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Detection of Antioxidant and Antimicrobial Components from Crude Extracts of *Hibiscus Arnottianus A. Gray* flowers using HPTLC-Bioautographic Technique

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Abstract: ***Hibiscus arnottianus belonging to family, malvaceae, is a herb used in traditional medicine for the treatment of inflammation and renal diseases. Methanolic extract of Hibiscus arnottianus flower was fractionated using different solvents of increasing polarity like petroleum ether, ethylacetate, methanol and water. The preliminary phytochemical screening showed the presence of carbohydrates, steroids, flavonoids, tannin. Methanolic extract of hibiscus arnottianus flower was screened for their antimicrobial and antioxidant activity. The test microorganisms includ Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Pseudomonas aeruginosa. It was concluded that Hibiscus arnottianus flowers extract was well effective against all these microorganisms. HPTLC-DPPH assays were used to identify the antioxidant activity of flower extract. This activity was estimated by HPTLC bioautographic method by using the mobile phase Chloroform:Ethyl Acetate:Methanol:Water (2.8:5.2:1.6:0.4 v/v/v/v). There was a successful chromatogram developed and detection of a band in the sample was observed. Later, the band showing antioxidant characteristic property was identified by DPPH Assay.***
Keywords: HPTLC- Bioautography, Antimicrobial, Antioxidant, DPPH, Hibiscus arnottianus

I. INTRODUCTION

Hibiscus arnottianus, commonly known as "White Hibiscus," is a species of flowering plant native to Hawaii. It belongs to the family Malvaceae. The plant is characterized by its large, showy white flowers with prominent yellow stamens. Hibiscus arnottianus is endemic to Hawaii. Hibiscus arnottianus A. grey, is an evergreen shrub or small tree generally 3-9 meters in height. The leaves are ovate 5 to 10 centimeters long and 4-7.5 centimeter broad, thin, green and slightly shiny on both surfaces, leafstalks are 2-4 centimeter, stipules are long pointed, shedding early. The faintly fragrant flowers have five white petals 8 to 11 cm long, 2.5 to 3.5 cm wide, with the calyx being 2.5 to 3 centimeters long. Anthers are arranged along the upper third of the white staminal column 10 to 14 cm long. This subspecies is distinguished from the other native Hawaiian members of its genus by its white petals and white staminal column [1]. Hibiscus has a long history of traditional use for various health purposes, hibiscus tea is often included in weight loss and management programs due to its diuretic properties and potential to inhibit the absorption of carbohydrate. Hibiscus flowers contain antioxidants, such as flavones like quercetin-3,5-diglucoside, quercetin-3,7-diglucoside, cyanidin-3,5-diglucoside and kaempeferol-3-xylosyl glucoside which can help combat oxidative stress and reduce inflammation in the body [2]. This antioxidant activity may contribute to various health benefits. The literatures indicates that hibiscus tea may help lower levels of LDL cholesterol and triglycerides, potentially reducing the risk of heart disease. Hibiscus extracts have been studied for their potential hepatoprotective effects, meaning they may help to protect the lever from damage caused by toxins or disease. Hibiscus arnottianus flowers are traditionally used in the treatment of various diseases for long time but no systematic phytochemical studies are reported for this plant. In recent years, thin layer chromatography-bioautography method has been widely and efficiently used for the screening and quantification of antioxidant compounds. Therefore, present investigation was planned to study the preliminary phytochemical detection and identification of antioxidant and antibacterial components in the flower of Hibiscus arnottianus using 2,2-dipheny-1-picryl-hydrazyl (DPPH)- HPTLC bioautographic method [3-7].

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II. MATERIALS AND METHODS

A. *Plant material*

The flower of Hibiscus arnottianus were collected from Bandra, Mumbai district, Maharashtra in September 2019. The plant was authenticated from blatter herbarium in St. Xaviers college, Mumbai, Maharashtra, India. The flower of the plant was shaded dried, powdered and passed through 70 mm mesh sieve and stored in an airtight container for further use.

