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Application of *Syzygium aromaticum*, *Ocimum sanctum*, and *Cananga odorata* essential oils for management of Ochratoxin A content by *Aspergillus ochraceus* and *Penicillium verrucosum*: An *in vitro* assessment in maize grains

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The study is directed to establish the minimizing effects of *Syzygium aromaticum*, *Ocimum sanctum*, and *Cananga odorata* essential oils on the growth and ochratoxin A (OTA) level of *Aspergillus ochraceus* and *Penicillium verrucosum* in maize grains. *S. aromaticum* essential oil (SAEO), *O. sanctum* essential oil (OSEO), and *C. odorata* essential oil (COEO) were extracted by hydro-distillation technique, and a total of 50, 44, and 48 chemical constituents were identified by gas chromatography-mass spectrometry (GC-MS), respectively.The SAEO and OSEO belong to the chemotype of eugenol, whereas, COEO was found to be the chemotype of thymol, limonene, and α-ylangene. The antifungal activity of essential oils (EOs) was determined by the micro-well dilution technique. The SAEO showed superior antifungal activity compared to OSEO, COEO, and synthetic antifungal agent nystatin, and its minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values against *A. ochraceous* and *P. verrucosum* were noticed as 1251 ± 42.32 and 1878 ± 28.47 μg/mL, and 0815 ± 22.69 and 1146 ± 51.19 μg/mL, respectively.The antifungal mechanism of EOs was unveiled by assessing the intracellular reactive oxygen species (ROS), ergosterol content, and membrane integrity. The antifungal investigations found that EOs caused fungal mortality by increasing the intracellular ROS, depleting ergosterol synthesis, and distracting membrane integrity. Finally, antifungal and antimycotoxin activity of EOs was demonstrated in maize grains. The SAEO, OSEO, and COEO have reduced the complete fungal growth and OTA level of *A. ochraceous* and *P. verrucosum* correspondingly at 2500 and 2500, 3500 and 2500, and 3500 and 3500 μg/g in maize. The EOs could act as natural antifungal agents; protect foodstuffs from fungal infection and mycotoxins during storage.

Keywords: *Aspergillus ochraceus*, Essential oils, Mycotoxins, Ochratoxin A, *Penicillium verrucosum*

Mycotoxins are derived from the Greek term ‘mukes’, which means fungi or slime molds, and the Latin word ‘toxicum’, which means poison. Mycotoxins are defined as fungal secondary metabolites when swallowed, breathed, and absorbed *via* the skin, they induce reduced performance, sickness, or death in people and animals. Toxic fungus and mycotoxins certainly appeared in the food chain some 10,000 years ago, when humans began cultivating food grains and storing grains from one harvest season to the next harvest season. In both human and animal diets, food grains have traditionally been the principal source of mycotoxins. When vast numbers of turkeys died after eating aflatoxins-contaminated peanut meal about four decades ago, significant scientific research on mycotoxins began[1,2].

Mycotoxins have become a worldwide epidemic that has affected a wide range of food commodities. Warmer, subtropical, and tropical regions have a higher prevalence of mycotoxins than temperate regions. Mycotoxins build up in food supplies both before and after harvest, according to climate factors[3]. The pre-harvest accumulation of mycotoxins has been linked to elevated humidity, pest devastation, and extended dry environments. During the post-harvest period, stored grain is in a vigorous condition and might turn out to be highly susceptible to molds and pests. The accumulation of mycotoxins is influenced by climate factors like temperature and moisture, geographic region, storage container form, and grain processing and distribution. Inadequate storage and handling techniques make processed foods susceptible to fungal infection and mycotoxins[3]. Fungal infection decreases nutritional value, color, and texture, as well as stimulates rancidity and decay of food, in addition

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to bringing the mycotoxins into the food chain and food web[1,2,4].

To date, around 400 mycotoxins have been found. The most prevalent mycotoxins detected in food include fumonisins, aflatoxins, zearalenone, ochratoxin A (OTA), and trichothecenes, which have deleterious impacts on humans and farm animals. Humans and agricultural animals are both susceptible to mycotoxins, which can cause serious health difficulties. When consumed, mycotoxins produce mycotoxicoses, but they can also be absorbed through the skin or breathed. Mycotoxicosis leads to liver, kidney, and hematological system toxicity, as well as immunological toxicity, neurotoxicity, reproductive toxicity, fetal toxicity and teratogenicity, and, most notably, carcinogenicity[1,5].

OTA is a secondary metabolite of certain *Aspergillus* and *Penicillium* species. Breakfast cereals and their derivatives, cocoa beans, fruits (grapes, apples, and oranges), cocktails, flavoring spices, and non-vegetarian foodstuffs all include OTA, a strong nephrotoxic[6]. The International Agency for Research on Cancer (IARC) has defined OTA as a Group 2B cancer-causing agent, claiming this could induce cancer in humans[7]. JECFA (The Joint FAO/WHO Expert Committee on Food Additives) and the EU (European Union) have established legal limits and HACCP processes for OTA in food and feed components as a result of the risk of OTA to mankind and livestock. The OTA limits for unprocessed cereals and food items prepared from unprocessed cereals are 5 and 3 µg/kg, respectively[8]. Management of mycotoxins requires periodic monitoring of mycotoxins in food sources. Accordingly, microbiologists, toxicologists, and food scientists have all been interested in OTA management in food sources.

