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Simultaneous extraction of four different bioactive compounds from *Garcinia indica* and their enrichment using Aqueous Two-Phase Systems

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ABSTRACT

Garcinia indica fruits contain several important bioactive compounds like anthocyanin, garcinol, isogarcinol and hydroxycitric acid. Simultaneous extraction of these bioactive compounds from the dried *G. indica* fruit by employing water, acidified water and an aqueous mixture of ethanol and propanol as solvents and subsequent enrichment using Aqueous Two-Phase Systems (ATPS) were studied. Aqueous 1-propanol (60% (v/v)) and aqueous ethanol (80% (v/v)) were found to be a superior solvent to extract anthocyanin, garcinol, isogarcinol and HCA compared to acidified water and water alone as solvent. The crude extract was further subjected to the PEG-salt and alcohol–salt based Aqueous Two-Phase Extraction (ATPE) for the simultaneous enrichment of garcinol, isogarcinol into top phase and anthocyanins and hydroxycitric acid towards the bottom phase. The ATPS containing ethanol and ammonium sulfate system with TLL of 38.60–43.28% was found suitable to enrich 86.33% of anthocyanins and 75.17% of HCA in salt-rich bottom phase and 96.39% of garcinol and 94.26% of isogarcinol in ethanol-rich top phase.

1. Introduction

Garcinia indica (GI) is a slow-growing slender tree belonging to the Guttiferae family found across the Asian, African and Polynesian countries. It is an underutilized tree and well distributed in rainforests of the Western Ghats of south India as well as in the north-eastern states of India (Chandran, 1996; Swami et al., 2014). The fruit of the GI (kokum fruit) is colored either dark purple or red tinged with yellow (Baliga et al., 2011). The weight of the fruits varies from 20 to 85 g and phytochemical studies have shown that kokum fruit rind contains about 2.4% (w/w) of anthocyanins (ACN) (Nayak et al., 2010a, 2010b), 10.3–12.7% (w/w) of hydroxyl citric acid (HCA) along with minor quantities of hydroxycitric acid lactone and citric acid (HCA) and about 1.5 wt% polyisoprenylated benzophenone derivative called Garcinol ($C_{38}H_{50}O_6$) (Nayak et al., 2010a). Anthocyanins (ACN) are safe hydrophilic water-soluble pigments known for their orange, red to blue color and their antioxidant and antimicrobial potential. The impurities such as sugars, sugar alcohols, organic acids, proteins, amino acids and water-soluble molecules present in the anthocyanin extract due to the non-selective nature of the solvents and process have a significant effect on ACN stability. Hence the further purification of ACN is desired to improve their biological activities and shelf-life. The solid-phase extraction (adsorption) (Buran et al., 2014; He et al., 2017; Heinonen et al., 2016; Jampani and Raghavarao, 2015; Yari and Rashnoo, 2017), membrane processes (Cissé et al., 2011; Husson et al., 2013) and Aqueous Two-Phase Extraction (Jampani and Raghavarao, 2015; Liu et al., 2013; Sang et al., 2018) were employed to purify the ACN, in recent times. Further, the isolation and quantification of ACN were well studied with capillary electrophoresis and chromatographic techniques like high-speed counter-current chromatography (HSCCC) and preparative HPLC (Chorfa et al., 2016; Jin et al., 2015; Valls et al., 2009; Xu et al., 2018).

The hydroxycitric acid (HCA) (1,2-dihydroxypropane-1,2,3-tricarboxylic acid), a derivative of citric acid is used as an anti-obesity ingredient due to their inhibitory effect on ATP-citrate lyase which is needed for the conversion of carbohydrates into fat (Jena et al., 2002a, 2002b). Moffett et al. (2001, 1996) have developed a process for the aqueous extraction of (−)-HCA from Garcinia rinds. A process for large-scale isolation of HCA from the fresh/dried rinds of *Garcinia* species and

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leaves of *Hibiscus* species was demonstrated by Ibnusaud et al. (2000a, 2000b). The osmotic membrane distillation (OMD) was used to concentre HCA by Ramakrishnan et al. (2008). Garcinol (GL), a yellow colour fat-soluble pigment, is yet another bioactive component present in the GI fruit. GL also found as its isomeric form called, isogarcinol (IGL). The GL and IGL have shown promising anti-oxidative, antiglycation, anti-inflammatory, antibiotic, anti-cancer, anti-bacterial, anti-ulcer and free radical scavenging activities (Liu et al., 2015; Tsai et al., 2014; Yamaguchi et al., 2000). GL is extracted from GI fruit rinds using the solvents ethanol, methanol, hexane, benzene, chloroform, and ethyl acetate and successively purified in the chromatographic processes (Kaur et al., 2012; Negi and Jayapraksha, 2006).

Even though extensive studies are reported for the extraction of ACN from GI, limited studies are available for the extraction of other valuable bioactive components like HCA, GL, and IGL. The development of economically feasible and mild downstream processes which can simultaneously extract and purify multiple compounds are in great demand. Aqueous Two-Phase Extraction (ATPE), a liquid–liquid extraction technique has been a versatile technique for the simultaneous recovery and purification of biomolecules and value-added products from complex biological sources. It is a single step unit operation, which selectively partitions and purifies a specific solute by concentrating in any one of the phases (Peters, 1987). Polymer (PEG)-salt phase systems are widely used for the partitioning of natural compounds like anthocyanins, betacyanins, and phycocyanins. ATPS made-up of PEG-4000/$MgSO_4$ and PEG-6000/$MgSO_4$ were reported as a most suitable system for the effective partitioning of ACN from the crude extract of red cabbage and Jamun fruits (Chandrasekhar and Raghavarao, 2015; Jampani and Raghavarao, 2015). The ATPS composed of short-chain alcohol (ethanol, 1-propanol, and 2-propanol) with inorganic phosphate and sulfate salts have been explored these days to purify natural compounds (Jiang et al., 2009) due to the advantages of being low-cost system and giving good resolution with higher yield and efficiency. These systems are able to recover the hydrophilic compounds as a result of lower solubility of the inorganic salt in alcohols and the salting-out effect (Wang et al., 2011, 2009). Further, the lower viscosity and surface tension of the phases, easy recovery of the alcohol and subsequent recycling and product recovery from the phases may solve the major scale-up issues (Liu et al., 2013; Reis et al., 2014). The ethanol–ammonium sulfate and propanol-magnesium sulfate systems are exploited to partition the anthocyanins from mulberry (Wu et al., 2011), purple sweet potatoes (Liu et al., 2013), blueberry fruits (Hua et al., 2013), grape juice (Wu et al., 2014) and kokum fruits (Nainegali et al., 2017).

