Comparative Assessment of Bacterial Contamination in Commercial Herbal Products of Lessertia frutescens


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Abstract: The present study aimed to assess commercial extracts of Lessertia frutescens L. for bacterial contamination and to test for the presence of the L-canavanine, a bioactive compound. The presence of L-canavanine was determined by thin layer chromatography method. Acinetobacter baumannii, Enterobacter sakazakii and Enterobacter cloacae were isolated from the dried powdered extracts only, following 24 h growth on nutrient agar. The average heterotrophic bacterial count was 9.5 x 10^6 c.f.u.g^-1 and complied with a regulatory standard, but the average coliform count was 5.95 x 10^6 c.f.u.g^-1, which is significantly higher (p < 0.001) than the permitted limit of 1 x 10^3 c.f.u.g^-1.

Key words: Cancer bush · Coliforms · Health care · Medicinal plants

INTRODUCTION

Herbal drugs have been used since ancient times as remedies and treatment for a range of diseases [1]. Western pharmaceutical drugs play a major role in modern medicine, but traditional medicines are used by approximately 60% of people in rural areas still make an important contribution in health care [2]. Herbal medicines are promoted as natural and safe and are therefore the preferred choice [3]. Furthermore, the high costs of anti-retroviral drugs and lack of knowledge about modern developments in HIV/AIDS treatment have caused patients to choose traditional cures [4].

Lessertia frutescens L., Fabaceae, commonly known as the cancer bush, is an attractive small shrub that reaches 1 m in height. The plant is distributed in Southern Africa countries such as South Africa, Namibia and Botswana [2]. It has been reported that Lessertia extracts plays a significant role in the treatment of HIV/AIDS [5], cancer [6], [7] and diabetes [8]. Extracts are also effective as anti-inflammatory agents [9], antioxidants [10] and anti-mutagenic agents [11]. The active ingredients in L. frutescens include L-canavanine, GABA (gamma amino butyric acid) and pinitol [2]. L-canavanine, a non-protein amino acid, is an L-arginine antagonist with antiviral properties against influenza viruses and retroviruses [12]. L-canavanine is also an inhibitor of nitric oxide synthase and therefore has possible application in the treatment of septic shock and chronic inflammation [13]. Because of its efficacy as a safe tonic for diverse health conditions, this medicinal plant has a long history of use by the people of Southern Africa [14]. The occurrence of coliforms in traditional medicines has been reported [15] and the presence of Salmonella spp., Klebsiella pneumoniae and Staphylococcus aureus was detected in extracts of Lessertia [16]. Furthermore, Bacillus diarrhoeal enterotoxins [17] and mycotoxins [16] were reported to occur in such preparations. Handling, collecting, sampling, preparation, storage and transportation of the plant material are different stages where contamination can occur [18]. Presently, the production and standardisation of traditional medicines is not regulated in South Africa. Consequently, traditional medicines are distributed without prior testing for safety and quality. In general, there is no information on the solvents used [19] and the quality or quantity of the active ingredients or on the extraction procedure. The vast majority of traditional medicines in the informal sector are produced in crowded areas [15] lacking in hygiene and sanitation. The hot and humid climate in the Durban area further facilitates microbial growth on

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substrates. The objective of this study was to determine the safety of commercially available material by examining the total heterotrophic as well as the total coliform count of extracts of *Lessertia*, in relation to the accepted national standards. Because of the concerns about depletion of indigenous plants, the bioactive compound was investigated in extract of *in vitro* grown plants as a possible alternative means of bio-production.

**MATERIALS AND METHODS**

**Sample Collection and Extracts Preparation for Microbial Analysis:** Dried powdered leaf samples and adaptogenic tonics (Phyto Force®) were obtained from three different suppliers in the Durban area. The dried material was identified and authenticated by Professor Ashley Nicholas, Ward Herbarium, University of KwaZulu-Natal. To avoid contamination, autoclaved plastic bags were used to package and transport the purchases to the laboratory and stored in a dark, cool place. The third sample used in the study was extracts made from 2 month old *in vitro* plants, acquired from the University of KwaZulu-Natal, Westville campus. (Parent plants for the *in vitro* regeneration were obtained from Silveryl Glen Nature Reserve, Durban, South Africa and were also identified by Professor Nicholas). The plantlets growing on MS medium [20], in sealed Magenta jars were transported across campuses in sealed containers.

Working under sterile conditions, three dried powder extracts were each prepared by mixing 0.6 g of powder in 30 ml of sterile distilled water. Three extracts from *in vitro* plants were each prepared by gently crushing 1.75 g fresh weight (FW) in 15.5 ml sterile distilled water. Three purchased bottles of the adaptogenic tonic (Phyto Force®) were used as is. All extracts were diluted to a concentration of 10⁻¹ as pre-testing revealed that bacterial growth was not evident at lower dilutions. Sterile distilled water was used as a control.

**Inoculation and Bacterial Identification:** Nutrient agar Biolab Merck® was used for the determination of the total heterotrophic bacterial count and Chromocult agar Merck® for determining the coliform count in the extracts. One hundred µl of each of the extracts was streaked across a culture plate and incubated in the dark at 37°C, for 24 h. One hundred µl of sterile distilled was streaked and incubated as the control. Four replicates per extract were prepared, rendering a group of 12 samples per extract.

