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Nutraceutical Potential of Traditional Medicinal Plant Knoxia Corymbosa Wild from Wayanad, Kerala

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Abstract: ***The nutrient content and the medicinal values of the traditional medicine Knoxia corymbosa wild (Rubiaceae family) has not been assessed so far. It is known for gastro- intestinal and skin treatments. The assessment of fatty acids and flavonoids, prominent secondary metabolite with nutraceutical and pharmaceutical potential, present in the species is done using solvent extraction and chromatographic techniques (GC-FID/MS, LC-MS/UV). Three major flavonoids, quercetin, myricetin and kaempferol which has high nutraceutical potential are observed. Palmitic acid, Palmitoleic acid, Stearic acid, Myristic acid, Oleic acid, Linoleic acid (ω-6) and Alpha-Linolenic acid (ω-3) are identified using internal standards. The mass spectral data gives the presence of gamma-linolenic acid and arachidonic acid and very long saturated fatty acids also. The ratio of ω-6/ ω-3 is found to be approximately 1. These results indicates that the species K. corymbosa is a potential source of flavonoids and fatty acids which results in high nutraceutical effects.***
Keywords: Fatty acids, Flavonoids, Nutraceutical, K. corymbosa wild, Traditional medicine

I. INTRODUCTION

Phytochemicals used for the treatment of various medical situations have made remarkable history. They have turned out to be the backbone of traditional/ tribal medicinal actions. A variety of phytochemical components have been already proved to possess good biological activities and there are still many to be identified and to be proven for its health effects. They act as refined alternatives to synthetic pharmaceutical drugs, which can effectively overcome the unwanted secondary effects and other complications. Many studies have been focussed and published on the nutritional content and the therapeutic effect owned by wild cultivated plants and fruits all over the world. Although plants have been in the leading role for the traditional medicinal uses for years with the set of unlimited sources of phytochemicals that are also used by modern therapeutic technology, it is important to identify and study the various potential compounds along with their biological properties from plants grown at different adaptations.

Strong recommendations are imputed for the consumption of nutraceuticals from plant origin for the improved health and to prevent various diseases. Nutrition in combination with pharmaceutical forms the nutraceuticals which are non-toxic food/ extract supplements which plays a major role in enhancing immunity and health condition and also in prevention and treatment of diseases [1], [2]. Pharmaceuticals and nutraceuticals are widely correlated to plant diversity and the rich bioactive plant compounds as they are defined to modulate one or more metabolic processes, thus boosting immune system. Flavonoids, polyphenolic secondary metabolites, forms a major and active nutraceutical from plant origin. They are high potent anti-oxidants and metal chelators [3]. Along with that, flavonoids also possess anti-inflammatory, hepatoprotective, anticarcinogen, anti-allergic and many other activities. From, earlier studies, researchers have proved that flavonoid rich diet could potentially improve the health system and the well-being, as they can directly show effect on neuronal communication and the betterment of age-related problems by altering the neuroprotective stress shock proteins and the signalling pathways [4]. Also, clinical studies reveal that flavonoids are that essential nutrients which are inevitable for different physical and pathological conditions of a full life cycle [5], [6]. As a result of these biological effects by flavonoids, they are prominent and known to be the disease preventing and health improvising dietary supplements. Fatty acids, part of the natural fats and oils, are viewed as an energy resource, nutraceutical supplement and its effective action towards human health and diseases have attracted considerable interest of research. Fatty acids being an essential component of lipids and the influential secondary metabolites, are capable to regulate the lipid metabolism, a disorder resulting in diabetes, cardiovascular diseases (CVD) and many other complications [7].

