Developing an environmentally friendly deodorizer and disinfectant for use in recreational vehicle toilets to protect septic tank and dump sites

By

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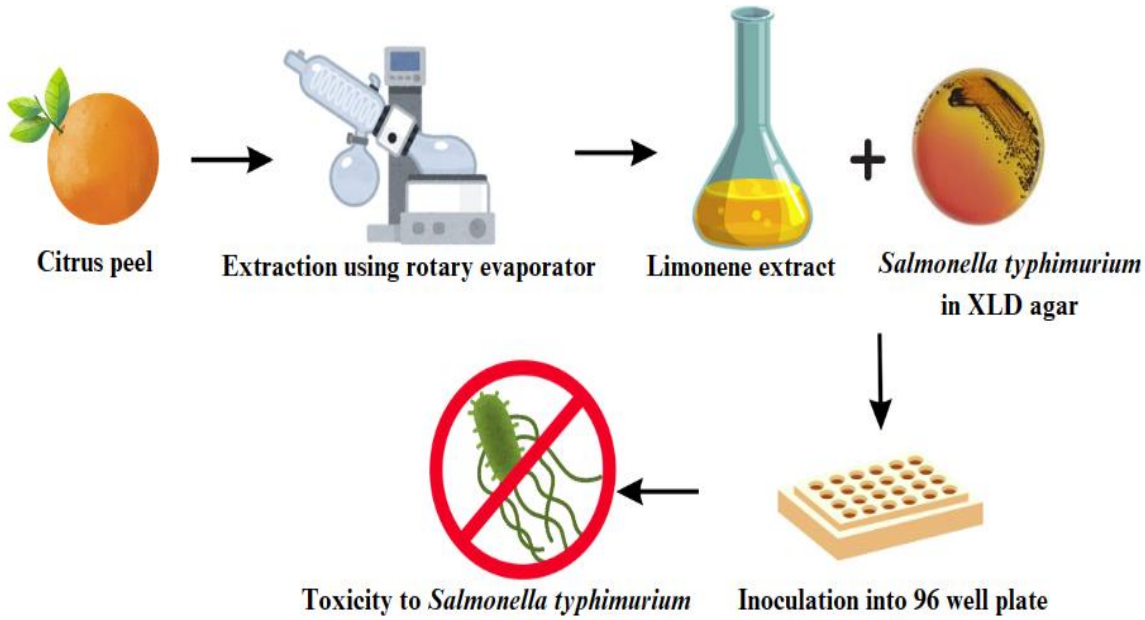
ABSTRACT

Outdoor recreation is one of the fastest growing sectors in the world which accounts for up to $11 billion in national GDP with over 900,000 recreational vehicle registrations in Australia. The wastewater generated by these recreational vehicles, may contain *Salmonella*, a pathogen that causes salmonellosis, a gastrointestinal disease with symptoms such as diarrhoea, fever, and abdominal cramps. Therefore, the wastewater in recreational vehicles is treated with disinfectants, surfactants, and deodorizers to minimize the microbial load of the water and to protect the environment around wastewater disposal sites such as septic tanks and dump sites. However, due to the unknown concentrations of the disinfectants used, their toxicity often remains undetermined. In this study, we evaluated the susceptibility of *Salmonella typhimurium* to a number of disinfectants such as Aqua Kem Blue, Portasol and limonene, an environmentally friendly disinfectant and deodorizer extracted from navel oranges. The minimum inhibitory concentrations of disinfectant were determined by using a Spectrophotometer and comparing the growth curves of *Salmonella typhimurium* to sterile control and a growth control*. Aqua Kem Blue and* Portasol showed toxicity towards *Salmonella typhimurium* at all concentrations from 0.2% to 100%; however, commercial Limonene extract showed toxicity at full strength, but its toxicity was reduced on further dilution.

Graphical abstract



DECLARATION

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed Madhura.N

Date: 06/05/2024

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Additionally, I would like to thank my family for encouraging me to pursue the degree enabling me to understand the dynamics of the biotechnology sector. I would like to offer a big thanks to my partner, Randall for ensuring my mental wellbeing during research work and thesis writing. I would like to acknowledge the support and resources provided by Flinders University.

