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Original Article

Investigation of Antimicrobial and Antioxidant Activity of *Tephromela atra* Lichen and its Chemical Isolates, α-Alectoronic Acid and α-Collatolic Acid

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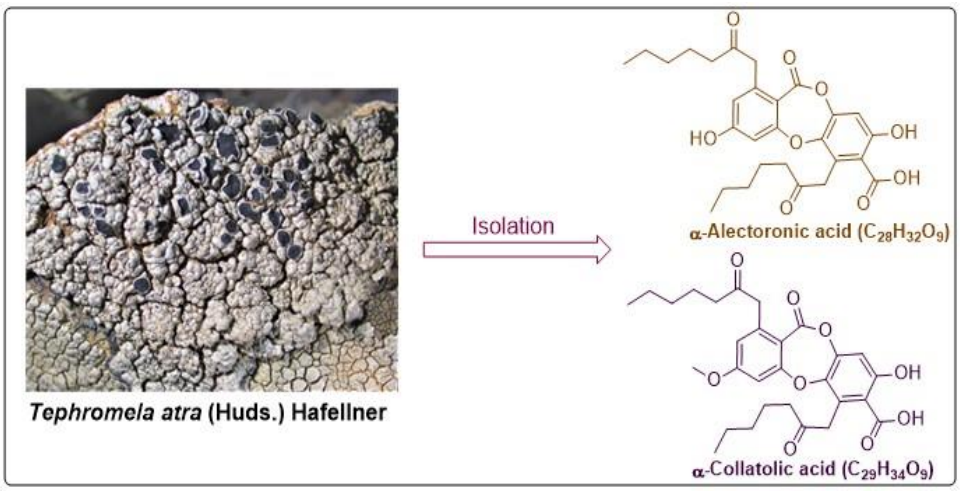
ABSTRACT

The lichens and their secondary metabolites have special interest since they can be used as an alternative to synthetic drugs in the treatment of various diseases. The present study was carried out to investigate *in vitro* antioxidant and antimicrobial activities of acetone, dichloromethane, petroleum ether, and methanol extracts of the lichens *Tephromela atra* and its major metabolites. α-alectoronic acid and α-collatolic acid were identified as bioactive substances in the lichens *Tephromela atra*. The antibacterial activity was investigated using disk diffusion method and the minimal inhibitory concentration values were evaluated against eleven species of bacteria, nine species of fungi, and four species of yeasts using broth microdilution method. The antioxidant activity of the methanol extracts of *Tephromela atra* was measured based on the ability to capture the free radical by using DPPH method. α-Alectoronic acid and α-collatolic acid extracts showed significant inhibitory activity against most of the tested bacteria, especially the lowest MIC values of both acids for *Bacillus subtilis*. In addition, the antioxidant activity was performed *in vitro* by measuring the free radical scavenging capacity of extracts on DPPH. The methanol extracts showed significant antioxidant activity and high DPPH scavenging activity with all concentrations. The results offer a perspective that *Tephromela atra* extracts and the natural substances obtained can be effectively used as a source of antimicrobial and antioxidant agents in the field of pharmaceutical industries.



Keywords: Lichens, *Tephromela atra*, α-Alectoronic acid, α Collatolic acid, Antimicrobial, Antioxidant.

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INTRODUCTION

For ages, scientists have been interested in the use of new remedies of natural origin for the treatment of plant, animal, and human diseases. It is a known fact that the long-term use of synthetic drugs brings some side effects and, most importantly, the inappropriate use of antibiotics makes the microorganism resistant to the drugs. [1-3] The lichens and their secondary metabolites are also an important group that can be used as an alternative to synthetic drugs in the treatment of various diseases.[4] Lichens are a unique group formed as a result of the symbiotic relationship between photosynthetic organisms (cyanobacteria or algae) and fungi. There are about 20.000 taxa spread all over the world.[5] Lichens are used in industry and medicine for various purposes and in many ways. It is known that some lichen samples have been used in the production of food, paint, alcohol, and perfume for a long time.[6, 7] The use of lichens in the treatment of various infectious diseases, especially in the field of traditional medicine, dates back to ancient times.[8-12] Many studies have shown that lichen metabolites have antimicrobial, antioxidant, anticarcinogenic, anti-inflammatory, antiproliferative, and cytotoxic effects.[13-19]

The present study aims to evaluate the antioxidant capacity and determine the *in vitro* antibacterial and antifungal activities of the acetone, dichloromethane, petroleum ether, and methanol extracts of the lichen *Tephromela atra*, and its major constituents (α-alectoronic acid and α-collatolic acid). *Tephromela* genus has about 25-30 species and the most common species is *Tephromela atra*, all these species share diagnostic anatomical characters with the type species. *Tephromela atra* shows a worldwide distribution. However, numerous other species have been reported. *Tephromela atra* lichen has been recorded in most of the provinces of Turkey in the last thirty years and the first studies that recorded *Tephromela atra* from Bozdag Mountains in Eskisehir province belong to Özdemir, (1991). In the present study various identification keys, flora books, and monographs were used for species identification. The lichen samples used in this study which collected from an open area on the siliceous rock in Bozdag mountain at Eskisehir, are compatible with the literature and had the same morphological characters. It is a cosmopolitan species that develops on siliceous and less calcareous, nutrient-rich rocks and walls, rarely bark and timber. Although there are some studies on the listed compounds (α-alectoronic acid and α-collatolic acid), there is no study on the biological activity and phytochemical contents of *Tephromela atra*.[20-23]

MATERIAL AND METHODS

Collection of lichen material

The lichen *Tephromela atra* (Huds.) Hafellner (Figure 1) was collected from an open area on the siliceous rock in Bozdağ mountain in Eskişehir province in September 2018, and the lichen samples have been deposited and stored in the herbarium of Biology Department, Eskisehir Technical University.

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Figure 1 Image of *Tephromela atra*

Preparation of the lichen total extracts

A dry sample of *Tephromela atra* was first grounded, and 20 g portions of the sample were separately treated with solvents (100 mL) with different polarities including petroleum ether, dichloromethane, acetone, and methanol. The mixtures were first sonicated in ultrasound apparatus at 38 °C for 20 minutes and then left at room temperature for 24 hours overnight. After the mixtures were filtered off, the filtrates were concentrated and dried under reduced pressure to give dry total extracts. The dry extracts were kept at 4 °C.

