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Research Article

Phytochemical Evaluation of Bark of *Chirbilva Holoptelea integrifolia* Planch.

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ABSTRACT

Purpose: This study serve the purpose of standardization of bark of *Chirbilva Holoptelea integrifolia* an Ayurvedic drugs mentioned in classics. **Methods:** Drug is studied both qualitatively and quantitatively. Bark was air-dried under net in sun and fine powdered. Fine powdered was taken for phytochemical analysis. **Results:** Phytochemical study reveals phytochemical standardization of plant material With preliminary phytochemical analysis of drugs, presence or absence of primary and secondary metabolites was exposed. Carbohydrates, protein, alkaloids and cardiac glycosides were slightly present in bark. Flavonoid was moderately present and contains important compounds such as gallic acid, quercetin catechin, rutin, hyperoside and chlorogenic acid. **Conclusion:** Finding of this study laid a specific background for further research on extracts of stem bark of *Holoptelea integrifolia* for identification, purification, isolation of compounds having anti-inflammatory, digestive, carminative, laxative, anthelmintic, depurative revulsive, hypolipidemic and urinary astringent properties.

INTRODUCTION

Holoptelea integrifolia planch is named as *Chirbilva* in Ayurveda used in diseases such as *Medoroga* (hypercholesterolemia), *Shotha* (oedema), *Krimi* (worms infestation), *Pleeha roga* (spleen disorders), *Shoola* (colic) etc. Its usable parts are *Twaka* (bark), *Pushpa* (flower), *Patra* (leaves) for both internal as well as external use.

It is a deciduous tree;[1] bark whitish grey with an offensive smell when freshly cut: wood light yellowish grey, moderately hard, little used except as fuel; leaves alternate, distichous entire penni nerved stipules lateral scarious; flowers polygamous or hermaphrodite in fascicles at the scars of the previous year's shoots, which are scaly but leafless; Perianth simple calycine 4-8 partite, lobes imbricate often unequal; stamens 4-8 erect: anthers hairy ovary stipitate compressed; ovule solitary pendulous, style short, bifid, the stigmatose; fruit dry indehiscent, samaroid flat the wing ovate, reticulate membranous; seeds flat, albumen 0; cotyledons longitudinally folded; radicle small superior. As mentioned in data base[2], the bark and leaves are *Tikta* (bitter), *Kashaya* (astringent), acrid and *Ushna veerya* (thermogenic). It has anti inflammatory, digestive, carminative, laxative, anthelmintic, depurative repulsive and urinary astringent properties. They are useful in inflammation, acid gastritis, dyspepsia, flatulence, colic, intestinal worms, vomiting, wounds, skin disease, vitiligo, leprosy, filariasis, diabetes, hemorrhoids', and rheumatism. *Chirbilva* is mentioned in all three *Samhitas*[3,4,5], Vangasena (12th A.D)[6] has mentioned the use of *Putikaranja* in the treatment of *Masurika*, references regarding *Chirbilva* also found in *Vachaspathya* (19th A.D) Bhattacarya taranatha taraka vacaspathi[7], Priya Nighantu (P.N.) (19A.D)[8], *Gunaratnamala* of Bhavmisra 17, Dravyaguna samgraha: (D.S) Bhagwan Dash & Lalit Kashap[9], Nighantu adarsha[10], and Dravyaguna Hastamalka[11] have detailed about the synonyms, *Guna-karma* etc of *Chirabilva*. According to Bhaisajyaratnavalli[12] it is

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used as an ingredient in *Gandhakajjalika*, *Mahasat Palakaghrita, Simhyamrita ghrita, Chirbilvadi kashaya, Chirbivadi Choorna, Puskaradi Kvatha, Mahapinda tailam.* According to A.P.I[13] it is used in *Piyusavalli rasa, Gandharvahastadi Kvatha Churna*. As usage of bark is more so phytochemical study of bark is taken.

Morphological characters of tree and macroscopical characters of bark of *Holoptelea integrifolia* (Roxb.) Planch.