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ABBREVIATIONS

IBC, Institutional biosafety committee

WHO, World health organisation

EU, European Union

H1N1, Hemagglutinin 1 neuraminidase 1

AgNPs, Silver nanoparticles

MIC, Minimum inhibitory concentration

ATCC, American Type Culture Collection

CO2, Carbon di oxide

INTRODUCTION

1.1. Epidemiology of Salmonella

Salmonella is a gram-negative bacterium that causes salmonellosis, a self-limiting gastrointestinal disease. This foodborne disease causes symptoms such as gastroenteritis, abdominal cramps, bloody diarrhoea, fever, myalgia, headache, nausea, and vomiting (Ehuwa et al., 2021). The disease results in 130,000 deaths every year (Murphy et al., 2017). The route of transmission majorly includes sources such as eggs, meat, dairy products, and water (Brenner et al., 2000). Although the majority of contamination is due to direct exposure to faeces, the main source of *Salmonella* outbreaks in Australia has been identified as eggs and raw egg products (IBC application #:2018-09).

Salmonella enterica serotypes cause diseases that lead to morbidity and mortality. The serotypes *Salmonella paratyphi* causes paratyphoid and *Salmonella enterica typhi* causes typhoid fever. The latter, *Salmonella typhimurium, was* the second most common motile serovar to cause infections in invertebrates and vertebrates including humans in 2021 (European Food Safety Authority, 2022). Humans are known to be the only host and reservoir harbouring *Salmonella typhi* which is spread by the oral faecal route either by direct contact or by contaminated water (WHO, 2022). As *Salmonella typhi* is present in faeces, the organism is expected to be found in wastewater and in surface waters in wastewater impacted locations such as septic tanks and dump sites.

Salmonella typhi shows optimal growth at 35 to 37°C, as such it may be found in enclosed areas such as recreational vehicle cassettes. Salmonella is a predominant pathogenic bacterium in wastewater. *Salmonella* in wastewater serves as an indicator organism to evaluate the presence of other microorganisms in a water source. Around 221 *Salmonella* strains have been isolated from wastewater treatment plants (Motlagh et al., 2019). The strains obtained with the usage of the disk diffusion method show antimicrobial resistance to tetracycline and sulfamethoxazole (Zhang et al., 2019). In further experimentation these bacterial growths were found to be inactivated at 1:200 dilution of citric acid-based detergents (Yi et al., 2022). Therefore, environmental surveillance of wastewater and wastewater-impacted surface waters is necessary to monitor pathogens such as *Salmonella typhi* (Zhou et al., 2023).

The wastewater produced by recreational vehicles may contain both black water which is wastewater from the toilets, and grey water which is wastewater from sinks, baths and showers containing *Salmonella* spp*, E. coli and Legionella* spp (Litwin et al., 2013). Disinfectants, surfactants and deodorizers are traditionally used to neutralise the pathogenic bacteria, however prior research investigating the minimum inhibitory concentration of these antimicrobials is limited. The lack of knowledge on proper disinfection practices for primary treatment used to treat these microbes represents a potential danger to the existing microbial community present in septic tanks and dump sites.

1.2. Hypothesis

The overarching hypothesis of this project is that an environmentally friendly disinfectant and deodorizer can be developed for use in recreational vehicles.

1.3. Aims

To achieve this hypothesis, three specific aims will be conducted.

Aim 1) Developing an environmentally sustainable deodorizer and disinfectant tailored for application within recreational vehicles.

Aim 2) Evaluating the susceptibility of *Salmonella* isolates to the environmentally sustainable deodorizer and detergent.

Aim 3) Evaluating the susceptibility of *Salmonella* isolates to a range of disinfectants.

1.4. Limonene as a disinfectant

Terpenes are the main constituent of essential oils making upto 20 to 60 different chemical compounds (Guimarães et al., 2019). The fruits commonly known as oranges produce citrus waste and due to its low pH and high water and organic matter contents is not legally disposable in the landfills in the EU (Siddiqui et al., 2022). Citrus wastes contain limonene, a cyclic terpene hydrocarbon that is being considered as an alternative disinfectant.

D-limonene or 1-methyl-4-(1-methylethenyl) is a monocyclic monoterpene, a major constituent of oils found in citrus plants, including oranges, lemons and limes. It exhibits antifungal and antibacterial properties, significantly reducing the growth of both gram-negative and gram-positive bacteria (Han et al., 2019). This is due to limonene's solubility in water where it can be found in its hydrophobic or hydrophilic isomers. It's chemical nature makes it an effective surfactant as it has the potential to penetrate and disrupt cellular membranes, causing membrane selective permeability leading to cell death.

