EXPLORING THE COSMETIC POTENTIAL OF ROOIBOS ANTIOXIDANTS

Rooibos is held in high esteem for its novel antioxidant constituents, however direct scientific evidence to support cosmetic application remains largely unexplored. The present in vitro study was undertaken to establish that rooibos antioxidants have adequate cellular bioavailability and are functional in combating oxidative stress within the cellular environment.

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Introduction

Over the past few years Rooibos tea has become known throughout the world as a product with many health benefits resulting in extensive research on the medicinal value of Rooibos. In contrast, introduction of rooibos as a cosmetic ingredient has not enjoyed the same level of scientific support. Rooibos is well-known as an antioxidant and is thus easy to positively associate with a myriad of ailments, including ageing, in which oxidative stress is believed to play a causative role. Given the key involvement of oxidative stress, many cosmetic products have been designed to contain natural product derived antioxidant ingredients including Green Tea, Resveratrol, Silymarin and α-Lipoic acid.

While the antioxidant activity of rooibos may not be unique, its antioxidant composition is, with some of its individual components very rare in nature. Two important antioxidant compounds, namely aspalathin and nothofagin may be the key to what makes Rooibos unique, with some sound scientific evidence to its therapeutic potential. It is however not the rarity of the Rooibos antioxidants nor the quantities of individual antioxidant compounds that defines its true antioxidant potential, rather it is the capacity for these compounds to enter into the cellular environment and to effectively counteract oxidative stress that is ultimately the crucial factor. For any antioxidant compound to have therapeutic value it must be able to 1) permeate into the cell where destructive free radicals reside, 2) effectively neutralise free radicals within the biological environment of the cell, and 3) successfully counteract the biological fate of oxidative stress, most notably cell death.

Here we report on our research findings to evaluate the cellular bioavailability and confirmation of biological activity of Rooibos antioxidants. We also attempt to place the antioxidant properties into context by comparing fermented Rooibos and aspalathin enriched green Rooibos to other antioxidants often used in cosmetics.

Ranking the antioxidant capacity of Rooibos based on chemical reactivity

The strength of an antioxidant is often reported as its capacity to neutralise free radicals through donating electrons. Methodology such as FRAP, DPPH scavenging activity and ORAC are commonly employed to rank the efficacy of antioxidants based on this principle. These relatively simple methods provide a good indication as to the chemical reactivity of antioxidant molecules and thus provide some idea on their potential to function as an antioxidant. Despite the phenomenal popularity of such methods, they unfortunately don't always relate well to the biological capacity of an antioxidant.

To characterise chemical reactivity of Rooibos and its comparators, Green Tea, resveratrol, silymarin, and α-lipoic acid, we made use of the FRAP and DPPH assay methods. As illustrated in figure 1, the FRAP activity for fermented Rooibos was substantially lower than that of green Rooibos which is in accord with the higher phenolic content of the green Rooibos extract. Expressing the data relative to the standard antioxidant Trolox, further illustrates this trend. To achieve the same antioxidant activity as 1g of Trolox requires approximately 55g of fermented Rooibos, 23g of green Rooibos and 20g of Green Tea. Of the standard antioxidants tested, resveratrol was the most potent (2g), while sylimarin and α-lipoic acid were substantially weaker (16g and 58g respectively). Using a different assay method, the DPPH assay, we could confirm similar trends (data not shown).

Taken together these results suggest that, measured outside of a cellular environment, resveratrol is the most potent of the tested cosmetic antioxidants, while the potency of green Rooibos is comparable to that of Green Tea, however both are substantially more active than fermented Rooibos. Although Green Tea is reported in the literature to be a more potent antioxidant than fermented Rooibos, to our knowledge this is the first comparison between Green Tea and green Rooibos. It is well published that Green Tea is an exceptional antioxidant and therefore one should acknowledge that green Rooibos has similar cosmetic potential. An additional factor to keep in mind is that toxicity may limit how much of an antioxidant substance is practical. Although

resveratrol proved highly efficient, its toxicity limits how much can be used and still maintain safety. Conversely Rooibos has been a popular health tea for many years, substantiating its safety for cosmetic advantage.

