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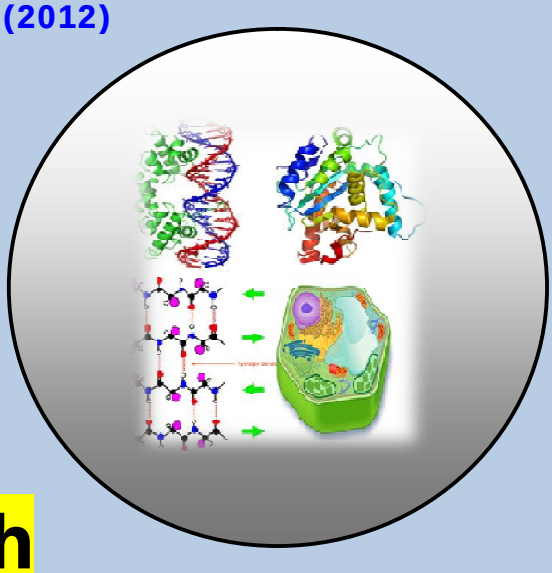

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HPLC Analysis of *in vitro* Raised Plants of *Murraya koenigii*

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ABSTRACT

The methanolic extracts of different in vitro raised plant materials of Murraya koenigii were run in HPLC using the Pronto SIL C18 ace EPS Column for qualitative and quantitative analysis of different methanolic extracts such as ADVS, ADVR, AXLS, AXLR. In present investigation the 100µl extracts was passed through the C-18 Column of HPLC which allowed the separation of many compound present in the sample. Characteristic HPLC profiles of methanolic extracts of some standard phenol (gallic acid), flavonoid (quercetin) and other compound (citric acid, ascorbic acid and BHT) having well known antioxidant activities were observed. The retention time, area of peak and percentage amount of compound available in the sample is shown in Tables. The Peak of chromatogram for ascorbic acid, BHT, citric acid, quercetin and gallic acid are shown. The data of chromatogram revealed that the absorption of 2498.68mAU (ascorbic acid), 385.63mAU (BHT), 219.47mAU (citric acid), 16.11mAU (quercetin) and 20.91mAU (gallic acid). The retention time, start time and area of peaks of fractioned compounds. HPLC analysis for quantitative analysis of phenols and flavonoids was conducted in order to separate the antioxidative components from in vitro raised plants parts of Murraya koenigii and compared them with the peaks of available commercial standard antioxidant compounds such as citric acid, ascorbic acid, BHT and quercetin. In the HPLC chromatograms the antioxidative compounds were identified by their Retention time by spiking with standards under the same condition. The compound number 5 was found in maximum quantity (42.24%) in the AXLR extract which has start time 3.81 minute and retention time 3.93 minute. The Retention time of peak number 3 (3.86minute) of (ADVS) is nearly similar with the Retention time of BHT (3.85minute) which has 51.69mAU absorption.

***Key Words:** Murraya koenigii, Flavonoid, Antioxidant, Phytochemicals, Citric acid, Ascorbic acid, BHT, Quercetin, HPLC, ADVS, ADVR, AXLS, AXLR, Pharmacological properties, Hyperglycaemia.*

