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This file also includes the supplementary data for the article, which is appended after the references section.

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SHORT COMMUNICATION

Valorisation of Whisky Distillery Waste as a Sustainable Source of Antioxidant and Antibacterial Properties with Neuroprotective Potential

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Abstract

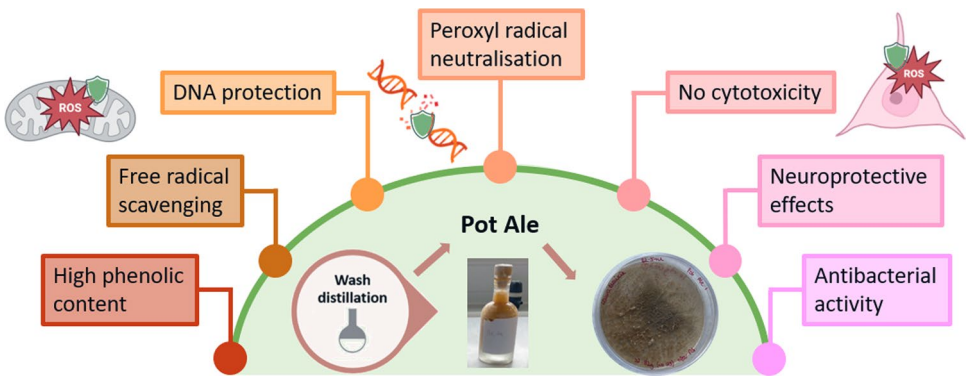
Purpose Waste by-products such as pot ale are abundantly produced during the whisky distillation process and are conventionally used as livestock feed, however a significant proportion continues to require land and sea disposal. Here, the novel potential of whisky by-products as antioxidant and antibacterial agents was investigated.

Methods The total phenolic content and antioxidant potential of waste by-products from whisky distillation were evaluated using FC and DPPH assays. Their DNA protective properties were assessed with gel electrophoresis. The cytotoxicity and cell protective effects of pot ale, in addition to its antibacterial activity, were also studied in this work.

Results Pot ale demonstrated the strongest antioxidant activity of the by-products tested and could reduce DNA damage by 52% at 0.5 mg/mL. Furthermore, pot ale was non-toxic in a neuroblastoma cell line up to 5 mg/mL and protected against the toxic effects of two inducers of oxidative stress (rotenone and hydrogen peroxide) by up to 1.25-fold. Pot ale also showed potent antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* with the minimum inhibitory concentration of 25 µg/mL and 1.56 µg/mL respectively.

Conclusion This work provides the first evidence of the potential of whisky by-products as antioxidants and antimicrobials with no adverse effects in cells, thereby promoting a circular economy.

Graphical Abstract



Keywords Antibacterial · Antioxidant · Pot ale · Valorisation · Waste · Whisky

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Statement of Novelty

This study presents the first evidence of the therapeutic potential of pot ale to treat conditions associated with oxidative stress and bacterial infection, and improve environmental sustainability.

Introduction

Oxidative stress contributes to ageing and is linked to the pathogenesis of various age-related health disorders, including cancer and neurodegenerative diseases. Antioxidants can scavenge and stabilise the highly reactive pro-oxidants which accumulate to induce oxidative stress, thus providing protection against oxidative injury [1]. A range of antioxidants are widely used for medicinal and nutraceutical purposes such as vitamin C, vitamin E, and glutathione [2]. Antioxidant supplements are generally derived from natural products. These antioxidants are typically polyphenols, which are naturally occurring compounds found abundantly in fruits, vegetables, cereals, and legumes [3, 4]. Natural products have also been increasingly researched as a source of antibacterials [5]. Antimicrobial resistance is a growing crisis linked to the increasing prevalence of bacterial infections with high rates of morbidity and mortality. Due to the rising number of potential applications of antioxidants and the urgent need for new antimicrobials, there has been a continuing effort to find sustainable sources of these bioactive compounds.

Whisky is a distilled alcoholic beverage made from water, barley (or other cereals), and yeast. During the whisky distillation process (Fig. 1), a number of by-products are generated. Traditionally, these by-products are used as livestock feed. More recently, distilleries are using these by-products as a source of renewable energy [6]. However, significant volumes of waste continue to be disposed of via land spreading or discharge to sea [7].

Among these by-products are pot ale, spent lees, and effluent. A typical distillery will generate approximately 10,000 m^3 of pot ale and 3,000 m^3 of spent lees for every million litres of alcohol produced in a year [8]. Pot ale is primarily water and yeast and barley residues, in addition to soluble proteins and carbohydrates, copper, and polyphenols. It is a brown liquid with an insoluble solid yeast fraction. Spent lees consists mainly of water, along with copper and low levels of organic acids and alcohols. Pot ale is the by-product of the first distillation stage: the wash distillation. Meanwhile, spent lees is produced during the second spirit distillation stage. Effluent is a combination of the waste by-products from the distillation processes.

Barley and its derived malt have high phenolic content with phenolic acids, proanthocyanidins, tannins, flavonols, chalcones, flavones, flavanones, and amino phenolic compounds contributing to the associated antioxidant activity [9]. Several studies have demonstrated that the malting process can affect the phenolic content with a significant increase in the levels of specific phenolic compounds during germination and kilning stages [10]. Both beer and whisky are typically produced from barley, and their phenolic content has been investigated. Lager beers have a phenolic content of 152–339 mgGAE/L [11], while a malt whisky had 180 mgGAE/L [12]. Furthermore, consumption of whisky (100 mL) was found to significantly increase plasma total phenol content and antioxidant capacity in healthy human subjects [12]. Phenolic compounds contribute smoky flavours and bitterness in whisky. The use of peat fires to dry the malted barley increases the phenolic content [13]. However, it is estimated that around 40–80% of the phenolic content of barley is lost during the production process. Therefore, the recovery of polyphenolics from distillery stillage has been proposed for application in food and medicine due to their potential health benefits [14].

