ORIGINAL ARTICLE

Physicochemical analysis of *Psophocarpus tetragonolobus* (L.) DC seeds with fatty acids and total lipids compositions

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Abstract *Psophocarpus tetragonolobus* (L.) DC. is a tropical legume with potential nutritional properties. In present study, the physical properties and proximate composition of the seeds were evaluated. Besides, the physico-chemical properties of fatty oil from fully mature seeds were also studied. The fatty oil compositions of immature, mature and fully mature seeds were evaluated by GC-FID, GC/MS and ^{1}H-NMR. The study revealed that, fatty oil from fully mature seeds contained high proportion of unsaturated fatty acids (75.5 %), whereas immature seeds contained higher percentage of saturated fatty acid (61.3 %). In addition, unsaponification matter (0.25 %) of fatty oil was identified as stigmasterol (66.4 %) and β-sitosterol (25.1 %). Total lipids of fully mature seeds were extracted and isolated as neutral, glyco- and phospholipids. Overall, the fatty oil of fully mature seeds was enriched with mono-unsaturated fatty acids (38.6 %) and poly-unsaturated fatty acids (36.9 %) without trans-fatty acids, thus meeting the edible oil standard.

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Introduction

Psophocarpus tetragonolobus (L.) DC. (Fam. *Fabaceae*) commonly called as winged bean, goa bean, asparagus bean or four-winged bean. It is a tropical legume cultivated in Africa, South Asia and the Western Pacific. The various plant parts such as leaves, pods, seeds and tubers are edible. It is a source of rich-dietary proteins and edible oils. The pods and seeds are rich in proteins and vitamins, thus have been used for pharmacological purposes (Anonymous 1975) There is a considerable interest in *P. tetragonolobus* seeds because of their high nutritional quality, mainly in terms of high proteins and fatty oil content. The seeds follow various post harvesting processes for isolation of fatty oil and its derivatives. Post harvesting processes of seeds are depending on their basic physical and chemical characteristics (Mohsenin 1986).

Ekpenyong and Borchers (1980) have studied physico-chemical properties including the fatty oil of Nigerian *P.tetragonolobus* seeds. Garcia and Palmer (1979) have carried out the similar study on five different varieties of *P. tetragonolobus* seeds including the Nigeria variety synonymously reported the chemical composition of fatty oil identified as oleic acid (35.04–41.01 %) and linoleic acid (15.28–31.77 %). However, no separation of linolenic acid and arachidic acid was reported. Khor and Chan (1988) had reported the change in lipids accumulation profile in seeds from three to seven weeks. Varriano-Marston et al. (1983) had studied the microscopic characteristics of seeds along with fatty acids and bound lipids composition. The major fatty acids identified in free and bound lipids were palmitic (7.47, 14.47 %), oleic (27.48, 29.95 %), linoleic (21.83, 29.2 %) and behenic (31.33, 22.93 %) acid, respectively.

Although, some preliminary work on chemical composition of *P. tetragonolobus* seeds had been done (Mohanty et al. 2013), but there was no reported work on physical properties along with systematic characterization of fatty oil. Therefore,

the present work includes various physical analyses of seeds along with chemical characterization of lipids. Similarly, chemical composition of fatty oils from immature seeds (3 weeks old), mature seeds (4 weeks old) and fully mature seeds (5 weeks old) were compared. Additional, proximate composition and inorganic element content of fully mature seeds were determined. The total lipids extracted from fully mature seeds was further fractionated into neutral, glyco- and phospholipids. Similarly, unsaponification material present in the fatty oil was isolated and analyzed.

Materials and methods

Collection of *P. tetragonolobus* seeds were done from National Bureau of Plant Genetic Resources, Akola, Maharashtra, India. The germplasm was maintained in the garden of National Botanical Research Institute, Lucknow. Fully mature seeds (10 kg approx) from these plants were collected and cleaned manually. The seeds were kept in an airtight plastic vessel and stored at 5 °C before use. Before starting a test, the seeds were allowed to warm up under ambient room conditions (22–25 °C, 30–40 % RH).

Various developmental stages of seeds are presented in Fig. 1a The panels (i), (ii), (iii) and (iv) represent second, third, fourth and fifth weeks of seed development named as undeveloped, immature, mature and fully mature seeds,

respectively. The sizes of the undeveloped seeds were very small and difficult to separate from pods (Online Resource). It was observed that, seeds at this stage hardly accumulate any fatty oil. Thus, we have taken 3 (immature), 4 (mature) and 5 (fully mature) weeks old seeds for fatty oil compositions study. The physical and physico-chemical studies were carried out using fully mature seeds.

Physical properties of seeds

The physical properties of *P. tetragonolobus* seeds were determined by the following methods:

To determine the size, seeds were randomly selected from the lots. The seed, in terms of the three principal axial dimensions i.e. length (L), breadth (B) and thickness (T), were measured using a vernier caliper with ±0.01 mm accuracy. Since, seed size was considered an important parameter in processing bulk samples. Hence, they were classified into three categories namely small, medium and large based on their length. On that basis, the dimension was set up by calculating the average dimension ($\dot{X}$) and standard deviation (σ_x). Then, the different seed sizes were defined on the basis of their specific (X) dimension, which satisfies the following inequalities (Sharma et al. 2011; Pradhan et al. 2010); small size: $X < \dot{X} - \sigma_x$, medium size: $\dot{X} - \sigma_x < X < \dot{X} + \sigma_x$, large size: $X > \dot{X} + \sigma_x$. Similarly, the arithmetic mean diameter (D_a),
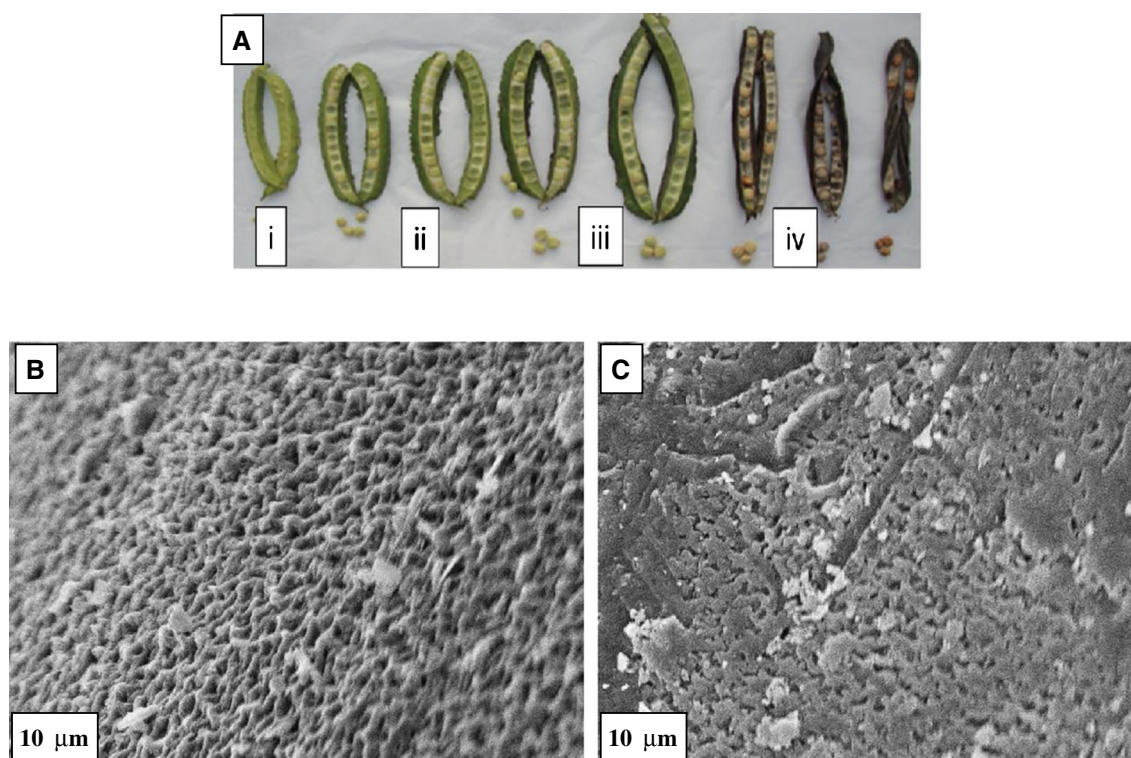

Fig. 1 **a** Different stages of *P. tetragonolobus* seed development, **b** Multi-reticulate finely interwoven pattern, **c** Coarsely interwoven secondary pattern recognize with 1000 × resolution

geometric mean diameter (D_g), sphericity (Ø), surface area (S), and aspect ratio (R_a) of the seeds were calculated by using the relationships described by Mohsenin (1986).

The unit mass of the randomly selected 100 numbers of seed samples were measured using an electronic balance with 0.001 g accuracy. The unit mass of 20 individual randomly selected seed samples were measured separately and multiplied by 50 to calculate 1000 seeds mass. Similarly, the bulk material (seeds) was put into a container of known volume (1000 cm³) and was weighted. Bulk density was calculated from the mass of the seeds and volume of the container. True density was determined using the toluene (C_7H_8) displacement method in order to avoid absorption of water during the experiment (Pradhan et al. 2010; Sharma et al. 2011). Toluene was used instead of water because of its low absorption by the seeds, low surface tension and also it filled even shallow dips in seeds due to its low dissolution number (Mohsenin 1986). True density was found as ratio of their masses to the volume of toluene displaced by the seeds. The volume of toluene displaced was calculated by immersing a weighted quantity of the seeds in toluene. Porosity (%) is indicated the amount of pores in bulk materials. It was calculated from bulk and true density using the relationship given by Mohsenin (1986).

Seed coat surface study

Dry seed was mounted on double adhesive carbon coated tape on an aluminum stub. The sample was coated with gold using Polaron E5001 scanning electron microscope coating system. The coated sample was viewed by SEM (SEM LEO 435VP, Carl Zeiss, Cambridge, UK) at 20 kV and 35 mm photography with digital imaging 1000x resolution.

Proximate composition of seeds

All the extractions and analysis have been carried out at least thrice and value reported along with the mean deviation. All the solvents used in the extraction and analysis purpose were reagent grade and distilled in the laboratory before experiments.

Proximate composition of seeds was determined as per the standard procedures. For moisture content, approximately 1 g of seeds was kept in an oven at 105 °C for 24 h. The loss of weight was indicated the moisture content of the seeds. Crude proteins were determined by nitrogen estimation method by Kjeldahl titration process. Crude fiber was determined by Ceramic fiber filter method (AOAC 962.09 1995). Ash content was determined in laboratory muffle furnace as per ASTM 3174–04 (2004) method. For determination of ash content, fresh seeds (1 g, approx) were taken in a crucible and placed in a muffle furnace at 550 °C for 12 h. After that,

the crucible was removed from the furnace and kept in a desiccator. The difference of weight was the ash content of the seeds.

Minerals in Ash

The minerals were extracted from the ash by adding 20 ml of 2.5 % HCl, heated in a steam bath to reduce the volume (~7 ml) and was transferred quantitatively to a 25 ml volumetric flask with de-ionized water. The analysis of some common elements present in the ash was determined by ICP-MS (PerkinElmer, Optima 5300 V, USA). A standard sample contained metals viz. Ag, Mg, Al, B, Ba, Bi, Ca, Mn, Fe, Cu, Zn, Sr, Cd, Co, Cr, Ga, Ir, K, Li, Na, Ni, Pb, Tl and Zn were used for calibration (signal intensity vs mass to charge ratio). A full scan m/z 40–250 was carried out for quantification study.

Condensed tannin content in the seed-coat

The total condensed tannin was determined using the colorimetric approach of modified vanillin-hydrochloric acid assay developed by Hagerman and Butler (1981). A calibration curve of catechin was prepared and the result was determined from regression equation of calibration curve (y=0.412x+ 0.068, R^2=0.968). When methanol used as an extraction solvent, the value was expressed as mg equivalent per ml of methanol. Dry powered seeds (40 mg) were suspended in methanol and then centrifuged. The supernatant was mixed with 5 ml of working vanillin-HCl reagent (one part vanillin solution and one part 8 % HCl solution in methanol). The solution was kept in incubator at 30 °C for 20 min and then absorbance was recorded at 500 nm on UV–VIS (Shimadzu, UV-1601, Japan). The value obtained was compared with the standard curve obtained from catechin equivalent.

