

Potential for *Moringa oleifera* oil and powder as an antibacterial product against gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*)

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Abstract

The development of novel treatments for bacterial infections is of growing importance to tackle the consequences of increasing antibiotic and multi-drug resistance. The current literature shows *Moringa oleifera* belonging to the Moringaceae family, has promise as an antibacterial agent so both oil and powder forms were tested against *Escherichia coli*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* (Abdallah *et al.*, 2023). The antibacterial activity of *M. oleifera*, was determined using disc diffusion assays on agar plates for all three bacterial strains, where no inhibition halos were present in the experimental conditions. Ethanol (95%) and water were used with the aim of extracting the active ingredients from the powder form in suspension over 7 days to examine antibacterial potential where at some conditions the *M. oleifera* appeared to act as a nutrient broth. Following completion of laboratory time available for this study, repeats could not be run to further explore the promising results seen and so the significance of any effect *M. oleifera* has on these bacterial strains requires further testing. Within the limitations of this study, it has been determined that *M. oleifera* leaf extract powder or oil has no antibacterial properties against *E. coli*, *P. fluorescens* or *P. aeruginosa*.

Keywords: antibacterial, essential oil, *Moringa oleifera*, gram negative bacteria

Introduction:

Antibiotic resistance is a severe and increasing issue globally, whereby a large number of species of bacteria have combated antibiotic treatment through evolution and transmission of resistance to other species (Allocati *et al.*, 2013). In the United States there are an estimated 2.8 million antibiotic resistant infections, leading to approximately 35,000 deaths per year (Morris *et al.*, 2020). Antibiotics work in different ways by blocking vital processes in bacteria, potentially killing it or preventing spread through the body and to other hosts. Factors which are propelling antibiotic resistance consist of increased consumption of antimicrobial agents and inappropriate usage, which is spread further by continuous migration of people and international tourism, additionally fuelling multi-drug resistance (Allocati *et al.*, 2013; Dubreuil *et al.*, 2013).

The World Health Organisation has identified MDR as one of the biggest global health issues of 2021 (Sofowora., 1999; Bassetti *et al.*, 2011; Worthington *et al.*, 2013; World Health Organisation., 2020). Multi-drug resistant (MDR) bacterial strains are rising globally due to the spread of genes located on mobile genetic elements, including plasmids, integrons, transposons and the combination of these genes with

chromosomally encoded resistance genes often leads to bacteria which are resistant to the main classes of available antimicrobials (Kaper *et al.*, 2004; Johnson *et al.*, 2009; Allocati *et al.*, 2013). Increasing instances of multi-drug resistance is an issue for human populations due to the accumulating number of bacterial infections which may not respond to antibiotic treatment effectively (Isitua *et al.*, 2016; Dzutam *et al.*, 2017).

Natural substances, such as tea tree oil, are being explored for use as antimicrobials with tea tree oil becoming well known for its antibacterial properties and consequently it has become commercially important for a variety of uses (Arweiler *et al.*, 2000; Lee *et al.*, 2013). Natural substances with the ability to exert antibacterial properties could provide wide-scale benefits, especially for immunocompromised individuals, without the need for the current intense drug development rates due to multidrug resistant bacteria and growing antibiotic resistance (Allocati *et al.*, 2013; Isitua *et al.*, 2016; Dzutam *et al.*, 2017).

Moringa oleifera is a fast-growing, drought-resistant tree within the Moringaceae family, native to the Indian subcontinent, known as the drumstick tree (Peter *et al.*, 2011; Chelliah *et al.*, 2017). It has been reported that *Moringa* leaves contain novel active compounds which induce an antibacterial effect and can therefore have the potential to overcome aspects of multi-drug resistance, demonstrating potential as a new infection-fighting strategy against pathogenic bacteria (Isitua *et al.*, 2016; Dzutam *et al.*, 2017). *M. oleifera* has been the subject of several studies on the basis of its antibacterial properties against both gram-positive and gram-negative bacteria, which so far have been conducted on bacteria implicated in water-borne diseases (Peter *et al.*, 2011; Chelliah *et al.*, 2017). An important factor that allows medicinal use of *M. oleifera* is the vast array of vital antioxidants, vitamins and minerals it contains, which can be sourced from almost every part of these trees for nutrition and other purposes, such as the carotenoids present in leaves (Razis *et al.*, 2014; Milla *et al.*, 2021). It has been found that the plant has a variety of properties including antibacterial, antioxidant, anti-inflammatory and water-purifying abilities of seeds, in addition to inhibitory properties also allowing for treatment of fungal infections (Abalaka *et al.*, 2012; Oluduro., 2012).

Escherichia coli is a gram-negative, rod-shaped member of the bacterial family *Enterobacteriaceae* which is commonly found in the lower intestine of warm-blooded animals (Allocati *et al.*, 2013). Most strains are harmless and are present in the gut of asymptomatic humans rarely causing illness, but virulent forms can cause severe diarrhea, meningitis and septicaemia, such as the O157:H7 strain which produces a toxin damaging the lining of the small intestine (Allocati *et al.*, 2013; Makvana *et al.*, 2015). Transmission of this bacteria comes from contaminated food and water or personal contact and when it leads to an infection, the severity of illness is affected by age, strength of the immune system, time of year and stomach acid levels. Treatment of these infections is at risk of being unsuccessful in the future due to antimicrobial resistance and the rise of multi-drug resistant strains of *E. coli* across Europe (Van der Bij *et al.*, 2005; Allocati *et al.*, 2013).

