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RESEARCH ARTICLE

Stem of Woody Pepper (*Piper pendulispicum* C. DC) as a Source of Phenolic Acids and Piperine

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Abstract

Woody pepper (*Piper pendulispicum* C. DC) is a medicinal spice recently reported from Andaman Islands apart from its natural distribution in Vietnam, Thailand *etc*. The species is unique as the thickened stem is used as a spice. As very limited information is available about the species, the present study was undertaken to understand the biochemical composition of its edible portion. Results revealed that stem thickness did not influence the moisture content and recovery of scraped stem. Piperine and phenolic acid profiling suggested that piperine content increased with an increase in stem thickness. Stem samples dried using vacuum drying had higher piperine content (7.665 mg/g) than those dried using oven drying (7.423 mg/g). Profiling of phenolic compounds revealed the presence of 18 phenolic compounds, ferulic acid being the dominant one. The concentration of ferulic acid was higher in thicker stems (867.945 µg/g) than in the thin stems (699.043 µg/g).

Keywords: Ferulic acid, Piperaceae, woody climber, tropical species, vacuum drying.

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Introduction

Members of the family Piperaceae are known to be rich sources of secondary metabolites (Tamuly *et al*., 2015) and hence several *Piper* species have been commercially used for various food, pharmaceutical and aroma industries (Salehi *et al*., 2019). However, of more than 1,000 reported *Piper* species so far (Parthasarathy *et al*., 2006), a large number of species have remained under-studied and thus less understood. Woody pepper (*Piper pendulispicum* C. DC) is an underutilized species of the Piperaceae family, which has recently been identified as a new addition to the Indian Flora (Waman *et al*., 2024). Further, this species has been found to be a potential crop for commercial cultivation in the warm and humid tropical Andaman and Nicobar Islands, India (Waman *et al*., 2019). It is also found distributed naturally in the Northern, North-eastern Thailand and Vietnam up to an altitude of 200-1200 m above mean sea level (Chaveerach *et al*., 2008).

Unlike the berries of black pepper, stem pieces of this vine species are used for imparting spiciness to the traditional food in the Andaman Islands (Waman *et al*., 2019; 2024). In Thailand, the species is one of the eight popular *Piper* species (Chaveerach *et al*., 2006), wherein stems of this species are often used in the preparation of local food- *kaeng khae* (Chaveerach *et al*., 2017) apart from its use as an ingredient in herbal tea (Adekoya *et al*., 2021). It is cultivated in home gardens of northern Thailand, while it is collected from wild stands in Loei province of North- eastern Thailand (Arisa *et al*., 2014). Ethanolic extract of *P. pendulispicum*

exhibited cytotoxic activity against cancerous cell lines (Mahavorasirikul *et al*., 2010). Systematic studies have not been conducted in woody pepper so far to understand its potential. Unfortunately, the natural population is dwindling due to destructive and unsustainable harvesting (Waman *et al*., 2019). In order to promote cultivation of such potential spice, knowledge about the biochemical aspects is important.

Piperine, an alkaloid with IUPAC name- (2E, 4E)-5-(benzo[d] [1,3] dioxol-5-yl)1-(piperidin-1-yl) penta-2,4-dien-1-one, has been regarded as one of the important bioactive compounds responsible for the pungent taste in *Piper* species (Takahashi *et al*., 2018; Weil *et al*., 2017). Piperine is known for its versatile properties including antioxidant, anti-tumor, hepato-protective, antidepressant, antimicrobial, immune-modulatory, anti-diarrheal, analgesic *etc*. (Chopra *et al*., 2016; Salehi *et al*., 2019; Shityakov *et al*., 2019; Tiwari *et al*., 2020). Further, piperine has proven to serve as a bio-enhancer, which enhances the efficiency and activity of other bioactive molecules and antibiotics (Shityakov *et al*., 2019). Several formulations including those based on Ayurveda and Homeopathy are being sold in the markets across the globe (Chopra *et al*., 2016), thereby suggesting the acceptability of this compound.

