plasma concentration of aspalathin. It is many fold more than the quantity of any other antioxidant present in a R_f extract. The amount of R_f we used to expose our cells in this study was equivalent of 1.9nM of aspalathin. It was the express intention of this study to investigate whether the quantity of antioxidants present in 500ml's of R_f tea would have physiological consequences on the BBB and thus be able to alleviate the ROS-induced effects of EtOH.

4.1.4 Viability

4.1.4.1 The effect of EtOH Treatment on cell viability:

Cells exposed to both concentrations of EtOH, resulted in a decrease in viability across all time-intervals from 24 to 96hrs (Fig 3.1.1). Previous work by Mentor (2015) utilizing similar concentrations of EtOH, showed no to very little changes in viability, however, mimicking the decrease in viability we observe at 48hrs. Metabolized EtOH is a primary contributor of ROS, which is exacerbated by increased CYP2E1 activity following moderate and excessive alcohol consumption (Manzo-Avalos and Saavedra-Molina, 2010). EtOH stimulates the enzymatic activity of cytochrome P450, which enhances excess ROS production as well as diminishes inherent cellular antioxidants which may reduce ROS (Wu and Cederbaum, 2003). Alcohol-induced ROS production impedes the body's natural defense mechanisms .EtOH may also initiate mitochondrial dysfunction and decreased energy conservation; therefore, it is not surprising that both physiological and supraphysiological EtOH concentrations resulted in a detrimental effect on viability. However, we still need to investigate the effects of EtOH on ATP production in our model. Alcohol metabolism produces NO, along with ROS by induction of NADPH/xanthine oxidase and nitric oxide synthase, adding to oxidative stress. Our data showed that the viability at 96hrs was significantly greater in response to EtOH than at 24hrs, indicating recovery of the bEnd5 cells. However, 24hr EtOH exposure induces a 96hr (4 day) negative effect on cell viability.

4.1.4.2 The effect of R_f treatment on cell viability:

A central component of the experimental design was to investigate the effects of R_f-derived antioxidants, equivalent to plasma levels of aspalathin after consuming 500ml's of R_f tea, on cell viability in mouse brain endothelial cells. We do however take cognizance of the fact that the rooibos extract used did not only contain aspalathin, but also other compounds that may work synergistically or antagonistically in our model. Nonetheless it was shown that aspalathin was the most prominent of all the compounds in the rooibos extract (Fig 3.8). We found that exposure to R_f, resulted in a decrease in cell viability between 24 and 48hrs, which recovered between 72 and 96hrs (Fig 3.1.2). Similarly, a study by Mentor (2015), using much higher concentrations also showed decreased viability at 24hrs, and increased viability at 96hrs. Our data indicated that the addition of R_f-derived antioxidants, equivalent of only 1.9nM aspalathin, can induce a physiological effect on the viability of BECs. This was particularly surprising as R_f is generally portrayed to have no adverse effects. Under normal conditions, ROS formation in cells is regulated by innate antioxidant systems, thus the addition of external antioxidants could induce an imbalance in the ROS-antioxidant system of the cell which may be reflected in the results seen in response to R_f. However, the recovery between the latter time intervals, 72 and 96hrs, suggests that the initial adverse effect on EC viability is dependent on inherent cellular mechanisms. Future studies are pivotal in measuring the antioxidant capacity of the bEnd5 cells used in this current study to determine the precise mechanism involved.

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4.1.4.3 The effect of EtOH and R_f treatment on cell viability:

When investigating if R_f could negate the effects of EtOH based on its antioxidant potential, we found that exposure to R_f reduced the effects of 25mM EtOH on bEnd5 cell viability at 72hrs, signifying that the EtOH-exposed bEnd5 ECs have significantly benefited as a result of the additional antioxidants. This is particularly important since this is a physiological dose

of EtOH. We further investigated if this was possible at a supraphysiological concentration, and found that the addition of R_f to 100mM EtOH exposed cells reversed the adverse effects of 100mM EtOH on cell viability at 72hrs (Fig 3.1.3.2). Overall, R_f, appeared to reduce and nullify the effects of both concentrations of EtOH at 72hrs, indicating that the alleviating properties of R_f-derived antioxidants requires a timeframe of at least 72hrs to diminish the effects of EtOH-induced ROS. This may be attributed to the metabolism of EtOH. Since its metabolite, acetaldehyde (AA) is equally as toxic; we hypothesize that this may be the reason for the delay in recovery in EtOH induced cells, even with the addition of additional antioxidants.

