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Antibacterial and Antibiofilm Activity of *Eucalyptus camaldulensis* Derived Fe_3O_4 Nano-particles against Foodborne Pathogens

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ABSTRACT: *Foodborne pathogens are zoonotic and multidrug resistant, which are not only affecting economy but also accountable for public health burdens. The present study was aimed to evaluate the efficacy of medicinal plant i.e., Eucalyptus camaldulensis extract mediated Fe_3O_4 and ZnO nanoparticles against the 27 food-borne pathogenic strains, isolated from milk, meat, dry fruits and vegetable samples collected from Multan, Pakistan. In phytochemical screening, the plant extract was found to contain numerous bioactive compounds including flavonoids, phenolic compounds, tannins, quinones, and anthocyanins. Fe_3O_4 NPs synthesized from Eucalyptus camaldulensis displayed the highest antibacterial activity with zones of inhibition of 12-13 mm against pathogens. Fe_3O_4 NPs were found to have highest anti-inflammatory potential with recorded percentage of 67 % at 40 µg/ml. Fe_3O_4 NPs also demonstrated the highest antibiofilm activity after 120 hours of incubation. For DDPH antioxidant assay, the highest antioxidant activity was displayed by Fe_3O_4 NPs and their absorbance recorded was 1.43. Therefore, Eucalyptus camaldulensis mediated Fe_3O_4 NPs proved as an effective and eco-friendly approach to combat multidrug resistance in bacterial infections through characteristic antibacterial, antibiofilm and antioxidant properties.*

Keyword: Foodborne pathogen, *Eucalyptus camaldulensis* extract, Nano-particles

INTRODUCTION

Food-borne illness is caused by a variety of pathogenic species like *Salmonella*, *Shigella*, *Vibrio Campylobacter*, *C. perfringens*, *Clostridium botulinum*, some *Escherichia coli* sero groups, *Bacillus cereus* and *Listeria monocytogenes* which are either taken intentionally or un-intentionally in body through different sources like raw or improperly cooked food, unprocessed dairy products, unhygienic food packaging and contaminated water etc. (Aladhadh, 2023). Immuno competent persons are not affected by them, but immuno suppressed patients, old people, young children, and pregnant women are susceptible to these pathogens. Later suffers from spontaneous abortion of the baby, septicemia, stillbirth and even death of the child. Pathogens are a small group of microbes, consisting of ≤10 species, responsible for severe illnesses due to annual global food poisoning (Elbehiry et al., 2023).

Variety of bacterial species present in raw milk cause zoonotic diseases like brucellosis, salmonellosis, tuberculosis and Q- fever (Alves de Aguiar Bernardo et al., 2021). Poultry is known as a prime source of pathogens which cause economic losses for the poultry industry and potential health risks like irritable bowel syndrome and long-term arthritis (Tarabees et al., 2017). Certain strains of *Salmonella* spp., and *Listeria monocytogenes* remain alive on dry fruits and vegetables for an extended period and are far more challenging to kill (Sheng and Wang, 2023). According to a report of WHO, diarrheal diseases, lower respiratory infection, malaria, HIV/AIDS and tuberculosis are top diseases associated with morbidity and mortality (Reygaert, 2018).

These pathogenic microbes are MDR because they have drug resistant genes which cause enzymatic inactivation, drug permeability reduction, antimicrobial target modification, and drug efflux. Moreover, pathogens form biofilms to protect themselves from the action of drugs, thus antibiotics are not effective to treat such infections (Castro-Vargas et al., 2020). Biofilm necessitates a physical surface to grow; formed on sutures, medical devices, catheters, dental implants, human and animal tissues, damp building materials, and aquatic habitats; and produce toxic substances (Dutt et al., 2022). Researchers are taking a look at different methods that could get rid of biofilm and so far, plant derived nano-particles appears to be the most successful way of preventing the formation of biofilm (Liu et al., 2023). Gene expression is increased in biofilm forming microbes; and extracellular

matrix secretion is a major factor which causes successful adherence, colony formation and maturation (Mohamad et al., 2023).

Medicinal plants possess therapeutic potential and thus are traditionally being used for phyto-therapy, aromatherapy, and home remedies (Villa et al., 2022). One of these plants is *Eucalyptus* having antibacterial properties. It contains compounds like eucalyptol and essential oils which not only kill bacteria like *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Trichophytonmentagrophytes* etc; but also inhibit biofilm formation. *Eucalyptus globulus* from *Myrtaceae* family, is a valuable source of many phytochemicals and has numerous applications ranging from medicinal to cosmetic. It is known to have antibacterial, anti-inflammatory and antioxidant properties (Surbhi et al., 2021).

