Agric. Sci. Digest., 39(1) 2019: 21-25
Print ISSN:0253-150X / Online ISSN:0976-0547

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
www.arccjournals.com

Detoxification assessment of Aflatoxin in *Aspergillus flavus* under the effect of *Calendula officinalis* Linn's methanolic extract

Jyoti Mishra[1], Maneesh Kumar*[2], Ajai Kishore Sharan[1] and Jainendra Kumar[3]

Department of Botany and Biotechnology,
Patliputra University, Patna-800 020, Bihar, India.

Received: 23-01-2019 Accepted: 01-04-2019 DOI: 10.18805/ag.D-4874

ABSTRACT

Calendula officinalis Linn (pot marigold) possess potential pharmacological activities which might be considered as an excellent cause, in scheming the aflatoxin biosynthesis in the aflatoxigenic fungi. The aim of the present study was to assess the antifungal activity of methanol extracts of the plant petals against biosynthesis of aflatoxin B1 (AFB1) in *Aspergillus flavus*. The antifungal potential of methanolic extract of *C. officinalis* was evaluated against the toxigenic fungus on Czapek Dox Agar (CDA) and Czapek Dox Broth (CDB) Media, using disc diffusion assay. The use of *C. officinalis* extract on the *A. flavus* culture showed the significant inhibition of AFB1up to 59.50% along with the reduction of mycelial growth to about 24.82%. Although, the treatment of the AFB1 with the extract for 5 hours at 80ºC which led to 78.48%. The work well established the inhibitory role of *C. officinalis* extract in order to reduce the AFB1 threat.

Keywords: Antifungal potential, Marigold, Medicine herb, Methiconol extract.

INTRODUCTION

Aflatoxin contamination in agricultural products is a serious global concern. *Aspergillus flavus*, an opportunistic fungal pathogen produces such highly toxic and mutagenic mycotoxins. These secondary metabolites are implicated as virulence factors in the biotic environment (Calvo *et al*. 2002). Normally, the pathogen colonization over organic subtracts and other food items, is much prone for infectivity for humans and animals (Singh *et al*. 2017). Preferentially, aflatoxin B1 (AFB1) abundantly produces by *A. flavus* through a complex series of oxidation-reduction reactions (Reddy and Waliyar 2000; Kumar *et al*. 2018c). As per health apprehension, the dietary exposure of AFB1 is major peril for the multifactorial etiology of hepatocellular carcinoma (HCC) in humans and potentially promotes viral infection i.e. chronic hepatitis B (HBV) infection (Fountain *et al*. 2015; Kumar *et al*. 2018b). During the immunosuppressive condition, the HBV infection sharpens the epoxidation potential of AFB1 to exo-8,9-epoxide. The highly unstable intermediate exo-epoxide binds with DNA to form trans-8, 9-dihydro-8-(N7-guanyl)-9-hydroxy-AFB1 (AFB1-N7-Gua) adduct, a pro-mutagenic DNA lesion in hepatocytes (Chemin *et al*. 1999; Kumar *et al*. 2018a). Such hepatocyte necrosis might increase the chance of mutation in p53 tumor suppressor protein at Ser^{249} amino acid residue in HCC (Rivkina *et al*. 1996).

Calendula officinalis Linn (Asteraceae) is a well known annual medicinal herb commonly used in anti-inflammatory and antifungal activities. The herb owns the property of hepatorenal-protection against aflatoxins (Hamzawy *et al*. 2013). Including this, organic constituents like carotenoids, sesquiterpenes glycosides, essential oils, flavonoids, xanthophylls, and volatiles, are found in the plant possess antimicrobial activities (Chalchat *et al*. 1991). Flavoxanthin, g-carotene, lycopene12, lutein, and rubixanthin are some carotenoids that have been cherished for its anti-viral and antifungal behavior. Different contents of phenolics and flavonoids have been also reported bearing antioxidant properties (Ercetin *et al*. 2012).

