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A Water Soluble Extract from *Uncaria tomentosa* (Cat's Claw) is a Potent Enhancer of DNA Repair in Primary Organ Cultures of Human Skin

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Cat's Claw (*Uncaria tomentosa*) water extracts, essentially free of oxindole alkaloids, have been shown to possess a broad spectrum of biological activity including DNA repair enhancement and antiinflammatory properties. These two biological mechanisms are key molecular targets to develop treatments that protect skin exposed to ultraviolet light from the sun. Because C-Med-100, a Cat's Claw water extract, is the only documented natural source of components that can up-regulate simultaneously both DNA repair and antiinflammation, its ability to modulate DNA repair in human skin organ cultures was undertaken. For this purpose skin cultures were treated with or without 5 mg/mL C-Med-100, irradiated with 0–100 mJ/cm^2 UVB, and microscopically analysed for necrosis as well as the level of pyrimidine dimers using immunofluorescent TT-dimer antibody staining. The data clearly demonstrated that co-incubation with C-Med-100 reduced skin cell death from UV exposure, and this protection was accounted for by a concomitant increase in DNA repair. Based on these results, it was concluded that C-Med-100 was a natural plant extract worthy of further consideration as a sunscreen product. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords: UV induced thymidine dimers; DNA repair; UV radiation protection; C-Med-100; quinic acid esters.

INTRODUCTION

Cat's Claw (*Uncaria tomentosa*) extracts have been used for centuries as ingredients of natural medicines originating primarily from use by Shaman priests in South American Indians. The first class of active ingredients identified in 1967 that supported a health benefit were the oxindole alkaloids (Keplinger *et al.*, 1999). However, in the late 1990s it was discovered that when Cat's Claw bark was prepared in a manner similar to South American Native Indian use, in which the plant parts are boiled in water overnight before being administered orally, these preparations had powerful properties to enhance DNA repair, avoid DNA damage accumulation, stimulate immune cell function, inhibit inflammatory responses, and thus contribute to anti-aging health benefits. The more recent Cat's Claw water extracts manifesting these properties such as C-Med-100, were essentially free of oxindole alkaloids (<0.05%, w/w) but contained more than 8% carboxy alkyl esters (CAEs) as their active ingredients (Sandoval *et al.*, 2002; Sheng *et al.*, 2000b).

The CAEs in C-Med-100 give profound nutritional support as a dietary supplement because they both enhance DNA repair and immune cell responses which in turn are critical physiological processes that regulate aging (Pero *et al.*, 2002; 2005; Sheng *et al.*, 2000a; 2000b; 2001 and as cited herein). Both these processes involve regulating the nuclear transcription factor kappa beta (NF-kB). NF-kB is well known to control both the nuclear events that salvage cells from apoptotic cell death as well as pro-inflammatory cytokine production (Beg and Baltimore, 1996; Wang *et al.*, 1996). Hence, water soluble commercial extracts such as C-Med-100 directly connect the induction of apoptosis and programmed cell toxicity to the inhibition of pro-inflammatory cytokine production and inflammation.

Thus the inhibition of NF-kB has antiinflammatory properties because it prevents over reaction of the inflammatory process that can be very harmful to normal tissues of the body. In addition because pro-inflammatory cytokines are a major source of endogenous free radical production in humans, NF-kB inhibition is also antimutagenic by reducing genetic damage that might accumulate over time if not removed by DNA repair processes. The health consequence is that aging is curtailed because fewer radicals are produced to

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damage the DNA and to inhibit its natural repair. So Cat's Claw water extracts such as C-Med-100® are registered with the Federal Trade Commission, and as such are registered nutritional supplements as anti-aging remedies. This is true because they have been shown in the literature to prevent free radical damage via NF-kB inhibition, induce functional differentiation and immune cell responsiveness via the apoptotic pathway, enhance DNA repair, reduce inflammation, inhibit cell growth without inducing cell death, and kill tumor cells, all of which are in turn primary factors of the aging process (Lamm *et al.*, 2001; Piscoya *et al.*, 2001; Sandoval *et al.*, 2000; 2002; Sandoval-Chacon *et al.*, 1998; Sheng *et al.*, 1998; 2000a; 2000b; 2001; Åkesson *et al.*, 2003a; 2003b; Cisneros *et al.*, 2005). Furthermore, daily oral doses of C-Med-100 of 250–700 mg in humans were consistently shown to have efficacy.

