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Biocompatibility Analysis of *Embelia ribes*–Loaded Hydrogel Using Cell Viability and Hemocompatibility Assays: An In Vitro Study

Dr Anjali Rathi , Dr Sindhu Ramesh

Department of Conservative Dentistry and Endodontics, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India

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KEYWORDS

Embelia ribes, hydrogel, hyaluronic acid, MTT Assay, Live-Cell Dead Assay

ABSTRACT:

Introduction: Embelia ribes Burm. F, also known as false black pepper, is used for an array of medicinal applications. Cell viability assays are essential for evaluating the biocompatibility of novel biomaterials intended for biomedical applications. A hydrogel was formulated incorporating Embelia ribes silver nanoparticles and hyaluronic acid, with hydroxy methylcellulose serving as a carrier.

Objectives: To assess the in vitro cytocompatibility of indigenously developed Embelia ribes-based hydrogel formulations, with and without silver nanoparticles, using cell viability and Hemocompatibility test on fibroblast cells to determine their potential suitability for biomedical and endodontic applications.

Methods Hydrogel Formulation primarily comprises seed formulation of Embelia ribes mediated silver nanoparticles (3%), Hyaluronic acid(3%), hydroxethyl cellulose (3%)in the ratio of 1:1:1. The hydrogel was then subjected to the above-mentioned biocompatibility tests.

Results: MTT and Live/Dead assays demonstrated that both *E. ribes* extract and *E. ribes*-AgNP hydrogels were highly biocompatible, maintaining fibroblast viability above 75% even at 300 µg/mL. Haemolysis analysis showed minimal red blood cell damage (<3%), indicating strong hemocompatibility. Notably, the *E. ribes*-AgNP hydrogel exhibited slightly enhanced cytocompatibility compared to the extract alone.

Conclusions: Excellent biocompatibility and hemocompatibility were proven by the E. ribes-AgNP hydrogel, which makes it a viable option for upcoming biomedical applications.

1. Introduction

The growing awareness of potential side effects spurred interest in customary herbal medicines. Embelia ribes is a woody shrub that is commonly known as Vidanga or false black pepper, plus Vaibidang in Sanskrit, and belongs within the family Myrsinaceae [1]. Known for its array of pharmacological properties, it is used to treat a range of conditions like diabetes, bronchitis, jaundice, rheumatoid arthritis, likewise to cure tumours as well as mental disorders. Seeds are used as both an antibiotic also as an anthelminthic, and as an antituberculosis [2]. They can also be used as an alternative stimulant. For its large contribution to the field of medicine, the Medicinal Board of the Government of India, New Delhi, recognised Embelia ribes as an important one out of 32 medicinal plants for its large-scale cultivation and labelled it Agro-techniques of Selected Medicinal Plants. Embelin, a meaningful chemical bioactive constituent, has medicinal and therapeutic abilities for revolutionary drugs to incorporate. Embelin is a quinone extract that occurs naturally and is found within the seeds of Embelia ribes. It also has anti-inflammatory, antibacterial, antioxidant, with antipyretic effects [3,4].

The broad and precise applications of nanotechnology in medicine, from contrast agents in imaging to carriers for drug and gene delivery into tumours, are driving the growth of nanomedicine. Nanoparticles with sufficient catalytic and absorptive capabilities range in size from 1

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to 100 [5]. There are NMs visible that vary in size from 2 nm to 30 nm. Since they are created in a way that is sustainable, green synthesis of nanomaterials is a great substitute for creating recyclable, non-toxic nanomaterials using techniques that produce little waste byproducts. The growth of metal and metal oxide-based nanomaterials depends on phytochemicals found in plant leaves, such as carboxylic and ascorbic acids, alkaloids, ketones, flavonoids, aldehydes, tannins, amides, and phenols [6]. These phytochemicals aid in the stability, biocompatibility, and eco-friendliness of the nanoparticles thus developed. Nanoparticles synthesized from plant sources utilize natural bio-reducing agents and are stabilized by phytochemicals acting as capping agents, which collectively improve their biocompatibility [7].

