Prediction of the antibacterial activity of garlic extract on *E. coli, S. aureus* and *B. subtilis* by determining the diameter of the inhibition zones using artificial neural networks

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A B S T R A C T

The aim of this study was to devise a model that predicts the inhibition zone diameter using artificial neural networks. The concentration, temperature and the exposure time of our extract were taken as input variables. The neural architecture model 3-13-3 and a learning algorithm Quasi-Newton (BFGS) revealed a positive correlation between the experimental results and those artificially predicted, which were measured according to a mean squared error (RMSE) and an R^2 coefficient of *E.coli* (RMSE=1.28; R^2=0,96), *S.aureus* (RMSE=1.46; R^2=0,97) and *B.subtilis* (RMSE=1.88; R^2=0,96) respectively . Based on these results, an external and an internal model validation were attained. A neuronal mathematical equation was created to predict the inhibition diameters for experimental data not included in the basic learning. Consequently, a good correlation was observed between the values predicted by the equation and those obtained experimentally, as demonstrated by the R^2 and RMSE values. The results regarding the sensitivity analysis showed that the concentration was the most determinant parameter compared to Temperature and Time variables.

Ultimately, the model developed in this study will be used reliably to predict the variation of garlic extract's inhibition diameter.

Keywords: Inhibition diameter, bacterial strain, neural networks, Prediction, Validation.

1. Introduction

For centuries, garlic (*Allium sativum*) has been known for its medicinal properties. Its anti-microbial activity is well known against a wide range of bacterial strains including: Gram-positive bacteria, *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli* (two types of bacteria commonly associated with the human environment) (Bakri & Douglas, 2005; Whitemore & Naidu, 2000; Zhang et al., 2016). The effectiveness of fresh garlic extract against a wide range of pathogenic bacteria has been well-established (Curtis, Noll, Störmann, & Slusarenko, 2004; Kumar, & Sharma, 1982), even amongst strains with an acquired resistance to antibiotics, such as *Staphylococcus aureus* (Curtis et al., 2004, Marchese et al., 2016). Because of its bactericidal capacity, garlic prevents bacteria from secreting toxins.

The main factors affecting stability and microbiological activity are temperature, pH, concentration and retention time. Since these factors can largely vary in food production and storage, mathematical models are needed to predict microbial behavior in the food matrix related to different microbial strains. In recent years, they have been growing interests in modeling food microorganisms (Keeratipibul, Phewpan, & Lursinsap, 2011), including modeling predictive microbiology, which generally focuses on the potential for alteration of food-borne pathogenic bacteria (Leroy, Degeest, & Vuyst, 2002).

In the present context, resides the importance of the Artificial Neurons Networks (ANN) in the bioactivity modelling making possible the prediction of microbial behaviour in specific operating conditions. (Marzouk & Elkadi, 2016). It is important to note that many studies have shown the benefits of the application of technical ANN compared to other statistical methods (Yamamura et al., 2008; Ross & McMeekin, 2003; Sagdic, Ozturk, & Kisi, 2012). In food microbiology, Artificial Neural Networks could be used to describe the effects of the interaction of environmental factors more precisely, such as temperature, pH, and storage time, as compared to conventional models predictive Microbiology (Lou & Nakai, 2000; Fang, Huang, Liu, Mei, & Chen, 2015). Predicting the bioactivity and the evaluation of the antibacterial activity of

compounds having a microbiological capacity was achieved by determining experimentally the diameters of the inhibition zones and the minimum inhibitory concentration (MIC). Ascertaining these parameters for different temperatures, times and concentrations using artificial neural networks (ANN), is an idea that has not yet been explored in this field.

The objective of this study is to examine the antibacterial activity of several samples of garlic extract, exposed for six hours at different temperatures on the growth of three bacterial strains, *staphylococcus aureus* (*S.aureus*), *Escherichia coli* (*E.coli*), and *Bacillus subtilis* (*B.subtilis*). The other objective is to establish a mathematical model that allows us to determine the diameter of inhibition zone of garlic extract on the three bacterial species. Furthermore, this model enables us to precisely determine the inhibitory zone for different values of concentrations, temperatures, and incubation times, which are not included in the database used in this model. The second objective is to validate this model (Artificial Neural Networks) in comparison with other observed results obtained experimentally.

