International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Isolation, Identification, and Bioactivity Evaluation of Novel Flavonoids *Alpinia Officinarum*

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ABSTRACT

In this study the ethanolic extract of *Alpiniaofficinarum* was analysed for its antibacterial and antioxidant activity. The sample was extracted with 70% ethanol with Soxhlet apparatus. The bioactive compounds in the extract were identified by qualitative and quantitative phytochemical analysis. The most prevalent phytochemicals in *A. officinarum* were identified to be flavanoids, phenols, saponins, tannins, anthocyanin, sterols, triterpenoids, and anthraquinones. The total flavonoid conent in extract of *Alpiniaofficinarum* was analysedby Aluminium chloride colorimetric technique with Quercitin as standard. The total flavonoid content in the ethanoilc extract was determined to be 63.60mg/g. The ethanol fraction of *Alpiniaofficinarum*was subjected to thin layer chromatography. The Quercitin, Rutin,Kaempferol, Narigenin revealed a single spot with R_f value of 0.35, 0.3, 0.57, 0.25 respectively and the flavonoid fraction revealed a single spot with R_f values in the range of 0.59. The $R_f$value of isolated compound correlated with Kampferol. 13C NMR can be used to analyse the anomeric configurations, linkage sites, and sequence of sugars in the flavonol glycosides. Compound was identified as kaempferol 3,7,di-O-L rhamnoside with two rhamnoses. The existence of signals at 136.5 and 163.5 in the 13C NMR spectra compound corroborated the glycosylation at the C-3 and C-7 sites. The antibacterial activity of the isolated compound was analysed by agar well diffusion assay and revealed that with increase in concentration, the zone of inhibition increased and the gram negative bacteria were more susceptible. The antioxidant capability of kaempferol 3,7,di-O-L rhamnoside was analysed by DPPH assay. The percentage of inhibition increased with increase in compound concentration. The compound was confirmed to have strong antioxidant activity with IC50 concentration of 253.83µg/ml.

KEYWORDS: *Alpiniaofficinarum*; Phytochemical; Flavanoid; kaempferol 3,7,di-O-L rhamnoside; Antibacerial activity; Antioxidant activity.

1. INTRODUCTION

The ability of plants to generate a wide range of chemical substances that are utilized to carry out diverse biological processes in the human body has been extensively debated(Süntar, 2020). Numerous of these phytochemicals have significant long-term health benefits, making them useful in the treatment of human ailments. Herbs have been used as a source of medicine and still are. Plants have been employed as the source of several biological chemicals that have been used to cure a variety of ailments. But further research is still required to assess the traits and negative consequences of the plants(Prakash & Sharma, 2014).

One of the biggest plant families, Zingiberaceae comprises several significant dietary and medicinal plants that are widely grown throughout many nations. *Curcuma longa*, *Curcuma zedoaria*, and *Alpiniagalanga* are three plant species in the Zingiberaceae family that have several biological properties, including antimicrobial, anti-allergic, and anti-itching properties(Alasmary et al., 2019).The most significant genera in the Zingiberaceae family that consists of rhizomes is*Alpinia*. These rhizomes are used in folk medicine to treat digestive system issues, coughs, bronchial ulcers, catarrh, rheumatism, foul breath, and catarrh(Kumar et al., 2013). Alpinia species have complex chemical profiles(S. Ghosh & Rangan, 2013), according to litterateurs.

Since ancient times, both Chinese and Ayurveda medicine have employed *AlpiniaofficinarumHance*, a member of the Alpinia Genus, as dietary spices. It revealed a wide range of chemical compounds that were correlated with a number of biological activities. *AlpiniaofficinarumHance's* rhizomes are the most crucial component of the plant; they have been shown to be quite efficient in treating digestive system illnesses as well as bacterial and fungal infections(Alasmary et al., 2019). Secondary metabolites called flavonoids have a benzopyrone ring with phenolic or polyphenolic groups attached at various places(Andrae-Marobela et al., 2013). Fruits, herbs, stalks, grains, nuts, vegetables, flowers, and seeds are the most typical places to find them(Sangeetha et al., 2016). These various plant parts' therapeutic usefulness and biological activity are due to the presence of bioactive phytochemical ingredients(Lata & Dubey, 2010). Over 10,000 flavonoid compounds have been found and isolated thus far(Khajuria et al., 2019).

