Advances in
Biotechnology & Microbiology
ISSN 2474-7637

Juniper Publishers
key to the Researchers

Research Article
Volume 18 Issue 3 - September 2024
DOI: 10.19080/AIBM.2024.18.555987

Adv Biotechnol Microbiol

Antifungal Effects of Rosmarinic Acid, α-Tocopherol and α-Tocopherol Acetate on *Aspergillus parasiticus* (NRRL 2999) and *Candida albicans* (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers

Vidya Chernapalli, Mir Zahoor Gul, Hanifa Mohammad Anwar, Naga Sai Susmitha Kuruganti and Karuna Rupula*

Department of Biochemistry, University College of Science, Osmania University, Telangana, India

Submission: August 23, 2024; **Published:** September 06, 2024

***Corresponding author:** Karuna Rupula, Department of Biochemistry, UCS, Osmania University, Telangana, India
Email: karunarupula@osmania.ac.in

Abstract

Fungal infections, primarily the ones associated with *Candida and Aspergillus* species are increasing every year and are challenging to eradicate despite the existence of antifungal drugs. For the past few decades, natural products have emerged as an essential source of antimicrobial agents particularly those derived from plants as promising alternatives to traditional drugs. They can selectively act on different targets with fewer side effects and moreover, phytotherapy is inexpensive. With this perspective, the present study reports the antifungal effect of selected natural compounds Rosmarinic acid, α-tocopherol and α-tocopherol acetate, on *Aspergillus parasiticus* (NRRL 2999) and *Candida albicans* (MTCC 183). The antioxidant capacity of these compounds was evaluated by DPPH assay that revealed Rosmarinic acid as a potent antioxidant than α-tocopherol and α-tocopherol acetate. Rosmarinic acid (RA), α-tocopherol (AT) and α-tocopherol acetate (ATA) showed significant antifungal activities against both *A. parasiticus* and *C. albicans* as studied by using well diffusion assay. RA exhibited significant inhibition against *A. parasiticus* and *C. albicans* with a minimum inhibitory concentration (MIC) of 15.62 μg mL^{-1} and 7.82 μg mL^{-1} respectively which is lower than the MICs of AT and ATA against the two fungi. RA, AT and ATA caused morphological changes in both *A. parasiticus* and *C. albicans* as studied by using scanning electron microscopy. The fungal growth biomarkers, chitin and ergosterol contents, were also lowered in all three antioxidants treated fungi with the potency of the compounds in inhibiting the chitin as well as ergosterol synthesis to be in the order of RA > ATA > AT. Thus, our experimental investigations suggest that RA, AT, an isoform of Vitamin E and ATA, a derivative of vitamin E are potent antifungal agents with RA being the most potent fungicidal compound.

Keywords: Antifungal; α-tocopherol; alpha tocopherol acetate; *Aspergillus parasiticus*; *Candida albicans*

Abbreviations: RA: Rosmarinic Acid; MIC: Minimum Inhibitory Concentration; ATA: Alphatocopherol Acetate; DMSO: Dimethyl Sulphoxide; DPPH: Diphenyl picrylhydrazyl; USDA: United States Department of Agriculture; IMTECH: Institute of Microbial Technology; MTCC: Microbial Type Culture Collection; YEPD: Yeast Extract Potato Dextrose; ZOI: Zone of Inhibition; CLSI: Clinical and Laboratory Standards Institute; NCCLS: National Committee for Clinical Laboratory Standards; GC: Growth Control; MC: Media Control; MIC: Minimum Inhibitory Concentration; DHE: Dehydro-Ergosterol; SD: Standard Deviation

Introduction

Fungi are eukaryotic, single cellular to multicellular varied clusters of living organisms distributed all over the biosphere. They are heterotrophic and differ in size, nature of life, and modes of reproduction. According to reports, more than 8000 species of fungi have been identified and classified with nomenclature [1]. *Aspergillus parasiticus* us is a saprophytic fungus that survives in soil and rotting plant material. It is one of the *Aspergillus* species, toxigenic that can produce aflatoxins B_1, B_2, G_1, & G_2 [2]. Other groups of fungi that produce aflatoxins include *A. flavus*

and *A. nomius* [3]. Aflatoxin consumption causes major toxic effects including carcinogenic, hepatotoxic, immunosuppressive, teratogenic, mutagenic, genotoxic and cytotoxic effects [4,5]. Additionally, in humans, aflatoxins are known to be associated with Indian childhood cirrhosis, Reye's syndrome, Kwashiorkor and respiratory diseases [6]. *A. parasiticus* breeds on several food and feed products, namely maize, rice, peanuts, cotton seeds, and milk, producing aflatoxins that cause food contamination [7].

The yeast *Candida albicans* is a dimorphic organism residing in healthy human gastrointestinal and urinogenital tracts. However, when the host defence mechanism of the host is compromised, it can cause severe infections [8]. The commensal or symbiotic *C. albicans* can transform into a pathogen in the presence of the virulence factors released by the organism such the hydrolases which allow it to penetrate the hosts, and in patients with comorbidities such as diabetes, alcohol consumption and smoking, antibiotic therapy, glucocorticoids, chemotherapy, radiotherapy, upper esophageal damage, and old age [9]. As *C. albicans* exhibits several morphological forms such as blastospores, pseudohyphae, and hyphae, it can spread to the skin and mucosal surface and may cause systemic infections in a variety of host niches.

Fungi possess a well-defined cell wall structure containing components that distinguish them from other eukaryotic cells and exhibit species-specific variations in their composition [10]. In fungal cell wall, is composed of different polysaccharides including chitin, cellulose, glucan, polyuronides and glycoproteins. The fungal cell wall. One of the chief constituents is chitin which is organized in the form of microfibrils and exhibits a significant contribution in maintaining the strength and integrity of the cell wall thus forming the major framework of the cell wall structure [11-13]. Chitin is a non-soluble polymer that contains β-1, 4-N-Acetylglucosamine (GlcNAc) monomers. Although it is found to be physically separated from the glucan within the cellular wall, it is chemically bonded to provide structural support [14,15]. In *Candida albicans*, chitin comprises 2-4% of its cell wall [15,16]. In filamentous fungi, chitin constitutes 10-30% of the dry weight of the cell walls. It is found in the spores and mycelia of the fungi. Chitin analysis was considered one of the significant methods for evaluating the fungal biomass. Polyoxins and Nikkomycins are promising antimycotics that target chitin biosynthesis.

The fungi cell membrane is composed of sterols and ergosterol is the most prevalent sterol. Sterols have a crucial role in maintaining fungal cell membranes by controlling their fluidity, heterogeneity, rigidity, resistance to water penetration, and overall integrity. The inhibition of the ergosterol biogenesis pathway results in reduced levels of cell membrane ergosterol levels thereby causing the growth inhibition of the fungi [17]. The azole based antifungal drugs mostly target the ergosterol biosynthetic enzyme lanosterol 14α-demethylase. Among the azoles fluconazole is widely applied as antifungal agent but several fungal pathogens are emerging resistant to it [18]. As the ergosterol biosynthetic pathway influences the viability of the fungal cells and as it functions as an antifungal drug target, the ergosterol biosynthetic pathway additionally plays a part in the emergence of resistance of the fungus to the antifungal drugs [19].

Several studies projected ergosterol as a primary index for fungal contamination [20,21] and is widely recognized as a significant indicator of fungal presence in culinary and agricultural settings [22]. Studies conducted on mycorrhiza showed that a combination of chitin and ergosterol analysis proved to be a reliable indicator of fungal biomass [23]. It is reported that fungal infections, majorly the infections caused by *Candida* species and *Aspergillus* species are associated with the death of over one million individuals annually [24]. These underrated infections are difficult to eliminate, and the death rate associated with these diseases is increasing although antifungal treatments are available [25].

Fluconazole and Amphotericin are the widely used anti-fungal drugs, fluconazole being the primary choice of drug [26]. Other antifungal azole drugs are also in use viz ketoconazole, voriconazole and itraconazole. But most of these drugs show side effects such as itching, rashes, abdominal pain, diarrhoea and liver damage. This problem is further aggravated by the incidence of resistance of *C. albicans* to the available conventional antifungal drugs [27].

Therefore, to combat the resistant fungi, it has become vital for the discovery of new antifungal drugs with minimal side effects. Our previous studies also establish that neem seed kernel extracts exhibit antifungal and anti-aflatoxigenic potentials and may find applications in formulating antimicrobial preparations for crop protection in field and for human and animal healthcare [28]. In this context, natural products are found to be promising alternatives for the treatment of fungal infections, few of which are in clinical use [12,29,30]. Rosmarinic acid is a phenolic acid compound extracted from plant species belonging to *Boraginaceae* and subfamily *Nepetoideae* of the *Lamiaceae* plant species. The RA is found more in *Liliaceae* family plant species namely *Rosmarinus officinalis, Perilla and Salvia* species [31]. *Rosmarinus officinalis* essential oil was found to show antifungal and anti-aflatoxigenic activity against *A. flavus. Salvia* species extract was also found to exhibit antifungal activity against different *Candida* species [32-34].

The role of vitamins in mitigating the detrimental effects of pathogenic fungi in chief crops and processed agricultural foods has been reported. In this regard, Vitamin E (α-tocopherol), a potential fat-soluble antioxidant compound sourced mainly from vegetable oils was found to exhibit a growth-inhibitory effect on *A. flavus* [35]. Vitamin E acetate, (an acetic ester of Vitamin E), α-tocopherol acetate (ATA) were reported to exhibit anti-inflammatory effects in an in vitro model of candidiasis [36]. Certain studies also reported the potency of Vitamin E in reducing

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

microbial adhesion especially established for *C. albicans*. These microbes are known to adhere and produce biofilm on a variety of biomaterials and causing infections [37]. Similarly, ATA is also reported to display anti-biofilm effects against various bacterial species by hindering the bacterial colonization of medical devices thereby reducing healthcare-associated infections [38]. The present study focused on analysing the effect of three selected antioxidants, RA, AT and ATA on *A. parasiticus* and *C. albicans* microbial growth.