As a potential household disinfectant, limonene has been known to show efficacy against the influenza A virus and H1N1 (Fadillah et al., 2022). Silver nanoparticles functionalized with D-Limonene, a component of limonene was used to determine the minimum inhibitory concentration (MIC) of *Klebsiella, E. coli, Enterococcus* and *Pseudomonas* with the nanoparticles at 5.6 Å~ 10−2% D-limonene and 4.7 Å~ 10−5% AgNP. The results constituted a minimum inhibitory concentration, which is a key indicator of an antimicrobial agent's potency against all the tested bacteria (Echeverry-Chica et al., 2022).

1.5. Determination of Minimum Inhibitory concentration (MIC)

The antibacterial activity of a disinfectant can be determined using the disk diffusion method, broth dilution method and dilution method. The dilution method is used to determine the minimum inhibitory concentration (MIC), which is the least amount of disinfectant required to inactivate the bacteria. The visible growth of microorganisms is evaluated by plotting the growth curve of the bacteria against a standard growth curve of microorganism grown without the presence of disinfectants. Broth mediums are used for the dilution method and with the disinfectants being tested at varying concentrations, usually a two or a ten-fold dilution. Broth dilution is cheaper and easier due to its use of broth instead of agar.

1.6. Significance in biotechnology:

In biotechnology, it is essential to establish the MIC of disinfectants as it establishes the lowest effective dosage for preventing microbiological development. MIC values are used to improve infection control, optimise disinfectant formulations and track germ resistance. MIC values also provide guidance for water and wastewater treatment plans in environmental health.

It is hoped that the development of an environmentally friendly deodorizer/disinfectant may protect the microbial community of septic tanks and dump sites by safeguarding the natural microbiota in the environment without causing toxicity to the flora and fauna around it.

2. MATERIALS AND METHODS

2.1. Extraction of limonene from navel oranges citrus peel:

The evaporation of ethanol, used as a solvent, along with distillation is carried out after grating the outer, orange-coloured rinds of two navel oranges which are then added to a round-bottomed flask containing ethanol. The rotary evaporator flask was heated so that distillation would proceed at a steady rate, approximately one drop of distillate per second. Anti-bumping granules were added to the flask and extraction was carried out by creating a vacuum whilst rotating the flask over boiling water at 100°C. At the end of the approximately 30 minutes long distillations, the distillate was collected and filtered multiple times using a 55mm LabServ® Qualitative filter paper to remove impurities. The filtrate was then tested against S.typhimurium by broth dilution. (Chapter 2.2) (Method modified from Andrew Thompson et al., 2018)