B. *Preparation of extract*

The shade dried flower powder, about 1.0 g, was weighted and sonicated with 10 cm^3 of methanol at room temperature for 1 hr. the extract was filtered through whattman no.1 filter paper then extract was stored at 4°C for further use.

C. *Proximate analysis*

Determination of extractive values, loss on drying, total ash value, water soluble ash value and acid-insoluble ash value were performed by using standard procedures [9]. Data were presented in table no.2.

D. *Preliminary phytochemical screening*

Preliminary phytochemical studies of various solvent extracts of H. arnottianus flowers were carried out by performing qualitative chemical test as per standard procedure [10]. The results are mentioned in table no.1

E. *Estimation of phytoconstituents*

Total alkaloid, total phenol, total flavonoid and total tannin contents were carried out by standard procedures [8]. Results were noted.

F. *DPPH-HPTLC Bioautographic Assay*

Chromatography was performed on 10 x 4 cm HPTLC plates coated with silica gel 60 F_{254} (Merk HX94930454). Samples (5µl) were applied as a band using Linomat 5 applicator (CAMAG, Switzerland). The plates were developed to a distance of 70 mm in an twin trough developing chamber (CAMAG) with chloroform-ethyl acetate-methanol-water (2.8:5.2:1.6:0.4, v/v/v/v) as developing solvent. The saturation time was 5 min. the plate was then dried for 3 min. After development, the HPTLC plate was immersed in 0.03% (m/v) DPPH methanolic solution. After derivatization, the plates were air-dried. Documentation of the chromatograms was carried out under 254 nm and white light using a CAMAG Scanner 3.

G. *Radical Scavenging Assay*

Radical scavenging activity of flower extract against DPPH was determined by using standard procedure [11]. DPPH react with antioxidant compound, which can donate hydrogen and reduce DPPH. The change in colour (from deep violet to light yellow) was measured at 517 nm on a UV visible light spectrophotometer. Radical scavenging activity was calculated by the following formula.

% Inhibition = $[(A_0 – A_1)/ A_0] \times 100$

A_0 = Absorbance of blank sample

A_1 = Absorbance of test extract sample

H. *HPTLC-Antimicrobial Bioautographic Assay*

The HPTLC-bioautographic method was employed for the determination of antibacterial activities of the methanolic flower extract of Hibiscus arnottianus. The extract was applied on 10 x 4 cm HPTLC silica gel 60 F_{254} (Merk HX94930454) plates using Linomat 5 applicator (CAMAG, Switzerland). The plates were developed in Twin trough chamber containing respective mobile phases. The developed plate was dried and to obtain well separated bands. The developed silica plates were placed into sterile petri plates. The culture of *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* were grown at 37°C at 24 hours. was mixed with Muller & Hinton agar medium and poured onto the sterile petri plate containing developed silica gel plate such that it will form a layer over the TLC plates. The plate was incubated at 37^0C for over 24 hours. After incubation the plates were observed by pouring INT (2-(-4-iodophenyl-)-3-(-4-nitrophenyl)-5-phenyl-2H-tetrazolium) dye in sterile conditions and further incubation of plates for 20 mins. For location and visualization of antibacterial substances, tetrazolium salts were usually used, which are converted by the dehydrogenases of living microorganisms to intensely colored formazan. After the treatment with INT dye the clear inhibition zones of active components will appear on the plate in comparison to the colored background.

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III. RESULT AND DISCUSSION

A. Preliminary phytochemical screening

Different solvents may yield extracts with varying compositions and properties hence various solvents like ethyl acetate, petroleum ether, methanol and water were used to prepare extracts of H. arnottianus. In present study the extractive values of petroleum ether, ethyl acetate, methanol and aqueous were found to be 33%, 32.4%, 42.8% and 24% w/w respectively. Preliminary phytochemical screening of various extracts of H. arnottianus flower reveals the presence of alkaloids, carbohydrates, amino acids, flavonoids, saponin, tannin. Amongst the various solvents methanol extract showed the presence of most of the secondary metabolites (Table no.1). The screening of phytoconstituents of H. arnottianus flower extract was performed on silica gel 60 F_{254} HPTLC plates. The Rf values of separated components were 0.014, 0.082, 0.321, 0.479, 0.0719, 0.761, 0.901, 0968 and 0.990 (Fig. 1). Possible components present in methanolic extracts were seen by TLC followed with exposure to iodine, dragondroff reagent, p-anisaldehyde-sulfuric acid and 10% ferric chloride (Fig.3).

