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Comprehensive Biocompatibility and Safety Evaluation of a Drug-Eluting Bioresorbable Sinus Scaffold (Mesire™ BRS) in Accordance with ISO 10993 Standards

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Abstract

Background: Mesire™ BRS is an innovative sinus scaffold system designed for use as a temporary implant to ensure the maintenance of patency of the Ostia of the sinuses and to provide localized administration of corticosteroids following FESS procedures.

Objectives: To conduct the biological evaluation of Mesire™ BRS in accordance with ISO 10993 standards.

Methods: A comprehensive biological assessment was performed, including *in vitro* assessment procedures like cytotoxicity in L-929 mouse fibroblasts. Other methods include *in vivo* assessment procedures such as skin sensitization in guinea pigs (Maximization Test), intracutaneous irritation in rabbits, acute systemic toxicity in mice, and 28 days sub-chronic toxicity in Sprague Dawley rats. Other procedures include the Genotoxicity test and the Pyrogenicity test, which are used to determine whether the material is capable of causing genetic toxicity and fever. Extraction of the samples was done by using polar and non-polar solvents.

Results: Mesire™ BRS had no cytotoxicity (grade 0 reactivity). Neither dermal sensitization nor intracutaneous irritation was observed from the reaction of the animals. There were no adverse effects, deaths, or changes in body weight, hematology, clinical chemistry, and histopathology in acute and sub-chronic systemic toxicity tests. In addition, the device was not Genotoxic and pyrogenic.

Conclusion: From the results obtained, it is proven that Mesire™ BRS is biocompatible and safe for its intended purpose.

Keywords: Biocompatibility, ISO 10993, Cytotoxicity, Sensitization, Systemic toxicity, Pyrogenicity

1. Introduction

Chronic rhino sinusitis is an inflammation of the paranasal sinuses that causes considerable suffering in patients due to its prevalence [1]. While conventional treatment methods are used to provide symptomatic relief to the affected individual, endoscopic sinus surgery has become increasingly popular where conventional treatment options fail to bring about any improvement [2]. Postoperatively, there are several issues that could develop as a consequence of the procedure, including mucosal inflammation, adhesions, ostial restenosis, and wound healing problems [3]. For this purpose, local drug delivery systems represent an

efficient approach to overcoming these obstacles and promoting wound healing after surgery [4]. The use of Bioresorbable drug-eluting implants is especially effective since they allow for delivering the mechanical and therapeutic effects at once, reducing the systemic delivery of the drug [5]. They facilitate sinus patency and inhibit inflammation and scar formation during the postoperative period [6]. The Bioresorbable Sinus Scaffold System has been created to fulfill these purposes, involving a Bioresorbable polymer scaffold of poly (DL-lactide-co-glycolide) (PLGA) and polyethylene [7]. Taking into consideration the fact that there might be extended exposure

with the mucous membranes, along with possible contact with extractable and leachable materials, it becomes absolutely necessary to carry out the assessment of biological safety^[8].

The ISO 10993 series is a set of standards designed to assess the biocompatibility of the medical device through *in vitro* and *in vivo* tests^[9]. In the present study, was tested for safety and biocompatibility in line with these standards, which included cytotoxicity, sensitization, irritation, systemic toxicity (both acute and sub-chronic), Genotoxicity and Pyrogenicity^[10].

2. Materials and Methods

2.1 Device Description

Drug Eluting Bioresorbable Sinus Scaffold System is a temporary, self-absorbing implant designed to keep the spaces within and around the sinuses during open sinus surgery. It consists of,

- a. Drug Eluting Bioresorbable Sinus Scaffold
- b. Delivery system

The drug is embedded in a bioabsorbable polymer matrix containing poly-(DL- lactide-coglycolide) and polyethylene glycol (inactive ingredients) which provides for gradual release of the drug. The scaffold delivers sustained release

of Mometasone Furoate USP directly to the sinus mucosa over a 30 day period prior to dissolving. A delivery system is supplied to access the sinus ostium and deploy the scaffold. The accessories/ loading tools; a funnel and pusher are supplied to load scaffold into delivery system.

The Drug Eluting Bioresorbable Sinus Scaffold System is intended to maintain patency of the frontal and maxillary sinus Ostia following sinus surgery and locally deliver steroids to the sinus mucosa. It separates/dilates mucosal tissues, prevents obstruction by adhesions/scarring, and reduces edema. The implant reduces the need for post-operative intervention such as surgical adhesion lysis and/or use of oral steroids.

The Stent is a bioabsorbable, drug-eluting implant indicated for use in adult patients (18 years and older) following sinus surgery. It is specifically designed for placement in the frontal and The openings to the maxillary sinuses will be created to maintain patency of the surgically formed sinus drainage route. The use of stent facilitates local and sustained delivery of mometasone furoate to the lining of the sinuses, thus minimizing the risk of developing inflammation and adhesions while reducing the need for further treatment that might include additional surgeries or steroid treatments. This device is beneficial to surgical results and in the long-term management of rhino sinusitis.



Fig 1: Figure of Drug Eluting Bioresorbable Sinus Scaffold System

2.2 Cytotoxicity Test

This study was carried out to assess the cytotoxic potential of the extract of Mesire™ BRS using L-929 mouse fibroblast cells through the Elution Method.

The test item extract was prepared using a surface area to volume ratio of 3 cm²/mL and was incubated at 37 ± 1 °C for 70 hours and 20 minutes with continuous agitation. This particular extraction process was chosen to ensure sufficient interaction between the test material and the extraction medium to facilitate the release of potential leachable substances. All the procedures were carried out in accordance with the safety and biosafety protocols established for the laboratory. The personnel handling the test item were provided with appropriate personal protective equipment, which included the use of sterile gloves, head covers, face masks, protective garments, and specific footwear/shoe covers. This was done with the aim of creating a controlled laboratory condition and at the same time avoiding any possible risks associated with inhalation, dermal contact, or ocular contact.

L-929 mouse fibroblast cells were cultured using 6-well plates and allowed to grow into a sub-confluent monolayer

prior to treatment. This was done to ensure the cells were actively proliferating and sensitive to the cytotoxic effects of the test substances. Once the confluence was established, the existing Minimum Essential Medium (MEM) was aspirated off and replaced with 1 mL of each respective material extract: the test item extract, negative control material extract (high-density polyethylene), and a positive control material extract (polyurethane). This was done to ensure the reproducibility and reliability of the results. Blank wells were also prepared using the extraction medium under the same conditions without the presence of any material to ensure the effects were not due to the extraction procedure itself.

After the incubation period of 48 hours and 30 minutes, the treated cultures were examined microscopically. In this context, the cellular responses were evaluated, focusing on the changes related to the morphology, lysis, and density of the cells. This qualitative approach was significant for the comparative analysis of the results, thereby facilitating the identification of the cytotoxic effects of the material within the established model.

Cells treated with the Mesire™ BRS extract contained

discrete intracytoplasmic granules without evidence of cell lysis or inhibition of cell growth, which translates to a reactivity grade of 0. Similarly, the cells of the blank and negative control groups contained discrete intracytoplasmic granules without evidence of cell lysis or inhibition of cell growth, thus a reactivity grade of 0. However, cells of the

positive control group showed almost complete destruction of the cell layers, which translates to a severe cytotoxicity response with a reactivity grade of 4.

Since the reactivity grade of the test item extract was less than 2, Mesire™ BRS was considered to be non-cytotoxic.

Table 1: Description and Justification of the Test System and Culture Conditions for *In Vitro* Cytotoxicity Assessment

Section	Description
Test System	Mammalian cell culture monolayer consisting of L-929 mouse fibroblast cells
Cell Line	L-929 mouse fibroblast cells
Justification for Selection	L-929 mouse fibroblasts are widely used and historically accepted for <i>in vitro</i> cytotoxicity assessment of biomaterials and medical devices. The cell line is well-characterized, reproducible, and recognized as a standard model for evaluating biological reactivity in extract-based assays.
Culture Medium	Minimum Essential Medium Eagle's (MEM) with L-Glutamine
Supplements	10% Fetal Bovine Serum (FBS); 1% Penicillin–Streptomycin
Culture Medium pH	7.32
Storage Conditions for Medium	Stored at 2–8 °C until use; equilibrated to room temperature prior to use

2.3 Test System Management

In this experiment, the use of L-929 mouse fibroblasts was justified because the cell line used proved to be free from contamination by Mycoplasma during initial cell culture analysis. These fibroblast cell cultures were grown in Minimal Essential Medium (MEM) at optimal conditions of 37 ± 1 °C under humidified air with $5 \pm 1\%$ of Carbon dioxide (CO₂), according to conventional mammalian cell culture conditions.

Cells were harvested by treating them with Trypsin-EDTA solution and then allowing them to centrifuge for 10 minutes at 800 rpm. For reproducibility purposes, the cells were sub-cultured for three times before carrying out the experiments. To prepare the cell suspension, cells were grown in the medium for an optimal number to achieve a cell concentration of 1×10^5 cells/mL. After this, 1mL of the suspension was added to each well of the six-well plate. Incubation was carried out at 37 ± 1 °C and $5 \pm 1\%$ CO₂ for 25 hours 36 minutes. This sub confluence of the cells, as a result of the pre-treatment, is significant to ensure that the cells behave uniformly in the experiments.

2.4 Extraction Procedure

2.4.1 Rationale for Selection of Extraction Criteria

According to ISO 10993-12:2021(E) and BS EN ISO 10993-12:2021 [11, 12, 13], the volume of the extraction vehicle was calculated on the basis of the standard surface area to volume ratio and the specifications provided for example, an extraction ratio of 3 cm²/mL was used in the study since the test item had a thickness greater than 0.5 mm. This method ensures that the concentration of the extract is representative of the clinical situation and meets international standards for the assessment of medical devices.

2.4.2 Rationale for Selection of Extraction Conditions

The test item, Mesire™ BRS, was designed for long-term contact, i.e., greater than 30 days, with muscle, subcutaneous tissue, and the sinus region. Therefore, the extraction process was carried out at 37 ± 1 °C for 70 hours and 20 minutes to mimic physiological conditions for the effective leaching of bioactive compounds.

The extraction process was carried out on the entire test item, with the addition of MEM culture media according to

the calculated surface area/volume ratio. For the extraction process, the negative and positive control samples were cut into pieces and sterilized using the autoclave method at 121 °C, 15 lbs pressure, for 15 minutes. In order to perform these experiments, a cell suspension was made which had a concentration of 10^5 cells/mL. After that, 1mL of suspension was poured onto each well of a six-well plate. These were incubated at 37 ± 1 °C and $5 \pm 1\%$ CO₂ for 25 hours and 36 minutes. The incubation period was sufficient enough to form a sub confluent monolayer of cells. Formation of this sub confluency among the cells as a result of pre-treatment is important because of the uniform behavior of the cells in experiments Table 2.

Table 2: Extraction Ratio and Volume

Treatment	Thickness (mm)	Extraction Ratio (cm ² /mL)	Quantity (cm ²)	Volume of Media (mL)
Blank	-	-	-	6
Negative control	>0.5	3	18	6
Positive control	<0.5	6	36	6
Test item	>0.5	3	52	17.333

2.4.3 Test Procedure

Sub confluent monolayers of L-929 cells were chosen for treatment to ensure maximum proliferation and minimal contact inhibition, following established *in vitro* protocols for cytotoxicity testing. Treatments, including the test item, positive control, and negative control, were performed in triplicate to ensure maximum reproducibility and to allow statistical evaluation of the results.

Every single well was appropriately labeled according to the experiment number, the replicate number, treatment type, and the date when the treatment was applied.

Then, the cultures were allowed to grow in an atmosphere of $5 \pm 1\%$ carbon dioxide at a temperature of 37 ± 1 °C for 48 h 30 min. Morphological alterations of the cellular monolayer were observed using microscopy in order to determine the cytotoxicity caused by the test item.

The cultures were then incubated at 37 ± 1 °C in a humidified atmosphere with $5 \pm 1\%$ CO₂ for 48 hours and 30 minutes. After that, the morphological changes in the cell monolayer were checked using microscopic examination to

identify the cytotoxic effects induced by the test item. Image of the test item, blank, and control wells were also captured and recorded refer to Figure 2.

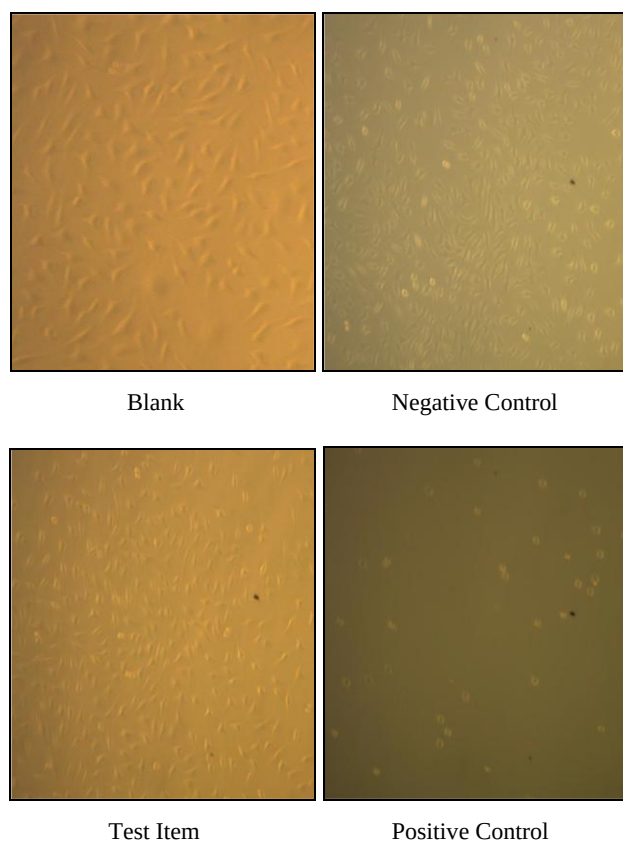


Fig 2: Images of the Test System Post Treatment

Table 3: Scoring for cytotoxicity was done based on the following criteria

Grade	Reactivity	Description of Reactivity Condition
0	None	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth.
1	Slight	Not more than 20% of the cells are round, loosely attached and without intracytoplasmic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
2	Mild	Not more than 50% of the cells are round, devoid of intracytoplasmic granules, no extensive cell lysis; not more than 50% growth inhibition observable.
3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50% growth inhibition observable.
4	Severe	Nearly complete or complete destruction of the cell layers.

2.4.4 Acceptance Criteria

Validity of the experiment was confirmed through the consistency of the data collected from the replicate wells since there were no variations noted. Validity of the experiment was confirmed because of the responses from the control groups. The negative control group had an

expected response since its grade was zero while the positive control group had an expected response since its grade was four.

2.5 Skin Sensitization

Sensitization potential of Mesire™ BRS was assessed using the Guinea Pig Maximization Test (GPMT), which is an established *in vivo* test system for the detection of delayed hypersensitivity reactions. The study was designed with four test groups: one polar solvent control (G1), one polar test item extract (G2), one non-polar solvent control (G3), and one non-polar test item extract (G4). Five animals were included in the control groups (G1 and G3), and the test groups (G2 and G4) had ten animals each, which is in accordance with the conventional GPMT test protocol.

Polar and non-polar extracts of the test item, along with their respective solvent controls, were prepared by employing an extraction ratio of 3 cm²/mL, which is surface area to volume, and then subjected to agitation at 37 ± 1 °C for 72 ± 2 hours. This extraction was meant to simulate conditions that would be clinically relevant, thereby ensuring maximum recovery of potentially leachable components.

