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RESEARCH ARTICLE

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Antibacterial activity of Chaga mushroom (*Inonotus obliquus*) extract against *Staphylococcus aureus* and *Salmonella typhimurium*: An *In Vitro* Study

ABSTRACT:

Inonotus obliquus (Chaga mushroom) is available as a herbal and edible mushroom widely distributed in different regions, especially temperate regions. Chaga parasites on the trunk of angiosperms and grows, forming an irregular black mass. The medical importance of Chaga has been reported from ancient cultures to data due to its activity against a wide range of diseases. In the present study, the ethyl acetate extract of the Chaga mushroom has been tested against two bacterial pathogens, *Staphylococcus aureus* ATCC25923 and *Salmonella typhimurium* ATCC14028. The antimicrobial activity of the extract showed high activity with inhibition zone diameters of 25 mm for *S. aureus* and 28 mm for *S. typhimurium* and minimum inhibitory concentration (MICs) of 3.125 µg/ml for both bacterial pathogens. GC-MS analysis of the extract revealed several compounds with seven major compounds; they are 3-pentanone, propyl butyrate, Ethyl propionate, Trimethylsilylmethanol, Neopentyl glycol, n-butyl acetate, and 1-butanol-3-methyl acetate. The antibacterial mode of action of Chaga extract was also elucidated by studying the effect of the extract on cell membrane permeability and recoded high effect on bacterial cell membrane permeability, which was confirmed by transmission electron microscope (TEM) images that revealed leakage of bacterial cell content outside the cells and death.

KEY WORDS:

Inonotus obliquus extract, Antibacterial activity, *Staphylococcus aureus*, *Salmonella typhimurium*, GC-MS, Cell membrane permeability, TEM.

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INTRODUCTION:

Inonotus obliquus "Chaga mushroom" is a terrestrial fungus that belongs to family Hymenochaetaceae and is widely distributed in different regions, especially in temperate and frigid regions, including Central Europe, North-East of Asia, and North America (Zhong *et al.*, 2009). On the bark of various angiosperms such as *Betula* spp. (birch) and *Fagus* spp. (Beech) Chaga parasites themselves develop shapeless black masses (Lee *et al.*, 2008). In many countries of Northern and Eastern Europe/Asia, this fungus has been used as a folk medicine for the treatment of different diseases such as liver-heart diseases, joint pains, dermatomycoses, different kinds of cancers, stomach diseases, and intestinal worms (Saar, 1991, Babitskaya *et al.*, 2002, Shashkina *et al.*, 2006, Lemieszek *et al.*, 2011, Shikov *et al.*, 2014, Koyama, 2017).

Chaga's scientific name is *Inonotus obliquus*, but other names have also been sporadically used, such as *Polyporus obliquus*, *Fuscoporia oblique,* or *Phaeoporus obliquus* (Reid, 1976, He *et al.*, 2001). Numerous studies have claimed several bioactivities of Chaga, including antimicrobial, antioxidant, anti-inflammatory, anticancer, and immunoregulatory (Shashkina *et al.*, 2006; Koyama *et al.*, 2008; Zhong *et al.*, 2009, Patel, 2015).

Multidrug resistance bacteria (MDR), have the ability to exhibit resistance against different antibiotics "Antibiotic resistance", which represent the main challenge in the infectious diseases treatment and leading to different clinical outcomes with increasing in mortality (Karaman *et al.*, 2017). In 2019, antibiotic resistance is listed as one of the top ten global health problems by the World Health Organization (WHO, 2018). Antibiotic resistance is responsible for a huge number of deaths every year in Europe, about 25000 deaths/year (Antibiotic resistance: Implication for global health and novel intervention strategies: workshop summary (Choffnes *et al.,* 2010). Also, it is responsible for large economic losses due to high productivity of new antibiotics, these losses reach to 1.5 billion euro per year. This challenge leads to use another source of antibiotics such as that extracted from natural sources.

Staphylococcus aureus is one of the most widespread and infamous bacterial pathogens; it causes many uncomplicated infections in the skin and invasive infections (Klevens *et al.*, 2007; Rasigade *et al.*, 2014). It is a main causative agent in many respiratory tract infections, such as Pneumonia, and is involved in cardiovascular infections, surgical sites, and nosocomial bacteremia (Tong *et al.*, 2015). Every year from 20 to 50 cases/100,000 infected by *S. aureus* bacteremia, 10 % to 30 % of these cases will die (van Hal *et al.*, 2012). In the U.S., the death caused by *S. aureus* infection was reported to be 20,000 (Kourtis *et al.*, 2019). More patients died due to infection with *S. aureus* than that caused by tuberculosis, viral hepatitis, and acquired immune deficiency syndrome (AIDS) (Klevens *et al.*, 2007; van Hal *et al.*, 2012). Other infections of *S. aureus*, such as severe skin infections including abscesses, infections of wound, and furuncles, do not represent a menacing of life but may be caused considerable pain (McCaig *et al.* 2006).

Salmonella typhimurium has many hosts, as humans cause gastroenteritis (Hohmann, 2001). The colonization of *S. typhimurium* in the intestinal tract depended on the inflammatory response (Stecher *et al.*, 2007; Winter *et al.*, 2010). The normal flora of the intestinal tract limits colonization and infection by bacterial pathogens (Buffie and Pamer, 2013; Olsan *et al.*, 2017) through different mechanisms. The intestinal inflammation causes dysbiosis that breaks the colonization barrier (Lupp *et al.*, 2007; Stecher *et al.*, 2007; Zeng *et al.*, 2017). Moreover, intestinal inflammation leads to changes in nutrient availability that are not present in the non-inflamed gut (Akira *et al.*, 2006; Stecher *et al.*, 2007). Thus, intestinal inflammation stimulation helps *S. typhimurim* to compete with the normal microbiota and secure sources necessary for their metabolism and its replication in the gut (Stecher *et al.*, 2007; Santos *et al.*, 2009; Winter *et al.*, 2010; Thiennimitr *et al.*, 2011). *S. typhimurim* is acquired by consuming contaminated water or food (Ohl and Miller, 2001). However, stomach acidity represents a good barrier against this bacterial pathogen (Giannella *et al.*, 1972), but consuming contaminated food in a large inoculum may result in infection (Galan, 2021). After consuming contaminated food, *S.typhimurim* enter the intestinal tract till it reaches the large intestine, where its replication occurs (Galan, 2021).