Microorganisms and media

The antimicrobial activity of lichen extract was studied on the following bacteria, yeasts, and filamentous fungi: *Listeria monocytogenes* ATCC 19111, *Bacillus cereus* ATCC 10876, *Bacillus subtilis* NRRL NRS-744, *Micrococcus luteus* NRRL B-4375, *Enterobacter aerogenes* NRRL 3567, *Streptococcus faecalis* NRRL B-14617, *Escherichia coli* ATCC 25922, *Proteus vulgaris* NRRL B-123, *Klebsiella pneumoniae* ATCC 700603, *Yersinia enterocolitica* Y53, and *Staphylococcus aureus* ATCC 6538 bacteria. *Penicillium notatum*, *Penicillium expansum*, *Penicillium citrinum*, *Fusarium solani* ATCC 12820, *Fusarium moniliforme* NRRL 2374, *Aspergillus fumigatus* NRRL 113, *Aspergillus flavus* ATCC 9807, *Aspergillus niger* ATCC 6275, *Aspergillus ochraceus* filamentous fungi. *Candida albicans* ATCC 90028, *Candida krusei* ATCC 6258, *Candida globrata* ATCC 90030, *Candida parapsilosis* ATCC 22019 yeasts. The microorganisms were obtained from Microbiology Research Laboratory, Department of Biology, Faculty of Science, Eskisehir Technical University University. Cultures of each of the bacteria were maintained on Mueller Hinton Agar (MHA) medium because it was standard and the best medium to use for susceptibility testing by Kirby-Bauer disc diffusion method,[24, 25] and the cultures of fungi were maintained on Sabouraud Dextrose Agar (SDA).

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In vitro antimicrobial activity

The total extracts, that were obtained from the aforementioned solvents, were again taken into 100 mL of its precedent solvents and the mixtures were homogenized using a vortex mixer and sonicator. Then 50 blank sterile antibiotic discs (7 mm diameter) were soaked into the 1 mL of these solutions and homogenized by shaking. The solvents on antibiotic discs were evaporated in a vacuum oven with shaking. The amount of total extracts, concentrations of solutions, and the amount of absorbed total extract on each disc were listed in Table 1.

Table 1 The amount of total extracts (mg), concentrations of solutions (mg/mL), and the amount of absorbed total extract on each disc (µg/disk). Mean value of 3 experiments ± standard deviation (SD).

Solvent	**Amount of total extract** (mg)	**Concentration** (mg/mL)	**Final concentration** (µg/disk)
Acetone	207.0	2.07	41.4 ± 1.2
Dichloromethane	181.8	1.818	36.36 ± 0.8
Petroleum Ether	28.1	0.281	5.625 ± 0.07
Methanol	484.6	4.846	96.9 ± 1.6

The anti-microbial activity of extracts was determined by using the disk diffusion method. For the disk diffusion method, antimicrobial susceptibility was tested on MHA solid media in Petri dishes. The bacteria from tested microorganisms were activated by incubating in 10 mL of Mueller-Hinton Broth (MHB) medium at 30-37° C for 24–48 hours. Yeasts were activated by incubating at SD Broth medium at 37°C for 24–48 hours. The concentration of activated bacteria and yeasts was adjusted according to McFarland No: 0.5 (10^8 CFU/mL). Filamentous fungi were developed on Potato Dextrose (PD) Agar for 10 days at 25 °C to obtain their spores, and 5 mL of sterile distilled water containing 0.1% Tween 80 was poured on the molds that developed. After waiting for 20-30 minutes, the spore suspension obtained was counted on the Thoma slide and the number of spores was determined and adjusted to 10^5 spores/mL.

The sensitivity of microorganisms to the different concentrations of acetone, dichloromethane, methanol, and petroleum ether extracts was tested by Kirby and Bauer disc diffusion method by measuring the zone of inhibition of the antibiotic disks (7 mm diameter) containing the extracts substance.[24, 25] The blank disc was used as a negative control on the plates. MH, SD, and PD agar were seeded with the suitable inoculum for bacteria, yeast, and filamentous fungi, respectively. Paper disks (7 mm) containing lichen extracts were positioned on the test plates using a pair of sterile forceps. Agar plates were cultivated for 24–48 h for bacteria at 37 °C and 5–7 days for filamentous fungi at 25 °C. Chloramphenicol and ketoconazole were used as controls for bacteria and fungi, respectively. Finally, the antimicrobial activities for different extracts were determined by observing the inhibitory zones around the disks.[26, 27] All experiments were performed in triplicate.

Isolation of α-alectoronic acid, and α-collatolic acid

The lichen (*Tephromela atra*) sample was first crushed, and then a 20 g portion of the dry sample was soaked into 250 mL of acetone. The mixture was placed in a sonicater for 30 min, then kept in dark at room temperature overnight. The mixture was filtered through a pad of silica (10 cm long) to remove physical dirt. The existing lichen acids in *Tephromela atra*

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extract were identified by comparing their R*f* values with reported values in A, C, and G eluent systems.[9, 28, 29] The major part of the solvent was evaporated under reduced pressure using a rotary evaporator. The lichen acids (Figure 2), α-alectoronic acid, and α-collatolic acid were isolated from the crude extract solution using preparative thin-layer chromatography (TLC) and a modified solvent G mix (toluene/ethyl acetate/formic acid (139: 83: 8 v/v/v)) as similarly described for the lichen, *Hypogymnia physodes* (L.) Nyl.[30] In a typical TLC separation, 50–100 mg of crude *Tephromela* extract was dissolved in acetone (10 mL) to prepare a stock solution. 2–3 mL of stock solution was applied onto preparative TLC plates (glass, Merck, 20x20 cm, and 0.25 mm thick silica-60 coated with F254, a fluorescent indicator). The crude-loaded TLC plates were developed twice using a modified solvent G system. The develoption and separation were monitored under UV light (254 nm) After satisfied develoption, the TLC plates were removed from the chamber. After the plates were dried, the lichen acid bands were separately scraped from the plates. The acid adsorbed silicas were extracted with acetone (2 x 50 mL). The silica part was removed with filtration. The solvents were evaporated. The residues were recrystallized over acetone to give pure products. The TLC separations were repeated until enough amount of compounds were obtained for antimicrobial tests. The characterization of α-alectoronic acid and α-collatolic acid was done based on High-Resolution Mass Spectroscopy (HRMS). HRMS analyses were performed with a Shimadzu LC-MS IT-TOF HRMS system. HRMS [ESI(+)-TOF] of α- alectoronic acid: *m/z* $[M + H]^+$ calcd. for $C_{28}H_{32}O_9$: 513.2119; found: 513.2117. HRMS [ESI(+)- TOF] of α-collatolic acid: *m/z* $[M + H]^+$ calcd. for $C_{29}H_{34}O_9$: 527.2276; found: 527.2273 (Supporting info.).

