Formulation and physicochemical characterization of an antibacterial black garlic (*Allium sativum)*-based nanogel with wound healing property

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Declaration of Originality and Compliance of Academic Ethics

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as a part of her M. Tech (Food Technology and Biochemical Engineering) studies.

All the information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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SYNOPSIS

The thesis entitled 'Formulation and physicochemical characterization of antibacterial black garlic (*Allium sativum)* extract-based nanogel with wound healing property' examines the effectiveness of alliin-rich black garlic extract as an antibacterial wound healing nanogel. When garlic is crushed or chopped, an enzyme called alliinase interacts with alliin to produce allicin, which is known for its antibacterial effects. Allicin is renowned for its potent antibacterial properties. Since allicin is generated from alliin, the substance ought to have antibacterial properties as well. As a result, this thesis attempts to investigate the antibacterial potency of alliin-rich black garlic extract on selected bacterial species and incorporating it into a gel matrix and exploring its potency as an effective antibacterial wound healing nanogel as no such research work has been done yet. The thesis has been divided into three chapters. Each chapter has been divided into sections for clarity.

Chapter 1 deals with the Introduction and Review of Literature where **Section 1.1** emphasizes on the chemical composition of alliin and preservation of alliin in the black garlic cloves, making it available for further study. This section also focuses on the composition of nanogel and its component's role in the gel formulation. The significance of current research has been emphasized in relation to the significance of alliin as a chemical. This section, mainly focuses on evaluating the antibacterial potency and wound healing characteristic of black garlic extract.

Chapter 2 deals with assessing the presence of alliin in black garlic extract (soxhlet extraction using green solvent) using the HPTLC system and then assessing for its antibacterial potential against two bacterial strains. **Section 2.1** summarizes the current work taken up for this investigation and its literature gap. **Section 2.2** elaborates on the **Materials and Methods** employed in this section's research. The preparation of the black garlic and the method suitable for alliin extraction are discussed in this section. This section also includes the process parameters employed for formulation of nanogel and illustrates the process

chosen to assess the antibacterial potency of nanogel loaded with black garlic extract. The process opted to characterize the nanogel, *in vitro* and animal studies are all discussed in this section. **Results and Discussion** regarding the yield of black garlic extract and the resultant nanogel are presented in **Section 2.3**. It discusses about the method used to produce a stable black garlic extract-based nanogel. It further confirms the antibacterial potency of the formulated nanogel and its physicochemical properties. The *in vitro* and animal studies further confirms its acceptability in terms of skin irritancy and antibacterial wound healing nanogel. **Section 2.4** presents the **Conclusion and Future Prospects** of this research. It provides an overall inference and the broad objectives of this project. The chapter explains the utility of the results obtained for the common people and industries. This chapter explains the benefit of black garlic extract-based nanogel using it with regards to its suitability and application and future prospects in this area.

CHAPTER 1

1.1

Introduction and Review of Literature

1.1.1 Introduction

Optimally fermented garlic produces black garlic, which is high in alliin possessing numerous properties such as antibacterial and antioxidant effects, while having reduced pungent odor and off-flavor (Zhang *et al*., 2016; Lu *et al*., 2017).Owing to its chemical structure, thermal decomposition temperature, water solubility, minimal inhibition concentration, accessibility and sensory, properties black garlic was chosen for this investigation. Black garlic extract has not been reported to be used for topical medication administration in the treatment of skin wounds. This work envisages to develop a non-toxic, highly viscous nanogel with the rheology modifier carbopol® 940 to facilitate topical application which would enable in increasing penetration of drugs/active substances into the skin thereby aiding in wound healing.

The underground bulb of garlic (*Allium sativum*) has been consumed by human as a spice and medicine since antiquity (Onyeagba *et al*., 2004;Singh *et al*., 2019;) for its strong antioxidant, anti-inflammatory, anti-spasmodic, anti-allergenic, anti-viral, anti-bacterial/fungal, anti-protozoal, anti-parasitic, anti-diabetic, anti-thrombotic, hypo-homocysteinemic, hypo-lipidemic, anti-hypertensive, and neuroprotective activities (de Rooij *et al.,* 1996; Cao *et al*., 1996; Yin *et al*., 1998; Osman *et al*., 2017; Dorrigiv *et al*., 2020;). The therapeutic effects of garlic is due to the interactions and biological activities of its bioactive components (Osman *et al*., 2017). The major bioactive components of garlic are its organosulfur compounds, such as diallyl thiosulfonate (allicin), diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), ajoene, s-allyl-cysteine, and alliin (Yoo *et al*., 2014; Mansingh *et al*., 2018). Among these major components, allicin is the by-product of alliin (Rola *et al*., 2007). The garlic cloves store alliin and alliinase separately, when crushed, the enzyme alliinase aids in the conversion of alliin into allicin giving garlic its distinctive smell (Amagase *et al*., 2001; Bhatwalkar *et al*., 2021). In a study conducted by Mouffok *et*

al. (2023), 50% garlic extract and 50% deep eutectic solvent containing alliin as the major antibacterial component of the extract, exhibited effective performance against 11 microbial strains, including gram-positive and gram-negative bacteria as well as a fungal strain frequently encountered in healthcare facilities. Both alliinase and allicin degrade at high temperature of ≥ 35°C (Bhattacharya *et al*., 2021), thus indicating high temperature fermentation arrests the conversion of alliin into allicin. Fermented garlic, known as black garlic are produced at high temperature (60 - 90°C) with a high humidity (70% - 90%) for a period of 8-9 days (Martínez-Casas *et al*., 2017) with the following changes in sensory properties when compared to fresh garlic: color (grows darker), taste (induces sweetness and sourness), texture (creates a jelly-like mouth feel and increases chewiness), flavor (reduces pungent odor and off-flavor), and other physicochemical properties (Borlinghaus *et al*., 2014; Zhang *et al*., 2016; Lu *et al*., 2017). Thus, alliin as one of the bioactive compound present in black garlic extract was chosen for this study as no work correlating this bioactive compound with this extract has been done. Study reported by Ejaz *et al*. (2009) suggested that an increase in wound healing, re-epithelialization and extensive dose-dependent neovascularization were found on chicken skin wounds exposed to garlic extract. Thus, a topical drug delivery system focusing on skin wound healing was chosen for this investigation to fill the gap due to lack of research describing topical application of an alliin-rich black garlic extract as an antibacterial wound healing agent.

Out of the various types of topical application, nanogel has drawn much attention due to its anticipated specificity of delivery mechanism. Nanogel system consisting of spherical particles can be altered as per requirement by changing their chemical composition, size, charge, porosity, amphiphilicity and softness (Kersey *et al*., 2012). As topical applications can be benefited greatly from short flow, non-drip characteristics of nanogel (Alam *et al*., 2020), highly viscous nanogel

using the effective rheology modifier Carbopol® 940 has been implemented to produce sparkling clear gel which are compatible with a wide range of drugs and active ingredients subsequently allowing enhanced penetration of drugs or active ingredients into the skin (Silna *et al*., 2016). Carbopol® 940 based nanogels are polymeric material that exhibits the ability to swell and retain a significant fraction of water within its structure, without dissolving in water (Sabir *et al*., 2019; Ismail *et al*., 2021). Hydrophilic functional groups connected to the polymeric backbone provide the nanogel their capacity to absorb water; cross-links between network chains giving them their capacity to resist dissolution (Sultana *et al*., 2013; Neamtu *et al*., 2017).

Therefore, the objective of the present study is to formulate an alliin-rich black garlic extract incorporated in a nanogel matrix based on Carbopol® 940 polymer and to investigate its physicochemical properties, *in-vitro* drug release and animal study focusing on its effectiveness as an antibacterial wound healing formulation.

1.1.2 Review of Literature

1.1.2.1 Composition of black garlic

Black garlic is produced when garlic spontaneously ferments in a controlled environment, such as one with controlled temperature and humidity (Zeng *et al*., 2013; Kim *et al*., 2016; Qiu *et al*., 2018). Due to the low quantities of allicin, which make black garlic rich in alliin, the characteristic garlic odor gets diminished after fermentation. Its components include allin, cycloalliin, s-allyl-L-cysteine, s-methyl-L-cysteine, s-ethylcysteine, s-1-proponyl-L-cysteine, s-allylmercapto-L-cysteine, fructosyl-arginine, and beta-chlorogenin. It also consists of L-arginine, L-cysteine, and L-methionine (Allison et al., 2006). Out of which the major bioactive components are its organosulfur compounds (Figure 1), such as diallyl thiosulfonate (allicin), diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS), ajoene, s-allyl-cysteine , alliin (Mansingh *et al*., 2018). Garlic undergoes enzymatic browning and Maillard reactions as a result of the heating process, which changes the color from white and yellow to dark brown (Kang *et al*., 2016).

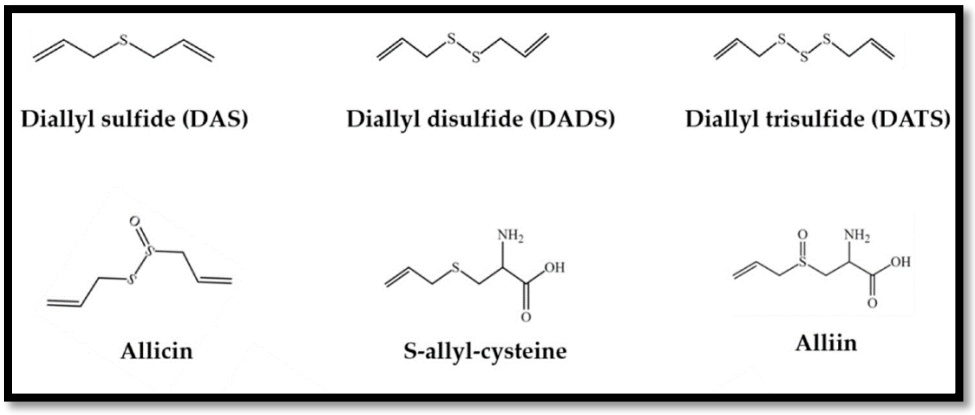

Figure 1: Major bioactive components of garlic

In comparison to fresh garlic (FG), black garlic (BG) has a lower moisture content and pH with higher levels of browning and high content of protein, lipid, carbohydrate, ash, total sugar, and reducing sugar. In general, black garlic has greater total and reducing sugar contents than FG, as well as higher levels of phenols, pyruvate, and flavonoids, but lower levels of allicin (Ryu *et al*., 2017). Four to eight times more s-allyl-cysteine is produced during the fermentation process of BG than in FG (Sasaki *et al*., 2007; Bae *et al*., 2012; Bae *et al*., 2014). With an increase in the concentration of amino acids like aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, methionine, isoleucine, leucine, tyrosine, and phenylalanine, BG's free sugar and mineral concentrations increase (Choi *et al*., 2008; Kanf *et al*., 2016).

1.1.2.2 Properties of black garlic extract

1.1.2.2.1 Anti-oxidant property

Black garlic extract (BGE) high in alliin is notable for having antioxidant properties. It is rich in phenolic, alkaloids, and flavonoids with -OH groups attached to the aromatic carbon rings that eases unpaired electrons (Lu *et al*., 2017). When compared to FG, the antioxidant activity of BG is the most potent, according to a research using the DPPH procedure, FG had a 20.21% antioxidant activity and BG had a 79.30% (Krovánková *et al*., 2018). 2,2′-azino-bis-3-ethylbenzothiazoline-6-ulfonic acid (ABTS) indicated 99.95% activity of BG in a study of free radical scavenging compared to FG's 35.92% (Toledano-Medina *et al*., 2016). . BG also has a positive impact on the body's ability to cope with stress, which is influenced by emotional and physiological circumstances. According to the research by Hosseini and Hosseinzadeh (2015), oral administration of BG to mice decreased the stress hormone corticosterone by 43% and increased serotonin and dopamine levels.

1.1.2.2.2 Anti-cancer activity

According to Almatroodi *et al*. (2019), BG has a strong therapeutic effect on a number of cancers including lung cancer, colon cancer, breast cancer, liver cancer, and leukemia. By raising the protein level of Bax while lowering the level of Bcl-2, BGE caused an increase in the protein expression of Bax/Bcl-2 (Ahmed, 2018). By blocking the phosphatidylinositol 3-kinase protein kinase B signaling pathway, it also decreased the HT29 colon cancer cell. It contributed to the expression of serine/threonine kinase and phosphorylated-serine/threonine kinase being down-regulated and phosphates being up-regulated (Liu *et al*., 2018).

1.1.2.2.3 Anti-microbial activity

High antibacterial properties of the BG as compared to the FG have been observed (Jeong *et al*., 2016).The study reported by Kang *et al*. (2017) on the antimicrobial activity of BG pomace extract was more effective against gram-positive bacteria (*Staphylococcus aureus* and *Listeria* sp.) as compared to gram-negative bacteria (*Escherichia coli* and *Salmonella* sp.). BG's antibacterial activity against *Streptococcus* mutans and *Enterococcus faecalis* significantly increased, according to a study of its antibacterial activity utilising the Kirby-Bauer diffusion method (Halimah *et al*., 2021).

1.1.2.2.4 Anti-inflammatory activity

Due to the presence of compounds including polyphenols, alkaloids, organic-sulfur components, polysaccharides, 5-HMF, melanoidins, and 2-linoleoylglycerol, BG has a strong anti-inflammatory effect (Najman *et al*., 2021). It prevented nitrogen-activated protein kinase enzymes from becoming phosphorylated (Mir *et al*., 2019).

1.1.2.2.5 Anti-allergy activity

BG is more effective as an anti-allergic agent against allergic reactions than FG as it inhibits immunoglobulin E-mediated allergic response in RBL-2H3 cells and *in vivo* passive cutaneous anaphylaxis, compared to FG's lower cytotoxicity and greater inhibitory effect (Yoo *et al*., 2014). According to Kim *et al*. (2012), BG exhibits anti-allergic properties that prevent the release of hexosaminidase from RBL-2H3 rat basophilic leukemia cells.

These results imply that using BGE, may inhibit the growth of skin pathogens and accelerate wound healing. In order to formulate a wound-healing antibacterial nanogel, the current work explores the antibacterial potency of this extract by soxhlet extraction using water as the solvent system.

1.1.2.3 Nanogel as a drug delivery system

According to Garg *et al*. (2012) nanogel are nanoparticles composed of a hydrogel containing cross-linked hydrophilic polymer particles in the nano-range. Nanogel are composed of biopolymers (Kabanov and Vinogradov 2009) or synthetic polymers that have undergone physical and chemical cross-linking. Nanogel are three-dimensional structures that can be used to entrap substances such as medicines, polymers, and liquid dispersion (Alvarez-Lorenzo *et al*., 2011; Vitiloiu and Leroux, 2008). According to Hayashi *et al*. (2004), nanogel have flexible size with a large surface area, and a high water content, which give them swelling and degrading properties. All biologically active substances and medications are delivered using nanogel in a regulated and sustained release method. Nanogel formulations are advantageous since a variety of polymer systems are readily available and their features can be easily fine-tuned as per requirement.

