ASSESSMENT OF THE BIOSAFETY OF THE BIOINSECTICIDE AGERIN ON DIFFERENT BIOLOGICAL SYSTEMS

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Biological pesticides are becoming recognized as an important factor in crops and forest protection and in insect vector control. These pesticides are natural, disease causing microorganisms such as viruses, bacteria and fungi, which infect specific pest group. Bacterial insecticides, especially *Bacillus thuringiensis*, have become important factors in insect control programs because of their efficacy and safety.

Bacillus thuringiensis (Bt.) 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, 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 *B. cereus*, a bacterial that causes food poisoning and to *B. anthracies*, the agent of the disease anthrax. Few studies have been conducted on the chronic health effects such as carcinogenicity or mutagenicity of *B. thuringiensis*. (Carrie, 1994). *B. thuringiensis* is a species of bacteria that has insecticidal properties affecting a selective range of insect orders. There are at least 34 sub species of *B. thuringiensis* (De Barjac *et al.*, 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 *Bacillus thuringiensis* in 1911 (Lambert and Peferoen, 1992).

It was not until 1958 that *B. thuringiensis* was used commercially in the United States (Jenkins, 1992) *Bacillus thuringiensis* products available in the United States are comprised of one var. *morrisoni*, which cause disease in moth and butterfly caterpillars; *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. (Entwistle, 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*, like many bacteria, form spores. Spores are the dormant stage of the bacterial life cycle, when the organism waits for better growing conditions. Unlike many other bacteria when *B thuringiensis* creates spores it also creates protein crystals. This crystal is the toxic component of *B. thuringiensis* after the insects ingest *B. thuringiensis*, the crystal is dissolved in the insect's alkaline gut. Then the insect's digestive enzymes break down the crystal structure and activates. Bt's 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 (BCMH, 1992).

The earliest tests done regarding Bt's toxicity were 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 *et al.,* 1995). Due to the chemical composition a certain affinity to DNA is likely to occur and, therefore, undesirable mutagenic properties of the toxin may exist (Frantivek *et al.,* 1989).

The objective of this study was to assess the potential mutagenic effect of AGERIN® bio-insecticide using different biological systems.

MATERIALS AND METHODS

The concentrations used of AGERIN® a commercial bio-insecticide (Bt) approved by both USA and Egyptian authorizes were 1.5, 2.5 and 3.5 g/L (The recommended dose is 2.5 g/L) to study their effect of mitotic and meiotic division of *Vicia faba* (Giza 3) which were supplied by Legume Crops Research Section, Agricultural Research Center. Giza, Egypt. In addition, mutagenic effects were assessed on Albino Swiss mice, Webster strain. Animals were raised in the animal house of the Department of Genetics, Faculty of Agriculture, Ain Shams University.

Cytological study

Mitotic in Vicia faba

Seeds of *Vicia faba* (Giza 3) were germinated in rolls of filter paper moisted with tap water at room temperature (20-25) .When the secondary roots reached 2-3 cm in length, three groups were treated with the a fore mentioned concentrations as well as a control. The roots were cut off after treatment for (24 h), fixed and stained using Feulgan squach method

(Sharma, 1980). Chromosomal abnormalities were scored in the prophase, metaphase, anaphase and telophase stages and in interphase cells. The data were statistically analyzed using t-test ($P<0.05$).

Meiotic study

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

Biochemical genetic studies

SDS-Protein electrophoresis

SDS-PAGE was performed on the protein of the samples according to the method of Laemmli (1970) as modified by Studier (1973). One dimensional SDS-PAGE was used to study proteins and separate them based on their molecular sizes. Proteins were fractionated on 15% acrylamide concentration.

Mice experiment

The mice were orally injected with either of the three concentrations of AGERIN® treatments, as well as, a control group, for two weeks. Then the mice were killed and the samples were taken from the liver for biochemical and molecular analysis.

Extraction of protein Form liver

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 treated samples 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 analysis was performed.

DNA isolation from liver tissues

DNA was isolated from liver tissue. About one gm of each mice liver. Liver as used to isolate the individuals genomic DNA, then the three replication of each untreated and treated groups were bulked together. Finally, three bulked samples were screened for the existence of p53 tumor suppressor gene mutation (Abdel-Tawab *et al.,* 1998).