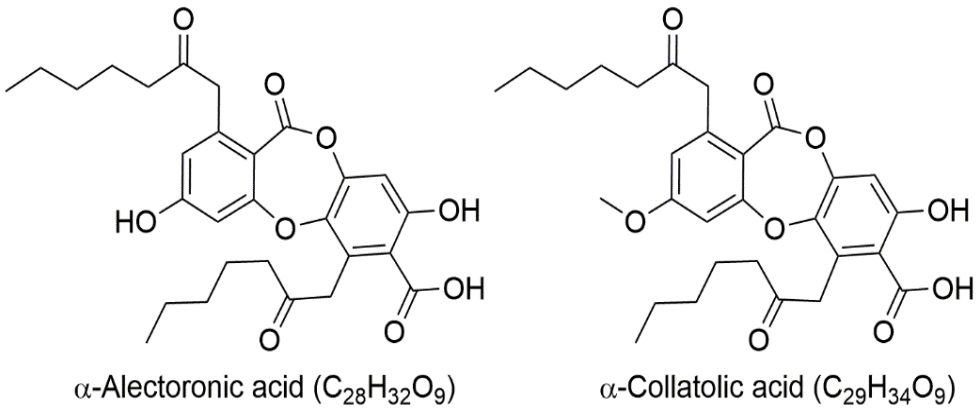

Figure 2 Chemical structures of α-alectoronic acid and α-collatolic acid.

Minimal inhibitory concentration (MIC) of α-alectoronic acid and α-collatolic acids

The minimal inhibitory concentrations of isolated lichen acids, α-alectoronic acids, and α- collatolic acids, were carried out using the broth micro-dilution method with 96-well microtiter plates. The concentrations of α-alectoronic acid and α-collatolic acid in the medium were adjusted in the range from 10 mg/mL and 19.5 µg/mL. Before the dilution process, lichen acids were dissolved in DMSO (20% in water) and their starting solutions were acquired first. The ten diluted test tubes were prepared with a two-fold serial dilution technique using MHB

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for bacterial cultures, SD broth, and PD broth. Then, 100 µl of each dilution plus 100 µL activated microorganism solution containing 10^8 cells/mL or fungal spore suspensions containing 10^5 spores/mL were transferred into microtitration plates and incubated for 24–48 h at 30–37 °C for bacteria/yeasts and at 28 °C for one week with daily observation for filamentous fungi. While the wells containing medium and microorganisms were used as the positive control group, the only medium-containing wells were used as a negative control. A third group was set up for control including ketoconazole for fungi and chloramphenicol for bacteria. Positive and negative results were evaluated according to turbidity that occurred after 24–48 h by comparing to the control wells. The lowest concentrations providing no observable growth of each microorganism were assigned as MIC. To determine the MIC values, 0.5% TTC (2,3,5-triphenyl tetrazolium chloride, Merck) aqueous solution was applied to indicate the lowest doses of concentration in which the microorganisms grew as the boundary dilution without color change.[31, 32]

Antioxidant activity by scavenging DPPH radicals

The antioxidant activity of the methanol extracts of *Tephromela atra* was measured based on the ability to capture the free radical by using 1,1-diphenyl-2-picryl-hydrazyl (DPPH).[16] The analysis was performed according to the reported method by Yu *et al.*, (2002).[33] First, 0.6 mM of DPPH in a methanol solution was prepared by dissolving of DPPH (0.024 g) into 100 mL of methanol. Then, 10 mL of this solution was diluted to 100 mL with methanol to prepare the final stock solution (0.06 mM) of DPPH, which was stored in dark at room temperature. Second, to prepare a sample solution of *Tephromela atra*, 5 mg methanol extract of *Tephromela atra* was dissolved into 100 mL methanol, which was lately diluted to prepare different concentrations ranging from 50 µg/mL and 1.56 µg/mL by the serial dilution method.[34] Third, for positive control, 250 µM ascorbic acid solution was prepared. 0.176 g of ascorbic acid was dissolved into 100 mL of water. Then, 500 µL of the concentrated solution was added to 500 µL of water. Finally, 50 µL of the last solution was added to 950 µL of water to prepare 250 µM of ascorbic acid as the final solution. A volume of 100 µL of each different concentration of *Tephromela atra* methanolic extract sample (1 mg/mL) was mixed with 200 µL of 0.06 mM methanolic solution of DPPH in a 96-well plate, the plate was incubated at room temperature in dark for 30 minutes, after that, the absorbance was measured at 517 nm on the spectrophotometer.[35] The measurements were repeated three times for each extract and the results were averaged. The free radical scavenger of the extract solutions is indicated as % inhibition of DPPH absorption. The calculation of the remaining DPPH was done as given in the following equation.

$$\text{DPPH scavenging effect (\% Inhibition)} = [(A_0 - A_1)/A_0)\ x\ 100]$$

A_0 = The absorption of the control sample.

A_1 = The absorption in the existence of all extract samples or references.

Statistical calculations were performed using the EXCEL and SPSS software package. One-way analysis of variance (ANOVA) was utilized to determine the statistical significance of extract antioxidant activity. All values are expressed as the mean value of three 3 experiments ± standard deviation (SD).

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RESULTS

The study includes the screening of the antibacterial and antifungal activity of *Tephromela atra* lichen acetone, dichloromethane, petroleum ether, and methanol extracts against eleven gram-positive and gram-negative bacteria, nine filamentous fungi, and four yeasts using the disk diffusion method (Table 2 and Table 3). The results in Table II indicate that the extracts of *Tephromela atra* have antibacterial activity against most of the tested microorganisms. The acetone and dichloromethane extracts are effective against all tested bacteria except for *K. pneumoniae*, while the petroleum ether extract showed activity against all tested bacteria except for *E. aerogenes*, *S. faecalis*, *K. pneumoniae*, and *Y. enterocolitica*. The methanol extract shows activity against all tested bacteria except for *E. aerogenes* and *Y. enterocolitica*. Except for the petroleum ether extract, other extracts show antibacterial activity against *S. faecalis* bacteria.