Freshly prepared nutrient agar cultures of dried powdered extracts were sent to the Department of Medical Microbiology at the University of KwaZulu-Natal for bacterial identification. Standard protocols for gram staining and the API 20 E (Biomerieux) kits were used to identify the bacteria. The number of visible colonies was counted under a magnifying glass stand, using a marker pen. The number of colony forming units (c.f.u) was calculated as: the number of counted colonies x volume of extract plated x dilution factor [21]. The colony forming units per g (c.f.u g⁻¹) was determined by dividing the c.f.u by the mass of the dried leaves used.

**TLC Analysis of L-Canavanine:** Dried powdered leaves and *in vitro* plants of *Lessertia* were prepared by soaking 1 g of dried powder in the following solvents: Extract A, in 20 ml of cold water for 1 h. Extract B, in 20ml methanol for 1 h. Extract C, in 20 ml ethanol for 1 h. Extract D, by boiling for 5 min in 20 ml sterile distilled water using a water bath. The adaptogenic tonics were used without any further preparation and were labelled Extract E. Extracts were prepared by gently grinding 1 g (FW) *in vitro* plants using a pestle and mortar and treatment as follows: Extract F, gently boiled for 5 min in 20 ml sterile distilled water, in a water bath. Extract G, soaked in 20 ml ethanol for 1 h. Extract H, soaked in 20 ml methanol for 1 h. All extracts were vacuum filtrated through Whatman No. 4 filter paper and then concentrated in a rotary evaporator under reduced pressure at 54°C. Using a fine glass capillary tube, exactly 5 drops of Extracts A-H were spotted on pre-coated aluminium plates, silica gel 60 F₂₅₄ (Merck®) and run in a solvent system comprised of n-butanol, acetone, ammonium hydroxide, hexane and water (37: 37: 19: 7). The L-canavanine (1 mg dissolved in 1 ml sterile distilled water) standard was included. The solvent front was pencilled before drying the plate. The air-dried plate was sprayed with 0.2% ninhydrin in acetone. The spots became visible as orange colourations after drying the plane in a laboratory oven at 110°C for 5 minutes (E-mail communication, Dr. J. Howard, Sigma-Aldrich Technical Service). The developed plate was immediately photographed using a Sony digital Cybershot camera.

**Statistical Analysis:** A non parametric Kruskal-Wallis Test run on the SPSS (Statistical Package for Social Science) software package version 15 was used to analyse the data. To statistically verify the data, a one sample T- test was performed where differences in the bacterial counts were compared with accepted South African standards.
RESULTS

Coliform identification on Chromocult agar plates was determined by the specific colour guidelines provided by the manufacturer. Following 24 h growth the presence of coliforms was indicated by the appearance of salmon-red and dark blue-purple colonies. Total heterotrophic bacteria and total coliform counts observed in extracts generated from powdered dried leaves were compared to the South African regulations, in accordance with the Foodstuffs, Cosmetics and Disinfectants Act of 1972 [22] governing microbiological standards. The total heterotrophic bacterial count was highest in extracts derived from dried powdered leaves. The counts (c.f.u.g⁻¹) ranged from 1.1 x 10⁶ to 2.65 x 10⁶ and were within the permitted level [22] of 1 x 10⁶ (Table 1). Bacterial growth was not recorded in extracts from in vitro plants, adaptogenic tonics and the control following 24 h incubation on nutrient agar. Coliform bacteria were observed in extracts derived from dried leaves only, following 24 h incubation on Chromocult agar. Extracts from in vitro plants, adaptogenic tonics and controls were bacteria-free and therefore not scored. The counts (c.f.u.g⁻¹) ranged from 7.1 x 10⁵ to 1.5 x 10⁶ (Table 1). Acinetobacter baumanii, Enterobacter sakazakii and Enterobacter cloacae were identified as the dominant contaminants of the dried plant material.

Using a non parametric Kruskal-Wallis Test on the numerical scores of total heterotrophic bacteria and coliforms obtained from the cultured extracts, the probability was determined at p = 0.013 (p < 0.05), which indicates that there was a significant difference between bacterial growth in the extracts. The mean rank value of the control, in vitro plant extracts and adaptogenic tonics data were all the same at 5.0, whereas the mean rank value for dried powder leaf extracts data was 11.0.

Table 1: Bacterial counts in extracts generated from dried powder, adaptogenic tonics and in vitro plants of L. frutescens compared to South African food regulations

<table>
<thead>
<tr>
<th>Total bacteria</th>
<th>SA Regulatory Standard (c.f.u.g⁻¹)</th>
<th>Sample range (c.f.u.g⁻¹)</th>
<th>Sample mean (c.f.u.g⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>Heterotrophic bacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dried powder extract</td>
<td>1 x 10⁶</td>
<td>1.1 x 10⁶ - 2.65 x 10⁶</td>
<td>9.5 x 10⁵ *</td>
</tr>
<tr>
<td>Adaptogenic tonic</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>In vitro plant extract</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
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<tr>
<td>Coliform bacteria</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Dried powder extract</td>
<td>1 x 10⁵</td>
<td>7.1 x 10⁵ - 1.5 x 10⁶</td>
<td>5.9 x 10⁴ b</td>
</tr>
<tr>
<td>Adaptogenic tonic</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>In vitro plant extract</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

* Data were analysed using a T-test, t = 29.91, df = 11, p < 0.001.