Pseudomonas fluorescens is a gram-negative, rod-shaped bacterium with the capacity to produce the antibiotic Mupirocin and is used in skin, ear and eye creams, ointments and sprays (Scales *et al.*, 2014). It is not typically pathogenic in humans but studies have found it to be present at low levels in microbiota of various body sites, including the lungs, which can be spread through transfusion of contaminated

blood products or equipment associated with intravenous infusions, despite being better known for its role in the soil and rhizosphere (Scales *et al.*, 2014). *P. fluorescens* is known for promoting plant health through encoded antimicrobial mechanisms, which can be applicable to mammalian cases with hopes of similar antimicrobial mechanism expression due to the presence of functional traits providing the ability to grow and survive in mammalian hosts, such as a type III secretion system, the ability to form biofilms and the potential for some strains to adapt to growth at higher temperatures (Scales *et al.*, 2014).

Pseudomonas aeruginosa is a gram-negative, aerobic, rod-shaped bacterium, commonly found in soil and ground water. It is an opportunistic pathogen which can cause disease in plants and animals but rarely affects healthy individuals (Pang *et al.*, 2019). However, it can be a leading cause of morbidity and mortality in immunocompromised humans and is difficult to eradicate due to its ability to resist antibiotic treatment due to intrinsic and acquired resistance mechanisms resulting in characteristics such as biofilm-mediated resistance and formation of multi-drug-tolerant persister cells (Pang *et al.*, 2019). *P. aeruginosa* displays resistance to a variety of antibiotics including aminoglycosides such as streptomycin and kanamycin leading to increasing challenges in treating these infections and making novel treatments essential (Pang *et al.*, 2019).

The aim of this study is to investigate the potential antibacterial properties of two forms of *M. oleifera*; an oil extraction and a powder. The *M. oleifera* extracts will be tested against *E. coli*, *P. fluorescens* and *P. aeruginosa*. Comparisons were made on the efficacy of each extract based on the bacterial growth seen when cultures were incubated in the presence of the extractions and conclusions were drawn about the antibacterial abilities of *M. oleifera*.

Methodology:

The antibacterial properties of *M. oleifera* as an oil and powder, against *E. coli*, *P. fluorescens* and *P. aeruginosa* were determined using a range of techniques and approaches. Bacterial strains were confirmed as gram-negative using the methodology of Bartholomew and Mittwer (1952).

Bacterial concentration determination:

To determine the concentrations of bacteria to be used a series of LB agar plates were made, poured and cooled in a sterile environment. Stock bacteria (10ml) of *E. coli*, *P. fluorescens* and *P. aeruginosa* were used to perform serial dilutions, with sterile water, of 10^{-1} to 10^{-5} , and 100 μ L of each concentration plated and incubated at 37°C overnight. After incubation the plates were removed for colony counts and concentration determination.

E. coli transformation:

To prevent contamination of plates and bacteria, *E. coli* (HB101) were transformed with pBR322 to express ampicillin resistance ('*bla*' gene) and plated on LB agar plates with 200 μ L of ampicillin (amp) added to 200ml media (1 μ L amp per ml media). The transformation protocol used 10^{-2} untransformed sample, 10 μ L of *E. coli* HB101 cells were added to 990 μ L of LB broth. Using a new tube, 5 μ L pBR322 plasmid DNA was added to the remaining 40 μ L of *E. coli* HB101 cells and placed on ice for 10 minutes. Cells were then incubated at 42 °C for 50 seconds using a heat block, then

placed back on ice for 2 minutes. 1mL of LB broth was added to the tube and incubated at 37 °C for 30 minutes. The recovered cells were added to the tube and spun for 2 minutes at 5000rpm which should lead to the cells forming a pellet at the bottom. The supernatant was removed without disturbing the pellet and the cell pellet was resuspended in 100µL of LB broth. 100µL of the cells was added to the surface of an LB plate containing ampicillin and spread under sterile conditions. They were incubated overnight at 37 °C and then moved to 4°C to prevent overwhelming bacterial growth.

Un-transformed cells were serially diluted and plated at concentrations 10^{-3} to 10^{-7} , transformed cells were also plated and incubated overnight at 37°C then moved to 4°C to prevent overwhelming bacterial growth.

Disc diffusion assay:

To examine if *M. oleifera* oil or the carrier oil (extra virgin olive oil) indicated antibacterial properties, different concentrations were made to perform disc diffusion assays (Table 1). If there were antibacterial properties, then it was determined at which percentage concentration these were. In addition to this a negative control of sterile water and positive controls with the antibiotics streptomycin and kanamycin were used. The positive control antibiotics were used as 6mm discs, all other conditions used 9mm sterile paper discs.

