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ORIGINAL ARTICLE

Biochemical Pathway of Depressive Disorder Biomarkers in Human Urine Post Consumption of Phoenix dactylifera L. using ^{1}H-NMR

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Abstract

Our previous study found potential health-promoting effects in Ajwa dates flesh due to the presence of phytochemicals. In this study, metabolomics study on the consumption of Ajwa dates flesh has been conducted on human urine samples using proton Nuclear Magnetic Resonance (^{1}H-NMR) coupled with Principal Components Analysis (PCA). The identified chemical shifts in ^{1}H-NMR spectra were compared with the reference spectra in the Human Metabolome Database (HMDB). The results showed the significant decrease of alanine, hippurate and citrate in urine samples within 24 hours after intake of Ajwa dates. These metabolites are known as the biomarkers of depressive disorder for human.

Keywords: Metabolomics, Human Urine, Ajwa dates, Proton-Nuclear Magnetic Resonance (^{1}H-NMR), Principal Components Analysis (PCA)

Introduction

Nutritional metabolomics have great potential as an effective tool in integrating the human metabolic response to dietary interventions, nutrition and lifestyle habits. Metabolomics analysis is normally performed on biofluids, like blood, urine and feces samples (Savorani et al., 2013). Consumption of nutrients and bioactive compounds from food component interact with numerous targets, metabolic functions and pathways in the organism and hereby has the potential to reduce or increase the risk of diseases (Salvamani et al., 2016). Targeted and untargeted metabolomics has been shown to generate global metabolic profiles (fingerprints) representative for dietary

intake (Radjursoga et al., 2017). Nutritional metabolomics discover new biomarkers of nutritional exposure, nutritional status and nutritional impacts on disease (Rezzi et al., 2007).

Sample collection for urine metabolomics is non-invasive technique, easy to obtain in large volume and can be collected at any time (Savorani et al., 2013). The 24 hours urine collection allowed the researchers to investigate the activity of gut microbiota in healthy people during day and night as it indicates the presence of biomarkers. Peralbo and Castro (2012) reported that there is obvious change in the activity of gut microbiota with the circadian rhythm which provides complete excretion.

^{1}H-NMR-based metabolomics is widely used compared to MS-based metabolomics since it can simultaneously display resonance peaks resulting from hundreds of metabolites (Duarte et al., 2014; Gu et al., 2007) and requires little or no sample preparation (Gu et al., 2007; Lehtonen et al., 2013), thus making it suitable for high-throughput analysis (Nguyen et al., 2016). Furthermore, Nguyen et al. (2016) stated that the advantages of ^{1}H-NMR-based metabolomics approach are highly reproducible and non-destructive. The combination of ^{1}H-NMR-based metabolomics with multivariate statistical analysis is a powerful approach for the study of metabolomics (Gu et al., 2007), which may help to uncover features of the biomarkers that are still hidden (Fanos et al., 2014).

Date fruits are considered as ideal food because it consists of high nutrients and provide beneficial effects on human health (Ahmed et al., 2013; Assirey, 2015). Our previous study for Ajwa dates flesh found that the fruit possess bioactive compounds; phenolic acids, fatty acids, flavonoids, procyanidin and sterols (Elma et al., 2018). Thus, the changes in human urine metabolites after the consumption of Ajwa dates were investigated to relate their beneficial for human body.

Materials and Methods

Chemical

Trimethylsilylpropanoic acid (TSP) was purchased from Merck while deuterated water (D_2O) was bought from Sigma-Aldrich.

Subjects

Volunteers between 20 and 25 years old with body mass index of 20-25 were selected. Ten healthy volunteers (5 females and 5 males) were enrolled with informed written consent. This study was approved by the Human Ethics Committee (HEC) of Universiti Sains Islam Malaysia (USIM/JKEP/2016-14). Subjects were required to fasting overnight and followed the control diet, which exclude fruits and vegetables, supplements and beverages such as tea, coffee, fruit juice or any bicarbonate drinks.

Urine Samples

The study was conducted for 4 days (Day 1 to day 4). Control urine samples were taken at Day 1 (7 am and 7 pm) and Day 2 (7 am) before the consumption of Ajwa dates and labelled as 0h. After the comsumption, urine samples were taken on Day 2 (11 am, 3 pm and 7 pm), Day 3 (7 am, 7 pm) and Day 4 (7 am and 7 pm). They were labelled as 4h, 8h, 12h, 24h, 36h, 48h and 60h, respectively. All samples were kept in a freezer at -20 $^{\circ}$C.