1.1.2.3.1 Advantages of nanogel

For a variety of reasons, nanogel are preferred to other drug delivery technologies, including (Gonçalves *et al*., 2010; Rigogliuso *et al*., 2012; Sultana *et al*., 2013; Soni *et al*., 2016):

1. High biocompatibility, making them a very promising drug delivery technology.
2. High biodegradability which prevents the buildup of nanogel material in the body's organs, which could cause toxicity and other negative effects.
3. Do not induce any immunological responses in the body as they are inert in the blood stream and the internal aqueous environment
4. Nano ranged size induces a number of effects such as:
    a) Superior permeation ability
    b) Passive and active drug targeting
    c) Potential to overcome the blood-brain barrier
    d) Improved capacity to reach places that hydrogels cannot reach on intravenous delivery
    e) Rapid adaptation to changes in the environment, including temperature and pH

1.1.2.4 Composition of nanogel

According to Khan *et al*. (2002) nanogel are essentially hydrogels of the nano regime made of a cross-linked network with a polymeric backbone. In addition to hydrogel characteristics, nanogel have an expanded range of nano-scale particle size, allowing them exceed the limitations of both micro-scale and macro-scale hydrogels. Due to their capacity to encapsulate a variety of drugs, nanogel is a promising technique that is currently being researched to accelerate wound healing (Grimaudo *et al*., 2019). Nanogel strategically administers anti-inflammatory drugs, antibacterial treatments, and essential growth factors to promote new tissue growth and blood vessel formation.

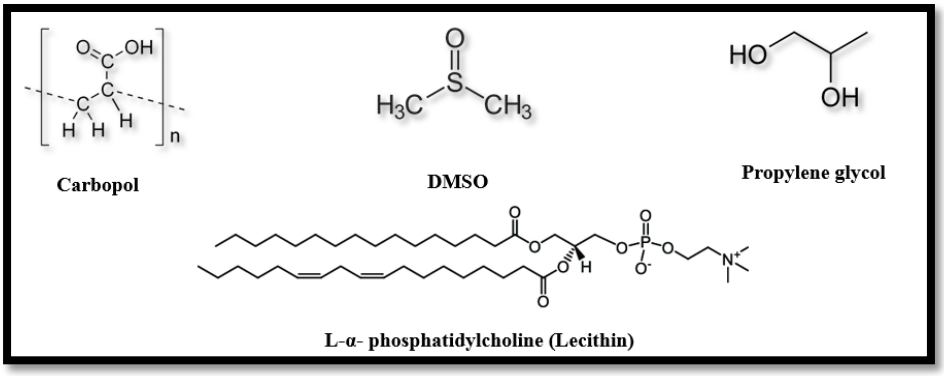

Figure 2: Structure of nanogel components

The following ingredients were used in the formulation of a stable nanogel:

- ✓ Carbopol 940 is a hygroscopic, mildly acidic synthetic polymer of acrylic acid (Figure 2) which is used as a gelling agent and aids in controlling the viscosity of gel preparations (Rowe *et al*., 2009). Due to this feature, it has been utilized as a bio-adhesive matrix for buccal, rectal, and nasal formulations as well as a gel matrix in suspensions and creams for external application (Nakajima *et al*., 1987; Wong *et al*., 1999). In order to create preparations that comply with the requirements for gel's physical properties, namely pH, viscosity, adhesion, spreadability, organoleptic and stability (Muramatsu *et al*., 2000), the concentration of carbopol 940 as a gelling-agent can be adjusted. The viscosity value of these preparations consequently influences the release of active drug substances.
- ✓ Dimethyl sulfoxide (DMSO) is an aprotic transparent solvent having a boiling point of 189°C at 1 atmosphere of pressure and a specific gravity of 1.0958 at 25°C (Booth and McDonald 1982). DMSO is a solvent for several herbicides, fungicides, antibiotics, and hormones. It can significantly increase their effectiveness and penetration into plants and trees, which increases plant growth by 15% to 20% (Garren, 1967; Kiehl, 1967; Leonard, 1967; Scuchetti, 1967; Smale *et al*., 1975). According to several studies (Weissman *et al*., 1967; David, 1972; Szmant, 1975),

DMSO can easily permeate the skin within five min of cutaneous application. This ability is believed to be a result of its ability to exchange and substitute for water in biological membranes. It is employed in clinical settings the carrier of antibacterial agents (Feldmen *et al*., 1975; Miranda-Tirado, 1975). As it speeds up the rate at which some compounds are absorbed through organic tissues, including skin, DMSO is primarily used in medicine as a topical analgesic (Hassan, 2014).

- ✓ Propylene glycol is a versatile non-toxic, antifreeze substance that attracts and locks in moisture and works as a preservative, making it a key ingredient in numerous foods, as well as cosmetic and skin care products (McGowan *et al*., 2018). It has been extensively used in skin preparations since 1932 either as a co-solvent for poorly soluble materials and to enhance drug permeation through the skin from topical preparations (Nicolazzo *et al*., 2005) and in different drug delivery systems as in liposomes where it can modulate biophysical properties of bilayer vesicles (Manca *et al*., 2014).
- ✓ Lecithin soya (30%) also known as L- α- phosphatidylcholine, the most abundant component of lecithin acts as an exceptional emulsifier. It can form lamellar mesophases and vesicles in an aqueous media (Mzutani and Nakamura. 1988) and also acts as filler particles which interact with the gel network reinforcing the network and therefore increasing the gel strength (Karleskind *et al*., 1995).

The following non-toxic, green and safe ingredients were used in this work in combination and ratios which have not yet been used for any formulation. As no work correlating alliin present in black garlic extract incorporated in a nanogel system has been reported, this work envisions in formulating black garlic extract-based nanogel.

1.1.2.5 Broad objective of the present investigation:

Formulation of stable black garlic extract-based nanogel which is rich in alliin (one of the organosulfur bioactive component) with a tentative shelf-life of 6 months having antibacterial wound healing property.

CHAPTER 2

Antibacterial and wound healing property of black garlic extract-based nanogel

2.1. Introduction

Fresh garlic (*Allium sativum*) when fermented for a set period of time at a controlled high temperature (60-90°C) and regulated high humidity (80-90%), yields black garlic. When compared to fresh garlic, black garlic does not have a strong unpleasant flavor and odor as it contains less amount of allicin, which gets converted through aging into antioxidant components including bioactive alkaloids and flavonoids (Yuan *et al*., 2016). The primary cause of the increased bioactivity of black garlic are due to the changes in physicochemical qualities. Black garlic extract has several functions, including anti-oxidative, anti-allergic, anti-diabetic, anti-inflammatory, and cancer protective properties (Yoo *et al*., 2014; Jeong *et al*., 2016). The antibacterial potential of alliin-rich garlic (raw) extract against few common human skin pathogens were reported by Mouffok *et al*. (2023); however, there is a lack of research on antimicrobial properties of alliin-rich black garlic extract. Till date, approximately 23 studies have been reported (Scopus) on black garlic and its components for its pharmacological and biological activities out of which 10 reports focus on the antibacterial potency of the black garlic extract, but none on alliin-rich black garlic extract. There is a gap in literature correlating these properties with topical skin application(s). The current investigation aims to explore the antibacterial potency of alliin-rich black garlic extract against *S. aureus* and *E. coli* to formulate a topical medication (nanogel) with promising wound-healing property.

Currently, to prevent infection and encourage quick healing, antibacterial medications, hydrogels, sponges, ointments, nanogels are being widely used for its efficient drug delivery system. Nanogel has the capability of overcoming the drawbacks of micro- and macro-scale hydrogels (Khan *et al*., 2020). Molecular composition, size, and design of nanogel can be modified and optimized to

produce a number of benefits, including stimuli responsive, efficient drug loading, and exceptional permeability over biological barriers, biocompatibility, and physical stability of the system (Hamidi *et al*., 2008). Carbopol® 940, a readily available polymer, which is non-toxic and non-irritating is frequently utilized for developing stable nanogel systems that aid in encapsulating an array of bioactive compounds.

Commercially available gels present in the market such as Hydroheal AM gel is formulated using non-green, toxic ingredient (colloidal silver) which on prolonged application to our skin leads to Argyria, a permanent bluish-gray skin discoloration. Additionally, it may interfere with drug absorption and lead to potential issues of kidney, liver, or nervous system (Anonymous, 2023a). The current investigation aims to use non-toxic, green ingredients in formulating a topical gel which are safe for long-term human usage.

Thus the specific objectives of the present investigation are:

1. To prepare black garlic from fresh garlic cloves
2. To perform soxhlet extraction using green solvent for black garlic powder
3. To quantify the amount of alliin present in black garlic extract using HPTLC analysis
4. To check the antibacterial potency of alliin-rich black garlic extract against bacterial strains
5. To formulate black garlic extract-based nanogel
6. To assess the antibacterial potency of the formulated nanogel
7. To carry out the physicochemical analyses of the formulated nanogel
8. To conduct skin irritancy study of the formulated nanogel on rabbits
9. To conduct wound healing study on rabbits using the formulated nanogel

To the best of our knowledge, no such study has yet been published

2.2

Materials and Methods

MATERIALS AND METHODS

2.2.1. Materials

Authenticated fresh garlic (*Allium sativum*) was procured from Jaydev Agro International Export Company, Maharashtra, India. Specialty Chemicals such as alliin (>98% HPLC grade) was procured from M/s Sigma-Aldrich, Munich, Germany, and L-α-Phosphatidylcholine (Lecithin Soya 30%) was purchased from M/s HiMedia, Maharashtra, India. Silica gel 60 (F_{254}) coated aluminium plates were obtained from M/s E-Merck, Maharashtra, India. Low density polyethylene (LDPE) pouches (dimensions 0.25 m × 0.18 m) and aluminum (Al) foil were purchased from Prince Plastic Pvt. Ltd., West Bengal, India. All other chemicals used were of analytical grade. Mueller Hinton (MH) broth (M/s HiMedia, Maharashtra, India), ATCC strains of *Candida albicans, Staphylococcus aureus, Escherichia coli*, MDR strain of *Escherichia coli* suspensions and Augmentin antibiotic discs were all availed from Peerless Hospital, West Bengal, India.

2.2.2. Methods

2.2.2.1. Fermentation of raw garlic

After procurement, the garlic samples were cleaned of dirt and placed in a rice cooker (Rize Excel 1.2 L, Kutchina, West Bengal, India) under warm mode (70°C) for a period of 8-9 days for controlled fermentation according to the method described by Lee *et al*. (2009).

2.2.2.2. Preparation of black garlic powder

Fermentation rendered the white garlic cloves black which were then peeled and cleaned. The obtained black garlic cloves were ground into powder using mortar pestle. The obtained powder was wrapped in an aluminum foil, followed by packing in LDPE pouch which was kept in a desiccator for future analyses.

2.2.2.3. Soxhlet extraction of alliin from black garlic powder

Extraction of alliin from black garlic powder (10 g) was carried out using Soxhlet extraction in three different green solvent systems, i.e., water (BG1), ethanol-water in the ratio 1:1 (BG2) and ethanol (BG3). Black garlic powder to the solvent systems were in the ratio 1:20 and each extraction was conducted for 2 h.

2.2.2.4. Concentration of the black garlic extracts (BGE)

BGE (BG1, BG2 and BG3) were concentrated in a rotary vacuum evaporator (M/s R1160 Superfit Continental, Maharashtra, India) at 45°C and 50 mbar Hg. The yield of the respective concentrated extracts were determined gravimetrically. Subsequently, the extracts were dissolved in distilled water and stored in amber colored glass vials at -18°C, until further analyses.

2.2.2.5. Physicochemical and antibacterial characterization of the extracts

2.2.2.5.1. Quantification of alliin using high-performance thin layer chromatography (HPTLC) method

HPTLC analyses of BGE samples were conducted to estimate their alliin contents in accordance to the method described by Kanaki and Rajani (2005). All extracts were applied in the form of bands, 8 mm wide with 13.6 mm spacing between consecutive bands, using a Camag Linomat V (M/s Camag, Switzerland) on Al TLC plates (200 mm × 100 mm), coated with silica gel 60 (F_{254}). The plates were sprayed with a saturated solution of ninhydrin reagent and kept at 100°C for 5 min in a hot air oven for color development. Densitometric analysis was carried out using Camag HPTLC unit (TLC scanner IV) at 450 nm employing VisionCATS 3.0.20196.1 software (Muttenz, Switzerland). The R_f value of alliin was found to be 0.82.

2.2.2.5.2. Antibacterial potency assessment of Std alliin and BGE

The *in vitro* antimicrobial assays were conducted at Peerless Hospital, West Bengal, India, under the supervision of professional microbiologists. For assessment of antibacterial potencies of Std alliin and BGE, the bacterial suspensions of ATCC strains of *Candida albicans* (ATCC 14053), *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922) were used (0.5 McFarland standard).

2.2.2.5.2.1. Determination of minimum inhibitory concentration (MIC)

MIC values were determined using a sterile 96-well microtitre plate (0.5 McFarland was considered as the opacity standard). Stock solutions of Std alliin (250 µg/mL), BG1 (60 µg/mL) and BG2 (80 µg/mL) were prepared by dissolving each in distilled water. From the apposite stock solutions, 100 µL of standard and black garlic extracts were added respectively to each well following micro-broth dilution method (Anonymous, 2023b) followed by loading of 10µL of bacterial suspensions to each well. The ingredients of each well were mixed thoroughly and the plate was incubated at 37°C for the next 24 h. The OD values of the mixture in the wells were measured twice, once at 620 nm immediately after addition of the bacterial suspensions and after 24 h of incubation using Multiskan™ FC Microplate Photometer (Model number: 51119000; Thermo Scientific™), from which MIC values were determined. The extract having the lowest MIC value was used for further analyses and was coded as BGE_{Best}.

2.2.2.6. Formulation of nanogels incorporating Std alliin and BGE_{Best}

Homo-polymeric synthetic hydrogels with physical cross-linking (Iizawa *et al*., 2007; Zhao *et al*., 2013) were formulated for Std alliin and BGE_{Best} nanogels. The hydrogels were prepared in two phases i.e., an organic and an aqueous phase. Average particle size of the hydrogels were reduced to nano-scale by employing two stage homogenization to obtain nanogels. The concentrations of

the ingredients (% w/w) used for the formulation of nanogels are listed in Table 1. For the organic phase, weighed amount of BGE_{Best} (based on the findings of MIC value) was dissolved in a mixture of DMSO and lecithin soya (30%). This mixture was vortexed for 20 min followed by addition of propylene glycol which was bath sonicated for 15 min. For the aqueous phase, Carbopol® 940 was stirred in distilled water using a magnetic stirrer for a duration of 1 h at 1200 rpm at 80 °C. The clear gel thus obtained was homogenized at 6000 rpm for 30 min followed by 8000 rpm for 45 min. During the second homogenization stage, 2 mL of organic phase was slowly added to the aqueous phase at an interval of 5 min. In the control nanogel no extract was added whereas in the Std nanogel, pure alliin standard dissolved in DMSO was added to the aqueous phase.