Contact to OTA must be kept as minimal as possible to keep individuals safe. There are several physical, chemical, and biological techniques for lowering mycotoxins in food at the moment. Chemicals such as citric acid, salicylic acid, ammonia, lactic acid, benzoic acid, and propionic acid have been shown to partially decontaminate mycotoxins; nevertheless, by-products of mycotoxins have been determined to be toxic, and their use is not deemed safe and accepted by consumers[9]. Physical decontamination tactics such as cold plasma, radiation, high hydrostatic pressure, and microwave have been proven to be effective in lowering fungal development and mycotoxin levels, but they come with limitations such as cost, difficulty in maintenance, and the need for professional competence[10,11]. Therefore, people, researchers, and the government are all interested in employing biological techniques to limit fungal growth and mycotoxin content in foodstuffs. In biological approaches, essential oils (EOs) have attracted a lot of interest as organic fungicides for preventing fungal development and mycotoxin content in foodstuffs due to their status of 'safe and green'. EOs of aromatic plants have inspired a wave of attention in the progress of bio-control herbal products because of their prospective antioxidant and antimicrobial effects. Particularly, governing organizations for example Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) have both permitted the usage of EOs in the food business[12].

The current investigation was directed to establish the minimizing effects of *Syzygium aromaticum*, *Ocimum sanctum*, and *Cananga odorata* EOs on the development and OTA level of *Aspergillus ochraceus* and *Penicillium verrucosum*. GC-MS analysis was used to profile out the chemical make-up of EOs.Micro-well dilution technique was used to test the minimizing impact of EOs on the growth of *P. verrucosum* and *A. ochraceus* in terms of MIC (minimum inhibitory concentration) and MFC (minimum fungicidal concentration). Finally, under *in-vitro* circumstances, the minimizing effect of EOs on the development and OTA level of *P. verrucosum* and *A. ochraceus* was demonstrated in maize.

Materials and Methods

Chemicals and reagents

0.22 µM syringe filter, Sabouraud dextrose agar (SDA),2'-7'dichlorofluorescin diacetate (DCFH-DA), Whatman no.1 filter paper, Sabouraud dextrose broth (SDB), peptone, and ochratoxin A (98% HPLC pure)were obtained from HiMedia, India.The OTA-specific immunoaffinity column from VICAM, Water Business, USA. Acetonitrile, Tween 80, dimethyl sulfoxide (DMSO), HPLC grade water, methanol, ethanol, and other chemicals of fine grade were obtained from Merck Millipore Corporation, India.

Fungi and cultural conditions

Positive fungal OTA chemotypes, *Aspergillus ochraceus* – ITCC 2454 and *Penicillium verrucosum* – ITCC 2986 were obtained from the culture collection centerIndian Type Culture Collection (ITCC), India. Following, fungi were grown for

3 days at 28°C on SDA Petri plates and maintained at 4°C[13]. On SDA Petri plates, the fungi were cultivated for 7 days at 28°C for confluence. The scarping technique was used to collect the fungal spores in peptone water (0.001% Tween 80), which were then counted using a hemocytometer, and the total fungal spore count was attuned to 10^6/mL[14].

Plant materials collection and EOs extraction

From January to March 2018, *Syzygium aromaticum* flower buds, *Ocimum sanctum* leaves, and *Cananga odorata* flower buds were collected in Ooty, India. Plants were authenticated, and the voucher was kept safe. The plant components were dried in the darkness for a few weeks before being ground into a fine powder with a blender. Following that, 250 g of plant extract powders were utilized for EOs extraction by hydrodistillation with a Clevenger-type device, in accordance withthe European Pharmacopoeia's procedure[15]. The moisture content of the EOs was desiccated over anhydrous sodium sulphate before being maintained at 4°C in brown glass jars.

Chemical profile of EOs

The chemical profile of *S. aromaticum*, *O. sanctum*, and *C. odorata* EOs were obtained using GC-MS PerkinElmer Clarus 600°C[16]. The Perkin Elmer Clarus 600 C equipped flame-ionization detector (FID), DB-5MS fused silica column (30 M × 0.25 mM; 0.25 µM film thickness), and TurboMass software application was utilized.

Briefly, essential oils were diluted in acetone (10 µL/mL) and injected into GC in a split-mode ratio of 1:30. The carrier gas was helium at a rate of 1 mL/min, and the operation temperatures were linearly programmed between 40 and 280°C at 4°C/min. Temperatures of the injector and sensor were kept at 250°C and 280°C, respectively. The mass spectra of essential oil components were scanned in the EI mode of 70 eV and the range of *m/z* 40-450. The chemical components of essential oils were determined using mass spectra (MS) in combination with the NIST/Wiley library and Adams' retention indices (RI) literature. GC peak regions were used to calculate the quantity of chemical components present in essential oil and expressed in percentage[16].

Antifungal activity of EOs

In vitro antifungal potential of *S. aromaticum* essential oil (SAEO), *O. sanctum* essential oil (OSEO), and *C. odorata* essential oil (COEO) was achieved on *P. verrucosum*– ITCC 2986 and *A. ochraceus* – ITCC 2454 in MIC and MFC values by well recognized and routinely used micro-well dilution technique[16].