INTRODUCTION

Plants play a dominant role in the introduction of new therapeutic agents, and also drugs from the higher plants continue to occupy an important niche in modern medicine. Many compounds used in today's medicine have a complex structure, and synthesizing these bioactive compounds chemically at a low price is not easy**.** With deforestation, medicinal wealth is rapidly lost, such that many valuable plants are threatened with extinction. Pharmaceutical companies depend largely upon materials procured from naturally occurring stands that are being rapidly. Medicinal plants are sources of important therapeutic aid for alleviating human ailments. With increasing realization of the health hazards and toxicity associated with the indiscriminate use of synthetic drugs and antibiotics, interest in the use of plants and plant-based drugs has revived throughout the world. However, a large number of medicinal plants remain to be investigated for their possible pharmacological value. Most of the pharmaceutical industry is highly dependent on wild populations for the supply of raw materials for extraction of medicinally important compounds. Due to a lack of proper cultivation practices, destruction of plant habitats, and the illegal and indiscriminate collection of plants from these habitats, many medicinal plants are severely threatened. Advanced biotechnological methods of culturing plant cells and tissues should provide new means of conserving and rapidly propagating valuable, rare, and endangered medicinal plants. Plants are a tremendous source for the discovery of new products of medicinal value for drug development. Today several distinct chemicals derived from plants are important drugs currently used in one or more countries in the world. Many of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. The evolving commercial importance of secondary metabolites has in recent years resulted in a great interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology. Plant cell culture technologies were introduced at the end of the 1960's as a possible tool for both studying and producing plant secondary metabolites. Different strategies, using an *in vitro* system, have been extensively studied to improve the production of plant chemicals. The focus of the present review is the application of tissue culture technology for the production of some important plant pharmaceuticals. Diabetes mellitus is a syndrome resulting from a variable interaction of hereditary and environmental factors and characterized by depleted insulin secretion, hyperglycaemia and altered metabolism of lipids, carbohydrates and proteins, in addition to damaged b-cells of pancreas and an increased risk of complications of vascular diseases. A number of pharmacological and chemical agents act as diabetogenic and produce variety of diabetic complications. Alloxan induction of diabetes is an experimental model widely used to study glycemic and lipidemic changes in plasma. Many species of plants and herbs are known to act as anti diabetic agents, but only a few of them have been investigated. *Murraya koenigii* (Rutaceae), popularly known as curry leaf is a medicinal plant that grows throughout the greater parts of India and SEA.

This species is known to possess anti-inflammatory, antidysenteric, antioxidant, antidiabetic and diverse pharmacological properties. Several studies also shown that curry leaf decreased blood glucose significantly in different animal models. *Murraya koenigii* leaves have been shown to prevent hyperglycemia and pancreatic damage induced by alloxan in rats. Dietary supplement with curry leaves has been shown to reduce total serum cholesterol, LDL + VLDL, increased HDL, decreased the release of lipoproteins into circulation and increased catalase activity in rats. The present study was carried out in Sprague-Dawley rats to explore the effects of *Murraya koenigii* aqueous leaf extract on plasma lipid profile changes associated with diabetes.

MATERIAL AND METHODS

HPLC Analysis

A duplicate of 15mg of freeze-dried sample was extracted in 15ml absolute methanol for one hour at room temperature. The filtrated samples were stored at –23 °C and used for HPLC analyses by KNAUER ASI ASIG**.** HPLC fingerprint is a powerful approach for the rapid identification of phytochemical constituents in botanical extracts, and it can be used to avoid the time-consuming isolation of all the compounds to be identified**.** Analytical studies for the determination of different compounds in different extracts of *Murraya koenigii* the chromatographic method HPLC was used because individual compounds can be separated and determined in one assay procedure. Using these analytical methods, the selection and modification for the chromatographic conditions were focused on the stationary phase, mobile phase, and the detector. C-18 or ODS column were used for analysis of extracts. Computer-based optimization programs have been followed in the wake of microcomputers. The concentration of the polymer peaks was quantified in mg QE g^{-1} DW by comparison to peaks of some standard antioxidant compounds such as gallic acid, BHT, ascorbic acid, qurcetine, and citric acid. Methanolic extracts of leaves and roots of tissue-cultured plants were prepared and 100µl extract was injected. Injection valves have been used successfully for sample delivery valves in HPLC. They ensure precise sample delivery in both partial and full sample loop-filling modes. In the LOAD position, the sample loop is filled; when switched to the INJECT position, the sample is injected. The eluent passes through the sample loop in the opposite direction to minimize band broadening. This is especially advantageous when sample loops are partially filled. When using partial loop filling, the highest degree of accuracy is obtained by filling less than 50% of the volume of the sample loop. Upon switching the valve into the INJECT position, the resulting reversal of the flow direction within the loop ensures that the sample is transferred completely into the column. When using full loop injection technique however, the highest degree of accuracy can be achieved by overfilling the loop three times. In this case, the eluent is completely pushed out of the loop by the sample and the reproducibility of the injection volume no longer depends on factors such as peak broadening or dispersion.