4.1.5 Cell proliferation

Cell proliferation was determined based on the number of live cells (Cadena-Herrera et al., 2015). The change in cell number between successive 24hr periods over 96hrs was used to calculate the rates of cell division. We compared the rates cell division in control populations of cells to those exposed to EtOH and R_f.

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4.1.5.1 The effect of EtOH treatment on cell proliferation:

Cell culture populations exposed to 25mM and 100mM EtOH had a significantly decreased rate of cell division based on the live cell number populations, than that of the untreated bEnd5 cells from 24 to 72hrs (Fig 3.2.1). Previously reported work by Mentor (2015) illustrated that similar concentrations also resulted in decreased cell proliferation at 72hrs, while at the other respective timelines, different findings to ours was noted. This may be attributed to the treatment protocol used, which is vastly different from ours (i.e. the exposure in our study was only for 24hrs). This was affirmed by the slopes of 25mM and 100mM EtOH (Fig 3.3.1), showing that the rates of cell division in EtOH-exposed cells were significantly suppressed in comparison to the control. The EtOH decrease in cell division is supported in

the literature where, Mikami et al. (1997) found that EtOH concentrations (25-200mM), suppressed cell proliferation.

4.1.5.2 The effect of R_f treatment on cell proliferation:

As part of the experimental design, we also investigated the effects of R_f derived antioxidants on the live cell number and cell division of bEnd5 cells. The live cell number was no different from the control at 24 and 48hrs, but resulted in a decrease at 72 and 96hrs. The latter results observed at 72 and 96hrs, respectively resulted in similar results in response to greater concentrations of R_f (Mentor, 2015). When analyzing the rate of cell division in response to R_f, we found that the rate in response to R_f was 0.226 cells/hr, which was significantly slower than the controls (0.435 cells/hr) (Fig 3.3.2). This data suggests that exposure to externally derived R_f antioxidants, may pose a long term detrimental effect to the proliferation of bEnd5 cells, possibly inhibiting or delaying cell proliferation. Since in a balanced cell system, there are equal amounts of oxidants and antioxidants, any addition of excess species that are physiologically not required by the cell may result in physiologically stressing the cell system. Therefore, it comes as no surprise that the additional R_f derived antioxidants displayed such an adverse effect in the bEnd5 ECs.

4.1.5.3 The effect of EtOH and R_f treatment on cell proliferation:

When analyzing the influence of R_f on the EtOH-induced effects on EC live cell number, and rate of cell division, we found that with the combinatorial exposure of EtOH and R_f, the R_f derived antioxidants partially reversed the effects of EtOH on the number of live cells in the experimental timeframe. Simultaneous exposure to R_f and 25mM EtOH, also partially reversed the effects of a physiological concentration of EtOH on the rate of cell division in bEnd5 cells. However, the exposure of R_f together with 100mM EtOH further exacerbated the decrease in the rate of cell division. The effects induced by the simultaneous administration of EtOH and R_f shows that the addition of

R_f-derived antioxidants is able to negate and nullify the EtOH induced ROS damage in the BECs but this imbalance of oxidants and antioxidants in the cell still has a detrimental effect on the cell division of these cells. It is expected that the greater concentration of EtOH will generate much larger concentrations of ROS. Thus, it is conceivable that the R_f concentration used in this study was not sufficient to completely nullify the EtOH-induced ROS.

4.1.6 Cell Toxicity

4.1.6.1 The effect of EtOH treatment on cell toxicity:

EtOH exposure increased cell toxicity in bEnd5 ECs. The production of AA by ADH, during the metabolism of EtOH, in ECs is the most probable explanation for the toxic effects. Not only is AA an extremely mutagenic and carcinogenic molecule, but it has the capacity to generate a large amount of excess ROS species during its metabolism (Sambuy, 2009). However, cell toxicity in response to 25mM and 100mM recovered to normal levels between 72 and 96hrs (Fig 3.4.1), which corroborates with the viability data shown in Fig 3.1.1.

