Phytoconstituents from the leaves of *Ageratina adenophora* (Sprengel) and their pharmacological activities

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ABSTRACT

The main objective of the current study was intended for isolation of bioactive compounds from the leaves of *Ageratina adenophora* (Sprengel) and their characterization. Crude extracts of *A.adenophora* were prepared using various solvents such as petroleum ether, chloroform, ethyl acetate, and methanol. The chloroform extract showed the presence of higher flavonoid content when compared with other solvent extracts. The chloroform extract was subjected to column chromatography and the eluted fractions were checked by thin layer chromatographywith different solvent ratio. Single compound was isolated which was subjected to spectral characterization. The characterization techniques confirmed that the isolated compound wasQuercetin. Petroleum ether and methanol extracts were subjected tofree radical scavenging activitieswhich suggests that petroleum ether and methanol extracts exhibited better activity and actsaneffective source of antioxidants and anti-cancer agents.

Keywords: Anti-cancer activity, Antioxidant activity, DPPH, Flavonoids, NMR, Quercetin.

INTRODUCTION

AgeratinaAdenophora (Sprengel) (*A.adenophora)*King & Robinson (synonym: Eupatorium adenophorum) is a perennial herbaceous invasive plant native to Mexico and Costa Rica [1]. The plant was introduced in India in the 19th century and since then widely proliferated in the hilly terrain of northern, north eastern himalayan region and other lower hilly regions of southern India[2].However, utilization of the weed gives reward over effortless control processes. As a natural plant, *A.adenophora* has some bioactive components. Anti-oxidant properties and anti-bacterial bioactivities of flavonoids 4'-methyl quercetagetin 7-O-(6''-O-E-caffeoyl glucopyranoside), quercetagetin 7-O-(6''-O-acetyl-b-D-glucopy-ranoside), and eupalitin 3-O-bD-galactopyranoside have been reported[3]. Previous bioassays have shown that caffeic acid has considerable anti-oxidative properties with both radical-scavenging and metal-chelating abilities[4].Eupalitin is a type of flavonoid that is an effective component of some medicines [5]. Mostly in nature; insects and microorganisms hardly ever affect *A. adenophora*. This suggests that the plant would have a rich defence system that includes bioactive chemicals, some of which may also have pharmaceutical value. This plant was previously mentioned in certain literature as being used in Nigeria and India as a traditional or folk medicine to cure a variety of human

ailments, such as fever, diabetes, inflammation [6, 7]. It has previously been reported that this plant contains various terpenoids, flavonoids, phenylpropanoids, coumarins, sterols, and alkaloids [8-10].with part of them exhibiting phytotoxic[11],allelopathic[12,13],antifungal[14]and antifeedant[15]activities.In recent study, certain bioactive substances from the aerial parts of this plant were also identified, including phytotoxic phenolics [16], antifungal monoterpenes [17], and antibacterial quinic acid derivatives [18].We further acquired as part of the effort to elucidate those potentially new and bioactive compounds in the aerial sections of *A. adenophora*. We discuss these compounds isolation, structural elucidation, in vitro antibacterial activity, and cytotoxicity toward different types of human cancer cell lines.

Materials and methods

Plant materials

The fresh leaves of*A.adenophora* were brought from Valparai Hills (10.3270° N, 76.9554° E), Tamil Nadu, India between the periods of February 2021. The plant specimen was identified andauthenticated by the Department of Botany, and the voucher specimen (21PCY16) was preserved in thePostgraduate Department of Chemistry, Nallamuthu Gounder Mahalingam College, Pollachi, Tamil Nadu, India.

Preparation of plant material

After collection and identification the fresh leaves were washed with water to remove the debris and dried in shade for two weeks and then powdered by using mixer grinder. The powdered plant material was tightly packed in air tight container for further process.

Chemicals used

Petroleum ether, methanol, ethyl acetate, chloroform, silica gel (column 100-120 mesh), silica gel (TLC 60-120 mesh), and all chemicals used were analytical grade.

