

## Review Article

# Problems of Reproducibility and Efficacy of Bioassays Using Crude Extracts, with reference to *Aloe vera*

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**ABSTRACT:** Aloe vera has a long history of medicinal usage and its biological activities have been well documented in a variety of bioassays. However, isolated Aloe vera leaf components generally do not display the same bioactivities, or have lower efficacies than crude juice/extracts. It is likely that several components work in a synergistic manner in the crude mixture, resulting in increased bioactivities. Furthermore, different laboratories often report varying bioactivities using the same extraction procedure on plant material from the same species. Individual Aloe vera cultivars may have widely varying levels of the bioactive phytochemicals. Due to the structure and chemical nature of many of the Aloe vera phytochemicals, it is likely that many of its reported medicinal properties are due to anti-oxidant or pro-oxidant effects. The anti-oxidant/pro-oxidant activities of many of Aloe vera's phytochemicals is dependent not only on their individual levels, but also on the ratios of various components, and on their individual redox states. Therefore, discrepancies between bioactivity studies are likely when using different crude mixtures. The potential differences between these crude mixtures need to be taken into account when analysing the reproducibility and efficacy of bioassays of crude extracts.

**KEY WORDS:** *Aloe barbadensis* Miller, Aloe vera, anti-oxidant, pro-oxidant, medicinal plant, crude extracts.

## INTRODUCTION

Plants have a long history of usage as medicinal agents and were the main source of medicines prior to the advances of modern medicine. In many developing countries, herbal medicinal systems remain important in the treatment of many ailments. Ayurvedic medicine is still commonly practiced within India with an estimated 85% of Indians still using crude plant preparations for the treatment of a wide variety of diseases and ailments.<sup>[1]</sup> Traditional Chinese medicine (TCM) and African medicinal systems also account for a major portion of health care in these regions. Even in countries where allopathic/Western medicine is dominant, much is also owed to plant medicinal systems. Furthermore, many users are returning to herbal medicinal systems due to the perception that natural medicines are often safer than allopathic drugs, as well as seeking treatments to diseases for which modern medicine does not yet have solutions.

Many of the prescription drugs currently marketed for a wide variety of ailments were originally isolated from plants or are

semi-synthetic analogues of phytochemicals. It has been estimated that approximately 25% of all prescription drugs currently in use are of plant origin.<sup>[2,3]</sup> Furthermore, approximately 75% of new anticancer drugs marketed between 1981 and 2006 were derived from plant compounds.<sup>[3]</sup>

Traditionally, plant based medicines have been used as crude formulations such as infusions, tinctures and extracts, essential oils, powders, poultices and other herbal preparations. The current trend is to isolate and characterise the individual phytochemical components with the aim of producing an analogue of increased bioactivity/bioavailability. Such studies have given rise to many useful drugs such as quinine (from *Cinchona* spp.) and digoxin (from *Digitalis* spp.) as well as the anticancer drugs vincristine and vinblastine (from *Vinca rosea*). However, the bioactivities seen for crude extracts are often much enhanced, or even totally different to those seen for the individual components.<sup>[4,5]</sup> Crude plant extracts may contain hundreds, or even thousands of different chemical constituents that interact in complex ways. Often it is not known how an extract works, even when its therapeutic benefit is well established.

The study of crude extracts is itself fraught with difficulties. Plants grown under varied conditions will often produce different phytochemical profiles, or at least different quantities of the individual components.<sup>[6,7]</sup> Similarly, different cultivars within a

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species may also produce different levels of other bioactive components or other constituents which enhance/counteract their medicinal activities.<sup>[8]</sup> Therefore, the bioactivity of crude extracts may be reliant on the conditions in which the plant grows, the season, and the individual plant itself. Other contributing factors may even include induced chemical defences against predators or pathogens. The extraction procedure, treatment and handling of crude plant extracts may also affect the condition and therefore the bioactivity/efficacy of the phytochemical components.

Most plant extracts contain a complex mixture of terpenes, phenolic compounds and alkaloids, many of which can undergo oxidation/reduction processes.<sup>[6]</sup> The alteration of the redox state may change the behaviour of phytochemicals. Indeed, the maintenance of cellular redox state has been associated with the treatment and prevention of many diseases and ailments including atherosclerosis, inflammatory injury and cancer,<sup>[9,10]</sup> cardiovascular disease<sup>[11]</sup> and neurological degenerative disorders.<sup>[12]</sup> Redox control is also linked with diabetes/anti-diabetic bioactivities<sup>[13]</sup> and has been associated with the reduction of obesity.<sup>[14]</sup> Anti-oxidants can directly scavenge free radicals, protecting cells against oxidative stress related damage to proteins, lipids and nucleic acids.<sup>[15]</sup> The following discussion will examine some problems associated with reproducibility and efficacy of using crude extracts in bioassays, with reference to the well characterised medicinal plant, Aloe vera.