In this assay, different quantities of EOs were added to fungal spore suspension of 10 µL (10^6 spores per mL) in a microtiter plate of 96-well and the total volume of the test sample was made up to 100 µL with SDB. The wells containing 0.001% Tween 80 and fungal spore suspension were considered as control. The antifungal compound nystatin was used as a reference. Next, test samples were incubated at 28°C. The MIC value of EOs was established as the concentration of EOs at which fungal development was absent ensuing of three days of culturing.

Following, 10 µL of the sample was taken from wells of the microtiter plate and carried out by spread plate on SDA Petri plates. The plates were incubated at 28°C for three days. The MFC values of EOs were defined as the dose of EOs at which fungal growth was not restored.

Antifungal mechanisms of EOs

In vitro antifungal activity of EOs was established by assessing the intracellular reactive oxygen species (ROS), ergosterol, and membrane integrity of fungi.

Estimation of intracellular ROS

2'-7'-Dichlorofluorescin diacetate (DCFH-DA) staining was used to investigate the effect of EOs on intracellular ROS-mediated fungal death. Briefly, various concentrations of EOs up to their MIC values were added to fungal spore suspension of 10 µL (10^6 spores per mL) and the overall volume was attuned to 100 µL with SDB in a 96-well microtiter plate. The control wells contained a suspension of fungal spores without EOs. The samples were cultured for three days at 28°C. Subsequently, DCFH-DA staining was performed at 5 µM[14]. The results of EOs treated test samples were reported with reference to control (100%).

Estimation of ergosterol

In this assay, the role of EOs in the depletion of ergosterol content of fungi and their impact on fungal death was revealed. Briefly, varying quantities of EOs (up to MFC values per mL) were introduced to 100 mL of SDB broth, inoculated with a fungal spore suspension of 10 µL (10^6 spores per mL) in 250 mL Erlenmeyer flask, and cultured for 7 days at 28°C. The control flasks contained fungal spore suspension without EOs. Following, ergosterol content was quantified using HPLC (Shimadzu, Japan)[14].

Estimation of membrane integrity

Propidium iodide staining technique was followed to evaluate the detrimental influence of EOs on the membrane integrity of fungal spores. In a 96-well microtiter plate, varied quantities of EOs up to their MIC values were added to a fungal spore suspension of 10 µL (10^6 spores per mL) and the total volume was completed to 100 µL with SDB. The control wells contained a suspension of fungal spores without EOs. The fungal test samples were cultured at 28°C for three days. Next, the propidium iodide staining assay was completed at 5 µM[10]. The test sample results were expressed in comparison to the control sample (100%).

***In vitro* antifungal and OTA inhibition activity of EOs in maize**

A local agricultural market provided freshly harvested and well-dried maize grains. Maize grains were sterilized right away in an autoclave at 121°C for 15 min under 15 pounds per square inch of pressure. The moisture content of maize grains was then eliminated using a hot-air oven at 55°C.

Following that, in a 500 mL Erlenmeyer flask, 100 g of maize grains were thoroughly mixed with different doses of EOs (up to MFC values) and 10 µL of fungal spore suspension (10^6 spores per mL) and incubated for 14 days at 28°C. The control group consisted of maize grains that had only been inoculated with fungi. After a time of incubation, maize grains were crushed into fine powder under hygienic practices. One gram of maize flour was serially diluted in peptone water, plated on SDA Petri plates, and cultured at 28°C for three days. The fungus's growth was determined in log CFU/g. To quantify OTA, a quantity of 10 g maize powder was subjected to immune affinity clean-up of OTA, and OTA was quantified by HPLC[16].

Statistical analysis

The studies have been carried out in triplicates, the collected data were analyzed using one-way ANOVA, and the results were reported as mean standard ± deviation. Dunnett's test was used to determine the statistical correlation between test and control samples. The $P \leq 0.05$ was considered significant and symbolized as '*'. Whereas, $P > 0.05$ was considered as non-significant and symbolized as '#'. GraphPad Prism trial version 8, San Diego, CA 92108, United States, was used for the aforementioned purpose.

Results

Chemical profile of EOs

In this objective, hydrodistillation was used to extract EOs from flower buds of *S. aromaticum*, leaves of *O. sanctum*, and flower buds of *C. odorata*. The dry weight of the plant material was used to compute the yield of EOs, which was expressed as (*w/w*). The yield of SAEO, OSEO, and COEO was determined as 1.71%, 1.92%, and 1.40% (*w/w*), respectively.

The obtained EOs were subjected to GC-MS analysis to determine their chemical make-up. The chemical compounds were identified based on the comparison of determined retention index (RI) with RI of literature of Adams, (2007). As well, the mass spectrum of the identified compounds was considered to determine the chemical constituents (Table 1).

Table 1 — Chemical constituents of *S. aromatic* essential oil (SAEO), *O. sanctum* essential oil (OSEO), and *C. odorata* essential oil (COEO) determined by GC-MS analysis.

