International Journal of Agricultural Technology 2014 Vol. 10(2): 367-381
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Assessment of The Biosafety of the Bioinsecticide-Agerin on Different Biological Systems

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El-Gawad, N. M. A. and Abdel-Aziz, S. H (2014). Assessment of the biosafety of the bioinsecticide-agerin on different biological systems. International Journal of Agricultural Technology 10(2):367-381.

Abstract In this research, the biosafety assessment of the bioinsecticide-Agrein was tested by the following concentrations 1-5, 2-5 and 3-5 g/l C_1,C_2 and C_3 respectively comparing with the control C_0 which showed that a great rate of aberration in most phases of mitotic division and the highest personality of aberration was 52%.. After the treatment of Vicia faba slower buds with the above concentrations of agrein 1-5 2-5 and 3-5 g/l C_1, C_2 and C_3 day after day, we found that the rate of aberration increases by increasment of the concentration in both of first and second meiosis and the highest percentage obtained was 46% with the higher concentration C_3 comparing with the control C_0. Agerin induced obvious alterations in the electrophoretic profiles of the seed proteins of Vicia faba. The maximum number of protein bands was 17 bands. Comparison between the treated samples and the control revealed the existence of some changes in the protein banding pattern among the treated samples. Treating the mice groups with the same doses of agrein,the electrophoresis of mice liver proteins indicate that the maximum number of bands was 24 bands and some protein bands existed in control C_0 and disappeared in the treated groups C_1,C_2 and C_3 and vice versa. Tumor suppressor gene p^{53} was affected by treating the mice with the three doses of agerin which revealed the occurrence of mutations.

Keywords: Agerin, biosafety.

Introduction

Biological pesticides are becoming recognized as an important factor in crops for forest protection and in insect vector control. These pesticides are natural compound resist the disease caused by some microorganisms. Bacterial insecticides, especially Bacillus thuringiensis (Bt.), have become important factors in insect control programs because of their efficacy and safety. *B. thuringiensis* is a living microorganism that kills certain insects and is used to kill unwanted insects in forests, agriculture and urban areas. In a purified form,

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some of the proteins produced by *B. thuringiensis* are actually toxic to mammals. However, in their natural form, acute toxicity of commonly-used *B. thuringiensis* varieties is limited to caterpillars, mosquito larvae, and beetle larvae. *B. thuringiensis* is closely related to *Bacillus cereus*, a bacterial that causes food poisoning and to *B. anthracies*, the agent of the disease anthrax. *Bacillus thuringiensis*, is facultatively anaerobic, endospore forming bacterium. It is characterized by its ability to form parasporal crystalline inclusions toxic to larvae of different insect orders. These proteinaceous are the basis for the commercial use of *B. thuringiensis* as a bioinsecticide, and since the begining of the 1950s, this bacterium has been used increasingly against various insect pests (Gert *et al*., 2002). Few studies have been conducted on the chronic health effects such as carcinogenicity or mutagenicity of *B. thuringiensis*. (Swadener, 1994). *B. thuringiensis* is a species of bacteria that has insecticidal properties affecting a selective range of insect orders. There are at least 34 subspecies of *B. thuringiensis* (De Barjac and Erachon, 1990) also called serotype or varieties) and probably over 800 strain isolates (Ellis, 1991) B. thuringiensis was first isolated in 1901 in Japan from diseased silk worm larvae. It was later isolated from Mediterranean flour moths and named *B. thuringiensis* in 1911 (Lambert and Peferoen, 1992). It was not until 1958 that *B. thuringiensis* was used commercially in the United States (Jenkins, 1992). *B. thuringiensis* products available in the United States are comprised of one var. morrisoni, which cause disease in moth and butterfly caterpillars and *B. thuringiensis* var. *israelensis* which causes disease in mosquito and black fly larvae; *B. thuringiensis* var. *aizawai* which cause disease in wax moth caterpillars and *B. thuringiensis* var. *tendebrionis*; also called var. Sandiego, which causes disease in beetle larvae. Farm's chemical hand book (1992) and (Entwistle *et al*., 1993). Other strains of *B. thuringiensis* have been discovered that exhibit pesticidal activity against nematodes, mites, flat warms, and protozoa (Feitelson *et al*., 1992).When conditions for bacterial growth are not optimal *B. thuringiensis*, form endospores. Which germinate at favurable conditions. Unlike many other bacteria when *B. thuringiensis* creates spores it also creates protein crystals. This crystal is the toxic component of Bacillus thuringiensis after the insects ingested it, the crystal is dissolved in the insect's alkaline gut. Then the insect's digestive enzymes break down the crystal structure and activates. *B. thuringiensis* insecticidal component called the delta endotoxin (δ-endotoxin). The delta endotoxin binds to the cells lining the midgut membrane and creates pores in the membrane, upsetting the gut's ion balance. The insect soon stops feeding and starves to death. If the insect is not susceptible to the direct action of the delta-endotoxin, death occurs after *B. thuringiensis* starts its vegetative growth inside the insect's gut. The spore