Extraction process

Powdered air-dried aerial parts of *A. adenophora* (3 kg) was extracted with petroleum etherby using soxhlet apparatus several times at room temperature until the color of the extract gets darken.In this method finely powdered leaf is packed with filter paper, which was placed in the thimble chamber of the soxhlet apparatus. After removal of petroleum ether by distillation process, the viscous concentrate was poured in Petri-dish to evaporate excess solvent. This process is done to remove fatty matters which are present in plant material. The final thick-sticky petroleum ether extract (15 g) was refrigerated using air-tight container for further analysis, after that chloroform extract was prepared by using the same plant material, same extraction process is repeated several times by using chloroform solvent. The final solvent free dark green residue chloroform extract (32 g) was refrigerated using air-tight container for further isolation process.

Likewise ethyl-acetate (2 g) and methanol (7 g) extracts were prepared and stored in refrigerator for further analysis.

Purification& Isolation by Silica Gel Column Chromatography

The chloroform extract was subjected to silica gel column chromatography. The silica gel powder was mixed with petroleum ether and poured into a clean silica gel column. Twice the column volume of petroleum ether was used as the eluent to stabilize the column, and the bubble-free column was used as the separation column. The thick sticky chloroform extract (30 g) were dissolved in chloroformin a ratio of 4:1, and it is mixed with silica gel finally the powdered sample was taken and slowly added to the silica gel column of the above cabinet. After loading, the valve was opened to allow the sample to enter the silica gel column. Methanol andchloroformwith different ratios like [10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10] (v/v) to give sub-fractions F21 –F52. Fraction F36 single spotted compound (1.2 g) was further subjected to Alumina pre-coated preparative plates by elution with (5:5) Chloroform andmethanol to afford a compound (9 mg). The separated and purified compound was given for spectral analysis.

Anti-Cancer Activity

MTT Assay

Anticancer activity of the compound was analyzed by MTT assay in MDA-MB-231. Breastcancer cell line purchased from National Centre for Cell Science, Pune, India. MDA-MB-2315 × 10^4 cells were cultured in 96-well plate (NuncMicroWell™) in complete Dulbecco'sModified Eagle Medium (DMEM) medium and incubated for 24 h at 37°C in 5% CO_2. Following 24 h of exponential growth, cells were exposed to different concentrations (mention concentrations µg/ ml) of compound in triplicates with vehicle control (0.05%DMSO served as negative control) and positive control (Etoposide in the concentration rangeof 1—1000 µM). 24 h of post-treatment, 10µL of 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) was added in each well and incubated for 4 hat 37°C. The resultant purple formazan crystals were solubilized in 150µL of DMSO. Thecolor development was recorded at a test wavelength of 570 nm and a reference wavelengthof 630 nm (Varioskan™ Flash Multimode Reader, Thermo Scientific, Switzerland). Thecytotoxicity in percentage was derived against control cells as 100%. The Cytotoxicity measurements were depicted in the dose-response curve. The IC_{50} value (half-maximal inhibition concentration) was determined using GraphPad Prism software version 9.1.0.221using nonlinear regression (curve fit). [19]

DPPH Assay:

Reagent preparation:

0.1mM DPPH solution was prepared by dissolving 4mg of DPPH in 100ml of ethanol.

Working procedure:

Different volumes of sample extracts with ethanol were made up to 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture was incubated in dark condition at room temperature for 20 min. After 20 min, the absorbance of the mixture was read at 517 nm by UV-Vis Spectrophotometer. 3ml of DPPH was taken as control.

$$\% \text{ RSA} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100 \quad (1)$$

Where, RSA is the Radical Scavenging Activity; Abs control is the absorbance of DPPH radical + ethanol; Abs sample is the absorbance of DPPH radical + sample extract. [20]

ABTSAssay:

The working solution of ABTS•+ radical was made by reacting ABTS (9.5 mL, 7 mM) with potassium persulfate (245 µL, 100 mM), and raising the volume to 10 mL with distilled water. The solution was kept in the dark at room temperature for 18 h, and then diluted with potassium phosphate buffer (0.1 M, pH 7.4) to an absorbance of 0.70 (±0.02) at 734 nm. Samples were prepared in methanol with dilutions 50–1250 µg/mL. A sample (10 µL) was placed in a test tube and mixed thoroughly with 2.99 mL ABTS radical working solution. Absorbance of the resulting clear mixture was recorded at 734 nm.

