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HPLC Method for Estimation of (-)-Epicatechin in Pterocarpus Marsupium Herbal Extracts and Pharmaceutical Dosage Formulations

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Abstract

A reversed-phase high performance liquid chromatographic separation and quantitative method using an aqueous acetic acid (mixture of acetonitrile and aqueous acetic acid) was developed to analyze procyanidin compound in extracts from the bark of *Pterocarpus marsupium* and pharmaceutical dosage forms. The concentrations of (-)-epicatechin in aqueous, 50% methanolic and methanolic extract of *Pterocarpus marsupium* are 13.14%, 17.46% and 9.15% (w/w), respectively. The method was validated with respect to recovery, precision and linearity. The recovery for (-)-epicatechin was 99.97%. The correlation coefficient of calibration curve was 0.9795. The LOD and LOQ were 4.24 µg mL^{-1} and 14.12 µg mL^{-1}, respectively. Statistical analysis of the data showed that the method is reproducible and selective for estimation of (-)-epicatechin. This chromatographic method is simple, sensitive and reproducible, ideally suited for rapid, routine analysis.

Keywords:

High-performance liquid chromatography; Method validation; *Pterocarpus marsupium*; (-)-epicatechin; Polyherbal formulations; Anti-diabetic

1. Introduction

Pterocarpus marsupium (PM; Bibla or Vijayasar/Bijasar in Hindi and Indian Kino in English) belongs to Fabaceae family and is a large tree common in central, western and southern parts of India and Sri Lanka. Various portions of the bark are used as astringent, anti-diarrheal, antacid, for the treatment of toothache and for the management of diabetes and leaves are used for boils, sores, and skin diseases [1]. The heartwood and bark have been traditionally used in the management of diabetes and hyperlipidemia. Its use in maintaining healthy blood sugar levels is further validated by preclinical and clinical studies. Many pharmacological studies have been conducted to account for the ancient reputation of its anti-diabetic potential [2-4]. Its water soluble active principle, epicatechin, has also been demonstrated in vitro for antidiabetic activity.

Its bioactive constituents include (-)-epicatechin (a flavonoid), marsupin (benzofuranone), and pterosupin (a dihydrochalcone) [5]. (-)-Epicatechin has been identified as the blood sugar lowering compound in the bark. The aqueous extract of stem bark was found to reduce the blood glucose level in alloxan-induced diabetic rats [6]. Many HPLC methods were reported for estimation of (-)-epicatechin [7-15].

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Despite many reports on the medicinal properties of *Pterocarpus marsupium,* no papers have been published on the determination of (-)-epicatechin in these material using HPLC. Therefore, an attempt has been made to develop accurate, specific, repeatable and robust HPLC method for the determination of (-)-epicatechin in various extracts of *Pterocarpus marsupium*.

2. Experimental

2.1. Plant material

Sample of dried bark was provided by Amsar Pvt, Ltd, Indore, India.

2.2. Reagents and chemicals

Acetonitrile (HPLC grade, Merck, Darmstadt) and acetic acid 99.8% was analytical grade. Water was purified using the Milli-Q plus purification system (Millipore, Bedford, USA). (-)-Epicatechin standard was purchased from Natural Remedies Pvt, Ltd, (Bangalore, India).

2.2. Instrumentation and chromatographic conditions

A Merck Hitachi chromatogram equipped with a pump (L-7100), thermostat controlled column chamber, rheodyne injection valve with a 20 µl sample loop and UV/vis detector (L-7400) , controlled by winchrome software. The column was RP-18 Lichrosphere 250 × 4 mm i.d. 5 µm particle diameter (Merck, Darmstadt and Germany), fitted with suitable guard column of C-18. The mobile phase finally adopted was solvent A (2.5% aqueous acetic acid) and solvent B (acetonitrile -2.5% aqueous acetic acid (80:20 v/v) [16]. An isocratic separation was performed using 80% solvent A: 20% solvent B with a flow rate of 1 mL min^{-1} and column compartment temperature of 25 ˚C. The detection wavelength was 280 nm, followed by washing and reconditioning the column. The chromatographic peaks of the analytes were confirmed by their retention times and UV spectra with those of the reference standards. Working standard solutions were injected into the HPLC, and peak area responses obtained. Standard graphs were prepared by plotting concentration (µg mL^{-1}) versus peak area. Epicatechin were quantified by external standard method.

