The antioxidant activity and cellular effects of the bioactive components of Flavonix® Cytoflamm Gel used in wound treatment

by

Petro Du Toit Bronkhorst

Submitted in fulfillment of the requirement for the degree of

MASTER OF SCIENCE

in the

SCHOOL OF MEDICINE
FACULTY OF HEALTH SCIENCES

of the

Department of Anatomy
University of Pretoria
South Africa

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SUPERVISER: PROF. MJ BESTER
Department of Anatomy
Degree: MSc ANATOMY with specialization in Human Cell Biology

ABSTRACT

Wound healing is one of the most complex processes in the human body consisting of several different phases namely inflammatory phase, proliferative phase, remodeling phase and contraction phase (Jurjus *et al*., 2007). Bioactive ingredients of Cytoflamm Gel (FCG), a complementary wound healing product are honey (H), oleuropein (OL), witch hazel (WH), xylitol (X), vitamin E (VE), aloe vera (AV) and farnesol (Fa). The aim of this study was to evaluate the antioxidant properties and cellular protective effects of FCG. This required the testing of each bioactive ingredient as well as related to antioxidant effects the interaction that occurs between ingredients.

This was achieved by measuring the antioxidant content and activity (chemical and cellular) of FCG and each ingredient at the concentrations found in FCG. Two groups of ingredients related to concentration were identified, major (H, OL and WH) and minor (X, VE, AV, Fa). A strategy was developed to determine the type of interaction; synergistic, additive or antagonistic that occurs between ingredients. For all samples, the total polyphenolic and flavonoid content (TPC and TFC) was determined. Antioxidant activity was measured using the 2,2'-diphenyl-2-picrylhydrazyl (DPPH), trolox equivalent antioxidant capacity (TEAC) both single electron transfer assays and the hydrogen atom transfer assay, as well as the oxygen radical antioxidant capacity (ORAC) assay. Cellular antioxidant protective effect/s was determined in the SC-1 cell line with the dichlorofluorescein diacetate assay with 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) as source of oxidative damage.

The high antioxidant content of FCG was due to major components OL and H contributing 84.08% and 81.70% respectively to TPC and TFC. VE, OL and H, contributed 44.56%, 32.06% and 11.56% (DPPH assay) and 56.44%, 15.12% and 13.80% (TEAC assay) respectively to antioxidant activity. With the ORAC assay, H, OL and WH were found to contribute equally 29.97%, 29.91% and 29.67% with a contribution of only 7.59% by VE. Strong synergism was found between H+OL, H+WH and OL+WH. In the *in vitro* SC-1 cell model, FCG, the antioxidant ingredients and mixtures showed significant cellular protective effects especially in combination with VE. Strong synergism was found between VE+OL, VE+OL+H and VE+OL+H+WH, indicating that effects may be related to antioxidant type and concentration although in some instances the effect of WH was antagonistic.

In conclusion, FCG and bioactive ingredients have significant levels of antioxidant activity and cellular protection against oxidative damage and this is due to synergism between antioxidant ingredients especially VE, OL and H.

DECLARATION

I, Petro Du Toit Bronkhorst, hereby declare that this research dissertation is my own work and has not been presented by me for any degree at this or any other University.

Sign: _____

On the _____ day of _____ 2014.

Department of Anatomy, School of Medicine,
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South Africa

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I cannot describe how thankful I am to have been able to reach this goal. I thank my Heavenly Father for giving me this opportunity. I am also thankful for the patience and the perseverance He has given me. I am especially blessed for everyone He has placed in my life that supports and loves me.

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LIST OF ABBREVATIONS AND SYMBOLS

℃	Degrees Celsius
%	Percentage
AAPH	2,2'-azobis-2-methyl-propanimidamide, dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACF	Aberrant crypt foci
ALL COMP	Flavonix® Cytoflamm Gel – Bioactive components
AMP	Antimicrobial peptide
ANOVA	Analysis of variance
AOM	Azoxymethane
AUC	Area under curve
AV	Aloe vera
bFGF	Basic fibroblast growth factor
BHT	Butylated hydroxytoluene
CALC	Sum of bioactive components
CAM	Complementary/alternative medicine
CE	Catechin equivalent
CO_2	Carbon dioxide
CQ_{10}	Co-enzyme CQ10, Ubiguinol
DCA	Deoxylic acid
DCFH-DA	Dichlorofluorescein diacetate
ddH_2O	Double distilled water
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
E	Expected
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
EGF	Epidermal growth factor
EMEM	Eagle's minimal essential medium
EPS	Extracellular polymeric substance
Fa	Farnesol
F-C	Folin-Ciocalteu

FCG	Flavonix® Cytoflamm Gel
FCS	Fetal calf serum
FP	Final product
g	Gram
GAE	Gallic acid equivalent
H	Honey
HAT	Hydrogen atom transfer
H_2O_2	Hydrogen peroxide
iNOS	Inducible nitric oxide synthase
$K_2S_2O_8$	Potassium peroxodisulfate
LDL	Low density lipoprotein
L-NAME	L-Nitro-arginine methyl ester
LNCaP	Epithelial cell line derived from a human prostate carcinoma
LPS	Lipopolysaccharide
LSD	Least significant difference
M	Molar
MCC	Medicines control council
mg	Milligram
mg/ml	Milligram per milliliter
MGO	Methylglyoxal
ml	Milliliter
mM	Millimolar
MRP	Maillard reaction product
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaOH	Sodium hydroxide
nm	Nanometer
NO	Nitric oxide
O	Observed
$O_2 \cdot^-$	Superoxide anion
OL	Oleuropein
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
®	Registered
RS	Revamil® source

ROS	Reactive oxygen species
SC-1	Mouse feral embryo cell line
Se	Selenium
SEM	Standard error of mean
SET	Single electron transfer
SOD	Superoxide dismutase
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TFC	Total flavonoid content
TGF-β(2)	Transforming growth factor-beta (2)
™	Trademark
TNF-α	Tumour necrosis factor-alpha
TPC	Total polyphenol content
µl	Microliter
µM	Micromolar
VE	α-tocopherol acetate (Vitamin E)
VEGF	Vascular endothelial growth factor
v/v	Volume per volume
WH	Witch hazel
w/v	Weight per volume
x	Times
X	Xylitol

CHAPTER 1: INTRODUCTION

Wound healing is one of the most complex processes in the body and a cascade of events is triggered when tissue damage occurs. This cascade of events consists of a series of actions which include: 1) inflammation, 2) epithelialization (proliferative phase), 3) connective tissue deposition (remodeling phase), and 4) contraction (Jurjus *et al*., 2007). These phases overlap each other and do not occur independently.

Several factors exists which can interrupt the cycle of wound healing. These factors include chronic inflammation, infections especially the formation of biofilms and oxidants, all of which cause cellular damage that impedes wound healing. To promote wound healing and prevent the above effects several wound treatment options are currently available. Four main classes of wound care dressings include the absorbent, hydrating, miscellaneous and biological wound dressings (Okan *et al*., 2007).

More advanced, and mostly biological based wound dressings include antibacterial wound dressings containing nano-silver technologies, bio-engineered wound dressings that can contain a wide range of growth factors such as platelet derived growth factor and epidermal growth factor, stimulating factors such as hyaluronic acid, collagen, glycosaminoglycans and antioxidants such as ascorbic acid and tocopherols.

Flavonix® Cytoflamm Gel is such a wound dressing which contains several bioactive components such as honey, oleuropein and aloe vera which has addressed several aspects of wound healing and these include antibacterial, antioxidant and anti-inflammatory activity (Dossier - Design History File: Medika SA. Prof Allen Widgerow, Celeste Smith). In addition, it was also developed to aid in the breakdown of biofilms usually present in chronic wounds that hinders the wound from going through the normal wound healing stages (James *et al*., 2008). Once the biofilms are cleared from the wound site, the anti-inflammatory and antioxidant properties can aid in the wound healing process. The anti-inflammatory components inhibit various precursors of inflammation and in doing so promote wound healing and reduces scar formation. Free radical damage is inhibited with the inclusion of sources of antioxidants such as honey, oleuropein and witch hazel as well as α-tocopherol acetate. The antioxidant effect of each of these components is well described (Fleming *et al*., 1973; Perugini *et al*., 2008; Puel *et al*., 2006; Pereira *et al*., 2007; Murray *et al*., 2008; Lin *et al*., 2003; Jabra-Rizk *et*

al., 2006; Brehm-Stecher & Johnson, 2003; Weber *et al*., 2008; Cugini *et al*., 2007; Lapczynski *et al*., 2008; Kuroda *et al*., 2007; Inoue *et al*., 2004; Söderling *et al*., 2008; Renko *et al*., 2008; Matilla *et al*., 2005; Masako *et al*., 2003; Lauk et al., 2003; MacKay, 2001; Choi *et al*., 2002; Reynolds *et al*., 1999; Moghbel *et al*., 2007) however little is known regarding the antioxidant capacity when combined in a complex preparation such as a wound dressing. The question is raised whether components have only an additive effect, or do synergetic interactions occur. In addition the inclusion of other components such as anti-bactericides and/or thickeners may have a detrimental effect on antioxidant activity.

Therefore, the main aim of this study was to determine the antioxidant content and activity of each ingredient of Flavonix® Cytoflamm Gel, alone or in combination. Then to determine the effect of other added components to the antioxidant activity of the antioxidants present in Flavonix® Cytoflamm Gel. Lastly to determine the ability of Flavonix® Cytoflamm Gel and the ingredients thereof, to protect cells such as fibroblasts that are present in the wound against oxidative damage, thereby promoting wound healing.

CHAPTER 2: LITERATURE REVIEW

2.1. INTRODUCTION

The terms "complementary medicine" or "alternative medicine" refer to a broad set of health care practices that are not integrated into the dominant health care system and may fall outside of one's own traditions (www.who.int). Complementary/alternative medicine (CAM) is widely available all over the world and is an ever growing market. Examples of complementary wound care products are Allyvan®, Acticoat®, Medihoney®, Adaptic®, Promogran®, including a locally developed product Flavonix® Cytoflamm Gel (FCG). Although regulations exist related to factors such as the production environment and quality control, factors related to the efficacy of such products is questioned as in many instances the development of such products is related to the activity of each individual ingredient and does not address the interaction between ingredients. For wound healing products this type of interaction may either increase wound healing effects or toxicity. These effects are a function of the type of ingredient and the concentration used.

Thus in many instances efficiency is related to antidotal effects and in most cases are rarely subjected to scientifically evaluation. This is mainly due to the complexity of the product or the non-existence of models to address the interactions of complex mixtures. FCG is such an example where components such as honey (H) and oleuropein (OL) contain many polyphenolics and other constituent molecules making it difficult to identify the specific source of activity. In addition, interactions such as synergism, additive and antagonism may occur between added molecules and/or ingredients.

The purpose of this section of this study is to review the scientific literature related to wound healing, treatment options specifically related to biological wound dressing products such as FCG and the ingredients thereof.

2.2. THE NORMAL WOUND HEALING PROCESS

According to the concise Oxford dictionary, the definition of a wound is as follows: "It is an injury done to living tissue by a cut or blow ect., especially beyond the cutting and

piercing of the skin". Thus, a wound by true definition is a breakdown in the protective function of the skin; the loss of continuity of epithelium, with or without loss of underlying connective tissue (i.e. muscle, bone, nerves) (Leaper and Harding, 1998) following injury to the skin or underlying tissues/organs caused by surgery, a blow, a cut, chemicals, heat/cold, friction/shear force, pressure or as a result of disease, such as leg ulcers or carcinomas (Hutchinson, 1992).

The normal wound healing process includes the inflammatory, proliferative and remodeling phases and is summarized in Figure 2.1 (Strodtbeck, 2001; www.emedicinehealth.com).

Inflammatory phase:

With injury a series of biochemical events occur. Firstly, platelets aggregate to form a clot and immediately blood vessels narrow which control and prevent any further blood loss. During this phase debris, bacteria and any other foreign objects are removed via phagocytosis. Specialized mediators such as notch-3, β-catenin and c-myc are also released which assist in the migration and differentiation of cells involved in the proliferative phase (Greaves *et al*., 2013; Strodtbeck, 2001; www.emedicinehealth.com).

Proliferative phase:

The proliferative phase, 3 - 28 days (Figure 2.1) after injury, involves the formation of new matrix and this process includes angiogenesis, epithelialization and the formation of granulation tissue, collagen deposition and wound contraction. New blood vessels that form during angiogenesis provide oxygen and nutrients to maintain new cellular growth and production of structural proteins such as collagen and fibronectin produced by fibroblasts. This extracellular matrix (ECM) provides the scaffolding for re-epithelialization resulting in the repair of the barrier function of the skin (Greaves *et al*., 2013**;** Strodtbeck, 2001; www.emedicinehealth.com).

Remodeling phase:

Fourteen to twenty one days (Figure 2.1) after the initial injury remodeling occurs. During this stage the collagen becomes more organized, the tissue becomes stronger and the blood vessel density is reduced. Over a period of 6 months, the area increases in strength, eventually reaching 70% of the strength of uninjured skin (Greaves *et al*., 2013; Strodtbeck, 2001; www.emedicinehealth.com).

A factor that is overlooked in the wound healing process is the ability of the body to try to repair itself as rapidly as possible. Neutrophils are released as soon as the trauma occurs and releases reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) (Al-Waili *et al*., 2011). These components are bactericidal and prevent infection from occurring (Al-Waili *et al*., 2011). Macrophages also migrate to the wound site in response to environmental stimuli. The macrophages release vascular endothelial growth factor (VEGF), an angiogenic factor critical for wound healing (Al-Waili *et al*., 2011). Thus the H_2O_2 increases the macrophage VEGF through an oxidant induction of the VEGF promotor (Al-Waili *et al*., 2011).

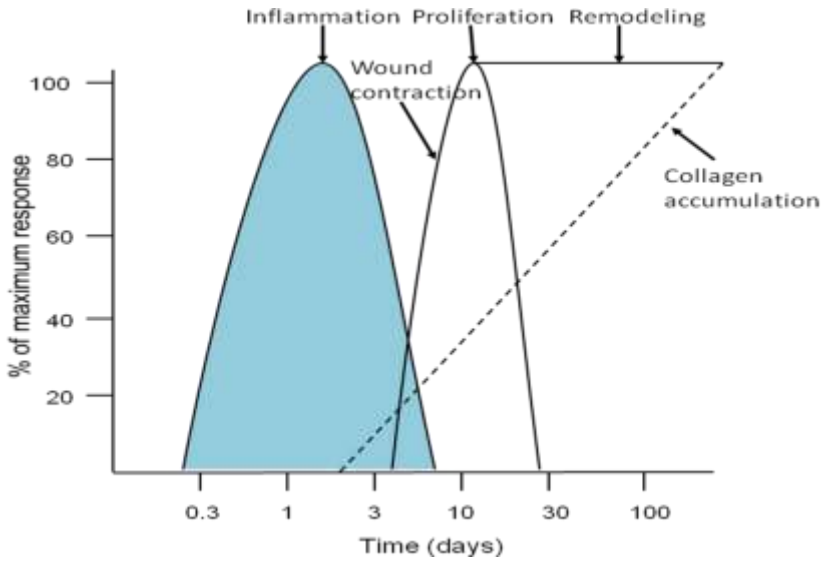


Figure 2.1: Wound phase over the required time period adapted from J Hutchinson (www.clinimed.co.uk/wound-care).

2.3. PROMOTION OF WOUND HEALING

Several wound treatment options are available that can shorten any of the phases in Figure 2.1 thereby promoting wound healing. A summary of the many options that are available is presented in Figure 2.2.

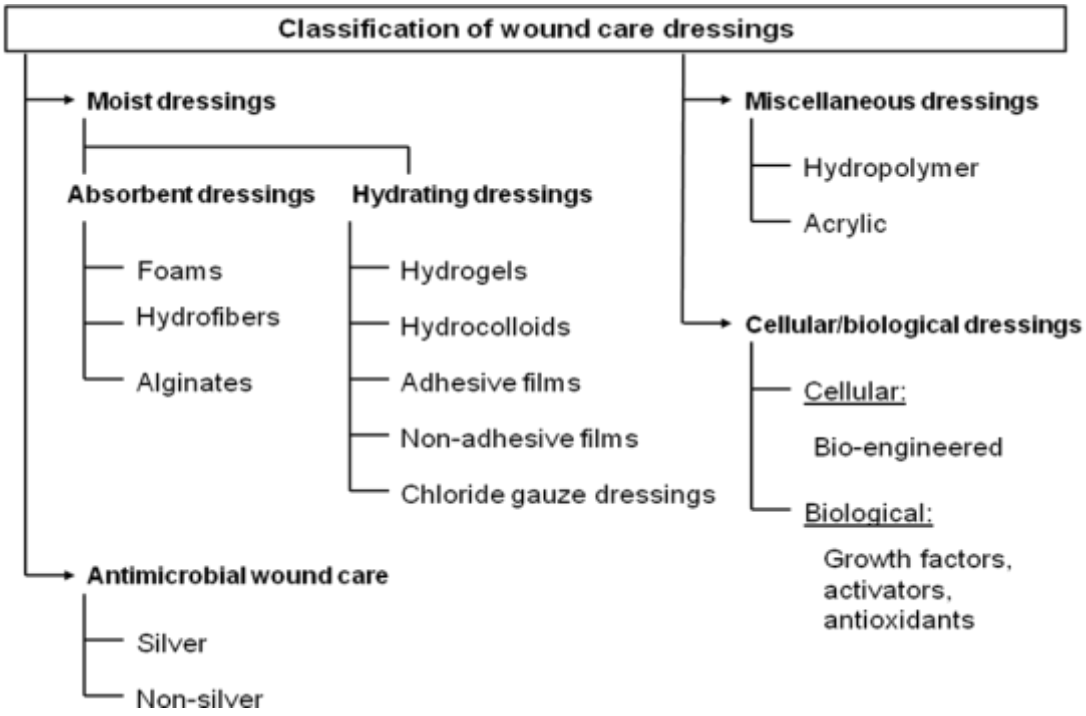


Figure 2.2: Summary of different types of wound dressings.

2.3.1. Current wound dressing treatment options

The choice of wound dressing used to promote wound healing depends on (i) localization of the wound, (ii) degree of infection, (iii) disease e.g. diabetic patient, (iv) type e.g. burn. The wound healing properties of the constituents of the biological wound dressing FCG will be evaluated in this study and therefore this type of wound dressing will be discussed in greater detail.

2.3.2. Cellular / biological dressings

Cellular / biological dressings are either completely based on natural occurring tissues and cells (cellular) or may contain ingredients that promotes the migration and growth of existing cell populations or establish within the wound site conditions that promotes wound healing (biological).

2.3.2.1. Cellular dressings

Three major biological dressings are available for the temporary closure of wounds namely (May, 1991): partial-thickness cadaveric human allograft skin, different forms

of partial-thickness antibiotic-treated porcine xenograft skin and human amnion. The advantages of these biological wound dressings is that they provide pain relief, assist in wound closure to reduce further fluid loss and minimize the risk of contamination. However, the disadvantages of biological dressings include transmission of infection, cost effectiveness and allogenicity of the patient (May, 1991).

Integra® and Apligraf® are examples of more complex dressings and are designed as skin substitutes or complete skin replacement. Integra® consists of chondroitan sulfate and reconstituted collagen backed by a polymer layer whereas Apligraf™ contains collagen seeded with cells such as fibroblasts or keratinocytes (Snyder *et al.,* 2011; Sefton *et al*., 1998). Both Integra® and Apligraf® are considered to be permanent wound closure products, whereas Biobrane® and TransCyte® are temporary skin substitutes composed of a silicone membrane to which peptides of porcine dermal collagen are bonded (Biobrane®) or impregnated with human fibroblast cells (TransCyte®) in a nylon mesh and should be removed after the specified time (Snyder *et al.,* 2011).

Closure of deep partial and full-thickness burns, and of chronic wounds can also be achieved by a direct application of keratinocytes in a cell suspension. The cell suspension is delivered within a fibrin spray which aids in the secure even placement of the keratinocytes. Examples of this keratinocyte fibrin spray product are ReCell®, CellSpray®, CellSpray®XP (Battler *et al.,* 2006).

2.3.2.2. Biological dressings

Biological dressings for the purpose of this review are defined as dressings prepared from animal, plant or synthetic materials with characteristics that promote cell migration and/or growth and in some instances create a wound environment that promotes wound healing i.e. reduces oxidative components. These kinds of dressings may contain a variety of active components such as growth factors: epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor-beta(2) (TGF- β(2)); antioxidants (vitamin A and C) to stimulate tissue repair and plant derived bioactive molecules such as hamamelitannins and oleuropein.

2.3.2.2.1. Growth factors

Growth factors are a subclass of cytokines that function as intercellular signals to permit cells to communicate with one another and specifically stimulate the migration and proliferation of cells and synthesis of new tissue. Growth factors are an essential part of the wound healing process and are thus involved in all three phases of wound healing. These are to attract immune cells to fight infection, stimulate and increase the production of connective tissue, assist in the angiogenesis process, to nourish the wound site and promote remodeling of the wound (http://www.janssen-ortho.com/joi/en/health/wound-know.asp). For example an animal derived collagen and other growth factors such as EGF are included in wound dressings to promote wound healing by providing an environment in which the growth of human epidermal keratinocytes and re-epithelialization is promoted.

2.3.2.2.2. Antioxidants

Other molecules added to biological wound dressing are antioxidants such as vitamin A and E. As this study will focus on the antioxidant activity of a biological dressing the role of antioxidants in biological dressings will be discussed in greater detail. Antioxidants are often added to biological dressings to fight against any excessive oxidation and therefore aiding in the wound healing process.

There are several different types of antioxidants that occur and can be divided into different groups (Vertuani *et al*., 2004) based on structure e.g. flavonoids or phenolic acids, or nature of activity e.g. enzymatic or non-enzymatic Table 2.1.

Table 2.1: Enzymatic and non-enzymatic antioxidants (Serem, 2011)

Enzymatic	Non-enzymatic
Superoxide dismutase	Antioxidant co-factors (Se, CQ_{10})
Glutathione peroxidase	Oxidative enzyme inhibitors (aspirin, ibuprofen
Catalase	Transition metal chelators (EDTA)
Thioredoxin	Radical scavengers (Vitamin C and E, EGCG)

These groups can then be further subdivided into their mechanism of action (preventative or chain breaking), the site of action (hydrophilic/lipophilic), the mode of action and lastly the chemical origin (natural or synthetic).

2.3.2.2.2.1. Enzymatic antioxidants

Superoxide dismutase (SOD), glutathione peroxidase and catalase are examples of enzymatic antioxidants (Table 2.1). These enzymes' main function is to neutralize the free radicals such as H_2O_2 and superoxide anion ($O_2^{\bullet-}$) (Vertuani *et al*., 2004) in the cytoplasm or blood. For example, superoxide dismutase catalyzes the conversion of superoxide radicals into H_2O_2 and oxygen.

2.3.2.2.2.2. Non-enzymatic antioxidants

Non-enzymatic antioxidants are free radical scavengers or serve as co-factors of antioxidant enzymes. Several co-factors, vitamins and antioxidant metals such as selenium are involved in the antioxidant effect which occurs in the mitochondria, cytoplasm or plasma membrane. There are two major routes of antioxidant effects. Either indirectly by participating in the regulation and expression of enzymes, or directly by an intrinsic free radical scavenging mechanism (Vertuani *et al*., 2004). An example of an indirect effect is selenium (Se) which is a cofactor of the antioxidant enzyme glutathioine peroxidase which removes damaging lipid hydroperoxide (Vertuani *et al*., 2004). Polyphenolics and vitamins such as ascorbic acid on the other hand directly scavenge radicals such as superoxide and peroxynitrite directly by recycling α-tocopherol which in turn prevents lipid oxidation (Vertuani *et al*., 2004).

2.3.2.2.3. Flavonix® Cytoflamm Gel

Flavonix® Cytoflamm Gel (FCG) is a chronic wound gel that contains several constituent molecules at different concentrations and these include an olive leaf extract, tocopherol acetate (vitamin E), Farnasol, Xylitol, *Hamamelis virginiana, Aloe barbadensis* and *Aloe barbadensis* honey (Table 2.2). These ingredients were chosen and added because of their reported anti-inflammatory, and antioxidant properties and their ability to break down biofilms (Fleming *et al*., 1973; Perugini *et al*., 2008; Puel *et al*., 2006; Pereira *et al*., 2007; Murray *et al*., 2008; Lin *et al*., 2003; Jabra-Rizk *et al*., 2006; Brehm-Stecher & Johnson, 2003; Weber *et al*., 2008; Cugini *et al*., 2007; Lapczynski *et al*., 2008; Kuroda *et al*., 2007; Inoue *et al*., 2004; Söderling *et al*., 2008; Renko *et al*., 2008; Matilla *et al*., 2005; Masako *et al*., 2003; Lauk et al., 2003; MacKay, 2001; Choi *et al*., 2002; Reynolds *et al*., 1999; Moghbel *et al*., 2007). In addition many of these ingredients are included to create a moist environment and control inflammation in acute or chronic wounds (Acton *et al*., 2008). Furthermore

several of the constituent molecules such as farnasol and xylitol prevent and heal wounds with a recalcitrant biofilm layer. Constituents in this wound gel also promotes cellular debridement in surrounding skin with a thick senescent epithelial cell layer and damage due to wound fluid contraction. In addition to the bioactive components listed in Table 2.2, several other components are added to thicken, add moisture and preserve FCG.

Molecules or extracts with reported antioxidant activity in FCG are aloe vera, honey, oleuropein, vitamin E and witch hazel (Table 2.2) (Fleming *et al*., 1973; Perugini *et al*., 2008; Puel *et al*., 2006; Pereira *et al*., 2007; Murray *et al*., 2008; Lin *et al*., 2003; Jabra-Rizk *et al*., 2006; Brehm-Stecher & Johnson, 2003; Weber *et al*., 2008; Cugini *et al*., 2007; Lapczynski *et al*., 2008; Kuroda *et al*., 2007; Inoue *et al*., 2004; Söderling *et al*., 2008; Renko *et al*., 2008; Matilla *et al*., 2005; Masako *et al*., 2003; Lauk et al., 2003; MacKay, 2001; Choi *et al*., 2002; Reynolds *et al*., 1999; Moghbel *et al*., 2007).

As this study will focus on the antioxidant capacity of FCG the antioxidant properties of the ingredients (Tale 2.2) that contribute to this antioxidant effect will be discussed in greater detail.

Table 2.2: The active ingredients in Flavonix® Cytoflamm Gel

Common name or synonym	Scientific/botanical name	Source	Chemical constituents	Reason for inclusion	Reference
Aloe Vera	*Aloe barbadensis*	Dried juice from leaves	Hydroxyanthrone	Anti-inflammatory Antimicrobial Cellular proliferation Antioxidant	Reynolds *et al*., 1999 Moghbel *et al*., 2007
Honey	*Aloe barbadensis* honey (crystallized)	Honey	Sugar mixture	Anti-bacterial effect Antioxidant	Molan, 1992 Overgaauw *et al*., 2005 Cooper *et al*., 2002 Molan, 2002 Hyslop *et al*.,1995 Cooper *et al*., 1999 Molan, 1999
Oleuropein	Olive (*Olea europaea*) leaf extract	Leaf	Olive leaves fructose, water	Anti-bacterial, Anti-inflammatory Antioxidant	Fleming *et al*., 1973 Perugini *et al*., 2008 Puel *et al*., 2006 Pereira *et al*., 2007
Vitamin E	Tocopherol acetate	Fruit and vegetables	Tocopherol	Antioxidant	Murray *et al*., 2008; Lin *et al*., 2003
Witch Hazel	*Hamamelis virginiana*	Fresh or dried leaves; bark	Hamamelitannin	Anti-inflammatory Antioxidant	Lauk et al., 2003 MacKay, 2001 Choi *et al*., 2002
Farnasol	3,7,11-trimethyl-2,6,10-dodecatrien-1-ol Farnasol Plus	Part of neroli oil and other essential oils	Essential oil	Bacteriostatic	Jabra-Rizk *et al*., 2006 Brehm-Stecher & Johnson, 2003 Weber *et al*., 2008 Cugini *et al*., 2007 Lapczynski *et al*., 2008 Kuroda *et al*., 2007 Inoue *et al*., 2004
Xylitol	Xylo-pentane-1,2,3,4,5-pentol	Fibres from fruits and vegetables	Sugar alcohol	Anti-bacterial	Söderling *et al*., 2008 Renko *et al*., 2008 Matilla *et al*., 2005 Masako *et al*., 2003

2.3.3. Antioxidant activity of the bioactive components of Flavonix® Cytoflamm Gel

2.3.3.1. Aloe vera

Aloe vera or *Aloe barbadensis Miller* is a cactus like, perennial plant that usually grows in hot and dry climates. Since Roman times and perhaps even before this, aloe vera has been used as a medicinal plant to treat different ailments (Morton, 1961, Crosswhite *et al*., 1984). Today the plant is readily used in cosmetic and healthcare products and is widely available (Reynolds *et al*., 1999). Aloe vera is a xenophyte type of plant and can grow to about 30 - 50 cm in length and 10 cm in breath. It has pointed and elongated leaves which are joined at the stem (World Health Organization 1999). The majority of the leaf volume consists of parenchyma (filet or pulp), which is a clear mucilaginous gel also known as aloe vera gel (Femenia *et al*. 1999; Femenia *et al*. 2003).

