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Research Article

ISOLATION, STRUCTURAL ELUCIDATION OF FLAVONOIDS FROM *POLYALTHIA LONGIFOLIA (SONN.) THAWAITES* AND EVALUATION OF ANTIBACTERIAL, ANTIOXIDANT AND ANTICANCER POTENTIAL

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ABSTRACT

Thin Layer Chromatography (TLC) analysis of Ethanolic Extract of *Polyalthia longifolia* leaves (EEPL) revealed the presence of flavonoids and the results were also confirmed with Reverse Phase High performance Liquid Chromatography (RP-HPLC) analysis. The Fourier Transform Infra Red (FT-IR) spectra were recorded for Preparative Thinlayer Chromatography (PTLC) isolated samples in the range of 4000 – 450 cm-1. The pronounced peaks belonging to the vibration of 3366 cm-1, 1650 cm-1and 679 cm-1 in the spectra corresponds to the characterized peaks of flavonoids. The High Performance Liquid Chromatography – Diode Array Detection – Electro Spray Ionization/ Mass Spectrometry (HPLC-DAD- MS (ESI+) analysis identified three flavonoids viz., Rutin, Chrysin and Daidzein-related isomer and an unknown flavonoid in the PTLC isolate of EEPL. *In vitro* anti-bacterial, anti-radical and MTT-based cytotoxicity activity against A549 human (lung carcinoma) cancer cell line study also strongly suggests that EEPL possess potent biological activity.

Keywords: *Polyalthia longifolia*, PTLC isolates, Flavonoids, Structural Elucidation, Cytotoxic action

INTRODUCTION

Polyalthia is a large genus of shrubs and trees found in tropic and sub-tropic regions. It belongs to the family of Annonaceae. The genus *Polyalthia* includes about 120 species occurring mainly in Africa, South and South-Eastern Asia, Australia, and New Zealand. India has 14 species of *Polyalthia*. *Polyalthia longifolia (Sonn.) Thwaites* is a tall, evergreen tree with a straight trunk and horizontal branches and is cultivated widely in India. *Polyalthia longifolia* which is found to be rich in anti- oxidant and anti microbial properties has been chosen for the present study. It is found to have an unique place in the traditional medicinal practice of Indian ayurvedic system and has been proved to be successful in the cure of various diseases like cancer, ulcer, inflammation, and hepatotoxicity. Researchers have reported that it possesses flavonoids, alkaloids, sesquiterpenes, diterpenes, saponins, quercetin, bulbocapnin, a-sitosterol, stigmasterol campest, enihalimane diterpenes, and sesquiterpenoids. Phytochemical studies on the hexane extract of the stem bark of *Polyalthia longifolia* have led to the characterization of clerodane and enihalimane diterpines, two of which have demonstrated significant antibacterial and antifungal activities [1]. Methanolic extracts of *Polyalthia longifolia* have yielded 20 known and two new organic compounds, some of which show cytotoxic properties [2]. Similarly, the ethanolic extract of *polyalthia longifolia* leaves have been shown to posses a potent nitric oxide radical scavenging activities [3]. The chloroform fraction of the *polyalthia longifolia* leaves extract was found to posses anti cancer potential towards various human cancer cell lines and in particular it has been found to posses maximum inhibition against colon cancer cells SW-620 [4]. It has been reported [5] that presence of quercetin, bulbocapnin, steroids (Stigmasterol, a- sitosterol and campesterol), in the leaves of *Polyalthia longifolia* contribute for the analgesic activities observed. It has been proved that its bark and leaves have antimicrobial, cytotoxic, hypoglycemic, hypertensive and anti hyperglycaemic actions. It has been proven to be useful to depress the heart functions, lower the blood pressure and stimulate respiration [5]. Subsequent investigations on this plant suggest the presence of antioxidant property [6]. The essential oils of the leaf and stem bark of *Polyalthia longifolia* (sonn.) *Thwaites* have been studied for their constituents by means of gas chromatography and gas chromatography/mass spectrometry [1]. In the present study an attempt has been made to isolate and elucidate the structure of flavonoids from ethanolic extract of *polyalthia longifolia* leaves and evaluate its anti-bacterial, anti-radical and cytotoxicity properties.

