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Sequence variants of *CYP345a1* and *CYP6a14* gene regions in *Tribolium castaneum* (Coleoptera: Tenebrionidae) adults treated with the novel characterızed *Bolanthus turcicus* (Caryophyllaceae) extract

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ABSTRACT

In this study, various doses of plant extracts that obtained from *Bolanthus turcicus* was applied to an important storage pest *Tribolium castaneum* adults. *Bolanthus turcicus* is an endemic species and spreads on the Hasan Mountain above Karkın town (Turkey, Aksaray province). The plant species was collected from June to July with the field study to be carried out in this region. Obtained extract of plant was analyzed by gas chromatography mass spectrometry (GC-MS) method. The doses were defined during the study and the concentrations that kill 50% and 99% of the population were determined after applications. After 24 h, DNA was isolated from live and dead individuals that obtained from LC_{50} and LC_{99} concentration applications and analyzed for Cytochrome P450-mediated detoxification resistance genes, *CYP345A1* and *CYP6A14* gene regions, by polymerase chain reaction (PCR). CYP genes in insects are known to be rapidly regulated when exposed to insecticides. In the study, in order to screen for 206 bp and 353 bp fragments of *CYP345A1* and *CYP6A14* genes in *T. castaneum* adults were amplified using specific primers, respectively. DNA direct sequencing was performed on each template using the forward primer. When compared to the control, it is believed that mutation differences in live and dead individuals according to the sequencing results obtained from survival and dead adults, may allow these genes to play a protective role against the toxic effect of *B. turcicus* extract.

Keywords: *CYP345a1*; *CYP6a14*; *Tribolium castaneum*; *Bolanthus turcicus*; detoxification

INTRODUCTION

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is a common and the most destructive stored-product insect pest worldwide and it causes serios economic loss to all kinds of stored cereal grains and products each year [1]. Larvae and adults

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of the insect damage grains and feed on these grains. Infested products may contain insect fragments, benzoquinones and residues after molting, so this insect has become economically important. Different kinds of pesticides have been used successfully for a long time to prevent this important pest [2]. Usage of these chemical pesticides have serious disadvantages, like toxicity to beneficial insects and human, induction of resistance in insects, environmental and health hazards [3]. Development of resistance in different strains of *T. castaneum* is a common problem to control of this pest [4].

Currently, the new trend is usage of biopesticides to minimize the use of harmful insecticides against pests in storage of cereals [5]. Botanical products are included in biopesticides and these products such as extracts and essential oils are environmentally safe, less hazardous, economic and easily available. These advantages make botanical products an alternative to chemical pesticides. Because of these properties, the effects of plant products such as toxicity, mortality, antifeedant, growth inhibitor, reduction of fecundity and fertility, on insects were investigated by many researchers [6].

Cytochrome P450s enzymes are wide and various group of enzymes that found in all domains of living things. These enzymes are especially an important system about the metabolism of endogenous compounds and xenobiotics like pesticides, mutagens, drugs, chemical carcinogens, etc. [7]. Thus, CYPs function as monooxygenases in the detoxification of endogenous compounds and xenobiotics and oxidize organic substrates. They can play antagonistic effect in biological systems. In arthropods, increasing activity of P450 is related with the metabolic detoxification of pesticides [8].

Development of molecular techniques have made easier to study complex gene families such as cytochrome P450 superfamily. In the National Center for Biotechnology Information (NCBI), there are more than 2000 insect P450s that are defined mostly from *Drosophila melanogaster* Meigen [9].

It is known that plant derived materials do not cause resistance in insects and not toxic to environment. Thus they are environmentally friendly and safe to non-target insects, especially natural enemies. Our study therefore has these objectives; 1) To evaluate the percent mortality of *B. turcicus* against *T. castaneum* adults. 2) To determine LD_{50} and LD_{99} concentartions of *B. turcicus.* 3) To determine the mutation differences in *CYP345A1* and *CYP6A14* genes in *T. castaneum* after application of plant extract.

MATERIALS AND METHODS

Insect culture: *Tribolium castaneum* adults were obtained from Kırşehir Ahi Evran University, Faculty of Agriculture, Department of Plant Protection. Insects were cultured on wheat flour and 5 % yeast (19:1, w/w) and maintained at constant regimes of 27±1°C and 70±5% relative humidity before use [10].

Plant material: *Bolanthus turcicus* is an endemic species and spreads in the Hasan Mountains in Turkey [11]. The plant species were collected from June to July by field work in this region. Samples were pressed and dried according to herbarium techniques, identified by *The Flora of Turkey* [12], and kept at the herbarium of the Bozok University Department of Biology (Yozgat, Turkey).

