Organic and Medicinal Chemistry
International Journal
ISSN 2474-7610

Research Article
Volume 11 Issue 4 - March 2022
DOI: 10.19080/OMCIJ.2022.11.555817

Organic & Medicinal Chem IJ

Preliminary Phytochemistry and High-Performance Thin-Layer Chromatography Fingerprint Profiling of the Seed Extracts of *Caesalpinia Bonduc*: A Pharmaceutically Significant Medicinal Plant

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Submission: February 28, 2022; **Published:** March 14, 2022

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Abstract

Caesalpinia bonduc (L.) Roxb. has been used in India and other tropical countries for several years to treat a variety of diseases and disorders. The present study was intended to investigate the preliminary phytochemicals and the High-Performance Thin Layer Chromatography (HPTLC) fingerprinting profile of C. *bonduc* seeds collected from the Ernakulam region, India. The various extracts (hexane, toluene, petroleum ether, ethyl acetate, chloroform, acetone, ethanol, methanol and water) obtained from the seeds were examined for the presence of different phytochemicals using standard methods. The HPTLC fingerprinting was carried out using a CAMAG HPTLC system equipped with a Linomat 5 applicator, TLC scanner 3, Reprostar 3 and WIN CATS-1.4.3 software. The phytochemical analysis study showed that ethanol, methanol, acetone and water extracts were rich in phenolic and glycoside compounds. At the same time, hexane extract recorded the presence of alkaloids, flavonoids and saponins. Tannins, steroids and terpenoids were absent in all the extracts. HPTLC fingerprinting of seed extracts revealed several peaks with Rf values in the range of 0.12 to 1.13. From the results, it was noted that the seed extracts of C. *bonduc* were a rich source of a variety of active secondary metabolites. This preliminary report may help other researchers to isolate and characterize the active secondary metabolites for bio-efficacy and bioactivity. Further, it can be concluded that HPTLC fingerprint analysis of seed extract of C. *bonduc* can be used as a powerful diagnostic tool for the correct identification of the plant and is helpful as a phytochemical marker analyzer.

Keywords: *Caesalpinia bonduc*, HPTLC, Phytochemistry, Seed, Secondary metabolites

Introduction

The importance of medicinal plants in drug development is known to humans for several centuries and we have used them for managing different diseases since the beginning of human history [1]. Traditional folk treatment based on wild plants has always guided researchers to search for novel medications to develop healthy life for humans and animals [2]. In addition, some medicinal plants still have masked parts within them that need to be scientifically evaluated for the development of novel medication [3]. Plant secondary metabolites have been extensively used as taxonomic characters for comparisons at all hierarchical levels, and certain classes of secondary metabolites, such as benzylisoquinoline alkaloids, betalaines, glucosinolates, iridoids, and polyacetylenes, have had a significant influence on the establishment of all recent angiosperm classification systems [4]. As a result, determining plant metabolites is critical. This also helps in the authentication of biological properties that a plant possesses. Several techniques are used to determine the number of phytoconstituents in any crude extract. Phytochemical screening is a method of bioactive compound identification that is unknown in plant extracts through qualitative analysis. Analysis of phytochemical is a preliminary stage in a phytochemical study that aims to provide an overview of the different classes of compounds contained in plants that are being studied. The phytochemical screening method looks at the color testing reaction using a color reagent [5].

Knowledge of the chemical components contained in medicinal plants needs to be studied. This information will be significant for synthesizing complex active ingredients of chemical compounds in medicinal plants. Phytochemical screening in medicinal plants, in addition to being used to identify active compounds that are beneficial to the body's health (positive effects of herbal medicines) can also be used to identify active compounds that cause toxins (negative effects of herbal medicines). This causes the phytochemical screening process of medicinal plants to be important before conducting further analysis [6-8]. The genus of Caesalpinia is widespread, with 500 species that have medicinal benefits based on their pharmacological activity. One of the medicinal plants from this genus is C. *bonduc* Linn. (C. bonducella) an Indian herb belonging to Family Caesalpiniaceae (Figure 1). It is found throughout India and other tropical countries [9]. It is commonly used as an antioxidant, antidiabetic, laxative, immune system modulator and in the treatment of rheumatoid arthritis. Two varieties of C. *bonduc* are found: the white and black varieties [10]. The name of the species, *bonduc* is derived from the Arabic word "*Bonduce*" which means little ball and also indicates the shape of the seed [11-12]. The synonyms of C. *bonduc* are C. bonducella (L.) Fleming and Caesalpinia crista auct. Amer. The main objective of our research work was to analyze the presence or absence of different phytochemicals and HPTLC fingerprinting in different extracts of C. *bonduc* seed.

