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Antifungal Activity of Rosemary Oil Extract against *Aspergillus flavus, Candida albicans, Epidermophyton floccosum,Trichophyton Verrucosum* Fungi and Its Effect on the *AFL.*1 Gene Expression in the *Aspergillus flavus* by RT.PCR

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Investigating about the extract of Rosemary in various groups of fungi and this extract's minimum effective deterrence density on types of fungi and also the survey of this extract in expressing the *AFL.1* gene in *Aspergillus flavus* (with the RT.PCR method) is the main target of this research. Rosemary is a very important medicinal herb. Although its antimicrobial effect is fully considered, but its effect on toxin-causing and pathogenic funguses is not studied very much. Therefore, considering the limitation of antifungal drugs, chemical effects, and drug resistance of them, it seems the access of reaching an effective herbal medicine really matters. Since the Aflatoxin is concerned in various food, livestock, pharmaceutical, and medical industries, this research illustrates the mchanism of growth containment by this fungus. First of all we cultivate *Aspergillus flavus* and *Candida albicans* in sabouraud dextrose agar and *Trichophyton verrucosum* and *Epidermophyton floccosum* (Dermatophytes) on S.C.C agar (mycosel agar) perimeter and then we put Rosemary impregnated paper disks on the surface of perimeter to determine the anti-fungal effect with disk difussion method and creation of inhibition zone then with the help of 10 standard sterile tubes we dilute Rosemary extract in the perimeter of sabouraud dextrose broth to gain this extract's effective concentration and finally Rosemary's effect on expressing the *AFL.1* gene was examined and eventually statistical analysis was done by the new version of "SPSS" software. Achieved results indicate that the extract of Rosemary on various types of fungi has an inhibitory effect that its effect is depended on its effective concentration so that with the increase of this extract's density, the fungal colony will be weaker and inhibited and the results of RT.PCR confirm this inhibitory effect on expressing the *AFL.R 1* gene which produces Aflatoxin in molecular level. The extract of Rosemary can have a considerable inhibitory effect on expressing the *AFL.1* gene and production of *Aspergillus flavus.*

Keywords: Rosemary oil extract**,** *Aspergillus flavus, Candida albicansi Epidermophyton floccosum, Trichophyton verrucosum* ¡ *AFL.R* Gene, RT.PCR Method.

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Human fungal diseases are known as one of the greatest phenomenon in 21st century. Most of the systemic fungal illnesses was described in early 1900s. This was the first time that the

scientists found out that lots of the specific areas' endemic fungus had pathogenicity abilities. Due to using of corticosteroids and anti-bacterial medicines in clinical medicine, increased the frequency of fungal diseases. The prescription of anti-bacterial factors and the usage of medical equipment in some patients that had a critical condition in terms of the immune system, made these patients more allergic to the fungal illnesses. Fortunately, nowadays a large number of antifungal drugs has been produced in the world; but the principal issue of drug resistance is the treatments. Also it's unfortunately just the beginning of dealing with Aflatoxin and there are lots of things to study on' Due to this reason, discovering new treatments or some materials that have antifungal effects is always the consideration of researchers in different fields. Our goal of studying is to survey antifungal effects of Rosemary's extract and this extract's impacts on expressing lethal poison of Aflatoxin[1,3]

Aspergillus fungus are spread as conidia in the environment and are a part of water and soil microflora that make extensive pollutions; these kinds of fungus are rather resistant facing heat and have high compatibility power. "Aflatoxins" are natural fungal poisons that their origins are *Aspergillus flavus* and *Aspergillus parasiticus*. These poisons can be carcinogenic if they enter the body with foodstuffs[3,10]

There are different kinds of Aflatoxins; like: Aflatoxin B1 & B2, or Aflatoxin G1 & G2. The importance of studying the Aflatoxin poison is that human is endangered by using unclean food poisoned by the growth of *Aspergillus* fungi and since prevention from the growth of fungal in foodstuffs in not easy, therefore preventing from fungal diseases in humans and animals is very difficult[5,24]

