MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society For Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

ENHANCEMENT OF ANTI-ADVANCE GLYCATION END PRODUCT FORMATION AND ANTIOXIDANT ACTIVITY OF SALAK PEEL EXTRACTS USING BETAINE-BASED DEEP EUTECTIC SOLVENTS

Raihan Farhana Zainudin[1], Mardiah Zainal Abidin[1], Aliana Najwa Hamsah Zollappi[1], Nor Liana Aqilah Mohd Yatim[1], Norain Mohd Tamsir[2], Muhajir Hamid[3], Mohd Ezuan Khayat[1*]

[1]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.
[2]Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.
[3]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia.

*Corresponding Author: m_ezuan@upm.edu.my

History
Received: 24th May 2020
Accepted: 6th August 2020

Keywords:

Salak peel; deep eutectic solvent; antioxidant; advance glycation end product.

Abstract

Advance glycation end products (AGEs) are harmful compounds produced through a non-enzymatic-glycation between proteins and sugars in the bloodstreams. Their accumulation triggers the production of reactive oxygen species which disturbs the functions of the cells and implicates in many diseases. In this study, we compared the anti-AGE and antioxidant properties of salak fruit peels extracted using four betaine-based deep eutectic solvents (DES) and a conventional solvent (aqueous ethanol). Betaine:sorbitol were found to be the most effective solvent for extracting polyphenols from salak peels while for flavonoid extraction, both betaine:sorbitol and betaine:propylene glycol exhibited the highest efficiency. The antioxidant activity of betaine:sorbitol extract was also found to be highest when assayed using FRAP assay while in another assay (DDPH assay), the antioxidant activity of betaine:citric acid:water extract was found to be the best. As for anti-AGEs property, the highest activity was found in betaine:sorbitol, betaine:glycerin and betaine:propylene glycol extracts. The flavonoids of the extracts showed high correlations with the anti-AGEs activities. However, they were found to be correlated only with the antioxidant activity obtained from FRAP assay but not from DPPH assay. It can be concluded from this study that the utilization of DES could yield better bio-activities of salak peel extracts.

INTRODUCTION

Fruits are among the most utilized commodities among horticulture crops [1]. With the increase of global population, the generation of fruit peel wastes can be massive and causes a serious environmental problem. Thus, recovering the bioactive compounds from the wastes for different industries such as pharmaceuticals can minimize the pollutions and losses. One of fruits that has attracted consumer interest is salak (*Salacca zalacca*) fruit due to its taste and nutritional values. The pulps are consumed in fresh fruit while the peels are commonly thrown away.

However, numerous studies have shown the salak peels also exhibit several bioactivities such as antioxidant and anti-diabetic properties [2]. The chemical constituent of salak peels shows they contain phenolic compounds such as gallic acid, chlorogenic acids, and quercetin [3]. Previously, these bioactive compounds from salak peels were successfully extracted using conventional solvents such as ethanol [3]. However, these solvents are highly flammable,

toxic and hazardous to the environment as their manufacturing depends on fossil resources [4]. As alternative, a new green solvent known as Deep Eutectic Solvent (DES) was introduced. Natural deep eutectic solvent is a mixture two or more components that are generally plant-based primary metabolites, such as organic acids, sugars, alcohols, amines, and amino acids, at particular molar ratio with greatly depressed freezing points relative to their components [5]. It possesses several advantages over the classical solvent such as having low toxicity and allowing the extraction of compounds with different polarities [6].

Type 2 diabetes mellitus is a metabolic disorder that characterized by elevated levels of blood glucose, as the result of the defects in insulin secretion, insulin action, or both [7]. The high level of glucose in the blood can lead to the formation of AGEs through a non-enzymatic-glycation between sugars and proteins [7]. The AGEs formation starts with the formation of the schiff bases and Amadori products as the result of the interaction between the carbonyl groups of glucose and amino groups of proteins. Further glycations lead the intermediates to undergo molecular rearrangements that resulting in AGEs generation [8]. The accumulations of AGEs in vessel wall are detrimental to human health. They have been shown to interact with the receptor for advanced glycation end products (RAGE) to produce reactive oxidative species (ROS) which can disturb the structures and functions of the cells [8]. Advance glycation end products have been implicated in many microvascular and macrovascular complications of diabetes such as retinopathy, nephropathy, neuropathy, ischemic heart disease, peripheral vascular disease, and cerebrovascular disease [9].

