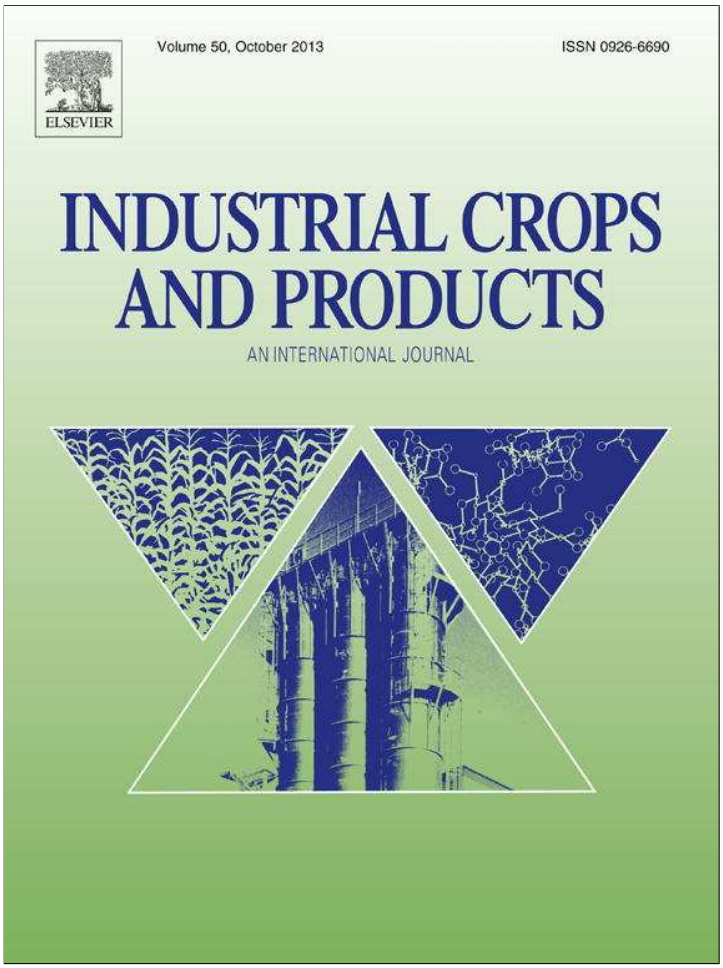
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Screening of phytochemical, physico-chemical and bioactivity of different parts of *Acmella oleraceae* Murr. (Asteraceae), a natural remedy for toothache

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ABSTRACT

Acmella oleraceae Murr. (Asteraceae) is a natural source of bioactive secondary metabolites which are responsible for an array of therapeutic properties. Therapeutic effects are mainly due to the secondary metabolites and antioxidant activity present in different parts of the plant. Purpose of this work was to compare the physico-chemical, phytochemical, total phenol content (TPC), total antioxidant capacity (TAC) and cytotoxicity of different parts of *A. oleraceae*. Physico-chemical and phytochemical parameters were performed according to the methods described in WHO guidelines. The TAC and TPC were determined using Ferric Reducing Antioxidant Power assay (FRAP) and modified Folin–Ciocalteu colorimetric method respectively. Presence of a prominent, bright light green color spot (R_f – 0.78) in TLC fingerprints was characteristic for flower extracts. The highest values for all the physico-chemical parameters, TAC and TPC were found in leaves while higher cytotoxocity was exhibited from flower extracts. Order of cytotoxicity was flower > leaf > stem. Presence of higher cytotoxicity in flower and leaf extracts scientifically validates the extensive use of flower and leaf in traditional systems of medicine in Sri Lanka. Information generated are vital important for the quality control and standardization of *A. oleracea* in order to validate/upgrade the Sri Lankan pharmacopeia.

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1. Introduction

Acmella oleraceae Murr. (Asteraceae) is a therapeutically important annual or short-lived perennial medicinal herb with yellow, non-fragrant flowers (Jayaweera, 1981). It has been widely used in Ayurveda and folk systems of medicine as an anti-inflammatory, antiseptic and anesthetic drug since historic times (Dias *et al.*, 2011). In traditional medicine, flowers have been chewed to relieve toothache and infection of throat and to paralyze the tongue (Jirovetz *et al.*, 2006). Moreover, the chloroform extract of *Acmella* species have been successfully used to inhibit tobacco-induced mutagenesis (Sukumaran and Kuttan, 1995). Biological properties of *S. acmella* mainly depend on N-Alkylamides, which are a group of bioactive molecules found in genera Spilanthes ((Keipert Ronald, 2009). The major constituent of these N-Alkylamides group is spilanthol (Fig. 1), the principal bioactive compound which has traditionally been used for the treatment of toothache and responsible for an array of bioactivities such as antioxidant, anti-inflammatory activities (Boonen *et al.*, 2010; Dias *et al.*, 2011), oral health care (Hebbar *et al.*, 2004), diuratic activity (Ratnasooriya *et al.*, 2004) and treatment for tooth ache (Ong and Nordiana, 1999). Moreover, Dolui and Debnath (1996, 2010) discovered that the methanolic extracts of leaf and flower extracts of *Acmella culva* possess a significant antifeedant activity against *Helopeltis theivora*. Flowers have been reported as the highest anti-inflammatory activity.

