

# Plant-Based Antimicrobial Studies – Methods and Approaches to Study the Interaction between Natural Products

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## Key words

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## Abstract

▼  
The therapeutic value of synergistic interactions has been known since antiquity, and many different cultural healing systems still rely on this principle in the belief that combination therapy may enhance efficacy. This paper intends to provide an overview, from an antimicrobial perspective, on the research undertaken and interactive principles involved in pharmacognosy studies. Methods used to determine antimicrobial interactions include basic combination studies, the sum of the fractional inhibitory concentration index ( $\Sigma$ FIC), isobole interpretations, and death kinetic (time-kill) assays. The various interactions are discussed with reference to molecules, different plant parts or fractions, different plant species, and combinations with nonbotanical antimicrobial agents. It is recommended for future development in the field of phytosynergy that consideration should be given to the selection criteria for the two inhibitors. A more conservative approach should be adopted when classifying synergy. When examining interactions in plant-based studies, antagonis-

tic interactions should not be ignored. Combinations involving more than two test samples should be examined where applicable, and very importantly, the mechanism of action of synergistic interactions should be given precedence. It is encouraging to observe the upsurge in papers exploring the complex interactions of medicinal plants, and undoubtedly this will become increasingly important in our continued quest to understand the mechanism of action of phytotherapy. The scientific validation of efficacious antimicrobial combinations could lead to patentable entities making research in the field of phytosynergy not only academically rewarding but also commercially relevant.

## Abbreviations

▼  
CFU: colony forming units  
FIC: fractional inhibitory concentration  
GC-MS: gas chromatography coupled to mass spectrometry  
MIC: minimum inhibitory concentration

## Introduction

▼  
The therapeutic value of synergistic interactions has been known since antiquity, and many different cultural healing systems have relied on this principle in the belief that combination therapy may enhance efficacy. The ancient texts pertaining to Ayurveda and traditional Chinese herbal medicine describe formulas consisting of complex herbal mixtures which may contain several plant-based ingredients [1]. African traditional healers rarely rely on a single plant for therapeutic regimens but often combine various plant parts and different species in order to achieve optimal results. The fundamental principle of aromatherapy is the combination of highly complex different es-

sential oils to achieve a therapeutic effect. The historical use and application of polyherbals has been carried down through the centuries, and today allopathic medicine commonly uses the very same principles to combine various molecules in single or separate dosage forms which are administered concomitantly. Recently, the application of combination therapy has gained a wider acceptance, especially in the treatment of infectious diseases. The World Health Organization, for example, has urged pharmaceutical companies to stop promoting the use of artemisinin derivatives in monotherapy. Instead, artemisinin combination therapy should be encouraged not only because it has a cure rate of 95% against the malaria parasite (*Plasmodium falciparum*) but may also con-

tribute to curb resistance. Multidrug therapy has become of paramount importance in the fight against multidrug resistant microbial strains. Without the current multidrug approach used to treat tuberculosis (isoniazid, rifampicin, pyrazinamide, and ethambutol), the mortality of infected patients could reach global epidemic proportions. Another renowned antimicrobial agent having a significant synergistic effect in combination is amoxicillin (a  $\beta$ -lactam antibiotic) and clavulanic acid. Clavulanic acid binds to  $\beta$ -lactamase producing microorganisms, which protects amoxicillin from  $\beta$ -lactamase attack, which in turn results in an extended spectrum of activity for amoxicillin.

The concept of antimicrobial synergy is based on the principle that, in combination, the formulation may enhance efficacy, reduce toxicity, decrease adverse side effects, increase bioavailability, lower the dose and reduce the advance of antimicrobial resistance [2–4]. New antimicrobial combination drugs which include natural product combinations have recently become a research priority. This approach has financial implications as reformulation of existing drugs or combinations may prove to be a more viable option, rather than developing a new drug which will require extensive clinical trials for verification. Furthermore, the ban imposed by the United States of America and European Union on the use of allopathic antimicrobials in livestock farming has led to the search for natural antimicrobial combinations that may impact positively on agricultural and livestock farming [4]. Even though the concept of interactive antimicrobial therapy is well practised in western medicine, and many anecdotal accounts of plants used in combination for the treatment of microbe-related infections are evident, the validation of this phenomenon in the field of pharmacognosy has been neglected. Medicinal plants offer a vast resource of natural compounds, and the exploration of the various levels of interaction that may exist include: constituents within a plant, interactions between different parts of a plant, between different plant species, or the interaction with non-plant-based antimicrobials. It is encouraging to observe that there has been a recent increase in the number of publications reporting on plant-based pharmacological interactions. The concept of synergistic principles from a pharmacological and/or phytotherapeutic perspective has been addressed in various reviews [5–8], but these have not specifically focused on the antimicrobial interactions. The literature on the proposed methods appears fragmented and often confusing, and several experimental designs have been proposed, which leaves the results inconclusive. This review intends to succinctly collate the available literature which has focused on interactive plant-based antimicrobial studies and to propose various methods and approaches which could be considered when embarking on research in this field.

### Experimental Approaches

The terminology defining the possible interactions that may occur are often subject to debate and interpretation [5,9–13], thus for the sake of this review, the associated terminology should be defined. The word “synergy” is derived from the Greek word “*syn-ergo*” meaning working together, and the resulting effect may be defined as a combination that is significantly greater than the sum of its parts. Synonyms used include “polyvalent activity” and “potentiation”. An “additive” or “summative” effect occurs when substances added together will improve or increase efficacy. A “noninteractive”, “indifferent” effect, or “zero interaction”

reflects an expected linear response when two agents are combined and show neither an additive nor antagonistic effect. “Antagonism” is a phenomenon where two or more agents in combination have an overall effect which is less than the sum of their individual effects [12,14,15]. For simplification, the terms “synergism, additive, indifferent, and antagonism” will be used to describe the types of interactions.

There are a number of different methodologies that have been proposed to express antimicrobial interactions. Many of these methods such as Etests, time-kill, and checkerboard methods have been comparatively evaluated [16–19]. Congruency in results for the evaluation of antibiotic synergy against *Acinetobacter baumannii* obtained between the three methods (Etests, time-kill, and checkerboard) varied between 51–72% [18]. Comparative results generated from the time-kill and checkerboard method presented only a 51% value of congruency. Lewis et al. [19] favoured the Etest where antimicrobials of fixed concentrations are impregnated on commercially available filter strips. This method, however, is not applicable for plant-based antimicrobial studies in which the test antimicrobial is not a commercially available sample at standard concentrations but an experimental plant sample whose preparation is dependent on the undergoing study. Various authors have expressed concern over the methods used to interpret synergy [5,11–13,20–23]. In an editorial published in the *Journal of Antimicrobial Chemotherapy* [10], the interpretation of interactive methods was debated and a more conservative analysis of synergistic interpretations encouraged (see section “The fractional inhibitory concentration index” for further discussions on this). Mathematical models and statistical approaches to validate antimicrobial interactions have been developed to allow for a more reliable and quantitative assessment of pharmacological interactions [24–27].

Considering plant-based antimicrobial studies, the use of different methodologies range from the most basic disc diffusion assays found in earlier ethnobotanical studies [28,29], to more recent studies incorporating the sum of the fractional inhibitory concentration index ( $\Sigma$ FIC) [30–33], time-kill methods [34–38], and isobologram studies [3,39–43].

### “Basic” combination studies

The simplest form of determining synergy is by means of diffusion assays. Each independent test sample (A or B) is placed in a well or on a disc. The combination (A + B) is placed on a separate disc and the inhibition zone of the combination comparatively examined with the independent test samples. Should the inhibition zone be larger in A + B than either A or B then synergistic interactions are noted. Should the inhibition zone be smaller in A + B than A or B independently, then antagonistic interactions are noted. Although simple, these assays are subject to many variables which may influence the results and should at the most be used as a qualitative guide only [40,44,45].