The study was conducted using the classical GPMT protocol, which includes an intradermal induction phase on Day 1, a topical induction phase on Day 8, and a challenge phase on Day 23. Before the commencement of each phase, the fur on the predetermined application sites was clipped approximately 24 hours in advance.

In the intradermal induction phase, the animals were given three pairs of injections (0.1 mL per injection) in the interscapular region. No dermal reactions were observed after 24 ± 2 and 48 ± 2 hours following the administration of the undiluted polar and non-polar extracts. In line with the GPMT protocol, and to increase the sensitivity of the test, animals were treated on Day 7 with 10% w/w sodium lauryl sulphate in Vaseline to the intradermal induction sites to cause mild local irritation.

Topical induction was carried out on Day 8, in which filter papers soaked in the corresponding extracts or controls were placed on the intradermal induction sites. Occlusive dressing was maintained on the animals for 48 ± 2 hours. Animals were observed after approximately 1 and 24 hours following the removal of the dressing. Erythema and edema reactions were not noted on any of the animals suggesting that there was no sensitization during the induction phase. The challenge phase testing was done at the same environmental conditions as those used on Day 21, where the patch occlusion period lasted 24 ± 2 hours. There were no dermal reactions noted on all the animals at 24 ± 2 and 48 ± 2 hours after the patch removal. The lack of coetaneous response to challenge, specifically for the test subjects, proves that Mesire™ BRS had no delayed contact sensitization effect.

The animals were observed daily for clinical signs, while mortality was observed twice daily. Sporadic, transient, equally distributed, isolated observations of clinical phenomena such as nasal discharge, soft stool, and Lacrimation in the control groups and the test groups were considered incidental, unrelated to the test article, and not indicative of dermal sensitization. Body weight changes, including the percentage body weight change from Day 1,

were also unaffected.

Overall, based on the results of the Guinea Pig Maximization Test, Mesire™ BRS did not show evidence of dermal sensitization potential. Thus, from the results obtained, it can be concluded that, depending on the test method used, the test substance will not lead to delayed-type hypersensitivity when applied dermal.

Throughout the experiment, the proper personal protective equipment was worn by all personnel involved. The following pieces of equipment include gloves, headgear, face masks, body suits, and the appropriate footwear. This was done to make sure that the individuals in the laboratory did not come into contact with the test substance via any of the three routes of exposure, namely inhalation, dermal, and ocular.

During the entire process of conducting the study, the right type of appropriate personal protective equipment was used to ensure the safety of the personnel. This included the use of gloves, head caps, face masks, body garments, and appropriate footwear. Such measures were taken to ensure that the personnel in the lab were not exposed to the test item through the routes of inhalation, dermal, or ocular exposure.

2.5.1 Selection of Solvents and Justification

The selection of extraction vehicles was based on international standards for biological evaluation of medical devices. Normal saline solution has been selected for this study as the polar extraction medium, being identical to 0.9% w/v sodium chloride injection solution, while the non-polar extraction medium selected was sesame oil. The choice has been made according to the recommendation of the International Organization for Standardization in its ISO 10993-12:2021 document, "Biological evaluation of medical devices – Part 12: Sample preparation and reference materials". This recommendation is identical to the European standard BS EN ISO 10993-12:2021 [14, 15].

The selection of two types of extraction mediums, polar and non-polar, will allow obtaining leachable compounds having different physicochemical properties; consequently, the biological risk assessment will be comprehensive. The use of normal saline solution and sesame oil conforms to the recommendations made within the standard concerning the relevant *in vivo* simulation.

2.5.2 Acclimatization

Healthy adult animals were acclimatized to the laboratory environment for a period of five days before the commencement of the treatment schedule. During this time, animals were observed once a day for clinical signs of suitability for the study. Extensive veterinary checks were performed on the animals both on the day of receipt and on the fifth day of acclimatization to assess the health of the animals and to exclude those with abnormal findings, which might interfere with the study results. Acclimatization of animals is a process aimed at reducing animal stress, which might cause variability in the results of animal studies.

2.5.3 Positive Control Response Validation

In the present study, a concurrent positive control was not

used. However, the reliability of the assay was ensured through its previous validation using 1-chloro-2, 4-dinitrobenzene (DNCB), which is a known sensitizing chemical. The outcome of the previous validation study, had demonstrated the responsiveness and adequacy of the system. The use of historical positive control data is acceptable in a laboratory setting provided the adequacy of the system had been demonstrated in the past.

2.5.4 Dose Selection and Justification

According to the guidelines provided by the International Organization for Standardization in the ISO 10993-10:2021 standard and its British/European equivalent BS EN ISO 10993-10:2023, the testing of extracts without any dilutions was deemed appropriate for conducting the sensitization tests. Testing extracts without any dilutions is viewed as a cautious approach aimed at ensuring the highest likelihood of identifying any potential sensitizing agents [16, 17].

2.5.5 Route of Administration and Justification

The test item extracts were injected intradermally in the induction stage while applied topically in the induction and challenge stages. The injection of the test item extracts is in accordance with the methodology for the evaluation of delayed dermal sensitization, as detailed in the standards set forth in ISO 10993-10:2021 and BS EN ISO 10993-10:2023 [17, 18]. The application of the test item extracts via the dermal route, incorporating intradermal and topical application, enhances the sensitivity of the assay through immune priming in both the induction and elicitation stages.

2.5.6 Extraction Preparation

2.5.6.1 Rationale for Selection of Extraction Criteria

The parameters for the process of extraction were established in compliance with the guidelines outlined in the ISO 10993-12:2021 (Biological Evaluation of Medical Devices – Part 12: Sample Preparation and Reference Materials) and the European standard (BS EN ISO 10993-12:2021) [18]. Since the thickness of the test item is greater than 0.5mm, the standard method of surface area-based extraction was followed. This means that the extracts were prepared in a ratio of 3cm²/mL in polar and non-polar solvents. This device is divided into smaller pieces in order to have complete immersion with the solvent.

2.5.6.2 Rationale for Selection of Extraction Conditions

The test item is intended to be in contact with the tissues for a period exceeding 30 days. This includes the muscle, the subcutaneous tissue, as well as the mucosal surfaces such as the sinus mucosa. For the purpose of the experiment, the extraction was performed at a temperature of 37 ± 1 °C for a period of 72 ± 2 hours. This temperature is the same as the normal human body conditions as well as the parameters set during the extraction process.

In the experiment, the extraction process was carried out by mixing the measured quantity of the polar solvent and the non-polar solvent in the measured quantity of the test item at a specific ratio of 3 cm²/mL. The extracts and the solvent controls were kept in the incubator shaker at 37 ± 1 °C and shaken continuously for 72 ± 2 hours.

Table 4: The test item and solvent controls were subjected to extraction

Type of extract	Test item (cm ²)	Volume of solvent to be added (mL)	Extraction conditions
Polar solvent Control	-	30.0	37±1 °C for 72±2hours
Polar Test Item Extract	52	17.3	
Non-Polar Solvent Control	-	30	
Non-Polar Test Item Extract	52	17.3	

The quality of the test item used for the test and prepared for the test may differ according to requirements. The information regarding preparation solution will be used in the raw data. The prepared solution will be used within 24 hours so that it does not get absorbed on the container or change composition.

Note: Images of the pre and post extraction of the test item and solvent control will be included and presented in the final report.

2.5.6.3 Preparation of Intradermal Injections

For Group G1

- Site 1 : 1:1 (v/v) mixture of Freund's Complete Adjuvant (FCA) in Polar Solvent Control Extract (2 mL+2 mL = 4 mL)
- Site 2 : Polar Solvent Control Extract (3 mL)
- Site 3 : 50% (v/v) of Polar Solvent Control Extract with 1:1 mixture (v/v) of Freund's Complete Adjuvant (FCA) in Polar Solvent Control Extract (2 mL+1 mL + 1 mL = 4 mL)

For Group G2

- Site 1 : 1:1 (v/v) mixture of Freund's Complete Adjuvant (FCA) in Polar Solvent Control Extract (2 mL+2 mL = 4 mL)
- Site 2 : Undiluted Polar Test Item Extract (3 mL)
- Site 3 : 50% (v/v) of Undiluted Polar Test Item Extract with 1:1 mixture (v/v) of Freund's Complete Adjuvant (FCA) in Polar Solvent Control Extract (2 mL+1 mL + 1 mL = 4 mL)

For Group G3

- Site 1 : 1:1 (v/v) mixture of Freund's Complete Adjuvant (FCA) in Non-Polar Solvent Control Extract (2 mL+2 mL = 4 mL)
- Site 2 : Non-polar Solvent Control Extract (3 mL)
- Site 3 : 50% (v/v) of Non-Polar Solvent Control Extract with 1:1 mixture (v/v) of Freund's Complete Adjuvant (FCA) in Non-Polar Solvent Control Extract (2 mL+1 mL + 1 mL = 4 mL)

For Group G4

- Site 1 : 1:1 (v/v) mixture of Freund's Complete Adjuvant (FCA) in Non-Polar Solvent Control Extract (2 mL+2 mL = 4 mL)
- Site 2 : Undiluted Non-Polar Test Item Extract (3 mL)
- Site 3 : 50% (v/v) of Undiluted Non-polar Test Item Extract with 1:1 mixture (v/v) of Freund's Complete Adjuvant (FCA) in Non-Polar Solvent Control Extract (2 mL+1 mL + 1 mL = 4 mL)

2.5.7 Preparation of Animals

For the induction phase, the fur of the animals is to be clipped from the interscapular region approximately 24 hours before the experiment, and the clipped fur is to cover

an area of at least 3 cm × 5 cm. For the challenge and re-challenge phase, the fur is to be clipped from the designated flank region, which covers an area of approximately 80 cm² (8 cm × 10 cm), approximately 24 hours before the experiment. Precautions are to be taken to ensure that the skin is not abraded during the process. The fur is to be clipped again, if necessary, during the experiment for the purpose of observation.

2.5.7.1 Treatment

The test comprised of three phases, viz.

- Induction: Intradermal injection
- Induction (Boosting): Topical application
- Challenge: Topical application

a) Induction: Intradermal Injection

Day 1: The animals were injected at the interscapular region with 0.1 mL per injection of 3 pairs of intradermal injections approximately 2 cm apart as per the section 6.8, so that one injection of each pair was on either side of the midline approximately 2 cm apart. Injections 1 and 2 were given near the head region at least 1 cm apart and injection 3 was given 2 cm apart towards caudal part of the body as shown in the figure 1. The injected sites were marked by permanent marker pen.

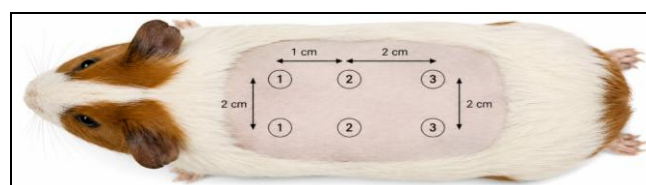


Fig 3: Site of intra-dermal injections in the Guinea pig for maximization test of Magnusson and Kligman.

- Site 1: 1st pair of injection
 Site 2: 2nd pair of injection
 Site 3: 3rd pair of injection

b) Induction: Topical Application

Day 7: The fur from the region of the intradermal induction sites on the back of the animal, i.e., the interscapular region, [(3 cm × 5 cm) or (15 cm² area)], will be closely clipped for the solvent control and treatment group animals. If the undiluted extract does not produce irritation in the intradermal induction at site 2, the topical induction site will be pre-treated with 0.5 mL of 10% w/w Sodium Lauryl Sulphate in Vaseline to produce local irritation 24 ± 2 hours before the topical induction. Note: Any SLS that remains will be removed.

Day 8: Filter paper of 2 cm × 4 cm will be saturated by soaking in the solvent control and the test item extract according to Section 7.8. The saturated filter papers will be topically applied to the previously injected sites of the

respective groups. The filter papers will be held in place by non-irritating adhesive tape. The test patch will be further covered by an occlusive dressing using crepe bandage. The test patch will be held in place for 48 ± 2 hours. The test patch will be removed after 48 ± 2 hours of the contact period. The area will be cleaned by using normal saline swabs.

c) Challenge: Topical Application

Day 22: The fur from the right flank region, which is not treated during the induction phase, will be clipped for all the animals.

Day 23: The filter paper (2 cm x 4 cm) is to be saturated by soaking with the respective solvent control and test item extract as per the section 7.8. The saturated filter paper is to be applied to the respective groups over the pre-clipped area. The test item extract is to be applied to the posterior part of the right flank region, and the solvent control extract is to be applied to the anterior part of the right flank region for the G1, G2, G3, and G4 group animals. The test patch is to be held in place with non-irritating adhesive tape and to be further wrapped with crepe bandage by an occlusive dressing. The test patch is to be held in its position for 24 ± 2 hours. After the 24 ± 2 hours of contact period, the test patch is to be removed, and the area is to be cleaned with normal saline swabs and dried with cotton.

2.5.7.2 Re-challenge: Topical Application

The re-challenge test was not conducted as there were no equivocal scores from the challenge phase treatment.

2.5.8 Observations: The following were observed during the experimental period.

2.5.8.1 Clinical Signs of Toxicity and Morality

The animals were observed once daily for clinical signs of toxicity and twice daily for mortality.

2.5.8.2 Skin Reactions Scoring

- **Intradermal Induction:** Scoring of the skin reactions were done at (24 ± 2) hours and (48 ± 2) hours after the induction (intradermal injection).
- **Topical Induction:** The scoring of the skin reactions occurred at (24 ± 2) hours and (48 ± 2) hours after removal of the test patch in the challenge phase.
- **Topical Challenge:** The scoring of the skin reactions occurred at (24 ± 2) hours and (48 ± 2) hours after removal of the test patch in the challenge phase.

The skin reactions were observed and recorded based on the grading scale by Magnusson and Kligman as presented in the table below:

Table 6: Magnusson and Kligman Grading Scale for the Evaluation of Challenge Patch Test Reactions

Patch Test Reaction	Grading Scale
No visible change	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and/or swelling	3

Note: After completion of the experiment, all the experimental animals were sent back to the animal facility.

2.5.8.3 Body Weight

Individual animal body weights were recorded at receipt, prior to initiation of the treatment (Day 1) and at termination.

2.6 Intracutaneous Test

Mesire™ BRS test item was tested for intracutaneous skin reactivity in New Zealand White rabbits in compliance with the principles laid out in ISO 10993-23:2021(E) and BS EN ISO 10993-23:2021^[19, 23]. Test material skin irritation potential was determined through intracutaneous skin irritation study involving use of three male New Zealand White rabbits.

On the day of application, 0.2 mL of the polar extract from the test substance was introduced intradermally into five different sites of the anterior part of the left side of the body of each test animal. In parallel, 0.2 mL of the polar solvent was injected into five sites of the anterior parts of the right side of the body. Afterwards, the same procedure was repeated for the non-polar extracts from the test material and its respective solvent, which were injected in five different sites of the posterior sides of the body of the animals.

Animals were observed once daily for toxic effects and twice daily for mortality. Dermal reaction sites were checked immediately after injection and then on days 1, 2, and 3 post-treatment. The weight of test animals was determined upon arrival to the laboratory, before treatment began (on Day 1), and then at the completion of observations (on Day 4). Euthanasia was done on the last day of testing by intravenous injection of sodium thiopental, followed by appropriate disposal of the test animals' carcasses.

During the whole course of the study, there were no deaths or symptoms showing signs of toxicity. No weight gain abnormalities occurred for all tested animals, as all animals displayed normal weight gain compared to Day 1. Moreover, there were no observations of treatment-related erythema or edema at any of the administration sites within the observation period of 72 hours. Since there were no signs of intracutaneous activity in all treated animals, the experiment ended after the observation period of 72 hours.

