भारतीय मानक
Indian Standard

IS 18192 : 2023

पारमपरिक औषधियों में परयोग हेतु अपामागर *(Achyranthes aspera* L.) पंचांग — ंविशिष्ट

Apamarga (*Achyranthes aspera* L.) Whole Plant for use in Traditional Medicine — Specification

ICS 11.120.10

भारतीय मानक ब्यूरो
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FOREWORD

This Indian Standard was adopted by Bureau of Indian Standards, after the draft finalized by Ayush Sectional Committee had been approved by the Ayush Division Council.

Apamarga is a stiff, erect herb of 0.3 m to 0.9 m height, found commonly as a weed throughout India up to 900 m. The plant has been extensively used in traditional medicine as an ingredient of different formulations.

Apamarga is having synonyms like *Mayuraka, Pratyakpushpa, Kharamanjari* and *Shikhari.* Some of the regional names are *Chirchita* (Assamese); *Apamg* (Bengali); Prickly Chaff Flower (English); *Aghedo* (Gujarati); *Chirchita* and *Latjira* (Hindi); *Uttarani* (Kannada); *Katalati* (Malayalam); *Aghada* (Marathi); *Puthakanda* (Punjabi); *Nayuruvi* and *Nayuruvic camulam* (Tamil); *Uttarenu* (Telugu); and *Chirchita* (Urdu).

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 the Ayurvedic Pharmacopoeia of India, Part 1, Vol 2, 1999; Ayurvedic Pharmacopoeia of India, Part 1, Vol 9, 2016; and Siddha Pharmacopoeia of India, Part 1, Vol 1, 2008 published by the Ministry of Ayush, Government of India. Inputs have also been derived from the information available in the public domain in print and electronic media including authoritative books.

In the formulation of this standard due consideration has been given to the provisions of the *Drugs and Cosmetics Act*, 1940 and rules framed thereunder. However, this standard is subject to the restrictions imposed under these Rules and Regulations, wherever applicable.

The composition of the committee responsible for the formulation of this standard is listed in Annex C.

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 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 shall be the same as that of the specified value in this standard.

Indian Standard

APAMARGA (*Achyranthes aspera* L.) WHOLE PLANT FOR USE IN TRADITIONAL MEDICINE — SPECIFICATION

1 SCOPE

This standard prescribes the requirements and methods of test for *Apamarga* which consists of dried whole plant of *Achyranthes aspera* L. (Family Amaranthaceae).

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 listed below:

IS No.	*Title*
IS 1070 : 1992	Reagent grade water — Specification (*third revision*)
IS 1797 : 2017	Spices and condiments — Methods of test (*third revision*)
IS 4333 : 2018	Methods of analysis for foodgrains: Part 1 Refractions (*third revision*)
IS 13145 : 2014	Spices and condiments — Methods of sampling (*second revision*)
IS 13859 :1993/ ISO 7513 : 1990	Instant tea in solid form — Determination of moisture content (loss in mass at 103 °C)
IS 16287 :2015/ ISO 16050 : 2003	Foodstuffs — 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
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

3 REQUIREMENTS

3.1 Description

3.1.1 *Macroscopic Examination of Apamarga Whole Plant*

The tap roots are cylindrical in shape, slightly ribbed, 0.1 cm to 1.0 cm in thickness, gradually tapering and rough due to presence of some root scars. Secondary and tertiary roots are present. Color is yellowish brown and the odour is not distinct. Stem measures 0.3 cm to 0.5 cm in cut pieces, yellowish brown in color, erect, branched, cylindrical, hairy, solid and hollow when dry. Leaf is simple, subsessile, exstipulate, opposite, decussate, obovate, slightly acuminate and pubescent due to the presence of thick coat of long simple hairs. Flowers are arranged in inflorescence of long spikes, greenish white in color, numerous, sessile, bracteate with two bracteoles, one spine lipped, bisexual, actinomorphic, hypogynous. Perianth segments are 5, free, membranous, contorted or quincuncial, stamens 5, opposite the perianth lobes, connate forming a membranous tube like structure, alternating with truncate and fimbriate staminodes, filament are short. Anther is two celled and dorsifixed. Gynoecium is bicarpellary and syncarpous. Ovary is superior, unilocular with single ovule, style single, stigma capitate. Fruit is an indehiscent, dry utricle enclosed within persistent perianth and bracteoles. Seed is sub cylindric, truncate at the apex, round at the base, endospermic and brown in color.

