The antibacterial activity of date syrup polyphenols against *S. aureus* and *E. coli*

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Abstract

Plant-derived products such as date syrup have demonstrated antibacterial activity and can inhibit bacteria through numerous different mechanisms, which may be attributed to bioactive compounds including plant-derived phenolic molecules. Date syrup is rich in polyphenols and this study hypothesized that date syrup polyphenols demonstrate inherent antimicrobial activity, which cause oxidative damage. This investigation revealed that date syrup has a high content of total polyphenols (605 mg/100g), and is rich in tannins (357 mg/100g), flavonoids (40.5 mg/100g) and flavanols (31.7 mg/100g) that are known potent antioxidants. Furthermore, date syrup, and polyphenols extracted from date syrup, the most abundant bioactive constituent of date syrup are bacteriostatic to both Gram positive and Gram negative *Escherichia coli* and *Staphylococcus aureus* respectively. It has further been shown that the extracted polyphenols independently suppress the growth of bacteria at minimum inhibitory concentration (MIC) of 30 mg/mL and 20 mg/mL for *E. coli* and *S. aureus*, and have observed that date syrup behaves as a prooxidant by generating hydrogen peroxide that mediates bacterial growth inhibition as a result of oxidative stress. At sub-lethal MIC concentrations date syrup demonstrated antioxidative activity by reducing hydrogen peroxide, and at lethal concentrations date syrup demonstrated prooxidant activity that inhibited the growth of *E. coli* and *S. aureus*. The high sugar content naturally present in date syrup did not significantly contribute to this effect. These findings highlight that date syrup's antimicrobial activity is mediated through hydrogen peroxide generation in inducing oxidative stress in bacteria.

Keywords: *Phoenix dactylifera.* L, date syrup, polyphenol, *S. aureus*

INTRODUCTION

Staphylococcus aureus is affiliated to chronic wounds that have a strong association with chronic inflammation leading to high morbidity (Orsi et al., 2002). Furthermore, the increase in antibiotic-resistant bacteria poses a threat to health care worldwide resulting in a revived interest in plant products as adjunct antimicrobial agents to control pathogenic microorganisms (Cowan, 1999). Naturally derived compounds such as aloe vera, honey and curcumin (De et al., 2009) are gaining popularity as alternative antimicrobial compounds. A major plant group used for traditional medicinal applications is *Phoenix Dactylifera* L, more commonly known as the date palm. Fruit of the date palm have been used customarily in the treatment of intestinal disturbances (Vyawahare et al, 2009). In Egypt, date palm pollen grains have historically been used to enhance fertility (Al Qarawii et al., 2003). Bauza (2002) has demonstrated that date palm kernels are included in medicinal skin treatment and nomadic tribes in the Middle East have been known to use traditional date syrup as an antimicrobial agent for wound healing (Tahraoui et al., 2003).

Date products such as date syrup are used in the food industry as a sweetening alternative and in the production of beverages and alcohol (Aboubacar et al., 2010). More than often, the perceived health benefits for the consumption and utilisation in date-derived medicinal concoctions are attributed to the bioactive and nutritious compounds found in date syrup and date fruit. Date syrup is a rich source of phenolic compounds which are known potent radical scavengers (Vayalil, 2002), various studies addressing the composition of date syrup have identified significant antioxidant potential (Guo et al., 2003) which may allude to the scientific basis of date fruit and date syrup's traditional medicinal application. Numerous phenolic compounds such as polyphenols and flavonoids are antibacterial as a result of their oxidizing potential (Daglia, 2012), which may offer a rationale for date fruit and date syrup's medicinal application as an antimicrobial agent.

Whilst it remains unclear as to precisely how the antioxidant scavenging potential contribute to the bacteriostatic and bactericidal activity of date syrup. Prooxidants are known to cause physiochemical and structural changes to microorganisms that results in growth retardation (Halliwell, 2008).

The challenge to this notion is the ability to determine by which mode of action date syrup inhibits microorganisms, and which bioactive compounds contribute to this effect. The topic of antioxidants as powerful scavengers of reactive oxygen species (ROS) has recently gained considerable attention in applied food microbiology, food science and technology and cell immunology. The antioxidant / prooxidant activity of secondary metabolites such as polyphenols can depend on factors such as pH, metal-reducing potential, chelating activity and solubility (Sakihama et al., 2002). Polyphenols have antioxidant activity (radical scavenging, and metal chelating activity) or prooxidant activity depending on environmental conditions, interaction, structural changes and exposure to microorganisms (Yordi et al., 2012). Polyphenols are able to act as prooxidants in systems that utilise redox active metals such as iron and copper. Binding of the polyphenol complex ligand to Fe^{3+}, the complex is able to reduce the iron to Fe^{2+} and is oxidised to a semiquinone, which is capable of reducing further Fe^{3+} oxidising the semiquinone to a quinone. The reduction of Fe^{3+} generates Fe^{2+} that consequently

participates in the Fenton reaction and results in reactive oxygen species (ROS) generation.