Preparation of Urine Sample

Samples preparation and ^{1}H-NMR analysis were performed as described by Walsh et al. (2007) with some modifications. Briefly, the urine samples were thawed at room temperature (25 °C) before the analysis. An aliquot of urine (1000 µl) was centrifuged at 5000 rpm for 5 min to remove the precipitate. The supernatant (500 µl) was mixed with internal standard TSP (10 µl; 5 mg of TSP dissolved in 1000 µl of D_2O) and D_2O (250 µl). Prepared sample was then well mixed and the samples (600 µl) were transferred into a 5 mm NMR sample tube. ^{1}H-NMR analysis was performed at Malaysia Genome Institute.

^{1}H-NMR Spectroscopy Analysis

NMR analysis of urine samples was carried out on a Bruker Ascend spectrometer (BRUKER Corporation, United State) operating at 700 MHz for ^{1}H and equipped with a cryogenically cooled probe. Samples are automatically delivered to the spectrometer via flow injection as the instrument also includes a 24 sample automation system. The acquisition temperature (TE) was set to 298 K. All spectra were obtained with 32 scans (NS) after application of 4 dummy scans (DS). The relaxation delay (D1) was set to 2 s with the acquisition time (AQ) of 1.46 s. Thirty-two Free Induction Decays (FIDs) (TD) were required into 32768 (32 K) complex data points using a spectral width (SWH) of 11194.03 Hz. An exponential line-broadening function of 0.3 Hz was applied to the FID prior to Fourier Transformation. All spectra were processed manually phased, calibrated and baseline corrected within TOPSPIN 1.3 software. Chemical shift was internally referenced to TSP (δ 0.0 ppm).

Statistical Analysis

ChenomX and Human Metabolome Database (HMDB)

Metabolites identification was accomplished by using ^{1}H-NMR while Chenomx NMR suite 8.2 (Chenomx Inc., Edmonton, Canada) was used for spectral binning (size of bin = 0.04 ppm) in multivariate data analysis. Prior to integration, the water region was removed. Chemical shifts in ^{1}H-NMR spectra were compared with reference spectra in the Human Metabolome Database (HMDB) (Wishart et al., 2013).

Chemometrics

The raw data from ^{1}H-NMR analysis was exported into the Microsoft Excel as excel spreadsheet (XLS) file. The informations exported into data matrix for ^{1}H-NMR analysis is the chemical shift (after binning) is exported to columset / loadings while urine time collection is exported to rowset / scores into the data matrix. The details in the center of this data matrix are the intensity of the metabolites peaks. The preprocessing method used for this multivariate data is the maximum normalization which to avoid the ambiguity of the peaks intensity in the resolved profiles. Then the Principal Components Analysis (PCA) was performed on the data set using Unscrambler 10.3 (CAMO Software, Norway) to cluster data into the scores and loadings plot.

Results and Discussion

Principal Components Analysis (PCA)

PCA model was used to assist in comprehending the clustering patterns of observation. PCA plots in Figure 1 illustrated that urinary metabolites at 0 hour has been clustered together with urinary

metabolites after 24 hours (24 – 60 hours), meanwhile all 4 hours, 8 hours and 12 hours are clustered together in the opposite side. One sample of 36 hours in the opposite site is outlier.