PCR analysis and condition for p53 gene

Three oligo-primers were synthesized with sequences corresponding to sequences in the exon 4 and 5 regions of GSTμ gene and a cloned related gene of the same multigene family (p53) 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$^{3`}$

RESULTS AND DISCUSSION

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 bio-insecticide, and since the beginning of the 1950s, this bacterium has been used increasingly against various insect pests. Gert *et al*. (2002) were suggested that the entire group represents a single species.

1. Cytological studies

Effect of AGERIN on mitotic division of Vicia faba

AGERIN induced a chromosomal aberration and abnormal cell division of the three treatments of faba bean root tips as compared with the control. The aberrations caused by treating the root tips 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 (C_3)

Table (1) also observed aberrations caused by treating the root tips with AGERIN. They were significantly different by t-test.

These results are in partial agreement with those obtained by El-Ashry (2003), 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, Frantivsek *et al.* (1989) disagreed with our result as they reported that using the *Drosophila* wing spot test. They have 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.

Sharma *et al.* (1977) reported that the β-exotoxin thuringiensin A and the protein subunit of the delta-endotoxin, both isolated from *Bacillus 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 different doses of AGERIN twice a 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 the second meiotic divisions. The maximum values of meiotic abnormalities were 46% which was ob-

served with the highest concentration (C_3) as compared to the control value.

Table (2) showed the studied aberrations which were significantly different by t-test ($p<0.05$). These results are in accordance with those of El Ashry (2003) 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) who found that the different tested concentrations of the two insecticides, parathyroid and catabron, have mutagenic activity at the cytological level in six cotton varieties. El-Sherbeny *et al*. (2002) also agreed with our results who reported that Cascade induced a significant percentage of abnormalities on meiosis of pollen grains of a variety of *Vicia faba* as pollen mother cells (PMCs) have some types of chromosomal abnormalities such as stickiness, lagging, bridges and spindle disturbance.

2. Biochemical genetic studies

2.1. SDS-protein electrophoresis in Vicia faba

The capability of AGERIN to induce cytological aberrations, denoting its possible mutagenic effect, the AGERIN also induced obvious alterations in gene expression as indicated by the electrophoretic profiles of the seed proteins of *Vicia faba* (Fig. 1 and Table 3). The maximum number of bands was 17. 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 C, C_2 and C_3 treatments, while it appeared in C_1. The figure also showed that band no. (3) was present in treatment C and C_1 and absent in C_2 and C_3. However, band no. (5) was distinguished by its appearance in C_2 and complete absence in C, C_1 and C_3. But band no. (12) was present in C_1 and C_3 and disappeared in C 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 C and C_1 but appeared in C_2 and C_3.

It is evident form Table (3) that inconsistent occurrence of protein profiles were not indication of a clear-cut effect of AGERIN on protein expressionin in *Vicia faba*. However both, bands number 12 and 15 showed that toxicity of AGERIN might have caused the appearance of each of these two bands as a result of treatment while such band was absent in their respective control.

1. SDS-protein electrophoresis in mice

Figure (2) and Table (4) showed liver protein banding patterns for the treated male with the three doses (C_1, C_2, C_3) and the control. From the figure the maximum number of bands was 25. Bands no. (4, 8, 15, 16 and 22) were absent in the control and present in each of the three concentrations. Bands No (11, 23 and 24) were found in the control and disappeared from the three treatments.

However, these results was contradicting with those of 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.

Joanne *et al.* (2005) identified current control of the sheep blowfly (*Lucilia cuprina*) relied on chemical insecticides, however, with the development of resistance and increasing concerns about human health and environmental residues, alternative strategies of controlling this economically important pest were required. They identified several isolates of *Bacillus thuringiensis* (Bt) 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, they identified the smaller crystal band (28 kDa) as a cytological (Cyt) protein. Upon solubilization and proteolytic processing by trypsin, the 130 kDa crystal protein yielded among others, a truncated 55-60 kDa toxin moiety which exhibited larvacidal activity against sheep blowfly. The amino-terminal sequence of the trypsin-resistant protein band revealed that this *Bt* endotoxin was encoded by a new cry gene. The novel cry protein was present in all the strains that were highly toxic in the larval assay. They also identified from one of the isolates, a novel secretory toxin with larvacidal activity.

