Original Research

Central and peripheral effects of *Sutherlandia frutescens* on the response to acute psychological stress

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Abstract

Sutherlandia frutescens is widely used in indigenous medicine for the treatment of stress- and anxiety-related disorders, and although anecdotal evidence has been scientifically confirmed, relatively little data are available on its potential mechanisms of action. We manipulated a rodent model of acute psychological stress by acutely administering a low dose (4 mg/kg body mass) of *S. frutescens* extract 30 min prior to stress exposure (1 h restraint), to elucidate both its central and peripheral mechanisms of action in the context of acute stress. After 1 h of exposure to stress, acute restraint resulted in a significant increase in plasma corticosterone levels (56 ± 33 versus 499 ± 50 ng/ml; $P < 0.0001$) and anterior pituitary adrenocorticotropic hormone (ACTH) levels (0.066 ± 0.017 versus $0.202 \pm 0.033\%$ fluorescent area; $P = 0.07$), while decreasing hippocampal glucocorticoid receptor (GR) and gamma-aminobutyric acid (GABA)(A)α1 receptor levels (both $P < 0.05$). While the low dose of *S. frutescens* administered did not seem to have an effect on the down-stream stress response, it abolished the stress-induced down-regulation of GR, in a manner independent of GABA(A)α1 receptor. Results suggest a non-sedative effect of low-dose *S. frutescens* and points to central mechanisms of action that is in support of the anecdotal claims for its effectiveness as complimentary treatment in chronic stress-associated diseases.

Keywords: Glucocorticoid receptor, gamma-aminobutyric acid receptor, ACTH, restraint

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Introduction

Depression, along with other anxiety-related ailments, is increasingly prevalent globally, and especially so in developing countries,[1] such as South Africa, where traditional healers outnumber conventional western health professionals by at least 10 to 1.[2] This statistic testifies to the importance of indigenous medicine and the validation of these practices. One of the most widely used plants, *Sutherlandia frutescens*, also known as cancer bush, has been used as traditional medicine by a wide variety of cultural groups for the treatment of cancer, stomach ailments, diabetes, stress, and anxiety. The native words for this plant, such as *motlepelo* (Sotho for 'bringing back the heart'), or *insiswa* (ancient Zulu word meaning 'he who dispels darkness'), clearly describe the traditional use of this plant in relieving anxiety and/or depression. Its traditional use in the treatment of stress has also been well-documented in a comprehensive review on the plant.[3]

Biochemical analysis of *S. frutescens* has identified several constituents which allude to a potential anti-stress effect, e.g. gamma-aminobutyric acid (GABA)—which has known mood-elevating effects—and pinitol, a known anti-diabetic and anti-inflammatory.[3] Despite its widespread use, limited physiological research data are available in the context of stress. Long-term oral administration of *S. frutescens* extract to rodents has been reported to significantly attenuate the corticosterone response on subsequent exposure to chronic intermittent restraint stress.[4] Subsequent *in vitro* studies suggested that the underlying mechanism may involve *S. frutescens*-induced suppression of the cytochrome P450, a catalyst of the corticosterone biosynthetic pathway.[5,6] However, this peripheral target cannot account for the acute mood-altering effects consistently reported by *S. frutescens* users, which seems to suggest a more acute effect than its established and scientifically proven endocrine anti-stress effect—perhaps a centrally active mechanism of *S. frutescens*. Anecdotally, *S. frutescens* has a calming effect, not unlike that ascribed to GABA,[7] so that central targets cannot be excluded at this point. It is possible, for example, that *S. frutescens*-derived GABA down-regulates stress perception-related neurotransmission and/or the response to stress, via inhibition of stress-related sympathetic signaling. This theory is further supported by reports of anti-convulsant properties of *S. frutescens* that is thought to be mediated by either acting

like GABA or by indirectly enhancing GABAergic inhibitory neurotransmission.[8] GABA does not cross the blood-brain barrier passively in any significant amount (while it is actively pumped out at much faster rate).[9,10] Therefore, such effects of *S. frutescens* would probably be dependent on some yet unidentified shuttle mechanism, for central delivery of significant amounts of ingested GABA. However, such a possibility cannot be excluded.