2.2.2.6.1. Quantification of alliin in Std and BGE_{Best} nanogels by HPTLC

For ascertaining the content of alliin in the prepared nanogels by HPTLC, 1 g of nanogel was dissolved in 2 mL distilled water. The solutions were vortexed and subsequently centrifuged (M/s R-8C Remi Laboratory Centrifuge, Maharashtra, India) for 5 min at 1500 x g at 25°C. The alliin content were determined using 30 µL of the diluted supernatant solutions, following the previously described procedure in section 2.2.2.5.1.

2.2.2.7. Physicochemical characterizations of the formulated nanogels

2.2.2.7.1. Specific gravity, pH, % weight loss on drying, spreadability, phase separation and hydrophilic-lipophilic balance (HLB) value of nanogels (control and BGE_{Best})

The physiochemical analyses of the nanogels included- determination of specific gravity by pycnometer, pH by digital pH meter (M/s Eutech Instruments, West Bengal, India); % loss on drying (at 105°C) by adopting the method reported by Buhse *et al*. (2005). The spreadability of the nanogels were assessed following the procedure adopted by Ghosh *et al*. (2016) using the equation:

$$S = (m.L)\ T^{-1} \quad (1)$$

where, S= spreadability of the nanogel; m= weight attached (66g); L= length of glass slide (7.5 cm); T= time taken

The viscosities of the nanogels were determined using Brookfield Digital Viscometer (M/s model: LVDVE230, Brookfield, USA) using spindle 4 (S64) at 25°C by plotting a graph of log shear vs log shear rate and the data were fitted into different model equations.

Centrifugation at 2500 x g (M/s Eltek, Maharashtra, India) and phase separation studies at 37°C and 55°C in water bath were conducted in accordance with Widodo *et al*. (2002). HLB values were determined according to Fennema (1996) using the following equation:

$$HLB = 20 - [1-(S/A)] \quad (2)$$

where, S= saponification value; A= acid value

2.2.2.7.2. Field emission scanning electron microscope (FE-SEM) analysis of nanogels

The surface morphology and average particle size of the nanogels were studied using FE-SEM (M/s INSPECT F50, FEI Company, Oregon, USA) at an operational voltage of 5 kV. The nanogels (control and BGE_{Best}) were dried and coated with gold using Q150R ES Coater (M/s Quorum Technologies Ltd., Kent, England) prior to the analysis.

2.2.2.7.3. Fourier transform infrared spectroscopy (FT-IR) and attenuated total reflectance (ATR) analyses of control and BGE_{Best} nanogels along with their components

FT-IR and ATR analyses were carried out to assess whether the constituents were incorporated in the nanogel matrix. FT-IR analysis of powdered samples (lecithin soya 30%, Carpobol® 940) were carried out by placing them on KBr pellets and subjected to FT-IR spectrometer (M/s PerkinElmer, Massachusetts, US). ATR analysis of liquid samples (DMSO, propylene glycol, Std alliin solution, BGE_{Best}, control nanogel, and BGE_{Best} nanogel) were conducted using a laser class I (light source) at 45° angle of incidence.

2.2.2.7.4. Release profile study of BGE_{Best} nanogel

The release profile of alliin from the BGE_{Best} nanogel was studied following the method described by Ghosh *et al*. (2016). 0.5 g of BGE_{Best} nanogel was added to 50 mL of phosphate buffer saline (PBS) solution (0.1 M, pH 7.2). The nanogel was continuously stirred using a magnetic stirrer maintaining a temperature of 34°C at 50 rpm. 3 mL aliquot was collected at an interval of 5 min for a period of 2 h and was replaced with same amount of fresh PBS solution. Alliin content in the nanogel was determined using the HPTLC method as described in section 2.2.2.5.1.

2.2.2.8. Analyses of antibacterial potency of Std alliin and BGE_{Best} nanogels

100 mL of MH agar plates were prepared by dissolving 3.8 g of MH agar powder (M/s HiMedia, Maharashtra, India) in sterile (autoclaved) distilled water.

2.2.2.8.1. Kirby-Bauer disk diffusion susceptibility test

The zones of inhibition against uniform suspensions of ATCC strains of *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922) and MDR strain of *Escherichia coli* (0.5 McFarland standard) were evaluated using the Kirby-Bauer disk diffusion susceptibility test (Hudzicki, 2009). Sterile non-toxic cotton swab (on a wooden applicator) soaked well in respective bacterial suspensions were spread three times by streak plate methods on the autoclaved MH agar plates. Sterilized antimicrobial discs (Whatman® antibiotic assay discs, 6 mm in diameter) coated with control, Std and BGE_{Best} nanogels were placed on the agar plates maintaining at least 24 mm distance from the edge of the plates. Augmentin antibiotic disc (6 mm), the standard drug for all the above-mentioned strains, was served as experimental control. The plates were kept at 37°C for overnight incubation, thereafter the diameters of zones of inhibition were measured following the protocol described by Hudzicki (2009).

2.2.2.9. Animal study of skin irritancy and wound healing study on rabbits

Skin irritancy test and assessment of wound healing efficacy of BGE_{Best} nanogel were carried out at the Clinical Research Centre, Jadavpur University, in collaboration with professional clinical pharmacologists. For the tests, four healthy New Zealand white Albino male rabbits, 6-8 months old, weighing between 1.52-1.78 kg were procured from M/s Reeta Ghosh Private Ltd., West Bengal, India. The animals were housed in individual cages and were acclimatized to the test environment (22ºC, 60-70% RH with 12 h light and 12 h dark cycle) for seven days. Prior to the experiments they were provided with *ad-labium* diet and drinking water (Teshome *et al*., 2008; Ghosh *et al*., 2016).

2.2.2.9.1. Skin irritation test of BGE_{Best} nanogel on rabbits

For safe future human usage, it was necessary to conduct the skin irritation test on a mammal such as rabbit following the OECD test guideline 404 (2014). On the day of the test, an area of 5 cm x 5 cm was meticulously shaved from the dorsolateral trunk of each rabbit without abrading the skin. Approximately, 1g of BGE_{Best} nanogel was evenly applied on the demarcated area and was monitored for a period of 72 h to test the skin irritability level. Skin responses, including erythema (redness) and edema (swelling) were evaluated in accordance with the Draize dermal irritation scoring system (Draize *et al*., 1944) - (0: no erythema or no edema; 1: barely perceptible erythema or edema; 2: well defined erythema or slight edema; 3: moderate to severe erythema or moderate edema; 4: severe erythema or edema). The primary irritation index (PII) was evaluated using the following equation:

$$PII = [(\Sigma \text{ ER at 24, 48 and 72 h}) + (\Sigma \text{ ED at 24, 48 and 72 h})]/n \quad (3)$$

where, n is the total number of observations ER- erythema and ED-edema. Negligible irritant (0.0–0.4); mild irritant (0.5–1.9); moderate irritant (2.4–4.9); severe irritant (5-8) (Amasa *et al*.,

2012). Until the experiment's conclusion, the animals were observed twice a day for morbidity and mortality and once a day for clinical toxicity signs.

2.2.2.9.2. Wound healing test on rabbits

2.2.2.9.2.1. Wound creation

Four New Zealand white Albino male rabbits (R1, R2, R3 and R4) were subjected to the wound healing experiment. The animals were anaesthetized using ether anesthesia and were secured to a sanitized table in their natural positions. Full thickness skin from the demarcated area was excised including the subcutaneous tissues (*Panniculus carnosus*) to create an epidermal wound (approximately 7.5±2 mm^2) following the method described by Morton (1972) on each rabbit. Following wound cleansing with sterile cotton, control nanogel, commercially available gel (positive control), and BGE_{Best} nanogels (2% and 4%) were applied to the wound area of R1, R2, R3 and R4, respectively. The treatment was continued until the wounds were completely healed.

2.2.2.9.2.2. Assessment of wound healing

The physical attributes of wound healing *viz*., wound closure, epithelialization time and scar features were studied from day 0 to day 6 by tracing the wound areas of R1, R2, R3 and R4, respectively. The degree of wound healing was calculated as percentage closure of the wound area from the original one using the following formula (Walker and Mason, 1968):

$$\text{Percentage closure} = [1-(A_d/ A_0)] \times 100 \quad (4)$$

where, A_0 is the wound area on day zero; A_d is the wound area on corresponding days.

2.2.2.10. Sensory evaluation of control and BGE_{Best} nanogels

Sensory evaluation of the nanogels were performed by a panel of semi-trained persons, at three stages of application on skin- ‘before rubbing and during pick up’, ‘during rubbing’ and ‘after-feel’ where parameters such as stiffness, grittiness, color, odor, homogeneity, stickiness, shine,

absorbance, skin feel, and spreadability were evaluated (Lukic *et al*., 2012). The evaluation was conducted by 6 panelists comprising of University Research Scholars in the age group of 25-34 years on day 1 and day 30. The panelists were acquainted with the various parameters of the sensory attributes of the nanogels. Evaluations were carried out in a well illuminated and ventilated room. Before application, the skin surface and fingers of the panelists were wiped with clean sterile cotton. The panelists applied the nanogels at the back of their hands using ten circular motions. The panelists were found to be non-allergic to the ingredients of the nanogel formulations and were free from any skin disease. The response of the panelists was recorded by rating the nanogels on a 9-point hedonic scale (9-like extremely and 1-dislike extremely) according to the method reported by Almeida *et al*. (2007).

2.2.2.11. Stability studies

The storage stability of nanogels were assessed for a period of 30 days and was checked for visual growth of mold. Assessments of antibacterial, physicochemical and sensory properties (at 23ºC, 70-75% RH) were carried out as discussed in sections 2.2.2.7.1 and 2.2.2.10.

2.2.2.12. Statistical analyses

In this investigation microbiological and physicochemical assays were conducted in triplicates and represented as mean ± S.D. of three independent analyses of three independent batches of samples. Statistical analyses was performed using one-way analyses of variance (ANOVA). A *p value* of 0.05 was used to verify the significance of all test assays. All statistical analyses were conducted using STATISTICA 8.0 software (Oklahoma, USA).

2.3

Results and Discussion

2.3.1. Black garlic powder obtained from fermented raw garlic

Garlic cloves after fermentation turned into a brownish black color (Figure 3a) with a decreased characteristic garlic odor. Fermented black garlic cloves yielded 95.67% of powder (Figure 3b).

2.3.2. Concentrated soxhlet BGE

The employed solvent systems produced 1.27-4.96 g of concentrated Soxhlet extracts. The lowest yield (1.27 g) was obtained while using ethanol as the solvent and that of the highest (4.96 g) while using ethanol: water (1:1). The solvent system as water yielded 4.54 g of extract, thus indicating that water and ethanol: water (1:1) having the highest yield.

2.3.3. Characterization of the Std alliin and BGE

2.3.3.1. Quantification of alliin in BG1 and BG2

Alliin contents of 1.8 mg/g and 1.322 mg/g were quantified for BG1 and BG2 respectively, with an R_f value of 0.82. Alliin could not be quantified in BG3 owing to its poor solubility in ethanol. BG1 had the highest content of alliin since the same is highly soluble in water (Iberl *et al*., 1990).

2.3.3.2. Antibacterial potency of Std alliin and BGE

The MIC values of Std alliin were 10µg/mL, 40µg/mL and 70 µg/mL for *Candida albicans, Staphylococcus aureus* and *Escherichia coli*, respectively. On an average, the MIC value of BG1 was 15µg/mL and that of BG2 was 25µg/mL for the above-mentioned strains (Figures 5). From this data, it can be interpreted that Std alliin has potential antibacterial property and out of the two extracts, BG1 had lower MIC value implying greater potential to inhibit the microbial growth. BG1 was used for further analyses and henceforth, referred as BGE_{Best}.

2.3.4. Nanogels incorporated with Std alliin and BGE_{Best}

Prepared Std nanogel (Figure 6) was odorless and clear in appearance whereas, BGE_{Best} nanogel (Figure 6) was pale yellow in color with translucent appearance and a slight garlic characteristic

odor to it.

2.3.5. Physicochemical properties of the formulated nanogels

2.3.5.1. Specific gravity, pH, % weight loss on drying, spreadability, phase separation and HLB value of nanogels (control and BGE_{Best})

The physicochemical analyses of the formulated topical nanogels are listed in Table 2. The pH values of 6.90 and 6.82 for control and BGE_{Best} nanogels respectively, were well within the range to be suitable for application as a topical medication, compatible with human skin (Opatha *et al*., 2022). Similar pH values were reported by Wadile *et al*. (2019) for their nanogel incorporated with itraconazole nanoparticles (pH=6.8); and by Ali *et al*. (2022) who reported a pH value of 6.87 for lidocaine wound-healing nanogel. The specific gravity of nanogels were 1.038 for control and 1.021 for BGE_{Best} nanogel, respectively, its % weight loss on drying were 2.31% for control and 1.5% for BGE_{Best} nanogel (dry basis). The spreadability of nanogel, a crucial aspect as it influences its viscosity and sensory acceptability (Miastkowska *et al*., 2020) were 15.92 g cm s^{-1} for control and 12.41 g cm s^{-1} for BGE_{Best} nanogel. Similar spreadability value was reported by Inamdar *et al*. (2018) for nanogel loaded with beta sitosetrol as its bioactive component.

The graph of log shear stress vs. log shear rate presented in Figure 7 indicated the nanogels to be non-Newtonian fluids. Model fitting exercise revealed that viscosity data to best fit the Modified-Casson equation:

$$\log \tau = \log \mu_{app} + n \log (\partial u/ \partial y) \quad (5)$$

where, τ is shear stress (dyne/cm); $\partial u/ \partial y$ is shear rate (sec^{-1}); μ_{app} is apparent viscosity (cP) and n is flow behavior index (dimensionless) (n = 1 for Newtonian fluid, n < 1 for a pseudoplastic fluid, n > 1 for a dilatant fluid).

The flow index (n < 1) using the above equation indicated the nanogels to have a pseudoplastic flow behavior. From equation 3, apparent viscosity for control nanogel was 18.92 cP and that of BGE_{Best} nanogel was 23.03 cP. The nanogels being shear thinning with appreciable spreadability were advantageous for effortless topical application. Similar flow behavior was reported by Agarwal *et al*. (2017) for semi-herbal clindamycin phosphate aloe-vera nanogel. No phase separation on centrifugation at 37 °C and 55 °C indicated the formulation to be properly homogenized. HLB values for the control nanogel was 17 and that for BGE_{Best} nanogel was 18.6 inferring the formulation to be hydrophilic (Fennema, 1996).