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In human diets, a variety of fatty acids are required which enters into the circulation after the intestinal absorption and are integrated into the tissues mainly as constituents of membranes. They are, therefore, considered as an important active ingredient in traditional and herbal medicinal and nutraceutical actions. In general, they contribute to various level of functions that includes reduction of the level of blood cholesterol, controlling of lipid and protein metabolism, increasing the immunization function and so on [8], [9]. Fatty acids are classified based on the presence of the number of double bonds found in them; saturated fatty acids (SFA) if no double bonds are present, monounsaturated fatty acids (MUFA) in case of presence of one double bond and poly unsaturated (PUFA) when more than two double bonds are there. Generally, fatty acids are considered as a nutritional food source which are found to be effective for the prevention of cardiovascular diseases [10-12]. The most important fatty acids needed for the human body metabolism are PUFA's: linoleic acid and linolenic acid which are omega 6 and omega 3 fatty acids, generally called essential fatty acids, could normalize the functioning of sub cellular and cellular level membranes [13]. Also, they can be used as a carrier to improve the therapeutic efficiency of anticancer drugs and will decrease the risk of CVD. SFA are associated with the factors affecting the cholesterol metabolism [14]. SFA increases the level of low-density lipoprotein (LDL) cholesterol level and the blood plasma cholesterol which in turn can lead to a risk of CVD. Studies show that Lauric acid is the strongest SFA that leads to the elevation of LDL to a greater extent [15]. Whereas Stearic acid does not show any effect on both LDL and HDL level of cholesterol [16].

Tropical and sub-tropical herbal plants are recognized to produce diverse collection of natural products which are capable of cancer prevention [17]. K. *corymbosa* is one among the widely used tribal medicine which belongs to the *Rubiaceae* family of medicinal plants. Though the plant is in use as tribal medicine the identification of the chemical constituents of K. *corymbosa* has not been fully attempted. In general, plants which belongs to the *Rubiaceae* family are widely known for its medicinal activities such as treatment for liver diseases, acute hypertension, diabetes mellitus, wound healing, respiratory and skin diseases, etc [18]. There are few reports about fatty acid profiling done on different species belonging to *Rubiaceae* family. Nauclea *latifolia*, a *Rubiaceaen* plant which has been used for hypertension and malaria are found to have palmitic acid, stearic acid, linoleic acid, behenic acid, oleic acid, erucic acid and arachidic acids as the major fatty acids [19]. Also, Desai et, al., (2011), reported the presence of oleic acid, palmitic acid, essential fatty acids and cis-10-pentadecanoic acid in the Morinda species *(Rubiacea*) [20]. The nutritive quality and health benefits of the plant species can be determined ideally by the presence of more unsaturated fatty acids than the saturated ones. However, there is no information regarding the fatty acid profile of K. *corymbosa*. Though GC/FID has been proven to be robust tool in FAME analysis, the lack of its selectivity of low abundant compounds, wrong identification in presence of contaminants and noisy background are still a concern in the usage. The MS coupled to the GC instrument have accomplished a wide range of qualitative analysis of FAME, since it gathers both the retention factor and the spectrometric details of every separated compounds. In spite of the limitations both FID and MS are still used by many researchers and laboratories for the FAME analysis and here both the technique has been used to get an overall detail and a comparative study on the fatty acids available in the species. Hence, the objective of the present study is to analyze the flavonoids and fatty acid profile of the plant species K. *corymbosa* using GC-FID and GC-MS.

II. MATERIALS AND METHODS

A. Plant Material

K. *corymbosa* is a subshrub growing in a wet tropical biome (Figure 1). The species grows to a height of 40-90 cm tall with a little branched stem and narrow lanced shaped leaves with velvety surfaces. The species have exceedingly small purple/ white -colored flowers, found dense at the end of the branches with a flowering season between August – October. World flora online reports 27 synonyms for the K. *corymbosa species* [21]. K. *corymbosa* was collected from the tribal locality of Valad in Wayanad, district Kerala. The plant samples were washed, freeze dried, powdered, and kept in moisture free condition.



