WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 11, Issue 6, 645-676. Research Article ISSN 2277– 7105

HEN'S EGG TEST – CHORIOALLANTOIC MEMBRANE (HET-CAM) TEST METHOD FOR EXAMINATION OF DERMAL OR CUTANEOUS IRRITATION OF ME.CP AS A POLYMER

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Article Received on
31 March 2022,

Revised on 21 April 2022,
Accepted on 12 May 2022

DOI: 10.20959/wjpr20226-23981

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ABSTRACT

Hen's egg–chorioallantoic membranes were used to screen for, information access and, evaluate anti-irritant properties of aqueous extracts of plants (HET–CAM tests), plant derived polymer gels, gums and, other acidic and alcoholic extracts in connection with searches for plant-derived substances that have topical anti-irritant action. Applicable in the medicinal chemistry in finding the potential drug effects in testing its teratogenic effect and, irritation in gastric or dermal membranes. This evaluation mainly focus on CAM-assay screening of plant extracts which could provide a useful information for identifying promising anti-irritant nature of plant aqueous, alcoholic, ethanolic, acetone extracts, exudate, gum, resins or polysaccharides, for the follow-up on clinical testing. The method would require materials with strong anti-irritant properties, and, would also have to avoid registering false negative data. The involved tests that were conducted, provided positive indications. Examination and, evaluation of the delays in the onset of three manifestations of significant membrane irritation–that includes: 1. vascular haemorrhaging, 2. membrane lysis and, 3. membrane coagulation; observed with the test substances (t.s.), in relative to the positive controls. Aqueous 10% laboratory prepared irritant, a commonly generative of skin irritation in the animal or human was used as irritant in the direct test as a standard; employed as the test irritant in this study which was evaluated. The ratio [irritation onset times after test substance pre-treatment]:[onset times without test substance pre-treatment] was used to measure the

anti-irritant and if any irritation inducing capacity of the test substances before starting any preclinical study and, evaluation of any significant measures.

KEYWORDS: HET-CAM, chicken cell embryo testing, irritation testing, skin irritation evaluation, artificial embryogenesis.

1. INTRODUCTION

Despite the fact that the hen's egg test on chorioallantoic membrane (HET-CAM) is a popular alternative toxicological method for determining ocular irritation potential, few studies have shown that it is useful for assessing the irritating qualities of vaccination adjuvants.

Objective: To study, examine and, evaluate the potential capacity of the '**Hen's egg test – Chorioallantoic membrane (HET-CAM) test method for examination of dermal or cutaneous irritancy or irritation of ME.CP as a polymer.'**

1.1. Snippet

A. Snippet: The hen's egg test-chorioallantois membrane (HET-CAM) assay, an alternative to the Draize eye irritation test, was developed by Luepke and has been improved on by means of a microscopic examination and the use of a test substance applicator (TSA). The TSA is a double Teflon ring in which a Perlon mesh is locked, and has several advantages over conventional protocols, reducing subjectivity of the method and avoiding the need for rinsing after treatment. It was confirmed by statistical analysis that the HET-CAM-TSA method can reproduce potential *in-vivo* irritant effects on the conjunctiva. The classification based on the *in-vitro* results was compared with four *in-vivo* classifications [MAS (maximal average score) with thresholds of 15.0 and 25.0; the Kay and Calandra method; and EC criteria]. Cooper's parameters (specificity, sensitivity and concordance with the Draize test) were calculated according to these four *in-vivo* classifications. When the most rigorous classification (MAS threshold of 15.0) was taken into account, a sensitivity of 80%, a specificity of 81.3% and a concordance with the Draize test of 80.4% were obtained for this set of 46 compounds.[20]

B. Snippet: GSK2894512 is a novel anti-inflammatory agent that is currently under development for the topical treatment of atopic dermatitis and chronic plaque psoriasis. This study will be a Phase I, single-centre, randomised, partial-blinded (evaluator blinded) study which consists of two parts (Part 1 and 2). Part 1 of this study will assess skin irritation

following a single application of GSK2894512 cream at 2 concentrations (e.g. 0.5% and 1%) and placebo by simple-patch test and photo-patch test under semi-occlusive conditions in 20 healthy volunteers. Part 2 of this study will assess skin irritation following repeat application at 0.5% and 1% of GSK2894512 cream and placebo for 7 days under non-occlusive condition in 6 healthy volunteers. The study will have Screening visit which will occur within 30 days from the Day 1 visit of each part. Eligible subjects will be able to participate either of Part 1 or Part 2. Subjects will visit the site on Day -1, and hospitalised until the end of all assessments on Day 4 (Part 1) or Day 7 (Part 2). Subjects will re-visit the site on Day 8 (Part 1) or Day 15 (Part 2) for follow-up assessments. This study will be the first to evaluate the safety, tolerability and pharmacokinetics of GSK2894512 cream after single and repeat application in subjects.[15]

C. Snippet: Allergic contact dermatitis; (ACD) is a prevalent skin condition that has serious social and economic consequences. Skin sensitisation, in contrast to irritation, is an adaptive immune response in which a delayed T-cell-mediated allergic response to chemically changed skin proteins occurs.[16]

D. Snippet: Comparison between HET-CAM protocols and a product use clinical study for eye irritation evaluation of personal care products including cosmetics according to their surfactant composition.

The hen's egg test on chorioallantoic membrane; (HET-CAM) is one of the most frequently used alternative tests for prediction of ocular irritation of cosmetic products. There are different HET-CAM protocols widely accepted, but there is no information about which of the protocols better correlates with the results obtained in product use clinical study under the conditions of use. Two Fix Time Methods (FTM) -Lüepke and the ICCVAM guideline - and two Reaction Time Methods; (RTM) -ECVAM DBALM Prot. No. 47 and No. 96- were employed to test 18 cosmetic products. Simultaneously, they were evaluated by an ophthalmological clinical test. A unified classification system was used, and products were classified into four irritation levels: non-irritant, weak, moderate and severe irritant. The duration of use (rinse-off or leave-on), and the concentration and type of surfactants were taken into account in the analysis. All the products that were classified as non-irritant by any HET-CAM protocols were also safe in the product use clinical study. The product that was found to be non-safe in the product use clinical evaluation was also unsuitable by most of the

HET-CAM protocols. These results were employed to develop an algorithm that allows selecting the appropriate HET-CAM protocol for each type of product to be tested.[21]

E. Snippet: [Study on using the hen's egg test-chorioallantois membrane as an alternative method of draize eye irritation test].

Objective: To establish and study the hen's egg test-chorioallantois membrane (HET-CAM) as an alternative method of Draize eye irritation test (Draize test).

Methods: 14 cosmetic ingredients were tested by the Hen's egg test-chorioallantois membrane score (HET-CAM score) and the chorioallantois membrane-trypan blue staining (CAM-TB) methods.

Results: Showed that compared with two kinds of scores in Draize test, i.e. Maximum average Draize total score (MAS) and score of 24 h after application (S24), the correlation coefficient between HET-CAM score and MAS or S24 was 0.847 or 0.779, while that between CAB-TB and MAS or S24 was 0.862 or 0.831 respectively. The results also showed that CAM-TB had a higher correlation with Draize test than HET-CAM score, partly because it is objective and quantitative. Also, the results showed that HET-CAM score had the greatest correlation with conjunctivae score of the three components, and so did the CAM-TB with corneal score.

Conclusion: It is suggested that the two types of HET-CAM can be used in a combined manner as an effective alternative method to Draize test.[22]

F. Snippet: Testing vaginal irritation with the Hen’s Egg Test-Chorioallantoic Membrane assay.

The HET-CAM (Hen’s Egg Test-Chorioallantoic Membrane) assay is an *in-vitro* alternative to the *in-vivo* Draize rabbit eye test. This qualitative method assesses the irritancy potential of chemicals. The chorioallantoic membrane responds to injury with an inflammatory process similar to that in the rabbit eye’s conjunctival tissue. Regarding topical toxicity assessment of medical devices, ISO 10993-10 states that any skin or eye irritant material shall be directly labelled as a potential vaginal irritant without animal testing, suggesting that the irritation potentials for the eye and the vaginal epithelia are similar. The aim of this work was to apply the HET-CAM assay to test the irritancy potential of vaginal formulations. Vaginal semisolid

medicines and lubricants currently marketed were tested along with the Universal Placebo formulation that has been shown to be clinically safe. Nonoxynol-9 (N-9), a known vaginal irritant, was enrolled as positive control (concentrations ranging from 0.001 to 100% (v/v)). The assay was conducted according to the ICCVAM – Recommended Test Method (NIH Publication No. 10-7553 – 2010). Formulations were then classified according to irritation score (IS), using the analysis methods (A) and (B). The studied vaginal formulations showed low potential for irritation. N-9 was classified as a severe irritant at concentrations above 2%, which is in line with clinical data, envisaging a possible in vitro/in vivo correlation. IS (B) was considered a more detailed classification output. Although still requiring further validation, the HET-CAM assay seems an ideal prospect for in vitro vaginal irritancy testing.

