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Detection of Mycotoxins from the ground nuts samples of Warangal District, Telangana State

Murali Krishna Thupurani[1], Rajender Vadlluri[2], Epur Manoj Kumar Reddy[3], B.S Anuradha[4]
Department of Biotechnology, Chaitanya Degree and Postgraduate College (Autonomous), Hanamkonda, Warangal, 506001-Telangana –India

Abstract: ***The main objective of this study is to conduct survey on ground nut fields and to detect the mycotoxin producing fungi and variety of mycotoxin. All the samples were collected from various systems such as freshly harvested groundnuts (FHG), farmer storage systems (FSS), wholesalers sample (WS), retailer (RS) from different regions of Warangal District. The study includes the screening of moisture content (m.c.) of the samples, mycological analysis, identification of the fungal genera, aflatoxin extraction and analysis by ELISA. The m.c. of samples collected during wet season from different storage systems ranges between 10.1 – 23.6. The freshly harvested ground samples exhibited highest m.c. percentage with 23.6%. Aspergillus spp. Fusarium sp., Cladospoium sp., Penicillium spp., Rhizopus stolonifer and yeasts are identified from samples collected. The total enumerated of isolated fungi from the collections as (89×10^{3}) CFU. High fungal contamination was observed with the sample kadiri-2(MK-374) The aflotoxin extraction using ELISA showed that Kadiri-2 (MK-374) resulted in high 8.2, ppb aflatoxin content.***

Key words: aflatoxin, mycotoxin, toxigenic fungi

I. INTRODUCTION

Mycotoxins are considered as secondary metabolites produced by toxigenic fungi. These fungi grow commonly in different agricultural crops and their products includes food and feed stuffs and been a potential threat to human beings and animals by causing serious health problems. The consumption of fungal contaminated foods such as fungal moulds, may lead to carry-over the mycotoxins in human being and animals [1].

According to the Council for Agricultural Science and Technology, globally 25% of crops are affected annually by mycotoxins (Trail et al., 1995)[2]. Currently, above 300 mycotoxins are identified from different sources. Among those aflatoxins are the major class of mycotoxins identified and characterized from the secretions of *Aspergillus sps* (Diener *et al.*, 1987; D'Mello and MacDonald, 1997)[3,4]. Using fluorescence and relative chromatography the major aflatoxins identified are B1, B2, G1, and G2, M1 and M2 (D'Mello and MacDonald, 1997)[4]. Among these Aflatoxins, B1 reported as most potent natural carcinogen (Squire, 1981)[5]. Telangana is well known state for large cultivation of groundnut crops in India. Approximately, 70-80 million tons (35-60 million hectares) of ground nuts are produced and exported to various regions of India especially from Warangal, Karimnagar, Mahaboobnagar districts. According to the Regional agricultural Research Station, Warangal, 1059 mm rain fall notices per year, which leads to development of suitable condition for the growth of fungal species and production of mycotoxins. Approximately, 565 Kg/hector in Kharif and 674 Kg/hector in Rabi is found as overall ground nut production in surrounding of Warangal district. With the above data of ground production in Warangal, the present study was framed out to for the detection of mycotoxin contamination in ground fields of Warangal district.

II. MATERIALS AND METHODS

During survey, we have collected (during August to November and October to March) different varieties of ground nut samples such as Kadiri-2 (MK-374), Kadiri-3 (Robout-33-1), Kadiri-4, Kadiri-5, Kadiri-6, Kadiri-7, Kadiri-8, Kadiri-9, Kadiri-71-1(Virginia Group), Abhaya (TPT-25), Greeshma, Icgv-91114, Jagtial-88 (JCG-88), Kalahasti (TCGS-320), Narayani (TCGS-29), Prasana (TCS-341), Rars-T-1, Rars-T-2, Tirupati-4 (TCGS-30), Jyoti, JM-2, JM-3, JM-24 from various District Mandals of Warangal (Table 1).

