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HPLC ANALYSIS AND ANTIOXIDANT ACTIVITIES OF *HOLOPTELEA INTEGRIFOLIA*

ALLI KAVITHA and MANGAMOORI LAKSHMI NARASU*

Centre for Biotechnology, IST. Jawaharlal Nehru Technological University,
HYDERABAD – 500072 (A.P.) INDIA

ABSTRACT

There has been a renewed interest in naturally-occurring antioxidants from fruits, vegetables and plants. This study is aimed at identifying antioxidant compounds present in the extracts of the *Holoptelea integrifolia*. The antioxidant properties of methanol, acetone and ethyl acetate extracts of *Holoptelea integrifolia* were determined using the 2,2-DPPH method, FRAP method and reducing power assay. The total phenolic content was estimated through the use of the folin-ciocalteu method. The total flavonoid content was determined according to Kim et al.[1] method. Ascorbic acid, Gallic acid and quercetin were used as standards for evaluating radical scavenging activity, total phenolic content (TPC) and total flavonoid content (TFC). Results derived from the 2,2-DPPH method, FRAP method and reducing power assay showed that the stem extracts of acetone had the highest antioxidant activity. The TPC and the TFC values also showed similar results. The HPLC analysis of the extracts also confirmed the trend derived from the assays. The HPLC analysis showed large quantities of gallic acid and rutin in plant extracts. Results showed that the stem extract of acetone and the leaf extracts from methanol had the highest antioxidant activity compared to other extracts. The present study is the first report on antioxidant properties and phytochemical analysis of *Holoptelea integrifolia*.

Key words: Antioxidant activity, 2,2-DPPH, FRAP, Reducing power assay, TPC, TFC.

INTRODUCTION

Reactive oxygen species (ROS) are forms of activated oxygen and include free radicals, such as superoxide and hydroxyl. The superoxide anion ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide are notable side products in metabolic reactions[2]. The oxidative damage caused by ROS tolipids, proteins and nucleic acids triggers various diseases including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, immune deficiency diseases and ageing[3,4]. Oxidative damage also causes a deterioration of food during storage[5]. In healthy individuals, however, the production of free radicals is

*Author for correspondence; E-mail: mangamoori@gmail.com; Mo.: 91-9490173899

balanced by the antioxidative defence system; however, oxidative stress is generated when equilibrium favours free radical generation as a result of the depletion of antioxidants. Ascorbic acid, α-tocopherol and phenolic compounds, which are present naturally in vegetables, fruits, grains and pulses, possess the ability to reduce oxidative damage[6]. The consumption of fruits and vegetables containing antioxidants offers protection against these diseases. Dietary antioxidants can augment cellular defences and help prevent oxidative damage to cellular components. In view of the adverse health effects of synthetic antioxidants, such as butylated hydroxyl toluene (BHT) and butylated hydroxyl anisole (BHA), there is considerable increase in the interest shown toward the isolation of naturally occurring bioactive molecules, which finds application in the food and pharmaceutical industry[7]. Also, research has investigated many other species of plants in an attempt to locate novel antioxidants[8-11]. A great number of plants worldwide have shown strong antioxidantactivity along with powerful scavenging activity against the free radicals[12,13].

Besides, phenolic compounds, flavonoids are also widely distributed amongst plants, and these have been reported to exert multiple biological effects such as-antioxidant-related, free radical scavenging, anti-inflammatory andanti-carcinogenic etc.[14] Natural antioxidants especially phenolics and flavonoids from tea, wine, fruits, vegetables and spices have already been commercially exploited either as antioxidant additives or as nutritional supplements[15]. As crude extracts of herbs and spices and other plant materials, rich in phenolics are of increasing interest in the food industry due to their ability to retard oxidative degradation of lipids and thereby, improve the quality and nutritional value of food. Therefore, the study of the importance and the role of phenolic compounds and flavonoids, as natural antioxidants have increased greatly. It is in view of this fact that the current study has been carried out on the *Holoptelea integrifolia*. Its vernacular name is Nemalichettu (Telugu) and it is commonly known as the Indian Elm tree. The *Holoptelea integrifolia* belongs to the family of the Ulmaceae and it is widely distributed across the tropical and temperate regions of the Northern Hemisphere including the Indian peninsula, China, Burma and Srilanka.

