Research Article
Volume 8:6, 2022

Journal of Pharmacognosy and Natural Products

ISSN: 2472-0992 Open Access

Comparison of Different Solvent and Extraction Methods for Isolation of Flavonoids Compound from Leaves of *Clerodendrum Infortunatum Linn*

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Abstract

Clerodendrum infortunatum Linn leaves of the Verbinaceae Family and contains biologically active substances. The aim of the current research was to determine best methods for extraction in different solvents and evaluation of different extraction methods for best of flavonoid compounds. *Clerodendrum infortunatum* Linn leaves was extracted with four different solvents. Extraction of the plant material with various organic solvents in increasing order of polarity with the help of Petroleum ether (60-80°), Chloroform, Acetone and Methanol solvents to the find out the percentage (%) yield of all the extracts. Thin layer chromatography study of each extract to know the number of components present in them. Extraction of flavonoid rich fraction with the help of (80%) ethanol (N.R Fransworth) by using different extraction methods then comparative study of percentage yield of total flavonoids like Maceration, Hot Continuous Percolation (Soxhlet Extraction), Microwave assisted Extraction and Ultrasonic Extraction (Extraction using Ultrasonic waves) and collect the fraction using column chromatography and Identification of isolated compound with the help of UV spectroscopy either Quercetin, Rutin or any other suitable flavonoid marker.

Keywords: Flavonoids • *Clerodendrum infortunatum* Linn leaves phytochemical screening • Different extraction methods • Different solvents TLC Rf value • Column chromatography • UV • Fraction

Introduction

Several species of leaves of *Clerodendrum infortunatum* Linn have been traditionally used over the centuries and their antioxidant and hepatoprotective potential has already been demonstrated. Various parts of the plant are used by the tribes in colic, scorpion stings, snakebites, tumors and some skin diseases. The leaves are slightly bitter but heal inflammation, skin diseases and are good against smallpox, treatment of bronchitis, asthma, febrile diseases of inflammation of the blood, burning sensation and epilepsy. The plant contains triterpenes, steroids and mainly it contains flavonoids. Antioxidant, antimalarial, antimicrobial, deworming and analgesic activity *Clerodendrum infortunatum* Linn well known for its traditional uses in various parts of the world, is commonly known as saraswaty leaf and other names are Bhant in Hindi, Bhagri in Sanskrit, Khanduchakka in Marathi and Bhatghetu in Bangali (Figure 1).

Plant profile

Figure 1. Leaves of *Clerodendrum infortunatum Linn*

Scientific classification

Kingdom: Plantae

Order: Lamiales

Family: Lamiaceae/Verbinaceae

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Received: 15-February-2022 Manuscript No. JPNP-22-51932; **Editor assigned:** 17-February-2022, Pre QC No. JPNP-22-51932 (PQ); **Reviewed:** 03-March-2022, QC No. JPNP-22-51932; **Revised:** 18-April-2022, Manuscript No. JPNP-22-51932 (R); **Published:** 27-April-2022, DOI: 10.37421/2472-0992.2022.8.192.

species: *C. infortunatum*

Botanical name: *Clerodendrum infortunatum*

Synonyms: *Clerodendrum infortunatum*, *Clerodendrumviscosum, Clerodendrumcalycinum*

Vernacular name: Khanduchakka, Bharangi, Bhagri

Materials and Methods

Extraction methods

Microwave-Assisted Extraction (MAE): MAE may be a technique that mixes microwave and ancient solvent extraction. In MAE, the extraction happens as a result of changes within the cell structure caused by magnetic waves. It is been projected that the extraction acceleration determined in MAE could also be because of the warmth and mass transfer gradients operating in the same direction. Exploitation microwaves for heating the solvents and plant tissues will increase the mechanics of extraction and various blessings are so obtained over traditional solvent extraction, together with shorter in the extraction of assorted compounds from natural sources.

Soxhlet extraction: Completely different elements those are utilized in soxhlet extraction are thimble, water cooling system, and reservoir, bypass tube, siphon tube and condenser is seen in fig. we'll take needed quantity of solid material of leaves confine thimble that is loaded into soxhlet vessel having flask containing extractor solvent. Solvent vapor moves up to the column and floods into the chamber housing the thimble of solid. Some part of non-volatile compounds dissolves in solvent. Method repeats repeatedly till we tend to get desired focused compounds in flask. Method has been done at boiling temperature of solvent and extraction has been drained 100 ml plant product for 3.5 hours.

Ultrasonic extraction method: Sonication is that the act of applying sound energy to agitate particles during a sample, for varied functions comparable to the extraction of multiple compounds from plants. In audible frequencies is (>20 kHz) are typically used, resulting in the method additionally being called ultrasonication or vibration extraction method.

Maceration: During this process solid ingredients are placed in closed instrumentation with full of the solvent and allowed to face for minimum of 3 days (3-7) days with frequent agitation, till soluble matter is dissolved. The mixture is then strained through sieves/nets the also the combined liquids clarified.