Flavonoids have a variety of health advantages, such as antiviral, anticancer, antioxidant, and anti-inflammatory effects. Additionally, they have cardio-protective and neuroprotective properties(Ullah et al., 2020). 69% of the 109 novel antibacterial medicines that were authorised between 1981 and 2006 were from natural sources (Newman & Cragg, 2016). Flavonoids are a significant category of phytochemicals that have been thoroughly investigated for their antibacterial effects(Cushnie & Lamb, 2005).Additionally, certain chemicals extracted from the rhizomes have demonstrated actions like anti-dermatophyte, anti-hepatotoxic, and antioxidant(Housman et al., 2014). Even though folklore medicine has been practiced by humans since ancient times, it continues to be based more on personal experience than on objective data(Darlington & Scott, 2020). Both the clinical studies using herbal remedies and the pharmacological investigation of natural compounds remain insufficient. The absence of standardized methods for assessing natural medicines is one issue that is preventing development. The current study focuses on isolation of flavonoid from *AlpiniaOfficinarum*and assessing its antibacterial and antioxidant capability.

2.MATERIALS AND METHODS

2.1. PLANT COLLECTION

*Alpiniaofficinarum*Hance rhizomes were acquired fromKolli hills, Tamil Nadu, India. The rhizomes were chopped, shade dried and grinded into fine powder for further study(Subramanian et al., 2008).

2.2. EXTRACTION AND ISOLATION

Using a Soxhlet extraction apparatus, the 2 kg of dried powder from the smaller galangal rhizomes was extracted with 70% ethanol. Following extraction, the solutions were filtered. The filtrates were

concentrated by evaporation under decreased pressure. Dried extract was stored in a 4°C refrigerator for use in a phytochemical examination later on (A. Ghosh et al., 2011). The filtered extracts were mixed and evaporated under reduced pressure. After this, the extract was dissolved in water and applied to Silica gel column (gel volume estimated to be 600 cm^3). The gel was then progressively eluted with water, 40%, 60%, 80%, and 100% MeOH to yield three fractions (Fr. 1-Fr. 5) based on their TLC patterns. After Sephadex LH-20 treatment on fraction 3 (13.1 g), four subfractions (SubFr 3-1–SubFr 3-4) were obtained. To obtain ten subfractions (3-2-1 to 3-2-10), subfractions 3-2 and 3-3 were blended and added to ODS CC (water, 10% - 100% MeOH). Subfraction 3-2-8 were each submitted to silica gel CC to produce compound, respectively (121 mg).The isolated compound were subjected to thin layer chromatography (Basri et al., 2017).

2.3. QUALITATIVE ANALYSIS OF PHYTOCHEMICAL

Standard screening test were carried out for various plant constituents. The dry extract was used for the phytochemical analysis for compounds which include tannins, alkaloids, steroids (Balamurugan et al., 2019), flavonoids(Lavanya et al., 2018), saponin(Gupta et al., 2021),steroids, phenol (Subash et al., 2013), Terpenoid(Menghani et al., 2012) and glycoside (Malik et al., 2016). The ethanol extract of plant sample (10mg/ml) was subjected to phytochemical examination.

2.4. QUANTITATIVE ANALYSIS OF FLAVONOIDS

Aluminium chloride colorimetric technique was used to determine the total flavonoid content (TFC) of each extract. In essence, methanol was used to dilute the extract sample until it contained 10 mg/mL. In order to create the calibration curve, quercetin (25–1000µg/mL) was diluted in methanol. 200µL of the extract or quercetin was diluted with 600µL of methanol, and 2.0 mL was combined with 40µL of 1M potassium acetate solution and 40µL of a 10% (w/v) aluminium chloride solution and allowed to stand for 30 mins. Then, a UV-VIS spectrophotometer was used to detect the mixture's maximum absorbance at 415 nm. TFC was calculated as mg QCE/g plant extract, which represents as mg quercetin equivalent per g of plant extract (Ferdous et al., 2018).