A. Sample Preparation

A total of twenty three samples, representing various varieties of ground nuts were used in the study. For each sample, 3 replicates

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were taken to prepare one composite sample. All the samples were sealed and stored at 3-5°C for mycoflora and mycotoxin determination. Samples were finely ground in a common household blender and rinsed in 85% alcohol. The powder stored at 4°C for further analysis.

1) *Moisture Content Analysis:* Percent moisture content of kernels was determined by the oven method (ISO 2014)[8]. Three replicates were used for each sample. The kernels were ground and dried in the oven at 130^0 C for 2 hours.

The moisture content was determined using the formula:

$$Moisture\ content = (Mo - MI)X\ \frac{100}{MO}$$

Where; Mo is the initial mass, in gram, of the test portion
Ml is the mass, in gram, of the dry test portion

B. Mycological analysis

1) Isolation of Sample-Borne Mycoflora: The isolation of fungi was carried out using the method previously described by Abdullah *et al.* (2002)[6]. 10 gm of each sample was decontaminated using 5-6% NaOCl (Sodium hypochlorite) for 1-2 min and rinsed with distill water. The disinfected samples are inoculated on the media that contain Czapek Dox Agar (CDA) supplemented with 0.5 mg chloramphenicol/mL to inhibit the bacterial growth. Three replicates were made and the plates were incubated at 25°C for one week. The fungi colonies were identified according to morphological and microscopic characteristics.

2) Standard Dilution Plate For Determination Of Colony-Forming Units: For fungal analysis, dilution method was used to determine total fungal counts in nut products samples. One grams of each composite sample (fine powder) were transferred into screw-capped medicinal bottle containing 9 mL of sterile distilled water and were mechanically homogenized at constant speed for 15 min. The sample-water suspension was allowed to stand for 10 min with intermittent shaking before being plated. Appropriate tenfold serial dilutions (1:10) were prepared and one mL portions of suitable dilutions of the resulting samples suspension (10^{-3}) were used to inoculate Petri dishes each containing 15 mL Potato Dextrose Agar (PDA). Plates were then incubated for 7 days at 28^{o}C. Three replicates plates per medium were used for each sample and the developing fungi were counted and identified according to several key processes. After incubation, the results were expressed in Colony-Forming Units (CFU) of samples; all plates were examined visually, directly and with a microscope [7].

III. DETERMINATION OF POTENTIAL TOXIGENIC FUNGI USING DRBC TEST

DRBC (Dichloran Rose Bengal chloramphenicol) is a selective medium that supports good growth of fungi. Dichloran reduces colony diameters of spreading fungi, rose bengal suppresses the growth of bacteria and restricts the size height of colonies of the rapidly growing moulds, chloramphenicol inhibits the growth of bacteria present in environmental and samples. The reduced pH of the medium from (7.2 to 5.6) helps inhibition of the spreading fungi [8,9]. The isolated fungi were inoculated in the solidified DRBC medium after incubation for 7 days at 25^{o}C search for pigmentation and color change observed due to toxigenic compound in compare to CDA medium control. DRBC containing compounds to inhibit or reduce spreading growth of moulds such as *Mucor* sp., *Rhizopus* sp., [10]. Dichloran and rose Bengal effectively slow down the growth of fast-growing fungi, thus readily allowing detection of other yeast and mold propagules, which have lower growth rates.

A. Aflatoxin Extraction and Analysis by ELISA

The homogenized samples (10 g) of each were taken in 50 mL of 70% methanol separately and blended individually for 3 min. Sample was filtered and used for analysis. Commercially available immunoassay kit Veratox for quantitative analysis of aflatoxin and ochratoxin test-NEOGEN Crop, Lansing, MI was used. The assay kit was based on Competitive Direct Enzyme Linked Immunosorbent Assay (CD-ELISA). The antibodies captured the analyte and conjugated to the enzyme (horse reddish peroxidase). Tetra methylbenzidine/hydrogen peroxide was used as a substrate for color development. Finally stopping solution was added to stop the reaction. The color intensity was inversely proportional to the mycotoxin concentration and measured with the ELISA reader. All necessary reagents were present in the kit. Concentration of mycotoxins was calculated by Log/logit Software Awareness

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Technology Inc. (Stoloff *et al*., 1991)[11].

