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ANTIBACTERIAL ANTIOXIDANT AND PHENOLIC FRACTIONS ANALYSIS OF *CAESALPINIA CRISTA* SEED COAT EXTRACT AND ITS DIFFERENT FRACTIONS

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Abstract

Methanolic crude extract from the seed coat of *Caesalpinia crista* was purified into different polyphenolic fractions. Solid Phase Extraction (SPE) was used for the purification of crude extract using C18 cartridges. Non-anthocyanin (fraction I) and anthocyanin (fraction II) are the two main fractions that were collected from crude extract and Fraction Ia (Phenolic acid), Fraction Ib (flavanols etc) and Ic (flavonols etc) are the three sub fractions collected from non-anthocyanin fractions. It was shown by the HPLC analysis of separated fractions that among the ten polyphenolic standards that were taken i.e. vanillic acid, syringic acid, coumaric acid, caffeic acid, delphinidin, myricetin, epicatechin, kaempferol, pelargonidin, cyanidin and their other derivatives were not the main constituent of the *C. crista* seed coat. Activity against the bacteria was determined against two Gram-positive and three Gram-negative bacteria by an agar well diffusion method. It was found that against a panel of bacteria the seed coat of *C. crista* was found active. Gram positive bacteria was more sensitive towards fraction Ic while fraction Ia was more active against gram negative bacteria. Antioxidant screening was carried out by using different methods such as reducing power, diphenyl-1-picrylhydrazyl and lipoxygenase assay. Study showed that the seed coat of *C. crista* has considerable antioxidant properties. Phenolic fractions's order of antioxidant activity was in accordance to their whole flavonoid and phenolic contents (except of anthocyanin fraction) i.e. Non anthocyanin>flavonols>flavanol> phenolic acid. The significant (p<0.01) correlation was found between the antioxidant activity and the total phenolic content. Current study suggests that along with the content of polyphenols, the antioxidant and antimicrobial activities depend also on the structure of polyphenols. Moreover, the seed coat of *C. Crista* is a potential source of antibacterial and antioxidant compounds.

Key words: *Caesalpinia crista*, Seed coat, Natural polyphenols, Antioxidant, Antibacterial, Solid phase extraction, HPLC.

Introduction

The great significance of natural bio active chemicals (antimicrobials, antioxidants etc) over synthetic compounds has attracted many researchers towards their endless exploration. The medicinal effect of plants and/or plant parts suggests that their cells are composed of such compounds that possess antibacterial, antifungal and antioxidant properties. Polyphenolics are the most copious secondary metabolites present in plant cells that have therapeutic effect against many diseases. Isolation and identification of such polyphenolic compounds from different plant parts has thus attracted many researchers for carrying their medicinal and health related research (Yaseen *et al.*, 2019; Mobin *et al.*, 2017a; Ajaib *et al*., 2017; Asif, 2015; and Ashraf *et al*., 2015).

The plant, *Caesalpinia crista* comes from the family of Caesalpiniaceae, is generally present in tropical areas, and is found almost in every part of India and Pakistan. The seeds of the plant are round, dry and hot. They are up to 1.2 cm in length. These seeds are protected in a glossy yet very hard coat that may be green or ash gray in color. The surface of the kernel is ridged and is furrowed and is about 1.23-1.75 cm in diameter (Preedy *et al*., 2011). Many parts of this plant have been conventionally used for many remedial purposes (Khare, 2004; Javed *et al*., 1994; Preedy, *et al*., 2011). Still the information about the *C. crista* seed outer coat polyphenolic and healing property is very inadequate.

Current study has focused on the seed coat polyphenolic of *C. crista* and their health associated functionalities. Being an active source of antimicrobial and antioxidant compounds, it could be used as a replacer of synthetic drugs. The research included the isolation and identification of polyphenolic compounds present in the seed coat of *C. crista* and their bio functional properties evaluation. Recent research shows that this plant's seed coat can be very effective for the separation of powerful antioxidant and antibacterial composites which can be used in different foods and medication schemes.

Experimental section

Standards and chemicals: The procurement of phenolic standards of p coumaric acid (PubChem CID: 637542), syringic acid (PubChem CID: 10742), vanillic acid (PubChem CID: 8468), and myricetin (PubChem CID: 5281672) were done from Sigma (St. Louis, MO, USA) while the purchasing of other phenolic standards that are; catechin (PubChem CID: 73160), malvidin (PubChem CID: 159287), petunidin (PubChem CID: 73386), epicatechin (PubChem CID: 72276), kaempferol (PubChem CID: 5280863), and delphinidin (PubChem CID: 68245) were done from ChromaDex (Irvine, California, US). The purchasing of HPLC grade solvents such as $C_4H_8O_2$, CH_3OH, and C_2H_3N were done from Fisher Scientific. Other ACS grade reagents like $AgNO_3$, H_3PO_4, KCL, NH_4, $ALCL_3$, NaOH, $C_2H_3NaO_2$, and HCL were bought from Merck and Fisher Scientific

Sample: The seeds of *C. crista* were collected from the Ghousia Dawakhana, Local market, Shah Faisal Colony Karachi-25 in March 2015. The taxonomist certified the samples and a voucher specimen (G.H. Nos. 94251) were deposited in the herbarium of Botany department, University of Karachi. The seeds were washed and dried (through air). The Black & Decker (US) grinder was used for grinding of pulse. The dried seeds of *C. crista* were exposed for one minute for the cracking of their external pelt. Outer seed coat from inside the legume was

manually removed. This was followed by the storage at -4ºC of the powdered seed coat of *C. crista* in a freezer.