3.1.2 *Microscopic Examination of Apamarga Whole Plant*

Transverse section (TS) of mature root shows 3 to 8 layered, rectangular, tangentially elongated, thin walled cork cells. The secondary cortex consisting of 6 to 9 layers of oval to rectangular, thin walled, parenchymatous cells having a few scattered single or groups of stone cells followed by 4 to 6 discontinuous rings of anomalous secondary thickening composed of vascular tissues. Small patches of sieve tubes are distinct in phloem parenchyma, demarcating the xylem rings. The xylem composed of usual elements. Vessels are simple and pitted. Medullary rays are 1 to 3 cells wide. Small prismatic crystals of calcium oxalate are present in cortical region and numerous in medullary rays.

TS of young stem shows 6 to 10 prominent ridges, which diminish downwards upto the base where it

becomes almost cylindrical. The epidermis is single layered, covered by thick cuticle having uniseriate, 2 to 5 celled covering trichomes and glandular trichomes with globular head and 3 to 4 celled stalk. Cortex is 6 to 10 layered, composed of parenchymatous cells. Most of them are containing rosette crystals of calcium oxalate. In the ridges, cortex is collenchymatous. The vascular bundles lie facing each ridge, capped by pericyclic fibres.

TS of mature stem shows lignified, thin walled cork cells. The pericycle is a discontinuous ring of lignified fibres. Vascular tissues show anomalous secondary growth having 4 to 6 incomplete rings of xylem and phloem. The secondary phloem consisting of usual elements form incomplete rings. Cambial strips are present between secondary xylem and phloem. Secondary xylem consists of annular, spiral, scalariform, elongately pitted vessels and elongated, lignified fibres. Pith is wide consisting of oval to polygonal, parenchymatous cells.

TS of petiole is crescent shaped in outline having single layered epidermis with thick cuticle. The ground tissues consisting of thin walled, parenchymatous cells containing rosette crystals of calcium oxalate. 4-5 vascular bundles are situated in mid region.

TS of midrib shows a single layered epidermis on both surfaces. The epidermis is followed by 4 to 5 layered collenchyma on upper side and 2 to 3 layered on lower side. Ground tissue consisting of thin walled, parenchymatous cells having a number of vascular bundles. Each vascular bundle shows thin layers of cambium below the xylem vessels followed by phloem and a pericycle represented by 2 to 3 layers of thick walled, non-lignified cells. Rosette crystals of calcium oxalate are found scattered in ground tissues.

TS of lamina shows single layered, tangentially elongated epidermal cells covered with thick cuticle having covering trichomes, similar to those of stem, on both surfaces. The mesophyll is differentiated into palisade and spongy parenchyma. Palisade is made up of 2 to 4 layered thick parenchyma, larger, slightly elongated in upper, while smaller and rectangular in lower surface. The spongy parenchyma is 3 to 5 layers thick, consisting of more or less isodiametric parenchymatous cells. The idioblast containing large rosette crystals of calcium oxalate are distributed in palisade and spongy parenchyma cells. Anisocytic and anomocytic stomata are present on both surfaces. Stomatal index is 4.5 to 9 on upper surface and 9 to 20 on lower surface. The palisade ratio is 7 to 11 and vein islet number is 7 to 13.

3.1.3 *Powder*

Apamarga whole plant crushed in the form of fine powder appears light yellow in color. The powder shows simple, multicellular, sharp or blunt ended, warty or smooth trichomes. Lower epidermal cells of leaf showing sinuous walls and upper with fairly straight walls are observed. Glandular trichomes with globular head of 3 or 4 cells, anisocytic and anomocytic stomata, thick walled and thin walled fibres with sharp or forked ends are found in the powder. The powder also shows cork tissue, fragments of pitted vessels, prismatic, rosette and sandy crystals of calcium oxalate scattered as such throughout or in idioblasts as in Fig. 1.