Sample loops are available for the analytical field in volume sizes ranging from 2 - 2500µl in stainless steel and PEEK. For preparative HPLC applications, sample loops are also available in stainless steel and PEEK in volumes up to 45 ml. The Pronto SIL C18 ace-EPS belongs to the new group of stationary RP-supports with Polar embedded groups. The packing is very stable over a wide pH range (1-10). In addition, it offers a maximum of hydrophobicity combined with a maximum of polar selectivity. The silanophilic activity of the support is very low. Ultra strong basic compounds such as amitriptyline can be eluted from the column at neutral pH values with excellent symmetrical peak shapes. The main application area of these packings is the pharmaceutical industry, where analytes often have basic or acidic groups. For the separation of these compounds these supports exhibit an enhanced polar selectivity. That means: In comparison to a classical bonded C18 column acidic compounds show a higher retention whereas basic compounds show a slight decrease of retention on an embedded polar column. The C18 ace-EPS - bonding type is available in several particle and pore sizes.

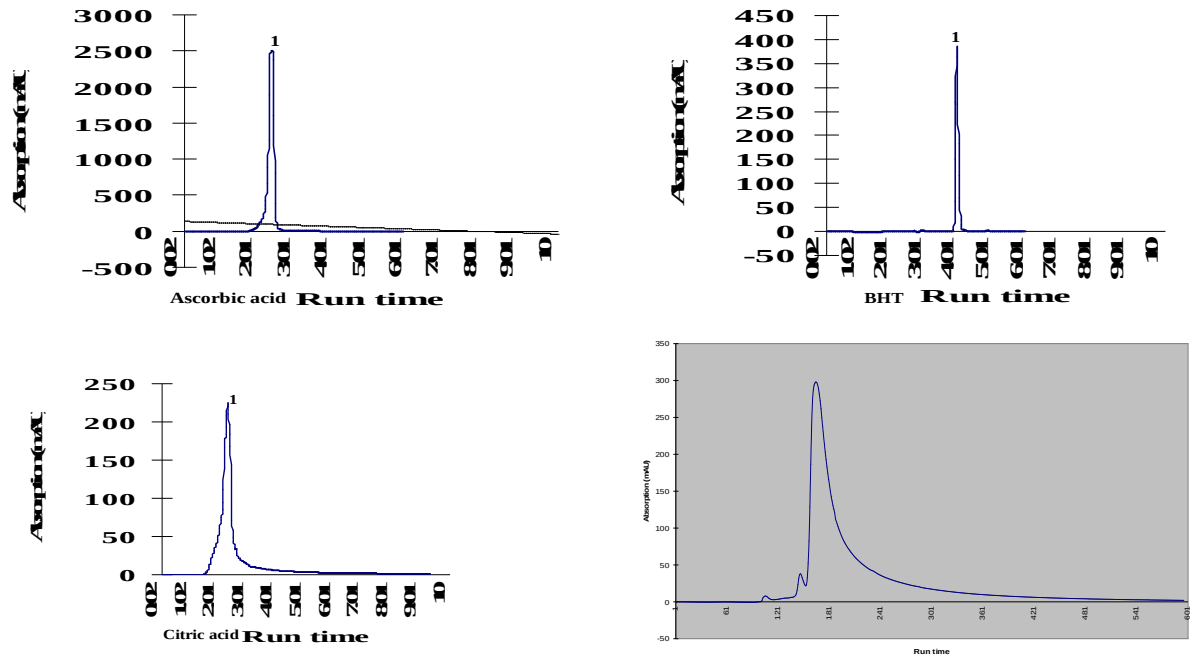

Figure. 1-4 HPLC profile of methanolic extracts of Standard antioxidants

(RESULTS) High performance liquid chromatography (HPLC) Analysis

The methanolic extracts of different plant materials were run in HPLC using the Pronto SIL C18 ace EPS Column for qualitative and quantitative analysis of different methanolic extracts such as ADVS, ADVR, AXLS, AXLR. In present investigation the 100µl extracts was passed through the C-18 Column of HPLC which allowed the separation of many compound present in the sample.

Characteristic HPLC profiles of methanolic extracts of some standard phenol (gallic acid), flavonoid (quercetin) and other compound (citric acid, ascorbic acid and BHT) having well known antioxidant activities were observed. The retention time, areas of peak and percentage amount of compound available in the sample are shown in Table-1. The peak of chromatogram for ascorbic acid (**Figure-1**), BHT (**Figure-2**), citric acid (**Figure-3**), quercetin (**Figure-4**) are shown. The data of chromatogram revealed that the absorption of 2498.68mAU (ascorbic acid), 385.63mAU (BHT), 219.47mAU (citric acid), 16.11mAU (quercetin) and 20.91mAU (gallic acid). The retention time, start time and area of peaks of fractioned compounds are shown in **Table-1**.

