AN INVESTIGATION ABOUT EFFICACY OF NOVEL HERMAL BASED COMPOSITES FOR DIFFERENT BIOMEDICAL APPLICATIONS

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MASTER THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by: **Mr. Hafiz Hassan Ali** reg no. 00000119227 Titled: **"An investigation about Efficacy of Novel Hermal based Polymeric Composition for Different Biomedical Applications"** be accepted in partial fulfillment of the requirements for the award of **MS Biomedical Sciences** degree. (Grade_____)

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DECLARATION

I certify that this research work titled "*An Investigation about Efficacy of Novel Hermal based Composites for Different Biomedical Applications"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources and properly acknowledged / referred.

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Hafiz Hassan Ali

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“Dedicated to my exceptional parents and adored siblings whose unconditional support, cooperation and motivation led me to this wonderful accomplishment.”

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Copyright Statement

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Hafiz Hassan Ali

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List of Abbreviations

PVA	Poly (vinyl alcohol)
CMC	Carboxymethyl cellulose
BSG	Basil Seed Gum
DMF	Dimethyl formamide
CG 31	Composite of CMC and Basil seed gum in the ratio 3:1
CGH 31	Composite of CMC and Basil seed gum with hermal seed extract in the ratio 3:1
PG 13	Composite of PVA and Basil seed gum in the ratio 1:3
PGH 13	Composite of PVA and Basil seed gum with hermal seed extract in the ratio 1:3
CPG 313	Composite of CMC, PVA and Basil Seed Gum in the ratio 3:1:3
CPGH 313	Composite of CMC, PVA and Basil Seed Gum with hermal seed extract in the ratio 3:1:3
EMB	Eosin Methylene Blue

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Abstract

In-vivo development of hydrogels and bio-materials is very contemporary issue nowadays, and a lot of literature is available on the advancements and procedures used to effectively decrease in-vivo rejection. Hydrogels are important for drug delivery and in-vivo prosthetic coatings. They are produced in different ratios of two complex polymers, depending upon the need of flexibility, strength, size, pore size and hydrophilicity. Basil seed gum having absorption property has been used for many pharmaceutical applications. CMC and PVA being biocompatible, has been used for wound dressing for a decade. In this project, the composites were synthesized in different ratios and optimized through swelling analysis. PVA/Gum 1:3, CMC/Gum 3:1, CMC/PVA/Gum 3:1:3 were best optimized composites. Hermal seed extract, a renowned natural anti biotic was added to the composites and checked for in-vitro anti-microbial activities using inhibition zone technique. Further analysis on live subjects was done by administration of bacteria in balb/c mice. Topical wound was induced on the back of mice. The composite was introduced to wound site and sewn. Blood sample analyses of pre and post insertion were compared and results were analysed. Introduction of Hermal seed extract composites exponentially decreased the bacterial activity in live samples.

Key Words: PVA, hermal seed extract, swelling analysis, *in vitro* studies, *in vivo* analysis inhibition zone balb/c mice, serial dilution

1. INTRODUCTION

For many years, people have placed substances on the skin for therapeutic effects and, in the modern era, a variety of topical formulations have been developed to treat local indications (Prausnitz and Langer 2008).

Topical drug delivery has made an important contribution to medical practice, but has yet to fully achieve its potential as an alternative to oral delivery and hypodermic injections (Prausnitz and Langer 2008).

Newer occlusive dressings speed up re-epithelialization, stimulate collagen synthesis, create a hypoxic environment at the wound bed to promote angiogenesis, decrease pH at wound surface and create an environment inhospitable to bacterial growth, which decreases the rate of wound infection (Paudel, Milewski et al. 2010). They have an edge over gauze dressings in terms of patient comfort, convenience and compliance as well as better cosmetic results because of reduced scarring.

In the past, traditional drug formulations (ointments, solutions, suppositories, emulsions) were used for treatment of various diseases. Newer drug design with essential characteristics like precise amount of drug release at a specific rate, or delivery of drug at pharmacological action site at a rate per the biological process are developed in last few years. When drugs are combined with macromolecular compounds the release rate of drugs in the body is modified and may result in prolonged action. Polymer-drug composites have been prepared in the past years which has attracted a lot of attention as they have many advantages over free drugs i.e. low drug consumption, prolonged action and sustained release of drug into the body(Buhus, Popa et al. 2007).

Natural polymers like PVA, CMC, gelatin, chitosan, etc. are widely used in pharmaceutical industry as adhesives, adjuvant and emulsifying agents (Akbari, Ghoreishi et al. 2015). These natural polymers possess water-binding capacity, gelation property, low cost and non-irritating as they are biocompatible in nature. They can be used as lining for artificial hearts and artificial skin, for preparation of contact lenses and biosensor membranes and drug delivery media (Gomes, Azevedo et al. 2012, Sim, Figueiras et al. 2012). Moreover, they also have functional groups that can be modified chemically thus provide a wide variety of

products with adjustable chemistries and properties (Gomes, Azevedo et al. 2012, Akbari, Ghoreishi et al. 2015).

The biological active components can be immobilized on substrates with support materials like water soluble polymers, hydrogels that possess swelling properties, fibrous or woven membranes and porous structures (Buhus, Popa et al. 2007).

PVA (Polyvinyl alcohol) is a most extensively used polymer for many biomedical applications because of its suitable physical and chemical properties, biocompatibility, easy degradation and non-toxic nature. It has been used for wound dressings, catheters, contact lenses and coatings for sutures (Walker, Young et al. 2007, Ghafoor, Ali et al. 2016)

On the other hand, Na-CMC is an amylose (carbohydrate) which consists of many hydroxyl and carboxylic groups that intent to introduce absorption behaviour in the polymer. The absorption of water and moisture results in many excellent properties like, high water content, good degradation and low cost (El Salmawi 2007).

Osmium basilicum or basil plant that is commonly found in Central and South America, Africa and Asia. For a long time, the seeds of this plant has been used for treatment of various diseases such as inflammation, dyspepsia, diarrhoea and colic ulcer and other diseases (Hosseini-Parvar, Matia-Merino et al. 2010). The polysaccharide layer in their outer pericarp of basil seed swells when they are soaked in water. Mucilage, extracted from these seeds are concentrated and dried and can be used further for various applications that include as a powerful candidate for various pharmaceutical formulations (Malviya, Srivastava et al. 2011). Prajapati et al. (Prajapati, Jani et al. 2013) studied the used of mucilage and gums for the making drug delivery systems (Akbari, Ghoreishi et al. 2015).

Hermal seeds have been used as a popular healing agent by people in since very long. These possess many therapeutic effects and is pharmaceutically beneficial because of the presence of secondary metabolites such as alkaloids, flavonoids, phenols, tannins, minerals and volatile oils (Ahmad, Hussain et al. 2013). Sharaf et al. and Prashanth and John reported the presence of alkaloids, flavonoids and anthraquinones as a major constituent in hermal seed extract (Sharaf, El-Ansari et al. 1997, Prashanth and John 1999).

The aim of this study was the synthetization and characterization of natural composite films that possess natural antimicrobial agent and can release it in a sustained manner. In this study,

CMC, PVA and Basil seed gum were used for the preparation of a nature composite and hermal seed extract as an anti-microbial agent. Swelling tests were done through gravimetric method and anti-bacterial activity of the composite was checked on agar discs through inhibition zones. Anti-bacterial activity in live subjects was checked through administration of composites in affected balb/b mice and comparison of bacterial count before and after administration.