Aloe vera consists of a complex mixture of active constituents which have been identified and includes saccharides, minerals, vitamins, enzymes, amino acids, anthraquiones, lignin, salicylic acids and saponins (Foster *et al.,* 2011). Anthraquinones especially barbaloin, also known as aloin A, are present in the leaf exudate which may be responsible for the cathartic effect and bitter taste of the exudate (Dagne *et al*., 2000; Boudreau and Beland, 2006). Aloin A, (Figure 2.3), is an antioxidant due to its phenolic acid precursor structure and its ability to inhibit free radical-mediated cytotoxicitiy (Cook and Samman, 1996). Aloin A and B are two diastereometic C-glucosylanthrones and are considered to be the components giving aloe its laxative effect. Aloesin, 8-C-beta-D-[2-O-(E)-cinnamoyl]glycopyranosyl-2-[(R)-2-hydroxy]propyl-7-methoxy-5-methylchromone exhibits free radical scavenging, antioxidant and anti-inflammatory properties (Krpetić, *et al*., 2009). Other contributing factors to the activity of aloe vera have been attributed to two polysaccharides namely, acetrylatedmannan and glucomannan and glycoproteins (lectins) found in the colourless leaf pulp (Krpetić, *et al*., 2009).

Antioxidant capacity of aloe vera have shown to have an effect on cell proliferation (Reynolds *et al*., 1999). Studies have shown that aloe vera gel promotes wound healing by stimulating fibroblast activity, and collagen production, resulting in an increase in granulation tissue (Reynolds *et al*., 1999). Another study showed the stimulation of

fibroblast growth in a synovial model as well as the enhancement of the wounds' tensile strength and the collagen turnover in the wound tissue (Choi *et al*., 2002).

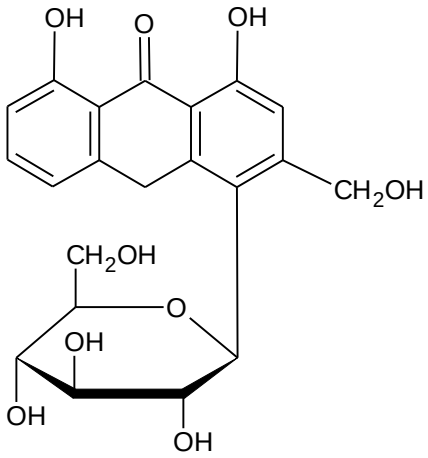


Figure 2.3: The chemical structure of aloin A. (www.sigmaaldrich.com)

2.3.3.2. Honey

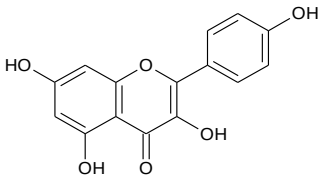
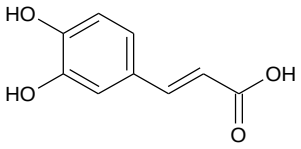
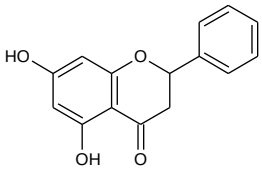
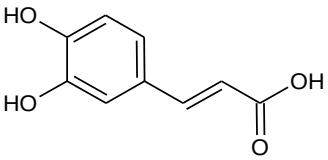
Honey can be described as a functional food since it have a potentially positive effect on health beyond basic nutrition. Honey has been used traditionally in wound healing and dates back to medical literature of Egypt, Greece and in the Ayurvedic traditions of India (Bodeker *et al*., 1999, Subrahmanyam, 1996). Therapeutic honeys are usually very viscous and are used "raw", which only means that the honey did not undergo heating treatment as do culinary honeys (Lusby *et al*., 2002).

Honey is a complex mixture consisting of a wide range of constituents e.g. hydrogen peroxide (H_2O_2), methylgyoxal (MGO), carotenes, phenolic compounds such as kaempferol, caffeic acid, ferriulic acid, quercetin, chlorogenicellagic acid benzoic acid, gallic acid and coumaric acid monosaccharides, polysaccharides, amino acids, vitamins and minerals (Figure 2.4) (Kwakman *et al.,* 2011), Maillard reaction product (MRP's), ascorbic acid and catalysts, giving it unique properties (Rice-Evans *et al*., 1997).

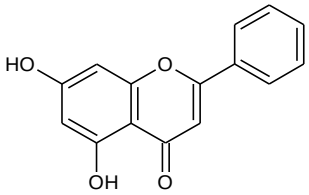
Honey is a complex mixture with molecules that target specific aspects of wound healing. The antibacterial activity of honey is attributed to the high sugar content, low pH (3.2 - 4.5) and the presence of MGO, H_2O_2, catalase, cationic antimicrobial peptide

(AMP) and bee defensins. Revamil® source (RS) honey and manuka medical grade honey, from New Zealand and Australia have been identified to have potent antibacterial activity. Researchers found that RS honey was specific against *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* and this activity was due to the presence of the antimicrobial peptide (AMP) bee defensin-1 and H_2O_2 (Kwakman *et al.,* 2011). In contrast, antimicrobial activity in manauka honey only showed activity against *B. subtilis* and antimicrobial activity was due to high levels of MGO (Kwakman *et al.,* 2011). In addition to the antimicrobial activity of H_2O_2, it also assists in wound debridement (Lusby *et al*., 2002). With the higher osmotic activity, due to a high sugar content, bacterial growth is also inhibited and furthermore honey creates a moist wound healing environment to aid in the wound healing process.

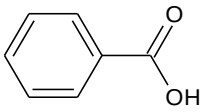
The antioxidant and anti-inflammatory effects of honey is due to the presence of flavonoids and phenolic acids and those most commonly found in European honey are presented in Figure 2.4.

Flavonoids	**Phenolic acids**
Kaempferol 	**Caffeic acid** 
Pinocembrin 	**Ferrulic acid** 

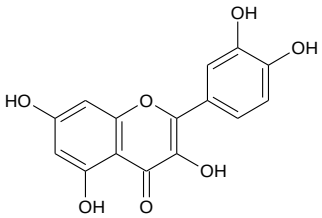
Chrysin



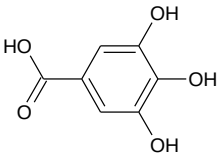
Benzoic acid



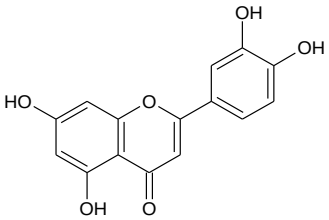
Quercetin



Gallic acid



Luteolin



Myrecitin

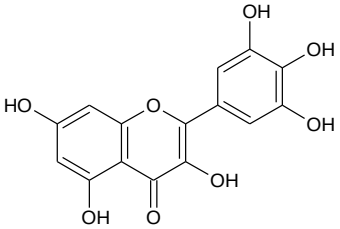


Figure 2.4: Most common flavonoids and phenolic acids found in European honeys. (Pyrzynska and Biesaga, 2009; Kaškonienė et al., 2009; Estevinho *et al*., 2008; Truchado *et al.,* 2009)

The antioxidant activity of honey from many different geographical regions has been determined (Silva *et al*., 2009). In a study done by Serem and Bester (2012) it was found that honey from southern Africa (such as that used in the preparation of FCG) had high antioxidant content and activity and showed high levels of cellular protection against oxidative damage in the SC-1 and Caco-2 cell lines (Serem and Bester, 2012).

Ranzato *et al*., studied the effect of acacia, buckwheat and manuka honeys in an *in vitro* model of HaCaT keratinocytes using the scratch wound and migration assays. This study showed an increase in re-epithelialization rates and chemoattractant effect in the presence of honey (Ranzato *et al*., 2012).

Ashokkumar and Sudhadiran, (2008), evaluated luteolin (a flavone also found in honey) for its ability to protect mouse colon against azoxymethane (AOM)-induced oxidative damage measured as the formation of aberrant crypt foci (ACF) (Ashokkumar and Sudhadiran, 2008). AOM caused the formation of an average of 42 ACF's per colon but when in combination with luteolin this decreased to 23 ACF's per colon (Ashokkumar and Sudhadiran, 2008). Similar results were obtained by Prabhu *et al.,* (2009) where the antioxidant astaxanthin was found to protect colon cells against oxidative damage induced by dimethylhydrazine (Prabhu *et al.,* 2009).

A study to determine the wound healing activity of acacia honey in a rat wound model was undertaken by Iftikhar *et al*., 2010. Honey was administered in different formulations, both topically as well as orally and both high and low concentration formulations had a significant effect on healing ($p < 0.05$). Noticeable changes include an increase in the area of epithelization as well as an increase in wound contraction, tissue granulation and skin-breaking strength (Iftikhar *et al*., 2010).

In a human clinical study, honey was used to treat 26 patients with postoperative wound infection in a randomized control study. Controls included an additional 24 wounds washed with 70% ethanol and treated with povidone iodine. In the honey treated group infections were eradicated and the wounds healed completely compared to the control group that took twice as long to heal (Al-Waili NS *et al*., 1999).

2.3.3.3. Oleuropein

The olive tree, *Olea europaea*, is widely grown in the Mediterranean areas and parts of Asia Minor (Waterman *et al*., 2007). The usages of the products of this plant date back to Biblical and Roman times and to Greek mythology where it have been used as emollients, laxatives, aphrodisiacs, sedatives, nutritive and tonics (Waterman *et al*., 2007). Hypertension, colic, rheumatic pain, paralysis, alopecia and sciatica all have been treated with olive products (Gilani *et al*., 2005). The biological benefits of olive oil

are believed to be due to constituent antioxidants (Waterman *et al*., 2007). Olive oil is a complex mixture with the major groups of compounds with anti-inflammatory and antioxidant health benefits being oleic acid, phenolics and squalene (Owen *et al*., 2000, Waterman *et al*., 2007). The phenolic constituents of olive oil can be divided into three categories, namely: lignans, secoiridoids and simple phenols. Ligstroside, (Owen *et al*., 2000) oleuropein, hydroxytyrosol and tyrosol are the key phenolics present in olive oil (Perona *et al.,* 2006). Oleuropein is a secoiridoid whereas hydroxytyrosol and tyrosol are simple phenols (Figure 2.5).

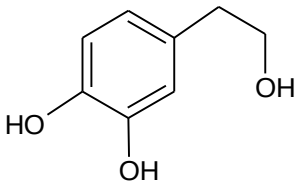
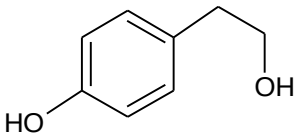
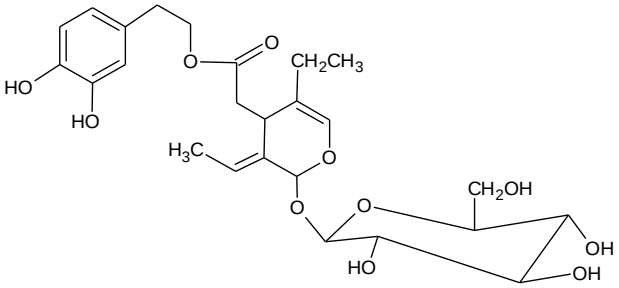
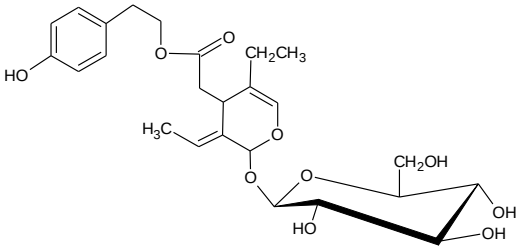
Hydroxytyrosol	**Tyrosol**
	
Oleuropein	**Ligstroside**
	

Figure 2.5: The chemical structure of the olive oil phenols: hydroxytyrosol; tyrosol; oleuropein and ligstroside.

Studies have shown that oleuropein enhances the response of macrophages to bacterial lipopolysaccharide (LPS) resulting in an increase in inducible nitric oxide synthase (iNOS) activity (Visioli *et al*., 1998). During acute sepsis and inflammation, there is an increase in the NO production released by macrophages and other cells (Visioli *et al*., 1998). The patho-physiological effects due to increased NO production by macrophages are still being debated (Visioli *et al*., 1998). Generally it is accepted that during inflammation and acute sepsis an adaptive response occurs to the antimicrobial effect of the macrophages. This is the inhibition of platelet aggregation, adherence and

thrombosis, as well as maintenance of proper perfusion and protection of the microvasculature (Visioli *et al*., 1998; Wong and Billiar, 1995).

Oleuropein and hydroxytyrosol inhibits low density lipoprotein (LDL) oxidation and scavenges free radicals (Owen *et al*., 2000, Visioli *et al*., 2002). Both oleuropein and hydroxytyrosol are more potent at the scavenging of free radicals than the exogenous antioxidants dimethyl sulfoxide (DMSO) and butylatedhydroxytoluene (BHT) and endogenous antioxidant vitamin E (Owen *et al*., 2000, Visioli *et al*., 2002). A variety of endogenous and exogenous radical and oxidants, namely hypochlorous acid, those generated by H_2O_2 (Owen *et al*., 2000) and xanthine/xanthine oxidase, have been showed to be scavenged by both catechols (Visioli *et al*., 2002).

In an animal model the effect of olive oil polyphenols on oxidation susceptibility of low density lipoprotein was studied. A decrease susceptibility of LDL to oxidation was detected in the presence of dietary polyphenol-rich extra virgin olive oil (Wiseman *et al*., 1996; Coni *et al*., 2000; De Pasquale *et al*., 1991). The mechanism of action has been described through the interaction of the iridoid part of the oleuropein molecule with the polyunsaturated fatty acid in the oil (Soler-Rivas *et al*., 2000). Some of the effects of oleuropein have been studied and noted that there is an increase in the levels of cholesteryl esters and a decrease in the cardiac levels of tricylglycerols and linoleic acid. An antioxidant effect was also exerted in the heart (Visioli *et al*., 1995; Maimeskulova *et al*., 1998). Relief from arrhythmias, increased blood flow to the coronary artery and reduced blood pressure are all cardiotonic effects noted in the animal models (Circosta *et al*., 1990; Petroni *et al*., 1995). Other studies reported the inhibition of platelet aggregation (Trovato *et al*., 1993) and hypoglycemic effects in animal models (Chimi *et al*., 1988).

In an animal study by Al-Azzawie and Alhamdani (2006) it was publicized that oleuropein acts as an effective antioxidant and hypoglycemic agent in alleviating oxidative stress and free radicals, but also enhances the enzymes degenses associated with diabetes. Singh *et al.,* (2008) did a randomized single blinded study involving 11 healthy male volunteers to investigate the effects of polyphenols in olive leaves on platelet function. The results showed that the polyphenols from the olive leaves greatly inhibits platelet aggregation *in vitro* possibly due to their ability to scavenge H_2O_2.

Literature on human clinical trials specifically related to oleiropein is not available since most clinical studies use olive oils. It should also be kept in mind that the oleuropein used in this study was an extract and thus may contain some of the other components found in olive leaves.

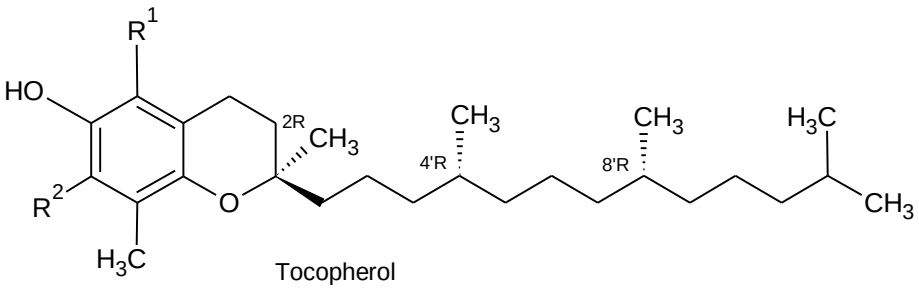
2.3.3.4. Tocopherol acetate (Vitamin E)

Vitamin E is found in many food sources including wheat germ oil, sunflower seed and almonds. In the human body vitamin E is found in the membranes, plasma and tissue (Shapiro *et al.,* 2001). Vitamin E is a fat-soluble antioxidant that comprises of eight naturally occurring structures, namely: α-, β-, γ-, δ-tocopherols and tocotrienols (Figure 2.6) (Shapiro *et al.,* 2001, Zingg, 2007). The main difference between the tocopherols and tocotrienols is that the tocopherols have a saturated phytyl-chain while the tocotrienols have an unsaturated phytyl-chain (Shapiro *et al.,* 2001). These chain breaking antioxidants ends the propagation of lipid peroxidation in membranes by rapidly scavenging the lipid-peroxyl radicals before they have time to react with other lipids (Shapiro *et al.,* 2001). Vitamin E's antioxidant function is membrane associated scavenging of peroxyl radicals (Traber and Atkinson, 2007).

Rosignoli *et al.* (2008) studied the effect of antioxidants on Caco-2 cells exposed to deoxylic acid (DCA), a bile acid that causes DNA damage. Both ß-carotene and α-tocopherol reduced the DNA damaging effects of DCA (Rosignoli *et al.,* 2008).

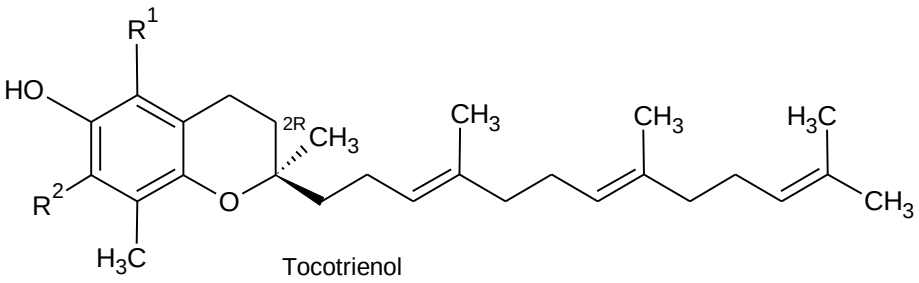
It has been suggested that vitamin E decreases excessive scar formation in chronic wounds but also play an important part as an anti-inflammatory agent. Vitamin E supplementation has been shown to be beneficial in wound healing (Arnold *et* al., 2006, Burgess, 2008). Some studies of vitamin E claim to shorten the wound healing time and improve the outcome pertaining to scars whereas other studies have shown that vitamin E has no effect when applied topically. Thus these results are inconclusive (Baumann e*t al.,* 1999, Pinnel, 1999, Mackay *et al*., 2003).

Basic structure of tocopherol



Tocopherol

Basic structure of tocotrienols



Tocotrienol

Figure 2.6: Structures of natural tocopherols and tocotrienols (Zingg, 2007).

2.3.3.5. Witch hazel

Witch hazel (*Hamamelis virginiana L.*) is native to damp woods in Canada and North America (Wang *et al.,* 2003*).* It is a small tree or deciduous shrub (Wang *et al.,* 2003*)*. Witch hazel is prepared as an extract, using the twigs, leaves and bark, and is commonly used as an astringent for people with oily skin as well as a treatment for sun burn and atopic eczema (Draelos, 2001). This astringent effect is probably due to the high tannin concentration of the plant (Draelos, 2001). In addition tannins also function as venous vasoconstrictors (Draelos, 2001).

A main component of the bark extract of witch hazel is hamamelitannin (2', 5-di-*O*-galloyl-hamamelose) (Draelos, 2001) (Figure 2.7). It has been reported by Habtemaraim *et al.* 2002 that hamamelitannin inhibits DNA fragmentation and tumour necrosis factor-α (TNF- α) – mediated endothelial cell death. At low concentrations, it has been found that against peroxides, hamamelitannin have a high protective activity against cell damage (Masaki *et al.*, 1995). *In vivo* studies have shown that witch hazel extracts inhibit human

leukocyte elastase, an enzyme which contributes to the degradation of connective tissue, as well as alpha-glucosidase (MacKay, 2001).

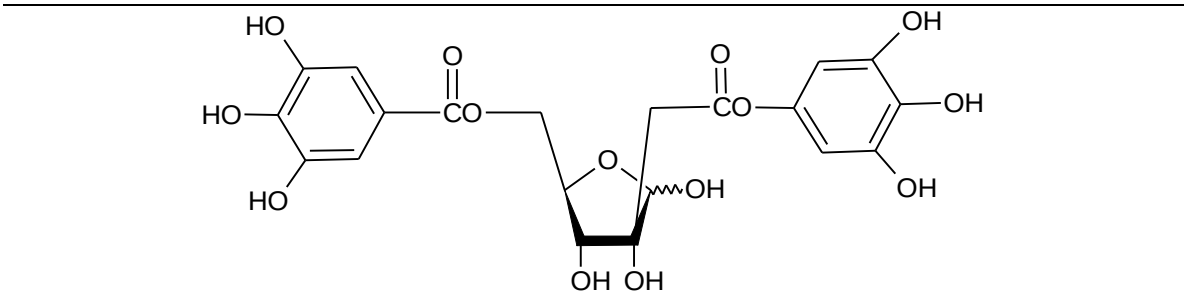


Figure 2.7: The chemical structure of Hamamelitannin. (Fleming, 1998)

2.3.3.6. Farnesol and xylitol

Biofilms are a complex structure consisting of colonies of bacteria that adhere to surfaces such as wounds. In this moist environment these biofilms are often associated with other organisms such as yeasts, fungi, and protozoa that secrete a mucilaginous protective coating called extracellular polymeric substance (EPS) (Donlan *et al*., 2002). Biofilms play an important role in the wound healing process. Both acute and chronic wounds initially go through the same wound healing stages but chronic wounds are difficult to treat as they are caught in a persistent inflammatory phase (Bjarnsholt *et al*., 2008). This phase usually is accompanied by high levels of bacteria which prolong the elevated expression of the inflammatory cytokines (Konturek *et al*., 2001, Power *et al*., 2001). Biofilm formation has four stages, namely: (I) the appearance of fibrin fibers, (II) formation of the fibrin net, (III) an increase in the number of bacteria and (IV) the covering of the bacteria with glycocalyx (Masako *et al*., 2005).

Farnesol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) is classified as a prenyl alcohol (Figure 2.8) and are major fragrance component in essential oils (Muramatsu *et al*., 2008). Farnesol have the capacity to inhibit the newly formed fibrin fibers (stages I – II) by inhibiting coagulase, and dissolving the fibrin fibers of the mature biofilm (Masako *et al*., 2005).

Xylitol (xylo-pentane-1,2,3,4,5-pentol) is a five-carbon polyalcohol and is found in fruits, berries and plants (Mattila *et al*., 2005) (Figure 2.8). Xylitol inhibits growth of *Streptococcus mutans* (Söderling *et al*., 2008). Xylitol have the ability to inhibit the

formation of glycocalyx possibly because xylitol cannot be fermented my most oral microorganisms (Masako *et al*., 2005). Both components have no reported radical scavenging activity.

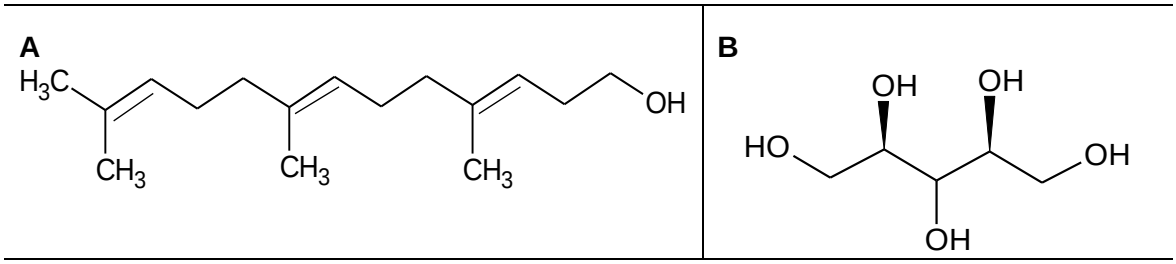


Figure 2.8: Chemical structure of A: farnesol (Muramatsu *et al*., 2008) and B: xylitol (www.medicinescomplete.com).

2.4. SYNERGISM, ANTAGONISM AND ADDITIVITY

The word synergy is derived from the Greek term synergos which means "working together (Segal-Horn, 2004). In nature the phenomena of synergism is ubiquitous ranging from the diverse groupings of quarks that produce neutrons and protons in physics to a simple example such as water, a compound of oxygen and hydrogen in chemistry to the mutual exchanges amongst genes in genomes, the synergies of scale in multi-cellular organisms and the division of work in bacterial colonies (Corning, 2003).

The same principle applies to the combination of different component where new products are formulated. Chemical synergism may make a compound either more or less toxic related to the safety of the product. On the other hand, pharmacological synergism may make a product more effective than it would have been with only the single component, making it an important concept to investigate when formulating any new product. Thus the same principle of synergism can be applied to this study and the questions can be asked whether or not the combinations of the different known antioxidant active components make the product more effective or is the different combination haltering the desired effect. The end result of the combination may either be synergistic, additive or an antagonistic effect on the total antioxidant capacity.

In addition when evaluating these effects several aspects should be considered for example structural differences of each component, mode of action, mechanism of action, concentration and effects on the target cell/organs.

Table 2.3: Definition of different types of combined effects (Borgert *et al*., 2004, Meek *et al*., 2011)

Type of combined effect	Subtypes	Synonyms	Effects observed
Non-interactive	Simple similar action Simple dissimilar action	Additive Independent action	Dose addition Response addition
Interactive	Synergy and Potentiation Antagonism		Greater than dose additively Less than dose additive effect

When the components in a mixture have the same mechanism and/or act in the same way it is described as a simple similar action (Table 2.3). Thus, the concentration/dose may show an additive effect when the components target the same cell and/or have the same mode of action and do not affect the activity of each other (Meek *et al.,* 2011, Price and Han, 2011).

A simple dissimilar action takes place when the nature of the site of action and/or mode differ between the components in the mixture but still do not have an effect on each other (Meek *et al*., 2011, Price and Han, 2011). A response to this is independent of the site or mode of action is additive.

Interactive effects occur when different components in a complex mixture interact with each other and the effect can be synergistic, potentiated or antagonistic. Synergism results in an enhanced effect; potentiation is an extension of the duration of the effect and lastly antagonism is a decrease in the observed effect (Table 2.3 and Figure 2.9).

Mathematically, additive effects is the sum of the individual effects of the components in the mixture and is equal to the total effect of the mixture (i.e. 1+1 = 2). When the effect shows a positive interaction in the mixture where the effect is greater than the sum of the individual components' effect is defined as synergism (i.e.1+1 > 2). On the other hand, antagonism occurs when the effect of the mixture is less than the sum of the individual component's effect (i.e. 1+1 < 2) (Warne, 2003) (Figure 2.9).

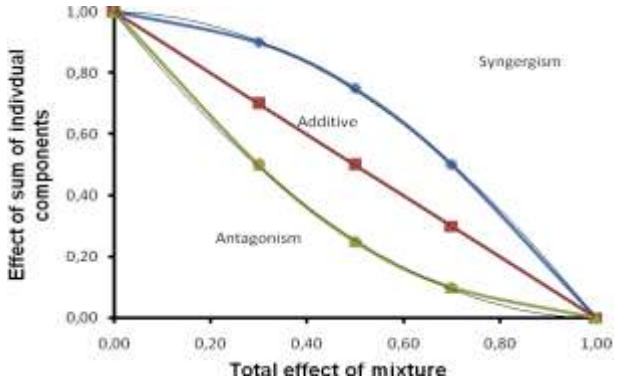


Figure 2.9: Graphical representation between syergistic, addivite and antagonistic effects where 1< x >1.

These effects are important to know especially when formulating a wound care product with many antioxidant components. Thus, for the best results in the final product, it will be of importance to formulate a product where the different components have a synergistic effect. The benefits of this would be more efficient and possibly more rapid wound healing as well as more cost effectiveness related to the formulation. Additive synergism related to antioxidant has been described by Freeman *et al*., 2010 for navel oranges by using the oxygen radical absorbance capacity (ORAC) assay. Combinations myricetin/luteolin, myricetin/quercetin and luteolin/quercetin all showed to have an additive ORAC. The reason behind this interaction may be due to the similarity between the structures where the donation and interaction of the electrons may be ineffective, as the electron may merely be donated backward and forward resulting in an additive effect (Freeman *et al*., 2010). Freeman also noticed that these compounds may interact independently with the peroxyl radicals until the ring structure is cleaved. In this study the interaction between the same type of antioxidant molecules, e.g. polyphenolics in honey, and different types of antioxidant molecules e.g. polyphenolics from honey and vitamin E will be investigated.

2.5. AIM OF STUDY

The aim of this study is to determine the antioxidant content and activity of the individual, and combinations of bioactive ingredients of a wound healing product FCG (FP). Then, to determine at a cellular level if high antioxidant activity translates into significant protection against oxidative damage.

Objectives:

a) To design a model to determine the interaction between major components of FCG.

b) To determine the total polyphenolic acid content and the total flavonoid content of (i) FCG (FP), (ii) FCG-bioactive formulation (ALL COMP), (iii) each individual bioactive component and (iv) combinations of each bioactive component.

c) To determine using the ORAC, DPPH and TEAC assays the antioxidant activity of (i) FCG (FP), (ii) ALL COMP, (iii) each individual bioactive component and (iv) combinations of each bioactive component.

d) To determine *in vitro* using the SC-1 fibroblast cell line if (i) FCG (FP), (ii) ALL COMP, (iii) each individual bioactive component and (iv) combinations of each bioactive component provide short term cellular protection against oxidative damage induced by AAPH.

e) To determine based on antioxidant activity and cellular protective effects if synergism occurs between ingredients of FCG.