The conventional techniques used to extract the ACN, HCA, GL, and IGL individually by losing other important compounds or simultaneous extraction of all the four components together and followed by sequentially fractionating or purifying the individual components may increase the processing cost. Even though the ACN was purified from different sources including GI, the literature failed to focus towards the purification of HCA, GL, and IGL from the GI. The present work attempts to extract four bioactive molecules like ACN, HCA, GL and IGL simultaneous from *G. indica*. The feasibility of ATPE and the preliminary partitioning behavior of ACN, HCA, GL, and IGL was verified in the system formed by PEG, ethanol, and 1-propanol with different salts.

2. Materials and methods

2.1. Plant material and chemicals

The kokum (*G. indica*) dried fruits were procured from the local market near Mangalore, India. HPLC-grade 1-propanol, methanol, acetonitrile, trifluoroacetic acid (TFA) were procured from Merck India Ltd. and standard anthocyanin-kuromanin chloride (cyanidin-3-O-glucoside), garcinol, isogarcinol, hydroxycitric acid, analytical grade polyethylene glycols of molecular weight PEG 6000, 1-propanol, ethanol were purchased from Sigma Chem. Co. USA. Glacial acetic acid, hydrochloric acid, sodium hydroxide and different salts ammonium sulfate, magnesium sulfate, sodium sulfate, zinc sulfate, trisodium citrate, sodium dihydrogen phosphate, dipotassium hydrogen phosphate, and diammonium hydrogen phosphate salts (purity > 99%) were purchased from Merck, Mumbai, India. All the chemical reagents used were of analytical grade. Double distilled water was used throughout the experiments.

2.2. Extraction studies

The seeds of the dried kokum fruits were removed and the rind parts (pulp) were considered for the studies. Four solvents like distilled water, acidified water (0.1% hydrochloric acid), 1-propanol and ethanol were considered for the extraction. The 5 g of fruit rinds were mixed with these solvents by maintaining a different solid–liquid ratio of 1:4, 1:6, 1:10, 1:20 w/v. Ascorbic acid (0.1%, w/v) was added to the extraction medium to inhibit the activity of polyphenol oxidase. The mixture was incubated about 24 h, ground using Morton piston and filtered with a muslin cloth to remove coarse particles. The filtrate was centrifuged at 10,000 rpm for about 20 min (Kubota 6930, Japan) to remove fine suspended impurities and the crude supernatant (a mixture of bioactive compounds) was stored at 4 °C.

2.3. Estimation of bioactive compounds by HPLC analysis

Detection of bioactive compounds such as anthocyanin, garcinol, isogarcinol and hydroxycitric acid and their quantification was performed with Shimadzu LC-20AD model high-performance liquid chromatography system (HPLC) (Shimadzu, Japan) using Reverse Phase C18 Shiseido Co., Ltd., Japan CAPCELL PAK C18 MGII S5 column (4.6 mm ID × 250 mm) with photodiode array UV–vis detector (SPD-M20A).

The modified method of Liu et al. (2013) was used to identify the ACN in kokum extract and samples. The Acetonitrile (solvent A) and 0.1% trifluoroacetic acid (TFA) in water (v/v) (solvent B) were used as mobile phases under binary gradient mode. The binary gradient is maintained as follows: 0–2 min — 95% B; 2–21 min — gradual decrease in B till 20% and remains constant till 23 min; 23–27 min — B increased from 20 to 95% B and finally constant B of 95% is maintained for equilibration till 35 min. The column temperature was 35 °C and absorbance was measured at 520 nm. The flow rate was maintained at 0.5 mL/min with a sample injection volume of 10 μL.

The chromatographic separation and quantitative analysis of GL (camboginol) and IGL (Isoxanthochymol) were performed using reverse phase (RP) C18 column with mobile phase consisting of acetonitrile–water (90:10 v/v, solvent A) and methanol–acetic acid (99.5:0.5 v/v, solvent B) in the ratio of 30:70 at a flow rate of 0.5 mL/min (Chattopadhyay and Kumar, 2006). The column temperature was maintained at 25 °C and GL and IGL were detected at 250 and 276 nm, respectively. The modified method of Jayaprakasha and Sakariah (2002, 1998) was used to determine the HCA. The HCA was eluted by 0.1% HCl in water with a flow rate of 0.3 mL/min under the isocratic condition for about 30 min at 40 °C and detected at 210 nm. The standard graphs were developed at different concentrations of standards ACN, GL, IGL and HCA for the estimation of their concentration in the samples.

Table 1 – List of ATPS considered for the selection of suitable ATPS.

ATP Systems	References
PEG6000-Magnesium sulfate ($MgSO_4$)	Salabat (2001), Zhang et al. (2013)
PEG6000-Ammonium sulfate $(NH_4)_2SO_4$	
PEG6000-Sodium sulfate (Na_2SO_4)	Glyk et al. (2014), Mirsiaghi et al. (2010)
PEG6000-Sodium citrate ($Na_3C_6H_5O_7$)	Zhang et al. (2013)
PEG6000-Di-potassium hydrogen phosphate (K_2HPO_4)	Guo et al. (2012), Nainegali et al. (2017)
PEG6000-Di-ammonium hydrogen phosphate ($(NH_4)_2$ HPO_4)	
1-Propanol–magnesium sulfate ($MgSO_4$)	Wang et al. (2011)
1-Propanol–zinc sulfate ($ZnSO_4$)	
1-Propanol–dipotassium hydrogen phosphate (K_2HPO_4)	Zafarani-Moattar et al. (2005)
Ethanol–dipotassium hydrogen phosphate (K_2HPO_4)	
1-Propanol–sodium citrate ($Na_3C_6H_5O_7$)	Khayati and Shahriari, (2016), Li et al. (2010), Wang et al. (2009), Wu et al. (2014)
Ethanol–ammonium sulfate $(NH_4)_2SO_4$	
Ethanol–sodium dihydrogen phosphate (NaH_2PO_4)	
1-Propanol–ammonium sulfate $(NH_4)_2SO_4$	
1-Propanol–sodium sulfate (Na_2SO_4)	

2.4. *Partitioning of bioactive compounds in PEG based ATPS*

The partitioning studies were conducted by preparing the system with a total weight of 10 g including 1 g of extract in a 15 mL Falcon centrifuge tubes by considering the phase diagrams of the identified systems listed in Table 1. The pH of the system was adjusted to 5 prior to the addition of extract since some of the bioactive components present in the GI extract were not stable beyond the pH of 5. The contents were mixed in a vortex mixer and allowed for phase separation. The top and bottom phase volumes and their weights were noted. The concentrations of the bioactive compounds such as ACN, GL, IGL, and HCA in both the phases of ATPS were quantified from the chromatogram obtained from the HPLC analysis and partition coefficient (K_{ACN}, K_{GL}, K_{IGL}, K_{HCA}) and Yield (Y_{ACN}, Y_{GL}, Y_{IGL}, Y_{HCA}) in top and bottom phases were calculated using Eqs. (1)–(3).

$$K = \frac{C_T}{C_B} \tag{1}$$

$$Y_t = \frac{C_T \quad V_T}{\left(C_T \quad V_T \quad + \quad C_B \quad V_B \right)} \times 100 \tag{2}$$

$$Y_b = \frac{C_B \quad V_B}{\left(C_T \quad V_T \quad + \quad C_B \quad V_B \right)} \times 100 \tag{3}$$

where, C_T and C_B represent the equilibrium concentrations of compounds in the top and bottom phase, V_T and V_B represent the volume of the top and bottom phase, respectively.