Table 1. Peak identification of methanolic extract of standard antioxidants.

Standards	Ret. time [min]	Start [min]	End [min]	Area [m AU*min]	Height [m AU]	% Area	% Height	Width [min]
Ascorbic acid	2.32	1.503	2.772	481.382	2498.681	100	100	0.158
BHT	3.85	3.740	4.442	38.330	385.634	100	100	0.092
Citric acid	2.28	1.434	3.069	80.957	219.477	100	100	0.264
Qurcetine	2.53	2.351	3.069	3.2647	16.115	100	100	0.164
Gallic acid	2.57	2.472	2.756	1.4629	20.913	100	100	0.060

AXLR

The chromatogram of roots of AXLS of *Murraya koenigii* showed total six peaks (**Table:-2 & Figure:-5**)**.** The compound number 5 was found in maximum quantity (42.24%) in the AXLR extract which has start time 3.81 minute and retention time 3.93 minute. The compound separated at peak number 1 was found minimum quantity (5.91%) in the extract which has the retention time 2.00 minute.

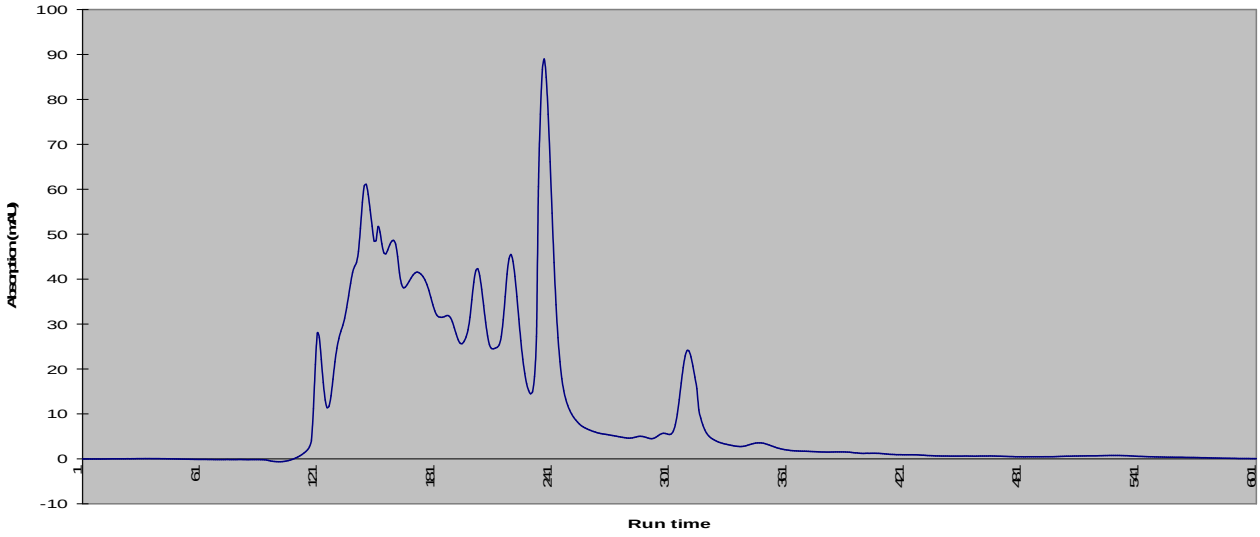

Figure 5. HPLC profile of methanolic extract of Root of AXLR.

Table. 2 Peak identification of methanolic extract of AXLR.

Peak No.	Ret. time [min]	Start [min]	End [min]	Area [m AU*min]	Height [m AU]	% Area	% Height	Width [min]
1	2.00	1.934	2.084	1.453	21.426	5.919	11.729	0.067
2	2.41	2.118	2.485	3.386	18.865	13.792	10.327	0.178
3	3.36	3.235	3.502	2.448	19.124	9.969	10.468	0.109
4	3.65	3.502	3.802	3.832	27.479	15.612	15.042	0.122
5	3.93	3.819	4.269	10.373	76.413	42.247	41.829	0.131
6	5.15	5.003	5.570	3.059	19.371	12.460	10.603	0.150

ADVR

The methanolic extract of roots of ADVR was analysed. The chromatogram of ADVR extract showed that five peaks were detected and the peak number 4 showed the maximum (32.90%) percent whereas the compound of peak number first was present in minimum (4.33%) quantity (**Figure:-6**). The retention time and start time of the compound in HPLC is presented in **Table:-3**. Amongst the five compounds separated in ADVR extract, the Retention time of compound of peak number 2 (2.31minute) with 11.73mAU absorption resembles with the peak of standard ascorbic acid. The percentage area of this compound of peak number 2 is 6.81% which has the retention time 2.31minute.