Since *S. frutescens* seems to be an indigenous medicine that is worthwhile investigating as a tool against stress, especially in developing countries where access to conventional pharmaceuticals is limited, and as the exact mechanisms of this plant's anti-stress action have not been comprehensively researched, the current study aimed to further elucidate both central and peripheral effects of *S. frutescens* using an *in vivo* model of stress. It is of importance to note that the acute time points purposely chosen for sample collection in this study was too early to indicate the systemic endocrine effects of acutely administered *S. frutescens*, which would only be evident at later time points. Rather, the aim of the current study was to elucidate acute central effects which may explain the anecdotes of an acute calming effect of the plant.

Materials and methods

Experimental animals

All protocols used were approved by the Animal Research Ethics Committee of Sub-Committee B of Stellenbosch University (ref no. 209B02001), and interventions carried out in accordance with ethical guidelines of the South African Medical Research Council. Research was conducted in accordance with the internationally accepted principles for laboratory animal care and use (NIH publication no.85-23, revised in 1985). Forty male Wistar rats (150–200 g) were purchased from a local breeding facility (University of Cape Town, South Africa) and housed in a small animal unit in standard rat cages. Rats were fed standard rat chow and tap water *ad libitum*, with temperature constant at 21°C and application of a reversed 12-h light/dark cycle (lights on at 19:00). Rats were allowed to acclimate to housing conditions for a period of three weeks prior to initiation of intervention protocols. During this time, all rats were purposefully handled and weighed daily, to accustom them to the investigators, thereby minimizing the confounding effects of potential experimental stressors not related to the protocol interventions.[11] Rats were divided into weight-matched groups of five animals per cage, and randomly assigned to four experimental groups ($n = 10$ per group): (a) control placebo (CP), control *S. frutescens* (CSu), stress placebo (SP), and stress *S. frutescens* (SSu).

S. frutescens treatment

In accordance with traditional use and effective doses previously determined to reduce chronic stress in rats, a warm water extract of dried *S. frutescens* (SU1; voucher specimen 4126 (University of Johannesburg herbarium)) leaves was prepared in boiling water (4 mg dried leaf material per milliliter water, infused with agitation for an hour) and filtered. Only one dose was chosen for supplementation, for two reasons: firstly, although different research groups employ different doses depending on the claimed effect to be investigated, previous publications in the context of stress[4,5] have shown that a dose of 4 mL/kg (warm water extract) effectively reduces stress in rodents. Therefore, it was not the aim of the current study to provide proof of efficacy, but rather to investigate central mechanisms of action of the supplement, using the dosage already known to be effective in our hands. Secondly, since the effective dose has been determined previously, it would be difficult to provide ethical justification for the use of more rats to cover more doses.

Rats were accustomed to the gavage procedure for a period of one week by daily gavage of tap water. For the intervention, *S. frutescens*-treatment groups were orally gavaged with 1 mL/kg body mass (i.e. 4 mg/kg body mass) once only. Placebo rats were gavaged with tap water only (1 mL/kg body mass).

Stress intervention

Stress group rats were individually restrained for a period of 60 min in purpose-made Perspex cages (dimensions 7 cm × 8 cm × 15 cm each), starting 30 min after *S. frutescens* treatment, and killed immediately on removal from these cages. Placebo rats were kept under normal housing conditions and in standard cages for this same time period. All rats were thus killed at a time point 90 min after gavage treatment. It is of importance to stress the fact that this study was not aimed to provide proof of effective stress relief by the supplement—this has been proven sufficiently before, as stated earlier. The aim of this study was to elucidate central mechanisms of action, and particularly at the level of glucocorticoid receptors (GR). In our opinion, no suitable reference drug was available for use in this particular model, which employed an acute protocol for detection of acute central effects of a drug that has known chronic peripheral effects. Therefore, we have not included a reference drug in the current study.