Phenolic compounds are among the most abundant and broad group of secondary metabolites, which are equally functional when present in the plant system or the products obtained after harvest. Several phenolic compounds are known to exhibit anti-inflammatory, antioxidant, cardio-protective, anti-allergic, vaso-dilatory, and antimicrobial properties (Giada and Mancini- Filho, 2006). In the case of the *Piper* species, piperine and phenolic compounds have been identified as the prime constituents contributing to the antioxidant activity (Takahashi *et al*., 2018). Drying methods and plant maturity have been considered important factors in determining the content of secondary metabolites in various plant species (Takahashi *et al*., 2018; Thamkaew *et al*., 2021). Some report also suggests use of dry powdered formulation in Thai medicines (Adekoya *et al*., 2021); however, no biochemical studies are available in this species (Arisa *et al*., 2014). Fresh produce of woody pepper is sold in the markets of Andaman Islands based on the stem thickness and hence, understanding the biochemical differences at different stem thickness (harvest stages) is required. The present study thus aimed at studying the effect of two drying methods on piperine content and phenolic compounds in the stems of woody pepper of different maturity.

Materials and Methods

Collection and primary processing of samples

Harvested stem pieces of woody pepper vines were collected from farmer's field in North and Middle Andaman District, India. The material was transported to the authors' laboratory at the Division of Horticulture and Crop Improvement, Port Blair, South Andaman Island, India for further processing. Stems were graded into two categories *viz*. thin (mean thickness 19.1 ± 0.52 mm) and thick (mean thickness 30.8 ± 0.74 mm), based on thickness. These pieces were then cut into small segments of equal length, and each segment was weighed using a digital weighing balance (Citizen India, CY-120). Outer layer of the vine comprising of dead tissues and adventitious roots was scraped off and weight of each segment was recorded to know the usable stem recovery percentage. Observations were recorded using ten replications. Moisture content was determined in triplicate and expressed as percentage.

Dehydration of samples

Samples were cut into small pieces using stainless steel secateurs (Falcon, India) and used for dehydration studies using two methods. In the first method, samples were dried in a hot air oven (50 °C), while in the second method, samples were dried in a vacuum tray dryer (RE 11142, Ruchi Enterprises, Delhi, India) at 50 °C temperature and -570 mm Hg vacuum. After drying, the samples were powdered using an electric grinder, sieved using 212-micron sieve and packed in air-tight glass containers with screw caps.

Phenolic acid profiling

Phenolic acid profiling in the samples was done using Waters Acquity UPLC H Class coupled with TQD MS/MS with methanol (80%) following the procedure described earlier (Weidner *et al*., 2000) with a slight alteration. Known quantity (500 mg) of the sample was homogenized in methanol (80%) followed by centrifugation (6,000 rpm for 10 min at 15°C) and the volume of the extract was made up to 20 ml. The extract was then evaporated at 45°C under vacuum to get near dried product. It was then diluted to 5 mL with water and was extracted thrice with ethyl acetate using a separating funnel. The aqueous layer was discarded and ethyl acetate extract was evaporated to dryness under vacuum. In the resultant dried extract, 4 mL of 2N NaOH was added and allowed for hydrolyzing overnight. It was then acidified to pH 2 using 2N HCl (5 mL) and again re-extracted with ethyl acetate (10 mL). The ethyl acetate layer carrying the phenolic acids was evaporated to complete dryness under vacuum and the residue was dissolved in MS grade methanol (2 mL). It was then filtered through 0.2µm nylon filter prior to injection in LC-MS/MS.

Phenolic acids were resolved on BEH-C18 (2.1 × 50 mm, 1.7 µm) analytical column (Waters India Ltd.) that was protected by Vanguard BEH C-18 (Waters, USA) guard column. The gradient flow of organic and aqueous phases with the flow rate of 0.3 mL/min was adopted. In the mobile phase, solvent A consisted of 0.1% formic acid in

water, while solvent B was 0.2% formic acid in methanol. The column was maintained at 25°C temperature during analysis. The injection volume used for analysis was 2µL. The eluted phenolic acids were pumped directly without any split into TQD-MS/MS system (Waters, USA) optimized for analysis of phenolic acids. The obtained values of phenolic acids were expressed in µg/g.

Estimation of Piperine

Known quantity (100 mg) of powdered sample was mixed with ethanol (80%), followed by sonication and centrifugation. The supernatant was collected and extracted once again with ethanol (80%) before making up the volume to 20 ml. From this, 0.1 ml volume was drawn; volume was made up to 2 ml with mobile phase and filtered through a nylon filter (0.2 µm) before injecting 2µl in LC-MS/MS for estimation.