germinates after the gut membrane is broken, it then reproduces and makes more spores. This body-wide infection eventually kills the insect (British Columbia Ministry of Health, 1992). The earliest tests done regarding *B. thuringiensis* toxicity was conducted using *B. thuringiensis* var. *thuringiensis*, a *B. thuringiensis* strain known to contain a second toxin called beta-exotoxin or (Thuringiensis). Thuringiensis is an adenosine derivative linked through a glucose moiety to the 5H position of phosphorallaric acid, secreted outside the bacterial cell (Gunnel Carlberg *et al*., 1995). Due to the chemical composition a certain affinity to DNA is likely occured and, therefore, undesirable mutagenic properties of the toxin may exist (Frantivek *et al*., 1969). The objective of this study was to assess the potential mutagenicety of agerin bio-insecticide using different biological systems.

Materials and methods

Bioinsectcide:

Common name	***Bacillus thuringiensis***
Use	Biological Insecticide
Chemical name	Active Ingridient is a Microbial Protein Insecticide
Trade name	"Agerin"
Formulation	Wettable Powder Potency 32.000 IU/mg
Cover the following crops	Cotton, Potato, Maize and Stored grains Mode of action : Stomach larvicide
Company	Agricultural Genetic Engineering Research Institute (AGERI)

Used of Agerin were 1.5, 2.5 and 3.5 gm/l C_1,C_2 and C_3 resbectively, The recommended dose is 2.5g/ml to study the mitotic and meiotic division in Vicia faba, and mutagenic effects on mice. Vicia faba seeds (Giza3) were supplied by Legume Crops Research Section, Agricultural Research Center, Giza, Egypt. Albino Swiss mice, Webster strain, were used in this study, Animals were raised in the animal house, Department of Genetics, Faculty of Agriculture, Ain Shams University, Egypt.

Cytological Study using Mitotic in Vicia faba

Seeds of Vicia faba Giza 3 were germinated; root tips were fixed and stained using Feulgan Squach method according to Sharma and Sharma (1980). Chromosomal abnormalities were scored in the four mitotic division stages. The data were recorded statistically analyzed using t-test using Duncan new multiple rang test $P<0.05$)

Meiotic Division Assay

Vicia faba flower buds were sprayed by the three concentrations of insecticide 1.5, 2.5 and 3.5 gm/l twice, daily. Control plants were sprayed with distilled water. Flower buds were gathered 24 hours after the last spray, then fixed immediately in Carnoy's fluied (3 ethyl alcohol; 1 glacial acetic acid) and then examined using the aceto-carmine smear method according to (Sharma and Sharma, 1980). Abnormalities were counted in the first and second meiotic division. The data were recorded and statistically analyzed using t-test.

Biochemical Genetic Studies

SDS-Protein Electrophoresis

SDS-PAGE was performed on the protein of the samples according to (Laemmli, 1970) as modified by (Studier, 1973). One dimensional SDS-PAGE was used to study the proteins banding patterns of control versus treated plants using 15 % acrylamide gel electrophoresis.

Effect of Agerin on Experimental Mice

The mice were orally injected with the three concentrations C_1,C_2 and C_3 of agerin treatments, as well as, a control groups, for two weeks. Then the mice were killed and the samples were taken from the liver to be prepared for biochemical and molecular analysis.

