

Total Polyphenolic Content, Antioxidant Activity and Chromatographic Profiling of Extracts of *Ardissia solanacea*

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ABSTRACT

Background: The current study focuses on the preliminary phytochemical analysis, antioxidant potential and identification of phytochemicals in *Ardissia solanacea* leaves extract using chromatography and HR-MS. **Materials and Methods:** The occurrence of phytoconstituents in different extracts of *Ardissia solanacea* were assessed by standard procedures. *In vitro* antioxidant potential was determined by Hydrogen peroxide and DPPH method. The extract was fractionated by Flash Chromatography. The fractions were analysed using HPTLC and further analyzed by HR-MS method to detect and characterize the active phytoconstituents present in it. **Results:** The highest concentration of flavonoids (371.91 ± 0.167 mg/g of Quercetin Equivalent), phenolics (10.138 ± 0.010 mg/g of Gallic acid Equivalent) and tannins (148.23 ± 0.510 mg/g of Tannic acid Equivalent) was observed in the ethanol extract. The antioxidant activity by DPPH and H_2O_2 assay demonstrated that the ethanolic extract (AsEt) showed highest anti-oxidant potential (74.21% and 83.16% respectively). The ASET was fractionated by using Flash chromatography. As a result, Out of 11 fractions, one fraction namely AsEt3 was selected further based on HPTLC results. The HR-MS analysis of ASET3 indicated presence of thirteen compounds wherein bergapten was predominantly identified. **Conclusion:** It was revealed that the leaves of *Ardissia solanacea* possesses high antioxidant potential. The known phytoconstituents namely quercetin and taxifolin along with one unknown compound were identified through the HR-MS study. Using mass library data, we report herein 2 flavonoids and 1 phenolic in the AsEt extract.

Keywords: *Ardissia solanacea*, Antioxidant activity, Flash chromatography, HR-MS analysis.

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INTRODUCTION

The herbal products have gained popularity as a potential therapeutic agents from the past decade. In developed countries, approximately 10-50% of people rely on herbal products for the treatment of various diseases. According to the Experts, the Global Herbal Medicine Market size is expected to reach \$ 39.52 bn. by the end of year 2026. The drastic increase in the use of herbal products is due to better tolerance as compared to synthetic drugs. Because of the natural origin, the herbal products are considered to be safer than synthetic products. The herbal medicines are prepared by using diverse portions of the plant like leaves, bark, seeds, oil, berries, and roots. However, the herbal drugs does not deliver the intended standards for purity or dosage due to lack systematic standardization, inadequate scientific evidence of their safety and efficacy. The lack of standardization of raw material, processing methods, and of final product; dosage preparation and non-availability of pre-set criteria's for high quality control are the major constraints for herbal product usage. Currently

measures are being undertaken to regularize the guidelines of herbal medicines to safeguard quality, safety, efficacy by using modern practices, applying suitable standards and GMP.^[1-6]

Ardissia solanacea (Primulaceae) known as *Shoebutton ardisia* is set up in all regions of India, Pakistan, Sri Lanka and Western China.^[7] From ancient times, the various parts of the plant such as leaves, fruit were used to treat several diseases because of rich content of phytoconstituents i.e. flavonoids, phenolics, alkaloids, etc.^[8] The fruits can be used to treat diarrhoea and dysentery,^[8] possesses stomachic, stimulant, astringent, diuretic property^[9] antidiabetic,^[10,11] antibacterial, antimicrobial,^[12] antioxidant,^[12] antispermatogenic and antisteridogenic,^[13] anti-inflammatory, antipyretic.^[14] Leaves possesses antibacterial, antioxidant, hepatoprotective, anti-inflammatory, insect antifeeding properties,^[15,16] anxiolytic, sedative, analgesic.^[17,18]

Thorough literature survey indicates that the plant has been explored for different pharmacological activities. However there are no reports of any study on the wound healing potential of the leaves. Also not much attention has being for the identification of possible phytoconstituents accountable for its pharmacological properties. Hence in the present study, the leaves extract of this plant was studied for presence of different phytoconstituents and their possible structures were identified by using different



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databases from massbank, Knapsack, metabolomics, metabolights etc.

