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Extraction of phenolic compounds with antioxidant potential from coconut (*Cocos nucifera* L.) testa and identification of phenolic acids and flavonoids using UPLC coupled with TQD-MS/MS

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ABSTRACT

Coconut testa, a brown skin covering of a coconut endosperm, is a rich source of phenolics. It is one of the by-products obtained in the coconut processing industries and currently underutilized despite being rich in phenolic compounds. A study was conducted to identify the suitable solvent system for maximum extractability of total phenolic content (TPC) and total flavonoid content (TFC) with antioxidant potential from the testa. Individual phenolic acids and flavonoids of different solvent extracts were also determined using UPLC-H class coupled with TQD-MS/MS. The TPC and TFC ranged from 4.9 to 167 mg GAE/g and 8.84–115 mg QE/g defatted testa, respectively; significant differences were observed for various solvent systems studied. Acidification of solvents significantly increased the extractability of TPC and reduced the extractability of TFC. A total of 28 phenolics comprising 16 phenolic acids and 12 flavonoids were identified. Protocatechuic acid, *p*-coumaric acid and ferulic acid are the major phenolic acids identified whereas, catechin, apigenin and kaempferol are the major flavonoids identified. In summary, this study proved that coconut testa is a natural source of multiple phenolics acids and flavonoids with potent antioxidant capacity, and it can be used as a natural source of antioxidants.

1. Introduction

Polyphenolic compounds are major secondary metabolites present in both edible and non-edible parts of plants. They range from simple phenolic acids to polyphenols such as flavonoids, tannins, anthocyanins, etc. (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). They function as antioxidants and have anti-tumor and anti-aging properties (Działo et al., 2016). Importance of natural phenolic compounds derived from plant materials is gaining interest among scientists, food manufacturers, and consumers' alike (Kähkönen et al., 1999). Consistent intake of raw vegetables and fruits rich in polyphenol compounds is believed to improve the conditions of degenerative diseases such as cancer, diabetes, hypertension and cardiovascular problems. It also can decrease the signs of aging, and improve physical fitness (Everitt et al., 2006). Hence, studies concerning commercial exploration and utilization of plant source antioxidants are of immense value (Alothman, Bhat, & Karim, 2009; Wijekoon, Bhat, & Karim, 2011).

Coconut (*Cocos nucifera* L.), which belongs to the family of Arecaceae (Palmae), is an important member of the monocotyledons, grown throughout the tropic and sub-tropic regions (Manivannan et al., 2018). Edible parts and various value-added products of coconut contain considerable amount of phenolics and flavonoid compounds with antioxidant potential (Arivalagan et al., 2018). One such material is coconut testa, a brown part covering coconut endosperm (kernel), i.e., brown skin. It is a co-product obtained during haustorium production and a by-product of coconut processing industries. Studies showed that coconut testa is a rich source of polyphenol compounds, and the hot processed virgin coconut oil (VCO) and oil extracted from coconut kernel with testa contain high amount of phenolic compounds (Appaiah, Sunil, Prasanth Kumar, & Gopala Krishna, 2014; Seneviratne, Chamil, & Sagarika, 2009). Despite being a rich source of polyphenol compounds, testa is currently underutilized and used as an animal feed. Identification of the individual polyphenol compounds and determination of antioxidant potential in the coconut testa is of prime important to exploit testa as a commercial source of polyphenol compounds with antioxidant potential.

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Due to the diverse nature and their complexing with carbohydrates, proteins and other phytochemicals, phenolic compounds of complex food matrices contain mixtures of different classes of phenolics that are soluble in the specific solvent system used (Khoddami, Meredith, & Thomas, 2013). The polyphenols or other bioactive compounds are extracted using water and organic solvents (ethanol, methanol, acetone, and diethyl ether). Among the various extraction methods employed, liquid-liquid extraction and solid-liquid extraction occupy the central place. For isolating polyphenol compounds from plant sources, solid-liquid extraction method is most commonly used (Alothman et al., 2009; Cottica et al., 2011). The percent recovery depends mainly on the type of solvent and the extraction methods being adopted, and it varies with the nature of both plant materials and the bioactive compounds present (Hayouni, Abedrabba, Bouix, & Hamdi, 2007; Sun & Ho, 2005; Turkmen, Sari, & Velioglu, 2006). Hence, it is very difficult to recommend a common extracting solvent for individual plant materials. Thus, the present study was aimed at evaluating the effect of various solvents on the extractability of phenolic compounds from coconut testa and to further test their antioxidant activity along with identification of individual phenolic acids and flavonoids using UPLC- MS/MS.

2. Materials and methods

2.1. Chemicals

All phenolic acid and flavonoid standards, DPPH (1,1-diphenyl-2-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), TPTZ (2,4,6-tris-2,4,6-tripyridyl-2-triazine), ABTS (2,2-azino-di-(3-ethylbenzothialozine-6-sulphonic acid) diammonium salt), potassium persulfate, and neocuproine (2,9-dimethyl-1,10-phenanthroline) were purchased from Sigma-Aldrich Co St. Louis, MO, United States of America. Analytical grade ethanol, methanol, acetone, acetic acid (glacial), sodium acetate, hydrochloric acid (conc.), ferric chloride, ammonium acetate, copper (II) chloride, Folin–Ciocalteu's phenol reagent and sodium carbonate were purchased from Merck KGaA, Darmstadt, Germany. Organic solvents used for the analysis were of chromatography/MS grade. Mobile phases were prepared using water purified in the Milli-Q (Millipore) system and filtered through membranes with a pore size of 0.45 µm.

2.2. Sample preparation

Three lots, containing 10 nuts each of West Coast Tall cultivar were obtained from Experimental Farm at ICAR-Central Plantation Crops Research Institute, Kasaragod, Kerala, India. Fruits were carefully dehusked, and kernel with testa was carefully removed from coconut shell using coconut de-sheller and testa was removed using the testa remover. The collected testa meal was dried in mechanical tray dryer at 50 °C and defatted using Soxhlet apparatus for 4 h using petroleum ether (boiling point 60–80 °C). The defatted meal was finely powdered using ball mill (Mixer Mill MM400, RETSCH GmbH, Germany) and screened through 200 mesh to get uniform particle size, and used for phenolics extraction using different solvent systems.

2.3. Solvent system and extraction procedure

Accurately weighed (1.0 g) defatted coconut testa meal powder was mixed with 10 mL of selected solvent in amber coloured centrifuge tube and extracted using rotospin shaker (Tarsons Products Pvt Ltd, Kolkatta, India) for 1 h at room temperature and 1 h at ultra-sonic bath (Riviera Glass Pvt Ltd, Mumbai, India) set at 60 °C for optimum extraction. Extracts were centrifuged and the supernatant was collected in amber reagent bottles. The residue was re-extracted twice using the same procedure as mentioned above. Filtrates collected from all the three successive extractions were pooled and used for analysis. In the extraction process, 14 different types of solvents comprising seven native solvents (water, methanol, 80% methanol, ethanol, 80% ethanol, acetone and 80% acetone) alone and acidified with 0.3 M HCl were used.

