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ORIGINAL ARTICLE

Comparative analysis of metabolites in contrasting chickpea cultivars

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Abstract

Chickpea (*Cicer arietinum* L.) is a good source of nutrients for animals and human consumption. In the present study, we analyzed the anthocyanin and total phenolic contents in two contrasting (desi and kabuli) chickpea cultivars. The quantification of anthocyanins showed higher amount in desi as compared to kabuli chickpea. The total phenolic contents was estimated in desi and kabuli chickpea using two different solvents (50% Acetone and 70% Methanol extracts) for coverage of all potential phenolic compounds. In continuation, desi chickpea culitvars (himchana and ICC4958) were found to be significantly higher total phenolic contents (in both solvent extracts) as compared to kabuli cultivars (JGK-03 and L-552). Higher phenolic contents was found to be directly correlated to higher anthocyanin contents in desi as compared to kabuli chickpea. The volatile organic compounds were also analyzed using gas chromatography mass spectroscopy technique in both cultivars. The significant compositional differences in volatile organic composition (polar and non-polar) of desi and kabuli cultivars were also found to be noticed using two different solvent extractions (methanol and chloroform). The comparative analysis of volatile organic acids in methanolic and chloroform extracts of desi cultivars (himchana and ICC4958), kabuli cultivars (JGK-03 and L-552) and between desi and kabuli cultivars was also carried out for in-depth understanding of the differential patterns of low molecular weight metabolites. Six metabolites were found to be common in all four selected cultivars in chloroform extracted samples, while four were found to be common in all four selected cultivars in methanolic extracted samples. The remaining detected metabolites are uncommon among different cultivars and represented as cultivar specific signatory metabolites. In conclusion, the present investigation revealed higher anthocyanin and phenolic contents in desi cultivars as compared to kabuli cultivars and differential accumulation of volatile organic compounds in chickpea cultivars. The metabolite alterations among desi and chickpea cultivars could be the potential attribute for diversity, resilience and commercial usuages.

Keywords Metabolites estimation · Chickpea · Desi and kabuli · GC–MS · Lipids · Phenolics · Anthocyanins

Abbreviations

GC	Gas chromatography
DMSO	Dimethyl sulfoxide
MS	Mass spectroscopy
FW	Fresh weight

Introduction

Chickpea (Garbanzo and Bengal gram bean) is considered as the third largest cultivated leguminous crop in the world and majorly grown in Indian subcontinent, Mexico, Australia, East Africa and the Mediterranean basin. Chickpea is good source of nutrients including proteins, carbohydrates, vitamins and minerals and acts as a good source of energy for human and animals (Wallace et al. 2016). The chickpea seed proteins have been documented for their potential role in anti-angiotensin-I converting enzyme, anticancer, anti-HIV-1 reverse transcriptase and antidiabetic (Ye and Ng

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2002). The lectin, carbohydrate binding protein has been characterized from wild chickpea with potential inhibitory action against human cancer cells (Gautam et al. 2018; Gupta et al. 2018). In addition, chickpea has very low glycemic index and contributes to significant reduction in serum total cholesterol (Wallace et al. 2016). Apart from analysis of proteins, carbohydrates and other important nutrient constituents, a limited literature is available for anthocyanins in chickpea. The leaf anthocyanin contents has been found to be an effective indicator for tolerance mechnism in chickpea (Bhasker et al. 2017). In other crops also, anthocyanin helps to mititage the influence of different stress conditions including abiotic and biotic stresses tolerance (Ahmed et al. 2015; Rubal et al. 2018).

On the basis of plant pigmentation in leaf and other parts of chickpea, different culitvars of chickpea easily grouped into two different types, desi and kabuli chickpea. Both chickpea types have also easily differentiate traits including size of seedling vigor, seed and pod sizes, number of seed/pod, seed color and texture, flower color, and others. The desi chickpea cultivar is generally grown in subtropics and adapted to winter sowing, while kabuli variety is cultivated into mediterranean region and adapted to spring sowing. Desi variety is mainly distributed in South and Southeast Asia, and can be cultivated to some extent in Ethiopia, Mexico, and Iran. On the other hand, kabuli chickpea is dispersed mainly in the West Asia and the Mediterranean region, and are well adapted to spring sowing at higher altitude (van der Maesen 1987). Kabuli chickpea is known to have greater tolerance against iron deficiency and is known to own more primary branches than desi chickpea (Hawtin and Singh 1979). The root anatomy of the kabuli chickpea has revealed the quick loss of cortical layers with the presence of large number of wider xylem vessels, which makes kabuli to use more water as compared to desi in addition to less resistance of water flow (Upadhyaya et al. 2008; Purushothaman et al. 2014). Coming to their evolutionary relationship, desi type is considered to be more primitive than kabuli (Gowda et al. 1987). Upadhyaya et al (2008) documented the frequency of common alleles between these two chickpea is in range of 47–54%. Both seem to be having mean gene diversity and kabuli is genetically more diverse than desi type chickpea. Among different recently introduced chickpea varieties, ICCV 10, JG 11 and ICCV 96029 (Desi) and KAK2, JGK 1, ICCV 2 and Vihar (Kabulis) are the most adaptable varieties in peninsular India. The desi and kabuli type chickpea cultivars have evolved in different environments experiencing relatively opposite temperature regimes and therefore greater adaptation to peninsular India and yield stability requires yet more genetic enhancement efforts with more alleles from the desi cultivar (Upadhyaya et al. 2008). The transcriptomic analysis from different cultivars from these two different grouped have also been investigated (Agarwal et al. 2012). A number of SSRs, SNPs and transcription factors, which are, involved in high intra-specific polymorphism between desi and Kabuli chickpea have also been studied (Agarwal et al. 2012). Genetic diversity among chickpea cultivars has also been assessed for several qualitative and quantitative traits (Sewak et al. 2012). In addition, the proteomic approach based study also revealed significant differences among the studied chickpea cultivars (Santos et al. 2017). Differences in their protein contents solely related to the genotypic diversity, varietal characteristics and region of cultivation. Apart from genetic and proteomic diversity among desi and kabuli cultivars, the comparative biochemical analysis was also investigated. Kabuli cultivar was found to be higher protein contents, fiber contents and lower water holding capacity as compared to desi chickpea cultivar (Ghribi et al. 2015; Macar et al. 2017). The neutral detergent fiber, crude fiber and total tannins were found to be higher in desi type chickpea as compared to kabuli chickpea. In contrast, crude proteins, non-fibrous carbohydrates and soluble sugars were found to be higher in kabuli chickpea seed (Maheri-Sis et al. 2008). Amino acid profiling among desi and kabuli chickpea cultivars has also been analyzed (Ghribi et al. 2015). The genetic variations in altered level of metabolites in drought tolerant and sensitive chickpea cultivars have been demostrated using un-targeted metabolite profiling (Khan et al. 2017). Khan et al. also highlighted pool of metabolites which are altered in light of drought conditions. Quantitative profiling of polar priamry metabolites with responses to salinity in two different desi chickpea cultivars has been reported using a developed and validated validated method for primary metabolites (Dias et al. 2015). The major classes of polar metabolites used for quantification in this work are sugars, sugar alcohol, sugar phospahtes and organic acid (Dias et al. 2015). In a different type of work, chickpea aromtic compounds have been identified using GC–MS and tested for quantify orientation behaviour of first instar *Heliothis armigera* (Rembold et al. 1989). Similary, non-targeted metabolite profiling of chickpea-*fusarium* interaction has been documented and identified differential modulation of disease resistance pathways using liquid chromatography mass sepctroscopy (Kumar et al. 2015). However, due to structural diveristy of metabolites, several extraction methods and even instrument protocols has been documented to analyze complex metabolites. GC–MS based metabolite analysis is best suitable for small organic molecules including fatty acids, alcohols and acids (Fiehn, 2016). The flavonoids and isoflavanoids were found to be the main pathogen responsive metabolites in plants including chickpea (Kumar et al. 2015).