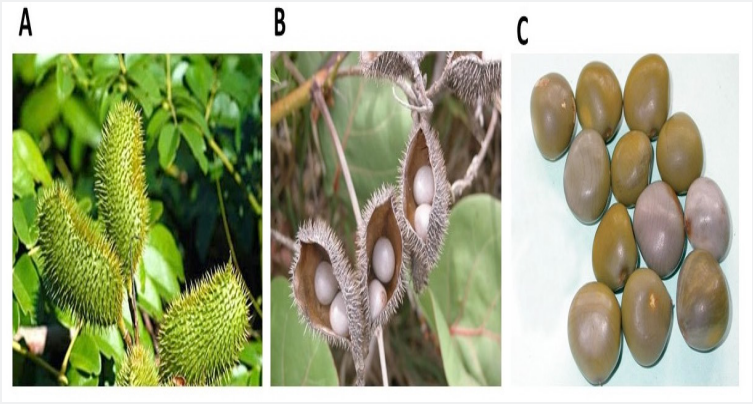

Figure 1: Bomduc (A)Fresh seeds (B) dry seeds contain pod and seed and (C) Seed kernels.

Materials and Methods

Plant materials-C. *bonduc* seed

Fresh seed of C. *bonduc* was collected from different regions of Ernakulam and identified. Voucher specimens were deposited in JNTBGRI, Trivandrum, Kerala, India. Throughout the study, the collection of the seeds was done with extreme care.

Preparation of crude extracts

The seeds were washed with distilled water and dried in the shade. These powdered dried samples were stored at 4°C until further use. The weighed seed powder (250 gm) was transferred to a thimble of Soxhlet apparatus for extraction. Crude extracts were made using the different solvents (750 ml) in the order of increasing polarity, such as hexane, toluene, petroleum ether, ethyl acetate, chloroform, acetone, ethanol, methanol and water respectively. To make the crude extract, the extracts were filtered through a fine muslin cloth and the clear filtrate was evaporated to dryness.

Preliminary phytochemical analysis

Detection of alkaloids

a) Hager's test: Two drops of Hager's reagent were added to one ml of seed extract. The formation of a yellow-colored precipitate indicates positive test alkaloids.

b) Dragendroff's test: To one ml of extract, two drops of Dragendroff's reagent was added. The formation of reddish orange precipitate indicates a positive test for alkaloids.

c) Wagner's Test: To one ml of extract, two drops of Wagner's reagent was added. The formation of reddish-brown precipitate indicates the presence of alkaloids.

d) Mayer's Test: To one ml of extract, two drops of Mayer's reagent was added carefully along the sides of the test tube. The appearance of whitish yellow precipitate indicates the presence of alkaloids.

Testing for flavonoids

Shinoda test: 1 mL of absolute ethanol and 3 drops of concentrated hydrochloric acid were carefully added to 0.5 mL of isopropyl alcohol-diluted extract. The presence of aurones and chalcones was indicated by the formation of a red colour. Metallic magnesium was added to cases where no colour change was observed. The presence of flavones and flavanols is indicated by the formation of orange, red, or magenta coloration.

Test for carbohydrates

A few drops of Benedict solution were added to the seed extracts. If it shows the brick red color, it confirms the presence of glucose and few drops of iodine were added in other extracts. The dark blue color confirms the presence of starch.

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Determination of total phenolic content

Total phenolic contents of each extract were determined using a Folin-Ciocalteu colorimetric method [13]. 1 mL properly diluted of each extract solution was mixed with 0.5 mL of Folin-Ciocalteu reagent. The reagent was pre-diluted, 10 times, with distilled water. After standing for 8 min at room temperature, 2 mL of (7.5% w/v) sodium carbonate solution was added. The solutions were mixed and allowed to stand for 30 min at room temperature and the absorbance was read at 760 nm spectrometrically.