In nanotechnology, various metals like Gold (Au), Silver (Ag), Copper (Cu), Zinc (Zn), Platinum (Pt), Iron (Fe), Nickel (Ni) and Cobalt (Co), are used to synthesize metallic and green nanoparticles, which have proven to be effective for antimicrobial therapy, and are involved in the treatment of cancer, bacterial infections and inflammation (Chandrakala et al., 2022). Iron oxide NPs are proven to be effective in eliminating various types of heavy metal ions and organic compounds (Tan et al., 2023). ZnO NPs are antimicrobial agents and one of the theories explains "Trojan Horse effect" for them which means nanoparticles are degraded inside the cellular lysosomes which results in the release of metal ions and other harmful elements that ultimately disrupt the cellular reproduction (Pushpalatha et al., 2022). This research was focused assess the antibacterial, anti-biofilm, antioxidant and anti-inflammatory potential of *Eucalyptus camaldulensis* extract mediated Fe_3O_4 and ZnONPs against the food-borne pathogens.

MATERIALS AND METHODS

Isolation and characterization of food-borne pathogens

Food samples of various types *i.e.*, milk, meat, raw vegetables, and dry fruits were collected from different areas of Multan Pakistan. A 25 g of each sample was added separately in 250 ml of water, blended for 5minutes and then filtered via sieve of 4 mm pore size. 10ml of each filtrate was then serially diluted upto 10^{-7}; spread on SS Agar and Nutrient Agar; and incubated for 24 hours at 37°C. Selected colonies were characterized morphologically and streaked on MacConkey and EMB agar to examine the colony appearance.

Isolated strains were then subjected to biochemical characterization following protocol given by Cappuccino and Welsh (2019).

Antimicrobial susceptibility and slime production test of food-borne pathogens

These tests were performed according to the protocols of Dela Cruz and Torres (2012), Ronavari et al. (2021) and Wilson et al. (2017) respectively. Antimicrobial susceptibility was checked against following antibiotics; carbapenem/CRO (30 µg), erthyromycin/E (10 µg), amikacin/Ak (30 µg), Penicillin G/P (10 units), tetracycline/TE (30 µg), levofloxacin/LEV (5 µg), amoxacillin/AML (10 µg), ampicillin/AMP (10 µg) and ciprofloxacin/CIP (5 µg).

Preparation of plant extract and nanoparticles

Fresh leaves of *Eucalyptuscamaldulensis* collected from the vicinity of Multan city (species identified and confirmed by The Department of Botany, The Women University Multan) were gently washed with tap water, dried at 60°C and powdered using mortar and pestle. 20 grams of Eucalyptus powder was then transferred to 100 ml distilled water and boiled at 80°C for 60 minutes in water bath. After that it was filtered through Whatman qualitative filter paper no. 1. Different concentrations of *Eucalyptus* aqueous extract *i.e.*, 2%, 4% and 8% were prepared and stored at 4°C till further use. Phytochemical screening of Eucalyptus aqueous extract was done according to the method described by Shaikh and Patil (2020) with slight modifications.

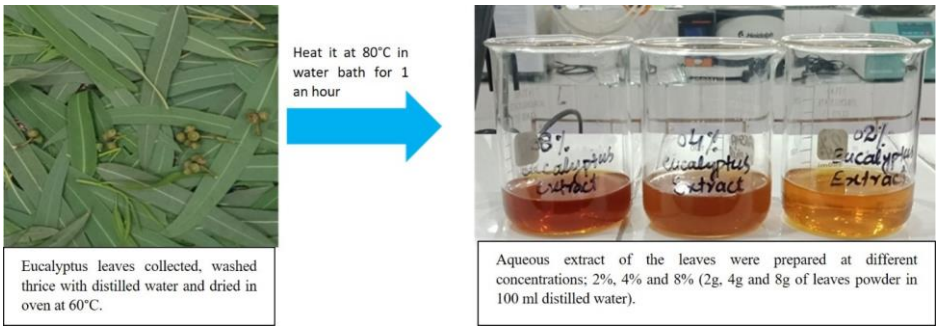


Fig. 1. Preparation of *Eucalyptus camaldulensis* aqueous extract from the collected plant leaves

Iron oxide nanoparticles were synthesized by using the method of Andrade-Zavaleta et al. (2022) with slight modifications. 0.1 M ferric chloride solution was prepared in distilled water and added to 2% aqueous extract of *Eucalyptus* in a 1:2 ratio. It was homogenized in rotary shaker for 30 minutes and then lyophilized for 3 hours, following Christ LCG lyophilization manual.

Zinc oxide nanoparticles were synthesized by using the protocol of Barzinjy and Azeez (2020) with slight modifications. 30 ml of the extract was added in beaker and heated at 60°C. 3g of zinc sulfate was then added and stirred continuously for about an hour until it turned into yellow paste. After that it was placed in oven at 400°C for two hours, and then rinsed with ethanol and distilled water repeatedly and the resulting powder was then dried in oven at 100°C.

Minimum inhibitory concentration of ZnO and Fe_3O_4 nanoparticles

MIC of the NPs was checked by using the protocol of Mann and Markham (1998) with slight modification. In this method, NPs of varying concentration were prepared as: 2.4, 1.2, 0.6, 0.3, 0.15, 0.075, 0.0375, 0.018 and 0.009 µg/ml and inoculated in the media. 20 µL of this was then added in 96 well flat bottom polystyrene plate and incubated for one day and read using microtiter plate reader. The absorbance was measured at 600 nm.

