KAVAKA 58 (4): 76-85 (2022) DOI: 10.36460/Kavaka/58/4/2022/76-85

Rationally designed peptide for rapid detection of ochratoxin in paper-based dot-blot assay and mitigation of toxicity

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(Submitted on September 07, 2022; Accepted on December 04, 2022)

ABSTRACT

Ochratoxin A (OTA) is a secondary metabolite produced by the *Aspergillus spp.* that contaminates a variety of food and feed. OTA is a nephrotoxic, hepatotoxic and carcinogenic mycotoxin. Here we developed a dot-blot assay for OTA detection using a specific 9-mer N-KSGSFNHPK-C peptide designed by rational strategy and computational modelling. The peptide binding to OTA was confirmed by fluorescence quenching (1.1-1.5 fold). The 9-mer peptide was conjugated to gold nanoparticles as labels for use as a detection agent in the dot-blot assay. The limit of detection for the developed dot-blot assay was 0.19 µg/kg. Further, OTA recovery from spiked wheat sample by dot-blot assay (90-96%) was comparable to HPLC method (97-99%). Furthermore, evaluation of developed assay with 146 samples of food and feed along with certified reference material demonstrated good sensitivity (74%) and specificity (99%), respectively. The assay displayed 94% assay accuracy with correlation of 0.75 with HPLC. Moreover, the addition of peptide reduced OTA toxicity (21-60%) to HepG2 cells. Therefore, an easy-to-use, rapid, and portable dot-blot assay can contribute in the sensitive detection of OTA and has potential for reducing the toxicity of OTA and ensures safe and healthy food.

Keywords: Ochratoxin, Gold nanoparticles, Rational design peptide, Dot-blot assay

INTRODUCTION

Ochratoxin A (OTA), is harmful secondary metabolite produced by *Aspergillus ochraceus* and *A. carbonarius* that is known to contaminate various food and feed materials (Duarte *et al.,* 2010; Zain*,* 2011). OTA contains a phenylalanyl group in its structure and binds competitively to phenylalanyl-tRNA synthetase enzyme (PheRS) leading to protein synthesis inhibition and toxicity. Consumption of OTA contaminated food causes nephrotoxicity, hepatotoxicity, carcinogenicity and increases the susceptibility to disease and death (Grollman and Jelaković, 2007; Wang *et al.,* 2016; Ostry *et al.,* 2017). FAO has estimated that 25% of crops are mycotoxin contaminated (Smith *et al.,* 2016; Human, 2018). In India, Food Safety and Standards Authority of India (FSSAI) has set a regulatory limit of 20 µg/kg for OTA for cereals and cereals products. Currently, OTA is detected by chromatographic and ELISA method (Chen *et al.,* 2012; Alhamoud *et al.,* 2019; Samuel *et al.,* 2021). However, these methods require laborious time-consuming procedures, trained personnel and sophisticated instruments and are not portable (Singh and Mehta, 2020). Therefore, an on-site, easy-to-use, and portable detection method for OTA can contribute in the rapid screening of food and feed samples.

Paper-based dot-blot and lateral flow assays that utilize easily synthesized gold nanoparticles (AuNPs) as a visual label are simple, fast, cost-effective and portable (Saha *et al.,* 2012; Nguyen *et al.,* 2020). Recently, dot-blot assay was reported for OTA detection with a cut-off of 50 µg/kg for rice and oats using AuNPs tagged with monoclonal antibodies (mAbs) (Chen *et al.,* 2020). Although, specific mAbs are a preferred recognition agent they are difficult to raise against toxin molecules and display a low stability (Le Basle *et al.,* 2020). Short amino acid peptides have potential to replace antibodies as an alternative recognition agent in paper-based assays. Peptides display advantages of easy, cheaper synthesis and good stability at different temperature and pH (Bazin *et al.,* 2013; Heurich *et al.*, 2013). Such peptides can be identified by *in silico* rational designing by computational method which is easy, fast and involves low-cost (Giraudi *et al.,* 2007; Vanhee *et al.,* 2011; Heurich *et al.,* 2013; Nandy and Basak, 2016; Śledź and Caflisch, 2018). Rational peptide designing interprets the structural information present in target and receptor complex by homology modelling and molecular docking (Kamagata *et al.,* 2019). *In silico* designing permits amino acids modifications and screening for binding energy by considering molecular interactions like H-bonding, hydrophobic interactions to generate a more stable receptor-ligand binding (Heurich *et al.,* 2013; Yan *et al.,* 2020; Liu *et al.,* 2022; Wang *et al.,* 2022). Recently, epitope-based peptide vaccine candidates for Zika virus and *Staphylococcus aureus* were developed through peptide designing (Alam *et al.,* 2016; Hajighahramani *et al.,* 2017).

Here, we describe the bioinformatic design of 9-mer peptide (KSG-SFNHPK), as a recognition agent for OTA detection with a dot-blot assay. The *in silico* binding interactions were confirmed in wet-lab by fluroscence quenching and ELISA. The 9-mer peptide was conjugated to AuNPs using streptavidin-biotin interaction to develop a sensitive dot-blot assay (LOD 0.19 µg/kg) that did not cross-react with other mycotoxins. Evaluation of dot-blot assay with certified reference material and 146 food and feed samples displayed a significant correlation to HPLC method. Further, the designed 9-mer peptide reduced OTA toxicity in HepG2 cells and can contribute to therapeutics. Therefore, the peptide-based dot-blot assay has potential for rapid, portable and sensitive detection of OTA contamination in food and feed.