Fig1: Knoxia *corymbosa* wild

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B. Qualitative Analysis of Flavonoids

The extracts for HPLC analysis were prepared as per [22], 40 ml of 62.5% aqueous methanol containing 2g/l TBHQ was added to 5mg of freeze-dried sample material. To this extract was added 10ml of 6M HCl with careful mixing. The extraction solution thus consisted of 1.2M HCl in 50% aqueous methanol (v/v). After refluxing at 900°C for 2hours with regular swirling, the extract was allowed to cool and subsequently made upto 100ml with methanol. Approximately 2ml was filtered through a 0.45µm filter for organic solvents prior to injection. The flavonoid standards of myricetin, quercetin, kaempferol, luteolin and apigenin (Sigma Aldrich) were dissolved in methanol to a concentration of 500 mg/l and stored at 40°C. Standard stock solutions were diluted in 20ml of 62.5% aqueous methanol to which 2g/l of TBHQ was added. To this solution was added 5ml of 6M HCl and the solution was subsequently made up to 50ml with methanol.

C. LC-MS/UV

Identification of flavonoids was performed on Shimadzu Prominent Liquid Chromatograph. Chromatographic separations were performed on a Supelco C-18 (25 cm× 4.6mm, 5µm) column under ambient temperature. The HPLC system was connected to LC20AD pump. The peaks were detected using UV-Visible detector SPD-20A at 370 nm. The working solutions were injected onto the column which was previously equilibrated with eluent for 60 minutes. The mobile phase consisting of methanol and 0.1% formic acid in low pressure gradient mode (0-5 min, 50% B; 5-11 min, 50-45% B; 11-25 min 45% B; 25-26 min 45-50% B; 26-40 min 50% B).

D. Preparation of Fatty Acid Methyl Esters (FAME)

The extraction of fatty acids was done using Harvey method [23]. The dried sample parts are mixed with 2:1 Dichloromethane (DCM): Methanol solvent mixture and was shaken for 72 hrs. The extract obtained was then saponified using 6% KOH in Methanol and refluxed for 4hrs at 70^{o}C. The residue is separated into neutral upper layer and bottom fatty acid layer with the addition of Hexanes. The fatty acid layer was acidified to pH 2 by the addition of 6M HCl and were extracted using (DCM). The polar lipid fraction containing the fatty acids was evaporated to dryness using rotary evaporation. To derivatize the fatty acids 12% BF3 in methanol is added to it and heated for an hour at 70°C. The methyl derivatives of fatty acids were partitioned from the reaction solution into DCM. The DCM layer was evaporated to dryness and the extract was re-dissolved in 1ml n-Hexane and subjected to two analytical platforms: GC-FID and GC-MS.

E. GC-FID Analysis of FAMES

FAME residues were dissolved in hexane and precaution was taken to maintain concentrations of FAME. The analysis was carried out by GC (Perkin Elmer Clarus 680 GC). FAME (1 µl) were analysed with a non-polar HP ultra-double-fused silica capillary column (30 m, 0.32 mm internal diameter, 0.25 mm film thickness) with helium as a carrier gas. The conditions used for GC analysis were injection temperature of 250 °C, flame ionization detector (FID) temperature of 260 °C and column temperature of 240 °C. The peaks were identified by comparing the retention time with authentic internal standards. The unknown FAME peak areas were computed, and relative percentage of individual fatty acid was calculated.

F. GC-MS Analysis

The same GC system was used for the MS analysis of FAME by equipping the GC instrument mentioned above with Perkin Elmer 600T Mass Spectrometer using a capillary column HP-5 MS (30 m × 0.25 mm × 0.25 mm). The temperature conditions for the chromatographic separation were same as mentioned in the case of GC-FID technique. Samples of 1 µL were administered in a 1:50 flow divider mode. Carrier gas flow rate through a column was 1.0 mL/min. Detection was held in the SCAN mode in the range of (38–400 m/z) and identified each component separated based on the comparison with those of the National Institute of Standards and Technology 08 mass spectral libraries.

III. RESULTS AND DISCUSSION

Majority of the nutraceuticals which are plant origin are claimed to possess wide range of therapeutic effects for various diseases. The flavonoids identified qualitatively from K. *corymbosa* relative to the internal standards are reported in Table I. Diets rich in flavonoids gets easily absorbed into the intestine with less side effects and are necessary for healthy aging and lifespan due to its effective down regulation in the degenerative chronic diseases [3].

