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IDENTIFICATION AND QUANTIFICATION OF PHYTOCOMPOUNDS IN ACALYPHA INDICA LEAVES

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ABSTRACT

Acalypha indica, a known medicinal plant in the treatment of various degenerative diseases and a potential source of valuable bioactives. The present study investigates the total phenolic and flavonoid content and also screen for phytocompounds in *A. indica* leaf extract obtained by two extraction methods, comparatively. Results indicated that the maceration extraction method (MEM) had highest total phenolic content and the sequential extraction method (SEM) showed highest total flavonoid content in methanolic extracts. RP-HPLC (Reversed Phase-HPLC) analysis revealed the presence of gallic acid, caffeic acid, 3,3´-Methylene-bis(4-hydroxycoumarin), syringic acid, ferulic acid, rutin and naringenin in methanolic extract. Major phenolic acid and flavonoid identified were gallic acid (119.7 mg kg^{-1} dry weight of extract) and naringenin (125.29 mg kg^{-1}) in *A. indica* extract, respectively. Thus, the results suggest that these identified phenolic compounds makes *A. indica* a good source of bioactive phenolic compounds and provide desirable health benefits beyond basic nutrition.

KEYWORDS: *Acalypha indica*; Total phenolic content; Total flavonoid content; phenolic acids; flavonoids; RP-HPLC

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INTRODUCTION

Phenolic compounds are the most abundant secondary metabolites synthesized in plants. Approximately, 8000 naturally occurring compounds belong to the category of "phenolics" and flavonoids forming the largest group among them[1]. Phenolics are able to act as antioxidants and thereby associated with the prevention of diseases such as CVDs, cancer and inflammation, which are thought to be induced by oxidative stress [2,3]. Flavonoids and other phenolic compounds are widely distributed and form important constituents of the human diet. Currently, the use of natural antioxidants, particularly phenolic compounds in food as well as preventive and therapeutic medicine, is gaining much recognition because of their nutraceutical and health benefits [4,5]. Many plants are reported to possess therapeutic properties and its bioactive compounds are investigated using various separation and identification techniques. Numerous extraction procedures have been described in the literature [6].*A. indica* is traditionally employed in folklore remedy for a wide spectrum of diseases [7] and is widely distributed in different regions of the world. Investigations on *A. indica* extract have been reported to possess various properties such as antimicrobial, antibacterial, antifungal, nitric oxide scavenging, larvicidal, ovicidal, antioxidant activities, venom neutralizing potential and post-coital anti-fertility activities [8,9,10,11,12,13,14,15]. The phytoconstituents identified in the leaves and twigs of *A. indica* are acalyphamide, acalyphamide acetate, aurantiamide, aurantiamide acetate, succinamide, acalyphol acetate, 2-methyl antaquinone, stigmasterol, β-sitosterol and its acetate. Flavonoids, notably the kaempferol glycosides, mauritianin, clitorin, nicotiflorin, biorobin, naringin, quercitrin and hesperitin have been isolated from the flowers and leaves. Studies on preliminary screening of phytochemicals have shown the presence of alkaloids, catachols, flavonoids, phenolic compounds, saponins, steroids, volatile oil and fatty acids [16].Recently, we reported that methanolic extract of *A. indica* has high antioxidant activity and also possesses cardioprotective activity against isoproterenol induced myocardial infarcted rats [7]. Though, *A. indica* has various therapeutic properties, the information related to their composition is limited. Thus, the present study is aimed to evaluate and quantify the phenolic compounds and flavonoid in *A. indica* leaf extract obtained by sequential extraction method (SEM) and maceration extraction method (MEM) using Reversed Phase- HPLC (RP-HPLC).

MATERIALS AND METHODS

(i) Plant Material

A. indica leaves were collected from Madurai Kamaraj University, Madurai (South India) vicinity during January, 2014. The plant was identified following the "Flora of presidency of Madras" by Gamble [17] and confirmed with the herbarium specimen at the Rapinat herbarium, St. Joseph's College, Tiruchirapalli, India. The voucher specimen was deposited at the herbarium of School of Biological Sciences, Madurai Kamaraj University. The leaves were washed with tap water and dried under shade for a week. The dried leaves were ground in a waring blender and used for extraction or stored at 4 ˚C until use.