Peter *et al*. (1985) reported that alkaline-dissolved crystal δ-endotoxin from "*Bacillus 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), they observed with mice and some insects, including some that are not sensitive to the toxin when administered orally, and some neuromuscular effects occurred 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 *Trichoplusiani*. The present results indicated that the 28 K polypeptide is the mammalian toxic component of BTI crystals.

DNA analysis in mice

Figure (3) shows that while the control treat meat exhibited the two characteristic fragments of the normal p53 at (~160 bp and 130 bp), each of the three concentrations of AGERIN exhibited only one band which indicates the occurrence of mutation due to the AGERIN effect. Such potential mutation could reflect serious hazards to those handling this bio-insecticide.

The general concession is that mutation in the p53 tumor suppressor genes 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 p53 is a G to T transversion in the 3rd 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).

These results are in agreement with Hussein and Cerutti (1993) who investigated the mechanism of formation of mutation in codon 249 of the p53 tumor suppressor gene in hapatocarcinogenesis. They suggested that both mutability on the DNA level and altered function of the mutant serine 249 p53 protein are responsible for the observed mutational hot spot in p53 in hepatpocellular carcinoma (HCC) from AFB_1.

Wu *et al.* (2000) indicated that deltamethrin leads to the persistent increase of p53 and Bax expression and transient elevation of Bcl-2 expression, resulting in an increased ratio of Bax to Bcl-2, which may contribute to apoptotic cell death in rat brain following deltamethrin treatment.

Mobio *et al*. (2003) suggested a possible loss of protective mechanisms (such as p53-dependent apoptosis cycle arrest) in FB_1-damage MEF cells and confirm that cells lacking mechanisms governed by p53 gene would susceptible to neoplastic cascade or mutation following DNA lesions induced by this mycotoxin.

Wang *et al.* (2005) reported that expression of p53 and Bax in each treatment group increased significantly compared with that in control group ($p<0.05$), with the exception of 0.1 microg/kg LR exposure group. Moreover, with exposure levels increasing the expression of p53 and Bax increased gradually; while no changes of the expression of Bel-2 were observed. They concluded that p53 and Bax may play important roles in microcystin LR induced apoptosis, but Bel-2 seems not to be involved in this process.

SUMMARY

Effect of AGREIN on the mitotic behavior was studied in *Vicia faba*. AGREIN was tested by the following concentrations "1.5, 2.5 and 3.5 " g/L which showed high rats of aberrations in most phases of mitotic division and the highest incidence of aberration was 26% after treating with the highest concentration comparing with the control. As for the effect of AGREIN on the meiotic behaviors of *Vicia faba*, the rate of aberration increased with increasing the concentration on both the first and the second meiosis and the highest percentage obtained was 46% with the highest concentration comparing with the control.

AGERIN induced considerable alterations in the electrophoritic profiles of the seed proteins of *Vicia faba*. The maximum number of bands was 17. Compari-

son between the treated samples and the control revealed the existence of some variations in the protein banding pattern between the treated samples on the mice treated with doses of "1.5, 2.5 and 3.5" g/L of AGREIN. The electrophoresis patterns of mice liver protein indicated that the maximum number of bands was 24 and some protein bands existed in control and disappeared in the treated groups and vice versa. Tumer suppressor gene p53 was affected by treating the mice with the three doses of AGERIN which revealed the occurrence of mutation. Such mutation could reflect a serious hazard to those handling this bio-insecticide.

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Table (1): Different types of abnormalities in root-tip cells after seed soaking for 24h of *Vicia faba* with different doses of AGERIN.