Testing vaginal irritation with the Hen's Egg Test-Chorioallantoic Membrane assay.[23]

G. Snippet: Comparison of the different protocols of the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) by evaluating the eye irritation potential of surfactants.

The Hen's Egg Test - Chorioallantoic membrane (HET-CAM) is a valid alternative method used to assess the potential for eye irritation from chemicals. This method is the only one that mimics the conjunctivae of the eye and aims to semi-quantitatively evaluate the irritant potential of a chemical on the chorioallantoic membrane surrounding the chicken embryo in egg by observing the irritation effects on the membrane immediately after the pure or diluted chemical is applied. The purpose of this study was to compare the different protocols of the HET-CAM, the French and German protocols, by evaluating the eye irritation potential of surfactants. The comparison led to the optimisation of the French protocol, generating an adapted one, to reduce subjectivity in the test evaluation, ensuring more accurate results and greater quality control. The comparison showed that there are no significant differences between the results obtained in the French and German protocols. HET-CAM is known to overestimate the results and to be able to accurately identify non-irritant products and it is a great candidate to be part of a Bottom-up test strategy. It also can be used in a battery of tests to completely replace rabbits.[24]

2. Skin irritation

Topical exposure to chemicals can lead to adverse skin effects. According to the severity and reversibility of effects one distinguishes skin corrosion (=skin burns) from skin irritation.

Corrosive substances irreversibly damage the skin beyond repair, while irritant substances lead to a reversible local inflammatory reaction caused by the innate (non-specific) immune system of the affected tissue: in response to chemically-induced tissue trauma and cell damage, skin cells release inflammatory mediators (chimokines and, cytokines) which increase the diameter and permeability of blood vessels, attract immune cells (e.g. mast cells, neutrophiles) to the site of injury and trigger the migration of immune cells through the endothelium into the tissue where they participate in antigen clearance and tissue repair.[8]

Moreover inflammatory mediators stimulate nerve endings leading to itching and stinging sensations.

2.1 Dermal sensitisation

A skin sensitiser is a substance that causes an allergic reaction when it comes into contact with the skin. (For more information, see section A.4 of Appendix A to 29 CFR 1910.1200.) Skin sensitisation, in contrast to skin irritation, is an immune response to past exposure to a chemical that causes an inflammatory skin reaction.[18]

Skin sensitiser also can be termed as: it means a chemical that will lead to an allergic response following skin contact.

Dermal irritation is the production of reversible damage of the skin following the application of a test chemical for up to 4 hours.

Dermal corrosion is the production of irreversible damage of the skin; namely, visible necrosis through the epidermis and into the dermis, following the application of a test chemical for up to four hours. Corrosive reactions are typified by ulcers, bleeding, bloody scabs, and, by the end of observation at 14 days, by discolouration due to blanching of the skin, complete areas of alopecia, and scars. Histopathology should be considered to evaluate questionable lesions.

Allergic contact dermatitis is characterised by a rash that is irritating but often painful. Raised lumps and blisters may appear on the rash. The reaction may occur immediately or up to 48 hours after your skin has been exposed to the substance that is causing it to respond. Hives are itchy, raised, flat bumps that can be sensitive.[19]

2.2 Dermal reaction: skin allergies

The material causes a hot, red itchy rash where it landed. This is referred to as contact dermatitis by your doctor. There are two kinds of them: Chemicals such as strong cleansers can induce irritant contact dermatitis. Allergic contact dermatitis is exactly what it sounds like: it's when your body reacts to an allergen.

2.3 Soap sensitivities

It's possible that the soap might dry out skin as it cleans. A harsh soap may not always leave a rash that can be seen, but it can leave a lasting itch after shower is over. Failing to wash all the soap residue off the skin after a shower can also be a source of itching and, discomfort. In such circumstances soap testing for safety use on skin is compulsory.

2.4 Other causes of skin irritation excluding a chemical irritant: Xerosis cutis

"Xerosis cutis" simply means that your skin is too dry. Soaking your skin in hot water for extended periods of time can strip your skin of its natural oils, irritating skin that already lacks moisture. Sometimes that results in itching after a shower. The itching may mostly happen on your feet or legs because those parts of your body have so much contact with the water.

Histopathological examination may be carried out to clarify equivocal responses.

The focus of this protocol is on the use of the HET-CAM test method for the detection of ocular corrosives and, severe irritants, as defined by the U.S. Environmental Protection Agency (EPA2003a), European Union (EU; EU 2001), and United Nations Globally Harmonised System (GHS) of classification and labelling of chemicals (UN 2007).[9,10,11,12,13]

HET-CAM test (hen's egg-chorioallantoic membrane test) is a test used to determine the irritation potential of substances and is an alternative test to the Draize rabbit eye test. In the test, incubated hen's eggs are opened carefully on day 9 and the chorioallantoic membrane (CAM) is exposed.

Test substances, pure or diluted solutions, are placed directly on the exposed CAM membrane (Figure 1) and, the membrane is inspected visually through a microscope. The time taken for the reaction to occur on the membrane is recorded during a 5 min period. The time period may vary on the capacity of the irritant potency of the isolate from the plant material or its any derived substances. The general reaction outcomes are haemorrhage as

seen in the (Figure 2), coagulation and, lysis. Based on the reaction time, an irritation score, I.S., was recorded or noted for any of its toxicological action, including skin inflammation which is calculated and, the substances are classified on the basis of the value of the score. Test solutions evaluation and, scoring pattern includes number rating based on the observation which include: 0–0.9 are classified as non-irritative, scores 1–4.9 as slightly irritative, scores 5–8.9 as moderately irritative and, scores 9–21 as strongly irritative.

The general test method used to evaluate the different substances used in dentistry such as extracts from different dental restorative materials, dental adhesives and primers, substances in toothpaste and hydrofluoric acid (H.F.). The HET-CAM test showed that a H.F. concentration of 0.05% was slightly irritative, 0.10% moderately irritative and 0.20–1% strongly irritative. In dentistry, H.F. is mainly used as a component for etching of ceramics and, if spilled on soft tissue it can give severe injuries. In a recent study, the HET-CAM test showed that H.F. combined with potassium fluoride (KF) to potassium hydrogen difluoride (KF·H.F.) reduces the irritation potential considerably.

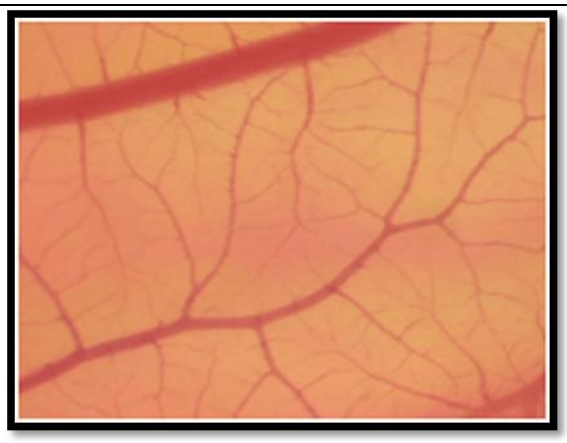


Figure 1: CAM membrane before exposure.

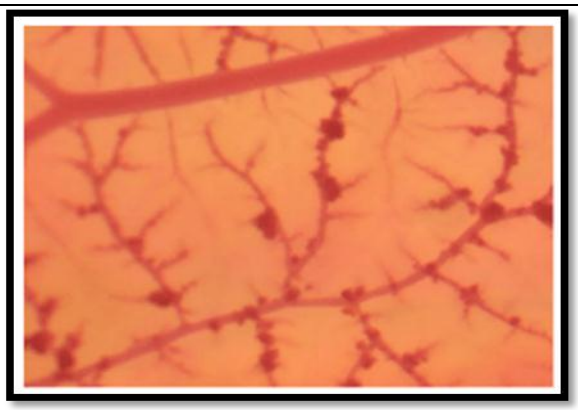


Figure 2: CAM membrane exposed to substance that gives haemorrhage.

