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Isolates Endophytic Fungi from Different Medicinal Plants and Check Its Antioxidant and Antidiabetic Activity

Nimiksha A Patel [1], Krishpa B Shah [2]
[1, 2] *Department of Microbiology, Dolat-Usha Institutes of Applied Sciences & Dhiru-Sarla Institute of Management & Commerce, Valsad, Gujarat, India*
[1, 2] *Veer Narmad South Gujarat University, Surat, Gujarat, India*

Abstract: ***Endophyte means "in the plant" [endon in Greek means within and phyton mean plant]. Endophytes are microbes that live in the plant tissues without causing any symptoms of disease. Endophytic fungi possess capacity to develop a relationship of symbiotic nature with their hosts. The endophytic fungi are source of bioactive compound which have found wide ranging pharmaceutical application. Endophytic fungi are highly diverse microorganisms which are chemical synthesizers inside host plants. A lot of work has been done on the bioactive potential of endophytes, such as antiviral, anticancer, antidiabetic and antimicrobial effects, but very little is known about their antioxidant capacity. In present study, isolation of endophytic fungi from different medicinal plants was collected out and its antioxidant and antidiabetic activity was checked. Six plant samples(Azadirachta indica [Neem], Bryophyllum [Elcho], Adhatoda vasica [Ardusi], Mentha spicata L. [Fudino], Momordica charantia [Karela], Ocimum sanctum [Tulsi]) were collected from local area (Pardi, Sukhlav, Borlai, Orvad) of Valsad district. Screening was carried out on sterile Sabouraud's Dextrose agar medium. Total eighteen endophytic fungi were isolated from indigenous medicinal plants, and fungal isolates were further characterized by mounting, observing physiological and morphological characteristics for successful isolation. Ethyl acetate extract of the endophytic fungal isolates were used for antioxidant and antidiabetic activity. The highest antioxidant activity was demonstrated by EF-12 & EF-18, and highest antidiabetic activity was demonstrated by EF-7, EF-12 and EF-18. These isolates were further subjected by phytochemical screening, the maximum compound present EF-12 was further analysed for to confirm the presence of various functional groups.***

Keywords: Medicinal plant, Endophytes, Antioxidants, Antidiabetic, Phytochemical, FT-IR.

I. INTRODUCTION

In 1809, The German botanist Johann Heinrich Ftiedrich Link first described Endophytes. Endophytes mean "Within plant". Endophytes are microbes that live in the plant tissues without causing any symptoms of disease [1]. Endophytic fungi possess capacity to develop a relationship of symbiotic nature with their hosts [2]. The endophytic fungi are source of bioactive compound which have found wide ranging pharmaceutical application such as anticancer, immunosuppressant, and antiviral [3]. The field of free radical chemistry is gaining more attention these days. Free radicals are reactive oxygen and nitrogen species which are generated by various physiological processes in the body. Uncontrolled generation of free radicals leads to attack on membrane lipids, proteins, enzymes and DNA causing oxidative stress and ultimately cell death. These ROS are responsible for many degenerative human diseases like diabetes mellitus, cancer, neurodegenerative disorders, Alzheimer's disease, Parkinson's disease, atherosclerosis, ageing and inflammatory diseases [4]. Endophytic fungi are highly diverse microorganisms which are chemical synthesizers inside host plants [5].

In present study, the leaves of different medicinal plants was used for isolation of endophytic fungi and ethyl acetate extract of endophytic fungal isolates were used for antioxidant, antidiabetic activity, phytochemical screening and FT-IR analysis.

II. MATERIAL AND METHOD

A. Collection of plant material

The leaves of medicinal plants like, *Azadirachta indica* [Neem], *Bryophyllum* [Elcho], *Adhatoda vasica* [Ardusi], *Mentha spicata L.* [Fudino], *Momordica charantia* [Karela], *Ocimum sanctum* [Tulsi] was collrcted from local area (Pardi, Sukhlav, Borlai, Orvad) of Valsad district.

B. Screening Of Endophytic Fungi

Leaves of healthy plants were collected and thoroughly washed with tap water followed by sterilized distilled water. They were surface sterilized by dipping into 70% ethanol for 2-3 min, rinsed with distilled water, and there after treated with 4% sodium hypochlorite for 1 min. Finally, the leaves were washed in sterile distilled water for 3 min. Further, the leaves were crushed with sterile distilled water using sterile mortal and pestle. About 0.1ml of crushed sample was taken and streak on Sabouraud's dextrose agar media and plates were incubated at room temperature. Observation was carried out daily until the growth of endophytic fungi was observed [6].

