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Comparative studies on phytochemistry, antioxidant and antibacterial activity of direct and sequential extracts of *Chromolaena odorata* leaves

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ABSTRACT

Phytochemistry, antioxidant and antibacterial activities of different solvent extracts of *Chromolaena odorata* leaves obtained by direct and sequential extraction were compared in this study. Antibacterial evaluation of the extracts was performed through a disc diffusion method. The results revealed that the solvents, Petroleum ether and Ethyl acetate showed the presence of a majority of the phytochemicals, Chloroform extract yielded maximum TPC and TAA and ethyl acetate yielded higher TFC when compared to other solvents. Ethanol and chloroform extracts showed lower concentrations of IC_{50} against DPPH and NO radicals respectively. Acetone recorded the maximum zone of inhibition against *Staphylococcus aureus* and *Pseudomonas aeruginosa*; ethyl acetate against *Bacillus subtilis* and Chloroform against *Streptococcus mutans.* Among the solvents studied for phytochemical content and bioactivities, ethanol showed a higher level of phytochemical extraction and better bioactivities. As a direct solvent extraction of *C. odorata* showed better efficiency when compared with sequential extraction, the study strongly recommends the use of a direct extraction method.

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INTRODUCTION

The use of plants in herbal medicine is popular, widespread and used by more than 80% of the total population in the world (Geneva, 2002). This is due to the compounds present in plants as secondary metabolites. Secondary metabolites are produced by plants as a defense mechanism as well as for their survival to forbid the competitors. The properties of plants related to the antioxidant, anti-inflammatory and antibacterial activities are due to the presence of the secondary metabolites, i.e. phenolic compounds, tannins and flavonoids (Amarowicz, 2007). Their role in the prevention of oxidative stress-related disorders is already suggested (Murugan and Parimelazhagan, 2014). However, extraction of plant materials is challenging, as plants possess myriads of compounds. Chigayo *et al.,* (2016) emphasized the importance of choice of extraction using a suitable solvent as an important step, as these biologically active compounds occur naturally in low concentrations. Differences in biological activities of plant extracts are mainly due to the extraction method (Hayouni *et al.,* 2007). The extraction yield and the presence of various antioxidant compounds depend upon the solvent used (Sultana *et al.,* 2007). Solvents play a

major role as the dissolution of phytocompounds depends on the polarity of the solvent.

This study is conducted to find a suitable solvent and extraction method for the isolation of phytocompounds from *Chromolaena odorata* (L.) R. M. King & H. Rob., commonly known as Siam weed. The weed is found in tropical and subtropical zones and dominates other weeds. *C. odorata* is a perennial herb, growing up to 2.5 m in height. The plant possesses soft stem, woody base; is hairy and glandular with a pungent, aromatic odor. *C. odorata* is widely used in folklore medicine. There are many reports on the pharmaceutical applications of *C. odorata* using different solvents (Hanh *et al.,* 2011; Akinmoladun *et al.,* 2010; Pandith *et al.,* 2012; Gade *et al.,* 2017). The medical importance of the weed is reviewed by Vijayaraghavan *et al.,* (2017). The major aim of the study is to compare the phytochemistry, the antioxidant and antibacterial activity of direct and sequential extracts of *Chromolaena odorata* leaves as the choice of solvents determines the presence of phytocompounds. As phytocompounds have a direct implication on medicinal value, solvents of different polarity, i.e., Petroleum ether, Chloroform, Ethyl acetate, Acetone and Ethanol were used for direct and sequential extraction of phytocompounds from the leaves of *Chromolaena odorata*. The extracts were studied for the estimation of total phenolics, flavonoid and antioxidant contents; free radical scavenging activity and antibacterial activity.