To address the problem, some synthetic compounds have been evaluated for their potentials of inhibiting AGEs formation. However, despite their inhibitory capacities against AGEs formation, none of them have passed the clinical trial so far due to relatively low efficacies, poor pharmacokinetics, and unsatisfactory safety [10]. Owing to these drawbacks, researchers turn their focus on natural compounds as a safer option. Polyphenols, particularly flavonoids, have often been reported to be able to inhibit AGEs formation. Moreover, they also possess antioxidant activity, which is important for combating ROS [10]. One of the sources of polyphenols is fruit peel, which can be utilised for the recovery of polyphenols for other industries such as pharmaceuticals [1].

In this study we were comparing the extraction of phenolic compounds and flavonoid from salak peels using ethanol and betaine-based DES. Moreover, the antioxidant and anti-AGE formation capacities of extracts from both solvents were evaluated. To our knowledge, this is the first time such comparative study was done on salak peels.

MATERIALS AND METHODS

Chemicals and Materials

Ethanol, methanol, sodium acetate (CH_3COONa) and Folin-Ciocalteau reagent were purchased from Merck (Darmstadt, Germany). Aluminium Chloride ($AlCl_3$), 2, 4, 6-Tris (2-pyridyl)-s-triazine (TPTZ), gallic acid, Iron (II) Sulphate (Heptahydrate) (FeSO4.7H2O), quercetin, bovine serum albumin (BSA), Sodium Carbonate (Na_2CO_3), and Sodium Azide (NaN_3) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Iron (III) Chloride ($FeCl_3$) was purchased from Fisher-Sientific (New Jersey, USA). Glacial acetic acid was purchased from HmbG Chemicals (Hamburg, Germany). Sodium Nitrite ($NaNO_2$) was purchased from Bio Basic (Toronto, Canada). Citric acid was purchased from Ungerer (New South Wales, Australia). Betaine was purchased from Cansun (Selangor, Malaysia). Sorbitol and glycerin were purchased from EvaChem (Selangor, Malaysia). The fruits of salak were bought from local market in Selangor, Malaysia. The fruit peels were dried for few days and grounded into a fine powder using a laboratory blender.

Preparation of DES

Betaine and HBDs (glycerin, propylene glycol, citric acid and sorbitol) were dried in incubator oven at 60 ˚C 24 hours before mixed at different molar ratio as shown in Table 1. The mixture was heated at 90 ˚C and stirred at 1000 x*g* until a homogeneous transparent colourless liquid was formed. DES solutions were kept in sealed glass tubes in the dark at room temperature until used.

Table 1: Betaine –based deep eutectic solvents (DES)

HBA	HBD	Molar ratio	Appearance
Betaine	Propylene glycol	1:4	Clear liquid
	Glycerin	1:3	Clear liquid
	Citric acid : Water	1:2:9	Clear liquid; Viscous
	Sorbitol	1:2	Clear liquid

Extraction of Salak Fruit Peels by Using Ultrasound-Assisted Extraction

The extractions were carried out according to a previously described method by Mouratoglou *et al.* with slightly modification [4]. Approximately 0.05 g of powdered salak fruit peels were mixed with 5 ml of solvent of interest (DES with 10 % of water or ethanol) in the falcon tubes. The mixtures were sonicated in ultrasound bath (Branson, USA) at 40 °C for 90 minutes. The mixtures were then centrifuged at 1000 x*g* for 10 minutes at room temperature using a bench top centrifuge (Universal 32, Hettich, Germany). The supernatant was collected and used for further analyses.