Chemical compound	RI[a]	Essential oils					
		SAEO		OSEO		COEO	
		RI[b]	%[c]	RI[b]	%[c]	RI[b]	%[c]
α-Thujene	924	922	0.12	923	0.19	922	0.02
α-Pinene	932	931	0.10	933	1.82	931	0.11
Camphene	946	947	1.31	945	1.29	948	0.14
Sabinene	969	971	0.82	970	0.13	970	0.06
β-Pinene	974	975	0.71	972	0.18	973	0.15
Myrcene	988	989	0.14	989	0.11	989	0.18
α-Phellandrene	1002	1003	2.90	1001	3.22	1001	1.29
δ-3-Carene	1008	1009	0.14	1006	0.78	1009	0.02
α-Terpinene	1014	1015	3.83	1015	10.03	1015	04.88
p-Cymene	1020	1022	1.10	1021	0.19	1019	0.22
o-Ocimene	1022	-	-	1024	0.21	-	-
Limonene	1024	1024	1.88	1025	0.07	1023	12.02
β-Phellandrene	1025	1026	10.59	-	-	1025	10.82

(*Contd.*)

Table 1 — Chemical constituents of *S. aromatic* essential oil (SAEO), *O. sanctum* essential oil (OSEO), and *C. odorata* essential oil (COEO) determined by GC-MS analysis — (*Contd.*)

Chemical compound	RI[a]	Essential oils					
		SAEO		OSEO		COEO	
		RI[b]	%[c]	RI[b]	%[c]	RI[b]	%[c]
(Z)-β-Ocimene	1032	-	-	1033	4.12	-	-
(E) -β-Ocimene	1044	-	-	1045	10.02	-	-
(3Z)-Octen-1-ol	1047	-	-	1048	0.22	1046	0.02
γ-Terpinene	1054	1.053	11.80	1055	3.39	1053	2.77
Acetophenone	1059	-	-	-	-	1060	0.14
p-Mentha-2,4(8)-diene	1085	-	-	-	-	-	-
Linalool	1095	1096	3.02	-	-	1096	11.09
n-Nonanal	1100	-	-	-	-	1101	0.19
exo-Fenchol	1118	-	-	-	-	-	-
trans-Pinocarveol	1139	-	-	1140	0.81	-	-
Camphor	1141	1142	0.88	1142	11.91	1142	0.22
p-Menth-8-en-3-ol	1150	1152	0.11	-	-	1151	0.31
Isoborneol	1155	1156	0.06	1157	0.10	1156	0.12
Pinocarvone	1164	1164	0.11	1163	0.03	1163	0.02
Borneol	1165	1167	0.31	1165	0.12	1165	0.19
Terpinen-4-ol	1174	1176	0.57	1176	1.31	1173	0.31
Isomenthol	1183	1184	0.23	1184	0.12	1182	0.82
α-Terpineol	1186	1188	0.39	-	-	1188	0.10
trans-Carveol	1215	1217	1.41	-	-	1216	0.81
Citronellal	1223	1224	0.93	-	-	-	-
Pulegone	1237	1237	0.80	-	-	-	-
Linalyl acetate	1254	1255	0.09	1255	0.21	-	-
Bornyl acetate	1287	1288	0.14	1288	0.09	-	-
Thymol	1289	1290	1.71	1290	0.34	1290	12.24
Carvacrol	1298	1299	1.14	1299	0.02	-	-
Isomenthyl acetate	1305	1307	0.88	1306	0.01	1307	0.19
δ-Elemene	1335	1336	0.06	1336	0.03	1336	0.21
α-Cubebene	1345	1346	0.19	-	-	1346	0.23
Eugenol	1356	1357	26.89	1357	21.01	1357	10.04
Cyclosativene	1369	-	-	-	-	-	-
α-Ylangene	1373	-	-	-	-	1373	11.93
α-Copaene	1374	1376	0.17	-	-	1374	0.01
Geranyl acetate	1379	-	-	-	-	-	-
β-Elemene	1389	1390	0.09	-	-	1390	1.21
β-Caryophyllene	1417	1416	20.16	1419	14.19	1418	1.29
β-Copaene	1430	1429	0.82	-	-	1431	0.11
α-Guaiene	1437	1438	0.15	-	-	1438	0.18
α-Humulene	1452	1453	0.18	1453	0.02	1453	0.21
Geranyl acetone	1453	-	-	-	-	-	-
Allo-Aromadendrene	1458	-	-	-	-	-	-
Ishwarane	1465	1466	0.12	1466	0.09	1466	0.29
Geranyl propionate	1476	-	-	-	-	-	-
Germacrene D	1484	1486	0.22	1487	10.81	1482	11.16
β-Selinene	1489	1490	0.11	1490	0.12	1490	0.14
Viridiflorene	1496	-	-	1497	0.05	-	-
α-Muurolene	1500	1501	0.41	1502	0.11	1501	0.19
γ-Cadinene	1513	1512	0.07	1512	0.05	1514	0.07
Geraniol isobutanoate	1514	-	-	1515	0.12	-	-
δ-Cadinene	1522	1523	0.52	1523	0.15	1523	0.02

(*Contd.*)

Table 1 — Chemical constituents of *S. aromaticum* essential oil (SAEO), *O. sanctum* essential oil (OSEO), and *C. odorata* essential oil (COEO) determined by GC-MS analysis — (*Contd.*)

Chemical compound	RI[a]	Essential oils					
		SAEO		OSEO		COEO	
		RI[b]	%[c]	RI[b]	%[c]	RI[b]	%[c]
Elemol	1548	1547	0.06	1549	0.02	1549	0.16
Elemicin	1555	1556	0.03	-	-	1556	0.11
Carotol	1594	1593	0.07	-	-	-	-
Cubenol	1639	-	-	1640	0.12	1640	0.08
Klusinone	1604	-	-	1605	0.18	-	-
Bulnesol	1670	1671	0.03	1671	0.03	1671	0.21
Total (%)			98.57		98.14		97.30