MATERIALS AND METHODS

Fungal growth and Aflatoxin B1: The toxigenic representative strain of *Aspergillus flavus* was procured for experimental analysis from the Department of Food Science & Technology, Pondicherry University, Pondicherry, India. The strain was revived, maintained and preserved in proper aseptic conditions.

Plant Material: The *Calendula officinalis* (Pot marigold) leaves sample was plucked from the garden of the Sanjay Gandhi Biological Park, Patna, Bihar, India and collected in a sterilized polybag for the study of biological effects on *Aspergillus flavus*. The sample was recognized and authenticated Department of Botany, Magadh University, Bodh Gaya, India.

Preparation of plant extract: The plucked leaves were properly rinsed under running tap water, followed by distilled

*Corresponding author's e-mail: kumar.maneesh11@gmail.com
[1]Department of Botany, Veer Kunwar Singh University, Ara- 802 301, Bihar, India.
[2]Department of Biotechnology, Magadh University, Bodh Gaya- 824 234, Bihar, India.
[3]Department of Botany and Biotechnology, Patliputra University, Patna-800 020, Bihar, India.

water and air dried for 72hours in an oven to achieve constant weight. The dried leaves were minced using laboratory blender (Yadav, Kumar and Jamal 2018). The methanol and aqueous extracts were prepared by macerating 50g of the powdered plant's sample in 100mL of absolute methanol. The macerate was first filtered through Whatman no.1 filter paper and then centrifuged at 4000g for 30min to obtained clear supernatant. The supernatant from the methanol extract was evaporated under a vacuum at 45ºC let to about 90% of the filtrate. Afterward, the sample was lyophilized properly to get dry extracts which were kept in the refrigerator (≤4ºC) in preserved in vials as the mother extract for further study (Kumar *et al*. 2017c).

Evaluation of fungal growth and AFB1 production: CDB medium (50ml) (Titan Biotech Ltd, New Delhi, India) was prepared in five different 250mL conical Erlenmeyer flasks and sterilized. In each media flask, 0.5, 1.0, 1.5, 2.0 and 2.5mg of lyophilized methanolic extract of *Calendula officinalis* were added to assess an antifungal activity. One medium containing flask without *C. officinalis* extract was also maintained as a control.

A week after harvesting *A. flavus* spores was counted using hemocytometer. The spore suspension (135spores/mL) was inoculated into each one flask. The inoculated flasks were incubated at 28±2°C for 10days. After the period of the incubation, the fungal mass in terms of dry mycelia weight and the amount of AFB1 production from each flask was measured.

To estimate the antifungal activity of the methanol extract of *C. officinalis* in CDB employed via diffusion method. The mycelial growth was filtered through Whatman no.1 filter paper (Kumar *et al*. 2017c). The wet weight mycelium measured and dried at 80°C in oven until it reached a constant weight. The percentage inhibition of mycelial growth was calculated by the following formula:-

$$\text{Percentage of inhibition of growth of test fungi} = \frac{DC - DT}{DC} \times 100$$

Where

DC =Average raise in mycelial growth in the control sample.
DT =Average raise in mycelial growth of the test sample.