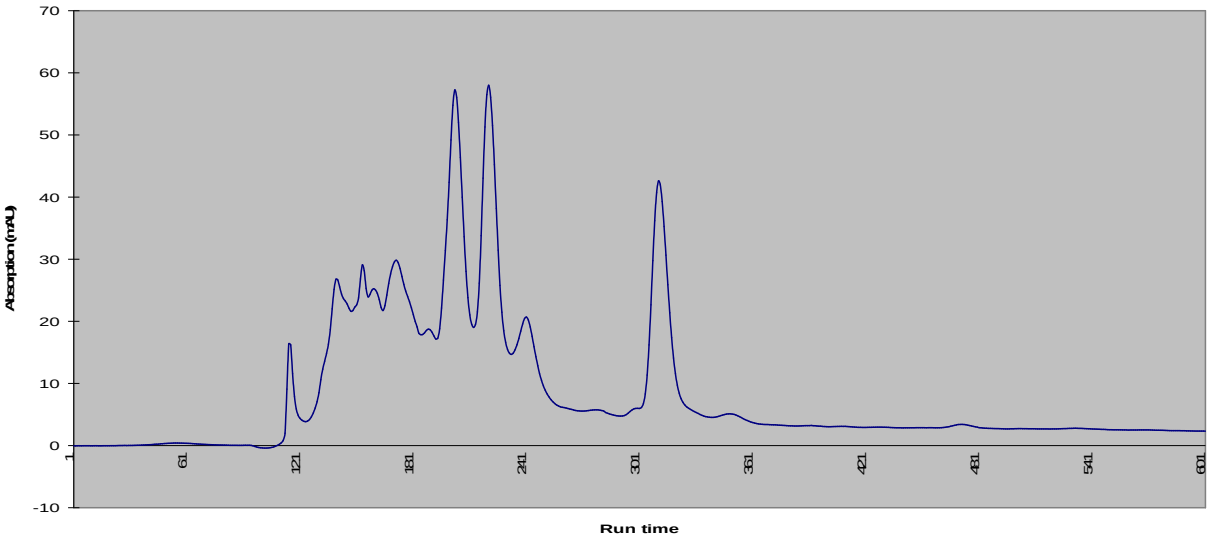

Figure 6. HPLC profile of methanolic extract of Root of ADVR.

Table 3. Peak identification of methanolic extract of ADVR.

Peak No.	Ret. time [min]	Start [min]	End [min]	Area [m AU*min]	Height [m AU]	% Area	% Height	Width [min]
1	1.901	1.851	2.051	1.011	14.818	4.336	9.793	0.057
2	2.318	2.135	2.451	1.590	11.731	6.816	7.753	0.120
3	3.369	3.219	3.536	6.338	41.497	27.172	27.424	0.135
4	3.669	3.536	4.286	7.675	45.312	32.903	29.945	0.139
5	5.170	4.837	5.637	6.711	37.954	28.770	25.083	0.156

ADVS

The ADVS of *Murraya koenigii* were analyzed. Samples of methanolic extracts of *in vitro* adventitious shoot were analysed and the results of retention time and start time are shown in **Figure:-7.** The HPLC chromatogram of ADVS showed that total four peaks. The Retention time of peak number 3 (3.86minute) is nearly similar with the Retention time of BHT (3.85minute) which has 51.69mAU absorption. The compound showed in peak number 2 was found in maximum quantity (69.98%) and its Retention time is 0.68minute (**Table;-4**). The compound in peak number 3 was observed to be present in minimum quantity (6.48%) in the ADVS extract.

