भारतीय मानक

Indian Standard

IS 18975 : 2024

पारम्परिक औषधियों में प्रयोग हेतु *मिलीफोलियम (अचिलिया मिलेफोलियम* एल.) *पंचांग* — विशिष्टि

Millefolium (*Achillea millefolium* L.) Whole Plant for Use in Traditional Medicine — Specification

ICS 11.120.10

भारतीय मानक ब्यूरो
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November 2024 **Price Group 10**

Homoeopathy Sectional Committee, AYD 07

FOREWORD

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

Millefolium is an erect, pubescent, 50 cm to 90 cm tall, perennial herb of the family Asteraceae. It is distributed in temperate zones of the Northern Hemisphere, Europe, Asia, and North America. This species is not native to India but has been introduced and cultivated in India and grows widely in India. In India, it is found in the Himalayan region of Jammu and Kashmir, Himachal Pradesh, Uttarakhand, at altitudes of 1 050 m to 3 600 m.

It thrives in hot, dry conditions, well-drained soil, and full sun to encourage compact growth and many flowers. It is used for making Mother tincture, dilutions or potencies in Homoeopathy, and as an ingredient of formulations in traditional Indian systems of medicine.

Millefolium is synonymous to *Common Yarrow, Blood Wart, Thousand Leaves Yarrow, and milfolia.* Some of the regional names are *Handrain, Puthkanda, Brinjasif* (Hindi), *Brinjosipha* (Sanskrit), *Rajmaari* (Marathi), *Achilliya* (Tamil), *Rajmari* (Konkani).

It contains 0.3 percent to 1.4 percent volatile oils, mainly achillicin (proazulene- biological marker), achillin, leucodin, and germacranolides (dihydro parthenolide, achillifolin, millefin); flavonoids (apigenin, luteolin, isorhamnetin, rutin); fatty acids (linoleic, palmitic, oleic); phenolic acids (caffeic, salicylic); sterols (β-sitosterol) etc.

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 formulating this standard, significant assistance has been derived from Homoeopathic Pharmacopoeia of India, Vol. IV, 1983, published by the Ministry of Ayush, Government of India. Inputs have also been derived from Indian Pharmacopoeia; The German Homoeopathic Pharmacopeia, 2000; British Homoeopathic Pharmacopeia Vol. IV, 2022; Homeopathic Pharmacopoeia of the United States, 2004 and 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 the restrictions imposed under these Rules and Regulations, wherever applicable.

The composition of the Committee responsible for the formulation of this standard is given in Annex D.

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.

Indian Standard

MILLEFOLIUM (*Achillea millefolium* L.) WHOLE PLANT FOR USE IN TRADITIONAL MEDICINE — SPECIFICATION

1 SCOPE

This standard prescribes the specific requirements and methods of testing for *Millefolium*, which consists of the whole plant of *Achillea millefolium* L. (Family Asteraceae).

2 REFERENCES

The standards listed in Annex A 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 these standards are encouraged to investigate the possibility of applying the most recent edition of these standards.

3 REQUIREMENTS

3.1 Description

3.1.1 *Macroscopic Examination of Millefolium Whole Plant*

The whole plant consists of dried pieces of stem, leaf, and flower. The stem is cylindrical, nodes are distinct, often attached with leaf traces, especially on young stem; externally yellowish green to pale brown; compound leaves, 2 to 3 pinnatisect, oblong-lanceolate, 5 to 10 × 0.65 to 1.95 cm, with numerous, linear, finely pointed pinnules; and many small flower heads surrounded by long hairs. It has a fragrant odour and bitter taste.

3.1.2 *Microscopic Examination of Millefolium Whole Plant*

The transverse section of the stem (Fig. 1A) is circular in outline with ridges and furrows; single-layered epidermis with oval to spherical cells, covered by thick cuticle with non-glandular and glandular trichomes as on the leaf; followed by hypodermis, which has 4 to 6 layers of collenchymatous cells, confined in ridges; cortex is parenchymatous, with 6 to 8 layers of polygonal cells with intercellular spaces; a single layered endodermis with casparian strip; the vascular bundles are conjoint, collateral, open, encapped by sclerenchymatous patches; phloem consisting phloem parenchyma, fibers and sieve elements; 2 to 3 layered cambium; large xylem, vessels and tracheids in radial rows; pith large and parenchymatous with intercellular spaces.