[a]Chemical compound's retention indices on DB-5MS column as per literature of Adams, (2007)
[b]Chemical compound's retention indices on DB-5MS column
[c]Individual chemical compound quantity expressed as a percentage

Table 2 — Antifungal activity of *S. aromaticum* essential oil (SAEO), *O. sanctum* essential oil (OSEO), and *C. odorata* essential oil (COEO) against *A. ochraceous* and *P. verrucosum* determined by micro-well dilution technique

Essential oil/standard antifungal agent	*A. ochraceus* – ITCC 2454		*P. verrucosum* –ITCC 2986	
	MIC (µg/mL)	MFC (µg/mL)	MIC(µg/mL)	MFC (µg/mL)
S. aromaticum	1251 ± 42.32	1878 ± 28.47	0815 ± 22.69	1146 ± 51.19
O. sanctum	1700 ± 41.64	2461 ± 56.09	1477 ± 11.59	1929 ± 39.51
C. odorata	1878 ± 39.41	2783 ± 48.12	1547 ± 51.73	2101 ± 41.39
Nystatin	1531 ± 10.17	1959 ± 21.19	1342 ± 19.88	1780 ± 58.15

A total of 50, 44, and 48 chemical constituents were identified in SAEO, OSEO, and COEO, respectively. Total constituents were occupied 98.57%, 98.14%, and 97.30%in SAEO, OSEO, and COEO, respectively. These results show that most of the chemical constituents were identified in EOs.

Antifungal activity of EOs

The micro-well dilution technique was used to evaluate the antifungal activity of EOs in terms of MIC and MFC. The SAEO, OSEO, and COEO, as well as synthetic antifungal agent nystatin, showed potent antifungal action against *P. verrucosum* and *A. ochraceous*, respectively (Table 2). EOs and standard antifungal agent nystatin showed superior antifungal action over *P. verrucosum* when compared to *A. ochraceous*. Antifungal activity of synthetic antifungal agent nystatin was noticed in terms of MIC and MFC against *A. ochraceous* and *P. verrucosum* as 1531 ± 10.17 and 1959 ± 21.19 µg/mL, and 1342 ± 19.88 and 1780 ± 58.15 µg/mL, respectively. While, SAEO showed superior antifungal activity compared to OSEOand COEO, and synthetic antifungal agent nystatin and its MIC and MFC values against *A. ochraceous* and *P. verrucosum* were noticed as 1251 ± 42.32 and 1878 ± 28.47 µg/mL, and 0815 ± 22.69 and 1146 ± 51.19 µg/mL, correspondingly.

Antifungal mechanism of essential oils

The antifungal mechanism of EOs was established in this investigation by relating the levels of intracellular ROS, membrane integrity, and ergosterol of EOs treated and untreated fungus (control).

DCFH-DA staining was employed in the study to demonstrate the effect of varying EO concentrations on the formation of intracellular ROS in fungi (Fig. 1A & B). The results demonstrated that intracellular ROS content in EOs-treated fungal samples was significantly higher than in control fungal samples, and that the increase in intracellular ROS in fungi was dose-dependent with EOs. The study found that intracellular ROS-mediated oxidative stress is the main reason for EOs' antifungal activity.

The role of EOs on fungi's ergosterol level was addressed in another antifungal mechanism investigation. In our study, when compared to a control sample, the ergosterol content of fungus was lowered by EOs treatment, and it was found to be dosage dependent on EOs concentration (Fig. 2A & B). The study revealed that inhibition of ergosterol level is one of the responsible factors for the antifungal activity of EOs.

Propidium iodide staining was used in the final research of antifungal assessment to investigate the

influence of EOs on the membrane integrity of fungal spores. In our research, EOs had a negative impact on the membrane integrity of fungal spores, which was found to be dose-dependent (Fig. 3A & B). According to the findings, EOs caused fungal death by interfering with membrane integrity, which could be owing to EOs-induced intracellular ROS-mediated lipid peroxidation and ergosterol loss.

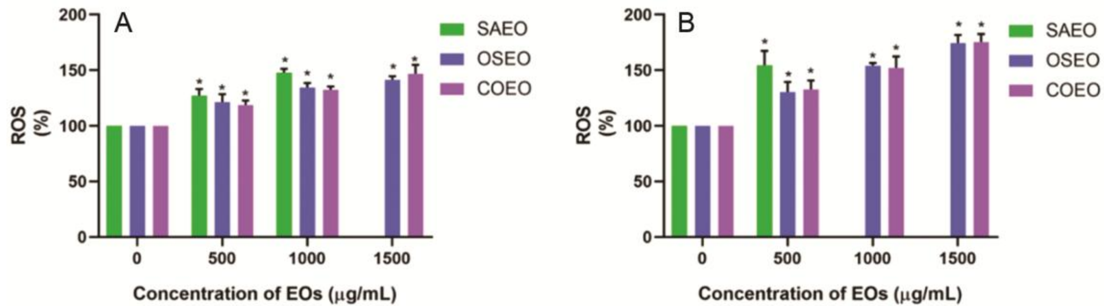

Fig. 1 — Dose-dependent effect of SAEO, OSEO, and COEO on the generation of intracellular ROS in (A) *A. ochraceous*; and (B) *P. verrucosum* determined by DCFH-DA staining. Dunnett's test was used to determine the statistical correlation between control and test samples and it was done within the respective study of essential oil treatments. The $P \leq 0.05$ was considered significant and symbolized as '*'. Whereas, $P > 0.05$ was considered as non-significant and symbolized as '#'

