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HPTLC Finger Print of Phytophenols and Flavonoids of *Artimisia Nilagirica* under Different Extraction Regimen

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ABSTRACT

To detect and estimate the phenolics and flavonoids of *Artimisia nilagirica* in different solvents under Cold Percolation (CP), Micro-Wave-Assisted Extraction (MAE) system and by HPTLC finger print analysis. Preliminary screening of phenolic components in hexane treated chlorophyll free extracts of solvent groups was studied qualitatively by specific chemical tests and total polyphenols and flavonoids were estimated quantitatively by UV-VIS-Spectrophotometer following CP and MAE system. Distinct chemo-profile of the extracts was analyzed by HPTLC technique with CAMAG TLC plate Heater-III, CAMAG-TLC scanner-3, Optical Filter K-400 and CAMAG *win CATS* planar chromatography manager software. Among two methods of extraction more components of polyphenols were detected and significantly higher contents of phenolics and flavonoids were estimated in MAE method than CP method. Out of two solvent types, organic solvent mixtures recovered significantly higher polyphenols and flavonoids in comparison to aqueous solvent where 80% methanol was to extract the maximum. HPTLC finger print analysis of phenolics revealed 7 (R_f 0.02- 0.93), 15 (R_f 0.01- 0.93), 16 (R_f 0.01- 0.89), 10 (R_f 0.01- 0.98) and 12 peaks (R_f 0.01- 0.93) for water, 80% methanol, ethanol, acetone and mixture solvents respectively under traditional CP method. But, in MAE method the peaks were 10 (R_f 0.01- 0.97), 19 (R_f 0.01- 0.92), 19 (R_f 0.01- 0.96), 16 (R_f 0.01- 0.99) and 16 (R_f 0.01- 0.95) respectively. Phyto-chemicals can be better screened qualitatively and estimated quantitatively in organic solvent extracts under MAE system by HPTLC finger print analysis than the aqueous solvent and CP method.

Keywords: *Artimisia nilagirica*, polyphenol, flavonoid, HPTLC finger print.

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INTRODUCTION

Endogenously generated Reactive Oxygen Species (ROS) and free radicals are fundamental to aerobic life and are cooperatively managed by oxidative defence mechanisms. Their additional burden acquired from within/ outside the body creates imbalance between pro-oxidant and antioxidant balance leading to oxidative stress and tissue injury[1]. It leads to age related molecular degenerations, angina, arthritis, ascites, atherosclerosis, some type of cancer, diabetes mellitus, fatty liver, haemorrhagic disease syndrome and Alzheimer's diseases in human and animals and even is involved in pathogenesis of several viral diseases including hepatitis, influenza and AIDS by transferring a normal benign RNA virus to a virulent one. Although, the detrimental effect of oxidative stress can be managed with some exogenous vitamins (A, E and C) and minerals (Se and Zn) but the risk of toxicity on over dosage cannot be over ruled. Natural anti-oxidants of herbal origin with free radical scavenging activity lack adverse effects and are cost effective. Diverged chemical composition of plants with phenolics and flavonoids is responsible to exhibit efficient antioxidant properties. In the need of safe natural anti-oxidants, scientific world emphasizes on phyto-constituents as lead molecules in development of future generation safe drugs.

Effect study of phyto-constituents can only be analyzed after extraction in suitable solvents[2]. Conventional techniques of extraction are prone to loss of constituents, limited to certain components, time and solvent consuming. Advanced technique of microwave assisted extraction (MAE) is promising with less time/ solvent consumption and more convenient for thermo-labile constituents[3]. As phyto-constituents vary in chemical characteristics, polarities and distribution in the plant matrix[4], their recovery at higher concentration depends on extraction techniques and the nature/ volume of solvent. Extraction, screening and detection of phyto-chemicals with different solvents/ techniques advocate better for the effect study. Following traditional Cold Percolation (CP) method with water cannot over rule the probability of potency suppression due to under recovery of active phyto-constituents. Microwave Assisted Extraction (MAE) method supersedes CP method in recovering these constituents. *Artimisia nilagirica* exhibits insecticidal[5], antifungal[6], antibacterial[7] activities and the shoot is used as anthelmintic, antiseptic, anti inflammatory, appetizer, expectorant, digestive tonic and even as anti-malarial drug by tribes of Odisha province. Therefore, the project aims to analyze the quality and quantity of active phenolic components of *Artimisia nilagirica* extracted in different solvents under MAE and CP method followed by HPTLC finger print analysis.

