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Research Article

INCORPORATION OF *CLITORIA TERNATEA* SEED EXTRACT INTO BIOPLASTIC SHEETS HAVING INDUCED PLASTICITY AND THEIR ANTIMICROBIAL ACTIVITY AGAINST MULTI-DRUG RESISTANT CLINICAL PATHOGENS

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ABSTRACT

The concept of bio-plastics having antimicrobial activity is becoming attractive in modern medical field. The objectives of our study were, to check the multi drug resistance of human bacterial pathogens, to prepare *C. ternatea* seed extract, phytochemical analysis and to demonstrate both antimicrobial activity and activity against genetic materials, to improve the plasticity of bio-plastic (Polyhydroybutyrate), to incorporate the seed extract into PHB sheets with induced plasticity and their killing effect on MDR pathogens. In methods, antibiotic susceptibility of the test pathogens was determined by Kirby Bauer technique. The antimicrobial activity of extracts was checked by well diffusion method and the extracts were treated directly with genetic materials and followed by agarose gel electrophoresis. The plasticity of PHB sheets was improved by PEG with the application of heat. The ethanolic extract was incorporated into PHB using PEG as a carrier in chloroform. A halo test and contact method was adopted to evaluate the antimicrobial activity of PHB. In results, ethanolic extract had shown good antimicrobial activity against MDR pathogens than methanolic extract, and the phytochemical analysis revealed the presence of antimicrobial compounds in the extract. The extract was effective against both DNA and RNA. The halo test was not effective and the contact method gave less than 30 bacterial colonies when plated with an elution made from antimicrobial agent incorporated PHB with bacterial pathogens. It can be concluded that the plant extracts are good antimicrobial agent, and the antimicrobial bio-plastic is effective under wet environment because positive contact method.

Keywords: Bio-plastic, Polyhydroybutyrate, MDR, Halo, PEG

INTRODUCTION

Infections that are acquired while a person is in a hospital, long-term care facility, or other health care setting have been a challenge for many years. Hippocrates, known as the father of medicine, used vinegar to irrigate open wounds and wrapped dressings around wounds to prevent further injury. The concept of wound healing remained a mystery, as highlighted by the famous saying by Ambroise Paré (French military surgeon, 1510-1590), "I dressed the wound. God healed it. The antibiotic, Penicillin first was used clinically in 1940 by Howard Florey and a new era in the management of wound infections commenced.

Unfortunately, eradication of the infective plague affecting surgical wounds has not ended because of the insurgence of antibiotic-resistant bacterial strains and the nature of more adventurous surgical intervention in immuno-compromised patients and in implant surgery. The confined population and the widespread use of antibiotics have led to the development of antibiotic-resistant strains. These strain methicillin resistant *Staphylococcus aureus* (MRSA), named after the antibiotic treatment that was developed in 1960 to treat penicillin-resistant strains. Infections caused by MRSA are frequently resistant to a wide variety of antibiotics and are associated with significantly higher rates of complications and death (morbidity and mortality)**[1]**.

The term 'antibiotic' was first coined by the French bacteriologist Vuillemon and it means (anti) against (biosis) life. It is ironical that while antibiotics have been useful to human kind in life threatening situations, today its indiscriminate use may prove to be against human life. Post-operative wound infections are the common problems which delay the recovery and often increases length of stay and require extra resources for investigations, management and nursing care. Therefore, its prevention or reduction is relevant to quality patient care. Studies support the concept that a reduction in post-operative wound infection is directly related to increased education and awareness of its causes. One of the best methods to prevent post-operative wound infections are to provide wound healing agents at the surgical sutures itself, the agent should act like an antibiotic, so that it can prevent the infections. Wound infections are also a common problem in developing countries such as South Asia countries due to poor hygienic practices and care to the wounds.

Use of polymers in surgical sutures, band aids and other medical devices have been attractive due to low density, resistance to chemical attack and resistance to weather / Ultra Violet. The harmful effects of synthetic plastics in the environment have been of increasing concern in recent years. The ecological awareness impelled development of new, eco-friendly materials, especially for single-use plastic items. One such material is a Pol-hyhydroxy-alkanoates (PHAs), a family of bacterial polyesters, are formed and accumulated by various bacterial species under balanced growth conditions. PHAs have thermo mechanical properties similar to synthetic polymers such as polypropylene, but are at present known as constituents of the bacterial storage polyesters. The first PHA detected and studied was PHB. It has recently received high attention in utilizing PHB as polymeric materials in medical field **[2]**.

From previous studies it is observed that, PHB does not produce any immune response and the body does not reject the implantation. Thus PHB is biocompatible and well suited for implantation medical uses. PHB has several medical applications such as durable bone implants, for dressing of wounds. Now a day's PHB coated or PHB surgical sutures are evolved. The main advantage of PHB sutures is that it is biocompatible and biodegradable and can be inserted into the human body and does not have to be removed again. This property is useful especially in open surgeries **[3]**.

But just PHB has no known antimicrobial effect which can prevent the post operative and wound infections. Medicines or antibiotics can be incorporated with the PHB sutures, so that it can prevent the infections at the wound. Antibiotics and chemicals incorporated sutures can be used. But due to the evolvement of MDR pathogens we cannot think about antibiotics incorporated sutures again.

In India it was a usual practice to use plant extracts for wound healing and as antibiotic. Now it is the time to think back and adopt our ancestor's practices. Innumerable numbers of plants are available which can act as, antimicrobial agent, anticancer agent, wound healing agent etc. As the plant extracts are natural compounds, they will be suitable than antibiotics and other human made chemicals. PHB can be incorporated with these plant

chemicals and can be used in sutures. But PHB having many applications in medical field like, band aids, surgical implants etc, and all these equipments can be incorporated with the plant extracts and we can expect a healthy surgery and hospital practices **[4,5,6]**.

