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REVIEW ARTICLE

Development of bio healing band aids using PHB-peg amalgamated with essential oil blends

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Abstract

Plastic waste exacerbates the major environmental issues that are now harming humans, animals, and the planet. PHB is the most promising alternative to plastic made from petrochemicals and biodegradable materials. This is due to their biocompatibility, biodegradability, UV resistance, and flexible properties, which make them an ecologically friendly substitute for synthetic polymers. The high degree of crystallinity and extreme stiffness and brittleness of PHB restrict its use. To get around this limitation, the PHB is fed plasticizers. Poly (ethylene glycol, or PEG), when mixed with other polymers, may have a plasticizing effect. Additionally, it is non-toxic and biodegradable. According to the literature, PEG reduces the interactions between PHB structures inside molecules. The aim of this work was to develop new antimicrobial films (PHB and PEG-based films), amalgamated with essential oils, and to determine its antimicrobial potential against five pathogenic microorganisms, namely *Staphylococcus aureus* MTCC 740, *Pseudomonas aeruginosa* MTCC 741 and hospital sample isolates of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. The essential oils used in this study are Garlic oil, Clove oil, Thyme oil and Eucalyptus oil. The methods performed are Bacterial staining, antimicrobial activity of Essential oils, Anti-Quorum sensing assay and *Violaceum* pigment extraction. Among four essential oils, Thyme oil incorporated with PHB and PEG based films showed better antimicrobial activity than the others.

Keywords: Essential oil, Plastic, Polyhydroxybutyrate (PHB), Polyhydroxyalkanoate (PHA), Polyethylene glycol (PEG)

Introduction

The grave environmental dangers that now threaten people, animals, and the ecosystem are being made worse by plastic waste. Depletion of non-renewable resources and corresponding greenhouse gas emissions, as well as a lack of efficient post-use circularity solutions and incorrect disposal of these non-biodegradable materials, are all directly causing serious environmental problems. Alternative biopolymers that are ecologically friendly and biodegradable, such as Polyhydroxyalkanoates (PHA) and its Homopolymer, Polyhydroxybutyrate (PHB) (Harris et al., 2021).

Alcohols, Aldehydes, Esters, Ethers, Ketones, Phenols, And Terpenes are only a few of the complex, volatile, and aromatic chemical components included in essential oil (Chen et al., 2017; Zhang et al., 2018). These essential oils, which

are often used in aromatherapy, are distilled from plant parts. The scientific literature has conducted in-depth examination of essential oils' impact on respiratory ailments. Numerous studies have looked at the use of essential oils for general health issues, antibacterial applications, as well as inhalation in traditional practices. Even if there has been a rise in interest and a chosen path in the area of essential oil research, the majority of the work that has been published is focused on determining the potential of a certain essential oil. But when used therapeutically, essential oils are often combined with other substances (Sharmeen et al., 2021; da Silva et al., 2020). This is because the practice of blending several essential oils to provide a more potent therapeutic effect is the foundation of aromatherapy. People who actively seek out CAM treatments to prevent or treat chronic infections spend $25 billion yearly, according to research on the use of complementary and alternative medicines in industrialized nations, including aromatherapy. Growing CAM therapy usage necessitates more study to assess the effectiveness, safety, and caliber of these processes and goods.

Numerous studies have been conducted on the anti-inflammatory effects of essential oils. Using essential oils with anti-inflammatory characteristics, which have been demonstrated to lower swelling and edema brought on by respiratory infections, may help with wheezing, congestion, and breathing issues. Although there is no evidence to support this claim, aromatherapy often uses essential oils for their anti-inflammatory properties. Since the inflammatory process plays a large part in respiratory tract infections, it is important to discover if utilizing a combination of essential oils may have an effect on the infection process (de L et al., 2020; Zhang et al., 2020).