In the present study, Chaga mushroom extract was tested against two bacterial pathogens, *Staphylococcus aureus* and *Salmonella Typhimurium,* and showed promising results in inhibiting these pathogens. GC-MS analysis of Chaga mushroom extract determined the major compounds that cause antimicrobial activity. The antibacterial mode of action of the section on cell membrane permeability was elucidated and confirmed by transmission electron microscope (TEM) images.

MATERIAL AND METHODS:

Extraction of the biologically active metabolites from chaga mushroom:

First, twenty grams of powder of chaga mushroom (was purchased from Chi Chaga Foods, Brownsburg, QC, Canada) was mixed with 400 ml of ethyl acetate, then incubated overnight at 50^{o}c and 200 rpm. After the incubation, the ethyl acetate extracted solution was separated from the powder by centrifugation. This step was repeated trice with the same method to increase the yield of the extracted active compounds (Nguyen *et al*., 2023). Finally, the supernatants resulting from centrifugation were collected and evaporated using a vacuum rotary evaporator to obtain the crude extract used in further experiments.

Assay of the extract's antibacterial activity:

The extract of the mushroom *I. obliquus* was tested to evaluate its antibacterial activities by the disc-diffusion method, The assay was carried out according to Clinical and Laboratory Standards Institute (CLSI). Two strains were studied, including one Gram-positive bacteria (*Staphylococcus aureus* ATCC25923) and one Gram-negative bacteria (*Salmonella typhimurium* ATCC14028). The bacterial strains' purity was tested by culturing the strains on nutrient agar medium at 37^{o}C for 24 hrs, then preserved on nutrient agar slants.

One mg of the chaga mushroom crude extract was dissolved in 5 ml DMSO; the paper

discs were then loaded with 5 µl of the extract. After that, the discs were placed upon Müller-Hinton (M.H.) plates inoculated with 0.5 McFarland standard of the tested bacterial pathogens. Plates were incubated at 37°C for 24 hrs, then the inhibition zone (mm) diameters were recorded. Positive control was treated with chloramphenicol. Negative control was treated with DMSO only. Minimum inhibitory concentration (MIC) was carried out in Elisa plate with starting concentration of 100 µg/ml and diluted in bifold dilution; Chloramphenicol was used as a positive control.

GC-MS analysis of chaga crude extract:

Active metabolites of chaga mushrooms were collected using ethyl acetate as an organic solvent. One milliliter of the dissolved chaga crude extract was injected in GC-MS-MS Triple Quad Agilent (7000). The analysis occurred using a G.C. (Agilent Technologies 7890A) interfaced with a mass-selective detector (MSD, Agilent 7000) equipped with a polar Agilent HP-5ms (5 % phenyl methyl polysiloxane) capillary column (30 m × 0.25 mm i.d. and 0.25 µm film thickness). The carrier gas was helium with a linear velocity of 1 ml/min and column temperature of 38°c. The compound's identification depended on its retention time and mass spectra compared with those of the authentic compounds reported in the literature.

Effect of Chaga extract on the permeability of cell membrane of *Staphylococcus aureus* & *Salmonella typhimurium*:

To assess the impact of Chaga extract on cell membrane permeability, the release of UV-absorbing substances from the cells was measured by recording the optical density at 260 nm using a UV spectrophotometer. Measurements were taken at various incubation intervals, ranging from 30 to 120 minutes, based on the methods of Mohamed *et al.* (2018) with slight modifications. The bacterial cultures were inoculated in M.H. broth and incubated at 37°C to obtain a suspension of 10^6 CFU/ml, then the bacterial culture (5 ml) was mixed with Chaga extract to reach a final concentration of 1/2 MIC of each bacterial strain used, then incubated at 37°C. Two negative controls and 1 positive control were used, sterile medium was used as a blank (first negative control), bacteria mixed with 1% DMSO (C_D) (second negative control), bacteria mixed with Chloramphenicol (C_{Ab}) with a final concentration of 1/2 MIC of each bacterial strain (positive control). Finally, samples from each control were treated at 0, 30, 60, 90, and 120 min, filtered with a 0.22 µm syringe filter, then measured at 260 nm. The experiments and all measurements were repeated thrice.

Transmission Electron Microscope (TEM):

At the candidate magnification, ultra-thin sections of bacterial pellets were examined by transmission electron microscope JEOL (JEM-1400 TEM). CCD camera model AMT took images of samples with 1632 × 1632 pixels. This camera uses a 1394 firewire board for acquisition. Samples were cut into slice tissue of 1 mm slices, then fixed in glutaraldehyde and osmium tetroxide, dehydrated in alcohol, and embedded in an epoxy resin. Sections were prepared by Leica Ultracut UCT ultramicrotome at approximately 500-1000 µm thickness, stained by toluidine blue (1X), then examined by camera Lica ICC50 HD.

Statistical analysis:

The present statistical analyses were executed using Statistical Package for Social Science (SPSS) software version 22. According to Kolmogorov–Smirnov test, data were normally distributed. Independent t-test was applied to illustrate the statistical differences in the studied parameters in each experimental group, as compared to the controls and nicotine-treated groups. $P<0.05$ represents significant differences. Data were displayed as mean ± standard error of the mean.

RESULTS AND DISCUSSION:

Antibacterial activity of chaga mushroom extract:

Antibacterial metabolites extracted from chaga mushrooms represent promising natural resources due to the large instance of novel antibacterial drugs with novel structures and mechanisms of action to compete for the resistance of bacteria against known antibiotics.

The results indicated that chaga mushroom extract exhibited high antibacterial activity against *S. aureus* and *S. typhimurium* with inhibition zone diameters of 25 ± 0.58 and 28 ± 0.567 mm, respectively, when compared with chloramphenicol antibiotic (control) that showed inhibition zone diameter of 19 ± 0.58 mm in case of *S. aureus* and 16±0.56 mm with *S. typhimurium*. Negative control was DMSO showed inhibition zone diameter of 6 ± 0.0 mm in case of both bacterial pathogens.