2.5. THIN LAYER CHROMATOGRAPHY

Quercetin, Narigenin, Kaempferol and Rutin were used as used as standard for identification of flavonoids. The following conventional solutions were made. In 10 ml of methanol, each standard flavonoid (5 mg) was dissolved. TLC plates used were silica gel-coated aluminium sheets measuring 10×10 cm from Merck. Standard flavonoid solutions were immediately spotted on the silica plates using a capillary tube and a mobile phase solvent of ethyl acetate, formic acid and distilled water (65:20:15, v/v/v) mobile phase. A tank was filled with the appropriate mobile phase. After then, it was anticipated that the solvent vaporization inside the operating tank would take at least 30 minutes to reach equilibrium.Then, in an even-running tank, a plate was created. The running process was permitted to ascend to a high point of up to 1.5 cm after leaving the mobile phase. The plates were then taken out and dried in a fume cupboard thereafter. Under UV light at 254 nm, colored and colorless bands appeared on the plate. 5% Ethanolicaluminium chloride was then applied to every place on the dry plate, and colorless dots appeared under the UV light at 365 nm. By comparing them to R_f values and conventional color features, flavonoids were identified (Victório et al., 2009).

2.6. NMR SPECTROSCOPY

NMR spectrometer system with Trimethylsilyl ether (TMS) as an internal standard was used to identify the carbon (67.5 MHz for 13C-NMR) in methanol, DMSO, CCl_4, and $CDCl_3$. Chemical shift measurements were reported in δ ppm(Ahmed et al., 2011)

2.7. BIOLOGICAL ACTIVITY

2.7.1 ANTIBACTERIAL ACTIVITY

The isolated compound was tested for antibacterial activity using the agar well diffusion technique. In order to adjust the turbidity to 0.5 McFarland standards, the test organisms *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 23235) were inoculated in Nutrient broth and cultured overnight at 37°C, yielding a final inoculum of 1.5×10^8 CFU/ml. The culture was spread on MHA plates. Compound at a concentration of 1000, 750, 500, 250 µg/ml with the positive control (amplicillin) for bacteria (30 mcg).Using a sterile cork-borer, infected medium were bored to create a well (6 mm). It was incubated for 18 to 24 hours at 37°C and kept undisturbed for 30mins. After incubation, the test compounds antibacterial activity was determined by observing at the plates for the development of a clear zone around the well. The observed zone of inhibition (ZOI) was in millimeters was assessed (Rao et al., 2010).

1.7.2. ANTIOXIDANT ACTIVITY

DPPH (2, 2-diphenyl-1-picrylhydrazyl) scavenging activity was measured with spectrophotometer method. 0.4 mM solution of DPPH in methanol was prepared. 100µl of DPPH solution was added to 100µl of sample with different concentrations (25 to 1000µg/ml). The mixture was shaken vigorously and kept at room temperature for 30 min. Then, absorbance was measured at 517 nm by using spectrophotometer. Each concentration was run in duplicates and mean value is determined. Ascorbic acid was used as the standard. The percent DPPH scavenging effect was calculated by using following equation:

$$\%\, Inhibition \;=\; [A0 - A1]/A0 \quad \times 100$$

Where A_0-Absorbance of control reaction

A_1- Absorbance in presence of test(Santos et al., 2012).

3. RESULT AND DISCUSSION

3.1. EXTRACTION YIELD

A total of 2.3 kg of *Alpiniaofficinarum* rhizome were gathered fromKolli hills, Tamil Nadu, India. The leaves were then extracted using 70% ethanol in the form of dark brown gum by soxhlet apparatus (Fig1). The extraction yield was calculated using formula and tabulated in Table 1

$$Percentage\ Yield\ (\%) \;=\; Dry\ weight\ of\ extract\ /\ Dry\ weight\ of\ plant\ material \;\times\; 100$$

Table 1: Extraction Yield of *Alpiniaofficinarum*

S.No	PLANT	EXTRACT	COLOUR	CONSISTENCY	EXTRACTION YIELD %
1	*Alpiniaofficinarum*	70% Ethanol	Brown	Gum	75.87

FIG1SOXHLET EXTRACTION OF ALPINIA OFFICINARUM

3.2. QUALITATIVE PHYTOCHEMICAL ANALYSIS

The qualitative phytochemical examination of *Alpiniaofficinarum* rhizome was conducted and the results were analysed. The ethanol extract of the plant was found to contain majority of phytochemical compounds which was tabulated in Table 2.The most prevalent phytochemicals in *A. officinarum* were discovered to be flavanoids, phenols, saponins, tannins, anthocyanin, sterols, triterpenoids, and anthraquinones(Fig 2). These findings imply that these plants may be a bioactive compounds with potential as medicinal agents (Alasmary et al., 2019). According to Fatemah A. et al.,2013, the phytochemical analysis of the *Alpiniaofficinarum*rhizomes revealed the presence of a variety of active bioactive compounds, including flavonoids,triterpenes, and sterols(Alasmary et al., 2019).The phytochemical analysis of rhizome extracts of *A. galangal* conducted by Kr et al., 2016revealed flavonoids, steroids, and terpenoids in the hexane extract; flavonoids, phenols, steroids, and terpenoids in the ethyl acetate extract, and all phytochemicals in the methanol extract (Kr et al., 2016).

