BUREAU OF INDIAN STANDARDS

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भारतीय मानक मसौदा

पारम्परिक औषधियों में प्रयोग हेतु डिजिटेलिस परप्यूरिया एल. पत्तियां – विशिष्टि

Draft Indian Standard

Digitalis Purpurea L. Leaves for use in Traditional Medicine - Specification

Homoeopathic Sectional Committee, AYD 07 **Last Date of Comments: 06-02-2025**

FOREWORD

(Formal clauses would be added later)

Digitalis purpurea L is a biennial, sometimes perennial herb, deciduous with branched tap root. It is native to Western Europe, Mediterranean region and northwest Africa; naturalized in Asia, South America, New Zealand, Canada, and much of the United States. It contains various cardiac and steroidal glycosides like digitoxin, digoxin, gitoxin and gitaloxin present in roots and leaves. It also contains digitoxigenin, gitogenin, gitalin, digicorin, inositol, luteolin, degalactotigonin and F-gitonin. It is used for making Mother tincture, dilutions or potencies in Homoeopathy and as an ingredient for formulations in traditional systems of medicine.

Digitalis purpurea commonly known as Digitalis or foxglove or purple foxglove (English); *Gant de Notre Dame* (French); *Fingerhu* (German). Some of the regional names are *Tilpuspi, Digitalis* (Hindi), and *Hritpatri, Tilapushpi,* (Sanskrit).

The standard is one of the series of standards being brought out on ingredients used in formulations of traditional medicine for the advantage of researchers, academicians, students, clinical practitioners and drug manufacturers

In the formulation of this standard, significant assistance has been derived from Homoeopathic Pharmacopoeia of India, Vol. 1, 1971; Vol. 10, 2013, Inputs have also been derived from the information available in the public domain in print and electronic media including authoritative books.

Also, due consideration has been given to the provisions of the *Drug and Cosmetics Act*, 1940 and the Rules 1945, framed thereunder, including the latest amendments. In case of any disparity, this standard is subject to therestrictions imposed under these Rules and Regulations, wherever applicable.

For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the result of a test or analysis, shall be rounded off in accordance with IS 2 : 2022 ‘Rules for rounding off numerical values (second revision)’. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

Draft Indian Standard

DIGITALIS PURPUREA L. LEAVES FOR USE IN TRADITIONAL MEDICINE - SPECIFICATION

1 SCOPE

This standard prescribes the specific requirements and methods of testing for *Digitalis purpurea*, which consists of the leaves of *Digitalis purpurea* L. (Family Scrophulariaceae).

2 REFERENCES

The standards listed below contain provisions which, through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards.

IS No.	***Title***
IS 1070 : 1992	Reagent grade water — Specification (third revision)
IS 16913 : 2018	Methods of test for cosmetics — Determination of heavy metals (Arsenic, Cadmium, Lead and Mercury) by atomic absorption spectrometry (AAS)
IS 17924 : 2022	Determination of pesticide residue in herbal materials
IS 13859: 1993/ISO 7513: 1990 IP (2.4.19)	Loss on Drying
IS 16287 : 2015/ISO 16050 : 2003	Determination of aflatoxin B1, and the total content of aflatoxins B1, B2, G1 and G2 in cereals, nuts and derived products — High performance liquid chromatographic method

3 REQUIREMENTS

3.1 Description

3.1.1 *Macroscopic Examination of Digitalis purpurea* L. *leaves*

Drug occurs in dried small pieces or whole shrivelled leaf; ovate-lanceolate to broadly ovate leaves, petiolate, winged petiole, 10-40 x 4-15 cm; margin is crenate to serrate or dentate; venation is pinnate, with a veinlet ending in each tooth of margin; each marginal tooth with a prominent hydathode; slightly pubescent upper surface, deep green - greyish; pubescent lower surface, pale green-gray; odour is somewhat tea-like, and tastes bitter.

3.1.2 *Microscopic Examination of Digitalis purpurea* L. *leaves*

Leaf: Vertical section shows single layered epidermis, rarely 2 layered, cells polygonal with thin cuticle, anticlinal walls slightly wavy on upper surface; covered with two types of trichomes: (a) non-glandular, uniseriate, 3 or 4 celled with an acute apex and warty (b) glandular trichomes, chiefly on veins of two types (i) single-celled stalk and bi-cellular or rarely unicellular head, (ii) uniseriate, multi-cellular stalk and unicellular head; mesophyll differentiated into a single layered palisade, occasionally 2 or 3 layered and 4-5 layers of spongy parenchyma; mid-rib convex or more protuberated on lower side, covered with aforesaid types of non-glandular and glandular trichomes; a layer of collenchymatous hypodermis present below lower epidermis; ground tissue of oval or polygonal parenchymatous cells; meristele large, arc-shaped with xylem towards upper side and phloem towards lower side and surrounded by a parenchymatous bundle sheath; stomata anomocytic, abundant on lower surface, a few on upper surface; stomatal index on lower epidermis 17.54 and on upper epidermis 16.12; palisade ratio 3.7-4.2; vein islet number 2-5.5 per sq. mm.

Petiole: Transection shows horse-shoe shaped in outline; epidermis, single layer of barrel-shaped cells, covered with thin cuticle; trichomes two types as on leaf; hypodermis 1 or 2 layers of collenchymatous cells; ground tissue broad, parenchymatous; large vascular bundle, conjoint, collateral, centrally placed, surrounded by a layer of parenchymatous cells and two small vascular bundles or vascular strands present in lateral projections.

3.1.3 *Powder*

Green or pale green, with tea-like odour and bitter taste; microscopy reveals fragments of polygonal epidermal cells with anomocytic stomata; a few fragments of broken or whole non-glandular, uniseriate trichomes and glandular trichomes; fragments of palisade and spongy parenchymatous cells with chloroplast; a few fragments of parenchymatous cells and tracheary elements with scalriform and annular thickenings.

