

Short communication

The effect of simulated gastrointestinal conditions on the antimicrobial activity and chemical composition of indigenous South African plant extracts

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Abstract

Few *in vitro* screening assays for biological activities of plant extracts consider the potential effect of the gastrointestinal system on orally consumed plant extracts. Crude water and methanol extracts of *Tarchonanthus camphoratus* (wild camphor) and *Agathosma betulina* ('buchu') were prepared and exposed to simulated gastric fluid and simulated intestinal fluid during dissolution studies to address this aspect. The crude extracts and resulting simulated gastric fluid and simulated intestinal fluid products were screened for antimicrobial activity against *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922) and *Proteus vulgaris* (ATCC 33420). The *T. camphoratus* crude extract exhibited antimicrobial activity which was reduced after exposure to simulated gastric fluid. After exposure to simulated intestinal fluid no antimicrobial activity was detected, which suggests chemical alteration or degradation of the active compounds. For *A. betulina*, the crude water extract and simulated gastric fluid product exhibited no antimicrobial activity, while the simulated intestinal fluid product exhibited antimicrobial activity. This suggests activation of antimicrobial constituents during exposure to simulated intestinal fluid. The chemical composition profiles of the crude extracts and products were determined by means of liquid chromatography coupled to an ultraviolet detector (LC-UV) and a mass spectrometer (LC-MS) to qualitatively assess the effect of exposure to simulated gastrointestinal conditions on the chemical composition of the extracts. In many cases, the peak area of compounds decreased after exposure to simulated gastric fluid and simulated intestinal fluid, while the peak area of other compounds increased. Thus, it can be deduced that the antimicrobial activity and chemical composition was altered after exposure to intestinal conditions during dissolution studies.

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1. Introduction

In South Africa, a country rich in plant diversity, approximately 3000 of the 30000 species of higher plants are used for medicinal purposes, many of which are endemic to the region. Of these, approximately 350–500 species are commonly used and traded in large quantities as medicines (Steenkamp et al., 2006). Both *Tarchonanthus camphoratus* L. (wild camphor bush) and *Agathosma betulina* (Bergius) Pill. (round leaf 'buchu') are indigenous South African plants and have been used extensively as part of a traditional herbal therapy to treat various diseases. *T. camphoratus* (Asteraceae), is a shrub or small tree that has a grayish appearance, hence the Afrikaans

vernacular name ("vaal" = gray, "bos" = bush). Infusions and tinctures of the leaves and twigs are used to treat gastrointestinal conditions including abdominal pain, headache, toothache, asthma, coughing, bronchitis and inflammation. The fresh or dried plants are burnt and the smoke or fumes are inhaled to treat asthma, headache, blocked sinuses, and rheumatism. Historically, the Khoi and San people smoked the dried leaves like tobacco, apparently with a slightly narcotic effect. *A. betulina* (Rutaceae) is an evergreen, multi-stemmed, perennial, woody shrub and is restricted in its natural distribution to the sandy mountain slopes of the Western Cape Province. 'Buchu' has remained one of the most popular herbal medicines in South Africa, with a reputation for treating kidney and urinary tract infections, stomach ailments, gout, for the symptomatic relief of rheumatism, and also for external application on wounds and bruises (Van Wyk et al., 1997).

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Due to the need for development of novel drugs there has been a renewed interest in ethnopharmacological research worldwide. Studies are generally of a screening nature and investigate biological activities such as antioxidant, antibacterial, anti-inflammatory and antifungal properties of extracts from medicinal plants usually based on ethnobotanical leads. The effect that the gastrointestinal environment may have on these orally consumed extracts is usually not explored even though it is well-known and accepted in biopharmaceutics that the gastrointestinal system plays a considerable part in the ultimate bioavailability of conventional medicines (Ashford, 2007; Hamman, 2007). It is a reasonable assumption that the same would apply to orally consumed herbal medicines. The human gastrointestinal tract transforms orally consumed substances into absorbable molecules and waste products by means of a digestive process. The fluids present in the gastrointestinal system contain various substances and enzymes which aids in the digestion process. The stomach has a pH between 1 and 2 due to the secretion of hydrochloric acid and contains the digestive enzyme pepsin. The pH of the small intestine ranges between 5.1 and 7.5 and contains numerous digestive enzymes (Mader, 1996; Hamman, 2007). It is well documented that some active pharmaceutical ingredients have to be protected, e.g. through enteric coating, against this harsh environment in order to be effective. The presence of hydrochloric acid and enzymes in the stomach can easily cause acid or enzyme catalysed hydrolysis of orally consumed compounds thereby resulting in chemical modification and inactivation. Pre-systemic metabolism and degradation in the gastrointestinal tract primarily results in reducing the bioavailability of medicines. However, in the case of pro-drugs these processes are essential for the active parent drug to become available for absorption. Medicines such as erythromycin stearate depend on degradation in the gastrointestinal tract to release the therapeutically active parent molecule. It exhibits limited solubility in gastric fluid but dissolves and dissociates readily in the intestinal fluid, liberating the free base which is absorbed (Proudfoot, 1996; Ashford, 2007). In this study the potential effect of the gastrointestinal system on various extracts was investigated by subjecting the extracts of two important South African medicinal plants to simulated gastrointestinal conditions.