MATERIALS AND METHODS

Collection of plant material and extraction

Healthy leaves of *Chromolaena odorata* (Asteraceae) were collected from Athani, Kerala, India in November 2016 and identified by the authors. The herbarium (BWCAH004) of the plant is deposited in the Department of Botany, Bharathi Women's College, Chennai, India. The leaves were dried under shade, at room temperature (25±2°C) and pulverized. Solvent extraction of the phytocompounds was performed by cold percolation method using the solvents petroleum ether, chloroform, ethyl acetate, acetone and ethanol, at a ratio of 1:10 (w/v), maintained at 25±2°C, for 48 h. The extracts were filtered, and the solvents evaporated, to obtain the crude extracts. The extraction was also sequentially performed by addition of solvents in the order of petroleum ether, chloroform, ethyl acetate, acetone and ethanol.

Phytochemical analysis

The presence of phytochemicals such as alkaloids, flavonoids, cardiac glycosides, saponins, tannins, phytosterols and terpenoids were conducted according to the methods prescribed by Trease and Evans (1997). The quantitative analysis on total phenolics, flavonoids and antioxidant contents were performed similarly to our previous study (Udayaprakash *et al.,* 2015).

Free radical scavenging activity

The free radical scavenging activity of the extracts was evaluated against 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Udayaprakash *et al.,* 2014) and nitric oxide (NO) radicals (Jagetia *et al.,* 2004) at varying concentrations of 1, 2, 3, 4 & 5 mg/ml. The percent inhibition of the radicals was calculated as:

Percent inhibition = [(Control absorbance – Test absorbance) / Control absorbance] × 100

The concentration at which 50% of radicals were inhibited (IC_{50}) was calculated from the linear plot obtained.

Antibacterial activity

The antibacterial activity was performed by disc diffusion method (Udayaprakash *et al.,* 2012). Varying concentrations (1.5, 2 and 2.5 mg/ml) of the extracts were loaded onto sterile discs (diameter 6 mm). The discs were placed over Nutrient agar plates swabbed with 24 hours cultures of *Staphylococcus aureus* (MTCC 96)*, Pseudomonas aeruginosa* (MTCC 741)*, Bacillus subtilis* (MTCC 441) and *Streptococcus mutans* (MTCC 497). Streptomycin was used as a positive control. The plates were incubated at 37±2°C for 24 h, after which the zone of inhibition was measured in mm.

RESULTS

Phytochemical analysis

The phytochemical analysis of *C. odorata* revealed the presence of different phytochemicals in solvents of different polarity. The solvents, petroleum ether and ethyl acetate, showed the presence of the majority of the compounds. Flavonoids were detected in all the extracts. Alkaloids were present in petroleum ether, chloroform and ethyl acetate extracts, indirect extraction as well as sequential extraction. Tannins were detected in direct extraction using petroleum ether, ethyl acetate and ethanolic extracts. However, they were absent in sequential extraction. Cardiac glycosides were detected only in petroleum ether extract and phytosterols in the extract of ethyl acetate under direct extraction method. Saponins were not recorded in any of the solvents. The various phytochemicals detected in the solvents through direct and sequential extraction are represented in Table 1.

Total Phenol Content (TPC): The solvent ethanol yielded high phenolic content of 162.11±0.29 mg TAE/g dw, followed by chloroform (158.43±0.39) and ethyl acetate (131.36±0.39), through direct extraction method. A similar trend was observed in the sequential extraction method. However, the

concentration was found to differ. The total phenolic content was reduced by nearly 50% in the sequential extraction method when compared to direct extraction. In sequential extraction, ethanol yielded the maximum content of phenolic compounds (83.41±0.44), followed by chloroform (73.19±0.49) and ethyl acetate (63.44±0.47). The total phenolic content recorded for different solvents using direct and sequential extraction methods from the leaves of *C. odorata* is presented in Fig. 1.