The MIC results revealed that the chaga mushroom extract recorded a maximum activity in inhibiting the growth of *S. aureus* at a low concentration of 3.125 µg/ml, where the MIC value for chloramphenicol (positive control) was 15.6 µg/ml. In the case of *S. typhimurium*, the MIC value was 3.125 µg/ml, and the MIC value of chloramphenicol was 3.9 µg/ml.

Milyuhina *et al.* (2022) reported that the methanol and ethanol extracts of chaga mushroom (*Inonotus obliquus*) had significantly increased the inhibition zones against *S. aureus* (22 ± 0.2), *V. parahaemolyticus* (20 ± 0.5), *Enterococcus* spp. (22 ± 0.2) and *Proteus* spp. (22 ± 0.2) when compared to the aqueous extract that

exhibited lower inhibition zones against the four strains of 12 ± 0.4, 12 ± 0.2, 13 ± 0.4, and 14 ± 0.4, respectively. These results indicated that the extraction by using organic solvents extracted large numbers of active metabolites such as flavonoids, terpenoids, glycosides, and carbohydrates than extraction by using an aqueous solvent, where water, in this case, doesn't extract a sufficient number of active metabolites necessary to inhibit the bacterial pathogen growth.

The antimicrobial activity of ethyl acetate extract of chaga tested against 4 pathogens (*Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa,* and *Candida albicans*) and showed high inhibition activity against pathogens (Upska *et al*., 2021).

GC-MS analysis of ethyl acetate extract from *Inonotus obliquus*:

The ethyl acetate extract of *Inonotus obliquus* was meticulously analyzed using GC-MS-MS to elucidate the diverse spectrum of bioactive mycochemicals. The analysis revealed 7 major compounds (3-pentanone, propyl butyrate, Ethyl propionate, Trimethylsilylmethanol, Neopentyl glycol, n-butyl acetate, and 1-butanol-3-methyl acetate) at different retention time. The first compound was 3-pentanone at RT 2.105 with the second largest area sum % of 10.47 (Fig. 1).

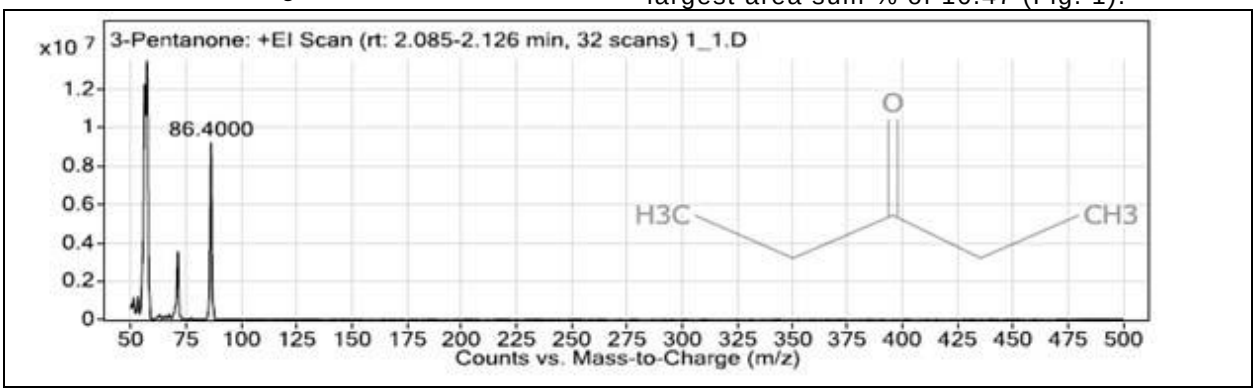

Fig. 1. GC-MS Chromatogram of the 3-pentanone compound.

The second one was propyl butyrate at RT 2.243, with the largest area sum % of 79.28 (Fig. 2).

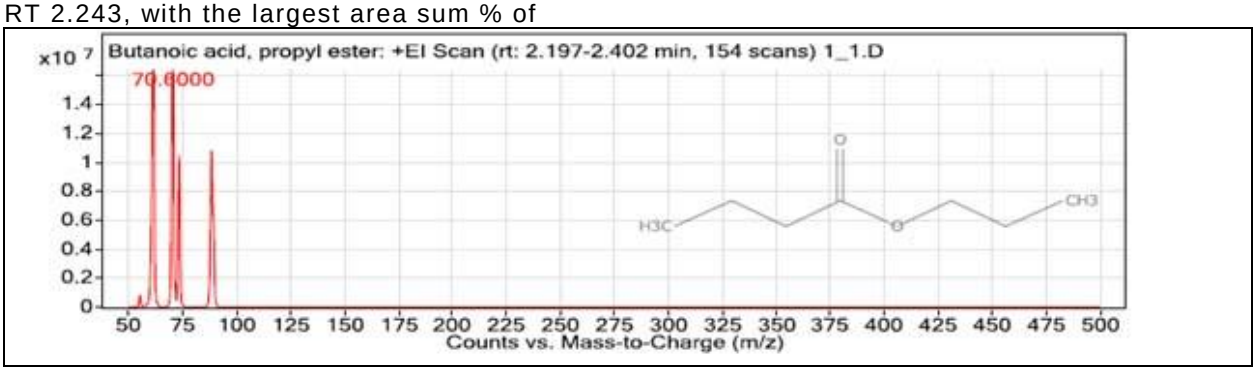

Fig. 2. GC-MS Chromatogram of propyl butyrate compound.

The third compound was Ethyl propionate which appeared at RT 3.377 and area sum % of 2.6 (Fig. 3).

