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Ultrasound-Assisted Extraction of Damsin and Neoambrosin from *Ambrosia maritima*: Optimization Using Response Surface Methodology

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Abstract

Damsin and neoambrosin are two important anticancer phytopharmaceuticals of *Ambrosia maritima* (Damsissa). Despite their importance, no report has yet discussed their extraction from plants on lab or industrial scales. Consequently, the present article aims to develop an ultrasonic-assisted extraction (UAE) model for their extraction on the lab scale. Box-Behnken model was applied to investigate the effects of ethanol strength (X_1: 40-100%), drug solvent ratio (DSR, X_2: 1:20-1:60), and time (X_3: 30-90 min) on the yield of damsin and neoambrosin in damsissa extract. Surprisingly, only ethanol strength was the significant factor in damsin yield with an optimum value of 55%. Time and DSR played no significant roles in damsin content. No statistically significant model could be deduced for neoambrosin. The model is a preliminary step to elucidate factors affecting the UAE of sesquiterpene lactones from plants and a preamble for its large scale green extraction.

Keywords: Damsin; Neoambrosin; Ultrasonic-assisted extraction; response Surface method; sesquiterpene lactones; Ambrosia.

1. Introduction

Natural products are still an important repertoire for lead pharmaceuticals especially anticancer drugs. Sixty percent of current anticancer are derived from natural products [1]. Sesquiterpene lactones (STL) are a large class of compounds distributed in several plant families. They act as potent anti-cancer, anti-inflammatory, antimicrobial, and molluscicidal [2]. Their anticancer activity is mediated through different targets, however, inhibition of NF- κβ was the main target [3-5]. STL act by inhibiting the activation of NF-κβ, binding of NF-κβ to DNA, interacting with DNA-NF-κβ complex [4, 6-8]. Efficacy of STL paved the way for testing them in clinical trials or to synthesize new drugs based on their nucleus [3, 5].

Ambrosia maritima, locally known as damsissa, is a weed growing on Nile river banks and Nile delta. It is traditionally used as diuretic, antidiabetic, anti-inflammatory, and molluscicidal [9]. Damsin and neoambrosin are two major STL found in genus *Ambrosia*. They had potent anticancer activity against many cancer cell lines and multi-drug resistant cells [9-13]. Similar to other STL, damsin and neoambrosin interact with NF-κβ by methylation of cysteine-38 and Cys-120 and rendering it unable to bind to DNA, consequently, halting the inflammation process, promote apoptosis of cancer cells [14, 15]. Moreover, they can interact with the DNA-NF-κβ complex [14]. Recently, Damsin and neoambrosin showed significant cytotoxic activity against multidrug-resistant cell lines by silencing several targets, c-Src kinase, STAT3, Akt and ABC transporters; therefore they are suggested as remedies for refractory tumors [9] Both compounds showed a potent antirheumatoid activity [16].

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Large scale extraction of STL is imperative to secure enough supply. Chemical synthesis of damsin required eighteen reactions and was found intricate because of the many chiral centres of the molecule [17-20]. Phytochemical isolation would be a cheaper and reliable alternative to chemical synthesis. Compared to conventional extraction techniques, ultrasound-assisted extraction is better in terms of saving time, energy, solvent volume, CO_2 emission [21, 22]. Although cavitation was known as the main mechanism of action of UAE, several other mechanisms are involved, e.g., fragmentation, erosion capillarity, detexturation and sonoporation [21]. Several factors affect the yield of the process, e.g., ultrasonic power, time, temperature, etc. [21, 22]. Response surface method (RSM) is a statistical method utilized to evaluate the effect of different variables on one or more responses of variables. This can be achieved from a lower number of experiments and in a shorter time compared to other optimization methods [23-25].

The current work is the first report about the extraction of damsin and neoambrosin from damsissa. Ultrasound-assisted extraction was utilized and response surface model was employed to optimize the extraction condition. Hopefully, the present work would be a step for scaling up the extraction of STLs from Damsissa as well as other plants rich in STL.

2. Experimental

2.1. Plant material & chemicals:

Ambrosia maritima aerial parts were collected from the Botanical Garden of Faculty of Pharmacy, Cairo University. Plant material of *A. maritima* was authenticated by Prof. Dr. Wafaa M. Amer, Botany Department, Faculty of Science, Cairo University. A voucher specimen was prepared and deposited in Cairo University herbarium (CAI). Another voucher specimen (13.06.2018) was deposited in the herbarium, Pharmacognsoy Department, Faculty of Pharmacy, Cairo University. Samples were powdered after being dried in shade.