MATERIAL AND METHODS

Molecular docking approach for rational designing of peptide for OTA detection

The binding interaction of OTA with phenylalanine tRNA synthase (PheRS) was reported by McMasters and Vedani, (1999) which showed the presence of AFFHWRER amino acids in the binding pocket. These interacting amino acids were utilized to design the peptide for OTA. Molecular docking software Autodock Vina 5.1 was used to study the

interaction of AFFHWRER amino acids with OTA. The amino acids sequence was linearized by generating its peptide in PDB format using Discovery studio2017R2. Using Autodock Vina, blind docking study was carried out of OTA and peptide, without bias. Low binding energy was used select to docking conformers. The interaction of OTA with AFFHWRER peptide residues was analyzed in detail with visualization tool Discovery studio. The possible putative peptides were generated by replacing the amino acid on basis of their binding interaction, hydrogen bonding, and hydrophobicity. The peptide characteristics like hydrophobicity index, solubility, isoelectric point (pI), molecular weight and grand average of hydropathy (gravy) value were determined by Pepcalc.com and Peptide Analyzing Tool, available online. The peptides were synthetically synthesized from BioResource Biotech Pvt. Ltd, India.

Fluorescence based peptide binding assay

100 µL of Biotin-KSGSFNHPK peptide (BioResource Biotech Pvt. Ltd, India) at a concentration of 10, 20, 30, 40 and 50 ng/mL in 1X PBS buffer, pH 7 were tested with 100 µL of 10, 20, 30, 40 and 50 ng/mL OTA (Sigma Aldrich) in 1X PBS buffer, pH 7. The mixture of peptide and OTA was incubated for 4 h in the dark at RT and analyzed by fluorimeter at Ex 380 nm and Ex 390-700 nm nm (Hitachi F-7000, Fluorescence Spectrophotometer). Peptide reactivity with OTA was assessed by comparing the max peak of free OTA to peptide bound OTA to observing the fluorescence quenching.

Synthesis of gold nanoparticles (AuNPs) and AuNPs-peptide conjugation with streptavidin-biotin binding

Colloidal AuNPs were synthesized from chloroauric acid (Spectrochem Pvt. Ltd, India) using the citrate reduction method (Frens, 1973) and adjusted to pH 7 using 2 M K_2CO_3. Then, AuNPs were functionalized with streptavidin (Sigma Aldrich) according to Hermanson (2008). Briefly, 30 µL streptavidin (1 mg/mL, Sigma, USA in 0.1 M sodium phosphate buffer, pH 7.4) was added to colloidal gold solution (0.5 mL) under stirring for 60 min at RT. The resultant mixture was centrifuged at 12,000 g for 10 min and the pellet was re-suspended in 0.1 M sodium phosphate buffer (PB, pH 7) and stored at 4°C until use.

The AuNPs-streptavidin-biotin-peptide conjugate was prepared by addition of 30 µL biotinylated peptide (10 µg/mL) to AuNPs-streptavidin solution (0.5 mL) and incubation at RT, 60 min. Later, 20 µL of 1% skimmed milk (Sigma Aldrich) was added and the mixture was incubated for 10 min and centrifuged at 8600 g, 15 min. The pellet was re-suspended in 25 µL of 0.5X PBS and stored at 4°C until use.

The bare and peptide-conjugated AuNPs were characterized for size by nano tracking analysis (NTA) using NanoSight LM20 (NanoSight, UK) and zeta potential using Malvern Zetasizer Nano ZSP (Malvern Panalytical Ltd, UK). Surface plasmon resonance measurement was performed at A520 nm using a Nanodrop spectrophotometer (Thermo Scientifc, USA) and gel retardation assay was performed using 2% agarose gel in 0.5X TAE buffer.

Development of dot-blot assay for detection of OTA and determination of its Limit of detection (LOD)

Dot-blot assay was performed with a flow through cassette module lined with absorbent pads and nitrocellulose membrane (NCM) (MDI, Chandigarh, India) pre-treated with citrate phosphate buffer. 5 µL OTA (50,100, 200, 400, and 800 ng/mL) was spotted on NCM and incubated for 1 h at 37°C. The membrane was blocked by 1% (w/v) skimmed milk for 10 min at 37°C. Further, 2 µL of AuNPs-peptide were added for the detection followed by washing thrice with washing buffer PBS containing 0.1% Tween 20 (PBST). The presence of antigen was demonstrated by formation of a red spot on the NCM.

The limit of detection (LOD) for the dot-blot assay was measured by ImageJ software. The spot intensities were recorded for 50 100 ng/mL OTA concentration by analyses of seven independent blots and normalized by the intensity of control. Normalized spot intensities were graphically plotted against antigen concentration and LOD of the developed dot-blot assay was determined by logarithmic linear graph. The LOD was calculated according to Little (2015) with the following formula:

$$LOD = 3.3 \times (\sigma/S)$$

Where σ is the standard deviation of normalized spot intensities and S is the slope of the sigmoidal curve.

Determination assay reproducibility of dot-blot assay

Assay reproducibility was determined by calculating the coefficient of variation (% CV) according to Jaedicke *et al.* (2012). Assay reproducibility was evaluated by calculating the intra-assay and inter-assay variation. Five replicates of each sample dilution were run within a same experiment and same day for the intra-assay variation. For inter-assay variation, the assay was performed on five different days for each sample dilution per experiment.