Table 2 Antibacterial activities of *Tephromela atra* extracts against different Gram-positive and Gram-negative bacteria according to the disk diffusion test. (+): show inhibitory against tested microorganism (-): show no inhibitory against tested microorganism. Mean value of 3 experiments ± standard deviation (SD).

Bacteria	**Acetone** (41.4 ± 1.2 µg/disk)	**Dichloromethane** (36.36 ± 0.8 µg/disk)	**Petroleum Ether** (5.625 ± 0.07 µg/disk)	**Methanol** (96.9 ± 1.6 µg/disk)
E. aerogenes	+	+	-	-
E. coli	+	+	+	+
P. vulgaris	+	+	+	+
K. pneumoniae	-	-	-	+
Y. enterocolitica	+	+	-	-
B. cereus	+	+	+	+
B. subtilis	+	+	+	+
L. monocytogenes	+	+	+	+
M. luteus	+	+	+	+
S. faecalis	+	+	-	+
S. aureus	+	+	+	+

The results in Table 3 show that all extracts have anti-yeast activity against *Candida krusei*. The petroleum ether extract shows antifungal activity against all tested filamentous fungi. On the other hand, the acetone extract has activity against *A. ochraceus*, *P. notatum*, *P. citrinum*, and methanol extract against *A. fumigatus*, *A. ochraceus*, *A. niger*, *and F. solani*. It is clearly seen that dichloromethane extract is effective on only bacteria rather than fungi, except for *C. krusei* yeast.

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Table 3 Antifungal activities of *Tephromela atra* extracts against different filamentous fungi and yeasts according to the disk diffusion test. (+): show inhibitory against tested microorganism (-): show no inhibitory against tested microorganism. Mean value of 3 experiments ± standard deviation (SD).

Microorganisms	**Acetone** (41.4 ± 1.2 µg/disk)	**Dichloromethane** (36.36 ± 0.8 µg/disk)	**Petroleum Ether** (5.625 ± 0.07 µg/disk)	**Methanol** (96.9 ± 1.6 µg/disk)
Yeasts				
C. globrata	-	-	-	-
C. parapsilosis	-	-	-	-
C. krusei	+	+	+	+
C. albicans	-	-	-	-
Filamentous fungi				
A. flavus	-	-	+	-
A. fumigatus	-	-	+	+
A. ochraceus	+	-	+	+
A. niger	-	-	+	+
F. moniliforme	-	-	+	-
F. solani	-	-	+	+
P. notatum	+	-	+	-
P. expansum	-	-	+	-
P. citrinum	+	-	+	-

Minimal Inhibitory Concentration values of α-alectoronic acid and α-collatolic acid (Table 4) are determined against the same test microorganisms using the broth microdilution method with 96-well microtiter plates.[25] The results indicate that α-alectoronic acid and α-collatolic acid of *Tephromela atra* have a significant effect against most of the microorganisms with different concentrations.

α-Collatolic acid exhibited equipotent activity to standard antibiotic chloramphenicol against *B. cereus* (MIC= 39 µg/mL) and *S. aureus* (MIC= 78.12 µg/mL). Moreover, it showed antibacterial activity against *B. subtilis* with half potency of chloramphenicol (MIC= 39 µg/mL). Comparing chloramphenicol, the highest antibacterial activity of α-collatolic acid was obtained against *Y. enterocolitica* (MIC= 39 µg/mL), while the lowest antibacterial activity was for *S. faecalis* (MIC= 1250 µg/mL).

α-alectoronic acid had equipotent activity to standard antibiotic chloramphenicol against *Y. enterocolitica*, *S. aureus* (MIC= 78.12 µg/mL) and *B. subtilis* (MIC= 19.5 µg/mL). The MIC value obtained against *E. areogenes* (MIC= 39 µg/mL), was lower than the chloramphenicol. However, it showed moderate antibacterial activity against *B. cereus*, *P. vulgaris* (MIC= 78.12 µg/mL), and *M. luteus* (MIC= 39 µg/mL) with a half potency of chloramphenicol. The lowest antibacterial activity of α-alectoronic acid was against *S. faecalis* (MIC= 312.5 µg/mL) similar to α-collatolic acid. As it can be seen from Table 4, no correlation has been observed between substances and activities of α- collatolic acid, and α-alectoronic acid. Table 5 contains the results of MIC values for α-alectoronic acid and α-collatolic acid extracts against filamentous fungi and yeasts.

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Table 4 MIC values (µg/mL) of α-alectoronic acid and α-collatolic acid of *Tephromela atra* against bacteria. Mean value of 3 experiments ± standard deviation (SD).

Microorganisms	**α-Collatolic acid**	**α-Alectoronic acid**	**Chloramphenicol**
Bacteria	**MIC (µg/mL)**	**MIC (µg/mL)**	**MIC (µg/mL)**
E. aerogenes	312.5 ± 2.1	39 ± 0.3	78.1 ± 0.6
E. coli	-	-	19.5 ± 0.2
P. vulgaris	156.2 ± 1	78.1 ± 0.6	39 ± 0.3
K. pneumoniae	-	-	78.1 ± 0.6
Y. enterocolitica	39 ± 0.3	78.1 ± 0.6	78.1 ± 0.6
B. cereus	39 ± 0.3	78.1 ± 0.6	39 ± 0.3
B. subtilis	39 ± 0.3	19.5 ± 0.2	19.5 ± 0.2
L. monocytogenes	-	-	39 ± 0.3
M. luteus	78,1 ± 0.6	39 ± 0.3	19.5 ± 0.2
S. faecalis	1250 ± 4.2	312.5 ± 2.1	39 ± 0.3
S. aureus	78.1 ± 0.6	78.1 ± 0.6	78.1 ± 0.6

Table 5 MIC values (µg/mL) of α-alectoronic acid and α-collatolic acid of *Tephromela atra* against fungi and yeasts. Mean value of 3 experiments ± standard deviation (SD).

