WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES
SJIF Impact Factor 6.647
Volume 6, Issue 10, 790-799 Research Article ISSN 2278 – 4357

HPTLC STUDY & *IN-VITRO* ANTIOXIDANT ASSAY OF 'PARUTTI VITAI CURANAM' – A SIDDHA DRUG

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Article Received on
29 July 2017,

Revised on 18 August 2017,
Accepted on 07 Sept. 2017

DOI: 10.20959/wjpps201710-10189

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ABSTRACT

Parutti Vitai Curanam, a siddha poly herbal formulation comprises of five herbal Ingredients. The present study is focused on the *In vitro* antioxidant assay where the hydroalcoholic extract of Parutti Vitai Curanam was examined for DPPH and superoxide radical scavenging activity, Pro-oxidant effect and total antioxidant capacity. The results revealed significant antioxidant activity when compared with standard. Further the HPTLC fingerprinting profile of the extract was established which is used as a diagnostic tool for quality evaluation and standardization of the drug.

KEYWORDS: Antioxidant, DPPH (1,1-diphenyl-2-picryl-hydrazyl), superoxide radical scavenging activity.

INTRODUCTION

The increasing awareness among the people towards natural products, natural medicine is attracting more attention than allopathic system. Moreover this system of medicine is pollution free and less toxic without side effects.[1]

An herbal based formulation improves the quality of human life through its potent natural antioxidants[2] and bioactive compounds.[3] They provide remedy for various chronic diseases and metabolic disorders which are multifactorial and therapeutic intervention.[4]

Antioxidants are compounds that inhibit or delay oxidation of other molecules by terminating initiation or propagation of oxidizing chain reaction. Free radicals are atoms or groups of

atoms with an odd (unpaired) number of electrons and can be formed when oxygen interacts with certain molecules. Once formed these highly reactive radicals can start a chain reaction. Free radicals can be hazardous to the body and damage all major components of cells, including DNA, proteins, and cell membranes.[5]

Radical scavenging antioxidants are particularly important in antioxidative defense in protecting cells from the injury of free-radical.[6]

Therefore, it is important to assess antioxidant activity of the plants used in the herbal medicine either to elucidate the mechanism of their pharmacological action or to provide information on antioxidant activity of these herbal plants.[7] The aim of the present study is to evaluate the antioxidant activity and to signify the use of HPTLC fingerprint profiles for qualitative identification of the drug.

MATERIALS AND METHODS

Collection of the Drug

The herbal ingredients (*Gossypium herbaceum*-seeds, *Piper longum*, *Limonia acidissima*-seeds,*Oryza sativa, Elettaria cardamomum)* for the drug, were procrured from the local markets, Chennai. All the ingredients were authenticated by Dr.Sunil Kumar, Research Officer (Pharmacognosy), Siddha Central Research Institute, Chennai

Preparation and Extraction of the Drug

The ingredients of the drug Parutti Vitai Curanam were dried and powdered separately. They were mixed together and the dried product was weighed and stored for further.75g of Parutti Vitai Curanam was soaked with 500ml 0f hydroalcohol (1:1) mixture for 48hours. The extract was filtered and concentrated using rotary evaporation under reduced pressure (100mbar) and reduced temperature (55 °C). It was transferred to a porcelain dish using minimum quantity of ethanol and dried over water bath to free ethanol.

Procedure

HPTLC Profile

Applied 10 and 15 µl of extracts on TLC plate using Camag's ATS4 applicator and developed by gradient mode the mobile phases, Methanol: Chloroform (50:50) (developed upto 30 mm from the bottom); Ethyl acetate : Hexane (70:30) (developed upto 50 mm from the bottom); Ethyl acetate: Toluene (50:50) (developed upto 70 mm from the bottom);

Toluene: Hexane (50:50) (developed upto 80 mm from the bottom) using the automated multiple developer (AMD2). After development, the plate was photodocumented using Camag's TLC Visualizer under UV 254 nm and UV 366 nm. The plate was then scanned using Camag's Scanner 4 at UV 254 nm (D2 lamp, Absorption mode) finger print profiles of the extracts were documented.

Estimation of Polyphenol content of Parutti Vitai Curanam

The amount of Polyphenol in the drug is determined by Folin-ciocalteau method. 300mg of drug was dissolved in 5ml of methanol: water: concentrated-Hydrochloric acid (60:40:0.3). The contents were filtered through Whatman No.1 filter paper. After 2 minutes 100 micro litre of 50% of Folins phenol reagent was added. The tubes were incubated at room temperature and the absorbance was read at 750 nm by using UV spectrophotometer.