Extraction of the fatty oils, bound lipids and total lipids

For isolation of fatty oil, the grounded seeds (200 g) was taken in a soxhlet extraction apparatus and extracted with hexane for 10 h. Then, the solution was filtered and followed by removal of solvent in a rotary evaporator at 40 °C and 256 mbar pressure. The oil samples were kept at 5 °C in a refrigerator for further analysis.

For isolation of bound lipids, 100 g of seed cake (after hexane extraction) was taken in a soxhlet extraction apparatus and extracted in butanol saturated with water for 10 h. Then, the solution was filtered and followed by removal of solvent in a rotary evaporator at 75 °C and 256 mbar pressure. The samples were kept at 5 °C in a refrigerator for further use.

For total lipids extraction from seed and seed cake, the grounded seeds (100 g) and 150 g of seed cake (after hexane extraction) were taken in soxhlet extraction apparatus and extracted with chloroform-methanol (2:1) for 10 h. Then, the solution was filtered and followed by removal of solvent in a rotary evaporator at 45 °C and 256 mbar pressure. The lipids samples of seeds and seed cake were kept at 5 °C in a refrigerator for further use.

The total lipids obtained from fresh seeds and seed cakes were separated into their corresponding neutral, glyco- and phospholipids by column chromatographic method. A column was set up by using 50 g of silica gel (100–200 mesh) in chloroform. Total lipids (5 g) was initially adsorbed in silica gel and then loaded into the column. Then, column was run in chloroform, acetone and methanol, successively. The fractions were concentrated in rotary evaporator under *vacuo* to obtain neutral, glyco- and phospholipids. Similarly, neutral, glyco- and phospholipids were isolated from fatty oil (10 g) and all the lipids were kept in refrigerator for further analysis.

Physico-chemical properties of fatty oil

Refractive index and specific gravity

Refractive index was determined by ATAGO refractometer (RX 7000 α) at 20 °C and specific gravity was measured by using Specific Gravitymeter (DA 500) at 20 °C.

Acid value, saponification value, unsaponification value and iodine number of fatty oil

The acid value, saponification value, iodine value and unsaponification value were determined using titration methods (IS: 586, 1986). Unsaponification value is the measure of non-lipids constituents (sterols, pigments, and hydrocarbons) in the oil. So, we have isolated unsaponifiable matter from 5 g of oil as per the procedure (Bodger et al. 1982).

GC-FID and GC/MS analysis of fatty acid methyl esters and unsaponified matter

The compositions of the oils were determined by GC-FID and GC/MS. The oils were converted to corresponding fatty acid methyl ester (FAME) as per our previous reported procedure (Sahoo et al. 2003; Rout et al. 2014).

GC analysis of FAMEs were carried out on a Agilent 4890D Gas Chromatograph equipped with a flame ionization detector (FID) using a polyethylene glycol coated FSCAP column (30 m × 0.25 mm × 0.25 μm film thickness; Supelcowax). Hydrogen was used as the carrier gas at column head pressure of 20 psi. Each sample (0.2 μl) was injected into the injection port of the GC using a split ratio of 50:1.

Temperature of the injector and detector was kept at 250 °C. Compound separation was achieved following a linear temperature program of 160 °C (1 min), 160 to 240 °C (2 °C/min), 240 °C (10 min), so the total run time was 51 min. Each sample was analyzed twice in GC; thus a total of six GC analyses were performed for extracts of all processes. Peaks were identified by co-elution of standard methyl ester samples procured from Sigma-Aldrich in the same GC conditions. GC/MS utilized a PerkinElmer autosystem XL GC interfaced with a Turbomass Quadrupole mass spectrometer based on the above oven temperature program. Injector, transfer line and source temperatures were 250 °C; ionization energy 70 eV; helium at 10 psi constant pressure; and mass scan range 40–500 amu. Characterization was achieved on the basis of retention time, elution order, calculated relative retention index using a homologous series of n-alkanes (C_{10}-C_{32} hydrocarbons, Polyscience Corp. Niles IL), mass spectral library search (NIST/EPA/NIH version 2.1 and Wiley registry of mass spectral data 7th edition). In similar procedure, the neutral lipids fractions were converted to corresponding methyl esters and analyzed by GC-FID and GC/MS. The peak identification is further confirmed by their calculated relative retention indices.

Similarly, the unsaponified material was dissolved in ether and subjected to GC-FID and GC/MS analysis. A same PerkinElmer GC, fitted with an Equity-5 column (60 m x 0.32 mm i.d., film thickness 0.25 μm) was used. The column oven was programmed from 160 °C to 240 °C at a rate of 2 °C/min, with initial and final hold times of 1 and 10 min, respectively, using hydrogen as carrier gas at a constant pressure of 10 psi, split ratio of 1:35, injector and detector (FID) temperatures were set up at 330 °C. Major peaks were identified by co-elution of standard samples procured from Sigma-Aldrich in the same GC conditions. GC/MS analysis was carried out on same PerkinElmer machine interfaced with a Turbomass Quadrupole mass spectrometer fitted with the same column and temperature programmed as above.

Calculation of relative retention indices (RRI)

RRI of the peaks was calculated as per our earlier publication (Rout et al. 2007). A standard mixture of normal saturated hydrocarbons (C_{10} to C_{32}) was injected in GC-FID under the same conditions of sample analysis and the retention times of these separated hydrocarbons were recorded. It observed that, RTs of saturated hydrocarbons follow a linear trend on a logarithmic scale and the values were multiples of 100.

^{1}H-NMR analysis

A Bruker Advance-300 was utilized for ^{1}H-NMR experiments with tetramethylsilane as an internal standard. About 8 mg of the sample was dissolved in $CDCl_3$ and spectral data were recorded.

TLC analysis

TLC analysis had been carried out as per the literature reported method (Khor and Chan 1988). The glyco- and phospholipids were analyzed by TLC for identification of compounds. Compounds were identified as per their R_f value and compared to the standard TLC analysis. The TLC was carried out by using solvent mixture diethyl ether/acetone/formic acid/water (40:50:1:0.5). Two dimensional TLC analyses had been carried out for better resolution and further information. For two dimensional TLC, dimension one was run by using chloroform/methanol/ammonia (65:30:4) and dimension two was run by using chloroform/methanol/acetic acid/water (170:25:25:6).