To synthesize the Aflatoxin by fungi there are more than 23 enzymatic reactions involved in the direction of this synthesis. The production of this poison is accomplished during some redox reactions. In the direction of intoxine, 23 genes based on 75 Kb gene cluster are involved that most of these genes are set up by a dedicated route of connecting-to-DNA proteins produced by *aflR* gene. The "*afl.R*" protein connects to a palindromic sequence (located in promoter of lots of involved-in-biosynthesis genes) and makes the transcription and synthesis of Aflatoxin to increase in *Aspergillus flavus* and *Aspergillus parasiticus* fungus. In other words it can be said that "*afl.R*" has a role in groups of intervening-in-transcription genes and producing Aflatoxin in *Aspergillus* fungus[1,27]

In some of the countries of the world, some cases about the outbreak of human Aflatoxicosis in effect of contaminated food's consumption are reported; but in developed countries, because of precise control systems, sale contaminated food is prohibited and the illness is rarely observed[3,4,5]

In 1988, The International Agency for Research on Cancer put Aflatoxin B1 (which is one of the most common produced poisons by *Aspergillus*) in the list of human carcinogens. The damaged which are caused by aflatoxins' consumption in human, like obviousness of Aflatoxin B1's role in cancer occurrence and Aflatoxin M1's resistance, which are available in milk (even against sterilization and pasteurization), indicate the necessity of looking for reason regarding the control of fungal poisons (especially the Aflatoxins) more than ever[3,31]

"Rosmarinus officinalis" (Rosemary) is of a family of Lamiaceae that has at least the minimum of 1% (Volume/Weight) oil. It is orally used in treatment of anxiety, headache, blood pressure, bloat, anorexia, and topically as a positional painkiller in the treatment of muscular pains and rheumatic diseases. Rosemary oil, which is a stimulus oil and is warm and incisive in terms of smell and effect, has an outstanding stimulant effect on central nervous system. Rosemary is one of the principal components of Saprophyte-Dermatophyte antifungals. However this rosemary's effect is along with 2 other herbal oils and an extracted compound from bacteria[7, 9, 10]

In 2013, a survey was done by Lopez-Reyes GJ and his co-workers with the aim of studying alcoholic extract's effect and some other herbal types that the researchers in this project used Rosemary's alcoholic extract and its volumetric percentage on saprophyte fungus. They indicated that antifungal features of these extracts that Rosemary was one of its most primary components is greatly depended on density and the times of extract's consumption on fungus; and this survey also indicated that the 10% (volume)

extracted extract has the maximum of antifungal feature[5]

In 2012, a research was done with the aim of rosemary's effect on destruction of fungal and bacterial biofilms by Chifiriuc and her co-workers. In this study, she indicated that magnetic nanoparticles that have rosemary, have a higher effect in the prevention of fungal biofilms' growth; also they destroy the previous formed biofilms to a large amount[6]

In 2011, M.moghtader and his co-workers indicated in a survey that carnosic extract of Rosemary has a potential protective effects against Aflatoxin poison which is originated from *Aspergillus flavus*; but he hasn't investigated or questioned the reason of this matter in this study. It seems that the reason of this matter would be Rosemary's effect on expressing this poison's gene[7]

Our intention in studying and reviewing the antifungal properties in three fungal groups that's mean *Aspergillus flavus* (saprophyte) *Epidermophyton floccosum* and *Trichophyton verrucosum* (dermatophyt) and *Candida albicans* (yeast) in the first level and also identifying the minimum amount of effective concentration on these kind of fungus and finally on reviewing the effects of this extract on expressing Aflatoxin gene with the method of Real-time PCR.

MATERIALS AND METHODS

Generally, the procedure of the task is based on providing the four kinds of studied Iranian strains fungus, cultivating them in the environments which have and which don't have rosemary's extract, calculating the MIC for the and finally checking the stability of them (RT.PCR).

Preparation of cultivation environment and Rosemary's extract

Firstly, sabouraud dextrose agar and sabouraud dextrose broth cultivation environment was permissibly and standardly prepared; sabouraud dextrose agar and sabouraud dextrose broth was purchased from Germany's MERCK Inc. Also the extract of rosemary was prepared standardly and under the certification of ministry of health from Barij Essence Co. The sufficient amount for making a sabouraud dextrose agar environment 65 gr.lit and for making the broth kind of it, 30 gr.lit will be needed which after weighing by the digital detailed scale, we clear that in in the distilled water and over the flame; then we autoclave that for about 15 minutes and distribute it in plates and pipes in fully-sterile condition; considering the fact that the whole process was done beside the flame and under the hood[7,8,34]