Table 2: Qualitative Phytochemical analysis of *Alpiniaofficinarum*

Plant extract	Phenol	Alkaloid	Flavonoid	Tannin	Glycoside	Saponin	Terpenoid	Steroid
Methanol crude	+	+	+++	++	+	+	+++	-

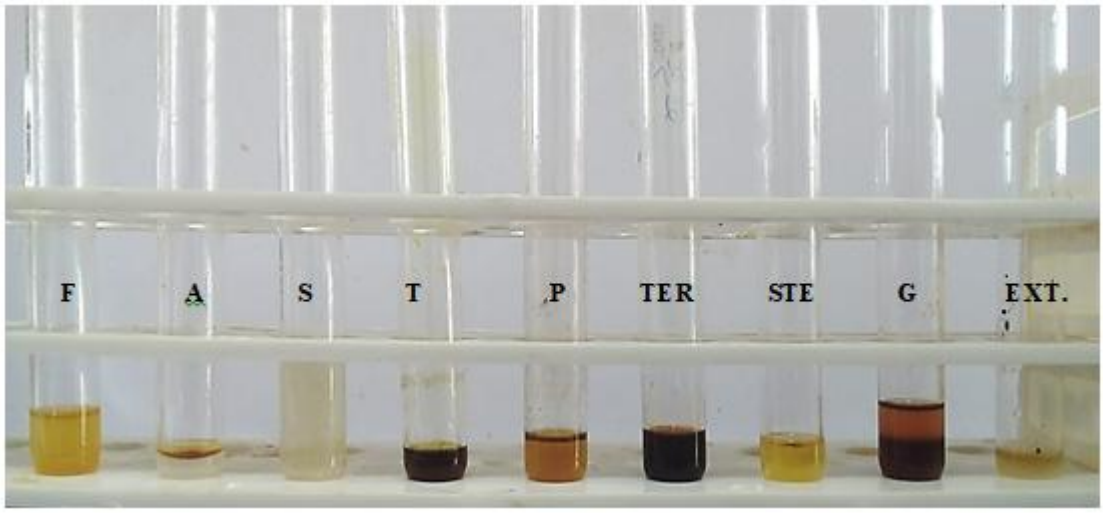

FIG 2 QUALITATIVE PHYTOCHEMICAL ANALYSIS

F- Flavonoids;**A**- Alkaloids;**S**- Saponins;**T**- Tannins; **P**- Phenols;**Ter**- Terpenoids ;**Ste**-Steroids;**G**- Glycosides ; **Ext**.- Extract

3.3. QUANTITATIVE PHYTOCHEMICAL ANALYSIS

Using standardized techniques, the quantity of phytochemicals present *Alpiniaoficinarum* was quantified with Quercetin as known standard with different concentration and represented graphically and measured at 415nm. Quercetin was used to create a standard curve, and linearity was attained between 25 and 1000µg/ml. Total flavonoid content was quantified as quercetin equivalent, in mg/g of the extract, using the standard curvey = 0.0039x + 0.0391, where y= Absorbance, x= Flavonoid content (Devi et al., 2018). The straight line equation y = 0.0039x + 0.0391(Fig 3)with a correlation coefficient was derived from the examination of the quercetin standard calibration curve. The regression equation is linear, and the concentration impacts the absorbance at 99%(Suzery et al., 2019). The total flavonoid content (TFC) was determined to be 63.60mg/g.The flavonoid concentration of *Alpiniagalanga* leaf extract was considerable (64.691.12 mg Quercetin equivalent/g of extract)(Singh et al., 2020). The result of present study was similar to previous analysis.