Determination of Total Phenolic Content

The protocol previously described by Wong-Paz *et al*. using Folin-Ciocalteu reagent was employed for the determination of total phenolic content (TPC) in the extracts [11]. Briefly, 20 µl of extracts or standard were incubated with 100 µl of 10 % (v/v) Folin-Ciocalteu reagent in 96-well plates. After 5 minutes of the incubation, 100 µl of 7.5 % Na_2CO_3 solution was added. The mixtures were then further incubated in the dark for 30 minutes at room temperature. At the end of the incubation period, the absorbance was measured at 750 nm using microplate reader (iMark^{TM}, Bio-Rad Laboratories, Inc.). The results were expressed as mg of gallic acid equivalent (GAE)/ g of the dried weight of the powdered peels.

Determination of Total Flavonoid Content

Total flavonoid content (TFC) of the extracts was measured using aluminium colorimetric method according to Chang *et al.* with slightly modification [12]. A hundred microliter of extracts were mixed with 10 µl of 5 % (w/v) sodium nitrite in 96-well microplate and incubated for 5 minutes. Then, 100 µl of 1 M NaOH and 30 µl of distilled water were added to the mixtures. Absorbance was read at 490nm using microplate reader. The results were expressed as mg of quercetin equivalence (GAE)/ g of the dried weight of the powdered peels.

Determination of Antioxidant Activity

1,1-diphenyl-2-picrylhydrazyl DPPH Assay

The DPPH free radical assay was carried out according to Wong-Paz *et al.* with a slight modification [11]. Briefly, 20 µl of each sample was mixed with 180 µl of 0.2 mM DPPH in 100% methanol in 96-well plate. Then, the plate was incubated in a dark room at room temperature for 30 minutes and the absorbance (A) was measured at 540 nm using the microplate reader (Infinite F50, Tecan). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity or percentage inhibition. The percentage inhibition was calculated by using the formula:

$$\% \text{ Inhibition} = ((Ac-As))/Ac \times 100$$

Where Ac represents the absorbance value of control reaction (containing all reagents except sample) and As represents the absorbance value of sample. All measurements were performed in triplicate and the data was the averaged.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was carried out according to Clarke *et al.* [13]. Briefly, 20µl of sample was mixed with 1180 µl of the FRAP reagents (300 mM acetate buffer, pH 3.6, 10 mM TPTZ, 20 mM $FeCl_3$) in 96-well microplate and incubated in dark at room temperature for 30 minutes. Then, the absorbance was measured at 595 nm using the microplate reader. The FRAP value was expressed as mmole of $FeSO_4$ equivalent/g of the dried weight of the powdered peels.

Determination of Anti-glycation Activity

The inhibition of the formation of AGE was determined by using BSA-fructose assay as described Justino *et al.* with slightly modification [14]. The BSA-fructose assay model evaluates all stages of protein glycation. The extracts were incubated in the dark with 50 mg/ml BSA and 1.25 M fructose containing 200 mM phosphate buffer, pH 7.4 and 0.02 % (w/v) sodium azide at 37 °C for 7 days. Then, 20% (w/v) trifluoroacetic acid was added to the mixture and centrifuged at 10,000 x*g* at 4 ˚C for 10 minutes. The pellet was resuspended in phosphate buffer, and the fluorescence intensity (350 nm_{ex}/420 nm_{em}) was measured using the fluorescence reader (Synergy^{HI}, BioTek, Inc.). Quercetin was used as a positive control and water as the blank. The results were presented as a percentage of glycation inhibition (GI), calculated according to the following equation:

$$GI\ (\%) = 100 - \frac{(F\ sample - F\ blank)}{(F\ control - F\ blank)} \times 100$$

Where *F sample* is fluorescence intensity in presence of extract, *F blank* is fluorescence intensity of water and *F control* is fluorescence intensity in the absence of extract.

