Published online in http://ijam.co.in
ISSN: 0976-5921
International Journal of Ayurvedic Medicine, 2013, 4(2), 113-121

Study of physicochemical properties of *Indrayava* (*Holarrhena antidysenterica* wall.) and its antibacterial effect on Enteropathogenic e-coli (EPEC) (in vitro)

Research Article

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Abstract

As stated in *Ayurvedic* texts *Indrayava* mainly useful in treatment of diseases like diarrhoea, dysentery etc. E-coli is most dangerous bacteria causing diarrhoea. *Ayurveda* has included all the microbes under the heading '*Krimi*'. As per *Bhavaprakasha Nighantu*, *Indrayava* having *Atisaraghna* and *Krimighna* property. Hence *Indrayava* having action on bacteria (*Krimi*) may have action on E.coli causing diarrhoea (EPEC). So it is necessary to do the physicochemical standardization of *Indrayava*, to study its antibacterial activity on EPEC (In-vitro), to determine minimum inhibitory concentration of *Indrayava* for antibacterial activity against EPEC. Materials used are self collected sample, clinically isolated EPEC. Method used for antibacterial susceptibility is disc diffusion method. After study result came are, foreign matter is negligible, moisture content is 8.57%, total ash is 4.61%, acid insoluble ash is 0.60%, water soluble ash is 6.67%, water soluble extract is 33.32%, alcohol soluble extract is 30.51% and pH is 5.42. *Indrayava* shows the antibacterial activity against Enteropathogenic E-coli (EPEC) in methanolic extract having minimum inhibitory concentration value 2.0gm/10ml.

Key words – *Indrayava*, *Atisaraghna, Krimighna, Krimi*

INTRODUCTION

Ayurveda is the flawless ancient science of life, the word '*Ayur*' literally means 'life' and '*Veda*', the 'science' or 'knowledge'. This system of medicine is based on holistic approach and origin of it can be traced to as early as dawn of the civilization and *Vedic* period. Its aim is not just the cure of disease but the maintenance of a positive healthy state of body, mind and spirit in a healthy environment and in harmony with the universe. It also provides way of living for prevention of disease. (1,2)

As traders are supplying raw materials, they are aware of knowledge of medicinal plants in terms of external appearances, similar looking drugs; hence they do the adulteration because of which patient's health is hampered. So the question arises about the safety and efficacy of the drug. Hence standardization is the key to overcome these problems. (3,4,5)

Indrayava is mainly useful in treatment of diseases like diarrhoea, dysentery etc. E-coli (EPEC) is most dangerous bacteria causing diarrhoea. *Ayurveda* has included all the microbes under the heading '*krimi*'. As stated in *Bhavaprakasha Nighantu, Haritkyadi varga shloka* no. 158-159, *Indrayava* is most commonly used as *Krimighna*. Hence

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Indrayava having action on bacteria (*Krimi*) may have action on E-coli (EPEC) causing diarrhoea.

AIMS AND OBJECTIVES

1. To assess the physicochemical parameters of *Kutaj beej* (*Indrayava*)
2. Lab practical tests to evaluate antibacterial susceptibility of *Indrayava* against EPEC
3. To determine minimum inhibitory concentration of *Indrayava* for antibacterial activity against EPEC.

MATERIALS AND METHODS

Collection of sample: -

The plants of the *Kutaj* were identified morphologically with the standard literature and the seeds of the plant were collected in Vidarbha region in Maharashtra state in India. The sample was collected in the month of July. The sample was allowed to dry on cotton cloth in a room (temp. between 30 0 C – 35 0 C-) in a such way that insect, flies and other contaminants should not damage it. The sample was powdered with *khalva* and passed through mesh of 72 no. size and packed in self sealed polythene based after labelling.(6,7,8,9)

(A) PHARMACOGNOSTICAL STUDY (10,11)

(1) Morphological Study:

Materials: The materials collected for the studies were.

Drug: Seeds of *Kutaj* (*Holarrhena antidysenterica* Wall.)

Equipments: Sense organs to determine the organoleptic characters of sample

Methods:

Organoleptic method- nature of the seeds, colours, taste, size, shape, odour, characteristics were studied.

(2) Microscopical study:

Materials: The materials collected for the studies were

Drug: Seeds of *Kutaj* (*Holarrhena antidysenterica* Wall.)

Equipments: Compound microscope, eye piece, camera lucida, glass slides, cover slips, watch glass, camel brush, mountain brush, filter paper, blades, spirit lamp, pipettes.