Microorganisms	**α-alectoronic acid**	**α-collatolic acid**	**Ketoconazole**
Fungi	**MIC (µg/mL)**	**MIC (µg/mL)**	**MIC (µg/mL)**
F. moniliforme	-	2500 ± 17.1	39 ± 0.3
F. solani	-	-	78.12 ± 0.6
P. citrinum	2500 ± 17.1	156 ± 1	78.12 ± 0.6
P. expansum	-	-	78.12 ± 0.6
P. notatum	2500 ± 17.1	625 ± 7.3	39 ± 0.3
A. niger	2500 ± 17.1	2500 ± 17.1	78.12 ± 0.6
A. flavus	1250 ± 4.2	5000	78.12 ± 0.6
A. ochraceus	2500 ± 17.1	625 ± 7.3	78.12 ± 0.6
A. fumigatus	-	-	78.12 ± 0.6
Yeasts			
C. albicans	2500 ± 17.1	156 ± 1	78.12 ± 0.6
C. krusei	5000	625 ± 7.3	78.12 ± 0.6
C. globrata	-	1000 ± 3.9	39 ± 0.3
C. paropilopsis	156 ± 1	156 ± 1	78.12 ± 0.6

When the antifungal activity results were evaluated, it was clearly seen that the obtained MIC values were quite high compared to the standard antibiotic ketoconazole. α-collatolic acid had moderate antifungal activity against filamentous fungi *P. citrinum* (MIC=156 µg/mL) and *C. albicans*, *C. paropilopsis* (MIC= 156 µg/mL) yeasts with half potency of ketoconazole. α-alectoronic acid also showed moderate antifungal activity against *C. paropilopsis* (MIC= 156

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µg/mL) with half potency of ketoconazole. From Tables 4 and 5, it was understood that the antimicrobial effects of these lichen acids were higher against bacteria than fungi.

The antioxidant activity of methanol extracts was measured by applying the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, and the free radical scavenger of the extract solutions was indicated as % inhibition of DPPH absorption. The DPPH scavenging effect (% inhibition) is calculated according to Equation 1. The results in Table 6 showed the absorbance values and DPPH scavenging 100% of various concentrations (µg/mL) of *Tephromela atra* methanolic extract at 517 nm by UV visible spectrophotometer, and the percentage scavenging activity of *Tephromela atra* methanol extract compared to a standard (Ascorbic acid) and shown in the same table. The extracts showed a high level of absorptions and scavenging effect, with all concentrations more than 50% DPPH scavenging (% inhibition). Methanol extract of *Tephromela atra* showed very good scavenging activity, it was further tested to determine the IC50. The percentage scavenging activity of *Tephromela atra* methanol extract compared to a standard ascorbic acid is shown in Table 6. Ascorbic acid was used as the standard. A calibration curve in Figure 3 was constructed using ascorbic acid with a concentration of 50 µg/mL. The assay was carried out in triplicates. The IC50 value of the extract was determined using the SPSS software. Methanol extracts of the tested lichen showed good scavenging activity on the DPPH radical. There was a statistically significant difference between extracts and control ($P < 0.07$). The scavenging effects of all lichen extracts in different concentrations were 63.0 – 70.0 %. The concentration of 50 µg/mL extracts from *Tephromela atra* showed the largest DPPH radical scavenging activity (70%) which was similar to the standard antioxidants, ascorbic acid (86%). The scavenging activity was also good for the concentration of 25 µg/mL (68%). The concentration of 1.56 µg/mL showed a slightly weaker DPPH radical scavenging activity (63%).

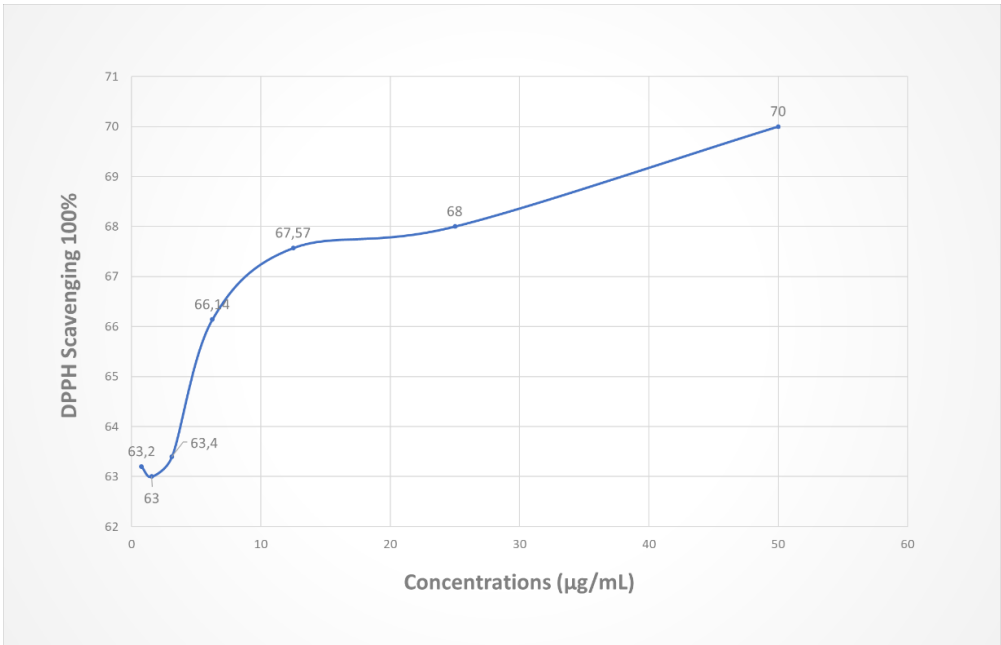

Figure 3 DPPH scavenging 100% to different concentrations (µg/mL) of *Tephromela atra* methanolic extract.

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Table 6 Absorbance and DPPH scavenging 100% of different concentrations (ug/mL) of *Tephromela atra* methanolic extract at 517 nm by UV visible spectrophotometer (DPPH scavenging assay).