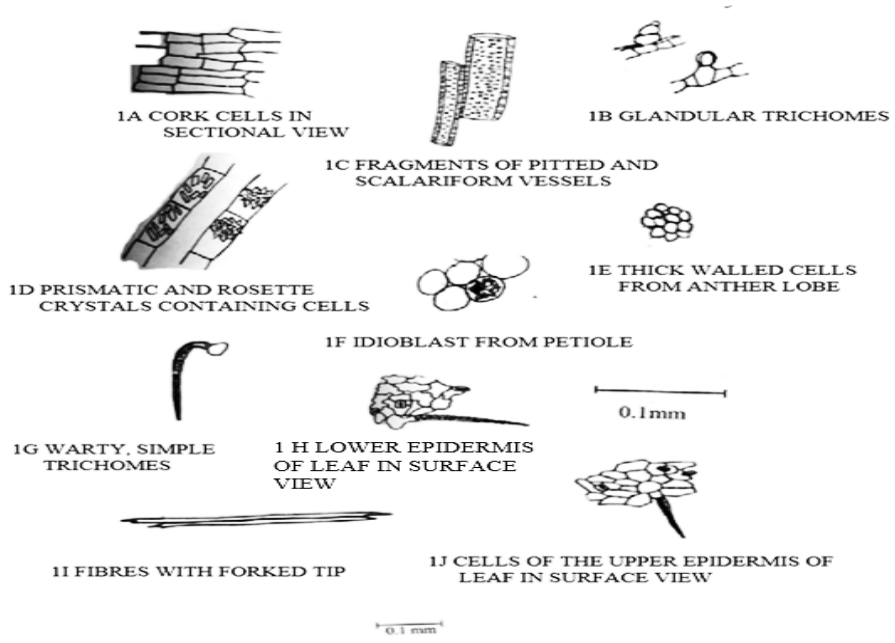

FIG. 1 POWDER OF *APAMARGA* (*Achyranthes aspera* L.) WHOLE PLANT

3.2 General

3.2.1 *Apamarga* whole plant shall be free from extraneous/artificial flavours.

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

3.2.3 If any pesticides other than those for which minimum requirements are given in Table 1 are applied to the herb before or after harvesting, those should also be tested. Their limit shall be calculated using the following formula:

$$\text{ADI} \times \text{M/MDD} \times 100$$

where

ADI = Acceptable daily intake of pesticide as published by Food and Agriculture Organization-World Health Organization (FAO-WHO), in milligrams per kilogram of body mass,

M = Body mass in kilograms (60 kg),

MDD = Maximum daily dose of the drug, in kilograms.

4 PACKING, STORAGE AND MARKING

4.1 Packing

Apamarga whole plant shall be packed in clean, sound and dry container made up 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

Apamarga whole plant shall be stored under conditions that prevent contamination and, as far as possible, deterioration. Storage area shall be clean, well ventilated, protected from light, moisture, insects and rodents.

4.3 Marking

The following particulars shall be legibly and indelibly marked or labelled on each pack:

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;
c) Batch number;
d) Net quantity;
e) State and country of production;
f) Date of packing (MM/YYYY);
g) Instructions for storage; and
h) Any other information requested by the buyer, such as the date of harvesting (MM/YYYY) (if known).

The above information, or part of it, may instead appear in the documentation after agreement between the buyer and the seller.

4.4 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.

5 SAMPLING

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

5.2 The samples of *Apamarga* whole plant shall be tested for ascertaining conformity of the material to the requirements in accordance with the relevant clauses given in col 4 of Table 1.

6 QUALITY OF REAGENTS

6.1 Reagents including pure chemicals used shall be of analytical grade.

6.2 Reagent grade water for laboratory use shall be as per IS 1070.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which effect the result of analysis

Table 1 Requirements for *Apamarga* (*Achyranthes aspera* L.) Whole Plant
(*Clauses* 3.2.2, 3.2.3 *and* 5.2)

Sl No. (1)	Characteristic (2)	Requirement (3)	Method of Test, Ref to (4)
i)	Foreign matter, percent by mass, *Max*	2.0	**6.2** of IS 4333 (Part 1)
ii)	Loss on drying, percent by Mass, *Max*	12.0	IS 13859
iii)	Total ash, percent by mass, *Max*	17.0	**6** of IS 1797
iv)	Acid insoluble ash, percent by mass, *Max*	5.0	**8** of IS 1797

Table 1 (*Continued*)

