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Original Article

# Optimization of Inhibitory Effects of *Thymus daenensis* Celak and *Zataria multifera* Boiss Essential Oils on *Candida albicans* Using Response Surface Methodology and Artificial Neural Network

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**Abstract**

Fungus *Candida albicans* has received much attention due to its oral, vaginal and/or systemic candidiasis. This study was undertaken to find out the optimum concentration of two essential oils of *Thymus daenensis* Celak. and *Zataria multiflora* Boiss that have been effective on the *C. albicans*. The essential oils (EOs) were obtained by hydrodistillation method from *T. daenensis* and *Z. multifera* and Time-dependent killing potential of essential oils were assessed in phosphate buffered saline solution containing the desired concentrations of the test agents and yeast suspension. Time of action, concentration of individual or EO mixture and *T. daenensis*:*Z. multifera* mass ratio predicted using response surface methodology (RSM) and artificial neural network (ANN). RSM and ANN techniques predicted the 0.8% as an optimum percentage concentration of EO mixture in oils ratio *T. daenensis*:*Z. multifera* 1:1, ensuring the highest antifungal effect of 95.8% and 96.4% after 20 h. Appraisal of the models through the coefficient of determination ($R^2$) and mean-square error (MSE) show that the ANN was superior ($R^2$= 0.994) to the RSM model ($R^2$= 0.957) in predicting the percentage of reduced cells. Our data confirmed that proper EOs mixtures may reduce the minimum effective dose of individual EOs.

*Keywords*: Artificial neural network (ANN), *Candida albicans*, Response surface methodology (RSM), *Thymus daenensis*, *Zataria multifera*

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## 1. Introduction

*Candida albicans* is the most common species of yeast in humans, particularly in immunocompromised patients, causing superficial to deep mycoses [1]. The spectrum of *Candida* infection is diverse, starting from asymptomatic colonization to pathogenic forms [2]. Oropharyngeal candidiasis is a common initial presentation in patients with AIDS. In patients receiving broad-spectrum antibiotics or undergoing cancer chemotherapy, *C. albicans* can enter the bloodstream to cause serious systemic invasive diseases [3]. For patients, disseminated candidiasis can be a serious disease, often resulting in death [4]. The treatment of these infections is still problematic. Only a few antifungal agents including azoles are currently available, although therapeutic options have increased considerably during the last decade due to the introduction of new agents, efficacy is not always optimal [5].

Prolonged use of antifungals has contributed to the development of drug resistance in *C. albicans* and other species [6, 7], also side effects that usually occur in patients during long term treatment. Essential oils (EOs) and extracts have been widely used for bactericidal, virucidal, fungicidal, and other effects in medicinal and cosmetic applications in a range of industries including pharmaceutical, cosmetic, agricultural, and food industries [8,9]. Natural antifungals still commonly identified from herbs and spices [10] and researches were made to find natural products that could safely be used to inhibit the growth of fungi and their mycotoxins production [11].

*Thymus daenensis* Celak. is one of the herb species of *Thymus* that is endemic to Iran belonging to the Lamiaceae family, Growing in many parts of Iran, and is extensively used in folk medicine [12]. Due to its high concentration of thymol, the plant's essential oil possesses high antimicrobial activities on human pathogenic strains [13]. Studies have shown that EOs containing thymol have used in medicine for their antimicrobial and disinfectant properties and also act as antibacterial additives in food and feed [14]. Notably, thymol has been successfully used *in vitro* against pathogenic fungi, including *C. albicans* [15]. Another plant *Zataria multifera* Boiss., being used as one of the most important spices in daily use by people all over the world [16]. A strong antimicrobial activity demonstrated for *Z. multifera*oils [13]. These oils alone can be used to treat various fungal diseases but their combination may enhance their efficacy.