2.6.1 Selection of Solvents and Justification

Guidelines for selecting suitable solvents for extraction were based on the ISO standards for the biological evaluation of medical devices, namely ISO 10993-12:2021 and European adoption BS EN ISO 10993-12:2021^[19, 23].

Following the instructions of the international standards, the combination of polar and non-polar solvents was used for extraction of possible substances from the test substance in order to guarantee maximum recovery of extractable substances having various physicochemical properties. As the polar solvent, an aqueous solution of 0.9% w/v sodium chloride for injection (normal saline) was chosen due to its physiological compatibility and capability to extract possible hydrophilic extractants. In turn, as a non-polar solvent, sesame oil was chosen because it could be used to extract possible lipophilic compounds.

Therefore, the combination of two types of solvents was recommended in the ISO standard in order to increase the chances for discovering all potentially leachable substances from the medical device materials. The chosen solvents

were thus appropriate for evaluating intracutaneous reactivity of the test substance.

2.6.2 Acclimatization

Young, healthy adult animals were acclimatized to laboratory conditions for five days before commencing the experiment. The observation of the animals' general health condition was carried out every day during the acclimatization period in order to establish any clinical conditions that may affect the experiment results.

All animals were examined by a veterinarian on the day of their arrival and at the end of five days of acclimatization to ascertain their fitness for experimentation. Only animals found healthy and without any abnormalities were used in the experiments.

2.6.3 Study Design

Intracutaneous injection of both the test item extracts and the respective solvent control extracts was done for each animal. The injection sites were different depending on whether the extract under evaluation was polar or non-polar. Polar test item extracts were intracutaneously injected to five sites in the anterior surface of the left side of the trunk. On the other hand, non-polar test item extracts were injected to five sites in the posterior surface of the left side of the trunk. Polar solvent control extracts were injected into five sites in the anterior surface of the right side of the trunk while non-polar solvent control extracts were injected to five sites in the posterior surface of the right side of the trunk.

Each injection was administered at 0.2 mL per injection site.

Table 7: The study was performed in three rabbits as per the following study design.

Number of Animals	Animals Number	Treatment	Region of Injection	Number of Injections	Volume per Injection (mL/site)
3	Nd0452 to Nd0454	Polar Test Item Extract	Anterior part of the Left Trunk	5	0.2
		Non-polar Test Item Extract	Posterior part of the Left Trunk	5	0.2
		Polar Solvent Control extract	Anterior part of the Right Trunk	5	0.2
		Non-polar Solvent Control extract	Posterior part of the Right Trunk	5	0.2

2.6.4 Extraction Procedure

2.6.4.1 Rationale for the Selection of Extraction Criteria

Extraction conditions have been chosen following the instructions provided by ISO 10993-12:2021 and its corresponding British Standard version BS EN ISO 10993-12:2021. This international standard document specifies that the extraction procedure should include standardized surface-to-volume ratios when preparing medical device extracts for biological assessment.

The test item possessed a thickness greater than 0.5 mm. In accordance with the ISO recommendations for devices of this thickness category, the extract was therefore prepared using a surface area to extraction vehicle ratio of 3 cm²/mL for both polar and non-polar solvents to ensure complete exposure of the device material to the extraction media, the whole device was cut into smaller pieces, thereby facilitating full submersion during the extraction process.

2.6.4.2 Rationale for the Selection of Extraction Conditions

The test item is intended to come into contact with muscle, subcutaneous tissue, and mucosal surfaces (specifically sinus mucosa) for a prolonged duration exceeding 30 days. Therefore, extraction conditions were selected to approximate physiological conditions that may influence the release of potential extractable substances from the device material.

As suggested by ISO guidelines, extraction took place at a temperature of 37 ± 1 °C over a period of 72 ± 2 hours. This is because the temperature is consistent with physiological conditions. As such, this would enable the possibility of evaluating any interaction that may occur between the tested items and adjacent biological tissues under such conditions.

2.6.4.3 Extraction Method

The required quantity of the test item was obtained, and the corresponding volume of extraction solvents was

determined based on the calculated surface area of the device material. Polar extraction was performed using normal saline (0.9% w/v sodium chloride solution), while sesame oil was used as the non-polar extraction medium. Both solvents were applied at an extraction ratio of 3 cm²/mL (surface area to extraction vehicle)

The test item extracts and their respective solvent control extracts were prepared separately. All extraction vessels were then placed in an incubator shaker and maintained under continuous agitation at 37 ± 1 °C for 72 ± 2 hours to facilitate the release of potential extractable substances from the test material.

The extraction conditions applied to the test item and solvent controls are summarized in the table 8 below.

Table 8: Extraction Method

Type of extract	Quantity of test item (cm ²)	Volume of solvent added(mL)	Extraction conditions
Polar solvent control	-	40.1	37±1 °C for 70 hours 57 minutes
Polar test item extract	104	34.7	
Non-Polar solvent control	-	40.0	
Non-polar test item extract	104	34.7	

2.6.4.4 Dose Selection and Justification

The dose administered in the current experiment was chosen based on the protocol suggested in ISO 10993-23:2021 and its European counterpart BS EN ISO 10993-23:2021. According to these protocols, undiluted extracts should be employed when investigating the capacity of an irritant to cause skin irritation in order to increase sensitivity.

Accordingly, the undiluted polar and non-polar extracts of the test item were administered at a volume of 0.2 mL per injection site. The use of undiluted extracts ensures that any potential irritant substances present in the extract are evaluated under conditions representing a worst-case exposure scenario.

2.6.4.5 Route of Administration and Justification

The extracts obtained from the test article and the solvents used to prepare them were administered using the intracutaneous route of administration. The selection of this route of administration was based on guidelines laid out in ISO 10993-23:2021 and BS EN ISO 10993-23:2021.

The intracutaneous route of administration is suggested for the assessment of irritancy of medical device extracts due to the ease of observing any inflammatory responses (such as erythema and edema) that can be induced by the leachable agents present in the test article.

2.6.4.6 Administration of Test Item

On the day of treatment, 0.2 mL of the polar test item extract was administered intracutaneously at five distinct sites on the anterior region of the left trunk of each rabbit. Correspondingly, 0.2 mL of the respective polar solvent control extract was injected at five sites on the anterior region of the right trunk of each animal.

The same procedure was subsequently repeated using the non-polar test item extract and the corresponding non-polar solvent control extract. These were administered at five sites on the posterior regions of the left and right trunks, respectively, thereby ensuring contra lateral placement of test and control injections within each animal.

All injection sites were clearly marked using a permanent marker to facilitate accurate identification and consistent evaluation of local skin reactions throughout the observation period.

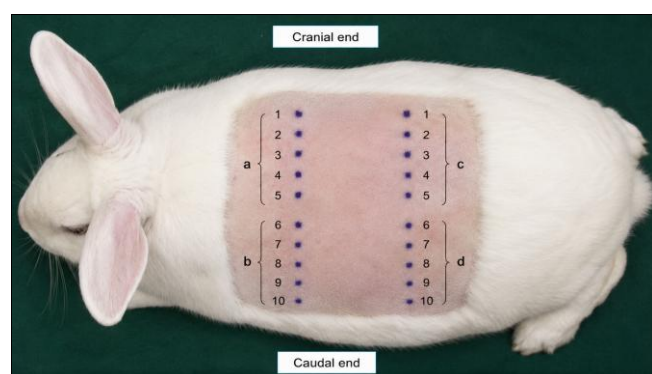


Fig 4: Injection Site

key

- 0.2 mL injections of polar extract of test item (Cranial end)
- 0.2 mL injections of non-polar extract of test item (Caudal End)
- 0.2 mL injections of polar solvent control (Cranial end)
- 0.2 mL injections of non-polar solvent control (Caudal End)

2.6.4.7 Observations

The following observations were conducted throughout the experimental period to evaluate the potential systemic and local effects associated with the administration of the test item extracts.

2.6.4.7.1 Clinical Signs of Toxicity and Mortality

The animals were examined once per day for symptoms

suggestive of systemic toxicity, as well as twice per day for mortality throughout the duration of the experiment. This examination involved evaluating the overall health status of the animals through observations regarding skin and coat changes, eye symptoms, respiration and circulation, as well as symptoms relating to the autonomic nervous system and the central nervous system. Somatomotor activities were also assessed to identify any possible deviations associated with exposure to the test substance extracts.

2.6.4.7.2 Intracutaneous Reactions

Immediate responses to local skin injection sites were assessed immediately after administration, and again after 24 ± 2 , 48 ± 2 , and 72 ± 2 hours of the administration of the substance. The main aim of the assessment was to assess the appearance of visible symptoms of skin irritancy, such as erythema and edema.

The intensity of the observed reactions in each injection site and at different observation times was recorded according to the scoring system provided in the ISO 10993-23:2021 and BS EN ISO 10993-23:2021 standards. The scoring system used for the assessment of the dermal reactions is presented in the table below.

If there are no delayed or progressive reactions observed, the observation will be terminated 72 hours after administration of the test substances.

Table 9: Grading System for Intracutaneous (Intradermal) Reactions

Intracutaneous Reactions	Numerical Grading
Erythema and Escher formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to Escher formation preventing grading of erythema	4
Oedema formation	
No oedema	0
Very slight oedema (barely perceptible)	1
Well-defined oedema (edges of area well-defined by definite raising)	2
Moderate oedema (raised approximately 1 mm)	3
Severe oedema (raised more than 1 mm and extending beyond exposure area)	4
Maximum possible score for irritation	8

No adverse changes were observed at the injection sites.

2.6.4.7.3 Body Weight

The weight of each individual animal was measured at the time of arrival, before treatment (day 1) and at the end of the experiment. Measuring body weight helped in determining the general state of health.

2.6.4.7.4 Pathology

Necropsy

When the experiment ended, all animals were humanely killed using sodium thiopentone through intravenous injection, and their bodies were properly disposed of. Necropsy was conducted to determine the gross pathology and gain additional knowledge regarding any toxicology effects.

2.6.4.7.5 Interpretation of Results

A quantitative evaluation was done for each administration site on all subjects at 24 (± 2 hours), 48 (± 2 hours), and 72 (± 2 hours) hours after the administration of the substances. The scores of erythema and edema for each test substance and its respective solvent control were added up, individually. They were later divided by 15 to find out the mean scores for both the test substance and the solvent control. This was achieved because 15 is equal to the sum of three time points per five injection sites. The score for reaction was obtained by deducting the mean score for the solvent control from that of the test substance. The criteria for conducting the experiment were met as the final test sample score was 0.

2.6.4.7.6 Amendments and Deviations

No amendments were implemented, and no procedural deviations occurred during the conduct of the study, ensuring adherence to the original experimental protocol.

2.7 Acute Systemic Toxicity

The test item Mesire™ BRS was assessed for acute systemic toxicity using Swiss albino mice and based on the guidelines provided in ISO 10993-11:2017(E) and BS EN ISO 10993-11:2018, which are related to biological evaluation of medical devices [23, 24]. A total of 20 male Swiss albino mice were randomly assigned into four groups to conduct this experiment. The polar and non-polar extracts of the test item were prepared using a surface area-to-volume extraction ratio of 3 cm²/mL, and were maintained under constant agitation using an incubator shaker at 37 $\pm$ 1 °C for 70 hours and 57 minutes.

Each group had five animals. Intravenous injection was done in case of polar solvent control (Group 1) and polar extracts of the test item (Group 2) while in case of non-polar solvent control (Group 3) and non-polar extracts of the test item (Group 4), the injection method used was Intraperitoneal with 50 ml/kg body weight dose volume.

Thereafter, observations were made at 30-40 minutes, 1 hour (± 10 minutes), 2 hours (± 10 minutes), and 4 hours (± 10 minutes) on day 1. Thereafter, daily observations were made from day 2-4 for clinical signs while observation was done twice daily for mortality. Body weight measurements were taken on the day of receipt before dosing on day 1, and days 2, 3, and 4.

Euthanasia of animals was performed by carbon dioxide asphyxiation and subsequently, necropsy was performed on all animals after conclusion of observation period. There were no treatment related clinical signs of toxicity and mortality in all groups.

In addition, there were no treatment-related changes in body weights and percentage change in body weights relative to Day 1. All animals showed physiologically normal weight gains throughout the observation period. Gross pathological evaluation of all the animals showed no treatment-related abnormalities.

2.7.1 Selection of Solvents and Justification for Selection of Solvents

The recommended use of solvents used for extraction agrees with that provided in the International Standard ISO 10993, Biological Evaluation of Medical Devices; the

recommendation in relation to the use of a selection of solvents used for extraction was made in document ISO 10993-12:2021(E), Biological Evaluation of Medical Devices-Sample Preparation and Reference Materials and its British equivalent, BS EN ISO 10993-12:2021 [25, 26]. For this reason, sodium chloride 0.9% solution, to be used for injectable solutions (normal saline solution), is used as a polar solvent and sesame oil is used as a nonpolar solvent.

These extraction solvents were recommended to be used in extraction tests to be used in biocompatibility evaluation tests owing to their potential to represent physiologically relevant polar and non-polar environments to effectively extract potential leachable substances from test materials. These solvents have been recommended for use in extraction tests for biocompatibility evaluation owing to their potential in representing physiologically relevant polar and non-polar environments. Therefore, the use of polar and non-polar solvents is essential in order to have a comprehensive evaluation of potential biological effects.

2.7.2 Extraction Procedure

According to the guidelines set out in Biological Evaluation of Medical Devices – Part 12: Sample Preparation and Reference Materials (ISO 10993-12:2021(E) and BS EN ISO 10993-12:2021) [25, 26], the standard approach to calculate the appropriate volume of extraction vehicle to be used was adopted. The test item is intended to have long-term contact (>30 days) with muscle tissue/subcutaneous tissue and mucosal tissue (sinus mucosa). Hence, extraction conditions have to be chosen to mimic the physiological conditions.

To mimic *in vivo* conditions, extraction was carried out at 37 $\pm$ 1 °C for 72 $\pm$ 2 hours, as suggested in the standard for devices intended to have prolonged tissue contact. The volumes of extraction vehicles to be used were calculated based on the surface area of the test item, using an extraction ratio of 3 cm²/mL.

The polar and non-polar extracts were prepared with normal saline (0.9% w/v NaCl) and sesame oil, respectively. The test item extracts and the solvent control solutions were prepared separately and maintained in an incubator shaker with continuous agitation at 37 $\pm$ 1 °C for 72 $\pm$ 2 hours.

2.7.3 Dose Selection and Justification for Selection

The undiluted polar and non-polar extract of the test item, as well as their respective solvent controls prepared using as extraction ratio of 3 cm²/mL, were administered using a dose volume of 50 mL/kg body weight. The dose volume was administered as a single dose per animal.

The dose volume is within the recommended limits for mice for intravenous and intraperitoneal routes of administration, as per ISO 10993-11, Biological Evaluation of Medical Devices – Part 11: Tests for Systemic Toxicity, and the standard BS EN ISO 10993-11. The use of undiluted extracts at the maximum recommended dose volume is necessary to provide adequate exposure to potential extractable.

2.7.4 Route of Administration and Justification for Selection

Polar test item extract and respective polar solvent control extract were given intravenously, and non-polar test item

extract and respective non-polar solvent control extract were given Intraperitoneal in accordance with ISO 10993 Part 11 - Tests for Systemic Toxicity and BS EN ISO 10993 Part 11 - Tests for Systemic Toxicity.

2.7.5 Administration of Test Item

The polar test item extract and respective polar solvent control extract was given intravenously with a dose volume of 50ml/kg body weight, and non-polar test item extract and respective non-polar solvent control extract was given Intraperitoneal with a dose volume of 50ml/kg body weight according to ISO 10993 Part 11 - Tests for Systemic Toxicity and BS EN ISO 10993 Part 11 - Tests for Systemic Toxicity.