Bacterial aerobic respiration produces oxygen (O_2) required in cellular energy production (Macvanin & Hughes, 2010). The incomplete reduction of O_2 by microorganisms during respiration generates ROS including hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). Bacteria that undergo aerobic respiration defend themselves against the oxidative stress associated with the accumulation of ROS such as exposure to polyphenols through several mechanisms one of which is the production of enzymes catalase and superoxide dismutase which combat ROS. Superoxide dismutase reduces OH^- to H_2O_2, and catalase consequently converts H_2O_2 to water and O_2. The detoxification process of ROS is efficient and with intracellular H_2O_2 concentration being controlled at a steady state value of 0.2 μM in *E. coli* (Brudzynski et al., 2011). Antioxidants such as polyphenols and flavonoids induce bacterial lysis through increased ROS and H_2O_2 production.

Given that date syrup is known to have various bioactive polyphenols, reported as potential antimicrobial agents, this study aimed to identify the bacteriostatic and bactericidal activity of date syrup against Gram positive and Gram negative bacteria and to establish whether this activity is influenced by a) date syrup phytochemical compounds, namely polyphenols, b) the susceptibility of bacteria to oxidative stress resulting from hydrogen peroxide generated and mediated by the presence of polyphenols, and c) osmolarity in regards to sugar content is the not the principal factor contributing to the antibacterial activity.

MATERIALS AND METHODS

STANDARDS, SOLVENTS AND REAGENTS

The following reagents were obtained from Sigma (Sigma Aldrich, United Kingdom): XAD-2 Resin, Folin-Ciocalteu reagent, Butylated hydroxytuolene, polyvinylpolypyrrolidone (PVPP), gallic acid and catechin. Acetone and methanol (HPLC grade), Xylenol orange, aluminium chloride, glucose, fructose and sucrose (analytical grade) and hydrogen peroxide were obtained from Fisher Scientific (UK) and 2,2' – diphenyl -1- picrylhydrazyl (DPPH) was purchased from Merck (Darmstadt, Germany).

DATE SYRUP PREPARATION FOR ANTIBACTERIAL TESTING

Date syrup was produced from the date fruit cultivar Khadrawi, belonging to the family *Arecaceae*, genus *Phoenix* and species *dactylifera* during the wet seasons of 2012-2013. The date syrup was raw and unprocessed; it was stored at 4 °C on receipt. The date syrup was unsterile and not immediately suitable for antibacterial susceptibility testing. Therefore, different sterilisation methods were undertaken to determine which method was the most ideal for date syrup with minimum effect on date syrup's constituents. Sterilisation of date syrup using the solvent acetone was determined to be most suitable. A 200 g of date syrup was mixed thoroughly and soaked in 200 mL acetone (Analytical grade) for 48 hours at room temperature. After 48 hours the homogenous mixture was filtered through Whatman No. 1 filter paper and the solvent was evaporated under rotary evaporation (Bibby RE-100, Bibby Scientific) at 40 °C to ensure all acetone was removed. Crude date syrup extract was rehydrated in nutrient broth medium and passed through a 0.22 µm filter (Millex-GV, Millipore, UK) and stored at -80 °C for analysis, the final concentration resulted in 50 mg / mL date syrup.

PREPARATION OF ARTIFICIAL DATE SYRUP

High Performance Liquid Chromatography (HPLC) analysis was conducted on the sugars present in date syrup to determine the percentage of individual sugar constituents. Artificial date syrup per 100 g was prepared by mixing 4.79 g sucrose (7.6% of total), 29.05 g fructose (46.13% of total) and 29.13 g glucose (46.3% of total) in sterile deionised water and warmed in a water bath at 50 °C for 10 minutes to ensure complete dissolving of sugars.

EXTRACTION OF FLAVONOID AND PHENOLIC FRACTION OF DATE SYRUP ON XAD-2 RESIN

Date syrup (50 g) was mixed with 250 mL of pH2 HCl water for 24 hours; the mixture was filtered through cotton wool to remove un-dissolved solid particles. XAD-2 resin (approximately 47 g) was initially conditioned in 2M HCl for 1 hour, conditioned by soaking in 1:1 methanol and water for pre-swelling overnight. The slurry with the resin was packed into a glass column (50 cm^3) and the solution removed for an approximate bed volume of 1 x 50 cm^3 and rinsed with 1 L of deionised water.