Basic minimum inhibitory concentration (MIC) assays may also be used to determine interactions. The microdilution method is undertaken, and combinations are comparatively assessed by incorporating the inhibitors at selected concentrations and combinations [46,47]. This arrangement of combinations formed by multiple dilutions is referred to as the checkerboard method.

Some combination studies have incorporated impedimetric methods extrapolating synergy by comparing growth as determined by optical density readings of single entities and comparing these growth rates with that found when exposed to test substances in combination [48,49]. One drawback in using such a

method is that the assessment of viability is not always accurate when relying on turbidometric readings.

### The sum of the fractional inhibitory concentration index ( $\Sigma$ FIC)

An algebraic equation to determine synergy by means of the  $\Sigma$ FIC is a widely accepted means of measuring interaction. The  $\Sigma$ 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 [50]. The  $\Sigma$ FIC is then calculated for each test sample independently as specified in the following equations:

$$FIC^{(i)} = \frac{\text{MIC (a) in combination with (b)}}{\text{MIC (a) independently}}$$

$$FIC^{(ii)} = \frac{\text{MIC (b) in combination with (a)}}{\text{MIC (b) independently}}$$

The sum of the FIC or FIC index is thus calculated as:

$$\Sigma FIC = FIC^{(i)} + FIC^{(ii)}$$

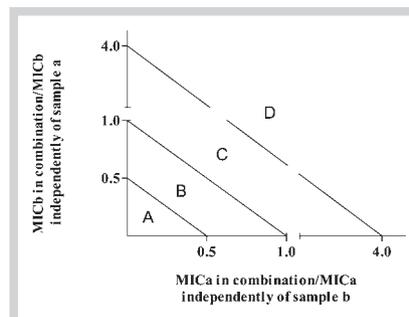
This basic equation has remained constant since inception. However, the interpretation has evolved and varies from author to author. The interpretation of the  $\Sigma$ FIC index as a numerical value is arbitrary, and the thresholds presented have stemmed from the need to critically assess interactions that are clinically significant [13]. The earlier interpretations by Berenbaum [50] were very broad taking into account synergistic interactions having  $\Sigma$ FIC values below one, antagonistic interactions above one, and additive interactions narrowly focused on one. These interpretations make it easy to analyse isobolograms and have been used in many papers describing interactions. A more conservative approach in describing interactions was recommended by Odds [10], with interpretations described as synergistic ( $\Sigma$ FIC  $\leq 0.5$ ), antagonistic ( $\Sigma$ FIC  $> 4.0$ ), and noninteractive ( $\Sigma$ FIC  $> 0.5-4.0$ ). The conservative approach takes into account inherent variations when performing MIC doubling dilution assays. Unfortunately, the “no interaction” range is a very broad one and makes no allowance for additive interpretations. A number of recent reputable studies, mainly reported in ISI antimicrobial journals, have incorporated an “additive” range into the interpretation for better clarification of the data set [4, 5, 13, 19, 42, 51–55]. In the critical review by Bell [13], the need to include a broader range to interpret pharmacological interactions was emphasised. Taking this into account, an additive range should be included, and it is suggested that the interpretation of either synergistic ( $\Sigma$ FIC  $\leq 0.5$ ), additive ( $\Sigma$ FIC  $> 0.5-1.0$ ), noninteractive  $\Sigma$ FIC ( $> 1.0-\leq 4.0$ ), or antagonistic ( $\Sigma$ FIC  $> 4.0$ ) should be used when describing *in vitro* antimicrobial interactions. A summary of the interpretative values given for the  $\Sigma$ FIC in accordance with the corresponding authors is given in **Table 1**. Irrespective of the variations in interpretation, most authors are in agreement that synergistic interactions should be considered only for  $\Sigma$ FIC values 0.5 and lower.

Although using  $\Sigma$ FIC calculations to determine interaction appears to be the simplest method, one needs to consider the limitations. The FIC method is based on the assumption that half the concentration will provide half the effect. However, this is not always the case, as two inhibitors may not always have identical dose responses [11]. The use of isobolograms which take into account combinations at various concentrations provide a more realistic means of measurement.

**Table 1** Classification of the  $\Sigma$ FIC in accordance with corresponding authors.

Interaction				References
Synergy	Additive	Indifference	Antagonism	
$\leq 0.7$	*	*	$\geq 1.3$	[133]
$< 1.0$	1.0	*	$> 1.0$	[50, 134]
$\leq 0.5$	$> 0.5-4.0$		$> 4.0$	[135]
$\leq 0.5$	1.0	*	$\geq 2.0$	[4]
$\leq 0.5$	$> 0.5-1.0$	$> 1.0- < 2.0$	$\geq 2.0$	[14]
$\leq 0.5$	*	$> 0.5- \leq 4.0$	$> 4.0$	[23]
$\leq 0.5$	$0.5- < 1.0$	$\geq 1.0- < 4.0$	$\geq 4.0$	[19]
$\leq 0.5$	*	$> 0.5-4.0$	$> 4.0$	[136]
$\leq 0.5$	*	$> 0.5-4.0$	$> 4.0$	[10]
$< 0.5$	$0.5-1.0$	$\geq 1.0-4.0$	$> 4.0$	[53]
$< 0.5$	$0.5- \leq 1.0$	$> 1.1- \leq 4.0$	$> 4.0$	[106]
$\leq 0.5$	*	$> 0.5- < 4.0$	$\geq 4.0$	[137]
$\leq 0.5$	$> 0.5-0.75$	$0.76-2.0$	$\geq 2.0$	[138]
$\leq 0.5$	$> 0.5-1.0$	$> 1.0- \leq 4.0$	$> 4.0$	[42], van Vuuren and Viljoen (recommended herein)

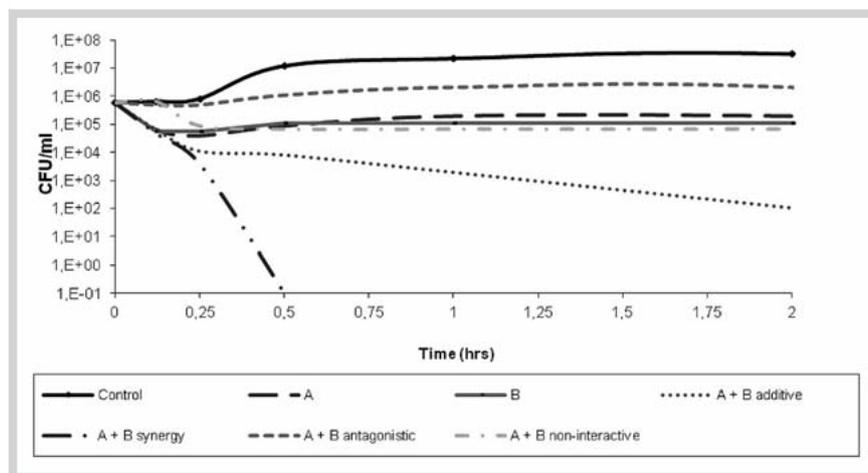
\* Not given by author



**Fig. 1** Isobologram which, if ratio points for two combined inhibitors fall in quadrants **A** depicts synergy; **B** an additive effect; **C** a non-interactive effect; and **D** an antagonistic interaction.

