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Efficiency of alcoholic cinnamon extract on ERG3and ERG11 genes in *Candida albicans* isolated from oral children candediasis

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***Abstract*---**In the current study, it was highlighted to know the inhibitory effect of the alcoholic extract of cinnamon bark Against Candida yeast As well as evaluating the effectiveness of this extract on the viability of cell membrane formation, By knowing the gene expression level of C.albiacns gene for yeast Using the polymerase chain reaction in time 50 samples of Candida were collected and diagnosed by diagnostic tests C.ablicans method on yeast (cinnamon) from the alcoholic extract of cinnamon bark qRT- PCR and through the use of Microdilution Method To find out the inhibitory effect of the extract on the membrane (measurement of the level of gene expression of the gene) 1 Able to form C.albiacns (51.6%) from the bio-yeast, and the results showed that The results also showed that the concentration of 150 mg / ml showed the highest inhibition area with a value of 22 . mm while the concentration of 12.5 mg/ml showed the lowest inhibition logic with a value of 9 mm, and so was done For the extract against yeast if its value was 12.5 mg / (MIC) Calculation of the minimum inhibitory concentration. It is shown that the concentration of 25 mg/ml is 1 and when calculating the gene expression level. (mg/ml respectively (for concentrations) 12.5,25,50,100,200,400 While the concentration of 12.5 mg/ml did not show a clear effect on the level of gene expression at We conclude from the current study an efficiency, P < its resistance with control at a probability level of 0.05 Alcoholic extract of pomegranate peels high in its effect on cellular biofilm.

Keywords*---Candida albicans*, candidiasis, cinnamon extract, genes.

Introduction

In C. albicans, ergosterol is synthesized from lanosterol, albeit through various enzyme reactions and requires oxygen at multiple steps. The biosynthesis of ergosterol consists of 20 enzymes encoded by the specific genes. The lanosterol 14α-demethylase enzyme, encoded by ERG11, is suggested to be major ratelimiting enzyme in ergosterol biosynthetic pathway [Lv QZ et al 2016]. The ever-increasing array of antifungal drug resistance of *C. albicans* continue to pose a serious life-threatening infection. Thus, it seems that there is important to find powerful methodical approaches that could be used to treat candida infections. The combination of two or more therapeutic antifungal drugs is a cornerstone for treatment of life-threatening infections. The use of combination strategy and the synergistic effect obtained by the combination of antifungal drugs has likely greatly improved efficacy of existing drugs such as azoles and reducing their harmful side effects on the host cell [Pianalto .,et al,2016].

The exact number of cases of candidiasis in the mouth, throat, and esophagus in the Countries is difficult to determine. This is because there is no national surveillance for these infections. The risk of these infections varies based on the presence of certain underlying medical conditions. For example, candidiasis in the mouth, throat, or esophagus is uncommon in healthy adults. However, they are some of the most common infections in people living with HIV/AIDS. In one study, about One third of patients with advanced HIV infection had candidiasis in the mouth and throat (Buchacz ,.et al, 2016).

The use of medicinal plants as a treatment option is an ancient practice and continues in the modern world. Ancient Asians including Sri Lankans, Indians, and Japanese people especially used these phytochemicals as their therapeutic agents. The use of medicinal plants as therapeutic alternatives has helped many populationsthat do not have any access to novel treatments anddrugs of high cost and low availability. So, the use of plants for the treatment of diseases/infections has been empirically employed worldwide. In the past few years, with scientific advances, more research was conducted to introduce new compounds with medicinal properties, allowing scientists to find new, effective, alternative medicinal compounds with low side effects (Wijesinghe ,et at 2020). Essential oils extracted from cinnamon leaves and bark were used as antimicrobial agents for centuries. Though this plant has been shown to be of great economic and pharmaceutical-medicinal interest (Ranasinghe Pet at 2013), proper evidencebased studies are not yet carried out to evaluate the efficacy of cinnamon oils as an antimicrobial therapeutic agent. Cinnamon oil from C. zeylanicum bark commonly presents antimicrobial and antifungal properties that have drawn great attention from many researchers (Kim et al., 2004). Increased fungal resistance to classical drugs, drug toxicity, and the costs involved justify the search for new approaches to developing antifungal drugs. Among those new approaches, essential oils are one of the most promising groups of natural compounds for the prevention and treatment of fungal infection (Silva et al., 2011). Essential oils derived from aromatic plants are well known in traditional medicine as antimicrobial agents and are characterized as food and feed preservatives, as inhibitors of mycotoxin production, and as antimycotic agents (Guimarães et al.,

2010). In the current study, the gene expression levels of ERG11 and ERG3gene were assessed by RTPCR in C. albicans treated with cinnamon alcoholic extract.