Tree

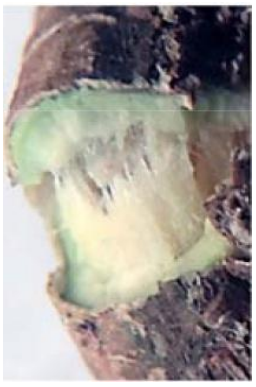
Fruits and Flowering Twig

Bark Pattern

Bark

Upper Surface

Inner Surface



Fracture

MATERIALS AND METHODS

Plant Material

The bark of *Holoptelea integrifolia* Planch. (Family- *Ulmaceae*) was collected from college campus of A.L.N. Rao Memorial Ayurvedic Medical College, Koppa. Taxonomical verification was done by botanist Prof. Radhakrishna Rao in A.L.N. Rao Memorial Ayurvedic Medical College. Ayurvedic medical from modern aspects Quality Control Laboratory at A.L.N. Rao Memorial by Dr. Prashant Kumar Jha.

Preservation of Samples

Holoptelea integrifolia bark was air-dried under net in sun and fine powdered. Fine powdered was taken for phytochemical studies.

Instruments and Chemicals

Formalin-aceto-alcohol (FAA) chloral hydrate and stained with phloroglucinol+HCl, saffranine green, iodine, sudan solution etc. Sony digital camera attached to BESTO RCM-20XL microscope, crucible, muffle furnace, dilute hydrochloric acid, Dragendorff's

reagent, 5% leadacetate solution, KOH solution, chloroform solution, H_2SO_4, 95% ethanol, glacial acetic acid, 5% $FeCl_3$, Folin-Ciocalteu reagent, Pre coated TLC plates (silica) of thickness 0.20mm, 20x20cm

Phytochemical Study

Physiochemical Analysis [14-17]

Physico-chemical and phytochemical parameters including qualitative tests, quantitative estimation, of total phenol, total flavonoids and thin layer chromatography of bark was done as prescribed in Indian pharamacopeia. Fluorescence tests were done using alkalies and acid for powder of both bark drugs. They were observed under visible light and under long UV.

Loss on Drying (LOD)

10 gram of drug sample was taken in a pre weighed dried petri dish. It was dried in an oven at 105°C until reaching a constant weight. The petri dish was taken out, self cooled and weighed immediately. The weight loss i.e., loss on drying was calculated and expressed as % w/w.

Ash Value (AV)

3 gram accurately weighed sample was taken in a pre weighed dried crucible. It was incinerated in a muffle furnace up to 450°C. The crucible was taken out, self cooled and weighed immediately. From the weight of the ash, the ash value was derived with reference to the air dried drug. It was calculated and expressed as %w/w.

Acid Insoluble ash

Ash obtained from total was boiled for 5 minutes with 25ml of dilute hydrochloric acid. Insoluble matter was collected on an ash less filter paper after filtering. The ash less filter paper was taken in crucible and was ignited for constant weight at 450°C. Percentage of acid insoluble ash was calculated with reference to the air dried drug.

Water Soluble Ash: The process was repeated as done for acid insoluble ash, only in place of acid hot water was used and soluble ash was determined after incineration.

Water Soluble Extractive (WSE)

5gm of the sample was weighed accurately. To it 100ml of distilled water was added and was kept covered for 24 hours. It was stirred intermittently for first six hours. Next day, it was filtered. Filtrate was taken in pre-weighed evaporating dish. The evaporating dish was placed on a water bath for evaporation of the water. After evaporation of the water, it was allowed for cooling and was kept in desssicator. Then it was weighed immediately. From the weight of the residue obtained, the percentage of water soluble extractive was calculated and expressed as % w/w.

Alcohol Soluble Extractive (ASE)

The method adopted for this experiment was same as that of water-soluble extract but by using methanol instead of water. Percentage of methanol soluble extract was calculated and expressed as % w/w.

Qualitative Tests [18,19]

Alkaloids

With Dragendorff's reagent, the alcoholic extract of the samples was taken in a watch glass, solvent was evaporated. It was added with 2N HCl and few drops of Dragendorff's reagent which gave orange brown precipitates. The aqueous extract of test samples was taken in test tubes, by adding 5% lead acetate solution, it gave precipitate which turned red on addition of KOH solution. Addition of excess KOH dissolved the precipitate.