### The isobole method

The isobole method of determining interaction is possibly one of the oldest methods used to express interactions, dating back to publications from 1870. Although well established, negative connotations have been associated with this method proving it unfavourable, until more recently when mathematical equations have been proposed to validate the results [25, 26]. It is presently the favoured method for interactive assessment [13, 15]. The principle is based on the fact that interactions may vary depending on the ratio in which the two inhibitors are combined. Although complicated, this method gives a more accurate assessment of each agent when studied in various combinations. The procedure involves the combination of two samples at various ratios. The MIC value for each sample is determined independently and comparatively assessed against the MIC value obtained in the ratio combination. This is expressed as a dose ratio response on an isobole graph. The adjoining line of the two axes indicates the individual doses and the isobologram can be interpreted by examining the data points of the ratios. The classical interpretation of the isobole is where the data points fall below the 1:1 line; synergy is expressed [50]. Antagonism is noted for data points falling above the 1:1 line, and an additive response is given when ratio points fall in the vicinity closest to or on the line. To standardise interactive values with the more conservative approach recommended by Odds [10], two additional lines are proposed, at the 0.5:0.5 and 4.0:4.0 axes (**Fig. 1**). These proposed



**Fig. 2** Time-kill method of interpreting interactions when two samples are combined.

additions will allow cross comparison between  $\Sigma$ FIC methods and isobole interpretations.

### Death kinetic (time-kill) assays

Time-kill studies provide descriptive information on the relationship between bactericidal activity and the concentration of test substance [27]. Even though the methodology is labour intensive and requires a number of steps where variables may be introduced, valuable information is given of the death kinetics over time. The time-kill method has been praised as one of the best methodologies to study synergy [48], even though earlier shunned by Berenbaum [56]. Further validation of the death kinetic method to assess synergy was given [57], and the method was commended from a clinical perspective. In an overview of the various methods to test antimicrobial synergy with conventional antibiotics, the time-kill method was found to be one of the most frequently employed, showed better sensitivities and greater reproducibility [19]. Briefly, the principle involves exposing the inhibitor to a selected pathogen and, at selected time intervals, aliquots are sampled and serially diluted. The dilutions are plated out, incubated at optimum conditions for the test organism, and the colony forming units (CFU) are counted and plotted logarithmically against time. Depending on the curve of the dose response, either an additive, synergistic, or antagonistic effect is noted (● Fig. 2). Antagonism in time-kill methods may be defined as at least a 100-fold increase in colony counts whereas synergism, a 100-fold decrease in colony counts [48].

In spite of the positive recommendation of this method to describe antimicrobial interactions, the method is not frequently used in plant-based studies. This is possibly due to the labourious nature of repetitive dilution sampling. The positive aspect of this method lies in the possibility to present a direct relationship in exposure of plant test material to a pathogen. A cidal effect is monitored over time which is not possible with the frequently used MIC assays.

### The Various Levels at Which Antimicrobial Interactions May Be Explored

#### Interaction between molecules

When examining the published literature and searching for scientific articles documenting the interactions between molecules, findings were predominantly focused on essential oil constitu-

ents. A quick review of some of our own studies demonstrates varied essential oil compositions ranging anywhere from 25 to over 173 compounds in any given plant [40]. It is thus not surprising that any of these compounds may interact to either enhance or reduce pharmacological effects. With the sophisticated gas chromatography coupled to mass spectrometry (GC-MS) and the multidimensional gas chromatography techniques available today, detection of any number of compounds in a given plant may be undertaken. Investigation of these interactions has thus become a more viable option than isolating compounds from extracts and investigating interactions. Another limitation of isolating compounds and investigating their interactive properties is that yields are usually insufficient. With many essential oil studies, the identified compounds are done using commercially available databases and retention indices.

In a review on synergism by Harris [58], the author documents on a number of earlier antimicrobial studies (between 1974–1996) on volatile constituents that demonstrate synergistic interactions between constituents, synthetic substances, and even ingredients within a formulation. Pattnaik et al. [59] noted that MICs from essential oils were in many cases lower than the major constituents independently, suggesting that synergy between constituents may be contributing to the enhanced activity. In another study, linalool was combined with methyl charvicol at v/v ratios of 1:0; 0.8:0.2; 0.6:0.4; 0.4:0.6; 0.2:0.8, and 0:1 [60]. It was observed that when these two monoterpene alcohols are combined, a higher efficacy is achieved, compared to when they are assayed independently. In another study, the interaction of the major essential oil constituents of four *Thymus* species was examined by the MIC checkerboard method [61]. Various interactions ranging from indifferent to synergistic were observed when combining carvacrol, thymol, 1,8-cineole, and *p*-cymene. No antagonism was noted, and the greatest synergistic interaction was observed with the thymol:1,8-cineole and thymol:*p*-cymene combination, having  $\Sigma$ FIC values of 0.125. In view of the international concern on the use of antibiotic growth promoters in animal feeds, it was interesting to note the application of combined essential oil constituents in controlling the antimicrobial populations in the pig gut. The combination of carvacrol and thymol demonstrated synergism, and recommendations for appropriate ratio studies to determine optimum synergistic effects were recommended [62].

In-depth isobologram interpretations have been undertaken on essential oil constituent interactions. Varied interactions were

noted in a study where the pharmacological interactions of a number of essential oil constituents were investigated [63]. Synergism was observed between (+)- $\beta$ -pinene and carvacrol as well as between  $\gamma$ -terpinene and geranyl acetate when tested against *Staphylococcus aureus*. (+)- $\beta$ -Pinene and (-)-menthone showed antagonism ( $\Sigma$ FIC value of 9.8), but interactions of (+)- $\beta$ -pinene with 1,8-cineole demonstrated synergy ( $\Sigma$ FIC value of 0.4), when tested together against *Candida albicans*. The combination of *trans*-geraniol and *E*- and *Z*-( $\pm$ )-nerolidol demonstrated an additive interaction against *Bacillus cereus*. For eugenol and *E*- and *Z*-( $\pm$ )-nerolidol, an indifferent interaction against *Escherichia coli* was noted. These results demonstrate varied interactions and not only synergism. All of these studies have one thing in common; they have focused on random essential oil constituent combinations.

Although there have been a number of papers that have focused on structure activity related antimicrobial studies of compounds within a plant [64–67], very little research has been conducted on how the co-occurrence of combined compounds contribute to efficacy. It has been questioned that independent activity related to one or two specific constituents is questionable and that synergistic functions between molecules are more probable [68]. In a study by Radulović [69], the major compound (68.6% salicylaldehyde) from *Filipendula vulgaris* was isolated and found to be less active than the whole essential oil. When combined in a 60:40 ratio with linalool (1.8% composition in *F. vulgaris* oil), strong synergistic activity was noted. Interestingly, when salicylaldehyde was combined with another essential oil component, methyl salicylate (2.4%) in a 60:40 ratio, antagonism was observed. We too have noted that synergistic interactions between molecules within a plant are evident. The two major essential oil components from *Osmitopsis asteriscoides* identified by GC-MS were (-)-camphor (12%) and 1,8-cineole (60%) representing 72% accumulatively. Time-kill studies were performed on the pathogen *C. albicans*, where (-)-camphor demonstrated negligible antimicrobial activity and 1,8-cineole indicated a cidal effect after 240 min. When these two major compounds were tested in combination, a synergistic effect was noted having a cidal effect at 15 min [35]. Prediction that synergistic interactions occur only between major constituents may not always be accurate. Earlier studies demonstrated that less abundant components may interact synergistically [70]. This has been noted in other studies where the  $\beta$ -triketone complex of manuka oil was found to have poor bactericidal properties [34]. Similarly, we found this to be evident when investigating the antimicrobial activity of the major constituents of *Artemisia afra*. The four major compounds (artemisia ketone, 1,8-cineole,  $\alpha$  and  $\beta$ -thujone, which accounts for 51.9% of the total composition) were investigated independently and in various permutations. Results showed minimal antimicrobial activity against *Klebsiella pneumoniae*. It was thus postulated that the minor compounds either independently or in combination contribute to the antimicrobial activity [37]. As noted in these interactions, when examining whole essential oils, predictions are complex and not only should major or minor compounds be considered, but one also needs to consider the stereochemistry of compounds. While it is known that biological activity is influenced by the enantiomeric configuration, the overall antimicrobial activity of different enantiomers may be additionally affected by interaction with other compounds. To demonstrate this, a study was undertaken on the different enantiomers of limonene in combination with 1,8-cineole. Isobologram plots for *S. aureus* demonstrated similar antagonis-

tic activity when exposed to the combinations of 1,8-cineole with (+) and ( $\pm$ )-limonene. However, with (-)-limonene, synergism was evident at selected ratios. Differences in activity were clearly noted with the other two pathogens studied (*Pseudomonas aeruginosa* and *Cryptococcus neoformans*), thus highlighting the significance of stereochemistry in antimicrobial combination studies.