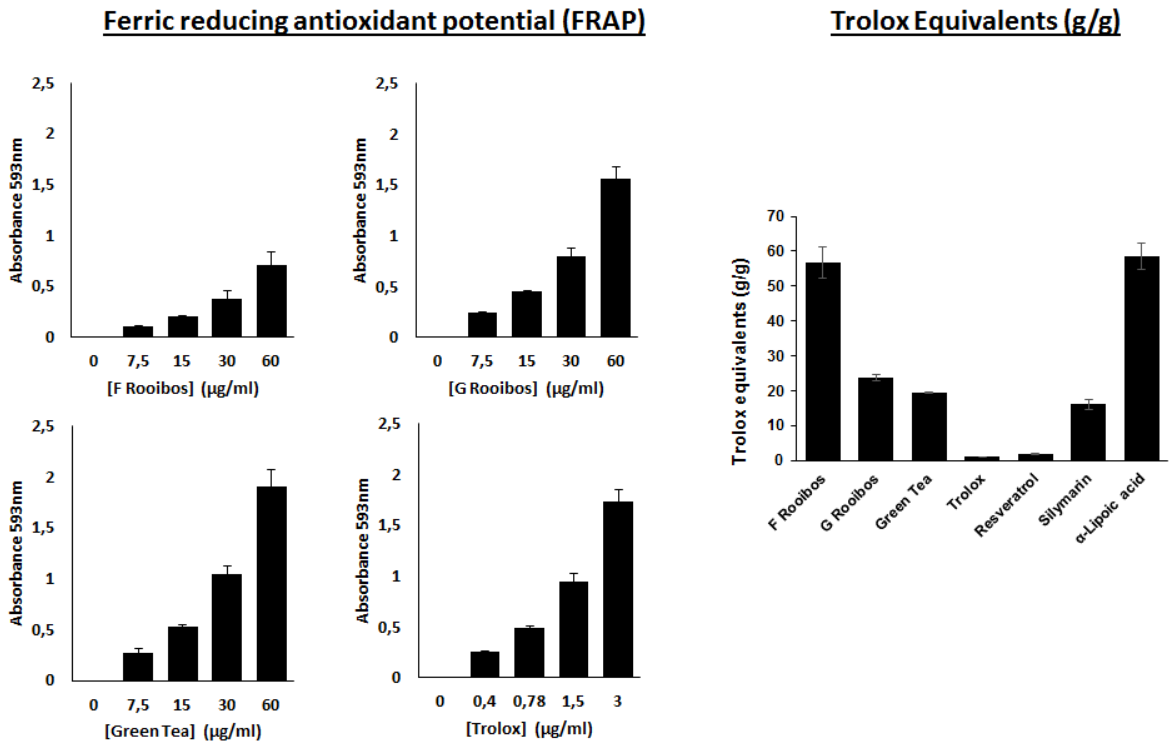

Figure 1: Antioxidant capacity of Rooibos and Green tea relative to other antioxidants as determined using the FRAP assay method. Illustrated are the dose responses for fermented Rooibos, green Rooibos, Green Tea and Trolox. Also indicated are the Trolox equivalents on a gram per gram dry mass basis. Data represents the mean from three independent experiments.

Confirming cellular bioavailability using CAPe and CAA assay methods

Cellular bioavailability can be defined as the rate and extent to which a therapeutic entity is taken up and becomes available to the intracellular site(s) for therapeutic action. It is clear that no matter how potent an antioxidant may be, if it does not reach the site where excess reactive oxygen species are produced it will have no benefit. There are principally two mechanisms which may apply for the cellular uptake of antioxidant molecules. Firstly, many compounds simply diffuse through or into the cell membrane based on a concentration gradient, a process known as passive diffusion. An alternative mechanism involves specific transporter proteins which recognise defined chemical structures and so drive these specific molecules into the cell. Usually this process requires energy and hence it is known as active diffusion.

Once inside the cell numerous fates await an antioxidant substance. They are often subject to metabolic conversion by enzymes which reside inside the cell resulting in changes in their chemical structure, which may subsequently alter their ability to function as an antioxidant. Metabolic inactivation of an antioxidant can rapidly render a potent antioxidant completely ineffective. Many cells also express drug efflux proteins which recognise foreign substances and eliminate them from the interior of the cell, thereby limiting their accumulation within the cell. Thus, certain antioxidants can be eliminated from the cell interior if they are recognised by these efflux systems. Together these factors ultimately define how much of a given antioxidant actually becomes available to combat cellular damage brought on by excess free radicals. Therefore, when defining the therapeutic value of an antioxidant substance it is imperative to also establish cellular bioavailability.

In order to extend beyond the simple FRAP and DPPH antioxidant tests we explored more complex and more biologically relevant cellular testing systems which incorporate elements of cellular bioavailability. Both the CAPe (cellular antioxidant protection in erythrocytes) and CAA (cellular antioxidant assay) are recognised methods to determine cellular antioxidant behaviour.