Aim and objectives

Study focused on the effects of cinnamon extract on identified that the ERG3 and ERG11 genes were also belonged to the "missing link" type of genes for the first time since their deletions were incapable of causing oral mucosal infection. To achieve this overarching aim the specific objectives were to:
* We are planning to investigate the gene expression for ERG3,ERG11 gene

Materials and Methods

A: Materials

NO	Material	Cat. No.	Company
1	GoTaq® 1-Step RT-qPCR System	A6020	Promega/USA
2	Primers	---	Macrogen/Korea
3	Easy-spin™ (DNA free) total RNA extraction Kit	17221	Intron/Korea

B: Equipments

NO	Material	Company	Country
1	Microfuge IB Centrifuge	Beckman Coulter	Germany
2	Dry microtubes incubator	Ae	UK
3	Mx3005P Stratagene Real-Timesystem	Agilent	USA
4	Kern PFB balance	Kern & Sohn	Germany
5	Pipettes	DARWELL	China

Total RNA extraction using Easy-spin™ (DNA free) total RNA extraction Kit

1. Preparation of 50-100 mg of fresh tissue.
2. Add 1ml of Lysis Buffer (easy-BLUETM reagent) and homogenize tissue sample using a homogenizer or equivalent.
3. Vigorously vortex in room temperature for 10sec.
4. Add 200µl of Chloroform and apply vortex.
5. After centrifuging the solution at 13,000 rpm (4℃) for 10 min, transfer 400µl of the upper fluid to an empty 1.5ml tube.
6. Add 400µl of Binding Buffer and mix it well by pipetting or gently inverting the 2-3 times. Do not centrifuge and leave it for 1min at room temperature.
7. Load the upper solution to the column, but do not load the whole upper solution because the maximum volume of the column reservoirs is 800µl. After loading the optimum of the upper solution to the column, and centrifuge at 13,000rpm for 30sec, discard the flow- through after centrifuging and place the spin column back in the same 2ml collection tube. And then repeat this step.
8. Add 700µl of Washing Buffer A to the column. Close the tubes gently, and centrifuge for 30 sec. at 13,000rpm to wash the column. Discard the flow-through and place the spin column back in the same 2ml collection tube.

9. Wash by adding 700µl of Washing Buffer B to the column and centrifuge for 30 sec. at 13,000rpm. Discard the filtrates and place the spin column back in the same 2ml collection tube.
10. Centrifuge for 1-2 min at 13,000rpm to dry the column membrane
11. Place the column in a clean 1.5ml microcentrifuge tube (not provided), and add 50µl of Elution Buffer directly onto the membrane. Incubate at RT for 1min, and centrifuge for 1min at 13,000rpm to elute.Preparation of primers According to instruction of the primer synthesis company, the primers (originally lyophilized), were dissolved in the free ddH2O to obtain a final concentration of 100 pM/µl which served as a stock solution that stored at -20 °C. A concentration of 10 pM/µl was prepared from the stock primers to be used as a work primer.

Primers used in this study
Primers of gene expression experiment

Organism	Target gene	Primer name	5'-3'	Reference
Candida albicans	ERG3	F	5' TCC AGT TGA TGG GTT CTT CC 3'	(Khodavandi A,et al 2011)
		R	5' GGA CAG TGT GAC AAG CGG TA 3'	
Candida albicans	ERG11	F	5' TGG AGA CGT GAT GCT G 3′	(Lee MK, *et al* .2004)
		R	5′ AGT ATG TTG ACC ACC CAT AA3′	
Candida albicans	ACT	F	5' GAG TTG CTC CAG AAG AAC ATC CAG 3'	(Khodavandi A, *et al* 2011)
		R	5' TGA GTA ACA CCA TCA CCA GAA TCC 3'	

Protocol of GoTaq® 1-Step RT-qPCR System for Real-Time qPCR (Gene expression assay):