Table 1: Dilutions of *M. oleifera* oil in extra virgin olive oil (carrier oil) totalling 1ml under sterile conditions for disc diffusion assay with *E. coli*, *P. fluorescens* and *P. aeruginosa*.

<i>M. oleifera</i> oil (µL)	Olive oil (carrier oil) (µL)
100	0
75	25
50	50
25	75
0	100

For each bacterial strain, two paper discs were soaked for 15 minutes in 10µL of the relevant concentration, excluding the preprepared antibiotic discs. One of these per *M. oleifera* concentration were added to a LB + ampicillin plate at each bacterial concentration for each of the bacteria (10^{-4} and 10^{-5} for pBR322 transformed *E. coli*, 10^{-3} and 10^{-4} on *Pseudomonas fluorescens*, 10^{-3} and 10^{-4} for *Pseudomonas aeruginosa*), then incubated at 37°C overnight. Following incubation inhibition halos were measured in millimetres with a ruler.

Bacterial refresh:

Under sterile conditions, one colony of the desired bacteria was touched with a sterile toothpick and added to 10ml LB broth and incubated at 37°C overnight. This was repeated weekly to ensure there were significant fresh living bacteria.

M. oleifera in suspension:

Six experimental conditions tested the antibacterial properties of *M. oleifera* extract powder (Table 2). These were made up in 20 ml universal tubes and stored in the shaking incubator at 200 rpm and 37 °C for 7 days. These solutions were then aliquoted into microcentrifuge tubes and spun at 13,000 rpm for 2 minutes. The supernatant removed and transferred to 5 ml bijou tubes (maximum of 3 ml supernatant in each bijou tube to ensure adequate oxygen for bacterial growth). Using these solutions, concentrations of ethanol extract were produced in 10ml of LB at 0.05 %, 0.5 % and 5 % for both ethanol extract dilutions. From each 1ml was removed from each for use as a reference sample, then 50µL of *E. coli* and 10µL ampicillin were added to all experimental concentrations and stored in the shaking incubator at 200 rpm and 37 °C, over the time course of the experiment. Readings were taken using the reference samples and experimental samples in a spectrophotometer using 1.5 ml cuvettes at 0 hours, 3 hours, 6 hours and overnight to detect changes in the amount of living bacterial cells. To generate a positive control 10 ml of LB had 50 µL of *E. coli* added. For negative controls 10 ml of LB had 95 % ethanol added at different volumes to generate 0.05 %, 0.5 % and 5 % concentrations.

Table 2: Conditions set up for the suspension with *M. oleifera* powder extract to which 30µL of *E. coli* was added to each at 0 hours

Dilutant (ml)	Amount of <i>Moringa oleifera</i> powder (g)
10ml Hot water	0.05
10ml Cold water	0.05
10ml ethanol (95%)	0.05
10ml Hot water	1
10ml Cold water	1
10ml ethanol (95%)	1

PCR:

A polymerase chain reaction (PCR) was conducted on the bacterial samples with the aim of confirming what bacterial species was present. Following PCR these samples were run on an agarose gel in order to confirm DNA presence in the sample. Samples with DNA present as confirmed by the gel electrophoresis were cleaned and sent for sequencing.

The sequencing results were run through BLASTn for analysis determining the most likely species based on comparisons with known sequences within the database.

Results:

Gram stains were conducted to confirm the gram status of the experimental bacteria strains (Figure 1). The gram stain for *M. luteus* was not as expected so a PCR was run, and the cleaned sample sent for sequencing in order to identify which bacteria is present in the sample. The PCR results were run through BLASTn and showed a high chance of this being *Pseudomonas aeruginosa* (gram-negative) rather than *M.*

luteus. These gram-negative bacteria were then tested with *M. oleifera* to investigate the potential antibacterial effects.

