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Proceeding Paper

Meta-Regression Modelling of the Inhibition Diameter Produced by Extracts of *Origanum*, *Syzygium* and *Citrus* as a Function of the Minimum Inhibitory Concentration †

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Abstract: Disk diffusion and minimum inhibitory concentration (MIC) are important methodologies for antimicrobial susceptibility testing. However, the two methods report the results of antimicrobial sensitivity in different ways (the first, in inhibition diameters, the second in concentrations). For this reason, it was questioned if a relationship could be found between inhibition diameter and MIC measurements. Such relationship was assessed by building a meta-analytical regression model utilising in-vitro data collected through systematic literature search on the susceptibility of pathogens to extracts of *Origanum*, *Syzygium* and *Citrus*.

Keywords: biopreservation; antimicrobial; mixed-effects model; meta-analysis

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1. Introduction

Antimicrobial agents have been extensively used in the food industry to inhibit foodborne bacteria and increase the shelf life of processed foods [1]. Many edible and medicinal plants, herbs, and spices used in traditional medicine have been studied and shown to possess antimicrobial functions, thus suggesting their suitability to act as antibacterial agents against food pathogens [1,2]. In this regard, extracts of *Origanum*, *Syzygium* and *Citrus* capable of preventing the growth of foodborne microorganisms have been tested in-vitro by numerous authors, using standardised protocols [3–5].

Distinct in-vitro antimicrobial susceptibility tests can be employed. The two major phenotypic methods of determining the susceptibility of a bacterial isolate to an antimicrobial agent are disk diffusion and minimal inhibitory concentration, MIC (which includes broth microdilution and agar dilution) [6].

Put simply, the disk diffusion method involves placing paper disks saturated with inhibitors of bacterial growth (plant extracts or essential oils, for example) on a lawn of bacteria seeded on the surface of an agar medium, incubating the plate overnight, and measuring the presence or absence of a zone of inhibition around the disks [6]. On the

other hand, MIC testing involves: (i) preparing a series of agar plates containing the antimicrobial agent in increasing concentrations and placing the bacterial suspension on each of the series of plates, when agar dilution method is used; or (ii) placing the bacterial suspension into a 96-well tray containing a liquid medium of predetermined, documented formulation with doubling dilutions of the antimicrobial agent to be tested, when broth microdilution is used [6].

Disk diffusion method reports antimicrobial activity in terms of inhibition diameter of the bacterium being tested (in millimetres), whereas MIC methods determine the lowest concentration of the assayed antimicrobial that inhibits the visible growth of that bacterium (in mg/mL, for example). This raises the question: can a correlation be found between inhibition diameter and MIC measurements, originated from different in-vitro methodologies?

To assess if such a relationship exists, through systematic literature search and meta-regression modelling, this study aimed to assess the susceptibility of pathogens to extracts of *Origanum, Syzygium* and *Citrus*, as well as the relationship between results originated from disk diffusion and minimum inhibitory concentration methodologies.

2. Materials and Methods

2.1. Data Collection and Description of the Data Set

An electronic, systematic literature search was carried out in Scopus, PubMed, Web of Science and SciELO databases to find original articles, published since 2000, reporting on the inhibition diameter, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Origanum*, *Syzygium* and *Citrus* extracts. The search aimed to find quality studies validated by the scientific community.

The bibliographic searches were conducted by properly applying the AND and OR logical connectors to combine terms regarding pathogens, biopreservatives, and terms referring to antimicrobial susceptibility methodologies, as follows: (*Listeria* OR *Salmonella* OR "*Staphylococcus aureus*" OR "*Escherichia coli*") AND (extract* OR antimicrobial OR "essential oil") AND (MIC OR MBC OR "agar diffusion" OR halo OR inhibition OR zone OR "minimum inhibitory concentration" OR "minimum bactericidal concentration") AND food.

Grey literature was not acquired to avoid data validity concerns and data duplication, since high-quality theses and reports are likely to be also published in peer-reviewed journals. Other meta-analysis studies and systematic reviews were also excluded. The criteria for inclusion were pre-set to providing information on: (i) extracts of the genus *Origanum*, *Syzygium* or *Citrus*; (ii) both MIC and inhibition diameter outcomes; (iii) antimicrobial effect against *L. monocytogenes*, *S. aureus*, shigatoxin-producing *E. coli*, *Campylobacter* spp. or *Salmonella* spp; and (iv) at least the dose at which the extract was tested and pathogen's inoculum size.