MATERIALS AND METHOD

Plant sample

Artimisia nilagirica collected from different places in Khurdha district of Odisha Province was identified and classified[8]. The whole shoot of the plant was collected at pre-flowering stage, cleaned, dried under shade and ground into fine structure for preparation of extracts.

Solvents

Gr-A: Distilled and deionised water (100%), Gr-B: Methanol: Water, Gr-C: Ethanol: Water and Gr-D: Acetone: Water (80:20%) each and Gr-E: Methanol: ethanol: acetone: water (30:30:30:10%)

Extraction

Extraction of polyphenols and flavonoids was conducted in two trials. In trial-I, extraction of phyto-constituents was done in MAE system by Multiwave 3000-801V (Anton Paar) digestion system[9] where 2 g of powdered plant shoot in 20ml of solvent was heated at 80^0 C for a period of 25 minutes followed by cooling for 15 minutes. In trial-II, extraction of phyto-constituents were made by CP method[10] where 2 g of powdered plant shoot in 40 ml of solvent was kept on magnetic stirrer at 10^0C temperature for a period of 24 hrs and then the supernatant was filtered by Whatman filter paper at room temperature to collect the residue free extract.

Hexane treatment

Equal volumes of filtered crude extract and hexane were mixed, kept for 2 minutes and aspirated the superficial fluid carefully to obtain chlorophyll free extract.

Qualitative detection of phyto-constituents

Phenolic components in shoot extracts were detected by chemical tests.

Test	Constituent	Protocol	Colour/ texture in +ve test
Salkowski	Terpenoids	1ml of extract+ 2ml of chloroform + 1 ml of conc. H_2SO_4.	Reddish brown at interface
$FeCl_3$	Tannins	1 ml extract + 1 ml of 0.1% $FeCl_3$.	Brown-green/ blue black
Shinoda's	Flavonoids	2 ml extract + Few drops of 1% NH_3 solution.	Yellow
Killer-killani's	Cardiac glycosides	5 ml of extract + 2 ml of glacial CH_3COOH containing 1 drop of 0.1% $FeCl_3$. It was added to 1 ml of conc. H_2SO_4.	Brown ring at interface
Frothing	Saponins	10 ml of extract + 5 ml of distilled water. The shacked froth was mixed with 3 drops of olive oil.	Formation of emulsion
Libarman-Burchard's	Steroids	1 ml of extract + 2 ml of acetic anhydride + 2 ml of conc. H_2SO_4.	Colour change from violet to green

Qunantitative estimation of Phenolics and flavonoids

The shoot extracts in different solvents were estimated for total polyphenols[11] and flavonoids[12] by chemical exposure followed by spectro-photometric assessment of optical absorbance.

HPTLC Finger printing of Phenolic components

Distinct chemo-profile of the extract was studied by HPTLC technique[13]. The silica gel TLC plates (EMerck, Germany) of $60F_{254}$ (20x10x 0.0002 cm) were spotted with 5 µl of extracts in the form of band in HPTLC glass syringe and dried at 60-70^{0}C by CAMAG TLC plate Heater-III for 60-90 seconds. The CAMAG Automatic Developing Chamber-2 was saturated with the 50 ml. mobile phase (Toluene: Ethyl acetate: Formic acid: (8.5: 1.0: 0.5) for 10 min at room temperature. The plates were exposed to mobile phase in the Chamber-2 till movement of solvent occurs up to 95% of the plate. Then the plates, after air drying for 10 minutes, were scanned by CAMAG-TLC scanner -3 in remission-absorbance mode at 254 nm, using optical filter K400, under control of Camag *win CATS* planar chromatography manager software with specific slit dimension (5× 0.45mm), the sample track and spot spectrum scanning speed (20 mm/ sec), band length (5mm) and distance between tracks (8.9mm).

