Genotoxicity Evaluation of the *Vitex agnus-castus* L. Essential Oil with the Yeast DEL Assay

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Abstract— The chasteberry (*Vitex agnus-castus*, VAC), which is endemic to Mediterranean Europe, is a member of the genus *Vitex* of Verbenaceae family. Its essential oil contains 1, 8-cineole, α-pinene, α-terpinyl acetate, (*Z*) -*β*-farnesene and this rich content of bioactive compounds makes this plant valuable for use in therapeutical applications. VAC has mostly been used in the treatment of women complaints such as premenstrual syndrome (PMS) and also due to the chemopreventive, immunomodulatory, tumoricidal, antimicrobial, antiepileptic and anti-inflammatory activites. Prior to using a plant material for any medicinal purposes it should be investigated that whether it has any negative effects on human health. Genotoxicity is one of the aspects that should be examined in this context and this term indicates the changes of DNA molecules in cells because of the various chemical, physical and biological factors. In order to evaluate the mutagenic and carcinogenic properties of the chemical substances or herbal extracts, the short-term genotoxicity test systems can be used. The yeast DEL assay is relatively more economical and advantageous of all, because it produces fast and reliable results. In this regard, the present study was conducted to investigate the mutagenic and carcinogenic properties of VAC essential oil in order to perform its safety assessment with the yeast DEL assay. For this purpose, the essential oil of VAC was extracted by Clevenger distillation method and its genotoxic properties were tested at 0.2, 0.4, 0.6, 0.8 and 1.0 µl/plate concentrations by using RS112 strain of *Saccharomyces cerevisiae*. According to the results, none of the tested concentrations showed any mutagenic and carcinogenic effects on eukaryotic yeast cells. In conclusion, the results have showed that the essential oil of VAC is genotoxically safe but the other toxicity and safety tests should be carried out to prove that it has not any adverse effects on human health.

Index Terms— *Vitex agnus-castus* L. essential oil, Yeast DEL assay, DNA deletions, Genotoxicity test, Mutagenicity, *Saccharomyces cerevisiae*, Short-term tests

1 INTRODUCTION

Natural products from plants, animals and also minerals have gradually become important for the treatment of human diseases. Active drug substances used in the treatment of existing diseases can be inadequate or ineffective in the treatment of some kind of certain diseases nowadays, so the discovery or development of new drugs is essential in the pharmaceutical field.

To standardize and evaluate drug properties of active plant-derived compounds or herbal drugs may start a new era of the existing healthcare systems in the future [1].

The chasteberry (*Vitex agnus-castus*, VAC) which is a member of the genus *Vitex* of Verbenaceae family, is a small tree or shrub and it is endemic to Mediterranean Europe and Central Asia [2]. Its essential oil contains 1,8-cineole, sabinene, α-pinene, α-terpinyl acetate, (*Z*)-*β*-farnesene (Fig. 1) and this rich content of bioactive compounds makes this plant valuable for use in therapeutical applications [3], [4], [5].

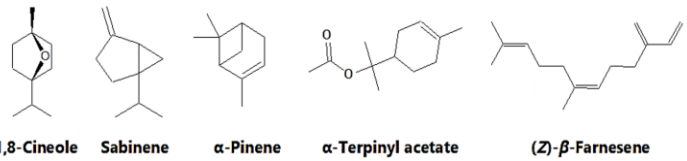

Fig. 1. Bioactive compounds of *Vitex agnus-castus* L. essential oil

V. agnus-castus L. has mostly been used in the treatment of premenstrual problems or hyperprolactinemia in women because of its hormone-like effect [6] and the other known biological activities of this plant are chemopreventive, immunomodulatory, tumoricidal, antimicrobial, antifungal, anti-inflammatory, antiepileptic, anticonvulsant, sedative, antinociceptive and tranquilizer activites [4].