Results and discussion

Physical properties

Seeds are commonly orbicular and measure (0.01–0.3) mm in SEM analysis (Online Resource). Multi-reticulate finely and coarsely interwoven cells were detected in seed-coat (Fig. 1b and c). The seed coat on either side of hilar region showed a different structure than that of hilum. As per Varriano-Marston et al. (1983), this was due to the presence of a single-layer of sclerides followed by columnar cells and crushed parenchyma cells underneath the seed-coat.

There were few seed-varieties of *P.tetragonolobus* contrasting in seed-coat colour from white to dark-brown. Colour of the seed-coat was directly proportional to the amount of condensed tannin content in the seed (Hagerman and Butler 1981). The amount of condensed tannin varied from (0.03–5.29) mg/g dry wt in testa of the seed coat. Condensed tannin, the polymers of flavan-3-ols might be forming complex chemically or physically with proteins (Hagerman and Butler 1981).

The average moisture content of the seed was 7.98± 0.09 %. The moisture was important for processing of seeds. The knowledge on seed morphology and size distribution is essential for adequate design of equipments for cleaning, grading and separation. About 70 % of seeds were medium sized with length ranging from 6.66 to 8.01 mm, while about 11 % and 19 % were large size (L>8.01 mm) and small size (L<6.66 mm) seeds, respectively (Online Resource). The average seed length, breadth and thickness were 7.34, 6.88 and 6.28 mm, respectively (Table 1).

The seed shape was determined in terms of its sphericity and aspect ratio. The sphericity and aspect ratio of seeds are measured 0.93 and 93.94 %, respectively (Table 2). Garnayak et al. (2008) have reported that, the grain considered spherical when the sphericity value was more than 0.70. In the present study, shape indices signified that *P. tetragonolobus* seed can be treated as a sphere. Considering the aspect ratio (relates the seeds breadth to length) and sphericity, it deduced that these seeds would roll freely on flat surfaces rather than slide. This tendency to either roll or slide is very important in designing of hoppers and dehulling equipments. Calculated surface area was 146.9 mm^2, which related to determining the shape of the seeds. Further, it designated how seeds behave on oscillating surfaces during processing.

Seed unit mass or one thousand seeds weight is useful in determining the equivalent diameter for theoretical estimation of seed volume, which helps to calculate aerodynamic forces requires in cleaning. Gravimetric properties like bulk and true density were used for designing the equipments related to aeration, drying, storage and transport. The bulk density and true density of seeds are calculated as 861.58 and 1162.95 kg/m^3, respectively (Table 2). These parameters are decided the storage and transport capacity of seeds. Porosity of the seeds was 25.68 %; this characteristic helps in remove the heavier foreign materials from seeds. It must be noted that porosity in seeds determines the airflow resistance during aeration and drying process.

Proximate analysis and ash content of seeds

The nitrogen content in the seed was 4.6±0.1, so the proteins percentage is 28.8 % (Table 2). However, its proteins and fat content was matched with soya bean (Cerny et al. 1971). Moreover, this is a possible potential source of vegetable proteins for supplementary feeding, thus seed might be used as a potential candidate in nutritional industry. Finally, total carbohydrate (32.2 %) in the seed was determined by percentage difference of moisture, ash, fat and fiber content as presented in Table 2. The seeds contained 30 mg of ash in 1 g of seed. The elemental analysis of ash indicated that, it contained significant amount (ppm) of K (13070.3±3.3), Mg (2207.2± 1.8), Fe (24.2±0.5), Mn (20.3±0.8), Sr (18.2±0.5), Cu (17.1± 0.6), B (15.7±0.5), Ba (14.0±0.5), Zn (7.0±0.4), Al (6.3±0.4) and Cr (4.1±0.3). These elements are very important micro nutrients, which might be recycled in soil for fertility. The elements such as Cd, Co, Ga, Li, Ni and Pb were present less than 1 ppm, whereas the elements like Al, Bi, Ca, Ir, Na and Tl were not detected in ICP-MS analysis.

Physico-chemical properties of seed oil

The hexane extracted fatty oil in mature seed was 13.6 (wt%). The acid value of the oil was 1.2 mg KOH/g oil. The saponification and unsaponification value of the oil were 172.6 mg KOH/g oil and 0.25 %, respectively. The iodine value of the oil was 127.7 g KOH/100 g oil. Iodine value indicated that, the oil contained more unsaturated fatty acids (USFA) and it did not solidify at room temperature. The ester value (171.4 mg KOH/g oil) is determined by the difference between saponification value and acid value.

Table 1 Size distribution of *P. tetragonolobus* seeds at moisture content of 7.98 % (w.b.)

Particulars	Size category			
	Ungraded	Small	Medium	Large
Length of seed, mm	5.90-9.00	<6.66	6.66-8.01	>8.01
Percentage of sample (by Number)	100	19	70	11
Average dimensions				
Length (L), mm	7.34 (±0.67)	6.37 (±0.19)	7.41 (±0.37)	8.53 (±0.31)
Breadth (B), mm	6.88 (±0.56)	6.13 (±0.25)	6.95 (±0.40)	7.71 (±0.22)
Thickness (T), mm	6.28 (±0.52)	5.77 (±0.32)	6.34 (±0.48)	6.85 (±0.17)
Arithmetic mean diameter, mm	6.88 (±0.55)	6.09 (±0.23)	6.90 (±0.37)	7.69 (±0.17)
Geometric mean diameter, mm	6.82 (±0.54)	6.08 (±0.23)	6.88 (±0.37)	7.66 (±0.17)

Calculation

The theoretical average molecular weight of the oil can be calculated from the following equation:

$$\text{Molecular weight} = (3000 \times 56.1/\text{ester value}) \qquad (1)$$

The calculated molecular weight is 980.1

Similarly, iodine value helped to calculate the theoretical average number of double bonds in one gram of oil and double bond per molecule.

$$\text{Number of double bonds in one gram of oil} = (\text{Iodine value}/126.9 \times 2) \qquad (2)$$

The double bond exist in one gram of oil is 5.0 mmole (approx). The average number of double bonds in one triglyceride molecule can be calculated from the following equation.

$$\text{Double bonds per molecule} =$$
$$(\text{no of double bonds in 1 g oil/no of mmoles of triglyceride}) \qquad (3)$$

The average no. of double bonds per molecule is 5.1. Calculated average number of double bonds indicated that the *P. tetragonolobus* oil contained more number of double bonds in the fatty acid chain in compared to the saturated fatty acids (SFA).

