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PHYTOCHEMICAL ANALYSIS, CHEMICAL CHARACTERIZATION AND ANTIOXIDANT ACTIVITY OF *HELICANTHUS ELASTICA* DESR.

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Abstract:
The objective of present studies deals with the Phytochemical analsis, chemical characterization and antioxidant activity of Helicanthus elastica Desr. Total phenolic content, Total flavonoid content, Total proanthocyanidin content of ethyl acetate fraction was determined. Antioxidant activity was carried out by Nitric oxide scavanging activity, DPPH scavenging activity, reducing power assay and anti-lipid peroxidation activity. Characterization of ethyl acetate fraction of H.elastica carried out by LC-MS/MS method. The HPTLC together with LC-MS/MS study confirm the presence of quercetin and epicatechin in ethyl acetate extract. Quantitative studies reveal that plant contains higher amount of phenolics such as flavonoids and proanthocynidins. Antioxidant activity in various in vitro models demonstrated that extract have significant radical scavenging as well as antioxidant activity which is consistent with its higher flavonoid content.

Key Words: *Helicanthus elastica, ethyl acetate fraction, characterization, flavanoid, antioxidant.*

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INTRODUCTION:

Parasitic plants host relationship was linked to three evolutionary pattern of insect response to the host plants. First, resistance to host chemistry, secondly use of host defence chemicals as feeding cues third is secondary utilization of host defence compounds. Thus chemical compound produced in parasitic plant are an interesting area for phytopharmacological investigation. *Helicanthus* is one of such interesting genus, from literature survey it is evident that no previous work on phytochemistry of this plant is reported. Even as some literature has accumulated on the medicinal properties of Loranthaceae plants as such very little information is available on *Helicanthus elastica* [1-4]. The chemotaxonomic tracing of other members of genus *Loranthus*, endemic to Western Ghat, is expected to yield useful pharmacological activities. In indigenous system of medicine, the leaves of plant is used to check abortion; also in vesicle calculi and kidney affections [5]. Plant has been investigated for its cytotoxic activity [6]. The decoction of plant is used by women as an antifertility agent [5]. The methanolic extract was reported to have potent as anti-inflammatory and anti-arthritic activity in experimental animals [7]. The extract also reported to have potent analgesic and anti-convolsant activity [8]. The plant reported to contain biologically active substances such as flavonoid (quercetin) and some cytotoxic proteins [5-6]. Therefore, present study was investigated phytochemistry of polyphenolics and characterization of this plant. Also, the plant has been investigated for its antioxidant activity.

MATERIALS AND METHODS:

Plant material

The mistletoe, *Helicanthus elastica* Desr. parasitic on *Syzygium cumini* Linn. (Myrtaceae) was collected from Western Ghats near Mahabaleshwar. The plant specimen (Voucher no. LOT-1) was authenticated at Botanical Survey of India, Pune. The dried plant material was powdered by using pulverizer and passed through sieve no. 20. Powder material was stored in airtight container until used with appropriate label.

Extraction of plant material

About 700 gm of powdered material was extracted with methanol (1.5 L×3) by cold maceration method for 24 h. The extract was collected; combined and concentrated using rotary vaccum evaporator which yields (9.48 % w/w) of extract. The aqueous extract was partitioned between ethyl acetate and n-butanol successively.

Quantitative phytochemical screening

Total phenolic content determination

Total phenolic content of ethyl acetate fraction of *Helicanthus elastica* Linn was determined using Folin–Ciocalteu reagent. In this method, the blue colour formed due to the polyphenol present in the extract was measured at 760 nm using UV spectrophotometer. The extract (0.1ml) was mixed with the Folin-Ciocalteu phenol reagent (0.2 ml), water (2 ml) and sodium carbonate (15 % w/v, 1 ml), and absorbance was measured at 760 nm after 2 h incubation period at 50^0C for 10 min. The total phenolic was expressed as µg gallic acid equivalent**.**

Total flavonoid content determination

Total flavonoid content of ethyl acetate fraction of *Helicanthus elastica* Linn was determined using method reported [9]. Sample solution (0.5 ml), ethanol (1.5 ml), Al $(NO_3)_3$ (0.1 ml, 10%), CH_3COOK (0.1 ml, 1 M) and H_2O (2.8 ml) were thoroughly mixed and kept at ambient temperature for 40 min. The absorbance of reaction mixture was measured at 415 nm. Total flavonoid content was calculated according to a standard curve established with quercetin. The total flavonoid was expressed as µg quercetin equivalent.

Total proanthocyanidin content determination

Total proanthocyanidin content of ethyl acetate fraction of *Helicanthus elastica* Linn was determined using method reported [10]. The proanthocyanidins were detected in TLC test; therefore total proanthocyanidins were determined using vanillin-H_2SO_4 method. Briefly, 1 ml of extract (100µg/ml) was mixed with 2 ml of freshly prepared vanillin solution (1% vanillin in 70% of H_2SO_4) and maintained for 15 min at 20 ^{0}C. The absorbance was measured at 500 nm. Calibration curved was drawn using aqueous solution of epicatechin (8-40 µg/ml) as a reference standard. The results were given in table 3. All experiments were performed in triplicate and mean values were used for calculation. Spectrophotometric determinations were performed using on Shimadzu UV-2450 spectrophotometer.

Antioxidant activity

Various methods have been used to monitor and compare the antioxidant activity of foods. These analytical methods measure the radical scavenging activity of antioxidants against free radicals like DPPH, hydroxyl radical or peroxyl radical (ROO), super oxide anion radical O_2, nitric oxide radical. The various methods used to measure the antioxidant

activity of various extracts compounds can give varying results depending on the specificity of the free radical being used as reactant.

Nitric oxide scavenging activity

The nitric oxide scavenging activity was determined according to method reported by [11]. Nitric oxide radicals were generated from sodium nitroprusside solution at physiological pH. Sodium nitroprusside (1 ml, 10 mM) was mixed with 1ml extract (10-100µg/ml) in phosphate buffer (pH 7.4). The mixture was incubated at 25^0C for 150 min. To 1 ml of incubated solution, 1 ml of Griess reagent (α-naphthyl-ethylenediamine dihydrochloride 0.1% in water and sulfanilamide 5% in H_3PO_4) was added and absorbance was read at 546 nm. The same reaction mixture without the extract or sample but equivalent amount of distilled water was served as control.