Statistical analysis

The data was subjected to analysis of variance to test the significance of difference of mean values between different groups following the method of Snedecor and Cochran[14] and by using Graph Pad *Instat* 3 software.

RESULTS AND DISCUSSION

Detection of phenolic compounds

The qualitative detection of phenolic compounds in chlorophyll free shoot extracts of *Artimisia nilagirica* in five different solvents (Table 1) reveals that, tannin, flavonoid, terpenoid, saponin and steroid are absent in CP method where as all the constituents under study exhibit positive result in MAE method leaving terpinoid for negative result at Gr-A. Organic solvent mixtures at Gr-B, C, D and E extract all the components except few in CP method in comparison to water at Gr-A. Methanol (80%) at Gr-B depicts better recovery of phyto-constituents as compared to rest of the solvents. MAE method of extraction and organic solvent mixtures particularly methanol (80%) supersedes the CP method and aqueous solvent in recovery of more Phenolic constituents.

Table 1: Screening of phenolic constituents in chlorophyll free extracts of *Artimisia nilagirica* in different solvents by CP and MAE method basing on the intensity of colour produced.

Methods	**CP**	**MAE**	**CP**	**MAE**	**CP**	**MAE**	**CP**	**MAE**	**CP**	**MAE**
Solvent groups	**Gr-A**		**Gr-B**		**Gr-C**		**Gr-D**		**Gr-E**	
Tannin	-	+	+	++	+	+	+	+	+	+
Flavonoid	-	+	+	++	+	+	+	+	+	+
Terpenoid	-	-	-	+	-	++	-	+	-	+
Cardiac glycosides	+	+	+	+	-	+	+	+	+	+
Saponins	-	++	-	+	-	+	-	-	-	-
Steroids	-	+	+	++	+	++	-	+	-	+

'+' denotes presence and '-' denotes absence of the component in the test sample

Estimation of phenolics and flavonoids under different methods

Table 2 compares the contents of total phenolics and flavonoids extracted from in five different solvents under MAE and CP methods. MAE method extracts significantly higher contents ($p<0.05$) of total phenolics in all the solvents except Gr-C and the maximum concentration (2.54 ± 0.17) is recovered in Gr-B solvent followed by C (2.05 ± 0.12), E (1.97 ± 0.07), D (1.86 ± 0.02) and A (0.92 ± 0.04) than those extracted under CP method. In addition, flavonoids extracted in MAE method depicts significantly higher contents ($p<0.05$) in Gr-A and B solvents only but methanol in Gr-B solvent extracts the maximum concentration (0.59 ± 0.03) followed by C (0.53 ± 0.06), E (0.47 ± 0.06), D (0.41 ± 0.05) and A (0.37 ± 0.05) in comparison to the corresponding contents observed in CP method. MAE method of extraction supersedes the traditional CP method in extracting significantly higher contents of phenolics and flavonoids.

Table 2: Total phenolics and flavonoids in chlorophyll free extracts of *Artimisia nilagirica* in different methods.

Solvent groups	**Total polyphenols (mg of Gallic Acid Equivalent, GAE / g of sample ± SE)**		**Total Flavonoids (mg of Rutin Equivalent, RE / g of sample ± SE)**	
	MAE	**CP**	**MAE**	**CP**
Gr-A	0.92 ± 0.04	0.61* ± 0.05	0.37 ± 0.05	0.19* ± 0.06
Gr-B	2.54 ± 0.17	1.67* ± 0.13	0.59 ± 0.03	0.36* ± 0.03
Gr-C	2.05 ± 0.12	1.76* ± 0.07	0.53 ± 0.06	0.38 ± 0.07
Gr-D	1.86 ± 0.02	1.53* ± 0.14	0.41 ± 0.05	0.34 ± 0.08
Gr-E	1.97 ± 0.07	1.56* ± 0.06	0.47 ± 0.06	0.37 ± 0.05

Superscripts between columns in the same parameter shows significant difference (*$p<0.05$) within a row.

Estimation of phenolics and flavonoids under different solvents

The contents of total phenolics recovered from different organic solvent extracts in Gr-B, C, D and E is significantly higher ($p<0.001$) than those in water at Gr-A in both the methods of MAE and CP. However, the maximum phenolics are extracted from Gr-B and Gr-C solvent in MAE and CP methods in comparison to other solvents respectively. Gr-B-Methanol (80%), extracts significantly higher ($p<0.05$) flavonoids than rest of the solvents in MAE method only (Table 3).