Composition of fatty oil

The fatty acid composition of the oil is presented in Table 3. The yield of oil was higher in fully mature seeds (13.6 %) in compared to the mature seeds (10.3 %). In total, more than 97 % of the fatty acids have been identified. The GC-FID chromatogram of fatty acids is presented in Fig. 2. There were five USFA detected in fully mature seed oil, which comprised

Table 2 Physical properties and proximate composition of *P. tetragonolobus* seeds

Physical properties	N	Mean	Minimum	Maximum	SD
Moisture content (w.b.), %	5	7.98	7.89	8.07	0.09
Oil Content, %	5	13.62	13.25	13.99	0.37
Sphericity	100	0.93	0.84	1.00	0.03
Aspect ratio, %	100	93.94	82.22	100.0	4.10
Unit mass, g	100	0.246	0.165	0.401	0.05
1000 seed weight, g	20	241.76	221.32	261.28	12.06
Surface area, mm^2	100	146.90	99.60	197.38	23.31
Bulk density, kg/m^3	10	861.58	843.67	892.03	26.51
True density, kg/m^3	10	1162.95	1101.89	1238.93	66.85
Porosity, %	10	25.68	24.03	28.00	2.07
Proximate composition					
Crude proteins	3	28.8	28.4	29.2	0.4
Crude fiber	3	6.5	6.2	6.8	0.3
Ash	3	4.3	4.1	4.5	0.2
Fat	3	16.8	16.5	17.1	0.3

N is the number of samples

Table 3 Fatty acids composition determined by GC-FID and GC/MS of different genotypes of *P. tetragonolobus* seeds

Fatty acids	Immature seeds	Mature seeds	Fully mature seeds	RRI cal[a]
Oil yield (%)	4.5±0.3	10.3±0.3	13.6±0.4	
Luric acid (12:0)	0.5±0.2	0.2±0.1	0.1	2009
Myristic acid (14:0)	1.0±0.3	0.4±0.2	<0.1	2106
Palmitic acid (16:0)	37.8±1.5	16.8±0.7	7.4±0.1	2210
Heptadecanoic (17:0)	2.1±0.4	0.2±0.1	<0.1	2307
Stearic (18:0)	14.5±1.0	8.8±0.4	5.3±0.3	2410
Oleic (18:1)	16.5±1.2	31.7±1.1	35.1±0.6	2433
Elaidic (18:1)	<0.1	<0.1	-	2450
Linoeic (18:2)	13.3±1.0	26.0±0.8	35.3±0.5	2480
Linolenic (18:3)	4.4±0.8	2.7±0.5	1.6±0.2	2539
Arachidic (20:0)	1.3±0.2	1.1±0.3	1.2±0.2	2606
Eicosenoic (20:1)	1.3±0.3	1.7±0.4	3.0±0.2	2625
Behenic (22:0)	2.4±0.6	5.1±0.7	7.0±0.4	2808
Erucic (22:1)	-	0.2±0.1	0.5±0.1	2825
Lignoceric (24:0)	1.4±0.3	1.5±0.3	0.8±0.2	2904
Cerotic (26:0)	0.3	0.6±0.3	0.1	2996
SFA[b]	61.3	34.7	21.9	
MUFA[c]	17.8	33.6	38.6	
PUFA[d]	17.7	28.7	36.9	
Total	96.8	97.0	97.4	

[a] Relative Retention Index Calculated, [b] Saturated fatty acids, [c] Monounsaturated fatty acids, [d] Polyunsaturated fatty acids

75.5 % of total oil composition. The percentage of USFA such as oleic (35.1 %), linoleic (35.3 %), eicosenoic (3.0 %) and erucic (0.5 %) were significantly higher in fully mature seeds. The percentage of SFA such as lauric (0.1 %), myristic (<0.1 %), palmitic (7.4 %), heptadecanoic (<0.1 %), stearic (5.3 %), lignoceric (0.8 %) and cerotic (0.1 %) were poor in fully mature seeds. It observed that linolenic acid, which was an important fatty acid recovered slightly less percentage in fully mature seeds (1.6 %), whereas present improved percentage in mature seeds (2.7 %) and immature seeds (4.4 %). Thus, decrease of linolenic acid contain with aging seeds was agreed with the earlier finding of Khor and Chan (1988). On contrary, the improved yield of fatty oil was obtained in fully mature seed, thus the total amount of linolenic acid was nearly close in compared to mature and immature seeds. The percentage of SFA, mono unsaturated fatty acid (MUFA) and poly unsaturated fatty acid (PUFA) are presented in the same table. It was observed that, SFA was in less percentage in fully mature seeds (21.9 %) and it gradually increased from mature seeds (34.7 %) to immature seeds (61.3 %). On the other hand, the MUFA (38.6 %) and PUFA (36.9 %) recovered improved percentage in fully mature seeds. The USFA identified in fully mature seeds was 75.5 %, whereas it was 62.3 % in mature seeds. The USFA was in very less percentage (35.5 %) in

immature seeds. The yields of fatty oil in fully mature, mature and immature seeds were 13.6, 10.3 and 4.5 %, respectively. Thus the amount of USFA in fully mature seeds was significantly higher as compared to the mature and immature seeds. On the other hand, parinaric acid (9, 11, 13, 15-octadecatetraenoic acid) was absent in our analyzed Indian origin winged bean. Cerny et al. (1971) have reported the high percentage of (2.5 %) parinaric acid (18:4) in fatty acid composition of *Psophocarpus palustris* (winged bean) seed oil. Parinaric acid is a toxic fatty acid and a potential anti-nutritional compound.

^{1}H-NMR analysis of fully mature, mature and immature seed oils are presented in Fig. 3. The oil is composed of fatty acids and esters of glycerol. The ^{1}H-NMR spectra of oils are characterized by overlapping signals originating from various fatty acids. In respect to the chemical similarity of different triglyceride esters, the signals resonate close together and build different clusters. The *P. tetragonolobus* oil composed of nine main signal clusters (Table 4). The observed chemical shift at δ: 5.2–5.4 ppm can be assigned to olefinic protons, which corresponding to USFA. The chemical shift at δ: 4.1–4.3 ppm arises from glycerol protons, thus did not give much information. The chemical shift at δ: 3.3-3.7 ppm arises from diglycerides. The chemical shift at δ: 2.7–2.8 ppm is due to protons attached to the bis-allylic carbons. Signals at δ: 2.2–2.3 ppm can be attributed to the methylene adjacent to the carbonyl groups. The chemical shift at δ: 2.0–2.1 ppm is due to protons attached to the allylic carbons. The chemical shift at δ: 1.6–1.7 ppm is due to β-carboxyl group. The signals due to further CH_2 groups are seen at δ: 1.2-1.4 ppm. The signals corresponding to terminal methylene and methyl hydrogens are appeared at δ: 0.97 and 0.87 ppm, respectively.