Detection of glycosides

Keller killiani test: About 0.5 g of the extract was treated with 2 ml of glacial acetic acid and a drop of 5% (w/v) FeCl3 was added to it. 1 ml of concentrated H2SO4 was added to it. The presence of brown ring at interface indicates the presence of glycosides.

FeCl3 (5%) test for tannins

About 0.5 ml of extracts was stirred with 10 ml distilled water. Production of a blue, blue-black, green or blue-green coloration or precipitate on the addition of 5% of $FeCl_3$ reagent was taken as evidence for the presence of tannins.

Testing for steroids and / or triterpenoids

Salkowski test: 2 mL of chloroform and 1 mL concentrated sulfuric acid were added to 10 drops of the extract dissolved in isopropyl alcohol, slowly until double phase formation. The presence of a dish-brown color in the middle layer was indicative of a steroidal ring.

Detection of saponins

Foam test: 100 mg of extract was diluted with distilled water to 20 ml. The suspension was shaken in a graduated cylinder for 15 min. Formation of 2 cm foam layer indicates the presence of saponins.

Test for proteins

Biuret test: A few mg of residue was taken in water and 1 ml of 1 % solution of sodium hydroxide was added followed by a drop of 1 % solution of copper sulphate. Development of violet–pink colour indicates the presence of proteins.

High performance thin layer chromatography (HPTLC) profile

HPTLC studies were carried out following the method of Harborne [14]and Wagner and Baldt [15]. CAMAG HPTLC system equipped with a Linomat 5 applicator, TLC scanner 3, Reprostar 3 and WIN CATS-1.4.3 software was used for HPTLC fingerprinting.

Sample Preparation

The extracts obtained was redissolved in 1mL of HPLC grade methanol, which was used for sample application on pre-coated silica gel 60F254 aluminum sheets (Merck, Germany).

Developing solvent system

A number of solvent systems were tried, for extracts, but a satisfactory resolution was obtained in the eluent containing Toluene: Chloroform: Methanol (8:3:1).

Sample Application

Application of bands of the different extract was carried out (4 mm in length and 1 µL in concentration to extract) using spray technique. The sample was applied in duplicate on pre-coated silica gel 60F254 Aluminum sheets (5 x 10 cm) with the help of Linomat 5 applicator attached to CAMAG HPTLC system, which was programmed through WIN CATS software.

Development of chromatogram

After applying the sample, the chromatogram was developed in Twin trough glass chamber 10x10 cm saturated with solvent Toluene: Chloroform: Methanol (8:3:1) extract for 15 minutes. The air-dried plates were viewed in ultraviolet light. The chromatograms were scanned by densitometer at 200 nm after spraying with anisaldehyde/sulphuric acid. The Rf values and fingerprint data were recorded by WIN CATS software.

Results

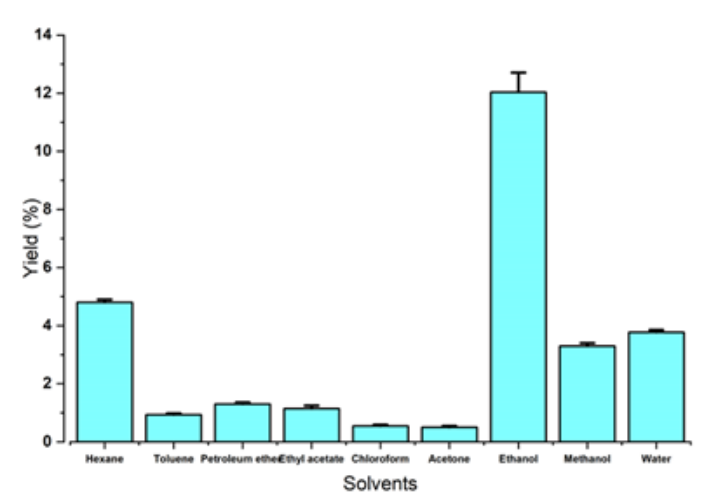

Figure 2: Yield of the extracts obtained from different solvents.

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The yield of the different extracts obtained from the Soxhlet extraction is shown in (Figure 2). From the results, it was clear that ethanol yielded the highest percentage of crude extract, followed by hexane, water and methanol. Acetone and chloroform yielded the lowest percentage of the crude extract. The color, and consistency, of all the extracts were also noted, and the results were summarized in (Table 1).