HYPOTHESIS: FCG and bioactive ingredients have significant levels of antioxidant activity and cellular protection against oxidative damage and this is due to synergism between antioxidant ingredients.

CHAPTER 3: MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Flavonix® Cytoflamm Gel raw material collection

Flavonix® Cytoflamm Gel was manufactured by Medika SA (Pty) Ltd., Irene, South Africa (SA). All components were purchased from the following companies and were kept onsite under controlled conditions at 20°C. Honey was purchased from Highveld Honey Farm, Benoni, SA while citric acid, xylitol and α-tocopherol acetate (vitamin E) were purchased from Chempure, Pretoria, SA. Oleuropein (Eurol BT), witch hazel, aloe vera and farnesol were purchased from Sharon Bolel, Edenvale, SA and trisodium citrate from Savannah Fine Chemicals (Pty) Ldt., Bedfordview, SA.

3.1.2. Equipment, reagents and disposable plastic ware

Equipment:

A BioTek plate reader purchased from Analytical and Diagnostic Products (ADP) Johannesburg, SA was used. Lambda LS5OB spectrophotometer from Perkin Elmer, Boston, MA, USA supplied by Separations Scientific, Honeydew, SA. Eppendorf pipettes from Eppendorf AG Hamburg, Germany were all supplied by the Scientific Laboratory Equipment Company (LASEC), Cape Town, SA. FLUOstar OPTIMA plate reader from BMG labtechnologies, Offenburg, Germany was used for all fluorescence based studies.

Reagents:

Folin-Ciocalteu's reagent, sodium carbonate anhydrous, gallic acid and catechin were obtained from the Sigma-Aldrich Company, Atlasville, SA. 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), 2, 2-diphenyl-2-picrylhydrazyl (DPPH), ABTS (2,2'-azo-bis (3-ethylbenzothiazoline-6-sulfuric acid) diamonium salt), flourescein sodium salt and potassium persulfate were obtained from Sigma-Aldrich Company, Atlasville, SA. The organic solvent methanol as well as sodium hydroxide (NaOH), sodium nitrite and aluminium chloride, all of analytic grade, were purchased from Merck Chemicals, Modderfontein, SA.

The media and supplements used were: Eagles Minimum Essential Medium (EMEM) powder, foetal calf serum (FCS) and antibiotic solution (containing, streptomycin, penicillin and fungicide) were obtained from Highveld Biological Company, Johannesburg, SA. Dulbecco's Modified Essential Medium (DMEM) and dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma-Aldrich, Atlasville, SA.

Water was double distilled and de-ionised (ddH_2O) with a continental water system and all medium, enzyme solutions and buffers were sterilized by filtration through a Millex 0.2 µm filter. Glassware was sterilized at 121°C for 20 min in a Prestige Medical Autoclave (series 2100).

Cell lines:

The mouse fibroblast (SC-1) cell line was obtained from Highveld Biological Company, Johannesburg, SA.

Disposable plastic ware:

Disposable plastic ware included: 96 and 48 well plates, 50 ml, 15 ml tubes and pipette tips (10, 25, 100, 200, and 1000 µl) and were obtained from Greiner Bio-one also supplied by LASEC, Cape Town, SA.

Laboratory facilities:

All research was conducted in the research facilities of the Departments of Anatomy and Pharmacology of the Faculty of Health Sciences, University of Pretoria.

3.2. SAMPLE PREPARATION

3.2.1. Flavonix® Cytoflamm Gel (FCG) is the commercial product that was provided by Medika SA (Pty) Ltd, Pretoria, SA. In addition to the bioactive ingredients added to FCG, (Table 3.2) it also contains glycerin and Natrasol 250 HR, which are thickening agents as well as a buffering system consisting of citric acid and trisodium citrate. Throughout this study this is referred to **FCG (FP)** (Table 3.1).

Table 3.1: Summary of important abbreviations

FCG	Flavonix® Cytoflamm Gel
FCG (FP)	Flavonix® Cytoflamm Gel (Final Product) - retail product
ALL COMP	Only bioactive ingredients at FCG concentrations, no thickners
CALC	Sum of individual effects generated from data

Table 3.2: Concentration of bioactive ingredients of FCG

Ingredient	Content in formulation (% v/v)
Major components	
Honey (H)	15.29%
Oleuropein (OL)	6.00%
Witch Hazel (WH)	12.95%
Minor components	
Xylitol (X)	5.00%
Vitamin E (VE)	0.10%
Aloe Vera (AV)	0.70%
Farnesol (F)	0.20%

3.2.2. Individual components

Individual components were divided into two groups (i) major components related concentration (> 5%) and known antioxidant activity (Pérez-Pérez *et al*., 2013; Bulotta *et al*., 2013; Thring *et al*., 2011) and minor components with low concentration < 5% and/or no reported antioxidant activity. Major components were H, OL and WH while the minor components were X, VE, AV and Fa (Table 3.2).

Major components were evaluated at concentrations 1 and 0.1 times the concentration in Table 3.2 while the minor components were evaluated at concentrations 10 times the concentration in Table 3.2. The final concentrations used for testing of individual components are presented in Table 3.3. This was compared to **ALL COMP** which is a mixture of all H, WH, OL, X, VE, AV, Fa as found in FCG. This mixture does not contain the others ingredients such as glycerin and Natrasol 250 HR. CALC is the calculated sum of individual effects generated from data.

Table 3.3: Concentrations evaluated for antioxidant content and activity

Ingredient	10x formulation	1x formulation	0.1x formulation
Honey		√	√
Oleuropein		√	√
Witch Hazel		√	√
Xylitol		√	√
Vitamin E	√		
Aloe Vera	√		
Farnesol	√		
ALL COMP			√

3.2.3. Major and minor component

To determine the contribution of each component to the antioxidant activity of complex mixtures is challenging. To investigate the interaction between the major antioxidant components but at the same time retaining the integrity of the product the following strategy was used. ALL COMP was prepared without H, OL and WH (single components), without H and OL, H and WH as well as H and OL (two components) and without H, OL and WH (three components) (see Table 3.4). This was used to determine the contribution of each component to total antioxidant activity. For example using the following calculation:

ALL COMP - ALL COMP without OL = contribution of OL to total antioxidant activity.

Whereas using ALL COMP without H & OL, ALL COMP without H & WH and ALL COMP without OL & WH the type of interaction was determined i.e. additive, synergistic or antagonistic.

Table 3.4: Formulations used to determine the effect of H, OL and WH alone and in combination

Samples	Measured effect
ALL COMP	**Minor components +H + OL + WH**
One component	
ALL COMP without H (All-H)	**Minor components + OL + WH**
ALL COMP without OL (All-OL)	**Minor components + H + WH**
ALL COMP without WH (All-WH)	**Minor components + H + OL**
Two components	
ALL COMP without H & OL (All-H-OL)	**Minor components + WH**

ALL COMP without H & WH (All-H-WH)	**Minor components + OL**
ALL COMP without OL & WH (All-OL-WH)	**Minor components + H**
Three components	
ALL COMP without H & OL & WH (All-H-OL-WH)	**Minor components**

Purple = major components, Black = minor components

A similar strategy was used to determine the contribution of the minor components to total antioxidant activity. All data generated related to minor components was expressed relative to VE, the only ingredient in minor component fraction with reported antioxidant activity (Ou *et al*., 2002).

Table 3.5: Formulations used to determine the effect of X, AV, VE and Fa alone and in combination

Samples	Measured effect
ALL COMP	**Major components + X + VE + AV + Fa**
One component	
ALL COMP without X (All –X)	**Major components + VE + AV + Fa**
ALL COMP without VE (All-VE)	**Major components + X + AV + Fa**
Two components	
ALL COMP without VE & Fa (All-VE-Fa)	**Major components + X + AV**
ALL COMP without VE & X (All-VE-X)	**Major components + AV + Fa**
ALL COMP without VE & AV (All-VE-AV)	**Major components + X + Fa**
Three components	
ALL COMP without VE & X & AV (All-VE-X-AV)	**Major components + Fa**
ALL COMP without VE &X & Fa (All-VE-X-Fa)	**Major components + AV**
ALL COMP without VE & Fa & AV (All-VE-Fa-AV)	**Major components + X**
Four components	
ALL COMP without VE & X & AV & Fa (All-VE-X-AV-Fa)	**Major components**

Purple = major components, Black = minor components

3.3. METHODS

The antioxidant content and activity as well as cellular protective effects of FCG, individual (Table 3.3), major (Table 3.4) and minor components (Table 3.5) was evaluated as summarized in Table 4.1 and Table 4.2, using the methods described below (Figure 3.1).

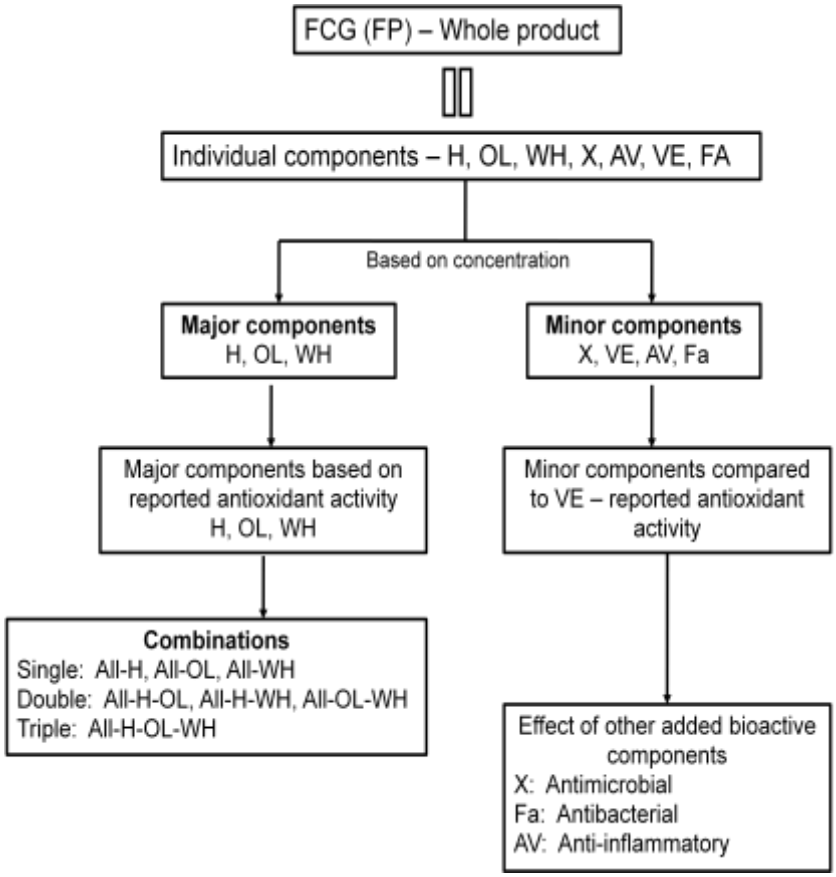


Figure 3.1: Summary of experiment procedure used for the determination of antioxidant content and activity.

3.3.1. ANTIOXIDANT CONTENT

3.3.1.1. Total polyphenolic content (TPC)

Total polyphenolic content (TPC) was determined using the Folin-Ciocalteu (F-C) method as described by Serem and Bester (2012) which has been modified for a 96 well format. Gallic acid (0 - 20 mg/ml) (see Figure 4.1.1.A) was used to prepare a standard curve. A 20 µl volume of each sample was added to each well of a 96 well microplate. To each well a 50 µl volume of F-C was then be added, followed by a 50 µl volume of a 7.5% sodium carbonate solution. The mixture was mixed well and the absorbance was read at 630 nm. TPC was expressed as mg gallic acid equivalents (GAE)/ml.

3.3.1.2. Total flavonoid content (TFC)

Total flavonoid content (TFC) was measured using a modified method as described by Serem and Bester (2012). Catechin (0 - 30 µg/ml) was used to prepare a standard curve. To 20 µl of each sample 30 µl of a 2.5% sodium nitrite, followed by a 20 µl of a 2.5% aluminum chloride and then 100 µl of a 2% NaOH solution was added. The mixture was mixed well and the absorbance was read at 450 nm using a BioTek plate reader. TFC was expressed as mg catechin equivalents (CE)/ml.

3.3.2. ANTIOXIDANT ACTIVITY

Antioxidant capacity can be classified (Zulueta *et al*., 2009) according to the reaction mechanism and there are basically two reaction types. The first type involves single electron transfer (SET) and the second type hydrogen atom transfer (HAT) (Huang *et al*., 2005). In the SET-based assays, the capacity of the antioxidant is measured via the reduction of an oxidant through a colour change when it is reduced (Zulueta *et al*., 2009). The sample's antioxidant concentration is directly linked to the degree of the colour change. In the greater part of the HAT assays, the antioxidant and substrate compete for the thermally generated peroxyl radicals through the decomposition of the azo-compound (Zulueta *et al*., 2009). The most popular SET and HAT methods used are the Trolox equivalent antioxidant capacity (TEAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and oxygen radical anti-oxidant capacity (ORAC) assays (Zulueta *et al*., 2009).

3.3.2.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay provides information on the reactivity of test compounds with a stable free radical. Because of its odd electron, DPPH gives a strong absorption at 517nm in visible spectroscopy (deep violet colour). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, resulting in decolorization (Ruso *et al*., 2004). ABTS is a stable nitrogen centered radical species. It is more versatile than DPPH, as both non-polar and polar samples can be assessed and spectral interference is minimized as the absorption maximum used is 760nm, a wavelength not encountered with natural products (Dastmalchi *et al*., 2007).

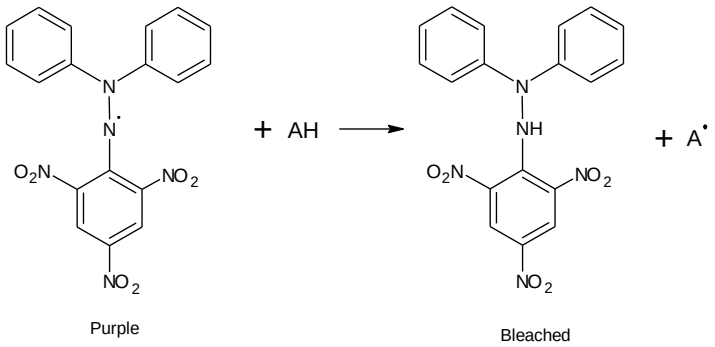


Figure 3.2: The DPPH radical reaction (Olajire *et al.,* 2011).

DPPH was carried out according to a modified method of Awika *et al*., (2003). Stock solutions of DPPH was prepared by dissolving 24 mg of DPPH in 100 ml methanol. The solution was then placed in a sonicator for 20 minutes. The working solution was then prepared by diluting 20 ml of stock solution with 80 ml methanol. Trolox (25 mg/ml) was used to prepare the standard curve. To 15 µl of each sample 285 µl of DPPH was added and then the microplate was then left to stand for 15 minutes in a dark place. To eliminate the possible effects of interference each sample served as its own control i.e. all components, no DPPH added. The plate was then read at 570 nm and the data was expressed as µM trolox equivalents (TE).

3.3.2.2. Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay is based on the scavenging of the ABTS·+ radicals cations by the anti-oxidant present in the sample. The maximum absorbance value of the ABTS·+ is 645nm, 734nm and 815nm and typically has a bluish-green colour (Re *et al*., 1993). The antioxidant compound in the sample captures the free radicals which translated into the reduction of the absorbance and the loss of colour, corresponding quantitatively to the concentration of the antioxidant present (Zulueta *et al*., 2009).

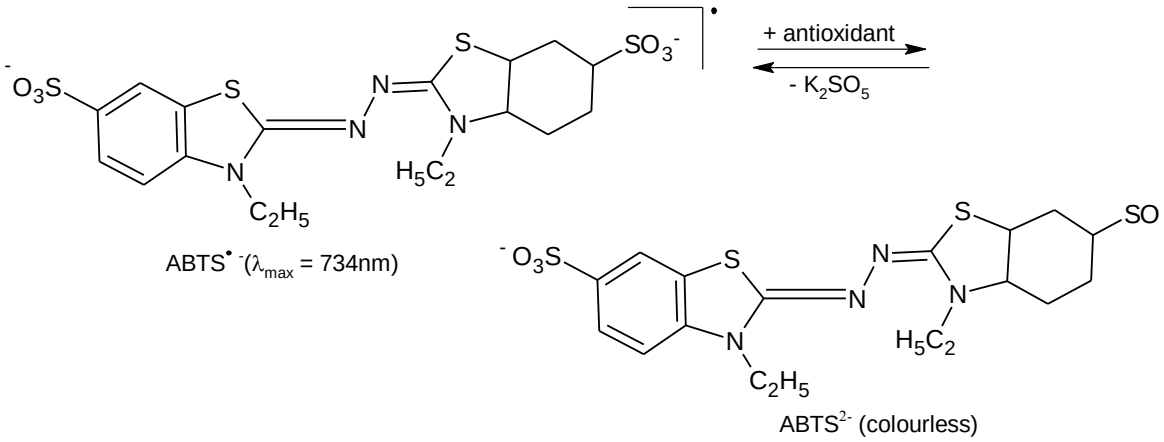


Figure 3.3: The reaction of the ABTS radical in the presence of the antioxidant compound during the ABTS assay (Zulueta *et al*., 2009).

The TEAC assay was done according to Awika *et al.*, (2003). The $ABTS^{\bullet+}$ was freshly generated by adding 3 mM of potassium peroxodisulfate ($K_2S_2O_8$) solution to 8 mM ABTS and the mixture was left to react in the dark for at least 12 hours at room temperature. The working solution was prepared by diluting ABTS stock solution with 0.2 M phosphate buffer, pH 7.4. Trolox was used as a standard, concentration range 0 - 1000 µM. A 2.9 ml volume of the working solution was added to 0.1 ml of each of the components at their different concentrations. The reaction mixtures was left to stand at room temperature and the absorbance readings were taken at 734 nm after 30 minutes for the samples and 15 minutes for the standards, using the Lambda EZ150 spectrophotometer. The results were expressed as µM TE.

3.3.2.3. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was initially developed by Cao *et al.*, 1993. The ORAC assay measures the decrease in the fluorescence of a protein. This is due to the loss of its confirmation when it suffers oxidative damage caused by a source of peroxyl radicals (Huang *et al*., 2005), namely those from 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) breakdown. The ability of the antioxidant to protect the protein against oxidative damage is measured. B-phycoerythrin was originally used as the protein but due to a series of disadvantages such as inconsistency between the batches and photosensitivity, the B-phycoerythrin has been replaced with fluorescein as the target molecule (Ou *et al*., 2001). These assays only measure the antioxidant potential of compounds and often this information is unrelated to the cellular effects.

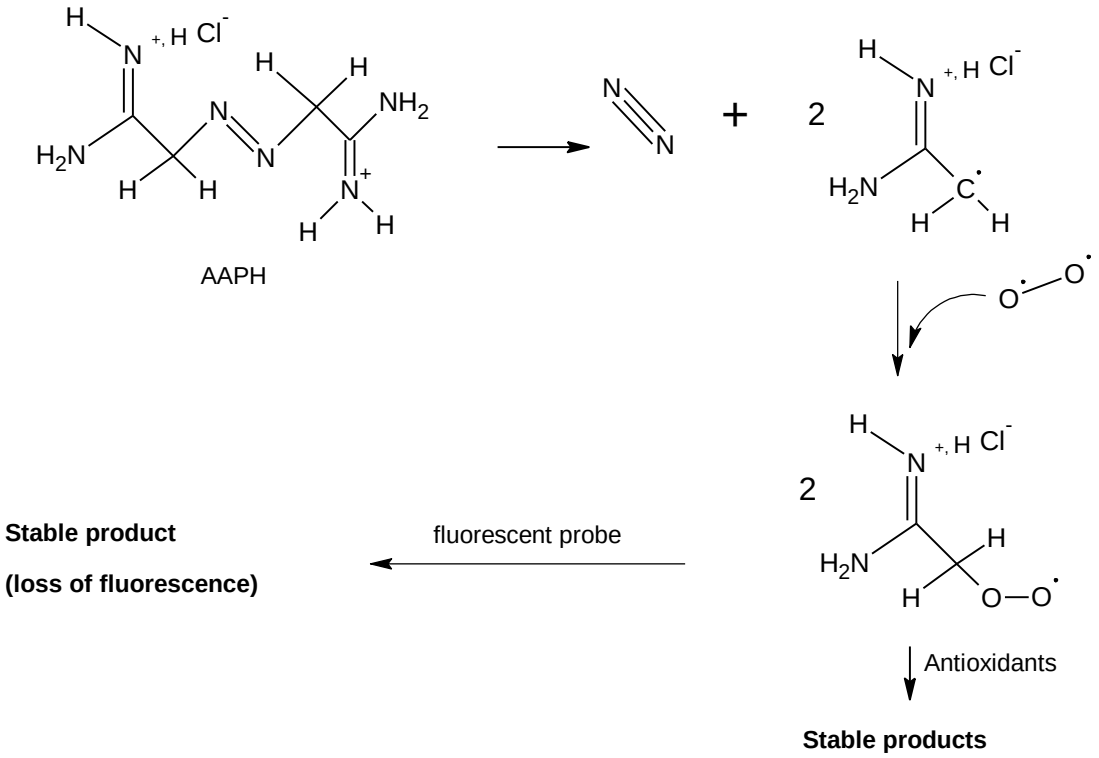


Figure 3.4: The reaction of the AAPH radical during the ORAC assay (Zulueta *et al*., 2009).

Procedures were based on a modified method of Ou *et al.*, (2002). AAPH was used as a peroxyl radical generator, trolox as standard (0 - 1000 µM) and fluorescein as the fluorescent probe. Phosphate buffered saline (PBS) was used as a blank. To 160 µl volume of 0.139 nM flourescein working solution, 40 µl of PBS, or trolox (serial dilution) or the prepared samples were added. This was followed by the addition of 40 µl of a 0.11 µM AAPH. Samples were mixed well and the microplate was placed into the plate reader and incubated at 37°C. The fluorescence was measured every 5 minutes for 4 hours. The assay protocol included: measurement start time of 0.0s, 10 flashes per cycle, 300 s cycle time, 485 nm for the excitation filter and 520 nm for the emission filter. The final ORAC values of the samples were calculated by using the net area under the decay curves (AUC). The data was expressed as µM TE.

3.3.3. CELLULAR ANTIOXIDANT PROTECTION

To investigate the cellular antioxidant effects the strategy presented in Figure 3.5 was used. The major difference between this strategy and that used for the determination of

antioxidant content and activity was that serial dilutions was used and the effect of VE was investigated in greater detail.

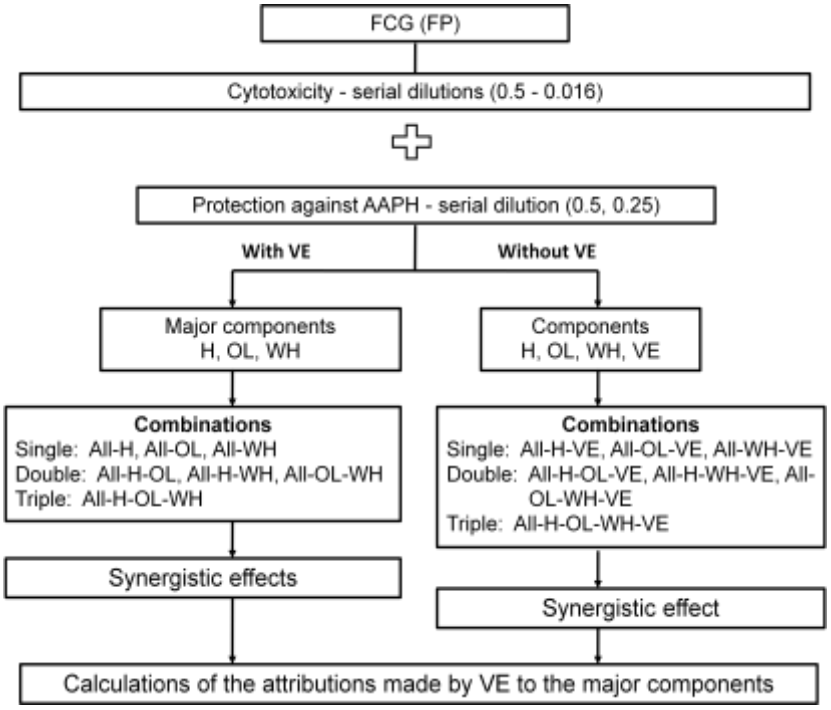


Figure 3.5: Summary of method used in cellular studies.

To investigate short term cytotoxicity and cellular protection aginst oxidative damage the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay was used. DCFH-DA is a non-fluorescent dye which can freely cross the cell membranes. In the cell through the action of the cellular esterases the diacetate group is removed. DCFH which is non-fluorescent is retained within the cell. With oxidative damage non fluorescent DCFH becomes fluorescent DCF. (http://www.biocompare.com). For instance, Song, *et al*., (2008) used this assay to assist in the determination of the effect of caffeic acid on skin-incised mice in the wound healing processes. In 2002, McCune and Johns used the DCFH-DA assay to determine the antioxidant activity in medicinal plants associated with the symptoms of diabetes mellitus used by the Indigenous Peoples of the North American boreal forest (McCune and Johns, 2002).

Likewise Serem and Bester (2012) used this DCFH-DA to determine the ability of 13 different honey samples to protect the SC-1 mouse fibroblasts and the Caco-2 colon adenocarcinoma cell lines against AAPH-induced oxidative damage. At a 2.5%

concentration in the SC-1 cell line 12/13 samples and 7/13 showed significant total and intracellular protection, respectively. In the Caco-2 cell line the effects were different to that found in the SC-1 cell line. At a 2.5% concentration, 11/13 samples showed total protection and this ranged from 100% to 30%. Overall, the Caco-2 cell line was more sensitive to the toxic effects AAPH than the SC-1 cell line. However in this study the SC-1 cell line was used as it is representative of a cell type name fibroblasts found in wound sites. Cytotoxicity and total cellular protective effects was determined for all samples.

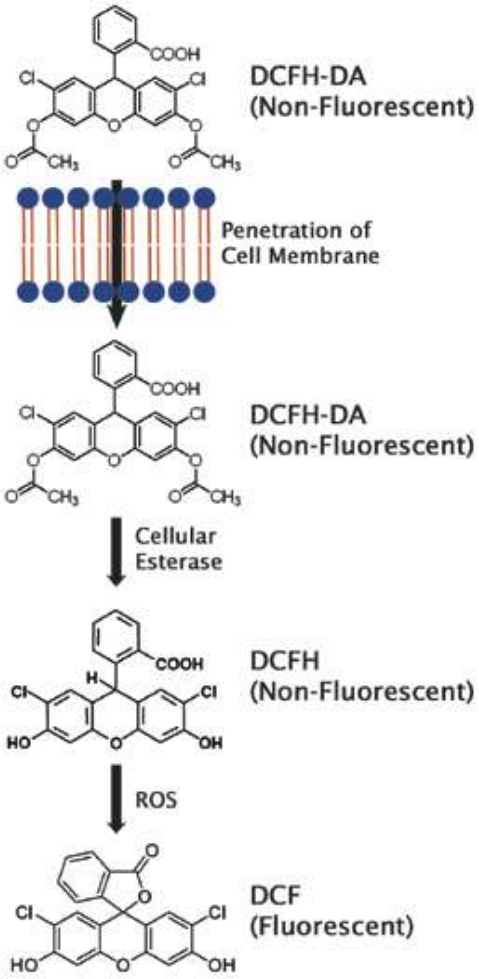


Figure 3.6: Overview of DCFH reaction (www.cellbiolabs.com).

The SC-1 cell line was maintained in EMEM supplemented with 10% fetal calf serum (FCS) and a 1% antibiotic solution containing streptomycin, penicillin and fungicide. SC-1 cells were plated at a cell concentration of $2x10^4$ cells per 100 µl in either 96 of 48 well flat bottom plates and were kept for 24 hours at 37°C and 5% CO_2 to allow cells to

attach to well surfaces before the cytotoxicity of cellular protection effects was determined.

3.3.3.1. Short term cytotoxicity related to oxidative damage

For the measurement of short term cytotoxicity as summarized in Table 3.6A, a 20 µM DCFH-DA solution was prepared as follows: a 1 mg/10 ml, 200 µM stock solution was prepared, from which a 20 µM working solution was prepared. A 40 µl volume of the DCFH-DA solution was added to each well containing cells and were incubated at 37^0C for 1 hour. The DCFH-DA was then removed and the wells were washed twice with PBS and then 40 µl of each sample was added. Immediately fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The change in fluorescence from 0 - 60 minutes was measured every 2 minutes using a FLUOstar OPTIMA plate reader. The gradient of the change in fluorescence was calculated, and the data were expressed as % damage where AAPH alone causes 100% damage.

3.3.3.2. Total protective effects

Total protective effects were evaluated as shown in Table 3.6B. For this 20 µM solution of DCFH-DA was prepared as described above. Volumes of 40 µl DCFH-DA solution was added to each well and cell culture plates were maintained for a further 1 hour at 37°C. The medium containing the DCFH-DA solution was carefully removed. Cell culture plates were then washed once with PBS, and plates were then be blotted dry. A volume of 40 µl each sample was then be added to each well of the cell culture plates followed by a 15 mM, 40 µl volume of AAPH, the AAPH having a final concentration of 7.5 mM. Change in fluorescence was measured immediately over 0 - 60 minutes, every 2 minutes. The gradient of the change in fluorescence was calculated, and the data were expressed as % damage where AAPH alone causes 100% damage.