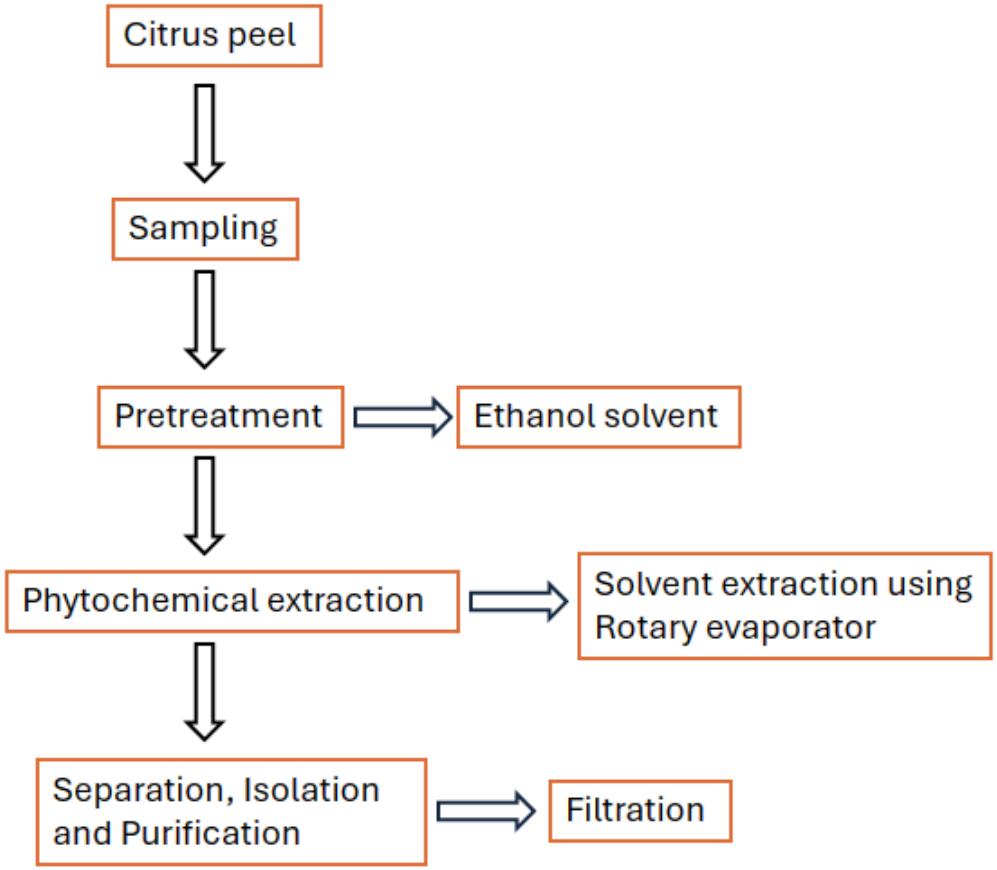

Figure 1: Flow chart of limonene extraction using a citrus peel; the citrus peel was sampled, pretreated with an ethanol solvent, solvent extracted using a rotary evaporator and filtered using a filter paper.

2.2. Minimum Inhibitory Concentration of Salmonella:

Table 1: Disinfectants tested against S.typhimurium. Including concentrations tested.

Disinfectant	Concentration
Commercial limonene	100%
	50%
	25%
	12.5%
	6.3%
	3.2%
	1.6%
	0.8%
	0.4%
	0.2%
Limonene extract	100%

	50%
	25%
	12.5%
	6.3%
	3.2%
	1.6%
	0.8%
	0.4%
	0.2%
Portasol	100%
	50%
	25%
	12.5%
	6.3%
	3.2%
	1.6%
	0.8%
	0.4%
	0.2%
Aqua Kem Blue	100%
	50%
	25%
	12.5%
	6.3%
	3.2%
	1.6%
	0.8%
	0.4%
	0.2%

Target pathogen isolates ATCC53647 of *S. typhimurium* were subjected to broth microdilution using a SPECTROstar® Nano 96 well plate. Ten working solutions were prepared using full-strength disinfectants/limonene in two-fold dilutions from 100% to 0.2% (Table 1). Each working solution was prepared at a concentration twice that of the final desired concentration. *S.typhimurium* was grown in nutrient broth at 37°C in a shaking incubator (New Brunswick™ Innova® 44/44R) until they reached the log growth phase and diluted using sterile broth to an OD 1.0 at 600 nm. Each target organism was measured in triplicate with each well containing 5 µL inoculum suspension, 190 µL broth and 5µL of disinfectant/limonene solution (Figure 2) (Chapter 3). A control series of inoculum suspension and sterile broth was measured to ensure growth of the organism. Each plate was sealed with a membrane and incubated for 24 hours at 37°C, shaking at 100 rpm and measured at 600nm every 30 min for turbidity and growth at each disinfectant/ limonene concentration.

3. RESULTS

3.1. Extraction of limonene from navel oranges citrus peel

The extract yielded from the rotary evaporator produced limonene, a bright yellow liquid with a citrus aroma.