2.3. Calibration curve of (-) epicatechin

Stock standard solution was prepared separately by accurately weighing 4.8 mg of (-)-epicatechin into a 100 mL volumetric flask and dissolving in 100 mL of 0.2% aqueous acetic acid with the aid of sonication. Working standard solutions, 2.4-19.2 µg mL^{-1}, were prepared by dilution with water from the stock standard solutions. The calibration curve was plotted by concentration vs. area.

2.4. Method validation

These parameters were also calculated from the data set obtained from a linear calibration curve of the working concentration in the range of 2.4-19.2 µg mL^{-1}. According to an ALAMIN program [17], analytical sensitivity (AS) is determined by the ratio of S_s /*b*, in which S_s is the residual standard deviation and b is the slope of the calibration curve. The limit of detection (LODapprox) is determined by the following equation:

$$LOD_{approx} = 3(S_s/b)\,[(n-2)/(n-1)]^{1/2}$$

Where n is the number of total measurements for each calibration set. The limit of quantitation $(LOQ)_{approx}$ is calculated by replacing 3 with 10 in the above equation.

The accuracy of the method was studied by performing experiments by standard addition technique. Three different levels (10, 20 and 30 μg mL^{-1}) of standards were added to a previously analyzed sample, each level being repeated thrice. Precision of an analytical method is expressed as S.D. and R.S.D. of series of measurement. It was ascertained by replicate estimation of the samples by proposed method. The precision of the developed method was also confirmed by different day's analysis. The similar process was repeated under the same set of condition for three days. To test the precision of the assay method, the standard solution and sample solutions was injected three times under the chromatographic conditions described above and areas were recorded. Same procedure was followed the next day and the concentration of the sample was calculated by comparing with the standard.

2.5. Stability

2.5.1. Standard solution stability

The stability of the (-)-epicatechin in the standard solution was also tested. The experiment showed stability of about 10 days when the standard of this compound was prepared with 0.2% aqueous acetic acid and kept at 15˚C.

2.5.2. Stability of sample solution

To verify samples stability throughout the analysis time and the behavior of the extracts of *Pterocarpus marsupium* under the studied conditions, the same sample was analyzed after every 30 min for 2hr when stored at room temperature. The results showed the same chromatographic profile during total analysis time and no degradations products were detected.

2.5.3. Analysis of (-)-epicatechin in herbal extracts

The bark sample of P*terocarpus marsupium* was ground to powder. The ground sample was extracted using different solvents and different extraction times. Among the solvents used for extraction were water, aqueous methanol (50%) and methanol. These solvents have been used for extraction of flavonols in various studies [18-19]. The supernatant was filtered and evaporated to make it concentrate. For HPLC analysis the concentrated extract (0.2g) was further extracted with 100 mL water with intermittent shaking. The sample was then centrifuged for 10 min at 16˚C. The supernatant was taken into a 100 mL volumetric flask and the extraction repeated 3 times with interval of 30 min. Make the final volume with 2.5% aqueous acetic acid. The extracts were filtered through ultipore filter (Pall life science) before the injection was made.

The test samples was passed through 0.2 μm filter using a syringe and 20μl of test solution was injected to HPLC using C-18 reversed phase column with a reversed phase guard column. The mobile phase consisted of solvent A: solvent B (80:20) in which solvent A was 2.5% aqueous acetic acid and solvent B was acetonitrile with 2.5% aqueous acetic acid in ratio 80:20 v/v. It was degassed using sonicator and filtered through 0.2 μm filter and used for separating the target marker with a flow rate of 1 mL min^{-1}.The chromatogram was scanned up to 20 min, which was detected at 280 nm, followed by washing and reconditioning the column. The analysis was repeated in triplicate.