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4.1.6.2 The effect of R_f treatment on cell toxicity:

Treatment of bEnd5 cells for 24hrs with R_f resulted in an increase in cell toxicity at all time-intervals (Fig 3.4.1), with decreased toxicity over the 96hr timeframe. This shows that cells exposed to R_f was on a trajectory in recovery and that the adverse effects in response to excess antioxidants is possibly short term. Also the maximum toxicity induced never exceeded 10%. This finding was similar to a study by Mentor (2015), whereby in response to greater concentrations of R_f, bEnd5 cells showed little to no toxicity.

4.1.6.3 The effect of EtOH and R_f treatment on cell toxicity:

When investigating the ability of R_f to reverse or reduce the effects of EtOH, we found that R_f exposure decreased the toxicity in cells exposed to the supraphysiological concentration of EtOH at 24 and 72hrs (Fig 3.4.1). Since EtOH metabolism is a source of ROS, it is believed that R_f may be displaying its antioxidant properties with its metal-chelating abilities and neutralization of singlet oxygen species and thus significantly reducing the oxidant damaging effects of alcohol to the BBB as we have observed by the reversal in cell toxicity, as well as live cell number and viability. The overall low toxicity to all compounds in our study indicates the robustness of bEnd5 cells to toxic insult.

4.1.7 Transcription

It has been premised in the literature that alcohol affects the permeability of the BBB. Haorah et al. (2008) postulated that the alcohol induced changes to BBB permeability was brought about by affecting TJ expression and thus paracellular pathway permeability. In this study, we found that treated and untreated bEnd5 cells did not express the TJ protein occludin. Steiner et al. (2011) also reported that bEnd5 cells lacked the localization of occludin to cellular junctions; however, Findley and Koval (2009) established that occludin-deficient mice are viable and exhibit normal barrier function. Furthermore, the bEnd5 cells used in this study has successfully expressed one of the critical proteins of brain endothelial cell TJs, claudin-5.

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4.1.7.1 The effect of EtOH treatment on transcription:

The transcription of claudin-5 in BECs exposed to 25mM and 100mM EtOH changed dramatically across the 96hr timeframe. In addition, as reported, the transcription of claudin-5 in response to the physiological concentration of EtOH was not statistically affected between 24 and 72hrs, but resulted in a decrease at 96hrs. Cells exposed to 100mM EtOH induced an irregular trend

in claudin-5 transcription, with a 4-fold increase in transcription at 24hrs, and statistical suppression at 96hrs (Fig 3.5.1). In addition, Haorah et al. (2008) established in a study using 50mM EtOH that the metabolism of EtOH resulted in the phosphorylation of TJ proteins, thereby altering TJ assembly; ultimately impairing BBB integrity, which is coherent with the suppression in claudin-5 transcription at 96hrs (Fig 3.5.1). Furthermore, it is plausible that the metabolite of EtOH, AA, also strongly contributes to the modifications in the molecular composition of TJs (Elamin et al., 2012).

4.1.7.2 The effect of R_f treatment on transcription:

When investigating the effects of R_f on claudin-5 transcription, we established that R_f resulted in an overall down-regulation of claudin-5 throughout the course of the experiment (Fig 3.5.2). To the best of our knowledge, the effects of R_f on claudin-5 transcription has never before been reported. However, green tea polyphenols (GTPs) was reported to also decrease claudin-5 expression similarly to what was observed in our study (Liu et al., 2013). Excess antioxidants may therefore compromise the paracellular pathway of the BBB.

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4.1.7.3 The effect of EtOH and R_f treatment on transcription:

While our study endorsed the data in the literature we further investigated whether this alcohol induced changes to BBB permeability could be reversed or neutralized by co-exposure to R_f. Both the physiological and the supraphysiological concentrations of EtOH on their own and in combination with R_f down-regulated claudin-5 mRNA transcription across the experimental timeframe with the exception of 72hrs. The simultaneous exposure of EtOH and R_f resulted in diminished claudin-5 transcription throughout the course of the experiment (Fig 3.5.3). Based on our hypothesis; we expected reversal or neutralization of the EtOH-induced ROS effects, thus, we were surprised to see that co-exposure led to an increased down-regulation of claudin-5 transcription. This indicated that the ROS and

antioxidant species affected the status of the TJs, by a yet unknown physiological molecular mechanism which exacerbated the suppression of claudin-5.

4.1.8 Permeability

Since TJs play a significant role in the regulation of paracellular permeability, we used the technique of measuring transepithelial electrical resistance (TEER) to approximate changes to monolayer permeability; hence the TEER of confluent monolayers of bEnd5 cells, were recorded in response to exposure of selected concentrations of EtOH, R_f, and the combinations thereof. The measured TEER is inversely proportional to permeability.