An ANN model was developed to predict the antibacterial properties (inhibition zones) and to set up an approach to analyze the predicted values of inhibitions diameters (d_{inh}) and the values obtained through different experiments. For this we opted for a model with three simultaneous outputs, d_{inh} *E.coli*, d_{inh} *S.aureus* and d_{inh} *B.subtilis*

The methodology adopted to meet the objectives of assessing the model to average statistical performance criteria were coefficient of determination, square root of the mean square error, and bias on the average. To test the robustness of the ANN model, we used data that is not part of the original database.

This model can be used to avoid the multiplicity of microbiological tests, which causes instability of biological material due to mutation of some bacterial strains. It can also solve the problems of anti-bacterial activity concentrations, which are difficult to use in practice, including very low and very high concentrations.

2. Materials and Methods

2.1. Preparation of garlic extract

Fresh garlic bulbs (*A. sativum L.)* were collected in the region of Benchicaou (Medea, Algeria). The cloves were peeled, sliced and ground into a paste and suspended in distilled water, for 24 hours at a temperature of 4 ± 0.5°C. The maceration obtained was filtered through a gauze and then through a 0.45 microns micro filter. The obtained aqueous extract of garlic was stored at 4 ± 0.5°C until it was used.

2.2. Reference strain preparation

According to the ATCC's recommendations: a seed lot system was followed whereby the reference strain is sub-cultured into several replicates at a time within a passage. Only 5 passages are permitted from the reference strain, therefore the risk of phenotypic alteration or genetic mutation is kept to a minimum.

Buffered sodium chloride-peptone solution Ph7.0 was used to prepare control suspensions. To obtain a spore suspension of *Aspergillus Brasiliensis* (ATCC 16404), 0.005 % of polysorbate 80, was added to the buffer solutions. This suspension may be maintained at 2-8°C for a limited period of time.

To check the operating conditions, a negative control obtained by substituting the diluent chosen in the preparation to be examined, consequently no microbial growth was observed. According to the (European Pharmacopoeia 2007), Non-compliant results required an investigation.

2.3. Microbiological method

We used the solid diffusion of depositing on the agar discs (6mm in diameter) impregnated with antimicrobial substances to be studied. In our study, we used the Tropic soy agar; strains have summers selected from the reference strains listed in the ATCC institutes (American Type Culture Collection), *E.coli* (ATCC 11105), *S.aureus* (ATCC 6633) and *B.subtilis* (ATCC 6538)

Clearly defined inhibition diameters that were obtained after suitable incubation time (18 hours).

We later used a series of six tubes in which we distributed our garlic extract. Each tube was brought to a selected temperature (4 °C, 25 °C, 37 °C, 42 °C and 75 °C) for a total period of 6 hours. We then prepared garlic extract solutions of decreasing concentrations from samples taken at intervals of one hour. Each solution was obtained by mixing 0.5 ml of the previous solution and 0.5ml of myristate, an organic solvent, used in microbiology, without antimicrobial effect (European Pharmacopoeia 2007), until the 15^{th} tube. The concentration decreased from the 1^{st} to the 15^{th} Tube to the order of 50% of a solution to another. Petri dishes were then prepared by depositing one blank disc on the surface of each one; they were inhibited by 25 µl (Microliters) of each of these concentrations of garlic extract to be tested. The same method was performed at different time intervals and temperatures of 18 °C, 50 °C and 90 °C for the same concentration of garlic extract.

2.4. Elaboration of the ANN model

Artificial neural networks (ANN) are nonlinear empirical models (Panagou, & Kodogiannis, 2009), In general, they are composed of many units (neurons) operating in parallel. The operation of this network is largely determined by the connections between these elements (Zilouchian & Jafar, 2001). These neurons are spread over three layers: an input layer, an output layer, and a hidden layer. The number of neurons of the input layer is related to the number of input variables and the number of neurons of the output layer is identical to the number of output variables. Between these two layers, there is at least one hidden layer with the number of neurons depends on the application of the network (Yolmeh, Najafi, Farhoosh, & Salehi, 2014). The Neuronal regression optimized by passing through the network architecture, which concerns the distribution of the database into three sets: (Learning, Test and Validation), the transfer functions, the number of neurons in the hidden layer and the training algorithm.