C. Study Of Colonial Characteristics And Mounting Of Endophytic Fungi

The isolated endophytic fungi were characterized morphologically by shape, colony colour, texture, topology, and microscopically [6].

D. Maintenance And Preservation Of Fungal Isolates

The purified endophytic fungal isolates were then transferred separately to Sabouraud's dextrose agar slants and maintained at 4˚C till further use [6].

E. Fermentation and Extraction

Total eighteen 100ml Erlenmeyer flask containing 50 ml of Sabouraud's dextrose broth was inoculated with isolated endophytic strain. Flask was incubated about on shaker at 120 RPM for 5 to7 days at room temperature. After observation of fungal growth, the fungal culture was filtered with whatmann filter paper no.1 to separated mycelium and filtrate. Culture filtrates were extracted with ethyl acetate (1:1 ratio) for around three times. Then the organic phase was allowed to evaporate by using hot plate and finally culture filtrate extract were stored for further use [7].

F. Antioxidant Activity

The antioxidant activity was determined by Reducing Power method for filtrate obtained after evaporation. The determination was carried out according to method by Oyaizu (1986). In this method, 0.5 ml of 0.2M phosphate buffer (pH 6.6) and 1ml of $K_3Fe(CN)_6$ (1%w/v) were added to 0.5ml of fungal extract. The resulting mixture was incubated at 50˚C for 20 min, followed by the addition of 1 ml of Trichloro acetic acid (10%w/v) to stop the reaction. The mixture was centrifuged at 1500 rpm for 10 min to collect the upper layer of the solution (1ml), mixed with deionised water(1ml) and 0.25ml of $FeCl_3$ (0.1 % w/v) and absorbance was then measured at 700nm against blank sample [8].

G. Antidiabetic Activity

Antidiabetic test was performed by alpha amylase inhibitory assay in which reaction mixture was prepared by adding 50µl of fungal extract, 200µl of phosphate buffer (pH 6.9) and 50µl alpha amylase. It was incubated at 37˚C for 10 min followed by addition of 50µl of 1% starch and incubation at 37˚C for 20 min. Reaction was terminated by adding 0.5 ml DNSA followed by incubation in boiling water for 5 min. The reaction mixture was diluted with 5ml of distilled water and absorbance was measured at 540nm. Blank tubes were prepared by replacing the enzyme solution with 50µl of buffer. Control representing enzyme activity was prepared in a similar manner without extract [9]

The AAI activity was calculated using the formula [9].

$$\%\text{inhibitory activity} = \text{Absorbance}540(\text{control}) - \text{Absorbance}540(\text{extract}) / \text{Absorbance}\ 540(\text{control}) \times 100$$

H. Phytochemical Screening

The ethyl acetate extract of the endophytic were checked for the presence of following secondary metabolites such as alkaloids, saponins, terpenoids, tannin, steroids, flavanoids by standard procedure [10].

1) *Tanins:* About 1ml to 2ml of fungal extract was stirred with 1ml of distilled water then adds few drops of 0.1% ferric chloride solution. Formation of brownish green colour, indicating the presence of tannins.
2) *Saponin:* About 1ml to 2ml of fungal extracts shakes vigorously with 1ml distilled water in test tube and warm. Formation of stable foam indication of presence of saponins.
3) *Flavonoids:* About 1ml to 2ml of fungal extract was added in 1ml of 10% lead acetate solution. Formation of yellowish white colour indicates the presence of Flavonoids.
4) *Terpenoids:* 1ml to 2ml fungal extract were mixed with 1 ml of chloroform and 1ml of H_2SO_4 was added and heated for 2 min. a greyish colour indicates the presence of terpenoids.

5) *Alkaloid:* Take 1 to 2ml of fungal extract was stirred with 2 ml of 1% HCL on a steam bath. Mayer's reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence alkaloids.
6) *Steroids:* Take 1 to 2ml of fungal extract dissolved with 2ml of chloroform and added equal volumes of concentrated H_2SO_4 to test tube by sides. The upper layer in the test tubes turns red and H_2SO_4 layer showed yellow with green fluorescence.