MATERIALS AND METHODS

Chemicals

The Quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and gallic acid have been procured from Sigma-Aldrich, USA. Folin-Ciocalteu reagent was purchased from Merck, Germany. All other chemicals and solvents used were of analytical grade and purchased locally.

Collection and Preparation of Plant Material

Fresh leaves of *Ardissia solanacea* were obtained from Tamil Nadu and authentication of the material was done at Sri Venkateswara University, Tirupati, wide Voucher no. 0663 dated 01/09/2017. The leaves were cleaned properly with water and dried up beneath the shadow at room temperature for one week. The dried material were powdered with mixer and set aside in sealed vessel in a cool, dark and dry place for added usage.

Standardization of plant material

The leaves possess dark green color, alternate, pinnate venation and obtuse apex.

Ash Analysis

The ash analysis was performed by the standard procedure explained by Kokate *et al.*^[19]

Solvent Extraction

The fatty material from the powdered sample was separated by extraction with petroleum ether. The Soxhlet extraction method was used to extract sample with solvents (ethyl acetate, ethanol, methanol, hydroalcoholic and water as the solvents). The concentrated extracts were obtained by using rotary vacuum evaporator and dried to obtain final extracts. The % yield of the extract was calculated.

Primary Phytochemical Assessment

The occurrence of numerous phytoconstituents in all the extracts were analysed by following standard procedures for the preliminary phytochemical screening.^[20]

Quantification of Phytochemicals

Determination of Phenolics

The improved Folin-Ciocalteu method was used to define the total phenolic content of the extracts.^[21] In this method, five ml of Folin-Ciocalteu reagent (formerly diluted with distilled water 1:10 v/v) was added to the 1 mg/ml extract and 4 mL (75 g/L) of sodium carbonate. The solution was shaken vigorously for about 15 sec, then kept undisturbed at 40°C for 30 min to acquire the color. The absorbance was recorded at 765 nm using

spectrophotometer. From the calibration curve, the equation obtained: $y = 0.0368x - 0.0337$ $R^2 = 0.9981$, was used to calculate the total phenolic content in terms of mg/g gallic acid equivalent of dry extract sample, where x is the absorbance and y is the Gallic Acid Equivalent (GAE).

Assessment of total flavonoid content (TFC) by aluminum chloride colorimetric method

TFC in crude extracts was estimated by the stated procedure of Ordonez *et al.*^[22] According to this method, the 1mg/mL quercetin stock solution was prepared in methanol and then dilutions were carried out to obtain the concentration in the series of 50-250 µg/mL. In the test tube, 0.5 mL of 2% AlCl₃ ethanolic solution was mixed with the diluted standard solutions of quercetin or plant extracts (0.5 mL) of different concentration. The mixture was set aside for 60 min at room temperature. The absorbance was recorded at 420 nm against blank using Double beam UV-Vis spectrophotometer. The quercetin calibration curve was used to compute the quantity of flavonoid present in extracts by using linear regression equation. The flavonoid concentration was described by way of mean ± SD ($n=3$) and stated as mg/g of Quercetin Equivalent (QE) of dry extract.

Assessment of free radical scavenging activity by DPPH method

The free radical scavenging activity of various crude extracts of *Ardissia solanacea* leaves and of standard BHT was assessed as per stated method.^[23] The dilutions of different crude extracts or standard BHT were done to get the concentration in the series of 20-100 µg/mL. 2 ml of 1.0 mmol/L DPPH solution was mixed with one ml of various extracts or standard BHT in each one of the test tube. The solutions were quickly mixed and set aside at 37°C for 30 min in dark. The absorbance was recorded at 517 nm by using UV-vis-spectrophotometer. The percent free radical scavenging activity of tested extracts and positive control BHT was calculated by using the following formula:

Free radical scavenging activity % = $\frac{Ac - As}{Ac} * 100$

Where Ac=Absorbance of control at 517 nm and As=Absorbance of sample.

The IC₅₀ (concentration of sample required to scavenge 50% of DPPH free radical) was determined from the curve of percent inhibitions plotted against the respective concentration.

