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Saliva interacts with hawthorn juice to reduce its antioxidant capacity

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Abstract

Hawthorn juice (HT), produced from the flowers, leaves and fruit of the hawthorn tree (*Crataegus spp.*), is easily available to purchase as a dietary agent. The aim of this study was to investigate the antioxidant capacity (AC) of HT in saliva using the Folin-Ciocalteu assay, both *in vitro* and following consumption. Healthy volunteers were recruited and gave informed written consent. Volunteers (n = 12) drank 10mL HT and provided saliva samples over 3 hours. There was a significant ($p < 0.01$) increase in AC of saliva immediately after drinking the juice, but not at later time points. In the *in vitro* study, volunteers provided saliva samples which were pooled and incubated with diluted HT (1/500, 1/200, 1/100 final dilutions in saliva). There was a highly significant interaction between HT and saliva ($p < 0.001$), whereby the AC of HT in saliva was less than the sum of the AC of saliva and the AC of HT in water. While the transient increase in AC following consumption may help prevent oral diseases, the interaction between saliva and HT (possibly due to protein-polyphenol interactions) may have implications for the bioavailability and functional activity of HT, both in the oral cavity and systemically.

Introduction

Hawthorn juice is a beverage derived from the flowers, leaves and fruit of the hawthorn tree (*Crataegus spp.*), which is commonly found in temperate regions of the Northern hemisphere. Many studies have investigated pharmacological effects of hawthorn, with some suggesting significant benefits, mainly in relation to cardiovascular disease (Pittler, Guo et al. 2008). However, hawthorn juice is easily available to purchase as a dietary agent, and so the physiological effects are of interest to ordinary consumers as well as to medical practitioners. When considering the possible physiological effects of a dietary agent, two issues are of central importance:

- The pharmacological properties of the constituents
- The oral bioavailability of the pharmacological constituents

The effects of hawthorn are generally attributed to its high polyphenol content, which are known antioxidants (Bernatoniene, Masteikova et al. 2008), and thus the capacity of hawthorn juice to reduce the Folin-Ciocalteu reagent in the well-known assay (Magalhaes, Santos et al. 2010), is used in this study as an indicator of pharmacological activity. Saliva is an important component of the food digestion process and it also can provide a window on aspects of systemic bioavailability (Idkaidek, Arafat et al. 2017). Therefore, through a study of the antioxidant capacity

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of a commercially-available hawthorn juice and its interactions with saliva, this study aims to further the knowledge on the physiological effects of hawthorn juice as a dietary agent.

Materials and Methods

Materials

Gallic acid, Folin-Ciocalteu reagent, Na_2CO_3, purchased from Sigma-Aldrich. Hawthorn juice purchased from a local healthfood store. Saliva collection vials and aids were purchased from Salimetrics.

Volunteer recruitment

Volunteers were recruited (using word-of-mouth and one in-class social media group) from the student population of NUI Galway. Volunteers were recruited separately for the two parts of the study ((1) consumption study and (2) *in vitro* study). Potential volunteers were given a Participant Information Sheet, and they gave informed written consent if they were willing to participate. All volunteers were aged between 18 and 25. Volunteers were asked to refrain from eating or from using oral hygiene products for 1 hour prior to the study. Volunteers were excluded if they had any medical condition or if they were currently suffering from any infection. For the consumption study, volunteers were excluded if they were currently in receipt of any medication, including over-the-counter preparations.

Saliva Collection and Handling

Saliva samples were collected using the passive drool method using a saliva collection vial and saliva collection aid. As soon as samples were collected, they were maintained on ice until they were transferred to storage at -20^{o}C. Samples were thawed at room temperature prior to analysis.

Analysis of the Antioxidant Capacity of Saliva and Hawthorn Juice

Antioxidant capacity was measured using the Folin-Ciocalteu (FC) assay as previously described (Magalhaes, Santos et al. 2010). Antioxidant capacity was determined as mg/L gallic acid equivalents using an equation obtained from a standard gallic acid curve. Antioxidant capacity was determined from triplicate measurements of three dilutions of hawthorn juice (1/100, 1/200 and 1/500).

Consumption Study

Volunteers were first asked to donate a saliva sample over 3 minutes and this was denoted as the time 0 sample. They then consumed 10mL of hawthorn juice, before proceeding to give a second saliva sample, beginning at the 5 minute time point. Volunteers proceeded to give four further saliva samples, at time points of 35, 65, 125 and 185 mins. Samples were separated into two aliquots prior to assay, and one aliquot was centrifuged for 15 minutes at 13,000 rpm in an Eppendorf bench-top centrifuge.

In Vitro Study

Volunteers were asked to donate one saliva sample, over a period of 5 minutes. Prior to assay, saliva samples from all volunteers were pooled together to ensure sufficient sample volume for the study. Hawthorn was used at final dilutions of 1/500, 1/200 and 1/100. Dilutions of 10X were made in distilled water and further diluted in either saliva (two aliquots) or distilled water. When hawthorn juice was added to the saliva or water, samples were incubated at room temperature for 5 minutes. One aliquot of saliva/juice was centrifuged (as above) prior to assay.