Statistical Analysis

The results were expressed as mean ± SEM of three replicated experiments. The data were analysed by One-Way ANOVA and the significance of the difference between means was determined by Tukey's multiple comparison test ($P < 0.05$) using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS AND DISCUSSION

Synthesis of DES

Deep eutectic solvents were proposed as alternatives to organic solvents in extraction of value-added products from agriculture wastes since they are generally safer and environmentally friendly [4][15]. On that ground, we attempted to synthesize DES that can efficiently recover bioactive compounds from fruit waste, salak peels, using substances that are cheap, abundant, and compatible with food and pharmaceutical formulations such as betaine, glycerin, citric acid and propylene glycol. In this study, DES were successfully synthesized by mixing HBA (betaine) with HBDs (glycerin, citric acid and propylene glycol) at molar ratio shown in Table 1. All DES were found to be stable in liquid form over time. Among the DES combination, betaine-citric acid was found to produce viscous solvent. In order to reduce its viscosity, the third component, water was added. Even the attempt succeeded to reduce the viscosity of the DES at molar ratio of 1:2:9 (betaine:citricacid:water), however, the viscosity was still higher compared to other DES. Since the high amount of water can destroy the hydrogen bond framework of DES, thus, no further addition of water was carried out [16]. In accordance, Aroso *et al.* observed similar high viscosity of betaine:citric acid DES, which required water as the third component in order for it to be working [17].

Extraction Efficiency of DES

The efficiency of the DES in recovering polyphenols from the fruit peels was evaluated on two indices, which are TPC and TFC. For the critical comparison, 70% (v/v) of aqueous ethanol was chosen as the control solvent since it was successfully used to extract polyphenols from salak peels [3]. As illustrated in Table 1A, the utilization of the DES except betaine:citric acid:water in the extraction yielded a higher TPC value compared to aqueous ethanol. The highest TPC was obtained with betaine-sorbitol followed by betaine:glycerin, betaine:propylene glycol, aqueous ethanol and betaine:citric acid:water with values ranged from 45.05±0.56 to 228.38 ±1.05mg GAE/g D.W. For flavonoid extraction, the utilization of betaine:sorbitol, betaine:propylene glycol and betaine:glycerin gave a higher TFC value compared to aqueous ethanol whereas betaine:citric acid:water gave a lower value. The highest TFC values were recorded in extracts with betaine:sorbitol and betaine:propylene glycol (138.91 ± 0.83 mg QE/ g D.W. and 136.16 ± 1.14 mg QE/ g D.W.; p>0.05) (Figure 1B).

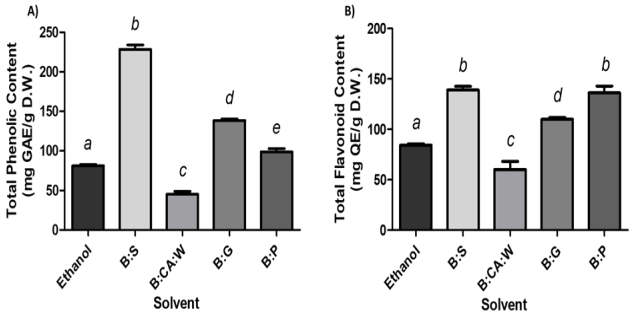

Figure 1. Effect of extraction solvents on A) total phenolic content and B) total flavonoid content of salak peel extracts. Data are mean ± SEM of 3 replicates. Different labels indicate the significant difference at P≤0.05 by one-way ANOVA with Tukey's post hoc test with water as control solvent. B:S : Betaine:sorbitol; B:CA:W : Betaine:citric acid:water; B:G : Betaine:glycerin; B:P : Betaine:propylene glycol.

The results show a great variation in extraction efficiencies of the DES used in the study. This may be explained by the different in their structures which influences their physico-chemical properties [6][18]. The DES containing organic acids are more polar than water, while the polarities of sugar and poly-alcohols containing DES are close to methanol [19]. It is known that the extraction efficiency increases when the polarity of DES used close to the polarity of targeted compounds [6]. Thus, the higher extraction efficiencies od most DES than aqueous ethanol suggest that their polarities are match better to the polarities of the polyphenols in salak peels as compared to ethanol. However, due to the limited information regarding the polarity of DES used in this study, thus the hypothesis remains to be elucidated. As for the low extraction efficiency of betaine:citric acid:water, it may be possibly caused by its high viscosity. The high viscosity of DES is the major drawback that restricts their use as extraction solvents as it hampers their penetrations into the extraction matrix [20]. Apart from that, the high-water content in the DES used to lower its viscosity may also reduce the intermolecular interaction between the solvent and polyphenols, thus reduced the extraction efficiency.