The chemical groups assigned in Table 4 gave separate signals, so that it is not possible to determine all single fatty acid components. But, it is possible to calculate the unsaturation proportion from signals (δ: 5.3, 2.0 ppm), which corresponds to the iodine number value (Sacchi et al. 1996). Furthermore, the proportion of linolenic acid can be calculated by consideration of the signal at δ: 0.97 ppm. The amount of PUFA can be determined from the signal δ: 2.8 ppm. The content of MUFA and SFA can also be calculated using various signals (δ: 2.3, 2.0, 0.87 ppm). Finally, three groups of relative integral of signals δ (5.3, 2.0), (2.8, 0.97), 1.6 were chosen, which reflected the amount of USFA, PUFA and fatty acid, respectively.

Therefore, it is clear from the results that the fully matured seeds contain more percentage of USFA and less SFA in comparison to mature and immature seeds. The results indicated that, fully mature seeds contained more USFA, corresponding to the signals appeared at δ: 5.3, 2.8, 2.0 and 0.97 ppm. On the other hand, fully mature seeds contained less SFA, which corresponded to the signals appeared at δ: 1.6 and 0.87 ppm. The triglycerides were in better percentage in
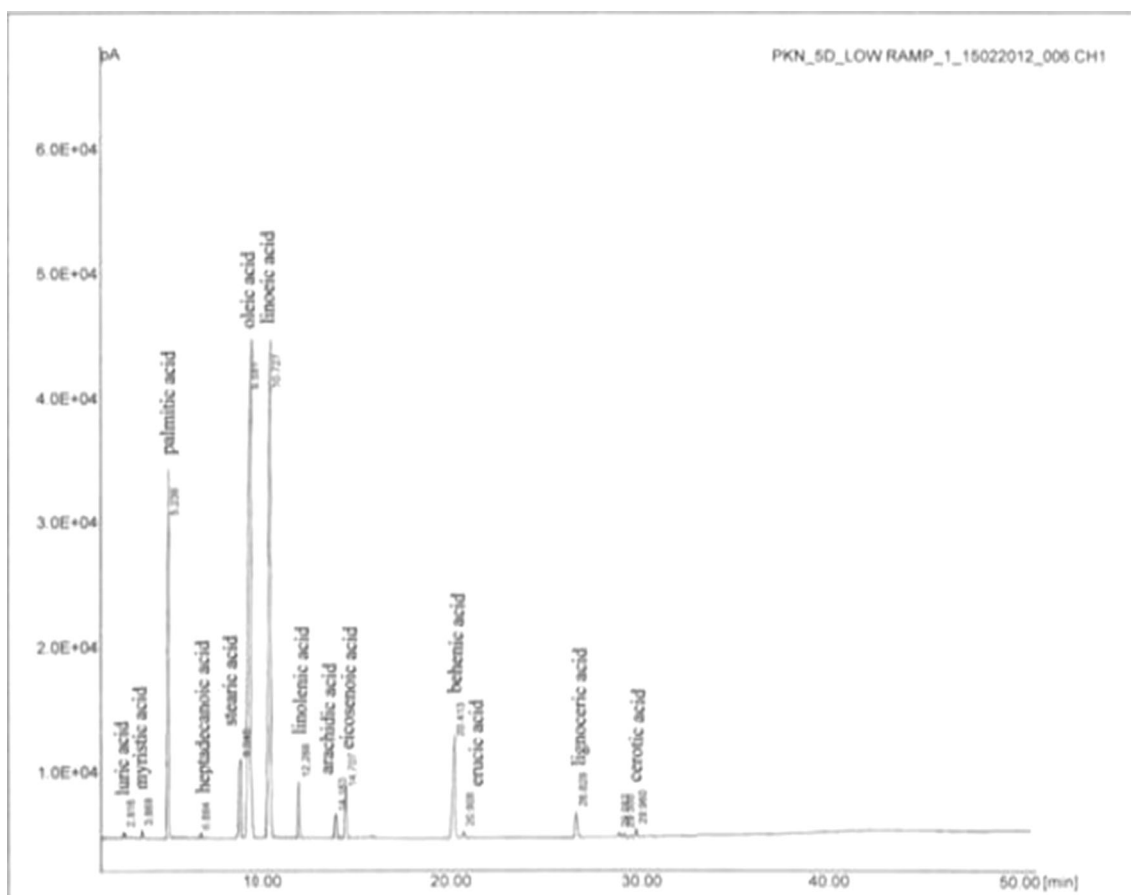
Fig. 2 GC-FID chromatogram of fatty oil

fully mature seeds, whereas diglycerides were detected in the early stage of the seed oil. Similarly, linolenic acid was presented in higher percentage in immature seeds, the results also agreed with the GC-FID analysis.

The oil gave negative Halphen test (AOAC 974.19 1995), which indicated the absence of cyclopropene acids. The absence of epoxy fatty acids was determined by HBr titer value according to Critchfield (2007). The fatty acid composition and wet chemical analysis indicated that, the oil did not contain any un-usual fatty acids. The only trans-fatty acid viz. elaidic acid was detected (<0.1 %) in oil recovered from early stage of seeds. Interestingly, this fatty acid was absent in mature seeds. On the other hand, high percentage of this trans-fatty acid was not recommended in food products. Thus, *P. tetragonolobus* oil is safe for consumption and suitable for edible purposes.

The refractive index (RI) of the oil extracted from fully mature seed was 1.46±0.005. The refractive index of oil depends on their molecular weight, fatty acid chain length, degree of unsaturation, and degree of conjugation. Triacylglycerols have higher refractive indices than do their constituent free acids. Refractive index values of edible oils generally vary in between 1.447 and 1.482. The obtained RI in between the standard values of major components such as glycerol

(1.473), oleic acid (1.459) and linoleic acid (1.466). The specific gravity of the oil was 0.915±0.005; the value came within the range of common edible oil (0.91–0.94).