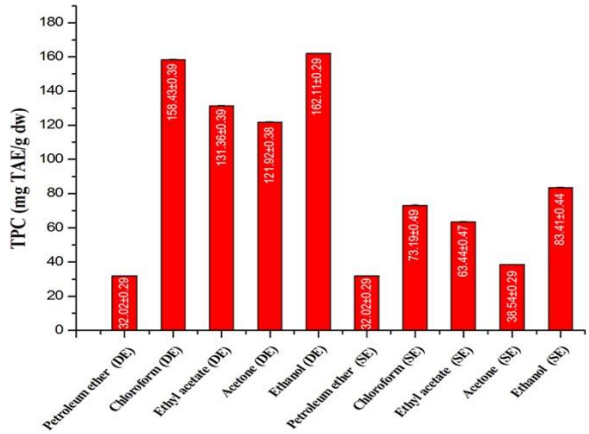

Figure 1: Total phenolic content recorded for C. odorata leaves

Total Flavonoid Content (TFC): The TFC was highest in the ethyl acetate extract in both direct (183.25±0.3 µg QE/g dw) and sequential extraction (138.25±0.42) methods. However, the concentration was low in sequential extraction. A similar trend was followed with the extracts of chloroform, acetone and ethanol which yielded more TFC through direct extraction when compared with sequential extraction. The total flavonoid content recorded for different solvents using direct and sequential extraction methods from the leaves of *C. odorata* is presented in Fig. 2.

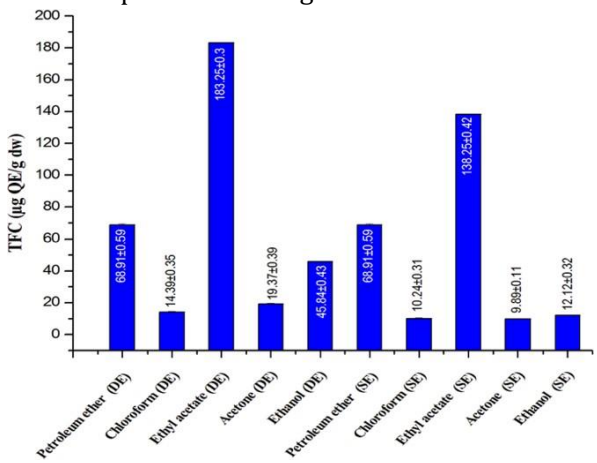

Figure 2: Total flavonoid content recorded for C. odorata leaves

Total Antioxidant Content (TAC)

The TAC reciprocated the results of TPC, in which the ethanol extract yielded a high concentration of TAC of 171.12±0.65mg TAE/g dw. The order of higher concentration of TAC recorded was ethanol, followed by chloroform, ethyl acetate and acetone in both direct and sequential extraction methods. However, the concentration of TAC recorded from direct extraction was more when compared to sequential extraction. The TAC recorded for different solvents using direct and sequential extraction methods from the leaves of *C. odorata* is presented in Fig. 3.

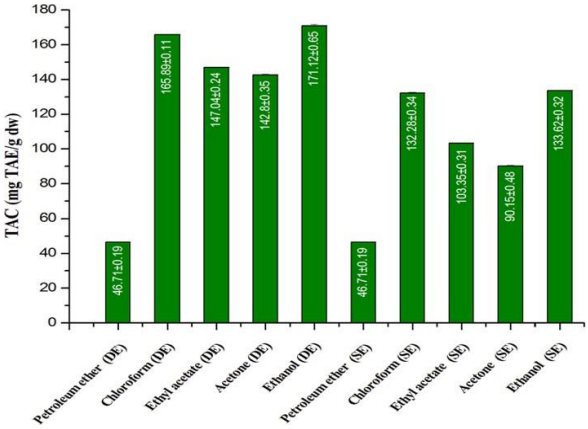

Figure 3: Total antioxidant content recorded for C. odorata leaves

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity

The DPPH radical scavenging activity of *C. odorata* leaves was found to differ with an increase in polarity. The ethanolic extract was the most efficient scavenger of DPPH radicals, with IC_{50} of less than 1 mg/ml, followed by acetone extract (2.45 mg/ml). On the contrary, the IC_{50} value was found to increase sequential extraction with subsequent solvents. When compared with the direct solvent extract, the IC_{50} values were found to be higher in sequentially extracted solvents. The scavenging activity of the different solvent extracts and the standard (BHT) against DPPH radicals is presented in Table 2.

