The influence of Rhus coriaria seeds Extracts on the Genetic Resistance of *Aspergillus amstelodami*

La influencia de los extractos de semillas de Rhus coriaria sobre la resistencia genética de *Aspergillus amstelodami*

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ABSTRACT

Introduction: This study aims to investigate the safe use of *Rhus coriaria* seed extracts (water and ethanol) genetically on fungi *Aspergillus amstelodami* because the extensive use of plant in many pharmaceutical and food fields. **Materials and methods:** In this study A1 (Wa1) strain of the fungus *A.spergillus amstelodami* was used in all genetic testing, *Rhus coriaria* seeds were obtained from the local markets and two types of extracts were prepared (water and ethanol extracts of *Rhus coriaria* seeds), two types of resistance mutants were isolated both spontaneous and induced by using mutation agent (nitrous acid). **Results and Discussion:** In this study 18 spontaneous resistance mutation were isolated in frequency 4.26×10^{-5} and 96 induced mutations were induced by nitrous acid in Frequency 39.76×10^{-5}at (MIC) 16 mg / ml of the ethanol extract, and 22 spontaneous mutations were isolate in frequency 4.59×10^{-5} and 91 nitrous acid induced mutation with an average recurrence 37.36×10^{-5} at (MIC) 25 mg / ml of water extract. **Conclusion:** We conclude that presence of resistant ability in *A. amstelodami* toward *Rhus coriaria* water and ethanol seeds extracts. Therefore, we suggest further thorough studies to detect the activity to plant extract in order to be use in agricultural pest control.

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INTRODUCCTION

Many organizations, including the World Health Organization (WHO), have been urged to investigate for plant extracts that are effective in the treatment of many infectious diseases as antibiotics[1] in spite of the development of antibiotics, but many infectious diseases still considered dangerous to humans through the emergence of resistant strains resulting from a genetic mutation in the target location where the active ingredient operates[2] therefore, the study of the genetic basis of resistance to different compounds are an important step to understanding the genetic basis of this resistance and helps to choose the right material to eliminate microorganisms. Due to the wide use of the plant of sumac in many pharmaceutical and food fields because its seeds contain active compounds such as Phenols, Tannins, Flavonoids, It has been used as an antibiotics in inhibiting the growth of fungi [3, 4, 5] these compounds are also antibiotics for many negative and gram positive bacteria [6, 7] and inhibitory to multiply a virus Herpes simplex and Influenza virus [8].

The plant of Sumac was known by word(Sumac) means dark red, back to the family (Anacardiaceae) [9] but the scientific name Rhus was derived from the Greek language, means "Red color", the plant of Sumac grow in the form of bushes that are automatically spread and adapted to live in different environments, It spreads in tropical temperate regions and in the Mediterranean region [10], There are more than 250 species and the Sumac Tanning (*Rhus coriaria*) the most common types, the term coriaria means its use in tanning and corium means the leather, the fruits of this type are characterized by flat kidney and mauve in a reddish brown color. Sumac is considered an economically important plant, it enters into many uses, including medical and industrial as a perfume and cosmetics industry as well as a major role in tanning and dyeing leather. The fruits of sumac contain the substance Tannins ,phenols and Flavonoids [6] in addition to containing citric acid, acetic and malic acid, which gives it acid taste, these compounds have given the Sumac medical importance as a holding, disinfectant and anti-inflammatory, It was used as a gargle to treat the problems of mouth and throat, as well as the use of sumac extracts as a fat and anti-heat and a position for bleeding and anti-diarrhea [9] and it was used in the treatment of burns and sores. The Aims of this study is to investigate the genetic resistance of mutations in fungus *Aspergillus amstelodami* towards the minimum inhibitory concentration (MIC) of the water and ethanol extract of *Rhus coriaria* seeds.

MATERIALS AND METHOD

Test organism

The A1 (Wa1) strain of the fungus *Aspergillus amstelodami* was used which was obtained from Prof. Dr. Sahi Jawad Dhahi, Science College, Department of Biology, University of Mosul.