Table 1. Phytochemical screening of different extracts of H. arnottianus flower

Phytochemical Components	Chemical Test	Petroleum Ether	Ethyl Acetate	Distilled Water	Methanol
Alkaloids	Mayer's reagent	-	-	-	+
	Wagners reagent	-	+	-	+
Amino acids	Milon's test	-	-	-	-
	Ninhydrine test	-	-	+	+
Carbohydrates	Molisch test	+	+	-	-
	Barfoed's test	-	-	+	+
Flavonoids	Shinoda	-	-	-	-
	Alkaline reagent	-	+	+	+
	Zinc hydrochloride	-	+	+	+
Cardiac glycosides	Killer killiani	-	-	-	-
	Legal's test/ Caumarin	-	-	-	-
Proteins	Biuret	-	-	-	-
Steroids & triterpenoids	Libermann-Burchard	+	-	-	-
	Salkowski	-	+	-	-
Saponin	Froth	-	-	+	+
Tannin	$FeCl_3$	-	-	+	+
	Lead acetate	+	-	+	+

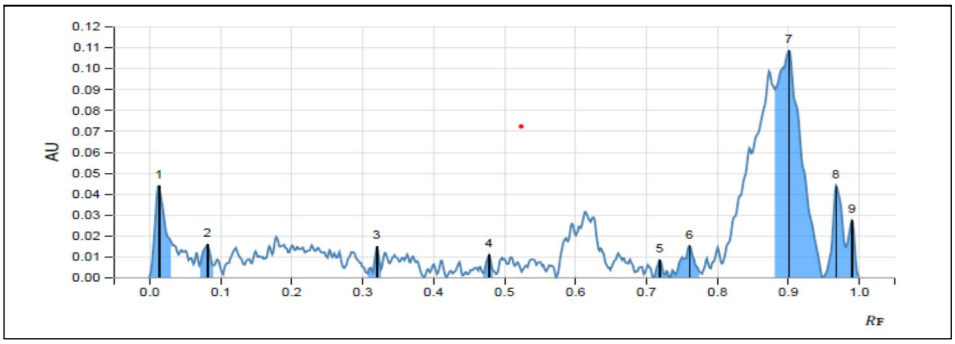


Fig. 1 HPTLC chromatograph of methanolic extract of H. arnottianus

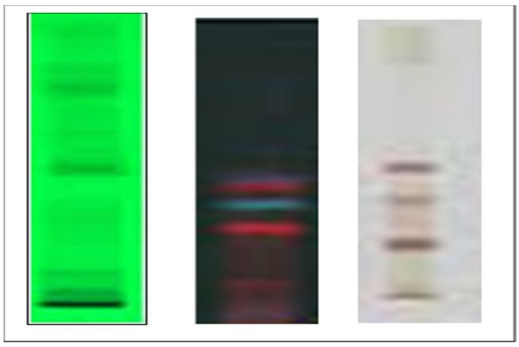
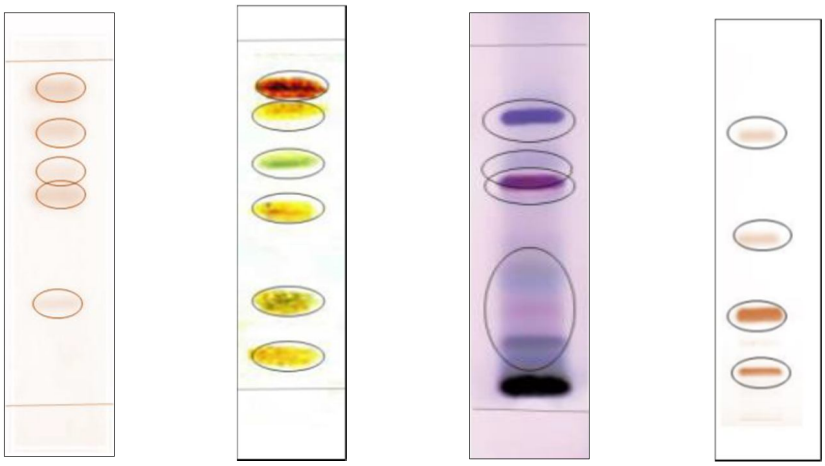