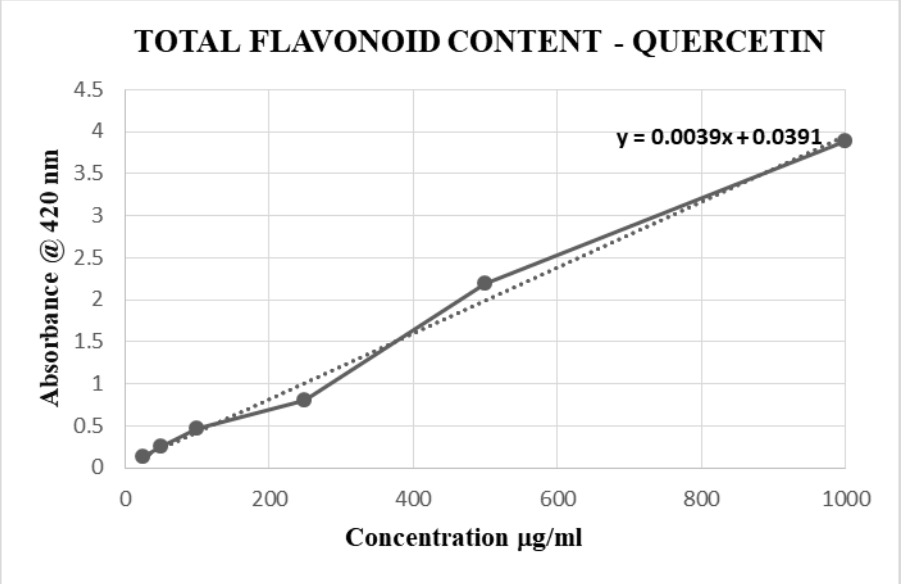

FIG 3GRAPHICAL REPRESENTATION OF TOTAL FLAVONOID CONTENT

3.4. TLC IDENTIFICATION

R_f values for standards and extracts (ethanol) applied on TLC plates were compared. Extracts and standard flavonoids components were determined using TLC analysis (Table 3 and Fig 4). Ehanol fraction of *Alpiniaofficinarum* were subjected to TLC and the results are shown in Figure 4. Standard quercetin produced a single spot with an R_f value of 0.35 (Kaya et al., 2012). The Quercitin, Rutin,Kaempferol, Narigeninrevealed a single spot with R_f value of 0.35, 0.3, 0.57, 0.25 respectively and the flavonoid fraction revealed a single spot with R_f values in the range of 0.59.When utilisingethyl acetate, formic acid and distilled water (65:20:15, v/v/v)as the mobile phase, the TLC revealed a single spot with $R_f$value of 0.59. The isolated substance was also put through TLC and contrasted with the reference flavonoid kaempferol. Ethyl acetate, formic acid and distilled water (65:20:15, v/v/v) make up the solvent system. 5% Ethanolicaluminium chloride is used as the spraying agent. To eliminate solvents from the prepared plate, a hot air oven drying process was performed at 100°C. Images at white light, UV 254 nm, and UV 366 nm were taken of the plate while it was within the photo documentation chamber. The isolated compounds are visible at R_f values of 0.59, which are quite close to the R_f value of normal kaempferol. Based on study conducted by Wang et al., 2012, kaempferol related compound was recognised as the isolated substance from *Alpiniaofficinarum* leaves(Wang et al., 2012). From the previous research, it was evident that isolated compound was kampferol.

Table 3: Quantitative flavonoid content analysis of *Alpiniaofficinarum*

FLAVONOIDS	R_F	COLOUR UNDER 366NM
Quercetin	0.35	Yellowish green
Kaempferol	0.57	Bluish fluorescence
Rutin	0.3	Dark yellow
Narigenin	0.25	Orange
Alpiniaofficinarum ethanol	0.59	Blue fluorescence