Sample collection

To avoid euthanasia-induced alterations in the central components of stress circuitry, decapitation was used as method of sacrifice. All animals were sacrificed between 8:00 and 10:00 to exclude influences of circadian rhythms on circulating hormone concentrations. Trunk blood was collected into heparinised tubes (Vacutainer, BD systems, Plymouth, UK). Blood tubes were kept on ice and centrifuged at 3000 rpm for 10 min at 4°C within an hour of collection. Plasma was aliquotted and stored at −80°C until subsequent batch analysis for testosterone, ACTH and corticosterone concentrations. Rat cerebrums were dissected out and midsections frozen in OCT mounting media, in liquid nitrogen-cooled isopentane. Whole adrenal and pituitary glands were also dissected out, cleaned and weighed on an electronic balance accurate to the nearest milligram. They were then fixed in 10% formal-saline for three days, after which it was processed into paraffin wax blocks using standard histological procedures. Between consecutive sacrifices all blood and

animal waste were removed and the working area disinfected to prevent an acute stress response to the odors of previously killed rats.

Sample analysis

Plasma samples were analyzed using EIA kits according to the manufacturers' instructions for ACTH (Biomerica, Newport Beach, CA, USA) corticosterone (Immunodiagnostic-systems, Boldon Business Park, UK), and testosterone (DRG Diagnostics GmbH, Germany). Lower detection limits for ACTH, corticosterone and testosterone kits were 1.5 pg/mL, 1.7 ng/mL, and 83 pg/mL, respectively.

Cerebral midsection samples were prepared into 10 μm cryosections using a cryostat (CM 1100, Leica Nussloch, Germany) and analyzed for expression of GABA(A)α1 receptor and GR using standard immunohistochemical techniques and fluorescent antibodies. GABA(A)α1 receptor was detected using a goat anti-human IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) followed by a Texas Red-conjugated secondary antibody (Santa Cruz Biotechnology Inc). The GR was detected using a rabbit polyclonal IgG raised against human GR (Santa Cruz Biotechnology Inc) and visualized by using a FITC-conjugated secondary antibody (Santa Cruz Biotechnology Inc).

Pituitary and adrenal tissue samples were cut into 5 μm sections using a rotary microtome (Leica RM 2125, Germany) and prepared for immunohistochemical analysis of ACTH expression as well as IL-6 expression. Immunoperoxidase staining of tissue sections was carried out using the avidin-biotin complex (ABC) staining system (Santa Cruz Biotechnology Inc) according to the manufacturers' instructions. Pituitary and adrenal samples were incubated with a monoclonal primary anti-rat ACTH IgG (Novus Biologicals, Littleton, CO, USA).

Digital images of the hippocampus (CA1 region), adrenal, and anterior pituitary were obtained for three fields of view per section, two sections per slide, at 40× magnification using a microscope (Nikon ECLIPSE E400; 400× objective used) equipped with a colour digital camera (Nikon DXM1200) and computer software for acquiring digital images (Simple PCI version 4.0, CImaging, Hamamatsu Corporation, Sewickley, PA, USA). All images were obtained using identical filters, exposure times and sensitivity settings. Images were analyzed using the software package Image J version 1.41O (Rasband, 1997–2009). The fluorescence unit obtained refers to the relative area of the image that fluoresced.

Statistical analysis

Less than 25% of samples returned plasma ACTH levels above the kit detection limit. Therefore, these data were excluded from further analysis. After confirming normal distribution of data using Levene's test for homogeneity of variances, differences between groups for all parameters were assessed by Factorial ANOVA and Fischer LSD *post hoc* tests (Statistica v.9, StatSoft Software). $P < 0.05$ was set as level of significance. All results are reported as means and standard errors of the mean (sem).

Results and discussion

In concurrence with previous results obtained by our group[4,12] as well as other research groups,[13,14] acute restraint in the current study resulted in a significant increase in plasma corticosterone levels (ANOVA mean effect of stress, $P < 0.0001$; Figure 1). Similarly, as shown before,[4] corticosterone levels of unstressed animals were increased ≈two-fold after *S. frutescens* administration, most likely due to its known insulin-like action.[15] In contrast to previous studies by our group—which used a *chronic* model of intermittent restraint stress[4,5]—the known anti-steroidogenic effects of *S. frutescens* on corticosterone release in restraint-exposed rats were not evident in this model, where assessments of hormone levels were made *immediately* after *acute* stress exposure. Given the differences in the magnitude of an acute versus chronic stress response, as well as differences in the timing of blood samples, this result is not unexpected. Firstly, the dosage of *S. frutescens*—as derived from previous work in a chronic model—may simply have been insufficient to fully counter the relatively larger corticosterone response seen acutely (8- to 12-fold increase) versus chronically (two-fold increase) in restraint stress models. However, this dose and the acute model were specifically chosen, since the aim was not to investigate acute anti-stress effects, but rather reasons for the occurrence of acute calming effects as reported in humans taking the chronic dose for management of chronic stress. Secondly, since an acute model was employed in order to facilitate elucidation of central effects of *S. frutescens* administration, timing of sample collection was not ideal for analysis of peripheral effects. Nevertheless, the peripheral steroid data are not sufficient evidence to exclude the possibility that the extract may aid in adaptation to stress at another level.