Medicinal value of the plant lies on the chemical active substances present in the plant. Thus, it is important to ensure the quality and the standards of herbal material. Although this plant possesses diverse range of therapeutic effects, information on quality control and standardization is yet to be investigated. Therefore, the present study was undertaken to establish quality control parameters for different parts of *A. oleraceae* through established protocols.

2. 2.Materials and methods

2.1. Plant materials

Leaves, stems, flowers were obtained from fully matured *A. oleraceae* plants maintained in similar soil and climatic conditions in

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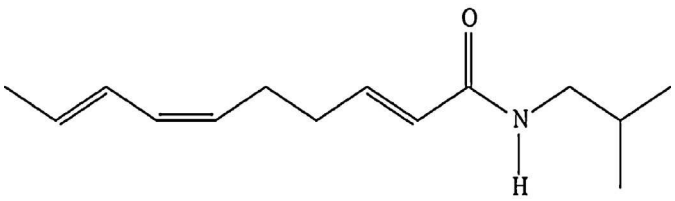


Fig. 1. Structure of spilanthol.

institutional research plots. Herbarium specimens were prepared and deposited in institutional herbarium (HTSSA-20). Collected samples were cut into pieces and air dried for three days at room temperature (28 ± 2 °C). These materials were ground to coarse powder using grinder (Janke and Kundel, Staufen, Germany). All solvents used for the extraction of phytochemicals were from Sigma Chemical Company (USA).

2.2. Determination of moisture content

Two grams of each sample was dried separately at 105 °C to a constant weight. Then it was cooled in a desiccator for 30 min and recorded the moisture content.

2.3. Quantification of total ash

Two grams of air-dried plant sample ignited at 500–600 °C until the sample turn into white color. After cooled down in a desiccator for 30 min total ash content was recorded.

2.4. Quantification of water-soluble ash

Previously ignited ash was boiled with 25 mL of distilled water for 5 min. Then it was filtered using a Whatman ashless filter-paper. Insoluble matter was washed with hot water and ignited for 15 min at 450 °C. The residue was allowed to cool in a desiccator for 30 min.

2.5. Quantification of acid-insoluble ash

The crucible containing ash was gently boiled with 25 mL of HCl. Insoluble matter was collected to Whatman ashless filter-paper and washed with hot water until the filtrate become neutral. Then the acid insoluble matter was transferred to original crucible and ignited to a constant weight at 450 °C. Then the residue was allowed to cool in a desiccator for 30 min.

2.6. Quantification of total extractable matter

2.6.1. Hot extraction method

Four grams of coarsely powdered samples of leaf, stem and flower were separately refluxed with 100 mL of methanol for 1 h. Then the mixture was filtered and total weight was re-adjusted by adding methanol. Then 25 mL of the filtrate was concentrated in a rotavapour (Buchi Rotavapour, Type-R-114A29 B-480, Switzerland) at 45 °C. The residue was dried at 105 °C for 6 h and allowed to cool for 30 min. The weight was recorded.

2.6.2. Cold maceration method:

Four grams of coarsely powdered, sample was macerated with 100 mL of methanol for 6 h. Then it was allowed to stand for 18 h and filtered. Then 25 mL of the filtrate was concentrated in a rotary evaporator at 45 °C. The residue was dried at 105 °C for 6 h, cooled in a desiccator for 30 min.

2.7. Sample preparation for chemical analysis

About 15 g of coarsely powdered plant material was extracted with 50 mL of methanol by using Soxhlet apparatus. The extract was concentrated at 45 °C using rotovapour (Buchi Rotavapour, Type-R-114A29 B-480, Switzerland).

2.8. Phytochemical screening

The phytochemical screening tests for alkaloids, flavonoids, saponins, steroid glycosides and tannins were performed according to the method described by Farnsworth (1966).