A similar experiment was performed whereby saliva was replaced with solutions of BSA (0.5, 1 and 1.5mg/mL). HT dilutions were prepared as above and diluted in BSA preparations. Samples were incubated at room temperature for 5 minutes prior to analysis.

Statistical Analysis

Data was calculated using Excel. For the consumption study, data was calculated as mean +/- s.e.m. of individual samples. For the *in vitro* study, mean +/- standard deviation of triplicate measurements from pooled saliva was calculated. Statistical analysis was performed using IBM SPSS Statistics 24.

Results and Discussion

Consumption Study

The hawthorn juice used in this study was measured as having an antioxidant capacity of 7219 mg/L +/- 1384, determined by the F-C assay as described in the Methods. There was a highly significant, though short-lived effect on the antioxidant capacity of saliva when 10mL HT was consumed by participants. There was a highly significant effect of HT consumption in both whole saliva [$F(5, 65) = 4.69$, $p = 0.001$] and centrifuged saliva [$F(5, 66) = 6.20$, $p < 0.001$]. Dunnett's post-hoc test showed that there was a highly significant increase in antioxidant capacity in the centrifuged ($p = 0.001$) and whole samples ($p = 0.004$) taken immediately following consumption, compared to baseline. This had returned to baseline levels 30 minutes later. This is in contrast to the study by Ginsburg (Ginsburg, Kohen et al. 2013), who showed that elevated antioxidant activity persisted in the saliva for up to 300 minutes after volunteers held a cinnamon extract in their mouth, and up to 120 mins after volunteers consumed an espresso drink. However, this

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study differed from ours in that our study the volunteers drank the HT directly, whereas in the Ginsburg study, volunteers held the agents in the mouth for 30s before either elimination or swallowing. There is no evidence of salivary excretion of polyphenols within the timeframe examined in our study.

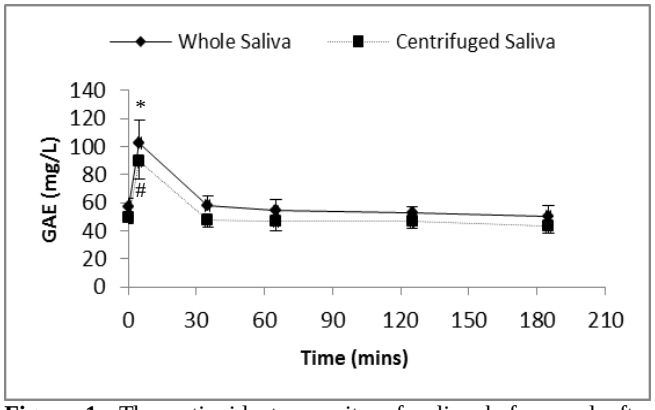

Figure 1: The antioxidant capacity of saliva before and after consumption of 10mL hawthorn juice. Juice was consumed after donation of the first saliva sample, and the second saliva sample was donated after consumption of the juice at the 5 minute time point. Data shown as mean +/- s.e.m. * $p < 0.01$ in Whole Saliva and # $p < 0.01$ in Centrifuged Saliva vs time 0; (n = 12).

In Vitro Study

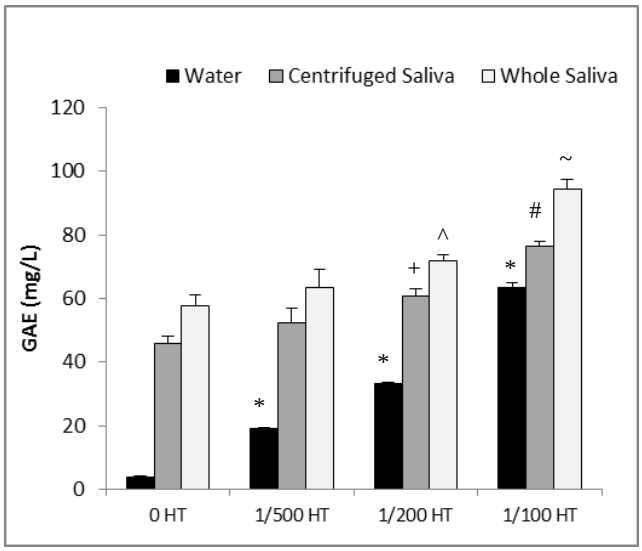

Figure 2: The *in vitro* antioxidant capacity of hawthorn juice (HT) following incubation in water and saliva. Data shows mean + s.d. of 3 replicates from samples pooled from 12 volunteers. * $p < 0.001$ vs 0HT in water; + $p < 0.01$ vs 0HT in centrifuged saliva; # $p < 0.001$ vs 0HT in centrifuged saliva; ^ $p < 0.01$ vs 0HT in whole saliva; ~ $p < 0.001$ vs 0HT in whole saliva.