(ii) Chemicals

HPLC grade methanol and glacial acetic acid were procured from Sisco Research Laboratories (Mumbai, India). Resveratrol, 3, 3' Methylene bis (4-hydroxyl coumarin), syringic acid, *p*-coumaric acid, umbelliferon, scopoletin and ferulic acid were purchased from Sigma Aldrich Inc. (St. Louis, MO, USA), and gallic acid from Himedia Laboratories (Mumbai, India). Quercetin, caffeic acid, salicylic acid, rutin and naringenin were purchased from Fluka (Buchs, Switzerland). All the other chemicals used in this study were of analytical grade.

(iii) Preparation of Extracts

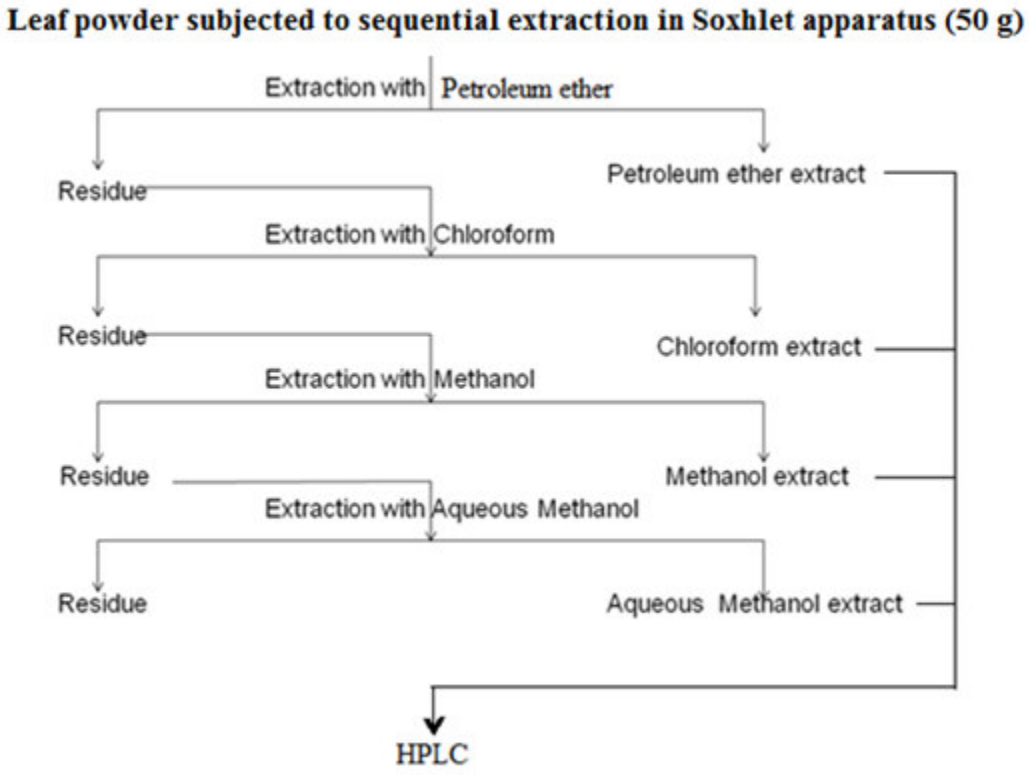
Sequential Extraction Method (SEM)

Air dried *A. indica* leaves were packed into a Soxhlet apparatus and extracted sequentially

with petroleum ether, chloroform, methanol and aqueous methanol (methanol:water-70:30), with their increasing order of polarity (Flow chart 1). Each extraction was performed using 200 ml of solvent for 6-12 h at its boiling temperature. The organic extracts were dried under reduced pressure on a rotary evaporator. The extracts were stored in a dark place at 4 ˚C, which were subjected to further analysis and performed in triplicates (n=3).