Liver Proteins Extraction

About 0.5 gm of liver tissue with 1 ml of distilled water was ground using a mortar and pestle until liquefying the tissues.The crude saps of the treated animals and control ones were centrifuged for 10 min at 12000 rpm at 4 °C.

Supernatants were transferred to new tubes and stored at -20°C until electrophoresis analysis was performed.

DNA Isolation From Liver Tissues

Total genomic DNA was extracted from Mice liver tissues according to (Dellaporta *et al*., 1983).

PCR Analysis and Condition For P^{53} Gene

Three oligo-primers were synthesized with sequences corresponding to sequences in the exon 4 and 5 regions of GSTμ gene and a recently cloned related gene of the same multigene family p^{53} as described by (Hollstein, et al. 1993). The sequences of these primers were as follows:

OLF_1: $^{5`}$CGCCATCTTGTGCTACATTGCCCG$^{3`}$
OLF_2: $^{5`}$ATCTTCTCCTCTTCTGTCTC$^{3`}$
OLF_3: $^{5`}$TTCTGGATTGTAGCAGATCA$^{3`}$
PCR products were dissolved in agarose gel electrophoreticaly

Results and discussions

Cytological Studies

Effect of Agerin on Mitotic Division of Vicia faba

Agerin induced a chromosomal aberrations and abnormal cell division of the three treatments C_1,C_2 and C_3 of Vicia faba root cell as compared with the control C_0. The aberrations caused by treating the root cells with agerin are shown in Table 1. The frequency of mitotic abnormalities increased by increasing the concentrations of the insecticide. The maximum value of mitotic abnormalities was 52 % after treating with the highest concentration 3.5 gm/l C_3. Table 1 also showed the mean and the standard error of the studied aberrations caused by treating the root cells with Agerin. They were significantly different by Duncan's new multiple range test.

These results are in partial agreement with that obtained by (El-Ashry, 2003a) who reported genotoxic effect of cadmium chloride ($CdCl_2$) in Vicia faba plant by studying its effect on root growth and mitotic division and abnormal mitosis. However, Frantisek, *et al*.(1989) disagreed with the obtained result as they reported that using the Drosophila wing spot test. They had not found any genotoxic activity of *B. thuringiensis* β-exotoxin. Both the pure β-exotoxin and commercial microbial insecticide Biotoxibacillin containing β-

xotoxin were negative in the induction of somatic mutations as well as miotic recombination. Also Sharma *et al*. (1977) reported that the beta-exotoxin thuringiensin A and the protein subunit of the delta-endotoxin, both isolated from *B. thuringiensis*, resulted in a depressive effect on mitosis in root-meristem cells of Allium cepa, possibly by prolonging the cell-generation time.

Effect of Agerin on the Meiotic Behavior of Vicia faba

The aberrations caused by spraying the flower buds with the used doses of agerin twice day are shown in Table 2 which demonstrated the cytological effect of agerin insecticide. The frequency of abnormal cells generally increased as the concentration of the insecticide increased. The three applied concentrations of Agerin induced a considerable frequency of chromosomal aberrations in both the first and second division represently 46% at concentration C_3 as compared to the control C_0. Table 2 represented the mean and the standard error of the studied aberrations which are significantly different by Duncan's new multiple range test ($p < 0.05$). These results are in accordance with those of El-Ashry (2003b) who reported that phosphamidon produced several types of chromosomal abnormalities in either mitosis or meiosis. These results also agree with those of Abd El-Salam *et al*. (2000). They found that the different tested concentrations of the two insecticides (Pyrethroid and catabron) have mutagenic activity at the cytological level in six cotton varieties. Also the same results given by El-Sherbeny *et al*. (2002) which found that Cascade induced a significant percentage of abnormalities on meiosis of pollen grains of variety of Vicia faba (G12a2) that Pollen mother cells (PMCs) have some types of chromosomal abnormalities such as stickiness, lagging, bridges and spindle disturbance