Hydrogen peroxide (H₂O₂) scavenging assay

The reported method of Dephour *et al.* was considered to evaluate the H₂O₂ scavenging ability of the *Ardissia solanacea*.^[23] The dilution of crude extracts and standard BHT were done to obtain the concentrations in the series of 20-100 µg/mL (20, 40, 60, 80, 100 µg/mL). In different test tubes, 1.4 mL of different concentrations of crude extracts or standard BHT solution along

with 0.6 mL of H₂O₂ was mixed and the absorbance of mixture was recorded after 15 min against a blank solution containing phosphate buffer (2 mmol/L pH 7.5) without H₂O₂ at 230 nm. The percentage of H₂O₂ scavenged was calculated using following formula:

$$\text{H}_2\text{O}_2 \text{ scavenge (\%)} = \text{OD control} - \text{OD test} / \text{OD control} * 100$$

Flash Chromatography analysis of Ethanolic extract (AsEt)

The flash chromatography system was used to fractionate AsEt extract. (Combiflash Companion, Teledyne ISCO company, USA and Interchim Flash Chromatography, France). The separation was carried out by using Rediseq (Teledyne ISCO Company, USA) 12 g column and the linear gradient mobile phase of toluene: ethyl acetate with flow rate of 25 mL/min. The scanning was done at 280 nm. As a result, the ethanolic extract yielded total 11 fractions. All the fractions were spotted on TLC plates and the plates were developed using the mobile phase as Toluene:ethylacetate:formic acid in a ratio of 5:4:0.2. The plates were developed in the solvent system and observed under UV cabinet at different wavelengths to detect the spots. The fractions were selected for further analysis by HPTLC method based on the R_f values of the spots observed.

HPTLC Fingerprint of fractions

In the current study, the qualitative assessment of extracts of leaves of *Ardissia solanacea* were performed for the presence of secondary metabolites using TLC (silica gel aluminum plate 60F₂₅₄, 0.5 mm). The mobile phase constituting Toluene: Ethyl acetate: Formic acid in a ratio of 5:4:0.2 was utilised as a solvent system.

The scanning of TLC plates at UV 254, 366 and 540 nm demonstrated presence of spots with different R_f values. The documentation of TLC is shown in Figures 3 and 4.^[24]

HR-MS analysis

For HR-MS analysis of fractions of *Ardissia solanacea* extract, samples were sent to the Central Instrumentation Facility (CIF), Savitribai Phule Pune University, Pune India. HR-MS analysis of ethanolic fractions were performed in dual (positive and negative) ion mode using Impact II UHR-TOF 1290 Mass Spectrometer System Bruker impact HD, technology generated ions by electrospray technique. Here, capillary tension was set at 4500 V, gas flow rate 7 L/min at a temperature of 200°C and a 1.7 Bar nebulizer gas flow pressure.

The 1 µL fraction of crude extracts (particle free) were introduced into injector with a split ratio 30:1. The full-scan mass spectrum was run within the scan range 50-1500 *m/z* to obtain the data. The *m/z* ratio was used to identify and characterize chemical

compounds present in fractions of crude extracts. The further confirmation was done by matching the mass spectra obtained with standard spectra existing in mass spectrum libraries.^[22]

RESULTS

Physico-chemical studies

Ash analysis of *Ardissia solanacea* leaves

Ash standards plays significant role in defining the quality and purity of crude drug, especially in powder form. The results of ash analysis was expressed in terms of % w/w and showed the total ash, acid insoluble ash and water soluble ash 11.5% w/w, 21.93% w/w and 6.5% w/w respectively.

Percentage yield of crude extracts

Various extracts of air dried powdered leaf of *Ardissia solanacea* were prepared by soxhlet extraction technique. The highest extractive value was found in ethanolic extract 6.72% followed by hydroalcoholic (4.37%), ethyl acetate (4.02%), methanol (1.45%) and aqueous (1.37%).