CAPe assay

The use of human red blood cells, also known as erythrocytes, in testing how well an antioxidant molecule can permeate through a cell membrane provides one of the simplest testing systems to investigate cellular bioavailability. Mammalian red blood cells offers a unique model since these cells are devoid of a nucleus thus eliminating the confounding contribution that cellular signalling may have. In addition, mature red blood cells lack mitochondria, a major source of ROS production, which interfere with the accuracy of determining how efficient an antioxidant protects against any given pro-oxidant. Prior to the assay, red blood cells are aged for a few weeks to deplete endogenous antioxidants and energy. Therefore, the system measures a combination of the capacity of an antioxidant to passively diffuse through a cell membrane and the antioxidant efficacy to inhibit ROS damage.

Figure 2 shows the results obtained for the CAPe method providing sound evidence that Rooibos antioxidants permeate through the cell membrane. The level of protection offered by the various samples tested corresponds well to the antioxidant capacity determined using the FRAP method (Figure 1) with the exception of Trolox and Resveratrol. While Trolox and Resveratrol were by far the most potent antioxidants in the FRAP assay, the magnitude of protection offered to the red blood cells does not reflect this. We suspect that there is a saturation level as to how much of a compound may permeate into the cell.

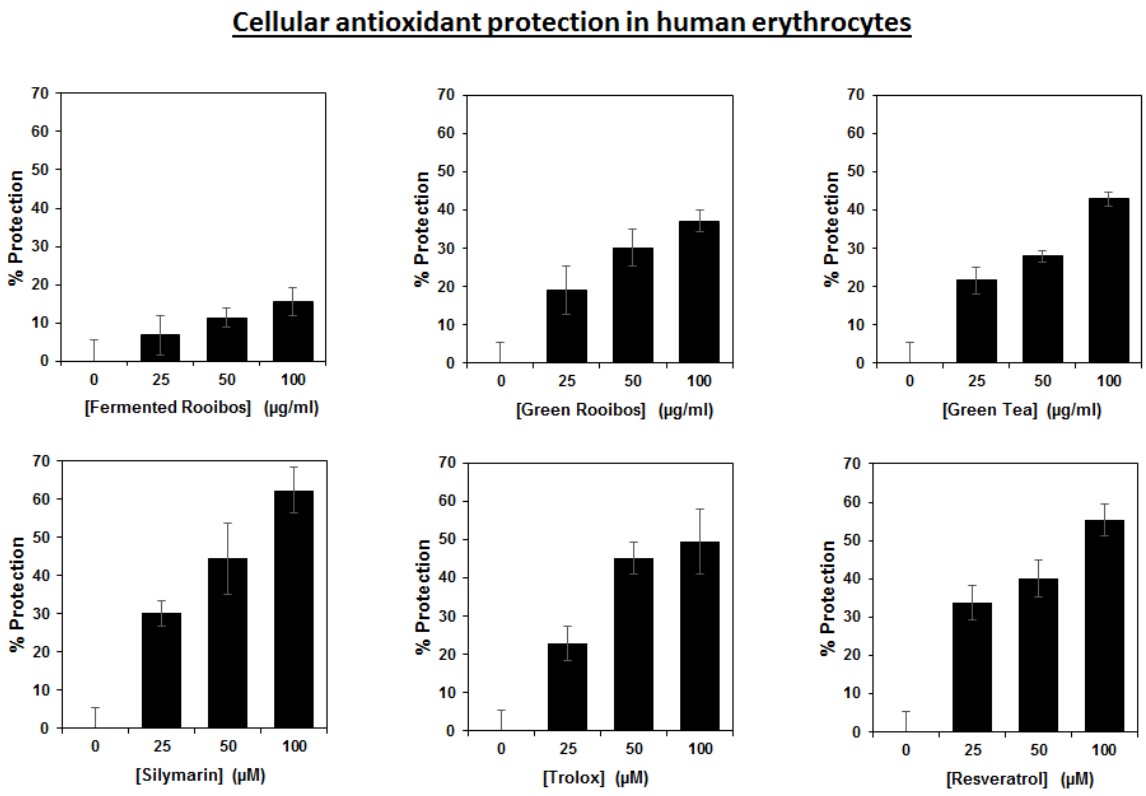

Figure 2: CAPe cellular bioavailability of Rooibos and Green tea relative to other antioxidants. Illustrated are the dose responses for fermented Rooibos, green Rooibos, Green Tea, Silymarin, Trolox and Resveratrol. Data represents the mean of three independent experiments.