Methods for isolation of chemical constituents

Preparative Thin Layer Chromatography (TLC)

Preparative TLC has been established to accelerate the separation of complex mixtures. In analytical TLC, microgram amounts of mixtures are separated. In this method, a thicker layer of absorbent is used for larger amounts. The separate bands of compounds are scraped off the plate and subjected to solvent extraction followed by the use of modern spectroscopic methods for structure-determination.

Column chromatography

This method is used to purify or isolate plant constituents from extracts of compounds partially purified using a column. This is the most preferred method for quantitative/preparative separation. Components measuring from a few micrograms to a few kilograms can be separated. In this method, the stationary phase, a solid adsorbent, is placed in a vertical glass column and a mobile phase, a liquid, is added at the top and flows through the column by gravity or external pressure.

Methods for characterization of chemical constituents

Ultraviolet Visible Spectroscopy (VS)

This is concerned with the study of absorption of UV and visible radiation. Radiation wavelength ranges from 200 nm-400 nm for UV light and 400 nm-800 nm for visible light. Thus, for colorless compounds, measurements are made in the range 200 nm-400 nm and for colored compounds; the range is 200-800 nm. In UV as well as visible spectroscopy, only valence electrons absorb the energy which causes the promotion of electrons of the sample molecule from ground state to higher energy states. This is called as transition due to which characteristic spectrum is obtained according to the structure of the compound.

Materials

The plant specimen was dried, identified and authenticated at Department of Botany, RashtrasantTukadojiMaharaj Nagpur University (RTMNU), and Nagpur with a specimen number 10156. The leaves of *Clerodendrum infortunatum* Linn were collected in the month of august from Department of Botany,RTMNU Nagpur.

Drying and size reduction

The leaves of Clerodendrucm *infortunum* Linn were shade dried under normal environmental conditions and then subjected for size reduction to coarse powder in a mechanical grinder.

Defatting of plant material

The plant material was shade dried for 10 days and powdered. The powdered plant material (250 g) was transferred into soxhlet extractor for extraction and defatted with the petroleum ether (60-80°C) (700 ml) at temperature of 60°C on heating mantle. The extraction was continued until the solvent in the thimble becomes clear indicating completion of extraction. The defatting was completed after 25-30 cycles. The defatted material was allowed for extraction.

Extraction from the defatted material

The material was extracted with reflux condenser by 80% ethanol. It was carried out for about 12 hours for complete extraction at 90°C

on a heating mantle. The solvent was distilled off and the extract was poured in petriplates and concentrated at room temperature. The extract was obtained as a greenish black gummy residue and was stored for further processing.

Phytochemical and Preliminary screening

Morphological characterization of extract: The extract was studied for morphological and physical characteristics. The morphological characteristic such as odor, color and taste of the leaves extract were examined.

Phytochemical screening

Solubility: Solubility of the ethanolic extract was determined by dissolving about 5-10 mg of extract in 5 ml of various solvents like chloroform, water, pet ether, methanol, ethanol, acetone

Preliminary test of hydroalcoholic extract: In these the various preliminary test were done to detect the presence of chemical constituent like alkaloids, flavonoids, carbohydrates, tannins, glycosides etc.

Tests for flavonoids

- **Shinoda test:** 1 g of the powder was extracted with 10 ml of ethanol (95%) for 15 min. on a boiling water bath and filtered. To the filtrate a small piece of magnesium ribbon and 3 drops of hydrochloric acid was added, formation of red colour indicated the presence of flavonoids.
- **Lead acetate test:** The test solution was mixed with lead acetate solution and observed for the formation of yellow precipitate that indicated the presence of flavonoids.
- **Alkali test:** Test solution was treated with increasing amounts of NaOH; and observed for the formation of yellow coloration which decolorizes after addition of acid that indicated the presence of flavonoids.

Test for carbohydrates

Molisch's test: Molisch's reagent (1 ml) was mixed with 2 ml of test solution, and then 1 ml of concentrated sulphuric acid was added. It was observed for the red to violet ring at the junction of the two liquids that indicated the presence of carbohydrates.

Test for glycosides

A small amount of extract was taken in 1 ml water. Then few drops of aqueous sodium hydroxide were added. Yellow precipitate was considered as an indication for the presence of glycosides in a boiling water bath. Brick red precipitate was considered as an indication for the presence of glycosides.

Test for proteins

Million's test: 3 ml of extract was mixed with 5 ml Million's reagent. White ppt. warm ppt turns brick red or the precipitate dissolves giving red colored solution.

Test for tannins

Potassium dichromate test: 5 ml of the extract was placed in a test tube and then 1 ml of 10% potassium dichromate solution was added. Red precipitate was considered as an indication for the presence of tannins and phenolic compounds.

Test for steroids

Salkowski reaction: 2 ml of the extract was placed in a test tube and then 2 ml of chloroform and 2 ml of concentrated sulphuric acid were added to it and shaken well. Chloroform layer appearing red and acid layer showing greenish yellow fluorescence was considered as an indication for the presence of steroids.