4.1.8.1 The effect of EtOH treatment on permeability:

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Studies have suggested that the mechanism by which alcohol affects the BBB is through the disruption of the endothelial TJ which then ultimately results in augmented BBB permeability (Haorah et al., 2005; Singh et al., 2007). Our findings have shown that both 25mM and 100mM EtOH decreased the resistance of the bEnd5 cells and increased the monolayer permeability. This decrease observed in response to the selected concentrations of EtOH comes as no surprise, since the overall trend observed in TEER (Fig 3.6.1) relates to events in transcription (Fig 3.5.1), where with the down-regulation of claudin-5 was observed. Interestingly, previous studies have demonstrated that EtOH concentrations of 50 and 100mM decreased TEER of a cell monolayer significantly in a dose dependent manner (Abdul Muneer et al., 2011). Abdul Muneer et al. (2011) also reported that EtOH decreased BBB electrical resistance while increasing BBB permeability, corroborating our findings.

There are two routes for substances to cross the BBB, the paracellular pathway and via the transcellular pathway. Similarly, transepithelial resistance is a function of both transcellular resistance in parallel with

paracellular resistance. Haorah et al. (2005) provided data which implicated alcohol affecting the myosin light chain kinase (MLCK), an enzyme integral to the structure of the intracellular cytoskeleton, which in turn led to compromising the paracellular permeability by phosphorylation of TJs. This provides a plausible explanation of why we observed decreases in TEER across our model of bEnd5 BECs.

4.1.8.2 The effect of R_f treatment on permeability:

Cell monolayers exposed to R_f also resulted in a decrease in TEER across the experimental timeframe of 96hrs (Fig 3.6.2). Given that the bEnd5 cell monolayers were exposed for 24hr before monitoring the permeability, we hypothesized that the R_f-derived antioxidants may pose a long term effect on the permeability of the BBB, as illustrated in the inability of the monolayer to return to control levels of permeability. In addition, the increase in permeability observed (Fig 3.6.2) corroborates the decrease observed in claudin-5 mRNA transcription in response to R_f. However, contrary to this finding, a study by Mentor (2015), illustrated an increase in TEER, which may likely be as a result of the much higher concentrations used.

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4.1.8.3 The effect of EtOH and R_f treatment on permeability:

The simultaneous exposure of EtOH and R_f to the bEnd5 cells was not able to reverse the effects of EtOH on TEER. Similarly R_f could not reverse the EtOH induced effects observed in the relative mRNA transcription of claudin-5 (Fig 3.5.3). This further validates the endorsement of the PCR data. Our hypothesis was that the R_f-derived antioxidants would neutralize EtOH-induced ROS, effectively reversing or nullifying, the effects of EtOH. Surprisingly, the addition of R_f exacerbated the effects of alcohol. Also, R_f on its own caused similar effects to alcohol by significantly decreasing the effects on permeability. Since the simultaneous exposure of R_f with EtOH was able to negate the effects of EtOH on the viability and proliferation of bEnd5 cells, we expected that it would produce a similar effect on claudin-5

transcription and bEnd5 permeability. On the contrary, as illustrated (Fig 3.5.3 and Fig 3.6.3), the addition of R_f was not able to reverse or reduce the EtOH-induced effects on these endothelial cells. This may likely be attributed to AA, a toxic metabolite of EtOH. We hypothesize that this highly toxic metabolite of EtOH may be influencing claudin-5 directly, since it has it is speculated that they do interfere with DNA and posttranslational modifications; ultimately signifying that AA may be more toxic than EtOH since it may act directly on proteins as well as via ROS.

4.1.9 Morphology of a monolayer of bEnd5 cells.

The ultrastructure of bEnd5 brain endothelial cells was investigated using high resolution SEM to visualize the fine morphological and molecular detail of the cells growing on cellulose covered inserts, and to compare the morphological effects of EtOH and R_f. Cells were processed for SEM at 96hrs. This enabled the verification of the bEnd5 monolayer and the analysis of the state of confluence of the layer.

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SEM is advantageous in the sense that the surface of a specimen is accessible for experimentation at a resolution and depth of field greater than that of the optical microscope (Nixon, 1971), allowing us to visualize structures impossible to see with the naked eye, such as the TJs discussed in our study.

