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Research Article

Pharmacognostical, Physico-chemical and Phytochemical Evaluation for Standardization of Three *Piper* species Used in Ayurvedic Medicine

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Abstract: The plant species of the family Piperaceae contain valuable natural compounds. Among the genera of Piperaceae family the Piper is an important genus which consists of 700 plant species growing in the different parts of the world. Many plants of this piper genus are the essential medicinal plants used in various traditional systems of medicine. Among those plants fruits of *Piper nigrum* L., fruits and root of *Piper longum* L. and stems and roots of *Piper retrofractum* Vahl are commonly used in the Sri Lankan traditional system and Ayurveda system of medicine. Piper species have demonstrated strong efficacy against both malignant and non-malignant diseases. Piperine is a valuable alkaloid found in the fruits and roots of *P. longum* (long pepper), *P. nigrum* (black pepper) and stems of *P. retrofractum*. The main objective of this study was to establish the physico-chemical, phytochemical and pharmacognostical profiles of *P. longum*, *P. nigrum* and *P. retrofractum* in order to develop standard parameters. Standardization parameters such as ash values, extractive values, heavy metals and qualitative phytochemical analysis were performed. Analysis of TLC and HPTLC fingerprint patterns of methanolic extract of 3 plants were also done. The results of ash and extractable values were comparable with the results in Ayurveda pharmacopeia of India. Phytochemical analysis reveals the presence of saponins, alkaloids, tannins, phenols, flavanoids, terpinoids, cardiac glycosides and steroids. Heavy metal concentrations were found to be within the limits. Results of these physico-chemical parameters, phytochemical, macroscopic and microscopic analysis can be used to assess the quality and detection of any adulteration for *P. nigrum, P. longum* and *P. retrofractum*.

Keywords: Standardization, Pharmacognosy, Physico-chemical, Phytochemicals

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INTRODUCTION

Medicinal plants have been the base of medicines since long time. The plant species of the family Piperaceae contain valuable natural compounds. Among the genera of Piperaceae family, Piper is an important genus which consists of 700 plant species growing in the various parts of the world (Parmer *et al*, 1997). Many plants of this piper genus are known as essential medicinal plants used in various traditional systems of medicines. Piper species have demonstrated strong efficacy against both malignant and non-malignant diseases (Mgbeahuruike *et al*, 2017). Piperine is a valuable alkaloid found in the fruits and roots of *Piper nigrum* L. (black pepper), *Piper longum* L. (long pepper), and stems of *Piper. retrofractum* Vahl. (Javanese long pepper) species. This alkaloid is responsible for the pungency along with chavicine, an isomer of piperine (Vasavirama & Upender, 2014). Scientific investigations have been confirmed that piperine exhibits potential anti-microbial, anti-oxidant, anti-inflammatory, anti-cancer, anti-depressant, anti-pyretic and analgesic activities (Taqvi *et al* 2008; Manoharan *et al* 2009; Parganiha *et al* 2011). The main objective of this study is to compare the pharmacognostical, physico chemical and phytochemical and HPLC profiles of 3 species of *P. nigrum, P. longum* and *P. retrofractum* commonly used in Sri Lankan traditional system of medicine and Ayurveda. *P. nigrum* is commonly known as black pepper and indigenous to South India. It is cultivated in Sri Lanka, India and Phillipine Islands. In Sanskrit, it is known as Maricha and it is a climbing perennial vine with much thickened nodes. Fruits are mainly used and occasionally roots are also used in Ayurvedic and traditional formulations. Black pepper is very useful in the treatment of intestinal worm infestations, asthma and chronic respiratory diseases. *P. longum* is a perennial herb with a thick, branched rootstock. In Sanskrit, it is known as pippali and it grows in Sri Lanka, warmer parts of India, Malay Peninsula and Philippine Islands (Jayaweera, 1981). The fruits and roots are the main used parts which are especially used in gastro intestinal tract diseases and respiratory tract diseases.*P. retrofractum* or chavya in Sanskrit is a plant cultivated in Sri Lanka, India and Malay islands and it is native to Java and Sumatra Islands (Jayaweera, 1981). *P. retrofractum* is a climbing perennial vine with cylindrical branched stems. The root is used for asthma, bronchitis, fever, piles and abdominal discomfort. It is sometimes used as a substitute for roots of *P. longum* (Sitaram & Chunekar, 2010).These 3 species are essential ingredients of various medicines in Sri Lankan traditional and Ayurveda system of medicine and possess various pharmacological actions such as antioxident, hepatoprotective, immunomodulatory, antipyretic, aphrodisiac, digestive, antimicrobial, antispasmodic actions (Zaveri *et al* 2010; Damanhouri & Ahmad, 2014; Manosi *et al* 2016). Furthermore, the main active ingredient of these 3 species is piperine. The Ayurvedic literature also reveals the actions of these 3 plants and categorized under same drug group as they give more or less similar actions. In the present study we have analyzed the pharmacognostical, physico-chemical, phytochemical and HPTLC fingerprint patterns to establish the identity and purity of plant raw materials as a important step of herbal drug standardization.