Antifungal agents given in combination may improve efficacy due to synergism, and because the dose can possibly be lowered, side effects could be reduced. Another advantage of combination therapy is the reduction of the

development of resistance and possibly a shorter duration of therapy [17]. However, Examination of the antifungal effect of EO mixture needs a process optimization. The Response Surface Methodology (RSM) is a set of statistical and mathematical techniques useful for determining the effects of response factor and the interactions among them. RSM is a faster and more efficient method for assembling research results than the classic one-variable at a time or full-factor experimentation [18]. At present, sample preparation by RSM is widely applied for analysis of foods and herbal medicines [19]. The artificial neural network (ANN) is method which also provides optimization and prediction of biological process based on set of the experimental data [20]. The ANNs capture high-dimensional inputs and generate relationships between the inputs and outputs from a training set.

Although the antifungal effect of the oils of *T. daenensis* and *Z. multifera* were well established, but there is a limited data available about antifungal activity of their mixtures, beside this the mixture of EOs and drugs may reduce the minimum effective dose and toxic side effects of antifungals [21, 22]. Keeping this in view we planned our study with the aims to test antifungal effect of individual *T. daenensis* and *Z. multifera* essential oils and their mixture on *C. albicans*; to optimize *in vitro* process variables (time of action, concentration of individual or EO mixture and *T. daenensis*:*Z. multifera* mass ratio) for antifungal effect of EO mixture using RSM and ANN; to compare data obtained by RSM and ANN for optimization of antifungal activity *in vitro*.

## 2. Materials and Methods

### *2.1. Plant Essential Oils*

Essential oils were extracted from fresh leaves of *T. daenensis* and *Z. multifera*. The plant powders (100 g) were subjected to hydrodistillation using a Clevenger-type apparatus for 3 h [5]. The collected essential oils were dried with anhydrous sodium sulphate, filtered and stored at 4-6°C [23].

### *2.2. Organisms and Media*

In this study CA01 (*C. albicans* ATCC 10231) obtained from the Biotechnology Research Center of Iran; and two clinical strains CA02 and CA03 from department of Plant Protection, Gorgan University of Agricultural Sciences and Natural Resources) were included. The test fungi were maintained on Sabouraud dextrose agar (SDA) slants at 4°C and subcultured in SDA for 48 h at 35°C. The count of yeasts was adjusted to yield $1.5\times10^8$ CFU using the standard McFarland counting method.

### *2.3. Time-dependent Killing Assessment of EOs*

Time-dependent killing potential of essential oils were assessed the method as adapted by Ahmad and Khan [23]. Briefly, twenty milliliters of phosphate buffered saline (PBS) solution containing the desired concentrations of the test agents were inoculated with 1 ml of yeast suspension. The

control solution contained PBS with yeast inoculums, but no essential oils. Immediately after inoculation, 100 µl was collected from the solutions for viable count ($y_0$). Test and control solutions were incubated at 37 °C for 48 h [23]. Viable counts were obtained from the test and control solutions at 2, 4, 6, 8, 12 and 24 h by plating 100 µl of 10-fold serial dilutions onto SDA plates and incubating at 37 °C for 48 h ($y_v$). Each experiment was performed in triplicate and the average colony was calculated. Antifungal effect is calculated according to Eq. (1) and represents the percentage of reduced cells ($y_r$):

$$y_r=y_0-y_v \quad (1)$$

*2.4. Models for Optimization of Antifungal Effect of EO Mixture*

*2.4.1. Optimization of antifungal effect of EO mixture using the RSM model*

RSM was adapted for the design of experimental combinations using Design-Expert version 10.0.4 to investigate the combined effect of the three independent variables namely the time of action, the percentage concentration of individual or mixture EOs and the *T. daenensis*:*Z. multifera* ratio. RSM makes it possible to represent independent process parameters in quantitative form [24] as:

$$y_r = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X^2 + \beta_{ij} X_i X_j \quad (2)$$

Where $y_r$ is the percentage of reduced cells, $X_i$ is the time of action of individual or mixture EOs, $X_j$ is the percentage concentration of individual or mixture EOs and m is the *T. daenensis*:*Z. multifera* mass ratio are the parameters of Eq. (2) obtained using the multiple nonlinear regression method by computer program (Design Expert 10.0.4). The coded and uncoded levels of the independent variables are shown in Table 1. A Box-Behnken factorial design with three factors and three levels was used for fitting a second-order response surface. Statistical significance of the independent variables and their interactions were estimated by the analysis of variance (ANOVA).