Antibacterial, anti-inflammatory, antibiofilm and antioxidant activity of plant extract and NPs Antibacterial effect of plant extract and nanoparticles

Agar well diffusion method was used to evaluate antibacterial activity of plant extract and NPs. For this purpose, isolate was spread on MHA using cotton swab, wells were made on agar using Pasteur pipette and 100 µL of the extract, iron oxide nanoparticles and zinc oxide nanoparticles were added in the wells. The plates were then incubated at 37°C for 24 hours. Then antibacterial activity was assessed by measuring the diameter of zones of inhibition in millimeter.

Anti-inflammatory assay

0.2% w/v BSA working solution was prepared and 5ml of this reagent was added in 50 µL of the different concentrations of the nanoparticles (200, 400, 800) µg/ml. Tubes were placed in a water bath at 75°C for 5 minutes and cooled at room temperature. Ascorbic acid was used as a standard control and absorbance was recorded at 600nm-660nm against the standard control.

Anti-inflammatory activity assay was performed in triplicates and results are described in terms of % activity, by using the following formula:

Anti-inflammatory assay (%) = (OD $_{control}$ −OD $_{sample}$) / OD $_{control}$ × 100]

Qualitative biofilm and anti-biofilm assay

Protocol of Mathur et al. (2006) was used with slight modifications to determine biofilm formation potential. BHI was prepared with 2% sucrose to check biofilm formation. In 96-well micro-titer plate, 180 µL of fresh broth was dispensed and 20 µL of bacterial culture (McFarland standard 0.5) was inoculated. Plate was then incubated at 37°C for (72, 120,168) hours. The test was performed in triplicate and repeated 3 times. Broth from each well was decanted and deionized water was used to wash planktonic cells from wells. The biofilms were fixed with 2% sodium acetate, stained with 0.1% w/v crystal violet and excess stain was rinsed off by thorough washing with deionized water. Plates were kept for drying and then read using microtiter plate reader at the OD of 570 nm.

Protocol of Kalishwaralal et al. (2010) was followed slight modifications for anti-biofilm assay. 160 µL of BHI broth was added in individual wells of 96-well-flat bottom TCP and inoculated with 20 µL of overnight culture (McFarland standard 0.5). A 20 µL of extract was then added and plate was incubated at 37°C for (72, 120 and 168) hours. The test was performed in triplicate and repeated 3 times. Further steps were performed as mentioned for qualitative biofilm assay.

Antioxidant assays

FRAP Assay: Protocol given by (Schlesier et al., 2002) was followed where five different concentrations of extract and NPs (20, 40, 60, 80 and 100)µg/ml were used in which potassium phosphate buffer (pH set at 6.6) was added following the addition of 2000 µL of the potassium ferric cyanide was added. The tubes were incubated in water bath at 50°C for 20 minutes. After that 2500µL of 10% tri-chloroacetic acid was added and subjected to centrifugation at 5000 rpm for 5 minutes. Afterwards 2 ml of supernatant was transferred to new tube and mixed with 200 µL of 0.1% ferric chloride and 2 ml of distilled water was added in the tubes. Left the tube left for 10 minutes and absorbance was recorded at 765 nm. Same procedure was performed with ascorbic acid which was used as a standard. The test was performed in triplicate and %age of the antioxidant activity was measured using following

formula: [FRAP (%) = FRAP $_{blank}$– FRAP $_{sample}$ / FRAP $_{blank}$ x 100].

RESULTS

Isolation and Characterization of food-borne pathogens

Food samples of various types (milk, meat, raw vegetables, and dry fruits) were collected from different areas of Multan Pakistan. A total of 27 strains were isolated and subjected to morphological and biochemical characterization with their results listed in Table 1.

Table 1: Morphological and biochemical characterization of food-borne pathogens

Strain isolated from food samples	Colony color on MacConkey Agar	Colony color on EMB Agar	Gram Staining	Catalase test	Oxidase test	Citrate test	Triple Sugar Iron (TSI) Test. *Y= yellow R=Red* ✓*=Yes,* ×*=No*				MR-VP test	
			Positive (+) Negative (-)				*Slant*	*Butt*	*CO_2*	*H_2S*	*Methyl-Red*	*Vogues-Proskauer*
S1D1	Light Pink	Pink	-	+	-	+	Y	Y	✓	✓	Red	Rose Red
S2D1	Light Pink	White	-	+	-	-	Y	Y	✓	✓	Pink	Yellow
S3D1	Yellow	-	+	+	+	-	R	Y	✓	×	Red	Rose Red
S4D1	Light Pink	Pink	-	+	+	+	R	O	✓	✓	Yellow-Orange	Rose Red
S5D1	Light Pink	Purple	-	+	+	-	R	Y	×	✓	Red	Rose Red
S6D1	Light Pink	Purple	-	+	-	-	Y	Y	✓	×	Pink-Red	Yellow
S7D1	-	White	-	+	+	+	R	R	×	✓	Red	Yellow