IV. RESULT AND DISCUSSION

Moisture content (m.c.) is the key factor for entering and development of fungi in the ground nuts (Christensen & Kaufmann 1975) [12]. The moisture content approximately 9.0-10.0% is favorable for invading of *Aspergillus flavus* in groundnuts. **Table 2 and 3** represents the percentage of m.c.'s and kernel damage of samples collected during the wet season. The percentage of moisture content and damaged kernels analysis was selectively conducted only. In the previous paper we have evaluated moisture content for few samples from overall 23 sample collection and now here we present the data of remaining samples. The m.c. of samples collected from Lingalaghanpur, Raiaparthy and Shyampet of different storage systems were found high 42.5%, 32.9%, 23.6 respectively (See table 2). However, freshly harvested ground samples showed highest m.c. percentage (Table 2).

The percentages of kernel damage of the samples collected during wet season are represented in Table 2. Samples collected from Narsampet, Dornakal, Maddur, Kesamudram and Eturnagaram were found high in kernel damage and ranges between 44.5-50.7, 35.5-40.9,44.5-48.1, 36.7-48.4, 41.4-50.2, 35.0-41.5, 33.5-41.8, 40.2-58.3, 38.0-41.9 of whole sale and retailer samples respectively.

The isolation and enumeration of surface fungal species we have used Czapek Dox Agar (CDA) media. The results of **Table 3** describes about the type isolated fungi using Agar Plate Method (APM) plated on CDA medium. The test was carried with two sets of sample that are off unsterilized and surface sterilized nut samples. Totally 12 samples were screened for enumeration of isolated fungal species. Based on morphological and cultural characteristics we have isolated six types of fungal genera and fourteen fungal species. The genera identified are *Aspergillus* spp. *Cladospoium* sp., *Fusarium* sp., *Rhizopus stolonifer*, *Penicillium* spp. and yeasts.

Among the fungi isolated *Aspergillus* spp was found in almost all the samples tested. Data of the current study correlates with the foundings of many investigations worked on seed pathology (Khomeiri *et al*. 2008; Sejiny *et al*. 1989)[13,14]. The result shown in Table 4 and 5 shows the range of fungal infection ground nut samples collected from different localities and different storage systems. Table 6 presents the total Colony Forming Units (CFU) of fungi isolated by standard plate technique. All the samples tested contain certain type fungi. The total enumerated of isolated fungi from the 12 collections was (175×10^{3}) CFU. High fungal contamination was observed with the sample Abhaya (TPT-25) (See table 6). The remaining samples were showed minimum, average and moderate fungal contamination. Among the fungal strains identified, *A. flavus* (16×10^{3}) was found with all most all samples collected. Following to *A. niger*, *yeast* (09×10^{3}), showed high infection in collected seeds comparing to remaining samples (See table 6). Our data is correlated with previously reported articles and revealed that, most of the dominant fungal species that infects peanut samples are *Aspergillus flavus*, *Aspergillus niger*, *Penicillium citrinum* (Rostami, *et al*., 2009; Magraby and Maraghy 1987; Maraghy 1988) [15-17]. Screening of toxogenic fungi using DRBC media is represented in table 7. The samples collected from different regions of Warangal District were showed for the presence of potential toxogenic fungi. The species identified are *A. niger, A. flavus, A. fumigates, A. candidus P. aethiopicum, P. fellutanum, P. citrinum, F. equiseti, R. stolonifer* and Yeast.

Aflatoxin extraction and analysis by ELISA revealed that, among the samples tested Kadiri-2 (MK-374) collected from Bhupalpalle region resulted in high 8.2 ppb aflatoxin content.

The present results are in correlation with other investigation reports published that ground samples are commonly infected by Aspergillus species that are responsible for production of afotoxin [18]. In accordance to the results obtained in the current study, here we report that the aflatoxin concentrations in the collected samples are found in safe limits for human consumption.

V. CONCLUSION

The samples collected from various regions of Warangal district were found for presence of toxoginic fungi and detected for the secretion of aflotoxin levels. However, the levels are found limit and safer for human consumption.