12. Program a real-time instrument for standard or fast mode one-step RT-qPCR (Table 2).
13. Thaw the components of the GoTaq® 1-Step RT-qPCR System, the RNA templates and the primer pair on ice, at room temperature or at 37°C. Immediately mix each thawed component thoroughly. If using a vortex mixer, mix at low speed to minimize aeration. Keep thawed reagents on ice.
14. Prepare the RNAsamples (mRNA[500fg–100ng])inwateroranother qPCR compatible diluent.
15. Combine reaction components (Table 1) in a non-stick, sterile tube on ice. Mix gently after each addition. Carefully pipet reaction volumes to plate on ice.
16. Transfer plate from ice into the pre-programmed instrument. Start the run immediately.
17. When the run is complete, collect the data and analyse the results.

Table 1: Preparation of Real-Time PCR solution

Components	Concentration	Volume (20µl)
GoTaq™ qPCR master mix, 2X	1X	10 µl
Forward primer	10 µM/µl	2µl
Reverse primer	10 µM/µl	2 µl
GoScript™ RT mix for 1-step RT-qPCR	1X	0.4 µl
ddH_2O	-	3.6 µl
RNA template	250 ng	2µl

Table 2: Real-Time PCR conditions
(According to the instruction ofGoTaq® 1-Step RT-qPCR System)

Stage	Ta (°C)	Time	Cycles
Reverse transcription	42	15 min	1
RT inactivation/Hot-start activation	95	10min	1X
Denaturation	95	10 sec.	40X
Annealing/data collection	60	30 sec.	
Extension	72	30 sec.	
Dissociation	72	2 min	1X

Sample Preparation

Firstly, cinnamon bark was washed and cut into small pieces. Cinnamon bark was dried using a drying cabinet at 40–42 C for 3–4 days. The dried plant material was ground into powder.

Preparation of Ethanol Cinnamon bark Extract

As much as 300 g of cinnamon bark crude (the powder of cinnamon bark) was weighed and placed into a flask. Ethanol solvent with a concentration of 96% was added to the flask until the plant materials were submerged by the solvent. The

extraction process took 3 h.Afterthat used filter paper wattman 1 to filtration the solution. The ethanol extract was combined and evaporated using a oven at 45 C. then preparation five concentration (12.5,25,50,100.200,400)mg/ml all concentration diluted by DEMSO.

Oral swab samples collection

Oral swab samples (50) were collected from patients and instructed on hand to collect sterile container. The samples were then transported to the laboratory with ice packs in sterile container. After that the samples were centrifuged at 2500g for 10 min. Four plates of Sabouraud's dextrose agar with the addition of 0.05 g/L chloramphenicol were inoculated: two plates were incubated at 25C◦ for 48 h and the other two at 35C◦ for 48 h. All the patients, age, gender, history of disease, and antibiotic uptake were recorded in a questioner sheet (Ellis, 1994).

Isolation and Identification
Culture

One-half ml of the collected specimens are inoculated onto sabouraud's dextroseagar. The inoculates are evenly spread with sterilized L- shape glass rod and incubated as duplicate culture and incubated separately at 25 C◦ and 37 C◦ to reveal dimorphism. While the swabs are streak onto sabouraud's dextroseagar and incubation. The cultures are examined microscopically, they were not considered negative for growth until after 4 weeks of incubation. Yeast colonies diagnosed depending on cultural characteristics on sabouraud's dextroseagar (Arther, 2004).

Figure (2)The swabs are streak onto sabouraud's dextrose agar and incubation

Results and Discussions
Expression profiles

The general expression levels for the Candida alb. Cell membrane building gene were determined by expression measurements and calculating the mean value for those. These were then compared between and within treated groups.