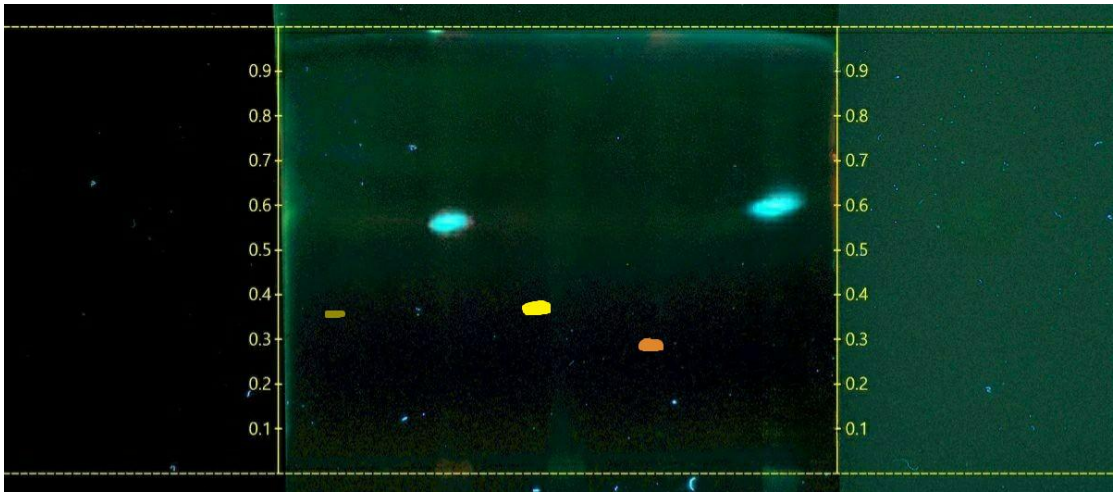

FIG 4 THIN LAYER CHROMATOGRAPHY

3.5. NMR SPECTROSCOPY

To isolate pure compound, the extract was repeatedly treated to column chromatography on Sephadex LH-20, ODS, and silica gel. *Alpiniaofficinarum* rhizome were air-dried and thoroughly extracted with ethanol. Column chromatography was used to analyse the remaining ethanol. Compound had free hydroxyl groups at C-5 and C-40 in place of the sugar substitutions at C-3 and C-7. 13C NMR can be used to determine the anomeric configurations, linkage sites, and sequence of sugars in the flavonol glycosides. Compound was identified as kaempferol 3,7-derivatives with two rhamnoses by analysing 13C NMR spectrum data.

The existence of signals at 136.5 and 163.5 in the 13C NMR spectra compound (Fig 5) corroborated the glycosylation at the C-3 and C-7 sites. Between 99.2 and 102.7, two anomeric carbon signals were detected. The remaining sugar carbons that appeared at 17.3 and 71.8 were identified using NMR data, which revealed that the remaining sugars were in the pyranose form. According to the spectrum information shown above, compound was identified to be kaempferol 3, 7-di-O-L-rhamnoside.

