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Sea buckthorn (*Hippophae rhamnoides* L.) oil exhibits antifungal activity against *Aspergillus flavus* via disrupting mitochondrial function

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Abstract: The present study sought to examine how sea buckthorn (SBT) oil impacts mitochondrial and overall functionality in *Aspergillus flavus*. In order to assess the effect of SBT oil, it was used to treat mycelia across a range of concentrations, after which mitochondrial structures were imaged via transmission electron microscopy (TEM). In order to explore the functional impact of this treatment, we additionally examined reactive oxygen species (ROS) production, malondialdehyde (MDA) levels, enzyme activity, and shifts in mitochondrial membrane potential ($\Delta\Psi m$) following treatment. We found that SBT oil induced mitochondrial damage in *A. flavus* in a dose-dependent manner, resulting in altered succinate dehydrogenase (SDH) and adenosine triphosphatase (ATPase) activity, reduced $\Delta\Psi m$ levels, and markedly elevated MDA and ROS levels. Together, these findings suggest that SBT oil can mediate antifungal activity against *A. flavus* through a mechanism associated with its ability to disrupt the tricarboxylic acid (TCA) cycle and mitochondrial potential, leading to MDA and ROS accumulation.

Keywords: sea buckthorn oil; mould; antifungal mechanism; mitochondria

Sea buckthorn (SBT) (*Hippophae rhamnoides* L.) is a shrub from the Elaeagnaceae family in the *Hippophae* genus (Geetha et al. 2003; Yildiz et al. 2012). The berries of this plant can be processed to produce SBT oil, which is rich in saturated fatty acids (palmitic acid, stearic acid), unsaturated fatty acids (eicosanoic acid, oleic acid, palmitoleic, linolic acid, alpha-linolenic acid, gamma-linolenic acid), sterols, approximately 14 vitamins: A, C, D, E, F, K, P, and B complex vitamins (B1, B2, B6), provitamin A, that is alpha- and beta-carotene, a mixture of other carotenoids, strong antioxidants (tocopherols, tocotrienols), flavonoids (approximately 36 types), fruit acids (malic acid and citric acids), phenolic compounds, approximately 11 mineral salts, including zinc, iron, calcium, selenium, copper, tannins, phospholipids, anthocyanins, steroids, sugars, pectins, and approximately 18 amino acids (Ranjith et al. 2006; Basu et al. 2007; Sajfrtová et al. 2010; Kuznetsova et al. 2010; Kagliwal et al. 2011; Yang and Kallio 2011; Yoon et al. 2012; Czaplicki et al. 2007). Sea-buckthorn oil contains approximately 190 bioactive substances. Therefore, seabuckthorn and its oil may be considered to be one of the most valuable natural products in the world. It supports the function of the immune system, helps to fight infections and microorganisms, improves circulation and heart function, prevents

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atherosclerosis, lowers the level of cholesterol in the blood, supports the function of the digestive system and metabolism, relieves the symptoms of chronic gastric ulcer disease and other diseases of the stomach, duodenum, pancreas, liver and intestines, prevents inflammations, improves the function of the brain and the nervous system, lowers the risk of malignant cancers, supports regeneration of the body after the chemotherapy and serious diseases, reenergises and revitalises, positively affects mood and has an antidepressant effect (Negi et al. 2006; Basu et al. 2007; Yen et al. 2008; Ting et al. 2011; Kim et al. 2012; Ito et al. 2014; Manea et al. 2014; Lotsch et al. 2015). Importantly, SBT oil is safe and beneficial when administered to humans (Upadhyay et al. 2009).

Aspergillus flavus produces powerful mycotoxins, including aflatoxin (Hua et al. 2018; Barakat et al. 2019), which is found in a variety of foods, such as corn, peanuts, cotton seeds, and other cereals worldwide. Roughly 4.5 billion people in developing countries are reported to be systematically exposed to uncontrolled amounts of aflatoxin (Lee et al. 2004; Thippeswamy et al. 2018). Aflatoxin can cause acute liver injury, cirrhosis, cancer, and other diseases (Barakat et al. 2019). Prior research suggests that SBT oil may possess antifungal activity (Gupta et al. 2011), but there have not been any thorough studies of the mechanistic basis for such activity conducted to date. SBT oil has the potential to alter endocellular organelle functionality, but whether this mode of action enables this oil to inhibit *A. flavus* growth remains to be established.