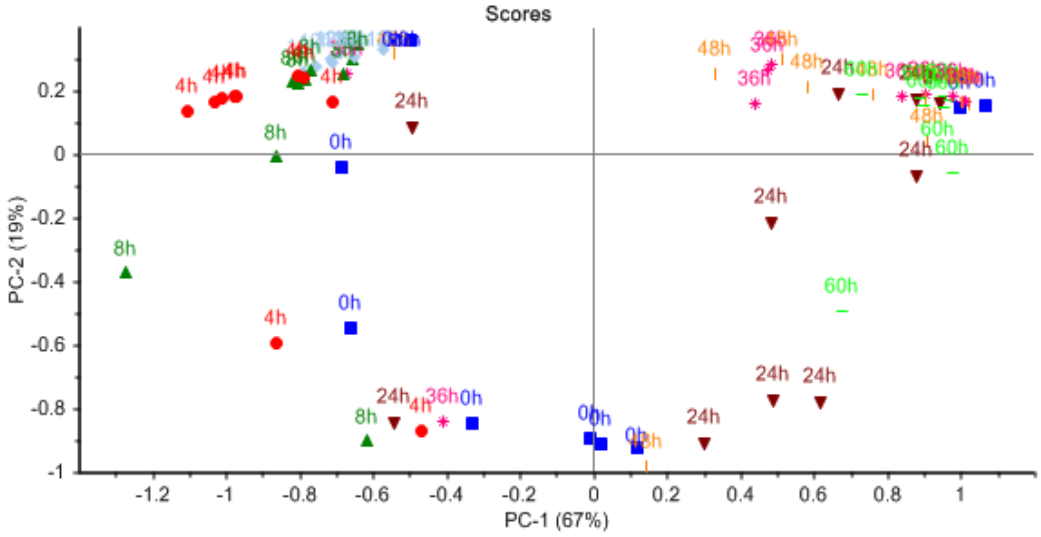

Figure 1. Scores Plot of ^{1}H-NMR Spectra from Day 1 to Day 4

Thus, ^{1}H-NMR spectrum for 0 hour to 24 hours was performed. The first principal component (PC1) and second principal component (PC2) accounted 58 % and 24 % of variance, respectively, was obtained from PCA to produce scores and loadings plot. Scores plot (Figure 2A) explains the distribution among the time of urine sampling (0 to 24 hours) whereas loadings plot (Figure 2B) elucidates the chemical shifts that contribute to respective time of sampling in scores plot.

PCA plots in Figure 2A illustrated a complete 24 hours cycle of the effect of Ajwa dates intake when the ^{1}H-NMR spectrum at 24 hours were found located near to the blank (0 hour) of the ^{1}H-NMR spectrum. Therefore, it indicates that the effect of Ajwa in human body is within 24 hours.

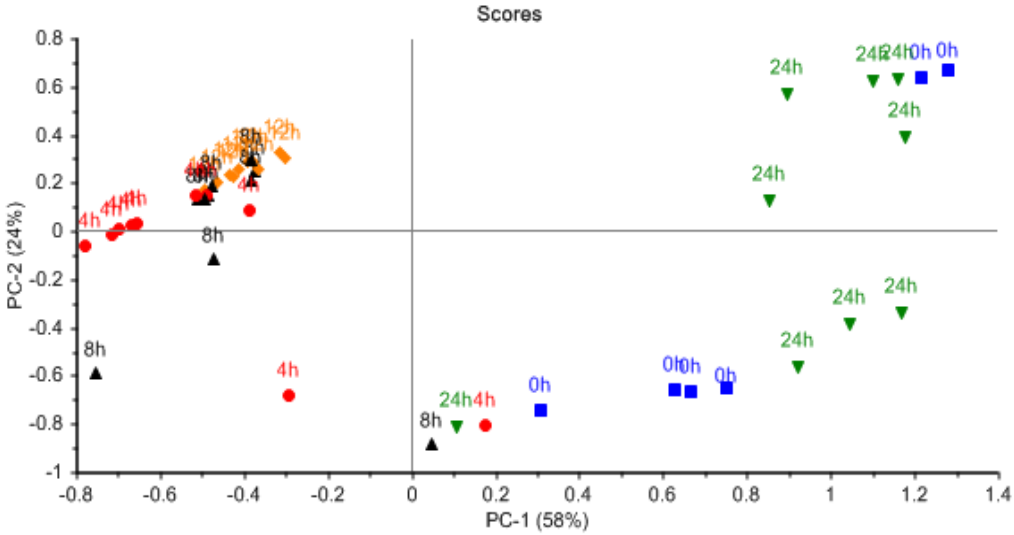

Figure 2A. Scores Plot of ^{1}H-NMR Spectra of Urine Samples Collected from 0h to 24h

Furthermore, the scattered spots of 4 hours group, was assumed due to different metabolism rate for each individual. On the contrary, spots in 12 hours group were highly aggregated compared to 8 hours, indicated that all respondents have reached the similar metabolism rate. Other chemical shifts are insignificant when lies close to origin.

Identification of Urine Metabolites (Metabolic Pathway)

PCA plots showed the metabolites change within 24 hours after consumption of Ajwa dates. Therefore, the focus is on the ^{1}H-NMR spectra from 0h to 24h. Chemical shifts in ^{1}H-NMR spectrum were compared with reference spectra in the Human Metabolome Database (HMDB) (Wishart et al., 2013). From ^{1}H-NMR spectra, the changes are mostly observed for peaks located in the region of 0.0 ppm – 4.0 ppm.

In the aliphatic region, citrate (2.57 and 2.72 ppm), taurine (3.42 and 3.25 ppm), alanine (1.48 ppm), lactate (1.32 ppm) and malonate (3.10 ppm) showed significant different from blank to 24 hours. There is also significantly different in aromatic region (6.5 ppm – 9.0 ppm) where there are relatively small peaks from hippurate (7.53, 7.62 and 7.82 ppm) which had been identified in all the spectra.