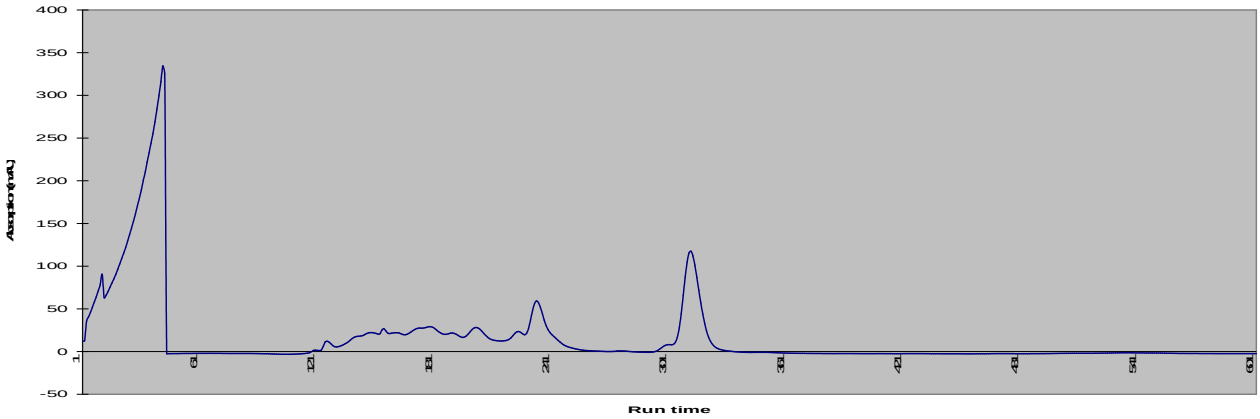

Figure 7. HPLC profile of methanolic extract of ADVS.

Table 4. Peak identification of methanolic extract of ADVS

Peak No.	Ret. time [min]	Start [min]	End [min]	Area [m AU*min]	Height [m AU]	% Area	% Height	Width [min]
1	0.166	0.016	0.200	8.687	80.952	6.8392	13.790	0.091
2	0.683	0.200	0.733	88.892	335.931	69.980	57.226	0.191
3	3.869	3.769	4.286	8.235	51.691	6.483	8.805	0.130
4	5.186	4.819	5.703	21.208	118.447	16.696	20.177	0.154

AXLS

The methanolic extract of AXLS of *Murraya koenigii* was injected in HPLC unit. Six peaks were observed in HPLC chromatogram (**Figure:-8**). The Retention time, start time and peak area of the compound in HPLC column was calculated and shown in **Table:-5.** The Retention time of AXLS were compared with the peaks of standards solutions. The chromatogram showed that AXLS extracts that the Retention time of peak number 5 is 3.85minute which has 53.48mAU absorption which was nearly similar to the BHT respectively. The percentage area of each peak represents the amount of each compound separated in HPLC in µgs/400µg fresh weight. The data of area of all the peaks are in **Table:-5.**

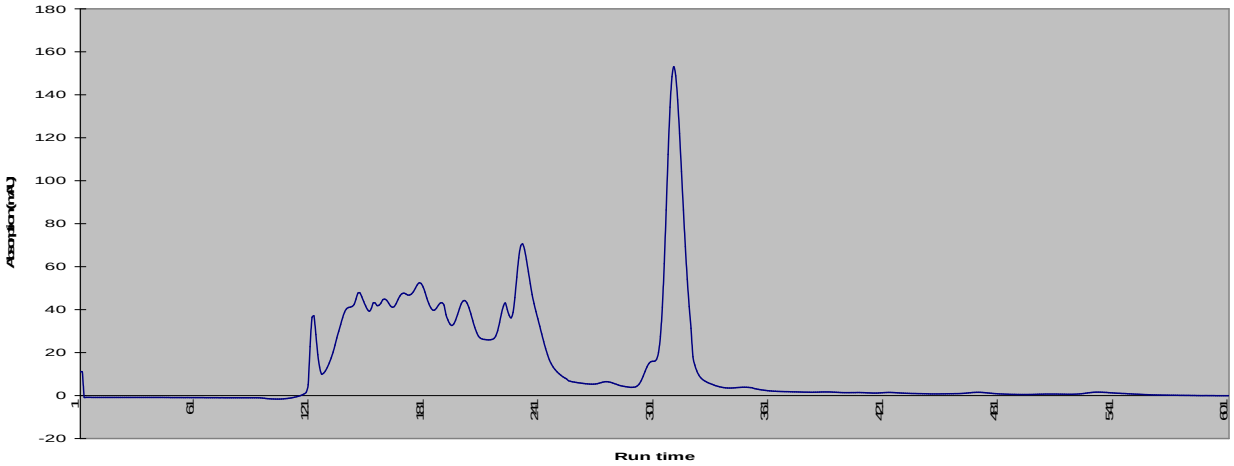

Figure 8. HPLC profile of methanolic extract of AXLS.