Different OTA concentration 50 800 ng/mL were used for detection with AuNPs-peptide and the normalized spot intensities were used to calculate the % CV as follows.

$$\% CV = SD/\bar{x} \times 100$$

Where,

% CV = coefficient of variation in %

SD = Standard deviation of normalized spot intensities

$\bar{x}$ = Mean of normalized spot intensities

Cross reactivity

The specificity of developed dot-blot assay was determined by testing the cross reactivity of 11-mer peptide against closely related mycotoxins of OTA. 50, 100 ng/mL of AFB1 and 250, 500, ng/mL of citrinin was checked in dot-blot assay.

Reverse phase HPLC for OTA detection

OTA was detected by reverse phase HPLC by HPLC-LC-

20AD (Shimadzu, Japan) using Discovery C18-bonded column. Acetonitrile: water: acetic acid (99:99:2 v/v/v) mobile phase was delivered at constant flow rate of 1 mL/min. The eluted samples were monitored using a fluorescence detector at 333 nm Ex and 460 nm Em. The minimum detectable concentration of OTA was determined by injecting different OTA concentration (10-500 ng/mL) in duplicate in the HPLC column under similar flow conditions. The chromatogram was recorded and processed by the software. The peak of OTA in the samples were identified by comparing the retention time with the standards of OTA and quantified by the comparing the integrated peak area with the standards of OTA.

Extraction and recovery of OTA from spiked wheat samples by HPLC and dot-blot assay

For OTA recovery study, wheat samples were collected randomly from local food stores and markets of Pune, Maharashtra, India. Briefly, 10 g was ground coarsely and OTA was extracted with acetonitrile-water (60/40, v/v) (25 mL) and chloroform according to the method in the previous section, and analysed by the dot-blot assay and HPLC. Wheat samples (10 g) showing no OTA contamination by HPLC were spiked with 100, 200, 400, 800 ng/mL of OTA. Spiked wheat samples were dried in a fume hood overnight and ground coarsely. Extraction from spiked wheat samples was carried out with 25 mL acetonitrile-water (60/40, v/v) by orbital shaking for 1h according to Bayman, *et al.* (2002). Extracts were centrifuged at 8000 rpm for 30 min and the supernatant was mixed with 15 mL chloroform. The organic phase was collected and dried overnight and solubilized using 1 mL methanol. The methanol solubilized sample was filtered with 0.22 µm syringe filter, to eliminate the impurities and stored at 4°C and detected by HPLC and dot-blot assay.

Evaluation of dot-blot assay with wheat samples and different wheat products

Wheat and wheat products (crushed wheat, course wheat powder, fine wheat powder, wheat flour) sample were collected randomly from local food stores of Pune, Maharashtra, India and subjected to OTA detection by HPLC and dot-blot assay. Since the developed dot-blot assay based on visual readout, the assay interpretation was performed by blinding the samples to the readers. The samples were denoted as 'true positive' (TP), 'true negative' (TN), 'false positive' (FP) or 'false negative' (FN) based on the comparison of our assay results with those from the HPLC method. The Detection sensitivity and specificity of the developed dot-blot assay were calculated according to FDA 2018 as follows:

Sensitivity (%) = TP/TP+FN×100

Specificity (%) = TN/TN+FP×100

Positive predicted value (%) = TP/TP+FP×100

Negative predicted value (%) = TN/TN+FN×100

Assay accuracy (%) = .TP+TN/TP+TN+FP+FN×100

Cohen's kappa coefficient of agreement (Cohen, 1960) was calculated to compare the two detection methods as follows:

Cohen's kappa = Pr(a)-Pr(e)/1-Pr(e)

where Pr(a) is the observed relative agreement among raters and Pr(e) is the theoretical probability of chance agreement expected among raters.

Cell viability assay

Human hepatocellular carcinoma cell line HepG2, obtained from (NCCS, Pune, Maharashtra) were grown as a monolayer culture in DMEM supplemented (Himedia) with 10% FBS (Himedia), 1% penicillin / streptomycin (Himedia), and 5 mM β-mercaptoethanol. After trypsinization, 1×10^4 cells were added in 96-well plate and incubated for 8 h at 37 °C in a CO_2 incubator. Cells were exposed to different concentrations of OTA (12.5-100 mM) for 24 and 48 h. Cell viability was determined using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay (Mosmann, 1983). In brief, 20 µL of 5 mg/mL MTT (Sigma Aldrich) in PBS was added to each well and the plates were incubated at 37°C for 4 h. The medium was removed and the purple crystals that formed were dissolved in DMSO (150 µL) and absorbance was measured at 570 nm, according to Mosmann (1983), using a microplate reader (MK3; Thermo) to calculate the percentage viability.

RESULTS

Molecular docking approach for rational designing of peptide for OTA detection

OTA interacts competitively with phenylalanyl -tRNA synthetase enzyme (PheRS) (**Fig. 1A**) (Ha, 2015; McMasters and Vedani, 1999). It was reported that A314, F260, F258, W149, Q204 involved in hydrophobic interaction while H178, R204 and R321 was involved in the H-bonding in the binding site of PheRS (**Fig. 1A**). During linearization for N-AFFHWRQR-C peptide, the positively charged amino acid was preferentially located at the C terminus of peptide to promote its interaction with OTA (**Fig. 1B i&ii**). A low binding energy -5.6 kcal/mol conformer was selected by molecular docking of peptide sequence and OTA (**Table 1**). Peptide hydrophobicity was high (26.85) and displayed hydrophobic interactions of F3, H4, and W5 amino acids with OTA while H-bonding was absent (**Fig. 1 A&B, Table 1**). The -CO group in isocoumarin ring of OTA interacted with the imidazole ring of H4, while the phenylalanine group of OTA interacts with F3 and W5 (**Fig. 1 A&B**). Further amino-acid modification was carried out to design a short peptide having maximum H-bonding, less hydrophobicity and good solubility.