The Hen's egg test-Chorioallantoic membrane (HET-CAM) method is used to test a wide range of substances and, mixtures. Testing of a broad variety of chemicals and formulations, using the Hen's egg test-Chorioallantoic membrane (HET-CAM) is one effective and, applicative method in clinical sciences and, clinical research, with adopted clinical research methods and, methodology that generally gives the idea of clinical evaluation based on the testing of any substances action with specific skin hyperactivity involving the skin rash. The Hen's egg test-Chorioallantoic membrane (HET-CAM) is one effective and, applicable method in clinical sciences and, clinical research, with adopted clinical research methods and,

methodology that generally gives the idea of clinical evaluation based on the testing of any substances action with the specific skin hyperactivity involving skin rash.

3. Precautions for the safety and, operation

All processes involving chicken eggs should adhere to the institution's rules and, regulations. Procedures for processing human or animal materials, such as: tissues and, tissue fluids, but not limited to them. The adoption of universal laboratory precautions, such as: the uses of laboratory gloves have been employed. Coats, eye protection and, gloves are all required. Additional precautions may be required for a specific investigation if they are available. Substances should be identified in the substance's material safety data sheet.

4. FDA. 2003. good laboratory practice for nonclinical laboratory studies. 21 CFR 58

(a) This part prescribes good laboratory practices for conducting nonclinical laboratory studies that support or are intended to support applications for research or marketing permits for products regulated by the, 'Food and Drug Administration', including food and colour additives, animal food additives, human and animal drugs, medical devices for human use, biological products, and electronic products. Compliance with this part is intended to assure the quality and integrity of the safety data filed pursuant to sections 406, 408, 409, 502, 503, 505, 506, 510, 512-516, 518-520, 721, and 801 of the Federal Food, Drug, and Cosmetic Act and sections 351 and 354-360F of the, 'Public Health Service Act'.

(b) References in this part to regulatory sections of the code of federal regulations are to chapter I of title 21, unless otherwise noted.

[43 FR 60013, Dec. 22, 1978, as amended at 52 FR 33779, Sept. 4, 1987; 64 FR 399, Jan. 5, 1999]

(c) Nonclinical laboratory study means in vivo or in vitro experiments in which test articles are studied prospectively in the test systems under laboratory conditions to determine their safety.

(d) The term does not include studies utilising human subjects or clinical studies or field trials in the animals. The term does not include basic exploratory studies carried out to determine whether a test article has any potential utility or to determine physical or chemical characteristics of a test article.

(e) Application for research or marketing permit includes

(1) A colour additive petition, described in part 71.

(2) A food additive petition, described in parts 171 and, 571.

(3) Data and, information regarding a substance submitted as part of the procedures for establishing that a substance is generally recognised as safe for use, which use results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food, described in §§ 170.35 and, 570.35.

(4) Data and, information regarding a food additive submitted as a part of the procedures regarding food additives permitted to be used on an interim basis pending additional study, described in § 180.1.

(5) An investigational new drug application, described in part 312 of this chapter.

(6) A new drug application, described in part 314.

(7) Data and, information regarding an over-the-counter drug for human use, submitted as part of the procedures for classifying such drugs as generally recognised as safe and, effective and, not misbranded as described in the part 330.

(8) Data and, information about a substance submitted as a part of the procedures for establishing a tolerance for unavoidable contaminants in food and, food-packaging materials, described in parts 109 and, 509.[13]

5. Sec. 58.49 Laboratory operation areas.

Separate laboratory space shall be provided, as needed, for the performance of the routine and, specialised procedures required by non-clinical laboratory studies.

5.a. [52 FR 33780, Sept. 4, 1987].

Sec. 58.83 Reagents and, solutions.

All reagents and, solutions in the laboratory areas shall be labeled to indicate identity, titer or concentration, storage requirements and, expiration date. Deteriorated or outdated reagents and, solutions shall not be used.

5.b. Sec. 58.130 Conduct of a nonclinical laboratory study

(a) The nonclinical laboratory study shall be conducted in accordance with the protocol.

(b) The test systems shall be monitored in conformity with the protocol.

(c) Specimens shall be identified by test system, study, nature, and, date of collection. This information shall be located on the specimen container or shall accompany the specimen in a manner that precludes error in the recording and, storage of data.

(d) Records of gross findings for a specimen from postmortem observations should be available to a pathologist when examining that specimen histopathologically.

(e) All data generated during the conduct of a nonclinical laboratory study, except those that are generated by automated data collection systems, shall be recorded directly, promptly and, legibly in the ink. All the data entries shall be dated on the date of entry and, signed or initialed by the person entering the data. Any change in entries shall be made so as not to obscure the original entry, shall indicate the reason for such change and, shall be dated and, signed or identified at the time of the change. In automated data collection systems, the individual responsible for direct data input shall be identified at the time of data input. Any change in automated data entries shall be made so as not to obscure the original entry, shall indicate the reason for change, shall be dated and, the responsible individual shall be identified.

[43 FR 60013, Dec. 22, 1978, as amended at 52 FR 33781, Sept. 4, 1987; 67 FR 9585, Mar. 4, 2002].[14]

5.c. REACH

Regulatory guidelines includes: **(EC 1907/2006)** aims to improve human health and, environmental protection by better and, earlier identification of chemical substances' intrinsic properties. Manufacturers and, importers are required to collect information on the properties of their chemical substances to ensure safe handling and, to register the information in a central database at the **European Chemicals Agency (ECHA),** in Helsinki.[1,2]

The classification of the *in vivo* data into eye irritation classes A (risk of serious damage to eyes), B (irritating to eyes) and NI (non-irritant). A data set for chemical/polymers/coating materials/API, etc., are analysed differencing its ability of irritation along with methods that includes three subsets: surfactants, water-soluble chemicals and, water-insoluble chemicals.[3]

REACH covers all chemical substances, not just those used in industrial processes, but also those found in our everyday life, such as: cleaning products, paints and, goods like, as: clothing, furniture and, electrical equipment. As a result, the regulation affects the majority of businesses in the EU.[4]

6. Helsinki, 4 April 2022 – The datasets contain information about the hazard properties of 19 substances from 153 tests. The information can contribute to the development of predictive computational testing models as well as other purposes.[5]
Candidate authorisation list of compounds of extreme concern. (Published in compliance with REACH regulation article 59(10)).

7. Notes:[6]

7.a. Authentic version: Candidate list is the only one that is considered authentic. Companies may be subject to urgent legal duties as a result of a substance's inclusion in the candidate list, including Articles 7, 31, and, 33 of the REACH regulation.

Each candidate list entry includes both the anhydrous and hydrated forms of a chemical. An entry's CAS number usually refers to the anhydrous form. The entry still applies to hydrated versions of the drug defined by other CAS numbers.

8. Other numerical identifiers: Where practicable, a non-exhaustive inventory of EC and/or CAS registry numbers describing substances or groupings of compounds believed to fall within the scope of the candidate list item is supplied for those entries with "-" in the EC number and CAS number fields. The "details" button on the selected entry provides access to this information.