I. Fourier transforms infrared spectrophotometric analysis

For the FT-IR study, spectrum FT-IR system (Shimadzu, IR Affinity 1, Japan), equipped with a DLATGS detector with a minor speed of 2.8 mm/sec. scan range: from 400-4000 cm^{-1} with a resolution of $4cm^{-1}$ was used. The ethyl acetate extract of the endophytes was prepared. The extract was evaporated by flash evaporator and it was mixed with a KBr salt, using mortar and pestle and compressed into a thin pellet. Infrared spectra were recorded on KBr pellet on a Shimadzu FTIR spectrometer 4000-500 cm^{-1} [11]. In this study, FT-IR technique was used to characterize the phytochemical compound, from Center Of Excellence-Molecular Lab, Vapi.

III.RESULTS AND DISCUSSION

A. Isolation And Screening Of Endophytes

In present study, total eighteen endophytic fungi has been isolated from the leaves of six different medicinal plants (*Azadirachta indica* [Neem], *Bryophyllum* [Elcho], *Adhatoda vasica* [Ardusi], *Mentha spicata L.* [Fudino], *Momordica charantia* [Karela], *Ocimum sanctum* [Tulsi]). The four isolation were found from *Azadirachta indica [*Neem] and *Momordica charantia [*karel]. The three isolates were isolated from *Bryophyllum* [Elcho] and *Ocimum sanctum* [Tulsi] and two isolates were found from *Adhatoda vasica [*Ardusi] and *Mentha spicata L. [*Fudino]. Whereas Different type of medicinal plant like *Azadirachta indica [* Neem*], Bryophyllum [*Elcho*], Vinca rosea* [Barmasi] and *Colotropis gigantean* [Akado] had leaves were used for source of endophytic fungi[9].

The endophytic fungi were isolate and purified on Sabouraud's dextrose agar medium (e.g.Fig.1 and 2) and were identified by microscopically (e.g Table I).

Fig.1 inoculation of sample for endophytic fungi.

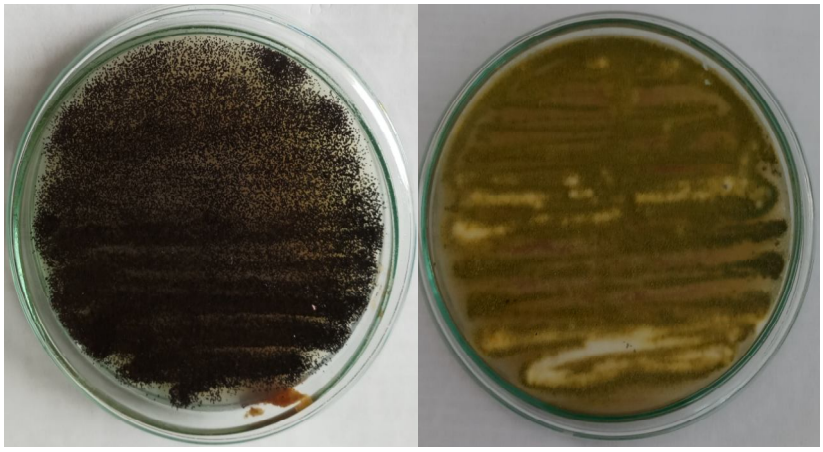

Fig. 2 purified of fungal isoltes.

TABLE I
Cultural and growth characteristics of fungal endophytes from collected medicinal plants for primary screening

Sr. no.	Sample	Isolates	Colony characteristics
1	*Adhatoda vasica* [Ardusi]	EF-1	Small, circular, flat and compact. dark green color colonies.
		EF-2	Large, circular, flat colonies are green color.
2	*Azadirachta indica* [Neem]	EF-3	Loose, fluffy, cottony white, blackish appearance.
		EF-4	Loose, large, green color colonies.
		EF-5	Small, flat and compact. Grey color colonies.
		EF-6	Loose, wooly, flat yellowish color colonies.
3	*Bryophyllum* [Elcho]	EF-7	Large, circular, flat colonies, black color spore.
		EF-8	Small, circular and greenish color colonies.
		EF-9	Small, flat and dark green color colonies
4	*Mentha spicata L.* [Fudino]	EF-10	Small, flat, compact and green color shade colonies
		EF-11	Large,circular and black color spore.
5	*Momordica charantia* [Karela]	EF-12	Large, circular, powdery colonies with dark brown color
		EF-13	Large, circular, compact black color colonies.
		EF-14	Small, round, green color colonies.
		EF-15	Large, circular, flat colonies were greenish yellow color
6	*Ocimum sanctum [*Tulsi*]*	EF-16	Small, circular, flat and light green color colonies.
		EF-17	Large, circular, flat and mehandi color colonies.
		EF-18	Small, fluffy, and greenish white colonies.