Under MAE method 80% methanol at Gr-B has greater effect on extraction of total phenolics and flavonoids.

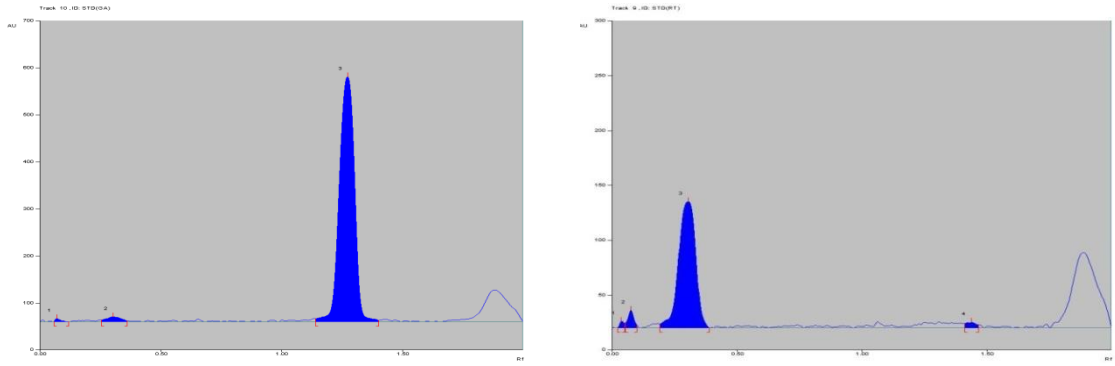
Table 3: Total phenolics and flavonoids in chlorophyll free extracts of *Artimisia nilagirica* in different solvents.

Methods	Constituents	Gr-A	Gr-B	Gr-C	Gr-D	Gr-E
MAE	Total polyphenols	0.92	2.54***	2.05***	1.86***	1.97***
	(mg of GAE / g)	± 0.04	± 0.17	± 0.12	± 0.02	± 0.07
	Total Flavonoids	0.37	0.59*	0.53	0.41	0.47
	(mg of RE/ g)	± 0.05	± 0.03	± 0.06	± 0.05	± 0.06
CP	Total polyphenols	0.61	1.67***	1.76***	1.53***	1.56***
	(mg of GAE / g)	± 0.05	± 0.13	± 0.07	± 0.14	± 0.06
	Total Flavonoids	0.19	0.36	0.38	0.34	0.37
	(mg of RE/ g)	± 0.06	± 0.03	± 0.07	± 0.08	± 0.05

Superscripts between columns shows significant difference (*p<0.05, ***p<0.001) within a row in comparison to Gr-A.