Table 1: Results of Phytochemical extraction.

SL. No	Solvent	Color of the extract	Consistency of the extract
1	Hexane	Pale yellow	Non-sticky
2	Toluene	Yellow	Non-sticky
3	Petroleum ether	Yellow	Non-sticky
4	Ethyl acetate	Yellow	Sticky
5	Chloroform	Brown	Sticky
6	Acetone	Brown	Sticky
7	Ethanol	Pale reddish brown	Sticky
8	Methanol	Brown	Sticky
9	Water	Dark brown	Non-sticky

Phytochemical study

The various seed extracts obtained after the extraction was subjected to preliminary phytochemical screening and presented in (Table 2). The phytochemical analysis study showed that ethanol, methanol, acetone and water extracts were rich with phenolic and glycosidic compounds. Whereas hexane extract recorded the presence of alkaloids, flavonoids and saponins. Tannins, steroids and terpenoids were absent in all the extract. Thus, the detected constituents in the seed extracts have good therapeutic values.

Table 2: Phytochemicals present in the different extract of C. *bonduc*.

Sl.No	Parameters	Hexane	Toluene	Chloroform	Pet. ether	Ethyl acetate	Acetone	Ethanol	Methanol	Water
1	Alkaloids									
	Dragendroff Test	Absent	Present	Present	Absent	Absent	Absent	Absent	Absent	Absent
	Hagers Test	Absent	Present	Present	Absent	Absent	Present	Absent	Absent	Absent
	Mayers Test	Present	Present	Absent	Absent	Present	Present	Absent	Absent	Absent
	Wagners Test	Present	Present	Absent	Absent	Present	Absent	Absent	Absent	Absent
2	Flavonoids	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
3	Carbohydrates	Absent	Absent	Absent	Absent	Present	Present	Present	Present	Present
4	Phenols	Absent	Present	Absent	Present	Present	Present	Present	Present	Present
5	Glycosides	Absent	Present	Present	Absent	Absent	Present	Present	Present	Present
6	Tannins	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
7	Steroids	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
8	Terpenoids	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
9	Saponins	Present	Present	Present	Present	Absent	Absent	Absent	Absent	Absent
10	proteins	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent

High-performance thin layer chromatography

Preliminary phytochemical analyses cannot predict the maximum number of compounds, as the nature of reagents etc., may influence it. Hence, we conducted an HPTLC analysis of different extracts obtained from the seed to determine the number of compounds. The same colors and Rf values under similar experimental conditions indicated the presence of the same compounds. The solvent system used for this particular study was the same as that used for TLC, which recorded a good separation of constituents in this specific solvent system. The chromatograms of the extracts at UV 254 nm and 366 nm revealed that the majority of the sample constituents were clearly separated without any tailing and diffuseness. The HPTLC chromatogram of extracts recorded at 254 nm, 366 nm was depicted in (Figure 3). The HPTLC fingerprint profiles, Rf values, and area obtained for extracts after scanning at UV 254 nm, 366 nm are given in (Table 3) and the corresponding HPTLC is presented in (Figure 4). In the HPTLC profile, Beta-sitosterol has been used as a standard compound. From the result, it was clear that Hexane, toluene, Ethyl acetate, acetone and methanol extract recorded the presence of Beta-sitosterol.

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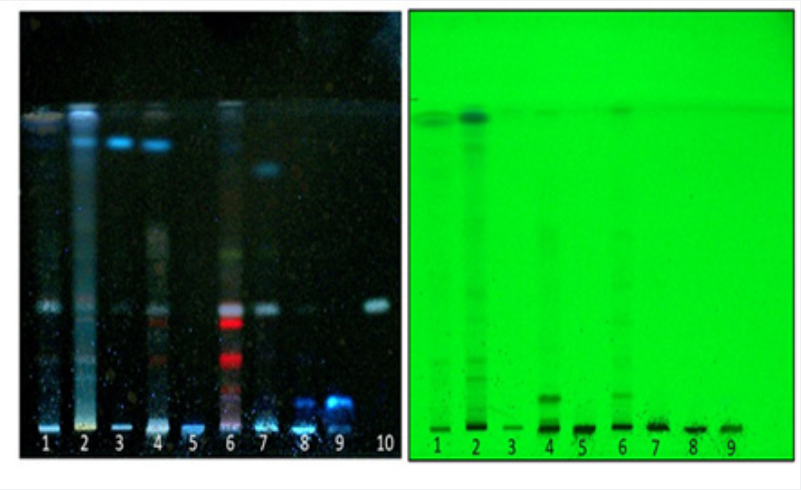

Figure 3: HPTLC chromatogram plate of the extracts.