2.9. Thin layer chromatography

The thin layer chromatography (TLC) was performed according to the method described in WHO guidelines (WHO, 1998) with some modifications. Pre-prepared methanol extracts of leaf stem and flowers of *A. oleraceae* were dissolved in 10 mL of methanol separately and 8 μL of the solutions of each extract was spotted on TLC plates (Pre-coated silica gel 60 A 20 × 20 cm; 0.2 mm thickness) and dried. Mobile phase consisted of chloroform: dichloromethane: cyclohexane: methanol (5:4:1:0.4). Spots were detected under UV 366 nm. Then plates were sprayed with Vanillin-Sulfuric acid and dried at 105 °C for 5 min.

2.10. Determination of total phenolic content

The total phenolic content was determined using a modified Folin–Ciocaltue method as described by Abeysinghe *et al.* (2007). Twenty milligrams of dried extract was dissolved in 5 mL of 80% methanol. 0.5 mL methanolic extract was mixed with 4 mL of distilled water and 0.5 mL of Folin–Ciocalteu phenol reagent and kept for 3 min. Then, 1 mL of saturated sodium carbonate was added and absorbance was measured at 760 nm using spectrophotometer (Shimadzu, UV Mini 1240, Japan) after incubated for 2 h in a water bath at 30 °C. Gallic acid was used as the standard solution.

2.11. Determination of total antioxidant capacity

Total antioxidant capacity was determined using Ferric Reducing Antioxidant Power (FRAP) assay as described by Benzie and Strain (1996). Twenty milligrams of dried extract was dissolved in 5 mL of 80% methanol. Methanolic extract (100 μL) of the extract was mixed with 900 μL of freshly prepared FRAP reagent of pH 3.6 containing 2.5 mL of 10 mol/L, 2,4,6-Tripyridyl-s-Triazine (TPTZ) solution in 40 mmol/L, HCl plus 2.5 ml of 20 mmol/L $FeCl_3$ and 25 mL of 300 mol/L acetate buffer. Absorbance of the reaction was measured at 593 nm using the spectrophotometer (Shimadzu, UV Mini 1240, Japan) after incubating for 4 min. The trolox was used as the standard solution.

2.12. Brine shrimp toxicity assay

Brine shrimp assay was carried out as described by Michael *et al.* (1956) with slight modifications. *Artemia salina* eggs were incubated in 500 mL of brine water under illumination at 28 °C for 24 h and larvae were transferred to 12 well plates containing 1 mL of aerated artificial brine water. The extracts of 3 different concentrations (5 ppm, 25 ppm and 50 ppm) were added into the wells and left for 24 h at 28 °C. Artificial brine water was used as the control. The number of death larvae was counted under light microscope (Olympus CX31, Japan).

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Table 1
Physicochemical parameters of different parts of *Acmella oleraceae*.

Part of the plant	Moisture content (%)	Ash content (%)			Extractable matter	
		Total ash	WSA	AIA	HEM (%)	CEM (%)
Leaf	10.48 ± 0.12[a]	18.31 ± 0.36 [a]	12.66 ± 0.23 [a]	0.93 ± 0.16 [a]	18.04 ± 1.78 [a]	17.38 ± 0.27 [a]
Stem	10.41 ± 0.02[a]	18.28 ± 0.26 [a]	10.64 ± 0.60[b]	0.78 ± 0.08 [a]	16.58 ± 0.10 [a]	16.54 ± 0.21 [a]
Flower	8.48 ± 0.36[b]	11.22 ± 0.14[b]	6.89 ± 0.12[c]	0.72 ± 0.09 [a]	10.17 ± 0.30[b]	9.05 ± 0.51[b]

Means followed by same letter in each column are not significantly different at 0.05 level. WSA = Water soluble ash; AIA = acid insoluble ash; HEM = hot extraction method; CEM = cold extraction method.

2.13. Statistical analysis

Results of physico-chemical parameters and antioxidant activity were analyzed by general linear model (GLM) ANOVA test followed by Duncan's Multiple Range Test (DMRT). LC_{50} values of brine shrimp toxicity assay were generated using probit analysis of Minitab 15 version and presented as means ± SE with 95% confidential level.

3. Results and discussion

Present work compared the physico-chemical (moisture, total ash, water soluble ash, acid insoluble ash and extractable matter contents) phytochemical (alkaloids, flavonoids, saponins, steroid glycosides and tannins), total phenol content (TPC), total antioxidant capacity (TAC) and cytotoxicity of different parts (leaf, stem and flower) of *A. oleraceae* as basic quality standards for herbal drug standardization process. As shown in Table 1, moisture content of all samples is almost ≤10%. However, moisture content of flower was significantly low (8.48 ± 0.36) from leaf and stem samples. Moreover, maintaining of moisture content below 10% is desired for herbal preparations. Present study reveals the total ash and water soluble ash contents were significantly ($p < 0.05$) lower in flowers compared to the leaves and stem (Table 1).