Here, we employed the comparative analysis of pigmentation related key metabolites, anthocyanins among desi (Himchana, ICC4958) and kabuli (JGK-03, L-552) cultivars. In continuation, comprehensive investigation of

metabolites among different chickpea cultivars using gas chromatogrpahy mass sepctroscopy (non-targeted metabolomics approach) was carried out that has not been performed till now with our best knowledge. In addition, we also demonstrated the applicability of two different solvents for extraction of metabolites. The pairwise comparison of different cultivars using different extraction solvents was also anlyzed. A large number of polar and non-polar metabolites also offers significant benefits of identification of volatile organic components in desi and kabuli cultivars. The anthocyanins content was found to be higher in desi chickpea as compared to kabuli chickpea and could be possible responsible for resistance tolerant ability of desi chickpea. In addition, it could be one of the important constituent of desi chickpea leaves that imparts the astrigency taste due to which it used widely as green vegetables. A few metabolites were also found to be cultivar specific and remaining are common among all selected cultivars.

Materials and methods

Selection of chickpea cultivars

Four chickpea cultivars, two desi (ICC4958 and Himchana) and two kabuli (JGK-03 and L-552) were used in the present study. The seeds of three chickpea cultivars were sowed in the field area of university campus during Nov 2017 (first week). The seed samples were harvested during the month of March in 2018. The seeds of L-552 were harvested during April, 2019 at Punjab Agriculatural University, Regional Research Centre, Bathinda. The seeds of different cultivars were photographed (Fig. 1).

Histology

Samples of seeds from late maturation stage were harvested from different cultivars (ICC4958 as desi and JGK-03 as kabuli) were harvested and fixed with FAA (Formaldehyde: Ethanol: Glacial acetic acid in ratio 1:1:18) for 24 h. Subsequently, samples were dehydrated in series of ethanol dilutions with internal of time. The dehydrated samples were given xylene infiltration in series of treatments and finally infilteration was carried out for preparation of wax blocks. Sections were prepared with 25 µm using Rotary Microtome machine and stained with methylene blue-safranin method. The selected slides among different replicates were observed under Olympus® CX series microscope at 4 × and 10 × resolution. Images of selected samples were captured using Cyber-Shot™ digital camera. The selected pictures from desi (ICC4958) and kabuli (JGK-03) cultivars were recolored to adjust the pictures for clarity of content (Fig. 2).

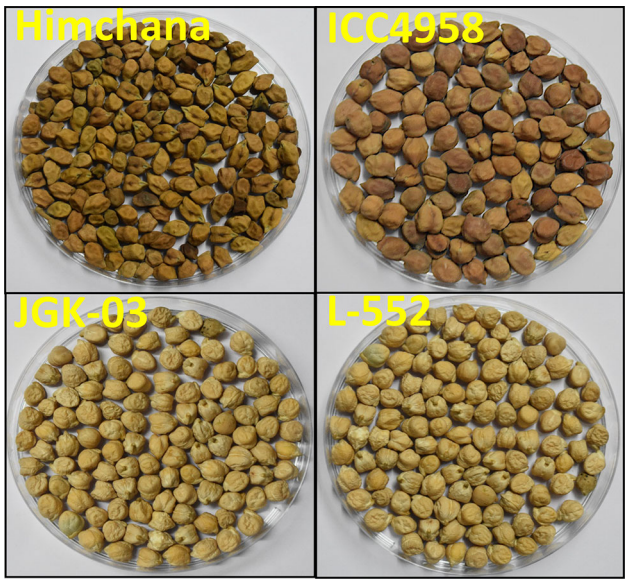

Fig. 1 The seed shape and seed color among different selected chickpea is easily distinguished

Estimation of anthocyanins content

The flowers from desi and kabuli cultivars were harvested from fully matured ramdomly selected chickpea plants and

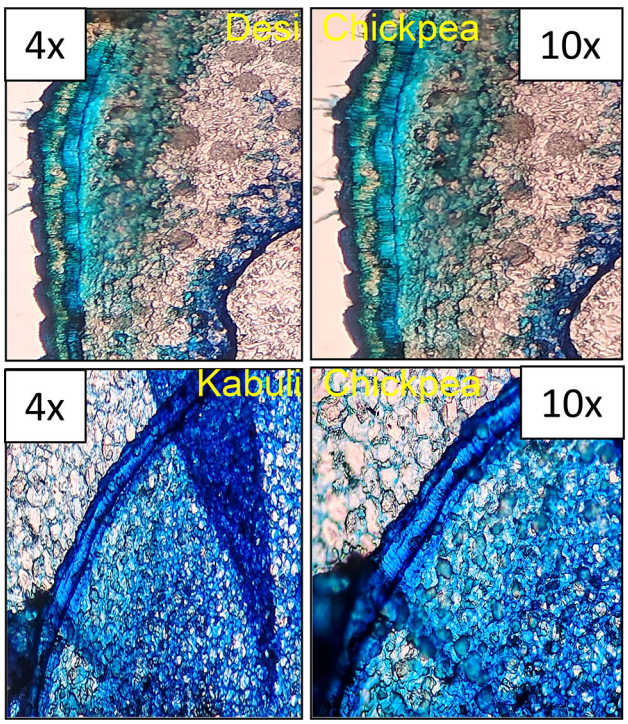

Fig. 2 The differences in anatomoy of seed coat of desi and kabuli cultivars. Both ×4 and ×10 are shown here

photographed. The anthocyanins content was estimated from selected flowers and separated pedicels. For estimation of total anthocyanins, three different developemtnal stages of leaf were selected to calculate overall accumulation of anthocyanins in foliar tissues of chickpea. The samples for anthocyanins estimation were collected from plant grown in growth room during the month of April, 2018 (Himchana, ICC4958 and JKG-03) and May, 2019 (L-552). The first emerged leaf was considered as the first leaf. With reference to first leaf, 3rd, 5th and 7th leaves were selected for the collectively estimation of total anthocyanins among different selected cultivars (Fig. 3).