For estimation of piperine, an analytical column BEH C18 (2.1 × 50 mm, 1.7 µm from Waters India Ltd.) with Vanguard BEH C18 (Waters, USA) guard column was used. Isocratic mode with a flow rate of 0.15 mL/min was used for analysis with a sample injection volume of 2µL. Mobile phase consisted of solvent A (Acetonitrile: water: Acetic acid:: 65:34.5:0.5) and solvent B (Methanol: Acetonitrile: Water :: 10:50:40). The column temperature was maintained at 25°C during analysis. Run time employed was 15 min. The eluted piperine by UPLC column effluent was pumped directly without any split into TQD-MS/MS (Waters, USA) system optimized for piperine analysis. Values of piperine were expressed in mg/g.

Results and Discussion

Recovery of stems and moisture content

Many *Piper* species are grown in tropical regions and are valued for their use as spices and medicines. Woody pepper is a unique species of the Piperaceae family in which stem is economically important. It is amongst the most important species in Thailand for food and medicinal purposes (Arisa *et al*., 2014). It is known to have creamish soft stems, which are commonly employed in the preparation of soup called '*kaeng khae*' in Thailand. During the present investigation, the thickness of thick samples ranged from 25.2 to 36.3 mm with a mean thickness of 30.8 mm (Table 1). The stems of thin group, on the other hand, had a mean thickness of 19.1 mm and a range of 11.9 to 24.6 mm. With the advancement of maturity, the thickness of the stem increases, and thicker stems fetch better prices in the local markets of Andaman Islands. Branches of primary and secondary origin generally have less thickness than the main stem. In the present case, most of the produce from the thick category was from main stem, while that from the thin category was from primary branches.

Table 1: Recovery (%) and moisture content (%) in woody pepper samples of different stem thickness

Sample	*Thickness (mm)*		*Recovery (%)*	*Moisture content (%)*
	Range	*Mean ± Sem*		
Thick	25.2 to 36.3	30.8 ± 0.74	75.9 ± 0.61	70.6 ± 0.19
Thin	11.9 to 24.6	19.1 ± 0.52	76.0 ± 1.22	70.4 ± 0.60

The outer bark of the species is rough and it produces heavy tufts of roots, especially on the nodes (Chaveerach *et al*., 2008). These roots facilitate clinging of vines, while growing on a support (Waman *et al*., 2019). However, while using the spice, such root masses are completely trimmed off and the outer layer of bark is scraped off using a stainless steel knife. To quantify the recovery of usable part from the produce, samples were weighed after scraping of the outer layer and percentage recovery was calculated. Interestingly, analysis of data suggested that the recovery of edible produce was not influenced by its stem thickness. Recovery of 75.9% was observed from stems of the thick category, while 76.0% recovery was observed in thin stems. Additionally, the moisture content in the edible part of the stem also remained on par in both samples. The moisture content of 70.4 to 70.6% was recorded in the peeled produce (Table 1).

Piperine content as influenced by stem thickness and drying methods

Fig. 1 depicts variations in the piperine content among the woody pepper samples dried using two different methods. Piperine content in the vacuum-dried sample was significantly higher (7.665 mg/g) than that observed in the oven-dried sample (7.423 mg/g). Many drying methods have commonly been applied for the dehydration of various spices and each of those methods is known to have its advantages and disadvantages (Thamkaew *et al*., 2021). In general, the suitability of the drying method varies with species. Experiment with *Piper nigrum* suggested significant role of the drying method on piperine content as oven drying improved piperine content, when compared with freeze-drying (Namjoyan *et al*., 2012). On the other hand, drying methods did not influence piperine content in *P. borbonense* (Weil *et al*., 2017). Maturity of plant part is one of the factors reported to influence the accumulation of piperine. In the present study, piperine content in the stem pieces of woody pepper was also influenced by the stem thickness used for estimation. Piperine content of 6.230 mg/g was observed in the sample of the thin category (Fig. 2), while it was significantly higher (6.654 mg/g) in the thick samples. Various biochemical and physiological changes in a plant system are known to vary with the species, plant part studied, position of the part on the plant, *etc*. (Salehi *et al*., 2019).