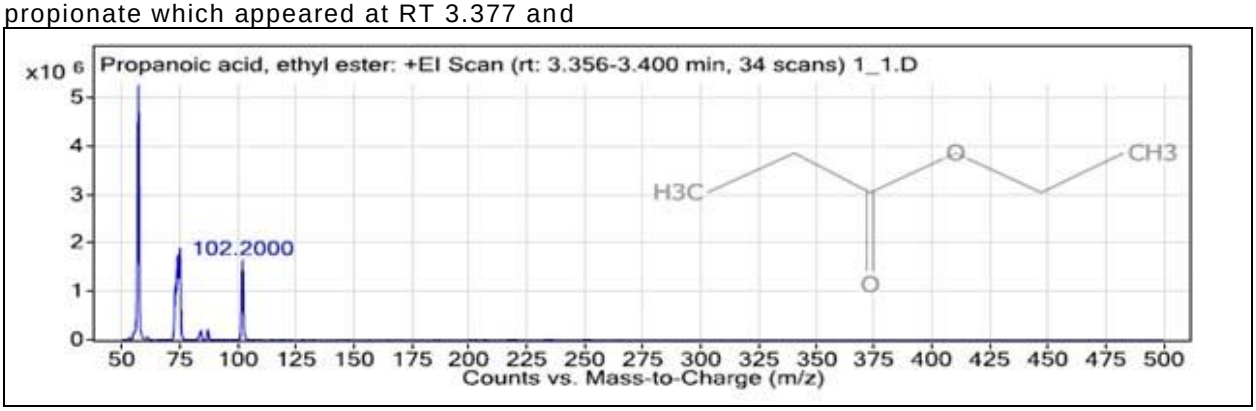

Fig. 3. GC-MS Chromatogram of Ethyl propionate compound.

The fourth compound was Trimethylsilylmethanol at RT 3.425 and area sum % of 2.61 (Fig. 4).

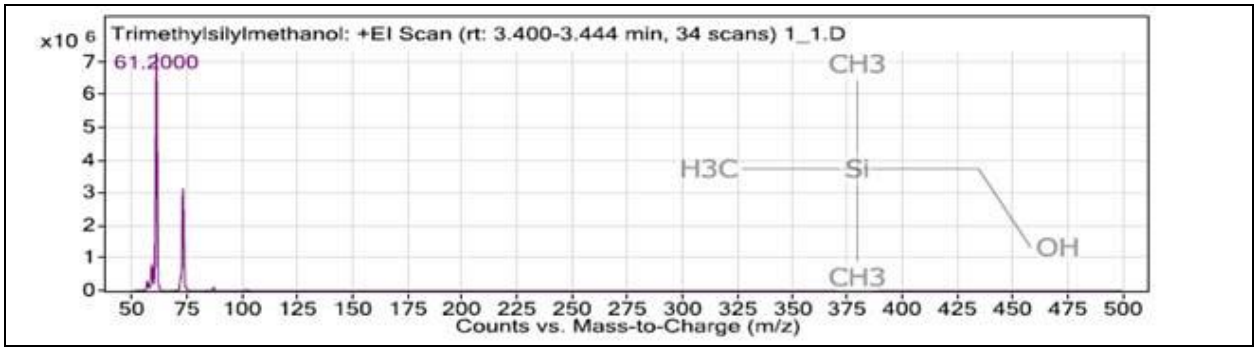

Fig. 4. GC-MS Chromatogram of Trimethylsilyl methanol compound.

The fifth compound was Neopentyl glycol at RT 4.632, with the least area sum % of 0.8 (Fig. 5).

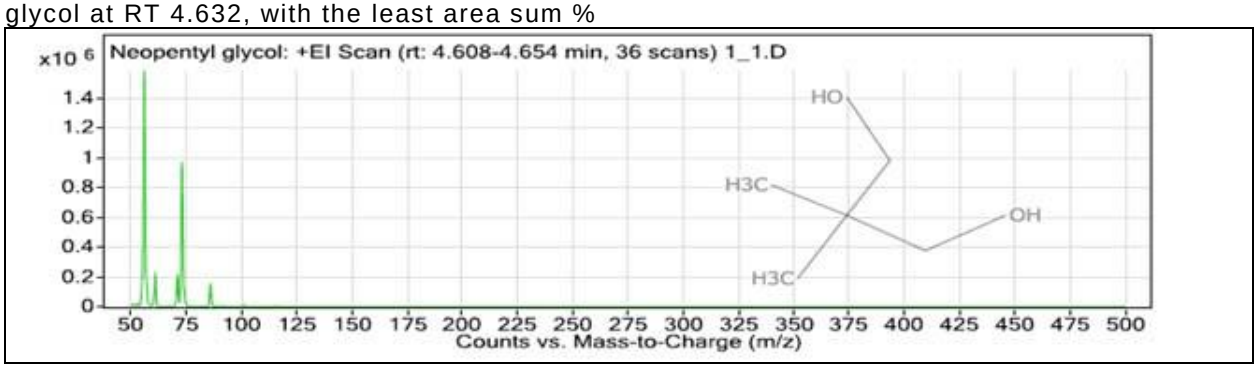

Fig. 5. GC-MS Chromatogram of Neopentyl glycol compound.

The sixth compound was n-butyl acetate at RT 5.686, an area sum % of 1.69 (Fig. 6).

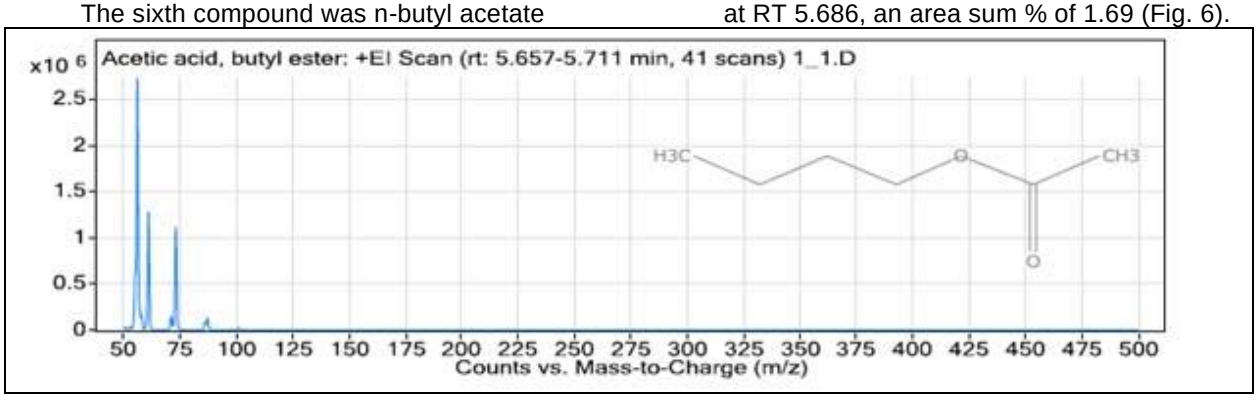

Fig. 6. GC-MS Chromatogram of n-butyl acetate compound.