Sl No. (1)	Characteristic (2)	Requirement (3)	Method of Test, Ref to (4)
v)	Alcohol-soluble Extractive, percent by mass, *Min*	2.0	**10** of IS 1797
vi)	Water soluble Extractive, percent by mass, *Min*	12.0	**11** of IS 1797
vii)	Oleanolic acid, percent, *Min*	0.002	Annex A
viii)	Aflatoxin (B1), ng/kg, *Max*	2.0	IS 16287
ix)	Aflatoxin (B1+B2+G1+G2), ng/kg, *Max*	5.0	IS 16287
x)	Lead, mg/kg, *Max*	10.0	IS 16913
xi)	Mercury, mg/kg, *Max*	1.0	IS 16913
xii)	Cadmium, mg/kg, *Max*	0.3	IS 16913
xiii)	Arsenic, mg/kg, *Max*	3.0	IS 16913
xiv)	*Staphylococcus aureus*, per g	Absent for extract and powder Absent for plant material	Annex B
xv)	*Pseudomonas aeruginosa*, per g	Absent for extract and powder Absent for plant material	Annex B
xvi)	*Salmonella* spp., per g	Absent	Annex B
xvii)	*Escherichia coli*, per g	Absent for extract and powder 10 for plant material	Annex B
xviii)	Total microbial plate count, per g, *Max*	10^5 for extract and powder 10^7 for plant material	Annex B
xix)	Total yeast and mould, per g, *Max*	10^3 for extract and powder 10^5 for plant material	Annex B
xx)	Pesticide residues		
1)	Alachlor (mg/kg) *Max*.	0.02	IS 17924
2)	Aldrin and Dieldrin (sum of) (mg/kg) *Max*.	0.05	
3)	Azinphos-methyl (mg/kg) *Max*.	1.0	
4)	Bromopropylate (mg/kg) *Max*.	3.0	
5)	Chlordane (sum of cis-, trans - and Oxythlordane) (mg/kg) *Max*.	0.05	
6)	Chlorfenvinphos (mg/kg) *Max*.	0.5	
7)	Chlorpyrifos (mg/kg) *Max*.	0.2	
8)	Chlorpyrifos-methyl (mg/kg) *Max*.	0.1	
9)	Cypermethrin (and isomers) (mg/kg) *Max*.	1.0	
10)	DDT (sum of p,p'-DDT, o,p'-DDT, p,p-DDE and p,p'-TDE) (mg/kg) *Max*.	1.0	
11)	Deltamethrin (mg/kg) *Max*.	0.5	

Table 1 (*Concluded*)

Sl No. (1)	Characteristic (2)	Requirement (3)	Method of Test, Ref to (4)
12)	Diazinon (mg/kg) *Max*.	0.5	IS 17924
13)	Dichlorvos (mg/kg) *Max*.	1.0	
14)	Dithiocarbamates (as CS_2) (mg/kg) *Max*.	2.0	
15)	Endosulfan (sum of isomers and endosulfan sulphate) (mg/kg) *Max*.	3.0	
16)	Endrin (mg/kg) *Max*.	0.05	
17)	Ethion (mg/kg) *Max*.	2.0	
18)	Fenitrothion (mg/kg) *Max*.	0.5	
19)	Fenvalerate (mg/kg) *Max*.	1.5	
20)	Fonofos (mg/kg) *Max*.	0.05	
21)	Heptachlor (sum of heptachlor and heptachlor epoxide) (mg/kg) *Max*.	0.05	
22)	Hexachlorobenzene (mg/kg) *Max*.	0.1	
23)	Hexachlorocyclohexane isomers (other than γ) (mg/kg) *Max*.	0.3	
24)	Lindane (γ-hexachlorocyclohexane) (mg/kg) *Max*.	0.6	
25)	Malathion (mg/kg) *Max*.	1.0	
26)	Methidathion (mg/kg) *Max*.	0.2	
27)	Parathion (mg/kg) *Max*.	0.5	
28)	Parathion-methyl (mg/kg) *Max*.	0.2	
29)	Permethrin (mg/kg) *Max*.	1.0	
30)	Phosalone (mg/kg) *Max*.	0.1	
31)	Piperonyl butoxide (mg/kg) *Max*.	3.0	
32)	Pirimiphos-methyl (mg/kg) *Max*.	4.0	
33)	Pyrethrins (sum of) (mg/kg) *Max*.	3.0	
34)	Quintozene (sum of quintozene, pentachloroaniline and methyl pentachlorophenylsulphide) (mg/kg) *Max*.	1.0	

ANNEX A

[*Table* 1*, Sl No.* (vii), *col* (4)]

IDENTIFICATION OF OLEANOLIC ACID

A-1 GENERAL

Apamarga contains not less than 0.002 percent of oleanolic acid when assayed.

A-2 THIN LAYER CHROMATOGRAPHY (TLC)

A-2.1 Apparatus

A-2.1.1 Thin layer chromatograph with a pre coated silica gel $60F_{254}$ plate.