## VARIABILITY IN BIOACTIVITY AND EFFICACY OF CRUDE ALOE VERA EXTRACTS

*Aloe barbadensis* Miller (commonly known as Aloe vera) has a long history of usage as a food, cosmetic and as a medicinal agent. Amongst its noted therapeutic activities, Aloe vera has been reported to have anti-bacterial,<sup>[16,17]</sup> anti-fungal,<sup>[16]</sup> anti-viral,<sup>[18,19]</sup> immune-stimulatory,<sup>[20]</sup> anti-inflammatory<sup>[21,22]</sup> and

anti-diabetes<sup>[23]</sup> bioactivities. However, many studies examining the therapeutic potential of Aloe extracts report conflicting results, showing either a lack of therapeutic bioactivity for some Aloe species,<sup>[24]</sup> or even toxicity associated with some Aloe vera preparations.<sup>[25-27]</sup>

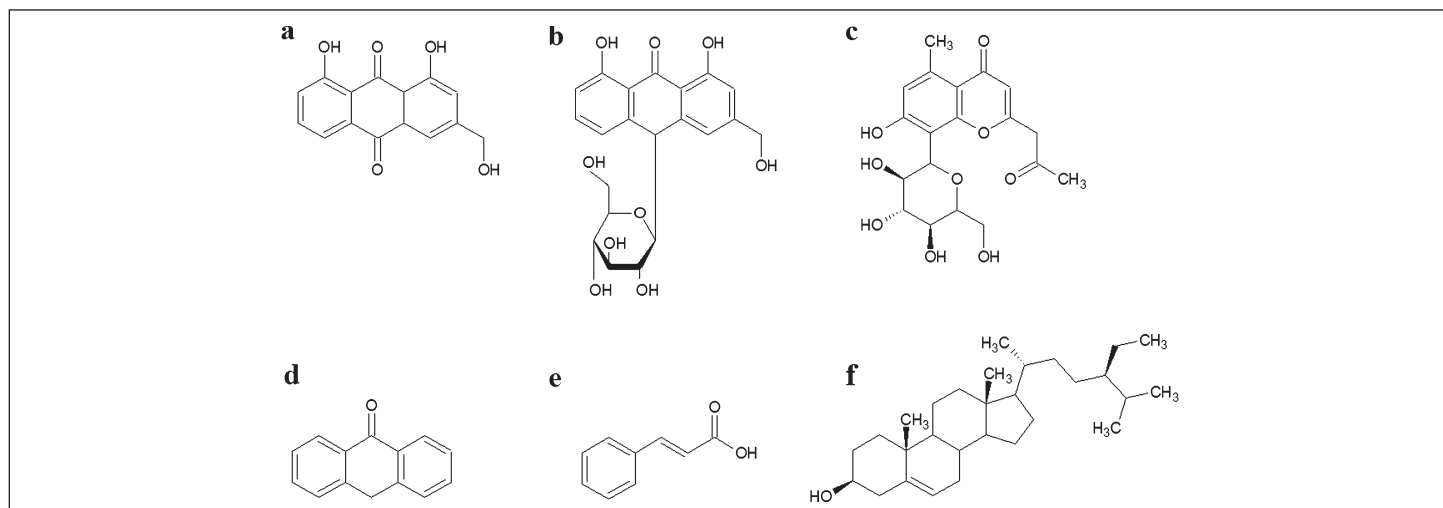
It is well known that plant age is an important determinant of Aloe vera bioactivities. With respect to anti-oxidant potential, the bioactivity has been shown to fluctuate within a given cultivar in relation to the age of the plant, with highest anti-oxidant levels reported for 3 year old plants.<sup>[28]</sup> This is complicated further as the relative levels of a plants anti-oxidant phytochemicals also fluctuates seasonally.<sup>[29]</sup> Furthermore, the phytochemical profiles of individual plants will change, dependent on a variety of other environmental and growth conditions.<sup>[6,7]</sup>

Plants may produce a wide variety of secondary metabolites which have no apparent role in primary plant growth or development processes. These molecules are often unique to plants from a single species and increase during times of high stress such as drought, fire and bacterial infection.<sup>[6]</sup> Therefore, whilst Aloe vera plant growth may be optimal during times of good growth conditions, it is likely that the level of useful bioactive phytochemicals will be elevated in conditions which stress the plant. Many of these secondary metabolites may exhibit anti-microbial, anti-oxidant, cytotoxic and other medicinally useful properties.<sup>[6]</sup>

## A. BARBADENSIS PHYTOCHEMISTRY

### Anthraquinones

Many bioactive phytochemical components have been isolated from Aloe vera leaves and their bioactivities extensively examined. In particular, the anthraquinones, anthrones and chromones have been particularly well studied and have been shown to be effective at counteracting various disease states.<sup>[21,30]</sup> The anthraquinones aloe emodin (Figure 1a) and aloin (Figure 1b)



**Figure 1:** Chemical structures for the anthraquinones (a) aloe emodin and (b) aloin, the chromone (c) aloesin, (d) anthrone, (e) cinnamic acid and (f)  $\beta$ -sitosterol.

are thought to exert their reported therapeutic potentials via an anti-oxidant mechanism. For example, aloe emodin has high inhibitory free radical scavenging activity and has been shown to act as an anti-oxidant by inhibiting lipid peroxidation.<sup>[31]</sup>

Interestingly, aloe emodin and aloin have been shown to be capable of behaving as either an anti-oxidant or as a pro-oxidant, with their action being dependent upon their concentration.<sup>[32]</sup> Aloe emodin exerts anti-oxidant behaviour at lower concentrations, yet acts as a pro-oxidant at high concentrations. In contrast, aloin has an anti-oxidant effect at higher concentrations, yet a pro-oxidant effect at low concentrations. Thus, the variable effects reported for crude Aloe vera extracts in various studies may be due to differing level of aloe emodin and/or aloin present in the extract.

