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Identification, isolation and characterization of *Aphanamixis polystachya* flavonoids by UV, IR and RP-HPLC

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Abstract

Objectives: The present study aims the phytochemical screening, isolation and characterization of isolated flavonoid fractions of the *Aphanamixis polystachya* (wall.) Parker stem bark extract, which is an important medicinal plant in all regions which could be further used as tool for standardization.

Methods: Successive extraction was carried out by Soxhlet extraction and maceration method. Further all extracts were studied for phytochemical screening for presence of various phytoconstituents. Total flavonoid content and phenolic content determination with standard $AlCl_3$ colorimetric method and Folin-Cioacalteu assay performed for all extracts. Ethanolic extract subjected for the column chromatography and fractions were collected further subjected for qualitative analysis by thin layer chromatography (TLC), UV-Vis Spectrophotometry, RP-High performance liquid chromatography (HPLC) and IR interpretation performed for structural elucidation.

Results: Phytochemical screening shown the presence of alkaloids, phenols, flavonoids, cardiac glycosides, saponin glycosides, anthraquinones, terpenoids and steroids. Total flavonoid and phenolic content were found highest in ethanolic extract. TLC, UV, HPLC and IR interpretation of isolated fraction confirmed the presence flavonoids when compared with of standard markers.

Conclusion: Study findings confirmed that *A. polystachya* stem bark extracts have the presence of flavonoids which has wide range of human health benefits. This study opens up the new ways to exploit a particular plant for various therapeutic and restorative purposes and the study will help in future for identifying this plant for further research.

Keywords: *Aphanamixis polystachya* (wall.) parker, column chromatography, flavonoids, RP-HPLC

Introduction

Traditional medicinal plants have wide variety of therapeutic effects due to the presence of specific phytoconstituents. *Aphanamixis polystachya* has potential therapeutic uses. It belongs to the Meliaceae family. *Aphnamixis polystachya* is also known by various synonyms like *Aglaia polystachya*, *Amoora rohituka*, *Andersonia rohituka*. The large deciduous tree (sometimes shrub), native to India. It grows about 20-30 m tall. Leaves are long about 30-60cm, odd –even pinnate, with 9-21 leaflets. Leaflets are ovate, elliptic or oblong-elliptic. Leaflet base is oblique. It has entire margin. Flowers are 6-7 mm in diameter with 3 bractioles. Flowers have 5 nearly curcular sepals, 1-1.5 mm across. Petals are 3-7 mm in diameter, concave. Anthers are 5-6, oblong. Grayish brown seeds & capsule becomes orange when matures. Flowering takes place in the month of May-September (Mishra A. P. and Saklani S., 2014) [1]. *Aphanamixis polystachya* has different local names, in Sanskrit Rohitaka, in Hindi Harinhara, in Bengali Tiktaraj, in Madras Rakta-rohida, in Tamil Semmaram, in Telugu Sevamanu. It has aperient action as per traditional use. It is also used for the treatment of liver and spleen diseases, enlarged glands and corpulence. (Dr. Nadkarni K. M. 2004) [2] *A. polystachya* bark is a strong astringent, used for the treatment of rheumatism, liver and spleen diseases and tumours. Study shows that *A. polystachya* stem bark extract has significant *in vitro*, antimicrobial, mild antifungal, antibacterial, cytotoxic as well as antioxidant effects. (Krishnaraju A. V., Rao C. V, 2009) [3]. Findings from present study selected plant are rich in the interesting compounds.

Material and Method

Collection of plant material

Plant part (Bark) of *Aphanamixis polystachya* were collected from district Nashik, Maharashtra. Botanical identification of plant was performed by Department of Agricultural Botany, K.K. Wagh College of Agriculture, Nashik, Maharashtra, India.

Extraction of plant material

Maceration and Soxhlet extraction are extraction techniques used for the extract preparation. Stem bark powder were kept in contact with distilled water in 1:10 ratio for 24 hr with occasional stirring then filtered and evaporated to get concentrated extract, similarly methanolic and hydroalcoholic extract prepared by using methanol and ethanol: water (1:1) respectively. Methanol recovered by

distillation for reuse. Ethanolic extract prepared by Soxhlet extraction (hot continuous extraction) procedure.The bark powder was kept for Soxhlet extraction by using ethanol as a solvent for 6 hrs. By distilling off the solvent, extracts were concentrated and then evaporated to dryness on the water bath. Percentage yield of aqueous, methanolic, hydroalcoholic and ethanolic extracts were obtained 8.4, 3, 9 and 2.4 % respectively. (Krishnaraju A. V. and Rao C. V. 2009., Khandelwal K. R. and Sethi V. K. 2004) [3, 4].

Phytochemical screening

Phytochemical screening of extracts was carried out to evaluate the presence of various active constituents, like alkaloids, flavonoids, anthraquinones, cardiac glycosides, steroids, terpenoids.