Determination of Flavonoid concentrations in Parutti Vitai Curanam

The content of the flavonoid in the examined drug was determined using spectrophotometric method. The drug contained 1ml of methanol solution of the drug in the concentration of 1 mg/ml and 1ml of 2% Aluminium chloride solution was dissolved in methanol. The sample were incubated for an hour at room temperature, the absorbance was determined using spectrophotometer 415nm.

Determination of DPPH radical scavenging activity

The free radical scavenging activity was determine by using 2,2-diphenyl-1-pincrylhydrazyl (DPPH) method, a simple method that has been developed to determined the antioxidant activity of foods utilizes the stable DPPH radical. The principle is explained following; the odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm and is purple in colour. The colour turns from purple to yellow as the molar absorptive of DPPH radical at 517 nm reduces the odd electron of DPPH radical undergoes pairing with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH. The colour developed is measured at 517nm the reagents of DPPH solution (100mM) was prepared by dissolving 33 mg of DPPH in 100 ml of ethanol.

Determination of Pro-Oxidant effect of Parutti Vitai Curanam

To determine the reducing power (Pro- oxidant) of the drug. The principle in which the substances which have reaction potential which reacts with potassium ferricyanide (Fe^{3+}) then reacts with ferric chloride which forms ferric-ferrous cyanide and has an absorption

maximum at 700 nm. The chemicals were prepared by dissolving 1g of Potassium ferricyanide in 100 ml of distilled water (1% w/v). Phosphate buffer (0.2 M, Ph-6.6) was prepared by dissolving 2.75 g of sodium dihydrogen phosphate and 5.365g of disodium hydrogen phosphate in 100 ml of distilled water. 10 g of Trichloro acetic acid (10%) was dissolved in 100 ml of distilled water and 0.1 g of Ferric chloride in 100 ml of distilled water.

Determination of Total Antioxidant capacity of Parutti Vitai Curanam

The total antioxidant activity of the drug was determined by reduction of Mo (VI) to Mo (V) by the samples and the subsequent formation of a green coloured Mo (V) / Phosphate complex in acidic PH. The reagents were prepared to 28mm of sodium phosphate was prepared by dissolving 84 mg of sodium phosphate in 25 ml of distilled water. 4mm of Ammonium molybdate was prepared by dissolving 124 mg of ammonium molybdate in 25 ml of distilled water and 0.6 mm of sulphuric acid was prepared.

100 µg of each drug in 0.3 ml of distilled water was added to 3 ml of molybdate reagent containing 0.6 mm sulphuric acid, 28 mm of sodium phosphate and 4 mm of ammonium molybdate. The tubes were incubated at 95°C for 90 minutes. Then the mixture was cooled at room temperature and the absorbance was measured at 695 nm. The results were expressed as equivalents of ascorbic acid.

Scavenging of Superoxide radical by alkaline DMSO method

The superoxide scavenging effect of the drug was studied using the alkaline DMSO method. The Reagents were prepared to 0.1 ml of NBT was prepared by dissolving 1 mg of NBT (Nitro blue Tetra Zolium) in 1 ml of DMSO (Dimethyl Sulfoxide). 1 ml of alkaline DMSO was prepared by dissolving 5 mm of sodium hydroxide in 1 ml of DMSO. The samples were dissolved in DMSO in various concentration were added with 0.1 ml of NBT (1 mg / ml of DMSO) and 1 ml of alkaline DMSO (5 mm of sodium hydroxide / ml of DMSO). Then the absorbance was measured at 560 nm using spectrophotometer.

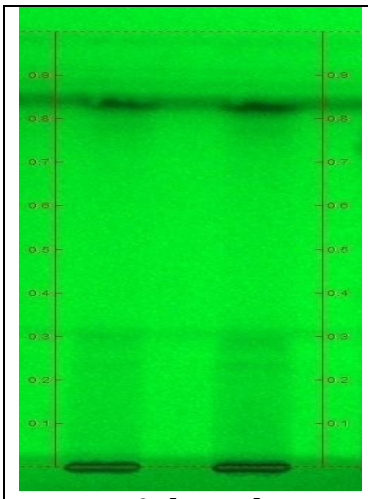
RESULT AND DISCUSSION

High Performance Thin Layer Chromatography (HPTLC)