Mitochondria are the primary source of intracellular energy, generating ATP via oxidative phosphorylation and the tricarboxylic acid (TCA) cycle (Nazaret et al. 2009; Li et al. 2017). In addition, mitochondria serve as essential regulators of cell survival, signal transduction, differentiation, and cell cycle progression (Nakayama and Otsu 2018). Mitochondrial dysregulation can dramatically impair cell fitness and can lead to cell death, thus making mitochondria ideal targets for antifungal compounds (Borgna et al. 2017). While SBT oil has been alleged to offer numerous clinical benefits, there have been few thorough studies of the mechanistic basis for such activity to date. As such, the present study was designed to explore the ability of SBT oil to disrupt *A. flavus* growth via adversely impacting mitochondrial functionality within these fungal cells.

Herein, we utilised transmission electron microscopy (TEM) to assess ultrastructural changes in *A. flavus* mitochondria following SBT oil treatment. In addition, we assessed the impact of SBT oil on mitochondrial membrane potential, reactive oxygen species (ROS) production, malondialdehyde (MDA) levels, and the activity of key enzymes related to the TCA cycle in treated *A. flavus* cells.

MATERIAL AND METHODS

Material. *A. flavus (*bio-52973*)* was obtained from the Agricultural Culture Collection of China. SBT oil was from Jinghua Tianbao Sea Buckthorn Oil Co., Ltd. (China).

Cell treatment and sample preparation. Two 2×2 cm^2 blocks of the activated solid *A. flavus* culture medium were added to sterile centrifuge tubes, washed with 10.0 mL of 0.9% sterile NaCl solution, and mycelia were scraped off the solid medium using a sterile inoculator (Bickman Biotechnology Co., Ltd., China). These were then filtered using four layers of sterile gauze to remove the excess mycelium and culture medium, yielding a spore suspension. Spore concentrations were then adjusted to 10^7 mL^{-1}, after which a 100 µL aliquot of this solution was mixed (Model 720210; Baden Medical Co., Ltd., China) with 45 mL of sterile potato dextrose broth medium and 5 mL of aqueous SBT oil (final concentration: 0, 24, 48, and 96 mL L^{-1}). Cultures without SBT oil were used as controls. Samples were then incubated for 3 days at 28 °C with constant shaking (150 rpm) (LRH-150-S; Forma Technology Co., Ltd., China). Mycelium was then isolated, rinsed three times using sterile 0.9% NaCl, frozen (GIPP5000 FDA; Shanghai Jipu Electronic Technology Co., Ltd., China), ground (BYrl-37; Bingyu, China) with liquid nitrogen, and used for downstream assays.

Isolation of mitochondria was performed following slightly modified version of a previous method (Li et al. 2017). Briefly, 2.0 g frozen samples were placed in sterile centrifuge tubes, washed twice with sterile buffer solution (20 mmol L^{-1} Tris-HCl, 5 mmol L^{-1} ethylenediamine tetraacetic acid disodium (Na_2EDTA), and 15% glucose; pH = 7.4), and then resuspended in 20.0 mL of the same sterile buffer solution (Model 720210; Baden Medical Co., Ltd., China). The samples were then ultrasonicated (SM-150A; SHUNMA Technology Co., Ltd., China) for 3 min (3 s ultrasonication with a 2 s interval period) in order to disrupt cell walls. Samples were then spun at low speed (3 000 rpm, 10 min) (JIDI-20D; Guangzhou Shundi Technology Co., Ltd., China), and supernatants were collected and added to sterilised centrifuge tubes which were then spun twice for 25 min at 4 200 rpm, with supernatants being discarded. Precipitated mitochondria were resuspended in 1.0 mL

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of sterile buffer and stored at –20 °C (BCD546WP-SCL; Zhuhai Gree Electric Appliance Co., Ltd., China). All preparation and extraction steps were conducted at 4 °C (CT15E; Hitachi, Ltd., Japan).

TEM analysis. Following collection from control or SBT oil (250 μg L^{-1})-treated cells, mitochondria were fixed (Model 720210; Baden Medical Co., Ltd., China) for 4 h with 2.5% glutaraldehyde. They were then imaged with a TEM (Model JEM-1230; Hitachi, Japan) at the Zhongke Baite Test Center of Beijing, China.