The present work dealt with the study of antioxidant activities of the methanolic, acetone and ethyl acetate extracts of *Holoptelea integrifolia* by using the reducing antioxidant power and the 2,2-DPPH radical scavenging assays. In addition, the study also measured the total content of phenolics and flavonoids from plant extracts.

EXPERIMENTAL

Plant material

Fresh parts of the *Holoptelea integrifolia* were collected from the Narsapur forest of

Andhra Pradesh, India. The plant was identified by Dr. Rasingham of the Botanical Survey of India, Hyderabad, India.

Chemicals

Methanol, acetone, ethylacetate, L-ascorbicacid, gallicacid, quercitin, 2,2-DPPH, potassium ferricyanide, phosphate buffer, trichloroacetic acid, ferri chloride ($FeCl_3$), folin-ciocalteu's phenol reagent, $NaNO_2$, $AlCl_3$, NaOH and double distilled water.

Preparation of crude plant extract

The fresh leaves, stem and root were taken and washed with the free-flowing, clean water and later cleansed further with distilled water. The washed leaves, stem and root were then, shade-dried to retain the active components of the plant material. After drying, the plant material was chopped into small pieces and then, powdered using the mortar and pestle. Thirty grams of powdered material was dissolved in 300 mL of the solvent in a glass stoppered round bottomed flask. The mixture was shaken well and kept at room temperature in a shaking incubator for 72 hrs. The extracts were filtered by using Whatman No. 1 filter paper. Then, the extracts were concentrated in a rotavapor at reduced pressure below 40^{o}C and evaporated to dryness in a vacuum oven at 40^{o}C. The extracts obtained were stored at 4^{o}C until further use.

Preliminary phytochemical screening

The screening of the phytochemical constituents was carried out with methanol, acetone and the ethyl acetate extracts. Various qualitative chemical tests were carried out to determine the presence of alkaloids, glycosides, phenols, resins, proteins, steroids, tannins, flavonoids, saponins and carbohydrates using the methods described by Harborne[16].

Antioxidant activity

Radical scavenging activity by the DPPH method

The determination of the free radical scavenging activity of the crude extracts was carried out using the assay described by Brand Williams et al.[17] after some modification. The crude extract of methanol, acetone and ethyl acetate concentrations of 0.01 mg/mL – 1.0 mg/mL was mixed with 2 mL of the 2,2-DPPH in methanol. The solution was incubated for 30 min at room temperature in dark before reading the absorbance (Ab) at 517 nm. As a positive control, 1 mL of methanol solution was used with the 2,2-DPPH in methanol. The radical solution (2 mL; 0.005 g per 100 mL methanol) was added to a test tube and 1 mL of the dissolved extract was added prior to the measurement. In its radical form, the 2,2-DPPH

absorbs light at 517 nm but upon reduction by an antioxidant or radical species, the absorption decreases. The decrease in absorbance was then, converted to percentage antioxidant activity using the formula shown below:

$$\% \text{ DPPH scavenging} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Ferric reducing antioxidant power (FRAP) assay

The total antioxidant potential of plant extracts was evaluated using FRAP assay by Benzie and Strain[18]. Briefly, the FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tri (2-pyridyl)-s-triazine in 40 mM HCl and 20 mM $FeCl_3$ solution in proportion of 10:1:1 (v/v), respectively. FRAP reagent was prepared fresh and was warmed to 37^{o}C before using. The reagent (1.5 mL) and plant extract (50 µL) were mixed thoroughly. The FRAP assay was based on the reducing power of an antioxidant. An antioxidant will reduce the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}), which increases the absorption at 593 nm, hence the absorbance was taken at 593 nm after 10 min. Standard curve was prepared using ferrous sulphate and the results were expressed as mmol Fe^{2+}.

Reducing power assay

The antioxidant potential of plant extracts can be investigated further by assessing the reducing power. The reducing power was determined according to the method used by Oyaizu[19]. Plant extracts of concentrations ranging from 0.01 mg/mL – 1.0 mg/mL were mixed with 2.5 mL of phosphate buffer (2.5 mL, 0.2 M, pH 6.6). Then, 2.5 mL potassium ferricyanide (1%) was added to the mixture. The mixture was incubated for 20 min at 50^{o}C temperature. After incubation, 2.5 mL of trichloroacetic acid (10%) was added to the mixture. The final mixture was then centrifuged at 3000 rpm for 10 min from the above mixture, and the upper layer of the solution (2.5 mL) was taken and diluted with 2.5 mL of distilled water and 0.5 mL of ferric chloride ($FeCl_3$, 0.1%). The absorbance of the mixture was measured at 700 nm. Increased absorbance of the reaction mixture indicated greater reducing power.