UTI is also a common infection which is faced by many of the women around us. The organism responsible for 80% of UTI is *Escherichia coli*. In menstrual period, women become more vulnerable than usual because of decreased resistance and the warm and humid pubes creates a good environment for the growth and infection of bacteria. The infections may be due to the irritation from the napkins. The materials of sanitary napkins produced by formal manufacturers are tested by special safety organizations so that there are basically no safety problems. But allergy to the napkins is a common problem faced by lot of women. Plastics are the main making material of the napkins, and we can think about PHB sheets incorporated napkins to avoid allergic reactions. When the PHB is incorporated with plant extracts that will add up its quality and it will get a killing effect on the *E. coli* and can prevent the UTI during menstruation **[7]**.

Since 1985, the trend has been towards thinner sanitary pads using less wood-based pulp and increased use of synthetic super absorbents made from petroleum. Aperture plastic film is mostly used as a cover on sanitary pads and liners today, and is often called the "Dry-weave top sheet". In reality, it is simply just loaded polyethylene film - or plastic with holes. European and North American consumption of this type of sanitary pad is the highest in the world, more than a third of total worldwide consumption of 45 billion units. Every year, in Britain alone, we would need to dig a hole 300 feet wide and 300 feet deep to bury the used sanitary pads and tampons that women throw away. Unfortunately, this synthetic material is being used more and more in other products such as baby wipes, wet wipes, feminine wipes, tumble dryer cloths, diapers, incontinence pads and moist toilet tissues. No doubt, all ending their "useful lives "flushed down the toilet or in a landfill site. With the flush, lots off pathogens are also adding to our environment.

In recent years, with the new development of PHB it is possible for these plastics to be replaced. This biodegradable material can be used for most products where plastics are being used to include: sanitary pads, liners, diapers and wet and dry wipes instead of the synthetics derived from petroleum used exclusively today. Antimicrobial or Antibacterial plastics represent a small segment of the plastics additives sector, and are projected to account for about 20% of the global plastics market in the near term. Traditionally, the industrial sector was considered the major hub for antifungal plastics, particularly with regard to construction and packaging industries. However, rising public awareness about contamination and infections, prompted significant demand for antibacterial plastic products in other markets such as healthcare and consumer products.

The United States stands tall as the largest global market, as stated by the new research report on Healthcare Antimicrobial Plastics. Asia-Pacific is poised to emerge as the fastest contender, expanding at the strongest CAGR of 16% through 2017. Asian markets such as China and India are potential markets for antimicrobial products due to factors such as high population density, and low income that lead to unhygienic conditions and possibility of greater microbes led infections. China along with US and Europe represents the largest worldwide markets for plastic additives.

The application of antimicrobial plastics in healthcare is expanding into medical implants and other biomedical devices. The need to prevent HAI (hospital-acquired infection) and related complications is a major impetus for the development of novel biocides, antimicrobial polymer technologies, and innovative applications deploying these solutions

Many microorganisms like *Bacillus* sp., *Azotobacter* sp., *Alcaligenes* sp., *Synechocystis* sp., *Pseudomonas* sp., *Ralstonia* sp., *Agrobacterium* sp., can accumulate a good amount of PHB in low cost. From previous studies it can see that *Rhizobium* sp. can accumulate PHB of 1.38 – 1.40 % (w/w) **[8]**, *E. aerogens* can accumulate 16.66-96.25 % (w/w) of PHB **[9]**. PHB production and extraction in high amount costs a lot. Based on this problem, lowering production cost is the only way to get PHB with lower price. One of factors included in production cost is raw material cost.

There are some disadvantages of using PHB as plastic material since its tendency to be brittle. But form previous studies it can be seen that by incorporating plasticizing materials like PEG, it is possible to improve the plasticity of PHB. Many studies are going on in incorporating PHV with PHB, which can reduce the brittleness of PHB **[9]**.

Organic-metallic biocides are the most commonly used additives for plastics, with the arsenic-based Oxybisphenox Arsine (OBPA) accounting for a major share of the global consumption. However, concerns about the long term environmental damage and human toxicity caused by heavy metals are leading to a shift in preference for more eco-friendly, natural and safe antimicrobials such as inorganic silver-based biocides. Further, the advent of more effective antimicrobials and technological advancements in recent years enable wider applicability to polymers with efficient antimicrobial properties. There is a growing demand in the medical and healthcare sector for non-toxic plastics with enhanced features such as transparency, durability, hardness and antimicrobial activity **[10]**.

The objective of our study is to induce both plasticity and antimicrobial activity to the bioplastic PHB with biocompatible compounds.

MATERIALS AND METHODS

Clitoria ternatea seeds

The fresh *Clitoria ternatea* seeds Chinnavedampatti, Coimbatore, and the species and genus were confirmed from Agricultural University, Coimbatore.

Bacterial and fungal strains

The clinical pathogens were obtained from the Kidney centre, Coimbatore and were preserved on agar slants at 4ºC.The various pathogens used were *Acinetobacter* sp.*, Escherichia coli, Escherichia coli* (mucoid), *Klebsiella* sp.*, Pseudomonas aeruginosa, Pseudomonas fluorescence, Proteus mirabilis, Staphylococcuc aureus, Staphylococcus epidermidis* and *Staphylococcos saprophyticus.*

Culture Media

All the culture media, used during the project were obtained from Himedia Laboratory pvt. Limited, Mumbai, India.

Reagents

The reagents including PHB powder used in the whole work were obtained from Department of Microbiology and Bioinformatics, CMS college of science and commerce, Coimbatore.

Antibiotic Susceptibility of test pathogens

Antibiotic susceptibility of the test pathogens was determined by Kirby Bauer technique. The different groups of antibiotics used against *Acinetobacter* sp.*, E. coli* and *P. mirabilis* (Gram negative Bacilli) are, Penicillins (Ampicillin), Amonoglucosides (Gentamycin), Tetracyclines (Tetracucline), Quinolones (Ciproflaxacin). The different groups of antibiotics used against *Klebsiella* sp. are, ß-lactam (Amoxicillin), Penicillins (Ampicillin). The different groups of antibiotics used against Pseudomonas sp. are, Amonoglucosides (Gentamycin), Amikacin, Quinolones (Ciproflaxacin), ß-lactam (Piperacillin). The different groups of antibiotics used against Staphylococcus sp. are, Chloramphenicol, Tetracyclines (Doxycycline and minocycline) and Oxacillin.