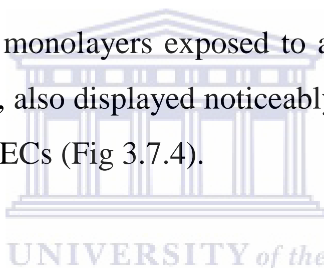
4.1.9.1 Cultural monolayers:

Untreated cells (controls) displayed intact primordial TJs between adjacent ECs, evident by their latero-apical location, and visibility of parallel strands present, while showing no compromised paracellular spaces (Fig 3.7.1 and Fig 3.7.2). The rationale for identifying these structures is based on their theoretical and reported location and morphology. Claudin-5 in particular, is located in the latero-apical membrane surface of adjacent ECs, and the extracellular components of the TJ protein, claudin-5 have two extracellular loops (Furuse et al., 1999; Ballabh et al., 2004; Schreibelt et al., 2007).

Knowing the structural location and morphology assisted in the identification of the paracellular spaces. Generally, the bEnd5 ECs in our study were homogenous, and, round-hexagonal in shape. In addition, intact monolayers displayed marginal folds, which are cell-overlapping areas between adjacent ECs. These findings, as observed in our cultural monolayers, corroborated previous published literature (MacCsallum et al., 1882; Dejana, 2004; Schrot et al., 2005).

4.1.9.2 The effect of EtOH treatment on the ultrastructure of bEnd5 cells:

Contrast to the control cells, bEnd5 monolayers exposed to 25mM EtOH resulted in the prominent appearance of compromised paracellular spaces between adjacent ECs, signifying impaired TJ integrity as well as diminished paracellular regulation of substances traversing the BBB (Fig 3.7.3). Similarly, bEnd5 monolayers exposed to a supraphysiological concentration of EtOH, 100mM, also displayed noticeably compromised paracellular spaces between adjacent ECs (Fig 3.7.4).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4.1.9.3 The effect of R_f treatment on the ultrastructure of bEnd5 cells:

Interestingly, BECs exposed to R_f mimicked the control cells by displaying no prominent compromised paracellular spaces, as well as cell overlapping between adjacent ECs (Fig 3.7.5 and Fig 3.7.6). It may be that the additional antioxidants administered to the BECs exert its effects by implicating the transcellular permeability rather than the paracellular pathways.

4.1.9.4 The effect of EtOH and R_f treatment on the ultrastructure of bEnd5 cells:

Similarly to the controls, cells exposed simultaneously to R_f and 25mM EtOH, displayed no compromised paracellular spaces (Fig 3.7.7), illustrating that the R_f-derived antioxidants were able to reduce the physiological EtOH-induced effects on the paracellular spaces. The combination of 100mM EtOH

together with R_f produced compromised paracellular spaces with stress fractures which resembled the results of exposure to only 100mM EtOH (Fig 3.7.8-3.7.10). This suggests that the simultaneous administration of additional antioxidants failed to reverse or prevent the EtOH-induced damage (at the 100mM level) to the TJs and ultimately paracellular permeability, which is in alignment with results observed in PCR and TEER. This is particularly important since in our study we observed that the effects of R_f treatment resembled data similar to ROS-implicated results.

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CHAPTER FIVE

Conclusion

Our findings in this study corroborated previous published literature in that EtOH disrupts TJ protein transcription, in particular, claudin-5, and in doing so led to altered BBB integrity. Furthermore, in alignment with these studies (Abdul Muneer et al., 2011), EtOH adversely affected permeability, by decreasing TEER measurements. This was further validated using high resolution scanning electron microscopy for the observation of paracellular spaces between adjacent BECs. It was also established in this study that both physiological and supraphysiological concentrations of EtOH, both, resulted in adverse effects on cell viability, cell proliferation, cell toxicity, and the rate of cell division. It is well established in the literature, that ROS, may be responsible for the EtOH-induced effects on the bEnd5 ECs. As a result, the underlining hypothesis for this project was that these EtOH-induced ROS effects could be reversed by the simultaneous exposure to R_f, an extract, with an established antioxidant capacity (Berker et al., 2013). Furthermore, the scope of the study limited the use of R_f to a concentration of aspalathin equivalent to that found in the plasma after the intake of 500ml's of R_f tea.