Culture media and culture conditions:

Culture media and culture conditions were used based on the method [12]. In our present study, we used Minimal medium (M), all tests were performed on this medium plus test materials.

Complete supplement (C) with a final concentration 5% (volume / volume) was added to the medium for supporting the growth, Sodium deoxycholate (D) with a final concentration 400 µg / mL also added to the medium counting the colony easily [13].

Preparation of extracts of *Rhus coriaria* seeds:

1. Collecting and classifying the plant of sumac: The seeds of the plant were obtained from the local markets, Plant was classified in the Museum of Science College / Department of Biology/ University of Mosul, the seeds were cleaned of

impurities and dust and kept in the refrigerator until the preparation of extracts.

2. Preparation of plant extracts of sumac seeds:

A. Ethanol extract: ethanolic extract was prepared based on the method described by [16] which modified from the original method described by [17]. One gram of dry ethanol extract powder dissolved in 5 ml of the Dimethyl sulfoxide (DMSO) to obtain a concentration of 200 mg / ml. the extract was sterilized by Pasteurization at 62 ° C for 20 minutes [15]. The samples were kept in freezer in glass bottles with a tight lid until they were used in the study.

B. Water extract: the seeds water extract of sumac was prepared depending on the method described by [14]. One g of water extract powder dissolved in 5 ml of distilled water to obtain a concentration of 200 mg / ml, the extract was sterilized with Pasteurization at 62 ° C for 20 minutes [15]. The samples were kept frozen in glass bottles with a tight lid until they were used.

Minimum Inhibitory Concentration (MIC):

The minimum inhibitory concentration of *Rhus coriaria* seeds extracts (water and ethanol) was determined by using the (M) medium containing the concentrations of each extracts separately, the concentration (0-12) mg / ml was prepared. Then different concentrations for each plant extract was inoculated by three strains A1 of tested fungi in a plate containing (M) medium plus a specific concentration of the studied material and three plates (R1, R2, R3) for each concentration. After four days of incubation, the colony diameters and the percentage of inhibition for each concentration were measured based on the following equation:

Percentage of inhibition =

Preparation of spore suspension:

The spore suspension of *A. amstelodami* was prepared from a four-day fresh culture incubated on the CM medium. By adding an appropriate amount of distilled water (about 10 ml) to the fungal culture, the spores were washed. After that the transferred to a sterile vial and shaked well to break the conidial chains, the suspension was filtered through sterile cotton to remove the Mycelial fragments and Cleistothecia, the leachate was considered as the non-dilute suspension (10^0 dilution) [12].

Isolation of mutant resistance:

Two types of resistance mutants were isolated, both spontaneous and induced by using mutation agent (nitrous acid) were performed, based on method described by [18] for each extract. The spore suspension is adjusted to 10^7 spore/ ml, then mutants was tested.

1. Isolation of spontaneous mutations:

Dishes containing the sterilized MD medium and the appropriate concentration of the water and ethanol extract of *Rhus coriaria* seeds were inoculated each by 0.1 ml prepared and adjusted spore suspension, and another 3 dishes containing the MD medium only as control were inoculated by 0.1 ml prepared and adjusted spore suspension [19].

2. Isolation of induced mutations:

The fungal suspension was treated by nitrous acid (known mutant) using the method described by [11] for this purpose 10 petri-dishes containing the MD medium and appropriate concentration of the water and ethanol extracts of *Rhus coriaria* seeds were inoculated, each by 0.1 ml / spore suspension which treated with nitrous acid [11] and 3 dishes containing the MD medium were inoculated by 0.1 mL of each adjusted spore suspention as control. The incubated for 3-4 days for control and 8-6 days for the treated plates with mutant resistance to detect their effects [19]. The frequency of induced and spontaneous mutations was estimated by:

Frequency of mutants = ×100

The population size expected from the spore suspension of both treatments was estimated as follows: Size of the expected population for each treatment = number of colonies on MD dishes × inverted dilution × number of dishes fertilized by non-dilute suspension (10^0 dilution) [20].

Statistical analysis:

The mean frequency and the standard error (SE) of the mutants were calculated for each transaction. The statistical analysis of the results was performed using a t-test at a significant level of ≤ 0.05 [21].