Fig. 2 HPTLC images of methanolic extract before derivatization



Iodine Vapor Dragendorff reagent p-Anisaldehyde – sulfuric acid Ferric Chloride

Fig. 3 HPTLC images of methanolic extract after derivatization

B. Physico-chemical parameters

Physico-chemical parameters including loss on drying, total ash content, acid insoluble ash, water soluble ash was found to be 87.66%, 4.77%, 3.15% and 0.54% (W/W) respectively (Table no. 2). Total alkaloid content was measured by using standard atropine in methanolic extract and expressed in terms of atropine equivalent as mg/g of extract (the standard curve equation: $Y = 0.0052x + 0.024$, $R^2 = 0.9846$) (Fig. 4) the concentration of alkaloid was 0.468 mg/g. The total phenolic content was examined using the Folin-ciocalteu's reagent is expressed in terms of gallic acid equivalent (the standard curve equation: $Y = 0.0056x + 1.5556$, $R^2 = 0.9138$). the concentration of total phenolic content was measured 13.89 mg/g (Fig. 5). Total tannin contents were examined using the Folin-ciocalteu's reagent is expressed in terms of gallic acid equivalent (the standard curve equation: $Y = 0.1124x + 0.068$, $R^2 = 0.9939$). the concentration of tannin was measured 0.471 mg/g in extract (Fig. 6). The concentration of flavonoids in methanolic extract of H. arnottianus was determined using spectrophotometric method with aluminium chloride. The content of flavonoids was expressed in terms of quercetin equivalent (the standard curve equation: $Y = 0.0064x + 0.1184$, $R^2 = 0.9797$). The concentration of flavonoids in methanol extract was 3.2 mg/g (Fig. 7).

Table no. 2 The physico-chemical analysis of H. arnottianus flowers

Sr. No.	Physico-chemical parameters	Values (gm%)
1	Loss on drying	87.66
2	Total Ash	4.77
3	Water soluble ash	0.54
4	Acid insoluble ash	3.15