Primary phytochemical assessment

The studies disclosed the occurrence of flavanoids, phenolics and alkaloids in the successive extracts. Along with this, ethanolic extract showed higher content of flavonoids, phenolics and tannins as compared to other extracts, whereas flavonoids were lacking in aqueous extract. Along with polyphenolics and flavonoids, other phytoconstituents such as amino acids, proteins, carbohydrates and alkaloids were also present in small concentration in the extracts.

Total phenolic content (TPC)

The TPC of the leaves extracts are quantified in relationships with GAE. The calibration graph of gallic acid was prepared by use of standard dilutions and the linear regression equation $y=0.0003x + 0.0515$, $R^2=0.9996$ obtained from the graph was used to estimate TPCs:

Where y is absorbance and x is the amount of gallic acid in µg.

Ethanolic extract showed highest amount of phenolic content (10.138±0.010 mg/g of Gallic acid Equivalent).

Total Flavonoid Content (TFC)

The TFCs of the extracts are stated by means of Quercetin Equivalent. The linear regression equation $y=0.0027x + 0.0967$, $R^2=0.9984$, was obtained from the standard plot of quercetin and used to calculate TFCs:

Where y is absorbance and x is the amount of quercetin in µg.

The maximal flavonoid content was obtained in ethanol extract i.e. 371.91±0.167 mg/g of Quercetin Equivalent.

Total Tannin content

The total tannin content of the extracts are described in terms of Tannic acid Equivalent. The linear regression equation obtained from the calibration graph of tannic acid was used to estimate total tannin content:

$$y = 0.0099x + 0.0189, R^2 = 0.998$$

Where y is absorbance and x is the amount of tannic acid in μg .

Ethanol extract has the highest value of total phenolic, flavonoid and tannin (148.23 ± 0.510 mg/g of Tannic acid Equivalent contents). This specifies the chemical compounds from *Ardissia solanacea* extracted well in ethanol solvent.

According to Pradeep Kumar *et al.*^[19] polyphenolic constituents possess redox properties like antioxidants. The high concentration of polyphenolic determines the characteristics of the crude extract. Most of the crude extracts obtained from various sources such as vegetables, herbs, fruits and other plant materials rich in polyphenolics are greatly used in the food industry for their antioxidative properties and health benefits.

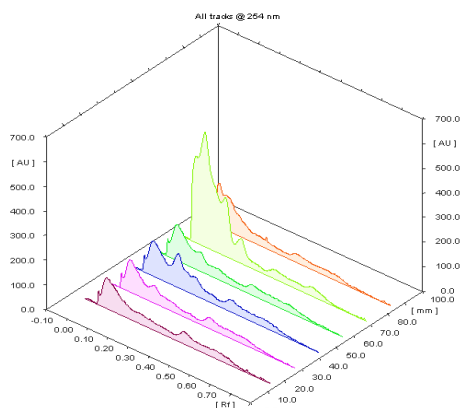


Figure 1: Qualitative TLC fingerprinting of fractions of ethanolic extract from AS leaves at UV-254nm..

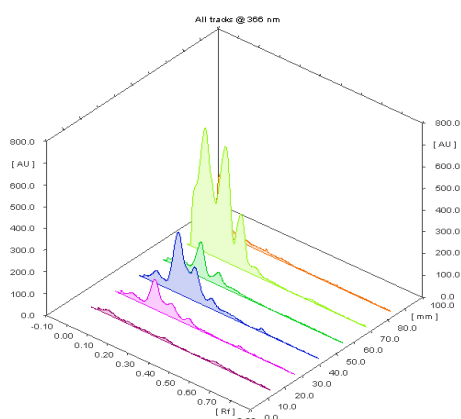


Figure 2: Qualitative TLC fingerprinting of fractions of ethanol extract from AS leaves at UV-366 nm.