MATERIAL AND METHODS

Collection and authentication of plant materials: The fruits of *P. nigrum, P. longum*, and the stems of *P. retrofractum* were collected from Western province, Sri Lanka in between June July 2018 and authenticated by Curator of National herbarium of Peradeniya, Sri Lanka. The raw materials were

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thoroughly washed with water to remove the impurities and dried under shade. The dried plant materials were powdered separately.

Pharmacognostical studies: Fruits of *P. longum*, *P. nigrum* and stem parts of *P. retrofractum* and were collected in fresh form and hand sections were taken. Anatomical studies were done according to the Indian Pharmacopoeial standard methods (Goveas & Abraham, 2014).

Physico-chemical studies: Physico-chemical parameters such as total ash, acid insoluble ash, water soluble ash, Heavy metals, water and methanol extractable matter were determined according to the WHO herbal drug standardization guidelines (WHO, 2011).

Preparation of extracts: Each plant material (200 g) was refluxed with methanol (750 ml) and water (750 ml) respectively for 6 hours and filtered. Each extract was dried by evaporation using a rotary evaporator (BUCHI Labortechnik AG, Flawil, Switzerland) and the obtained methanol and water extracts were kept in a refrigerator for further investigations.

Qualitative analysis of phytochemicals: Phytochemical analysis of hot methanol and hot water extracts of 3 piper species were performed as per the standard methods, to detect the various phytochemicals such as saponins, alkaloids, tannins, flavonoids, terpenoids and cardiac glycosides (Goveas & Abraham, 2014).

Determination of chromatographic HPTLC fingerprint profile of methanolic extract of 3 piper species
One gram of each methanol extract was dissolved in 5 ml of methanol and spotted (10 µl from each extract) on a pre-coated TLC plate. Then TLC fingerprint profiles of *P. nigrum, P. longum* and *P. retrofractum* were developed by using hexane: ethyl acetate and dichloromethane in a ratio of 5:4.5 v/v and along with standard marker compound piperine 5 µl (10 mg/ml). TLC fingerprint profiles of three extracts and piperine were scanned using a HPTLC (CAMAG HPTLC (Switzerland) comprising CAMAG Linomat5 applicator, CAMAG TLC scanner3, Wincats software, Version 1.44).

Statistical analysis: Results were expressed as mean $\pm$ (S. E. M.). Statistical analysis of the data was performed with one-way analysis of variance (ANOVA) (IBB SPSS Statisticals22). Significant different were indicated by p values lower than 0.05)

RESULTS

Macroscopical features of P. nigrum : Fruits of *P. nigrum* were grayish-black to black in colour, hard, wrinkled, 0.3-0.5 cm in diameter with pungent aromatic odour and pungent taste.