DPPH scavenging activity

The DPPH radical scavenging activity of the ethyl acetate fraction was performed in flat bottom polystyrene 96-well micro titer plates using reported method. with some modifications. Briefly, to 100 µl of extract (10-100µg/ml) in ethanol were added 25µl of DPPH (1 mM in ethanol) and 75 µl of ethanol to give a final volume of 200µl. The resultant mixture was briefly shaken and maintained at room temperature in the dark for 30 min. At the end of this period the absorbance of mixture was measured at 517 nm using Microtiter Plate Reader (PowerWave XS, Bio-tek, USA). The percent antiradical activity was calculated by using following formula. The antioxidant activity of the extract was expressed as IC_{50} value was defined as concentration (in µg/ml) of extracts that inhibits the formation of DPPH radicals by 50 %.

Determination of reducing power assay

To 1ml of different concentrations of extract (10-100µg/ml), 2.5ml of phosphate buffer (0.25 M) and 2.5 ml of potassium ferricyanide (1% w/v) were added. The mixture was incubated at 50^0C for 20min. After incubation 2.5ml of TCA was added to the reaction mixture, which was then centrifuged at 3000 rpm for 10 min. Then 2.5ml of supernatant was taken and to it add 2.5ml of distilled water and 0.5ml of ferric chloride solution (0.1% w/v) and the absorbance was measured at 700 nm.

Anti-lipid peroxidation activity using rat brain homogenate

To the 100µl of rat brain homogenate 10µl of different concentration (10-100µg/ml) of samples were added. Lipid per oxidation was stimulated by adding 10µl of 100µM Fe^{3+} (ammonium ferric sulphate) solution and the mixture was incubated for 30min. at 37°C.

The reaction was stopped precipitating the proteins using sodium dodesyl sulphate (SDS) solution. Further oxidation was stopped by addition of 750µl of 20%w/v acetic acid (adjusted to pH 3.5 using NaoH) solution. The precipitated proteins were then removed by centrifugation at 10,000 rpm for 15min. 500µl of the clear supernatants were heated in glass tubes with an equal volume of TBA solution (0.8%w/v) at 95 ºC for 30min. Samples were cooled on ice and 100µl of each sample was pipette into 96 well micro titer plate and abs

Characterization of ethyl acetate fraction of *H.elastica* by LC-MS/MS

Mass Spectrometry

The MS spectra were obtained using, chemical ionisation (APCI), at electron energy of 80V, at source temperature 190°C. The ion energy at 1.7V and at pressures < 1.7e – 4 Torr and 1.1e – 5 Torr penning was used for MS analysis.

Quantification of epicatechin by HPTLC

Quantification of epicatechin in ethyl acetate fraction of *Helicanthus elastica* was carried out by using HPTLC (Camag, Switzerland) using pre-coated silica gel plates (Merck).The chromatogram was evaluated densitometrically using win CAT software and the proanthocyanidins were traced by using Vanillin-HCl.

Quantification of Quercetin by HPTLC:

Quantification of Quercetin in Ethyl acetate fraction of *Helicanthus elastica* was carried out by using HPTLC (Camag, Switzerland) using pre-coated silica gel plates (Merck).The chromatogram was evaluated densitometrically using win CAT software and the flavonoids were traced by using Natural product reagent.

RESULTS AND DISCUSSION:

Total phenolic content determination

Calibration curve of standerd Gallic acid was obtained where graph was plotted by keeping concentratuin in x- axis and absorbance in y axis as shown in figure 1. The total phenolic was expressed as µg gallic acid equivalent was given in table 1**.**

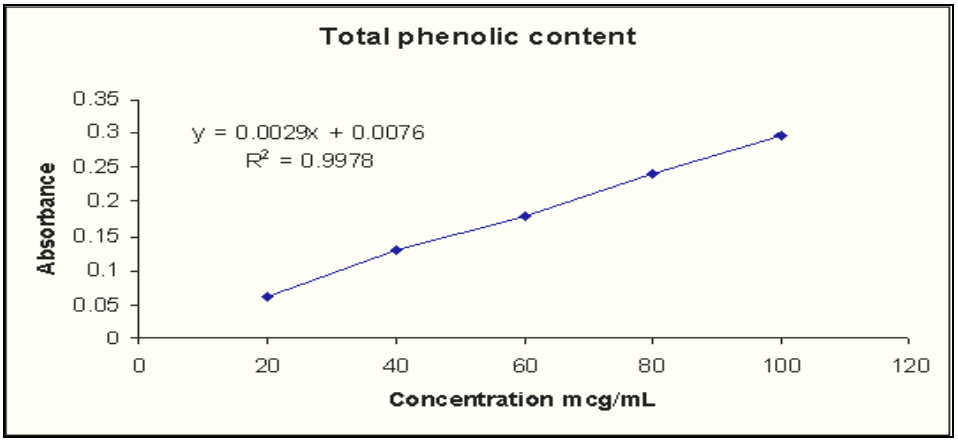


Fig. 1: Calibration curve for gallic acid

Table 1: Total phenolic content

Sample HE-J Concentration (µg/mL)	Total Phenolics (µg gallic acid equivalent)			Mean±SEM
	I	II	III	
100	80.65	80.10	80.41	80.38±0.15
200	161	160.79	161.24	161±0.13
400	314.51	315.62	314.34	314.8±0.4

Values are expressed as mean ± SEM, n=3.

Total flavonoid content determination

Calibration curve of standard quercetin was obtained where graph was plotted by keeping concentratuin in x- axis and absorbance in y axis as shown in figure 2 . The total phenolic was expressed as µg quercetin equivalent was given in table 2.

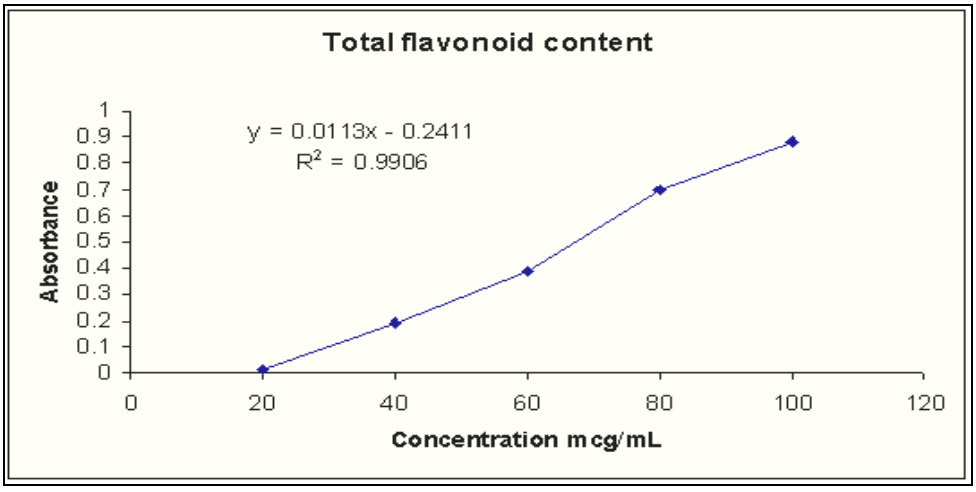


Fig. 2: Calibration curve for quercetin

Table 2: Total flavonoid content

Sample HE-J Concentration (µg/mL)	Total Flavonoids (µg Quercetin equivalent)			Mean± SEM
	I	II	III	
100	52.44	53.28	52.36	52.69± 0.21
200	102.66	103	103.07	102.91 ± 0.12
400	207.35	207.23	208.15	207.5 ±0.28

Values are expressed as mean ± SEM, n=3.