Ethanol, HPLC grade was purchased from Thermo Scientific, Germany. Deionized water prepared in the institute. Damsin and neoambrosin authentic materials were previously isolated and authenticated [16, 26].

2.2. Model construction

Box-Behnken design was constructed for the optimization of ultrasound-assisted extraction of sesquiterpene lactones from *A. maritima*. Three parameters were selected for optimization of the extraction process, namely, Ethanol ratio (Ethanol %, X1), Drug solvent ratio DSR (X2), and extraction time (X3). Three levels for each independent variable were selected as in table 1

The design consisted of 15 runs; however, 14 were used in analysis after the omission of an outlier. The model proposed for each response Y was

$$Y = \beta 0 + \beta 1X1 + \beta 2X2 + \beta 3X3 + \beta 12\, X1X2 + \beta 13\, X1X3 + \beta 23\, X2X3 + \beta 11\, X12 + \beta 22\, X22 + \beta 33\, X32 \quad \textit{(equation 1)}$$

Y is the response, i.e., damsin or neoambrosin content (µg/g dry powder). **X1, X2, X3** are the independent variables, namely, ethanol percentage, DSR, and time, respectively. ***β0*** is the intercept***. β1, β1, β1***, are the linear coefficients**. β12, β13, β23***, are the interaction coefficients; ***β11, β22, β33,*** are the quadratic coefficient terms.

Box-Behnken design was set up using JMP software Statistical DiscoveryTM from SAS (Germany). The established model was validated by Regression value (R2), p-value ($p<0.05$), and lack of fit testing. Significance of the tested independent variables and interactions were determined form Pareto-graph, ANOVA testing.

2.3. Extraction process:

Different weights of damsissa powder (0.33–1.00 g) powder were extracted in 20 ml solvent (40-100% ethanol) in an ultrasonic bath for different durations (30-90 min) according to individual experiments (Table 1). Extraction was performed in Elmasonic S, 0.8 l with an ultrasonic frequency of 37 kHz (Elma Schmidbauer GmbH, Germany) at room temperature. Samples were extracted in 50 ml falcon tubes, then centrifuged, aliquots of 2 ml were separated and filtered through a membrane filter (0.45 mm). Volumes of 100 µl were directly injected in HPLC.

Table 1: Real and coded values of UAE independent variables

	-1	0	1
Alcohol %, X_1	40	70	100
Drug solvent ratio (DSR, X_2)	20	40	60
Extraction time (X_3).	30	60	90

2.4. HPLC analysis

Damsin and neoambrosin as well as the different extracts were analysed as previously performed but with slight modifications [16]. HPLC Agilent 1200 infinity instrument was equipped with an autosampler and DAD detector. The mobile phase system consisted of solvent A & B, water containing 0.1% formic acid and acetonitrile, respectively. The separation was in gradient mode on LiChrospher 100 RP-C18 column, preceded by RP-C18 guard column, and started by using solvent B at 45% for 2 min, then, it was gradually increased to attain 58% at 19 minutes then increased to 100% at 21 minutes. Afterward, the column was washed using 100 % MeOH for 2 min. Quantification was determined at wavelength 240 nm. Standard curves for damsin and neoambrosin were set up in a concentration range of 3.125-100, 1.65-50mg/ml, respectively.

3. **Results and Discussion**

Few reports had studied the UAE of sesquiterpene lactones from plants. One report evaluated the extraction of alantolactone and isoalantolactone [27]. In the other report, UAE was done as a preparatory step before percolation to extract artemisinin [28]. Neither of the two reports had utilized the RSM to mathematically model the extraction process. A recent study utilized RSM to optimize the extraction of cyanopicrin [29]. The biological significance of damsin and neoambrosin necessitated the search for a green isolation technology as a preliminary step before large scale production. HPLC analysis showed that damsin and neoambrosin were major components of the ethanolic extract of damsissa (Figure 1).