2. Materials and methods

2.1. Plant material

A. betulina leaves (dried) were purchased at a community pharmacy and the aerial parts of *T. camphoratus* was collected at the Walter Sisulu Botanical Garden and the leaves were air-dried. Voucher specimens (AV 961 and AV 1395) were deposited in the Department of Pharmaceutical Sciences, Tshwane University of Technology.

2.2. Extract and final product preparation

The dried plant material was ground to a fine powder and extracted with water and methanol. Extraction of the material was conducted in a water bath (Scientific 32 l) at 40 °C for 3 h. This

was repeated three times to maximise extraction efficiency. The methanol extracts were dried by evaporation and the water extracts were freeze-dried (Jouan LP3 freeze-drier). These extracts were subjected to simulated gastrointestinal conditions using a six-station Pharmatest Type PTW 5 dissolution apparatus set up according to the British Pharmacopoeia (BP, 1988) specifications. The paddle apparatus was set to 37 °C with a paddle speed of 150 rpm. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as prescribed in the United States Pharmacopoeia (USP, 1990). However, the composition of the SGF was slightly altered for this study. Hydrochloric acid was replaced with trifluoroacetic acid (TFA) (Sigma Aldrich) as it is volatile thereby eliminating the possible interference of hydrochloric acid with subsequent antimicrobial experiments. The experimental procedures and gastrointestinal fluids are also used in dissolution experiments on conventional medicines. The dissolution times of 2 h for the gastric simulation (stomach) and 6 h for the intestinal simulation (small intestines) were selected to simulate the transit time of orally consumed substances in the respective gastrointestinal segments. Agitation of the dissolution media with the paddle simulated the normal peristaltic movements of the gastrointestinal tract. After 900 ml of dissolution medium were added to the dissolution flasks and the temperature was stabilised at 37 °C, the paddles were lowered and 500 mg of each plant extract were added to the different gastrointestinal fluids. After the appropriate time for each simulated fluid had elapsed the total contents of the flasks were dried by evaporation. The extracts were re-dissolved in 30 ml of distilled water, 10 ml of methanol were added and sonicated (Sonorex digital 10p) for 10 min at 37 °C and 100% power to dissolve solids. Proteins (added as enzymes in the simulated fluids) were removed by complexation with the addition of 30 ml of acetonitrile placed in flasks in a water bath (Scientific 32 l) at 60 °C for 10 min. The products were filtered (Whatman® no. 41) and dried by evaporation. For each plant, a crude water and methanol extract, a water and methanol SGF product and a water and methanol SIF product were obtained for testing purposes.

2.3. Antimicrobial activity

The minimum inhibitory concentration (MIC) for each sample was determined using the micro-titre plate method (Eloff, 1998). Samples were screened against two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212) and two Gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Proteus vulgaris* (ATCC 33420). Pathogen selection was undertaken on the basis of commonly occurring infectants of the gastrointestinal tract. Microbial suspensions in Tryptone Soya broth (TSB) were prepared according to a McFarland No. 0.5 standard (approximately 1×10^8 CFU/ml). The plant extracts were dissolved in sterile water to a concentration of 64 mg/ml. The *T. camphoratus* methanol extract did not dissolve completely in water and was subsequently dissolved in dimethylsulfoxide (DMSO) and sterile water (1:1). The 96-well micro-titre plates were aseptically prepared in a horizontal laminar air flow cabinet. Sterile water aliquots (100 µl) were pipetted into all the wells and 100 µl of each test sample were added to the top