Skin is the largest organ of the body and it can be viewed as the primary defensive barrier protecting the rest of our internal organs from the environment. Consistent with this specialized role in biological function, the skin has the capacity to absorb nutrients, metabolites or toxins from inside or outside the body. Consequently the earliest manifestations of aging usually occur in the skin.

Skin aging can be defined as the accumulation of molecular modifications occurring over time that are not removed by faithful repair processes, especially those involving the macromolecules of the dermis (Giacomoni, 1992). A prominent molecular mechanism gaining widespread scientific acceptance is the microinflammatory model of skin aging (Giacomoni *et al.*, 2000). Accordingly surface peroxide accumulation attributed to environmentally induced impairment of defensive enzymes increases the rate at which the skin elasticity and thickness are modified. On the contrary, topical application of antioxidants decreases DNA damage and other subsequent macromolecular damage, thereby presenting an effective treatment modality. The postulated sequence of events is that surface peroxides damage the skin cells causing them to secrete prostaglandins and leucotrienes which then induce skin mast cells to liberate histamine and tumor necrosis factor alpha (TNF_{alpha}) that in turn stimulates the release of P-selectins and the new synthesis of intracellular molecule-1 (ICAM-1) in skin endothelial cells. Thus, the microinflammatory skin aging model predicts that Cat's Claw water extracts (such as C-Med-100) would be a highly effective anti-aging treatment of skin because of its already demonstrated multifactorial mechanisms, i.e. NF-kB inhibition blocking inflammatory cytokine production (e.g. TNF_{alpha}), antioxidant properties via electrophilic scavenging (Pero *et al.*, 1996) and NF-kB inhibition reducing surface peroxides, and DNA repair enhancement reducing macromolecular expression and damage. This paper challenges the hypothesis that water extracts of Cat's Claw, when applied topically in primary skin organ cultures, do in fact enhance the protection of skin from ultraviolet (UV) exposure.

MATERIALS AND METHODS

Source of water extracts of *Uncaria tomentosa* (Cat's Claw). The primary historic medicinal preparation of *Uncaria tomentosa* involves heating bark to near boiling temperatures covered in water overnight, decanting the partially evaporated water extract and drinking it as a tea. Whereas such ethnopharmacological preparations have been shown repeatedly to be efficacious, high concentrations of tannins have contributed significantly to their toxic side effects after oral administration. However, this problem has been circumvented in water extracts of *Uncaria tomentosa* (Cat's Claw) destined for human consumption by utilizing C-Med-100® supplied by CampaMed, Inc and Optigenex, Inc (New York, NY) for these studies. C-Med-100® was manufactured by a proprietary ultrafiltration process (U.S. patent 6,361,805 B2) that was standardized to contain about 8% CAEs having no components >10000 MW and was essentially free of indole alkaloids (0.05%) (Sheng *et al.*, 2000b; Sandoval *et al.*, 2002). Standardization of the CAE content was accomplished using UV absorption at 200 nm and quantified against dioctyl phthalate or colormetrically by the Bartos reaction (Sheng *et al.*, 2005). In order to stabilize chemically the manufacturing process the water extracts were spray dried on maltodextrin. It was this formulation of C-Med-100 that was used in our studies.