FIG. 1 RAW DRUG OF DIGITALIS PURPUREA LEAVES

FIG. 2 POWDER OF DIGITALIS PURPUREA LEAVES

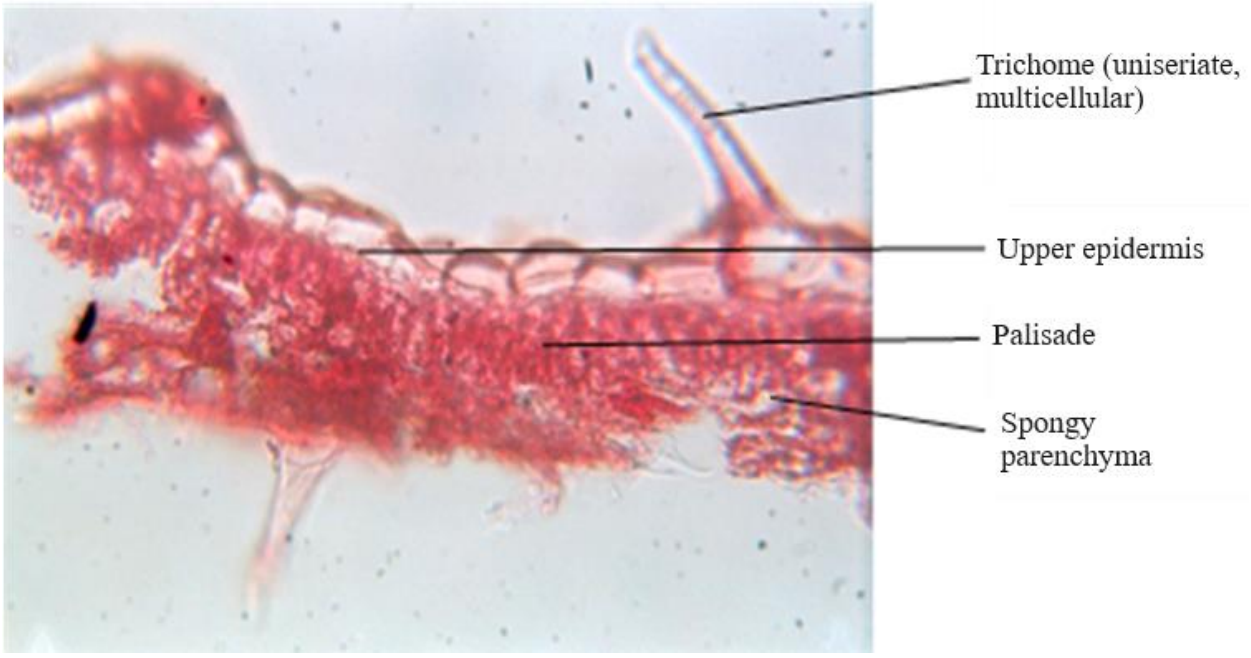


FIG. 3 TRANSVERSE SECTION OF LEAF OF DIGITALIS PURPUREA THROUGH LAMINA

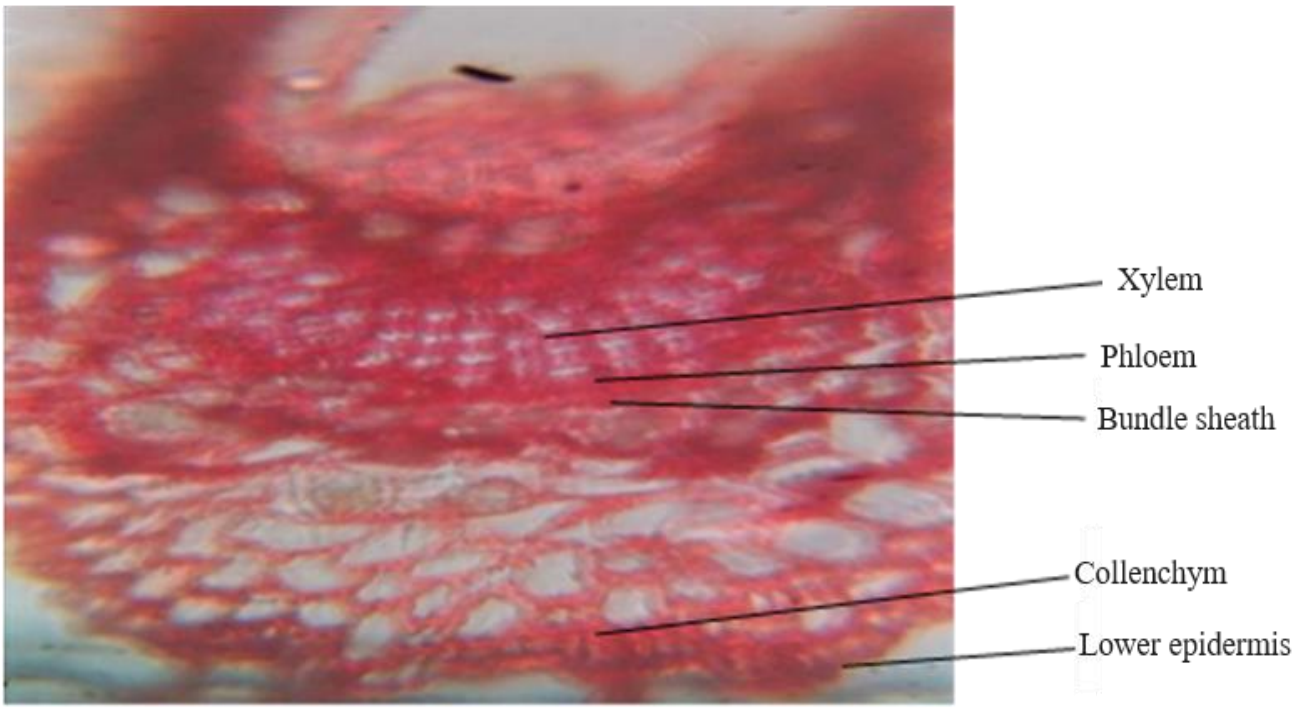


FIG. 4 TRANSVERSE SECTION OF LEAF OF DIGITALIS PURPUREA THROUGH MIDRIB

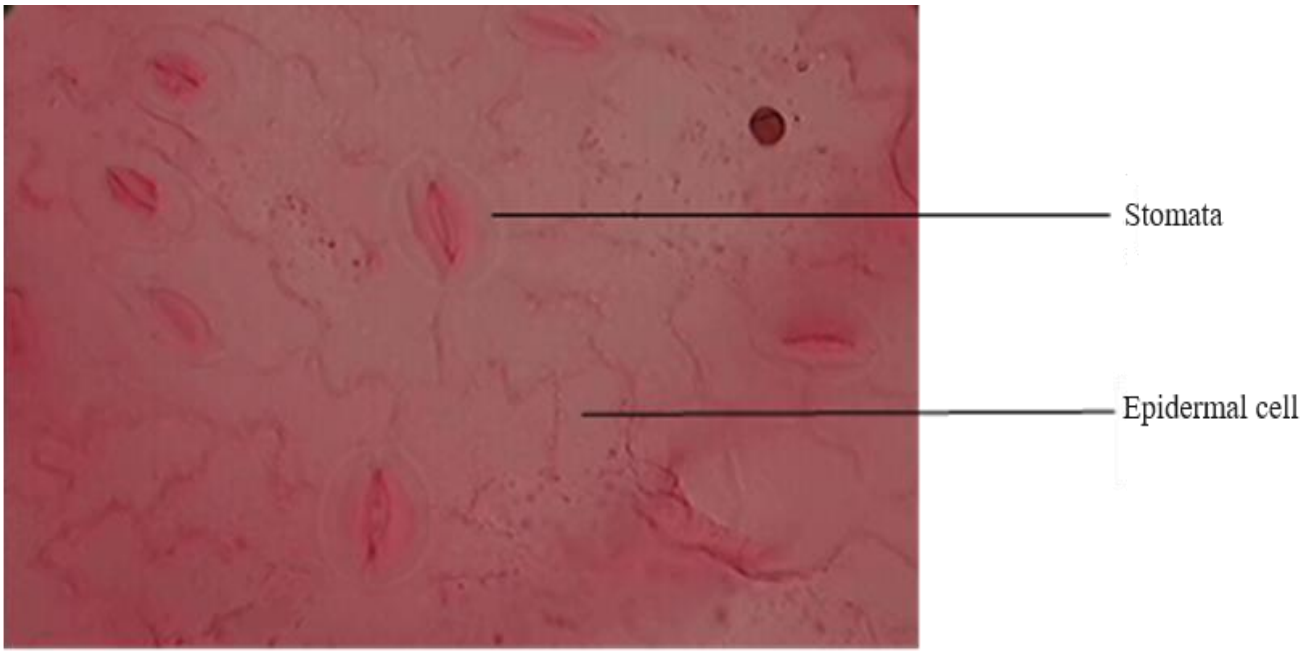


FIG. 5 ANOMOCYTIC STOMATA IN DIGITALIS PURPUREA (SURFACE VIEW)

3.2 General Specifications

3.2.1 *Digitalis purpurea* plant shall be free from extraneous/artificial flavours.

3.2.2 *Digitalis purpurea* plant shall comply with physical, chemical and microbiological requirements given in Table 1.