Concentration µg/mL	Absorbance (517 nm)	DPPH Scavenging 100%
0.78	0.257	63.2
1.56	0.259	63.0
3.13	0.256	63.4
6.25	0.237	66.1
12.5	0.227	67.1
25.0	0.224	68.0
50.0	0.210	70.0
Ascorbic acid	0.700	86

DISCUSSION

Increasing the number of antibiotic-resistant microorganisms due to unconscious use reduces the effectiveness of existing antibiotics. Further screening and investigations of natural pharmaceutical raw materials will help to reduce drug-resistant problems. Unlike plant species, lichens as a symbiotic organism, synthesize a variety of chemical metabolites in large amounts. Lichens have also gained special importance since the lichen's acids and other metabolites are mostly unique and not found in other plant groups.[25] So, recent studies have been focused on lichen extracts, their metabolites, and their pharmacological uses. As a result, many lichen metabolites have been characterized and their biological activities are inspected. It is known that some of these metabolites are intrinsic antibiotics protecting lichen organisms from micropredators.[21,36-38] Huge amounts of research have been done in many countries about the antibiotic properties of lichen metabolites. Today, many lichen substances have been identified as antibiotics.[38] For example, it is determined that atranorin, gyrophoric acid, fumarprotocetraric acid, lecanoric acid, physodic acid, protocetraric acid, stictic acid, usnic acid, *etc*. have relatively strong antimicrobial effects against various bacteria and fungi, as plant, animal and human pathogens.[2, 38-40]

In this study, acetone, dichloromethane, petroleum ether, and methanol lichen extracts obtained from the lichen *Tephromela atra* were investigated for antibacterial and antifungal activity against a wide range of microorganisms using the disk diffusion method. Also, α-alectoronic acid and α- collatolic acid same lichen were isolated from the same lichen using TLC plate technique. The minimal inhibitory concentration (MIC) values of isolated metabolites against several microorganisms was determined using broth microdilution method with 96-well microtiter plates. The antioxidant efficiency of the methanolic extracts of the same lichen was analyzed by using the DPPH free radical method.

All of the extracts have shown activity against *E. coli, P. vulgaris, B. cereus, B. subtilis, L. monocytogenes, M. luteus, S. aureus*. Only methanolic extract is effective to *K. pneumonia* and also acetone and dichloromethane extracts are effective to *E. aerogenes* and *Y. enterocolitica.* On the other hand, the results show that the *Tephromela atra* petroleum ether extract has greater antifungal activity against most of the tested fungi than acetone, methanol, and dichloromethane extracts. The dichloromethane extract of *Tephromela atra* did not show a

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significant effect against most of the yeast and filamentous fungi examined in this study except against *C. krusei* ATCC 6258.

S. aureus has been listed on top as a dangerous pathogen that has been responsible for nosocomial and community-acquired infections for the last 30 years.[41-42] Vancomycin is the only option for the treatment of *S. aureus* infections, and it is the main reason for seeking new alternatives. In our study *S aureus* ATCC 6538 type was also used as a test microorganism. It is worthful to mention that all different extracts of *Tephromela atra* examined in our study show antagonist activity on *S. aureus*, which is clinically important, as seen in Tables 3 and 4. The *Tephromela atra* extracts, α-alectoronic acid, and α-collatolic, are examined against a wide range of microorganisms, and their minimum inhibition concentrations are determined. The results show that α-alectoronic acid and α-collatolic acid of *Tephromela atra* have a significant effect against all tested microorganisms in different concentrations. In light of the remarkable information of this study, it is determined that *Tephromela atra* extracts have impressive antibacterial activity against gram-positive bacteria and inhibit the growth of gram-negative bacteria. Also, all extracts have remarkable anti-yeast activity against *C krusei*. MIC values of α-alectoronic acid and α-collatolic acid are also significant in different concentrations against tested microorganisms, besides their radical-scavenging effect, and antioxidant activity.

The biological activities of α-alectoronic acid and α-collatolic acid, isolated from *Parmotrema* species, were recently reported by Rajan *et al.* (2016).[23] In their study, α-alectoronic acid and α- collatolic acid had antibacterial activity against *S. aureus* (MIC value 500 µg/mL) and *B. subtilis* (MIC value 125 µg/mL). The MIC values that we obtained were lower against *S. aureus* bacteria than in this study. And so, we expanded our study to include a wide range of fungi, and yeasts, besides bacteria. In a study by Piovano *et al.*, (2002),[20] the MIC value of α-collatolic acid was found above 250 µg/mL against *Candida albicans, Saccharomyces cerevisae, Cryptococcus neoformans, Aspergillus flavus, A. niger, Microsporum canis* C 112, *Microsporum gypseum C* 115, *Trichophyton mentagrophytes* ATCC 9972, *T.rubrum* C 113 and 12.5 µg/mL for *Staphylococcus aureus* (methicillin-sensitive), *Staphylococcus aureus* (methicillin-resistant). The results obtained from this study support the results of our study. In another study by Verma *et al.*, (2011),[22] some lichen compounds including α-collatolic acid and α-alectoronic acid isolated from different lichens (*C. ochrochlora*, *P. nilgherrensis*, and *P. sanctiangelii*) demonstrated moderate to strong bactericidal activity with low MIC values; α-alectoronic acid showed MIC 21.9 µg to 162.1 µg/mL and α-collatolic acid showed MIC value of 8.6–245 µg/mL against examined species. These values also supported our study.

The production of harmful free radicals which overtake the antioxidant defense capacity of the body results in the increase of oxidative stress. The production of these reactive oxygen species is partly due to environmental factors such as pollution, temperature, excessive light intensities, and nutritional limitations.[43] These highly reactive free radicals are known to be responsible for some human diseases like cancer and cardiovascular diseases.[44] Therefore there is a need for an external source of antioxidants to fight the impact of the reactive free radicals, and lichens have been shown to possess promising antioxidant activity.[45] In this study, the methanol extracts of *Tephromela atra* showed high percentage scavenging of DPPH free radicals, and the DPPH radical scavenging was compared to standard antioxidants (ascorbic acid), and the results showed that the methanol extracts of *Tephromela atra* had similar activity

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to standard antioxidants and possessed promising antioxidant activity, also the data from this study indicate that the extracts can be a potential lead for research and development of active agents against infectious diseases and free radical-induced oxidative stress. Further studies should be done to search for new compounds from lichens that exhibit strong antioxidant activity.