*2.4.2. Optimization of Antifungal Effect of EO Mixture on Using the ANN Model*

Multi-layer perceptron network (MLP) based on back propagation learning rule was developed using MATLAB R20012a. The obtained experimental data (antifungal effect

**Table 1.** Experimental range and levels of independent variables.

| Variables | Symbols | Range and levels | | |
|---|---|---|---|---|
| | | -1 | 0 | 1 |
| Time (h) | *t* | 0 | 12 | 24 |
| Percentage of concentration of EOs | *C* | 0.12 | 0.5 | 1 |
| EOs ratio | *m* | 1:0 | 1:1 | 0:1 |

of EO mixture on *C. albicans* at different time points for different concentrations of individual or mixture EOs) were used to develop the ANN with Levenberg–Marquardt (LM) algorithm. ANN included three layers of neurons (information): input, hidden, output (Table 2). Three input neurons were the input data vectors viz. time interval (t) and the concentration of individual EO or mixture Eos (C), and the *T. daenensis*:*Z. multifera* ratio (m). The output layer was a single neuron, the percentage of reduced cells ($y_r$).

The number of neurons in the hidden layer and the parameter α (momentum coefficient) were determined by calibration through several run tests. At the first step, the number of hidden layer was fixed to 1, because neural network with one hidden layer and sufficient neurons can model every complex non-linear problem [25]. Thereafter, by choosing various number of neurons in hidden layer, the root mean square error (RMSE) of predicted values were computed and the variation of RMSE of prediction against the number of neurons in hidden layer were plotted. ANN with LM algorithm consisted of three phases: training, validation, and testing [20]. The use of the ANN with LM has comprised of three phases based on a set of input and output data of the design of experiments with replication. Which was divided into three subsets: training (70% of the total data), testing (15% of the total data), and validating (15% of the total data).

### *2.4.3. Statistical Evaluation of Mathematical Models*

Mathematical methods (Section 2.4.1 and 2.4.2) were statistically evaluated by

**Table 2.** Artificial neural network (ANN) parameters for predicting the effect of essential oils (EOs) mixture on *Candida albicans* cells.

| Model | Property | Value/comment |
|---|---|---|
| ANN | Algorithm | Levenberg-Marquardt back-propagation |
| | Minimized error function | MSE |
| | Learning | Supervised |
| | Input layer | Normalized |
| | Data division | random |
| | Hidden laye | Tansig transfer function |
| | Output layer | Purelin transfer function |
| | Number of iteration | 500 |
| | Step size for gradient descent | $1.00e^{-5}$ |
| | Number of input neurons | 3[a] |
| | Number of hidden neurons | 1-15 |
| | Number of output neurons | 1[b] |

a Time (t), concentration of EO mixture (C), and EOs ratio (m).

b Percentage of reduced cells ($y_r$).

determining the mean square error (MSE), coefficient of determination ($R^2$), and, the mean relative error percentage (MRPD) as calculated according to the following equations:

$$MSE = \frac{1}{n}\sum_{i=1}^{n}(y_{a,i} - y_{p,i})^2 \quad (3)$$

$$R^2 = \frac{\sum_{i=1}^{n}(y_{p,i} - y_{a,i})^2}{\sum_{i=1}^{n}(y_{p,i} - y_m)^2} \quad (4)$$

$$MRPD = \frac{100}{n}\sum_{i=1}^{n}\left|\frac{y_{p,i} - y_{a,i}}{y_{a,i}}\right| \quad (5)$$

Where $y_{p,j}$ and $y_{a,i}$ are the calculated and experimental values of the percentage of reduced cells, $y_m$ is the mean value of the percentage of reduced cells, and n is number of experimental measurements.