In-vitro antioxidant activity

The antioxidant activity of the extracts of *Ardissia solanacea* leaves was studied by the DPPH and H_2O_2 assay method. The scavenging effect of leaves extracts are stated by means of % inhibition and are

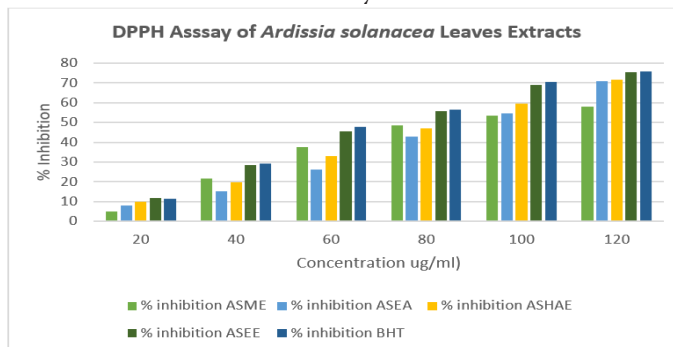


Figure 3: Percentage inhibition of DPPH free radical by crude extracts/ BHT at 517 nm (n=3).

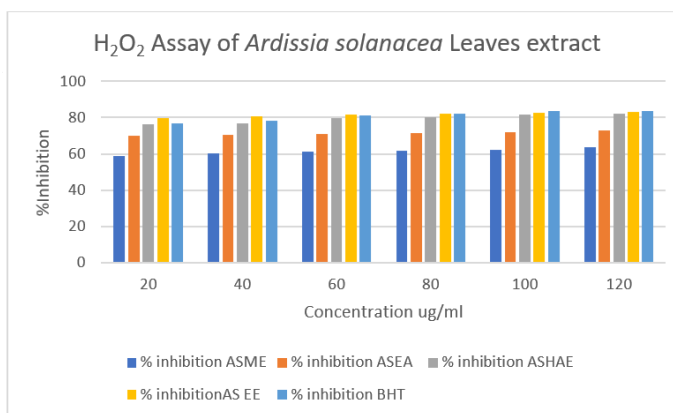


Figure 4: Percentage inhibition of H_2O_2 radical by crude extracts/ BHT at 230 nm (n=3).

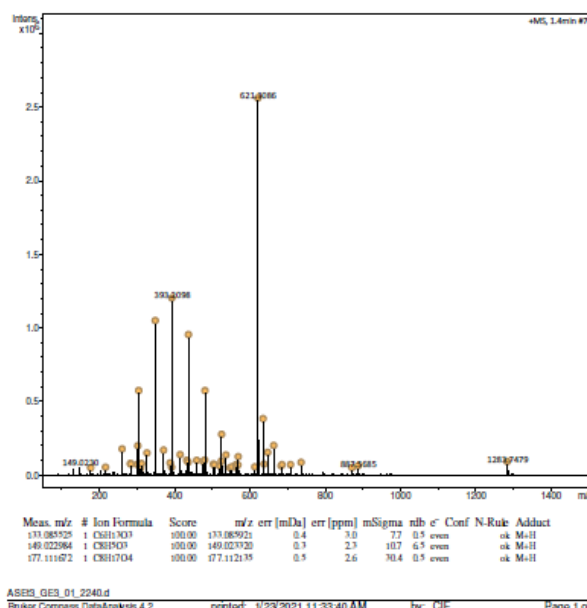


Figure 5: HRMS spectra of AS et3 fraction.

Table 1: IC₅₀ value of Standard BHT and *Ardissia solanacea* extracts by H₂O₂ assay.

Extract	Standard BHT	Methanolic	Ethyl acetate extract	Hydroalcoholic	Ethanollic
IC ₅₀	53.09	75.83	68.98	61.76	52.73

Sl. No.	No. of Peaks	R _f values
AsEt1	4	0.03, 0.07, 0.45, 0.66
AsEt2	6	0.02, 0.07, 0.18, 0.26, 0.45, 0.63
AsEt3	7	0.02, 0.06, 0.18, 0.26, 0.35, 0.45, 0.66
AsEt4	6	0.01, 0.06, 0.17, 0.26, 0.43, 0.61
AsEt5	6	0.04, 0.08, 0.18, 0.26, 0.43, 0.61
AsEt6	6	0.03, 0.07, 0.27, 0.41, 0.42, 0.60

Sl. No.	No. of Peaks	R _f values
AsEt1	4	0.02, 0.05, 0.33, 0.60
AsEt2	6	0.01, 0.05, 0.13, 0.24, 0.33, 0.58
AsEt3	6	0.03, 0.12, 0.24, 0.32, 0.45, 0.57
AsEt4	3	0.03, 0.12, 0.22
AsEt5	3	0.13, 0.22, 0.31
AsEt6	3	0.01, 0.08, 0.26

Table 2: Chemical composition of various fractions of *Ardissia solanacea* leaves extract by HR-MS analysis.