Compound interaction studies on nonessential oil components include the combined effects of cinnamaldehyde with catechin, quercetin, or eugenol, tested against wood decay fungi with the aim to provide a rational in natural wood preservation. Varied interactions were noted ranging from synergistic to antagonistic when tested against the two fungal test organisms *Lenzites betulina* and *Laetiporus sulphureus* [71]. Another example where poorer antimicrobial activity is noted for the isolated compound rather than the combined compounds was reported in a study of linoleic and oleic acid, isolated from *Helichrysum pedunculum*, which were found to have higher activity against *S. aureus* and *Micrococcus kristinae* in combination (MIC value 0.05) than independently (MIC values 1.00) [72]. More recently [73], it was demonstrated that the biological effects observed for the major compounds of *Ocimum gratissimum* were not responsible for the overall effect of the essential oil. These types of studies reinforce the concept of a multi-targeted approach in therapeutic strategies and prove the hypothesis formulated by Tyler [74], that searching for potent antimicrobial compounds is becoming more and more improbable and that research should be moving towards the investigation of a combination of substances to achieve efficacy.

#### Interactions between different plant parts or fractions

Although it is evident that the many constituents within plants interact, it should also be noted that other interactions may occur between groups of molecules or fractions of the plant. For many species there is a strong distinction in the chemistry between the subterranean and above ground plant organs. If, for example, roots and leaves are combined, then the number of “active” compounds may be increased, and possibly an increased chance of synergistic interactions may occur. This may be why there are numerous anecdotal reports of plants used in combination therapy, i.e., when plant parts such as roots and leaves are combined and used in therapeutic regimens [75–79]. Even though the ethnobotanical use of many plants incorporates mixes of the different plant parts, there has been very little scientific evidence to support such interactive efficacies. There have been numerous screening studies that have investigated the antimicrobial activity of different plant parts such as fruit, leaf, root, barks, seeds, etc.; however, these have been investigated separately and not in combination [80–85]. Our studies on the various plant parts of *Croton gratissimus* was undertaken on the ethnopharmacological basis that these various parts are often used in combination. The root, leaf, and bark extracts were investigated singularly and combined in various ratios to establish possible interaction. The MIC value (0.4 mg/mL),  $\Sigma$ FIC (0.4), and isobologram results (all ratios depicting synergy) for *C. neoformans* validate the traditional use of a root: leaf combination [41]. Up until recently, no other studies, other than our own work, could be found where different parts of the same plant are combined and investigated for antimicrobial efficacy [41,86,87]. It is encouraging to see such studies now being published, in which the individual and combined phenolics within the *Olea europaea* plant extract have been studied [88]. The results indicated that the combined phenolics had sig-

nificantly higher antimicrobial activity than the individual phenolics investigated within the plant. Only one earlier study could be found that partially addresses the interactions that may occur between plant fractions. Garlic oil and allyl alcohol, both derived from *Allium sativum*, were combined and their interaction evaluated against *Candida utilis*. Isobolograms were used to interpret synergistic  $\Sigma$ FIC values of between 0.37–0.42 [89].

Aromatic plants have an additional component of chemical complexity—the volatile constituents. In healing rituals, the volatiles may be administered selectively (inhalation) or the volatiles and nonvolatiles may be applied collectively, e.g., a poultice placed directly onto a wound or the alcoholic extraction (tincture) of crude plant material. It has been well recorded that extracts of aromatic plants have superior activity over the essential oils [41, 90, 91]. In our own studies, we explored the possible interaction between the volatile and nonvolatile fractions to yield greater antimicrobial activity. To test this hypothesis, a number of plants were investigated, i.e., three *Pelargonium* species (*P. graveolens*, *P. quercifolium*, *P. tomentosum*) [92], *Plectranthus grandidentatus* [40], three *Salvia* species (*S. africana-caerulea*, *S. africana-lutea*, and *S. lanceolata*) [86], and *Tarchonanthus camphoratus* [87]. These studies examined the need for coexistence of volatile and nonvolatile constituents to enhance antimicrobial efficacy. It is widely accepted that the administration of an infused oil may act as a penetrative enhancer [1], and possibly the synergistic interactions noted may be a result of improved solubility and bioactivity of the active principles.

#### Interactions between different plant species

Currently available on the market are phytomedicines which are sold as whole extract combinations, for example, *Ginkgo biloba* with *Echinacea*. It is believed that synergistic interactions are responsible for their therapeutic efficacy [15]. Many traditional healing practises prescribe plant combinations from different species to treat diseases. A comprehensive study has been undertaken on the ethnobotanical use of plant mixtures, in which 170 plant species from Cuba were examined for their combined medicinal use. Sixty-one combinations were attributed to anti-infective applications [93]. In African traditional medicine it is well known that traditional healers often combine various plant species in order to enhance efficacy. A number of instances where plants have been combined for the treatment of microbe related infections have been found in the ethnobotanical literature. Some documented accounts include the combination of *Portulaca quadrifida* with *Monadenium lugardiae* to treat stomach complaints, *Trichilia emetica* with *Cyathula natalensis* for leprosy, and *Momordica foetida* with *Pittosporum viridiflorum* for boils. Various combinations of *C. gratissimus* have been used. Accounts of the administration with other species have been noted, e.g., for the treatment of swellings, the bark of *C. gratissimus* is combined with the root of Amaryllidaceae species and rubbed into incisions. Also noted is the use of the bark of *C. gratissimus* and *Ocotea bullata* in combination, which are powdered and blown into the womb to treat uterine disorders [76].

Given the fact that it is common traditional practice to combine medicinal plants, it was surprising to find so little published on plant to plant interactions. Previous studies include a study on the combination of *Thymus vulgaris* with *Pimpinella anisum*, two plants combined in Iraqi folk medicine. Both essential oils and methanol extracts were studied against nine test organisms, and predominantly additive interactions were noted [94]. Tea tree (*Melaleuca alternifolia*) and lavender (*Lavandula angustifolia*) es-

sential oils were combined and tested against the dermatophytes *Trichophyllum rubrum* and *Trichophyllum mentagrophytes* var. *interdigitale*. Various combinations were prepared, and results presented in isobolograms, demonstrating an antimycotic effect [95].