2.5. *Partitioning of bioactive compounds in ethanol–ammonium sulfate system*

The binodal curve of the ethanol–ammonium sulfate ATPS was developed at 30 °C (303.15 K) using cloud point titration method (Kaul, 2000). The binodal data were correlated by Eq. (4) (Wang et al., 2009) and the constants were obtained through the regression analysis ($a_1 = 0.75918$, $a_2 = 0.08385$, $b_1 = 0.32753$, $b_2 = 0.04413$ and $c = -0.21043$) with R^2 of 0.99.

$$w_1 = a_1 \exp(-w_2/b_1) + a_2 \exp(-w_2/b_2) + c \tag{4}$$

where, w_1 is the mass fraction of ethanol, w_2 is the mass fraction of $(NH_4)_2SO_4$, Six different mixture points of ethanol–ammonium sulfate (w/w) on 10 g basis were considered for the partitioning studies due to the narrow biphasic area of the system (Qin et al., 2017). About 1 g of crude extract was loaded to all the ATPSs. After the phase separation, the mass of the top (m^t) and bottom phases (m^b) were determined. The equilibrium compositions of the phases were calculated by solving the Eqs. (5)–(8) using MATLAB (Wang et al., 2011, 2009). The tie-line length (TLL) and the slope (S) at different compositions were also calculated using the equations Eqs. (9) and (10).

$$w_1^t = a_1 \exp(-w_2^t/b_1) + a_2 \exp(-w_2^t/b_2) + c \tag{5}$$

$$w_1^b = a_1 \exp(-w_2^b/b_1) + a_2 \exp(-w_2^b/b_2) + c \tag{6}$$

$$m^t.\sqrt{(w_2^t - w_2)^2 + \left(w_1^t - w_1\right)^2} = m^b.\sqrt{(w_2^b - w_2)^2 + \left(w_1^b - w_1\right)^2} \tag{7}$$

$$\frac{w_1 - w_1^t}{w_2 - w_2^t} = \frac{w_1 - w_1^b}{w_2 - w_2^b} \tag{8}$$

$$TLL = \sqrt{(w_1^t - W_1^b)^2 + \left(w_2^t - w_2^b\right)^2} \tag{9}$$

$$S = \frac{w_1^t - w_1^b}{w_2^t - w_2^b} \tag{10}$$

where $w_1{}^t$, $w_1{}^b$, $w_2{}^t$, and $w_2{}^b$ represent the equilibrium compositions (in mass fraction) of ethanol (1) and $(NH_4)_2SO_4$ (2), in the top (t), and bottom (b) phases, respectively. w_1, w_2 represent the total compositions of ethanol (1) and $(NH_4)_2SO_4$ (2), respectively. m^t and m^b are mass of top and bottom phases, respectively.

3. Results and discussion

3.1. *Crude extraction of bioactive compounds*

The simultaneous extraction of ACN, GL, IGL and HCA from the dried rinds of GI was tried initially with the pure solvents like water, acidified water, ethanol and 1-propanol (Fig. 1(a–d)). The solid–liquid (S/L) ratio of the extraction process also studied for all the solvents. The S/L was varied as 1:4, 1:6, 1:10

