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Chemical standardization of leaf extract of *Coccinia grandis* (L.) Voigt (Cucurbitaceae) of Sri Lankan origin

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Abstract
New drug leads from natural products either as pure compounds or as standardized herbal preparations provide a plethora of management opportunities in modern medicine. *Coccinia grandis* (Linn.) Voigt (Cucurbitaceae) has been widely used for the management of diabetes mellitus in Sri Lankan traditional medicine. The present investigation was to standardize the leaf extract of *C. grandis* through standard *in vitro* techniques. The determination of physicochemical parameters and antioxidant activity, analysis of selected heavy metals, phytochemicals and development of thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) fingerprints were carried out using standard protocols. The analysis of physicochemical parameters showed that the *C. grandis* leaves were consisted of 14.2% moisture, 19.5% total ash, 13.3 % water soluble ash, 1% acid insoluble ash. In contrast, selected heavy metals were absent in the plant material. TLC and HPLC fingerprints clearly demonstrated the proportional differences of these chemical constituents. In conclusion, the quality control parameters obtained from this study can be used as reference standards for the leaf of *C. grandis* of Sri Lankan origin.

Keywords: *Coccinia grandis*, HPLC fingerprint profile, physicochemical analysis, phytochemical analysis, TLC fingerprint profile

1. Introduction
Natural products have long been an inspiration for the development of novel drugs. Indeed, medicinal plant extracts are undeniably the best sources for diversity that have been used for the discovery of novel therapeutics. Plant based drugs are used worldwide to treat a myriad of ailments in humans since time immemorial. The use of herbal medicines has increased remarkably in line with global trend of people returning to natural therapies [1]. However, one of the impediments in the acceptance of the herbal extracts among general public is lack of chemical standardization [2].

New drug leads from natural products either as pure compounds or as standardized herbal preparations provide a plethora of management opportunities in modern medicine. It is estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants [3, 4]. Non availability of pharmacopoeial standardization for plant based medicines is one of the major draw backs that often over shadow the time tested healing properties of plant medicines [5]. Therefore, chemical standardization of plant extracts is essential after confirming the bioactivities by *in vivo* experimental procedures as it strengthens their proven in *vivo* safety and efficacy.

As in many Asian countries in the world, Sri Lanka constitutes an apt example where medicinal plants are widely used in everyday life for culinary purposes and as medicinal remedies. A huge scientific literature has been focusing on bioactivities of Sri Lankan medicinal extracts over the past recent years. However, chemical standardization were carried out for a tiny minority of plant species [6-8].

Coccinia grandis (Linn.) Voigt (Cucurbitaceae) is an edible perennial climber distributed in tropical Asia, commonly found in Sri Lanka, India and Pakistan. Every part of this plant is valuable in medicine and various decoctions have been prepared in traditional Sri Lankan medicine for the treatment of various skin diseases, diabetes mellitus, urinary tract infections, bronchitis, itchy skin eruptions and ulcers [9]. Further, leaf extract of *C. grandis* is widely used by traditional physicians for the management of hyperglycemia and related microvascular complications associated with diabetes mellitus [10]. To date, the acute and long term antihyperglycemic activity of *C. grandis* at the optimum therapeutic dose (0.75 g/kg) and pancreatic mechanism of antidiabetic activity was scientifically proven in streptozotocin induced (STZ) diabetic rats by our research group [11, 12]. To date, complete chemical standardization of the leaf extract of *C. grandis* was not reported.

The aim of the present study was to standardize the leaf extract of *C. grandis* of Sri Lankan origin through standard *in vitro* techniques.

2. Materials and Methods

2.1 Chemicals and instruments

All chemicals and solvents were of analytical grade and used without any purification. UV visible spectrophotometer (Gallenkamp PLC, UK) and rotatory evaporator (Buchi, B-480) were used in the standardization of *C. grandis* extract.

2.2 Plant material

Leaves of *C. grandis* were collected during May-June 2015 from the Southern region of Sri Lanka. Botanical identity was determined by the descriptions given by Jayaweera [9] and confirmed by comparing authentic samples at the National Herbarium, Royal Botanical Gardens, Peradeniya, Sri Lanka. A voucher specimen was preserved at the Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Sri Lanka (Attanayake/2011/ 01).

2.3 Preparation of the plant extract

Plant parts were cut into small pieces and air dried. Plant material (60 g) was boiled in 1.9 L distilled water and the final volume was reduced to 240 mL. The hot water extract was freeze-dried and stored at 4 °C until use.

2.4 Determination of physiochemical parameters

Physicochemical parameters were determined according to methods described in the guidelines of WHO [13].