Microscopical features of P. nigrum : Fruit consists of a thick pericarp for about one third of fruit and an inner mass of perisperm. Pericarp consists of epicarp, mesocarp and endocarp; epicarp composed of single layered, tubular cells forming epidermis. Mesocarp is wide and composed of parenchymatous cells and oil cells. Testa of the endocarp is single layered, yellow coloured, thick

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walled sclerenchymatous cells. Perisperm contains parenchymatous cells having olio resins and packed with abundant, oval to round shape, simple starch grains (Figure 1).

Macroscopic features of P. longum : Fruiting spike of *P. longum* is greenish-black to black, cylindrical, 2.5 to 4.5 cm long, 0.4 to 0.8 cm thick, consisting of minute sessile fruits, arranged around an axis. Surface of the fruit is rough and composite. Broken surface shows a central axis and minute fruits arranged around an axis. Taste is pungent and producing numbness on the tongue; odour, aromatic.

Microscopic features of P. longum : Transverse section of the fruit shows 6-8 fruitlets arranged in circle on a central axis. The outer epidermal layer consist irregular cells which are filled with yellow brown content. Mesocarp consists of large parenchymatous cells, irregular in shape and thin walled, filled with starch grains. Stone cells present in mesocarp and endocarp and seed coat fused to form a deep zone, outer layer of this zone composed of thin walled cells and colourless, inner layer composed of tangentially elongated cells. Most of endocarp filled with starch grains, round to oval shape (Figure 2).

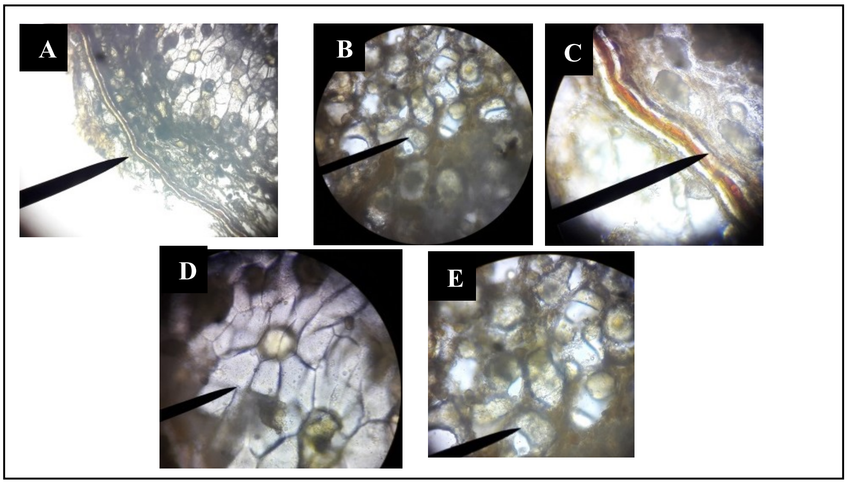

Figure 1 **:** Microscopic features of *P. nigrum* fruit A- Pericarp & perisperm (LP), B- Mesocarp cells and oil cells, C- Testa & perisperm (HP), D- Olio resin inside the seed, E - Starch granules

Macroscopic features of P. retrofractum : Drug consists of dried cut pieces of stem of variable length, cylindrical and somewhat twisted, grayish brown. The surface of the stem is smooth with few longitudinal wrinkles, nodes and internodes distinct in the stem; peppery odour and acrid taste.

Microscopic features of P. retrofractum : Stem shows thin cork consisting of rectangular cells and secondary cortex a wide zone, consisting of round, oval thin walled parenchymatous cells with prominent intercellular spaces. Simple starch grains, pitted vessels, trachides, vascular bundles consisting of spiral vessels are present (Figure 3).

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Ash values and extractive values: Total ash, acid insoluble as, water soluble ash, hot and cold water extractive values, hot and cold methanol extractive values were determined for three piper species. The results were summarized in Table 1 and 2.