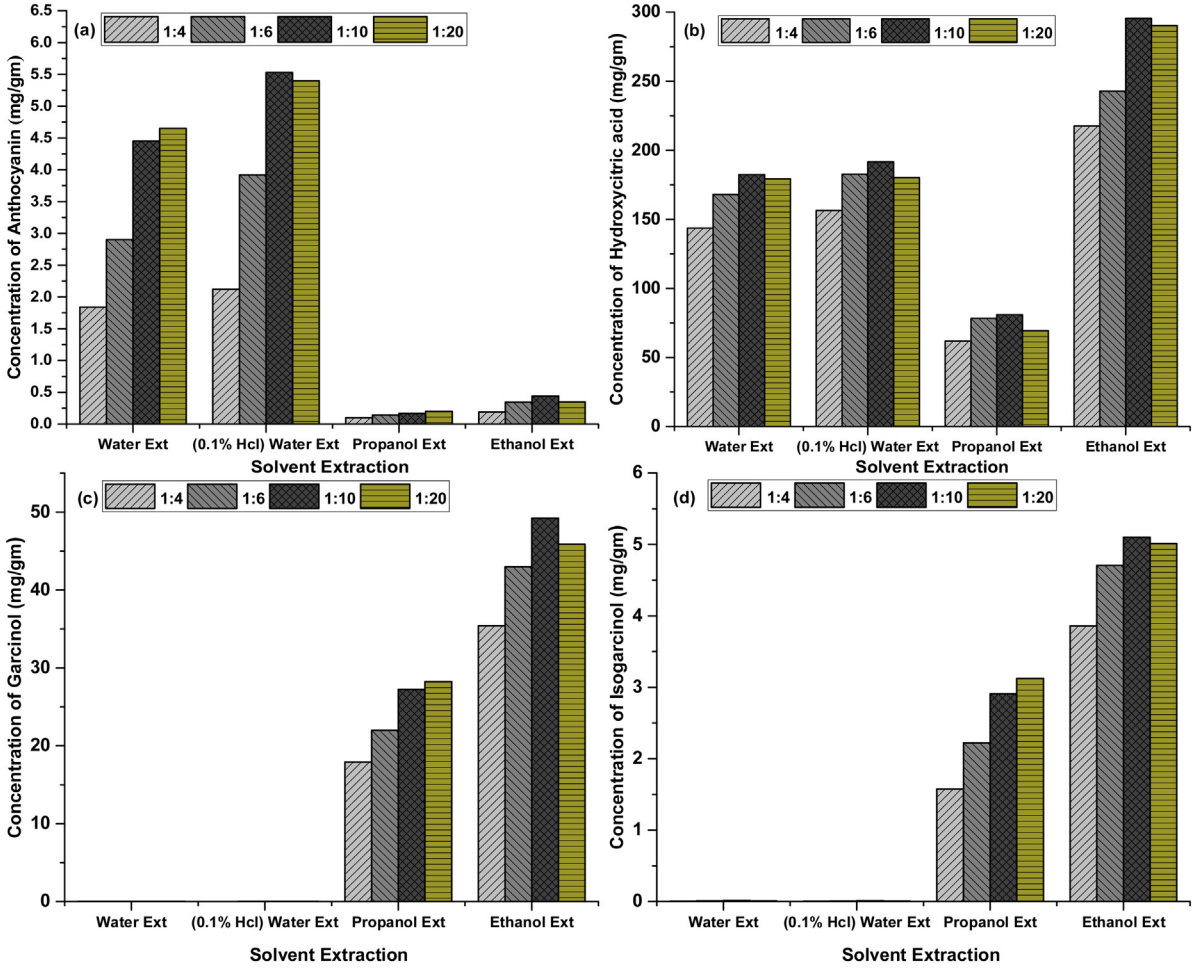

Fig. 1 – Extraction of anthocyanin (a), HCA (b), Garcinol (c), Isogarcinol (d) with different extraction mediums like water, acidified water, 1-propanol and ethanol at different solid–liquid ratios.

and 1:20 and the results are presented in (Fig. 1(a–d)). It was observed from Fig. 1 that the significant amount of ACN (5.53 mg/g) and HCA (191.66 mg/g) were extracted in water and acidified water due to their hydrophilic nature. Comparatively no or lesser quantity of hydrophobic molecules, GL and IGL, were extracted to water. However, the ethanol and 1-propanol could able to extract the GL and IGL with the lower concentration of ACN and HCA due to their reduced polarity and the hydrophobicity of the GL and IGL. However, all the three components together (GL—49.23 mg/g, IGL—5.10 mg/g and HCA—295.43 mg/g) except ACN (0.44 mg/g) were extracted in the pure ethanol system. The 1-propanol system could able to extract all the four components together at lower concentration itself when compared to the extraction of hydrophilic molecules (ACN and HCA) with water and acidified water and hydrophobic molecules (GL and IGL) with ethanol. Increase in concentration of all the four components was observed with increasing S/L ratio till 1:10, but significant increase in concentration was not observed beyond the S/L ratio of 1:10. The maximum concentration of ACN (5.53 mg/g) was observed at S/L ratio of 1:10 with acidified water. However, the ethanol extract at 1:10 S/L ratio provided the maximum concentration of GL (49.23 mg/g), IGL (5.10 mg/g) and HCA (295.43 mg/g). The S/L ratio studies revealed that the maximum concentration of bioactive components in the extract was possible with the S/L ratio of 1:10 irrespective of the solvents used for the extraction. Hence the S/L ratio of 1:10 was kept as constant for further studies.

3.2. *Aqueous solution of a solvent for extraction*

Even though all the four bioactive compounds can be simultaneously extracted in a lower concentration with 1-propanol and ethanol, the extraction of diversified components from GI may be improved by modifying the hydrophobicity and polarity of the solvents using the aqueous mixture of 1-propanol and ethanol at various concentrations. The extraction of ACN was found to increase with increasing concentration of 1-propanol and reached the highest concentration of 109.57 mg/g at 50% 1-propanol (Fig. 2), when compared to the water and acidified water extraction which showed about 6.6 mg/g. However, the ACN concentration was found to reduce gradually with increasing 1-propanol concentration beyond 50%. The aqueous ethanol also showed a similar trend for the extraction of ACN (Fig. 3). The highest concentration of ACN extracted (43.44 mg/g) with 60% ethanol in water solution as solvent. The acidified water extraction yielded up to 247.17 mg/g of HCA and a slightly higher concentration of 266.71 mg/g was achieved with 50% 1-propanol (Fig. 2). Water as a pure solvent and 20–80% 1-propanol extraction showed not much variation in the extraction efficiency of HCA but best productivity was found at 50% 1-propanol. As shown in Fig. 3, the HCA concentration increases with increasing concentration of ethanol and maximum concentration of 399.74 mg/g of HCA was extracted with pure ethanol (100%). However, a significant increase in HCA was not observed (364.47 mg/g HCA) beyond 40% of ethanol in water (Fig. 3).

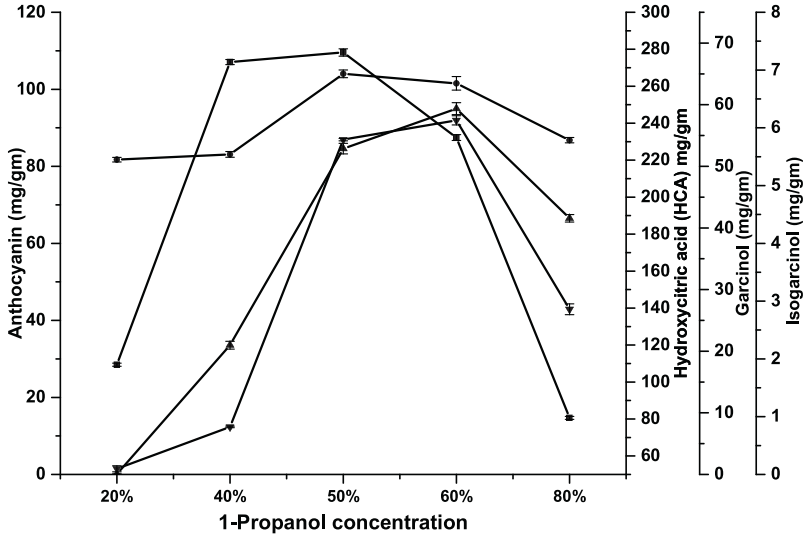

Fig. 2 – Extraction of bioactive compounds from fruits of GI (kokum) with various concentrations of 1-propanol in water. The extraction efficiency of 1-propanol for ACN (■), HCA (●), GL (▲), and IGL (▼).

