

Study of In Vitro digestion on antioxidant capacity of three different varieties of *Phoenix dactylifera*

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ABSTRACT

In this study red color dates developed by AAU, Anand using tissue culture technique was used and its antioxidant capacity was checked against yellow and red color dates available at local markets of Anand. The main aim of the study was to evaluate the antioxidant capacity of three different varieties of *Phoenix dactylifera* by conventional extraction and In Vitro gastrointestinal methods. The three different dates were procured, pre processed and later the samples were stored and then analyzed for total phenolics, flavonoid and total antioxidant capacity (FRAP, DPPH RSA, ABTS+ RSA) and Reducing Power assay. The red date developed by AAU, showed the highest total phenolics content when extracted using methanolic solvent. Further, the In Vitro gastrointestinal model proved that the antioxidant capacity of three different varieties of date fruit was reduced as flavonoid content was reduced as compared to conventional extraction methods. In conclusion, polyphenolic compounds were not completely degraded and made easy accessible in the In Vitro gastrointestinal model.

Keywords: *Phoenix dactylifera*, In Vitro gastrointestinal model, antioxidants, free radicals.

INTRODUCTION

Fruits are often ingested in many different ways like fresh, dried, frozen, canned and also as fruit juices (Ramulu, 2003). Mosele et al, 2015 reported that, 5-9 servings of fruits and vegetables per day as per U.S. Department of Agriculture tend to have the ability to lower the risks of many chronic diseases like cardiovascular diseases, cancer, metabolic syndromes, degenerative diseases, maintenance of optimal body function (Mosele et al, 2015). The above statements are evident because, fruits and vegetables contain natural phenol compounds like catechin, gallic acid, lutein, rutin, etc.

Phoenix dactylifera (date palm fruits) is also widely consumed like other fruits, all over the globe either in fresh state or dried state (Murugan et al, 2016).

Date palms are ancient plants cited from Vedas and Ramayana eras on this planet. They are generally rooted in Asia, Middle East, Africa and Arabian Peninsula (Nasir et al, 2015). This *P. dactylifera* passes through several stages to be termed as a mature fruits (Fig.1) (Ghnimi et al, 2017).

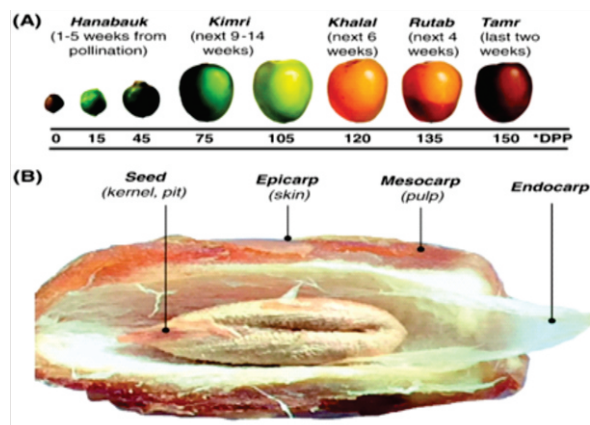


Fig.1 – Different fruiting stages of date.

Source – Ghnimi et al (2017) [5]

Abbreviations: GAE –gallic acid equivalents, RE – rutin equivalents, TAC – total phenolic content, TE –trolox equivalent

The date fruits are the richest source of carbohydrates, dietary fibers, essential vitamins and minerals. In addition to its dietary use, they also possess pharmacological benefits used to treat a variety of ailments in the various traditional systems of medicine. Phytochemicals analyzed by Baliga et al (2011) (Baliga et al, 2011), has revealed that the

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fruits contain anthocyanins, phenolics, sterols, carotenoids, procyanidine and flavonoid compound known to possess multiple beneficial effects.

In recent years, Anand Agricultural University (AAU), Anand Gujarat, has developed a new variety of red date fruit palm using a tissue culture technique which are sweeter than the variety grown in Kutch and now it is commercialized and farmers also started cultivating it. Recently these tissue culture date fruits are been sold in the markets of Anand. The sources report that tissue culture date fruits as well as date palm variety grown in Kutch are not subjected for the study of In Vitro gastrointestinal digestion for antioxidant capacity (Anonymous, 2011) (Shah, 2014).