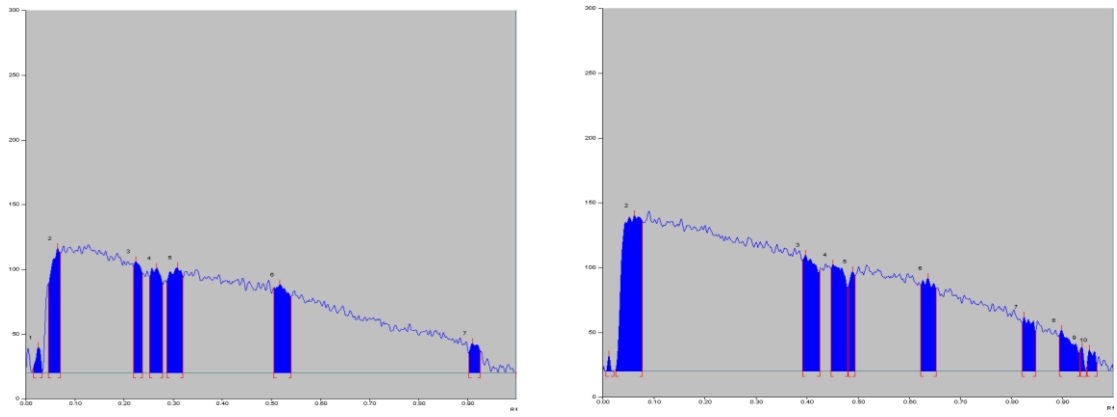
HPTLC finger print analysis

The chromatogram at Figure 1a and 1b presents the spots of Gallic Acid (R_f 1.19- 1.35) and Rutin (R_f 0.23- 0.35) standard for polyphenols for flavonoids respectively. The shoot extracts of *Artimisia nilagirica* under HPTLC finger print depicts variable number of peaks under MAE and CP method in different solvents. CP Method exhibits 7 (R_f 0.02- 0.93), 15 (R_f 0.01- 0.93), 16 (R_f 0.01- 0.89), 10 (R_f 0.01- 0.98) and 12 peaks (R_f 0.01- 0.93) for Gr-A, B, C, D and E solvents (Table 4 and Figure 2a, 3a, 4a, 5a and 6a) respectively. In MAE method the number of peaks in these respective solvent groups (Table 5) are 10 (R_f 0.01- 0.97), 19 (R_f 0.01- 0.92), 19 (R_f 0.01- 0.96), 16 (R_f 0.01- 0.99) and 16 (R_f 0.01- 0.95) as presented in Figure 2b, 3b, 4b, 5b and 6b. MAE method supersedes CP method in exhibiting more number of peaks in all the aqueous and organic solvent mixture groups and organic solvent groups in B, C, D and E extract more variants of polyphenols and flavonoids than in aqueous Gr-A to present more number of peaks.



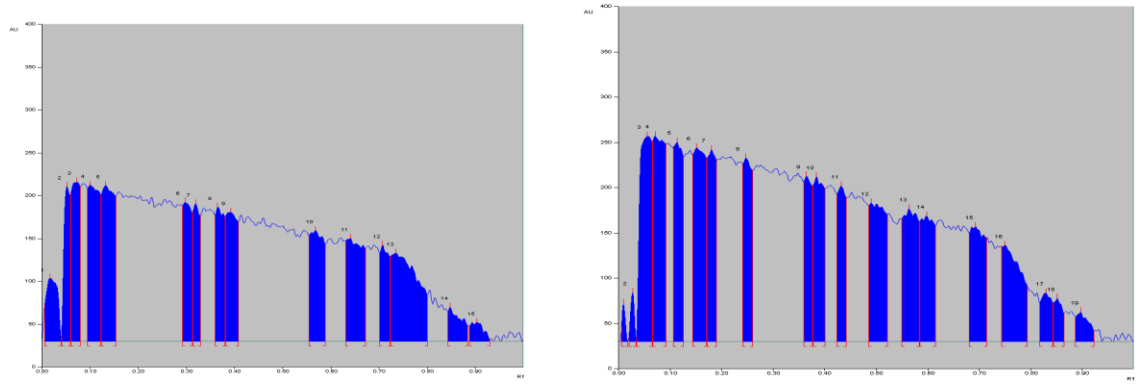
(a) Gallic Acid Standard 100mcg/ ml **(b) Rutin Standard 100mcg/ ml**

Figure 1: HPTLC chromatogram of Gallic acid and Rutin Standard



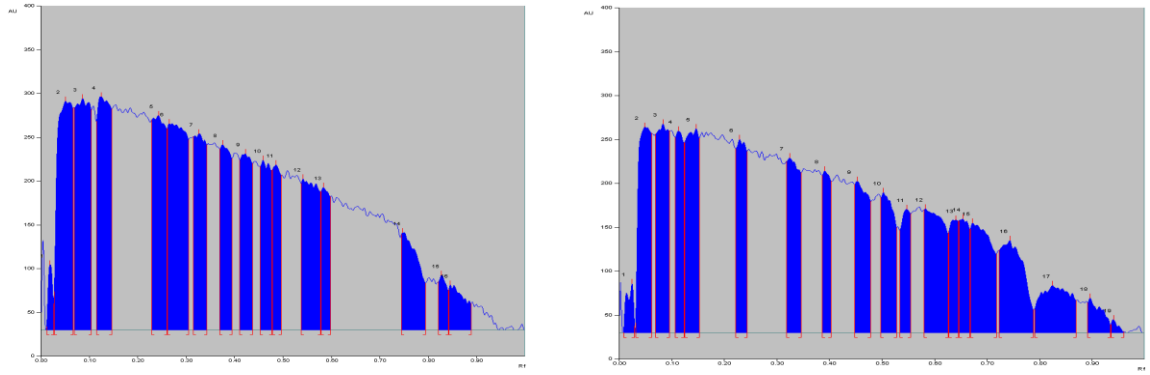
(a) Cold Percolation (CP) method (b) Micro-wave assisted Extraction (MAE) method