2.6. Analysis of (-)-epicatechin in marketed formulations

Polyherbal formulations were finely powered and accurately weighed equivalent to 200mg of *Pterocarpus marsupium* in each formulation. Each of weighed formulation was extracted with 100 mL of water for 30 min in ultrasonic bath; filtered it through Whatmann

filter paper. The extraction was repeated for 3 times as above. Combined the filtered extract, added 2.5 % aqueous acetic acid and finally the volume was made upto 100 mL with the same solvent, these solutions were used for analysis. This solution was filtered through 0.2 µm filter and used for HPLC analysis.

3. Results and Discussion

After trying a number of assay conditions and mobile phases and the information collected from the related research papers the following mobile phase was considered best suited for the analytical characterization of chemical marker. The mobile phase consisted of solvent A: solvent B (80:20) in which solvent A was 2.5% aqueous acetic acid and solvent B was acetonitrile with 2.5% aqueous acetic acid in ratio 80:20 v/v. The standard solution and the test solution were injected in HPLC. It showed peak at retention time 4.61 for epicatechin (Fig. 2A) at room temperature.

The present HPLC method for estimation of (-)-epicatechin showed a good correlation coefficient in the concentration range 2.4-19.2 µg mL^{-1} with respect to the peak area. Within this interval the calibration curves (Fig.1) were linear with correlation coefficient > 0.9795 R^2=0.9594 is considered very significant (ANOVA, P is 0.0035), 95% confidence interval.

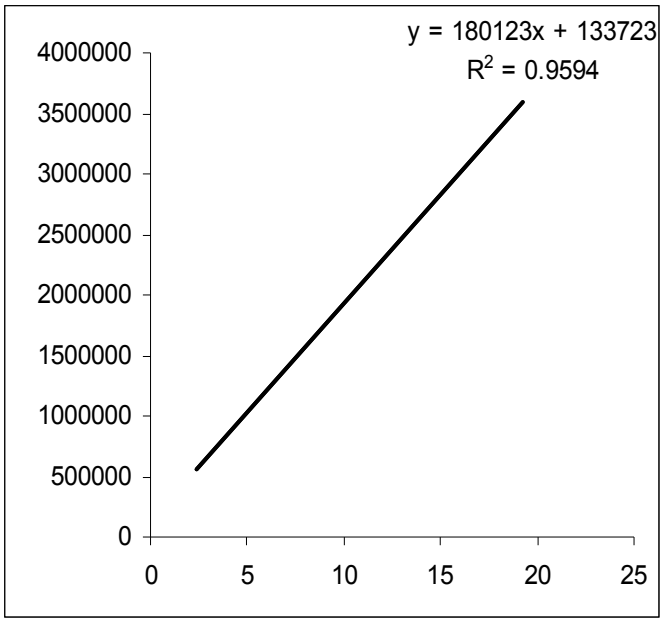


Fig. 1. Calibration curve of (-)-epicatechin between concentration and area at λ_{max} 280 nm

The accuracy of the method was determined by recovery experiments. The recovery studies were carried out 3 times and the percentage recovery were calculated and presented. From the data obtained, recoveries of added standard drugs were found to be accurate (Table 1). Three repeated standard and sample solutions of each extracts and formulations were made and using linear regression equation the actual amount and % RSD were calculated and presented. From the data obtained, the developed HPLC method was found to be precise.

Analytical sensitivity (AS) is determined by the ratio of S_s /*b*, in which S_s is the residual standard deviation and b is the slope of the calibration curve. The AS was found to be 1.63 µg mL^{-1}. The LOD and LOQ of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed methods. The LOD is the smallest concentration of the analyte that gives a measurable response. The LOD and LOQ were found to be 4.24 µg mL^{-1} and 14.12 µg mL^{-1}, respectively, which indicate adequate sensitivity of the method. The LOD and LOQ values determined are effected by the

separation conditions i.e. the choice of solvents, additives and the nature of the stationary phase and the analyte; instrumentation and detection wavelength and data system solvents other than AR grade solvent can result in large changes in single to noise ratio due to base line noise and drift.