Digestion of food is an intricate procedure where, the complex food is broken into simple form which gets assimilated into the blood. "In Vivo gastrointestinal digestion" means digestion within human body. The study of In Vivo GI model is difficult hence; scientists have developed "In Vitro gastrointestinal model" which is a replica of human GI where, all the digestion processes are mimicked with the help of commercial enzymes, digestive juices and acids in controlled environment (Ulleberg *et al*, 2011).

In view of the above, the date palm fruits available in market which includes the yellow and red varieties of Anand local markets and tissue culture dates of AAU are not been studied for In Vitro digestion, hence the present study was planned.

MATERIALS AND METHODS

2.1 Preparation of sample

The red (V-1) and yellow (V-2) varieties of date fruit were procured from local market of Anand. The date fruit of tissue culture variety (V-3) were procured from AAU. Procured date fruit of all the three varieties were washed with tap water, air dried, separated from branches and weighted accurately, counted the number of fruits. Later the fruits were cut and seeds were separated, fruit pulp and seeds were weighted. Fruits were dried at 60°C for 2-3 days and stored at -20°C till the analysis.

2.2 Extraction of Samples

2.2.1 Extraction of sample by conventional method

A known amount of sample was taken and then it was extracted by using 80% methanol which was acidified to pH 2.0. The extracted samples were stored at -20°C in appendroff tubes, for further analysis to be carried out (Seerano *et al*, 2007) [10].

2.2.2 Extraction of sample by In Vitro gastrointestinal model (Bhatt and Patel, 2013)

Bhatt and Patel, 2013 gave the procedure of In Vitro gastrointestinal model, wherein a known amount of sample were taken and were subjected to enzymatic digestion like "In Vivo digestion." For enzymatic digestion, the samples were incubated in the following way –

Pepsin (0.6 ml of 300 mg/ml solution in a buffer of 0.2M HCl-KCl, pH – 1.5 at 40°C



Pancreatin (3 ml of 5 mg/ml solution in 0.1M Phosphate buffer, pH-7.5 at 37°C for 6 hours)



Lipase (6 ml of 7mg/ml solution in 0.1 M Phosphate buffer at 37°C for 6 hours)



Bile extracts porcine (6 ml of 17.5 mg/ml solution in 0.1M Phosphate buffer, pH-7.5 at 37°C for 6 hours)



α Amylase (3 ml of 120 mg/ml solution in 0.1M Tris Malate buffer pH-6.9 at 37°C for 16 hours)

Later the samples were centrifuged for 15min at 6000rpm's and then the supernatant was collected. Obtained residues were washed twice with the help of distilled water and then all the supernatants were pooled. The supernatant were incubated with 300μl of amyloglycosidase for 45min at 60°C. Then the volume was made up to known amount. For control, similar treatment was carried out, except addition of enzymes to the buffer.

2.3 Measurement of phenols by FolinCiocalteu Assay (Singleton *et al*, 1999)

Folin Ciocalteu method was used to determine total phenol content of the sample obtained from conventional method and In Vitro digestion model. A known sample aliquot was taken and volume was made up to 1 ml. 0.5 ml of FC reagent was added to the samples and were vortexed well and were incubated for 1 minutes at 37° C . Then 10 ml of 7.5% sodium carbonate was added and incubated at 37°C for up till 1 hour. A blue complex was developed which was read at 750 nm. Different aliquots of

known concentration of Gallic acid were taken as standard. For blank, standard and samples were not added and was treated like sample.

2.4 Estimation of Flavonoid (Zhishen *et al*, 1999)

Different aliquots of sample and standard rutin were taken and volume was made up to 5 ml with distilled water. 0.3 ml of 5% sodium nitrate was added and then incubated at 37°C. Later 0.6 ml of 10% aluminum chloride was added and mixture was incubated at 37°C for 6 minutes. Then 2 ml of 1N sodium hydroxide was added and 2.1 ml of distilled water was added. The pink color complex was obtained which was read at 510 nm. For blank, sample and standard were not added, and it was treated as sample.

2.5 Measurement of FRAP (Ferric reducing antioxidant power assay) (Benzie *et al*, 1999)

Different aliquots were taken and volume was made up to 300µl with distilled water. 1.8 ml of FRAP reagent was added and then incubated at 37°C for 10 minutes. The developed blue color was read at 593 nm, trolox was used as standard. For blank, sample and standard was not used, it was treated like sample.