In this work, 450 experimental datasets were selected to form the final database (Supplementary File S1). A multilayer perceptron network (MLP) was chosen for modeling. This is an artificial neural network of anticipation, owing to its excellent nonlinear generalization capabilities (Rafei,

Sorkhabi, & Mosavi, 2014; Wang, Zhang, Wang, Han, & Kong, 2014; Hamadache et al., 2016a). The input vector was composed of the following variables: concentration, temperature and time. The output vector represents three outputs, which are as follows: inhibitions diameters of our garlic extract on *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*.

Model performance was evaluated with the coefficient of determination R^2 between predicted and experimental values (Yang, Yang, Liu, & Hoogenboom, 2014), and by the square root of mean squared error RMSE (Chai & Draxler, 2014; Teke, Yıldırım, & Çelik, 2015) calculated by the following equation (1)

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n} (Y_i^{\exp} - Y_{i=1}^{pred})^2}{n}} \quad (1)$$

In this equation, n is the number of compounds in the dataset, and y_i^{exp} , y_i^{pred} are the experimental and the predicted values, respectively.

2.5. Model validation

In order to validate the model, two developed basic principles were commonly used, Internal validation and external validation (Hamadache et al., 2016b), they were judged by the following statistical parameters: $RMSE_{ext}$, $RMSE_{int}$, Q^2_{ext} and Q^2_{int} (Zhou et al., 2015), and calculated using the following equations:

$$RMSE_{ext} = \sqrt{\frac{\sum_{i=1}^{n} (Y_{test} - \hat{Y}_{test})^2}{n}} \quad (2)$$

$$RMSE_{int} = \sqrt{\frac{\sum_{i=1}^{n} (Y_{train} - \hat{Y}_{train})^2}{n}} \quad (3)$$

$$Q^2_{ext} = 1 - \frac{\sum (Y_{test} - \hat{Y}_{test})^2}{\sum (Y_{test} - \bar{Y}_{train})^2} \quad (4)$$

$$Q^2_{int} = 1 - \frac{\sum (Y_{train} - \hat{Y}_{train})^2}{\sum (Y_{train} - \bar{Y}_{train})^2} \quad (5)$$

In these equations, n is the number of compounds in the dataset, y_{test} the experimental value of the test set, $\hat{y}_{test}$ the predicted value of the test set, $\hat{y}_{train}$ the predicted value of the training set, $\bar{y}_{train}$ the mean experimental value of the training set and y_{train} the experimental value of the training set. Great importance is attributed for these terms of validation. A value of $Q^2_{int} > 0,5$ is generally considered satisfactory, and a value greater than 0.9 is seen as excellent (Eriksson et al., 2003). Furthermore, in order to judge the quality of the model, it was a necessity to conduct further experiments to have a generalization of the real predictive ability of the model. For this, we have operated on other experimental values in temperature (T=18°C, T=50°C and T=90°C) and time (t=0.5h, t=1.5h, t=2.5h, t=3.5h, t=4.5h, t=5.5h and t=6.5h), which were randomly selected, in the same operating conditions and which were not part of the database used for model development. This type of validation was evaluated by the coefficient of determination Q^2 (Robustness term) and the root mean square error RMSE.

A local and global sensitivity analysis approach was utilized to assess the impact of input parameters on antibacterial activity of garlic extract. The sensitivity values were obtained from Statistica 10.0.228.8.

3. Results and Discussion

3.1. Microbiological results

The results obtained (Supplementary File S1) show that the media temperature influences the inhibition diameter and consequently the antibacterial activity of garlic. The inhibition zones became increasingly important as the temperature rises from 25°C to 37°C. This demonstrates the great antibacterial effect of the garlic extract with increasing temperatures. However, increasing the temperature above 42 °C causes a reduction of the antibacterial activity of our extract, marked by a reduction of inhibition zones. Our results are in agreement with other authors, e.g. Jansen, Muller, and Knobloch (1989), Rybak, Calvey, and Harnly (2004), Lagunas, and Castaigne (2008) and Guo et al. (2012).

3.2. Model development and validation

The objective of this phase was to find the optimal architecture of the neural network so that the influence of temperature, time of conservation and concentration of garlic extract on antibacterial activity could be predicted using diameters of inhibition zones.