The quality and quantitative analysis of AFB1 concentration extracted from CDA culture media, later evaluated with help of thin layer chromatography (TLC) and high-pressure liquid chromatography (HPLC). The detection procedure performed as mentioned in Kumar *et al*. 2017a. According to Turner *et al*. (2009), the dry remnants, treated with 20ml chloroform was used and allows the chloroform (Himedia, Mumbai, India) extract to evaporate at normal room temperature till complete dryness. The residue was again treated with 1ml of chloroform. 60µl of the chloroform extract was loaded and analyzed on the TLC plates (silica gel on aluminum foil with F 254 from Fluka, Munich, Germany) to confirm the presence of aflatoxin presence (Turner, Subrahmanyam and Piletsky 2009). Ten grams of fungal culture were blended with the mixture of 50ml methanol (55%), and 20ml petroleum ether for 10minutes continuously. The blended mixtures were kept for 30minutes undisturbed, and then, were filtered by using Whatman no.1 filter paper that removes all mycelium and other cell debris. In new falcon tubes, 25ml aqueous methanol phase and 25ml chloroform were added and gently shook for 2mins to mix appropriately (Trombete *et al*. 2014). The falcon tubes were left to separate for the next 5minutes and aflatoxins were concentrated for HPLC detection. Elution of the matrix loaded in the column was carried out with chloroform: methanol (11.76:0.24) at the rate of 5ml/min. 5ml of the fraction was collected from both aerobic and anaerobic samples and monitored for the absorbance of aflatoxins at 360nm in UV spectrophotometer, and followed by HPLC for further confirmation. The C18 Polaris column (Ace, 250mm x 4.6mm, 5µm) was used for the stationary phase. The samples were run with an isocratic mobile phase consisting of deionized water: methanol: acetonitrile (70:20:10) at a flow rate of 1ml/min (Rajarajan, *et al.,* 2013). The absorption of samples in HPLC was detected using a fluorescence detector with excitation at 360nm and emission at 450nm.

AFB1 degradation using *Calendula officinalis* extract at the different time interval: A pure AFB1 was incubated with extract of *C. officinalis* in varying time interval as well as temperature. In this, 5µg AFB1 has been incubated with 2.0mg of the plant extract for 5hours at room temperature (25°C). At every hour the samples were extracted with the help of chloroform and analyzed as mention in (Kumar *et al*. 2017a; Kumar *et al*. 2017b). Likewise, the effect of temperature on the sample was also tested. The samples were incubated for 25, 30, 45, 65, 80°C for 3hrs. Double distilled water in place of *C. officinalis* extract was taken as control and incubated in similar conditions. All the works related to spectroscopy were performed using UV-Vis Spectrophotometer (Perkin Elmer).

The quantities of aflatoxins were calculated by the following formula:

$$\text{Aflatoxin concentration (µg//g)} = \frac{D \times M}{E \times L} \times 1000$$

Where, D =Absorbance, E =Molar extinction coefficient of aflatoxins, M =Molecular weight of aflatoxins, L =Path length.

Statistical analysis: The fungal mycelial production and growth pattern assay were done under the effect of selected plant extract was further examined by repeated measurement of variance, and these parameters were studied by performing one-way ANOVA of maximum values under significant of $P<0.05$. The mycelial growth and aflatoxin production values are expressed as mean + Standard Deviation (SD).

RESULTS AND DISCUSSION

Mycelial Growth and AFB1 Production by A. flavus under the effect of extract of *C. officinalis*: *C. officinalis* possesses a wide variety of phytochemicals and pharmacological activities, so it can be considered as an excellent source of new drugs. Many reports are available on the various *Calendula* species which possess high efficacy of anti-bacterial, anti-fungal, antihelminthic, anti-molluscan and anti-inflammatory properties with no toxicity. The plant constitutes major components including triterpendiol esters, saponins, and flavonoids including rutin and hyperoside that might trigger the inhibition of AFB1. Form the growth investigations; we observed that the aqueous extract of *C. officinalis* in treated culture medium diminished the mycelium growth and restrained aflatoxin B1 (AFB1) biosynthesis in *Aspergillus flavus* in comparison to the control is shown in Fig.1. The difference which monitored in between treated and the control values were seen more important at the lower amounts of *C. officinalis* extract. The dry mycelium weight (DMW) correspondingly decreases with the AFB1 production as the amount of *C. officinalis* extract increases. The maximum reduction seen in DMW and AFB1 production found 2.5mg under effect of *C. officinalis*. Later, HPLC analysis was also performed to validate these results. Interestingly, the chromatographic analysis of the effect of the plant extracts drastically diminished the fluorescence of AFB1 spot on TLC plant under Ultra Violet (UV) radiation. The inhibition (percentage) of mycelial growth and AFB1 (Table.1) and the differences with control are found to be statistically significant, $P<0.05$. As a result, it is interesting to note that the AFB1 production was inhibited along with the inhibition of fungal growth by 22.36% of 1.5mg of *C. officinalis* extract in the growth medium. Kumar *et al*. 2017d had earlier demonstrated variations in the inhibition AFB1 using of powdered leaves of *Zea mays*. Later, Mahoney *et al*. (2010) used plant antioxidant against of AFB1 production without affecting the mycelial growth of fungi.