Chemicals: Phloroglucinol, Chloral hydrate, Conc. HCl. Glycerin, Iodine.

Methods:

1) Section Method
2) Staining Process Method

(B) PHYSICO-CHEMICAL STUDY (11,12,13)

Foreign matter
Moisture content
Total ash value
Acid insoluble ash value
Water soluble ash value
Water soluble extractive value
Alcohol soluble extractive value
pH value

(C) PHYTOCHEMICAL STUDY (14,15,16,17)

1) Solubility of *Indrayava*

Materials: Funnels, beaker, filter paper, test tube, fine powder of *Indrayava*

Solvents: 1. Water 2. Ethanol 3. Chloroform

Methods:

Test for reducing sugars:

Benedict's test: Mix equal volume of test solution and Benedict's reagent. Heat in boiling water bath --- solution appears green in colour.

Fehling's test: Add 1ml test solution and Fehling's solutions; boil in water bath— yellow ppt.

Test for starch:

Iodine test: Mix 3ml of test solution and few drop of dilute iodine solution -- blue colour.

Published online in http://ijam.co.in
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Test for mucilage:
Add 1ml of test solution and 1ml of Ruthenium red solution ------red colour.

Test for proteins:
Biuret test: 1ml of test solution and few drops of Biuret reagent---pink colour.
Millon's test: 1ml of test solution and 2ml of Millon's reagent---- white ppt.
Xanthoprotein test: Mix 3ml of test solution with 1ml conc. Sulphuric acid---- white ppt. is formed.

Test for amino acids:
3ml of test solution and 3 drops 0.1% ninhydrin solution, heat in boiling water bath for 10mins. ----- Purple colour.

Test for oils:
Take the drug powder in the filter paper and press it----filter paper gets permanently stained with oils.

Test for steroids:
Salkowski test: 5ml of test solution and 2ml of chloroform and 3ml conc. sulphuric acid. Shake well ---brown ring.

Test for cardiac glycosides:
Killer- killiani test: 2ml of test solution. Add glacial acetic acid, a drop of ferric chloride and conc. sulphuric acid------a reddish brown ring.

Test for anthraquinone glycosides:
Borntrager's test: 3ml test solution, add dil. sulphuric acid. Boil and add filter. Cool and add benzene. Shake well. Separate the organic layer. Add ammonium hydroxide solution ---- pink colour.

Test for cyanogenetic glycosides:
Guignard test: Test solution, picric acid and sodium carbonate-----red colour.

Test for saponins:
Foam test: Shake the test solution with water vigorously. Allow it to stand – persistent foam observed.

Test for flavonoids:
1ml test solution and 5ml of sodium hydroxide------yellow colour, decolourises after addition of acid.

Test for alkaloids:
Add 2ml of test solution and a few drops of Dragendorff's reagent--orange brown ppt.
Add 2ml of test solution and a few drops of Wagner's reagent---reddish brown ppt.
Add 2ml of test solution and a few drops of Mayer's reagent ----- white ppt.

Test for tannins:
Ferric chloride test: 1ml test solution and a drop of ferric chloride solution ----blue ppt.
Lead acetate test: 1ml test solution and 1ml of lead acetate solution ------white ppt.
{Reference: Kokate C.K., 1999; Harbone, J.B., 1973}

(D) EXPERIMENTAL WORK (18,19,20)
To evaluate the antibacterial activity of *Indrayava* (*Holarrhena antidysenterica* Wall.) the following various materials were use
Materials:
A) Drugs:
1. Methanolic extract 2. Water extract 3. Ethanolic extract of *Indrayava*
B) Micro organisms
Clinically isolated E-coli (EPEC) bacterias
C) Equipments:
1. Distillation apparatus,
2. Water bath
3. Petri dish
4. Borer
5. Loops and loop holder
6. Hot air oven
7. Auto clave
8. Incubator
9. Spirit lamp
10. Cotton

11. Digital balance
12.Test tubes

Method:

Preparation of plant extracts: 2.5gm of samples were extracted with water, ethanol and methanol. The extracts obtained from the above were used for testing antimicrobial efficacy.

Cultural media: Standard Nutrient agar Petri plates were prepared for the growth of bacterial cultures.

Test culture: Escherichia coli.