The samples were prepared by simple washing with distilled water and freeze dried. Then 100 mg of freeze dried powder was homogenized in 5 ml solution (95% methanol: 1NHCl in 9:1 ratio). Samples were incubated at 4°C, for 4 h and centrifuged at 10,000 rpm for 30 min duration. The supernatants were used for measurement at 530 nm and 657 nm by using a UV double beam spectrophotometer (UV-1800, Shimadzu, Japan) (Yin et al. 2012; Kumar et al. 2013) and further calculation was carried out using given formula as

$$Qanthocyanin = (A_{530} - 0.25 \times A_{657}) \times (Weight\ of\ the\ plant\ tissue\ used)^{-1}$$

$Q_{anthocyanin}$ is the corrected value of absorption correlated linearly with the concentration of anthocyanins as described earlier by Yin et al (2012). All samples from different cultivars were analyzed in biological triplicates.

Estimation of total phenolic contents

For the estimation of total phenolic contents in chickpea seeds, healthy and uniform shaped seeds among different cultivars were selected and grinded individually into fine powder with liquid nitrogen. 10 mg of powdered samples were dissolved in two different solvents (50% acetone and 70% methanol) separately and extracted as details provided by Segev et al. (2010, 2011). Samples were mixed and incubated overnight for complete extraction. Then samples were centrifuged at 3000 rpm for 10 min. By taking supernatant, Folin–Ciocalteu assay was performed for the total phenolics estimation (Xu and Chang 2007). Sample were analyzed at 765 nm in UV spectrophotometer. Total phenolic contents was calculated as gallic acid equivalents (mg of GAE/gm sample) using the standard calibration curve (y = 0.2119x − 0.0327 and R^2 = 0.9882).

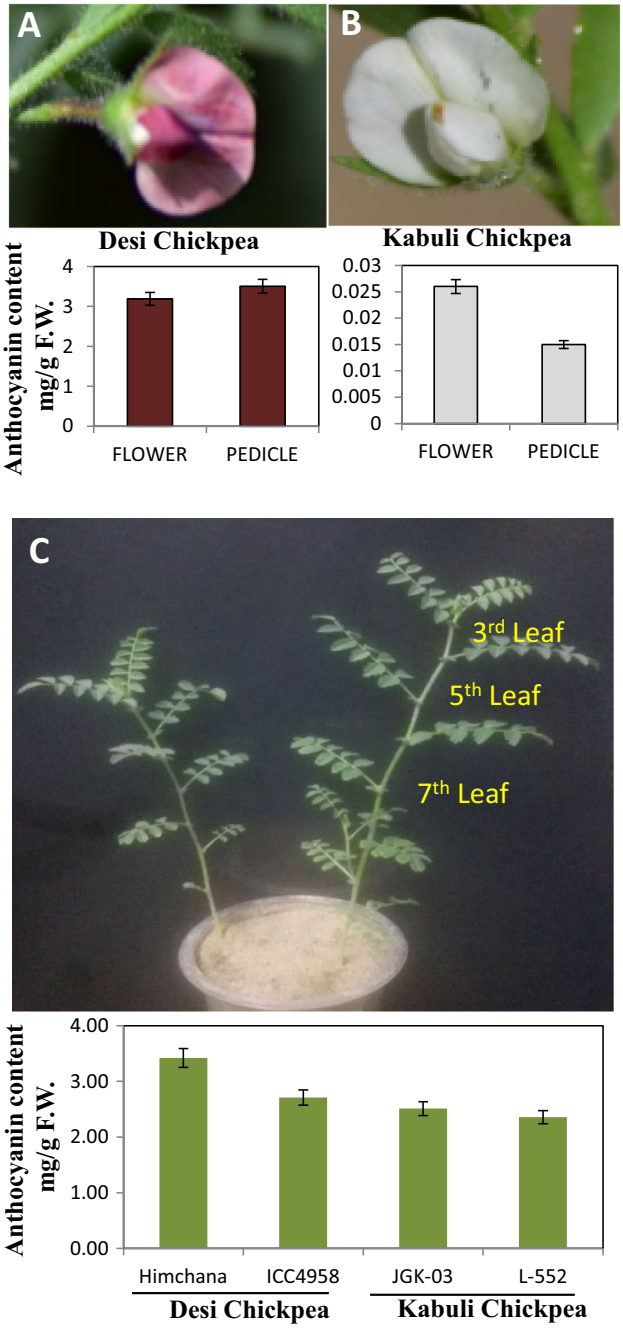

Fig. 3 Comparison of anthocyanin contents in chickpea cultivars. **a** The pink flower and dark red pedicel are from desi cultivars and anthocyanin content is found to be higher in pedicel as compared to flower. **b** White flower has very low amount of anthocyanin content as compared to desi cultivars. It has also very low amount in pedicel even as compared to white flower. **c** The chickpea plant grown in laboratory with clear represetation of 3rd, 5th and 7th leaves with reference to first leaf. The unit used to exressed anhocyanin contents is mg/g fresh weight of sample

Sample preparation and GC–MS analysis

The selected numbers of healthy uniform shaped seeds from different cultivars were grounded to fine powdered sample using liquid nitrogen. The samples were lyophilized and separately extracted (100 mg each) in two different selected solvents (100% chloroform and 100% methanol) of 10 ml each, and incubated for overnight duration. The incubated samples were then sonicated for one hour duration at 45 °C using ultra sonicator. The sonicated samples were filtered twice by using Whatman filter paper. The extracted solvents were completely dried using rotor evaporator (LabIndia) and dissolved in respective solvents (one ml each) again. Samples were subsequently filtered using filter syringe and the filtrate was taken to GC vials for analysis by GCMS-QP2010 ultra, Shimadzu. To analyze the samples, the temperature of column and injection set at 40 °C and 250 °C respectively. The flow control mode was kept in linear velocity (split injection mode ratio: 5). The column was allowed to flow at 1.24 ml/min with helium gas. Oven temperature was programmed as follows: 40 °C for 3 min and then increased to 220 °C at the rate of fourfold and for 5 min and then further increased to 250 °C at the rate of 15 and for 5 min. For MS analysis, the ion source temperature was set to 200°C and interface temperature was set to 260 °C. Mass by charge ratio (m/z) was 50-800 m/z, means starting ratio was 50 and ending ratio was 800. The data was analyzed with the help of chromatograms obtained from the same.

