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Standardization and Quality Evaluation of *Cassia surattensis* seed extract.

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ABSTRACT

Standardization and quality control of herbal products used for the development of medicinal materials is important. The objective of the present study is to standardize the *Cassia surattensis* seed extract. Various chromatographic and spectral fingerprinting techniques, and heavy metal analysis and microscopy of seed were used to standardize the *C. surattensis* seed. The findings of the current study can be useful to progress and surge further scientific investigation on the seeds of this species. Pharmacognostic investigation of the fresh and anatomical sections of the seeds of *C. surattensis* was carried out. Pharmacognostic evaluation confirmed the authenticity of the seeds which involved parameters like gross morphology, microscopy of the seeds and functional group analysis by Fourier Transform Infrared (FTIR) spectroscopy. The chromatographic and spectroscopic evaluation provided the qualitative and semi quantitative information about the main active constituents present in the seed extract. The concentrations of heavy metals determined in the *C. surattensis* seed extract were well below the permissible limit. Standardization of *C. surattensis* is needed to ensure their safe and quality control for effective use.

Keywords: Authenticity, *Cassia surattensis*, HPTLC, Standardization.

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INTRODUCTION

There is much interest created by natural resources such as medicinal plants for their antioxidant activity and as a hepatoprotective agent. The genus *Cassia*, comprising about 600 species widely distributed worldwide is well known for its diverse biological and pharmacological properties. *Cassia surattensis* belongs to the family Fabaceae. They are distributed throughout Malaysia. These flowering plants are widely grown as ornamental plants in tropical and subtropical areas. This plant species has been traditionally used in many countries for food and medicinal use. No local uses were known for *C. surattensis*, but the bark and leaves are said to be antibleorrhagic. A property also mention for a decoction of the roots. The Balinese rub the leaves of *C. surattensis* into both internal and external cooling medicine [1, 2].

It is very important that a system of standardization is established for every plant medicine in the market because the scope for variation in different batches of medicine is enormous. Standardization has been the key factor in the field of natural products for their safe and effective use. World Health Organization (WHO) encourages, recommends and promotes traditional /herbal remedies innational health care programmes because these remedies are easily available at low cost, safe and people have faith in them. The WHO assembly in number of resolutions has emphasized the need to ensure quality control of traditional medicinal plant products by using modern techniques and applying suitable standards [3].

For these reasons a report of pharmacognostic parameters for the identification of seeds of *C. surattensis* and standardization of its extract form by using physical methods and chromatographic fingerprints. Standardized plants material with pharmacological activities which enables the quality of the material to be determined can be highly attractive to the researcher for the development of pharmacological products such as antimicrobial and antioxidant agents.

METHODOLOGY

Chemicals and reagents

All the chemicals and reagents used were of analytical grade, purchased from Sigma Chemical Co. (St Louis, MO, USA) or Merck (Darmstadt, Germany).

Plant material samples collection

Plant material samples of *C. surattensis* seeds were obtained from Universiti Sains Malaysia and identified at School of Biological Sciences, Universiti Sains Malaysia. Pods were dried and seeds were removed from the pods. The seeds were washed with water to remove dirt prior to the drying process. Dried seeds of *C. surattensis* were powdered and used for standardization methods.

Extraction

The dried sample (approximately 100 g) was added to methanol (300 mL) and soaked for 4 days at room temperature ($30 \pm 2^\circ\text{C}$). The suspension was stirred from time to time to allow the seed powder to fully dissolve in the methanol. Removal of the sample from the solvent was done by filtration through cheesecloth followed by filter paper (Whatman No. 1); the filtrate was concentrated under a vacuum to one-fifth its volume using a rotary evaporator at 60°C and then sterilized by filtration using a 0.22- μm membrane. The thick paste obtained was further dried in an oven at 40°C . The resultant extract was kept at 4°C for further analysis. The methanol was used for the extraction in this study to mimic the usage of water by the traditional healers to prepare plant extracts as a decoction. Water and methanol have the highest polarity in the polar protic solvents group. Moreover, the usage of methanol makes the process of evaporation easier compared to water.

Macroscopic and microscopic

The macroscopic features of the seeds were determined using the methods of Evans [4]. Anatomical sections, a surface preparation of the fresh seed sample for the microscopy was carried out according to methods outlined by Brain and Turner [5]. Morphological examination of the *C. surattensis* was used to

identify the seed structures. Seed tissue was fixed in 10% buffered formalin. After fixation, the tissue was dehydrated in a graded series of alcohols, cleared in xylene and embedded in paraffin wax. Multiple 5 mm sections from the block were mounted on slides and stained with 0.5% methylene blue and examined under a light microscope [6].

