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HPTLC and HPLC analysis of *T. chebula* extracts prepared using microwave and ultrasonication assisted extraction methods

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Abstract
Objective: The medicinal value of the plants lies in the bioactive phytochemical constituents that produce definite physiological effects on human body. These natural compounds form the base of modern drugs. Thus, it is very important to develop effective and selective extraction methods for the recovery of these compounds. The aim of this study is to prepare *Terminalia chebula* extracts were using the microwave and ultrasonication assisted extraction methods and to determine the efficiency of the extraction method by a comparative study
Methods: *Terminalia chebula* extracts were prepared using methods of MAE and UAE. The extracts were analysed by HPLC. The tannin and gallic acid content of the extracts were determined and compared with that of the conventional extract. Fingerprint profiling of the extracts using HPTLC was also done.
Results: The study revealed an increase in the concentration of tannin and gallic acid establishing the superiority of the microwave and sonication extractions compared to the conventional extraction method. The HPTLC profile of the conventional, microwave and sonication extracts of *T. chebula* was compared and the difference in the peak display of the extracts is indicative of additional compounds being extracted as well as an increase in the concentration of certain compounds
Conclusion: Thus it can be concluded that microwave and ultrasonication assisted extraction methods can be useful tool for phytochemical extraction.

Keywords: *Terminalia chebula*, Microwave, Ultrasonication, HPLC, HPTLC

1. Introduction
The fruit of *Terminalia chebula Retizus* (Combretaceae) commonly known in India as Harad (Sanskrit: Haritaki) is found throughout India and Southeast Asia. *T. chebula* has been reported to an exhibit variety of biological activity including anticancer, antioxidant, antidiabetic, antibacterial, antiviral, purgative, and astringent and blood purifier. In *T. chebula*, 33% of the total phytoconstituents are hydrolysable tannins (which may vary from 20-50%) and are responsible for different pharmacological activities [1-6].
Growing interest in plant secondary metabolites and their diverse pharmacological effects has prompted the need to review the traditional phytochemical extraction technologies and develop new economical, efficient and rapid extraction technologies for enhancing the concentration of phytochemicals. Since the general methods of extraction had several drawbacks in terms of the yield, amount of solvent and time for extraction certain new extraction methods including microwave assisted extraction (MAE), ultrasonication assisted extraction(UAE), supercritical fluid extraction etc. are gaining importance [7].
Microwave Assisted Extraction (MAE) of constituents from plant material has shown tremendous research interest and potential in the recent years. Microwaves are non-ionizing electromagnetic waves of frequency between 300 MHz to 300 GHz. Inside the plant cell when the moisture gets heated due to microwave effect it evaporates generating tremendous pressure on the cell wall due to swelling of the plant cell. The pressure pushes the cell wall from inside, stretching and then ultimately rupturing it, which facilitates leaching out of the active constituents from the ruptured cells to the surrounding solvent, thus improving the yield of phytoconstituents [8].
Ultrasonication Assisted Extraction (UAE) uses high frequency sound waves (20–50 kHz) to create minute pores in the cell wall of plant cells and thereby release the phytochemicals from plant materials. This extraction process is fast compared with traditional laboratory methods because of particle disruption of the plant material [3].This extraction methodology was used for the isolation of essential oils, polysaccharides and bioactive phytochemicals including menthol, cardiac glycosides, pyrethrins and camptothecin [9].

2. Materials and Methods

2.1 Collection and Authentication

The sundried fruits of *T. chebula* was provided by Konark Herbals and Healthcare, Mumbai and authenticated by the Botany Department of the College. The plant material was powdered and the extracts were prepared.

2.1.1. Chemicals: Chemicals and solvents were purchased from Merck, India and were of either analytical or HPLC grade.

2.1.2. Conventional organic solvent extraction [10]: 50g of dried and powdered plant material of *T.chebula* was added to 500ml of methanol and left overnight. The extracts were filtered using Whatman no.1 filter paper and used for further studies after evaporating the solvent under vaccum in a rotary evaporator. The residues were dissolved in DMSO and stored at 4 ^{o}C.

2.1.3. Microwave assisted extraction (MAE): 50g of dried and powdered plant material was exposed to microwave energy for a time duration from 5 minutes at 100% power and then added to 500ml of the methanol and left overnight.