2.6 Measurement of Reducing power assay (Oyaizu M, 1986)

Different aliquots were taken and mixed with 2.5 ml of 0.2M Phosphate buffer, pH 6.6 and 2.5 ml of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 3000rpm's for 10 minutes. 2.5 ml of the supernatant was taken and it was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride was added and the absorbance was measured at 700 nm. Different aliquots of Trolox were taken and treated as standard. For blank, sample and standard were not taken, and it was treated like sample.

2.7 Estimation of ABTS radical scavenging assay (Huang *et al*, 2005)

The ABTS radical cation [2,2-Azinobis (3-ethyl benzothiazolin-6-sulfonic acid) diammonium salt] were generated by reacting ABTS and potassium persulfate after incubation at room temperature in the dark for 16 hours. The solution of ABTS was diluted with ethanol to an absorbance of 0.7 at 734 nm. A known sample aliquot was taken and volume was made up to 1 ml with ethanol. Later 1 ml of ABTS reagent was added and the content was vortexed for 10 seconds. The resultant oxidized mixture was measured at 734 nm and was compared with standard trolox. The blank was performed like sample, without standard and sample aliquot.

2.8 Estimation of DPPH radical scavenging (Brand Williams *et al*, 1995)

The antioxidant activity of the extract, on the basis of the scavenging activity of 2,2-diphenyl -1- picryl-hydrazyl (DPPH) free radical were determined of the samples. 3 ml of DPPH of optical density 1 at 513 nm in methanol was added to different aliquots of sample after making up to the volume 1 ml with methanol. The absorbance was measured at 517nm after incubation at 37°C for 20 minutes. For standard, trolox was used, and was treated like sample. The blank was carried out same like sample, without standard and sample.

Statistical Analysis

All the analysis were done in duplicates of each sample and are presented as Mean $\pm$ S.D by using ANOVA with two way analysis with replication (MS Excel) and SPSS version:20.

RESULTS AND DISCUSSION

The present study was carried out on the "Effect of In Vitro digestion on antioxidant capacity of fresh date fruits, procured from local markets (V-1 and V-2) and tissue culture variety (V-3) from Anand Agricultural University. To understand the results, the samples are given the codes as follows –

- S-1:- conventional extracted samples
- S-2:- control samples without addition of digestive enzymes
- S-3:- samples after gastric digestion
- S-4:- samples obtained after small intestinal digestion.

3.1 Total Phenols

The total phenolics content was found highest in V-3. It was lowest in V-2. The different varieties of date fruit have shown a significant ($P \leq 0.05$) effect on the total phenolics content in conventional extract as well as In Vitro digested samples. The total phenolics content was significantly ($P \leq 0.05$) effected by the extraction methods that are conventional extract and In Vitro extracts. The interaction between different varieties of date fruit and methods of extraction didn't show any significant effect on the total phenolics content of date fruits. The ranking of the phenolics content of the date fruits is V-3 > V-1 > V-2 as shown in the Table-1.

3.2 Flavonoid Content

The flavonoid content was found highest in V-3. It was found lowest in V-2. The different varieties of date fruit have shown a significant ($P \leq 0.05$) effect

on the flavonoid content in conventional extract as well as In Vitro digested samples. The flavonoid content was significantly ($P \leq 0.05$) effected by the extraction methods that are conventional extract and In Vitro. The ranking of the flavonoid content of date fruits is V-3 > V-1 > V-2 as shown in Table -2.

3.3 Total Antioxidant Capacity

3.3.1 FRAP (Ferric reducing antioxidant power)

The FRAP was found highest in V-3. It was lowest in V-2. The different varieties of date fruits has shown a significant ($P \leq 0.05$) effect on the FRAP in conventional extract as well as In Vitro digested samples. The FRAP was significantly ($P \leq 0.05$) effected by the extraction methods that are conventional extracts and In Vitro. The interaction between variety of date fruit and methods of extraction didn't show any significant effect on FRAP of date fruits. The ranking of the FRAP of the date fruits is V-3 > V-1 > V-2 as shown in Table – 3.