Materials and methods

Chemicals and Reagents

Rosamarinic acid [(RA) (R)-O-(3,4-Dihydroxycinnamoyl)-3-(3,4- dihydroxyphenyl) lactic acid, Alpha Tocopherol [(AT) 2,5,7,8-Tetramethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol, 5,7,8-Trimethyltocol, D-α-Tocopherol, Vitamin E] and Alphatocopherol acetate [(ATA) Vitamin E acetate, all-rac-α-Tocopherol acetate], Fluconazole, Dimethyl Sulphoxide (DMSO), 2,2- Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, St. Louis, USA. Tween-20 was obtained from Loba Chemie, Mumbai, India; All other chemicals and reagents used were of analytical grade

Fungal Strains and Media

Aspergillus parasiticus (NRRL 2999), a toxic fungal strain, was obtained from the United States Department of Agriculture (USDA), located in Peoria, Illinois, in the USA. The culture was inoculated and maintained on PDA slants at a temperature of 28°C for 8 days in a BOD incubator on PDA slants (Remi Laboratory Instruments, Mumbai, India), and was sub-cultured regularly.

Candida albicans (MTCC 183), a pathogenic strain, was purchased from the Institute of Microbial Technology (IMTECH), Chandigarh. The *C. albicans* were cultured and maintained on YEPD agar medium in the laboratory as specified in the Microbial Type Culture Collection and Gene Bank (MTCC) protocol.

Potato Dextrose Agar (PDA) medium

The PDA agar (3.9 %) media was prepared, and the pH was adjusted to 6.0. Fractions of 20 mL medium were distributed into 100 mL Erlenmeyer flasks, plugged with cotton and autoclaved at 103 kPa pressure, 121°C for 15 min [39].

Yeast Extract Potato Dextrose (YEPD) agar medium

Yeast agar medium with yeast extract 0.3 % (w/v), peptone 1% (w/v), dextrose 2 % (w/v) and agar 1.5 % (w/v) were dissolved in Milli-Q water and prepared as per the growth medium details specified by the MTCC and autoclaved at 103 kPa pressure, 121°C for 15 min (According to MTCC media composition).

YES, aqueous medium

YES, a medium containing 2% (w/v.) yeast extract, and 15% (w/v) sucrose was prepared, and the pH was adjusted to 6.0. Then 20 mL portions of the broth were transferred into conical flasks of 100 mL capacity, plugged with cotton, and sterilized for 15 min at 121°C, 1.05 kg cm^{-2} of pressure in an autoclave [40].

YEPD broth

YEPD broth with yeast extract 0.3 % (w/v), peptone 1% (w/v) and dextrose 2 % (w/v) was prepared as per the growth medium details specified by the MTCC. The 20 mL aliquots of the broth were transferred into Erlenmeyer flasks of 100 mL, plugged with cotton, and sterilized in an autoclave for 15 min at 121°C and 1.05 kg cm^{-2} (103 kPa) of pressure.

Preparation of the RA, AT and ATA solutions

Dimethyl sulfoxide (DMSO) was used as a solvent for the preparation of antioxidant solutions. The RA stock was prepared at 1.0 mg mL^{-1} while AT and ATA stock solutions were prepared at concentrations 100.0 mg mL^{-1}.

Estimation of antioxidant activity of the selected antioxidants: DPPH assay

Rosmarinic acid, AT and ATA, each of the concentrations, 0.5µg mL^{-1}, 1.0µg mL^{-1}, 2.5µg mL^{-1}, 5µg mL^{-1}, 10µg mL^{-1}, 25µg mL^{-1}, and 100µg mL^{-1} were added individually to a solution of 0.002% of DPPH in methanol. The solutions were kept for incubation at room temperature for a duration of 30 min in the absence of light, and the absorbance measurement was conducted at a wavelength of 517 nm against a blank containing 0.002% of DPPH. Ascorbic acid at varied concentrations (0.5-100µg mL^{-1}) was used as a reference standard. Triplicate assays at each of the concentrations for the test compounds were carried out and the absorbance was measured. The percentage (%) of inhibition of DPPH at each of the concentrations of the test samples were computed utilizing the subsequent equation:

$$\% \text{ Inhibition} = [(A_C - A_S)/ A_C] \times 100$$

Where Ac denotes the absorbance of the control and As denotes the absorbance of the sample. The concentration of the sample that was required to scavenge 50% of DPPH (IC_{50}) was calculated [41].

Preparation of inoculum

The *A. parasiticus* cultures maintained on PDA slants were used to prepare fungal spore inoculum in 0.01% Tween 20 [40]. The spore suspensions containing 1×10^6 spores were aseptically inoculated into the Erlenmeyer flasks comprising 20 mL of the YES medium prepared. The culture flasks were maintained in a cooling incubator at a temperature of 28±1°C under stationary conditions. The fungus was grown over a duration lasting 12 days. A loop of *C. albicans* culture was inoculated in the YEPD agar slants and incubated at 4°C. Subsequently, the cells were inoculated into 100 mL of freshly prepared YEPD media and incubated for a duration of 48 h at a temperature of 30°C. To obtain the cell suspension at 1.5 x 10^6 CFU mL^{-1}, 30°C grown microbial cultures were diluted adequately in sterile YEPD broth to the 0.5 McFarland standard [42].

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

Antifungal Assay: Well Diffusion Method

The well diffusion assay method was used to measure the antifungal efficacy of RA, AT, and ATA against the two fungal strains, *A. parasiticus* and *C. albicans*. The spread plate technique was used to inoculate the PDA plates with 1 x 10^6 fungal spores of *A. parasiticus* in a volume of 30 µl. In the case of *C. albicans* cell suspension at 1.5 x 10^6 CFU ml^{-1} was applied to the YEPD agar plates. The wells with a diameter of 8 mm were made in the plates. The wells were loaded with the test compounds in various concentrations namely RA in the concentrations of 1.5, 10, 25, and 50µg mL^{-1}; while AT and ATA were at higher concentrations of 0.5, 1.0, 5.0 and 10 mg mL^{-1}.s

The negative control wells were maintained with DMSO. As a positive control, the reference standard antibiotic Fluconazole (8.0µg mL^{-1}) was used. The treated PDA plates were maintained at a temperature of 28±1°C under stationary conditions and the *A. parasiticus* fungus was permitted to proliferate for 7 days. On the other hand, for *C. albicans*, the test plates were subjected to incubation for a duration of 48 h at 30°C in stationary conditions. The experiments were carried out in triplicates for each of the selected compounds and the zone of inhibition (ZOI) was quantified in mm by subtracting the well diameter from the overall diameter of the inhibition zone [43].

Determination of MIC by Micro broth dilution method

Using the broth micro-dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, previously the National Committee for Clinical Laboratory Standards [NCCLS]), the fungicidal activity of RA, AT, and ATA against *A. parasiticus* and *C. albicans* was assessed [44,45].

The sterile polystyrene microtiter plate wells were loaded with various concentrations of the compounds and YES broth (100µL) containing 1 x 10^6 spores of *A. parasiticus*. The plates were subjected to a 48h stationary incubation period at 28±1°C. In the case of *C. albicans*, the wells after being loaded with varying amounts of the test compounds, were inoculated with 100 µl of YEPD broth containing 1 x 10^6 CFU mL^{-1} of *C. albicans* and then the plates were kept for incubation for a duration of 48 h at 30±1°C. The fungal suspension without the test compounds was included in each dilution series as the growth control (GC) wells. Each dilution series also included media control (MC) wells that contained only the YES media free of A. parasiticus, YEPD media devoid of *C. albicans* and the test compounds that served as negative controls. An ELISA microplate reader (Micro Scan, MS5608A, ECIL, India) was used to measure the absorbance of each well. The minimum inhibitory concentration (MIC) values were monitored visually by measuring the turbidity that appeared in the wells and recording the absorbance at 546 nm. The MIC is characterized as the minimum concentration of the test substances that showed complete inhibition of the fungal growth in comparison to the controls. The experiment was carried out in triplicates for each of the test compounds.

Scanning Electron Microscopy Analysis

Using SEM with an accelerating voltage of 18 kV, the morphological alterations in *A. parasiticus* and *C. albicans* treated with the test drugs were observed.

A. parasiticus

➢ The *A. parasiticus* fungal mycelial mat, formed in YES media after 7 days of incubation, was treated with RA, AT and ATA at their respective MIC concentrations and a negative control without the test compounds was also maintained. The treated and the control samples were incubated at 28±1°C for 24 h under stationary conditions. The samples were fixed overnight in a solution of glutaraldehyde [2.5% (v/v)] in 0.1M sodium phosphate buffer at a pH of 7.2. The mycelia were dehydrated using a sequence of water-acetone solutions with increasing acetone concentration (10% increments from 30-90%) for 60 min each. They were then exposed to 100% acetone for 180 min and left overnight in 100% acetone. Finally, the mycelia were immersed in hexamethyldisilazane and then preserved at 4°C. The carbon tape was covered with mycelium, dried in the air, and then coated with gold using a sputtering process. The sputtered sample was dried under vacuum and installed for imaging under the Scanning Electron Microscope (JEOL, JCM 6000 plus). The samples were observed in the magnification range of: x 1500 to 3000 [46].

