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A Biological Approach to Control Aflatoxins by *Moringa Oleifera*

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Abstract: This present research work has been designed to evaluate medicinal plant *moringa oleifera* leaves against *A. flavus* and *A. parasitiucus*, to minimize the fungus growth and aflatoxin production in spice sample during storage. Biological procedures have been used so far for controlling aflatoxins in the spice samples. The spice sample was inoculated with *A. flavus* and *A. parasitiucus* were stabilized with *M. oleifera* and stored at specific moisture (16%) and temperature (28°C) conditions for 8 months. The aflatoxins significantly increase during incubation period ($p<0.05$) and also increased by inoculation with both fungal strains in both spice samples. The contaminated spices the antifungal agents (*Moringa oleifera* leaves) were added to control the aflatoxins production and to investigate their efficiency. After regular intervals, samples were drawn and levels of aflatoxins were estimated by HPLC. The investigated medicinal plants *M. oleifera* extract were found to be more effective as they fully inhibited (100%) aflatoxin production throughout the entire storage period. Overall results of the present study showed that *M. oleifera* can be used effectively to make agents to control aflatoxin production in spice sample. It was observed that *Moringa oleifera* at all steps to retard aflatoxin production for eight months were found to be very excellent ($p<0.05$) potential agents.

Keywords: *Moringa Oleifera*, Aflatoxins, A. Flavus, A. Parasitiucus

1. Introduction

Aflatoxins are a group of naturally occurring mycotoxins that are produced by two closely related fungi *Aspergillus flavus* and *Aspergillus parasitiucus*. They are highly toxic, carcinogenic and mutagenic in humans and animals. Aflatoxins contaminate the food during growth, harvest and storage. These fungus grow in soil, grains and also in decaying vegetation [1, 2]. These natural food contaminants are found in more than hundred kinds of agriculture products and in different kinds of food [7]. These fungi can grow under favourable temperature and humidity conditions on different in improperly stored commodities and foodstuffs like maize, nuts, wheat, and in spices [3, 4].

Aflatoxins enter into the food supply when contaminated food is processed where they have been transferred in both animals and human food. For example, its toxicity is 416 times of melamine, 68 times in arsenic and ten times of in potassium cyanide [8]. Moreover, the carcinogenicity is 10000 times of Benzene Hexachloride (BHC) than that of dimethylnitrosamine. Fungus *A. flavus* shows large variation to produce aflatoxins. Carcenogenic *A. flavus* strains usually produce only two types of aflatoxins, AFB1 and AFB2, but four toxins could be produced by strains of *A. parasiticus* [5, 6]. Aflatoxins are divided into two groups on the basis of their chemical structure; difuro-coumarocyclopentenone and the difuro coumarolactone. Where difuro coumaro cyclopentenone contains Aflatoxin B_1 and Aflatoxin B_2, however difuro-coumarolactone possesses AFG_1 and AFG_2. These two are less toxic as compared to Aflatoxin B_1 [3]. AFM_1 and M_2 are the milk toxins and are the metabolites of AfB_1 and AfB_2, respectively [17, 18].

The plants have been playing a vital role all over the world for humans. *Moringa oleifera* plant leaves can be used as an antiaflatoxigenic agent. In *M. oleifera* antifungal agents are present as an alternate to prevent from fungal contamination in food [19, 20]. Medicinal plants *M. oleifera* were investigated for inhibition of AFTs in spice sample. The aim

of this research was to evaluate medicinal plants potential to inhibit aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasitiucus* during storage. The treatment efficiency was evaluated on the basis of AFT inhibition and nutritional value of the stored spice sample over the period of eight months of storage [21].

2. Materials and Methods

Plant collection, Pretreatment and Extraction preparation:

Moringa oleifera leaves were obtained from the vicinity of Lahore Garrison University, Lahore, Pakistan. *Moringa oleifera* leaves were washed and then dried at 40°C in an oven (Memmert, Germany). By using a commercial blender the dried leaves were grounded into fine powder, and then this material used for extraction. The grounded samples were stored in polythene bags at 4°C for further analysis.

Five solvent systems (100% methanol, 80% methanol, Absolute ethanol and Aqueous ethanol and distilled water) were used for bioactive compounds extraction. In this regard powdered leaves (20g) were extracted for 8 hr with 200mL water in an orbital shaker. What man No. 1 filter paper was used for separation of extract from residue. With the same solvent system the residues were extracted twice and at 45°C extract were dried. To calculate the yield the dried extracts were weighed [12].