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Flavonoids as a group is reported to have vasorelaxant actions which results in hindrance to human platelet accumulation [1]. Studies shows that among the group, quercetin, kaempferol and myricetin were effective inhibitors for platelet aggregation in cats and dogs [24]. Quercetin exerts a protective effect in hepatic ischemia reperfusion injury and tissue damage and also protect LDL from oxidative modifications [25]. Myricetin acts as a critical nutritional component of diet by providing protection in immunological systems and thereby maintains a good health. Quercetin, kaempferol and myricetin are important flavonoids with high nutraceutical and therapeutical functions that includes mainly antioxidative, anticarcinogen and anti-inflammatory effects.

Table I: Results of Flavonoids from K.*corymbosa*

Flavonoids	t_R(min)	Molecular formula
Myricetin	4.048	$C_{15}H_{10}O_8$
Quercetin	4.897	$C_{15}H_{10}O_7$
Kaempferol	5.883	$C_{15}H_{10}O_6$

The esterified fatty acids obtained in the plant species are separated and analysed using two analytical detectors (FID and MS) coupled with GC and the chromatogram obtained in both the analytical technique is given in Figure 2. The quantitative fatty acid composition determined using FID tool corresponding to the internal standards are given in Table II. A total of thirteen fatty acid peaks were obtained out of which seven fatty acids including saturated fatty acids (SFA), monounsaturated (MUFA) and poly unsaturated (PUFA) fatty acids were identified using the internal standard FAME mixtures. The area percentage given is based on the total fractions observed in the GC. The SFA were found to be the prominent with palmitic acid (C:16) being the major fatty acid with an area percentage of 14.26% compared to the other fatty acids. The two main fatty acids that has been identified are PUFA's i.e., ω-6 linoleic acid (C 18:2) and ω-3 linolenic acid (C 18:3), essential fatty acids, and they constitute a percentage of 12.28% of the total energy source. The palmitoleic acid (ω-7) and the oleic acid (ω-9) are the two MUFA's observed in the plant species.

Table II: GC-FID results of fatty acid profile of K. *corymbosa*

Peak	Fatty acid	t_R (min)	Type of acid	Area%
1	C14-Myristic acid	35.176	Saturated	0.94
2	C16-Palmitic acid	41.704	Saturated	14.26
3	C16:1- Palmitoleic acid	42.249	Mono-unsaturated	7.39
4	C18- Stearic acid	46.737	Saturated	1.86
5	C18:1- Oleic acid	47.092	Mono-unsaturated	1.98
6	C18:2- Linoleic acid	49.097	PUFA- Essential	6.21
7	C18:3- Linolenic acid	51.421	PUFA- Essential	6.07

Based on previous studies, a diet rich in SFA could promote to CVD by affecting the plasma cholesterol level in human body. Though SFA acts as an energy source, the nutritionists have recommended to maintain a diet having SFA up to 10% of the total energy source to prevent high level of cholesterol situation [26]. A comparable amount of essential fatty acids presence indicates a good sign of nutraceutical potential of the plant species. Intake of adequate level of fatty acids is important for the pregnant women during the prenatal period for ensuring the normal brain development of the growing foetus [27]. The unsaturated fatty acids ω3, ω6 and ω9 plays an important role in human body as it involves in the functions of cell membranes, in regulating fat metabolism and the cholesterol removal from the body by converting it into cholic acids [9], [28]. Also, these unsaturated fatty acids show anti-inflammatory actions, immunomodulatory functions and helps in improving blood circulation in nervous system. The ratio of unsaturated to saturated fatty acids and the ω6 to ω3 fatty acids were found to be 0.8 and 1.02, gives the comparative detail of the nutritive value. The dietary ratio of ω6 to ω3 are taken as important parameter in human diet since it has been a major controlling factor of metabolic syndrome, insulin sensitivity and in lipid profiles. World Health Organization has recommended the ratio of n6 to n3 PUFA to be in between 1 and 4:1. Many studies have already reported the preventive role of fatty acids especially PUFA in coronary heart diseases, hypertension, rheumatoid arthritis, cancer, and other inflammatory problems.