2.7.6 Observations

The following observations were made during experimental period.

2.7.6.1 Clinical Signs Observation

At 30 to 40 minutes, 1hour (+/- 10 minutes), 2hours (+/- 10 minutes) and 4 hours (+/- 10 minutes) after treatment (Day1) all animals were observed for signs of clinical toxicity and death. This evaluation occurred daily for days 2, 3 and 4, where the animals were observed daily for signs of clinical toxicity and twice daily for sign and death. No signs of clinical toxicity were observed to have occurred in animal prior to Day4, therefore the study terminated.

2.7.6.2 Body Weight

Body weight was measured on receipt, on the day before test item treatment and on each day of the first three days after treatment (days 2, 3, and 4) during the observation period.

2.7.6.3 Pathology

- a. **Necropsy:** Finally, at the end of the observation period, all the animals were humanely euthanatized by carbon dioxide asphyxiation, subjected to necropsy, and gross pathological examination.
- b. **Histopathology:** Histopathological examination of the tissues was not done since there were no gross lesions in the animals.

2.7.6.4 Interpretation of results

The sample meets the criteria of the test as:

1. Animals have not shown any signs of toxicity compared to their respective solvent control group animals.
2. There were no mortalities, convulsions, or prostrations in all the tested animals.
3. There was no treatment-related loss of body weight during the study.

2.8 Pyrogen Test

The test item, Mesire™ BRS obtained., was subjected to Pyrogenicity testing, and the results were determined in rabbits in accordance with the requirements specified in USP General Chapter <151> and ISO 10993-11:2017(E) [27, 28]. The extract of the test item was prepared in 0.9% normal saline at 37 ± 1 °C for 71 hours and 19 minutes, with a surface area to extraction medium ratio of 3 cm²/mL. The extract was visually inspected at the end of the extraction

period. There was no suspended particulate matter in the extract. There was no change in the physical appearance of the test item. Hence, the extract was considered suitable for administration without any further alteration.

The animals used in the main test were based on the outcome of the preliminary screening. Three rabbits with control temperature variation within 1 °C, with a baseline temperature < 39.65 °C, were used for the Main Test I. During the test period, the animals were restrained about an hour prior to recording the baseline temperature, and food and water were withheld in accordance with the test requirements. The baseline temperature was recorded within 30 minutes prior to dosing.

The extract was pre-warmed and kept at 37 ± 2 °C prior to administration. The test extract was intravenously administered via the marginal ear vein at 10 mL/kg body weight, and the administration was completed within 10 minutes from the start of injection. Rectal temperatures were recorded at 30 ± 2 minutes after dosing for 1 to 3 hours.

The animals were monitored for clinical signs of toxicity once a day, and for mortality, twice a day. There were no clinical signs of toxicity and mortality. Body weights were taken from each rabbit at receipt and on the day of Main Test I.

None of the rabbits showed an individual increase in temperature of 0.5 °C or greater above the respective control temperature. Therefore, according to the criteria for acceptance established in USP General Chapter <151>, the test item was considered non-pyrogenic.

2.8.1 Extraction Procedure

As per "Biological Evaluation of Medical Devices – Part 12: Sample Preparation and Reference Materials", ISO 10993-12:2021(E) and BS EN ISO 10993-12:2021 [28, 29], the extract was prepared using a surface area to extraction medium ratio of 3 cm²/mL in normal saline (0.9% w/v NaCl). As the thickness of the test item is above 0.5 mm, and the test item is expected to make contact with muscles/subcutaneous tissues as well as the mucosa for an extended period of time (>30 days), the most appropriate temperature at which to carry out the extraction process was selected as 37 ± 1 °C for a duration of 72 ± 2 hours.

The required amount of the test substance was obtained, and depending on the surface area of the test substance, the correct volume of normal saline (0.9% w/v NaCl) was used to obtain a dilution ratio of 3 cm²/mL (surface area/volume). The test item was cut into smaller pieces to ensure complete submersion in the extraction medium. The whole device was used for the extraction process. The test item extract was then placed in an incubator shaker and maintained under agitation at 37 ± 1 °C for 72 ± 2 hours to facilitate the extraction process.

2.8.2 Route of Administration and Justification for Selection

The test item extract was administered through intravenous route as per guideline, USP 44-NF 39 General Chapters: <151> Pyrogen test and 21 CFR Part 610 [29, 30].

2.8.3 Dose Selection and Justification for Selection

The test item extract (pre heated to 37 ± 2 °C and maintained at 37 ± 2 °C during administration) was injected at a dose

volume of 10 mL/kg body weight as per guideline USP 44-NF 39 General Chapters: <151> Pyrogen test ^[31].

2.8.4 Preliminary Test

Two days before the main test, rabbits were first screened through a preliminary test to select suitable animals. They were fasted overnight for about 16 hours before being restrained for temperature measurement, although water was allowed during this fasting period. During the actual test, both feed and water were withheld. About one hour before recording temperature, the rabbits were placed in restrainers, and temperature probes were inserted into the rectum to a depth of at least 7.5 cm and left in place to stabilize. The first temperature measurement was considered as the control, and subsequent measurements were made at an interval of 30±2 minutes within the span of 1 to 3 hours thereafter. Only those animals whose control temperatures did not exceed 39.65 °C, and had variations among themselves not exceeding 1 °C, were used in the experiment proper.

2.8.5 Main Test

Main test I was conducted by using 3 rabbits. Main test II was not conducted since individual animal response did not exceed 0.5 °C in any of the 3 animals.

2.8.5.1 Procedure

The animals were fasted overnight for approximately 17 hours before being restrained for temperature measurement, and fasting continued until completion of the test, although water was allowed during the fasting period; however, both feed and water were withheld during the test itself. Body weights were recorded prior to restraining, and about one hour before temperature measurement, the animals were placed in restrainers, with temperature probes inserted into the rectum of the rabbits to a depth of at least 7.5 cm and left to stabilize. After stabilization, the control temperature was recorded, and the test item extract was administered within 30 minutes of this recording. The extract, preheated and maintained at 37±2 °C, was injected intravenously through the marginal ear vein at a dose volume of 10 mL/kg body weight, with the administration completed within 10 minutes. Following injection, body temperature was measured at intervals of 30±2 minutes between one and three hours.

2.8.6 Observations

The following observations were made during experimental period.

2.8.6.1 Clinical Signs of Toxicity and Morbidity/Mortality

Rabbits were observed once daily for clinical signs of toxicity and twice daily for morbidity/mortality throughout the experimental period.

2.8.6.2 Body weight

Body weight of animals was taken on the day of receipt and on the day of main test before restraining the animals.

2.8.6.3 Determination of Rectal Temperature

The maximum temperature of each rabbit was defined as the

highest value recorded during the three-hour observation period, with measurements taken at intervals of 30±2 minutes after administration of the test item extract. The response for each rabbit was calculated as the difference between this maximum temperature and its corresponding control temperature.

2.8.7 Procedure for Interpretation of Results

Any decrease in temperature is considered as zero. If the increase in temperature above the respective control temperature in all three animals of Main Test I is less than 0.5 °C, the test item is considered to meet the requirements for absence of Pyrogen. However, if any individual rabbit shows a temperature rise of 0.5 °C or more above its control temperature, a second test (Main Test II) must be conducted using five additional animals. The material is ultimately considered free from Pyrogen if no more than three out of the total eight rabbits (from both Main Test I and II) show an individual temperature rise of 0.5 °C or more, and the sum of the maximum temperature increases of all eight rabbits does not exceed 3.3 °C.

2.9 Sub Chronic

The possible health hazards of Mesire™ BRS were evaluated through a 28-day repeated-dose exposure study on male and female Sprague Dawley rats. The evaluation aimed at determining the possible health hazards that may result from the exposure of the test subjects to the polar and non-polar extracts of the test item when administered intravenously and Intraperitoneal. This method of evaluation is often employed in the evaluation of the toxicological properties of biomedical materials to ascertain the possible biological responses that may result from the leachable materials.

The total number of rats used in the experiment was twenty-four rats, with each group consisting of 12 male rats and 12 female rats. The rats were segregated into two major groups, namely the group that served as solvent control group (G1) where the rats were treated using polar as well as non-polar extraction vehicle; and the second one that was referred to as the treatment group (G2). There were six males and six females in each group. Extracts from the test item were prepared using a polar solvent, 0.9% w/v sodium chloride for injection, normal saline, and a non-polar solvent, sesame oil. These extracts were prepared at 37 ± 1 °C, 72 ± 2 hours, and under continuous agitation in an incubator shaker at a ratio of 3 cm²/mL this was done to ensure that was an easy release of extractable substances. These extracts, both polar and non-polar, were given to the rats in the treatment group, while the rats in the control group were given extracts prepared from the same solvents.

During the entire period of the experiment, the test subjects were monitored for signs of systemic toxicity. These signs were monitored by daily observation of clinical signs, twice-daily observation of mortality and morbidity, and once-a-week measurement of body weight and food consumption. The subjects were also subjected to detailed clinical observation once a week. Ophthalmoscopic examination was conducted during acclimatization and after the completion of the experiment. Blood and urine samples were collected for hematological, biochemical, and urinalytic studies on Day 29. After collection of samples

from all the organisms, the organisms were then euthanized using CO₂ inhalation followed by Gross pathological examinations, and the main internal organs removed for Histopathological examinations.

While conducting the experiment, there were some clinical symptoms like soft faeces, lethargy, dehydration, piloerection, and nasal discharge that appeared in some animals in control and test groups. However, these were not related to treatment as these signs were isolated and seen in various groups and sexes. What is more interesting is that there were no mortality or clinical signs related to treatment with test item extracts throughout the treatment period. Although some reductions were seen in body weight, percent body weight gain, and feed consumption during the experimental period, these were not related to treatment as similar trends were seen in control and treated groups. There were no abnormal Ophthalmoscopic findings in either group after the examination at the termination of the treatment period. Additionally, analysis of hematological, clinical chemistry, and urinalysis parameters did not reveal any toxicologically significant changes related to the administration of test item extracts. These results are consistent with the findings of no treatment-related changes in gross pathological examination of tissues in both sexes. These results collectively suggest that repeated dose administration of polar and non-polar extracts of Mesire BRS in Sprague Dawley rats did not demonstrate evidence of systemic toxicity after a 28-day treatment period.

2.9.1 Selection of Solvents and Justification for Selection of Solvents

Solvent used for polar extract was 0.9% w/v sodium chloride for injection (normal saline) as per the standard "Biological Evaluation of Medical Devices - Part 12: Sample Preparation and Reference Materials" according to international standard ISO 10993-12:2021(E), while non-polar extract was performed using sesame oil as per BS EN ISO 10993-12:2021.

2.9.2 Acclimatization

Healthy and young animals were acclimatized for six days to experimental room conditions prior to dosing and were observed for clinical signs once daily. Veterinary examination of all the animals was performed on the day of receipt and day 5 of acclimatization.

2.9.3 Grouping

The animals received for the experiment were weighed and arranged in ascending order of their body weights. These body weight stratified animals were distributed to all the experimental groups using Microsoft Excel Spreadsheet, such that body weight variation of animals selected for the experiment did not exceed $\pm 20\%$ (Male: -8% to $+7\%$, Female: -4% to $+4\%$) of the mean body weight of each sex. The grouping was done one day prior before the initiation of treatment. Body weights of the animals were analyzed statistically for mean body weight to rule out the statistically significant difference between groups within each sex.

2.9.4 Route of Administration and Justification for Selection

The polar test item extract and respective polar solvent

control extract (Normal Saline) were administered through intravenous route and non-polar test item extract and respective non-polar solvent control extract (Sesame Oil) were administered through intraperitoneal route. Polar and non-polar extracts of test item were administered into the same animal as exposure to polar and non-polar leachable are concurrent in clinical conditions. Hence, dual routes of parenteral administration method were selected as per ISO 10993-11:2017(E).

2.9.5 Extraction Preparation

2.9.5.1 Rationale for the selection of extraction criteria

The test item, had the thickness of more than 0.5 mm and therefore, Extracts were prepared in 3 cm²/mL surface area/volume extraction ratio in the polar and non-polar solvents as per "Biological Evaluation of Medical Devices - Part 12: Sample preparation and reference materials", ISO 10993-12:2021(E) and BS EN ISO 10993-12:2021.

2.9.5.2 Rationale for the selection of extraction conditions

The test item, Mesire™ BRS comes in contact with muscle and mucosa for long term (more than 30 days) duration of tissue contact. The test item was extracted at 37 ± 1 °C for 72 ± 2 hours in both polar and non-polar solvent. After completion of the extraction period, extract was decanted to a sterile vessel and stored at room temperature.

The required quantity of the test item was received and according to the surface area of the test item, the required volume of the polar solvent [Normal Saline (0.9% w/v NaCl)] and non-polar solvent (Sesame oil) were added. The test item was cut into pieces to ensure the complete submersion of test item in the solvents.

2.9.6 Dose Selection and Justification for Selection

The undiluted extracts of test item and solvent control for polar and non-polar extracts were administered at the dose volume of 10 mL/kg body weight and 5 mL/kg body weight respectively. Dose volume is selected as per Annex H, Sub-chronic rat - Dual routes of parenteral administration, as per the guidelines ISO 10993 Part 11 - Tests for Systemic Toxicity and BS EN ISO 10993 Part 11 - Tests for Systemic Toxicity ^[32, 33].

2.9.7 Administration of Test Item

The polar test item extract and respective polar solvent control extract were administered through intravenous route at dose volume of 10 mL/kg body weight and non-polar test item extract and respective non-polar solvent control extract were administered through Intraperitoneal route at dose volume of 5 mL/kg body weight as per the guideline ISO 10993 Part 11 - Tests for Systemic Toxicity and BS EN ISO 10993 Part 11 - Tests for Systemic Toxicity ^[32, 33].

2.9.8 Frequency of Test Item Administration

The test animals were dosed with the polar test item extract, and the control animals were dosed with polar solvent control extract, intravenously 7 days/week over the duration of the study (i.e. 28 doses). For no polar test samples, the same test animals were dosed with the non-polar test item extract, and the control animals were dosed with the non-polar solvent control extract, Intraperitoneal every third day

(Day 1, 4, 7, 10, 13, 16, 19, 22, 25, 28) over the duration of the study (i.e. 10 doses).

2.9.9 Observations

The following observations were done during the experimental period.

2.9.10 Clinical Signs of Toxicity and Mortality

The animals were observed for the clinical signs of toxicity once daily after the administration of solvent/test item and twice daily for mortality and morbidity.

2.9.11 Detailed Clinical Examination

The animals were subjected to detailed clinical examination on Days 1, 7, 14, 21 and 28. These observations were made outside the home cage and preferably at the same time. Signs included, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity such as Lacrimation, piloerection, pupil size, and unusual respiratory pattern.

2.9.12 Body Weight

Individual animal body weight was recorded at receipt, on treatment day 1 (prior to treatment) and at weekly intervals thereafter. Fasting body weights of all animals were recorded at terminal sacrifice.

2.9.13 Feed Consumption

Cage wise feed consumption was measured at weekly intervals, coinciding with the body weights of the respective animals. Average feed intake per rat (g/rat/day) was calculated by using the amount of feed given, left over, spillage in each cage, number of day's interval and the number of rats surviving in each cage.

2.9.14 Ophthalmoscopic Examination

Ophthalmoscopic examination was carried out once during acclimatization (Pre-treatment) and last week of treatment for all the animals.