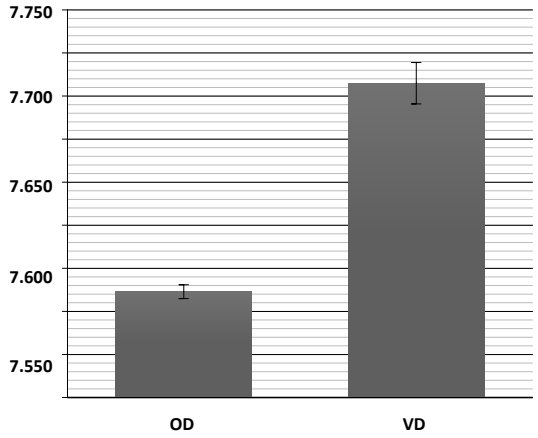

Fig. 1: Piperine content (mg/g) as influenced by drying methods

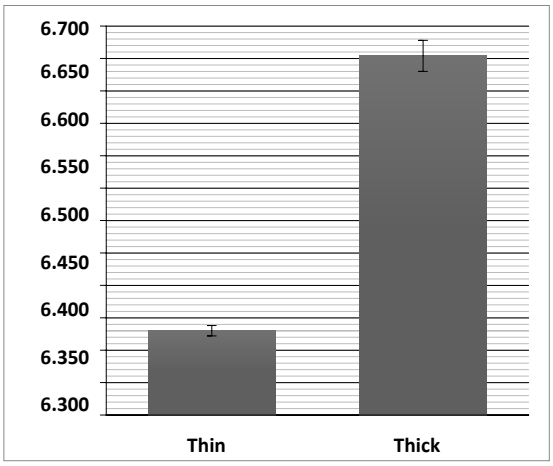

Fig. 2: Piperine content (mg/g) as influenced by stem thickness

Piperine has been reported to occur in various plant parts such as fruits of *P. nigrum* (Kotte *et al*., 2014), *P. borbonense* (Weil *et al*., 2017), *P. retrofractum* (Takahashi *et al*., 2018), *P. longum* (Khound *et al*., 2017), the stem of *P. retrofractum* (Roy *et al*., 2018) and multiple parts of *P. nigrum*, *P. guineense* and *P. longum* (Adosraku *et al*., 2013; Prasad *et al*., 2018; Semler and Gross, 1988). Also, the concentration of secondary metabolites varies among different parts of a plant e.g. piperine was detected in the leaves of a female plant in *P. longum* while it was absent in the leaves of a male plant (Prasad *et al*., 2018). Accumulation of various bioactive molecules in the plant tissues has been reported to vary with the stage of tissue/ plant part's growth. In *P. retrofractum* fruits (Takahashi *et al*., 2018), piperine content decreased from the green mature stage (23.59 mg/g-FW) to the red mature stage (12.97 mg/g-FW). These reports support the variations observed for piperine content in the present study.

Higher piperine content in the thick stem could be taken as an indicator of its spiciness as used for other species (Takahashi *et al*., 2018; Weil *et al*., 2017). Several bioactive constituents are lost during processing (Cascais *et al*., 2021); hence, such higher levels of bioactive molecules could be of advantage during the preparation of processed products. Such additional quantities of piperine could help in the maintenance of the optimum level of spiciness in the processed products as well. Hence, use of thicker stems could be advocated for the preparation of products requiring higher levels of piperine.

Phenolic acid profiling as influenced by stem size

Phenolic acid composition of woody pepper was influenced by the maturation of the stem *i.e.* size of the stem used for analysis. In general, eighteen compounds were present in woody pepper of both classes (Table 2). Significantly higher quantities (1142.860 mcg/g) of phenolic acids were found in thin stem than its thicker counterpart (1113.099 mcg/g). Study on ingredients of a Thai medicine (Adekoya *et al*., 2021) was conducted which revealed low content of phenols in the stem of *P. pendulispicum* collected from Thailand (0.06 mg/g total phenolic content, GAE) when compared with the present study. Geographical region, genetic variations and other unknown factors could be considered responsible for this variation. Reduction in phenolic content was evident in present study with increase in stem thickness. An earlier study in the fruits of *P. retrofractum* suggested reduction in total phenolic content with maturity from green mature to red mature stage (Takahashi *et al*., 2018), while the antioxidant activity of leaves of *P. sarmentosum* increased with maturity from young to middle age followed by drop at full maturity (Ibrahim and Sayidah, 2019). This suggests that the maturity of plant part has a role in the accumulation of phenolic compounds, though the contents might vary with the species and plant part. Of the 18 compounds, 14 compounds were found in significantly higher quantities in thin stems, two compounds (Ferulic acid and Salicylic acid) were relatively higher in thick stems, while the concentration of Gentisic acid and Chlorogenic acid remained statistically similar. In both the stem sizes, Ferulic acid was found to be the dominant phenolic acid, followed by *p*-Coumaric acid in woody pepper. The concentration of Ferulic acid was significantly higher (867.945 mcg/g) in thick stems than in thin stems (699.043 mcg/g) studied. It means Ferulic acid constituted about 61.17% of the total phenolics in thin stems, while its share was 77.98% in thick stems. The second dominant compound *i.e. p*-Coumaric acid was comparatively higher (188.510 mcg/g) in thin samples than its thick counterpart (99.620 mcg/g).

