Isolation, characterization and pharmacological screening of active constituents from *Stevia rebaudiana* and *Rhizophora mucronata.*

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List of Abbreviations

Abbreviation	Elaboration
NMR	Nuclear Magnetic Resonance Spectroscopy
HSQC	Heteronuclear Single Quantum Correlation
HMBC	Heteronuclear Multiple Bond Correlation
COSY	Correlation Spectroscopy
IC_{50}	Half Maximal Lethal Concentration
R_f	Retardation Factor
PTLC	Preparative Thin Layer Chromatography
TLC	Thin Layer Chromatography
DMSO	Dimethyl Sulphoxide
MeOH	Methanol
MeOD	Deuterated Methanol
EtOAc	Ethyl acetate
$CHCl_3$	Chloroform
$CDCl_3$	Deuterated Chloroform
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl Tetrazolium Bromide
DPPH	2,2-diphenyl-1-picrylhydrazyl (DPPH)
DHFR	Dihydrofolate Reductase
FBS	Fetal Bovine Serum
CARS	Centre for Advanced Research in Sciences
PCR	Polymerase Chain Reaction

EAC	Ehrlich Ascites Carcinoma
DNA	Deoxyribonucleic Acid
cDNA	Complementary Deoxyribonucleic Acid
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
ALP	Alkaline Phosphatase
HeLa	Human Cervical Carcinoma
DMEN	Duleco's Modified Eagles Medium
WHO	World Health Organization

ABSTRACT

The current study was designed to isolate and characterize some bioactive secondary metabolites from two plants of *Stevia rebaudiana* Bertoni. (Family- Asteraceae*)* and *Rhizophora mucronata* Lam. (Family- Rhizophoraceae*)* by using repeated chromatographic and spectroscopic techniques, targeting their antioxidant, analgesic, antihyperglycemic, antimicrobial, and anticancer properties through *in vitro, in-vivo* and *in silico* approaches. The leaves from two plants were extracted with methanol and then fractioned by organic solvents *n*-hexane, dichloromethane, and ethyl acetate to obtain three fractions which were HSR, DSR and ESR from *Stevia rebaudiana* (SR) and three fractions HRM, DRM and ERM from *Rhizophora mucronata* (RM*)*. All the fractions from *Stevia rebaudiana* gave strong antioxidant, analgesic, and antidiabetic activities whereas, fractions from *Rhizophora mucronata* gave prominent antioxidant effect but exhibit mild analgesic, antimicrobial and antidiabetic effects. A total **fourteen (14)** compounds were isolated from the two plants. A total nine known compounds were isolated from *Stevia rebaudiana* and five components from *Rhizophora mucronata.* The structure of the isolated compounds were identified by studying 1D and 2D spectral feature with comparin the published data and characterized as 5-*O*-caffeoyl quinic acid (**1**), *trans*- syringin (**2**), luteolin (**3**), apigenin (**4**), quercitrin (**5**), 1,8-dihydroxy-3,6-dimethylanthracene-9,10-dione (**6**), jhanol (**7**) and jhanidiol (**8**), decanoic acid **(9)** and compounds from *Rhizophora mucronata* were β-amyrin **(10)**, 2-oxo-14,15-bisnor-3,11 *E*-Kalavadien-10, 13, 17-triol **(11),** β-sitosterol **(12)** , rutin **(13)**, and *N*-trans-para-caffeoyl-tyramine **(14)**. Among the five compounds isolated from *Rhizophora mucronata*, compound 11 was a new compound characterized by 1D, 2D and Mass spectroscopy. A promising antioxidant effects was observed by the compounds **1, 3, 4, 7, 9** of *Stevia rebaudiana* except compound **2** when compared to the standard drug with IC_{50} value 6.10 μg/mL. Antimicrobial assay was done by disc diffusion method where the isolated compounds (**1, 2, 3, 4, 7** and **8)** from *Stevia rebaudiana* gave mild to moderate antibacterial activity against selected both Gram (+)ve and Gram (-)ve strains. Among the isolates, compound **1** exhibited highest antibacterial activity with zone of inhibition 12-15mm with comparison of standard drug ciprofloxacin. The *in-vitro* cytotoxic effect of each isolate from *Stevia rebaudiana*

was done by MTT assay in HeLa cell line and compound **1** (5-*O*-caffeoyl quinic acid) showed a higher anti-proliferative effect (IC_{50} value was 181.3 µg/ ml) than other compounds **2, 4, 5**, and **6** from *Stevia.* Finally, the *in vitro* bioactivities of the isolates from *Stevia rebaudiana* were also supported by molecular docking studies. The computational study demonstrated that the isolated compounds exerted stronger affinity compared to the standard drugs towards the binding sites of dihydrofolate reductase (DHFR), glutathione reductase, and urase oxidase. The *in-vitro* cytotoxicity activity of the isolates **10 (**β-amyrin), **11** (2-oxo-14,15-bisnor-3,11 *E*-Kalavadien-10, 13, 17-triol), and **13 (**rutin) from *Rhyzophora mucronta* was also done by MTT assay in Ehrlich:s Ascites carcinoma (EAC cell) cell line and the compounds showed inhibitory activity in dose dependent manner having IC_{50} value of the three compounds **10, 11** and **13** were 139.8, 112.01 and 144.92 µg/ml respectively. Based on this *in-vitro* cytotoxicity result, *in-vivo* anticancer activity of the three compounds **10, 11** and **13** was evaluated against EAC cell line in mice model and bleomycin was used as standard drug. It was observed that the maximum cell growth inhibition was given by the compounds **11** which was 58.7% compared to standard drug bleomycin which showed 84.83% cell growth inhibition. A promising apoptotic cell morphological changes were observed using optical and fluorescence technique by these three compounds. EAC cells treated with the three compounds for five consecutive days to examine expression pattern of apoptosis regulatory gene showed upregulation of Bax, P^{53}, Cyt C, TNF-alpha, Cas-3, Cas-8, Cas-9 and down regulation of Bcl-2 and NF-κB which indicated that the apoptosis induced by compounds was mediated by extrinsic and mitochondrial pathway. In the current study, we also examine blood, liver, and kidney parameters treated by the compounds especially compounds **10** and **11** regain the healing process as well as these compounds have no toxic effects to this organ.

1. Introduction

1.1 Background

Now a days multiple health disease is challenge for the world. Different type of herbal medicine from plants have been used to manage life threatening diseases as a therapeutic weapon. Hence, due to the development of scientific research and technology lots of plants are used to treat disease [1]. Traditional medicinal system is not sufficient due to high cost of medication, population rise, poor supply of drugs. Besides, drawbacks of synthetic drugs inadequate used of recently developed drugs against infectious disease, drug from plant source is the choice of medicine to manage disease for human beings [2]. According to WHO, worldwide about 80% of people depend on herbal medicine to manage their health [3]. Medicinal plants are more safe, minor toxic and less side effect rather than compound from synthetic drugs [4]. The present study deals with separation and identification of secondary metabolites which are pharmacologically active through several chromatograph and spectroscopic methods from two medicinal plants. Plants are-

1) *Stevia rebaudiana* (Bertoni.)

2) *Rhizophora mucronata* (Lam.)

1.2 Objective of the study

The main objective of the study was to isolate, identify and characterize the active constituents from the two plants.

The specific objectives are-

- Extraction of *Stevia rebaudiana* and *Rhizophora mucronata.*
- Quarantine of bioactive secondary metabolites from *Stevia rebaudiana* and *Rhizophora mucronata* using different chromatography methods.

- Identification and quantification of the pharmacologically active components by several Spectroscopy method such as UV, Nuclear Magnetic Resonance and Mass Spectroscopy.

- The isolated compounds will be used to examine molecular docking studies. In this study three receptors such as dihydrofolate reductase (DHFR), glutathione reductase, and urase oxidase active site were used to assist the *in-vitro* bioactivities.

- Lastly evaluation of *in-vitro* and *in-vivo* biological investigation of the components of *Stevia rebaudiana* and *Rhizophora mucronata* using different pharmacological screening methods.

2.Plant Profile

2.1 Plant Profile of *Stevia rebaudiana:*

Stevia rebaudiana (Bertoni) is a small tree widely known as sugar leaf or candy leaf belongs to **Asteraceae** family which is pharmacologically very important due to its zero calorie sweetener properties [5].

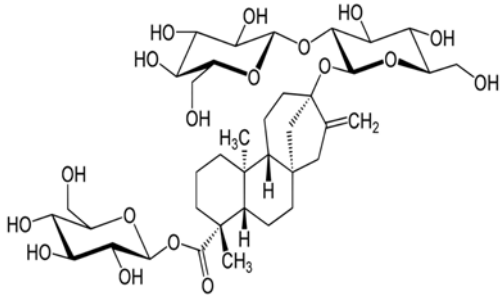
2.1.1 Distribution:

Stevia rebaudiana (Bertoni), member of Asteraceae family, which is a perennial shrub, native to South America. Nowadays, this plant is cultivated globally mostly developing country because of its medicinal value [6]. This plant is indigenous to Brazil and Paraguay and humid and wet environment is the optimum condition where this perennial sweet herb is distributed [7]. Because of its natural, non-toxic, and non-mutagenic sweetener properties, this plant has gained popularity towards research scientist as well as for economic reason. Stevia plant is 200-300 times sweeter than that of sucrose due to the presence of several steviol glycosides in its leaf, worth of use in the food and drug industry as an alternative source of sugar [8].

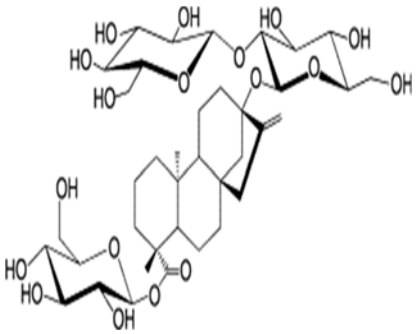
2.1.2 Chemical constituents:

- Three glycosides namely stevioside, rebaudioside A and C; are the main sweetening constituents whose common structures are *ent*-kaurene diterpene steviol skeleton [9-11].
- This plant also contains some other glycosides like staviolbioside, isostaviol, labdane-type diterpenes sterebins E, H, N and I [12-13].
- Apart from glycosides, the lead components for antioxidant property namely several phenolic compounds were also delineated [14-15].

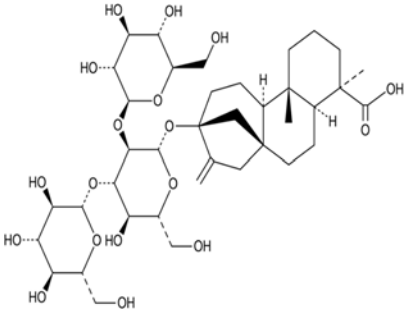
- The key components of potential free radical scavenging properties and other numerous diseases are chlorogenic acids, esters molecules of polyphenols, including hydroxycinnamic acids with quinic acid which components are isolated from the leaves of *Stevia rebaudiana* [16].



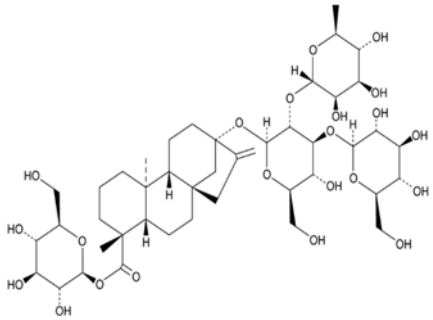
Stevioside



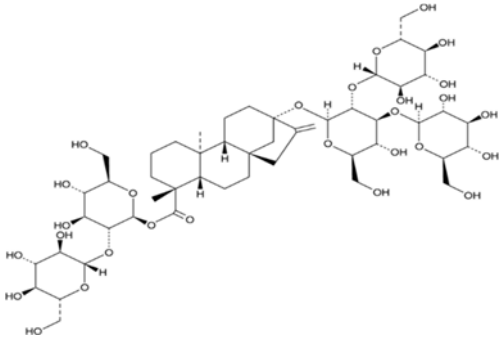
Rebaudeside A



Rebaudeside B



Rebaudeside C



Rebaudeside D

Figure 2.1: Major chemical constituents of *Stevia rebaudiana*.

2.1.3 Traditional and medicinal uses:

- In a world ravaged by diabetes, stevia is likely to become a staple herb to replace sugar. Stevia contains a series of glycoside that are up to 300 time as sweet as sucrose which is a popular tonic for treatment of diabetes and prophylactic for prediabetes by improving insulin sensitivity within the cell [17].

- Stevia is considered as a safe and natural sugar alternative for the preparation of confectionary and bakery, soft drink, tea coffee and ice-cream. The USA Food and Drug administration approved stevia as sucrose substitute because it is drastically changing the health risk that is related to sugar [18].

- Other than the industrial application, Stevia has been used traditionally and medicinally to treat various diseases like diabetes, hypertension, cancer, infection, and obesity [19-21].

- Stevia was also reported to have kidney and liver protecting effects along with increasing the insulin level in diabetic rat model [22].

- This plant has great role to treat in blood pressure regulation, inflammatory bowel disease, renal function, glucoregulation, tumor and obesity has been reported by a comprehensive review [23].

2.1.4 Literature review:

- Studies on antimicrobial and anticancer potentials are very limited although several bioactivities of *Stevia rebaudiana* extracts and some of the glycoside constituents have been reported. To address antimicrobial and anticancer potentials of the crude extracts, a very few reports are found [24-25].

- Recently reported antimicrobial and antioxidant properties of different extracts of *Stevia rebaudiana* in this regard [26].
- Stevia and stevioside are used to treat diabetes, high blood pressure [27].
- An investigation published that Stevia leaf extract used to reduces plaque acidogenicity and cariogenic microflora [28, 29].
- It has been reported that stevia glycoside used to dilation of blood vessel by increasing sodium excretion through urinary output [30].
- A study reported that steviosides and their metabolites has anti-inflammatory and immunomodulatory effects [31].

2.1.5 Photographs of *Stevia rebaudiana*:

Figure 2.2: Stevia leave and whole plant.

2.2 Plant preview *Rhizophora mucronata:*

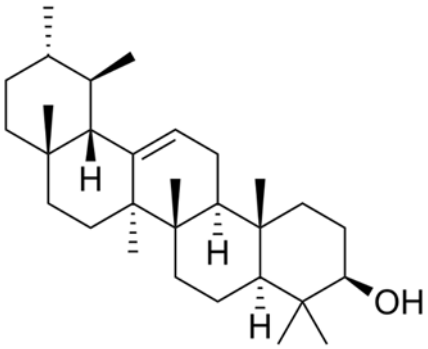
Rhizophora mucronata (Lam.) is a small to medium size tree which is about 20 to 25 meters height on the banks of river [32].

2.2.1 Distribution of *Rhizophora mucronata*:

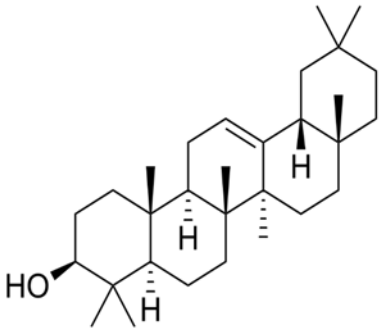
Rhizophora mucronata (Rhizophoraceae) commonly known as Asiatic mangrove largely distributed to tropical and subtropical coastal regions [33]. In tropical region mangrove forests are very important for ecologically, socio-economically, and social-cultural reasons. The coastal area of Sundarban is the largest mangrove forest in Bangladesh [34].

2.2.2 Chemical constituents of *Rhizophora mucronata*:

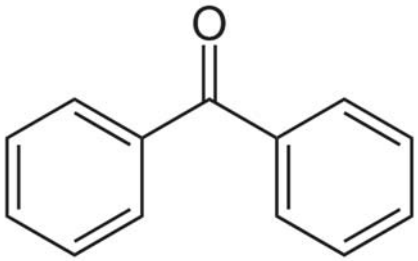
Andaman and Nicobar are renowned for its mangrove plants which contain xanthone, lichixanthone along with atranorin, α-amyrin, β-amyrin, palmitone, β-sitosterol, dimyristyl ketone [35]. It has been reported that betanidine alkaloids isolated from the leaf extract of *Rhizophora mucronata* [36] and it also contained tannin as catechin and epigalocathecin gallate [37] and 20% total dietary fiber obtained from both mature and immature fruits of *R mucronata* [38]. Diterpene rhizophorin B, C, D and E have been isolated from this mangrove [39-40].



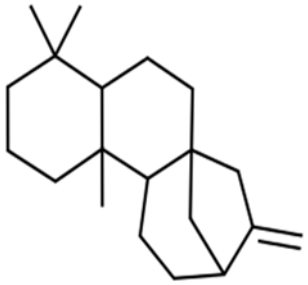
α-amyrin



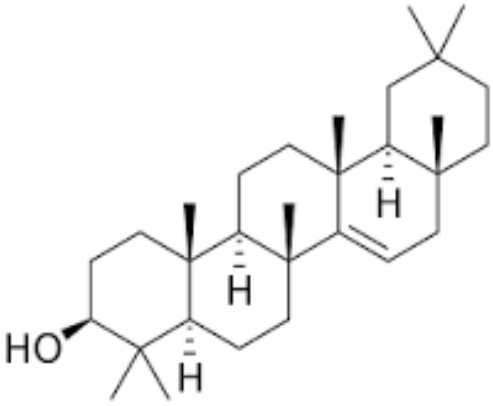
β-amyrin



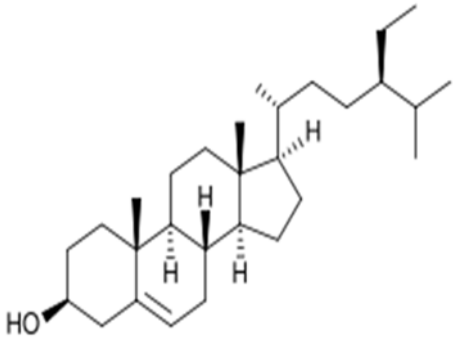
Benzophenone



Kaur-16-ene



Taraxerol



β-Sitosterol

Figure 2.3: Major chemical constituents of *Rhizophora mucronata*

2.2.3 Traditional uses of *Rhizophora mucronata*:

- The mangrove plants are used as an antinociceptive, anti-inflammatory, and antipyretic agent in Bangladesh [41] and as an anti-inflammatory and anti-diarrheal agent in Myanmar [42].

- Mangrove plant *Rhizophora mucronata* have ability to adjust salinity changes. *Rhizophora mucronata* mangrove has a rough, thick wax layer which can protect against excessive radiation from sunlight and can prevent transpiration which assist to preserve water in the leaf's tissue. Besides Ion toxicity, metabolism malfunction, physiological dehydration may be happened due to salinity changes which is maintain by such type of mangrove plant. Local Thai people used the mangrove bark extract used to manage diarrhea, nausea, vomiting and as an anticoagulant agent for uncured wounds [43-44].

- In Malaysia, *Rhizophora mucronata* locally known as bakau kurap which is used in charcoal industry to produce tannins [45].

- This mangrove is gaining interest by Indian people day-by-day to cure diseases like flatulence, epilepsy, smallpox, diabetic, asthma, rheumatism, stomach pains, fevers, malaria, cholera, hepatitis, cancer, ulcer and wounds. [46-47].

2.2.4 Literature review of *Rhizophora mucronata*:

The biological literature of *Rhizophora mucronata* are given bellow-

- *Rhizophora mucronata* has anticancer, antioxidant, antimicrobial, antidiabetic, and anti-inflammatory activities [48].

- The fruit flour has potential α-glucosidase inhibitory activity, and this plant is also famous due to its anti-HIV activity. [49].

- It has been reported that leaf extracts of *Rhizophora mucronata* is potent larvicides which is ecologically sound and to control blood sucking pests and this plant also demonstrated for haematuria and hemorrhages [50].

- The capability of free radical scavenging as well as restore defacement or injure liver of lab experimental rat was done by CCl_4 hepatotoxins. Rutin is a flavonoid glycoside isolated from *Rhizophora mucronata* which was known as vitamin P. Rutin has ability to decrease capillary fragility, permeability and retinal hemorrhage and increase venous blood flow [51].

2.2.5 Photographs of the *Rhizophora mucronata*:

Figure 2.4: Whole plant, leaves and flower of *Rhizophora mucronata*.

3. Material and Methods

3.1. Materials and instruments:

Methotrexate, ciprofloxacin, fluconazole, bleomycin, glibenclamide and ascorbic acid were obtained as generous gift from Eskyf Pharmaceutical Limited; Bangladesh and authorized suppliers were supplied DME medium, 10% fetal bovine serum and HELA cell was maintained in CARS. To execute the cytotoxicity, test a number of instruments were used namely trinocular microscope with camera (Olympus, Japan), biological biosafety cabinet (NU-400E, Nuaire, USA), hemocytometer (Nexcelom, USA) and CO_2 incubator (Nuaire, USA). First plant freshly leave of *Stevia rebaudiana* was collected from BRAC cultivation center, Dhaka, Bangladesh in March 2018 and 2nd plant *Rhizophora mucronata*was collected from mangrove Sundarbans Bangladesh in February 2019. The Principal Scientific Officer, Bangladesh National Herbarium, Mirpur, Dhaka identified the exsiccated plant samples who provided a voucher specimen (accession number: DACB-38588 and DACB-47468) for future reference. DMSO, DAPI, trypan blue dye, sodium carbonate was obtained from Merk (Darmstadt, Germany), 2, 7- dichlorofluorescent diacetate was acquired from Sigma Aldrich scriptase, dNTPs and oligo (dT) primers were purchased from TIANGEN Biotech (Beijing, China).

For elucidation the structure of isolated compounds a Burker AMX-400 operating at 400 MHz was used for ^{1}H-NMR and 100MHz ^{13}C NMR spectra respectively. Coupling constants (*J*) are expressed in Hertz (Hz) and tetramethylsilane as internal reference was used to measure chemical shift expressed as in δ (ppm) scale. Pre-coated TLC (Silica gel 60 F_{254}- MerkKGa) plates were utilized to carry out analytical thin layer chromatography (TLC) and plates were observed under UV light (254 nm). Silica gel (Kieselgel 60, 230-400 mesh, Merck KGaA, Dermstadt, Germany) column chromatography and size exclusion chromatography using Sephadex LH-20 were used to perform separation technique. Glass (20 × 20 cm) surface coated with slurry of silica gel were used to prepare Preparative TLC plates.

3.2. Fractionation of methanolic extracts:

Fresh and dried leaves of *Stevia rebaudiana* (1 kg) were soaked with 70% methanolic solution (methanol and water 70:30, total volume was 2L) and agitated and stirred time to time for 7 days at room temperature. Mature green and fresh leaves of *Rhizophora mucronata* were collected from the coastal area of Sundarbans, Bangladesh and the plant was then dried and grinded to make fine powder and were extracted with pure methanol (100%, total solvent 2 L for 1kg) with frequent agitation for a week at room temperature. The Whatman filter paper no. 1.was used to filter the whole mixer through clean, white cotton. The solvent of the filtrate was evaporated by a rotary evaporator to obtain concentrated methanol extracts of *Stevia rebaudiana* (100 gm) and *Rhizophora mucronata* (60gm). The plants extract was fractionated by n-hexane, dichlormethane and ethyl acetate [52] and the fractionated crude extracts were dried. After drying total three extracts for SR namely *n*-hexane *Stevia rebaudiana* (HSR), dichloromethane *Stevia rebaudiana* (DSR) and ethyl acetate *Stevia rebaudiana* (ESR) and the % of yield were 0.08% HSR, 0.10% DSR and 0.15% ERM respectively. Similarly, three extracts namely *n*-hexane *Rhizophora mucronata* (HRM), dichloromethane *Rhyzophora mucronata* (DRM) and ethyl acetate *Rhizophora mucronata* (ERM) and and the % of yield were 4.5% HSR, 4.09% DSR and 7.2% ERM respectively.

3.2.1 Phytochemical Screening:

Phytochemical analysis was performed conformity with the standard methods outlined by Nayek and Pereira [53].

Test of alkaloid (Hager's reagent)

1% solution of picric acid was mixed with all the crude extracts from two plants formation of crystalline yellow precipitate indicates the presence of alkaloid.

Test of saponins

50mg of crude extracts from two plants was boiled with 5ml water for 2 minutes. Mixer was cooled and mixed vigorously and left it for 3minutes. The presence of saponin was confirmed by formation of frothing.

Test of flavonoids

The fractions (small amounts) from two plants were dissolved with methanol and added a few drops of concentrated hydrochloric acid and a very small amount of Mg ribbon to the solution. Immediate development of a red color indicated the presence of flavonoids.

Test of tannins (Ferric chloride test)

At first crude extracts were dissolved with water and then boiled. After boiling it was filtrated by filter paper and the filtrate was collected. 2 ml filtrate was taken from there in a test tube and a few drops of 5% $Fecl_3$ was added to it and changing the color of the solution to dark green color indicated the presence of tannins.

Test of steroidal triterpenoids

Crude extracts were dissolved in acetic anhydride and boiled and cooled. After that at the side of test tube concentrated sulfuric acid was added. Formation of brown layer at the junction of two layer indicated the presence of steroids and formation of deep red color indicated the presence of triterpenoids.

3.2.2 Isolation of compounds from *Stevia rebaudiana:*

ESR was chosen for further isolation and purification based on the preliminary antimicrobial screening. For convenient application in column chromatography (CC), the ESR fraction (2gm) was dissolved in methanol along with little amount of silica gel (mesh size 60-120, 3gm) to get a dry sample. A clean and dry glass column (3 × 40 cm) packed with silica gel (60-120 mesh size) was loaded with the dry sample and ethyl acetate: acetic acid: water (8:1:1) with increasing polarity solvent system was chosen for elution and volume of each elution was 10 ml which was observed by thin layer chromatography (TLC). A total 15 fractions were obtained after TLC pooling and mixed 5-10 number fractions and ethyl acetate: acetic acid: water (8:1:1) were used to perform repeated preparative Thin Layer Chromatography (PTLC) to yield 5-*O*-caffeoyl quinic acid (**1**) 16 mg, *trans*-syringin (**2**) 20 mg, luteolin (**3**) 17mg and apigenin (**4**) 15 mg. A yellowish crystal of

compound of quercitrin **5** (7 mg), white powder of compound **6** or proposed chemical name 1,8-dihydroxy-3,6-dimethylanthracene-9,10-dione (5mg) with R_f values of 0.3 and 0.5 respectively afforded by another PTLC which was carried out using ethyl acetate: acetic acid: water (6:2:2) system.

Figure 3.1: Column Chromatography

Again, the *n*-hexane fraction (HSR) was subjected to silica column chromatography using the solvent system of *n*-hexane and ethyl acetate at a ratio 9:1 for elution. The test tubes number 46-53 gave same spot which were identified as jhanol (**7**, amount 30 mg), 50-60 were same band characterized as jhanidiol **(8,** amount 25mg) and 65 -68 numbers have identical spot which was known as decanoic acid (**9,** amount 7mg) on TLC plate respectively.

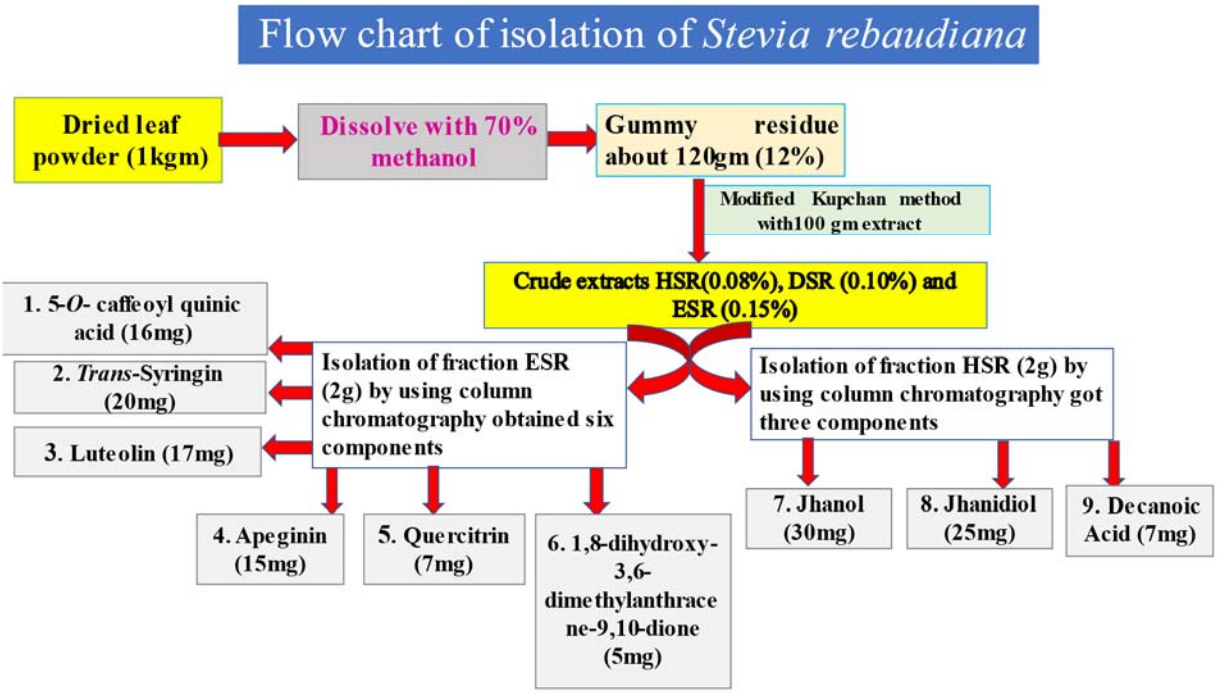


Figure 3.2: Diagram representing isolation of *Stevia rebaudiana* (Bert.)