2.4. Determination of total phenolic and flavonoid content

Determination of total phenolic content (TPC) in the extract was done by Folin-Ciocalteu (FC) assay as described by Singleton, Orthofer, and Lamuela-Raventos (1999). Gallic acid was used as a standard, and a five-point calibration curve in the range of 10–50 µg/mL ($y = 0.0137x - 0.0075$ with R^2 of 0.9994) was constructed. Total phenolic content was expressed as mg gallic acid equivalent (mg GAE) per g dry weight of defatted testa meal. The total flavonoid content (TFC) was determined according to Zhishen, Mengcheng, and Jianming (1999). Quercetin was used as a standard, and a five point calibration curve in the range of 10–50 µg/mL was constructed ($y = 0.0032x + 0.0016$ with R^2 of 0.9985), and the results were expressed as mg of quercetin equivalent (mg QE) per g of the sample.

2.5. Determination of antioxidant potential

The DPPH and ABTS radical scavenging activities [Scavenging concentration (SC_{50})] of the different solvent extracts of testa were determined by the methods of Brand-Williams, Cuvelier, and Berset (1995) and Arnao, Cano, and Acosta (2001), respectively. In both methods, SC_{50} value signifies the concentration of test samples to scavenge 50% of the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical and Trolox served as a positive control and the results were expressed in µmol TE/g dry defatted coconut testa. The FRAP (Ferric Reducing Antioxidant Power) assay was done according to Benzie and Strain (1996) and CUPRAC (Cupric ion reducing antioxidant capacity) assay was done according to the method of Apak, Guclu, Ozyurek, and Karademir (2004) and the results were expressed in µmol TE/g dry defatted coconut testa.

2.6. Identification of phenolics extracted from different solvents from coconut testa using UPLC-H class coupled with TQD-MS/MS

The phenolic acids and flavonoids were isolated from different solvent extracts as described by Weidner, Amarowicz, Karamac, and Fraczek (2000) and Chen, Zuo, and Deng (2001). After removing the respective solvent completely by vacuum evaporator, the residue of each solvent extract was being hydrolysed by 2N NaOH followed by acidic hydrolysis with 4N HCl, the free phenolic acids and flavonoids were extracted using ethyl acetate and evaporated to complete dryness in vacuum at 40 °C, and the residue was dissolved in 2 mL MS grade methanol filter through 0.2 µm nylon filter prior to injecting in LC-MS/MS. An Acquity UPLC-H class along with TQD-MS/MS from M/S Waters, USA equipped with ESI source, degasser, quaternary pump, automatic injection system (0–10 µL) with a diode array detector and a temperature control column compartment for analytical column was used in the phenolic acids and flavonoids identification. The overall system was controlled by the Mass lynx software. The individual mass spectra of phenolic acids and flavonoids were obtained by collision induced decomposition fragmentation of molecules using negative ionization mode (ES^-). The most abundant forms of de-protonated $[M-H]^-$ molecules were identified as precursor ions of the corresponding phenolic acids and flavonoids to develop the multiple reaction monitoring (MRM) methods for further analysis (Tables 1 and 2).

The mobile phase used in the study consisted of an aqueous phase of 0.1% formic acid in water (A) and organic phase of 0.2% formic acid in methanol (B), with gradient elution (Table 3) at the flow rate of 0.3 mL/min. The analytical column used was 2.1 × 50 mm UPLC BEH- C18 (Waters) with 1.7 µm particles, which was protected by a Vanguard BEH C-18, 2.1 × 5 mm with 1.7 µm particles guard column (Waters). The column temperature was maintained at 25 °C. The sample injection volume was 4 µL each time for both phenolic acids and flavonoids. The

Table 1
List of quantified phenolic acids and MRM parameters (precursor and product ions *m/z*, cone voltage, collision energy ion mode and retention times).

Compound	Formula Mass	Precursor/Parent m/z	Cone Voltage	Product/Daughter m/z	Collision Energy	Ion Mode	RT (min)
Caffeic acid	180	178.9	30	135.05	16	ES^-	4.88
2,4-dihydroxybenzoic acid	154	152.9	28	109.0[a]	12	ES^-	4.25
				65.02	18		
Chlorogenic acid	354	352.97	22	191.10	18	ES^-	4.62
Ferulic acid	194	192.9	26	134.02[a]	14	ES^-	6.13/6.50
				178.06	12		
				149.07	10		
Gallic acid	170	168.9	28	125.03	12	ES^-	0.92
Gentisic acid	154	152.9	24	108.98	12	ES^-	2.86
o-coumaric acid	164	162.9	22	119.06	12	ES^-	6.91
p-coumaric acid	164	162.9	24	119.05	14	ES^-	5.67
4-hydroxy benzoic acid	138	136.9	26	93.01	12	ES^-	3.14
Protocatecuic acid	154	152.9	26	109.05	16	ES^-	1.91
Salicylic acid	138	136.9	28	93.10	14	ES^-	7.04
Syringic acid	198	196.97	26	153.02[a]	12	ES^-	5.37
				121.04	18		
				182.07	10		
trans-Cinnamic acid	148	146.9	26	103.05	10	ES^-	9.12
Vanillic acid	168	166.97	26	123.07[a]	12	ES^-	4.63/5.34
				152.07	12		
				108.01	20		
Sinapic acid	224	223.11	32	164.10[a]	14	ES^-	6.37/6.61
				149.06	20		
3-hydroxy benzoic acid	138	137.02	28	93.05	16	ES^-	4.79

[a] Quantifier m/z; RT – Retention Time.

metabolites eluted were pumped directly without any split into the TQD-MS/MS system, which was optimized for the phenolic acids and flavonoids analysis with source temperature 135 °C, and disolvation gas flow and temperature at 650 L/h and 350 °C, respectively. The calibration curve for phenolics acid and flavonoid standards were made using different concentrations. Individual phenolic acids and flavonoids were identified and quantified by MRM method in LC-MS/MS using their parent mass *m/z* and most abundant fragmented daughters. The

Table 2
List of quantified flavonoids and MRM parameters (precursor and product ions *m/z*, cone voltage, collision energy ion mode and retention times).

Compound	Formula Mass	Precursor/Parent m/z	Cone Voltage	Product/Daughter m/z	Collision Energy	Ion Mode	RT (min)
Apigenin	270	268.97	46	117.08[a]	36	ES^-	7.01
				148.61	24		
				107.04	30		
Catechin	290	289.03	34	245.23[a]	12	ES^-	0.9
				109.12	24		
				125.14	20		
Hesperetin	302	300.97	42	164.05[a]	20	ES^-	6.49
				286.15	16		
				151.03	24		
Kaempferol	286.23	284.97	52	145.50	36	ES^-	6.9
				93.05[a]	32		
Leutoline	286	284.97	54	132.95[a]	34	ES^-	7.37
				150.99	26		
Myrcetin	318	317.03	42	151.06[a]	28	ES^-	5.79
				179.06	18		
				137.06	30		
Neringenin	272	271.03	34	151.00[a]	16	ES^-	6.82
				119.08	22		
				107.03	22		
Quercetin	302	301.03	36	151.12[a]	20	ES^-	6.69
				179.19	16		
Rutin	610	609.10	60	300.20[a]	42	ES^-	5.3
				271.11	58		
Umbelliferone	162.14	161.04	42	133.07[a]	18	ES^-	2.55
				105.07	20		
				77.06	24		
Epicatechin	290	289.05	36	109.08[a]	22	ES^-	1.47
				123.08	28		
				151.09	20		
Epigallocatechin	306	305.05	34	125.10[a]	20	ES^-	0.79
				164.70	18		
				179.09	16		

[a] Quantifier m/z; RT-Retention Time.