The filtered date syrup solution was passed slowly through the packed resin column, followed by 250 mL of acidified water (pH2), deionised water (300 mL) and phenolic fractions were finally eluted with 300 mL pure methanol. A 50 mL of collected methanol extract was concentrated to dryness under vacuum at 40 °C, re-dissolved in water and stored at -80 °C for analysis, and the remaining methanol extract was stored at -80 °C for and dissolved accordingly for antibacterial analysis.

DETERMINATION OF ANTIOXIDANT ACTIVITY

QUANTIFICATION OF TOTAL PHENOL CONTENT

The total phenolic content of date syrup was determined by the Folin-Ciocalteu colorimetric assay based on the procedure previously identified by Al-Farsi et al., (2005). Gallic acid was used as a spectrophotometric standard (0-100 mg/mL) and results were expressed and means ± SD mg of gallic acid equivalents (GAE) per 100 g of date syrup. Measurements were taken in triplicate.

TOTAL FLAVONOID CONTENT

Total flavonoid content was measured by the aluminium chloride colorimetric assay described by Zhishen et al., (1999). Absorbance was measured at 510 nm against a blank control. Total flavonoid content was expressed as mg GAE per 100 g date syrup.

TOTAL FLAVANOL CONTENT

Total flavonol content was adapted from the method described by Jimoh et al., (2010); 200 µl of date syrup (25 mg/mL) was mixed with 250 µl of 2% $AlCl_3$ and 250 µl of 5% sodium acetate solution. Mixtures were sealed and incubated for 2.5 hours at room temperature. The absorbance was measured at 440 nm and results were expressed as mg of catechin equivalents per 100g of date syrup (mg catechin / 100 g date syrup).

TOTAL TANNIN CONTENT

The total tannin content was determined by the Folin-Ciocalteu method after the removal of tannins by their adsorption to the insoluble matrix polyvinylpolypyrrolidone (PVPP). This method was based on Hagerman et al., (2000) and Kchaou et al., (2013); 1mL of date syrup extract (25 mg/mL) was added to 100 mg of PVPP and incubated for 15 minutes at 4 °C. The mixture was vigorously shaken and centrifuged for another 15 minutes at 13,000 g, where the supernatant was collected and non-adsorbed phenolics were subjected to the Folin-Ciocalteu assay for total phenolic content. Results were subtracted from total phenolic content and total tannins was expressed as mg GAE / 100 g fresh weight.

TOTAL CAROTENOID CONTENT

Total carotenoids were extracted according to the method of Talcott & Howard (1999), working under red light and in dark conditions total carotenoids were calculated using the following equation and expressed as mg per 100g of date syrup:

Total carotenoids = $[(OD)(V)(10^6)/(A^{1}\%)(100)(W)]$

Where OD = absorbance at 470 nm, V = volume of sample extract, $A^{1}\%$ = the average extinction coefficient for a 1% mixture of carotenoids at 2500, and W= sample weight in g.

TOTAL ANTHOCYANIN CONTENT

Total anthocyanin was determined and calculated according to the pH-differential method as described by Giusti & Wrolstad (2001). Total anthocyanin content was expressed as mg/100 g of date syrup and calculated according to the following two equations:

1. The difference in absorbance between the two anthocyanin extracts were calculated by:
   $\Delta A = (OD_{510}\ pH\ 1.0 - OD_{700}\ pH\ 1.0) - (OD_{510}\ pH\ 4.5 - OD_{700}\ pH\ 4.5)$
2. The monomeric anthocyanin pigment concentration in the original sample is expressed as cyaniding3-glucoside equivalents and calculated on the basis of the following formula:

$$[(\Delta A)(MW)(DF)(V)(100) / (\varepsilon)(L)(W)]$$

Where MW = molecular weight of cyaniding3-glucoside (449.2 g/mol), DF = dilution factor, V = final volume in mL, ε = molar extinction coefficient for cyaniding3-glucoside (26,900), L = cell path length of 1 cm and W = sample weight in g.