Total proanthocynidin content determination

Calibration curve of standard epicatechin was obtained where graph was plotted by keeping concentration in x- axis and absorbance in y axis as shown in figure 3 The total phenolic was expressed as µg quercetin equivalent was given in table 3

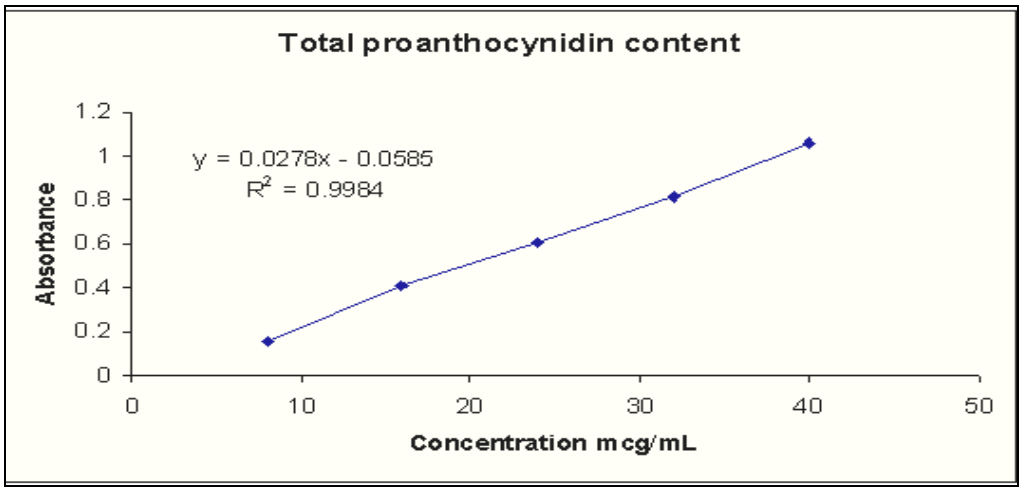


Fig. 3: Calibration curve for epicatechin

Table 3: Total proanthocynidin content

Sample Concentration HE-J (µg/mL)	Total Proanthocynidins (µg epicatechin equivalent)			Mean± SEM
	I	II	III	
100	24.61	24.68	25.00	24.76 ± 0.12
200	49.27	49.66	49.35	49.43± 0.11
400	102.85	102.52	102.69	102.68 ± 0.055

Values are expressed as mean ± SEM, n=3.

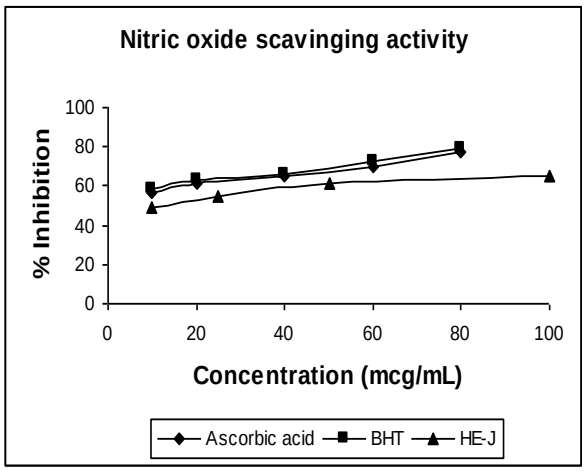
Antioxidant activity of extract

The results of antioxidant activity tested in various *in vitro* models demonstrated that extract have concentration dependent radical scavenging as well as antioxidant activity.

The results of antioxidant activity in various *in vitro* models demonstrated that extract have significant radical scavenging as well as antioxidant activity which is consistent with its higher flavonoid content [12].

Table 4: IC_{50} value of extract in different *in vitro* antioxidant models

Sample	IC_{50}			Reducing power assay Absorbance Mean ± SEM at10 µg/ml
	Nitric oxide scavenging activity	DPPH scavenging activity	Anti-lipid peroxidation activity	
Ascorbic acid	8.8	9.27	9.39	0.1875 ± 0.0006
BHT	8.48	7.89	7.86	0.2032 ± 0.0006
HEJ	10.18	8.17	8.18	0.3216 ± 0.0007



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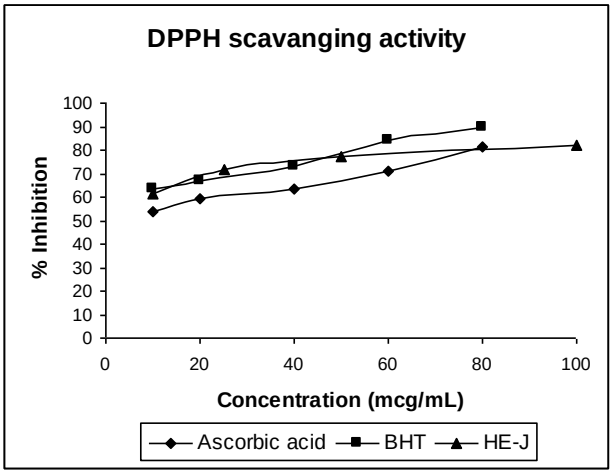

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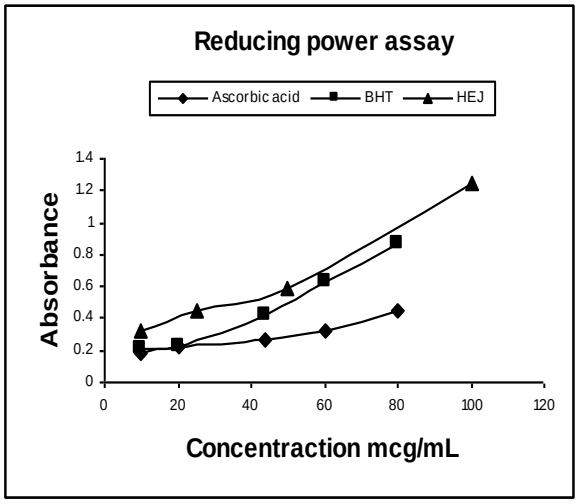