Extractable matter percentage is one of the parameters used for the characterization of botanical drugs (Gami and Parabia, 2010). Comparatively higher extractable matter contents were observed in the extracts of all plant parts (leaf, stem and flower) obtained through hot extraction method over the cold extraction method. This may be due to enhancement of extraction process in hot extraction procedure. However, significantly lower extractable matter contents were observed in flower extracts in both hot (10.17 ± 0.30) and cold extractions (9.05 ± 0.51). Presence of lower extractable matter in flower extracts might be due to the presence of some waxes and heavy hydrocarbons. The similar pattern has been observed by Stashenko *et al.* (1996), who observed the lower extractable matter in flower extracts of *Spilanthes americana* due the presence of some waxes and heavy hydrocarbons. Physico-chemical parameters such as moisture content, total ash content, water soluble ash content, acid insoluble ash content and extractable matter content play an important role in quality control and standardization by means of stability, purity and

Table 2
Qualitative phytochemical parameters of *Acmella oleraceae*.

Phytochemicals	Part of the plant		
	Leaf	Stem	Flower
Alkaloids	+	+	+
Flavonoids	+	+	+
Saponins	+	+	+
Steroid Glycosides	+	+	+
Tannins	+	+	+

+ Presence; – Absence.

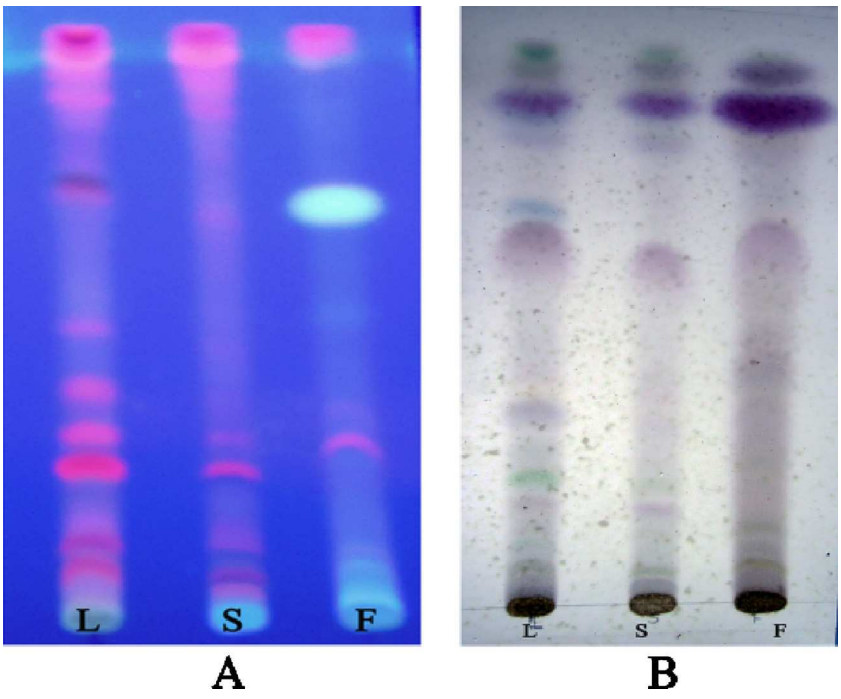


Fig. 2. Thin layer chromatogram of different parts of *Acmella oleraceae*. (A) Under UV 366 nm; (B) After spraying Vanillin sulfuric acid; L – Leaf; S – stem; F – flower.

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Table 3
Contents of total phenolics and total antioxidant capacity (TAC) and cytotoxicity of *Acmella oleraceae*.