A-2.1.2 *Analytical Balance*

A-2.1.3 *Standard Glassware*

A-2.1.4 *Water Bath*

A-2.2 Reagents

A-2.2.1 *Reference Standard* — Oleanolic Acid, of known purity

A-2.2.2 *Hydrochloric Acid* — AR or Equivalent Grade

A-2.2.3 *Methanol* — AR or Equivalent Grade

A-2.2.4 *Anhydrous Sodium Sulphate* — AR or Equivalent Grade

A-2.2.5 *Ethyl Acetate* — AR or Equivalent Grade

A-2.2.6 *Toluene* — AR or Equivalent Grade

A-2.2.7 *Formic Acid* — AR or Equivalent Grade

A-2.2.8 *Sulphuric Acid* — AR or Equivalent Grade

A-2.2.9 *Chloroform* — AR or Equivalent Grade

A-2.2.10 *Water* — HPLC Grade

A-2.3 Procedure

A-2.3.1 *Solvent System*

Dissolve ethyl acetate, toluene and formic acid in the ratio of 45.0 : 0.5 : 0.1.

A-2.3.2 *Preparation of Test Solution*

Extract 2 g of substance by refluxing with 50 percent methanol (50 ml x 3) for a period of 30 min each, cool and filter. Combine all the filtrates, concentrate and transfer to a 100 ml round bottomed flask. Add 20 ml of 2 M methanolic hydrochloric acid and reflux at 60 ºC to 70 ºC on a water bath for 3 h, cool and transfer the solution to a separating flask, extract with chloroform (25 ml x 3). Combine all the organic extracts and wash gently with water. Pass the combined chloroform extract through anhydrous sodium sulphate and evaporate the chloroform under vacuum. Dissolve the residue obtained in 10 ml of methanol.

A-2.3.3 *Preparation of Standard Solution*

Dissolve 10 mg of oleanolic acid RS in about 10 ml of methanol.

A-2.3.4 *Estimation*

Apply 10 µl each of the test and standard solutions 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. Spray the plate with 10 percent methanolic sulphuric acid reagent and heat at 105 °C till the color of the spots/bands appear without charring. The chromatographic profile of the test solution shows a band corresponding to that of the standard solution as in Fig. 2.

A-3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

A-3.1 Apparatus

A-3.1.1 The high performance liquid chromatograph equipped with UV-VIS detector. The operative condition suggested below are typical which can be changed provided the standardization is done:

a) Column – 100 mm x 3 mm, 2.5 micron SS packed with C18

b) Detector – UV (205 nm)

c) Mobile Phase – Filtered and degassed mixture of 33 volumes of phosphate buffer (prepared by dissolving 0.14 g of potassium dihydrogen orthophosphate in 900 ml of water, adding 1 ml of orthophosphoric acid and making up

the volume to 1 000 ml) and 67 volumes of acetonitrile.

d) Flow Rate – 0.3 ml per min

e) Injection Volume – 20 µl

f) Temperature – ambient

A-3.1.2 *Microlitre Syringe* — 50 µl capacity

A-3.1.3 *Analytical Balance*

A-3.1.4 *Standard Glassware*

A-3.1.5 *Water Bath*

A-3.2 Reagents

A-3.2.1 *Reference Standard* — Oleanolic Acid, of known purity

A-3.2.2 *Potassium Dihydrogen Orthophosphate* — AR or Equivalent Grade

A-3.2.3 *Orthophosphoric Acid* — AR or Equivalent Grade

A-3.2.4 *Methanol* — AR or Equivalent Grade

A-3.2.5 *Chloroform* — AR or Equivalent Grade

A-3.2.6 *Water* — HPLC Grade

A-3.2.7 *Acetonitrile* — HPLC Grade

A-3.3 Procedure

A-3.3.1 *Test Solution*

Take about 2 g, accurately weighed substance being examined and reflux with 50 percent methanol (50 ml x 3) on a water bath for 30 min each, cool and filter. Combine all the filtrates, concentrate and transfer to a 100 ml round bottomed flask. Add 20 ml of 2 M methanolic hydrochloric acid and reflux at 60 °C to 70 °C on a water bath for 3 hours, cool and transfer the solution to a separating flask, extract with chloroform (25 ml x 3). Combine all the organic extracts and wash gently with water, pass the combined chloroform extract through anhydrous sodium sulphate and evaporate the chloroform under vacuum. Dissolve the residue obtained in 5 ml of methanol, transfer to a 10 ml volumetric flask and make up the volume. Filter through 0.42 µm membrane.

A-3.3.2 *Standard Solution*

Take about 10 mg, accurately weighed, oleanolic acid RS in a 100 ml volumetric flask and dissolve in 50 ml of methanol and make up the volume with methanol. Filter through 0.42 µm membrane.

A-3.3.3 *Estimation*

Inject 20 µl of the standard solution and record the chromatogram. Inject 20 µl of the test solution, record the chromatogram and measure the response for the analyte peak.