Table 1:

Substance name	Description	EC No.	CAS No.	Reason for inclusion
(±)-1,7,7-trimethyl-3-[(4-methylphenyl) methylene]bicyclo[2.2.1] heptan-2-one covering any of the individual isomers and/or combinations thereof (4-MBC)		-	-	Endocrine disrupting properties (Article 57(f) - human health)
(±)-1,7,7-trimethyl-3-[(4-methylphenyl) methylene]bicyclo[2.2.1]heptan-2-one		253-242-6	36861-47-9	Endocrine disrupting properties (Article 57(f) - human health)
(1R,3Z,4S)-1,7,7-trimethyl-3-(4-methylbenzylidene) bicyclo[2.2.1]heptan-2-one		-	852541-21-0	Endocrine disrupting properties (Article 57(f) - human health)
(1S,3E,4R)-1,7,7-trimethyl-3-(4-methylbenzylidene) bicyclo[2.2.1]heptan-2-one		-	852541-30-1	Endocrine disrupting properties (Article 57(f) - human health)
(3E)-1,7,7-trimethyl-3-(4-methylbenzylidene)bicycle [2.2.1]heptan-2-one		-	1782069-81-1	Endocrine disrupting properties (Article 57(f) - human health)
1,3,5-Tris(oxiran-2-ylmethyl)-1,3,5-triazinane-2,4,6-trione (TGIC)		219-514-3	2451-62-9	Mutagenic (Article 57b)
2,2-dimethylpropan-1-ol, tribromo derivative (TBNPA)		253-057-0	36483-57-5	Carcinogenic (Article 57a)
4-isododecylphenol		-	27459-10-5	Toxic for reproduction (Article 57c)# Endocrine disrupting properties (Article 57(f) - environment)#Endocrine disrupting properties (Article 57(f) - human health)

6,6'-di-tert-butyl-2,2'-methylenedi-p-cresol		204-327-1	119-47-1	Toxic for reproduction (Article 57c)
Boric acid (H3BO3), disodium salt		-	22454-04-2	Toxic for reproduction (Article 57c)
Boric acid, sodium salt		215-604-1	1333-73-9	Toxic for reproduction (Article 57c)
Hexahydro-3-methylphthalic anhydride		260-566-1	57110-29-9	Respiratory sensitising properties (Article 57(f) - human health)
Hexahydro-4-methylphthalic anhydride		243-072-0	19438-60-9	Respiratory sensitising properties (Article 57(f) - human health)
Hexahydromethylphthalic anhydride	including cis- and trans- stereo isomeric forms and all possible combinations of the isomers	-	-	Respiratory sensitising properties (Article 57(f) - human health)
Orthoboric acid, sodium salt		237-560-2	13840-56-7	Toxic for reproduction (Article 57c)
Phenol, 4-isododecyl-		-	27147-75-7	Toxic for reproduction (Article 57c)# Endocrine disrupting properties (Article 57(f) - environment)#Endocrine disrupting properties (Article 57(f) - human health)
Phenol, tetrapropylene-		-	57427-55-1	Toxic for reproduction (Article 57c)# Endocrine disrupting properties (Article 57(f) - environment)#Endocrine disrupting properties (Article 57(f) - human health)
tris(2-methoxyethoxy)vinylsilane		213-934-0	1067-53-4	Toxic for reproduction (Article 57c)

9. REACH information requirements:[7]

Annex VII of the REACH Regulation includes a requirement for *in-vitro* tests for skin corrosion (section 8.1.1) and, for skin irritation (8.1.2). An overview of the available internationally validated *in-vitro* methods is presented in Table 1. An *in-vivo* skin irritation study shall only be considered at Annex VIII level (section 8.1) in case the *in-vitro* skin corrosion and, irritation tests are not applicable for the substance or the results obtained are not adequate for classification and, risk assessment.

The *in-vitro* test methods can be summarised as follows: Test method EU B.46 / OECD TG 439 – *In-vitro* skin irritation: Reconstructed Human Epidermis Test Method (RHE) is an in vitro assay that allows distinction between irritants (CLP Cat. 1/Cat. 2) and, substances not classified. Note, in case information is only available from this test method and the outcome is positive i.e. Cat. 1/Cat. 2, an in vitro skin corrosion study is needed to assess if the substance is corrosive or irritant. The revised test guideline (OECD, 2013) includes a new "me-too" test method. The 2015 revision further included a reference to the Integrated Approach to Testing and Assessment (IATA) Guidance Document and introduced the use of an alternative procedure to measure viability. Test method EU B.40bis / OECD TG 431 – In vitro skin corrosion: Reconstructed Human Epidermis Test Method (RHE) is an in vitro assay that allows distinction between corrosives (CLP Cat. 1) and non-corrosives. The revised test guideline (OECD, 2013) includes sub-categorisation of corrosives, i.e. Cat. 1A and Cat. 1B/C (of CLP). No distinction between categories 1B and 1C can be made. In addition, the revised test guideline (2014) contains instructions how to address chemicals that directly reduce the viability dye (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or interferes with the optical density measurements (colourants). The 2015 revision further includes a reference to the IATA Guidance Document and introduces the use of an alternative procedure to measure viability. The 2016 revision improves the capacity of these methods for the correct prediction of subcategory 1A.

10. OECD/OCDE: 404.

OECD Guideline for the testing of chemicals. Acute dermal irritation/corrosion.[17]

11. Definitive using of *in-vitro* methods

Testing for skin corrosion/irritation must always start with *in-vitro* test methods, in case new testing is required. *In-vivo* testing is only needed if *in-vitro* methods are not suitable for the substance or if the results of the in vitro tests are not adequate for classification and risk

assessment. If the results of the first in vitro test allow a decision on the classification, a second test does not need to be conducted; see Figure 1, "Top-down and bottom-up approaches", below. Certain steps need to take place before any testing (*in-vitro* or *in-vivo*) is conducted as described in the introductory paragraph to Annex VII, i.e. assessment of all available information which could be e.g. existing *in-vitro, in-vivo* and human data.

If a conclusion on the classification cannot be made based on existing information, the following test(s) needs to be performed:

1) Skin corrosion, *in-vitro*

2) Skin irritation, *in-vitro*.

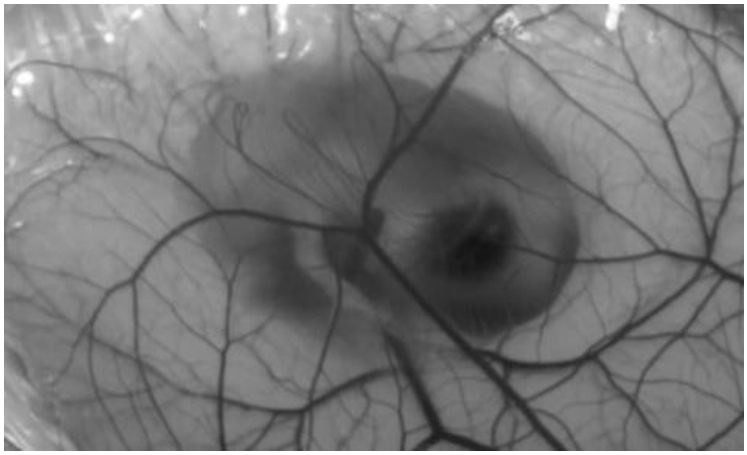


Figure 3: Day 9 of the fertilised egg exposed to artificial incubation in an incubating machine.

Testing strategies such as: the top-down and bottom-up approaches may be applied, based on presumed properties (see Figure 1). In case only one *in-vitro* test is needed to conclude on the skin corrosion/irritation potential, an adaptation statement shall be submitted for the second test (both *in-vitro* corrosion and, irritation tests are standard information requirements).

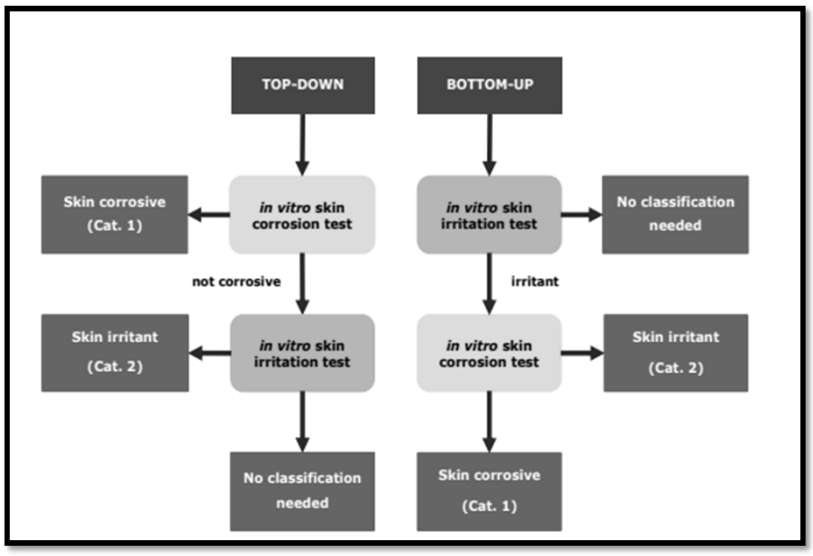


Figure 4: Top-down and, bottom-up approaches. A top-down approach should be applied when it is presumed that the substance is irritant or corrosive (based on existing information), and, a bottom-up approach when that is not the case. The rationale of this approach is that in case the first in vitro test confirms the presumption, then in many cases a conclusion on classification can be made and further testing is not necessary.