2. LITERATURE REVIEW

The use of natural polymers in many biomedical applications is an emerging field of current research because of their biocompatible nature. During the last twenty years, biodegradable polymers are used for many applications. The degradation process is carried out by microorganisms or the by chemical decomposition by the body fluids. In the degradation process the complex organic molecules are converted into simpler ones (Chandra 1982, Nair and Laurencin 2007).

Degradable polymers are being used for drug delivery in various areas of research especially through skin which is very challenging. Advanced technologies have resulted in several drugs being administered subcutaneously including hydrophilic drugs, hydrophobic drugs having small molecules and macromolecules. This has advantage over tradition dosage routes i.e. convenient delivery of drug, pain free self-administration. It also eliminates the disadvantage of multiple dosage regimes, regular application of drug and maintaining a constant drug concentration in plasma in case of oral dosing or injections (Paudel, Milewski et al. 2010).

In the past, traditional drug formulations (ointments, solutions, suppositories, emulsions) were used for treatment of various diseases. Newer drug design with essential characteristics like precise amount of drug release at a specific rate, or delivery of drug at pharmacological action site at a rate per the biological process are developed in last few years. When drugs are combined with macromolecular compounds the release rate of drugs in the body is modified and may result in prolonged action. Polymer-drug composites have been prepared in the past years which has attracted a lot of attention as they have many advantages over free drugs i.e. low drug consumption, prolonged action and sustained release of drug into the body (Buhus, Popa et al. 2007).

2.1 Properties of Poly (vinyl alcohol) (PVA)

PVA is a water soluble and biocompatible polymer (Chiellini, Corti et al. 2003) which forms stable hydrogels and elastic gels through repeated freezing and thawing method or crosslinking chemically or physically (Nuttelman, Mortisen et al. 2001). For the use of PVA in pharmaceutical and in the areas of medicine, it must be crosslinked through chemical or physical means. Crosslinking can also be done by irradiation (Peppas and Merrill 1977, Shaheen and Yamaura 2002). The PVA hydrogels can be cross linked by functionally

crosslinking agents such as formaldehyde, glutaraldehyde, acetaldehyde and other monoaldehydes. In the presence of sulfuric acid, methanol these cross-linking agent forms acetal bridges between the hydroxyl group of PVA chains. However, the residual amounts of crosslinking agents are present in the PVA hydrogel. Removal of these residue material is very time-consuming process. However, when these residues are no removed PVA cannot be further used for biomedical or pharmaceutical applications. The crosslinking agents contain toxic chemicals that are released when they are in contact with the body. The crosslinking methods can be replaced with electron beam or gamma irradiations (DİLAVER 2011).

PVA (Polyvinyl alcohol) is a most extensively used polymer for many biomedical applications because of its suitable physical and chemical properties, biocompatibility, easy degradation and non-toxic nature. It has been used for wound dressings, catheters, contact lenses and coatings for sutures (Walker, Young et al. 2007, Ghafoor, Ali et al. 2016).

2.2 Properties of Carboxymethyl Cellulose (CMC)

Cellulose is expected to become the main chemical resource of the future considering it is the most widely available renewable resource on earth. (Schurz 1999, Eichhorn, Young et al. 2005). In addition to this there is an increasing demand for sustainable and environmentally friendly products and cellulose is a great precursor for the development of new functional materials for a broad range of applications (Klemm, Heublein et al. 2005). There is immense potential to prepare hydrogels using cellulose because of the abundant hydroxyl groups present in cellulose. There are many favourable properties associated with hydrogels such as biodegradability, hydrophilicity, biocompatibility, non-toxicity, low cost and transparency. Due to their attractive structures these hydrogels have wide applications such as in tissue engineering (Vinatier, Gauthier et al. 2009), controllable delivery system (Chang, Duan et al. 2010), blood purification (Ye, Watanabe et al. 2003), sensor (Sannino, Pappada et al. 2007), and chromatographic supports (Xiong, Zhang et al. 2005).

As mentioned earlier the numerous hydroxyl groups in cellulose make hydrogen bonding easy, therefore cellulose hydrogels can be easily prepared via physical cross-linking using cellulose solutions. However, this same phenomenon of extended hydrogen bonding makes dissolution of cellulose very difficult in common solvents (Edgar, Buchanan et al. 2001). To overcome this issue water soluble cellulose derivatives can be used which have the added advantage of being biocompatible. These derivatives can be used as thickeners, emulsifiers,

binding agents, suspension aids, film formers, surfactants, stabilizers and lubricants. They can also be used as additives in pharmaceutical, food and cosmetic industries (Weng, Zhang et al. 2004).

One prominent example of such a derivative is Carboxymethyl cellulose (CMC). CMC is a non-toxic, highly biocompatible and biodegradable derivative. It is a water soluble, low cost ionic polysaccharide that contains abundant carboxyl and hydroxyl groups. Another added feature is its ability to exhibit pH sensitivity (Charpentier, Mocanu et al. 1997, Mitsumata, Suemitsu et al. 2003).

One of the most widely studied conversion methods is carboxymethylation. Carboxymethylation is simple and leads to a variety of products with promising features. The general procedure involves the activation of the polysaccharide with aqueous alkali hydroxide usually sodium hydroxide. Monochloroacetic acid or its sodium salt is then used for further conversion to yield carboxymethyl polysaccharide derivative the process which is generally known as the Williamson ether synthesis (Heinze 2005). CMC also behaves as a typical polyelectrolyte and is therefore also categorized as a cellulose ether.

2.3 Properties of Basil Seed Gum

A novel hydrocolloid is extracted from seeds of basil herb and is known as Basil seed gum (BSG) or alternatively as Ocimum bascillum. It is commonly used as a thickening and gelling agent in the food industry (Hosseini-Parvar, Matia-Merino et al. 2010). BSG contains xylan, glucan and glucomannan and is therefore referred to as hetero-polysaccharide (Rafe and Razavi 2013). Chemically BSG is comprised of two major fractions i.e. an acid stable core and a linked xylan with acidic side chains. The acid stable core galactomannan (43%) has a glucose/manose ratio of 10:2. The acidic side chains make up the acid soluble portion. There is also a minor glucan fragment that comprises 2.31% of the total structure (Mirhosseini and Amid 2012).

Basil (O. basilicum) is an herb that is found abundantly in central and South America, Asia and Africa. Basil seeds have been used traditionally to treat several diseases such as diarrhoea, dyspepsia, colic ulcer, inflammation etc. (Hosseini-Parvar, Matia-Merino et al. 2010). The seeds are black, oval and covered in a polysaccharide layer. This outer layer turns into

mucilage when the seeds are soaked in water. This mucilage can then be extracted, dried or concentrated to be used in further applications (Akbari, Ghoreishi et al. 2015).