Direct Degradation of AFB1 under treatment with C. *officinalis* Extract: Direct degradation of AFB1 was also observed by incubating a pure form of AFB1 with methanolic extract of *C. officinalis*. The gradual reduction in the

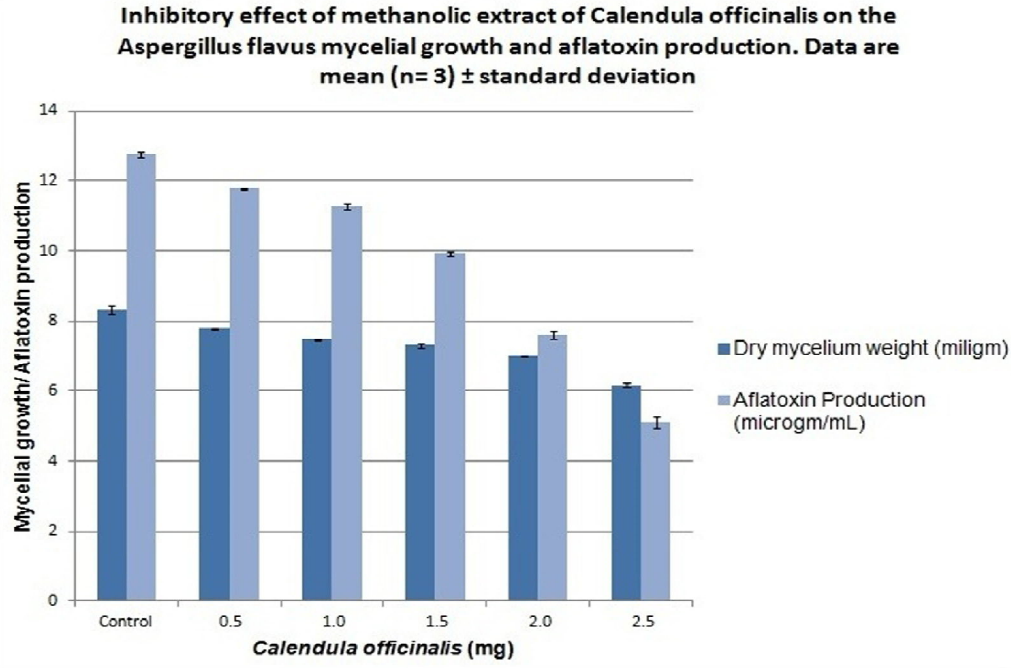

Fig 1: More than 50% reduction of aflatoxin production with increase of dosages of *Calendula officinalis* methanolic extracts.

Table 1: The effect of *Calendula officinalis* extract on the growth and AFB1 production by *Aspergillus flavus*.

***Calendula officinalis* extract in 10mL culture medium (mg)**	**DMW (gram)**	**AFB1 (µg/mL)**	**Percentage Inhibition (%)**	
			Mycelial Growth	**AFB1 production**
Control	8.18±0.154	12.79±0.115	00.00	00.00
0.5	7.87±0.083	11.84±0.057	03.79	07.40
1.0	7.51±0.084	11.26±0.117	08.19	11.96
1.5	7.28±0.122	09.93±0.098	11.00	22.36
2.0	7.02±0.061	07.62±0.122	14.18	40.42
2.5	6.15±0.128	05.18±0.165	24.82	59.50