Hydrogels comprise a three-dimensional (3D) network which can absorb a large amount of water and swell in the water due to their hydrophilic groups such as such as -NH2, -COOH, -OH, -CONH2, etc. Chemical or physical crosslinking of natural or synthetic polymer chains can be used to design the hydrogels [8]. They can be soft and flexible due to water absorption. Over the past 60 years, hydrogels have been engineered to be implantable, injectable, and sprayable for many organs and tissues. The sources of hydrogels can be divided into natural, synthetic, or semi-synthetic polymers [9]. Naturally derived hydrogels (natural hydrogels) include cellulose, chitosan, collagen, alginate, agarose, hyaluronic acid, gelatin, and fibrin etc [10].

Hyaluronic acid (HA) is a non-sulphated glycosaminoglycan composed of repeating units of the disaccharide β-1,4-D-glucuronic acid–β-1,3 N-acetyl-D-glucosamine. This polysaccharide is naturally found in the human body, especially in connective tissues, skin, and synovial joint fluids. HÀ is an essential component of the ECM, in which its structural and biological properties mediate its activity in cellular signalling, wound repair, morphogenesis, and matrix organization [11]. HA displays physicochemical properties, such as high-water retention and viscoelastic properties, which make it the candidate of choice for bio-applications in several fields of medicine. They can be modified as per the application [12,13].Smart hydrogels are composed of functional groups on the polymer backbone of the structure, which result from noncovalent bonding, such as hydrogen bonding, hydrophobic interactions. When smart hydrogels are exposed to environmental factors, the porosity and hydrophilicity of the hydrogel can regulate the loading and release of drugs in a controlled manner. Because of this self-regulating behaviour, smart hydrogels are promising candidate materials for drug delivery systems [14]. Cellulose is a common, naturally occurring polymer of glucose; however, it is insoluble in water, as well as many other organic solvents. Cellulose-based derivatives have been developed to improve the solubility of cellulose, including methylcellulose (MC), hydroxypropyl methylcellulose (HPMC), ethyl cellulose (EC), and hydroxyethyl cellulose (HEC).HEC has been extensively used as a thickener, stabilizer, or coating in several application fields [15].

The MTT assay [short for (*3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* assay] is a colorimetric assay used to assess cell metabolic activity, which correlates with cell viability, proliferation, and cytotoxicity. A popular technique for assessing cell viability and cytotoxicity is the live/dead cell assay, which separates live cells from dead ones using enzyme activity and membrane integrity [16]. Two fluorescent dyes are usually used: one that is cell-permeable (calcein-AM, for example) and stains living cells green, and another that is membrane-impermeable (ethidium homodimer), which labels dead cells red. This assay offers a rapid and accurate evaluation of cell health in response to different therapies [17].

A hemocompatibility test's main objective is to determine whether a substance or medical equipment is compatible with blood. This entails determining whether the substance or apparatus reacts negatively with blood components. The objective of the current study is to evaluate the cytocompatibility and Hemocompatibility of Embelia ribes incorporated hydroxyethyl-hyaluronic acid-based Hydrogel.

2. Objectives

The objective of the current study is to assess the in vitro cytocompatibility of indigenously developed Embelia ribes-based hydrogel formulations, with and without silver nanoparticles, using cell viability and Hemocompatibility test on fibroblast cells to determine their potential suitability for biomedical and endodontic applications.

3. Methods

The dried seeds of Embelia ribes, a well-known medicinal plant traditionally used in Ayurvedic formulations, were obtained from the Indian Medical Practitioners Co-operative Pharmacy and Stores (IMPCOPS), located in Chennai, Tamil Nadu, India. The authenticated seeds were thoroughly cleaned to remove any debris or foreign material and then shade-dried to preserve their phytochemical constituents [18].The dried material was coarsely powdered using a mechanical grinder. The powdered sample was subjected to cold maceration in ethanol (or aqueous/ethanol mixture,

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depending on the study design) at room temperature for 72 hours with intermittent shaking to enhance extraction efficiency. After maceration, the extract was filtered using Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator to obtain a thick, semi-solid crude extract. The final extract was stored at 4°C in airtight containers until further use for pharmacological or biochemical studies [19].