Biochemical Genetic Studies

SDS-Protein Electrophoresis in Faba bean

The capability of agerin to induce cytological aberrations, denoting its capability to cause mutagenic effect, the agerin also induced obvious alterations in the electrophoretic profiles of the seed proteins of *Vicia faba* (Fig. 1 and Table 3). The maximum number of protein bands was 17 bands. Comparison between the treated samples and the control revealed the existence of some changes in the protein banding pattern among the treated samples. Band no. 4 was distinguished by its complete absence in treatments C_0, C_2 and C_3 , whereas it presented in treatments C_1, also band no. 3 was presented in treatments C_0 and C_1 and absent in treatments C_2 and C_3. However, band No 5 is

distinguished by its appearance in treatment C_2 and complete absence in treatments C_0, $C_1$and C_3. But band no. 12 was presented in treatments C_1 and C_3 and disappeared in treatments C_0 and C_2. It was also noted that band no. 8 is considered to be prominent band. The figure also showed that band no. 15 was absent in treatments C_0 and C_1 but appeared in treatments C_2 and C_3.

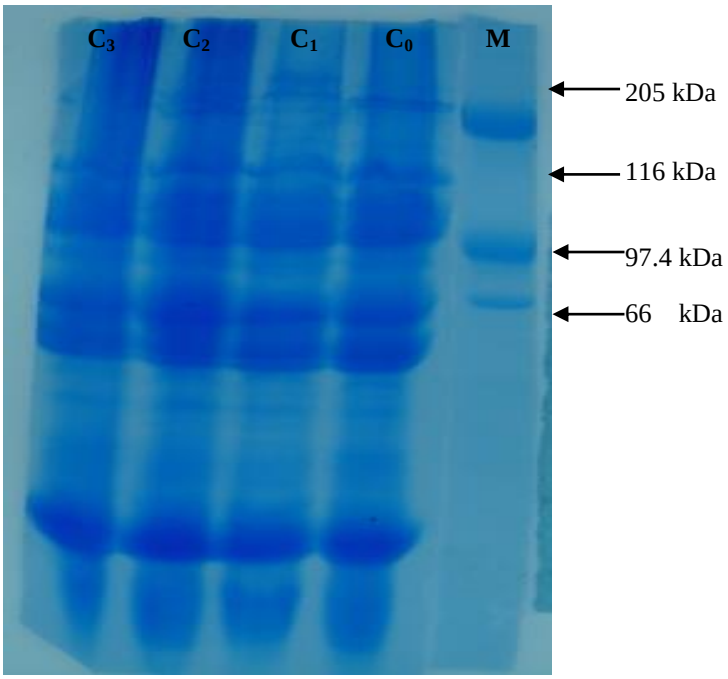

Fig. 1. SDS-PAGE bands of total protein for the control (C_0) and the three treatments (C_1,C_2 and C_3) with agerin in Vicia faba (from right to left).

Table 3. SDS-PAGE bands of total protein for the control (C_0) and the three treatments (C_1, C_2 and C_3) of Vicia faba with Agerin

Band No.	M.wt.	C_0	C_1	C_2	C_3
1	205	-	-	-	-
2		+	-	+	+
3		+	+	-	-
4	116	-	+	-	-
5		-	-	+	-
6		+	+	+	+
7		+	+	+	+
8	97.4	+	+	+	+
9		+	+	+	+
10		+	+	+	+
11		+	+	+	+
12		-	+	-	+
13		+	+	+	+
14		+	+	+	+
15	45	-	-	+	+
16		+	+	+	+
17		+	+	+	+

SDS-Protein Electrophoresis in Mice

Figure 2 and Table 4 showed the liver protein banding patterns for the control C_0 treated mice with the three doses C_1 , C_2, and C_3. From the figure the maximum number of protein bands was 24bands. The bands no. 4, 8, 15, 16 and 22 were absent in the control C_0 and present in each of three concentrations used C_1 , C_2, and C_3 . Bands no 11, 23 and 24 were found in the control C_0 and disappeared from the three C_1,C_2 and C_3 treatments.