MATERIALS AND METHODS

Plant material

The leaves of the plants were collected from the Medicinal garden of Kamaraj College of Engineering and Technology (KCET), Virudhunagar, Tamil Nadu, India, identified by an expert taxonomist and confirmed by Botanical Survey of India (BSI), Southern Circle, Coimbatore, India. The voucher specimen of the sample (SAM-03) was deposited at the Department of Biotechnology, Kamaraj College of Engineering and Technology, Virudhunagar, Tamilnadu, India.

Sample preparation

The leaves were air-dried and powdered. The extract of sample was prepared by soaking 10g of dried powdered sample in 100ml of Ethanol for 12 h in an orbital shaker. The extracts were filtered using Whatman filter No.1. The ethanol was then removed under reduced pressure at 40^0C to obtain the dry extract.

Identification of flavonoids by TLC

TLC was performed according to [7], on 20 × 20 cm plates precoated with silica gel G. The concentrated extracts were spotted on the lower left of the TLC plate and the diameter of the spot in each chromatogram was normally about 5mm.The EEPL and reference standard rutin were run one dimensionally in the mobile phase solvent (ethyl acetate - methanol- water, 5:1:5, v/ v/ v) at room temperature. Identification of the flavonoids in the extracts was done under UV light after the application of ammonia [8].

Isolation of flavonoid by PTLC

The PTLC (20 X 20 cm, 0.5mm thickness of 45gm of Silica gel G/80 ml water) was performed [9] similar to TLC to isolate flavonoid related compounds, the only difference is being an increase of optimal extract volume and the adsorbent bed thickness. From the PTLC plate, flavonoids were scraped out and diluted in water. The diluted mixture was then centrifuged at 10000 rpm for 10 min at 4^0 C. The supernatant containing flavonoids were collected, lyophilized and utilized for further experimental (FT-IR and HPLC-DAD-ESI/MS) analysis.

FT-IR analysis

The PTLC leaf isolates was mixed with 200 mg KBr (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 4000-450 cm^{-1} in FT-IR spectroscopy (Perkin Elmer FT-IR Spectrometer, USA).

HPLC-DAD-ESI/ MS analysis

This analysis was performed using an LC-DAD- MS (ESI+) system with HPLC coupled Diode array detector thermo Finnigan LCQ advantage max ion trap mass spectrometer. The separation was performed on Thermo Hypersil gold, 150 x 4.6, 5µm, RP-18 column at a flow rate of 80µl/min. 20 µl sample was introduced into the Electro Spray Ionization (ESI) source through finnigan surveyor auto sampler. The mass spectra were scanned in the range 100-1000 Da and the maximum ion injection time was set to 200ns. Ion spray voltage was set as 5.3 kv and capillary voltage as 40 v. The following gradient program was used: The mobile phase A was made up of acetonitrile while B was made of 0.1% formic acid (pH 4.0, adjusted with ammonium hydroxide) aqueous solution. The gradient elution was performed at 0.5 ml/min with an initial condition of 12% of mobile phase A and 88% of mobile phase B for 10 min. The mobile phase A was increased to 25% at 60 min and linearly increased to 60% at 80 min and then increased to 100% at 85 min. The flow rate was 1 ml/min and injection volume was 10 μl. The eluates were monitored by PDA (Multi wavelength) detector at 357 nm. The MS scan ran upto 60 min and the data reductions were performed by Xcalibur 1.4 SRI.

Screening for antibacterial activity

The antibacterial activity of EEPL and PTLC isolates were analysed by using [10] disc diffusion method. Sterile antibiotic disc from HiMedia was used for the present investigation. 100 µl of the EEPL and PTLC were incorporated to the sterile disc individually. Precautions were taken to prevent the flow of the solvent extract from the disc outer surface. Commercial amikacin antibiotic disc was used as positive control and absolute ethanol used as the negative control. The discs were placed on the agar plates on which the bacteria were inoculated and spread uniformly and incubated at 37° C for 24 hr. The diameter of inhibition zone was measured in mm.