Total flavonoid content determination

$AlCl_3$ colorimetric method was used for determination of total flavonoid content. Rutin used as a standard. In 10 ml volumetric flask, 50µl of crude extract (1 mg/ml ethanol) were made up to 1 ml using methanol, to it added 4 ml of distilled water, followed by addition of 0.3 ml of 5 % Sodium nitrite, then 0.3 ml of 10% Aluminium chloride was added after 5 minutes. After 6 minutes incubation at R.T. 2 ml of 1 M Sodium hydroxide was added to the reaction mixture. Immediately the final volume was made up to 10 ml with double-distilled water.

The mixture was allowed to stand for 15 min. Absorbance of sample was measured against the blank at 510 nm using a UV-Visible spectrophotometer (Shimadzu). From the calibration curve of standard (rutin), total flavonoid content calculated, and the results were expressed as mg rutin equivalent per g dry weight. (Baba S. A., and Malik S. A. 2015) [6]

Total phenol content determination

Folin-Ciocalteu's assay performed to determine the total phenol content using garlic acid as standard. In the procedure, 0.5 ml of plant extracts were mixed with 1.5 ml Folin-Ciocalteu's reagent (FCR) diluted 1:10 v/v then after 5 minutes,1.5 ml of 7% sodium carbonate solution was added.

The final volume of the tubes was made up to 10 ml with distilled water and allowed to stand for 90 minutes at R.T. Absorbance of sample was measured against the blank at 750 nm using a spectrophotometer (Shimadzu).

All the experiment was repeated three times for precision and values were expressed in mean ± standard deviation in terms of phenol content (Gallic acid equivalent, GAE) per g of dry weight. (Mathur R. and Vijayvergia R. 2017) [7] Boonyen C. and Chaisuksant R. 2009) [8].

Column chromatography for isolation of flavonoid fraction

For column chromatography, ethanolic extract was subjected in silica gel (60-120 mesh) glass column. 50 gm of activated silica gel (60-120 mesh) was used as stationary phase, then about 5 gm of crude extract was loaded onto the column and eluted with mobile phase Acetone (90): Chloroform (10), Acetone (80): Ethyl acetate (20), Acetone (70): Ethanol (30), Acetone (50): Methanol (50), Methanol (100) with increasing polarity. Total 10 fractions were obtained which were further studied by TLC.

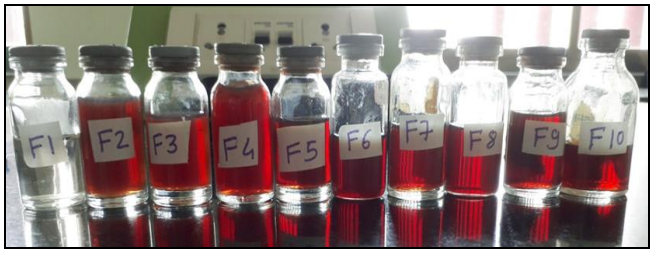

Fig 1: Fractions obtained from column chromatography

Thin layer chromatography

TLC carried out with silica gel 60 F254 plate performed for the all the fractions collected by column chromatography, and by using mobile phase which was ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:27). And, brown spots were detected. Fraction 7, 8 shown Rf value matched with the standard. Fractions shown Rf value matching with the marker were collected and further used for UV, HPLC and IR analysis. (Wagner H. and Bladt S. 2005).

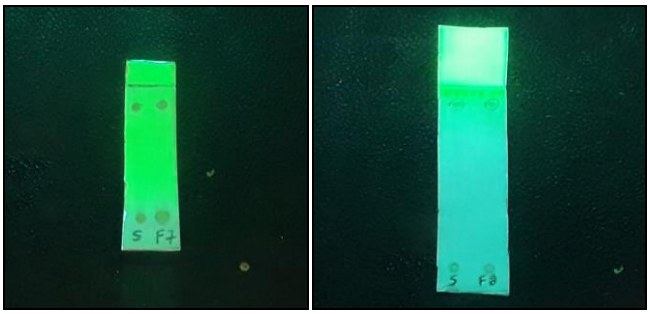

Fig 2: TLC of fractions F7 and F8 (compared with standard flavonoid)

Characterization by UV, IR and RP-HPLC analysis technique by using standard flavonoids

UV-VIS analysis

For identification of flavonoids present in the isolated fractions of *Aphanamixis polystachya* UV-visible spectra were performed. The qualitative UV-VIS profile of isolated fractions of *Aphanamixis polystachya* was taken at the wavelength of 200 nm to 800 nm and compared with standard rutin UV-VIS profile. The profile of isolated fraction7 showed the peaks at 663,358 and 257 nm with the absorption 0.003, 1.458 and 1.771 respectively. The profile of isolated fraction 8 showed the peaks at 747, 656, 364, 303 and 256 nm with the absorption 0.003, 0.004, 2.050, 0.822 and 2.262 respectively. The UV-VIS profile of the standard flavonoids that is quercetin and rutin shows peak at 375 and 257 respectively. (Chaudhari S. P., and Bangar J.V. 2014). The UV-VIS spectra concluding that isolated fraction contains flavonoids by comparing with standard markers. UV-VIS qualitative spectrophotometry is limited due to inherent difficulties in assigning absorption peaked in complex media analysis. Thus, UV-VIS findings must be supplemented with some other analytical technique such as HPLC etc, to enable proper extract characterization and constituent identification. (Jain P. K., and Bhawsar J. 2016).