Before using a plant material for any medicinal purposes, it should be investigated that whether it has any negative effects on human health. Genotoxicity is one of the aspects

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that should be examined in this context and this term indicates the changes of DNA molecules in living cells because of the various chemical (e.g., metals, pesticides), physical (e.g., radiation) and biological (e.g., defects in DNA metabolism and/or repair) factors [7]. DNA damage occuring in a somatic cell may result in a somatic mutation, which may lead to be the initial steps in the development of cancer [8].

The short-term genotoxicity test systems can be used in order to evaluate the safety of environmental chemicals and consumer products and also to explore the mechanism of action of known or suspected carcinogens [9]. The yeast DEL assay is relatively more economical and advantageous among these test systems. Because of being a sensitive and spesific assay for detecting the carcinogens, it has widely been used to investigate the genotoxicity of agricultural chemicals, environmental contaminants and pharmaceuticals. It also allows for investigating the mechanism and genetic control of homologous recombination and genomic instability. Similarly to *Ames*/*Salmonella*, the yeast DEL assay can be used to detect the mutagenic and carcinogenic properties of the chemical substances or herbal extracts by giving more accurate results [10].

In the present study, *in-vitro* mutagenic and carcinogenic properties of VAC essential oil were investigated with the intention of performing its safety assessment by using yeast DEL assay.

2 MATERIALS AND METHODS

2.1 Chemicals

In order to perform this yeast-based genotoxicity tests, the known mutagen ethyl methanesulfonate (EMS) and the other pure chemicals, including *D*-glucose, *L*-amino acids were purchased from Sigma-Aldrich (St. Louis, MI, USA), Merck and Fluka. Bacto agar was obtained from Difco (Detroit, MI, USA).

2.2 Collection of Plant Material

Vitex agnus-castus L. fruits were collected from Alanya province (Antalya) in Turkey in October 2018. The collected samples were dried and stored in a clean, dry and dark environment during the laboratory transfer process.

2.3 Preparation of Essential Oil

The extraction of the essential oil of *Vitex agnus-castus* were done by boiling approximately 20 g of fruits in 350 ml of distilled water in a Clevenger apparatus for 5 hours. The oil was seperated, stored in sealed glass containers at +4 °C in the dark. The total yield was 0.12 mL per 20 g of plant material.

2.4 Preparation of Media and Solutions

The media and solutions used in this study were prepared according to the described procedures by Brennan and Schiestl [10].

2.4.1 Yeast extract adenine dextrose (YPAD) plates

1% Yeast extract, 2% peptone, 2% dextrose, 48 mg/mL adenine sulfate, 2% bacto agar dissolved in distilled water and autoclaved at 121 °C for 15 min. before pouring.

2.4.2 Liquid inoculation media (Liquid SC-Leu)

0.67% Yeast nitrogen base, 2% *D*-glucose and 1.2 mL of the amino acid mixture (minus leucine) were dissolved in 600 ml of distilled water and autoclaved at 121 °C for 15 min.

2.4.3 Synthetic complete (SC) agar plates

0.67% Yeast nitrogen base, 2% *D*-glucose, 2% bacto agar and 1.2 mL of the following amino acid mixture were added to 600 ml of distilled water: 1.8 g *L*-adenine hemisulphate, 1.2 g *L*-arginine-hydrochloric acid (HCl), 1.2 g *L*-histidine-HCl, 6.0 g homoserine, 1.8 g *L*-isoleucine, 1.8 g *L*-leucine, 1.8 g *L*-lysine, 1.2 g *L*-methionine, 3.0 g *L*-phenylalanine, 2.4 g *L*-tryptophan, 1.8 g *L*-tyrosine, 1.2 g uracil, 9.0 g *L*-valine. The resulting mixture was autoclaved at 121 °C for 15 min. and poured into plates.

2.4.4 Dropout agar plates

SC medium lacking leucine (SC-Leu), SC medium lacking histidine (SC-His) and SC medium lacking adenine (SC-Ade)

2.5 Yeast Strain and Growth Conditions

The diploid mutant *Saccharomyces cerevisiae* RS112 strain which was sent by Dr. Robert Schiestl to our laboratory was used in the application of the yeast DEL assay. The genotype of this strain is *MAT*a/α *ura*3-52/*ura*3-52 *leu*2-3,112/*leu*2-Δ98 *trp*5-27/*TRP*5 *arg*4-3/*ARG*4 *ade*2-40/*ade*2-101 *ilv*1-92/*ILV*1 *HIS*3: pRS6/*his*3-Δ200 *LYS*2/*lys*2-801 [10].