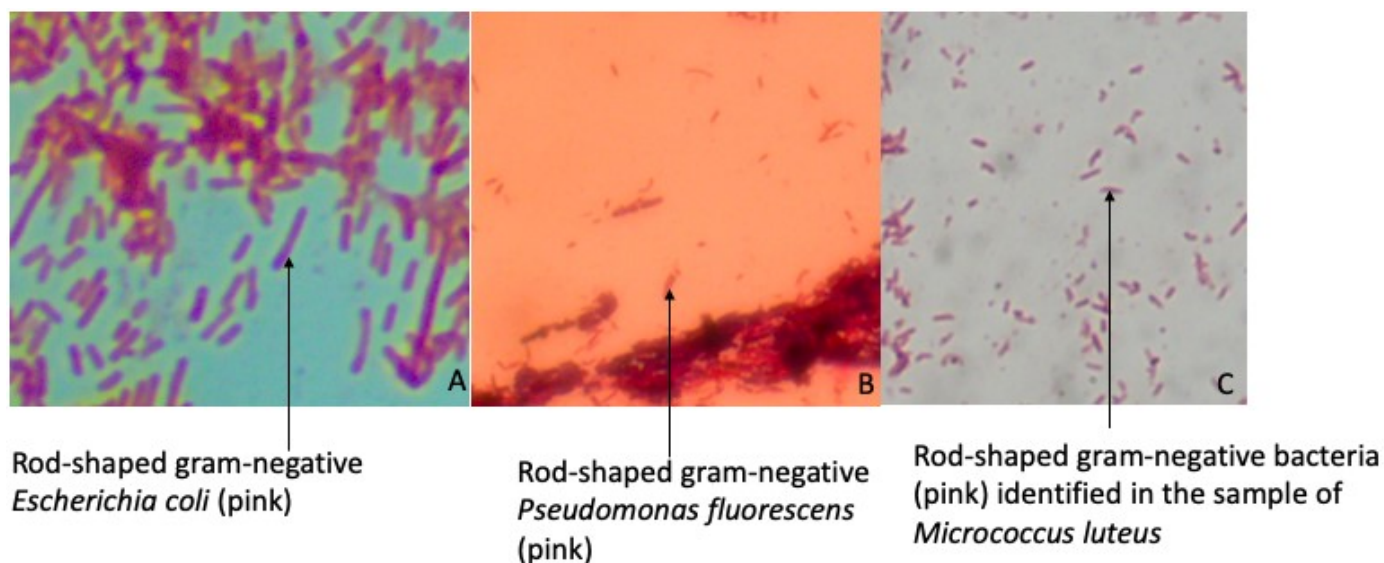


Figure 1: Gram stains for samples labelled as *Escherichia coli* (A), *Pseudomonas fluorescens* (B) and *M. luteus* (C) showing as gram-negative, *M. oleifera* oil and powder will be tested on these bacteria. Images collected using microscope (1000X) and ToupView.

Serial dilutions of each species of bacteria were used to determine which bacterial concentrations to proceed with. Following incubation these indicated contamination, seen by more than the desired concentration and species being present and so all of these samples were autoclaved. Focus then turned to *E. coli* which was transformed with pBR322 to express ampicillin resistance from the 'bla' gene, and plated on LB + ampicillin plates, to prevent anything other than the specified bacterium growing, which yielded successful serial dilutions (Table 3, Figure 2).

Transformed <i>E. coli</i> dilution	Colony counts
10^{-1}	Too many to count
10^{-2}	Too many to count
10^{-3}	Too many to count
10^{-4}	755
10^{-5}	95
10^{-6}	10
10^{-7}	1
10^{-8}	1

Table 3: Serial dilution colony counts from *E. coli* transformed with pBR322 to express ampicillin resistance ('bla' gene), plated on LB agar plates with ampicillin, after incubation for 24 hours at 37°C.

The concentrations used to test the antibacterial properties of *M. oleifera* were 10^{-4} and 10^{-5} as they have the best countable colonies (Table 3, Figure 2). By running

both dilutions, it accounts for any variation in incubation time, so there will be a suitable number of colonies to test the oil against. The dilution series was conducted to 10^{-8} to ensure there was a good spread of countable colonies and to show the spectrum of dilutions.

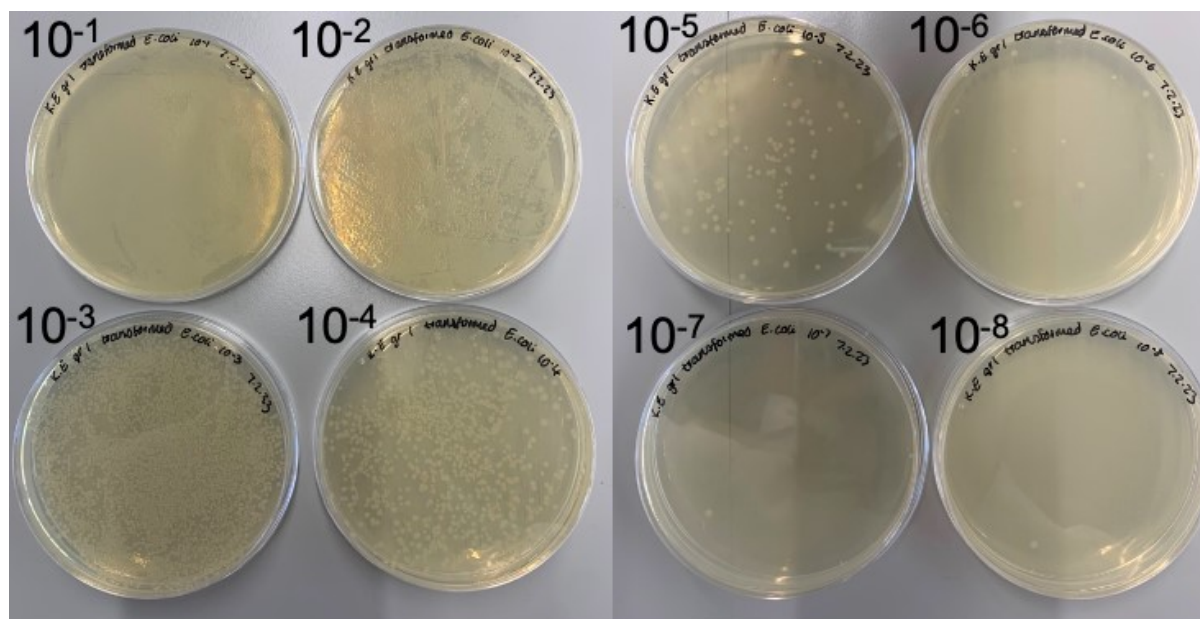


Figure 2: The plates from the serial dilution of transformed *E. coli* with pBR322 to express ampicillin resistance on LB agar plates with ampicillin. Colony counts were conducted from these plates to determine the concentrations of bacteria to test moringa oil against (Table 3).