The vertical section of the leaf (Fig. 1B) shows single layered epidermis, barrel-shaped cells, a few oval to spherical, covered by thick cuticles; trichomes non-glandular, uniseriate, macroform, conical and glandular trichomes with multicellular stalk and unicellular globose head; palisade is 2 to 3 layered, loosely arranged, extending into midrib region; spongy parenchyma with 3 to 4 layers of compactly arranged cells; vascular bundle is conjoint, collateral and enclosed by a parenchymatous sheath; phloem consists of phloem parenchyma, fibers and sieve elements with companion cells; xylem consists of vessels, tracheids, arranged in radial rows; secretory duct is present above the vascular bundle, lined with epithelial cells. In surface view, epidermal cells are polygonal anisodiametric, wavy to sinuate with cuticular striation; leaf amphistomatous with anomocytic stomata, stomatal index 7.38 on the abaxial side and 8.78 on the adaxial side; palisade ratio 2 to 3; vein–islet number 3 to 4.5 per sq. mm.

Transection of the petiole (Fig. 1C) shows V-shape in outline; single layered epidermis with thick cuticle, small, polygonal epidermal cells; trichomes as on leaf; 1 to 2 layered, collenchymatous hypodermis; cortex has 3 to 5 layers of polygonal, thin-walled, anisodiametric, parenchymatous cells; vascular bundles are 3 to 5, conjoint, collateral, open, encapped on both sides by sclerenchymatous patches and arranged in an arc, with larger middle bundle compared to the lateral ones; xylem consists of xylem parenchyma, vessels and tracheids; phloem with phloem parenchyma and sieve elements with companion cells.

3.1.3 *Powder*

Greenish-brown, coarse, fragrant powder with bitter taste. Microscopy shows fragments of epidermal cells with anomocytic stomata broken or whole uniseriate, macroform, conical trichomes, and a few glandular trichomes; fragments of the thin-walled parenchymatous cell, tracheary elements with spiral an d annular thickenings, and a few pollen grains (Fig. 2).