The percent antioxidant activity of the sample was determined using the following formula:

$$\%\text{Antioxidant activity} = [(A_c - A_s)/A_c] \times 100 \quad (2)$$

Where A_c and A_s are the absorbance of the control and sample, respectively. The control was prepared by adding 10 µL of methanol in place of the sample. [21]

Results

The petroleum ether and chloroform soluble fractions of the methanol extract of the aerial parts of *A.adenophora* were isolated and purified by repeated column chromatography (CC) and TLC to afford one known compound. By comparing their NMR and MS data with those reported in literatures, the known compound were identified as Quercetin (Fig 1).

Compoundwas isolated as a yellow amorphous powderand determined to have amolecular formula of $C_{15}H_{10}O_7$, its melting point of 315°C,The IR spectrum exhibits characteristic absorption bands at 3454 $cm^{-1}$for OH group and 1643 $cm^{-1}$for α β-unsaturated carbonyl group, absorption at 1514 cm^{-1} for C=C group and at 1 cm^{-1}267 for C-O group (Fig 2). The ^{1}H NMR spectrum (400 MHz, $CDCl_3$ -d6) indicated a 3, 5, 7, 3′, 4′-pentaoxygenated flavone. The spectrum exhibited a characteristic proton signal at δ 12.48(1H, br.s), 10.81 (1H, br.s), 9.59 (1H, br.s), 9.36 (1H, br.s), and 9.30 (1H, br.s) (Fig 3), corresponding to five free hydroxyl at C-3, C-5, -7, C-3' and C-4' carbon atoms. The aromatic protons exhibited one ABX coupling system at δ 7.54 (1H, dd, J = 7. 8, 2.0 Hz) for H-6′, δ 7.66 (1H, d, J =2.0 Hz) for H- 2′, δ 6.87 (1H, d, J = 7.8

Hz) for H-5′. The other AX coupling system at δ 6.17 (1H, d, J =2.0 Hz) and δ 6.39 (1H, d, J = 2.0 Hz) was assigned to H-6 and H-8 protons, respectively. The ^{13}C NMR spectrum (100 MHz, $CDCl_3$ -d6) gave 15 carbon signals which indicated the presence of 15 carbon atoms due to the flavonolskeleton (Fig 4). By comparison with the ^{13}C NMR spectral data of quercetin, the ^{13}C NMR spectral features were very similar to those of quercetin. In considering the 3′, 4′-dihydroxyl flavone system, studies revealed that the δC value for 4′ appeared further downfield than that of 3′, therefore, the δC 145.00 and 147.70 were assigned to C-3′and C-4′, respectively (Fig 5).