Table 1 Requirements for Digitalis purpurea leaves
(Clauses 3.2.2 and 5.2)

S. No.	Characteristic	Requirement	Method of Test, Ref to
(1)	(2)	(3)	(4)
1.	Foreign matter, percent by mass, *Max*	2	6.2 of IS 4333 (Part 1)
2.	Loss on drying, percent by mass, *Max*	10	IS 13859
3.	Total ash, percent by mass, *Max*	5	6 of IS 1797
4.	Acid insoluble ash, percent by mass, *Max*	0.6	8 of IS 1797
5.	Alcohol soluble Extractive, percent by mass, *Min*	15	10 of IS 1797
6.	Water soluble Extractive, percent by mass, *Min*	40	11 of IS 1797
7.	**Identification tests**		
i.	General Identification	Should pass the test	Annex A
ii.	HPTLC or TLC	Should complies	Annex A
8.	**Microbial load**		
i.	*Staphylococcus aureus*, per g	Absent	Annex B
ii.	*Pseudomonas aeruginosa*, per g	Absent	Annex B
iii.	*Salmonella Spp.*, per g	Absent	Annex B
iv.	*Escherichia coli*, per g	Absent	Annex B
v.	Total microbial plate count, per g, *Max*	10^5 for extract and powder 10^7 for plant material	Annex B
vi.	Total yeast and mould, per g, *Max*	10^3 for extract and powder 10^5 for plant material	Annex B
9.	**Pesticide residues**	Limit (mg/kg)	
i.	Alachlor, mg/kg, *Max*	0.02	IS 17924
ii.	Aldrin, dieldrin (sum of), mg/kg, *Max*	0.05	IS 17924
iii.	Azinphos-methyl, mg/kg, *Max*	1.0	IS 17924
iv.	Bromopropylate, mg/kg, *Max*	3.0	IS 17924
v.	Chlordane (sum of cis-, trans- and oxythlordane), mg/kg, *Max*	0.05	IS 17924
vi.	Chlorfenvinphos, mg/kg, *Max*	0.5	IS 17924

vii.	Chlorpyrifos, mg/kg, *Max*	0.2	IS 17924
viii.	Chlorpyrifos-methyl, mg/kg, *Max*	0.1	IS 17924
ix.	Cypermethrin and isomers, mg/kg, *Max*	1.0	IS 17924
x.	DDT (sum of p,p' -DDT, o,p'-DDT, p,p' DDE and p,p'-TDE), mg/kg, *Max*	1.0	IS 17924
xi.	Deltamethrin, mg/kg, *Max*	0.5	IS 17924
xii.	Diazinon, mg/kg, *Max*	0.5	IS 17924
xiii.	Dichlorvos, mg/kg, *Max*	1.0	IS 17924
xiv.	Dithiocarbamates (as CS_2)	2.0	IS 17924
xv.	Endosulfan (sum of isomers and endosulfan sulphate, mg/kg, *Max*	3.0	IS 17924
xvi.	Endrin, mg/kg, *Max*	0.05	IS 17924
xvii.	Ethion, mg/kg, *Max*	2.0	IS 17924
xviii.	Fenitrothion, mg/kg, *Max*	0.5	IS 17924
xix.	Fenvalerate, mg/kg, *Max*	1.5	IS 17924
xx.	Fonofos, mg/kg, *Max*	0.05	IS 17924
xxi.	Heptachlor (combined residues of heptachlor and its epoxide to be determined and expressed Milled as Heptachlor), mg/kg, *Max*	0.05	IS 17924
xxii.	Hexachlorobenzene, mg/kg, *Max*	0.1	IS 17924
xxiii.	Hexachlorocyclohexane isomers (other than γ), mg/kg, *Max*	0.3	IS 17924
xxiv.	Lindane (Gamma Hexachlorocyclohexane), mg/kg, *Max*	0.6	IS 17924
xxv.	Malathion, mg/kg, *Max*	1.0	IS 17924
xxvi.	Methidathion, mg/kg, *Max*	0.2	IS 17924
xxvii.	Parathion, mg/kg, *Max*	0.5	IS 17924
xviii.	Parathion Methyl, mg/kg, *Max*	0.2	IS 17924
xxix.	Permethrin, mg/kg, *Max*	1.0	IS 17924
xxx.	Phosalone, mg/kg, *Max*	0.1	IS 17924
xxxi.	Piperonylbutoxide, mg/kg, *Max*	3.0	IS 17924
xxxii.	Pirimiphos- methyl, mg/kg, *Max*	4.0	IS 17924
xxiii.	Pyrethrin (sum of), mg/kg, *Max*	3.0	IS 17924
xxiv.	Quintozene (sum of quintozene, pentachloroaniline and methyl Pentachlorophenyl sulphide), mg/kg, *Max*	1.0	IS 17924
10.	**Heavy Metals**		
i.	Lead (as Pb)	10.0 mg/kg, max	IS 16913
ii.	Arsenic (as As)	3.0 mg/kg, max	IS 16913
iii.	Cadmium (as Cd)	0.3 mg/kg, max	IS 16913
iv.	Mercury (as Hg)	1.0 mg/kg, max	IS 16913
11.	**Mycotoxin Parameters:**		
i.	Total Aflatoxin (B1+B2+G1+G2) ng/kg, *Max*	5	IS 16287
ii.	Aflatoxin B1 ng/kg, *Max*	2	IS 16287

NOTE — QUALITY OF REAGENTS

1. Reagents, including pure chemicals used, shall be of analytical grade.
2. Reagent grade water for laboratory use shall be as per IS 1070.
3. 'Pure chemicals' shall mean chemicals that do not contain impurities that affect the analysis results.

4 PACKING, STORAGE AND MARKING

4.1 Packing

Digitalis purpurea leaves shall be packed in clean, sound, and dry containers made of metal, glass, food-grade polymers, wood, or jute bags. The wooden boxes or jute bags shall be suitably lined with moisture-proof lining, which does not impart any foreign smell to the product. The packing material shall be free from any fungal or insect infestation and should not impart any foreign smell. Each container shall be securely closed and sealed.

4.2 Storage

Digitalis purpurea leaves shall be stored under conditions that prevent contamination and, as far as possible, deterioration. The storage area shall be clean, well-ventilated and protected from direct sunlight, moisture, insects and rodents. A leaflet containing instructions for storage shall be enclosed with each packing.

4.3 Marking

The following particulars shall be legibly and indelibly marked or labeled on each pack of *Digitalis purpurea* plant:

a) Name of the material, including part of the plant, botanical name and trade name or brand name, if any;
b) Name and address of the producer or packer, including contact details;
c) State and country of production (if known);
d) Batch number;
e) Net quantity;
f) Date of packing (MM/YYYY);
g) Date of expiry or use before (MM/YYYY);
h) QR code for authentication (optional);
j) Instructions for storage; and k) Any other information requested by the buyer, such as the date of harvesting (MM/YYYY) (if known).

5 SAMPLING

5.1 Representative samples of the material shall be drawn and tested for conformity to the specifications prescribed in IS 13145.

5.2 The samples of *Digitalis purpurea* leaves shall be tested for ascertaining conformity of the material to the requirements in accordance with the relevant clauses given in column 4 of Table 1.

6 BIS Certification Marking

The product(s) conforming to the requirements of this standard may be certified as per the conformity assessment schemes under the provisions of the Bureau of Indian Standards Act, 2016 and the Rules and Regulations framed there under and the product(s) may be marked with the Standard Mark.

ANNEX-A

IDENTIFICATION OF DIGITALIS PURPUREA (LEAVES)
[Table 1, Sl No. (7 & 8)]

A-1: GENERAL IDENTIFICATION (REACTION TEST):

Test-A (*Ref. GHP***)**

Solution **S**: To 10 ml of the mother tincture, add 20 ml of water and 10 ml of lead acetate solution (10 % w/v solution of lead acetate in carbon dioxide free water). Shake, leave to stand for 5 min and filter. Shake the filtrate with 2x 15 mL portions of ethyl acetate and evaporate the combined organic phases to dryness under reduced pressure at a temperature not exceeding 50˚. Dissolve the residue in 1 mL of mixture of equal volumes of ethyl acetate and methanol (stock solution) following tests to be performed.

A. Carefully evaporate 0.2 mL of stock solution to dryness on a water bath. Dissolve the residue in 0.2 mL of dinitrobenzoic acid solution (2% w/v solution of dinitrobenzoic acid in ethanol 95%) and add 0.2 mL of dilute sodium hydroxide solution. A reddish-violet colour is produced.

B. Carefully evaporate 0.1 mL of stock solution to dryness on a water bath and add 0.3 ml of mixture of 2 ml of *acetic anhydride* and add 0.3 ml of *sulphuric acid*. A yellow colour and then a muddy green colour is produced.