### Other phenolic Aloe vera constituents

Similar pro-oxidant effects have been reported for other anti-oxidant phytochemicals including flavonoids,<sup>[33]</sup> tannins<sup>[34]</sup> and curcumin.<sup>[35]</sup> Previous studies have shown that transition metal ions, such as copper or iron, can enhance the conversion of the anti-oxidant to the pro-oxidant state.<sup>[36,37]</sup> The pro-oxidant/anti-oxidant effect of plant extracts is due to a balance between the free radical scavenging activities and reducing power of their phytochemical components. This can be explained using the anti-oxidant vitamin ascorbic acid as an example. Although ascorbic acid has well characterised anti-oxidant bioactivities, it is also known to act as a pro-oxidant at high concentrations.<sup>[38]</sup> This is due to the greater reducing power of ascorbic acid compared to its free radical scavenging activity. In the presence of transition metal ions, ascorbic acid will function as a reducing agent, reducing the metal ions. In the process, it is converted to a pro-oxidant. Therefore, high dietary intake of ascorbic acid in individuals with high iron levels (e.g. premature infants) may result in unexpected negative health effects due to the induction of oxidative damage to susceptible biomolecules.<sup>[39-41]</sup>

The anti-oxidant activity of aloesin (Figure 1c) and other chromones has also been extensively described.<sup>[42,43]</sup> In contrast, a literature search did not reveal any studies examining the potential pro-oxidant activity of these compounds. One study reported several chromones to have higher reducing power than ascorbic acid.<sup>[42]</sup> The relatively high reducing power of ascorbic acid is believed to be responsible for its ability to function as a pro-oxidant.<sup>[38]</sup> It is therefore possible that aloesin and other Aloe vera chromones may have a similar anti-oxidant/pro-oxidant profile to ascorbic acid (i.e. anti-oxidant activity at lower concentrations and pro-oxidant activity at higher concentrations). However, it must be emphasised that this possibility is based on the reported higher reducing power of the chromones compared to their free radical scavenging activity<sup>[42]</sup> and has not been adequately tested.

Cinnamic acid (Figure 1e) and its derivatives are phenolic molecules which are present in many fruits, vegetables and whole grains, as well as in Aloe vera leaves. Studies indicate that cinnamic

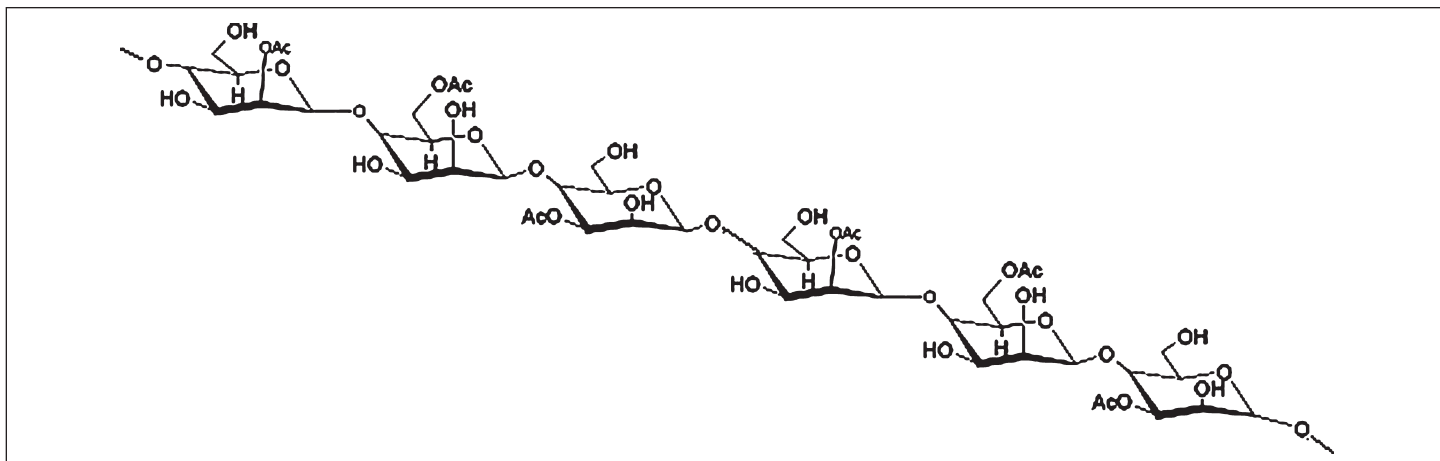
acid derivatives also have concentration dependent anti-oxidant/pro-oxidant activities. Cinnamic acid derivatives behave as anti-oxidants at lower concentrations, but convert to pro-oxidants at concentrations above 5  $\mu\text{M}$ .<sup>[44]</sup>

In contrast, Yen et al. (2000) demonstrated that the chemical structure of anthrone (Figure 1d) predisposes it to function as an electron acceptor (electrophile), hence as a strong anti-oxidant, independent of its concentration within an extract.<sup>[31]</sup> It therefore remains possible that Aloe vera extracts with high concentrations of anthrone may maintain anti-oxidant potential, even under conditions which would otherwise predispose the extract to function as a pro-oxidant. For example, Aloe vera extracts containing high aloe emodin and low aloin concentrations (both of which favour pro-oxidant bioactivity) may still function as an anti-oxidant if high enough levels of anthrone are present to maintain the redox state of these anthraquinones. Conversely, low levels of anthrone may predispose an extract to display pro-oxidant activities. It is therefore likely that the redox character of an extract is not only dependent on the levels of the different phytochemicals present, but also on the ratios of several important components within the mixture.