Table 3
LC-MS gradient elution pattern for phenolic acid and flavonoids profiling.

Time	Mobile phase A	Mobile Phase B
0 min	90%	10%
2.5 min	90%	10%
4.0 min	70%	30%
5.0 min	60%	40%
10.0 min	80%	20%
12.0 min	80%	20%
14.0 min	90%	10%

Mobile phase A - 0.1% formic acid in water; mobile phase B - 0.2% formic acid in methanol (B).

MS chromatogram and MS/MS fragmentation pattern for selected phenolic acids and flavonoids are given in supplementary material 1 and 2, respectively.

2.7. Statistical analysis

All the experiments were conducted on triplicate samples. The effect of different extraction solvents on TPC and TFC with antioxidant potential was evaluated by analysis of variance (ANOVA) using SAS (SAS., 2012). Values are expressed as means ± standard deviation. Duncan's Multiple Range Test (DMRT) was used to determine the significant differences ($p < 0.05$) between the means of parameters analyzed. Pearson's linear correlation was performed to indicate the measure of correlation and strength of the relationship between TPC and antioxidant potential of different solvent extracts. Multivariate analysis was carried out by applying principal component analysis.

3. Results and discussion

A study was conducted to optimize the solvent system to get the maximum extraction of phenolics with maximum antioxidant potential from coconut testa. Different solvents having varying degrees of polarity were used for the extraction of phenolics and flavonoids with antioxidant potential with and without acidification. Individual phenolic acids and flavonoids of different solvent extracts were also identified and quantified using Acquity UPLC-H class coupled with TQD-MS/MS from M/S Waters, USA with ESI source. Significant differences were observed for the parameters studied.

3.1. Effect of polarity and acidification of solvent on extraction of total phenolic in coconut testa

Total phenolic content (TPC) of each solvent extracts were determined using FCR method (Singleton et al., 1999). The results obtained for each solvent with and without acidification are given in Table 4. The TPC varied between 4.89 mg GAE/g for acetone to 167 mg GAE/g for 80% acidified acetone. The phenolic extraction ability was found higher for solvents with acidification, indicating that most of the phenolics in the testa are in bound form.

3.1.1. Polarity

Methanol, ethanol, and acetone yielded less amount of TPC compared to their aqueous counterparts. Among the solvents, extractability of phenolics was found more for methanol (55.6 mg) followed by ethanol (22.2 mg). While acetone extracted significantly less phenolics (4.89 mg). Among the aqueous forms of solvents, aqueous acetone extracted more phenolics (98.5 mg) than aqueous methanol (89.7 mg) and ethanol (72.7 mg). The results suggest that the change in polarity of the solvents had a significant effect on extractability. The addition of water in the solvent system enhanced the yield, and it could be due to higher solubility of sample matrices like protein and carbohydrates in aqueous solvent system which in turn enables the release of the phenolic compounds to the medium (Zielinski & Kozłowska, 2000). The superiority of aqueous acetone in extracting phenolics has been documented in earlier studies *viz.*, 80% aqueous acetone was found suitable for maximum extraction of phenolic compounds of barley varieties with higher antioxidant activity (Zhao et al., 2006); 70% acetone is an efficient solvent system for extracting phenolic compounds from vegetables (Sulaiman, Sajak, Supriatno, & Seow, 2011); 50% acetone and methanol reported for the optimal extraction of phenolics from bungakantan (*Etlinger aelatior* Jack.) (Wijekoon et al., 2011). Similarly, Chavan and Amarowicz (2013) used the different solvent systems for extraction of phenolics, tannins, and sugars from beach pea (*Lathyrus maritimus* L.), and observed that acetone-water system yielded a higher amount of phenolics and condensed tannins than the ethanol-water or methanol-water system. In earlier studies, total phenolic content in the coconut testa was reported as about 1.9 mg GAE/100 g (80% methanol as a solvent), 1.3 g/100 g (ethanol extract) and 0.78–2.41 mg GAE/g sample (water extract of fermented coconut testa) by Appaiah et al., 2014, Appaiah, Sunil, Krishna, & Suresh Kumar, 2016 and Razak, Jamaluddin, Rashid, Sharifudin, & Long, 2016, respectively.

3.1.2. Acidification

In this study, 0.3 M HCl was used for the acidification of solvents, and results showed that the acidification of solvents significantly increased the amount of total phenolic compounds extraction compared to solvents without acidification (Table 3). Among the acidified solvents, 80% acetone with HCl could extract more TPC (167 ± 1.1 mg/g) followed by ethanol with HCl (110 ± 1.6 mg/g). Acidified water could extract less amount of TPC (51.0 ± 1.3 mg/g). The difference in the phenolic yield due to acidification was found to be high for acetone (16.2 times) followed by ethanol (4.9 times) and methanol (2.5 times). On the other hand, similar extraction yield difference was less for aqueous solvents with acidification. It is evident that the testa contained more of conjugated phenolics complexes (for example phenolic glycosides), which otherwise go unextracted using solvents without any acidification. Acids like HCl, formic acid, and acetic acid were used to enhance the extraction efficiency of solvents. Earlier studies showed that acidification with HCl and acetic acid not only enhanced the extraction ability of the solvent but also stabilized the anthocyanins and increase their antioxidant activity (Kalt, McDonald, & Donner, 2000; Michiels, Kevers, Pincemail, Defraigne, & Dommes, 2012). Similarly, acidification by formic acid (5% v/v) resulted in a significant increase in TPC and total flavonoids content and its antioxidant activity as measured by FRAP and ORAC methods compared with unacidified water (Vuthijumnok, Abdul-Lateef, & Julian, 2013).

3.2. Effect of polarity and acidification of extracting solvents on the total flavonoids content in coconut testa

Total flavonoid content (TFC) for each solvent (water, methanol, ethanol, acetone ± 0.3 M HCl) were determined using aluminium chloride method and expressed as mg quercetin equivalent (QE). Flavonoid content varied between 8.84 mg and 115 mg QE/g testa (Table 3). Among the solvents used (without acidification), 80% methanol yielded the maximum amount of flavonoids (115 mg) followed by methanol (78.1 mg) and 80% acetone (74.2 mg). The acetone was found to be a poor solvent for extraction of flavonoids (8.84 mg). Except for ethanol and acetone, acidification caused significant reduction in flavonoids extraction compared to their counter parts without acidification. The reduction was about three times in case of acidified 80% methanol and 80% acetone (40.2 and 28.0 against 115 and 74.2 mg/g, respectively), whereas, two times for acidified 80% ethanol (20.9 against 48.6 mg/g). Similar results were obtained for flavonoids in the acidified extract of flowers and leaves of gardenia (*Gardenia jasminoides* Ellis). It was reported that strong acid hydrolyzed the glycosidic bond and covalent bonds in flavonoids during extraction, so flavonoids degraded into fragments which do not react with $AlCl_3$ (Saptarini,

Table 4
Total phenolic and flavonoid content and antioxidant potential of different solvent extracts of coconut testa.