*Artemisia afra* is one of the oldest and most widely used plants in African traditional medicine [96, 97]. It is commonly used to treat respiratory infections such as coughs, colds, lung inflammation and often combined with plants such as *Lippia javanica*, *Agathosma betulina*, *Osmitopsis asteriscoides*, *Eucalyptus globulus*, *Zanthoxylum capense*, *Leonotis microphylla*, *Tetradenia riparia*, and *Allium sativum* [75, 76, 98]. In spite of these numerous reports in the ethnobotanical literature, very little research has been dedicated to validate these combinations. In our own combination studies on anti-infective African traditional medicines, *A. afra* was combined with *L. javanica*. The objective was to scientifically validate the concomitant use of these two coveted ethnomedicinals to treat respiratory infections. A time-kill assay was undertaken against the respiratory pathogen *K. pneumoniae*. Essential oil obtained from *L. javanica* (0.25%) and *A. afra* (0.25%) were run independently and in combination (*L. javanica* and *A. afra* together totalling 0.25%). *Artemisia afra* when studied independently showed initial microbial destruction within one hour, but regrowth after 24 h. For the *L. javanica* oil at 0.25%, death kinetics was observed within 40 min, but regrowth after four hours. When the two plants were combined, a bactericidal effect was maintained for the full 48 hours of testing. This synergistic effect scientifically validates the combined use of *L. javanica* and *A. afra* for the treatment of respiratory infections associated with *K. pneumoniae* and corroborates the traditional use of these two plants when administered in combination [40]. More recent results of the combination of the essential oils of *A. afra* with three medicinal aromatic plants, *Agathosma betulina*, *Eucalyptus globulus*, and *O. asteriscoides*, displayed predominantly additive interactions [99]. In an earlier combination study, we investigated the ethnobotanical use of *Salvia chamelaeagnea* in combination with *Leonotis leonurus* to treat respiratory infections. Individual extracts and a combination of the aerial parts of *S. chamelaeagnea* and *L. leonurus* were evaluated for the *in vitro* antibacterial activity ( $\Sigma$ FIC index presented as data points in isobolograms). When the two extracts were combined, synergistic actions were observed for the Gram-positive bacteria while antagonism, synergism, and/or additive actions were observed for the various ratios tested on studies with the Gram-negative bacteria [39].

Plant combinations with the potential to increase preservative efficacy in foods have recently been given more attention. The trend toward a more natural and greener approach to consumerism together with the economic benefit that may concur make this area of research attractive. Thus, a number of studies have been undertaken on food systems in the hope to achieve better antimicrobial effects in combination. A disc diffusion study was undertaken where *Cinnamomum cassia* was combined with *Allium tuberosum* and the fruit of *Cornus officinalis* in a triple combination at varying ratios i.e., 1:1:1, 8:1:1, 6:6:1, 1:6:6, 1:8:1, and 3:1:2. The combination 8:1:1 (*C. officinalis*: *C. cassia*: *A. tuberosum*) was found to possess antimicrobial efficacy against a wide range of test organisms. When applied to food systems, the combination retained antimicrobial activity [100]. Another interactive study with effective food preservation as an outcome was undertaken using isobolograms to interpret interactions. Fractions of *Eucalyptus dives* and *Coriandrum sativum* were combined and investigated against 12 test organisms. Of all the

test organisms, *Yersinia enterocolitica* was the most susceptible to synergism. Other interactions varied between additive to antagonistic [101]. More recently, *Origanum vulgare* and *Thymus vulgaris* were combined and found to have an additive effect against food spoilage bacteria [102]. In another study, the interactive combination of cranberry, blueberry juice, and grape seed extract was antimicrobially tested against *Helicobacter pylori*. This study was undertaken on the assumption that a diet rich in phytochemicals may act prophylactically to ward off infection. Of the five different combinations formulated, the permutation having cranberry juice extract (75%) with blueberry juice extract (10%) and grape seed extract (15%) demonstrated the highest synergy [103]. Multiple combination studies are challenging due to the endless number of permutations which exist to produce complex formulation. The development of predictive software using factorial designs to optimize experimental design may offer a solution to simplify the complexity.

A study on the combined effect of *Origanum vulgare* and *Vaccinium macrocarpon* was undertaken using the disc diffusion assay. Antimicrobial activity against *Vibrio parahaemolyticus* was best noted in a 1:1 combination [36]. Rosemary and clove essential oils have been combined and the antimicrobial efficacy reported using both MIC methods and time-kill studies. This comprehensive study was undertaken on a number of different pathogens and various MIC ratios depicted mostly additive effects against the test bacteria; *Staphylococcus epidermidis*, *S. aureus*, *Bacillus subtilis*, *E. coli*, *Proteus vulgaris*, and *P. aeruginosa*. The fungal test organisms, however, demonstrated either synergy (*C. albicans*) or antagonism (*A. niger*). The time-kill studies reported that combinations in lower concentrations were not sufficient to produce a cidal effect. Only concentrations twice that of the MIC value had a lethal effect [104]. Using synergistic principles, some plants were evaluated for the prevention of *Cassava* root rot during storage. Garlic, *Landolphia owerrience*, and *Garcinia kola* were investigated independently and in various 1:1 combinations. The combination of garlic with *G. kola* demonstrated the highest inhibition preventing rot during 14 days of storage [105]. Essential oil combinations of oregano (*Origanum vulgare*) with basil (*Ocimum basilicum*), lemon balm (*Melissa officinalis*), marjoram (*Origanum majorana*), rosemary (*Rosmarinus officinalis*), sage (*Salvia triloba*), and thyme (*T. vulgaris*) have been investigated against *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa*. All interactions either demonstrated an additive or indifferent effect in the MIC checkerboard method [106]. Using time-kill methods, a study was undertaken on *Melaleuca alternifolia* oil which was blended with a polar fraction of manuka (*Leptospermum scoparium*). Death kinetics demonstrated synergistic interactions [34]. Interactions between the isolated compound polygodial and plant species such as *Perilla frutescens* and *Licaria puchuri-major* have yielded varied results depending on the pathogen studied [107, 108]. Another study focusing on selected unrelated compounds and the combination thereof was undertaken whereby the antimicrobial action of *Staphylococcus aureus* produced a synergistic effect when berberine, a common alkaloid found in a variety of plant species, was combined with 5'-methoxyhydnoicarpin [109].

After reviewing the literature and examining the scientific interactive antimicrobial studies presented, it is clear that reports on antagonistic interactions seem to be largely ignored or possibly rejected by phytotherapy journals. It is thus encouraging to see that a study that focused on the antagonistic effects of two herbal extracts (*Rhizoma Coptidis* and *Fructus Evodiae*) in a traditional

Chinese medicine formula have recently been published. Findings suggest that the two samples have opposing effects [110].

### Antimicrobial plant interactions with nonbotanical antimicrobial agents

Of all the interactions studied, research into phytoconstituents in combination with nonbotanical chemical entities has been the most widely studied. Studies range from plant interactions with preservatives to interactions with conventional antimicrobials. ● **Tables 2 and 3** record these studies including a summary with the salient outcomes achieved. Interactive interpretation is given according to the respective authors. For many of the plants whose allopathic combination studies were reviewed, either an additive or synergistic effect is presented. Very few reports have documented antagonism despite the fact that it is not unusual to encounter adverse drug reactions with herbal medicines [15]. In our own studies we assessed the interaction between a selection of popular commercial oils (*Melaleuca alternifolia*, *Thymus vulgaris*, *Mentha piperita*, and *Rosmarinus officinalis*) and conventional antimicrobials (ciprofloxacin and amphotericin B). The initial objective was to determine if a synergistic pattern predominates as noted in other similar studies reported in literature. Whilst some synergistic and additive interactions were evident between essential oils and antimicrobials, antagonistic interactions were also highlighted. It was interesting to note that when *Melaleuca alternifolia* (tea tree) oil which is often recommended for treatment of skin ailments, was combined with ciprofloxacin and tested against *Staphylococcus aureus*, antagonism was noted for all ratios in the isobologram [111]. This study highlights that caution should be adhered to when combining natural products with allopathic antimicrobials and addresses the proposal by Cuzzolin et al. [112], that there is a need for more systematic interactive studies to be undertaken to identify unfavourable combinations. Many of the methods employed to depict synergy between plants and nonbotanical components other than conventional antibiotics (● **Tables 2 and 3**) are based on the experimental methods described herein. However, some studies have used alternate approaches to prove synergistic interactions. Such studies include the investigation of synergistic effects between catechin, an extract of green tea which was combined with ciprofloxacin using *in vivo* studies on a rat model. It was confirmed that the combination resulted in a statistically significant decrease in bacterial growth [113]. Kurita and Koike [114] examined the combination of ethanol, sodium chloride, or acetic acid with 19 essential oil components. Using an agar dilution method incorporating various combinations, the interactions were analysed over a 20-day period. Studies were undertaken with seven fungal species. Generally, a synergistic effect was noted when the variables were combined in pairs, threes, or altogether. In another study, synergistic interactions were also determined over a 21-day period against *Penicillium notatum*, however, with the incorporation of volatile compounds in an atmospheric jar. Synergistic interactions were noted for six combinations (ethanol: carvacrol; sulphur dioxide: carvacrol; sulphur dioxide: isothiocyanate; sulphur dioxide: cinnamaldehyde; isothiocyanate: cinnamaldehyde; cinnamaldehyde: carvacrol) [115]. A recent study demonstrated synergistic interactions with carvacrol. These combinations comprised of carvacrol with ciprofloxacin and carvacrol with amphotericin B against *Bacillus cereus* and *C. albicans*, respectively. Additionally, eugenol with ciprofloxacin or amphotericin B was synergistic when tested against *E. coli* and *C. albicans*, respectively [63].