The last compound was 1-butanol-3-methyl acetate at RT 7.641 and an area sum % of 1.11 (Fig. 7).

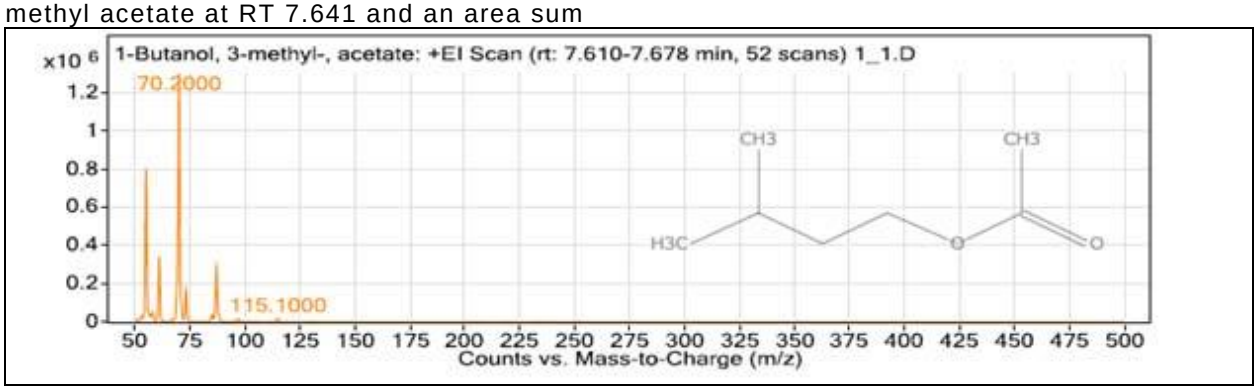

Fig. 7. GC-MS Chromatogram of 1-butanol-3-methyl acetate compound.

The present comprehensive GC-MS-MS analysis of the ethyl acetate extract from *Inonotus obliquus* identified seven major mycochemicals, consistent with other studies highlighting the rich diversity of bioactive compounds in this mushroom species (Chang *et al.*, 2022).

3-pentanone, identified with a 10.47% area sum at RT 2.105, has been previously reported as a volatile component of other

fungal species and has shown antimicrobial properties (Nishino *et al*., 2013). Although its role in *Inonotus obliquus* has not been explicitly defined, it is an interesting area for future research.

The most abundant compound we identified was propyl butyrate (79.28% area sum at RT 2.243). While this compound has been less extensively studied in mycological contexts, it has been associated with various appealing organoleptic properties in food science, suggesting the potential utility of *I. obliquus* extracts in food and beverage applications.

Ethyl propionate and Trimethylsilyl-methanol were detected at RT 3.377 and 3.425, respectively, each accounting for roughly 2.6% of the area sum. Ethyl propionate is known for its fruity smell and is used as a flavoring agent in food industries. Trimethylsilylmethanol, although less explored, is a commonly used sialylation agent in GC-MS.

The remaining compounds - Neopentyl glycol, n-butyl acetate, and 1-butanol-3-methyl acetate - were present in smaller proportions (0.8%, 1.69%, and 1.11%, respectively), but their identification underscores the chemical complexity and potential bioactivity of *I. obliquus* extracts. For example, n-butyl acetate and 1-butanol-3-methyl acetate are known for their characteristic fruity and floral notes and are used in food and cosmetics (Segal *et al.*, 2020; Liu *et al.*, 2022).

Th present results demonstrate the diverse chemical composition of Inonotus obliquus and establish a basis for future exploration of the biological properties and potential uses of these bioactive compounds.

Effect of chaga mushroom extract on cell membrane permeability of bacterial pathogens:

Chaga extracts demonstrating high antibacterial activity were further characterized to elucidate the mode of action of antibacterial activity. The results in figure 8a&b showed that UV-absorbing release materials were significantly increased after treatment of *S. typhimurium* and *S. aureus* with the chaga extract and chloramphenicol at 1/2 MIC concentration of both.

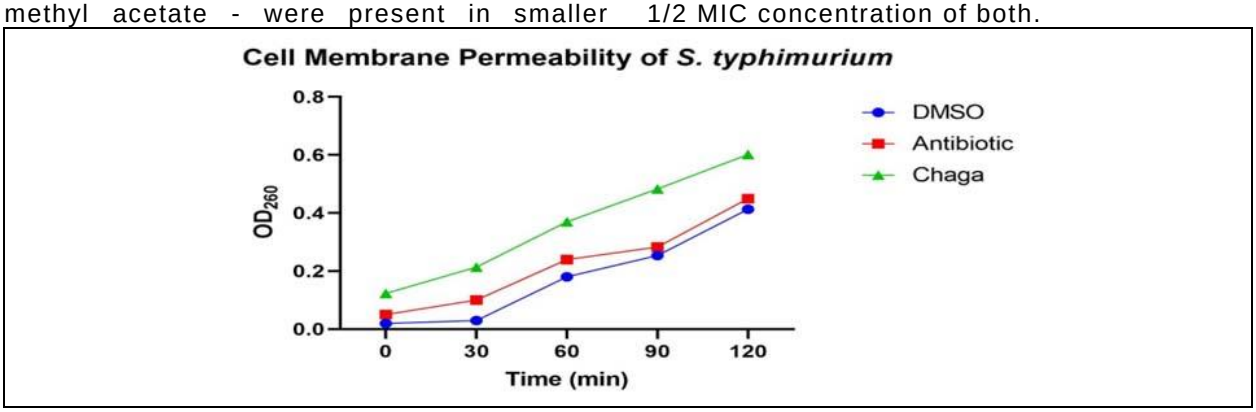

Fig. 8a. The effect of chaga mushroom extract on cell membrane permeability of *S. typhimurium.*