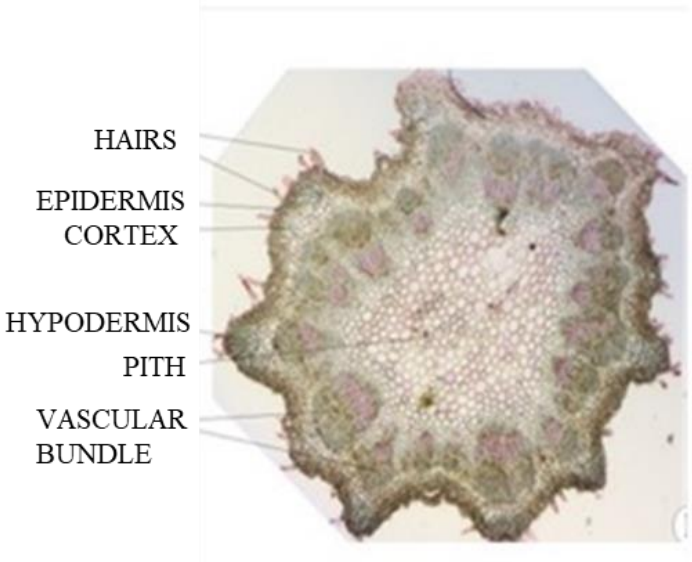

FIG. 1 A TRANSECTION OF THE STEM

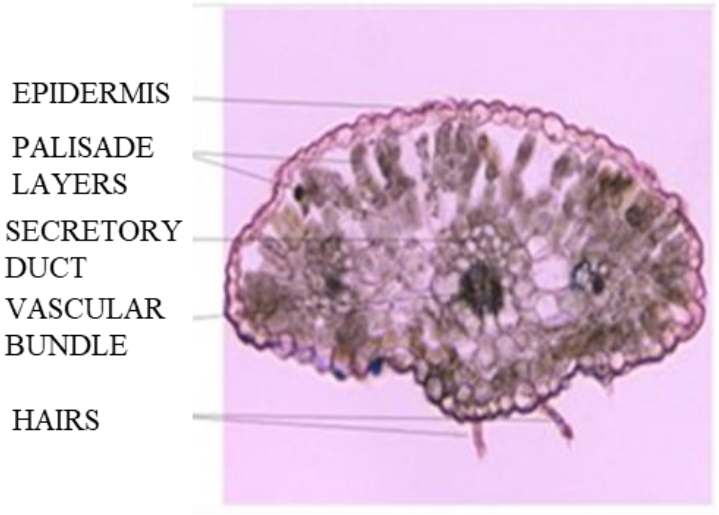

FIG. 1B VERTICAL SECTION OF THE LEAF

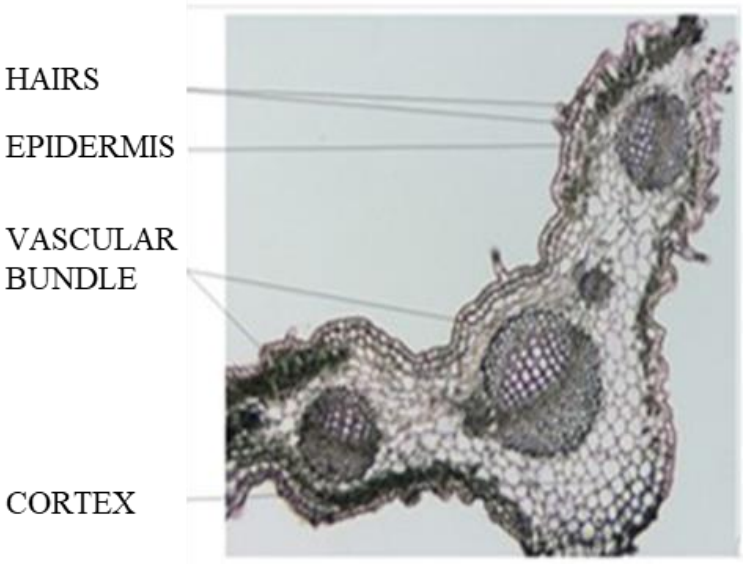

FIG. 1C TRANSECTION OF PETIOLE

3.2 General

3.2.1 *Millefolium* whole plant shall be free from extraneous/foreign matter.

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

3.2.3 If any pesticide other than those for which minimum requirements are given in Table 1 have been applied to the herb before or after harvesting, those should also be tested.

4 PACKING, STORAGE AND MARKING

4.1 Packing

Millefolium whole plant 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

Millefolium whole plant 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 *Millefolium* whole 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).