2.1. Spice Sample Treatment

2.1.1. Collection of Spice Samples

We will take 1Kg of each kind of freshly prepared anti-toxin free spice samples i.e. black pepper and cumin was obtained from market, Lahore.

2.1.2. Pre-treatment and Inoculation

The spice samples were dried at 60°C in an oven. The spice samples were divided separately into 4 lots. Every lot having 200 gm of spice sample. Each 4 lots of spice were autoclaved separately. With sterilized distilled water the spice samples were moistened (16%). Spice samples inoculation was done by adding 4mL of spores of *Aspergillus flavus* and *Aspergillus paraciticus* to each bag one by one. Then it was mixed thoroughly in air laminar flow chamber [13].

2.1.3. Natural Treatments for the Stabilization of Spice Samples

Powdered form of *Moringa oleifera* leaves and 3 different concentrations of 5%, 10% and 15% w/w of spice sample were added separately to each plastic bag. It containing 200gm of inoculated spices and thoroughly shakes it. Untreated spice sample (without *Moringa oleifera*) was used as control from each type of spice. The spices samples control and treated having 16% moisture were stored at 28°C for a period of 8 months.

Spice samples were analysed after regular interval of time for aflatoxin by HPLC technique. The difference in the inhibitory effect of treatments was estimated in amount of aflatoxins produced in treated samples as compared to control on production of aflatoxins [13, 14].

2.2. Aflatoxins Extraction and Analysis

For the extraction of aflatoxins from spices the [19] method was used. In a conical flask about 5g sample was taken. Then mixed it with 20 mL of Acetonitrile: water (84:16) which is an extraction solvent. At ambient conditions shake it for 90 minutes in an orbital shaker. The filteration of extract was done with Whatman filter paper No. 4**.**

2.3. Derivitization of Aflatoxin

To increase aflatoxin recovery and detection pre-column derivatization of aflatoxin was done. To the dried vial 200µL n-hexane was added and then for 30s vortexed it. Then TFA (50µL) was added. Sample was used for HPLC analysis and again vortexed it for 20s.

The instrument was validated before analyzing the spice samples of the present study to check its accuracy, LOD and precision. Solutions of aflatoxins in acetonitrile with concentration of 0, 0.05, 0.1, 0.5, 1.0, 5.0, 10µgL^{-1} by using a series of calibration curve were drawn [15, 17].

$$\text{Inhibition percentage}=[Y\text{-}X/Y] \times 100$$

2.4. Conditions for Analysis of HPLC

For aflatoxins analysis the conditions used were as:

System controller unit	SCL-10A
Shimadzu HPLC system	LC-10A
Dual pumps	LC-10AS
Column oven	CTO-20A (Shimadzu, Japan)
Discovery C-18 Column	250 mm × 4.6 mm (Supelco, Bellefonte, PA, USA)
Column Temp	30-35°C
Total quantity Injected	20 µL
Acetonitrile: methanol: water	22.5: 22.5:55
Flow rate of sample	1.5mL min^{-1}
Reverse phase (HPLC)	SPD-10A (190-600
For Detection	RF-530 (Japan)
Excitation	360 nm
Wavelength of emission	440 nm

2.5. Statistical Analysis

The data was reported as mean ± SD and experiments were performed in triplicate (n=3). For analysis of variance procedures of ANOVA were performed.

3. Results

In this research paper the spices (black pepper and cumin) were inoculated for the production of aflatoxins with purified fungal strains. Then in the contaminated spices the antifungal agents (*Moringa oleifera* leaves) were added to control the aflatoxins production and to investigate their efficiency. At specific moisture (16%) and temperature (28°C) the spices