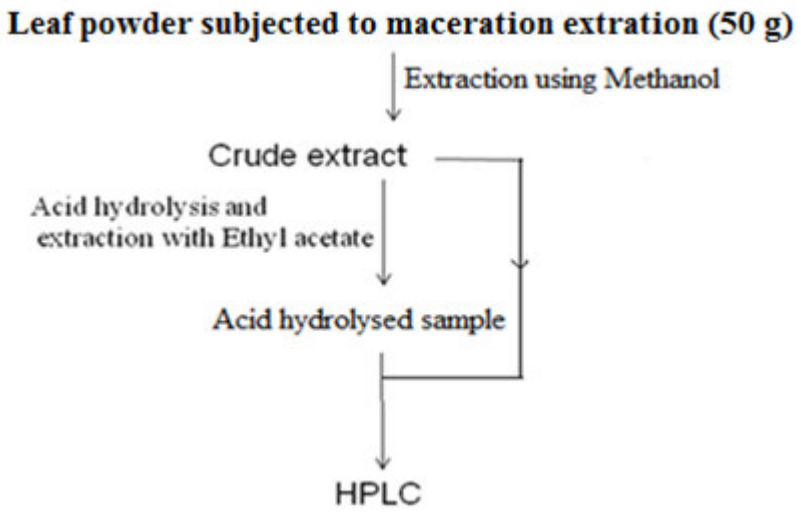
Maceration Extraction Method (MEM)

Air dried A. *indica* leaves were soaked in methanol in an Erlenmayer flask and left to macerate (methanol) in the dark for five days at room temperature. The extracts (n=3) obtained were filtered and



Flow chart 1

Systematic representation of preparation of various extracts of *Acalypha indica* leaves by sequential extraction method.



Flow chart 2

Systematic representation of preparation of various extracts of *Acalypha indica* leaves by maceration extraction method. concentrated under reduced pressure on a rotary evaporator. The extracts were stored in a dark place at 4 ˚C until analysis (Flow chart 2).

(iv) Determination of Total Phenolic and Flavonoid Content

The total phenolics content (TPC) of extracts obtained by SEM and MEM was determined by Folin and Ciocalteau method [19]. 0.5 ml of each extract was mixed with 2.5 ml of Folin and Ciocalteau Reagent (diluted 1:10, v/v) followed by 2 ml of sodium carbonate (7.5%, v/v) solution. The absorbance was measured at 765 nm using UV-Vis spectrophotometer after 90 min of incubation at 30 ˚C. Gallic acid was used as standard. Results were expressed as milligrams of gallic acid equivalent per gram (mg GAE/g) of dried extract. The total flavonoid content (TFC) of extracts acquired by SEM and MEM were estimated by modified aluminum chloride colorimetric method of Woisky and Salatino [20]. 10 mg of quercetin (standard) was dissolved in 80% ethanol and further diluted to 25, 50 and 100 µg/ml. 0.5 ml of diluted standard solutions and extracts were separately mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After 30 min incubation at room temperature, the absorbance of the mixture was read at 415 nm on UV-Vis spectrophotometer. 0.1 ml of 10% aluminum chloride was substituted by the same amount of distilled water in blank. The results were expressed as milligram of quercetin equivalent per gram (mg QE/g) of dried extract.

(v) RP-HPLC Analysis

The SEM and MEM extracts were filtered using 0.45 µm filter and subjected to RP-HPLC using Shimadzu HPLC instrumentation (Shimadzu corporation Kyoto, Japan) with LC 8A pump, SPD-M20A PDA detector, CTO-20AC prominence column oven and FRC-10A fraction collector. Separations were performed on a 250 x 4.6 mm i.d., reversed-phase C18 analytical column (Phenomenex, Torrance, CA, USA) operated at room temperature at a flow rate of 1 ml/min and injection volume of 25 µl. Detection of compounds was carried out at a wavelength of 254 nm. Elution was done according to modified method of Yumrutas et al. [21] with solvent A [acetic acid/water (2:98, v/v)] and solvent B [methanol/acetic acid/water (90:2:8, v/v/v)] using a isocratic condition of 0% B concentration up to 1 min and the proportion of solvent B was increased to 100% (gradient) in 30 min. 10 min re-equilibration time was allowed between each injection. All the standards and samples were injected in triplicate (n=3).