Little research has been carried out into the potential of the recovery of polyphenols from distillery stillage and their associated antioxidant activity. However, a recent study reported the extraction of phenolic compounds from spent

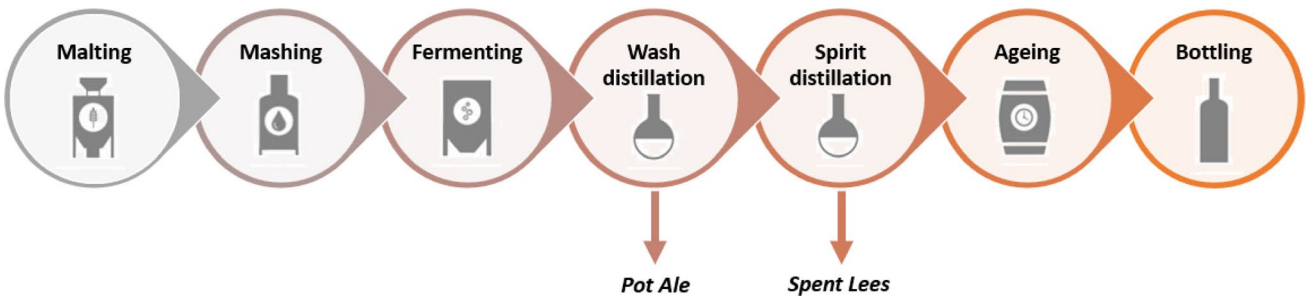

Fig. 1 Whisky preparation process

grain (which is a by-product of the mashing stage) and its associated antioxidant activity [15]. Spent grain extracts which had been subjected to ultrasound-assisted pre-treatment had a total phenol content of up to 2.11 mg GAE/g dry spent grain and could scavenge 78.30% of radicals in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. P-coumaric acid was found to be the major phenolic constituent of spent grain, followed by rosmarinic acid. Furthermore, stillage from a distillery which manufactures alcohol from wheat and rye cereals was used to source phenolic acids, including ferulic acid and p-coumaric acid, with a total polyphenol content of 3.73 mg GAE/g [16]. There has also been success in the recovery of antioxidants for winery wastewater valorisation [17]. Extracts of a synthetic winery wastewater sample demonstrated antioxidant activity with a DPPH inhibition value of up to 85.68%.

The antimicrobial capacity of whisky by-products has also not been widely researched and reported. However, waste from the production of beer and wine has demonstrated promising antibacterial activity. A 3% w/v crude extract of brewery waste reduced the growth of both Gram-positive and Gram-negative bacteria, which the authors attributed to the phenolic content of the extract [18]. Silva et al. [19] also reported that polyphenols (e.g. phenolic acids, flavonoids, quinones) are responsible for the antibacterial properties of winemaking by-products, and structural variation of the phenolics in a sample can affect the antimicrobial activity. Pinot noir wine pomace demonstrated MIC_{50} values of 25 mg/mL and 0.78 mg/mL for *E. coli* and *S. aureus* respectively [20]. Typically, natural extracts are more effective against Gram-positive bacteria (*S. aureus*) rather than Gram-negative bacteria (*E. coli*) which have a strong hydrophilic outer membrane that does not facilitate penetration of lipophilic polyphenols [21].

The aim of this work was to study the antioxidant and antimicrobial potential and neuroprotective properties of samples from the whisky preparation process. While research has been carried out on the recovery of polyphenolics from other distillery by-products, this study is the first of its kind to investigate the antioxidant and antibacterial capacity of pot ale. Initially, the antioxidant capacity and total phenolic content of the whisky by-products were evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Folin–Ciocalteu (FC) assays respectively. The DNA protective properties of the by-products were then tested using gel electrophoresis techniques with plasmid DNA (pBR322) and the oxidative stress inducer, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). The waste product from the distillation process with the strongest antioxidant and protective activities was identified based on these studies. The oxygen radical absorbance capacity (ORAC) assay was employed to further evaluate the antioxidant activity, and the cytotoxicity of the by-product was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay. The neuroprotective effects of the whisky by-product were studied against inducers of oxidative stress-related toxicity in a cell-based model. Finally, the antibacterial potential of the by-product against Gram-positive and Gram-negative bacteria was assessed using the agar well diffusion method.

Materials and Methods

Materials

The samples for analysis were provided by Glen Wyvis distillery, Dingwall, Scotland. Pot ale was taken from the first wash distillation phase, while the spent lees was collected from the second spirit distillation stage. The effluent was a mixture of the waste by-products from the distillation processes. The whisky by-products were collected in 200 mL glass bottles and stored at − 20 °C prior to use. All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) and Fisher Scientific (Waltham, MA, USA) unless otherwise stated and were of analytical grade.

Sample Preparation

The samples were freeze-dried for 72 h to remove all water (Modulyo Freeze Dryer, Edwards) and stored at − 20 °C. Prior to testing, stock solutions were prepared by dissolving the resulting residues from freeze-drying in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL. The stock solutions of effluent and pot ale samples were turbid due to the insoluble solid yeast fraction [7]. Therefore, they were sterile-filtered (0.22 µm) and centrifuged to remove the undissolved solids. Series of dilutions in water were prepared for each sample, and phosphate buffer for the ORAC assay. For the cell-based assays, a stock solution of pot ale was prepared in DMSO at a concentration of 500 mg/mL and dilutions were prepared in media.

Measurement of pH of Pot Ale

Pot ale has a high concentration of volatile organic acids and typically has a pH of around 3.6–4.1 [7]. The pH of the pot ale sample from Glen Wyvis was measured directly using a pH meter (Denver Instruments Basic) and found to have a pH of around 4.0. Following freeze-drying and preparation of a stock solution in DMSO (500 mg/mL), the pH of 5 mg/mL solutions diluted in water and serum-free media were measured. This concentration represented the highest concentration which was tested on cells in this work, with a DMSO content of 1%. The 5 mg/mL solution in water had a pH of around 5.0, while the solution in serum-free media

had a pH of around 7.0. Therefore, the dilution series of pot ale for the cell experiments was prepared in serum-free media to buffer the pH and ensure the cells were not exposed to acidic conditions.

Evaluation of Antioxidant Activity (DPPH Assay)

The free radical scavenging activity of the whisky by-products was evaluated using the DPPH assay according to Payet et al. [22] with minor modifications. From each dilution series of the samples in water (0.625–20 mg/mL), 50 µL was transferred to the corresponding well in a 96-well plate. Water (50 µL) was used as a negative control with DMSO content equivalent to the highest DMSO concentration in the sample solutions. DPPH solution (100 µL, 0.1 mM in methanol) was added to each well followed by a 30-min incubation in the dark. The absorbance was then measured at 490 nm using a Bio-Rad iMark microplate reader. The results were expressed as an IC_{50}, which corresponds to the concentration at which 50% of the DPPH free radicals were scavenged.