Preparation of extracts of *Clitoria ternatea* seeds

Ethanolic extracts were prepared by maceration of dried powdered seeds (3 g) in ethanol solvent (30 ml) for 3 days at 27ºC. The mixture was then centrifuged at 5000 rpm for 15 min. The macerated extracts were then filtered through No. 1 Whatman filter paper. The supernatant was collected and allowed to dry on a Petri dish and mixed with minimum amount of 95% Ethanol and stored at 4ºC for further use. Methanolic extracts were also prepared as ethanolic extract, by using methanol instead of ethanol in the procedure.

Qualitative phytochemical screening

The extracts were subjected to qualitative phytochemical testing for the detection of major chemical groups **[11, 12, 13, 14, 15, 16]**

Antimicrobial activity of *C. ternatea* seeds extract

Well diffusion method

Antibacterial and antifungal activities of seed extracts were investigated by the well diffusion method. Muller Hinton agar plates were prepared and the homogenous inoculums of bacteria and fungus (3 h old culture) was made and swabbed on the agar plates. Under aseptic condition, wells were bored in agar media using sterile cork borer (3 mm diameter) and 30 µg of each samples were pipette out into wells. All the plates were left at room temperature for 30 min to allow diffusion of the extracts and then incubated at 37°C for 24 h and zones of inhibition were measured. The sensitivity of the microorganism species to the plant extracts was determined by measuring the sizes of inhibitory zones (including the diameter of wells) on the agar surface around the wells. The results are reported as the mean of zones of inhibition ± standard deviation.

Inhibitory action of *C. ternatea* seed ethanolic extracts on genetic material

About 50 ml of LB broth was prepared, inoculated with *Escherichia coli* culture and incubated at 37ºC for 24 h at shaker incubator. DNA isolation was carried out by Potassium acetate method and RNase enzyme was not added in any of the steps during isolation. The isolation was confirmed by performing agarose gel (0.8 %) electrophoresis. The isolated DNA was stored at 4ºC in 40X TE buffer for later use. From the stored DNA 50 µl of DNA solution was taken into a fresh sterile vial and mixed with 25 µg *Clitoria ternatea* extract (in powder form). The mixture was mixed thoroughly by single spin in centrifuge and incubated at 37ºC for 6 h. After incubation the DNA sample was run by agarose gel electrophoresis with Ethidium bromide as DNA staining dye. The gel was observed for the presence or absence of DNA bands under UV transilluminator.

Development of PHB sheets

The PHB (5 g) was dissolved in chloroform (95 ml) under stirring for 8 min at 70ºC to ensure complete dissolution. Additional heating for 5 min at 70ºC was done. The solution was subsequently stored at room temperature for 20 min and vigorously stirred and resulting viscous solution (20 ml) was cast in Petri plates and dried at room temperature till the sheet was formed. Aging was done for 2 weeks for stabilization.

Modification of plasticity of PHB sheets

The PHB sheets (5 g) were re-dissolved in chloroform (95 ml) under stirring and the plasticizer PEG-300 (10% w/w) was added to the mixture and stirred for 8 min at 70ºC to ensure complete dissolution. And the sheets were prepared according to the procedure as previously said.

Incorporation of *C. ternatea* seed extract to PHB sheets

Since the ethanolic extract has shown good antimicrobial activity against the pathogens, we selected ethanolic extract for further work. About 5 g of modified PHB (with PEG) was taken in clean conical flasks with 95 ml of chloroform. Crude ethanolic extract (in powder form) of *Clitoria ternatea* seeds which was 50 % weight of the PHB was taken was added to the same conical flasks and stirred for 8 min at room temperature to ensure complete dissolution. 20 ml of the contents in the conical flasks were poured into each clean sterile Petri plates and the chloroform was allowed to evaporate till the PHB sheet was formed. The PHB sheets were carefully removed and aging was done at room temperature.

Antimicrobial activity of *C. ternatea* seed extract incorporated PHB sheets

There are no suitable methods to evaluate the effect of antimicrobial activity of PHB sheets. But there are some official methods to investigate the effect of antimicrobial activity of paper, fiber, plastic plate and so forth. We also adopted these methods to evaluate the antimicrobial activity of the PHB sheets.

First, we adopted 'Halo Test'. Generally, this method is adopted to evaluate the effect of antimicrobial activity of fibers. In this method, the test piece puts on agar containing microorganisms. After incubating in proper time and temperature, no microorganism area (halo) expand around test piece. The size of halo indicates strength of antimicrobial activity and the diffusion of antimicrobials from film. We placed the film which was cut into circle having 6mm diameter on a solid agar containing bacterial culture. The agar plates are incubated at 35°C for 24 h. After then, we observed if Halo expand around the PHB sheets or not.

Second, we adopted 'Contact Method'. Generally, this method is adopted to evaluate the effect of antimicrobial activity of plastic plate and metal plate. In this method, bacterial culture is put on sample containing antimicrobials and blank sample. After incubating in proper time, temperature and humidity, the culture can be eluted and plated. The colonies can be counted in each plate. By counting and comparing the microbial colonies it is possible to understand that whether PHB sheets having antimicrobial activity. We placed the PHB sheets of dimension 30 mm × 30 mm. and 0.4 ml of test organism was spread on 2 sets of triplicates of these layers. At zero hour, a set of the triplicates was eluted separately using maximum 10 ml of a suitable neutralizer (bisulfate) and directly plated onto plate count agar. The remaining triplicate was incubated for 24 h at 37ºC and then eluted and plated separately. The same procedure was repeated for untreated PHB sheets. The results were observed and compared **[17]**.