When considering more upstream events in the response to stress, consistent patterns were observed for central parameters, which strongly suggests that even a relatively low dose of *S. frutescens* indeed had an effect on the acute response to stress. Firstly, when considering averaged organ mass for both right and left adrenal glands, adrenal mass tended to increase as a result of stress ($P = 0.06$), with *S. frutescens* seeming to act synergistically to strengthen this response (Figure 2). Similarly, at the level of the pituitary, stress again tended to increase organ mass ($P = 0.08$), again with a significant synergistic

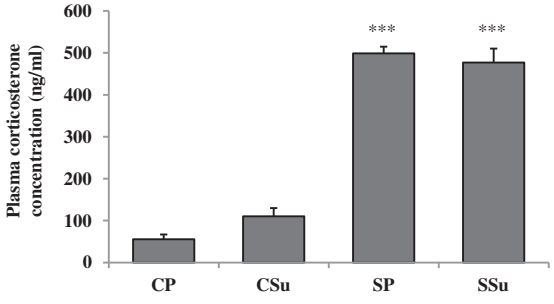

Figure 1 Effect of acute restraint on circulating corticosterone levels in rats after administration of placebo or 4 mg/kg body mass *S. frutescens*

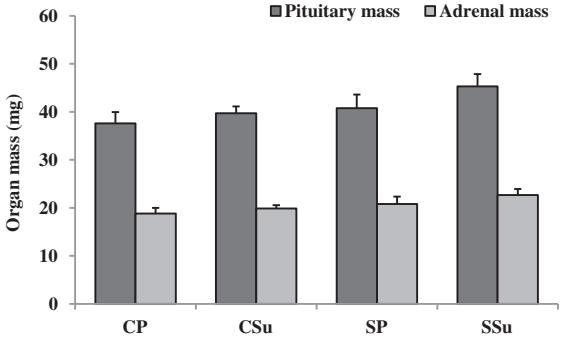

Figure 2 Rat pituitary and adrenal mass after *S. frutescens* administration and/or acute restraint stress exposure

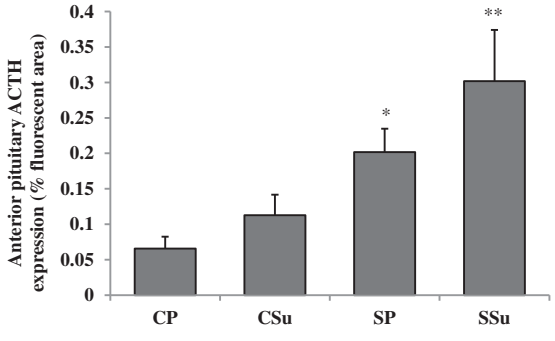

Figure 3 ACTH expression in the rat anterior pituitary in response to acute restraint stress in the presence or absence of *S. frutescens*

function for *S. frutescens* (Figure 2). While ACTH at the level of the adrenal was similar for all groups (data not shown), ACTH expression in the anterior pituitary was increased in stressed groups, again with indications of a synergystic effect of *S. frutescens* (Figure 3). These data are difficult to interpret in isolation, given the time point of sample collection. However, when considered in conjunction with results obtained even further upstream, at the level of the hippocampus, significant beneficial effects of *S. frutescens* become evident.