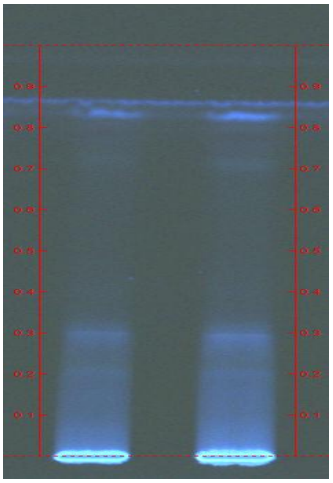
The HPTLC fingerprinting patterns of hydroalcoholic extract of Parutti vitai curanam was developed at 254nm, 366nm and derivatized at 520nm. The solvent systems, Methanol: Chloroform (50:50), Ethyl acetate: Hexane (70:30), Ethyl acetate: Toluene (50:50), Toluene:Hexane (50:50)efficiently resolved the components present in the extract. HPTLC

photo documentation profile of the hydroalcoholic extract of Parutti vitai curanam at 254nm, 366nm and after derivatisation is given in Fig.1. On observation 3 bands appeared under UV at 254nm with R_f 0.23, 0.29 and 0.78. TLC pattern at 366nm showed 2 bands at R_f value 0.29 and 0.72. The 3D chromatogram of the methanol extract of Parutti vitai curanam at 254nm and the HPTLC Fingerprinting profile are given in Fig.2 and Fig.3 respectively and the Rf value and percentage area of the peaks are shown in Table 2.

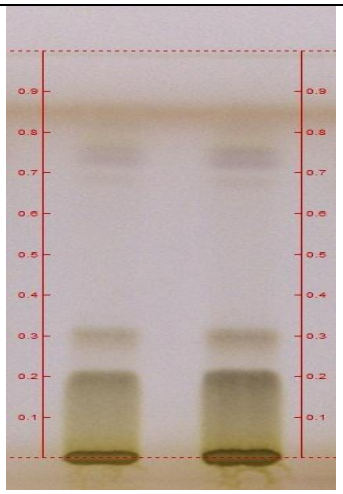
Table.2: Peak Table at 254 nm



10µl 15µl



10µl 15µl



10µl 15µl

Under UV 254 nm		Under UV 366 nm		520 nm (Derivatized)	
Rf	**Colour**	**Rf**	**Colour**	**Rf**	**Colour**
0.23	Green	0.29	blue	0.19	Black
0.29	Green	0.72	blue	0.30	Grey
0.78	Green			0.68	Light yellow
				0.75	Grey
				0.78	Grey

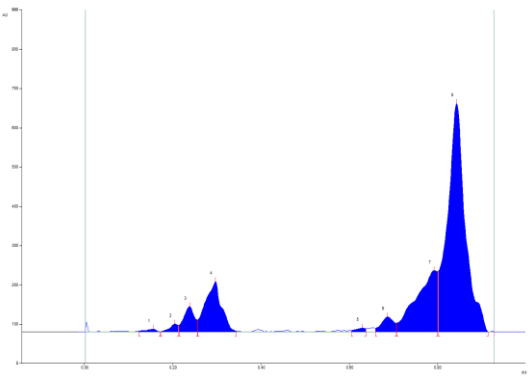
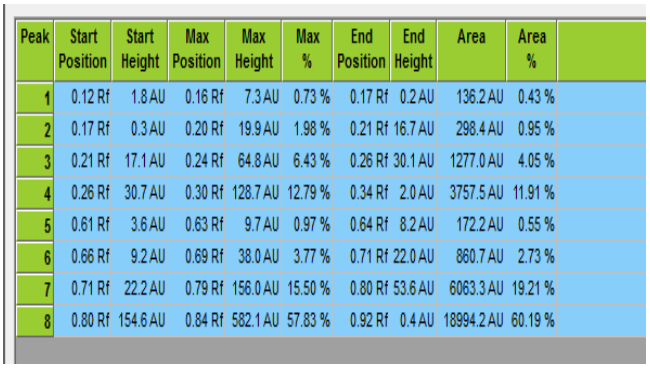


Fig.1: HPTLC photo documentation of parutti vitai curanam.



Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	0.12 Rf	1.8 AU	0.16 Rf	7.3 AU	0.73 %	0.17 Rf	0.2 AU	136.2 AU	0.43 %
2	0.17 Rf	0.3 AU	0.20 Rf	19.9 AU	1.98 %	0.21 Rf	16.7 AU	298.4 AU	0.95 %
3	0.21 Rf	17.1 AU	0.24 Rf	64.8 AU	6.43 %	0.26 Rf	30.1 AU	1277.0 AU	4.05 %
4	0.26 Rf	30.7 AU	0.30 Rf	128.7 AU	12.79 %	0.34 Rf	2.0 AU	3757.5 AU	11.91 %
5	0.61 Rf	3.6 AU	0.63 Rf	9.7 AU	0.97 %	0.64 Rf	8.2 AU	172.2 AU	0.55 %
6	0.66 Rf	9.2 AU	0.69 Rf	38.0 AU	3.77 %	0.71 Rf	22.0 AU	860.7 AU	2.73 %
7	0.71 Rf	22.2 AU	0.79 Rf	156.0 AU	15.50 %	0.80 Rf	53.6 AU	6063.3 AU	19.21 %
8	0.80 Rf	154.6 AU	0.84 Rf	582.1 AU	57.83 %	0.92 Rf	0.4 AU	18994.2 AU	60.19 %

Fig.2: HPTLC fingerprint profile of Parutti vitai curanam at 254 nm.