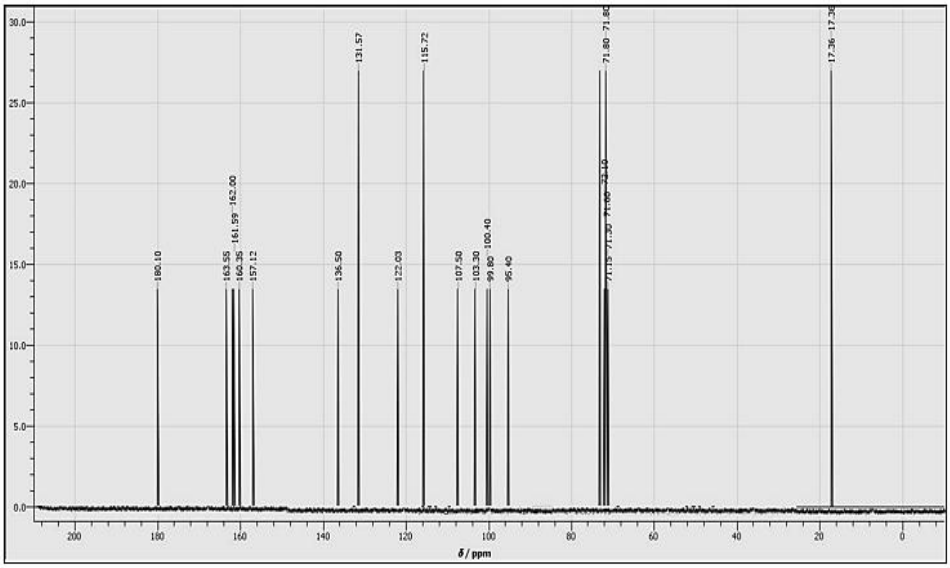

FIG 5 C-13 NMR SPECTRUM OF ALPINIA OFFICINARUM

3.6. BIOLOGICAL ACTIVITY

3.6.1. ANTIBACTERIAL ACTIVITY

The antibacterial activity of kaempferol 3,7-di-O-L-rhamnoside was analysed at different concentration against Gram negative strain and Gram positive strain. The activity of the compound increased with increase in concentration. Ampicillin was used as standard positive control. The zone of inhibition of the compound at various concentrations was represented in table 4. The antibacterial activity extracted compound showed better activity against Gram positive strain (*S.aureus*) than Gram negative strain (*E.coli*). Gram-negative bacteria are often more resistant to antibiotics than Gram-positive bacteria, which has been explained by the presence of an outer-membrane permeability barrier, which limits antimicrobial agent access to their targets within the bacterial cell.Plants are known to generate flavonoids in response to infection. Their ability to react with extracellular and soluble proteins, as well as complex with bacterial cell walls, is most likely responsible for their activity(Cowan, 1999).Luteolin (*C. scapigera*), which contains a hydroxyl group at the 3' position, was found to have antibacterial efficacy against *S. mutans*, *S. aureus*, and *C. tropicalis* strains, but apigenin had minimal impact(Schinor et al., 2007).*Flavobacteriumsp.* was resistant to all phenolic chemicals tested (caffeic,gallic,rutin,vanillic acid, and quercetin of different wine), while *Escherichia coli* was the most vulnerable(Vaquero et al., 2007).Flavonoids in *Calycotomevillosa*, including as genistein,chrysin,quercetin-4'-methyl ether and luteolinwere found to be responsible for the inhibition of *Bacillus lentus*, *Escherichia coli*, and *Klebsiellapneumoniae* growth(Loy et al., 2001). The results of current study were in accordance with previous researches. Fig 5 and 6 shows the susceptibility of organism at different concentration.

TABLE 4: ZONE OF INHIBITION

CONCENTRATION(µg)	Zone of Inhibition	
	E.coli	***S.aureus***
250(A)	-	-
500(B)	-	11
750(C)	13	15
1000(D)	17	19
Ampicillin (30µg)(E)	19	22

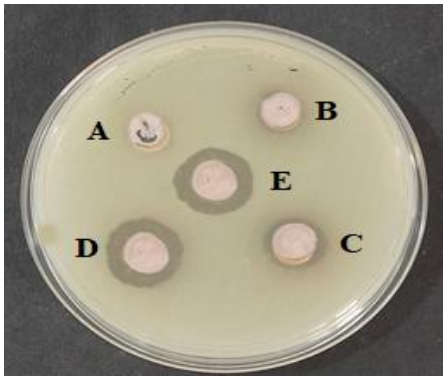

FIG 5 ZONE OF INHIBITION AGAINST E.COLI

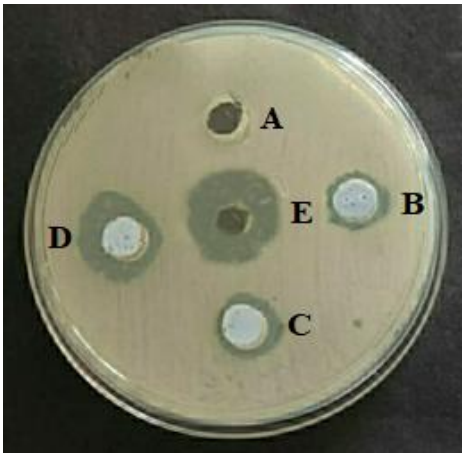

FIG 6 ZONE OF INHIBITION AGAINST S.AUREUS

3.6.2. DPPH ASSAY

.The free radical scavenging activities of isolated compound were shown in Table 6 and Fig 8. The antioxidant effect of ascorbic acid (Standard) was represented in Table 5 and Fig 7. The sample tested showed 65.04% inhibition at the highest concentrations of 1 mg/ml. The isolated compounds showed a IC50 concentration of 253.83 µg/ml(Table 7).Flavonoids with their ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide anion radical and hydroxyl radicalsare potential antioxidants (Pietta et al., 1998). Kaempferol-3-O-glucoside, which was isolated from numerous other plant species, was discovered to have strong antioxidant properties (Pourmorad et al., 2006). The dihydroflavonol derivative of quercetin-3-O-rhamnoside, taxifolin-3-O-rhamnoside, demonstrated higher radical-scavenger activity. Phenolic substances have been partially implicated in the reduction of risks of oxidative stress-related illnesses such cancer, osteoporosis, cardiovascular and neurological diseases(Arts & Hollman, 2005).