Test for saponins

Foam test: 1 ml of the extract was placed in a graduated cylinder and was diluted to 20 ml with distilled water and shaken gently for 15 min, persistent foam was considered as an indication for the presence of saponin glycosides.

Thin Layer Chromatography Profile

Sample preparation for TLC

Few ml of extract was dissolved in 10 ml of methanol, and sonicated as required for spotting in TLC.

Preparation of plate

The absorbent used for TLC was silica GF254 (Merck Chemical, Mumbai). About 25 g of silica gel GF254 was taken in glass mortar and about 35 ml of distilled water was added to it. The mixture was stirred using glass pestle until it became homogenous. Then an additional 15 ml of distilled water was added to it with stirring. This suspension was transferred to 150 ml glass flask fitted with plastic stopper and was shaken vigorously for about 2 minutes. The suspension was then spread immediately on TLC plate by pouring technique.

Drying and storage of plates

The freshly coated plates were air dried until the transparency of the layer was disappeared. The plates were then dried in hot air oven and were activated at 110-120°C for 30 minutes prior to sample application. This removed the water and frees active sites of absorbents which should react with the sample to be separated. Thus, better resolution and separation can be achieved.

Application of sample

Capillaries were used to apply the samples to the TLC plates. Samples were applied in the form of spots.

Chromatographic chamber, condition of saturation and development of TLC

Chromatographic rectangular glass chamber (16.5 cm × 29.5 cm) was used in the experiment. To avoid insufficient chamber saturation and the undesirable edge effect, smooth sheet of filter paper

approximately of 15 × 40 cm size was placed in the developing chamber in U shaped and allowed to be soaked in the developing solvent. After being thus moistened, paper was then pressed against the wall of chamber so that adhered to the walls. The experiment was carried out at room temperature in diffused daylight.

Development of solvent system

The extract was tried with several developing solvent systems 47, but satisfactory resolution was obtained in a mixture of Toluene: Methanol for alkaloids and Ethyl acetate: Glacial acetic acid: Formic acid: Water (100:11:11:26) for flavonoids. Various solvent systems which were tried for the separation are enumerated in (Table 1).

Various solvent system trial for flavonoids

Solvent system	Ratio	Observation
Ethyl acetate: benzene: methanol: water	75:5:10:10	Tailing effects
Ethyl acetate: Methanol: Distilled water: Formic acid	50:10:10:10	Spots were separated
Toluene: Ethyl acetate: Formic acid	2.111227	Spots were separated
Toluene: Chloroform: Acetone	1.684433	Partial separation
N-Butanol: Acetic acid:water	0.334838	Partial separation
Ethyl acetate: Acetone: Formic acid Water	50:30:10:10	Tailing effects
Chloroform: Toulene: Ether: Acetic acid	60:60:15:5	Spots were separated
Toluene: Ethyl acetate: Diethylamine	7.5:1.5:1	No separation

Table 1. Solvent developed for flavonoids

Determination of Rf alue

The Rf value of the isolated spots were determined by using the following formula.

Rf value=Distance travelled by band/Distance travelled by solvent

Identification of spots

The spots were examined under UV light (254 nm) and in the iodine chamber for the identification of the separated spots.

Extraction of the plant material with various organic solvents

Extraction of the plant material with various organic solvents in increasing order of polarity with the help of following solvents to find out the percentage (%) yield of all the extracts.

- Petroleum ether (60^0-80^0)
- Chloroform
- Acetone
- Methanol
- The TLC study of each extract to know the number of components present in them.

Chemical evaluation of plant material

Preliminary phytochemical screening of each extracts to know the various classes of chemical constituents present in them.

Different Extraction Methods

Maceration

- In this process, the whole or coarsely powdered crude drug was placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 13 days with frequent agitation until the soluble matter has dissolved.
- The mixture then was strained, the marc (the damp solid material) was pressed, and the combined liquids clarified by filtration or decantation after standing.

Hot continuous extraction (Soxhlet)

- In this method, the finely ground crude drug was placed in a porous bag or “thimble” made of strong filter paper, which was placed in chamber E of the Soxhlet apparatus. The extracting solvent in flask Awas heated and its vapors condense in condenser D. The condensed extract drips into the thimble.
- When the level of liquid in chamber E rises to the top of siphon tube C, the liquid contents of chamber E siphon into flask A.
- These processes were continuous and were carried out until a drop of solvent from the siphon tube does not leave residue when evaporated.
- The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent.
- This affects tremendous economy in terms of time, energy and consequently financial inputs.
- At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale.

Ultrasound extraction (Sonication)

- The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation.
- Although the process is useful in some cases, like extraction of *rauwolfia* root, its large-scale application is limited due to the higher costs.
- One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules

Microwave-Assisted Extraction (MAE)

- Microwave-Assisted Extraction (MAE) is a relatively new technique that combines microwave and traditional solvent extraction.
- In MAE, the extraction occurs as the result of changes in the cell structure caused by electromagnetic waves. It has been proposed that the extraction acceleration observed in MAE may be due to the heat and mass transfer gradient working in the same direction.
- Soxhlet extraction, Maceration, Microwave assisted synthesis and Sonication are the basic extraction methods.