Evaluation of release of *C. ternatea* seed extract from the PHB

The antimicrobial agent incorporated PHB (cut into square shapes, 3×3 cm) was put into water and kept at 23ºC and the solution was observed for every 15 min for plant extract. Since the plant extract is colored it is possible to understand its release into the surrounding medium (water) by the color change in the medium **[17]**.

RESULTS

Antibiotic susceptibility of test pathogens

The antibiotic susceptibility of the test pathogens was performed (Table 1, Table 2, Table 3 & Table 4). From the tables it is clear that, the pathogens are resistant to the recommended commercial antibiotics. The resistance may be due to the production of bacterial enzymes that can destroy the antibiotics or due to the bacterial efflux pump that expels the antimicrobial agent from the cell.

Table 1: It shows Antibiotic susceptibility of gram - ve Bacilli

Bacterial pathogens tested (gram-ve Bacilli)	Zone of inhibition by Antibiotics in mm			
	Amp (10µg/ml)	Gent (10µg/ml)	Tetra (30µg/ml)	Cipro (5µg/ml)
Acinetobacter	11±1	12	10±2	13
E. coli	13	10±2	11.5±0.5	12±1
P. mirabilis	13	11.5±0.5	12±2	13.5±0.5

Table 2: It shows Antibiotic susceptibility of *Klebsiella* sp.

Bacterial pathogens tested	Zone of inhibition by Antibiotics in mm	
	Amox (10µg/ml)	Amp (10µg/ml)
Klebsiella sp.	8	9.5±0.5

Table 3: It shows Antibiotic susceptibility of *Pseudomonas* sp.

Bacterial pathogens tested	Zone of inhibition by Antibiotics in mm			
	Gent (10µg/ml)	Ami (30µg/m)	Cipro (5µg/ml)	Piper (10µg/ml)
Pseudomonas sp.	10±1	12	15	15.5±0.5

Table 4: It shows Antibiotic susceptibility of *Staphylococcus* sp.

Bacterial pathogens tested (Staphylococcus sp)	Zone of inhibition by Antibiotics in mm			
	Chlo (30 µg/ml)	Doxy (30 µg/ml)	Mino (30 µg/ml)	Oxa (1 µg/ml)
S. aureus	8±1	14	14	15.5±0.5
S. epidermidis	12	13	12±1	13±1

Qualitative phytochemical screening

The results of the qualitative phytochemical screening in the seed extract of *C. ternatea* are as shown in Table 5. These findings have provided a general understanding of the antimicrobial properties of the extracts tested in this study. From this analysis we understood that, *C. ternatea* seeds are with alkaloids, carbohydrates, proteins and amino acids, phytosterols, phenolic compounds, gum, mucilage, oils, fat, phlobotannins and terpenoids. These compounds may contribute to the antimicrobial activity of *C. ternatea* seeds.

Table 5: It shows Phytochemical analysis of *C. ternatea* seed extracts

S. No.	Tests	Result
1	**Alkaloids**	
	Dragendroff's test	+ (Yellow precipitate formation)
	Mayer's test	+ (White precipitate formation)
	Wagner's test	+ (Reddish brown precipitate formation)
2	**Carbohydrates**	
	Molish's test	+ (violet ring at interface)
	Fehling's test	+ (Red precipitate formation)
3	**Saponins**	
	Foam test	- (No layer of foam)
4	**Proteins and Aminoacids**	
	Million's test	- (No precipitate)
	Biuret test	+ (Pink color in ethanol layer)
	Ninhydrin test	+ (Purple color formation)
5	**Phytosterol**	
	Libermann-Burchards method	+ (No colour change)
6	**Phenolic compounds**	
	Ferric chloride test	+ (Dark green color formation)
	Gelatin test	+ (White precipitate formation)
	Lead acetate	+ (Bulky white precipitate formation)
	Alkaline reagent test	+ (Yellow fluorescence formation)
	Mg and HCl test	+ (Color change from Pink to crimson)
7	**Gum and mucilage**	+ (White precipitate formation)
8	**Oils and Fat**	+ (Partial neutralization of alkali)
9	**Tannins**	- (No precipitate)
10	**Phlobatannins**	+ (Red precipitate)
11	**Terpenoids**	+ (Reddish brown color at the interface)

Antimicrobial Activity of *Clitotia ternatea* seed extract

The ethanoilc and methanolic extracts of the seeds of *Clitoria ternatea* were tested for their antibacterial activity against different pathogenic drug resistant clinical isolates and zone of inhibition against the selected strains by well diffusion was determined. The seed was found to possess powerful antibacterial activity against *Acinetobacter* sp., *P. mirabilis* and *S. aureus*. From the tables 7 & 8, it is clear that ethanolic extract is more powerful than the methanolic extracts. The crude ethanolic extract from seeds of *C. ternatea* showed maximum zone of inhibition (21.5 ± 7.5 mm) against *Acinetobacter* sp. at 0.75 mg concentration and minimum with *P. fluorescence* of (7 mm) among the bacterial pathogens.

Table 6: It shows antibacterial activities (as zone of microbial growth inhibition in mm[a]) of Ethanolic extract of *Clitoria ternatea* seeds

Bacterial Pathogens	E[b]	ECt[c] (mg/L)
Acinetobacter sp.	-	21.5 ± 7.5
E. coli	-	12.5 ± 2.5
E. coli (mucoid)	3.5 ± 0.5	7 ± 1
Klebsiells sp.	6.5±0.5	10
P. aeruginosa	9.5±0.5	10.5 ± 0.5
P. fluorescence	12±3	7
P. mirabilis	12±3	21.5 ± 0.5
S. aureus	5.5±0.5	19.5 ± 0.5
S. epidermidis	8	13.5 ± 0.5
S. saprophyticus	4.5±0.5	14.5 ± 1.5

[a] Values for zone of growth inhibition are presented as mean ± SD; [b] Zone of inhibition by Ethanol (95%)

[c] Zone of inhibition by Ethanolic extract of *Clitoria ternatea* seeds; - Inhibition zone was not noted