Table 1. Recovery studies (n = 3)

S. No.	Herbal extract/ Formulation	Amount of (-) epicatechin Present (μg)	Amount of (-)-epicatechin added (μg)	Recovery[a], %	%RSD	SE
1	Aqueous methanol (50%)	289.7	10, 20 and 30	98.5± 0.38	0.0039	0.219
2	Methanol	400.1	10, 20 and 30	98.1± 0.49	0.0050	0.285
3	Aqueous	210.4	10, 20 and 30	98.0± 0.44	0.0045	0.252
4	Formulation-I	10.32	10, 20 and 30	98.4± 0.29	0.0029	0.167
5	Formulation-II	7.75	10, 20 and 30	98.0± 0.17	0.0018	0.100
6	Formulation-III	4.52	10, 20 and 30	98.3± 0.23	0.0023	0.133

a – Average of three different quantities of (-)-epicatechin added (10, 20 and 30 μg), RSD – Relative standard deviation, SE – Standard error.

The precision (Table 2) of the methods were studied by carrying out experiments by changing conditions. It was observed that there were no marked changes in the chromatograms. The values obtained demonstrated the suitability of the system for the analysis of the above drug system suitability parameters might fall within ± 3% standard deviation range, during routine performance of the method.

Table 2. Precision of the HPLC method (n = 3, 5 μg spot^{-1})

	Days	% (-)-Epicatechin		
		Aqueous methanol (50%)	Methanol	Aqueous
Herbal Extracts	Day 1st	13.16	17.63	9.15
	Day 2nd	13.11	17.32	9.08
	Day 3rd	13.02	17.05	8.98
	Mean	13.10	17.33	9.07
		Formulation I	Formulation II	Formulation III
Marketed formulation	Day 1st	10.30	7.75	4.52
	Day 2nd	10.28	7.73	4.49
	Day 3rd	10.28	7.72	4.47
	Mean	10.29	7.73	4.49

An identified peak of (-)-epicatechin at retention time 4.60 was observed in the chromatogram of the extracts along with other components. There was no interference in analysis from the other components present in the extracts (Fig. 2A). The concentrations of (-)-epicatechin in aqueous, 50% methanolic and methanolic extract of *Pterocarpus marsupium* were found to be13.14%, 17.46% and 9.15% (w/w), respectively. An identified peak of (-)-epicatechin at retention time 4.60 was observed in the chromatogram of sample solutions extracted from formulations. There was no interference in analysis from the other active components and excipients present in the formulations (Fig. 2B). The total (-)-Epicatechin content in pharmaceutical formulation-1, formulation-2 and formulation-3 were found to be

0.021%, 0.019% and 0.019% (w/w), respectively. The percentage recovery from the formulations was found to be 98.0 to 98.4%.