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

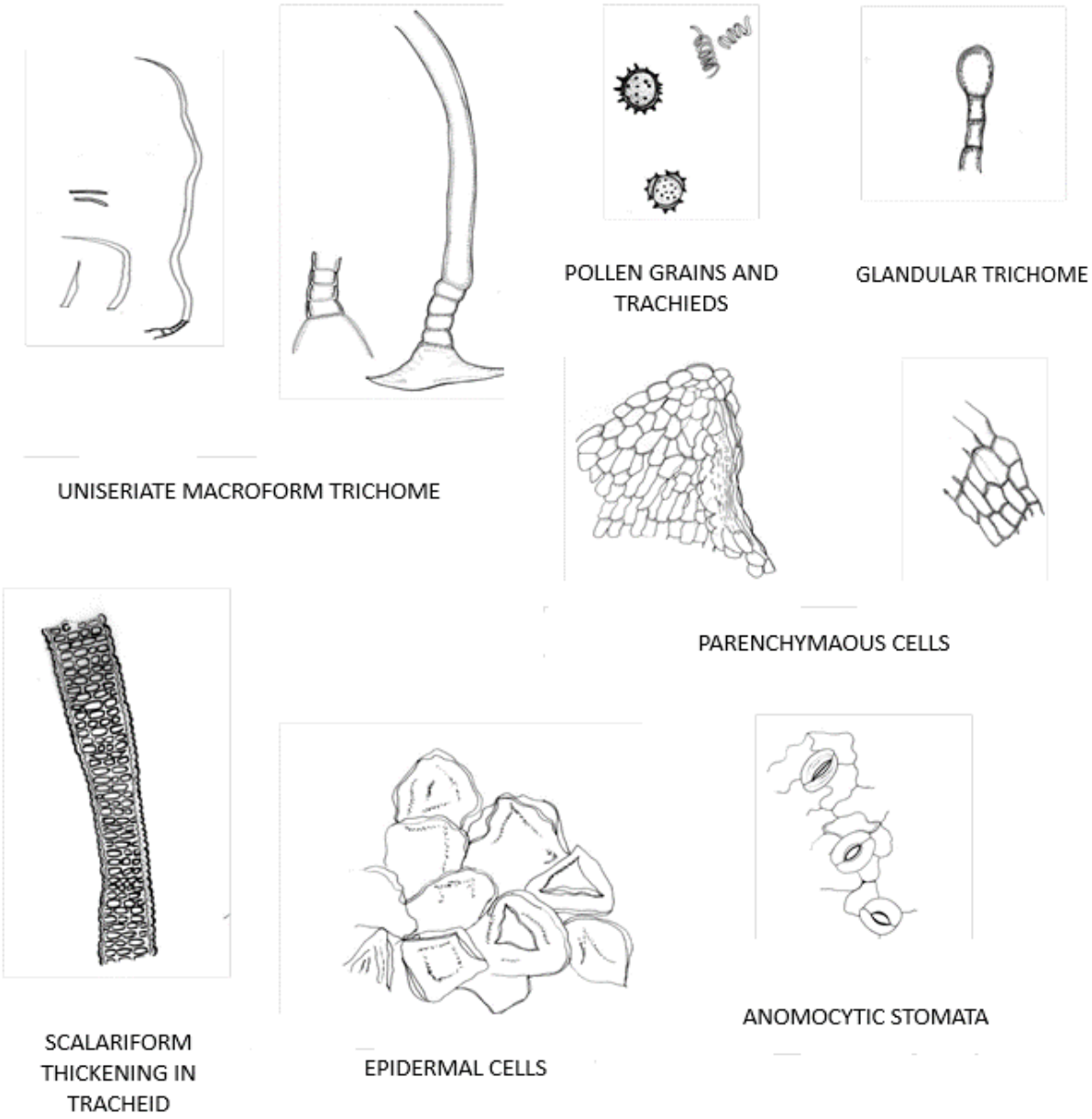

FIG. 2 POWDER OF MILLEFOLIUM

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 *Millefolium* whole plant shall be tested to ascertain the material's conformity to the requirements as per the relevant clauses in col (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.

Table 1 Requirements for *Millefolium* (*Achillea millefolium*) Whole Plant

(*Clauses* 3.2.2, 3.2.3 *and* 5.2)

SI No.	Characteristic	Requirement	Method of Test, Ref. to
(1)	(2)	(3)	(4)
i)	Foreign matter, percent by mass, *Max*	2	**6.2** of IS 4333 (Part 1)
ii)	Loss on drying, percent by mass, *Max*	12 (for dried herbs) 55 (for fresh herbs)	IS 13859
iii)	Total ash, percent by mass, *Max*	10	**6** of IS 1797
iv)	Acid insoluble ash, percent by mass, *Max*	2.5	**8** of IS 1797
v)	Alcohol Soluble Extractive, percent by mass, *Min*	3.5	**10** of IS 1797
vi)	Water soluble Extractive, percent by mass, *Min*	18	**11** of IS 1797
vii)	Thin layer chromatography identification	Should comply	Annex B
viii)	**Assay** (Total flavonoids expressed as luteolin-3',7-di-O-glucoside), percent by mass, *Min*	0.5	Annex B
ix)	Aflatoxin B_1 ng/kg, *Max*	2	IS 16287
x)	Total Aflatoxin (B_1+B_2+G_1+G_2) ng/kg, *Max*	5	IS 16287
xi)	Lead (as Pb) mg/kg, *Max*	10.0	IS 16913
xii)	Arsenic (as As) mg/kg, *Max*	3.0	
xiii)	Cadmium (as Cd) mg/kg, *Max*	0.3	
xiv)	Mercury (as Hg) mg/kg, *Max*	1.0	
xv)	*Staphylococcus aureus*, per g	Absent	Annex C
xvi)	*Pseudomonas aeruginosa*, per g	Absent	
xvii)	*Salmonella Spp.*, per g	Absent	
xviii)	*Escherichia coli*, per g	Absent	
xix)	Total microbial plate count per g, *Max*	10^5 for extract and powder 10^7 for plant material	
xx)	Total yeast and mould per g, *Max*	10^3 for extract and powder 10^5 for plant material	
xxi)	Pesticide residues		IS 17924
	a) Alachlor, mg/kg, *Max*	0.02	
	b) Aldrin, dieldrin (sum of), mg/kg, *Max*	0.05	
	c) Azinphos-methyl, mg/kg, *Max*	1.0	
	d) Bromopropylate, mg/kg, *Max*	3.0	
	e) Chlordane (sum of cis- trans- and oxythlordane), mg/kg, *Max*	0.05	
	f) Chlorfenvinphos, mg/kg, *Max*	0.5	
	g) Chlorpyrifos, mg/kg, *Max*	0.2	
	h) Chlorpyrifos-methyl, mg/kg, *Max*	0.1	
	j) Cypermethrin and isomers, mg/kg, *Max*	1.0	
	k) DDT (sum of p, p'-DDT, o, p' DDT, p,p'-DDE and p, p'-TDE) , mg/kg, *Max*	1.0	
	m) Deltamethrin, mg/kg, *Max*	0.5	
	n) Diazinon, mg/kg, *Max*	0.5	
	p) Dichlorvos, mg/kg, *Max*	1.0	
	q) Dithiocarbamates (as CS_2), mg/kg, *Max*	2.0	