Table 1: Physico-chemical parameters of *P. nigrum* , *P. longum*, and *P. retrofractum*

Plant	Parameters		
	Total ash content	Water soluble ash content	Acid insoluble ash content
P. nigrum	5.5 ± 0.0 %	3.5 ± 0.0 %	0.3 ± 0.0 %
P. longum	5.1 ± 0.1 %	3.1 ± 0.0 %	0.5 ± 0.0 %
P. retrofractum	5.7 ± 0.0 %	2.6 ± 0.1 %	0.2 ± 0.0 %

n=3, Results are expressed as mean ± S.E.M. ($p<0.05$)

Table 2: Extractable matters of *P. nigrum, P. longum*, and *P. retrofractum*

Plant	Solvent – water		Solvent - Methanol	
	Cold	Hot	Cold	Hot
P. nigrum	9.8 ± 0.3 %	16.7 ± 0.2 %	15.4 ± 0.7 %	22.9 ± 1.0 %
P. longum	16.0 ± 0.6 %	28.5 ± 0.3 %	12.1 ± 0.2 %	18.2 ± 0.3 %
P. retrofractum	7.9 ± 0.6 %	14.8 ± 0.6 %	7.7 ± 0.2 %	12.5 ± 0.2 %

n=3, Results are expressed as mean ± S.E.M. ($p<0.05$)

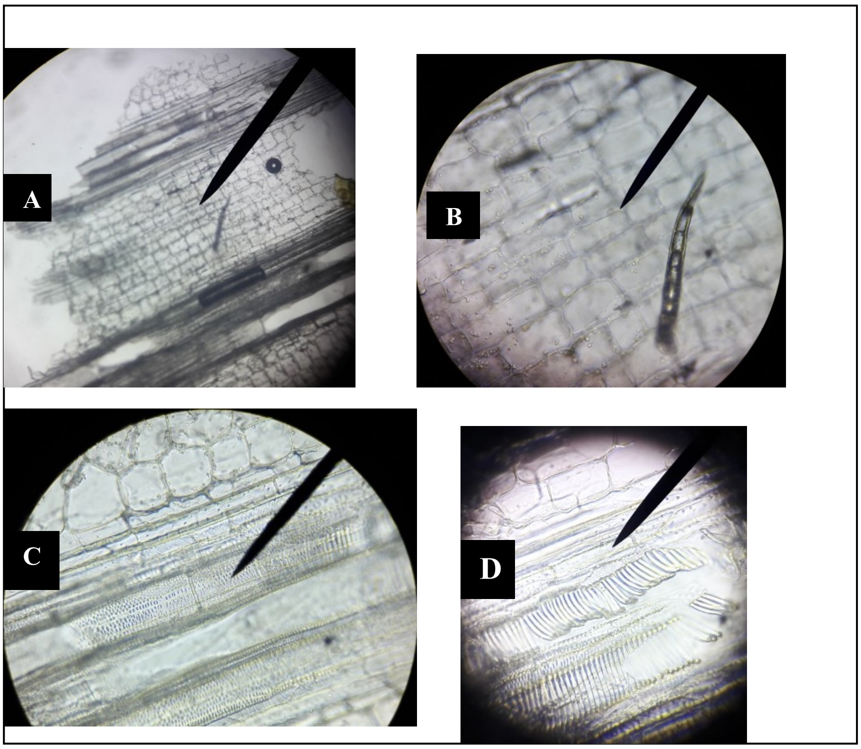

Figure 3: Microscopic features of *P. retrofractum* stem A-LS of stem, B-trachides, C-pitted vessels, D-spiral vessel

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Determination of heavy metal residues: The Inductively Couple Plasma Spectrometry (ICP-MS) was used to analyze the heavy metals (Pb, Cd, As, Hg) in the powder of *P. nigrum* fruits, *P. longum* fruits and the *P. retrofractum* stem parts. The level of Pb concentration of *P. nigrum* and *P. retrofractum* were found to be 0.4 and 0.2 mg/kg respectively. It was much lower than acceptable limit of 10 mg/kg by WHO guidelines. Cd was found to be 0.06 mg/kg in *P. nigrum* was lower than the acceptable limit of 0.3 mg/kg as set by WHO guidelines 2007. As and Hg were not detected (<0.05) in *P. nigrum* and *P. longum* samples. As and Hg were found to be 0.06 mg/kg in *P. retrofractum* sample. Both of these metals were found to be within permissible limits of 0.5 ans 1.0 mg/kg respectively (Table 3).