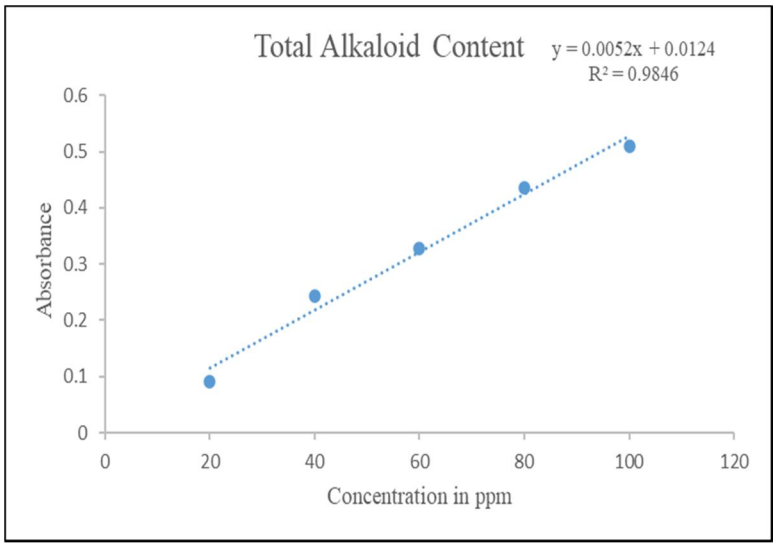


Fig. 4 Calibration curve of total alkaloid content

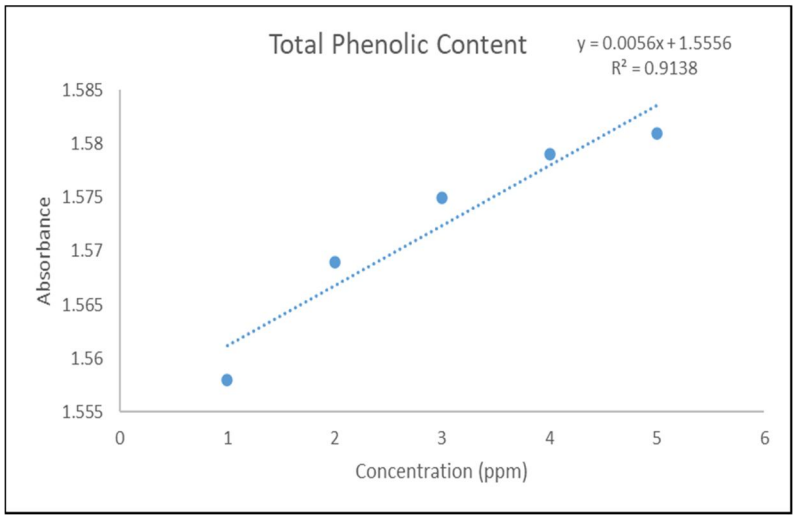


Fig. 5 Calibration curve of total phenolic content

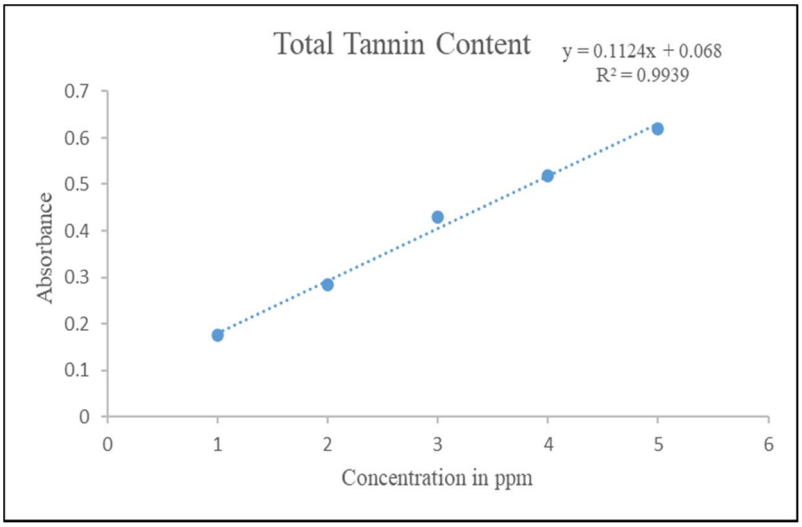


Fig. 6 Calibration curve of total tannin content

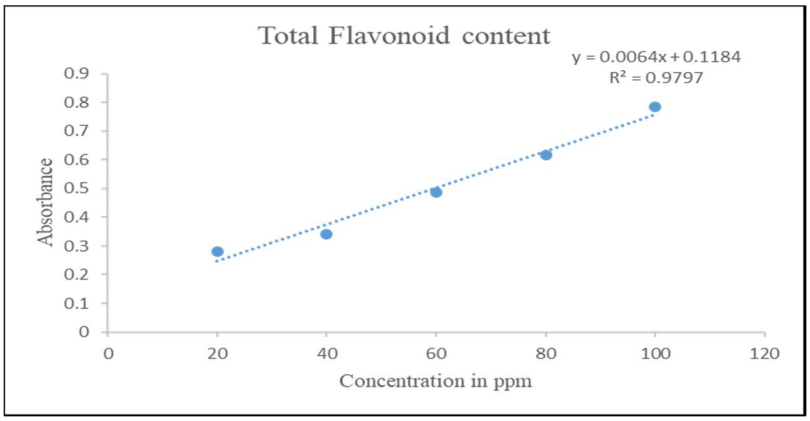


Fig. 7 Calibration curve of total flavonoid content

C. Antioxidant assay

The methanolic flower extract was found to posses antioxidant molecules which has been evidenced through the HPTLC-bioautography analysis for antioxidants (Fig. 8a). The Rf value of antioxidants compounds are 0.662, 0.753 (Fig. 8b). DPPH free radical scavenging activity exhibited scavenging potential of methanolic flower extract of H. arnottianus was 11.90 %.