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Table 1: Survey of ground nut fields for the collection of ground seeds from various Mandals of Warangal District

S.No	Mandal	Area (Hectares) Kharif	Rabi	variety
1.	Bhupallpalle	33.87	25.5	Kadiri-2 (MK-374)-2015
2.	Cheriyal	41.96	52.6	Narayani (TCGS-29)-2015
3.	Dornakal	38.59	30.1	Kadiri-4-2015
4.	Duggondi	38.0	36.8	Rars-T-1-2015
5.	Eturnagaram	38.7	44.9	Jyoti-2015
6.	Kesamudram	40.5	41.4	JM-24-2015
7.	Lingalaghanpur	55.5	64.2	Abhaya (TPT-25)-2015
8.	Maddur	76.1	84.8	Kadiri-7-2015
9.	Narsampet	52.5	59.1	Kalahasti (TCGS-320)-2015
10.	Raiparthy	24.6	----	Tirupati-4 (TCGS-30)-2015
11.	Shyampet	39.1	20.6	Kadiri-71-1(VirgniaGroup)-2015
12.	Wardhannapet	45.7	22.3	Greeshma-2015

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Table 2: Moisture content and percentages of damaged kernels of ground nut seeds collected during wet season.

Date and Place of collection	Source of sample	Nature of sample	Sample Code	(%) Moisture Content	(%) Kernel Damage
20th June 2015 Bhupalpalle	Farmer Storage sample (FSS)	Shelled	MK-M-S-15-II-2015	10.1-12.0	20.3-31.9
14th Jan, 2015 Dornakal	Wholesaler sample (WS)	Unshelled	MK-M-US-6-II-2015	11.0	44.5-48.1
	Retailer sample (RS)	Unshelled	MK-M-US-21-II-2015	16.1	36.7-48.4
8th Feb, 2015 Eturnagaram	House Kitchen (HK)	Unshelled	MK-M-US-8-II-2015	11.9	38.0-41.9
25th Mar, 2015 Maddur	Wholesaler sample (WS)	Unshelled	MK-M-US-27-II-2015	10.1	41.4-50.2
	Retailer sample (RS)	Unshelled	MK-M-US-19-II-2015	15.9	35.0-41.5
15th May,2015 Wardhannapet	Wholesaler sample (WS)	Unshelled	MK-M-US-15-II-2015	13.1	22.1-38.0
	Retailer sample (RS)	Unshelled	MK-M-US-2-II-2015	11.8	37.0-45.8
12th July, 2016 Lingalaghanpur	Freshly Harvested Ground nut (FHG)	Shelled	MK-M-S-5-II-2015	42.5	----------
20th June 2015, Shyampet	Farmer Storage sample (FSS)	Shelled	MK-M-S-15-II-2015	23.6	25.8-35.5
14th Jan, 2015 Kesamudram	Wholesaler sample (WS)	Unshelled	MK-M-US-6-II-2015	10.8	33.5-41.8
	Retailer sample (RS)	Unshelled	MK-M-US-21-II-2015	15.0	40.2-58.3
8th Feb, 2015 Duggondi	House Kitchen (HK)	Unshelled	MK-M-US-8-II-2015	19.0	36.0-51.0
25th Mar, 2015 Narsampet	Wholesaler sample (WS)	Unshelled	MK-M-US-27-II-2015	12.7	44.5-50.7
	Retailer sample (RS)	Unshelled	MK-M-US-19-II-2015	15.5	35.5-40.9
15th May,2015 Cheriyal	Wholesaler sample (WS)	Unshelled	MK-M-US-15-II-2015	14.0	28.0-38.3
	Retailer sample (RS)	Unshelled	MK-M-US-2-II-2015	10.1	27.6-35.0
12th July, 2016 Raiaparthy	Freshly Harvested Ground nut (FHG)	Shelled	MK-M-S-5-II-2015	32.9	----------

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Table 3: Surface fungal genera and species isolated from the ground seeds using Agar Plate Method with and without treatment of sodium hypochlorite.