These results were contradicting with that obtained by Ankrah *et al*. (1993) who reported that serum total protein and albumin levels were not affected by the exposure to aflatoxin B_1 and G_1 in mice via their feed. In this respect also Joanne *et al*. (2005) identified current control of the sheep blowfly Lucilia cuprina relies on chemical insecticides, however, with the development of resistance and increasing concerns about human health and environmental residues, alternative strategies of control this economically important pest are required. In this study, they have identified several isolates of *B. thuringiensis* collected from various Australian soil samples that produce crystals containing 130 and 28 kDa proteins. These isolates were highly toxic to feeding larvae in both in vitro bioassays and in vivo on sheep. By N-terninal amino acid sequencing, we identified the smaller crystal band (28 kDa) as a cytological

(Cyt.) protein. Upon solubilization and proteolytic processing were shown by trypsin, the 130 kDa crystal protein

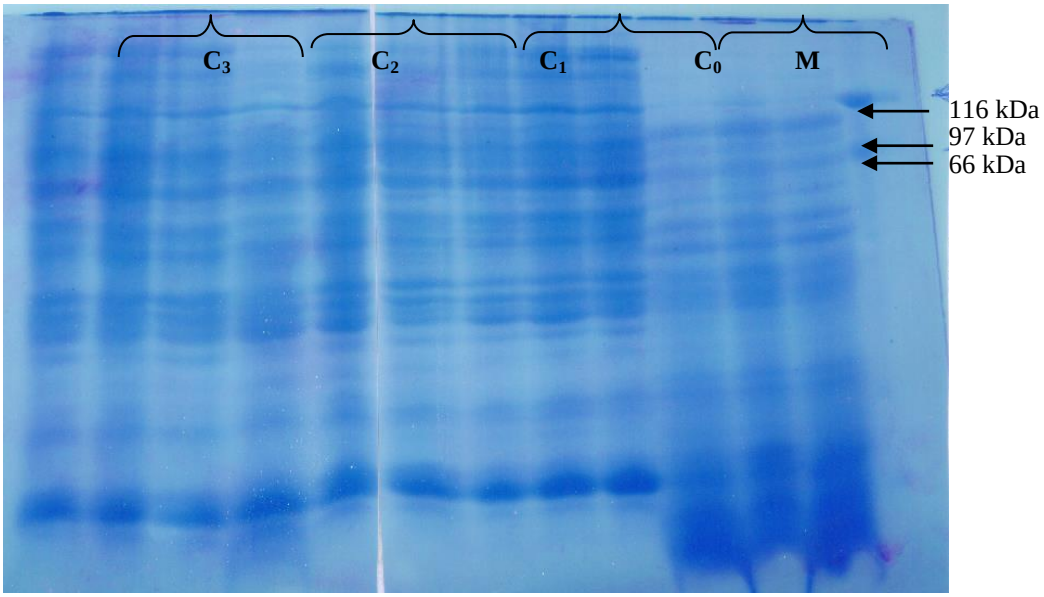

Fig. 2. SDS-PAGE bands of total protein for the control (C_0) and the three treatments (C_1,C_2 and C_3) with agerin in mice (from right to left).

Table 4. SDS-PAGE bands of total Protein for control C_0 and three treatments C_1 ,C_2 and C_3 of mice treated with agerin

Band No.	conc. M.wt.	C_0			C_1			C_2			C_3		
1		+	+	+	+	+	+	+	+	+	+	+	+
2		+	+	+	+	+	+	+	+	+	+	+	+
3		+	+	+	+	+	+	+	+	+	+	+	+
4		-	-	-	+	+	+	+	+	+	+	+	+
5		+	+	+	+	+	+	+	+	+	+	+	+
6		+	+	+	+	+	+	+	+	+	+	+	+
7		+	+	+	+	+	+	+	+	+	+	+	+
8		-	-	-	+	+	+	+	+	+	+	+	+
9		+	+	+	+	+	+	+	+	+	+	+	+
10		+	+	+	+	+	+	+	+	+	+	+	+
11		+	+	+	-	-	-	-	-	-	-	-	-
12		+	+	+	+	+	+	+	+	+	+	+	+
13		+	+	+	+	+	+	+	+	+	+	+	+
14		+	+	+	+	+	+	+	+	+	+	+	+
15		-	-	-	+	+	+	+	+	+	+	+	+
16		-	-	-	+	+	+	+	+	+	+	+	+
17		+	+	+	-	-	-	-	+	+	+	+	+
18		+	+	+	+	+	+	+	+	+	+	+	+
19		-	-	-	+	+	+	-	-	-	-	-	-
20		+	+	+	+	+	+	+	+	+	+	+	+
21		+	+	+	+	+	+	+	+	+	+	+	+