Various studies have focused on the use of mucilage for the development of drug delivery systems. Prajapati et al. (Prajapati, Jani et al. 2013) reviewed how mucilage, natural gums and their altered forms can be used in the pharmaceutical industry to develop drug delivery systems. Mucilage polysaccharide from waste of *Abelmoschus esculentus* for biomedical applications has been characterized by Archana et al. (Archana, Sabina et al. 2013). Characterization and in vitro drug release studies of *Terminalia catappa* gum were carried out by Meka et al. (Meka, Nali et al. 2012). Srinivas et al. (Srinivas, Prakash et al. 2003) study focused on O. basilicum as disintegrates in the formulation of dispersible tablets.

2.4 Properties of Hermal Seed

Pegnum hermala or Hermal Seeds are widely distributed in most parts of the world including the Middle East, Pakistan and India. Moreover, they have now been introduced in America and Australia too (Asghari and Lockwood 2002, Yousefi, Ghaffarifar et al. 2009). By burning the seeds of *P. harmala* it can be used as a disinfectant and antiseptic and such is the practice in Iran (Fathiazad, Azarmi et al. 2006, Arshad, Zitterl-Eglseer et al. 2008).

Hermal seeds have the potential to be used in the treatment of a variety of diseases such as asthma, jaundice, colic and lumbago. It is also used a s a stimulant emmenagogue (Bukhari, Choi et al. 2008).

The seeds and the roots are most beneficial because they contain the active compounds which have been characterized as alkaloids (Mirzaie, Nosratabadi et al. 2007). Anti-tumor activity of P.harmala has also been identified (Goel, Singh et al. 2009). In addition to showing anti-tumor activity P.harmala also has anti-histaminic (Asghari and Lockwood 2002), vasorelaxant effect (Asghari and Lockwood 2002), wound healing (Derakhshanfar, Oloumi et al. 2010), anti-oxidant activity (Astulla, Zaima et al. 2008), leukemic healing (Zaker, Oody et al. 2007), immuno- modulator properties (Astulla, Zaima et al. 2008) and anti-inflammatory properties (Muhi-eldeen, Al-Shamma et al. 2008). In addition to the above-mentioned properties this plant has exhibited antifungals and antibacterial properties too. (Shonouda, Osman et al. 2008).

3: MATERIALS AND METHODS

3.1 Materials

Hermal and Basil seeds were purchased from local market (Islamabad, Pakistan) and suitable seeds were collected and cleaned from sand and dust. Polyvinyl alcohol (PVA) (Mol. Wt. 72000 g/mol) was obtained from AppliChem Panreac (Darmstadt, Germant). Carboxymethyl cellulose sodium salt was purchased from Daejung Chemical Co. (Siheung, Korea). Ethanol (Mol. Wt. 46.07g/mol) used for extraction was obtained from Merck KGaA (Darmstadt, Germany). Sodium Chloride (NaCl) (Mol. Wt. 58344 g/mol), Sodium Hydrogen carbonate (NaHCO3) (Mol. Wt. 84.01g/mol) and Sodium Phosphate monobasic dehydrate (NaH2PO4) (Mol. Wt. 156.01 g/mol) purchased from Sigma-Aldrich (USA) whereas Potassium Chloride (KCl) (Mol. Wt. 74.55g/mol) purchase from Haque Chemicals (Pakistan) were used for the preparation of Pseudo-extracellular Fluid (PECF). For anti-bacterial and anti-fungal Testing Tryptone (BioWorld, USA), yeast extract (MERCK, Germany), nutrient agar (MERCK, Germany), were used. Doubly distilled water was used for preparation of solutions.

3.2 Extraction from Plant material

3.2.1 Basil Seed Gum Extraction:

Basil seeds were soaked and swelled in Distilled water in a ratio 1:50 at room temperature for 2-3 hours. The seeds were blended until the mucilage is smooth and squeezed out through double folded muslin cloth. The extracted gum was further used for preparation of composite films (Razavi, Mortazavi et al. 2009).

3.2.2 Hermal Seed Extraction:

10g dry seeds were ground and soaked in 100 ml 96 % ethanol. Extraction was done in water bath at 30o C for 4-5 days with continuous stirring at 150 rpm (Arshad, Zitterl-Eglseer et al. 2008). The extract was filtered and dried at 40^{o}C. The dried extract was then dissolved in DMF and used in composite films.

3.3 Solution Preparation

Carboxymethyl Cellulose (CMC) 1% solution was prepared by dissolving 1g CMC in 100ml distilled water at 70oC. CMC was added slowly with continuous stirring for even distribution and avoiding formation of clumps.

Polyvinyl Alcohol (PVA) 5% solution was prepared by adding 5g of PVA in 100ml distilled water at 120oC and stirred for 15-20 min until clear solution was obtained.

Hermal Extract Solution was prepared by dissolving 10g extracted powder in 50ml Dimethyl Formamide (DMF) purchased from TEDIA Company Inc., USA.

3.4 Synthesis of Composite Films

Different composites were synthesized by mixing 1% CMC, 5% PVA and Basil Seed Gum (BSG) in varying ratios. PVA/BSG and CMC/BSG composites were prepared in 3 different ratios i.e. 1/1, 3/1 and 1/3 whereas CMC/PVA/BSG composite was prepared in ratios 3:1:1, 3:1:2 and 3:1:3. The ratios were mixed such that final volume remains 40ml in all composites. The mixture was then poured in petri-plates and air dried for 2 days. The harvested films were than used for further testing.

3.5 Synthesis of Composite Films with Hermal Extract

16ml of hermal extract solution was added slowly, in intervals, to the composites of (a) PVA/BSG, ratio 1:3 (PG13) (b) CMC/BSG, ratio 3:1 (CG31) and (c) CMC/PVA/BSG, ratio 3:1:3 (CPG313) and mixed manually with a glass stirrer to avoid clump formation. The mixture was casted in petri-plates and air dried for 2-3 days. The resultant films (PGH 13, CGH 31, and CPGH 313) were used for further testing.

3.6 Swelling Analysis of Composite Films

Swelling analysis of the films was done by Gravimetric method i.e. by immersing a small piece (1cm x 1cm) of the film in fluid of pH 5.5 at 37^{o}C and measuring the weight at intervals. The dry weight of pieces was measured initially. The weight of the film was measured after

an interval of 10 min for 80 mins. Weak Acidic solution was prepared by dissolving 0.68g NaCl, 0.22g KCl, 2.5g CH_3COOH, 0.35g NaH_2PO_4 and keeping the pH to 5.5.

3.7 *In-vitro* Analysis of the Composite Films

Disc Diffusion method was used to evaluate the anti-microbial activity of the composite films.

3.7.1 Disc Diffusion Tests:

To investigate the anti-bacterial activity of the films sterilized nutrient broth agar was used as a growth medium for bacterial strains of *Pseudomonas aeruginosa (P. aeruginosa), Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus).* Film discs of 6mm diameter were placed on the inoculated plates and incubated at 37^{o}C and results were recorded after 24 hours of incubation (Monte, Abreu et al. 2014).

During anti-microbial testing CPG-313 (without hermal seed extract) was used as a negative control and commercial tetracycline (30ug) disc were used as positive controls. The bacterial culture strains were provided by UG Industrial Lab, ASAB, NUST, Islamabad.