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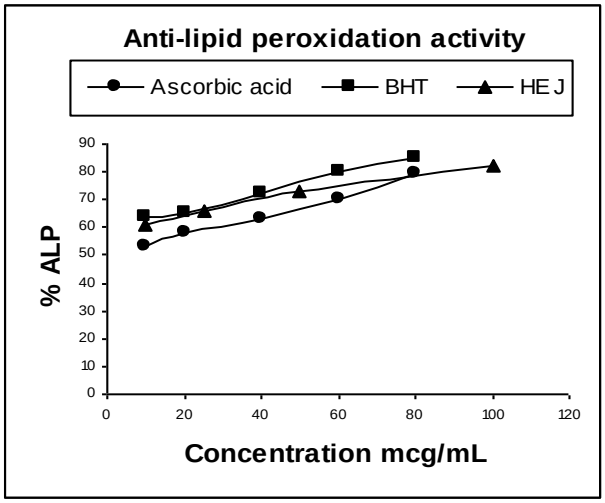
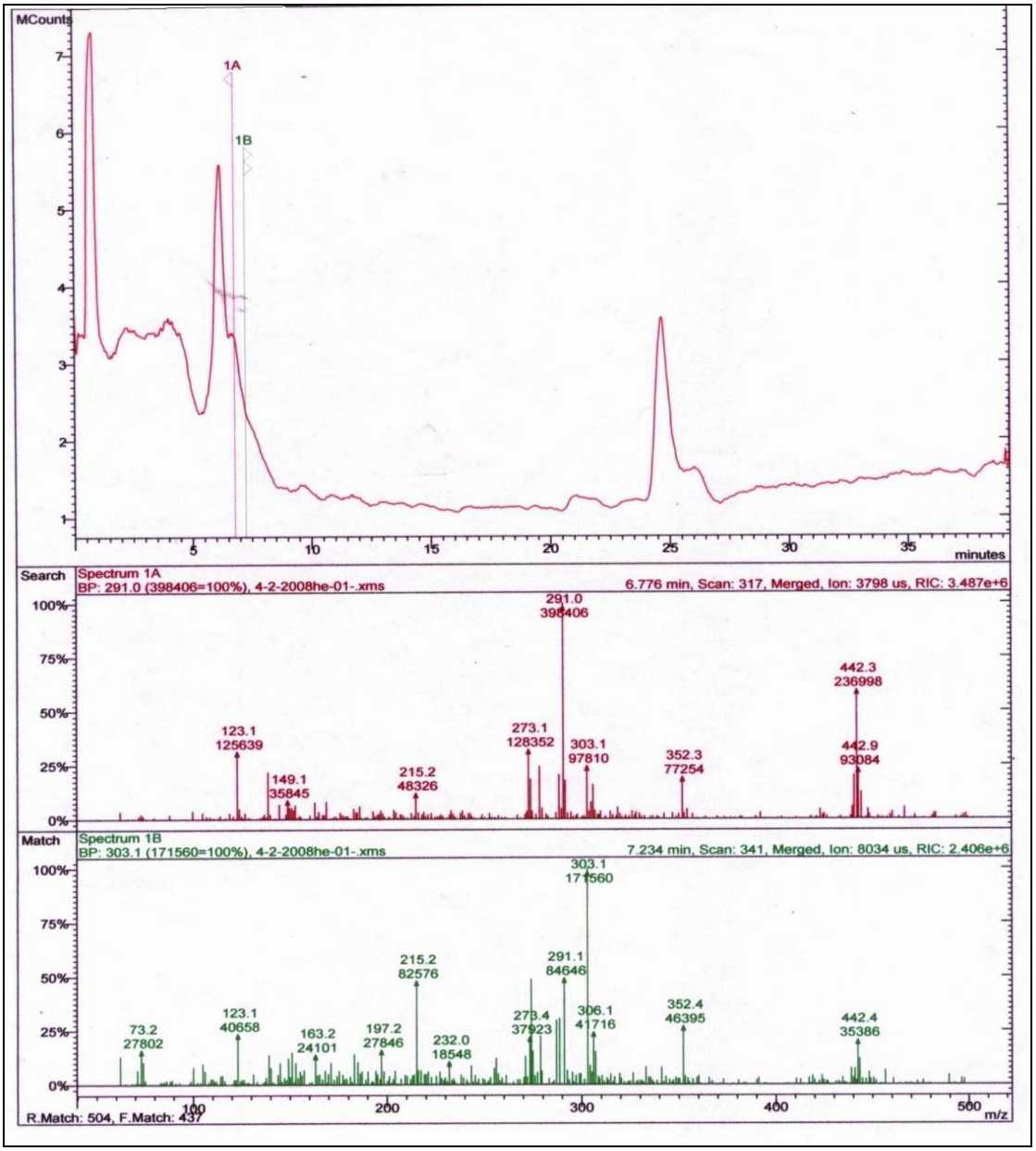


D

Fig. 4: A. Nitric oxide scavenging activity
B. DPPH scavenging activity
C. Reducing power assay
D. Anti-lipid peroxidation activity

Characterization of ethyl acetate fraction of *H.elastica* by LC-MS/MS Mass Spectrometry

The results of Characterization of ethyl acetate fraction of *H.elastica* by LC-MS/MS Mass Spectrometry is given in following table and graph