A significant degree of toxicity has also been linked to essential oils, particularly when compared to other organic goods. The United States Food and Drug Authority (US, FDA) only accepts individual agents not mixtures on its list of items that are "Generally Regarded As Safe" (GRAS) (Abdelaal et al., 2021; Barua et al., 2020). It is possible for the interaction of the blended essential oils to enhance toxicity or to reduce these effects. In particular when breathed, small amounts of essential oils have the potential to produce hazardous consequences. Despite these results, essential oils continue to be combined through cutaneous or inhalation administration and are the most widely utilized type of supplementary therapy. Additional study is necessary to prove the therapeutic efficacy of utilizing essential oils through the respiratory system since there isn't enough proof that doing so will have anti-inflammatory and anti-bacterial benefits, and doing so also has a higher risk of toxicity.

Therefore, the present study aims at development of bio healing band aids using PHB-PEG incorporated with essential oil blends.

Literature Review

In a study by Zhang et al., 10 Essential Oil (EO) components were evaluated at sub-MIC concentrations against the generation of biofilms against *Erwiniacarotovora* and *Pseudomonas fluorescens*, two organisms that cause vegetable rotting. "*Chromobacteriumviolaceum CV026*" was selected as the Quorum Sensing (QS) biosensor bacteria (CV026). Researchers detected the production of Exopolysaccharide (EPS), the formation of biofilms, and motility. All of the EO components under investigation were found to inhibit QS, including CV026, *"E. carotovora", and "P. fluorescens"*. All EO components inhibited biofilm development by blocking their mobility and EPS production. Salicylic acid and thymol demonstrated the greatest inhibitory effects on respective biofilm formation processes of "*P. fluorescens" and "E. carotovora"*. Initially, the QS inhibitor hexanal was shown to limit the growth of biofilm. In the food industry, utilizing anti-QS EO components to prevent the establishment of biofilms may improve the safety and quality of vegetables (Zhang et al., 2018).

In research by Olivero-Verbel and coworkers, the technique put out by (Choo et al. 2006) and McLean et al. was followed while conducting the assays (2004). The mutant CV026 was capable of producing violacein when C6-HSL was added externally. The cells were treated concurrently with various doses of the oils, and the amount of violacein production was quantified to examine the inhibitory effects of *Lippiaalba* essential oils on this activity. Using the disc diffusion technique in Mueller-Hinton agar, the antibacterial activity of "*L. alba"* essential oils against "*S. aureus ATCC 25923"* was evaluated. To reactivate the bacterium, LB was used to grow the strain S. aureus ATCC 25923. 5 liters and 10 liters of each pure essential oil were placed in wells before the bacterium was infected all over the agar plate's surface. Next, the plates were incubated. The most powerful QS inhibitors were found to be two of *L. alba's* essential oils. Small impacts on cell proliferation were also seen for both oils. Additionally, "*S. aureus ATCC 25923"* was most effectively inhibited by the

geranial chemotype oil. These findings imply that L. alba essential oils have potential as QS modulators and have antibacterial effects on S. aureus (Olivero-Verbel et al., 2014).

Turco et al. looked at the state-of-the-art in PHA processing, providing light on environmentally friendly and economically viable custom solutions intended to modulate and enhance polymer capabilities. They looked at several trailblazing instances in this area and then discuss the opportunities and difficulties associated with using them. A PHA-based industry's development also necessitates the creation of cost-effective manufacturing methods, thus this research will look at documented instances of this economic element and the most recent advancements in process sustainability (Turco R et al., 2021).

A focus on starch-and-polymer blends was made when Zhong et al. examined the functioning of biodegradable polymers. The food packaging industry has a serious challenge with microbial activity as well. A useful option for protecting food against the development and spread of microbes is therefore provided by utilizing antimicrobial chemicals or polymers to build barrier-enhanced or active packaging materials. A discussion of the mechanical, barrier, and other properties of biodegradable polymers is also included (Zhong et al., 2020).

Methodology

Design

In the present study, hospital isolates of pathogenic bacteria are collected and then used for testing the antimicrobial activity of essential oils. Antimicrobial activity of essential oils was carried out which was then followed by Anti-quorum sensing assay. Furthermore, the preparation of blends of PHB and PEG was performed and the evaluated the antimicrobial activity of PHB films incorporated with essential oils.