CONCLUSION

The antimicrobial effects of these important acids isolated from *Tephromela atra* show strong antimicrobial effects against a broad spectrum of microorganisms and *in vitro* antioxidant ability on DPPH. Our results give a perspective that *Tephromela atra* extracts and the isolated metabolites can be effectively used as a source of antimicrobial and antioxidant agents in the field of pharmaceutical industries. The bioactive metabolites from *Tephromela atra* can be alternative remedies to synthetic drugs in the pharmaceutical industry and are considered promising therapeutic agents from natural sources. By expanding this type of work, it will be possible to determine the biological activities of lichen species-metabolites and investigate their possible uses in medicine, pharmacology, and industrial areas.

MAIN POINTS

1. The present study determines the antibacterial and antifungal activities of the *Tephromela atra*, lichen extracts, and its isolated major constituents (α-alectoronic acid and α-collatolic acid), as well as evaluates the antioxidant capacity.
2. Although there are some studies on α-alectoronic acid and α collatolic acid, isolated in different lichens, there is no study on the biological activity and antioxidant capacity of lichen *Tephromela atra*.
3. The MIC values of isolates were evaluated against a broad range of microorganisms including eleven species of bacteria, nine species of fungi, and four species of yeasts.
4. α-Alectoronic and α-collatolic acid extracts showed significant inhibitory activity against most of the tested bacteria, especially the lowest MIC values of both acids for Bacillus subtilis.
5. Our results provide a future perspective that *Tephromela atra* extracts and the isolated metabolites might be effectively used as antimicrobial and antioxidant agents in pharmaceutical areas.

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Contributions: All authors participated in this study. All authors read and approved the final manuscript.

Ethics Declarations

Conflict of interest: The authors declare that they have no conflict of interest.

Consent for publication: All authors confirmed the publication of the manuscript.

Ethical approval: Our research does not need ethical approval.

Human and animal rights: This article does not contain any studies on human or animal subjects.

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Table 1 The amount of total extracts (mg), concentrations of solutions (mg/mL), and the amount of absorbed total extract on each disc (µg/disk). Mean value of 3 experiments ± standard deviation (SD).

Solvent	**Amount of total extract** (mg)	**Concentration** (mg/mL)	**Final concentration** (µg/disk)
Acetone	207.0	2.07	41.4 ± 1.2
Dichloromethane	181.8	1.818	36.36 ± 0.8
Petroleum Ether	28.1	0.281	5.625 ± 0.07
Methanol	484.6	4.846	96.9 ± 1.6

Table 2 Antibacterial activities of *Tephromela atra* extracts against different Gram-positive and Gram-negative bacteria according to the disk diffusion test. (+): show inhibitory against tested microorganism (-): show no inhibitory against tested microorganism. Mean value of 3 experiments ± standard deviation (SD).

Bacteria	**Acetone** (41.4 ± 1.2 µg/disk)	**Dichloromethane** (36.36 ± 0.8 µg/disk)	**Petroleum Ether** (5.625 ± 0.07 µg/disk)	**Methanol** (96.9 ± 1.6 µg/disk)
E. aerogenes	+	+	-	-
E. coli	+	+	+	+
P. vulgaris	+	+	+	+
K. pneumoniae	-	-	-	+
Y. enterocolitica	+	+	-	-
B. cereus	+	+	+	+
B. subtilis	+	+	+	+
L. monocytogenes	+	+	+	+
M. luteus	+	+	+	+
S. faecalis	+	+	-	+
S. aureus	+	+	+	+

Table 3 Antifungal activities of *Tephromela atra* extracts against different filamentous fungi and yeasts according to the disk diffusion test. (+): show inhibitory against tested microorganism (-): show no inhibitory against tested microorganism. Mean value of 3 experiments ± standard deviation (SD).

Microorganisms	**Acetone**	**Dichloromethane**	**Petroleum Ether**	**Methanol**
Yeasts	(41.4 ± 1.2 µg/disk)	(36.36 ± 0.8 µg/disk)	(5.625 ± 0.07 µg/disk)	(96.9 ± 1.6 µg/disk)
C. globrata	-	-	-	-
C. parapsilosis	-	-	-	-
C. krusei	+	+	+	+
C. albicans	-	-	-	-
Filamentous fungi				
A. flavus	-	-	+	-
A. fumigatus	-	-	+	+
A. ochraceus	+	-	+	+
A. niger	-	-	+	+
F. moniliforme	-	-	+	-
F. solani	-	-	+	+
P. notatum	+	-	+	-
P. expansum	-	-	+	-
P. citrinum	+	-	+	-

Table 4 MIC values (µg/mL) of α-alectoronic acid and α-collatolic acid of *Tephromela atra* against bacteria. Mean value of 3 experiments ± standard deviation (SD).

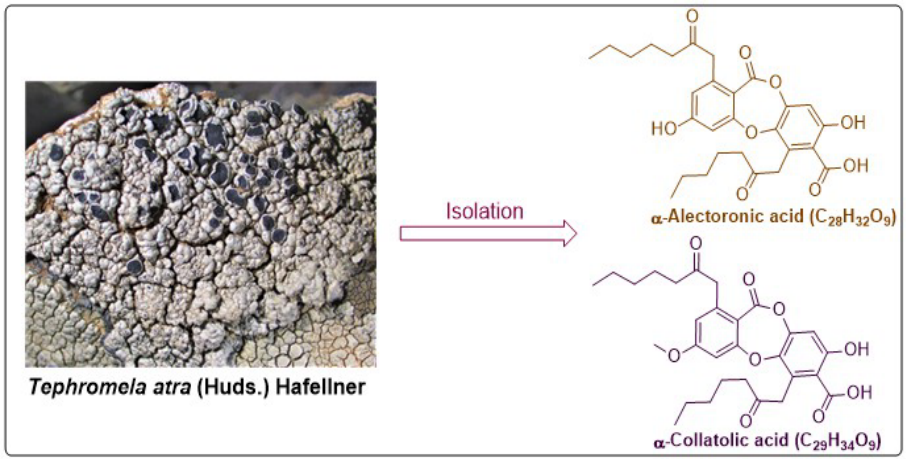
Microorganisms	**α-Collatolic acid**	**α-Alectoronic acid**	**Chloramphenicol**
Bacteria	**MIC (µg/mL)**	**MIC (µg/mL)**	**MIC (µg/mL)**
E. aerogenes	312.5 ± 2.1	39 ± 0.3	78.1 ± 0.6
E. coli	-	-	19.5 ± 0.2
P. vulgaris	156.2 ± 1	78.1 ± 0.6	39 ± 0.3
K. pneumoniae	-	-	78.1 ± 0.6
Y. enterocolitica	39 ± 0.3	78.1 ± 0.6	78.1 ± 0.6
B. cereus	39 ± 0.3	78.1 ± 0.6	39 ± 0.3
B. subtilis	39 ± 0.3	19.5 ± 0.2	19.5 ± 0.2
L. monocytogenes	-	-	39 ± 0.3
M. luteus	78,1 ± 0.6	39 ± 0.3	19.5 ± 0.2
S. faecalis	1250 ± 4.2	312.5 ± 2.1	39 ± 0.3
S. aureus	78.1 ± 0.6	78.1 ± 0.6	78.1 ± 0.6

Table 5 MIC values (μg/mL) of α-alectoronic acid and α-collatolic acid of *Tephromela atra* against fungi and yeasts. Mean value of 3 experiments ± standard deviation (SD).

