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Original article

Antimutagenic activity of *rosmarinus officinalis* L. by Ames test

A. Tajehmiri[a], M. Ghasemi[b], F.P. Sabet[b], M.M. Chakoosari[b], N. Abdolahzadegan[b,*]
[a]*Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, IRAN.*
[b]*Department of Microbiology, College of Basic Science, Lahijan Branch, Islamic Azad University, Lahijan, IRAN.*

*Corresponding author; Department of Microbiology, College of Basic Science, Lahijan Branch, Islamic Azad University, Lahijan, IRAN.

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ABSTRACT

Plants are an important source of substances which are claimed to induce anti-inflammatory and antioxidant effects. Rosemary (*Rosmarinus officinalis* L.) is a medicinal plant that has many uses in traditional medicine. In this study, of *R. officinalis* leaves extracts were screened for their antimutagenic activity against sodium azide by Ames test. The results showed that of *R. officinalis* leaves extracts can inhibit mutagenic agent of sodium azide. Rosemary leaves extracts with the inhibition of 42.92% sodium azide, showed high potential in decreasing mutagenic agent. Antimutagenic activity was increased significantly when there were liver microsome extract (S_9).

1. Introduction

In the last three decades, although pharmacological industries have produced number of new-antibiotics, but microbial resistance to these antibiotics has increased because of genetic ability of the bacteria to acquire and transmit the resistance against therapeutic agents. Herbal drugs have been used since ancient times as remedies for various diseases across the world. Traditional medicinal plants always play a positive role in the prevention or control of diabetes, heart disease and various types of cancers. Various drugs are prepared singly or in combination of medicinal plants and they are even used as principal raw material for the other medicines (Darsanaki and Parsa Lisar, 2014). Using plant compounds as a source of anticancer agents was initially performed by Hartwell in 1967; he used Podophyllotoxin and its derivatives as anticancer agents. Results from epidemiological studies as well as laboratory investigation suggest an inverse relationship between dietary intake of

phytochemicals and human cancer risk (Shams et al., 2012). Humans are continually exposed to a variety of natural and artificial mutagens generated by industrial and environmental activities. Each factor that causes removal, inhibition and inactivation of mutagen substances is rewarding. Today, bacteria are being used for the assessment of antimutagenic activities of different compounds in a short-time with excellent results. One of the methods used for assessing the mutation prevention properties of a compound in bacteria is the Ames test. Ames test is a worldwide short-term bacterial reverse mutation test specifically designed for screening a variety of new chemical substances and drugs that can produce genetic damage that leads to gene mutations. The Salmonella strains used in the test have different mutations in various genes in the histidine operon, each of these mutations is designed to be responsive to mutagens that act via different mechanisms (Shams et al., 2012; Mortelmans and Zeiger, 2000; Chalvo et al., 2004). Rosemary (*Rosmarinus officinalis* L.) which has long been known as a spice and medicinal herb belongs to the *Lamiaceae* family (Eva et al., 2003). Rosemary herbs have been widely used in the traditional medicine and cosmetics. They are also used as flavouring agents in foods. *R. officinalis* essential oil is also important for its medicinal uses and its powerful antibacterial, cytotoxic, antimutagenic, antioxidant, antiphlogistic and chemopreventive properties (Hussain et al., 2010). In this study, rosemary leaf ethanolic extracts were screened for their antimutagenic activity against sodium azide by Ames test in presence and absence of S_9

2. Materials and methods

2.1. Plant material

Fresh leaves of *R. officinalis* were collected from Guilan Province, North of Iran and dried in a place not exposed to sun light. Then the dried leaves were crushed to powder. Powdered leaves (Fifty gram) were extracted with ethanol by the aid of a Soxhelet apparatus. Finally, the obtained solution was passed through Whatman No.1 filter paper and stored at 4°C for further antimutagenic activity study.

Bacterial strains: Histidine dependent strain of *S. typhimurium* TA100, developed by Dr. Ames of the University of California, Berkeley, USA, was cultured in a nutrient broth (Merck; Germany). The overnight culture was used for strain identity confirmation.

2.2. Strain TA100 identity assays

Histidine requirement: The media conclude bacteria were incubated for 18h at 37ºC. Then, 0.1 ml of this media was added to histidine and biotion culture (minimal medium having a little histidine and biotin). Also, 0.1 ml *S. typhimurium* TA100 was added to biotin medium (minimal medium having biotin and lacking histidine) as control plate. All plates were incubated for 48h at 37ºC.

Rfa mutation: Sensitivity to crystal violet was tested. A 100 µl sample of the overnight bacterial culture was inoculated in 2 ml of melted and cooled top agar and spread over an agar nutrient plate. A disk dipped in crystal violet was later placed on this plate and after a18 h period, a bright zone was observed around the disk, an indication of the lack of cell growth due to the Rfa mutation.

UVrB mutation: This test is used to confirm UV sensitivity. After culture the bacteria on plate, a half of one was covered with aluminum foil, and it was exposed to UV radiation 8 seconds. Then, the plate was incubated for 18 h at 37ºC.

R-factor assay: This test is used to show resistance factor against ampicillin. The absence of zone of growth inhibition around the disk was an indication of ampR and a proof for the presence of the R-factor in the bacterial strain.

Preparation of the rat microsomal liver enzyme (S_9) and mutagen substances: A broad range of carcinogenic agents require metabolic activation for recognition. In this investigation, 2 male rats (body weight~200g), were used. Rats were starved for 24 hours in order to get the titer of the liver enzymes to their highest levels. Animals were sacrificed by cervical dislocation and the livers were collected, homogenized in 0.15 M KCl. Livers were cut into pieces using sterile scissors and smashed prior to a 10 min centrifugation at 9000g. The supernatant (S_9) was stored at -80°C. The antimutagenic assay was performed in the presence and absence of S_9. Chemical mutagen, sodium azide was purchased from Merck Company.