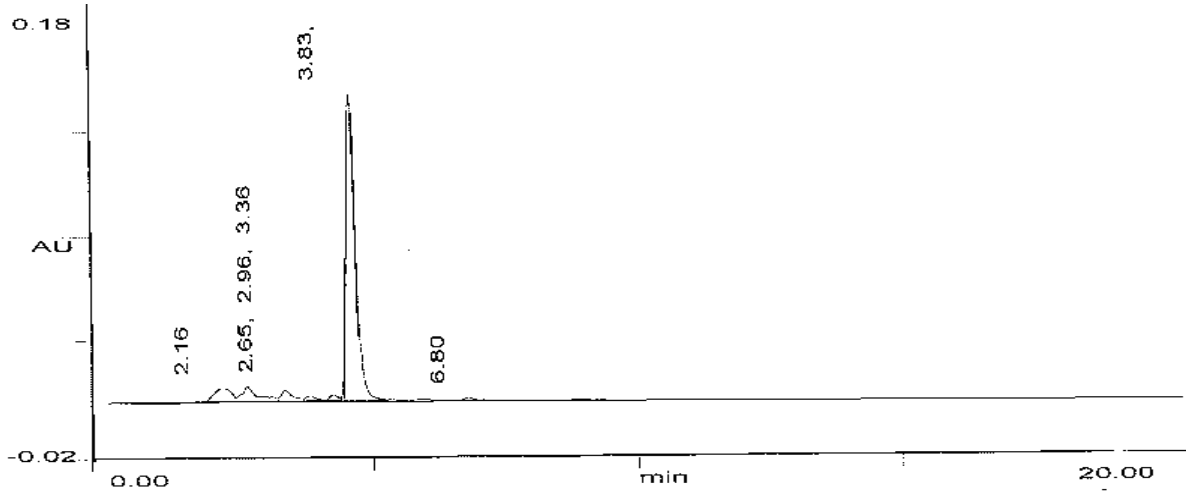
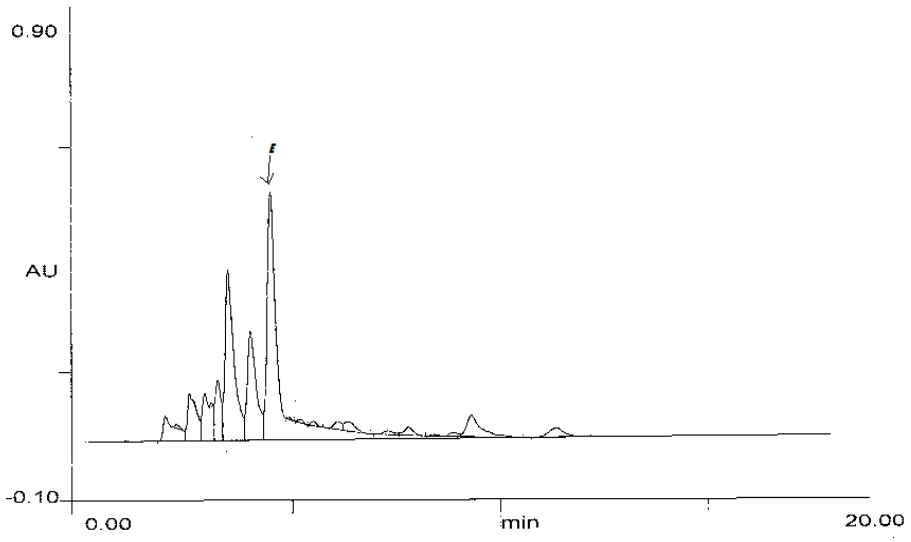
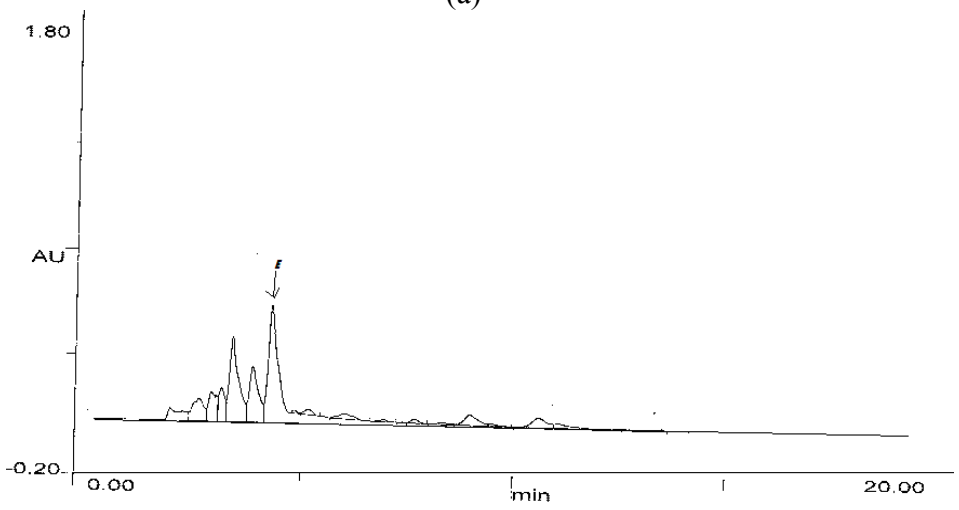


Fig. 2A– Chromatogram of standard solution of (-)-epicatechin (48 µg mL^{-1}),



(a)