row. Serial dilutions were made by transferring 100 µl each time to obtain concentrations ranging from 16–0.125 mg/ml. The bacterial culture (100 µl) was added to all wells, except the TSB control row. A sterile film was used to cover all the wells to eliminate evaporation and the plates were incubated at 37 °C for 24 h. As an indicator of bacterial growth, 40 µl of a 0.4 mg/ml *p*-iodonitrotetrazolium violet (INT) solution (Sigma Aldrich) was added to all the wells and left for 6 h to develop. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibited growth of the microorganism detected visually, that is a well that shows no red discolouration (Matasyoh et al., 2007). The broad-spectrum antibiotic, ciprofloxacin (Merck) was used as a positive control at a starting concentration of 0.1 mg/ml. Negative controls (SGF, SIF and DMSO) were included to assess whether they had any impact on the antimicrobial activity. Test microorganisms without inhibitor were included in each assay to ascertain microbial viability. MIC values were determined at least in duplicate.

2.4. LC-UV-MS analysis

The detection of chemical constituents present in the crude extracts and simulated gastric fluid and simulated intestinal fluid products was performed using a Waters Alliance 2690 HPLC system (Phenomenex Aqua C18, 4 µ, 250 × 2 mm) coupled to a 996 photodiode array (PDA) detector and a Waters API Qautro Micro MS detector. The PDA detector was set to scan a wavelength range of 100–1400 nm. The MS detector was operated in electron impact mode with the capillary voltage at 3.5 kV, the cone voltage at 15 (positive switching — ES⁺) and 25 (negative switching — ES⁻). The flow rate of the HPLC was 0.3 ml/min, the nebuliser flow rate 50 l/h and the desolvation gas flow rate 380 l/h. The source temperature was 120 °C and the desolvation temperature 380 °C. The mobile phase used consisted of 0.1% formic acid (solvent A) and acetonitrile (solvent B) and gradient elution was used over a 40 min period. The test samples were dissolved in distilled water to a concentration of 50 mg/ml. The Masslynx™ (Version 4.0) software package and Microsoft® Excel (2003) were used to analyse and graphically display the results. The LC-MS results confirmed

the LC-UV results but for the sake of brevity the former is omitted in further discussions.

3. Results and discussion

3.1. *T. camphoratus*

3.1.1. Antimicrobial activity

Sterile water and broth with bacterial culture exhibited growth indicating viability of the microorganism. The antibiotic control, ciprofloxacin, was active against all the test organisms (Table 1). The wells of the Tryptone Soya broth (TSB) control column remained clear after incubation indicating sterility of the TSB. The DMSO/water (1:1) solvent exhibited poor antimicrobial activities (16 mg/ml) and did not influence the assay results. The simulated gastric fluid and simulated intestinal fluid control samples did not exhibit any antimicrobial activity at the highest concentration tested (16 mg/ml).

A summary of the MIC results against all the organisms is displayed in Table 1. The crude methanol and water extracts of *T. camphoratus* showed good (2 mg/ml) to poor (>16 mg/ml) antimicrobial activity against the test microorganisms. Recently, Braithwaite et al. (2008) reported antimicrobial activity of 2 mg/ml for a methanol extract against *S. aureus* which correlates with the result from this study. They also reported activity of 0.5 mg/ml for the acetone extract, 4 mg/ml for the essential oil and 0.62 mg/ml for the inhaled smoke fraction. For the *T. camphoratus* extracts that exhibited antimicrobial activity in this study, the activity decreased in all cases after exposure to simulated gastric fluid with the exception of the water extract which remained constant at 8 mg/ml against *E. coli*. It can be deduced that on exposure to conditions that are similar to those in the stomach, the antimicrobially active compounds are degraded. After exposure to simulated intestinal fluid, no activity was noted for any of the extracts against any of the microorganisms. This indicates that orally ingested wild camphor loses all antimicrobial activity when exposed to conditions similar to those found in the small intestines. These results lend even more scientific credibility to the use of

Table 1
Summary of MIC results (mg/ml) for *T. camphoratus* crude extracts, simulated gastric fluid and simulated intestinal fluid products against four different microorganisms.