FT-IR

For analysis, dried extracts were used. FT-IR spectra were collected using FTIR spectrophotometer, model IRAffinity-1, with Configuration of Full mid-IR wave number from 7800 cm-1 to 350 cm-1, spectral resolution of 0.5 cm-1. Selectable moving mirror speed of 2.0, 2.8, 5.0 and 9.0 mm/sec. Built-in validation conforming to ASTM and EP, with IR solution software, from Shimadzu.

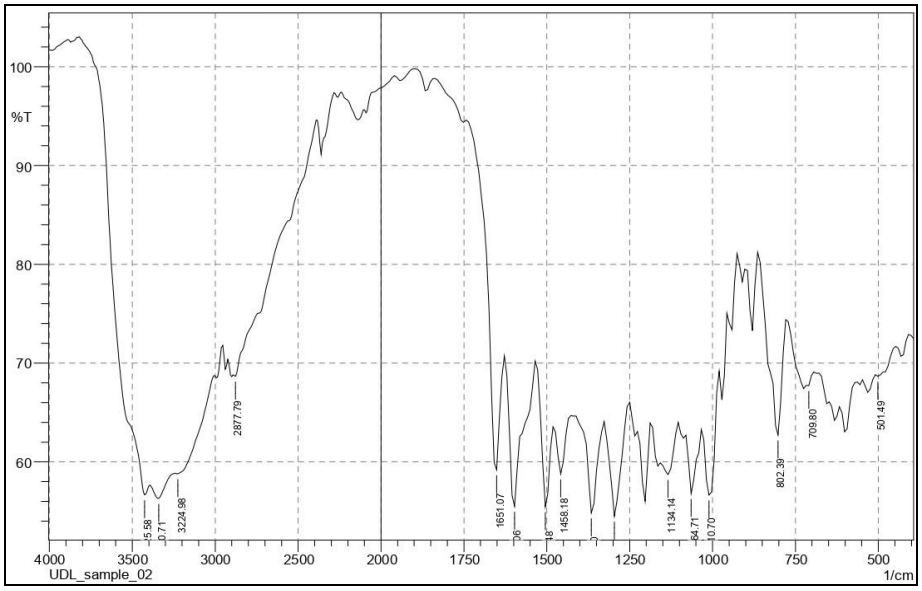

Fig 3: (A) IR spectra of standard Rutin

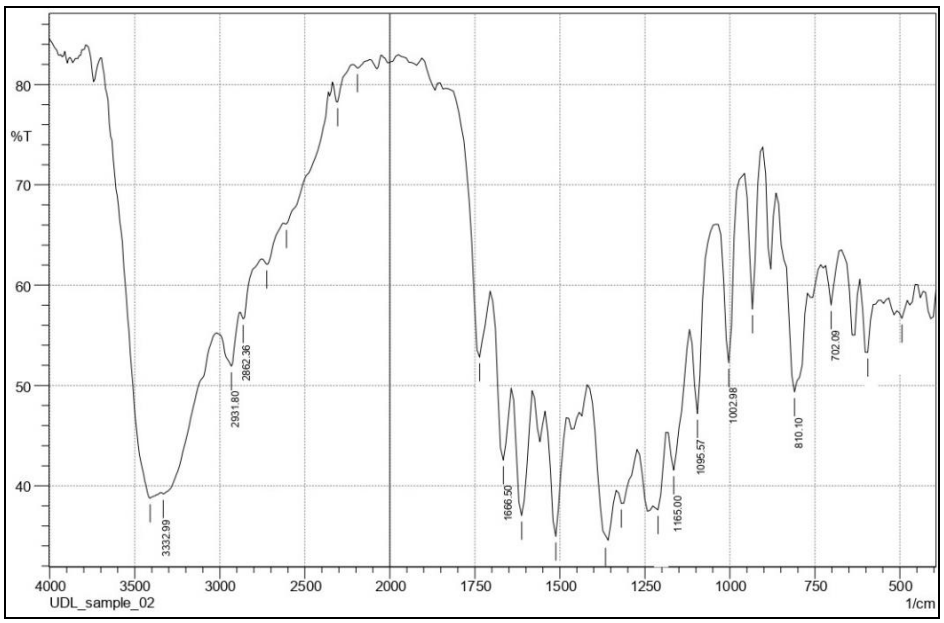

Fig 3: (B) IR spectra of standard Quercetin

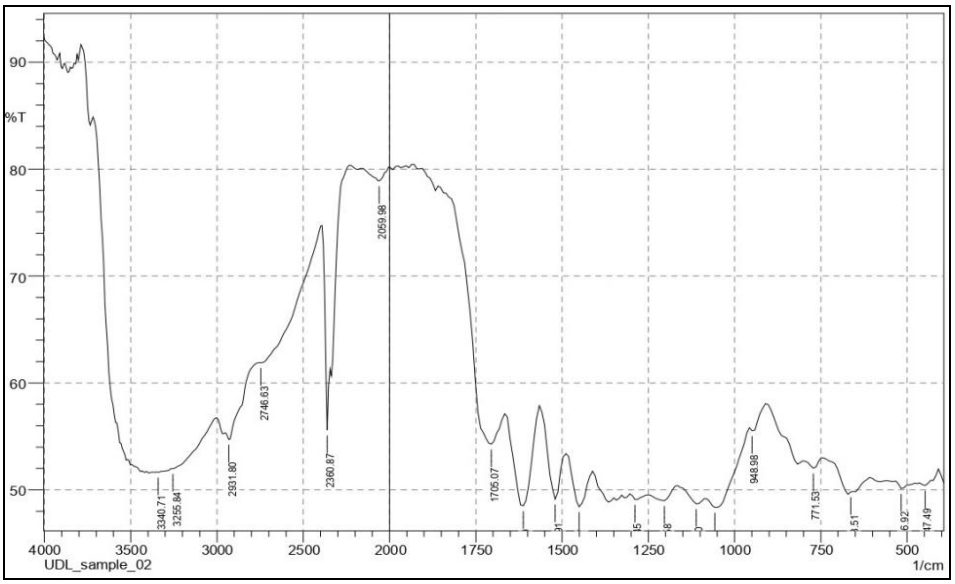

Fig 3: (C) IR spectra of Fraction 7

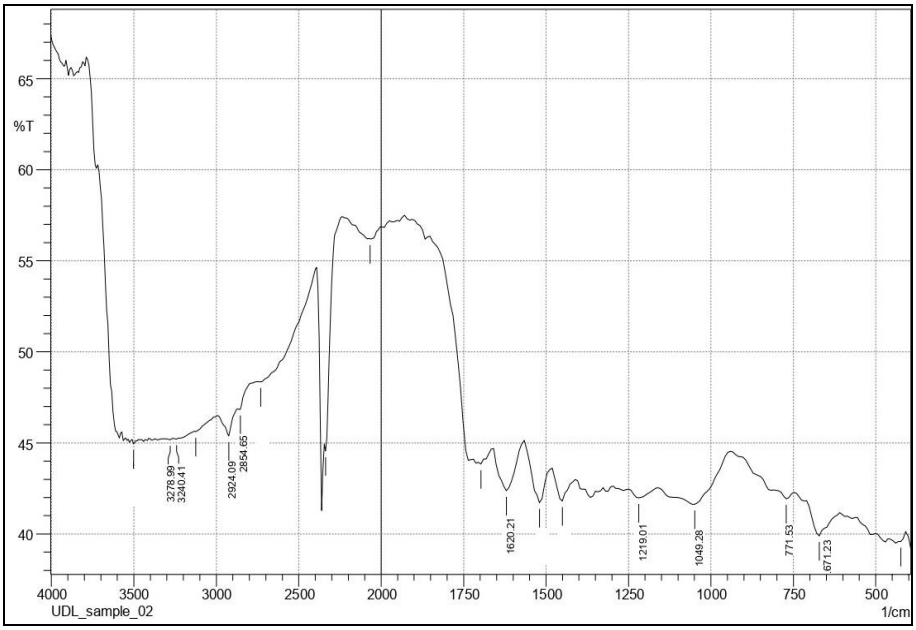

Fig 3: (D) IR spectra of Fraction 8

Instrumentation and condition of RP-HPLC system

HPLC Binary Gradient System (Model no. HPLC 3000 series) was used for herbal extract sample analysis. HPLC system is equipped with an autosampler, column compartment, pump and UV-3000-M detector. The column used for the herbal extract analysis was comosil C-18 (250mm × 4.6ID , paricle size 5 µ). The samples concentration used were 1000 µg/ml using methanol as a solvent. The standard concentration used was 30 µg/ml with methanol as solvent. Mobile phase used was methanol: water (75:25). Injection volume and flow rate were 20µl and 0.8 ml/min respectively. UV visible spectra were recorded over range of 200-400 nm. Chromatograms were acquired at 256 nm. Analysis was carried out with the help of HPLC workstation software.

Result and Discussion

Phytochemical screening

Phytochemical screening tests for aqueous and methanolic extracts of *Aphanamixis polystachya* showed the active phytochemical classes as cardiac glycosides, alkaloids, reducing sugars, phenols and flavonoids as presented in Table 1.