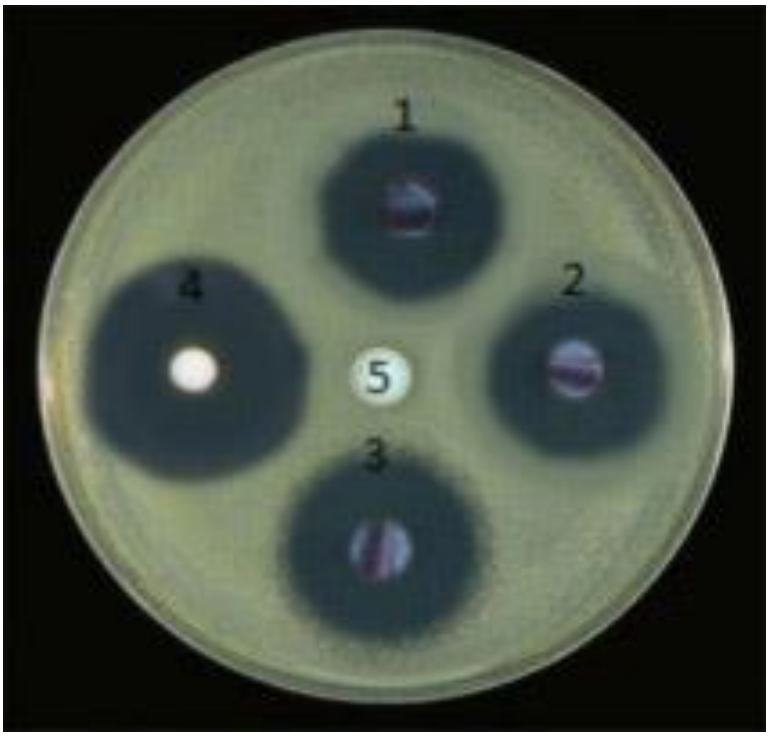


Figure 1. Disc diffusion test to analyse inhibition zones created by composite films. 1=CPGH-313, 2=CGH-31, 3=PGH-13, 4=Tetracycline, 5=CPG=313

3.8 In-vivo Analysis of Composite films

In-vivo analysis was done to analyse the anti-microbial activity of composite films in live tissue.

3.8.1 Mice Preparation

To carry on in-vivo analysis, blab/c mice were selected as test subjects. 2 weeks old mice were procured from National Institute of Health, Islamabad, and their feed was adjusted. After a week, they were administered with a (10uL bacteria + 90L saline) cocktail of *Escherichia coli*, *Pseudomonas Aeruginosa* and Multi drug Resistant *Staphylococcus aureus (MRSA).*

Anaesthesia was prepared using 30uL Xylaze, 1 mL Keta max and 9mL de-ionized water. It administered on the portal vein of mice. A topical wound was introduced on dorsal side left bottom of mice, and composite was placed there. 100 µL blood was taken from the site. Wound was sewn afterwards.

Figure 2. Balb/c mouse injected with hermal seed composite and sewn. Blood sample of 100uL litre taken from mouse before administration of composite and after 24 hours of composite administration.

3.8.2 Bacterial Analysis

Blood Sample from before the composite interaction was serially diluted to 10^5 and spread on selective agar to count the colonies. Two blood samples (one from primary site and another from heart) were taken after 24 hours and were serially diluted to 10^5 (Maitreya J. 2010). The samples were spread on selective media in controlled conditions.

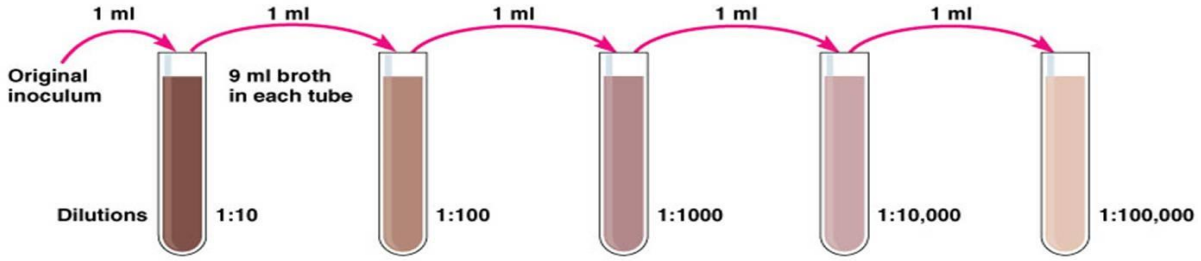


Figure 3. Shows serial dilution of samples

Dilutions	10^1	10^2	10^3	10^4	**10^5**	10^6
CFU	Too many	Too many	>200	121	**39**	4

Table 1. shows serial dilution results of E. coli samples

Eosin Methylene Blue was used for selection of *E. coli* as it allows growth of gram negative bacteria. It gives *E .coli* a dark purple colony, while giving other gram negative bacteria a bright colour colony. MacConkey Agar was used for *Pseudomonas Aeruginosa* as it gives large colourless colonies in yellow to it while giving others colonies in pink. Mannitol Salt agar only allows growth of MRSA, giving it small colourless colonies.

3.8.3 Eosin Methylene Blue Agar

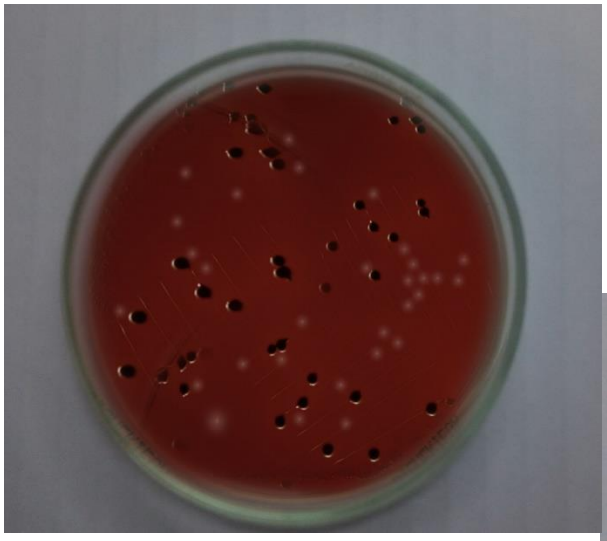
Eosin Methylene Blue Agar is a selective agar for gram-negative bacteria such as *E. coli.* It contains toxic dyes against gram-positive bacteria. Methylene Blue and Eosin are blended in a ratio of 1:6. Among gram negative bacteria, it differentiates them on basis of lactose fermentation. Microbes that ferment lactose make the environment acidic and increase absorption of dye by the colonies. This makes small dark purples fermented colonies e.g. *E. coli.* Bacteria that don't ferment lactose deamine the area and increase the pH, so colonies don't absorb the dye. They provide large smudged light coloured colonies, e.g. *P. aeruginosa* (Levine M 1918).