Triterpenoids

Liebermann-Burchard reaction: A chloroform solution of test drugs was taken in a test tube. On addition of acetic anhydride and conc. H_2SO_4 the solution turned green, orange, blue or purple red color.

Carbohydrate

Fehling Test: To 2ml of solution of both drugs, equal volume of Fehling A and Fehling B mixture were added. It was place in boiling bath for 5 minutes. A red precipitate was formed.

Flavonoid

Shinoda Test: To dry powder of both species, 5ml of 95% ethanol was added. It was followed by few drops conc. HCl and 0.5gm magnesium turnings. Pink colour observed was positive test.

Saponins

Foam test: The dry powder was vigorously shaken with water. Persistent foam indicated the presence of saponins.

Cardiac glycosides

Keller Killiani test: To 2ml aqueous extract of both species glacial acetic acid was added. It was followed by one drop of 5% $FeCl_3$ and conc. H_2SO_4. Appearance of reddish brown colour at junction of the two liquid layers and changing of upper layer bluish green was positive result.

Fluorescence Tests

Fluorescence tests were done using alkalies and acid for powder of both bark drugs. They were observed under visible light and under long UV.

Determination of Total Phenolic Content[20]

The amount of total phenolics in hydro-alcoholic extracts of bark of *Holoptelea integrifolia* was determined with the Folin-Ciocalteu reagent. Gallic acid (Merck) was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE). For this purpose, the calibration curve of gallic acid was drawn. 1ml of standard

solution of concentration 0.01, 0.02, 0.03, 0.04 and 0.05mg/ml of gallic acid were prepared in methanol. Concentration of 0.1 and 1mg/ml of plant extract were also prepared in methanol and 0.5ml of each sample were introduced into test tubes and mixed with 2.5ml of a 10 fold dilute Folin- Ciocalteu reagent and 2ml of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 minutes at room temperature before and the absorbance was at read at 760 nm. UV-Visible spectrophotometer of Systronic 106 was used for determination.

Concentration (mg/100ml)	Absorbance
0.5	0.236
1	0345
1.5	0.402
2	0.472
2.5	0.573
3	0.657

Determination of the Total Flavonoid[21]

It was determined by Chang et al. method. Flavonols in hydro-alcoholic extracts of bark of *Holoptelea integrifolia* was expressed as quercetin equivalent. Quercetin (Merck) was used to perform the calibration curve. Standard solutions of 2, 4, 6, 8, and 10mg per 100ml were used in 70% solution of ethanol in water (V/V). Hydro-alcoholic extracts of bark was obtained using the powder of bark. Hydro-alcoholic extracts consisted of 70% absolute alcohol and 30% water.

1ml of sample (hydro-alcoholic extracts and quercetin) was mixed with 3ml 95% ethanol (V/V), 0.2 ml 10% aluminum chloride (m/V), 0.2ml of 1 mol L–1 potassium acetate and 5.6ml water. The same volume of distilled water in blank substituted a volume of 10% (m/V) aluminum chloride. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 420nm. UV-Visible spectrophotometer of Systronic 106 was used for determination.

Sample Number	Concentration (mg/100ml)	Absorbance
1	2	0.236
2	4	0.451
3	6	0.662
4	8	0.869
5	10	1.12

Thin layer Chromatography (TLC) (Reference: Quality Control Methods for Medicinal Plants by WHO)

Materials: Pre coated TLC plates (silica) of thickness 0.20mm, 20x20cm, applicator, glass chamber, oven, solvents, spraying reagents.

Sample Preparation: Hydro-alcoholic (70% methanol and 30% water) extract of barks and reference compounds was used. Gallic acid, quercetin, rutin, catechin, hyperoside, and chlorogenic acid of Sigma brand was used. This was dissolved in Hydro-alcohol with specified ratio of 7:3 for spotting.