Anti radical activity by $ABTS^+$ method

A method developed by [11] was followed for the Anti radial activity by ABTS method determination of anti radical scavenging activity. The ABTS radical cation, was generated by reacting 7 mm ABTS stock solution with 2.45 mM potassium per sulphate solution and the mixture was kept in the dark at room temperature for 12-16 hours before use. The ABTS solution was diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm. An aliquot of 100 µl of sample at different concentrations (0.2-2.0 µg/ml) was added to 0.9 ml of diluted ABTS. After 6 min, the absorbance was measured at 734 nm. 1.0ml of $ABTS^+$ solution alone was used as a control. The inhibition of ABTS radical scavenging in percentage was expressed by the following formula, I_{ABTS^+} % = $[(A_{control} - (A_{sample} - A_{blank}))/A_{control}] \times 100$ (%). Rutin was used as the reference compound.

Cytotoxicity activity by MTT assay

The cytotoxic assay [12] was carried out using 0.1 ml of cell suspension, containing 10,000 cells seeded in each well of a 96 well microtiter plate (Nunc and Tarsons). Fresh medium containing different concentration of the extracts were added 24 hours after seeding. Control cells were incubated without the test extracts and Minimum Essential Medium (MEM). The microtiter plates were incubated at 37^0C in a humidified incubator with 5% CO_2 for a period of 72 h. four wells were used for each concentration of the extracts. Morphological changes in the cells were inspected daily and observed for microscopically detectable alterations. The cytopathic effect was observed. The 50 percent cytotoxic concentration (CTC_{50}) was determined by MTT assay.

RESULT AND DISCUSSION

Thinlayer Chromatography (TLC) analysis

In TLC, due to the adsorption process [9] the solutes of the flavonoids present in the EEPL were distributed throughout the silica gel. When, ammonia solution [8] was sprayed onto the silica plate, the flavonoid in the EEPL reacts with ammonia solution to give a yellowish green patch under the UV exposure. On comparison of the retention factor of EEPL with the reference standard (Rutin), the authenticity of the extracted sample was found. The R_f value of Rutin and EEPL were found to be 0.676 and 0.658 respectively. Medicinal plant research is aimed at the identification of naturally occurring substances. Identification of bioactive compounds from plant material will play a central role in the development and modernization of herbal medicine. Thin layer chromatography (TLC) has frequently been used for the separation and the quantitative or semi-quantitative analysis of natural compounds. It can also be used to control isolation in column chromatography and identity of the compounds. TLC has some advantages such as rapidity, easiness and cheapness. This method does not require complex instrumental equipment and it is more sensitive than paper chromatography (PC). An observation made by [13] regarding the identification of flavonoids under far UV light has been well documented and agreed with the present results. It has been reported [14] that essential amino acids from *polyalthia longifolia* seeds were qualitatively analyzed by paper chromatography. Previous report [7] suggested the presence of rutin related compound in the ethanolic extact of *Polyalthia longifolia (sonn.) Thawates* by using TLC.

Preparative Thin layer chromatography (PTLC) analysis

The green coloured patches of flavonoids in the PTLC plate alone were scraped out for further qualitative analysis. In industrial scale, to isolate and purify the bioactive compounds in bulk amounts, preparative is the only choice for increasing the yield. Most of the researchers adopt the column chromatography for the isolation and purification of plant metabolites. Hence PTLC was chosen because of its simplicity, rapid detection, single step purification, cost effectiveness and ability to separate the possible flavonoids present in the EEPL with shorter time than column chromatography [15]. It is often used for preparative applications on scales from micrograms up to milligram as a pilot technique for preparative column chromatography. PTLC can be used not only for isolation but also for on-line purification of biomolecules like lipids, chlorophylls, waxes, tannins, sugars from the plant source. Recently, quercetin related flavonoids were [13] isolated from *Citrullus colocynthis (Linn.) Schrad* using silica gel mediated PTLC technique.