EVALUATION OF ANTIOXIDANT ACTIVITY

DPPH RADICAL SCAVENGING ACTIVITY

Date syrup's anti radical scavenging capacity was assessed based on the scavenging activity of the stable free radical 2,2' – diphenyl -1- picrylhydrazyl (DPPH). Briefly, 100 µl of different date syrup concentrations (5-50 mg/mL) dissolved in deionised water were aliquoted into a 96-well plate (Costar), 50 µl of ultrapure (ELGA) water was added followed by 50 µl of 400 µm of DPPH (in absolute ethanol). The plate was sealed and shaken for 5 minutes and subsequently incubated in the dark for 25 minutes at room temperature. Absorbance was measured spectrophotometrically at 490 nm against a blank solution. The commercially available antioxidant butylated hydroxytuolene (BHT) was used as a positive control (10 mg/mL in ethanol) and the percentage inhibition activity was calculated based on the following equation and expressed as % antioxidant activity:

$$[OD_1 - OD_2 / OD_1\ 100]$$

where OD_1 is absorbance of blank control and OD_2 is absorbance of sample extract

ANTIBACTERIAL SUSCEPTIBILITY TESTING

BACTERIAL STRAINS

Escherichia coli (reference strain NCTC 10418) and *Staphylococcus aureus* (reference strain NCTC 13142) were used throughout the study. Cultures were grown aerobically in nutrient broth (NB) (Fluka) for 24 hours at 37 °C to promote planktonic growth.

MINIMUM INHIBITORY CONCENTRATION (MIC) & MINIMUM BACTERICIDAL CONCENTRATION (MBC)

MIC for date syrup and extracted date syrup polyphenol against *E. coli and S. aureus* was determined using a broth-micro dilution method and spectrophotometric assay. Minimum inhibitory concentrations were determined in sterile 96 well round bottomed polystyrene microtitre plates (Corning Costar Ltd, NY, USA) in accordance to methods of the Clinical and Laboratory Standards Institute (CLSI, 2012), MIC was determined by serial dilution (5-50 mg/mL in increments of 5 mg/mL). Bacterial inoculum corresponding to 0.5 McFarland standard of pre-culture (16 hour at 37° C and equivalent to 10^6 colony forming units (CFU)) was added to test samples at each concentration. Samples were measured in triplicate. Plates were incubated at 37 °C for 24 hours and turbidity was measured spectrophotometrically at 650 nm in a plate reader (SPECTROstar Nano, BMG Labtech). The MBC was assessed in accordance to CLSI (2012) standards whereby those wells described for the MIC above, showing no apparent growth were streaked onto nutrient agar (NA) (Fluka). Plates were incubated overnight at 37° C, the plates with the lowest concentration of date syrup and date syrup polyphenol sample showing no growth following incubation overnight was recorded as the MBC. Tetracycline was used as an antibiotic control with a stock concentration of 33 µg/ml.

MEASUREMENT OF H_2O_2 CONCENTRATION

The generation of hydrogen peroxide in nutrient broth medium (NB) without bacterial cells (cell free medium) after the addition of date syrup, date syrup polyphenols or artificial date syrup for 1 hour at 37 °C was measured by the ferrous ion oxidation-xylenol orange (FOX) assay as described by Packer & Sies (2001), and Maeta et al., (2007). Date syrup was prepared fresh in nutrient broth medium corresponding to concentrations sub-lethal (15 mg/mL) and lethal (30 mg/mL) to bacteria as identified in minimum inhibitory concentration studies. Date syrup polyphenols and artificial date syrup were prepared at concentrations of 30 mg/mL to investigate their independent effect on H_2O_2 production. A working FOX reagent was prepared from two separate reagents; reagent 1 consisting of 4.4 mM BHT in methanol and reagent 2 compromised of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulphate in 250 mM H_2SO_4, reagents were prepared fresh daily for each assay.

Samples of date syrup, extracted date syrup polyphenols or artificial date syrup (90 µl) were mixed with 10 µl of methanol, vortexed and left to incubate at room temperature for 30 minutes, 900 µl of working FOX reagent was added to each sample assayed in triplicate and incubated for another 30 minutes followed by centrifugation at 15,000 g for 10 minutes. Absorbance was read at 560 nm against a methanol blank containing the necessary amount of sample to correct for background associated with sample. The FOX assay was calibrated using standard H_2O_2, diluted from stock (500uM) and its concentration assessed using molar extinction coefficient of 43 M^{-1} cm^{-1} at 240 nm.