A multilayer perceptron (MLP) of a three-layer network, an input layer, a hidden layer and an output layer is adopted in this work. The number of neurons in the hidden layer, learning algorithms and the transfer function have been optimized after many tests. The optimal network was chosen following the values of the mean square error (RMSE) and the coefficient of determination (R^2). The results obtained in this optimization (Tables 1, 2) show that BFGS (Quasi-Newton) is an acceptable learning algorithm that can be applied with a distribution of the database as follows: 70% training, 15% for the test and 15% for the validation set. In addition, the transfer functions, which have given good results, are the hyperbolic tangent function for the whole "input layer - hidden layer" and the logistic sigmoid function for the whole "hidden layer - layer output". The determination coefficient R^2 reaches 0.98 and a mean square error was less than 1.90 regarding the three strains studied. Furthermore, the number of neurons in the hidden layer was equal to thirteen. Finally, the architecture {3-13-3} of the neural network has been selected (Figure 1). This network will be used to predict new data.

Based on these results, we can say that we have established a neuronal model with three outputs and predicted the zones inhibition diameters of the garlic extract with regard to the three strains *E. coli, S. aureus. B. Subtilis*. The results of selected optimal parameters were used to develop a non-linear model of ANN networks (table 1).

In Figure 2 (a, b, c) the values shown of experimental inhibition diameter (d_{inh}) as a function of those produced by the selected network for all three strains. We note a significant correlation with the coefficient of determination R^2 greater than 96% and an RMSE less than 1.90.

To establish the performance of the developed model, the parameters of the internal and external validation were calculated and reported in Table 3. As can be seen in this Table, the nonlinear ANN model has given good results with impressive values of the determination coefficient (R^2) and a better robustness (Q^2) for assemblies training and test sets. The values of the diameters of

inhibition produced for all three strains are close to the corresponding experimental values. These results indicate that this model not only gave good results during its development, but also provides an excellent predictive power. The results also suggest that there is a nonlinear correlation between the antibacterial activity (represented by the diameter of inhibition) and the studied parameters, mainly the temperature, storage time and concentration of the aqueous garlic extract samples. Therefore, the resulting ANN model can be effectively used to predict the antibacterial activity of aqueous extract of garlic on the strains used and at different temperatures, storage time, and the concentration of samples.

This will allow us to generate a large database where we can model the antibacterial activity by neural networks.

3.3. Application of mathematical model equation

The architecture of the network developed in this study is a multi-layer perceptron {3-13-3}. The network is composed of three inputs (E_i, i = 1 à 3), an output (d_{inh}) for each strain and thirteen neurons in the hidden layer. The two transfer functions used in this study are the hyperbolic tangent and logistic sigmoid function. Their mathematical equations (6 and 7) are given below:

Hyperbolic tangent: $$f(x) = \frac{e^{x} - e^{-x}}{e^{x} + e^{-x}} \quad (6)$$

Logistic sigmoïd: $$f(x) = \frac{1}{1 + e^{-x}} \quad (7)$$

The mathematical equation of the model (8), which can be used subsequently for the prediction of inhibition diameter d_{inh} is shown below:

$$d_{inh} = \frac{1}{1 + \exp\left(-\sum_{j=1}^{13} W_{kj}\left(\frac{\exp\left(\sum_{i=1}^{3} W_{ji}E_i + b_j^H\right) - \exp\left(-\sum_{i=1}^{3} W_{ji}E_i + b_j^H\right)}{\sum_{i=1}^{3} W_{ji}E_i + b_j^H\right) + \exp\left(-\sum_{i=1}^{3} W_{ji}E_i + b_j^H\right)}\right) + b_k^0\right)} \quad (8)$$

W_{KJ} represents the weight associated with each output connection k / j hidden neuron, E_i the inputs, b^H_J biais associated to hidden neuron j, W_{ji} the weight associated with each input connection i / j hidden neuron, and b^0_k designates the bias associated with the output neuron k

To validate the model and verify its predictive power, this equation was used for a set of 59 experimental values that were not used in the training and test sets. The results are shown in Figures (3a to 3c). A dotted line indicates the best linear fit. As seen in these figures, there is good correlation between the results of our mathematical equation and experimental data. Furthermore, as demonstrated by the statistical parameters (R^2 and RMSE) calculated for the results of the equation are reported in Table 4, the results show that the predicted values are very close to the observed values.