Isolation and characterization of plant extract: The collected samples were dried and pulverized with the aid of a disintegrant and 10 g of the sample was weighed and dissolved in 200 ml of solvent (distelled water). Extraction was carried out for 12 hours with the help of a soxhlet apparatus. All the samples obtained were then lyophilized and stored at +4 ^{0}C until tested. For the distillation process, 200 g of the plant sample was taken in powder form and dissolved in 3 L of water. Distillation was carried out for 3 hours with the aid of a Clevenger type apparatus. Cold water circulation ensures continuous cooling of the system. At the end of

the period, the condensed sample was collected [13]. The collected sample was stored at +4^{0}C for GC-MS analysis and insecticidal activity assays. GC-MS analysis result was shown in Table 1.

Table 1. Percentages of compounds detected by GC-MS in *Bolanthus turcicus* extract

Group	Chemical compound	*B. turcicus* %
Alkanes	Eicosane	2.36
	Nanodecane	14.72
	Docosane	1.34
	Hexacosane	6.78
Acids	Palmitic acid	20.12
	Strearic acid	11.56
	Oleic acid	36.88
Alcohols	Geraniol	2.29
	Stearyl alkol	2.49
Monoterpenoid	α-Citronellol	1.42

Insect bioassays: The plant extract was tested for its insecticidal effect against adult stage of the *T. castaneum*. Adults were placed in 1000 ml glass jars. Six replicates were used for each concentration and ten adults were used for each replicates. The plant extract was applied with filter paper at different doses and placed the bottom of the jar's cover. Adults were exposed to extract vapor with different doses (125-2000 µL L^{-1} air) for 24 h. After 24 h, adults were removed from the jar and mortalities were determined. The LC_{50} and LC_{99} (the concentrations that killed 50% and 99% of the treated insects, respectively), were defined. LC_{50} and LC_{99} values were calculated by Probit analysis [14]. The control group was kept on the same conditions but no extract application was made. Mortality rates recorded daily. 24 hours after application, DNA isolations were made from living and dead individuals obtained from LC_{50} and LC_{99} concentration applications.

DNA extraction from Insect samples: Genomic DNA was isolated from insect samples of both controls and treated samples with plant extract using E.Z.N.A.® Insect DNA Kit (Omega BIO-TEK) according to manufacturer's instructions.

Sequence slignment and confirmation of mutations in *CYP345A1* and *CYP6A14* genes in *T. castaneum*: DNA amplification was carried out on a BIORAD-PCR system in a 50 µl reaction mixture containing PCR buffer (Thermo Fisher Scientific) 25 mM magnesium chloride (Thermo Fisher Scientific), 200 µM dNTP (Thermo Fisher Scientific), 10 pmol of *CYP345A1* (Forward: 5'-CGG TTC AGT CGT TTG GTT GT-3', Reverse: 5'-ATT TGC CAC TGG ACA CGT TC-3') and *CYP6A14* genes primers (Forward: 5'-AGG CAC AAT CAG CCA AGG TT-3', Reverse: 5'-ATT TCC TTT TGG GGA CGC GA-3' Macrogene Company), 0.5 U Taq DNA polymerase (Thermo Fisher Scientific) and 50 ng genomic DNA. The PCR cycling conditions consisted of an initial denaturation step at 95 ^{0}C for 5 min followed by 35 cycles of 94 ^{0}C for 1.30 min, 58.5 ^{0}C for 1,30 min, 72 ^{0}C for 1,30 min and final extension step at 72 ^{0}C for 5 min. An agarose gel was made to see whether the target sequence was amplified as a result of the reaction. The PCR product were separated on a 1.5 % agarose gel electrophoresis at 120 V, stained with ethidium bromide (0.5 µg/ml) and and photographed using MiniLumi DNR Bio-Imaging Systems. Primer pairs for each gene region were designed according to sequence information of gene regions. PCR products on the agarose gel elctrophoresis are indicated in (Fig. 1 of supplementary file). The amplified PCR samples were analyzed by DNA sequencing by the Macrogene Company (Korea) using the one-way sequencing method. Sequence results were compared with the control group to determine mutations.

RESULTS

As a result of the toxicity studies, doses that killed 50% and 99% of adult insects (LC_{50}, LC_{99}) were calculated by Probit analysis (Table 2). DNA was isolated from the surviving and dead individuals after the application at the respective doses without sex discrimination and PCR was performed with primers determined for *CYP354A1* and *CYP6A14* gene regions.

Table 2: LC_{50} and LC_{99} values of *Bolanthus turcicus* essential oil against adults of *Tribolium castaneum*

Duration (24 hours)	N	LC_{50}, µl/L air	LC_{99}, µl/L air	df	χ^2
Adult	10	251.6	1990.8	4	7.573[a]

N: Number of the tested adult. a: Since the goodness-of-fit chi-square was significant ($P < 0.05$), a heterogeneity factor was used in the calculation of confidence limits.

The resulting gel images of the surviving and dead individuals from LC_{50}, LC_{99} dose applications of *CYP354A1* and *CYP6A14* gene regions are shown in the supplementary file. After DNA sequence analysis, a region of 206 bp in the *CYP345A1* gene region and 353 bp in the *CYP6A14* gene region was screened and mutations were determined.

