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In vitro evidence of phyto-synergy for plant part combinations of *Croton gratissimus* (Euphorbiaceae) used in African traditional healing

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ABSTRACT

Aim of the study: Despite the extensive traditional use of Croton *gratissimus* Burch. var. *gratissimus* for medicinal purposes, scientific studies validating the therapeutic properties of this indigenous plant are lacking. As the bark, roots and leaves of C. *gratissimus* are used separately as well as in combination, this study focused on determining antimicrobial efficacies of the plant parts independently and in combination to assess possible pharmacological interactions (e.g. synergy, antagonism).
Material and Methods: The hydro-distilled leaf essential oil and extracts of bark, root and leaf were comparatively assessed for antimicrobial activity by means of microdilution minimum inhibitory concentration (MIC). The fractional inhibitory concentrations (FIC) were determined for the leaf and root (1:1), bark and root (1:1), leaf and bark (1:1) combination. Isobolograms were plotted to demonstrate interactions between various ratios of the roots and leaves.
Results: The MIC and FIC results indicated variable efficacies for the various plant part combinations, the greatest of which was noted for *Cryptococcus neoformans* in the root and leaf combination (MIC 0.4 mg/ml and FIC of 0.4). Isobolograms indicated the greatest synergy for *Bacillus* cereus, *Candida albicans* and *Cryptococcus neoformans*.
Conclusion: The observed synergistic interactions clearly indicate that the reductionist approach may often be short-sighted and that biological activity may be improved through combination therapy, where different complex metabolic pools collectively contribute to the enhanced effect.

1. Introduction

Many phytomedicines on the market today such as *Ginkgo biloba* and *Echinaceae purpurea* are sold as whole extracts and it is believed that the synergistic interactions between the constituents are responsible for the therapeutic efficacy (Williamson, 2001). It is also known that many traditional healers rely not only on a single plant extract for therapeutic regimens but often combine various plant parts and even different species in the belief that efficacy may be enhanced. While literature cites the traditional use of plant combinations for the treatment of microbe related infections, there has been very little scientific evidence to support this specific mode of traditional use. Some *in vitro* antimicrobial combination studies have been undertaken to validate the role of synergism in phytotherapy. Efficacy studies on extracts (Kang et al., 1992), essential oils (Lachowicz et al., 1998) and in combination with conventional antimicrobials (Scott et al., 2004; Shin and Kang, 2003; Filoche et al., 2005) have been undertaken but not in great depth. In the review by Williamson (2001) on synergy, the need for further interactive investigations was emphasized.

Despite the extensive traditional use of *Croton gratissimus* Burch. var. *gratissimus* (Euphorbiaceae) for medicinal purposes (Watt and Breyer-Brandwijk, 1962; Van Wyk et al., 1997; Van Wyk and Gericke, 2000), scientific studies validating the therapeutic properties of this indigenous plant are lacking. One of the vernacular (Afrikaans) names for *Croton gratissimus* is "koorsbessie" ("koors" = fever) suggesting that the plant is used as a pyrogenic. All parts of the plant have reputed medicinal value (Van Wyk et al., 1997; Hutchings et al., 1996). The leaves are aromatic and often used as infusions to treat coughs. Decoctions of roots have been used to treat chest complaints, coughs, fever and sexually transmitted diseases such as syphilis (Von Koenen, 2001). The bark is most frequently used to treat bleeding gums, abdominal disorders, skin inflammation, earache and chest complaints (Hutchings et al., 1996; Von Koenen, 2001). The combinations of roots and bark to treat respiratory disorders have also been reported (Von Koenen, 2001). Reports on the co-administration with other species (Hutchings et al., 1996) have also been noted, i.e. for the treatment

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of swellings the bark of *Croton gratissimus* is combined with the root of Amaryllidaceae species and rubbed into incisions. The powdered bark of *Croton gratissimus* together with bark of *Ocotea bullata* is used to treat uterine disorders. As the bark, roots and leaves of *Croton gratissimus* are used separately as well as in combination, this study focused on determining antimicrobial efficacies of the plant parts independently and in combination to assess possible pharmacological interactions (e.g. synergy, antagonism).