ANALYSIS OF BACTERIAL SURVIVAL

E. coli and *S. aureus* were cultured in NB medium at 37 °C for 16 hours in accordance to CLSI, (2012) standards, date syrup, date syrup polyphenols or artificial date syrup with or without 100 U/mL catalase or H_2O_2 (1 mmol/L) were added to nutrient broth medium and allowed to equilibrate for 4 hours. This was followed by inoculation with bacteria corresponding to 10^6 CFU/mL (0.5 McFarland). After incubation at 37 °C for 4 hours with shaking, cells were diluted (10^{-1} – 10^{-8}) and enumerated using the surface drop count method to determine CFU.

STATISTICAL ANALYSIS

All data were expressed as mean ± SD of independent triplicates unless otherwise stated. One way ANOVA with Tukey's *post-hoc* analysis was used for multiple comparisons within groups of normally distributed data Statistical analysis was performed using GraphPad Prism® Version 6 software and results were significant at $p < 0.05$ and $p < 0.01$.

RESULTS

The antimicrobial activity of date syrup might be associated with the presence of antioxidative compounds in date syrup that possess bioactive behaviour. It was hypothesised that the phytochemical compounds present in date syrup may be involved in redox reactions mediated by the production of H_2O_2 that results in bacterial inhibition providing justification for date syrup's traditional medicinal application.

Determination of date syrup antioxidant behaviour

The determination of secondary metabolites as antioxidants is outlined in **Table 1**. In comparison to previous literature investigating date fruit and date syrup (Dhaoudi et al., 2012; Abbès et al., 2013; Al-Farsi et al., 2007) the results are in agreement that date syrup has sufficient secondary metabolites that are typically associated with bioactive behaviour and radical scavenging (Vayalil, 2012).

As the concentration of date syrup and date syrup polyphenols (PPDS) increases, so does the percentage antioxidant behaviour, which is a demonstration of the free radical scavenging activity (**Figure 1**). However this antioxidant behaviour was only evident up until a concentration of 60-70% date syrup, therefore at a greater concentration of date syrup, the antioxidant power began to decline.

Antibacterial susceptibility testing

Date syrup and extracted date syrup polyphenols were investigated for their antibacterial activity. The bacteriostatic activity of date syrup and date syrup polyphenols was tested against *E. coli* and *S. aureus* and represented as the MIC. The MIC of date syrup and date syrup polyphenols is outlined in **Table 2.** Date syrup's MIC for the tested bacteria was determined at 30 mg/mL. For extracted date syrup polyphenols, the MIC was 30 mg/mL for *E. coli* and 20 mg/mL for *S. aureus*, these results are not significantly different ($p<0.05$) from date syrup's MIC suggesting that both date syrup and date syrup polyphenols exert the same effect in retarding bacterial growth.

Furthermore, it was found that treatment of both *E. coli* and *S. aureus* with the different date syrup treatments using concentrations of date syrup corresponding to sub-minimum inhibitory concentration (MIC) (15 mg/mL), above MIC (30 mg/mL), extracted date syrup polyphenol (PPDS) and a concentration of artificial date syrup sugar (consisting of 7.6% w/v sucrose, 46.13% w/v fructose and 46.3% w/v glucose) (Sugar) significantly decreased the survival rates as represented in **Figure 2**.

To assess whether extracted date syrup polyphenol (PPDS) derived hydrogen peroxide was responsible for the suppression of *E. coli* and *S. aureus* growth, the effect of catalase on the antibacterial activity of the different date syrup treatments including extracted date syrup polyphenol and artificial date syrup sugar was examined. The addition of 100U/mL catalase restored the growth of *E. coli* significantly ($p<0.05$) and *S. aureus* medium containing different date syrup treatments as outlined in **Figure 2**. This suggests that H_2O_2 mediates the antibacterial activity of date syrup.

Hydrogen peroxide mediates the antimicrobial action of date syrup

To obtain evidence that H_2O_2 is generated by date syrup, the hydrogen peroxide production was determined in nutrient broth (NB) medium at concentrations of date syrup corresponding to 15 mg/mL date syrup, 30 mg/mL date syrup, extracted date syrup polyphenol (PPDS) and a concentration of artificial date syrup sugar (Sugar) corresponding to the MIC, this was achieved by the FOX method an assay sensitive to hydrogen peroxide production by measuring the formation of a complex between xylenol orange and ferric ion as identified in **Figure 3**. The addition of 100 U/mL catalase on the hydrogen peroxide activity of date syrup was also further investigated, the enzyme catalase quenches the generation of H_2O_2 and the addition of catalase significantly (p <0.05) decreased the hydrogen peroxide generated.