2.9.15 Clinical Pathology

At the end of treatment, all animals were fasted overnight (approximately 16 hours) before blood collection. Water was provided *ad libitum* during fasting period. Blood samples were collected from the animals separately into the tubes containing K3-EDTA for hematology, lithium heparin for clinical chemistry and 3.2% sodium citrate for Prothrombin time and activated partial thromboplastin time parameters. Blood samples were collected from retro-orbital plexus puncture method under mild Isoflurane anesthesia with the help of a fine capillary tube. The following haematology and clinical chemistry parameters were analyzed.

a. Haematology

The following haematology parameters were estimated using automated Advia®2120i Hematology System (Siemens Limited).

Table 10: Haematology Parameters

Parameters	Units
Hemoglobin concentration (HGB)	g/dL
Haematocrit (HCT)	%
Erythrocyte count (RBC)	10 ⁶ cells/ μ L
Total leukocyte count (WBC)	10 ³ cells/ μ L
Mean corpuscular volume (MCV)	fL
Mean corpuscular hemoglobin (MCH)	pg
Mean corpuscular hemoglobin concentration (MCHC)	g/dL
Platelet count (PLT)	10 ³ cells/ μ L
Mean platelet volume (MPV)	fL
Absolute reticulocyte counts	10 ⁹ cells/L
Reticulocyte count	%
Differential leukocyte count (DLC)	%
Absolute differential leucocytes count (DLC)	10 ³ cells/ μ L

The following coagulation parameters were analyzed using the STA satellite max coagulation analyzer (Stago Diagnostic)

Table 11: Coagulation Parameters

Parameters	Units
Prothrombin Time (PT)	Secs
Activated Partial Thromboplastin Time (APTT)	Secs

b. Clinical Chemistry

The following clinical chemistry parameters were analyzed using the Rx Imola Chemistry Analyzer (Randox Laboratories).

Table 12: Clinical chemistry Parameters

Parameters	Units
Alanine aminotransferase (ALT)	U/L
Aspartate aminotransferase (AST)	U/L
Alkaline phosphatase (ALP)	U/L
Gamma-glutamyl transpeptidase (GGT)	U/L
Total Protein	g/dL
Albumin	g/dL
A/G Ratio	g/dL
Total bilirubin	mg/dL
Fasting Glucose	mg/dL
Total Cholesterol	mg/dL
Blood Creatinine	mg/dL
Blood Urea Nitrogen (BUN)	mg/dL

The following electrolytes parameters were analyzed using the Easylyte® plus Na/K/Cl analyzer (Medica Corporation).

Table 13: Electrolytes Parameters

Parameters	Units
Sodium (Na)	mmol/L
Potassium (K)	mmol/L
Chloride (Cl)	mmol/L

c. Urinalysis

Urine was collected and analyzed from all rats on day 29. The animals were kept in urine collection cages for overnight (approximately 16 hours). Animals were not

given access to feed but water was provided *ad libitum* during their stay in urine collection cages. The volume of urine collected (mL), appearance and the color were examined by physical evaluation.

The urine was analyzed using Uri plus - 600 Urine analyzer (Dirui Industries) for the following Parameters.

Table 14: Urine Analyzed

Parameters	Units
Blood	Ery/ μ L
Bilirubin	mg/dL
Urobilinogen	mg/dL
Ketones	mg/dL
Protein	mg/dL
Glucose	mg/dL
Leucocytes	Leu/ μ L

In addition, nitrite, pH and specific gravity were analyzed. After analysis of the above parameters, the urine was subjected for centrifugation at 2000 rpm for 5 minutes. Then the urine was subjected for microscopic examination for urinary sediments.

Pathology

- a. **Gross Necropsy:** On day 29, all the animals were subjected to necropsy and detailed gross pathology examination. The animals were fasted overnight (water was provided *ad libitum*) prior to scheduled necropsy. On the day of terminal sacrifice, the body weights of all the fasted animals were recorded prior to necropsy. The animals were euthanized using carbon dioxide asphyxiation followed by exsanguinations. The gross pathological changes were recorded for each rat. Gross pathological examination of external surfaces, external orifices, abdominal, thoracic and cranial cavities, organs and tissues of each animal were conducted.
- b. **Tissue Collection:** The following Tier I organs /tissues were collected, trimmed off any adherent tissue, as appropriate. All the tissues mentioned below, from all animals, were preserved in 10% v/v Neutral Buffered Formalin and Modified Davidson's fixative for eyes and testes. Testes was preserved in modified Davidson's fixative for at least 24 hrs and then transferred to 10% NBF. Eyes with optic nerve was preserved in modified Davidson's fixative for at least 24 hrs and then transferred to 50% isopropyl alcohol.
- c. **Organ Weights:** The weighing organs from all animals at the scheduled sacrifices were trimmed off any adherent tissue, as appropriate and weighed wet as soon as possible to avoid drying. Relative organ weights were calculated against fasting body weight.
- d. **Histopathological Examination:** Histopathological examination was performed on a limited number of potential target organs/tissues using the tiered approach from the solvent control and test item extract groups. As there was no treatment related changes noticed in Tier I organs/tissues and therefore the Tier II organs/tissues were not evaluated. These tissues were embedded in paraffin wax, sectioned and stained with hematoxylin and eosin.
- e. **Bone Marrow Smear Examination:** Bone marrow smear (from one femur) was prepared for all the

animals at the time of necropsy. Since, treatment related changes were not observed in hematology parameters and/or microscopic changes in certain tissues/organs such as thymus, spleen and lymph node; bone marrow smears were not evaluated at the discretion of Study Director/Study Pathologist.

2.9.16 Statistical analysis

The computer printout of the data (in the form of appendix) was verified with the original raw data. After verification, the data was subject to various statistical analyses for the parameters like body weight, body weight gain, organ weights, food consumption, hematology, clinical chemistry, and other toxicology parameters were analyzed using validated SPSS software, version 27.

Student' t-test' was performed for different treatment groups compared with the respective control group. All statistical analysis and comparisons were evaluated at 95% level of confidence. Further, hypotheses testing was done at two-sided $p < 0.05$ level. Whenever feasible, the tail probabilities associated with each hypothesis being tested were provided. Statistically significant ($p < 0.05$) changes obtained from the aforementioned tests were designated by the superscripts throughout the report as stated below: Statistically significant ($p < 0.05$).

2.10 Genotoxicity Test

The test item, namely Mesire™ BRS was subjected for Mutagenicity in Bacterial Reverse Mutation Test.

The test item extracts were prepared at an extraction ratio of 3 cm²/mL (Surface Area/Volume) as per "Biological Evaluation of Medical Devices" Part 12:2021 (E), Sample preparation and reference materials ISO 10993-12:2021. The prepared extracts were kept in an incubator shaker for agitation at 37 $\pm$ 1 °C for 70 hours and 08 minutes for extraction. The precipitation test was performed for polar and Non-polar 100% Test Item Extract. There was no precipitation observed for 100% polar Test Item Extract and 100% Non-polar Test Item Extract.

Test Item Extract was subjected for *Salmonella typhimurium* tester strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2 uvrA (pKM101) and was subjected for Test Item Extract and plate was used in mutation assay for the identification of their mutagenic effects.

For the mutation assay, extracts of the test item (polar and non-polar) were tested for mutagenic potential using the limit concentration of 100% extract/plate, both in the presence and absence of metabolic activation. Solvent control Normal Saline and DMSO, along with positive control substances 2-Nitrofluorene, Sodium Azide, 9-Aminoacridine, and 4-Nitroquinoline N-oxide for trials "without metabolic activation" and 2-Aminoanthracene for trials "with metabolic activation," were tested concurrently. The experiment was carried out in two trials for this study in triplicates by plate incorporation method and Preincubation method. From the experimental result obtained, it was clear that the average number of revertants colonies obtained at the limit concentration was comparable to that of the solvent control in trials, plate incorporation method, and Preincubation method, in the presence and absence of metabolic activation. There was no appreciable increase in the average number of revertants colonies observed for the

limit concentration of polar and non-polar test item extracts in both plate incorporation method and Preincubation method. The number of revertants colonies observed in positive control substances was found to give 3.6-14.5 fold increase.

2.10.1 Test System

The Bacterial Reverse Mutation Test is quite popular in assessing the potential of certain chemical compounds to cause genetic mutations. Conducted following the OECD guideline 471 [34].

2.10.1.1 Allows the use of auxotrophic strain of bacteria

Histidine strains of *Salmonella Typhimurium* and Tryptophan strains of *Escherichia coli*.

- TA98 and TA1537: Frame shift mutation
- TA100, TA 1535 and *Escherichia coli* WP2 uvrA (pKM101): Base pair substitution

2.10.1.2 Justification for Test system selected

The following strains are recommended by the regulatory agencies:

Salmonella typhimurium -TA98, TA100, TA1535, TA1537. *Escherichia coli* -WP2 uvrA (pKM101).

2.10.2 Storage of the Test System

Stock cultures of the test organisms used in the testing were maintained in a frozen permanent stock using Oxoid Nutrient Broth No. 2 media at $-80 \pm 10^\circ\text{C}$ within the testing laboratory. For lab use, master plates containing each test organism were maintained on minimal glucose agar media, stored refrigerated under $2-8^\circ\text{C}$, and used within three months of preparation date.

2.10.3 Genetic Characterization of Test System

The requirements for growth and the genetic characteristics of strains such as histidine requirement, sensitivity to UV light, and resistance of tester strains TA98, TA100, TA1535, and TA1537 to ampicillin and rfa mutation of *Salmonella typhimurium* tester strains were examined, along with the number of spontaneous revertants, after the preparation of the master plates. Similarly, the requirements for growth and the genetic characteristics of strains such as tryptophan requirement, sensitivity to UV light, and resistance of *Escherichia coli* WP2 uvrA (pKM101) to ampicillin were examined, along with the number of spontaneous revertants, after the preparation of the master plates. The same tester strains were used for the experiment.

2.10.4 Outline of the Method

The Bacterial Reverse Mutation Test is based on the principle that the test will be able to detect the mutations that reverse the existing mutations in the tester strains and hence regain the functional potential of the bacteria to produce an essential amino acid (Histidine/Tryptophan). The Revertant bacteria are identified by their ability to grow in the absence of the amino acid (Histidine/Tryptophan) in the culture medium as required by the parent tester strain. In the first trial, the bacterial cultures were exposed to the test item, solvent, and positive controls in the presence and

absence of an exogenous metabolic activation system. These bacterial cultures are then combined with soft agar and plated immediately onto minimal glucose agar medium, viz., his for *Salmonella typhimurium* and trp for *Escherichia coli* (WP2 uvrA (pKM101)).

2.10.5 Preparation of S9 Homogenate and Activation Mixture

Sodium phenobarbitone and β -naphthoflavone induced rat liver S9 homogenate was used as the metabolic activation mixture. Sodium phenobarbitone and β -naphthoflavone induced rat liver S9 homogenate was used. The S9 homogenate was prepared from male Wistar rats induced with intraperitoneal injection of sodium phenobarbitone and β -naphthoflavone at 16 mg/mL and 20 mg/mL respectively for 3 days prior to sacrifice. The S9 homogenate was prepared and stored in the test facility at $-80 \pm 10^\circ\text{C}$ until use. Each batch of S9 homogenate was assessed for sterility by streaking the supernatant fluid on nutrient agar plates and incubated at 37°C for overnight, and the protein content (BCA Method) and its ability to metabolize promutagens 2-Aminoanthracene and Benzo (a) pyrene to mutagens using *Salmonella typhimurium* TA100 tester strain were also evaluated.

S9 homogenate was kept in frozen condition and thawed just prior to use. It was mixed with co-factor solution containing 4mM Nicotinamide Adenine Dinucleotide Phosphate (NADP) disodium salt, 5 mM Glucose 6-phosphate, 8 mM MgCl_2 , and 33 mM KCl in PBS at pH 7.24 for plate incorporation and Preincubation method.

2.10.6 Procedure to Calculate the Mass of Extracted Residuals

The pre-test was performed to calculate the mass of the extracted residuals of the test item. Acetone and Methanol and were used as common extraction solvents.

The percentage of extractable with each of these solvents was calculated individually.

Quantity of 0.22638 g and 0.24872 g of test item was added to 10 mL of acetone and methanol respectively in suitable containers. The test item was extracted for 22 hours and 2 minutes at 24°C with constant agitation. At the end of the incubation of test item extraction, no visible degradation, visible particles and no change in extract color was observed. Hence residue collection was done for both acetone and methanol extract of the test item.

The mass of the extracted residuals for acetone was calculated using the below mentioned formula.

$$W_R = m_2 - m_1 \\ = 0.00167$$

Where,

m_1 = is the mass of the empty flask;

m_2 = is the mass of the flask after evaporation of the extract.

$$\%_e = W_R / m_3 \times 100 \\ = 0.74\%$$

Where,

$\%_e$ = is the percentage of extractable;

m_3 = is the mass of the test item prior to extraction.

The mass of the extracted residuals for methanol was calculated using the below mentioned formula.

$$W_R = m_2 - m_1$$

$$= 0.002$$

Where,

m_1 = is the mass of the empty flask;

m_2 = is the mass of the flask after evaporation of the extract.

$$\%_e = W_R/m_3 \times 100$$

$$= 0.80\%$$

Where,

$\%_e$ = is the percentage of extractable;

m_3 = is the mass of the test item prior to extraction.

2.10.6.1 Acceptance Criteria

For the devices with a mass <0.5 g, the percentage of extractable in the test item is $\geq 1\%$.

For the devices with a mass ≥ 0.5 g, the percentage of extractable in the test item is $\geq 0.5\%$.

2.10.7 Results of Residue Extracted

Device with a mass 0.31297 g, the percentage of extractable in the test item is for Acetone as a solvent is 0.34%.

Device with a mass 0.34833 g, the percentage of extractable in the test item is for Methanol as a solvent is 0.92%.

The percentage of extractable did not meet the criteria mentioned above, the extract Were prepared using Method C.

2.10.8 Extraction Solvents

Normal saline (polar solvent) and DMSO (non-polar solvent) were used as extraction solvents for extracting samples of the test item and the extraction medium without test item was used as solvent control.

2.10.8.1 Justification for Selection of Extraction Solvents

The normal saline is selected for polar and dimethyl sulphoxide for non-polar extraction solvent as per the guideline International Standard ISO 10993 "Biological Evaluation of Medical Devices"- Part 12:2021(E), Sample preparation and reference materials.

2.10.9 Test Item Extraction Procedure - Method C

In accordance with "Biological Evaluation of Medical Devices - Part 12: Sample preparation and reference materials", ISO 10993-12:2021(E), BS EN ISO 10993-12:2021, standard surface area was used for calculating the volume of vehicle for extraction. Also, test item is of thickness more than 0.5mm. Hence, test item extract was prepared using an extraction vehicle volume to surface area ratio of 3 cm²/mL. Rationale for selection of extraction condition.

Test item comes in contact with muscle/subcutaneous tissue & mucosa (Sinus Mucosa) for long term i.e., more than 30 days. Hence, for exaggerated conditions of 37 $\pm$ 1 °C for and 72 $\pm$ 2 hours, extraction was selected.

The whole device was used for extraction. The quantity of the polar solvent (Normal Saline) and non polar solvent (Dimethyl Sulphoxide) required based on the surface area of the test item is added at an extraction ratio of 3 cm²/mL. The test item extracts and blank were prepared separately and

kept in an incubator shaker with agitation at 37 $\pm$ 1 °C for 70 hours and 08 minutes for extraction.

2.10.10 Precipitation Test

The solution of test item extract with respective solvent has been considered as 100% extraction solution. From the above solution of test item extract with respective solvent, 100 μ L of test item extracts (polar and non-polar) were mixed with 2 mL of molten soft agar and spread onto MGA plates. The plates were incubated for 2 hours at 37 $\pm$ 1 °C.