Sample Collection	Czapex dox agar	
	Untreated	Treated
Bhupalpalle		
1. Kadiri-2 (MK-374)	*A. candidus, A. flavus,*	*A. flavus*
2. Narayani (TCGS-29)	*A. flavus, A. tamari,*	*A. flavus*
Dornakal		
3. Kadiri-4	*A. niger,A. flavus,*	*A. niger*
4. Rars-T-1	*A. tamarii, A. candidus*	*A. candidus*
Eturnagaram		
5. Jyoti	*A. wentii, A.fumigates,*	*R. stolonifer*
6. JM-2	*A. fumigatus, A. niger*	Yeast
Maddur		
7. Kadiri-8	*A. ochraceus, A. niger*	*F. equiseti*
8. Kadiri-9	*P. aethiopicum, A.candidus*	*P. fellutanum*
Wardhannapet		
9. JM-24	*P. citrinum, A. fumigatus*	*A. tamarii*
10. Abhaya (TPT-25)	*A .fumigates,A niger*	*P. citrinum*
Lingalaghanpur		
11. Kadiri-7	*A. niger, A. candidus*	*A. candidus*
12. ICGV-91114	*A. candidus, A. flavus,*	*P. aethiopicum*
Shyampet		
13. Jagtial-88 (JCG-88)	*A. flavus, A. niger*	*A. fumigatus*
14. Kalahasti (TCGS-320)	*A. ochraceus, A. candidus*	Yeast
Kesamudram		
15. Kadiri-3(Robout-33-1)	*A. candidus, A. flavus,*	*A. niger*
16. Prasana (TCS-341)	*A. ochraceus, A. candidus*	*A. flavus*
Duggondi		
17. Kadiri-5	*A. flavus, A. tamarii*	*A. tamarii*
18. Rars-T-2	*A. tamarii, A. niger*	*A. ochraceus*

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Narsampet		
19. Tirupati-4 (TCGS-30)	*P. citrinum, P. aethiopicum*	*A. tamarii*
20. Kadiri-6	*A. niger, A. flavus ,*	*A. candidus*
Cheriyal		
21. Kadiri-71-1(Virginia Group)	*A. ochraceus, A. candidus*	*R. stolonifer*
22. JM-3	*A. fumigates A. flavus*	*P. fellutanum*
Raiaparthy		
23. Greeshma	*A. niger,A. flavus*	*A. flavus*

Table 4: Range of fungal infection ground nut samples collected from different localities and different storage systems

	Bhupalpalle		Dornakal		Eturnagaram		Maddur		Wardhannapet		Lingalaghanpur	
	D	W	D	W	D	W	D	W	D	W	D	W
	WS	RS	WS	RS	WS	RS	WS	RS	WS	RS	WS	RS
Fungi												
A. flavus	45-49	31-38	40-51	52-59	58-63	78-85	50-55	40-47	33-40	52-60	28-35	43-49
	51-66	48-55	31-54	70-82	63-86	32- 41	19-25	29-32	33-37	39-43	48-57	17-25
A. niger	35-41	40-53	50-69	44-55	63-68	54-71	38-42	59-66	52-68	35-41	44-50	26-34
	29-38	50-61	32-45	50-56	25-29	35-43	20-32	37-48	22- 30	66-75	27-33	48-60
A. fumigates	19-29	33-36	38-45	22-31	35-38	49-66	51-54	57-59	30-39	10-20	58-66	50-58
	35-36	27-35	41-50	52-61	42-48	40-49	25-32	26-35	30-37	50-69	48-50	54-63
A. candidus	67-85	41-50	80-89	61-65	35-42	75-89	68-70	55-61	35-37	37-43	48-60	69-78
	44-52	40-51	70-81	36-40	33-40	35-49	30-36	28-33	20-25	19-28	58-61	70-88
F. equiseti	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-
R. stolonifer	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-
P. fellutanum	15-20	12-19	14-17	10-15	05-11	20-25	14-20	18-20	19-25	30-35	44-53	40-55
	33-40	47-56	38-39	30-41	16-20	08-16	15-22	10-18	09-25	08-15	04-09	18-23
P. aethiopicum	-	-	-	-	-	-	-	-	-	-	-	-