Table 7: It shows antibacterial activities (as zone of microbial growth inhibition in mm[a]) of Methanolic extract of *C. ternatea* seeds

Bacterial Pathogens	M[d]	MCt[e]
Acinetobacter sp.	11±2	9.5±0.5
E.coli	-	17±3
E. coli (mucoid)	-	6
Klebsiella sp.	9	6
P.aeruginosa	-	7.5±1.5
P. fluorescence	-	-
P. mirabilis	8.5±1.5	16.5±0.5
S. aureus	5.5±0.5	-
S. epidermidis	8±1	-
S. saprophyticus	-	5

[a] Values for zone of growth inhibition are presented as mean ± SD; [d] Zone of inhibition by Methanol (95%)

[e] Zone of inhibition by Methanolic extract of *Clitoria ternatea* seeds; - Inhibition zone was not noted

Inhibitory action of *C. ternatea* seed ethanolic extracts on genetic material

The DNA band was observed in the control lane whereas the lane with the sample (DNA and plant extract) has not shown any bands. RNA band is present only in the control lane and absent in sample lane (Figure 1). From this study it is inferred that phytochemicals can interfere with the DNA replication and RNA so that it can prevent the cell multiplication. Many studies have shown that inside the human body, the phytochemicals can bind to the cell wall and can prevent adhesion of pathogens.

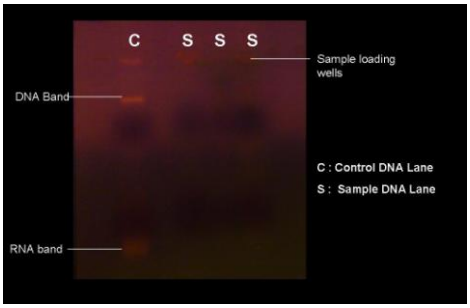

Fig. 1: It shows inhibitory action of *C. ternatea* seed extracts on genetic material

Development of PHB sheets

Since the PHB dissolves in organic chloride solutions, we used chloroform to dissolve PHB and make it into a solution. The application of heat can melt the PHB, thus it was possible for us to make the PHB into smooth sheets. The produced PHB sheets were brittle and translucent.

Modification of Plasticity of PHB sheets

The hot solution of PHB in chloroform is added with PEG with constant stirring. The resulted viscous solution was cast into sheets. The PHB sheets modified with PEG were translucent and flexible.

Incorporation of *C. ternatea seed* ethanolic extract to PHB sheets

The Antimicrobial PHB sheets were prepared as the procedure. Since the ethanolic extract with ethanol will not completely dissolve in chloroform, the ethanol was completely evaporated from the extract before adding into the chloroform with PHB and PEG. Evaporation of ethanol will avoid interference of ethanol in the antimicrobial activity of extract. That is, the ethanol may or may not induce the antimicrobial activity of the antimicrobial agent, so it will not be possible to understand the exact activity of the agent. Re-dissolving of modified PHB sheets in chloroform for incorporation of antimicrobial agent avoided application of heat, and the antimicrobial agent remained effective. The application of heat can destroy the phtyochemical compounds in ethanolic extract.

The produced sheet was translucent, flexible and desirable light greenish yellow in color due to the plant extract.

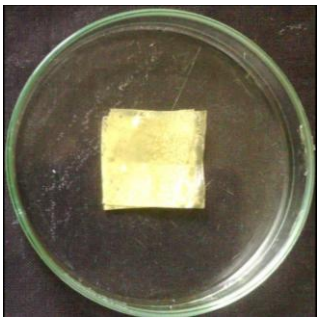

Fig. 2: It shows *C. ternatea* seed extract incorporated PHB sheets (cut into square shape)

Antimicrobial activity of *C. ternatea* seed extract incorporated PHB sheets

Halo test

No microorganism area (Halo) indicates strength of antimicrobial activity, and the diffusion of antimicrobial from the PHB. In our study, the halo expanded around the treated PHB discs only in some plates (≤7 mm) and the halo were not much effective like when the antimicrobial agent, the plant extract alone tested. But it was clearly visible that there was no growth under or on the PHB discs in any of the plates and the control had growth under the discs. These results indicate that the treated PHB having antimicrobial activity but the strength of the activity could not be investigated through this method. Therefore to demonstrate the antimicrobial activity of treated PHB it is necessary to do "contact" method.

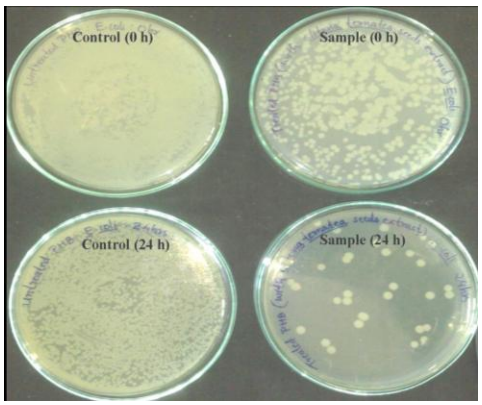

Fig. 3: It shows plates showing microbial colonies after incubated (0 and 24 h) with PHB and treated PHB

Contact Method

The result by contact method is shown in Figure 3. The elution made from PHB after 0 and 24 h of incubation, when plated showed that PHB does not have any antimicrobial activity, since the plates were

TNTC. The elution made from treated PHB after 0 and 24h of incubation, when plated showed that the PHB having antimicrobial activity, since the colonies were less than 30 in number in the plate with 24 h incubated elution.

Evaluation of release of antimicrobial agent from the PHB

The solution in which the PHB was kept obtained a desirable green/greenish yellow color of the phytochemical which indicates that the positive halo test and contact method were caused by the plant extract. This is also strongly recommended that under wet environment, treated PHB is effective against pathogens.