B- 1 THIN LAYER CHROMATOGRAPHY (TLC)

B-1.1 Apparatus

B-1.1.1 *Thin Layer chromatography with a Precoated Silica Gel 60 F_{254} Plate*

B-1.1.2 *Analytical Balance*

B-1.1.3 *Standard Glassware*

B-1.1.4 *Water Bath*

B-1.2 Reagents

B-1.2.1 *Chloroform – AR or Equivalent Grade*

B-1.2.2 *Ethanol – AR or Equivalent Grade*

B-1.2.3 *Standard Digitoxin – AR or Equivalent Grade*

B-1.2.4 *Methanol – AR or Equivalent Grade*

B-1.2.5 *Ethyl acetate – AR or Equivalent Grade*

B-1.2.6 *Anisaldehyde sulphuric acid–AR or Equivalent Grade*

B-1.2.7*Purified Water- IP grade or Equivalent grade*

B-1.3 Procedure

B-1.3.1 *Preparation of Hydro-alcoholic extract*

To 5 g of the substance being examined, add 24 ml of ethanol and 29 ml of purified water. Allow to stand for twenty four hours, shake and filter. Evaporate 25 ml of Mother Tincture on water bath to remove alcohol and extract with 3x20 ml of chloroform. Combine and concentrate chloroform layer to 2 ml. Carry out TLC of chloroform extract in silica gel 60 F_{254} pre- coated plate.

B-1.3.2 *Preparation of standard Digitoxin*

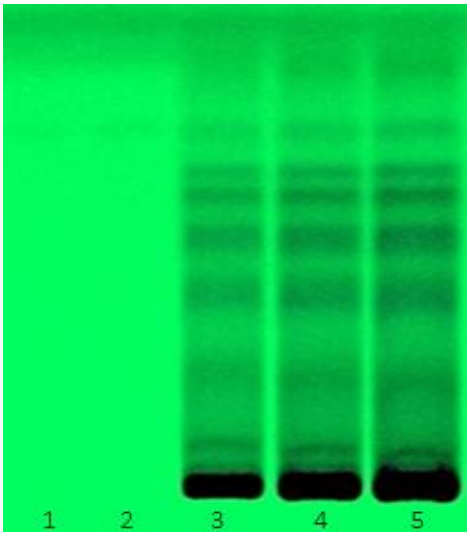
Dissolve 5 mg of Digitoxin in 10 ml methanol in a volumetric flask and sonicate for 10 min to prepare a working standard of Digitoxin with a concentration of 1 mg/ml.

B-1.3.3 *Mobile phase*

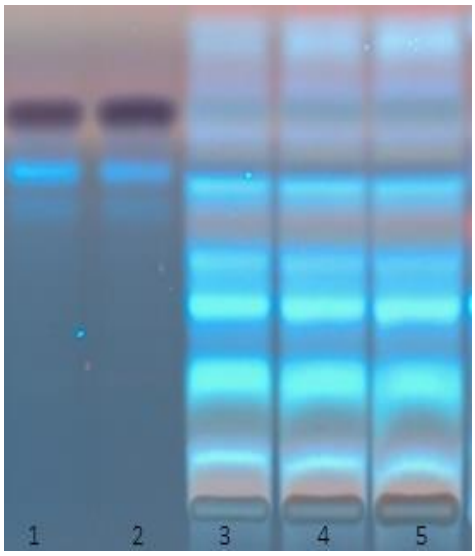
Ethyl acetate: Methanol: Water (8:1.5: 1, v/v/v)

B-1.3.4 *Estimation*

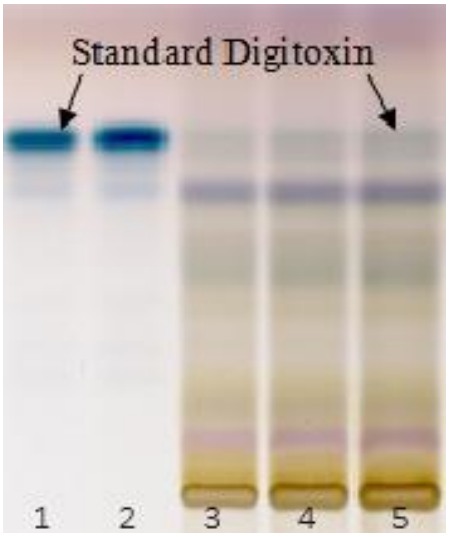
A. Apply 10µl each test solution on a TLC plate as bands of 10 mm. Develop the plate to a distance of 80 mm from the line of application. Dry the plate in air and examine under UV light 254 nm five brown spots appear at R_f. 0.22, 0.37, 0.46, 0.55 and 0.64
B. Under UV light 366 nm, six blue spots appear at R_f 0.15, 0.31, 0.47, 0.56 and 0.64 (Standard digitoxin).
C. After derivatization with Anisaldehyde sulphuric acid reagent, three spots appear at R_f. 0.19 (purple), 0.54 (purple) and 0.64 (blue) (standard digitoxin).



UNDER 254 NM
TLC PROFILE OF *DIGITALIS PURPUREA*



UNDER 366 NM
TLC PROFILE OF *DIGITALIS PURPUREA*



AFTER DERIVATIZATION WITH *ANISALDEHYDE SULPHURIC ACID* REAGENT
TLC PROFILE OF *DIGITALIS PURPUREA*

ANNEX B

[*Table* 1, *Sl No.* (XIV to XX), *Col* 4]

MICROBIAL LIMIT TESTS

A-1 GENERAL

The tests are designed for the estimation of the number of viable aerobic microorganisms present and for detecting the presence of designated microbial species in the extract. The term 'growth' is used to designate the presence and presumed proliferation of viable microorganisms.

A-2 APPARATUS

A-2.1 Oven for Dry Sterilization
A-2.2 Autoclave for Wet Sterilization
A-2.3 Incubator
A-2.4 Water Bath
A-2.5 pH-meter
A-2.6 Sterile Membrane Filters, 50 mm in Diameter
A-2.7 Colony Counting Equipment
A-2.8 Analytical Balance
A-2.9 Standard Glassware

A-3 MEDIA

Culture media may be prepared as given below or dehydrated culture media may be used provided that, when reconstituted as directed by the manufacturer, they have similar ingredients and / or yield media comparable to those obtained from the formulae given below.

Where agar is specified in a formula, use agar that has a moisture content of not more than 15 percent. Where water is called for in a formula, use purified water. Unless otherwise indicated, the media should be sterilized by heating in an autoclave (15 psi) at 121 °C for 15 min. In preparing media by the formulas given below, dissolve the soluble solids in the water, using heat if necessary, to effect complete solution, add solutions of 0.1N hydrochloric acid or 0.1N sodium hydroxide in quantities sufficient to yield the required *p*H in the medium when it is ready for use. Determine the *p*H at 25°C± 2°C.

A-3.1 Baird Parker Agar Medium

A-3.1.1 *Composition*

Pancreatic digest of casein	10.0 g
Beef extract	5.0 g
Yeast extract	1.0 g
Lithium chloride	5.0 g
Agar	20.0 g
Glycine	12.0 g
Sodium pyruvate	10.0 g
Water	1000 ml

A-3.1.2 Preparation

Suspend the components in 1000 ml of water, heat with frequent agitation and boil for 1 min. Sterilize, cool in between 45 °C to 50 °C, add 10 ml of a one percent w/v solution of sterile potassium tellurite and 50 ml of egg yolk emulsion. Mix thoroughly, but gently and pour into plates. (Prepare the egg yolk emulsion by disinfecting the surface of whole shell eggs, aseptically cracking the eggs, and separating out intact yolks into a sterile graduated cylinder. Add sterile saline solution, get a 3 to 7 ratio of egg yolk to saline. Add to a sterile blender cup and mix at high speed for 5 seconds). Adjust the pH after sterilization to 6.8 ± 0.2.