NO radical scavenging activity

The nitric oxide radical scavenging activity of *C. odorata* leaves was found to be efficient in the ethanol extract followed by ethyl acetate through direct extraction method, which exhibited IC_{50} of 1.81 mg/ml and 2.48 mg/ml respectively. When compared with the direct solvent extracts, the IC_{50} values were higher in sequentially extracted solvents. The scavenging activity of the different solvent extracts and the standard (Ascorbic acid) against NO-radicals in the present study is presented in Table 3.

Table 1: Qualitative analysis of the phytochemicals in C. odorata leaves

Extraction method	Solvent	Phyto chemical	Alkaloids	Flavnoids	Cardiac glycsides	Saponins	Tannins	Phyto sterols	Terpe-noids
Direct	Petroleum ether		+	+	+	-	+	-	+
	Chloroform		+	+	-	-	-	-	-
	Ethyl acetate		+	+	-	-	+	+	+
	Acetone		-	+	-	-	-	-	+
	Ethanol		-	+	-	-	+	-	+
Sequential	Petroleum ether		+	+	+	-	+	-	+
Chloroform		+ + -		-	-	-	-		
	Ethyl acetate		+	+	-	-	-	-	-
	Acetone		-	+	-	-	-	-	+
	Ethanol		-	+	-	-	-	-	-

+ denotes presence - denotes absence

Table 2: Percent inhibition and IC50 recorded by C. odorata against DPPH free radicals

Extrac-tion method	Solvent	Concentration (mg/ml)	1	2	3	4	5	IC_{50} (mg/ml)
Direct	Petroleum ether		2.22	16.67	27.78	38.89	55.56	4.69
	Chloroform		17.78	46.67	50	66.67	78.89	3
	Ethyl Acetate		16.67	37.78	43.33	57.78	64.44	3.52
	Acetone		36.67	47.78	55.56	61.11	72.22	2.45
	Ethanol		62.22	66.67	73.33	75.56	85.56	<1
Sequen-tial	Petroleum ether		2.22	16.67	27.78	38.89	55.56	4.69
	Chloroform		0	3.33	25.56	47.78	71.11	4.1
	Ethyl Acetate		0	31.11	38.89	48.89	60	3.78
	Acetone		3.33	5.56	20	26.67	33.33	>5
	Ethanol		1.11	3.33	17.78	21.11	23.33	>5
		Concentration (mg/ml)	0.1	0.2	0.3	0.4	0.5	IC_{50} (mg/ml)
Standard Butylated Hydroxy Toluene(BHT)			77.96	78.22	78.79	79.18	79.38	<0.1

Antibacterial activity

Although all the extracts showed a zone of inhibition against the bacteria studied, the solvent acetone recorded the maximum zone of inhibition against *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The ethyl acetate extract of *C. odorata* recorded the maximum zone of inhibition (24 mm) against *Bacillus subtilis* at the concentration of 2.5 mg/disc. The most efficient extract against *Streptococcus mutans* was that of chloroform. The extracts obtained through the same solvents by sequential extraction method yielded lower zone of inhibition when compared to the direct solvent extracts. The zone of inhibition recorded for different concentration of the extracts and streptomycin against the bacteria studied is presented in Table 4.

DISCUSSION

The importance of medicinal plants or the plants used in herbal medicine is primarily due to their phytochemical components or secondary metabolites which contribute to their bioactivities. The presence of compounds like polyphenols, flavonoids, tannins and saponins are responsible for the bioactivities of the plants. In this study, the plant, *Chromolaena odorata* showed the presence of different compounds like alkaloids, flavonoids, tannins and terpenoids. However, the presence of the components was found to differ according to the solvent used. The solvents petroleum ether and ethyl acetate were observed to isolate more compounds when compared with other solvents. Similarly, isolation of compounds was also found to differ according to the extraction method. Fewer compounds were detected in the sequential extraction method when compared with the direct extraction method. Secondary metabolites vary with chemistry, dynamic structure and complex matrix (Wernisch and Pennathur, 2016), which is the reason for the dissolution in specific solvents.