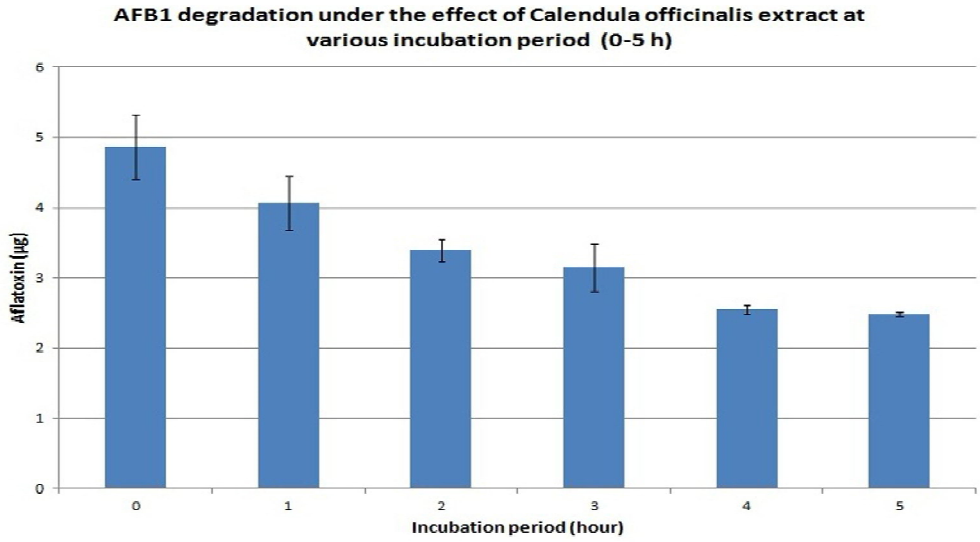

Fig 2: The progressive degradation of aflatoxin on incubating the culture along with the methanolic extracts of *Calendula officinalis.*

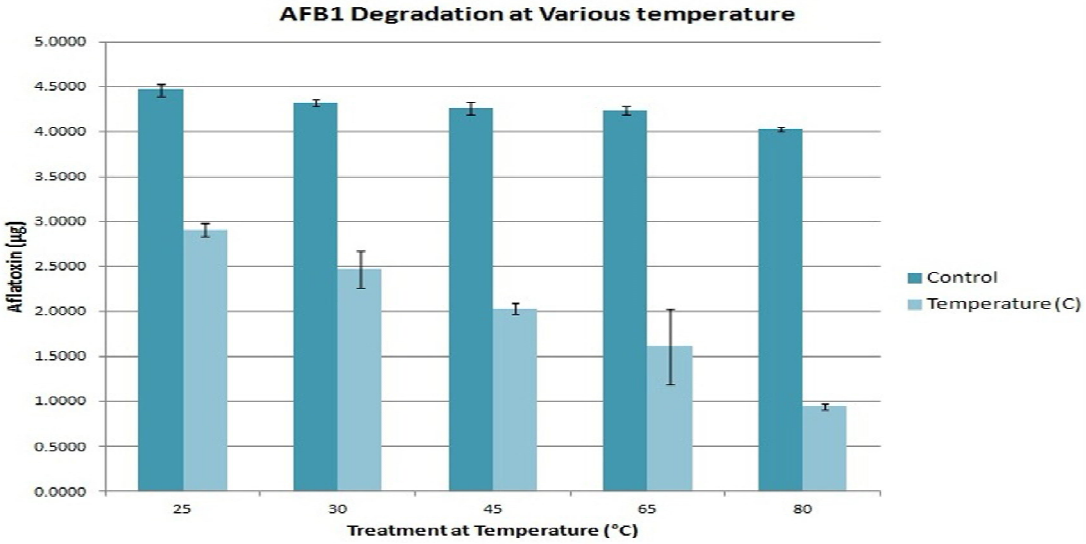

Fig 3: Effect of temperature on degradation of aflatoxin.

Table 2: Degradation of AFB1with *C. officinalis* different temperature for 3 hours.