Antioxidant Activity of Salak Peel Extracts

The utilization of different solvents in the extractions is likely to affect the antioxidant properties of the extracts as well. To confirm that, the antioxidant activities of the salak peel extracts were evaluated using DPPH and FRAP assays. DPPH assay represents the ability of antioxidant to reduce free radicals via electron and hydrogen transfer mechanisms while FRAP assay reflects the ability of antioxidant to reduce the radicals through electron transfer only [21].

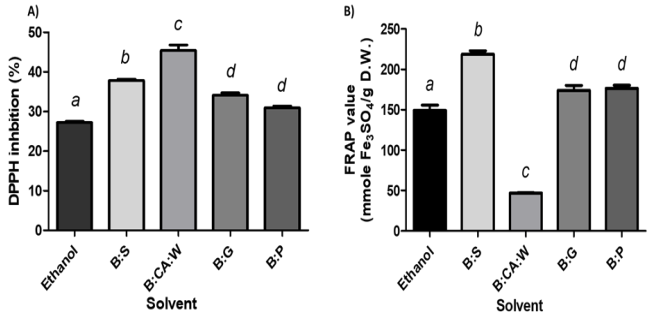

Figure 2. Effect of extraction solvent on A) DPPH antioxidant activity and B) FRAP antioxidant activity of salak peel extracts. Data are mean ± SEM of 3 replicates. Different labels indicate the significant difference at P≤0.05 by one-way ANOVA with Tukeys's post hoc test. B:S : Betaine:sorbitol; B:CA:W : Betaine:citric acid:water; B:G : Betaine:glycerin; B:P : Betaine:propylene glycol.

Indeed, as shown in (Figure 2A), the DPPH scavenging activity of the extracts was varied according to the solvents used. The highest percentage of DPPH radical inhibition was found in the betaine:citric acid:water extract (45.44±0.51%) followed by betaine:glycerin extract (37.81±0.27%), betaine:glycerin and betaine:propylene glycol extracts (37.81±0.27%; 34.12± 0.34%; p>0.05), and aqueous ethanol extract (30.93 ± 0.30%). These show that the application of DES yielded a higher scavenging activity than aqueous ethanol. Interestingly, the high scavenging activity of betaine:citric acid:water extract seems not associated to its low TPC and TFC. In contrast, several studies have indicated the DPPH scavenging activities of plant extracts were positively related to their TPC and TFC [22][23]. Such discrepancy may be caused by the synergetic action of the DES component citric acid that enhanced the DPPH scavenging activity of the extract. It was previously reported that the addition of citric acid has increased the DPPH scavenging activity of ascorbic acid [24]. As expected, the results from Pearson's correlation test show that the DPPH scavenging activity of the extracts is not correlate with their TPC (p>0.05, r=0.003) and TFC (p>0.05, r=0.132) (Table 2).

Table 2: Correlation between total phenolic and total flavonoid contents with antioxidant activity and anti-AGEs.

	Correlation coefficient (r^2)		
	Antioxidant (FRAP)	Antioxidant (DPPH)	Anti-AGEs
TPC	No correlation	No correlation	No correlation
TFC	0.880	No correlation	0.968

In FRAP assay, the rank of FRAP values was as follows: betaine:sorbitol extract (218.67±0.91mmoles Fe_3SO_4/g D.W.) > betaine: propylene glycol and betaine: glycerin extracts (176.62±0.85mmoles Fe_3SO_4/g D.W. and 174.13±1.08 mmoles Fe_3SO_4/g D.W.; p>0.05) > aqueous ethanol extract (149.24 ± 1.12 mmoles Fe_3SO_4/g D.W.) > betaine:citric acid:water extract (46.89±0.30mmoles Fe_3SO_4/g D.W.) (Figure 2B). It is compelling to learn that the high scavenging activity of betaine:citric acid:water extract in DPPH assay was absent in FRAP assay. This may possibly happen due to the compounds in betaine:citric acid: extract were also reducing the free radicals via hydrogen donation, which can only be assessed through DPPH assay.