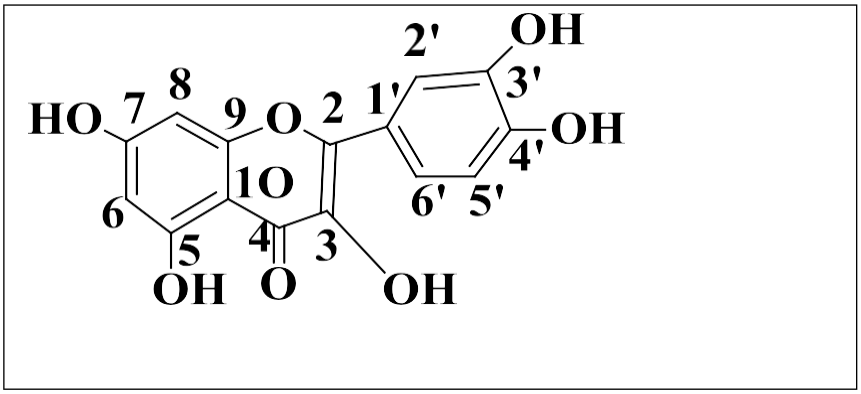


Fig:1 Structure of Quercetin

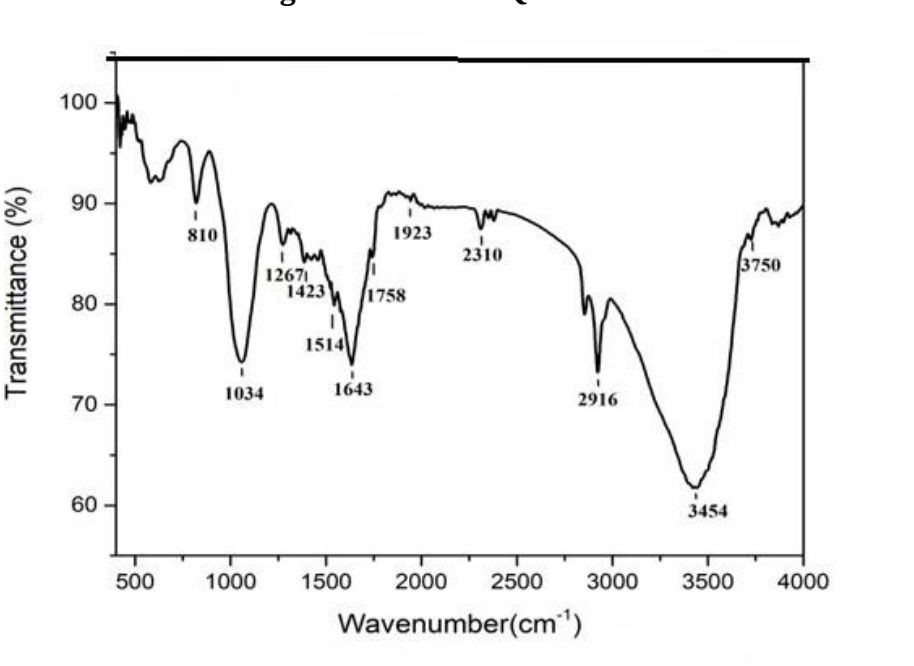


Fig:2 IR Spectrum for Quercetin

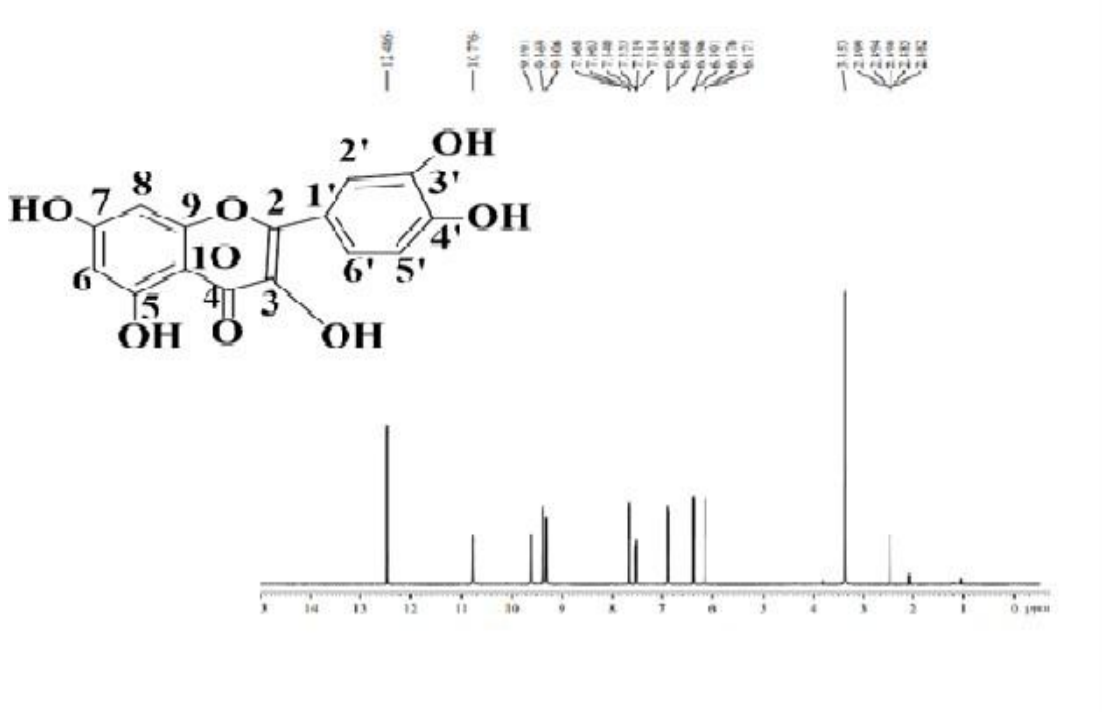


Fig:3^{1}H NMR Spectra for Quercetin in $CDCl_3$

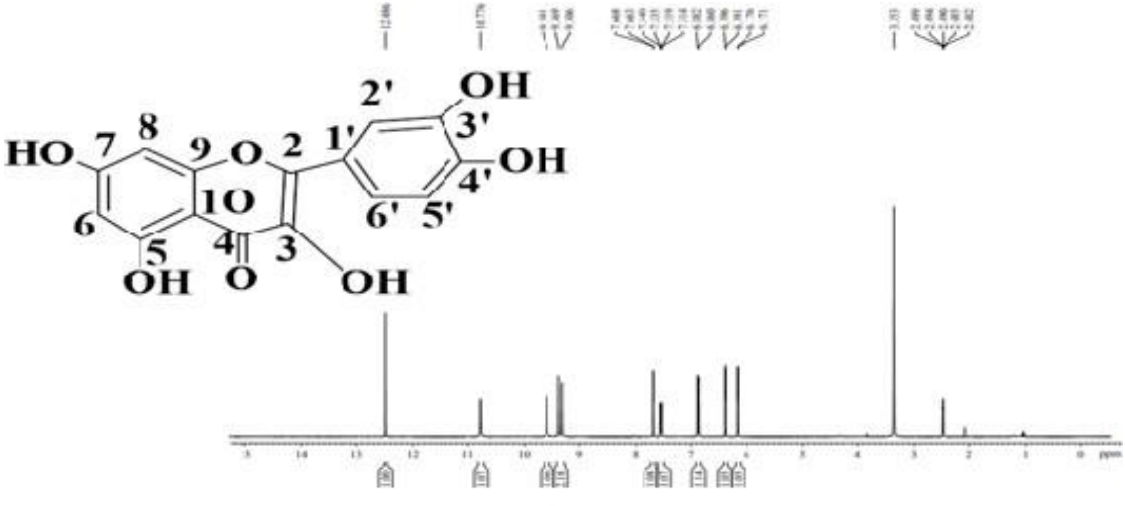