Transformation of *E. coli* with pBR322 (Table 3) prevented contamination (Figure 2).

To test the *M. oleifera* oil against *E. coli*, different *M. oleifera* and olive oil concentrations were made (Table 1), and a disc diffusion assay performed to show if there were any initial antimicrobial effects.

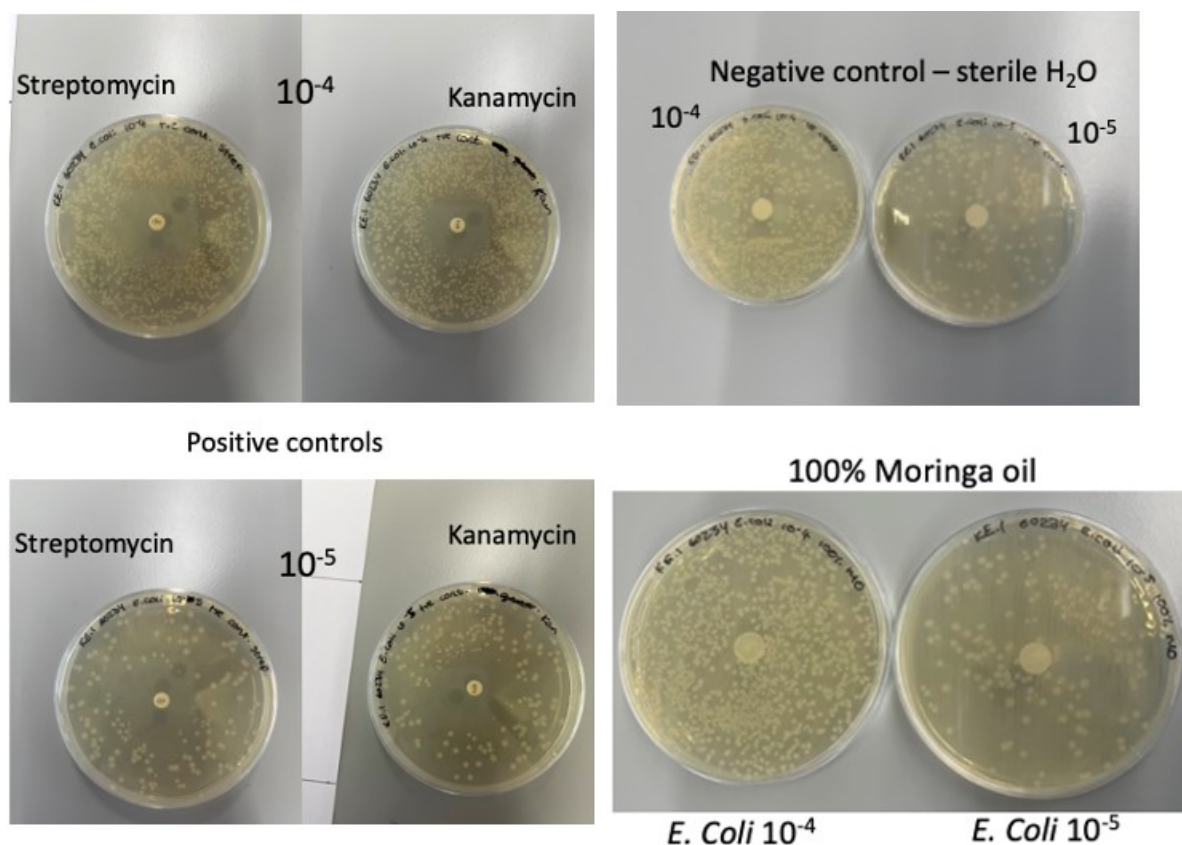


Figure 3: The results from the disc diffusion assay on *E. coli* (10^{-4} , 10^{-5}) with 100% concentration of *M. oleifera* oil, in addition to positive controls of streptomycin and kanamycin and negative control of sterile water on ampicillin LB plates.

There was no antimicrobial activity seen from any concentration of *M. oleifera* oil on *E. coli* at 10^{-4} or 10^{-5} , as no inhibition halos were present following 24 hours of incubation (Figure 3). Throughout the transformation the dilutions remained consistent, showing a high standard of practical working.

Positive and negative controls were used alongside the experimental series, with positive controls including streptomycin and kanamycin 6mm discs, and the negative control using sterile water on a 9mm sterile paper disc (Figure 3). The positive and negative controls acted as expected, with the antibiotics producing inhibition halos and the negative control having no effect, so the results obtained from the disc diffusion (Figure 3) were due to the effect of the *M. oleifera* oil and were unlikely to be influenced by other factors. Streptomycin at 10^{-4} had an inhibition halo diameter of 27mm and at 10^{-5} this was 29mm. Kanamycin at 10^{-4} had an inhibition halo diameter of 27mm and at 10^{-5} this was 29mm, so both have similar inhibitory impact on *E. coli* growth.