Based on Pearson's test, a high positive correlation was obtained between the ferric reducing power and TFC (p < 0.05, r = 0.879). In contrast no correlation was calculated between ferric reducing power and TPC (p > 0.05, r = 0.635) (Table 2). The results suggest that the extracts from salak peels that enriched in flavonoids can be expected to exert stronger ferric reducing power. Previously, Kottaras *et al.* were reported a similar observation where the barley bran extract that showed highest TFC among the studied cereal wastes extracts also exhibited the strongest ferric reducing power [15]. However, in contrast, Chavan *et al.* reported a low correlation between total flavonoid and ferric reducing power in the extracts of three *Ceropegia* spp. [25].

Advance Glycation End Product Inhibition Effect of Salak Peel Extracts

The anti-AGE formation ability of salak peel extract was first reported in this study. The effect of various extractants against AGE formation was investigated by detecting the fluorescence emission generated by glycated BSA. Based on Figure 3, all extracts showed positive influences on the inhibition of AGE formation. Betaine:sorbitol extract (89.02±0.31%), betaine:propylene glycol extract (85.03±2.00%) and betaine:glycerin extract (81.95±0.15%) were shown to be the most effective AGE formation inhibitors (p>0.05 between their AGE formation inhibitions) whereas aqueous ethanol extract (71.67±3.71%) and betaine:citric acid:water extract (50.89±0.85%) possess the lowest ability to inhibit AGE formation.

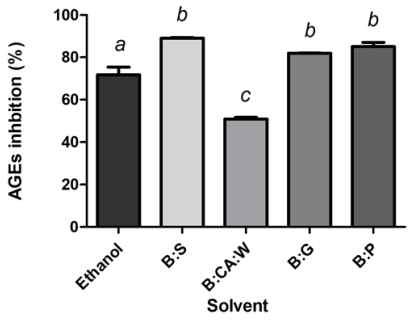

Figure 3: Effect of extraction solvent on AGEs inhibition of salak peel extracts. Data are mean ± SEM of 3 replicates. Different labels indicate the significant difference at P≤0.05 by one-way ANOVA with Dunnet's post hoc test with water as control solvent. B:S : Betaine:sorbitol; B:CA:W : Betaine:citric acid:water; B:G : Betaine:glycerin; B:P : Betaine:propylene glycol.

From the data, it is shown that betaine:sorbitol was one of the efficient DES for extracting AGE formation inhibitor from salak peels. However, it should be considered that sorbitol is the intermediates for AGE formation in polyol pathway. In the pathway, sorbitol is converted to fructose to form the highly reactive dicarbonyl compounds which can be further glycated into AGE [7].

According to Pearson's test (Table 2), a positively correlation was found between AGE formation inhibition and TFC (p>0.05, r^2=0.968) and between AGE formation inhibition and ferric reducing power (p<0.05, r^2=0.635). On the other hand, no correlation was obtained between AGE formation inhibition and TPC (p>0.05, r^2=0.968) and between AGE formation inhibition and DPPH scavenging activity (p<0.05, r^2=0.635) (Figure 2). These observations suggest that the AGE formation inhibition activity of the extracts was attributed to their flavonoids. Furthermore, the inhibition of AGE formation by the extracts is most likely to involve the electron transfer rather than hydrogen transfer mechanism, as occurs in FRAP assay. In accordance, the AGE formation inhibition of *Scutellaria alpina* L. and *S. altissima* L. extracts were also found to be more correlated to TFC and ferric reducing power than DPPH scavenging activity [26].

CONCLUSION

Our study shows for the first time that extract from salak peels possess anti-AGEs property. We also further investigate the effect of different solvents used as extractants on the bio-activities of the extracts. From the results, it can be concluded that the usage of DES in the extraction of salak peels increased the yield of polyphenols and flavonoids. Furthermore, the anti-AGE and antioxidant activities of the extracts were also higher when DES were utilised. From the correlation study, it is clear that the increase of anti-AGEs activity in the extracts was due to the higher extraction yields of flavonoids from the peels. However, there are mix conclusions can be drawn for the correlation between the flavonoids with the antioxidant activities of the extracts. The antioxidant activity measured with FRAP assay showed a correlation with the flavonoid contents while the same activity measured using DPPH assay showed no correlation with these compounds. It might be happened due to the ability of these assays to measure different antioxidant mechanisms thus resulting in different measurement of the activity. Overall, it can be summarised that the implementation of DES in salak peel extraction process can enhance their anti-AGEs and antioxidant activities. In future, the optimisation of other parameters in the extraction process should be carried out in future to further increase the bioactivities of salak peel extract.