2. Materials and methods

2.1. Plant collection, preparation of the crude extracts and distillation of essential oils

To rule out any possible chemotypic variation, the plant material (leaf, bark and root) were collected from a single tree in the wild on the western slopes below 'Diep-in-die-Berg' in the Tshwane area of South Africa. Extracts were prepared by submerging the dried macerated plant material (leaves 17.1 g, bark 22 g and root 24.4 g) in a 1:1 mixture of methanol and chloroform (approximately 300 ml) overnight. After filtration, the solvent was evaporated using a rotary evaporator. The yield for the root, leaf and bark extracts were 4.34%, 4.04% and 6.41% (w/w) respectively. Leaves (470.8 g, wet weight), were hydro-distilled in a Clevenger-type apparatus and the essential oil (0.3%, w/w) collected after 3 h. A voucher specimen (PB975) was deposited at the Department of Pharmaceutical Sciences, Tshwane University of Technology.

2.2. Antimicrobial aspects

Culture inoculum, media preparation and assays were undertaken according to the NCCLS guidelines (2003). The minimum inhibitory concentration (MIC) method was adopted from Eloff (1998) as it has been refined for plant-based studies as well as Carson et al. (1995), which makes provision for the volatility of essential oil samples. All stock cultures were obtained from the National Health Laboratory Services with the exception of *Candida albicans,* which was obtained from the South African Bureau of Standards. Table 1 lists the cultures with corresponding reference numbers used in this study. Micro-well MIC's were performed with nine test organisms on the essential oil, leaf, bark and root extracts singularly and in combination. Commercial antimicrobials (ciprofloxacin for bacteria and amphotericin B for yeasts) at starting concentrations of 0.01 and 0.10 mg/ml respectively were included as positive controls in all MIC repetitions to validate microbial sensitivity. Assays were repeated to ensure standard error variation of not more than one dilution factor was obtained.

Once the independent MIC was determined for the essential oil, leaf, bark and root extracts, the synergistic or antagonistic interaction between plant parts was investigated using two approaches. Firstly, stock solutions (64 mg/ml) of each individual extract was prepared and mixed in the following combinations: leaf and root (1:1), bark and root (1:1), leaf and bark (1:1) and leaf, bark and root (1:1:1). An MIC was determined for these combinations to establish any interaction affecting the antimicrobial activity. The fractional inhibitory concentration (FIC) was calculated for the leaf with bark combination, bark with root combination, and leaf with root combination. The FIC is expressed as the interaction of two agents where the concentration of each test agent in combination is expressed as a fraction of the concentration that would produce the same effect when used independently (Berenbaum, 1978). The FIC index (Schelz et al., 2006) is determined as the correlation between the two combined test substances and may be classified as either synergistic ($\leq$0.5), additive (>0.5 to 1.0), indifferent (>1.0 to <4.0) or antagonistic ($\geq$4.0).

The second part of this study involved combining the extracts of the roots and leaves in nine ratios, i.e. 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, and 10:90. The MIC was determined for all ratios and the two extracts independently. The MIC values (mg/ml) were plotted on an isobologram, allowing for a graphical representation of the interaction of the various combinations. The isobologram can be interpreted by examining the data points of the ratios where the MIC for each concentration is determined in relation to the independent MIC's (shown as a straight line) and extrapolating synergy (below the line), antagonism (above the line) and additive interaction in the vicinity closest to or on the line. Conventional antimicrobials (not shown on isobologram) were included in all repetitions. Tests were undertaken in triplicate and the mean values plotted on the isobologram. Only values within the 0–1.25 range were plotted on the isobologram.

3. Results

Previous studies on *Croton* species report positive antimicrobial activity for the essential oils (Martins et al., 2000; Alviano et al., 2005). In this study moderate activity (4–11 mg/ml) for the essential oil against six pathogens is noted (Table 1). Three pathogens (*Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli*) showed poor susceptibility (13–32 mg/ml) towards the essential oils (Table 1). The leaf, bark and root extract showed higher efficacies than the oils against all the pathogens studied (Table 1). All three Gram-negative organisms (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) showed highest susceptibility to the leaf extracts with the bark and root having equivalent susceptibility patterns. Both yeasts indicated a relatively consistent MIC value for all three extracts: *Candida albicans* (5–6 mg/ml) and *Cryptococcus neoformans* (2 mg/ml).