Fig:4^{13}C NMR Spectra for Quercetin in CDCl3

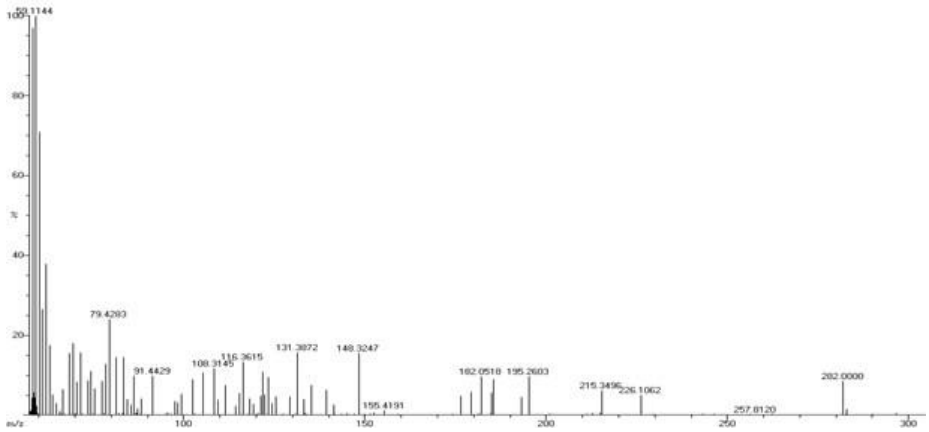


Fig:5 MassSpectra forQuercetin in $CDCl_3$

Control Treated

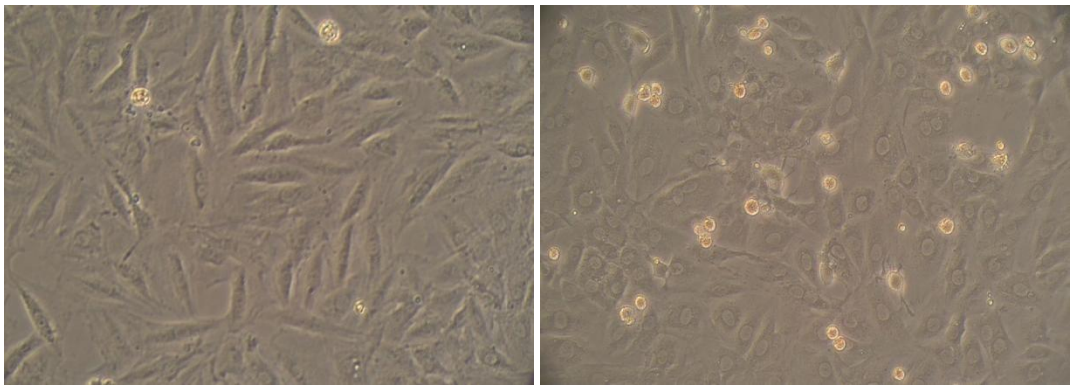
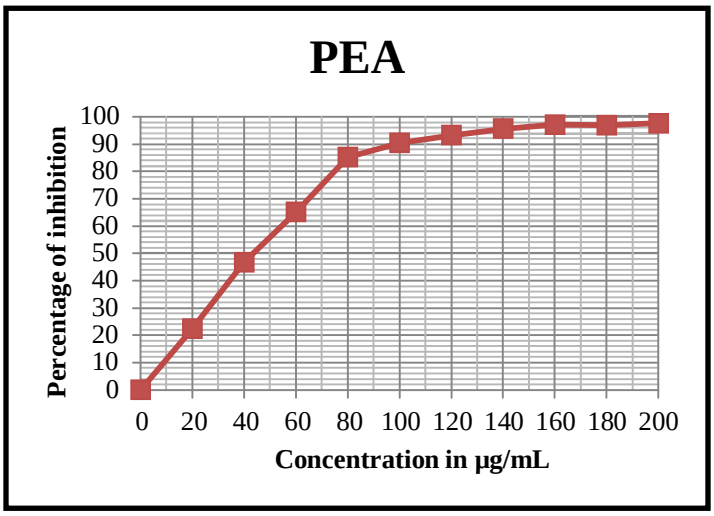


Fig: 6Anti-cancer activity of petroleum ether extract of *A.adenophora* leaves