The untargeted qualitative analysis of FAME from the plant species was analysed using GC-MS and are reported in Table III. The GC-MS data has given a total of 17 esterified fatty acids out of which seven are in accordance with the FID data. The MS spectral

data displays a list of eleven SFA, two MUFA and four PUFA based on the comparison of molecular ion (m/z) with the data in NIST spectral library. The two other major fatty acids which failed to get detected in FID data are γ-Linolenic acid (GLA) and arachidonic acid (ARA) which are ω6 PUFAs and GLA serves as a precursor for the ARA formation. ω3 and ω6 fatty acids which are having opposing effects are the precursors for the signalling molecules or lipid mediators called eicosanoids. These molecules derived from the ω3 fatty acids exhibits anti-inflammatory action while those from ω6 have pro-inflammatory and immunoactivity properties [29], [30]. α-linolenic acid (ALA) obtained from plant sources are an important factor in human diet since it plays the role as precursor to produce long chain n3 fatty acids such as eicosapentanoic acid (EPA) and docosahexanoic acid (DHA), important for the cardiovascular and neuronal systems [31], [32]. WHO and Food and Agricultural organization (FAO) has recommended the neonates formula powder (non-breast feeding) for premature babies who has insufficiency in arachidonic acid for the better growth and development [33]. LA and GLA, most crucial element in human diet, since the deficiency in ARA could adversely affect the complete metabolic processes. The fatty acids containing more than 20 carbon atoms, very long saturated fatty acids (VLSFA), have found to be associated with lower risk in the heart failure, mortality and type 2 diabetes, includes arachidic acid and lignoceric acid [34].

Comparing with the earlier studies of fatty acid composition on *Rubiaceae* families, there are some differences both qualitatively and quantitatively. Based on the study on Nuclea *latifolia*, KBIRU found that the major fatty acid from this African grown species were oleic acid (23.46%), palmitic acid (20.47%), behenic acid (12.74%) and linoleic acid (10.52%) [19]. The species shows a higher percentage of SFA than the unsaturated fatty acids. Similarly, SFA, MUFA and PUFA percentage in Morinda species of *Rubiaceae* family were found to be 19.7%, 17.5% and 62.7% respectively in *M. pubescens*, whereas in *M. Citrifolia it was* 18.8%, 14.9% and 66.6% respectively [20].

Table III: Results of FAME analysis using GC-MS

Peak	t_R (min)	FAME	Chemical nomenclature
1	24.29	Capric Acid	C 10:0
2	35.61	Myristic Acid	C 14:0
3	38.34	Pentadecanoic Acid	C15:0
4	41.13	Palmitic Acid	C 16:0
5	42.05	Palmitoleic Acid	C16:1
6	43.59	Heptadecanoic Acid	C17:0
7	46.07	Stearic Acid	C 18:0
8	47.46	Oleic Acid	C 18:1 n9
9	49.53	Linoleic Acid	C 18:2 n6
10	50.75	Arachidic Acid	C 20:0
11	50.99	Gamma Linolenic Acid	C 18:3 n6
12	51.87	Alpha- Linolenic Acid	C 18:3 n3
13	52.86	Heneicosanoic Acid	C 21:0
14	56.41	Arachidonic Acid	C 20:4 n6
15	57.1	Tricosylic Acid	C 23:0
16	59.22	Lignoceric Acid	C 24:0
17	61.64	Pentacosylic Acid	C 25:0