C. albicans

➢ *C. albicans* cell suspension (10 mL) bearing a concentration of 1x10^6 CFU mL^{-1} was subjected to incubation at 30°C, for 24 h on a YEPD broth containing RA, AT, and ATA at their MIC and the control group (i.e., the medium containing no test compounds). The test compound-treated yeast samples were cautiously washed with 0.1 M phosphate buffer (pH 7.2). Using 2% Osmium tetroxide, the post-fixation was carried out for 2 h at room temperature. Preliminary dehydration was carried out by placing specimens in a series of ethanol gradients. The first step of dehydration was done two times for 10 min using 50% and 70% ethanol, the second dehydration was carried out again two times but with 95% ethanol for 5 min and finally 100% ethanol two times for 1 min. Subsequently, the samples were dehydrated with acetone, two times for 30s each till they were dried by the critical point method in liquid CO_2. Later, the specimens were subjected to gold spluttering and the sputtered samples were examined under a scanning electron microscope [47].

Chitin analysis

Extraction of chitin from *A. parasiticus* and *C. albicans*

A 20 mL of YEPD broth taken in separate conical flasks, were inoculated with an individual *Candida* colony from a YEPD agar plate harboring culture and incubated overnight. To the culture the test compounds were added at their MIC; RA (7.82 µg mL^{-1}), AT (200 mg mL^{-1}) and ATA (100 mg mL^{-1}). Conical flasks with only

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

the YEPD broth and *C. albicans* devoid of any test compounds were considered as controls. Similarly, *A. parasiticus* spore suspension in the volume of 100 µL containing $1x10^6$ spores was inoculated for seven days at 28±1°C in 20 mL of YES medium containing RA, AT, and ATA at their respective MIC values, i.e., RA at 15.62 µg mL^{-1}, AT at 200 mg mL^{-1}, and ATA at 200 mg mL^{-1}. Control samples were devoid of any test compounds. After the incubation period of 24 h and 7 days for *C. albicans* and *A. parasiticus* respectively, the samples were subjected to the process of centrifugation at 2,700 rpm for five minutes and subsequently, the cell pellet's net wet weight was calculated and the pellet was suspended in one mL of 4M HCl and boiled for 4 h [48,49]. The hydrolysate obtained was further analyzed for the chitin content.

Estimation of chitin content in *A. parasiticus* and *C. albicans*

The number of amino sugar content within the hydrolysate obtained was estimated by the modified Elson-Morgan method [48], wherein N-acetyl-D-glucosamine (subjected to hydrolysis using the identical parameters mentioned earlier) served as a standard. The hydrolysates obtained were mixed with 19 mL of fresh distilled water to achieve a final concentration of 0.2 M HCl. Following this, 1 mL of acetyl acetone reagent, which consists of 2% (v/v) acetyl acetone and 1.25 M Na_2CO_3, was added to the 0.5 mL portion of the hydrolysates. The solutions were then subjected to incubation at a temperature of 90°C for 60 min and later were allowed to cool to the ambient temperature and thereafter treated with ten ml of C_2H_5OH and 1 mL of Ehrlich's reagent. The resulting mixture was subjected to incubation at the ambient temperature for a duration of 60 min. The amount of hexosamine in the processed samples was determined spectrophotometrically by measuring OD at 530 nm with a spectrophotometer. All the sample analyses were carried out in triplicates. Glucosamine hydrochloride (0.020mg) was employed as a standard. A correction factor of 0.829 is required to represent values of the unknown/test sample in terms of free hexosamine.

Hexosamine (mg) in the sample =

(Optical density of the unknown)/ (optical density of the standard) X (0.020) (0.829).

$M = m \times 203/179$

Where, m = mass of glucosamine as calculated by analysis

203 = molecular weight of acetylglucosamine anhydride (g mol^{-1})

179 = molecular weight of glucosamine (g mol^{-1}).

Ergosterol analysis

Extraction of ergosterol from *A. parasiticus* and *C. albicans*

Extraction of ergosterol from fungal cultures was achieved successfully following the standard protocols [50,51]. A 100µL spore suspension of containing $1x10^6$ spores of the fungus *A. parasiticus* was inoculated in 20 mL of YES medium containing RA, AT and ATA at their respective MIC concentrations i.e., RA of 15.62 µg mL^{-1}, AT of 200 mg mL^{-1} and ATA of 200 mg mL^{-1}. These cultures were kept for incubation for a period of seven days at a temperature of 28±2°C in a BOD incubator (Remi Laboratory Instruments, Mumbai, India). Samples without the compounds were considered as controls. In case of *Candida*, a single colony from an overnight YEPD agar plate culture was used to inoculate 20 ml of YEPD broth in flasks maintained separately each for control and RA (7.82 µg mL^{-1}), AT (200 mg mL^{-1}) and ATA (100 mg mL^{-1}) containing media. The cultures were incubated for 24 h at 30°C in a BOD incubator. After incubation, the cultures were harvested by centrifugation at 2,700 rpm for five minutes and cell pellets net weights were calculated after removing excess moisture. The samples obtained from *Aspergillus* and *Candida* were treated with 5 mL and 3 mL of 25% potassium hydroxide in alcohol solution respectively. The mixtures were then vigorously stirred for 2-3 min and kept for incubation at a temperature of 85°C for a duration of 4 h and 1 h for *Aspergillus* and *Candida* samples respectively. The sterols were extracted by combining a mixture of 2 mL of sterile distilled water and 5 mL of n-heptane for *Aspergillus* samples and in the case of *Candida*, 3 mL of n-heptane and one mL of sterile distilled water were added. The mixtures were then vigorously vortexed for 2-3 min following incubation, and the layer of heptane was transferred to a sterile borosilicate glass tube, screw-capped and preserved at a temperature of -20°C.

Estimation of ergosterol content in *Aspergillus parasiticus* and *C. albicans*

The n-heptane fractions of *A. parasiticus* and *C. albicans* cultures were analyzed using UV-vis spectrophotometry between 230 and 300 nm. Ergosterol displays absorbance at 282 nm and 24 [28] dehydro-ergosterol (DHE), the late sterol intermediate shows absorbance at 230 nm wavelength and 282 nm wavelength in the n-heptane layers [50]. Using the measurements of absorbance and moist weight of the initial pellet, the amount of ergosterol was determined as a percentage of the cell's wet weight. The following formula is used to determine the amount of ergosterol:

%Ergosterol + %24(28) DHE = $[(A_{281.5}/290) \times F]$/pellet weight,

%24(28) DHE = $[(A_{230}/518) \times F]$/pellet weight,

%Ergosterol = [%Ergosterol + %24(28) DHE] -%24(28) DHE

Where F = factor for dilution in ethanol; 290 = percent extinction coefficient (E) values per centimeter determined for crystalline ergosterol in an absolute alcohol solvent; 518 = percent extinction coefficient (E) values per centimeter determined for 24 [28] DHE in an absolute alcohol solvent [52]. All the analyses of the samples were carried out in triplicates.

Statistical analysis

The experimental investigations were carried out in

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

triplicates. The values were reported in mean±standard deviation (SD). The GraphPad prism software (version 9.3) was used for the statistical analysis and plotting of graphs. A 'p' value less than 0.05 was deemed to be statistically significant.

Results

DPPH Assay

The change in absorbance observed by the reduced DPPH was employed to gauge the antioxidant capacity of the test samples. The results depicted a decrease in absorbance caused by varying concentrations of RA, AT, ATA and ascorbic acid. Ascorbic acid was used as a reference standard for the analysis. The analysis revealed that among the three compounds selected for the study, RA is more potent than AT and ATA as graphically represented in (Figure 1). The IC_{50} values for RA, AT and ATA were found to be 4.32±0.04 µg, 13.31±0.23µg and 16.49±0.17µg respectively when compared with that of ascorbic acid which was,, found to be 3.32±0.06 µg and is presented in (Table 1).

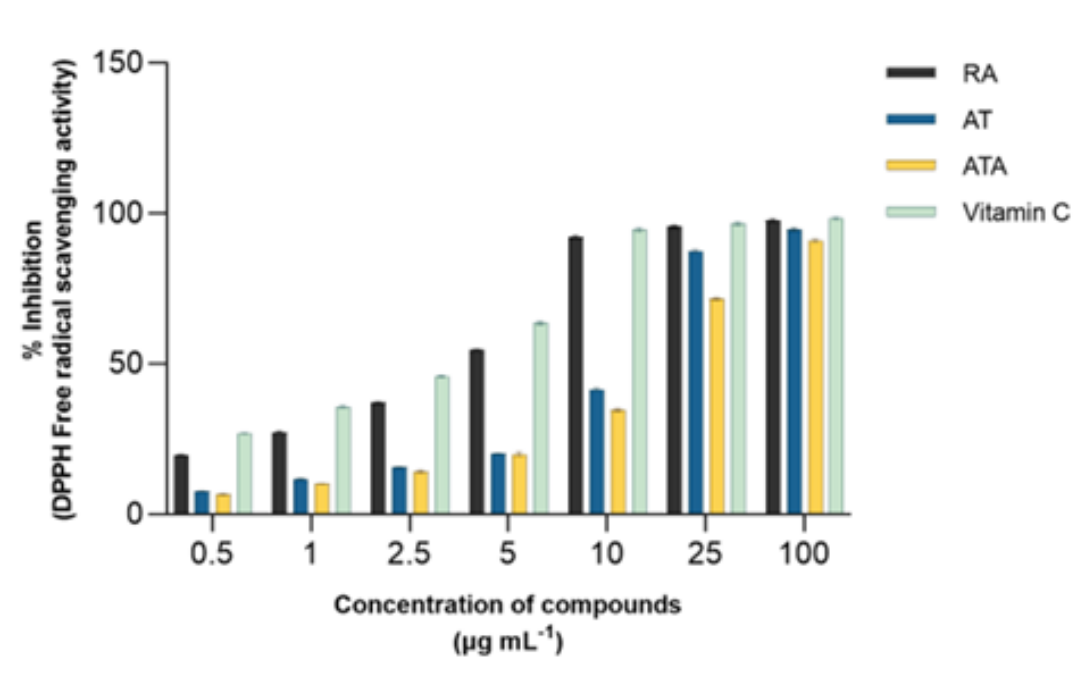

Figure 1: Antioxidant potentials of Rosamarinic acid, alpha tocopherol and alpha tocopherol acetate.