Figure 2: HPTLC chromatogram of aqueous (Gr-A) extract of *Artimisia nilagirica* shoot



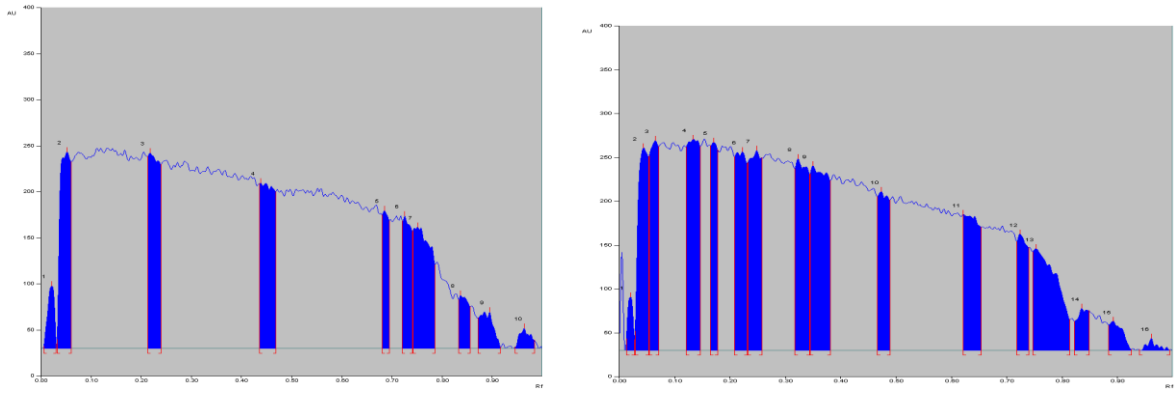
(a) Cold Percolation (CP) method (b) Micro-wave assisted Extraction (MAE) method

Figure-3: HPTLC chromatogram of 80% methanol (Gr-B) extract of *Artimisia nilagirica* shoot



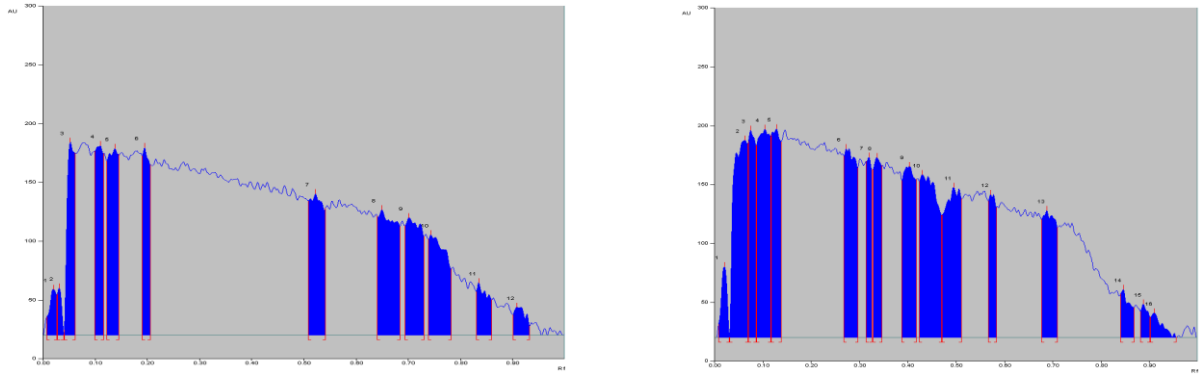
(a) Cold Percolation (CP) method (b) Micro-wave assisted Extraction (MAE) method