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Prior to co-exposure of R_f and EtOH, we investigated the effects of only R_f on the bEnd5 ECs. As observed with SEM, cells exposed to R_f mimicked the controls, illustrating that R_f does not have a detrimental effect on the morphology of ECs and their supporting structures. Exposure to R_f initially resulted in decreased viability but recovered within 96hrs. In terms of live cell numbers, R_f led to diminished numbers of live cells at the later time intervals but remained similar to controls up to 48hrs. The decrease in the number of live cells is corroborated by the rate of cell division in response to R_f, which was significantly slower than untreated bEnd5 ECs. For the first time according to our knowledge, our study established, that similarly to EtOH, R_f administered on its own resulted in

detrimental effects on bEnd5 ECs, as noted when investigating cell toxicity, claudin-5 transcription and permeability across monolayers of bEnd5 ECs.

Overall, we established that EtOH is detrimental to the integrity of bEnd5 ECs, and that the addition of external antioxidants can partially alleviate excess ROS-induced effects. However, morphologically, the addition of R_f derived antioxidants only reversed the effects of a physiological concentration EtOH. Surprisingly, the simultaneous exposure of R_f together with EtOH exacerbated the adverse effects on claudin-5 transcription as well as permeability. The detrimental effects of alcohol exposure may be due to the effects of ROS, as well as the toxic effects of metabolites of EtOH, such as acetaldehyde. These dual effects may have obscured the sole protective effects of R_f against ROS. We therefore hypothesize that this may be a plausible explanation, since the addition of R_f derived antioxidants reversed and reduced the effects of EtOH on cell viability, cell toxicity, and cell proliferation.

Limitations in this study include insufficient analysis on the antioxidant status of the model used in this study, as well as the effects of the experimental compounds on ATP production. To better elucidate the mechanisms underlying the effects of EtOH and R_f on the BBB, future perspectives would include optimizing our bEnd5 model and cell cycle analysis to better analyze cell proliferation and cell division.

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APPENDICES

APPENDIX A

Measurement of flavanols (Analysed by the Oxidative Stress Research Centre at CPUT)

Principle of flavanol measurements

The Flavanol analysis was conducted using 4-dimethylaminocinnamaldehyde (DMACA). DMACA reacts with flavanols to generate a significant blue colour, which is interpreted at a wavelength of 640nm.

Chemical preparation

For the quantification of flavanols, chemicals used were: 32% hydrogen chloride-methanol (HCl-MeOH), and 250ml HCl (Saarchem, Cat no.100319 LP) which was added to 750ml MeOH (Saarchem, Cat no.4164080 LC) and thoroughly combined. A total of 0.25g DMACA (Merck, Cat no.822034) was weighed and added to a 500ml HCl-MeOH mixture, and further mixed until dissolved. The standard, catechin, was employed for the measurement of flavanols. Furthermore, 1mM of catechin hydrate standard (Sigma-Aldrich, Cat no.C1251) was set by allowing 0.0145g of catechin hydrate to dissolve in 50ml MeOH. In preparation of the control, 0.0029g catechin hydrate was dissolved in 50ml of MeOH. All solutions were prepared daily for the respective analysis, and subsequently stored at -40°C.

Sample analysis

50µl of the standard catechin, control, and sample was added to the designated wells of a clear 96-well plate. To initiate the reactions, 250µl DMACA was added to all the wells. Subsequently, the plate incubated for 30

min at RT and the resulting absorbance was analysed at a wavelength of 640nm using a Multiskan^{TM} spectrum plate reader.

APPENDIX B

Measurement of flavonols (Analysed by the Oxidative Stress Research Centre at CPUT)

Principle of flavonol measurements

Quercetin was employed in the flavonol analysis as the standard for determining total phenolic content at a wavelength of 360nm.

Chemical preparation

Chemicals utilized for the measurement of flavonols were as follows: 10% EtOH, 95% EtOH, and 0.1% HCl (Saarchem, Cat no.100319 LP) combined in 95% EtOH and 2% HCl. The standard solution was prepared by dissolving 4mg quercetin (Sigma-Aldrich, Cat no.Q0125) in 50ml of 95% EtOH, while for the control sample, a total of 1.5mg quercetin was dissolved in 50ml 95% EtOH. Intermittently, the samples were stored at -40°C.