were stored for eight months. It was observed that *Moringa oleifera* at all steps to retard aflatoxin production for eight months were found to be very excellent (p<0.05) potential agents. Aflatoxins analysis was carried out and the spice samples were drawn out at regular interval. In the present study four mycotoxins (B1, B2, G1 and G2) are present in aflatoxins. These are produced by two closely linked fungi *A. parasiticus* and *A. flavus*. Toxigenic strains of *A. Flavus* produce only two types of aflatoxins, B1 and B2. Strains of *A. parasiticus* produce aflatoxins of four types (B1, B2, G1 and G2). It is in agreement with study of Dorner 2004 and Varga et al. (2003) who reported that *A. parasiticus* produces AFB1, B2 and AFG1, G2, while *A. flavus* produces only of two types AFB1and B2. Most carcinogenic and poisonous form is AFB1 [9]. In the present study, and all *Moringa oleifera* extract showed a good result against *A. paraciticus* than *A. Flavus* regardless of spice samples. By done a Statistically analysis shown that in treated samples and control in aflatoxins production by *A. Flavus* and *Paraciticus* there was significant difference (p<0.05). The aflatoxins significantly increase during incubation period (p<0.05) and also increased by inoculation with both fungal strains in both spice samples. In inhibiting the production of aflatoxins a significant difference present among treatments [11-13]. The inhibitory effect of plant *Moringa oleifera* leaves on production of aflatoxins by *Aspergillus flavus* and *Aspergillus paraciticus* in cumin and black pepper incubated of eight months for incubation period is given in tables 1 and 2. A variable inhibitory effect was showed by all the treatments in concentration dependent manner on aflatoxin production over control. The Higher the inhibitory effect, the concentration of plant extract applied will also be greater. *Moringa oleifera* leaves were found to be very outstanding (p<0.05) potential agents at all levels in cumin over a storage period of eight months to control aflatoxin production. It was observed that *Moringa oleifera* at all steps to retard aflatoxin production for eight months were found to be very excellent (p<0.05) potential agents [12].

Table 1. *Aflatoxin content (ppb) in cumin treated with Moringa oleifera leaves at incubation period of 1-8 month.*

***M. oleifera* (g/100gm)**		***A. flavus*^{X}**		***A. paraciticus*^{Y}**			
		B1^a	**B2^b**	**B1^a**	**B2^c**	**G1^b**	**G2^d**
Month 1	00	8.12±0.02	3.27±0.04	5.261±0.06	2.23±0.04	3.14±0.01	1.29±0.02
	05	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	10	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	15	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Month 2	00	12.71±0.02	5.56±0.03	9.78±0.01	4.57±0.06	5.23±0.05	3.14±0.04
	05	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	10	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	15	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Month 3	00	20.78±0.07	8.73±0.05	11.43±0.04	5.61±0.05	7.86±0.05	3.39±0.09
	05	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	10	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	15	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Month 4	00	29.71±0.06	13.34±0.05	19.58±0.07	9.37±0.04	13.43±0.03	4.24±0.03
	05	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	10	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	15	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Month 5	00	40.12±0.08	22.48±0.09	31.43±0.08	13.53±0.05	20.52±0.03	7.23±0.24
	05	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	10	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	15	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Month 6	00	70.81±0.09	37.72±0.04	46.19±0.05	22.41±0.64	32.13±0.07	12.47±0.04
	05	3.24±0.05	1.29±0.03	0.00±0	0.00±0	0.00±0	0.00±0
	10	2.51±0.05	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	15	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Month 7	00	101.81±0.09	76.72±0.04	85.19±0.05	41.41±0.64	60.13±0.07	21.47±0.04
	05	5.79±0.01	3.71±0.09	3.16±0.05	1.04±0.04	1.65±0.03	0.34±0.02
	10	3.41±0.06	1.41±0.02	1.50±0.03	0.77±0.03	0.98±0.02	0.21±0.03
	15	1.30±0.03	0.20±0.03	1.18±0.02	0.24±0.02	0.54±0.03	0.00±0
Month 8	00	221.81±0.09	126.72±0.04	165.19±0.05	81.41±0.64	116.13±0.07	21.47±0.04
	05	7.37±0.06	5.31±0.01	4.56±0.01	2.49±0.03	3.53±0.04	1.41±0.04
	10	3.58±0.01	3.34±0.05	3.15±0.01	1.47±0.01	2.70±0.03	0.57±0.02
	15	1.22±0.03	1.02±0.02	2.02±0.04	1.18±0.04	1.32±0.04	0.21±0.03

Values are mean ± SD of three samples analysed individually in triplicate at $p<0.05$. Superscript small alphabets within the column indicated significant difference ($p<0.05$) among different aflatoxins. X and Y represent effect of fungal strains on aflatoxin production.