After the conformation of His^- and Ade^- auxotrophic phenotypes, the stock yeast cultures were prepared in 30% aqueous glycerol solution and stored at -70 °C. The working cultures were also prepared by the direct inoculation of the frozen stock cultures to YPAD medium and the prepared cultures were incubated at 30 °C for 3 days.

2.6 Viability Assays and Determination of Test Concentrations

The toxicity of *Vitex agnus-castus* essential oil on *S. cerevisiae* RS112 strain was evaluated according to the experimental procedure as described [11] in detail. Thus, the test concentrations were identified as 0.2, 0.4, 0.6, 0.8 and 1.0 µL/plate. According to the results, the shape of yeast cells were proper, they grew normally, the spontaneous colony formation were within a normal range and there was no significant decrease in the cell survival. It was concluded that the specified test concentrations and experimental conditions did not induce any toxic and other adverse effects toward the yeast cells.

2.7 Mutagenicity Test

The yeast mutagenicity assay was performed as described [10]. EMS (100 µg/plate) was used as positive control. The essential oil was dispersed in distilled water and the resulting mixture was used as negative control during the experiment.

In the mutagenicity test performed with *S. cerevisiae* RS112, the auxotrophic yeast strain was grown in liquid SC-Leu medium for 24 h at 30 °C with agitation (250 rpm). After diluting the culture to 2×10^7 cells/mL in fresh liquid SC-Leu medium, 500 µL of yeast culture, 100 µL of *Vitex agnus-castus* essential oil at different concentrations (2.0, 4.0, 6.0, 8.0 and

10.0 µL/mL in distilled water) were added to 4.35 mL of liquid SC-Leu medium. The tubes were incubated for 16-18 h at 30 °C with shaking at 250 rpm with 45° angle from the horizontal to ensure adequate agitation. In the end of this period, the yeast cells were counted with a hemocytometer and precipitated at 3200*g* for 3 min by centrifugation, washed with 5 mL of distilled water three times and finally resuspended in 5 mL of sterile distilled water. Thereafter, the dilution tubes were prepared for treated and control culture by using sterile distilled H_2O and transferred into sterile microcentrifuge tubes. 100 µL of diluted yeast cultures were inoculated on SC-His to assess deletion (DEL) recombination frequency and also on SC-Ade medium to assess gene conversion frequency. Colonies were counted after 3 days of incubation at 30 °C.

For the purpose of assessing the results of the mutagenicity assay, the plate incorporation method was used as mentioned [12], [13], [20]. When observed a dose-response relationship and two-fold increase in the number of revertants at least in one concentration, it is considered that the test material has mutagenic effects on the eukayotic yeast cells [14], [15], [16], [21].

2.8 Statistical Analysis

The results were expressed as means ± S.D. (standart deviation) and also the average and standart error of the three experiments with duplicate plates/doses. Analysis of variance (ANOVA) was conducted and Tukey's test were used to determine the significant difference between groups. The statistical significance level was set to $P< 0.05$ [17].

3 RESULTS AND DISCUSSION

According to the viability assay results, the five concentration levels ranging from 0.2 to 1.0 µL per plate were defined as applicable for the test material. Besides, none of the tested concentrations (0.2, 0.4, 0.6, 0.8, 1.0 µL/plate) of *Vitex agnus-castus* essential oil showed any mutagenic and carcinogenic activity in the mutagenicity assay performed with mutant *S. cerevisiae* RS112 strain (Table 1).

In the literature, *Vitex agnus-castus* L. essential oil has known to possess a wide range of biological activities and there have been several studies to determine these bioactivities in order to scientifically confirm its traditional use [4, 6, 18]. Its importance is based on the rich content of bioactive compounds that may have a therapeutic value in the treatment of current diseases [3], [19], [22].