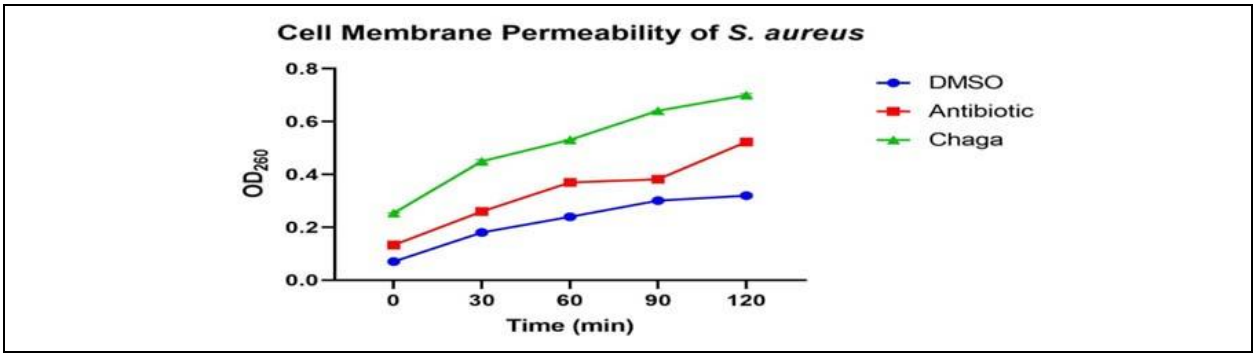

Fig. 8b. The effect of chaga mushroom extract on cell membrane permeability of *S. aureus.*

Compared with the positive control (containing bacteria with antibiotics), there was a two-fold increase approximately after 30 min of treatment in both bacterial pathogens. After 30 min of treatment, the $O.D_{260}$ values rapidly increased from 0.123 and 0.254 at 0 min to 0.214 and 0.45 and then increased gradually to reach 0.602 and 0.7 after 120 min of treatment for *S. typhimurium* and *S. aureus*, respectively. There was a significant difference between chaga mushroom extract-treated- and chloramphenicol-treated-bacterial pathogens.

Mohamed *et al.* (2018) by the same method, elucidating the antibacterial mode of action of oil derived from *Syzygium aromaticum* flower (clove) and reported an increase by twofold in the $O.D_{260}$ after the treatment at 10, 20, and 30 min. In contrast, after 40 min, a four-fold increase was

observed after the treatment of bacterial pathogens by the oil.

TEM analysis of the treated bacterial pathogens:

TEM images of bacteria treated with 1/2 MIC concentration of chaga extract showed shrinkage of the cell content in some cells and leakage of cell content in others, where the cells appeared empty from the cytoplasmic content (Figs 9-a & 10-a), as the extract affected cell membrane permeability causing loss of cell wall integrity followed by the releasing of cytoplasmic content and finally cell lysis occurred. In contrast, TEM images of control showed that cells appeared complete without any leakage of cytoplasmic content (Figs 9-b & 10-b).

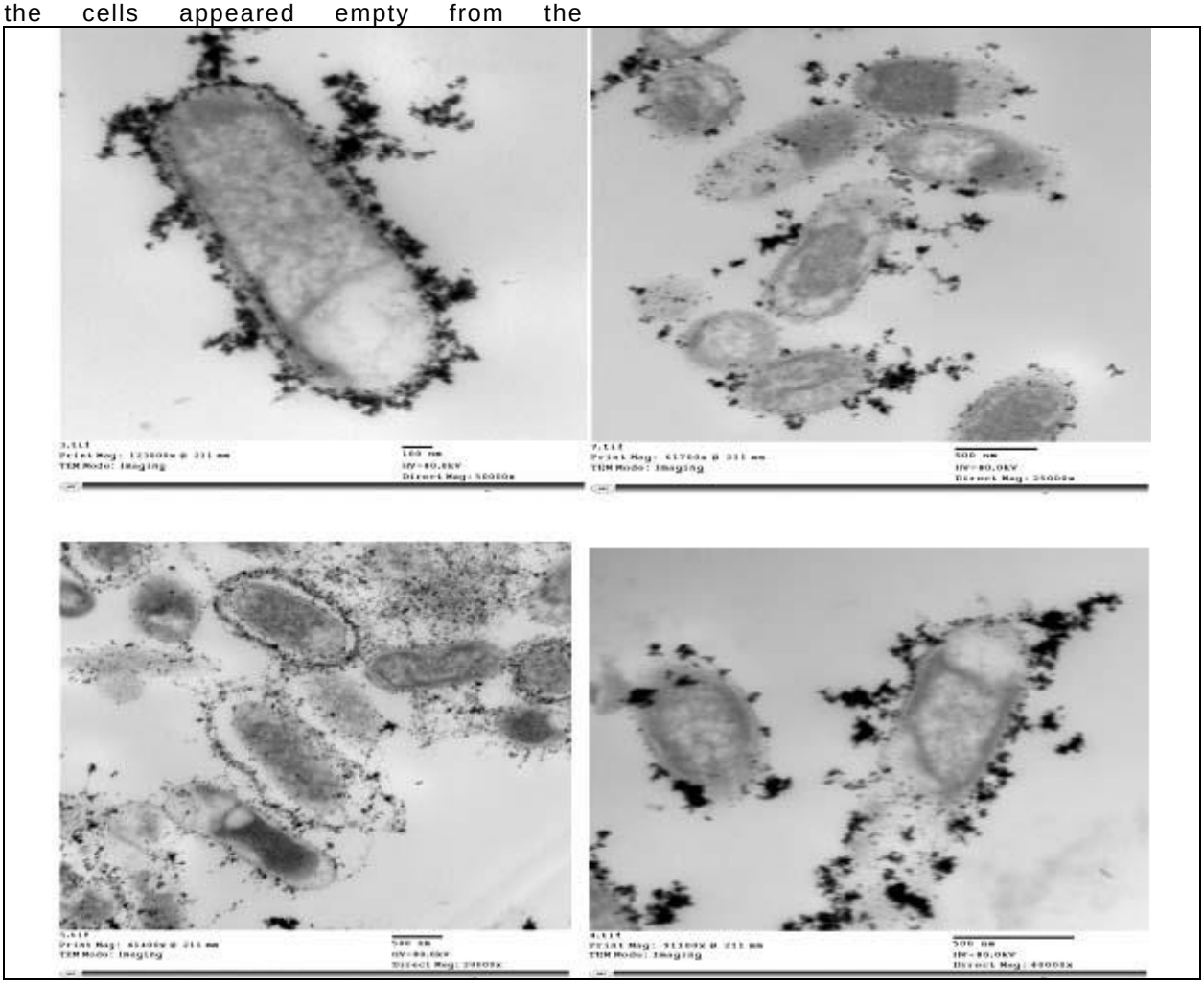

Fig. 9a. TEM images of *S. typhimurium* after treatment with chaga mushroom extract.