Table 3.6: Testing strategy for the oxidant and antioxidant effects in SC-1 cell line

A. Cytotoxicity			
Cells + DCFH-DA	Uptake, 1 hour →	Add samples or AAPH	Immediately measure change in fluorescence, 1h

B. Total antioxidant protection

Cells + DCFH-DA	Uptake, 1 hour →	Add samples +AAPH	Immediately measure change in fluorescence, 1h

3.3.4. Synergistic effect calculation

The theoretical scavenging activity/expected (E) was calculated as the sum of the scavenging activity of the individual components. The experiment scavenging activity is the experimental/observed (O), of ALL COMP - ALL COMP without OL = contribution of OL to total antioxidant activity. This provides the expected effects of OL. The synergistic effect was calculated as Synergism = O/E > 1. For an antagonistic effect, antagonism = O/E < 1. Where no effect occurred, E = O. This has been refined taking into consideration biological variations as 0.90 > x < 1.10 as additive, x > 1.10 as synergism and x < 0.90 as antagonism.

3.3.5. Data management and statistical analysis

All data is an average of three experiments and each measurement was done at least in triplicate, thereby generating 9 data points. The results are expressed as mean ± standard error of mean (SEM) of three experiments where each experiment point is the average of 3 assays.

Comparisons between groups data were statistically evaluated using analysis of variance (ANOVA), using samples as independent variables and the values determined as dependent variables. Fisher's least significant difference (LSD) test was used for comparison of means using Statistica software Version 9.0 (StatSoft, Tulsa, OK).

CHAPTER 4: RESULTS

The purpose of this study is to determine the antioxidant content and activity as well as the cellular effects of the bioactive components of FCG (FP). The synergic, additive and/or inhibitory effects between components were also evaluated.

FCG consists of a complex mixture of bioactive components. In scientific literature, usually the interaction of only two or three major components (Lila, 2009; Wang *et al.,* 2011) are considered whereas a lot more information could be gathered by studying the effects of all components. In a complex mixture, different components may have no effect, i.e. effect or may interact with each other antagonistically or synergistically.

In this study, the antioxidant content and activity of the whole product Flavonix® Cytoflamm Gel (final product) ((FCG (FP)), as used commercially for wound healing, was evaluated. This was then followed by the evaluation of each component at the concentration as found in the FCG formulation. This was compared to ALL COMP, the sum of each bioactive component. These seven components were honey (H), oleuropein (OL), witch hazel (WH), xylitol (X), farnesol (Fa), aloe vera (AV) and vitamin E (VE). These individual components were then divided into two groups based on concentrations in FCG (FP) (i) major components, concentration > 5% and reported antioxidant activity and these were H, OL, WH and (ii) minor components, concentration < 5% and these were, VE, AV, Fa and X.

To investigate the interaction between the major antioxidant components, based on the antioxidant activity of single components or activity as reported in the scientific literature but at the same time retaining the integrity of the product, the following strategy was used. FCG (FP) minus glycerin, citric acid and natrasol was prepared and throughout this study was known as ALL COMP which is a solution at the same concentrations in FGC (FP) containing H, OL, WH, VE, AV, X and Fa. Solutions were prepared without each of the major antioxidant bioactive components (All-H, All-OL and All-WH), without two components (All-H-OL, All-O-WH, All-H-WH) and then without all three components (All-H-WH-OL) (Figure 4). Likewise solutions of ALL COMP were prepared without the minor antioxidant bioactive components and these were single (All-VE), two (All-VE-Fa, All-VE-AV and All-VE-X), three (All-VE-X-AV, All-VE-Fa-X and All-VE-Fa-AV) and four

components (All-VE-AV-Fa-X). Minor effects were determined relative to VE known antioxidant although only present at 0.1%.

From the data generated the contribution of each component to the antioxidant content (TPC and TFC), antioxidant activity (DPPH, TEAC and ORAC) as well as cellular effects (DCFH-DA), could be determined.

4.1. ANTIOXIDANT CONTENT

4.1.1. Total polyphenolic content (TPC)

4.1.1.1. Standard curve and serial dilutions

Polyphenolics such as that present in H (Figure) and OL (Figure 2.5) are a major group of compounds that contribute to antioxidant activity and TPC was determined with the Folin–Ciocalteu assay. A standard curve of 0 – 20 mg/ml gallic acid was prepared with a $R^2 = 0.994$ (Figure 4.1.1.A).

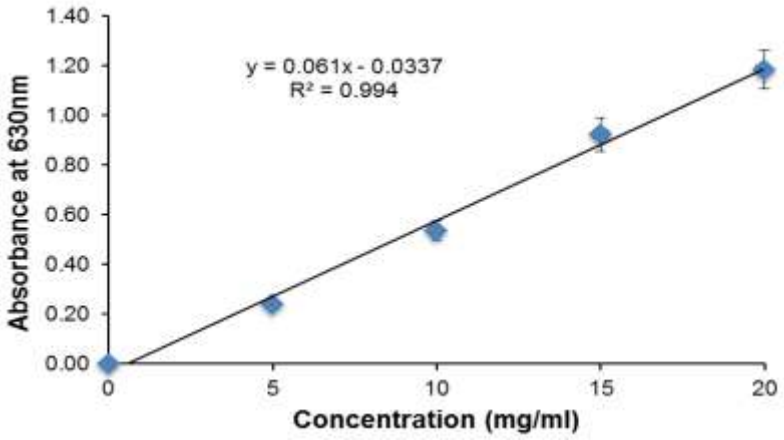


Figure 4.1.1.A: Standard curve for TPC determination with the Folin–Ciocalteu assay. Data expressed as gallic acid equivalent (GAE). Data is an average of three experiments ± SEM.

To determine the polyphenolic content of FCG(FP) a 0.50 – 0.01 times serial dilution was prepared. With increasing dilutions a linear decrease in TPC was obtained with $R^2 = 0.9769$ (Figure 4.1.1.B). From the line equation, it can be calculated that the TPC content of FCG is equivalent to 9.875 mg GAE/ml. This implies that FCG contains significant amounts of polyphenolics, which may translate into significant levels of antioxidant activity.

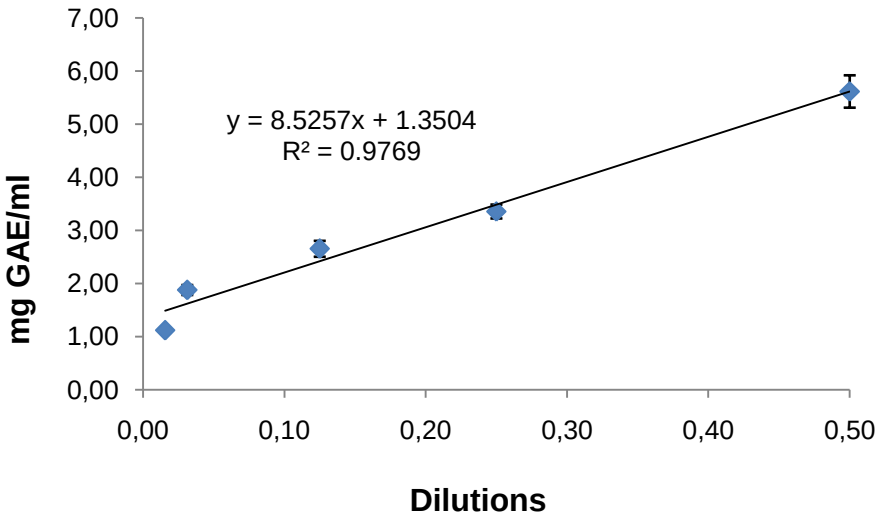


Figure 4.1.1.B: TPC content of serial dilutions of FCG was determined and expressed as mg GAE/ml. Data is an average of three experiments ± SEM.

4.1.1.2. Individual components

The TPC of each individual component at the concentrations found in FCG (FP) was evaluated. Major components were evaluated at 1x and 0.1x the concentrations found in FCG (FP) while minor components were evaluated at 10x concentrations found in FCG (FP). Of the major components, H and OL showed the highest polyphenolic content with 6.957 and 7.845 mg GAE/ml respectively. WH had a low TPC value of 0.605 mg GAE/ml. Minor components X, VE, AV and Fa were found to have minimal TPC content of 0.574, 0.587, 0.598 and 1.234 mg GAE/ml respectively. Therefore the major components which contribute to TPC in FCG (FP) are H and OL (Figure 4.1.1.C). The sum of TPC at concentrations in FCG (FP) was calculated (CALC) and was found to be significantly more than that found in FCG (FP) indicating that the non-active components such as citric acid, trisodium citrate, glycerin and natrasol may interfere with F-C reaction.

In summary, the individual components in FCG (FP) with highest TPC was H and OL.

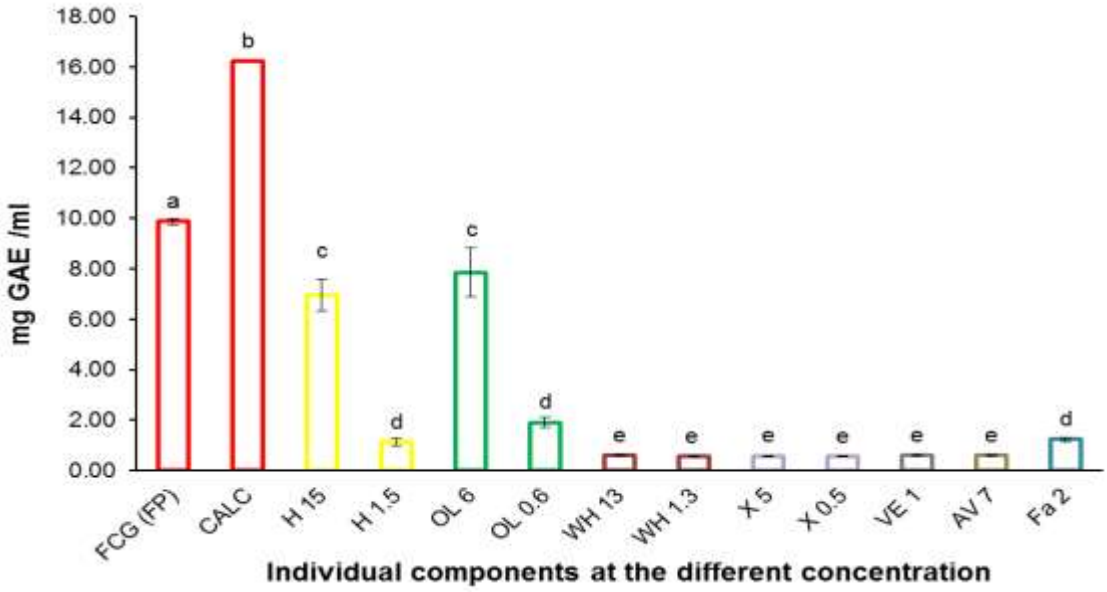


Figure 4.1.1.C: TPC of each individual component (major components at 1x and 0.1x concentrations and minor components at 10x concentrations) was expressed as mg GAE/ml. Data is an average of three experiments ± SEM. Means of individual components with different letters are significantly different.

4.1.1.3. Major components

The effect of antioxidant components both major and minor were evaluated as described earlier in this chapter and the data is presented in Figure 4.1.1.D for the major components and for the minor components in Figure 4.1.1.E, respectively.

All samples for major and minor components were evaluated at 7 x less than for the individual components (Figure 4.1.1.D and Figure 4.1.1.E). Removal of H and OL results in a significant decrease in TPC from 2.596 to 1.368 mg GAE/ml and 2.596 to 1.630 mg GAE/ml for H and OL respectively. Removal of both decreases TPC from 2.596 mg GAE/ml to 0.154 mg GAE/ml. WH does not contribute significantly to TPC when compared to ALL COMP. Both H and OL (All-H-OL) contributes 84.08 % of TPC.

Evaluation of all major components, OL, H and WH reveals that only OL and H contributes significantly to TPC whereas WH has no significant effect. This confirms the finding when individual components were evaluated.

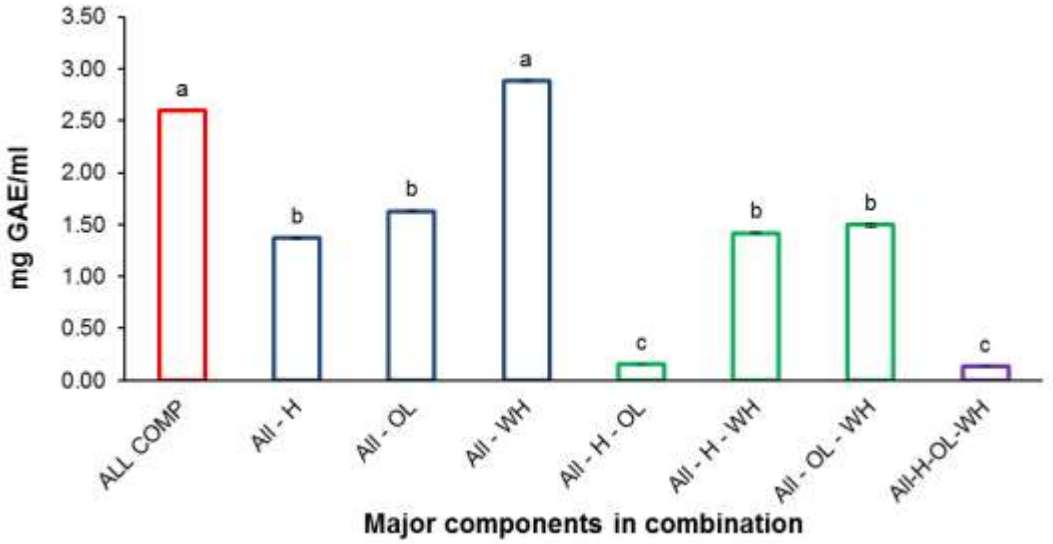


Figure 4.1.1.D: The contribution of the major components to the TPC of FCG (FP) expressed as mg GAE/ml. The effect of single components (All-H, All-OL and All-WH), two components (All-H-OL, All-H-WH and All-OL-WH) and three components (All-H-OL-WH) was evaluated. Data is an average of three experiments ± SEM. Means of individual components with different letters are significantly different.

4.1.1.4. Minor components

Using the same strategy as for the major components, the effect of the minor components was determined. Although low concentrations are present in FCG (FP) some of these components have been reported to have significant antioxidant activity (Chapter 2). Biologically the most relevant is VE. Based on this all effects were evaluated relative to VE. In the absence of VE, an increase in TPC is seen for All-VE-Fa, All-X-VE and All-Fa-VE-AV. Components X, AV and Fa have no significant effect on TPC. Components VE and Fa are not polyphenolic, therefore possible reasons for the increase in TPC will be discussed in Chapter 5.

With the removal of minor components VE, Fa, X and AV a small but significant increase in TPC compared to ALL COMP was measured and this, although significant, only accounts for 14.56% of TPC content.

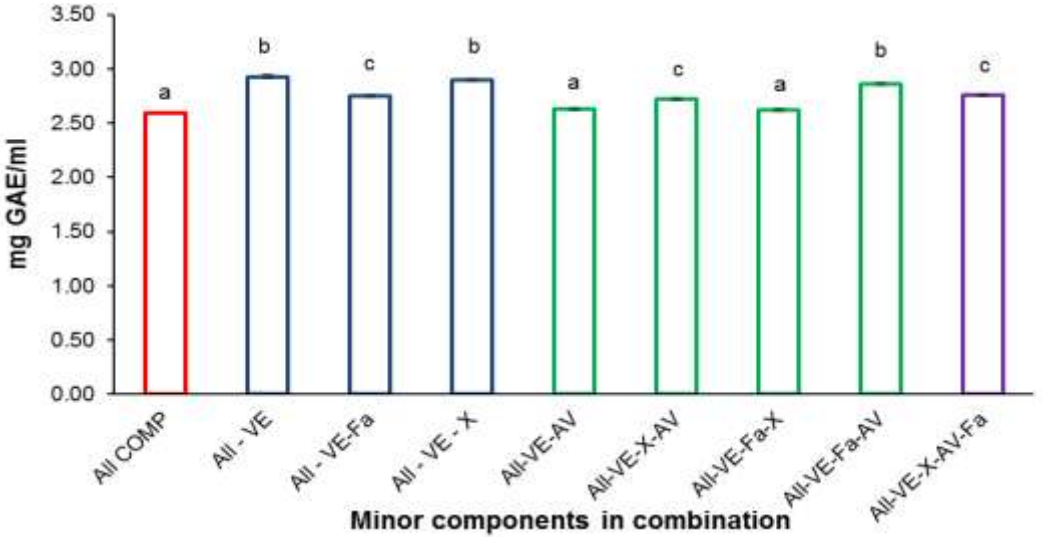


Figure 4.1.1.E: The contribution of the minor components to the TPC of FCG (FP) expressed as mg GAE/ml. The effect relative to VE relative for Fa, X and AV was evaluated. Data is an average of three experiments ± SEM (< 1%). Means of individual components with different letters are significantly different.

4.1.1.5. Summary

The contribution of H, OL, X, Fa, WH, AV and VE to the TPC of FCG (FP) was calculated and was found to be 45.89%, 38.19%, 8.82%, 4.70%, 1.36%, 1.04% and 0% respectively, with major components H, OL and WH (although the effect of WH being minimal) contributing 85.44% of TPC.

4.1.2. Total flavonoid content (TFC)

4.1.2.1. Standard curve and serial dilutions

TFC was measured with an aluminum chloride colorimetric assay. A standard curve was prepared from 0 – 30 µg/ml with a $R^2 = 0.999$ (Figure 4.1.2.A).

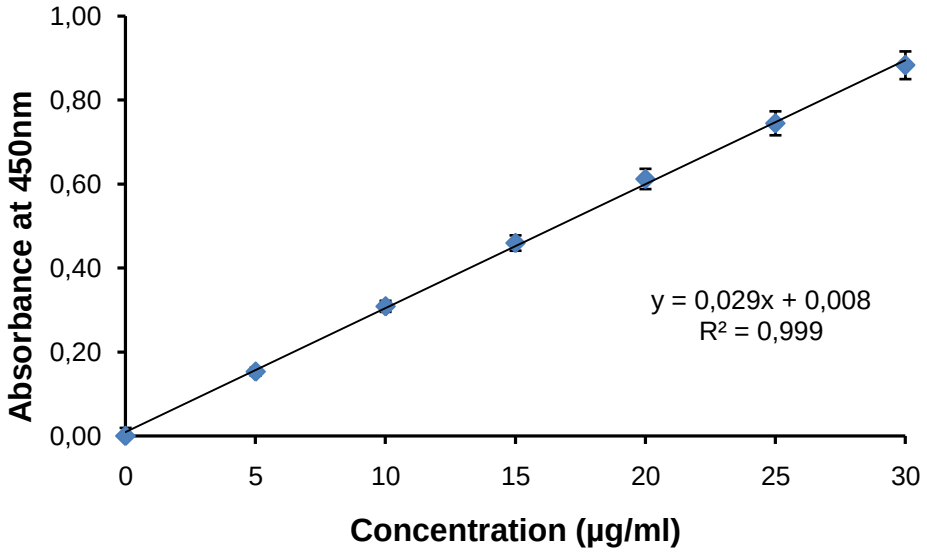


Figure 4.1.2.A: Standard curve for TFC determination with the aluminum chloride colorimetric assay. Data expressed as catechin equivalents µg CE/ml. Data is an average of three experiments ± SEM.

A serial dilution of 0.50 – 0.01 of FCG (FP) was prepared and a linear equation was obtained with R^2 = 0.994 (Figure 4.1.2.B). From the line equation, it can be calculated that FCG (FP) is equivalent to 9.148 µg CE/ml. The serial dilution indicates that FCG (FP) contains significant flavonoid content implying associated antioxidant activity.

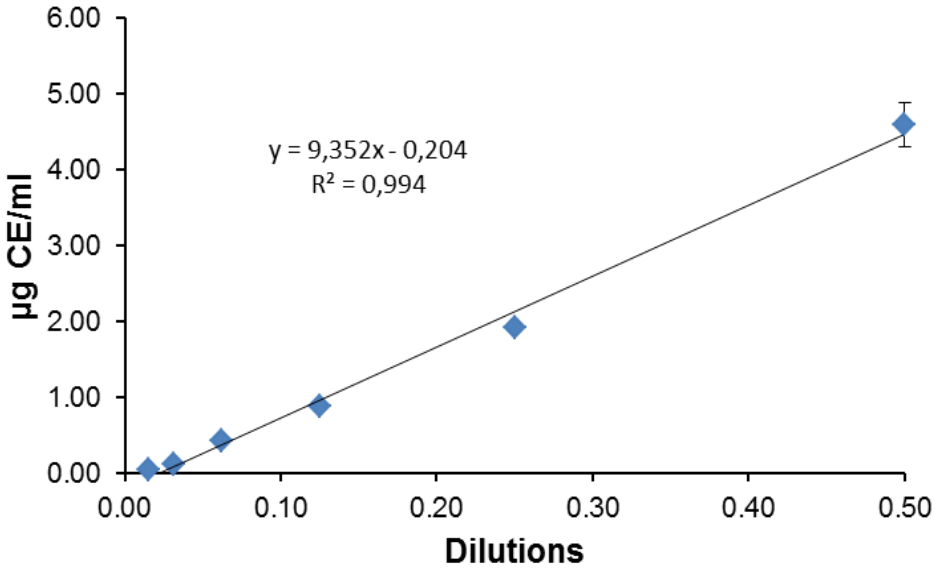


Figure 4.1.2.B: TFC content of serial dilutions of FCG (FP) was determined and expressed as µg CE/ml. Data is an average of three experiments ± SEM.

4.1.2.2. Individual components

Similar to TPC, the TFC of individual components was determined. The TFC of H, OL and Fa was 0.836, 3.912 and 1.356 µg CE/ml, respectively. The contribution of WH, X, VE and AV in TFC to FCG (FP) was minimal. Therefore, the major components which contribute to TFC are components H and OL (Figure 4.1.1.C). The sum of TFC at concentrations found in FCG (FP) was calculated (CALC) and was found to be significantly less than that found in FCG (FP) indicating possible interference of non-bioactive FCG (FP) in the TFC such as was found for TPC.

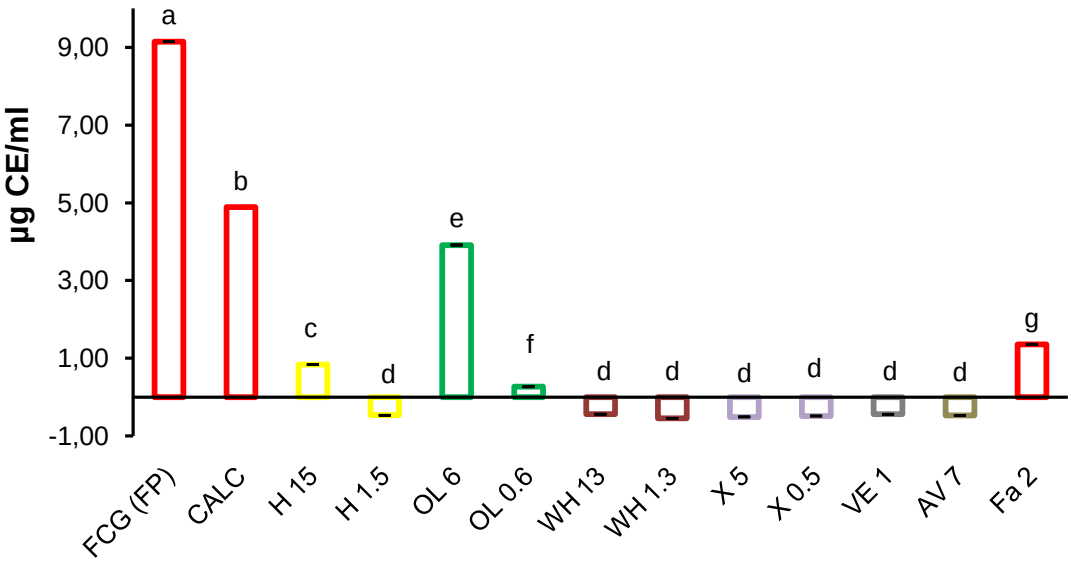


Figure 4.1.2.C: TFC of each individual component (major components at 1x and 0.1x concentrations and minor components at 10x concentrations) was expressed as µg CE/ml. Data is an average of three experiments ± SEM. Means of individual components with different letters are significantly different.

4.1.2.3. Major components

The contribution of each the major components to TFC was evaluated as described for TPC and these results are presented in Figure 4.1.2.D. The components OL and H contributed 0.91 and 0.562 mg CE/ml to TFC and in combination contributes to 81.70 % of total TFC compared to ALL COMP. WH also did contribute to measured TFP with a contribution of 6.86%.

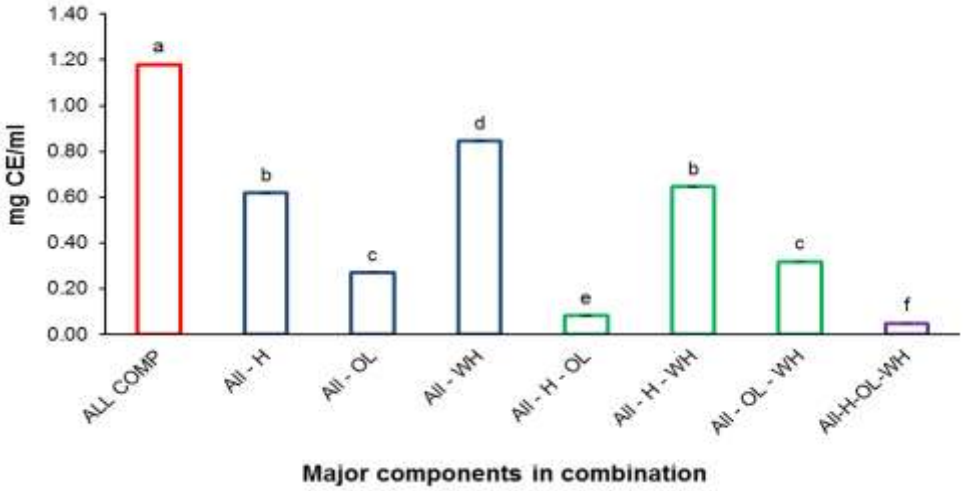


Figure 4.1.2.D: The contribution of the major components to the TFC of FCG (FP) expressed as mg CE/ml. The effect of single components (All-H, All-OL and All-WH), two components (All-H-OL, All-H-WH and A-OL-WH) and three components (All-H-OL-WH) was evaluated. Data is an average of three experiments ± SEM. Means of individual components with different letters are significantly different.

4.1.2.4. Minor components

The contribution of the minor components to the TFC of FCG (FP) was further evaluated. All-VE differed significantly from ALL COMP. The absence of X and AV did not have an effect on TFC. The removal of Fa from All-VE-Fa, All-VE-Fa-X and All-VE-Fa-X-AV caused a significant decrease in TFC and this may be due to the presence of Fa, an acrylic sesquiterpene alcohol that does give a positive reaction with the TFC method as seen in Figure 4.2.1.C.

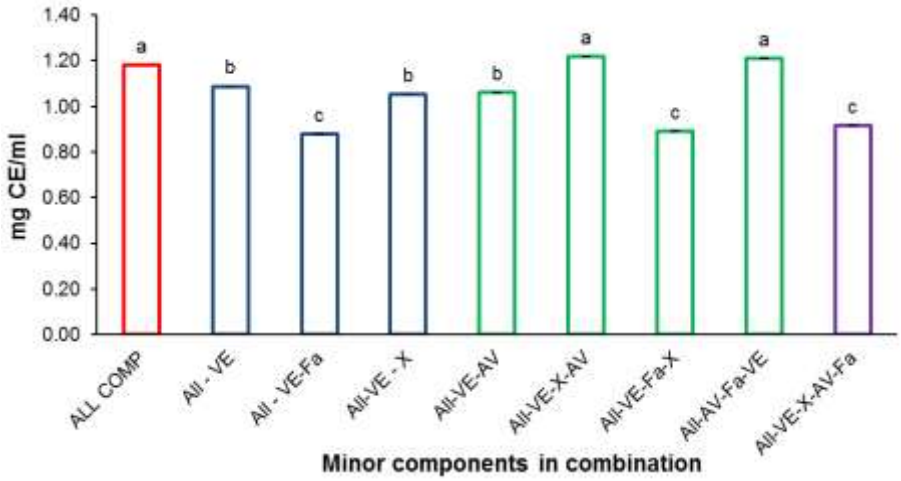


Figure 4.1.2.E: The contribution of minor components to TFC of FCG (FP) expressed as mg CE/ml. The effect relative to VE relative to Fa, X and AV was evaluated. Data is an average of three experiments ± SEM. Means of individual components with different letters are significantly different.

4.1.2.5. Summary

Contribution of all components to TFC relative to FCG (FP) are OL, H, WH, VE, AV, X and Fa with 47.70%, 32.84%, 10.94%, 3.91%, 2.37%, 2.24% and 0% respectively, with the major components H, OL and WH contributing 91.48% of TFC.

Table 4.1: Summary of contribution of individual components to TPC and TFC content

Components	TPC (%)	TFC (%)
Major components		
H	45.89	32.84
OL	38.19	47.70
WH	1.36	10.94
TOTAL	**85.44**	**91.48**
Minor components		
VE	0.00	3.91
X	8.82	2.24
AV	1.04	2.37
Fa	4.70	0.00
TOTAL	**14.56**	**8.52**

4.2. ANTIOXIDANT ACTIVITY

4.2.1. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

4.2.1.1. Standard curve and serial dilutions

The DPPH assay was done according to the modifications of Serem and Bester (2012). A standard curve of 0 – 800 µM TE with R^2 = 0.9972 (Figure 4.2.1.A) was prepared.