Evaluation of Total Reducing Capacity (FC Assay)

The total phenolic content of the whisky by-products was studied using the FC assay, based on the method described by Waterhouse [23] with minor modifications. From each dilution series of the samples in water (0.625–20 mg/mL), 25 µL was transferred to the corresponding well in a 96-well plate along with 200 µL water. Water (25 µL) was used as a negative control with DMSO content equivalent to the highest DMSO concentration in the sample solutions. FC reagent (20 µL) was added to each well and incubated at room temperature for 3 min. Finally, sodium carbonate solution (25 µL, 20%) was added to all wells, and the plate was incubated at 37 °C for 30 min. The absorbance was then measured at 750 nm using a Bio-Rad iMark microplate reader. A calibration curve of gallic acid was constructed (0.02–0.625 mg/mL), in order to calculate the results as gallic acid equivalents using Eq. (1) below, where 'c' is the concentration determined using the calibration curve, 'V' is the volume used, and 'M' is the total mass of sample present in one well.

$$\mathrm{C(mgGAE/1g)} = \mathrm{c(mg/mL)} * \left(\frac{\mathrm{V(mL)}}{\mathrm{M(g)}} \right) \tag{1}$$

Evaluation of DNA Protective Properties (Gel Electrophoresis)

The inhibition of supercoiled plasmid DNA breakage was measured by gel electrophoresis as described by Shahidi et al. [24] with minor modifications. The samples were prepared by mixing phosphate buffer (6 µL) with 8 µL of each sample solution (0.625–10 mg/mL), 8 µL AAPH solution (10 mM) and 1 µL pBR322 DNA plasmid solution (0.5 µg/µL). A DNA control was prepared by mixing DNA plasmid solution (1 µL) with phosphate buffer (22 µL), and an AAPH control was prepared with DNA plasmid (1 µL), AAPH (8 µL) and phosphate buffer (14 µL). The samples were incubated in the dark for 1 h at 37 °C, followed by the addition of loading dye (2 µL) to each tube. Finally, each sample (10 µL) was loaded onto a 0.7% agarose gel prepared in 50 mL TAE (1X) with 5 µL GelRed Nucleic Acid Gel Stain (Biotium). The gel underwent electrophoresis for 70 min at 80 V (Life Technologies Horizon 58 gel tank and Thermo EC 105 power pack), then visualised and imaged under ultraviolet (UV) light (Peqlab Fusion FX7 with Fusion 15.11 software). Using ImageJ software, the intensity of each band in the gel was quantified and applied in the following Eq. (2).

$$\text{DNA protection} = \frac{\text{band intensity of DNA with sample and stressor}}{\text{band intensity of DNA without stressor}} * 100 \tag{2}$$

Evaluation of Antioxidant Activity (ORAC Assay)

The capacity of the whisky by-products to prevent the oxidative degradation of a fluorescent probe was investigated using the ORAC assay as described by Huang et al. [25] with minor modifications. Dilution series of pot ale and Trolox from 500 mg/mL (1.9–1000 µg/mL) and 10 mM (0.625–31.25 µg/mL) DMSO stock solutions respectively were prepared in phosphate buffer (75 mM, pH 7.4), and 25 µL of each solution was transferred into the corresponding well of a black-walled 96-well plate. Phosphate buffer (25 µL) was added to the control wells (blank and no AAPH controls). Sodium fluorescein solution (150 µL, 25 nM) was added to each well, and the plate was incubated for 30 min in the dark at 37 °C. Next, 25 µL of AAPH solution (0.15 M) was added to the blank control and sample wells. For the control with no AAPH, 25 µL of phosphate buffer was added instead. The fluorescence was measured every 2 min for 2 h (485 nm excitation, 525 nm emission) using a BioTek Synergy HT microplate reader. To analyse the results, the following Eqs. (3) and (4) were used where the area under the curve is 'AUC' and the fluorescent measurement at the corresponding time is 'fxmin'.

$$\mathrm{AUC} = 0.5 + \frac{\mathrm{f2min}}{\mathrm{f0min}} + \frac{\mathrm{f4min}}{\mathrm{f0min}} + \frac{\mathrm{f6min}}{\mathrm{f0min}} + \cdots \frac{\mathrm{f120min}}{\mathrm{f0min}} \tag{3}$$

$$\text{Net AUC} = \text{AUC of sample} - \text{AUC of blank} \tag{4}$$

For each compound and Trolox, a graph of net AUC was plotted against concentration. The linear section of the graph

was isolated and the slope for each compound was compared to that of Trolox to obtain the Trolox equivalents using Eq. (5) below, where 'c' is the concentration determined using the calibration curve, 'V' is the volume used, and 'M' is the total mass of sample present in one well.

$$C(\mu molTE/1g) = c(\mu mol/L) * \left(\frac{V(L)}{M(g)}\right) \quad (5)$$

General Cell Culture Techniques and Treatment

The neuroblastoma SH-SY5Y cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC). MTT was purchased from Acros Organics, and PBS was sourced from Oxoid. Cells were cultured as previously described by Smith et al. [26]. The cells were maintained in DMEM medium (Gibco DMEM, high glucose, pyruvate) with 10% foetal bovine serum (FBS), 1% non-essential amino acids (NEAA), and 1% penicillin/streptomycin (Pen/Strep). Cells were grown in a humidified incubator at 37 °C and 5% CO_2. Cells were passaged at around 80% confluence by discarding the old media, washing with PBS, and trypsinising at 37 °C for 4 min. Trypsin was deactivated with fresh supplemented media and the cells were centrifuged (1500 rpm for 5 min). The supernatant was discarded and the cells were suspended in fresh media. Cells were counted using a haemocytometer and seeded in plates or flasks for further experiments.

Stock solutions of rotenone were prepared at 5 mM in DMSO on the day of the experiment. Prior to cell exposure, the stock solutions were diluted to the desired concentration with serum-free media. Solutions of hydrogen peroxide were prepared in serum-free media at the desired concentration from a 30% w/v solution.

Cell Toxicity (MTT Assay)

The method used to evaluate the toxicity of pot ale was as described by Barron et al. [27] with minor modifications. The cells were seeded in 96-well plates (10,000 cells in 100 µL) and incubated for 24 h at 37 °C to allow the cells to attach. A dilution series of pot ale (0.02–10 mg/mL) was prepared in serum-free media from a 500 mg/mL stock solution in DMSO and added to the corresponding wells (100 µL). Serum-free media alone was included as a negative control. The plate was then incubated for a further 24 h. The media was removed from the wells, and sterile-filtered (0.22 µm) MTT solution (100 µL, 1 mg/mL in serum-free media) was added to each well. The plates were incubated for 4 h at 37 °C, after which the MTT solution was removed and DMSO was added to the wells (200 µL). The plate was shaken for 5 min in the dark at room temperature, then the absorbance was read at 595 nm using a BioTek Synergy HT microplate reader. The percentage absorbance, or cell viability, was calculated using Eq. (6) below.

$$\text{Absorbance (\% relative to control)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} * 100 \quad (6)$$

Cell Protection (MTT Assay)

The protective effects of pot ale against rotenone (1 µM for 24 h) and hydrogen peroxide (400 µM for 24 h) toxicity were evaluated in SH-SY5Y using the MTT assay. Briefly, 10,000 SH-SY5Y cells (100 µL) were seeded in each well of a 96-well plate and allowed to attach for 24 h. A dilution series of pot ale (0.003–15 mg/mL) was prepared in serum-free media from a 500 mg/mL stock solution in DMSO, and 50 µL of each was added to the corresponding wells with 50 µL of serum-free media in the control and stressor control wells. The plate was incubated for a further 24 h. The cells were then exposed to each stressor (50 µL) using the conditions provided above with serum-free media in the negative control wells. After 24 h, the media was removed and the MTT assay was performed using the method described in the previous "Cell Toxicity (MTT Assay)" section to assess the cell viability. Results were expressed as percentage of cell viability relative to untreated cells using Eq. (6) in "Cell Toxicity (MTT Assay)" section.