A-3.2 Bismuth Sulphite Agar Medium

A-3.2.1 Composition

Solution (1)

Beef extract	6 g
Peptone	10 g
Agar	24 g
Ferric citrate	0.4 g
Brilliant green	10 mg
Water	1000 ml

Solution (2)

Ammonium bismuth citrate	3 g
Sodium sulphite	10 g
Anhydrous disodium hydrogen phosphate	5 g
Dextrose monohydrate	5 g
Water	100 ml

A-3.2.2 Preparation

Suspend the components of Solution 1 in 1000 ml of water. Heat to boiling to dissolve the medium completely. Sterilize by maintaining at 115 °C for 30 min. Suspend the components of Solution 2 in 100 ml of water. Heat to boiling to dissolve the medium completely. Do not autoclave. Add 1 volume of solution 2 to 10 volumes of solution 1 previously melted and cooled to a temperature of 55 °C. Bismuth Sulphite Agar Medium should be stored at 2°Cto 8°C for 5 days before use.

A-3.3 Brilliant Green Agar Medium

A-3.3.1 Composition

Peptone	10.0 g
Yeast extract	3.0 g
Lactose	10.0 g
Sucrose	10.0 g
Sodium chloride	5.0 g
Phenol red	80.0 g
Brilliant green	12.5 mg
Agar	12.0 g
Water	1000 ml

A-3.3.2 Preparation

Mix the components, allow to stand for 15 min, sterilize by maintaining at 115 °C for 30 min and mix before pouring.

A-3.4 Buffered Sodium Chloride Peptone Solution pH 7.0

A-3.4.1 Composition

Potassium dihydrogen phosphate	3.56 g
Disodium hydrogen phosphate	7.23 g
Sodium chloride	4.30 g
Peptone (meat or casein)	1.0 g
Water	1000 ml

A-3.4.2 Preparation

Mix the components and heat if necessary to dissolve the medium completely. 0.1 to 1.0 percent w/v Polysorbate 20 or polysorbate 80 may be added. Sterilize by heating in an autoclave at 121°C for 15 min.

A-3.5 Casein Soyabean Digest Agar Medium

A-3.5.1 Composition

Pancreatic digest of casein	15.0 g
Papaic digest of soyabean meal	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1000 ml

A-3.5.2 Preparation

Suspend the components in water. Heat to boiling to dissolve the medium completely. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.3 ± 0.2.

A-3.6 Cetrimide Agar Medium

A-3.6.1 Composition

Pancreatic digest of gelatin	20.0 g
Magnesium chloride	1.4 g
Potassium sulphate	10.0 g
Cetrimide	0.3 g
Agar	13.6 g
Glycerin	10.0 g
Water	1000 ml

A-3.6.2 Preparation

Dissolve the components in 1000 ml of water. Heat to boiling for 1 min with shaking. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.0 to 7.4.

A-3.7 Deoxycholate Citrate Agar Medium

A-3.7.1 Composition

Beef extract	5.0 g
Peptone	5.0 g
Lactose	10.0 g
Trisodium citrate	8.5 g
Sodium thiosulphate	5.4 g
Ferric citrate	1.0 g
Sodium deoxycholate	5.0 g
Neutral red	0.02 g
Agar	12.0 g
Water	1000 ml

A-3.7.2 Preparation

Mix the components and allow to stand for 15 min. Gently boil with continuous stirring and continue boiling until solution is complete. Cool to 80 °C, mix, pour and cool rapidly.

Care should be taken not to overheat Deoxycholate Citrate Agar during preparation. It should not be re-melted and the surface of the plates should be dried before use.

A-3.8 Fluid Casein Digest Soya Lecithin Polysorbate 20 Medium

A-3.8.1 Composition

Pancreatic digest of casein	20 g
Soya lecithin	5 g
Polysorbate 20	40 ml
Water	1000 ml

A-3.8.2 Preparation

Dissolve the pancreatic digest of casein and soya lecithin in water, heating in a water bath at 48 °C to 50 °C for about 30 min to effect solution. Add polysorbate 20, mix and dispense as desired. Sterilize at 121 °C for 15 min in an autoclave.

A-3.9 Fluid Lactose Medium

A-3.9.1 Composition

Beef extract	3.0 g
Pancreatic digest of gelatin	5.0 g
Lactose	5.0 g
Water	1000 ml

A-3.9.2 Preparation

Suspend the components in 1000 ml water. Heat if necessary to dissolve the medium completely. Sterilize at 121 °C for 15 min in an autoclave. Cool as quickly as possible after sterilization. Adjust the pH after sterilization to 6.9 ± 0.2.

A-3.10 Lactose Broth Medium

A-3.10.1 Composition

Beef extract	3.0 g
Pancreatic digest of gelatin	5.0 g
Lactose	5.0 g
Water	1000 ml

A-3.10.2 Preparation

Suspend the components in water and heat if necessary to dissolve the medium completely. Sterilize at 121 °C for 15 min in an autoclave. Adjust the pH after sterilization to 6.9 ± 0.2.

A-3.11 Levine Eosin Methylene Blue Agar Medium

A-3.11.1 Composition

Pancreatic digest of gelatin	10.0 g
Dibasic potassium phosphate	2.0 g
Agar	15.0 g
Lactose	10.0 g
Eosin Y	400 mg
Methylene blue	65 mg
Water	1000 ml

A-3.11.2 Preparation

Dissolve the pancreatic digest of gelatin, dibasic potassium phosphate and agar in water with warming and allow to cool. Just prior to use, liquify the gelled agar solution and the remaining ingredients, as solutions, in the following amounts and mix. For each 100 ml of the liquified agar solution use 5 ml of a 20 percent w/v solution of lactose, 2 ml of a 2 percent w/v solution of eosin Y and 2 ml of a 0.33 percent w/v solution of methylene blue. The finished medium may not be clear. Adjust the pH after sterilization to 7.1±0.2.

A-3.12 MacConkey Agar Medium

A-3.12.1 Composition

Pancreatic digest of gelatin	17.0 g
Peptone (meat and casein, equal parts)	3.0 g
Lactose	10.0 g
Sodium chloride	5.0 g
Bile salts	1.5 g
Agar	13.5 g
Neutral red	30 mg
Crystal violet	1 mg

Water	1000 ml

A-3.12.2 Preparation

Boil the mixture of solids and water for 1 min to effect solution. Sterilize at 121 °C for 15 min in an autoclave. Adjust the pH after sterilization to 7.1 ± 0.2.

A-3.13 MacConkey Broth Medium

A-3.13.1 Composition

Pancreatic digest of gelatin	20.0 g
Lactose	10.0 g
Dehydrated ox bile	5.0 g
Bromocresol purple	10 mg
Water	1000 ml

A-3.13.2 Preparation

Suspend the components in 1000 ml of water and heat if necessary to dissolve the medium completely. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.3 ± 0.2.

A-3.14 Mannitol Salt Agar Medium

A-3.14.1 Composition

Pancreatic digest of gelatin	5.0 g
Peptic digest of animal tissue	5.0 g
Beef extract	1.0 g
D-Mannitol	10.0 g
Sodium chloride	75.0 g
Agar	15.0 g
Phenol red	25 mg
Water 1000 ml	

A-3.14.2 Preparation

Mix the components, heat with frequent agitation and boil for 1 min to effect solution. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.4 ± 0.2.

A-3.15 Nutrient Broth Medium

A-3.15.1 Composition

Beef extract	10.0 g
Peptone	10.0 g
Sodium chloride	5 mg
Water	1000 ml

A-3.15.2 Preparation

Dissolve the components with the aid of heat. Adjust the pH to 8.0 to 8.4 with 5M sodium hydroxide and boil for 10 min. Filter and sterilize by maintaining at 115 °C for 30 min and adjust pH to 7.3 ± 0.1.

A-3.16 Nutrient Agar Medium

Nutrient broth gelled by the addition of 1 to 2 percent w/v of agar.