Fourier Transform Infrared (FTIR) spectroscopy

The methanol extract of *C. surattensis* seed extract was mixed with KBr salt, using a mortar and pestle, and compressed into a thin pellet. Infrared spectra were recorded on a Shimadzu FTIR Spectrometer 8000 series, between 4,000–500 cm^{-1} . All determinations were performed in triplicate [6].

High Performace Thin Layer Chromatography (HPTLC)

A HPTLC method for the separation of the active constituents in methanolic extract of *C. surattensis* seeds has been developed and HPTLC of these extracts on silica gel 60 F₂₅₄, glass plate (Merck) by syringe and using solvent system toluene : ethyl acetate : formic acid : methanol (3:3:0.8:0.2 v/v) was performed. The plate was observed after drying under visible light-254 nm and UV 366 nm and the HPTLC fluorescence image documented.

High Performance Liquid Chromatography (HPLC)

For HPLC analysis, 0.1 g of dried materials was mixed with 5 ml of 100 % methanol. The sample-methanol mixture was placed into an ultrasonic bath until it well dissolves. Then the sample-mixture was filtered and the filtrate was put in vial for HPLC analysis. The HPLC analysis of methanolic extract was carried out with Model HPLC (Waters 2695 separation). The separation was performed on Column Novapak C18 4.6 X 250 mm. The mobile phase consists of water to formic acid, A (99 : 1 v/v) and water to methanol to formic acid, B (49 : 50 : 1 v/v) and the elution were performed at a flow rate of 1 ml/min. The samples were run for 15 min and detection was done at 370 nm by Waters 2996 PDA Detector. All chromatographic data were recorded and processed using Empower Software System.

Heavy Metal Analysis

Atomic absorption spectrometry (AAS) was used for the determination of the amount or concentration of specific heavy metals in *C. surattensis* seeds extract. AAS uses the phenomenon that atoms in the ground state absorb light of a specific wavelength that is characteristic of the particular atom when the light passes through an atomic vapour layer of the element to be determined [7]. The determination of Lead (Pb), Cadmium (Cd), Arsenic (As) and Mercury (Hg) were performed on a Perkin-Elmer 200 atomic absorption spectrophotometer under optimized measurement conditions using hollow cathode lamps according to the standard method in the British Pharmacopoeia 2011 [8].

RESULTS AND DISCUSSION

Herbarium

A voucher herbarium specimen is a pressed plant deposited in a herbaria for future reference of the plant. Deposition of voucher specimen in herbaria will facilitate the research activity and will be useful to verify the identity of the plant used in a study. Hence, in this study a *C. surattensis* herbarium was prepared (Figure 1). *C. surattensis* is a recognized source of useful plant for medicinal usage, but authenticity of specimens is important to avoid the acceptance of wrong plant materials for the drug development activities. Therefore, the correctly identified herbarium specimens are useful as references for plant identification and for the determination of plant locations and ranges, abundance, habitat, and flowering and fruiting periods.

Pharmacognostical study

Description of the seed includes physical appearance of the seed by visual examination under sunlight and artificial light sources similar to daylight was reported in this study. Moreover, the features of seed were examined by using the microscope.

Macroscopic characteristics of seeds

The seeds of *C. Surattensis* (Figure 2) were observed to be transverse, oblong, discoid, 4-5 mm long, 2.5-3.5 mm wide, 3-5 seeds in a pod and shiny dark brown in colour. Testa is hard smooth and glossy in appearance.