Table 2. *Aflatoxin content (ppb) in black pepper treated with Moringa oleifera leaves at incubation period of 1-8 month.*

***M. oleifera* (g/100gm)**		***A. flavus*^{X}**		***A. paraciticus*^{Y}**			
		B1^a	**B2^b**	**B1^a**	**B2^c**	**G1^b**	**G2^d**
Month 1	00	7.49±0.28	2.58±0.07	4.54±0.23	1.35±0.07	2.42±0.02	0.95±0.03
	05	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	10	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	15	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Month 2	00	11.23±0.06	4.46±0.03	9.53±0.04	3.47±0.06	5.41±0.02	1.17±0.04
	05	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	10	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	15	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Month 3	00	18.41±0.02	7.65±0.04	10.48±0.01	4.18±0.01	8.67±0.02	2.05±0.09
	05	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	10	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	15	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Month 4	00	27.71±0.04	12.37±0.03	17.14±0.07	7.59±0.08	12.59±0.03	3.21±0.06
	05	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	10	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	15	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Month 5	00	37.42±0.07	21.78±0.08	32.29±0.04	13.21±0.02	23.76±0.01	5.72±0.04
	05	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	10	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
	15	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0	0.00±0
Month 6	00	67.58±0.01	34.17±0.03	48.83±0.04	20.18±0.03	30.21±0.01	9.29±0.09
	05	3.40±0.04	1.51±0.07	3.36±0.07	1.29±0.04	1.77±0.04	0.91±0.03
	10	1.42±0.06	0.13±0.06	2.32±0.05	0.44±0.01	1.29±0.03	0.19±0.04
	15	0.49±0.05	0.00±0	1.01±0.02	0.00±0	0.71±0.05	0.00±0
Month 7	00	73.58±0.01	38.17±0.03	58.83±0.04	22.18±0.03	35.21±0.01	8.29±0.09
	05	23.30±0.04	8.51±0.07	4.45±0.07	1.38±0.04	2.73±0.06	0.79±0.03
	10	17.52±0.06	5.23±0.06	3.32±0.05	0.37±0.01	1.69±0.04	0.07±0.02
	15	6.43±0.05	2.30±0.01	1.21±0.02	0.01±0	0.73±0.02	0.00±0
Month 8	00	203.58±0.01	138.17±0.03	168.83±0.04	62.18±0.03	133.21±0.01	28.29±0.07
	05	56.04±0.04	14.11±0.07	41.05±0.07	22.38±0.04	29.73±0.06	1.79±0.03
	10	23.52±0.06	12.13±0.06	31.32±0.05	16.97±0.01	27.09±0.04	0.67±0.02
	15	11.23±0.05	8.88±0.01	12.31±0.02	7.88±0.03	11.13±0.02	0.24±0.02

Values are mean ± SD of three samples analysed individually in triplicate at $p<0.05$. Superscript small alphabets within the column indicated significant difference ($p<0.05$) among different aflatoxins. X and Y represent effect of fungal strains on aflatoxin production.