(vi) Hydrolysis of Extract

The extract obtained by MEM was hydrolyzed according to the method described by Chin et

al. [18]. In summary, 25 ml of acidified methanol containing 5.5% (v/v) hydrochloric acid was added to extract (5 g) and the mixture was refluxed in a water bath at 90 °C for 30 min. After cooling, the pH was adjusted to 2 with 0.1 M hydrochloric acid and centrifuged at 5000 rpm for 10 min. The supernatant collected were further extracted using ethyl acetate (1:1), the upper layer was collected and evaporated to dryness using a rotary evaporator. The resulting substance was dissolved in 100% methanol. The sample was then sonicated for 5 min to remove any air present and finally filtered through a 0.45 µm filter (Millipore) before RP-HPLC analysis (n=3).

(vii) Preparation of Standards and Quantification

For HPLC analysis, the stock solution of the standards was prepared at a concentration of 1 mg/ml and standard curve was obtained using working standards at a range of 25-200 µg/ml. Each compound was identified based on co-chromatography with authentic standards (retention time) and quantified by comparing its peak area against the standard curve obtained specifically for the reference standard solutions containing the respective compound.

(viii) Statistical Analysis

Statistical analysis was carried out using SPSS 16.0 software (version 10.0, SPSS Inc., USA). All the data were expressed as mean±standard deviation. The student's *t*-test was used to assess the differences between means. P value of <0.05 was considered to be statistically significant. All the samples were measured in triplicates.

RESULTS AND DISCUSSION

1. *Total phenolic and flavonoid contents*

In this study, four solvents such as petroleum ether, chloroform, methanol and aqueous methanol were employed to determine the most efficient extraction solvent for phenolic acids and flavonoids in SEM. MEM extract was prepared using methanol only. We had reported that 70% methanolic extract obtained by Soxhlet extraction contains high phenolic content qualitatively [7]. Thus, in order to confirm whether the methanolic extract by SEM or MEM was better in phenolic compound extraction, the study concentrated only on methanolic extracts rather than extracts of other solvent. The efficacy of phenolic compound and flavonoid content extracted from leaves was determined using TPC and TFC in the extracts. TPC is expressed as mg of gallic acid equivalent per g of extract (GAE) (the standard curve equation: y=0.01999x, r^2=0.9971) and TFC is expressed as mg of quercetin equivalent per g of extract (QE) (the standard curve equation: y=0.004x, r^2=0.909). Table 1 shows the TPC and TFC of various extracts of *A. indica* from SEM and MEM. TPC of extracts were found in the order of methanol (MEM) > methanol (SEM) > aqueous methanol > petroleum ether > chloroform, while the TFC were methanol (SEM) > petroleum ether > methanol (MEM) > chloroform > aqueous methanol.

Table 1
Total phenolic and flavonoid content in A. indica extracts obtained by two different extraction methods

Extracts	Solvent	TPC (mg of GAE/g of dried extract)	TFC (mg of QE/g of dried extract)
Sequential extraction	Petroleum ether (100%)	20.0 ± 0.0	70.7±5.3
	Chloroform (100%)	10.0 ± 0.0	30.0±0.0
	Methanol (100%)	306.7±30.5	81.0±7.0
	Aqueous Methanol (70%)	60.0± 0.0	6.7±5.7