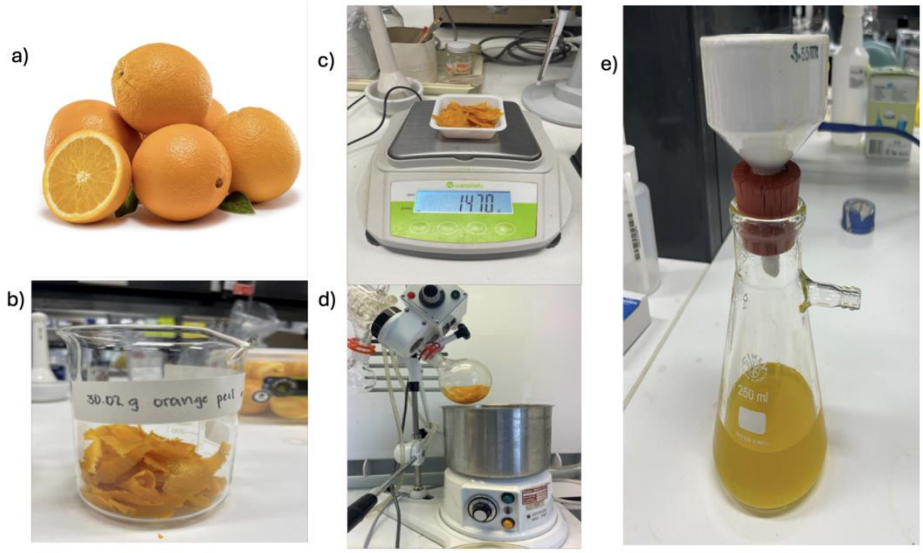

Figure 2: The above photographs show the extraction of Limonene from navel oranges. 2a) navel oranges obtained from Woolworths website. 2b) and c) navel orange peel being weighed and added to a beaker. 2 d) the extraction of limonene from citrus peel using a roto Vap by constant agitation and heating of beaker containing citrus peel and ethanol as a solvent. 2 e) shows the final extract of limonene after straining the extract with filter paper.

3.2. Minimum Inhibitory Concentration test of Salmonella typhimurium against limonene extract and disinfectants:

The minimum inhibitory concentration of limonene extract tested against S.typhimurium in a (SPECTROstar® Nano over 12 hours at 37°C showed no toxicity to the microbe at all concentrations. The growth curve showed linear curves similar to the growth curve hence proving zero toxicity against the microorganism.
The 96 well plates were inoculated with controls sterile control, containing broth and growth controls containing broth and salmonella. The disinfectants were loaded at varying concentrations with a 1:2 dilution.

Table 2: Layout for concentrations of disinfectants/ Limonene tested in triplicates and the controls.

	1	2	3	4	5	6	7	8	9	10	11	12
A	100	50	25	12.5	6.3	3.2	1.6	0.8	0.4	0.2	SC	SC
B	100	50	25	12.5	6.3	3.2	1.6	0.8	0.4	0.2	SC	SC
C	100	50	25	12.5	6.3	3.2	1.6	0.8	0.4	0.2	SC	SC
D	100	50	25	12.5	6.3	3.2	1.6	0.8	0.4	0.2	SC	SC
E	100	50	25	12.5	6.3	3.2	1.6	0.8	0.4	0.2	SC	SC
F	100	50	25	12.5	6.3	3.2	1.6	0.8	0.4	0.2	SC	SC
G	GC	GC	GC	GC	GC	GC	GC	GC	GC	GC	GC	GC
H	GC	GC	GC	GC	GC	GC	GC	GC	GC	GC	GC	GC

Figure 3: Figure showing 96 well plate layout with sample 1 in lanes A, B and C with concentrations 100-0.2% and sample 2 in lanes D, E and F with concentrations 40-0%. Lanes G and H contain *Salmonella* spp (growth control) and Lane 11 & 12 contains broth (sterile control).

3.2.1. Commercial limonene vs S.typhimurium:

The minimum inhibitory concentration of limonene extract tested against S.typhimurium in a SPECTROstar® Nano over 12 hours at 37°C showed that the commercial limonene was toxic to the microbe at full strength. Further dilution showed no toxic effects.

The sterile control broth, showed no growth due to the lack of microbial growth, whereas the growth control showed positive results with a linear growth curve.