Aloe vera leaves also contains a number of other medicinally important phytochemicals including  $\beta$ -sitosterol (Figure 1f) and  $\beta$ -sitosterol glucosides. These phytosterols have been shown to promote arterial endothelial cell proliferation.<sup>[45]</sup> They also promote the expression of proteins involved in angiogenesis and thus have potential applications in the management of chronic wounds. Recently,  $\beta$ -sitosterol has also been trialled for the treatment of breast cancer<sup>[46]</sup> and diabetes,<sup>[47]</sup> although the efficacy is still under investigation. It appears that these therapeutic bioactivities may be due, at least in part, to their redox state of the molecule. A recent study has indicated that  $\beta$ -sitosterol treatment results in glutathione reduction as well as maintaining the anti-oxidant enzymes superoxide dismutase and glutathione peroxidase in a reduced state.<sup>[48]</sup> This bioactivity in turn is related to the redox state of the sterol. Interactions between the various components within the crude extracts may also play a role in converting otherwise anti-oxidant molecules into pro-oxidants in the extract or vice versa.

### Non-phenolic components

Other phytochemical components of Aloe vera leaf extracts include acemannan (Figure 2), a long chain polymer of  $\beta$  (1 $\rightarrow$ 4) linked galactomannan saccharides.<sup>[49,50]</sup> Acemannan has been reported to accelerate wound healing,<sup>[51-54]</sup> activate macrophages<sup>[55,56]</sup> and have synergistic anti-viral activity in combination with azidothymidine and acyclovir.<sup>[19]</sup> It has been reported that acemannan also has anti-oxidant properties and that these properties may be responsible for its therapeutic activities.<sup>[57]</sup> Furthermore, the anti-oxidant potential of Aloe vera polysaccharides is dependent upon the concentration of the molecule and the degree of acetylation of the monomeric units.<sup>[58]</sup> High polysaccharide concentrations ( $>8 \text{ mg mL}^{-1}$ ) were found



**Figure 2:** The structure of acemannan (a major polysaccharide component of Aloe vera leaves) consists of a polymer of  $\beta$  (1 $\rightarrow$ 4) linked galactomannan sugars.

to be necessary for Aloe vera polysaccharides to display anti-oxidant activity. The same study also showed that increased acetylation enhances the anti-oxidant activity of Aloe vera polysaccharides.<sup>[58]</sup> However, the polysaccharide components within Aloe vera leaves are not constant. Instead, the composition and concentration of the polysaccharides fluctuate with changes in the growing environment and conditions.

Aloe vera leaves also contain inorganic minerals in variable concentrations (e.g. calcium, magnesium, zinc, iron and copper). As previously discussed, the redox state of many Aloe vera phytochemicals is affected by the presence of metal ions, converting otherwise anti-oxidant components into pro-oxidants. Thus, Aloes growing in soil containing elevated levels of metallic ions would be expected to have higher concentrations of metal ions, and thus tend towards pro-oxidant rather than anti-oxidant bioactivities. Other molecules (such as vitamins, amino acids and proteins) may also have an effect on the redox state of the phytochemical components.

## MEDICINAL EFFECTS OF ALOE VERA REQUIRING MULTIPLE PHYTOCHEMICALS

The multitude of phytochemicals present in an Aloe vera crude extract not only affect each others redox state and ability to function as an anti-oxidant/pro-oxidant, but several phytochemicals may also be required for different aspects of the same therapeutic effect. Some of the medicinal properties associated with plant extracts require the concerted action of several bioactivities. The following discussion examines several therapeutic properties of Aloe vera extracts that require the synergistic action of several bioactivities, each of which may be reliant on multiple phytochemicals. This is by no means a complete examination of the therapeutic properties of Aloe vera extracts, but instead serves to illustrate the difficulties of assigning a therapeutic effect to a single component. Similarly, it further illustrates the

problems with reproducibility when analysing crude extracts by bioassay due to differences in the levels of specific phytochemicals, their redox state, and their ratio to other components.

### Anti-inflammatory Activity

Inflammation is a complex response by the body to injury. It typically follows a variety of insults including burns, wounds, bites and stings etc. It is characterised by a wide variety of symptoms<sup>[59]</sup> including:

- Swelling. Injury may result in increased capillary permeability which allows leukocyte migration and fluid accumulation in the damaged tissue. This accumulation results in the swelling characteristic of inflammation.
- Redness and heat are caused by vasodilation, reducing blood pressure and increasing circulation.
- Pain is a complex reaction resulting from the release of short peptides and prostaglandins.

These inflammatory processes require the cellular release of several classes of molecules. Vasoactive substances (e.g. bradykinin, prostaglandins and vasoactive amines) are required to dilate blood vessels, opening junctions between cells to allow leukocytes to pass through capillaries. Any compound capable of blocking these vasoactive substances would potentially have a therapeutic effect on the symptoms of inflammation.  $\beta$ -sitosterol is the most abundant phytosterol in Aloe vera extracts.  $\beta$ -sitosterol stimulates smooth muscle cells to release of prostacyclin ( $\text{PGI}_2$ ).<sup>[60]</sup> However,  $\beta$ -sitosterol treatment blocks the release of  $\text{PGI}_2$  and prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ) from macrophages.<sup>[60]</sup> Thus,  $\beta$ -sitosterol treatment would be expected to affect vasodilation and, therefore, have a therapeutic effect on inflammation. The Aloe vera leaf chromone aloesin, and its derivatives, inhibit cyclooxygenase-2 and thromboxane  $\text{A}_2$  synthesis through their anti-oxidant activities.<sup>[61,62]</sup> Thus, Aloe vera chromones produce anti-inflammatory effects. In contrast, anthraquinones have been shown to stimulate  $\text{PGE}_2$  release<sup>[63]</sup> and would, therefore, be expected to promote pro-inflammatory activity.