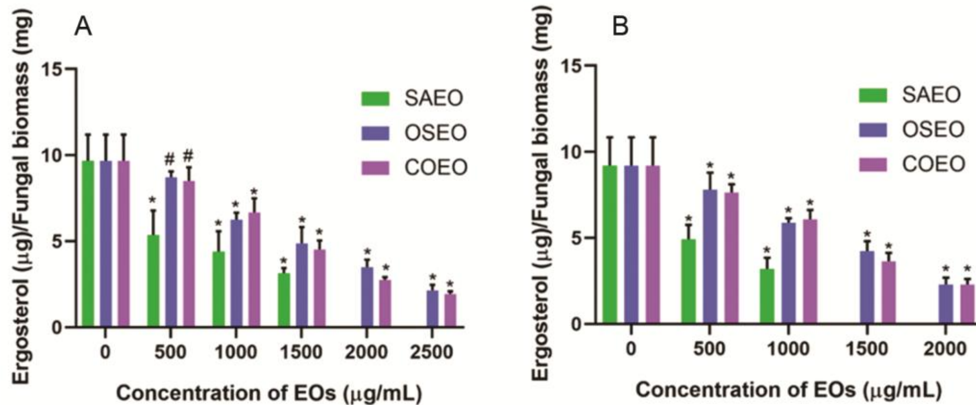

Fig. 2 — Dose-dependent effect of SAEO, OSEO, and COEO on ergosterol content of (A) *A. ochraceous*; and (B) *P. verrucosum*. Dunnett's test was used to determine the statistical correlation between control and test samples and it was done within the respective study of essential oil treatments. The $P \leq 0.05$ was considered significant and symbolized as '*'. Whereas, $P > 0.05$ was considered as non-significant and symbolized as '#'

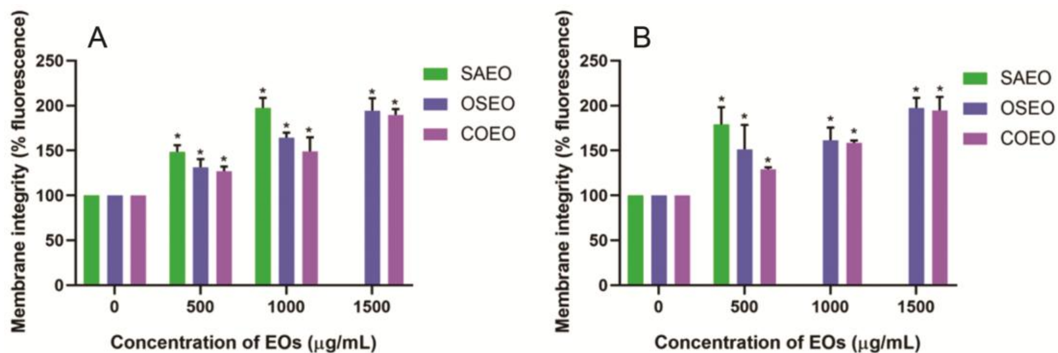

Fig. 3 — Dose-dependent effect of SAEO, OSEO, and COEO on membrane integrity of (A) *A. ochraceous*; and (B) *P. verrucosum* spores determined by propidium iodide staining. Dunnett's test was used to determine the statistical correlation between control and test samples and it was done within the respective study of essential oil treatments. The $P \leq 0.05$ was considered significant and symbolized as '*'. Whereas, $P > 0.05$ was considered as non-significant and symbolized as '#'

Table 3 — Inhibitory effect of *S. aromaticum* essential oil (SAEO), *O. sanctum* essential oil (OSEO), and *C. odorata* essential oil (COEO) on the growth of *A. ochraceous* and *P. verrucosum* in maize grains

Essential oil	*A. ochraceus* – ITCC 2454(log CFU/g)					*P. verrucosum* – ITCC 2986 (log CFU/g)				
	0 (control)	500 µg/g	1500 µg/g	2500 µg/g	3500 µg/g	0 (control)	500 µg/g	1500 µg/g	2500 µg/g	3500 µg/g
S. aromaticum	7.19 ± 0.81	5.93 ± 0.44*	2.15 ± 0.09*	Nil[&]	Nil[&]	6.39 ± 0.64	3.56 ± 0.32*	1.92 ± 0.03*	Nil[&]	Nil[&]
O. sanctum	7.19 ± 0.81	6.21 ± 0.61*	3.67 ± 0.34*	1.81 ± 0.28*	Nil[&]	6.39 ± 0.64	4.16 ± 0.78*	1.31 ± 0.50*	Nil[&]	Nil[&]
C. odorata	7.19 ± 0.81	6.03 ± 0.97*	4.28 ± 0.56*	1.68 ± 0.22*	Nil[&]	6.39 ± 0.64	4.20 ± 0.15*	2.78 ± 0.89*	1.04 ± 0.21*	Nil[&]