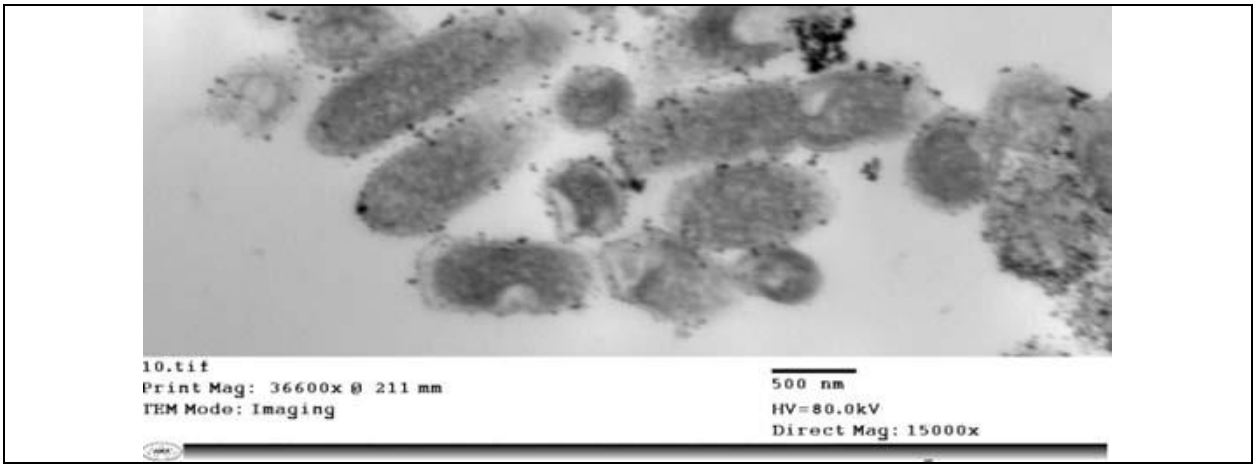

Fig. 9b. TEM images of *S. typhimurium* before treatment with chaga mushroom extract.

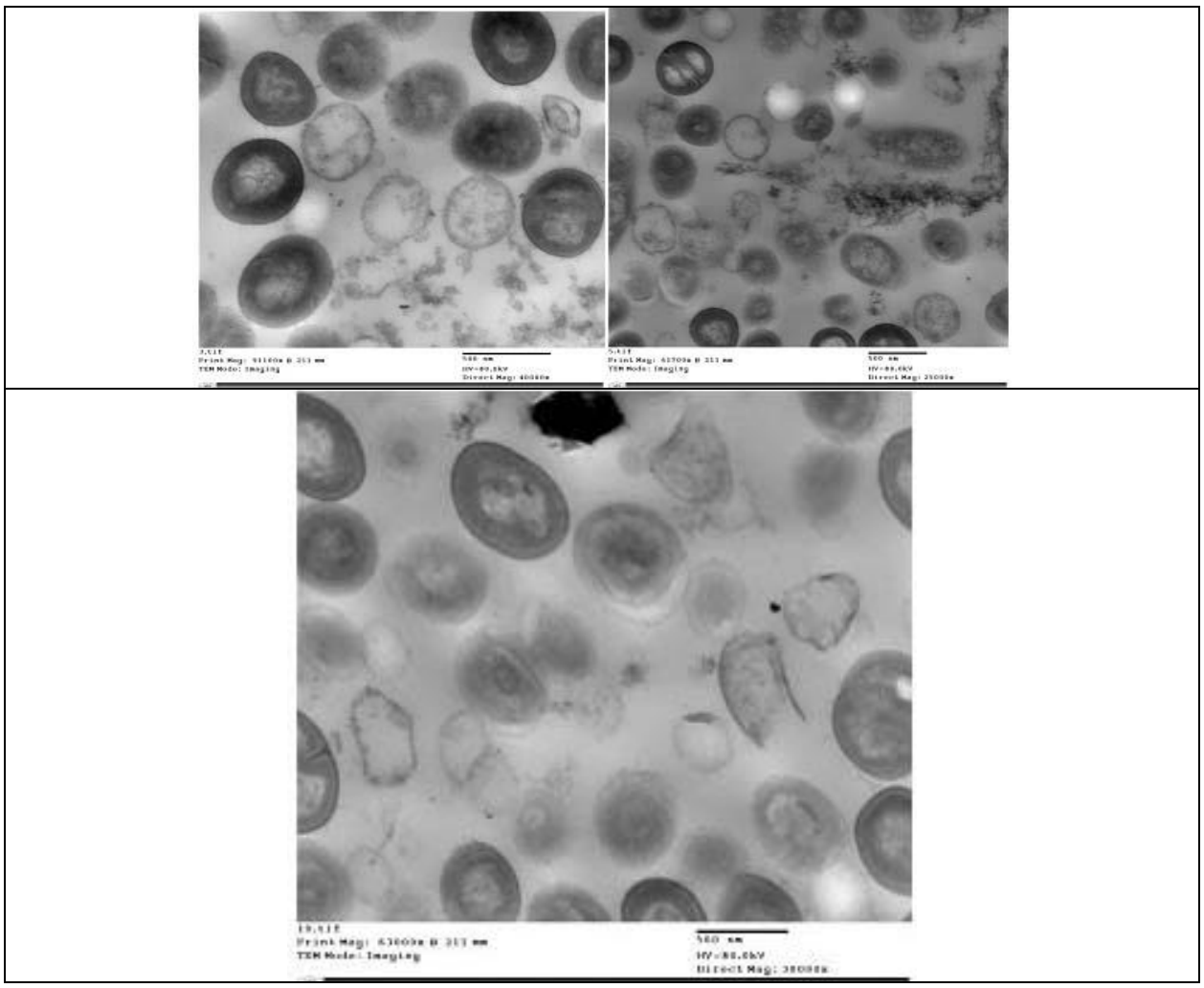

Fig. 10a. TEM images of *S. aureus* after treatment with chaga mushroom extract.