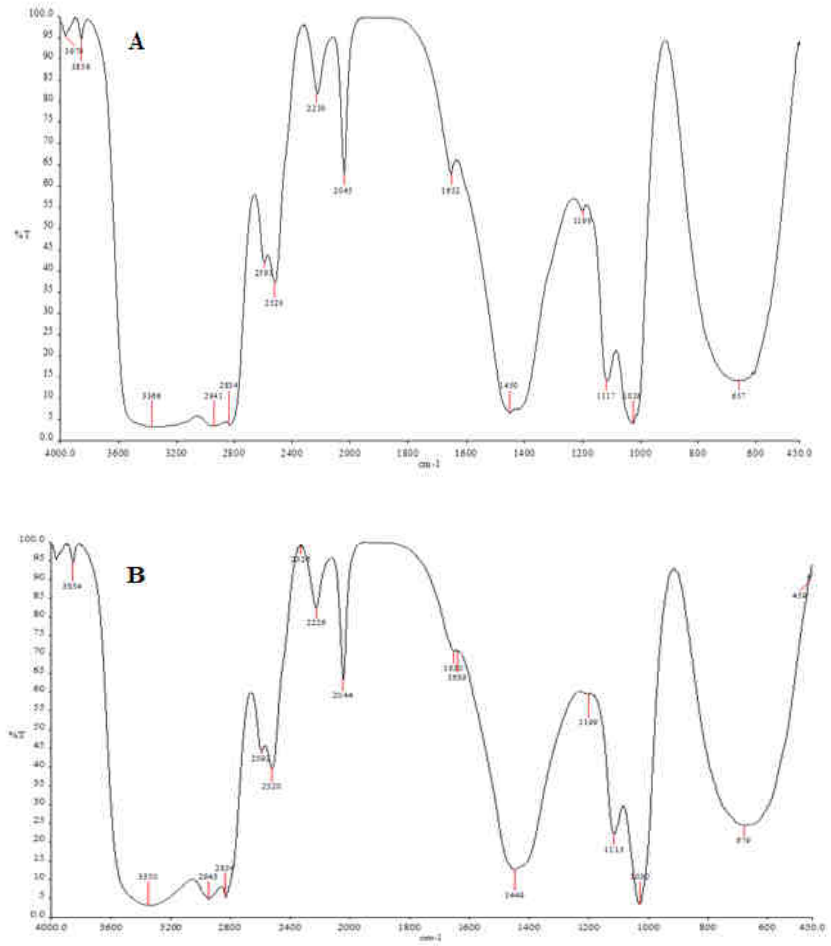
Fourier Transform Infrared (FT-IR) analysis

FT-IR plays a vital role for identification of functional group present in the naturally occurring substances and Chemical analysis of particular analyte. The functional group present in the analyte will make vibrations of specific wave numbers. FT-IR fingerprinting provides the chemical characterization of the PTLC isolates and rutin. The spectral analysis of rutin and PTLC of EEPL showed the following (Fig. 1) wave numbers with functional groups: 1450/1449 cm^{-1} indicating C-H bend stretching alkanes, 3366/3350 cm^{-1} indicating OH stretched phenols , 2833/2834 cm^{-1}indicating H-C=0 stretched aldehydes, 2941/2943 cm^{-1}indicates C-H stretched alkanes, 2230/2326 cm^{-1}indicates C(triple bond) N stretched nitriles, 1652/ 1650 cm^{-1} indicating conjugated dye and 679/657 indicating =C−H out of plane (loop) bending or aromatic mono substituted benzene (C-H) bond [17], 1030/ 1026 cm^{-1} indicatingC-N stretched aliphatic amines. Fig 3 shows the possible tentative functional group and their corresponding wave numbers, which indicated presence of alkanes, alkenes, amines and nitriles probably in different amount. The fingerprint also proved the presence of OH stretched phenol, conjugated dye and monosubstituted benzene, which are common functional groups for all the flavanoid reported in plant world. Recently, FT-IR associated KBr pellet method [18] was used, for the determination of flavonoids from the ethyl acetate, methanol and water extract of *Uncaria gambir*.