The peptidase bradykinase has been isolated from Aloe vera leaves and has been shown to break down the vasoactive peptide bradykinin.<sup>[64,65]</sup> As bradykinin treatment results in vasodilation, hydrolysing this protein would result in decreased vasodilation and, therefore, inhibit leukocyte passage and fluid leakage from the capillaries into the surrounding tissue. Aloe vera leaf bradykinase would, therefore, be expected to contribute to the therapeutic effects on the symptoms of inflammation.

Chemotactic factors, including several proteins and peptides, are required to increase cell motility, especially the motility of leukocytes during inflammation. Blocking these chemotactic factors, or blocking their effects, prevents inflammatory swelling. Several compounds in Aloe vera extracts have been shown to be capable of blocking chemotaxis. Anthraquinones suppress cytolytic T-lymphocytes in favour of suppressor cells.<sup>[66,67]</sup> Furthermore, anthraquinones decrease cytokine production and IL-2 mRNA expression in activated T lymphocytes,<sup>[68]</sup> thereby decreasing chemotaxis. More recent studies have demonstrated that the anthraquinone emodin decreases plasma levels of the cytokines IL-2 and TNF- $\alpha$ , whilst increasing IL-10 (which itself down-regulates IL-2 and TNF- $\alpha$  cytokine activity).<sup>[69]</sup> None of these studies, however, examined the relationship of the redox state of the anthraquinones with these effects. Furthermore, these studies have not rigorously examined the effects of a range of doses of these phytochemicals.

In contrast, Aloe vera polysaccharides (including acemannan) have a stimulatory effect on chemotaxis. Acemannan exposure stimulates cytokine production and activates lymphocytes.<sup>[70,71]</sup> Specifically, pure acemannan isolated from Aloe vera leaves has been shown to stimulate macrophages to release IL-1, IL-6, interferon, GM-CSF and TNF- $\alpha$  in vitro.<sup>[72]</sup> Similarly, Aloe vera lectins stimulate cytokine production. Aloctin A, the best characterised of the Aloe lectins, has been shown to stimulate the production of IL-2<sup>[73]</sup> and to enhance the production and activation of macrophages.<sup>[73]</sup> Therefore, Aloe vera extracts contain both chemotactic stimulatory and inhibitory compounds. The chemotactic effect of Aloe vera extracts would, therefore, be dependent on the levels and ratios of the factors affecting chemotaxis as well as their redox state.

Aloe vera extracts contain multiple active phytochemicals. It is likely that several of these may be required to address different aspects of the inflammatory process. Failure to consider this is likely to be responsible for past ambiguities about the efficacy of Aloe extracts in relation to its anti-inflammatory activity.

### Antiseptic activity

The interruption of the external epidermal barrier by a wound, burn or other such event allows microbes to enter and infect the wound. The invasion of microorganisms may cause or intensify inflammation (described in section 4.1.) and may hinder wound healing (described in section 4.3) and/or cause disease.

Aloe vera leaf extracts have been previously shown to display good anti-bacterial<sup>[16,74,75]</sup> and anti-fungal bioactivities.<sup>[16,76]</sup>

Early anti-bacterial studies of Aloe vera extracts have provided confounding and even contradictory results. Some of these studies indicate that the bioactive agent(s) are anthraquinones,<sup>[77,78]</sup> whilst other studies found Aloe vera anthraquinones to be inactive as anti-bacterial agents.<sup>[79]</sup> Numerous subsequent studies have demonstrated the anti-bacterial activity of isolated anthraquinones from Aloes<sup>[80,81]</sup> and various other plant species.<sup>[82-84]</sup> Whilst the mechanism of anti-bacterial activity is still subject to investigation, it has been suggested that aloe emodin and aloesin function by inducing bacterial membrane disruption.<sup>[80]</sup> This study also determined that the form of aloe emodin and aloesin tested also affects their anti-bacterial activity. It was demonstrated that anthraquinone loaded liposomes had strong anti-bacterial activity, whilst the purified free anthraquinones did not. It is, therefore, possible that some of the observed differences in the anti-bacterial activities of anthraquinones and Aloe vera extracts may be due to the form of anthraquinones that the bacteria were tested against. Whilst this study showed that anti-bacterial activity is dependent on the form of anthraquinone tested, the effect of concentration was not extensively examined. MIC values were determined by testing across a range of concentrations, although only relatively low concentrations were tested. It is possible that higher concentrations may have a very different effect, analogous to the concentration effects already described for anthraquinone anti-oxidant/pro-oxidant activity.

Other Aloe vera components have also been implicated in the antibacterial activity of leaf extracts. A recent study tested anthraquinone free leaf extracts and isolated components.<sup>[85]</sup> This study showed that cinnamic acid, coumaric acid, ascorbic acid and pyrocatechol purified from Aloe vera gel all display good anti-bacterial activity, especially towards Gram-positive bacteria. It was postulated that the phenolic anti-bacterial agents functioned by disrupting bacterial cell membranes, as well as by denaturing bacterial proteins. Furthermore, cinnamic acid is known to block bacterial glucose uptake and ATP production,<sup>[86]</sup> therefore, inhibiting bacterial growth. Coumaric acid has been shown to inhibit bacterial enzymatic activity.<sup>[87]</sup> A number of other phenolic components were also found to have low to moderate anti-bacterial activity.

In addition to direct inhibitory effects on bacteria, Aloe vera components may also function by selectively modulating the cells of the immune system (described in detail in section 4.4). Furthermore, acemannan also inhibits bacteria adhering to epithelial cells and establishing an infection.<sup>[88]</sup> It is likely that the anti-bacterial activity of Aloe vera extracts in vivo is due to the synergistic effects of multiple bioactive components, functioning through several mechanisms.