Extraction: Sultana *et al.*, in 2009 proposed a method which with some upgrading was used for the *Caesalpinia crista* seed coat extract. In short, to get the 80 mesh particle size the seed coats that were collected were ground in a grinder. Methanol with a ratio of sample: solvent 1:10 (CH_30H: H_20, 80:20 v/v) was used to unstiffen the crushed particles at an ambient temperature for about twenty-four hours (30±2ºC), What man No.1 filter paper was used to separate the filtrate from the residue. The rotary evaporator (Buchi, R-200, Flawil, Switzerland) was used to concentrate the combined extract at 45ºC under a vacuum. For more analysis, the extract that was concentrated previously was kept at -4º C until they are used.

Fractionation

Kim and Lee in 2002a and Oszmianski and Lee in 1990 offered a method that was used here for obtaining the seed coat extract of *C. crista* fraction's i.e., non-anthocyanin and anthocyanin. The fractionation of polyphenolics from the outer peel of *C. crista* is illustrated in Figure S1 (supporting material available online) (Mobin *et al.*, 2017b).

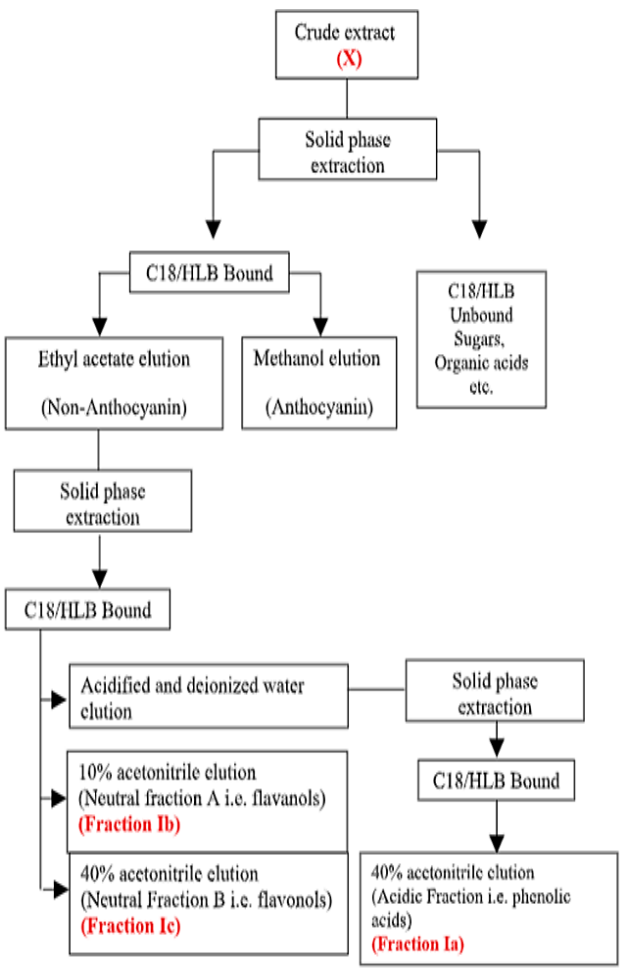

Fig. S1. Purification and isolation of different polyphenols from the seed coat of *C. crista*.

Identification and quantification

HPLC analysis: DAD-HPLC (LC series 1260 infinity, agilent technology) was used for polyphenolic identification. Fractions I and II were filtered and 20 µm of each fraction were injected. The maintenance of the reverse-phase C18 column (five-micrometer × two fifty-millimeter × 4.6- millimeter) was done at temperature 23ºCelsius. Kim & Lee, in 2002b offered a method that was used for setting the conditions for HPLC examination. In short, the solvents were (A) $(NH_4)H_2PO_4$ 50 mM with pH 2.6, (B) acetonitrile/ $(NH4)H_2PO_4$ 80:20 (v/v) with pH 2.6 and (C) H_3PO_4 200 mM with an acidic pH of 1.5. The following is the ratio at which gradient's flow rate was fixed at a one milliliter per minute:

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	100	0	0
4	92	8	0
10	0	14	86
22.5	0	16.5	83.5
27.5	0	25	75
50	0	80	20
55	100	0	0
60	100	0	0

The characteristic of UV–Vis spectra and retention times were used for recognizing the polyphenolic composites

Total phenol content (TPC): The method by Gutfinger (1981) was used to estimate total phenolic content. Results were defined on DM of extract as milligram of GAE per gram.

Total flavonoid content (TFC): Dewanto *et al.*, (2002) method was used to measure the total flavonoid content. If briefly described, 4ml of water was used to dilute each fraction and later 0.3 ml of $NaNO_2$ (5%) was added to it. From 10% aluminum chloride 0.3 ml was added after 5 minutes, this was followed by the addition of two milliliter of sodium hydroxide of molarity 1.0. Afterward, two and a half milliliters of H_2O was added to it. At 510 nm the absorbance was read. The standard used was Quercetin.