The peak of alanine was compared with reference spectra in HMDB and was supported by Marques et al. (2016) which stated that the alanine-CH_3 doublet signal resonating at 1.47 ppm is prominent in the ^{1}H-NMR spectra. Both alanine and taurine are classified as amino acids and also very similar in chemical structure (Nishigawa et al., 2018). Taurine is the end product of metabolic degradation of cysteine. In various stress levels, disrupted taurine metabolism was observed. Increased excretion of taurine was noticed in rats after being exposed to chronic radiation stress, immobilization stress and continuously fasting stress (Feng et al., 2016). Besides, Holmes et al. (1998) reported that the elevation of taurine in urine has been concomitant with hepatoxicity. In our study, the taurine peaks showed no changes in intensity from blank to 24 hours in all samples. Table 1 showed the summary of the urine metabolites change at different sampling time.

Table 1. Summary of the Urine Metabolites Change at Different Sampling Time

Metabolites	**Chemical Shifts, δ (ppm)**	**Changes of the urine metabolites at different sampling time**				
		0H	**4H**	**8H**	**12H**	**24H**
lactate	1.32	+	-	-	-	-
alanine	1.48	+	-	-	-	+
citrate	2.57, 2.72	+	-	-	-	+
malonate	3.10	+	-	-	-	+
taurine	3.42, 3.25	nc	nc	nc	nc	nc
hippurate	7.53, 7.62, 7.82	+	-	-	-	+

(nc)= no changes, (+)= appear/identified, (-)= decreased, (nd)= not detected

The level of alanine, hippurate, malonate and citrate were significantly decreased after the consumption of Ajwa dates, but were elevated after 24 hours. These endogenous metabolites were reported as biomarkers of depressive disorder where the concentration of these metabolites increased with depression (Tian et al., 2016). Thus, this could be suggested that Ajwa dates have the ability to reduce the level of depression. Figure 3 shows the overview of metabolic pathway related to metabolites change after Ajwa dates intake.