Mitochondrial enzyme activity analysis. A crude enzyme solution was prepared from 0.2 g of wet mycelia by washing them twice in water, grinding them with liquid nitrogen, combining them with 1.8 mL of 0.9% NaCl, spinning at 4 °C, and collecting supernatants for enzymatic activity analyses. The activity of adenosine triphosphatase (ATPase), succinate dehydrogenase (SDH) samples were assessed using commercial kits based on provided directions. Samples were analysed in triplicate.

MDA level measurement. Commercial kits (Nanjing Jiancheng Bioengineering Research Institute, China) were used to analyse MDA levels in triplicate samples based on provided directions.

Analysis of oxygen species production. A modified version of a previously published protocol (Tian et al. 2012) was used to measure ROS production. Briefly, *A. flavus* spores were isolated as above and suspended at 10^6 spores mL^{-1}. The fluorometric 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) dye was then used to measure endogenous ROS production by treating spores with SBT oil for an appropriate period of time and then adding DCFH-DA (10 μM) for 1 h at room temperature. Cells were then spun for 12 min at 5 000 rpm, washed three times in a phosphate buffer (pH 7.0), and resuspended in 1 mL of phosphate buffer. A microplate reader (Infinite M200 Pro; Tecan, Switzerland) was then used to measure fluorescence in individual samples with excitation and emission wavelengths of 485 nm and 525 nm, respectively. Triplicate samples were analysed.

Mitochondrial membrane potential measurement. Following a 3 day culture, fungal spores were collected via the addition of phosphate-buffered saline (PBS) (2%) to individual dishes, scraping gently with an L-shaped spreader (Bio-Mark; Guangzhou Biaomi Biotechnology Co., Ltd., China), and resuspending spores at 4×10^6 spores mL^{-1}. A 200 μL volume of this sample was then combined with 3.8 mL of PBST and mixed (Model 720210; Baden Medical Co., Ltd., China) with 0, 24, 48, or 96 mL L^{-1} SBT oil. Samples were then incubated for 12 h at 25 °C with constant rotation (150 rpm). Spores were then isolated, washed thrice in PBS, resuspended at 4×10^6 spores mL^{-1}, and mixed with 100 ng mL^{-1} rhodamine 123 for 30 min at 28 °C protected from light. Spores were then next spun for 10 min at 5 000 rpm, washed thrice in PBS, and resuspended in 500 uL PBS. Fluorescence in individual samples was then measured using excitation and emission wavelengths of 485 nm and 525 nm, respectively. Triplicate samples were analysed.

Statistical analyses. Data were analysed via one-way analysis of variance (ANOVA) and Duncan's multiple range tests, with $P < 0.05$ as the significance threshold.

RESULTS AND DISCUSSION

SBT induces ultrastructural changes in the mitochondria of *A. flavus*. We began by assessing whether SBT oil treatment induced ultrastructural changes in *A. flavus* mitochondria via TEM. We found that there were clear structural differences when comparing control untreated mitochondria to those from SBT oil-treated cells. Control cells exhibited mitochondria with clearly defined inner and outer membranes and turgid cristae that appeared normal (Figures 1A, 1B). In contrast, mitochondria in SBT oil-treated cells exhibited clear signs of vacuolation, abnormal vesicle formation, and breakdown of the mitochondrial matrix structure (Figures 1C, 1D). This thus suggested that SBT oil can induce significant mitochondrial damage in *A. flavus* cells.

Few studies to date have explored the mechanistic basis for the antimicrobial activity of SBT oil. However, there is prior evidence suggesting that this oil can impair the growth of fungi and bacteria (Eccleston et al. 2002). In the present study, we observed clear signs of mitochondrial structural damage in *A. flavus* cells treated with SBT oil (Figure 1). This is in line with the results of a prior study wherein tea tree oil was shown to disrupt mitochondrial morphology and structural integrity when used to treat *Botrytis cinerea*, thereby exerting an antifungal effect (Li et al. 2017). Similarly, citral can impair the growth of *Penicillium digitatum* by interfering with mitochondrial structural integrity, leading to the development of depressions in the mitochondrial surface, potentially causing functional alterations within these organelles (Zheng et al. 2015).