Table 1: Phytochemical screening tests for aqueous and methanolic extracts of *A. polystacya* stem bark

Phytochemical compounds	Aqueous extract	Methanolic extract	Ethanolic extract	Hydroalcoholic extract
Cardiac glycosides	+	+	+	+
Saponin glycoside	+	+	+	+
Alkaloids	+	+	+	+
Amino acids	-	-	-	-
Starch	-	-	-	-
Flavonoid	+	+	+	+
Steroids	+	+	+	+
Phenols	+	+	+	+
Reducing sugars	-	-	-	-
Volatile oil	-	-	-	-
Tannin	+	+	+	+
Anthraquinones	+	+	+	+
Terpenoids	+	+	+	+

Total flavonoid content

The total flavonoid content for different extracts of *A. polystachya* stem bark presented in table no. 3 as well as absorbance of standard compound (Rutin) is shown in this Table 2 at different concentrations.

Table 2: Absorbance of standard compound (Rutin) at λ max = 510nm

Rutin concentration(µg/ml)	Absorbance at λ max=510nm
5	0.038
10	0.087
20	0.162
40	0.253
100	0.559

Standard curve of Rutin indicated the equation of y = 0.0053x+0.034and R^2 =0.9938 clarified in Fig.13.

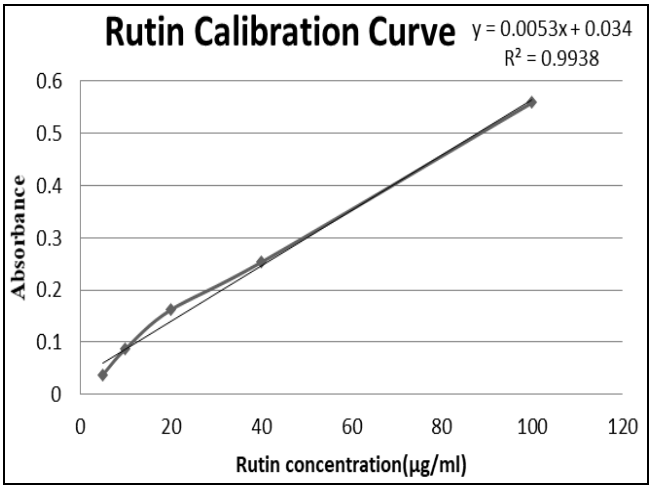

Fig 4: Standard calibration curve of Rutin

Table 3: Total flavonoid content in different *A. polystachya* stem bark extracts

A. Polystachya extract	Total Flavonoid content (mg of RE/ gm of dry weight) ± SD
Aqueous extract	1.756 ± 0.607
Methanolic extract	7.79 ± 0.892
Ethanolic extract	12.006 ± 1.22
Hydroalcoholic extract	6.26 ± 0.416

Total phenolic content

The total phenolic content for different extracts of *A. polystachya* stem bark presented in table no. 5 as well as absorbance of standard compound (Gallic acid) is shown in this table no.4 at different concentrations.

Table 4: Absorbance of standard compound (Gallic acid) at λ max = 750 nm

Gallic acid concentration(µg/ml)	Absorbance at λ max= 750nm
2	0.017
4	0.032
6	0.048
8	0.062
10	0.075

Standard curve of gallic acid indicated the equation of y = 0.0077x and R^2 = 0.9947 clarified in Fig.14.

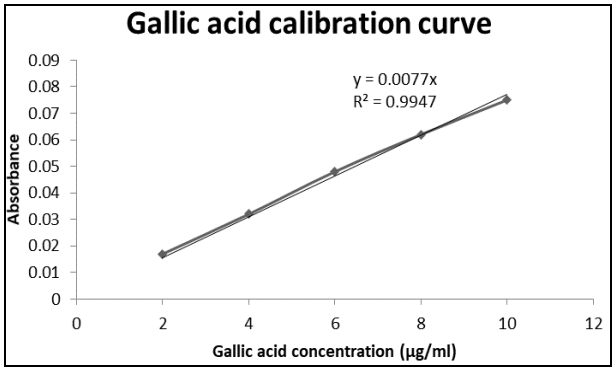

Fig 5: Standard calibration curve of Gallic acid

Table 5: Total phenolic content in different *A. polystachya* stem bark extracts

A. polystachya extract	Total phenolic content (mg of GAE / gm of dry weight) ± SD
Aqueous extract	41.66±0.288
Methanolic extract	47.16±1.25
Ethanolic extract	48.83±0.288
Hydroalcoholic extract	43±0.5

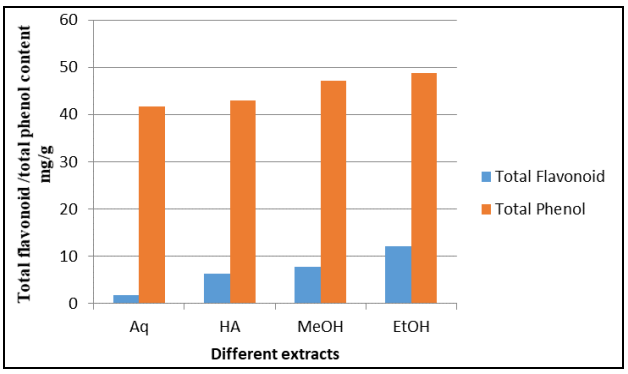

Fig 6: Total flavonoid and total phenolic contents of different *A. polystachya* stem bark extracts

Table 6: UV-VIS peak values of isolated fraction 7 and 8.