Definition of the active ingredient of Cat's Claw water extracts as quinic acid esters. Identification of the CAEs as quinic acid esters and the only bioactive ingredients found in C-Med-100 have been reported in detail elsewhere (Sheng *et al.*, 2005). Basically the chemical evidence was that the only bioactive identified by TLC in C-Med-100 was water soluble and located at R_f = 0.3–0.4 on silica gel 60 F_{254} plates chromatographed in 1% ammonia in ethanol. It had a UV absorption maximum at 200 nm, absorbed onto charcoal, and was sensitive to base hydrolysis yielding quinic acid analysed by mass spectral and NMR analyses. Furthermore, when quinic acid and C-Med-100 were evaluated *in vitro* they both similarly inhibited NF-kB (Åkesson *et al.*, 2003a; 2005). Moreover, when evaluated *in vivo* in rodent models quinic acid and C-Med-100 had a similar efficacy in treating chemotherapy-induced leucopenia, and in increasing spleen cell counts by inhibiting growth without cell death (Åkesson *et al.*, 2003a; 2003b; 2005). The present study shows more direct evidence that the only bioactive present in C-Med-100 is the quinic acid esters.

For this purpose, the study used inhibition of the proliferation in Con A (2.5 μg/mL) mitogen-stimulated primary microcultured spleen cells treated with C-Med-100 for 48 h which (i) either had been pre-treated with 0.625 M NaOH for 2 h before neutralization or (ii) not, but with the same amount of salt (NaOH + HCl). The C-Med 100 preparation (E-44038, Campamed) was diluted in saline (stock solution C-Med-100 = 40 mg/mL) freshly prepared. Next 1 mL of C-Med 100 (stock solution) was mixed with 1 mL 0.625 M NaOH and incubated for 2 h at room temperature before neutralization with HCl to pH = 7. A control solution of 1 mL of 0.625 M NaOH was directly neutralized with HCl (pH = 7) and after this 1 mL of C-Med-100 was added before 2 h incubation at room temperature. After ± NaOH treatment the concentration of C-Med-100 was adjusted so that all samples had the same concentrations equal to 12.4 mg/mL before the samples were further diluted

in RPMI culture media, added to the cell cultures for analyses of biological activity by normal spleen cell toxicity as described in detail previously (Åkesson *et al.*, 2003b). This bioassay of growth arrest without cell death has been used by our laboratory to estimate increased cell survival because of the prolonged period of time that spleen cells need to repair cellular damage before having to enter cell division.

Organ culture of human skin cells. The experimental conditions for growing human organ cultures of skin for these studies were carried out under a research contract using a novel LSE (living skin equivalent) culture medium developed by Organogenesis, Inc. (Canton, Mass.)

Selection of starting dose of C-Med-100. The starting dose of C-Med-100 was 5 mg/mL or 0.5% w/w because the range 0.1%–1% was shown previously to be maximally effective in penetrating the stratum corneum of skin or living skin equivalents, which in turn led to a general efficacious administration of natural compounds/extracts when using our testing procedures (Laboratory of Skin Biology Group, The Estee Lauder Companies Inc).

Histopathological determination of sunburned skin cells in human organ cultures after *in vitro* exposure to ultraviolet radiation. Excised portions (8 mm) were taken from living human skin equivalent cultures (Organogenesis, Inc) and established over transwell membrane plates for 24 h. These excised portions were pre-treated topically with C-Med-100 at 5 mg/mL (in sterile H_2O) for 6 h. After this post-incubation, the excised skin portions were irradiated with ultraviolet light (UVB) at 0, 50, 75 and 100 mJ/cm^2 which entailed only a few minutes exposure of the not occluded UVB light in a 1 cm area of the culture containing the skin sample. These UVB doses were pre-determined to induce a dose dependent induction of sunburned cells. The skins were then re-treated with C-Med-100 for another 24 h post-incubation. The skin equivalent cultures were fixed in formalin and stored at −4 °C. Subsequently the samples were sent to Paragon Biotech for H&E staining. Microscopic views (400× magnification) were randomly selected from each sample and counts of the sunburned cells identified as necrotic stained cells were made.