3.2.3 Isolation of compounds from *Rhizophora mucronata:*

To isolate pure components from DCM extract (1g) size exclusion chromatography Sephadex LH-20 was used. The solvent systems used in the size exclusion chromatography were petroleum ether and ethyl acetate (80:20) with increasing polarity. The volume collected in each test tube was 3ml. The promising fractions according to the TLC results were pooled together and further subjected to PTLC with the solvent system toluene and ethyl acetate (9.5:0.5). The PTLC provided three compounds white crystal of β-amyrin **(10)** 32mg, yellow powder of 2-oxo-14,15-bisnor-3,11 *E*-Kalavadien 10, 13, 17-triol **(11)** 28mg, and white crystal of β-sitosterol **(12)** 7mg.

Figure 3.3: Sephadex Chromatography

Column chromatography was subjected by 1gm ERM fraction to separate pure components. Therefore, methanol solvent was used to dissolve the ERM fraction and then small amount of silica gel (mesh size 60- 120, 1 g sample) was added and mixed properly to get dry, fine sample. This dry sample was loaded on with small amount of silica gel (mesh size 60- 120, 1 g sample) and ethyl acetate: acetic acid: water (9:0.5:0.5) in order of increasing polarities and eluted to the column and eluent (10 ml) was collected which was observed by TLC. Identical TLC features were combined and about 10 fractions were acquired. After that re-chromatographed by preparative thin layer chromatography (PTLC) to impart yellow crystalline powder of compound **13** (27 mg) which was identified as rutin and white amorphous powder of compound **14** (6mg) which is *N*-trans-para-caffeoyl-tyramine.

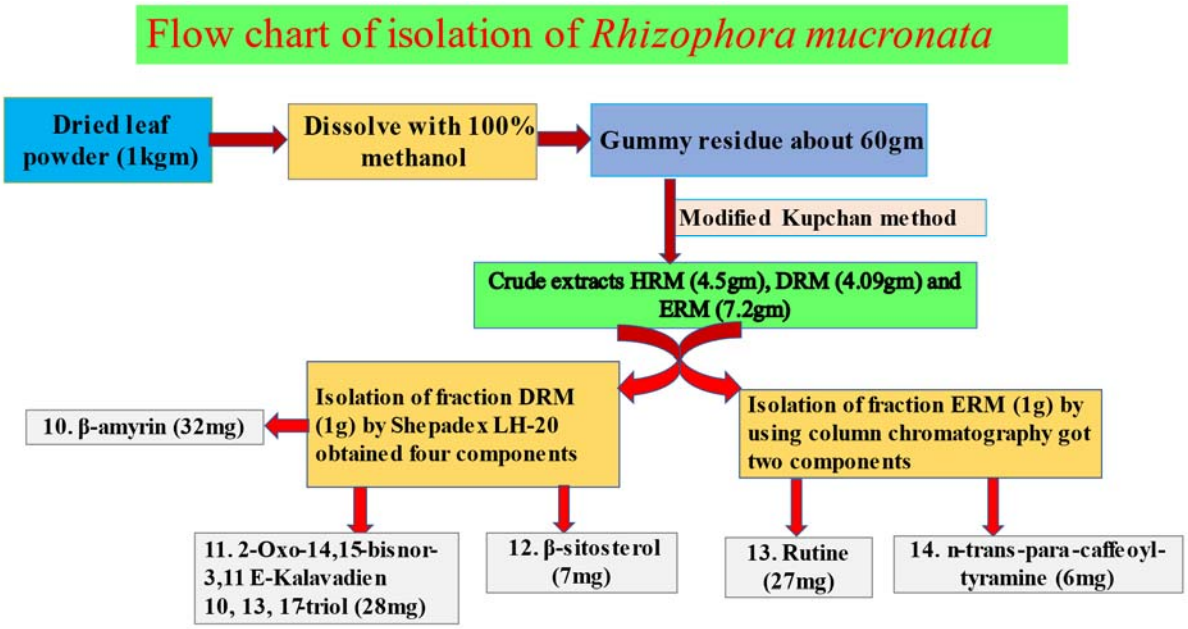


Figure 3.4: Diagram representing isolation of *Rhizophora mucronata*(Lam.)

3.3. Experimental animals:

About 110 mature male Swiss albino mice (average wt. 25- 30g) were procured from the department of Pharmacy of Jahangirnagar University, Dhaka, Bangladesh. During the study period standard laboratory conditions such as temperature 25±2^{0}C; humidity 5±5% and 12h dark/light cycle was used to maintain the experimental animals. To maintain the hydration condition stopped to supply food and water 12 hours before the experiment.

The aspect of Swiss Albino mouse is given below-

1. Scientific Name: *Mus musculus*
2. Life Span: 2-3 Years
3. Optimal Temperature: 18-27 ^{0}C
4. Optimal humidity range: 30-70%
5. Age at onset of puberty: 28-30 days
6. Estrus (heat) cycle length: 4-5 days
7. Estrus length: 12 hours

3.4 DPPH free radical scavenging assay:

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay method was done described by Brand-Williams et al. [54] to examine the antioxidant activity of the crude extracts and isolated compounds of both plants. Spotting on a TLC plate with the diluted stock solution and appearance of the compounds at distinct positions on the plate when the proper solvent system was run over the plate. 0.02% (w/v) DPPH in ethanol sprayed to the plate at room temperature. The potential antioxidant property of these compounds was observed by changing the color due to reduce of DPPH by the effect of these compounds.

To assay potential antioxidant property of these compounds by scavenging DPPH free radical, the spectroscopic method was further applied [26]. At first serial dilution (500, 100, 50, 10, 5, 1 µg/mL) of the samples as well as ascorbic acid as standard was prepared with the volume 2ml of each sample and 2 ml freshly prepared DPPH solution (0.004% w/v) were mixed carefully with each concentration of sample and ascorbic acid [55]. Then all the solution incubated for 30 mins in a dark place at room temperature and recorded the UV absorbance of these mixers at 517 nm wavelength where methanol was used as blank solution. The % inhibition or percentage of DPPH scavenging was estimated by applying the following equation:

$$\% \text{ Scavenging} = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100\%$$

Where A_{blank} = absorbance of the methanol only containing DPPH and

A_{sample} = Absorbance of the test samples and ascorbic acid after reaction with DPPH solution. To get accurate result the experiment was done 3 times and ploted a graph percent of inhibition against sample concentration. IC_{50} value (50% inhibition concentration) was computed from the graph.

3.5 Analgesic Test:

Acetic acid writhing model of mice was used for analgesic test of the crude extracts of both plants. A total of 25 mice were used for this experiment and divided into five groups; five animals encompass to each group. 1% tween in saline was used as vehicle received by Group I, Group II was given standard drug diclofenac-Na, Group III, IV and V received *n*-hexane, dichloromethane,

and ethyl acetate fractions of both plants respectively. The dose of standard drug diclofenac-Na was 10 mg/kg i.p and extracts were 200mg/kg. 0.1 ml of 1% acetic acid was administered intraperitoneally which was used for induction of peripheral pain. Intraperitoneal administration of 0.1 ml of 1% acetic acid 30 mins after administration of extracts and vehicle but 15 minutes before injection of acetic acid diclofenac sodium was administered intraperitoneally. After 5 mins interval the body contraction of mice referred to as writhing was counted for 10 mins [56].

3.6 Anti-hyperglycemic Test:

Standard method was used to perform anti-hyperglycemic test. All the mice were fractionated into five groups and five animals encompass to each group for both plant extracts. 0.5% methyl cellulose was taken by Group I (control group), 5 mg/kg of glibenclamide (as reference drug), was given to Group II and *n*-hexane, dichloromethane, and ethyl acetate fractions of both plants (dose of 200 mg/kg) were received by Group III, IV and V respectively. After fasting 16 hours, streptozotocin (60mg/kg) was introduced intraperitoneally to all mice for induction of diabetes which was initially dissolved in saline.Tail-vein blood of all groups mice were used for determination of blood glucose level by glucometer which was 0-day result, and it is known diabetes if higher than 11.5 mmol/L glucose was present in blood. For 7 days standard drug glibenclamide and extracts of both plants were administered once daily to corresponding mice groups. Glucose levels of blood were determined by a glucometer at the 1^{st}, $3^{rd,}$ and 7^{th} days [57].

3.7 Antimicrobial assay:

The fractions and purified components of SR and RM were subjected to examine of antimicrobial activity and minimum inhibitory concentration (MIC) using different strains by disc diffusion technique [58]. Antimicrobial assays were carried out in Biomedical research laboratory, University of Dhaka, Bangladesh. Five Gram-positive bacteria (*Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea*) and eight Gram-negative strains (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Salmonella typhi*, *Shigella boydii*, *Shigella dysen.teriae*, *Vibrio mimicus*, *Vibrio parahemolyticus)* along with three unicellular fungi were (*Candida albicans*, *Aspergillus niger*, *Saccharomyces cerevacae*) were used

to perform the test. The fractions HSR, DSR and ESR from SR and HRM, DRM and ERM from RM were given at the dose of 500 µg/disc by a micropipette. The pure isolated compounds **1, 2, 3, 4, 7**, and **8** from *Stevia rebaudiana* were introduced at the concentration of 250µg/disc with the help of micropipette. For antimicrobial test, ciprofloxacin (5 µg/disc) were used as positive controls and fluconazole (5 µg/disc) were applied for antifungal activities. The mean diameter was taken after finishing the experiment in thrice.

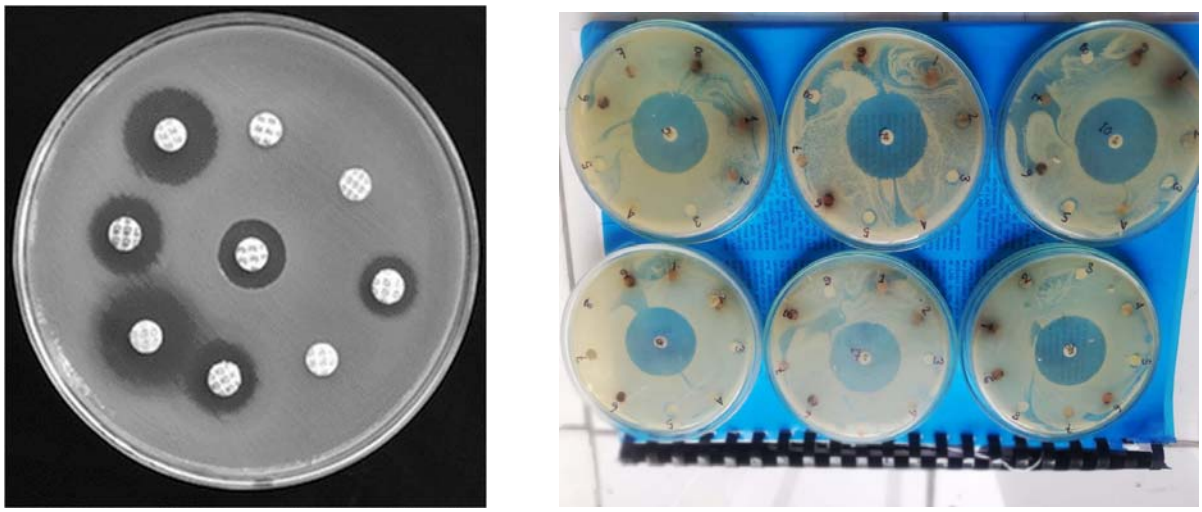


Figure 3.5: Antibacterial activities of the extracts and sample.

3.8 MTT cell viability assay against HeLa cell:

The crude extracts from both plants and pure compounds **1, 2, 4, 7** and **8** from *Stevia rebaudiana* were subjected to perform *in-vitro* cytotoxicity test in human cervical carcinoma cell line (HeLa cell line) at Centre for Advanced Research in Sciences (CARS), Dhaka, Bangladesh. Hela cells were maintained by DMEN (Dulbeco's modified eagles' medium) which is composed by 1% penicillin-streptomycin (1:1), 0.2% gentamycin, and 10% fetal bovine serum (FBS). A series of concentration (62.5, 125 and 250 µL/mL) of standard drugs methotrexate (for *Stevia rebaudiana)* and bleomycin (for *Rhizophora mucronata)* were used as positive control to estimate the cell proliferative test. 96-well plates with $2x10^4$ cells per well (100 µL) were made to implant HeLa

cells and a humidified atmosphere of 5% of CO2 at 37^0C conditions were maintained to incubate the cells for 24 hrs. Hence at first test samples (five components **1, 2, 4, 7** and **8** from SR and fractions from SR and RM) at the concentration (62.5, 125, 250 and 500 µL/mL) were dissolved in 2.5% DMSO, then incubation was done for 48 hr while 2.5% DMSO was used as negative control to compare the effect. A non-radioactive colorimetric Cell Proliferation and Cytotoxicity assay kit (Sigma-Aldrich, USA) called Kit-8 was used for counting the cell viability [59]. This test was done for each sample two times.

Figure 3.6: MTT colorimetry assay by using Laminar airflow.

3.9 Molecular docking study:

The molecular docking study of the six isolated compounds **1, 2, 3, 4, 7** and **8** from SR were performed by the widely used software packages (PyRx, PyMoL 2.3, Discovery Studio 4.5, and Swiss PDB viewer).

3.9.1 Target protein selection:

The ability of efficacy of the isolated compounds such as antibacterial, anticancer and antioxidant activities were executed by docking analysis. Based on the biochemical pathways and previous evidence target protein dihydrofolate reductase (DHFR) has been selected for studying of antibacterial and anticancer activities [60-61]. The structure of the dihydrofolate reductase (DHFR) enzyme basically three-dimensional (3D) structure was salvaged from the protein data

bank (https://www.rcsb.org) with PDB ID 4M6J. According to evaluate the potential antioxidant property, the same database (https://www.rcsb.org) were applied for computational docking of another for computational docking [62]. The PDB format were chosen to save all the protein/ receptor. Water and any unwanted ligand/residue were removed from the collected proteins by administrating in PyMoL 2.3. The non-polar hydrogen atoms were included to organize all the biomolecules. Swiss PDB viewer, an energy minimization program was utilized to and kept to the lowest energy further analysis.

3.9.2 Ligand preparation:

The PubChem database (https://pubchem.ncbi.nlm.nih.gov/) were used to retrieve the stated or structure of the compounds (**1, 2, 3, 4, 7** and **8**) in namely, 5-*O*-caffeoyl quinic acid, syringin, luteolin, apigenin, jhanol, and jhanidiol, respectively. Standard drugs, including ciprofloxacin, methotrexate, and ascorbic acid assigned to the ligands were loaded for studying the comparative binding affinity to the target macromolecules which was stored in 3D SDF format. Discovery studio 4.5 serially was applied including their PubChem CID to log in the ligands. In this study, for enhancing the docking accuracy Pm6 semi-empirical method were followed to optimize all the phytochemicals [63-64].

3.9.3 Ligand-protein interaction:

The possible binding profiling of the isolated compounds with their binding affinities to the target molecules could be predicted from the sketched of the current computer-aided ligand-protein interaction. The molecular drug-protein linking process was performed by a highly advanced PyRxAutodock Vina and molecular docking was accomplished by semiflexible modeling. Firstly, the protein has been loaded and formatted to target macromolecule**.** Firstly, the protein has been packed and configured to selected macromolecule. To ascertaining target-specific binding of the ligands selected the amino acids with their ID based on literature study. The site targeted docking was plucked by some amino acid such as 4M6J target, Ala9, Ile16, Leu93, Ser92, Arg91, Arg77, Glu78, Ser76, Leu75, Lys54, Val120, Ser119, Lys55, Thr56, Ser 118, Gly117, and for 1R4U, Arg 176, Val 227, Gln 228, Asn254, His 256. [60-61]. The isolated natural compounds with 3GRS

protein selected of Val 102, Lys 127, Asn 129, Lys 143, Gly 148, Ser 145, Ser 147, optimal hit during the docking was maintained against these selected macromolecules by importing all the PDB files of the ligands and after that the PyRxAutoDock Vina software was used to minimize into pdbqt format with Open Bable tool. On the contrary, the active binding sites of the protein was kept inside the middle of the box by creating the grid box. During docking with DHFR protein the grid mapping was set as center: X=7.4850, Y=7.3500, Z=-18.6398, and dimension: X= 43.8582, Y=51.2793, Z=48.8257. Moreover, the grid mapping was firmed center: X = 27.4790, Y = 50.2748, Z = 40.1613, and dimension: X = 31.7158, Y = 37.2601, and Z = 42.9295 for 1R4U protein while the grid map was kept for 3GRS center: X = 74.0693, Y = 51.4437, Z = 25.0217, and dimension: X = 24.7046, Y = 25.0, Z = 29.9240. During the docking process, the remaining frameworks were arranged as default settings. After that AutoDock Vina (version 1.1.2) was used to perform the computer-aided molecular docking of the ligands as well as maintained all associate condition [65]. Lastly, BIOVIA Discovery Studio version 4.5 was used to hypothesis the best-fitted structures with 2D and 3D configurations in all the docking analysis.

3.9.4 Experimental animals:

About 110 mature male Swiss albino mice (average wt. 25- 30g) were procured from the Department of Pharmacy of Jahangirnagar University, Dhaka, Bangladesh. During the study period standard laboratory conditions such as temperature 25±2^{0}C; humidity 5±5% and 12h dark/light cycle was used to maintain the experimental animals. To maintain the hydration condition supply food and water were stopped 12 hours before the experiment.

3.10 Anticancer activity:

In-vitro and *in-vivo* each method was used to determine anticancer activity of isolated compounds. A compound has anticancer effect or not might be obtained idea from *in-vitro* assay whereas.

in-vivo method gives further idea about its toxicity and therapeutic index.

3.10.1 Reagents and Solvents:

Analytical grade chemicals were used for experiments. Solvents were purchased from Active Fine (Bangladesh) and dimethyl sulphoxide (DMSO) was obtained from BDH(England). Hoechst 33342 was purchased from Sigma Aldrich (USA), M-MLV reverse transcriptase, dNTPs and oligo (dT) were obtained from Tiangen (Beijing, China). Beside these some special chemicals and reagents needed for hematological and histopathology studies have been properly cited at corresponding places.

3.10. 2 Instrumentation:

Hemocytometer:

A Neubauer hemocytometer (Germany) was utilized to. count tumor cells, WBC and RBC. The following equation was used to determine the subsequent cell concentration per ml.

Cells per ml=The average count per square × dilution factor × 10^4

Microscope

Motic Advanced system microscope (B, series) utilizing Motic J1.0 software was utilized for visualization of samples. Magnifaction of 10x40x was used. Histopathological analysis was carried out using the microscope.

Hemoglobinometer

Hellige Sahli's Hemometer No. 304-B Helige (USA) was ulilized to measure concentration of hemoglobin.

Nanodrop

Nanodrop 2000 spectrophotometer (Thermo Scientific) was applied to assess the goodness and concentration of RNA and at 260 and 280 nm wavelength, absorbance was measured.

Fluorescence microscope

The cellular histological change of EAC cells was examined by a fluorescence microscope Olympus i.X71, Korea.

Gel documentation System

The gel documentation system was used to examine the isolated DNA, RNA and PCR components by electrophoresis on 1% agarose gel including 0.1µg/ml ethidium bromides.

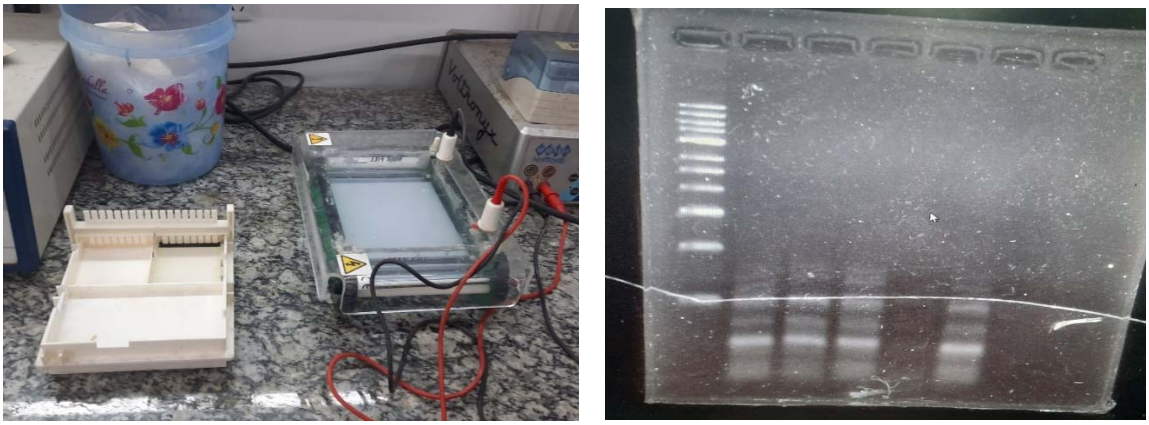


Figure 3.7: 1% gel electrophoresis

3.10.3 Thermal Cycler: Polymerase chain reaction (PCR):

Polymerase chain reaction was used for cDNA amplification associate with gene specific oligoes and to accomplish amplification thermal cycler (gene Atlas 482, Japan) was used.

3.10.4 Transplantation of tumor:

Ehrlich ascites carcinoma (EAC) cells were acquired from Indian Institute of Chemical Biology, Kalkata, India. The standard technique of Alam was followed for culture and aspiration EAC cells maintenance. 6-7 days aged ascites tumor-bearing mice were used for inoculation of cultured EAC cells. 0.9% normal saline was used to dilute the drawing cell. Approximately $1x10^6$ cells/ml was adjusted by a hemocytometer after dilution. Suspension of about 0.1ml of tumor cell containing $1x10^6$ tumor cells intraperitoneally [66].

3.10.5 *In-vitro* anticancer activity against on EAC cell:

In-vitro study was executed by MTT (3- [4,5-dimethylthiazole-2yl]-2,5-diphenyl tetrazolium bromide) colorimetric technique [67]. EAC cells about 1x 10^6 cells in 200 µl RPMI-1640 mixer were put in all of the 96-well plates and the compounds **10**, **11** and **13** at different concentrations (500, 250, 125, 62.5 and 31.25 µg/ml) was added in DMSO. Bleomycin (250, 125, 62.5 and 31.25 µg/ml) was utilized as standard and in control group DMSO was used for EAC cell. The experiment was carried out three times to reduce investigational errors. EAC cells were incubated in CO_2 incubator with 80% humidity for 24h at 37^0C. Then, aliquots were removed from each well and after that 180 µL of phosphate-buffered saline (PBS) and 20 µL (5mg mL^{-1} in PBS) of MTT was added and retained at 37^0C for 8hr into dark place. After that aliquots aspiration was done from each well, absolute isopropanol about 200 µL which was acidified with 0.1N HCl were put in and after that incubation was done for 60min at 37^0C. Finally, absorbance was computed at 570 nm. The cell growth inhibition (%) was quantified with the formula (A-B) x100/A,

where A and B are in absence and presence of the cellular homogenate of the drugs, respectively.

3.10.6 *In-vivo* cell growth inhibition:

For *in-vivo* evaluation five groups of *Swiss albino* mice (n=5) were used. On the first day, all mice were intraperitoneally injected about 1x 10^6 cells. After 24 h of tumor inoculation, treatment was carried out for five days. Group I was control group received only 10% DMSO, Group II received bleomycin at a dose of 0.3 mg/kg /day; while Group III, IV and V have received 5mg/kg b.wt/day drugs of **10, 11 and 13** respectively. On the six day all mice were sacrificed, and aspiration of total tumor cell was done intraperitoneally by normal saline (0.98%). The viable EAC cells of both control and treated mice were identified by applying typhan blue and calculated with the help of hemocytometer. The following formula was used to calculate growth inhibition [68].

$$\% \text{ Cell growth inhibition} = (1 - T/C) \times 100,$$

Where T = treatment group and C= control group.

Figure 3.8: Aspiration of mice to collect the EAC cell.

3.10.7 Examination of cellular histopathological change and nuclear deformation of EAC cells induced by isolated compounds:

Cellular morphologic changes induced by isolated compounds were monitored. Morphological inspection of Group I (control group which were untreated EAC cells) and Group I, Group II and Group III (compounds **10, 11,** and **13** induced EAC cells) were carried out using the fluorescence microscope Olympus iX71, Korea. EAC cells were accumulated after 5 days of treatment from both control and drug induced mice. The collected EAC cells were stained by DAPI dye [69].

Fluorescent microscopy protocol

1. After sacrificing both treated and control mice, EAC cells (1ml) were collected from them.
2. Cells were centrifuged at 2000 rpm for 3 minutes.
3. Throughout the supernatant.
4. 1ml of cold phosphate buffered saline (10mM) was added.
5. Cells were washed by centrifugation at 2000 rpm for 2-3 minutes.
6. Supernatant was discarded.
7. 1 ml PBS (10mM) and 5µl of Hoechst 33342 dye were added at the tube.
8. It was kept at 37 ^{0}C for 10 minutes at water bath (in dark place).
9. Again, cells were centrifuged at 1000 rpm and supernatant was rejected.
10. PBS (200 µl) was added for dilution and slide was prepared for observation under fluorescent microscope.

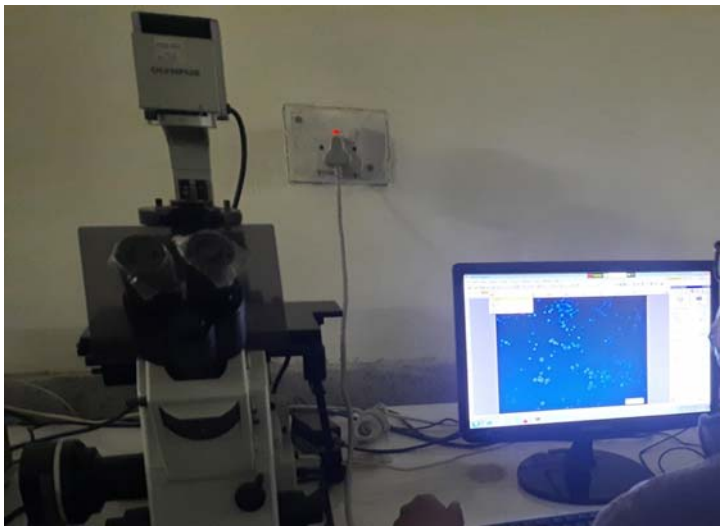


Figure 3.9: Morphological observation of EAC cell by flurocence microscope and optical microscope.

3.10.8 DNA fragmentation test:

Firstly, total genomic DNA was separated from all group of mice to perform this study by utilizing TIANamp Genomic DNA kit (Tiangen, Beijing, China). DNA fragmentation test was determined according to the guideline provided in the kit. Afterward the isolated DNA were subjected to electrophoresis system with the help of 1% agarose for 60 min at 100 V and lastly UV light illuminator, a gel documentation method was used to observe the bands. (Protein simple) [70]. The procedure is described below-

1. Treatment and control cells were taken and centrifuged at 10,000 rpm (~11,200xg) for 1 min. The flow-through is rejected and cell pellet was resuspended in 200 µl buffer GA.
2. 20 µl Proteinase K were added, mixed thoroughly by vortex.
3. 200 µl Buffer GB were put on the suspension and vortex was used for proper mixing and incubated at 70^0 C for 10 min to yield a homogenous solution. Precisely, the 1.5ml micro centrifuge tube was centrifuged to discard drops from the inside of the lid.
4. 200 µl ethanol (96 – 100%) were mixed to the sample, and blend thoroughly by vortex for 15 seconds.

5. Spin Column CB3 (in a 2ml collection tube) was used to pipette out the sample from step 3 and centrifuged at 12000 rpm (~13,400 x g) for 30 s followed by discarding the flow through and collection tube was used to place the spin column.
6. Then 500 µl Buffer GD were added to Spin Column CB3 and centrifuged at 12000 rpm (~13,400 x g) for 30 seconds. After that discarded the flow through and collection tube was used to place the spin column.
7. 600 µl Buffer PW were added to Spin Column CB3 and centrifuged at 12000 rpm (~13,400 x g) for 30 seconds. Subsequently the flow throw was discarded, and collection tube was used to place the spin column.
8. Step was repeated twice.
9. Centrifuged at 12000 rpm (~13,400 x g) for 30 seconds and dry the membrane completely.