3.8.4 MacConkey Agar

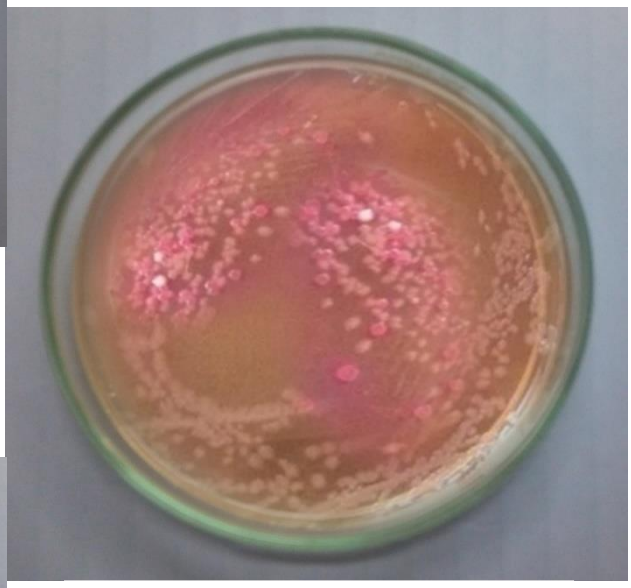
MacConkey agar is a differential and selective cultural medium that is used for microbes to isolate enteric and gram negative bacteria. It differentiates between them based on fermentation of lactose. The bile salts and crystal violet decrease the outburst of gram positive bacteria. It also allows to select and isolate gram negative microbes. Bacterial species which have the ability of lactose fermentation can easily be isolated with the help of carbohydrate lactose, In this case, pH turns to neutral red. The acidic pH of bacteria such as *P. aeruginosa* keeps it yellow and slightly colourless (Anderson et. al. 2013).

3.8.5 Mannitol Salt Agar

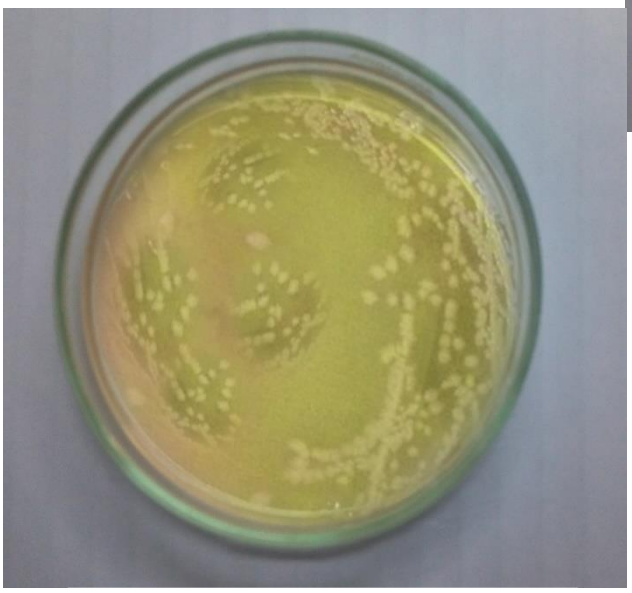
Mannitol salt agar is a differential and selective while common growth medium for microbes. It enhances the exponential increment in growth of certain bacteria while inhibiting the growth of others. High concentration of sodium chloride, 7.5-10% makes it a good selective growth medium for gram positive bacteria like *S. Aureus,* as a high concentration of NaCl inhibits a variety of bacteria. The medium is selective for staphylococci species that ferment mannitol as well. Bacteria that contain carbohydrate mannitol make it phenol red, for the sake of acidic pH that is produced by staphylococcus species other than S. aureus. Yellow colonies are produced by S. aureus while small pink colonies are produced by other species. The organisms that ferment mannitol form a by-product that is acidic and changes the colour of phenol red in the medium to slightly pale yellow colour. This isolates pathogenic species of bacteria, Gram negative bacteria can't grow on the medium, so it is best for the isolation of *Staphylococcus aureus* species in the experiment (Bachoon et. al 2008)



(a) Eosin Methylene Blue Agar



(b) MacConkey Agar



(c) Mannitol Salt Agar

Figure 4. shows multiple aspects of selective agars. (a) Eosin Methylene Blue which is used to differentiate E. coli in dark purple.(b) MacConkey Agar isolates P. aeruginosa with colourless colonies.(c) Mannitol salt agar isolates gram positive bacteria and shows colourless colonies of S. Aureus

3.8.6 Sample Comparison

The samples from before the composite interaction were compared with primary and secondary site and were plotted in graph. Each colony formed on nutrient agar is called colony forming unit (CFU). To analyse the bacterial amount, following formula was used.

No. of bacteria per ml= cfu /ml = (no. of colonies x dilution factor) / volume of culture plate

No. of bacteria per ml= $(39 \times 10^{5})/25$

No. of bacteria per ml= 1,56,000

4: RESULTS AND DISCUSSION

4.1 Swelling Results of the Composite films

4.1.1 Swelling Result of Composites without Hermal Seed Extract

Swelling analysis of individual components (CMC, PVA, Basil seed Gum) and the 3 compositions was done through Gravimetric method (figure 2). The swelling tests confirm the hydrophilic nature of the composite material. It can be observed however, that the ratio of gum in the composites affects the swelling profile. When swelling of the components was done, CMC possess higher degree of swelling as compared to gum which possess a complex polysaccharide structure, while PVA was degraded during the same course of time.