| Sample | MIC (mg/ml) | | | |
|---------|---|---|------------------------------------|------------------------------------|
| | <i>Staphylococcus aureus</i> ATCC 25923 | <i>Enterococcus faecalis</i> ATCC 29212 | <i>Escherichia coli</i> ATCC 25922 | <i>Proteus vulgaris</i> ATCC 33420 |
| Crude W | 16 | 2 | 8 | 8 |
| SGF W | >16 | 16 | 8 | >16 |
| SIF W | >16 | >16 | >16 | >16 |
| Crude M | 2 | >16 | 4 | 4 |
| SGF M | >16 | >16 | 8 | >16 |
| SIF M | >16 | >16 | >16 | >16 |
| GC | >16 | >16 | >16 | >16 |
| IC | >16 | >16 | >16 | >16 |
| AC | 0.625 µg/ml | 2.5 µg/ml | 0.63 µg/ml | 0.25 µg/ml |
| DMSO | 16 | 16 | 16 | 16 |

Abbreviations: W — water extract/product; M — methanol extract/product; GC — simulated gastric fluid control; IC — simulated intestinal fluid control; AC — antibiotic control (0.1 mg/ml); DMSO — dimethyl sulfoxide control; SGF — simulated gastric fluid; SIF — simulated intestinal fluid.

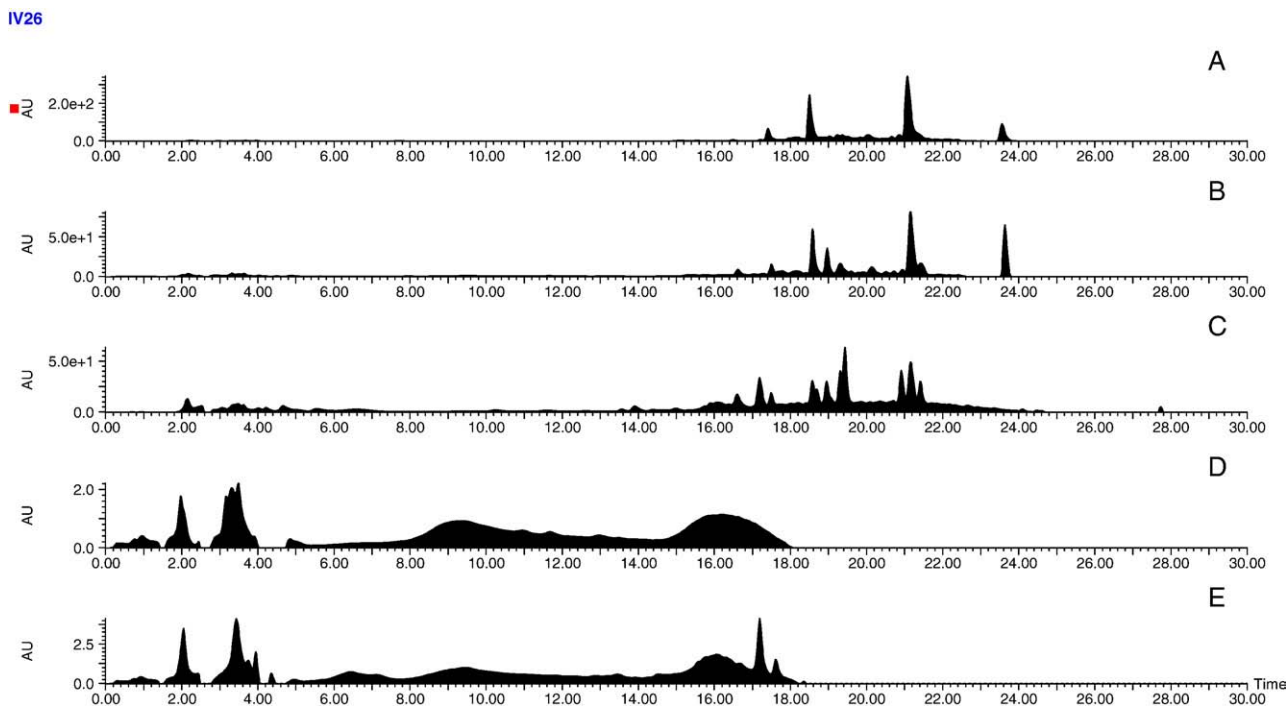


Fig. 1. LC-UV chromatograms for *T. camphoratus* crude methanol extract and products. (A) Crude extract, (B) simulated gastric fluid product, (C) simulated intestinal fluid product, (D) simulated gastric fluid control, and (E) simulated intestinal fluid control.