CAA assay

In recognition of the fact that cellular bioavailability extends beyond fundamental permeation through a cell membrane, researchers propose the requirement for a more elaborate system to accurately predict cellular bioavailability. Using a cell type representative of human skin, we explored the use of MRHF cells as a model to investigate cellular bioavailability of Rooibos. Unlike the red blood cell model, these cells are expected to contain the full complement of features which define bioavailability including cellular uptake (passive an active diffusion), metabolism, drug efflux proteins and cellular signalling networks. To confirm the functionality and accuracy of this model we first tested two known antioxidants with different bioavailability profiles and could indeed demonstrate the expected outcome (data not shown).

Next, we tested Rooibos, Green Tea and three standard antioxidants (Silymarin, Resveratrol and Trolox). As with the CAPe model, we found that Green Rooibos and

Green Tea produced a similar response and was also greater in magnitude than that seen with fermented Rooibos (Figure 3). Both Silymarin and Resveratrol were strongly active, however Trolox, our most potent antioxidant used in this study, yielded a rather unexpected weaker response. Perhaps an indication that, at least in part, Trolox is weakly permeable through the cell membrane, a feature which may relate to the high water solubility of this compound, or that Trolox is subjected to metabolic inactivation by enzymes or expulsion as a foreign substance by efflux proteins.

Taken together the data obtained from the CAPe and CAA provide strong evidence that the antioxidant components of both fermented and Green Rooibos can accumulate within the cell and are thus available to function as an antioxidant in close vicinity to where oxidative stress is initiated. This is an important consideration for any substance which must act as therapeutic antioxidant. As the name suggests, reactive oxygen species are highly reactive and as such are characterised by extremely short life span, rapidly reacting with cellular components. Therefore, antioxidants need to be close by if they are to prevent cellular damage.

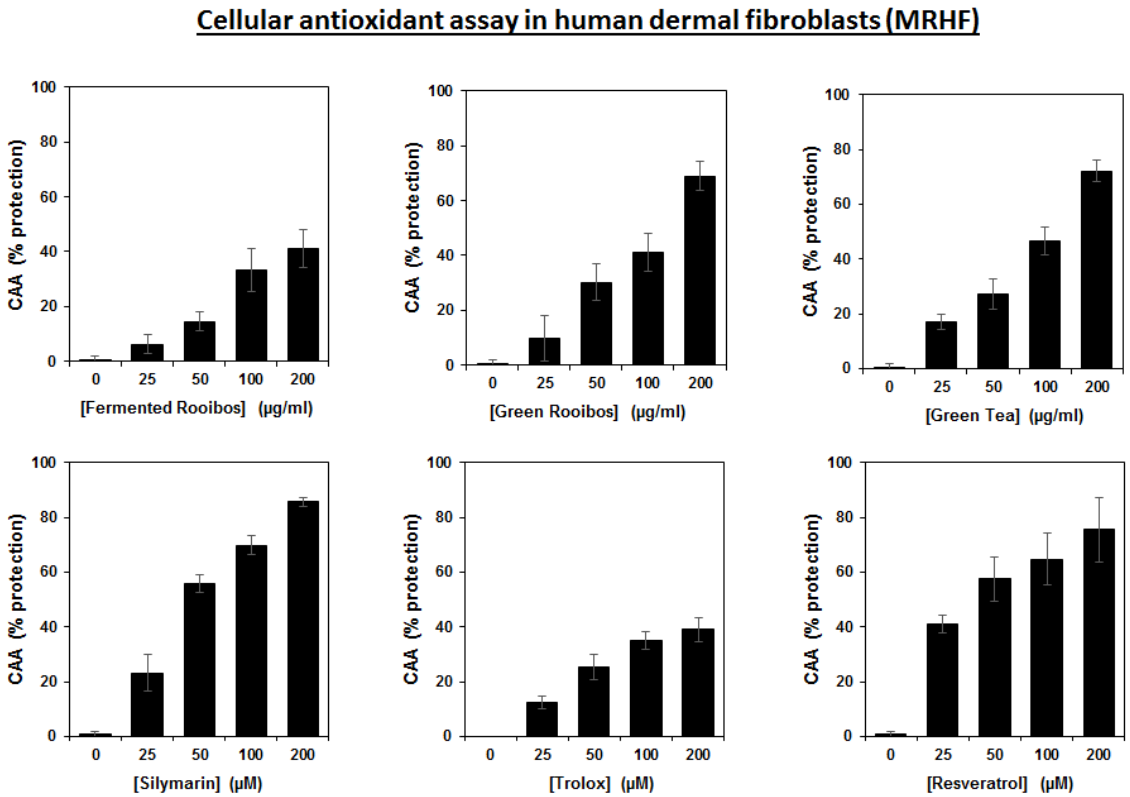

Figure 3: CAA cellular bioavailability of Rooibos and Green tea relative to other antioxidants. Illustrated are the dose responses for fermented Rooibos, green Rooibos, Green Tea, Silymarin, Trolox and Resveratrol. Data represents the mean from three independent experiments.