HPTLC fingerprint profile of the hydroalcoholic extract of Parutti vitai curanam was developed after derivatising and scanned at 254nm. There were eight peaks observed of R_f

values at 0.16, 0.20, 0.24, 0.30, 0.63, 0.69, 0.79, 0.84. Out of these, three peaks (R_f 0.30, 0.79, 0.84) were found to be more prominent.

Estimation of polyphenol content of parutti vitai curanam

The Polyphenol content of the extract of Parutti vitai curanam were detected and compared with Gallic acid. In this method, Gallic acid was used as standard. The percentage of inhibition at various concentration (10-50µg) of drug as well as standard were calculated and plotted in graph(Fig.4). The control value at 750nm is 0.340. The test drug shows higher inhibition 38.8% at 50 µg sample concentration in 750nm. The standard Gallic acid exhibits 12.9% of inhibition at 50 µg. In lower concentration 10 µg, the test drug shows 29.7% of inhibition.

S.NO	CONCENTRATION (µg)	ABSORBANCE VALUE at 750nm		PERCENTAGE OF INHIBITION	
	Standard/ Drug	Standard	Drug	Standard	Drug
1	10	0.337	0.239	0.8	29.7
2	20	0.330	0.238	2.9	30
3	30	0.305	0.222	10.2	34.7
4	40	0.302	0.219	11.1	35.5
5	50	0.296	0.208	12.9	38.8

Determination of flavonoid concentrations in parutti vitai curanam

The Flavonoid concentration of the extract of Parutti vitai curanam were detected and compared with Quercitin. In this method, Quercitin was used as standard. The percentage of inhibition at various concentration(1-5µg) of drug as well as standard were calculated and plotted in (Fig.5). The control value at 415nm is 0.339.The test drug shows higher inhibition 44.2% at 5µg sample concentration in 415nm. The standard Quercitin exhibits 44.2% of inhibition at 5 µg. Inlower concentration 1µg, the test drug shows 11.5% of inhibition.

S.NO	CONCENTRATION (µg)	ABSORBANCE VALUE at 415nm		PERCENTAGE OF INHIBITION	
	Standard/ Drug	Standard	Drug	Standard	Drug
1	1	0.268	0.300	20.9	11.5
2	2	0.261	0.253	23	25.3
3	3	0.220	0.228	35.1	32.7
4	4	0.219	0.214	35.3	36.8
5	5	0.189	0.189	44.2	44.2

Determination of DPPH radical scavenging activity

The DPPH radical scavenging activity of the extract of Parutti vitai curanam were detected and compared with Quercitin. In this method, Quercitin was used as standard. The percentage

of inhibition at various concentration(5-25µg) of drug as well as standard were calculated and plotted in graph (Fig.6). The control value at 517nm is 0.166. The test drug shows higher inhibition 63.2% at 25 µg sample concentration in 517nm. The standard Quercitin exhibits 91.5% of inhibition at 25 µg. Inlower concentration 5 µg, the test drug shows 40.3% of inhibition.

S.NO	CONCENTRATION (µg)	ABSORBANCE VALUE at 517nm		PERCENTAGE OF INHIBITION	
	Standard/ Drug	Standard	Drug	Standard	Drug
1	5	0.022	0.099	86.7	40.3
2	10	0.019	0.094	88.5	43.3
3	15	0.018	0.076	89.1	54.2
4	20	0.014	0.073	91.5	56.0
5	25	0.014	0.061	91.5	63.2

Determination of pro-oxidant effect of parutti vitai curanam

The prooxidant effect of the extract of Parutti vitai curanam were detected and compared with Quercitin. In this method, Quercitin was used as standard. The percentage of inhibition at various concentration (5-25µg) of drug as well as standard were calculated and plotted in graph (Fig.7). The control value at 700nm is 1.506. The test drug shows higher inhibition 98% at 25µg/ml sample concentration in 700nm. The standard Quercitin exhibits 97.4% of inhibition at 25µg. In lower concentration 5 µg, the test drug shows 96% of inhibition.