Figure 1: The *Cassia surattensis* herbarium

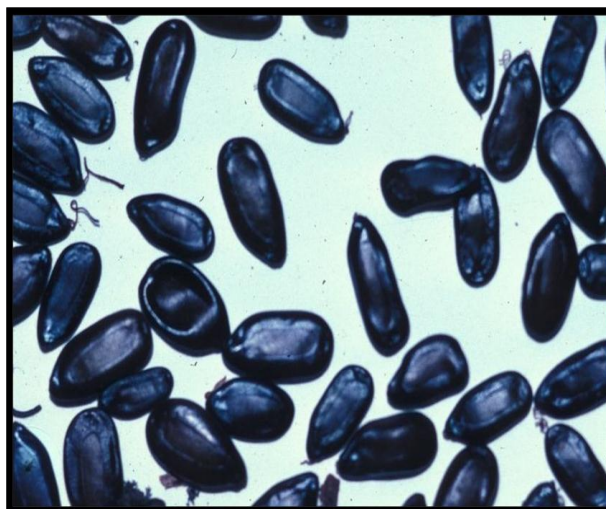


Figure 2: The *Cassia surattensis* seeds

Microscopic characteristics of seeds

The photomicrograph of the longitudinal section of the seed of *C. Surattensis* (Figure 3) revealed the presence of the palisade layer, subepidermal layer, tangentially elongated parenchyma cells and endosperm. Cotyledon positioned in the middle and surrounded by seed coat and endosperm. The light microscopic method offers several advantages over conventional authentications of the medicinal plants including the effectiveness, simplicity, and low cost, and has been widely adopted as an official method in many international herbal pharmacopoeias [9].

Fourier Transform Infrared (FTIR) spectroscopy

The FTIR analysis of plant extract gives information about the distribution of functional groups and provides a basis for comparison of compositional differences between various plants samples. FTIR is perhaps the most powerful tool for identifying types of chemical bonds (functional groups). The wavelength of light absorbed is characteristic of the chemical bond as can be seen in this annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined [10].

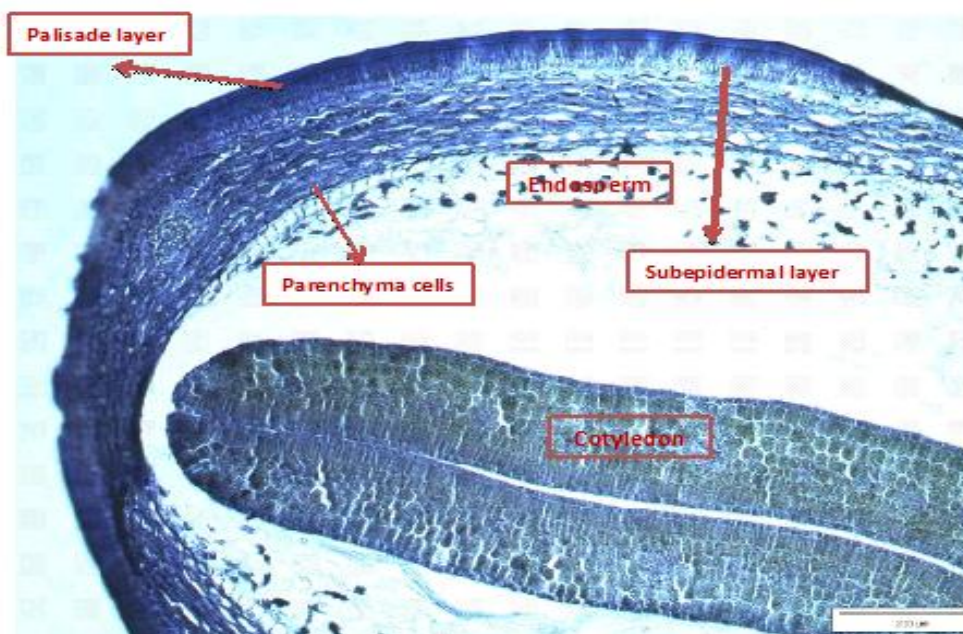


Figure 3: The longitudinal section of the seed of *Cassia surattensis*

C. surattensis seeds extract was analysed for functional group analysis using FTIR. The size of the peaks in the spectrum is a direct indication of the amount of compound present. The results of functional group analysis using FTIR demonstrated that the existence of various characteristic functional groups in the *C. surattensis* seed (Figure 4). Seven major peaks in the range $1,000 - 1,600 \text{ cm}^{-1}$, $1,645 - 2,000 \text{ cm}^{-1}$ and $2,925 - 4,000 \text{ cm}^{-1}$ were observed in the FTIR spectra. Therefore, for future *C. surattensis* methanolic extraction, this FTIR spectrum can be used for comparison. Thus, the intuitive evaluation method is to compare the similarities and/or differences in the shape of the FTIR fingerprints which can be used to ensure that the functional groups in the new extract are present in reproducible manner [9].