HPLC-DAD-ESI/ MS analysis

The HPLC-DAD- ESI/MS analysis further confirmed presence of flavonoids in PTLC isolate. Liquid chromatography (LC) coupled to mass spectrometry (MS) has been demonstrated to be a powerful tool for the identification of natural products in crude plant extracts owing to their soft ionization which favours the analysis of flavonoids, in addition to its high sensitivity and specificity. The purpose of this research was to identify possible flavonoids present in *Polyalthia Longifolia* leaves. The molecular masses of flavonoids were assigned by ESI/MS and subsequent structure characterization

was carried out, purely based on Knowledge acquired from other studies. Negative ion mode was selected because previous reports [19] suggested that negative mode was more sensitive than positive mode for identification of flavonoids. The compounds finally identified in PTLC isolates (Fig. 2) were Rutin: 609.2 M/H^-, Chrysin [20] : 255.2 M/H^-, Daidzein-related isome [21] :253.2 $M/H^-$and unknown: 243.2 M/H^-. Generally during the usage of APCI or ESI mass spectrum for the analysis of chemical mixture, the chemical structure can be converted as fragmented ions from the original chemical backbone. Hence the flavonoid can easily undergo the fragmentation of ions from the parent ion due to its high sugar moiety. It has been [22] carried out LC/MS analysis on several flavonoid glycosides has revealed the fragmentation mode by fast atom bombardment method. It has been [23] has successfully explained the fragmentation pattern of rutin obtained from *Ginkgo biloba* oral capsules. In this mechanism rutin (m/z 609.12) gave rise to intense ions at *m/z* 301 and m/z 179 corresponding to the loss of the rutinose unit and subsequent retrocyclization of the C-ring (between bonds 1 and 2) leading to the A^- fragment. The present results are in accordance with the above said report. The Parent ion m/z 253.2 and fragment ion m/z 227.1 (Fig.2 B), correlated well with the previous results [21]. The overall results have proved that the EEPL possess appreciable quantity of flavonoids. It has beenreported [24] that the presence of new clerodane-type-hydroxybutenolide diterpene from the bark of *polyalthia longifolia var. angustifolia*. LC/MS and NMR analysis of ethanolic extract of *Polyalthia longifolia var. pendula* revealed the presence of quercetin, quercetin-3-O-β-glucopyranoside, kaempferol-3-O-α-rhamnopyranosyl-(1→6)-β-galactopyranoside, kaempferol-3-O-α-rhamnopyranosyl-(1→6)-β-glucopyranoside, rutin and allantoin [25]. However this is the first detailed chromatographic analysis of flavonoids in the EEPL that need to be investigated further.