Histopathological determination of DNA repair of thymine dimers (TT-dimers) in organ cultures of skin cells exposed to ultraviolet radiation. Again excised portions (8 mm) were taken from living human skin equivalent cultures (Organogenesis, Inc) and established over transwell membrane plates. These excised portions were pre-treated topically with C-Med-100 at 5 mg/mL (in sterile H_2O) for 6 h. After the post-incubation, these excised portions were UVB irradiated at 0, 50, 75 and 100 mJ/cm^2. One set of skins was fixed immediately after UVB irradiation to determine the TT-dimer induction. Another set of skins was then re-treated with C-Med-100 at 5 mg/mL. Following a 24 h post-incubation, the skin equivalents were fixed in formalin and stored at −4 °C. These samples were then sent to Paragon Biotech for immunostaining of the TT-dimers. Histopathological examination was carried at 400× magnification. Random microscopic views were selected from each sample and counts of cells expressing the TT-dimer staining were calculated. The TT-dimer levels of each section were expressed as the number of cells expressing the immunofluorescent TT-dimer antibody tagged stain divided by the total number of cells in that section.

Statistics. Comparison of mean differences between two groups was made by *t*-test statistics. Population differences in the distribution of TT-dimer containing and non-containing TT-dimer cells were analysed by Chi Square distribution analyses.

RESULTS

Definition of the active ingredient in C-Med-100 being evaluated for DNA repair enhancement

Chemical studies reported earlier and reviewed herein define the active ingredients of C-Med-100 to be of the general class CAEs and more specifically quinic acid esters (QAEs). They are water soluble and as such are directly available for absorption. In addition, when administered orally QAEs are susceptible to acid hydrolysis by the gut or enzymatically by intestinal microflora which in turn may metabolically convert QAEs to the free quinic acid forms. That this seems to be the case, is recorded in Fig. 1. Here the efficacy of C-Med-100 to enhance spleen cell growth arrest without cell death was shown to be dependent on base hydrolysis. Clearly it can be seen that as the quinic acid

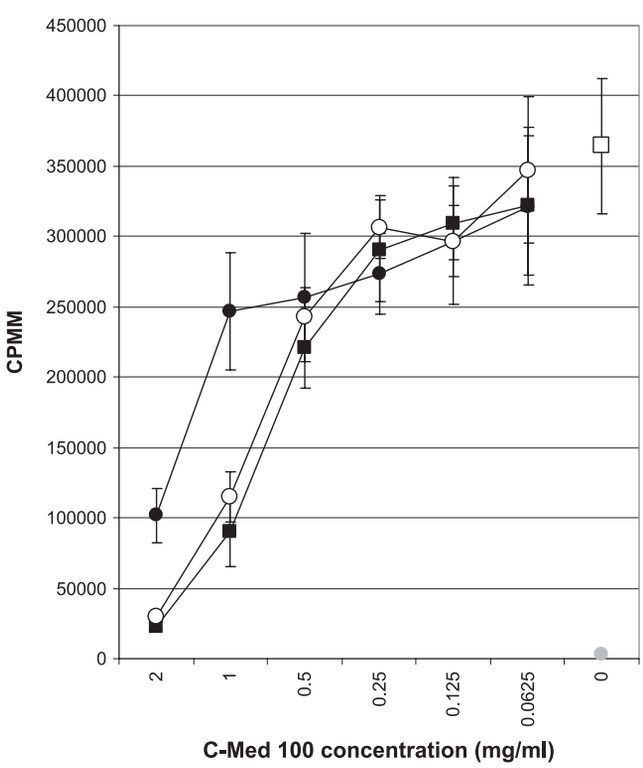

Figure 1. Proliferation of stimulated primary C57BL/6 mouse spleen cells treated for 48 h with 2.5 μg/mL Concavalin A in microtiter plate cultures, quantified and data processed as previously described elsewhere (Åkesson *et al.*, 2003a). ■, C-Med (untreated); ●, C-Med + 0.625 M NaOH (2 h); ○, 0.625 m NaOH + HCl + C-Med; □, positive control; ●, negative control.

esters became hydrolysed by 0.625 M NaOH into quinic acid and its alcohol subunit the biological activity began to disappear in a dose dependent manner at about 0.5 mg/mL. These data were interpreted as strong evidence that the water soluble quinic acid esters are at least one of the primary bioactive ingredients of C-Med-100, responsible for the enhanced cellular repair activity of this particular class of natural products; i.e. water extracts of *Uncaria*.

