of Infection and Public Health, 2020. 13(12): p. 1907-1911.

[3] Desforges, M., et al., Human coronaviruses and other respiratory viruses: underestimated opportunistic pathogens of the central nervous system? Viruses, 2020. 12(1): p. 14.

[4] Viegas, C., et al., Aspergillus spp. and azole-resistance characterization on filtering respiratory protective devices from waste sorting industry. 2020.

[5] Ikwegbue, P.C., et al., Interplay between heat shock proteins, inflammation and cancer: a potential cancer therapeutic target. American journal of cancer research, 2019. 9(2): p. 242.

[6] Arias, M., et al., Preparations for invasion: modulation of host lung immunity during pulmonary aspergillosis by gliotoxin and other fungal secondary metabolites. Frontiers in immunology, 2018. 9: p. 2549.

[7] Kolahi, M., et al., Oxidative stress induced by cadmium in lettuce (Lactuca sativa Linn.): Oxidative stress indicators and prediction of their genes. Plant Physiology and Biochemistry, 2020. 146: p. 71-89.

[8] Katt, W.P., M.A. Antonyak, and R.A. Cerione, Opening up about tissue transglutaminase: when conformation matters more than enzymatic activity. Med one, 2018. 3(6).

[9] Dewidar, B., C. Meyer, and S. Dooley, TGF-β in hepatic stellate cell activation and liver fibrogenesis—updated 2019. Cells, 2019. 8(11): p. 1419.

[10] Verzella, D., et al., Life, death, and autophagy in cancer: NF-κB turns up everywhere. Cell death & disease, 2020. 11(3): p. 1-14.

[11] Bhat, A.A., et al., Tight junction proteins and signaling pathways in cancer and inflammation: a functional crosstalk. Frontiers in Physiology, 2019. 9: p. 1942.

[12] Wang, S.-T., et al., Tea polyphenols and their chemopreventive and therapeutic effects on colorectal cancer. World journal of gastroenterology, 2020. 26(6): p. 562.

[13] Lawani-Luwaji, E.U., Differential expression of long and short isoforms of tissue transglutaminase 2; Implications for cisplatin resistance in an in-vitro MCF-7 breast cancer model. 2019, Anglia Ruskin University.

[14] Wouters, A., et al., The intriguing interplay between therapies targeting the epidermal growth factor receptor, the hypoxic microenvironment and hypoxia-inducible factors. Current pharmaceutical design, 2013. 19(5): p. 907-917.

[15] Gundemir, S., et al., Transglutaminase 2: a molecular Swiss army knife. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 2012. 1823(2): p. 406-419.

[16] Labib, P.L., G. Goodchild, and S.P. Pereira, Molecular pathogenesis of cholangiocarcinoma. BMC cancer, 2019. 19(1): p. 1-16.

[17] Sieniawska, E., et al., Plant-based food products for antimycobacterial therapy. EFood, 2020. 1(3): p. 199-216.

[18] Süntar, I., et al., Pharmacological and chemical features of Nepeta L. genus: Its importance as a therapeutic agent. Phytotherapy Research, 2018. 32(2): p. 185-198.

[19] Naghdibadi, H., et al., An overview on two valuable natural and bioactive compounds, thymol and carvacrol, in medicinal plants. Journal of Medicinal Plants, 2017. 16(63): p. 1-32.

[20] Salehi, M., M.R. Naghavi, and M. Bahmankar, A review of Ferula species: Biochemical characteristics, pharmaceutical and industrial applications, and suggestions for biotechnologists. Industrial Crops and Products, 2019. 139: p. 111511.

[21] Saez, N.J. and V. Herzig, Versatile spider venom peptides and their medical and agricultural applications. Toxicon, 2019. 158: p. 109-126.

[22] Leontopoulos, S., et al., Bioactivity potential of polyphenolic compounds in human health and their effectiveness against various food borne and plant pathogens. A review. J. Food Biosyst. Eng, 2017. 7: p. 1-19.

[23] Devi, R., et al., Fungal secondary metabolites and their biotechnological applications for human health, in New and Future Developments in Microbial Biotechnology and Bioengineering. 2020, Elsevier. p. 147-161.

[24] Bonifácio, B.V., et al., Antifungal activity of a hydroethanolic extract from Astronium urundeuva leaves against Candida albicans and Candida glabrata. Frontiers in microbiology, 2019. 10: p. 2642.

[25] Abbas, S. and S. Malla, Cytotoxicity and expression studies of angiogenesis-promoting genes in cancer cell lines under the treatment of cancer candidate drugs. Asian J Pharm Clin Res, 2019. 12(5): p. 130-134.

[26] Praditya, D., et al., Anti-infective properties of the golden spice curcumin. Frontiers in microbiology, 2019. 10: p. 912.

[27] Ficociello, G., et al., Anti-candidal activity and in vitro cytotoxicity assessment of graphene nanoplatelets decorated with zinc oxide nanorods. Nanomaterials, 2018. 8(10): p. 752.

[28] Guillin, O.M., et al., Selenium, selenoproteins and viral infection. Nutrients, 2019. 11(9): p. 2101.

[29] Shrestha, R., et al., Fungus-derived hydroxyl radicals kill hepatic cells by enhancing nuclear transglutaminase. Scientific reports, 2017. 7(1): p. 1-11.

[30] Kawai, T. and S. Akira, Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity, 2011. 34(5): p. 637-650.