Table 1
The MIC values (mg/ml) for the extracts, independently and in combination for *Croton gratissimus* with the FIC[a] where two extracts were combined

Pathogen	Essential oil	Leaf	Bark	Root	Leaf, bark and root (1:1:1)	Leaf and bark (1:1)	Bark and root (1:1)	Leaf and root (1:1)	Control[b]
Staphylococcus aureus (ATCC 12600)	13.3	2.3	0.6	0.4	0.3	2.0 (4.2)	0.4 (1.6)	1.0 (2.9)	1.0×10^{-3}
Enterococcus faecalis (ATCC 29212)	10.6	6.0	6.0	2.6	1.3	4.0 (1.4)	1.3 (0.7)	1.5 (0.8)	0.2×10^{-3}
Bacillus cereus (ATCC 11778)	6.0	1.5	0.9	0.8	0.3	1.0 (1.8)	0.3 (0.7)	0.7 (1.3)	0.5×10^{-3}
Staphylococcus epidermidis (ATCC 2223)	32.0	4.0	0.8	4.0	1.0	4.0 (6.0)	3.0 (4.5)	2.0 (1.0)	1.6×10^{-3}
Escherichia coli (ATCC 11775)	16.0	2.0	4.0	4.0	2.0	3.0 (2.3)	1.5 (0.8)	4.0 (3.0)	0.1×10^{-3}
Klebsiella pneumoniae (ATCC 13883)	8.0	4.0	6.0	6.0	2.0	4.0 (1.7)	3.0 (1.0)	2.0 (0.8)	0.2×10^{-3}
Pseudomonas aeruginosa (ATCC 9027)	8.0	1.0	2.0	2.0	0.6	2.0 (3.0)	1.5 (1.5)	0.5 (0.8)	0.3×10^{-3}
Candida albicans (ATCC 10231)	8.0	6.0	6.0	5.3	1.0	4.0 (0.7)	>8 (>8)	2.0 (0.8)	1.3×10^{-3}
Cryptococcus neoformans (ATCC 90112)	4.0	2.0	2.0	2.0	0.6	4.0 (2.0)	0.8 (0.8)	0.4 (0.4)	0.6×10^{-3}

[a] FIC is shown in brackets for the leaf and bark combination, bark and root combination, and leaf and root combination.
[b] Control is ciprofloxacin and amphotericin B served as controls for bacteria and yeasts respectively.

When leaf, bark and root extracts are studied in a 1:1:1 ratio, antimicrobial efficacy is either enhanced (lower MIC value) or equivalent (equal MIC value) for all pathogens except for *Staphylococcus epidermidis* where the MIC in combination is lower than the leaf and root assayed separately, but higher than the MIC obtained for the bark extracts (Table 1).

When leaf and bark extracts were combined, efficacy was enhanced only for *Enterococcus faecalis* and *Candida albicans*. Antagonism (FIC ≥ 4) was noted for two of the nine pathogens studied. The bark and root combination had a lower MIC than when investigated independently for *Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Cryptococcus neoformans*. The FIC for all these pathogens (with the exception of *Pseudomonas aeruginosa*), indicated an additive (FIC 0.5–1) effect when combined in a 1:1 ratio. For *Staphylococcus aureus* (FIC 1.6) and *Pseudomonas aeruginosa* (FIC 1.5) no significant interaction was noted. The greatest antagonism (FIC ≥ 4) was noted for *Staphylococcus epidermidis* (FIC 4.5) and *Candida albicans* (FIC > 8).

When the leaf and root were combined in a 1:1 ratio, most of the test pathogens (*Enterococcus faecalis*, *Bacillus cereus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Candida albicans* and *Cryptococcus neoformans*) had MIC's lower in combination (higher antimicrobial efficacy) than when investigated independently. The FIC for most of these pathogens showed an additive effect. A synergistic effect (FIC 0.4) was only demonstrated with studies on *Cryptococcus neoformans*.