Fraction	Molecular Formula	m/z	Chemical/compound name	Chemical category
AsEt3 fraction	C ₁₅ H ₁₁ O ₇	305.15	Quarcetin	flavonoid
	C ₁₅ H ₁₁ O ₇	303.049	Taxifolin	flavonoid
	C ₂₀ H ₃₉ NO	309.30	1-Hexadecanoylpyrrolidine	N-acyl pyrrolidones
	C ₅₅ H ₇₄ N ₄ O ₅	870.56	Pheophytin-a	pheophytin
	C ₂₀ H ₂₅ NO ₇	391.16	O-acetylerucifoline	Alkaloid

inhibition. At a concentration of 120 µg/mL, the extracts showed best inhibition activity. The ethanolic extract showed highest amongst them followed by hydroalcoholic, ethyl acetate and methanolic extract respectively as compared to standard BHT.

The crude extracts showed IC₅₀ value for the H₂O₂ scavenging activity in the sequence of methanolic > ethyl acetate > hydroalcoholic > ethanolic extract significantly different from the standard BHT. The 120 µg/mL concentration showed maximum antioxidant activity by H₂O₂ assay. The results are tabulated in Table 1.

Flash chromatography and HPTLC fingerprint

The flash chromatographic system was used to separate and collect the fractions. The fractions were further analysed by HPTLC method with mobile phase Toluene: Ethyl acetate: Formic acid (5:4:0.2 v/v/v). The TLC plates were scanned at different wavelengths i.e., 254 nm and 366 nm.

The HPTLC analysis revealed several bands with different R_f values. The scanning of plates was done for determining metabolites such as flavanoids, polyphenolics etc. The flash separation of the AsEt extract yielded total 11 fractions. As per the R_f values of bands observed, the fractions from AsEt 7-11 did not show any bands and total six fractions of AsEt namely AsEt 1,2,3, 4, 5, 6 were selected for further analysis.

The results of the HPTLC analysis are shown in Figure 1 and 2 respectively.

Based on the results obtained from the TLC and HPTLC data, the AsEt3 was further processed to HRMS analysis for identification of possible phytoconstituents.

HR-MS Analysis

The chemical compositions of the one fraction of *Ardissia solanacea* was identified by HR-MS analysis (Figure 5). The