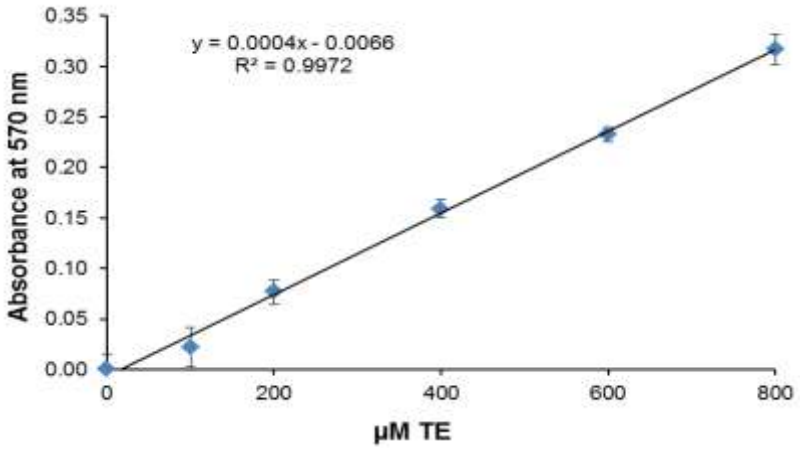


Figure 4.2.1.A: Standard curve for antioxidant activity determined with the DPPH assay. Data expressed as µM trolox equivalents (TE). Data is an average of three experiments ± SEM.

A 0.50 – 0.01 times serial dilution of FCG (FP) was prepared and with increasing dilutions a linear decrease in µM TE was obtained with R^2 = 0.982 (Figure 4.2.1.B). From the line equation, it can be calculated that the antioxidant activity of FCG (FP) is equivalent to 1533.47 µM TE. This implies that FCG (FP) has a significant amount of antioxidant activity measured with the DPPH assay. The gradient does not go through zero since the lowest concentration of 0.05 still showed significant levels of antioxidant activity.

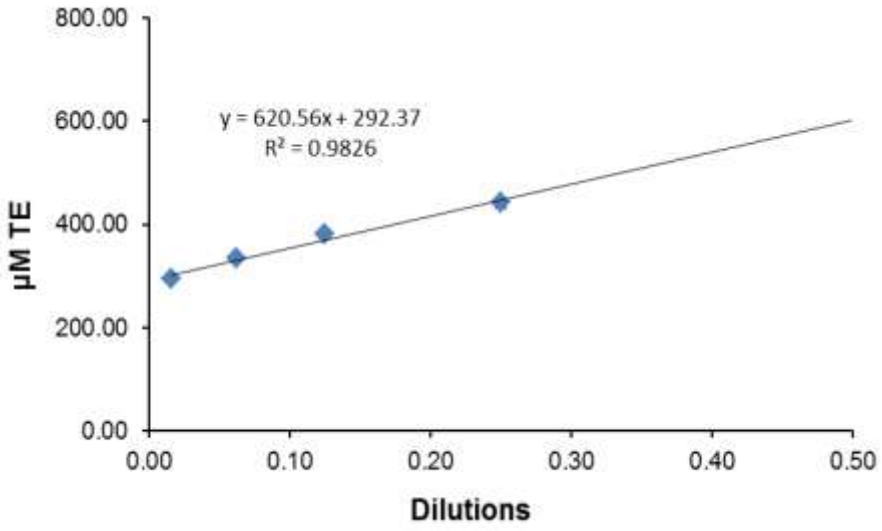


Figure 4.2.1.B: Antioxidant activity, DPPH assay of serial dilutions of FCG (FP) was determined and was expressed as µM TE. Data is an average of three experiments ± SEM.

4.2.1.2. Individual components

Similar to TPC and TFC antioxidant activity, DPPH was determined for each individual component. From Figure 4.2.1.C, The antioxidant activity for H and OL was 213.72 and 780.62 µM TE respectively. But no significant antioxidant activity was found for WH, X, VE, AV and Fa with values 1.92, 11.22, 2.89, 13.86 and 10.81 µM TE, respectively. Therefore, the major components which contribute to antioxidant activity, using the DPPH assay in FCG (FP) are H and OL (Figure 4.2.1.C). There is a significant difference between FCG (FP) and CALC with CALC greater than FCG (FP). Possible antagonistic effects due to the presence of other added compounds to FCG(FP) may account for this effect and this will be discussed further in Chapter 5.

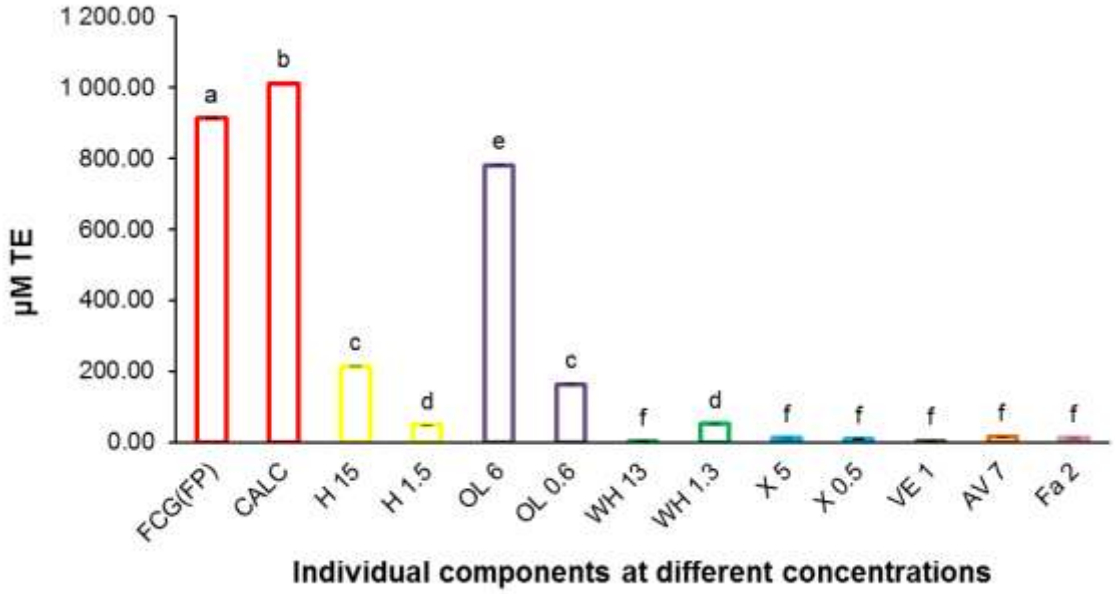


Figure 4.2.1.C: Antioxidant activity, DPPH of each individual component (major components at 1x and 0.1x concentrations and minor components at 10x concentrations), relative to FCG (FP) expressed as µM TE. Data is an average of three experiments ± SEM. Means of individual components with different letters are significantly difference.

4.2.1.3. Major components

The contribution of the major components was firstly evaluated with the DPPH assay (Figure 4.2.1.D). Compared to ALL COMP, All-H and All-OL with 139.67 and 89.00 µM TE contributed significantly to total antioxidant activity. In combination All-H-OL contributed 63.81% activity of the total antioxidant activity found in ALL COMP. The component WH did not contribute significantly to the measured antioxidant activity. Remaining activity was due to VE and Fa, see Section 4.1.3.4.

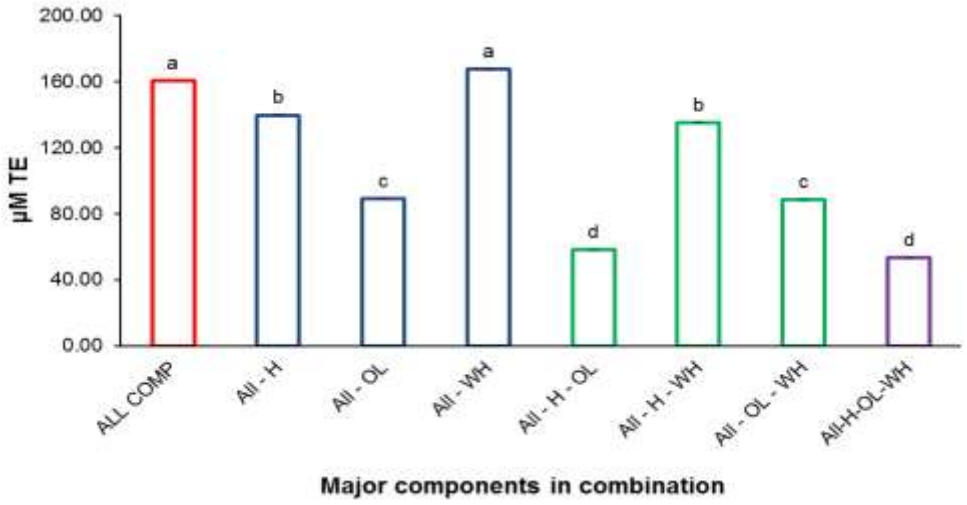


Figure 4.2.1.D: The contribution of the major components to the antioxidant activity measured with the DPPH assay relative to ALL COMP expressed as TE. The effect of single components (All-H, All-OL and All-WH), two components (All-H-OL, All-H-WH and All-OL-WH) and three components (All-H-OL-WH) was evaluated. Data is an average of three experiments ± SEM. Means of individual components, different letters show significantly differences.

4.1.3.4. Minor components

Similar to TPC and TFC to determine the antioxidant activity, the same experimental design was followed. In the absence of VE, the antioxidant activity was significantly less than ALL COMP. All-VE-Fa also showed a significant difference compared to ALL COMP. However the effect of Fa was not observed for any of the other combinations.

Evaluation of all components reveals that of the major component that contributes to antioxidant activity measured with the DPPH assay is OL and then H. Minor components VE and interestingly Fa, not a known did in some instances show antioxidant activity.

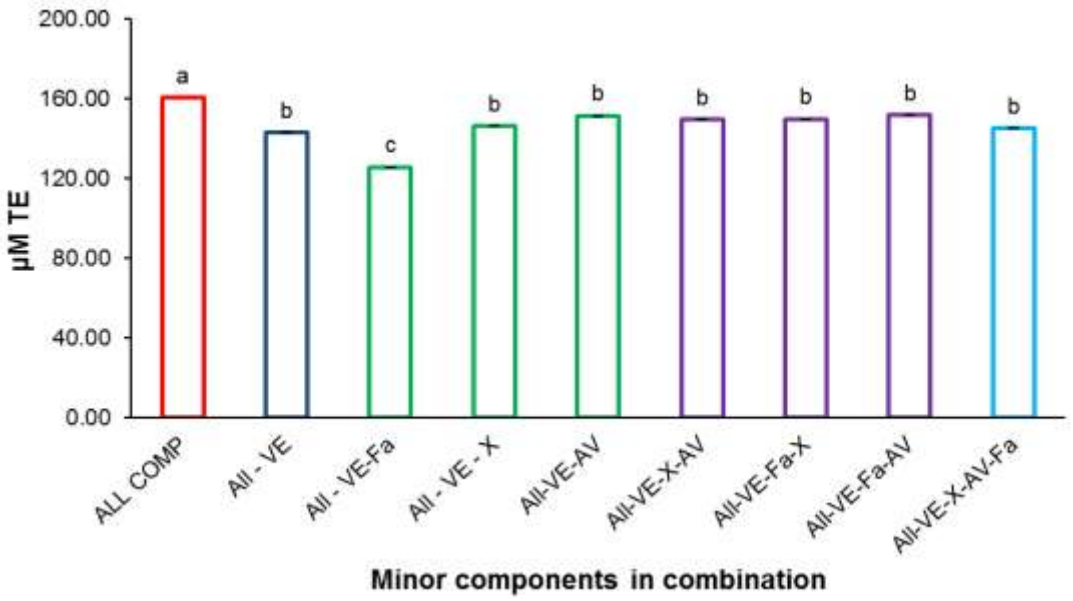


Figure 4.2.1.E: The contribution of the minor components to antioxidant activity, DPPH assay relative to ALL COMP, expressed as µM TE. The effect relative to VE relative to Fa, X and AV was evaluated. Data is an average of three experiments ± SEM. Means of individual components with different letters are significantly different.

4.1.3.5. Summary

Contribution to antioxidant activity in the DPPH assay of all components VE, OL, H, X, Fa, AV and WH are responsible for 44.56%, 32.86%, 11.63%, 6.55%, 4.17%, 0.24% and 0% respectively. Differences between FCG (FP) and CALC, with FCG (FP) < CALC indicate a possible antagonistic effect or inhibition of DPPH reacting with components of FCG (FP). Major components H, OL and WH contribute 44.49% of total activity and although a minor component VE contributes 44.56% of measured activity, indicating possible synergism between components.

4.2.2. Trolox equivalent antioxidant capacity (TEAC)

4.2.2.1. Standard curve and serial dilutions

The TEAC assay was done according to the Arnae *et al*. (2001) method. A standard curve was prepared from 0 – 800 µM TE with R^2 = 0.989 (Figure 4.2.2.A).

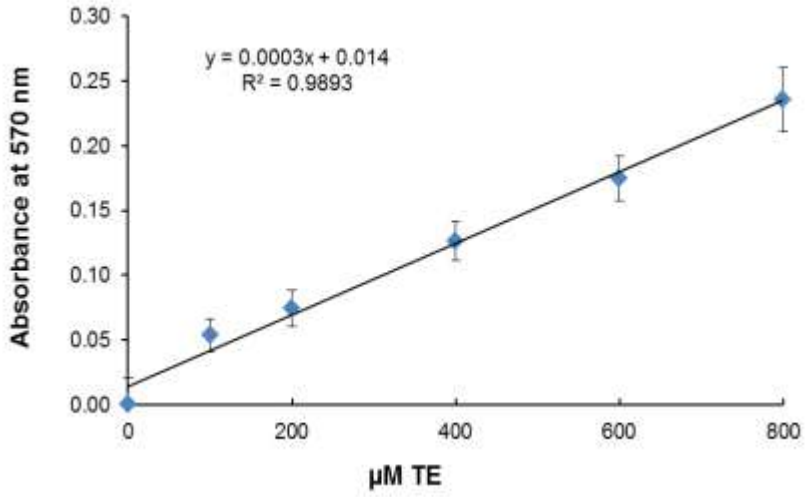


Figure 4.2.2.A: Standard curve for antioxidant activity determined with the TEAC assay. Data expressed as µM trolox equivalents (TE). Data is an average of three experiments ± SEM.

A 0.25 – 0.05 times serial dilution of FCG was prepared and with increased dilutions a linear decrease in TEAC was obtained with R^2 = 0.966 (Figure 4.2.2.B). From the line equation, it can be calculated that FCG (FP) is equivalent to 4673.80 µM TE. This implies that FCG contains significant amounts of antioxidant activity measured with the TEAC assay. As for the DPPH assay, the line gradient does not go through zero since the lowest concentration of 0.05 show a significant level of antioxidant activity.

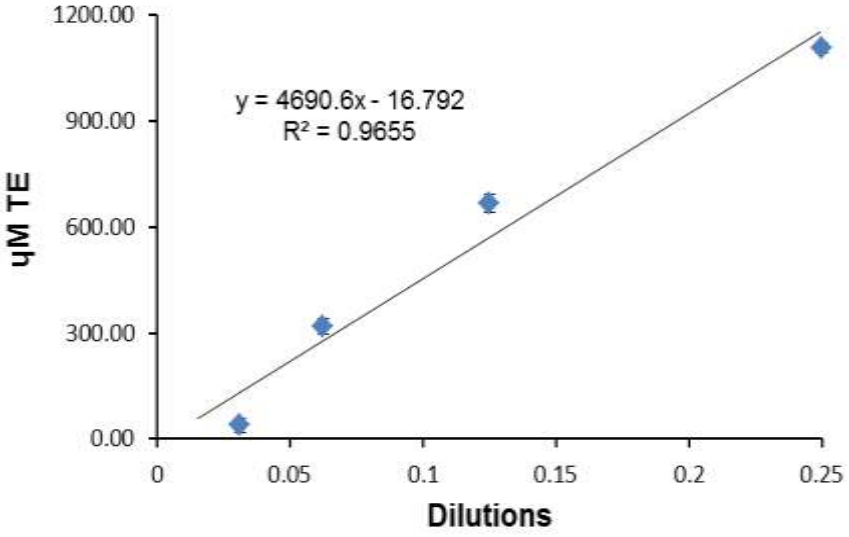


Figure 4.2.2.B: Antioxidant activity, TEAC assay of serial dilutions of FCG (FP) was determined and was expressed as µM TE. Data is an average of three experiments ± SEM.

4.2.2.2. Individual components

Similar to DPPH antioxidant activity, TEAC was determined for each individual component. From Figure 4.2.2.C, it is observed that H and OL contained 897.59 and 1376.85 µM TE respectively. But no significant antioxidant activity in TEAC was found for WH, X, VE, AV and Fa with values 32.59, 8.15, 18.33, 84.07 and 84.07 µM TE, respectively. Therefore, the major components which contribute to antioxidant activity measured with the TEAC assay in FCG (FP) are H and OL (Figure 4.2.2.C). There is no significant difference between FCG (FP) and CALC.

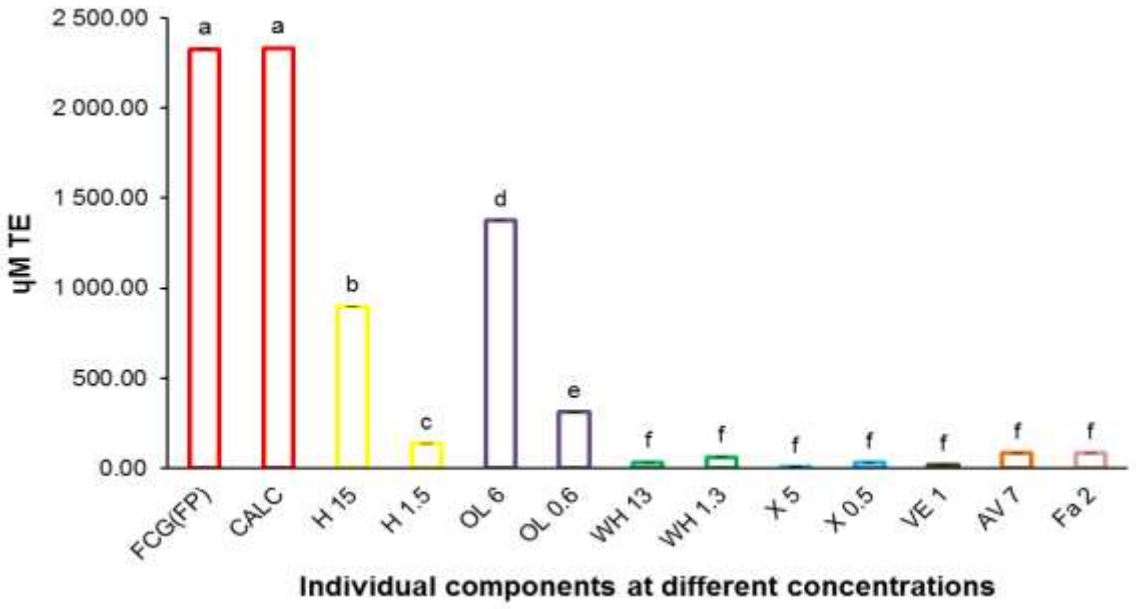


Figure 4.2.2.C: Antioxidant activity, TEAC of each individual component (major components at 1x and 0.1x concentrations and minor components at 10x concentrations) relative to FCG (FP) expressed as µM TE. Data is an average of three experiments ± SEM. Means of individual components with different letters are significantly different.

4.2.2.3. Major components

The contribution of the major components was evaluated with the TEAC assay (Figure 4.2.1.D). Compared to ALL COMP, All-H, All-OL and All-WH with 272.40, 254.35 and 271.56 µM TE respectively contributed significantly to total antioxidant activity. In combination All-H-OL contributed to 109.50% activity of the total antioxidant activity found in ALL COMP. Although WH (All-WH) contributes 271.56 µM TE to the total antioxidant activity when combined with H and OL no significant antioxidant effects was observed.

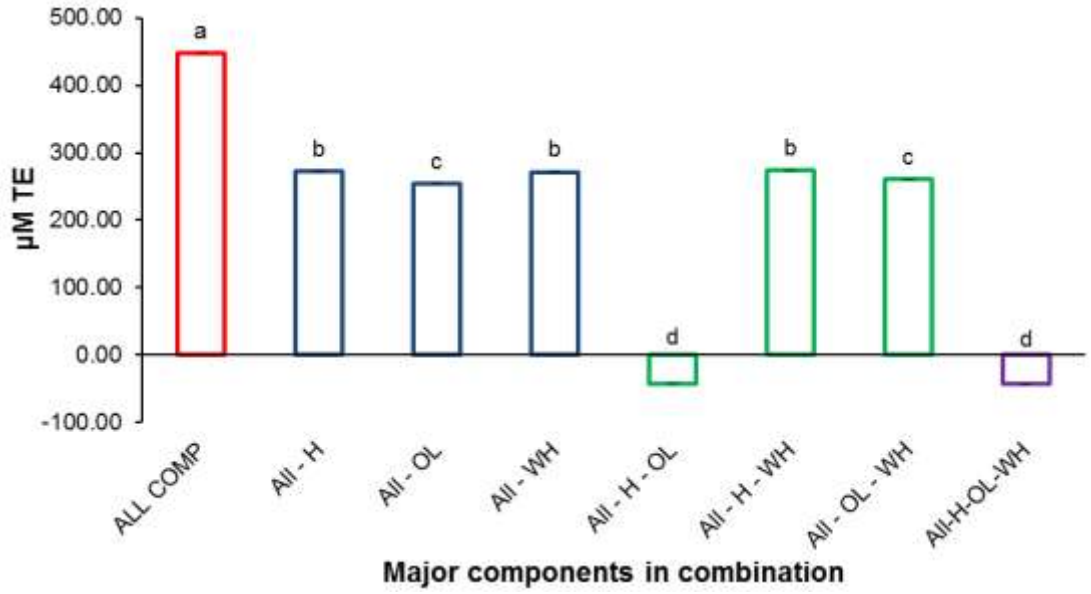


Figure 4.2.2.D: The contribution of the major components to the antioxidant activity measured with the TEAC assay relative to ALL COMP expressed as µM TE. The effect of single components (All-H, All-OL and All-WH), two components (All-H-OL, All-H-WH and All-OL-WH) and three components (All-H-OL-WH) was evaluated. Data is an average of three experiments ± SEM. Means of individual components, different letters are significantly different.

4.2.2.4. Minor components

The effect of the minor components were evaluated with the TEAC assay. The component VE caused 181.11 µM TE decrease in measured antioxidant activity. The components Fa, AV and X did not further contribute significantly to antioxidant activity measured with the TEAC assay.

In summary, using the TEAC assay, OL, H, WH as well as VE contributes significantly to antioxidant activity when measured with the TEAC assay.

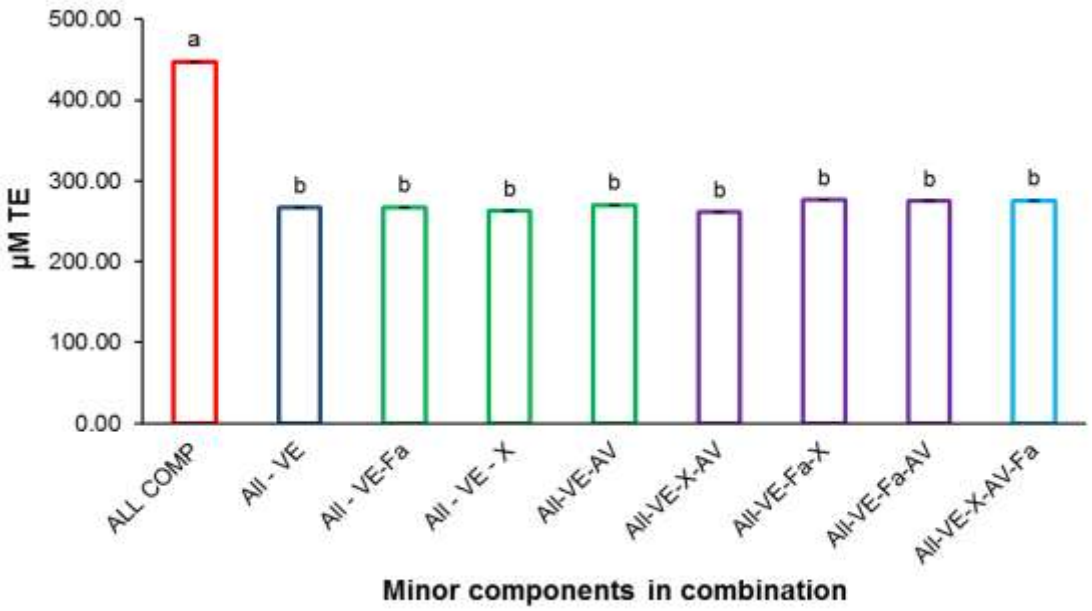


Figure 4.2.2.E: The contribution of the minor components to antioxidant activity, TEAC assay relative to ALL COMP expressed as µM TE. The effect relative to VE for Fa, X and AV was evaluated. Data is an average of three experiments ± SEM. Means of individual components with different letters are significant different.

4.2.2.5. Summary

Contribution to antioxidant activity of all components VE, OL, WH, H, AV, Fa and X is responsible for 56.44%, 15.12%, 13.80%, 13.74%, 0.56%, 0.34% and 0%, respectively. No significant difference were found between FCG (FP) and CALC. Major components H, OL and WH contribute 42.65% of activity and the minor component VE 56.44% of activity.

4.2.3. Oxygen radical antioxidant capacity (ORAC)

4.2.3.1. Standard curve and serial dilutions

The ORAC assay was then used to determine antioxidant capacity. And a standard curve was prepared with a final concentration of 0.25 mg/ml trolox which was represented a linear equation from 0 – 1000 µM TE with R^2 = 0.985 (Figure 4.2.3.A).

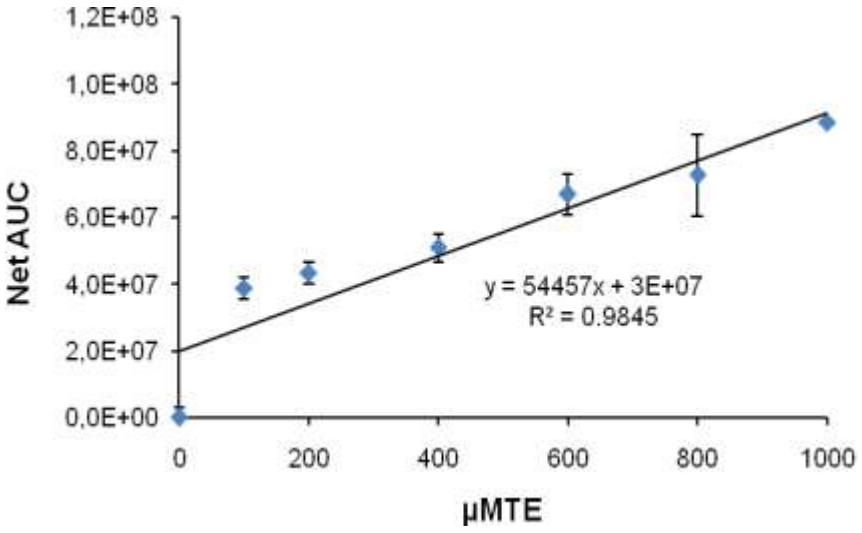


Figure 4.2.3.A: Standard curve for antioxidant activity determined with the ORAC assay. Data expressed as µM trolox equivalents (TE). Data is an average of three experiments ± SEM.

A 0.50 – 0.01 times serial dilution was prepared and with increasing dilutions a linear decrease in antioxidant activity was observed with $R^2 = 0.833$ (Figure 4.2.3.B). From the line equation, it can be calculated to be equivalent to 1.10E+0.9 µM TE. This implies that FCG contains significant amounts of antioxidant activity measured with the ORAC assay.

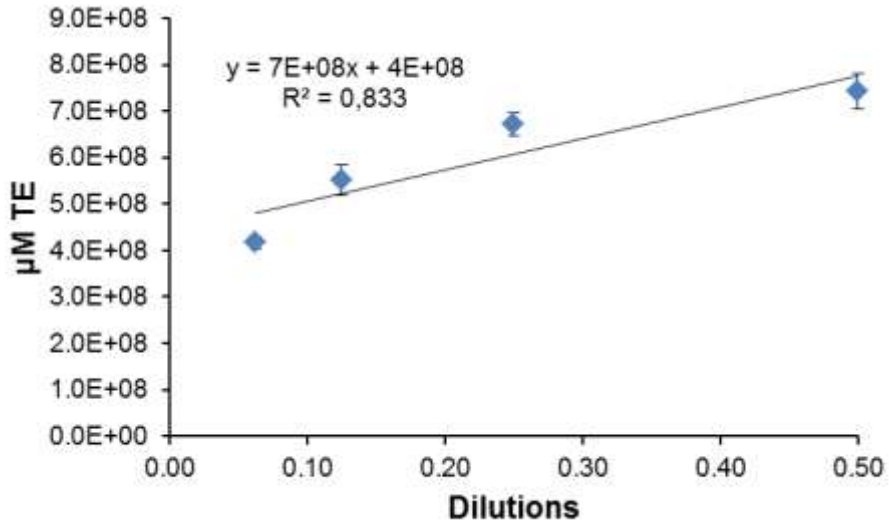


Figure 4.2.3.B: Antioxidant activity, ORAC assay of serial dilutions of FCG (FP) was determined and was expressed as µM TE. Data is an average of three experiments ± SEM.