Toxicity of topically applied C-Med-100

The simulated topical dose of 5 mg/mL C-Med-100 used throughout our organogenic human skin culture experiments had no toxicity associated with it in the presence or absence of ultraviolet irradiation, when evaluated by histopathologic examination of cell necrosis. The 5 mg/mL C-Med-100 dose extrapolates to an *in vivo* oral dose of 5000 mg/kg, and in other *in vivo* studies acute oral doses as high as 8000 mg/kg were also shown to be not toxic (Sheng *et al.*, 2000a), thus confirming the observed lack of toxicity. The skin models used in this study have been validated against human whole skin and have had a similar response to UVB for sunburn cell formation. The UVB doses were not occluded and delivered in a 1 cm area. Although the presence of sunburned cells was evident within the dose range of UVB used in this study, however, there was no evidence that C-Med-100 enhanced any skin toxic reaction either alone or in combination with UVB. On the contrary, C-Med-100 protected skin against sunburn induced cell death induced by UVB (Fig. 2). The toxic effects of systemic uptake of C-Med-100 and the cumulative effects of repeated UVB dosing were not evaluated in this study.

Sunburn protection by C-Med-100 treatment of skin exposed to ultraviolet light (UVB)

Organogenic human skin cultures were exposed to a dose range of 0, 50, 75 and 100 mJ/cm^2 of UVB followed by a 24 h incubation in the presence or absence of 5 mg/mL C-Med-100. The numbers of necrotic cells killed by the UVB (i.e. sunburned cells) were evaluated from direct histopathological examination per 400× magnification field utilizing a light microscope. According to this protocol, it was possible to determine whether C-Med-100 could reduce UVB irradiation cell death. Whereas 50 and 75 mJ/cm^2 of UVB could not be shown to induce any sunburned cells even without any C-Med-100 treatment, there were significant increased numbers of sunburned cells after 100 mJ/cm^2 of UVB, and C-Med-100 reduced this toxicity by about 95% after treatment with C-Med-100 for 24 h (Fig. 2).

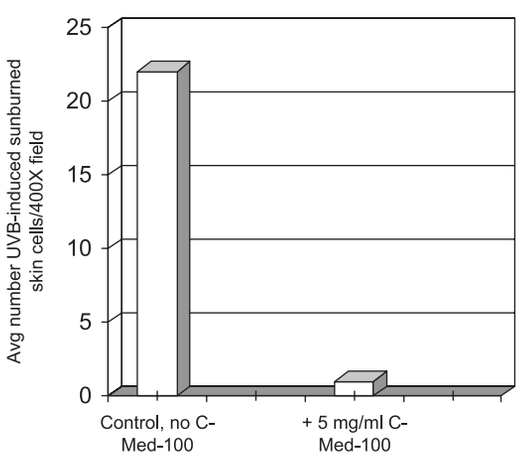

Figure 2. The number of sunburned (necrotic) skin cells per microscopic field at 400× magnification 24 h after exposure to 100 mJ/cm^2 of UVB irradiation and ±5 mg/mL C-Med-100. A *t*-test comparison of the average number of sunburned cells ± C-Med-100 was statistically significant ($p \leq 0.001$).

Evidence for DNA repair enhancement in organogenic human skin cultures treated with C-Med-100

The lethal effects of UVB after *in vitro* exposure of cells in tissue culture is well known and the leading hypothesis of why skin sunburned cells and neoplasms are induced (Al Mahroos *et al.*, 2002). This study has already confirmed that skin equivalents maintained in organogenic culture not only generate UVB-induced sunburn (necrotic) cells but their formation is prevented by treatment with C-Med-100 (Fig. 2). Next the study sought to determine if the UVB-induced cytotoxicity, and its prevention by C-Med-100 treatment, could be explained by enhanced removal of TT-dimers: i.e. the DNA lesion caused by UVB irradiation (Durbeej and Eriksson, 2003). In order to shed light on this hypothesis, it was first important to determine that C-Med-100 itself did not affect the level of TT-dimers present in UVB-induced DNA damage. Although a dramatic increase in cells expressing TT-dimers after UVB irradiation was observed in both sets of living skin equivalent cultures, there was no difference in the levels of TT-dimers induced due to the presence of C-Med-100. At 75 mJ 67% of keratinocytes in a selected 400× microscopic field expressed TT-dimers in the C-Med-100 treated set compared with 71% in the control set. Furthermore, at 100 mJ 65% of keratinocytes in a selected microscopic field expressed TT-dimers in the C-Med-100 treated set compared with 67% in the control set (Fig. 3).