2.3.5.2. Micro structure of nanogels

The FE-SEM analyses revealed that the control and BGE_{Best} nanogels had a smooth spherical surface morphology. Average particle size was 286.06 nm and 217.77 nm for control and BGE_{Best} nanogels, respectively (Figure 8). From the findings it can be inferred that the average particle size of the formulated nanogels ranged in the nano-scale (217-286 nm) and can be nomenclatured as nanogel.

2.3.5.3. FT-IR and ATR spectra

The FT-IR and ATR spectra were analyzed using the wavenumbers reported by Dyer (2012). The transmission (%T) peaks of BGE_{Best} was similar to Std alliin solution. Transmission peaks of nanogels was similar to those ingredients present in the formulation. The FT-IR and ATR analysis affirmed that the Soxhlet extract (BG1) was rich in alliin and was successfully incorporated in the nanogel matrix.

The functional groups and bonds present in the samples are presented in Table 3. The spectra indicated the presence of O-H stretching (3628, 3348 cm^{-1}), C-H stretching (2853 cm^{-1}), C=C alkene stretching (1651 cm^{-1}), C-O-C stretching (1249, 1225 cm^{-1}) and C-F stretching (1044, 1016 cm^{-1})

confirmed the presence of Std alliin in BGE_{Best} nanogel along with the other ingredients of the formulation.

2.3.6. *In vitro* release profile of BGE_{Best} nanogel

The findings of BGE_{Best} nanogel indicated a 75% burst release of alliin at 5 min indicating a high release rate which is advantageous for instant drug delivery on topical application. Similar burst release has been observed by Gu *et al*. (2007) for PNIPAM-co-acrylic acid hydrogels in the submicron range.

2.3.7. Antibacterial potencies of formulated nanogels

2.3.7.1. Kirby-Bauer disk diffusion susceptibility test

Std alliin nanogel exhibited a zone of inhibition of 7 mm for *Staphylococcus aureus* and that for BGE_{Best} nanogel was 10 mm. For *Escherichia coli*, the zone of inhibition was 7 mm for Std alliin nanogel and 8 mm for BGE_{Best} nanogel and that for MDR strain of *Escherichia coli* it was 6 mm for Std alliin nanogel and 7 mm for BGE_{Best} nanogel (Figure 10). From these findings it can be inferred that BGE_{Best} nanogel has a greater antibacterial potency when compared to Std nanogel, making the test nanogel a feasible formulation.

2.3.8. Animal study on skin irritancy and wound healing property on rabbits

2.3.8.1. Skin irritation test of BGE_{Best} nanogel

PII scores of nanogel for erythema and edema were zero, indicating that during the course of the study, there were no signs of skin irritation at the test location (Figure 11; Table 4). Post application of the nanogel on skin surfaces, the rabbits did not exhibit any clinical signs of toxicity or fatality. Therefore, it can be concluded that the BGE_{Best} nanogel had no adverse irritancy effect when applied to the skin of rabbits and thus could be applied topically to evaluate its efficacy in epidermal wound healing.

2.3.8.2. Wound healing study on rabbits

Figure 12 portrays the epidermal wound healing experiment using positive control gel, control nanogel and BGE_{Best} nanogels (2% and 4%) on rabbits R1, R2, R3 and R4, respectively. Post topical application, epithelialization of tissue over the wounds (in R3 and R4) commenced on day 2 and by day 6, 67% and 77% of wounds were healed in R3 and R4, respectively; which was found to be almost similar to the wound healing efficacy of the positive control gel which exhibited a wound closure of 86% by day 6 in R1. Besides, the control nanogel exhibited no signs of wound closure in R2, indicating that the control nanogel on its own did not participate in the wound healing process and thereby confirming the wound healing property of BGE_{Best} nanogels. The wound healing efficacy of 4% BGE_{Best} nanogel in R4 was found to be better in comparison to 2% BGE_{Best} nanogel in R3 as the healing process was accelerated in R4. Additionally, 2% BGE_{Best} nanogel and 4% BGE_{Best} nanogel protected the wound from external infection. No scar was left behind after the wound healed completely on day 7 in R4 establishing BGE_{Best} nanogel (4%) to be a promising antibacterial topical nanogel with accelerated wound healing property. A study reported by Ahmed *et al*. (2019) revealed accelerated wound healing of rabbits using *Centella asiatica* hydrogel, similar to our findings.

2.3.9. Sensory evaluation of control and BGE_{Best} nanogels

From the sensory evaluation of nanogels, the panel preferred the non-sticky consistency and shiny appearance of BGE_{Best} nanogel. Both the control and BGE_{Best} nanogels were highly spreadable, moderately slimy and very shiny with moderate absorbance. The findings suggested that the panel validated the formulation as a topical nanogel with moisturizing attributes. The BGE_{Best} nanogel was favored for its color, odor, spreadability, absorption, and non-irritancy, as proven in the radar plot by the panelists on comparison with control nanogel (Figure 13).

The shear thinning behavior of BGE_{Best} nanogel contributed to its spreadability, resulting in good absorption and high primary and secondary skin feel (Brummer *et al*., 1999). The property of a nanogel to spread without being sticky has been attributed to a good quality and it allows formation of a homogenous layer on skin without being highly adhesive (Ghosh *et al*., 2016). The safety of nanogel for human use was further validated by the skin irritancy test on rabbits as has been discussed in section 2.3.8.1. The present study conclusively demonstrated the potential of black garlic based-nanogel for topical application. These attributes agree well with pharmaceutical recommendations for effective topical delivery of active ingredients through the human skin as reported by Kulawik-Pióro *et al*. (2019) who studied the quality of barrier creams with similar attributes.

2.4

CONCLUSION

AND

FUTURE PROSPECTS

This work is the first to describe the antibacterial efficacy of black garlic extract (rich in alliin) loaded in a nanogel. The resultant yellow tinted black garlic extract-based nanogel exhibited antibacterial potency against common bacterial pathogens *S. aureus* and *E. coli*. The nanogel formulation produced homogenous, spherical, lump-free particles in the nanometer range with a smooth surface morphology. With a pH range of 6.82 to 6.90, BG1 outperformed BG2 in terms of drug content and MIC value. The formulated nanogel can be used for topical application with ease owing to its excellent spreadability and viscosity values. The spreadability value of 12.41 g cm s^{-1} for BGE_{Best} nanogel along with its pseudoplastic behavior validate the nanogel for topical application. The formulation was confirmed to be in the nano-range by FE-SEM analysis with 217.77 nm average particle size of the formulated nanogel. The integration of allin-rich BGE_{Best} into the nanogel matrix along with its other components was validated by FT-IR and ATR analyses. Skin irritation and wound healing study using the BGE_{Best} nanogel on rabbits were found to be positively impacted. The wound did not dry up or heal on application of control gel, indicating that the addition of the extract accelerated the healing process. The study also demonstrated that the percentage of wound closure increased with the increase in the drug content (BGE_{Best}). The short-term stability, skin irritancy, and wound healing studies of the formulation with significantly positive results, demonstrate the viability of the nanogel. The favorable reactions of the panelists to the BGE_{Best} nanogel further confirmed its acceptability.

The future scope of the nanogel application against additional potent bacterial skin infections may be explored. Other types of hydrogel can also be formulated using natural polymers (apart from synthetic polymer i.e, Carbopol® 940) such as chitosan, pectin, gelatin, agar to formulate an eco-friendly nanogel. Other pharmacological and biological properties of black garlic extract (anti-oxidative, anti-inflammatory and anti-allergic properties) can be used for the formulation of

hydrogel, ointment etc. The mechanism underlying these findings may also be investigated for future research.

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Abbreviation

ANOVA- Analysis of variance

BG- Black garlic

BG1- Soxhlet extraction of black garlic extract using water

BG2- Soxhlet extraction of black garlic extract using ethanol: water (1:1)

BGE- Black garlic extracts

BGE_{Best} – Black garlic extract with the lowest MIC value

CFU- Colony forming unit

DMSO- Dimethyl sulfoxide

FG- Fresh garlic

FE-SEM- Field emission scanning electron microscope

FT-IR- Fourier transform infrared

HPTLC- High performance thin layer chromatography

MH- Mueller hinton

MIC- Minimum inhibitory concentration

OECD - Organisation for economic co-operation and development

PBS- Phosphate buffer saline

RH- Relative humidity

Std- Standard

TLC- Thin layer chromatography

Table 1- Composition of nanogel formulation

Phase	Ingredients	Quantity (% w/w)	Functions
Organic Phase	Lecithin Soya (30%)	0.5	Emulsifier; increases gel strength
	DMSO	25.5	Solvent for drug; enhances permeation of drug through skin
	Propylene Glycol	74	Moisturizer; enhances permeation of drug through skin
Inorganic Phase	Carbopol® 940	2	Gelling agent
	Water	98	Solvent for polymer (Carbopol® 940)

Table 2: Physicochemical analyses of formulated topical nanogels

PROPERTY		CONTROL NANOGEL	BGE_{Best} NANOGEL
Appearance		Clear	Pale yellow
Specific gravity		1.038	1.021
pH (25˚C)		6.90	6.82
% weight loss on drying (dry basis)		2.31	1.5
Spreadability (g cm/s)		15.92	12.41
Centrifuge test		No phase separation	No phase separation
Phase separation at	37°C	No phase separation	No phase separation
	55°C	No phase separation	No phase separation
HLB value		17	18.6
Apparent viscosity		18.92 cP	23.03 cP
Average particle size (FE-SEM)		286.06 nm	217.77 nm

Table 3: FT-IR spectrum of Carpobol® 940, DMSO, propylene glycol, lecithin soy (30%), Std alliin, BGE_{Best}, control nanogel and BGE_{Best} nanogel

Carbopol® 940 (Wave no in cm^{-1})	**DMSO** (Wave no in cm^{-1})	**Propylene glycol** (Wave no in cm^{-1})	**Lecithin soy (30%)** (Wave no in cm^{-1})	**Std alliin** (Wave no in cm^{-1})	**BGE_{BEST}** (Wave no in cm^{-1})	**Control nanogel** (Wave no in cm^{-1})	**BGE_{BEST} nanogel** (Wave no in cm^{-1})
3118: O-H stretching	3442: O-H stretching	3307: O-H stretching	3445: O-H stretching	3331: O-H stretching	3259: O-H stretching	3272: O-H stretching	3628,3348: O-H stretching
1713: C=O ketone stretching	2995: =C-H stretching	2969,2929, 2875: carboxylic acid OH stretching	2925: C-H stretching	**2920, 2836: C-H stretching**	**2948: C-H stretching**	2130: C-H aldehydic stretching	**2853: C-H stretching**
1454: CH_3 stretching	2912: C-H stretching	1455, 1411: C=C aromatic	1651,164 , 1634 : C-C multiple bond stretching	**1646 : C=C alkene**	**1641**, 1405: **C=C alkene stretching**	1635, 1420: C=C aromatic	**1651: C=C alkene stretching**
1114,117,1247: C-O-C stretching	1435: C=C aromatic	1375,1331: CH_3 stretching	1732: ester stretching	1575, 1540, 1452, 1417: C=C aromatic	1118, 1013: C-O-C stretching	1077, 1039: C-O-C stretching	1249,1225: C-O-C stretching
801,648: C-Cl stretching	1040, 1017: C-F stretching	1232, 1135, 1076, 1036: C-O-C stretching	1466: C-H_2 bending	**1015: C-F stretching**			1044, **1016: C-F stretching**
	760: C-Cl stretching		1377: CH_2-O-P-O				
			1226,108 : C-N				

Table 4: Skin reaction of rabbit on application of BGE_{Best} nanogel

Time	Skin reaction	
	Erthyema	Edema
24 h	0	0
48 h	0	0
72 h	0	0

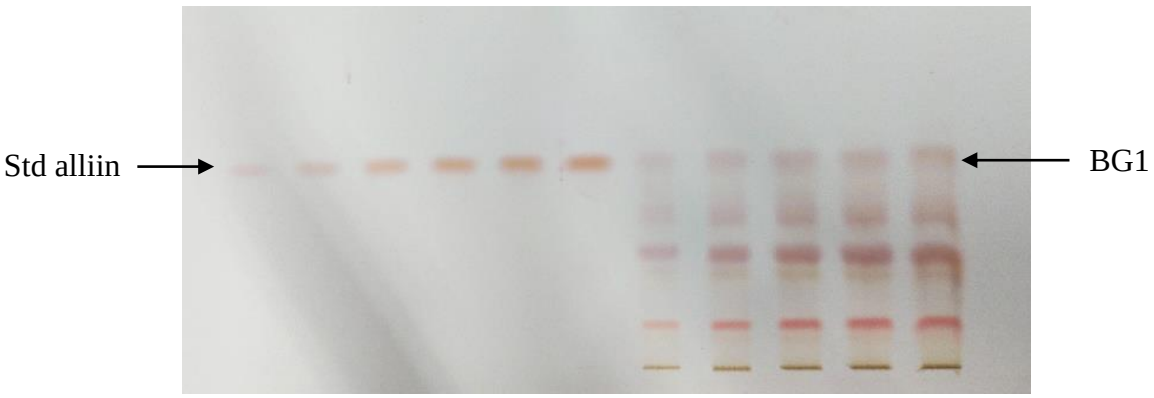
Primary Irritation Index (PII): 0/36 = 0

Category of irritation based on PII: Non-irritant

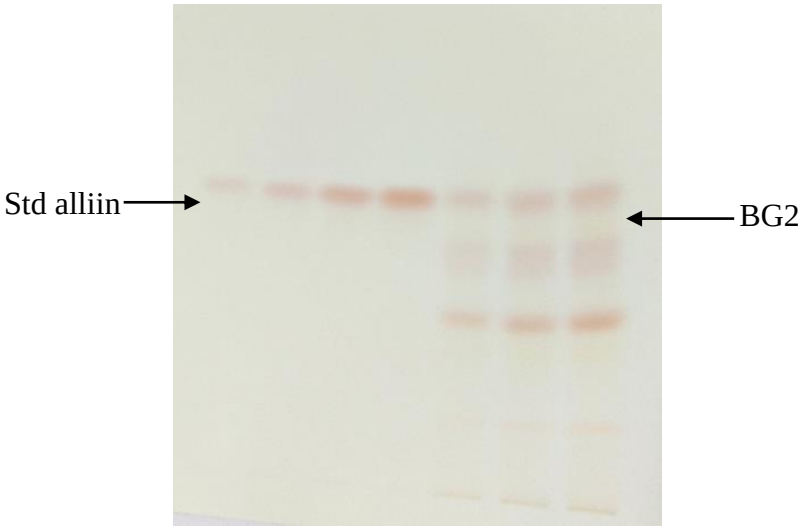
a)

b)

Figure 3 a) Black garlic cloves on fermentation for a period of 8-9 days and b) black garlic powder obtained on grinding.