† Data were analysed using a T-test, t = 3.37, df = 11, p < 0.001.

Fig. 1: Analysis by TLC of extracts from dried leaves (1-4), adaptogenic tonic (5) and in vitro plants (6-8) of L. frutescens. Lane 1: Extract A (cold water); Lane 2: Extract B (methanol); Lane 3: Extract C (ethanol); Lane 4: Extract D (boiled water); Lane 5: Extract E (adaptogenic tonic); Lane 6: Extract F (boiled water); Lane 7: Extract G (ethanol); Lane 8: Extract H (methanol); Lane 9: L-canavanine standard in water
Data were further analysed using a T test (one tailed) to compare the heterotrophic and coliform counts in the extracts, with South African regulatory food standards [22]. A difference was found at \( p < 0.001 \) signifying that the average total heterotrophic count was significantly lower than the acceptable limit of \( 1 \times 10^5 \text{ c.f.u.g}^{-1} \) product. However, the average coliform count was highly significant at \( p < 0.001 \), compared with the regulatory limit of \( 1 \times 10^5 \text{ c.f.u.g}^{-1} \) product (Table 1).

The presence of L-canavanine was detected as a bright orange spot following spraying with 0.2% ninhydrin and was evident in all the samples (Fig. 1). The Rf value for L-canavanine was 0.25. The clear presence and apparent stability of L-canavanine in the in-vitro extracts (Extracts F, G and H- Fig. 1) indicate that in vitro plant material of Lessertia could be used as an alternative. Furthermore, L-canavanine could be produced in suitable quantities by the use of in vitro protocols and other plant biotechnologies.

**DISCUSSION**

The findings of the present study confirmed the presence of harmful bacteria in commercially available herbal products of Lessertia, i.e. dried powdered leaves. By contrast the adaptogenic tonics, which are also commercially available, were shown to be free of bacterial contamination. The tonics were preserved in ethanol, (as indicated on the label) which would clearly have an inhibitory effect on bacterial growth. Although the average total heterotrophic bacterial count was high \( (9.5 \times 10^5 \text{ c.f.u.g}^{-1}) \) in extracts made from powdered leaves, there was compliance with regulatory standards, which is \( 10^5 \text{ c.f.u.g}^{-1} \) of product [22].

In the present study, *A. baumannii*, *E. cloacae* and *E. sakazakii* were detected in the dried powder extracts and are coliforms usually associated with the environment surrounding plants, animals and humans [23]. *A. baumannii* and *E. cloacae* have been linked with nosocomial infections in neonatal intensive-care units in association with multi-drug resistant strains [24,25]. Furthermore, *E. sakazakii* has been implicated in outbreaks of sepsis, meningitis, cerebritis or enteritis in patients of all age groups. *E. sakazakii* has also been isolated in infant formula and powdered milk [26]. The stationary phase of *E. sakazakii* cells were shown to be more resistant to osmotic and dry stress than *E. coli*, *Salmonella* and other strains of the Enterobacteriaceae [27]. This may give an indication that the high tolerance to desiccation provides a competitive advantage for *E. sakazakii* in dry environments, as occurrence was reported in milk powder and in the dried powdered leaf extracts of the present study. Furthermore, strains of *A. baumannii* were also recorded to survive dry conditions [28]. The average total coliform count in the dried powder extracts was beyond the acceptable limit of \( 1 \times 10^5 \text{ c.f.u.g}^{-1} \). Hence, species resistant to dry conditions were able to tolerate desiccation during the preparation of the dried powder. Dangerous coliforms are life-threatening and may be exceedingly so in the immune compromised such as HIV/AIDS sufferers. On the other hand, the adaptogenic tonics were observed to be free of bacterial contamination as a result of the preservative and antibacterial effect of ethanol. Analysis by TLC revealed the presence of L-canavanine in all extracts, suggesting that ethanol is a satisfactory storage medium.

Methanol, ethanol and water proved equally suitable extractants for L-canavanine from fresh, dried or liquid material of Lessertia as was indicated by TLC. Aqueous extracts in this study were prepared similarly to decoctions of Lessertia which are traditionally used as a medicine (instructions on dried powder packets) [2]. It is possible therefore those aqueous decoctions possess L-canavanine. The potential of a water extract of Lessertia as an effective hypoglycaemic agent was reported [8]. Inhibition of pregnenolone and progesterone metabolism was significantly higher in the aqueous extract [29] and hot water extracts possessed significant reactive oxygen species scavenging properties [30]. However, whilst aqueous decoctions almost certainly possess L-canavanine, microbial growth in dried powder represents a potential problem on storage and subsequently harmful effects on human health.

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**REFERENCES**