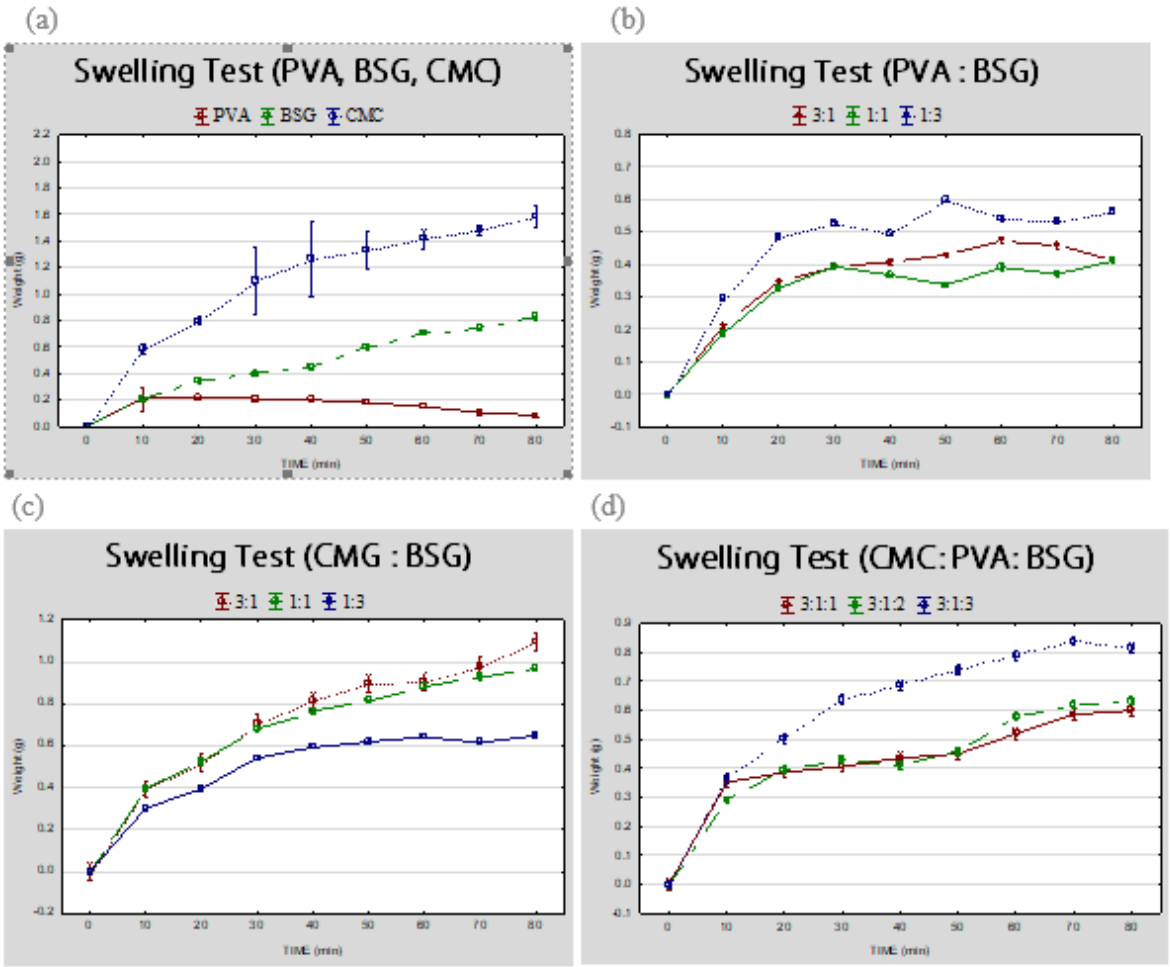


Figure 5. Graphical Representation of increase in weight of the films when submerged in PECF Solution. Figure 2(a) shows individual swelling results of PVA, CMC and BSG. Figure 2(b) shows swelling results for composites of PVA and BSG. Figure 2(c) shows swelling results for CMC and BSG composites, while figure 2(d) shows composite results for CMC, PVA and BSG all combined.

In PVA/Gum swelling profile, it can be observed that when the ratio of gum was increased (PG13) the swelling of the film was also increased. It may be attributed to the amine groups present in gum which are present in more amount in case of higher gum ratio (Akbari, Ghoreishi et al. 2015). While when the amount PVA was increased (PG31), the downward curve shows the rapid degradative nature of the film. The structural integrity of the film was compromised which is indicated by the presence of a hump in the curve. PVA is degrading rapidly leaving behind gum which doesn't retain film forming property.

The swelling curve of the CMC/Gum composition shows that increasing CMC ratio increase the PECF uptake of the composition. The ratio 3:1 (CG31) hold more fluid because of free carboxyl and hydroxyl groups in the composite contrary to gum (Charpentier, Mocanu et al. 1997, Mitsumata, Suemitsu et al. 2003). The CG31 curve also shows stable increase in weight during latter half of the time.

All the three components under study were mixed together in different ratios as shown in bottom right graph. Here the ratio 3:1:3 (CPG313) hold more PECF uptake ability than rest of the ratios. This shows increase in weight at constant speed which will help in sustained release of drug in later section. The carboxyl and hydroxyl groups; present in abundance in CMC and gum; are responsible for this property.

4.1.2 Swelling Result of Hermal Seed Composites

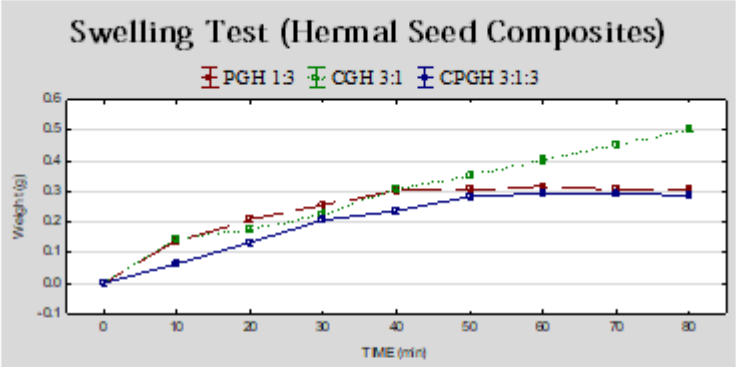


Figure 6. Graphical Representation of increase in weight of the films with Hermal Seed Extract PGH 1:3, CGH 3:1 and CPGH 3:1:3 when submerged in 5.5 pH PECF Solution

Hermal extract was added in the composite ratios (CG31, PG13, CPG313) showing greatest swelling results as mention above. As in can be seen in figure 2, CG31-H illustrates maximum increase in weight in 80 minutes and PG13-H has reached to its equilibrium state but the swelling curve of CPG313-H shows lesser uptake of PECF solution when compared with the other two. This may be because the oligosaccharide layer of basil seed gum binds strongly with polymers with carboxyl and hydroxyl groups by forming H- bonds (Archana, Sabina et al. 2013).

4.2 *In-vitro* Results of the Composite Films

4.3.1 Antimicrobial Testing

The composite films, placed on bacterial inoculated plates, gave zone of inhibitions that were recorded after 24 hours. Hermal extract released from the surface of the films contributed to the formation of zone of inhibitions. The maximum antibacterial activity was observed by PVA/Gum composite (PG13-H), as PVA is degrades quickly releasing hermal extract. Also, the structure of the films does not remain integrated and gum starts dispersing after the degradation of PVA during swelling of the films. While CG31-H has second largest zone of inhibitions with good structural integrity. CPG313-H formed smallest zones as compared to the other two because of the formation of hydrogen bonding between the gum and the carboxyl and hydroxyl groups of the polymers as well as of hermal extract (as discussed in swelling and FTIR analysis).

Arshad et al. demonstrated the Anti-bacterial activity of ethanoic extract of hermal seed and the zone of inhibition of *E. coli* 11–21 mm, *Staphylococci* sp. 14–18 mm was recorded. The solvent used for extraction do not have any anti-bacterial activity (Arshad, Zitterl-Eglseer et al. 2008). In the current research the mean diameter of the zone of inhibition of the composite films is shown in the table 1.

Table 2 In-vitro Results of the Composite films with Hermal Extract

Zone of Inhibition (mm)				
	PGH-13	**CGH-31**	**CPGH-313**	**Tetracycline**
MRSA	20.057	19.9999	18.7777	19.8923
P. aeruginosa	20.00011	17.011	15.988	20.20666667
E. coli	22.474	21.94	19.205	23.00112