Sr. No.	Isolated Fraction 7		Isolated Fraction 8	
	Wavelength	Absorbance	Wavelength	Absorbance
1.	663.00	0.003	747.00	0.003
2.	358.00	1.458	656.00	0.004
3.	257.00	1.771	364.00	2.050
4.	-	-	303.00	0.822
5.	-	-	256.00	2.262

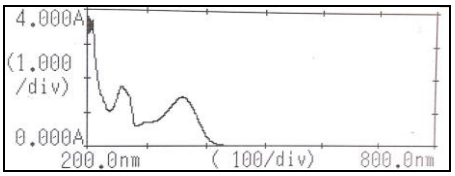

Fig 7: (A) UV-VIS spectra of Isolated fraction 7

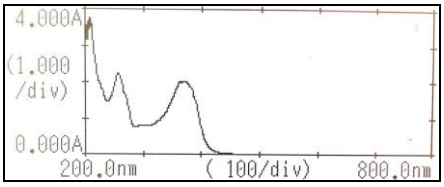

Fig 7: (B) UV-VIS spectra of Isolated fraction 8

IR interpretation

IR spectra of extract compared with the markers.IR interpretation found that ranges of the peaks in the spectra of flavonoid fraction, matches with the spectra of the markers.

Table 7: IR interpretation

Sr. No.	Standard peak	Observed peak of standard (Rutin)	Observed peak of standard (Quercetin)	Observed peak of fraction 7	Observed peak of fraction 8	Characteristic bond	Characteristic functional group
1.	3400-3200	3340.71 3224.98	3332.99	3340.71 3255.84	3278.99 3240.41	H-bonded	Alcohols, Phenols
2.	3000-2850	2877.79	2931.80 2862.36	2931.8	2924.09 2854.65	-CH_3 Stretch	Alkane
3.	1600-1680	1651.07	1666.50	1612.49	1620.21	C=C Stretch	Alkene
4.	1000-1300	1296.16 1134.14 1064.71 1010.7	1165.00 1095.57 1002.98	1288.45 1203.58 1111 1056.99	1219.01 1049.28	O	Ether
5.	900-690	802.39 709.8	810.10 702.09	771.53	771.53 671.23	Out of plane bend	Aromatic

HPLC analysis

HPLC qualitative analysis confirmed the results obtained by the UV, IR, TLC. HPLC qualitative analysis confirmed the presence of flavonoids in the stem bark extract of *Aphanmixis polystachya*.

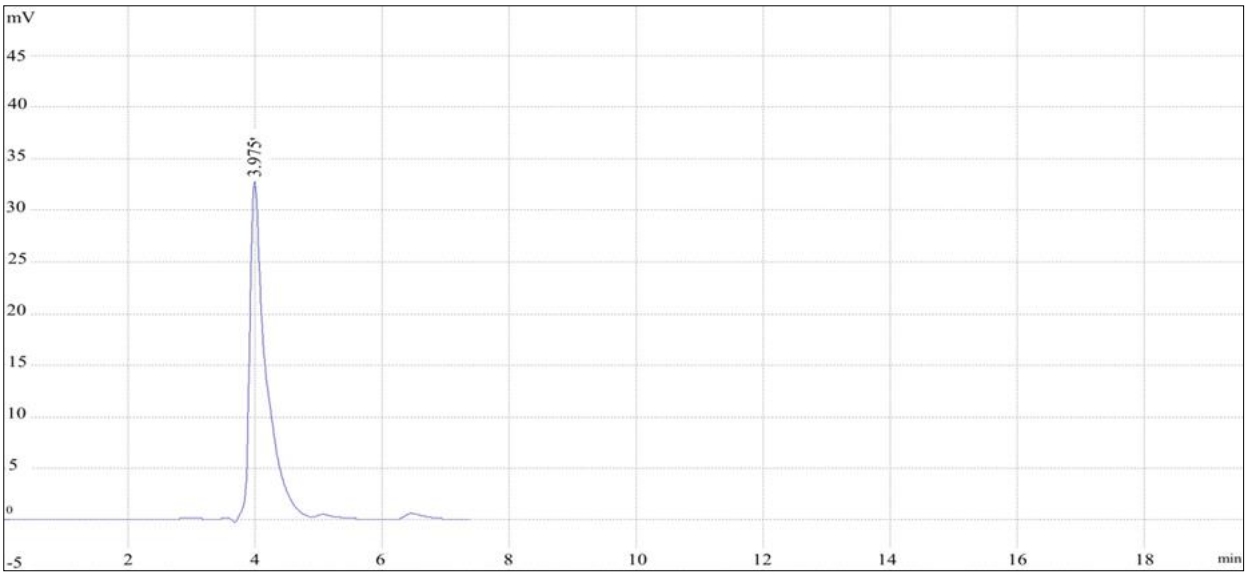

Fig 8: (A) HPLC chromatogram of standard (Rutin)