SI No.	Characteristic	Requirement	Method of Test, Ref. to
(1)	(2)	(3)	(4)
r)	Endosulfan (sum of isomers and endosulfan sulphate, mg/kg, *Max*	3.0	IS 17924
s)	Endrin, mg/kg, *Max*	0.05	
t)	Ethion, mg/kg, *Max*	2.0	
u)	Fenitrothion, mg/kg, *Max*	0.5	
w)	Fenvalerate, mg/kg, *Max*	1.5	
y)	Fonofos, mg/kg, *Max*	0.05	
z)	Heptachlor (combined residues of heptachlor and its epoxide to be determined and expressed Milled as Heptachlor), mg/kg, *Max*	0.05	
aa)	Hexachlorobenzene, mg/kg, *Max*	0.1	
bb)	Hexachlorocyclohexane isomers (other than γ), mg/kg, *Max*	0.3	
cc)	Lindane (γ Hexachlorocyclohexane), mg/kg, *Max*	0.6	
dd)	Malathion, mg/kg, *Max*	1.0	
ee)	Methidathion, mg/kg, *Max*	0.2	
ff)	Parathion, mg/kg, *Max*	0.5	
gg)	Parathion Methyl, mg/kg, *Max*	0.2	
hh)	Permethrin, mg/kg, *Max*	1.0	
jj)	Phosalone, mg/kg, *Max*	0.1	
kk)	Piperonyl butoxide, mg/kg, *Max*	3.0	
mm)	Pirimiphos- methyl, mg/kg, *Max*	4.0	
nn)	Pyrethrin (sum of), mg/kg, *Max*	3.0	
pp)	Quintozene (sum of quintozene, pentachloroaniline, and methyl pentachlorophenyl sulphide), mg/kg, *Max*	1.0	

NOTES — 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.

ANNEX A

(*Clause* 2)

LIST OF REFERRED STANDARDS

IS No.	*Title*
IS 1070 : 2023	Reagent grade water — Specification (*fourth revision*)
IS 1797 : 2017	Spices and condiments — Methods of test (*third revision*)
IS 4333 (Part 1) : 2018	Methods of analysis for foodgrains: Part 1 Refractions (*third revision*)
IS 13859 : 1993/ ISO 7513 : 1990	Instant tea in solid form — Determination of moisture content (loss in mass at 103 °C)
IS 13145 : 2014	Spices and condiments — Methods of sampling (*second revision*)
IS 16287 : 2015/ ISO 16050 : 2003	Foodstuffs — Determination of aflatoxin B_1, and the total content of aflatoxins B_1, B_2, G_1 and G_2 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

ANNEX B

[*Table* 1, *Sl No.* (vii) *and* (viii)]

THIN LAYER CHROMATOGRAPHY OF MILLEFOLIUM (*Achillea Millefolium* L.)

B-1 GENERAL IDENTIFICATION (REACTION TEST)

Test A

To 2.0 g of the powdered herbal drug, add 25 ml of ethyl acetate, shake for 5 min, and filter. Evaporate to dryness in a water bath and dissolve the residue in 0.5 ml of toluene. To 0.1 ml of this solution, add 2.5 ml of dimethylaminobenzaldehyde solution and heat on a water bath for 2 min. Allow to cool. Add 5 ml of light petroleum and shake the mixture vigorously. The aqueous layer shows a blue or greenish-blue colour. (Dimethylaminobenzaldehyde solution: Dissolve 0.25 g of dimethylaminobenzaldehyde in a mixture of 5 g of phosphoric acid, 45 g of water, and 50 g of anhydrous acetic acid.)

Or

Test B

To 1 ml of the Hydro-alcoholic extract in a test tube, add 0.3 ml of dilute sodium hydroxide solution. A yellow colour is produced. Warm on a water bath. The colour of a moistened red litmus paper placed over the mouth of the test tube changes to blue.

B-2 THIN LAYER CHROMATOGRAPHY (TLC)

B-2.1 Apparatus

B-2.1.1 *Thin Layer Chromatograph with a Precoated Silica Gel* 60 F 254 *Plate*

B-2.1.2 *Analytical Balance*

B-2.1.3 *Standard Glassware*

B-2.1.4 *Water Bath*

B-2.2 Reagents

B-2.2.1 *Toluene* — AR or equivalent grade

B-2.2.2 *Ethyl Acetate* — AR or equivalent grade

B-2.2.3 *Ethanol* — AR or equivalent grade

B-2.2.4 *Chloroform* — AR or equivalent grade

B-2.2.5 *Anisaldehyde Sulphuric Acid* — AR or equivalent grade

B-2.2.6. *Purified Water* — IP grade or equivalent grade

B-2.3 Procedure

B-2.3.1 *Preparation of Hydro-alcoholic extract*

To 5g of the substance being examined, add 30 ml of ethanol and 20 ml of purified water. Allow to stand for twenty-four hours, shake, and filter.

B-2.3.2. *Chloroform Extraction*

Evaporate 25 ml of alcoholic extract/tincture in a water bath to remove alcohol and extract with 3 × 20 ml of chloroform. Combine and concentrate the chloroform layer to 2 ml. Carry out TLC of chloroform extract of the hydroalcoholic extract on silica gel 60 F 254 pre-coated plate.

B-2.3.3 *Mobile Phase*

Toluene: ethyl acetate (9 : 1, *v/v*).