DISCUSSION

Now a day, evolvement of MDR pathogens is a common problem faced by Asian countries, due to excessive use of antibiotics. Asian countries are extensively depending on the antibacterial drug/agent incorporated medical devices, cloths, cotton etc because of poor hygienic practices and contaminated environment. The term "resistance" is used to describe a relative insensitivity of a microbe to an antimicrobial drug as tested in vitro and compared with other isolates of the same species. In contrast, clinical failure describes failure of an appropriate therapy for a certain indication to result in a clinical response. The reason for clinical failure may be, for example, antifungal resistance, but other causes, such as an impaired immune function, poor bioavailability of the drug given, or an accelerated metabolism of the drug, are possible causes of treatment failure

Therefore, whenever an antimicrobial agent incorporated thing is produced, it is better to use clinical pathogens which are resistant to common antibiotics (MDR pathogens) instead of using commercially available microorganisms. Because by using an antimicrobial agent which can kill only common clinical pathogens may not effective in killing the resistant pathogens. The above said reasons explain why we selected MDR (Multi Drug Resistant) pathogens for testing the effectiveness of a new antimicrobial agent or product.

There are a number of ways by which microorganisms are resistant to antimicrobial agents. These include: 1) the bacteria produce enzymes that either destroy the antimicrobial agent before it reaches its target or modify the drug so that it no longer is recognized by the target; 2) the cell wall becomes impermeable to the antimicrobial agent; 3) the target site is altered by mutation so that it no longer binds the antimicrobial agent; 4) the bacteria possess an efflux pump that expels the antimicrobial agent from the cell before it can reach its target; and 5) specific metabolic pathways in the bacteria are genetically altered so that the antimicrobial agent cannot exert an effect. Bacteria also can acquire resistance to antimicrobial agents by genetic events such as mutation, conjugation, transformation, transduction and transposition.

So in our work, to check the antibiotic susceptibility we selected clinical pathogens which are isolated from the infected, post operative wounds, UT etc. of different patients who are failed to respond to the common antibiotics. To reveal the multi drug resistance of gram negative bacilli we used the antibiotics Ampicillin (10µg), Gentamycin (10µg), Tetracycline (30µg) and Ciproflaxacin (5µg). These antibiotics are usually used against the gram negative Bacilli. When Ampicillin (10µg) gives a zone of inhibition of ≤ 13mm, it is considered that the test pathogen is resistant to the antibiotic. In our work Ampicillin has given ≤ 13mm zone of inhibition against the test gram negative Bacilli. Like Ampicillin, when Gentamycin (10µg), Tetracycline (30µg) and Ciproflaxacin (5µg) give zone of inhibition ≤ 12mm, 14mm, ≤15mm respectively, it is considered that the test pathogen is resistant to the antibiotics. From the Table 1 it is clear that the gram negative test Bacilli are resistant to the commercial antibiotics.

To test the multidrug resistance of *Klebsiella* sp. we used the antibiotics such as Amoxicillin (10µg) and Ampicillin (10µg), which gave a zone of inhibition ≤ 13mm and ≤ 11mm respectively indicates the resistance as shown in Table 2. To test the multidrug resistance of Pseudomonas sp. we used the antibiotics, Gentamycin (10µg), Amikacin (30µg), Ciproflaxacin (5µg), Piperacillin (10µg), which gave zone of inhibition ≤ 12mm, ≤ 14mm, ≤ 15mm, ≤ 17mm respectively indicates the resistance which are recorded in the Table 3. To test the multidrug resistance of *Staphylococcus* sp. we used the antibiotics such as Chloramphenicol (30µg), Doxycycline (30µg), minocycline (30µg) and Oxacillin (1µg), which gave the zone of inhibition ≤ 12mm, ≤ 14mm, ≤ 14mm and ≤ 17mm respectively indicates the resistance as shown in Table 4. From these findings we can understand that the clinical pathogens which are used in the work are MDR pathogens.

The possible applications of bacterial PHB is directly connected with their properties such as biological degradability, thermoplastic characteristics, piezoelectric properties, and depolymerization of PHB to monomeric D(-)-3- hydroxybutyric acid **[18]**. Unfortunately PHB is a highly crystallin, stiff, but brittle material. When spun into fibers it behaves as a hard-elastic material. Copolymers like PHBV or mcl-PHAs are less stiff and brittle than PHB, while retaining most of the other mechanical properties of PHB **[2]**.

In our work, when the PHB powder was made into sheets. The sheets were brittle and translucent. These properties reduce the applications of PHB in various fields. So the PHB sheets were modified with plasticizer. Plasticizing effect could acquire by using PEG. Since PHB is soluble in chloride organic solvents, heating with chloroform produced PHB solution which could easily cast to produce PHB sheets. Additional heating for 5 minutes allowed concentration of the solution by partial evaporation of the chloroform. The viscous solution of PHB can be mold into sheets. Drying process ensured the complete evaporation of the volatile chloroform and the aging process gives stabilization. The modified PHB sheets were translucent and flexible. From the previous studies it is estimated that stronger plasticizing effect could be acquire by lower molecular weight plasticizer and the biodegradation will also get increased. Through our work we are recommending that it is possible to increase the plasticizing effect of PHB.

The blood-compatibility of PHB-PEG was evaluated by means of platelet clotting time test and exploring its morphological changes in a study **[9]**. The results showed that PEG played an important role in resisting platelet adhesion. The Chinese Hamster Lung (CHL) fibroblast cells cultured on the matrix spread and proliferated well. These results indicated that PHB exhibited satisfying cell-compatibility and the addition of PEG also could improve the cell-compatibility of PHB.

The demand of antimicrobials in the plastic business is growing. Certain microbes are harmful to humans and if left unchecked will proliferate to cause infections and diseases. There is a particular need to control these in sensitive environments such as hospitals where acquired infections including MRSA can prove to be fatal. Microbes can also be responsible for a wide range of undesirable effects such as product deteriation and discoloration, malodour and food contamination to name but a few. Antimicrobial additives to plastics provide an extra layer of protection when combined with normal cleaning procedures. So through our work we attempted to find an antimicrobial agent which can induce antimicrobial activity to the PHB sheets. Wide use of antibiotics as antimicrobial agents has lead to the evolvement of MDR pathogens. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. There are more than thousand known phytochemicals. It is well-known that plants produce these chemicals to protect itself but recent research demonstrate that they can protect humans against diseases **[19]**.