SBT oil alters SDH enzyme activity in *A. flavus*. We next examined the impact of SBT oil on SDH enzymatic activity, which was 476.10 ± 6.16 U mg^{-1} protein in control untreated samples (Figure 2). In contrast, samples treated with 24, 48, and 96 mL L^{-1} SBT ex-

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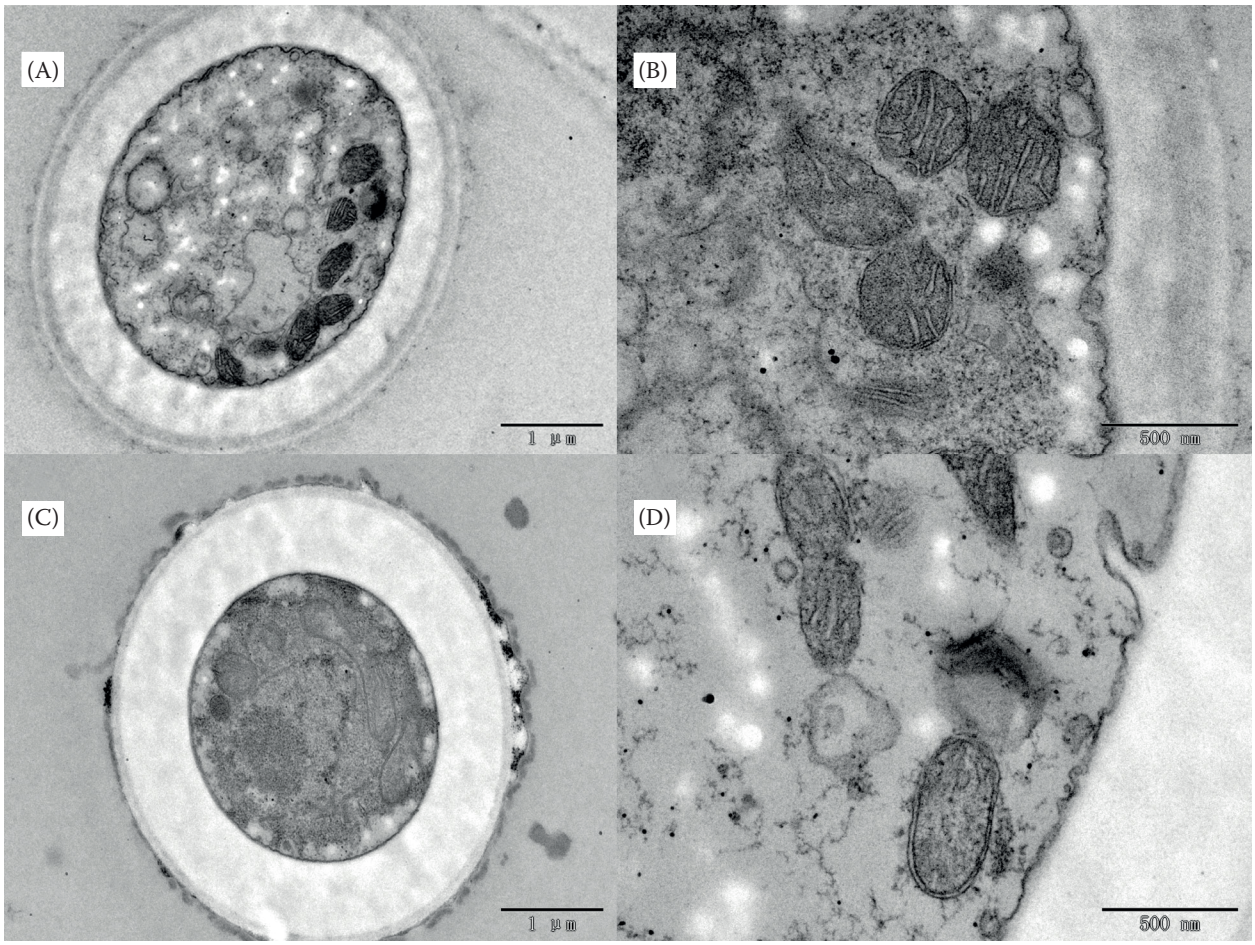

Figure 1. Effects of sea buckthorn (SBT) oil on mitochondrial ultrastructure: (A) *Aspergillus flavus* (control), scale bar: 1 μm, (B) *A. flavus* (control), scale bar: 500 μm, (C) *A. flavus* treated with SBT oil (24 mL L^{-1}), scale bar: 1 μm, and (D) *A. flavus* treated with SBT oil (24 mL L^{-1}), scale bar: 500 μm

hibited reduced SDH activity levels of 293.80 ± 3.50, 238.72 ± 5.47, and 179.78 ± 5.41 U mg^{-1} protein, respectively. This thus suggests that SDH enzymatic activity decreased in a dose-dependent manner in response to SBT oil treatment.