Ames test: Three plates (main plate containing *R. officinalis* leaves extracts with positive and negative control) were used synchronously. This test was carried out in the basis of described Ames test.

Procedure in presence of liver microsome (S_9): In this assay 0.5 ml of rosemary leaf extract is mixed 0.1 ml of the overnight culture *S. typhimurium* TA100 and 0.1 ml of sodium azide in test-tube containing 3ml top agar. Then, 0.1ml of histidine and biotin 0.5 mM solution and 0.5ml of liver microsome extract (S_9) were added. After were poured on glucose minimal medium and incubated for 24h at 37˚C.

Positive control: The mixture of 0.1 ml of overnight cultured *S. typhimurium* TA100, 0.1 ml of sodium and were poured in test-tube containing 3ml top agar. Then, 0.1ml of histidine and biotine 0.5 mM solution and 0.5ml of liver microsome extract (S_9) were added, after shaking for 3 minutes, the test-tube contents was poured on glucose minimal medium and incubated for 24h at 37˚C.

Negative control: The mixture of 0.1ml of overnight cultured *S. typhimurium* TA100, 0.1ml of DMSO, 0.1 ml of histidine and biotine 0.5 mM solution and 0.5 ml of liver microsome extract (S_9) were added to 3ml of top agar. After shaking for 3 minutes, it was poured on glucose minimal medium and incubated for 24h at 37˚C.

Procedure in absence of liver extract (S_9): All the steps in this stage are the same as previous part. But, here, it was not used from liver microsome extract (S_9).

Inhibitory percentage calculation: The calculation percentage of inhibition was done according to the formula given by Ong et al: Percentage inhibition = [1-T/M] ×100 where T is number of revertants per plate in presence of mutagen and test sample and M is number of revertants per plate in positive control. The number of spontaneous revertants was subtracted from numerator and denominator. The antimutagenic effect was considered moderate when the inhibitory effect was 25-40% and strong when more than 40%. Inhibitory effects of less than 25% was considered as weak and was not recognised as positive result (Ong et al., 1986). Statistical analyses were performed using SPSS software.

3. Results and discussion

In accordance with the *S. typhimurium* TA100 strain genotype, the presence of colony in biotin-histidine medium and absence one in control biotin medium show that these strains are dependent to histidine. The existence of inhibitory zone around the disk indicates that the bacteria do not grow and the Rfa mutation was occurred. This mutation can causes relative decreasing of lipopolysaccharide barriers and then, increase cell wall permeability for bigger molecules. If the inhibitory zone is not presence around the disk, the bacterium has R-factor plasmid and also, lack of growth in radiated culture region indicates that uvr B mutation was ocurred.

The results of antimutagenic activity of *R. officinalis* leaves extracts were shown in table 1. Antimutagenic activity was increased significantly when there were S_9. *R. officinalis* leaves extracts with the inhibition of 42.92% sodium azide, showed high potential in decreasing mutagenic agent.

Table 1
Antimutagenic effect of *R. officinalis* leaves extracts against sodium azide.

Revertant colony	***S. typhimurium* / S_9^-**		***S. typhimurium* /S_9^+**	
Sampels	**Revertants (CFU/plate)**	**Inhibition (%)**	**Revertants (CFU/plate)**	**Inhibition (%)**
Positive control (sodium azide)	400	-	473	-
Negative contorol (DMSO)	45	-	65	-
R. officinalis leaves extracts	245	38.75%	270	42.92%

Cancer is considered as one of the main causes of mortality throughout the industrial world in the present century (Issazadeh et al., 2012). Plants have long been used in the treatment of cancer (Shoeb, 2006). The use of antimutagens and anticarcinogens in everyday life is the most effective procedure for preventing human cancer and genetic disease. Ames test was used to determine antimutagenic of olive leaf. This method is very fast and economical and used to identify antimutagenic and mutagenicity of agents. In this study, Rosemary leaves extracts with the inhibition of 42.92% sodium azide, showed high potential in decreasing mutagenic agent. In Study by Wang et al, (2012) *R. officinalis* L. essential oil showed greater activity than its components in both antibacterial

and anticancer test systems, and the activities were mostly related to their concentrations. In Study by Hussain et al, (2010) *R. officinalis* essential oil exhibited antiproliferative, antioxidant and antibacterial activities. In Study by Golshani and Sharifzadeh (2014), ethanol extract of rosemary leaves at concentrations of 400 mg/ml was active against *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus* and *Escherichia coli*. Minimum inhibitory concentration of the extract on the growth of these bacteria from 6.25 mg/ml to 100 mg/ml was change. Also MBC of extract showed range from 12.5 to 200 mg/ml respectively. In Study by chobba et al, (2012) the *R. officinalis* essential oil showed excellent activity against *Staphylococcus aureus*, followed by *Staphylococcus epidermidis* and *Staphylococcus aureus* 25923, with strong inhibition zones of 38.00, 29.40 and 26.00 mm, respectively. Cytotoxicity assays involved the application of an MTT testing method against HeLa cell lines. The results yielded high IC50 value values of up to 26,77 µg/ml. It can be concluded that Rosemary leaves should have more effective place in treatment because of antimutagenic and anti-carcinogenic. Of course, more comprehensive researches are needed for indicating scope and exact mechanism of this function.

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