10. A new clean 1.5 ml micro centrifuge tube was used to keep the Spin Column CB3. At the middle of the membrane poured the pipetted 50-200 µl Buffer TE directly followed by incubation at 15-25°C and then centrifuged at least 2 min at 12,000 rpm (~13,400 x g) to collect DNA.

3.10.9 RNA isolation and preparation of cDNA:

RNA simple Total RNA kit (Tiangen, Beijing, China) was used to separate RNA based on the processor guidelines. To estimate the concentration and purity of RNA Nanodrop 2000 spectrophotometer (Thermo scientific) was used. Reverse transcription reaction mixture (20 µL) containing oligo dT (2µL), M-MLV reverse transcriptase (1 µL), dNTPs (2 µL), 5x1st stand buffer (4 µL), and required amount dH_2O to make 20µL volume was needed to synthesis cDNA.

1. 500 µl of EAC cell were taken in 1ml RZ buffer in different eppendorf tubes.
2. The test tube was incubated at 15-30^{0}C for 5 minutes.
3. 200 µl chloroform was added and incubates at room temperature after vortexing for 15seconds and then mixed with RT.
4. Centrifuged the sample at 12,000 rpm at 4^{0}C for 10 min.
5. Upper aqueous phase was pipetted out in a new tube because RNA remains exclusively in the upper aqueous phase.
6. Half of the pure ethanol of total aqueous phase was added and mixed by tapping the tube.
7. The solution was taken in the CR3 column.

8. The column with collection tube was centrifuged at 12,000 rpm for 30 sec at 4^0C.
9. 500 µl RD was added and centrifuged at 12,000 rpm for 30 sec. Supernatant was rejected from the collector.
10. 700 µl RW was added and centrifuged at 12,000 rpm for 30 sec. Supernatant was rejected from the collector.
11. 500 µl RW was added and centrifuged at 12,000 rpm for 30 sec. Supernatant was rejected from the collector.
12. Empty column was centrifuged at 12,000 rpm for 2 min to dry the membrane.
13. 50µl RNase free water were added and incubated at room temperature for 2 min.
14. Finally, the column placed in 1.5ml RNAse free tube and centrifuge at 12,000 rpm for 2 mins at 4^0C to elute the RNA.
15. Extracted RNA was analyzed to observe under UV-trans illuminator (Alphalmager Mini System, Protein simple) by electrophoresis containing 1% agarose gel with EtBr (ethidium bromide) staining.

3.10.10 Reverse transcription (RT) of RNA:

After total RNA extraction, cDNA was prepared for gene expression analysis 20µl of total cDNA was prepared by using following reverse transcription reaction parameters [71].

Table 3.1: Recipe used in cDNA preparation.

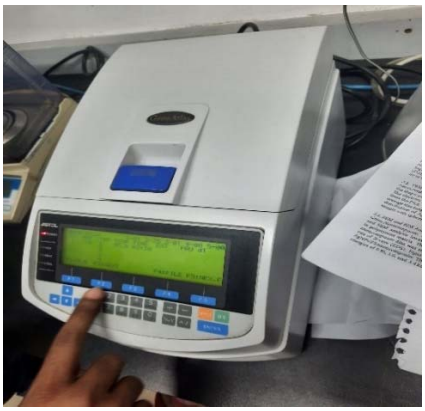
Name of chemical	Amount
mRNA (3µg)	5 µl
Oligo dt	2 µl
RNase inhibitor	1 µl
dNTPs 10 nM	2 µl
dH_2O	5 µl
5X First strand buffer	4 µl
M-MLV (reverse transcriptase	1 µl
Total	20 µl

3.10.11 Reaction parameter of RT:

1. mRNA, oligo dt, dNTPs and dH_2O were added in the PCR tube.
2. It was heated at 70 ^{0}C for 5 minutes and placed in ice for 5 minutes instantly.
3. 4 µl of 5x First strand buffer were added to the tubes.
4. Then 1 µl M-MLV (reverse transcriptase) was added.
5. It was incubated at 42^{0}C for 50 minutes.
6. Finally heated at 95 ^{0}C for 5min to inactivate the RNA polymerase.

3.10.12 PCR amplification of gene related to apoptosis:

Different genes of 25 reaction volume were prepared for PCR amplification. Gene Atlas 482 (Japan) thermal cycle was used for amplification. Different annealing temperature was used for different gene expression.



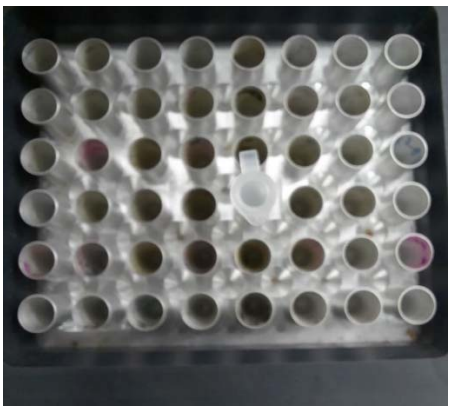


Figure 3.10: PCR amplification

Table 3.2: Reaction parameter of PCR:

Segment	Replication	Step	Temperature (^{0}C)	Time (min)
1	1	1	95 (activation of enzyme)	2.10
2	36	1	95 (denature)	0.45
		2	48-55 (annealing)	0.45
		3	72 (extension)	0.45
3	1	1	72(elongation)	5.00
		1	20 (hold)	10.0

Table 3.3: Recipe for PCR analysis:

Components	Volume
DNA polymerase (5x)	2.50µl
dNTP (2.5 mM)	1.00µl
Template	1.00µl
Forward Primer	1.00µl
Reverse Primer	1.00µl
DNA polymerase	.25µl
Distilled Water	18.25µl
Total	25.00µl

3.10.13 Gene expression analysis:

Real-time polymerase chain reaction (real-time PCR) technique was used to analysis gene expression [72]. In brief, gene specific oligos was used to amplify cDNA by reverse transcriptase polymerase chain reaction (RT-PCR). 25 µL reaction mixtures contained of IX Taq polymerase buffer, 25pmol each of forwarded and reverse primers, 2.5mM of each dNTPs, and 0.25 µL of platinum Taq polymerase (Tiangen, Beijing, China). The subsequent oligo nucleotide (IDT, Singapore) was used. GAPDH (housekeeping gene) was used as reference by comparing with other specific gene. The primer sequences and their thermal cycle conditions of the genes such as GAPDH, P53, BAX, Bcl-2, Cas-3, Cas-8 and Cas 9 that were used in this experiment are given in **Table 3.4**. GAPDH and other genes were amplified with PCR and the PCR products were staining with EtBr for electrophoresis in 1% agarose gel and finally visualized and photograph was pulled under UV light illuminator.

Table 3.4: Prime sequence and PCR protocol used for the RT-PCR assay.

Target genes	Prime sequence	AT, amplification (bp)	PCR protocol
GAPDH (F)	5′-GTGGAAGGACTCATGACCACAG-3′	52 ^{0}C, 350 bp	Program was set at 95 ^{0}C for 3 mins, 95 ^{0}C for 1 min, 52-58^{0}C (changeable annealing temperature for each oligo) for 1 min, 35 cycles followed by 72 ^{0}C for 1 min, 72 ^{0}C for 10 min, and finally hold at 72 ^{0}C
GAPDH (R)	5′-CTGGTGCTCAGTGTAGCCCAG-3′	
P^{53} (F)	5′-GCGTCTTAGAGACAGTTGCCT-3′	53^{0}C, 500 bp
P^{53} (R)	5′-GGATAGGTCGGCGGTTCATGC-3′	
BcL-2 (F)	5′-GTGGAGGAGCTCTTCAGGGA-3′	53 ^{0}C, 150 bp
BcL-2 (R)	5′-AGGCACCCAGGGTGATGCAA-3′	
Bax (F)	5′-CGCCCACCAGCTCTGAGCAGA-3′	50 ^{0}C, 350 bp
Bax (R)	5′-GCCACGTGGGCGTCCCAAAGT-3′	
CASEPASE-8(F)	5′-CTGCTGGGGATGGCCACTGTG-3′	55 ^{0}C, 600 bp
CASEPASE-8(R)	5′-TCGCCTCGAGGACATCGCTCTC-3′	
CASEPASE-3(F)	5′-TTAATAAAGGTATCCATGGAGAACACT-3′	55 ^{0}C, 300 bp
CASEPASE-3(R)	5′-TTAGTGATAAAAATAGAGTTCTTTTGT-3′	
CASEPASE-9(F)	5′-ATGGACGAAGCGGATCGG-3′	52 ^{0}C, 490 bp
CASEPASE-9(R)	5′-CCCTGGCCTTATGATGTT-3′	
Cyt-C (F)	5′-CCAGGTATACAAGCAGGTGTGCTC-3′	50 ^{0}C, 150 bp
Cyt-C (R)	5′-CATCATTAGGGCCATCCTGGAC-3′	
NF-κB (F)	5′-AACAAAATGCCCCACGGTTA-3′	55 ^{0}C, 125 bp
NF-κB (R)	5′-GGGACGATGCAATGGACTGT-3′	

Abbreviations: AT, annealing temperature; F, forward; PCR, polymerase chain reaction; R, reverse; RT-PCR, reverse transcriptase polymerase chain reaction.

3.10.14 blood, liver and kidney parameters check:

To check blood, liver, kidney parameters mice were sacrificed, and blood were collected from the aorta of heart of the mice. Firstly, all mice were divided into six groups and each group comprises five mice. Groups were Group-1 positive control group which group of mice did not contain EAC cell, Group-2 EAC cell caring control group, Group-3 received standard drug bleomycin (0.3mg/kg) and Group-4, Group-5 and Group-6 received compounds **11, 12**, and **13** at the dose 5 mg/kg b.wt. respectively. The blood parameters such as white blood cell (WBC), red blood cell

(RBC), and hemoglobin (Hb) were checked by standard procedure [73]. A hemocytometer was used to count total WBC and RBC and amount of hemoglobin (%Hb).

3.10.14.1 Hemoglobin content:

Reagent-N/10 HCl solution

Procedure:

Shali's hemometer was used to measure the amount of hemoglobin content. Blood was taken with the assistance of a 3ml syringe (up to the preference label) from mice and a cuvette was used to transfer blood in hemometer accommodating a small quantity of N/10 HCl solution. After 5 minutes the color of the blood was monitored and changing of color was observed by a hemometer. Hemoglobin content in g/dL was measured from the cuvette reading.

3.10. 14.2 Total WBC counting

Reagent: Aqueous acetic acid solution (2% v/v) and aqueous methylene blue (.3% w/v)

Procedure: 1ml WBC counting fluid was mixed with 10 µl non-coagulated blood. Neubauer hemocytometer was used to check resultant mixture and a microscope was used to compute the number of cells. The dilution factor was 100 and calculated number of WBC/ml.

3.10.14.3 Total RBC

Reagents: The reagents which mixed to count RBC were Tri-sodium citrate (8%), formaldehyde (37% formalin), distilled water.

Method:

At first blood was non-coagulated and 1ml blood was taken by a micropipette and added sterile water 1000 times for counting RBC. The counting technique of RBC was same as WBC with the help of hemocytometer.

3.10.14.4 Biochemical parameters:

The liver parameters such as ALT (ALT-Alanine Aminotransferase), AST (ASP-Aspartate aminotransferase) and ALP (ALP-Alkaline Phosphatase) and total bilirubin were assessed by enzymatic (NADH without P-S'-P) and conventional diazo system, employing the Abbot Architect Plus ci4100 (bilirubin, ALT, AST) and Siemens Dimension RL Max (ALP) analyzer. Blood samples were collected from mice to perform this test by the aortic rupture after six-day inoculation of EAC-cell, then centrifuged at 4000 rpm for 10 min and collected the serum and maintained at -20^0C otherwise utilized immediately. Here mice were divided into six groups and groups were Group I was normal control group without bearing EAC cell, Group II which was control group containing EAC (did not contain any drug) , Group III containg standard drug bleomycin at the dose of 0.3mg/kg/day, and Group IV, Group V and Group VI were receiving compounds **10, 11**, and **13** respectively at the concentration of 5mg/kg b.wt/day.

3.10.14. 5 Histopathological study of liver and kidney:

According to standard protocol, liver and kidney were procured from all the groups mouse on the sixth day [74]. The hepatotoxicity and nephrotoxicity were performed at the department of pathology, Rajshahi Medical college; Rajshahi, Bangladesh. PBS (phosphate buffer solution) containing 10% formalin was used to attach strongly of liver and kidney from each group mice. Running tap water was used to wash the tissue and normal grade of isopropanol was used to dry the sample and finally washed with xylene.

Figure 3.11: Liver and kidney of mice store in 70% alcohol.

Molten paraffin wax was used to embed the tissue. After that the mid organ level of liver and kidney incise different transverse sections (5m) with the help of microtome machine and hematoxylin and eosin (HE) was used to stain the sample and were observed under Mitotic Advanced system microscope (B, series) with the help of Mitotic J1.0 software in a Macintosh computer.

Figure 3.12: Mitotic Advanced system microscope. **Figure 3.13: Rotary Microtone.**

3.11 Statistical Study:

All experiment was accomplished three times and represented as mean ±SEM. One-way ANOVA followed by Dunnet test using SPSS 16 software to carry significance test between control and treatment group where *p value < .05 was considered as statistically significant. All the *in-vitro* and *in silico* experiments were performed three times, and the result were displaying mean ± Standard Error of Mean (SEM). Here, below 1% were considered for the standard errors and the analysis was conducted in MS Excel 2019 version.

4. Results

4.1 Percent of yield and phytochemical screening of the crude extracts

4.1.1 Percent of yield of the crude extracts from two plants:

70% methanolic solution was used for extraction of dried fine powder of *Stevia rebaudiana* (1kg) and *Rhizophora mucronata* was soaked with 100% methanol and fractionation. The percent of yield of the fractions were (w/w) 8% n-hexane *Stevia rebaudiana* (HSR), 10% dichloromethane *Stevia rebaudiana* (DSR) and 15% ethyl acetate *Stevia rebaudiana* (ESR) respectively. In case of *Rhizophora mucronata*, the % of yield was HRM 4.5%, DRM 4.09% and 7.2% ERM.

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4.1.2 Phytochemical Screening Test Result of Extracts:

Al the fractions HSR, DSR and ESR from *Stevia rebaudiana* and HRM, DRM and ERM from *Rhizophora mucronata* were subjected for phytochemical analysis which demonstrated the presence of alkaloids, saponins, flavonoids, tannins and triterpenoids. It is presented in **Table 4.1** and **4.2**.

Table 4.1: Phytochemical analysis results of crude extracts HSR, DSR ESR from *Stevia rebaudiana.*

Plant Extract	Alkaloids	Saponins	Flavonoids	Tannins	Triterpenoids
HSR	+	+	+	+	+
DSR	+	+	+	+	+
ESR	+	+	+	+	+

Table 4.2: Phytochemical analysis results of crude extracts HRM, DRM and ERM from *Rhizophora mucronata*.

Plant Extract	Alkaloids	Saponins	Flavonoids	Tanins	Triterpenoids
HRM	+	+	+	+	+
DRM	+	+	+	+	+
ERM	+	+	+	+	+

4.2 Results of biological activity of the fractions

4.2.1 Antioxidant activity test result:

4.2.1.1 Antioxidant test result of crude extracts of *Stevia rebaudiana*.

The DPPH assay revealed basic features on the antiradical activity of *Stevia rebaudiana*. Antioxidant activity of HSR, DSR, ESR and ascorbic acid is represented in the **Figure 4.1**. The IC_{50} results of HSR, DSR, ESR and standard drug were accounted for the inhibition of 11.59, 44.6, 10.39 and 6.45 µg/ml respectively.

Antioxidant Effect of SR crude extracts and Ascorbic Acid on DPPH

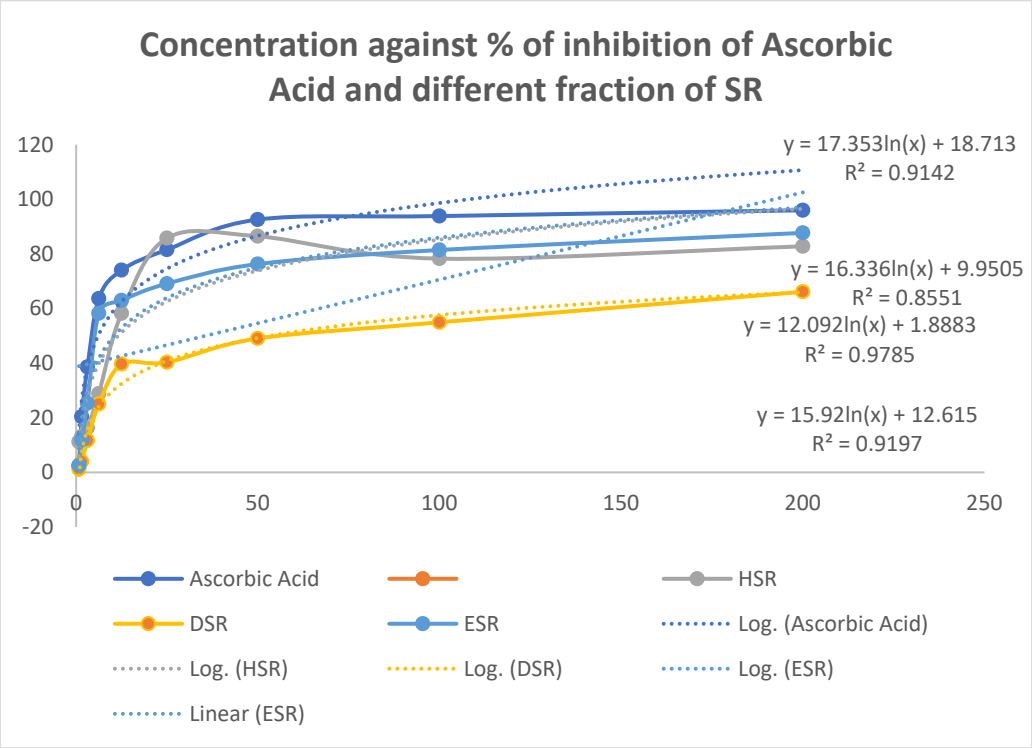


Figure 4.1: The percent of inhibition against concentration of HSR, DSR, ESR extract and ascorbic acid.

Table 4.3: IC_{50} values of HSR, DSR, ESR extract and Ascorbic Acid.

Test Sample	Regression line	R^2	IC_{50} µg/ml
Ascorbic Acid	y = 17.353 In(x) + 18.713	0. 9142	6.45
HSR	y = 16.1336 In(x) + 9.9505	0.8551	11.59
DSR	y = 12.092In(x) + 1.8883	0.9785	44.6
ESR	y = 15.92In(x) + 12.615	0.9197	10.39

*Ascorbic acid used as standard

4.2.1.2 Antioxidant effect of crude extracts of *Rhizophora mucronata:*

All the fractions exhibited promising antioxidant effect, among them DRM and ERM fractions have shown high free radical scavenging properties (11.7 and 10.41 µg/mL) compared to the IC_{50} value of reference drug ascorbic acid is 6.05 µg/mL (**Table 4.4).**

Table 4.4: DPPH scavenging activity of HRM, DRM and ERM fractions and ascorbic acid.

Compound Code	R^2	IC_{50} (µg/mL)
Ascorbic acid	0.9142	6.05
HRM	0.8551	44.59
DRM	0.9785	11.7
ERM	0.9197	10.38

4.2.2 Analgesic test result:

4.2.2.1 Analgesic activity of the fractions HSR, DSR and ESR of *Stevia rebaudiana*

The peripheral antinociceptive activity of the crude extracts HSR, DSR and ESR was done by acetic acid induced writhing model in mice. It has been shown that the fractions HSR, DSR and ESR of *Stevia rebaudiana* revealed prominent anti-inflammatory activity 62.21%, 67.32% and 65.34% inhibition of writhing in mice model respectively compared to standard drug receiving group (Group-II) which was 81.53% (**Table 4.5**).

Table 4.5: Analgesic activity of HSR, DSR and ESR of *Stevia rebaudiana* in mice.

Groups	Dose (mg/kg)	No of Writhing	% of Protection
Group-l	Vehicle	35.2± 2.7	-
Group-ll	10	6.5±1.43*	81.53
Group-lll	200	13.3 ± 1.81*	62.21
Group-lV	200	11.5±3.14*	67.32
Group-V	200	12.2±2.54*	65.34

Values are expressed in Mean ± SEM, (n=5). 1% Tween 80 in saline was given in Group-I and 10mg/kg Diclofenac-Na injected to Group-II, SR extracts HSR, DSR and ESR was received by Group-lll and Group-lV and Group -V at the dose 200 mg/kg body weight. *p<0.05 indicate significance compared with control group.

4.2.2.2 Analgesic activity of the fractions HRM, DRM and ERM of *Rhizophora mucronata*:

The analgesic activity of the crude extracts HRM, DRM and ERM demonstrated that all the fractions did not show significance anti-nociceptive activity or displayed insignificant activity when compared to standard drug receiving group (Group-II).

Table 4.6: **Analgesic activity of HRM, DRM and ERM of acetic acid induced writhing model in mice.**

Groups	Dose (mg/kg)	No of Writhing	% of Protection
Group-l	Vehicle	35.2± 2.7	-
Group-ll	10	6.5±1.43*	81.53
Group-lll	200	29.9 ± 5.2	15.05
Group-lV	200	31.2±4.33*	11.36
Group-V	200	32.4±6.33*	7.95

Values are expressed in Mean ± SEM, (n=5). Group-l received 1% Tween 80 in saline and Group-ll received 10mg/kg Diclofenac-Na, group-lll and group-lV and group -V received RM extracts HRM, DRM and ERM at the dose 200 mg/kg body weight. *p<0.05 indicate significance compared with control group.

4.2.3 Antihyperglycemic test results:

4.2.3.1 Antihyperglycemic test results of the fractions of HSR, DSR and ESR from *Stevia rebaudiana.*

The crude extracts HSR, DSR and ESR manifested prominent antihyperglycemic effect. DSR and ESR fractions (Group IV and group V) exhibited significant glucose lowering effect of 58.08% and 55.24% respectively (at the dose 200 mg/kg body weight) which is comparable to the effect obtained by standard drug glibenclamide (Group II) showed 71.5% hypoglycemic effect (**Table 4.7**).

Table 4.7: Antihyperglycemic effect of fractions HSR, DSR and ESR on streptozotocin induced diabetic mice.

Groups	Blood Glucose level (mmol/L)- Mean± SEM					
	0 day	**1st day**	**3rd day**	**5th day**	**7th day**	**% of lowering effect**
Group- I (Diabetic control)	20.9±4.4	18.3±2.7	16.3±4.3	20.1±3.2	21.8±5.2	----
Group-II (Diabetic standard)	18.6±4.3	12.4±4.2	10.8±4.6	8.7±2.2	5.3±1.7*	71.5
Group-III (Diabetic HSR)	16.3±1.8	15.8±6.4	13.1±2.6	11.6±4.2	10.8±3.4	33.7
Group-IV (Diabetic DSR)	19.8±5.5	16.6±2.4	12.2±3.9	10.1±3.3	8.3±2.8*	58.08
Group-V (Diabetic ESR)	18.1±2.2	16.3±3.3	13.3±3.4	9.7±2.2	8.1±3.2*	55.24

Values are expressed in Mean ± SEM. Control group received 0.5% Methyl cellulose and standard group received glibenclamide 5 mg/kg and fractions HSR, DSR and ESR were given 200 mg/kg.

4.2.3.2 Antihyperglycemic test results of the *Rhizophora mucronata* fractions:

The antihyperglycemic effect of fractions HRM, DRM and ERM of *Rhizophora mucronata* has been represented in the **Table 4.8**. Here all three fractions (at the dose 200 mg/kg body weight) did not show remarkable antidiabetic effect when compared to diabetic standard group (Group II).

Table 4.8: Antidiabetic effect of fractions HRM, DRM and ERM on streptozotocin induced diabetic mice.

Groups	Blood Glucose level (mmol/L)- Mean± SEM					
	0 day	1st day	3rd day	5th day	7th day	% of lowering effect
Group- I (Diabetic control)	20.9±4.4	18.3±2.7	16.3±4.3	20.1±3.2	21.8±5.2	----
Group-II (Diabetic standard)	18.6±4.3	12.4±4.2	10.8±4.6	8.7±2.2	5.3±1.7*	71.5
Group-III (Diabetic HRM)	17.3±1.8	14.8±3.2	15.1±1.4	16.2±3.1	16.6±1.1	4.04
Group-IV (Diabetic DRM)	18.3±2.2	17.7±2.8	16.2±1.9	16.9±1.7	16.5±2.6	9.8
Group-V (Diabetic ERM)	18.4±1.9	15.5±2.5	15.4±3.8	16.1±1.7	16.8±3.6	8.6

Values are expressed in Mean ± SEM. 0.5% Methyl cellulose received by control group and glibenclamide 5 mg/kg received by reference group and fractions HRM, DRM and ERM were given to Group-III, Group-IV and Group-V respectively at the dose 200 mg/kg.

4.2.4 Results of antimicrobial activity:

4.2.4.1 Antimicrobial activity of different fraction of *Stevia rebaudiana*

Disc diffusion method was used to examine antimicrobial effect of HSR, DSR and ESR on pathogenic bacteria and fungal growth at concentration of 500µg/ disc (**Table 4.9)**. HSR, DSR and ESR depicted good inhibitory effect with zone of inhibition ranges from 9.8 to 15.5 mm for Gram (+)ve and 8.9 to 16.8 mm Gram (-)ve strains and 16.2-23.3 mm zone of inhibition was observed in case of reference drug ciprofloxacin at a concentration of 5µg/ml which explained that ESR and DSR fraction accomplished appreciable antimicrobial activity against both Gram (+)ve and Gram (-)ve strains and HSR fraction showed comparing mild effect. Moreover, there was no antifungal activity observed by all the fractions from *Stevia rebaudiana.*

Table 4.9: Antimicrobial activity of the fractions of SR against Gram-positive, Gram-negative bacteria and fungus.

Pathogens	Zone of Inhibition (mm)			
	HSR (500µg/ml)	DSR (500 µg/ml)	ESR (500 µg/ml)	Ciprofloxacin (5 µg/ml)
Bacillus cereus	10.2±0.20	14.6±0.8	14.7±0.46	19±0.12
Bacillus megaterium	15.3±0.34	13.7±0.27	15.5±0.24	21±0.33
Bacillus subtilis	13.8±0.26	11.3±0.44	13.6±0.37	19.7±0.33
Staphylococcus aureus	11.3±0.65	12.8±0.18	12.8±0.46	17.6±0.25
Sarcina lutea	14.7±0.14	9.8±0.30	12.5±0.27	22.4±0.34
E coli	13.6±0.22	10.3±0.58	10.8±0.48	16.3±0.54
P aeruginosa	10.7±0.45	11.4±0.36	11.6±0.33	17.3±0.44
S paratyphi	12.5±0.25	9.8±0.27	16.3±0.23	23.3±0.48
S typhi	11.3±0.29	11.7±0.47	10.4±0.21	16.2±0.41
S boydii	13.3±0.21	11.3±.23	16.8±0.20	22±0.74
S dysenteriae	15.6±0.65	12.1±0.67	13.8±0.14	18.7±0.46
V mimicus	14.9±0.33	8.9±0.11	15.1±0.22	21±0.53
V parahemolytcs	11.6±0.11	8.3.1±0.33	13.7±0.12	18±0.23

Values are expressed in Mean ± SEM. Dose of ciprofloxacin was 5 µg/disc whereas dose of fractions were 500µg/disc.

4.2.4.2 Antimicrobial activity of different fraction of *Rhizophora mucronata*:

The antimicrobial efficacy of different fraction of HRM, DRM and ERM of *Rhizophora mucronata* is displayed in **Table 4.10**. HRM fraction gave mild antibacterial activity against gram positive bacteria *B. cereus, B. megaterium, B. subtilis, S. aurius, Sarcina lutea,* ERM fraction showed mild to moderate activity against some gram-positive bacteria *B. cereus, B. megaterium, B. subtilis* but inactive on *S. aurius, Sarcina lutea.* On the other hand, DRM fraction was found to be active against *S. aurius, Sarcina lutea* (**Table 4.10**). All the three fractions were ineffective against the gram-negative bacteria.

Table 4.10: Antimicrobial activity of test samples of *Rhizophora mucronata*.