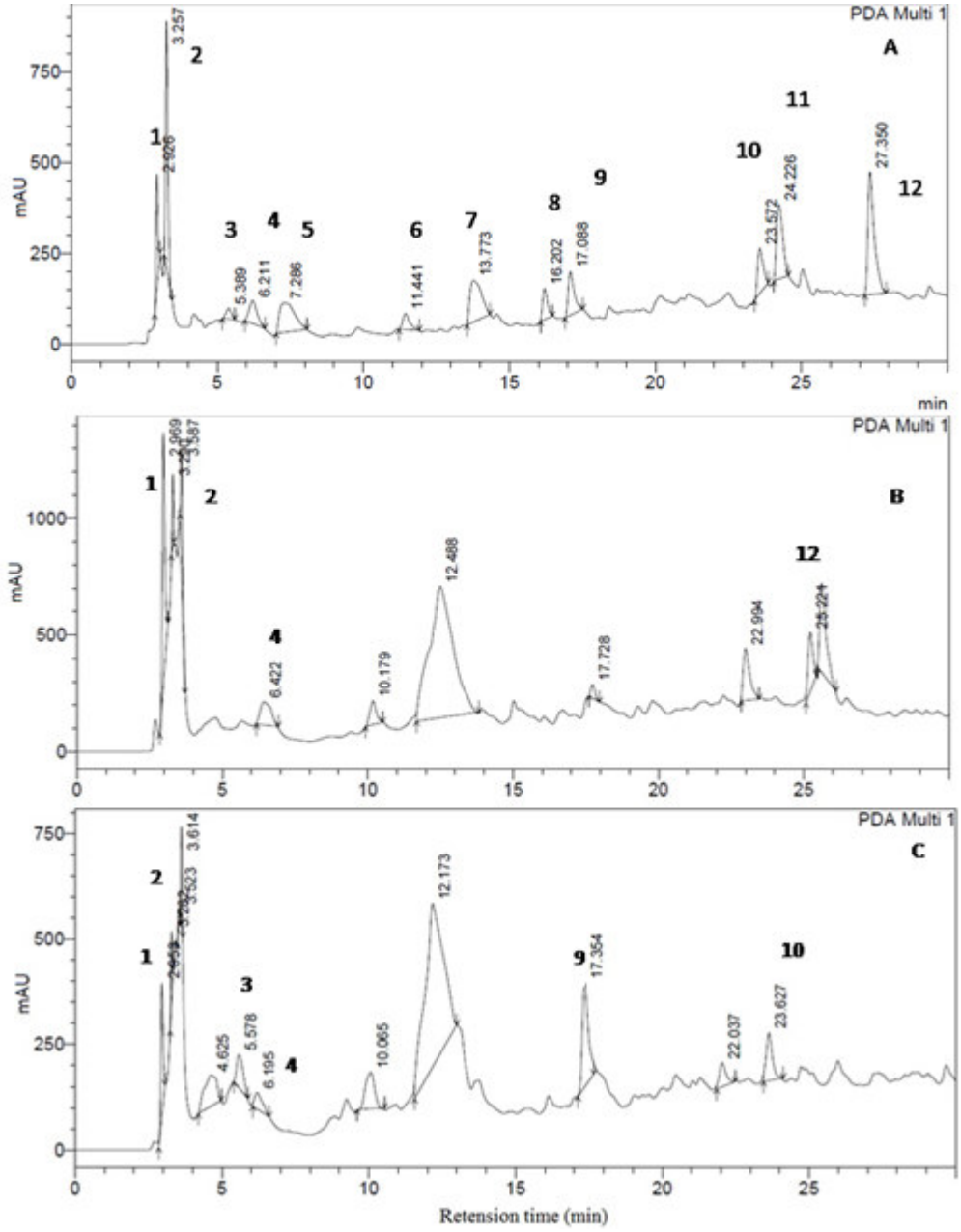
Each value represents the mean ± S.E.M of three replicates; TPC- Total phenolic content; TFC- Total flavonoid content; GAE- gallic acid equivalent; QE- Quercetin equivalent.

The highest TPC was recorded in methanolic extract (373.5 mg GAE/g of dried extract) obtained by MEM and the lowest was found in chloroform extract (10 mg GAE/g of dried extract) by SEM. Whereas, highest TFC was observed in methanolic extract (81.0 mg QE/g of dried extract) and the lowest content in aqueous methanolic extract (6.7 mg QE/g of dried extract). The result of the present study showed that the maximum TPC was recorded in the methanolic extract of *A. indica*, which is well correlated with the results of previous report suggesting that the *A. indica* (ethanolic extract) to be one among 19 investigated plants having high phenolic content. Surprisingly, the TPC of methanolic extract observed in this study was found to be greater than the TPC of ethanolic extract reported earlier by Marwah et al. [12]. Recently, Saha et al. [22] reported a low TPC and high TFC were obtained in ethanolic extract of *A. indica* leaves. The highest TPC was observed in methanolic extracts obtained by MEM than in SEM, might be due to loss or denaturation of active compounds due to heating process in SEM. However, the result of TFC was found vice-versa. Phenolic acids and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals [23]. The compounds such as flavonoids, which hold hydroxyls groups, are responsible for the radical scavenging activity in the plants [24]. Thus, MEM with methanol was better in phenolic content extraction efficacy, while SEM with methanol was better in flavonoid extraction. Therefore, methanolic extracts of SEM and MEM were chosen for further RP-HPLC analysis.