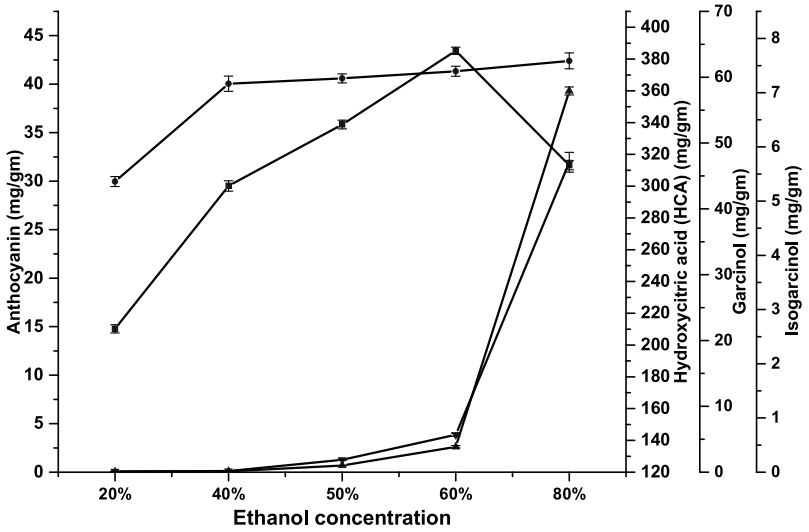

Fig. 3 – Extraction of bioactive compounds from fruits of GI (kokum) with various concentrations of ethanol in water. The extraction efficiency of ethanol for ACN (■), HCA (●), GL (▲), and IGL (▼).

The presence of GL and IGL were not noticed in the aqueous extract of GI, however, the extraction efficiency was found to increase with increasing concentration of 1-propanol in the solvent medium. The effective extraction was observed from 40% to 80% of 1-propanol with the increasing GL concentration from 20.98 to 59.36 mg/g and IGL of 0.82 and 6.13 mg/g at 60% 1-propanol. Further decrease in extraction was found beyond 60% 1-propanol as seen in Fig. 2. On the other hand, a lower concentration of GL (3.85 mg/g) and IGL (0.69 mg/g) were extracted till 60% ethanol in water mixture and which increased further with higher ethanol content. The maximum extraction reached 57.90–59.34 mg/g of GL and 5.72–5.89 mg/g of IGL concentrations at 80 and 100% ethanol (Fig. 3). Almost, similar extraction efficiency was observed in the case of GL and IG for the solvent–water mixture, which is 60% 1-propanol and pure ethanol systems (Figs. 2 and 3).

The hydrophilic molecules ACN and HCA were get extracted with a polar solvent, water or acidified water. But the hydrophobic molecules GL and IGL were got extracted by decreasing the polarity of the solvent through the addition of ethanol and propanol. It was observed that the higher ethanol concentration (80–100%) was required to extract maximum concentration of all the bioactive molecules from the GI when compare to 1-propanol (50–60%) due to the lower polarity of 1-propanol than the ethanol. The equal quantity of water and 1-propanol present in the mixture (approx. 50%) promote the simultaneous extraction of all the bioactive components since the diversified molecules interact with the aqueous and non-aqueous part of the solvent mixture at different intensity.

3.3. *HPLC analysis of extracts*

Each extract was analyzed for four different bioactive components separately as per the HPLC methods explained. The chromatograms obtained with each type of solvent extract for specific bioactive compound are combined along with the chromatogram of standards and presented as a single graph for the comparison purpose (Fig. 4). Two peaks were observed for ACNs namely cyanidin-3-glucoside (retention time 19.098 min) and cyanidin-3-sambubioside (retention time 18.752 min) which was in accordance with other reports (Nayak et al., 2010b) (Fig. 4a). The peaks for both free and lactone form of HCA were obtained at 8.689 and 9.017 min, as

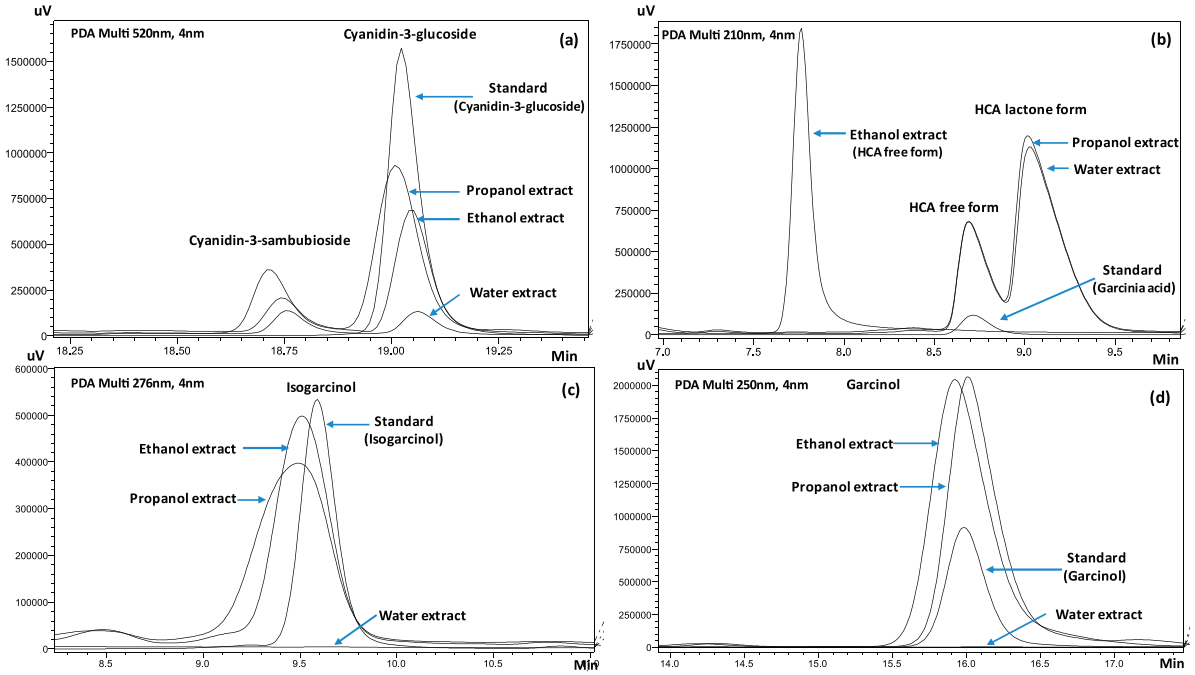

Fig. 4 – The chromatograms of ACNs (a), HCA (b), IGL (c) and GL (d), for the standards and crudes obtained using different solvents.

reported in the literature for water and propanol extract. Yet, the peak for the free form of HCA alone was detected in the ethanol extract with the retention time of 7.763 min (Fig. 4b). Garcinia acid as a standard displayed the retention time of 8.437 min. The free form of HCA in the ethanol extract was eluted much before (7.763 min) the elution time of the standard. The standard molecules of IGL and GL eluted at 9.535 and 15.983 min, respectively (Fig. 4c and d). Water extract of GI failed to display any peak for GL and IGL (Fig. 4c and d). The GL and IGL were eluted at 9.489 and 15.922 minutes for GL and IGL, respectively for the GI extracts obtained using ethanol and 1-propanol as solvents.

