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Effect of Phytochemicals in *Phoenix Dactylifera* L. on Human Body Using LC-QTOF-MS

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Abstract

The presence of nutrients and phytochemicals including alkaloids, sterols, phenols and flavonoid in *Phoenix dactylifera* L. has been related to the health-benefits of date fruits consumption. Metabolomics study has been conducted to explore the human urine metabolome modifications after date fruits intake. After overnight fasting, urine samples were collected before the ingestion at 0h and every 4 hours after the consumption of date fruits at 4-24-h. Samples were analysed using LC-QTOF-MS, followed by Principal Component Analysis (PCA). Results revealed the changes of urinary metabolome during the 24 h after date fruits intake. Some phytochemicals, including alkaloids, sterols, phenols and flavonoids were appeared and disappeared after date fruits intake. These findings confirm that metabolomics is an effective tool that can be practiced in order to further discover the metabolism of phytochemicals and its relation with human health.

Keywords: *Ajwa dates, Human urine, Liquid chromatography mass spectrometry (LCMS), Metabolomics, Principal component analysis (PCA)*

Introduction

Metabolomics investigations attempt to detect and profile the changes in metabolites, which reflect changes in metabolic pathways and may provide information concerning a disease state or the biological stress of an organism[1] or for discovery of biomarker[2]. There are two metabolomics based analysis; nuclear magnetic resonance (NMR-based) and mass spectrometry (MS-based). 1H-NMR-based is widely used in metabolomics study as it can simultaneously display resonance peaks resulting from hundreds of metabolites[3-4], requires little or no sample preparation[4-5] and highly reproducible and non-destructive[6]. Meanwhile, many researches using MS-based analysis due to the high sensitivity and selectivity. For instance, Liquid Chromatography Mass Spectrometry (LCMS) could be benefited from lower detection limits and improved MS data quality due to reduced background noise[7-8] stated that Gas Chromatography Mass Spectrometry (GCMS) gives high resolution chromatographic separation and wide applicability through derivatization.

In our previous study using 1H-NMR-based metabolomics indicated that a complete recovery of 24 hours after the Ajwa dates intake as a result of the urinary metabolome 24 hours after consuming Ajwa dates back to blank (0 hour). In other words, the effect of ajwa dates remained in body within 24 hours (unpublished data). Thus, in order to study the beneficial effect of Ajwa dates in human health, metabolomic study on the consumption of Ajwa dates flesh has to be performed. The aim of this study is to investigate the changes of metabolites in urine after the consumption of Ajwa dates using LC-QTOF-MS.

Methodology

Ten healthy volunteers (5 females and 5 males) between 20 and 25 years old with body mass index of

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20-25 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 follow the control diet, which exclude fruits and vegetables, supplements and beverages such as tea, coffee, fruit juice or any bicarbonate drinks. Equal meals were given to subjects during the experiment after considering their food allergy.

The urine samples were obtained before consumption of Ajwa dates as blank (0h). İmmediately, all subjects consumed 7 Ajwa dates that were given. Urine were collected at 4, 8, 12 and 24 hours after the consumption of Ajwa dates, which were at 11 am, 3 pm, 7 pm and 7 am (the next day), respectively. All the collected urine were stored in -20 ^{0}C. This design was followed Lopez et al (2014)[9] with some modifications. Organic solvent used in this study is methanol (Merck, Darmstadt, Germany).

This method was followed by previous research[9] with some modifications. Briefly, the urine samples were thawed before the analysis and vortexed using vortex mixer to homogenize the sample. An aliquot of urine (2.0 mL) was filtered using syringe filter (0.25 µm) and then centrifuged at 13 000 rpm for 5 min to remove the precipitate. The supernatant (1.0 mL) was transferred into a vial (2.0 mL) and diluted with an organic solvent; methanol (0.5 mL) and then well mixed. The prepared sample directly injected to LC-QTOF-MS.

Urine analysis was performed on ACQUITY UPLC I-Class system (WATERS Corporation, MA, USA) instrument. Compounds were chromatographically separated using a column ACQUITY UPLC HSS T3 (100 mm x 2.1 mm x 1.8 µm), maintained at 40 °C. A linear binary gradient of water (0.1 % formic acid) and acetonitrile was used as mobile phase A and B, respectively. The mobile phase composition was changed during the run as follows: 0 min, 1% B; 0.5 min, 1% B; 16.00 min, 35% B; 18.00 min, 100% B; 20.00 min, 1% B. The flow rate was set to 0.6 mL/min and the injection volume was 1.0 µL. The UHPLC system was coupled to a Vion IMS QTOF hybrid mass spectrometer, equipped with a Lock Spray ion source. Data were acquired in high-definition MS^E (HDMS^E) mode in the range m/z 50 - 1500 at 0.1 s/scan. Thus, two independent scans with different collision energies (CE) were alternatively acquired during the run: a low-energy (LE) scan at a fixed CE of 4 eV, and a high- energy (HE) scan where the CE was ramped from 10 to 40 eV. Argon (99.999%) was used as collision-induced-dissociation (CID) gas.