Preparation of discs: Discs of 5mm diameter were prepared from Whatman's filter paper no.41 (ash less) were cut out with a punch press and were soaked in water, alcohol, methanol for some time and then dried. Few of these discs were used as standard discs and the remaining discs were transferred to the above plant extracts for thorough moistening. They were maintained for 48 hrs so that maximum amount of extract or active principle in it was impregnated on each disc. These discs were used for antimicrobial efficacy.

About 0.1ml of 8 hrs old culture was placed in each nutrient agar plate with a Pasteur pipette. The plates were then gently rotated to spread the inoculums uniformly. Then the impregnated discs were placed on the media with a sterile forceps; 3-4 discs impregnated with plant extract.

The discs were then pressed gently on the surface so that they are not shifted from position subsequently and firmly affixed to the plate. This reacts to the uniform diffusion. All this operation was carried out aseptically. The plates were then incubated at 35-370c for 24hrs.

The experiments were performed in triplicates and the average zones of inhibition were recorded.

(Chandrakant R.K., 2007; Mandal P., Sinha Babu, S.P., and Mandal, N.C., 2005; Kavitha, D., 2004; Khan, M.R., Kikhara, M. and Omoloso, A.D., 2001; Nair, A. and Bhide, S.V., 1996; John, B.H., 1989; Kirti, S.L., 1985; Banerjee, Anup and Nigam, S.S., 1978, 197)

(E) Determination of Minimum Inhibitory Concentration (MIC)(21,22,23)

Materials

Plant extract : Methanol extract of *Indrayava*

Organism used: Escherichia coli (EPEC)

Preparation of the sample solution:

2.00gm of plant extract was taken in vials separately. Then 10ml methanol was added.

Preparation of inoculums:

The E. coli was grown at 37 degree Celsius in nutrient agar medium and was diluted in nutrient broth medium in such a manner that the suspension contains about 107 / ml. This suspension was used as the inoculums.

Procedure:

1. Twelve test tubes were taken, nine of which were marked 1, 2, 3, 4, 5, 6, 7, 8, 9, and the rest were assigned as TM(medium), TME(Medium + extract) and TMI(Medium + Inoculum).
2. 4 ml of nutrient broth medium was poured to each of the 12 test tubes.
3. These test tubes were cotton plugged and sterilized in an autoclave for 15 Ibs/ sq.inch pressure.
4. After cooling 2ml of the sample solution was added to the 1st test tube and mixed well and then 2ml of this content was transferred to the test tube.
5. The content of the second test tube was mixed well and again 2ml of this mixture was transferred to the 3rd test tube. This process of serial dilution was continued up to the 9th test tube.
6. 10µl of properly diluted inoculum was added to each of 9 test tubes and mixed well.
7. To the control test tube TME, 2ml of the sample was added, mixed well and 2ml of this mixed content was

Published online in http://ijam.co.in
ISSN: 0976-5921
International Journal of Ayurvedic Medicine, 2013, 4(2), 113-121

discarded to check the clarity of the medium in presence of diluted solution of the compound.

8. 10µl of the inoculum was added to the control test tube TMI, observe the growth of the organism in the medium.
9. The control test tube TM, containing medium only was used to confirm the sterility of the medium.
10. All the test tubes were incubated at 370 c for 18 hours.

Result:

A) ORGANOLEPTIC CHARACTERS

Shabda : *Jvalankalin – Char-Char, Bhanguratva* : *Abhangur*
Sparsha : *Kathin, Ruksha, Khara*
Rupa : *Light yellowish brown*
Rasa : *Tikta, Katu, Kashaya*
Gandha : *Mrudu*

B) PHARMACOGNOSTIC STUDY

1) Macroscopic characters: - Elongated, margins curved inside, one side convex and other side concave, 1 to 2cm long, 0.2 to 0.3cm thick.

2) Microscopic characters:

Testa: It is single layered radially arranged, compact parenchymatous cells filled with brown content. This layer is surrounded on the upper surface by papillose cells which looks like a trichome.

Tegmen: Testa is followed by two layers of small rounded to irregular cells; few of them show the presence of prismatic shaped calcium oxalate crystals.

Just below these layers are the collapsed nucellar cells filled with brown content.

Endosperm: It is with outer and inner tangentially elongated epidermal cells covered with cuticle. They are 5-6 layered, rounded to polygonal, compactly arranged parenchymatous cells. These cells contain aleurone grains and abundant oil globules.