Evaluation of Antibacterial Potential (Agar Well Diffusion Assay)

The antibacterial potential of pot ale was determined by the Clinical and Laboratory Standards Institute (CLSI) standard method CLSI M07-A9 [28]. Gram-positive bacteria *Staphylococcus aureus* NCTC 6571 (SA) and Gram-negative bacteria *Escherichia coli* NCTC 4174 (EC) were cultured in Mueller Hinton broth (MHB) overnight (24 h, 37 °C, 75 rpm). Bacterial suspensions were prepared at 10^8 CFU/mL, 0.5 McFarland's standard in MHB, and 100 µL inoculums were used to prepare bacterial lawn plates in Mueller Hinton agar. Using a sterile cork borer (9 mm diameter), wells were made in the agar plates containing bacteria. A dilution series of pot ale (0.19–200 µg/mL) was prepared in sterile MHB and sterile-filtered (0.22 µm). From each dilution, 50 µL of pot ale was added to the corresponding wells (in triplicate) with 50 µL of sterile MHB and Gentamicin (100 µg/mL) as negative and positive controls respectively (see Supplementary Data for results of positive control). The plates were refrigerated for 30 min to allow the pot ale to diffuse into the agar, then the plates were incubated at 37 °C for 24 h. The antibacterial activity was determined

Table 1 Solid content of whisky by-products following freeze-drying

Sample	% w/v
Spent lees	< 0.01
Effluent	8.45
Pot ale	2.91

by measuring the zone of inhibition that had developed following the incubation period (see Supplementary Data for images of zones of inhibition from agar well diffusion assay). The minimum inhibition concentration (MIC) was considered to be the lowest concentration of pot ale which inhibited bacterial growth.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9 using one-way ANOVA with Tukey's multiple comparisons test, where significant differences are indicated as $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$. Results are shown as mean ± standard deviation (SD) of three independent experiments. All calibration curves and calculations for the following assays are included in the Supplementary Data.

Results and Discussion

Sample Preparation

The whisky by-products were freeze-dried for 72 h to obtain a solid residue. Following the freeze-drying process, the solid content for each whisky by-product could be calculated as % w/v. The results are detailed below in Table 1.

Spent lees had a low solid content, while effluent had the greatest mass of solids remaining following freeze-drying. The low solid content of spent lees is as expected due to its collection point at the second distillation stage where little solid would remain. Pot ale had around 25% less solids compared to the effluent. Since pot ale is collected at the first distillation phase, more solid residue is present at this stage (e.g. yeast residues) compared to the second distillation phase from which spent lees is taken.

Evaluation of Antioxidant Activity (DPPH Assay)

Initially, the DPPH assay was used to assess the capacity of the whisky by-products to scavenge free radicals and thus act as antioxidants. The assay is based on a colour change from purple to yellow which is the result of the reduction of the DPPH free radical by an antioxidant, and this can be measured using UV/Vis spectrometry. The results of the DPPH assay are shown below in Fig. 2, and expressed as IC_{50} values in Table 2.

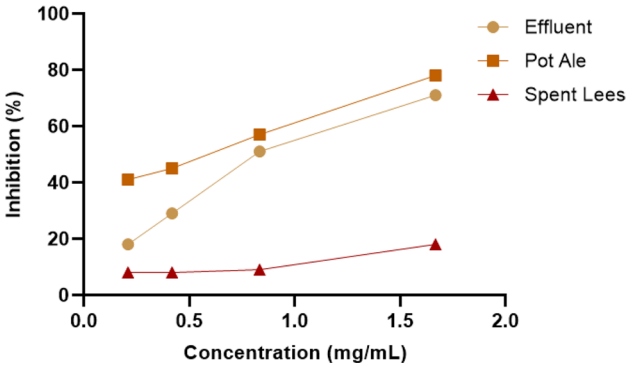

Fig. 2 Antioxidant potential of whisky by-products evaluated using the DPPH assay. Results are shown as mean of three independent experiments and expressed as percentage of inhibition

Table 2 Antioxidant potential of whisky by-products evaluated using the DPPH assay

Sample	IC_{50} (mg/mL)
Spent lees	> 6
Effluent	0.82 ± 0.06
Pot Ale	0.65 ± 0.06

Results are shown as mean ± standard deviation (SD) of three independent experiments and expressed as IC_{50} values

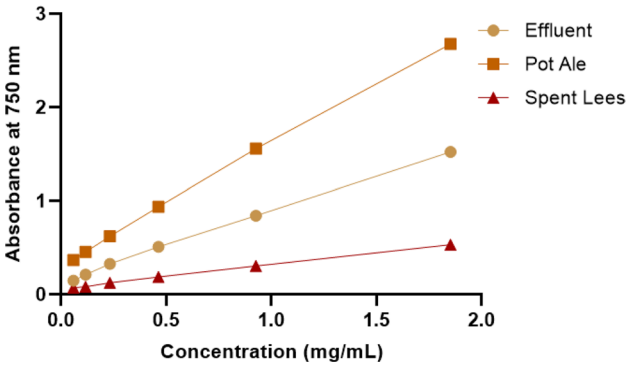

Fig. 3 Total reducing capacity of whisky by-products evaluated using the FC assay. Results are shown as mean of three independent experiments and expressed as absorbance at 750 nm

Spent lees exhibited negligible antioxidant activity at the concentrations tested. Effluent and pot ale demonstrated antioxidant potential by inhibiting DPPH radicals. The pot ale sample, taken from the wash distillation stage of the whisky distillation process, showed the strongest antioxidant activity with the lowest IC_{50} value (0.65 mg/mL) and the highest percentage inhibition of the DPPH radical.

Table 3 Total reducing capacity of whisky by-products evaluated using the FC assay

Sample	Gallic acid equiv (mgGAE/1 g)
Spent lees	1.57 ± 0.18
Effluent	22.82 ± 3.8
Pot ale	46.73 ± 6.8

Results are shown as mean ± standard deviation (SD) of three independent experiments and expressed as gallic acid equivalents

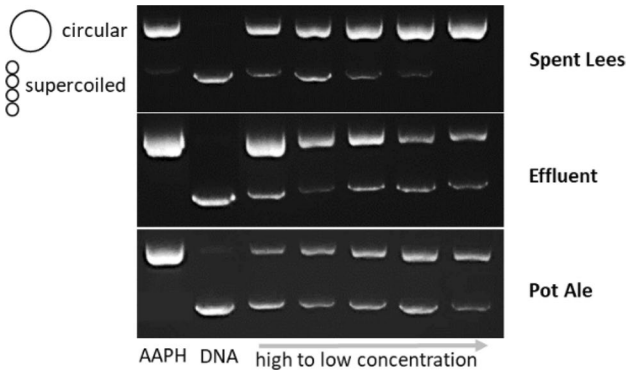

Fig. 4 DNA protection assay using gel electrophoresis with whisky by-products. Supercoiled DNA is the undamaged form, while the open circular conformation is the result of oxidative stress-induced damage

Evaluation of Total Reducing Capacity (FC Assay)

The FC assay is conventionally used as a measure of the total phenolic content by analysing the reducing capacity, and therefore the antioxidant potential, of a sample. A colour change from yellow to blue is measured during this assay, with the intensity of the blue colour corresponding to the phenolic content of the sample. The results of the FC assay are shown in Fig. 3, and expressed as gallic acid equivalents in Table 3.