Mitochondrial dehydrogenase is a key metabolic regulatory enzyme (Li et al. 2017). SDH catalyses the oxidation of succinate to fumarate via transferring electrons from succinate to ubiquitin (Kobayashi et al. 2002). In their prior study, Li et al. (2017) found that tea tree oil can interfere with mitochondrial SDH, thereby mediating antifungal activity. Consistent with this, several studies have linked mitochondrial dysfunction to SDH activity (Ranjith et al. 2006). The treatment of *Candida albicans* with plagiochin E can also disrupt dehydrogenase activity, thereby impairing fungal growth (Wu et al. 2009). Treatment of *A. flavus* cells with *Anethum graveolens* L. essential oil can disrupt the cell membrane and impair SDH activity within these cells (Tian et al. 2012). In the present study, we found that SBT oil was also able to impair SDH activity in a dose-dependent fashion (Figure 2), consistent with a model wherein this oil can disrupt mitochondrial membrane permeability and thereby impair mitochondrial functionality.

SBT oil alters ATPase activity in *A. flavus*. When we assessed ATPase activity in our *A. flavus* samples, we found that control cells exhibited a Na^+-K^+ ATPase activity level of 28.68 ± 5.21 U mg^{-1} protein (Figure 3). Following treatment with 24, 48, and 96 mL L^{-1} SBT oil, these values rose to 14.01 ± 2.03, 10.22 ± 0.36, and 7.97 ± 0.25 U mg^{-1} protein, respectively. We additionally assessed Ca^{2+}-Mg^{2+}ATPase activity, which in control cells was found to be 22.59 ± 3.01 U mg^{-1} protein, whereas after treatment with 24, 48, and 96 mL L^{-1} SBT oil these values fell to 12.06 ± 2.03, 7.30 ± 2.53, and 4.78 ± 1.15 U mg^{-1} protein, respectively. Most notably, at an SBT oil concentration of 96 mL L^{-1}, these

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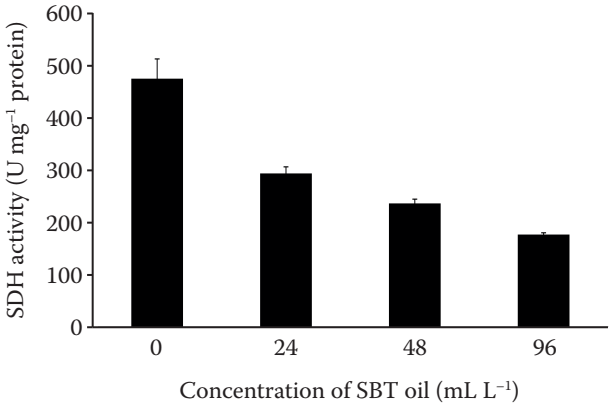

Figure 2. Effects of sea buckthorn (SBT) oil on succinate dehydrogenase (SDH) activities in *Aspergillus flavus* mitochondrion

U – enzyme unit (the amount of enzyme that can convert 1 μmol substrate in 1 min)

Na^+-K^+ATPase and Ca^{2+}-Mg^{2+}ATPas activities were reduced by 72.21% and 78.84%, respectively (both $P < 0.01$) relative to control cells.

The TCA cycle is a primary mechanism whereby mitochondria generate cellular energy (Fernie et al. 2004). ATPases mediate ATP decomposition (Huang et al. 2003). In their study, Li et al. (2017) found that tea tree oil was capable of inhibiting the TCA cycle within *B. cinerea* cells, resulting in reduced ATPase activity. We also found that SBT oil suppressed ATPase activity when it was used to treat *A. flavus* (Figure 3). Such reduced ATPase activity will result in the impaired utilisation of ATP as an energy source within fungal cells, thereby impairing growth and survival.