Total anthocyanin content (TAC): The method reported by Wrolatad *et al.*, (1995) for the total monomeric anthocyanin content was used. To adjust sample pH 1 and pH 4.5, buffer KCL and CH_3COONa_2 were added to the sample and upheld for a time period of fifteen minutes. By using spectrophotometer, the pH maintained sample's absorbance was evaluated at 520 nanometer & 700 nanometers. Results are calculated using following equations;

$$A = (A520 - A700)\,pH1.0 - (A520 - A700)\,pH4.5 \quad Eq\ (1)$$

$$TAC\left(\frac{mg}{L}\right) = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1} \quad Eq\ (2)$$

where,
A= absorbance
ε= cyanidin-3-glucoside molar extinction coefficient (449.2g)
DF= dilution factor
MW= molecular weight

Antibacterial activity

Microbial strains: The *Staphylococcus aureus* (ATCC 25931), *Escherichia coli* (ATCC 25922), *Salmonella typhi* (6539), *Pseudomonas aeruginosa* (27853) and *Bacillus subtilis* (ATCC 6633) strains were used. These strains were voluntarily provided by the Department of Microbiology, University of Karachi.

Zone of inhibition: The agar diffusion method proposed by Ortega & Julian, 1996 and Ferreira *et al*., 1996 was used to determine the activity of *Caesalpinia crista* seed coat extract and fractions against a range of bacterial strains. Syringe filter of 0.45 micrometer was used to filter the seed coat extract & its fractions. One milliliter from each strain culture suspension, which is having 25 percent transmittance at wavelength of 530 nanometers, was introduced to hundred milliliters of antibiotic number eleven agar at 45 ^{O} Celsius and mixed finely. Immunized agar of approximately 25 milliliters was transferred in a petri and kept for hardening. This was followed by making holes of diameter 8 millimeter with 6 millimeters interior diameter via sterile borer. Finally, the excerpt and its fractions of 100 microliters were then transferred into their particular patent well and nurtured at 37^{o} Celsius for twenty-four hours. The samples were examined 3 times and the outcomes were described in triplets. When the incubation period ends after one day, each extract and fraction's produced inhibition in millimeter were calculated and defined in terms of inhibition%. As a reference, the standard antibiotic. Gentamicin with 0.3% inhibition% was used.

Antioxidant action: The *C. crista* seed coat extract antioxidant action was evaluated according to following methods. Each method was briefly explained in our previous research (Mobin *et al.*, 2017b).

Reducing power: Yen *et al.*, in 2000 offered a technique that was used with minor upgradation for the determination of reducing power.

Free radical scavenging activity: Hossain *et al*., in 2011 offered a technique namely; 2,2-diphenyl-1-picrylhydrazyl, that was used for the evaluation of the antioxidant by radical scavenging activity of the *C. crista* seed coat extract and fractions.

Lipoxygenase analysis: The lipoxygenase inhibition activity was determined by the method proposed by Maharvi *et al*., 2008.

Statistical analysis: The ANOVA was performed by using SPSS software (version 17.0). The sections were examined three times, and the outcomes (vital level $p<0.05$and $p<0.01$) were expressed as the means ± standard error. Correlations were established using Pearson test.

Results and Discussion

Total phenolic content, Total flavonoid content, and HPLC examination: The total phenolic content in the eighty percent methanolic *C. crista* seed coat extract was found to be 103.1 mg GAE/gm of dried extract, which was higher as compared to the total content of phenol in the whole seed reported previously i.e. 62.50 mg GAE /gm of ethanolic extract (Shukla *et al*., 2009), 50.23 mg GAE /gm and 106.83 mg QE/gm in the methanolic extract (Mandal *et al*., 2011). This variation in total phenolic and flavonoid content could be due to the different solvents type since the extraction of compound containing polyphenolic depends on the number of parameters like method of extraction, solvent type, the assessing bases i.e. either dry or wet as proposed by the Cheng *et al*., in 2012. Furthermore, the previous studies are on the *C. crista* whole seed while in the present study the sample used is the external rigid coat of *C. crista* seed. The statistical study (ANOVA) indicated that the total phenols and flavonoid content of *C. crista* seed coat extract and its fractions have a significant difference ($p<0.05$). The phenolic content was found to be more in the crude seed coat extract as it is the sum of phenolic composites together existing in the coat of seed.

Table 1 showed that the *C. crista* seed coat largely consisted of polyphenolic compounds such as flavonol, flavanol etc other than anthocyanins. Excluding anthocyanin fraction, the order in which TPC was found was of Ic>Ib>Ia (Table 1).

Table 1. Total content of phenols, flavonoids and anthocyanin in the crude extract and fractions isolated from crude extract of *C. crista* seed coats.

Crude/Fractions	TPC (GAE/ gm)	TFC (QE/ gm)	TAC (CYN-3-GLU/100gm)
Extract	103.11 ± 1.1^{f}	45.12 ± 0.1^{e}	23.5 ± 0.4^{b}
Non Anthocyanin (I)	89.1 ± 1.5^{e}	42.8 ± 1.8 ^{d}	ND
Anthocyanin (II)	21.7 ± 4.8^{d}	20.02 ± 1.83^{c}	20.12±0.3^{a}
Acidic (Ia)	10.18 ± 0.83^{a}	-	ND
Neutral A (Ib)	13.4 ± 0.3^{b}	9 ± 0.4^{a}	ND
Neutral B (Ic)	16.74 ± 0.6^{c}	11± 0.0 ^{b}	ND

(-) estimated value

^{a} Values are mean and ± standard deviation of three separate determinations. Values in the same column with different alphabet superscripts are significantly different at ($p<0.05$)

Table 2. Retention time and wavelength of the phenolic standards analyzed by DAD-HPLC.