RESULTS

The results of the determination of the minimum inhibitory concentration (MIC) for the studied extracts,(Tables 1,2) showed that the diameter of the *A.astelodami* fungi colonies decreases with increasing concentration of extracts in the medium severally, there is a positive correlation between the increasing in concentration of the extracts and the percentage of inhibition the percentage of inhibition ranged between 16.42% and 100% at concentrations 1 and 16 mg / ml of ethanol extract, respectively, and an inhibition ratio between 16.43% and 100% at concentrations 1 and 25 mg / ml of water extract, respectively. This result indicating that the water extract of *Rhus coriaria* seeds has given a less inhibitory effect compared to the inhibitory effect of the ethanol extract of *Rhus coriaria* seeds on the growth of *A. amstelodami* which was shown in table (1, 2).

This result matches with the results of several studies that indicated the efficacy of the *Rhus coriaria* seeds as an antifungal, including the fungi under study [4, 5, 22]. This activity is due to the containment of the seeds of sumac on many active compounds such as Tannins and Phenol [23, 24, 25]. Which have antifungal activity against many fungi [26, 27].

Table 1. The effect of different concentrations of ethanol extract of *Rhus coriaria* on the colony diameters of *A. amstelodami.*

Ethanol extract concentration mg/ml	Average Colonies diameter (cm)	Percentage of inhibition %
0	2.07	-
1	1.73	16.42
2	1.63	21.26
3	1.6	22.70
4	1.53	26.09
5	1.5	27.54
6	1.42	31.4
7	1.27	38.64
8	0.93	55.07
9	0.73	64.73
10	0.6	71.01
11	0.43	79.23
12	0.3	85.5
13	0.2	90.3
14	0.13	93.71
15	0.03	98.6
16	0	100

Table 2. The effect of different concentrations of water extract of *Rhus coriaria* on the colony diameter of *A.astelodami*

Water extract Concentrations (mg/ml)	Average Colonies diameter (cm)	Percentage of inhibition %
0	2.07	-
1	1.73	16.43
2	1.7	17.87
3	1.47	28.99
4	1.36	34.29
5	1.3	37.2
6	1.16	43.96
7	1.1	46.85
8	0.96	53.62
9	0.86	58.45
10	0.83	59.90
11	0.73	64.73
12	0.6	71.01
14	0.43	79.22
16	0.36	82.60
18	0.26	87.43
20	0.13	93.71
22	0.03	98.6
25	0	100

The current study recorded isolation of 18 spontaneous mutations and 96 mutations induced by nitrous acid resistant to the ethanol extract of *Rhus coriaria* seeds which induced by the treatment of *A.astelodami* fungal colony with a lower inhibitory concentration of 16 mg / ml (Table 1), and the calculation of the size of the conidial population for each treatment showed that the average frequency of spontaneous mutants was 4.26×10^{-5} while the average frequency of induced mutants was 39.76×10^{-5} (Table 3,4). Based on the statistical analysis the results show that there is significant differences between the mean frequency of spontaneous mutants and the nitrous acid induced mutations resistance to the extract of *Rhus coriaria* seeds. The calculated value of t_4 (8.236) was greater than the t_4 tabular value (4.032) at the probability level of 0.05(Table 4).

Table 3. The population size (× 10^{-5}) and the number of mutants (spontaneous and induced by nitrous acid) resistant to the ethanol extract *Rhus coriaria* seeds and their frequency (× 10^{-5}) observed between fungal conidia of *A. amstelodami* .

Treatment	Average of			Average of			Average of		
	Expected population size	number of resistance mutants	Frequency of resistance mutants	Expected population size	number of resistance mutants	Frequency of resistance mutants	Expected population size	number of resistance mutants	Frequency of resistance mutants
0	118.8	6	5.0	179	6	3.35	135	6	4.44
HNO_3	78.9	38	48.16	83.34	31	37.19	79.52	27	33.95

0: Without treatment and their frequencies considered frequencies of spontaneous mutants. HNO_3: Treatment with nitrous acid and their frequency considered the frequencies of induced mutants.