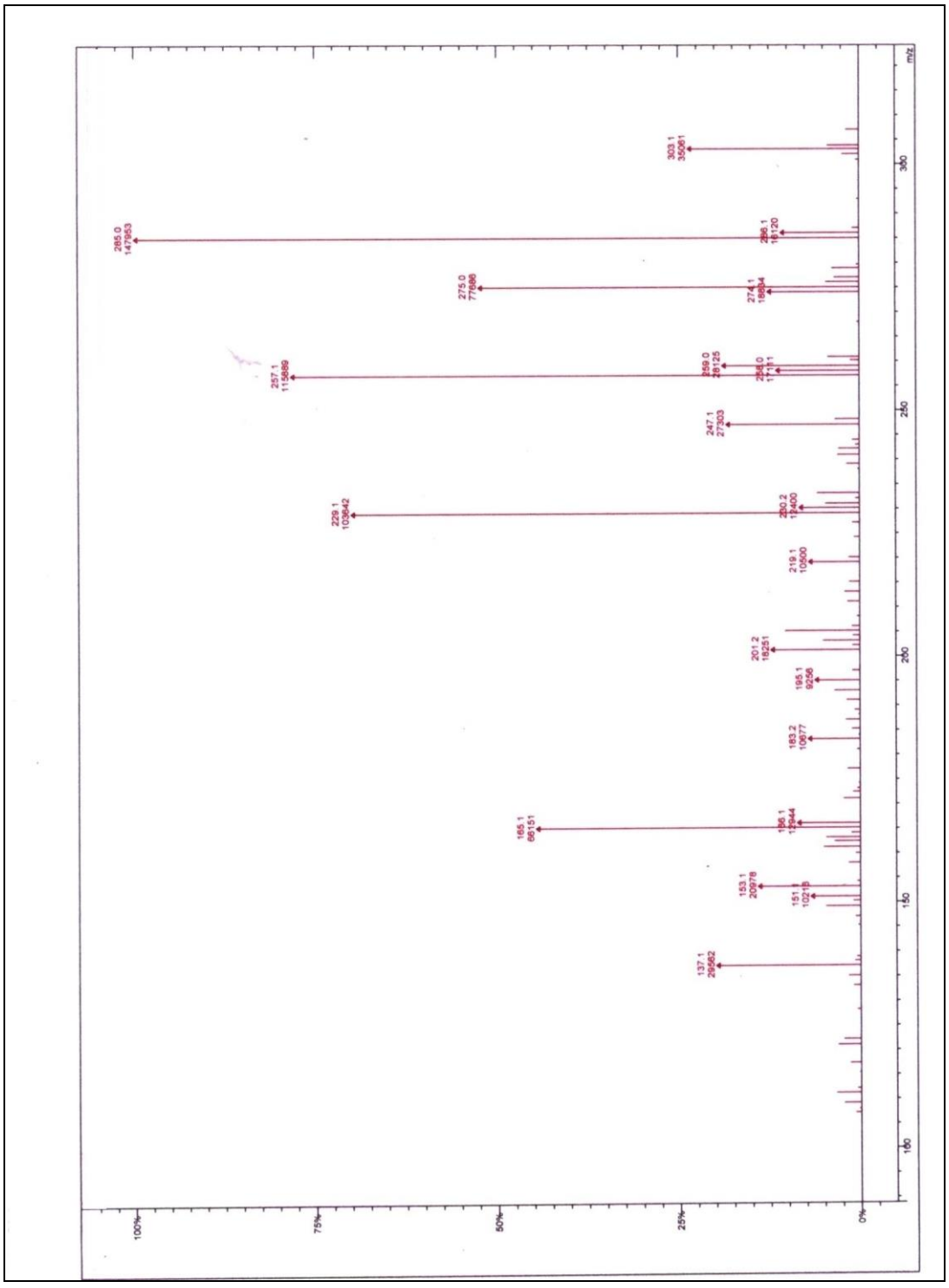


Fig. 5: MS/MS of compound 1

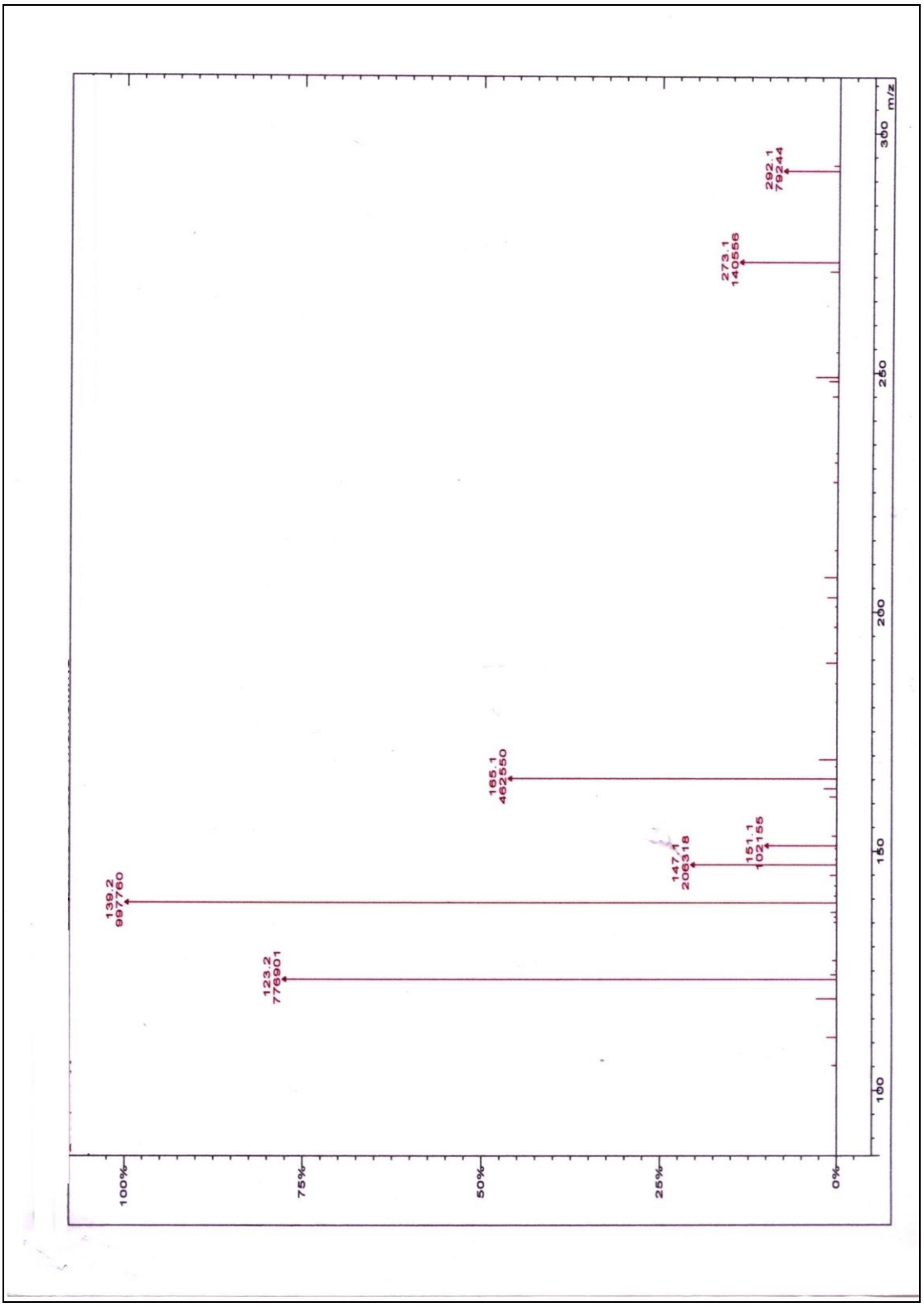


Fig. 6: MS/MS of compound 2

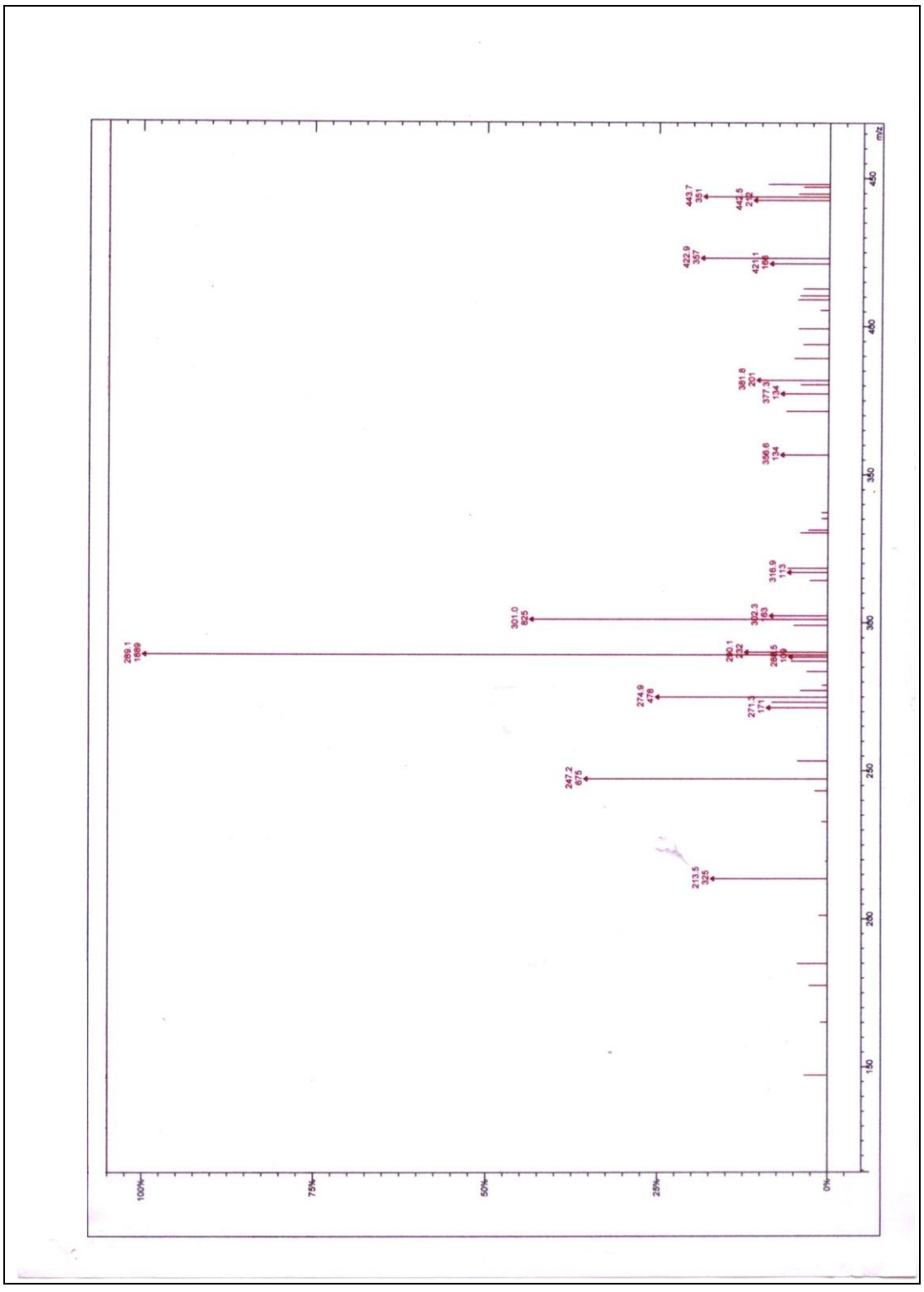


Fig. 7: MS/MS of compound 3

MS fragmentation pattern of some flavonoids

Table 6: MS fragmentation pattern of some flavonoid aglycones