Table 1: Inhibitory Concentration (IC_{50}) value of the compounds tested on DPPH

Test sample	IC_{50} value (µg)
Rosmarinic acid (RA)	4.32±0.04*
α-tocopherol (AT)	13.31±0.23*
α- tocopherol acetate (ATA)	16.49±0.17*
Ascorbic acid	3.32±0.06

Each value is expressed as a mean ± standard deviation (n = 3). Significant p value (*p<0.05) were obtained by Student's t test analysis.

Well Diffusion Assay

All three samples RA, AT and ATA exhibited antifungal effects on *A. parasiticus* and *C. albicans* as depicted in (Figure 2) by the agar well diffusion method. The ZOI in mm at different concentrations of RA, AT and ATA were measured and the results are depicted in (Table 2). The highest concentrations of RA, AT and ATA used for the assay are 50 µg mL^{-1}, 10mg mL^{-1} and 10mg mL^{-1} respectively. At the highest concentrations chosen, RA, AT and ATA exhibited ZOI of 20.00, 15.67 and 16.33 mm against *A. parasiticus* respectively. In the same way, RA, AT and ATA exhibited ZOI of 15.67, 12.00 and 11.67 mm against *C. albicans* respectively (Table 2). Dimethyl sulphoxide which was used as a solvent for preparing various concentrations of the test compounds was considered as

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

a negative control and did not show any inhibition zones in both the microbial species. The antibiotic fluconazole used as positive control displayed 21.80 and 16.67 mm ZOI against *A. parasiticus* and *C. albicans* respectively. The zone of inhibition visualized is depicted in (Figure 2.1 & 2.2).

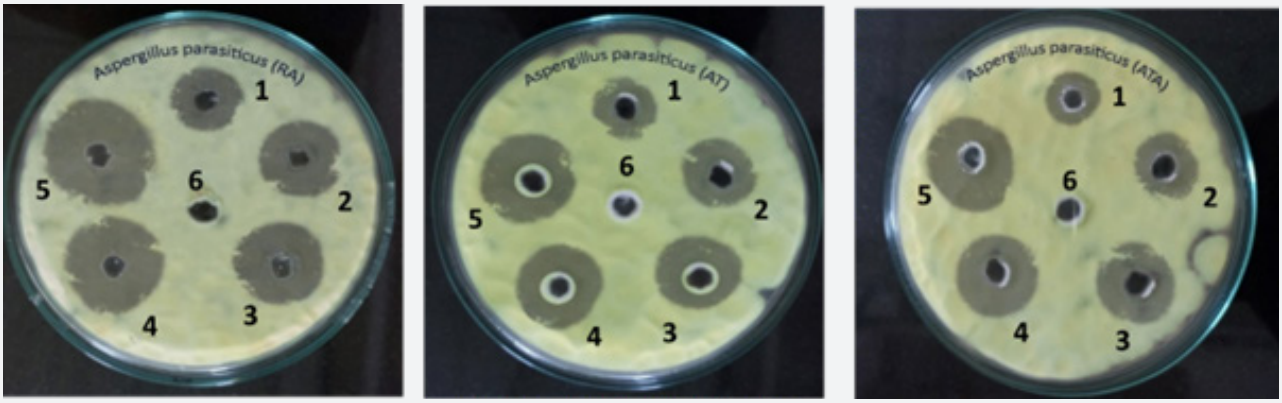

Figure 2.1: Agar well diffusion assay depicting the antifungal effects of (A) RA (B) AT (C) ATA on A. *parasiticus* NRRL 2999

(A) RA concentrations in different wells: 1. 1.5µg mL-1; 2. 10µg mL-1; 3. 25µg mL-1; 4. 50µg mL-1; 5. Fluconazole 8µg mL-1; 6. DMSO
(B) AT concentrations in different wells: 1. 0.5mg mL-1; 2. 1mg mL-1; 3. 5mg mL-1; 4. 10mg mL-1; 5. Fluconazole 8µg mL-1; 6. DMSO
(C) ATA concentrations in different wells: 1. 0.5mg mL-1; 2. 1mg mL-1; 3. 5mg mL-1; 4. 10mg mL-1; 5. Fluconazole 8µg mL-1; 6. DMSO

Table 2: Antifungal potentials (Zone of inhibition-ZOI) of RA, AT and ATA determined by agar well diffusion analysis

Test Samples	Concentration	Zone of inhibition (mm)	
		A. parasiticus	*C. albicans*
RA	1.5 (µg mL^{-1})	15	10.33
	10.0 (µg mL^{-1})	16.67	13
	25.0 (µg mL^{-1})	18.67	14.67
	50.0 (µg mL^{-1})	20	15.67
AT	0.5 (mg mL^{-1})	10	5
	1.0 (mg mL^{-1})	13	6.33
	5.0 (mg mL^{-1})	14.67	9.33
	10.0 (mg mL^{-1})	15.67	12
ATA	0.5 (mg mL^{-1})	11	6.67
	1.0 (mg mL^{-1})	13	8.33
	5.0 (mg mL^{-1})	14.33	9.67
	10.0 (mg mL^{-1})	16.33	11.67
Fluconazole	0.5-1.0 (µg/mL^{-1})	21.8	16.67
Positive control			
DMSO	2% (v/v)	ND	ND
Negative control			

RA= Rosmarinic acid, AT= α-tocopherol, ATA= α- tocopherol acetate, ND= Not detected

MIC Determination

The minimum inhibitory concentration (MIC) of RA, AT and ATA against *A. parasiticus* and *C. albicans* were determined based on the absorbance recorded in the fungal cultures and are depicted in (Figure 3). The positive control chosen for the analysis was the standard drug fluconazole. The MICs of RA, AT and ATA against *A. parasiticus* were 15.62 µg mL^{-1}, 200 µg mL^{-1} and 100 µg mL^{-1} respectively and against *C. albicans* were 7.82 µg mL^{-1}, 200 µg mL^{-1} and 100 µg mL^{-1} respectively (Table 3). The MIC was lowest

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

for RA against both *A. parasiticus* and *C. albicans*. The MIC values obtained indicated that RA is more potent against *C. albicans* and the most effective compared to the other two antioxidants used in the study. Interestingly, the MIC of AT (200 mg $mL^{-1)}$ was observed to be the same for both *A. parasiticus* and *C. albicans*. Likewise, the MIC of ATA (100 mg mL^{-1}) was found to be identical for both fungal species. However, ATA displayed lower MIC than AT indicating that the acetate form of AT holds more ability in showing fungal inhibitory activity.

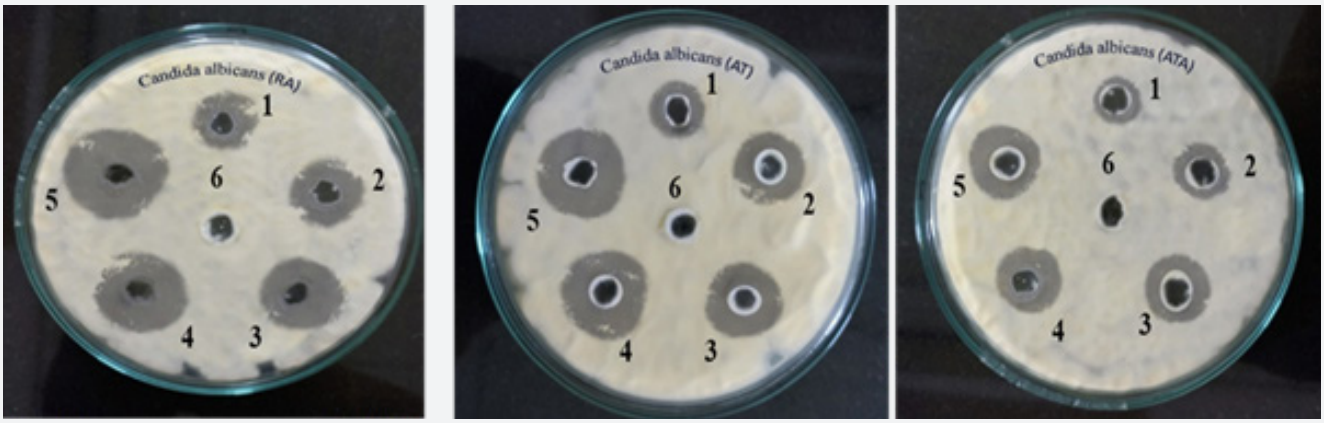

Figure 2.2: Agar well diffusion depicting the antifungal effects of (A) RA (B) AT (C) ATA on C. *albicans* MTCC 183

(A) RA concentrations in different wells: 1. 1.5µg mL-1; 2. 10µg mL-1; 3. 25µg mL-1; 4. 50µg mL-1; 5. Fluconazole 8µg mL-1; 6. DMSO
(B) AT concentrations in different wells: 1. 0.5mg mL-1; 2. 1mg mL-1; 3. 5mg mL-1; 4. 10mg mL-1; 5. Fluconazole 8µg mL-1; 6. DMSO
(C) ATA concentrations in different wells: 1. 0.5mg mL-1; 2. 1mg mL-1; 3. 5mg mL-1; 4. 10mg mL-1; 5. Fluconazole 8µg mL-1; 6. DMSO

Table 3: The minimum inhibitory concentrations (MICs) of the compounds against the fungal organisms

Test Samples	*A. parasiticus*	*C. albicans*
RA	15.62 (µg mL^{-1})	7.82 (µg mL^{-1})
ssAT	200 (mg mL^{-1})	200 (mg mL^{-1})
ATA	100 (mg mL^{-1})	100 (mg mL^{-1})