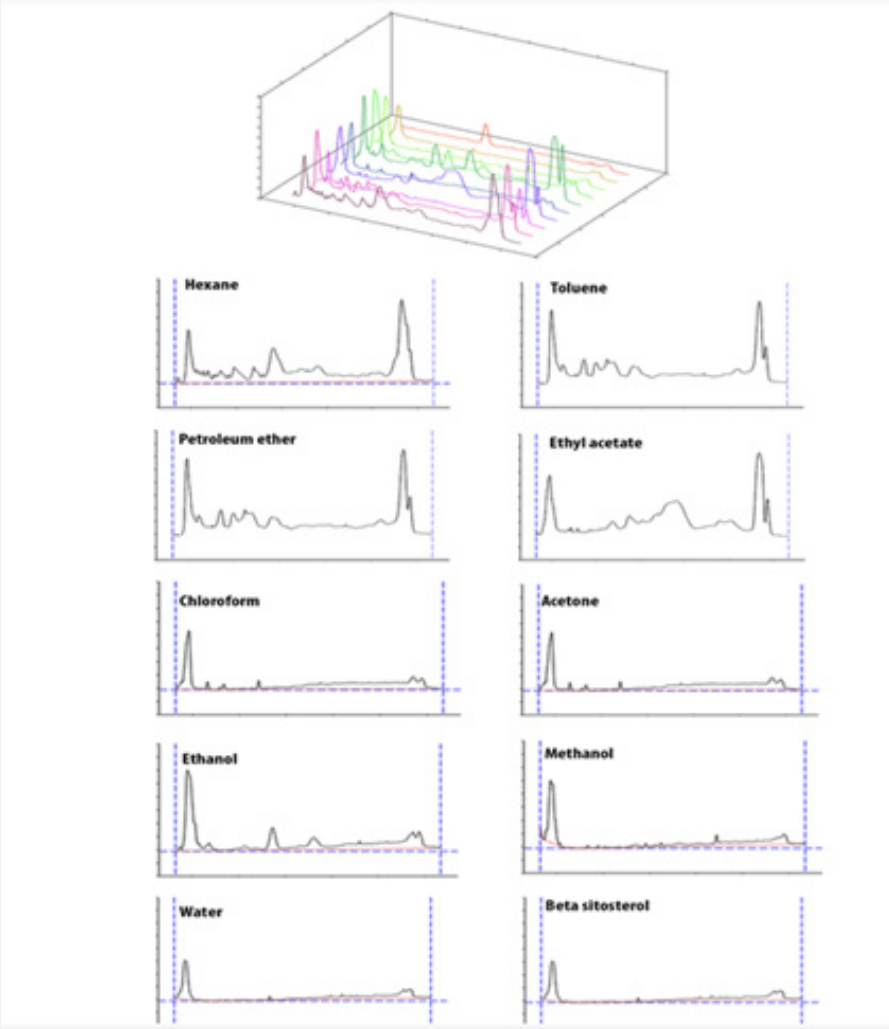

Figure 4: Three-dimensional plot of fingerprint showing different peaks of phytoconstituents.