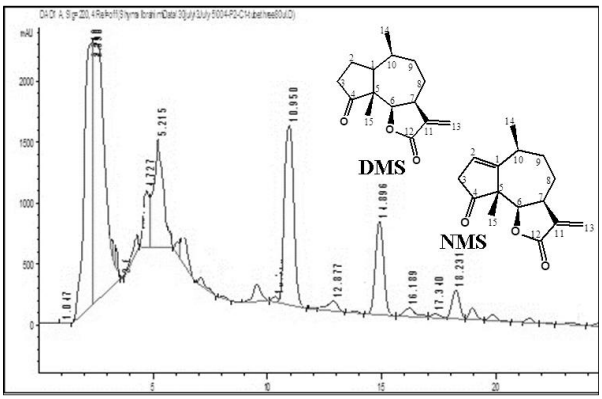

Fig. 1: HPLC chromatogram of Damsissa extract. Inserts show the chemical structure of damsin and neoambrosin. DMS, damsin; NMS, neoambrosin**.**

3.1. Effect of extraction variables on damsin content.

Damsin was quantified from 47 to 723 µg/g DW. The highest content was obtained when extraction was performed using 70% ethanol for 90 min and DSR of 1:60. The lowest yield was obtained when the powder was extracted with 100% ethanol for 60 min and at DSR of 1:20 (Table 2). UAE of damsin can be modelled, its model had a satisfactory R^2 of regression at $p<0.05$ and there was no lack of fit (Table 3). The prediction model for Damsin (Eq.2) showed a higher positive coefficient for ethanol percent factor (X1) compared to other factors main effects. ANOVA testing of individual variables revealed that only ethanol strength was the most significant parameter, it affected damsin content at first order and quadratic terms (Table 3). Other factors like DSR and extraction time have a negative impact on damsin extraction process. However, their effects were not significant. Besides, interaction between factors was also trivial by comparison. Except for ethanol strength, no other parameters had a significant effect on the UAE of damsin. The response surface model showed that decreasing the alcohol strength positively affected the damsin content to the optimum value of 55%. Variation in DSR has no effect (Figure2).

Considering only the significant variables, the predictive equation which can be utilized to optimize the UAE of damsin from damsissa was introduced (Eq.3).

$$\textit{Damsin (mg/g)} = 129.22 + 22.22X_1 - 5.55X_2 - 4.15X_3 + 0.06X_1X_2 - 0.02X_1X_3 + 0.03X_2X_3 - 0.22X_1^2 + 0.04X_2^2 + 0.05X_3^2 \quad (Equation\ 2)$$

$R2 = 0.97$, $p\ value < 0.008$

$$\textit{Damsin (mg/g)} = -85.49 + 25.75X_1 - 0.24X_1^2 \quad (Equation\ 3)$$

$R2 = 0.89$, $p\ value < 0.0001$

To further validate the model, the extraction procedure was repeated in triplicate using ethanol strength of 55%, as deduced from the response surface curve (Figure 2). However, extraction time and DSR were kept to lower levels due to their insignificance, 30 min, 1:20, respectively. The model predicted extraction of 587.25 µg/g damsin. Experimental result showed close values (610.74 ± 34.4 µg/g) which verify the validity of the model.

Although STL (SLC) is not polar, higher solubility in EtOH/water mixtures could be attributed to several factors. Water tends to increase tissue swelling; hence, a larger surface area of plant material is exposed to solvent. Moreover, bubble formation helps in the diffusion of hydrophobic parts of the

molecule in the hydrophilic aqueous solvents due to the formation of gas-liquid interfaces [30, 31]. Higher yields of alantolactone were achieved with 70% ethanol [27]. Similarly, 60% isopropanol was the best solvent to extract rebaudioside A from *Stevia* and at a short time (18 min) [32]. Sonication time, DSR had no significant effect in the UAE of gallic acid, punicalin, and punicalagin from pomegranate peels [33].

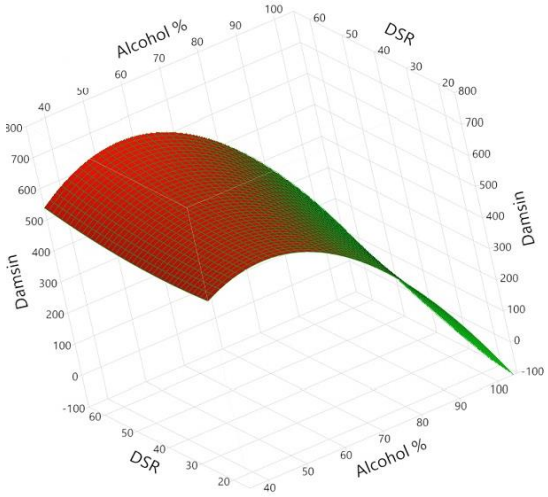

Fig.2: Response surface plot for the effect of Alcohol %/DSR on damsin content.