[&]Fungal growth was absent. Dunnett's test was used to determine the statistical correlation between control and test samples and it was done within respective study of essential oil treatments. The $P \leq 0.05$ was considered as significant and symbolized as '*'. Whereas, $P > 0.05$ was considered as non-significant and symbolized as '#'

Table 4 — Inhibitory effect of *S. aromaticum* essential oil (SAEO), *O. sanctum* essential oil (OSEO), and *C. odorata* essential oil (COEO) on ochratoxin A (OTA) level of *A. ochraceous* and *P. verrucosum* in maize grains

Essential oil	*A. ochraceus* – ITCC 2454(OTA µg/g)					*P. verrucosum*–ITCC 2986(OTA µg/g)				
	0 (control)	500 µg/g	1500 µg/g	2500 µg/g	3500 µg/g	0 (control)	500 µg/g	1500 µg/g	2500 µg/g	3500 µg/g
S. aromaticum	6.53 ± 0.96	5.31 ± 0.92*	1.94 ± 0.30*	Nil[&]	Nil[&]	5.11 ± 0.37	2.72 ± 0.19*	1.41 ± 0.03*	Nil[&]	Nil[&]
O. sanctum	6.53 ± 0.96	6.01 ± 0.58[#]	3.21 ± 0.63*	1.04 ± 0.06*	Nil[&]	5.11 ± 0.37	3.01 ± 0.47*	1.68 ± 0.84*	Nil[&]	Nil[&]
C. odorata	6.53 ± 0.96	5.66 ± 0.89*	3.90 ± 0.22*	1.10 ± 0.07*	Nil[&]	5.11 ± 0.37	4.38 ± 0.70[#]	3.55 ± 0.59*	1.03 ± 0.18*	Nil[&]

[&]OTA was absent. Dunnett's test was used to determine the statistical correlation between control and test samples and it was done within respective study of essential oil treatments. The $P \leq 0.05$ was considered as significant and symbolized as '*'. Whereas, $P > 0.05$ was considered as non-significant and symbolized as '#'

***In vitro* antifungal and OTA inhibitory activity of EOs**

In a real food sample, maize grains, the antifungal and OTA production inhibitory action of EOs was established. The dilution plating method was applied to determine the fungus development in maize grains. Whereas, OTA concentration in maize grains was determined by HPLC. The EOs were exhibited potential activity in safeguarding the maize grains from fungi and OTA contamination, and these findings in line with the micro-well dilution technique's *in vitro* antifungal evaluation (Tables 3 & 4). The SAEO exhibited superior inhibitory activity on development and OTA production of *P. verrucosum* and *A. ochraceus* in maize grains over other two essential oils, *i.e.*, OSEO and COEO. The SAEO, OSEO, and COEO have inhibited the complete fungal growth and OTA content of *A. ochraceus* and *P. verrucosum* correspondingly at 2500 and 2500, 3500 and 2500, and 3500 and 3500 µg/g. The COEO had the least inhibitory effect on fungus development and OTA formation in maize grains.

Discussion

The chemical profile of studied EOs, *i.e.*, SAEO, OSEO, and COEO was revealed by GC-MS analysis. The major chemical compounds in SAEO were β-phellandrene (10.59%), γ-terpinene (11.80%), eugenol (26.89%), and β-caryophyllene (20.16%), and results concluded that SAEO belongs to the chemotype of eugenol. Several previous studies have suggested that EOs derived from buds and leaves of *S. aromaticum* contains eugenol as the primary component, which supports our findings[17,18]. The major chemical compounds in OSEO were α-terpinene (10.03%), (E) -β-ocimene (10.02%), camphor (11.91%), eugenol (21.01%), β-caryophyllene (14.19%), and germacrene D (10.81%) and it concluded that OSEO belongs to eugenol chemotype. In accordance with our results, several earlier reports showed that EOs obtained from *O. sanctum* contain eugenol as one of the major compounds and are responsible for various bio-potentials such as antioxidant, antimicrobial, and anticancer[19,20]. The major chemical constituents in COEO were limonene (12.02%), β-phellandrene (10.82%), linalool (11.09%), thymol (12.24%), eugenol (10.04%), α-ylangene (11.93%), and germacrene D (11.16%) and results concluded that COEO belongs to chemotype of thymol, limonene, and α-ylangene. Thus, obtained results showed that COEO contains a variety of chemical compounds that were quite highly responsible for biological functions[11]. However, chemical constituents of EOs are quite difficult to evaluate because of their complex and diversified character, as well as the wide range of quantities seen in plants based on their genome, nutrition, geographical distribution, and finally extraction technique adopted[21]. Therefore, the chemical profile of SAEO, OSEO, and COEO was diverse when compared to existing reports.

To support our antifungal research outcome, an earlier report confirmed the antifungal action of SAEO against diverse fungi such as A. *niger*,

A. oryzae, and *A. ochraceus* by the agar diffusion method[22]. In addition, some other earlier reports such as showed a wide spectrum of antifungal activity of SAEO against *A. flavus*, *P. citrinum*, *R. nigricans*, *A. parasiticus*, and *F. oxysporum*[23,24,25]. Similarly, OSEO has shown potent antifungal activity on *F. graminearum*[19] and *Lasiodiplodia theobromae*[26]. These earlier reports on the antifungal activity of SAEO and OSEO concluded that eugenol was the foremost compound responsible for antifungal activity. In our research, eugenol was the most abundant chemical in SAEO and OSEO, and it may be the source of antifungal activity. The COEO is well known for treatment for cold, inflammation, asthma, and other respiratory diseases[27]. Recently, few reports have shown the antifungal activity of COEO against *F. graminearum*[11] and *A. flavus*[28]. These previous reports and our present reports were coherent and suggested that the antifungal activity of COEO could be due to thymol, limonene, Germacrene-D, linalool, and eugenol[27].