S. NO	CONCENTRATION (µg)	ABSORBANCE VALUE at 700nm		PERCENTAGE OF INHIBITION	
	Standard/ Drug	Standard	Drug	Standard	Drug
1	5	0.100	0.059	93.3	96.0
2	10	0.082	0.048	94.5	96.8
3	15	0.051	0.040	96.6	97.3
4	20	0.041	0.035	97.2	97.6
5	25	0.039	0.029	97.4	98.0

Determination of total antioxidant capacity of parutti vitai curanam

The Total antioxidant capacity of the extract of Parutti vitai curanam were detected and compared with Ascorbic acid. In this method, Ascorbic acid was used as standard. The percentage of inhibition at various concentration (0.5-2.5µg) of drug as well as standard were calculated. The control value at 695nm is 0.290. The test drug shows higher inhibition 15.8% at 2.5 µg sample concentration in 695 nm. The standard Ascorbic acid exhibits 16.55% of inhibition at 2.5 µg. In lower concentration 0.5µg, the test drug shows 4.4% of inhibition.

S. NO	CONCENTRATION (µg)	ABSORBANCE VALUE at 695nm		PERCENTAGE OF INHIBITION	
	Standard/ Drug	Standard	Drug	Standard	Drug
1	0.5	0.287	0.276	1.03	4.4
2	1	0.282	0.275	2.7	5.1
3	1.5	0.272	0.269	6.2	7.2
4	2	0.262	0.259	9.6	10.6
5	2.5	0.242	0.244	16.55	15.8

Scavenging of superoxide radical by alkaline dmso method

The superoxide radical scavenging of the extract of Parutti vitai curanam were detected and compared with Ascorbic acid. In this method, Ascorbic acid was used as standard. The percentage of inhibition at various concentration (0.5-2.5µg) of drug as well as standard were calculated and plotted in graph (Fig.9). The control value at 560nm is 0.485. The test drug shows higher inhibition 50.1% at 2.5 µg sample concentration in 560nm. The standard Ascorbic acid exhibits 52.5% of inhibition at 2.5 µg. In lower concentration 0.5 µg, the test drug shows 43.5% of inhibition.

S.NO	CONCENTRATION (µg)	ABSORBANCE VALUE at 560nm		PERCENTAGE OF INHIBITION	
	Standard/ Drug	Standard	Drug	Standard	Drug
1	0.5	0.285	0.274	41.2	43.5
2	1	0.274	0.272	43.5	43.9
3	1.5	0.251	0.270	48.2	44.3
4	2	0.243	0.266	49.8	45.1
5	2.5	0.255	0.242	52.5	50.1

SUMMARY AND CONCLUSION

The study was performed to evaluate the *Invitro* antioxidant activity and HPTLC study of the hydroalcoholic extract of Parutti vitai curanam. Standardization is an essential measure for quality, purity and sample identification. The preliminary antioxidant analysis and the developed HPTLC chromatogram obtained from this study helps in identification and standardization of Parutti vitai curanam. HPTLC finger printing profile establishes the identity and purity of the drug Parutti vitai curanam.

Antioxidant slows down the oxidative damage of our body. Antioxidants act as free radical scavengers preventing and repairing damages. The antioxidant activity of Parutti vitai curanam was tested by Total antioxidant capacity, Prooxidant effect, DPPH free radical scavenging activity and Superoxide scavenging activity by alkaline DMSO method.

DPPH radical is scavenged by antioxidants through the donation of proton forming the reduced DPPH. DPPH was used to determine the proton radical scavenging action of the extract of Parutti vitai curanam and ascorbic acid (standard), because it possess a proton free radical and shows a characteristic absorbance at 517nm.

Superoxides are known to be very harmful to the cellular components. Superoxide free radical was formed by alkaline DMSO which reacts with NBT to produce coloured complex. The hydroalcoholic extract of Parutti vitai curanam scavenges superoxide radical and shows a characteristic absorbance at 560nm. Similarly, the Total antioxidant capacity of the extract of Parutti vitai curanam was compared with Ascorbic acid at 695nm and that of the prooxidant effect of the extract of Parutti vitai curanam was compared with standard quercitin at 700nm from which a standard calibration graph was determined.

There is a strong need for effective antioxidants from natural sources as alternatives to synthetic food additives in order to prevent deterioration of food. The results of the present study showed that the extract of Parutti vitai curanam exhibits significant antioxidant activity, which indicates that Parutti vitai curanam is a significant source of natural antioxidant. However further studies are required to isolate the marker compounds.

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