To test if this was the same for other gram-negative bacteria, *P. fluorescens* and *P. aeruginosa* were tested in the same way at their respective determined concentrations.

M. oleifera oil did not show any antibacterial properties across a range of bacterial strains, so *M. oleifera* leaf extract powder was tested for antibacterial properties against *E. coli* in suspension at a variety of experimental conditions in bijou tubes.

Due to contamination from an unknown source the hot and cold-water samples in the bijou tubes could not provide spectrophotometer readings, due to solid matter being present and so were autoclaved. The ethanol extract conditions were not contaminated so were used for the next stage of investigation where these were used to generate the 0.05%, 0.5% and 5% ten-fold concentrations in LB.

The spectrophotometer readings can be interpreted into levels of bacteria present as a higher Abs reading is letting less light through, so the sample is cloudier indicating that more bacteria are present. Similarly, where more light is let through and the reading is lower, there are fewer bacteria present.

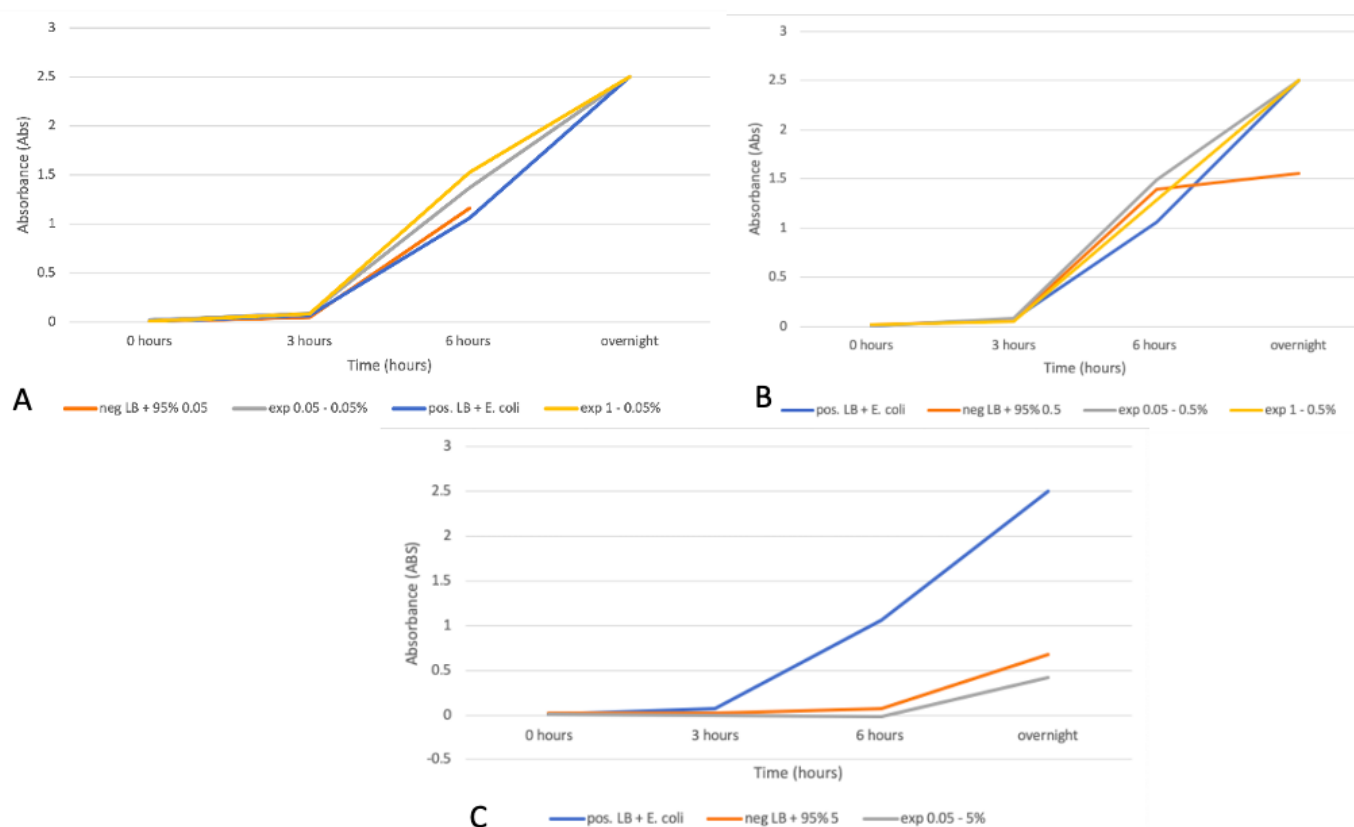


Figure 4: Absorbance readings of 0.05% (panel A), 0.5% (panel B) and 5% (panel C) concentrations consisting of 10ml LB and 0.05g, 0.5g and 5g respectively of *M. oleifera* extract including positive and negative controls

Absorbances for all conditions were comparatively low from 0 to 3 hours showing that rates of bacterial growth were low. From 3 hours onwards absorbance readings increased for tested concentrations with 0.05% of *M. oleifera*, meaning levels of living bacteria increased. This was also conducted at 0.5 % and 5 % of *M. oleifera* extract in 95 % ethanol.