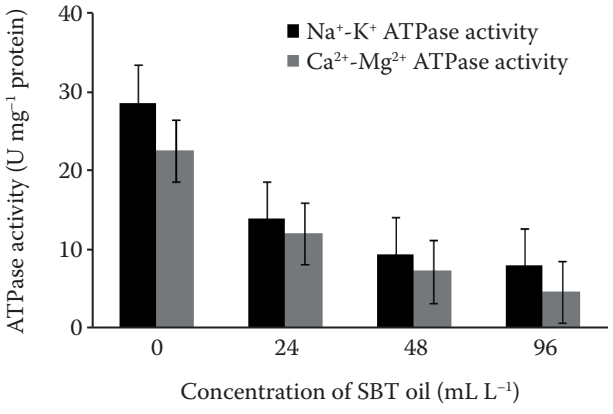

Figure 3. Effects of sea buckthorn (SBT) oil on ATPase activity in *Aspergillus flavus* mitochondrion

ATPase – adenosine triphosphatase; U – enzyme unit (the amount of enzyme that can convert 1 μmol substrate in 1 min)

SBT oil alters MDA levels in *A. flavus*. We next assessed MDA levels in *A. flavus* cells following SBT oil treatment (Figure 4). At baseline, we detected MDA levels of 0.04 ± 0.00 nmol mg^{-1}. Treatment of these cells with 24, 48, and 96 mL L^{-1} SBT oil resulted in increases in these levels to 0.05 ± 0.00, 0.07 ± 0.00, and 0.10 ± 0.00 nmol mg^{-1}, with all of these values being significantly higher than in control cells ($P < 0.01$). Most notably, the levels in cells treated with 96 mL L^{-1} SBT oil were 150% higher than levels in control cells ($P < 0.01$).

MDA levels in cells are commonly measured in order to gauge levels of lipid peroxidation, thereby offering insight into the degree of oxidative injury to the cell plasma membrane (Kong et al. 2012). The combined treatment of *Penicillium expansum* with cinnamaldehyde and citral can drive MDA accumulation (Yuan et al. 2018). Gao et al. (2016) also found that thymol was able to promote MDA accumulation in *Fusarium graminearum*. In line with these results, we determined that SBT oil was able to promote MDA accumulation in *A. flavus* cells (Figure 4), suggesting that SBT oil can disrupt mitochondrial membranes by reducing membrane mobility.

SBT oil alters oxygen species accumulation in *A. flavus*. We next examined how SBT oil treatment affected ROS accumulation within *A. flavus* cells, revealing a dose-dependent increase in these levels in response to treatment (Figure 5). Specifically, relative to normalised levels in untreated cells (100.00 ± 0.58%), treatment with 24, 48, and 96 mL L^{-1} of SBT oil led ROS level to increase to 199.94 ± 0.58, 299.94 ± 0.008, and 439.89 ± 1.58%, respectively, with all values being higher than those in control cells ($P < 0.01$).

ROS are primarily produced by the mitochondrial respiratory chain, with this production being enhanced in damaged cells (Tian et al. 2012). ROS can adversely impact cellular viability by damaging nucleic acids, enzymes, and the cellular membrane (Kobayashi et al. 2002). Such ROS accumulation is closely associated with apoptosis, as are other key morphological changes including nuclear fragmentation, chromatin condensation, and phosphatidylserine externalisation (Pozniakovsky et al. 2005). Borate has been shown to promote mitochondrial damage and associated ROS accumulation in *Colletotrichum gloeosporioides* spores (Shi et al. 2012), while plagiochin E can promote such ROS accumulation in *C. albicans* (Wu et al. 2009). Citral can disrupt the mitochondrial electronic respiratory chain in the mitochondria of *P. digitatum*, thereby increasing oxidative stress (Zheng et al. 2015). Simi-

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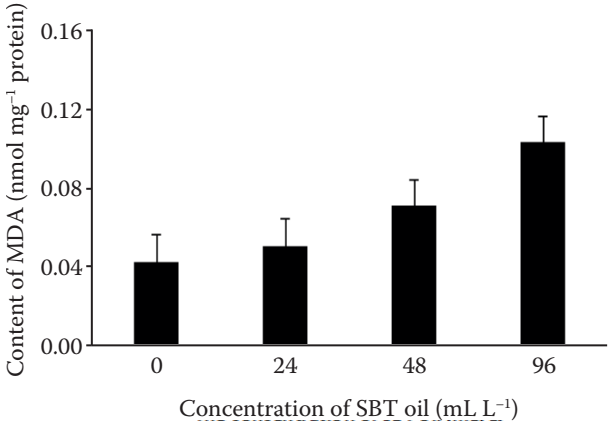

Figure 4. Effects of sea buckthorn (SBT) oil on malondialdehyde (MDA) content in *Aspergillus flavus* mitochondrion