No.	Compound	Retention time (min)	Identification wavelength (nm)
1.	vanillic acid	21.6	280
2.	syringic acid	22.6	280
3.	caffeic acid	22	320
4.	epicatechin	24.8	280
5.	delphinidin	29.9	520
6.	p coumaric acid	33.5	280
7.	cyanidin	33.9	520
8.	pelargonidin	39.9	520
9.	myricetin	45	370
10.	kaempferol	54.9	370

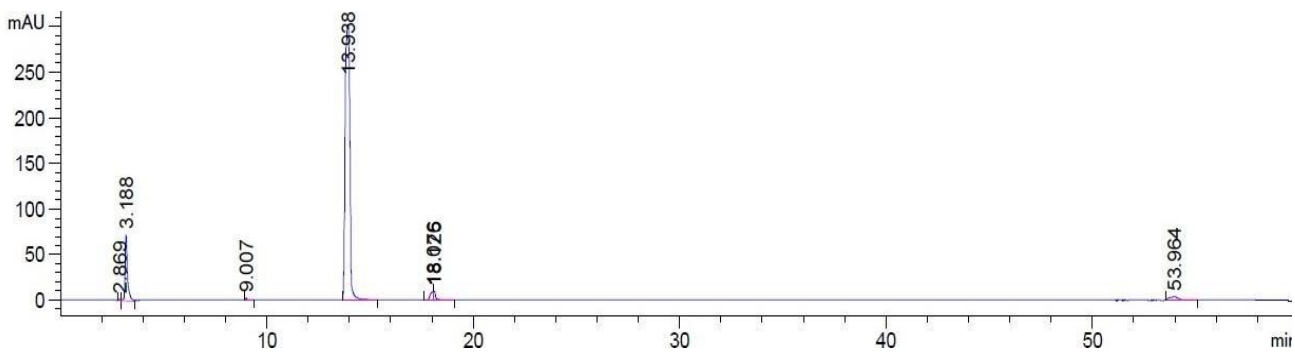

Fig. 1. HPLC chromatogram of the anthocyanin fractions of the *C. crista* seed coat monitored at 520 nm.

The isolated fractions of anthocyanin and non-anthocyanin from *C. crista* seed coat by using C18 cartridges when analysed through DAD-HPLC, among the taken standards (Table 2) no phenolic acid compounds have been determined. The current researches shows that the seed coat of *C. crista*, which on the basis of previous researches are actually the main source of diterpenoids and has not comprised of following polyphenols such as syringic acid, gallic acid, coumaric acid, caffeic acid, myricetin, epicatechin, delphinidin, kaempferol, cyaniding, pelargonidin and their derivatives. However, the chromatogram of anthocyanin fraction monitored at 520 nm wavelength is illustrated in Figure. 1. On the basis of a research on *Caesalpinia* genus, in 100 species of plant around 280 compounds have been recognized. Diterpenoid is one of the prominent constituents (Wu *et al*., 2011). From the seed coat of *C. crista* many terpenes are also being isolated (Kalauni *et al*., 2005; Linn *et al*., 2005; Kalauni *et al*., 2004; Jiang *et al*., 2001). Perhaps from the species of *crista* no phenolic compound has been found yet. However, work on the total phenolic and flavonoid content on the species *crista* has already been reported (Mandal *et al*., 2011; Jana *et al*., 2011). On the basis of current study, the presence of polyphenols is also supported by our research. However, no such polyphenol has been determined among the ten tested standards in the seed coat of *C. crista*. The reported flavonoid from certain species of *Caesalpinia* are: 7-Dihydroxy-3-(4-hydroxybenzyl)-chroman-4-one, 3,4,7-Trihydroxy-3-(4-hydroxybenzyl)-Chroman, 3'-Deoxy Sappanol, 7-Hydroxy-3-(4-hydroxybenzylidene)-chroman-4-one, 3, 3'-Deoxy-4-O-methyl sappanol, 3-Deoxy Sappanone B, 4-O-Methyl Sappanol, 4-O-Methyl Episappanol, Sappanone B have been identified in different part of species *Sappan* (Namikoshi *et al*., 1987a; Namikoshi, *et al*., 1987b; Nguyen *et al*., 2005; Jeong *et al*., 2009; Fu *et al*., 2008), Bonducellin, Isobonducellin, 2'-Methyl Bonducellin, 8-Methoxy Bonducellin, 4'-Methyl Isoliquiritigenin, 5,7-Dimethoxyflavone, 5,7-Dimethoxy-3', 4'-(methylenedioxy)-flavanone, Dihydrobonducellin, s2'-Methyl Dihydro Bonducella are dominated in the different plant part of species *Pulcherrima* (McPherson *et al*., 1983;Srinivas *et al*., 2003; Zhao *et al*., 2004; Rao *et al*., 2005) whereas 8-Methyl Iso Bonducellin, 8-Methoxy Bonducellin, Isoliquiritigenin, Intricatinol, Eucomin, Liquiritigenin are reported in aerial part of species *Millettii* (P. Chen *et al*., 2007).

Antibacterial activity: Against th**e** pathogenic and food borne bacteria **t**he antibacterial potential of fractions isolated and crude extract from the seed coat of *C. crista* was determined. In Table 3 the results are shown. The antibacterial activity was determined against Gram-positive (*B. subtilis, S. aureus*) and Gram-negative (*P. aeruginosa, E. coli, S.typhi)* bacteria by agar well diffusion method.