2.1.4. Ultrasonication assisted extraction (UAE): 50g of dried and powdered plant material was added to 500ml of methanol and sonicated for time duration of 5 minutes and left overnight.

The extract of *T.chebula* prepared using MAE and UAE was then filtered using Whatman no.1 filter paper. The post treatments of the extracts were same as that of the conventional extract.

2.1.5. Tannin assay for *Terminalia chebula* by Indigo carmine method[11]: The amount of tannins in the plant extracts was determined by the Indigo carmine method. To 1ml of sample, 2.5ml of indigo carmine solution and 75ml distilled water was added. This mixture was titrated against 0.04N $KMnO_4$ solution ("A" ml). To determine the volume of $KMnO_4$ ("B" ml) used for non-tannin compound, each sample of 5ml quantity was mixed with 2.5ml of 2% gelatin solution and 5ml of the acidic NaCl solution. After shaking the mixture for 15min, it was filtered through Whatman filter paper no.1. 2.5ml of filtrate was mixed with same volume of the indigo carmine solution and 75ml H_2O. This mixture was again titrated against 0.04N $KMnO_4$ solution. The percentage of tannin is calculated as:

%Tannin (as gallotannic acid) = (A-B) x 100 x ('g' of tannin / ml of KMnO4)

ml of sample solution

Where, A = Total tannin material; B = Non tannin material; A-B = True tannin material; 1ml of KMnO4 = 0.0042g of tannin (as gallotannic acid)

2.2. Screening of Extracts

Plant extracts prepared using the methods of microwave and ultrasonication were screened for the presence additional compounds and an increase in yield of compounds in comparison to the conventional extracts by carrying out:

2.3. HPTLC Analysis

The fingerprint profile of the plant extracts was developed by performing the densitometric HPTLC analysis. Samples were loaded as 8 mm band length in the 10 x 10 Silica gel 60F TLC plate using CAMAG LINOMAT 5 instrument with a 100µl Hamilton syringe and run in developing chambers saturated with the respective mobile phase upto a solvent front of 80mm. The plates were visualised in a photo documentation chamber CAMAG Visualizer: 150503. Images were captured (at 254nm and white light after derivatization) and the plates scanned to display the Peak table and Peak densitogram. The analysis of *Terminalia chebula* extracts was done as per the Indian Herbal Pharmacopoeia (2002) using the mobile phase of Mobile phase: Toluene: Ethyl acetate: Formic acid: Methanol (3: 3: 0.8: 0.7) and the Derivatization agent of Alcoholic Ferric chloride

2.4. HPLC analysis [12]

HPLC analysis of the plant extracts were carried out in the Mobile phase: methanol: acetic acid: deionized water (15:5:80).The Column used was C18 silica column (4.6mm x 150mm), 5µm particle and the conditions maintained were as follows: Flow rate: 1ml/min., Detector: UV detector at 280 nm, Injection volume: 20µl. From the chromatograms obtained, the retention time and peak characteristics were noted.

3. Results and Discussion

3.1 Estimation of tannin content

The tannin content of the extracts prepared using the conventional method, MAE and UAE under optimized conditions were estimated by the Indigocarmine method. The tannin content of the conventional extract was found to be 20.63±1.2% in terms of gallotannic acid. A similar value of tannin content was also reported by Chia Ling Chang (2012). The tannin content of the extracts prepared using MAE and UAE were found to be 35.2±1.7% and 25.8 ± 1.52% respectively in terms of gallotannic acid. Thus, extraction methods of microwave and ultrasonication resulted in a 14.56±0.5% and 5.16±0.12% increase in the yield of tannins.

3.2 HPTLC of extracts of *T. chebula*

For the HPTLC analysis the extracts of *T. chebula* was run in a mobile phase of Toluene: Ethyl acetate: Formic acid: Methanol (3: 3: 0.8: 0.7). The samples were loaded in the order of conventional extract, microwave extract, Ellagic acid and ultrasonication extract. The plates were visualized at 254 nm and after derivatization with alcoholic ferric chloride reagent.

At 254nm, the Rf values obtained are as shown in **Table 1 - 3**. From the tabulations it can be summarized that the samples when loaded in a volume of 2 µl, the chromatograms showed the presence of 7, 9 and 6 peaks for the conventional, microwave and sonication samples respectively **(Fig.1)**.

