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Antimutagenic Activity of Olive Leaf Aqueous Extract by Ames Test

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Abstract

Medicinal plants are an important source of substances which are claimed to induce anti-inflammatory and antioxidant effects. The leaves of the olive tree (*Oleaeur opaea*) have been used for medicinal purposes since ancient times and research has suggested that olive leaf extracts have antimicrobial and antimutagenic properties. In this study, Olive leaf aqueous extracts were screened for their antimutagenic activity against sodium azide and 2-nitrofluorene by Ames test in presence and absence of rat microsomal liver enzyme (S_9). Each assay was performed in triplicates simultaneously and the percentage of inhibition was determined using the formula [1-T/M] ×100. The results showed that olive leaf aqueous extracts can inhibit mutagenic agents of sodium azide and 2-nitrofluorene. Antimutagenic activity was increased significantly when there were S_9. Olive leaf extracts with the inhibition of 54.21% sodium azide and 51.62 %, 2-nitrofluorene showed high potential in decreasing mutagenic agents.

Keywords: *Salmonella typhimurium TA100*, sodium azide, Ames test, 2-nitrofluorene, olive leaf extract

Introduction

Mutation is an important factor in carcinogenesis. Therefore, the incidence of cancer may be reduced by decreasing the rate of mutation. The best way for humans to decrease the rate of mutation is to avoid exposure to or ingestion of mutagens and carcinogens [10]. Since ancient times, the medicinal properties of plants have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities [20]. Phenolic compounds and flavonoids are also widely distributed in plants which have been reported to exert multiple biological effects, including antioxidant, free radical scavenging abilities, anti-inflammatory and anti-carcinogenic etc [17,20]. 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 [24]. Results from epidemiological studies as well as laboratory investigation suggest an inverse relationship between dietary intake of phytochemicals and human cancer risk [25]. The olive leaf is the first botanical mentioned in the Bible [9]. The leaves of the olive tree (*Oleaeur opaea*) have been used for medicinal purposes since ancient times and research has suggested that olive leaf extracts have antibacterial, anti-inflammatory and antioxidant properties. Now it also appears that a supplement containing olive leaf extract could help lower blood pressure and cholesterol [5,9,21]. 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 [24]. 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 [16]. 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 [12, 16] (Table 1).

Table 1: List of *Salmonella typhimurium* strains used in Ames test

Strain	**Amino acid marker**			**other relevant mutations**	
	histidine mutation	type of mutation	main DNA target	cell-wall	DNA-repair
*Salmonella typhimurium*TA97	*hisD6610*	Frameshift	*rfa*	GC	*uvrB*
*Salmonella typhimurium*TA98	*hisD3052*	Frameshift	*rfa*	GC	*uvrB*
*Salmonella typhimurium*TA100	*hisG46*	Base pair substitution	*rfa*	GC	*uvrB*
*Salmonella typhimurium*TA102	*hisG428*	Base pair substitution	*rfa*	AT	-

In a comparative study, it was concluded that systems exploiting *Salmonella typhimurium* TA100 in the assays are most capable in identifying the mutagenic capacity of different chemicals. On the other hand, mouse hepatic homogenate, containing microsomal enzymes including cytochrome P450 has anticancer properties. Therefore, in cases where an antioxidant compound shows a synergistic effect with the anticancer activity of cytochrome P450, an anticancer activity can also be assigned to this compound. Many reports proved that some vegetables and fruits contain phytochemicals, which have bioactive effects. Thus, these naturally occurring phytochemicals have been received much attention [16,24]. Morita *et al*, demonstrated of antimutagenic factors in several vegetables and fruits [15]. In this study, Olive leaf aqueous extracts were screened for their antimutagenic activity against sodium azide and 2-nitrofluorene by Ames test in presence and absence of S_9.

Materials and Methods

Preparation Aqueous Extract of Olive Leaf (OLE):
Olive leaves used in this study were collected in winter 2011 from Gilan province (Northern Iran). Leaves were washed to remove impurities such as dust and then dried in an air oven for 3 days. Then, they were ground by grinder and sterilized with Tendalization method. One liter water was added to 50 grams powder obtained from leaves and put on the shaker to be solved thoroughly. Finally, obtained solution was passed through filter [14].

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.