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P. citrinum -

Yeast -

D- Dry sample, W- Wet sample, WS- Wholesale Sample, RS-Retail Sample

Table 5: range of fungal infection ground nut samples collected from different localities and different storage systems

	Shyampet				Kesamudram				Duggondi Raiaparthy				Narsampet				Cheriyal			
	D		W		D		W		D		W		D		W		D		W	
	WS	RS	WS	RS	WS	RS	WS	RS	WS	RS	WS	RS	WS	RS	WS	RS	WS	RS	WS	RS
Fungi																				
A. flavus	65-79	41-52	30-62	59-66	61-66	71-75	60-65	68-74	48-55	50-62	38-39	44-52	56-69	59-65	40-48	80-88	60-66	52- 61	39-42	29-35
A. niger	32-39	45-56	70-78	44-48	56-70	30-39	35-42	30-35	28-32	35-41	30-40	41-46	52-68	58-69	55-69	60-79	45-55	30-38	28-33	67-70
A. fumigates	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. candidus	74-79	52-60	60-71	66-73	75-80	59-69	60-68	59-65	40-45	49-53	51-60	45-63	53-70	55-60	73-80	45-35	38-44	49-55	58-70	49-63
F. equiseti	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R. stolonifer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. fellutanum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aethiopicum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

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P. citrinum -

Yeast 53-59 45-50 60-71 58-63 57-60 45-50 56-60 65-73 48-55 56-65 65-70 35-53 40-60 35-48 49-55 65-75 58-70 69-79 35-45 58-63 30-45 28-33 38-41 47-56

D- Dry sample, W- Wet sample, WS- Wholesale Sample, RS-Retail Sample

Table 6: Fungal isolates using standard dilution plate method and the Colony Forming Units

Sample	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	TCFU
A. flavus	0	0	5	1	2	1	0	2	1	1	1	2	16
A .niger	2	0	0	0	2	1	3	1	1	1	0	0	11
A. umigates	0	1	0	0	2	1	1	0	2	1	0	0	08
A. candidus	0	0	0	2	2	0	0	1	0	1	2	0	08
R. stolonifer	1	1	1	0	0	2	0	0	0	1	1	0	07
P.fellutanum	0	0	1	0	0	1	1	0	0	2	1	1	07
P. citrinium	1	1	1	2	3	1	0	0	0	0	1	0	10
F. equiseti	0	2	1	1	0	0	0	0	1	1	1	0	07
Yeast	2	1	0	0	0	0	1	1	1	1	2	2	11
Total	8	6	9	7	11	7	6	6	6	9	9	5	89×10^3

Samples 1-12: Kadiri-2 (MK-374), Narayani (TCGS-29), Kadiri-4 ,Rars-T-1 , Jyoti, JM-24, Abhaya (TPT-25), Kadiri-7, Kalahasti (TCGS-320), Tirupati-4 (TCGS-30), Kadiri-71-1(Virginia Group), Greeshma

Table 7 Determination of aflotoxin content in fungal culture by DRBC agar media

No	Fungi	Dichloran rosebengal chloramphenicol agar media
1.	*A. flavus*	Positive
2.	*A. candidus*	Positive
3.	*P. fellutanum*	Negative
4.	*F. equiseti*	Negative
5.	*R. stolonifer*	Positive
6.	*Yeast*	Negative
7.	*P. aethiopicum*	Negative
8.	*P. citrinum*	Negative
9.	*A. niger*	Positive
10.	*A. fumigates*	Positive

Table 8 Total aflatoxin content in collected samples by EISA method

NO	Sample	aflatoxin content (ppb) OD	Results

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1.	Kadiri-2 (MK-374)	2.891	8.2
2.	Narayani (TCGS-29)	0.231	3.7
3.	Rars-T-1	0.493	0.9
4.	Kadiri-4	1.369	1.4
5.	Kadiri-7	1.282 2.630	1.9 7.0
6.	Kadiri-71-1(Virginia Group)	0.521	0.4
7.	Greeshma	2.145	5.9
8.	Kalahasti (TCGS-320)	1.553	1.4
9.	Jyoti	2.331	6.5
10.	JM-24	1.854	2.3
11.	Kalahasti (TCGS-320)		
12.	Tirupati-4 (TCGS-30)	1.991	1.1