Solvent system	TPC	TFC	DPPH	ABTS	CUPRAC	FRAP
Water	$34.9^{j} \pm 1.1$	$48.9^{d} \pm 1.6$	$149^{g} \pm 7$	$382^{h} \pm 8$	$569^{i} \pm 27$	$180^{i} \pm 7$
Acidified Water	$51.0^{i} \pm 1.3$	$12.4^{i} \pm 0.3$	$143^{g} \pm 19$	$121^{i} \pm 7$	$421^{j} \pm 41$	$140^{j} \pm 8$
Methanol	$55.6^{i} \pm 1.4$	$78.1^{b} \pm 2.0$	$173^{gf} \pm 21$	$459^{gf} \pm 25$	$681^{h} \pm 9$	$207^{h} \pm 12$
Acidified Methanol	$141^{b} \pm 7.2$	$42.2^{e} \pm 2.1$	$367^{bc} \pm 41$	$709^{c} \pm 14$	$1583^{b} \pm 22$	$509^{b} \pm 5$
Methanol 80%	$89.7^{f} \pm 2.7$	$115^{a} \pm 3.4$	$215^{f} \pm 37$	$650^{d} \pm 31$	$1027^{f} \pm 11$	$318^{f} \pm 7$
Acidified Methanol 80%	$118^{c} \pm 4.9$	$40.2^{e} \pm 1.7$	$344^{cd} \pm 36$	$648^{d} \pm 5$	$1421^{c} \pm 27$	$438^{c} \pm 7$
Ethanol	$22.2^{k} \pm 0.7$	$30.1^{g} \pm 0.9$	$72.6^{h} \pm 7$	$146^{i} \pm 6$	$396^{j} \pm 6$	$169^{i} \pm 14$
Acidified ethanol	$110^{d} \pm 1.6$	$33.1^{f} \pm 0.5$	$308^{de} \pm 50$	$523^{e} \pm 28$	$1250^{d} \pm 12$	$395^{d} \pm 8$
Ethanol 80%	$72.7^{h} \pm 1.9$	$48.6^{d} \pm 1.3$	$183^{gf} \pm 20$	$417^{gh} \pm 7$	$831^{g} \pm 34$	$260^{g} \pm 4$
Acidified ethanol 80%	$93.9^{ef} \pm 5.3$	$20.9^{h} \pm 2.4$	$290^{e} \pm 27$	$488^{ef} \pm 19$	$1199^{e} \pm 43$	$361^{e} \pm 5$
Acetone	$4.89^{l} \pm 0.42$	$8.84^{j} \pm 0.8$	$20.3^{i} \pm 4$	$23.6^{j} \pm 15$	$11.7^{k} \pm 3$	$6.28^{k} \pm 2$
Acidified Acetone	$78.7^{g} \pm 5.8$	$14.6^{i} \pm 1.1$	$269^{e} \pm 23$	$453^{gf} \pm 36$	$847^{g} \pm 21$	$251^{g} \pm 6$
Acetone 80%	$98.5^{e} \pm 0.6$	$74.2^{c} \pm 0.9$	$396^{b} \pm 9$	$836^{b} \pm 65$	$1251^{d} \pm 15$	$431^{c} \pm 15$
Acidified Acetone 80%	$167^{a} \pm 1.1$	$28.0^{g} \pm 0.2$	$476^{a} \pm 35$	$1178^{a} \pm 46$	$2035^{a} \pm 22$	$710^{a} \pm 7$
CD	5.6	2.7	46.3	47.0	40.3	14.1

Three independent experiments were performed and data are presented as mean ± SD in one gram of dried defatted testa sample; TPC- Total phenolic content; TPC was expressed as mg GAE - Gallic acids equivalent (GAE); TFC- Total flavonoid content; TFC was expressed as mg QE - Quercetin equivalent. DPPH and ABTS values are expressed as μmol trolox equivalent/g dry defatted testa sample, obtained from a trolox solution having a free radical scavenging activity (SC_{50}) equivalent to that of sample. FRAP and CUPRAC values are expressed as μmol trolox equivalent/g dry defatted testa sample, obtained from a trolox solution having reducing power equivalent to that of sample. CD- Critical Difference at 5% level of significance; Different letters in each column after the mean value are significantly different at 5% level of significance according to Duncan's multiple range test (DMRT).

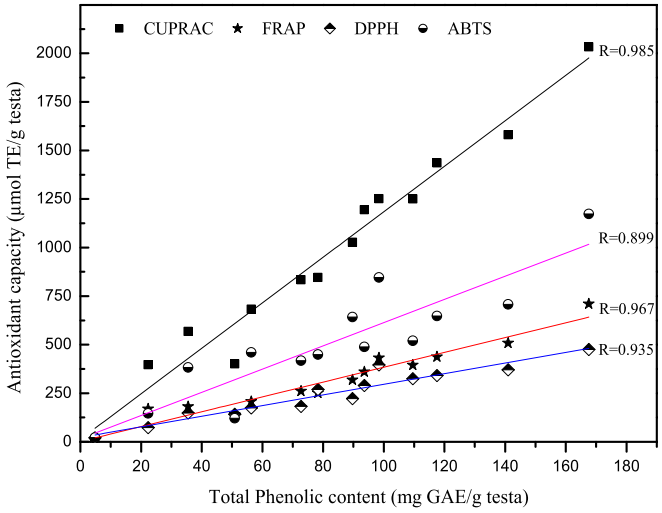

Fig. 1. Relationship between total phenolic content and antioxidant capacity measured by DPPH, ABTS, FRAP and CUPRAC methods in different solvent extracts of coconut testa. GAE- Gallic acid equivalent; TE- Trolox equivalent. R- Pearson's correlation coefficient ($p < 0.05$).

Herawati, & Permatasari, 2016). The lower and higher pH affects the extraction of flavonoids and other compounds with antioxidant potential from Thai hot curry paste extracts (Settharaksa, Jongjareonrak, Hmadhlu, Chansuwan, & Siripongvutikorn, 2012). Mello, Kakuda, and Hubinger (2011) reported that the reduction in pH of the extraction medium significantly reduced the total flavonoids content in propolis.

3.3. Antioxidant capacity of coconut testa

For evaluating the effectiveness of antioxidants, different methods were used based on their specific chemical properties. Since multiple reaction characteristics and mechanisms are usually involved, it is difficult to accurately measure the antioxidant potential of the mixed system using single assay. Thus, we used four complementary methods *viz.* DPPH and ABTS radical scavenging activity, FRAP, and CUPRAC reducing power – based on the single electron transfer mechanism to evaluate the antioxidant activity due to their simplicity, stability, and accuracy. The results obtained in the study were expressed as μmolTE per g testa sample and given in Table 4.