In this study, the levels of intracellular ROS, membrane integrity, and ergosterol of EOs-treated fungus (test sample) and untreated fungus (control sample) were compared to establish the antifungal mechanism of studied EOs. Intracellular reactive oxygen species (ROS) are key regulatory molecules in the biological system and could impair the cellular proteins, lipids, and DNA, as well as trigger oxidative stress-mediated apoptosis through caspase activation[29]. In our study, intracellular ROS was increased after EOs treatment, and oxidative stress-mediated apoptosis could be the main cause for the antifungal action of EOs. To support our research outcome, earlier investigations concluded that EOs of *Curcuma longa, Cinnamomum verum, and Cymbopogon citratus* could induce fungal death by a generation of intracellular ROS[30,31].

Another antifungal mechanism study examined the effect of EOs on fungi's ergosterol levels. In our investigation, the ergosterol content of fungus was reduced by EOs treatment when compared to a control sample, and it was revealed to be dose-dependent on EOs concentration. To support our research outcome, earlier investigations demonstrated that EOs could inhibit ergosterol biosynthesis, and thus, could reduce the fungi's capacity to grow and produce mycotoxins[14,32]. In fungal cell membranes, ergosterol is the most abundant sterol, and it regulates permeability and fluidity. The low level of ergosterol could cause the death of the fungi by damaging the fluidity and permeability of the cell membrane. Therefore, researchers' main target for the development of novel antifungal agents is inhibition of ergosterol biosynthesis[33]. Our study determined that inhibition of ergosterol biosynthesis is one of the responsible factors for the antifungal and antimycotoxin activities of EOs.

In the final investigation of antifungal assessment, propidium iodide staining was utilized to investigate the effect of EOs on the membrane integrity of fungal spores. Membrane integrity is critical for cell survival, defects of which cause disturbs in the fluidity and various metabolic processes of the cells[34]. The EOs exhibited a deleterious effect on the membrane integrity of fungal spores, which was dose-dependent. In accordance with our results, previous reports of Kalagatur *et al*. and Khan *et al*. showed that detrimental membrane integrity caused by essential oils and high-pressure processing is responsible for fungal death[10,19,34]. According to our findings, EOs caused fungal mortality by interfering with the membrane integrity. The membrane integrity could have been disturbed due to the lipid peroxidation action of intracellular ROS and the loss of ergosterol content caused by EOs. Altogether, antifungal investigations found that EOs caused fungal mortality by increasing the intracellular ROS, depleting ergosterol synthesis, and disrupting membrane integrity.

The antifungal and OTA production inhibitory effects of EOs were demonstrated in a real food sample of maize grains. The EOs showed potential effectiveness in preventing the fungal growth and OTA level in maize grains. Few earlier investigations on the use of EOs for limiting the fungal development and mycotoxins in real-time food samples were supported our findings. The earlier report demonstrated the application of SAEO in controlling the growth and OTA of *Aspergillus* spp. in peanut meal, rice, oats, and wheat grains[35-37].

In the case of OSEO, earlier reports have recognized the antifungal and antimycotoxin activity (zearalenone) of OSEO against *F. graminearum* in maize and determined that eugenol was responsible for antifungal and antimycotoxin activity[19]. Also, earlier investigations showed that COEO was highly effective in limiting the development and mycotoxins formation of *F. graminearum* and *A. flavus* during the storage of foodstuffs[11,28].

Moreover, in support of the study's findings, a previous report revealed that cinnamaldehyde, eugenol,

and citral could be possible natural counter agents for OTA level during the storage of the food sources[32]. Similarly, an earlier report of Komala *et al*. determined the inhibitory effect of eugenol against aflatoxin B1 content in stored sorghum grains[38]. In our study, SAEO, OSEO, and COEO were found to contain a high amount of eugenol. Therefore, the studied SAEO, OSEO, and COEO have shown strong inhibitory activity on the development and OTA content of *P. verrucosum* and *A. ochraceous* in our study.

Conclusion

According to findings, EOs are rich in naturallybeneficial antimicrobial compounds. The SAEO and OSEO belong to the chemotype of eugenol, whereas, COEO was found to be the chemotype of thymol, limonene, and α-ylangene. In a micro-well dilution approach, the EOs demonstrated substantial growth inhibitory effect on *A. ochraceous* and *P. verrucosum*, with potent MIC and MFC values. The antifungal mechanisms experiments demonstrated that EOs had significant antifungal action due to an increase in intracellular ROS, membrane disruption, and ergosterol depletion. In the final study, EOs were found to have a strong inhibitory effect on the development and OTA level of *P. verrucosum* and *A. ochraceous* in maize. According to the findings, EOs could act as natural antifungal agents; protect foodstuffs from fungal infection and mycotoxins during storage.

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Conflict of interest

There is no conflict of interest

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