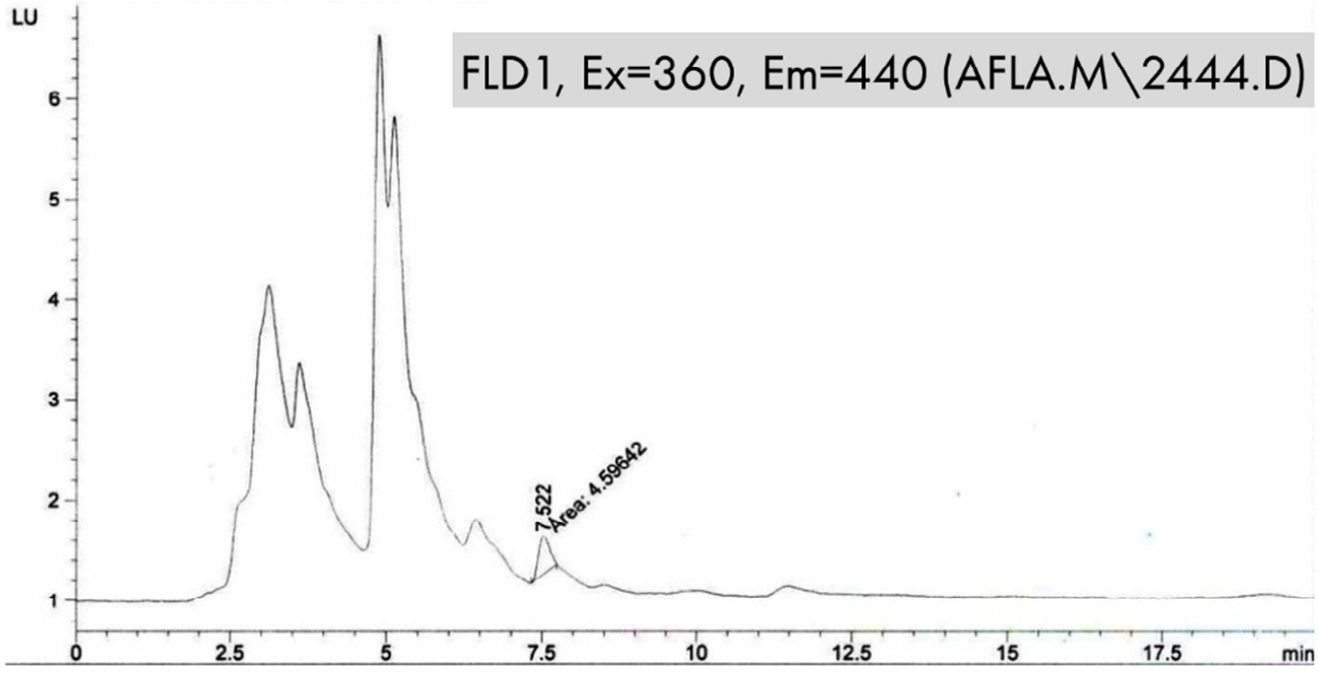

Figure 1. *Typical HPLC chromatogram showing the separation of aflatoxin in standard.*

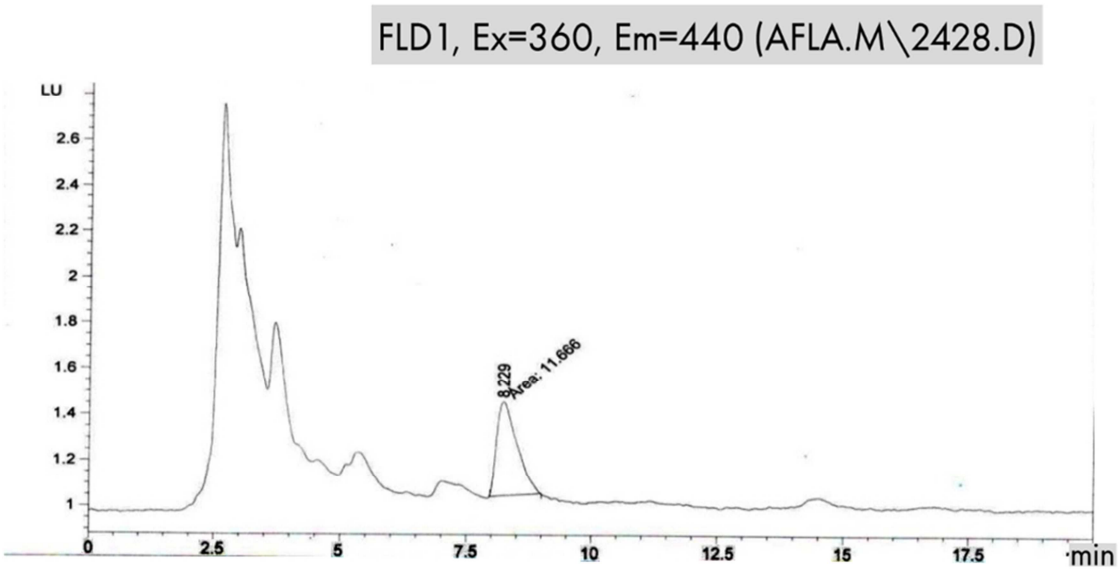

Figure 2. *Typical HPLC chromatogram showing the separation of aflatoxin in cumin.*