2. *Identification of phenolic compounds*

During the last decade, HPLC has been the analytical technique that has dominated the separation and characterization of phytocompounds. HPLC techniques offer a unique opportunity to separate simultaneously all components together with their possible derivatives or degradation products. The introduction of reversed-phase columns has considerably enhanced HPLC separation of different classes of phenolic compounds [1]. In the present study, phenolic acids (gallic acid, caffeic acid, 3,3' Methylene bis (4-hydroxyl coumarin), syringic acid, salicylic acid, *p*-coumaric acid, resveratrol and ferulic acid), flavonoids (quercetin, rutin and naringenin) and coumarins (umbelliferon, scopoletin) were screened in the extracts obtained by SEM and MEM using RP-HPLC analysis. The phenolic compounds were identified with respect to the retention time of authentic standards and

quantified by comparing its peak area against the standard curve obtained specifically for the HPLC standards (Fig. 1.A). The concentration of identified compounds in *A. indica* extracts is summarized in Table 2. Methanolic extracts of *A. indica* from both extraction methods were used in this study. RP-HPLC analysis resulted in the identification of phenolic acids such as gallic acid, caffeic acid and syringic acid in methanolic extracts obtained by both SEM (Fig.1.B) and MEM (Fig. 1.C). While, 3,3' Methylene bis



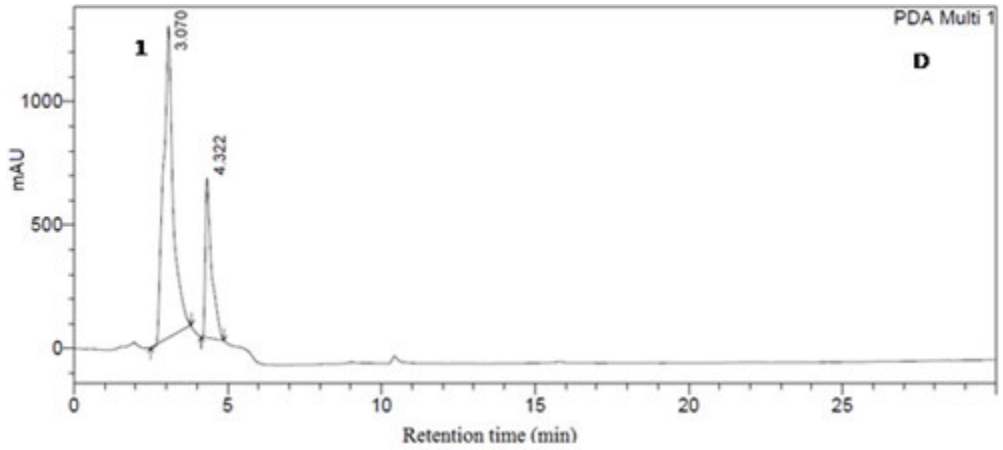


Figure 1

Reversed phase-high performance liquid chromatogram of A) Standard phenolic compounds. Peaks are indicated as follows: 1) Gallic acid, 2) Caffeic acid, 3) 3,3' Methylene bis (4-hydroxyl coumarin), 4) Syringic acid, 5) Salicylic acid, 6) p-Coumaric acid, 7) Umbelliferone, 8) Scopoletin, 9) Ferulic acid, 10) Rutin, 11) Resveratrol, 12) Naringenin, 13) Quercetin. B) *A. indica* extract from sequential extraction method. C) extract from maceration extraction method. (D) Extract from maceration extraction method after acid hydrolysis. Typical HPLC chromatogram recorded at 254 nm.