Clitoria ternatea (Family- Leguminoceae, previously known as Papillioneceae) is a perennial twining herb. Leaves of this herb are imperipinnate, petioles are 2-2.5 cm long. Flowers are axillary, solitary, standard bright or blue or sometimes white, with an orange centre; seed are 6-10, yellowish brown and smooth **[20,21]**. It originated from tropical Asia and later was distributed widely in South and Central America, East and West Indies, China and India, where it has become naturalized. Native to the island of Ternate in the *Molluca archipelago*, this species is now widely grown as ornamental, fodder or medicinal plant **[22]**. From previous work it is known that the *C. ternatea* seeds having antimicrobial activity. We prepared both ethanolic and methanolic extracts of *C. ternatea* seeds and analyzed its antimicrobial activity against the multi drug

resistant clinical pathogens we selected. The phytochemical analysis was also done for the extracts to understand the phytochemical components which really cause the antimicrobial effect **[20]**.

The results of the qualitative phytochemical screening of *C. ternatea* are as shown in Table 5. These findings had provided a general understanding of the antimicrobial properties of the extracts tested in this study **[23]**.

The selected clinical pathogens were collected from the patients who failed to respond to common antibiotics. The results of the antimicrobial screening assay of the seed extract *C. ternatea* are shown in Tables 6, 7, 8 & 9. The extract was found to be active on almost all microbial strains tested. By comparing the effectiveness, we can understand that the ethanolic extract is more effective against the pathogens than the methanolic extract. And the extracts are more effective against fungal pathogens, when compared to bacterial pathogens. When the commercial antibiotics failed to show any zone of inhibition for fungal pathogens, the extract could show a maximum zone of inhibition (23±8 mm) against *Candida* sp. In the study of Chauhan *et al.* (2012), it was reported that crude extract from seeds of *C. ternatea* showed maximum zone of inhibition (22±0.5 mm) against *E. coli* at 0.75 mg concentration and minimum with *M. flavus* of (14±1 mm) and the callus extract showed maximum zones of inhibition (16±2mm) against *S. typhi* while the lowest with *E. coli* and *S. aureus* (12±1 mm and 12±0.9mm) respectively. Alcoholic and Aqueous extracts from in vitro raised calli were tested for antibacterial activity by agar well diffusion method against Gram-negative bacteria. Antibacterial activity was shown against *Salmonella* sp. and *Shigella dysenteriae*; organisms causing enteric fever. In addition, the methanol crude extracts showed anti-bacterial activity against *K. pneumonia* and *P. aeruginosa* **[24]**.

To understand the ability of the extract to denature the genetic material we directly treated the DNA and RNA with extract powder. The examination of DNA and RNA in the agarose gel after treatment revealed the absence of DNA and RNA in the treated samples.

Less research has been done with antimicrobials incorporated PHB, especially in medical filed. In a study Fluorazamide is used as an antimicrobial agent. FZ incorporated PHB was coated on to the surgical sutures. But in US the FZ chemical has been banned due to its adverse effects. Many researches have released based on antibiotics incorporated sutures. But due to the development of MDR pathogens and adverse effect, now less interest has been shown by people towards antibiotics incorporated surgical sutures and medical implants **[10]**.

In our work, we tried to incorporate the *C. ternatea* extract to see whether its antibiotic effect can be active even after incorporated with the PHB sheets. To incorporate the ethanolic extract with the PHB, we can convert PHB into liquid form by chloroform. The PHB along with seed extract powder with PEG in chloroform can produce a homogenous solution of PHB, PEG and seed extract. The chloroform can be evaporated from the homogenous solution and can be molded into sheets. PEG can act as carrier for plant extract and a plasticizing agent for PHB. Thus the produced PHB is flexible and having antimicrobial agent.

It is essential to understand whether the incorporated antimicrobial agent is effective in killing the pathogens which comes in contact with the PHB. Usually antimicrobial activity of plastic sheets or films can be understood by 'halo' test. 'Halo' means 'no microorganism area'. The plastic sheets can be cut into discs and placed onto agar plates with microorganisms. The antimicrobial agent in the sheet discs can inhibit the growth of microbes on or under the discs. But the diffusion of antimicrobial agent from the sheets through the agar can increase the no microorganism area around the discs. In our test, halo was appeared only some plates. But it was clearly observable that in whichever plates the halo could not expand around the PHB discs there was no growth below or above the disc. These results indicate that the treated PHB having antimicrobial activity but the strength of the activity could not be investigated through this method. There are two possible explanations; one possibility is that the effect was limited and the other possibility is that the antimicrobial agent failed to diffuse through the agar. Therefore to demonstrate the antimicrobial activity of treated PHB, we adopted "contact" method. From the Figure 3, it is clear that the pathogens are almost killed after incubating for 24 h on treated PHB sheets and the plates spread with elution of sheets had less than 30 colonies. But in blank PHB the pathogens were survived and the plates were TNTC. We used bisulfate solution to elute the remaining bacterial culture from the PHB sheets, which can act as a neutralizer, so that during the time period of elution and plating the pathogens are not supposed to reproduce. Because in between reproduction of pathogens will reduce the accuracy of the experiment.

To evaluate the dissolving ability of the extract from PHB, to the surrounding medium we put the antimicrobial incorporated PHB sheets cut into square shapes and put into water. The water was observed for color change. Since the plant extract is colored it is possible to understand whether it is getting dissolved from PHB into the water. We observed the water in every 15 minutes and the water was getting the color, light greenish yellow. As time increased the color of the water was also get increased slowly, which indicates that there is a controlled release of extract from the PHB. This also explains the reason of positive result of halo test and contact test were due to the seed extract. This is also strongly recommended that under wet environment, treated PHB is effective against pathogens.