Confirming that Rooibos antioxidants are biologically active

In the cell-based methods described above, oxidative stress is induced by a once off bolus exposure of an exogenously added oxidant and the capacity of an antioxidant to inhibit cellular damage is indirectly quantified based on a fluorescent indicator. While this format is acceptable to determine cellular uptake and accumulation of an antioxidant molecule, it does not necessarily confirm biological relevance as neither stressor nor fluorescent marker occur naturally. From a biological perspective oxidative stress occurs in response to a continual low-grade exposure to ROS and excess ROS can damage multiple cellular constituents to differing degrees. To mimic this situation in an *in vitro* setting we treated cultured dermal fibroblasts with a chemical compound (AAPH) which has been shown to induce the activity of an oxidant forming enzyme called NADPH oxidase with subsequent long-term induction of oxidative stress and cell damage even after removal of the inducer. As a more appropriate end point indicator of oxidative stress we measured cell death, the ultimate consequence of unrepaired cell damage. In this model both stressor and end point measures reflect what we may expect to occur naturally, as for example during the ageing process.

Our results from this model indicate that all tested tea extracts provided dose dependent protection against cell death in MRHF cells, with green Rooibos providing comparable protection to Green Tea and superior protection relative to the standard antioxidant compounds tested. This not only confirms the cellular bioavailability of Rooibos antioxidants, but also proves that these antioxidants do function at a biochemical level and we can thus conclude the Rooibos antioxidants are indeed biologically active within the cellular environment. These findings also corroborate our earlier studies on the wound healing properties of Rooibos where we report that both fermented and green Rooibos extracts attenuate apoptotic cell death in dermal fibroblasts subjected to oxidative stress (Pringle *et al*., 2018). Using a different model, namely that of 3T3-L1 preadipocytes exposed to nutrient starvation-induced oxidative stress, we found Rooibos also decreased apoptotic DNA fragmentation.