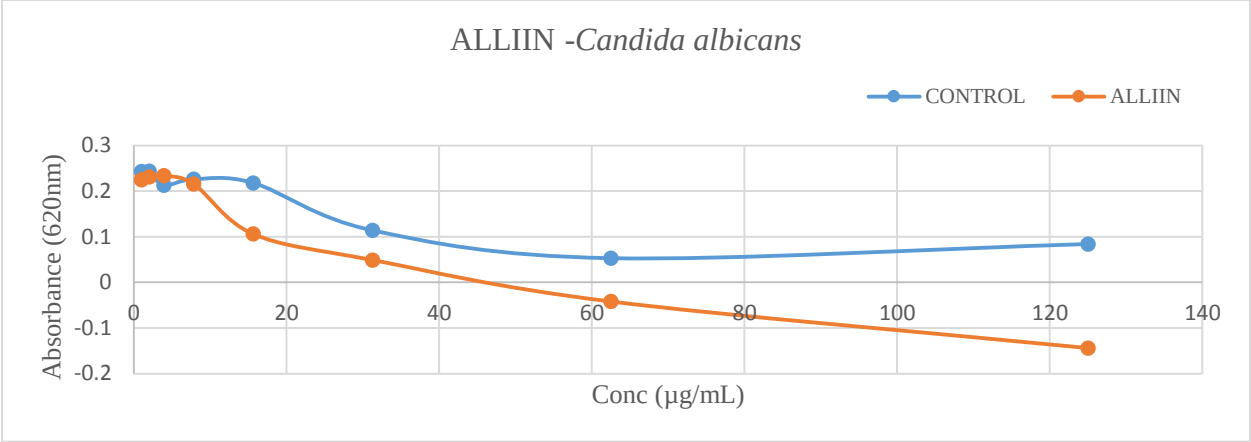


a)

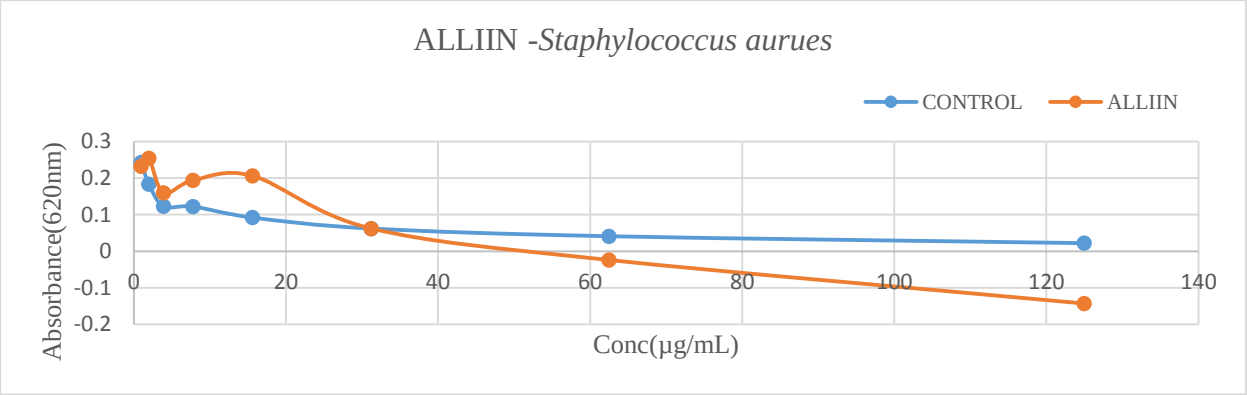


b)

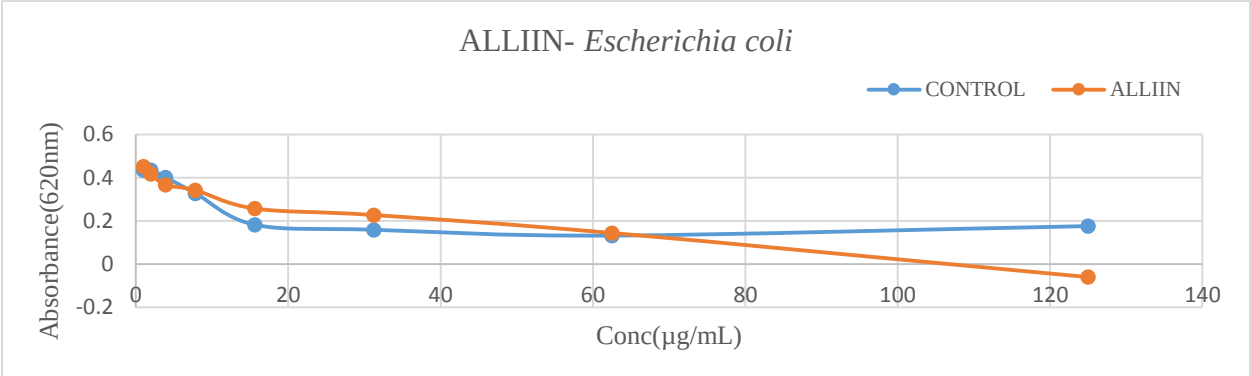
Figure 4: Plate developed for a) BG1 and b) BG2 extracts along with Std alliin for HPTLC run



a)

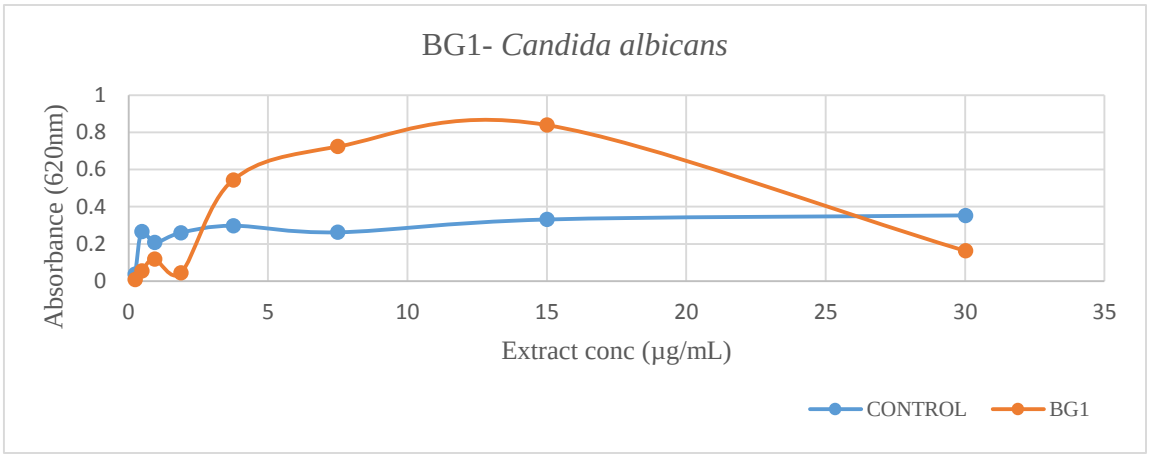


b)

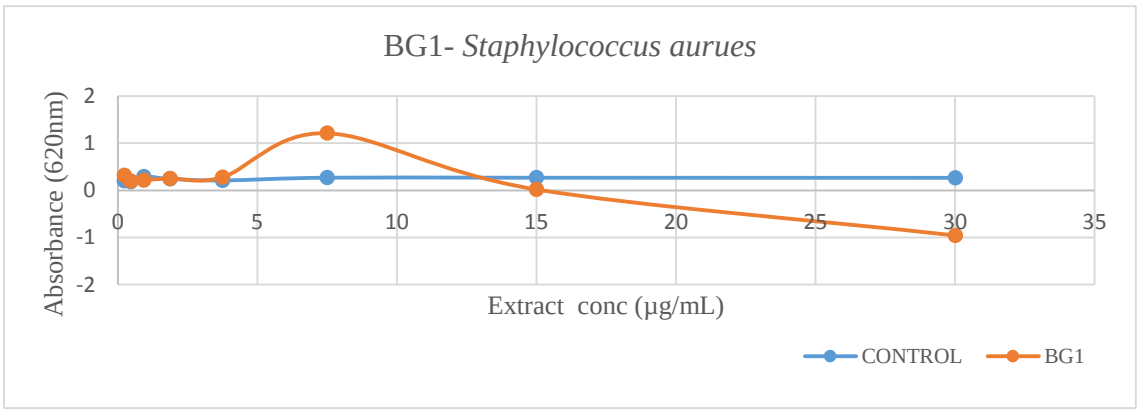


c)

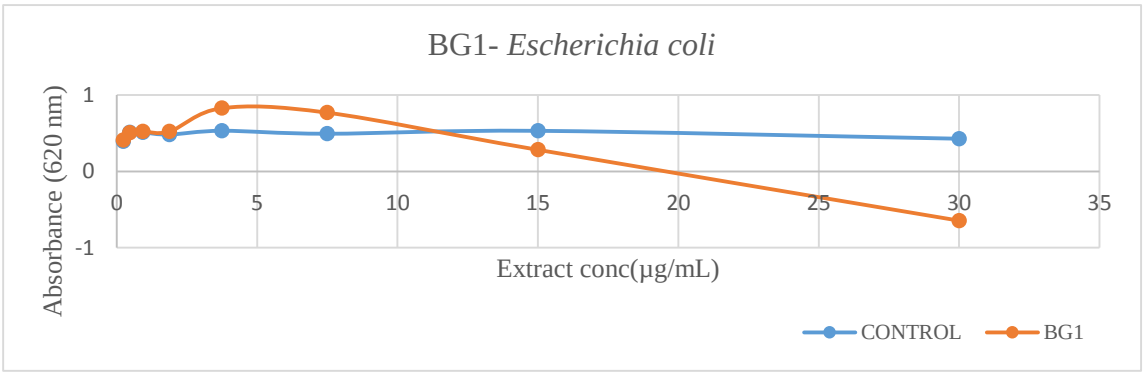
Figure 5 a: MIC value graph for Std alliin against a) *Candida albicans* b) *Staphylococcus aureus* c) *Escherichia coli*



a)

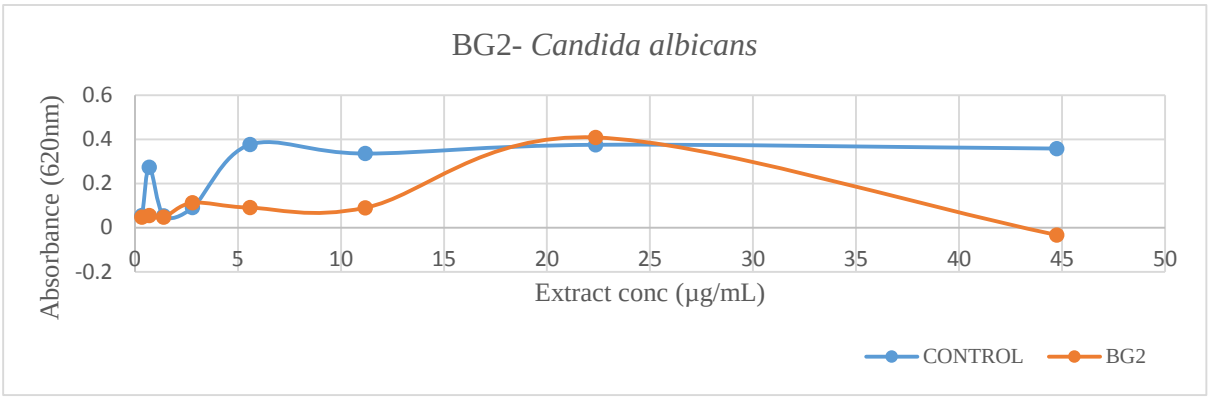


b)

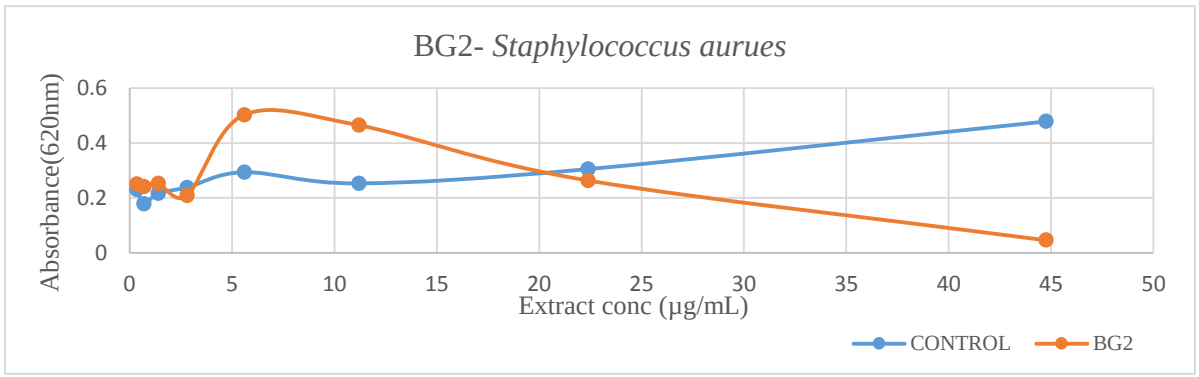


c)

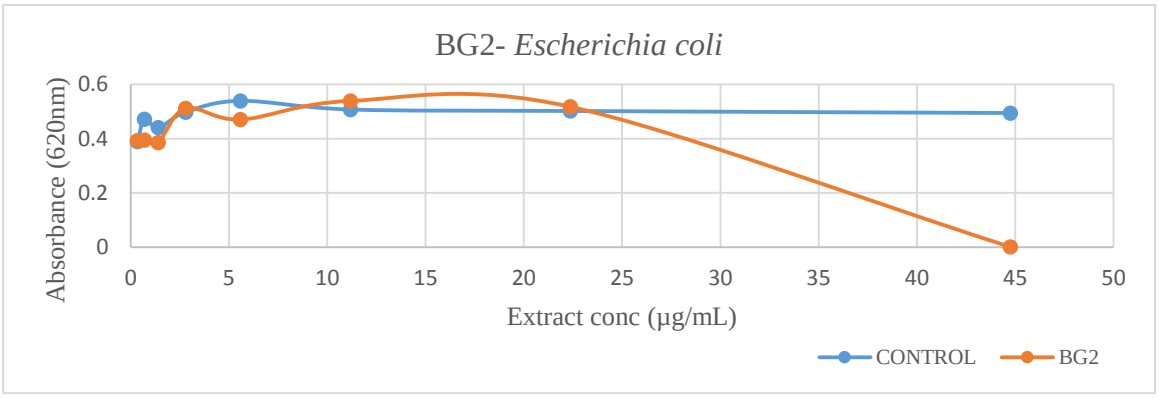
Figure 5 b: MIC value graph for BG1 extract against a) *Candida albicans* b) *Staphylococcus aureus* c) *Escherichia coli*



a)