Table 5. Peak identification of methanolic extract of AXLS.

Peak No.	Ret. time [min]	Start [min]	End [min]	Area [m AU*min]	Height [m AU]	% Area	% Height	Width [min]
1	2.034	1.968	2.101	2.032	31.352	4.276	10.984	0.062
2	2.435	2.135	2.518	3.579	14.611	7.531	5.119	0.220
3	3.352	3.235	3.552	1.881	14.439	3.958	5.058	0.127
4	3.702	3.552	3.753	2.204	22.174	4.638	7.768	0.067
5	3.853	3.753	4.270	10.056	53.487	21.159	18.739	0.172
6	5.170	4.820	5.654	27.773	149.362	58.434	52.329	0.163

DISCUSSION AND CONCLUSION

The investigation provides the HPLC analysis of all the samples and established a correlation between quantitative analysis of antioxidant activity of extracts of different plant parts and identification of compounds having antioxidant by using known antioxidant compounds in HPLC analysis.

HPLC analysis to identify the specific compound present in the extract having antioxidant activity with the help of chromatogram of known standard compounds run in the HPLC unit. In the early days of HPLC it was stated that the liquid chromatography gives accurate and specific results, it is slow relative to total phenol assay procedures requires expensive equipments and specialized skills. The introduction of enhanced resolution and increased automation has resulted in HPLC becoming the most popular analysis method for plant phenolics (Robards et al., 1997; Waksmundzka-Hajnos, 1998). The phenolic compounds of natural origin have the positive property to being solution in polar solvents. This leads to possibility of using HPLC in their analysis sufficient retention being achieved. Present study describes and compares the HPLC profiles detected at 254nm of fresh weight of methanolic extracts of different parts of *Murraya koenigii*. In addition to antioxidative compounds (citric acid, ascorbic acid quercetin and BHT) many other peaks were simultaneously separated and main components identified by comparing the peaks of unknown samples with available commercial standards such as citric acid, Ascorbic acid, BHT and Quercetin. Potential sources of antioxidant compounds have been searched from several types of plant materials (Ramarathnam, et al., 1997) using different methods. In present investigation the quantitative analysis of total phenols, flavonoids and antioxidant activities of extracts were carried out and a modified gradient HPLC analysis of same samples for identification and quantification of phenols and flavonoids in the plant samples was used. The study was conducted on 4 samples representing plant tissues of *in vitro* raised parts of *Murraya koenigii*. Although reports are available on quantitative analysis of antioxidant vitamins such as lutein ά tocopherol and β carotene from fresh curry leaves using Reversed phase HPLC (Palaniswany, et al., 2003) but in this study the HPLC analysis for quantitative analysis of phenols and flavonoids was conducted in order to separate the antioxidative components from different parts of *in vitro* plantlets of *Murraya koenigii* and compared them with the peaks of available commercial standard antioxidant compounds such as citric acid, ascorbic acid, BHT and quercetin. The present study was also conducted of *in vitro* raised plant parts of *Murraya koenigii* for analyze the availability or synthesis of bioactive compounds. In the HPLC chromatograms the antioxidative compounds were identified by their Retention time by spiking with standards (citric acid, ascorbic acid, BHT and quercetin) under the same conditions. In the HPLC chromatograms the antioxidative compounds were identified by their Retention time by spiking with standards under the same condition. The compound number 5 was found in maximum quantity (42.24%) in the AXLR extract which has start time 3.81 minute and retention time 3.93 minute. The Retention time of peak number 3 (3.86minute) of ADVS is nearly similar with the Retention time of BHT (3.85minute) which has 51.69mAU absorption. The compound showed (**Table;-4**) in peak number 2 was found in maximum quantity (69.98%) and its Retention time is 0.68minute. The compound in peak number 3 was observed to be present in minimum quantity (6.48%) in the ADVS extract.

LIST OF ABBREVIATIONS: *in vitro* adventitious shoot (ADVS), root of *in vitro* adventitious shoot (ADVR), *in vitro* axillary shoots (AXLS), root of *in vitro* axillary shoot (AXLR), South East Asia (SEA), Advanced Scientific Instruments, Germany (ASIG), High-performance liquid chromatography (HPLC),

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