The growing importance of medicinal plants requires to ensure their safety, quality and effectiveness properties [1]. For this purpose, some kind of pharmacological and toxicological tests should be done prior to the therapeutic use of any chemical substances or plant extracts. Thus, the aim of the current study was the determination of potential toxicological effects of *Vitex agnus-castus* essential oil using yeast DEL assay.

In an *in-vitro* yeast mutagenicity test system, essential oil constituents can act in four different ways on the living organisms like the other synthetic or natural substances. These effects can be directly mutagenic, comutagenic, promutagenic and antimutagenic [17]. Some constituents may have one or more genotoxic effects, such as changing the deletion and gene conversion frequencies at the same time in the yeast cells, depending on the tested concentrations. However, the mutagenic effects could also be observed in a non-concentration-dependent manner [21], [23], [24]. In our study, the genotoxicity of *Vitex agnus-castus* essential oil was evaluated for five different concentrations. The results showed that there had no dose-response relationship and no two-fold increase in the number of the revertants at specified concentration levels in this short-term mutagenicity assay.

TABLE 1
THE MUTAGENICITY ASSAY RESULTS OF THE *VITEX AGNUS-CASTUS* ESSENTIAL OIL FOR *S. CEREVISIAE* RS112 TESTER STRAIN

Test Items	Concentration	Number of Revertants *S. cerevisiae* RS112			
		Number of DEL Events		Number of ICR[a] Events	
		Mean±S.D.	M/NM[b]	Mean±S.D.	M/NM[b]
VAC Essential Oil	0.2 µL/plate	59.17±3.31	NM	28.33±2.42	NM
	0.4 µL/plate	59.67±2.66	NM	30.17±2.14	NM
	0.6 µL/plate	62.00±1.79	NM	29.50±2.74	NM
	0.8 µL/plate	61.83±2.79	NM	29.83±2.40	NM
	1.0 µL/plate	59.67±4.37	NM	30.17±1.83	NM
EMS[c]	100 µg/mL	152.33±6.25		94.67±3.50	
Distilled H_2O[c]	1.0 µL/plate	62.00±3.10		30.33±2.66	

[a]ICR: Interchromosomal rearrangement; [b]M: Mutagenic and NM: Non-mutagenic; [c]EMS was used as positive control for S. cerevisiae RS112 strain. Distilled H_2O was used as negative control.

The yeast DEL assay is generally considered safer, more sensitive and specific for detecting mutagenic and carcinogenic agents than the other short-term genotoxicity test systems such as *Escherichia coli* WP2 and *Ames/Salmonella*. In order to investigate the mutagenesis mechanisms of any chemical substances or herbal extracts with the yeast DEL assay, at least one or more known mutagens should be used during the experiments. In this study, EMS was used as positive control on the purpose of inducing DNA damage in the yeast cells. It is an alkylating agent and also it increases the rate of genetic mutation by interfering with the function of nucleic acids. Because of having the ethylating effect of DNA molecules, it damages the genetic material and leads to several genomic instabilities such as mutations, single-stranded breaks and chromosomal aberrations in the living organisms. In this context, regarding to the five different concentration levels of *Vitex agnus-castus* essential oil, none of the mutagenic and carcinogenic effects causing deletion or gene conversion events in DNA molecules of *S. cerevisiae* RS112 strain were observed.

4 CONCLUSION

In conclusion, the essential oil of *Vitex agnus-castus* can be considered as genotoxically safe at the tested concentrations because it did not show any mutagenic and carcinogenic activ-

ity on eukaryotic *S. cerevisiae* RS112 strain. These findings indicate that *Vitex agnus-castus* L. essential oil may have a potential use for therapeutic applications and this study are beneficial in terms of phytotherapeutic drug discovery and development research, but the long-term *in-vitro* toxicity tests and *in-vivo* experiments should still be carried out to prove that it has not any toxic and/or adverse effects on human health.

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