3.4. *Screening of ATP systems*

The ATPS formed with PEG, ethanol, and 1-propanol–salts (Table 1) were individually studied for the simultaneous partitioning of similar molecules in either of the phases of ATPS. The ACN is found to be stable in the acidic pH and hence a grey colour precipitation was observed in the ATPS whose natural pH was greater than 5 with the addition of crude. The systems formed with the salts like K_2HPO_4, $(NH_4)_2HPO_4$, $Na_3C_6H_5O_7$ showed a pH greater than 5 and lead to the formation of precipitation. The pH of such systems was adjusted to 5 before the addition of crude to the system. The stable systems formed without any precipitation was identified and the partition coefficients of ACN, HCA, GL, and IGL in the respective system along with the yield were reported in Table 2 and 3. The crude extract contains all the valuable bioactive components (ACN, HCA, GL, and IGL), which was obtained during the extraction with 55% 1-propanol as a solvent, was considered for the partitioning experiments.

3.4.1. *Partition characteristics of PEG-based ATPS*

The partitioning characteristic of the bioactive compounds was initially analyzed in the ATPS formed with PEG6000 with different salts and found that the ACN was partitioned in the PEG-rich upper phase by leaving the HCA in the salt-rich bottom phase and failed to partition the GL and IGL into any of the phases. The HPLC analysis of GL and IGL in both the phases confirms the non-presence of GL and IGL. The PEG-$MgSO_4$/$(NH_4)_2SO_4$ systems partitioned the ACN in the PEG-rich phase with partition coefficients of 24.56, 9.057 and yield of 96.98%, 92.31%, respectively at the native pH of the system. However, the ATPS formed by Na_2SO_3, $Na_3C_6H_5O_7$ with PEG 6000 provided higher partition coefficients of 48.39 and 36.32 with nearly 97% yield in the top phase at the adjusted pH of 5. The ATPS formed with the salts like K_2HPO_4 and $(NH_4)_2HPO_4$ were partitioned lesser ACN in the top phase (Table 2). It is preferred to partition the HCA in the bottom phase to obtain the HCA free ACN as a by-product. However, the HCA was not significantly partitioned in any of the phases of the systems considered except the system formed with Na_2SO_3 and $Na_3C_6H_5O_7$. The salts like Na_2SO_3, $Na_3C_6H_5O_7$ can be a better option to partition ACN in top and HCA in bottom phases because the ATPS retains 74.96% and 81.16% HCA molecules in bottom phase with a lower partition coefficient of 0.40 and 0.20, respectively. Even though both ACN and HCA are considered as hydrophilic components, ACN is less hydrophilic than HCA because of the presence of the aromatic ring. Hence, HCA prefers bottom phase compared to ACN and was strongly interacted with salt-rich phase than the ACN. The higher concentration of HCA and the limited availability of solvent water for ACN in the bottom phase promote the partitioning of ACN in the PEG-rich top phase. The obtained results are in accordance with the observation made by Chandrasekhar and others for the ACN partitioning from red cabbage and Jamun fruit in PEG-4000/$MgSO_4$ and PEG-6000/$MgSO_4$ ATPS (Chandrasekhar and Raghavarao, 2015; Jampani and Raghavarao, 2015). As the PEG-salt systems failed to partition GL and IGL because of its hydrophobic and insoluble nature, these systems may be suitable for the simultaneous partitioning of HCA and ACN into the bottom and top phases, respectively. Specifically, PEG 6000-Na_2SO_3 and PEG 6000-$Na_3C_6H_5O_7$ ATPS may be extended for the better yield of HCA and ACN after the systematic analysis and optimiza-

Table 2 – Screening of PEG-Salt systems for partitioning of bioactive compounds from *Garcinia indica* (GI).

ATP systems (% w/w)	Initial pH	Top phase		Bottom phase	
		Anthocyanin		HCA	
		K_{ACN}	Y_{ACN}	K_{HCA}	Y_{HCA}
PEG-Magnesium sulfate (12.5%–8%)	4.8	24.563	96.98	0.764	52.46
PEG-Ammonium sulfate (12.5%–10%)	4.9	9.057	92.31	0.938	47.35
PEG-Sodium sulfate (12.5%–9%)	5.5	48.388	97.53	0.402	74.96
PEG-Trisodium citrate (12.5%–10%)	7.8	36.322	97.67	0.201	81.16
PEG-Di-potassium hydrogen phosphate (12.5%–10%)	8.1	4.564	85.09	0.844	48.67
PEG-Di-ammonium hydrogen phosphate (12.5%–9%)	7.9	1.564	65.08	0.981	46.68

tion of different variables including, tie line length and phase volume ratio.

3.4.2. *Partitioning characteristics of alcohol based ATPS*

The alcohol-based ATPS are considered to improve the simultaneous recovery and purification of all the bioactive components. Three salts namely, $(NH_4)_2SO_4$, K_2HPO_4 and NaH_2PO_4 only able to form the ATPS with ethanol (Table 3). In general, the hydrophobic molecules (GL and IGL) preferred to be in the alcohol-rich top phase and the hydrophilic molecules preferred to be in the salt-rich bottom phase during the partitioning. Hence the yield of GL and IGL were considered in the top phase and ACN and HCA in the bottom phase of the ATPS.

The ATPS consisting of $(NH_4)_2SO_4$ and $ZnSO_4$ showed higher yield for ACN about 87.54 and 85.30% with 0.12 and 0.26 partition coefficient, respectively. At the adjusted pH (5≤) condition, the 1-propanol–$Na_3C_6H_5O_7$ was found to be next better choice to partition the ACN to bottom phase when compared to the ATPS of Na_2SO_3, $MgSO_4$ (Table 3). The ATPS formed with K_2HPO_4 and 1- propanol failed to partition the ACN in the bottom phase. However, more than 90% of HCA was partitioned in the bottom phase of all the ATPS studied other than the K_2HPO_4–1-propanol system. The lowest partitioning coefficient (<0.042) with the highest yield of HCA (>96%) in the bottom phase was observed for the ATPSs formed with $Na_3C_6H_5O_7$ and $(NH_4)_2SO_4$. HCA strongly interacts with the salts and hence the solubility of HCA in the free water of bottom phase increases. The sulfate salts were found to be more hydrophilic than the phosphate and citrate salts and hence the hydrophilic components like ACN and HCA are retained in the bottom phase. However, a considerable amount of ACN was expelled into the top phase in the ATPSs formed by Na_2SO_3 and $MgSO_4$ due to the lesser volume of free water and a larger amount of salts present in the bottom phase.