The preparation of fungal strains

We provide various types of four studied Iranian and environmental fungus that's mean 1. *Aspergillus flavus* (saprophyte) 2. *Epidermophyton floccosum* and *Trichophyton verrucosum* (dermatophytes) and 4. *Candida albicans* (yeast) from Karaj's center of research and scientific studies which is certified by ministry of health and Higher Education and in fully-sterile condition and beside the flame we added sabouraud dextrose agar and sabouraud dextrose broth to the environment for the growth and then for the growth and making a colony, and incubated at 25- 30°C. *Candida albicans* yeast colony and *Aspergillus flavus* mold appear only after some days and the colonies of *Trichophyton verrucosum* and *Epidermophyton floccosum* dermatofity appear after some weeks. For the microscopic study, the procedure was operated with the split or teased mount method[7,8,34]

Cultivation of fungus near the Rosemary xtract

We should cultivate each one of the intended fungus both by the side of Rosemary's extract and also without the extract. The whole process should be done by the disk-fusion in a fully-sterile environment. It means that with the help of Rosemary's extract and applying it in a paper disk (and with the temperature of 30°C), we cultivate that on the cultivation environment in bulk to see its inhibitory effect on blight halos on fungus and around the disks.(8,14)

MIC method

The second phase of work is the MIC for each of these four types of funji.

At this stage we calculate the minimum inhibitory concentration of Rosemary extract on fungal growth. In other words, we want to calculate how much can dilute the Rosemary extract, while still not lost its antifungal effect. To do this we can use 10 standard sterile tube test after coding tubes from 1 to 10 in each tube pour 2cc of liquid food (sabouraud dextrose broth), and 1cc is a fungus

suspensine in distilled water(Mackfarland standard) to tube one , we add 1cc of Rosemary extract and passage 1cc of liquid from tube 1 to 10 and so we dilute the Rosemary extract and for equaliry of each tube volumes , throw away 1cc of last tube . The tubes incubated at 25-30 ° C . fungi growth in the vicinity of the extract will dull and lack of growth will remain transparent medium . Transparency or opacity of the mediums indicate , the growth or lack of growth of fungi surely the primary tubes from 1 onwards with high concentration of extracts , will be transparent that mean non growth and tube 10 to one due to low concentration of Rosemary will be cloudy and with associated of fungal growth . now compare tubes together , the density of the transparent tubes the minimum effective concentration and growth retardation former MIC for a fungicide that Rosemary extract consider . so if we more diluted extract , miss another anti fungal effect. (10,14) With the surveyed research in 2006 in Argentina, methanol extract of rosemary was studied and its effective ingredients was announced carnosic acid (30%), carnosol (16%) and Rosmarinic acid (5%). In this survey the effective MIC of Rosemary was calculated for yeasts and gram-negative and gram-positive bacteria. They announced 2 to 60 mgr. / milt. for gram-negative and 2 to 15 mgr. / milt. for gram-positive bacteria and 4 mgr. / milt.for yeasts[10]

Doing RT.PCR on Aspergillus flavus

Finally the 3rd step is to so RT.PCR on *Aspergillus flavus* fungus.

The first step in order to examine of quantitative amount is to express the purification of an RNA protein. In this survey, for purification of the RNA, the materials were used from Bio Basic company's Rapid Fungal RNA Extraction Kit (cat no: FT71416). The followings explain the process:

Table 1. Primer in *afl*R Gene expression(like primer for beta-actin

The piece	The direction of the	The sequence of
aflR	F	5' ATGGTAGCAGTAGCGTCTCC 3'
	R	5' TTCCGTGTTCCATTGACTGC 3'
B-actin	F	5' CAAGAGATGGCCACGGCTGCT 3'
	R	5' TCCTTCTGCATCCTGTCGGCA 3'

Table 2. Temperature conditions to evaluate the expression (Real-time PCR)

Cycle	Time	T°C	Step
35	30 sec	94	Denature
	30 sec	60	Annealing
	30 sec	72	Extension
1	10 min	72	Final extension
	-	4	

1. We take 100 to 500 mgs of fungal samples and frozened by liquid nitrogen quickly. Then put frozen samples in a porcelain morta and crush them and transferring the resulted powder.
2. Add 1 ml lysis buffer and close the door of micro-tube the mixing them by inversion.
3. In order to ensure complete lysis of yeast cells incubated the mixture for 5 minutes at room temperature.