Pathogens	**Zone of Inhibition (mm)**			
	HRM (500µg/ml)	**DRM. (500µg/ml)**	**ERM (500µg/ml)**	**Ciprofloxacin (5 µg/ml)**
Gram positive				
B. cereus	9.1±0.22	-	10.3 ±0.44	20.3±0.44
B. megaterium	8.3±0.12	-	8.3±0.21	22.3±0.21
B. subtilis	10.3±0.2	-	12.3±0.11	23.3±0.11
S. aureus	11.2 ±0.1	9.7±0.44	-	21.0±0.45
Sarcina lutea	10.1±0.22	10.1±0.22	-	23.0±0.22
Gram negative				
E. coli	-	-	-	21.2±0.23
P. aeruginosa	-	-	-	12.4±0.14
S. paratyphi	-	-	-	18.5±0.44
S. typhi	-	-	-	17.7±0.14
S. boydii	-	-	-	18±0.18
S. dysenteriae	-	-	-	19.5±0.33
V. mimicus	-		-	18.3±0.42
V. parahemolyticus	-		-	20.0±0.41

Values are expressed in Mean ± SEM. Dose of ciprofloxacin was 5 µg/disc whereas dose of fractions were 500µg/disc.

4.2.5 Evaluation of *In-vitro* anticancer property by crude extracts:

4.2.5.1 *In-vitro* cytotoxic activity of HSR, DSR and ESR against HeLa cell

The crude extracts HSR, DSR ESR from Stevia showed promising *in-vitro* cytotoxic activity against HeLa cell which is represented in the **Table 4.11**. Among the three fractions ESR fractions gave most potential inhibitory effect and the IC_{50} value was 147. µg/ml against HeLa cell. The morphological feature of the cell treated by crude extracts are displayed in **Figure 4.2**.

Table 4.11: *In-vitro* cytotoxic activity of HSR, DSR and ESR from *Stevia rebaudiana* on HeLa cell line.

Compound No.	Concentration (µg/ml))	% of inhibition against in HeLa cell line	IC_{50} value (µg/ml)
Control (DMSO)	2.5	-	-
Methotrexate	62.5	47.15	71.08
	125	61.52	
	250	87.23	
HSR	62.5	28.24	252.95
	125	47.43	
	250	55.13	
	500	63.88	
DSR	62.5	20.4	250.88
	125	48.4	
	250	54.2	
	500	70.8	
ESR	62.5	72.81	147.54
	125	58.11	
	250	66.24	
	500	72.81	

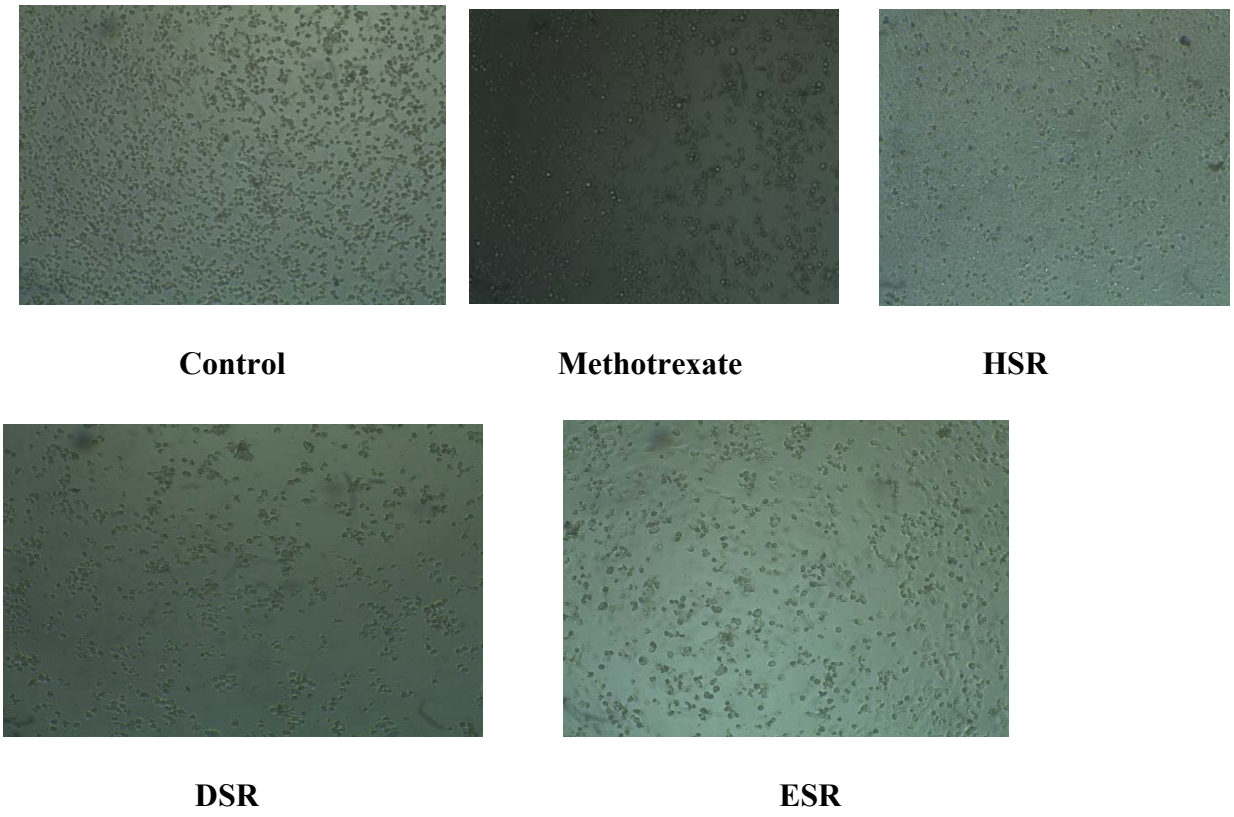


Figure 4.2: Cytotoxicity activity of control, standard and fractions at the concentration of 500 µg/ml observed by an advanced system of microscope.

4.2.5.2 *In-vitro* cytotoxic activity of HRM, DRM and ERM against HeLa cell:

The investigation of *in-vitro* cytotoxic activity of crude extracts against HeLa cell is presented on the **Table 4.12**. Among the different extracts DRM fractions showed highest inhibitory effect with the IC_{50} value of 89.08 µg/ml compared to standard drug bleomycin (IC_{50} value of 35.3 µg/ml) against HeLa cell. The morphological status of the cell is presented in **Figure 4.3**.

Table 4.12*: In-vitro* cytotoxic activity of HRM, DRM and ERM from RM on HeLa cell line.

Compound No.	Concentration (µg/ml))	% of inhibition against in HeLa cell line	IC_{50} value (µg/ml)
Control (DMSO)	**2.5**	**--**	**--**
Bleomycin	62.5	30.7	35.3
	125	70.4	
	250	92.8	
HRM	62.5	20.4	250.88
	125	48.4	
	250	54.2	
	500	70.8	
DRM	62.5	41.8	89.08
	125	54.3	
	250	70.5	
	500	76.8	
ERM	62.5	32.6	326.30
	125	45.5	
	250	52.8	
	500	68.9	

Note: DMSO = Dimethyl sulfoxide

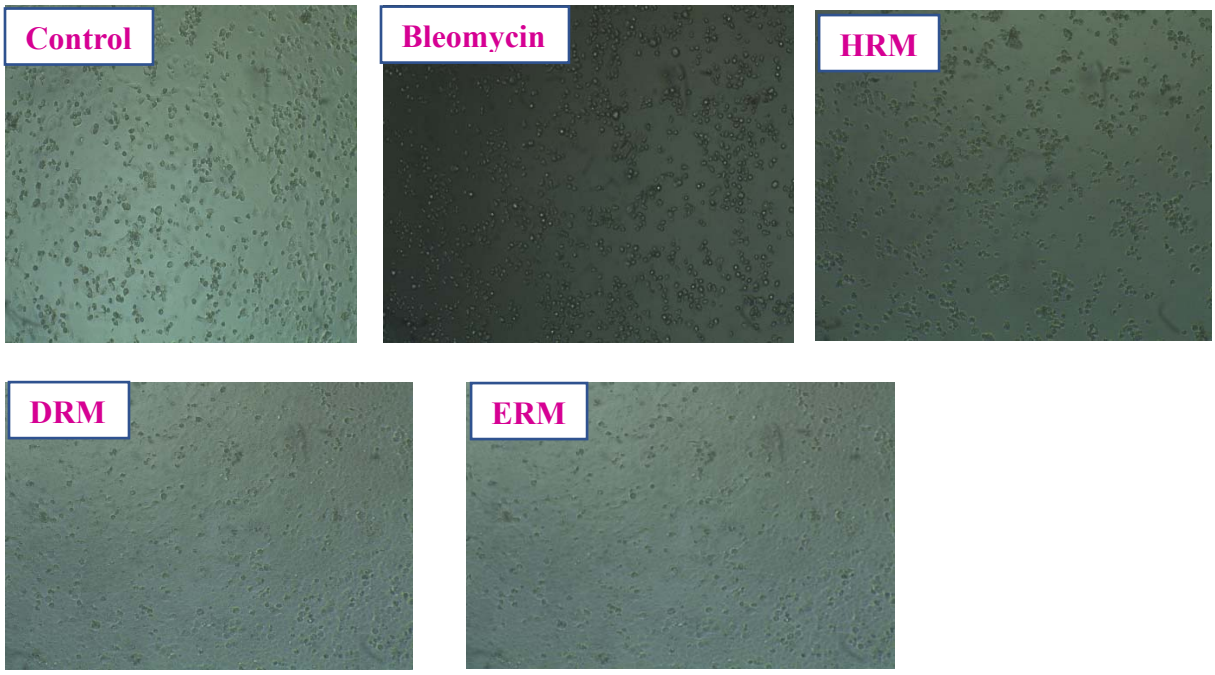


Figure 4.3: Cytotoxicity activity of control, standard and fractions from RM at the concentration of 500 µg/ml observed by an advanced system of microscope.

4.3 Structural determination of isolated compounds

4.3.1 Characterization of isolated compounds:

Isolation of the pure compounds from HSR and ESR fractions of *Stevia rebaudiana* and DRM and ERM fractions from *Rhizophora mucronata* was accomplished by different separation techniques. A total nine compounds were isolated from *Stevia rebaudiana* and a total five compounds were isolated were from *Rhizophora mucronata* via chromatographic methods.

4.3.2 Spectral data:

The structure of the isolated compounds by studying 1D and 2D spectral feature with comparing the published data and characterized as 5-*O*-caffeoyl quinic acid (**1**), *trans*-syringin (**2**), luteolin (**3**), apigenin (**4**), quercitrin (**5**), physcion (**6**), jhanol (**7**) and jhanidiol (**8**), decanoic acid **(9)** and compounds from *Rhizophora mucronata* were β-amyrin **(10)**, 2-oxo-14,15-bisnor-3,11 *E*-Kalavadien 10, 13, 17-triol **(11),** β-sitosterol **(12)** , rutin **(13)**, and N-trans-para-caffeoyl-tyramine **(14)**.

4.3.2.1 Characterization of compound 1 as 5-*O*-Caffeoyl quinic acid isolated from *Stevia rebaudiana* (Bert.).

Compound **1** isolated by silica column from the fraction no.5-10 of ESR crude extracts as a white crystal and it gave deep reddish type color after spraying with methanol in 10% sulfuric acid. The R_f value of this compound was 0.51 in the solvent system ethyl acetate: acetic acid: water (7:2:1). This compound was soluble in methanol.^{1}H NMR and ^{13}C NMR was used for identification and characterization of the compound.

The ^{1}H NMR of the compound **1** gave caffeoyl signal at δ 7.04, 6.77 and 6.92 ppm resonance at 2', 5' and 6' and also displayed signal at 5.34, 3.74 and 3.66 ppm correspond to -OH, H-3 and H-1 respectively which is characteristic of quinic acid.

The ^{13}C NMR spectrum (**Figure 4.4-4.6**) displayed 18 carbon and indication of C-C double bond due to resonance of over δ 100 ppm. Therefore, signals at 126.2, 114.3, 145.3, 148.2, 115.1 were

assigned for C-1' to C-6' indicate the signal for aromatic ring. The spectrum at δ164.34 ppm might be assigned to carbonyl group. The spectral NMR data are presented in **Table 4.13** and compared with reference data of 5-*O*-Caffeoyl quinic acid [75] from which it was concluded that the compound **1** identified as 5-*O*-Caffeoyl quinic acid.

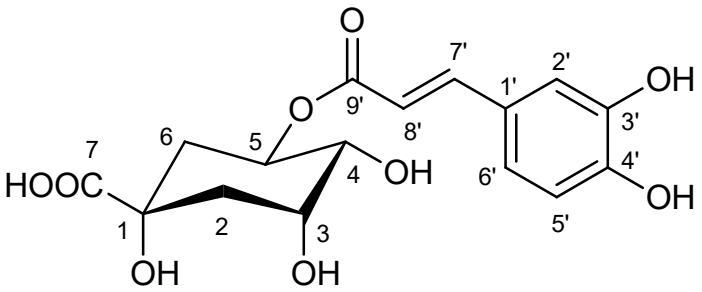


Figure 4.4: Structure of Compound 1 (5-*O*-Caffeoyl quinic acid)

Table 4.13: ^{13}C NMR at 100 MHz and ^{1}H NMR at 400 MHz of compound 1 in CD_3OD reference to TMS.

Position	Compound 1 ‘		5-*O*-Caffeoyl quinic acid [75] (Solvent- CD_3 OD)	
	δ_C	δ_H	δ_C	δ_H
1	71.2	-	73.6	12.41 245 (1H, *brs*, 7-H)
2	36.4	-	37.3	
3	67.7	3.74 (1H, *ddd*, *J*= 7.6, 2.9, 2.8 Hz)	68.2	3.94 (1H, *ddd*, = 7.7, 2.9, 2.9 Hz)
4	69.3	3.66 (1H, *dd*, *J* = 6.9, 2.9 Hz)	70.5	3.58(1H, *dd*, *J* = 6.9, 2.9 Hz)
5	70.7	-	71.0	5.08 (1H, *ddd*, *J* = 6.9, 6.8, 4.2 Hz)
6		-	36.4	
7	173.8	-	175	12.41(1H, *vbrs*)
1'	126.2	-	125.7	

2'	114.3	7.04 (1H, *d*, *J* = 2.0 Hz)	114.9	7.04 (1H, *d*, *J* = 2.0 Hz)
3'	145.7		145.6	
4'	148.2		148.4	
5'	115.9	6.77 (1H, *d*, *J* = 8.0 Hz)	115.9	6.77 (1H, *d*, *J* = 8.2 Hz)
6'	121.6	6.92 (1H, *dd*, *J* = 8.0, 2.0 Hz)	121.5	6.98 (1H, *dd*, *J* = 8.2, 2.0 Hz)
7'	145.4	7.58 (1H, *d*, *J* = 15.9 Hz)	145.0	7.43 (1H, *d*, *J* = 15.9 Hz)
8'	113.8	6.24 (1H, *d*, *J* = 15.9 Hz)	114.4	6.16 (1H, *d*, *J* = 15.9 Hz)
9'	167.0		165.9	
	-	5.34 (1H, *m* OH)		5.54 (1H, *brs*, OH)
	-	2.05 (1H, *m*, H-2$_{ax}$)		2.03 (1H, *dd*, *J* = 13.1, 2.9 Hz)
	-	2.01 (1H, *m*, H-6$_{ax}$)		2.01 (1H, *dd*, *J* = 13.3, 6.8 Hz)
	-	1.96 (1H, *m*, H-6$_{eq}$)		1.96 (1H, *dd*, *J* = 13.3, 4.2 Hz)
	-	1.90 (1H, *m*, H-2*eq*)		1.80 (1H, *dd*, *J* = 13.1, 7.7 Hz, H-2*eq*)

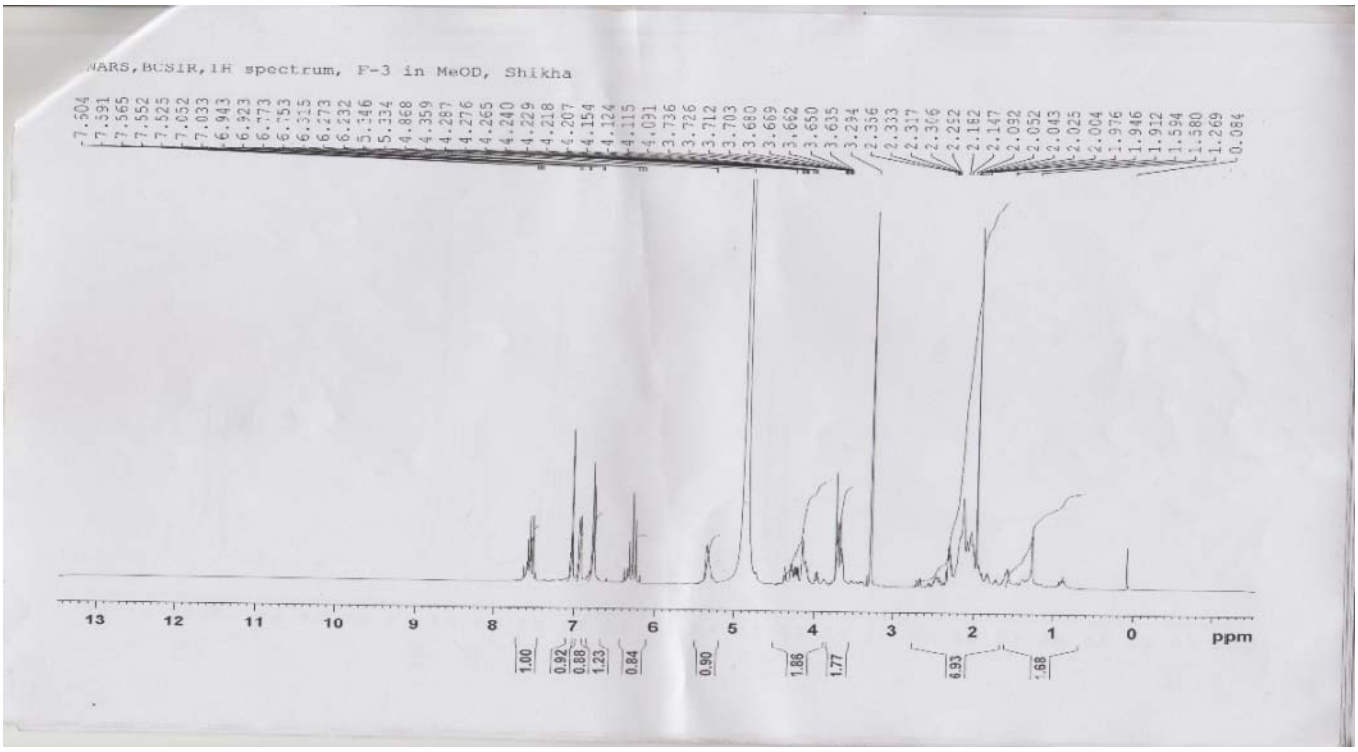


Figure 4.5: ^{1}H-NMR spectra of compound 1 (5-*O*- Caffeoylquinic acid)

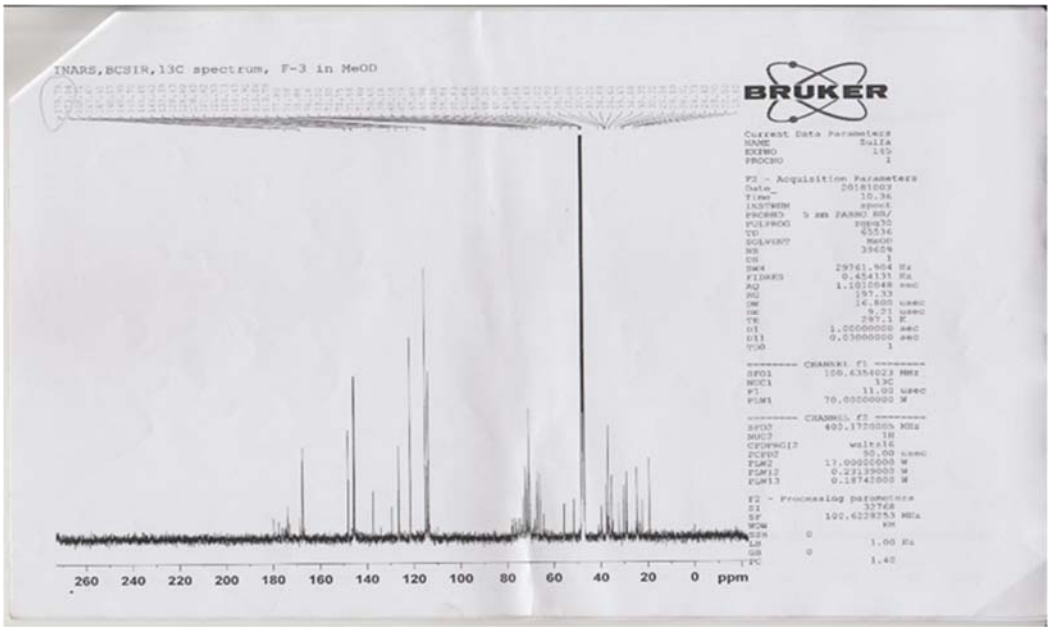


Figure 4.6: ^{13}C-NMR spectra of compound 1 (5-*O*- Caffeoylquinic acid).

4.3.2.2 Characterization of compound 2 as *Trans*-syringin isolated from *Stevia rebaudiana* (Bert.).

Column chromatography was used to separate compound **2** as white crystalline solid from ESR fraction. The TLC result gave radish y.ellow color when sprayed with vanillin-sulfuric acid followed by heating at 110^0C for 5-10 mins and the R_f value was 0.6 in ethyl acetate: acetic acid: water (7:2:1). The compound is soluble in methanol. ^{1}H NMR was used for structure elucidation of the compound. A typical singlet was found at δ 6.65ppm integrating two protons indicating the presence of a symmetric tetra substituted aromatic ring. This also indicated that a sugar unit (β-D glucopyronoside) is attached with the aromatic ring.

Two methoxy group attributed as doublet at 3.75ppm represented 6H. It also displayed two characteristic doublets of triplets at δ 6.42 and δ 6.23 ppm assigned to olefinic proton at H-7 and H-8 (**Figure 4.7**) which also suggested presence of a trans-disubstituted double bond directly linked to a methylene group attached. According to this data (**Table 4.14**) and comparing with reference data [76], it was confirmed that the compound **2** is *Trans*-syringin (**Figure 4.7)**.

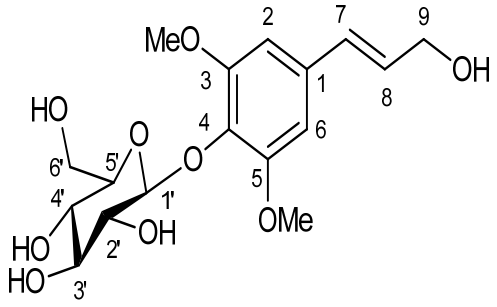


Figure 4.7: Structure of Compound 2 (*Trans*-Syringin)

Table 4.14: ^{1}H NMR spectra data at 400 MHz of compound 2 in CD_3OD reference to TMS.

Position	Compound 2	*Trans*-syringin [76] (Solvent- CD_3 OD)
	δ_H	δ_H
1		
2	6.65 (1H, *s*)	6.75 (1H, *s*)
3	--	3.86 (3H, *s*)
4	--	--
5		3.86 (3H, *s*)
6	6.65 (1H, *s*)	6.75 (1H, *s*)
7	6.42 (1H, *d*, *J*=15.0 Hz)	6.55 (1H, *d*, *J*=15.0 Hz)
8	6.23 (1H, *dt*, *J*=15 and 6.0 Hz)	6.32 (1H, *dt*, *J*=15.9 and 5.4 Hz)
9	4.11 (1H, *dd*, *J*=6.0 and 2.0 Hz)	4.22 (2H, *dd*, *J*=5.7 and 1.2 Hz)
1'	4.78 (1H, *d*, *J*=8.0 Hz)	--
2'	3.1 (1H, *m*)	3-3.80 (1H, *m*)
3'	3.41 (1H, *m*)	3-3.80 (1H, *m*)
4'	3.48 (1H, *m*)	3-3.80 (1H, *m*)
5'	3.32 (1H, *m*)	3-3.80 (1H, *m*)
6'	3.65 (1H, *dd*, *J*=12 and 6.0 Hz) 3.5 (1H, *dd*, *J*=12 and 2.0 Hz	3.64 (1H, *dd*, *J*=11.7 and 5.1 Hz) 3.77 (1H, *dd*, *J*=12 and 2.4 Hz)

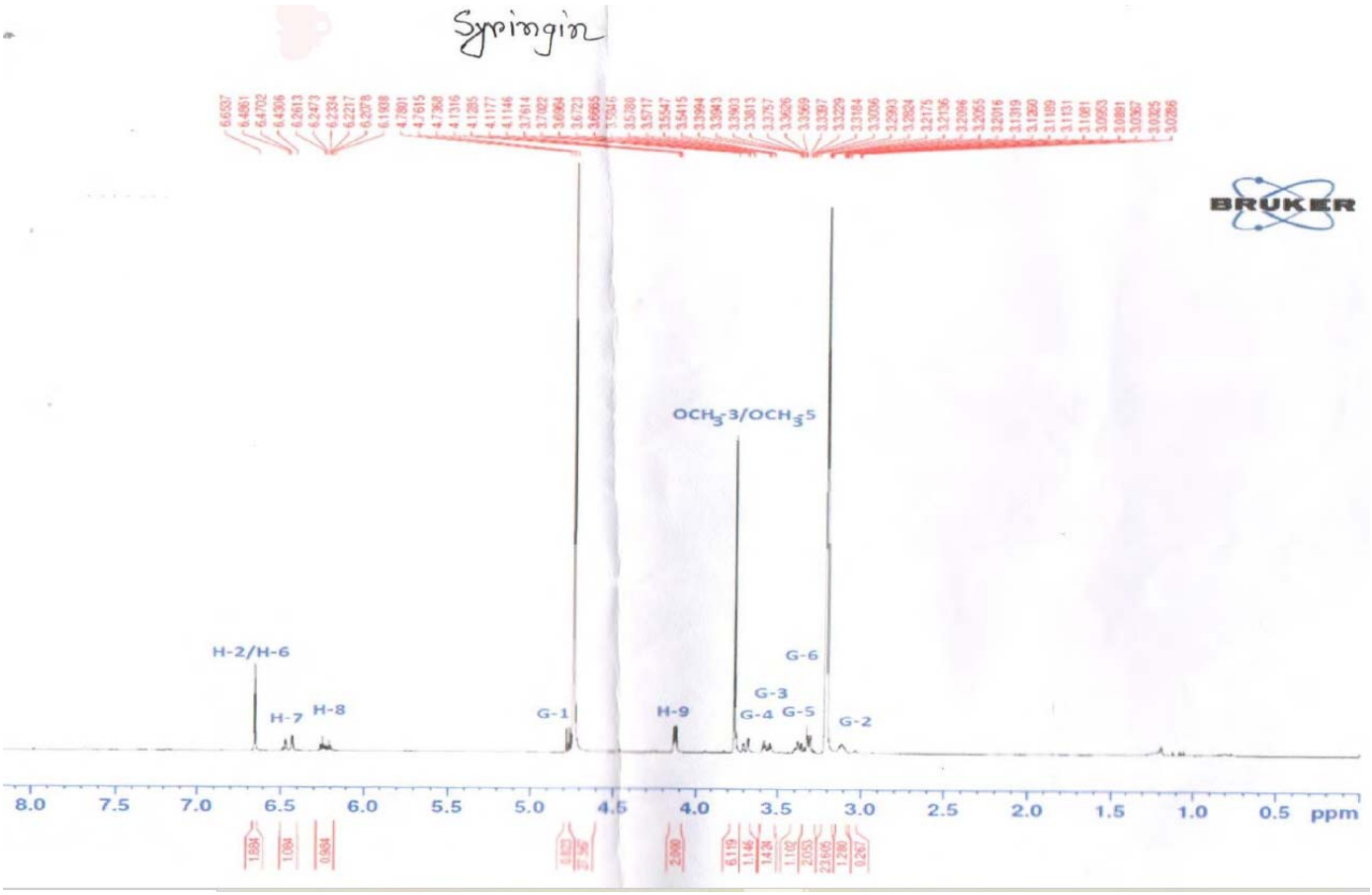


Figure 4.8: ^{1}H-NMR spectrum of compound 2 (*Trans*-Syringin).

4.3.2.3 Characterization of compound 3 as luteolin isolated from *Stevia rebaudiana* (Bert.).

Column chromatography was used to separate compound **3** as white crystalline solid from ESR fraction. The TLC result gave radish yellow color when sprayed with vanilline-sulfururic acid followed by heating and the R_f value was in ethyl acetate: acetic acid: water (8:1:1) solvents system which is soluble in methanol. ^{1}H NMR (400 MHz, CD_3OD) was used for structure elucidation of the compound. Compound **3** also gave two meta couple at δ 6.11, 6.34 and 7.30.