Results showed that the *C.crista* all fractions (excluding anthocyanin) and seed coat extract have shown considerable antibacterial activity. Against *S. typhi* and *S. aureus* a zone of inhibition was found significantly considerable while against *B.subtilis, E. coli* and *P. aeruginosa* activity was moderate by crude extract. The crude extract from the seed coat of *C. crista* was found to be more active against all the bacterial strain tested with zone of inhibition ranged between 15-30 mm than the whole seed crude extract of *C. crista* (inhibition zone

ranged 5-15 mm) as reported by Khan *et al.,* 2011. Thus the isolation of antibiotic seed coats of these plants could be a better source than a whole seed which minimizes the yield of other compounds that reduces the antibacterial effect. The phenolic compounds are well known for their antimicrobial activity (Pereira *et al.,* 2007). Depending on what is the nature of microbes and phenolic assembly itself, the act against every individual class of microbes of phenolic is completely dissimilar. This different act of phenolic against each class of microbes is linked to the inhibition of enzymes which entirely subjected to the rate of diffusion of the phenolic composite into the cells of microbes or it could also be due to some other fluctuations in the membrane permeability as proposed by the Moreno *et al. in* 2006.

Results suggest that the crude extract fractionations have better antibacterial results against every selected strain. Apart from, the antibacterial activity (p<0.05) of all other fractions were higher than the crude extract. So it can be concluded from the outcomes that the antibacterial activity of polyphenols present in a crude extract of seed was lower than the fractions consisting of a particular class of polyphenols at considerable quantity. The antibacterial activity of fractions Ia, Ib and Ic imitative from the extract of seed coat to every selected species was substantial. Anthocyanin i.e. fraction II showed antibacterial activity against *Staphylococcus aureus*. Antibacterial activity of fractions came out in order as: Ic> Ib> Ia for gram+ bacteria while for gram- bacteria, the order turned out as: Ia > Ic> Ib. As per results, Ic showed more activity against gram positive strains on the other hand, Ia showed least activity against gram- bacteria. Difference in antibacterial of different fractions of phenolic might be due the reason that it might be different against the microbes depending on the nature of microbes and the assembly of polyphenolic compounds. In 1998, Helander *et al.,* described that against the lipophilic polyphenolic compounds, the complexity of the outer membrane of gram negative strains acts as a protective wall. Therefore, the Ia fraction's higher antibacterial property in comparison to other fractions was because of the reason that it has less lipophilic structure of the phenolic acid. Due to less lipophilic structure of phenolic acid in fraction Ia, the membrane permeability increases & therefore the movement of phenolic acids (Ia) in comparison to the other fractions i.e. Ib which was flavanol & Ic which was flavonol inside the cell was more. Once entered inside the bacterial cell, the phenolic acid successfully prevents the growth of microbes by reacting with protein and nucleic acid, interfering with the electron transfer and/or other activity of enzymes as proposed by the Dorman & Deans, 2000.

Table 3. Antimicrobial activity of seeds coat extract and fractions of *C. crista*[a].

Sample	Zone of inhibition (mm)				
	S. typhi	***B. subtilis***	***E. coli***	***P. aeruginosa***	***S. aureus***
Extract	26 ± 0.3^{d}	15 ± 0.1^{c}	15 ± 0.1^{b}	18 ± 0.5^{c}	30 ± 0.2^{c}
Non anthocyanin(I)	30 ± 0.3^{e}	21 ± 0.2^{e}	27 ± 0.3^{d}	26 ± 0.2^{e}	36 ± 0.2^{e}
Anthocyanin(II)	-	-	-	-	20 ± 0.1^{b}
Acidic (Ia)	25 ± 0.2^{c}	10 ± 0.2^{a}	20 ± 0.4^{c}	22 ± 0.1^{d}	27 ± 0.9^{a}
Neutral A (Ib)	15 ± 0.1^{a}	12 ± 0.1^{b}	10 ± 0.1^{a}	12 ± 0.1^{a}	23 ± 0.2^{c}
Neutral B (Ic)	17 ± 0.5^{b}	19 ± 0.4^{d}	10 ± 0.5^{a}	13 ± 0.1^{b}	18 ± 0.3^{d}

[a] Values are mean and ± standard deviation of three separate determinations. Values in the same column with different alphabet superscripts are significantly different at (p<0.05)

Table 4. Correlation analysis between phenolic content and antioxidant activity of *C. crista* seeds extract and fractions.

	Pearson coefficient (r)	
	DPPH	**RED**
TPC	0.8*	0.66*

*Correlation is significant at 0.05 (p two-tailed) PHEN-phenanthroline activity; RED-reducing power; DPPH- α, α, diphenyl –β, picrylhydrazyl

Antioxidant capacity: The potential of Antioxidant was determined by using various different techniques such as reducing power, radical scavenging activity and lipoxygenase assay. According to the result the crude seed coat extract of *C. crista* had considerable inhibition of free radical DPPH i.e. 70%. The scavenging activity of free radicals of samples can be evaluated easily by the DPPH method, which is mainly based on the presence of antioxidants in a sample. By giving its electron, the antioxidants counterbalance the free radical DPPH. The reducing power of crude extract was found to be 75 percent of BHT using the reduction method of potassium ferricyanide. According to the Elmastaş *et al.,* this is found to be linked with the presence of compounds that can denote their H atom to a free radical and split up the sequences of oxidation reactions. Phenols are substantiated as powerful antioxidants in any medium i.e. food or/and isolated (Yu & Ahmedna, 2013; Abideen *et al*., 2015; Qasim *et al*., 2016). They maintained the antioxidant system either by chelating metals or other pro oxidant factors or by scavenging free radicals. Crude extract of Caesalpinia *crista*'s substantial reduction potential was because of the incidence of polyphenols which had yielded antioxidant effects synchronously. Crude extract and fractions reducing power were discovered in need of identical BHA. The link among the antioxidant capacity and phenolic content appeared to be positive (p<0.01) as shown in Table 4.