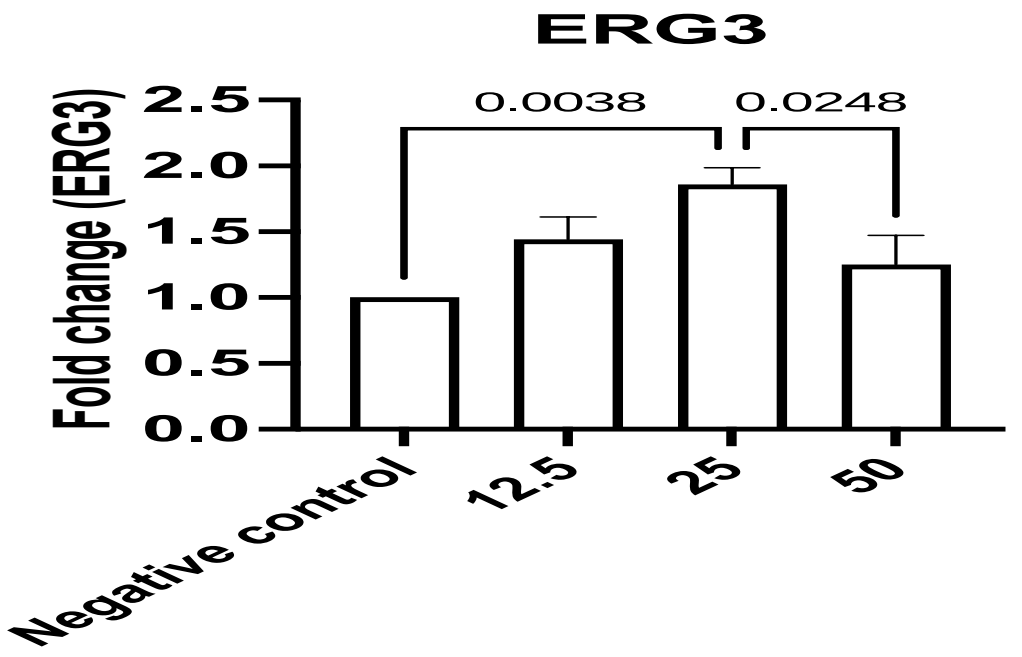

Figure (3) In this figure explain Compared the NEGATIVE CONTRROL by used DEMSO only with all concentration (12.5,25,50)mg/ml in order to investigate whether the affected the overall gene expression of ERG3 levels in the candida growth in these concentration showed the no effect in all concentration on ERG3 gene whene compared with negative control. Statistics using GRAPH PAD PRISM 6 software. Gene ERG3