## 3. Results and Discussion

### *3.1. Optimization of antifungal effect of the EO mixture using the RSM model*

The responses of the percentage of reduced cells obtained from the experiments are listed in Table 3. The second order regression equation has provided the best description of the antifungal effect as a function of time (t), concentration of EO mixture (C), and EOs ratio (m), which can be predicted by the following equation (Eq. 6):

$$y_r = -62.64 + 5.515t + 75.08C + 16.71m + 1.60tC + 0.018tm - 7.14Cm - 0.141t^2 - 20.50C^2 - 1.125m^2 \quad (6)$$

Statistical testing of the model was performed with the Fisher's statistical test for analysis of variance (ANOVA). The results of ANOVA for reduced cells are shown in Table 3. The *p*-value of this model was very low (<0.0001), indicating that the model was significant at the 95% confidence level. The non-significant value 0.5438 for lack of fit shows that the quadratic model was valid for the present study [26]. The natural logarithm (ln) of the residual SS (sum of square) against confidence interval value (λ) is one, dip suddenly with a minimum in the region of the best optimum value 0.85 (Figure 1). The data do not require a transformation, as current value of confidence interval it contains (λ) is very close to the optimum value [24]. The model shows the minimum and maximum confidence interval values of 0.39 and 1.43, respectively (Figure 1).

A significant effect on the percentage of reduced cells was observed: individual independent variables (time of action, concentration of individual or mixture EOs, *T. daenensis*:*Z. multifera* mass ratio), interaction of the time of action and concentration of individual or mixture EOs as well as the square of the time. Other multiple interactions (time of action and *T. daenensis*:*Z. multifera* ratio and concentration of individual or mixture EOs and *T. daenensis*:*Z. multifera* mass ratio) and squares (concentration of

**Table 3.** Analysis of variance of response surface methodology (RSM) method for antifungal effect of the essential oils (EOs) mixture on *Candida albicans* cells.

| Source of variance | Sum of squares | Degrees of freedom | Mean square | F-value | p-value |
|---|---|---|---|---|---|
| Model | 25378.30 | 9 | 2819.81 | 66.93 | <0.0001* |
| t | 5839.64 | 1 | 5839.64 | 138.80 | <0.0001* |
| C | 5316.89 | 1 | 5316.89 | 126.19 | <0.0001* |
| m | 1429.81 | 1 | 1429.81 | 33.94 | <0.0001* |
| tC | 316.16 | 1 | 316.16 | 7.50 | 0.0108* |
| tm | 0.37 | 1 | 0.37 | $8.859E^{-3}$ | 0.9257 |
| Cm | 113.81 | 1 | 113.81 | 2.70 | 0.1119 |
| $t^2$ | 995.58 | 1 | 995.58 | 23.63 | <0.0001* |
| $C^2$ | 78.93 | 1 | 78.93 | 1.87 | 0.1824 |
| $m^2$ | 8.27 | 1 | 8.27 | 0.20 | 0.6616 |
| Residual | 1137.59 | 27 | 42.13 | | |
| Lack of fit | 1113.09 | 26 | 42.81 | 1.75 | 0.5438 |
| Pure error | 24.50 | 1 | 24.50 | | |
| Core total | 26515.90 | 36 | | | |
| Adeq Precision | 28.958 | | | | |
| $R^2$ | 0.9571 | | | | |

(t-time; C-concentration of EO mixture; m- *T. daenensis*:*Z. multifera* ratio)

*Statistically significant at the confidence level of 95%.