After assessing all the information from the 70 publications initially retrieved, eleven studies published from 2010 until 2021 qualified for inclusion [7–17]. From the selected studies, information on the study identification, country of the study, plant, part of the plant used, extraction method, temperature and solvent, bacterium, strain, antimicrobial susceptibility testing method (disk diffusion or MIC), dose applied ("LogDose"), inoculum level, inhibition diameter value (ID, mm) and MIC value ("LogMIC"; ml/mg for extracts, μL/mL for essential oils) were collected. MIC units were all converted to ml/mg for extracts and μL/mL for essential oils. At total of 162 entries made up the meta-analytical data for modelling.

2.2. Meta-Regression Modelling

A mixed-effects linear model with weights was adjusted to the data set to evaluate the antimicrobial effect of *Origanum* (n = 145), *Syzygium* (n = 10) and *Citrus* (n = 7) extracts on the inhibition diameter (ID) of various pathogens.

Moderators are study characteristics that can be selected and codified from the primary sources in an attempt to explain the between-study variability in effect size. Due to lack of or uneven data, not all variables extracted could be evaluated as moderators in the meta-regression. In this meta-analysis, the moderators defined encompassed: minimum inhibitory concentration, extract dose and bacterium.

The meta-regression model adjusted to the meta-analytical data was of the form:

$$ID_{ik} = (\beta_0 + u_i) + \beta_1 LogDose + \beta_2 LogMIC + \beta_{3k} Bacterium_k + \varepsilon_{ik} \quad (1)$$

In the above equations, β_0 is an intercept, β_1 and β_2 are the effect of a one log increase in extract dose and of a one log increase in minimum inhibitory concentration, respectively, on the inhibition diameter, and β_{3k} is the set of fixed effects of the k types of bacteria (a class variable consisting of the levels: *L. monocytogenes, S. aureus, Salmonella,* STEC, *C. jejuni*). Extract dose and minimum inhibitory concentration were natural-logarithm transformed to normalise data distribution and reduce heteroscedasticity.

The error term ε_{ik} accounts for the variability between studies i and pathogens k. The remaining unexplained variability was extracted by placing between-study heterogeneity in the intercept (u_i), which was assumed to have a normal distribution with mean zero and variance τ^2.

In order to obtain precise estimates of the antimicrobial effect on pathogen inactivation and reflect quality of research design, different weights were assigned to each primary study according to the study size.

Model parameters, as affected by moderators, were calculated from the fitted meta-regressions, and the significance of moderators was evaluated by analysis of variance (α = 0.05). No effect of type of plant was found and hence such a moderator was removed. The meta-regression model described was fitted using the *lme* (linear mixed-effects models) function from the *nlme* package implemented in R software (version 3.6.2, R Foundation for Statistical Computing, Vienna, Austria) [18].

3. Results and Discussions

The fitted parameters of the meta-regression modelling the inhibition diameter produced by extracts of *Origanum*, *Syzygium* and *Citrus* as a function of the minimum inhibitory concentration, extract dose and bacterium are presented in Table 1.

A clear tendency for microbial inhibition is observed when *Origanum*, *Syzygium* and *Citrus* extracts are tested in-vitro against pathogens, as revealed by the negative intercept in Table 1, thus supporting the potential of such herbs against microbial spoilage in foods. Further insight on the variables affecting inhibition diameter measurements is provided by analysis of the remaining parameters.

The significant positive estimate of "Log Dose" (18.00 [SE = 0.227], $p < 0.0001$) indicates a tendency for greater inhibition diameter as the extract dose applied increases, a result otherwise expected. The negative estimate of "Log MIC" (−5.554 [SE = 0.181], $p < 0.0001$) implies that this moderator and inhibition diameter are inversely correlated: the higher the minimum inhibitory concentration, the less effective is the plant extract to inhibit bacterial growth, so a smaller inhibition diameter would result when testing such plant extract at that concentration using the disk or well diffusion methods. Although this relationship may seem theoretically evident, it should be noted that it concretised despite the various factors affecting such measurements. The inverse correlation between inhibition diameter and "Log MIC" is also depicted by the negative slope in the scatter plot of Figure 1.