4.2.3.2. Individual components

Similar to the TEAC antioxidant activity, ORAC was determined for each individual component. From Figure 4.2.3.C, it is observed that H and OL, WH and X show antioxidant activity of 19.86, 19.86 and 19.51 µM TE respectively. Component VE shows minimal antioxidant activity with the ORAC assay but this may be due to a solubility problem that occurred. Components AV and Fa also showed antioxidant activity with values of 10.90 and 19.85 µM TE respectively. There is a significant difference between FCG (FP) and CALC indicating a possible synergistic effect between antioxidant components of FCG (FP). This effect will be further discussed in Chapter 5.

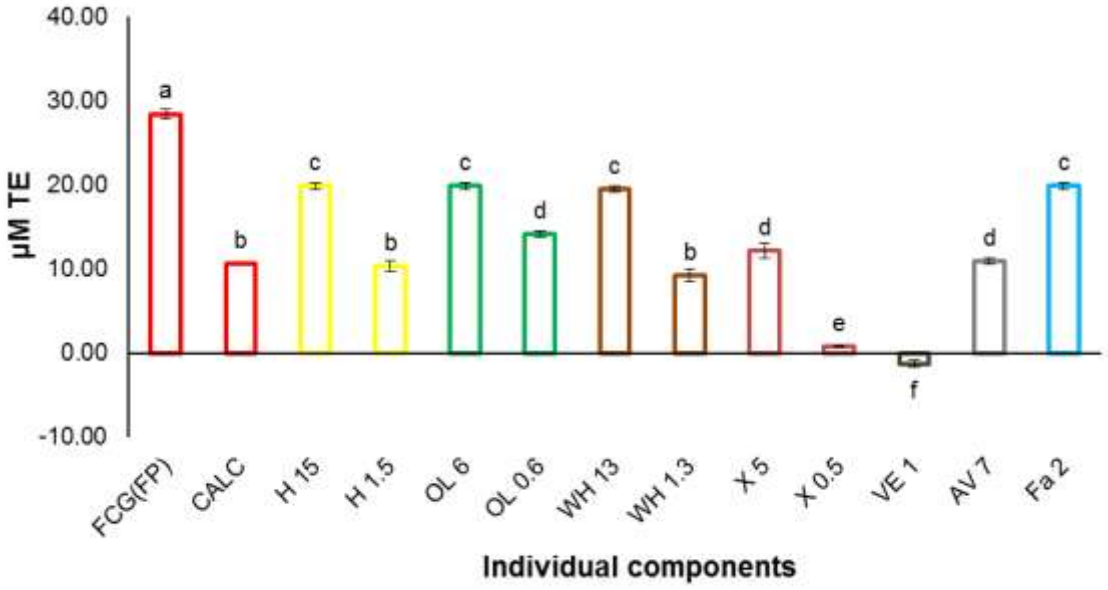


Figure 4.2.3.C: Antioxidant activity, ORAC of each individual component (major components at 1x and 0.1x concentrations and minor components at 10x concentrations), expressed as µM TE. Data is an average of three experiments ± SEM. Means of individual components with different letters are significantly different.

4.2.3.3. Major components

As expected, the removal of H, OL as well as WH results in a significant decrease in the antioxidant activity with values of 0.80, 0.95 and 1.62 µM TE respectively. With All-WH compared to All-H-WH, WH does not significantly contribute to the antioxidant activity in combination in the ORAC assay. Although, when WH is in combination with OL, it shows

a synergistic effect which will be discussed in Chapter 5. Thus, all major components H, OL, and WH contributes significantly to the antioxidant activity in the ORAC assay. In combination All-H-OL-WH contributed to 110.98% activity of the total antioxidant activity found in ALL COMP. Although at the concentrations used a negative ORAC value may indicate a pro-oxidant effect, indicating antioxidant/pro-oxidant effects is concentration dependent.

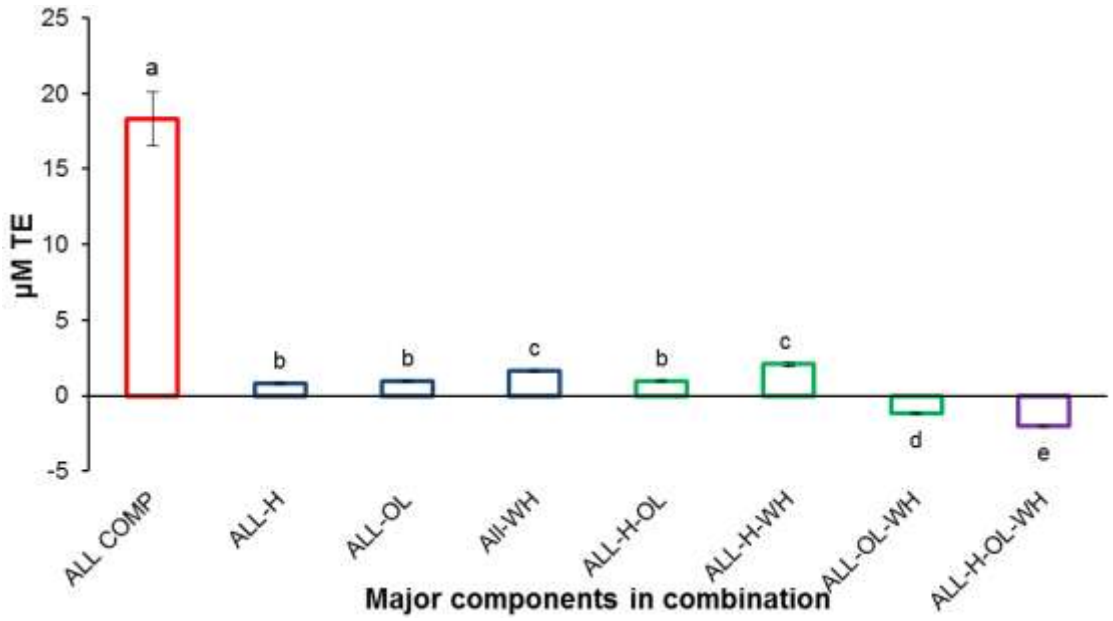


Figure 4.2.3.D: The contribution of the major components to the antioxidant activity measured with the ORAC assay relative to ALL COMP expressed as µM TE. The effect of single components (All-H, All-OL and All-WH), two components (All-H-OL, All-H-WH and All-OL-WH) and three components (All-H-OL-WH) was evaluated. Data is an average of three experiments ± SEM. Means of individual components, different letters are significantly different.

4.2.3.4. Minor components

The component VE contributed significantly to the antioxidant activity of ALL COMP. From Figure 4.2.3.E indications are that Fa and AV also contribute to antioxidant activity.

The ORAC assay identifies that OL, H and WH contribute significantly to antioxidant activity measured with the ORAC assay. Minor components VE, Fa and AV, as for TEAC assay also shows antioxidant activity with the ORAC assay.

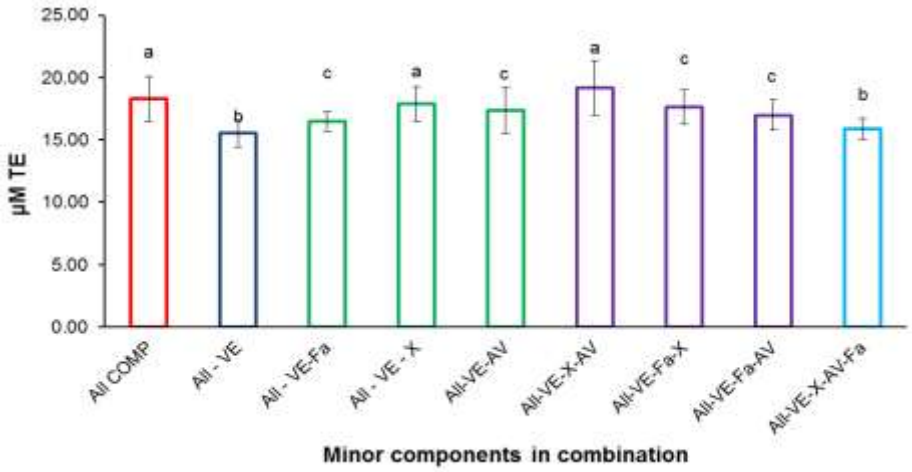


Figure 4.2.3.E: The contribution of the minor components to antioxidant activity, ORAC assay relative to ALL COMP, expressed as µM TE. The effect relative to VE relative to Fa, X and AV was evaluated. Data is an average of three experiments ± SEM. Means of individual components with different letters are significantly different.

4.2.3.5. Summary

Contribution to the antioxidant activity of all components H, OL, WH, VE, Fa, AV and X are 29.97%, 29.91%, 29.67%, 7.59%, 2.06%, 0.80% and 0% respectively, with H, OL and WH contributing 80.55% of total activity and VE 7.59%. As FCG (FP) is statistically greater than CALC, indications are that synergism has occurred between the antioxidant components of FCG (FP).

All data for antioxidant activity is presented in Table 4.2. The DPPH, TEAC and ORAC assays identify H and OL as the major components contributing to antioxidant activity while DPPH and TEAC assays identify that VE although a minor added component contributes 44.56% and 56.44% of activity respectively.

Table 4.2: Final summary of percentage contribution of each component to antioxidant activity

Component	DPPH (%)	TEAC (%)	ORAC (%)
Major components			
H	11.63	13.74	29.97
OL	32.86	15.12	29.91
WH	0	13.80	29.67
TOTAL	**44.49**	**42.66**	**89.55**

Minor Components			
VE	44.56	56.44	7.59
AV	0.24	0.56	0.80
X	6.55	0	0
Fa	4.17	0.34	2.06
TOTAL	**55.52**	**57.34**	**10.45**

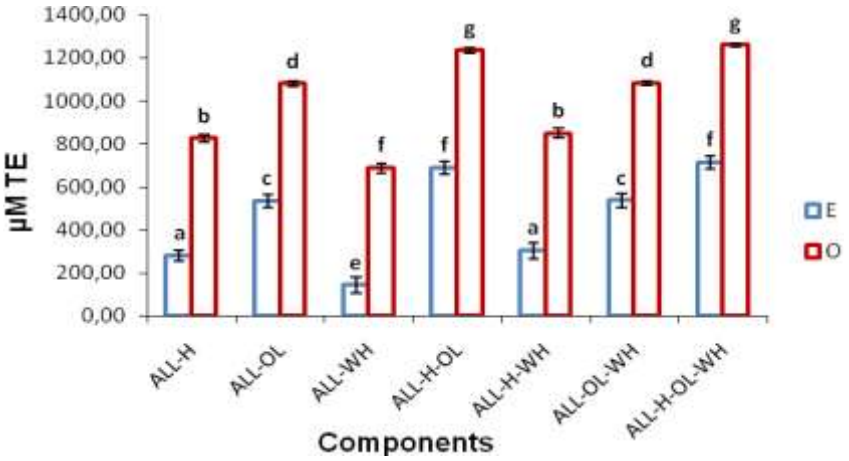
4.3. ANTIOXIDANT SYNERGISTIC EFFECTS

Synergistic effects were further evaluated for the major components that show significant levels of antioxidant content or activity. From the data of single components the sum of the total effect of components in combination can be calculated. This is then compared to the observed (O), experimental (E) values. Liu *et al.* (2008) calculated E/O = 1 as an additive effect, < 1 antagonistic effect and > 1 a synergistic effect. This has been refined for this study taking into consideration biological variations as $0.90 > x < 1.10$ as additive, $x > 1.10$ as synergism and $x < 0.90$ as antagonism. Parker *et al*. (2010) described that these effects are only significant if the differences between E and O are statistically significant with $p < 0.05$. The fold increase was then calculated. These effects were determined using data generated from the DPPH, TEAC and ORAC assays.

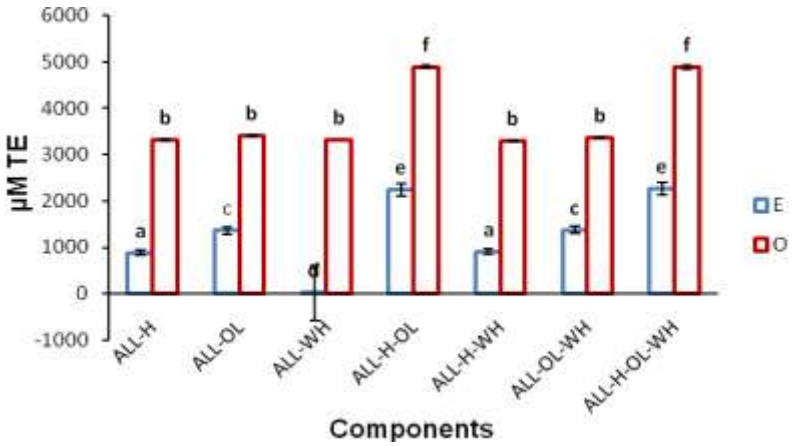
Synergistic antioxidant effects with O > E were observed between all components evaluated H, OL, and WH determined with the DPPH, TEAC and ORAC assays (Figure 4.3.1). The fold difference between the observed and expected values was calculated (Table 4.3). The largest synergistic effect/fold increase was observed between H+OL and WH+OL (DPPH assay), WH+OL (TEAC assay) and H+WH and WH+OL (ORAC assay) indicating interactions between H+OL and OL+WH.

For the DPPH and TEAC assays the major contribution was for OL with values of 2.79 and 3.67 respectively. While for the ORAC assay OL and WH contributed equally to antioxidant activity.

A) DPPH



B) TEAC



C) ORAC

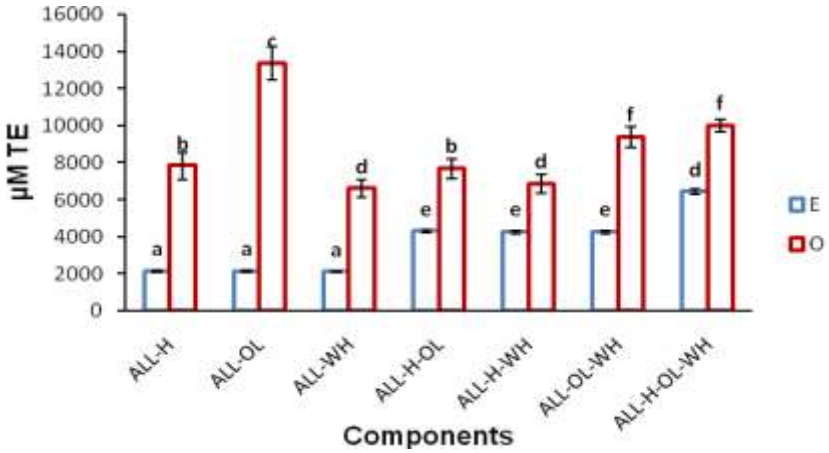


Figure 4.3.1: Synergistic effect in (A) DPPH assay; (B) TEAC assay; (C) ORAC assay between experimental (E) and observed (O) effects reported as total µM TE contributing to activity.

Table 4.3: Fold of increase (E/O) in antioxidant activity between H, OL, and WH with synergism at values >1.1

Sample	Effect	DPPH	TEAC	ORAC
All-H	WH+OL	**2.94**	**3.75**	**3.63**
All-OL	H+WH	2.01	2.50	6.20
All-WH	H+OL	**4.83**	*186.43**	3.11
All-H-OL-WH	VE, Fa, AV, X	1.76	2.16	1.55
All-WH-H	OL	**2.79**	**3.67**	**1.78**
All-WH-OL	H	2.01	2.18	1.60
All-H-OL	WH	1.79	2.44	**1.78**
All-H-OL-WH	VE, Fa, AV, X	1.76	2.16	1.55

* Not reliable

4.4. CELLULAR ANTIOXIDANT EFFECTS, H, OL AND WH

To investigate the cellular antioxidant effects a slightly different strategy was used. Instead as for antioxidant content and activity a single concentration was used a serial dilution was used as antioxidants in cellular models depending on concentration will either be antioxidant or pro-oxidant. For this purpose the fibroblast SC-1 cell line was used and the procedures followed were as summarized in Figure 4.4.

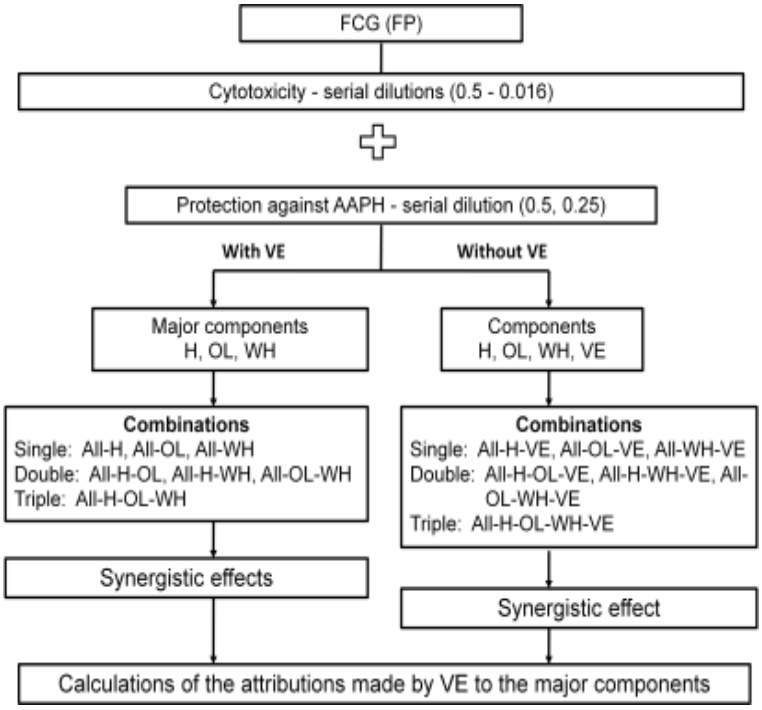


Figure 4.4: Summary of method used in cellular studies

Firstly, cellular protective effect of FCG (FP) was evaluated at dilution 0.125 and 0.25. As shown in Figure 4.4.1, AAPH as oxidant alone causes 100% damage and FCG (FP) in the absence of AAPH causes no damage (no pro-oxidative effect). FCG (FP) at a 0.125 and 0.25 dilution in combination with AAPH inhibits AAPH induced oxidative damage by > 95%.

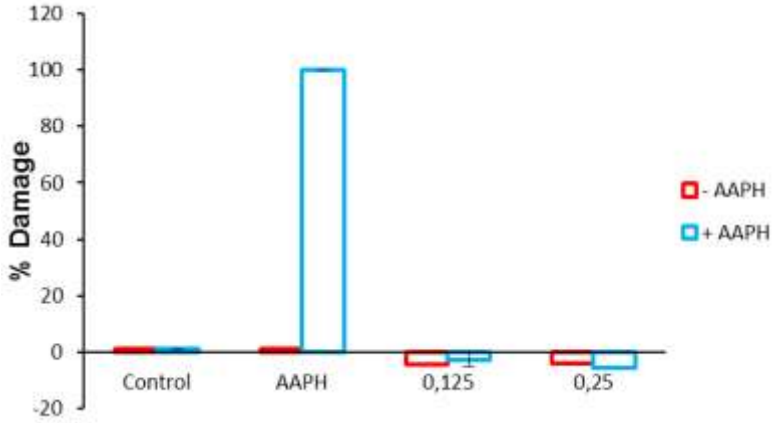


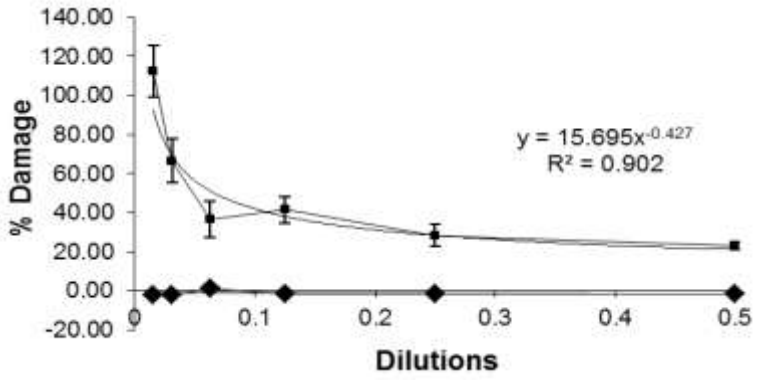
Figure 4.4.1: Cellular protective ability, DCFH-DA assay of 0.125 and 0.25 dilution of FCG (FP). Data expressed relative to AAPH alone causing 100% damage.

The effect of All-H, All-OL, All-WH, (Figure 4.4.2.A); All-H-OL All-H-WH, All-OL-WH (Figure 4.4.2.B) and lastly All-H-OL-WH (Figure 4.4.2.C) was then evaluated, using serial dilutions, 0 - 0.5. For single components, All-H, All-OL and All-WH alone in the absence of AAPH at all dilutions causes no damage to SC-1 cells. The antioxidant effect was evaluated as follows. If % fluorescence at the lowest concentration was > 100% this indicates a pro-oxidant effect. If % fluorescence at the highest concentrations (lowest dilutions) was < 100% indicates an antioxidant effect. A curve fit with a high correlation > 0.75 indicates a dosage effect.

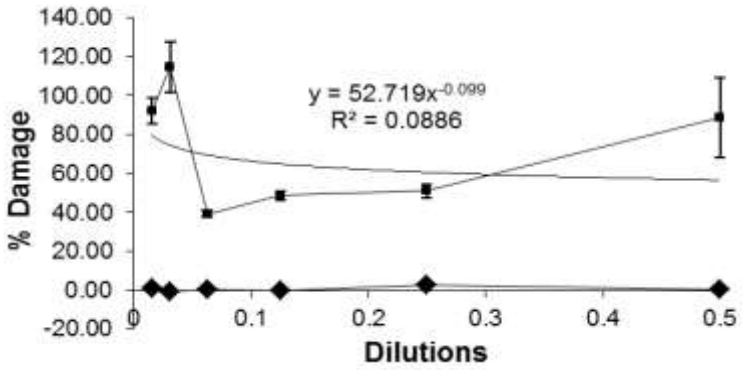
In the presence of AAPH, at the lowest dilution a pro-oxidant effect (>100% damage) was observed for All-H and All-OL at a dilution of 0.031% and this was 116% and 120% (at 0.06%) respectively.

A dosage effect was observed for All-H, with a R^2 = 0.902 (Figure 4.4.2.A), for All-OL with a poor correlation of R^2 = 0.088 (Figure 4.4.2.B.) and for All-WH a dosage effect with R^2 = 0.955 (Figure 4.4.2.C). From this data WH and OL, as well as H and OL provide SC-1 cells protection against AAPH induced oxidative damage.

A: All-H



B: All-OL



C: All-WH

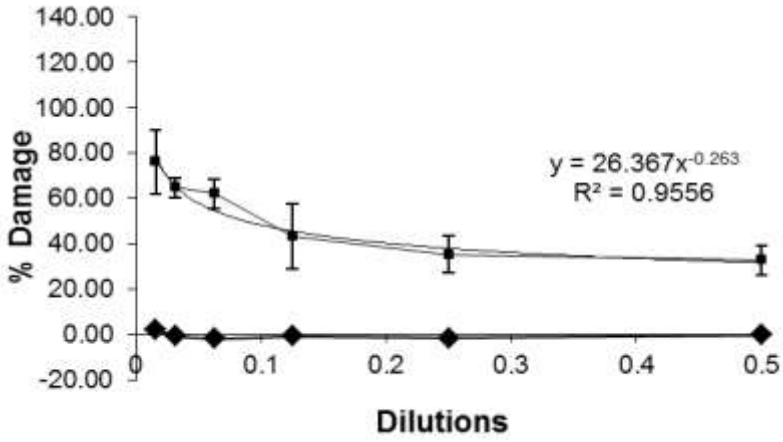
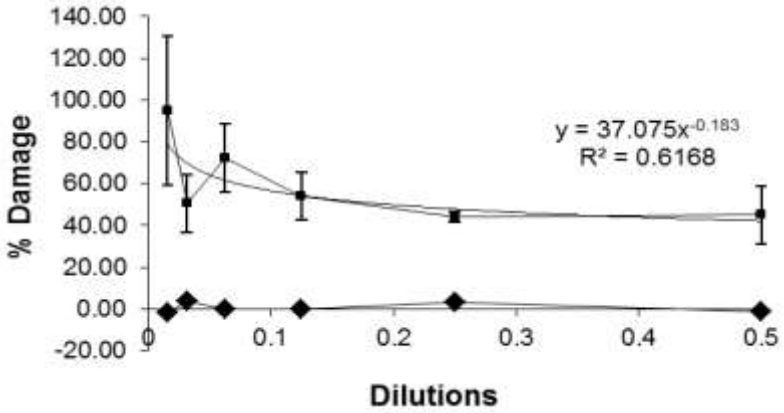
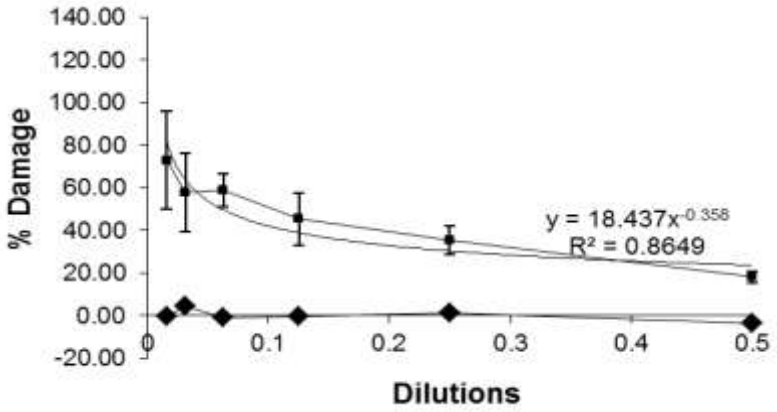


Figure 4.4.2: Cellular protective ability, DCFH-DA assay of serial dilution of major component, A: All-H, B: All-OL and C: All-WH. Data expressed relative to AAPH alone causing 100% damage. Data is an average of three experiments ± SEM.

A: All-H-OL



B: All-H-WH



C: All-OL-WH

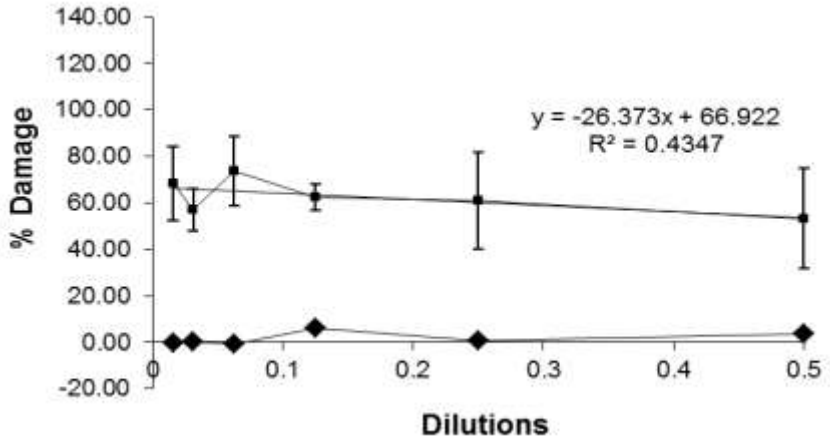


Figure 4.4.3: Cellular protective ability, DCFH-DA assay of serial dilution of major component, A: All-H-OL, B: All-H-WH and C: All-OL-WH. Data expressed relative to AAPH alone causing 100% damage. Data is an average of three experiments ± SEM.

For two component combinations, All-H-OL, All-H-WH and All-OL-WH alone in the absence of AAPH at all dilutions causes no cytotoxic effect to SC-1 cells. In the presence of AAPH the following effects were observed. A dosage effect with for All-H-OL, with a R^2 = 0.616 (Figure 4.4.3.A), for All-H-WH of R^2 = 0.864 (Figure 4.4.3.B.) and for All-OL-WH a dosage effect with R^2 = 0.434 (Figure 4.4.3.C). The best effect was observed for All-H-WH and is due to the presence of OL. Even at the lowest dilution All-H-OL showed 40% protection compared to 25% and 5% for All-H-WH and All-OL-WH. This indicates that there may be a strong interaction between OL and VE.

Finally the effects of three major components in combination namely All-H-OL-WH was evaluated (effect of minor components such as VE) and this data is presented in Figure 4.4.4.

All-H-OL-WH

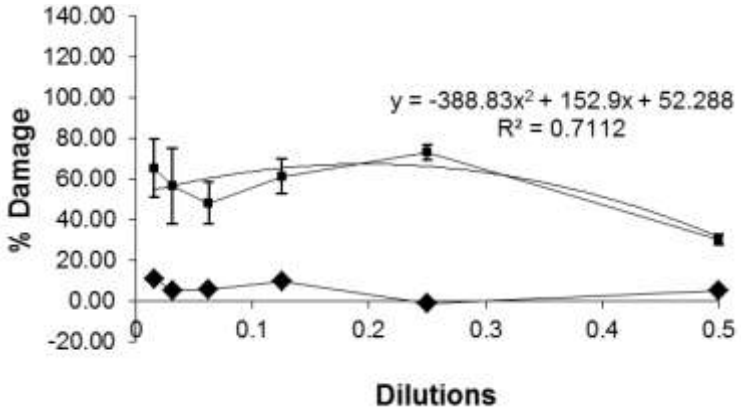


Figure 4.4.4: Cellular protective ability as percentage damage, DCFH-DA assay of serial dilution of major component, All-H-OL-WH. Data expressed relative to AAPH alone causing 100% damage. Data is an average of three experiments ± SEM.