TABLE 5: DPPH ASSAY – ASCORBIC ACID

CONCENTRATION OF ASCORBIC ACID	PERCENTAGE OF INHIBITION
1000	91.34
500	69.37
250	54.96
100	40.79
50	34.72
25	29.53

TABLE 6: DPPH ASSAY – COMPOUND

CONCENTRATION OF SAMPLE	PERCENTAGE OF INHIBITION
1000	65.04
500	58.03
250	48.50
100	37.48
50	34.17
25	27.48

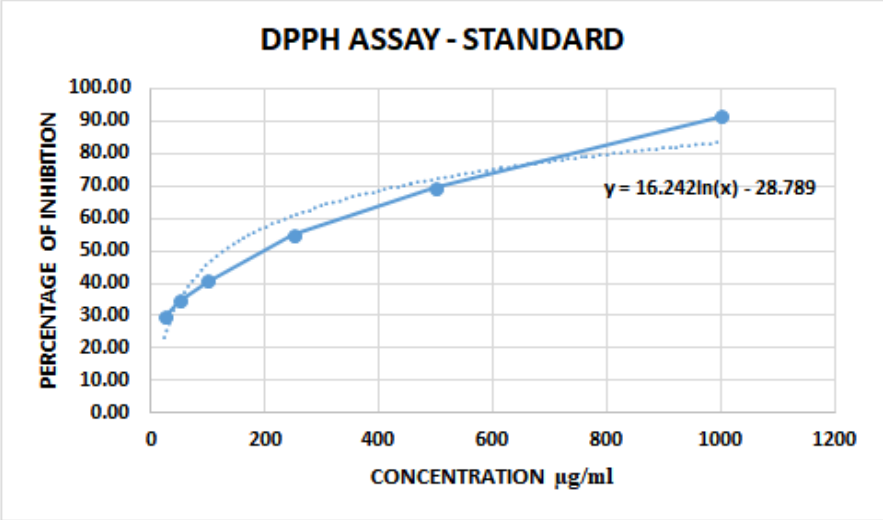

FIG 7 DPPH ASSAY – ASCORBIC ACID

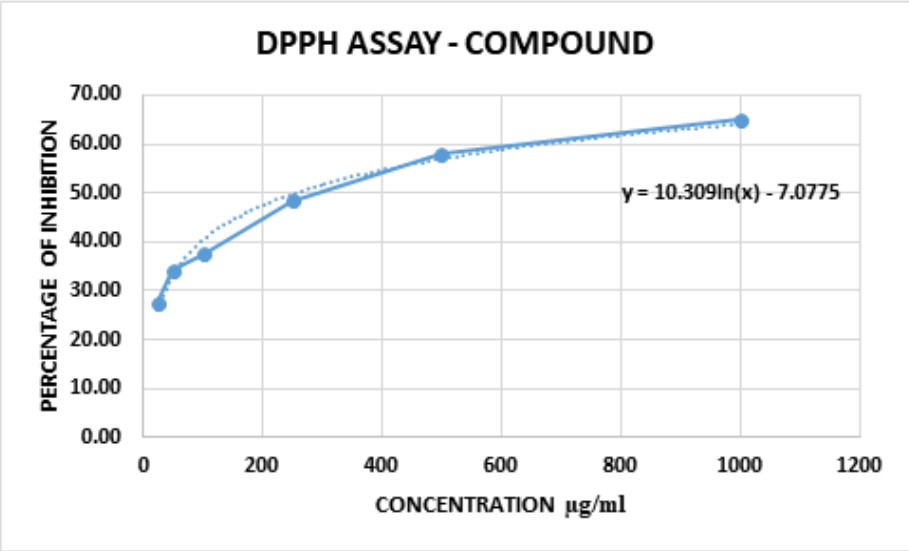

FIG 8 DPPH ASSAY – SAMPLE

TABLE 7: IC 50 VALUES OF STANDARD AND COMPOUND

MINIMUM INHIBITORY CONCENTRATION	
STANDARD	127.86
COMPOUND	253.83

4. CONCLUSION

The conclusion of the current study is that ethanolic extract of *Alpiniaofficinarum* had abundant flavonoid content. The NMR spectra revealed the compound to be kaempferol 3,7-di-O-L-rhamnoside. The compound showed excellent antibacterial and antioxidant activity. The study of mechanism of action has to be investigated. There is vast scope with future studies of *Alpiniaofficinarum*. With magnificent antioxidant activity, anticancer activity of the compound can be studied in future research.

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