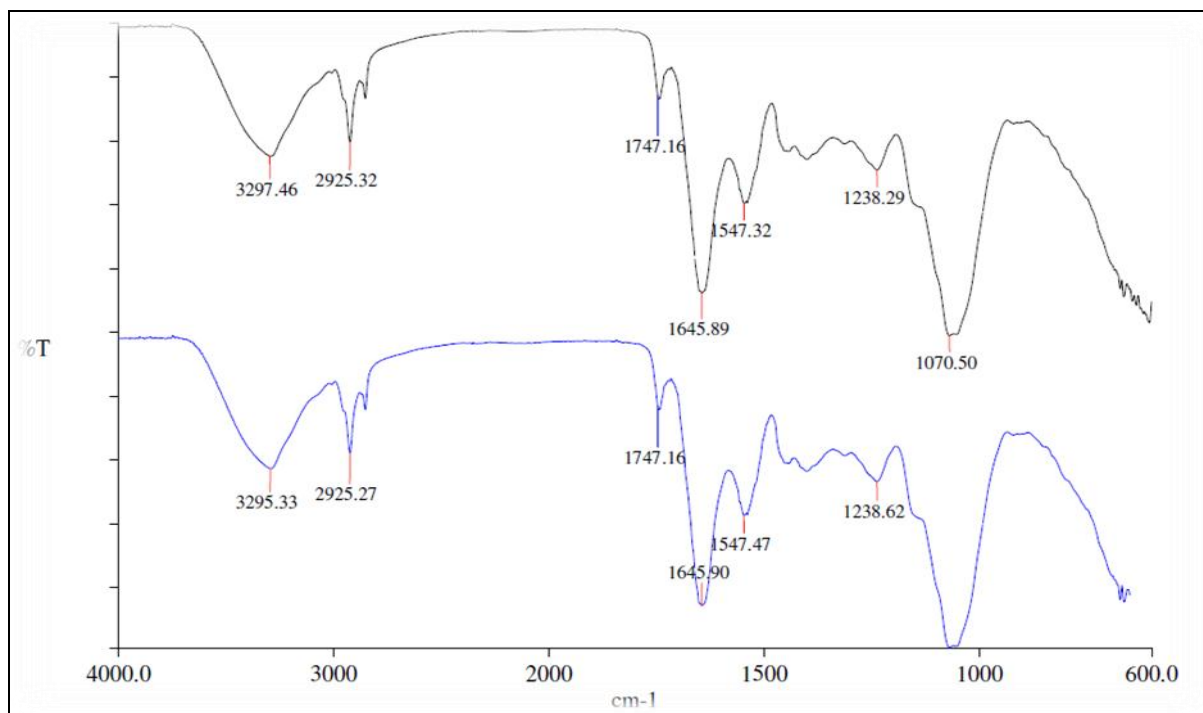


Figure 4: FTIR spectra of *Cassia surattensis* seed extract, cm^{-1}

Chromatographic techniques

Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they might represent appropriately the chemical integrities of the herbal medicines and its products and therefore be used for authentication and identification of herbal plant [11]. Chromatographic techniques such as (i) High Performace Thin Layer Chromatography (HPTLC) and (ii) High Performance Liquid Chromatography (HPLC) were used in this study for separation of chemical components in *C. surattensis* seeds extract.

High Performace Thin Layer Chromatography (HPTLC)

High Performace Thin Layer Chromatography (HPTLC) finger prints of the seed extract has been carried out and the result provide referential information for standardization. The HPTLC method for routine quality control of present species can be carried out using this method for different extracts of plant parts and serve in qualitative, quantitative and was appropriate for standardization of the *C. surattensis* seed. The HPTLC fingerprint is also suitable for rapid and simple authentication and comparison of subtle differences among samples of identical plant resource [12].

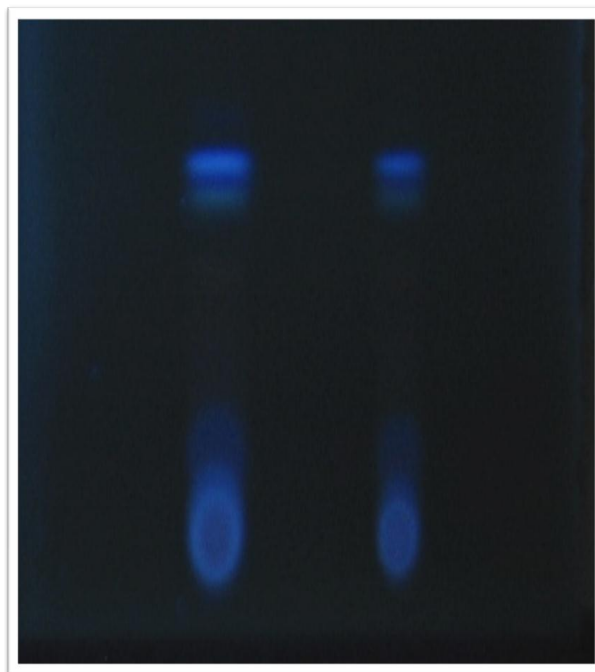


Figure 5: HPTLC fluorescence image of *Cassia surattensis* seed extract observed at 366 nm