Cotyledons: Two foliaceous convolute cotyledons are present. Each cotyledon is single layered tabular epidermal cells towards the dorsal side and rectangular cells towards ventral side. They are covered with cuticle. The central cells are polygonal, compactly arranged, with oil globules and prism shaped calcium oxalate crystals. There are poorly developed vascular bundles at the groove region.

Powder Study:

Calcium oxalate crystals: abundant, prism shaped calcium oxalate crystal measuring 0.8 -1 µm in length and 1.2 -1.5 µm in breadth.

Parenchymatous cells of cotyledon: polygonal, with starch grains, measuring 2-6µm in diameter.

Oil globules: round in shape, measuring 1-2 µmin diameter

Papillose cells: broken pieces of elongated cells measuring 1.4 µm in diameter.

Cells of endosperm: the cells are polygonal, compactly arranged, parenchymatous, measuring 1.3-1.7 µm in diameter.

Cells of tegmen: irregular to polygonal fragments of parenchymatous cells measuring 0.9-1.6 µm in diameter.

C) PHYSICOCHEMICAL VALUES

a) Foreign matter	:	Nil
b) Moisture content	:	08.57 %
c) Total ash	:	04.61 %
d) Acid insoluble ash	:	00.60 %
e) Water soluble ash	:	06.67 %
f) Water soluble extract	:	33.32 %
g) Alcohol soluble extract	:	30.51 %
h) pH value	:	05.42

D) PHYTOCHEMICAL STUDIES

Reducing sugar, amino acids, alkaloids, tannins, proteins, cardiac glycosides, Anthraquinone Glycosides, oils, flavonoids are present in water, Ethanol & chloroform extract and saponins present only in water extract. Starch, mucilage, steroids are absent in all the three extracts.

E) ANTIBACTERIAL ACTIVITY

Table1. Showing antibacterial susceptibility against EPEC

Name of organism	Extract	Diameter of zone of inhibition (mm)

E coli	Water	-
	Ethanol	-
	Methanol	12

F) MINIMUM INHIBITORY CONCENTRATION (MIC) VALUE AGAINST EPEC

Table 2 showing MIC value against EPEC

No. of test tubes	Nutrient broth medium added (ml)	Diluted solution of plant extract (gm/10 ml)	Inoculum added µl	Observations
1	4	0.1	10	+
2	4	0.5	10	+
3	4	1.00	10	+
4	4	1.5	10	+
5	4	2.00	10	-
6	4	2.1	10	-
7	4	2.2	10	-
8	4	2.3	10	-
9	4	2.5	10	-
TME	4	0.1	10	-
TMI	4	0	10	+
TM	4	0	10	-

'+' Indicates growth '-' Indicates no growth

In E. coli the growth of the organism was observed in the test tube no. 4, indicating that the MIC value of the plant extract was 2.00 gm /10ml.

Discussion:

1. Holarrhena antidysenterica is a drug which is easily available. It is also being sold in market. Indrayava is being used in many diseases as per *Ayurvedic* classics. It is mainly useful in treatment of diseases like diarrhoea, dysentery etc. *Indrayava* is a *sangrahi*. It is most commonly used as *krimighna*. *Ayurveda* has included all the microbes under the heading *krimi*. *Indrayava* is *kriminashaka* as explained earlier. Hence *Indrayava* having action on bacteria may have action on E.coli. E-coli is most dangerous bacteria causing diarrhoea. Day by day this bacteria is resistant to most of the higher antibiotics like Ampicillin, Tetracycline, Cotrimoxazole etc. In modern drug study, the preclinical study is essential to carry out the clinical trials. Antibacterial activity is part of it. Hence it is a need of today's era to evaluate the antibacterial activity of *Indrayava* against E.coli.

2**.** Result shows that the rasa of *Indrayava* is *Tikta, Katu, Kashaya, Veerya* is *Sheet* and *Vipaka* is *Katu*. The drug is sparingly soluble in water, alcohol, oil and ghee (*ghrit*).

3. *Indrayava* is elongated; margins curved inside, one side convex and other side concave, 1 to 2cm long, 0.2 to 0.3cm thick. Microscopic characters show testa, tegmen, collapsed nucellar cells, endosperm containing aleurone grains and abundant oil globules, two foliaceous convolutes cotyledons.

4. Powder study reveals abundant, prism shaped calcium oxalate crystal, parenchymatous cells of cotyledon, oil globules, papillose cells, cells of endosperm, cells of tegmen.