Stress lowered hippocampal GR levels (Figure 4), a result supported by previous studies by our group and others which showed similar results in liver tissue after chronic restraint.[12,16,17] This well-characterized adaptation of GR to *chronic* psychological stress[18] has also been illustrated after acute exposure to stress, in a paper reporting decreased GR mRNA levels within an hour after the onset of acute stress.[19] The current study is the first to demonstrate that this change is carried through to protein level, with decreased GR levels measured 1 h from onset of restraint stress. In the context of chronic stress, the down-regulation of GR levels is possibly an adaptive process to protect hippocampal neurons from the known glucocorticoid-mediated[20] and GR-dependent[21–23] neurodegeneration. Although more research is required before the exact benefit of down-regulated hippocampal GR in the context of acute stress can be speculated on, an obvious reason could be the maintenance of HPA-axis activation by preventing negative feedback by cortisol via GR binding, to sustain the cortisol response to the acute stressor, probably mainly for metabolic purposes. However, with repeated exposure to such acute stressors, an undesired outcome of this GR down-regulation could be decreased glucocorticoid sensitivity. Our novel finding that *S. frutescens* abolished this down-regulation of GR (Figure 4) may therefore indicate a protective function. Although the acute stress response is not hindered, *S. frutescens* may be limiting its duration by facilitating negative feedback. This effect is supported by lower basal corticosterone levels after *S. frutescens* treatment in chronically stressed rats.[5] By limiting the duration of the stress response, glucocorticoid insensitivity in the longer run may be largely prevented, which is in accordance with indigenous knowledge that it is beneficial in countering long-term effects of stress,[3] as well as development of the metabolic syndrome, which is also associated with glucocorticoid dysregulation.[24] This current result, together with those of previous studies using chronic models, suggests that low doses of *S. frutescens* may be sufficient to effect positive long-term adaptive changes. However, its use as an acute anxiolytic treatment in the context of acute stress is not supported by current *in vivo* data. More specifically, not sufficient data are available on the appropriate dosage to be used in the context of acute stress.

Another effect of *S. frutescens* at the hippocampal level, the decrease of GABA(A)α1 receptor levels, provides further information on the potential mechanism of action of *S. frutescens*. The anecdotal calming effect of *S. frutescens* along with its GABA content[25] suggest an ability of the extracted plant constituents to somehow increase hippocampal GABA content, resulting in increased inhibitory (GABAergic or GABAergic-like) neurotransmission. Since acute stress has been shown to down-regulate GABA receptor function,[19,26] it would be expected that *S. frutescens* administration would attenuate the stress-induced down-regulation and maintain or increase GABA(A) receptor levels and thus GABAergic function within the CA1 region of the hippocampus.

However, in the current study, a decrease was detected in the level of specifically the α1 subunit of GABA(A) receptors in the CA1 region of the hippocampus. This may seem to argue against an anxiolytic effect for *S. frutescens*. However, the stress-induced decreases in GABA(A) receptor levels reported in the literature related to *total* GABA(A) receptor levels. It is therefore not clear which subunits of the GABA(A) receptor (α1, α2 or α3) was implicated in the simultaneous decrease in GR levels reported in the same study.[19] In the current study, GABA(A)α1 was not responsible for the maintenance of GR levels in stressed *S. frutescens*-treated rats, as it was decreased below control levels. This suggests that increased levels of the α2 and/or α3 subunits had likely facilitated the maintained GR levels seen. Since the GABA(A)α1 receptor subunit is the one associated with sedation,[27] the current result is a desired outcome for all potential users of *S. frutescens*, as it provides scientific evidence that consumption of low doses of