On derivatizing the HPTLC plates with alcoholic ferric chloride, blue and brown spots were observed. The chromatograms were scanned at 520nm. The Rf values are as shown in **Table 4 - 6**. On loading 5 µl of the extracts, the conventional extract showed the presence of 5 peaks whereas the extracts prepared using microwave and sonication showed the presence of 6 peaks each **(Fig.2)**.

3.3. HPLC

HPLC analysis quantified the Gallic acid content in the different extracts. The chromatograms of the *T. chebula* extracts and standard Gallic acid are as shown in **Graph 1 - 4**. The amount of Gallic acid in the conventional, microwave and sonication extracts were estimated to be 0.307g%, 0.421g% and 0.353g%. The difference in the concentration of Gallic acid in the extracts confirm that the extraction procedure

influences the extraction of phytochemicals and MAE and UAE methods are superior compared to the conventional extraction procedures for yielding extracts rich in Gallic acid.

4. Conclusion

On a comparative study of the microwave assisted extraction and ultrasonication assisted extraction with the conventional organic solvent extraction method it can be concluded that both microwave and sonication assisted extraction methods proved to be effective in increasing the yield of tannins and gallic acid. Also a difference in the peak characteristics is noticed for the microwave and sonication extracts compared to the conventional extract this could be because the cell wall of plant cells are ruptured as an result of microwave irradiation and ultrasonication thereby influencing the release of phytochemicals into the solvents. Polyphenols and tannins are plant secondary metabolites and are very important by virtue of their antimicrobial and antioxidant activity. *T. chebula* extracts prepared using microwave and ultrasonication would therefore have a higher therapeutic efficiency due to high increased phytochemical yield and the presence of additional compounds, thus increasing their application in the field of herbal medicine Therefore MAE and UAE are procedures that have a high efficiency of extraction and thus can be applied for the extraction of other plant materials.

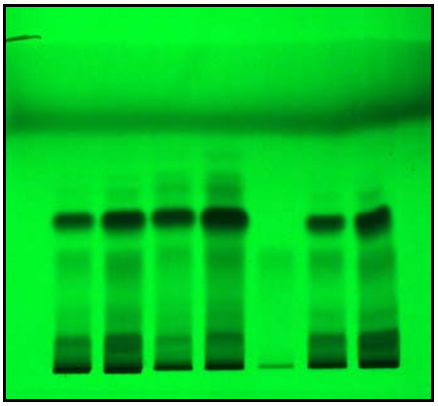


Fig 1: HPTLC of *T. chebula* extracts at 254nm
Key: Left to Right; Lane 1-Conventional extract (2µl), Lane 2 - Conventional extract (5µl), Lane 3- Microwave extract (2µl), Lane 4- Microwave extract (5µl), Lane 5- Ellagic acid (2µl), Lane 6- Ultrasonication extract (2µl), Lane 7 - Ultrasonication extract (5µl).

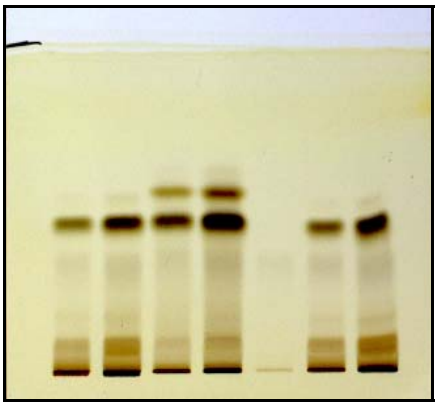


Fig 2: HPTLC of *T. chebula* extracts after derivatization
Key: Left to Right; Lane 1- conventional extract (2µl), Lane 2- conventional extract (5µl), Lane 3- Microwave extract (2µl), Lane 4- Microwave extract (5µl), Lane 5-Ellagic acid (2µl), Lane 6 - Ultrasonication extract (2µl), Lane 7 -Ultrasonication extract (5µl).