	MS/MS of standard Quercetin
1.	303
2.	285
3.	257
4.	229
5.	275
6.	247
7.	165
8.	137
9	153
10	149

Sr no.	MS/MS of isolated compound no.1
1.	303.1
2.	285
3.	275
4.	257.1
5.	229.1
6.	247.1
7.	165.1
8.	153.1
9	137.1

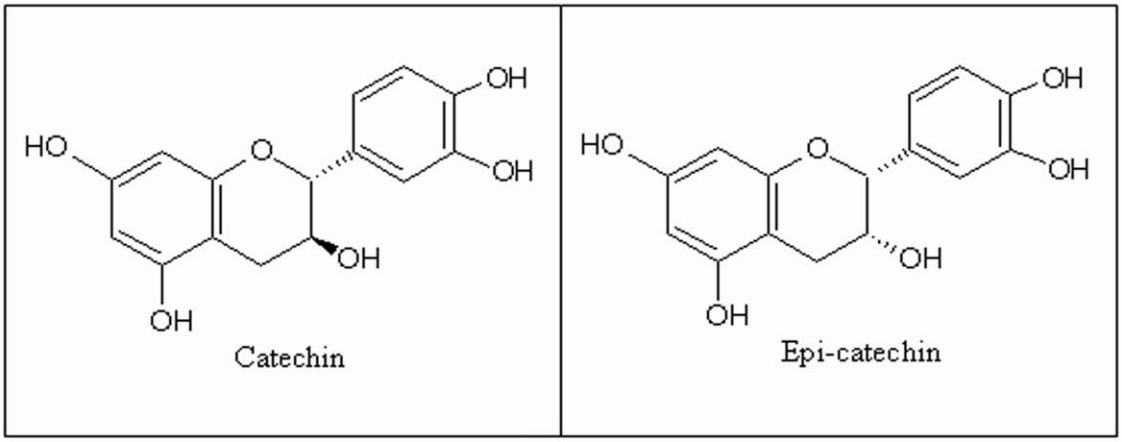
Table 7: MS fragmentation pattern of some flavanols