Microorganisms	**α-alectoronic acid**	**α-collatolic acid**	**Ketoconazole**
Fungi	**MIC (μg/mL)**	**MIC (μg/mL)**	**MIC (μg/mL)**
F. moniliforme	-	2500 ± 17.1	39 ± 0.3
F. solani	-	-	78.12 ± 0.6
P. citrinum	2500 ± 17.1	156 ± 1	78.12 ± 0.6
P. expansum	-	-	78.12 ± 0.6
P. notatum	2500 ± 17.1	625 ± 7.3	39 ± 0.3
A. niger	2500 ± 17.1	2500 ± 17.1	78.12 ± 0.6
A. flavus	1250 ± 4.2	5000	78.12 ± 0.6
A. ochraceus	2500 ± 17.1	625 ± 7.3	78.12 ± 0.6
A. fumigatus	-	-	78.12 ± 0.6
Yeasts			
C. albicans	2500 ± 17.1	156 ± 1	78.12 ± 0.6
C. krusei	5000	625 ± 7.3	78.12 ± 0.6
C. globrata	-	1000 ± 3.9	39 ± 0.3
C. paropilopsis	156 ± 1	156 ± 1	78.12 ± 0.6

Table 6 Absorbance and DPPH scavenging 100% of different concentrations (ug/mL) of *Tephromela atra* methanolic extract at 517 nm by UV visible spectrophotometer (DPPH scavenging assay).

Concentration μg/mL	**Absorbance (517 nm)**	**DPPH Scavenging 100%**
0.78	0.257	63.2
1.56	0.259	63.0
3.13	0.256	63.4
6.25	0.237	66.1
12.5	0.227	67.1
25.0	0.224	68.0
50.0	0.210	70.0
Ascorbic acid	0.700	86



Graphical Abstract
181x88mm (300x300 DPI)

Figure 1 Image of *Tephromela atra*
357x278mm (118x118 DPI)

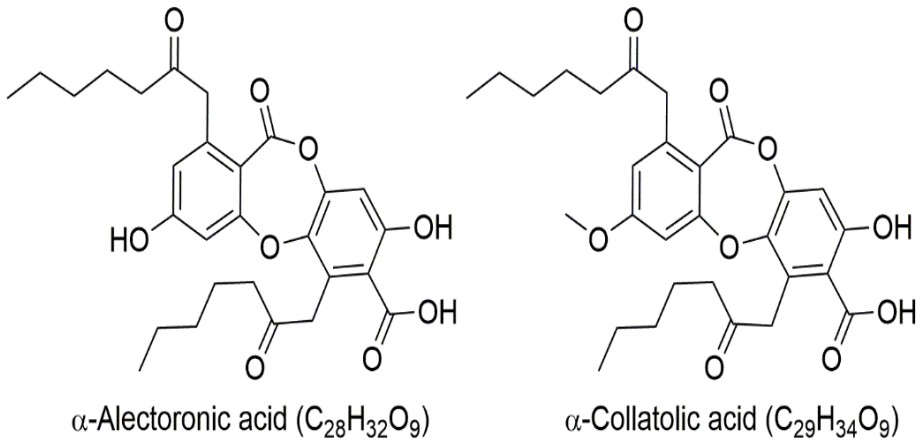


Figure 2 Chemical structures of α-alectoronic acid and α-collatolic acid.

120x46mm (300x300 DPI)

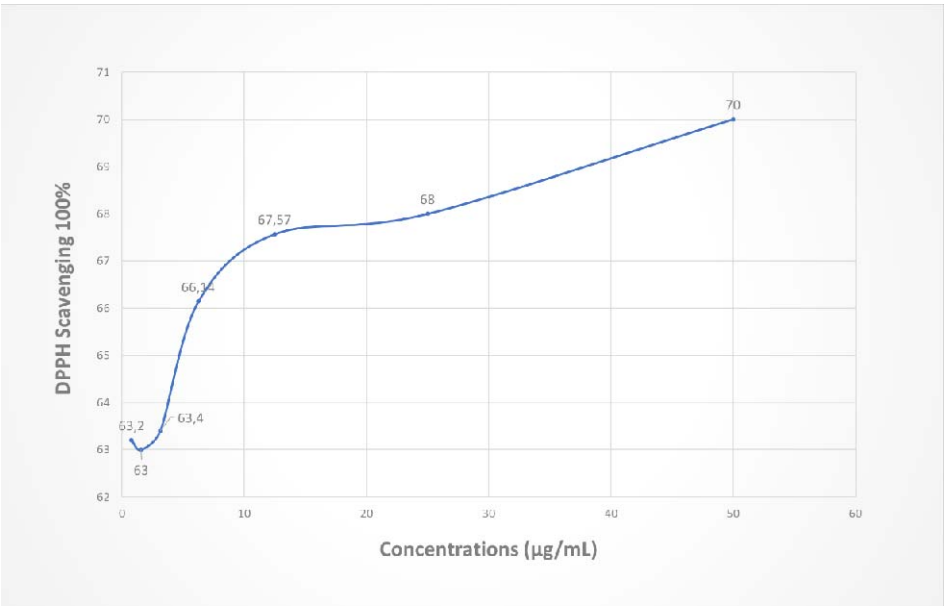


Figure 3 DPPH scavenging 100% to different concentrations (µg/mL) of *Tephromela atra* methanolic extract.

458x294mm (118x118 DPI)