While these results give an indication of interaction between the plant parts, the methodology is restricted to a 1:1 combination only. Isobolograms of the leaf and root combination (based on the traditional use) were thus undertaken which considered more than one ratio of the test substances. The isobolograms for all ten pathogens are shown in Figs. 1–3.

The isobologram combination studies for the Gram-positive organisms do not show any specific pattern for *Bacillus cereus*, where synergy was observed for four ratios in the isobologram where the leaf material were equivalent and in majority. The FIC index (Table 1) for *Bacillus cereus* shows indifference (1.3) for the leaf and root combination (1:1). MIC data for *Bacillus cereus* indicate that the combined MIC (0.7 mg/ml) is lower than the MIC for leaves (1.5 mg/ml) and MIC for roots (0.8 mg/ml). The *Staphylococci* having FIC values of 2.9 and 1.0 for *Staphylococcus aureus* and *Staphylococcus epidermidis* respectively (Table 1) show similar concentration dependent additive to indifferent profiles, with the 1:1 ratio of the isobole curve being on the line (Fig. 1). Results obtained for both combination methods are consistent for *Staphylococcus epidermidis*. For *Enterococcus faecalis*, most ratios (Fig. 1) where the leaf ratio was higher than the root, an additive profile predominated. Where the root ratio is higher than leaf ratio and where ratios are in equilibrium a synergistic profile mostly predominated. The 1:1 ratio (Table 1) indicates how efficacy is enhanced when comparatively observing the independent profiles. The FIC (0.8) for *Enterococcus faecalis* indicates an additive effect in the 1:1 combination thus corroborating results obtained in the isobologram study.

The isobolograms for the Gram-negative test organisms: *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli* all indicate a ratio dependent pattern (Fig. 2). All ratios mainly fall within the additive profile range. The 1:1 combination (leaf and root) tested on *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* indicated an additive effect when the FIC is calculated (Table 1).

Both yeasts (*Candida albicans* and *Cryptococcus neoformans*) investigated in the combination studies showed synergism for most

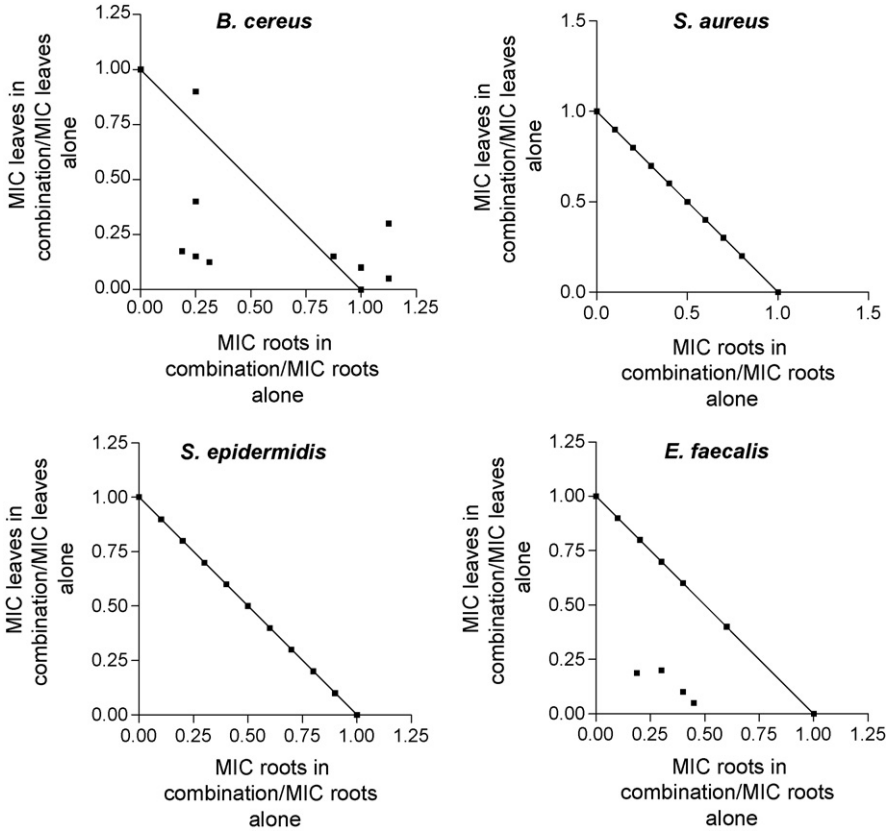

Fig. 1. The isobologram of leaf and root combination for the Gram-positive test organisms (*Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis* respectively).