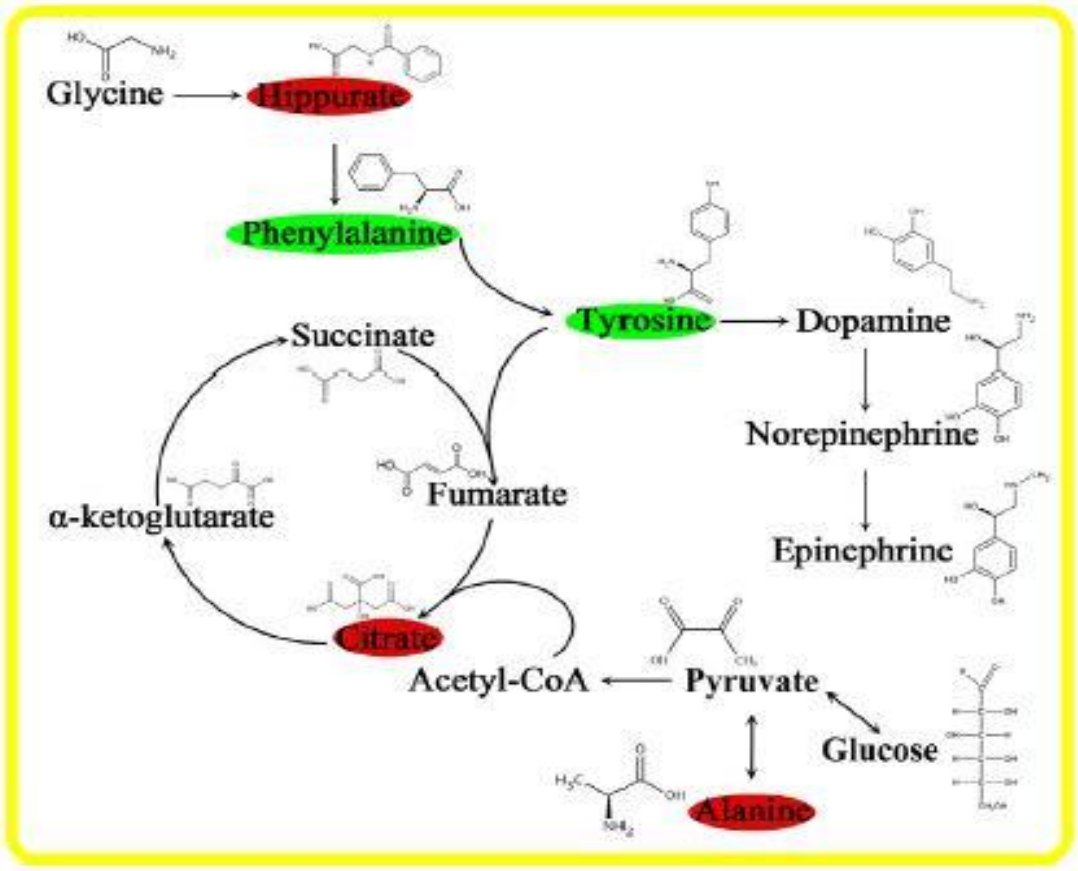

Figure 3. An Overview of Metabolic Pathway Related to Metabolites Change after Ajwa Dates Intake (Tian et al., 2015)

Generally, alanine is produced from pyruvate by reversible transmination reactions and highly produced in muscle, which functioning as a major energy source. According to Tian et al (2015), alanine is a regulator in glucose metabolism. Therefore, if the alanine is fully utilized, the level of alanine would decreases as well as the depression level. Most organisms will go through cellular respiration to produce ATP in the presence of oxygen. However, generally human body will develop a way called fermentation to create ATP even the absent of oxygen. This method could only works in anaerobic conditions, which is there is no air. Glucose still goes through glycolysis which creates the pyruvic acid and 2 ATP, but in order to regenerate more NAD the pyruvic acid is then broken down into lactic acid. Although the lactic acid fermentation takes places in muscle, however, as the lactic acid starts to build up in your muscle, your muscle starts to cramp up. Figure 4 shows the metabolic pathway of lactate and pyruvate.

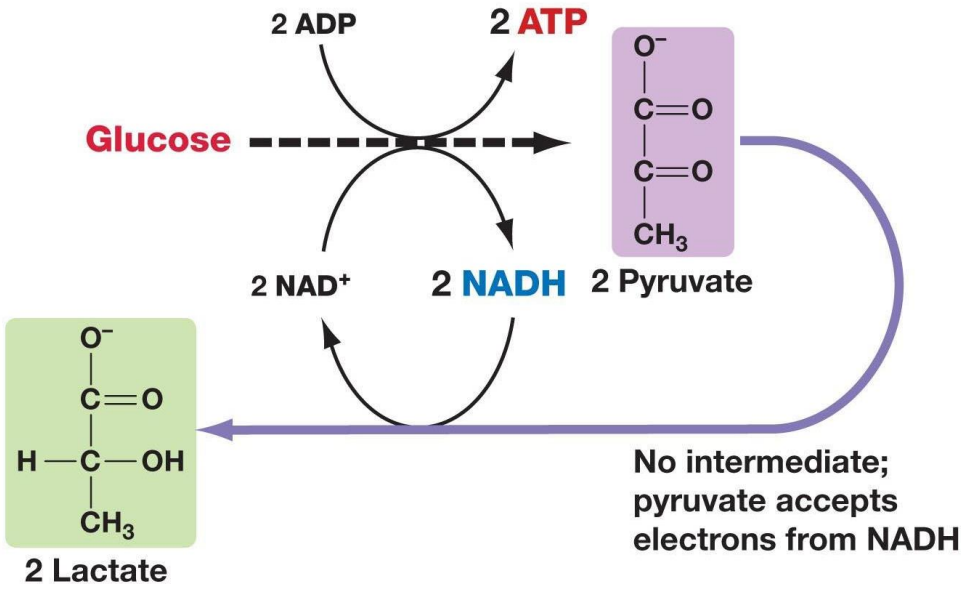

Figure 4. Relation of Lactate and Pyruvate in the Presence of Glucose

In addition, several studies reported that the enrichment of lactate, which is an organic molecule produced by most tissues in human body, was an indication of the increased in anaerobic glycolytic activity (Heather et al., 2013), related to diabetes and microalbuminuria (Chou et al., 2015), and major depressive disorder (Chen et al., 2017). Meanwhile, McNiven et al. (2011) stated that colon and stomach tumour tissues have high level of lactate compared to the healthy tissues. In this study, lactate peak was decreased after 4 hours of Ajwa dates consumption. This can be acknowledged as a good effect of Ajwa dates due to the decreasing level of lactate as shown in ^{1}H-NMR spectra. Summary of the urine metabolites change at different sampling time are tabulated in Table 1.

Conclusion

The urinary metabolites change in ^{1}H-NMR spectra within 24 hours post consumption of Ajwa dates were found to decrease the level of alanine, hippurate and citrate. Those metabolites are the biomarkers of depressive disorder which reduce the stress level for human.

Acknowledgments

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