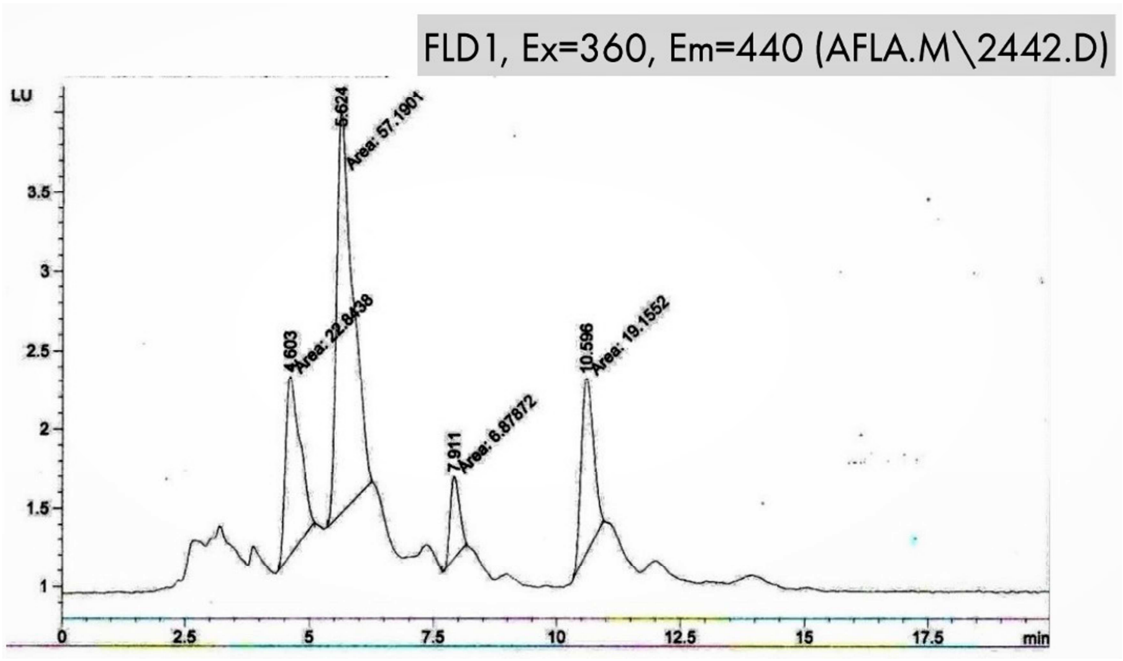

Figure 3. *Typical HPLC chromatogram showing the separation of aflatoxin in black pepper.*

4. Discussions

Aflatoxogenic spices can be harmful to all humans as well as to the consumers because of the toxins contained in different foodstuff. The use of plant leaves and their extract and certain herbal medicines may give an alternative way to prevent from aflatoxin production and fungal growth. *Moringa oleifera* leaves contain flavonoids, vitamin C, phenolics, flavonoids. It is nutrient dense and enzymatically active plant [9]. All parts of *M. oleifera* plant like flowers, leaves, seeds and bark are an excellent source of natural antioxidant and possess high nutritional value but in this leaves of *Moringa oleifera* were used to control aflatoxin production [10, 18].

The components present in other plants have beneficial effects but toxic effects of certain plant components have also been reported, i.e., the oestrogenic effect of isoflavonoids or the toxicity of coumarins. But *M. oleifera* is a complete nutritive plant containing various minerals including iron, magnesium and calcium. It is also a good way of getting various beta-carotene, amino acids and phenolics [21].

M. oleifera plant leaves can be used as an anti-aflatoxigenic agent. Aflatoxins cause food spoilage on large scale. To secure human and economy, antifungal agents present an alternate to prevent from fungal contamination in food and its extract having alot of bioactive compounds to control aflatoxin production. Spice samples showed a slight change due to AFTs production in the proximate composition and this slight decrease in proximate contents because AFTs

affect proximate contents and ultimately, lead to bad nutritive quality [16].

5. Conclusion

Despite all efforts, it has been very difficult to control the exposure of man and animals to aflatoxins, because of their natural occurrence in the environment. Although the prevention of aflatoxin contamination by inhibiting the fungal growth in food and spices is the best practice. Aflatoxins are produced by two fungi *Aspergillus flavus* and *Aspergillus parasitiucus*. Biological procedures have been used for controlling aflatoxins in the spice samples. The inhibitory effect of plant *Moringa oleifera* leaves on production of aflatoxins by *Aspergillus flavus* and *Aspergillus paraciticus* in cumin and black pepper incubated of eight months for incubation period. The fungal strains also used to minimize the fungus growth and aflatoxin production in spice sample during storage. These were stabilized with *M. oleifera* leaves and stored for 8 months at specific moisture (16%) and temperature (28°C). After regular intervals, samples were drawn and levels of aflatoxins were estimated by using HPLC. The investigated medicinal plant leaves of *M. oleifera* were fully inhibited (100%) aflatoxin in cumin and black pepper production for eight months.

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