Smart Hydrogel Formulation-

A solution of 1 millimole (mM) of silver nitrate in 90 millilitres (ml) was utilized for the nanoparticle synthesis process. The analysis of the synthesized nanoparticles was conducted using UV-visible spectroscopy. Smart Hydrogel Formulation primarily comprises seed formulation of *Embelia ribes* mediated silver nanoparticles (3%), Hyaluronic acid(3%) , hydroxypropyl-methyl cellulose (3%)in the ratio of 1:1:1. The obtained Hydrogel was evaluated for biocompatibility.

Cell Viability Assessment

a. MTT Assay

MTT reagent was applied to the cells following the treatment period. Viable cells used mitochondrial enzymes to convert the yellow MTT to purple formazan crystals, which were then dissolved in DMSO and measured with a spectrophotometer set to 570 nm. Both E. ribes extract and E. ribes-AgNPs were observed over a 24-hour period, according to the L-929 fibroblast cell assay.

Viability remained above 70% even at the highest dosage, however there was a slight, dose-dependent decrease as treatment concentrations increased from 50 to 300 µg/ml, whereas the control group maintained almost 100% viability. Interestingly, compared to the extract alone, E. ribes-AgNPs demonstrated marginally improved cytocompatibility. These results validate the formulations' safety and suitability for use in wound healing, tissue engineering, and other biomedical domains [20].

b. Live - Dead Cell Assay

On the two days following incubation, live/dead staining was carried out using *Embelia ribes* hydrogel. A Live/Dead Viability/Cytotoxicity kit (Calcein-AM dye, Invitrogen, USA) was used in accordance with the manufacturer's instructions, with minor adjustments, to visualize live and dead cells. In a nutshell, the stem cells were sown at a density of 1x106 cells/well in 6-well plates. Following a 24-hour incubation period, a Calcein-AM dye was applied, incubated for 30 minutes, and then rinsed with 1x PBS. Additionally, inverted phase contrast fluorescent microscopy (Invitrogen, evos) was used to view the cells [21]. Calcein-AM was the only stain used on viable cells that showed green fluorescence. A ratio of live to dead cells was computed for every cell state after counting the live and dead labelled cells by hand.

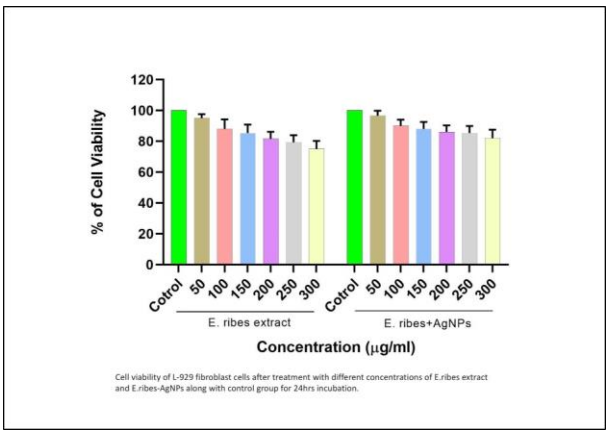
Hemocompatibility test

Haemolysis percentages at a concentration of 200 µg/mL were used to evaluate the hemocompatibility of the hydrogel formulations that were developed. The hydrogel and hydrogel-loaded silver nanoparticles (Hydrogel+AgNPs) were treated with red blood cells (RBCs) for two hours at 25°C. Normal saline was utilized as the negative control (NC) to determine baseline erythrocyte stability, and distilled water was utilized as the positive control (PC), signifying 100% haemolysis. To guarantee statistical reliability and reproducibility, the experimental design contained triplicate measurements. By comparing the absorbance of the supernatant from each sample to that of the positive control, the haemolysis % was determined [22].

4. Results

MTT Assay

The control group, which received no treatment, showed 100% cell viability, serving as the baseline for comparison. Both the *E.ribes* extract and E. ribes+AgNPs formulations maintained high levels of cell viability across all concentrations tested, indicating low cytotoxicity.