REFERENCES

1. Sagar, N.A., Pareek, S., Sharma, S., Yahia, E.M. and Lobo, M.G. 2018. Fruit and Vegetable Waste: Bioactive Compounds, Their Extraction, and Possible Utilization. *Comprehensive Reviews in Food Science and Food Safety*, 17(3): 512–31.

2. Saleh, M.S., Siddiqui, M.J., So'ad, S.Z., Murugesu, S., Khatib, A. and Rahman, M.M. 2018. Antioxidant and Alpha-Glucosidase Inhibitory Activities and Gas Chromatography-Mass Spectrometry Profile of Salak (Salacca Zalacca) Fruit Peel Extracts. *Pharmacognosy Research*, 10(4): 385–90.

3. Kanlayavattanakul, M., Lourith, N., Ospondpant, D., Ruktanonchai, U., Pongpunyayuen, S. and Chansriniyom, C. 2013. Salak Plum Peel Extract as a Safe and Efficient Antioxidant Appraisal for Cosmetics. *Bioscience, Biotechnology and Biochemistry*, 77(5): 1068–74.

4. Mouratoglou, E., Malliou, V. and Makris, D.P. 2016. Novel Glycerol-Based Natural Eutectic Mixtures and Their Efficiency in the Ultrasound-Assisted Extraction of Antioxidant Polyphenols from Agri-Food Waste Biomass. *Waste and Biomass Valorization*, 7(6): 1377–87.

5. Liu, Y., Friesen, J.B., McAlpine, J.B., Lankin, D.C., Chen, S.N. and Pauli, G.F. 2018. Natural Deep Eutectic Solvents: Properties, Applications, and Perspectives. *Journal of natural products*, 81(3): 679–690.

6. Skarpalezos, D,. and Detsi, A. 2019. Deep Eutectic Solvents as Extraction Media for Valuable Flavonoids from Natural Sources. *Applied Sciences*, 9(19): 4169.

7. Nowotny, K., Jung, T., Höhn, A., Weber, D. and Grune, T. 2015. Advanced Glycation End Products and Oxidative Stress in Type 2 Diabetes Mellitus. *Biomolecules*, 5(1): 194–222.

8. Schmidt, A.M., Hori, O., Brett, J., Yan, S.D., Wautier, J.L. and Stern, D. 1994. Cellular Receptors for Advanced Glycation End Products. Implications for Induction of Oxidant Stress and Cellular Dysfunction in the Pathogenesis of Vascular Lesions. *Arteriosclerosis and Thrombosis*, 14(10):1521–1528.

9. Cade, W. T. 2008. Diabetes-Related Microvascular and Macrovascular Diseases in the Physical Therapy Setting. *Physical Therapy*, 88(11): 1322–35.

10. Odjakova, M., Popova, E., Al Sharif, M. and Mironova, R. 2012. Plant-Derived Agents with Anti-Glycation Activity Plant-Derived Agents with Anti-Glycation Activity. *Glycosylation*, 223–256.

11. Wong-Paz J.E., Muñiz-Márquez, D.B., Aguilar-Zárate, P., Rodríguez-Herrera, R., Aguilar, C.N. 2014. Microplate Quantification of Total Phenolic Content from Plant Extracts Obtained by Conventional and Ultrasound Methods. *Phytochemical Analysis*, 25(5): 439–44.

12. Chang, C.C., Yang, M.H., Wen, H.M. and Chern. J.C. 2002. Estimation of Total Flavonoid Content in Propolis By. *Journal of Food and Drug Analysis*, 10(3): 7802.