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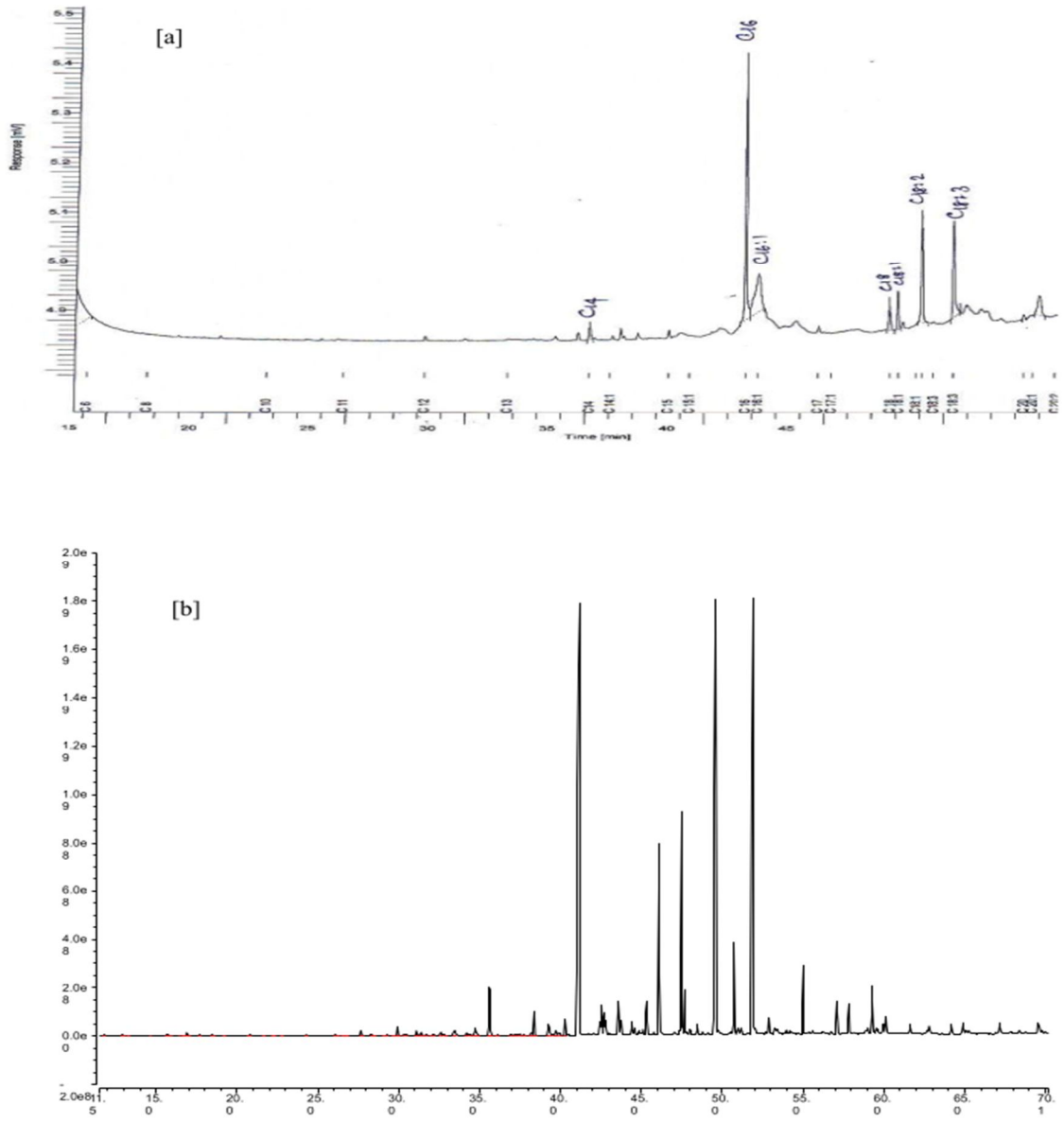


Fig 2. Chromatograms of FAME analysis: (a) GC-FID, (b) GC-MS

IV. CONCLUSION

Nutraceuticals, the non-toxic supplements for improving the metabolic processes and health conditions could range from isolated nutrients, herbal products or processed foods. Fatty acids and flavonoids act as the nutritional source in the human diet and are identified as a major ingredient in ethnic medicines. From the present study, it can be concluded that K. *corymbosa* has got a diverse source of fatty acid profile, major fatty acids observed being unsaturated fatty acids with PUFA as prominent suggesting that the plant could be of great use in nutritional pharmacological purposes. The ratio of n6 to n3 PUFA implies a good healthy diet. The major flavonoids identified were potent in both therapeutical and nutraceutical level due to its high antioxidant activity.