2.4.1 Moisture content

The powdered material (1.0 g) was placed in an aluminum moisture dish and dried to a constant weight in an oven at 100-105 °C. The weight loss of the sample was calculated using the below mentioned formulae.

$$\% \text{ Moisture content} = \frac{\text{Weight loss}}{\text{Weight of sample}} \text{ x } 100\%$$

2.4.2 Total ash content

The powdered material (2.0 g) was accurately weighed in a previously ignited crucible. The material was spread in an even layer and ignited by increasing the heat to 500-600 °C in a muffle furnace until it was white, indicating the absence of carbon. The crucible was cooled in a desiccator and weighed. The content of total ash in the dried material was calculated using the below mentioned formulae.

$$\% \text{ Total ash} = \frac{\text{Total ash weight}}{\text{Weight of sample}} \text{ x } 100\ \%$$

2.4.3 Acid-insoluble ash content

HCl (2M, 25.0 mL) was added to the crucible containing the total ash, covered with a watch glass and boiled gently over a flame for 5 min. The watch glass was rinsed with 5.0 mL of hot water and the rinsed contents were added to the crucible. The acid insoluble matter was collected on a filter paper and washed with hot water until the filtrate was neutral. The filter paper containing the acid insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to constant weight. The percentage of acid insoluble ash was calculated using the below mentioned formulae.

$$\% \text{ Acid insoluble ash} = \frac{\text{Weight of acid insoluble ash}}{\text{Weight of sample}} \text{ x } 100\%$$

2.4.4 Water soluble ash content

Water (25.0 mL) was added to the crucible containing the total ash and boiled for 5 min. The water insoluble matter was collected on an ashless filter paper and washed with hot water. The filter paper containing the water insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to a constant weight. The weight of this residue was subtracted from the weight of total ash and the content of water soluble ash calculated.

$$\% \text{ Water soluble ash} = \frac{\text{Total ash weight-Water insoluble residue}}{\text{Weight of sample}} \text{ x } 100\%$$

2.4.5 Heavy metal analysis

Presence or absence of heavy metals mercury (Hg), lead (Pb), cadmium (Cd) and arsenic (As) were determined according to Sri Lanka Standard (SLS) and Association of Analytical Communities (AOAC) standards [14, 15].

2.5 Determination of antioxidant activity

2.5.1 2, 2′-diphenyl-2-picrylhydrazyl hydrate (DPPH)

The total antioxidant activity was measured by the DPPH radical scavenging assay method [16]. The radical scavenging activity of plant extract against stable DPPH radical (DPPH*) was determined. When DPPH* reacts with an antioxidant compound in the plant extract, the antioxidant donates hydrogen and reduced. The change in color from deep violet to light yellow was measured spectrophotometrically at 517nm. Briefly, 0.004% DPPH solution was prepared with ethanol and the absorbance of the DPPH solution alone was measured ($A_{control}$) at 517 nm. Ethanol was used as the sample blank. A volume of 1.0 mL of each extract at different concentrations (1-500 µg/mL) was added to 3.0 mL of 0.004% ethanol DPPH solution. The mixture was shaken vigorously, allowed to stand at 25 °C in dark for 30 min. The decrease in absorbance of the resultant solution was measured spectrophotometrically at the same wave length (A_{sample}). L-Ascorbic acid was used as the reference compound. The antioxidant activity is expressed in terms of IC_{50} (concentration of the extract /reference compound required to inhibit DPPH radical formation by 50%).

% DPPH radical scavenging activity= $(A_{control}-A_{sample})/A_{control}$ x100%

Where, $A_{control}$ represents the absorbance of the control without the plant extract/reference compound, A_{sample}represents the absorbance of the plant extract/reference compound.

2.6 Qualitative and quantitative analysis of chemical constituents

2.6.1 Phytochemical screening

Preliminary qualitative phytochemical screening for the presence of alkaloids, cardenolide glycosides, phenols, flavonoids, phytosterols, saponins, tannins, reducing sugars, and proteins was carried out by the reported protocol [17].

2.6.2 Total polyphenol content

Total polyphenol content was measured using Folin-Ciocalteu colorimetric method [18]. Plant extract (1. 0 mL of 0.05 g/mL) was mixed with 1.0 mL of 95% ethanol, 5.0 mL of distilled water and 0.5 mL of 50% Folin-Ciocalteu reagent. The mixture was allowed to react for 5 min and 1.0 mL of 5% sodium carbonate was added. Thereafter, it was thoroughly mixed and placed in dark at room temperature (27 °C) for one hour and the absorbance was measured

spectrophotometrically at 725 nm. Quantification was done with respect to the standard curve of gallic acid (0-50 µg/mL). The results were expressed in gallic acid equivalents mgGAE/g of the dry weight.