3.3.1. Effect of extracting solvents on the radical scavenging activity measured by DPPH and ABTS methods in coconut testa

Antioxidant potential as measured by DPPH method varied from 20.3 ± 4 μmol TE/g (acetone extract) to 476 ± 35 μmol TE/g testa (acidified 80% acetone extract). Acidification of solvents caused about 2.1, 1.54, 4.44, 1.59, 13.2 and 1.2 fold increases in antioxidant activity measured by DPPH method for methanol, 80% methanol, ethanol, 80% ethanol, acetone and 80% acetone, respectively compared to solvents with acidification. The ABTS radical assay measures the scavenging of free radicals as the discoloration of the ABTS blue reactant. Acidified 80% acetone showed highest ABTS reducing ability (1178 ± 46 μmol TE/g), followed by 80% acetone (836 ± 65 μmol TE/g) and acidified methanol (709 ± 14 μmol TE/g). Absolute acetone extract showed lesser ABTS radical reducing ability (23.6 ± 15 μmol TE/g). The values obtained for ABTS radical scavenging activity was significantly high compared to DPPH radical scavenging activity. ABTS method offers a number of advantages over the DPPH assay, as it can be used in a wide pH range, but DPPH method is limited to higher pH applications. In addition, ABTS is soluble in aqueous and organic solvents; hence, the assay has been used to measure both hydrophilic and lipophilic antioxidants by carrying out the assay in buffered media and organic solvents, respectively (Cano, Hernandez-Ruiz, Garcia-Canovas, Acosta, & Arnao, 1998).

3.3.2. Effects of extracting solvents on the reducing power measured by FRAP and CUPRAC methods in coconut testa

Acidified 80% acetone extract of testa showed better ferric reducing ability (710 ± 7 μmol TE/g) compared to other extracts, while absolute acetone extract showed less reducing ability (6.28 ± 2 μmol TE/g). The values obtained for antioxidant activity of coconut testa was higher for CUPRAC compared to other methods studied *viz.* DPPH, FRAP, and ABTS; it varied from 11.7 ± 3 μmol TE/g for acetone extract to 2035 ± 22 μmol TE/g for acidified 80% acetone. Acidification of methanol, ethanol, and acetone caused 2.32, 3.16 and 70.4 fold increase in CUPRAC values, respectively. In CUPRAC method, the redox reaction was carried out at a pH 7 as opposed to the acidic conditions (pH 3.6) of FRAP. At more acidic conditions than the physiological pH, the reducing capacity may be suppressed due to protonation of antioxidant compounds. However, in more basic conditions, proton dissociation of phenolics might enhance a sample's reducing capacity (Apak et al., 2004).

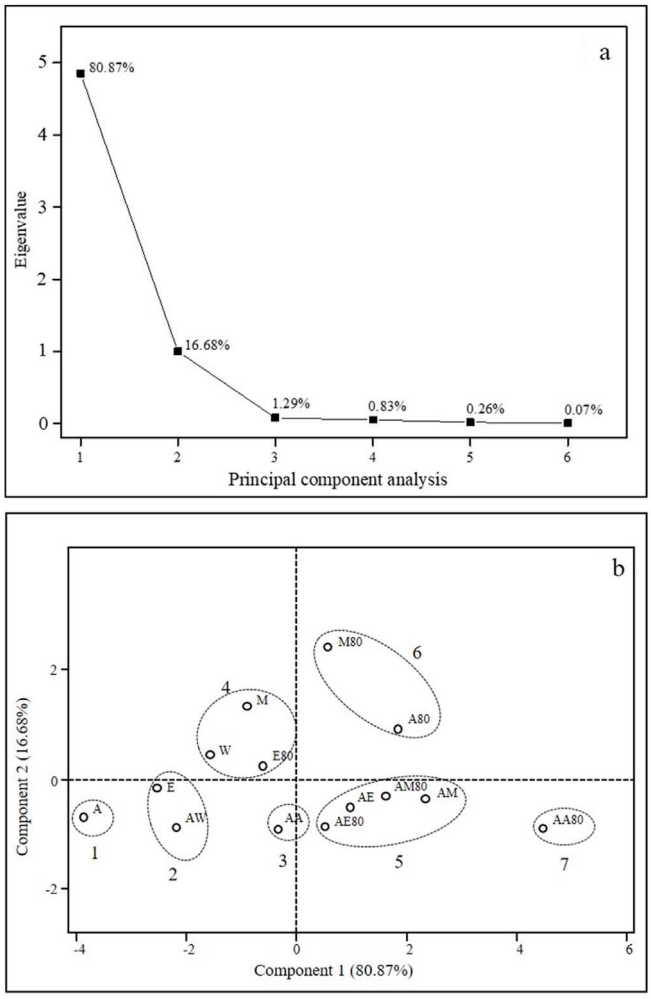

Fig. 2. Principle component analysis of TPC, TFC and antioxidant potential in different solvent extracts of coconut testa. 2a. Eigenvalue of each components for different solvent extracts of coconut testa. 2b. Components score plot for PC1 and PC2 for TPC, TFC and antioxidant potential of different solvents extracts of coconut testa. W- Water; AW-Acidified Water; M-Methanol; AM-Acidified Methanol; M80%-Methanol 80%; AM80%-Acidified Methanol 80%; E-Ethanol; AE-Acidified ethanol; E80%-Ethanol 80%; AE80%-Acidified ethanol 80%; A-Acetone; AA-Acidified Acetone; A80%-Acetone 80%; AA80%-Acidified Acetone 80%.

3.4. Correlation and principal component analysis

The presence of polyphenolic compounds contributes to the total antioxidant potential of the coconut testa. The relationship between TPC and antioxidant potential measured by DPPH, ABTS, FRAP, and CUPRAC was studied using Pearson's linear correlation ($p < 0.05$). Significant positive correlation with high Pearson's correlation coefficient (R) between TPC and antioxidant potential measured by various methods indicated the major role of TPC in antioxidant potential in the different solvent extracts of coconut testa (Fig. 1). The principal component analysis (PCA) revealed that two principal components (PCs) accounted for 97.54% of the total variability for TPC, TFC and antioxidant potential measured by DPPH, ABTS, FRAP and CUPRAC methods in the different solvent extracts of coconut testa (Fig. 2). The first PC had highest eigenvalue (4.85), and accounted for 80.9% of the total variability in the whole data set, while the second PC had eigenvalue of 1.00 and accounted for 16.7% of the total variability. The remaining 4 PCs had lesser eigenvalues (< 1) and accounted for less variability (< 1.3%) and become insignificant.

From the component plot of PC1 and PC2, which together accounted for 97.6% variability, seven distinct groups were identified (Fig. 2). Coconut testa extract obtained using acidified 80% acetone occupied the first group with high TPC and antioxidant potential measured by DPPH, ABTS, FRAP and CUPRAC methods, while the group 2 had 4 extracts obtained using acidified methanol and ethanol (both aqueous and non-aqueous forms) with comparatively high amount of all the parameters studied. Extracts obtained using aqueous methanol and acetone placed in the third group with comparatively high amount of flavonoids followed by other constituents studied. Testa extracts of water, methanol, and aqueous ethanol with comparatively lesser amount of TPC and antioxidant potential compared to group 1 to 3 belonged in the group 4. Group 5 and 6 had the extracts obtained using acidified acetone and acidified water and ethanol. The extract obtained using acetone alone placed in the group 7 with less amount of TPC, TFC, and antioxidant activity.