A-3.4 Calculation

Calculate the content of oleanolic acid in the substance being examined from the peak response of analyte as in Fig. 3. The test is not valid unless the relative standard deviation for replicate injections is not more than 2.0 percent.

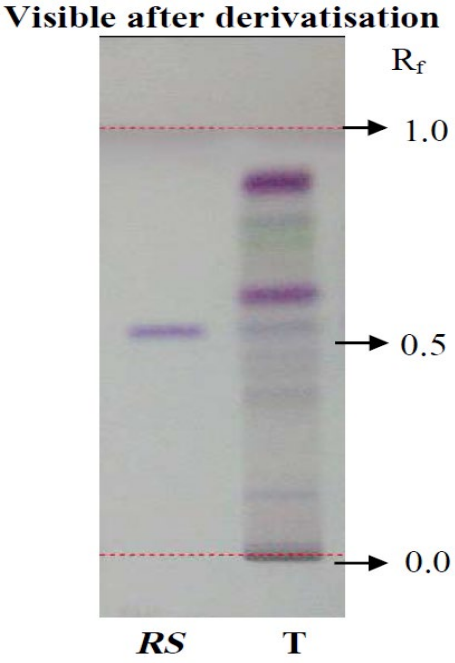

FIG. 2 THIN LAYER CHROMATOGRAM OF *APAMARGA* (*Achyranthes aspera* L.) WHOLE PLANT
RS: OLEANOLIC ACID, T: TEST SOLUTION

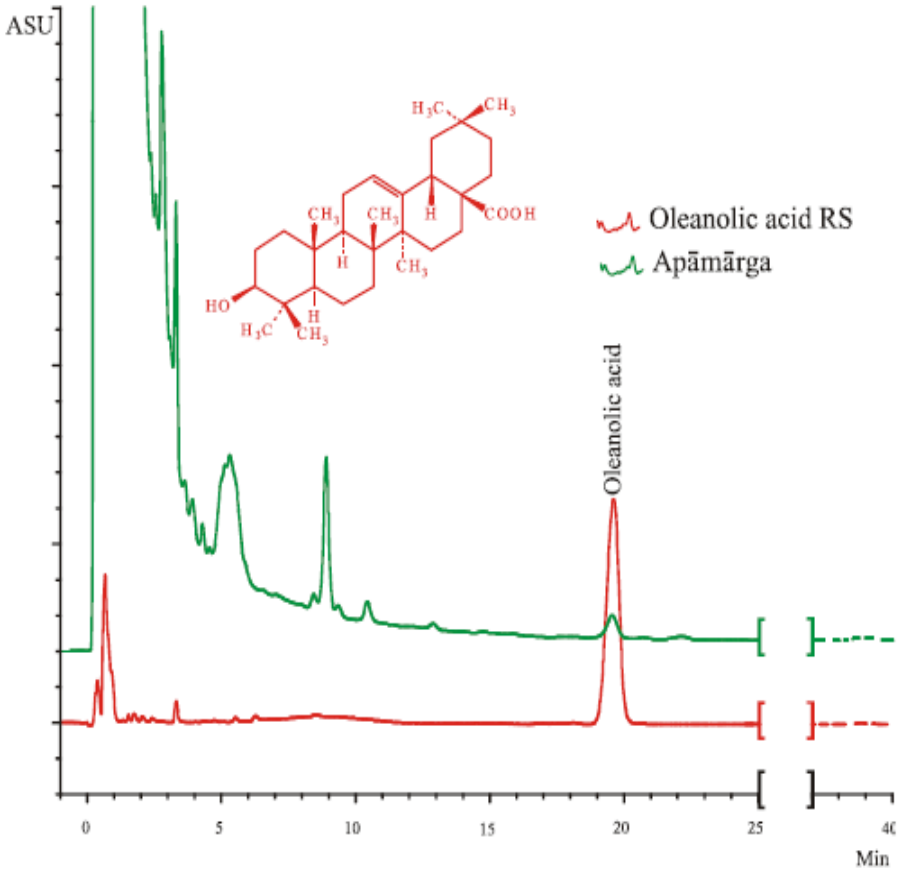

FIG. 3 HPLC CHROMATOGRAM OF *APAMARGA* (*Achyranthes aspera* L.) WHOLE PLANT WITH OLEANOLIC ACID AS RS

ANNEX B

[*Table* 1, *Sl No.* (xiv), (xv), (xvi), (xvii), (xviii), (xix), *col* (4)]

MICROBIAL LIMIT TESTS

B-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.