Table 1: Peak list and R_f value of the chromatogram of *Cassia surattensis* seed extract at 366 nm.

Peak	Start R_f	Start Height	Max R_f	Max Height	Max %	End R_f	End Height	Area	Area %
1	-0.07	2.5	-0.01	285.7	32.82	0.00	277.6	9740.2	33.19
2	0.01	277.7	0.02	283.4	32.55	0.13	17.7	12322.9	41.99
3	0.14	16.8	0.14	18.2	2.09	0.18	6.1	414.9	1.41
4	0.19	6.9	0.25	16.8	1.93	0.29	2.1	837.7	2.85
5	0.30	2.2	0.32	40.0	4.59	0.35	1.7	591.2	2.01
6	0.41	0.7	0.43	87.7	10.08	0.45	6.9	1224.8	4.17
7	0.45	7.2	0.48	55.6	6.39	0.50	9.1	977.5	3.33
8	0.50	9.6	0.53	16.8	1.93	0.58	0.2	598.5	2.04
9	0.60	0.4	0.64	51.5	5.92	0.71	13.0	2101.7	7.16
10	0.81	14.5	0.82	14.8	1.70	0.89	1.3	534.8	1.82

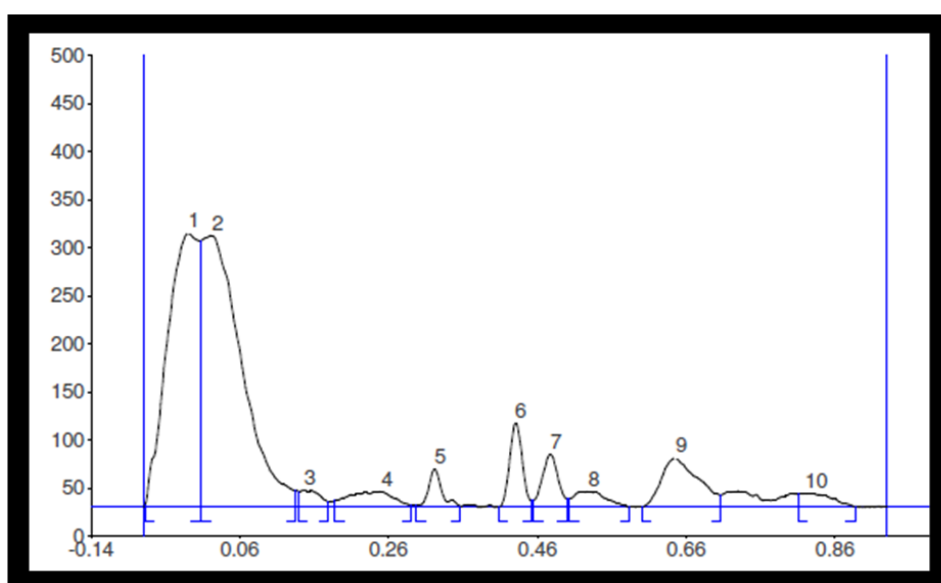


Figure 6: Typical HPTLC densitogram of *Cassia surattensis* seed extract at 366 nm

Figure 5 indicate that the plant extract constituents were clearly separated without any tailing and diffuseness. It is evident from Table 1 that in the methanol extract of *C. surattensis* seed there are 10 spots at the following R_f values of 0.01, 0.02, 0.14, 0.25, 0.32, 0.43, 0.48, 0.53, 0.64, 0.82 as shown in Figure 6, indicating the occurrence of at least 10 different chemical components in methanol extract. It is also clear from Table 1 and the chromatogram as shown Figure 5 that out of 10 components, the component with R_f values of 0.02 and 0.64 were found to be more predominant as the percentage area is more with 41.99% and 7.16% respectively. And remaining components were found to be very less in quantity as the percentage area for all the spots was less than 4.17%. Characteristic HPTLC fingerprinting of particular plant species will not only help in the identification and quality control of a particular species but also provide basic information useful for the isolation, purification, characterization and identification of marker chemical compounds of the species. Thus the HPTLC results will provide sufficient information about therapeutic efficacy of the plant part and also in the identification, standardization and quality control of medicinal plant.