Treatment Temperature (°C)	AFB1treated with double distilled water (µg)	AFB1 treated with *C. officinalis* Extract (µg)	Percentage Inhibition (%)	
			Control(Without any treatment)	Treated with *C. officinalis* Extract
25	4.46±0.16	2.88±0.18	0.00	35.43
30	4.36±0.07	2.48±0.44	2.24	44.20
45	4.31±0.17	2.06±0.17	3.36	53.81
65	4.29±0.12	1.63±0.48	3.81	63.45
80	4.03±0.03	0.96±0.03	9.64	78.48

quantification of AFB1 was measured by using UV spectroscopy with increasing incubation time. The maximum 48.35% degradation was seen after 5 hours of incubation at 25ºC (Fig.2). Also, the significant amount of AFB1 degradation was increased 4 hours onwards, while no change was observed in the control.

The temperature was also taken into consideration to check its degradation effect on AFB1 biosynthesis using the extract of *C. officinalis*. The quantitative study of AFB1 degradation under the effect of temperature was performed with UV-Vis Spectrophotometer. The degradation of 78.48%

was obtained while treated with 5µg of AFB1 with 2.0mg the plant extract at 80°C for 5hours (Fig 3). The percentage inhibition of AFB1 under different temperature was mentioned in Table 2.

CONCLUSION

The study illustrated that the methanolic extract of *C. officinalis* can be effectively used to reduce the impact of AFB1. The plant aqueous extract also potentially inhibits the AFB1 biosynthesis synthesis in the aflatoxigenic strain of *A. flavus*. This also explains the metabolites which are present as constituents in the plant have desirable properties that can safely be used against the mycotoxigenic activities with the concern fungi. Hence, *C. officinalis* could be a better alternative which is frequently used for toxin degradation. It could also conquer the undesirable effect of chemical preservatives.

REFERENCES

Calvo AM, Wilson RA, Bok JW, Keller NP (2002) Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews*. **66**(3):447-459.

Chalchat J, Garry RP, Michet A. (1991). Chemical composition of essential oil of *Calendula officinalis* L.(Pot Marigold). *Flavour and Fragrance Journal*. **6**(3):189-192.

Chemin I, Ohgaki H, Chisari F, Wild C. (1999) Altered expression of hepatic carcinogen metabolizing enzymes with liver injury in HBV transgenic mouse lineages expressing various amounts of hepatitis B surface antigen. *Liver International*. **19**(2):81-87.

Ercetin T, Senol FS, Orhan IE, Toker G. (2012) Comparative assessment of antioxidant and cholinesterase inhibitory properties of the marigold extracts from *Calendula arvensis* L. and *Calendula officinalis* L. *Industrial Crops and Products*. **36**(1):203-208.

Fountain JC, Scully BT, Chen ZY, Gold SE, Glenn AE, Abbas HK, Lee RD, Kemerait RC, Guo B. (2015) Effects of hydrogen peroxide on different toxigenic and atoxigenic isolates of *Aspergillus flavus*. *Toxins*. **7**(8):2985-2999.

Hamzawy MA, El-Denshary ES, Hassan NS, Mannaa FA, Abdel-Wahhab MA. (2013) Dietary supplementation of *Calendula officinalis* counteracts the oxidative stress and liver damage resulted from aflatoxin. *ISRN Nutrition* **2013**.

Kasiram K, Sakharkar P, Patil A. (2000) Antifungal activity of *Calendula officinalis*. *Indian Journal of Pharmaceutical Sciences*. **62**(6):464.

Kumar M, Topno RK, Kumar H, Kumar J. (2018a) Biological conversion of Carcinogenic Aflatoxin B1 to AFB1-8, 9-exo-epoxide and its potential prevention. *International Journal of Research and Analytical Reviews*. **5**(4):935-946.

Kumar M, Sahoo G, Topno R, Pandey K, Ravidas V, Dikhit M, Kumar H, Das P. (2018b) Molecular interaction of antifungal compounds with fungal type I Polyketide synthase and preventing hepatocarcinogenesis. *International Journal of Infectious Diseases*. **73**:285. DOI: https://doi.org/10.1016/j.ijid.2018.04.4065.