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Samples analysis

12.5µl of the standard quercetin, controls and samples was added to the designated wells of a clear 96-well plate. Furthermore, a 12.5µl solution comprising of 0.1% HCl and 95% EtOH was added to each well. Subsequently, 225µl of 2% HCl was added to each. The plate incubated for 30 min at RT, after which, the Multiskan^{TM} spectrum plate reader was used to measure the absorbance at the wavelength of 360nm.

APPENDIX C

Measurement of phenolics (Analysed by the Oxidative Stress Research Centre at CPUT)

Principle of polyphenolic measurements

This analysis employs the Folin Ciocalteu reagent using gallic acid as the standard in measuring total polyphenols in a sample.

Chemical preparation

Chemicals employed in the quantification of polyphenolic content comprised of: 10% EtOH and Folin Reagent (Merck, Cat no.109001) and 7.5% sodium carbonate ($NaCO_3$) (Sigma-Aldrich, Cat no.223530), kept at RT. In preparation of gallic acid (Sigma-Aldrich, Cat no.G7384), 40mg was dissolved in 50ml of 10% EtOH to generate a 800mg/L stock concentration. In preparing the control, 10mg of the gallic acid was dissolved in 50ml of 10% EtOH solution to generate a final concentration of 200mg/L. Solutions were prepared daily on the respective day of analysis and stored at -40°C.

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Sample analysis

25µl of the standard gallic acid, controls, as well as the samples (in triplicate) was added to the designated wells of a clear 96-well plate (Lasec SA, Cat no.PGRE655180). A total of 125µl Folin reagent was added to all wells. The plate incubated for 5 min at RT, before 100µl Na_2CO_3 was added to each well. This was followed by further 2hr incubation in the dark at RT before measuring absorbance with a Multiskan^{TM} plate reader at 760-765nm.

APPENDIX D

Oxygen radical absorbance capacity assay (ORAC) (Analysed by the Oxidative Stress Research Centre at CPUT)

Principle of the ORAC assay

The results signify the ORAC value, which refers to the net protection area under the quenching curve of β-PE (fluorescein) in the presence of an antioxidant. Furthermore, the ORAC value is determined by dividing the area under the sample curve by the area under the trolox curve, with both areas being amended by subtracting the area under the blank curve. A single ORAC unit is allocated as the net protection area provided by 1µM Trolox in final concentration.

Chemical preparation

Chemicals utilized in the ORAC assay were namely: hexane (Saarchem, Cat no.2868040 LC) kept at RT, acetone/water/acetic acid (AWA) solution, which was made up of 700ml acetone (Saarchem, Cat no.1022040 LC), 295ml dH_20 and 5ml glacial acetic acid. A 75mM phosphate buffer with a pH 7.4 was prepared and consisted of two solutions.

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For the preparation of solution 1, 1.035g sodium di-hydrogen orthophosphate-1-hydrate ($NaH_2PO_4.H_2O$) (Sigma-Aldrich, Cat no.S9638) was weighed and mixed in 100ml double distilled water (ddH_2O). For the preparation of solution 2, 1.335g di-sodium hydrogen orthophosphate dehydrate ($Na_2HPO_4.2H_2O$), (Merck, Cat no.5822880EM) was weighed and mixed in 100ml of ddH_2O. An amount of 18ml (solution 1) was combined with 82ml (solution 2), to obtain a 75mM, pH 7.4 phosphate buffer. The fluorescein sodium salt stock solution ($C_{20}H_{10}Na_2O_5$) was kept in the dark at 4°C (Sigma-Aldrich, Cat no.F6377). Of the $C_{20}H_{10}Na_2O_5$ solution, 0.0225g was weighed and left to dissolve in 50ml phosphate buffer. 25mg/ml AAPH (2, 2'-Azobis (2-methylpropionamidine) dihydrochloride (Sigma-Aldrich, Cat

no.440914) .0.5M Perchloric acid (PCA) (Saarchem, Cat no.494612), comprised of 195ml dH_20 mixed with 15ml 70% PCA. Trolox (Sigma-Aldrich, Cat no.238831) was utilized as a standard in the measurement of ORAC., whereby 500µM of Trolox standard concentration was generated by mixing 0.00625g of 6-Hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Sigma-Aldrich, Cat no.238831) in a 50ml phosphate buffer thoroughly. In preparing the Trolox control (250µM), 0.00312g of 6-Hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid was dissolved in 50ml phosphate buffer. The samples were prepared on ice daily, and stored at -40°C on the day of analysis.