The AFFHWRQR peptide was modified by replacing A1 and F2 with S, a polar amino acid for reduction in hydrophobicity (**Table 1**). In addition, N was introduced after F2 to permit its side chain to form H-bond with the OTA and help in the

affinity binding (**Fig. 1 C i&ii, Table 1**). Further W5, R6 and Q7 were replaced with P5, as proline introduces a kink in the peptide. Further, the R8 was replaced with K6 due to formation of a groove like structure by its side chain to stabilize peptide-OTA binding (**Fig. 1 C i&ii, Table 1**).

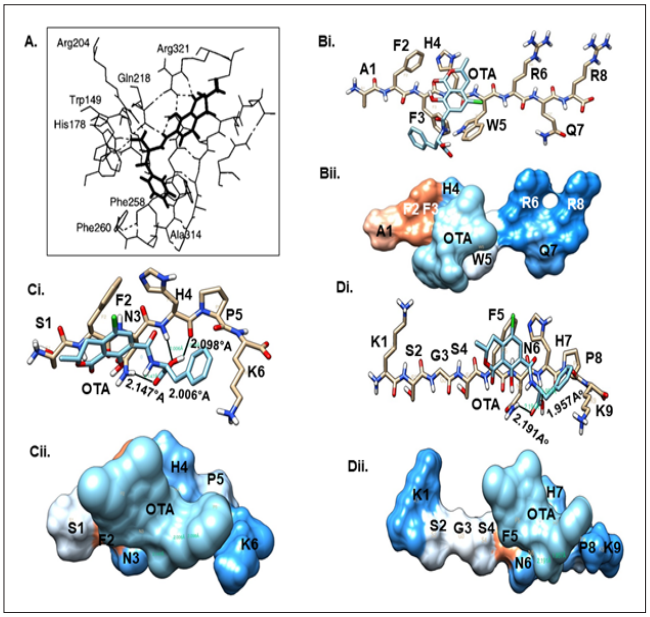

Fig. 1: Peptide designing for OTA by computational modelling **(A)** Binding interaction of OTA and phenyl alanine t-RNA synthetase by molecular docking using Autodock vina 5.1. and visualized by Discovery Studio and Chimera **(B i)** AFFHWRQR amino acid residues present in the binding site represented with the stick structure and **(B ii)** solid surface structure by Chimera **(C i)** Binding interaction of hexa-peptide (N-S-F-N-H-P-K-C) and OTA, stick structure by Discovery Studio and **(C ii)** solid surface structure by Chimera. **(D i)** Binding interaction of modified 9-mer peptide (N-K-S-G-S-F-N-H-P-K-C) and OTA, stick structure solid surface structure by Chimera, **(D ii)** solid surface structure.

The designed SFNHPK peptide showed the presence of 3 H-bonds with N3 (2) and H4 (1) and a low binding energy of - 5.6 kcal/mol (**Fig. 1 C&D, Table 1**). The OTA is in non-covalent Van der Waals interaction with S1, H4, and P5 of peptide. N3 and H4 improve the affinity binding of OTA with designed peptide by H-bonding (**Fig. 1 C i&ii, Table 1**). The P5 residue formed a kink while S1 and K6 side-chain provided a groove for OTA binding while F2 residue enhanced binding by hydrophobic interaction (**Fig. 4 C i&ii**) Hydrophobicity (7.08) was considerably lower than the original peptide implying a good water-solubility. The pI of this peptide was 10.1, suggesting peptide good solubility ≤ 7 pH. Further, the grand average of hydropathy (GRAVY) value (- 1.70) also suggested a good water solubility (**Table 1**).

The hexapeptide (SFNHPK) was modified with a 3-mer linker sequence biotin-KSG at the N' terminus of peptide for conjugation to the streptavidin functionalized AuNPs and to avoid steric hinderance during interaction of the conjugate with OTA (**Fig. 1 D i&ii**), The binding interaction of modified 9-mer peptide with OTA displayed a low stable binding energy of -5.6 kcal/mol similar to that of the unmodified hexapeptide (-5.6 kcal/mol, **Fig. 4 D i&ii, Table 1**). The grand average of hydropathy value (GRAVY value -1.70) and hydrophobicity index of 9-mer peptide (7.64) were calculated by Peptide Analyzing Tool and Pepcal which suggested good water solubility. The pI of 10.6 also confirmed good solubility at pH ≤ 7 (**Table 1**). These results were similar to the unmodified hexapeptide having good water solubility (**Table 1**). The molecular docking studies predicted a good binding affinity of the 9-mer peptide with OTA which serve as a good bio-recognition molecule for t he intended detection assay.