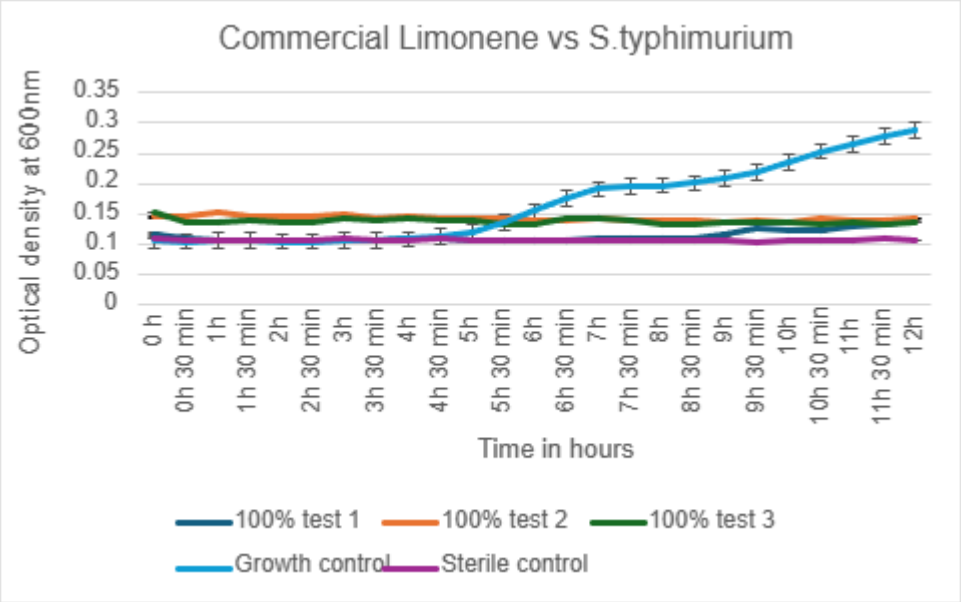

Figure 4: The line graph above represents the growth curves of 100% limonene from 3 tests, with lines coloured navy, orange and green representing samples containing broth, *S.typhimurium* and limonene. The purple line represents the sterile control containing only broth, whilst the blue line represents the growth control containing broth and *S.typhimurium.*

3.2.2. Limonene extract vs S.typhimurium:

The minimum inhibitory concentration of limonene extract tested against S.typhimurium growth in a SPECTROstar® Nano 96 well plate over 12 hours at 37°C showed no toxicity to the microbe at all concentrations. The growth curve showed a linear trend similar to the growth control, hence proving zero toxicity to the microorganism.
The sterile control, broth showed no growth due to its lack of microbial growth, whereas the growth control showed positive results with a linear growth curve.

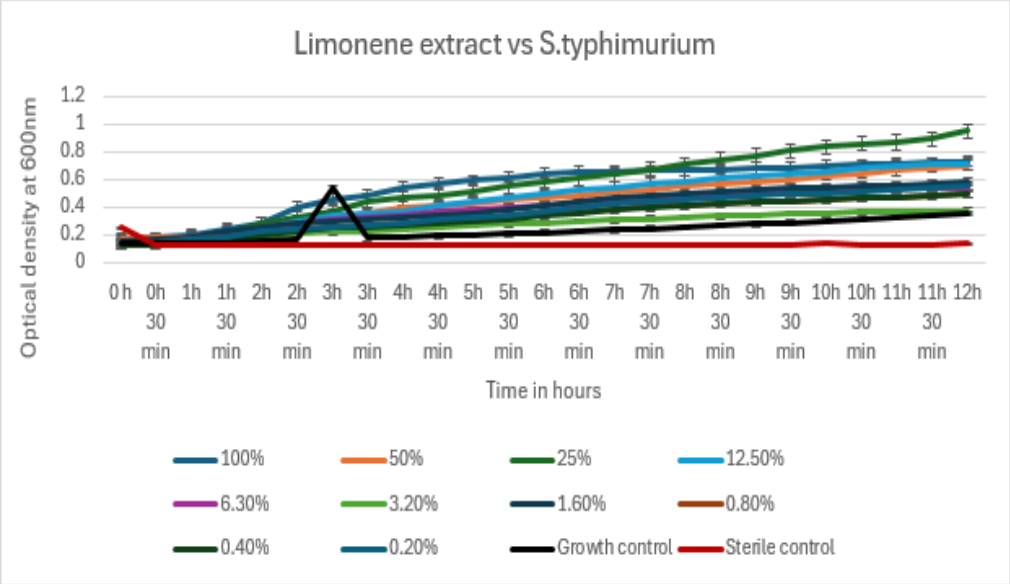

Figure 5: The line graph above represents the growth curves of limonene extracts in concentrations 100 to 0.2% containing broth, *S. typhimurium* and limonene. The red line represents the sterile control containing only broth, whilst the black line represents the growth control containing broth and *S. typhimurium.*

3.2.3. Portasol vs S.typhimurium

The minimum inhibitory concentration of Portasol (Chemtech portasol toilet sanitiser) tested against S.typhimurium grown in a SPECTROstar® Nano 96 well plate over 12 hours at 37°C showed that the Portasol was toxic to the microbe at a 0.2% concentration. The growth curves at all concentrations at a 1:2 dilution generated a decreasing growth curve and hence proving the toxicity of Portasol to *S. typhimurium* at all concentrations. The sterile control, broth generated no growth curve due to the lack of microbial growth in the sample, whereas the growth control showed positive results with a linear growth curve.