The multivariate analysis has been done using Unscrambler 10.3 (CAMO Software, Norway) which is a complete multivariate data analysis software solution, equipped with powerful methods including Principal Component Analysis (PCA), Partial Least Square (PLS), clustering and classification. The Unscrambler 10.3 is an ideal tool to analyze any sort of multivariate data quickly, easily, and accurately.

Results and Discussion

From our previous GCMS results, Ajwa dates flesh was found to have possible pharmacological functions such as antioxidants, antimicrobial, anticancer, antifungal, antidiabetic, anti-inflammatory, anticonvulsant and anti-asthmatic[10]. According to previous study[11], phytochemicals that rich in dates pulp are like phenolics, sterols, carotenoids, anthocyanins, procyanidins and flavonoids. The ratio and concentrations of these constituents depend on the type of the fruit, stage of fruitpicking, location and soil conditions. A research[12] who studied Algerian dates stated that the results obtained for phenolic content were much higher than Omanian dates. Another study[13] indicated that mono and di-glycosides identified in dates fruits were mostly flavonol derivatives (quercetin) and flavone derivatives (luteolin and apigenin).

In this study, compounds that had been identified in Ajwa date flesh using LC-QTOF-MS are quercetin, apigenin, caffeic acid, rhoifolin, crocetin, procyanidin, cyanidin, ferulic acid, glucoside, glucopyranoside, trimethylgallic acid, quercimetrin, proanthocyanidin, pyrogallic acid, coumatic acid, luteolin, β-sitosterol, decaffeolaceoside, elaeocyanidin and glucosyringic acid.

Metabolomics originally refers to the sum of the pool of cell metabolites[2]. This brief review of result focuses on untargeted metabolomics using LC-QTOF-MS. Investigation on the urinary metabolome could further confirm the beneficial effect of Ajwa dates in human health. There are a number of metabolites identified in 0h (blank) only such as flavonoids; Cnidimol F[14], Kushenol I, Kushenol M, Kushenol T, Liquiritin[15], Pachypodol and Sanggenon J, alkaloids; Ephedradine B[16], Isomaistemonine[17], Melicopidine[18], Piperyline and Pellitorin, phenolics; Feruperine and

Glabrol. Other known compounds that identified in blank urine are Isoanhyoicaritin and Riboflavin[19].

Urinary metabolome that appeared after 4h consuming Ajwa dates flesh are flavonoids; 2-Methoxykurarinone, Bavachin (Corylifolin), Kaempferol-3-O-α-L-arabinoside[20], Leachianone G[21] and Licoflavone , alkaloids; Codonopsine and Nigeglanine. Other compounds that identified after 4h Ajwa dates intake are Ophiopogonanone B and Retusine. Meanwhile, 2 alkaloids; Loganin[22,25] and Polycanthine, 1 phenolic compound; Eugenyl glucoside and 1 sesquiterpene; Pseudosantonin were identified in 8h after consumption of Ajwa dates flesh. Geniposidic acid and Paeonilactone A is other compound that found in the same urine time collection (8h). And there is only 1 known compound that appeared after 12h consuming Ajwa dates flesh which is Methyl lucidenate Q[6].

Most urinary metabolome appeared after 24h Ajwa dates flesh consumption. There are 32 urinary metabolome including alkaloids, phenol, amino acid, flavonoids, terpene glycoside and terpenoid. Other known compounds found to be appeared in 24h after Ajwa dates intake are Ajugasterone C-2,3,20,22-diacetonide, Andropanolide, Bruceine H[14], Citric acid, Cyclo(Ala-Ile), Dehydroabietic acid[4], Desmodimine, Glucosyringic acid, Glutamine, Heterodendrin, Kusulactone, Linustatin, Methyl-α-D-fructofuranoside, Methyl-β-D-fructofuranoside, Morusimic acid F, Mudanpioside F, Paeonisuffrone, Paeonolide, Pterodontriol D, Ranunculin, Schizonepetoside E, Scutellone E, Sibiricaphenone[23,26-27] and Vitamin B5. Alkaloids that identified are Coniferol[24], Gentianine and Picrasidine P. Meanwhile other compounds are phenol; Ginkgolic acid, flavonoid; Lutonarin, amino acid; Tyrosine, terpene glycoside; trans-Carveol-6-β-glucopyranoside and terpenoid; Xanthatin.

Some peaks were spotted appeared and disappeared in the chromatogram. There also peaks that belong to certain compounds remain in almost all the time collection (0h to 24h). The compounds named 6-Hydroxykynurenic acid, Adenosine, Coixol, Cuscohygrine, Dictysine, Evoxanthine, Isoxanthohumol, Lindelofine, Magnocurarine, Neokurarinol, Oxymaistemonine, Uridine and Xanthosine (black color in Figure 1).