Graph 1 : Represents a quantitative assessment of the biocompatibility of fibroblast L929 cells following 24 hours of exposure to various formulations, including E. ribes extract and E. ribes-mediated silver nanoparticles (AgNPs), at a concentration of 200 µg/mL. The percentage of viable cells was determined and compared across five treatment groups: untreated control, Triton X-100 (positive cytotoxic control), hydrogel alone, hydrogel incorporated with E. ribes extract, and hydrogel loaded with E. ribes-AgNPs.

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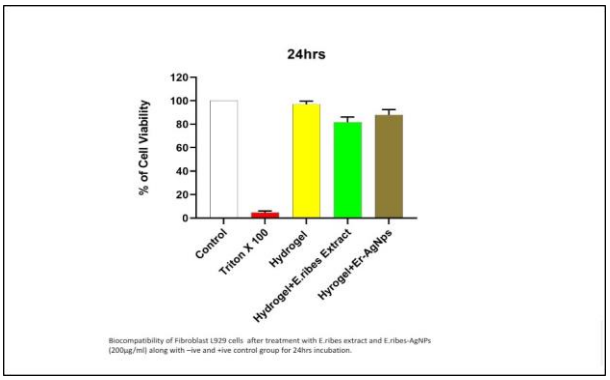
At the lowest concentration (50 µg/mL), cell viability was over 96% for both groups, while at the highest concentration (300 µg/mL), viability slightly declined but remained above 75%, with E. ribes+AgNPs showing consistently higher viability than the extract alone at each dose.



Graph 2 : Biocompatibility of Fibroblasts L929 cells after treatment with E.ribes extract and E. ribes- AgNPs (200µg/mL) along with negative and positive control groups for 24 hrs. incubation.

Live- Dead Cell Assay

Figure 1 presents a comparative evaluation of cell viability and morphology under different treatment conditions using phase contrast microscopy and live/dead fluorescence staining. The experimental groups include a control (untreated cells), hydrogel alone, hydrogel combined with Er-extract, and hydrogel incorporated with Er-mediated silver nanoparticles (Er-AgNPs). In the phase contrast images (top row), cells across all groups display normal morphology, appearing elongated and evenly distributed, with no signs of shrinkage or detachment, indicating that none of the treatments adversely affected cell structure.

The haemolysis percentage was calculated by comparing the absorbance of the supernatant from each sample to that of the positive control. Both the hydrogel and Hydrogel+AgNPs samples exhibited haemolysis rates well below the acceptable limit of 5%, indicating excellent hemocompatibility and potential suitability for biomedical applications. Haemolysis percentages indicate the degree of red blood cell (RBC) damage, with higher percentages showing more haemolysis.

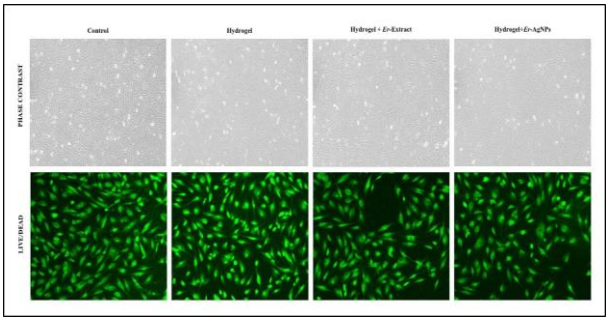
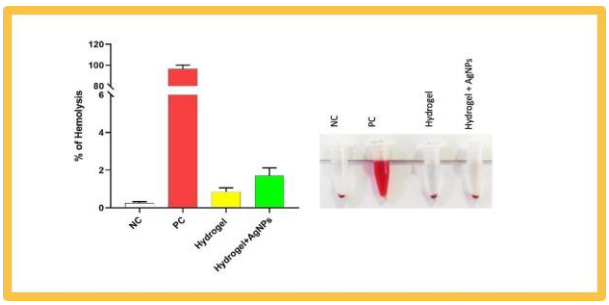


Fig 1 : Morphological evaluation of the Biocompatibility (Phase contrast) and cell proliferation levels (Live/Dead) with E.ribes extract and E.ribes-AgNPs (200µg/ml) along with –ive and +ive control groups for 24 hrs incubation, using phase contrast microscopy & Fluorescent microscopy (20x objective).