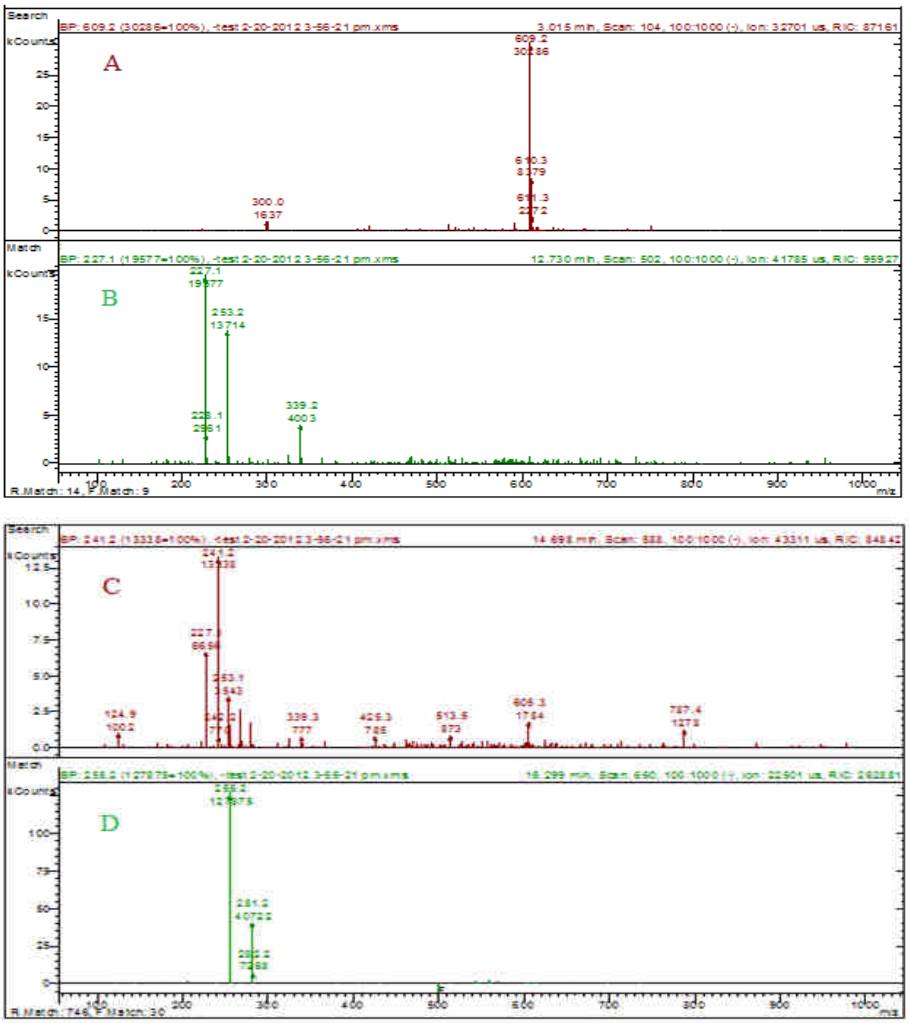
A-Rutin**; B-** PTLC isolate

Fig. 1: FT-IR spectrum of reference standard rutin and PTLC isolate of EEPL

Antibacterial Study

The antibacterial activity of EEPL and PTLC isolate against *Escherichia coli*, *Klebsiella aerogenes*, *Proteus vulgaris, Pseudomonas aeruginosa, Serratia marcescenes, Salmonella typhi, Shigella flexneri, Bacillus Subtillis, Streptococcus pyogenes* and *Staphylococcus aureus* are shown in Table 1.

Both Gram positive and Gram negative bacteria used in the present study were very sensitive to the EEPL. PTLC isolate showed appreciable level of potency against growth of *E.coli*, *Klebsilla aerogenes*, *Pseudomonas aerogenes*, *Salmonella typhi, Shigella flexneri* and *Staphylococci aureus.* This could be attributed to the presence of flavonoids such as Rutin, Chrysin and Daidzein-related isomers. The overall results suggest that EEPL has significant antimicrobial potency, when compared with amikacin. In recent years, it has been established that the plants which naturally synthesize and accumulate some secondary metabolites like alkaloids, flavonoids, glycosides, tannins and volatile oils, posses anti microbial activity. It has also been reported [26] that flavonoids posses anti bacterial, anti viral and anti fungal activities against various pathogenic strains. It has been reported [27] that methanol, acetone and 1, 4-dioxan fractions of *Polyalthial longifolia* leaves have antibacterial activity against most of the gram positive bacterial strains. It has been proved [28] that the methanolic, ethanolic, ethylacetate and petroleum ether crude extract of *Polyalthia Longifolia* leaves posses antibacterial activity. Methanolicextracts of leaves, stem, twigs, green berries, flowers, roots, root-wood and root-bark [29] of *Polyalthia longifolia var. pendula,* were tested for their antibacterial and antifungal potentials.



A-Rutin, **B**- Daidzein-related isomer, **C**- Chrysin, **D**-Unknown

Fig. 2: HPLC-DAD- ESI/MS spectrum of PTLC isolate of EEPL.

Table 1: Antibacterial potency of EEPL, PTLC isolates, Rutin and Amikacin

Bacterial sp.,	**Antibacterial Activity**					**Zone of Diameter (mm)***				
	Antibiotic (Amikacin)	**EEPL**	**Rutin**	**PTLC isolate**	**Control (Ethanol)**	**Antibiotic (Amikacin)**	**EEPL**	**Rutin**	**PTLC isolate**	**Control (Ethanol)**
Escherichia coli	+	+	+	+	*R*	18 ±0.23	16±0.56	12±0.44	7 ±1.18	R
Klebsiella aerogenes	+	+	+	+	R	17±0.67	17±0.20	21 ± 0.52	6±0.67	R
Proteus Vulgaries	+	+	+	*R*	*R*	19±0.55	14 ±1.18	20 ± 0.12	R	R
Pseudomonas aeruginosa	+	+	+	+	R	17± 0.23	15 ±0.99	17±1.04	11±4.01	R
Serratia marcescenes	+	+	+	*R*	*R*	17 ± 0.11	18 ±0.21	20±2.07	R	R
Salmonella typhi	+	+	+	+	*R*	22 ± 3.09	20 ±1.45	15±0.11	9±0.3	R
Shigella flexneri	+	+	+	+	*R*	18 ±0.67	16 ± 0.56	12 ±4.13	7±1.54	R
Bacillus Subtillis	+	+	+	*R*	*R*	20 ± 0.78	17 ± 2.87	13±0.65	R	R
Streptococcus pyogenes	+	+	+	*R*	*R*	18 ± 0.54	22 ±0.51	15±1.98	R	R
Staphylococcus aureus	+	+	+	+	*R*	18 ±0.08	R	11±3.09	6±3.7	R