**Table 2** The combination of plants with conventional antibiotics.

Plant derived test substance	Non-plant derived test substance	Test organism	Interaction	References
<i>Santolina chamaecyparissus</i>	clotrimazole	<i>Candida albicans</i>	synergistic when comparing MIC data	[139]
<i>Agastache rugosa</i> and major compound estragole	ketoconazole	<i>Blastoschizomyces capitatus</i>	isobologram depicting synergy	[140]
Bidwillon isolated from <i>Erythrina variegata</i>	mupirocin	<i>Staphylococcus aureus</i>	FIC values range between 0.5–1	[30]
Pomegranate extract	chloramphenicol	<i>Staphylococcus aureus</i>	FIC values range between 0.03–1	[31]
	gentamicin		FIC values range between 0.13–4	
	ampicillin		FIC values range between 0.03–1	
	tetracycline		FIC values range between 0.03–1	
	oxacillin		FIC values range between 0.03–1	
<i>Mentha piperita</i> essential oil and menthol	ampicillin	<i>Escherichia coli</i>	FIC values range between 1–2	[53]
	erythromycin		FIC values range between 1–2	
	gentamicin		FIC values range between 1–1.25	
	oxytetracycline		FIC values of 0.5	
<i>Kola nitida</i>	ciprofloxacin	<i>Escherichia coli</i>	potentiation for all antibiotics tested	[141]
	pefloxacin			
	levofloxacin			
<i>Cassia fistula</i> fruit solution	amoxicillin	<i>Salmonella enterica</i> (48 isolates)	FIC method indicated synergism for 80% strains tested; no antagonism noted	[142]
Essential oils from <i>Cedrus atlantica</i> , <i>Styrax tonkinensis</i> , <i>Juniperus communis</i> , <i>Lavandula angustifolia</i> , <i>Melaleuca alternifolia</i> , <i>Pelargonium graveolens</i> , <i>Pogestemon patchouli</i> , and <i>Rosmarinus officinalis</i>	ketoconazole	<i>Aspergillus niger</i>	FIC indices ranging from 0.52–1	[143]
	amphotericin B	<i>Aspergillus flavus</i>		
<i>Pelargonium graveolens</i> and main constituents citronellol, geraniol, triacetin	norfloxacin	<i>Bacillus subtilis</i>	FIC indice 0.5	[144]
		<i>Bacillus cereus</i>	FIC indices 0.5; synergy in isobole method for plant oil and norfloxacin	
		<i>Staphylococcus aureus</i> (2 strains)	FIC indices ranging from 0.37–0.5; synergy in isobole method for plant oil and norfloxacin	
		<i>Escherichia coli</i>	FIC indice 0.57	
<i>Allium</i> species (essential oils)	ketoconazole	<i>Trichophyllum</i> spp.	FIC indices ranging from 0.09–0.75	[145]
$\alpha$ -Mangostin isolated from <i>Garcinia mangostana</i>	ampicillin	<i>Enterococcus faecalis</i> (8 strains)	FIC values range between 0.5–1	[146]
	gentamicin	<i>Staphylococcus aureus</i> (9 strains)		
<i>Catha edulis</i>	tetracycline	<i>Streptococcus oralis</i>	4-fold potentiation	[147]
	tetracycline	<i>Streptococcus sanguis</i>	2-fold potentiation	
	penicillin G	<i>Fusobacterium nucleatum</i>	4-fold potentiation	
Eight Chinese medicinal plants	penicillin G	resistant and standard strains of <i>Staphylococcus aureus</i>	% inhibition of combination varies between < 1 (synergistic) to 75.4	[148]
	gentamicin		% inhibition of combination varies between < 1 (synergistic) to 104.5	
	ciprofloxacin		% inhibition of combination varies between < 1 (synergistic) to 107.3	
	ceftriaxone		% inhibition of combination varies between < 1 (synergistic) to 71.5	
Sophoraflavanone G isolated from <i>Sophora flavescens</i>	gentamicin	11 strains of oral bacteria	FIC values range between 0.28–0.75	[149]
15 traditional Indian plants	tetracycline	<i>Staphylococcus aureus</i> and <i>Escherichia coli</i>	synergism or "neutralism" when investigating inhibition zones	[150]
	ciprofloxacin			
<i>Melaleuca alternifolia</i> , <i>Origanum vulgare</i> , and <i>Pelargonium graveolens</i>	amphotericin B	5 different <i>Candida</i> strains	isobolograms demonstrating <i>P. graveolens</i> oil with amphotericin B as the most synergistic combination	[151]
Galangin isolated from <i>Alpinia officinarum</i>	gentamycin	<i>Staphylococcus aureus</i>	FIC values range between 0.18–0.255	[32]
<i>Thymus eigii</i>	vancomycin and erythromycin	13 test organisms	antagonism determined by zone inhibition	[152]
<i>Rhus coriaria</i> , <i>Psidium guajava</i> , <i>Lawsonia inermis</i> , <i>Sacropoterium spinosum</i>	oxytetracyclin	<i>Staphylococcus aureus</i>	synergy determined by zone inhibition	[153]
	gentamicin		synergy/antagonism determined by zone inhibition	
	enrofloxacin		antagonism determined by zone inhibition	
	sulphadimethoxin		synergy determined by zone inhibition	
<i>Melaleuca alternifolia</i> , <i>Thymus vulgaris</i> , <i>Mentha piperita</i> , and <i>Rosmarinus officinalis</i> essential oils	ciprofloxacin and amphotericin	<i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , and <i>Candida albicans</i>	antagonism mainly noted with amphotericin B: essential oil combination	[111]