Table 1: Peptide list showing rationally designed peptide for OTA

S.No	Peptide sequence	Binding energy	H-bond present	Hydro-phobicity	Solubility in water	Gravy value	Mol.wt g/mol	pI
1.	AFFHWRQR	-5.6	-	26.85	Good	-1.15	1147.31	12.5
2.	SFNHPK	-5.6	3	7.08	Good	-1.70	728.81	10.1
3.	KSGSFNHPK	-5.6	2	7.64	Good	-1.70	1001.11	10.6

Fluorescence based peptide binding assay

The binding of 9-mer peptide and OTA was assessed by the fluorescence based binding assay. Different concentration of free OTA (10 100 ng/mL) showed 510-5000 AU fluorescence (**Fig. 2**) Fluorescence quenching during OTA-peptide binding suggested that there is a good binding between the OTA and the 9-mer peptide. The OTA-peptide displayed 1.1-to 1.5-fold reduction in the fluorescence intensity as compared to only OTA.

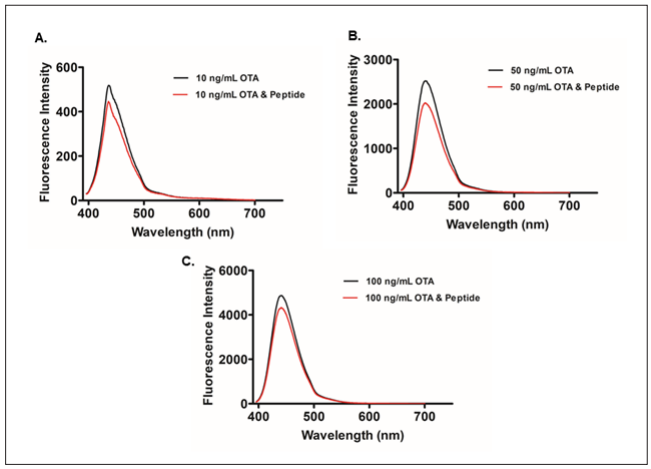

Fig. 2: OTA fluorescence quenching assay. Fluorescence intensity of OTA and fluorescence quenching by addition of 9-mer peptide in 1:1 ratio (A) 10 ng/ml (B) 50 ng/ml (C) 100 ng/ml

Synthesis colloidal AuNPs, their characterization and conjugation with biotinylated 9-mer peptide

AuNPs were synthesized by citrate reduction method and displayed a characteristic wine-red colour with a surface plasmon absorption peak at A 520 nm (**Fig. 3 A&B**). The colloidal AuNPs were 35 ± 5 nm in size by Nano Tracking

Analysis (NTA) (**Fig. 3C**). The size analyses by zeta sizer were in agreement with NTA data while the zeta potential was - 30.8 ± 5 mV.

AuNPs were conjugated with biotinylated 11-mer peptide (biotin-KSGGGSNLHPK) using streptavidin-biotin binding (**Fig. 3D**). First, streptavidin coating of AuNPs was optimized at saturation concentration of 30 µg AuNPs-streptavidin (**Fig. 3D**). AuNPs-streptavidin binding was confirmed by red to blue peak shift (520 nm to 529 nm) of surface plasmon resonance (**Fig. 3E**).

In the next step, streptavidin functionalized AuNPs were conjugated to biotinylated 9-mer peptide (30 µg) at 1:1 ratio (biotinylated peptide: streptavidin, wt/wt), respectively. The AuNPs-streptavidin-biotin-peptide conjugate was 50 ± 12 nm in size. The increase in size was attributed to the binding of biotinylated peptide to the streptavidin functionalized AuNPs surface. AuNPs-streptavidin-biotin-peptide conjugate binding was confirmed by red to blue peak shift (520 nm to 530 nm) of surface plasmon resonance (**Fig. 3E**). Conjugation of AuNPs-peptide conjugate was further confirmed with gel retardation assay, which showed decreased mobility in comparison to bare AuNPs.

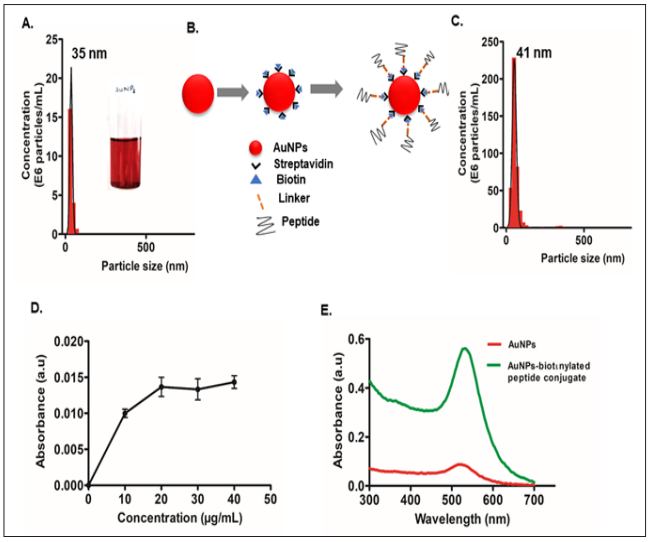

Fig. 3: Characterization of AuNPs and AuNPs peptide conjugate (A) AuNPs showing the wine-red color and particle size by Nano Tracking Analysis (NTA), (B) Schematic showing the conjugation of colloidal AuNPs with biotinylated peptide using biotin streptavidin binding. (C) particle size of 9-mer conjugated AuNPs by NTA analysis (D) Saturation curve for conjugation of biotinylated peptide with AuNPs (E) UV-Vis spectrum shift for bare AuNPs and conjugated AuNPs

Standardization of dot-blot assay for OTA detection with AuNPs bound 9-mer peptide conjugate

The AuNPs bound 9-mer peptide conjugate was used as a label for standardization of dot-blot assay for OTA detection, visually by the appearance of a red spot **(Fig. 4A)**. **Fig. 4B** showed visual increase in spot intensities corresponding to the increasing toxin concentrations 50 - 800 ng/mL on NCM. Further, measurement of spot intensities by ImageJ software showed a significant increase in colour development with OTA concentrations (**Fig. 4C**). The assay showed a good linear correlation (R2= 0.99) when the normalized spot intensities were plotted against the OTA concentrations (**Fig. 4D**). The limit of detection (LOD) for the developed dot-blot assay was 0.19 µg/kg as determined by logarithmic linear graph (**Fig. 4E**).