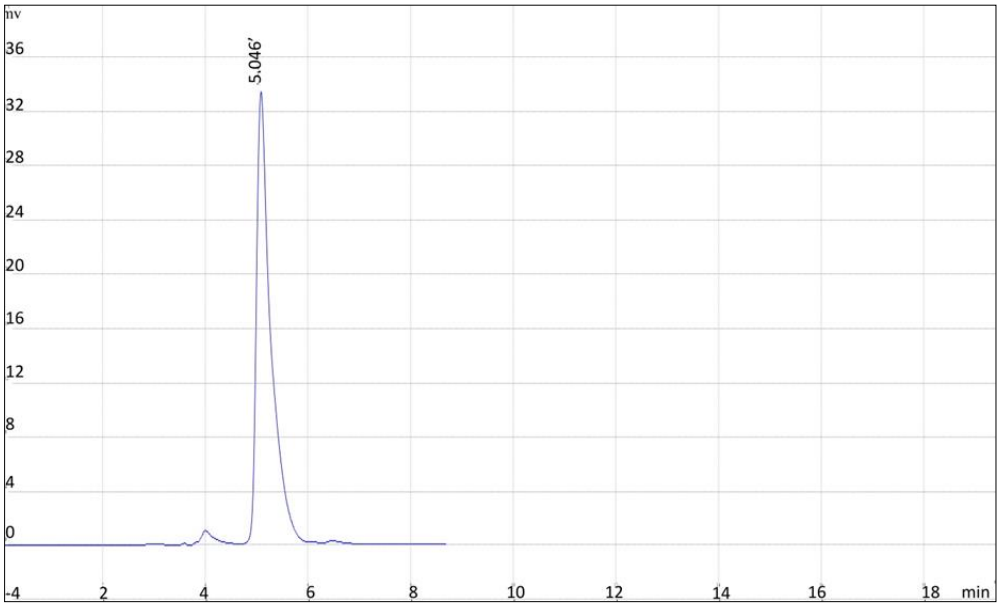

Fig 8: (B) HPLC chromatogram of standard (Quercetin)