B-2 APPARATUS

B-2.1 Oven for Dry Sterilization

B-2.2 Autoclave for Wet Sterilization

B-2.3 Incubator

B-2.4 Water Bath

B-2.5 *p*H-meter

B-2.6 Sterile Membrane Filters, 50 mm in Diameter

B-2.7 Colony Counting Equipment

B-2.8 Analytical Balance

B-2.9 Standard Glassware

B-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 0.1 N hydrochloric acid or 0.1 N 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 ± 2) °C.

B-3.1 Baird Parker Agar Medium

B-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	1 000 ml

B-3.1.2 *Preparation*

Suspend the components in 1 000 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 *p*H after sterilization to 6.8 ± 0.2.

B-3.2 Bismuth Sulphite Agar Medium

B-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	1 000 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

B-3.2.2 *Preparation*

Suspend the components of Solution 1 in 1 000 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 °C to 8 °C for 5 days before use.

B-3.3 Brilliant Green Agar Medium

B-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	1 000 ml

B-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.

B-3.4 Buffered Sodium Chloride Peptone Solution *p*H 7.0

B-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	1 000 ml

B-3.4.2 *Preparation*

Mix the components and heat if necessary to dissolve the medium completely. 0.1 percent 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.

B-3.5 Casein Soyabean Digest Agar Medium

B-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	1 000 ml

B-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 *p*H after sterilization to 7.3 ± 0.2.

B-3.6 Cetrimide Agar Medium

B-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	1 000 ml

B-3.6.2 *Preparation*

Dissolve the components in 1 000 ml of water. Heat to boiling for 1 min with shaking. Sterilize at 121 °C for 15 min in an autoclave and adjust the *p*H after sterilization to 7.0 to 7.4.

B-3.7 Deoxycholate Citrate Agar Medium

B-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	1 000 ml

B-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.

B-3.8 Fluid Casein Digest Soya Lecithin Polysorbate 20 Medium

B-3.8.1 *Composition*

Pancreatic digest of casein	20 g
Soya lecithin	5 g
Polysorbate 20	40 ml
Water	1 000 ml

B-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.

B-3.9 Fluid Lactose Medium

B-3.9.1 *Composition*

Beef extract	3.0 g
Pancreatic digest of gelatin	5.0 g
Lactose	5.0 g
Water	1 000 ml

B-3.9.2 *Preparation*

Suspend the components in 1 000 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 *p*H after sterilization to 6.9 ± 0.2.

B-3.10 Lactose Broth Medium

B-3.10.1 *Composition*

Beef extract	3.0 g
Pancreatic digest of gelatin	5.0 g
Lactose	5.0 g
Water	1 000 ml

B-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 *p*H after sterilization to 6.9 ± 0.2.

B-3.11 Levine Eosin Methylene Blue Agar Medium

B-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	1 000 ml

B-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 *p*H after sterilization to 7.1 ± 0.2.

B-3.12 MacConkey Agar Medium

B-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	1 000 ml

B-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 *p*H after sterilization to 7.1 ± 0.2.

B-3.13 MacConkey Broth Medium

B-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	1 000 ml

B-3.13.2 *Preparation*

Suspend the components in 1 000 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 *p*H after sterilization to 7.3 ± 0.2.

B-3.14 Mannitol Salt Agar Medium

B-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	1 000 ml

B-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 *p*H after sterilization to 7.4 ± 0.2.

B-3.15 Nutrient Broth Medium

B-3.15.1 *Composition*

Beef extract	10.0 g
Peptone	10.0 g
Sodium chloride	5 mg
Water	1 000 ml

B-3.15.2 *Preparation*

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

B-3.16 Nutrient Agar Medium

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

B-3.17 Pseudomonas Agar Medium for Detection of Flourescein

B-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 sulphate hepta hydrate	1.5 g
Glycerin	10.0 ml
Agar	15.0 g
Water	1 000 ml

B-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 *p*H after sterilization to 7.2 ± 0.2.

B-3.18 Pseudomonas Agar Medium for Detection of Pyocyanin

B-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	1 000 ml

B-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 *p*H after sterilization to 7.2 ± 0.2.

B-3.19 Sabouraud Dextrose Agar Medium

B-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	1 000 ml

B-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 *p*H after sterilization to 5.6 ± 0.2.

B-3.20 Sabouraud Dextrose Agar Medium with Antibiotics

To 1 000 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.

B-3.21 Selenite F Broth

B-3.21.1 *Composition*

Peptone	5 g
Lactose	4 g
Disodium hydrogen phosphate	10 g
Sodium hydrogen selenite	4 g
Water	1 000 ml

B-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.