S1D2	-	Green	-	+	+	-	R	Y	✓	×	Yellow-Orange	Yellow
S2D2	-	Green	-	+	-	-	Y	Y	✓	×	Pink-Red	Yellow
S3D2	-	Pink	-	+	+	+	R	R	✓	×	Pink-Red	Rose Red
S4D2	White	Wheat	-	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S5D2	-	-	+	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S1D3	-	-	+	+	-	+	R	R	×	✓	Yellow-Orange	Yellow
S2D3	-	-	+	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S3D3	-	-	+	+	+	+	R	R	×	✓	Pink-Red	Rose Pink
S4D3	White	Pink	-	+	+	+	R	R	×	✓	Red	Pink
S5D3	Colorless	Pink	-	+	-	+	R	R	×	✓	Orange	Yellow
S6D3	Light Pink	Pink	-	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S7D3	Baby Pink	Pink	-	+	+	+	R	R	✓	✓	Pink-Red	Rose Pink
S8D3	-	-	+	+	+	+	R	R	×	✓	Pink-Red	Pink
S1D4	White	White	-	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S2D4	Colorless	Pink	-	+	+	+	R	R	×	✓	Orange	Yellow
S3D4	-	-	+	+	+	+	R	R	×	✓	Pink-Red	Rose Pink
S4D4	Wheat	Purple	-	+	+	+	R	R	×	✓	Yellow-Orange	Yellow
S5D4	yellow	Pink	-	+	-	+	Y	Y	✓	×	Pink	Rose Pink
S6D4	-	-	+	+	+	+	B	R	×	✓	Pink-Red	Yellow

S7D4	-	Green	+	+	-	-	R	Y	✓	×	Yellow-Orange	Rose Pink

Antimicrobial susceptibility and slime production test of food-borne pathogens

Certain microorganisms produce (proteolytic extracellular enzyme) gelatinase, which is involved in liquefaction process and hydrolyzes protein to amino acids. Even extremely low temperatures of 4°C won't be able to recover the gel characteristic once this degradation has taken place. In this study, only strains S3D1, S5D1 and S7D1 gave the positive results which means that they have the enzyme gelatinase. For slime production test, the appearance of black coloredcolonies in the presence of Congo red dye and sucrose indicated the strong slime production and results are given in Table 2. Strain S4D1 showed the maximum sensitivity against ceftriaxone (CRO). Most strains were resistant to penicillin, tetracycline and erythromycin. Most bacterial strains were sensitive to ciprofloxacin, amikacin and levofloxacin as shown in Table 3.

Table 2: Slime production test of food-borne pathogens

Strain	*Congo Red Agar without Sucrose*	*Congo Red Agar with Sucrose*	Strain	*Congo Red Agar without Sucrose*	*Congo Red Agar with Sucrose*	Strain	*Congo Red Agar without Sucrose*	*Congo Red Agar with Sucrose*
S1D1	Light Pink	Black	S3D2	Baby Pink	Red	S7D3	Pink	Light Red
S2D1	Red	Black	S4D2	Pink	Black	S8D3	Pink	Light Red
S3D1	Red	Red	S5D2	Baby Pink	Red	S1D4	Red	Orange
S4D1	Light Red	Black	S1D3	Pink	Pink	S2D4	Red	Orange
S5D1	Bright Red	Red	S2D3	Pink	Orange	S3D4	Light Pink	Red
S6D1	Baby Pink	Light Black	S3D3	Pink	Light Red	S4D4	Red	Red
S7D1	Baby Pink	Black Color	S4D3	Pink Color	Light Red	S5D4	Light Pink	Black
S1D2	Red	Red Color	S5D3	Pink	Pink	S6D4	Peach	Black

S2D2	Baby Pink	Black Color	S6D3	Color Pink Color	Light Red	S7D4	Peach	Black

Table 3: Antibiotic sensitivity profiling of food-borne pathogens

Strain	**Antibiotic Sensitivity profiling (millimeter)**								
	P	**E**	**CRO**	**AMP**	**CIP**	**AK**	**LEV**	**AML**	**TE**
S1D1	R	R	R	R	R	S	S	S	R
S2D1	R	R	S	R	S	S	S	R	R
S3D1	R	S	S	R	S	S	S	R	R
S4D1	S	S	S	S	S	S	S	S	S
S5D1	R	S	S	R	S	S	S	R	R
S6D1	S	S	S	R	S	S	S	R	S
S7D1	R	S	S	R	S	S	S	S	S
S1D2	R	S	S	S	S	S	S	R	S
S2D2	S	S	S	S	S	S	S	S	S
S3D2	R	S	S	R	S	S	S	R	S
S4D2	R	S	S	R	S	S	S	R	S
S5D2	R	S	R	R	S	S	S	R	R
S1D3	R	R	R	R	S	S	S	R	R
S2D3	R	R	R	R	S	S	S	R	R
S3D3	R	R	R	R	S	S	S	R	R
S4D3	R	R	R	R	S	S	S	R	R
S5D3	R	R	R	R	R	R	R	R	R
S6D3	R	R	R	R	S	S	S	R	R
S7D3	R	R	R	R	S	S	S	R	R
S8D3	R	R	R	R	S	S	S	R	R
S1D4	R	R	S	R	S	S	S	R	R
S2D4	R	R	R	R	S	S	S	R	R
S3D4	R	R	R	R	S	S	S	R	R
S4D4	R	R	S	R	S	S	S	R	R
S5D4	R	R	S	R	S	S	S	R	R
S6D4	R	R	S	R	S	S	S	R	R
S7D4	R	R	S	R	S	S	S	R	R