Table 5. Antioxidant screening of C. crista seeds coat extract and its fractions [a].

Fractions	DPPH (percent inhibition)	Reducing activity (percent compared with BHT)	Lipoxygenase (% inhibition)
Extract	70.1 ± 0.3 [f]	75 ± 0.5 [f]	72 ± 0.1 [f]
Non-Anthocyanin (I)	64.3 ± 1.1 [e]	70 ± 0.2 [e]	60 ± 0.1 [e]
Anthocyanin (II)	34.2 ± 1.2 [b]	61 ± 0.6 [b]	52± 0.1 [b]
Acidic (Ia)	39.1 ± 1.9 [a]	49± 0.1 [a]	63 ± 0.2 [a]
Neutral A (Ib)	57.5 ± 1.5 [c]	55 ± 0.3 [d]	59 ± 0.2 [d]
Neutral B (Ic)	62.8 ± 1.3 [d]	65 ± 0.4 [c]	68 ± 0.1 [d]

[a] Values are mean and ± standard deviation of three separate determinations. Values in the same column with different alphabet superscripts are significantly different at ($p<0.05$)

The fractions show variations in the antioxidant activity which are found significantly different from each other ($p<0.05$) (Table 5). *Caesalpinia crista* crude extract fractions (I> Ic> Ib> II> Ia) antioxidant sequence can be defined on the foundation of structures of polyphenolic and entire contents of it. Fraction I had the highest antioxidant capacity due to the greater content of phenolic compounds that were producing synergistic effects. Fraction Ic's had higher antioxidant capacity shown in comparison to the fraction Ib and fraction Ia, which could be due to the bases of the relation of structure function of polyphenols. The structures of polyphenols not only give the total amount of polyphenols, but also vital in telling the antioxidant capacity. As per Nascimento *et al.*, the antioxidant capacities of phenols with mono electron donating system are less than the phenols with polysubstituted hydroxyl groups. Fraction Ic's higher activity might be due to the occurrence of flavonol (quercetin etc) in it. The polysubstituted hydroxyl groups and conjugation in the aromatic rings of polyphenols in fraction Ic, increased its reduction and free radical scavenging capacity. Fraction Ia gave less antioxidant activity merrily because it is made up of phenolic acids that are mono substituted hydroxyl phenols i.e. hydroxycinnamic and hydroxybenzoic acid. However, the degree of antioxidant of phenolic acid is determined by the number and position of hydroxyl groups. The glycosylation of anthocyanin might result in its low antioxidant potential of fraction II when compared with other fractions. According to a research by Heinonen & Hopia (1999) the glycosylation of flavonoids at a position 3 in aromatic rings reduces the antioxidant activity. The outcome of our experiment indicates the lipoxygenase inhibition which means that the lipoxygenase enzyme in the existence of crude extract and fractions of *Caesalpinia crista* had reduced the oxygenation of linoleic acid. Moreover, the order was found the same as in previous antioxidant assays.

Conclusion

The seed coat of *Caesalpinia crista* could be a significant source of polyphenols as they play an important part against reactive oxygen types and not only that, they also have a wide range against illness triggering bacteria. As per the recent studies the *Caesalpinia crista*'s seed coat can be highly effective to treat a number of human diseases as a natural means of preservative. Nevertheless, in order to discover its further potential biological and industrial uses, more study needed to be carried out on *Caesalpinia crista* seed coats.

References

Abideen, Z., M. Qasim, A. Rasheed and M.Y. Adnan. 2015. Antioxidant activity and polyphenolic content of Phragmites karka under saline conditions. *Pak. J. Bot.*, 47(3): 813-818.

Ajaib, M., A. Zubairia and M.S. Faheem. 2017. Cocculus laurifolius: a rich antimicrobial, antioxidant and phytochemical source. *Pak. J. Bot*, 49(1): 337-344.

Ashraf, M.W., M. Bilal and M. Iqbal. 2015. Antiglycation activity of vegetables aqueous and methanolic extracts. *Curr. Sci., Perspectives*, 1: 12-15.

Asif, M. 2015. Antiviral and antiparasitic activities of various substituted triazole derivatives: A mini. *Chem. Int.*, 1(2): 71-80.

Cheng, V.J., A.E.D.A. Bekhit, M. McConnell, S. Mros and J. Zhao. 2012. Effect of extraction solvent, waste fraction and grape variety on the antimicrobial and antioxidant activities of extracts from wine residue from cool climate. *Food Chem*., 134(1): 474-82.

Dewanto, V., X. Wu, K.K. Adom and R.H. Liu. 2002. Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *J. Agric. Food Chem*., 50(10): 3010-3014.

Dorman, H. and S. Deans. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol*., 88(2): 308-316.

Elmastaş, M., L. Gulcin, S. Beydemir, K. Irfan and Y. Hasan. 2006. A study on the *In vitro* antioxidant activity of juniper (*Juniperus communis* L.) fruit extracts. *Anal. Lett.*, 39(1): 47-65.

Ferreira, M.J.U., A. Daurte and J.R. Ascenso. 1996. Antimicrobial and phytochemical studies of *Euphorbia tuckeyana*. *Fitoterapia*, 67(1): 85-86.