3.3.2 DPPH Radical Scavenging Assay (DPPH)

DPPH radical scavenging ability was highest in V-3. The V-2 variety showed the lowest activity. The different varieties of date fruit has shown significant ($P \leq 0.05$) effect on the DPPH RSA through conventional extract and In Vitro digested samples. The DPPH RSA was significantly ($P \leq 0.05$) effected by the extraction methods that are conventional extracts and In Vitro extracts. The ranking of the DPPH RSA of the date fruits is V-3 > V-1 > V-2 as shown in Table -4.

3.3.3 ABTS+ Radical Scavenging Activity (ABTS+ RSA)

The ABTS+ RSA was highest in V-3. The V-1 variety showed the lowest ability to scavenge ABTS+ radicals. The different varieties of date fruits had shown significant ($P \leq 0.05$) effect on the ABTS+ RSA through conventional extracts and In Vitro digested samples. The ABTS+ RSA was significantly ($P \leq 0.05$) effected by the extraction methods that are conventional extracts and In Vitro. The interaction between varieties of date fruits and methods of extraction didn't show any significant effect on ABTS+RSA. The ranking is V-1 < V-2 < V-3 as shown in Table – 5.

3.4 Reducing Power Assay (RPA)

The RPA was found highest in V-3. The V-2 variety showed the lowest. The different variety of date fruits has shown a significant ($P \leq 0.05$) effect on reducing power in conventional and In Vitro extracts. After In Vitro digestion V-1 showed the lowest and V-2 showed highest reducing power. The interaction between three different varieties of date fruit and methods of extraction didn't show any significant effect on the reducing power. The ranking for reducing power is V-2 < V-1 < V-3 as shown in Table – 6.

Fresh dates are rich in polyphenols like benzoic acids and cinnamic acids which possess antioxidant properties that have a significant role in biological system. Murugan et al (2016) reported in his study that after small intestinal digestion of date fruit there is an increase in catechin and quercetin polyphenols contents and p-Coumaric acid and ferulic acid were undetectable [3]. Studies also stated that methanolic extract had higher polyphenolics content rather than obtained after small intestinal digestion.

Table 1 : The Total Phenolics content of conventional extract and In Vitro digested extracts of three different varieties of date fruits (mgGAE/100 gm extracts).

Samples	Extraction Method				F-Value
	S-S-1-1	S-2S-2	SS-3-3	S-4	
V-1	690.5 ± 17.2	458.1 ± 52.6	459.3 ± 27.9	1468.0 ± 24.2	1820.55*
V-2	360.9 ± 28.7	254.3 ± 16.2	487.4 ± 12.7	1053.4 ± 32.	
V-3	1009.4 ± 60.0	336 ± 43.84	495.8 ± 18.1	1337.2 ± 30.5	
F-Value			280.34*		96.33
Interaction					

- Values are Mean ± S.D of four observations
- *Indicates a significant difference at $P \leq 0.05$

Table : 2 The Flavonoid content of conventional extract and In Vitro digested extracts of three different varieties of date fruits (mgRE/100 gm extracts).

Samples	Extraction Methods				F-Value
	S-1	S-2	S-3	S-4	
V-1	466.4 ± 34.1	189.8 ± 6.9	181.5 ± 44.0	305.4 ± 37.4	432.25*
V-2	235.4 ± 24.0	106.0 ± 11.9	106.5 ± 10.9	223.6 ± 47.3	
V-3	867.4 ± 24.0	123.4 ± 17.8	156.6 ± 10.7	235.1 ± 23.7	
F-Value			147.21*		112.4

- Values are Mean ± S.D of four observations
- *Indicates a significant difference at $P \leq 0.05$.

Table : 3 The Ferric Reducing Antioxidant Power of conventional extract and In Vitro digested extracts of three different varieties of date fruits (mgTAC/100gm extracts).

Samples	Extraction Methods				F-value
	S-1	S-2	S-3	S-4	
V-1	1077.1 ± 197.9	415.6 ± 80.3	352.2 ± 137.1	1821.9 ± 168.5	257.64*
V-2	648.2 ± 36.9	261.2 ± 23.5	311.3 ± 76.7	1445.7 ± 69.21	
V-3	1822.7 ± 126.1	310.3 ± 87.9	473.7 ± 76.7	353.5 ± 20.3	
F-value	24.04*				106.94
Interaction					

- Values are Mean ± S.D of four observations
- *Indicates a significant difference $P \leq 0.05$.