Total flavonoid content

Total flavonoid content was determined by using aluminium chloride colorimetric assay[1] with some modifications. 50 µL of plant extracts was added to the 250 µL of distilled water. Then, 15 µL of 5% $NaNO_2$ was added to the mixture, followed by 30 µL of 10% $AlCl_3$ was added. The mixture was incubated at room temperature (25^{o}C) for 5 min. Then, 100 µL of 1 M NaOH was added to the mixture. The mixture was diluted with 55 µL of

distilled water and the mixture was thoroughly vortexed while the absorbance was measured at 510 nm. Gallic acid was used as the standard and the absorbance values were expressed as mg Gallic acid equivalents. All the measurements were taken in triplicate and then, the mean values were calculated.

Total phenolic content

The total phenolic content was determined spectrophotometrically by the Folin-Ciocalteu method described by Singleton et al.[20] with some modifications. Briefly, 0.2 mL of sample was mixed with 1 mL of 1:10 Folin-Ciocalteu reagent (Sigma-Aldrich Chemie, Steinheim, Germany) for 5 min and 0.8 mL of 7% Na_2CO_3 was then, added to the mixture. After 2 hr of incubation in the dark at room temperature, the absorbance of the reaction mixture was measured at 760 nm. The total phenolic content was expressed as mg gallic acid equivalents GAE/100 g using the Gallic acid calibration curve (10-100 mg/L).

Phytochemical analysis with HPLC

Profiling of major phytochemical compounds (phenols and flavonoids) was determined by using high performance liquid chromatography (HPLC) analysis. Profiling was carried out based on retention time. HPLC analysis was carried out using a Shimadzu HPLC system consisting of SPD-M10AVP photodiode array detector with class VP software, C18 chromatographic separation column, UV-Vis detector and pump. The elution was performed isocratically with mobile phase consisting of acetonitrile and water (7:3) and filtered through a membrane of 0.45 µm and the flow rate was 1 mL/min. The injection volume of the sample was 20 µL. A wavelength of 215 nm was used for the flavonoids and 254 nm was used for profiling phenols. Stock solution of standard compounds gallic acid and rutin at various concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 µg/mL) was injected into the HPLC system and calibration curves were established. Concentration of the compounds was calculated from the peak area. The results were expressed as mg/mL of gallic acid and rutin.

RESULTS AND DISCUSSION

Preliminary phytochemical screening

The results of the phytochemical screening of extracts using the methods described above indicated the presence of phenols, flavonoids, terpenoids and tannins. The tests also revealed the presence of essential oils and proteins. Saponins were not present in any of the extracts. Phenols and steroids were not present in root extracts. Ethyl acetate extracts showed a marked absence of flavonoids. Presence of proteins was tested through the xanthoproteic test and the ninhydrin test. Xanthoproteic test showed the presence of proteins

Table 1: Phytochemicals in each extract based on preliminary screening

Phytochemical	Leaves			Stem			Root		
	Methanol	Acetone	Ethyl acetate	Methanol	Acetone	Ethyl acetate	Methanol	Acetone	Ethyl acetate
Alkaloids	+	-	-	+	+	-	+	+	-
Phenols	+	+	+	+	+	-	-	-	-
Flavonoids	+	+	-	+	+	-	+	+	-
Saponins	-	-	-	-	-	-	-	-	-
Terpenoids	+	+	-	-	+	-	+	+	+
Diterpenoids	+	+	+	+	+	+	+	-	+
Glycosides	+	-	+	+	-	+	+	-	-
Steroids	+	+	+	-	+	+	-	-	-
Xantho protein	+	+	+	+	-	-	+	+	+
Ninhydrin	+	-	-	+	-	-	+	+	-
Carbohydrates	+	+	+	+	+	+	+	+	+

in all samples of the root and the leaves whereas ninhydrin test indicated the presence of protein in all methanol extracts and the acetone extract of the root. The ethyl acetate extracts showed negative results for the alkaloid test. Table 1 shows the scale of presence of phytochemicals in each extract. Preliminary phytochemical analysis of *Holoptelea integrifolia* indicated the presence of various phytochemicals responsible for antioxidant activity. Phenols and flavonoids are major phenolics that possessed antioxidant activity. Plant samples also possessed terpenoids and proteins.