Table 3: Percent inhibition and IC50 recorded by C. odorata against NO free radicals

Extraction method	Sol-vent	Concentration (mg/ml)	1	2	3	4	5	IC_{50} (mg/ml)
Direct	Petroleum ether		43.93	45.71	47.42	50	51.70	4
	Chloroform		43.45	44.05	48.21	51.43	51.67	3.94
	Ethyl Acetate		48.69	50.47	51.79	52.74	54.17	1.81
	Acetone		17.86	33.93	53.57	60.71	69.64	3.22
	Ethanol		48.21	49.64	50.47	51.74	52.97	2.48
Sequential	Petroleum ether		43.93	45.71	47.42	50	51.70	4
	Chloroform		44.53	46.67	49.62	52.86	54.81	3.11
	Ethyl Acetate		45.83	46.43	47.62	50.47	54.05	3.55
	Acetone		47.97	48.57	49.05	49.53	50.36	4.58
	Ethanol		45	47.14	47.50	50.36	51.79	3.98
Standard		Concentration (mg/ml)	0.1	0.2	0.3	0.4	0.5	IC_{50} (mg/ml)
Ascorbic acid			69.95	72.62	76.16	79.63	81.29	<0.1

Table 4: Zone of inhibition (mm) recorded for the bacteria against different solvent extracts of leaves of C. odorata

Extraction	Solvent	Concentration (mg/ml)	*S. aureus*	*P. aeruginosa*	*B. subtilis*	*S. mutans*
Direct	Petroleum ether	1.5	-	-	11	-
		2	-	-	15	-
		2.5	11	9	17	11
	Chloroform	1.5	9	9	11	11
		2	11	17	13	13
		2.5	15	17	17	15
	Ethyl Acetate	1.5	7	13	20	7
		2	7	17	22	9
		2.5	13	17	24	13
	Acetone	1.5	11	17	15	7
		2	13	19	15	9
		2.5	15	21	17	13
	Ethanol	1.5	-	7	11	7
		2	9	7	15	11
		2.5	13	9	17	15
Sequential	Petroleum ether	1.5	-	-	11	-
		2	-	-	15	-
		2.5	11	9	17	11
	Chloroform	1.5	9	9	11	9
		2	11	11	13	11
		2.5	13	13	15	15
	Ethyl Acetate	1.5	-	9	9	-
		2	11	11	15	7
		2.5	15	15	17	15
	Acetone	1.5	-	13	-	-
		2	-	15	-	-
		2.5	7	17	7	7
	Ethanol	1.5	-	7	7	-
		2	-	7	9	-
		2.5	-	9	13	-
Standard	Streptomycin	1.5	11	13	21	11
		2	13	17	23	13
		2.5	15	19	25	15

Phenolic compounds are the secondary metabolites synthesized by plants. They possess different biological properties such as antioxidant, anti-aging, anti-inflammatory, cardiovascular protection and cell proliferation. They are found to be potential agents to control the Alzheimer's, Parkinson's and remove oxidative stress. The biological activities of plants are due to the intrinsic reducing capacity of polyphenolic compounds present in plants (Han *et al.,* 2007). High phenolic content is known to be beneficial, as the phenolic compounds quench primary oxidants or free radicals (Chigayo *et al.,* 2014). The present study revealed that high concentration of total phenolic compound was extracted using ethanol when compared to other solvents. Siddhuraju and Becker (2003a) stated that methanol and ethanol are effective solvents to extract phenolic compounds and are safe for human consumption (Shi *et al.,* 2005). It is reported that higher amount of phenolics are extracted in polar solvents (Sultana *et al.,* 2007); Siddhuraju and Becker, 2003b; Anwar *et al.,* (2006). In the present study, the phenolic content is found to be higher in direct extraction method when compared to that of sequential extraction. The lower concentrations of phenolics extracted under sequential extraction are due to the removal of partial phenolics with the previous solvents.