Two way ANOVA showed that there was a highly significant effect of hawthorn juice on antioxidant capacity [$F(3, 24) = 380.3$; $p < 0.001$], and a highly significant effect of saliva [$F(2, 24) = 705.4$; $p < 0.001$]. There was also a highly significant interaction between saliva and hawthorn juice [$F(6, 24) = 16.4$; $p < 0.001$]. This was borne out by One Way ANOVAs followed by Dunnett's post-hoc tests, which showed that while the overall effect of hawthorn juice was highly significant in all cases, the impact of hawthorn juice was greater in water than in saliva, whereby the 1/500 HT dilution was significantly different from 0HT in water, but not in saliva. Table 1 further illustrates this blunting effect, as the effect of HT in saliva is considerably less than in water, when the mean antioxidant capacity of saliva alone is subtracted from the mean antioxidant capacity of HT in saliva.

	Water	C. Saliva	W. Saliva	0.5mg/mL BSA	1mg/mL BSA	1.5mg/mL BSA
1/500 HT	100%	41.2%	37.5%	78.8%	78.4%	67.1%
1/200 HT	100%	50.4%	48.0%	82.4%	76.6%	75.2%
1/100 HT	100%	50.9%	61.2%	88.6%	74.5%	82.2%

Table 1: The mean antioxidant capacity of hawthorn juice (HT) is expressed as % control (where control is the effect in water), following subtraction of the relevant salivary antioxidant capacity (mean value) in each case. The first 3 columns show data represented in figure 2, and the latter 3 columns represent a separate experiment.

In contrast to this study, Fibach and Ginsburg (Fibach and Ginsburg 2015) demonstrated that the antioxidant activity of a fermented papaya preparation was significantly greater in saliva, and the authors suggested that saliva may play a role in increasing the availability of polyphenols from the preparation. The varying results may be due to the different preparation and plant source being used in their study. More in line with our findings however, are the studies which show that tea polyphenols such as catechin and epigallocatechin can interact with proteins such as albumin and casein to reduce the antioxidant function of the polyphenols (Arts, Haenen et al. 2002, Bourassa, Cote et al. 2013). The flavonoids catechin and epicatechin are dominant constituents in the fruit of *Crataegus monogyna* (Bernatoniene, Masteikova et al. 2008) and therefore it is conceivable that the constituents of hawthorn juice may bind to salivary proteins, reducing their antioxidant capacity. Indeed a number of studies have investigated the interactions between polyphenols and salivary proteins (Faurie, Dufourc et al. 2016). This possible interaction between HT and salivary proteins would have important implications, not only for functioning in the oral cavity, but also for systemic function, where protein/polyphenol interaction could limit polyphenol availability. As epigallocatechin gallate (EGCG) has previously been shown to bind to both bovine serum albumin (BSA) and human serum albumin (HSA) (Nozaki, Hori et al. 2009), and albumin is an important protein component of saliva, the interaction of HT with BSA was also examined. While BSA itself

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demonstrated reducing capacity in the F-C assay (data not shown), this study showed a significant interaction between BSA and HT [$F(9, 32) = 8$; $p < 0.001$]. Table 1 shows that when the mean antioxidant activity of BSA alone was subtracted at each level, the antioxidant capacity of HT was less than in water. While this supports the hypothesis of an interaction between HT polyphenols and salivary proteins, further studies would need to be done.

In this study both whole saliva and centrifuged saliva were analysed for antioxidant capacity (for both the consumption study and the *in vitro* study). The reason for analysing both was because there are advantages and disadvantages to using whole saliva. Whole saliva is the most representative of the *in vivo* milieu. However, it is difficult to accurately pipette and analyse due to the presence of mucopolysaccharides which make it highly viscous. Also, there may be food particles and cell squames present that could lead to inconsistencies in assay results from whole saliva. While we found that the measured antioxidant capacity was slightly higher overall in the whole saliva than the centrifuged saliva (in both the consumption study and the *in vitro* study), the interactions between HT and saliva were observed from both whole and centrifuged saliva (*in vitro* study). Less blunting of the effect of 1/100 HT *in vitro* may have been observed in the whole saliva (Table 1), but the significance of this, if any, cannot be deduced from this study.

Conclusion

The aim of this study was to address an important issue for consumers and for medical practitioners – the physiological functions of a widely-available, off-the-shelf preparation of hawthorn juice. This study has shown that consumption of this beverage can greatly enhance the antioxidant capacity of saliva for a short time, and thus could have potential benefits for the prevention of oral disease such as periodontitis. Perhaps this effect could be enhanced by retaining the juice in the mouth for a longer time. Also, this study demonstrated that there is an interactive effect between saliva and hawthorn juice, possibly due to interaction of polyphenols with salivary proteins such as albumin. Further studies would need to be done to investigate this possible interaction of hawthorn juice with salivary proteins.

Conflict of Interests

The authors have no conflict of interests to declare. There is no affiliation with the manufacturers or the sellers of the product in the study, and no commercial funding has been received.

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