Radical scavenging activity by the 2,2-DPPH

Stable radical DPPH has been widely used in the determination of the antioxidant activity of plant extracts. A reduction of the 2,2-DPPH radicals can be observed by a decrease in the absorbance at 517 nm. The results are shown in Table 2. Percentage inhibition values are calculated by considering methanol as the control. The results indicate that acetone extracts have a high free radical scavenging properties as compared to the other extracts and among different parts of plant, it is the leaves that have shown better free radical scavenging activity. The free radical scavenging activity of 2,2-DPPH method and reducing power assay was also confirmed the antioxidant activity of methanol, acetone and ethyl acetate extracts of *Holoptelea integrifolia*'s leaves, stem and root. The acetone extract of the stem and leaf extracts of all the solvents showed high percentage inhibition compared to other extracts.

Table 2: Percentage of 2, 2- DPPH radical scavenging activity

Plant part	% Inhibition		
	Methanol	**Acetone**	**Ethyl acetate**
Leaves	75.78	89.2	87.49
Stem	74.6	88.9	79.7
Root	78.5	79.3	76.4

Ferric reducing antioxidant power (FRAP) assay

FRAP assay is used to determine antioxidant power of plant extracts. The results are shown in Table 3. Calibration curve of ferrous sulphate standard is calculated as $y = 5.326 x + 0.843$, $R = 0.977$. Acetone extract of stem has highest antioxidant power of 0.308 mmol Fe^{2+} and antioxidant power of stem ranged from 0.176 to 0.308 mmol Fe^{2+}, leaf extracts antioxidant power ranged from 0.108 to 0.247 mmol Fe^{2+} and root extracts have antioxidant power from 0.074 to 0.112 mmol Fe^{2+}. Results from FRAP assay showed that acetone

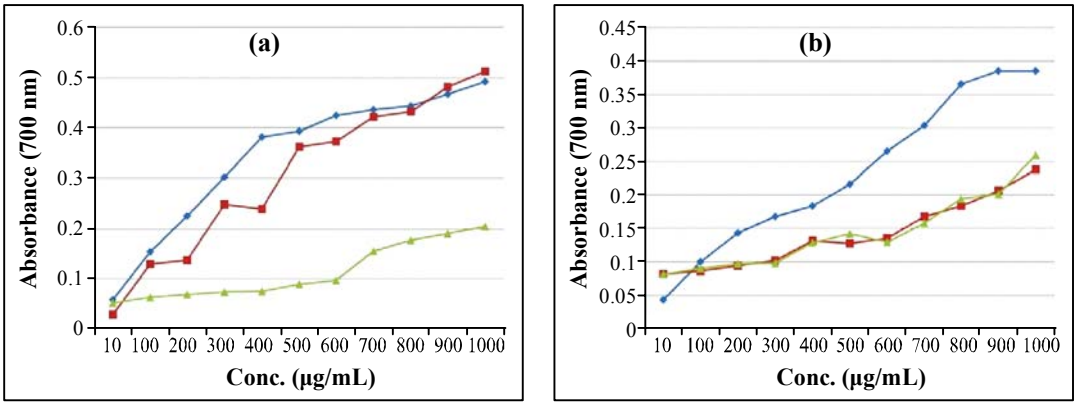
extract of stem has higher antioxidant power and ethyl acetate extract of root has lower antioxidant power. Among the plant parts, stem extracts had higher antioxidant power. These results were similar to that of 2, 2-DPPH results.

Table 3: Antioxidant power measured by FRAP assay

Plant part	Antioxidant power expressed as mM Ferrous sulphate		
	Methanol	Acetone	Ethyl acetate
Leaves	0.183 ± 0.026	0.247 ± 0.013	0.108 ± 0.0089
Stem	0.219 ± 0.009	0.308 ± 0.022	0.176 ± 0.042
Root	0.098 ± 0.012	0.112 ± 0.009	0.074 ± 0.0092

Reducing power assay

The reducing power assay is used to further investigate the antioxidant activity of the extracts. In the reducing power assay, the antioxidants in the extracts would result in a reduction of the Fe^{3+} to Fe^{2+}. The reducing power of the extract would serve as indicator of its potential antioxidant activity[21]. The reducing power of all the extracts was determined by observing the absorbance values. The absorbance values were presented in Fig. 1. An increase in absorbance values at 700 nm indicated an increase in antioxidant activity. At 1000 µg, highest absorbance was observed in the methanol extract from the leaves while the least was found in the ethyl acetate extract of the root. Overall, the leaf extracts showed higher absorbance numbers compared to the other extracts. The reducing power assay showed trends almost similar to that of 2,2-DPPH, except that the reducing power of Acetone extracts of the stem was lower than the other extracts.