Sample collection

Hospital isolates "*Escherichia coli*", "*Pseudomonas aeruginosa*", "*Staphylococcus aureus*" and "*P.aeruginosa MTCC 741*", "*S. MTCC 740*" were used for antimicrobial activity testing. The bacterial cultures were grown on the nutrient agar medium and were maintained at 4°C. The bacterial cultures were subculture once in 15 days to maintain viability.

Data analysis

Sudan black B staining: The manufacture of PHA by *Bacillus megaterium Ti3* was identified using *Carbolfuchsin* and *Sudan black* B stain. *Carbolfuchsin* stain was used to determine if the isolate generates PHA intracellularly. Carbolfuchsin stain was used to stain each isolate for 45 seconds on a thin smear. In isolates that were able to manufacture it, dark-colored PHA granules were visible intracellularly. The existence of bacteria that make PHA was confirmed further using the Sudan black b staining method. It was created using a Sudan black B stain 0.3% solution (w/v) in 60% ethanol. The cultures were spread and heated to adhere them to the glass slides. After cleaning with water and counterstaining for 5 minutes with 0.5% safranin, the samples were viewed under a 100X light microscope after being stained for 10 minutes with Sudan black B solution.

Antimicrobial activity of essential oils: The essential oils in this research had their potential to fight against microbes tested. We utilized "clove oil", "garlic oil", "eucalyptus oil", and "thyme oil" as our essential oils. With the use of the well diffusion technique, the essential oils' antibacterial activity was identified. The following concentrations of analytical grade ethanol (v/v, alcohol/oil) were used to dilute the essential oils: 1/0, 1/1, 1/10, and 1/20. Mueller Hinton agar plates were created under aseptic circumstances, and bacteria homogenously inoculated and swabbed on the plates. Wells were then drilled in the agar medium using a sterile cork borer. Zones of inhibition were evaluated after all the bacterium plates had been cultured at 37°C for 24 hours.

Anti-quorum sensing assay: The ability of the essential oils to detect Anti-Quorum activity was tested. Thyme oil, Eucalyptus oil, and clove oil were among the utilized essential oils. The well diffusion technique was used to assess the essential oils' anti-quorum sensing potential. Analytical-grade ethanol was used to dilute the essential oils at concentrations of 1:100 and 1:1000.

Aseptic techniques were used to make Mueller Hinton agar plates. Using sterile swabs and 100 microliters of the culture, a homogenous inoculum of *Chromobacteriumviolaceum* was created and inoculated on the agar plates. The swabs were then used to distribute the plates. A sterile cork borer was used to drill four wells in agar medium. One component cinnamon oil was mixed with one thousand parts of pure alcohol to create the positive control. Zones of inhibition on all the bacterium plates were assessed after a 24-hour incubation period at 37°C.

***Violaceum* pigment extraction:** Each essential oil dilution was prepared by diluting them in absolute alcohol. The dilutions prepared were 1-part oil in 200 parts alcohol, 400 parts alcohol, 600 parts alcohol and 800 parts alcohol. 50 microliters of each of these dilutions of each oil were mixed with 5 ml of LB broth. Subsequently 50 ml of overnight cultures were inoculated in each tube. Positive control was prepared by adding 1-part dilution of cinnamon oil in 200 parts alcohol. Negative control was prepared by adding 50 ml of culture to 5 ml broth without any oil. Tubes were incubated for 24 hours.

200 microliters of culture were taken in the Eppendorf vials, it was centrifuged at 13,000 rpm for 5 minutes. It was thoroughly mixed with 10% SDS for 5 seconds using a vortex. This is then incubated at room temperature for 5 minutes. 900 microliters of saturated butanol solution were added (butanol and water mixed in the ratio 5:1) to the lysate and thoroughly mixed using a vortex for 5 seconds. It was centrifuged at 13,000 rpm for 5 minutes.