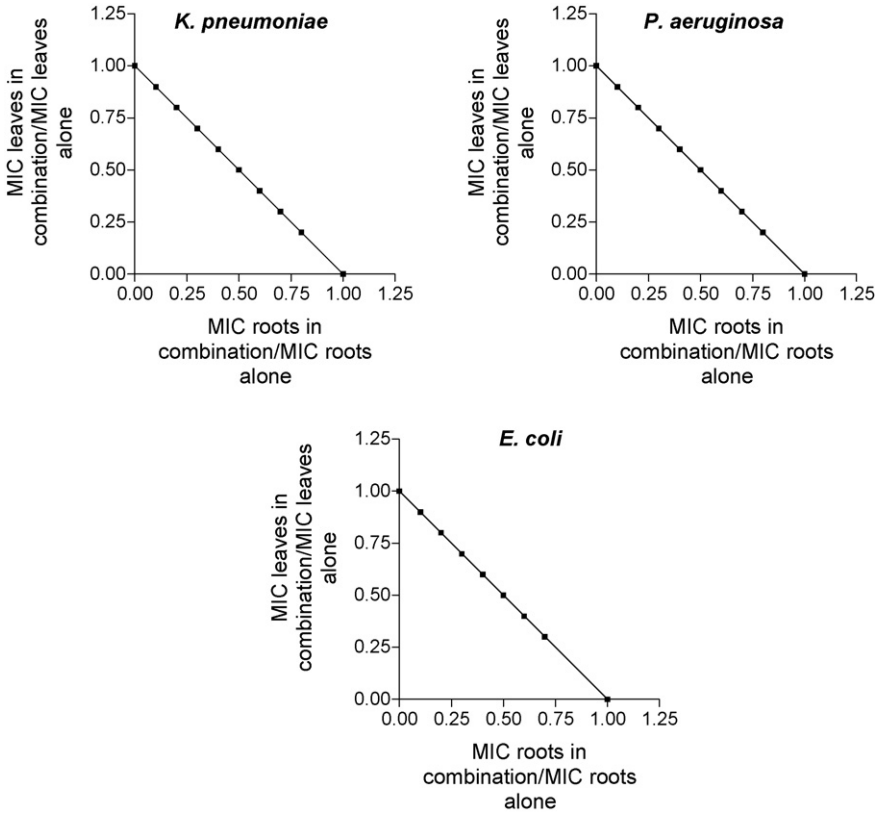

Fig. 2. The isobologram of leaf and root combination for the Gram-negative test organisms (*Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli* respectively).

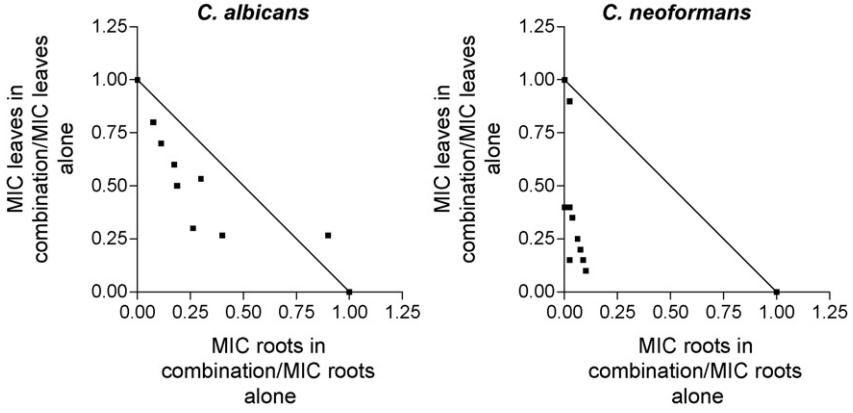

Fig. 3. The isobologram of leaf and root combination for the yeasts *Candida albicans* and *Cryptococcus neoformans*.

ratios when observing the isobologram data (Fig. 3). The FIC for *Candida albicans* (0.8) indicates an additive effect whereas for *Cryptococcus neoformans* the FIC (0.4) was synergistic. In both 1:1 FIC combination studies and isobologram ratios, the leaf/root combination against *Cryptococcus neoformans* indicates the greatest synergistic pattern.