2.6.3 Total flavonoid content

The total flavonoid content of plant extracts was determined using aluminum chloride colorimetric method of Koksal and Gulcin [19] based on the method of Chang *et al.* [20]. The plant extract
(0.50 mL) was mixed with ethanol 95% (1.5 mL) followed by aluminium chloride 10% (0.10 mL), potassium acetate 1M (0.10 mL) and distilled water (2.8 mL). The resultant mixture was incubated at 27 °C for 30 minutes. The absorbance of the reaction mixture was measured spectrophotometrically at 415 nm. The flavonoid content was calculated using standard calibration of quercetin solution in range of 0-50 µg. The results are expressed as micrograms of quercetin equivalent (QE)/g of the dry weight.

2.6.4 Development of Thin Layer Chromatography (TLC) profile

Dichloromethane (50 mL) was added to a beaker containing approximately 10 g of the sample and stirred well for 4 hours. Then the solution was filtered and evaporated using a rotory evaporator. The residue was dissolved in 5 mL of dichloromethane and the extract (7 µL) was spotted on a TLC plate. R_f values and the colour of the spots were recorded. Silica gel-GF_{254} was used as the absorbent. TLC fingerprint profile was developed by using methanol, dichloromethane and cyclohexane in a ratio of 0.1:1:1 (v/v/v). Spots were observed under UV (both 254 nm and 366 nm) light and they were visualized using vanillin sulphuric acid.

2.6.5 Development of high performance liquid chromatography (HPLC) profile

Each diluted dichloromethane extract (10 mg in 5 mL) was purified using Sep-pak C18 cartridge and HPLC fingerprint profile was developed using the following conditions. Injection volume: 20 µL; Apparatus: Shimadzu LC-10 ADvp pumps and Shimadzu SPD –M10 Avp uv/vis photodiode array detector; Column: Inertsil 5U ODS-2 reverse phase column (250 mm x 2.6 mm); Solvent system: Acetonitrile: Water (40:60); Flow rate: 1mL/min and detection at 254 nm.

2.7 Statistical analysis

The replicates of each sample were used for statistical analysis and the values were expressed as mean ± standard deviation.

3. Results

3.1 Physiochemical properties

The results of the physiochemical parameters are shown in Table 1. Any of the tested heavy metals including mercury (Hg), lead (Pb), cadmium (Cd) and arsenic (As) was not detected in the leaf extract of *C. grandis*.

3.2 Antioxidant activity

The IC_{50} of the aqueous leaf extract of *C. grandis* and ascorbic acid in DPPH assay was 45.05±0.85 µg/mL and 4.52±0.11 µg/mL respectively.

3.3 Qualitative and quantitative analysis of chemical constituents

The phytochemical analysis conducted on *C. grandis* extract revealed that the polyphenolic compounds, alkaloids flavonoids, saponins and sterols/ triterpenoids are present whereas cardenolide glycosides, anthracene glycosides and cyanogenic glycosides were absent. The polyphenol and flavonoid contents of aqueous extract of *C. grandis* were 8.59 mgGAE/g of the dry weight ±1.3 and 1.6±0.1 (QE)/g of the dry weight respectively. As shown in Figure 1, TLC fingerprint profile of *C. grandis* showed eight prominent spots bearing R_f values of 0.06, 0.21,
0.38, 0.48, 0.53, 0.58, 0.73, 0.88 and seven prominent spots bearing R_f values of 0.21, 0.29, 0.40, 0.16, 0.53, 0.51, 0.73 under 254 nm and 366 nm respectively. In addition, HPLC fingerprint is shown in Figure 2.

Table 1: Physicochemical parameters of *C. grandis*

Parameter	Percentage (%)
Total ash content	19.5
Water soluble ash content	13.3
Acid insoluble ash	1
Moisture content Heavy metals [a]	14.2
Mercury (Hg)	Not detected
Lead (Pb)	Not detected
Cadmium (Cd)	Not detected
Arsenic (As)	Not detected