A singlet at 6.44 ppm, one multiplate at 7.30 corresponding to an ortho and meta coupled aromatic proton which is distinctive for a 5,7,3′,4′- tetra substituted flavone. By comparing with the reported proton NMR data [77], compound **3** (**Table 4.15**) was identified as luteolin (5,7,3′,4′- tetra substituted flavone).

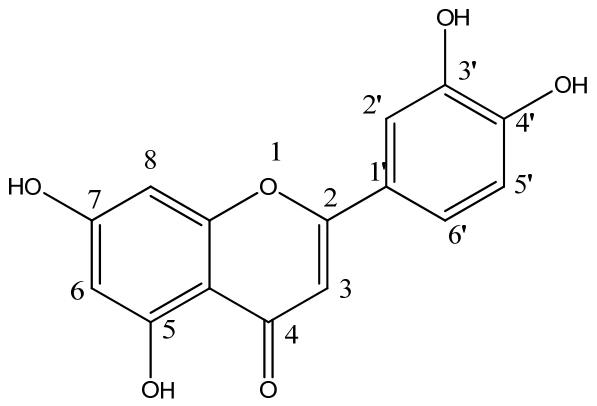


Figure 4.9: Structure of Compound 3 (Luteolin)

Table 4.15: ^{1}H NMR at 400 MHz in CD_3OD reference to TMS of Compound 3.

Position	**Compound 3**	**Luteolin [77] (Solvent- CD_3OD)**
	δ_H	**δ_H**
1	**--**	--
2	**--**	--
3	6.48 (1H, *s*)	6.53 (1H, *s*)
4	**--**	--
5	**--**	--
6	6.21 (1H, *d*, *J*=1.8 Hz)	6.19 (1H, *d*, *J*=1.8 Hz)
7	**--**	**--**
8	6.44 (1H, *s*)	6.43 (1H, *d*, *J*=1.43 Hz)
1'	--	--
2'	7.40 (1H, *m*, overlapped)	7.36 (1H, *d*, *J*=1.8 Hz)
3'	--	--
4'	--	--
5'	6.84 (1H, *d*, *J*=7.5 Hz)	6.88 (1H, *d*, *J*=9 Hz)
6'	7.40 (1H, *m*, overlapped)	7.39 (1H, *dd*, *J*=9 and 1.9 Hz)

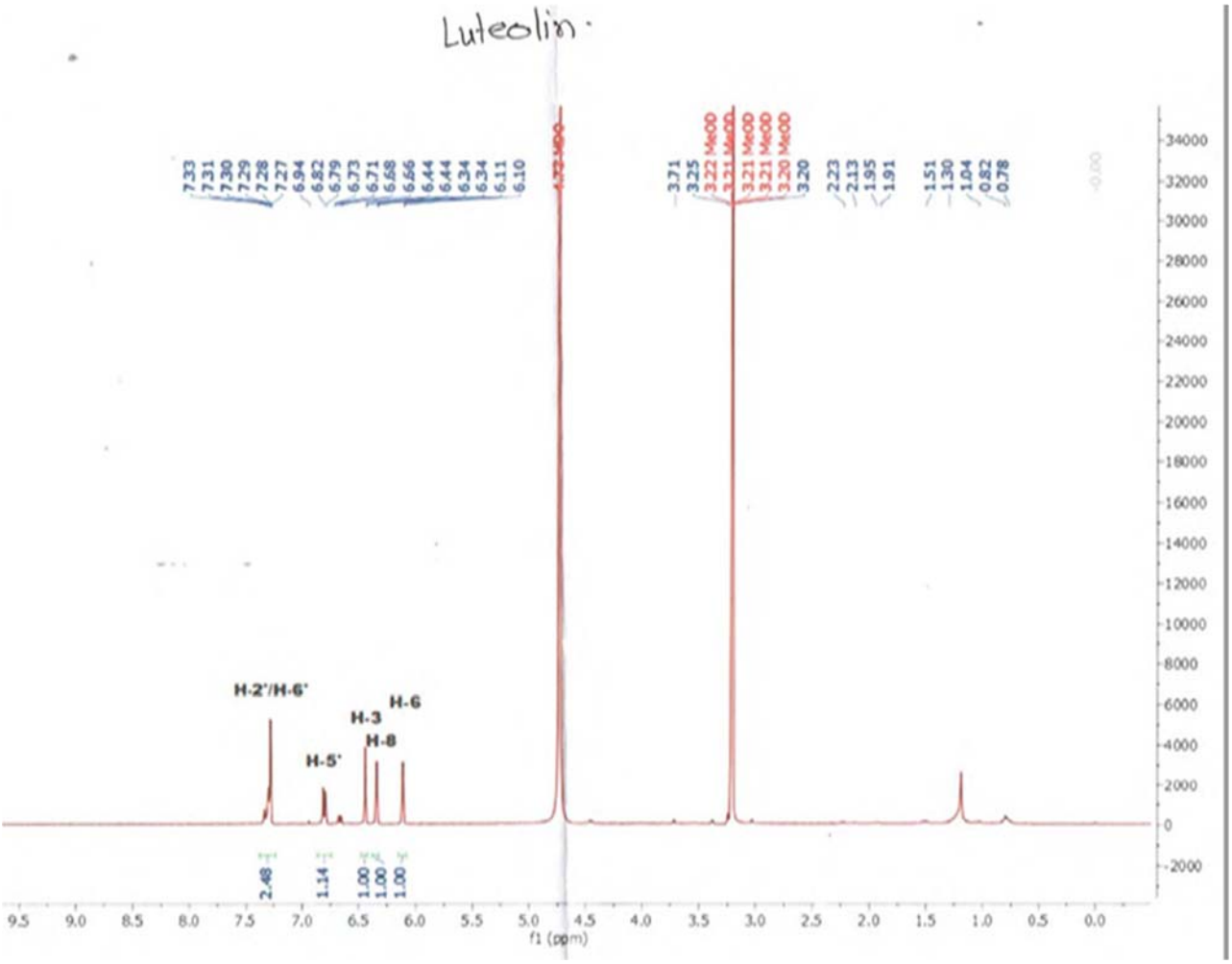


Figure 4.10: ^{1}H-NMR spectrum of compound 3 (luteolin).

4.3.2.4 Characterization of compound 4 as apigenin isolated from *Stevia rebaudiana* (Bert.).

ESR fraction was subjected for column chromatography which gave compound **4**, as a white crystalline solid by the solvent system of ethyl acetate: acetic acid: water (8:1.5:.5) with the R_f value 0.46. The TLC result gave radish yellow color when sprayed with vanillin -sulfuric acid followed by heating which is soluble in methanol. ^{1}H NMR was used for structure elucidation of the compound.

Compound **4** has a pair of doublets at δ 6.94 for H-3′ /H-5′ and 7.63 ppm assigned to H-2′/H-6′ protons and a pair of meta coupled at δ 6.4 and 6.64 ppm to H-6 and H-8 protons which indicate aromatic singlets described by ^{1}H NMR spectrum. The NMR spectra also displayed a singlet at δ 6.8 ppm corresponding to H-3 proton which is identical for a 5,7,4′- trisubstituted flavone. Based on this spectral data and by balancing with published spectra [78], compound **4 was** confirmed as apigenin (5,7,4′-trihydroxyflavone).

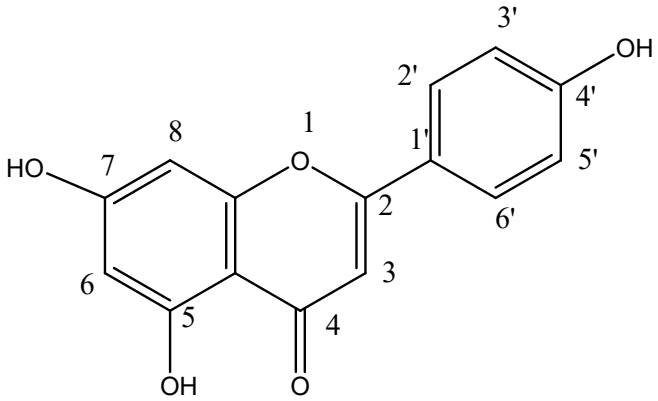


Figure 4.11: Structure of Compound 4 (Apigenin)

Table 4.16: ^{1}H NMR at 400 MHz of Compound 4 in CD_3OD reference to TMS.

Position	**Compound 4**	**Apigenin [78] (Solvent- CD_3 OD)**
	δ_H	**δ_H**
1	**--**	--
2	**--**	--
3	6.8 (1H, *s*)	δ6.62 (1H, *s*)
4	**--**	--
5	**--**	--
6	6.4 (1H, *s*)	6.23 (1H, *br s*)
7	**--**	**--**
8	6.64 (1H, *s*)	δ6.48 (1H, *br s*)

1'	--	--
2'	7.63 (2H, *d*, *J*=8.6 Hz)	7.88 (2H, *d*, *J*=8.6 Hz)
3'	6.94 (1H, *d*, *J*=8.6 Hz)	6.95 (2H, *d*, *J*=8.6 Hz)
4'	--	--
5'	6.94 (1H, *d*, *J*=8.6 Hz)	6.95 (2H, *d*, *J*=8.6 Hz)
6'	7.63 (2H, *d*, *J*=8.6 Hz)	7.88 (2H, *d*, *J*=8.6 Hz)

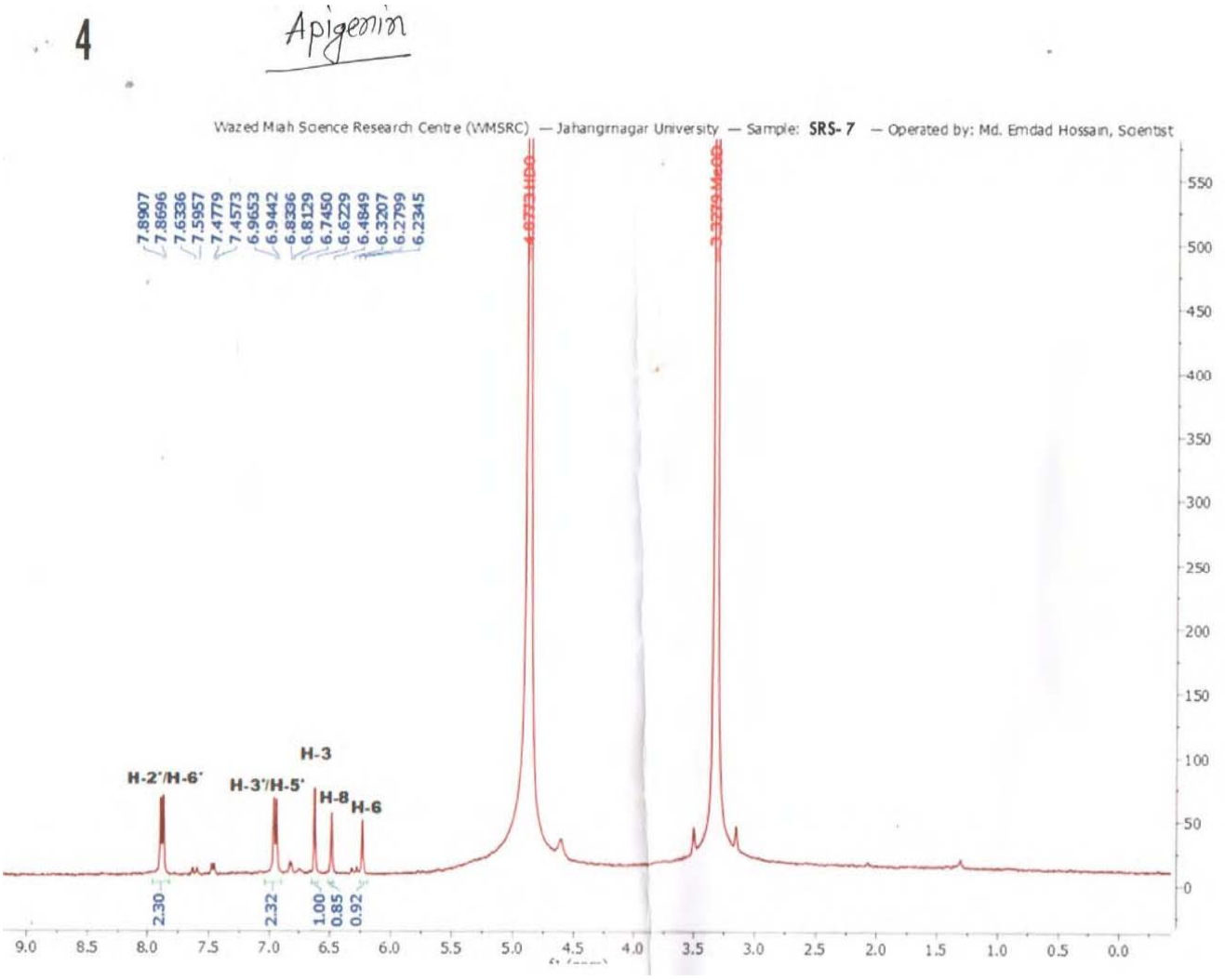


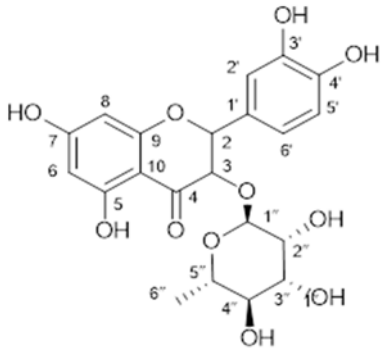
Figure 4.12: ^{1}H-NMR spectrum of compound 4 (apigenin).

4.3.2.5 Characterization of compound 5 as quercitrin isolated from *Stevia rebaudiana* (Bert.).

Compound **5** was separated by silica column chromatography as white powder from SR plants. TLC result gave radish black color when sprayed with vanillin-sulfuric acid followed by heating and the R_f value was 0.71 in ethyl acetate: acetic acid: water (7:2.5:0.5) solvents ratio which is soluble in methanol.^{1}H NMR and ^{13}C NMR was applied for identification and characterization of this component.

Compound **5** showed two meta-coupled hydrogen at δ 6.19 ppm and δ 6.37 ppm corresponding to H-6 and H-8 protons indicating an aromatic doublet. Another meta-coupling was observed between the protons of H-2' and H-6' at δ 7.35 and δ 7.30 ppm. An ABX-type proton signals substituted at δ 7.35, 7.30, 6.90 which resembles that H-3’ and H-4' substituted aromatic ring C. A sp3- hybridized methine proton signal at δ 5.34 and 6 carbon signals range from 17.4-102.1 represents a glycosidic linkage in flavonoid ring. Three proton doublet signals at 0.82 represented a characteristic peak of rhamnose was observed.

The ^{13}C NMR of compound **5** exhibited a signal at δ 177.9 ppm which indicated carbonyl group at the position of C-4 and other peaks at δ 93.6-164.6 ppm which revealed the double bond in aromatic ring. According to the carbon and proton NMR data (**Table 4.17**) as well as with published data [79], the structure of compound **5** (**Figure 4.13**) confirmed as the known compound flavonoid glycoside quercitrin which is constructed a flavonoid quercetin connected to rhamnose at the position of C_3 via C-O-C bonding.



Quercetrin

Figure 4.13: Structure of Compound 5 (Quercitrin)

Table 4.17: ^{13}C NMR at 100 MHz and ^{1}H NMR at 400 MHz of compound 5 in CD_3OD reference to TMS.

Position	Compound 5		Quercitrin [79] (Solvent- CD_3 OD)
	δ_C	δ_H	δ_H
1	--	--	--
2	148.4	--	--
3	134.8	--	--
4	177.9	--	--
5	161.8	--	--
6	98.7	6.19 (1H, *d*, *J*=1.5 Hz)	6.2 (1H, *d, J*=1.9 Hz)
7	164.6	--	--
8	93.6	6.37 (1H, *d*, *J*=1.5 Hz)	6.4(1H, *d, J*=1.9Hz)
9	157.8	--	--
10	104.4	--	--
1'	121.4	--	--
2'	115.6	7.35 (1H, *d*, *J*=1.5 Hz	7.5 (1H, *d, J*=2 Hz)
3'	157.2	--	--
4'	145.1	--	--
5'	115.1	6.9 (1H, *d, J*= 7.5 Hz,)	6.9 (1H, *dd*, *J*=8.4 and 2.0 Hz)
6'	121.6	7.30 (1H, *dd*, *J*=8.0 and 1.5 Hz)	7.7(1H, dd, *J*=8.4 and 2.0 Hz)
1"	102.1	5.34 (1H, *br. s*)	--
2"	170.6	--	--
3"	70.7	--	--
4"	71.9	--	--
5"	17.4	--	--
6"	70.5	0.82 (3H, d, *J*=3.5 Hz)	--

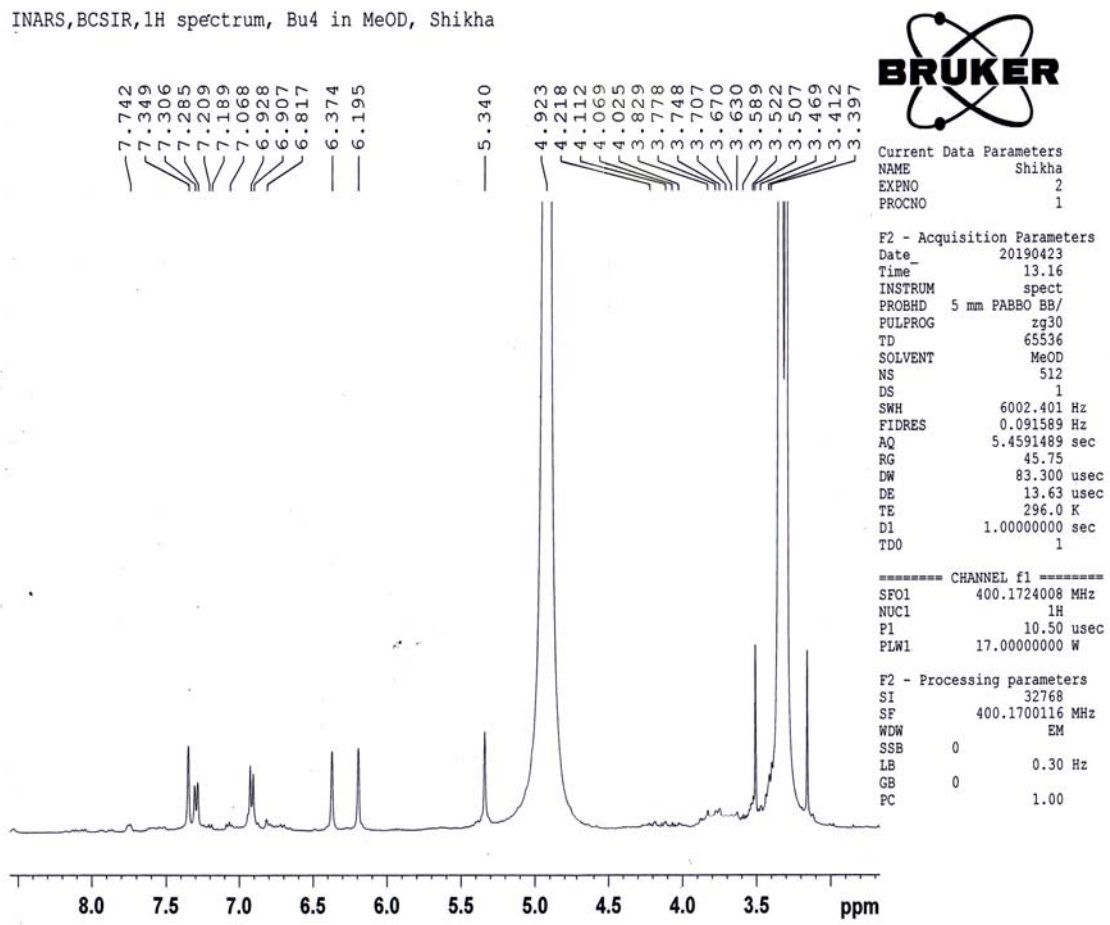


Figure 4.14: ^{1}H-NMR spectrum of compound 5 (quercitrin).

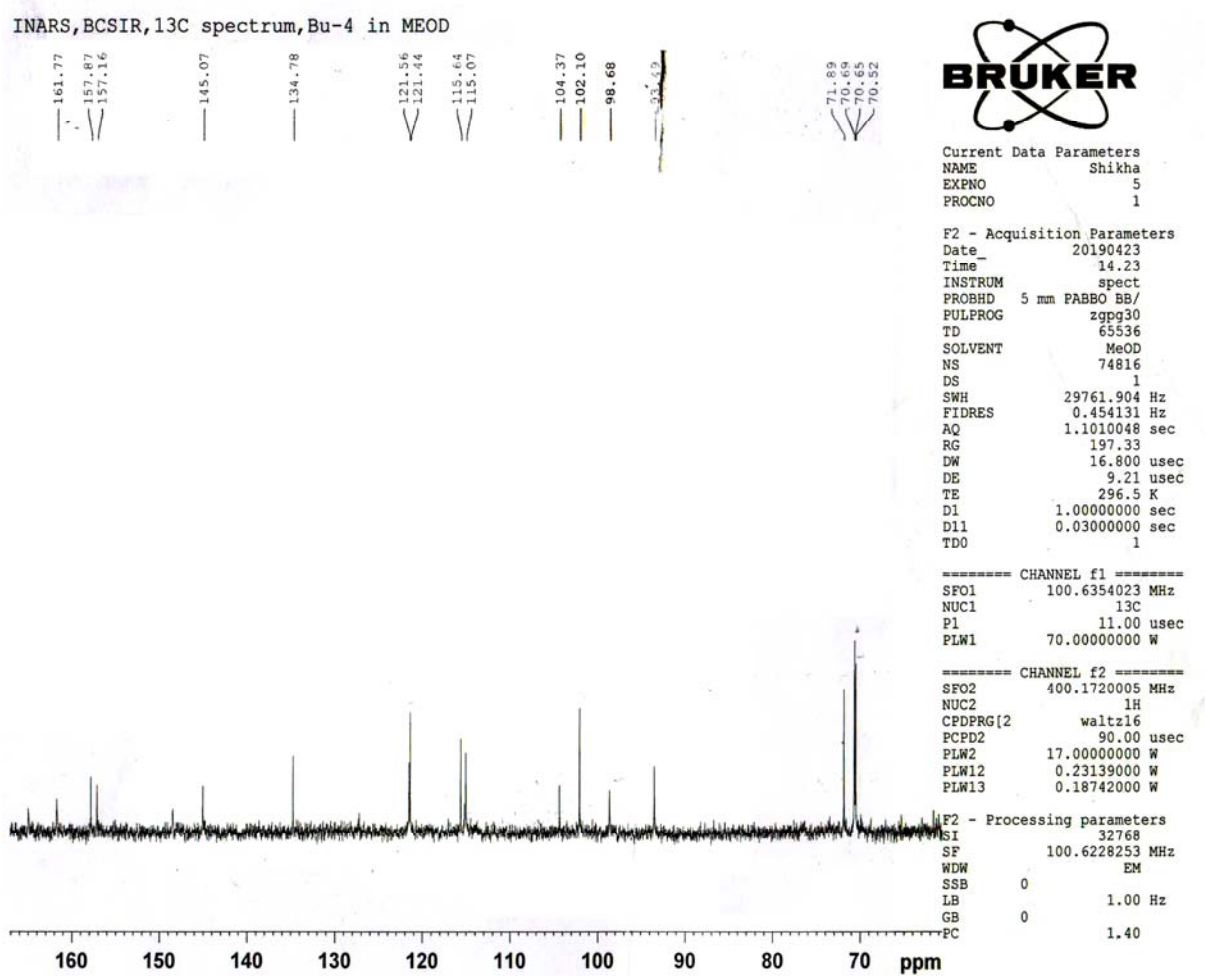


Figure 4.15: ^{13}C NMR spectrum of compound 5 (quercitrin).

4.3.2.6 Characterization of compound 6 isolated from *Stevia rebaudiana* (Bert.).

Compound **6** was separated from ESR fraction of *Stevia rebaudiana* plant subjected by silica gel column chromatography as a yellow crystalline powder. The TLC showed deep red color after spraying with 10% sulfuric acid in methanol. The R_f value was 0.72 in ethyl acetate: acetic acid: water (8:1.5:0.5) solvents system and the compound was found to be soluble in methanol.

The ^{1}H NMR spectrum of compound **6** shows only three signals among which a pair of meta-coupling protons at δ 6.96 (1H, *d*, *J*=2.7 Hz) and δ 7.75 (2H, *d*, *J*=2.6 Hz) along with a sp3 hybridized methyl peak at 1.98(6H, *s*). The integration of meta-coupled protons shows 2H each

and methyl protons 6H. The above spectral features suggested an anthraquinone derivative in which symmetrical structural features can be proposed. Similar kind of anthraquinone derivatives namely chrysophenols were reported earlier [80]. So, the proposed chemical name of the compound would be 1,8-dihydroxy-3,6-dimethylanthracene-9,10-dione. Due to small number of yields, ^{13}C-NMR and Mass spectra could not be performed.

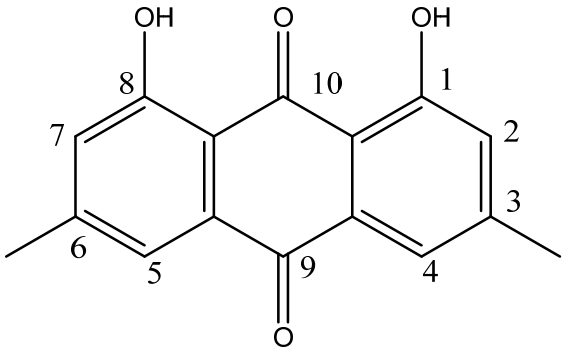


Figure 4.16: Structure of Compound 6 (1,8-dihydroxy-3,6-dimethylanthracene-9,10-dione).

Table 4.18: ^{1}H NMR at 400 MHz of Compound 6 in CD_3OD reference to TMS.

Position	**Compound 6**
	δ_H
1	**--**
2	6.96 (1H, *d*, *J*=2.7 Hz)
3	1.98 (3H, *s*)
4	7.75 (1H, *d*, *J*=2.6 Hz)
5	7.75 (1H, *d*, *J*=2.6 Hz)
6	1.98 (3H, *s*)
7	6.96 (1H, *d*, *J*=2.7 Hz)

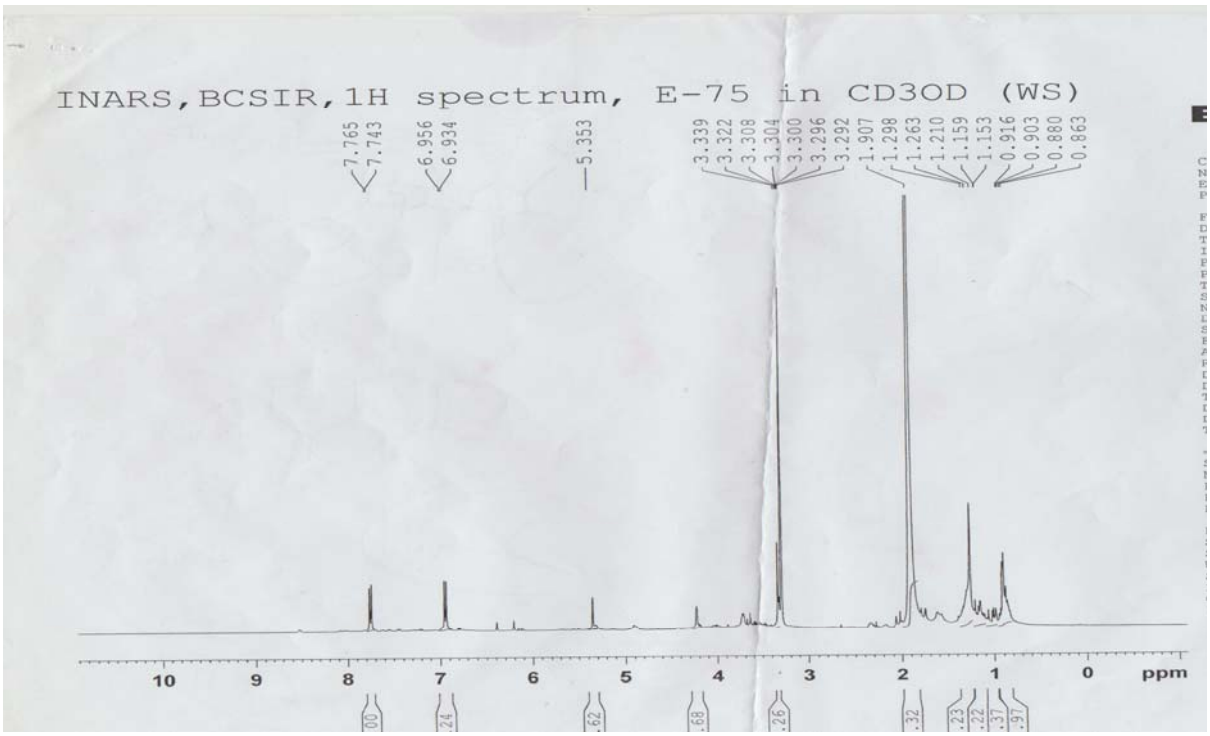


Figure 4.17: ^{1}H-NMR spectrum of compound 6 ((1,8-dihydroxy-3,6-dimethylanthracene-9,10-dione).