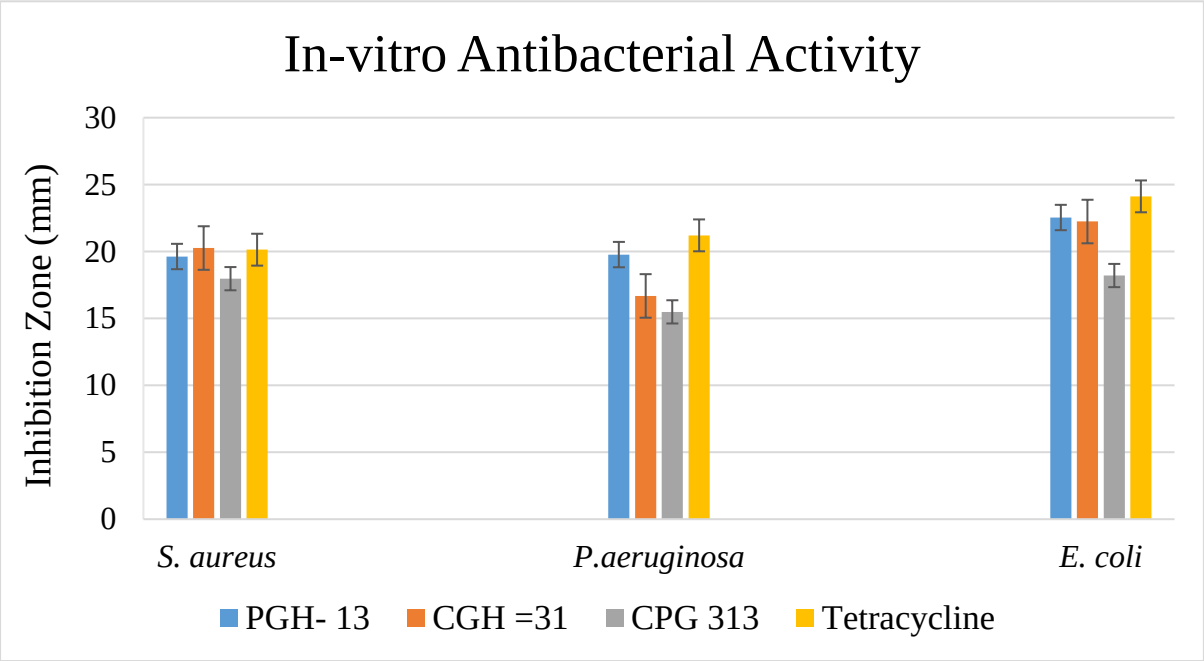


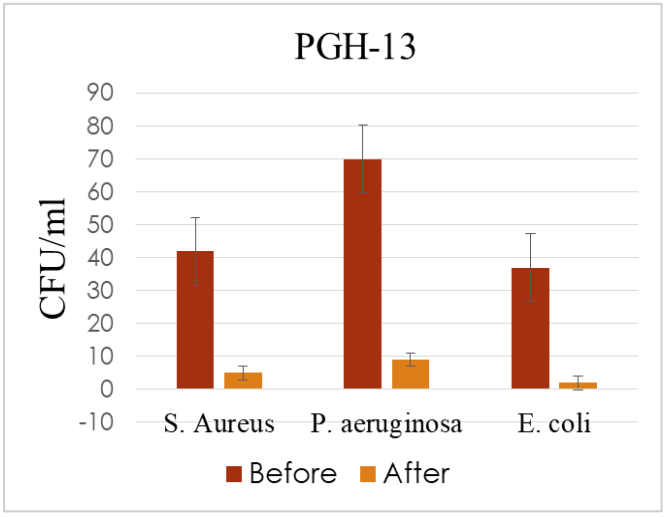
Figure 7. Graphical representation of antibacterial activity of different concentrations of composite films. Y axis shows zones in mm while x axis shows different bacterial strains used.

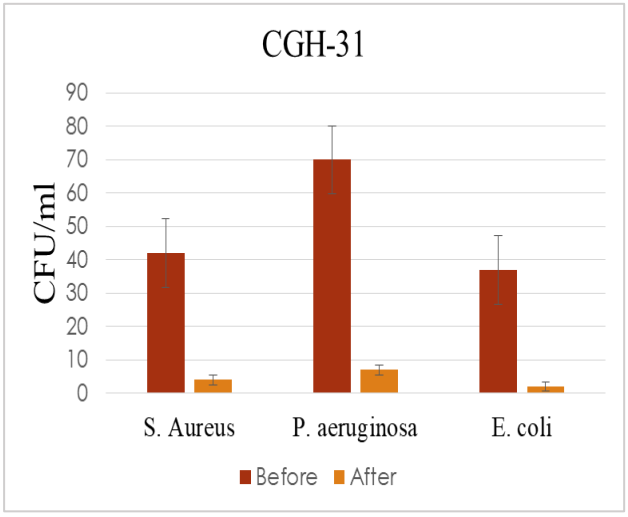
4.4 *In-vivo* Results of Composite Films

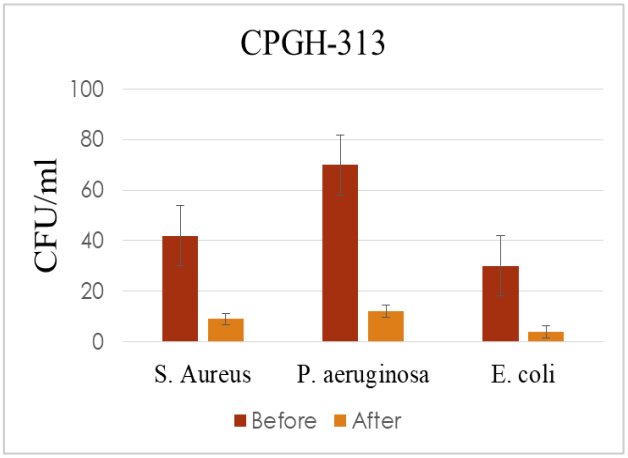
3 weeks old balb/c mice were used in in-vivo analysis of anti-microbial composite. Bacterial Count from pre administration and post administration were compared and analysed.

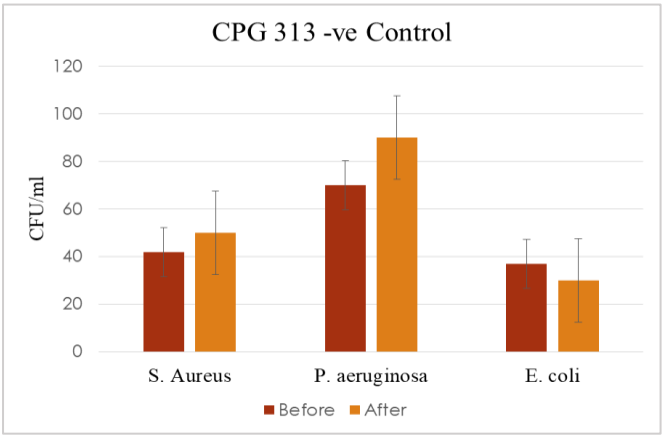
Table 3 In-vivo Results of the Composite films with Hermal Extract. Pre= Before treatment, Post 1= Primary site of wound, Post 2= Secondary site of wound

Colony forming Units									
	PGH-13			**CGH-31**			**CPGH-313**		
	Pre	**Post 1**	**Post 2**	**Pre**	**Post 1**	**Post 2**	**Pre**	**Post 1**	**Post 2**
MRSA	42	4	14	42	3	29	42	9	35
P. aeruginosa	69	9	24	69	6	29	69	11	43
E. coli	37	3	12	37	2	8	37	4	13