*R=Resistant, S=Sensitive

Preparation of plant extract and synthesis of metallic NPs

Plant extract was prepared from the leaves of *Eucalyptus camaldulensis* and subjected to phytochemical screening. Carbohydrates, reducing sugars, flavonoids, phenolic compounds, tannins and quinones were found in the extracts (Table 4).

Table 4: Phytochemical profiling of *Eucalyptus camaldulensis* leaves extract

Compound	Observation	Results
Alkaloids	Yellow color appeared	-
Carbohydrates	A violet color ring formation	+
Reducing Sugars	Green color appeared	+
Glycosides	Yellow color appeared	-
Proteins and Amino Acids	Yellow color appeared	-
Flavonoids	An intense yellow color	+
Phenolic Compounds	Black color appeared	+
Tannins	Formation of emulsion	+
Quinones	Red color appeared	+
Anthocyanin	Yellow color	-

***+=Positive, -=Negative**

MIC determination of Iron Oxide Nanoparticles

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent (such as nanoparticles) that inhibits the visible growth of a microorganism. The lowest MIC value recorded for the Iron Oxide nanoparticle was 0.04 microgram / milliliter against S5D1.

Antibacterial, anti-inflammatory, antibiofilm and antioxidant activity of extract and NPs

Order of antibacterial activity from highest to lowest, with recorded zones of inhibition was found as Fe_3O_4 NPs (12-13mm), ZnO NPs (7-8mm) and *Eucalyptus* extract (2-3mm) respectively. So Fe_3O_4 nanoparticles performed the maximum antibacterial activity (Fig 2 (a)). While Fe_3O_4 NPs were also found to have highest anti-inflammatory potential with recorded percentage of 67% at 40 µg/ml (Fig 2 (b)). Fe_3O_4 NPs also performed the highest antibiofilm activity after 120 hours of incubation (Fig 2 (c)). For DDPH assay, the highest antioxidant activity was performed by Fe_3O_4 NPs and their absorbance recorded was 1.43. In FRAP antioxidant assay, the highest

activity was performed by *Eucalyptus* (Fig. 3). extract with recorded absorbance of 1.07