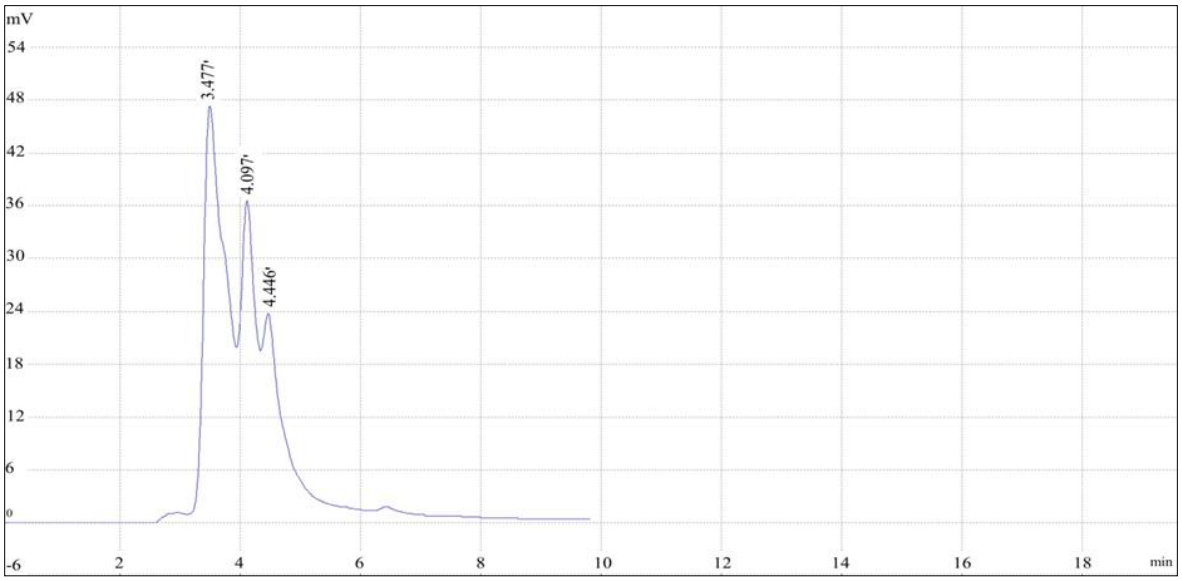

Fig 8: (C) HPLC chromatogram of fraction 7

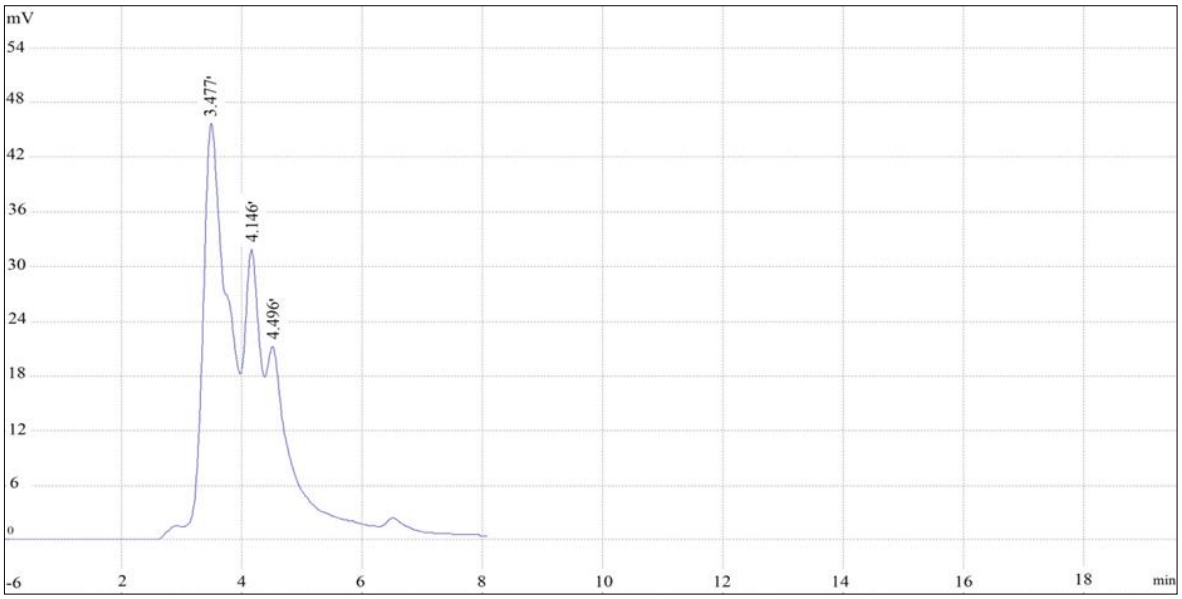

Fig 8: (D) HPLC chromatogram of fraction 8

Discussion

Aphanmixis polystachya is widely used medicinal plant. According to the literature survey, stem bark is traditionally used in the treatment of number of diseases. So, the stem bark extracts were prepared and studied further for identification of the bioactive phytoconstituents. Mainly the stem bark extract popularly known for antioxidant activity, which is responsible for number of disease treatments. The present study is focused on the bioactive compounds present in the stem bark of the A. polystachya. The phytochemical screening showed that the *Aphanamixis polystachya* stem bark extract contains a mixture of phytochemicals as alkaloids, cardiac glycosides, saponin glycosides, tannin, anthraquinones, terpenoids, flavonoids, phenolic compounds. Flavonoids have the antioxidant activity and highly active against free radical. In the recent era, the no. of disease is the result of oxidative stress in the body. Free radicals' generation is the cause of oxidative stress. Flavonoids are free radical scavenger leading to antioxidant activity. So, we have focused on the flavonoids of extracts. Total flavonoid content and total phenol content study of aqueous, ethanolic, methanolic and hydroalcoholic extract indicated that the ethanolic stem bark extract has the highest contents of flavonoids and phenol. Thus, ethanolic extract were chosen for the column chromatography to isolate the fractions, for further characterization by UV, IR and HPLC study. Collected fractions were subjected to TLC. Fraction shown R_f value similar to the standard, proceeded for the UV, IR and HPLC study. UV – visible spectrophotometer shown the presented the wavelengths of quercetin and rutin in fractions. Advanced analytical techniques thus applied to confirm presence of flavonoids in fraction collected by using markers (standard rutin and quercetin). IR interpretation shown the similar IR ranges of fractions with that of the standards. HPLC confirmed the presence of flavonoids in the fraction as HPLC chromatogram of fraction displayed area which was found near with the markers. This study can help to find out the novel therapeutic applications, preparation of novel dosage form, identification of exact class and mechanism of action along

with therapeutic use of bioactive phytoconstituents identified by the phytochemical screening. Structural elucidation and quantification by advanced analytical techniques is needed.

Conclusion

Phytochemical study identified the no. of phytoconstituents in the stem bark extract. Targeted flavonoid content was found highest in the ethanolic extract which further subjected to column chromatography. Two fractions on comparison with markers by TLC were found containing flavonoids further proceeded for characterization by UV, IR and RP-HPLC by using markers (standard quercetin and rutin) confirmed the presence of flavonoids in the isolated fraction of stem bark extract.

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Conflict of Interest

The Authors declares that there is no conflict of interest.

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