3.5. Identification of phenolics extracted from different solvents from coconut testa using UPLC-H class coupled with TQD-MS/MS

The two major family compounds, phenolic acids and flavonoids were identified by UPLC-H class coupled with TQD-MS/MS analysis in multiple reaction-monitoring (MRM) mode. The analysis of different solvent extracts gave twenty eight phenolic compounds including sixteen phenolic acids and twelve flavonoids. The results have revealed significant differences in the phenolic profile among the different

Table 5
Phenolic acids in different solvent extracts of testa (μg/g defatted testa sample).

Phenolic acids	W	AW	M	AM	M80%	AM80%	E	AE	E80%	AE80%	A	AA	A80%	AA80%
Chlorogenic acid	0.03	0.10	0.07	0.16	0.38	0.03	0.09	0.16	0.10	0.12	0.16	0.31	0.16	0.07
Vanillic acid	11.5	36.0	26.6	208	91.0	203	74.2	216	96.2	69.3	21.0	591	205	411
Syringic acid	4.08	9.64	1.66	31.4	10.5	21.8	7.81	21.2	14.0	12.5	3.70	43.3	13.8	21.1
Ferulic acid	23.0	806	495	14052	8204	5753	2698	10502	3711	3950	503	26341	5655	8592
Caffeic acid	53.3	942	412	416	899	842	718	366	288	338	7.11	771	199	654
Gallic acid	50.3	132	23.8	2375	524	259	18.5	217	132	207	45.0	514	130	5654
p-Coumaric acid	248	1678	556	6910	5190	5748	3198	5834	2634	6982	1477	16596	7225	15778
o-Coumaric acid	214	473	69.7	1091	321	662	222	472	250	477	89.2	1727	835	2745
2,4-Dihyroxybenzoic acid	15.6	2.40	44.4	24.0	22.8	79.2	26.4	142	275	70.8	15.6	400	4.80	13.2
Gentisic acid	62.1	8.27	128	16.6	ND	335	ND	203	8.27	141	29.0	70.3	37.2	33.1
Protocatechuic acid	873	4079	65.5	8380	6109	10258	6088	18180	7695	9638	681	18162	2533	34015
transCinnamic acid	1058	1247	735	189	347	1296	1041	1536	1725	845	1027	560	124	412
4-Hydroxybenzoic acid	52.2	98.5	19.9	1264	573	820	253	1030	567	803	189	3094	662	1257
Salicylic acid	54.0	576	197	918	719	810	966	1014	672	840	543	2642	1326	1157
3-Hyroxybenzoic acid	4.58	16.7	6.77	212	75.4	129	33.5	54.6	82.4	137	17.5	430	97.7	213
Sinapic acid	6.31	11.8	11.8	362	188	108	44.9	393	100	101	11.8	838	153	281

W- Water; AW-Acidified Water; M-Methanol; AM-Acidified Methanol; M80%-Methanol 80%; AM80%-Acidified Methanol 80%; E-Ethanol; AE-Acidified ethanol; E80%-Ethanol 80%; AE80%-Acidified ethanol 80%; A-Acetone; AA-Acidified Acetone; A80%-Acetone 80%; AA80%-Acidified Acetone 80%; ND-not detectable.

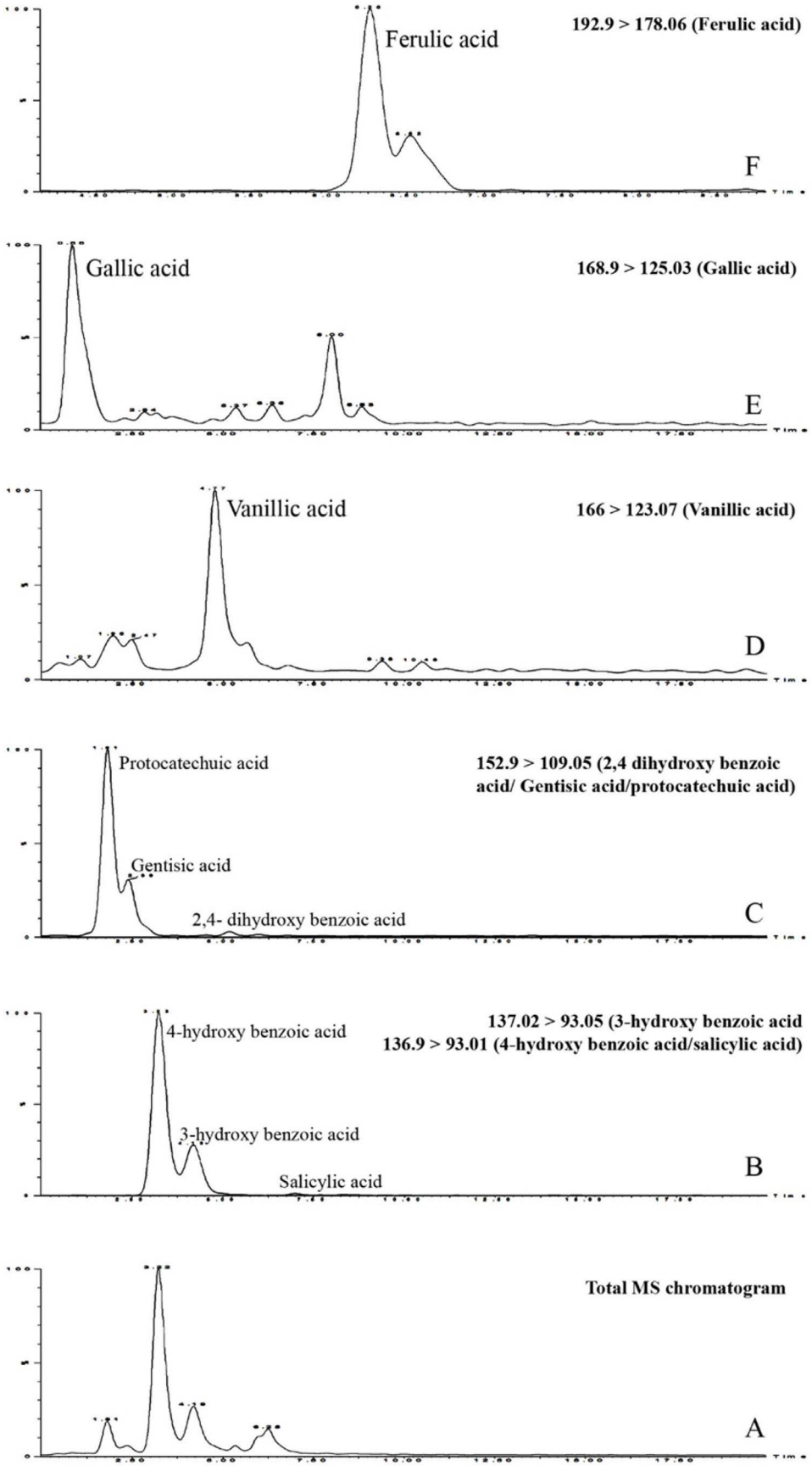

Fig. 3. Chromatogram of selected phenolic acids identified from coconut testa using LC MS/MS. A- Total MS chromatogram of coconut testa phenolic acids; B- MS/MS chromatogram of 3-hydroxy benzoic acid, 4-hydroxy benzoic acid and salicylic acid; C – MS/MS chromatogram of 2,4-dihydroxy benzoic acid, gentisic acid and protocatechuic acid; D – MS/MS chromatogram of vanillic acid; E- MS/MS chromatogram of gallic acid; F- MS/MS chromatogram of ferulic acid.