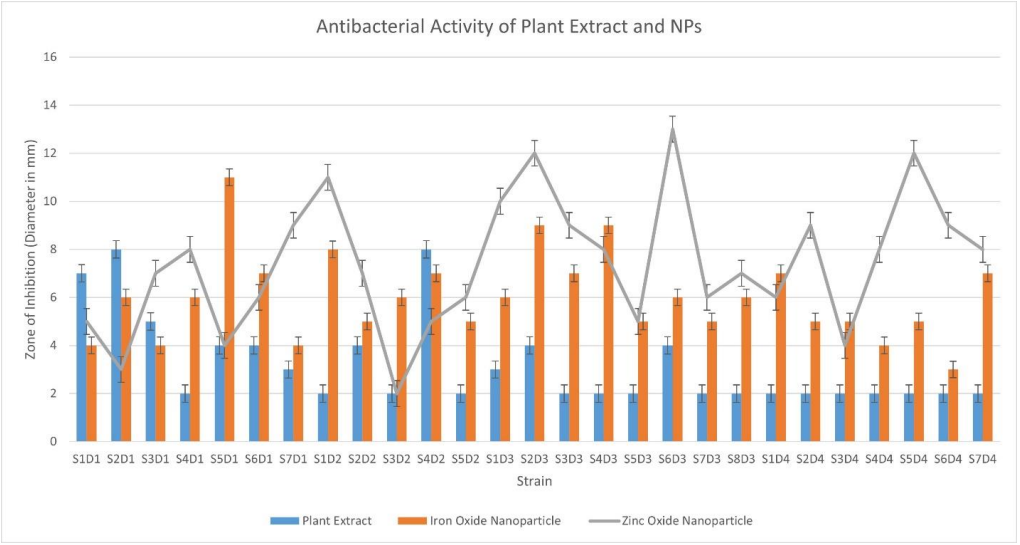


Fig. 2a. Antibacterial activity of plant extract and NPs

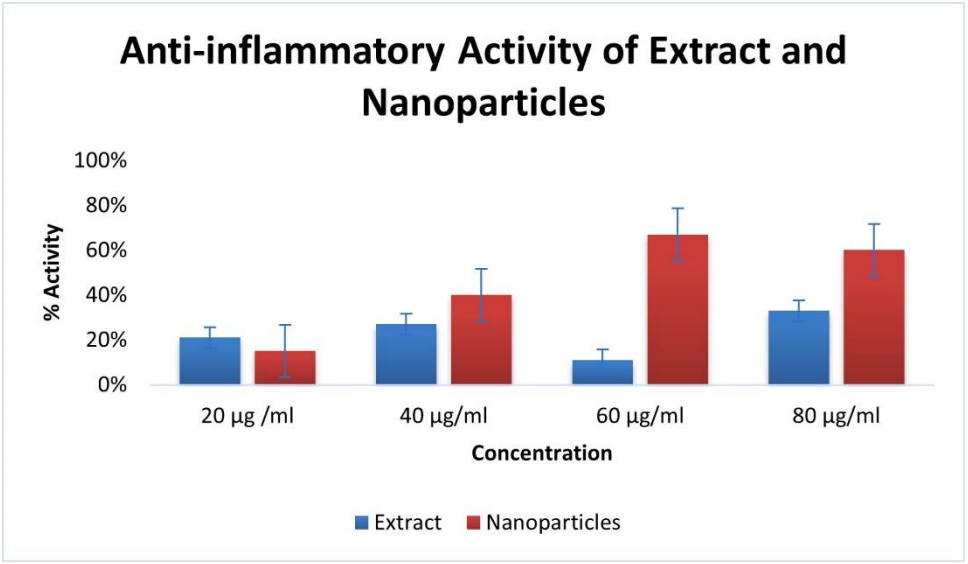
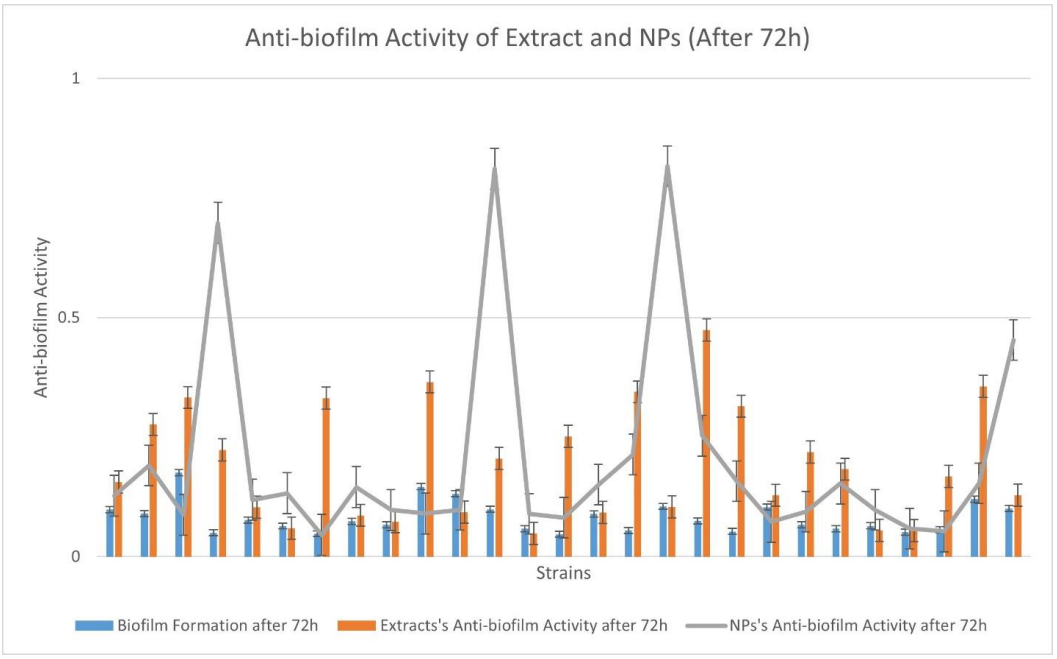