Column Chromatography

Sample preparation for column

30 g of hydroalcoholic extract were weighed and taken for column chromatography. The extract was mixed with petroleum ether to make the slurry which was poured into column. Preparation of Column chromatography

A small precleaned and dried glass with asbestos column (about 60 × 3.5 cm) was used for the column chromatography. Silica gel 60-120 mesh size (Merck Chemicals, Mumbai) which was preactivated at 110-120°C was placed at the bottom and then silica gel was taken and mixed with extract and poured. This was allowed to settle down in column.

Sample application

For sample application, cotton plug were placed over the layer of packed stationary phase to avoid disturbances of column.

Gravity elution of mobile phase

Gravity elution is a simple method where mobile phase is allowed to run under gravity. In order to fractionate the compounds, the mobile phases with non-polar to polar solvents in different proportion were used for the column chromatography. The flow rate of the mobile phase through the column was adjusted at 25 ml/15 min by the knob. Each 25 ml eluted solution was collected in conical flask and distilled for getting the fraction. Each fraction were collected in test tubes and stored for further processes.

The different organic solvents i.e. non-polar to polar in variable mixtures of solvents were eluted from column. The various solvents like petroleum ether-benzene, Benzene-chloroform, Chloroform-ethyl acetate, Ethyl acetate-acetone, Acetone-methanol and Methanol-glacial acetic acid in variable proportions as 100%, 9:1, 8:2, 7:3, and 1:1 is used.

Thin layer chromatography of each fraction was carried out to find out the homogeneity of each fraction by using number of developing solvent system such as benzene, benzene :ethyl acetate(8:2), ethyl acetate: acetone (8:2), acetone: methanol (9:1). The detecting reagent used was iodine vapors, 50% sulphuric acid and ferric chloride solution (5% w/v in 90% alcohol). The chromatographic pattern of each fraction was studied carefully and the fraction (belonging to the same eluting solvent) which gave identical pattern in respect of color in RF values were mixed.

The fraction 31-60 showed the orange-brown sticky compound, having the same Rf value in the TLC. The remaining fractions were concentrated to a small volume and attempt was made to get crystalline compound by keeping them undisturbed in freeze or at room temperature for some time. By doing so, the fraction no.95-101, 102-107, 108-115, 116-130, 131-142, 143-150, 151-159, 160-165, and 166-170 gave dark brown crystalline compounds. The fraction having crystal in the solution was taken and then the solution was decanted. The crystals were then scraped out separately, and wash quickly with warm petroleum ether (60-80°), benzene, and n-hexane. The crystal obtained after washing was shining brown. This compound was designated as the compounds 1) 95-101, 2) 116-130, 3) 143-150, 4) 171-180 it was kept aside for characterization and identification (Table 2).

The different fraction showed the presence of crystals as follows:

Column solvent system	Fractions collected No.	TLC mobile phase			Spots
Petroleum ether to Benzene	46023	Benzene: (40:30:30)	Chloroform:	Methanol	No spot
Benzene to Chloroform	26-30,31-36,37-42,43-48,	Benzene: (40:30:30)	Chloroform:	Methanol	Show spots
Chloroform to Ethyl acetate	49-55,56-62,63-67,68-71,72-78	Benzene: (16:9:5)	Chloroform:	Methanol	Show spots
Ethyl acetate to acetone	79-81,82-86,87-91,92-95,96-100,101-1 03	Benzene: (16:9:5)	Chloroform:	Methanol	Show spots

Acetone to Methanol	104-107,108-115,116-122,123-126,127-133,134-138,139-143,144-146	Benzene: (14:7:8)	Chloroform:	Methanol	Show spots
Methanol to glacial acetic acid	147-148,149-152,153-157,158-160,161-164,165-171,172-180	Benzene: (16:9:5)	Chloroform:	Methanol	Show spots

Table 2. Fractions collected from Clerodendrum leaves extract and its TLC mobile phase.

Characterization and Identification of the fractions

About 180 fractions were collected from *Clerodendrum infortunatum* extract and solvent [31,32] fractions were characterized by the chromatographic patterns. The *clerodendrum* extract is a complex mixture of various phytochemical and this mixture is difficult to separate completely using column chromatography due to its sticky nature. Therefore, plant extract was fractionating by using different organic solvents. The chromatographic patterns of the fractions were carefully studied and the fractions which showed similar TLC pattern were collected together. The isolated fractions were studied for their chemical structure by using chemical test, UV spectroscopy.

Chemical Test of Isolated Fraction

Test for Flavonoid

Shinoda test: 1 g of the fraction was extracted with 10 ml of ethanol (95%) for 15 minutes on a boiling water bath and filtered. To the filtrate a small piece of magnesium ribbon and 3 drops of hydrochloric acid was added, formation of red color indicated the presence of flavonoids.

Lead acetate test: The fractioned compound was mixed with lead acetate solution and observed for the formation of yellow precipitate that indicated the presence of flavonoids.