4. Discussion

Research on synergy relating to antimicrobial activity has in the past been very vaguely defined and conclusions are often based on results obtained using one experimental procedure (Lambert et al., 2003). Synergy as determined by the FIC method varies in interpretation of results and may either be expressed as an FIC of <1 (Berenbaum, 1978) or ≤0.5 (ESCMID, 2000). Similarly an isobole on the line may be interpreted as either additive (Berenbaum, 1978) or no interaction (Williamson, 2001). It was therefore decided that the most comprehensive and most recent definition (Schelz et al., 2006) would be used to classify FIC synergy or antagonistic interactions. Until recently the checkerboard MIC method and the calculation of FIC indices were the only methods adopted for antimicrobial studies investigating synergy (Ryan et al., 1981; Lambert et al., 2003). Variation may be encountered when looking at different methods to assess synergistic and antagonistic interactions. This was highlighted by Odds (2003), wherein it was stated that errors in reproducibility using MIC methods are common and researchers were encouraged to consider more sophisticated approaches to measure synergy. Recently, a mathematical 3-dimensional isobologram approach for the examination of antimicrobial agents was

presented by Boucher and Tam (2006) validating isobolograms as an effective tool for the examination of antimicrobial combinations. Thus, the synergistic investigation of *Croton gratissimus* was based on more than one method. The results obtained for both methods were not always in agreement. Of the nine pathogens investigated in the combination studies, six showed congruency in both methods with *Staphylococcus aureus* showing partial congruency.

It is clear however, that despite the variability, combining various plant parts of *Croton gratissimus* enhances efficacy. This is demonstrated when comparing the MIC's of different plant part combinations in either a 1:1 or 1:1:1 ratio (Table 1). The combination of leaf and root demonstrates the greatest efficacy of the 1:1 combinations having five additive profiles and one synergistic profile for the nine test pathogens studied. The benefit of combining roots and leaves of *Croton gratissimus* is also apparent in the isobologram studies where most ratios show an additive or close to an additive profile for most pathogens studied. Note that the leaf:root combination has been cited in the ethnobotanical literature for the treatment of respiratory conditions. Furthermore, the respiratory pathogen (*Cryptococcus neoformans*) showing the highest synergistic profile (MIC 0.4; FIC 0.4) correlates with diseases for which the plant is traditionally used.

5. Conclusion

It is common to observe in ethnopharmacological and natural products literature in general, that researchers embark on the laborious process of isolating molecules from an active extract. Often, the crude extract is then assayed in parallel with the isolated molecules only to conclude that the extract is more active than the isolated molecules. In many of such studies, synergy is then speculated and offered as an explanation for the 'anomalous' results but not further explored.

Rios and Recio (2005) recommended that a high priority should be given to further studies involving plant combinations and Gilani and Rahman (2006) amplified that there is a scarcity of ethnopharmacognosists performing much needed studies into herbal combinations. This aspect has probably been neglected due to the more complex methodologies involved to examine possible synergistic interactions. The use of more intricate methods such as the isobologram is a novel way to demonstrate such interactions. Furthermore, journal editors and reviewers often insist that molecules are isolated from an active crude extract and the biological activity confirmed as this is seen to add 'scientific credibility' to the study. Although this 'one molecule one target' approach is justified in many cases, the value of multi-molecule therapy cannot be ignored and this application is becoming increasingly important in the treatment of some of the most virulent diseases such as tuberculosis and malaria. This study (albeit *in vitro*), using the ethnomedicinally important plant *Croton gratissimus* as a model, clearly provides evidence that certain plant part combinations are more efficacious, granting scientific credibility to the documented traditional use.

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