The levels of H_2O_2 increased significantly as the concentration of date syrup increased, this was also evident with extracted date syrup polyphenols. This demonstrated that the addition of catalase had an effect on hydrogen peroxide activity regardless of date syrup treatment and concentration, and this effect was further corroborated (**Figure 2A** and **2B**) with the addition of catalase in the presence of bacteria. Date syrup sugar appears to generate the least hydrogen peroxide and is influenced least by catalase activity suggesting no direct effect in date syrup's antimicrobial activity in inhibiting *E. coli* and *S. aureus*.

When *E. coli* cells were treated with hydrogen peroxide, the addition of 15 mg/mL concentration of date syrup appeared to behave as an antioxidant, as outlined in **Figure 4**, indicating that this concentration of date syrup in conjunction with hydrogen peroxide reduced any excessive accumulation of hydrogen peroxide that would otherwise be lethal (**Figure 4A**). Interestingly, a 15 mg/mL concentration of date syrup appeared to enhance the growth of bacterial cells implying antioxidative behaviour, this result was supported by previous MIC's whereby this concentration is not inhibitory to bacteria and it is possible that the % antioxidant activity as outlined in **Figure 1** is not strong enough to inhibit bacterial growth.

Date syrup and date syrup polyphenol where then further evaluated for synergistic activity with hydrogen peroxide as identified in **Figure 5**.
Date syrup and extracted date syrup polyphenol at the highest antioxidant activity potential, function as a prooxidants in inhibiting *E. coli*, whereas at a lower concentration it behaves as an antioxidant in allowing bacteria to survive, which corresponds to the MIC values.

Discussion

This study demonstrated that date syrup, and date syrup polyphenols, the most abundant bioactive constituent in date syrup, have antibacterial activity against the disease causing pathogens *E. coli* and *S. aureus*. The study has also shown that the extracted polyphenols retard bacterial growth and has observed that date syrup behaves as a prooxidant by generating hydrogen peroxide that mediates bacterial growth inhibition as a result of oxidative stress. Furthermore, low concentrations of date syrup demonstrated

antioxidative activity by reducing hydrogen peroxide, whereas at optimal bacterial growth and weakly alkaline conditions date syrup demonstrated prooxidant activity that inhibited the growth of *E. coli* and *S. aureus*. The osmolarity as a result of the high sugar content naturally present in date syrup did not significantly contribute to this effect. These findings highlight that date syrup and date syrup polyphenols interaction with bacteria are involved in prooxidant mediated bacterial inhibition.

The determination of secondary metabolites as antioxidants is outlined in **Table 1**. In comparison to previous literature investigating date fruit and date syrup (Dhaoudi et al., 2010; Abbès et al., 2013; Al-Farsi et al., 2007), the results indicate that date syrup contains secondary metabolites that are associated with bioactive behaviour (Vayalil, 2012).

The extent to which the bacterial growth was inhibited by date syrup and date syrup polyphenols was related to the content of redox active phenolic compounds and H_2O_2. These results support the assertion that the structural interaction between these bioactive compounds is responsible for growth inhibition beyond an osmotic effect of sugars alone. This offers a new possibility that redox active phenolic compound present in date syrup; date fruit and other antioxidant rich fruits are active intermediates contributing to microbial impairment.

Although polyphenols and individual phenolic compounds have long demonstrated antioxidant behaviour, the present study has demonstrated that date syrup and date syrup polyphenols inhibit the growth of *E. coli* and *S. aureus* by generating H_2O_2. Furthermore, it has also been demonstrated that date syrup polyphenols, one of the most abundant constituents in date syrup, function similarly to whole date syrup suggesting that date syrup polyphenols are the major constituents contributing to date syrup's antibacterial activity (**Figure 2** and **3**). The capacity for date syrup and date syrup polyphenols to generate H_2O_2 in culture medium is consistent with current literature (Yamamoto et al., 2004; Liu et al., 2013; Nakagawa et al., 2004) describing H_2O_2 generation in various mediums, implying that organic components of medium (such as vitamins, proteins, and inorganic salts) do not directly affect date syrup and date syrup polyphenol mediated H_2O_2 generation.

Critically, it was also observed that the contribution of osmolarity of date syrup had no significant influence on its antibacterial activity with MIC for artificial date syrup being higher than date syrup or extracted date syrup polyphenols (**Figure 2A** and **2B**).