Fig. 2b. Anti-inflammatory activity of plant extract and NPs



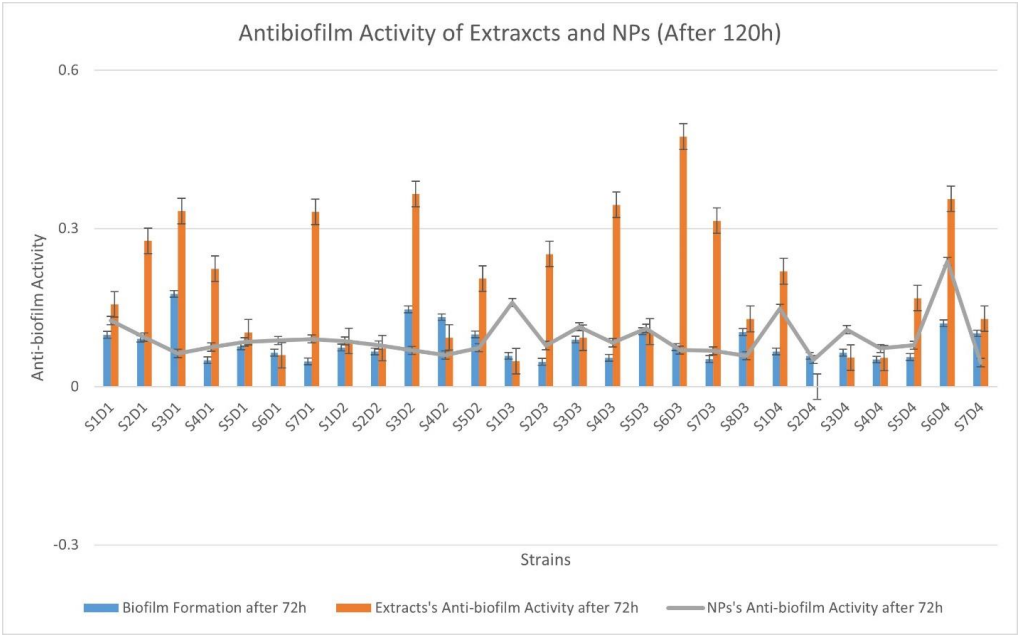


Fig. 2c and 2d: Anti-biofilm activity of plant extract and NPs (After 72 and 120 hours)

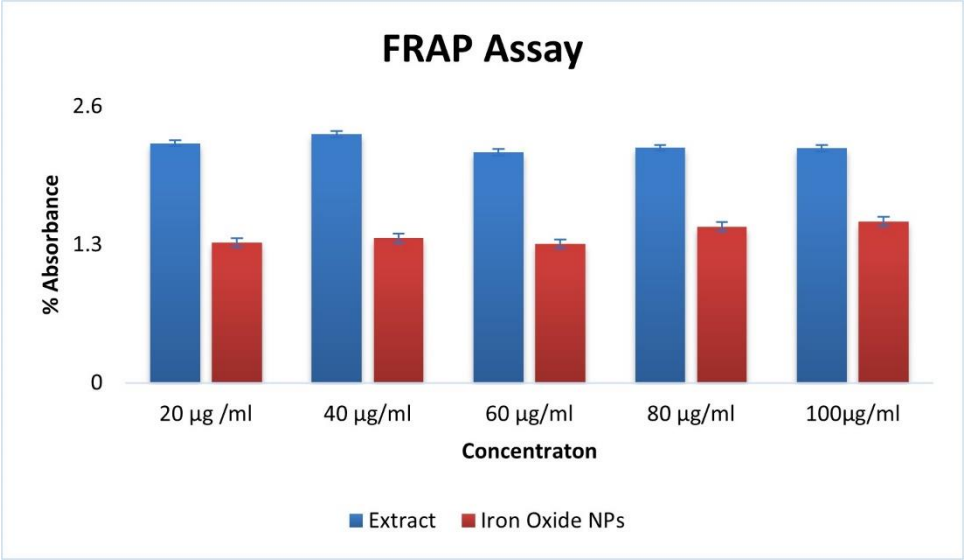


Fig. 3. Antioxidant potential of plant extract and Fe_3O_4 NPs

DISCUSSION

Food-borne pathogens are one of the main cause of infections, encountered in the population of any age group. Drug resistance is continuously booming in microbes and there are multiple factors involved in this phenomenon like the biofilm formation, excess dosage, and misuse of antibiotics. More than 80% of the bacterial species live by the formation of biofilm, which is extracellular polymeric substance, forms a protective layer around microbial communities and helps the bacteria to escape from action of drug or antibiotics. Thus, the infections are becoming difficult to treat. As biofilms are formed in five different steps, so if at any step its formation is interrupted by a strong agent, it will ultimately lead to stop their growth. Under the consequence of these prevailing circumstances, sources other than antibiotics are thought to be potential to ameliorate the infections. More than 5,700 species of the medicinal plants are there in Pakistan. Nanotechnology and medicinal plants are found to be very effective in this regard and are thus being employed for treatment purposes. Nanotechnology is a promising tool for treating the ailments and infections, using nanosized materials applicable in medicine, agriculture, dairy industry and fisheries, drug delivery system and fabric industries etc. Nanoparticles can either be metallic in nature or can be synthesized from plants or they can be bio-metallic NPs i.e., they are synthesized from microbes like bacteria. Aim of this study was the isolation of foodborne pathogens and evaluation of

the efficacy of plant extract and metallic nanoparticles against them effect.