The total lipids isolated from the *P. tetragonolobus* seed, hexane extracted seed cake and hexane extracted fatty oil were taken for further study. The total lipids were fractioned into neutral, glycol- and phospholipids. The compositions of neutral lipids of each category along with the fatty acid composition of bound lipids are presented in Table 5. The neutral lipids isolated from hexane extracted oil contained poor percentage of palmitic acid (7.7 %) and behenic acid (7.3 %). On the other hand, neutral lipids of oil contained improved percentage of oleic acid (35.2 %), linoleic acid (35.4 %), linolenic acid (1.9 %), eicosenoic acid (3.1 %) and erucic acid (0.7 %) in comparison to the neutral lipids of seeds and cake. The SFA, MUFA and PUFA of neutral lipids fractions are presented in the same table. The neutral lipids isolated from oil contained poor percentage of SFA (22.1 %), whereas improved percentage of MUFA (39.0 %) and PUFA (37.3 %) in comparison to the neutral lipids isolated from seeds and cake. The bound lipids contained higher percentage of palmitic acid (12.0 %), oleic acid (35.6 %), elaidic acid (1.7 %), linoleic acid (34.6 %) and linolenic acid (2.6 %), whereas total yield was very poor (0.23 %). The elaidic acid was not detected in neural lipids
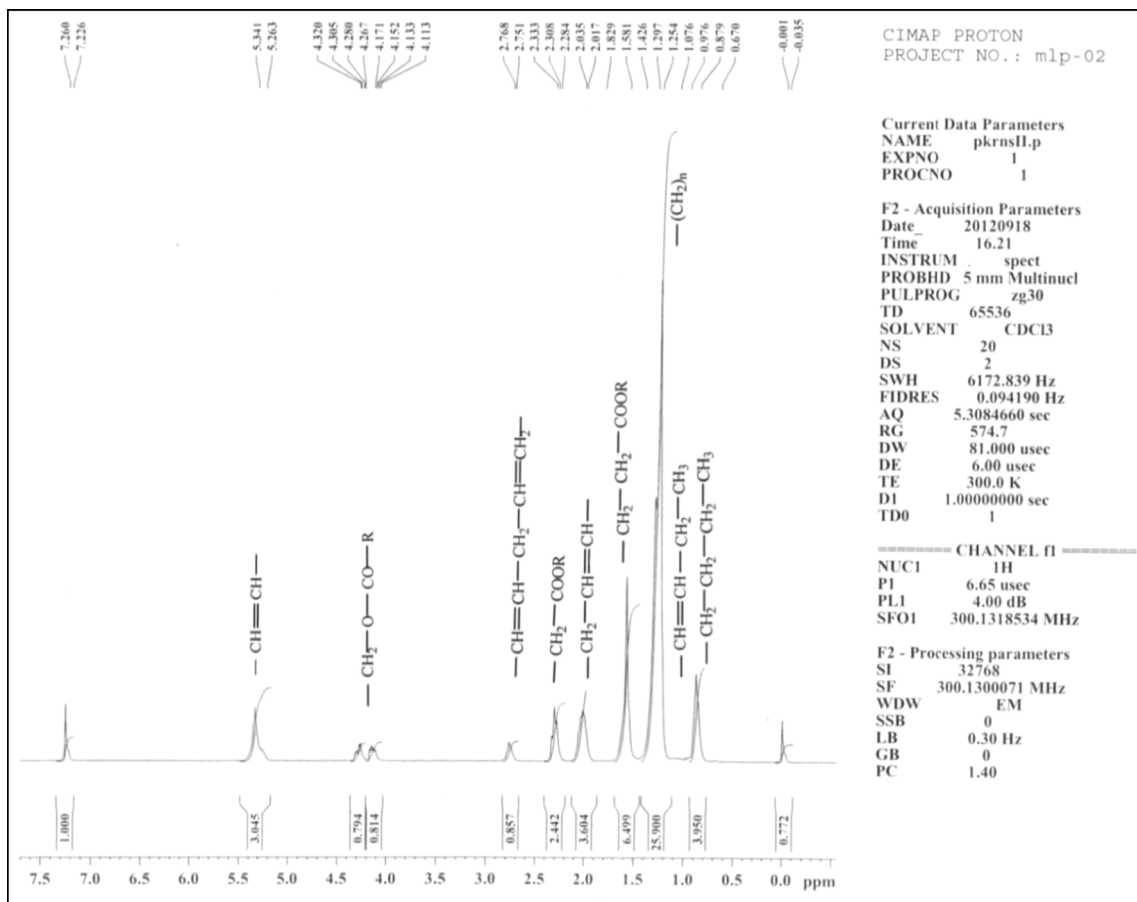
Fig. 3 ¹H-NMR spectrum of fatty oil

isolated from oil, whereas it was present significant percentage (1.2–1.7 %) in neutral lipids of seeds (1.2 %), cake (1.5 %) and also in bound lipids (1.7 %).

Bound lipids contain improved percentage of USFA (77.4 %) such as oleic, elaidic, linoleic and linolenic as presented in Table 5. On the other hand, fewer percentage of SFA

(20.0 %) was detected in bound lipids in compared to the extractable lipids. So, it concludes that, USFA are more bound in nature and it might be complexed chemically or physically with carbohydrate or proteins. Therefore, a part of USFA having bound temperament and could not be readily extracted with non-polar solvents (hexane).

Table 4 Fatty acids composition determined by ¹H-NMR of different genotypes of *P. tetragonolobus* seeds

δ ppm	Immature seeds (%)	Mature Vseeds (%)	Fully mature seeds (%)	Proton types	Compounds
5.3	1.7±0.04	4.7±0.05	6.4±0.05	olefinic	unsaturated fatty acids
4.3	1.1±0.03	1.1±0.02	1.7±0.02	glycerol	triacylglycerols
4.1	1.0±0.02	1.2±0.02	1.7±0.03	glycerol	triacylglycerols
3.7	4.0±0.03	3.0±0.04	-	-	diglycerides
3.3	1.6±0.02	-	-	-	diglycerides
2.8	0.2±0.01	1.2±0.2	1.8±0.02	diacyl	linolic acid
2.3	5.0±0.04	4.9±0.05	5.0±0.05	α-carboxyl	acyl chains
2.0	3.5±0.06	6.3±0.1	7.5±0.1	α-olefinic	unsaturated fatty acids
1.6	25.0±0.2	19.2±0.2	14.5±0.1	β-carboxyl	acyl chains
1.3	48.2±0.2	50.3±0.3	53.2±0.3	methylene groups	acyl chains
0.97	0.2±0.1	0.1	<0.1	methyl groups	linolenic acid
0.87	8.3±0.2	7.8±0.1	7.9±0.1	methyl groups	All acyl chains except linolenic
Total	99.8	99.8	99.8		