Method: Dried TLC chamber was taken and was saturated for 30 minutes with respected solvent systems for specific *Kashaya*. Vacuum grease was smeared on the lid so that chamber became air tight. A piece of tissue paper was kept immersed in the chamber for perfect saturation of solvent system. Now the preheated pre coated TLC plates were taken and spotted with the help of applicator 1cm away from the base, space of 2cm was maintained between each spots. The spotted plate was dried and gently immersed in TLC chamber in such a way that solvent had uniform linear contact with the plate. Now the chamber was closed. The chromatogram was developed at room temperature by allowing the solvent to ascend the specified distance. Now, the plate was removed from chamber. The position of the solvent front was marked. All the plates were viewed under long UV without using spraying agent except *Pippali*. For *Pippali*, Anisaldehyde Sulphuric acid was used as spraying agent.

Solvent System: Ethyl acetate: Formic acid : Acetic acid : Water (10:1.1: 1.1: 2.6)

Spraying Agent: Iodine

Observation: The centre of each spot was marked with a needle. The distance from centre of each spot to the point of application was measure and recorded.

Resolution factor (RF value) = a/b

Where, a = Distance between the point of application and the centre of the spot of the material being examined.

DISCUSSION

Phytochemical Study

Physico-chemical Parameters

Bark of *Holoptelea integrifolia* assessed with physico-chemical parameters viz., loss on drying, total ash, acid insoluble ash, water soluble ash and extractive values (both alcohol soluble and water soluble). Loss on drying at 105°C was seen 7.3% in H.integrifolia. This is amount of moisture present with powder of bark. Water holding capacity brings more moisture content with material. Total ash was found 6.3%. It means inorganic salts present with drug, either in free form or in compound form along with organic or inorganic compounds were slightly more corresponding to quantity of Total ash. Out of obtained ash, that soluble with water was found 3.4% in *Holoptelea* where as ash insoluble with acid (6N HCl) was 1.3%. Solubility with water and alcohol (methanol) was 13.40% for *Holoptelea* whereas

alcohol soluble extractives were found to be 8.30% for this bark.

Qualitative Tests

With preliminary phytochemical analysis of drugs, presence or absence of primary and secondary metabolites was exposed. The intensity of colour might be giving an idea about the quantity of specific metabolite. Carbohydrates, protein, alkaloids and cardiac glycosides was slightly present in bark. Flavonoid was moderately present. Quantity of tannin was abundantly found where as test for anthraquinone glycoside was less intense in *Holoptelea*. Steroids and triterpenoids was also found abundant.

Fluorescence Tests

Fluorescent study was done with alcohol, acid, alkali and water. Reactions with compounds (metabolites) present with drugs give various color reactions. Powder of bark was mud colored in visible light whereas milky white in long UV. Color differences were noted in almost all cases under visible light and under long UV except with 10% HCl, where colour observed under visible light was same for bark being creamish yellow. Even for same solvent under long UV, color was different being yellow respectively. With water it was yellow respectively under visible light and long UV for *Holoptelea*, it was brick red. With 10% alkali (NaOH), colour exhibited for *Holoptelea* was orange and green under visible light and under Long UV in sequence. With oxidizing acid like nitric acid and sulphuric acid, the colour was pale yellowish-green, bright yellow and yellowish green, pale yellow respectively under visible light and under long UV for *Holoptelea*. Ammonia gave creamish-yellow colour under visible light and green colour under Long UV. The colour obtained with methanol was straw colour and white respectively under visible light and under long UV for *Holoptelea*.

Quantitative Estimation

Quantitative estimation was done for total phenol and total flavonoids of bark. Total phenol was found 152 mg per gram for *Holoptelea*. Quantity of flavonoid is 86mg/gram.

Thin Layer Chromatography

Thin Layer Chromtaography of hydro-alcoholic extracts of barks of *Holoptelea* was done with reference to standard compounds of gallic acid, quercetin, and catechin. Rf values of these standards were respectively 0.19, 0.34 and 0.52 for gallic acid, quercetin and catechin under ethyl acetate/formic acid/acetic acid/water with ratio of 10/1.1/1.1/2.6. Intensity of colour under long UV before derivatization with iodine was more intense in *Holoptelea* for compounds of Rf of gallic acid for compounds of Rf of quercetin and catechin. Compounds with Rf values 0.03, 0.20, 0.32, 0.40, 0.52, 0.60, 0.67 and 0.76 were seen under long UV for *Holoptelea*. After derivatization with iodine, intensity of colour was less in *Holoptelea* for Rf 0.76. Under visible light after derivatization compound with Rf 0.45 were clearly seen in *Holoptelea* was pinkish touch for quercetin and catechin. After derivatization compounds were seen.