Fig. 1. Antifungal activity of Rosemary oil extract against of fangi

4. Then adding 200 microliter of chloroform to the tube and mixing by inversion.
5. Then centrifuge the micro-tube at 4C and 12000g power for 5 minutes and transferring the supernatant to a new RNase & DNase free micro-table.
6. Add 1.3 volume of cold ethanol to the solution and after vortexing for 5 minutes at 2C we incubate the solution.
7. The centrifuge the micro-tube at 4C and 12000g power for 5 minutes and throw away the supernatant and keep the sediment.
8. Add 1 ml cold 70% ethanol to precipitate and mixing by conversion 10 times. Then again centrifuge the micro-tube at 4C and 12000g power for 1 minute and throw away the supernatant.
9. Repeating step 8 on the sediment once again.
10. Dry the sediment at room temperature for 2 to 5 minutes.
11. Add 30 to 50 micro-liters of RNase & DNase free water to the sediment[1,2,27]

Checking the quality and quantity of extracted mRNA

After extracting RNA, the quality and quantity with the spectrophotometric method was evaluated by Bio photometer (Eppendorf) and electrophoresis gel.

Spectrophotometric method: this method, a quantitative method which can achieve constriction and purity of samples using by optical absorption at a wavelength of 200 nm. Also by using spectrometric method we can determine the amount of impurities due to the presence of protein or DNA in mRNA solution. To do this at same optical density at 280 nm was measure and the A260/280 ratio was calculated by the device. I pure RNA sample, A260/280 ratio equals 2+-0.15 and in pure DNA sample, ratio equals 1.8+- 0.15. Less calculation ratio, more polluting with protein. Thus, two microliters of RNA were dissolved in 98 ml distilled water and was absorbed by the device using the vortex wane mixed with distilled water was zero, then cuvette is filled with a solution containing RNA and RNA related to the optical density in one device, was measured and then the amount of DNA after 50 precision coefficient was corrected, and was calculated according to the following equation:

RNA concentration = accuracy x 40 x OD260 nm

Electrophoresis gel method**:** when electrophoresis RNA sample, all electrophoresis equipment was incubated for 15 minutes in NaOH (0.5%) solution and after a complete washing with water, was fixed with a 3% solution of hydrogen peroxide and incubated for 15 minutes.

Finally, all equipment with water treated with diethyl carbonate pursuant washed and dried in the oven[2,27], Quantification of the expression through quantitation real-time RT-PCR:

The general principles of conventional PCR method is similar with the exception that in this technique, the template is CONA and quantity along with the progress of reaction is detectable by PCR. This is due to the presence of compounds in the PCR reaction which show the variable behavior of fluorescent light emission in both connected and separated from the DNA. This essay is a single stage and two-stage two general form can be doer which the choice depends on general conditions survey in this study, we used a two-step[2,27]

(Two Step Quantitative Real-Time PCR) 2 step procedure

In this method, from the RNA mdecules, cDNA made and next the cDNA is used as a role mode for PCR. The advantages of this method include the preparation and storage of cDNA, is more appropriate than creating RNA samples[2,27]

cDNA preparation

To convert RNA to cDNA to reverse transcription enzyme. DNTPs, we need special buffer and primer to be able to hybrid with RNA and by the enzyme DNA dependent RNA polymerase sequence to be made to create the file cDNA. The primer for first strand synthesis used cDNA can be specifically designed to be a hybrid gene or RNA that is publican everyone in connected. In this study, using a kit (Revert aid™ first Strand cDNA Synthesis kit; Ferments catNO: K1622)

cDNA manufacturing process was carried out as follows: RNA unit in micro tubes that remote the DNA contamination left behind, 0.5 ug Primer shed and was incubated for 5 minutes at 70C and immediately placed on ice and 4 ul of 5x buffer, 2 ul of 10 milligram dNTPs mix and 20 units ribonucleic inhibitor added and then incubated for 5 min at 27C. Adding 200 units of Revert aid ™ M Reverse Transcriptase enzyme and then incubation for one

hour at 42C in order to build cDNA and finally incubation for 10 min at 70C to stop the reaction.