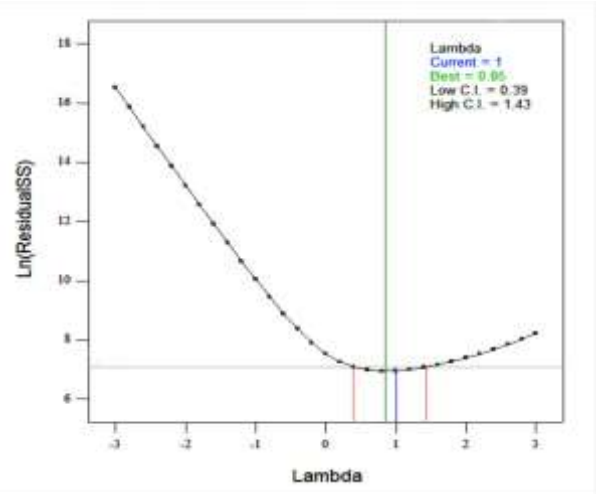


**Figure 1**. Box–Cox plot for power transforms.

individual or mixture EOs, *T. daenensis*:*Z. multifera* mass ratio) had minor importance with no significant effects ($P \leq 0.05$, Table 3). A coefficient $R^2$ of 0.957 was showing a strong correlation between the percentage of reduced cells and independent variables (time, EO

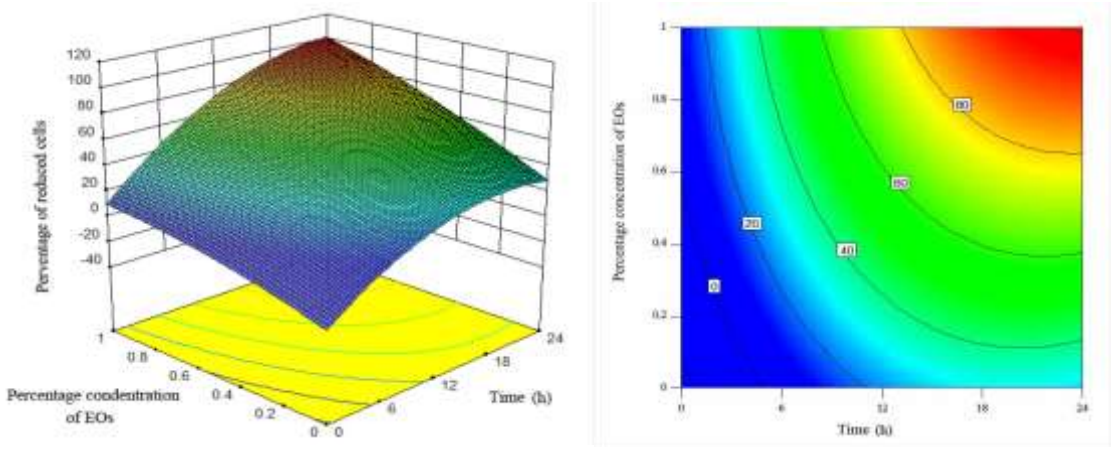


**Figure 2**. Response surface methodology (RSM): the response surface (left) and contour (right) plots for the predicted percentage of reduced cells as a function of the time of action and the percentage concentration of individual or mixture EOs at the ratio of 1:1.

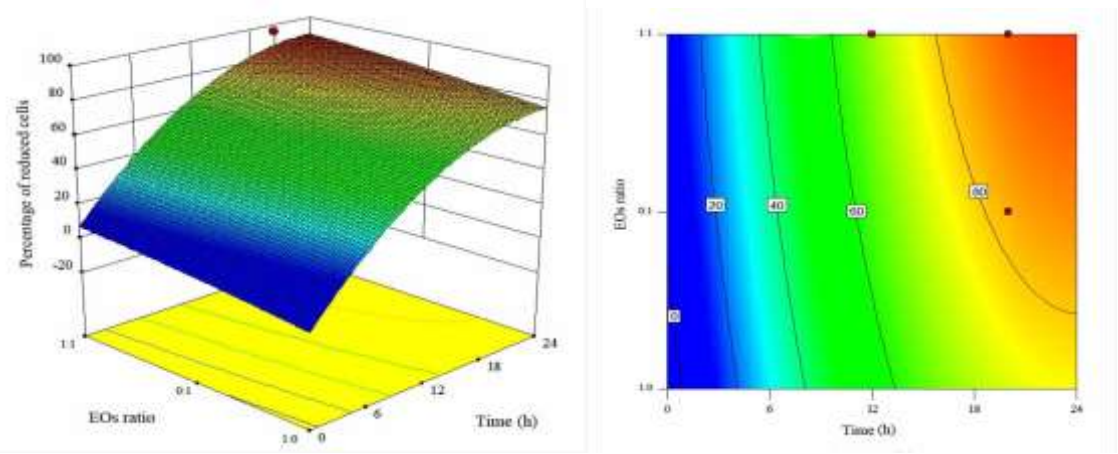


**Figure 3**. Response surface methodology (RSM): the response surface (left) and contour (right) plots for the predicted percentage of reduced cells as a function of the time of action and the EOs of 0.8.