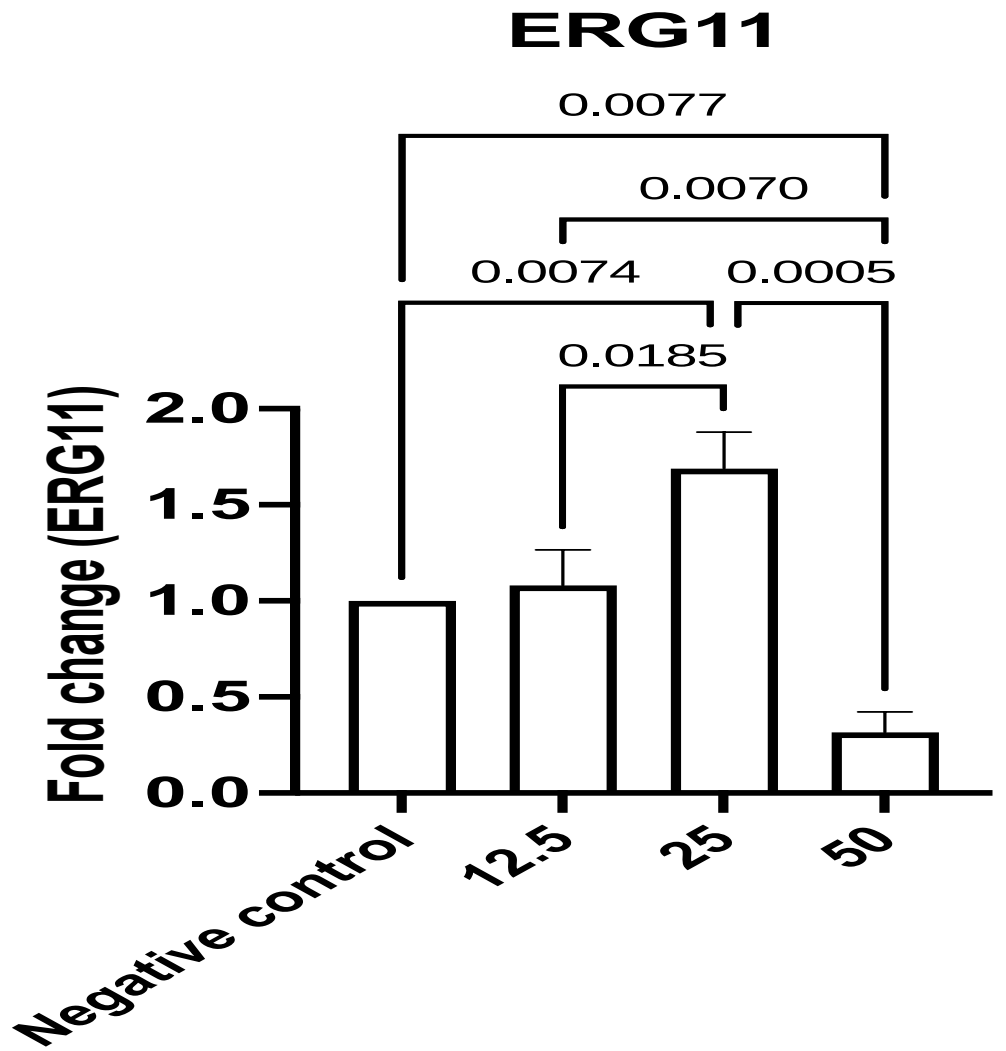

Figure (4) In this figure demonstrated the group treated with 50 mg /ml of plant alcoholic extract was best effect on decrease the EGR11 gene expression when compared with the negative control and also when compared with the other treated groups (12.5- and 25) mg /ml compared the NEGATIVE CONTRROL by used DEMSO only with all concentration (1/100,1/200,1/400)mg/ml in order to investigate whether the affected the overall gene expression of ERG3 levels in the candida growth . Figure (1 and 2) showed seemed lower expression ERG3 gene in all concentrations of extract significantly difference between by given DEMSO and different concentration of extract respectively (**Figure 5**).

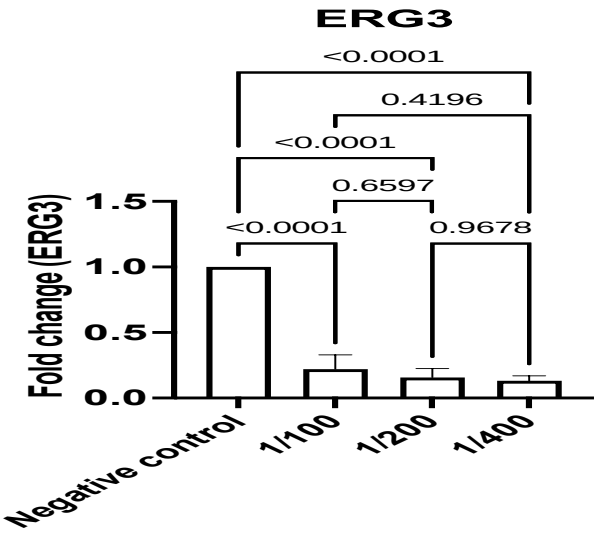

Figure (5) showed extremely Higher of ERG3 gene expression in (-) control (DMSO.) When compared with CONCENTRATIONS OF EXCTRACT. Statistics using GRAPH PAD PRISM 6 software. Gene ERG3