Test for Steroids

Salkowski reaction: 2 ml of the fractioned compound was placed in a test tube and then 2 ml of chloroform and 2 ml of concentrated sulphuric acid were added to it and shaken well. Chloroform layer appearing red and acid layer showing greenish yellow fluorescence was considered as an indication for the presence of steroids.

Thin Layer Chromatography (TLC)

Spots of isolated fractions and standards were applied on the TLC plate and mixture of were used as mobile phase Rf value of the isolated fraction were noted.

UV-Visible Spectroscopy

About 10 mg of the isolated fractions from column chromatography was dissolved in 100 ml of methanol, 1 ml from the resulting solution was pipetted out and diluted up to 10 ml with methanol and analyzed using Ultraviolet and Visible Spectroscopy (UV-1800 UV Spectrometer, Shimadzu) in the range of 200-400 nm. Methanol was used as a blank. Similarly, dilution were made for all the isolated fractions and analyzed.

Results and Discussion

Morphological characteristics of hydroalcoholic extract

Color, odor and taste were shown in Table 3 and Hydroacholic extract shown in Figure 2.

Sr. No.	Test	Characteristics of Hydro Alcoholic Extract
1	Color	Greenish black
2	Odour	Characteristics
3	Taste	Sweet

Table 3. Morphological characteristics of hydroalcoholic extract of leaves.

Figure 2. Hydroacholic Extract.

A Table 3 show the color of hydroalcoholic extract was greenish-black, odour was characteristics and sweet taste.

Phytochemical and Preliminary Screening of the Extract

Solubility

Solubility of the extract in various solvents is shown in Table 4.

Sr. No.	Solvent	Solubility
1	Chloroform	Soluble
2	Water	Soluble
3	Petroleum ether	Sparingly soluble
4	Methanol	Soluble
5	Ethanol	Soluble
6	Acetone	Soluble

Table 4. Solubility of the Hydro alcoholic Extract.

The hydroalcholic extract of *Clerodendrum infortunatum Linn* was soluble in chloroform, water, methanol, ethanol, acetoneand sparingly soluble in petroleum ether.

Chemical test for hydroalcholic extract

Chemical test for hydroalcholic extract shown in Table 5, (Figures 3 and 4).

Test	Observation	Inference
Shinoda test	Magenta red color is observed	Flavonoids are present
Lead acetate test	Yellow precipitate are formed	Flavonoids are present
With sodium hydroxide	Yellow coloration (Thus decoloration after addition of HCL) (acid)	Indicating presence of flavonoids

Table 5. Chemical test for hydroalcholic extract.

TLC (Rf value) of hydro alcoholic extraction

For Value of the hydroalcolic extract shown in Table 6.

SR. NO.	Solvent System	No. of Spot	RF Value
1	Ethyl acetate : Benzene : Methanol : Water (75:5:10:10)	Two	0.96 and 0.49
2	Ethyl acetate : Formic acid : Methanol : Water (50:5:2:4)	Two	0.91 and 0.60
3	N-butanol : Acetic acid : Water (8:2:10)	Two	0.96 and 0.37
4	Ethyl acetate : Acetone : Formic acid : Water (50:30:10:10)	Two	0.86 and 0.36
5	Chloroform : Toluene : Ether : Acetic acid (60:60:15:5)	Two	0.42 and 0.45
6	Toluene : ethyl acetate : formic acid (50:40:10)	Two	0.32,0.75

Table 6. For Value of the hydroalcolic extract.

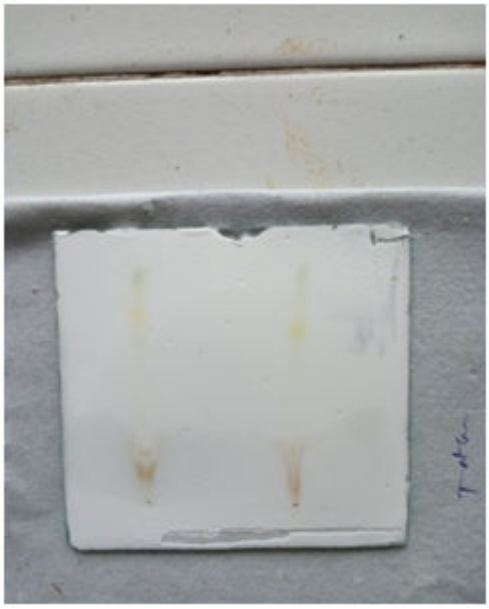


Figure 3. Hydroalcoholic extracts.

Toluene:ethylacetate:formicacid (50:40:10) Chloroform:Toluene:Ether:Aceticacid (60:60:15:5).

Extraction and Chemical Evaluation of Plant Material

Extraction of the plant material with various organic solvents in increasing order of polarity with the help of following solvents (Table 7 and Figure 5).