Assay reproducibility

The assay precision and reproducibility with AuNPs bound 9-mer peptide for OTA detection was calculated as % CV, which ranged from 1.9-6.5 % and 2.2 - 8.0 % for intra- and inter-assay variations, respectively. These values were in the acceptable range of ≤ 15% according to the US Food and Drug Administration (FDA) which indicate a high level of assay reproducibility (FDA guidelines for bioanalytical method validation, 2018).

Cross-reactivity

Cross-reactivity of AuNPs-peptide was tested with aflatoxin B1 and citrinin (**Fig. 4 F, G&H**). The AuNPs-peptide did not cross react with aflatoxin B1 upto 100 ng/mL. Citrinin did not show cross-reactivity upto 500 ng/mL (**Fig. 4 I & J**).

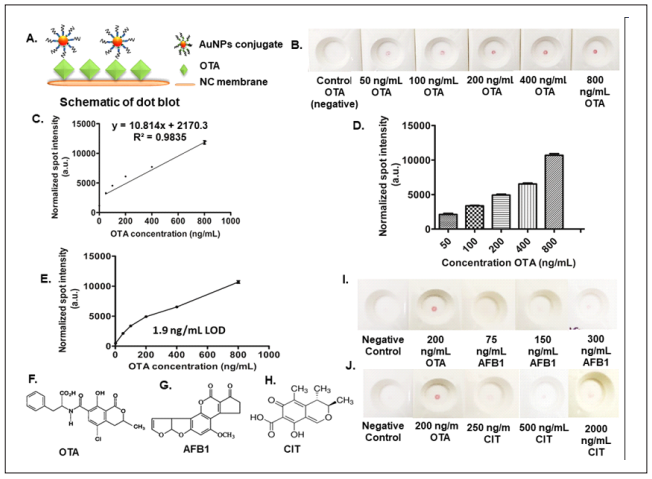

Fig. 4: Development of dot-blot assay for OTA detection (A) Schematic of peptide-based dot-blot assay (B) Dot-blot assay for detection of OTA, red spot intensity increases with increasing in concentration of OTA (C) Spot intensity measured by ImageJ software and normalized by the negative control (D) Standard graph for OTA detection by dot-blot assay (E) LOD measurement for the developed dot-blot assay. (F, G and H) Structures of OTA, AFB1 and citrinin. (I and J) Cross- reactivity test of 9-mer peptide in the dot-blot assay with AFB1 and citrinin, respectively.

Recovery of OTA from spiked wheat samples

OTA was extracted from spiked wheat samples and detected with the developed dot-blot assay and HPLC. Standard OTA concentrations (1-500 ng/mL) were detected by HPLC at the retention time (RT) of 9.2 min and gave a linear response with the high correlation coefficient r = 0.9998. In dot-blot assay, the spot intensities were measured with ImageJ software in comparison to negative control to determine the concen-

tration of OTA and showed a recovery of 90-96% **(Fig.5A)**. The detection of OTA recovered from spiked wheat samples was 98-99% by HPLC method **(Fig. 5A)**.

Evaluation of dot-blot assay

The developed dot-blot assay was evaluated by analyses of CRM and 146 samples (i. whole wheat, ii. crushed wheat, iii. course wheat powder, iv. wheat flour, v. refined wheat flour and, vi. Peanut, vii. grapes, viii. dog feed) for the occurrence of OTA in comparison to the standard RP-HPLC method (**Fig. 5 B, Table 2**). CRM sample detected by HPLC showed < 0.6 µg/kg OTA concentration. The dot blot assay showed a faint spot below the detection limit of assay that confirmed very low concentration of OTA in CRM. Among 146 samples, 118 samples tested negative for OTA and 7 showed the presence of OTA (≤ 5 µg/kg) by both methods (**Table 2**). Only 7 samples give false negative results, with dot-blot as compared to HPLC (**Table 2**).

The developed dot-blot assay showed diagnostic sensitivity and specificity of 74 and 99%, respectively and an assay accuracy of 94% (**Table 2**). The positive and negative predictive values obtained were 95 and 94%, respectively. Further, a significant correlation coefficient for dot-blot and HPLC methods was obtained (R2=0.75) indicating substantial agreement of the two methods (**Fig. 5 C**). The Cohen's kappa coefficient was 0.94 showing a substantially agreement of the two methods.