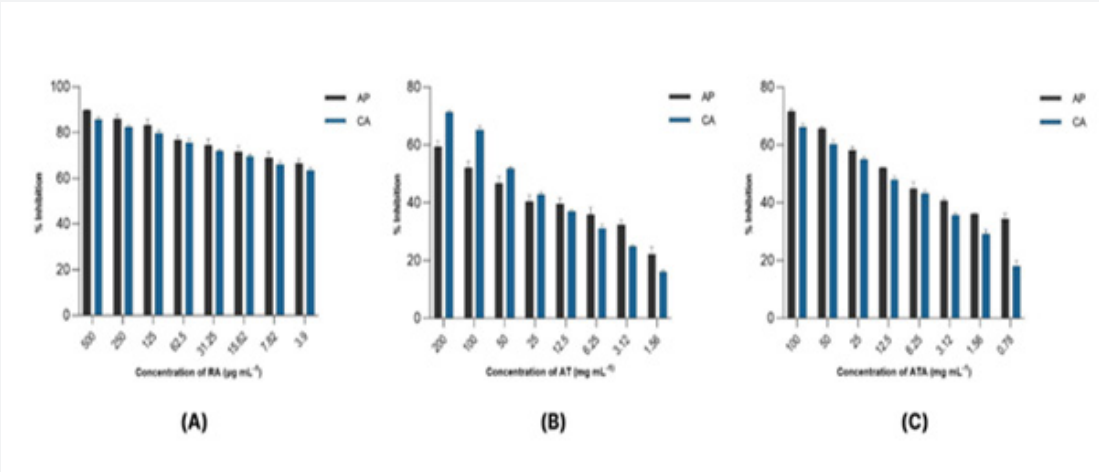

Figure 3: Inhibitory effects of the compounds on *A. parasiticus* and *C. albicans* Fungal growth
A) RA, B) AT and C) ATA

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

Scanning Electron Microscope studies

The effect of RA, AT and ATA at their MIC on the morphology of *A. parasiticus* was examined using SEM investigation. The SEM photographs demonstrated that in untreated control, the fungi mycelium was normal while in the treated samples, the mycelium was irregular, shrunken and damaged as depicted in (Figure 4). Similarly, the SEM analysis of the *C. albicans* at MIC of RA, AT and ATA-treated cells showed irregularities on their surface and had scars indicating morphological abnormalities compared to the control which appeared normal as depicted in (Figure 5).

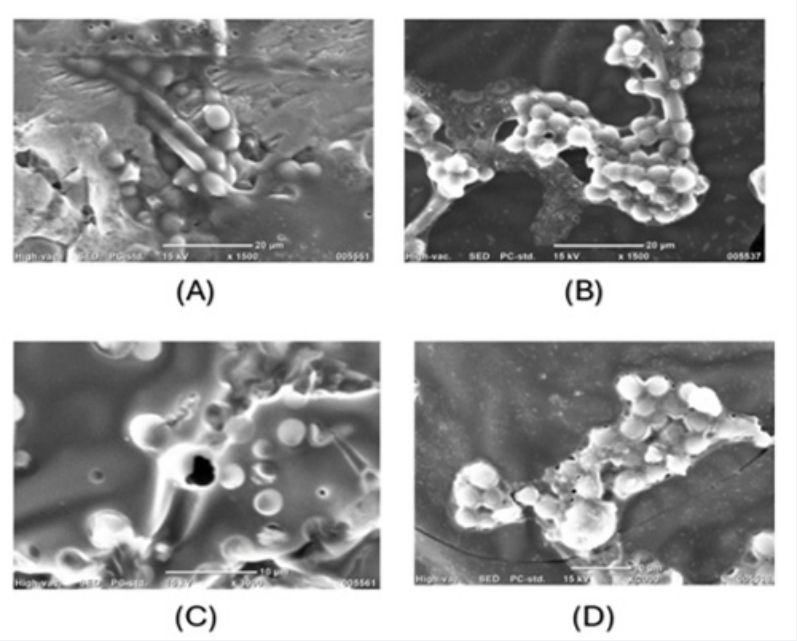

Figure 4: SEM analysis of *A. parasiticus*: (A) Control and in (B) RA (C) AT (D) ATA treated samples.

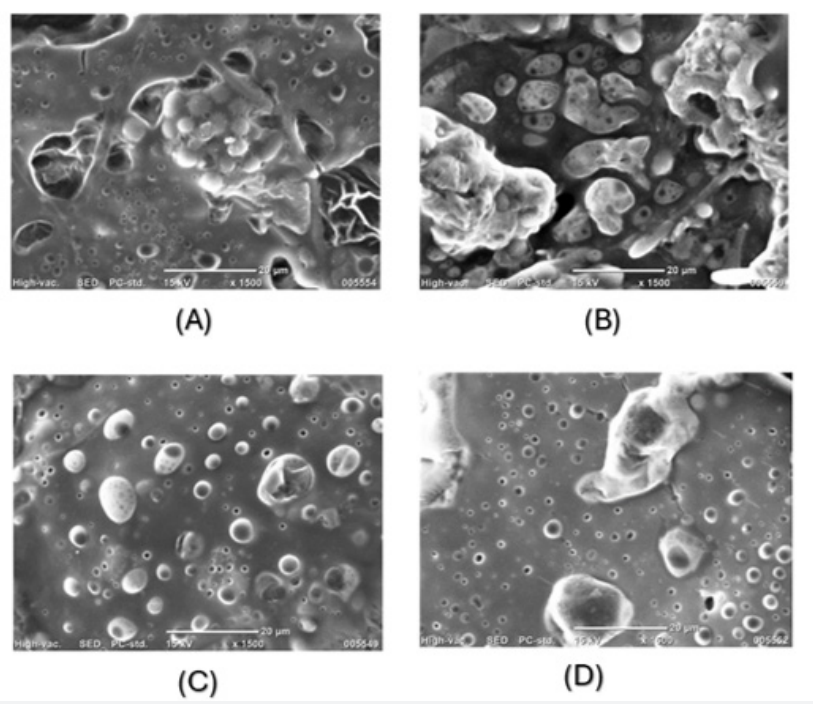

Figure 5: SEM analysis of *C. albicans* in (A) Control and in treated (B) RA (C) AT (D) ATA samples

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

Chitin analysis

The fungal samples were analyzed for chitin as per Yabe et al., 1996. The results observed are presented in (Figure 6). The chitin content in the untreated control sample was 1.454±0.039 mg g^{-1}. In RA, AT and ATA exposed fungal cells the chitin content was estimated to be 0.868±0.087 mg g^{-1}, 1.340±0.077 mg g^{-1} and 1.179±0.067 mg g^{-1} respectively. Thus, the fungal samples treated with the compounds under the study exhibited a decrease in the chitin levels in comparison to the control with a p value of statistical significance i.e. < 0.05. Further, the potency of the test compounds in influencing the chitin synthesis was found to be in the order, RA>ATA>AT. Similarly, the chitin levels were also evaluated in *C. albicans* samples which were treated with RA, AT and ATA at the specified MIC. The chitin levels in the untreated control yeast samples were estimated to be 2.414±0.463 mg g^{-1}. Rosmarinic acid, AT and ATA downregulated the chitin levels in *C. albicans,* and the chitin levels recorded were 1.320±0.175 mg g^{-1}, 1.844±0.103 mg g^{-1} and 1.778±0.234 mg g^{-1} respectively. RA was found to be the most potent followed by ATA and AT.

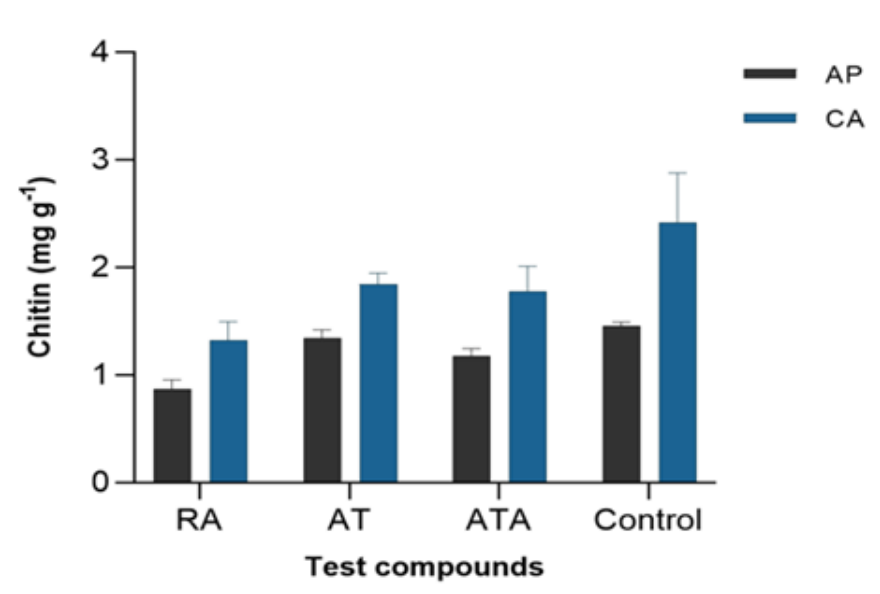

Figure 6: Analysis of chitin content in *A. parasiticus* (NRRL 2999) and *C. albicans* (MTCC 183)

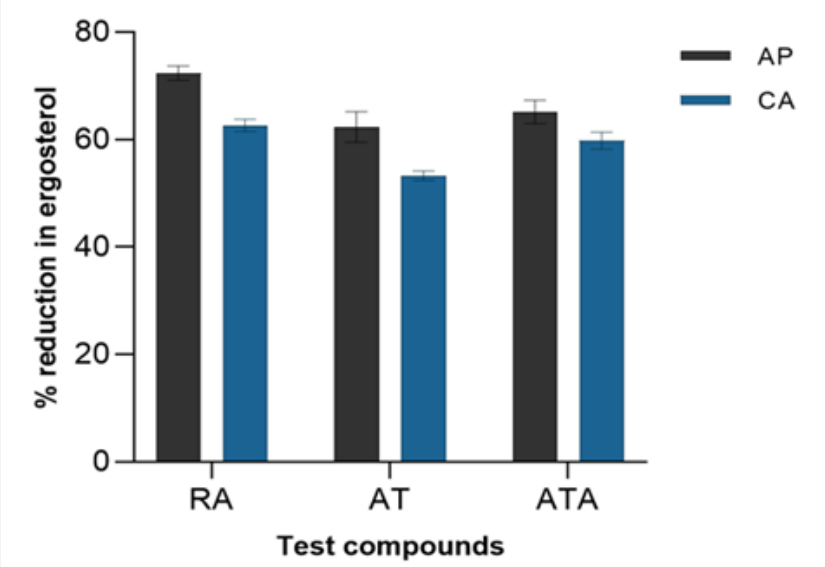

Figure 7: Analysis of ergosterol in A. *parasiticus* (NRRL 2999) and *C. albicans* (MTCC 183).