A-3.17 Pseudomonas Agar Medium for Detection of Flourescein

A-3.17.1 Composition

Pancreatic digest of casein	10.0 g
Peptic digest of animal tissue	10.0 g
Anhydrous dibasic potassium phosphate	1.5 g
Magnesium sulphatehepta hydrate	1.5 g
Glycerin	10.0 ml
Agar	15.0 g
Water	1000 ml

A-3.17.2 Preparation

Dissolve the solid components in water before adding glycerin. Heat with frequent agitation and boil for 1 min to effect solution. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.2 ± 0.2.

A-3.18 Pseudomonas Agar Medium for Detection of Pyocyanin

A-3.18.1 Composition

Pancreatic digest of gelatin	20.0 g
Anhydrous magnesium chloride	1.4 g
Anhydrous potassium sulphate	10.0 g
Agar	15.0 g
Glycerin	10.0 ml
Water	1000 ml

A-3.18.2 Preparation

Dissolve the solid components in water before adding glycerin. Heat with frequent agitation and boil for 1 min to effect solution. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.2 ± 0.2.

A-3.19 Sabouraud Dextrose Agar Medium

A-3.19.1 Composition

Dextrose	40 g
Peptic digest of animal tissue and Pancreatic digest of casein (1:1)	10 g
Agar	15 g
Water	1000 ml

A-3.19.2 Preparation

Mix the components and heat to boiling to dissolve completely. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 5.6 ± 0.2.

A-3.20 Sabouraud Dextrose Agar Medium with Antibiotics

To 1000 ml of Sabouraud Dextrose Agar Medium, add 0.1 g of benzylpenicillin sodium and 0.1 g of tetracycline HCL or alternatively add 50 mg of chloramphenicol immediately before use.

A-3.21 Selenite F Broth

A-3.21.1 Composition

Peptone	5 g
Lactose	4 g
Disodium hydrogen phosphate	10 g
Sodium hydrogen selenite	4 g
Water	1000 ml

A-3.21.2 Preparation

Suspend the components in water and mix well. Warm to dissolve the medium completely. Distribute in sterile containers and sterilize by maintaining at 100 °C for 30 min.

A-3.22 Fluid Selenite Cystine Medium

A-3.22.1 Composition

Pancreatic digest of casein	5.0 g
Lactose	4.0 g
Sodium phosphate	10.0 g
Sodium hydrogen selenite	4.0 g
l-Cystine	10.0 mg
Water	1000 ml

A-3.22.2 Preparation

Suspend the components in water and heat in flowing steam for 15 min. Adjust the final pH to 7.0 ± 0.2. Do not sterilize.

A-3.23 Tetrathionate Broth Medium

A-3.23.1 Composition

Beef extract	0.9 g
Peptone	4.5 g
Yeast extract	1.8 g
Sodium chloride	4.5 g
Calcium carbonate	25.0 g
Sodium thiosulphate	40.7 g
Water 1000 ml	

A-3.23.2 Preparation

Dissolve the solids in 1000 ml of water and heat the solution to boil. On the day of use, add a solution prepared by dissolving 5 g of potassium iodide and 6 g of iodine in 20 ml of water.

A-3.24 Tetrathionate Bile Brilliant Green Broth Medium

A-3.24.1 Composition

Peptone	8.6 g
Dehydrated ox bile	8.0 g
Sodium chloride	6.4 g
Calcium carbonate	20.0 g
Potassium tetrathionate	20.0 g
Brilliant green	70 mg
Water	1000 ml

A-3.24.2 Preparation

Suspend the components in 1000 ml of water. Heat just to boiling. Do not autoclave or reheat. Adjust the pH so that after heating it is 7.0 ± 0.2.

A-3.25 Triple Sugar Iron Agar Medium

A-3.25.1 Composition

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Lactose	10.0 g
Sucrose	10.0 g
Dextrose monohydrate	1.0 g
Ferrous sulphate	0.2 g
Sodium chloride	5.0 g
Sodium thiosulphate	0.3 g
Phenol red	24 mg
Water	1000 ml

A-3.25.2 Preparation

Mix the components in 1000 ml of water, allow standing for 15 min. Heat to boiling to dissolve the medium completely. Mix well and distribute into test tubes and Sterilize by maintaining at 121 °C for 15 min. Allow the medium to stand in a sloped form with a butt about 2.5 cm long.

A-3.26 Urea Broth Medium

A-3.26.1 Composition

Potassium dihydrogen orthophosphate	9.1 g
Anhydrous disodium hydrogen phosphate	9.5 g
Urea	20.0 g
Yeast extract	0.1 g

Phenol red	10 mg
Water	1000 ml

A-3.26.2 Preparation

Mix the components, sterilize by filtration and distribute aseptically in sterile containers.

A-3.27 Vogel Johnson Agar Medium

A-3.27.1 Composition

Pancreatic digest of casein	10.0 g
Yeast extract	5.0 g
Mannitol	10.0 g
Dibasic potassium phosphate	5.0 g
Lithium chloride	5.0 g
Glycerin	10.0 g
Agar	16.0 g
Phenol red	25.0 mg
Water	1000 ml

A-3.27.2 Preparation

Suspend the components in 1000 ml of water. Boil the solution of solids for 1 min. Sterilize at 121 °C for 15 min in an autoclave. Cool to 45 °C to 50 °C and add 20 ml of 1 percent w/v sterile solution of potassium tellurite. Adjust the pH after sterilization to 7.0 ± 0.2.

A-3.28 Xylose Lysine Deoxycholate Agar Medium

A-3.28.1 Composition

Xylose	3.5 g
l-Lysine	5.0 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	80 mg
Agar	13.5 g
Sodium deoxycholate	2.5 g
Sodium thiosulphate	6.8 g
Ferric ammonium citrate	800 mg
Water	1000 ml

A-3.28.2 Preparation

Suspend the components in 1000 ml of water. Heat with frequent agitation until the medium boils. Do not overheat or sterilize. Transfer at once to a water bath maintained at about 50 °C and pour into plates as soon as the medium has cooled. Adjust the final pH to 7.4 ± 0.2.

A-4 SAMPLING

Use 10 ml or 10 g specimens for each of the tests specified in the Indian Standard.

A-5 PRECAUTIONS

The microbial limit tests should be carried out under conditions designed to avoid accidental contamination during the test. The precautions taken to avoid contamination must be such that they do not adversely affect any microorganisms that should be revealed in the test.

A-6 PRELIMINARY TESTING

Prior to doing the tests, inoculate diluted extracts being examined with separate viable cultures of *Escherichia coli, Salmonella species, Pseudomonas aeruginosa* and *Staphylococcus aureus*. This is done by adding 1 ml of 24 h broth culture containing not less than 1000 microorganisms to the first dilution (in buffer solution pH 7.2, fluid soyabean casein digest medium or fluid lactose medium) of the test material and following the test procedure. If the organisms fail to grow in the relevant medium the procedure should be modified by (a) increasing the volume of diluent with the quantity of test material remaining the same, or (b) incorporating a sufficient quantity of a suitable inactivating agent in the diluents, or (c) combining the afore mentioned modifications so as to permit growth of the organisms in the media. If inhibitory substances are present in the extracts, 0.5 percent of soya lecithin and 4 percent of polysorbate 20 may be added to the culture medium. Alternatively, repeat the test as described in the previous paragraph, using fluid casein digest soya lecithin polysorbate 20 medium, to demonstrate neutralization of preservatives or other antimicrobial agents in the test material. Where inhibitory substances are contained in the extracts and the latter is soluble, the membrane filtration method described under Total Aerobic Microbial Count may be used. If in spite of incorporation of suitable inactivating agents and a substantial increase in the volume of diluent it is still not possible to recover the viable cultures described above and where the article is not suitable for applying the membrane filtration method, it can be assumed that the failure to isolate the inoculated organism may be due to the bactericidal activity of the product. This may indicate that the article is not likely to be contaminated with the given species of microorganisms. However, monitoring should be continued to establish the spectrum of inhibition and bactericidal activity of the article.