+ Growth inhibition; R –Resistance; *Mean ± SEM, n = 3, zone includes disc diameter 7mm; Rutin/Amikacin/ PTLC isolate/EEPL (100 µg/disc)

Anti radical activity against ABTS radical

Although various assays were reported to estimate the free radical scavenging activity [33], one common method is ABTS. When ABTS encounters a proton- donating substance such as an antioxidant, the radical would be scavenged and the absorbance is reduced. Based on the principle, the antioxidant activity of the test compound can be expressed as its ability in scavenging the ABTS radical. ABTS assay was performed for EEPL, PTLC isolate and rutin. Fig. 3 shows percentage inhibition for EEPL, PTLC isolate and Rutin at 734 nm, which depicts the scavenging activity of the ABTS radicals. The inhibitory constant (IC_{50}) of EEPL, PTLC isolate and rutin was found to be 10.91±0.080µg/ml, 30.08± 0.071µg/ml and 14.67±0.023µg/ml respectively. Recently it has been reported [6] that the antioxidant potential of ethanolic leaf extracts of *Polyalthia longifolia (Sonn.) Thwaites* against DPPH assay. PTLC isolate fraction shows lower % inhibition of ABTS radicals and hence a higher IC_{50}.This could be attributed to the limited rutin related flavonoid entities present in the PTLC isolate. These results suggest that EEPL might contain other unidentified flavonoids that enriched its anti-radical activity. Further studies are required to evaluate the structure of phytoconstituents present in EEPL.

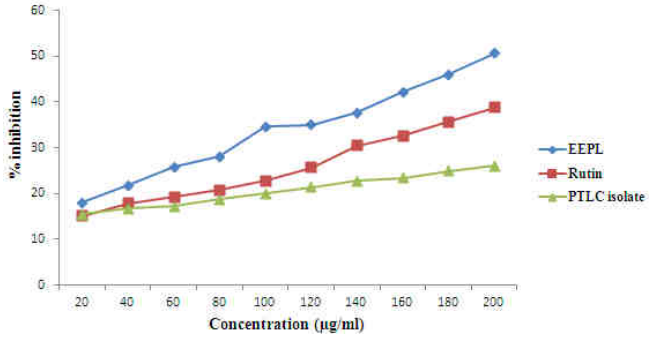


Fig. 3: Anti radical activity of EEPL, PTLC isolate and Rutin against ABTS radical

Cytoxicity activity against A549 Human lung cancer cell line

Cancer is a public health problem all over the world [30]. Large number of plants and their isolated constituents has been shown to possess potential anti-cancer activity. EEPL, PTLC isolate and rutin showed (Fig. 4) *in vitro* cytotoxicity against A549 human lung cancer cell line. EEPL and PTLC isolate showed better toxicity than the reference standard rutin. Cytotoxic Concentration (CTC_{50}) values of EEPL, PTLC isolate and rutin were found to be 210±5.14 µg/ml, 230±10.62 µg/ml and 270±3.001µg/ml respectively. CTC of >270 µg/ml showed 100% toxicity of the A549 lung cancer cells (Fig. 5).