Samples were collected from different food items like meat, milk, vegetables, dry fruits etc. They were serially diluted and spread on two different media (Nutrient Agar and SS Agar). A total of twenty-seven strains were isolated which were streaked and re-streaked to obtain pure colonies. Their colony morphology was observed on different media like EMB and MacConkey Agar. They were then subjected to biochemical characterization. Isolated strains belonged to *Salmonella, Shigella, Enterobacter aerogenes, Pseudomonas aeruginosa, Staphylococci, Escherichia. coli, Klebsiella pneumoniae,* Coagulase positive *Staphylococci* and non-pathogenic *Shigella spp.* Similar results were reported in literature by (Abdaslam et al., 2014; Al-Hilua & Al-Shujairib, 2020; Bantawa et al., 2019; Dib et al., 2018; Tassew et al., 2010).

Gelatin hydrolysis test was performed. Certain microorganisms produce (proteolytic extracellular enzyme) gelatinase, which is involved in liquefaction process and hydrolyzes protein to amino acids. In this study only strains S3D1, S5D1 and S7D1 gave the positive results which means that they have the enzyme gelatinase. Biofilm formation ability of the strains was checked in 96 well micro titer plate. The strains growing could form biofilm in the wells of polystyrene plate. Biofilm or slime producing ability of the strains were checked by Congo red agar method, the slime production was best as the media is supplemented with sucrose. The slime producing ability of the strain depends on the availability of the sucrose. Without sucrose there were a less amount of slime is produced. For slime production test, Black coloration of strain in the presence of Congo red dye and sucrose indicates the strong slime production of the strain. Strain S4D1 showed the maximum sensitivity against ceftriaxone (CRO). Antimicrobial susceptibility test was done by Kirby-Bauer Disc Diffusion method to check if isolated strains were resistant to antibiotics. Most strains were resistant (R) to the thus these were found to be the most exhausted antibiotic which did not give any zone of inhibition. However, CRO, ciprofloxacin, erythromycin and Levofloxacin were the antibiotics for which most strains were found susceptible. Strains were also found sensitive to antibiotics like amoxicillin, ampicillin, and amikacin. Similar results were reported by (Ardila & Vivares-Builes, 2022; Ben Mhenni et al., 2023; Pino-Otín et al., 2022; Singhal et al., 2023).

Eucalyptus plant was selected in this study as it was reported in literature ((Efdi et al., 2023; (Mousa et al., 2023; Serag et al., 2023; Song et al., 2022) that this medicinal plant has important application in the treatment of foodborne pathogens. Extract was prepared from the leaves of selected plant and then phytochemical screening of this extract was done. It was found to have Carbohydrates, Reducing sugars, Flavonoids, Phenolic Compounds, Tannins and Quinones. Similar results were also cited in previous studies (Kwansa-Bentum et al., 2023; Lenny et al., 2023; Obembe, 2023). Two different types of metallic nanoparticles were prepared i.e., ZnO NPs and Fe_3O_4 NPs. It was reported in literature that these had antibacterial, anti-inflammatory, antibiofilm and antioxidant properties (Gul et al., 2023; Hamk et al., 2023; Lee et al., 2023; Murali et al., 2023; Mushtaq et al., 2023; Patil et al., 2023; Smaoui et al., 2023). MIC of Fe_3O_4 NPs was also evaluated, and it was found to be 0.004 µg/ml which was the lowest concentration of an iron oxide nanoparticle that is required to inhibit the growth of a specific bacterial culture.

If we talk about antibacterial activity, the order observed for it, with (mm) diameter of zones of inhibition was as: Fe_3O_4 NPs(11-13mm) >ZnO NPs (7-8mm) >*Eucalyptus* extract (2-3mm). So, metallic nanoparticles showed high antibacterial activity in comparison to plant extract and Fe_3O_4 NPs were found as best candidate for this activity. That's why, Fe_3O_4 nanoparticles were selected for further studies. The anti-inflammatory activity of the extract and nanoparticles was also analyzed. The results revealed that Fe_3O_4 NPsare the best anti-inflammatory compounds. The anti-inflammatory activity of the nanoparticles was seen to be directly proportional to the concentration and highest activity observed was 67% at 40 µg/ml. Anti-biofilm assay was performed using 96-well micro titer plate. 10 µL of the stress was given to the biofilm growing strains and anti-biofilm activity was checked. Fe_3O_4 NPs also performed the highest antibiofilm activity after 120 hours of incubation. Antioxidant assays (FRAP and DPPH) were performed. In FRAP assay, absorbance is directly proportional to antioxidant activity while in DDPH assay it is inversely proportional. In FRAP antioxidant assay, highest activity was performed by *Eucalyptus* extract with recorded absorbance of 1.07. For DDPH assay, the highest antioxidant activity was performed by Fe_3O_4 NPs and their absorbance recorded was 1.43.

CONCLUSION

Fe_3O_4 NPs synthesized from *Eucalyptus camaldulensis* extract have displayed the largest zone of inhibition, the highest percentage of anti-inflammatory activity, the highest antioxidant activity, and the maximum biofilm inhibition after 120 hours of incubation against foodborne pathogens. It is therefore concluded that Eucalyptus mediated iron oxide nanoparticles are potent effective alternative cutting edge therapeutic agents against foodborne pathogens.

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