4.3.2.7 Characterization of compound 7 as jhanol isolated from *Stevia rebaudiana* (Bert.).

Compound **7** was isolated from *n*-hexane fraction of *Stevia rebaudiana* which was performed by column chromatography and selected solvents were *n*-hexane and ethyl acetate (ratio 9:1). It gave white needle crystal which showed reddish color after spraying on TLC. The R_f value was 0.55 in n-hexane: ethyl acetate (9:1) solvents system which was found to be soluble in $CDCl_3$.

The doublets at 3.43 (1H, *d*, *J*=11 Hz) and 3.13 (1H, *d*, *J*=11 Hz) in ^{1}H NMR spectra of compound **7** suggested that it contains a primary alcohol group. The ^{1}H NMR also represented a distinctive signal at 5.17 (1H, *dd*, *J*= 18 and 2 Hz) and 4.93 (1H, *q*, *J*=12 and 2 Hz) which confirmed the presence of vinyl group at H-15. A total four methyl singlets at 1.31 (3H, *s*), 1.35 (3H, *s*), 0.85 (3H, *s*) and 0.75 (3H, *s*) corresponding to H-16, H-17, H-18 and H-20 respectively. Hence compound **7** might be belonging to the 8,13-epoxylabdene series.

The ^{13}C NMR revealed that the compound **7** showed resonance near 25.1, 27.5, 15.8 & 15.2 ppm corresponding to four methyl group at C-17, C-18, C-19 and C-20. The observation of ^{13}C NMR spectra displayed 20 carbons which indicated that the compound **7** could be a diterpene. Based on above spectral features (**Table 4.19**) the structure (**Figure 4.18**) of compound **7** was elucidated as jhanol as compare with the published data for this compound [81,82].

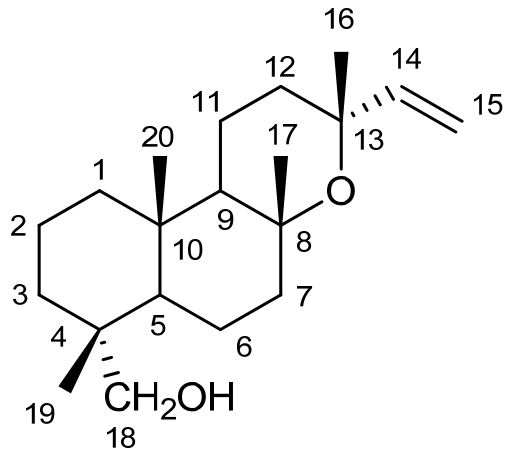


Figure 4.18: Structure of Compound 7 (Jhanol)

Table 4.19: ^{13}C NMR at 100 MHz and ^{1}H NMR at 400 MHz of Compound 7 in $CDCl_3$ reference to TMS.

Position	Compound 7		Jhanol [81, 82] (Solvent- $CDCl_3$)	
	δ_C	δ_H	δ_C	δ_H
1	37.6	--	38.49	--
2	17.2	--	17.83	--
3	36.9	--	35.6	--
4	35.8	--	36.89	--
5	49.9	--	49.64	--
6	19.7	--	19.6	--
7	42.4	--	42.88	--
8	73.3	--	73.23	--
9	55.6	--	55.57	--
10	35.2	--	37.57	--
11	17.8	--	15.28	--
12	35.9	2.34 (2H, *m*)	35.27	--
13	75.0		74.96	--
14	147.5	5.91 (1H, *dd*, *J*=17 and 12 Hz)	147.81	5.92 (1H, *q*, *J*=17 and 11 Hz)
15	110.4	5.17 (1H, *dd*, *J*= 18 and 2 Hz) 4.93 (1H, *q*, *J*=12 and 2 Hz)	110.32	4.95 (1H, *q*, *J*=11 and 2 Hz) 5.15 (1H, q, *J*=17 and 2 Hz)
16	27.5	1.31 (3H, *s*)	28.5	0.80 (3H, *s*)
17	25.1	1.35 (3H, *s*)	25.47	0.88 (3H, *s*)
18	72.8	0.85 (3H, *s*)	72.05	1.31 (3H, *s*)
19	15.8	3.43 (1H, *d*, *J*=11 Hz) 3.13 (1H, *d*, *J*=11 Hz)	17.8	3.47 (1H, *d*, *J*=11 Hz) 3.15 (1H, *d*, *J*=11 Hz)
20	15.2	0.75 (3H, *s*)	15.77	1.35 (3H, *s*)

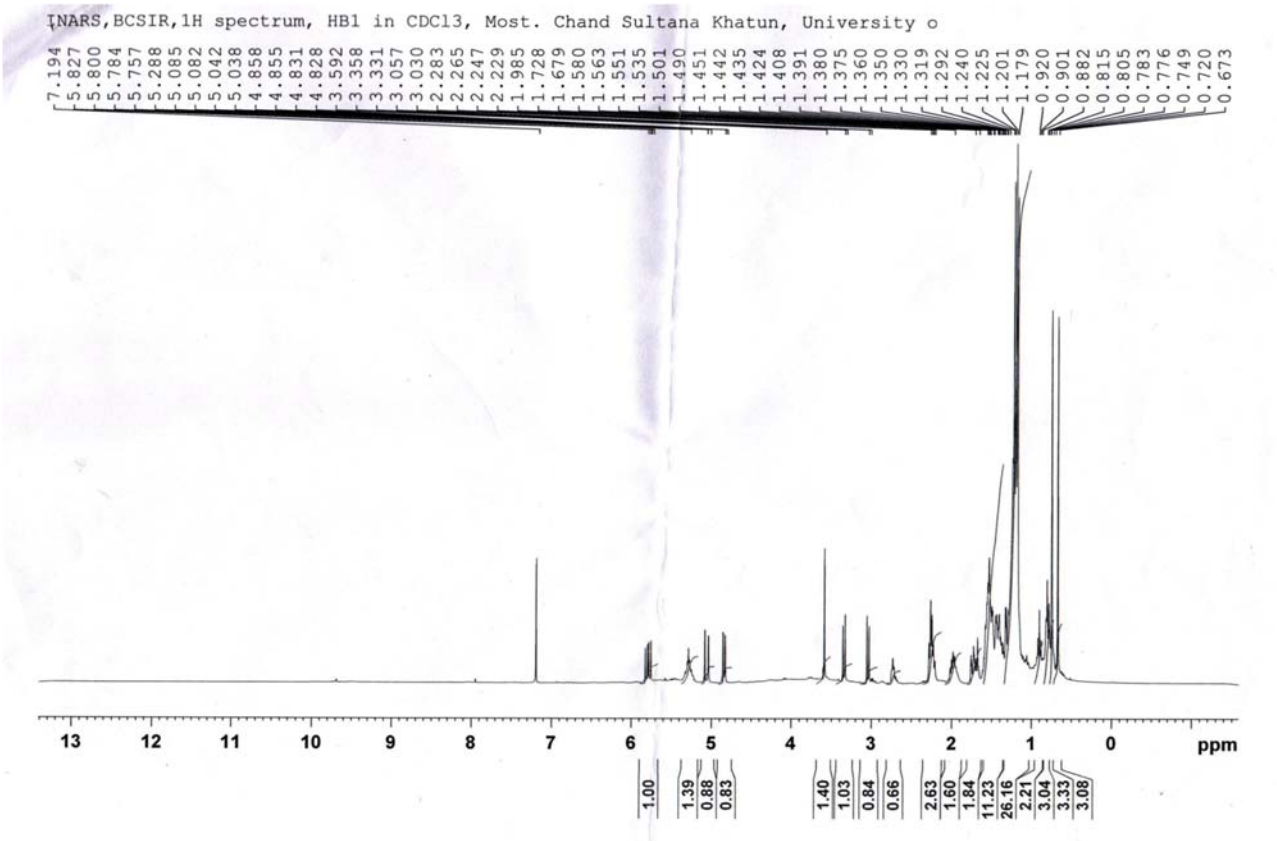


Figure 4.19: ^{1}H-NMR spectrum of compound 7 (Jhanol)

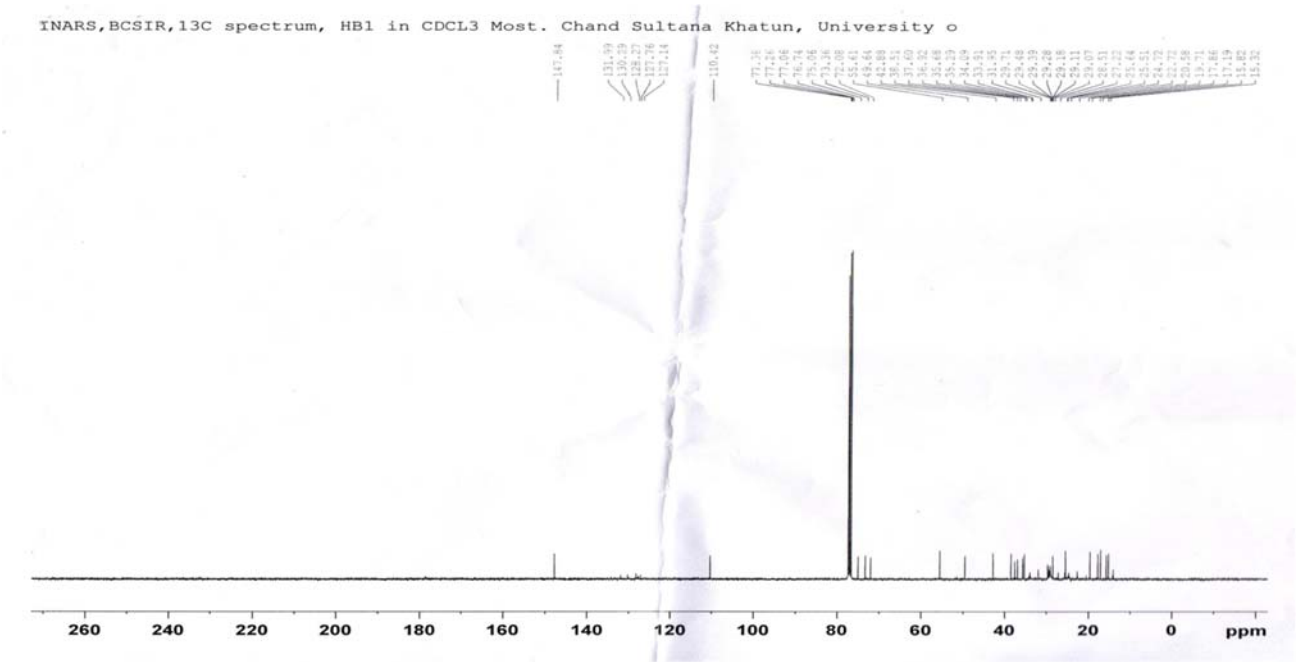


Figure 4.20: ^{13}C-NMR spectrum of compound 7 (Jhanol)

4.3.2.8 Characterization of compound 8 as jhanidiol isolated from *Stevia rebaudiana* (Bert.).

Compound **8** was isolated from *n*-hexane fraction of *Stevia rebaudiana* which was performed by column chromatography and selected solvents were n-hexane and ethyl acetate (ratio 9:1). It gave white needle crystal which showed reddish color after spraying on TLC which was soluble in $CDCl_3$.

The ^1H NMR spectrum of the compound **8** was almost same with jhanidiol, only difference is that compound **8** gave singlet at 78.5 ppm assigned to C-1, which suggest that compound **8** contain both primary and secondary alcohol. The complete NMR data are presented in **Table 4.20**.

According to ^1H NMR & ^{13}C NMR spectral features of compound **8** was deduced as jhanidiol, by comparing with the reported data for this compound [82]. Jhanidiol isolated from *Stevia rebaudiana* for the first time.

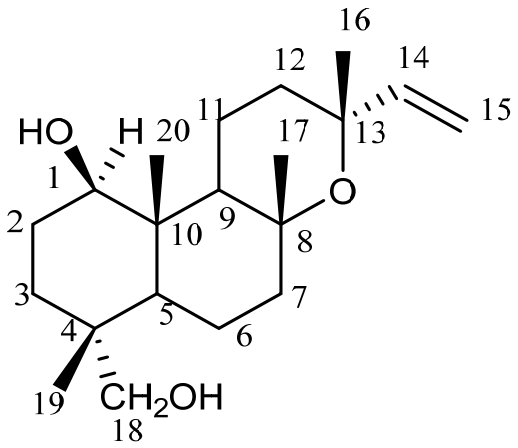


Figure 4.21: Structure of Compound 8 (Jhanidiol)

Table 4.20: ^{13}C NMR at 100 MHz and ^{1}H NMR at 400 MHz of compound 8 in $CDCl_3$ reference to TMS.

Position	Compound 8		Jhanidiol [82] (Solvent- $CDCl_3$)	
	δ_C	δ_H	δ_C	δ_H
1	78.5	--	79.4	--
2	17.2	--	28.89	--
3	36.1	--	34.04	--
4	36.8	--	36.22	--
5	50.2	--	49.26	--
6	20.03	--	19.73	--
7	42.66	--	42.77	--
8	75.8	--	74.80	--
9	56.1	--	55.86	--
10	36.9	--	42.91	--
11	19.1	--	18.49	--
12	35.9	--	35.30	--
13	73.3	--	73.28	--
14	148.7	5.95 (1H, *dd, J*=17 and 12 Hz)	147.96	5.92 (1H, q, *J*=17 and 11 Hz)
15	111.3	5.15 (1H, *dd*, *J*= 17 and 2 Hz) 4.95 (1H, *q*, *J*=12 and 2 Hz)	110.28	5.15 (1H, dd, *J*= 17 and 2 Hz) 4.95 (1H, q, *J*=12 and 2 Hz)
16	28.2	1.35 (3H, *s*)	28.81	1.34 (3H, *s*)
17	26.1	1.31 (3H, *s*)	25.69	1.28 (3H, *s*)
18	66.9	0.80 (3H, *s*)	72.49	0.78 (3H, *s*)
19	18.2	3.45 (1H, *d*, *J*=11) 3.14 (1H, *d*, *J*=11)	16.91	3.45 (1H, *d*, *J*=11) 3.14 (1H, *d*, *J*=11)
20	15.7	0.88 (3H, *s*)	11.71	0.92 (3H, *s*)

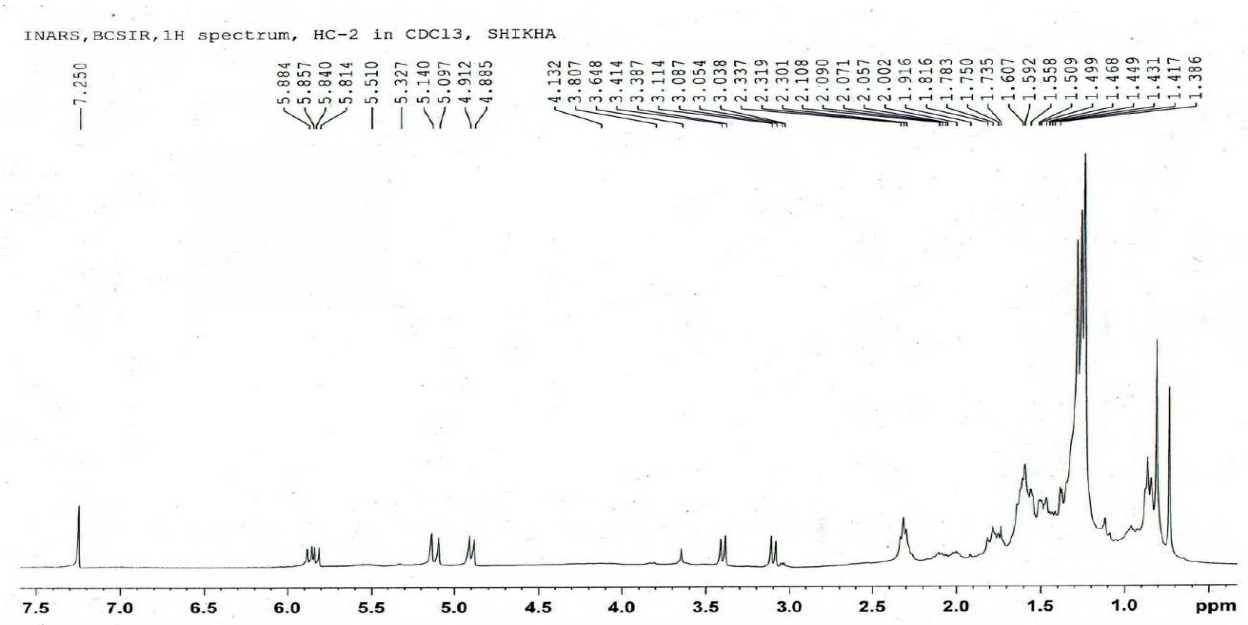


Figure 4.22: ^{1}H-NMR spectrum of compound 8 (Jhanidiol).

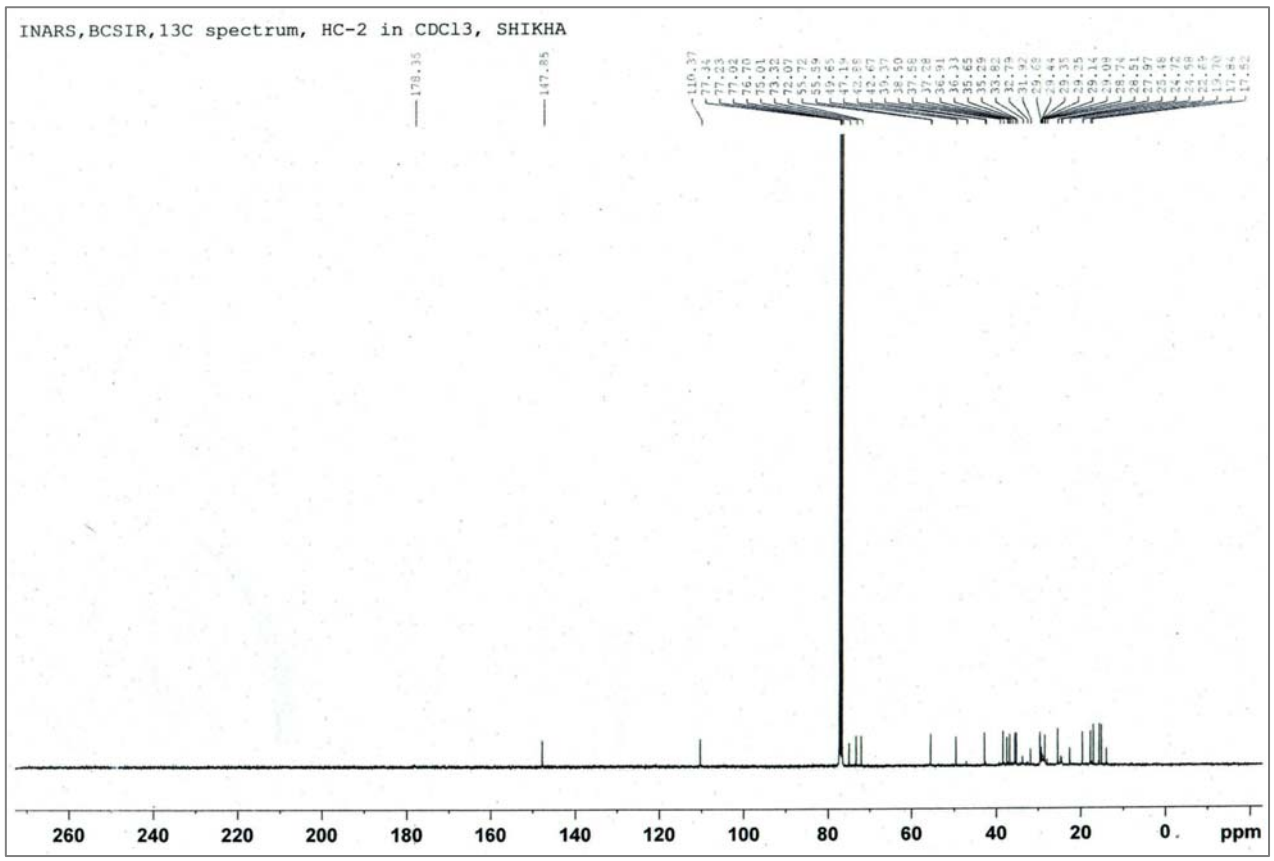


Figure 4.23: ^{13}C-NMR spectrum of compound 8 (Jhanidiol).

4.3.2.9 Characterization of compound 9 as decanoic acid isolated from *Stevia rebaudiana* (Bert.).

Compound **9** was isolated from *n*-hexane fraction of *Stevia rebaudiana* by silica column chromatography using solvent system of n-hexane and ethyl acetate at a ratio of 9:1. It gave white needle crystal which showed reddish color after spraying with vanillin-sulfuric acid on TLC. The R_f value was 0.42 in *n*-hexane: ethyl acetate (9:1) solvents system and the compound found to be soluble in $CDCl_3$.

The ^{1}H NMR spectrum of compound **9** explained that it gave distinctive peak a singlet at δ2.26 assigned for H-14 and multiplet at δ 4.15 for H-15. Besides it resonated at δ 1.80, 1.2 and 1.25 ppm corresponding for H-1, H-2 and H-3. All this document explained that compound **9** is a fatty acid decanoic acid (**Table 4.21** and **Figure 4.24**) when compared to reported data [83].

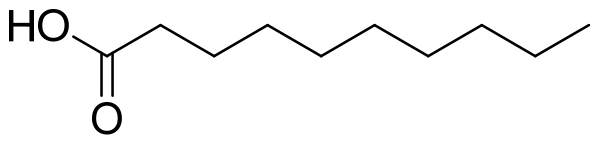


Figure 4.24: Structure of Compound 9 (Decanoic acid)

Table 4.21: ^{1}H NMR at 400 MHz of compound 9 in $CDCl_3$ reference to TMS.

Position	**Compound 9**	**Decanoic Acid [83] (Solvent- $CDCl_3$)**
	δ_H	**δ_H**
1	1.80 (2H, *m*)	2.10 (2H, *m*)
2	1.20 (2H, *m*)	1.70 (2H, *m*)
3	1.25 (2H, *m*)	1.11 (2H, *m*)
4	--	--
5	--	--
6	--	--
7	--	--
8	--	--
9	--	--
10	--	--
11	--	--
12	--	--
13	--	--
14	2.26 (1H, *s*)	2.80 (1H, *s*)
15	4.15 (1H, *m*)	4.55(1H, *m*)

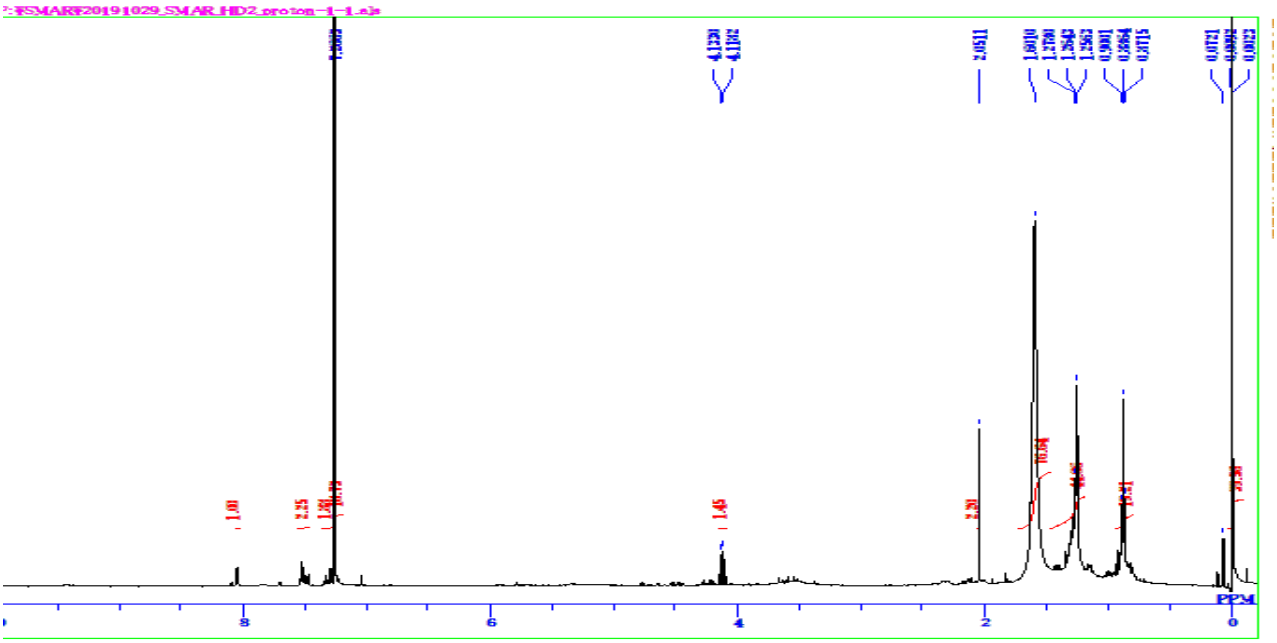


Figure 4.25: ^{1}H-NMR spectrum of compound 9 (Decanoic Acid).

4.3.2.10 Characterization of compound 10 as β-amyrin isolated from *Rhizophora mucronata* (Lam.).

Compound **10** was isolated from DRM fraction of *Rhizophora mucronata* by size exclusion chromatography using sephadex LH-20 as a white needle crystal which showed purple, pink color after spraying on TLC. The R_f value was 0.65 in *n*-hexane: ethyl acetate (7:2) solvents system and the compound found to be soluble in $CDCl_3$.

Eight single peaks for methyl group, one olefinic signal at 5.5 (1H, *t*, *J* = 3.5 Hz) and an oxygenated peak at 3.2 (1H, *dd*, *J* = 1.49 Hz) in ^{1}H NMR spectrum of compound **10**, all suggested that compound **10** olealane type triterpenoid. The double doublet at 3.2ppm also indicative for the hydroxyl group at H-3 (**Figure 4.27)**.

The ^{13}C NMR spectra of compound **10** showed that it contains total 30 carbon and a framework of pentacyclic carbon and at C-12 position double bond appeared (**Figure 4.28**). The above spectroscopic method and by comparing with published data [84] explained that compound **10** is confirmed as β-amyrin.

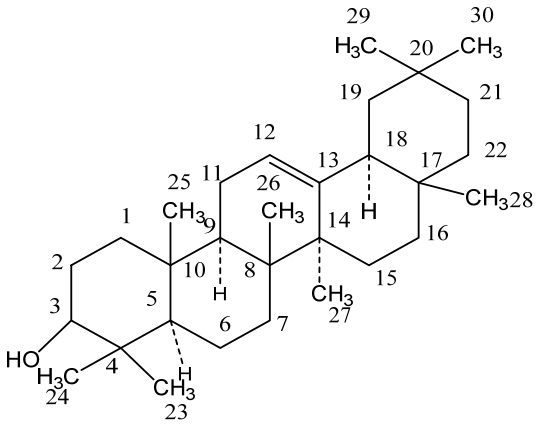


Figure 4.26: Structure of Compound 10 (β-amyrin)

Table 4.22: ^{13}C NMR at 100 MHz and ^{1}H NMR at 400 MHz of Compound 10 in $CDCl_3$ reference to TMS.

Position	Compound 10		β-amyrin [84] (Solvent- $CDCl_3$)	
	δ_C	δ_H	δ_C	δ_H
1	37.7	--	38.89	1.55 (Hb-1) 1.49 (Ha-1)
2	28.07	--	27.44	1.52 (Hb-2) 1.55 (Ha-2)
3	--	3.2 (1H, *dd*, *J* = 1.49 Hz)	79.24	3.20 (1H, *dd*, *J* = 1.49 Hz)
4	39.0	--	38.99	--
5	54.5	--	55.37	0.71
6	18.8	1.53 (1H, *d*, *J* = 2.0 Hz	18.58	1.53 (Hb-6), 1.30 (Ha-6)
7	40.0	--	32.85	--

Table 4.22: ^{13}C NMR at 100 MHz and ^{1}H NMR at 400 MHz of Compound 10 in $CDCl_3$ reference to TMS.