Finally for All-H-OL-WH, alone in the absence of AAPH at all dilutions evaluated caused minimal damage (< 10%) to SC-1 cells. In the presence of AAPH in this group, a dosage effect is observed with R^2 = 0.711 (Figure 4.4.4) even at the lowest dilution a 35% protection was observed for All-H-OL-WH. This implies that the minor components such as VE also contribute significantly to the antioxidant activity of FCG (FP).

From the percentage damage the percentage protection can be calculated which is 100 - % damage. This makes interpretation of data easier. From the % damage data the % protection was calculated at 0.25% and 0.125% for data generated (Figure 4.4.4) and is presented in Figure 4.4.5. and Table 4.3 and this represents the observed values. Concentrations of 0.125% and 0.25% is same as that used for FCG (FP), Figure 4.4.1. Expected values were calculated from the effect of single components (e.g. All-H and All-OL see M*H (1.67 at 0.25%) and M*OL (27.16 at 0.25%), calculated expected (E= 28.85). This data was used to calculate if synergism occurs between components when evaluated in a cellular model.

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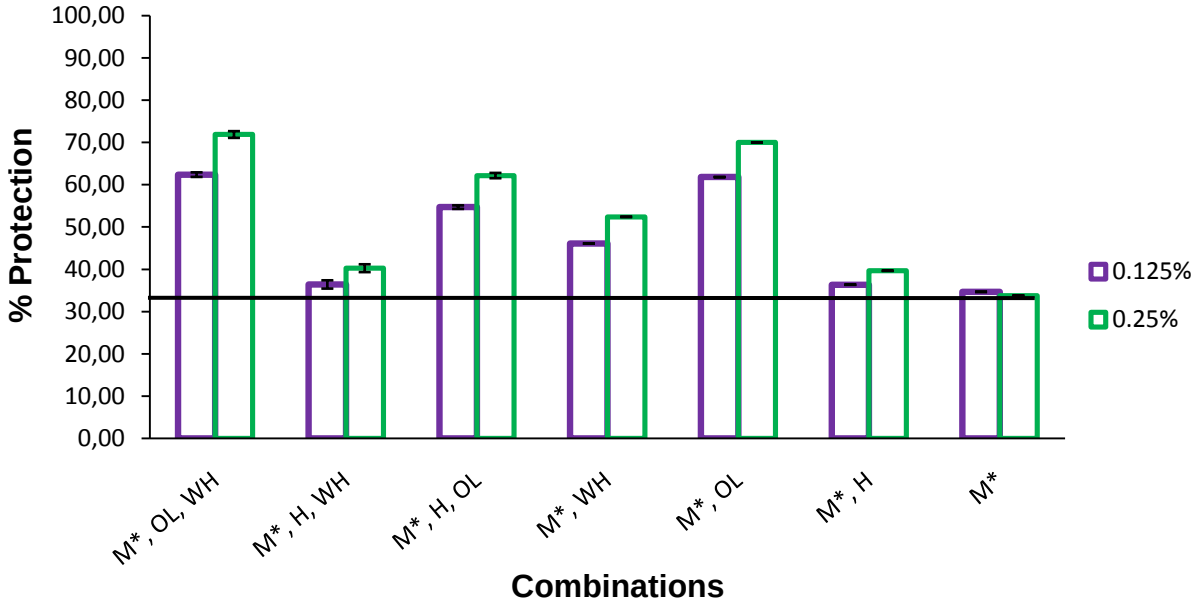

Figure 4.4.5: Summary of contribution to cellular protection by H, OL and WH in the presence of minor components. Purple represents a dilution of 0.125% and green a dilution of 0.25% as reported for total activity of FCG in Figure 4.4.1. The line represents the contribution of the effect of the minor components.

Table 4.4: Summary of the cellular protective ability of the major components against AAPH derived from Figure 4.4.5.

Effect	O (0.25%)	O (0.125%)	E (0.25%)	E(0.125%)	E/O(0.25%)	E/O (0.125%)	EFFECT
M*, OL, WH	27,74	38,12	38,58	54,89	1,39	1,44	**SYN**
M*, H, WH	1,76	6,49	13,11	24,51	7,45	3,78	**SYN**
M*, H, OL	20,05	28,41	28,85	42,14	1,44	1,48	**SYN**
M*, WH	11,42	18,63					
M*, OL	27,16	36,27					
M*, H	1,69	5,88					
M*	0,00	0,00					

In the presence of minor components which include VE synergism was found for minor components plus H, OL and WH. The contribution of minor components to % protection was 35%.

4.5. CELLULAR SYNERGISTIC EFFECTS, CONTRIBUTION OF VITAMIN E

The previous section showed that minor components contributed significantly to cellular antioxidant activity, in this section specifically the contribution of VE will be investigated. This is also based on the results of the antioxidant activity assays (Table 4.1). The contribution of VE to cellular protection was evaluated at a dilution of 0.1% (E/O) and

synergistic effects were re-evaluated taking VE into account. This single dilution was chosen as in Figure 4.4.2 and 4.4.3 no pro-oxidant effect was found. All samples evaluated showed no oxidative effect (green bars). In the presence of AAPH, VE in combination with H, OL and WH significant levels of cellular protection.

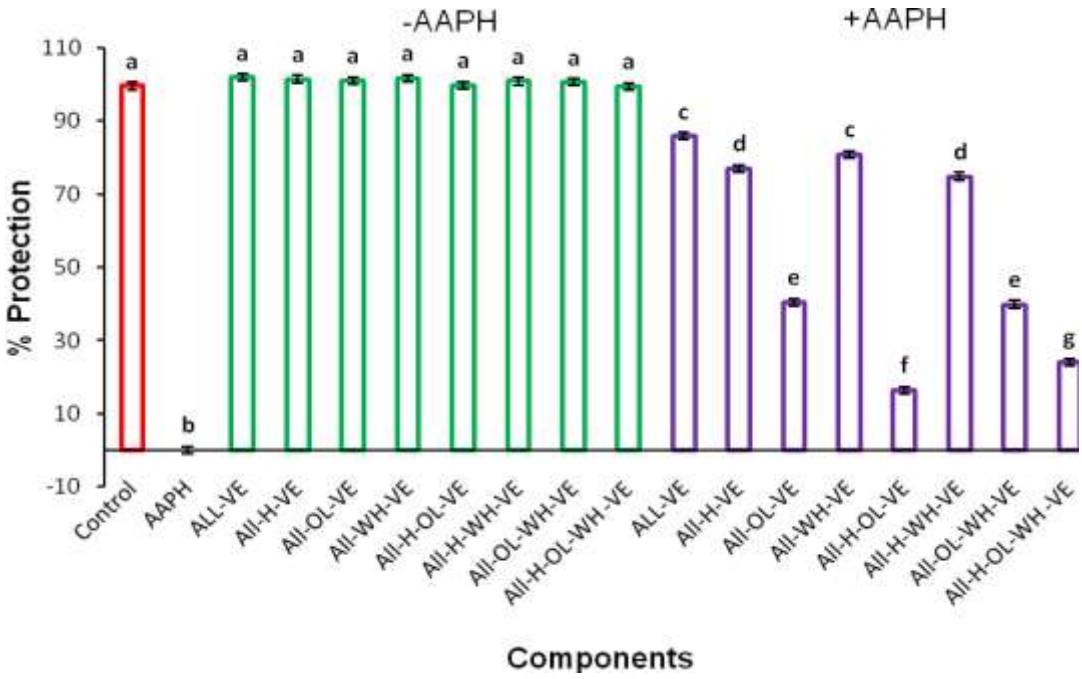

Figure 4.5.1: Vitamin E and the effect of major components. Green columns represents combinations without AAPH, purple columns with AAPH. Data expressed relative to AAPH alone causing 100% damage.

The greatest effect was observed for All-VE, All-VE-WH and All-VE-WH-H which indicates that OL contributes the most to cellular protection when in combination with VE.

To determine the cellular effects of the other major components at a 0.1 dilution, the percentage protection for each component in the presence of VE was calculated from the line equations from the graphs, Figure 4.4.2 - 4.4.4. These results were then compared to Figure 4.5.1 when VE is absent taken into account and is presented in Figure 4.5.2.

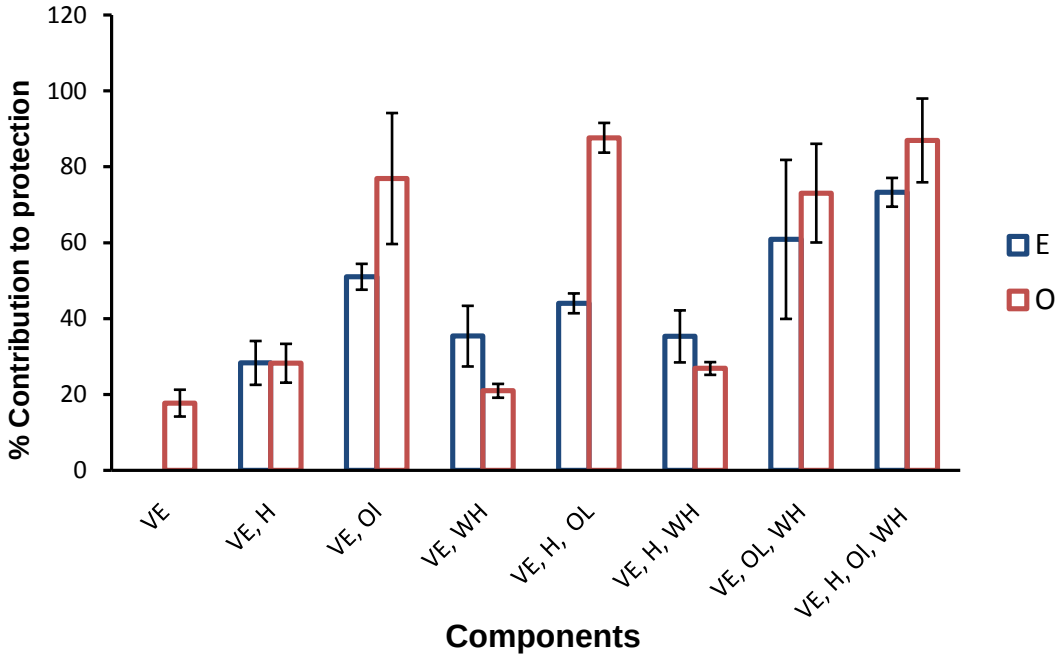

Figure 4.5.2: Synergism between VE and major components between experimental (E) and observed (O).

The greatest % protection was observed for VE in combination with H and OL with OL contributing the most to cellular protection when in combination with VE. Removal of VE, H and OL indicates that these three compounds are responsible for 85% of cellular protection. Cellular protection studies confirm that VE, H and OL act together to protect cells against oxidative damage similar to the findings of the antioxidant assays. All data was then re-evaluated taking VE into account. Synergistic effects were recalculated as done previously and this data is presented in Table 4.5.

Table 4.6: Cellular synergism between components VE included (from Figure 4.5.2).

Sample	O	E	E/O	Effect
SINGLE +VE				
H	28,34	28,25	1.00	ADD
OL	51,03	76,92	**1,51**	**SYN**
WH	35,39	20,99	0,59	ANT
DOUBLE + VE				
OL, H	44,03	87,65	**1,99**	**SYN**
H, WH	35,33	26,87	0,76	ANT
OL, WH	60,88	73,07	1,20	**SYN**
TRIPPLE +VE				
H, OL, WH	73,29	86,96	1,17	**SYN**

From this data synergism occurs between VE and OL, H and WH. In contrast for VE combined with WH, WH and H an antagonistic effect was observed.

In conclusion OL promotes synergism between VE and other components whereas WH inhibits this effect with antagonistic results.

CHAPTER 5: DISCUSSION

5.1. INTRODUCTION

Chronic wounds occur in the aged, patients with AIDS, diabetes and arterial disease. Treatment options are limited mainly due to the difficulty in healing these wounds which has a direct impact on treatment costs. Wound healing is a complex process involving inflammation, cellular proliferation, remodelling and contraction. In chronic wounds this normal process is disrupted and/or inhibited and thus the wound stays in the inflammatory phase. Strategies for the treatment of chronic wounds are to eradicate infection and then to stimulate cellular regrowth. When developing a wound care product several aspects need to be addressed which includes the sterility of the product; reactivation of cellular growth; added protection against micro-organisms; and increased connective tissue production in an oxidant free environment (Fonder *et al.,* 2008).

FCG was developed as a complimentary wound care product and the formulation of this product consists of ingredients with antimicrobial (including biofilm breakdown), anti-inflammatory and antioxidant activity. FCG consists of several bioactive ingredients including honey, oleuropein, witch hazel, xylitol, farnesol, aloe vera and vitamin E. The inactive components comprises of Natrasol™ 250 HR which acts as the thickening agent, glycerin a moisturizing agent, citric acid and trisodium citrate which forms part of the buffering system and additional citric acid to lower the pH. Details related to the reasons for the addition of each component is presented in Table 2.2.

To optimize a complex formulation such as FCG careful consideration of overall safety related to toxicity, optimal activity related to bacterial killing, antioxidant and anti-inflammatory activity as well as cost must be taken into consideration. Interactions between components such as antagonism and/or synergism must also be taken into account. Each component is a complex mixture of molecules and these can interact with each other as well as other components, e.g. between the many polyphenolics present in honey and/or the polyphenolics in honey and those present in oleuropein.

5.2. EXPERIMENTAL DESIGN

The main aim of this project was to evaluate a complex mixture containing molecules with diverse function and the various interaction between these molecules related to a specific activity e.g. antioxidant activity is challenging. This becomes even more complex if antibacterial and antioxidant activity are evaluated simultaneously.

Firstly, it was determined if molecules with antioxidant activity when added as part of a complex mixture such as FCG (FP), loose activity i.e. activity is inhibited by other components, retain activity i.e. have an additive effect, or have a synergistic anti-oxidant effect i.e. increased activity due to interactions between polyphenolics and other antioxidants such as vitamin E.

Secondly this study was an ideal opportunity to construct a model to evaluate complex mixtures since there is a lack in this area of research. Most research done is where a single ingredient is taken as was done by Freeman *et al*., 2010 in which the component with the highest activity is combined with another component/s to create two combinations (x + y) or three combinations (x + y + z). No literature is available where a complex product such as FCG is evaluated where the product consists of 4 or more ingredients and where each component was chosen for a specific type of activity e.g. antibacterial, anti-inflammatory and antioxidant.

Different strategies can be used to determine antioxidant content and activity. For example Wang *et al.* (2011) determined whether synergistic, additive and antagonistic antioxidant effects occurred in food mixtures. For individual components the TPC, FRAP, DPPH and ORAC was determined and then combinations of two food components were evaluated. Likewise the scavenging activity and synergistic effects of lycopene, vitamin E, vitamin C and β- carotene was determined by Liu *et al*., (2008). In a similar way Freeman *et al*., (2010) studied the synergistic and antagonistic interactions between the phenolic compounds in navel oranges. All of these authors added each component together in different combinations. Each started by measuring the antioxidant content of the individual components and then of each mixture. For example Freeman *et al*. (2010) determined Difference = (combined abcd) – (a+b+c+d). Statistical differences indicated whether a synergistic or antagonist effect had occurred. In this study a complex mixture of 7 compounds, each containing several antioxidant components are combined in a

wound healing product namely Flavonix Cytoflamm Gel (FCG). The more components/ingredients the greater is the number of combinations that need to be evaluated and in this instance is 2^7 = 64 combinations.

To develop a strategy for the evaluation of a complex mixture such as FCG only antioxidant activity was considered in this study. Firstly the antioxidant activity of FCG as a final wound healing product was evaluated to confirm activity. Then each single component at the concentrations as found in the formulation was evaluated to confirm activity. Related to concentration and activity the major and minor components were identified. The contribution of each of the major and minor components was investigated by preparing the formulation with only the bioactive components which included antioxidant, antibacterial and anti-inflammatory activity as found in the wound healing dressing. Then related to the formulation the ingredients were divided into two groups major and minor components relate to activity/concentration. A formulation was then prepared missing one, two and three components. This strategy takes into account the effect of other components that although may have little or no activity that may inhibit, mask or alter activity. These include other components added with antimicrobial activity e.g. xylitol and farnesol, anti-inflammatory activity e.g. aloe vera and other components such as thickeners e.g. Natrasol™ 250 HR and buffer salts e.g. citric acid with trisodium citrate. This will eventually assist in the reformulation of the product according to this information to obtain an economical product with maximal wound healing properties.

This study was started by determining the antioxidant content (TPC and TFC) and activity (DPPH, TEAC and ORAC) of the FCG in each individual component present in FCG. Then the activity was determined of a complex mixture of all bioactive antioxidant components (ALL COMP). Based on these results two groups were identified (i) with antioxidant activity but present at a concentration of > 5% (major components) and (ii) with known antioxidant activity but present at a concentration less than 5% (minor components). Minor components may be present at low concentrations but may show increased bioactivity as was found for VE. Mixtures were prepared missing one (eg., All-H), two (eg., All-H-OL), three (eg., All-H-OL-WH) of the major components, but still in the presence of the minor components and then mixtures were also prepared missing one (eg., All-VE), two (eg., All-VE-AV), three (eg., All-VE-AV-X) and four (eg., All-VE-AV-X-Fa) of the minor components, but still in the presence of the major components. Lastly using this data synergistic, antagonistic and additive effects could be determined. This

determines if the antioxidant activity measured individually is retained in complex mixtures. The effect of each molecule may also be increased due to synergism, may be unchanged or reduced due to decomposition, reaction compounds, interference and antagonistic effects.

This cellular antioxidant effect of each mixture was further evaluated but as antioxidants have been found to have a pro-oxidant effect the effect of serial dilutions was evaluated rather than a single concentration as was done for antioxidant content and activity. In addition the type of interaction between components was determined and the contribution of VE was evaluated in greater detail.

5.3. ANTIOXIDANT CONTENT

Firstly the total polyphenolic content (TPC) and total flavonoid content (TFC) was determined. Chemically, polyphenols are described as compounds which have one or more hydroxyl group attached to a benzene ring (Fraga *et al.,* 2010). Polyphenolics can be divided into 4 major groups: the key classes are phenolic acid and flavonoids and the lesser known classes are the stilbenes and lignans (Tapiero *et al.,* 2002). Flavonoids are the most abundant polyphenols with a C6-C3-C6 structure consisting of two aromatic rings (A and B) and are linked through a three carbon chain which is structured as an oxygenated heterocycle (ring C) (Tapiero *et al.,* 2002, Fraga *et al.,* 2010). Flavonoids can then be subdivided into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones according to the degree of oxidation of the oxygen heterocycle (Tapiero *et al.,* 2002). Quercetin, myricetin, kaempferol and fisetin forms part of the flavonol subdivision (Tapiero *et al.,* 2002). Stilbenes consist of two aromatic rings linked through a two carbon bridge with a double bond which gives it a C6-C2-C6 structure (Fraga *et al.,* 2010). The main compound of this division is resveratrol (Fraga *et al.,* 2010). Tannins are oligomers and polymers of flavonoids and can be subdivided into two groups: condensed tannins and hydrolyzable tannins. Condensed tannins also known as proanthocyanidins and procyanidins are not only defined based on the monomeric structure but also the type of linkage found between the monomers (Fraga *et al.,* 2010). Hydrolyzable tannins on the other hand are polymers which are hydrolyzed by acids into a central core consisting of polyol and a phenolic carboxylic esterifying totally of partially the core molecule (Fraga *et al.,* 2010).

FCG consists of 2 types of components single molecule namely vitamin E (VE), xylitol (X) and farnasol (Fa) with a well-defined structure and complex mixtures and these are honey (H), oleuropein (OL) witch hazel (WH) and aloe vera (AV). The concentration and type of the polyphenolic anti-oxidant components in H, OL and WH is a function of geographical localization grown as well as seasonal conditions and season harvested. Of these H, OL and WH and VE have the reported antioxidant activity (Chau *et al.,* 2013; Bulotta *et al*., 2013; Thring *et al*., 2011; Burton and Traber, 1990).

Honey is a complex mixture of carotenes, phenolic compounds, Maillard reaction product (MRP's) as well as ascorbic acid and (Rice-Evans *et al*., 1997). Phenolic acids found in honey include caffeic, ferriulic, chlorogenicellagic and coumaric acid. The flavonoids include hesperetin, galangin, kaempferol, pinobanksin, chrysin, myricetin, apigenin, luteolin, quercetin and pinocembrin (Blasa *et al*., 2007) (Figure 2.4).

Oleic acid, phenolics and squalene are major components of olive oil (Owen *et al*., 2000) and components of all three groups have been shown to have antioxidant activity (Waterman *et al*., 2007). The phenolic constituents of olive oil are the lignans, secoiridoids and simple phenols. Ligstroside, (Owen *et al*., 2000) oleuropein, hydroxytyrosol and tyrosol are the key phenols present in olive oil (Perona *et al.,* 2006) (Figure 2.5). The hydrolysis of oleuropein (Waterman *et al*., 2007) results in the formation of tyrosol, hydroxytyrosol and ethanol (Martinez-Dominques *et al*., 2001).

Witch hazel (*Hamamelis virginiana L.*) is derived from the twigs, leaves and bark of a small tree or deciduous shrub native to damp woods in Canada and North America (Wang *et al.,* 2003*).* A main component of the bark extract of witch hazel is hamamelitannin (2', 5-di-*O*-galloyl-hamamelose) (Draelos, 2001), a polyphenolic derivative (Figure 2.7).

Active constituents in aloe vera have been identified which includes saccharides, minerals, vitamins, enzymes, amino acids, anthraquiones, lignin, salicylic acids and saponins (Foster *et al.,* 2011). Anthraquinones especially aloin A (Figure 2.3) are present in the leaf exudate which may be responsible for the cathartic effect and bitter taste of the exudate (Dagne *et al*., 2000; Boudreau and Beland, 2006). Aloins are generally referred to as polyphenolic compounds because these molecules are derived for a phenolic acid precursor and may inhibit free radical-mediated cytotoxicitiy and lipid peroxidation due to their antioxidant tendencies (Cook and Samman 1996). Aloins A

and B are two diastereometic C-glucosylanthrones and are considered to be the components giving aloe its laxative effect. Aloesin, a 5-methylchrome (typically C-glucosylated) exhibits free radical scavenging, antioxidant and anti-inflammatory activities (Krpetić, *et al*., 2009). Other contributing factors to the activity of aloe vera have been attributed to two polysaccarides namely, acetrylatedmannan and glucomannan and glycoproteins (lectins) found in the colourless leave pulp (Krpetić, *et al*., 2009).

Farnesol (Figure 2.8: A), a natural hydrophobic organic compound, is an acrylic sesquiterpene alcohol and can be found in many essential oils e.g., from *plucheadioscoridis*, *zeamays* and *pittosporumundulatum* (Derengowski *et al*., 2009). Its main use is in perfumery to emphasize the odours of sweet floral perfumes but new literature arises investigating its quorum-sensing abilities (Trimble and MacFarland, 1885). Several sesquiterpene alcohol molecules such as bisabolol those found in chamomile and rose have been shown to have antioxidant activity. (Braga *et al*., 2009; de Oliveira Júnior *et al*., 2013).

TPC in each ingredient was measured spectrometrically with the Folin-Ciocalteu reagent (Arbianti *et al.,* 2007*).*

The contribution of H, OL, X, FA, WH, AV and VE to the TPC of FCG (FP) was calculated and was found to be 45.89%, 38.19%, 5.78%, 8.82%, 4.70%, 1.36% and 0% respectively, with major components H, OL and WH contributing 85.44% of TPC.

FCG (FP) had a TPC of 9.875 µg GAE/100g and CALC was 16.22 µg GAE/100g. Since CALC shows a higher value than FCG (FP) it may indicate a possible interference of the non-bioactive components, that result in an underestimation of TPC. This may be due to the presence of Natrasol™ 250 HR, citric acid, trisodium citrate and glycerin. TPC's mechanism involves a basic redox reaction where the phenols are oxidized by the phosphotungstate-phosphomolybdate complex (Folin, Ciocalteu, 1927). Natrasol™ 250 HR is a water soluble polymer and consists of hydroxyethyl celluloses and therefore would not cause any interference. FCG also contains glycerin and Natrasol™ 250 HR which increases viscosity which may limit the accessibility of phosphotungstate-phosphomolybdate to phenolic residues and therefore may account for CALC being greater than FCG (FP).

As shown in Chapter 4, the individual components with the highest TPC values was OL and H. The TPC value for OL was 7.85 µg GAE/100g (or 1.3 µg GAE/1g of a 100% solution). The high value of OL is due to the polyphenols present in the mixture such as oleuropein and possibly its hydrolysis products, hydroxytyrosol and tyrosol reacting with the phosphotungstate-phosphomolybdate complex. The only reported study related to the TPC of OL was undertaken by Dekanski *et al*., 2009, and these researchers reported a TPC of 197.8 ± 11.3 µg GAE/g for a methanolic solution of a dry extract with TPC being 18 – 26% of the total extract. This study shows that OL contains polyphenolics that will react with F-C reagent but does not provide information on content as a methanolic extract was used compared to a water extract in this study.

The TPC value for H was 6.96 µg GAE/100g at a 15% concentration (0.46g/100g**). The high value of H is due to the polyphenols present in the mixture such as caffeic acid, benzoic acid and gallic acid reacting with the phosphotungstate-phosphomolybdate complex. This value is similar to the value of 0.67 ± 0.06 g/100g found by Serem *et al*., (2012) for honeys from the southern Africa regions. When compared to manuka honey found in New Zealand, the TPC is twice is as high with a value of 1.12 ± 0.31 g/100g. Prior *et al*., (2005) reported that besides phenols the F-C reagents may react with other antioxidants such as carbohydrates, proteins, amino acids, nucleotides, unsaturated fatty acids, amines, ketones, vitamins, aldehydes, thiols and proteins. Since honey has a high carbohydrate (polysaccharides, monosaccharides) content this may be one of the contributors to the TPC value.

As shown in Chapter 4, the evaluation of the major components revealed that both OL and H contributed significantly to TPC while WH had no significant effect and this may be related to the complex structure of tannins and procyanadins.

Evaluation of the minor and major components showed that the major components OL and H contributed 38.18% and 45.89% respectively and this accounted for 84.07% of TPC. The remaining 15.4% of activity was due to WH, AV and FA. Components X, AV and Fa had no significant effect on TPC taking into consideration that the concentrations of the minor components are low. Components VE and Fa are not polyphenolic compounds but showed to have an increase in TPC. Both these components are lipid-soluble antioxidants with a phytyl tail. The free hydroxyl group on the aromatic ring in VE and at the end of the phytyl tail of Fa may be responsible for this increase where the

** For conversion: 1ml honey is equivalent to 1.4g (Serem and Bester (2012)). This applies to all experiments.

hydrogen from these groups assist in the redox reaction with the phosphotungstate-phosphomolybdate complex (Kamal-Eldin *et* al., 1996)

The flavonoids are subgroup of the polyphenolics and have been categorized according to their chemical structure into flavonols, isoflavones, flavones, catechins, flavanones, chalcones and anthocyanidins (Cook and Samman, 1996). The aluminum chloride colorimetric method can be used to measure total flavonoid content.

Contribution of all components to TFC relative to FCG (FP) are OL, H, WH, VE AV and Fa with 47.70%, 32.84%, 10.94%, 3.91%, 2.37%, and 0% respectively, with the major components H, OL and WH contributing 91.48% of TFC.

FCG (FP) had a TFC of 9.148 mg CE/100g and CALC of 4.884 mg CE/100g. Increased values for TFC of FCG (FP) and CALC indicate that other minor components react with the TFC. This may be due to the presence of Natrasol™ 250 HR, structurally with hydroxyl groups attached to a benzene structure (Figure 5.1) . These hydroxyl groups have high reactivity and interact with the free radicals (Nijveldt *et al*., 2001).

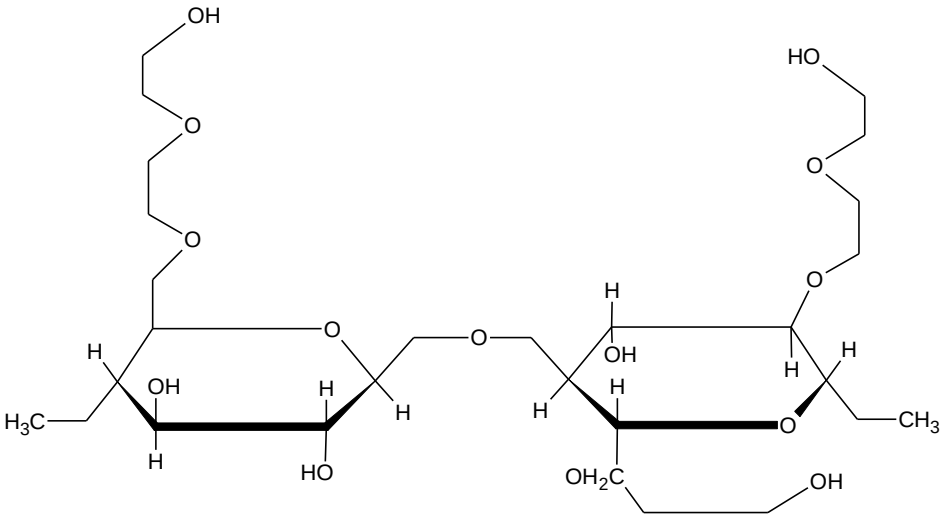

Figure 5.1: Structure of Natrasol™ 250 HR (Aqualon, 1999).