It is reported that flavonoids are a class of secondary plant metabolites with significant antioxidant and chelating properties, which is due to the presence of hydroxyl groups (Sharififar *et al.,* 2009). These compounds are found to have healing properties and are effective against most of the disease-causing organisms (Khanam *et al.,* 2015); Pandey, 2015). Flavonoids are extracted at high concentration in ethyl acetate extract in both direct extraction and sequential extraction method. This was followed by petroleum ether and ethanol. The dissolution of flavonoids is found to be in partially polar solvents when compared with phenolics, which are extracted in high polar solvents.

The presence of phenols and flavonoids are the major contributors to the concentration of total antioxidants. Among different phytocompounds, phenolics are the major antioxidant compounds (Liu *et al.,* 2009). This is evident in this study, where the total antioxidant content reciprocated the results of total phenolics recorded. Similarly, the maximum concentration of total antioxidants was recorded by the solvent ethanol, in both direct extraction and sequential extraction method. The extracts recording higher levels of total phenolic content also exhibit greater reducing activity or greater antioxidant activity (Sultana *et al.,* 2007; Siddhuraju and Becker, 2003b; Cheng *et al.,* 2006).

The IC_{50} value of a compound is inversely proportional to its antioxidant activity. The extract which scavenges 50% of the DPPH and NO radicals is represented as its IC_{50} value. It is considered that an extract is active when its IC_{50} value is <5 mg/ml (Abdillah *et al.,* 2015). In this study, the ethanol extract of *C. odorata* has recorded 1 mg/ml as its IC_{50} against DPPH. All other extracts, i.e. Acetone, Chloroform, Ethyl acetate and Petroleum ether have recorded less than 5 mg/ml as their IC_{50} value. This shows that the plant *C. odorata* is a potent antioxidant. However, the same extracted through sequential extraction method yielded more than 5 mg/ml as its IC_{50}. Thus, it is evident that the extraction through the direct method is most preferential when compared with sequential extraction method. Similar results are observed in scavenging of NO radicals.

Melinda *et al.,* (2010) reported high phenolic content and free radical scavenging activity of the methanolic extract of *C. odorata* and stressed the need to study using different solvents. The antibacterial activity of different solvent extracts of *C. odorata* leaves has been recorded (Irobi, 1992; Irobi, 1997; Sukanya *et al.,* 2011; Vital and Rivera, 2009). In the present study, the acetone extract obtained by direct extraction method showed antibacterial activity against *S. aureus* and *P. aeruginosa*, which is similar to the activity exhibited by the standard. Ethyl acetate and chloroform extracts exhibited antibacterial activity against *B. subtilis* and *S. mutans* respectively. The bioactivity of plants differs according to the extraction methods (Altemimi *et al.,* 2017); different solvents (Ngo *et al.,* 2017) and also due to the environment and climatic factors (Jeyaseelan *et al.,* 2012). In the present study, the differences in phytochemical analysis and the bioactivity of the solvent extracts of *C. odorata* is attributed to the varying extraction methods and the solvents used.

CONCLUSION

From the study, it is evident that the extracts obtained through direct extraction of *C. odorata* possessed the majority of the phytochemicals, and exhibited high yield of TPC, TFC and TAA when compared with sequential extraction. Similarly, the extracts of direct extraction method were efficient in scavenging free radicals and possessed better antibacterial compounds when compared to sequential extraction. The study also suggests that the extraction of phytocompounds is better with polar solvents when compared with another solvent system. Hence, the study strongly recommends the use of a direct extraction method and highly polar solvents.

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