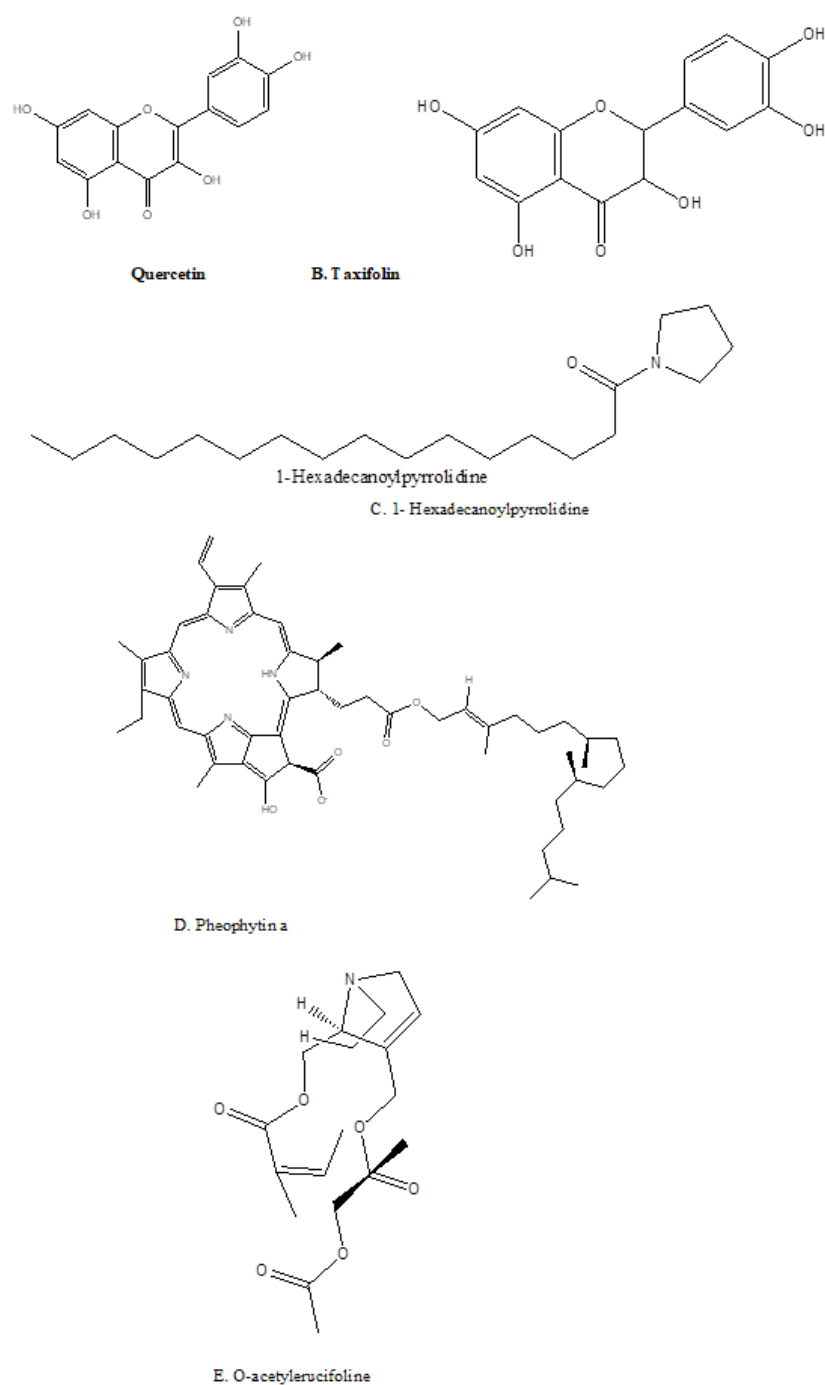


Figure 6: Putative Chemical structures of compounds identified in AsEt3 fraction of *Ardissia solanacea* Leaves extracts (A.) Quercetin, (B.) Taxifolin, (C.) 1-Hexadecanoylpyrrolidine, (D.) Pheophytin a (E.) O-acetylerucifoline.

phytoconstituents were identified based on their mean m/z values, MS/MS fragments with standard compounds, metabolite class or reported data in references or mass bank (<http://www.massbank.jp/QuickSearch.html>). Five compounds were identified. Details of identified compounds are provided in Table 2.

Putative Chemical structures of compounds identified in AsEt3 fraction of *Ardissia solanacea* Leaves extracts Figure 6 (A.)

Quercetin, (B.) Taxifolin, (C.) 1-Hexadecanoylpyrrolidine, (D.) Pheophytin a (E.) O-acetylerucifoline. AsEt-3 showed presence of Quercetin and taxifolin flavonoid which is already reported in the literature at m/z 305.15 and 303.049 in the HR-MS study respectively.^[20] The AsEt3 fraction showed the presence of 01 new alkaloid at m/z 391.16 identified as O-acetylerucifoline.

CONCLUSION

Based on the preliminary screening of extracts of *Ardissia solanacea*, the ethanolic extract showed the presence of highest concentration of polyphenolics which contribute to antioxidant potential of it. The newer identified molecules may be further isolated and purified through various techniques and studied for their pharmacological properties.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

AsEt: *Ardissia solanacea* ethanolic extract; **AsEt3:** *Ardissia solanacea* ethanolic extract fraction 3; **TPC:** Total Phenolic Content; **TFC:** Total Flavanoid Content

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