Fu, L.C., X.A. Huang, Z.Y. Lai, Y.J. Hu, H.J. Liu and X.L. Cai. 2008. A new 3-benzylchroman derivative from Sappan Lignum (*Caesalpinia sappan*). *Molecules*, 13(8): 1923-1930.

Gutfinger, T. 1981. Polyphenols in olives oils. *J. Amer. Oil Chem. Soc*., 58(11): 966-968.

Helander, L.M., H.-L. Alakomi and K. Latva-Kala. 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. *J. Agric. Food Chem*., 46: 3590-3595.

Hopia, A. and M. Heinonen. 1999. Antioxidant activity of flavonol aglycones and their glycosides in methyl linoleate. *J. Amer. Oil Chem. Soc*., 76(1): 139-144.

Hossain, M.S., M.B. Alam, M. Asadujjaman, M. Islam, M.E. Mazumdar and M. Haq. 2011. Antidiarrheal, antioxidant and antimicrobial activities of the *Musa sapientum* seed. *Avicenna J. Med. Biotech.*, 3: 95-105.

Javed, I., M.S. Akhtar, Z.U. Rahman, T. Khaliq and M. Ahmad. 1994. Comparative anthelmintic efficacy and safety of *Caesalpinia crista* seed and piperazine adipate in chickens with artificially induced *Ascaridia galli* infection. *Acta Vet. Hung*, 42(1): 103-109.

Jana, K., K. Chatterjee, K.M. Ali, A. Ghosh, T.K. Bera and D. Ghosh. 2011. Antioxidant potential of hydro-methanolic extract of seed of Caesalpinia bonduc: An in vitro study. *J. Adv. Pharm. Technol. Res*., 2(4): 260.

Jeong, G.S., D.S. Lee, T.O. Kwon, H.S. Lee, R.B. An and Y.C. Kim. 2009. Cytoprotective constituents of the heartwood of Caesalpinia sappan on glutamate-induced oxidative damage in HT22 cells. *Biol. Pharm. Bull.*, 32(5): 945-949.

Jiang, R.W., S.C. Ma, P.P.H. and T.C. Mak. 2001. New antiviral cassane furanoditerpenes from caesalpinia m inax. *J. Nat. Prod.*, 64(10): 1266-1272.

Khan, H.U., I. Ali, A.U. Khan, R. Naz and A.H. Gilani. 2011. Antibacterial, antifungal, antispasmodic and Ca++ antagonist effects of *Caesalpinia bonducella. Nat. Prod. Res.*, 25(4): 444-449.

Khare, C.P. (Ed.). 2004. Indian herbal remedies: rational Western therapy, ayurvedic, and other traditional usage, Botany. *Springer science & business media*.

Kalauni, S.K., S. Awale, Y. Tezuka, A.H. Banskota, T.Z. Linn and S. Kadota. 2005. New cassane-type diterpenes of Caesalpinia crista from Myanmar. *Chem. Pharm. Bull.*, 53(2): 214-218.

Kalauni, S.K., S. Awale, Y. Tezuka, A.H. Banskota, T.Z. Linn and S. Kadota. 2004. Cassane-and Norcassane-Type Diterpenes of *Caesalpinia crista* from Myanmar. *J. Nat. Prod.*, 67(11): 1859-1863.

Kim, D.O. and C.Y. Lee. 2002a. Extraction and isolation of polyphenolics. Current protocols in food analytical chemistry, Unit 11.2, John Wiley & Sons Inc

Kim, D.O. and C.Y. Lee. 2002b. HPLC separation of polyphenols. Current protocols in food analytical chemistry, Unit 11.3, John Wiley & Sons, Inc.

Linn, T.Z., S. Awale, Y. Tezuka, A.H. Banskota, S.K. Kalauni, F. Attamimi and S. Kadota. 2005. Cassane-and Norcassane-Type Diterpenes from Caesalpinia crista of Indonesia and their antimalarial activity against the growth of plasmodium falciparum. *J. Nat. Prod.*, 68(5): 706-710.

Mandal, S., B. Hazra, R. Sarkar, S. Biswas and N. Mandal. 2011. Assessment of the antioxidant and reactive oxygen species scavenging activity of methanolic extract of *Caesalpinia crista* leaf. *J. Evid. Based Complementary Altern Med*, https://doi.org/10.1093/ecam/nep072

Maharvi, G. M., S. Ali, N. Riaz, N. Afza, A. Malik, M. Ashraf & M. Lateef. 2008. Mild and efficient synthesis of new tetraketones as lipoxygenase inhibitors and antioxidants. *J. Enzyme Inhib. Med. Chem*., 23(1): 62-69.

McPherson, D.D., G.A. Cordell, D.D. Soejarto, J.M. Pezzuto and H.H. Fong. 1983. Peltogynoids and homoisoflavonoids from *Caesalpinia pulcherrima*. *Phytochemistry*, 22(12): 2835-2838.

Mobin, L., S.A. Saeed, R. Ali, S.G. Saeed and R. Ahmed. 2017a. Antibacterial, antioxidant and phenolics compound analysis of *Abrus precatorius* seed coat extract and its different fractions. *Pak. J. Bot*., 49(6): 2499-2506.

Mobin, L., S.A. Saeed, R. Ali, S.G. Saeed and R. Ahmed. 2017b. Antibacterial and antifungal activities of the polyphenolic fractions isolated from the seed coat of *Abrus precatorius* and *Caesalpinia crista*. *Nat. Prod. Res.*, 1-5. doi: 10.1080/14786419.2017.1378217.