Cell viability assay of OTA and its photodegradation products

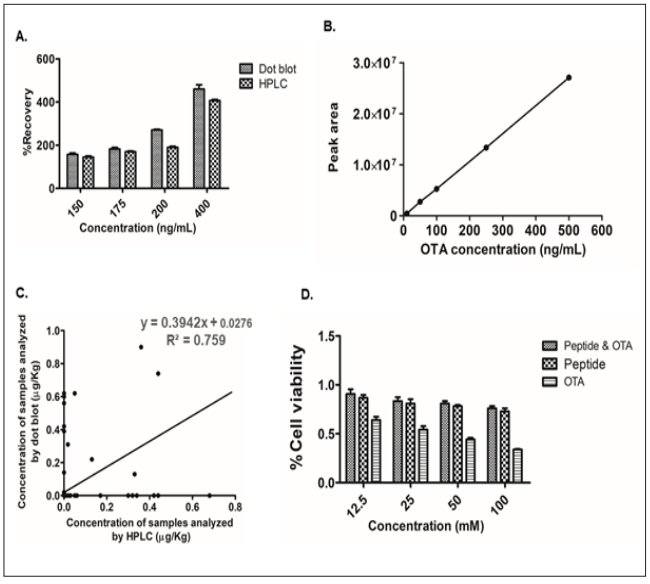

Fig. 5: Evaluation of developed dot-blot assay. A) Percent recovery for OTA by dot-blot assay and HPLC. B) Standard curve of OTA detection by HPLC. C) Correlation between dot-blot assay and HPLC by linear regression curve. D) % Cell viability of HepG2 cells at different concentration of peptide and OTA, only peptide and only OTA.

Cell viability in the presence of different concentration of OTA, 9-mer peptide and OTA-peptide was assessed using the MTT assay. Following incubation for 24h, the viability of $HepG_2$ cells decreased in a concentration-dependent manner with increasing concentrations of OTA from 12.5 to100 µM. The mixture of OTA-peptide showed good viability of $HepG_2$ cells as compare to only OTA suggested peptide reduce the toxic effect of the OTA by binding to it and possibly served as a therapeutic peptide for OTA (**Fig. 5D**).

Table 2: Evaluation of the dot-blot assay in detecting OTA from food and samples as compared to HPLC

No. of samples tested by the dot-blot assay for AFB_1 detection				
Positive	Negative	False positive	False negative	Total
21	125	0	0	146
No. of sample tested by HPLC				
Positive	Negative			Total
20	118	1	7	146
Statistical parameters for evaluation				
Sensitivity (%)			74	
Specificity (%)			99	
Positive predicted value (%			95	
Negative predicted value (%)			94	
Assay accuracy (%)			94	
Cohen's kappa coefficient			0.94	

DISCUSSION

OTA is a toxic food and feed contaminant which is a causative agent of nephrotoxicity, carcinogenicity and mutagenicity in humans and animals (Bui-Klimke and Wu, 2015). Most methods used for OTA detection depend on instrumental analyses by thin-layer liquid chromatography, high pressure liquid chromatography, ultra-performance liquid chromatography-tandem mass spectrometry and immuno-assays such as ELISA (Li *et al.,* 2022). Although these methods provide a quantitative analysis, the complex preparation, time-consuming procedures, and expensive equipment are not suitable for routine use in basic laboratories and on-site detection (Turner *et al.,* 2009; Meulenberg*,* 2012; Zhang *et al.,* 2018). Therefore, paper-based assays like LFA and dot-blot are simple, fast and portable immunochromato-graphic assays that provide a better alternative for on-site detection with low operational costs (Laura *et al.,* 2011; Bahadir and Sezgintürk, 2016; Zhang *et al.,* 2018).

Usually, paper-based assays for OTA detection are developed with antibodies, as recognition agent in direct or indirect formats (Hong *et al.,* 2022). However, antibodies are toxic, expensive and difficult to generate against mycotoxins due to their low molecular weight (Sun *et al.,* 2021). Peptides provides a better alternative as they have several advantages

like they can be easily synthesized, functionalized and are specific and stable (Bazin *et al.,* 2013; Giraudi *et al.,* 2013; Heurich *et al.,* 2013). *In silico* peptide designing by homology modelling can help in selection of the specific and accurate peptide for the target mycotoxin (Usmani *et al.,* 2018). Peptide designing by *in silico* rational approach is fast, easy, and has a simple selection procedure (Basu *et al.,* 2021).

In this paper we describe the rational designing of specific peptide for OTA, using bioinformatics tools with target protein phenylalanine t-RNA synthetase (PheRS). OTA binds competitively to the PheRS and inhibits protein synthesis. The interaction of PheRS and OTA reported by McMasters and Vedani (1999) was utilized for specific peptide designing. The interaction showed participation of A314, F260, F258, H178, W149, R204, Q218, and R321 amino acids in the binding site of PheRS. It was reported that A314, F260, F258, W149, Q204 involved in hydrophobic interaction while H178, R204 and R321 was involved in the H-bonding.

The AFFHWRQR peptide was linearized and modified to yield the short peptide sequence SFNHPK. The peptide displayed low binding energy and high affinity with 3 H-bonds with N3 (2) and H4 (1), and a low binding energy of -5.6 kcal/mol. The peptide had good solubility, low hydrophobicity and pI 10.1 suggested good solubility at ≤ 7 pH. The molecular docking performed with Autodock vina 5.1. The interaction was visualized with Discovery studio2017R2 and Chimera. The 3-mer linker amino acid sequence was added at the N-terminus of peptide sequence to avoid steric hindrance when bound to AuNPs as the colorimetric visual label.

In silico studies of the 9-mer peptide (KSGSFNHPK) displayed a good binding affinity with OTA. Molecular docking showed low-binding energy (-5.6 kcal/mol) and hydrogen bonding of peptide N6 and H7 with OTA. The peptide had good solubility, low hydrophobicity and pI 10.6 suggested good solubility at ≤ 7 pH same as hexamer peptide. The wet lab experiment by OTA fluorescence quenching by 9-mer peptide also confirmed the binding interaction of designed peptide and OTA.