Statistical analysis

All the experiments were performed in three biological replicates and the results were expressed as arithmetic mean ± standard deviation.

Results and discussion

Desi cultivar has more thickened seed coat as compared to kabuli cultivar

The seed coat is an important feature and contributes in nutritive value, cooking time and processing quality of chickpea seed. The seed coat is documented only 4.9 (mean seed coat percentage) in kabuli chickpea as compared to 14.2 in desi chickpea as mentioned by Purushothaman et al. (2014). In present study, it is also documented that kabuli chickpea (JGK-03), has a much thinner seed coat overall in comparison to the desi (ICC4958) cultivar, except for the hypodermal region (Fig. 2). Persistant occurance of hypodermal at later stage of seed development, contributes to more thickness in desi seed coat as compared to kabuli cultivar. More accumulation of metabolites may also contribute toward rigidity of seed coat in desi cultivar (Fig. 2). The seed coat thickness is an important parameter of seed coat architecture that is evolved in adapt to diverse environment and reproductive strategies (Coen and Magnani 2018). The seed coat of desi chickpea is also darkened in color due to two subsequent reactions including biosynthesis of proanthocyanidins (polymers of monomers), and their subsequent oxidation to reactive quiones. These two features, higher fractions of seed coat and accumulation/deficient of proathocyanidins (PAs) determine the different usages of both dark and kabuli chickpea cultivars as well as different demand in commercial market. The desi chickpea is more suitable to feed animals as high energy and protein containing feed in animals diet to support milks, meat, and/or egg production after heat treatment. Due to high accumulation of phenolic compounds majorly included anthocyanins and PAs, dark seed coat chickpea also possesses a high anti-oxidant activity. In addition, the desi chickpea is usually prepared for human consumption by dehulling and splitting, while kabuli may be processed into flour without dehulling due to structure and content variations in seed coat. In continuation, the in-vitro nutrient digestibility value of chickpea grain is also reported more in kabuli cultivar as compared to desi cultivar (Maheri-Sis et al. 2008). The quality of chickpea is also largely based upon the color, texture and brightness of the seed coat, which differs in kabuli and desi cultivars. In addition, as discussed previously, darkened chickpea is responsible for increased cooking times and reduced palatability, and responsible for rejection in direct human consumption in the market. Thus, the higher accumulation of secondary metabolites and more rigidity of desi cultivar has different usuage as compared to kabuli cultivar.

Desi cultivar has higher anthocyanins than kabuli cultivar

The aerial plant parts of himchana and ICC4958 contain anthocyanins and their flower pigmented with pink or purple color. However, kabuli aerial parts are green that lacks anthocyanins pigmentation and their flower are white in color (Upadhyaya et al. 2008). The pink flowers from desi cultivar were found to be with higher anthocyanins content as compared to white flower from kabuli cultivar. Similary, the pedicel from pink and white flowers were also contained similar pattern of anthocyanins accumulation as in flowers (Fig. 3a, b). Interestingly, the anthocyanins content in pedicel portion was found to be higher than pink flower of the same cultivar (desi) (Fig. 3a, b). The anthocyanins content in kabuli cultivar was very low as compared to desi cultivar (Fig. 3). The anthocyanins and

related chemicals have also been linked with pathogen related responses in plant and protect the plant from infections mediated by pathogenic organisms (Kumar et al. 2018). It has also been established that these non-photosynthetic pigements have the potential to offer many functions concurrently. Thus, the total anthocyanins content was measured in selected cultivars, being an important metabolite in many plants including chickpea. Overall, the total anthocyanins content was found to be higher in the desi cultivar as compared to the kabuli cultivar (Fig. 3). In conclusion, the desi cultivar has been found to be accumulated higher anthocyanins content than kabuli cultivar. Among different cultivars, himchana has 1.259 fold higher anthocyanins content than ICC4958 in the leaf samples and both himchana and ICC4958 have 1.36 and 1.08 fold higher anthocyanins content as compared to the kabuli cultivar, JGK-03 in the leaf sample. Among all cultivars, L-552 was found to be reported with lesser amount of anthocyanins content (Fig. 3). Hughes et al. (2005) also documented higher accumulation of anthocyanins in younger tissues as compared to older tissues and supporting our designing's for selection of specific leaves for estimation of anthyocyanins content. The younger leaves have immature photosynthetic pigments which could undergo photodegradation and anthocyanins play protective role to protect these pigments (Hoch et al. 2003). But when plant gain photosynthetic maturity, photoprotection is no longer required and being a greater metabolic investment, the synthesis of secondary key metabolites, synthesis or accumulation of anthocyanins declined. This observation has also been validated by the hypothesis as mentioned that the reason of higher anthocyanins in himchana is due to its origin in higher altitudes that is directly related to environment of lower temperature and higher degree of photodegradation (Hoch et al. 2003). These findings could also encourage to understand the cultivar specific behaviour of biosynthesis and accumulation of anthocyanins in chickpea because anthocyanins pigmentation could be an important trait for improving the adaptability potential of chickpea against various environmental constraints.