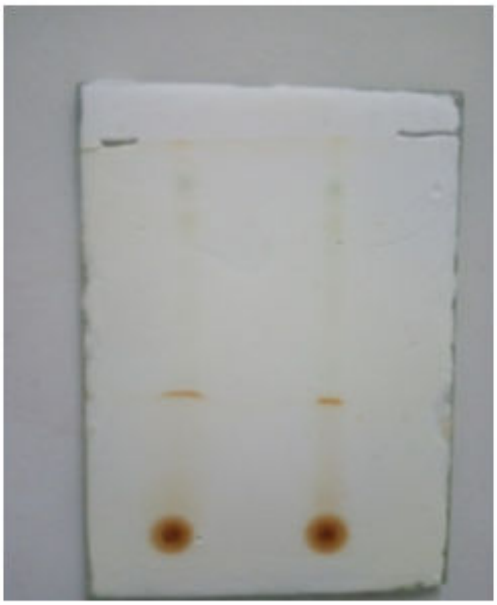


Figure 4. 2-Hydroalcoholic Extracts.

- Petroleum ether (60-80)
- Chloroform
- Acetone
- Methanol

Solvents	% yield
Petroleum ether (60-80)	0.0243
Chloroform	0.0222
Acetone	0.023
Methanol	0.0237

Table 7. Percentage yield of various organic solvents.

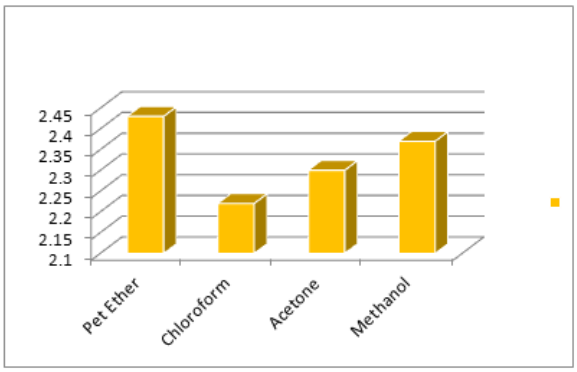


Figure 5. Percentage yield of different types of solvent.

Different solvent systems were tried for the complete separation of the phytochemical present in the hydroalcoholic extract. The Extraction of the plant material with various organic solvents in increasing order of polarity was identify the more percentage yield was found to be petroleum ether as compare to chloroform, acetone, methanol.

Chemical Test of different extraction

Chemical Test of different extraction shown in Table 8.

Test	Observation	Inferences
1)Petroleum ether (60-80)		
a)Salkowaski test		
Test sample (1-2 mg)was dissolved in 1 ml ofchloroform ($CHCl_3$) and 1 ml of concentrated H_2SO_4 was added	Green color appeared in chloroform layer	Sterol present
2) Chloroform	Green color appeared in chloroform layer	Sterol present
a)Salkowaski test Test sample (1-2 mg) was dissolved in 1 ml of chloroform ($CHCl_3$ and 1 ml of concentrated H_2SO_4 was added		
b) Shinoda test: Small quantity of test residue was dissolved 5 ml of ethanol (95% v/v) and treated with few drops of conc. HCl and 0.5 g o g magnesium metal.	Brownish black color are form	Flavonoids are absent
c) Molisch test : Sugar test molisch reagent (1 ml)was mixed with 2 ml of test solution and then 1 ml of conc. H_2SO_4 was added.	Green ring at the junction of two liquid	Carbohydrate are absent
3)Acetone	Blue-black color are observed	Tannins are present
a) Test for Tannins		
1) Ferric chloride :- Test sample (2 ml) was mixed with $FeCl_3$ solution (5%)		
2)Lead acetate: Test solution was mixed with lead acetate solution and observed for the formation of white ppt	White ppt are form	Tannins are present
b) Shinoda test: Test for flavonoids small quantity of test residue was dissolved 5 ml of ethanol (95% v/v) and treated with few drops of conc. HCl and 0.5 g o g magnesium metal	Brown color are observed	Flavonoids are absent
4) METHANOL	Blue-black color obsered	Tannins are present
a) Test for Tannins		
1) Ferric chloride: Test sample (2 ml) was mixed with $FeCl_3$ solution (5%)		
2)Lead acetate:- Test solution was mixed with lead acetate solution and observed for the formation of white ppt	White ppt appeared	Tannins are present
b) Test for flavonoids	Magenta-red color are observed	Flavonoids are present
(Shinoda test) Small quantity of test residue was dissolved 5 ml of ethanol (95% v/v) and treated with few drops of conc. HCl and 0.5 g magnesium metal		

Table 8. Chemical Test of different extraction.

The different solvent extracts of *Clerodendrum infortunatum* Linn in pertoleum ether and Chloroform shown the presence of steroids whereas extracts in acetone and methanol shown the presence of flavonoids and tannins (Table 9 and Figure 6).

SR. NO.	Organic Solvent	Solvent System	No. of Spot	RF Value
1	Petroleum Ether	Benzene : Ethyl Acetate (8:2)	Two	0.84, 0.95
2	Chloroform	Ethyl Acetate : MeHO (7:3)	Two	0.18, 0.84
3	Acetone	Ethyl Acetate : MeHO (7:3)	Two	0.80,0.78
4	Methanol	Ethyl acetate : formic acid : glacial acetic acid : water (100:11:11:26)	Two	0.90,0.86

Table 9. TLC characterization of different extract.