concentration, and *T. daenensis*:*Z. multifera* ratio). The value of Adeq Precision, which measures the signal to noise ratio, was much higher than 4, so the model could be used to navigate the design space. The high value of 75.08 for coefficient of concentration of EO mixture (Eq. 6) illustrates the significant, positive effect of variable on the percentage of reduced cells.

Response surface profiles and percentage of reduced cells as a function of two variables, while the third was held at a constant level as shown in figure 2-4. These graphs were suitable to represent optimization process, since they allow defining the optimal conditions for achieving the maximum percentage of reduced cells. From response surfaces it can be observed that the antifungal effect rapidly increased with the increase of

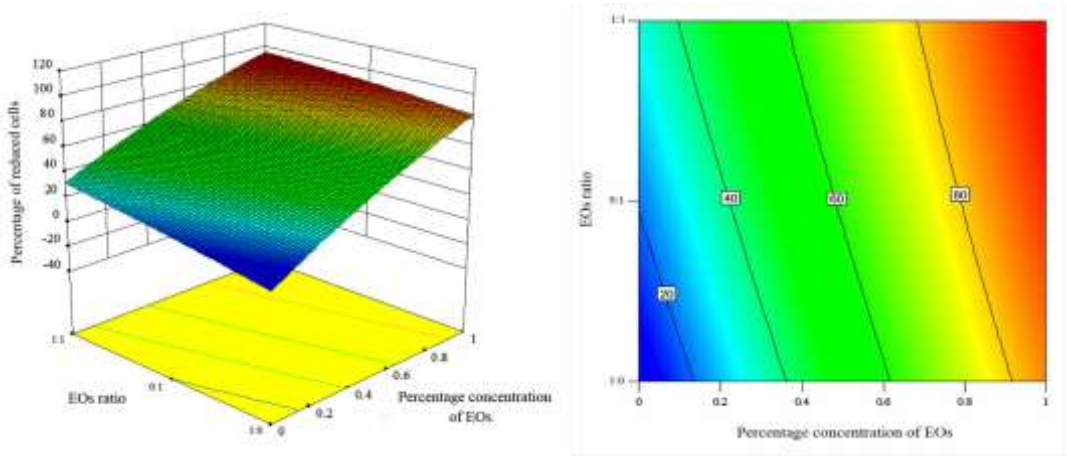


**Figure 4**. Response surface methodology (RSM): the response surface (left) and contour (right) plots for the predicted percentage of reduced cells as a function of the EOs ratio and the percentage concentration of EOs at the time of action of 20 h.

the time and the concentration of EOs mixture (Figure 2). This could be attributed to the positive interaction of the concentration of EOs in mixture towards the antifungal effect. The shape of the contours confirmed the significant effect of interaction of the time and the concentration of EOs mixture on the percentage of reduced cells (Figure 2). The effect of the time and the *T. daenensis*:*Z. multifera* ratio on the percentage of reduced cells is shown in figure 3. The percentage of reduced cells generally increased, reached a plateau and then declined slightly with increasing mass fraction of individual EO in mixture. Figure 4 shows that percentage of reduced cells decreased with increase of fraction of *T. daenensis* independently of percentage concentration of EO mixture at the time of 20 h. The shape of the contours confirmed no significant interaction between the percentage concentration of EO mixture and the *T. daenensis*:*Z. multifera* ratio (Figure 4). The RSM model has predicted that the percentage of reduced cells of 95.8% can be obtained within 12 h under the following conditions of action: the percentage concentration in EO mixture of 0.6%and the *T. daenensis*:*Z. multifera* mass ratio of 1:1. The predicted value of the percentage of reduced cells was close to the experimental value of the percentage of reduced cells (100%) under the same conditions of action.