In an effort to determine if C-Med-100 could influence the level of DNA repair induced by UVB, the levels of cells expressing TT-dimers in the 24 h post-irradiation group were compared with the 0 h group reported in Fig. 4. This was done by subtracting the 0 h level of UVB-induced TT-dimers from the level remaining after 24 h incubation and calculating the percent reduction. It was shown that at 75 mJ, a 79% reduction of cells expressing TT-dimers in the C-Med-100 treated skin was observed, while only a 54% reduction was observed in the control group not receiving any C-Med-100 (Fig. 4). The data were even more pronounced at the higher UVB irradiation dose of 100 mJ. In this case, a 73% reduction of cells expressing TT-dimers in C-Med-100 treated skin was observed while only an 11% reduction was observed in the control group not receiving C-Med-100 (Fig. 4). Taken together it was concluded that these data were of particular importance because they supported that the higher the UVB exposure dose the greater the DNA repair enhancement

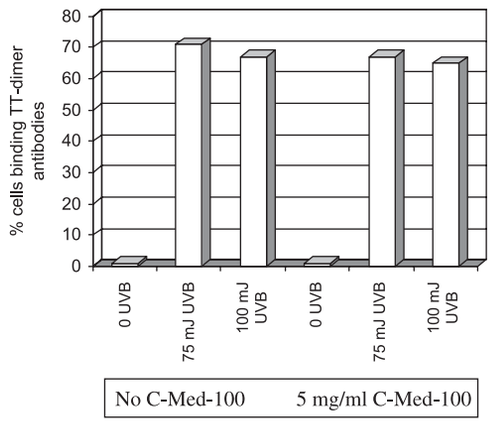

Figure 3. The lack of any significant effect on formation of TT-dimers in skin organogenic cultures exposed to UVB irradiation in mJ/cm^2 in the presence of C-Med-100. Skin cultures were treated with ±5 mg/mL C-Med-100 and then the keratinocytes were immediately analysed histopathologically for the presence of TT-dimers using fluorescent-tagged antibodies. The number of cells containing any stain per microscopic field was recorded. The 0 h UVB exposed cultures served as the positive control showing that normal cells did not contain significant TT-dimers unless irradiated. Chi Square analysis showed significantly different distributions of TT-dimer containing cells ($p \leq 0.01$) compared with non TT-dimer containing cells.

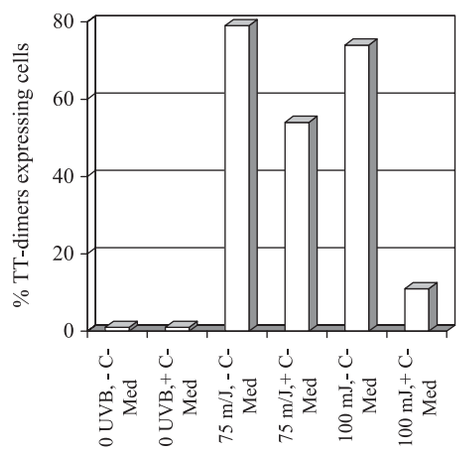

Figure 4. Demonstration that human skin organogenic cultures exposed to UVB in mJ/cm^2 were enhanced to repair TT-dimer DNA lesions in the presence of C-Med-100. The levels of TT-dimers per microscopic field were estimated at 0 h and 24 h after UVB irradiation ±5 mg/mL C-Med-100 as described in Materials and Methods. The percent reduction in TT-dimers was calculated and found to be even more significantly reduced compared with the untreated controls (i.e. no C-Med-100) when analysed by *t*-test statistics ($p \leq 0.001$).

became. Hence, the protection from sunlight was enhanced 26% at 75 mJ (79%–54% = 26%) and 63% at 100 mJ (73%–11% = 63%) by inclusion of 5 mg/mL C-Med-100 as a DNA repair enhancing treatment. The data support that protection from UV induced DNA damage was dose dependent and directed to where it was most needed, i.e. when the TT-dimer DNA damage was the highest.