Table 3: Determination of heavy metals in *P. nigrum, P. longum*, and *P. retrofractum*

Plant	Concentration (mg/kg)			
	Pb	Cd	As	Hg
P. nigrum	0.40	0.06	Not detected (<0.05)	Not detected (<0.05)
P. longum	Not detected (<0.05)	Not detected (<0.05)	Not detected (<0.05)	Not detected (<0.05)
P. retrofractum	0.20	Not detected (<0.05)	0.06	0.06

Qualitative analysis of phytochemicals: Phytochemical analysis of *P. nigrum, P. longum* and *P. retrofractum* revealed the presence of saponins, alkaloids, tannins, steroids, flavanoids, terpinoids, phenols, steroids and cardiac glycosides (Table 4).

Table 4: Phytochemical screening of *P. nigrum, P. retrofractum, and P. longum*

	P. nigrum		*P. retrofractum*		*P. longum*	
	Hot water extraction	Methanol extraction	Hot water extraction	Methanol extraction	Hot water extraction	Methanol extraction
Saponins (Frothing test)	+	+	+	+	+	+
Alkaloids						
*Mayer's test	+++	+++	+++	+++	+++	+++
*Picric acid test	-ve	+	-ve	+	-ve	+++
*Wagner test	+	+++	+++	+++	++	+++
Tannins						
*Ferric cl.test	+++	+++	+++	+++	+++	+++
*Pb acetate test	++	++	+++	+++	+++	+++
Steroids	++	+++	+	+++	++	+++
Flavanoids	+	+++	-	+++	-	+++
Terpenoids	+	+++	+++	+++	++	+++
Phenols	+++	+++	+++	+++	+++	+++
Cardiac glycosides	++	-ve	+	-ve	++	-ve

***-ve:** Negative, **+:** Positive in low concentration, **++** Positive in moderate concentration**, +++** positive in high concentration*

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TLC and HPTLC: Figure 4 illustrates the TLC fingerprint profiles of *P. nigrum, P. longum* and *P. retrofractum.* The active ingredient piperine exhibited a sharp spot bearing a Rf value of HPTLC Chromatograms demonstrated number of peaks at 254 nm for *P. nigrum, P. longum*, and *P. retrofractum* respectively (Figure 5).