Table (4): The average frequency of mutant (spontaneous and induced by nitrous acid) (× 10^{-5}) resistance to the ethanol extract *Rhus coriaria* seeds observed between fungal conidia of *A. amstelodami.*

Treatment	Average frequency of mutants ± Standard error	Calculated t4 value*
0	4.26± 0.47	B
HNO_3	39.76± 4.29	8.236

0: Without treatment and their frequencies considered frequencies of resistance mutants. HNO3: Treatment with nitrous acid and their frequency considered the frequencies of induced mutants. *: significant at probability level 0.05.

The results revealed that the water extract of *Rhus coriaria* seeds at 25mg/ml have greater effects on the colony diameter of the *A. amstelodami* which inhibit the fungal growth completely, from the result 22 spontaneous resistance mutations and 91 nitrous acid induced mutations with average frequency of mutant 4.59×10^{-5} and 37.36×10^{-5} respectively (Table 5,6), the statistical analysis indicated that there is significant differences between the mean frequency of spontaneous mutants and the nitrous acid induced mutations resistance to the water extract of *Rhus coriaria* seeds. The calculated value of t_4 (18.619) was greater than the t_4 tabular value (4.032) at the probability level of 0.05 (Table 6).

Table (5): The effect of water extract *Rhus coriaria* seeds on population size (× 10^{-5}) and the number of resistant mutants (spontaneous and induced by nitrous acid) on *A. amstelodami* .

Treatment	Average of			Average of			Average of		
	Expected population size	number of resistance mutants	Frequency of resistance mutants	Expected population size	number of resistance mutants	Frequency of resistance mutants	Expected population size	number of resistance mutants	Frequency of resistance mutants
0	170	8	4.70	164	7	4.26	145.2	7	4.82
HNO_3	85.5	30	35.08	80.2	29	36.15	78.3	32	40.86

0: Without treatment and their frequencies considered frequencies of resistance mutants. HNO_3: Treatment with nitrous acid and their frequency considered the frequencies of induced mutants. *: significant at probability level 0.05.

Table (6): The average frequency of mutant (spontaneous and induced by nitrous acid) (× 10^{-5}) resistance to the water extract *Rhus coriaria* seeds observed between fungal conidia of *A. amstelodami* .

Treatment	Average frequency of mutants ± Standard error	Calculated t4 value*
0	4.59±0.168	B
HNO_3	37.36±1.753	18.619

0: Without treatment and their frequencies considered frequencies of resistance mutants. HNO3: Treatment with nitrous acid and their frequency considered the frequencies of induced mutants. *: significant at probability level 0.05.

DISCUSSION

The results shown in the tables (3,4,5,6,) indicates decrease a low frequency of spontaneous mutants compared to frequency of induced mutations resistance to both extracts ,this confirmed by [18] which

mentioned that the nitrous acid is a mutagenic factor , However, this does not negate the appearance of spontaneous mutants in the fungi *A. amsteloda mi* , which begins with a single spore, and with the doubling of the DNA and the occurrence of the divisions becomes a mutant colony resistance that spreads its spores in the air causing long-term danger especially when resisting the active compounds found in extracts of *Rhus coriaria* seeds which are considered important as antifungal agents in the medical field or as fungicides in the agricultural field [22, 27, 28, 29, 30].

In the previous results we note that the tested fungus *A. amstelodami* was able to give strains or mutants resistant to the ethanol and water extract of the of *Rhus coriaria* seeds at appropriate inhibitory concentrations studied, although the number of resistance spontaneous mutants is much less than the frequen-

cy of induced mutants and this is expected because the frequency of spontaneous mutations is very low [31, 32].

CONCLUSIONS

Rhus coriaria ethanol and water seeds extracts has inhibitory effect on *A. amstelodami*. The tested fungus *A. amstelodami* was able to give strains or mutants resistant to the ethanol and water extract of the of *Rhus coriaria* seeds at appropriate inhibitory concentrations studied, although the number of resistance spontaneous mutants is much less than the frequency of induced mutants. There is significant differences between the frequency of spontaneous mutants and the nitrous acid induced mutations.

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