TT-dimers are well known to induce lethal damage in skin cells as measured by necrotic cell death. In this study sunburned cell formation was used as a standard type assay for damage to human skin. Our data showing that enhanced DNA repair of TT-dimers causing lethal cellular damage also in turn reduced the number of sunburned cells (i.e. necrotic cell death) (Figs 2–4). This was taken as strong evidence that C-Med-100 indeed reduced cell toxicity by enhancing the removal of TT-dimer DNA lesions.

DISCUSSION

As there was no significant difference in the induction of TT-dimers between the C-Med-100 treated and untreated skins (Fig. 3), it was concluded that C-Med-100 does not directly reduce UVB induced DNA damage *per se*. However, C-Med-100 treatment was found significantly to increase DNA repair in UVB-irradiated living skin equivalents and indirectly reduce DNA damage (Fig. 4). These data significantly contributed to our earlier observation of C-Med-100 reducing the number of UVB-induced sunburned cells (Fig. 1). Furthermore, they were also consistent with other published work that demonstrated that oral consumption of C-Med-100 increased DNA repair in humans and animals (Sheng *et al.*, 2000a; 2001). In showing the efficacy of topically applied C-Med-100 to increase DNA repair, the study additionally validates our own data by also concomitantly reducing sunburned cell formation in living skin organogenic cultures. Hence, it was concluded that C-Med-100 (i.e. a Cat's Claw water extract), or its documented bioactive components (i.e. quinic acid analogs) might potentially become useful in providing sunscreen protection as natural repair enhancing products.

Herbal topical treatments for human skin disorders such as eczema, dermatitis, acne, rashes, skin cancers/infections, skin quality, psoriasis or sun protection are well documented in the literature (Blumenthal, 2003; Capasso *et al.*, 2003). Most of the plant extracts known to have biological activity when administered topically are rich in tannins providing 'astringent' properties and also contain other antiinflammatory ingredients as well (Capasso *et al.*, 2003). Thus botanical extract products are a major source for skin care products because they are supported and validated by the well-accepted microinflammatory model of skin aging (Giacomoni *et al.*, 2000). Some of the more widely used plant sources for skin care are aloe, chamomile, evening primrose, marigold, witch hazel, eucalyptus and tea tree (Blumenthal, 2003; Capasso *et al.*, 2003). Essentially there have been no published scientific reports that topically applied Cat's Claw extracts could protect skin from DNA damage or otherwise improve skin quality. There have been three non-peer reviewed reports that root extracts of Cat's Claw administered topically did help to retard skin viral infections, but no scientific validation of these claims has been forthcoming (Blumenthal, 2003). Furthermore, these unsubstantiated reports used aqueous-acid extracts of Cat's Claw roots and were standardized to the % POA content (pentacyclic alkaloids). Under these chemical conditions the POA are derivatized to salts rendering them soluble in water. C-Med-100, AC-11 and other non-acidified Cat's Claw water extracts that were used in the studies reported here contained only trace amounts of alkaloids such as POA (<0.05%, Sheng *et al.*, 2000b; Sandoval *et al.*, 2002), and so consequently POA could not have been the active ingredients, explaining the data presented in this study.

The CAEs are the proposed active ingredients for Cat's Claw water extracts and not POA (see review in Introduction). Alpha hydroxy acids and their analogs such as CAEs are quite ubiquitous in nature, and recently have become quite popular in the topical treatment of several skin conditions (Hunt and Barnetson, 1992). In parallel to this scientific awareness, is the fact that the alpha hydroxy acid, quinic acid, has been identified as an important *in vivo* active ingredient of C-Med-100 (Åkesson *et al.*, 2005; Sheng *et al.*, 2005). Taken together our data (Figs 1–4) support the continued research and development of CAEs as an efficacious treatment for skin disorders, either as an active ingredient of plant extracts or as isolated and identified individual components.

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