A-7 TOTAL AEROBIC MICROBIAL COUNT

Pre-treat the extracts and raw materials being examined as described below.
NOTE –The raw materials need to be ground as a coarse powder before analysis.

A-7.1 Water Soluble Products

Dissolve 10 g or dilute 10 ml of the extract preparation being examined, unless otherwise specified, in buffered sodium chloride peptone solution pH 7.0 or any other suitable medium shown to have no antimicrobial activity under the conditions of test and adjust the volume to 100 ml with the same medium. If necessary, adjust the pH to about 7.

A-7.2 Products Insoluble in Water (Non Fatty)

Suspend 10 g or 10 ml of the extract preparation being examined, unless otherwise specified,

in buffered sodium chloride peptone solution pH 7.0 or any other suitable medium shown not to have antimicrobial activity under the conditions of the test and dilute to 100 ml with the same medium. If necessary, divide the preparation being examined and homogenize the suspension mechanically. A suitable surface active agent such as 0.1 percent w/v of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary, adjust pH of the suspension to about 7.

A-7.3 Fatty Products

Homogenise 10 g or 10 ml of the extract preparation being examined, unless otherwise specified, with 5 g of polysorbate 20 or polysorbate 80. If necessary, heat to not more than 40 °C. Mix carefully while maintaining the temperature in the water bath or in an oven. Add 85 ml of buffered sodium chloride peptone solution pH 7.0 or any other suitable medium shown to have no antimicrobial activity under the conditions of the test, heated to not more than 40 °C if necessary. Maintain this temperature for the shortest time necessary for formation of an emulsion and in any case for not more than 30 min. If necessary, adjust the pH to about 7.

A-7.4 Examination of the Sample

Determine the total aerobic microbial count in the extract being examined by any of the following methods.

A-7.5 Membrane Filtration

Use membrane filters 50 mm in diameter and having a nominal pore size not greater than 0.45 μm the effectiveness of which in retaining bacteria has been established for the type of preparation being examined. Transfer 10 ml or a quantity of each dilution containing 1g of the preparation being examined to each of two membrane filters and filter immediately. If necessary, dilute the pretreated extract preparation so that a colony count of 10 to 100 may be expected. Wash each membrane by filtering through it three or more successive quantities, each of about 100 ml, of a suitable liquid such as buffered sodium chloride peptone solution pH 7.0.For fatty substances add to the liquid polysorbate 20 or polysorbate 80.Transfer one of the membrane filters, intended for the enumeration of bacteria, to the surface of a plate of casein soyabean digest agar and the other, intended for the enumeration of fungi, to the surface of a plate of Sabouraud dextrose agar with antibiotics.

Incubate the plates for 5 days, unless a more reliable count is obtained in shorter time, at 30 °C to 35 °C in the test for bacteria and 20 °C to 25 °C in the test for fungi. Count the number of colonies that are formed. Calculate the number of microorganisms per g or per ml of the extract preparation being examined, if necessary count bacteria and fungi separately.

A-7.6 Plate Count for Bacteria

Using Petri dishes 9 to 10 cm in diameter, add to each dish a mixture of 1 ml of the pretreated extract preparation and about 15 ml of liquified casein soyabean digest agar at not more than 45 °C.

Alternatively, spread the pretreated extract preparation on the surface of the solidified medium in a petri dish of the same diameter. If necessary, dilute the pretreated extract preparation as described

above so that a colony count of not more than 300 may be expected. Prepare at least two such petri dishes using the same dilution and incubate at 30 °C to 35 °C for 5 days, unless a more reliable count is obtained in a shorter time. Count the number of colonies that are formed. Calculate the results using plates with the greatest number of colonies but taking 300 colonies per plate as the maximum consistent with good evaluation.

A-7.7 Plate Count for Fungi

Proceed as described in the test for bacteria but use Sabouraud dextrose agar with antibiotics in place of casein soyabean digest agar and incubate the plates at 20 °C to 25°Cfor 5 days, unless a more reliable count is obtained in a shorter time. Calculate the results using plates with not more than 100 colonies.

A-7.8 Multiple Tube or Serial Dilution Method

In each of fourteen test tubes of similar size place 9.0 ml of sterile fluid soyabean casein digest medium. Arrange twelve of the tubes in four sets of three tubes each. Put aside one set of three tubes to serve as controls. Into each of three tubes of one set ("100") and into fourth tube (A) pipette 1 ml of the solution of suspension of the test specimen (extract) and mix. From tube A pipette 1 ml of its contents into the one remaining tube (B) not included in the set and mix. These two tubes contain 100 mg (or 100 µl) and 10 mg (or 10 µl) of the specimen respectively. Into each of the second set ("10") of three tubes pipette 1 ml from tube A, and into each tube of the third set ("1") pipette 1 ml from tube B. Discard the unused contents of tube A and B. Close well and incubate all of the tubes.

Following the incubation period, examine the tubes for growth. The three control tubes remain clear. Observations in the tubes containing the test specimen, when interpreted by reference to Table 2, indicate the most probable number of microorganisms per g or per ml of the test specimen.

Table 2
Most probable Total Count by Multiple Tube or Serial Dilution Method
(Clause A-7.8)

S.No.	**Observed Combination of Numbers of Tubes Showing Growth in Each Set**			**Most Probable Number of Microorganisms per g or per ml**
	Number of mg (or ml) of Specimen per Tube			
	100 (100 µL)	10 (10 µL)	1 (1 µL)	
(1)	(2)	(3)	(4)	(5)
i.	3	3	3	>1100
ii.	3	3	2	1100
iii.	3	3	1	500
iv.	3	3	0	200
v.	3	2	3	290
vi.	3	2	2	210
vii.	3	2	1	150

viii.	3	2	0	90
ix.	3	1	3	160
x.	3	1	2	120
xi.	3	1	1	70
xii.	3	1	0	40
xiii.	3	0	3	95
xiv.	3	0	2	60
xv.	3	0	1	40
xvi.	3	0	0	23

A-8 TESTS FOR SPECIFIED MICROORGANISMS

A-8.1 Pre-treatment of the Extract Sample Being Examined

Proceed as described under the test for total aerobic microbial count but using lactose broth or any other suitable medium shown to have no antimicrobial activity under the conditions of test in place of buffered sodium chloride peptone solution pH 7.0.

A-8.2 Escherichia coli

Place the prescribed quantity in a sterile screw capped container, add 50 ml of nutrient broth, shake, allow to stand for 1 h (4 h for gelatin) and shake again. Loosen the cap and incubate at 37 °C for 18 to 24 h.

A-8.2.1 Primary Test

Add 1 ml of the enrichment culture to a tube containing to 10 ml MacConkey broth and a Durham tube. Incubate at 42 to 44°C for 24-48 hours. If the contents of the tube show acid and gas production in Durham tube, carry out the secondary test.

A-8.2.2 Secondary Test

Add 0.1 ml of the contents of the tubes containing (a) 5 ml of MacConkey broth and (b) 5 ml of peptone water. Incubate in a water bath at 43.5 °C to 44.5 °C for 24 h and examine tube (a) for acid and gas and tube (b) for indole. To test for indole, add 0.5 ml of Kovac's reagent, shake well and allow to stand for 1 min. If a red colour is produced in the reagent layer indole is present. The presence of acid and gas and of indole in the secondary test indicates the presence of Escherichia coli.

Carry out a control test by repeating the primary and secondary tests, adding 1.0 ml of the enrichment culture and a volume of broth containing 10 to 50 Escherichia coli (NCTC 9002) organisms, prepared from a 24 h culture in nutrient broth, to 5 ml of MacConkey broth. The test is not valid unless the results indicate that the control contains Escherichia coli.

A-8.2.3 Alternative Test

By means of an inoculating loop, streak a portion from the enrichment culture (obtained in the previous test) on the surface of MacConkey agar medium. Cover and invert the dishes and incubate.