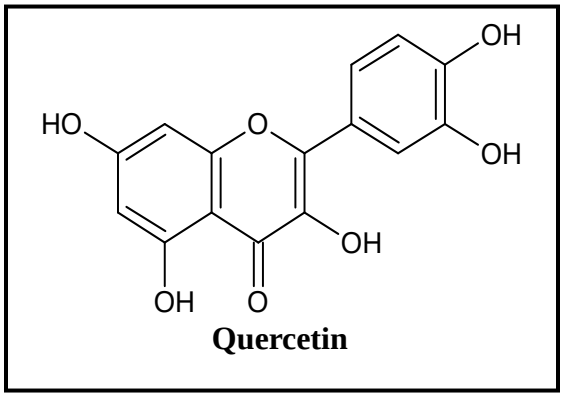
Sr no.	MS/MS of standard Epicatechin
1.	291
2.	273
3.	249
4.	123
5.	139
6.	165

Sr no.	MS/MS of isolated compound no. 2
1.	292.1
2.	273.1
3.	165.1
4.	151.1
5.	139.2
6.	123.2

The LC-MS and MS-MS data thus confirm the presence of quercetin and epicatechin and / or catechin in ethyl acetate fraction of *H. elastica*.

Structure of flavonoids characterized from ethyl acetate fraction of *H. elastica*





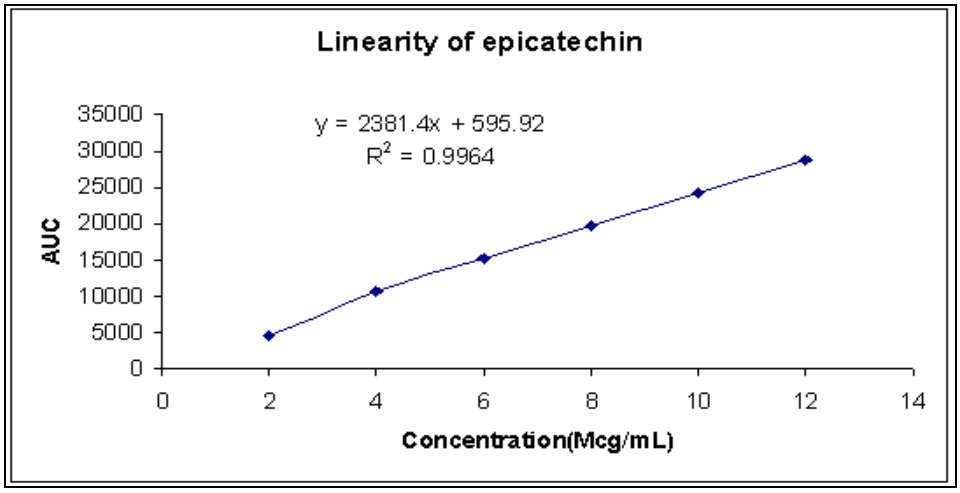


Fig. 8 Linearity of epicatechin

Table 8: R f values and peak areas in HPTLC of epicatechin

Track no.	Standard	Conc.(µg/ml)	Max. Rf	AUC
1	Epicatechin	2	0.64	4537.9
2		4	0.64	10790
3		6	0.65	15251.1
4		8	0.66	19871.7
5		10	0.67	24292.4
6		12	0.67	28852.3

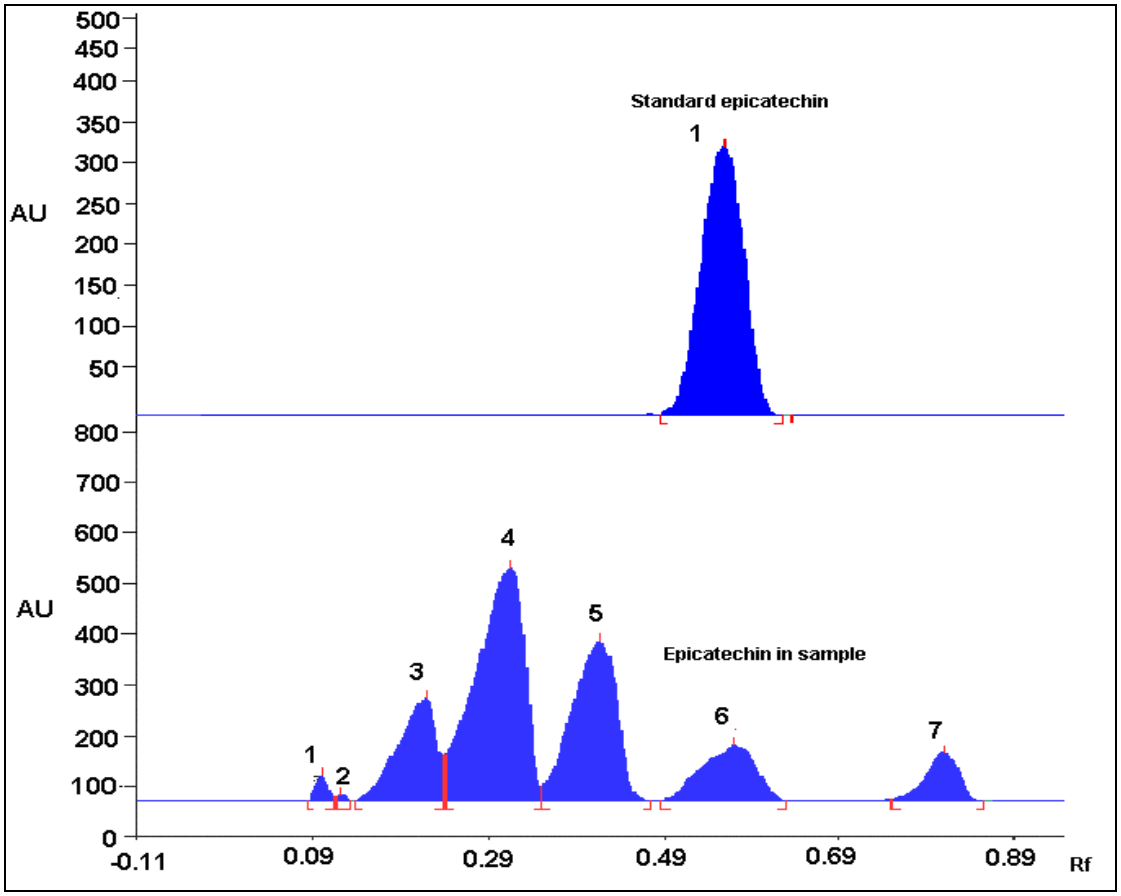


Fig. 9: HPTLC of ethyl acetate fraction of *H. elastica* for epicatechin

Table 9: R f values and peak areas for epicatechin in HPTLC of ethyl acetate fraction of *Helicanthus elastica*