Moreno, S., T. Scheyer, C. Romano and A. Vojnov. 2006. Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. *Free Radic. Res.*, 40(2): 223-231.

Namikoshi, M., H. Nakata and T. Saitoh. 1987a. Homoiso flavonoids from Caesalpinia sappan. *Phytochemistry*, 26(6): 1831-1833.

Namikoshi, M., H. Nakata, H. Yamada, M. Nagai and T. Saitoh. 1987b. Homoisoflavonoids and related compounds. II. Isolation and absolute conFigurations of 3, 4-hydroxylated homoisoflavonoids and brazilins from *Caesalpinia sappan* L. *Chem. Pharm. Bull*., 35(7): 2761-2773.

Nascimento, A.P., T.C.E.S. Nascimento, N.S.M. Ramos, G.R. Silva, J.E.G. Gomes, R.E.A. Falcao, K.A. Moreira, A.L.F. Porto and T.M.S. Silva. 2014. Quantification, antioxidant and antimicrobial activity of phenolics isolated from different extracts of *Capsicum frutescens* (Pimenta Malagueta), *Molecules*, 19(4): 5434-5447.

Nguyen, M.T.T., S. Awale, Y. Tezuka, Q. Le Tran and S. Kadota. 2005. Xanthine oxidase inhibitors from the heartwood of Vietnamese *Caesalpinia sappan*. Chem. and Pharmaceutical Bull., 53(8):984-988.

Ortega, M.G. and H.R. Julian. 1996. Antimicrobial agents in *Dalea elegant*, *Fitoterapia*, 67(1): 81-85.

Oszmianski, J. and C.Y. Lee. 1990. Isolation and HPLC determination of phenolic compounds in red grapes. *Amer. J. Enol. Vitic*., 41(3): 204-206.

Chen, P. 2007. Institute of medicinal plant development, Chinese academy of medical sciences and peking union medical college, Beijing, 2007.

Preedy, V.R., R.R. Watson and V.B. Patel. (Eds.). 2011. Nuts and seeds in health and disease prevention. Academic press.

Pereira, A.P., I.C.F.R. Ferreira, F. Marcelino, P. Valentão, P.B. Andrade, R. Seabra, L. Estevinho, A. Bento and J.A. Pereira. 2007. Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L. *Cv.* Cobrançosa) leaves. *Molecules*, 12(5): 1153-1162.

Qasim, M., I. Aziz, M. Rasheed, B. Gul and M.A. Khan. 2015. Effect of extraction solvents on polyphenols and antioxidant activity of medicinal halophytes. *Pak. J. Bot.*, 48(2): 621-627.

Roy, S., R. Acharya, N.C. Mandal, S. Barman, R. Ghosh and R. Roy. 2012. A comparative antibacterial evaluation of raw and processed *Guñjā* (*Abrus precatorius* Linn.) seeds. *Ancient Sci. Life*, 32(1): 20-23.

Rao, Y.K., S.H. Fang and Y.M. Tzeng. 2005. Anti-inflammatory activities of flavonoids isolated from *Caesalpinia pulcherrima*. *J Ethnopharmacol*, 100(3): 249-253.

Shukla, S., A. Mehta, J. John, S. Singh, P. Mehta and S.P. Vyas. 2009. Antioxidant activity and total phenolic content of ethanolic extract of *Caesalpinia bonducella* seeds. *Food Chem. Toxicol*., 47(8): 1848-1851.

Srinivas, K.V.N.S., Y.K. Rao, I. Mahender, B. Das, K.R. Krishna, K.H. Kishore and U.S.N. Murty. 2003. Flavonoids from *Caesalpinia pulcherrima*. *Phytochemistry*, 63(7): 789-793.

Sultana, B., F. Anwar and M. Ashraf. 2009. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. *Molecules*, 14(6): 2167-2180.

Wu, M., Y.F. Wang, M.L. Zhang, C.H. Huo, M. Dong, Q.W. Shi and H. Kiyota. 2011. Chemical constituents of plants from the genus Caesalpinia. *Chem. Biodivers*, 8(8): 1370-1399.

Wrolstad, R.E., V. Hong, M.J. Boyles and R.W. Durst. 1995. Use of anthocyanin pigment analysis for detecting adulteration in fruit juices. In: *Methods to Detect Adulteration in Fruit Juice and Beverages*, Vol. I (Eds.): Nagy, S. and R.L. Wade. Ag Science Inc., Auburndale, Fla.

Yaseen, G., M. Ahmad, S. Shinwari, D. Potter, M. Zafar, G. Zhang and S. Sultana. 2019. Medicinal plant diversity used for livelihood of public health in deserts and arid regions of Sindh-Pakistan. *Pak. J. Bot*., 1(51): 657-79.

Yen, G.C., P.D. Duh and D.Y. Chuang. 2000. Antioxidant activity of anthraquinones and anthrone. *Food Chem*., 70(4): 307-315.

Yu, J. and M. Ahmedna. 2013. Functional components of grape pomace: Their composition, biological properties and potential applications. *Int. J. Food. Sci. Tech.*, 48(2): 221-237.

Zhao, P., Y. Iwamoto, I. Kouno, Y. Egami and H. Yamamoto. 2004. Stimulating the production of homoisoflavonoids in cell suspension cultures of *Caesalpinia pulcherrima* using cork tissue. *Phytochemistry*, 65(17): 2455-2461.

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