Figure 6. TLC of different extract.

The characterization of different extraction and showing Rf value near to each other of TLC and showing two spots.

Different extraction methods

Extraction of plant material with the help of following extraction methods to determine the percentage yield of total flavonoids.

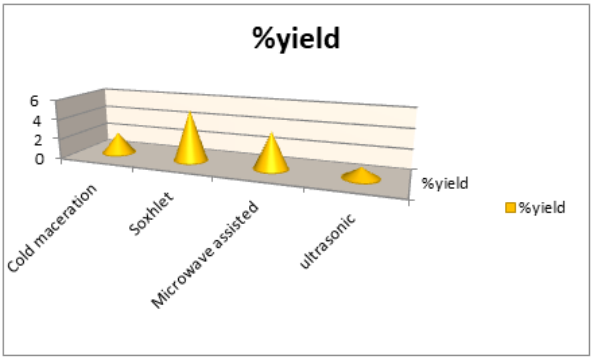
- Cold maceration
- Hot continuous percolation (Soxhlet extraction)
- Microwave assisted extraction
- Ultrasonic extraction

Percentage yield of each extraction methods

Percentage yield of each extraction methods shown in Table 10 and Figure 7.

Methods of extraction	Weight of plant material (g)	(%) yield
Cold maceration	25 g	0.0216
Soxhlet extraction	25 g	0.0516
Microwave assisted extraction	25 g	0.0364
Ultrasonic extraction	25 g	0.0104

Table 10. Percentage yield of each extraction methods.



The soxhlet extraction was more percentage yield of each compare to the other extraction methods.

Figure 7. Percentage yield of each extraction methods.

Column chromatography

Figure 8. Column Chromatography.

The different fractions was collected in the column chromatography (Figure 8).

Fraction Analysis

Preliminary test for fractions

Preliminary test of fractioned compounds was shown in Table 11.

Solvent system (Fraction No.)	Test for Flavonoid	Test for Steroids	Test for Carbohydrate
Ethyl acetate : acetone (1:1) (79-103)	+	-	+
Acetone : Methanol (1:1) (104-146)	+	-	-
Acetone : Methanol (1:1) (147-158)	+	+	+
Methanol -1 (158-171)	+	-	-
Methanol : Glacial Acetic Acid (9:2)	+	-	+
171-180			

Table 11. Preliminary test for isolated fractions.

The ethyl acetate: acetone showed the presence of Flavonoids, Carbohydrate. The acetone: methanol shown the presence of flavonoids. The acetone: methanol fraction shown presence of flavonoids, steroids, fatty acid, carbohydrates.

TLC of isolated fractions

The Rf value of the isolated fractions with standard is mentioned the spots was detected by using iodine chamber illustrated (Figure 9 and Table 12).

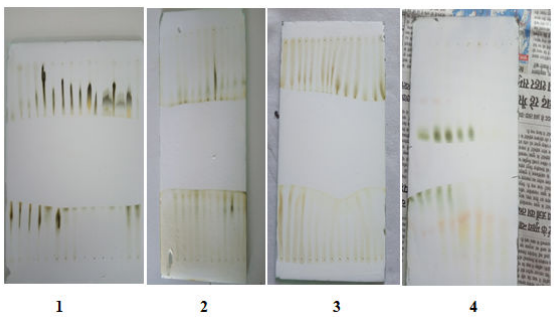


Figure 9. TLC of isolated compounds.

TLC of Isolated Fractions with Standards

Sr. no.	Samples	Number of spots	Rf value
1	Ethanol extract	6	0.75, 0.32,0.42,0.45
2	Rutin	1	0.32
3	Quercitin	1	0.75
4	Isolated fraction 1	1	0.74,0.72
5	Isolated fraction 2	1	0.33
6	Isolated fraction 3	1	0.75

Table 12. Rf values of ethanol extract, standards, isolated compounds.

The isolated fraction 1 and 3 showed the same Rf value as that of quercitin. The isolated fraction 2 shown same Rf value as that of rutin.

TLC of isolated fractions with standards

Comparative TLC of isolated compound and standard quercetin (Figure 10 and Table 13).

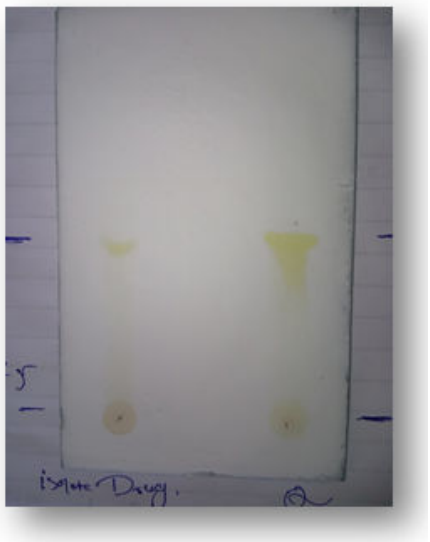


Figure 10. Comparative TLC.