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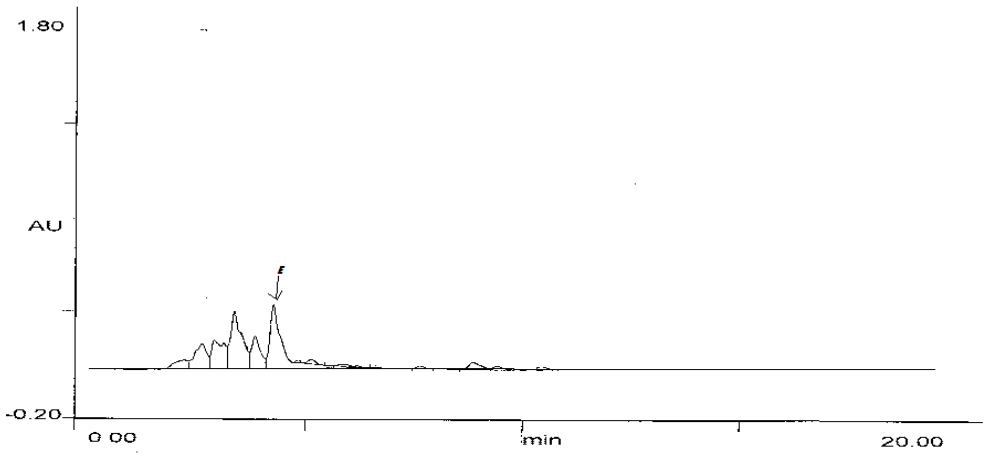
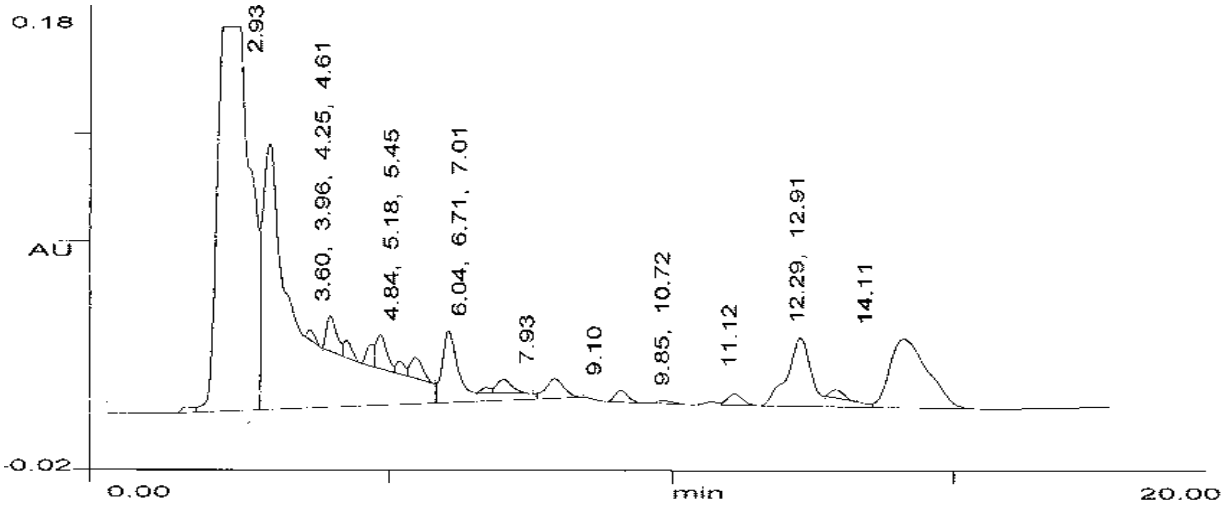
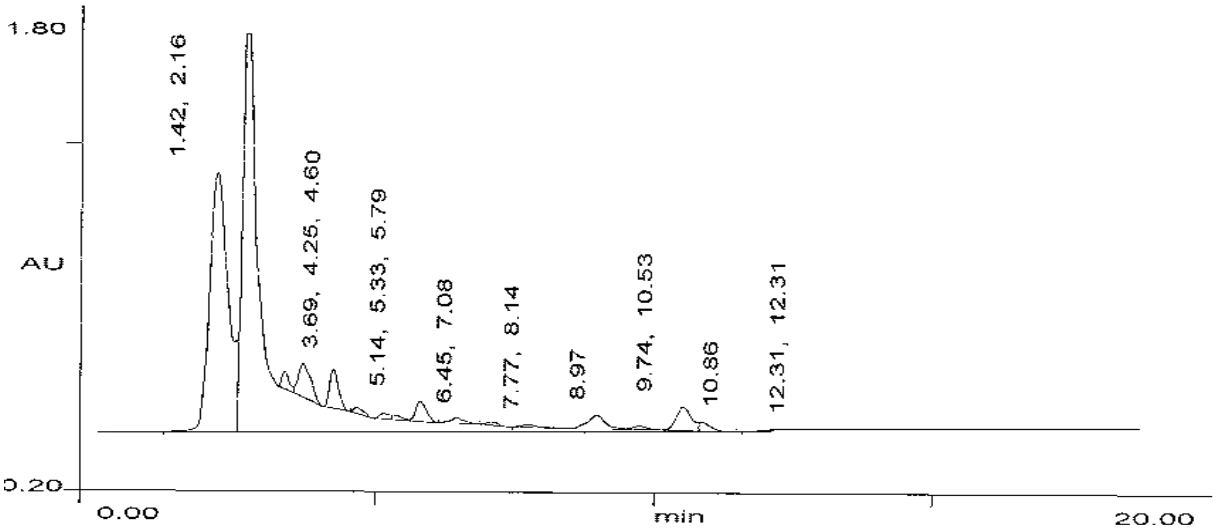