13. Clarke, G., Ting, K.N., Wiart, C. and Fry, J.. 2013. High Correlation of 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical Scavenging, Ferric Reducing Activity Potential and Total Phenolics Content Indicates Redundancy in Use of All Three Assays to Screen for Antioxidant Activity of Extracts of Plants from the Malaysian Forest. *Antioxidants*, 2(1): 1–10.

14. Justino, A.B., Miranda, N.C., Franco, R.R., Martins, M.M., Silva, N.M.D. and Espindola, F.S. 2018. Annona Muricata Linn. Leaf as a Source of Antioxidant Compounds with in Vitro Antidiabetic and Inhibitory Potential against α-Amylase, α-Glucosidase, Lipase, Non-Enzymatic Glycation and Lipid Peroxidation. *Biomedicine and Pharmacotherapy*, 100: 83–92.

15. Kottaras, P., Koulianos, M. and Makris, D.P. 2017. Low-Transition Temperature Mixtures (LTTMs) Made of Bioorganic Molecules: Enhanced Extraction of Antioxidant Phenolics from Industrial Cereal Solid Wastes. Recycling, 2(1): 3.

16. Dai, Y., Witkamp, G.J., Verpoorte, R. and Choi, Y.H.. 2015. Tailoring Properties of Natural Deep Eutectic Solvents with Water to Facilitate Their Applications. *Food Chemistry*, 187: 14–19.

17. Aroso, I.M., Paiva, A., Reis, R.L. and Ana Duarte, R.C. 2017. Natural Deep Eutectic Solvents from Choline Chloride and Betaine – Physicochemical Properties. *Journal of Molecular Liquids*, 241: 654–61.

18. Radošević, K., Ćurko, N., Srček, V.G., Bubalo, M.C., Tomašević, M., Ganić, K.K. and Redovniković, I.R. 2016. Natural Deep Eutectic Solvents as Beneficial Extractants for Enhancement of Plant Extracts Bioactivity. *LWT - Food Science and Technology*, 73: 45–51.

19. Dai, Y., Spronsen, J.V., Witkamp, G.J., Verpoorte, R. and Choi, Y.H. 2013. Natural Deep Eutectic Solvents as New Potential Media for Green Technology. *Analytica Chimica Acta*, 766: 61–68.

20. Ruesgas-Ramón, M., Figueroa-Espinoza, M.C. and Durand, E. 2017. Application of Deep Eutectic Solvents (DES) for Phenolic Compounds Extraction: Overview, Challenges, and Opportunities. *Journal of Agricultural and Food Chemistry*, 65(18): 3591–3601.

21. Prior, R.L., Wu, X. and Schaich, K. 2005. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *Journal of Agricultural and Food Chemistry*, 53(10): 4290–4302.

22. Aryal, S., Baniya, M.K., Danekhu, K., Kunwar, P., Gurung, R. and Koirala, N . 2019. Total Phenolic Content, Flavonoid Content and Antioxidant Potential of Wild Vegetables from Western Nepal. *Plants*, 8(4): 96.

23. Piluzza, G. and Bullitta, S. 2011. Correlations between Phenolic Content and Antioxidant Properties in Twenty-Four Plant Species of Traditional Ethnoveterinary Use in the Mediterranean Area. *Pharmaceutical Biology*, 49(3): 240–47.

24. Lo Scalzo, R. 2008. Organic Acids Influence on DPPH Scavenging by Ascorbic Acid. *Food Chemistry*, 107(1): 40–43.

25. Chavan, J. J., Gaikwad, N.B., Kshirsagar, P.R. and Dixit. G.B. 2013. Total Phenolics, Flavonoids and Antioxidant Properties of Three Ceropegia Species from Western Ghats of India. *South African Journal of Botany*, 88: 273–77.

26. Grzegorczyk-Karolak, I, Gołąb, K., Gburek, J., Wysokińska, H. and Matkowski A. 2016. "Inhibition of Advanced Glycation End-Product Formation and Antioxidant Activity by Extracts and Polyphenols from *Scutellaria Alpina* L. and *S. Altissima* L. *Molecules,* 21(6): 1–10.