12. Methodology

12.1. Supplies, materials and, equipment-requirements

12.1.1. Procurement of chicken eggs

Commercial sources should be used to obtain fertile Q-White Leghorn chicken eggs. Fresh (less than seven days old), viable, fertile and, clean eggs weighing 50 to 60 grams should be utilised. Before using eggs, they should be candled and, nonviable or damaged eggs should be discarded. Eggs that are excessively deformed or have cracked or thin shells should not be utilised. Transporting eggs should be done in a way that does not compromise embryo viability or development.

Figure 5: Incubation of the egg at appropriate temperature.

12.1.2. Requirements along with equipment

Micropipette(s) and, disposable tips appropriate for suggested amounts. Deionised/distilled water. Dentist's revolving saw blade. Incubator with an automatic rotating device. Stop clock or electronic chronometer. Standard general biological laboratory equipment and, supplies (e.g., micro-centrifuge tubes for material volume measurement), if needed. Tapered forceps. Volumetric flasks. Candling light. Parenteral drug association (PDA), Pre-filled syringes and, injection devices.

Figure 6, a: Micro syringe pipette.

Figure 6, b: Parenteral drug association (PDA), Pre-filled syringes and, injection devices.

Figure 6, c: Prefilled syringe for injection or dosing.

12.1.3. Solutions

When it comes to storage temperature and, shelf life of stock solutions, the manufacturer's guidelines should be followed. Volumetric solutions should be prepared.

1. Sodium chloride (NaCl) 0.9 percent (w/v) in deionised/distilled water.
2. 0.1 N sodium hydroxide (NaOH) in deionised/distilled water.
3. 1 percent (w/v) sodium dodecyl sulphate (SDS) in deionised/distilled water.

12.1.4. Preparation of the substance to be tested

Unless dilution is justified, all test chemicals should be examined undiluted. If dilution is necessary, the diluent should be 0.9 percent NaCl or Olive oil, depending on the solubility of the material. It should be justifiable to use a different solvent. The dilutions of the test should be made on the same day as the test. Test chemicals or formulations that are paste, particle, or granular should be examined without dilution. After the mild compression of the particles in a measuring container, solid test substances should be ground to a fine dust to acquire a volume of 0.3 mL. (e.g., micro-centrifuge tube).

13. Controls

13.1. Negative control

Each experiment should contain a 0.9 percent NaCl negative control to provide a baseline for the assay endpoints and, to guarantee that the assay conditions do not improperly cause an irritating reaction. The assay conditions do not inappropriately result in an irritant response.

13.2. Solvent control (if appropriate)

13.2.1. Controlling solvents (if appropriate)

If the test material is diluted in olive oil, it should be used as a controlled substance to give a baseline for the assay endpoints and, to ensure that the assay conditions do not cause an irritating response. If a solvent other than 0.9 percent NaCl or olive oil is used, both the solvent and, the 0.9 percent NaCl should be used as controls to ensure that the alternative solvent does not cause any irritation in the subject.

13.2.1.1. Positive control

Each experiment should include: a known ocular or potent skin irritant to ensure that the desired response is elicited or induced. If the HET-CAM assay is only being used to detect corrosive or severe irritants, the positive control should be a chemical (e.g., 1 percent SDS, NaOH and, other laboratory standard skin irritant prepared using laboratory chemicals), that

causes a severe reaction both *in-vivo* and, in HET-CAM. The degree of the severe response, on the other hand, should not be excessive in order to measure variability in the positive control response over time. The availability of high quality in vivo data should be used to identify positive control test chemicals.

14. Experimental design

14.1. Treatment groups

Each group should have at least minimum of three eggs (negative and, positive controls, test substance and, if included, benchmark and, solvent controls). Eggs from the same hen should be randomised as much as possible amongst the treatment groups.

14.1.1. CAM preparation

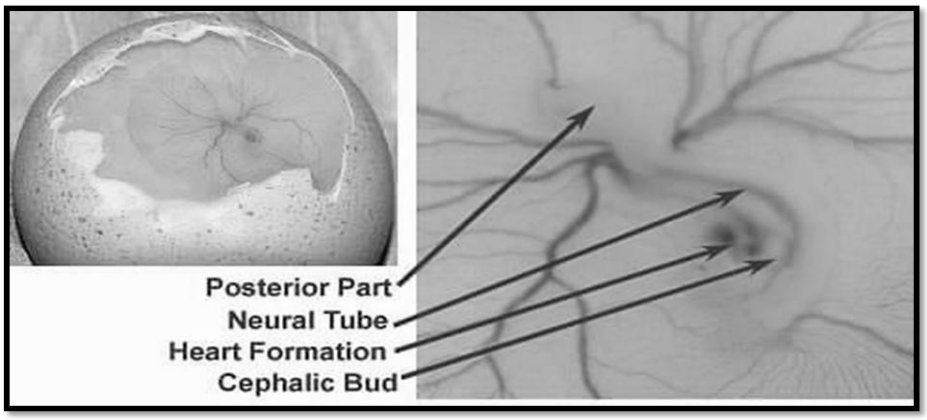


Figure 7: Partial deshelled end of the egg and its parts explained.

a. Select fresh (not older than 7 days), clean, fertile 50-60 g White Leghorn chicken eggs. Candle the eggs and discard any eggs that are nonviable or defective. Excessively misshapen eggs or eggs with cracked or thin shells should not be used. Shaking, unnecessary tilting, knocking, and all other mechanical irritation of the eggs should be avoided when preparing.

b. Place eggs in an incubator with a rotating tray. Incubate eggs at 38.3 ± 0.2°C and 58 ± 2% relative humidity when incubating in a still-air incubator or at 37.8 ± 0.3ºC and 58 ± 2% relative humidity when incubating in a forced-air incubator. Hand rotate eggs five times per day until day 8.

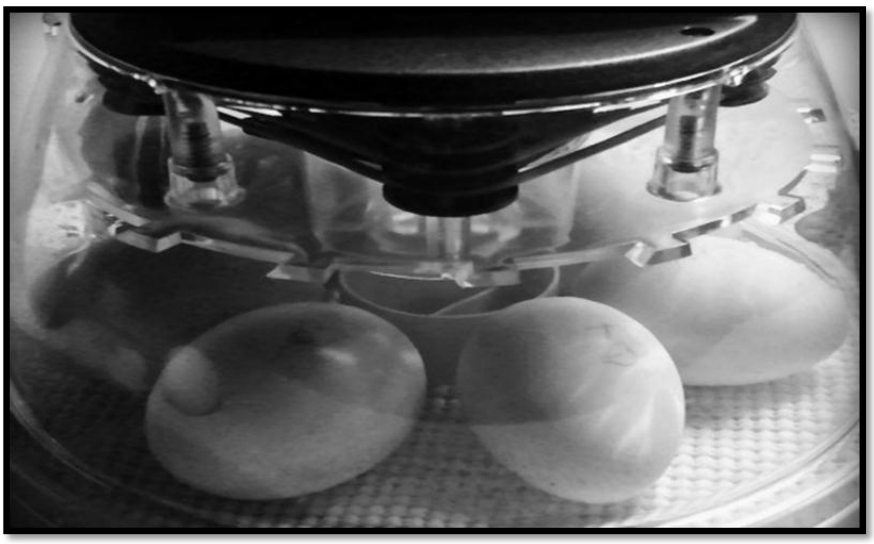


Figure 8: Incubation process of the fertilised egg to be developed at optimum condition outside the chicken reach using an incubator.

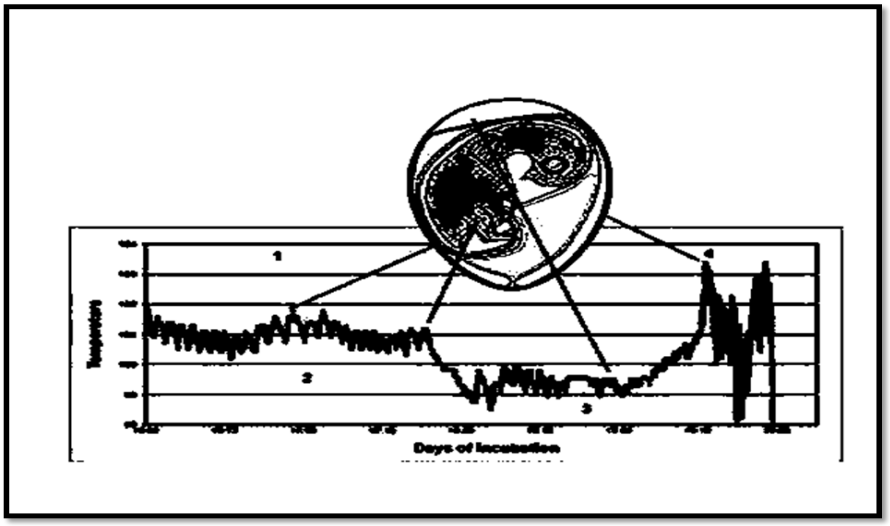


Figure 9: Graph of study of days of incubation of the egg.

c. Candle the eggs on incubation day 8 and, isolate or remove any nonviable or defective eggs. Eggs are returned to the incubator (without hand rotation) with the large end of the eggs upwards for an additional day.