Real-time PCR

In this project SYBR iscript ™One-stepRT.PCR made by (cat No. 170-88) BIORAD Company is used. Summary: the name called RNA obtained from fungi defectively in this kit were used. The content of this site, including reverse transcriptase, an enzyme and a PCR Master Uix. In a nonspecific band first in Thermocycler device. Temperature suitable conditions for primer concentration was obtained and then optimized according to conditions, PCR reaction in Real-time device were performed[2,41]

PCR products obtained from the first stage (optimization)

The temperature and concentration of the gel in several stages to test parts and under the temperature conditions for amplification of target mRNA and affair of beta-action gene, was appropriate.(table1)

After several round table temperature gradient (From 54 to 62) and the concentration gradient was obtained primer. Although PCR Patter above the two tubes extend from the initial model obtained total RNA in response to Tube using the BIORAD kit will definitely not be involved. But estimates close to that loses. Then after several stages in the PCR reaction in Real-Time device, high concentrations were obtained when similar pattern to ensure the obscene of non-specific band Melting care of the process as well as 1% gel electrophoresis was used in the machine.

Quantitative analysis of expansion levels between the pipes meet affair fungal growth A*spergillus* MIC test

With due attention to quiddity of the research that is a proportional study between fungus growth possessing vessels , crude data analysis from RT-PCR quantitative was used by pfaffl . In this method a particular gene's illustration alterations can be measured in

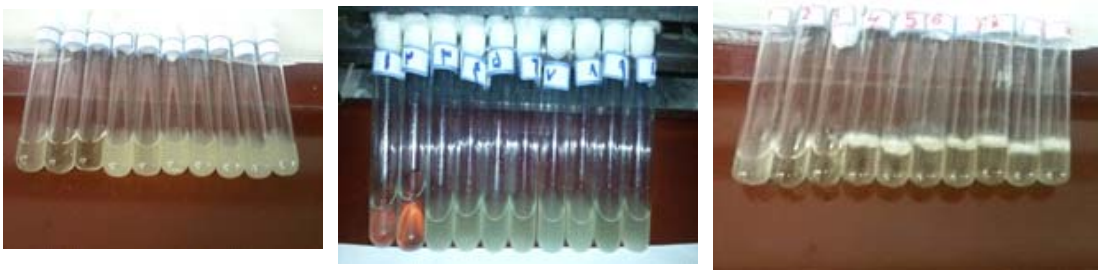


Fig.2. The results of calculating the MIC for the fangi

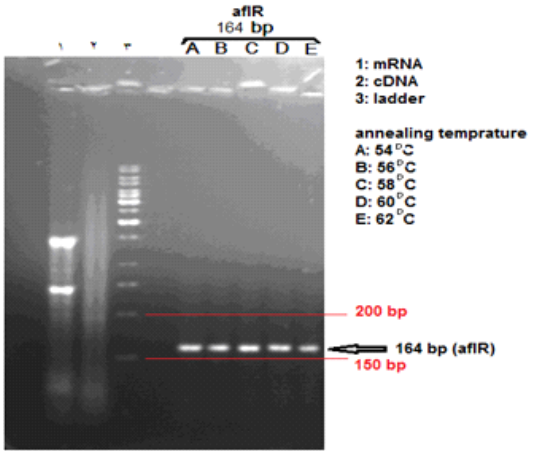


Fig. 3. Temperature Electrigram for propagating *aflR* Gene

comparison with a reference gene using the formula below:

$$\text{Expression Ratio} = E_{\text{target}}^{(\text{Ct Control} - \text{Ct Target})} / E_{\text{Reference}}^{(\text{Ct Control} - \text{Ct Target})}$$

in which E is the PCR's utility reference and Ct is number of the cycle that interjects the fluorescence amount alteration graph of each sill model line . (2,10,14)

Mindfully to the above facts, for smidgen analysis of *afIR* illustration amount between *Aspergillus flavus* growth unit vessels, in MIC test we have to just ascertain the utility basis of PCR for *afIR* gene and even beta-actin gene as a reference via exclusive standard graph for each

gene and then measure the allocated Ct average of both *afIR* and Beta-actin in *Aspergillus flavus* growth possessing vessels in MIC test and use it in pfaffl equation. In this way we can estimate alteration amount in patient group in comparison to control group.