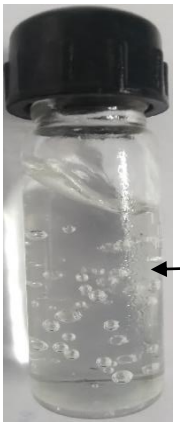


b)



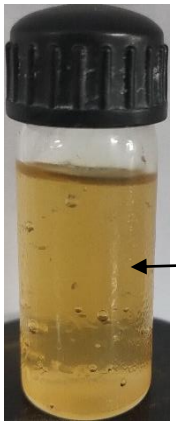
c)

Figure 5 c: MIC value graph for BG2 extract against a) *Candida albicans* b) *Staphylococcus aureus* c) *Escherichia coli*



Clear Std alliin nanogel

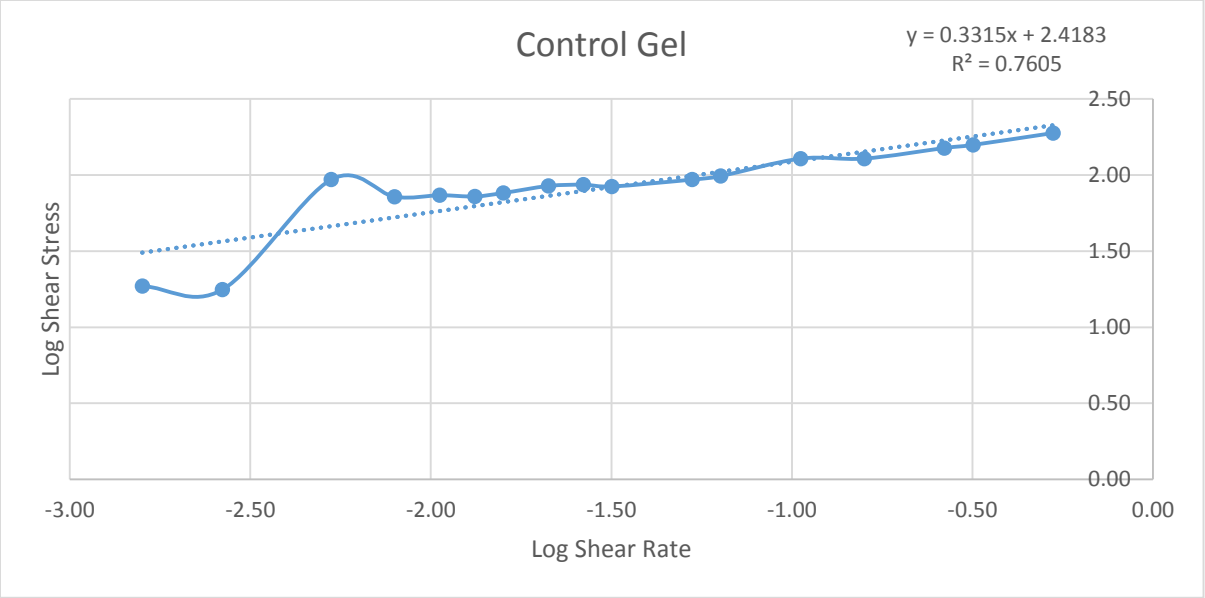
a)



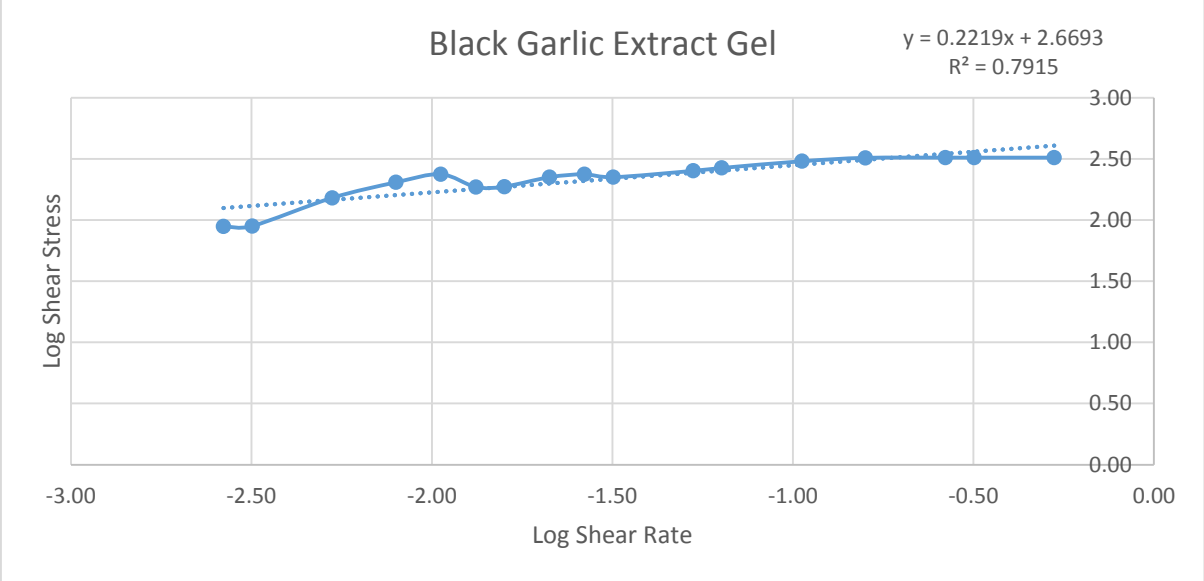
Pale yellow translucent BGE_{Best} nanogel

b)

Figure 6: a) Std alliin nanogel and b) BGE_{Best} nanogel

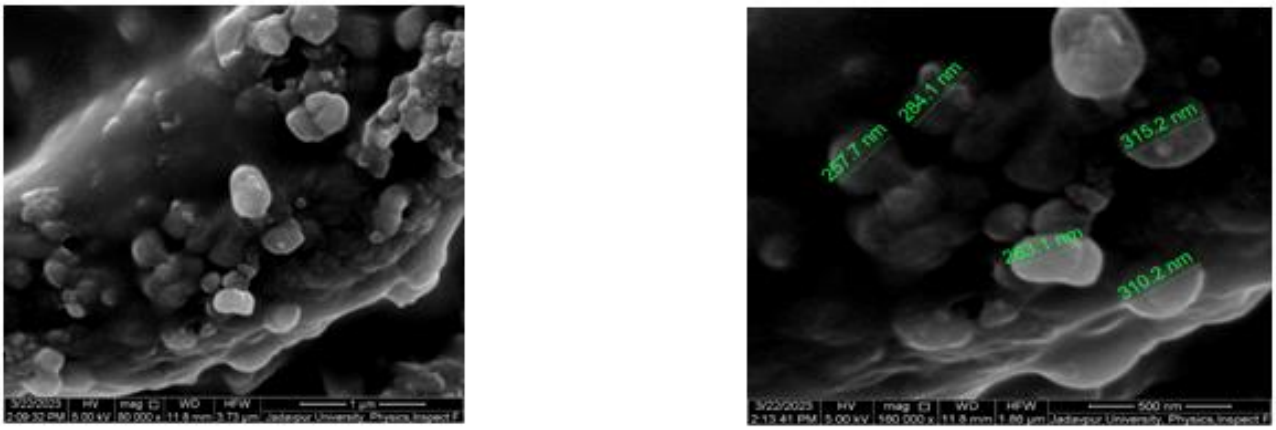


a)

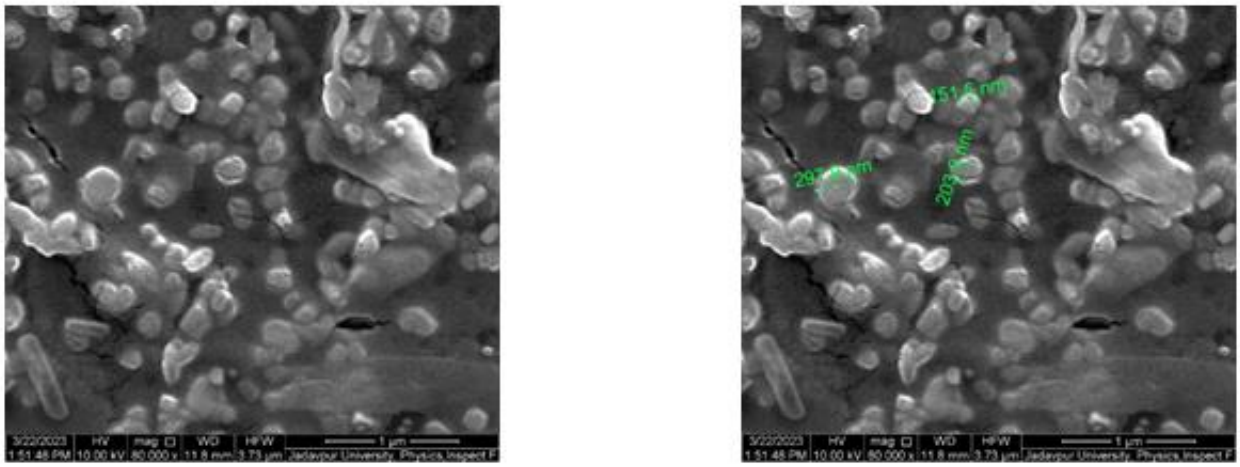


b)

Figure 7: Viscosity graph of log shear stress vs log shear rate for a) control nanogel b) BGE_{Best} nanogel

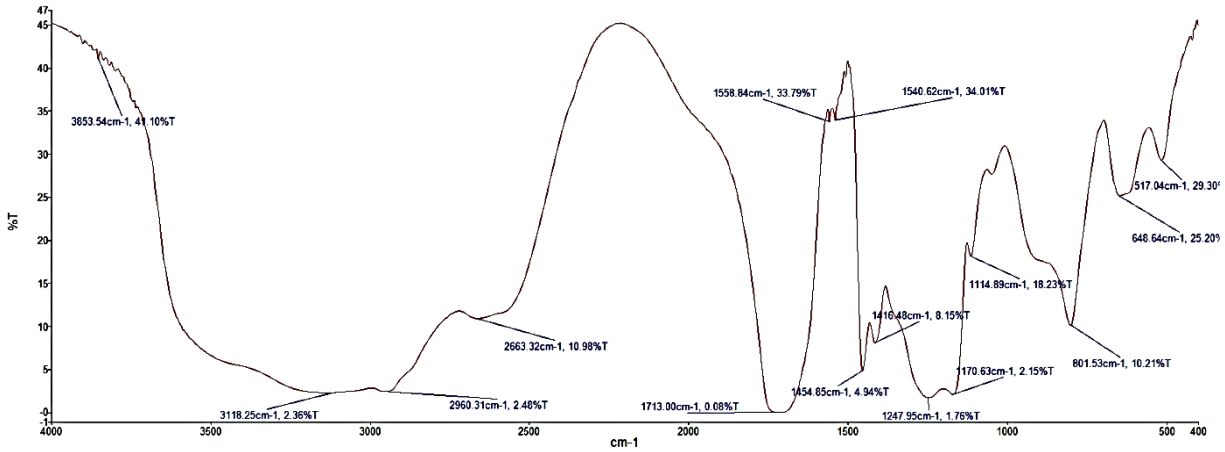


a)

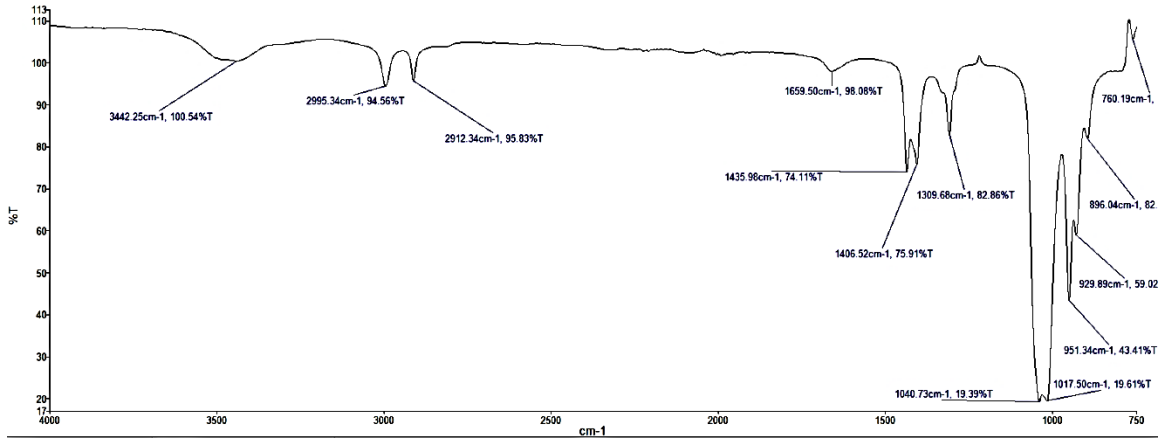


b)

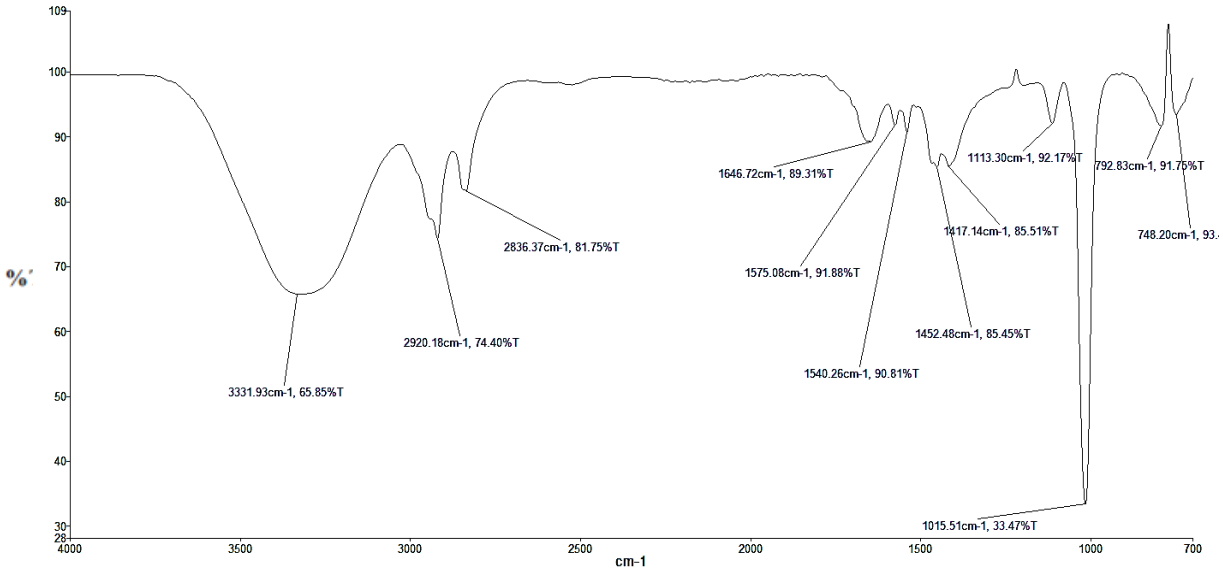
Figure 8: FE-SEM results for the micro structure of a) control nanogel and b) BGE_{Best} nanogel