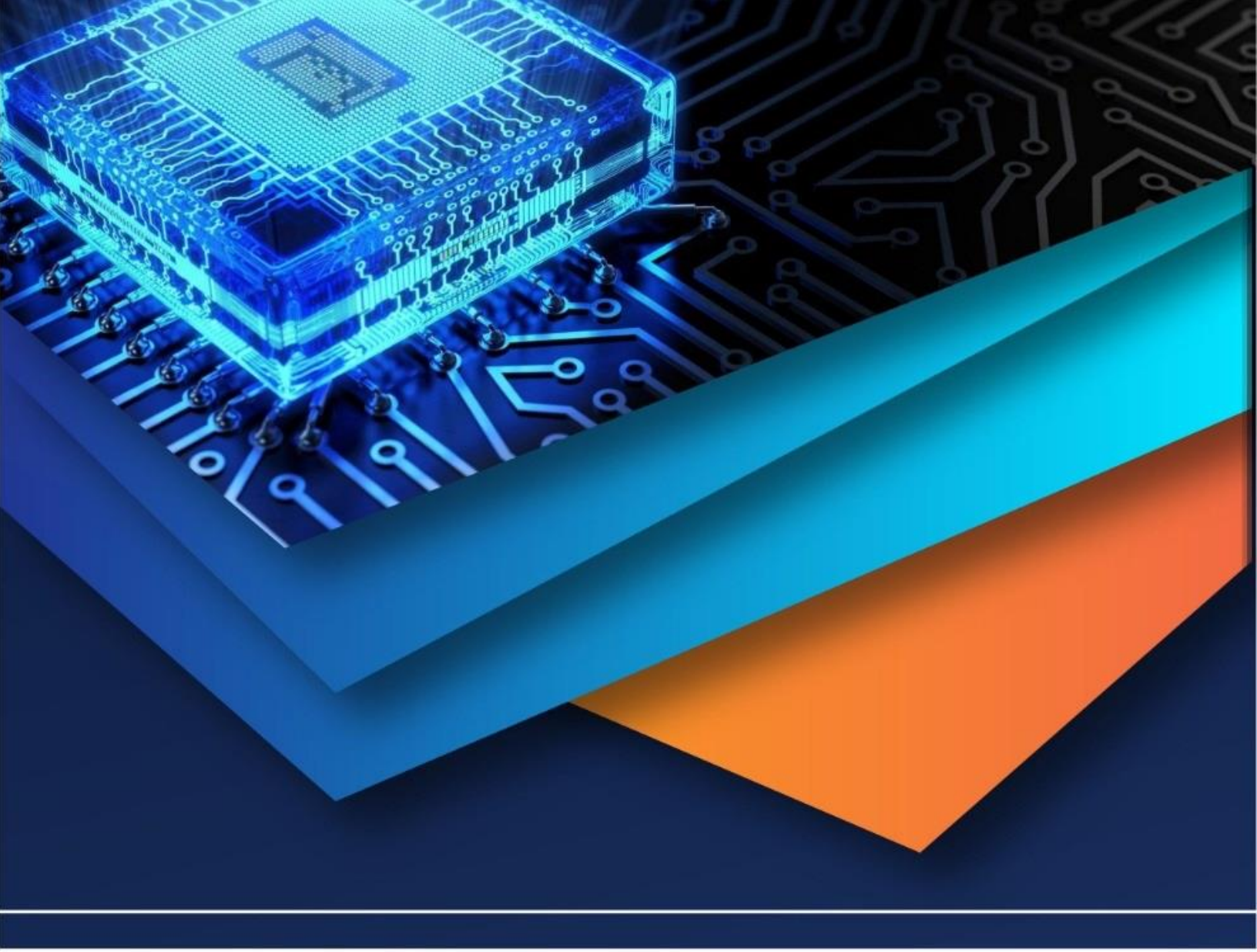
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