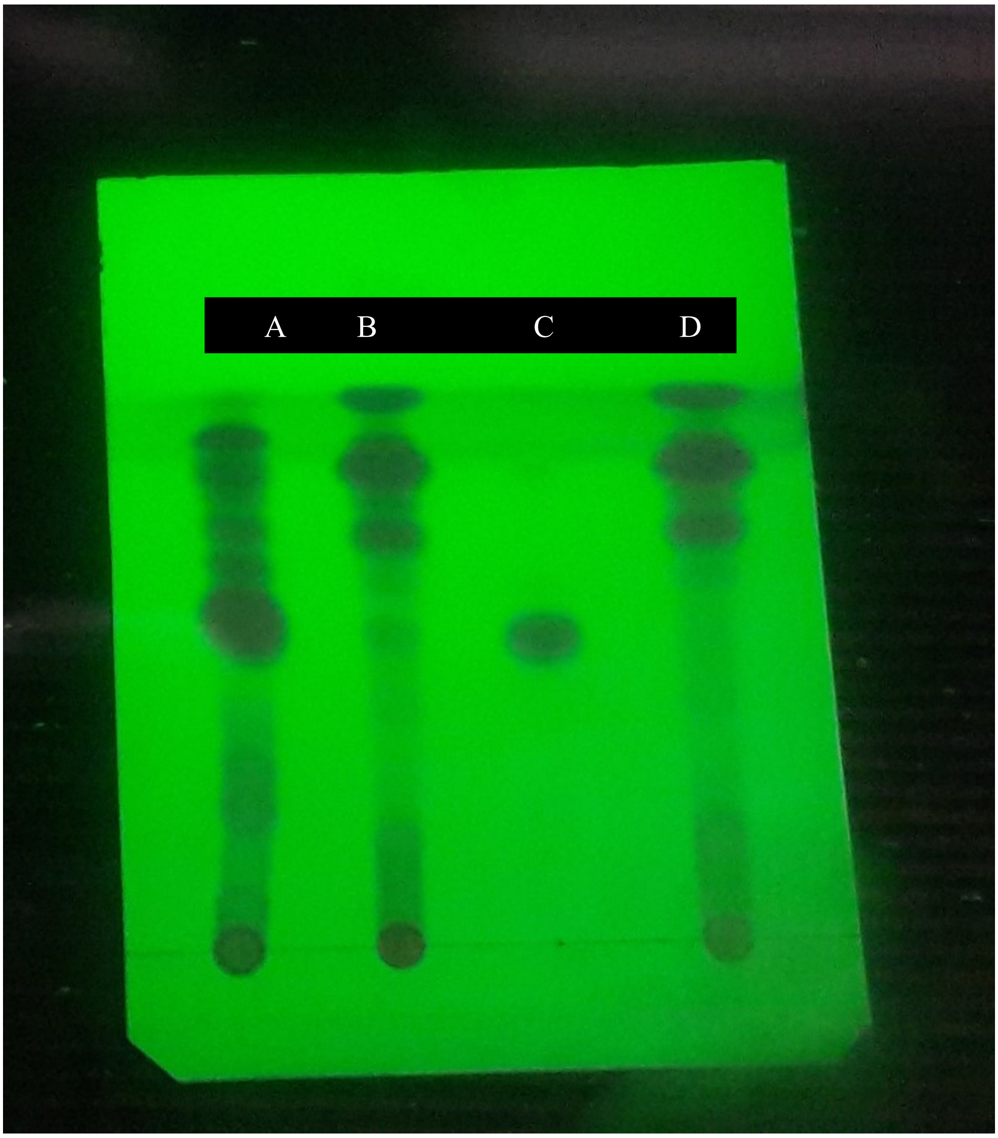

Figure 4: A: *P. nigrum* , B: *P. retrofractum* , C: Piperine, D: *P. longum*

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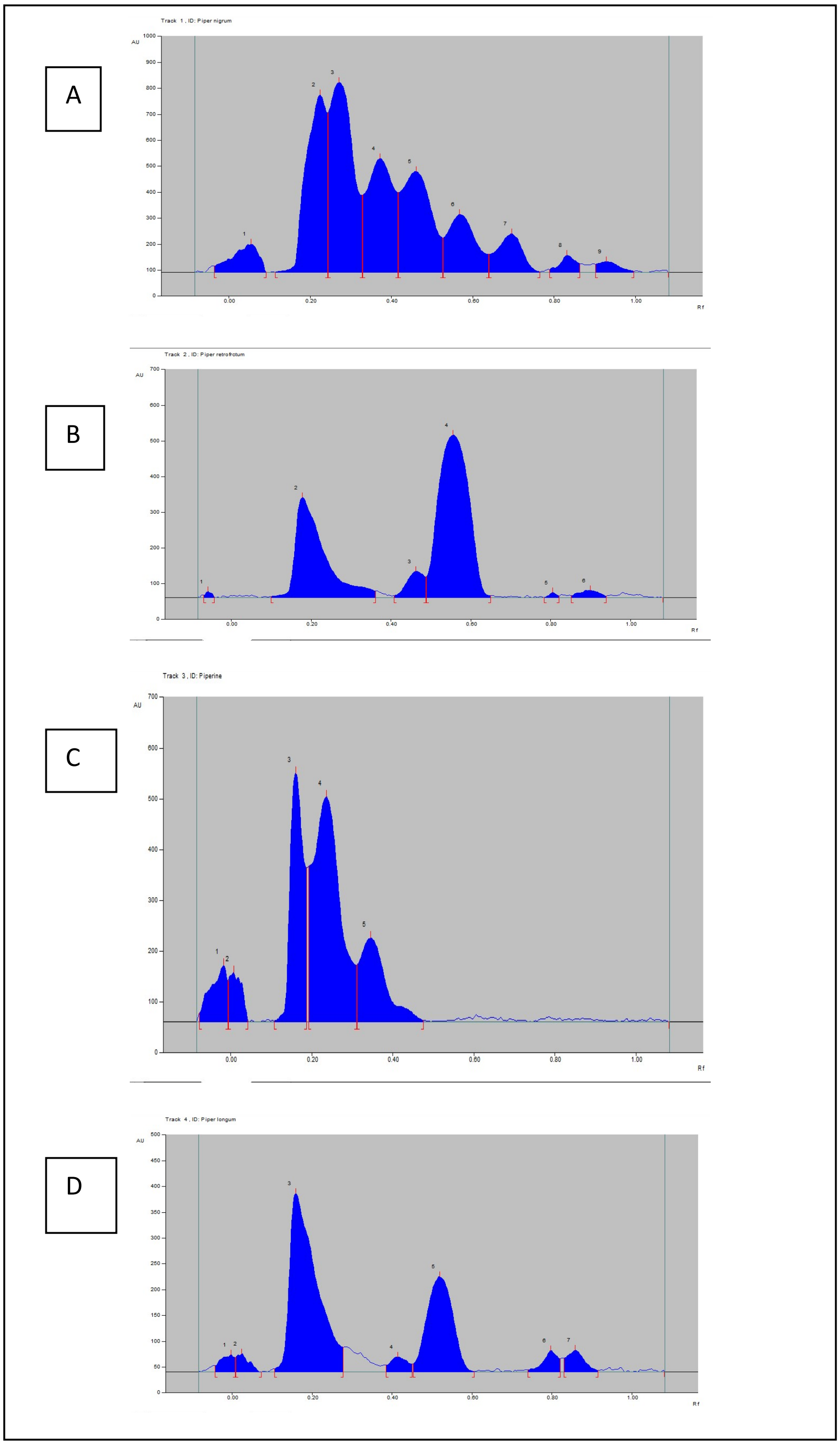

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Figure 5: HPTLC fingerprint patterns of three piper species with the Piperine standard
A: *P. nigrum* **,** **B:** *P. retrofractum* **,** **C:** Piperine**,** **D:** *P. longum*

DISCUSSION

Standardization of herbal medicines is necessary to obtain quality and effective drugS. Bioassays related to herbal drugs play a significant role in the process of herbal drug standardization. Pharmacognostical studies, detection of various physico-chemical parameters such as ash values, extractive values in different solvents and phytochemical parameters are being used to standardize the herbal drugs (Folashade *et al* 2012). At the beginning of the manufacturing process of the herbal drugs, pharmacognostical studies are more important to identified genuine plant materials under raw material standardization. Currently, herbal materials are easily adulterated with low quality plant materials due to the high demand of natural medicines. This condition also worsens by the incorrect identification of plant materials due to lack of knowledge about the medicinal plants. Therefore, an examination to determine sensory, macroscopic and microscopic characteristics is the first step towards establishing the identity and degree of purity of plant materials and it should be carried out before any tests are undertaken (WHO, 2011).