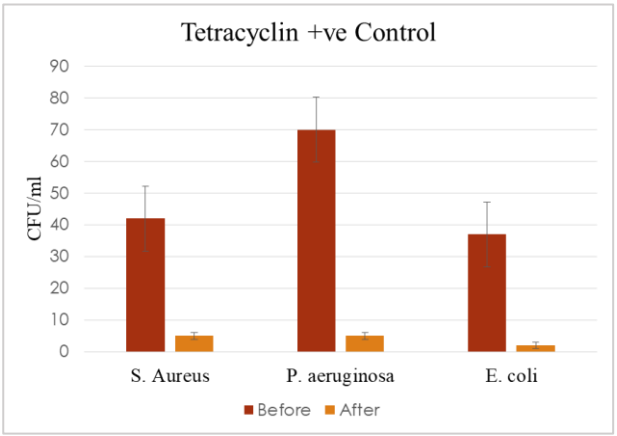
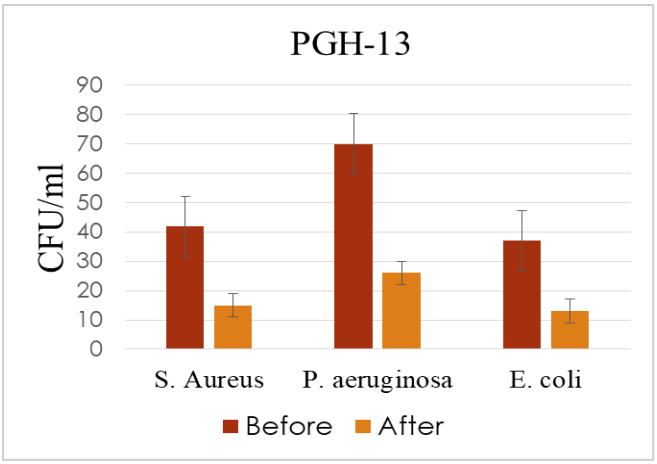
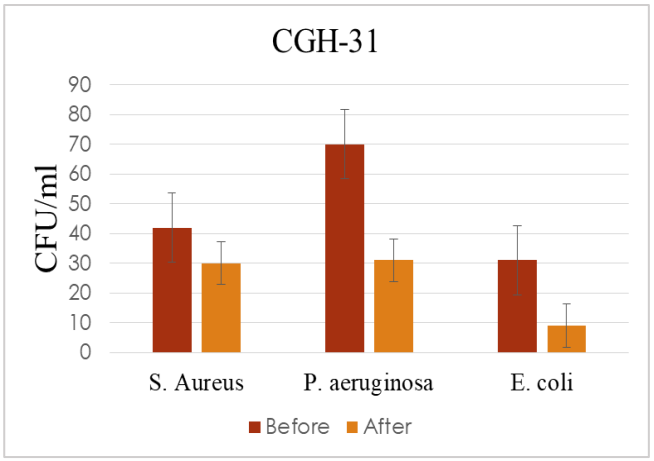


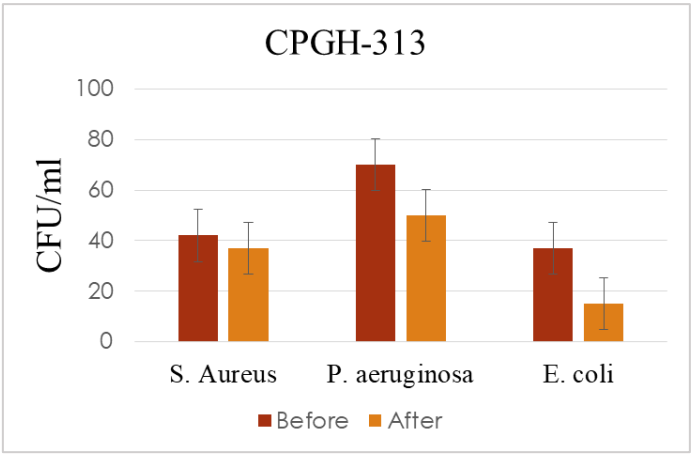
Figure 8. Graphical representation of antibacterial activity of different concentrations of composite films in-vivo at primary site. Y axis shows colony forming units per ml while x axis shows the effect before and after administration of composite. Figure 5(a) shows PGH 1:3, figure 5(b) shows CGH3:1, Figure 5(c) shows CPGH 3:1:3, figure 5(d) shows negative control i.e. without hermal seed extract and figure 5(e) shows positive control on tetracycline drug used.

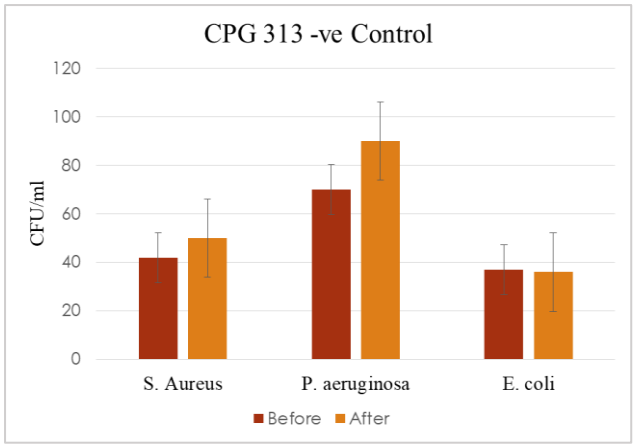
Table 4. In-vivo Results of the negative and positive controls. Pre= Before treatment, Post 1= Primary site of wound, Post 2= Secondary site of wound

Colony Forming Units						
	Negaive Control			**Positive Control**		
	Pre	**Post 1**	**Post 2**	**Pre**	**Post 1**	**Post 2**
MRSA	42	48	45	42	4	11
P. aeruginosa	69	88	80	69	4	19
E. coli	37	25	22	37	2	12









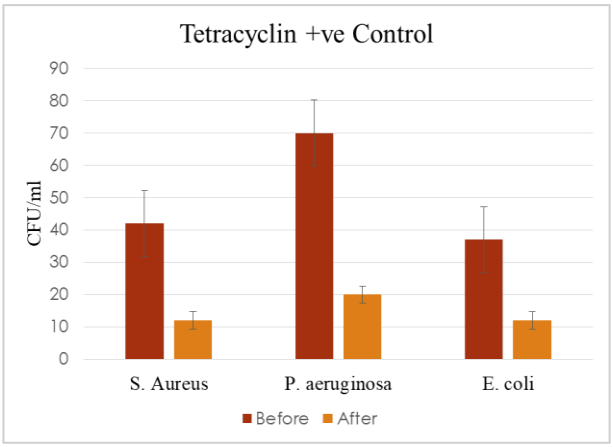


Figure 9. Graphical representation of antibacterial activity of different concentrations of composite films in-vivo at primary site. Y axis shows colony forming units per ml while x axis shows the effect before and after administration of composite. Figure 5(a) shows PGH 1:3, figure 5(b) shows CGH3:1, Figure 5(c) shows CPGH 3:1:3, figure 5(d) shows negative control i.e. without hermal seed extract and figure 5(e) shows positive control on tetracycline drug used.

In-vivo analysis shows decrease in microbial flora over the period of 24 hours. There was no effect of CPG-313, the composite without hermal seed extract. Microbes continued to flourish in presence of negative control. All the composites are highly effective at site of wound administration. CGH-31 showed best results and diminished colony forming units for three bacterial species. Considerable diminishing at secondary site is observed. CGH-31 showed bets results for secondary sites as well.

5. CONCLUSION

This study focused on the application of the composite for topical and subcutaneous applications where accumulation of exudate causes maceration and enhances bacterial growth. Hermal extract can be very suitable to counter this problem. A detailed analysis on swelling and property of Basil seed gum composites with PVA, CMC and hermal extract was done. Antimicrobial activity on diffusion discs as analysed. According to antibacterial and swelling tests results, CMC/Gum 3:1 with hermal extract showed sustained activity. Activity at primary site showed enhanced ant bacterial activity. Results on secondary site showed promising result in circulatory system of mice.

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