%T
cm-1
3853.54cm-1, 41.10%T
3118.25cm-1, 2.36%T
2960.31cm-1, 2.48%T
2663.32cm-1, 10.98%T
1713.00cm-1, 0.08%T
1558.84cm-1, 33.79%T
1540.62cm-1, 34.01%T
1454.85cm-1, 4.94%T
1416.48cm-1, 8.15%T
1247.95cm-1, 1.76%T
1170.63cm-1, 2.15%T
1114.89cm-1, 18.23%T
801.53cm-1, 10.21%T
648.64cm-1, 25.20
517.04cm-1, 29.30

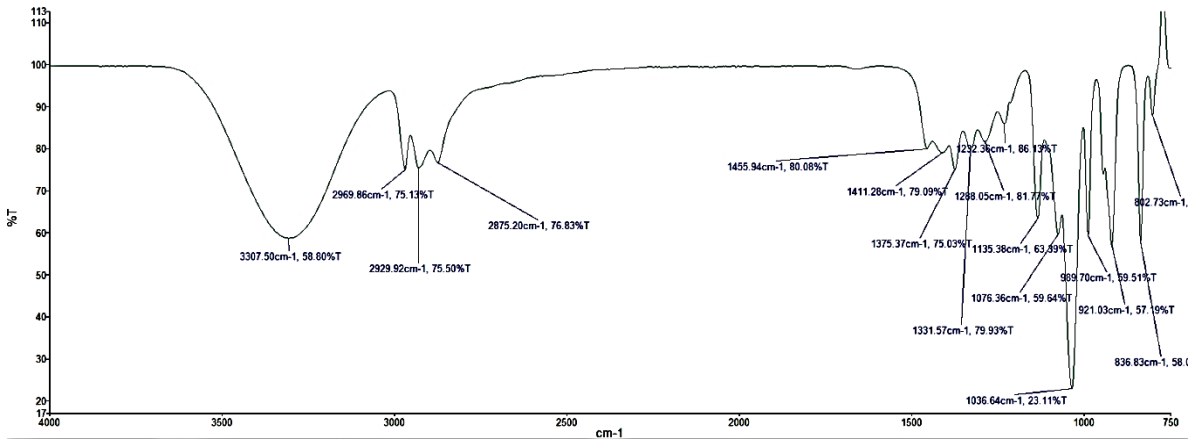
a)


%T
cm-1
3442.25cm-1, 100.54%T
2995.34cm-1, 94.56%T
2912.34cm-1, 95.83%T
1659.50cm-1, 98.08%T
1435.96cm-1, 74.11%T
1406.52cm-1, 75.91%T
1309.68cm-1, 82.86%T
1040.73cm-1, 19.39%T
951.34cm-1, 43.41%T
929.89cm-1, 59.02
896.04cm-1, 82
760.19cm-1,
1017.50cm-1, 19.61%T

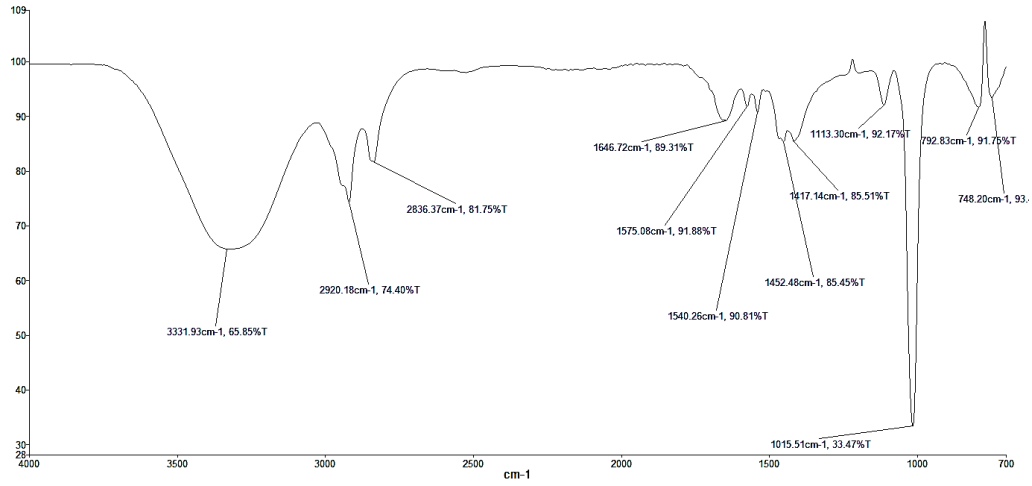
b)


%
cm-1
3331.93cm-1, 65.85%T
2920.18cm-1, 74.40%T
2836.37cm-1, 81.75%T
1646.72cm-1, 89.31%T
1575.08cm-1, 91.88%T
1540.26cm-1, 90.81%T
1452.48cm-1, 85.45%T
1417.14cm-1, 85.51%T
1113.30cm-1, 92.17%T
1015.51cm-1, 33.47%T
792.83cm-1, 91.75%T
748.20cm-1, 93.

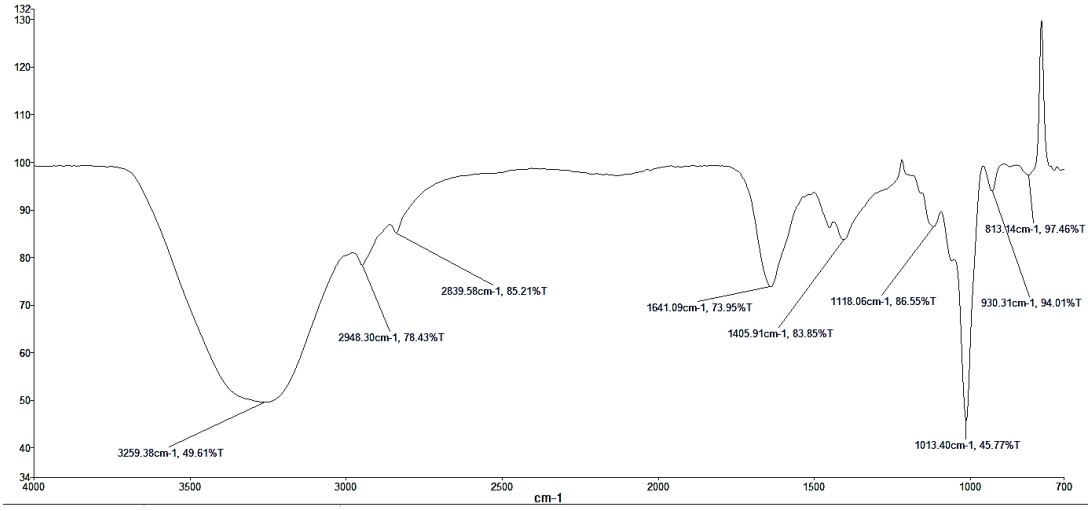
c)


%T
cm-1
3307.50cm-1, 58.80%T
2969.86cm-1, 75.13%T
2929.92cm-1, 75.50%T
2875.20cm-1, 76.83%T
1455.94cm-1, 80.08%T
1411.28cm-1, 79.09%T
1375.37cm-1, 75.03%T
1331.57cm-1, 79.93%T
1232.36cm-1, 86.13%T
1135.38cm-1, 63.39%T
1076.36cm-1, 59.64%T
1036.64cm-1, 23.11%T
989.70cm-1, 59.51%T
921.03cm-1, 57.19%T
836.83cm-1, 58.

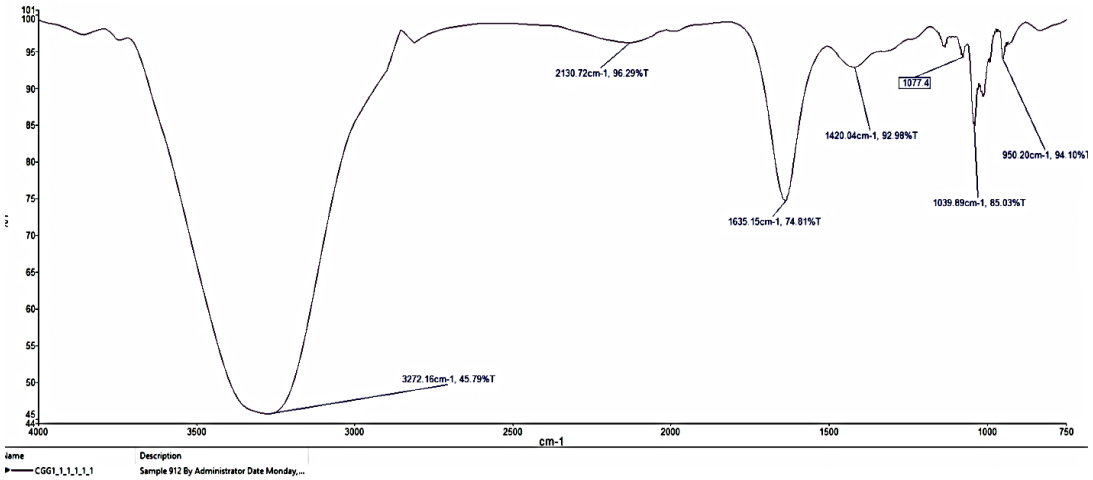
d)


3331.93cm-1, 65.85%T
2920.18cm-1, 74.40%T
2836.37cm-1, 81.75%T
1646.72cm-1, 89.31%T
1575.08cm-1, 91.88%T
1540.26cm-1, 90.81%T
1452.48cm-1, 85.45%T
1417.14cm-1, 85.51%T
1113.30cm-1, 92.17%T
1015.51cm-1, 33.47%T
792.83cm-1, 91.75%T
748.20cm-1, 93.
cm-1

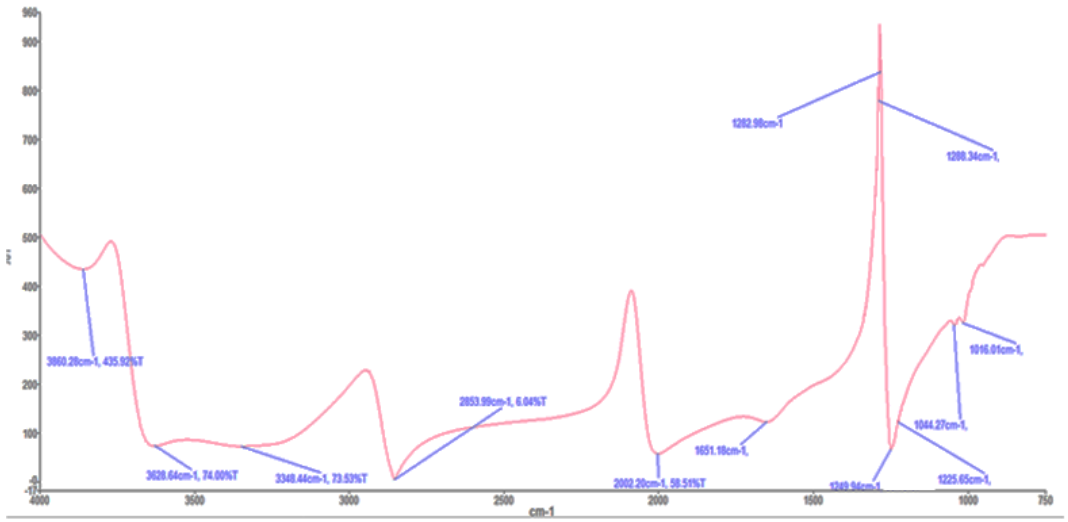
e)


3259.38cm-1, 49.61%T
2948.30cm-1, 78.43%T
2839.58cm-1, 85.21%T
1641.09cm-1, 73.95%T
1405.91cm-1, 83.85%T
1118.06cm-1, 86.55%T
1013.40cm-1, 45.77%T
930.31cm-1, 94.01%T
813.14cm-1, 97.46%T
cm-1

f)

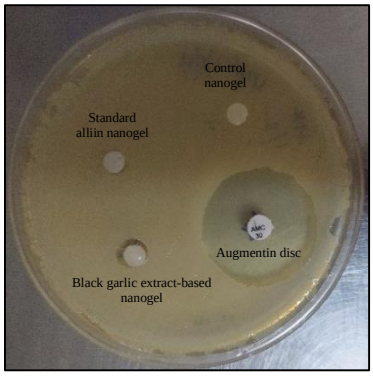


g)

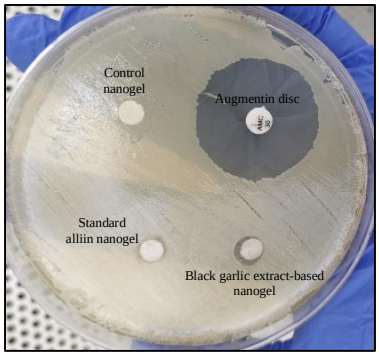


h)

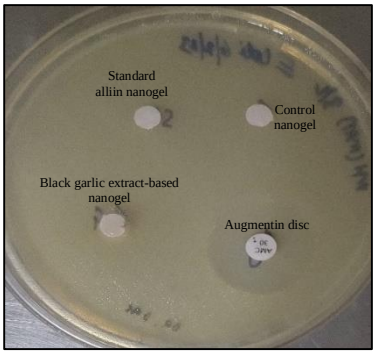
Figure 9: FT-IR analyses of the components of formulated nanogel. a) Carbopol 940 b) DMSO c) lecithin soya 30% d) propylene glycol e) Std alliin f) BGE_{Best} g) control nanogel h) BGE_{Best} nanogel



a)

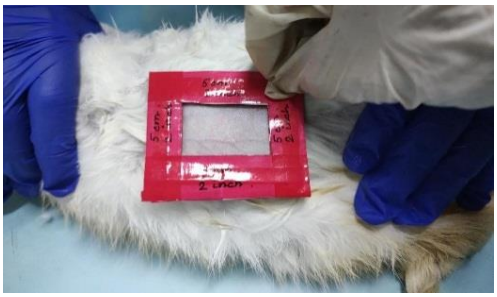


b)