In addition to natural enemies, aromatic plants and their extracts and essential oils are intensively investigated in order to determine their pesticidal properties [15]. Essential oils are seen as a good alternative to fumigant pesticides because of their environmental safety characteristics. Fumigation is used as an effective method to reduce stored product pests. For this reason, many researchers have investigated the insecticidal activity of essential oils against different storage pests [6, 16].

In our study, it was for the first time determined the fumigant toxicity effect of *B. turcicus*, an endemic plant for our Country, on the adults of an important storage pest, *T. castaneum*. We detected 10 different constituents of which Oleic acid (36.88%) was the main component of the *B. turcicus* extract. Don Pedro found that oleic and linoleic acids were active in reducing progeny development in *Callosobruchus maculatus*. Oleic acid are generally more toxic to insects than those of stearic acid [17]. Silva et al. determined the oleic acid and linoleic acid in *Solanum lycocarpum* fruit. Thay found that the oils, fatty acids, and methyl esters of *S. lycocarpum* have greatest larvicidal effect against *Culex quinquefasciatus* [18].

Therewithal, we also have detected geraniol and citronellal in this plant extract that were known to be incecticidal and repellent effects on insects. Pinheiro et al. presented that the major component of *Citronella* grass was geraniol and caused mortality in *Frankliniella schultzei* and *Myzus persicae* [19]. Sfara et al. found that geraniol and menthyl acetate caused a repellent effect on nymphs of *Rhodnius proxilus* [20].

Insecticide resistance developed by product pests has become a serious problem. It is also possible that this resistance mechanism may be improved against plant-based insecticides. The cytochrome P450 (CYP) genes constitute one of the largest gene families with representatives in all living organisms, including bacteria, fungi, plants and animals. In insects, CYP enzymes are usually associated with the metabolism of either endogenous or exogenous compounds. Although the physiological importance of up-regulation or overexpression in insects is uncertain, up regulation is multifaceted in environmental adaptation or is thought to play a role as a protective mechanism by which the organism can detoxify xenobiotics. Indeed, CYP-mediated detoxification is considered to be an important resistance mechanism in insect populations that can result in significant high resistance to many insect pesticides. Until today, thousands of CYP genes have been identified in total in insects, and this number is rapidly increasing as more insect genomes are sequenced. The upregulation of CYP genes mediated by insecticides and other xenobiotic compounds has been reported in many insect species. The presence of genome sequences of many insects have facilitated the identification of new CYP

genes and the identification of upregulated CYP genes at the genomic scale. In our study, T. castaneum's Georgia 2 strain (*T. castaneum* strain Georgia GA2 linkage group LG5, Tcas5.2, whole genome shotgun sequence) sequence information was utilized. Mutations were detected by comparing the sequence information from the NCBI with the relevant sequences.

When resistant and susceptible insects are exposed to the sublethal dose of phosphin, the expression of *CYP6A14* and *CYP346B1* genes has been reported to be significantly increased in resistant insects than susceptible ones [21]. In our study, it was determined that the sequence results of the *CYP6A14* region obtained from the dose killing 99% of the adults exposed to the plant extract resulted in a considerable amount of mutation when compared with the control. Similarly, when the sequence results of the *CYP345A1* region are compared with the control, it is noted that deletion and insertion occur in a considerable amount, especially in the sequence results obtained from the dose killing 99% of the adults. In comparison of nucleotide sequences of *CYP345A1* with control in dead *T. castaneum* from LC_{99} concentration application, 171 mutations were determined that consist deletions, insertions and point mutations. Least number of mutations were obtained from comparison of nucleotide sequences of *CYP6A14* with control in live and dead *Tribolium castaneum* from LC_{50} concentration application. The mutation started at nucleotide position 21967 of *CYP345A1* sequence and ended at nucleotide position 22192 in dead *T. castaneum* from LC_{50} concentration application (Given in Supplementary data).

It is clear that more than one mechanism can exist in a single insect genome; whereas structural (amino acid changes) mutations continue to be the subject of debate relative to the regulatory (transcriptional) mutations. However, the possibility of a large number of different mutational events (eg; deletions, insertions, duplications) that form a series of complex alleles in a single locus has been largely overlooked.

In our study, we compared two CYP genomic regions of living and dead individuals from doses that kill 50% and 99% of adults with *T. castaneum* when we apply plant extract and found that there was a significant amount of mutation, especially in LC_{99}-derived individuals. These mutations suggest that the *CYP345A1* and *CYP6A14* genes may play a protective role against the toxic effect of the extract. The study has provided very specific results for the first time study of *B. turcicus* ektsract against a pest and for the first time *T. castaneum* CYP genes are being studied in Turkey. The results will be the basis for future work and further analysis of the CYP genes and, at the same time, the inclusion of up-regulation studies of the gene regions, allowing for a detailed analysis of the resistance mechanisms in the pest insects.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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