As with the results obtained from the DPPH assay, spent lees was observed to be the least active sample while pot ale demonstrated the greatest total reducing capacity, and therefore is likely to contain the highest phenolic content of the three by-products. Effluent exhibited around half the total reducing capacity of pot ale, which reflects the reduced antioxidant activity of effluent compared to pot ale in the DPPH assay.

Alternative waste by-products from the food and drink industries which have been extensively researched as a source of antioxidants include rapeseed pomace [29]. Extracts of rapeseed pomace have been studied in similar assays and exhibited an IC_{50} between 49 and 180 µg/mL in the DPPH assay and a total reducing capacity of 25–55 mgGAE/1 g in the FC assay. This is an improvement of around 3–13 times in activity over the pot ale sample for the DPPH assay. Meanwhile, the range of total reducing capacity is similar between rapeseed pomace extracts and the whisky by-products.

Evaluation of DNA Protective Properties (Gel Electrophoresis)

The capacity of the whisky by-products to protect DNA from oxidative stress-induced damage was assessed using gel electrophoresis (Fig. 4). As a result of plasmid DNA strand breakages by peroxyl radicals generated by AAPH, the supercoiled form is broken into open circular or linear formations. This conformational change of the plasmid DNA results in delayed movement through agarose gel. Therefore, the undamaged and damaged forms of the plasmid DNA can be separated and quantified.

The results from this assay are expressed below in Table 4 as the percentage of DNA protection at 0.5 mg/mL of each sample.

Again, the results from this assay reflect the trend for the previous assays whereby spent lees showed minimal activity and pot ale was able to protect DNA to the highest degree of the three samples at 0.5 mg/mL. Therefore, due to the capacity of pot ale to act as an antioxidant, pot ale is able to protect DNA from oxidative stress-induced damage by 52.1%.

In the aforementioned study with rapeseed pomace [29], extracts were reported to have 75% protection of DNA at a concentration of 20.9 µg/mL, which is around 25 times less concentrated than the 0.5 mg/mL concentration used for the whisky samples in this work. Notably, the various extraction methods used for the rapeseed pomace samples prior to testing were compared and were found to affect the resulting antioxidant activity. In particular, the Soxhlet and ultrasound-assisted extraction techniques resulted in rapeseed pomace extracts with stronger antioxidant activity. Therefore, in a similar manner, it is likely that an improvement

Table 4 DNA protective properties of whisky by-products evaluated using gel electrophoresis

Sample	Protection at 0.5 mg/mL (%)
Spent lees	15.8 ± 0.1
Effluent	35.9 ± 4.8
Pot ale	52.1 ± 5.5

Results are shown as mean ± standard deviation (SD) of three independent experiments and expressed as percentage of DNA protection

in activity could be observed if extraction techniques were applied to the whisky by-products.

Evaluation of Antioxidant Activity (ORAC Assay)

Based on the previous results, pot ale was selected for further study as it demonstrated the strongest antioxidant activity and DNA protective properties of the three whisky by-products tested. The capacity of pot ale to protect a fluorescent probe from peroxyl radical-induced oxidative damage was evaluated using the ORAC assay. While both the DPPH and ORAC assays are used to assess antioxidant activity, the ORAC assay is considered to be more biologically relevant as it is carried out at 37 °C in aqueous solutions at a neutral pH which more closely represents the body environment. The results of the ORAC assay were calculated as mean ± standard deviation (SD) of three independent experiments and expressed as Trolox equivalents.

Pot ale demonstrated strong antioxidant activity in the ORAC assay with a value of 698.87 ± 60.45 µmolTE/1 g. This indicates that the phenolic compounds present in this by-product of the whisky distillation process can neutralise peroxyl radicals.

In comparison, rapeseed pomace extracts gave values of 1618–2825 µmolTE/g dry weight in the ORAC assay [29] which is around 2–4 times more active than the pot ale studied in this work.

Cell Toxicity (MTT Assay)

The pot ale sample was then tested in cells to assess the toxicity of this by-product in a biological system. The neuroblastoma cell line, SH-SY5Y, was used for the cell-based experiments in this work. To assess the cytotoxicity of pot ale, the MTT assay was employed which measures cell viability. The MTT assay measures metabolic activity through the conversion of a tetrazolium salt to a formazan product by mitochondrial enzymes in viable cells. The formazan product is purple and can be measured using UV/Vis spectrometry. The results of the MTT assay for cell toxicity are expressed as percentage of cell viability (Fig. 5).

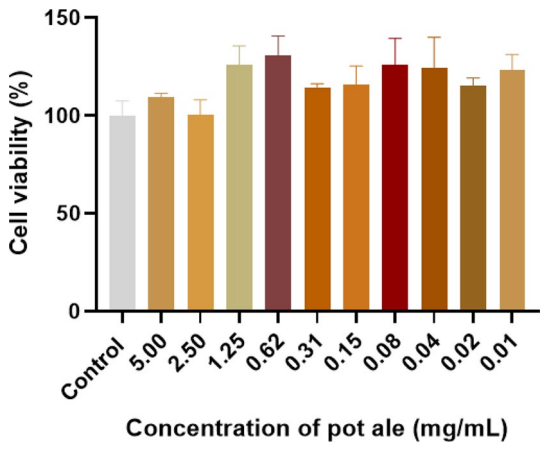

Fig. 5 Toxicity of pot ale evaluated using the MTT assay. Results are shown as mean ± standard deviation (SD) of three independent experiments and expressed as percentage of cell viability

Pot ale did not induce any toxic effects in cells at concentrations up to 5 mg/mL. Therefore, pot ale solutions with a maximum concentration of 5 mg/mL could be used for the cell protection experiments.

Cell Protection (MTT Assay)

The protective effects of pot ale against inducers of oxidative stress in a biological system were assessed using the MTT assay. The cells were pre-treated with various concentrations of pot ale, then exposed to rotenone (1 µM) or hydrogen peroxide (400 µM) to induce oxidative stress-related toxicity. The cell viability was then measured to assess the protective capacity of pot ale. The results of the MTT assay for cell protection are expressed as percentage of cell viability (Fig. 6).