Figure 10: Candle the eggs upon the incubation on day 8 to check the embryo development in the egg shell.

d. Take out the eggs from an incubator on day 9 that is to be used for in the assay. Candle eggs and, discard any nonviable or defective eggs.

Figure 11: Candle the eggs upon the incubation on day 8 to check the embryo development in the egg shell.

e. Mark the air cell air-sack of the egg. Cut the section marked as the air cell with a rotating dentist saw blade and then pare it off. Care should be taken when removing the eggshell to ensure that the inner membrane is not injured. **ICCVAM** *In-vitro* Ocular Evaluation Report B-34.

Figure 12: Partial deshelled egg with injecting the sample directly in the systemic circulation along with standard irritant and, matrix substance or polymer substances.

f. Moisten the inner membrane with 0.9% NaCl. A disposable glass pipette can be used to apply the solution. Place the egg into the incubator for a maximum of 30 minutes.

g. Remove or take out the eggs from the incubator, prior to its use in the assay, and decant the 0.9% NaCl solution. Carefully remove the inner membrane with forceps, ensuring that the inner membrane is not injured.

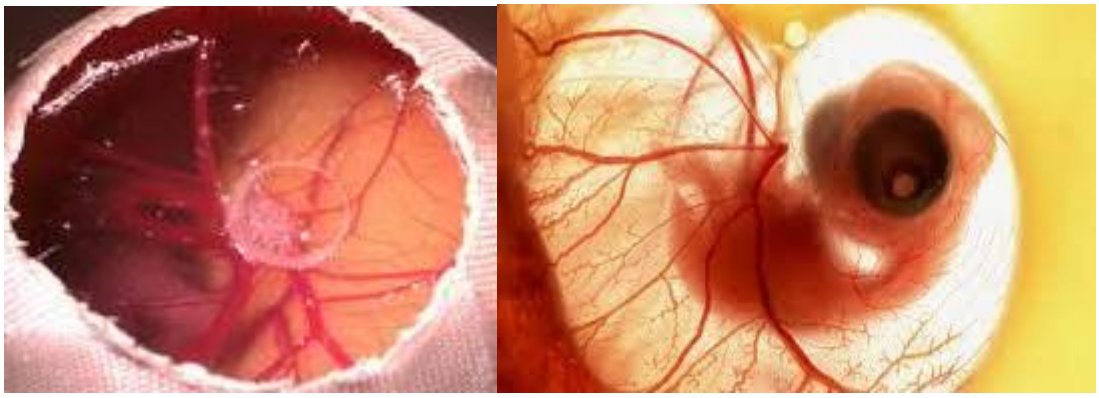


Figure 13: Partial deshelled egg with distinct embryo.

15. Eggs that have been treated with test substances

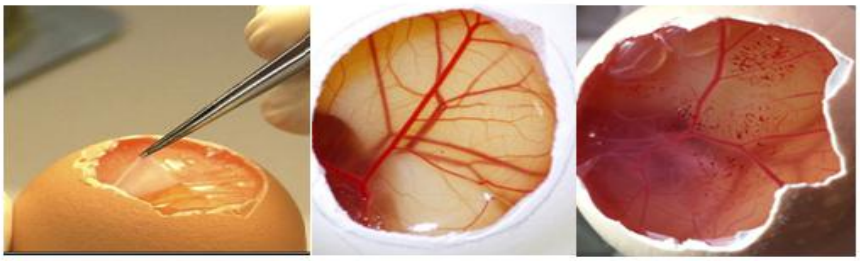
15.1. Paste test ingredients or formulations substance for examination

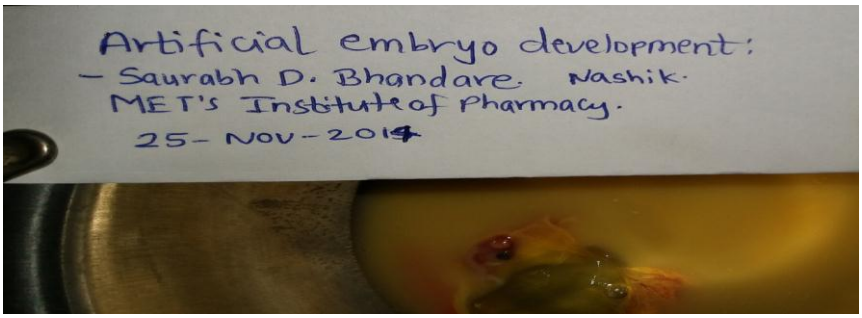
Apply 0.3 mL of paste substances or formulations directly to the CAM, making sure to cover at least 50% of the CAM surface area. When the total weight of the test substance at this

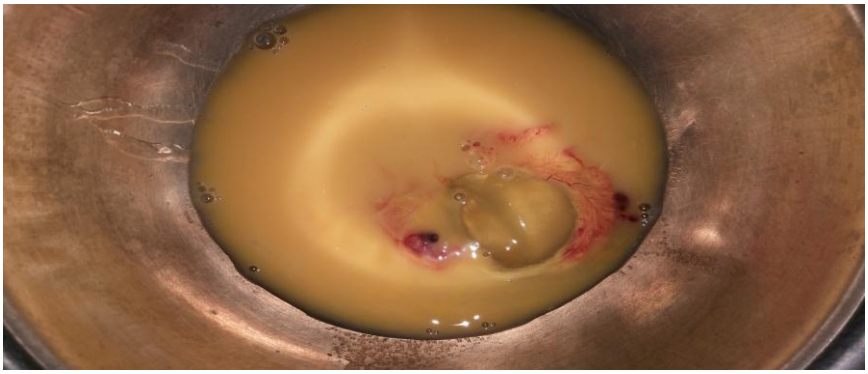
volume is larger than 0.3 g, use 0.3 g of the paste test substance. The weight of the test material should be noted in either situation.

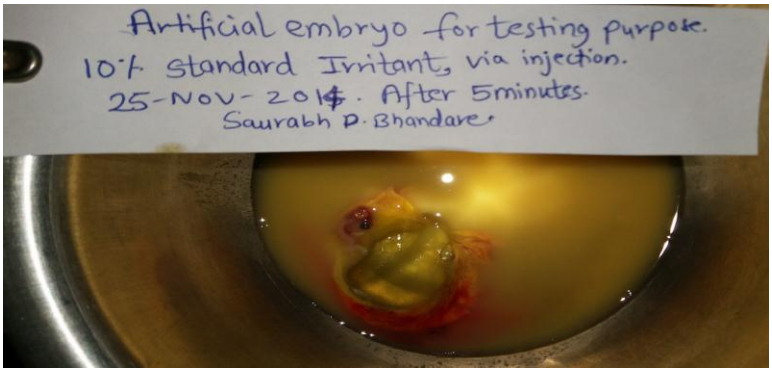
15.2. Observations

Over a 300-second period, observe the reactions on the CAM. The time it takes for each of the listed endpoints to appear should be tracked and documented in seconds.