Table 6
Flavonoids in different solvent extracts of testa (μg/g defatted testa sample).

Flavonoids	W	AW	M	AM	M80%	AM80%	E	AE	E80%	AE80%	A	AA	A80%	AA80%
Catechin	35.3	22.5	6.4	202	12.8	3451	2462	2822	2077	844	1278	1233	205	96.3
Umbelliferone	0.3	0.4	0.1	0.6	0.1	0.2	0.6	1.0	0.2	0.4	0.2	0.2	0.2	0.3
Luteolin	82.7	38.6	226	66.2	33.1	38.6	49.6	49.6	55.1	77.2	11.0	11.0	11.0	60.6
Rutin	107	81.7	14.2	7.1	39.1	46.2	14.2	14.2	14.2	32.0	103	103	24.9	32.0
Hesperetin	435	37.8	75.7	208	360	643	1022	416	851	75.7	246	227	37.8	37.8
Myricetin	161	15.4	92.3	69.2	38.4	146	15.4	277	138	69.2	53.8	53.8	53.8	30.8
Quercetin	39.6	8.8	30.8	39.6	48.4	30.8	30.8	48.4	101	35.2	48.4	44.0	66.0	96.8
Apigenin	387	157	305	239	107	2999	3782	3881	404	115	49.4	49.4	33.0	57.7
Naringenin	20.9	40.0	91.4	24.8	20.9	286	24.8	17.1	3.8	85.7	19.0	20.9	7.6	101
Kaempferol	3090	4516	2139	2139	1189	1187	3566	2377	2615	9271	1902	2139	1426	3090
Epicatechin	33.1	141	16.5	33.1	141	33.1	174	66.1	16.5	41.3	49.6	57.9	107	33.1
Epigallocatechin	35.7	7.9	7.9	19.8	43.6	313	63.5	27.8	27.8	7.9	15.9	19.8	23.8	51.6

W- Water; AW-Acidified Water; M-Methanol; AM-Acidified Methanol; M80%-Methanol 80%; AM80%-Acidified Methanol 80%; E-Ethanol; AE-Acidified ethanol; E80%-Ethanol 80%; AE80%-Acidified ethanol 80%; A-Acetone; AA-Acidified Acetone; A80%-Acetone 80%; AA80%-Acidified Acetone 80%.

solvent extracts.

3.5.1. Phenolic acids

Sixteen phenolic acids were identified from testa extracted with different solvent systems and identity was confirmed using commercial standards (Table 5). The MS chromatogram of selected phenolic acids is given in Fig. 3. Among them, three phenolic acids belong to mono-hydroxy benzoic acid derivatives (salicylic acid, 4-hydroxy benzoic acid, and 3-hydroxy benzoic acid); four phenolic acids belong to di-hydroxy benzoic acid derivatives (protocatechuic acid, vanillic acid, 2,4-dihdroxy benzoic acid, gentisic acid); two trihydroxy benzoic acid derivatives (gallic acid and syringic acid); seven phenolic acids belong to hydroxyl cinnamic acid derivatives (chlorogenic acid, ferulic acid, caffeic acid, *p*-coumaric acid, *o*-coumaric acid, sinapic acid and trans-Cinnamic acid).

Among the mono hydroxyl benzoic acid derivatives, the concentration of salicylic acid was high followed by 4-hydroxy benzoic acid and 3-hydroxy benzoic acid. A significant difference was observed for phenolic acids content between different solvent systems used. Salicylic acid content ranged between 54.0 μg/g for water and 2642 μg/g for acidified acetone. 4-hydroxy benzoic acid content ranged between 19.9 μg/g (ethanol) and 3094 μg/g (acidified acetone). Acidification of solvents significantly increased extraction of phenolic acids when compared to the solvents without acidification. Among the dihydroxy benzoic acid derivatives, protocatechuic acid was found predominant phenolic acid followed by vanillic acid. Protocatechuic acid content in the acidified 80% acetone was most among the phenolic acids identified (34015 μg/g). Acidification of solvents increased the extraction of protocatechuic acid compared to other dihydroxy benzoic acid derivatives. Gentisic acid was found in very less concentration and ranged from 8.27 μg/g for acidified water to 335 μg/g for 80% acidified methanol, and was not detectable in 80% methanol and ethanol.

Gallic acid was predominant in the testa among the trihydroxy benzoic acid derivatives and ranged between 23.8 μg/g for methanol and 5654 μg/g for 80% acidified acetone. Syringic acid was found to be very less and ranged from 1.66 μg/g from methanol to 43.3 μg/g for acidified acetone. Among the seven hydroxyl cinnamic acid derivatives trans-Cinnamic acid and ferulic acid content was most followed by *p*-Coumaric acid. Chlorogenic acid content was found very less and ranged between 0.03 μg/g for water extract and 0.31 μg/g for acidified acetone extract. Gallic acid, hydroxyl benzoic acid, coumaric acid, caffeic acid, ferulic acid, and cinnamic acid were identified from coconut testa by HPLC method (Appaiah et al., 2014). Similarly gallic acid, protocatechuic acid, vanillic acid, chlorogenic acid, *p*-hydroxy benzoic acid, caffeic acid, syringic acid, ferulic acid, *p*-coumaric acid, and elagic acid are reported in virgin coconut oil and coconut oil cake indicating that coconut is rich source of dietary polyphenols (Seneviratne & Dissanayake, 2008, Seneviratne, Prasadani, & Jayawardena, 2016; Marina, Che man, Nazimah, & Amin, 2009; Janu et al., 2014). The total number of phenolic acids reported in the present study is more than the earlier reports. The study demonstrated that most of the phenolic acids are extracted in high amount when acetone and 80% acetone acidified with 0.3 M HCl was used as a solvents. It indicates most of these compounds are in bound form and are freely soluble in acetone when released by hydrolysis. Thus acetone acidified with HCl is found suitable solvent system for extraction of phenolic acids present in the coconut testa for commercial exploitation.

3.5.2. Flavonoids

Twelve flavonoids were identified in the different solvent extracts of coconut testa (Table 6), and MS chromatogram of selected flavonoids was given in Fig. 4. Among the twelve flavonoids, three belong to flavan-3-ol (catechin, epicatechin, and epigallocatechin); three belong to flavonol (myristin, quercetin, and kaempferol); one belongs to flavonol glycoside (rutin); two belong to flavone (leteolin and apigenin); two belong to flavanone (naringenin and hesperetin) and one belongs to coumarin derivative (umbelliferone). Among the flavan-3-ols identified, catechin was found maximum followed by epicatechin and epigallocatechin. Methanol yielded less catechin (6.42 μg/g) and acidified 80% methanol extracted maximum amount of catechin (3451 μg/g). Except for methanol and ethanol, acidification of other solvents caused a significant reduction in catechin extraction. Similarly, extraction of epicatechin was also influenced by the acidification, acidification of 80% methanol, ethanol and 80% acetone caused significant reduction of epicatechin content. Only acidified 80% methanol extracted maximum amount of epigallocatechin (313 μg/g) and other solvents extracted very less amount of epigallocatechin ranging from 7.93 μg/g to 51.6 μg/g testa sample.