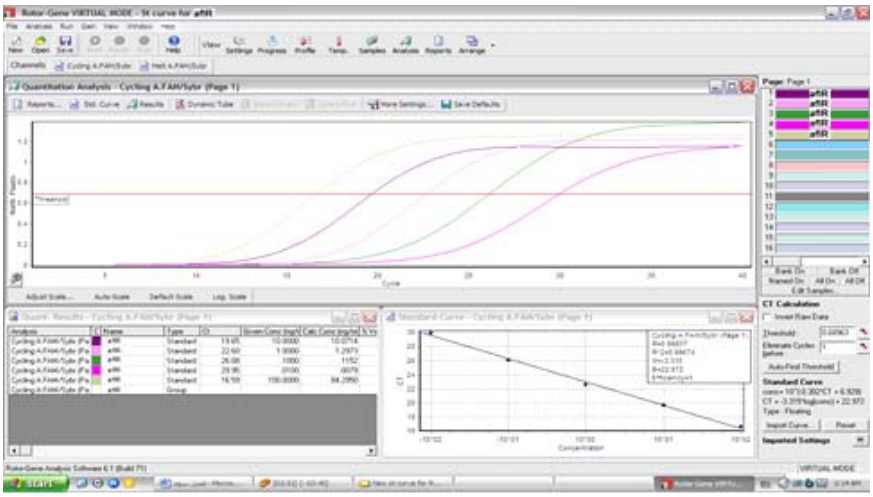
RESULTS

The outcomes of this investigation contain important materials such as:

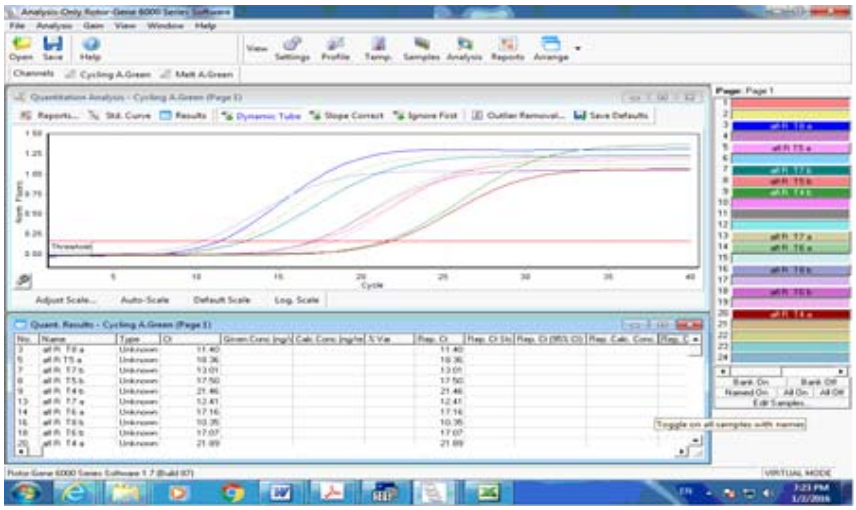
The herbaceous extract of Rosemary has such an effective and desired deterrence on different kinds of fungus such as mycellial fungus, yeast fungus and dermatophyte fungus that the diagonal of bright anti-growth haloes around the Rosemary soaked disks have such desired size and quality and are similar to bright haloes of anti biotical anti growth in Antibiogram method that we can ascertain the desired deterrence effect of Rosemary extract. The average diagonal of bright anti growth haloes are about 18-20 mm.

Thus the obtained images from vicinal blur vessels and bright vessels MCI calculation are perfectly shown for each fungus and can exactly calculate the minimum density of desired deterrence rosemary extract.

Therefore the minimum density of



Graphs1. Efficiency determination results for *afIR* propagation using normal graphs



Graphs 2. The CT's average amount appointment for two simultaneous MIC tests in growth possessing vessels for 4 up to 8 for *afIR* gene

deterrence rosemary extract or MCI for yeast fungus is approximately 4 to 6 mg per liter, mycelial fungus 3 to 5 mg per liter and dermatophyte fungus is 4 to 6 mg per liter.

The Annealing temperature optimization result for propagating *afIR*

In this level RNA based cDNA and pair designed primers are used for measuring the amount of *afIR* illustration.

In thermal range of 54 to 62 that was applied with the scale of 2, PCR's reaction for *afIR* propagation was done with a good and almost equal quality and quantity. Therefore at the end of the studies that was the *afIR* illustration amount that was done by Real time PCR, 60°C temperature was used as Annealing temperature. It should be remained that in upon PCR reactions, cDNA was used as temperature and a pair of *afIR* primer. The PCR's result *(afIR* gene) was formed exclusively and without extra band in the mentioned area. The recent fact creates the desired situation in Real Time PCR.