B-2.3.4 *Estimation*

Apply 10 µl 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 the air and examine it under 366 nm. Spray the plate with a solution of anisaldehyde sulphuric acid reagent. Heat the plate at 110 °C for about 5 min or till the bands are clearly visible. The chromatogram obtained in the test solution shows under UV light 254 nm (Fig. 3), four bands appear at Rf. 0.31, 0.45, 0.75, and 0.84 (All brown), and under UV light 366 nm (Fig. 4), seven bands appear at Rf. 0.32 (blue), 0.38 (red), 0.45 (brown), 0.55 (red), 0.64 (blue), 0.73 (brown), and 0.83 (brown). After derivatization with Anisaldehyde sulphuric acid reagent, four bands appear at Rf.0.27 (blue), 0.57, 0.68 (blue), and 0.85 (purple) (Fig. 5).

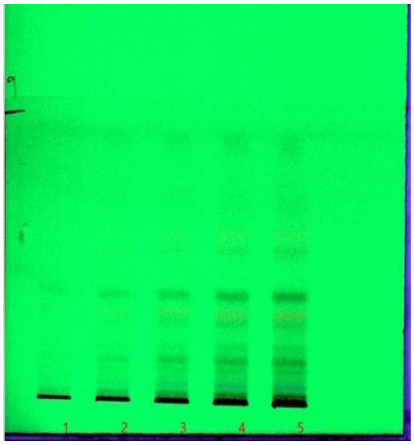

FIG. 3 UNDER 254 nm
TLC PROFILE OF ACHILLEA MILLEFOLIUM RAW DRUG

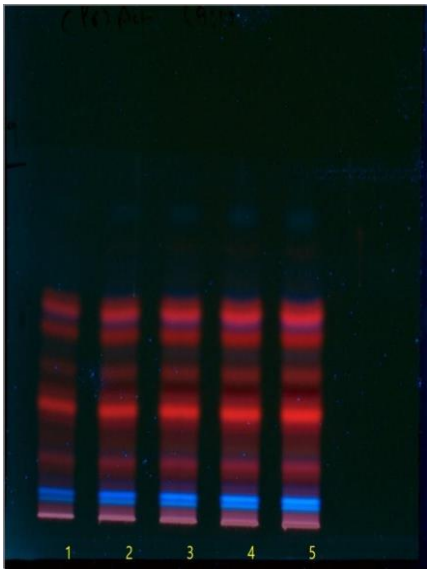

FIG. 4 UNDER 366 nm
TLC PROFILE OF ACHILLEA MILLEFOLIUM RAW DRUG

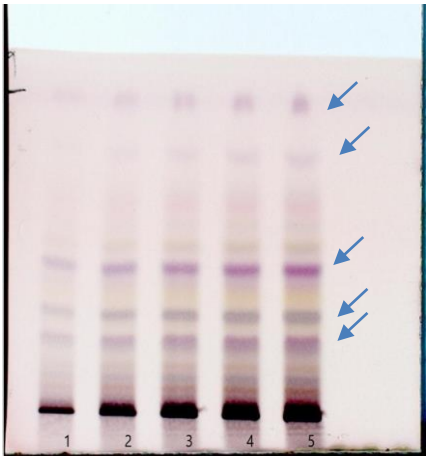

FIG. 5 AFTER DERIVATIZATION WITH ANISALDEHYDE SULPHURIC ACID REAGENTS
TLC PROFILE OF ACHILLEA MILLEFOLIUM RAW DRUG
TRACK 1 : 2 µl; TRACK 2 : 4 µl; TRACK 3 : 6 µl; TRACK 4 : 8 µl; TRACK 5 : 10 µl

B-3 THIN LAYER CHROMATOGRAPHY (TLC)

B-3.1 Apparatus

B-3.1.1 *Thin Layer Chromatograph with a Precoated Silica Gel* 60 F 254 *Plate*

B-3.1.2 *Analytical Balance*

B-3.1.3 *Standard Glassware*

B-3.1.4 *Water Bath*

B-3.2 Reagents

B-3.2.1 *n-Hexane* — AR or equivalent grade

B-3.2.2 *Ethyl acetate* — AR or equivalent grade

B-3.2.3 *Methanol* — AR or equivalent grade

B-3.2.4 *Anhydrous formic acid* — AR or equivalent grade

B-3.2.5 *Diphenylboric acid aminoethyl ester* — AR or equivalent grade

B-3.2.5 *Polyethylene glycol* 400 — AR or equivalent grade

B-3.2.6 *Purified water* — IP grade or equivalent grade

B-3.3 Procedure

B-3.3.1 *Preparation of Hydro-alcoholic extract*

To 5 g of the substance being examined, add 30 ml of ethanol and 20 ml of purified water. Allow to stand for 24 h, shake, and filter.