RESULTS

Phytochemical study

Physico-chemical parameters

Parameters	***Holoptelea integrifolia* bark Powder**
Total ash	6.3%
Water soluble ash	3.4%
Acid insoluble ash	1.3%
Loss on Drying (LOD)	7.3%
Water Soluble Extractive (WSE)	13.40%
Alcohol Soluble Extractive (ASE)	8.30%

Qualitative Tests

Table showing the results of qualitative phytochemical study of crude bark Course powder of *Holoptelea integrifolia*

Phytochemicals	***Holoptelea integrifolia* Bark**
Carbohydrate	++
Protein	+
Alkaloids	+
Cardiac glycosides	++
Flavonoids	++++
Tannins	++++
Anthroquione glycoside	++
Steroids	++++
Triterpenoids	+++

Fluorescence Test

Showing Observation of Fluorescent study of *Holoptelea integrifolia* bark powder

Chemical Treatment	In Visible	In U.V
Bark powder	Mud color	Milky white
Sample powder + water	Yellow	Greenish yellow
Sample + 10% NaoH	Orange	Green yellow
Sample + 10% HCL	Creamish yellow	Yellow
Sample + 10% HNO3	Pale yellowish brown	Bright yellow
Sample + 10% NH3	Yellowish green	Green
Sample + 10% MeoH	Straw color	White

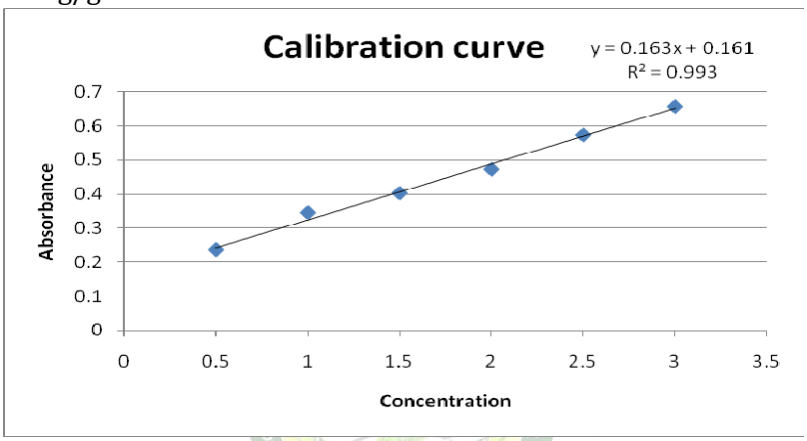
Determination of the Total Phenols

Bark of *Holoptelea integrifolia*: 152 + 21.55mg/gm

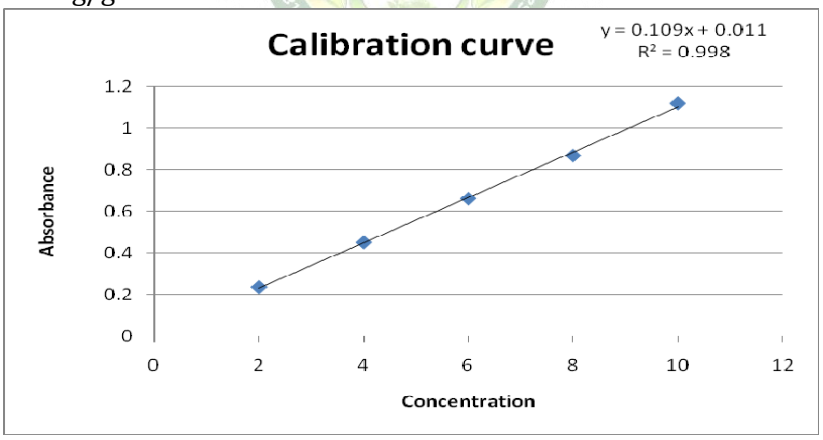
Quantitative Estimation *Holoptelea integrifolia*

Total Phenol 152 + 21.55mg/gm

Total Flavonoids 80 + 12.24mg/gm



Total Flavonoids 80 + 12.24 mg/gm



Thin layer Chromatography (TLC): First System

Solvent system: Ethyl acetate: Formic acid: Acetic acid: Water (10: 1.1: 1.1: 2.6)

Spraying agent: Iodine

T.L.C. profile of the *Holoptelea integrifolia* Planch.