22	-	-	-	+	+	+	+	+	+	+	+	+
23	+	+	+	-	-	-	-	-	-	-	-	-
24	+	+	+	-	-	-	-	-	-	-	-	-

In this respect also Peter *et al*. (1985) reported that alkaline-dissolved crystal δ-endotoxin from "*B. thruingiensis*" var. israelensis (serovar H14) was injected into "mice" and seven species of insects representing the orders Lepidoptera, Orthoptera, Coleoptera, Hemiptera, and Diptera. High in vivo "toxicity" at 1 to 5 ppm (μg toxin/g body wet wt), was observed with mice and some insects, including some that are not sensitive to the toxin when administered orally. Neuromuscular effects were observed when the toxin was injected directly into the body cavity of the test animals. Biochemical studies suggested that different protein fragments within the crystal δ-endotoxin may be responsible for the majority of the mosquito larvacidal activity and the neurotoxic symptoms observed in larvae of *Trichoplusia ni* presented results indicated that the 28 K polypeptide is the mammalian toxic component of BT_1 crystals.

DNA Analysis in Mice

Figure 3 showed that while the control treatment C_0 exhibited the two characteristic bands of the normal p^{53} at 160 bp and 130 bp, each of the three concentrations C_1, C_2 and C_3 of agerin exhibited only one band which indicates the occurance of mutation due to the agerin effect. Such potential mutation could reflect (a) serious hazards to those handling this bioinsecticide. The general conclusion is that mutation in the P^{53} tumor suppressor gene is closely linked with the high incidence of several types of mammalian cancer including human and mice (Hollstein *et al*., 1991). Cancer is now recognized as a genetic disorder at the cellular level that involves the mutation of a small numbers of genes. Many of these genes normally act to suppress or stimulate progression through the cell cycle, and loss or inactivation of these genes causes uncontrolled cell division and tumor formation mutation in P^{53} is a G to T transversion in the 3^{rd} nucleotide of codon 249 (Hsu *et al*., 1991). This specific G to T transversion is consistent with the occurrence of DNA damage induced by AFB_1 since the mutagenic metabolite induced this type of base change (Foster *et al*., 1983). The same patients of AFB_1 adducts in liver DNA and serum albumin, and the polymorphism of glutathione-s-transferase μ, and enzyme was reported to be involved in the detoxification of AFB_1 (Leu *et al*., 1991).

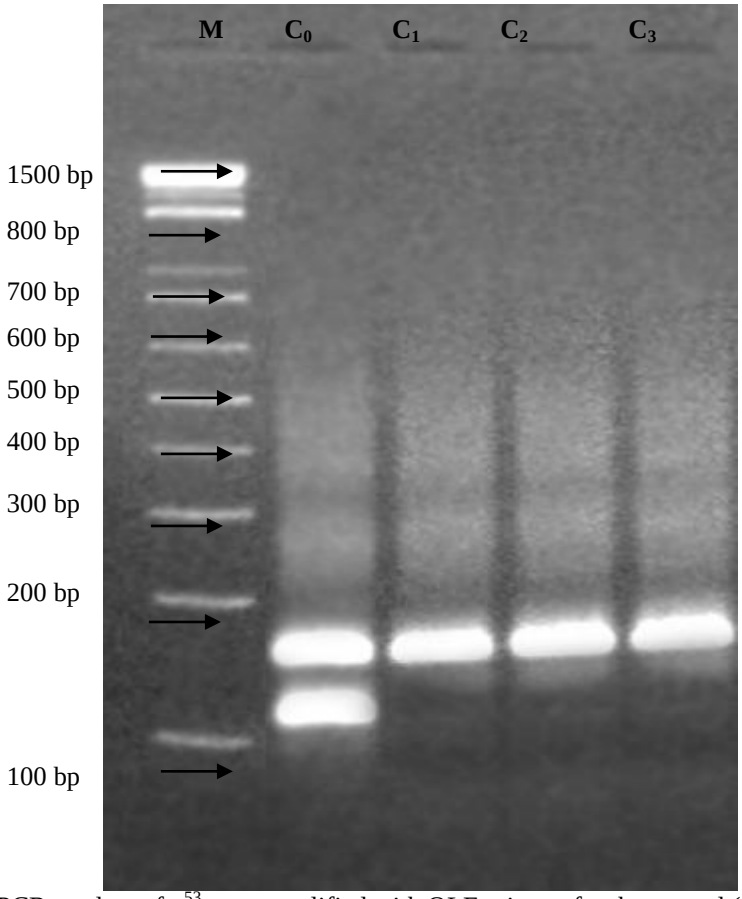

Fig. 3. PCR product of p^{53} gene amplified with OLF primers for the control (C_0) and the three treatments (C_1,C_2 and C_3) with agerin in mice (from left to right).