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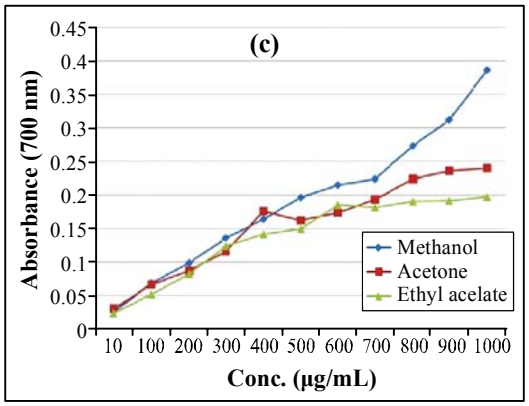

Fig. 1: Graph showing absorbance at 700 nm in reducing power assay (a) Reducing power of leaf extracts (b) Reducing power of stem extracts (c) Reducing power of root extracts

Total flavonoid content and total phenolic content

The results of the evaluation of the total flavonoid content of the examined plant extracts have been presented in Table 4. The total flavonoid content has been expressed as a Quercetin equivalent concentration mg/mL by plotting the Quercetin calibration curve ($y = 0.11568\ x - 0.679$, $r^2 = 0.9887$). The TFC values of all extracts ranged from 2.4 QE mg/mL to 8.96 QE mg/ML. The results show that acetone extracts have high TFC values and ethyl acetate leaves and root have lower values of TFC.

Table 4: TPC and TFC values expressed as gallic acid and quercetin equivalent concentrations

	TPC as GA concentration mg/mL			TFC as quercetin concentration mg/mL		
	Methanol	**Acetone**	**Ethyl acetate**	**Methanol**	**Acetone**	**Ethyl acetate**
Leaves	7.9	6.4	2.11	8.94	5.9	3.11
Stem	4.81	8.32	3.9	7.92	8.96	4.87
Root	5.129	2.632	1.8	3.59	2.94	2.4

The total phenolic content evaluated by the Folin-Ciocalteu method is presented in Table 4. The TPC values are expressed as Gallic acid equivalent concentrations mg/mL. The Gallic acid calibration curve ($y = 0.08418\ x + 0.1169$, $r^2 = 0.99976$) was plotted by taking the absorbance readings for Gallic acid from 0.1 mg/mL to 1.0 mg/mL.

TPC values of the extracts ranged from 1.8 to 8.32 GAE mg/mL. The results show that acetone extracts had high TPC values compared to other extracts. TPC and TFC content measured using the methods followed by Singleton[20] and Kim et al.[1] has revealed high TPC and TFC content in the acetone extracts of the stem and the leaf extracts of methanol. Lower TPC for root samples supported the negative results for phenols in the preliminary screening.

HPLC analysis

The results from the HPLC analysis showed considerable quantities of flavonoids and phenols in these extracts. Phenolic content was high in the methanol leaf extract (6.8 mg/mL) and low in the ethyl acetate leaf extract (0.2 mg/mL). The flavonoid content was high in the methanol root extract (22 mg/mL) and low in the acetone extract of the stem (0.5 mg/mL). Plant samples showed significant quantities of Rutin and Gallic acid. Fig. 2a-2c show the overlay of chromatograms from the HPLC analysis with Rutin, Gallic acid, and Catechol. The HPLC chromatograms showed the large quantities of Gallic acid and Rutin in the *Holoptelea integrifolia* plant extracts. The chromatograms also showed other phenols and flavonoids in all the extracts. TPC and TFC from the HPLC analysis showed the same trends as the values derived from the methods followed by Singleton[20] and Kim et al.[1] Isolation and purification of compounds revealed in the HPLC analysis ascertains the claims of the good antioxidant activity. Antioxidant activities of ethanol extract of stem of *Holoptelea integrifolia* were reported by Saraswathy[22] and this study confirms reported properties across various extracts and in leaves, roots. Leaf and stem extracts had higher anti-oxidant activity, which was supported by higher TPC, TFC values and results from 2,2-DPPH, reducing power assay.