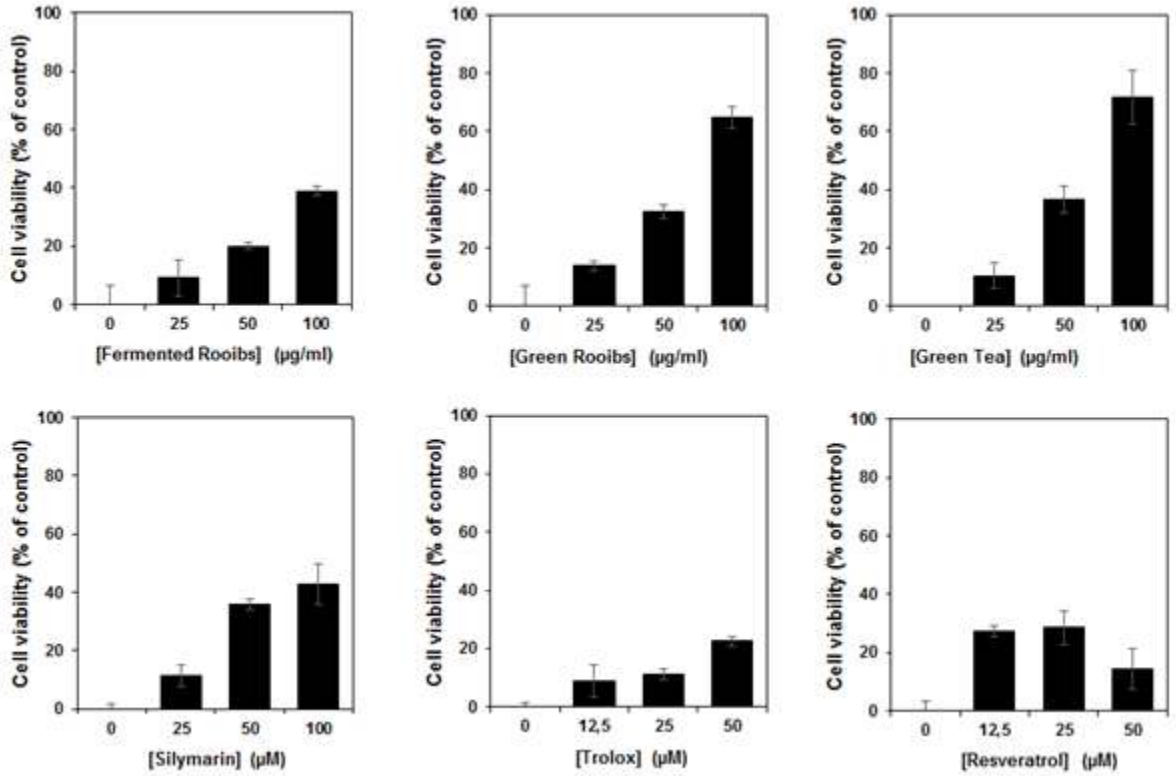

Figure 4: Confirming that Rooibos antioxidants are biologically active in stressed MRHF cells. Cells were pre-treated with the indicated concentrations of test sample and then treated with AAPH to induce a state of chronic oxidative stress. Cell viability was determined 24 hours later using the MTT viability assay. Illustrated are the dose responses for fermented Rooibos, green Rooibos, Green Tea, Silymarin, Trolox and Resveratrol

Rooibos can enhance the intrinsic antioxidant network in dermal fibroblasts

Most cells are naturally endowed with an elaborate antioxidant network comprising antioxidant enzymes, low molecular weight antioxidant molecules and an intricate signalling system aimed at maintaining redox homeostasis. It is only when this antioxidant network fails that oxidative stress ensues and ultimately progresses to the demise of cellular function and disease. It is well known that certain environmental signals can activate this intrinsic antioxidant network and in so doing enhance the cells own capacity to resist oxidative stress.

A number of studies have shown that Green Tea polyphenols induce key components of the intrinsic antioxidant network most likely via the NRF2 signalling pathway. Some

researchers however caution that many plant polyphenolic compounds turn pro-oxidant in cell culture medium due to the high free iron content of commercial media, a situation different to that found in the body. To circumvent this problem it has been recommended that the addition of pyruvate to the growth medium can counteract the pro-oxidant activity of plant phenolics. Simply put, plant antioxidants become strong pro-oxidants in some types of commercial culture medium and the cells subsequently respond by activating their own defence mechanism. Currently there is no evidence that plant antioxidants function in this manner in the body and would further appear unlikely as the free iron concentration *in vivo* is very low.

To test the capacity of Rooibos extracts to enhance cellular fitness against oxidative stress we pre-treated MRHF cells, for 24 hours, with varying concentrations of Rooibos in the presence of pyruvate. Residual extract was then removed by washing the cells with extract free medium. Cells were then exposed to glucose oxidase induced oxidative stress for 4 hours and the capacity for the cells to survive measured. The findings presented below in figure 5 indicate that both Rooibos and Green Tea can enhance cellular resistance to oxidative stress as is evident through increased cell survival. Interestingly, fermented Rooibos produced a much more prominent response in this aspect relative to its capacity as a direct antioxidant, providing confirmation that these effects are indeed independent of the direct antioxidant reactivity of extract components. Although Silymarin produced a robust effect, Resveratrol and Trolox appeared to exacerbate oxidative stress induced cytotoxicity thus yielding a negative response. Again, this contrasts with their exceptional antioxidant activity. From this we may conclude that in addition to its direct free radical scavenging activity (antioxidant activity), Rooibos can further enhance the intrinsic cellular capacity to resist oxidative stress. This appears to be independent of the aspalathin concentration as fermented Rooibos also produced a robust response.