Kaempferol content in the testa was found significantly high compared to myricetin and quercetin among the three flavonols identified. Extraction ability of acidified 80% ethanol was found high for kaempferol extraction (9271 μg/g) followed by acidified water (4516 μg/g). Rutin is the only flavonol glycoside identified in this study. Water and acetone extracted significantly high amount of rutin (107, 103 μg/g, respectively) compared to other solvents. Among the two flavones identified, apigenin was found maximum in coconut testa. Acidified ethanol, ethanol, and acidified 80% methanol extracted considerably high amount of apigenin (3881, 3782 and 2999 μg/g, respectively) compared to other solvents. Methanol was found to be a suitable solvent for extraction of luteolin (226 μg/g), whereas 80% acetone was found poor extractor of luteolin (11.0 μg/g). Among the flavanones, hesperetin found higher in coconut testa compared to naringenin. Both ethanol and 80% ethanol were found suitable solvent system for extraction of hesperetin, while, acidified 80% methanol was found suitable for naringenin extraction. Umbelliferone was the only coumarin derivative identified in testa and it was present in very low quantity

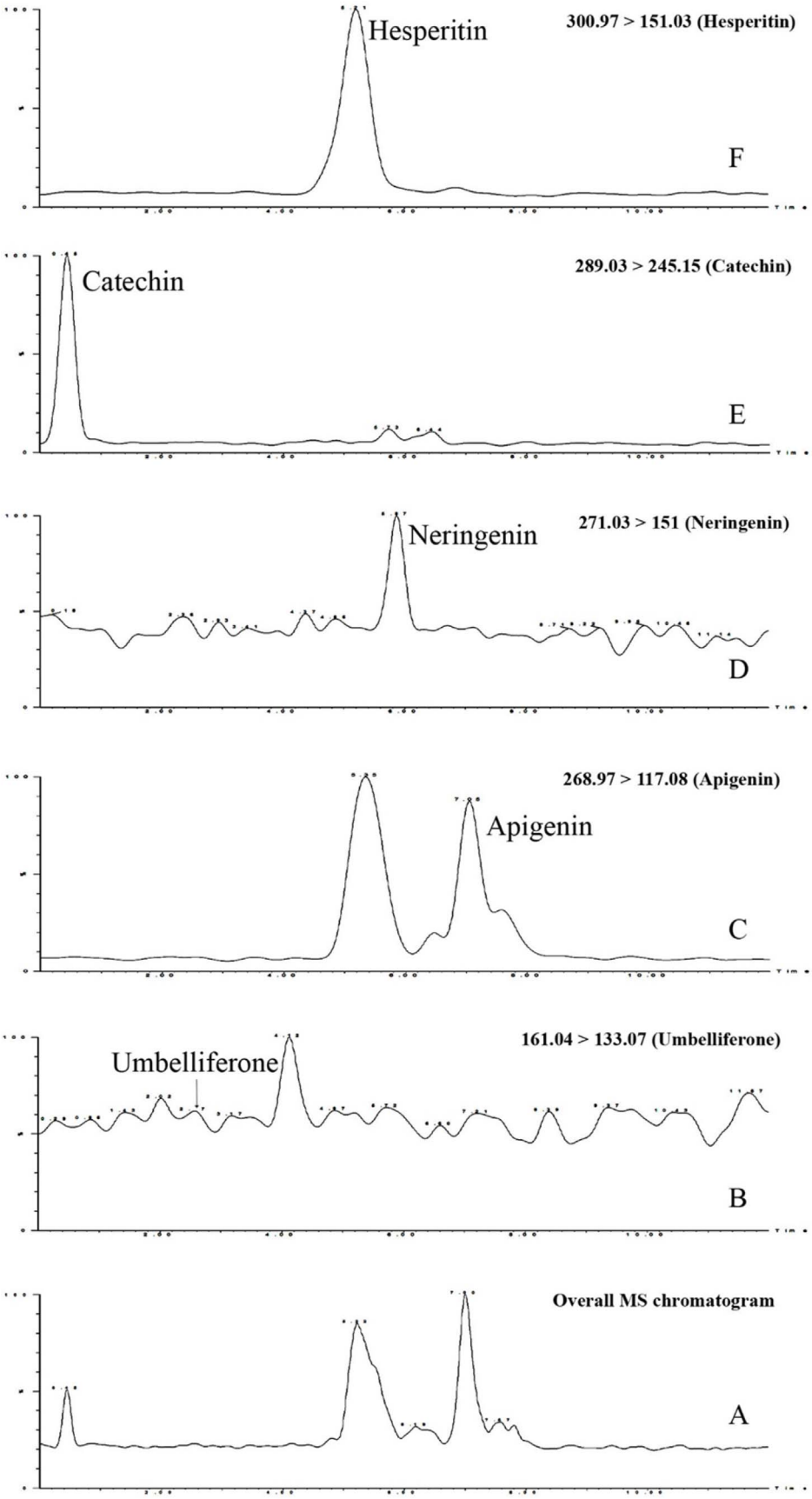

Fig. 4. Chromatogram of selected flavonoids identified from coconut testa using LC MS/MS. A- Total MS chromatogram of coconut testa flavonoids; B- MS/MS chromatogram of umbelliferone; C – MS/MS chromatogram of apigenin; D – MS/MS chromatogram of neringenin; E- MS/MS chromatogram of catechin; F- MS/MS chromatogram of hesperitin.

compared to other flavonoids identified and it ranged between 0.10 and 0.98 μg/g testa.

Phenolics and flavonoids have versatile functions like antioxidants, anti-tumor, anti-aging, etc. Natural phenolics acts as functional food and provide specific health benefits (Löliger, 1991). Consistent intake of raw vegetables and fruits rich in polyphenol compounds is believed to improve the conditions of diseases such as cancer, diabetes, hypertension and cardiovascular problems, it also can decrease the sign of aging, and improve physical fitness (Działo et al., 2016; Pandey & Rizvi, 2009). Commercial exploration and utilization of plant source antioxidants are of immense value (Alothman et al., 2009; Rice-Evans, Miller, & Paganga, 1997; Wijekoon et al., 2011). The present study revealed that coconut testa has numerous health promoting phenolic acids and flavonoids.

4. Conclusion

The study showed that, depending upon the type of solvent, the quantity of phenolics, flavonoids and antioxidant compounds being extracted also vary. Eighty percent acetone (v/v) acidified with 0.3 M HCl is the suitable solvent system for maximum extraction of phenolics, and antioxidant compounds, while 80% methanol was suitable solvent system for maximum extraction of flavonoids. In conclusion, coconut testa is proved to be a natural source of multiple phenolics acids and flavonoids with potent antioxidant capacity and these natural compounds can replace the synthetic antioxidants in food formulations that can be used as a natural source of antioxidants. Additionally, further research is warranted to explore the health benefits of bioactive compounds identified in the coconut testa for their better utilization.

Conflict of interest

Authors do not have any conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2018.02.024.

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