against MCF-7cell lines by MTT assay



IC_{50} =56.95 ± 5.37 µg/ml

Fig:7Anti-cancer activity of petroleum ether extract of *A.adenophora* leaves against MCF-7 cell lines by MTT assay

Control Treated

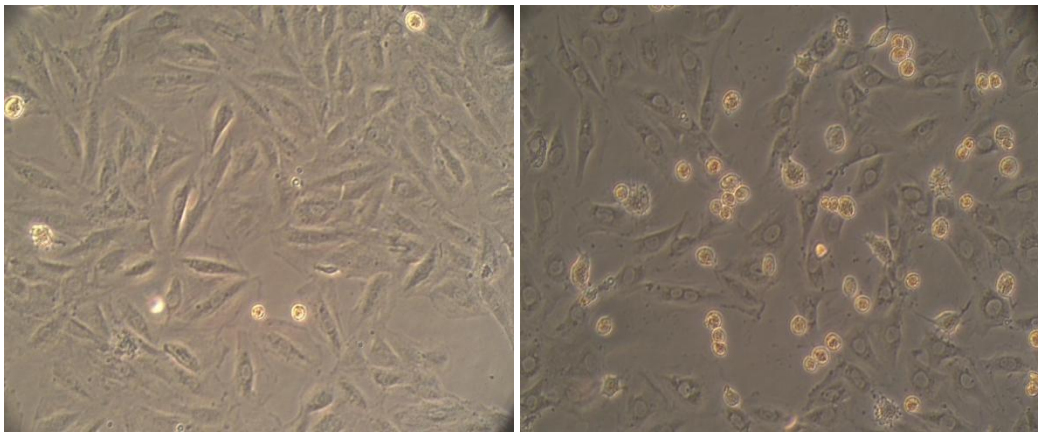
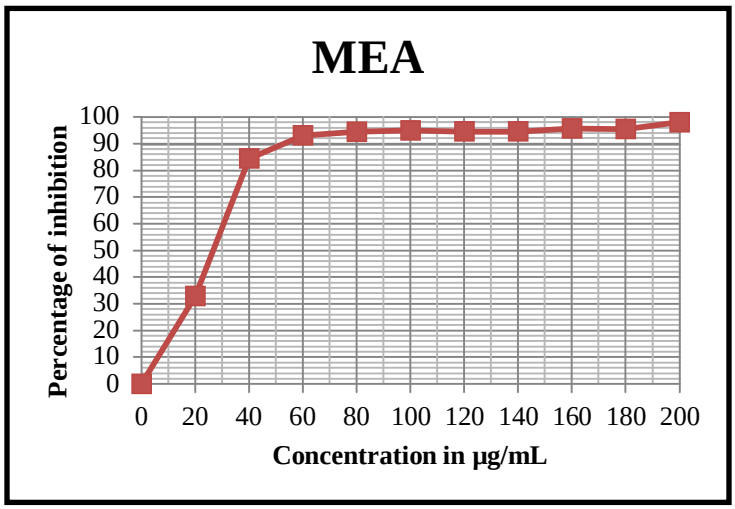