Figure-4: HPTLC chromatogram of 80% ethanol (Gr-C) extract of *Artimisia nilagirica* shoot



(a) Cold Percolation (CP) method (b) Micro-wave assisted Extraction (MAE) method

Figure-5: HPTLC chromatogram of 80% acetone (Gr-D) extract of *Artimisia nilagirica* shoot



(a) Cold Percolation (CP) method (b) Micro-wave assisted Extraction (MAE) method

Figure 6: HPTLC chromatogram of solvent mixture (Gr-E) extract of *Artimisia nilagirica* shoot

Table 4: R_f values (Start and End Position) of peaks in different solvent extracts of *Artimisia nilagirica* under CP method of extraction.

Peaks	Gr-A		Gr-B		Gr-C		Gr-D		Gr-E	
	Start	End	Start	End	Start	End	Start	End	Start	End
1	0.02	0.03	0.01	0.04	0.01	0.03	0.01	0.03	0.01	0.03
2	0.05	0.07	0.04	0.06	0.03	0.07	0.03	0.06	0.03	0.04
3	0.22	0.24	0.06	0.08	0.07	0.10	0.21	0.24	0.04	0.06
4	0.25	0.28	0.10	0.12	0.12	0.15	0.44	0.47	0.10	0.11
5	0.29	0.32	0.12	0.15	0.23	0.26	0.68	0.69	0.12	0.14
6	0.51	0.54	0.29	0.31	0.26	0.30	0.72	0.74	0.19	0.21
7	0.90	0.93	0.31	0.32	0.32	0.34	0.74	0.79	0.51	0.54
8	-	-	0.36	0.38	0.37	0.40	0.83	0.86	0.64	0.68
9	-	-	0.38	0.41	0.41	0.44	0.87	0.92	0.69	0.73
10	-	-	0.56	0.59	0.45	0.48	0.94	0.98	0.74	0.78
11	-	-	0.63	0.67	0.48	0.50	-	-	0.83	0.86
12	-	-	0.70	0.72	0.54	0.58	-	-	0.90	0.93
13	-	-	0.72	0.80	0.58	0.60	-	-	-	-

14	-	-	0.84	0.88	0.75	0.79	-	-	-	-
15	-	-	0.89	0.93	0.82	0.84	-	-	-	-
16	-	-	-	-	0.84	0.89	-	-	-	-

Table 5: R_f values (Start and End Position) of peaks in different solvent extracts of *Artimisia nilagirica* under MAE method of extraction.

Peaks	Gr-A		Gr-B		Gr-C		Gr-D		Gr-E	
	Start	End	Start	End	Start	End	Start	End	Start	End
1	0.01	0.02	0.01	0.02	0.01	0.03	0.01	0.02	0.01	0.03
2	0.03	0.08	0.02	0.03	0.03	0.06	0.03	0.05	0.03	0.07
3	0.39	0.42	0.04	0.07	0.07	0.10	0.06	0.07	0.07	0.09
4	0.45	0.48	0.07	0.09	0.11	0.12	0.12	0.15	0.09	0.12
5	0.48	0.49	0.11	0.13	0.13	0.15	0.17	0.18	0.12	0.14
6	0.62	0.65	0.14	0.17	0.22	0.24	0.21	0.23	0.27	0.30
7	0.82	0.85	0.17	0.19	0.32	0.35	0.23	0.25	0.31	0.32
8	0.89	0.93	0.24	0.26	0.39	0.40	0.32	0.34	0.33	0.35
9	0.94	0.95	0.36	0.38	0.45	0.48	0.34	0.38	0.39	0.42
10	0.95	0.97	0.38	0.40	0.50	0.53	0.47	0.49	0.42	0.47
11	-	-	0.42	0.44	0.53	0.56	0.62	0.65	0.47	0.51
12	-	-	0.48	0.52	0.58	0.63	0.72	0.74	0.57	0.58
13	-	-	0.55	0.58	0.63	0.65	0.75	0.81	0.68	0.71
14	-	-	0.58	0.61	0.65	0.67	0.82	0.85	0.84	0.87
15	-	-	0.68	0.71	0.67	0.72	0.88	0.93	0.88	0.90
16	-	-	0.74	0.79	0.72	0.79	0.94	0.99	0.90	0.95
17	-	-	0.82	0.84	0.79	0.87	-	-	-	-
18	-	-	0.84	0.86	0.89	0.94	-	-	-	-
19	-	-	0.89	0.92	0.94	0.96	-	-	-	-