Doses gm/l	Total no. of counted cells	Chromosomal Aberration									% of abnormalities
		Break	Sticky	Bridge	Lagger chromo-some	Micro nuclei	Ring	Disturbed chromosome	Fragmentation	Total no. of ab-erration cells	
C_o	50	2	-	-	-	-	-	-	-	2	4
C_1 1.5 gm/l	50	3	2	3	1	1	-	1	-	11	22*
C_2 2.5 gm/l	50	7	-	2	-	1	-	5	3	18	36*
C_3 3.5 gm/l	50	10	3	4	-	-	1	6	2	26*	52**

* and ** significant at 5% and 1% using t test

Table (2): Different types of abnormalities in the meiosis of *Vicia faba* after spraying of the flower buds with different doses of AGERIN twice in the field.

Doses gm/l	Total no. of counted cells	Chromosomal Aberration									% of abnormalities
		Break	Sticky	Bridge	Lagger chro-mosome	Micro nuclei	Ring	Disturbed chromosome	Fragmentation	Total no. of ab-erration cells	
C_o	50	-	-	-	-	-	-	-	-	-	-
C_1 1.5 gm/l	50	2	3	2	2	-	1	-	1	11	22*
C_2 2.5 gm/l	50	3	3	1	-	2	1	-	3	13	26*
C_3 3.5 gm/l	50	6	3	2	-	1	2	3	6	23	46**

* and ** significant at 5% and 1% using t test

Table (3): SDS-PAGE bands of total protein for the control and the three treatments of *Vicia faba* treated with different concentrations of AGERIN.

Band No.	M. wt.	C	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		+	+	+	+

Table (4): SDS-PAGE bands of total protein for control and three treatments of Mice treated with different doses of AGERIN.

Band No.	M.wt.	C			C_1			C_2			C_3		
1		+	+	+	+	+	+	+	+	+	+	+	+
2		+	+	+	+	+	+	+	+	+	+	+	+
3		+	+	+	+	+	+	+	+	+	+	+	+
4	116	-	-	-	+	+	+	+	+	+	+	+	+
5		+	+	+	+	+	+	+	+	+	+	+	+
6		+	+	+	+	+	+	+	+	+	+	+	+
7		+	+	+	+	+	+	+	+	+	+	+	+
8	97	-	-	-	+	+	+	+	+	+	+	+	+
9		+	+	+	+	+	+	+	+	+	+	+	+
10		+	+	+	+	+	+	+	+	+	+	+	+
11	66	+	+	+	-	-	-	-	-	-	-	-	-
12		+	+	+	+	+	+	+	+	+	+	+	+
13		+	+	+	+	+	+	+	+	+	+	+	+
14		+	+	+	+	+	+	+	+	+	+	+	+
15		-	-	-	+	+	+	+	+	+	+	+	+
16		-	-	-	+	+	+	+	+	+	+	+	+
17		+	+	+	-	-	-	-	+	+	+	+	+
18		+	+	+	+	+	+	+	+	+	+	+	+
19		-	-	-	+	+	+	-	-	-	-	-	-
20		+	+	+	+	+	+	+	+	+	+	+	+
21		+	+	+	+	+	+	+	+	+	+	+	+
22		-	-	-	+	+	+	+	+	+	+	+	+
23		+	+	+	-	-	-	-	-	-	-	-	-
24		+	+	+	-	-	-	-	-	-	-	-	-

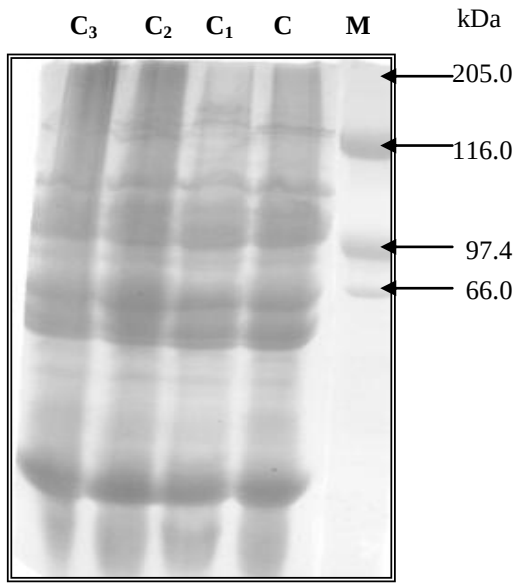

Fig. (1): SDS-PAGE bands of total protein for the control (C) and the three concentrations C_1, C_2, C_3 of AGERIN in *Vicia faba* (from right to left).