The 9-mer peptide was conjugated to gold nanoparticles label using biotin-streptavidin binding interaction for its use in the development of a detection assay. Gold nanoparticles (AuNPs) are extensively used as detection labels due to their small size, easy synthesis, functionalization and visual detection. The dot-blot assay showed a LOD of 0.19 µg/kg for OTA detection. Li *et al.* (2016) developed a multi-analyte LFA for the detection of deoxynivalenol (DON), T2 toxin and fumonisin B1 (FB1) using specific antibodies. The LOD for the detection assay was 242 µg/kg for DON, 17.8 µg/kg for T2 and, 331.5 µg/kg for FB1. The dot-blot assay showed negligible cross-reactivity with co-occurring mycotoxins such as AFB1 and CIT. Moreover, the developed dot-blot assay had low intra- (1.8- 9.5%) and inter- (2.3-7.9%) assay variations. The % CV in of the detection assay was less than 21% for reported LFA.

Further, the mAbs-based commercially available detection assays includes Ochra-V AQUA LFA from VICAM (US, https://www.vicam.com) which has 0-30 ppb limit of quantitation for a wide range of food materials detected by reader for the strip intensities. Recently, Fadlalla *et al.* (2020) develop a competitive format of LFA for the detection of OTA and OTB using colloidal AuNPs (CAuNPs) and gold nanoflowers (AuNFs). Single mAb was used a recognition agent for the detection of OTA and OTB in different food and agricultural products. The LOD of the assay was 5 and 1 µg/mL for CAuNPs and AuNFs, respectively. The % CV of the reported LFA was 2.182 % in spiked samples.

OTA extraction from food and feed material was achieved by organic solvents like acetonitrile and chloroform. OTA is non-polar in nature its showed good solubility in acetonitrile and chloroform. Therefore, these solvents are appropriate for the extraction of OTA from food and feed materials (Rahmani *et al.,* 2009; Turner *et al.,* 2009). The OTA percent recovery from spiked wheat samples suggested that dot-blot assay (90 - 96%) was in agreement with the standard HPLC (97 - 99%) method. The recovery of OTA from spiked wheat samples were comparable to the reported recoveries for OTA. Lippolis *et al.* (2017) reported 86 and 95% average recovery values of OTA from rye and rye crispbread respectively. The extraction was carried out by the acetonitrile/water (60/40) by solid phase extraction. Shahdeo *et al.* (2021) reported an aptamer based diagnostic assay for detection of OTA in microfluidics fabricated device. For detection extraction was carried out from spiked corn and groundnut samples with % recovery ranges from 43-87%.

146 samples of food and feed materials including wheat and wheat product (whole wheat, ii. crushed wheat, iii. coarse wheat powder, iv. fine wheat powder, v. wheat flour) peanut, grapes, and dog feed along with CRM was evaluated for OTA detection by the developed dot blot assay. All samples were analyzed by the dot-blot assay and standard HPLC method. The developed dot-blot assay displayed high (94%) accuracy, good sensitivity (74%) and specificity 99%. The positive and negative predictive values were 95%, and 94%, respectively. Cohen's kappa coefficient (0.94) and correlation coefficient (0.75) indicated good agreement between the dot-blot assay and the standard HPLC method for OTA detection. These results demonstrated that the developed dot-blot assay would be reliable for detection of OTA in food and feed samples.

The binding of OTA with 9-mer peptide was also utilized to study the cell viability of liver (HepG2) cells in presence of OTA. The mixture of OTA-peptide showed good viability of HepG2 cells as compare to only OTA suggested peptide reduce the toxic effect of the OTA by binding to it and possibly served as a therapeutic peptide for OTA. Agarwal *et al.* (2020) reported a protective efficiency of N-Acetyl-L-Tryptophan against OTA induced toxicity was estimated

using embryonic kidney cells. The % viability was 89% in comparison to 50% in only OTA.

CONCLUSION

We have developed a specific 9-mer peptide-based dot-blot assay for the detection of OTA. The specific peptide was designed *in silico* using bioinformatics tools. The 9-mer peptide were successfully tagged with AuNPs and an on-site, rapid, sensitive paper-based dot-blot assay was developed for OTA detection in food and feed materials. The dot-blot assay displayed a LOD of 0.19 µg/kg and did not display cross-reactivity with AFB_1 and CIT. The coefficient of variation for inter- and intra- assay were in acceptable range according to FDA standards. Further, the developed assay is specific, sensitive and accurate and does not require any sample pre-treatment, trained personnel and sophisticated instrument. The evaluation of food and feed materials including certified reference materials suggested this method can be used for screening of large number of samples in industries and the laboratories. The dot-blot assay is simple, cost-effective, rapid and feasible for the small labs. In future the peptide-based detection can be further used for other mycotoxins detection in multiplexed paper-based format. Further peptide-based dot-blot assay can be used for the detection of other food contaminants and in biomedical field as a point-of-care test.

ACKNOWLEDGEMENT

SR acknowledges the fellowship from Department of Science and Technology Inspire Programme. VG thanks Department of Biotechnology for the project grant (BT/PR10455/PFN/20/869/2013). The authors thank Dr. Vikram Lanjekar, Bioenergy Group, Agharkar Research Institute for the HPLC analyses.

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