Numerous studies have demonstrated that plant extracts contain diverse bioactive components that can control fungal cell growth [9]. A strong antimicrobial activity has been demonstrated for both *T. daenensis* and *Z. multifera* EOs [13]. Optimization of antifungal effect of the EOs mixture using these models have been implicated and investigated by many researchers. In a study, applying RSM model for Food decay and contamination by fungi has represented a health risk for consumers due to the potential for fungi to produce mycotoxins and causes considerable economic losses [10]. The results of Rajkovic

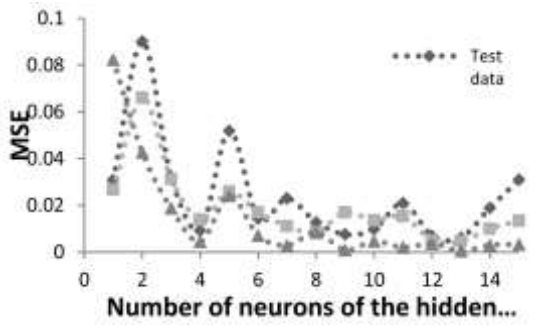


**Figure 5**. Variation in the mean square error (MSE) values with changing the number of neurons in the hidden layer.

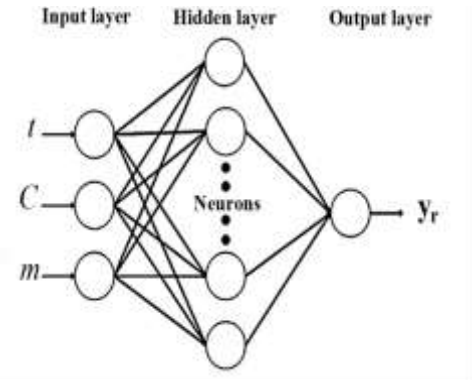


**Figure 6**. Architecture of the developed multilayer artificial neural network (ANN) (t-time of action; C-concentration of EOs; m-EOs ratio; $y_r$- percentage of reduced cells).

*et al*. [22] also developed the RSM model for the optimization of *Thymus vulgaris* L. and *Cinnamomum cassia* L. mixture on *Aspergillus flavus in vitro* and show the model useful for prediction of antimicrobial effects of EOs and their mixtures. However, validation of mixtures for optimum antifungal effects of *T. daenensis* and *Z. multifera* using RSM and ANN has not yet been reported.

### *3.2. Optimization of antifungal effect of the EO mixture using the ANN model*

The antifungal effect of the EOs mixture predicted by the ANN is consisted of one output and three input layer neurons. The optimum number of hidden neurons was found to be 13 when the lowest values of MSE for training, testing, and validation were determined (0.00048; 0.00047; 0.0062). Figure 5 shows the change of the value of MSE with the number of neurons in hidden layer. The network predictive ability was measured by $R^2$ and MSE. As shown in figure 6, the ANN with the 3-13-1 (Figure 6) topology was found to have the highest R-values (0.994). The excellent fitting of experimental data was achieved with a rather simple back ANN propagation performed by using the LM algorithm. This implies that empirical models derived from ANN can be used adequately. The optimum set of the independent variables, namely: the time, the concentration of EO mixture and the *T. daenensis*:*Z. multifera* mass ratio to the maximum the percentage of reduced cells, was determined by applying the ANN model.