Before Spray			After Spray		
R_f	In UV	In Visible	R_f	In UV	In Visible
0.036	Pale pink	Florescent pale green	0.036	-	Pale yellow
0.22	Red	Pale yellow	0.22	-	Creamish grey
0.33	Yellow	Green	0.33	-	Pale yellow
0.40	Red	Pale pink	0.40	-	Pale yellow
-	-	-	0.45	-	Yellowish grey green

0.52	Florescent green	Pale green	0.52	-	Grey
0.67	Yellow	Pale green	0.67	-	Yellow
0.75	Yellow	Yellow	0.75	Pale blue	Yellowish grey green

Gallic acid (G)

Before spray			**After Spray**		
Rf	**In UV**	**In Visible**	**Rf**	**In UV**	**In Visible**
0.20	Pale yellow	-	0.20	-	Purple

Quercetin (Q)

Before spray			**After Spray**		
Rf	**In UV**	**In Visible**	**Rf**	**In UV**	**In Visible**
0.34	Pale yellow	Pale yellow	0.34	-	Pale yellow

Catechin (C)

Before spray			**After Spray**		
Rf	**In UV**	**In Visible**	**Rf**	**In UV**	**In Visible**
0.52	-	Pale yellow	0.52	-	Pale pink

Thin Layer Chromatography: Second System (Plate Number: 6)

Solvent system: : Ethyl acetate : Formic acid : Acetic acid : Water(10: 1.1: 1.1: 2.6)

Spraying agent: Iodine

Holoptelea integrifolia

Before spray			**After Spray**		
Rf	**In UV**	**In Visible**	**Rf**	**In UV**	**In Visible**
0.036	Pale pink	Florescent pale green	0.036	-	Pale yellow
0.22	Red	Pale yellow	0.22	-	Creamish grey
0.33	Yellow	Green	0.33	-	Pale yellow
0.40	Red	Pale pink	0.40	-	Pale yellow
-	-	-	0.46	Pale blue	Yellowish grey green
0.52	Florescent green	Pale green	0.56	Pale blue	Grey
0.67	Yellow	Pale green	0.67	-	Yellow
0.75	Yellow	Yellow	0.70	blue	Yellowish grey green
0.81	-	grey	0.81	Florescent blue	-

Hyperoside

Before spray			**After Spray**		
Rf	**In UV**	**In Visible**	**Rf**	**In UV**	**In Visible**
0.67	Pale florescent blue	-	0.67	florescent blue	Pale creamish grey green