Absorbance for all experimental conditions increased slightly from 0 to 3 hours, followed by a sharper increase to 6 hours which was also reflected by the positive control, excluding the negative control at 0.5g in 95 % ethanol, which began to plateau from 6 hours. The increase in readings indicates that bacteria within the sample were alive and reproducing. The negative control showed a less rapid level of survival from 6 hours to overnight, showing some reduction in rate. This is not likely to be significant due to there not being repeats present and also due to the initial increase in bacterial levels from 0 to 6 hours.

The experimental 5% from 1g *M. oleifera* in 95% ethanol was excluded due to excess powder in suspension so readings could not be taken. This concentration was plated on LB + Amp plate to show if there were any inhibitory growth effects of the *M. oleifera* on *E. coli*. Following incubation, this sample showed no inhibitory effect against *E. coli*. The experimental 0.05g per 10ml LB broth at 5% had the lowest absorbance reading after the overnight readings. At 3 and 6 hours there was a reduction in absorbance so bacteria levels were being reduced, these values then increased overnight but remained lower than all other conditions, showing some level on inhibitory effect of *M. oleifera* on *E. coli*. However, this cannot be deemed a significant effect as there are no repeats allowing for statistical analysis.

As the disc diffusion showed no initial antibacterial activity, the powder form of *M. oleifera* was tested in suspension and following this there was no laboratory time or availability remaining within which to run further repeats and thus statistical testing of the data was not possible.

Discussion:

Several bacteria have combated antibiotic treatment through evolution and resistance transmission between species, which has led to the increase in the issue of antibiotic resistance globally. According to the World Health Organisation 88% of all countries use traditional medicines which have been an integral resource for health in many worldwide communities using plant extracts and active substances, with over 40% of pharmaceutical formulations based on natural products, and development continuing at the WHO Global Centre for Traditional Medicine (Sofowora., 1999; Gupta *et al.*, 2014; World Health Organisation, 2022). Between 1981 and 2010 of the 1073 new chemical entities in the group of small molecules which had been approved, only 36% were purely synthetic, while more than half were derived or inspired by nature (Atanasov *et al.*, 2015; Newman and Cragg., 2012). Due to this, it is important to investigate novel perspectives, particularly natural substances, for treating infections either alone or as complementary medicine due to the increasing instances of antibiotic and multi-drug resistance. In recent years, tea tree oil has become increasingly popular in cosmetics for anti-acne and activity against skin irritation (Arweiler *et al.*, 2000; Lee *et al.*, 2013). It has also been a focus of studies into the therapeutic potential of tea tree oil for scabies, finding that the cumulative effects may successfully reduce the associated bacterial complications (Thomas *et al.*, 2016). As a result of the increased commercial awareness of the abilities of tea tree oil as an antibacterial, further investigations have taken place into additional natural substances with a range of success in the findings. One of the natural substances with apparent success as an antibacterial, antifungal and anti-cancer treatment is *M. oleifera* (Abalaka *et al.*, 2012; Oluduro, 2012). The full potential and practical applications of *M. oleifera* are still currently being investigated, but with further research there is potential for it to become as commercial as tea tree oil in a variety of forms for various treatments or preventatives.

During this study a range of approaches, bacterial strains, concentrations and forms of *M. oleifera* were used in order to conduct a thorough investigation into the antibacterial properties of *M. oleifera*, with the gram status proven for each bacterium used for investigation (Figure 1). It was found by this study that commercial *M. oleifera* oil has no significant antibacterial properties against *E. coli*, *P. fluorescens* or *P. aeruginosa* in disc diffusion assays at relevant concentrations, and no antibacterial

properties against *E. coli* when tested as extract powder in suspension with ethanol (95%) at 0.05%, 0.5% or 5%.

The positive controls used in the disc diffusion aspect of the study (Figure 3) were the antibiotics streptomycin and kanamycin. These are known to be effective against *E. coli* and the *E. coli* strain used was made ampicillin resistant, using pBR322, and grown on ampicillin LB plates. These antibiotics showed clear evidence of being effective against the pBR322 transformed *E. coli* (Figure 3). The negative control used was sterile water which gave the expected results showing no antibacterial effect against *E. coli*, similarly to the *M. oleifera* oil which showed no inhibition properties (Figure 3). The same conclusions can also be drawn for *P. aeruginosa* and *P. fluorescens*. The differences between these findings and those present in current literature may be due to the product used, as Ablaka (2012) found *M. oleifera* leaf extract to have activity against *E. coli*, whereas this study used oil and extract powder, which may account for differences showing that not all forms have the active compound present and so cannot be broadly known as an antibacterial natural substance.

It has been shown that *M. oleifera* oil has no apparent antibacterial effect against the *E. coli* strain HB101 (Figure 3), which contradicts many articles in literature focused on the antibacterial properties of *M. oleifera* (Peter *et al.*, 2011; Peixoto *et al.*, 2011; Ablaka *et al.*, 2012; Chelliah *et al.*, 2017). These results are due to the lack of effect of *M. oleifera* oil rather than issues with the bacteria as the positive and negative controls show the expected results (Figure 3).