Polyphenols are able to inhibit microorganisms and the antimicrobial activity of polyphenols is dependent on their chemical structure and environmental conditions (Almajano et al., 2007). This study investigated whether date syrup and extracted date syrup polyphenols function as an antioxidant or as an antimicrobial. The naturally weak acidic date syrup (pH 5.1) at low concentrations behaved as an antioxidant and protected both *E. coli* and *S. aureus* from H_2O_2 induced oxidative damage, whereas at MIC concentrations date syrup and extracted date syrup polyphenols demonstrate prooxidant activity (**Figure 4** and **5**) thus behaving as an antimicrobial. The exact mechanism

contributing to this effect remains unclear but polyphenols exist as esters of organic acids and can be readily bound to protein (Kroll et al., 2003); the interaction of polyphenols with proteins present in the bacteria result in ionic bonding and hydrogen bonding interactions (Canillac & Mourey, 2004) this will alter protein activity in the microorganism and make it more susceptible to treatment, but will also influence the antioxidant activity of polyphenols (Rawel et al., 2002; Rawel et al., 2001).

These observations could be the result of changes to the proteins on and within the bacteria as a result of the interaction with date syrup and date syrup polyphenols, making it more susceptible to attack and oxidative stress. Oxidants such as polyphenols cause oxidative stress and as aerobic bacteria, both *E. coli* and *S. aureus* have evolved intricate molecular mechanisms in response to oxidative stress by the activation of several stress genes (Macvanin & Hughes, 2010; Brudzynski et al., 2012).

Oxidative stress and damage is often associated with DNA damage due to the breakdown of fragments in DNA and further transcriptional changes in antioxidant associated genes such as superoxide dismutase and catalase (Brudzynski et al., 2012), which are induced and influenced by H_2O_2. The *oxyR* and *perR* genes control the expression of inducible forms of *katG*, and *ahpCF* genes, which function to homeostatically control the concentration of H_2O_2 once it becomes too high. Therefore it can be suggested that the antibacterial activity of date syrup mediated by hydrogen peroxide will most likely demonstrate transcriptional changes associated with antioxidant genes and oxidative stress genes.
In agreement with previous literature (Liu et al., 2013; Brudzynski et al., 2012; Chen et al., 2012) pre-treatment of date syrup and extracted date syrup polyphenols with catalase to remove H_2O_2 reduced the bacteriostatic activity of date syrup to a conservative level (**Figure 2A** and **2B**), this was particularly significant between 15 mg/mL and 30 mg/mL date syrup and was independent of the initial H_2O_2 concentration (**Figure 3**) thus suggesting that H_2O_2 generated as a result of date syrup induces antibacterial activity.

It has been recently documented that date syrup is an antioxidant fruit with specific compounds possessing antioxidant activity (Dhaouadi et al., 2010; Cadenas & Packer, 2005), of date syrup constituents the polyphenol compounds are renowned for their antioxidant behaviour, **Figure 1** illustrates this behaviour. As shown in **Figure 1** the antioxidant behaviour of both date syrup and extracted date syrup polyphenols increase linearly as the concentration increases (p <0.05). This assay revealed two particular insights; firstly, there was no significant difference between date syrup antioxidant activity and date syrup polyphenol antioxidant activity which suggests that the polyphenols in date syrup compromise predominantly the bioactive constituents and these bioactive compounds influence H_2O_2 in mediating it as an antimicrobial agent. Secondly, the increase in antioxidant behaviour (activity) was observed repeatedly up until 60% (corresponding to 30 mg/mL), further supporting the role of date syrup and date syrup polyphenols as both antioxidants and prooxidants in antibacterial activity. At a concentration corresponding to 15 mg/mL, which is sub-lethal MIC, date syrup demonstrates antioxidative behaviour signifying that it scavenges any free radicals and reduces H_2O_2 generated thus allowing bacterial cells to proliferate and grow. This is

evident in the antibacterial results in both the presence and absence of catalase, signifying that this concentration is not lethal to bacteria implying minimal stress responses are activated by bacteria at this concentration. Previous research conducted on date syrup and date fruit support this finding (Abbès et al., 2013; Kchaou et al., 2013; Martín-Sánchez et al., 2014; Procházková et al., 2011).