[a]Limit of detection (mg/kg); Pb: 0.50; Cd: 0.10; As: 0.05; Hg: 0.05

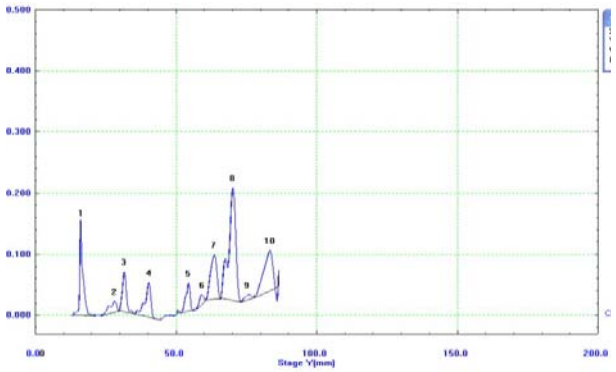

Fig 1: TLC densitogram at 254 nm

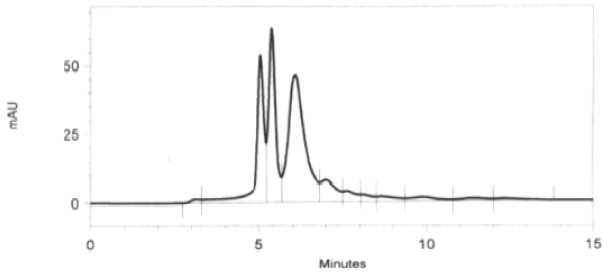

Fig 2: HPLC fingerprint profile of *C. grandis* at 254 nm

4. Discussion

Standardization of crude plant extracts is massively wide and deep. This involves the process of prescribing a set of standards or inherited characteristics, constant parameters, definitive quality and quantitative values that carry an assurance of quality, safety and reproducibility [3, 21]. By way of chemical standardization, basically the plant extracts are evaluated for their physiochemical properties and identification of phyto-constituents through analytical methods. Among the existing analytical methods, chromatographic methods are the mainstream such as thin layer chromatography (TLC) and high performance liquid

chromatography (HPLC). In the present investigation, the determination of physiochemical parameters and antioxidant activity, analysis of selected heavy metals, phytochemicals and development of thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) fingerprints were done in order to standardize the aqueous leaf extract of *C. grandis*.

The quantitative determination of pharmacognostic parameters is useful for implementing standards for medicinal plants. Total ash is particularly important in the evaluation of the purity and the quality of a plant. The ash value was determined by three different methods; total ash, acid insoluble ash, and water soluble ash. The total ash method is employed to measure the total amount of material remaining after ignition [22]. The total ash usually consists of carbonates, phosphates, silicates, and silica, which include both physiological ash and non-physiological ash. The physiological ash derives from the mineral components of the plant itself. However, the plant may contain foreign matter adhered to it by contact with the soil and sand. This foreign matter is known as the non-physiological ash. A high ash value is an indication of contamination, substitution, adulteration, or carelessness in developing nutraceuticals for marketing [23]. Acid insoluble ash indicates contamination with silica, for example, earth and sand. Water soluble ash is the part of the total ash content, which is soluble in water. It is a good indicator of the water soluble salts in the plant [23]. In the present study, very low amount of acid insoluble ash content and moderate level of total ash content indicates the purity of *C. grandis* leaves that could be useful in developing nutraceuticals or dietary adjuncts for the early management of diabetes mellitus and its' complications. Upper limits of Hg, As, Pb and Cd for any raw herbal material should be below 0.2, 5, 10 and 0.3 ppm respectively [13]. Interestingly, heavy metals were not detected in *C. grandis* leaves (minimum detection levels of Hg, As, Pb and Cd are 0.01, 0.05, 0.05 and 0.01 ppm respectively). Therefore, this may be one of the indicators to prove the safety of the plant for human consumption.

The chemical approaches facilitate the study of the total antioxidant activity of medicinal plant extracts and the precise mechanisms of action of antioxidants. Thus far, numerous studies on antioxidant properties of many plant species have been conducted using different assay methods.

However, DPPH assay is widely used to determine the antioxidant activity of plant extracts [24, 25]. Water soluble antioxidant potentials are determined based on the traditional method of intake of plant extracts therapeutically. The IC_{50} values of extracts were calculated for DPPH assay to compare the antioxidant activities of the extract and the standard compound at different concentrations as described by many authors [26, 27]. The leaf extract of *C. grandis* and the standard compound exhibited concentration-dependent radical scavenging activities in the DPPH assay. The DPPH radical scavenging activity was expressed in IC_{50} value to obtain a more precise single value over a range of concentration of plant extracts. The results clearly demonstrated that aqueous leaf extract of *C. grandis* showed relatively high radical scavenging activity in DPPH assay. However, the IC_{50} of standard compound (L-Ascorbic acid) as a powerful antioxidant was more pronounced in DPPH scavenging assays and the value was comparable with previous studies [28]. The DPPH assay is reported to be a direct and reliable method for the determination of radical scavenging activity where the structure of electron donor (e.g. plant extract) is not known. DPPH assay method can afford data on reduction potential of the sample and hence can be helpful in comparing the reduction potential of unknown compounds. In addition the high amount of polyphenol compounds and the presence of flavonoids and saponins may lead to high antioxidant activity (low IC_{50}) as shown in the DPPH assay.

5. Conclusions

The physiochemical parameters and the antioxidative activity of *C. grandis* can be used as reference standards for the assessment of quality control parameters of the plant. Furthermore, the data obtained from the standardization, confirmed the safe consumption of *C. grandis* as a dietary adjunct and the use in the development of pharmaceutical products and nutraceuticals for the management of diabetes mellitus and its' associated complications.

6. Acknowledgement

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