Part of the plant	Total phenolics (mg GAE per g of dry matter)	TAC (mg TE per g of dry matter)	Brine Shrimp Toxicity Assay	
			LC_{50} (ppm) (after 24 h)	95% confidence level
Leaf	7.59 ± 1.26 [a]	5.29 ± 0.85 [a]	15.89 ± 3.46 [a]	8.30–22.92
Stem	1.65 ± 0.35 [b]	1. 42 ± 0.40 [b]	23.87 ± 4.28 [b]	15.39–34.07
Flower	5.34 ± 0.75 [c]	3. 42 ± 0.59 [c]	9.75 ± 1.79 [a]	6.16–13.66

Means followed by same letter in each column are not significantly different at 0.05 level; GAE = Gallic acid equivalent; TE = trolox equivalent; LC = lethal concentration.

phytochemical composition of an herbal drug (Gami and Parabia, 2010). Since the ash represent the physiological (derived from plant itself such as calcium oxalate and silicate) and non-physiological (accumulated from external environment such as sand, soil, adulterants etc.) impurities, determination of these parameters are vital important in order to maintain the purity of herbal medicines (Gami and Parabia, 2010; Kunle *et al.*, 2012).

As shown in Table 2, Current study exhibited the presence of major phytochemicals (alkaloids, flavonoids, saponins, steroid glycosides and tannins) in all three main parts (leaf, stem and flower) of *A. oleraceae*. Preliminary investigation of phytochemicals is important for the quantitative estimation and locating of pharmacologically active chemical compounds (Sharanabasappa *et al.*, 2007). Presence of biologically active phytochemicals which act as stimulant, and local anesthetic properties and uses of areal parts, flower heads, roots and whole plant of *A. oleraceae* have been investigated (Leng *et al.*, 2011).

Thin layer chromatography (TLC) is the widely used analytical method in herbal drug standardization process due to its simplicity and cost effectiveness. As shown in Fig. 2,

TLC finger print profiles observed under UV 366 nm exhibited the higher number of spots in leaf extracts (12 spots) followed by stem (10 spots) and flowers (8 spots). A prominent, bright light green color spot (R_f – 0.78) under UV 366 nm was characteristic for flower sample while it was not observed in leaf and stem extracts. After spraying the color reagent (vanillin sulfuric acid) 12 spots for leaf and 8 spots for stem and flower were observed. A blue color spot (R_f – 0.67) was observed only in leaf sample. However, some common spots for all samples were observed in TLC chromatogram.

The phenolic contents were significantly higher in leaves and flowers while the lowest value was observed in stems. The order of increase was leaf > flower > stem with the values of 7.59 ± 1.26, 5.34 ± 0.75 and 1.65 ± 0.35 mg Gallic acid equivalent (GAE)/g dry matter respectively. Our results are in agreement with Shanthi and Amudha (2010), who evaluated the phenolics contents of inflorescence, leaves and whole plant extracts *Spilanthes culva.*

The total antioxidant capacity (TAC) of tested extracts was also exhibited in same order as observed in TPC. The TAC was also significantly different among leaf, stem and flower. The values of TAC were 5.29 ± 0.85, 3.42 ± 0.59 and 1.42 ± 0.40 mg/TE/DM/g for leaf, flower and stem samples, respectively. This study exhibited the significantly high TAC and TPC in leaves and flowers. Antioxidants and phenolics are secondary metabolites which are mainly responsible for the defense mechanisms of a plant (Pourmorad *et al.*, 2006). As shown in Table 3 all the parts of *A. oleraceae* exhibited brine shrimp toxicity. Order of potency was flower > leaf > stem. Flowers displayed very potent cytotoxicity. Since brine shrimp toxicity assay has a good correlation with cytotoxic activities, it is commonly used as a preliminary tool for screening cytotoxicity in plant crude extracts (Vivas *et al.*, 2005). Extensive use of flowers and leaves of *S. acmella* in traditional systems of medicine for tooth ache (Jayaweera, 1981) and modern scientific investigations such as extraction of spilanthol (Dias *et al.*, 2011); transdermal behavior of spilanthol (Boonen *et al.*, 2010) antifeedant activity (Dolui and Debnath, 2010); and cytotoxicity (Sharmin *et al.*, 2012); anti-inflammatory and diuretic activity (Ratnasooriya *et al.*, 2004) have been reported. Therefore, results of the present study clearly validate the extensive use of flower and leaf of *A.oleraceae* in traditional systems of medicine and pharmaceutical industry.

4. Conclusions

The present study compared physico-chemical parameters, phytochemical analysis, antioxidant activity and cytotoxicity of leaf, stem and flower extracts of *A. oleraceae* for the first time in Sri Lanka. Presence of higher, phenolics content, antioxidant capacity and brine shrimp toxicity in leaf and flower extracts scientifically validates the use of leaves and flowers in traditional systems of medicine in Sri Lanka

Information generated through the present study could be effectively used for the quality control and standardization process of different parts of *A. oleracea* in order to validate/upgrade the Sri Lankan pharmacopeia.

Conflicts of interests

Authors declare that there is no conflict of interest.

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