Fig: 8Anti-cancer activity of methanol extract of *A.adenophora* leaves againstMCF-7 cell lines by MTT assay



IC50 = 23.77 ± 0.39 µg/mL

Fig:9Anti-cancer activity of methanol extract of *A.adenophora* leaves against MCF-7 cell lines by MTT assay

Anti-Oxidant Activity

Table: 1 DPPH Assay for Quercetin, Methanol extract and Petroleum ether extract

Treatments	Concentration (µM)					IC_{50} (µM)
	10	**25**	**50**	**75**	**100**	
Standard (%) Ascorbic acid	10.2	18.0	35.1	73.5	89.2	**13.8**
Quercetin (%)	9.2	18.8	36.0	57.1	74.6	**66.7**
Methanol extract (%)	7.6	16.0	31.8	52.5	68.4	**73.4**
Petroleum ether extract(%)	5.2	11.6	26.3	39.7	55.8	**91.5**

Table: 2 ABTS AssayforQuercetin, methanol extract and petroleum ether extract

Treatments	Concentration (µM)					IC_{50} (µM)
	10	**25**	**50**	**75**	**100**	
Standard (%) Ascorbic acid	9.0	15.2	34.4	69.9	87.5	**14.4**
Quercetin (%)	8.6	16.0	33.8	55.0	70.3	**70.1**
Methanol extract (%)	7.0	15.7	32.5	50.2	65.3	**76.2**
Petroleum ether extract(%)	4.8	11.0	23.1	36.0	51.0	**100.5**

Discussion

Isolation of flavonoid is the core target in our research. For the first time in Western Ghats region we isolated a flavonoid quercetin and its anti-oxidant activity has been studied which is shown in table 1 and 2. Simultaneously the anticancer activity and antioxidant activity of methanol extract and petroleum ether extract of *A.adenophora* has been studied.

In current years, researchers have successively isolated many compounds from *A.adenophora*, including 1-dotriacontanol ($C_{32}H_{66}OCH_3$–$(CH_2)_3O$–CH_2OH), sitosterol($C_{29}H_{50}O$), stigmasterol ($C_{29}H_{48}O$), taraxasterylpalmitate ($C_{46}H_{80}O_2$), taraxasteryl acetate ($C_{32}H_{52}O_2$), 7-hydroxycoumarin ($C_8H_6O_3$), caryophyllene ($C_{15}H_{24}$), and other sesquiterpenoids[22]. High purity caffeic acid also isolated by using HSCCC (High speed counter current chromatography)[23]. Step-wise elution mode is successfully used in HSCCC to improve the purification of natural products and isolated five Bio-active components like caffeic acid, 40 -methyl quercetagetin 7-O-(600-O-E-caffeoylglucopyranoside), quercetagetin 7-O-(600-O-acetyl-b-D-glucopyranoside), eupalitin 3-O-b-D-galactopyranoside, and eupalitin[24]. The hepatotoxic components, including 9-oxo-10, 11-dehydroageraphorone, 10Hα-9-oxo-ageraphorone and 10Hβ-9-oxoageraphorone were discovered[25].

In-vitro Antioxidant Assays

DPPH radical has been used to evaluate antioxidants for their free radical quenching activity. The antioxidant mechanism of isolated compound quercetin was evaluated for free radical scavenging activity against DPPH. The antioxidant activity of the isolated quercetin with respect to the standard molecule (ascorbic acid) was calculated in table 1 & 2. The decrease in the absorbance of the DPPH radical with respect to the ascorbic acid is caused by antioxidant activity through the reaction between antioxidant quercetin and free radical results in the scavenging of radical by hydrogen donation[26]. Previous reports have also mentioned the efficient antioxidant activity of the compound quercetin[27, 28]thus the isolated compound quercetin showed potential antioxidant activity.