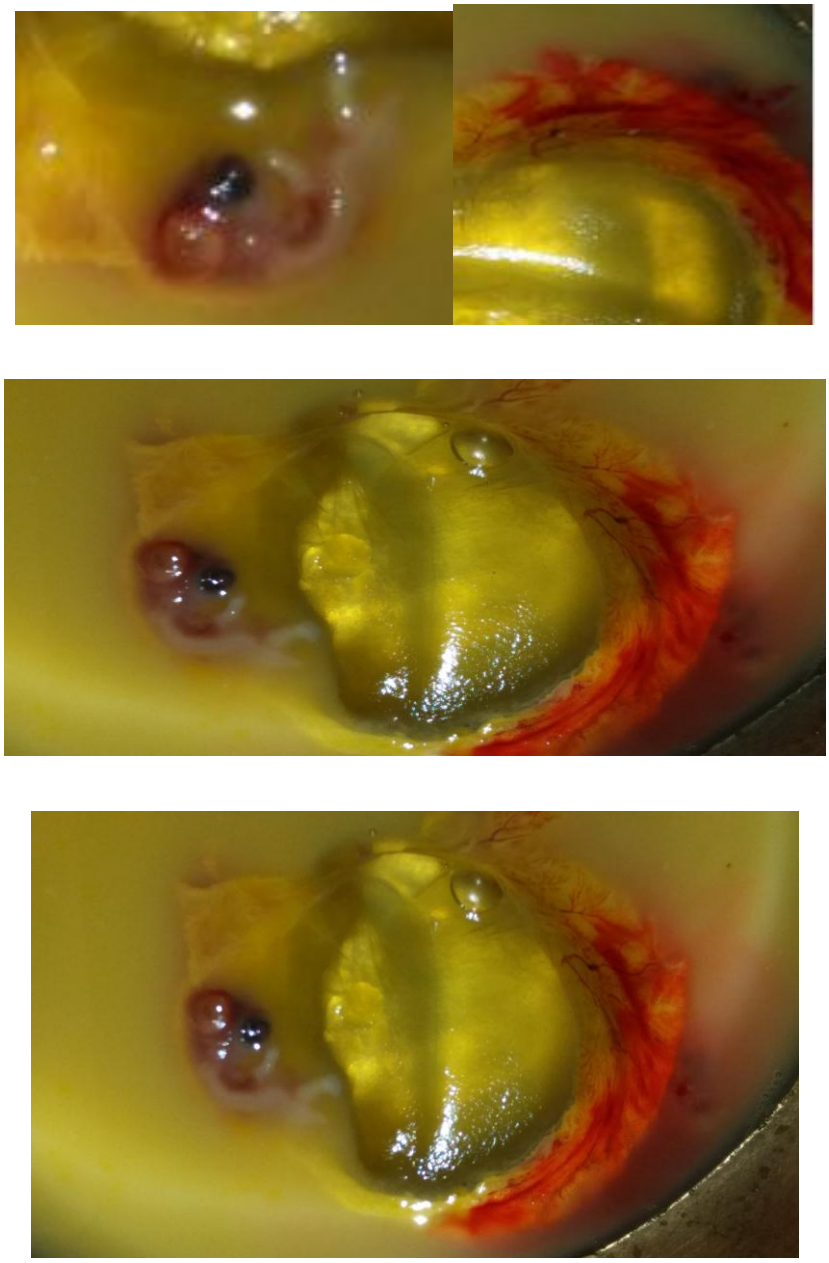


Figure 14: Observation and recording the score.

15.2.1. The following endpoints should be monitored

Hemorrhage (bleeding from the vessels),

Vascular lysis (blood vessel disintegration),

Coagulation (intra- and extra-vascular protein denaturation).

15.2.2. Explaination

Hemorrhage (bleeding from the blood vessels),

Liquefaction of the blood vessels (that is blood vessel disintegration),

The process of coagulation (intra- and, extra-vascular protein denaturation.)

Hemorrhage time = observed start (in seconds) of hemorrhage reactions at CAM Lysis time = observed start (in seconds) of vessel lysis at CAM Coagulation time = observed start (in seconds) of coagulation formation on CAM.

Additional data and information may be valuable in conducting retrospective research and doing further analyses. Reference images for all endpoints should be available to increase the possibility of reproducible outcomes.

15.2.3. Examination and, evaluation of the test results

The IS(A) analytic approach, which is based on developing each of the three HET-CAM endpoints at predetermined time intervals of 0.5, 2, and 5 minutes, is the ICCVAM-recommended HET-CAM methodology for prospective investigations (Luepke 1985).

With a range of one to twenty-one, the numerical time-dependent scores for lysis, bleeding, and coagulation (Table-1) are added to yield a single numerical value representing the irritation potential of the test substance.

16. Scoring scheme for irritation testing with the HET-CAM test method

Table: 2.

		Score	
Effect	**0.5 min**	**2 min**	**5 min**
Lysis	**5**	**3**	**1**
Hemorrhage	**7**	**5**	**3**
Coagulation	**9**	**7**	**5**

For retrospective analyses, data from the HET-CAM test method protocol using the IS(B) analysis method (ICCVAM 2006) could be converted to fixed time points similar to those used for the IS(A) analysis method.

17. Criteria for an acceptable test

A test is considered acceptable if the negative and positive controls each induce a response that falls within the classification of non-irritating and severely irritating, respectively. Historical control studies indicate that using 0.9% NaCl, as a negative control, the IS value was 0.0. Historical control studies indicate that using 1% SDS and 0.1 N NaOH, as positive controls, the IS values ranged between 10 and 19, respectively.

18. RESULTS

Tabulation of data from individual test samples (e.g., irritancy scores for the test substance and, the various controls, including data from replicate repeat experiments as appropriate and, means and, ± the standard deviation for each test). For testing including the testing or use of standard irritant all irritancy test have been positive as shown in the pictures in the observation table.

Table 3:

		Score	
Effect	**0.5 min**	**2 min**	**5 min**
Lysis	**Nil/0.**	**Nil/0.**	**Nil/0.**
Hemorrhage	**Nil/0.**	**Nil/0.**	**Nil/0.**
Coagulation	**Nil/0.**	**Nil/0.**	**Nil/0.**

19. Skin irritation study: 1. HET CAM test incubated hen's eggs were subjected for to HET CAM Test and, observed for either coagulation or haemorrhage or haemolysis after 300 seconds of application of a test substance. Coagulation was observed within 9 seconds of application in the case of positive control while no coagulation or any viable reaction was observed in case of negative control and, test substance, (t.s.). Observations made for HET CAM test.

20. Other understanding factors that depends on the study

1. Fresh frozen plasma (FFP),	**2.** Standard laboratory tests (SLT),
3. Randomised controlled trial (RCT),	**4.** aPTT' (activated partial thromboplastin time),
5. 'INR' (international normalised ratio),	**6.** Viscoelastic test (VET),
7. Tissue factor (TF),	**8.** von Willebrand factor (VWF),
9. Endogenous thrombin potential (ETP),	**10.** Prothrombin time (PT),
11. Maximum clot firmness (MCF).	**12.** Microliter dosing syringe (MDS).

21. Other related experimental images

(1) Underdeveloped embryo/non fertilised egg type, which failed to generate or develop embryo after laboratory incubation of the eggs.
(2) Such egg proteins obtained from unfertilised eggs were collected and utilised for other laboratory evaluation purposes or research in achieving pharmaceutical study related properties involved in pharmacokinetics and, pharmacodynamics of a drug substances.

22. CONCLUSION

A scoring notation was devised for this, which treats the delay parameters as independent effects of individual. Most tested plant extracted and, derived polymeric material showed no significant irritant or anti-irritant effects. Among the apparently anti-irritant plant extracts (approx. 10% of all those tested), most showed their greatest effect against haemorrhaging. Lesser but still readily measurable effects against membrane lysis and, coagulation were also observed in nearly all the apparently anti-irritant extracts. Two of the tested extracts proved to be membrane irritants. Some key CAM assay results were compared with results obtained in direct tests on human skin using the same test irritant as prepared in the laboratory. In these comparative tests an essentially similar pattern of efficacy was obtained with the plant extract and, polymer was best in the CAM screenings, outperforming the benchmark anti-irritant determination and, evaluation of polymer that are examined to be non-irritant. From these initial results it appears that physiological CAM assays may prove useful in screening natural materials for anti-irritant properties, as alternatives to mechanism-dependent biochemical assays, or procedures that includes expensive direct screening tests on human subjects.