In terms of relative concentrations, thin samples had *o*-Coumaric acid (68.400 mcg/g), *t*- Cinnamic acid (41.310 mcg/g), Caffeic acid (39.570 mcg/g), Gallic acid (35.569 mcg/g), Sinapic acid (25.148 mcg/g) and Vanillic acid (19.944 mcg/g); while thick samples showed the presence of *t*-Cinnamic acid (35.923 mcg/g), Caffeic acid (29.229 mcg/g), *o*-Coumaric acid (25.100 mcg/g) and Gallic acid (23.026 mcg/g) as other constituents. Minor constituents were also present in relatively smaller quantities in both

Table 2: Phenolic acid content (µg/g) in woody pepper as influenced by stem thickness

Phenolic acids	*Thin*	*Thick*
Benzoic acid	5.418 ± 0.0272	2.932 ± 0.0161
p-hydroxy benzoic acid	0.689 ± 0.0026	0.349 ± 0.0022
Salicylic acid	1.917 ± 0.1062	2.639 ± 0.0058
3-Hydroxy benzoic acid	2.070 ± 0.0220	0.876 ± 0.0168
t-Cinnamic acid	41.310 ± 0.6008	35.923 ± 0.1601
2,4-dihydroxybenzoic acid	7.278 ± 0.1648	1.300 ± 0.0140
Gentisic acid	2.893 ± 0.1722	2.988 ± 0.0643
Protocatechuic acid	3.381 ± 0.0727	2.018 ± 0.1187
p-Coumaric acid	188.510 ± 0.6655	99.620 ± 0.8803
o-Coumaric acid	68.400 ± 0.7558	25.100 ± 0.0944
Vanillic acid	19.944 ± 0.1179	11.394 ± 0.0367
Gallic acid	35.569 ± 0.1983	23.026 ± 0.0822
Caffeic acid	39.570 ± 0.2219	29.229 ± 0.5244
Ferulic acid	699.043 ± 1.1475	867.945 ± 1.7603
Syringic acid	1.657 ± 0.0067	0.965 ± 0.0062
Sinapic acid	25.148 ± 0.0820	6.743 ± 0.1316
Ellagic acid	0.061 ± 0.0012	0.051 ± 0.0045
Chlorogenic acid	0.001 ± 0.0001	0.001 ± 0.0001
Total	1142.860 ± 1.3978	1113.099 ± 0.1767

Table 3: Phenolic acid content (µg/g) in woody pepper as influenced by drying methods

Phenolic acids	*Oven drying*	*Vacuum drying*
Benzoic acid	5.254 ± 0.0636	4.076 ± 0.0374
p-hydroxy benzoic acid	1.159 ± 0.0356	0.701 ± 0.0076
Salicylic acid	3.877 ± 0.0791	4.321 ± 0.0380
3-Hydroxy benzoic acid	0.601 ± 0.0018	1.510 ± 0.0103
t-Cinnamic acid	98.235 ± 0.9480	63.798 ± 0.5229
2,4-dihydroxybenzoic acid	6.889 ± 0.0925	13.161 ± 0.0826
Gentisic acid	12.538 ± 1.1917	3.777 ± 0.1799
Protocatechuic acid	5.882 ± 0.2835	4.699 ± 0.1108
p-Coumaric acid	161.626 ± 2.1589	101.016 ± 2.4899
o-Coumaric acid	31.743 ± 0.5920	25.146 ± 0.0529
Vanillic acid	14.677 ± 0.1078	14.657 ± 0.2774
Gallic acid	32.338 ± 0.1611	41.502 ± 0.1439
Caffeic acid	45.374 ± 0.1298	64.110 ± 1.5065
Ferulic acid	938.796 ± 1.8019	771.576 ± 4.3171
Syringic acid	1.419 ± 0.0152	0.743 ± 0.0016
Sinapic acid	39.845 ± 0.1151	27.039 ± 0.8282
Ellagic acid	0.063 ± 0.0047	0.040 ± 0.0021
Chlorogenic acid	0.001 ± 0.0001	0.001 ± 0.0000
Total	1400.317 ± 3.7595	1141.873 ± 6.4196