After the incubation period of 2 hours, the plates were observed for the presence of precipitation.

2.10.11 Tester Strain Cultures

The culture of Salmonella typhimurium TA98, TA100, TA1535, TA1537 and Escherichia coli WP2 uvrA (pKM101) strains of respective genotype master plates were grown in Oxoid broth no.2 separately for 15 hours and 15 minutes for plate incorporation and Preincubation methods at 37 $\pm$ 1 °C in the shaking water bath and the cell density was adjusted to approximately 18 $\times$ 10⁸ cells/mL.

2.10.12 Mutation Assay

2.10.12.1 Extraction Control

Polar solvent (normal saline) and non-polar solvent (DMSO) was used as a solvent.

2.10.12.2 Positive Controls

DMSO was used to prepare the stock and/or dilutions of the following positive controls:

Table 15: Positive Controls

Strain	Metabolic Activation	Positive controls	Concentration (μ g/plate)
<i>Salmonella typhimurium</i> TA98	+	2-Aminoanthracene	4
	-	2-Nitrofluorene	2
<i>Salmonella typhimurium</i> TA100	+	2-Aminoanthracene	4
	-	Sodium azide	1
<i>Salmonella typhimurium</i> TA1535	+	2-Aminoanthracene	4
	-	Sodium azide	1
<i>Salmonella typhimurium</i> TA1537	+	2-Aminoanthracene	4
	-	9-Aminoacridine	50
<i>E. coli</i> WP2 uvrA (pKM 101)	+	2-Aminoanthracene	30
	-	4-Nitroquinoline N-oxide	5

2.10.13 Test Concentrations

The limit test was conducted with 100% test item extract/plate test sample. Since there was no cytotoxicity or mutation observed with 100% test item extract/plate test sample for both polar and non-polar extracts, there was no need for further study with five different lower concentrations of the test item.

2.10.14 Performance of the assay

Labeling: The Petri-dishes were labeled with study number, strain number, treatment group, metabolic activation (with or without), experiment date, replicate number, and trial number. Three replicate plates for solvent control, positive

control, and one treatment level were maintained in the study.

Plate Incorporation Method - Trial I

The first trial was conducted using direct plate incorporation method.

Table 16: Preincubation Method - Trial I

In presence of metabolic activation	In absence of metabolic activation
2.0 mL soft agar containing histidine - biotin/tryptophan	2.0 mL soft agar containing histidine - biotin/tryptophan
500 μ L S9 mix	500 μ L Phosphate buffer saline (PBS)
100 μ L polar/non-polar test item extracts/solvents/ appropriate positive controls	100 μ L polar/non-polar test item extracts/solvents/ appropriate positive controls
100 μ L of bacterial culture	100 μ L of bacterial culture

For plate incorporation method, limit concentration of test item is 100% extract solution/plate, solvent controls, and positive controls. The tester strains along with S9/PBS were added with 2 mL soft agar containing histidine-biotin for *Salmonella typhimurium* and tryptophan for *Escherichia coli*. The mixture was vortexed and added to MGA plates. 100 μ L of the test item extract was added to the plates with the tester strains, such as *Salmonella typhimurium* TA98, TA100, TA1535, TA1537, and *Escherichia coli* WP2 uvrA (pKM101) with/without metabolic activation. The plates were incubated at $37\pm 1^\circ\text{C}$ for 65 hours and 32 minutes. The condition of the bacterial background lawn for the test item cytotoxicity using the code system and Revertant colonies for a specific tester strain within the limit concentration of 100% test item extract solution/plate were manually counted.

Table 17: Preincubation Method - Trial II

In presence of metabolic activation	In absence of metabolic activation
500 μ L S9 mix	500 μ L Phosphate buffer saline (PBS)
100 μ L polar/non-polar test item extracts/solvents/ appropriate positive controls	100 μ L polar/non-polar test item extracts/solvents/ appropriate positive controls
100 μ L of bacterial culture	100 μ L of bacterial culture

Preincubation method was carried out with limit concentration of 100% extract solution/plate, solvent control, and positive controls. The tester strains, along with S9/ PBS, and these test constituents were placed in sterile test tubes, which were incubated using an incubator shaker operating at 100 ± 5 rpm, with the temperature maintained between $37\pm 1^\circ\text{C}$ for a period of 20-25 minutes. After the incubation, these test constituents were added to 2 mL of soft agar containing histidine-biotin for *Salmonella Typhimurium*, while tryptophan was added for *Escherichia coli*, after which the mixture was vortexed and placed on MGA plates. The plates were incubated for a period of 64 hours and 50 minutes at $37\pm 1^\circ\text{C}$. Evaluation of the bacterial background lawn was performed for the presence of cytotoxicity of the test item using the code system, while revertant colonies for a given strain in the limit concentration of 100% extract were counted manually.

2.10.14.1 Viable Counts

The bacterial suspension of each of the tester strains was diluted up to a maximum of 10^{-7} in PBS and 1000 μ L of the diluted suspension of each of the tester strains was plated onto nutrient agar plates in triplicate. The plates were incubated at $37\pm 1^\circ\text{C}$ for 65 hours and 32 minutes for the plate incorporation and Preincubation method. After incubation, the number of colonies in each plate was counted manually and expressed as the number of Colony Forming Units per mL (CFU/mL) of bacterial suspension.

2.10.14.2 Quality Control Plates

Quality control plates with 100% polar and non-polar test item extracts; solvents (normal saline and DMSO), S9 mix, PBS, soft agar, DMSO, and MGA plate were incubated at $37\pm 1^\circ\text{C}$ for 65 hours and 32 minutes. The plates were maintained on the day of the conduct of the mutation assay.

2.10.15 Observations

2.10.15.1 Effect of Bacterial Background lawn

The condition of the bacterial background lawn was evaluated for evidence of the test item cytotoxicity using the code system which is as follows.

Table 18: Cytotoxicity using the code system

Code	Definition	Characteristics
0	No lawn (Absent)	Distinguished by a complete lack of any background lawn compared to solvent control plates
1+	Very thin lawn (Extremely reduced)	Distinguished by an extreme thinning of the background lawn compared to solvent control plates
2+	Thin lawn (Moderately reduced)	Distinguished by a marked thinning of the background lawn compared to solvent control plates
3+	Slightly thin lawn (Slightly reduced)	Distinguished by a noticeable thinning of the background lawn compared to solvent control plates
4+	Thick lawn (Normal)	Distinguished by a healthy background lawn compared to solvent control plates

2.10.15.2 Counting of Revertant colonies

The revertants colonies of each tester strain within limit concentration were counted manually.

2.10.15.3 Viable Counts

Viable counts of various bacterial strains on nutrient plates were counted manually.

2.10.15.4 Quality Control Plates

Control plates containing S9 mix, PBS, 100% polar/non-polar test item extracts, DMSO, solvent controls (normal saline + DMSO), soft agar, and MGA plates were evaluated to check for any signs of contamination.

2.10.16 Evaluation of Results

The requirements for a positive result are, there should be a dose related increase in the mean revertants per plate of at least that one tester strain over a minimum of two increasing doses of the test item, either in the presence or absence of the metabolic activation system.

The test will be considered positive if the increase in mean revertants at the limit dose tested is equal to or greater than

2 times the mean vehicle control value in *Salmonella typhimurium* strains TA98, TA100, and *Escherichia coli* WP2 uvrA (pKM101) or equal to or greater than 3 times the mean vehicle control value in tester strains TA1535 and TA1537.

Equivocal response is defined as the biologically significant increase in the revertant numbers which are partially meeting the requirements for evaluation as positive. It could be the dose responsive increase which did not attain the respective thresholds as mentioned above or the non-dose responsive increase which was equal to or greater than the respective thresholds as mentioned above. The response was considered as negative if it was neither positive nor equivocal.

Based on the above definition, the test item was considered as negative.

2.10.17 Acceptance criteria

The Bacterial reverse mutation test is acceptable as it fulfilled the following criteria:

The positive control substances were found to have produced a significant increase in mutant colony frequencies.

The spontaneous reversion rates in the solvent control are within the range of in-house historical control data and preferably within the range reported in the literature and as per new batch of certificate of analysis received by the vendor.

The tester strains must have met all required genetic characterization.

The Tester Strains used were in the approximate range of 1×10^9 to 2×10^9 cells/mL.

Cytotoxicity is defined as greater than 50% reduction in mean revertants per concentration relative to the mean solvent/vehicle control value, and/or a thinning of the bacterial background lawn.

2.11 Implantation Study

The test item Mesire™ BRS was used to evaluate degradation and local effect likely to occur after muscle implantation in New Zealand White Rabbits.

Before implantation (day 1), fur on the dorso-lateral sides of the trunk region of animals was removed by clipping closely using an electric hair clipper. Abrasion to the skin was avoided while removing fur. The clipped area was swabbed using 5% (w/v) povidone-iodine solution and allowed to dry before implantation.

The test item and reference item were cut into pieces of 15 mm length (01 strip) and was implanted. The test item and reference item were handled in such a way that it was not contaminated in any way before and during implantation.

All surgical equipment and HDPE film used for implantation were autoclaved for about 15 minutes at 121 °C and 15 psi to maintain sterility. Before implantation, animals were anaesthetized using Xylazine and Ketamine at the dose of 5 mg/kg and 35 mg/kg respectively by intramuscular injection.

The test item and the reference item were implanted in 09 animals in a total of 36 test item and 36 reference item sites respectively. The test item and the reference item were implanted in the par vertebral muscle of the animal. A non-reactive location marker HDPE film was implanted along

with the test item and the reference item in the animal.

Four strips of test item in the form of pieces of 15mm length were implanted in the animal on the right side of the spine of the animal, approximately 25 to 50mm from the midline and parallel to the spinal column, and approximately 25mm apart from each implant. The reference item was implanted in the opposite muscle of the animal. The incision was made on both sides of the back of the animal through the skin parallel to the lumbar region of the vertebral column. The test item and the reference item were placed in the incision made parallel to the Para vertebral muscle of the animal. The glass bead sterilizer was used for the sterilization of surgical equipment. The muscle and the skin incisions were closed using absorbable and non-absorbable suture materials respectively.

All the animals were observed once a day for clinical signs of toxicity and twice a day for mortality during the entire experimental period. The body weight of the animals was recorded at the time of receipt, on the day of implantation, i.e., Day 1, and after the completion of each observation period.

All the animals were sacrificed after the completion of each observation period, i.e., 2 weeks, 8 weeks, and 13 weeks, by intravenous injection of sodium thiopentone and subjected to macroscopic and microscopic examinations. The microscopic examination of the tissue after implantation was evaluated semi-quantitatively according to ISO 10993, Part 6:2016 (E).

No treatment-related clinical signs of toxicity and mortality were observed in all the animals during the entire experimental period. However, clinical signs of toxicity, i.e., wound and scab, were observed in all the animals. These clinical signs were considered to be due to the surgical procedure.

No macroscopic changes were observed in the implantation sites of all the animals.

2.11.1 Histological Scoring at the end of 2 weeks

Microscopically, the implantation sites of both reference item and test item group showed the presence of remnants of implanted material.

Cell response in terms of lymphocytic infiltration was graded to be score 2 (5 to 10 per high powered field) at both reference item implantation sites and test item implantation sites.

Cell response in terms of macrophage infiltration was graded to be score 1 (1 to 5 per high powered field) to score 2 at both reference item implantation sites and test item implantation sites.

Tissue response in terms of fibrosis was graded to be score 1 (narrow band) at both reference item implantation sites and test item implantation sites.

The response at each implantation site was determined and average score for test item and reference item site was calculated. Average score for reference item site was subtracted from average score of test item site to arrive at histological evaluation score of the test item. The histological evaluation score was calculated to be 0.40 at the end of two-week study period.

2.11.2 Histological Scoring at the end of 8 weeks

There was no evidence of test item material at test item

implantation sites at the end of 8 weeks interim sacrifice. However, minimal to mild inflammatory reactions were observed, and this was considered to be due to the implantation of location marker.

There was no evidence of reference item at reference item implantation sites at the end of 8 weeks interim sacrifice. However, minimal to mild inflammatory reactions were observed, and this was considered to be due to the implantation of location marker.

The response at each implantation site was evaluated, and the average score for test item and reference item sites was calculated.

The average score for reference item site was subtracted from the average score of test item site to arrive at histological evaluation score of test item. The histological evaluation score was calculated to be 0.00 at the end of 8 weeks.

2.11.3 Histological Scoring at the end of 13 weeks

No evidence of test item material was found at test item implanted sites at the end of thirteen weeks. However, minimal to mild inflammatory reactions were observed, and this was due to the implantation of location marker.

No evidence of material was found at reference item implanted sites at the end of thirteen weeks. However, minimal to mild inflammatory reactions were observed, and this was due to the implantation of location marker.

The response at each implantation site was evaluated, and the average score was calculated for test item and reference item sites. The average score for reference item sites was subtracted from the average score for test item sites to calculate the histological evaluation score of test item. The histological evaluation score was calculated to be 0.00 at the end of thirteen weeks.

2.11.4 Acclimatization

Healthy young adult animals were subjected to acclimatization for a period of eight days. The animals were observed once a day for clinical signs. Veterinary examination of all the animals was carried out on the day of receipt.

2.11.5 Study Design

The study was performed in 09 rabbits as per the following study design.

Table 19: Study Design

Duration of Implantation	Number of Animals	Animal Number	Total No. of Implants		Site of Implantation
			Test Item (Right)*	Reference Item (Left)*	
Early time frame (2 Weeks)	03	Nd2014 to Nd2016	12	12	Para vertebral Muscle
Mid time frame (8 Weeks)	03	Nd2017 to Nd2019	12	12	
Late time frame (13 Weeks)	03	Nd2020 to Nd2022	12	12	

*: 4+4 Implants/Animal

2.11.6 Route of Implantation and Justification for the Choice

Test material and the reference substance have been implanted in the Para vertebral muscle in accordance with the International Standard ISO 10993 "Biological Evaluation of Medical Devices" Part 6 Tests for local effects after implantation ISO 10993-6:2016(E) Third Edition: 2016-12-01^[35].

2.11.7 Preparation of Animals

The dorso-lateral aspects of the trunk region of the animals were removed by clipping closely with an electric hair clipper before implantation (day 1). Care was taken to see that no abrasion to the skin was done during this procedure. The area was then swabbed with 5% povidone-iodine solution and allowed to dry.

2.11.8 Preparation of Test Item and Reference Item

The test item and reference item were cut into pieces of 15 mm length (01 strip) and were implanted. The test item and reference item were handled in such a way that they were not contaminated in any way prior to and during implantation.

Test item and reference item were cut into pieces of 15 mm in length. The pieces were implanted. The test item and reference item were aseptically handled in such a manner that they were not contaminated in any way prior to or during implantation.

All surgical equipment and HDPE materials used for implantation were sterilized by autoclave for about 15

minutes at 121 °C and 15 psi to maintain sterility. Rabbits were anaesthetized prior to implantation using Xylazine and Ketamine in a dose of 5 mg/kg and 35 mg/kg respectively by intramuscular route.

The test item and the reference item were implanted into the 09 animals, making a total of 36 test item sites and 36 reference item sites. The test item and the reference item were implanted into the Para vertebral muscle. A non-reactive location marker, HDPE film, was implanted together with the test item and the reference item.