Anti-fungal activity has received less attention, although some studies have demonstrated the ability of Aloe vera extracts to

inhibit fungal growth.<sup>[16,76,89]</sup> Anthraquinones, especially aloe emodin and aloesin, were implicated in this anti-fungal activity,<sup>[76]</sup> however, the identity of anti-fungal components and their mechanisms of action have not been extensively examined. Similarly, the anti-viral activity of Aloe vera leaf extracts has been demonstrated,<sup>[18,90]</sup> although detailed purification, identification and mechanistic studies are required.

## Wound Healing

Whilst anti-inflammatory and anti-microbial bioactivities are complex processes requiring the synergistic action of several bioactivities, wound healing is more so. Wound healing, a relatively well studied therapeutic property of Aloe vera, is the result of several bioactivities including:

- Inflammation, which has summarised in section 4.1.
- Antiseptic bioactivity, which has summarised in section 4.2.
- Cell growth and proliferation
- Matrix remodelling

The growth of endothelial, epithelial and fibroblast cells are critical in wound healing. As a first step in wound healing, a fibrin clot is formed as a temporary repair. This step is vital as it helps avoid microbial infection which may retard the healing process. The wound is subsequently invaded by a variety of cell types, some of which stimulate an inflammatory response, and others which are directly involved in the repair mechanism.<sup>[91]</sup> The effects of Aloe vera extract components on inflammation processes and chemotaxis have already been summarised in section 4.1. Wound repair itself occurs in three phases: the migration of epithelial cells and fibroblasts to the wound site, proliferation of cells and cellular maturation. It is likely that the wound healing effect of Aloe vera extracts involves the synergistic action of multiple components on several pathways.

Aloe vera anthraquinones reportedly possess contradictory effects on cell growth and proliferation. For instance, Aloe emodin has been shown to stimulate a 2.5 fold increase in rat hepatocyte DNA synthesis with a corresponding increase in cell growth.<sup>[92]</sup> Additionally, aloe emodin has been shown to protect hepatocytes from apoptosis.<sup>[69]</sup> In contrast, other studies have shown aloe emodin to induce apoptosis in pro-myeloleukemic HL-60 cells<sup>[93]</sup> and human lung squamous cell carcinoma,<sup>[94,95]</sup> and to inhibit human neuroectodermal tumour growth.<sup>[96]</sup> Some studies have postulated that the pro-apoptotic effect of aloe emodin is due to an induction of caspase 3 activity, together with a decrease in the levels of the anti-apoptotic protein Mcl-1.<sup>[93]</sup> Another study has implicated caspase 8 mediated cleavage in the apoptotic activity of emodin.<sup>[97]</sup> Studies into the pro-apoptotic mechanism of aloe emodin are ongoing. Similarly, anthrones have also been shown to induce cell death. In a recent study, an anthrone from the Ethiopian medicinal plant *Kniphofia foliosa* was shown to induce rapid death in mouse and human cancer cells via necrosis.<sup>[98]</sup>

Other Aloe vera phenolic compounds have also been implicated in the wound healing effects of Aloe vera extracts.  $\beta$ -sitosterol and  $\beta$ -sitosterol glucosides promote endothelial cell proliferation and angiogenesis,<sup>[45]</sup> although their activity appears to be dependent on its redox state.<sup>[48]</sup> The reduced sterol has anti-oxidant activity and stimulates wound healing processes, whilst oxidised sterols are pro-oxidants and induce cell death.  $\beta$ -sitosterol and  $\beta$ -sitosterol glucosides, therefore, have potential applications in wound management in their reduced state. The Aloe vera chromone aloesin has also been reported to stimulate cellular proliferation.<sup>[51,61,99]</sup> It is possible that the proliferative effect of aloesin is due to its high anti-oxidant activity.<sup>[42,43]</sup> In contrast, cinnamic acid has been shown to down-regulate expression of cell proliferation and anti-apoptotic gene products, although the effects of both high and low concentrations were not examined.<sup>[100,101]</sup>

The redox environment affects cellular signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, regulation of the cell cycle, ligand binding, DNA binding and nuclear translocation, and therefore ultimately cell proliferation/death.<sup>[102,103]</sup> Transcription factors are active in their reduced form and their translocation to the nucleus is also redox dependent.<sup>[104]</sup> A reducing environment favours cellular proliferation whilst an oxidising environment results in an increase in reactive oxygen species, initiating cell death.<sup>[105,106]</sup> Therefore, extract conditions favouring anti-oxidant activity (e.g. low aloe emodin, high aloin, low cinnamic acid, low ascorbic acid, low transition metal and high anthrone concentrations) would be expected to favour cellular proliferation whilst conditions favouring pro-oxidant activity (e.g. high aloe emodin, low aloin, high cinnamic acid, high ascorbic acid, high transition metal and low anthrone concentrations) would favour cell death.

The non-phenolic components, particularly acemannan, have also been shown to have a role in wound healing. For example, the stimulation of gingival fibroblast proliferation has been demonstrated when treating oral wounds with high doses of acemannan.<sup>[107]</sup> This stimulatory effect was found to be due to an induction in expression of the growth factors KGF-1, VEGF and an increase in collagen expression. This study only examined the effects of relatively high concentrations of acemannan, in the range that would correlate to anti-oxidant activity. As lower concentrations may correlate to pro-oxidant activities, it is possible that the induction of fibroblast proliferation may not be seen at these concentrations. Indeed, as lower concentrations of acemannan correspond to pro-oxidant effects, it is possible that at lower concentrations, cell death may be induced. The concentration dependent redox effect of acemannan may also contribute to the discrepancies seen between proliferative studies of Aloe vera extracts.