There was no much difference in CTC_{50} of EEPL and PTLC isolate. This could be attributed by rutin related compounds present in the EEPL. An interesting observation made in the present study is that almost the PTLC isolate fraction and EEPL were on port contribute other with respect to their Cytoxicity potential, hence the PTLC isolate fraction compounds like Daidzin, Rutin, Chyrisin needs to be explored for their anti-cancer potential. Previous report has revealed the presence of several cytotoxic compounds like halimane diterpene, 3β, 5β, 16α-trihydroxyhalima-13(14)-en-15, 16-olide and a new oxoprotoberberine alkaloid, (−)-8-oxopolyalthiaine from *Polyalthia longifolia* var. *pendula* [31]. Similarly it has been screened [32] the ethanolic extract of stem bark of *Polyalthia longifolia* Benth and Hook (Annonaceae) for its *in vitro* and *in vivo* antitumor activity. In this study, *Polyalthia longifolia* extract showed concentration-dependent cytotoxicity in Ehrlich's Ascites Carcinoma (EAC), Dalton's ascites lymphoma (DLA), HeLa and MCF-7 cells with IC50 values of 45.77 and 52.52, 25.24 and 50.49 µg/ml respectively. There are no earlier reports on anti-cancer activity of *Polyalthia longifolia* leaves extract or its isolated compounds against A549 lung cancer cell line. Therefore, anti-cancer/antitumor effect of crude extract of this plant needs to be evaluated in order to discover potential anti-cancer agents.

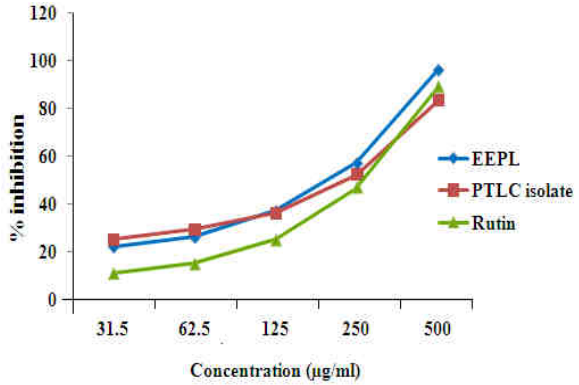


Fig. 4: Cytotoxicity activity against A549 Human Lung Carcinoma cell line

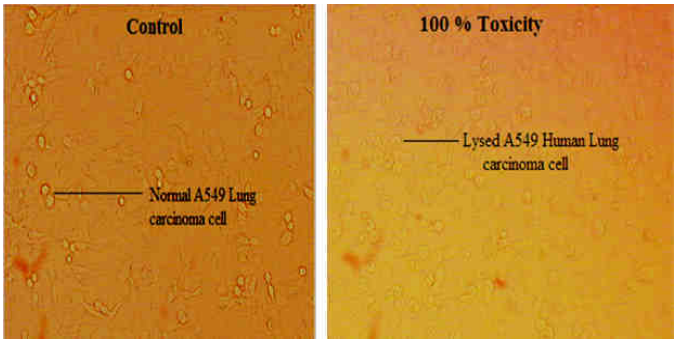


Fig. 5: Lysed and normal A549 human lung cancer cells

CONCLUSION

In the present study, Rutin related compounds present in the EEPL were identified by TLC and isolation was done by the PTLC, which was further confirmed in RP- HPLC, FT-IR, and by comparing with the reference standards (Rutin). Chromatogram of HPLC-DAD-ESI/MS revealed the presence of an unknown flavonoid in the PTLC of EEPL and hence further NMR studies are required to predict the flavonoid structure. Antibacterial study suggests that EEPL has potent antibacterial activity than rutin and Amikacin. PTLC isolate reveals appreciable antibacterial potency however this was found to be lesser when compared to EEPL. This could be attributed to limited flavanoid compounds in the isolate. Anti radical study suggests that EEPL posses potent anti radical effect than rutin. PTLC isolate reveals lower anti-radical activity against ABTS radicals. This anti-radical activity could be attributed to rutin related flavonoids in the PTLC. Cytotoxic study suggests that PTLC isolate has potent cytotoxic effect than rutin and an equivocal activity when compared to EEPL extract, against A-549 lung carcinoma cells. This result strongly suggests that rutin related compounds in the EEPL could have contributed to its cytotoxic potential. The presence of flavonoids in the EEPL may contribute to many medical applications, and hence EEPL could be used as a nutraceutical product.

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