Upon examination, if none of the colonies are brick red in colour and have a surrounding zone of precipitated bile the sample meets the requirements of the test for the absence of Escherichia coli. If the colonies described above are found, transfer the suspect colonies individually to the surface of Levine eosin methylene blue agar medium, plated on petri dishes. Cover and invert the plates and incubate. Upon examination, if none of the colonies exhibits both a characteristic metallic sheen under reflected light and a blue black appearance under transmitted light, the sample meets the requirements of the test for the absence of Escherichia coli. The presence of Escherichia coli may be confirmed by further suitable cultural and biochemical tests.

A-8.3 Salmonella

Transfer a quantity of the pretreated preparation being examined containing 10 g or 10 ml of the product to 100 ml of nutrient broth in a sterile screw capped jar, shake, allow to stand for 4 h and shake again. Loosen the cap and incubate at 35 °C to 37 °C for 24 h.

A-8.3.1 Primary Test

Add 1.0 ml of the enrichment culture to each of the two tubes containing (a) 10 ml of selenite F broth and (b) tetrathionate bile brilliant green broth and incubate at 36 °C to 38 °C for 48 h. From each of these two cultures subculture on at least two of the following four agar media: bismuth sulphate agar, brilliant green agar, deoxycholate citrate agar and xylose lysine deoxycholate agar. Incubate the plates at 36 °C to 38 °C for 18 to 24 h. Upon examination, if none of the colonies conforms to the description given in Table 3, the sample meets the requirements of the test for the absence of the genus Salmonella. If any colonies conforming to the description in Table 3 are produced, carry out the secondary test.

Table 3
Interpretation of the Confirmatory Tests for Salmonella
(Clause A-8.3.1)

S.No.	Medium	Description of Colony
(1)	(2)	(3)
i.	Bismuth sulphite agar	Black or green
ii.	Brilliant green agar	Small, transparent and colorless, or opaque, pinkish or white (frequently surrounded by a pink or red zone)
iii.	Deoxycholate citrate agar	Colorless and opaque, with or without black centers
iv.	Xylose lysine deoxycholate agar	Red with or without black centres

A-8.3.2 Secondary Test

Subculture any colonies showing the characteristics given in Table 3 in triple sugar iron agar by first inoculating the surface of the slope and then making a stab culture with the same inoculating needle, and at the same time inoculate a tube of urea broth. Incubate at 36 °C to 38 °C for 18 to 24 h. The formation of acid and gas in the stab culture (with or without concomitant blackening) and the absence of acidity from the surface growth in the triple sugar iron agar, together with the

absence of a red color in the urea broth, indicate the presence of Salmonella. If acid but no gas is produced in the stab culture, the identity of the organisms should be confirmed by agglutination tests.

Carry out the control test by repeating the primary and secondary tests using 1.0 ml of the enrichment culture and a volume of broth containing 10 to 50 Salmonella abony (NCTC 6017) organisms, prepared from a 24 h culture in nutrient broth, for the inoculation of the tubes (a) and (b). The test is invalid unless the results indicate that the control contains Salmonella.

A-8.4 Pseudomonas aeruginosa

Pretreat the preparation being examined as described above and inoculate 100 ml of fluid soyabean casein digest medium with a quantity of the solution, suspension or emulsion thus obtained containing 1 g or 1 ml of the preparation being examined. Mix and incubate at 35 °C to 37 °C for 24 to 48 h. Examine the medium for growth and if growth is present, streak a portion of the medium on the surface of cetrimide agar medium, each plated on petri dishes. Cover and incubate at 35 °C to 37 °C for 18 to 24 h.

If, upon examination, none of the plates contains colonies having the characteristics listed in Table 4for the media used, the sample meets the requirement for freedom from Pseudomonas aeruginosa. If any colonies conforming to the description in Table 4are produced, carry out the oxidase and pigment tests. Streak representative suspect colonies from the agar surface of cetrimide agar on the surfaces of Pseudomonas agar medium for detection of fluorescein and Pseudomonas agar medium for detection of pyocyanin contained in petri dishes. Cover and invert the inoculated media and incubate at 33 °C to 37 °C for not less than 3 days. Examine the streaked surfaces under ultra violet light. Examine the plates to determine whether colonies conforming to the description in Table 4 are present. If growth of suspect colonies occurs, place 2 or 3 drops of a freshly prepared 1 percent w/v solution of N,N,N1,N1-tetramethyl-4-phenylenediamine dihydrochloride on filter paper and smear with the colony. If there is no development of a pink color, changing to purple, the sample meets the requirements of the test for the absence of Pseudomonas aeruginosa.

Table 4
Interpretation of the Confirmatory Tests for Pseudomonas aeruginosa
(Clause A-8.4)

S.No.	Selective Medium	Characteristic Colonial Morphology	Fluorescence in UV Light	Oxidase Test	Gram Stain
(1)	(2)	(3)	(4)	(5)	(6)
i.	Cetrimide agar	Generally greenish	Greenish	Positive	Negative rods
ii.	Pseudomonas agar medium for detection of fluorescein	Generally colorless to yellowish	Yellowish	Positive	Negative rods
iii.	Pseudomonas agar for detection of pyocyanin	Generally greenish	Blue	Positive	Negative rods

A-8.5 Staphylococcus aureus

Proceed as described under Pseudomonas aeruginosa, if upon examination of the incubated plates, none of them contains colonies having the characteristics listed in for the media used, the sample meets the requirements for the absence of Staphylococcus aureus. If growth occurs, carry out the coagulase test. Transfer representative suspect colonies from the agar surface of any of the media listed in Table 5 to individual tubes, each containing 0.5 ml of mammalian, preferably rabbit or horse, plasma with or without additives

Table 5
Interpretation of the Confirmatory Tests for Staphylococcus aureus
(Clause A-8.5)

S.No.	Selective Medium	Characteristic Colonial Morphology	Gram Stain
(1)	(2)	(3)	(4)
i.	Vogel johnson agar	Black surrounded by yellow zones	Positive cocci (in clusters)
ii.	Mannitol salt agar	Yellow colonies with yellow zones	Positive cocci (in clusters)
iii.	Baird parker agar	Black, shiny, surrounded by clear zones of 2 to 5 mm	Positive cocci (in clusters)

A-8.6 Validity of the Tests for Total Aerobic Microbial Count

Grow the following test strains separately in tubes containing fluid soyabean casein digest medium at 30 °C to 35 °C for 18 to 24 h or, for Candida albicans, at 20°C for 48 h.

Staphylococcus aureus (ATCC 6538; NCTC 10788)
Bacillus subtilis (ATCC 6633; NCIB 8054)
Escherichia coli (ATCC 8739; NCIB 8545)
Candida albicans (ATCC 2091; ATCC 10231)

Dilute portions of each of the cultures using buffered sodium chloride peptone solution pH 7.0 to make test suspensions containing about 100 viable microorganisms per ml. Use the suspension of each of the microorganisms separately as a control of the counting methods, in the presence and absence of the preparation being examined, if necessary.

A count for any of the test organisms differing by not more than a factor of 10 from the calculated value for the inoculum should be obtained. To test the sterility of the medium and of the diluent and the aseptic performance of the test, carry out the total aerobic microbial preparation. There should be no growth of microorganisms.

A-8.7 Validity of the Tests for Specified Microorganisms

Grow separately the test strains of Staphylococcus aureus and Pseudomonas aeruginosa in fluid soyabean casein digest medium and Escherichia coli and Salmonella typhimurium at 30 °C to 35 °C for 18 to 24 h. Dilute portions of each of the cultures using buffered sodium chloride peptone solution pH 7.0 to make test suspensions containing about 103 viable microorganisms per ml. Mix equal volume of each suspension and use 0.4 ml (approximately 102 microorganisms of each

strain) as an inoculum in the test for E. coli, S. typhimurium, P. aeruginosa and S. aureus, in the presence and absence of the extract preparation being examined, if necessary. A positive result for the respective strain of microorganism should be obtained.