Table 1: HPTLC profile of Conventional extract of *T. chebula* at 254nm

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.06	4.2	0.10	198.7	13.56	0.12	0.7	4582.7	6.41
2	0.14	0.5	0.17	43.4	2.96	0.21	0.4	1136.6	1.59
3	0.24	4.7	0.33	158.7	10.83	0.38	0.1	8354.1	11.68
4	0.38	0.3	0.45	644.7	43.99	0.50	38.8	25569.2	35.74
5	0.51	41.7	0.53	53.6	3.66	0.55	22.7	1470.6	2.06
6	0.55	22.6	0.57	38.1	2.60	0.59	26.4	843.9	1.18
7	0.59	25.7	0.74	328.2	22.40	0.91	1.5	29580.8	41.35

Table 2: HPTLC profile of Microwave extract of *T. chebula* at 254nm

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.07	12.7	0.07	12.9	0.84	0.08	0.1	63.0	0.08
2	0.08	0.6	0.11	152.0	9.92	0.15	0.1	3016.8	4.00
3	0.15	0.2	0.17	16.5	1.08	0.19	0.6	239.8	0.32
4	0.19	0.9	0.21	14.2	0.93	0.24	0.5	204.2	0.27
5	0.25	0.4	0.36	130.4	8.51	0.40	46.4	6890.4	9.14
6	0.40	46.5	0.47	643.1	41.97	0.51	120.4	29960.0	39.74
7	0.51	120.6	0.55	179.4	11.71	0.62	13.1	8289.6	11.00
8	0.62	13.7	0.65	63.3	4.13	0.67	52.9	1687.8	2.24
9	0.68	52.3	0.75	320.5	20.92	0.93	2.7	25034.8	33.21

Table 3: HPTLC profile of Ultrasonication extract of *T. chebula* at 254nm

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.09	0.1	0.12	227.5	15.07	0.15	0.1	5540.3	7.18
2	0.17	0.6	0.20	55.6	3.68	0.23	0.3	1143.4	1.48
3	0.26	2.6	0.35	197.6	13.09	0.40	18.2	10400.7	13.49
4	0.40	18.3	0.46	612.2	40.55	0.52	21.9	24972.2	32.38
5	0.52	22.2	0.53	38.2	2.53	0.56	0.3	708.6	0.92
6	0.61	0.0	0.75	378.7	25.08	0.93	5.9	34354.2	44.55

Table 4: HPTLC profile of Conventional extract of *T. chebula* after derivatization

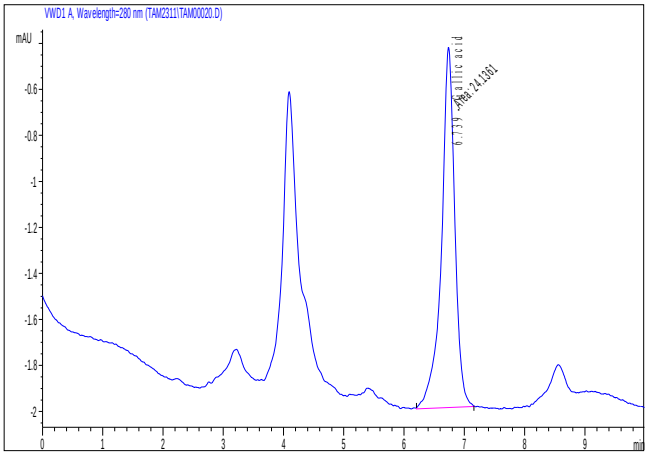
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.10	25.1	0.11	44.0	6.83	0.12	1.3	415.8	1.80
2	0.14	0.0	0.17	22.2	3.45	0.20	0.2	576.1	2.49
3	0.24	0.4	0.32	54.6	8.48	0.38	6.2	3236.4	14.01
4	0.38	6.5	0.45	482.1	74.85	0.49	14.6	17793.5	77.04
5	0.49	14.7	0.53	41.1	6.38	0.56	1.7	1074.6	4.65

Table 5: HPTLC profile of Microwave extract of *T. chebula* after derivatization

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.10	3.3	0.12	53.1	4.75	0.14	0.4	737.4	1.68
2	0.16	2.8	0.17	11.2	1.00	0.19	4.6	172.1	0.39
3	0.24	0.2	0.34	74.7	6.69	0.38	50.2	4690.1	10.70
4	0.38	50.4	0.46	551.4	49.34	0.51	105.9	24725.8	56.41
5	0.51	107.4	0.54	389.5	34.85	0.59	34.8	12675.3	28.92
6	0.59	35.0	0.60	37.8	3.38	0.65	0.1	828.4	1.89