Pot ale protected cells against the toxic effects of both hydrogen peroxide and rotenone. At 0.1 mg/mL, 1 mg/mL, and 5 mg/mL, pot ale significantly improved cell viability by reducing the effects of hydrogen peroxide-induced stress. While no statistically significant improvement was observed in cell viability, pot ale did demonstrate low levels of protection against rotenone-induced stress. Overall, pot ale demonstrated moderate protection against oxidative stress-related toxic effects induced by two different stressors. In addition to the DNA protection results, this work indicates that pot ale can protect against stress via an antioxidant mechanism.

While rapeseed pomace extracts could protect cells from hydrogen peroxide-induced oxidative stress, these extracts were toxic in cells at higher concentrations with 50% cell viability at 2.75 mg/mL [29]. Therefore, whisky by-products (in particular, pot ale) demonstrate promising antioxidant activity and protective effects with minimal cytotoxicity. Further work to generate extracts of pot ale and identify the phenolic compounds present in the samples is recommended.

Evaluation of Antibacterial Potential (Agar Well Diffusion Assay)

The antibacterial properties of pot ale were assessed using the agar well diffusion assay. Agar plates containing *E. coli* (Gram-negative) or *S. aureus* (Gram-positive) were incubated with various concentrations of pot ale, and the diameter of the treated zones where bacterial growth was inhibited were measured to quantify the antibacterial properties of the

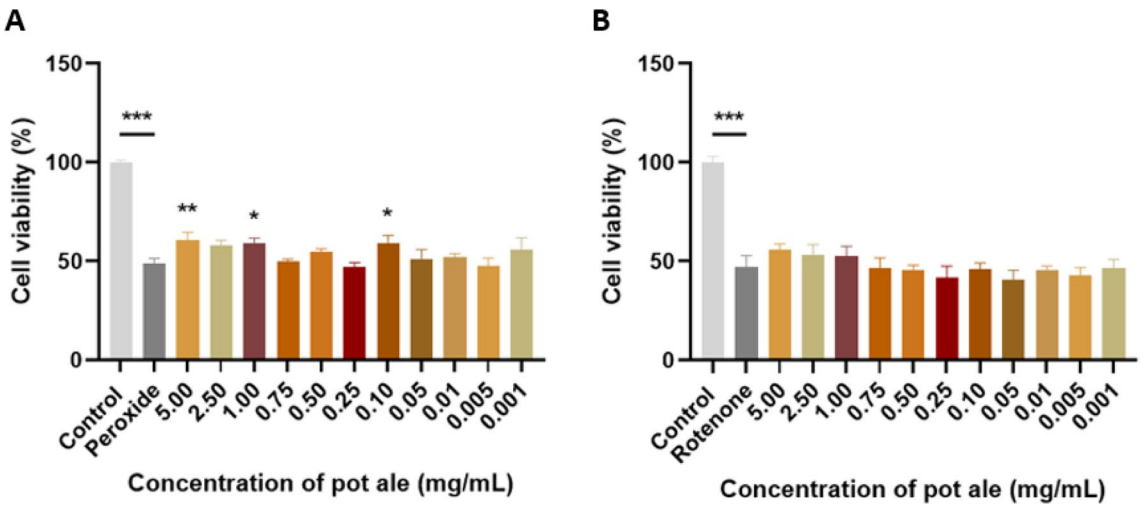

Fig. 6 Protective effects of pot ale against **A** hydrogen peroxide and **B** rotenone evaluated using the MTT assay. Results are shown as mean ± standard deviation (SD) of three independent experiments and expressed as percentage of cell viability. For statistical analysis, $*p<0.05$, $**p<0.01$, $***p<0.001$ (one-way ANOVA, Tukey's multiple comparisons test) for comparison between control and stressor control, and between stressor control and each compound

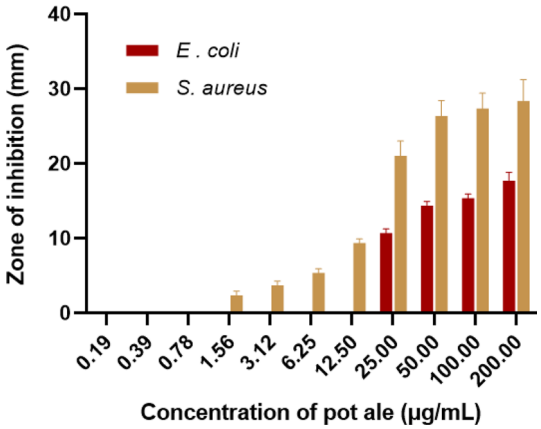

Fig. 7 Antibacterial activity of pot ale against *E. coli* and *S. aureus* evaluated using the agar well diffusion assay. Results are shown as mean ± standard deviation (SD) of three independent experiments and expressed as zone of inhibition in millimetres (mm)

whisky by-product. The results of the agar well diffusion assay for antibacterial potential are expressed as zone of inhibition in millimetres (mm) (Fig. 7).

Pot ale was effective against both *E. coli* and *S. aureus* with inhibition zones of 17.7 mm and 28.3 mm respectively at 200 µg/mL. In addition, the MIC of pot ale was calculated as the minimum concentration at which bacterial growth was inhibited. This value was 1.56 µg/mL for *S. aureus* and 25 µg/mL for *E. coli*. These results show that pot ale was more effective as an antibacterial against Gram-positive bacteria (*S. aureus*) compared to Gram-negative bacteria (*E. coli*). This finding supports previous research which demonstrated that Gram-negative bacteria was more resistant to natural extracts than Gram-positive bacteria [21]. The reduced susceptibility of Gram-negative bacteria to the antibacterial properties of pot ale is likely a result of their additional outer membrane.

Previous research has classified the antibacterial potential of plant extracts based on their MIC values with strong antimicrobials having a MIC below 500 µg/mL, moderate antimicrobials with a MIC between 600 and 1500 µg/mL, and weak antimicrobials with a MIC of above 1600 µg/mL [21]. Based on this classification, pot ale has potent antibacterial activity. In comparison, a Merlot pomace extract had a MIC value of 625 µg/mL for *S. aureus* and 1000 µg/mL for *E. coli* with inhibition zones of 12 mm and 7 mm respectively [21]. The authors suggested the antimicrobial activity of the winemaking by-product could be attributed to the phenolic content of the extract. Given that pot ale was determined to have a high total phenolic content of 46.73 mgGAE/1 g ("Evaluation of total reducing capacity (FC assay)" section), it is likely that these bioactive constituents are contributing to the antibacterial properties of the whisky by-product. These polyphenolic compounds may be having a synergistic effect along with other active antimicrobial components in pot ale, such as zinc and copper [7].