B-3.22 Fluid Selenite Cystine Medium

B-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	1 000 ml

B-3.22.2 *Preparation*

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

B-3.23 Tetrathionate Broth Medium

B-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	1 000 ml

B-3.23.2 *Preparation*

Dissolve the solids in 1 000 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.

B-3.24 Tetrathionate Bile Brilliant Green Broth Medium

B-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	1 000 ml

B-3.24.2 *Preparation*

Suspend the components in 1 000 ml of water. Heat just to boiling. Do not autoclave or reheat. Adjust the *p*H so that after heating it is 7.0 ± 0.2.

B-3.25 Triple Sugar Iron Agar Medium

B-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	1 000 ml

B-3.25.2 *Preparation*

Mix the components in 1 000 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.

B-3.26 Urea Broth Medium

B-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	1 000 ml

B-3.26.2 *Preparation*

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

B-3.27 Vogel Johnson Agar Medium

B-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	1 000 ml

B-3.27.2 *Preparation*

Suspend the components in 1 000 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 *p*H after sterilization to 7.0 ± 0.2.

B-3.28 Xylose Lysine Deoxycholate Agar Medium

B-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	1 000 ml

B-3.28.2 *Preparation*

Suspend the components in 1 000 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 *p*H to 7.4 ± 0.2.

B-4 SAMPLING

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

B-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.

B-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 1 000 microorganisms to the first dilution (in buffer solution *p*H 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.

B-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.

B-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 *p*H 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 *p*H to about 7.

B-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 *p*H 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 *p*H of the suspension to about 7.

B-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 *p*H 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 *p*H to about 7.

B-7.4 Examination of the Sample

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

B-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 *p*H 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.

B-7.6 Plate Count for Bacteria

Using Petri dishes 9 cm 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.

B-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 °C for 5 days, unless a more reliable count is obtained in a shorter time. Calculate the results using plates with not more than 100 colonies.

B-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.

B-8 TESTS FOR SPECIFIED MICROORGANISMS

B-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 *p*H 7.0.

B-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 h to 24 h.

B-8.2.1 *Primary Test*

Add 1.0 ml of the enrichment culture to a tube containing 5 ml of MacConkey broth. Incubate in a water bath at 36 °C to 38 °C for 48 h. If the contents of the tube show acid and gas, carry out the secondary test.

Table 2 Most probable Total Count by Multiple Tube or Serial Dilution Method

(*Clause* B-7.8)

Sl No.	Observed Combination of Numbers of Tubes Showing Growth in Each Set Number of mg (or ml) of Specimen per Tube			Most Probable Number of Microorganisms per g or per ml
	100 (100 µl)	10 (10 µl)	1 (1 µl)	
(1)	(2)	(3)	(4)	(5)
i)	3	3	3	> 1 100
ii)	3	3	2	1 100
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

B-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*.

B-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.

B-8.3 ***Salmonella***

Transfer a quantity of the pretreated preparation being examined containing 1 g or 1 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.

B-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 h 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.

B-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 h 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 not valid unless the results indicate that the control contains *Salmonella.*

B-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 h 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 h to 24 h.

If, upon examination, none of the plates contains colonies having the characteristics listed in Table 4 for the media used, the sample meets the requirement for freedom from *Pseudomonas aeruginosa*. If any colonies conforming to the description in Table 4 are 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.*

B-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.

Incubate in water bath at 37 °C examining the tubes at 3 h and subsequently at suitable intervals up to 24 h. If no coagulation in any degree is observed, the sample meets the requirements of the test for the absence of *Staphylococcus aureus.*

B-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 h 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 *p*H 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 count method using sterile buffered sodium chloride peptone solution *p*H 7.0 as the test preparation. There should be no growth of microorganisms.

B-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 h to 24 h. Dilute portions of each of the cultures using buffered sodium chloride peptone solution *p*H 7.0 to make test suspensions containing about 10^3 viable microorganisms per ml. Mix equal volume of each suspension and use 0.4 ml (approximately 10^2 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.

Table 3 Interpretation of the Confirmatory Tests for *Salmonella*
(*Clause* B-8.3.1 *and* 8.3.2)

Sl 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

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

Sl 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

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

Sl 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)

ANNEX C

(*Foreword*)

COMMITTEE COMPOSITION

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Panel for Herbal materials and related subjects, FAD 26/Panel 1

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Bureau of Indian Standards, New Delhi	DR RAGHAVENDRA NAIK DR SNEH LATA JAIN
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