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Table 3: HPTLC finger printing data of the extract.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
Hexane										
1m	-0.04	26	-0.01	415.1	26.59	0.03	105.5	7676	16.43	unknown
2m	0.21	60.3	0.22	122.2	7.83	0.29	23.4	3503.4	7.5	unknown
3m	0.29	23.7	0.32	121.1	7.76	0.36	44.5	2708.1	5.8	unknown
4m	0.37	56.4	0.43	269.4	17.26	0.49	74	10954.2	23.44	Beta sitosterol
5m	1.03	85.8	1.09	633.3	40.57	1.16	12.2	21886.5	46.84	unknown
Toluene										
1m	-0.05	0	-0.01	594.8	26.51	0.04	105.7	11563	20.4	unknown
2m	0.12	74.1	0.16	197.1	8.79	0.2	62.3	5315.6	9.38	unknown
3m	0.2	61.3	0.23	171.2	7.63	0.26	113.4	4232.4	7.47	unknown
4m	0.26	114.9	0.29	196.9	8.78	0.37	63.8	9043	15.96	unknown
5m	0.39	58.6	0.43	139.5	6.22	0.51	54.1	6975	12.31	Beta sitosterol
6m	1.04	85.8	1.1	652.6	29.09	1.12	194	15991.7	28.22	unknown
7m	1.13	198.5	1.13	291.4	12.99	1.21	3	3554.8	6.27	unknown
Petroleum ether										
1m	-0.04	20.6	-0.01	306.7	47.46	0.03	14.2	3627.5	31.05	unknown
2m	1.05	28.5	1.1	339.6	52.54	1.24	1.1	8056.8	68.95	unknown
Ethyl acetate										
1m	-0.07	17.3	-0.01	480.5	28.26	0.04	36.9	12087.4	18.16	unknown
2m	0.28	56.8	0.32	114.9	6.75	0.36	58.2	4015.5	6.03	unknown
3m	0.37	61.9	0.41	161.6	9.5	0.46	105.7	6580.7	9.89	Beta sitosterol
4m	0.58	184	0.66	281.3	16.54	0.75	60.4	21988.5	33.03	unknown
5m	1.05	49.6	1.09	662.3	38.94	1.23	1.1	21891.5	32.89	unknown
Chloroform										
1m	-0.07	17.3	-0.01	480.5	28.26	0.04	36.9	12087.4	18.16	unknown
2m	0.28	56.8	0.32	114.9	6.75	0.36	58.2	4015.5	6.03	unknown
3m	0.37	61.9	0.41	161.6	9.5	0.46	105.7	6580.7	9.89	Beta sitosterol
4m	0.58	184	0.66	281.3	16.54	0.75	60.4	21988.5	33.03	unknown
5m	1.05	49.6	1.09	662.3	38.94	1.23	1.1	21891.5	32.89	unknown
Acetone										
1m	-0.04	63.2	-0.01	630.6	24.05	0.02	72.4	9944	17.42	unknown
2m	0.05	70.8	0.09	197.9	7.54	0.12	49.8	4477	7.84	unknown
3m	0.37	72.2	0.4	295.3	11.26	0.45	70.4	8381.9	14.68	Beta sitosterol
4m	0.57	112.6	0.6	308.7	11.77	0.64	120	9857.1	17.26	unknown
5m	1.07	180.9	1.1	630.6	24.05	1.13	218.5	17348.7	30.38	unknown
6m	1.13	265.7	1.14	559.4	21.33	1.25	0.2	7088.7	12.42	unknown
Ethanol										
1m	-0.06	32.5	-0.02	612.8	61.31	0.07	33.1	15767.7	55.34	unknown
2m	0.36	9.1	0.41	171	17.11	0.46	11.6	3813.3	13.38	Beta sitosterol
3m	0.57	23.8	0.61	90.3	9.04	0.68	20.4	3178.1	11.15	unknown
4m	1.07	54.5	1.14	125.3	12.54	1.24	2.3	5735.3	20.13	unknown
Methanol										

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1m	-0.08	0	-0.03	469.3	85.85	0.03	0.7	9519.2	73.45	unknown
2m	1.05	32.5	1.14	77.4	14.15	1.23	4	3440.8	26.55	unknown
Water										
1m	-0.06	13	-0.03	307.5	81.78	0.03	0	6349.4	69.85	unknown
2m	1.07	26.8	1.14	68.5	18.22	1.16	11.7	2741.1	30.15	unknown