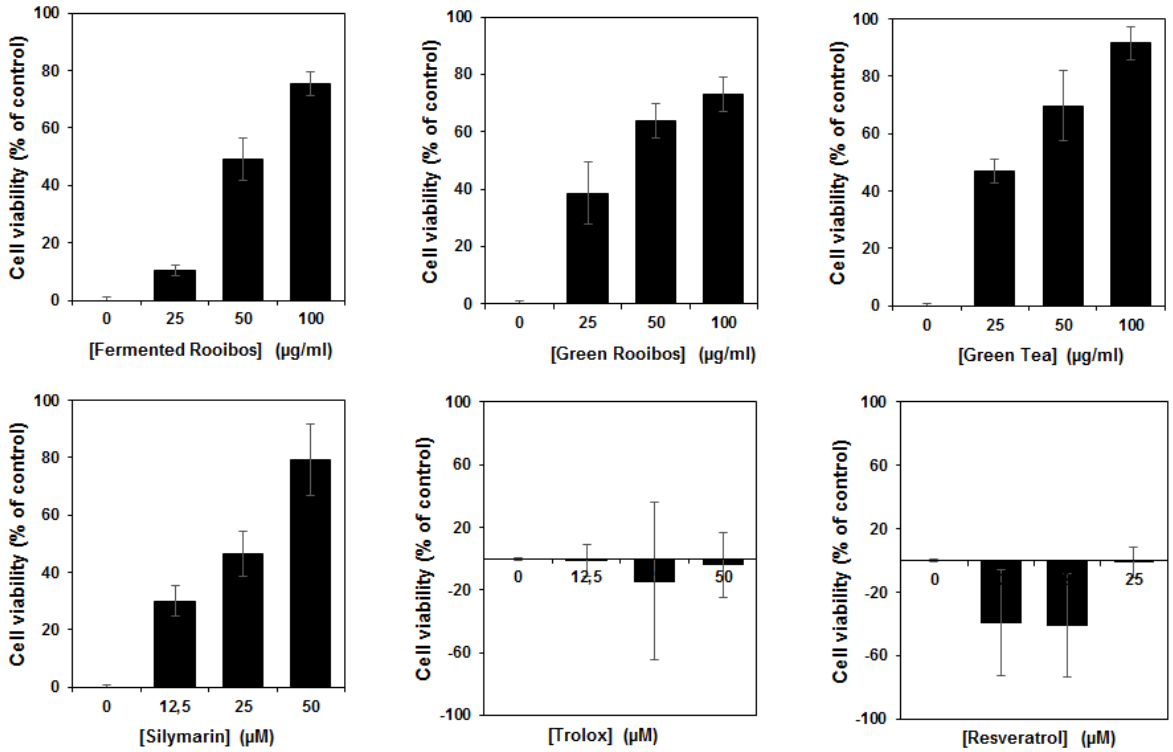

Figure 5: Activation of the intrinsic antioxidant network by Rooibos, Green Tea and Silymarin. Cells were pre-treated for 24 hours with the various extracts or silymarin and their resistance to oxidative stress assessed by measuring cell viability after a 4 hour treatment with glucose oxidase. LDH release was used as a marker of cell death. Illustrated are the dose responses for fermented Rooibos, green Rooibos, Green Tea, Silymarin, Trolox and Resveratrol.

Conclusion

Oxidative damage by reactive oxygen species is a major contributory factor in skin ageing and as a result intervention using antioxidants has become a prominent strategy against ageing and other skin diseases. Although Rooibos is well-known for its antioxidant activity, it is uncertain to what extent Rooibos antioxidants are active within the cell. Using various *in vitro* model systems, we have characterised the cellular bioavailability and biological activity relative to other known cosmetic antioxidants.

In South Africa Rooibos has been a popular cosmetic ingredient and health tea for many years, substantiating its safety. Initially the fermented form of Rooibos was used, however the fermentation process drastically reduces the antioxidant activity. To improve the antioxidant activity, especially with the cosmetic market in mind, an optimised extraction procedure was devised to yield and extract with superior antioxidant activity primarily due to the higher aspalathin content. To date there has been little concrete scientific evidence to support the superiority of this form, especially toward skin health.

Using a number of mechanistically distinct cell models, we confirm that Rooibos antioxidants have the capacity to permeate into the cell interior, where damaging free radicals are produced, and function within this environment to limit the negative impact of oxidative stress. Our results confirm that both the antioxidant capacity and the cellular bioavailability of Green Rooibos in combating oxidative stress is far greater than that of fermented Rooibos and is comparable to Green Tea. Perhaps more important, Green Rooibos is shown to be active within the cell to a greater extent than some better-known antioxidants. This however, is in line with the hypothesis that antioxidant combinations can provide superior activity relative to a single compound, despite exceptional strength of some individual antioxidants. Plant extracts, such as those derived from Rooibos and Green Tea, contain a plethora of compounds with diverse antioxidant properties which together can provide additive and/or synergistic effects. From a biological perspective it is clear that the different cellular compartments within a cell present distinct physical/chemical environments making it unlikely that a single antioxidant can be completely effective. For example,

antioxidants which target the lipid-rich domain of the cell membrane must possess sufficient lipid solubility to penetrate and function as lipid peroxidation inhibitors. This contrasts with the hydrophilic cell interior where lipid soluble antioxidants would be ineffective. Therefore, many researchers are at one that the multi-component nature plant extracts are likely to hold more therapeutic/cosmetic benefits than formulations based on single active constituents. Our findings illustrated in this report clearly support this concept and highlight Rooibos as a suitable antioxidant cosmetic ingredient.