Conclusions

Antioxidants and antimicrobials are widely used in various industries, therefore isolating these bioactive agents from waste could provide important health benefits and improve environmental sustainability. Here, by-products of whisky

distillation were evaluated for their potential as sources of antioxidant and antibacterial properties. Pot ale exhibited the strongest antioxidant potential and protected DNA from oxidative stress-induced damage. Pot ale demonstrated no cytotoxicity up to 5 mg/mL, with moderate protective effects against oxidative stress-related toxicity in cells. Furthermore, pot ale could inhibit the growth of both Gram-positive and Gram-negative bacteria. To our knowledge, this is the first evidence of the antioxidant and antibacterial activity of pot ale. Overall, this work illustrates the value of pot ale as a sustainable source of polyphenols.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12649-023-02292-4.

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Data Availability Our manuscript has supplementary data included as electronic materials.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Valorisation of whisky distillery waste as a sustainable source of antioxidant and antibacterial properties with neuroprotective potential

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Supplementary Data

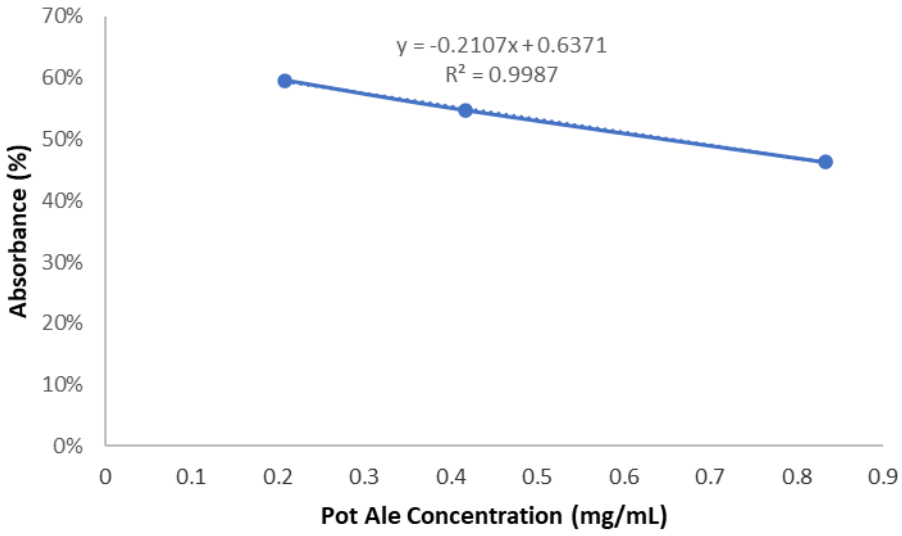

Fig. S1 Determination of DPPH IC_{50} for pot ale.

The calculation to determine the IC_{50} of pot ale was as follows:

$$y = -0.2107x + 0.6371$$

$$y = -0.2107 * \text{DPPH IC50} + 0.6371$$

$$\text{DPPH IC50} = (0.5\ (50\%) - 0.6371)/-0.2107$$

$$\text{DPPH IC50} = 0.65\ \text{mg/mL}$$

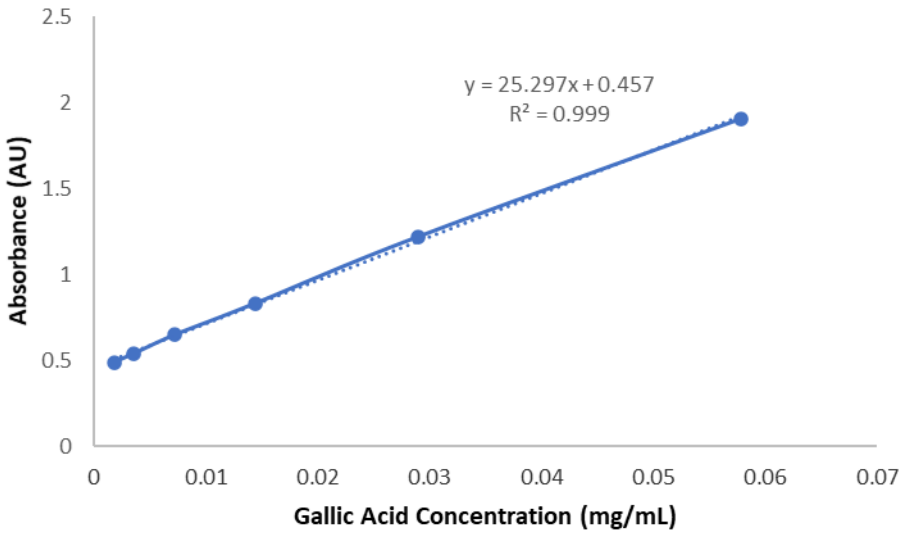

Fig. S2 Calibration curve of gallic acid for FC assay.

The calculation to determine the gallic acid equivalents of pot ale was as follows:

$$\mathrm{C(mgGAE/1g)} = \mathrm{c(mg/mL)} * (\frac{\mathrm{V(mL)}}{\mathrm{M(g)}})$$

$$\mathrm{C(mgGAE/1g)} = 0.043\ \mathrm{mg/mL} * (\frac{0.025\ \mathrm{mL}}{0.000023\ \mathrm{g}})$$

$$\mathrm{C(mgGAE/1g)} = 46.73\ \mathrm{mgGAE/1g}$$

The calculation to determine the percentage of DNA protection by pot ale was as follows:

$$\text{DNA protection} = \frac{\text{band intensity of DNA with sample and stressor}}{\text{band intensity of DNA without stressor}} * 100$$

$$\text{DNA protection} = \frac{4357.19}{8599.17} * 100$$

$$\text{DNA protection} = 51\%$$

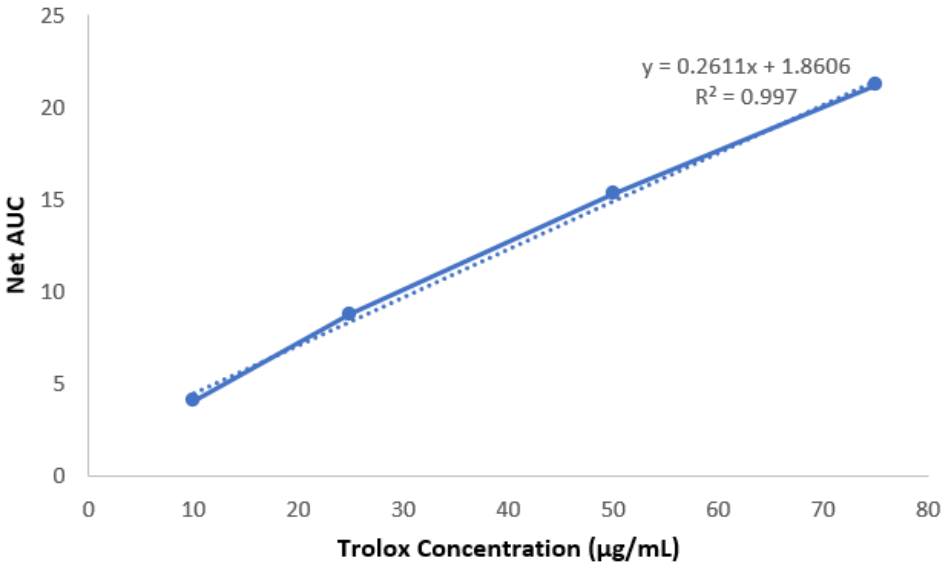

Fig. S3 Calibration curve of Trolox for ORAC assay.