Track no.	Sample	Conc.(µg/ml)	Max. Rf	AUC	Mean ±SEM
1	HE J	10	0.63	5963.9	5715.23± 128.87
2	HE J	10	0.64	5649.6	
3	HE J	10	0.66	5532.2	

Table 10: Epicatechin in ethyl acetate fraction of *Helicanthus elastica*

Sr.No.	Total Proanthocyanidin Content (µg %)	Epicatechin (µg %)
1	24.76	17.3

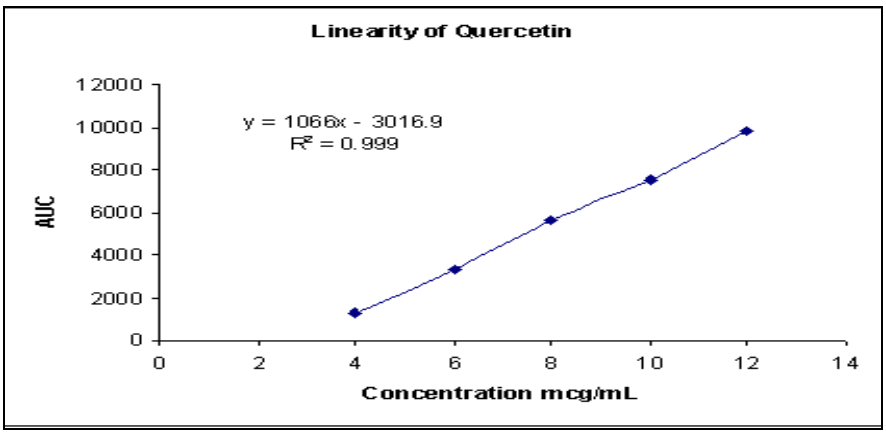


Fig.10: Linearity of quercetin

Table 11: R $_f$ values and peak areas in HPTLC of quercetin

Track no.	Standard	Conc.(µg/ml)	Max. Rf	AUC
1	Quercetin	4	0.85	1301.8
2		6	0.85	3296.1
3		8	0.84	5600.1
4		10	0.83	7497.2
5		12	0.84	9861.6

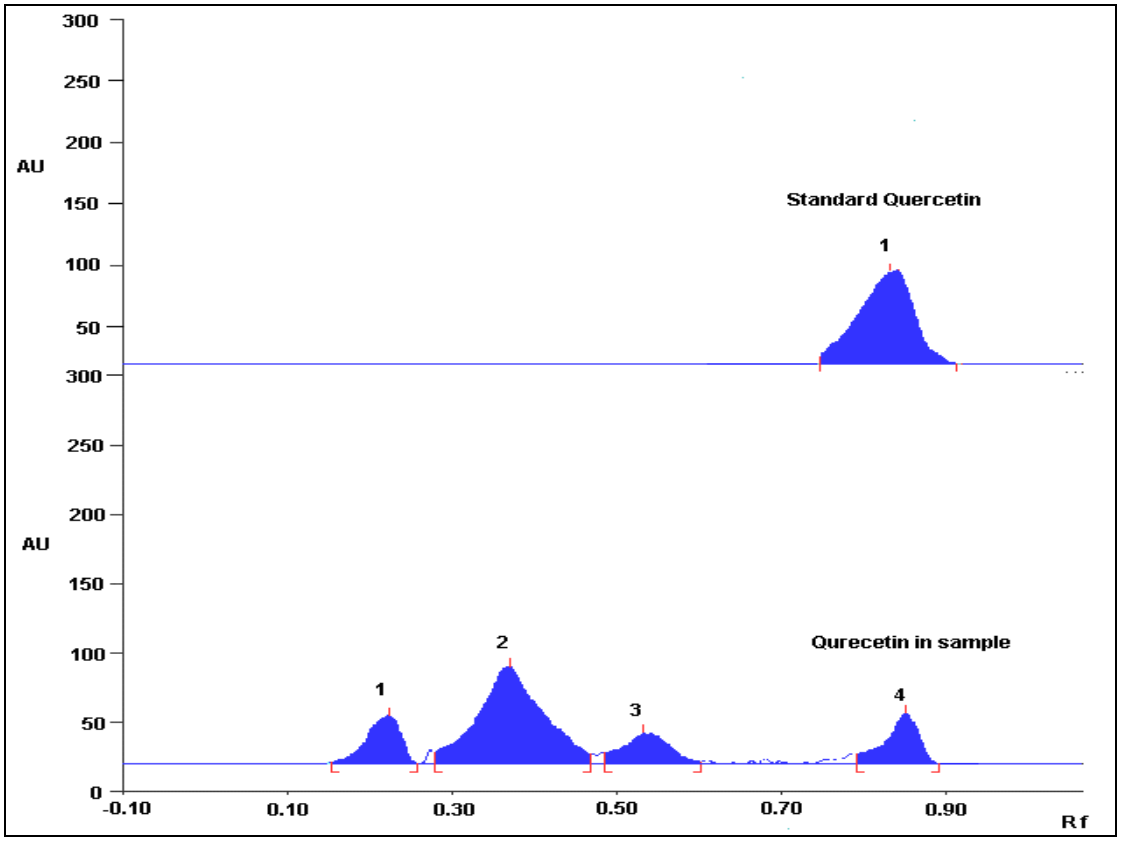


Fig. 11: HPTLC of ethyl acetate fraction of *Helicanthus elastica* for quercetin

Table 12: R $_f$ values and peak areas for quercetin in HPTLC of ethyl acetate fraction of *Helicanthus elastica*

Track no.	Sample	Conc.(µg/ml)	Max. R_f	AUC	Mean ±SEM
1	HE J	10	0.89	1415.6	1468.6± 31.75
2	HE J	10	0.89	1465	
3	HE J	10	0.88	1525.4	

Table 13: Quercetin in ethyl acetate fraction of *Helicanthus elastica*

Sr.No.	Total Flavonoid Content (µg%)	Quercetin (µg%)
1	52.69	19.58

CONCLUSION:

Quantitative studies reveal that plant contains higher amount of polyphenolics such as flavonoids and proanthocynidins. The HPTLC together with LC-MS/MS study confirm the presence of quercetin and epicatechin in ethyl acetate extract. The results of antioxidant activity tested in various *in vitro* models demonstrated that extract have concentration dependent radical scavenging as well as antioxidant activity. The results of antioxidant activity in various *in vitro* models demonstrated that extract have significant radical scavenging as well as antioxidant activity which is consistent with its higher flavonoid content

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