As well as requiring cellular growth and proliferation, wound healing also requires matrix remodelling. Aloe vera gel extracts have been shown to stimulate and speed up the production of hyaluronic acid and dermatan sulphate.<sup>[52]</sup> Activities of the enzymes

$\beta$ -glucuronidase and N-acetyl glucosaminidase are increased during wound healing, resulting in increased carbohydrate turnover at the site of the wound. Other studies also demonstrated that wounded diabetic rats treated with Aloe vera gel show increased collagen formation<sup>[53]</sup> and cross linking.<sup>[54]</sup> It is evident that a synergistic action is required by several Aloe vera extract components on multiple wound healing associated bioactivities. The reported discrepancies between different studies may be due to differences in concentrations, ratios and redox states of these components.

### Immunomodulation

Manipulation of the immune system has therapeutic potential in the treatment of a variety of diseases. Aloe vera leaf extracts have been reported to have both good immuno-stimulatory<sup>[20]</sup> and immune-suppressive activities (as reviewed in Boudreau and Beland<sup>[108]</sup>); however, rigorous scientific examination of these effects is limited. Much of the studies into the immune-modulatory potential of Aloe vera extracts have focused on the immuno-stimulatory effects, particularly of the polysaccharide components. Whilst numerous Aloe vera polysaccharide components have been shown to have immune-modulatory effects,<sup>[109-111]</sup> acemannan has been particularly well studied. The immune-modulatory effects of acemannan are thought to be due to activation of macrophage cells and antigen processing. The activated macrophages secrete cytokines including IL-1, IL-6, interferon, GM-CSF and TNF- $\alpha$  in vitro.<sup>[72]</sup> The release of these cytokines is itself associated with further pathology through the induction of inflammation. Acemannan also enhances macrophage sensitivity to IFN- $\gamma$ , inducing apoptosis.<sup>[20]</sup> Neither acemannan nor IFN- $\gamma$  was capable of inducing apoptosis alone. Instead, a synergistic effect is required and this effect appears to function through the inhibition of the expression of Bcl-2 proteins.<sup>[20]</sup>

Studies have also highlighted the immune-modulatory properties of the smaller phenolic components of Aloe vera leaves. Aloe emodin and other anthraquinone derivatives have been shown to have an immune-suppressive effect by blocking lymphocyte proliferation.<sup>[66,67]</sup> Emodin also reduced IL-1, IL-2 and IL-2 receptor expression.<sup>[66]</sup> It was suggested that emodin suppresses both macrophages and lymphocytes. Further studies have identified 37 other anthraquinones with the ability to block cytolytic T lymphocyte induction and the ability to prevent antibody production.<sup>[67]</sup> The effect of concentration and the ratio between anthraquinones were not tested in these studies.

It has been postulated that Aloe vera extracts may exert immune-modulatory effects through their functioning as anti-oxidants, inhibiting/stimulating the production of free radicals.<sup>[28]</sup> Treating streptozotocin induced diabetic<sup>[112]</sup> or gamma-irradiated rats<sup>[113]</sup> with Aloe vera leaf extracts reduces lipid peroxidation and the formation of hydroperoxides whilst increasing the levels of anti-oxidant enzymes (e.g. reduced glutathione, glutathione peroxidase, glutathione-S-transferase, catalase, superoxide dismutase) in the liver, lungs and kidney. Similarly, Aloe vera gel has been shown to inhibit ROS production in colorectal mucosa

cells.<sup>[114]</sup> Interestingly, this study found the Aloe gel extract lacks this activity at either higher or lower concentrations, indicating a concentration dependence similar to that reported for the redox effects of Aloe vera components.<sup>[32]</sup> It is, therefore, possible that the variable immune-modulatory effects reported for Aloe vera extracts in different studies may be due to the concentrations, ratios and redox states of several important compounds in the tested extracts, with extract conditions favouring anti-oxidant bioactivity resulting in immune-stimulation. Conversely, conditions favouring pro-oxidant activity would be expected to result in immune-suppression, although this has not been extensively tested.

### Anti-Diabetic activity

Diabetes mellitus refers to a group of metabolic disorders that result in increased blood glucose concentrations, either because the pancreas does not produce enough functional insulin (type 1 diabetes), or because cells do not respond to the insulin which is produced (type 2 diabetes). The causes of diabetes mellitus include the auto-immune destruction of pancreatic cells,<sup>[115]</sup> viral infections,<sup>[116]</sup> genetic and environmental factors,<sup>[117]</sup> insulin or insulin receptor gene mutations<sup>[118]</sup> and altered pancreatic prostaglandin metabolism.<sup>[119]</sup> Diabetes has significant health effects, impacting on the quality of life and life expectancy of those suffering with it.

A number of studies have indicated the beneficial effects of Aloe vera extracts in diabetic patients.<sup>[23,120]</sup> Administration of Aloe vera extracts to streptozotocin-induced diabetic rats resulted in a decrease in blood glucose and a corresponding increase in liver glycogen.<sup>[120]</sup> The maintenance of glucose homeostasis by Aloe vera extracts in diabetic rats was shown to involve a number of mechanisms. Aloe vera extract treatment altered the activities of multiple enzymes: glycogen phosphorylase activity was decreased and glycogen synthetase increased, resulting in increased hepatic glycogen stores.<sup>[120]</sup> Hexokinase activity and mRNA levels were decreased in diabetic rats,<sup>[121]</sup> yet treatment with Aloe vera extract returned these parameters towards normal levels.<sup>[120]</sup> Similarly, increased lactate dehydrogenase, glucose-6-phosphatase and fructose-1, 6-bisphosphatase activities were seen in diabetic rats.<sup>[122]</sup> Aloe vera extract treatment significantly restored these enzyme activities.<sup>[120]</sup>