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

Ergosterol analysis

The ergosterol levels were evaluated spectrophotometrically in *A. parasiticus* cultures treated with RA, AT and ATA at their respective MICs and incubated for seven days. The results revealed that the selected compounds had a negative impact on the ergosterol synthesis thereby decreasing the ergosterol level in comparison to the control sample. Rosmarinic acid significantly ($p<0.05$) decreased the ergosterol levels by 72% in comparison with the control and showed a better potency than AT and ATA. The α-tocopherol (AT) and ATA were significantly able to reduce the ergosterol content by 62% and 65% respectively compared to the control fungal cells as depicted in (Figure 7). Likewise, RA, AT and ATA were tested at their MICs against *C. albicans* incubated for 48h, to analyze their potency in altering the amount of ergosterol in the *C. albicans* cellular membrane. All the selected compounds were able to lower the ergosterol levels in *C. albicans*. The percentage decline in the amount of total ergosterol was 63%, 53 % and 60% in the presence of MICs of RA, AT and ATA respectively.

Discussion

The *Candida* genus is the major pathogen in the spread of yeast-based infections in humans among which *C. albicans* is the predominant (90%) virulent species [15,53]. *Aspergillus* species are the common fungal organisms for the incidence of disorders in various plants and contaminate the plant products directly or mediated through mycotoxins [54]. Antimycotics, the antifungal agents employed in the management of fungal infections are limited and are comparatively less than their antibacterial counterparts. The mechanisms through which antifungal drugs target are also associated with side effects and the fungi have developed alternative ways to adjust. The resistance to antifungal drugs is one of the chief reasons for advancing the research studies of antifungal medication development. Hence, it is challenging to generate drugs with maximum selectivity and fewer side effects on humans. All these create a need for the emergence of designing novel antifungal drugs of natural origin [19,55]. Our earlier study demonstrated the binding affinity of Rosmarinic acid, α-tocopherol and their derivatives with CYP51 of *Candida* spp. and their drug-likeliness potencies in in silico [56].

Rosmarinic acid (RA) and plant extracts containing RA such as extracts of rosemary, are the most widely utilized natural antioxidants that are included in food products [57]. Alpha-tocopherol is a biologically active compound and is one of the most potent lipophilic antioxidants that has a significant role in protecting cells from reactive oxygen species [58]. Similarly, an investigation revealed that α-tocopherol and α-tocopherol acetate have comparable effects as free radical traps in rat liver microsomes that are supplemented with tert-butyl hydroperoxide [59]. Based on the earlier reports, in the present work, DPPH analysis was carried out to understand the antioxidant abilities of purified RA, AT and ATA. The DPPH analysis revealed that RA possesses a significantly higher antioxidant capacity than AT and ATA with IC_{50} of 4.322±0.043µg. This result is supported by an earlier study that reported, RA possesses higher antioxidant potency in comparison to α-tocopherol, BHT, TBHQ and BHA and can be a better natural substitute for the antioxidants used in the food industry [60].

Rosemary extracts contain Rosmarinic acid as one of the primary bioactive materials showing antibacterial properties [61]. The essential oil of rosemary was reported to show fungicidal effect against *C. albicans* and A. niger [62]. Rosmarinic acid is one of the major phenolics found in mints namely *Mentha piperata* and *Mentha spicata* [63]. Mentha species essential oil showed inhibitory efficacy against *C. albicans* and *Aspergillus* spp. α-tocopherol is an important constituent of Ficus carica leaves accounting for an amount of 57 mg 100 g^{-1} of dried leaves [64] and leaf extracts of *Ficus carica* exhibited antifungal activity against the *Aspergillus* species [65]. Similarly, other studies indicated that methanolic fractions of *Ficus carica* displayed a 100% inhibitory effect on *C. albicans* [66]. In this aspect, the well diffusion assays carried out in our study demonstrated that all three selected antioxidants namely RA, AT and ATA had significant inhibitory activity ($p<0.05$) on the growth of *A. parasiticus* and *C. albicans*.

There were significant variations in the MIC values exhibited by RA, AT and ATA. The MIC of RA was less in both the fungal species in comparison with AT and ATA and the MIC of RA in *C. albicans* (7.82 µg mL^{-1}) was less in comparison to *A. parasiticus* (15.62 µg mL^{-1}). The S. *montana* and S. *subspicata* extracts, containing Rosmarinic acid as the most prevalent phenolic compound with a concentration ranging from 1.11% to 3.31% (w/w), were found to be effective against clinical isolates of *Candida* spp. with MIC values ≤ 32.9 µg mL^{-1} [67]. Similarly, *Cynara cardunculus* extracts were found to contain phenolic compounds and flavonoids as the major bioactive compounds, Rosmarinic acid being one of the phenolic compounds. The ethanolic extracts of the artichoke showed antifungal activity against eight species of mycotoxigenic fungi with MIC values of 0.87-4.16 mg mL^{-1} [68]. There are no studies to date about the effect of purified RA on *A. parasiticus*, however, there are limited studies that reported the antimicrobial potency of purified RA on *C. albicans*. Further studies revealed that RA possesses antimicrobial potential against 11 *Candida* strains with MIC ranging from 100-200 µg mL^{-1} [69].

Both AT and ATA showed the same MIC values against *A. parasiticus* and *C. albicans*. Earlier, a research group reported that whole ground black cumin followed by black cumin oil had higher α-tocopherol content which inhibited *A. flavus* and its aflatoxin production [70]. Additionally, *Curtisia dentata* acetone extract (30%) showed antifungal activity against *A. flavus* & *A. ochraceous* with MIC of 0.63 mg mL^{-1} and 0.08 mg mL^{-1} respectively. Further, the phytochemical analysis of the extract revealed vitamin E as one of the major constituents [71]. When compared, our studies suggest that since *A. parasiticus* is a highly toxigenic strain the

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

concentration of compounds needed to inactivate it might be higher than on other fungal species.

All three antioxidants, RA, AT and ATA damaged the morphology of *A. parasiticus* and *C. albicans* with irregularities and scars on the surface as examined by SEM. Similar observations were reported wherein it was shown that *Salvia officinalis*, a good source of many biologically active compounds particularly Rosmarinic acid as one of the phytochemicals that was used to synthesize zinc oxide nanoparticles cause morphological changes in the *C. albicans* clinical isolates [72]. The SEM analysis revealed the development of lesions and furrows on the cell wall and cell membrane leading to cell death. The nanoparticles stabilized by the phytochemicals were also able to disrupt the cell membrane by inhibiting the production of ergosterol.

It is well established that chitin analysis can be used as a suitable marker for the assessment of fungal growth in various samples. The hydrolysis of chitin polymer leads to the formation of N-acetyl-D-glucosamine and its colorimetric estimation was formerly used as a biomarker for fungal contamination [22]. In the fungal cell membrane, the ergosterol biosynthetic pathway is the major antifungal drug target. Ergosterol is a major component of the fungal cell membrane and has been established as a good indicator of fungal growth owing to its correlation with the metabolically active biomass [73]. Thus, not only the determination of ergosterol as biomarker for fungal contamination allow for monitoring changes during fungal growth in food samples but also its biosynthesis pathway is a target for the major antifungal drugs [22]. In the present study, the chitin and ergosterol were extracted from *A. parasiticus* and *C. albicans* cultures and quantified spectrophotometrically.

Rosmarinic acid at MIC of 15.62µg mL^{-1} lowered the chitin levels by 40% and ergosterol levels by 72% in *A. parasiticus*. In *C. albicans*, RA reduced the ergosterol by 63% and the chitin content was lowered by 45%. Studies by earlier researchers revealed that phenolic compounds extracted from plants exhibit antifungal activity by blocking ergosterol synthesis [74,75]. These compounds were found to diffuse through the cell membrane and enter the cell where they alter the synthesis of chitin and ergosterol by interfering in the biosynthetic pathways. Essential oil from Coriandrum sativum was reported to be effective against moulds and lead to the decrement of the ergosterol amount in the fungal cell membranes [76]. A decrease in the cell wall chitin levels in *C. albicans* when treated with *Cleome viscosa* essential oil was also reported [77].

So far there have been no studies that evaluated the direct effect of AT and ATA on fungal chitin and ergosterol synthesis. In this perspective, our study demonstrated that AT and ATA lowered the chitin levels in *A. parasiticus* and *C. albicans* with the reduction of chitin content being significantly more in *A. parasiticus* treated compounds than the *Candida albicans*. Between α-tocopherol and α-tocopherol acetate, the ATA was more potent in reducing the chitin content in both *A. parasiticus* and *C. albicans.* Similarly, AT and ATA also affected the ergosterol synthesis in both the fungi under the study with ATA reducing the ergosterol content significantly than AT. Similar observations were reported about the essential oils of *Mentha piperata* that contained α-tocopherol and Rosmarinic acid as two of the major constituents and the leaf oils showed antifungal activity by lowering the ergosterol levels [73].