3.2. Effect of extraction variables on neoambrosin content

Neoambrosin was extracted at variable quantities ranging from 24 to 193µg/g. Its maximal level was attained when powder extracted with 40% ethanol for 90 min at DSR of 1:40. The minimal yield of Neoambrosin was attained when ethanol 100% was utilized for 90 min at DSR 1:40 (Table 2). Although this tremendous difference can be directly related to ethanol strength, ANOVA testing of individual variables of the polynomial model shows no significance (Table 3). This is probably due to the lower content of neoambrosin compared to damsin in the studied plant. Therefore, there is no significant difference in the extraction content between ethanol percent levels. The positive impact of ethanol content on neoambrosin yield can be deduced from its response surface model (Figure 3). The figure also shows small curvature of the quadratic term nonsignificant effect. DSR ratio did not affect neoambrosin yield (Figure 3).

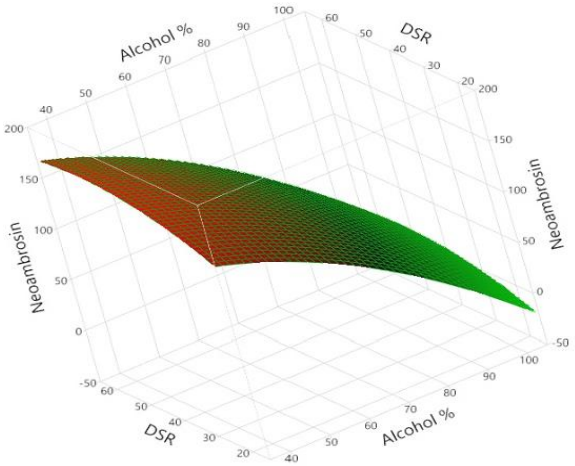

Fig. 3: Response surface plot for the effect of Alcohol %/DSR on neoambrosin content

Table 2 Box-Behnken design with coded and real values as well as the observed responses in different extracts

Run	Alcohol Percent (A%), X_1 (%)	Drug solvent ratio, (DSR) (1-X2)	Time, X_3 (min)	Damsin (µg/g powder)*	Neoambrosin (µg/g powder)
1	1 (100)	0 (40)	1 (90)	104.44	24.246
2	0 (70)	-1 (20)	-1 (30)	432.53	63.019
3	0 (70)	-1 (20)	1 (90)	539.75	110.94
4	1 (100)	0 (40)	-1 (30)	101.24	18.595
5	0 (70)	1 (60)	-1 (30)	555.57	112.34
6	-1 (40)	0 (40)	1 (90)	623.84	193.27
7	0 (70)	0 (40)	0 (60)	506.38	113.48
8	1 (100)	-1 (20)	0 (60)	47.884	19.723
9	-1 (40)	0 (40)	-1 (30)	565.77	155.63
10	-1 (40)	1 (60)	0 (60)	525.44	148.23
11	0 (70)	0 (40)	0 (60)	538.87	121.68
12	-1 (40)	-1 (20)	0 (60)	524.47	141.53
13	0 (70)	0 (40)	0 (60)	474.59	90.563
14	0 (70)	1 (60)	1 (90)	723.32	135.55

Table 3: Estimated regression coefficient and ANOVA of the fitted second-order polynomial models of the investigated parameters

	Damsin	Neoambrosin
source	***p*-value <0.05**	
Model	**0.008**	**0.018**
Lack of fit	0.139	0.333
A% (X_1)	**0.032**	0.749
DSR (X_2)	0.603	0.413
Time (X_3)	0.505	0.696
X_1X_2	0.470	0.987
X_1X_3	0.675	0.463
X_2X_3	0.645	0.565
X_1^2	**0.004**	0.229
X_2^2	0.708	0.490
X_3^2	0.297	0.562

4. Conclusions

The conclusions section should come in this section at the end of the article, before the acknowledgements. Due to the promising potential of DMS and NMS as an anticancer, a UAE method was studied as a prelude for large scale extraction of theses valuable bioactive compounds. UAE of natural products is a highly evolving technology because of its eco-friendly and economic advantages. It was found that only the ethanol strength was the most decisive factor affecting the yield of damsin, where 55% of ethanol was the best percent. This is the first report of the UAE of pseudoguaianolide STLs from damsissa. It could serve as a basis for scaling up and also for extraction of other important sesquiterpene lactones from plants.

5. Conflicts of interest

"There are no conflicts to declare".

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