Desi cultivar has higher total phenolic contents than kabuli cultivar

The comparative evaluation of anthocyanins further encourages to check the total phenolic contents of dried seed powder from different chickpea cultivars. This experiment was also performed using two different extracting solvents for checking the effect of different extraction solvent on the estimation of total phenolic contents. The extraction of dried seed was carried out with 50% acetone and 70% methanol as referred earlier (Abozed et al. 2014; Ngo et al. 2017). The desi cultivar, ICC4958 was found to be higher amount of phenolic contents followed by himchana and JGK-03. The estimation of total phenolic contents in selected cultivars using 50% acetone was recorded as 29.8 μg/mg of freeze dry weight (FW) in himchana, 31.21 μg/mg of FW (ICC4958), 13.68 μg/mg of FW (JGK-03) and 23.24 μg/mg of FW (L-552). Similarly, total phenolic contents using 70% methanol extraction were recorded as 15.29 μg/mg of FW (himchana), 13.688 μg/mg of FW (ICC4958), 11.769 μg/mg of FW (JGK-03) and 12.67 μg/mg of FW (L-552). The comparative data of phenolic contents among different cultivars using two different extractions are also shown in Table 1. Our results are consistant with previous report of documentation of higher phenolic contents in desi as compared to kabuli chickpea (Macar et al. 2017). In continuation, Maheri-Sis et al. (2008) also found that desi cultivar contains more phenolic compounds than kabuli cultivar and documented that there was no significant differences in total phenolic contents of different genotypes of desi and kabuli itself. Himchana has the highest concentration of anthocyanins among the three cultivars but surprisingly total phenolic contents was found to be higher in ICC4958. As anthocyanin compounds also belong to the group of phenolics, the variation in results could be due to overaccumulation of non-anthocyanin phenolics compound in ICC4958. Overall, this data helps to understand that desi cultivar has higher phenolic contents including anthcoyanins as compared to kabuli cultivar. In conclusion, the total phenolic contents could be the reason for specific colour and taste of the chickpea seeds (Table 2).

Volatile organic components (VOCs) analysis in desi and kabuli cultivars

Chickpea is a good source of important vitamins such as riboflavin, niacin, thiamine, folate and vitamin E, which are abundant in both kabuli and desi cultivars. The health benefits of chickpea seeds were also documented against human diseases including cardio-vascular diseases, Type 2

Table 1 Total phenolic contents in selected chickpea cultivars

Chickpea cultivars	Total phenolic acids (μg/mg of FW samples)	
	50% acetone	70% methanol
Desi		
Himchana	29.80	15.29
ICC4958	31.21	13.68
Kabuli		
JGK-03	13.68	11.77
L-552	23.24	12.67

Table 2 Metabolites detected in the chloroform extract of all the four cultivars: Himchana, ICC4058, JGK-03 and L-552. 'D' denotes detection and 'ND' denotes non detection of metabolite(s)

Sl. no.	Metabolites	Himchana	ICC4958	JGK-03	L-552
1.	Cyclopropane	D	ND	D	ND
2.	Dodecane	D	D	D	D
3.	Tetradecane	D	ND	D	ND
4.	Phenole	D	D	D	D
5.	Hexadecane	D	D	D	D
6.	e-15-heptadecenal	D	D	D	ND
7.	Heptadecane	D	D	D	D
8.	Dibutyl phthalate	D	D	D	ND
9.	Pentadecanoic acid	D	D	ND	ND
10.	1-heneicosanol	D	ND	D	ND
11.	Nonadecane	D	ND	D	D
12.	Octadecanoic acid	D	D	D	D
13.	Heptadecyltrifluroacetate	D	D	ND	ND
14.	Eicosane	D	D	D	D
15.	5-eicosene	ND	D	D	D
16.	7,9-di-tri butyl-1-oxaspiro(4,5) decane	ND	D	D	D
17.	Palmidrol	ND	D	D	ND
18.	2-Bromo dodeccane	ND	ND	D	D
19.	n-Hexadecanoic acid	ND	ND	D	D
20.	Cyclohexanol	ND	ND	D	D

diabete, digestive diseases and some types of cancer (Jukanti et al. 2012). In addition, chickpea seed is also rich in nutritionally important unsaturated fatty acids including linoleic and oleic acids. Among sterol, β-Sitosterol, campesterol and stigmasterol are important documented sterols in chickpea oil (Hirdyani 2014). Chickpea also contain several another phytochemical compounds including phenolic compounds, soya saponins and volatile aliphatic hydrocarbons (Srivastava and Vasishtha 2012). Both desi and kabuli cultivars have different nutrient compositions and subsequently have different commercial or domestic usuages.

A number of analytical methods have been used for metabolomics in plants, among them, the metabolic profiling of the non volatile compounds involved in primary plant metabolism is usually carried out using GC–MS. The compounds detected by GC–MS are low molecular weight volatile compounds and are also involved in important fundamental processes including signaling mechanisms, inter-organims and act as taste of the biological food item(s). This approach has been successfully utilized in various plant species including tomato (Tikunov et al. 2005). In present research, chemical constituents of desi and kabuli chickpea cultivars have been anlyzed using two different solvent extracts (chloroform and methanol). The chromagram of different samples extracted in either methnaol or chloroform are given in Fig. 4. To get the better coverage of metabolites extracted from solvent of different polarity, two different solvents were used.

Differential patterns of metabolites in desi and kabuli cultivars using different solvent extractions

In the chloroform extraction of himchana sample, a total of 24 peaks of putative metabolites were detected. Among them, fatty acids (8), alkanes (11), acidic compounds (2) were identified as significant metabolites (Supplementary Table 1). One ketone, phenol and alcohol molecules were also other identified metabolites (Fig. 5A). E-15-heptadecinal, a fatty acid which were detected as major metabolite followed by phenol and E-14-hexadecenal metabolites. In another desi cultivar (ICC4958), a total of 31 peaks of putative metabolites were detected (Supplimentary Table 2). Among them, fatty acids (8), alkanes (13), alkene (4) were putatively majorly identified metabolites. The others remaining putatively metabolites were identified as phenol, pathalic acid, alkaloid, carbohydrate, ester and acetate (Fig. 5a). The major detected metabolite was identified as E-15-heptadecinal, followed by phenol and 5-eicosene. In chloroform extraction of JGK-03, total 28 peaks were putatively identified (Supplimentary Table 3). The fatty acids (7), alkanes (14), alkene (2) were identified as major metabolites. Phenol, ester, ketone, alcohol and triterpene were identified as remaining putatively identified metabolites (Fig. 5b). The major detected metabolites in chloroform extraction of JGK-03 was E-15-heptadecinal followed by 5-eicosene and phenol. In chloroform extract of L-552, total 35 peaks were putatively identified. The fatty acids (13), alcohol (8), alkanes