The absence of red fluorescence suggests minimal or no cell death, highlighting the biocompatibility of the hydrogel formulations, including those modified with Er-extract and Er-AgNPs. Overall, these results demonstrate that the hydrogels, particularly when combined with bioactive plant extract and nanoparticles, maintain high levels of cell viability, supporting their potential for safe use in biomedical applications.

Hemocompatibility test

The results in the graph 3 below revealed that the NC showed minimal haemolysis (0.56 ± 0.12%), confirming its non-hemolytic nature, while the PC caused extensive haemolysis (98.42 ± 2.11%), indicating complete lysis of RBCs. In contrast, the hydrogel and Hydrogel+AgNPs samples exhibited low haemolysis values of 1.87 ± 0.31% and 3.24 ± 0.45%, respectively, indicating good hemocompatibility.



Graph 3 : The image illustrates the haemolysis assay of hydrogel and hydrogel+AgNPs at 200 µg/mL, compared to positive (Triton X-100) and negative (normal saline) controls. The bar graph shows negligible haemolysis for both formulations (<3%), confirming excellent hemocompatibility.

Statistically, both the hydrogel and Hydrogel+AgNPs showed a significant difference in haemolysis compared to the NC group ($p < 0.01$) but remained well below the

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threshold of 5% considered safe for biomedical applications.

5. Discussion

The study's results demonstrate the E. ribes-based product's exceptional biocompatibility. The plant extract and its silver nanoparticle (AgNP) formulation were both well tolerated by L929 fibroblast cells, as demonstrated by the MTT assay. Even at the highest tested dose (300 µg/mL), cell viability remained high (above 75%). Remarkably, when compared to the extract alone, the E. ribes-AgNP group continuously maintained marginally higher cell viability. The combined advantages of bioactive plant phytochemicals and the regulated activity of AgNPs may be the cause of this, as they both contribute to a more balanced and less cytotoxic environment for the cells [23].

These findings are substantially supported by phase-contrast images and Live/Dead cell assay observations. Similar to untreated control cells, cells exposed to the various formulations maintained their uniform distribution and displayed healthy, elongated forms. The fact that there were no noticeable red fluorescence signals further suggests that there was not much cell death, confirming that fibroblasts can safely come into touch with these hydrogels [24].

Crucially, the hemocompatibility test verified that the hydrogel and hydrogel-AgNPs formulations did not significantly harm red blood cells, with haemolysis values remaining well below the recognized 5% threshold. This is an essential component of any material intended for biomedical applications, especially those involving tissue repair or wound healing where direct blood contact is frequent. Overall, the MTT, Live/Dead, and hemolysis test findings indicate that E. ribes-functionalized hydrogels have outstanding hemocompatibility and biocompatibility, particularly when paired with AgNPs. Stability and cellular tolerance are certainly enhanced through the presence of phytochemicals as natural reducing and capping agents. These characteristics make the formulations attractive options for biomedical uses, including localized drug delivery devices, regeneration scaffolds, and wound dressings [25].

The present study demonstrates that hydrogel formulations involving Embelia ribes (E. ribes) extract and silver nanoparticles (AgNP) exhibit good hemocompatibility and biocompatibility, making them feasible choices for biomedical applications. Haemolysis tests revealed minimal damage to red blood cells, well within safe limits, while the MTT and Live/Dead cell assays verified good cell viability and healthy fibroblast morphology. The stability and compatibility of the nanoparticles were certainly enhanced through the phytochemicals' synergistic role as natural reducing and capping agents. All things taken into account, the findings suggest that hydrogels derived from E. ribes, specifically those functionalized via AgNPs, provide an efficient and bioactive platform suitable for applications like tissue regeneration, wound healing, and other biological domains needing substances that promote cellular health.

The E. ribes-AgNP hydrogel system's long-term stability, biodegradability, and controlled release characteristics require more investigation. Clinical research and in vivo assessments are necessary to confirm its safety, effectiveness in wound healing, and capacity for tissue regeneration in physiological settings.

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