The analysis of the anatomical features of *P. nigrum*, *P. longum* and *P. retrofractum* may help to establishing the botanical identity among the Family piperaceae. Identification of plant materials can be done accurately by using comprehensive histology (Metcalfe & Chalk, 1979). This applies in identification of adulterants and substitutes of the raw materials and help to detect genuine raw materials. Piper is a valuable herbal medicine as well as a spice and other two species also very valuable herbal medicines in Ayurveda and Sri Lankan traditional system of medicine. Therefore, correct pharmacognostical identification is need of time. This pharmacognostical study will provide reference for identification of 3 piper spices.

P. longum showed the highest percentage of water extractable matter in both cold (16.0 $\pm$ 0.6 %) and hot (28.5 $\pm$ 0.3 %) conditions whereas *P. nigrum* showed the highest percentage of methanol extractable matter in both cold (15.4 $\pm$ 0.7 %) and hot (22.9 $\pm$ 1.0 %) conditions.

The ash values help to determine if there are any contaminants such as soil and sand. The water soluble ash indicates the amount of inorganic elements in the sample and acid insoluble ash indicates the presence of silica in the plant materials. The high values of acid insoluble ash point toward the high contaminants with soil materials. The acid insoluble ash contents of 3 plant materials of this study were measured as 0.26 + 0.0 %, 0.48 $\pm$ 0.0 % and 0.19 + 0.0 % in *P. nigrum, P. longum* and *P. retrofractum* respectively which was comparable with Ayurveda pharmacopieal reference values. *Piper* species are often cultivated for their fruits, stems and roots and they are rich in bioactive compounds. Bioactive compounds such as alkaloids, saponins, tannins, phenols, cardiac glycosides, flavanoids, and terpenoids have been widely reported to be present in fruits, seeds, roots and stems (Mgbeahuruike *et al* 2017). The pharmacological activities of these phytochemicals comprise health benefits which help to preventing from diseases and cure the diseases (Kumar *et al* 2011). The quantitative analysis of the phytochemicals in hot water and hot methanol extracts of *P. nigrum* and *P. longum* fruits and *P. retrofractum* stems showed the presence of the alkaloids, saponins,

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tannins, flavanoids, terpinoids, phenols, steroids and cardiac glycosides. Phytochemical analysis is very important to determine the quality of drug and to detect therapeutic efficacy.

At present TLC and HPTLC techniques are commonly used for quality assessment in Ayurvedic preparations. These methods widely employed in pharmaceutical industry in process of identification, development and in quality control of herbal products (Soni & Navad, 2010). However, HPTLC is a modern adaptation of TLC with better and advance separation efficiency and detection limit (Rakesh *et al* 2013). Results of HPTLC fingerprint patterns of methanolic extracts of 3 species showed the presence of various chemical compounds. Further in this study more concern was focused to compare the piperine content of the three plants. Hence the comparative HPTLC fingerprint patterns give an idea about the content of piperine present in three piper species which demonstrate the peaks relate with piperine are comparable with the peaks of three piper species. Also, it illustrates that the fruits of *P. nigrum* and *P. longum* have more piperine content than the stem parts of the *P.retrofractum* species.

Medicinal plants can present health risks due to the presence of toxic metals such as Pb, Cd, As and Hg which are hazardous to humans (Singh & Ajay, 2011). According to the WHO guidelines Herbal medicines should be free from heavy metals or within the limits of heavy metals. Heavy metals are stored in different parts of the plant which enter through the biological cycle of the plant (Emamverdian & Ding, 2017). The concentrations of Pb, Cd, As and Hg analysis in the extracts of fruits of the *P.nigrum*, *P. longum* and stems of *P. retrofractum* using the atomic absorption spectrophotometer were found to be within the acceptable limits.

CONCLUSION

Results of physico-chemical parameters, phytochemical, macroscopic and microscopic analysis can be used to assess the quality and detection of any adulteration for *P. nigrum* , *P. longum* and *P. retrofractum* .

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DECLARATION OF CONFLICT OF INTEREST

We have no conflict of interest to declare

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