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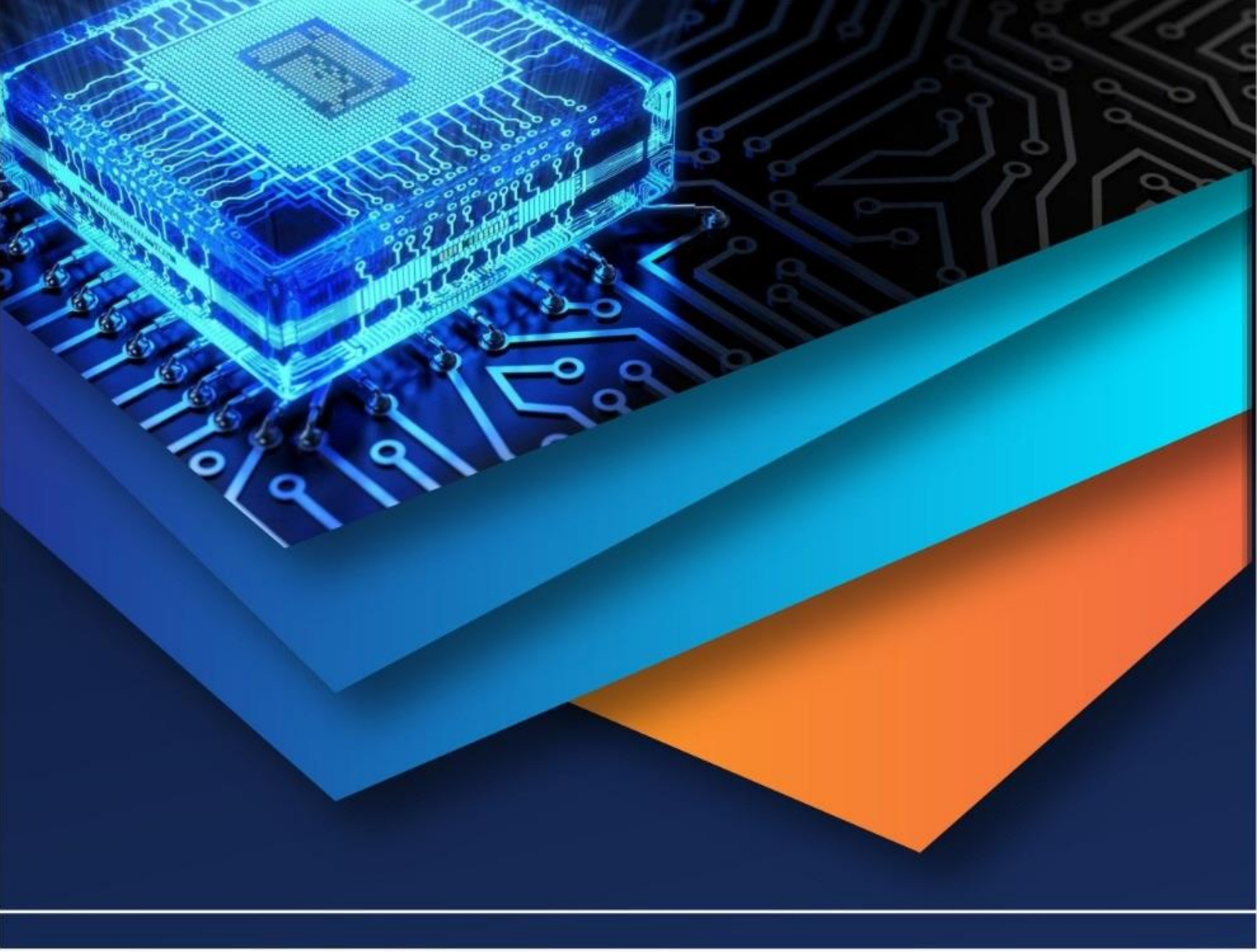

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