Four pieces of test item, each of 15 mm in length, were implanted on the right side of the spine of all the animals, approximately 25 to 50 mm from the midline and parallel to the spinal column, and approximately 25 mm apart from each implant. The reference item was implanted on the opposite muscle of all the animals. Incision was made on either side of the back of all the animals through the skin parallel to the lumbar region of the vertebral column. The test item and reference item were implanted to the incision made parallel to the Para vertebral muscle of all the animals. Glass bead sterilizer was used for surgical equipment sterilization. Absorbable and non-absorbable sutures were used for closing muscle and skin incisions respectively.

2.11.9 Justification for the selection of the sites for implantation: Muscle implantation sites were selected as per the guidelines given in the International Standard ISO 10993 'Biological Evaluation of Medical Devices' Part 6:2016(E) According to ISO 10993 Part 6 Annex B Tests for muscle tissues effect after implantation.

2.11.10 Pre and Post-operative Care

Meloxicam (0.3 mg/kg – subcutaneous injection) was given on day 01, post-operative day 02 to day 04. Antiseptic [5% (w/v) povidone-iodine solution] was applied to the implant site from day 02 to day 24.

2.11.11 Observations

The following observations were made during the experiment.

2.11.12 Clinical Signs of Toxicity and Mortality

The rabbits were observed once daily for clinical signs of toxicity and twice daily for mortality throughout the experimental period.

2.11.13 Body Weight

The body weight was recorded at receipt, prior to test item implantation (Day 1) and at termination of observation periods (Each Necropsy time points).

2.11.14 Anatomic Pathology

2.11.14.1 Necropsy

At the end of observation period (2 weeks, 8 weeks and 13 weeks), all the animals were sacrificed by intravenous administration of sodium thiopentone and subjected to necropsy and complete gross pathological examination.

2.11.14.2 Macroscopic Assessment of Implanted Sites

No other gross changes noted at implanted sites of both test item and reference item and in all other tissues.

2.11.15 Implant Retrieval and Tissue Collection

At the end of each observation period (2, 8 weeks and 13 weeks), a total of 36 implantation sites along with implants from reference item and total of 36 implantation sites along with implants from test item sites were retrieved from the left and right Para vertebral muscles of the animals, respectively. The retrieved implantation sites from all the animals were preserved in 10% Neutral Buffered Formalin (NBF).

2.11.16 Microscopic Assessment

The implanted sites were preserved in 10% Neutral Buffer Formalin embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. The scoring was evaluated semi-quantitatively as per the ISO 10993-6:2016(E) [36].

The biological response parameters, which were assessed and recorded as appropriate,

- The extent of fibrosis/fibrous capsule (layer in micrometers) semi quantitatively and inflammation;
- The degeneration as determined by changes in tissue morphology;
- The number and distribution as a function of distance from the material/tissue interface of the inflammatory cell type's namely polymorph nuclear neutrophilic leucocytes, lymphocytes, plasma cells, eosinophils, macrophages and multinucleated cells;
- The presence, extent and type of necrosis;
- Other tissue alterations such as vascularization, fatty infiltration, granuloma formation and bone formation;
- The material parameters such as fragmentation and/or debris presence form and location of remnants of

degraded material;

- The quality and quantity of tissue in growth, for degradable implant materials.

2.11.17 Grading of implantation sites

The evaluation was semi-quantitative as per ISO 10993-6:2016(E) depending upon the type of reaction shown by the implant.

Depending on the scoring of implantation sites, the test sample was graded as follows when compared to the reference item samples:

- Minimal or no-reaction (0.0 up to 2.9)
- Slight reaction (3.0 up to 8.9)
- Moderate reaction (9.0 up to 15.0)
- Severe reaction (>15)

3. Results and Discussion

3.1 Cytotoxicity

When the L-929 mouse fibroblast cells were exposed to the test item extract, discrete intracytoplasmic granules were observed without any evidence of cell lysis, reduction in cell growth, or an overall reactivity score given a Grade 0. When the L-929 mouse fibroblast cells were exposed to the test item extract, discrete intracytoplasmic granules were observed without any evidence of cell lysis, reduction in cell growth, and overall reactivity score given a Grade 0. According to the grading criteria for cytotoxicity test results, reactivity less than 2 is indicative of a non-cytotoxic effect. Thus, the test item extract can be considered a non-cytotoxic substance.

Similar results were also obtained in the blank and negative control wells, wherein discrete intracytoplasmic granules were observed without any evidence of cell lysis, reduction in cell growth, and overall reactivity (Grade 0). However, the cells exposed to the positive control substance showed severe disruption in cell morphology with almost complete destruction of the cell monolayer, which is consistent with a Grade 4 reactivity score.

The results were consistent with the previous studies using L-929 fibroblasts as a standard cell line for the *in vitro* cytotoxicity test. This indicates the suitability of the cell line for the evaluation of the biocompatibility of the material using the ISO 10993 protocol. The significant difference between the negative and positive controls also indicates the accuracy of the experimental design and results.

Table 20: Individual Results of Cytotoxicity Test

Treatment	Microscopic Observations		
	Replicate	Grade	Reactivity
Blank	1	0	None
	2	0	
	3	0	
Test Item	1	0	None
	2	0	
	3	0	
Negative Control	1	0	None
	2	0	
	3	0	
Positive Control	1	0	None
	2	0	
	3	0	

3.2 Skin Sensitization

3.2.1 Clinical Signs of Toxicity and Mortality

No clinical signs of toxicity and mortality were observed in any of the animals in all the groups. However, in animal No. Gg4140, (G1) nasal discharge from day 08 to 12 was observed. In animal No Gg4146 (G2) soft stool from day 07 to 10 was observed. In animal No Gg4155, (G3) Lacrimation from day 14 to 18 was observed. In animal No Gg4159, (G4) nasal discharge from day 07 to 10 was observed. These isolated incidences of clinical signs observed were considered as incidental and not related to any treatment effects.

3.2.2 Skin Reactions Scoring

3.2.2.1 Intradermal Induction (Day 1)

No treatment-related skin reactions were observed at injection site 2 after intradermal injection at 24 ± 2 and 48 ± 2 hours of observation in group animals of G1, G2, G3, and G4. However, skin reactions like Moderate and confluent erythema to Discrete or patchy erythema were observed at injection site 1 after intradermal injection at 24 ± 2 and 48 ± 2 hours of observation.

At 24 ± 2 and 48 ± 2 hours, skin reactions like Discrete or patchy erythema were observed after intradermal injection at injection site 3 in all group animals of G1, G2, G3, and G4. The change observed, i.e., Erythema, at site 1 and 3 is expected due to intradermal Injection of Freund's Complete Adjuvant (FCA). Hence, this change observed is Considered nonspecific to test item related response or sensitization.

3.2.2.2 Topical Induction (Day 8)

No treatment-related skin reactions were observed at approximately 1 hour and 24 hours after removal of the test patch in any of the animals of all the groups tested.

3.2.2.3 Challenge (Day 23)

No treatment-related skin reactions were observed at (24 ± 2) and (48 ± 2) hours after removal of the test patch in any of the animals of all the groups tested.

3.2.2.3.1 Body Weight

No treatment-related changes were observed in the body weights and percent change in body weights with respect to Day 1. Normal physiological increases in the body weights of the animals were observed.

3.3 Skin Irritation

3.3.1 Clinical Signs of Toxicity and Mortality

During the experiment, no signs of treatment-related toxicity and death were observed in the animals. The

physiological state of all subjects was normal during the entire observation period, which proves that the administered substance did not cause systemic toxicity.

3.3.2 Intracutaneous Reactions

The analysis of the reaction in the intracutaneous zone showed no signs of erythema or edema formation in any injection area. Thus, the extract of the test substance did not cause irritation of the local skin area.

3.3.2.1 Body Weight

The assessment of body weight and changes in its percentage value relative to the initial value on day one showed no treatment-related changes. The body weight of all animals increased normally during the entire treatment period, indicating the lack of adverse health.

3.3.2.2 Evaluation of Results

Quantitative estimation of the reactions in the intracutaneous zone resulted in zero average scores for both the test substance extract and the solvent used. Thus, the total score of the reaction of the test substance amounted to zero, calculated by the difference between the average values.

Table 21: Clinical Signs of Toxicity and Mortality Record

Animal No.	Dose (mL/site)	Sex	Clinical Signs of Toxicity on Days			
			1	2	3	4
Nd0452	0.2	Male	N	N	N	N
Nd0453	0.2	Male	N	N	N	N
Nd0454	0.2	Male	N	N	N	N

N: Normal

Table 22: Skin Reaction Scoring Record

Animal No.	Dose (mL/Site)	Sex	Observation Time	Erythema	Oedema	Total Score
Nd0452	0.2	Male	24 hr	0	0	0
			48 hr	0	0	0
			72 hr	0	0	0
Nd0453	0.2	Male	24 hr	0	0	0
			48 hr	0	0	0
			72 hr	0	0	0
Nd0454	0.2	Male	24 hr	0	0	0
			48 hr	0	0	0
			72 hr	0	0	0

Mean Score (Blank control) = Total score of 3 animal/3

Final test sample score = Mean score of the test item sample –

Mean score of the blank control = $0/3 = 0 = 0-0$

Mean Score (Test item sample) = Total score 3 animal /3 = $0 = 0/3 = 0$

0 = No erythema / No oedema

Table 23: Body Weight (kg) and Percent Change in Body Weight with Respect to Day 1

Animal No.	Dose (mL/site)	Sex	Body Weight (kg) on Day		Percent Change in Body Weight with Respect to Day 1
			1	4	
Nd0452	0.2	Male	2.16495	2.27910	5.27264
Nd0453	0.2	Male	2.64510	2.78013	5.10491
Nd0454	0.2	Male	2.24869	2.39408	6.46554
		Mean	2.35291	2.48444	5.61436
		±SD	0.25648	0.26245	0.74190
		n	3	3	3

3.4 Acute Systemic Toxicity

3.4.1 Clinical Signs of Toxicity and Mortality and Body weight: No treatment related clinical signs of toxicity and mortality were observed in any of the animals. There were no treatment related changes observed in body weight and percent change in body weight with respect to day 1. All the

animals revealed physiologically normal Increase in the body weight during the observation period.

3.4.2 Pathology

No gross pathological changes were observed in any of the animals.

Table 24: Clinical Signs of Toxicity and Mortality Record

Group	Description	Animal Numbers	Day 1 (30–40 min)	1 hrs	2 hrs	4 hrs	Day 2	Day 3	Day 4
G1	Polar Solvent Control (Males)	Me5421–Me5425	N	N	N	N	N	N	N
G2	Polar Test Item Extract (Males)	Me5426–Me5430	N	N	N	N	N	N	N
G3	Non-Polar Solvent Control (Males)	Me5431–Me5435	N	N	N	N	N	N	N
G4	Non-Polar Test Item Extract (Males)	Me5436–Me5440	N	N	N	N	N	N	N

N: Normal; mins: minutes, hr/hrs-hour/hours dose volume administered (ml), body weight (g) and percent c in with respect to day

Table 25: Gross Pathology Findings

Group, Sex & Group Description	Animal No.	Fate	Gross Pathology Findings
G1, Males & Polar Solvent Control Extract	Me5421-Me5425	TS	NAD
G2, Males & Polar Test Item Extract	Me5426-Me5430	TS	NAD
G3, Males & Non-Polar Solvent Control Extract	Me5431-Me5435	TS	NAD
G4, Males & Non-Polar Test Item Extract	Me5436-Me5440	TS	NAD

TS: Terminal Sacrifice; NAD: No Abnormality Detected

3.5 Pyrogen Test

3.5.1 Clinical Signs of Toxicity and Morbidity/Mortality

No treatment related clinical signs of toxicity and mortality were observed in the animals.

3.5.2 Determination of Rectal Temperature

3.5.2.1 Preliminary Test

The Maximum control temperature variation observed in the selected animals was 0.02 °C and the temperature of the rabbits did not exceed 39.65 °C.

3.5.2.2 Main Test – I

The test substance was administered to three male subjects

at 17.5 – 17.9 mL and their initial body temperatures ranged from 39.01 °C to 39.72 °C. After administering the extract, the subjects had slight changes in body temperature, which included increases of 0.25 °C, 0.21 °C.

3.5.2.3 Interpretation of Results

No rabbit showed an increase in individual temperature of 0.5 °C or more above the respective control temperature in main test I.

Hence the test item meets the requirements of the Pyrogen test and is considered to be non-pyrogenic.

Table 26: Temperature Record for Preliminary Test

Animal No.	Sex	Temperature (°C)					
		Control Temperature	1 Hour	1½ hrs	2 hrs	2½ hrs	3 hrs
*Nc9806	M	39.36	39.44	39.47	39.55	39.60	39.56
Nc9807	M	39.75	39.85	39.87	39.95	40.01	39.97
Nc9808	M	40.62	40.48	40.62	40.48	40.51	40.33
*Nc9809	M	39.35	39.45	39.47	39.57	39.62	39.57
*Nc9810	M	39.37	39.48	39.49	39.60	39.65	39.60
Nc9811	M	39.52	39.54	39.58	39.66	39.70	39.72

*: Represents the animals selected for the main study; hrs: hours, M: Males

3.6 Sub Chronic Test

3.6.1 Clinical Signs of Toxicity, Detailed Clinical Examination and Mortality:

All the animals which were treated with polar, non-polar solvent control extracts and polar, non-polar test item extracts observed to be normal throughout the experimental period. No mortality and no treatment-related clinical signs were observed throughout the treatment period however clinical signs as such soft stool in 21 animals, slight lethargy in 2 animals, slight dehydration in 1 animal, piloerection in 1 animal and nasal discharge in 1 animal were observed. These isolated clinical signs in G1 and G2 group animal of both the sex were considered as incidental and not related to treatment.

Detailed clinical Examination was carried out on day 7, day 14, and day 21 and on day 28. Soft stool was observed on day 7 in 2 animals, on day 14 in 12 animals, on day 21 in 2 animals and on day 28 in 4 animals. These clinical signs were considered to be related to intraperitoneal administration of sesame oil which was used as non-polar solvent for extraction.

3.6.2 Body Weight and Percent Change in Body Weight with Respect to Day 1:

No treatment related changes in body weight and percent change in body weight were observed in both the sex when compared to polar and non-polar solvent control extracts. However, a statistically significant ($p < 0.05$) decrease in body weight on day 14, day

21 and day 28 in G2 male and G2 female animals and a statistically significant ($p < 0.05$) decreases in change in body weight on day 1-14, day 1-21 and day 1-28 in G2 male and

G2 female animals respectively were noted. This could be due to as there was a concomitant variation in feed consumption during the specific period.

Table 27: Summary of Percent Change in Body Weight with Respect to Day 1 record

Group, Sex & Group Description		Percent Change in Body Weight (%) during Days			
		1 to 7	1 to 14	1 to 21	1 to 28
G1, M & Polar and Non-polar solvent control extract	Mean	18.46	39.88	56.83	69.06
	±SD	2.73	6.39	7.15	10.04
	n	6	6	6	6
G2, M & Polar and Non-polar test item extract	Mean	15.64	31.21*	37.14*	51.06*
	±SD	2.51	4.92	15.06	15.75
	n	6	6	6	6
G1, F & Polar and Non-polar solvent control extract	Mean	11.71	20.89	29.44	42.29
	±SD	4.71	5.92	6.40	7.38
	n	6	6	6	6
G2, F & Polar and Non-polar test item extract	Mean	8.26	12.72*	18.21*	28.54*
	±SD	3.51	2.82	5.35	5.93
	n	6	6	6	6

3.6.3 Feed Consumption

No treatment related changes in feed consumption (g/rat/day) in polar and non-polar test item extracts were observed when compared with respective polar and non-polar solvent control extracts groups in both the sex. However, a statistically significant ($p < 0.05$) decrease in feed consumption during week 3 was observed in G2 male animals and a statistically significant ($p < 0.05$) decrease in feed consumption during week 4 was observed in G2 female animals. This could be due to as there was a concomitant variation in body weight and change in body weight during the specific period.