Table 2

Concentration of phenolic acids and flavonoids in A. indica extracts obtained by different extraction methods using RP-HPLC

No.	Compound	Retention time (min)	Content of compounds (mg kg^{-1} dry weight) In Methanolic extract by SEM	Content of compound (mg kg^{-1} dry weight) in Methanolic extract by MEM	Content of compund (mg kg^{-1} dry weight) in Methanolic extract by MEM after acid hydrolysis
1	Gallic acid	3.0±0.1	119.7±8.6	32.5±2.3	290.5±41.8
2	Caffeic acid	3.3±0.0	79.8±4.4	4.7±0.1	ND
3	3,3' Methylene bis (4-hydroxyl coumarin)	5.5±0.1	ND	103.7±6.5	ND
4	Syringic acid	6.3±0.1	66.8±4.0	19.4±2.0	ND
5	Salicylic acid	7.3±0.1	ND	ND	ND
6	p-Coumaric acid	11.5±0.1	ND	ND	ND
7	Umbelliferone	13.8±0.1	ND	ND	ND
8	Scopoletin	16.3±0.1	ND	ND	ND
9	Ferulic acid	17.2±0.2	ND	63.6±3.3	ND
10	Rutin	23.6±0.0	ND	30.9±2.2	ND
11	Resveratrol	24.3±0.1	ND	ND	ND
12	Naringenin	25.2±0.0	125.3±8.3	ND	ND
13	Quercetin	27.4±0.1	ND	ND	ND

ND: Not detected; SEM- sequential extraction method; MEM- Maceration extraction method. All analyzes were mean of triplicate measurements ± standard deviation.

(4-hydroxyl coumarin) and ferulic acid was detected only in the extract obtained by MEM. Out of four flavonoids investigated, two flavonoids viz. naringenin and rutin were identified in the extract of SEM and MEM, respectively. The analysis showed the absence of coumarins such as umbelliferone and scopoletin. Hence, the results of the RP-HPLC analysis revealed that gallic acid (119.7 mg kg^{-1} dry weight) was the major phenolic acid and naringenin (125.29 mg kg^{-1} dry weight) was the major flavonoid, identified and quantified in *A. indica* leaf extract by SEM. The phenolic compounds like salicylic acid, *p*-coumaric acid, resveratrol, quercetin were not detected, which might be due its low abundance in the extract or lost during extraction. In the present study, few major peaks detected in RP-HPLC profile were unidentified, which limit the study. Acid hydrolysis is the most common means of releasing phenolic acids. Hydrolysis is frequently used to remove sugar moieties from glycosides (disrupt glycoside or sulphur linkages) by acidic/ basic or enzymatic [1]. As the RP-HPLC analysis of extract obtained by MEM (possess high TPC and TFC) showed major unidentified peaks (Fig. 1.C), the extract was subjected to acid hydrolysis and further RP-HPLC analysis to identify bound phenolic acids. The RP-HPLC analysis of acid hydrolysed extract resulted in two major peaks, of which the peak corresponding to a retention time of 3.0 min was identified as gallic acid based on the retention time of standard. The other peak at 4.3 min did not correspond to the retention time of HPLC standards analyzed and was unidentified (Fig. 1.D). The content of gallic acid was found higher (290.45 mg kg^{-1} of dry weight) in the extract of acid hydrolysis than the extract without hydrolysis (32.54 mg kg^{-1} of dry weight).

CONCLUSION

According to the results, MEM and SEM has better phenolic acid and flavonoid extraction efficacy from *A. indica* leaves, respectively. Methanol has greater value towards phenolic acid and flavonoid extraction when compared to other solvents. The results revealed the presence of phenolic acids such as gallic acid, 3,3´-Methylene-bis (4-hydroxycoumarin), ferulic acid, caffeic acid and syringic acid and flavonoid such as rutin and naringenin were identified by RP-HPLC analysis. The results of the study suggested that *A. indica* is a rich source of phenolic compounds that leads to play a protective role against various degenerative diseases. However, further studies involving complete characterization and bioassay analysis of phenolic compounds in the leaf extract of *A. indica* are necessary in finding the active compound for its cardioprotective effect using *in vitro* and *in vivo* studies. Thus, the solvent and extraction system should modify the extraction efficacy and further it emphasizes the importance of a suitable solvent to achieve maximum phenolic compound extraction and identification of active components in the extracts.

ABBREVIATIONS

RP-HPLC – Reversed Phase-HPLC
PDA – Photo Diode Array
TPC – Total Phenolic Content
TFC – Total Flavonoid Content
GAE – mg of gallic acid equivalent per g of extract
QE – mg of quercetin equivalent per g of extract

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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