The 1-propanol–$(NH_4)_2SO_4$ ATPS showed the highest partition coefficient of GL (89.61) and IGL (9.57) with a yield of 98.28% and 85.89%, respectively. The 1-propanol–$MgSO_4$ (or) $ZnSO_4$ ATPSs also showed a promising partitioning coefficient and yield of both compounds in the top phase. Conversely, the 1-propanol–$Na_3C_6H_5O_7$ and 1-propanol–K_2HPO_4 ATPSs failed to partition the GL and IGL into the top phase effectively. The hydrophobic nature and insolubility of the GL and IGL in water resulted for the better interaction and affinity with alcohol through hydrogen bonds promoted the partitioning coefficient of GL and IGL in the top phase. Further, the lower concentration of propanol in the bottom phase for the dissolution of GL and IGL also helped to improve the partitioning coefficient and yield in the top phase. The screening of ATPS using 1-propanol with different salts revealing that the ATPS formed with sulfate salts are better for the differential partitioning of ACN and HCA. These systems may be further optimized for the better yield and partitioning coefficients of ACN, HCA, GL, and IGL. However, the detailed studies on the implementation of second step ATPS may be required to fractionate the ACN and HCA separately from the bottom phase obtained in the present ATPE.

When the ethanol is used as a phase forming component with different salts, ethanol–$(NH_4)_2SO_4$ showed a yield of 67.54% ACN in bottom phase with the partition coefficient of 0.72 and partitioned about nearly 90% of the GL and IGL into the top phase. However, both the ATPSs formed by phosphate salts (NaH_2PO_4 and K_2HPO_4) were efficient to partition the hydrophobic molecules, GL and IGL, with a yield of more than 90% (Table 3). But the salt phase of these systems showed a poor affinity towards the hydrophilic molecules and caused a poor partitioning coefficient for ACN and HCA. The lesser availability of free water in the bottom phase of the phosphate salts ATPSs might be the major reason for the lesser partitioning coefficient of the hydrophilic molecules (ACN and HCA). The stronger affinity of GL and IGL to the ethanol leads to the partitioning of GL and IGL into the ethanol rich top phase. Among the three salts studied, ethanol–$(NH_4)_2SO_4$ could able to partition all the four molecules simultaneously into different phases. Meanwhile, ethanol–K_2HPO_4 ATPS was found to be a promising system to partition the hydrophobic compounds towards top phase, but then the ACN and HCA present in the bottom phases should be removed out at a later stage using different purification strategies including ATPS.

3.5. *Differential partitioning behavior of bioactive compounds in ethanol–ammonium sulfate system*

Ethanol being a safe/cost effective solvent and used in food industries and the good salting-out ability of the $(NH_4)_2SO_4$ salt (Khayati and Shahriari, 2016), the partition characteristics at different concentrations were studied by considering the phase diagram of the ATPS. Ethanol–$(NH_4)_2SO_4$ system was well studied in alcohol–salt ATPS category for ACN partitioning (Hua et al., 2013; Liu et al., 2013; Sang et al., 2018; Wu et al., 2011, 2014). The total system concentration should be prepared at different combination within the $(NH4)_2SO_4$ concentrations ranging from 15 to 26% and ethanol from 20 to 30% due to the narrow two-phase area in the phase diagram (Qin et al., 2017). In general, the equilibrium characteristics of the ATPS will be represented through tie line length (TLL). The total concentration of the system laying at different points on a specific tie line results in a constant equilibrium concentration of the components (salt and alcohol) in the equilibrated top and bottom phases (nodes of the tie line). The partitioning characteristics of the solutes in the ATPS hence depends on the equilibrium concentration of the phase forming components, which offer a specific level of different attractive forces

Table 3 – Screening of 1-propanol–salt and ethanol–salt systems for partitioning of bioactive compounds from *Garcinia indica* (GI).

ATP systems (% w/w)	Initial pH	Bottom phase				Top phase			
		Anthocyanin		HCA		Garcinol		Isogarcinol	
		K_{ACN}	Y_{ACN}	K_{HCA}	Y_{HCA}	K_{GL}	Y_{GL}	K_{IGL}	Y_{IGL}
Propanol–magnesium sulfate (25%–15%)	4.3	0.74	64.50	0.170	90.24	38.72	96.87	10.67	89.51
Propanol–ammonium sulfate (25%–15%)	4.9	0.12	87.54	0.042	96.78	89.61	98.28	9.57	85.89
Propanol–sodium sulfate (25%–15%)	5.6	0.74	67.22	0.079	95.86	36.89	96.14	8.75	85.51
Propanol–trisodium citrate (25%–15%)	7.9	0.58	82.73	0.040	97.92	5.57	75.01	1.97	51.51
Propanol–zinc sulfate (25%–15%)	5.4	0.26	85.30	0.064	95.98	39.01	96.23	16.01	91.30
Propanol–dipotassium hydrogen phosphate (25%–12%)	8.1	1.15	57.10	0.45	82.12	1.67	25.05	1.00	16.66
Ethanol–ammonium sulfate (25%–22%)	4.6	0.72	67.54	0.51	74.36	13.12	89.75	12.38	89.19
Ethanol–sodium dihydrogen phosphate (25%–20%)	4.7	1.16	57.53	1.40	23.59	6.80	90.29	7.41	91.03
Ethanol–dipotassium hydrogen phosphate (22.5%–20%)	7.6	1.83	40.52	1.63	13.66	69.13	98.95	50.12	98.56

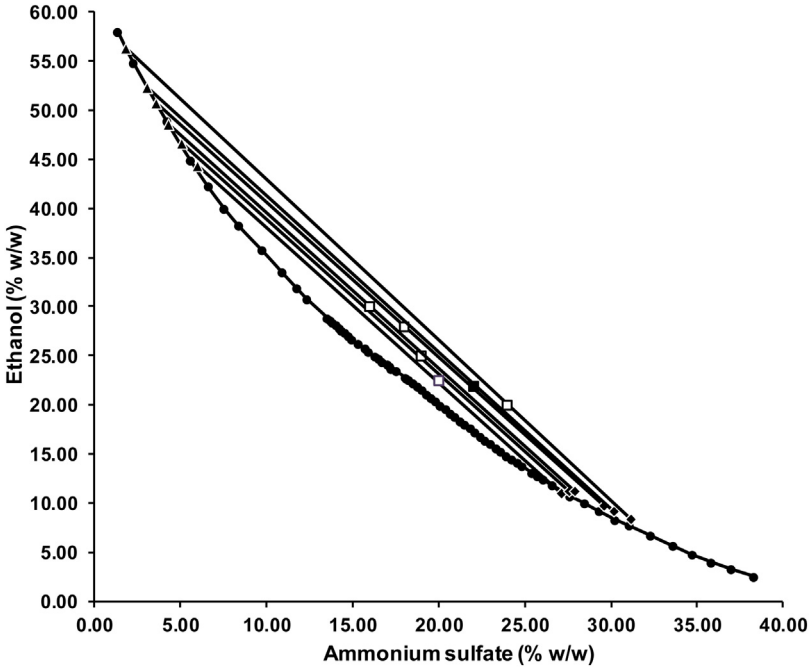

Fig. 5 – Phase diagram of ethanol-ammonium sulfate system at 303.15 K with Tie-lines. The binodal curve is represented by (●), whereas (▲) represents the equilibrium concentrations in the top phase, (♦) equilibrium concentrations in bottom phase and (□) total compositions of ATP system respectively.