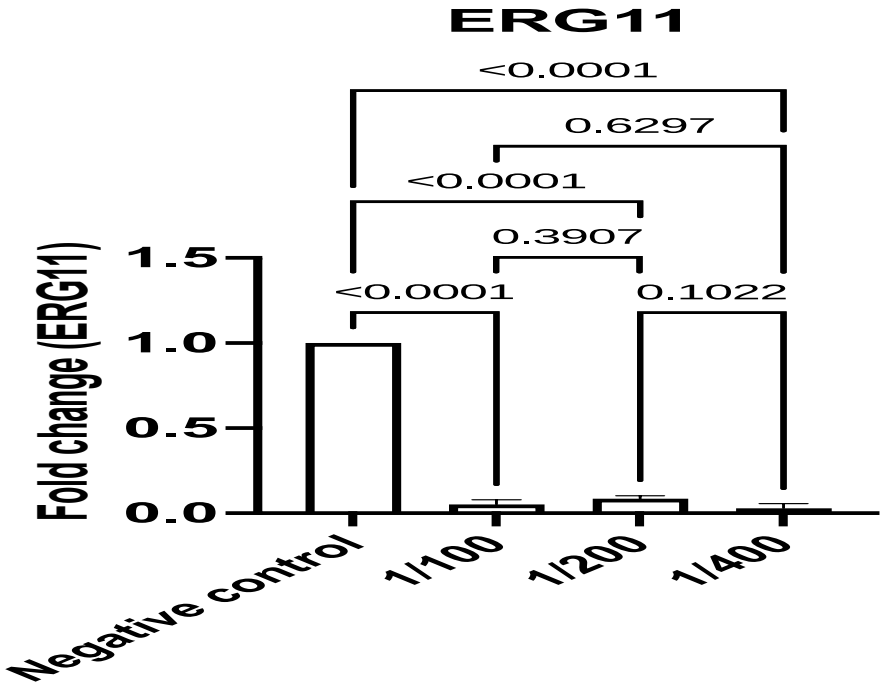

Fig.6: showed highly different significant compared between negative control (DMSO) and all concentration of extract and non significant within concentration of extract for ERG11 Gene expression

One possible hypothesis to explain the effect has been proposed ,Antibiotic of yeast albicans.C by alcoholic extract of plant of cinnamon in the pH dependence of the biofilm Where the biofilm is formed in a neutral pH, but it is possible to change in PH value When adding the plant extract, the PH may be too high or too low (basic). or acidic) may be accompanied by changes in microbial cellular metabolism and surface properties Thus, there will be interference in the process of adhesion to the surfaces of the host (Bott, 1995). For example, a solution of hypochlorite produces an alkaline pH (11 < PH) and thus increases the electrical repulsion between yeast cells and the surface of the denture material And then it will lead to the removal and dissolution of the cell membranes. (Fukuzaki, 2006). Studies have indicated that there are many genes that are closely related to membrane formation C albicans.C from these genes (HWP (Protein Wall Hyphal) and Phospholipase B(PLB) Gene and Secreted Aspartyl Protease (ASP) The Agglutinin - Like Sequence gene, the Lipease (Lip) gene and the gene (Nailis, et. al., 2010). The reason for choosing the ERG3,ERG11 gene for the current study is its relationship to encoding proteins (Hoyer, 2001), and because the gene expression of the ERG3,ERG11 genes is highly regulated in all parts of the body Patterns or modalities of cell membrane development (Nailis, et. al., 2010) ,where (O'Connor,etal., 2004)) that the gene expression level of the erg3.erg11 gene is high up regulation during biofilm formation. The results of the current study in the high gene expression of the erg3 gene in the control sample O'Connor,etal.,(2004) results with matching of fold change).

This study supports the view that carvacrol exerts its antifungal activity on the cell membrane of C. albicans by inhibition of ergosterol biosynthesis. Whether these events reflect the potential of Cinnamomum alcoholic xtract for inhibition of ergosterol biosynthesis in C. albicans which differentially expresses a specific gene, requires further dissection. In addition, ERG3 and ERG11 genes could be probable molecular targets for Cinnamomum alcoholic xtract in C. albicans. Greater knowledge of molecular mechanisms of antifungal effects may serve as a guide for future in the development of new therapeutic strategies.

On the other hand, the phenols (flavonoids) available in cinnamon bark have an effect on enzyme inhibition As oxidizing compounds, it probably reacts with the sulfate group Or through a lot of non-specialized interactions with (SH) proteins It greatly affects the nature of change, (Masson and Wasserman, 1987) Proteins and membrane damage through their binding to the active sites of cellular enzymes by The hydroxyl groups in it that have the ability to form hydrogen bonds with those sites are more And then it may inhibit one or more of the substrate (Plazar, etal., 1986). The necessary metabolic reactions controlled by these enzymes affect the cell Effect of making microbial flavonoids, possibly flavonoids.

Conclusion

1. We conclude the yeast isolated from the mouth (C.albicans)and Around the teeth is the most common type of Candida.
2. The alcoholic extract of cinnamon bark is highly effective and has a high inhibitory effect against yeast

3. Through its inhibitory effect on the gene encoding proteins Its resistance to traditional antifungals.
4. Which showed a decrease in the level of gene expression in both ERG3, ERG11 genes.

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