The antioxidant activity of Rooibos within a cellular environment is two-fold;

(i) by performing free radical scavenging activity to combat oxidative stress and counteract cell death – the biological fate of oxidative stress, and

(ii) similar to the mechanism performed by Green Tea, by stimulating the intrinsic antioxidant network, which cells rely on to naturally to protect against oxidative stress.

Interestingly our results show that Rooibos is more effective than Trolox (soluble Vitamin E) in respect of entering a cell and performing antioxidant activity inside a cell. Moreover, unlike Rooibos, the standard antioxidants Resveratrol and Trolox do not exhibit the ability to enhance cellular resistance to oxidative stress and, in contrast to their exceptional antioxidant activity, they appear to exacerbate oxidative stress-induced cytotoxicity, at least under our experimental conditions. This dichotomous antioxidant mechanism exhibited by tea antioxidants should not be overlooked when considering their cosmetic potential. Multi-component antioxidant mixtures are no doubt better suited to the complexity of a biological system where homeostasis is achieved through diverse and often unrelated mechanisms. In the past, enthusiasm towards developing therapeutic products derived from complex mixtures has been discouraged due to limited patentability and difficulty to standardise natural products, however this does not distract from their exceptional efficacy.

In favour of using tea polyphenols for combating oxidative stress, our results show that dermal fibroblasts with oxidative stress-induced cell damage, when treated with tea extracts, exhibit protection against cell death; Green Rooibos demonstrates comparable protection to Green Tea and superior protection relative to Trolox,

Resveratrol and other standard antioxidants, reinforcing the superior performance of tea antioxidants within a cell environment.

It is important to emphasise that combating oxidative stress represents only one aspect of skin health, albeit possibly one of the best researched aspects, other mechanisms can also significantly contribute to the cosmetic value of Rooibos. Previous and ongoing independent research at the Nelson Mandela University has highlighted at least two additional features that make Green Rooibos an exciting anti-ageing component. Firstly, we have shown that both fermented and Green Rooibos attenuate protein glycation, a contributory factor to poor skin health, typically associated with diabetics, smokers and aged skin (Pringle et al., 2018). Collagen is a protein with an exceptionally long half-life (many years), subsequently glycated collagen can accumulate substantially over time ultimately giving rise to chronic inflammation and destruction of a functional collagen matrix, thereby exacerbating the aged appearance of skin. We found that Green Rooibos was significantly better than fermented Rooibos in attenuating protein glycation.

Secondly, and perhaps the most novel aspect of our research regarding Rooibos is its potential role in protecting and repairing dysfunctional preadipocytes (progenitors to mature fat cells), a recognised, but as yet poorly researched therapeutic target against age related diseases. As we age the layer of fat beneath our skin dramatically declines which accentuates fine lines and wrinkles. Research indicates that preadipocyte dysfunction is the predominant factor responsible for this event. To evaluate the therapeutic potential of Rooibos to protect and restore preadipocyte function, we developed a cell based model simulating preadipocyte dysfunction and explored the effects of both fermented and Green Rooibos on key features of preadipocyte function (Hattingh et al 2019). In this model cells are “aged” by chemically disabling mitochondrial function, a feature characteristic of aged cells. Our findings demonstrate that Rooibos, and in particular Green Rooibos, significantly improves the capacity of these mitochondrially depleted and dysfunctional preadipocytes, most notably improved proliferation and energy metabolism. This raises the possibility of protecting subcutaneous adipose tissue from ageing characteristics, restoring fat mass and subsequently the potential to de-accentuate the appearance of fine lines and wrinkles. To the best of our knowledge this is the first study reporting a therapeutic ingredient

with the potential to restore preadipocyte function, making Rooibos a novel cosmetic ingredient in this regard.

Studies by other research groups that also bear relevance to the cosmetic potential of Rooibos include Gelderblom et al., who indicate that rooibos plays a role in photo-protection for skin health. Extended studies will no doubt continue to uncover new therapeutic properties of Rooibos and the mechanisms by which they operate.

In conclusion, the translation of Green Rooibos antioxidant to cellular bioavailability and biological activity is aligned with that of Green Tea and superior to other well-known cosmetic antioxidants. This combined with the substantiated safety of rooibos, as well as its other therapeutic properties, should help recognise Rooibos as a true and powerful cosmetic ingredient, particularly for anti-ageing. Aspalathin-enriched Green Rooibos has been used throughout this research, as well as in our previous published work, and data obtained supports the superiority of this form for cosmetic application.