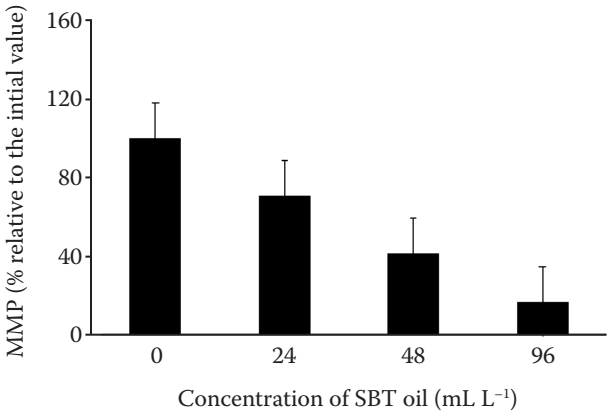

Figure 6. Effects of sea buckthorn (SBT) oil on mitochondrial membrane potential (MMP) levels in *Aspergillus flavus* mitochondrion

lar ROS accumulation was observed in *A. flavus* after treatment with *A. graveolens* L. essential oil (Tian et al. 2012). The combination of both cinnamaldehyde and citral treatment is also capable of inducing ROS accumulation in *P. expansum* (Gao et al. 2016), while tea tree oil has similar effects in *B. cinerea,* resulting in the induction of oxidative damage (Li et al. 2017). Herein, we found that SBT oil treatment was able to promote ROS accumulation in *A. flavus* (Figure 5), potentially explaining the resultant mitochondrial dysfunction and oxidative damage observed in the present study.

SBT oil alters mitochondrial membrane potential in *A. flavus*. Lastly, we examined the impact of SBT oil on mitochondrial membrane potential in *A. flavus* cells, revealing that this potential decreased in a dose-dependent manner in response to treatment (Figure 6). Specifically, after a 12 h treatment this membrane potential fell from 100.00 ± 1.00 to 70.84 ± 0.58, 41.66 ± 0.58, and 16.66 ± 0.58%, respectively, across the tested dose range, with these values being below those in control cells ($P < 0.01$).

A loss of mitochondrial membrane potential is a common feature of cellular apoptosis and has been detected in response to treatment with certain compounds (Yan et al. 2015). For example, in *Saccharomyces cerevisiae* the trypanocidal compound 2,5-bis(4-amidinophenyl) furan was shown to significantly reduce mitochondrial membrane potential upon treatment (Lanteri et al. 2004). In the present study, we similarly found that SBT oil markedly reduced $\Delta\psi m$ values in *A. flavus* cells (Figure 6), with this loss of potential coinciding with increasing intracellular ROS accumulation (Figure 5). ROS production within cells can result in the opening of transition pores within the mitochondrial membrane, thereby increasing its permeability and leading to depolarisation, $\Delta\psi m$ loss, and consequent apoptotic cell death. Indeed, excess ROS generation is a primary inducer of $\Delta\Psi m$ depolarisation in many apoptotic systems (Yan et al. 2015).

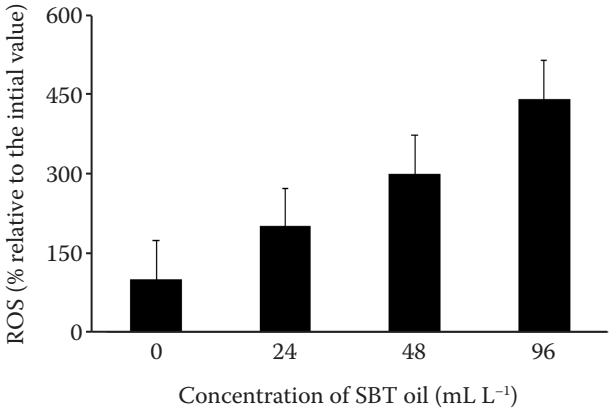

Figure 5. Effects of sea buckthorn (SBT) oil on oxygen species levels in *Aspergillus flavus* mitochondrion

ROS – reactive oxygen species

CONCLUSION

Together, our results suggest that SBT oil can disrupt mitochondrial structural and functional integrity in *A. flavus* cells. Specifically, we found that SBT oil impairs the activity of enzymes associated with mitochondrial functionality and the TCA cycle, reduces mitochondrial membrane potential, and induces increases in endocellular ROS and MDA levels. These findings represent an important step towards a com-

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prehensive understanding of the mechanisms whereby SBT oil can serve as an antifungal agent that can combat *A. flavus*.

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