Table 1. Meta-regression model on inhibition diameter produced by extracts of *Origanum* (n = 145), *Syzygium* (n = 10) and *Citrus* (n = 7) plants, as a function of the minimum inhibitory concentration (ml/mg for extracts and μm/mL for essential oils), extract dose (%) and bacterium. Number of observations (n) per factor level, heterogeneity analysis and *p*-value of the publication bias test are shown.

Parameter	Estimate [1]	Standard Error	*p*-Value	n	Heterogeneity Analysis [2]
Intercept	−1.515	6.499	0.816		
Log Dose	18.00	0.227	<0.0001		$\tau^2 = 33.96$
Log MIC	−5.554	0.181	<0.0001		$s^2 = 29.34$
Bacterium					$I^2 = 53.6\%$
L. monocytogenes	1.319 [b]	0.150	<0.0001	43	$\tau^2_{res} = 17.75$
S. aureus	2.668 [c]	0.146	<0.0001	37	$R^2 = 47.7\%$
Salmonella	2.429 [c]	0.141	<0.0001	41	
STEC	−0.411 [a]	0.234	<0.0001	9	Publication bias
C. jejuni	-	-	-	32	$p = 0.254$

[1] Different superscript letters indicate significant differences in the estimates between bacteria; [2] Heterogeneity analysis encompasses within-study variability (s^2), between-study variability of the null model (τ^2), intra-class correlation (I^2), residual between-study variability (τ^2_{res}), and between-study variability explained by significant moderators (R^2).

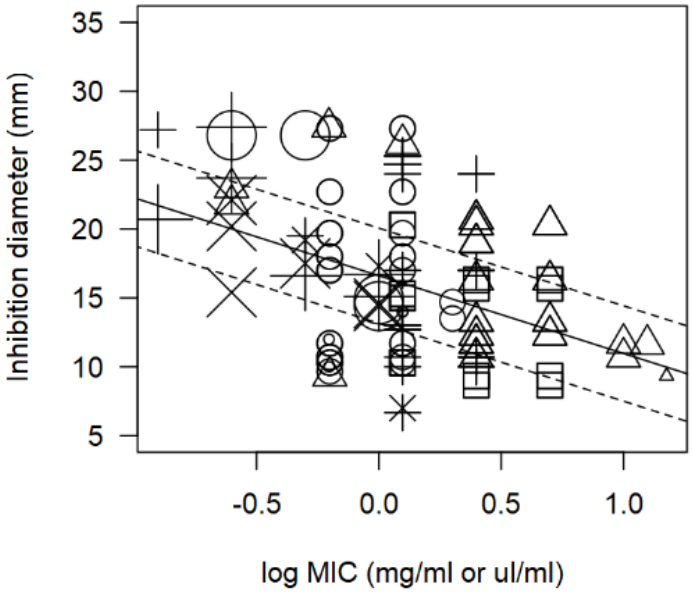

Figure 1. Scatter plot depicting the effect ($p < 0.001$) of the logarithm of the minimum inhibitory concentration of extracts of *Origanum* (n = 145), *Syzygium* (n = 10) and *Citrus* (n = 7) plants on inhibition diameters for each bacterium. Markers symbolise bacterium: □ = *C. jejuni*, ○ = *L. monocytogenes*, Δ = *S. aureus*, + = *Salmonella*, × = STEC; and marker size is proportional to study size.

From the results of Table 1, it is also possible to observe that distinct pathogens present different inhibition diameters when the same plant extract is applied at the same dose ($p < 0.0001$), as demonstrated by the various mean values of the "Bacterium" moderating variable. For better understanding of the meta-regression results, it is important to note that the estimate for *C. jejuni* is considered the "base value" for inhibition diameter, with mean zero, and the estimates for other microorganisms reflect deviations from that base value, with negative and positive estimates below and above the base value, respectively. Lower estimates suggest smaller inhibition diameters, while higher estimates indicate larger inhibition diameters. In this sense, STEC appears as the most resistant pathogen

(−0.411 [SE = 0.234]), followed by *C. jejuni*, *L. monocytogenes* (1.319 [SE = 0.150]), and *Salmonella* (2.429 [SE = 0.141]) and *S. aureus* (2.668 [SE = 0.146]), the latter two not being significantly different. Although no differences were found between the estimated inhibition diameters for *S. aureus* and *Salmonella*, these pathogens differed from the remaining bacteria ($p < 0.05$).