Phyto-polyphenols are secondary metabolites/ their derivatives/ isomers of flavones, isoflavones, flavonols, catechins and phenolic acids for which over 8,000 structural variants are distributed in different parts of plants. As phenolic components vary in structure, physical and chemical properties their solubility becomes different according to polarity of solvents. Most of the phenolic components are soluble in organic polar solvents for which methanol, ethanol, acetone in Gr-B, C and D extracts more principles from herb shoot and water at Gr-A being non-polar solvent fails to recover all the phenolic compounds in qualitative detection[15]. Less recovery of phenolics in pooled solvent at Gr-E may be due to incorporation of less (10%) water in polar solvents which fails to modify solubility of certain components but, their presence/ absence may be due to variation in physical and chemical behaviour in a particular solvent[16, 17].

The polarity of solvents and physical/ chemical properties of active components play a crucial role on contents of phenolic compounds to be extracted. But, method of extraction along with physical conditions of exposure adds to their yield. CP method involves exposure of the solvent at 10^0C for 24 h where the effects of more temperature and pressure on polarity of solvent are

ignored thereby; it fails to recover more phenolics and flavonoids in all the polar and non-polar solvents. This may be the reason of estimating significantly ($p<0.05$) low total polyphenol and flavonoids in all the solvent extracts under CP method. On the other hand, MAE is an advanced technique where extraction of bio-active compounds is associated with solvent type and concentration[2, 18]. Dominance of polar organic solvents (Gr-B, C and D) and their mixture (Gr-E) in extraction and estimation of significantly higher ($p<0.05$) phenolics and flavonoids than that of aqueous one (Gr-A) in MAE method is due to intervention of microwave energy, higher temperature and pressure[19, 20].

Physical/ chemical property and polarity of solvents affect percolation in CP method and microwave energy in MAE method respectively. Therefore, polar solvents in Gr-B, C, D and E extracts significantly higher phenolics ($p<0.001$) than those in water at Gr-A. This is because water being non-polar solvent remains unaffected to microwave energy. But, water in organic solvents (Gr-B and C) increases yield of phenolics by protecting and preventing them from being oxidized by phenol-oxidase enzymes[21] and by modifying the solvent polarity, so significantly higher ($p<0.05$) contents of these constituents are estimated in polar organic solvents in association with water irrespective of methods[22].

Significantly higher contents of phenolics and flavonoids in organic solvents at Gr- B, C, D and E in MAE method under HPTLC analysis contribute to exhibit more number of spots than the non-polar solvent at Gr-A and than those in CP method. Increased number of peaks in organic solvent groups may be due to presence of phenolic and flavonoid components like quercetin, sebiferine and litseferine which are extracted under protection cover of water in polar solvents. Besides, the variations in number of spots of phenolics between solvents may be due to their occurrence as glycosides/ aglycones with lower R_f values (0.00-0.25) than oligo-hydroxylated and meth-oxylated components (0.5-0.75). The existence of least common number of peaks in all the solvents is due to occurrence of quercetin-3-glucosides (R_f-0.33), quercetin-3-arabinosides (R_f-0.35), quercetin-3-rhamnoglucosides (R_f-0.29) and Kaempferol glucoside (R_f- 0.54) for their compatible R_f values observed in the study. On the other hand, less peaks in water at Gr-A and in CP method may be attributed to low extraction/ missed phenolic components on the basis of polarity of solvent and lack of temperature and pressure effect.

CONCLUSION

Among two methods of extraction more components of polyphenols were detected qualitatively and significantly higher contents of phenolics and flavonoids were estimated quantitatively in

advanced MAE method than traditional CP method. Out of four types of solvents, organic solvent mixtures recovered significantly higher polyphenols and flavonoids in comparison to aqueous solvent where 80% methanol proved greater effect on extraction. HPTLC finger print analysis exhibited more spots in all the solvent extracts under MAE than CP method.

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