As shown in the results the individual components with the highest TPC values were OL, H and Fa. The TFC value for OL in this study was 3.91 mg CE/100g. The high value of OL is due to the flavonoids present in the mixture such as luteolin 7-O-glucoside reacting with the aluminum chloride. In a study done by Abaza *et al*., 2011, these authors have

reported a TFC value of 21.47 ± 2.56 mg CE/g for a 80% methanolic solution for a pure dry extract. This can be calculated as ± 3.5 mg CE/g at a 6% concentration and this indicates that the flavonoids in the OL used in this study are mostly lipophilic (Ghanbari *et al*., 2012).

The TFC value for H was 0.84 mg CE/100g at a 15% concentration. The high value of H is due to the flavonoids present in the mixture such as luteolin, quercetin, kaempferol and chrysin reacting with the aluminum chloride. This is similar to the value of 15.41 ± 2.40 mg CE/100g found by Serem and Bester, (2012) for honeys from the southern Africa regions (1.03 mg CE/100g at a concentration of 15%) When compared to manuka honey found in New Zealand, the TPC is triple the amount with a value of 46.00 ± 13.1 g/100g.

Fa measured 1.356 mg CE/ml for TFC. Although not a flavonoid the reactivity of Fa may be related to the sesquiterpene alcohol structure of this molecule. This value may be due to the added hydroxyl group to the alcohol structure which binds to the aluminum chloride to form an acid stable complex thus giving it the higher TFC value.

The major component WH showed a negative value when tested individually however was found to contribute 10.94% to the total flavonoid content. The reason for this is unknown. In contrast Fa showed to have a relative high TFC, but the final contribution to the total flavonoid content was minimal and this was due to the low concentration in the final product. The major components contributing to TFC were All-OL and All-H. OL and H contributed 47.70% and 32.84% respectively to TFC, with a total contribution of 80.54%.

Minor components X, VE and AV showed to have no significant effect when in combination. What was interesting to see was that All-VE-Fa, All-VE-Fa-X and All-VE-Fa-X-AV showed a decrease in TFC and this may be due to the effect Fa, as described previously.

In conclusion OL and H contributed the main content in both TPC and TFC and were 45.89% (H), 38.19% (OL) and 47.70% (OL), 32.84% (H) respectively.

5.4. ANTIOXIDANT ACTIVITY

Antioxidant activity was determined using two electron transfer assays (DPPH and TEAC) and a single HAT assay (ORAC assay). Three different assays were used as there are methodological differences between these assays and thus reported significantly different antioxidant activities when using these assays. In a study done by Prior *et* al., reported that these effects are due to the type of antioxidant, structure and properties, partition coefficient and solubility, and system solvent. The mechanism and efficacy of antioxidants are determined by two major factors, namely ionization potential and bond dissociation energy (Prior *et al.,* 2005; Wright *et al.,* 2001).

Contribution to antioxidant activity in the DPPH assay of all components VE, OL, H, X, Fa, AV and WH are responsible for 44.56%, 32.86%, 11.63%, 6.55%, 4.17%, 0.24%, and 0% respectively. Differences between FCG (FP) and CALC, with FCG (FP) < CALC indicates a possible antagonistic effect or inhibition interference of the DPPH by components of FCG (FP). Major components H, OL and WH contribute 44.49% to total activity and although a minor component VE contributes 44.56% of activity.

FCG (FP) had a DPPH value of 912.800 µM TE and CALC was 1010.235 µM TE. Since CALC shows a higher value than FCG (FP) it may indicate interference between antioxidant components of FCG (FP) and the non-bioactive components such as Natrasol (thickening agent) or glycerin as these component may interfere with the accessibility of the bioactive components.

As the polyphenolic and flavonoid content of H and OL was the highest of all the components it is expected that the antioxidant activity of these two components will be related to these finding. Thus again, OL and H showed to have the highest antioxidant activity for the DPPH assay.

The DPPH value for OL was 780.62 µM TE at a 6% concentration. In a study done by Abdel-Sattar *et al.,* 2012, the authors reported a DPPH of IC_{50} of 60.2 µg/ml (3.85 µmol TE/g) for a 80 % methanolic solution. This highlights a common problem related to the reporting of antioxidant activity where data especially for the DPPH assay is reported as µM TE, IC_{50} or % inhibition (Yuting *et al*., 1990; Pourmorad *et al.,* 2006*).*

The DPPH value for H was 213.72 µM TE, 15% solution, final antioxidant activity is 1420 µM TE or 1.014 µmol TE/g. Serem and Bester (2012) obtained a value of 0.42 µmol TE/g and the antioxidant activity measured in this study is within the range for honey from southern Africa.

The major components that contributed to antioxidant activity calculated from All-H and All-OL was for OL and H with 32.86% and 11.63% respectively, accounting for 44.49% of DPPH measured activity. The remaining 55.51% of activity was due to WH, AV, FA and VE. There is no significant difference between ALL COMP and All-WH, indicating that WH did not contribute to the total antioxidant activity. All-H-OL (WH is the main major component) again shows no antioxidant activity. The antioxidant activity occurring is mainly due to VE (44.56%) and in confirmed in Figure 4.2.1.D.

Akap *et al*., 2013, reported that the number of OH groups present in a structure influences the radical protection ability in the DPPH assay and with increased esterification of the OH groups on the chromanol ring activity is reduced (see Figure 2.7).

VE, a minor component showed to have contributed significantly with 44.56%. VE is a fat-soluble antioxidant that comprises of eight naturally occurring structures, namely: α-, β-, γ-, δ-tocopherols and tocotrienols (Shapiro *et al.,* 2001, Zingg, 2007). The chroman head of the phenolic group which is located in the lipid membrane near the aqueous phase gives VE the antioxidant moiety (Afri *et al*., 2004).

The DPPH value for VE was 142,93 µM TE at a 1% concentration. Hussain *et al*., 2009 found the standard VE had a DPPH value of IC_{50} 14.4 mg/ml. Apak *et al*. (2013) reported a antioxidant activity of < 0.1 µmol TE per µmol vitamer for α-TA. Calculated activity in the present study is 0.0064 µmol TE per µmol vitamer relative to α-TA. Both studies indicate that VE has low activity, however in combination with other components e.g polyphenolics a strong synergistic effect is observed.

Contribution to antioxidant activity for the TEAC assay of all components VE, OL, WH, H, AV, Fa and X is responsible for 56.44%, 15.12%, 13.80%, 13.74%, 0.56%, 0.34% and 0% respectively. Major components H, OL and WH contributed 42.65% of activity and VE 56.44%.

FCG (FP) had a TEAC value of 2328,21 µM TE and CALC was 2333.83 µM TE, thus no significant difference between FCG (FP) and CALC which may mean that there seems to be no interactions from the non-bioactive constituents.

As with DPPH, H and OL are again the dominant major components in expressing antioxidant activity. The major component WH did not show any significant antioxidant activity.

The TEAC value for OL was 1376,85 µM TE at a 6% concentration measuring the ability of OL to reduce the $ABTS^{\bullet+}$ radicals. In a study done by Abdel-Sattar *et al.,* 2012, these authors reported a TEAC of 1080 µmol TE/g for an 80 % methanolic solution again indicating the majority of polyphenolics in OL are lipopholic.

The TEAC value for H, 15% solution was 897,60 µM TE or 4.27 µmol/g. Serem and Bester (2012) reported a range of 5.36-20.84 µmol/g for honey from southern Africa. The antioxidant activity of the honey used in this study was just below the reported range of honey from southern Africa. WH antioxidant activity was 32,60 µM TE at a 13% concentration. Thring *et al.,* 2009 reported a value of 13.15 µmol for a 1% solution of WH, as this data is not expressed as µM comparisons related to activity is difficult.

The major components that contributed reflected similar activity in All-OL, All-WH and All-H was for OL, WH and H, 15.12%, 13.80% and 13.74% respectively, accounting for 42.66% of TEAC measured activity. The major component WH when combined with H and OL had no significant antioxidant effect. The remaining 57.34% of activity was due AV, FA and VE. The minor component VE was shown to have contributed significantly, 56.44% to activity. The minor components AV, X and Fa did not show significant contribution to the antioxidant activity relating to the inability of these components to scavenge the $ABTS^{\bullet+}$ radicals.

The TEAC value for VE was 142,93 µM TE at a 1% concentration. Zou *et al.,* 2011 found the VE had a TEAC value of 89.0 ± 0.10 µM TE/g. Since both the DPPH and TEAC assays are SET-assays, the same conclusion can be followed with regards to VE as discussed at the DPPH assay.

The ORAC assay was initially developed by Cao, Alessio and Cutler (Cao *et al*., 1993) and is a HAT based assay.

Contribution to the antioxidant activity of all components H, OL, WH, VE, Fa, AV and X was 29.97%, 29.91%, 29.67, 7.59%, 2.06%, 0.80% and 0% respectively, with H, OL and WH contributing 80.55% of total activity and VE 7.59%. FCG (FP) had a ORAC value of 28.36 µM TE and CALC was 10.62 µM TE. The FCG (FP) value is three times the value of CALC, implying synergism between these components.

All the major components i.e., H, OL and WH showed to contribute significantly to the antioxidant activity in the ORAC assay. The TEAC value for H, 15% solution was 897,60 µM TE or 4.27 µmol/g. Serem and Bester (2012) reported a range of 5.36-20.84 µmol/g for honey from southern Africa. The antioxidant activity of the honey used in this study was just below the reported range of honey from southern Africa.

The ORAC value for H was 19.86 µM TE at a 15% and 19.86 µM TE at a 6% concentration. This can be calculated to be 9.45 and 23.64 µmol/g for the 15% and 6% solutions respectively which is in the range for southern Africa honey where Serem and Bester (2012) measured values of 3.71-49.26 µmol/g. In a study done by Wojcikowski *et al.,* 2007, they reported an ORAC of 261.53 ± 17.01 µmol TE/g for an aqueous methanolic solution, however this represents a highly purified fraction containing only polyphenolics.

The ORAC values for WH and Fa was 19.51 µM TE at a 13% concentration and 19.85 µM TE at a 2% concentration respectively. Related ORAC data could not be found.

As expected the absence of H, OL and WH showed a significant decrease in the antioxidant activity. The major components that contributed reflected similar activity in All-OL, All-WH and All-H was for H, OL and WH, 30.21%, 29.99% and 28.96% respectively, accounting for 89.16% of ORAC. When WH was evaluated in combination with H, there seem to be no significant contribution from WH. However, when in combination with OL, a synergistic effect was observed. This may be related to the type of polyphenolics, where H contains predominantly caffeic acid, caffeic acid phenyl esters, chrysin, galangin, quercetin, kaempferol, acacetin, pinocembrin, pinobanksin, and apigenin (Jaganathan and Mandal, 2009) and olive oil oleuropein, hydroxytyrosol, kaempferol, apigenenin, verbascoside, oleic acid, caffeic acid, quercetin, luteolin and rutin (de Block *et al*., 2013)

The remaining 10.84% of the total antioxidant activity was due AV, FA and VE. The component VE showed to contribute the highest value of the minor components at 7.88% of the antioxidant activity. The ORAC value for VE was 1536,183 µM TE at a 1% concentration for the ORAC assay. This value may be inaccurate as it was noted that the plate had totally precipitated and the values may not reflect the true antioxidant activity for VE. This highlights the importance of using more than one antioxidant assay as well as the solvent used where in the DPPH assay, methanol is used as an excellent solvent for VE.

Correlation between DPPH, TEAC and ORAC

As mentioned earlier in this chapter, both DPPH and TEAC are SET-reaction assays and ORAC a HAT-reaction assay. This would create an expectation that the values between DPPH and TEAC assay would be relatively similar as seen with VE.

Minor components contributed 55.52%, 57.34% and 10.45% to the total antioxidant activity in DPPH, TEAC and ORAC respectively. Although VE is present in low concentrations, it showed to have the most antioxidant activity in the minor component group with 44.56%, 56.44% and 7.59% antioxidant activity in DPPH, TEAC and ORAC respectively. Getoff (2001) confirmed that vitamins C, vitamin E and β-carotene can initiate a synergistic effect on a given cytostatica, for example mitomycin, by a strong increase of its activity when tested *in vitro* using *E. coli* bacteria and cultured cancer cells.

For this reason VE was further evaluated in a cellular model to establish the interaction of VE with the major components.

5.5. ANTIOXIDANT SYNERGISTIC, ADDITIVE AND ANTAGONISTIC EFFECTS

Interactions between components of a complex mixture can be synergistic, additive or antagonistic. A synergistic effect takes place when the effect is greater than the total amount of the individual components (Wang *et al*., 2011). On the other hand of the spectrum, an antagonistic effect takes place when the effect is less than the total amount of the individual components (Wang *et al*., 2011). When a combination provides the total amount of the effect of the individual components, it is referred to as an additive effect (Wang *et al*., 2011). These effects are important to know especially when

formulating a product. Thus, for the best results in the final product, it will be of importance to formulate a product where the different components have a synergistic effect and increased antioxidant capacity. This will be greatly beneficial for the wound environment and therefore will greatly add to the wound healing process.

From the data of single components the sum of the total effect of components in combination was calculated. This was then compared to the observed (O), experimental (E) values. Liu *et al.* (2008) calculated E/O = 1 as an additive effect, < 1 antagonistic effect and > 1 a synergistic effect. Parker *et al*. (2010) described that these effects are only significant if the differences between E and O are statistically significant with $p < 0.05$. If differences were significant the fold increase was calculated. Antioxidant synergistic effects were determined using data generated from the DPPH, TEAC and ORAC assays.

In Figure 4.3.1.A, synergism was observed for all samples in the DPPH assay where the difference between E and O was noticeable. Data evaluation revealed that WH does not contribute significantly to the antioxidant activity in the DPPH assay since (ALL-H and ALL-H-WH), (ALL-OL and ALL-OL-WH) and (ALL-H-OL and ALL-H-OL-WH) shows no significant differences where WH was present as seen in the DPPH assay (individual components). This may be due to the complexity of the structure and the number of hydroxyl groups present in the structure.

In Figure 4.3.1.A, synergism was observed for all samples in the DPPH assay where the difference between E and O was noticeable. No synergism was observed for WH but a strong synergistic interaction was observed between H + OL. H and OL contributed 11.83% and 32.86% each to antioxidant activity and this translates for H and WH in a two fold increase in antioxidant activity whereas OL alone is responsible for a threefold increase in antioxidant activity.

The antioxidant activity of phenolics is ascribed to the number and position of substituted hydroxyl or methoxyl groups and glycosylation around the flavonoid skeleton (Heim *et al*., 2002, Montoro *et al*., 2005). When the structures of the phenolics with one aromatic ring and antioxidant capacity is compared, the number of hydroxyl groups correlated positively with antioxidant capacity against DPPH (Palafox-Carlos *et al*., 2012). The kind of interaction depends greatly of the specific type of antioxidant interacting in the system and the experimental or environmental conditions. It is suggested that the phenolic acids

are capable not only to donate hydrogen atoms to the radical, but they are also able to donate electrons to regenerate other pro-oxidant phenols. This regeneration mechanism maximizes the antioxidant capacity of the system to reduce free radicals. According to Leopoldini *et al*., (2004) phenolic compounds are capable to transfer electrons to other phenolics or antioxidants, promoting their chemical regeneration.

The number and pattern of hydroxyl substitutions on the B-ring are associated with the highest antioxidant activity (Cao *et al*., 1997). The structural differences existing between H and OL could be responsible for the opposite effect (antagonistic effect) of interaction in these mixtures. It is possible that in the interactions between the flavonoids a hydrogen-bonding between the flavonoids may occur, decreasing the availability of the hydroxyl groups, which may in turn reduce the possibility of interaction with the radical DPPH.

As stated earlier, both DPPH and TEAC assays are SET-assays, thus the correlation in the two assays are estimated to be the same. Again, as in the DPPH assay, synergism is observed in all the samples as the difference between O and E was evaluated (Figure 4.3.1.B). Again it was noticed that WH played a small role in the antioxidant activity as there was no significant difference between ALL-H-WH and ALL-H-OL-WH. The groups ALL-H, ALL-OL, ALL-WH, ALL-H-WH and ALL-OL-WH all showed no significant difference which may cause us to believe that the TEAC assay became saturated and were not able to function properly.

As seen in the DPPH assay, the TEAC assay (Table 4.2) has a ratio effect where H + OL is the highest. This may be due to the high antioxidant content for both of these components. Again, H and WH showed to have 2 x the fold increase in antioxidant activity whereas OL has 3 x the fold increase. This can also be related back to why H + OL has the highest ratio effect and the fact that H and OL contributed 11.83% and 32.86% each to antioxidant activity.

As observed in both DPPH and TEAC assays again there can be synergism observed in all the samples as the difference between O and E was observed (Figure 4.3.1.C). Again WH seems not to contribute significantly to the antioxidant activity in combination with OL, but the opposite effect occurs when in combination with H as seen in Figure 4.3.1.C where ALL-OL has the highest activity. H alone has a higher antioxidant activity

when compared to when it is in combination with OL, giving it an antagonistic effect between the two components.

As pointed out in chapter 2, ORAC is an HAT-assay, and we suspected that the ratio between H, OL and WH would differ as seen in Table 4.2. Again All-OL showed to have the most antioxidant activity as confirmed by Figure 4.3.1.C. The fold increases for All-WH-H, All-WH-OL and All-H-OL were all only an 1 x increase.

5.6. CELLULAR ANTIOXIDANT EFFECTS

Of the constituents of FCG (FP): OL, WH, H and VE showed to have significant cellular effects. All the components have been tested on some sort of cellular model such as OL which was known for its anti-tumor/anti-metastatic effect in breast and prostate cancers (Acquaviva *et al*., 2012, Hassan *et al*., 2012); H has shown a high degree of intracellular protection against oxidative damage in fibroblast cell lines (Serem, 2011); VE in combination with selenium, an antioxidant, potentiates vitamin E-induced inhibition of LNCaP (epithelial cells derived from a human prostate carcinoma) cells (Venkateswaran *et al*., 2004), and WH has a protective function in red blood cells in radical-induced hemolysis and on the other hand also inhibited the proliferation of tumoral SK-Mal 28 melanoma cells (Touriño *et al*., 2008). It has also been reported by Habtemaraim (2002) that hamamelitannin inhibits DNA fragmentation and tumour necrosis factor-α (TNF- α) – mediated endothelial cell death. At minimum concentrations, it has been found that against peroxides, hamamelitannin has a high protective activity against cell damage (Masaki *et al.*, 1995).

FCG (FP) a wound healing product containing all the above components in this study was found not to cause oxidative damage/not cytotoxic and was able to protect SC-1 cells against oxidative damage. The contribution of individual components was then evaluated. From this data an expected cellular protective effect could be calculated for different combinations such as H+OL, OL+WH, H +WH as well as H+OL+WH. This was compared to experimentally generated data. This observed value was then used to calculate if synergism had occurred between major components. Synergism was found for all combinations and indications were that the minor components contributed to this effect.

Little information is available related to antioxidant synergistic effects but synergistic effects related to antibacterial and anticancer activity has been described for natural products or derived products in combination with drugs. Jenkins and Cooper (2012) showed Manuka honey in combination with ocacillin restored oxacillin susceptibility to methicillin-resistant *Staphylococcus aureus* (MRSA) whereas Fernandez-Cabezudo *et al*., 2013 concluded that when paclitaxel (mitotic inhibitor used in cancer chemotherapy) in combination with honey was intravenously injected in a melanoma mouse mode, it showed an anti-cancerous effect. Carrera-González *et al.,* 2013 studied the ability of oleuropein aglycone to exhibited synergistic antitumor effects when concurrently given to breast cancer cells chronically exposed to trastuzumab (a monoclonal antibody that interferes with the HER2/neu receptor used in the treat of metastasized breast cancer).

5.7. VITAMIN E

Of the minor components VE has extensively reported antioxidant activity (Niki *et al.,* 1995). VE was also evaluated on cellular level due to the high antioxidant activity shown in DPPH and TEAC assays. In a cell based model system with AAPH as a source of oxidative damage, VE and OL showed strong synergistic protective effects while the effect of VE and WH was antagonistic. In a study done by Podhaisky *et al.,* 1997, the authers reported that vitamin E, at sub-threshold concentrations, had a potentiating effect on endothelial protection by aspirin. Leung *et al.,* 1981 observed a synergistic effect between vitamins C and E in the delay of malondialdehyde production during Fe ^{2}+-catalyzed peroxidation of rat liver microsomes and phospholipid liposomes.

In conclusion, FCG and bioactive ingredients, H, OL, WH and VE have significant levels of antioxidant activity and cellular protection against oxidative damage and this is due to synergism between antioxidant ingredients. This is the first study where synergistic effects have been described between H, OL, WH and VE, however the precise mechanism still needs to be elucidated.

CHAPTER 6: CONCLUDING DISCUSSION

6.1. Rationale for study

Flavonix Cytoflamm Gel was classified as a complementary medicine and thus may not have been subjected to stringent laboratory based evaluations for i) activity, ii) toxicity and iii) efficacy. To aid in this process the purpose of this study can be divided into 3 segments. Firstly, it was an ideal opportunity to develop a model to evaluate complex mixtures since there is a lack in this area of research. Most research done is where a single ingredient is taken, for instance an orange, and the different components within the orange with the highest antioxidant activity is used to create combination with either just two components (x + y) or three components (x + y + z). No literature is available where a complex product such as FCG is evaluated, where the product consists of 4 or more ingredients and where these ingredients can be broken down into smaller active components, and where the effect would have as a whole or the interaction between each component.

Secondly was to determine FCG antioxidant content and then the antioxidant activity thereof using different assays. FCG itself was also tested as a whole. Individual components and combinations were evaluated. In this study the product in question consisted of 7 bioactive components. The components were divided into 2 groups, namely the major and minor bio-active components. To obtain the different combinations, it was thought that instead of adding each component and then determining the effect, it would be more time saving to remove an individual component out of the combination and then the contribution of each component to total activity. Another factor contributing to the activity of these combinations were whether they interact with each other namely in a synergistic, additive or antagonistic manner.

Thirdly, it was important for this study to determine the effect of the FCG, the individual components as well as the combination on a cellular level. Cytotoxicity of FCG and its components were determined as well as the cells ability to protect itself against radical oxidation. The synergistic, additive and antagonistic effect of the combination was also calculated to determine the best combination to use to prevent any cellular damage.
Cytotoxicity, and the product's ability to protect the cells at different concentrations was determined.

6.2. SUMMARY OF RESULTS

FCG is a commercially based product as a complementary medicine. Due to the lack of testing method and knowledge of possible interactions for complex multi-component products, the method in this thesis has proved to be very useful.

FCG consists of seven bioactive components namely H, OL, WH, X, VE, AV and Fa. Concentrations were used related to the original formulation with x > 5% to be major components and x < 5% to be minor components. Combinations with the major components were made where one, two or three of the components were left out of the sample to study the effect of the remaining components i.e. xyz - y = xz. The same principle were applied to the minor components. Thus the interactions between the components could also evaluated by using this method to determine whether a synergistic, additive or antagonistic effect exists.

With the individual components and combination the antioxidant content was determined by using the TPC and TFC assays. In TPC and TFC H, OL and WH showed to have the most polyphenolic and flavonoid content (> 85% and 91% total content respectively) and this is again reflected in the combinations where these components were present.

The same samples were then taken to determine the antioxidant activity by using the DPPH, TEAC and ORAC assays. It could then be determined if significant levels of TPC and TFC translated into antioxidant activity. Major components H, OL and WH (DPPH; TEAC and ORAC assay) as well as the minor component VE (DPPH and TEAC assay only) presented with high levels of antioxidant activity. It was thus decided to include VE in the cellular studies. In the DPPH, TEAC and ORAC assays all combinations showed a synergistic effect.

A cellular assay was undertaken to determine whether the individual components and combinations with high antioxidant activity translates into significant levels of cellular antioxidant activity. This was undertaken *in vitro* using the SC-1 cell line with 2,2'-azobis(2-amidinopropane) dihydrochlorde (AAPH) as the source of oxidative damage. Serial dilutions of all components and combinations were prepared and tested on the SC-1 cell line for toxicity. Effects were measured using the dichlorofluorescein diacetate assay (DCFH-DA). All the samples showed minimal cytotoxicity with All-H and All-H-WH showing the greatest protective effect. Secondly, the contribution of VE to cellular

protection was evaluated. In this study, VE in combination with H, WH or H+WH showed increased synergistic protection.

In conclusion the antioxidant activity and cellular protection of FCG against oxidative damage was due to strong synergistic interactions between H, OL, WH and VE where the type of antioxidant and the concentrations of different components were related to the antioxidant and protection effects.

6.3. IMPLICATION OF STUDY

This is the first study in which this strategy of deducting components out of a mixture rather than to add them have been used to evaluate the antioxidant activity of a complex mixture. Using this method synergism between different components could be evaluated. This method worked well especially with the high number of components that had to be tested in combination. Thus, this method can be used to optimize the ingredient content by using bioassay directed optimization and this method can be also be applied to determine the antibacterial activity and anti-inflammatory of FCG and many other complementary medicine products that are usually complex.

6.4. LIMITATIONS OF STUDY

Some limitations was detected during the course of the study:

The effect of each of the components (H, OL, WH, VE, X, AV and Fa) on cellular structure and morphology is unknown.

Only ingredients from a single source, a single batch of each ingredient and a single batch of FCG (FP) was used. Thus these results are only pertaining to this specific source and batch that was obtained. However, this strategy of testing can be applied to different batches of FCG and the individual components which can make an essential contribution to quality control.

In FCG quality control is an essential part of product evaluation. Liquid chromatography tandem mass spectrometry (LC-MS/MS) can be used to identify and quantify the constituent molecules of each ingredient. This especially is important in the case of plant derived products where the concentration of the bioactive component is a function of seasons, geographical location and source.

As this is a wound healing product, this product was only evaluated using fibroblasts and this does not reflect the complexity of skin that contains keratinocytes, melanocytes, Merkel's and Langerhan's cells.

In addition VE contributed significantly to antioxidant activity and cellular protective effects when combined with other antioxidants. This means that VE although added at very low concentrations compared to H, WH and OL had a major effect on total activity which could not be predicted.

6.5. FUTURE DIRECTIONS

The main aim in the business world is to develop a "must have" product that is effective, safe in every aspect and cost effective to the company. To do so the following suggestion can be taken into consideration to create a product that satisfy every intended used to the maximum as described by the manufactures of FCG (FP):

Future directions should include the identification of the bioactive molecules in H, OL, WH, VE, Fa, X and AV. With all molecules identified, combinations of bioactive molecules can be prepared to determine synergistic effects between these molecules. Then this information can be used to formulate a product with the highest degree of efficacy related to antioxidant, antimicrobial and anti-inflammatory properties. In addition the toxicity of these combinations must also be determined. A disadvantage of this type of evaluation is that a more defined product is usually more expensive due to the cost of the purified individual components.

That the effect of H, OL, WH, VE, Fa, X and AV be investigated in a cell line such as keratinocytes and/or fibroblast cells using scanning electron microscopy. Fluorescence can be used to determine if H, OL, WH, VE, Fa, X and AV can protect the keratinocytes and/or fibroblast cells against AAPH induced appoptosis.

The methodology used in this thesis can be reapplied to evaluate the antimicrobial activity of FCG against common skin pathogens such as *Staphylococcus epidermidis* and *S.aureus* including biofilm formation. Cell based studies can be followed by animal studies where for example in a rat model, burn wounds can be inoculated with *S. epidermidis* and *S. aureus*. The wounds would then be treated with FCG and the

"golden standard" in the market such as Medihoney® Gel. In this single model antibacterial, anti-inflammatory and antioxidant activity can be evaluated by measuring levels of bacteria, anti-inflammatory and antioxidant markers in the wound exudate.

Effects of other nutraceutical ingredients such as plant extracts, retinoic acid, essential oils, and alkaloids can be evaluated which has recently been found by Tsala *et al*., (2013) to have wound healing properties. The effect of these ingredients in combination with those found in FCG can be evaluated using the model and techniques presented in this thesis to develop a better wound healing product.

Anti-inflammatory effects can also be investigated using the RAW 264.7 cell model to evaluate the effect on inflammatory biomarkers. In a study done by Huo *et al*., 2013, gossypol, a polyphenolic compound derived from cotton plants, was tested on inflammation in lipopolysaccharide (LPS) stimulated RAW 264.7 cells and LPS induced *in vivo* lung injury model. The signaling pathways and pro-inflammatory effect of gossypol was evaluated by enzyme-linked Western blot and immunosorbent assay. The same could be done for this product and its components.

Other cellular models can be used to evaluate FCG more extensively especially related to wound healing using isolated human keratinocytes and fibroblasts in the scratch wound model. This can be followed up in a study using the pig model as earlier for the rat model. The advantage of this model is that histologically the skin of the pig is similar to human and healing can be further evaluated with different staining methods and immunocytochemistry. Besides using UMF manuka honey, Apinate or Medihoney® Gel as controls, the wound healing properties of FCG can be compared to drug based products such as Acticoat®, an antimicrobial wound dressing.

Further studies will involve clinical trials. After obtaining clearance from the Medicines Control Council (MCC) as well as ethical clearance the modified FCG could then be tested on patients with chronic wounds in a setting such as the diabetes clinic of Steve Biko Academic Hospital.

In conclusion, laboratory based studies have shown FCG to have significant antioxidant activity and cellular protection properties that will contribute to wound healing.

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