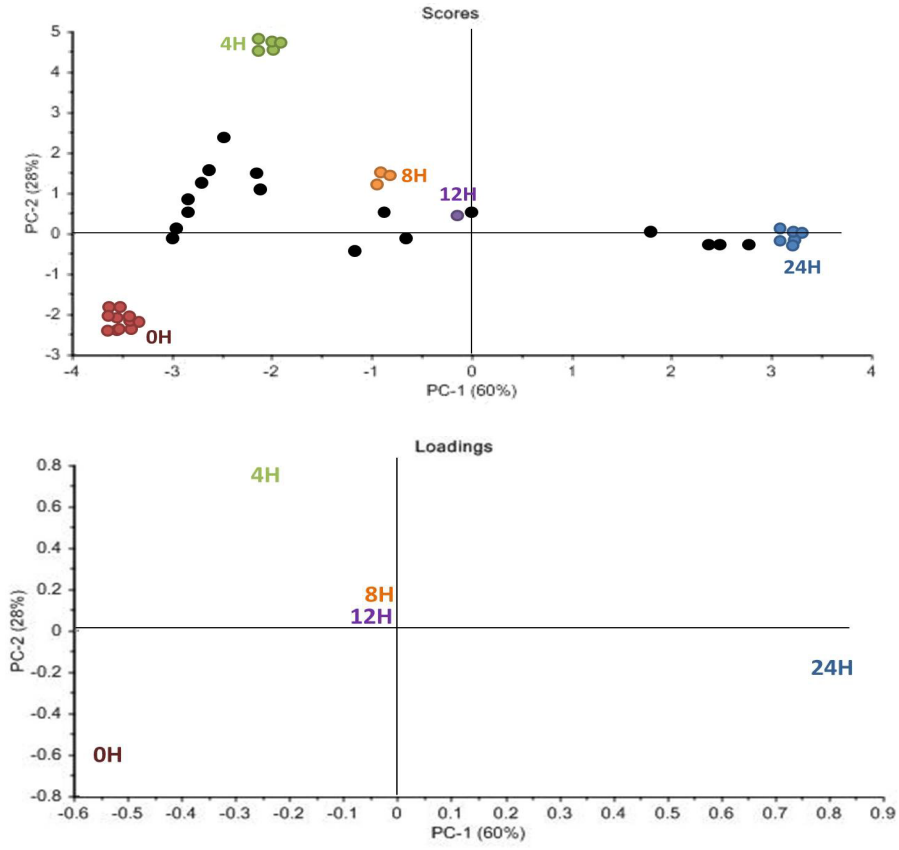

Figure 1: Scores and Loadings Plot of Urinary Metabolomes 24h after Consuming Ajwa Dates Flesh in Positive Ionization

All healthy respondents are required to follow the given diet schedule in which, they are allowed to drink plain water only during one week experiment, the same dietary intake (breakfast, lunch and dinner). No fruits, vegetables or beverages are allowed during experiment. They also required to fasting overnight. It is because we have to lessen the consequence of the metabolites change from other sources. Compared to urinary metabolome identified in 0h, more urinary metabolome are appeared after the consumption of Ajwa dates flesh. The metabolism pathway of the urinary metabolome before and after Ajwa dates intake should be investigated to study the beneficial effects of Ajwa dates to human body.

One of the techniques in chemometric had been used in this study is Principal Component Analysis (PCA). This technique is a powerful tool to help us to comprehend the multivariate data. The scores plot elucidating the urinary metabolome and the loadings plot is the urine time collection. The urinary metabolome that identified only in respective time collection has been highlighted. As illustrated, the scores and loadings plot pattern is similar. Urinary metabolome that had been circled in scores plot are belongs to each time collection in loadings plot, accordingly. The metabolome that scattered in between time collection are showing that metabolome had been identified in both or more time collection.

Conclusion

Phytochemicals that rich in Ajwa dates flesh are phenolics, sterols, carotenoids, anthocyanins, procyanidins and flavonoids. Results revealed the changes of urinary metabolome during the 24 h after date fruits intake. Some phytochemicals, including alkaloids, sterols, phenols and flavonoids were appeared and disappeared after date fruits intake. Investigation on the urinary metabolome could further confirm the beneficial effect of Ajwa dates in human health. These findings confirm that metabolomics is an effective tool that can be adept in order to further discover the metabolism of phytochemicals and its relation with human health.

Recommendations: In future, there should be a study that focuses on discovery of the effects of dates flesh which differ in quantity of date fruits.

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Ethical Clearance: This study was approved by the Human Ethics Committee (HEC) of Universiti Sains Islam Malaysia (USIM/JKEP/2016-14)

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Conflict of Interest: Nil

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