Tables 1 & 2 illustrate the results of the DPPH and ABTs assays carried out to assess methanol and petroleum ether extracts antioxidant abilities. The antioxidant ability of *A.adenophora* extracts was compared with commercial standard ascorbic acid. With IC_{50} values of 73.4 mg/ml for methanol extract followed by IC_{50} values of 91.5 mg/ml for petroleum ether extract and 13.8 mg/mL for commercial standard ascorbic acid, methanol extract demonstrated the strongest radical scavenging activity against DPPH radical and ABTs assays which showed the IC_{50} value of methanol extract is 76.2 mg/mL followed by IC_{50} value of petroleum ether extract is 100.5 mg/mL for standard Ascorbic acid is 14.4 μg/mL respectively.The results indicate that the methanol extract exhibited the strongest scavenging activities as compared to those of Petroleum ether extract.

In Previous study, the methanol extract of *A. adenophora* leaves were evaluated for their in-vitro antioxidant activity based on DPPH (2,2-diphenyl1-picrylhydrazy) and hydrogen peroxide radical scavenging activity. In the scavenging assays, the extract showed a significant DPPH activity as compared to the standard butylated hydroxyl toluene (IC_{50} for *A. adenophora* was 92.791 and for butylated hydroxyl toluene was 68.043). Similarly, they also showed a comparable H_2O_2 scavenging activity as compared to the standard ascorbic acid (*A. adenophora* = 79.32%, Ascorbic acid = 86.84%) [29]

Anticancer activity

The results of the anti-cancer potential of methanol and petroleum ether extracts were presented in this study in Fig 6, 7, 8, & 9. By using the MTT assay, it was determined whether the Jurkat E6.1 cancer cells were still viable after being incubated with various quantities of the extracts the leaves of *A.adenophora* (10–50 g/mL). Our findings revealed that high cytotoxicity was considerably and concentration-dependently induced by the extracts. The extracts had the strongest anticancer properties against the Jurkat E6.1 cell line and Etoposide with IC_{50} values comprised of 56.95 ± 5.37 µg/ml for petroleum ether extract followed by IC_{50} = 23.77 ± 0.39 µg/mL for methanol extractand 30 µg/mL respectively. The outcomes demonstrated the methanol extract of *A.adenophora* could be used as a potential source of substitute medicine for treating cancer.

There are limited results on the anticancer activity of extracts of *A.adenophora* leaves. It has been reported that, when the crude 9-oxo10, 11- dehydroageraphorone (euptox A), a cadeninesesquiterpene from *A. adenophora*, was tested for its cytotoxicity to human lung cancer A549 cells, Hela cells & Hep-2 cells in-vitro by 4, 5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, it was found that euptox A had significant antitumor activity against the three tumor cell lines in-vitro in a dose-dependent manner. When the concentration of euptox A was at 500 µg/mL, the percent inhibition of human lung cancer A549 cells, Hela cells, and Hep-2 cells were 76.42, 68.30 and 79.05%, respectively while the 50% inhibitory concentration (IC_{50}) of euptox A for the three tumor cell lines were 369, 401 and 427 µg/mL (A549, Hela and Hep-2 cells, respectively). This study suggested that euptox A may be considered as a potential candidate for developing a novel low toxicity antitumor agent [30].

Conclusion

From the present work, the compound quercetin has been isolated successfully from the chloroform extract leaves of *A.adenophora*. It is found that solvent plays the main role in the extraction of plant constituents. The isolated constituent of quercetin was identified through FT-IR, NMR and mass spectroscopy. The isolated compound quercetin showed increased antioxidant activity with an increase in the treated concentrations. The in-vitro anti- cancer activity and anti-oxidant activities results suggested that the petroleum etherand methanol extracts of *A. adenophora* might serve as antioxidants and potent anti-cancer activity. Whereas, the methanol extract showed a stronger cytotoxicity than petroleum ether extract.

Disclosure statement

No potential conflict of interest was reported by the authors.

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