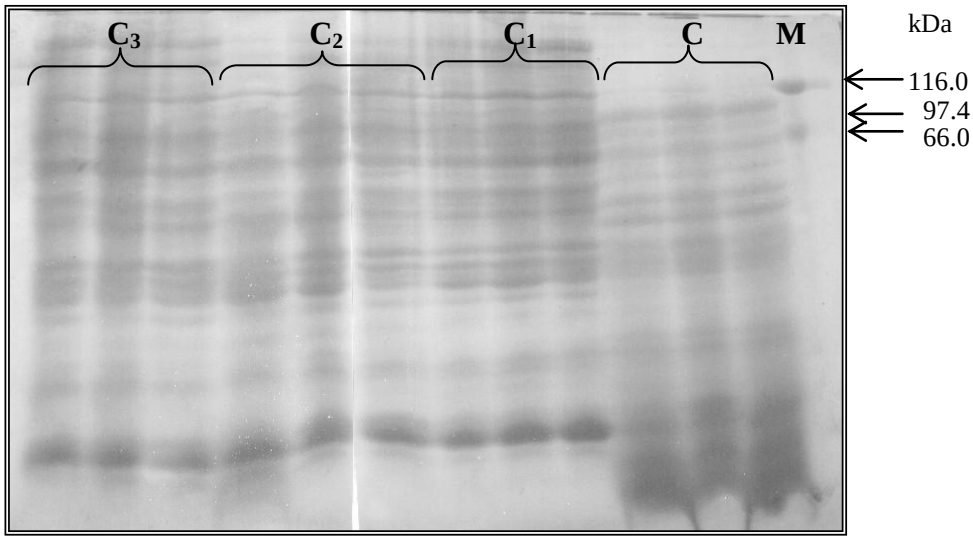

Fig. (2): SDS-PAGE bands of total protein for the control and the three treatments with AGERIN in mice (from right to left).

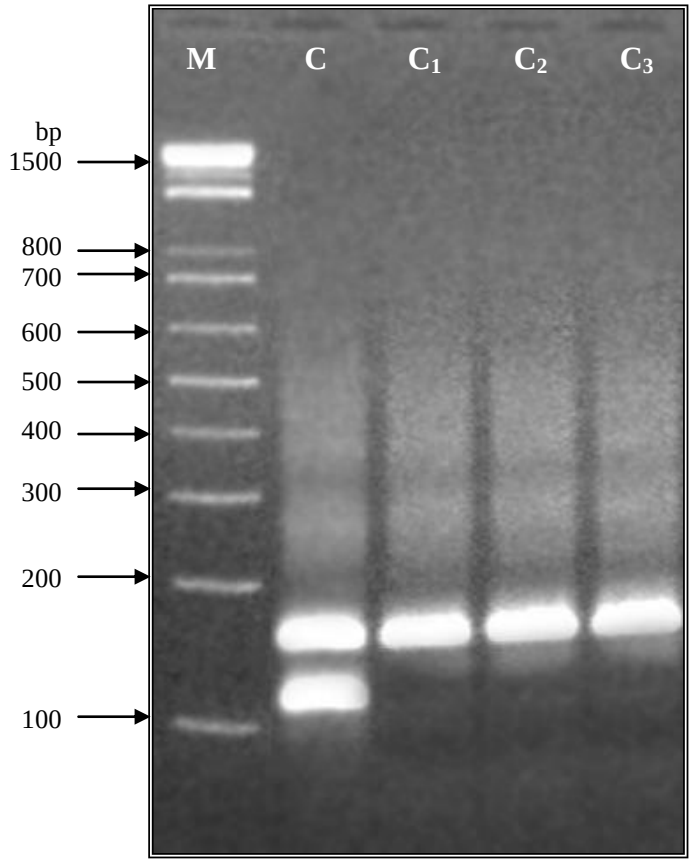

Fig. (3): PCR product of p53 gene amplified with OLF primers for the three groups treated with the three doses of AGERIN and the control.