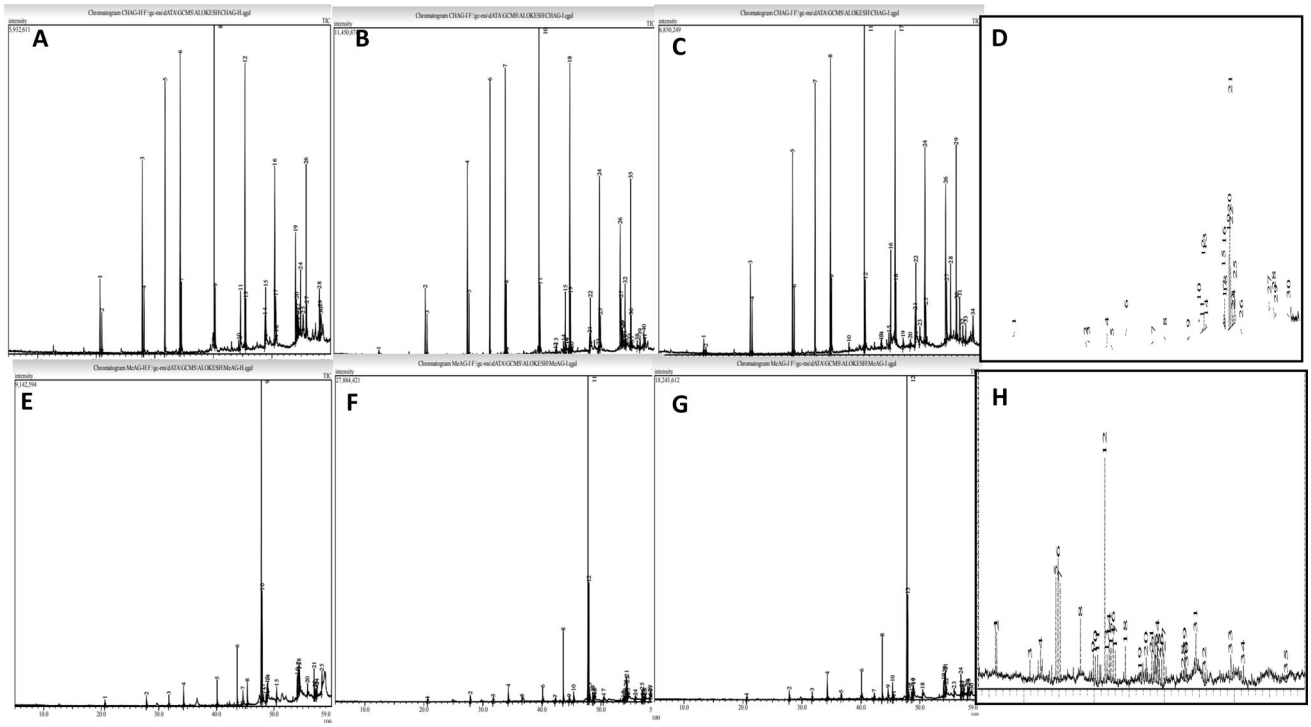

Fig. 4 Chromatograms of chlroform extract of himchana (**a**), ICC4958 (**b**), JGK-03 (**c**), L-552 (**d**) and methanolic extract of himchana (**e**), ICC4958 (**f**), JGK-03 (**g**) and L-552 (**h**)

(8) and alkenes (4) were identified as major metabolites (Supplimentary Table 4). The remaining putatively identified metabolites were enlisted as docecane and phenol (Fig. 5b). The major detected metabolites were identified as 2-undecanethiol, phenol and 1-decanol. Similary, the transcripts level of genes involved in fatty acid metabolism during seed development was also found to be higher in kabuli as compared to desi chickpea (Garg et al. 2017).

In the methanol extraction of himchana, a total number of 24 putatively metabolites were identified (Supplimentary Table 5). Among them, fatty acids (15), alkanes (3), vitamin E (2) were majorly identified metabolites. The remaining metabolites were identified as phenol, ketone, acid and alkene (Fig. 5a). The major detected metabolites in methanolic extraction of himchana were as 9–12-octadecadienoic acid and 10-octadecanoic acid. In same nature of extraction from ICC4958, total 28 putatively metabolites were identified (Supplimentary Table 6). The fatty acid (18), alkenes (2), vitamin E (2) and acids (2), ketones (2) and one metabolite of phenol and steroid were enlisted as identified metabolites (Fig. 5a). The 9,12-Octadecadienoic acid and 10-octadecanoic acid were also majorly detected metabolites in methanolic extraction of ICC4958. A total of 29 putatively metabolites have been identified in methanolic extract of JGK-03 (Supplimentary Table 7). Among them, fatty acids (16), alkanes (3), acid compounds (4), vitamin E (2) and, one metabolite each of phenol, ketone, alcohol and alkaloid were identified (Fig. 5b). The major detected metabolites were identified as 9-12-octadecadienoic acid and 10-octadecanoic acid in methanolic extraction of JGK-03. In methanolic extraction of L-552, putatively 30 metabolites have been identified. The fatty acids (13), alkanes (6), phenol (3) and alcohol (2) were identified as majorly documented metabolites (supplimentary Table 8). The other documented metabolites were enlisted as benzene,1,-azido, phthalic acid, and benzoic acid (Fig. 5B). In L-552, octadecadienal, 17-octadecynoic acid and pentadecanoic acid were majorly identified metabolites (Table 3).

The present work revealed that there is no significant variation between fatty acid contents extracted using chloroform solvent in selected desi cultivars. However it was documented in selected kabuli cultivars (Fig. 5a, b). However, this variation was documented much more in methanolic extraction from desi and kabuli cultivars. The highest number of fatty acid metabolites were enlisted in ICC4958 (26) and L-552 (26) and followed by JGK-03 (23) and himchana (22) (Fig. 5). This observation could be based upon cultivar variations (Srivastava and Srivastava 2004). In continuation, environmental conditions have also been found to be strongly affected the fatty acid composition and genotype × environment interaction that was found to be a major source of variation (Gül et al. 2008). The volatile compounds have also been comparatively