continued next page

**Table 2** The combination of plants with conventional antibiotics. (continued)

Plant derived test substance	Non-plant derived test substance	Test organism	Interaction	References
<i>Croton zehntneri</i>	gentamicin and tetracycline	<i>Staphylococcus aureus</i> and <i>Pseudomonas aeruginosa</i>	activity increased by 42.8% against <i>P. aeruginosa</i> in combination	[154]
<i>Thespesia populnea</i>	oxytetracycline	12 bacterial strains	highest synergy noted for <i>Shigella boydii</i> using the disc diffusion method	[155]
<i>Origanum vulgare</i> , <i>Pelargonium graveolens</i> , and <i>Melaleuca alternifolia</i>	nystatin	5 different <i>Candida</i> strains	<i>O. vulgare</i> essential oil and nystatin indicate most prominent synergy with FIC indices between 0.11 and 0.17	[156]
<i>Rhus coriaria</i> , <i>Sacropoterium spinosum</i> , <i>Rosa damascene</i>	oxytetracycline, penicillin G, cephalixin, sulfadimethoxine and enrofloxacin	<i>Pseudomonas aeruginosa</i> (3 clinical strains)	synergy	[157]
<i>Ocimum sanctum</i> essential oil	fluconazole and ketoconazole	16 fluconazole-resistant <i>Candida</i> isolates	FIC values ranging from mostly synergistic (0.25–0.50) to indifferent (0.52–0.93)	[158]
<i>Melaleuca alternifolia</i> oil	tobramycin	<i>Escherichia coli</i> and <i>Staphylococcus aureus</i>	synergy demonstrated with time-kill methods	[159]
Punicalagin isolated from <i>Punica granatum</i>	fluconazole	<i>Candida albicans</i>	synergy demonstrated with disc diffusion, MIC, and time-kill methods	[160]
Eugenol, thymol, carvacrol, cinnamaldehyde, allyl, and isothiocyanate	tetracycline, ampicillin, penicillin G, erythromycin, bacitracin, and novobiocin	<i>Salmonella typhimurium</i> , <i>Escherichia coli</i> , <i>Streptococcus pyogenes</i> and <i>Staphylococcus aureus</i>	FIC indices mostly indicating synergy with strongest synergy noted (FIC 0.11) with carvacrol: penicillin G combination against <i>S. aureus</i>	[161]
<i>Myrtus communis</i> essential oil	amphotericin B	<i>Candida albicans</i> and <i>Aspergillus niger</i>	FIC indices and isobologram indicate synergy	[162]
<i>Thymus maroccanus</i> and <i>Thymus broussonetii</i>	amphotericin B and fluconazol	<i>Candida albicans</i>	synergistic FIC indices ranging between 0.27–0.49	[163]

An area that has been sorely neglected is the incorporation of botanicals within formulations to achieve an enhanced naturally subsidised pharmaceutical product. Nostro et al. [116] examined the synergistic interactions of *Calamintha officinalis* with EDTA in cream formulations. More recently, *Artemisia afra*, *Eucalyptus globulus*, and *Melaleuca alternifolia* were encapsulated into diastearoyl phosphatidylcholine and diastearoyl phosphatidylethanolamine liposomes. The  $\Sigma$ FICs were calculated in order to determine if the incorporation of essential oils would enhance the antimicrobial activity of the formulation. Synergistic to additive interactions were noted for encapsulated *E. globulus* ( $\Sigma$ FIC values 0.25–0.45) and *M. alternifolia* ( $\Sigma$ FIC values 0.26–0.52) formulations [33].

## Future Considerations

▼  
**The reductionist approach in studying natural products**  
 For decades, phytochemists have been isolating natural products in the hope to find an antimicrobially active molecule comparable in activity to the allopathic antimicrobials available today. Yet, no single plant-derived antibacterial has been commercialised [117]. Perhaps this reductionist approach which limits complexity and variability is somewhat short-sighted when we consider the convolution of plants and the many compounds (major and minor) that may contribute to the overall activity of the plant. Allopathic medicine has realised the importance of studying the interaction between molecules for several years. Typical titles would include papers such as “*In vitro* synergy studies based on tazobactam/piperacillin against clinical isolates of metallo- $\beta$ -lactamase-producing *Pseudomonas aeruginosa*” [118] and “Synergic activity, for anaerobes, of trovafloxacin with clindamycin or metronidazole: checkerboard and time-kill meth-

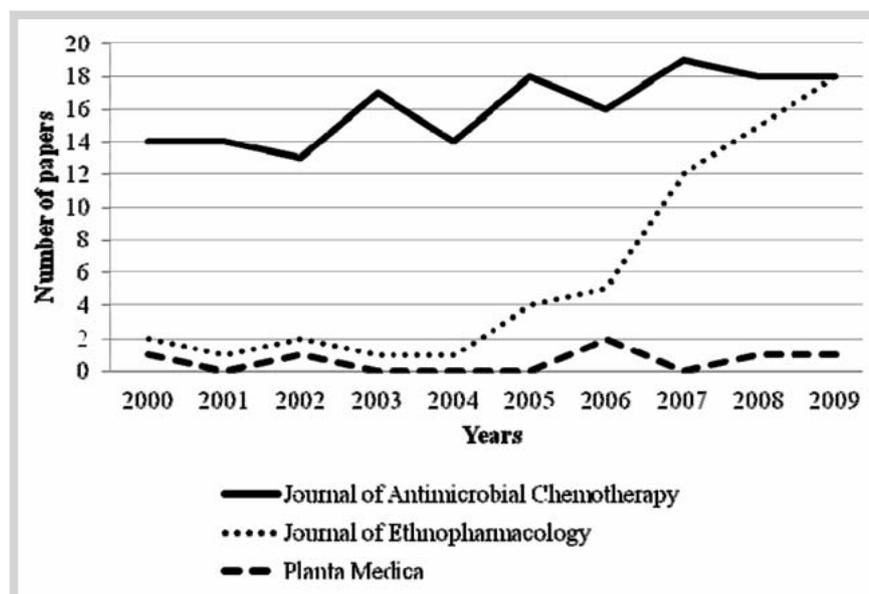
ods” [119]. ◉ **Fig. 3** shows the number of papers reporting on pharmacological interactions for three journals (two plant based and one antimicrobial) over a ten-year period. The Journal of Antimicrobial Chemotherapy has consistently published a number of papers on this subject over the ten-year period (a total of 161). Ironically, the Journal of Ethnopharmacology and Planta Medica only carried 61 and six papers, respectively, over the same period. This is ironic for several reasons. The very nature of ethnopharmacology is based on traditional healing practices where crude extracts are administered, not molecules, and hence one cannot ignore the possible interactions between the various constituents and different species. Furthermore, researchers working in the field of ethnopharmacology often justify their projects based on traditional practices, but often the methodology followed is divorced from the real-life application in a traditional setting. Although it remains a rewarding and challenging exercise to search for the active principles in complex crude mixtures, it is not surprising that so many papers following this reductionist approach conclude that “the crude extract was more active than the isolated molecules”.

## Natural products and antimicrobial mode of action

The mode of action of conventional antimicrobials, both independently and in combination therapy, have been extensively studied. The mechanisms by which agents act on the cell wall interfere with biological pathways, and other more complex interactions have also been explored [57]. With respect to natural product combinations, the mechanism of synergy may be attributed to complex multi-target effects, pharmacokinetic or physiochemical properties, neutralization principles, or even therapeutic approaches [121]. Gilbert and Alves [120] have hypothesised on the efficacy of whole plant extracts rather than isolated molecules. Furthermore, an extensive review on possible modes of ac-