Other focuses during the study: Further work remains to extend the CAM screening approach to irritants other than standard test solution but included an urgent appeal for an urgent study assistance in research work of a junior colleague under the guide Dr. G.S. Deokar and, with my senior mentor assistance and, guidance in the study in my part time, as directive from Dr. G.S. Deokar which involved in the evaluation of the study of irritation potential of Calatropis procera and, to access its quantitative and, qualitative powers of prediction of topical anti-irritancy. This also included systemic toxicity evaluation of plant material Calatropis procera carried on mice with lethal dosing the mice followed with chemical induced euthanasia; induced with the help of laboratory assistant, followed by dissection functional of each of its organs for study purpose, that included: liver, kidney, intestine, heart and, brain, also reproductory organs of both sexes with its evaluation under the microscope. This study was done as per obtained certificates from the animal ethics committee. Animal Ethics

Committees (AECs), including the permission for conducting experiments involving use of animals. Recommendation for import of animals for use in experiments and, experiment conduct on animals. **Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) on an Institutional Animal Ethics Committee (IAEC)** is the wellbeing and welfare of the animals housed or kept for experiments / breeding. Irritation with standard irritant have shown to have the development of: 1. Hemorrhage (bleeding from the vessels), Vascular lysis (blood vessel disintegration), Coagulation (intra- and extra-vascular protein denaturation).

22.1. Study Discussion

In the case of artificial embryo development, artificial fertilisation is a critical step to ensure that a complete embryo is generated and developed as per requirements and, that no naturally unfertilised eggs are present, resulting in lower experimental counts of the embryo after the incubation to assure total size and numbers.

A very significant aspect of this study is the meticulous development of an artificial embryo in a surgical steel cup/bowl or a kidney dish or an emesis basin to further evaluate specific pharmacological and, narcotic medications tests for future study purposes for any API or unqualified new drug study or evaluation purposes.

This technology can be used to construct artificial organs for testing purpose, including laboratory testing animals, such as: the heart, kidneys, and, eyes. In artificial embryo genesis, the teratogenic effects of unknown study medications or API during the first developing stages and, thereafter can be conveniently examined. The study of its genetics, teratogenic effects of medications on the body, chemically induced gene specific, gene deficiency and, critical evaluation are equally important and, vital parts of the research. These tools can also be used to investigate nutrition and, good embryo development. The outer membrane and, artificial drug introduction in the important organs of a developing embryo, as well as the period of genetic division; pharmaceutical ingredient interference, and, the pregnancy safety of a drug substance, are all majour research focuses, interests and, topics, along with the irritation study.

Teratogenic drugs: *A teratogen is* ***an agent that can disturb the development of the embryo or fetus****. Teratogens halt the pregnancy or produce a congenital malformation (a birth defect). Classes of teratogens include radiation, maternal infections, chemicals, and drugs.*

‘*This study is a part of drugs and, excipient safety and toxicity study on an artificial chicken embryo developed outside or in-vitro in the laboratory with necessary and, desired interventions in the embryo development phases as per desired or necessary requirements.’*

'Such data obtained of a particular drug and recipient, during the study are also applicable in the filing of NDA, reports submission to the FDA, etc.'

23. ACKNOWLEDGEMENT

The researcher, author of this work would like to express his gratitude to the “World Journal of Pharmaceutical Research,” and, Dr. Valentina Petkova, (Ph.D., M.Sc.Pharm., M.Sc.P.H.), Faculty of Pharmacy, Medical University, 2 Dunav str. Sofia, Bulgaria; Vice Dean of International Integration Bulgaria; for recognising and, publishing this work in the journal.

24. CONFLICT OF INTEREST

None. The researcher declares that there is no conflict of interest in the publication of this research paper and, that all the individual permissions had been granted to publish this research work either individually or with publishing partners. The research was carried out during the year 2013-2015, before publishing this research work, it was under study and investigation for analysing and obtaining significant scientific information on the ability and stability of polymer as a source of drug delivery in critical and acute cases of fungal infections in both humans and animals. The entire research study was carried out to meet the requirement of majour research work that was a part of curriculum and, required to be produced to fulfil the grades in the examination.

25. REFERENCES

1. ECHA: European Chemicals Agency.
2. How to use new or revised in vitro test methods to address skin corrosion/irritation (Revised in November), 2017.
3. https://echa.europa.eu/documents/10162/21650280/oecd_test_guidelines_skin_irritation_en.pdf/cfbe42b4-0708-4241-93dd-8bbee15a2c23 (Re-accessed on: 18-April-2018.). REACH (EC 1907/2006).
4. https://ec.europa.eu/environment/chemicals/reach/reach_en.htm (Re-accessed on: 18-April-2018.)
5. https://lendseed.comvl%3D4485241/cl=30/nw=1/rpsv/cw/vhosts/oecdjournals/1607310x/v1n4/contp1-1.htm (Re-accessed on: 18-April-2018.).

6. https://echa.europa.eu/regulations/reach/understanding-reach (Re-accessed on: 18-April-2018.).
7. https://echa.europa.eu/-/pharmaceutical-industry-provides-unpublished-data-on-chemical-substances (Re-accessed on: 17-April-2022.).
8. https://echa.europa.eu/web/guest/candidate-list-table (Re-accessed on: 17-April-2022.).
9. https://echa.europa.eu/documents/10162/21650280/oecd_test_guidelines_skin_irritation_en.pdf/cfbe42b4-0708-4241-93dd-8bbee15a2c23 (Re-accessed on: 18-Jan-2018.).
10. https://joint-research-centre.ec.europa.eu/eu-reference-laboratory-alternatives-animal-testing/alternative-methods-toxicity-testing/validated-test-methods/skin-irritation_en(Re-accessed on: 18-Jan-2018.).
11. EPA. Label Review Manual: 3rd ed. EPA737-B-96-001. Washington, DC:U.S. Environmental Protection Agency, 2003.
12. EPA. Good Laboratory Practice Standards. 40 CFR 792. EPA, 2003b.
13. Good Laboratory Practice Standards, 2003; 40 CFR 160.
14. EU. Commission Directive 2001/59/EC of 6 August 2001 adapting to technical progress for the 28th time Council Directive 67/548/EEC on the approximation of the laws, regulations and Appendix B – ICCVAM-Recommended Protocols B-37 administrative provisions relating to the classification, packaging and labelling of dangerous substances. Official Journal of the European Communities, 2001; L255: 1–333.
15. FDA. Good Laboratory Practice for Nonclinical Laboratory Studies, 2003; 21 CFR 58.
16. Luepke N. Hen's egg chorioallantoic membrane test for irritation potential. Food Chem Toxicol, 1985; 23: 287–291.
17. https://clinicaltrials.gov/ct2/show/NCT02637206 (Re-accessed on, 18-Jan-2016.).
18. https://pubmed.ncbi.nlm.nih.gov/18509253/ (Re-accessed on: 18-Jan-2016.).
19. https://ntp.niehs.nih.gov (Re-accessed on: 18-Jan-2016.).
20. https://www.schc.org (Re-accessed on: 18-Jan-2016.).
21. https://intermountainhealthcare.org (Re-accessed on: 18-Jan-2016.).
22. Gilleron., L., Coecke., S. and Sysmans., M., Evaluation of a modified HET-CAM assay as a screening test for eye irritancy. *Elsevier journal of toxicology in vitro*, 1996; 10(4): 431-446.
23. Rivero MN, Lenze M, Izaguirre M, Pérez Damonte SH, Aguilar A, Wikinski S, Gutiérrez ML. Comparison between HET-CAM protocols and a product use clinical study for eye irritation evaluation of personal care products including cosmetics according to their

surfactant composition. Food Chem Toxicol, 2021 Jul; 153: 112229. doi: 10.1016/j.fct.2021.112229. Epub 2021 Apr 19. PMID: 33887397.

24. Liao Y, Wang X, Zhang L, Li G. [Study on using the hen's egg test-chorioallantoic membrane as an alternative method of draize eye irritation test]. Wei Sheng Yan Jiu, 2004 May; 33(3): 279-83. Chinese. PMID: 15211792.
25. Palmeira-de-Oliveira R, Monteiro Machado R, Martinez-de-Oliveira J, Palmeira-de-Oliveira A. Testing vaginal irritation with the Hen’s Egg Test-Chorioallantoic Membrane assay. ALTEX, 2018; 35(4): 495-503. doi: 10.14573/altex.1710091. Epub 2018 Mar 11. PMID: 29534246.
26. De Araujo Lowndes Viera LM, Silva RS, da Silva CC, Presgrave OAF, Boas MHSV. Comparison of the different protocols of the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) by evaluating the eye irritation potential of surfactants. Toxicol In Vitro, 2022 Feb; 78: 105255. doi: 10.1016/j.tiv.2021.105255. Epub 2021 Oct 29. PMID: 34743969.