B-3.3.2 *Preparation of Reference Solution*

Dissolve 5 mg of luteolin and 5 mg of chlorogenic acid in 20 ml of methanol.

B-3.3.3 *Mobile phase*

Anhydrous formic acid: ethyl acetate: toluene (20 : 40 : 40 *v/v/v*).

B-3.3.4 *Estimation*

Apply 10 μl each of the test solutions (Hydro-alcoholic extract) and reference 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 the air and examine it under 366 nm. Spray the plate first with a 10 g/l diphenyl boric acid aminoethyl ester solution in methanol. Then, spray with a 50 g/l solution of Polyethylene glycol 400 in methanol. Allow to dry and examine under ultraviolet light at 365 nm.

The chromatogram of the reference solution shows the blue-green chlorogenic acid zone in the lower third and an orange luteolin zone in the upper third. The chromatogram of the test solution shows a yellow-green to green-blue zone in the lower third at about the height of the chlorogenic acid reference substance, two green-blue zones varying with more or less intensity in the middle third, an orange zone above the luteolin reference substance in the upper third and a greenish-yellow zone above the previous zone. Other faint fluorescent zones may also be present (Fig. 6).

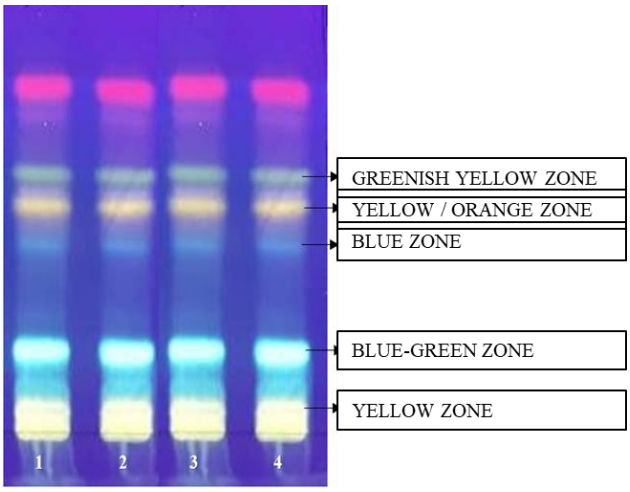

FIG. 6 AFTER DERIVATIZATION WITH NP/PEG REAGENTS TLC PROFILE OF ACHILLEA MILLEFOLIUM RAW DRUG

B-4 QUANTITATIVE ANALYSIS (ASSAY) FOR TOTAL FLAVONOIDS

Methodology: Ultraviolet and visible absorption spectrophotometry

Stock solution: To 10 g of powdered herb, add 100 ml of ethanol (60 percent *v/v*), allow to stand for 2 h, and shake it for 30 min. Then, sonicate and filter.

Test solution: In a 25.0 ml volumetric flask, introduce 2.0 ml of stock solution, 2.0 ml of a 20 g/l solution of aluminium chloride in methanol, and dilute to 25.0 ml with methanol.

Compensation liquid: In a 25.0 ml volumetric flask, introduce 2.0 ml of stock solution and dilute to 25.0 ml with methanol.

Twenty-five minutes after the last addition of the reagent, measure the absorbance of the test solution at 390 nm, compared with the compensation liquid.

Calculate the percentage content m/m of total flavonoids, expressed as luteolin-3',7-di-O-glucoside ($C_{27}H_{30}O_{16}$; Mr 610), from the expression:

$$\frac{A \times 1250}{196 \times m}$$

That is taking the specific absorbance to be 196 nm.

where

A = absorbance of the test solution at 390 nm; and

m = mass, of the sample in grams.

ANNEX C

[*Table* 1, *SI No.* xv), xvi), xvii), xviii), xix), *and* xx)]

MICROBIAL LIMIT TESTS

C-1 GENERAL

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

C-2 APPARATUS

C-2.1 Oven for Dry Sterilization

C-2.2 Autoclave for Wet Sterilization

C-2.3 Incubator

C-2.4 Water Bath

C-2.5 *p*H-meter

C-2.6 Sterile Membrane Filters — 50 mm in diameter

C-2.7 Colony Counting Equipment

C-2.8 Analytical Balance

C-2.9 Standard Glassware

C-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 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 °C ± 2 °C.

C-3.1 Baird Parker Agar Medium

C-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

C-3.1.2 *Preparation*

Suspend the components in 1 000 ml of water, heat with frequent agitation, and boil for 1 min. Sterilize, cool between 45 °C to 50 °C, and 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 and get a 3 : 7 ratio of egg yolk to saline. Add to a sterile blender cup and mix at high speed for 5 s). Adjust the *p*H after sterilization to 6.8 ± 0.2.