**Table 3** The combination of plants with nonbotanical agents other than conventional antibiotics.

Plant derived test substance	Non-plant derived test substance	Test organism	Interaction	Reference
<i>Origanum vulgare</i> and <i>Vaccinium macrocarpon</i>	lactic acid	<i>Vibrio parahaemolyticus</i>	total time-kill inhibition throughout 10 h tested	[36]
<i>Ocimum basilicum</i> (anise variety)	5% NaCl	<i>Lactobacillus curvatus</i>	synergy using indirect impedance method	[60]
Thymol	potassium sorbate	<i>Escherichia coli</i>	FIC values range between 0.5–1.1	[52]
		<i>Listeria innocua</i>	FIC values range between 0.3–0.8	
		<i>Salmonella typhimurium</i>	FIC values range between 0.5–1.0	
		<i>Staphylococcus aureus</i>	FIC values range between 0.3–0.8	
Carvacrol		<i>Escherichia coli</i>	FIC values range between 0.5–0.8	
		<i>Listeria innocua</i>	FIC values range between 0.4–0.8	
		<i>Salmonella typhimurium</i>	FIC values range between 0.4–0.6	
		<i>Staphylococcus aureus</i>	FIC values range between 0.4–0.9	
<i>Rosmarinus officinalis</i>	butylated hydroxyanisole	<i>Escherichia coli</i> and <i>Staphylococcus aureus</i>	synergy for most points depicted on the isobolograms	[43]
Oregano & oregano:cranberry extracts	sodium lactate (1–2%)	<i>Listeria monocytogenes</i>	total time-kill inhibition between days 10–15	[38]
Carvacrol, thymol, and eugenol	nisin	<i>Listeria monocytogenes</i>	optical density readings indicate reduction in growth	[123]
	diglycerol fatty acid esters		varied interaction depending on compound studied	
<i>Melaleuca alternifolia</i> , <i>Leptospermum scoparium</i> , and <i>Leptospermum morrisonii</i>	chlorhexidine digluconate	<i>Streptococcus mutans</i> and <i>Lactobacillus plantarum</i>	CFU reduction 4- to 10-fold depending on the combination	[126]
Thymol, carvacrol, citral, eugenol, geraniol	acetic, citric, lactic, and pyropolyphosphoric acids	<i>Salmonella typhimurium</i>	no synergistic effects noted	[164]
Oils of fennel, anise, and basil	benzoic acid and methyl-paraben	<i>Listeria monocytogenes</i> and <i>Salmonella enteritidis</i>	synergy detected in five of the 16 combinations studied	[165]
<i>Thymus vulgaris</i> , <i>Rosmarinus officinalis</i> , <i>Origanum vulgare</i>	lactic acid	<i>Listeria monocytogenes</i>	synergy at lower concentrations with highest synergistic activity noted for thyme: lactic acid and rosemary: lactic acid combinations	[166]
Eucalyptus oil, tea tree oil, and thymol	chlorhexidine digluconate	<i>Staphylococcus epidermidis</i>	thymol: chlorhexidine digluconate combination demonstrated the most synergistic interactions with FIC values of 0.25	[127]
Nine different plant essential oils	enterocin AS-48	<i>Listeria monocytogenes</i>	reduction in CFU for combinations	[167]
<i>Origanum vulgare</i> subsp. <i>hirtum</i>	nisin	<i>Salmonella enteritidis</i>	combination of <i>O. vulgare</i> essential oil at 0.9% and nisin at 1 000 IU/g demonstrated the best synergistic activity over time	[168]

**Fig. 3** Number of papers reporting on pharmacological interactions for three journals (Journal of Antimicrobial Chemotherapy, Journal of Ethnopharmacology, and *Planta Medica*) over a ten-year period.

tion of natural products with allopathic antimicrobials has been undertaken [122]. Targets include receptor site modification, enzymatic degradation, reduced accumulation of drug within the bacterial cell, decreased membrane permeability, and efflux pumps. Even though these possible modes of action have been addressed, supporting studies to confirm these mechanisms are sorely lacking, especially with respect to combination therapy. When reviewing the literature on phytotherapeutic combinations, the elucidation of mode of action for both inhibitors are rarely reported in spite of the authors' efforts to include this as recommendations for further study [52, 101, 123, 124]. In a study on the synergistic interaction of *Punica granatum* (methanol extract) with a range of antibiotics, the authors allude to the mode of action whereby the extract plays a role in efflux inhibition enhancing the uptake of a conventional drug [31]. In another study on the combination of an isoflavanone from *Erythrina variegata* with mupirocin, the mechanism of action is thought to involve bacterial cell membranes; however, further studies are recommended for confirmation [30]. One notable study that focused on mode of action with respect to combined inhibitors was the investigation of berberine with 5'-methoxyhydnocarpin where the mode of action was attributed to the effect of 5'-methoxyhydnocarpin blocking the Nor A pump and thus potentiating the antibiotic action of berberine [109]. This valuable insight into specific modes of action should be encouraged in future endeavours, keeping in mind that exploring this area of research may be extremely complex. One needs to consider the variability of possible interactions that may occur within this phytochemical pool, not only within a single extract but in various combinations. Furthermore, predominant mechanisms of action may be potentiated by other less effective modes of action [7] and vice versa.

### Biofilm inhibition from combined phytomedicinals

Plant-based antimicrobial studies on planktonic microorganisms have been given extensive priority. The inhibition of biofilms, whether on independent or combined plant inhibitors, however, has been largely neglected. Combination studies are sparse, and the only interaction predominant in the literature is the combination of phytomedicinals with chlorhexidine digluconate, a skin antiseptic commonly used in clinical settings. Studies include the combined effect of *Eucalyptus* essential oil and the monoterpene 1,8-cineole with chlorhexidine digluconate, for which mainly synergistic interactions were found against *C. albicans*, *E. coli*, *P. aeruginosa*, *S. aureus* (including a methicillin resistant strain) biofilms [125]. Previous studies have shown that a number of essential oils together with chlorhexidine digluconate are effective in inhibiting biofilm cultures [126, 127].

In another biofilm combination study, two diterpenoids, salvipisone and aethiopinone, isolated from the roots of *Salvia sclarea* and combined with beta-lactam antibiotics, demonstrated synergy. It was postulated that the mechanism of action may be due to cell surface hydrophobicity or cell wall permeability [128]. Studies such as this provide not only valuable information on biofilms but also offer explanations on possible modes of action.

### Conclusions

There has been a recent increase in awareness towards the concept of synergy within phytomedicine, as noted in a number of review style articles [6, 15, 120, 121]. In conjunction with earlier publications on synergistic principles [9, 56, 122, 123, 129, 130],

the validation of multiple phytotherapy has provided a much needed platform with which to expand future research in this area. In particular, research into antimicrobial combinations may yield new developments that may address the ever increasing concern towards antimicrobial resistance. It has been shown that resistance to crude extracts occurs less than resistance to single actives [131]. Thus, the search for single targeted molecules may not yield long-term solutions in combating antimicrobial resistance. For plants to rely on a single compound in their biochemical warfare with pathogens would be equivalent to relying on the "single golden bullet" approach, and thus, as researchers investigating the activity of single compounds, we would be ignoring the evolutionary approach that plants may have developed various metabolic mechanisms for the production of structurally and functionally diverse compounds to overcome emerging resistance. To ensure future success in natural product research, we encourage interactive phytochemical studies with existing practices in the hope that developments may be used as a foundation and driving force in the much needed discovery of novel chemotherapeutic agents.

It is recommended that future development in the field of phyto-synergy should consider:

- ▶ The selection criteria for the two inhibitors. This should be clearly defined and justification should be given for the choice of test substances to be examined in combination.
- ▶ Classification of synergistic interactions should be more conservatively evaluated taking into account inherent doubling dilution variations noted in MIC methodology.
- ▶ Even though it is popular to report synergistic interactions, antagonism should be given the same priority.
- ▶ Combinations involving more than two plant entities should be examined where applicable.
- ▶ Combination studies involving biofilm inhibition should be considered.
- ▶ Articles addressing the mechanism of action of synergistic interactions should be given precedence. It is encouraging to note that some attention to this has been given [6, 120]. The criteria addressed include receptor or site modification, enzymatic degradation, accumulation of antibiotic within bacterial cell, decreased outer membrane permeability, and efflux pumps. This area of research has been greatly neglected as many studies just report on the interactions that occur.

With such validations in place, the justification and development of antimicrobial combinations could lead to patentable entities making research in the field of phytosynergy commercially relevant [132]. New techniques, such as metabolomics and the dual applications of chemometric data analysis methods, are providing the researcher with new tools to explore this fascinating phenomenon which will undoubtedly become increasingly important in our continued quest to understand the mechanism of action of complex herbal preparations.

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