The calculation to determine the Trolox equivalents of pot ale was as follows:

$$\mathrm{C(\mu molTE/1g)} = \mathrm{c(\mu mol/L)} * (\frac{\mathrm{V(L)}}{\mathrm{M(g)}})$$

$$\mathrm{C(\mu molTE/1g)} = 44.67\ \mu\mathrm{mol/L} * (\frac{0.000025\ \mathrm{L}}{0.0000015625\ \mathrm{g}})$$

$$\mathrm{C(\mu molTE/1g)} = 714.86\ \mu\mathrm{molTE/1g}$$

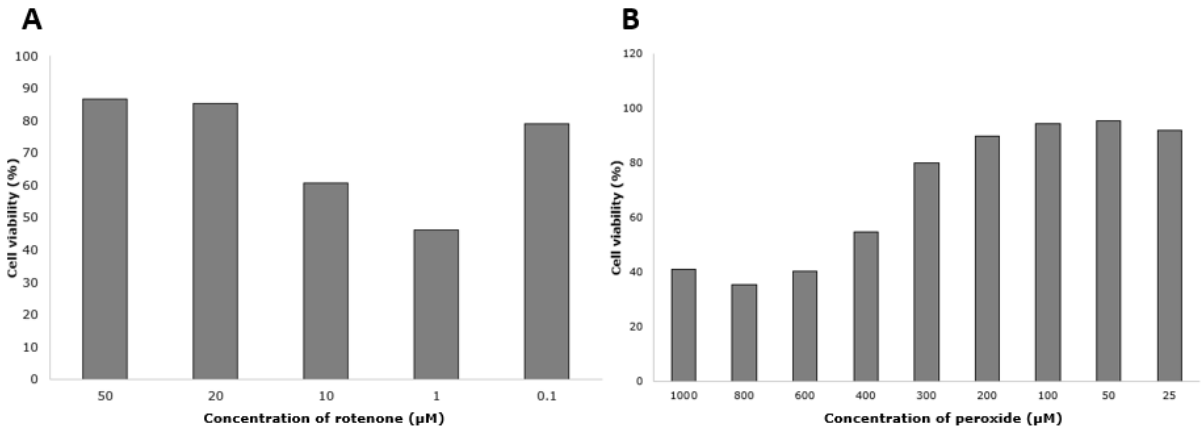

Fig. S4 Determination of MTT IC_{50} for (A) rotenone and (B) hydrogen peroxide.

The calculation to determine the cell viability was as follows:

$$\text{Absorbance (\% relative to control)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} * 100$$

$$\text{Absorbance (\% relative to control)} = \frac{0.241}{0.503} * 100$$

$$\text{Absorbance (\% relative to control)} = 47.9\% \text{ cell viability}$$

Table S1 Antibacterial activity of the positive control, Gentamicin at 100 µg/mL, against *E. coli* and *S. aureus* evaluated using the agar well diffusion assay. Results are shown as mean ± standard deviation (SD) of three independent experiments and expressed as zone of inhibition in millimetres (mm).

Gentamicin (100 µg/mL)	Zone of inhibition (mm)
E. coli	34.33 ± 1.15
S. aureus	37.67 ± 4.04

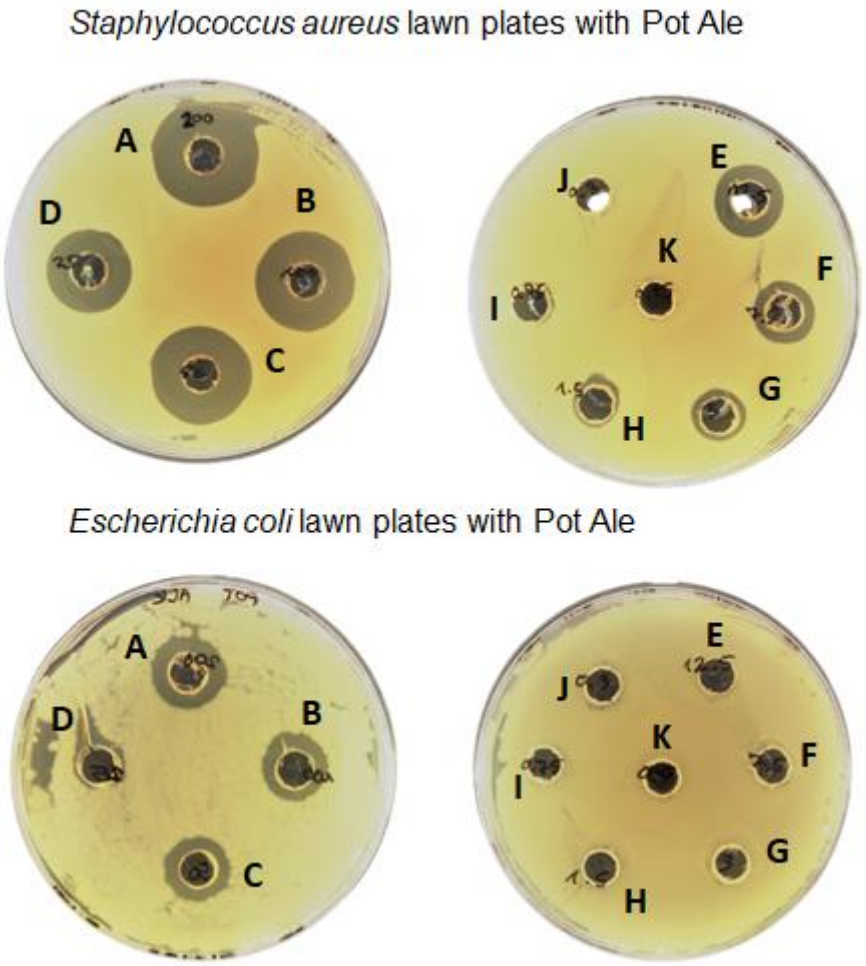

Fig. S5 Agar well diffusion assay showing antimicrobial activity of pot ale with zones of inhibition on *S. aureus* and *E. coli* lawn plates (A: 200 µg/mL, B: 100 µg/mL, C: 50 µg/mL, D: 25 µg/mL, E: 12.5 µg/mL, F: 6.25 µg/mL, G: 3.12 µg/mL, H: 1.56 µg/mL, I: 0.78 µg/mL, J: 0.39 µg/mL of pot ale, and K: negative control of sterile autoclaved Mueller Hinton broth).