Conclusion

Our present study revealed that Rosmarinic acid (RA), α- tocopherol (AT) and α-tocopherol acetate (ATA) exhibited antifungal activities against *A. parasiticus* and *C. albicans*. Among the three antioxidants, RA was found to be more potent than AT and ATA and *C. albicans* was more susceptible. The morphological damage and the inhibitory effects on chitin and ergosterol in both the fungal organisms due to the compounds were significant. These findings suggest that all the selected antioxidants are potent antifungal agents and among the three antioxidants, RA could serve as a valuable natural alternative to the commercial chemical antifungal drugs in usage. Further, in vivo investigations are needed to explore the antifungal potential of RA, AT and ATA and to decipher the mechanisms by which the selected test compounds could target the chitin and ergosterol biosynthetic pathways. Additionally, the synergistic effect of RA, AT and ATA can be studied and may hold the potential for formulating topical treatments alongside the existing medications or on par with the existing antifungal drugs. Such a formulation could provide clinicians with additional tools for managing the diseases caused by *A. parasiticus* and *C. albicans*. Moreover, the topical sprays of the concoctions of the selected antioxidants may protect the crops affected by the *Aspergillus* spp. thus evading the deleterious effects of the fungi on the crops and their yield.

Author's Contribution

VC carried out all the experimentation, acquisition and analysis of data and drafting of the manuscript. MZG assisted with the acquisition and analysis of data and drafting of the manuscript. HMA and KNS assisted in the drafting of the manuscript. KR conceived, designed, and supervised the study and revised the manuscript. All authors have read and approved the final manuscript.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgements

All the authors are thankful to the Department of Biochemistry, Osmania University, Hyderabad. VC is also thankful to the Department of Biochemistry, Government City College, Hyderabad for the support during the Ph.D. studies.

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

References

1. Sun XM, Zhang XH, Wang HY, Cao WJ, Yan X, et al. (2002) Effects of sterigmatocystin, deoxynivalenol and aflatoxin G1 on apoptosis of human peripheral blood lymphocytes in vitro. Biomed Environ Sci BES 15(2): 145-152.
2. Chang PK (2001) Authentication of Aspergillus parasiticus strains in the genome database of the National Center for Biotechnology Information. BMC Res Notes 14(1): 111.
3. Coppock RW, Christian RG, Jacobsen BJ (2018) Aflatoxins. In: Veterinary Toxicology [Internet] Ppp: 983-994.
4. Horn BW, Dorner JW (2011) Evaluation of different genotypes of nontoxigenic *Aspergillus flavus* for their ability to reduce aflatoxin contamination in peanuts. Biocontrol Sci Technol 21(7): 865-876.
5. Benkerroum N (2020) Chronic and Acute Toxicities of Aflatoxins: Mechanisms of Action. Int J Environ Res Public Health 17(2): 423.
6. Dhanasekaran D, Shanmugapriya S, Thajuddin N, Panneerselvam A (2011) Aflatoxins and Aflatoxicosis in Human and Animals. In: Guevara-Gonzalez RG, editor. Aflatoxins-Biochemistry and Molecular Biology.
7. Yooussef MM, Pham Q, Achar PN, Sreenivasa MY (2016) Antifungal activity of essential oils on Aspergillus parasiticus isolated from peanuts. J Plant Prot Res 56(2): 139-142.
8. Whiteway M, Bachewich C (2007) Morphogenesis in *Candida albicans*. Annu Rev Microbial 61(1): 529-553.
9. Talapko J, Juzbašić M, Matijević T, Pustijanac E, Bekić S, et al. (2021) Candida albicans-The Virulence Factors and Clinical Manifestations of Infection. J Fungi 7(2): 79.
10. Mitchell TG (2012) Fundamental Medical Mycology. Emerg Infect Dis 18(8): 1393.
11. Brown HE, Esher SK, Alspaugh JA (2019) Chitin: A "Hidden Figure" in the Fungal Cell Wall. In: Latgé JP, editor. The Fungal Cell Wall [Internet]. Cham: Springer International Publishing Pp: 83-111.
12. Curto MÁ, Butassi E, Ribas JC, Svetaz LA, Cortés JCG (2021) Natural products targeting the synthesis of β (1,3)-D-glucan and chitin of the fungal cell wall. Existing drugs and recent findings. Phytomedicine 88: 153556.
13. Adams DJ (2004) Fungal cell wall chitinases and glucanases. Microbiology 150(7): 2029-2035.
14. Lorand T, Kocsis B (2007) Recent Advances in Antifungal Agents. Mini-Rev Med Chem 7(9): 900-911.
15. Zida A, Bamba S, Yacouba A, Ouedraogo-Traore R, Guiguemdé RT (2017) Anti- Candida albicans natural products, sources of new antifungal drugs: A review. J Mycol Médicale 27(1): 1-19.
16. Garcia-Rubio R, De Oliveira HC, Rivera J, Trevijano-Contador N (2020) The Fungal Cell Wall: Candida, Cryptococcus, and Aspergillus Species. Front Microbiol 10: 2993.
17. Lv Q zhen, Yan L, Jiang Y ying (2016) The synthesis, regulation, and functions of sterols in *Candida albicans:* Well-known but still lots to learn. Virulence 7(6): 649-659.
18. Onyewu C, Blankenship JR, Del Poeta M, Heitman J (2003) Ergosterol biosynthesis inhibitors become fungicidal when combined with calcineurin inhibitors against Candida albicans, Candida glabrata, and Candida krusei. Antimicrob Agents Chemother 47(3): 956-964.
19. Ivanov M, Ćirić A, Stojković D (2022) Emerging Antifungal Targets and Strategies. Int J Mol Sci 23(5): 2756.
20. Rodrigues ML (2018) The Multifunctional Fungal Ergosterol. mBio 9(5): e01755-18.
21. Pasanen AL, Yli-Pietilä K, Pasanen P, Kalliokoski P, Tarhanen J (1999) Ergosterol Content in Various Fungal Species and Biocontaminated Building Materials. Appl Environ Microbiol 65(1): 138-142.
22. Kosuri T, Nayak S, Gul MZ, Rupula K, Beedu SR (2018) TLC-Digital Image-Based Fluorometric Analysis of Ergosterol and Chitin Content in Food Grains Artificially Infested with *Aspergillus flavus* and Fusarium verticillioides. Food Anal Methods 11(5): 1267-1280.
23. Ekblad A, Wallander H, Näsholm T (1998) Chitin and ergosterol combined to measure total and living fungal biomass in ectomycorrhizas. New Phytol 138(1): 143-149.
24. Pathakumari B, Liang G, Liu W (2020) Immune defence to invasive fungal infections: A comprehensive review. Biomed Pharmacother 130: 110550.
25. Janbon G, Quintin J, Lanternier F, d'Enfert C (2019) Studying fungal pathogens of humans and fungal infections: fungal diversity and diversity of approaches. Microbes Infect 21(5-6): 237-245.
26. Abrantes PMDS, McArthur CP, Africa CWJ (2014) Multi-drug resistant oral Candida species isolated from HIV-positive patients in South Africa and Cameroon. Diagn Microbiol Infect Dis 79(2): 222-227.
27. Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS (2017) Azole Antifungal Resistance in Candida albicans and Emerging Non-albicans Candida Species. Front Microbiol 7: 2173.
28. Bhootham V, Zahoor Gul M, Velpula S, Rao Beedu S, Rupula K (2020) Screening of Anti-aflatoxigenic Potential of Neem Seed Kernel Extracts in Solid Culture Media. Adv Biotechnol Microbiol 16(1): Pp.
29. Bouz G, Doležal M (2021) Advances in Antifungal Drug Development: An Up-To-Date Mini Review. Pharm Basel Switz 14(12): 1312.
30. Marena GD, Ramos MADS, Carvalho GC, Junior JAP, Resende FA, et al. (2022) Natural product-based nanomedicine applied to fungal infection treatment: A review of the last 4 years. Phytother Res 36(7): 2710-2745.
31. Petersen M, Abdullah Y, Benner J, Eberle D, Gehlen K, et al. (2009) Evolution of rosmarinic acid biosynthesis. Phytochemistry 70(15-16): 1663-1679.
32. Salari S, Bakhshi T, Sharififar F, Naseri A, Ghasemi Nejad Almani P (2016) Evaluation of antifungal activity of standardized extract of Salvia rhytidea Benth. (Lamiaceae) against various Candida isolates. J Mycol Médicale 26(4): 323-330.
33. Bomfim NDS, Kohiyama CY, Nakasugi LP, Nerilo SB, Mossini SAG, et al. (2020) Antifungal and antiaflatoxigenic activity of rosemary essential oil (*Rosmarinus officinalis L.*) against *Aspergillus flavus*. Food Addit Contam Part A 37(1): 153-161.
34. Zhang J, Cui X, Zhang M, Bai B, Yang Y, et al. (2022) The antibacterial mechanism of perilla rosmarinic acid. Biotechnol Appl Biochem 69(4): 1757-1764.
35. Farina C DH, Jing R B, Franco G T (2014) Effects of Vitamins A, C and E on Growth and Colonial Morphology of Aspergillus flavus. Int Sci Community Assoc 3(10): 52-59.
36. Barros S, D Ribeiro AP, Offenbacher S, Loewy ZG (2020) Anti-Inflammatory Effects of Vitamin E in Response to Candida albicans. Microorganisms 8(6): 804.
37. Banche G, Bracco P, Allizond V, Bistolfi A, Boffano M, et al. (2015) Do Crosslinking and Vitamin E Stabilization Influence Microbial Adhesions on UHMWPE-based Biomaterials? Clin Orthop 473(3): 974-986.

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

38. Vergalito F, Pietrangelo L, Petronio Petronio G, Colitto F, Alfio Cutuli M, et al. (2019) Vitamin E for prevention of biofilm-caused Healthcare-associated infections. Open Med 15(1): 14-21.