Gram-negative bacteria (STEC and *C. jejuni*) are generally more resistant to natural extracts than Gram-positive bacteria, with some exceptions [19], and this was the case in our study. The exception was for *Salmonella*, which revealed lower or similar resistance to the plant extracts than the Gram-positive bacteria tested (*L. monocytogenes* and *S. aureus*, respectively).

The goodness-of-fit of the model was assessed by plotting the predicted inhibition diameter against the observed, as shown in Figure 2.

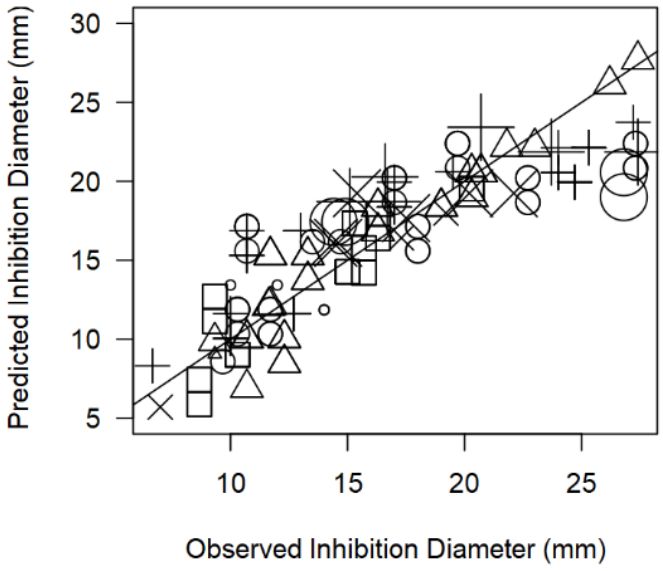

Figure 2. Scatter plot of the observed inhibition diameters produced by extracts of *Origanum* (n = 145), *Syzygium* (n = 10) and *Citrus* (n = 7) plants versus values predicted by the meta-regression model (R = 0.860), with 45° reference line. Markers symbolise bacterium: □ = *C. jejuni*, ○ = *L. monocytogenes*, Δ = *S. aureus*, + = *Salmonella*, × = STEC; and marker size is proportional to study size.

Whereas the goodness-of-fit (R = 0.860) indicates a satisfactory correlation between predicted and observed values, which supports the robustness and usefulness of the model produced, the model hinted that there is still some residual variability to be explained that the model cannot account for, as heterogeneity analysis showed that moderators explain 47.7% of the between-studies variability (R^2, Table 1).

This value suggests that other factors may affect the ID measurements. Possible sources of variation could be the extracts specificities (origin, stage of development, part of the plant used, etc.), the inoculum size and strain used, for example, or other non-quantifiable sources. Information was collected regarding some of these possible sources of variation (inoculum size, for example) and introduced in the dataset, however, due to lack of or uneven data, not all variables extracted could be evaluated as moderators. This R^2 value signals that disk diffusion methodologies may not be appropriate to compare results from different studies, as the inhibition diameter measurements could be affected by errors and variations in the protocols, impacting on the degree of extract diffusion within the agar matrix.

Lastly, regarding publication bias, with a *p*-value of 0.254, they were undetected in the model produced.

4. Conclusions

Literature data was used to build a meta-analytical regression model capable of evaluating if a correlation between the results obtained through disk diffusion and minimum inhibitory concentration methodologies exists.

The results of the modelling showed that inhibition diameter and minimum inhibitory concentration measurements are inversely correlated. It also summarised the reduction in *L. monocytogenes, S. aureus, Salmonella,* STEC and *C. jejuni* populations in-vitro attained by *Origanum*, *Syzygium* and *Citrus* extracts, and elucidated inhibitory effectiveness by extract dose. Furthermore, the model revealed that various factors may affect the measurements of inhibition diameter, so caution must be taken when comparing results from different studies using the disk diffusion methodology.

In this sense, the outcomes of this meta-regression model support the potential of *Origanum*, *Syzygium* and *Citrus* extracts to inhibit or slow the growth of bacteria and provide insight on the variables affecting inhibition diameter measurements.

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Conflicts of Interest: The authors declare no conflict of interest.

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