B. Antioxidant Activity

The ethyl acetate extract of endophytic fungi were studied for antioxidant activity. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound. The maximum Reducing power was given by metabolites from ethyl acetate extract of endophytic fungal isolates EF-12 and EF-18 with absorbance of 0.82 and 0.69 at concentration 500µl/ml respectively (e.g. Fig.3). It was evident that isolate EF-12

Fungal extracts of CPIMR-2 (Penicillium sp.) and CPIL-1 (Aspergillus sp.) were observed positive for reducing power. The methanolic extracts of both the fungi, i.e., Aspergillus species (CPIL-1) and Penicillium species (CPIMR-2) showed a potent reducing power. Maximum reducing power was observed at a concentration of 0.1 mg/ml in both the extracts. Among the two endophytes, CPIL-1 exhibited high reducing power [12].

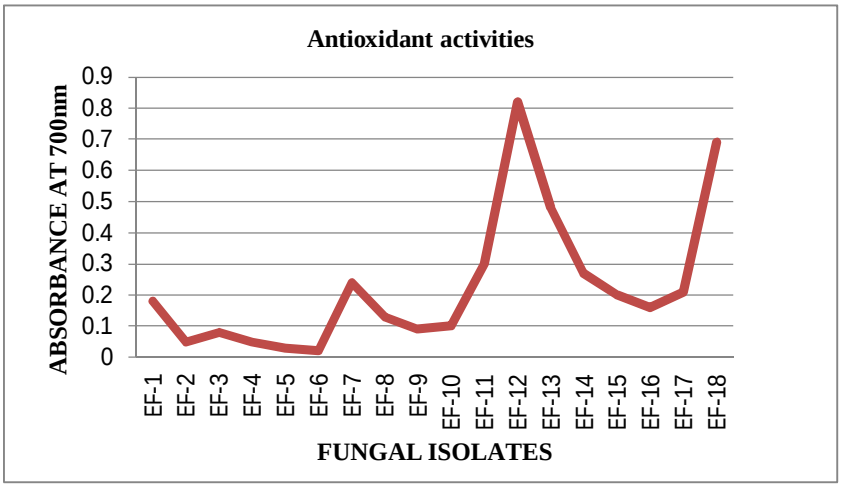

Fig.3 Reducing power activities of metabolites from ethyl acetate extract of endophytic fungal isolates.

C. Antidiabetic activity

α- amylase inhibitors are also called as starch blockers as they prevent or slow the absorption of starch into the body mainly by blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose, maltriose and other simple sugars.

In present study, the maximum % inhibition was given by metabolites from ethyl acetate extract of endophytic fungal isolates EF-7, EF-12 and EF-18 with % inhibition of 72.72%, 82.95% and 73.86% respectively, at concentration of α-amylase 0.05mg/ml (e.g. Fig. 4). A higher % inhibition value indicates the more inhibition of α-amylase enzyme.

α-amylase inhibition studies demonstrated that the crude extract from Nigrospora showed 75.76% inhibitory activity on α-amylase. There are several possible mechanism through medicinal plant can act to control the blood glucose level. One such mechanism is that an alteration of activity of enzyme involved in glucose metabolism. The α-amylase inhibitor act as an anti-nutrient that obstruct the digestion and absorption of carbohydrates [13].