T. camphoratus as an effective antimicrobial through smoke inhalation therapy as reported by Braithwaite et al. (2008).

3.1.2. LC-UV analysis

The LC-UV chromatograms for the crude methanol extract, SGF and SIF products and SGF and SIF control samples are displayed in Fig. 1. Comparing the peak area per compound in the extract and each of the products it is clear that the chemical composition of these extracts changed after exposure to simulated gastrointestinal conditions. For example, at retention times 23.56–23.63 min the peak area for the simulated gastric fluid product was smaller than the crude extract peak area and no corresponding simulated intestinal fluid product compounds were detected at these retention times. In the case of simulated intestinal fluid this compound was degraded to below the detection limit of the apparatus. At retention times 18.49–18.58

and 21.07–21.16 min the peak area decreased in order from the crude extract to the simulated gastric fluid product to the simulated intestinal fluid product. Thus, it can be deduced that chemical degradation or alteration takes place on exposure to simulated gastric as well as simulated intestinal fluid.

3.2. *A. betulina*

3.2.1. Antimicrobial activity

A summary of the MIC results against all the organisms is displayed in Table 2. The antimicrobial activity for the *A. betulina* crude extracts was poor (8–16 mg/ml) against all the microorganisms. Moolla and Viljoen (2008) reported antimicrobial activity of 4 mg/ml for a dichloromethane:methanol (1:1) extract of *A. betulina* against *S. aureus*. The most interesting result from this study was that the SIF water extract products

Table 2

Summary of MIC results (mg/ml) for *A. betulina* crude extracts, simulated gastric fluid and simulated intestinal fluid products against four different microorganisms.

| Sample | MIC (mg/ml) | | | |
|---------|---|---|------------------------------------|------------------------------------|
| | <i>Staphylococcus aureus</i> ATCC 25923 | <i>Enterococcus faecalis</i> ATCC 29212 | <i>Escherichia coli</i> ATCC 25922 | <i>Proteus vulgaris</i> ATCC 33420 |
| Crude W | >16 | >16 | >16 | 8 |
| SGF W | >16 | >16 | 16 | >16 |
| SIF W | 8 | 8 | >16 | >16 |
| Crude M | >16 | 16 | 8 | 16 |
| SGF M | >16 | >16 | >16 | >16 |
| SIF M | >16 | >16 | >16 | >16 |
| GC | >16 | >16 | >16 | >16 |
| IC | >16 | >16 | >16 | >16 |
| AC | 1.25 µg/ml | 1.25 µg/ml | 0.63 µg/ml | 0.25 µg/ml |

Abbreviations: W — water extract/product; M — methanol extract/product; GC — simulated gastric fluid control; IC — simulated intestinal fluid control; AC — antibiotic control (0.1 mg/ml); control; SGF — simulated gastric fluid; SIF — simulated intestinal fluid.