samples. The results obtained here are in agreement with the earlier study in which eighteen phenolic compounds were reported from the leaves of *Ficus carica* (Nadeem and Zeb, 2018). They have reported variations in the composition of phenolic compounds with the advancement of maturity. However, the dominant compound (Caftaric acid) remained the same during all stages of development, which was also the case in woody pepper.

Phenolic acid profiling as influenced by drying methods

Use of an appropriate drying method has been emphasized for drying herbs to obtain products with better phenolics and antioxidant activities (Orphanides *et al*., 2013). Hence, the effect of two drying methods was studied which suggested a significant role of the drying method on the concentration of phenolic acids in woody pepper. In general, oven-dried samples had higher quantities of phenolics (1400.317 mcg/g) than vacuum-dried samples (1141.873 mcg/g) (Table 3). This result is in line with a report on green tea in which higher total phenolic content was observed in oven-dried samples (Roshanak *et al*., 2016). Also, relative concentrations of 11 compounds were higher in oven-dried produce, while the concentration of five compounds (Caffeic acid, Gallic acid, 2,4-dihydrobenzoic acid, Salicylic acid, and 3- hydroxyl benzoic acid) was higher in vacuum dried produce. The varied composition of phenolic compounds in response to different processing methods has been reported earlier (Brandão *et al*., 2021).

Ferulic acid, the dominant constituent of woody pepper stem, was in higher quantities (938.796 mcg/g) in oven-dried (OD) produce than in vacuum dried (VD) (771.576 mcg/g). Similarly, *p*-Coumaric acid was present in a significantly higher amount (161.626 mcg/g) in OD than in VD samples. In OD, compounds present in their order of relative abundance were *t*- Cinnamic acid (98.235 mcg/g), Caffeic acid (45.374 mcg/g), Sinapic acid (39.845 mcg/g), Gallic acid (32.338 mcg/g), *o*-Coumaric acid (31.743 mcg/g) and Gentisic acid (12.538 mcg/g) apart from other minor constituents. On the other hand, compounds such as Caffeic acid (64.110 mcg/g), *t*-Cinnamic acid (63.798 mcg/g), Gallic acid (41.502 mcg/g), Sinapic acid (27.039 mcg/g), *o*-Coumaric acid (25.146 mcg/g), Vanillic acid (14.657 mcg/g) and 2,4-dihydroxy benzoic acid (13.161 mcg/g) were found in the vacuum dried samples. These findings are in line with the earlier report in which the significant influence of drying methods on the concentration of phenolic compounds (caffeic acid and gallic acid) and antioxidants was reported in spearmint (Orphanides *et al*., 2013).

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is one of the potent bioactive molecules reported to occur in a number of plants (Zhao and Moghadasin, 2008). The compound has been considered a ubiquitous phenolic derivative of cinnamic acid (Rosazza *et al*., 1995). Owing to its proven benefits, it has been considered an important constituent in the food, nutraceutical, and pharma industries (Kumar and Pruthi, 2014).

Conclusion

The present study could generate valuable information to understand the biochemical composition of this lesser-known plant genetic resource. It was observed that the maturity of the stem, as judged by its thickness in the market, did not influence moisture content and scraping percentage; however, piperine content increased with an increase in stem thickness. Ferulic acid was identified as the most abundant phenolic acid in the stems of both sizes. The use of vacuum drying resulted in high piperine recovery, while oven drying showed higher recovery of phenolic compounds. These studies would form the basis for utilizing this medicinally important and antioxidant-rich spice.

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Competing Interests

None declared.

Authors' Contributions

AAW: Conceptualization, exploration and collection of material, draft preparation; PB: processing of samples and data analysis; RKD: processing of samples; ANL: Biochemical analysis; KSS: Biochemical analysis. All the authors read and approved the manuscript**.**

Availability of Data and Material

All the relevant data has been included in the manuscript.

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