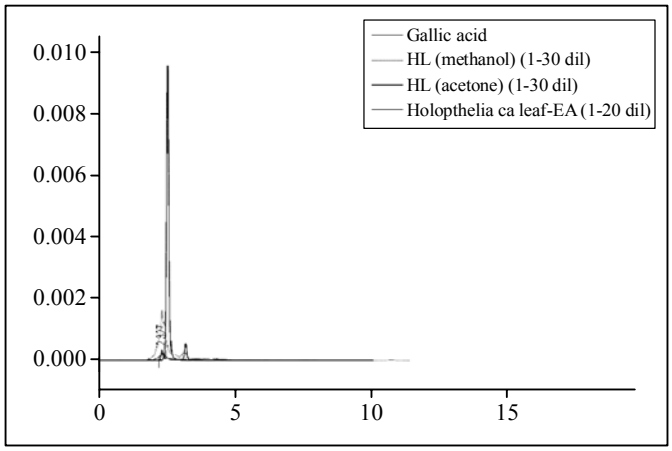

Fig. 2a: HPLC chromatograms at 254 nm for phenols with gallic acid as standard for overlay

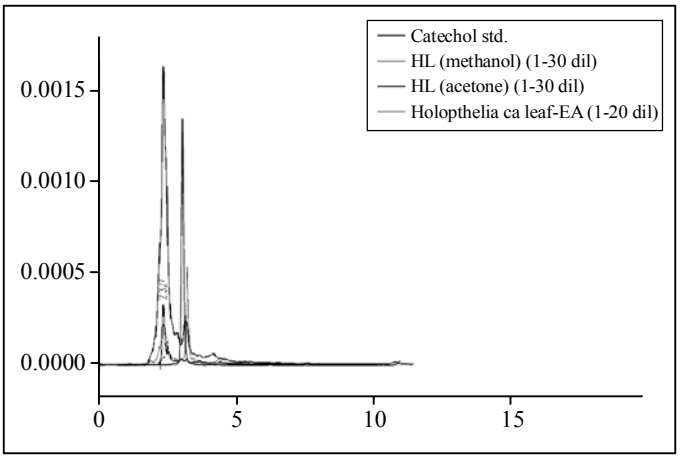

Fig. 2b: HPLC chromatograms at 254 nm for phenols with catechol as standard for overlay

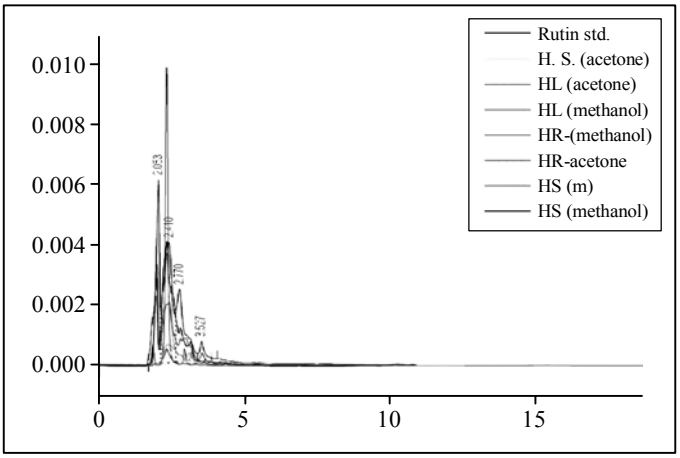

Fig. 2c: HPLC chromatograms at 215 nm for flavonoids with Rutin as standard for overlay

Root extracts showed lesser antioxidant activity compared to leaves and stem. Isolation of other major compounds would help in investigating presence of new compounds in *Holoptelea integrifolia* plant extracts.

CONCLUSION

Leaf and stem extracts had higher anti-oxidant activity, which was supported by higher TPC, TFC values and results from 2,2-DPPH, reducing power assay. Root extracts

showed lesser antioxidant activity compared to leaves and stem. Isolation of other major compounds would help in investigating presence of new compounds in *Holoptelea integrifolia* plant extracts.

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