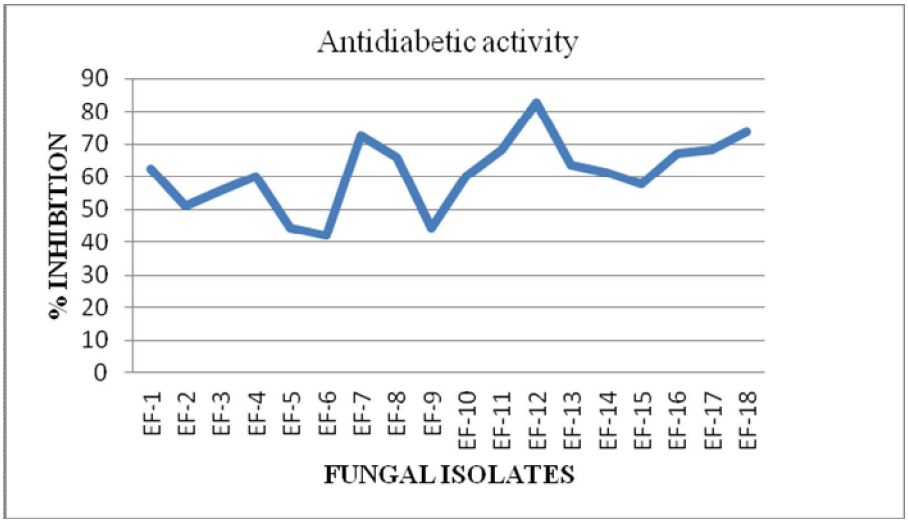

Fig. 4 Alpha amylase inhibitory activities of metabolites from ethyl acetate extract of endophytic fungal isolates.

D. Phytochemical Screening

Among eighteen isolates, EF-12 gave the highest antioxidant activity and EF-7 EF-12 and EF-18 gave the highest antidiabetic activity, thus these isolates were selected for phytochemical screening. In present study, the qualitative phytochemical analysis indicated that the ethyl acetate extract of endophytes contains tennin, flavonoid, terpenoid and alkaloid (e.g. TABLE IV).

The extracts of endophytes revealed the presence of saponins, phenol compounds, anthraquinones, flavonoids, steroids, cardiac glycosides and tannins. Extracts from cultures of seven different fungal species of endophytic fungi gave a wide variety of biological activities. Endophytes, A. *niger*, *Penicillium* sp., *Trichoderma* sp. and plant extracts has showed presence of all compounds (of saponins, phenolic compounds, anthraquinones, steroids, cardiac glycosides and tannins), whereas other endophytesalso have all the phytochemical except tannins. The presence of anthraquinones was observed in only threeendophytes (A. *niger*, *Penicillium* sp. and *Trichoderma* sp.) and flower extract [14].

TABLE II
Phytochemical test for fungal endophytes.

Sr No.	Phytochemical test	EF-7	EF-12	EF-18
1	Tennin test	Positive	Positive	Positive
2	Saponin test	Negative	Negative	Negative
3	Flavonoid test	Positive	Positive	Positive
4	Terpenoid test	Positive	Positive	Negative
5	Alkaloid test	Negative	Positive	Negative
6	Steroid test	Negative	negative	Negative

E. FT-IR analysis

Among three isolates, EF-12 showed maximum number of phytochemical compounds in phytochemical screening, thus phytochemicals produced by these fungi was characterized by FTIR method. The FTIR analysis of this EF-12, revealed that distribution of functional groups within the phytochemicals such as Carboxylic, Methylene, Alkyne, Tertiary amides, Aliphatic amines and Aliphatic bromo groups. The presence of various functional groups may be attributed to the existence of a variety of potential phytochemicals.

FT-IR spectra of culture filtrate extract of both fungal isolates indicate the presence of alkyl halides, carboxylic acids, aliphatic amines, alkanes, primary amines, esters, aromatics, nitro compounds, and aldehydes. FT-IR analysis of the two endophytic isolates (Penicillium funiculosum and Trichoderma viride) revealed the distribution of functional group within the organic fractions [15].

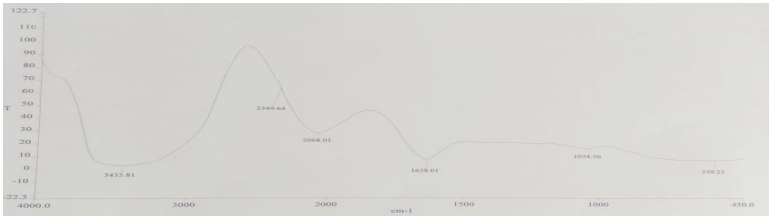

Fig. 3 FT-IR analysis of metabolites from ethyl acetate extract of fungal endophytes.