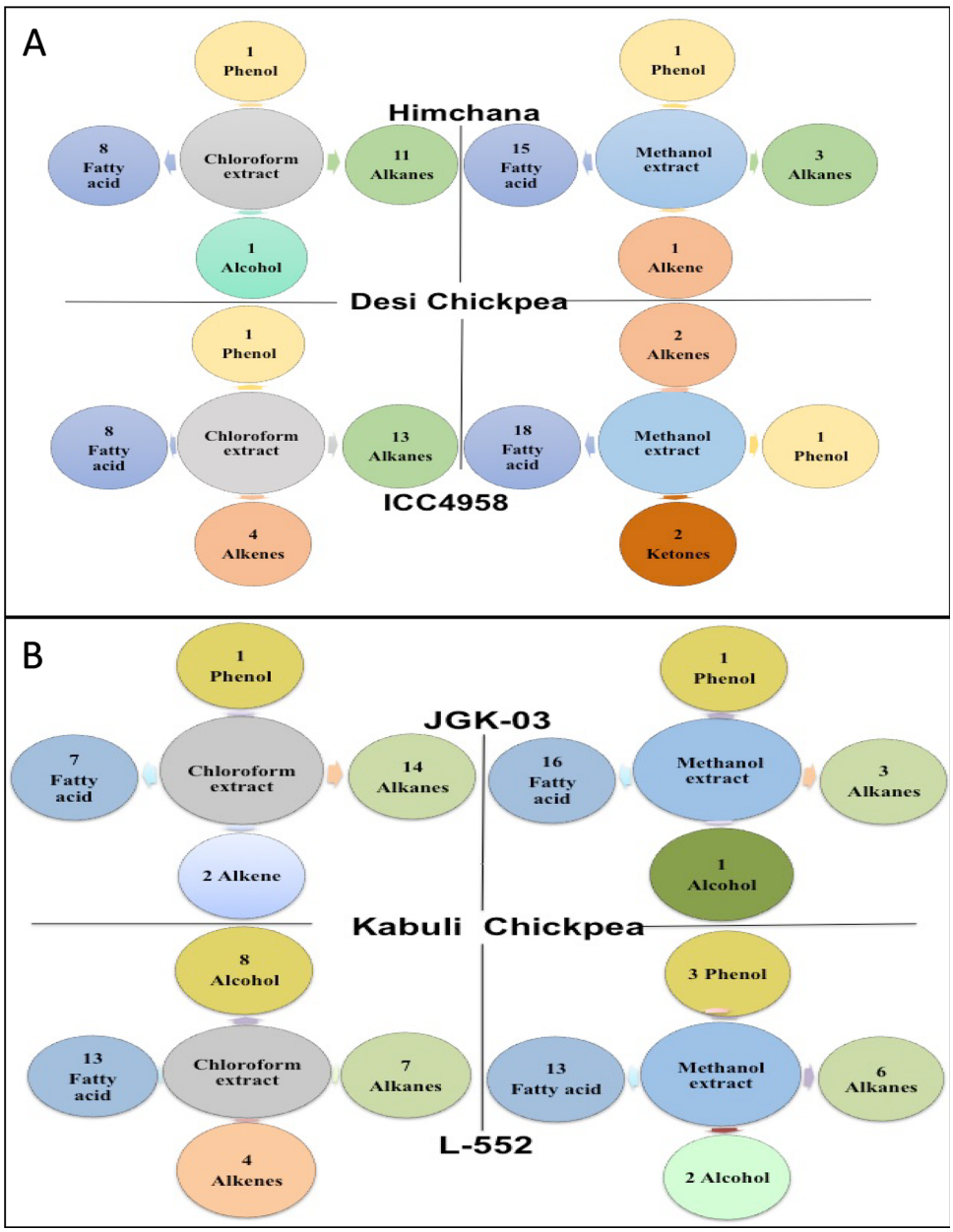

Fig. 5 The distribution of putatively identified metabolites among different cultivars. **a** Different metabolites in desi cultivars using both solvent extracts. **b** different metabolites in kabuli cultivars using both solvent extracts

identified and a significant variations have been documented among desi and kabuli cultivars (Fig. 6a, b). Similary, Aprea et al (2015) reported that the significant variations in volatile compounds have been influenced by numerous factors including genotype, climate, soil, ripeness, and many other variables that impact on odour and flavor features.

Common patterns of metabolites in desi and kabuli cultivars using different solvent extractions

Two different solvents of different polarity were used for the extraction of metabolites from seed samples of desi and kabuli cultivars. The selection of methanol and chloroform solvents for present work has been used as recommended by Čertík et al (1996). The former was used to extract highly polar metabolites and later was used for moderately or non-polar metabolites. In present work, 20 and 31 putatively identified metabolites were exclusively documented in desi and kabuli cultivars, respectively in chloroform extracted samples. In more specific detail, 3, 12, 3 and 25 putatively metabolites were documented in himchana, ICC4958, JGK-03 and L-552 cultivars in chloroform extracted samples. Apart from exclusively detected metabolites, six metabolites have been documented as common metabolites in all selected cultivars (Fig. 6a). Similary, Among desi cultivars, 13 putatively metabolites have been documented as common metabolites. In selected

Table 3 Metabolites detected in the methanol extract of all the four cultivars: Himchana, ICC4058 and JGK-03. 'D' denotes detection and 'ND' denotes non detection of metabolite(s)

Sl. no.	Compound	Himchana	ICC4958	JGK-03	L-552
1.	Cyclopropane	D	ND	D	ND
2.	Dodecane	D	D	D	D
3.	Tetradecane	D	ND	D	ND
4.	Phenole	D	D	D	D
5.	Hexadecane	D	D	D	D
6.	e-15-heptadecenal	D	D	D	ND
7.	Heptadecane	D	D	D	D
8.	Dibutyl phthalate	D	D	D	ND
9.	Pentadecanoic acid	D	D	ND	ND
10.	1-heneicosanol	D	ND	D	ND
11.	Nonadecane	D	ND	D	D
12.	Octadecanoic acid	D	D	D	D
13.	Heptadecyltrifluroacetate	D	D	ND	ND
14.	Eicosane	D	D	D	D
15.	5-eicosene	ND	D	D	D
16.	7,9-di-tri butyl-1-oxaspiro(4,5) decane	ND	D	D	D
17.	Palmidrol	ND	D	D	ND
18.	Hexadecanoic acid	D	D	D	D
19.	9,12-Octadecanoic acid	D	D	D	D
20.	10-Octadecanoic acid	D	D	D	D
21.	3-cyclopentayl propanoic acid	D	D	D	ND
22.	Heptadecanoic acid	D	D	ND	ND
23.	5-Octadecane	ND	D	D	ND
24.	E-14 Hexadecanol	ND	D	D	ND
25.	Methyl stearate	ND	D	D	ND
26.	n-Hexadecanoic acid	D	ND	D	D
27.	Isopropyl linoleate	D	ND	D	ND

kabuli cultivars, total 9 metabolites (six common in all selected culitvars and 3 common among both kabuli culitvars) were identified in all the two selected cultivars of kabuli chickpea using chloroform as solvent extract (Fig. 6a). These nine putatively identified metabolites were also been found to be non-polar or moderately polar metabolites..The remaining metabolites were found to be documented with differential patterns in either any two or three combination of selected desi and kabuli cultivars. In conclusion, more diversity was documented in ICC4958 and L-552 in chlorofrom extracted samples.