High Performance Liquid Chromatography (HPLC)

For standardization of *C. surattensis* seed extract, HPLC is a sensitive and accurate tool that widely used for the quality assessment of plant extract and its derived product/formulation [13]. HPLC fingerprints is a convenient method to identify the presence of numerous chemicals constituents present in the *C. surattensis* seed extract.

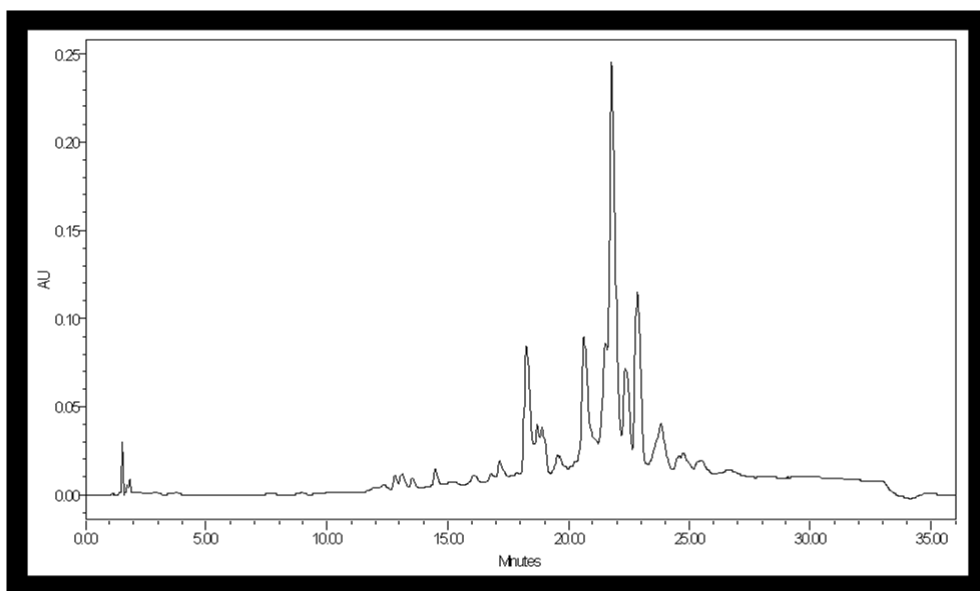


Figure 7: HPLC chromatogram of *Cassia surattensis* seed extract at 370 nm

Results of HPLC analysis (Figure 7) of *C. surattensis* seed extract, at 370 nm, shows the presence of various chemicals constituents as evidenced by the chromatogram obtained at various retention times (i.e. 18.50, 19.00, 20.50, 21.50, 22.50, 23.00 and 24.00). Moreover, the compound with retention times of 21.50 found to be the main constituents in methanolic seed extract.

Heavy Metal analysis

The heavy metals content analysis is one of the criteria for the use of plant material in the production of traditional medicines and herbal infusions. Therefore, the quality control of heavy metals in medicinal plants and their products should be made such to ensure safety and efficacy of herbal products [14]. The heavy metal analysis of mercury, lead, arsenic and cadmium levels in the *C. surattensis* seed extract was determined using AAS. The residues of heavy metals [Lead (Pb), Cadmium (Cd), Arsenic (As) and Mercury (Hg)] in *C. surattensis* seed extract were below the allowed limits according to WHO standard (Table 2) [15].

CONCLUSION

Although *C. surattensis* a very common plant found as a road side tree in most parts of Malaysia and has been used extensively for its medicinal purposes since ages, there are very few scientific studies conducted to support its usefulness. This systematic study gives a scientific proof with respect to its pharmacological standardization and created standards that can serve as an important source of information to identity and to determine the quality and purity of the plant material in future studies.

Table 2: Heavy metal analysis of *Cassia surattensis* seed extract

PARAMETER	RESULTS	SPECIFICATION
LEAD	0.279 ppm	Not more than 10 ppm
CADMIUM	0.047 ppm	Not more than 0.3 ppm
ARSENIC	0.216 ppm	Not more than 5.0 ppm
MERCURY	0.069 ppm	Not more than 0.5 ppm

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