CONCLUSION

In the present study, we have tried to give plasticity and antimicrobial activity to the bioplastic, PHB. From the above mentioned methods and their results it can be conclude that plant extracts are good options as antimicrobial agent to get incorporated with the bioplastics and thus the application of PHB can be improved by incorporating plasticizers and antimicrobial agent to the PHB. Since PHB is biodegradable, effective in wet environment and is incorporated with antimicrobial agent which is effective against infections, we are recommending this antimicrobial PHB in sanitary napkins, surgical sutures and medical implants.

REFERENCES

1. Blot SI, Vandewoude KH, Hoste EA, Colardyn FA: Outcome and attributable mortality in critically Ill patients with bacteremia involving methicillin susceptible and methicillin-resistant *Staphylococcus aureus*. Arch. Intern. Med. 2002;162(19): 2229-35.
2. Abbate M, Martuscelli E, Ragosta G, Scarinzi G: Tensile properties and impact behavior of poly (D(-)3-hydroxybutyrate)/rubber blends. J. Mater. Sci. 1991; 26(4): 1119-1125.
3. Chen GQ, Wu Q: The application of polyhydroxyalkanotes as tissue engineering materials. Biomaterials. 2005;26: 6565-6578.
4. Chauhan N, Rajavaidhya S, Dubey BK: Pharmacognostical, phytochemical and pharmacological review on *Clitoria ternatea* for antiasthmatic activity. Int. Pharm. Pharm. Sci. Res. 2011;3(2): 398-404.
5. Ogston A: On Abscesses, Classics in Infectious Diseases, Rev. Infect. Dis. 1984;6(1): 122–28.
6. Cimolai: MRSA and the environment: implications for comprehensive control measures. Eur. J. Clin. Microbiol. 2008;27(7): 481-93.
7. Nazime: Production of PHB by some *Rhizobium* sp. bacteria. Turk. J. Biol. 2009;26: 215-219.
8. Ceyhan N, Guven: Polyhydroxybutyrate production from domestic waste water using *Enterobacter aerogens* 12Bi strain. Afr. J. Microbiol. Res. 2011;5(6): 690-702.
9. Cheng GX, Cai ZJ, Wang L: Biocompatibility and biodegradability of poly(hydroxybutyrate)/poly(ethylene glycol) films. J. Mater. Sci. 2003;14: 1073-8.
10. Fedorov MB, Vikhoreva GA, Yu K, Borisova TI, Vinokurova GA, Bonartseva: Structure and strength properties of surgical sutures modified with a polyhydroxybutyrate. Fiber Chemistry 2006;48(6): 471-475.
11. Kommu S, Gowrishankar NL, Kamala D, Saritha B, Srinivasulu B, Naresh B et al.: Evaluation of wound healing activity of methanolic extract of *Balanites aegyptiaca* L. leaves. Int. J. Pharm. Pharmaceut. Sci. 2013;5(2): 52-53.

12. Shyamkrishnan M, Dhanalakshmi P, Yamini SG, Gopalakrishnan S, Manimaran A, Sindhu S et al.: Evaluation of phytochemical constituents and antioxidant activity of Indian medicinal plant *Hydnocarpus pentandra*. Int. J. Pharm. Pharmaceut. Sci. 2013; 5(2): 453-458.
13. Wani SA, Ashfaq M, Shah KW, Sing D: Phytochemical screening of methanolic extracts of *Podophyllum hexandrum* royle and *Rheum emodi* wall. J. Curr. Chem. Pharm. Sc. 2012;2(2): 125-128.
14. Egwaikhide PA, Gimba CE: Analysis of phytochemical content and antimicrobial activity of *Plectranthus glandulosis* whole plant. Middle East J. Sci. Res. 2007;2(3-4): 135-138.
15. Vijayalakshmi R, Ravindhran R: Preliminary comparative phytochemical screening of root extracts of *Diospyrus ferrea* (wild). Bakh and *Aerva lanata* (L.) Juss. Ex Schultes: Asian J. Plant Sci. Res. 2012;2(5): 581-587.
16. Anyiin TAT, Yakumbur DT: Phytochemical screening and antimicrobial activity of stem bark extracts of Antidesma Venosum: J. Nat. Prod. Plant. Resour. 2012;2(3): 427-430.
17. Hasegawa Y, Kimura T: Antimicrobial activity of novel packaging material made from biomass plastics and shell (scallop) powder. Int. Commission. Agr. Biosystems. Eng. 2010;3: 113-126.
18. Lafferty RM, Korsatko B, Korsatko B, Korstako W: Microbial production of poly-3-hydroxybutyric acid. Biotechnol. Spec. Microb. Process. 1988;136-176.
19. Selvamaleeswaran, Wesely EG: Comparative study of primary metabolites in different plat parts of *Clitoria ternatea.* J. Chem. Pharm. 2011;3(4): 614-617.
20. Amol P Vijay R: Evaluation of Invitro antioxidant activity of seeds of blue and white flowered varieties of *Clitoria ternatea.* Int. Pharm. Pharm. Sci. Res. 2011;3(4): 0975-1491.
21. Thevissen K, Osborn RW, Achand DP, Brockaest WF: Specific binding sites for an antifungal plant defense from *Dahlia (Dahlia merckii*) on fungal cells ar*e* required for antifungal activity. Mol. Biol. Plant microbe. Interact. 2000;13, 54- 61.
22. Malabadi RB, Nataraja K: Shoot regeneration in leaf explants of *Clitoria ternatea* L. cultured *in vitro*. Phytomorphology 2001;51: 169-171.
23. Kamilla L, Mnsor SM, Ramanathan S, Sasidharan S: Antimicrobial Activity of *Clitoria ternatea* (L.) Extracts. Pharmacologyonline 2009;1: 731-738.
24. Chauhan N, Rajavaidhya S, Dubey BK: Pharmacognostical, phytochemical and pharmacological review on *Clitoria ternatea* for antiasthmatic activity. Int. Pharm. Pharm. Sci. Res. 2011;3(2): 398-404.