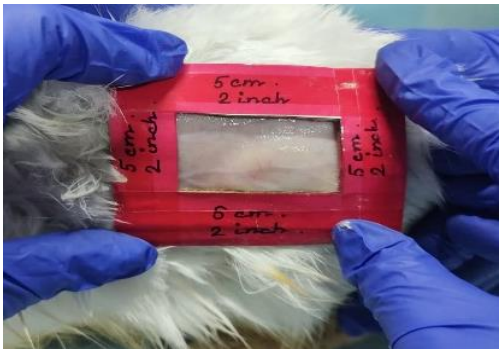
c)

Figure 10: Zones of inhibition plates for Std alliin and BGE_{Best} nanogels against a) *Staphylococcus aureus,* b) *Escherichia coli and* c) *MDR strain of Escherichia coli.*

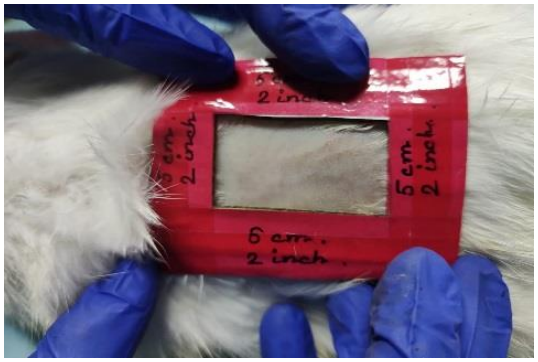


a)

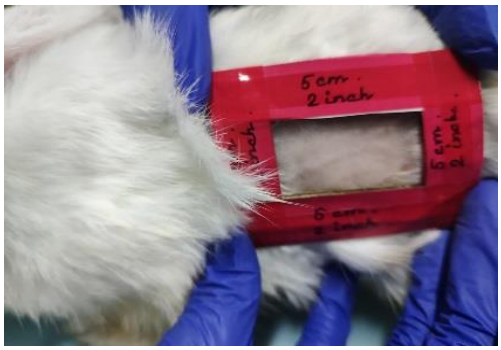
b)



c)



d)



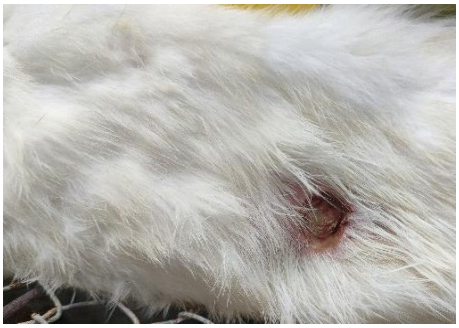
e)

Figure 11: Skin irritancy test of BGE_{Best} nanogel on the skin of dorsolateral trunk of rabbit. a) before application b) application of nanogel c) after application at 0 h d) after application at 24 h e) after application at 72 h.

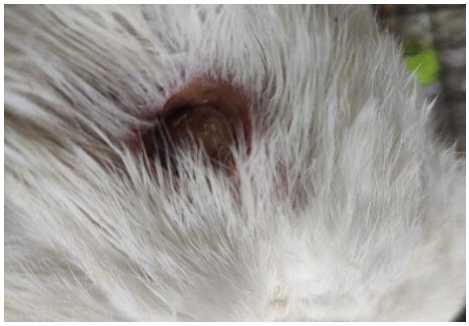
a) i)

a) ii)

a) iii)

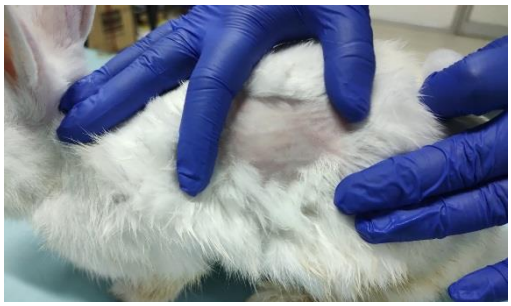


a) iv)

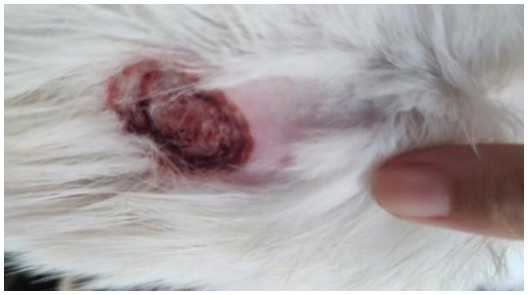


a) v)

Figure 12 a: Wound healing study on rabbit R1 using commercially available gel as positive control i) before wound creation ii) wound created (day 0 wound area) iii) application of the ointment iv) wound closure on day 1 v) wound closure on day 6.

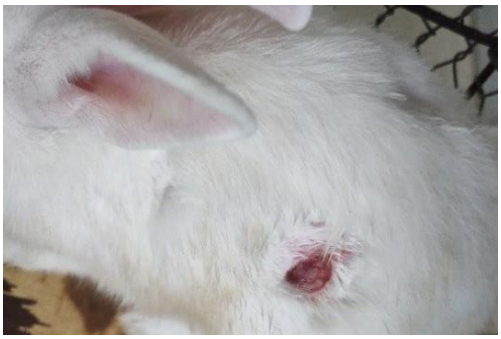


b) i)

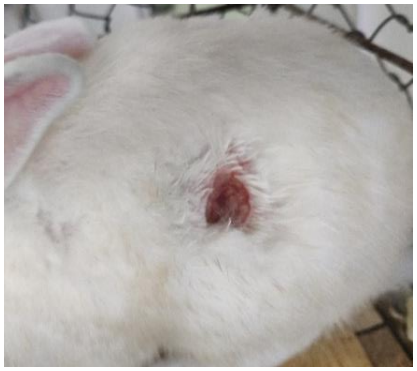


b) ii)

b) iii)



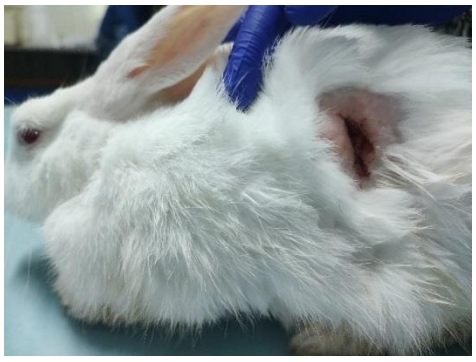
b) iv)



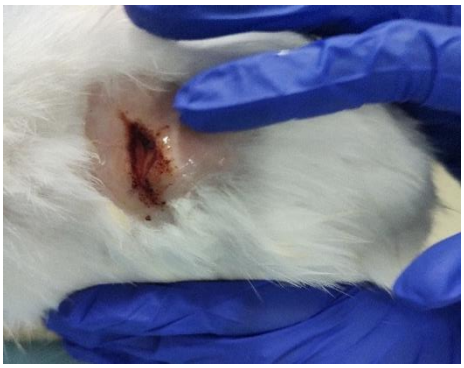
b) v)

Figure 12 b: Wound healing study on rabbit R2 using control nanogel i) before wound creation ii) wound created (day 0 wound area) iii) application of the nanogel iv) wound closure on day 3 v) wound closure on day 6.

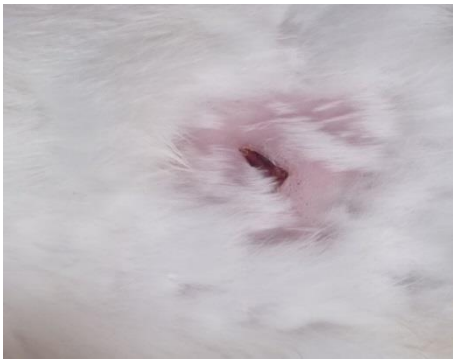
c) i)



c) ii)



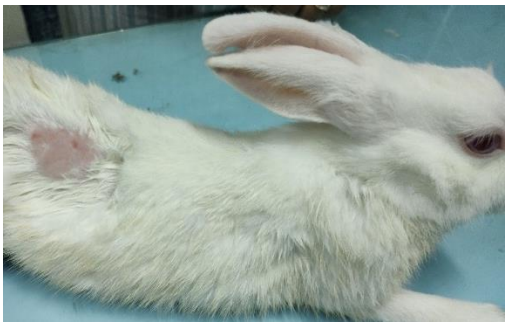
c) iii)



c) iv)

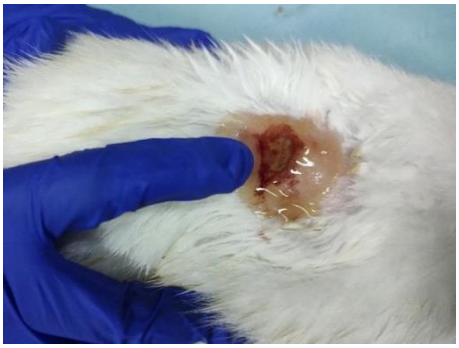
c) v)

Figure 12 c: Wound healing study on rabbit R3 using 2% BGE_{Best} nanogel i) before wound creation ii) wound created (day 0 wound area) iii) application of the nanogel iv) wound closure on day 1 v) wound closure on day 3.

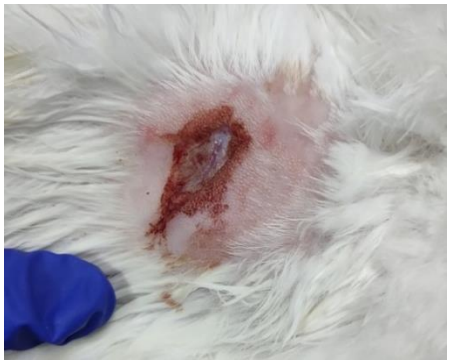


d) i)

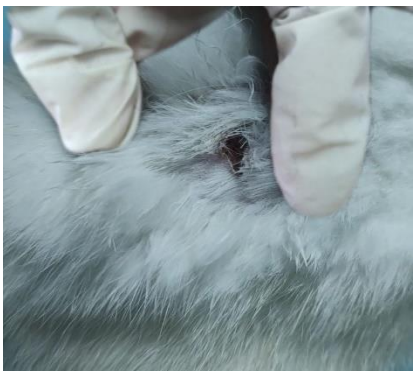
d) ii)



d) iii)

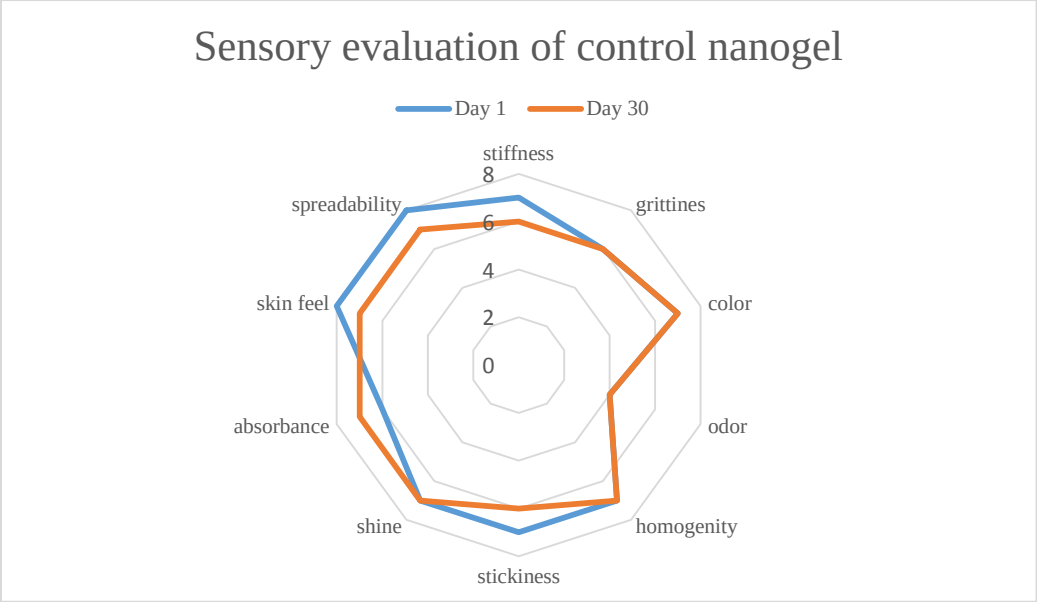


d) iv)

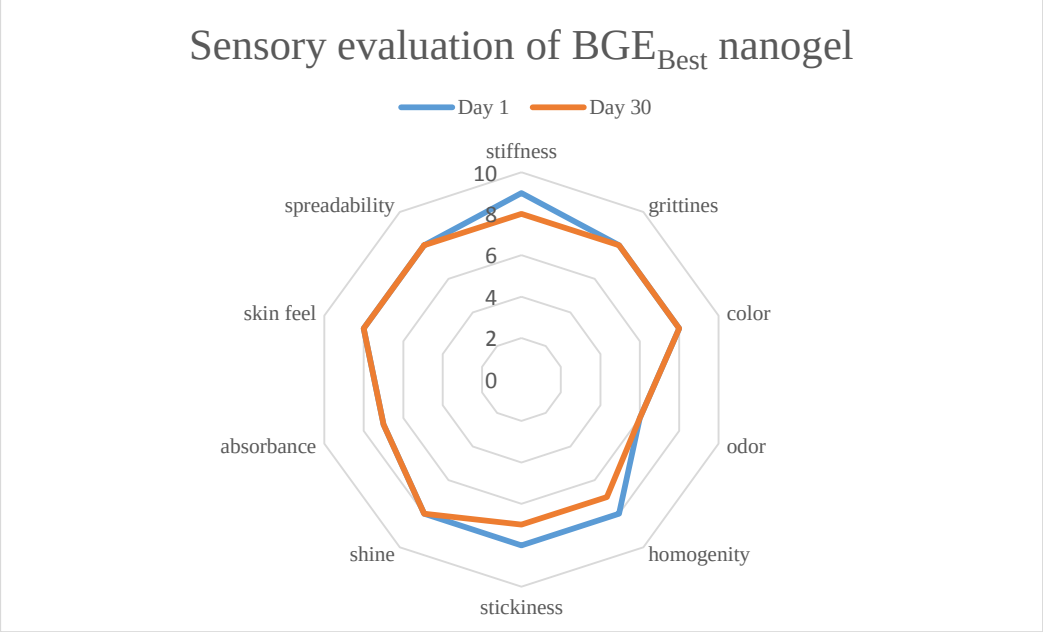


d) v)

Figure 12 d: Wound healing study on rabbit R4 using 4% BGE_{Best} nanogel i) before wound creation ii) wound created (day 0 wound area) iii) application of the nanogel iv) wound closure on day 3 v) wound closure on day 6.



a)



b)

Figure 13: Radar plot of hedonic scores obtained by sensory analyses of a) control nanogel b) BGE_{Best} nanogel on day 1 and day 30