Discussions

Plants owe their therapeutical potential to the presence of secondary metabolites. In plants, the medicinal value of secondary metabolites is due to the presence of chemical substances that produce a definite physiological action on the human body [16]. The present study carried out on plant parts revealed the presence of medicinally active constituents. Preliminary phytochemical screening of extracts from the seed of C. *bonduc* has been carried out. The results of phytochemical analysis indicated the presence of phytochemical constituents contained in extracts of the seed of C. bonduc. The secondary metabolites components contained in the extracts of medicinal plants include the following compounds: alkaloids, saponins, terpenoids, steroids, tannins, flavonoids, etc. The preliminary phytochemical screening of the seed extract of C. *bonduc* recorded the presence of various phytochemicals like phenols, glycosides, alkaloids etc. Phenolic compounds possess diverse biological activities, for instance, antiulcer, anti-inflammatory, antioxidant, cytotoxic and antitumor, antispasmodic, and antidepressant activities [17]. Flavonoids have attracted a great deal of attention due to their potential health benefits. Over the past few years, several experimental studies have demonstrated the biological and pharmacological properties of many flavonoids especially their antimicrobial activity, anti-inflammatory, antioxidant and anti-tumour effects, which are associated with free radical-scavenging action [18]. Flavonoids have also been reported to possess hypoglycemic and anti-diabetic effects [19]. Flavonoids have antioxidant activity, protect cells against oxidative damage and reduce the risk of developing certain types of cancer [20]. Alkaloids are secondary metabolites known to be produced by plants and are of considerable pharmaceutical importance since they are used as drugs for the treatment of several diseases [21]. In the present study, alkaloid presence was confirmed in methanol extracts by quantitative analysis. HPTLC is a valuable tool for investigating herbal products concerning different aspects of their quality [22]. HPTLC fingerprint technique can be used as a powerful diagnostic tool to identify plants precisely. It is useful as a phytochemical marker and a good estimator of genetic variability in plant populations. The presence or absence of a chemical constituent is helpful in the placement of the plant in taxonomic categories [22]. HPTLC profile differentiation is such an important and powerful procedure, and it has often been employed for this purpose. HPTLC fingerprinting has proved to be a linear, precise, accurate method for herbal identification and can be used further in authentication and characterization of medicinally important plants. The developed HPTLC fingerprints will help the manufacturer with quality control and standardization of herbal formulations. Such fingerprinting is useful in differentiating the species from the adulterant and acts as a biochemical marker for various medicinally significant plant in the pharmaceutical industry and plant systematic studies [23]. HPTLC is an invaluable quality assessment tool for evaluating botanical materials, and it allows for more versatile analysis than ordinary TLC methods, as the spots are well resolved. Though further work to characterize the other chemical constituents and perform a quantitative estimation with marker compounds is necessary, this data can also be considered along with the other values for fixing standards for this plant.

Conclusion

The present study revealed that C. *bondoc* is a medicinal plant rich in secondary metabolites like alkaloids, terpenoids, flavonoids, etc. The preliminary research on the seed extract possessed significant secondary metabolites. Preliminary phytochemical and phytochemical analysis reports can be helpful to reinforce and authenticate the drug. Finally, it can be concluded that HPTLC fingerprint analysis of C. *bondoc* seed extract may be utilized as a diagnostic tool for proper plant identification and as a phytochemical marker.

Acknowledgements

The authors would like to thank the Managing Director and Director Pankajakasthuri Herbals India Pvt. Ltd., Poovachal, Kattakada, Trivandrum, India for providing the essential infrastructure, amenities and financial support for this study. The authors also thank Dr. Suresh Kumar C MD (Ayurveda), Professor, Department of Shalyatantra, Pankajakasthuri Ayurveda Medical College & P.G. Centre, Killy, Kattakada, Thiruvananthapuram, Kerala, India and Prof. (Dr.) K.G. Revikumar, Former Chief & Head, Clinical Pharmacy, Govt. Medical College Hospital, Trivandrum, Kerala, India for their valuable technical advice. The authors would like to express their gratitude to CARe KERALA, Koratty, Thrissur, Kerala, India, for her invaluable technical assistance to conduct the experiments.

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DOI: 10.19080/OMCIJ.2022.11.555817

How to cite this article: Shan S, Srinivasakumar K, Amiya B, Sreemoy K D, Hareendran N. Preliminary Phytochemistry and High-Performance Thin-Layer Chromatography Fingerprint Profiling of the Seed Extracts of Caesalpinia Bonduc: A Pharmaceutically Significant Medicinal Plant. Organic & Medicinal Chem IJ. 2022; 11(4): 555817. DOI: 10.19080/OMCIJ.2022.11.555817