Table 5 Total lipids composition of *P. tetragonolobus* fully mature seeds

Lipids	Total lipids of seeds	Total lipids of cake	Fatty oil	Bound lipids
Yields (%)	16.8±0.3	3.6±0.4	13.6±0.4	0.23±0.03
Lipids fractions	NL[a]	GL[b]	PL[c]	NL[a]
GL[b]	PL[c]	NL[a]	GL[b]	PL[c]
Yields (%)	86.5	6.1	7.3	12.5
39.8	47.7	97.4	1.9	0.7
Fatty acids compositions	NL of total lipids of seeds (%)	NL of total lipids of cake (%)	NL of total lipids of oils (%)	NL of bound lipids (%)
Luric acid (12:0)	0.1	0.2±0.05	0.1	0.1
Myristic acid (14:0)	<0.1	0.1	0.1	0.1
Palmitic acid (16:0)	12.7±0.3	14.7±0.4	7.7±0.3	12.0±0.5
Heptadecanoic (17:0)	0.1	0.2±0.05	0.1	0.2±0.05
Stearic (18:0)	4.1±0.3	4.7±0.3	5.0±0.4	4.4±0.5
Oleic (18:1)	29.8±0.9	29.4±1.1	35.2±1.3	35.6±1.5
Elaidic (18:1)	1.2±0.2	1.5±0.4	-	1.7±0.4
Linoeic (18:2)	32.2±1.6	30.8±1.5	35.4±1.2	34.6±1.4
Linolenic (18:3)	1.8±0.2	1.7±0.3	1.9±0.2	2.6±0.2
Arachidic (20:0)	1.0±0.2	0.8±0.1	1.1±0.2	0.1
Eicosenoic (20:1)	2.4±0.2	2.2±0.3	3.1±0.3	2.3±0.2
Behenic (22:0)	8.3±0.5	8.5±0.6	7.3±0.6	2.3±0.2
Erucic (22:1)	0.5±0.1	0.5±0.1	0.7±0.2	0.6±0.1
Lignoceric (24:0)	2.9±0.2	2.9±0.3	0.7±0.3	0.8±0.1
Cerotic (26:0)	0.1	0.2±0.1	<0.1	-
SFA	29.3	32.3	22.1	20.0
MUFA	33.9	33.6	39.0	40.2
PUFA	34.0	32.5	37.3	37.2
Total	97.2	98.4	98.4	97.4

[a] Neutral Lipids, [b] Glyco Lipids, [c] Phospho Lipids

The glycolipids detected in normal TLC analysis were acylstreolglyceride (R_f: 0.1), sterol esters (R_f: 0.9), whereas glycolipids detected in two dimensional TLC were sterolglycoside and digalactosyl diglyceride. The phospholipids were more prominent in two dimensional TLC analyses. The compounds detected were phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine, sulpholipids, phosphatidylinositol, phosphatidylserine. Phospholipids are important biological compounds found in the membranes of organelles (e.g. mitochondria), plant and animal cells, including plasma, mitochondria, chloroplast and bacterial membranes. Phosphatidyl-choline, as well as phosphatidyl-ethanolamine, phosphatidyl-inositol, and glycolipids are listed as components of commercial lecithin (Patil et al. 2010). The non-toxicity of lecithin leads to its use with food, as an additive or in food preparation.

The yield of un-saponifiable material (white solid) was 14 mg from 5 g of the oil. Unsaponified material was analyzed by GC-FID and GC/MS and four compounds were identified. The compounds were 3-β-acetoxy-5-cholenic acid (0.7±0.1 %), stigmasterol (66.4±0.5 %), β-sitosterol (25.1±0.4 %) and stigmasta-3,5-dien-7-one (1.0±0.2 %) (Online Resource). The major compounds were stigmasterol and β-sitosterol comprised more than 90 % of the unsaponified matter. On contrary, a very negligible amount of un-saponifiable material was co-extracted along with the fatty oil. Thus, the hexane extracted oil also needs refining to ensure its safe use in food products (Rout et al. 2014). Over all, the present analysis of *P.* tetragonolobus oil contained 75.5 % USFA and 21.9 % SFA, whereas soybean oil contained around 86 % of USFA and 14 % SFA (Garcia and Palmer 1979). Thus *P. tetragonolobus* fatty oil may be fulfilled a gap of high demand soybean oil.

Conclusions

The present work enumerates the physico-chemical study of *Psophocarpus tetragonolobus* seed and its lipids. The engineering aspects of seeds were evaluated and their importance was highlighted in relation to post harvest processing. The

fully mature seeds contain ~13.6 % of fatty oil and remaining cakes are enriched in proteins (28.8 %) and carbohydrates (33.2 %). Cakes might be used for high quality cattle and poultry feed. The most practiced industrial process for isolation of fatty oil is non-polar solvent (hexane) extraction. The hexane extracted fatty oil agrees with all edible oil characteristics in terms of physico-chemical analysis as well as fatty acid compositions. Elaidic acid and parinaric acid, which are anti-nutritional trans-fatty acids absent in the hexane extracted fatty oil. On the other hand, the fatty oil contained very little percentage (0.25 %) of non-fatty acid compounds such as stigmasterol, β-sitosterol, etc. Thus, hexane extracted oil is suitable only for edible purpose after refining by standard procedure such as alkali-deguming method. Refined oil must be followed all the existing safety norms. The remains left, after hexane extraction i.e. de-fatted seed cake contained improved percentage of proteins, carbohydrate, thus can be effectively utilized as a nutritious food supplement. The total lipids, comprised of glyco- and phospholipids, were identified by standard TLC methods. These glyco- and phospholipids might be used in high value pharmaceutical applications. It is proposed that, the edible oil must be separate out using hexane followed by treatment of cake with chloroform-methanol to recover the glyco- and phospholipids for value addition. Further work on extraction of fatty oils using pressurized solvents is on progress.

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