39. Liu Y, Tortora G, Ryan ME, Lee HM, Golub LM (2002) Potato Dextrose Agar Antifungal Susceptibility Testing for Yeasts and Molds: Evaluation of Phosphate Effect on Antifungal Activity of CMT-3. Antimicrob Agents Chemother 46(5): 1455-1461.

40. Tanuja K, Hemalatha K, Karuna R, Sashidhar Rao B (2010) Effect of various surfactants (cationic, anionic and non-ionic) on the growth of Aspergillus parasiticus (NRRL 2999) in relation to aflatoxin production. Mycotoxin Res 26(3): 155-170.

41. Gul MZ, Bhakshu LM, Ahmad F, Kondapi AK, Qureshi IA, et al. (2011) Evaluation of Abelmoschus moschatus extracts for antioxidant, free radical scavenging, antimicrobial and antiproliferative activities using in vitro assays. BMC Complement Altern Med 11(1): 64.

42. Al Marby A, Ejike C, Nasim M, Awadh Ali N, Albadani R, et al (2016) Nematicidal and antimicrobial activities of methanol extracts of seventeen plants, of importance in ethnopharmacology, obtained from the Arabian Peninsula. J Intercult Ethnopharmacol 5(2): 114-121.

43. Sánchez E, Heredia N, García S (2005) Inhibition of growth and mycotoxin production of *Aspergillus flavus* and Aspergillus parasiticus by extracts of Agave species. Int J Food Microbiol 98(3): 271-279.

44. Schwab U, Gilligan P, Jaynes J, Henke D (1999) In Vitro Activities of Designed Antimicrobial Peptides against Multidrug-Resistant Cystic Fibrosis Pathogens. Antimicrob Agents Chemother 43(6): 1435-1440.

45. CLSI C and LSI (2024) Performance Standards for Antimicrobial Susceptibility Testing. 30th ed. S.l.: CLINICAL AND LABORATORY.

46. Malkapur D, Devi MS, Rupula K, Sashidhar RB (2017) Biogenic synthesis, characterisation and antifungal activity of gum kondagogu-silver nano bio composite construct: assessment of its mode of action. IET Nanobiotechnol 11(7): 866-873.

47. Staniszewska M, Bondaryk M, Swoboda-Kopec E, Siennicka K, Sygitowicz G, et al. (2013) Candida albicans morphologies revealed by scanning electron microscopy analysis. Braz J Microbiol 44(3): 813-821.

48. Yabe T, Yamada-Okabe T, Kasahara S, Furuichi Y, Nakajima T, et al. (1996) HKR1 encodes a cell surface protein that regulates both cell wall beta-glucan synthesis and budding pattern in the yeast Saccharomyces cerevisiae. J Bacteriol 178(2): 477-483.

49. Wang L, Wang S, Yang S, Guo X, Lou H, et al. (2012) Phenolic alkaloids from the aerial parts of Dracocephalum heterophyllum. Phytochemistry 82: 166-171.

50. Tian J, Ban X, Zeng H, He J, Chen Y, et al. (2012) The Mechanism of Antifungal Action of Essential Oil from Dill (Anethum graveolens L.) on Aspergillus flavus. PLoS ONE 7(1): e30147.

51. Rajput SB, Karuppayil SM (2013) Small molecules inhibit growth, viability and ergosterol biosynthesis in Candida albicans. SpringerPlus 2(1): 26.

52. Arthington-Skaggs BA, Jradi H, Desai T, Morrison CJ (1999) Quantitation of Ergosterol Content: Novel Method for Determination of Fluconazole Susceptibility of *Candida albicans*. J Clin Microbiol 37(10): 3332-3337.

53. Sardi JCO, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJS (2013) Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol 62(1): 10-24.

54. Perrone G, Gallo A (2017) Aspergillus Species and Their Associated Mycotoxins. In: Moretti A, Susca A, editors. Mycotoxigenic Fungi [Internet]. New York, NY: Springer New York Pp: 33-49.

55. Feucht C, Patel DR (2011) Principles of Pharmacology. Pediatr Clin North Am 58(1): 11-19.

56. Chernapalli V, Rokkala SC, Bhootham V, Rupula K (2023) In silico Studies on the Binding Affinity of Rosmarinic Acid, Alpha Tocopherol and their Derivatives with CYP51 of Candida Species and their Drug Likeliness Potencies. Appl Biol Res 25(3): 291-302.

57. Choi SH, Jang GW, Choi SI, Jung TD, Cho BY, et al. (2019) Development and Validation of an Analytical Method for Carnosol, Carnosic Acid and Rosmarinic Acid in Food Matrices and Evaluation of the Antioxidant Activity of Rosemary Extract as a Food Additive. Antioxidants 8(3): 76.

58. Yao Z, Zhang Y, Li H, Deng Z, Zhang X (2015) Synergistic effect of Se-methylselenocysteine and vitamin E in ameliorating the acute ethanol-induced oxidative damage in rat. J Trace Elem Med Biol 29: 182-187.

59. Gonzalez Flecha BS, Repetto M, Evelson P, Boveris A (1991) Inhibition of microsomal lipid peroxidation by α-tocopherol and α-tocopherol acetate. Xenobiotica 21(8): 1013-1022.

60. Guitard R, Paul JF, Nardello-Rataj V, Aubry JM (2016) Myricetin, rosmarinic and carnosic acids as superior natural antioxidant alternatives to α-tocopherol for the preservation of omega-3 oils. Food Chem 213: 284-295.

61. Moreno S, Scheyer T, Romano CS, Vojnov AA (2006) Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radic Res 40(2): 223-231.

62. Jiang Y, Wu N, Fu YJ, Wang W, Luo M, et al. (2011) Chemical composition and antimicrobial activity of the essential oil of Rosemary. Environ Toxicol Pharmacol 32(1): 63-68.

63. Zaidi S, Dahiya P (2015) In vitro antimicrobial activity, phytochemical analysis and total phenolic content of essential oil from Mentha spicata and Mentha piperita. Int Food Res J 22(6): 2440-2445.

64. Konyalıoğlu S, Sağlam H, Kıvçak B (2005) α-Tocopherol, Flavonoid, and Phenol Contents and Antioxidant Activity of Ficus carica. Leaves. Pharm Biol 43(8): 683-686.

65. Ullah NB (2021) omparative antifungal activity of wild and cultivated leaves of ficus carica linn. j med sci 29: 03.

66. Aref HL, Salah KBH, Chaumont JP, Fekih A, Aouni M, et al. (2010) In vitro antimicrobial activity of four Ficus carica latex fractions against resistant human pathogens (antimicrobial activity of Ficus carica latex). Pak J Pharm Sci 23(1): 53-58.

67. Kremer D, Košir I, Končić M, Čerenak A, Potočnik T, et al. (2015) Antimicrobial and Antioxidant Properties of *Satureja Montana* L. and S. Subspicata Vis. (Lamiaceae). Curr Drug Targets 16(14): 1623-1633.

68. Shallan MA, Ali MA, Meshrf WA, Marrez DA (2020) In vitro antimicrobial, antioxidant and anticancer activities of globe artichoke (Cynara cardunculus var. scolymus L.) bracts and receptacles ethanolic extract. Biocatal Agric Biotechnol 29: 101774.

69. Ivanov M, Kostić M, Stojković D, Soković M (2022) Rosmarinic acid-Modes of antimicrobial and antibiofilm activities of a common plant polyphenol. South Afr J Bot 146: 521-527.

70. Elsamra IA, Shama SM, Hamza AS, Youssef NH, Youssef MS, et al. (2012) Effect of some mould inhibitors and herbal plants on mycotoxins production by *Aspergillus flavus* and *Fusarium verticilloides* in vitro and in stored corn grains. Arch Phytopathol Plant Prot 45(15): 1861-1878.

71. Dikhoba PM, Mongalo NI, Elgorashi EE, Makhafola TJ (2019) Antifungal and anti-mycotoxigenic activity of selected South African medicinal plants species. Heliyon 5(10): e02668.

72. Abomuti MA, Danish EY, Firoz A, Hasan N, Malik MA (2021) Green Synthesis of Zinc Oxide Nanoparticles Using Salvia officinalis Leaf Extract and Their Photocatalytic and Antifungal Activities. Biology 10(11): 1075.

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987

73. Samber N, Khan A, Varma A, Manzoor N (2015) Synergistic anti-candidal activity and mode of action of *Mentha piperita* essential oil and its major components. Pharm Biol 53(10): 1496-1504.

74. Simonetti G, Brasili E, Pasqua G (2020) Antifungal Activity of Phenolic and Polyphenolic Compounds from Different Matrices of Vitis vinifera L. against Human Pathogens. Molecules 25(16): 3748.

75. Zhou X, Zeng M, Huang F, Qin G, Song Z, et al. (2023) The potential role of plant secondary metabolites on antifungal and immunomodulatory effect. Appl Microbiol Biotechnol 107(14): 4471-4492.

76. Das S, Singh VK, Dwivedy AK, Chaudhari AK, Upadhyay N, et al. (2019) Encapsulation in chitosan-based nanomatrix as an efficient green technology to boost the antimicrobial, antioxidant and in situ efficacy of Coriandrum sativum essential oil. Int J Biol Macromol 133: 294-305.

77. Mishra B, Mishra AK, Kumar S, Mandal SK, Nsv L, et al. (2021) Antifungal Metabolites as Food Bio-Preservative: Innovation, Outlook, and Challenges. Metabolites 12(1): 12.

DOI: 10.19080/AIBM.2024.17.555987

How to cite this article: Vidya C, Mir Zahoor G, Hanifa Mohammad A, Naga Sai Susmitha K, Karuna R, et al. Antifungal Effects of Rosmarinic Acid, α-Tocopherol and A-Tocopheryl Acetate on Aspergillus Parasiticus (NRRL 2999) and Candida Albicans (MTCC 183) and Assessment of Chitin and Ergosterol Biomarkers. Adv Biotech & Micro. 2024; 18(3): 555987. DOI: 10.19080/AIBM.2024.17.555987