IV. CONCLUSION

The present work was carried out to isolation of endophytic fungi from different medicinal plants and showed the presence of its antioxidant, antidiabetic activities and revealed the production of various phytochemicals. Thus endophytes from medicinal plants are remarkable natural source of biologically active metabolites. Further investigation will focus on the structure elucidation of bioactive compound and its effective application strategies and the influence of environmental factors when used in different field conditions.

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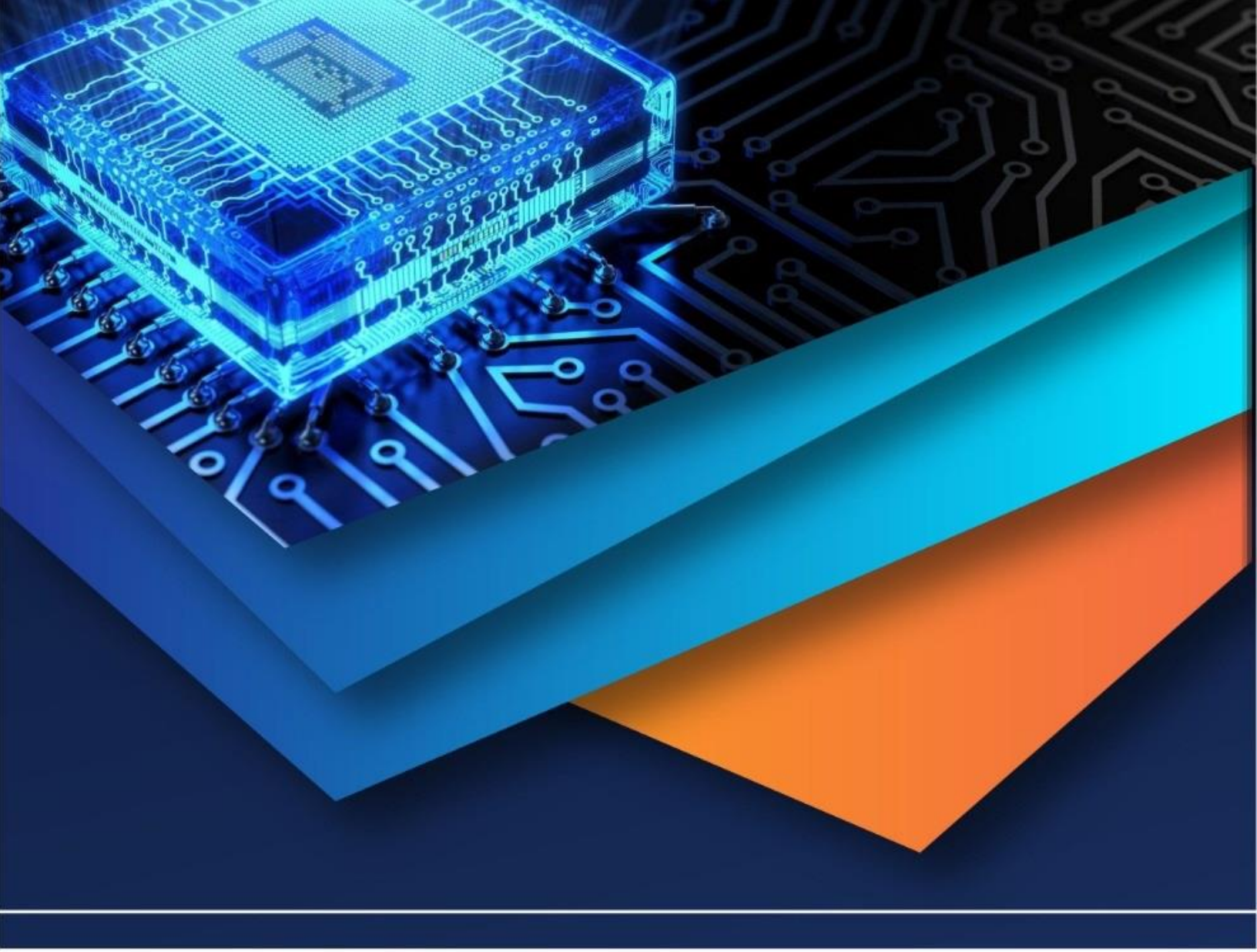

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