3.6.4 Ophthalmoscopic Examination: No treatment related ocular changes were observed in any of the animals.

3.6.5 Clinical Pathology

3.6.5.1 Haematology

The hematological parameters in both the male and female groups that received treatment were fairly equal to the control group values. There is a significantly lower ($p < 0.05$) total Leucocyte count in males after treatment compared to their baseline level; for women, there is a significantly higher mean corpuscular hemoglobin concentration after receiving treatment compared to their own baseline values.

3.6.5.2 Clinical Chemistry

The clinical chemical parameters of both the treated male and female groups were similar to those of the control group. In males, only the level of calcium was significantly reduced ($p < 0.05$). However, other parameters such as glucose, Creatinine, cholesterol, triglycerides, protein, liver

enzyme levels, and alkaline phosphatase levels did not differ among the three groups. This finding was regarded as incidental, as no pathological changes were seen in the kidney.

3.6.5.3 Urinalysis: There were no treatment related changes in urinalysis observed in any of the groups.

3.6.6 Pathology

3.6.6.1 Gross Pathology

There were no test item-related gross observations in this study. Gross changes such as white deposits on pancreas were observed in all animals across the groups. However, single incidence of white deposits on Epididymides & liver were observed in two male rats of G1 group. These changes were considered to be related to administration of sesame oil which was used as a non-polar solvent extract.

3.6.6.2 Fasting Body weights, Organ weights and its Ratios:

No treatment related changes in organ weight (both absolute and relative) were observed in any of the groups. However, a statistically significant ($p < 0.05$) decrease in relative weight of thymus in G2 female animals and increase in relative weight of brain in G2 female animals and a statistically significant ($p < 0.05$) decrease in absolute organ weight of thymus, adrenals and heart in G2 female animals were observed. And a statistically significant ($p < 0.05$) decrease in fasting body weight was observed in males and females of G2 group animals. The noted change was considered to be incidental as there were no associated corresponding changes observed in histopathology of thymus, adrenals and heart.

Table 28: Summary of Absolute Organ Weights (G) Record

Group, Sex & Group Description		Adrenals	Brain	Epididymides	Heart	Kidneys	Liver	Spleen	Testes	Thymus
G1, M & Polar and Non-polar solvent control extract	Mean	0.0600	1.8801	0.9984	1.0300	1.9987	8.4570	0.8591	3.3113	0.3686
	±SD	0.0085	0.1024	0.1567	0.1000	0.1964	0.7147	0.3258	0.2571	0.1140
	n	6	6	6	6	6	6	6	6	6
G2, M & Polar and Non-polar test item extract	Mean	0.0532	1.8316	0.8612	0.9509	1.8422	7.8767	0.9595	2.9768	0.2701
	±SD	0.0065	0.0861	0.1590	0.0910	0.1802	0.6084	0.6871	0.3020	0.0700
	n	6	6	6	6	6	6	6	6	6

M: Male; SD: Standard Deviation; n= Number of animals F: Female; SD: Standard Deviation; n= Number of animals; *: Statistically significant ($p < 0.05$)

Table 29: Summary of Organ Weight Relative to Fasting Body Weight (%) Record

Group, Sex & Group Description		Fasting Body Weight (g)	Adrenals	Brain	Epididymides	Heart	Kidneys	Liver	Spleen	Testes	Thymus
G1, M & Polar and Non-polar solvent control extract	Mean	250.02	0.0240	0.7551	0.4012	0.4139	0.8004	3.3906	0.3462	1.3289	0.1462
	±SD	17.56	0.0034	0.0662	0.0671	0.0503	0.0698	0.3121	0.1374	0.1252	0.0411
	n	6	6	6	6	6	6	6	6	6	6
G2, M & Polar and Non-polar test item extract	Mean	221.22*	0.0243	0.8362	0.3916	0.4310	0.8367	3.5862	0.4579	1.3570	0.1256
	±SD	25.61	0.0039	0.0954	0.0788	0.0180	0.0749	0.3556	0.3639	0.1752	0.0436
	n	6	6	6	6	6	6	6	6	6	6

M: Male; SD: Standard Deviation; n= Number of animals; *: Statistically significant ($p < 0.05$)

3.6.6.3 Histopathology

There were no test item-related microscopic findings in both the sexes. Lipogranuloma observed on the serosal surface of the adrenals, Epididymides, liver, pancreas, spleen and ovaries did not affect the normal architecture of the organ/s and was observed in both non-polar solvent control and non-polar test item extract administered groups. This finding was considered to be related to intraperitoneal administration of sesame oil which was used as non-polar solvent for extraction. Presences of Intraperitoneal lipogranuloma do not have any significant clinical implications and were considered as non-adverse findings. Few other microscopic findings observed in this study like, mononuclear cell infiltrations in lungs, congestion in spleen and all other findings were considered as incidental as they occurred randomly across the dose groups including concurrent controls and/or were expected for laboratory rats.

3.7 Genotoxicity Test

3.7.1 Precipitation Test

The test item resulted in no precipitation observed in 100% polar test item extract and 100% non-polar test item extract.

3.7.2 Mutation Assay

3.7.2.1 Plate Incorporation Method

The concentration tested at 100% extract/plate showed very close resemblance to the solvent control [normal saline (polar solvent) and DMSO (non-polar solvent)] in both with and without metabolic activation. There was no appreciable increase in number of Revertant colonies and no change in bacterial background lawn as compared to that of the solvent control, among the tester strains. The specific positive controls tested simultaneously produced approximately 3.6 to 13.7 fold increase in mean number of revertants as compared to the solvent control.

3.7.2.2 Preincubation Method

The concentration tested at 100% extract/plate showed very close resemblance to the solvent control [normal saline

(polar solvent) and DMSO (non-polar solvent)] in both with and without metabolic activation. There was no appreciable increase in number of revertants colonies and no change in bacterial background lawn as compared to that of the solvent control, among the tester strains. The specific positive controls tested simultaneously produced approximately 3.6 to 14.5 fold increase in the mean number of revertants as compared to the solvent control.

3.7.2.3 Viable count

Each tester strain was diluted to 10^{-7} and plated on nutrient agar. Post incubation, plates were observed and the colonies were counted manually for Colony Forming Units (CFU). Each tester strains resulted in acceptable range of 1×10^9 CFU/mL to 2×10^9 CFU/mL

3.7.2.4 Quality Control

Plates Control plates with S9 mix, PBS, DMSO, solvent controls (normal saline and DMSO), soft agar, 100% test item extract (polar and non-polar) and MGA plate were checked for contamination. No microbial contamination was observed.

3.7.2.5 Interpretation

The test item, Mesire™ BRS, polar and non-polar extracts were assayed for Bacterial Reverse Mutation Test at the limit concentration of 100% extract solution/plate using Salmonella typhimurium strains; TA98, TA100, TA1535, TA1537 and Escherichia coli WP2 uvrA (pKM101). In the two trials (plate incorporation and pre incubation method) conducted, with metabolic activation (+S9) and without metabolic activation (-S9), the number of revertants colonies in the limit concentration (100% extract/plate) of both polar and non-polar extracts did not increase appreciably over the solvent control, while the positive controls tested simultaneously showed 3.6 to 14.5 fold increase in the number of revertants colonies/plate, when compared to their respective solvent control.

Table 30: Summary of Colony Counts of Revertants - Trial-I

Treatm ent	Test Concentration (%Extract/plate)		No. of Revertants (Mean of 3 Plates)									
			With S9					Without S9				
			Salmonella typhimurium				E. coli WP2 uvrA (pKM 101)	Salmonella typhimurium				E. coli WP2 uvrA (pKM101)
			TA 98	TA 100	TA 1535	TA 1537		TA 98	TA 100	TA 1535	TA 1537	
Solvent Control	100 µL of Normal Saline	Mean	31.7	111.7	21.3	11.0	77.3	27.3	100.7	19.7	9.3	73.0
		±SD	4.5	3.1	2.5	2.0	4.5	3.1	5.5	3.5	2.5	4.6
	Lawn Intensity		4+,4+, 4+	4+,4+, 4+	4+,4+, 4+	4+,4+, 4+	4+,4+, 4+	4+,4+, 4+	4+,4+, 4+	4+,4+, 4+	4+,4+, 4+	4+,4+, 4+
Mesire ^T ^M BRS	100 µL of	Mean	32.3	110.0	20.7	10.7	78.3	28.7	103.3	18.7	8.0	71.3
	100% Polar Extract	±SD	5.7	3.0	4.0	3.1	2.1	2.5	5.5	2.1	2.0	3.2
	Fold Increase		1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	0.9	1.0
	Lawn Intensity		4+,4+, 4+	4+,4+, 4+	4+,4+, 4+	4+,4+, 4+	4+,4+, 4+	4+,4+, 4+	4+,4+4+	4+,4+, 4+	4+,4+, 4+	4+,4+, 4+
Positive Control	100 µL of	Mean	378.7	399.7	146.3	122.3	377.7	375.0	391.3	144.0	116.7	373.0
	Respective Positive Control	±SD	12.0	7.5	11.7	9.1	12.5	12.5	6.5	11.5	6.5	12.1
	Fold Increase		12.0	3.6	6.9	11.1	4.9	13.7	3.9	7.3	12.5	5.1
	Lawn Intensity		4+4+, 4+	4+4+, 4+	4+4+, 4+	4+4+, 4+	4+4+, 4+	4+4+, 4+	4+4+, 4+	4+4+, 4+	4+4+, 4+	4+4+, 4+

Values of Revertants are in Mean±SD Positive controls:

With S9:

For Salmonella typhimurium TA98, TA100, TA1535 and TA1537 = 4 µg/plate of 2-Aminoanthracene for Escherichia coli WP2 uvrA (pKM101) strain +S9= 30 µg/plate of 2 - Amino anthracene

Without S9:

For TA98: 2 µg/plate of 2-Nitrofluorene for TA100 and TA1535: 1µg/plate of Sodium azide. For TA1537: 50 µg/plate of 9 Aminoacridine for Escherichia coli WP2 uvrA (pKM101): 5 µg/plate of 4-Nitroquinoline N-oxide.

Lawn intensity: 4+ = Thick lawn: Distinguished by a healthy (Normal) background lawn compared to solvent control plates.

Note: Positive control values are from the polar extraction treatment and are included in the non-polar extraction treatment for comparison with the treatment groups for fold increase.

Lawn intensity: 4+= Thick lawn: Distinguished by a healthy (Normal) background lawn compared to solvent control plates.

Table 31: Individual Viable Counts of Tester Strains

Name of the Strain	Required CFU/mL	No. of Colonies of 10-7 Dilutions in triplicates			Average No. of Colonies	Obtained CFU/mL (1 to 2 ×10 ⁹ Cells/mL)
		R1	R2	R3		
Salmonella typhimurium TA98	1-2×10 ⁹	163	177	184	175	1.75×10 ⁹
Salmonella typhimurium TA100	1-2×10 ⁹	147	168	155	157	1.57×10 ⁹
Salmonella typhimurium TA1535	1-2×10 ⁹	180	171	150	167	1.67×10 ⁹
Salmonella typhimurium TA1537	1-2×10 ⁹	154	167	182	168	1.68×10 ⁹
Escherichia coli WP2 uvrA (pKM101)	1-2×10 ⁹	174	181	153	169	1.69×10 ⁹

R: Replicate

3.8 Implantation Study

3.8.1 Clinical Signs of Toxicity and Mortality

No treatment related clinical signs of toxicity and mortality were observed in any of the animals during the experimental period. However, clinical sign such as wound and scab was observed in all animals. These isolated clinical signs noticed in all animals were considered due to surgical procedure.

In G1 group animals severe to moderate and slight wound due to surgery was observed from day 2 to day 15 observation.

In G2 group animals severe to slight wound due to surgery was observed from day 2 to day 23 and Scab was observed from day 23 to 28.

In G3 group animals severe to slight wound due to surgery was observed from day 2 to day 22 and scab was observed from day 22 day 27.

3.8.2 Body Weight

No changes were noted in body weights and percent change in body weight with respect to Day 1 in all the groups.

3.8.3 Pathology

3.8.3.1 Macroscopic Observation

At the end of observation period (2 weeks), the implantation sites (par vertebral muscle) of test item and reference item revealed presence of implanted material. However, at both 8-weeks interim sacrifice and 13-weeks terminal sacrifice, no evidence of the implanted material was observed at the implantation sites (par vertebral muscle).

There were no other gross changes noted at implanted sites of both test item and reference item and in all other tissues.

3.8.3.2 Microscopic Observation

3.8.3.2.1 Histological Scoring at the end of 2 weeks

Microscopically, the implantation sites of both reference and test item group revealed presence of remnants of implanted material.

Microscopically, cell response in terms of lymphocytic infiltration was graded to be score 2 (5 to 10 per high powered field) at both reference item implantation sites and test item implantation sites.

The cell response in terms of macrophage infiltration was graded to be score 1 (1 to 5 per high powered field) to score 2 at both reference item implantation sites and test item implantation sites.

The tissue response in terms of fibrosis were graded to be score 1 (narrow band) at both reference item and test item implantation sites.

The response at each implantation site was determined and average score for test item and reference item site was calculated. Average score for reference item site was subtracted from average score of test item site to arrive at histological evaluation score of the test item. The histological evaluation score was calculated to be 0.40 at the end of two-week study period.

3.8.3.2.2 Histological Scoring at the end of 8 weeks

At the end of eight-week interim sacrifice, there was no evidence of test item material at test item implantation sites. However, minimal to mild inflammatory reactions observed and this reaction was attributed to implantation of location marker.

At the end of eight -weeks interim sacrifice, there was no evidence of reference item at reference item implantation sites. However, minimal to mild inflammatory reactions observed and this reaction was attributed to implantation of location marker.

The response at each implantation site was determined and average score for test item and reference item site was calculated.

Average score for reference item site was subtracted from average score of test item site to arrive at histological evaluation score of the test item. The histological evaluation score was calculated to be 0.00 at the end of eight-week study period.

3.8.3.2.3 Histological Scoring at the end of 13 weeks

At the end of thirteen-week terminal sacrifice, there was no evidence of test item material at test item implanted sites. However, minimal to mild inflammatory reaction observed and this reaction was attributed to implantation of location marker.

At the end of thirteen-week terminal, there was no evidence of material at reference item implanted sites. However, minimal to mild inflammatory reaction observed and this reaction was attributed to implantation of location marker.

The response at each implantation site was determined and average score for test item and reference item site was calculated. Average score for reference item site was subtracted from average score of test item site to arrive at histological evaluation score of the test item. The histological evaluation score was calculated to be 0.00 at the end of thirteen-week study period.

4. Conclusion

Safety and biocompatibility of Mesire™ BRS were confirmed in accordance with the international standard during various laboratory investigations. It was found out to be non-cytotoxic for cells, non-sensitizing for guinea pigs and non-irritant for the rabbit skin. These findings indicate that there is little possibility of cellular injury, allergic or irritant reactions caused by the material under study. Moreover, there were no evidences of system toxicity when

the substance was introduced to animals either orally, Intraperitoneal or intravenously. In addition, there was no fever response in test animals, which proved the non-pyrogenic action of the substance. Finally, it was found out that Mesire™ BRS is non-mutagenic for bacteria. During implantation experiments on rabbits, tissue reaction caused by the introduction of the material was negligible and absorption of the product occurred without any negative effects.

5. COI (Conflict of Interest)

All the authors are an employee of Meril Medical Innovations Private Limited, Vapi, Gujarat – India and declare no competing interests related to this study.

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