Glycosylation of blood proteins including haemoglobin, albumin and lipoproteins is also characteristic of diabetes mellitus.<sup>[123]</sup> Under the hyperglycaemic conditions of diabetes mellitus, blood glucose interacts with specific amino acids on the proteins surface, forming glycosylated protein products which may undergo a series of further chemical modifications resulting in the production of advanced glycation end products (AGE).<sup>[124]</sup> The binding of AGEs to their receptors results in altered cell signalling which in turn results in free radical production.<sup>[125]</sup> Indeed, diabetes mellitus has been shown experimentally to be associated with an increase in free radical formation and an associated decrease in anti-oxidant potential.<sup>[126,127]</sup> Studies have directly linked oxidative stress with

the impaired maintenance of glucose homeostasis and the enhanced lipid peroxidation seen in diabetes mellitus.<sup>[127]</sup> Furthermore, increased total anti-oxidant levels have been measured in the blood and saliva of diabetic patients, further supporting the proposed role of oxidative stress in diabetes mellitus.<sup>[128]</sup>

Oxidative stress induction has also been suggested to be the common link between the diverse medical complications (including cardiovascular disease, renal and neural degeneration, impaired vision and erectile dysfunction) seen in diabetes mellitus.<sup>[129,130]</sup> Therefore, treatment with anti-oxidants would be expected to counteract many of these complications. Aloe vera has a number of compounds (both phenolics and non-phenolic compounds) that can act as anti-oxidants (as described in section 3. - *A. barbadensis* phytochemistry). As many of these compounds can potentially behave as either anti-oxidant or pro-oxidant dependant on their concentration, redox state and ratio between compounds, it is not surprising that studies using Aloe vera crude extracts to treat diabetes mellitus have had mixed success.

### Anti- Cancer activity

The growth and development of healthy cells depends on fine regulation of growth promoting and inhibiting pathways. Proto-oncogenes and tumour suppressor genes are responsible for encoding proteins that regulate cell division/cell cycle, as well as for the repair of damaged DNA and cell programmed death by apoptosis. Mutations within these genes have been implicated in the onset of cancer.<sup>[131]</sup> Such mutations result in cells that no longer require external signals to proliferate. Furthermore, these cells fail to recognise signals that restrict cell division, resulting in uncontrolled cell growth. In tumour genesis, multiple genes may be altered and transmitted to daughter cells, which subsequently escape normal growth restraints and form a tumour, which may be benign or malignant.

The induction of oxidative stress has been linked with several types of cancer.<sup>[132,133]</sup> Chromosome instability is also a common feature of many of the cancers that have been linked with oxidative stress, suggesting that increased oxidative stress may contribute to development of genetic instability. Oxidative stress leading to genetic instability may result in the emergence of new tumour phenotypes. In such populations, a decrease in apoptosis but an increase in tumour growth and subsequent tumour progression is observable.

Currently used anti-cancer agents (e.g. doxorubicin, daunorubicin, mitomycin C, etoposide, cisplatin, arsenic trioxide, ionising radiation, photodynamic therapy) depend exclusively, or in part, on the production of ROS for cytotoxicity. Sensitivity of tumour cells to oxidative stress and/or apoptosis may affect treatment success.<sup>[134,135]</sup> Studies indicated that WEHI7.2 mouse thymoma cells over expressing catalase (CAT38) or thioredoxin (THX) were resistant to glucocorticoid-induced apoptosis in vitro.<sup>[136,137]</sup> This suggested that glucocorticoid-induced apoptosis occurred by a ROS dependant/independent mechanism. It was observed

that average tumour weights increased in severe combined immune-deficient (SCID) mouse tumour xenografts from cells over expressing catalase or thioredoxin.<sup>[137]</sup> Tumours from both transfectants contained fewer apoptotic cells but mitotic cell numbers were similar. This suggested that anti-oxidant over expression resulted in increased tumour size due to a decrease in apoptosis.

The cell proliferation/apoptosis inducing abilities of Aloe vera extracts and isolated components have been described in Section 4.3. Briefly, ROS based tumour therapy may induce regression in apoptosis/oxidant sensitive tumour cells. Thus, if Aloe vera components were present in concentrations and ratios consistent with pro-oxidant activity, the extract would induce apoptosis and, therefore, would have anti-cancer activity. If the levels of components were consistent with a reducing environment, anti-oxidant activity would result and the extract would not have anti-cancer activity. Conversely, should the protocol be repeated on a tumour with apoptotic resistant/oxidant resistant cells, the converse would apply and tumour progression would be likely.

## CONCLUSIONS

The problems associated with reproducibility and efficacy of bioassays using Aloe vera juice and/or crude extracts illustrates some of the difficulties encountered in natural products research. Individual extract batches may vary widely with regards to individual phytochemical profiles, ratios between various components, and the redox state of these components. These variances may have profound effects on the reported bioactivities and are likely to account for the reported discrepancies between different studies bioassaying crude mixtures. Despite these difficulties, the use of crude extracts is often necessary as the individual components often do not show the same bioactivities, or have different efficacy to crude extracts. This is true for Aloe vera. Aloe vera juice, or Aloe vera crude extracts, often display higher efficacy than the purified components. It is likely that the biological activity of Aloe vera is a synergistic and perhaps additive action of the different classes of compounds found within the plant, rather than a single constituent or just a few compounds. Furthermore, these compounds are required in the correct levels/ratios/redox states for bioactivity to be observed.

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