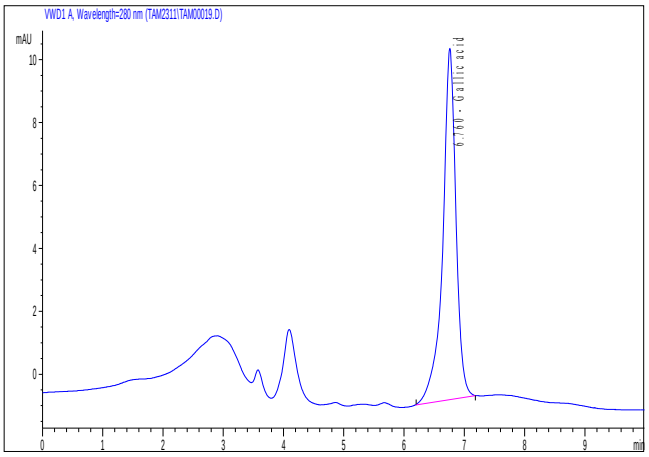
Table 6: HPTLC profile of Ultrasonication extract of *T. chebula* after derivatization

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.10	6.1	0.12	74.0	14.02	0.14	0.7	1360.0	6.32
2	0.16	0.5	0.18	27.2	5.15	0.21	0.0	565.7	2.63
3	0.25	0.1	0.34	56.7	10.73	0.38	33.5	3391.2	15.76
4	0.39	33.7	0.46	339.1	64.19	0.51	12.7	15657.3	72.77
5	0.51	12.9	0.53	19.6	3.70	0.57	0.2	450.8	2.10
6	0.63	0.1	0.65	11.6	2.21	0.66	1.1	91.9	0.43



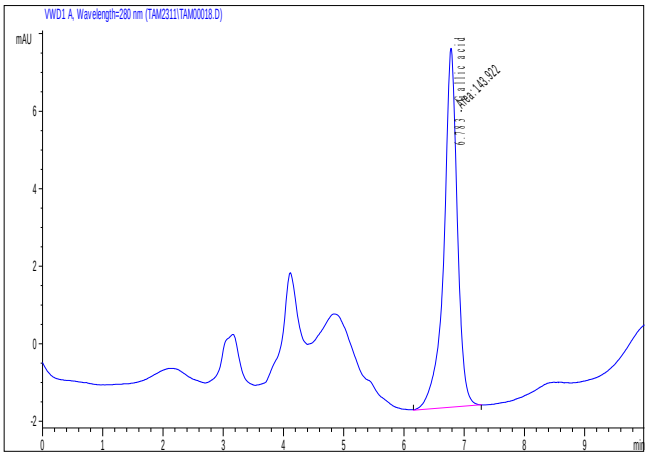
Graph 1: HPLC chromatogram of *T.chebula* conventional extract

Sr. No.	Time (mins)	Area	Height	Width	Area%	Symmetry
1	6.739	124.1	1.6	0.2569	100.000	1.126



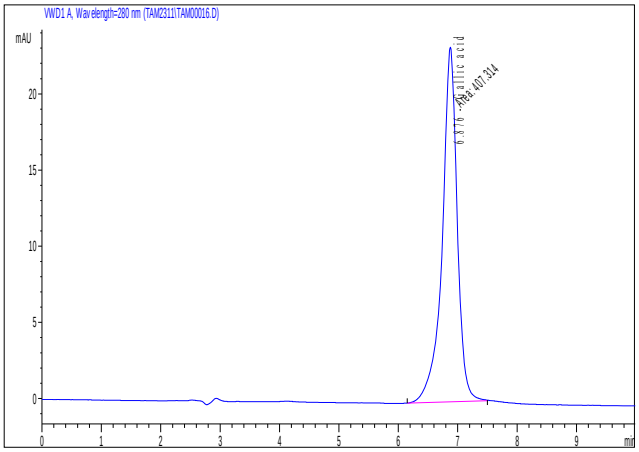
Graph 2: HPLC chromatogram of *T. chebula* microwave extract

Sr. No.	Time (mins)	Area	Height	Width	Area%	Symmetry
1	6.76	170.1	11.2	0.2229	100.000	1.176



Graph 3: HPLC chromatogram of *T. chebula* ultrasonication extract

Sr. No.	Time (mins)	Area	Height	Width	Area%	Symmetry
1	6.783	143.9	9.3	0.259	100.000	1.134



Graph 4: HPLC chromatogram of Gallic acid

Sr. No.	Time (mins)	Area	Height	Width	Area%	Symmetry
1	6.876	403.1	23.2	0.2549	100.000	1.186

5. Acknowledgement

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