The smidgen analysis results for *afIR* illustration amount between *Aspergillus flavus* growth unit vessels in MIC test

For smidgen analysis of a gene's illustration amount by RT-PCR technique, calculating two proficient characters (E^2) and a cycle in which given fluorescence amount passes the sill line(CT) for mentioned gene's in growth possessing vessels and for reference gene is necessary. In the next level with the usage of this parameters and pfaffl equation, the *afIR* gene's illustration alteration amount that is filled normally is gained. The results of studies for *afIR* illustration amount's smidgen analysis between *Aspergillus flavus* growth possessing vessels in MIC test are shown below.

Efficiency determination results for *afIR* propagation using normal graphs

In diagram no.1 cDNA density slope as Template is from 0.001 to 100 units. Important obtained parameters from this graph are E that is usage alliance coefficient between standard tenderness and M which is the standard line slope. The parameters which are obtained from this section will be used for studding for illustration amount of *afIR* between fungus growth possessing vessels. The equation for calculating E by the software of device is: E=10^ (-1/m). when E=1 it means that the PCR utility is 100% but in pfaffl equation when the amount of PCR's product is each cycle is twice as much as the previous cycle PCR's utility is 100%. Therefore for changing the E which is obtained from Rotor Gene 3000 to the E which can be used in pfaffl equation we have to just total 1 with the amount given for the E by the device. For example when the amount of the E given by the Rotor Gene 3000 is 1,it should be used (1+1)=2 in pfaffl equation.

The CT's average amount appointment for two simultaneous MIC tests in growth possessing vessels

for 4 up to 8 for *afIR* gene

The information obtained from these graphs in Quant resalts window verifies the CT's amount for *afIR* in 4 to 8 vessels in which fungus have grew and done delicately. The average amount of each vessel's CT is used in calculation.

Primarily and extremity nucleotides of *afIR* gene

F: 435 ATGGTAGCAGTAGCGTCTCC 454

R : 599 TTCCGTGTTCCATTGACTGC 580

The pfaffl equation for studding the alteration's amount of a gene is shown below:

$$\text{Expression Ratio}_{aflR} = E_{aflR}^{(Ct\ tube\ 8 - Ct\ tube7)} / E_{B\text{-}actin}^{(Ct\ tube8\ -\ Ct\ tube7)}$$

By replacing the 3rd table's information in this equation illustration alteration amount for afIR genes in diverse vessels of MIC test is obtained.

Due to the almost equal average of CT for ß-Actin gene in no. 7 and 8 vessels and also CT's outside difference for afIR gene in no. 7 and 8 vessels we could understand that the marked effect of rosemary extract repressing aflatoxin's production and repressing the illustration of afIR gene was initiated from the vessel no. 7 and in no. 4 vessel in addition to repressing the afIR's illustration, ß-Actin gene's CT increases too which is the critical density of rosemary extract and prevents the fungus growth in no. 3 vessel in which the density of extract is increased. Therefore afIR's gene repression is between the densities of no. 4 to 7 vessels and the repressing of no. 8 vessel is shown below:

Illustration alteration amount of afIR gene from vessel 8 to 7 is: $\text{Expression Ratio}_{aflR} = 3.58 / 1.006 = 3.55$

Illustration alteration amount of afIR gene from vessel 8 to 6 is: $\text{Expression Ratio}_{aflR} = 75.6/$

1.24=60.51

Illustration alteration amount of afIR gene from vessel 8 to 5 is: Expression Ratio $_{aflR}$ =133.43/ 2.24=59.5

Illustration alteration amount of afIR gene from vessel 8 to 4 is: Expression Ratio $_{aflR}$ =1782.88/ 270.12=6.6

The obtained results show that the repressing power of rosemary extract in *aflR* gene illustration is Aflatoxin in *Aspergillus flavus* fungus and molecule level it is understand that the extract's repressing power is related to it's density.

Proposals

Due to the obtained results of this study that show the repressing power of the Rosemary extract on different groups of fungus and rarity and price of anti fungus medicals that have bad chemical effects we can trust Rosemary extract as an anti fungus medical and we can change the situation from laboratory's to body situation. what we can extract from this study is that medical factories specially anti fungus medical drugs can use Rosemary extract for fungus sickness by examining them first on animals and then on humans.

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