Despite apparent antioxidative activity, this was diminished at concentrations of 60%, above 60% it acts as a prooxidant suggesting high concentration of date syrup and date syrup polyphenols are required to achieve prooxidant mediated bacterial inhibition. It is possible that the prooxidant activity and subsequent H_2O_2 generation are affiliated with the presence of metal ions. The co-incubation of bacteria with date syrup polyphenols may disrupt bacterial respiration by sequestering metal ions leading to generation of H_2O_2. As a traditional medicinal application, this provides a preliminary scientific basis for date syrup's medicinal use as an antimicrobial agent and it's potential for future bacterial infection treatment. This observation is supported by previous literature highlighting the closely related relationship in polyphenols behaving as prooxidants and antioxidants, suggesting that dietary polyphenols exhibit both antioxidative and prooxidative properties under certain conditions such as pH, metal reducing potential, solubility and a natural defence in response to attack (Perron & Brumaghim, 2009; Sakihama et al., 2002; Procházková et al., 2011). This implies that prooxidant environment is beneficial, since, by imposing a mild degree of oxidative stress, the levels of antioxidant defenses and xenobiotic-metabolizing enzymes might be raised, leading to protection through cytotoxicity in inhibiting microorganisms (Halliwell, 2008).

Conclusion

It has been demonstrated for the first time that date syrup and date syrup polyphenols are able to inhibit Gram negative *E. coli* and Gram positive *S. aureus* by generating H_2O_2, and that date syrup polyphenols are active intermediates directly involved in inducing oxidative stress in bacteria as a result of hydrogen peroxide generation. These results confirm the critical relationship between antioxidants and prooxidants of date syrup polyphenols in bacterial growth and bacterial inhibition. It has also been shown that the high content of naturally occurring sugars in date syrup do not significantly contribute to its antibacterial activity. These results confirm the critical role of the relationship of antioxidants and prooxidants of date syrup polyphenols in bacterial inhibition and as an antimicrobial agent.

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673 **Table 1. Antioxidant determination of date syrup. Results are expressed as means ±**
674 **SD mg/100g of fresh date syrup weight**
675

	Date syrup (mg/100g)
Total phenol content	605.1± 31.6
Tannins	357.4 ± 18.7
Flavanoids	40.5 ± 28.9
Flavanols	31.7 ± 8.6
Anthocyanins	6.63 ± 1.9
Carotenoids	1.59 ± 0.1

676
677

Table 2. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of date syrup, and extracted date syrup polyphenols necessary to inhibit microbial growth in vitro expressed in mg/mL.

Microorganism	Date Syrup		Date syrup polyphenols	
	mg / mL (SD)			
	MIC	MBC	MIC	MBC
E. coli	30 (± 0.83)	40 (± 0.97)*	30 (± 0.11)	32 (± 0.73)*
S. aureus	30 (± 0.76)	35 (± 0.54)*	20 (± 0.82)	23 (± 0.94)*

Mean and (SD) of results are expressed as three independent experiments in triplicates.
*Significant differences between each treatment and microorganism indicated as $p<0.05$.

Legend Titles

FIGURE 1. Comparison of the antioxidant potential of date syrup (DS) (round grey scale dot) and extracted date syrup polyphenol (PPDS) (black triangle) against the commercially available antioxidant butylated hydroxytuolene (BHT) (black dot). Results are expressed as mean ± SD.

FIGURE 2. Inhibitory effects of catalase on the antibacterial action of date syrup. Date syrup treatments; Date syrup (DS), extracted date syrup polyphenols (PPDS) and artificial date syrup sugar (Sugar) were added to a) *E. coli* and b) *S. aureus* cell suspensions with or without 100 U/mL catalase. After being incubated at 37 °C for 4h with shaking, cell viability was determined using surface drop count methods and expressed as viability in colony forming units (CFU). Data is mean ± SD of three independent experiments. Significant differences between treatment groups are indicated as $^{**}p < 0.05$.

FIGURE. 3. H_2O_2 generation by different sample treatments of date syrup (Date syrup (DS), extracted date syrup polyphenols (PPDS) and artificial date syrup sugar (Sugar)) and the effect of 100U/mL catalase on the production of hydrogen peroxide by date syrup. The concentration of H_2O_2 in the medium was immediately determined by the FOX method 1h after the addition of different date syrup treatments to NB medium (pH7.5). Data is mean ± SD of three independent experiments. Significant differences between treatment groups are indicated as $^{***}p < 0.05$.

FIGURE 4. Evaluation of antioxidant activity of date syrup treatments.
a. 15 mg/mL date syrup (DS) b. 30 mg/mL date syrup and c. polyphenol date syrup (PPDS)(30 mg/mL).
Date syrup treatments were added to NB medium and incubated at 37 °C with *E. coli* for 4h with shaking, cell viability was determined using the surface drop count method and expressed as viability in colony forming units (CFU). Significant differences between treatment groups are indicated as $^{**}p < 0.01$.

FIGURE 5. **Evaluation of the synergistic effect of date syrup and H_2O_2 on cellular viability.** Significant differences between treatment groups are indicated as $^{****}p<0.01$.