(c)

Fig. 2B - Chromatogram of extract of *Pterocarpus marsupium.* (a) Aqueous methanol (50%): (b) Methanol: (c) Aqueous. Peak identification: ***E***: (-)- epicatechin.



(a)



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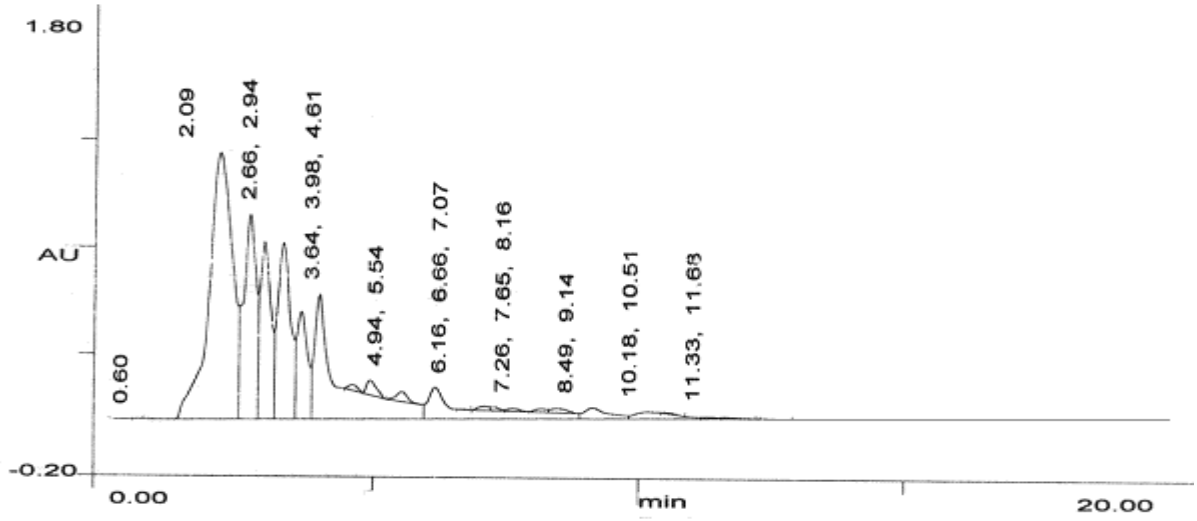

(c)

Fig. 2C - Chromatogram of (-)-epicatechin containing pharmaceutical formulations. (a) Formulation I (b) Formulation II (c) Formulation III

4. Conclusion

From the above studies, it can be concluded that HPLC method can be successfully used for estimation of (-)-epicatechin in extracts and polyherbal formulations. The developed HPLC method for this estimation of (-)-epicatechin is accurate, linear, rugged, simple and rapid. Statistical analysis proves that the method is reproducible and selective for the analysis of (-)-epicatechin.

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