8	48.7	--	40.81	--
9	38.7	1.98 (1H, *s*)	47.43	1.95
10	25.9	--	37.15	--
11	--	1.99 (1H, *s*)	23.75	1.84
12	--	5.5 (1H, *t*, *J* = 3.5 Hz)	121.93	5.16 t (1H, *t*, *J* = 3.5 Hz)
13	25.9	--	145.41	--
14.	41.3	--	41.92	--
15.	27.15	--	26.36	--
16	33.1	--	27.14	--
17	38.99	--	32.70	--
18	34.8	2.01 (1H, *s*)	47.84	1.89
19	15.6	2.10 (1H, *s*)	47.03	1.59
20	28.00	--	31.03	--
21	33.7	1.66 (1H, *m*)	37.35	1.66
22	31.3	--	34.94	--
23	--	0.79 (1H, *s*)	15.71	0.77 (1H, *s*)
24	--	.89 (1H, *s*)	26.21	0.98 (1H, *s*)
25	--	.93 (1H, *s*)	28.31	0.92 (1H, *s*)
26	--	89 (1H, *s*)	15.80	0.94 (1H, *s*)
27	--	1.12 (1H, *s*)	17.01	1.11 (1H, *s*)
28	--	1.3 (1H, *s*)	28.62	0.81 (1H, *s*)
29	--	0.85 (1H, *s*)	33.56	0.85 (1H, *s*)
30	--	0.85 (1H, *s*)	23.91	0.85 (1H, *s*)

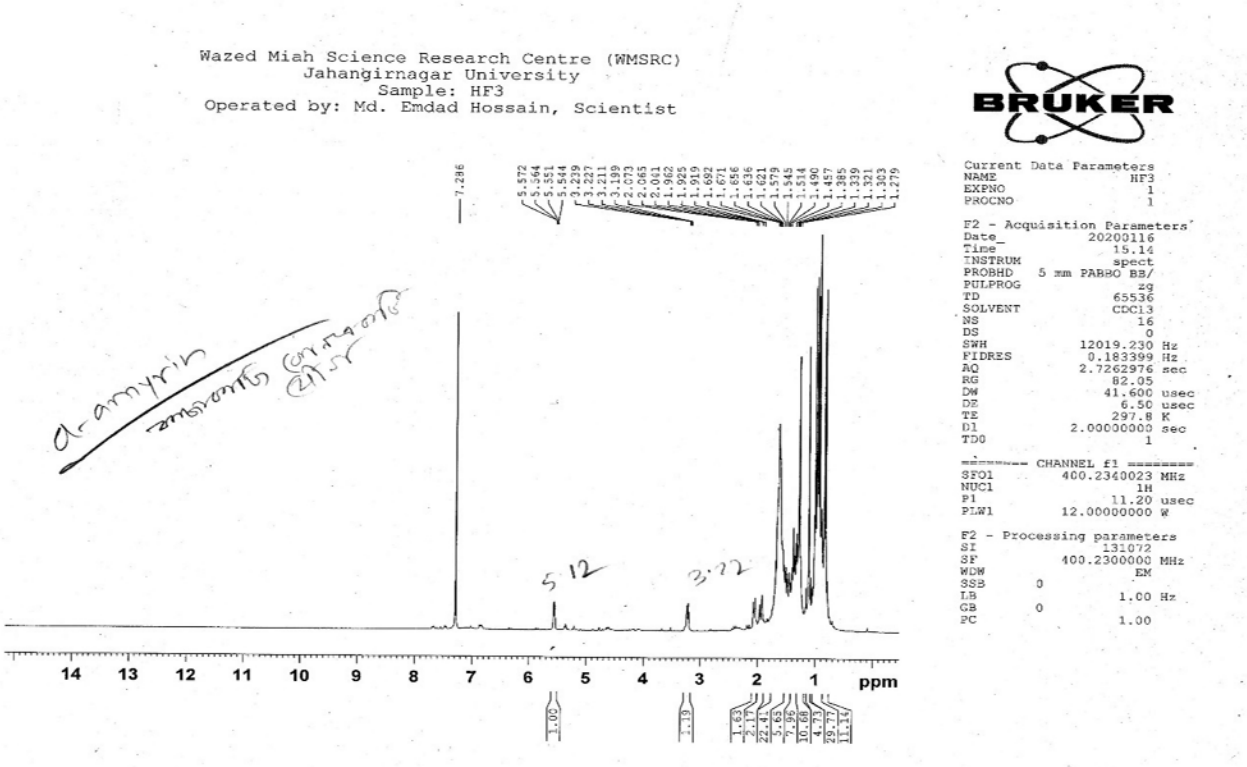


Figure 4.27: ^{1}H-NMR spectrum of compound 10 (Beta amyrin).

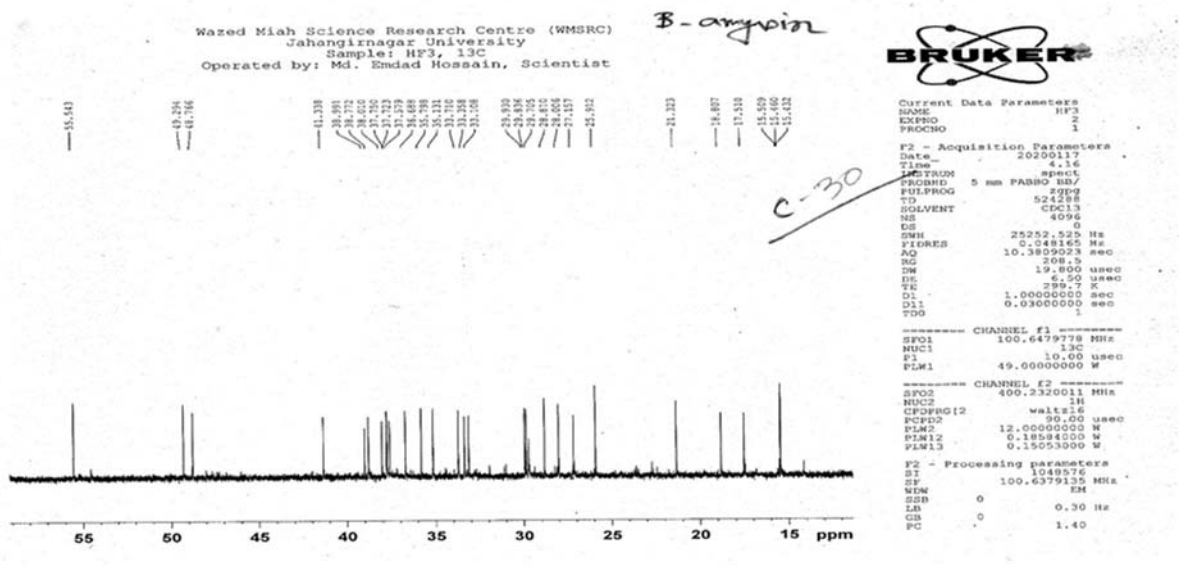


Figure 4.28: ^{13}C-NMR spectrum of compound 10 (Beta amyrin).

4.3.2.11 Characterization of compound 11 isolated from *Rhizophora mucronata* (Lam.).

The size exclusion chromatography using sephadex LH-20 was used to isolate the compound **11** from DRM fraction and identified as a new compound from the following data. The compound **11** accounted as light yellowish amorphous powder and the molecular formula is **C_{18} H_{28} O_4.** designated from the HR-ESIMS the quasimolecular ion [M+ CH_3CN] $^+$ peak at m/z 349.1747 and the calculated m/z [M+ CH_3CN] $^+$ is 349.1753 **(Figure 4.38**). After spraying vanilline-sulfuric acid solution on the TLC it gave yellow colour which turned into deep yellow color after heating 80^0C for 1min. The R_f value was found 0.47 in toluene: ethyl acetate (4:7) solvents system and the compound found to be soluble in $CDCl_3$. The structure of the compound was elucidated by extensive NMR studies like ^{1}H NMR, ^{13}C NMR, HSQC, DEPT, HMBC and COSY spectra.

The ^{1}H NMR spectrum of compound **11** revealed two doublets at δ2.29 and 2.50 which could be assigned for H-1. Additionally, two methyl protons (δ_H 1.06, δ_C 23.78; δ_H 1.13, δ_C 22.9), an olefinic methyl proton at δ_H 1.94, δ_C 18.86), and an olefinic one proton signal at δ_H 5.96 (δ_C 127.0) can be assignable to a bicyclic diterpenoid moiety. A quaternary carbon signals appeared at δ_C 79.08 indicated hydroxyl group attached to equatorial position at C-10. The position of the methyl groups was confirmed by HSQC data. The above partial arrangement was further confirmed by HMBC correlation of the methyl protons such as H-19 to C5, H-18 to C-4 and H-20 to C-9. Two vicinal coupled protons at δ5.83 and double doublet at δ5.90 in the ^{1}H NMR spectra are designated for SP^2 hybridized double bond and protons are located between H-11 and H-12 which was confirmed by COSY signals. The coupling constant (J) between H-11 and H-12 was found 15.6 Hz resembling the configuration as *E*. Two multiplets at δ 3.80 and δ 4.00 ppm indicated aliphatic alcohols at position of H-17.

The ^{13}C NMR revealed that the compound **11** represented 18 carbon resonance that was pillar of molecular formula and a bisnor-type diterpenoid was demonstrated of this spectral characteristic [85]. About 14 carbons out of 18 is attached with proton which was confirmed by DEPT and HSQC experiment. The carbon spectra also gave distinctive chemical shift which are about δ127 and 162.5 assigned to C-3 and C-4 respectively. It has been shown that the chemical shift of C-2 demonstrated greater value 197.85 ppm which indicate C-2 is oxygenated which is further confirmed by HMBC correlation of H-1 with carbonyl carbon. The ^{13}C NMR also resonate to

higher region at 129.04 and 135.7 ppm corresponding to C-11 and C-12. The sp^2 carbons at δ129.04 and 135.7, two sp^3 carbons δ 68.09 and 24.03 along with doublet methyl protons δ 1.33 allowed us to enumerate a partial structure of -CH=CH-C(OH)H-CH_3. Similar side chain was reported earlier in 14, 15-bisnor-3,11 *E*-kolavadien-13-one except a reduced form a carbonyl group at C-13 [86,87]. The side chain was found to be attached to C-9 of the bicyclic part of the diterpenoid which was confirmed by HMBC correlation of H-11 to C-10. All this evidence indicates that compound **11** can be deduced as unusual C-18 clerodane type diterpenoids containing 2-oxo kolavenic skeleton [88]. C-18 bisnor-type clerodane diterpenoid with lack of two carbon is very rare in nature. 14, 15-bisnor-3, 11 *E*-kolavedein-13-one was reported earlier from *P. viridis* [88] and *P. longifolia* [89]. Another unusual bisnor-clerodane diterpenoid was reported from *Polyalthia simiarum* [90]. To the best of our knowledge, this is the third report of naturally occurring C-18 bisnor-clerodane diterpenoid from this species. Accordingly, the 1D and 2D NMR data and comparing with same type of diterpene [91] it was confirmed that it is diterpene **2**-oxo-14,15-bisnor-3,11 *E*-Kalavadien-10, 13, 17-triol and was named as Mehejabaien A.

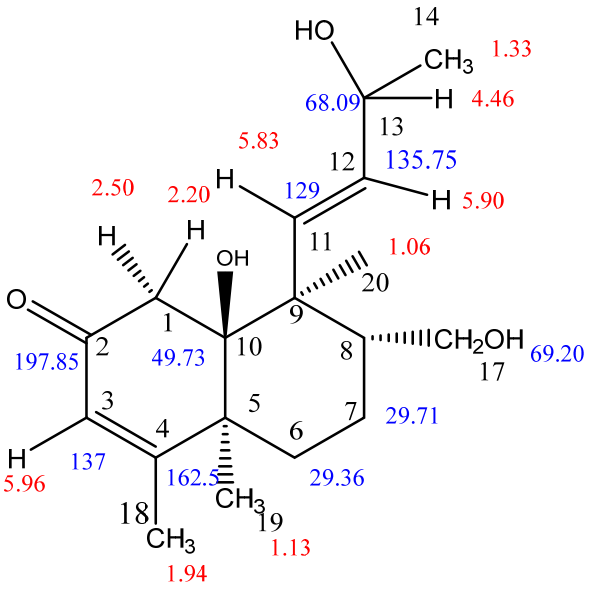


Figure 4.29: Structure of Compound 11 (2-oxo-14,15-bisnor-3,11 *E*-Kalavadien -10, 13, 17-triol).

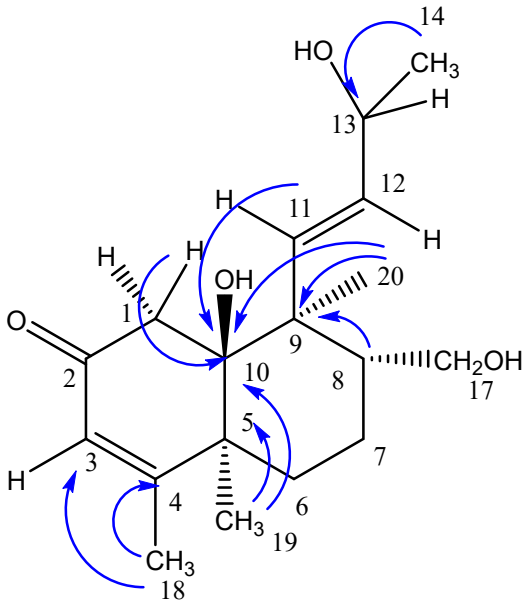


Figure 4.30: Key HMBC (blue) correlation for compound 11.

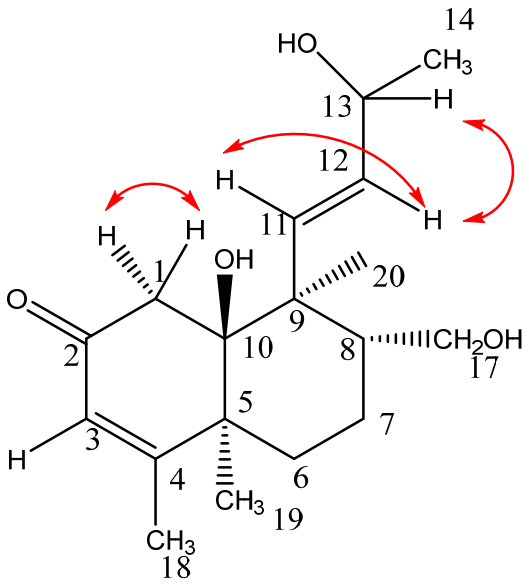


Figure 4.31: Key COSY (Red) correlation for compound 11

Table 4.23: ^{13}C NMR at 100 MHz and ^{1}H NMR at 400 MHz of compound 11 in $CDCl_3$ reference to TMS.

Position	**Compound 11**		
	δ_C	**δ_H**	HMBC
1	49.73	2.29 (1H, *d*,16.8 Hz), 2.50 (1H, *d*,17.2Hz)	**10**
2	197.85	**--**	**--**
3	127.0	5.96 (1H, *s*)	**--**
4	162.5	**---**	**--**
5	41.16	**---**	**--**
6	29.36	Not detectable	**--**
7	29.71	Not detectable	**--**
8	31.93	1.30 (1H, *m*)	**9**
9	22.7	--	**--**
10	79.08	---	**--.**
11	129.04	5.83 (1H, *d*, *J* = 15.6 Hz)	**10**
12	135.7	5.90 (1H, *dd*, *J* = 15.6 and 4.2 Hz)	**--**
13	68.08	4.46 (1H, *m*)	**13 and 12**
14	24.03	1.33(3H, *d*, 6.4Hz)	**13**
17	64.18	3.80(1H, *m*), 4.00(1H, *m*)	**--**
18	18.86	1.94 (3H, *s*)	**3 and 4**
19	22.90	1.13(3H, *s*)	**4, 5, and 6**
20	23.78	1.06(3H, s)	**9, 10 and 11**

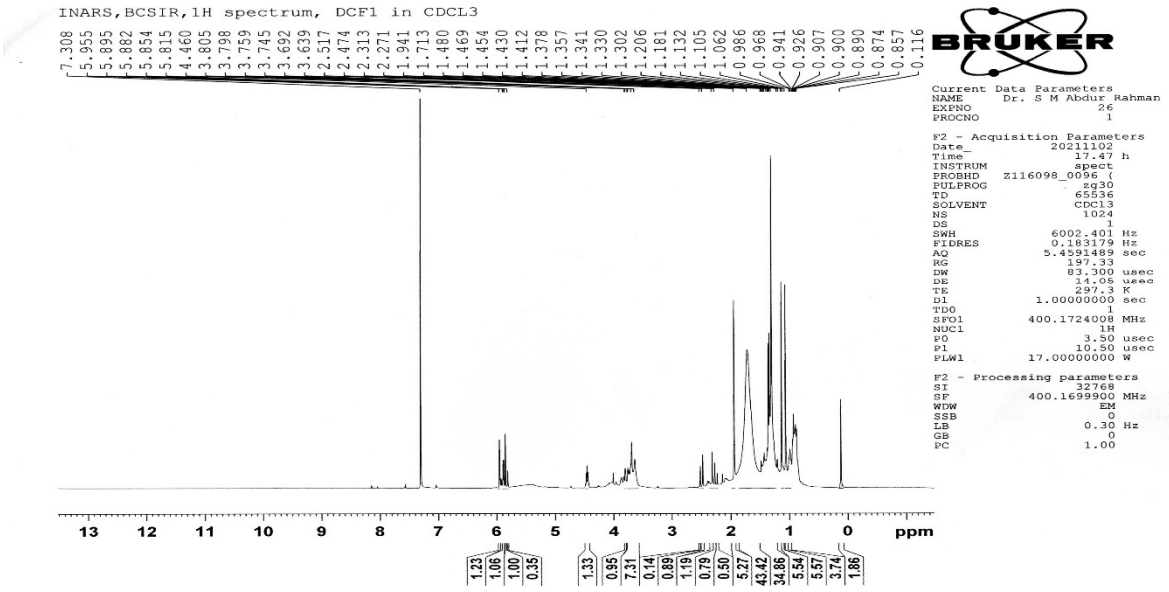


Figure 4.32: ^{1}H-NMR spectrum of compound 11 (2-oxo-14,15-bisnor-3,11 *E*-Kalavadien- 10, 13, 17-triol).

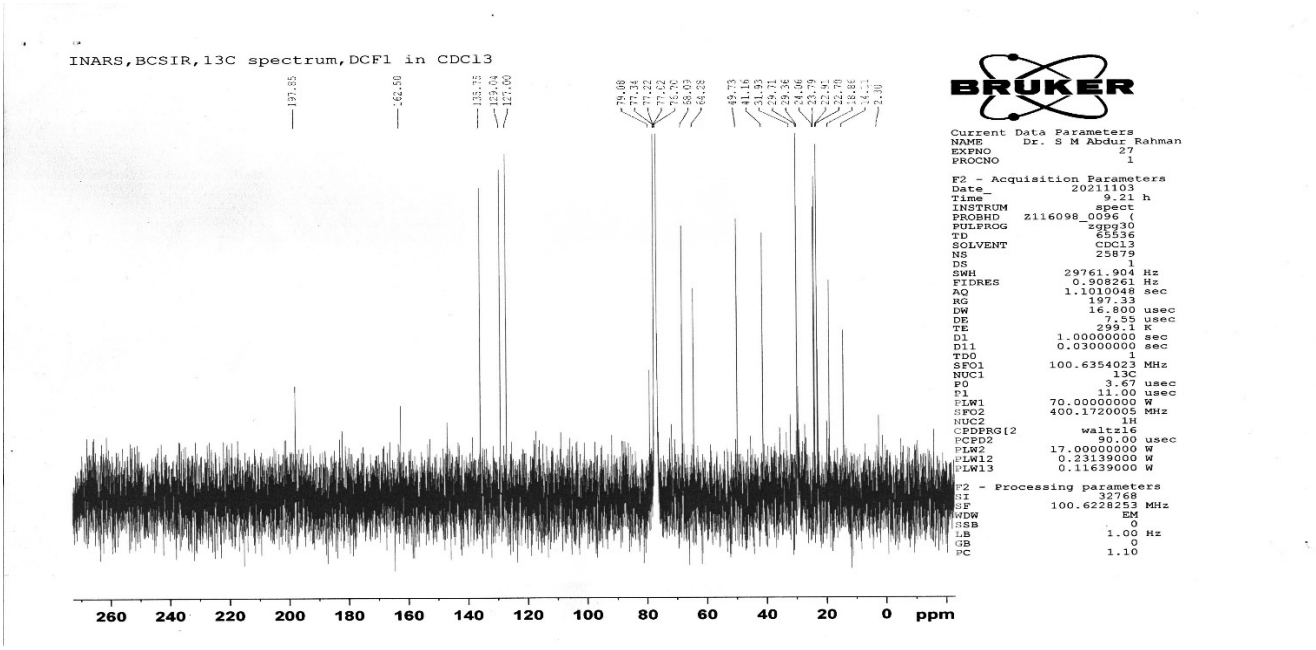


Figure 4.33: ^{13}C-NMR spectrum of compound 11 (2-oxo-14,15-bisnor-3,11 *E*-Kalavadien-10, 13, 17-triol).

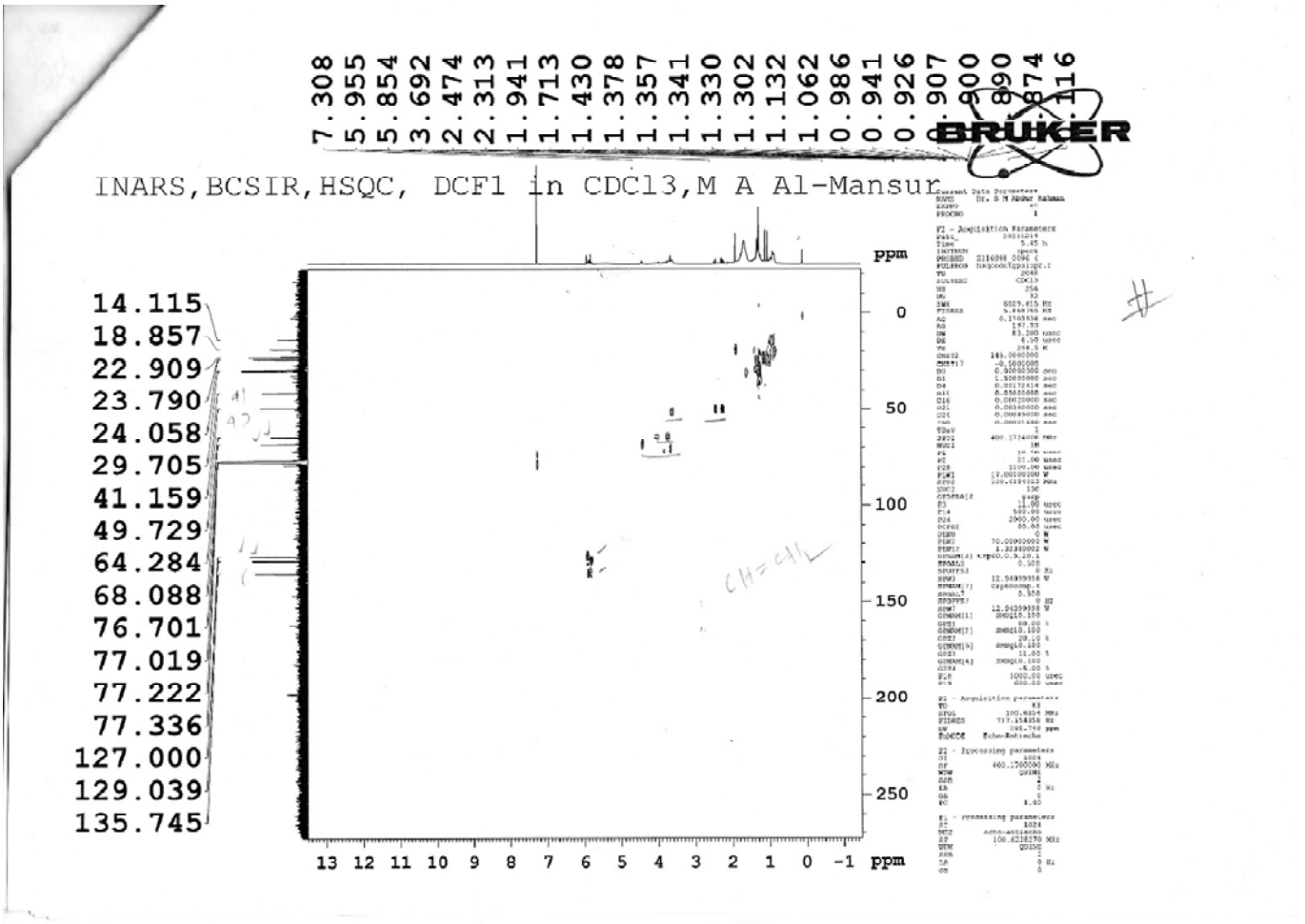


Figure 4.34: HSQC spectrum (400 MHz, $CDCl_3$) of compound 11 (2-oxo-14,15-bisnor-3,11 *E*-Kalavadien- 10, 13, 17-triol).

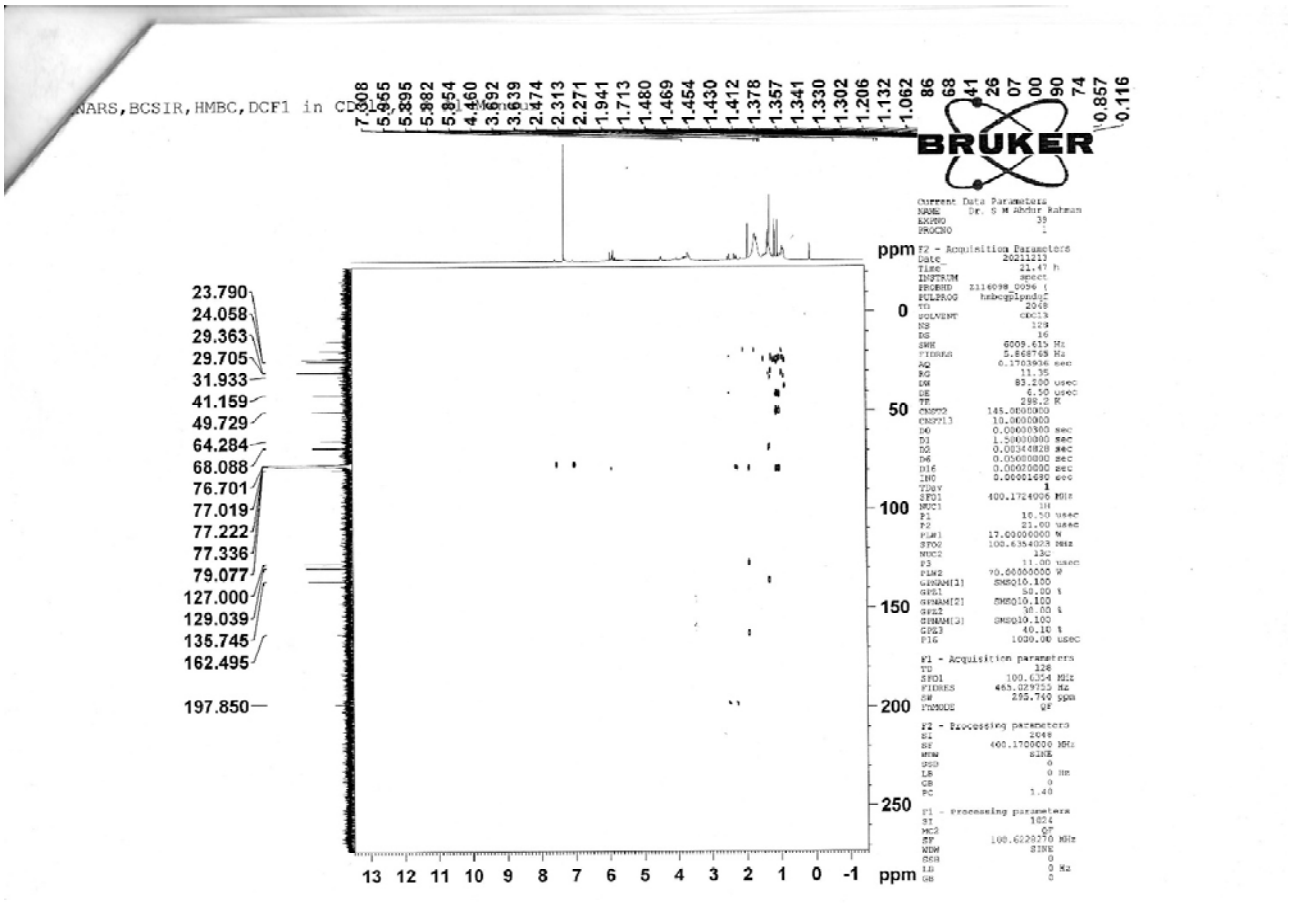


Figure 4.35: HMBC spectrum (400 MHz, $CDCl_3$) of compound 11 (2-oxo-14,15-bisnor-3,11 *E*-Kalavadien -10, 13, 17-triol).