Table 4 – Phase equilibrium compositions for the ethanol + $(NH_4)_2SO_4$ + H_2O system at 303.15 K.

Composition of ATPS (wt%) (C_2H_5OH/$(NH_4)_2SO_4$)	TLL (%)	Top phase (wt%)		Bottom phase (wt%)		Slope
		W_1	W_2	W_1	W_2	
23/20	38.60	44.32	6.00	12.02	27.14	−1.581
25/19	41.37	46.61	5.08	11.69	27.27	−1.576
30/16	43.28	48.61	4.33	11.94	27.32	−1.574
28/18	48.55	50.71	3.59	9.72	29.6	−1.528
22/22	50.44	52.33	3.05	9.55	29.78	−1.600
20/24	56.29	56.36	1.81	8.31	31.14	−1.638

for the partitioning. Hence, the effect of phase forming components ethanol–$(NH_4)_2SO_4$) concentration on the partitioning of ACN, HCA, GL, and IGL were studied by varying the TLL. The phase diagram, including the binodal curve and the tie lines, considered in the partitioning process was developed and presented in Fig. 5. The liquid–liquid equilibrium (LLE) compositions of the ethanol–$(NH_4)_2SO_4$)–water system was calculated by simultaneously solving the binodal equations, lever arm rule and the mass balance equations Eqs. (4)–(8) using MATLAB (Wang et al., 2011, 2009) (Table 4). The equilibrium concentrations were further utilized to calculate the TLL using Eq. (9) (Table 4).

The partition coefficient of all the four components found to increases with increasing TLL till 48 (Fig. 6) and remained constant with further increase in TLL to 50 and 58. However, the K_{GL} and K_{IGL} were found to increase up to the TLL of 43.28 and remain constant (Fig. 6) which indicates that the higher ethanol concentration in the top phase may not require to

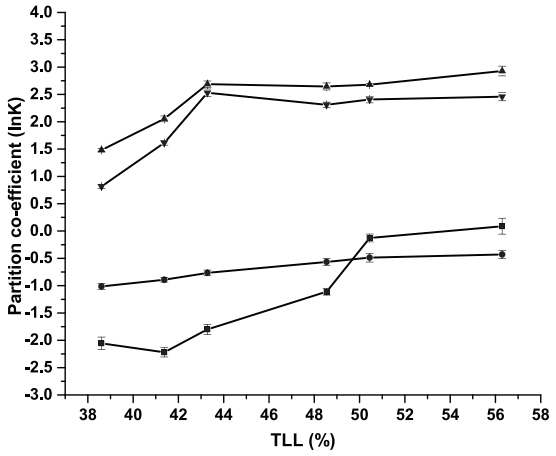

Fig. 6 – Effect of TLL of ethanol and ammonium sulfate ATPS on the partition coefficient of ACN (■) and HCA (●) in bottom phase and GL (▲) and IGL (▼) in top phase.

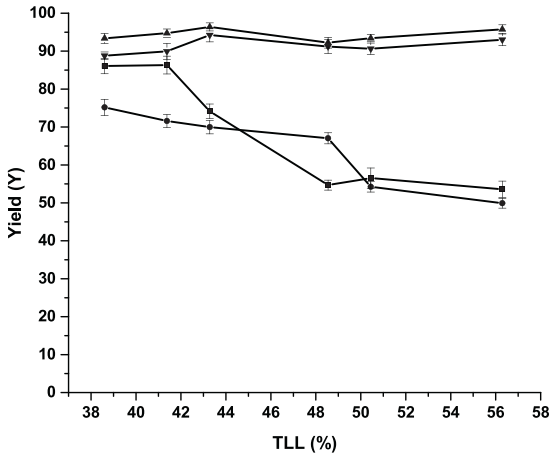

Fig. 7 – Effect of TLL of ethanol–ammonium sulfate ATPS on the % yield of ACN (■) and HCA (●) in bottom phase and GL (▲) and IGL (▼) in top phase.

partition the GL and IGL into the top phase. The lower TLL is enough to partition ACN and HCA compared to GL and IGL. The K_{ACN} and K_{HCA} also found to increase with increasing TLL due to the rejection of ACN and HCA from the bottom phase to top phase. The GL and IGL yield were not influenced by the TLL due to the increased solubility of GL and IGL into the ethanol, however the ACN and HCA yield in the bottom phase was gradually decreased with increasing TLL (Fig. 7), since the higher salt concentration in the bottom phase at higher TLL excluded the solutes, ACN and HCA, to the top phase. Further, the bottom phase exhibits the lower solute equilibrium concentrations due to the non-availability of free water solvent. The solvent water was utilized for the dissolution of higher concentration of phase forming a salt, $(NH_4)_2SO_4$. The higher yield of ACN (86.33%) and HCA (75.17%) in bottom phase (Fig. 7) with a partition coefficient of $K_{ACN} = 0.109$ and $K_{HCA} = 0.363$ and GL (96.39%), IGL (94.26%) yield in top phase were observed between the TLL of 38.60 and 43.28 (Figs. 6 and 7).

4. Conclusion

The valuable bioactive compounds present in the fruit of *G. indica* (GI) were explored for the simultaneous extraction. The aqueous mixture of solvents, 1-propanol (50–60%) and ethanol (80%) were identified as the solvent mixture, which can simultaneously extracts all the bioactive components namely ACN, HCA, GL and IGL. The solvent to solid ratio studies revealed that the ratio of 1:10 able to provide the maximum yield of bioactive components in the crude extract. PEG-salt systems failed to partition the GL and IGL, however, the ACN and HCA were partitioned to the top and bottom phases of the ATPS, respectively. The alcohol-based ATPS showed the simultaneous partitioning of GL and IGL to the top phase and ACN and HCA to the bottom phase. Ethanol-ammonium sulfate system was considered to study the effect of TLL on the partition coefficient and yield of all the four bioactive components and found that the ATPS with lower TLL values between 38.60 and 43.28 showed better yield of ACN (86.33%), HCA (75.17%) in bottom phase and GL (96.39%), IGL (94.26%) in top phase. The present work paved a way to extract all the four bioactive components simultaneously from GI fruit and simplify the initial partitioning of biomolecules using ATPE for the easier purification.

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