The butanol and water got separated into 2 different layers, the butanol was extracted and transferred to separate vials. The OD of this butanol was taken at 440 nm, to check the amount of pigment extracted using microtiter plates.

Preparation of blends of PHB and PEG: PHB was dissolved in chloroform and turned into a solution. Heat application may cause the PHB to melt, enabling the creation of smooth sheets from the PHB. The PHB sheets that were created were fragile and transparent. By including PEG during dissolution, this was avoided.

When formed into sheets, PHB powder is transparent and fragile. These characteristics limit the use of PHB in numerous sectors. Consequently, polyethylene glycol was used as a plasticizer to modify the PHB sheets (PEG 300). PHB is soluble in organic chloride solvents, therefore boiling chloroform to a high temperature created a PHB solution that was readily cast to create PHB sheets. By partially evaporating the chloroform during a further five minutes of heating, the solution may be concentrated. PHB's viscous solution may be formed into sheets via moulding. The drying procedure makes sure that the flammable chloroform is completely evaporated, while ageing provides stability. The altered PHB sheets had a transparent and pliable appearance.

PEG-modified PHB sheets have a transparent, flexible appearance. Different essential oil quantities were added to the PHB-PEG sheets, and their antibacterial effectiveness was evaluated against hospital isolates of "*P.aeruginosa*", "*S.aureus*", and "*E.coli*", including "*P.aeruginosa MTCC 741*" and "*S.aureus MTCC 740*".

Antimicrobial activity of PHB films incorporated with essential oils: Contact methods were used to test the antimicrobial activity of these essential oil- incorporated PHB+PEG blends as well as plain blends against Hospital isolates of "*Pseudomonas aeruginosa*", "*S.aureus*" and "*E.coli*", "*P.aeruginosa MTCC 741*", "*S.aureus MTCC 740*".The discs were placed on duplicate petri plates containing these organisms and incubated for 0 and 24 hours each. Plates were checked for zones of inhibition.

Results and Discussion

Antibiotic sensitivity test

Antibiotic Sensitivity Test of Hospital isolates of "*P.aeruginosa*", "*S.aureus*" and "*E.coli*", "*P. aeruginosa MTCC 741*", "*S.aureus MTCC 740*" was performed to check for their susceptibility against various antibiotics.

Hospital isolates of *P.aeruginosa, S.aureus* and *E.coli* exhibited resistance to almost all of the antibiotics except for Gentamicin which was effective against Hospital isolates of "P*. aeruginosa*" and "*E. coli*". Chloramphenicol was effective against *E.coli*. The hospital isolates showed higher resistance to antibiotics when compared to the MTCC cultures indicating that it has a higher degree of pathogenicity as shown in Tab. 1.

Table 1: Zone size of isolates in mm against antibiotics

	A (10 mcg)	Cpm (30 mcg)	Cl (30 mcg)	Ce (30 mcg)	C (30 mcg)	Clp (5 mcg)	S (10 mcg)	G (10 mcg)	TE (30 mcg)	E (15 mcg)
S.aureus	22	31	21.5	25.5	28	30	19	25	31.5	26
	R	S	S	S	S	S	S	S	S	S
P.aeruginosa	0	6.5	18	18	0	26.5	12.5	23.5	10	8.5
	R	R	I	R	R	S	I	S	R	R
S.aureus (Clinical isolate)	14.5	18.5	14.5	14.5	0	14	0	14	11.5	8.5
	R	R	I	I	R	R	R	I	I	R
P.aeruginosa (Clinical isolate)	0	0	20.5	13.5	0	30.5	16	21	8.5	9.5
	R	R	I	R	R	S	R	S	R	R
E.coli	0	7.5	0	0	24.5	9	8.5	14.5	11	15
	R	R	R	R	S	R	R	S	R	R

Antimicrobial sensitivity assay

In addition to being employed in herbal medicine and a variety of culinary preparations as flavour enhancers, thyme and clove oil extracts are known to have some antibacterial properties. The extracts include thymol, terpenes, eugenol, flavones, glycosides of phenolic monoterpenoids, aliphatic alcohols, and other antibacterial compounds, albeit the exact mechanisms of action of these substances remain unknown. Following *S.aureus*, thyme oil had the highest antibacterial efficacy against *E. coli*, with the largest zone of inhibition.