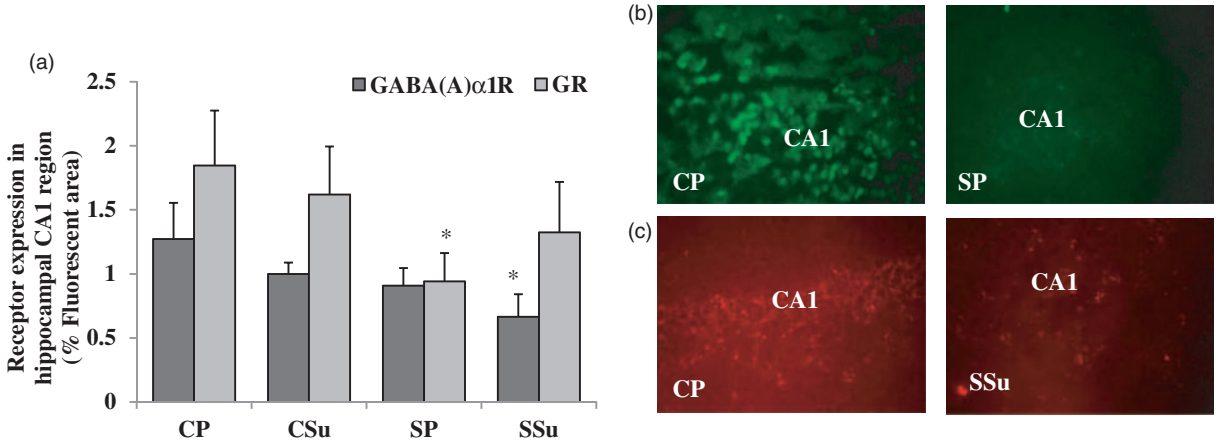

Figure 4 Hippocampal GR and GABA(A)α1R expression in rats, in response to acute restraint with or without prior administraton of *S. frutescens* (a), with representative images of the GR (b), and GABA(A)α1R expression (c) in the CA1 region of the hippocampus (magnification 400× for GR and 200× for GABA(A)α1R). (A color version of this figure is available in the online journal.)

S. frutescens is unlikely to have a sedative effect. Unfortunately, levels of the α2 and α3 receptor subunits were not assessed due to limited antibody availability. If future studies can provide evidence of increased levels of the α2 and α3 subunits of the GABA(A) receptor, this would also further support the anecdotal anti-stress action of *S. frutescens*, as these subunits are associated with anxiolytic effects.[27]

Interestingly, inhibition of GABA(A)1α receptor-mediated synaptic transmission has also been observed in hippocampal CA1 neurons after acute cannabinoid administration.[28] Cannabinoids such as tetrahydro-cannabinol are the primary mood-elevating elements found within marijuana, a shrub which is also known for its anxiolytic effects. The inhibitory effects of cannabinoids on GABAergic neurons are shown to be mediated through cannabinoid-receptor 1 (CB1) and likely involves down-regulation of presynaptic voltage-dependent Ca^{2+} channels.[29–32] Even though the exact mechanisms of this CB1 mediated inhibition of hippocampal GABAergic neurons are still unknown, it could be postulated that similar mechanisms could be at work after *S. frutescens* administration. It is thus also possible that *S. frutescens* mediates its mood-elevating effects in a similar fashion as marijuana, which is postulated to decrease inhibitory GABAergic neurotransmission in the hippocampus and amygdala, causing an increased glutamatergic outflow which in turn activates the mesolimbic dopamine pathway (the primary reward pathway).[33]

In conclusion, our results indicate that the relatively low dosage of *S. frutescens*, which has been proven to be an effective treatment for chronic stress, could not counter the acute stress response to restraint in rodents at the peripheral level. It therefore remains to be seen whether a higher acute dosage might show anti-stress benefit in the context of acute stress, in a study designed for this purpose, to allow for assessment of suitable peripheral parameters at more appropriate time points. However, we have elucidated novel central mechanisms of action of *S. frutescens*, which further support anecdotal claims for its effectiveness as complimentary treatment in chronic stress- and anxiety-associated conditions. In particular, the effects on GABA receptor levels that were elucidated here warrants further study into the potential of *S. frutescens* as a long-term anxiolytic. Future investigations should include quantification of changes in all subunits of the GABA(A) receptor using robust, modern techniques such as proteomic analysis to further strengthen data reported here. Furthermore, the potential of *S. frutescens* to increase central GABA delivery has implications not only for treatments targeting anxiety, but also for pathological states such as Huntingtons' chorea, Alzheimer's and Parkinson's disease as well as epilepsy, all of which are associated with abnormal GABA systems.[34] For these reasons, it is of specific interest to further elucidate the 'shuttle' mechanism via which *S. frutescens* achieves its central effects on the GABA system.

Author Contributions: The project was conceptualized by CS. Experimental work was conducted by MJVV and supervised by CS. Data analysis and manuscript preparation were performed jointly by CS and MJVV.

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