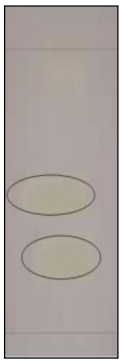


Fig. 8a HPTL images of DPPH assay

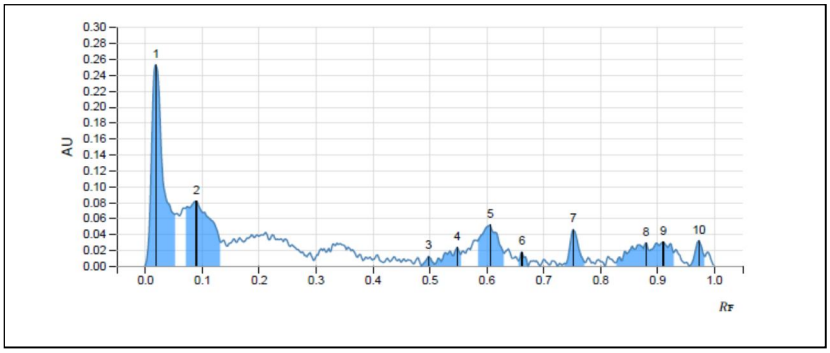
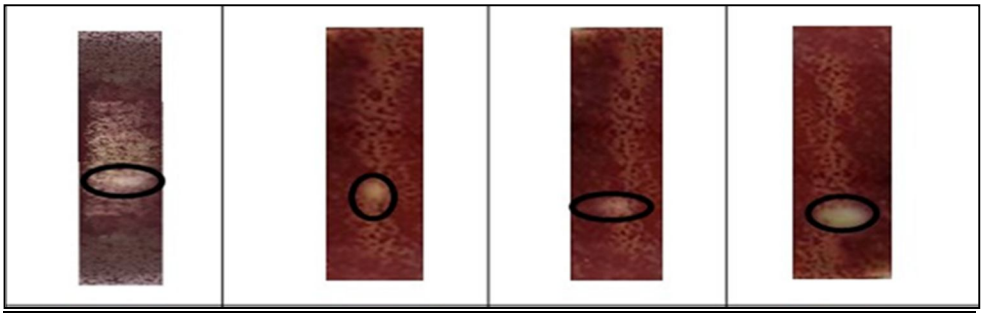


Fig. 8b HPTLC chromoagraph of DPPH assay

D. Antimicrobial assay

The extract of H. arnottianus flower showed zone of inhibition against *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa* (Fig. 9). The Rf values of antimicrobial active compound was 0.753.

The bioautography showed that the compounds responsible for antioxidant activity and antimicrobial activity could be polyphenols and flavonoids (Fig.1). The polyphenols of H. arnottianus with retention factor of 0.753 shows both antioxidant and antimicrobial properties although flavonoids with retention factor 0.632 shows only antioxidant activity.