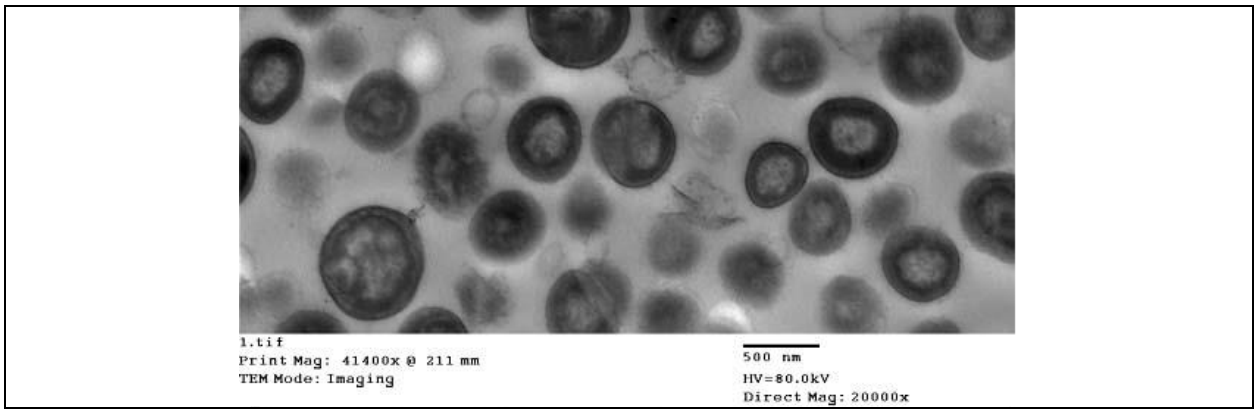

Fig. 10-b. TEM images of *S. aureus* before treatment with chaga mushroom extract.

TEM analysis confirmed the antibacterial activity of chaga mushroom extract, which causes cell lysis and death of bacterial pathogens. These results recommended the use of chaga extract as an alternative source for antibiotics extracted from natural source in competing antibiotic resistance of bacterial pathogens.

CONCLUSION:

This study's findings reveal the diverse mycochemical composition of the ethyl acetate extract of chaga mushroom (*Inonotus obliquus*), and its considerable antibacterial activity against two important bacterial pathogens. The mode of action of antibacterial activity was elucidated, and reported the effect of chaga extract on the cell membrane permeability of the bacterial pathogens was. As suggested by these results, the potential therapeutic advantages of the ethyl acetate extract of chaga mushroom (*Inonotus obliquus*) underline the importance of continued research into its bioactive compounds and other mechanisms of action. Such studies could further clarify

the potential applications of chaga extract in antibacterial therapies.

In summary, the present findings substantiate the potent bioactivity of the ethyl acetate extract of chaga mushroom (*Inonotus obliquus*), highlighting its potential as a source of novel antibacterial agents. Future investigations could help elucidate the other mechanisms underlying these bioactivities and potentially contribute to developing new therapeutic strategies based on *I. obliquus* bioactive compounds.

Declarations:

Ethics approval and consent to participate:

Not applicable.

Consent for publication:

All authors agree to submit this manuscript for publication.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interest:

Authors have no competing interest to declare.

Funding:

Not applicable.

Author's contributions:

The manuscript conceptualization, SMSA; methodology, MMA and SMSA; data analysis, MMA and SMSA; writing the original draft preparation, SMSA; the manuscript review and editing, MMA and SMSA. All authors have read and agreed to the published version of the manuscript.

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النشاط المضاد للبكتيريا لمستخلص فطر الشاجا (*Inonotus obliquus*) ضد المكورات العنقودية والسالمونيلا تيفيموريوم : دراسة في المختبر

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فطر الشاجا (*Inonotus obliquus*) هو فطر عشبي صالح للأكل منتشر في نطاق واسع في مناطق مختلفة، وخاصة المناطق المعتدلة. تتطفل الشاجا على جذع كاسيات البذور وتنمو لتشكل كتلة سوداء غير منتظمة، تم الإبلاغ عن الأهمية الطبية لفطر الشاجا بسبب نشاطها ضد مجموعة واسعة من الامراض. في هذه الدراسة، تم اختيار مستخلص اسيتات الإيثيل من فطر الشاجا ضد إثنين من مسببات الأمراض البكتيرية، المكورات العنقودية الذهبية ATCC25923 والسلمونيلا تيفيموريوم ATCC14028 . أظهر النشاط المضاد للميكروبات للمستخلص نشاطا عاليا حيث بلغ قطر منطقة التثبيط 25ملم لبكتيريا *S. aureus* و28 ملم لبكتيريا *S. typhimurium* وأقل تركيز مثبط (MICs) قدره 3.125ميكروجرام/مل لكلا البكتيريتين الممرضة. كشف تحليل GC-MS للمستخلص عن عدة مركبات تحتوي على سبعة مركبات رئيسية ; هم 3-بنتانون، بروبيل بوتيرات, بروبيونات الإيثيل ، تريميثيل سيليميثانول ، نيوبنتيل جلايكول، اسيتات ن-بوتيل و1-بيوتانول3-اسيتات ميثيل. تم أيضا توضيح طريقة عمل مستخلص الشاجا المضاد للبكتيريا من خلال دراسة تأثير المستخلص على نفاذية الخلية وإعادة ترميز التأثير العالي على نفاذية غشاء الخلية البكتيرية، وهو ما تم تأكيده من خلال صور المجهر الإلكتروني النافذ (TEM)التي كشفت عن تسريب محتوى الخلايا البكتيرية خارج الخلايا والموت.