Sample analysis

12ul of the standard Gallic acid, controls and samples (triplicate) was added to the designated wells of a clear 96-well plate. 10µl was added to a volume of 2ml phosphate buffer before being diluted. The final volume of the assay was 200ul. The Multiskan^{TM} plate reader was employed to measure results utilizing an excitation wavelength of 485nm and emission wavelength of 530nm.

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APPENDIX E

Ferric reducing antioxidant power assay (FRAP) (Analysed by the Oxidative Stress Research Centre at CPUT)

Principle of the FRAP assay

The FRAP assay utilizes an oxidation/reduction reaction to evaluate the ability of a sample to reduce Fe^{III} to Fe^{II}. Antioxidants give electrons the same way as reductants in oxidation/reduction reactions, thus it is proposed that the FRAP assay is an optimal assay for assessing antioxidant capacity. On the other hand, it does not directly calculate the antioxidant capacity of potential antioxidants, and as there are no free radicals introduced into the system, it lacks methods for comparing antioxidant capacities towards different types of radicals.

Chemical preparation

For the chemical preparation of the FRAP assay, the following was used: 300mM acetate buffer, pH 3.6 containing 1.627g sodium acetate and 16ml glacial acetic acid (Saarchem, Cat no.1021000), as well as distilled water (dH_20), to a final volume of 1L. 40mM HCl and 10mM TPTZ (2, 4, 6-tri [2-pyridyl]-s-triazine) (Sigma-Aldrich, Cat no.T1253) containing 0.0093g TPTZ and 3ml of 40mM HCl. In addition, a 20mM iron (III) chloride hexahydrate (Sigma-Aldrich, Cat no.F2877) solution was produced with 0.054g $FeCl_3 \cdot 6H_2O$ and 10ml dH_20. L-ascorbic acid (Sigma-Aldrich, Cat no.A5960) was utilized as a standard for this assay. The stock solution of this standard comprised of 1mM L-ascorbic which was attained by dissolving 0.0088g of ascorbic acid in 50ml dH_20. The solutions were prepared daily for the chemical analyses.

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Sample analysis

The FRAP solution was prepared by combining the following reagents: 30ml acetate buffer, 3ml TPTZ solution, 3ml $FeCl_3$ solution, and 6.6ml dH_20. 10µl of the standard ascorbic acid, controls and samples (triplicate) was added to the designated wells of a clear 96-well plate. Subsequently, a volume of 300µl FRAP reagent was added to all wells, generating a final volume of 310µl. The plate was placed in the incubating oven for 30 min, set at 37°C and analysed using a Multiskan^{TM} plate reader at a wavelength of 593nm.

APPENDIX F

ABTS (2, 2'-azino-di-3-ethylbenzthialozine sulphonate) Trolox equivalent antioxidant capacity (TEAC) assay (Analysed by the Oxidative Stress Research Centre at CPUT)

Principle of the ABTS (TEAC) assay

ABTS radical cation scavenging assay was established for the determination of the total antioxidant status (TAS) of body fluids. $ABTS^{\bullet+}$, a stable and long-lived cation comes about when methymyoglobin, activated to its ferryl state by hydrogen peroxide incubated with ABTS. Antioxidants are suggested to either scavenge the $ABTS^{\bullet+}$ or impede the radical generating process.

Chemical preparation

7mM ABTS diammonium salt, prepared 24hrs prior to assay (Sigma-Aldrich, Cat no.A1888) and 140mM potassium-peroxodisulphate, freshly prepared (Merck, Cat no.105091) were required for this assay. Incubation was in the dark at RT. The standard utilized in the ABTS assay was Trolox. The standard (1mM) was prepared by combining 0.0125g Trolox (Sigma-Aldrich, Cat no.238831) to 50ml EtOH. In addition, 200µM of the control was prepared by mixing 0.0025g Trolox with 50ml of EtOH. Samples were set up on ice and stored at -40°C.

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Sample analysis

25µl of the standard Trolox, controls and samples (triplicate) was added to the designated wells of a clear 96-well plate. EtOH was used to dilute the ABTS solution, 1ml of ABTS to 20ml of EtOH. Furthermore, the absorbance was read at approximately 2 (±0.1). 300µl taken from the ABTS solution, was added to each well. The plate incubated at RT for 30 min, using a Multiskan™ plate reader with a wavelength of 734nm and temperature of 25°C.