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, 5 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 S9. Two chemical mutagen, sodium azide and 2- nitrofluorene were purchased from Sigma and Merck company [8,12, 16].

Ames test

Three plates (main plate containing olive Leaf extract with positive and negative control) were used synchronously. This test was carried out in the basis of described Ames test [12].

Procedure in presence of liver microsome (S_9): In this assay 0.5 ml of olive leaf extract is mixed 0.1 ml of the overnight culture *S. Typhimurium* TA100 and 0.1 ml of our mutagenic substances including sodium azide and 2-nitrofluorene 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 37ºC.

Positive control: The mixture of 0.1 ml of overnight cultured *S. typhimurium* TA100, 0.1 ml of mutagenic substances including sodium azideand 2-nitrofluorene were prepared 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 37ºC.

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 37ºC.

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 [18]. Statistical analyses were performed using SPSS software.

Results

In accordance with the *Salmonella 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 showed that olive leaf aqueous extracts can inhibit mutagenic agents of sodium azide and 2-nitrofluorene (Table 2, 3). Antimutagenic activity was increased significantly when there were S_9. Olive leaf extract with the inhibition of 54.21% sodium azide and 51.62% 2-nitrofluorene showed high potential in decreasing mutagenic agents.

Table 2:Antimutagenic effect of olive leaf extract against sodium azide

Revertant colony / Sampels	*S.typhimurium* + S_9^-		*S.typhimurium* + S_9^+	
	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)
Positive control (sodium azide)	392	-	463	-
Negative contorol (DMSO)	53	-	75	-
Olive leaf extract	232	40.81%	212	54.21%

Table 3:Antimutagenic effect of olive leaf extract against 2-nitrofluorene

Revertant colony / Sampels	*S.typhimurium* + S_9^-		*S.typhimurium* + S_9^+	
	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)
Positive control (2-nitrofluorene)	413	-	523	-
Negative contorol (DMSO)	53	-	75	-
Olive leaf extract	264	36.07%	253	51.62%

Discussion

Cancer is considered as one of the main causes of mortality throughout the

industrial world in the present century. Scientists believe that damage to the genetic material, changes in DNA sequence and continuity, mutation in genes and other genetic changes in chromosomal structures play important roles in carcinogenesis [24]. The use of anti-mutagens and anti-carcinogens in everyday life is the most effective procedure for preventing human cancer and genetic disease [10]. Ames mentioned that some natural substance contain factors, which act to lower the mutation rate either by inactivating mutagens or interfering in the process of mutagenesis [12]. Ruan *et al*, reported that antimutagenic substances may prevent cancer because they can destroy mutagens both inside and outside body cells, and block mutagens that damage DNA and cause mutations in cells [23]. OLE is very effective activity against various diseases, such as coronary artery disease, hypertension, high cholesterol level, arrhythmia, cancer, diabetes, overweight, osteoporosis, herpes, colds and some bacterial, fungus and yeast infections [22]. 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. Atawodi studied antioxidant activity of African herb including African olive leaf and found that its antioxidant activity is good [2]. Also, studies have referred anti-carcinogenic and antimutagenic properties of olive and its oil [3,4]. Escrich *et al*, reported the effect of olive oil on colon cancer and identified antioxidants and phenolics as well as the MUFAs (monounsaturated fatty acids) as important reducing agents in the incidence of colon cancer [7]. Owen *et al*, reported use of olive oil in a diet can be an effective way in preventing cancer [19]. Menendez *et al*, showed that oleuro peinaglycone is the most potent phenolic compound in decreasing breast cancer cell viability [13]. Also, the investigations on other plants antimutagenic properties show that, grape skin extract has high antioxidant effects due to presence of much phenolic compounds [6]. Loots *et al*, researches suggested that Aloe vera extracts in human and animals show antioxidant properties [11]. Adhami *et al*, refers anticarcinogenic of green tea extract impacts on prostate cancer [1]. In this study, consideration of antimutagenic and anti-carcinogenic of olive leaf aqueous extracts compared with positive controls (sodium azide and 2-nitrofluorene) indicates good antimutagenic properties olive leaf extracts. Olive leaf as an ever-green leaf is available in all year seasons easily and is inexpensive raw material and full of phenolic compounds. It can be concluded that olive leaf 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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