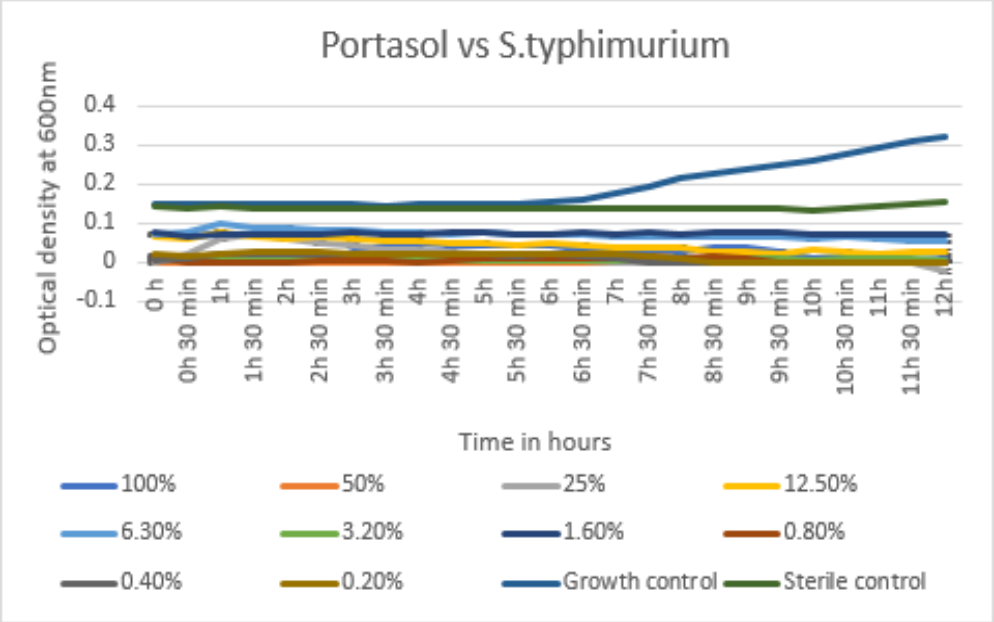

Figure 6: The line graph above represents the growth curves of Portasol disinfectant in concentrations 100-0.2% containing broth, *S. typhimurium* and Portasol. The green line represents the sterile control containing only broth whilst the blue line represents the growth control containing broth and *S. typhimurium*.

3.2.4. Aqua Kem Blue vs S.typhimurium

The minimum inhibitory concentration of disinfectant, Aqua Kem Blue (Thetford Aqua Kem Blue toilet additive) tested against S.typhimurium grown in a Spectrostar SPECTROstar® Nano 96 well plate over 12 hours at 37°C showed that the Aqua Kem Blue was toxic to the microbe at 0.2% concentration. The growth curves at all concentrations at a 1:2 dilution generated a straight line similar to that produced by the sterile control, hence proving the toxicity of Aqua Kem Blue to *S. typhimurium.* The sterile control, broth showed no growth curve due to the lack of microbial growth, whereas the growth control showed positive results with a linear growth curve.