### *3.3. Comparison of RSM and ANN models performance*

For the accuracy of the models, $R^2$ and MRPD from RSM and ANN were evaluated. The results showed that both models gave good predictions due to the values of $R^2$ (0.957 and 0.994 for RSM and ANN, respectively). However, ANN showed superiority over RSM due to smaller value of MRPD obtained (±11.7% and ±0.1% for RSM and ANN, respectively). Randomly scattering of points

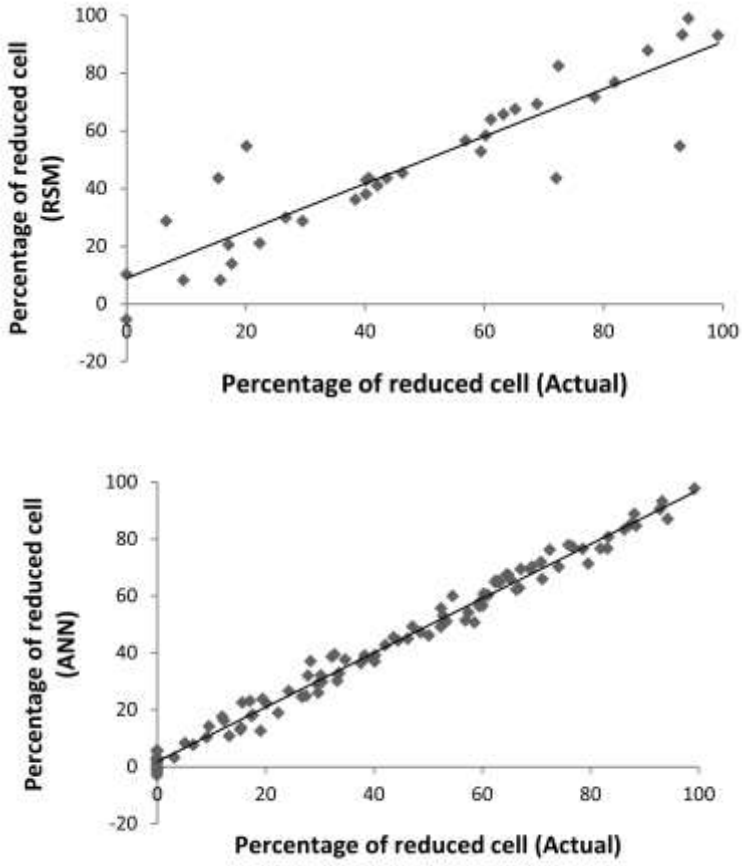


**Figure 7**. The comparison of experimental and calculated values of the percentage of reduced cells: response surface methodology (RSM) model (7.A) and artificial neural Network (ANN) model (7.B).

around the diagonal could be observed by comparing the predicted and actual values of the antifungal effect (Figure 7). An excellent agreement between the predicted and experimental values of the antifungal effect of the developed ANN is shown in figure 7B. Additionally, the relative deviations between the predicted and experimental values of the percentage of reduced cells at the optimal conditions of the models are ±12.48 and ±0.58 for RSM and ANN, respectively. Conclusively, ANN was better than RSM in the modeling and optimization studies of antifungal effect. ANN model superiority in relation to the RSM model has been shown in some previous studies by many researchers [22, 27].

## 4. Conclusion

From the data above, we can conclude that *T. daenensis* shows potent antifungal effects against clinical *C. albicans* isolates, when combined with *Z. multifera*, although it was showing weak antifungal activity when used alone (data not shown)Our findings have confirmed that proper EOs mixtures may reduce the minimum effective dose of individual EOs. The applied model could be

suitable for the determination of the most efficient application of EOs not only in terms of antifungal but their other activities important for agriculture and medical applications. Using RSM and ANN techniques, the optimum percentage of the concentration mixture EO was found to be 0.5%, with *T. daenensis*:*Z. multifera* mass ratio of 1:1. Both RSM and ANN ensured the highest antifungal effect of 95.8% and 96.4% in 65 min, respectively. Both models are useful for optimization the antifungal effect in vitro but ANN applied for experimental design is more accurate than RSM due to its lower value of MRPD. Therefore, ANN can be generally used for optimization and prediction of *in vitro* antimicrobial effects of oils. Our findings can be exploited to achieve effective formulation of oils especially *T. daenensis* and *Z. multifera*in combination therapy against candidiasis.

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