C-3.2 Bismuth Sulphite Agar Medium

C-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

C-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.

C-3.3 Brilliant Green Agar Medium

C-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

C-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.

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

C-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

C-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.

C-3.5 Casein Soyabean Digest Agar Medium

C-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

C-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 to 7.3 ± 0.2 after sterilization.

C-3.6 Cetrimide Agar Medium

C-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

C-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.

C-3.7 Deoxycholate Citrate Agar Medium

C-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

C-3.7.2 *Preparation*

Mix the components and allow them to stand for 15 minutes. Gently boil with continuous stirring and continue boiling until the 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.

C-3.8 Fluid Casein Digest Soya Lecithin Polysorbate 20 Medium

C-3.8.1 *Composition*

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

C-3.8.2 *Preparation*

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

C-3.9 Fluid Lactose Medium

C-3.9.1 *Composition*

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

C-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.

C-3.10 Lactose Broth Medium

C-3.10.1 *Composition*

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

C-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.

C-3.11 Levine Eosin Methylene Blue Agar Medium

C-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

C-3.11.2 *Preparation*

Dissolve the pancreatic digest of gelatin, dibasic potassium phosphate, and agar in water with warming and allow it 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.

C-3.12 Mac Conkey Agar Medium

C-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

C-3.12.2 *Preparation*

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

C-3.13 Mac Conkey Broth Medium

C-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

C-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 to 7.3 ± 0.2 after sterilization.

C-3.14 Mannitol Salt Agar Medium

C-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	5 mg
Water	1 000 ml

C-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 to 7.4 ± 0.2 after sterilization.

C-3.15 Nutrient Broth Medium

C-3.15.1 *Composition*

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

C-3.15.2 *Preparation*

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

C-3.16 Nutrient Agar Medium

The nutrient broth is gelled by adding 1 percent to 2 percent w/v of agar.

C-3.17 Pseudomonas Agar Medium for Detection of Flourescein

C-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 heptahydrate	1.5 g
Glycerin	10.0 ml
Agar	15.0 g
Water	1 000 ml

C-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 to 7.2 ± 0.2 after sterilization.

C-3.18 Pseudomonas Agar Medium for Detection of Pyocyanin

C-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

C-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 to 7.2 ± 0.2 after sterilization.

C-3.19 Sabouraud Dextrose Agar Medium

C-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

C-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 to 5.6 ± 0.2 after sterilization.

C-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.

C-3.21 Selenite F Broth

C-3.21.1 *Composition*

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

C-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.

C-3.22 Fluid Selenite Cystine Medium

C-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

C-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.

C-3.23 Tetrathionate Broth Medium

C-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

C-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.

C-3.24 Tetrathionate Bile Brilliant Green Broth Medium

C-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

C-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.

C-3.25 Triple Sugar Iron Agar Medium

C-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

C-3.25.2 *Preparation*

Mix the components in 1 000 ml of water and allow to stand for 15 min. Heat to boiling to dissolve the medium completely. Mix well, 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.

C-3.26 Urea Broth Medium

C-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

C-3.26.2 *Preparation*

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

C-3.27 Vogel Johnson Agar Medium

C-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

C-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.

C-3.28 Xylose Lysine Deoxycholate Agar Medium

C-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

C-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.

C-4 SAMPLING

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

C-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.

C-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 aforementioned 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 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 the 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.

C-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.

C-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.

C-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 the pH of the suspension to about 7.

C-7.3 Fatty Products

Homogenize 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 the formation of an emulsion and, in any case, for not more than 30 min. If necessary, adjust the *p*H to about 7.

C-7.4 Examination of the Sample

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

C-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 1 g of the preparation being examined to each of the 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 a 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.

C-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.

C-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.

C-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 the 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* C-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

C-8 TESTS FOR SPECIFIED MICROORGANISMS

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

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

C-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.

C-8.2.1 *Primary Test*

Add 1.0 ml of the enrichment culture to a tube containing 10 ml MacConkey broth and a Durham tube. Incubate at 42 °C to 44 °C for 24 h to 48 h. If the contents of the tube show acid and gas production in the Durham tube, carry out the secondary test.

C-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*.

C-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 the 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.

C-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.

C-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.

Table 3 Interpretation of the Confirmatory Tests for Salmonella

(*Clause* C-8.3.1 *and* C-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

C-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 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*.

C-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 the 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 ultraviolet 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*.

C-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, and 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 4 Interpretation of the Confirmatory Tests for *Pseudomonas aeruginosa*

(*Clause* C-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* C-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 mm to 5 mm	Positive cocci (in clusters)

C-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.

S*taphylococcus 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 preparation. There should be no growth of microorganisms.

C-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 volumes 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 microorganisms should be obtained.

ANNEX D

(*Foreword*)

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