These results also agreement with Hussein and Cerutti (1993) they investigated the mechanism of formation of mutation in codon 249 of the P^{53} tumor suppressor gene in hepatocarcinogenesis. They suggested that both mutability at the DNA level and altered function of the mutant serine 249 P^{53} protein are responsible for the observed mutational hot spot in p^{53} in hepatpocellular carcinoma (HCC) from AFB_1, contaminated areas. Also these results agreement with Wu *et al*. (2000) and Wang *et al*. (2005) they investigated that deltamethrin leads to the persistent increase of p^{53} expression which may contribute to apoptotic cell death in rat brain following deltamethrin treatment. Also agreement with Mobio *et al*. (2003) , Wang *et al*. (2005) and Tong *et al*. (2006) they suggested that the human DNA context of the p^{53} gene alone may not be the sole determinant of AFB1-induced mutagenesis. Furthermore, humanized p^{53} appears not to be as effective as murine. Also Fu *et al*. (2005) and Penttinen *et al*. (2007) they found that same results among

mutation p^{53} tumor suppressor gene significantly both in vitro and in vivo . In addition the spores of *Streptomyces californicus* alone induced a 1.5-fold increase in DNA damage compared to control, dose dependent p53 accumulation and also extensive cytotoxicity. Also Farazi *et al*. (2006) reported that, in the setting of intact telomeres, p53 mutation had no effect on hepatocarcinogenesis, whereas in the setting of telomere disfunction, p^{53} mutation enabled advanced hepatocellular carcinoma of the wild-type p^{53} alleile in the late generation mTerrt(-/-)p^{53}(+/-)mice, suggesting that reduced levels of p^{53} potently enable hepatocellular carcinoma progression in the setting of telomere disfunction.

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(Received 11 January 2014; accepted 28 February 2014)

Table 1. Different types of abnormalities in root-tip cells after seed soaking for 24h of Vicia faba with the control C_0 and three doses C_1,C_2 and C_3 of agerin

Doses gm/L	**Total No. of counted cells**	**Chromosomal Aberration**								**Total No. of aberration cells**	**% of abnorm-alities**	**Mean ±Std. Error**
		Break	Sticky	Bridge	Lagger chromosome	Micro nuclei	Ring	Disturbed chromosome	Fragmentation			
C_o	50	2	-	-	-	-	-	-	-	2	4	-1.1250±1.12748
C_1 1.5 gm/l	50	3	2	3	1	1		1	-	11	22	1.1250±1.12748(*)
C_2 2.5 gm/l	50	7		2	-	1		5	3	18	36	2.0000 ±1.1274.8(*)
C_3 3.5 gm/l	50	10	3	4	-	-	1	6	2	26	52	3.0000±1.12748

(*) Significantby Duncan test

Table 2. Different types of abnormalities in the meiosis of Vicia faba after spraying of the flower buds with the control C_0 three doses C_1 , C_2 and C_3 of agerin twice in the field

Doses gm/L	**Total No. of counted cells**	**Chromosomal Aberration**								**% of abnorm-alities**	**Mean ±Std. Error**
		Break	Sticky	Bridge	Lagger chromosome	Micro nuclei	Ring	Disturbed chromosome	Fragmentation		
C_o	50	-	-	-	-	-	-	-	-	-	-1.3750±0.68547
C_1 1.5 gm/l	50	2	3	2	2	-	1	-	1	22	1.3750±0.68547(*)
C_2 2.5 gm/l	50	3	3	1	-	2	1	-	3	26	1.6250 ±0.68547(*)
C_3 3.5 gm/l	50	6	3	2	-	1	2	3	6	46	2.8750±0.68547(*)

(*) Significant by Duncan test.