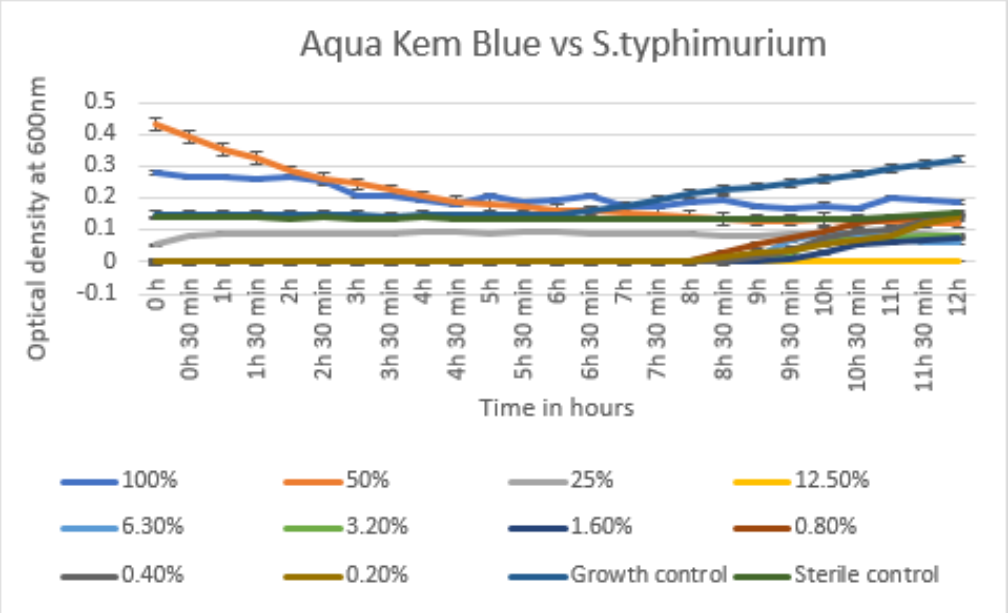

Figure 7: The line graph above represents the growth curves of Aqua Kem Blue disinfectant in concentrations 100 to 0.2% containing broth, *S. typhimurium* and Aqua Kem Blue. The green line represents the sterile control containing only broth whilst the blue line represents the growth control containing broth and *S. typhimurium*.

4. DISCUSSION

4.1. Extraction of Limonene from navel oranges citrus peel:

The limonene extract obtained depends on the solvent used, extraction time and technique used for the extraction. The extract obtained from the rotary evaporator using ethanol as a solvent produced 100% yield assuming the concentration of the citrus peel extract in ethanol solution was 30g/100ml. However, the peel might not have completely dissolved leading to a lower final concentration. The contaminants of citrus peels impact the purity of the product that can be analysed using a Gas chromatography-mass spectroscopy.
Extraction techniques such as steam distillation has proven to yield 90% of yield in one hour, Soxhlet extraction with 100% yield and molecular distillation with 98.7% (Table 3).

Table 3: Techniques of limonene extraction with fruit extracted using, extraction time, yield obtained and references.

Technique	Fruit	Extraction time	Yield	References

Steam distillation	Citrus peel	1 hour	90%	Wilkins et al., 2007
Soxhlet extraction	Citrus	187 minutes	100%	Jha et al., 2019
Molecular distillation	Lemon	1 hour	98.7 %	Rossi et al., 2011

The use of solvents greatly impacts the final yield, the use of ethanol as a solvent in comparison to hexane as a solvent reduced the environmental impacts and provided safety due to the toxicity of hexane.

4.2. Minimum Inhibitory Concentration test of Salmonella typhimurium against Limonene extract and disinfectants:

Effectiveness of disinfectants is a function of both time and concentration and depends on the growth phase of the *S.typhimurium.* The haphazard use of disinfectants in undetermined concentrations results in toxicity to the environment. The present study tried to investigate the minimum inhibitory concentration of disinfectants and an environmentally friendly disinfectant/deodorizer limonene. The results from the MIC tests indicated that Limonene and disinfectants have varying effectiveness again *S.typhimurium*. The results showed that the limonene extract obtained from rotary evaporation showed no toxicity to *S.typhimurium* however the limonene extract used commercially as a solvent (Gilly's Citrus Thin Pure D-Limonene) tested against S.typhimurium showed toxicity at full strength.
Traditional disinfectants such as Portasol and Aqua Kem blue demonstrated lower MIC values, indicating higher potency against *S.typhimurium.*

4.3. Limitations of this study and future work

Limitations of this study include the specificity of the *Salmonella* strain (ATCC5364) used for the identification of the minimum inhibitory concentration of the disinfectant as the strain isolated from the recreational vehicles could exhibit resistance to the disinfectants used.
The purity of limonene extracted depends on the fruit used such as citrus and lemons. The purity can be identified by using analysis such as Gas chromatography- mass spectrometry.
The use of Mueller-Hinton agar provides the standardised results for conduction of minimum inhibitory concentration testing of disinfectants against *Salmonella.* The use of specific agar ensures consistent results and higher reproducibility.
Conduction of experiment in multiple replicates under varying conditions such as variable temperature, contact time and CO2 would provide results for the susceptibility of bacteria under varying environmental conditions. The conduction of minimum bactericidal concentration to prove the efficacy of the disinfectants is essential for proving the minimum inhibitory concentration of disinfectant/limonene.

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