In methanolic extraction samples, a total of 15 and 21 putatively identified metabolites were exclusively documented in desi and kabuli cultivars. In cultivar specific, himchana, ICC4958, JGK-03 and L-552 have exclusively detected 4, 6, 2, and 12 metabolites respectively (Fig. 6b). metabolites were documented as common metabolites in desi chickpea cultivars (Fig. 6b). In addition, among these common 12 metabolites, 4 metabolites were also documented as common metabolites with kabuli cultivars. Among desi cultivars, 12 metabolites were found to be common of all detected metabolites in himchana and ICC4958. Similarly, 13 metabolites were documented as common among kabuli cultivars. In all selected cultivars, four metabolites were found to be documented as common metabolites. The different cultivars, as previously documented, both ICC4958 and L-552 have more diversity in term of votalite organic acids. Similary at molecular level, appraox. 50% total estimated genes exhibit differential expression in kabuli (JGK-03) as compared to desi (himchana) cultivar (Garg et al. 2017). In conclusion, this approach identified an optimal protocol consisting of 60 identified nonpolar metabolites and 74 polar metabolites, and will potentially reduced the variations associated with combining metabolite profiles from different samples for untargeted analysis. In conclusion, both cholroform and methanol extracted samples were documented with differential pattern of metabolites. This observation provided the importance of selection of apporapriate solvent for any metabolite analysis.

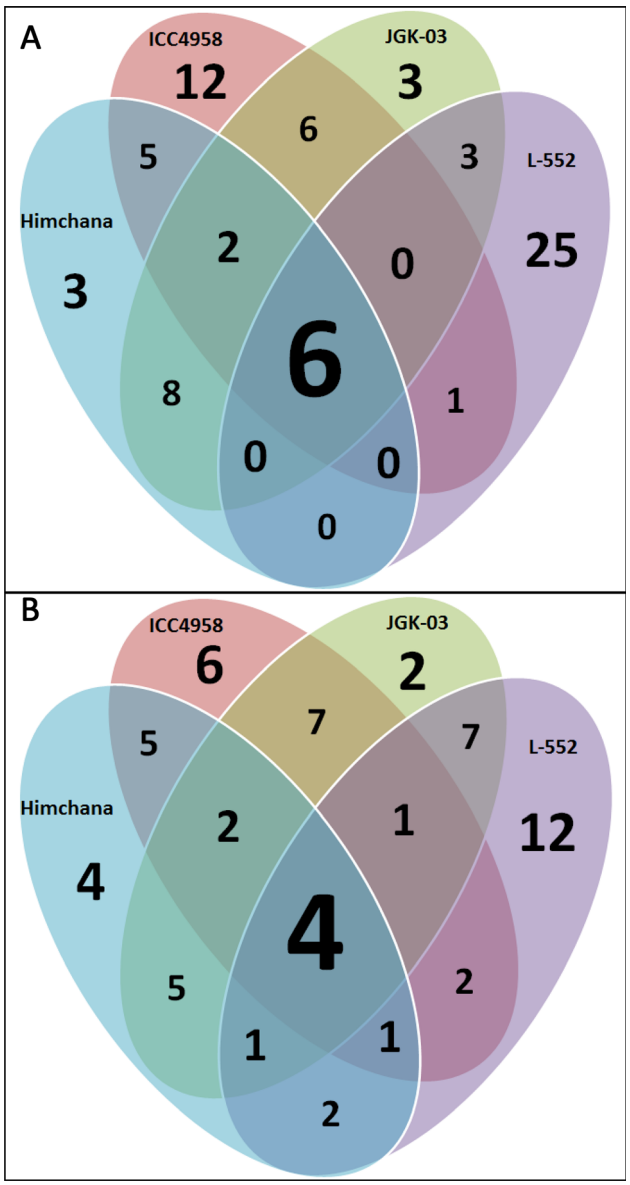

Fig. 6 The common metabolites among all selected cultivars, desi (himchana and ICC4958) and kabuli (JGK-03 and L-552) cultivars using methanolic and chloroform extracts

Common patterns of metabolites in all selected four chickpea cultivars in either single or both extraction solvents

Only six metabolites were detected in chloroform extract, which were found to be common in all desi and kabuli cultivars. All other putatively identified metabolites, 68 are differential documented in different cultivars. In methanolic extract, only four metabolites were found to be common in both desi and kabuli cultivars while 60 are differential documented in different cultivars. Among them, one metabolite (phenol) was only detected both solvent extract in all cultivars. In chloroform extract, other identified metabolites are docecane (lipids), hexadecane (alkane), heptadecane (alkane), eicosane (alkane), octadecanoic acid (fatty acid) (Table 4). While In methanolic extract, apart from phenol, hexadecanoic acid (fatty acid), 9,12-octadecadienoic acid (fatty acid) and 10-octadecenoic acid (fatty acid) were also documented.

In conclusion, in chloroform extract of all desi and kabuli cultivars, the most abundant common metabolites belong to alkane class while in methanolic extract, fatty acids are most common metabolites. Both monounstaurated and polyunsaturated metabolites are predominant in plants (Scheuerbrandt and Bloch 1962). Apart from these common metabolites, all others metabolites are differntially identified in different cultivars and proved that metabolites measuremnet by GC–MS is subjected to different solvents as well as different chickpea cultivars.

Conclusion

The estimation of anthocyanins content was found to be higher in desi cultivar as compared to kabuli cultivar. Similary, the total phenolic contents was also found to be higher in desi cultivar as compared to kabuli cultivar. A number of metabolites were also commonly detected among selected desi and chickpea cultivars. All uncommon putatively metabolites are highly specific to a particular cultivar. Thus, metabolite analysis of most prominent secondary metabolites in chickpea plant and volatile organic components, could reveal the key cultivar specific metabolites and further integrated approaches including genomics, transcriptomics and advanced metabolomics tools could pave the way to undersand the metabolite diversity in chickpea that is creating the differences among desi and kabuli cultivars.

Table 4 Common metabolites detected in all cultivars in chloroform and methanolic extracts, respectively

Solvent extract	Compound		Category
Chloroform	1.	Dodecane	Lipids
	2.	Phenol	Phenol
	3.	Hexadecane	Alkane
	4.	Heptadecane	
	5.	Eicosane	
	6.	Octadecanoic acid	Fatty acid
Methanol	1.	Phenol	Phenol
	2.	Hexadecanoic acid	Fatty acid
	3.	9,12-Octadecadienoic acid	
	4.	10-Octadecenoic acid	

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Author's contribution VK conceived and designed present research. AG, AD and VK conducted experiments. AG, AD, PB, JNB and VK analyzed data. AD, PB and VK wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest This article contains unpublished materials and original. Authors confirmed that all authors have read and approved the manuscript and there is not ethical issue involved and declare that there is no conflict of interest.

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