Table : 4 The DPPH RSA of conventional extract and In Vitro digested extracts of three different varieties of date fruits (mgTE/100 gm extracts).

Sample	Extraction Methods				F-value
	S-1	S-2	S-3	S-4	
V-1	227.6 ± 31.6	61.5 ± 4.2	89.9 ± 7.1	197.3 ± 13.3	323.11*
V-2	128.5 ± 16.5	64.7 ± 7.0	90.7 ± 5.6	142.0 ± 14.9	
V-3	307.2 ± 8.5	44.4 ± 4.8	117.7 ± 4.6	175.3 ± 21.1	
F-Value		62.84*			40.36

- Values are Mean ± S.D of four observations
- * Indicates a significant difference at $P \leq 0.05$

Table : 5 The ABTS+RSA of conventional extract and In Vitro digested extracts of three different varieties of date fruits (mgTE/100 gm extracts)

Sample	Extraction Methods				F-value
	S-1	S-2	S-3	S-4	
V-1	90.0 ± 6.0	249.5 ± 10.9	141.9 ± 2.6	195.7 ± 14.3	216.93*
V-2	155.2 ± 41.4	450.8 ± 31.6	122.3 ± 10.1	325.0 ± 42.9	
V-3	193.1 ± 51.5	407.2 ± 37.5	137.3 ± 0.4	398.7 ± 3.0	
F-Value		78.89*			16.91

- Values are Mean ± S.D of four observations
- * Indicates a significant difference at $P \leq 0.05$.

Table : 6 The Reducing Power Assay of conventional extract and In Vitro digested extracts of three different varieties of date fruits (mgTE/100 gm extracts).

Sample	Extraction Methods				F-value
	S-1	S-2	S-3	S-4	
V-1	1844.1 ± 106.1	1651.1 ± 409.3	1266.9 ± 134.7	1493.1 ± 153.1	44.33*
V-2	1140.3 ± 94.9	1079.2 ± 119.1	1092.3 ± 39.7	1687.3 ± 137.9	
V-3	2011.6 ± 155.4	1152.9 ± 40.6	854.7 ± 110.7	1682.72 ± 141.3	
F-Value Interaction			18.47*		16.56

- Values are Mean ± S.D of four observations
- * Indicates a significant difference at $P \leq 0.05$.

The possible reason for the decrease of polyphenolics In Vivo after small intestinal digestion is, the phenolics activity with other dietary constituents released during digestion, such as iron, other minerals, dietary fiber or proteins may be present which are known to impair polyphenols solubility and bioavailability (Chen *et al*, 2016) the possible reason for the decrease in phenolics after small intestinal digestion after In Vitro digestion is difficult to justify (Chen *et al*, 2016).

The results of the present study indicated that there is a significant decrease in the bioavailability of flavonoids, the possible reason for In Vivo may be, due to the decreased absorption through gut barrier, and excreted through bile or metabolized by the colonic microflora of our own body tissues. Several other factors are also known to affect the quantity of polyphenols profile; one of the major factors is the geographical origin as well as the degree of ripeness of the fruit which also contribute to the presence of flavonoids content (Vinha *et al*, 2005) (Scalbert and Williamson, 2000). Kosinska-Cagnazzo *et al* (2015) reported that during In Vitro digestion of fruits containing anthocyanins pigment (V-1 and V-3), when subjected with pepsin during gastric digestion, it showed no significant effect on the total anthocyanins content but, during small intestinal digestion, there was a significant decrease; this may be possible due to the alkaline medium where, anthocyanins lose their stability (Kosinska-Cagnazzo *et al*, 2015).

The result of the present study showed that antioxidants present in three different varieties of date fruit may have been destroyed, inhibited, transformed or otherwise far less available during the intestinal phase of digestion.

CONCLUSION

From the results obtained for various biochemical parameters for "Effect of In Vitro digestion on

antioxidant capacity of three different varieties of *Phoenix dactylifera*" it is concluded that bioavailability of dietary polyphenolics after subjecting to gastrointestinal digestion was easy. The red date fruit developed using tissue culture technique (V-3) showed the highest total phenolics content when extracted using methanolic solvent. Further, the In Vitro gastrointestinal model proved that the antioxidant capacity of three different varieties of date fruit was reduced, as flavonoids content was reduced as compared to conventional extraction method.

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