5. The drug is standard as all the tests show result within the normal limit as per Ayurvedic Pharmacopoeia of India vol.III.

6. Drug show antibacterial activity against E-coli in methanolic extract only.

7. Minimum inhibitory concentration for the antibacterial activity against E-coli (EPEC) in methanolic extract is 2gm/10ml.

8. So, extract of *Indrayava* powder is effective against Enteropathogenic Escherichia Coli (EPEC) in methanolic extract at the minimum inhibitory concentration of 2gm/10ml which is already mentioned in *ayurvedic* text the *Krimighna* property and antidiarrhoeal property of *Indrayava*.

9. The further research is required for providing efficacy of the drug in animals and then patients

Conclusion:

From the discussion it is concluded that the self collected sample of *Indrayava* is standard with respect to its pharmacognostical, physicochemical, phytochemical test. Antibacterial study with reference to E.coli shows the positive results in methanolic extract of *Indrayava*. It does not show zone of inhibition in water and ethanolic extracts. This is due to the active constituents which are dispersible in methanol may have antibacterial action against EPEC. The other constituents which are soluble in water and ethanol may not have antibacterial activity against EPEC. The minimum inhibitory concentration requires for antibacterial activity of *Indrayava* against EPEC is 2.00gm/10ml.

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Acknowledgements:
With the blessing of God Dhanwantari I completed my research project in a comprehensive manner. I sincere thanks to my father Mr. Sudhakarrao and my mother Mrs Rekha for their constant support and admonition, this work cannot be completed without their help. Last but not the least the whole credit goes to my sincere efforts.

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Published online in http://ijam.co.in
ISSN: 0976-5921
International Journal of Ayurvedic Medicine, 2013, 4(2), 113-121

Fig. 1

Fig.2

Fig. 1&2 Showing Macroscopic Characters

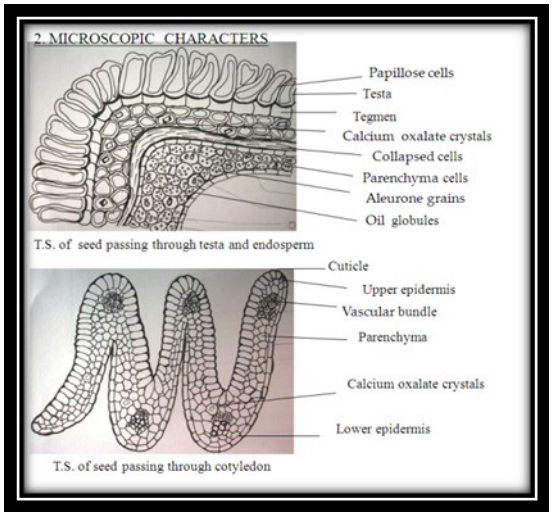

Fig. 3 Showing Microscopic Characters

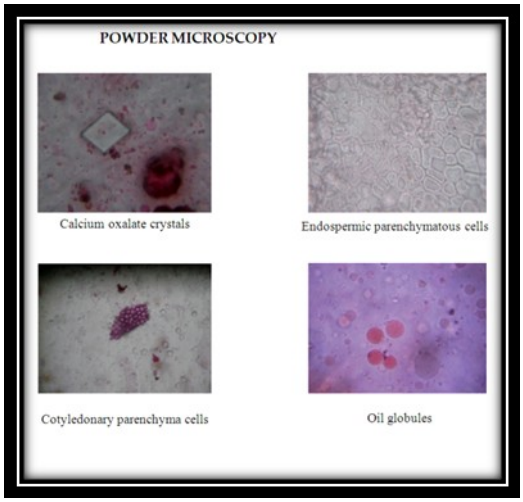

Fig. 4 Showing Powder Study

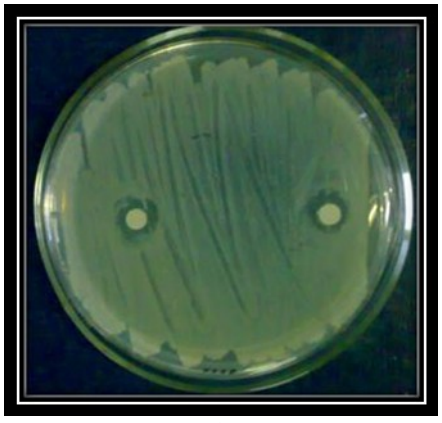

Fig 5 Zone Of Inhibition

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