Escherichia coli	Staphylococcus aureus	Bacillus subtilis	Pseudomonas aeruginosa

Fig. 9 HPTLC images of antimicrobial assay

IV. CONCLUSION

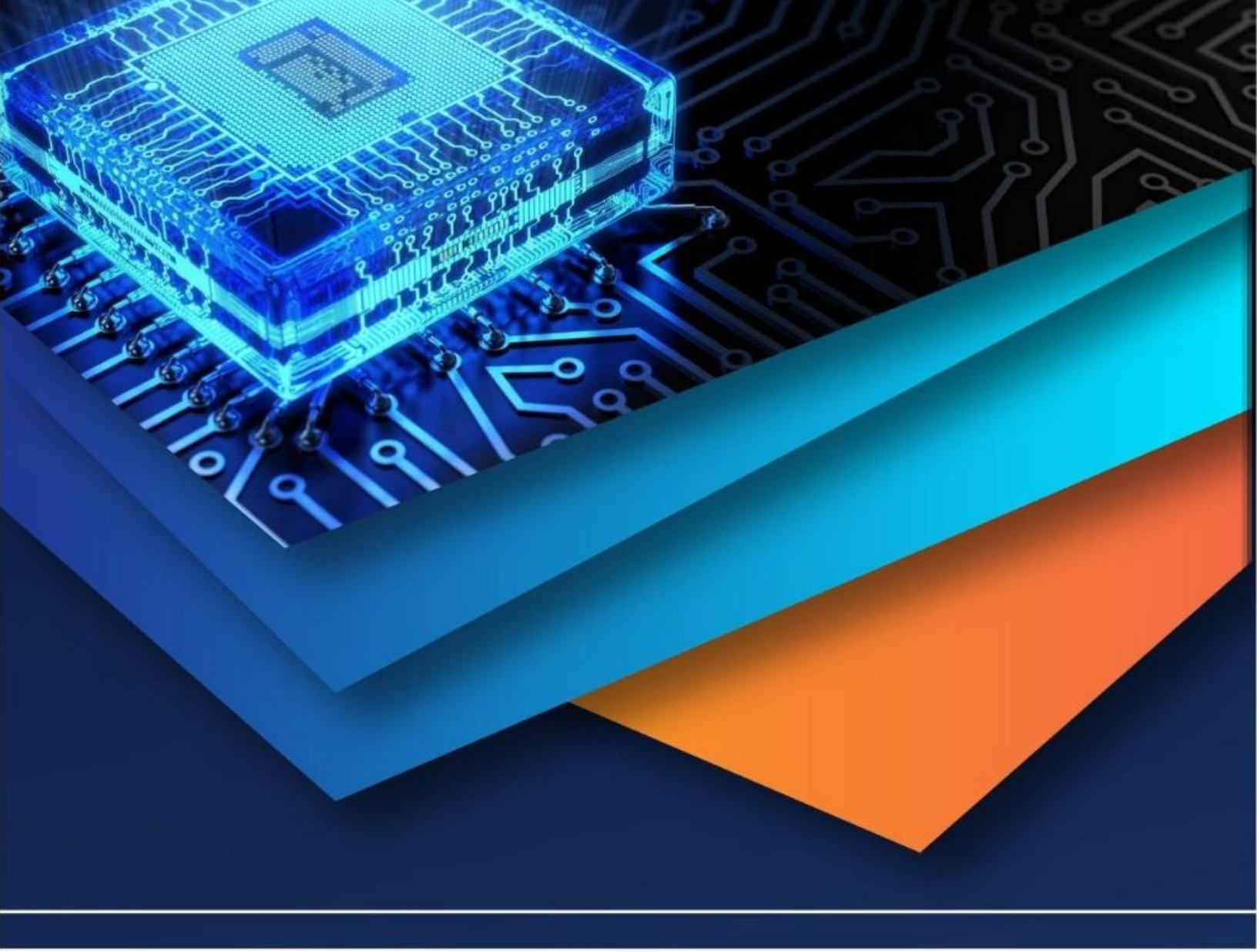
The flowers of H. arnottianus were proven to have both antioxidant and antimicrobial activity and it has been confirmed through HPTLC guided identification. These findings will be useful towards further isolation of polyphenols and flavonoids from methanolic extract of H. arnottianus flower.

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