Kumar H, Saw SK, Kumari P, Kumar M, Kumar M, Kumar J, Sahoo GS (2018c) Understanding the Role of aflS Gene Expression in *Aspergillus flavus* and *Aspergillud oryzae* Regarding the Aflatoxin Production. *International Journal of Research and Analytical Reviews*. **5**(4):148-157.

Kumar M, Rana S, Kumar H, Kumar J, Mansuri R, Sahoo GC. (2017a) Inhibition of product template (PT) domain of aflatoxin producing polyketide synthase enzyme of *Aspergillus parasiticus*. *Letters in Drug Design & Discovery*., **14**(7):811-818. DOI : 10.2174/1570180814666170203144914.

Kumar M, Kumar H, Kumar S, Kumar K, Jha S, Kumar S. (2017b) Inhibitory effect of the acetone extract of maize leaves on the mycelial growth of *Aspergillus parasiticus* along with its aflatoxins production. *International Journal of Advanced Research*. **5**(11):751-757. DOI: 10.21474/IJAR01/5827.

Kumar M, Rana S, Kumar H, Kumar P, Dikhit MR, Mansuri R, Kumar J, Sahoo GC. (2017c) Computational, structural and functional aspects of hypothetical protein of *Aspergillus flavus* Pheromone Receptor Pre-A (PRP-A). *Journal of Applied Pharmaceutical Science*. **7**(07):089-097. DOI: 10.7324/JAPS.2017.70714

Kumar H, Kumar M, Kumar B, Bharti S, Kumar P, Kumar M, Kumar J. (2017d). Effect of acetone extract of *Zea mays* L. leaves on the growth of *Aspergillus flavus* and aflatoxin production. *International Journal of Advanced Research*. **5**(8):1667-1671.

Mahoney N, Molyneux RJ, Kim JH, Campbell BC, Waiss AC, Hagerman AE. (2010) Aflatoxigenesis induced in *Aspergillus flavus* by oxidative stress and reduction by phenolic antioxidants from tree nuts.*World Mycotoxin Journal*. **3**(1):45–48.

Rajarajan P, Rajasekaran K, Devi N. (2013) Aflatoxin contamination in agricultural commodities.

Reddy S, Waliyar F. (2000) Properties of aflatoxin and it producing fungi. *Aflatoxin. Int. Crops Res. Inst. for the Semi-Arid Tropics.*

Rivkina M, Cote PJ, Robinson WS, Tennant BC, Marion PL. (1996) Absence of mutations in the p53 tumor suppressor gene in woodchuck hepatocellular carcinomas associated with hepadnavirus infection and intake of aflatoxin B1. *Carcinogenesis*. **17**(12):2689-2694.

Singh R, Rai TS, Sharma NS, Arora AK, Kaur P. (2017) Evaluation of a Real time polymerase chain reaction assay for the detectionof aflatoxin/sterigmatocystin producing strains of *Aspergillus* spp. *Indian Journal of Animal Research*. 51(4):676-8. DOI: 10.18805/ijar.v0iOF.7263

Trombete FM, de Ávila Moraes D, Porto YD, Santos TB, Direito GM, Fraga ME, Saldanha T (2014) Determination of aflatoxins in wheat and wheat by-products intended for human consumption, marketed in Rio de Janeiro, Brazil. *Journal of Food and Nutrition Research*. **2**(10):671-674.

Turner NW, Subrahmanyam S, Piletsky S (2009) Analytical methods for determination of mycotoxins: a review. *Analytica chimica acta*. **632**(2):168-180.

Yadav, D.P., Kumar, M. and Jamal, M.A., (2018) Larvicidal & Juvenomimetic property of Withania somnifera extracts against larvae of *Tribolium confusum* Duavl. *International Journal of Research and Analytical Reviews*.