Alternatively, it has been found that *M. oleifera* oil specifically does not show any antibacterial activity against 12 pathogenic bacterial strains via the disc diffusion method (Azzay *et al.*, 2015; Boulal *et al.*, 2021), which supports the findings of this study regarding the antimicrobial effects from the oil form (Figure 3). These findings provide this study with greater backing due to supporting each other for *M. oleifera* oil both commercially in this study in addition to being directly produced from a plant as per Azzay (2015) and Boulal (2021).

Following on from the disc diffusion method of investigation, an *M. oleifera* extract powder was commercially sourced and tested in suspension. During this aspect of the study, the hot and cold-water conditions were contaminated, which potentially originated from the powder as the experimental set-up was under sterile conditions (Figure 4). Due to this contamination, only the *M. oleifera* suspended in ethanol was able to be tested and so to prevent further contamination, ampicillin was added to all conditions. This ethanol condition was run over a time course with *E. coli* and different *M. oleifera* concentrations. In several experimental conditions the *M. oleifera* appeared to act as a nutrient broth, encouraging bacterial growth in suspension showing similar trends to the positive control, as spectrophotometer reading values unexpectedly increased, meaning more bacteria were present in the samples over the time course (Figure 4). This is possible as the plant which this originated from is rich in macro and micro nutrients and so can support bacterial growth (Oyeyinka *et al.*, 2018). Bacterial growth was slower at 5% of 0.05 concentration of *M. oleifera* experimental condition so there were some inhibitory properties, however this is not sufficient evidence for the extract powder to possess antibacterial properties in these conditions (Figure 4).

This study as a whole found that there were no antibacterial properties of commercially available *M. oleifera* oil or powder, which was examined through a

diverse array of approaches (Figure 4). However, had this natural substance been proven to be antibacterial, there may be potential for perturbation of the gut microbiome of the consumers, which could have detrimental effects on their health. An extension of this study could examine the gut microbiome of individuals who regularly consume *M. oleifera* in the form of tea or tablets to examine potential negative impacts of consumption of natural products in this way. To test this faecal samples would be collected from individuals who regularly consume *M. oleifera* and then DNA could be extracted and sequenced for analysis of which bacteria are present, compared to individuals who don't consume *M. oleifera*. Tablet forms of *M. oleifera* may also have different effects to the oil or powder and so could be investigated in this way, in addition to a survival study in stomach acid should be conducted to investigate if the product makes it to the gut alive.

An additional direction for further investigation is the collection of product directly from the *M. oleifera* plant, producing an extract powder or oil to investigate if there is a difference in the antibacterial properties of this method compared to commercially available options as in this study. This study was limited by time and products available and so commercially available products were tested, where much of the other literature had access to the plant and were able to produce their own products, with some finding contradicting results, potentially as a result of this difference. Wild grown *M. oleifera* has been found to have higher antibacterial and antifungal activity compared to the commercial products, supporting this study, however the commercial version has advantages as an edible plant used to fight malnutrition in developing countries (Fernandes *et al.*, 2021).

This project originally set-out to investigate if there was any difference in the antibacterial properties of *M. oleifera* powder or oil between gram-positive and gram-negative bacterial strains. As the gram status was a key aspect of this, gram stains were conducted to confirm this prior to investigation, showing that the *M. luteus* sample was gram-negative rather than positive. As this result was not as expected for this bacterium, the species was sequenced and showed *Pseudomonas aeruginosa* (a gram-negative bacterium) as the most likely match. This unexpected discovery resulted in the investigation no longer being a comparison of gram status due to time limitations, but remained in-depth regarding gram-negative strains including *P. aeruginosa* which exhibits high levels of MDR and so antibacterial properties of natural substances against this would be exceptionally beneficial. This means that further investigation could take place into the effects of *M. oleifera* on gram-positive bacterial strains. In addition to this, a wider range of bacterial strains could be subjected to testing in a similar manner including pathogenic strains to portray the potential of real-world application through use in addition to antibiotics, if antibacterial properties are found.

This study has been a comprehensive, in-depth study into the antibacterial properties of *M. oleifera* as a powder and an oil, whereby no antibacterial properties were identified. The results of this study are supported by Azzay (2015) and Boulal (2021) where when the oil form was tested as a disc diffusion assay and no antibacterial properties were established. Despite this, much of the published literature shows that a leaf extract powder form exhibits antibacterial behaviours contrasting this study, although this study tested products which were commercially available to the general public rather than direct plant extracts. These findings fit in research by testing commonly available products, as the plant is not widely available in the UK and that this can affect efficacy of the potential properties. In terms of other

natural substances in oil form or different bacteria, these findings are not widely applicable as each natural substance is individual and will have varying effects on alternative bacterial strains, due to containing different natural substances in fluctuating levels. There is also further research required to be able to investigate use as treatment of bacterial infections and the effects these may have within use on humans or higher levels of model organisms.

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