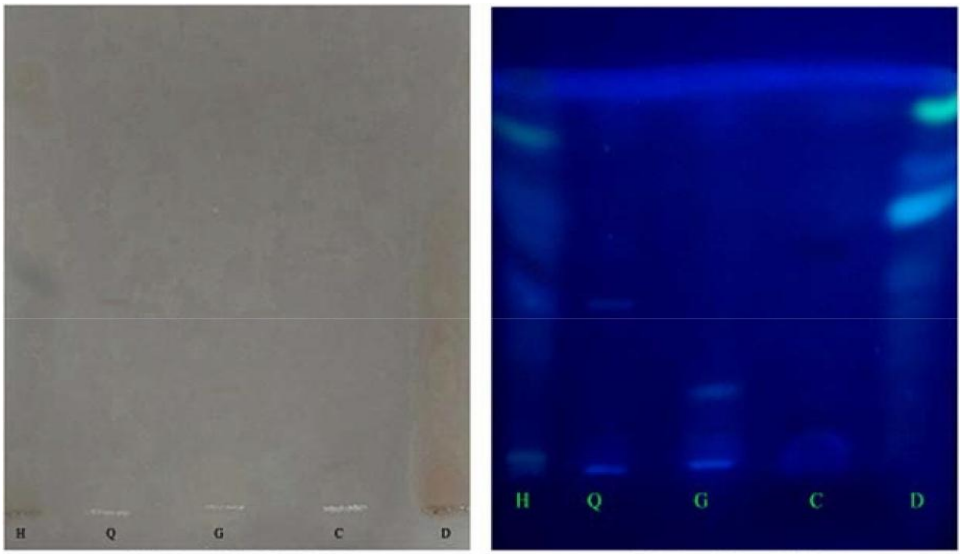
Rutin

Before spray			**After Spray**		
Rf	**In UV**	**In Visible**	**Rf**	**In UV**	**In Visible**
0.41	blue	-	0.41	Florescent blue	Grey green

Chlorogenic Acid

Before spray			**After Spray**		
Rf	**In UV**	**In Visible**	**Rf**	**In UV**	**In Visible**
0.53	Bright florescent blue	-	0.53	Blue	Creamish grey

Thin Layer Chromatography of Bark of *Holoptelea integrifolia* with reference to Quercetin (Q), Gallic acid (G) and Catechin(C)

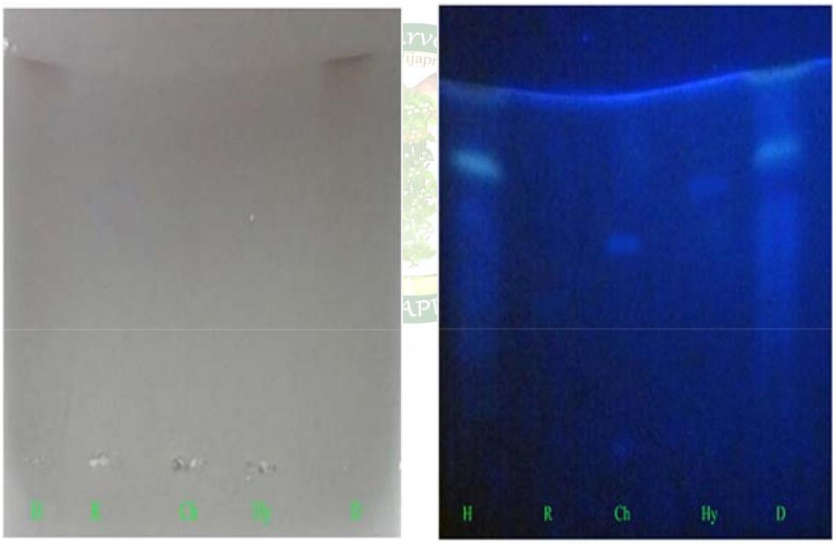


Under Visible Light **Under Long UV**

Before Derivatization

Thin Layer Chromatography Of Bark Of Holoptelea integrifolia With Reference to Rutin(R),Chloregenic acid(G) and Hyperoside(C)

Under Visible Light



Before Derivatization **Under Long UV**

CONCLUSION

Ash values and extractive values were observed in bark. Qualitative tests exposed almost intensity of colours after adding reagents. Differences were observed with fluorescent tests as with NaOH. *Holoptelea* bark exhibited green yellow color under long UV. Differences in colour with alkalies, acids and alcohol were might due to difference in quantity of coloring materials present in bark. On quantitative estimation of secondary metabolites, total phenol were found more with *Holoptelea* bark while total flavonoids were slightly found. In system I, total seven spots were observed under visible light or under long UV for *Holoptelea* bark. Compound with Rf 0.67 was observed in *Holoptelea* bark whereas compound with Rf values 0.38, 0.45 and 0.62 were absent *Holoptelea* bark. For reference compounds in mentioned solvent system, gallic, quercetin and catechin was having Rf values 0.20, 0.34 and 0.52 respectively in system I while rutin, hyperoside and chlorogenic acid were having Rf values 0.41, 0.67 and 0.53 in sequence. In system II, total eight spots were observed under visible light or under long UV for *Holoptelea* bark.

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