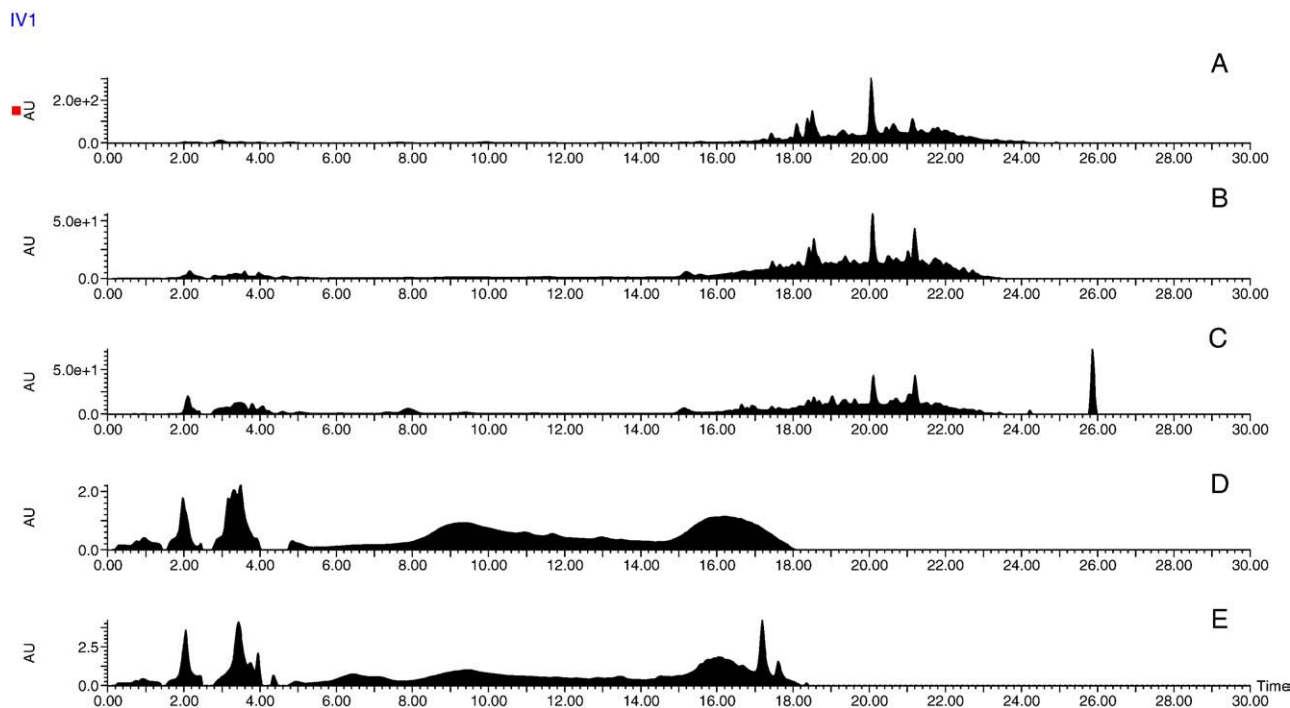


Fig. 2. LC-UV chromatograms for *A. betulina* crude water extract and products. (A) Crude extract, (B) simulated gastric fluid product, (C) simulated intestinal fluid product, (D) simulated gastric fluid control, and (E) simulated intestinal fluid control.

exhibited antimicrobial activity of 8 mg/ml against *S. aureus* and *E. faecalis* while the crude water extract did not display any antimicrobial activity against these microorganisms. This suggests that the antimicrobially active constituents display behaviour similar to that of a pro-drug. Activation of some of the phytoconstituents only takes place after the extract has been exposed to an environment similar to that of the small intestines.

3.2.2. LC-UV analysis

Fig. 2 displays the LC-UV chromatograms for the crude water extract, SGF and SIF products and SGF and SIF control samples. The peak area values decreased from the crude extract to the simulated gastric fluid product and further for the simulated intestinal fluid product for the compounds that eluted at retention times 18.50–18.55 and 20.05–20.11 min. This implies that more degradation occurs in the small intestine environment for these specific constituents, which may have a profound effect on bioavailability of these constituents. This was noted as the general trend for most of the extracts. At retention time 21.13–21.21 min the peak area decreased slightly more from the crude extract to the simulated gastric fluid product than from the crude extract to the simulated intestinal fluid product indicating greater sensitivity towards simulated gastric fluid or better stability in the intestinal fluid. This indicates that there would be even less of this compound available for absorption in the small intestine after transit through the stomach than if it was protected in the stomach (by enteric coating for example) and only liberated in the small intestines. New compounds that were not present in the crude extract were detected at retention times 24.23 and 25.86 min in the simulated intestinal fluid products. This corresponds to LC-MS results and it was speculated that these compounds may be

responsible for the antimicrobial activity that is exhibited after exposure to the simulated intestinal environment.

These results therefore indicate that the chemical composition and antimicrobial activity was altered after exposure to simulated gastrointestinal conditions during dissolution studies. This implies that orally ingested extracts of *T. camphoratus* and *A. betulina* will behave similarly *in vivo*, highlighting the importance of investigating the effect of the gastrointestinal system for all medicinal plants intended for oral administration. This is one of the first steps towards validating their extensive use by the public. More importantly, it is evident that results of published screening assays may lead to overestimation of antimicrobial activity since the effect of the gastrointestinal system is not considered. Alternatively, weak antimicrobial activity noted during a screening assay generally negates further exploration of a plant or extract which may have been activated when exposed to gastrointestinal tract conditions.

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