A: Isolated compound

B: Quercetinstd

Sr. No.	Sample	Mobile phase	No. of spots	Rf value
1	Isolated compound	Toulene: Ethyl acetate; formic acid	2	0.84, 0.64
2	Standard quercetin	Toulene: Ethyl acetate; formic acid	1	0.84

Table 13. Rf value of isolated compound and standards.

The solvent system used for isolated compound was toulene, ethyl acetate, formic acid. The isolated compound shown the same Rf value as that of standard quercetin.

UV visible spectroscopy of isolated fractions

UV spectrum of all isolated fractions and standards are illuminated in figures. The major peaks of isolated compounds from column are in the UV range and mentioned (Figure 11 and Table 14).

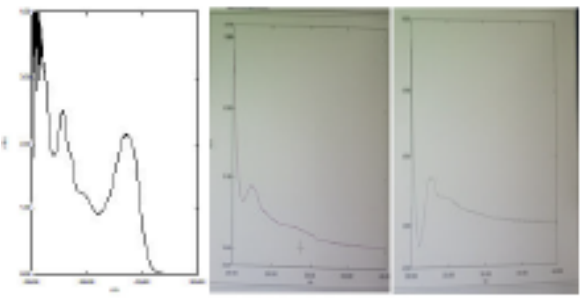


Figure 11. UV Spectra of QuercetinStd/UV spectra of 104-146 fraction/147-158.

Sr. No.	Sample	Mobile phase	No. of spots	Rf value
1	Isolated compound	Toulene: Ethyl acetate; formic acid	2	0.84, 0.64
2	Standard quercetin	Toulene: Ethyl acetate; formic acid	1	0.84

Table 14. λmax of the isolated fraction using UV visible spectroscopy.

On the basis of λmax of standard compared to λmax of isolated fractions, fraction 104-146 was found to be quite comparable with that of quercetin.

Conclusion

The leaves of *Clerodendrum infortunatum* Linn were collected, dried and authenticated. The dried parts were pulverized to make coarse materials which were used for experimental work. Total one kg of plant material was accurately weighed and according to the plan of work the leaves of the *Clerodendrum infortunatum* Linn defatted with petroleum ether. The defatted marc was then refluxed with 80% ethanol for 12 hours. The ethanolic extract was then subjected for evaluation. The prelimnary phytochemical screening showed that the leave extract showed the positive test in Molisch test, Shinoda test, Alkali test, Lead acetate test and salkowski test. Rf value of leave extract was calculated using various mobile phase like Ethyl acetate : formic acid : glacial acetic acid : water (100:11:11:26), Toluene : ethyl acetate : formic acid (50:40:10), Chloroform : Toluene : Ether : Acetic acid (60:60:15:5) Extraction of the plant with various organic solvents in increasing order of polarity with the help of various solvents were performed and the percentage yield was calculated. The prelimnary phytochemical study of the extract of different solvent was done and also the Rf value was calculated by using thin layer chromatography. The comparative study of the leave extract with the standard of Quercetin was performed and the Rf value was calculated.

The column chromatography of the 80% ethanolic extract over silica gel afforded various compounds (fractions) which was identified as flavonoids, long chain of fatty acids, sterols, etc. The TLC studies, UV spectroscopic study were also carried out on fractions and both these studies confirmed that the flavonoids, steroids and fatty acids.

Extraction of plant material with different extraction methods to determine the percentage yield of total flavonoids.

- Cold maceration
- Hot continuous percolation (Soxhlet extraction) more percentage yield
- Microwave assisted extraction
- Ultrasonic extraction

In the present study it was concluded that-

- presence of flavonoids in 80% hydroalcoholic extract.
- The column chromatography of 80% hydroalcoholic extract yielded compounds like flavonoids, steroids etc.
- The flavonoids rich fraction (compound) was compared with a standard flavonoids i.e. quercetin, which showed a comparable results.
- The extraction of plant material with various organic solvents in increasing order of polarity was identifty the more percentage yield was found to be petroleum ether.
- The extraction of plant material with different extraction methods. Thesoxhlet extraction were more percentage yield was found by compare to the other extraction methods.

Acknowledgement

I Acknowledged to Dr. AbhayIttadwar (Principal) and My Guide Dr. ShekharWaikar of Gurunanak College of Pharmacy, Nagpur for allowing me to research work in the institute setup.

I dedicate thise research work publication under the guidance of Dr. Mudhada sir (Principal) Agnihotri College of Pharmacy, Wardha and my Colleagues for their valuable time and guidance.

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How to cite this article: Daf Abhijit N, Waikar Shekhar B, Madavi Akshay N, and Kapse Akash S, et al. "Comparison of Different Solvent and Extraction Methods for Isolation of Flavonoids Compound from Leaves of *Clerodendrum Infortunatum* Linn." *J Pharmacogn Nat Prod* 8 (2022): 192.