This type of validation is necessary for the model to have practical application. (Bagheri, Mirbagheri, Bagheri, & Kamarkhani, 2015; Luccarini et al., 2010).

The nonlinear model MLP-ANN gives good results for this type of prediction with an optimal coefficient of determination R^2 (Threshold value R^2 >0, 6) (Abbasi, & Eslamloueyan, 2014), and root mean square error RMSE. These values indicate that the MLP-ANN model is reliable not only because we has obtained good results in the development of the model, but also good interpolation capacity (Figure.3). These results allow us to predict the evolution of the inhibition diameters zones of garlic extract in different temperature and different incubation times without going through the individual experiments.

4. Analysis of the input variables for the prediction of the inhibition zone diameters

After validating the prediction power of the neural network model, a sensitivity analysis was utilized to provide better understanding of possible relationships between input and output variables of the prediction model. There are two types of sensitivity analysis: the local sensitivity analysis and the global sensitivity analysis.

4.1. *Local sensitivity analysis*

The local sensitivity of an input is measured by varying its value of an infinitesimal amount in 10 different equidistant points, encompassed by a minimum (point 1) and a maximum (point 10) (STATISTICA 10.0.228.8).

The Studied neural network (Figure 4) can be very sensitive to small concentration variations in a certain zone of the input space (point 1). Whereas, that very input is negligible with a positive or negative influence on the output value (low sensitivity) in other points in space that are close to the input value; mainly, large variations in this latter can bring little change in the predictions of the network. However, local sensitivity measures may present a type of behavior that could not be interpreted based on how important (influential) an input variable is at a given point in space (Rodriguez-Fernandez, Banga, & Doyle, 2011).

These results show that the concentration is non-linearly dependent (interactions) with time and with temperature at the same time. However, in the local sensitivity approach, a single factor is disrupted while all other factors are fixed to evaluate the variation of the production (Yi, Zou, & Guo, 2016).

Therefore, it is advantageous to evaluate not only the response to changes of a single input but also the response to simultaneous variations of all inputs. This indicates that the network is better made when all variables were considered.

4.2. Global sensitivity analysis

This method explores the influence of a factor throughout the full multi-dimensional space, it evaluates the effect of a parameter while all other parameters vary simultaneously (Laoun, Naceur, Khellaf, & Kannan, 2016; Younes, Delay, Fajraoui, Fahs, & Mara, 2016). The advantage of this approach is that it provides information regarding the interaction of various model parameters. A coefficient of higher global sensitivity corresponds to the highest influence on the output variables (Benković et al., 2015). Figure 5 shows that the concentration of garlic extract was the most influential parameter (sensitive) affecting the inhibition diameter zone, followed by temperature. It is also found that the conservation duration (Time) is relatively the

least sensitive parameter. These results indicate that the antibacterial activity of garlic extract on the three bacterial strains *E. coli*, *S. aureus*, *B. subtilis*, is mainly controlled by the concentration and temperature of garlic extract.

5. Conclusion

The application of artificial neural network enabled the prediction of antibacterial activity of garlic extract in three different strains of bacteria. For this purpose, one model with three different outputs was designed. This study consists of 450 samples of extract, modeled for their antibacterial activity based on the artificial neural network (Multilayer Perceptron: MLP-ANN). All the internal and external validation methods indicate that the results of the constructed neural model were robust and satisfactory as shown by the values of R^2, Q^2 and RMSE respectively. A neuronal mathematical equation predicted the inhibition diameters for a series of 59 samples, which were not included in the training set. A good correlation was observed between the values predicted by the equation and those obtained experimentally. Furthermore, the sensitivity results reveal a dependence upon the different input parameters, and the most influential on power output are the garlic extract concentration and temperature. Moreover, the model developed could be used to predict the inhibition diameter of garlic extract using three bacterial strains. These results offer new perspectives, particularly in the field of microbiology, since they could be applied to other concentrations, temperatures, and time values, without resorting to experiments.

Conflict of interests

The authors declare that there is no conflict of interests.

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