Anti-quorum sensing assay

Quorum Sensing (QS) is a bacterial communication system that allows bacteria to exhibit multiple survival or virulence features, resulting in increased resistance. *Chromobacteriumviolaceum* is a regularly utilised strain that emphasises bioactive compounds' anti-QS activity. In this study, we intended to investigate whether three Essential Oils (EO) may have anti-QS action.

Quorum Sensing (QS) regulates the transcription of particular genes important for antibiotic synthesis, biofilm development, cell division, bioluminescence, and other functions in *C. violaceum*.

Thyme Oil was the most effective against *C. violaceum* in terms of anti-quorum sensing activity. The QS mechanism is reliant on the synthesis, release, and absorption of Autoinducers (AIs) in the environment, the concentration of which is proportional to the density of secreting bacteria. This intercellular communication is facilitated by AIs, extracellular signalling molecules that accumulate in the environment in proportion to cell density. "*Agrobacterium*", "*Aeromonas*", "*Burkholderia*", "*Chromobacterium*", "*Citrobacter*", "*Enterobacter*", "*Erwinia*", "*Hafnia*", "*Nitrosomonas*", "*Obesum bacterium*", "*Pantoea*", "*Pseudomonas*", "*Rahnella*", "*Ralstonia*", "*Rhodobacter*", "*Rhizobium*", "*Serratia*", and "*Yersinia*" have all been studied for the QS phenomenon.

Sudan black staining

Using sudan black staining, the strain "*Bacillus megaterium*" was tested for the presence of PHB granules in this investigation. When 48 hours of cultures were treated to sudan black staining, the bacterial smear revealed the presence of PHB granules of black colour inside pink colour vegetative cells as shown in Fig. 1.

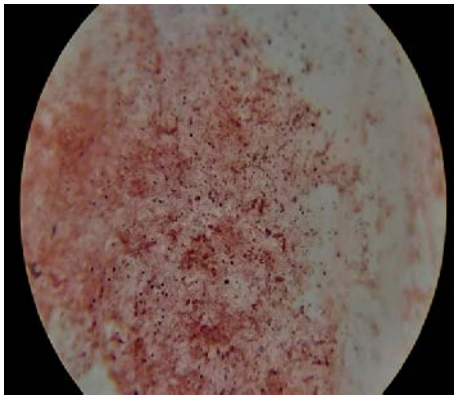

Figure 2. Sudan Black Staining of *Bacillus megaterium* showing PHB granules.

Antimicrobial assay of PHB films loaded with essential oils

Non-clinical S*. aureus* was shown to be the most sensitive to PHB-PEG thyme oil blends at doses of 0.02%, 0.03%, 0.04%, and 0.05% in this investigation. The films, comprising 10%, 20%, and 30% (w/w) essential oils, were microbiologically evaluated against six species using the agar-diffusion technique. The microbiological growth of any microbe examined was not inhibited by the films containing melaleuca and citronella. In contrast to the films containing 20% and 30% cinnamon, which prevented the development of practically all bacteria except *Salmonella sp*.

Conclusions

Due to the antibiotic resistance shown by different organisms it has become one of the major concern in the modern days, this is the reason why natural substances came into the picture, essential oils extracted from different plant sources have been recorded to show good antimicrobial activity, in this present study, an attempt was made to show some of the antimicrobial sensitive essential oils and blended it with PHB-PEG blends to prepare bio healable band aids which is economic as well as doesn't get resisted by the MDR organisms.

The PHB films were incorporated with essential oils of garlic, clove, thyme and eucalyptus. As the films incorporated with thyme presented the highest antimicrobial activity followed clove against *Staphylococcus aureus* MTCC 740, *Pseudomonas aeruginosa* MTCC 741 and hospital samples *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*, while others that did not present any antimicrobial activity for the studied microorganisms.

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