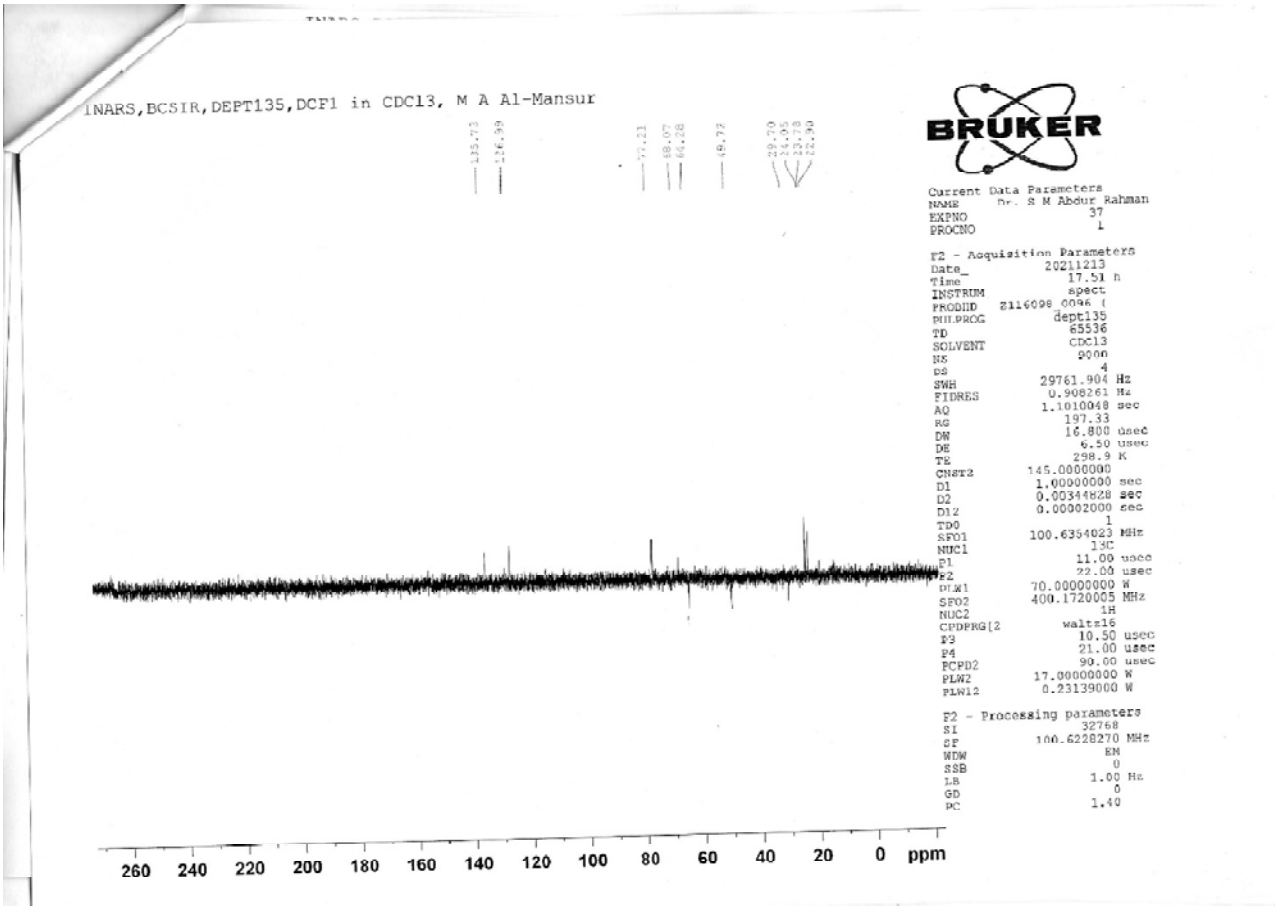


Figure 4.36: DEPT-135 spectrum (400 MHz, $CDCl_3$) of compound 11 (2-oxo-14,15-bisnor-3,11 *E*-Kalavadien -10, 13, 17-triol).

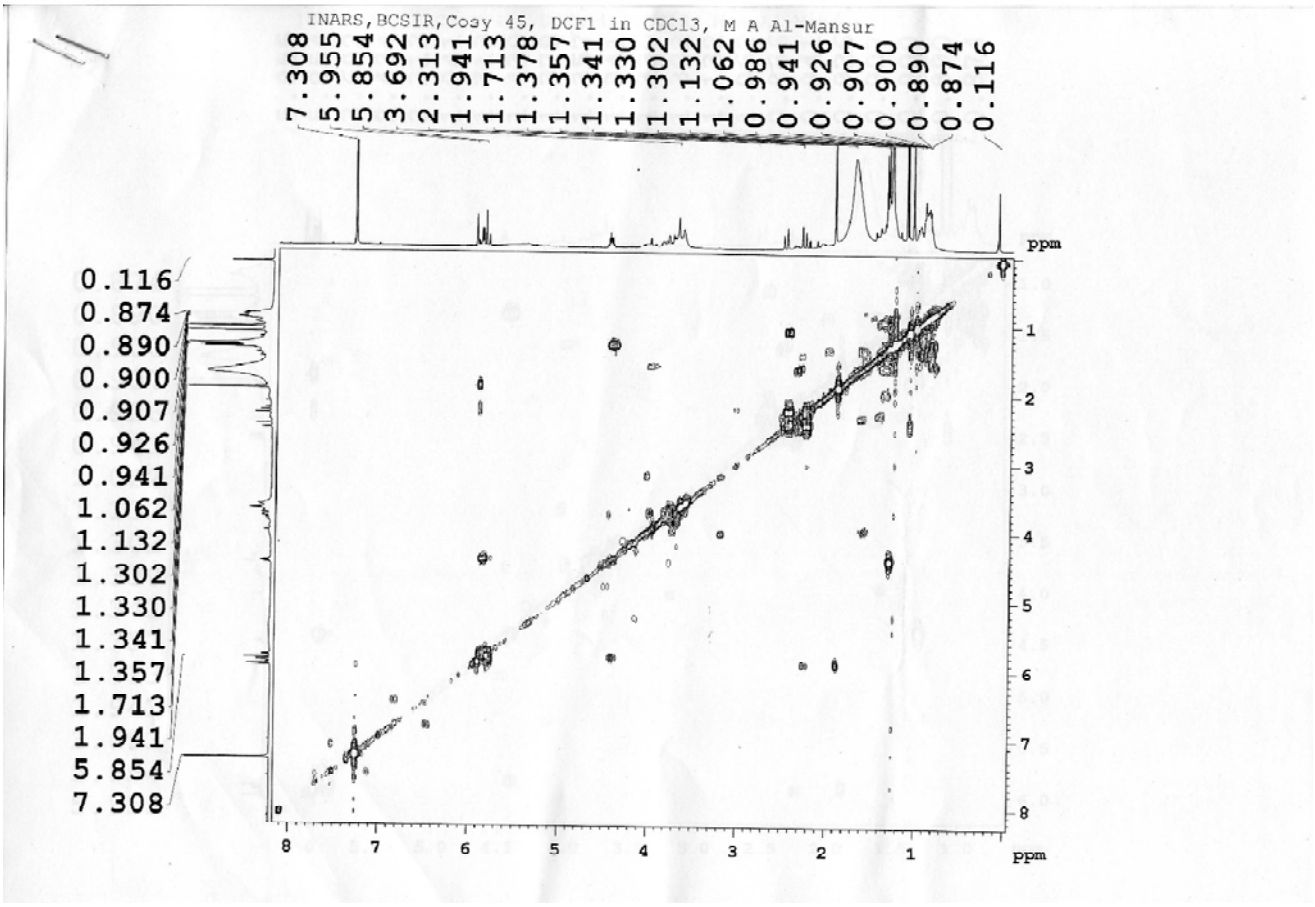


Figure 4.37: COSY spectrum (400 MHz, $CDCl_3$) of compound 11 (2-oxo-14,15-bisnor-3,11 *E*-Kalavadien -10, 13, 17-triol).

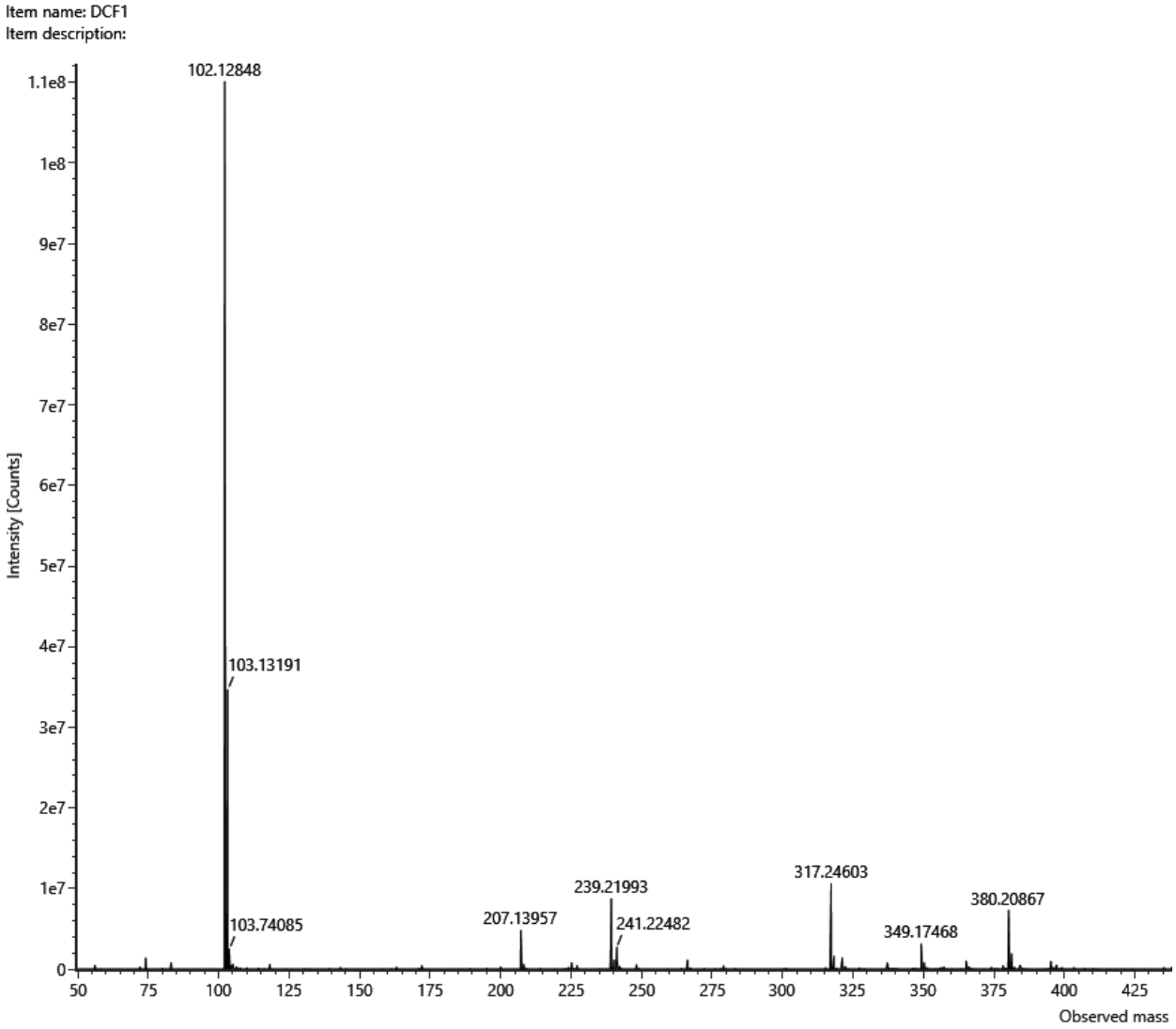


Figure 4.38: HR-ESIMS of compound 11 (2-oxo-14,15-bisnor-3,11 *E*-Kalavadien- 10, 13, 17-triol).

4.3.2.12 Characterization of compound 12 as β-Sitosterol isolated from *Rhizophora mucronata* (Lam.).

Compound **12** was isolated from DRM fraction of *Rhizophora mucronata* (RM) by sephadex LH-20, a size exclusion chromatography as a white needle crystal which showed deep purple color after spraying by vanillin-sulfuric acid on TLC. The R_f value was 0.65 in *n*-hexane: ethyl acetate (8:2) solvents system and the compound **12** was found to be soluble in $CDCl_3$.

The multiplet at δ3.32ppm assign for H-3 in ^1H NMR spectra of compound **12** exhibited the posture and abundance which indicated steroidal center. The observation showed that the characteristic signal of olefinic H-6 of the steroidal skeleton was given at δ5.32 (*t*, 1H). The spectrum also described that signal at δ 1.10 (*s*,3H) and δ 0.98 (*d*,3H) ppm corresponding to a double tertiary methyl group at C-18 and C-19 respectively.

Based on spectral data (**Figure 4.40**) the identity of the compound **12** was confirmed as β-Sitosterol as compared with published report [92] (**Table 4.24**).

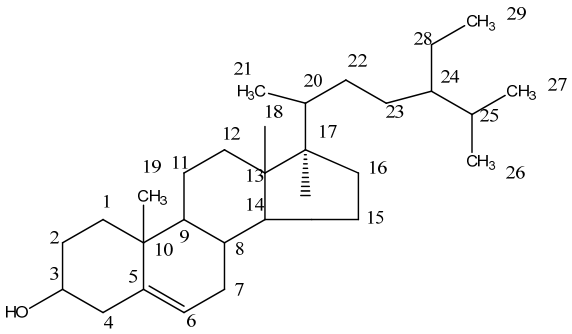


Figure 4.39: Structure of Compound 12 (β-Sitosterol)

Table 4.24: ^{1}H NMR at 400 MHz of Compound 12 in $CDCl_3$ reference to TMS.

Position	Compound 12	β-Sitosterol [92] (Solvent- $CDCl_3$)
	δ_H	δ_H
1	--	--
2	--	--
3	3.32 (*m*, 1H)	3.54 (1H, *m)*
4	--	--
5	--	--
6	5.32 (*t*, 1H)	5.36 (1H, *br s*)
7	1.86 (*m*, 2H)	--
8	1.66 (*m*, 1H)	--
18- CH3	1.10 (*s*,3H)	0.70 (3H, *s*)
19- CH3	0.98 (*d*,3H)	0.91 (3H, *s*)
21	0.92 (*d*,3H)	0.93 (*d*, *J*=6.5 Hz)
26	0.83 (*d*, 2H),	0.82 (*d*, *J*=6.5 Hz)
27- CH3	0.95 (*d*, 2H)	0.84 (*d*, *J*=6.5 Hz)
29- CH3	0.87 (*m*,1H)	0.87 (*t*, *J*=7.6 Hz)

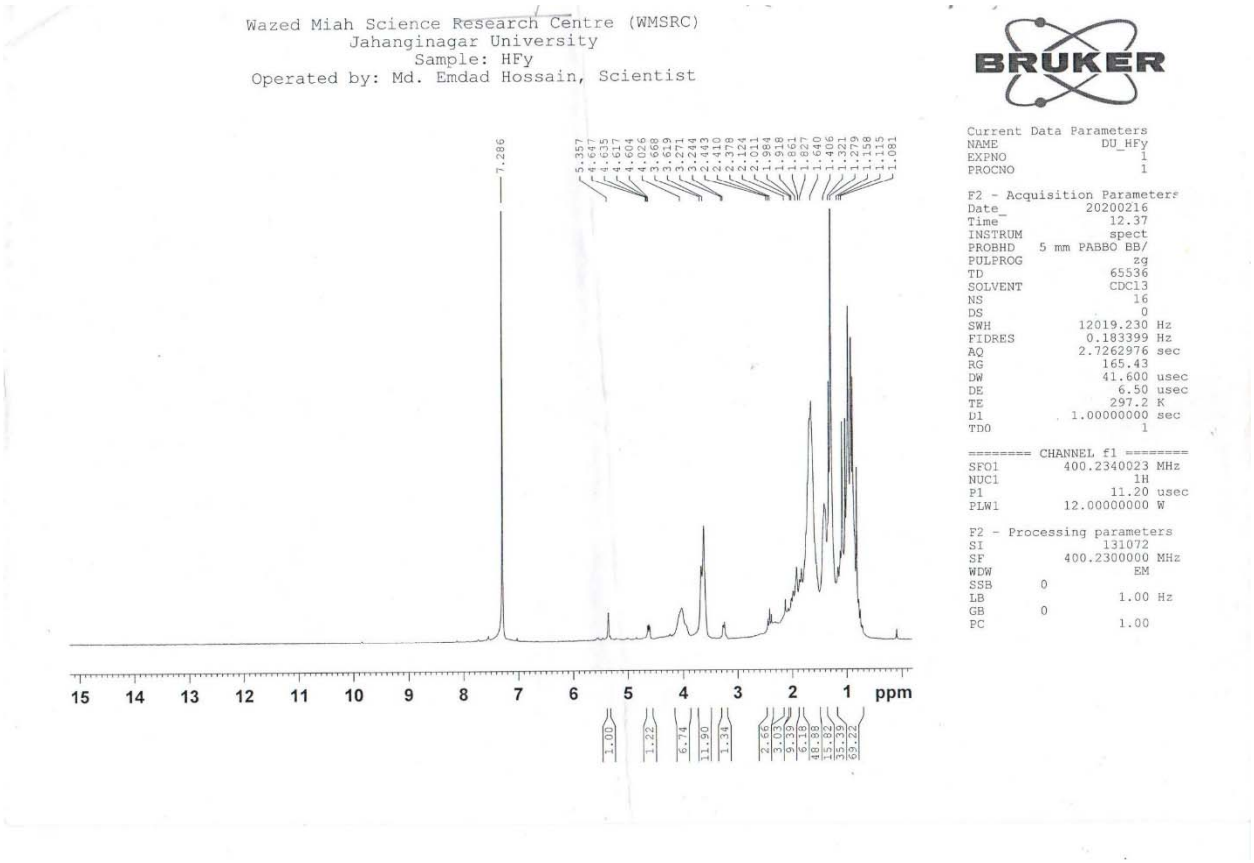


Figure 4.40: ^{1}H-NMR spectrum of compound 12 (β-Sitosterol)

4.3.2.13 Characterization of compound 13 as rutin isolated from *Rhizophora mucronata* (Lam.).

Compound **13** was obtained from ESR fraction of *Rhizophora mucronata* using silica column chromatography as a yellow crystalline powder. The TLC result gave yellow color when sprayed with vanilline-sulfuric acid followed by heating and the R_f value 0.51 was in ethyl acetate: *n*-hexane (7:3) solvents system and the compound is soluble in methanol.

The ^{1}H NMR spectrum of the compound **13** exhibited characteristic signals at δ 5.14 (1H, *d, J*= 7.8), δ 5.15 (1H, *d, J*= 7.8) and δ 3.88 (1H, *dd, J*=11and 1.3 Hz), δ 3.7 (1H, *dd, J* = 12 and 4.9 Hz) assigned for H-1" and H-6"a and H-6"b respectively (**Table 4.25**) indicated that quercitrin skeleton substitute a glucose unit and signal at H-5'"a (δ2.78, 1H, *m*) and H-5'"b (δ 2.78, 1H, *m*) suggested a rhamnose proton. The spectra also revealed that it has two pair of meta couple at the position of H-5, H-7 and H-4', H-5' (δ 6.5, *d, J* = 8.5, Hz).

Although all signals were not found but the remaining resonance is strong. Based on this spectral feature (**Figure 4.42**), compound **13** was characterized as rutin, a flavonoid glycoside by comparing with published data [93].

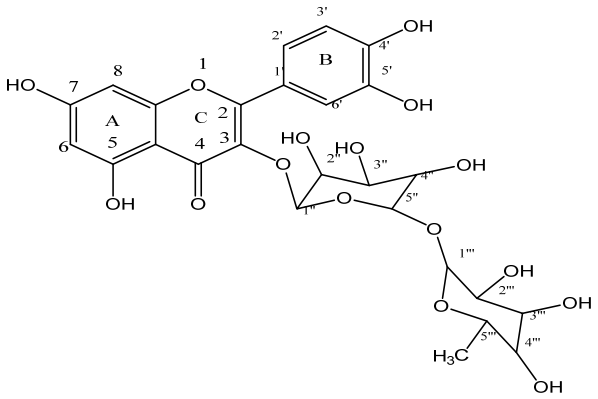


Figure 4.41: Structure of Compound 13 (Rutin)

Table 4.25: ^{1}H NMR at 400 MHz of Compound 13 in $CDCl_3$ reference to TMS.

Position	Compound 13	Rutin [93] (Solvent- CD_3OD)
	δ_H	δ_H
1	--	--
2	--	--
6	--	6.19 (1H, *d, J* = 1.8 Hz)
7	--	--
8	6.4 (1H, *d, J* = 2.3 Hz)	6.38 (1H, *d, J* = 2.3 Hz)
2'	6.9 (1H, *d, J* = 1.8 Hz)	7.66 (1H, *d, J* = 1.8 Hz)
5'	6.5 (1H, *d, J* = 8.5, Hz)	6.86 (1H, *d, J* = 8.5 Hz)
6'	7.77 (1H, *dd, J* = 8.4 and 1.6 Hz)	7.64 (1H, *dd, J* = 8.5 and 1.5Hz)
1"	5.14 (1H, *d, J*= 7.8) 5.15 (1H, *d, J*= 2.3)	5.10 (1H, *dd, J* = 7.9 Hz) 6.38 (1H, *d, J*= 2.3 Hz)
2"	3.61 (1H, *dd, J*=7.8 Hz)	--
6"a	3.88 (1H, *dd, J*=11and 1.3 Hz)	3.79 (1H, *d, J* = 11 and 1.2 Hz)
6"b	3.7 (1H, *dd, J* = 12 and 4.9 Hz)	3.63 (1H, *dd, J* = 12.9 and 4.9 Hz)
8"	3.5 (1H, *m*)	--
1"'	4.5 (1H, *d, J* = 1.8 Hz)	4.51 (1H, *d, J*=1.8 Hz)
2"'	3.66 (1H, *dd, J*= 3.7 and 1.8 Hz)	3.63 (1H, *dd, J*=3.7 and 1.8 Hz)
4"'	2.45 (1H, *d, J* = 9.8 Hz)	3.53 (1H, *d, J*=9.8 and 1.8 Hz)
5"'a	2.78 (1H, *m*)	3.61 (1H, *m, J*=7.8 Hz)
5"'b	2.78(1H, *m*)	3.28 (1H, *m*)
6"'	1.12 (1H, *d, J*= 6.7 Hz).	1.12 (1H, *d, J*=6.7 Hz)

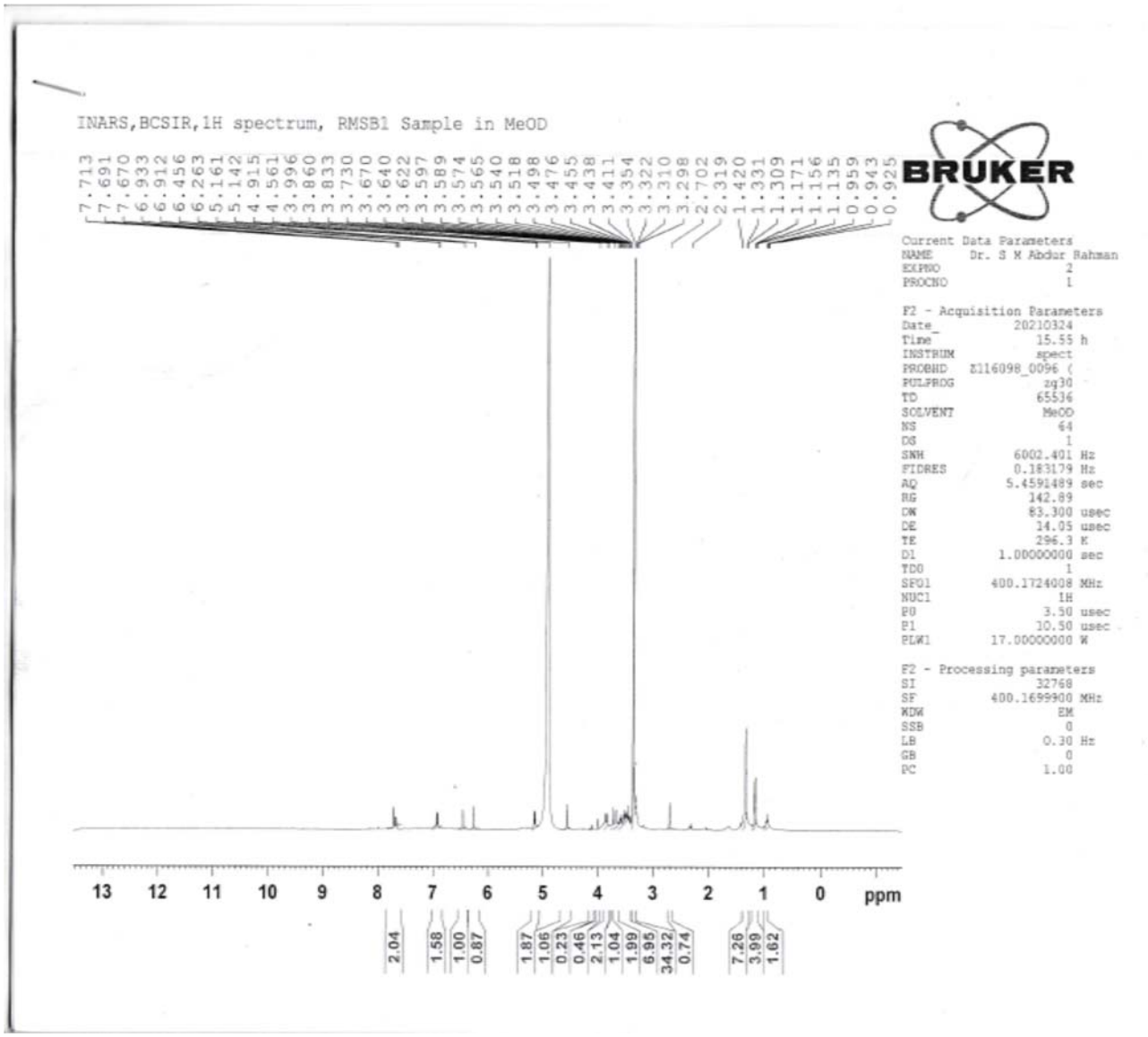


Figure 4.42: [1]H-NMR spectrum of compound 13 (Rutin)

4.3.2.14 Characterization of compound 14 as *N*-trans-para-caffeoyl-tyramine isolated from *Rhizophora mucronata* (Lam.).

Compound **14** was isolated from ERM fraction of RM by silica column chromatography as a white amorphous powder. The TLC result gave reddish black color when sprayed with vanillin-sulfuric acid followed by heating and the R_f value was 0.37 in ethyl acetate: *n*-hexane (7:3) solvents system and compound **14** is soluble in methanol.

The resonances at 6.9 and 6.78 ppm in the ^{1}H NMR spectrum of compound **14** indicated for caffeoyl group. The characteristic peak at 6.35 (*d*, 1H) corresponding peak at H-3 and H-5 indicate tyrosine amino acid signal. Above all this evidence indicate the compound **14** known as *N*-trans-para-caffeoyl-tyramine (**Table 4.26** and **Figure 4.44**) as compared with the published document [94].

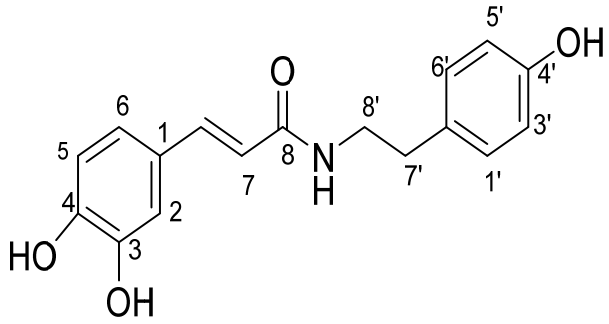


Figure 4.43: Structure of Compound 14 (*N*-trans-para-caffeoyl-tyramine)

Table 4.26: ^{1}H NMR at 400 MHz of compound 14 in $CDCl_3$ reference to TMS.

Position	Compound 14	*N-trans*-para-caffeoyl-tyramine [94] (Solvent- CD_3OD)
	δ_H	δ_H
1	--	--
2	6.9 (1H, *d, J*=1.4 Hz)	7.01 (1H, *d, J*=1.4 Hz)
3	6.78 (1H, *d, J*=2.3 Hz)	--
4	--	--
5	6.78 (1H, *d, J*=8.1 Hz)	6.74 (2H, *d, J*=8.4 Hz)
6	6.9 (1H, *dd, J*=8.4, 2.2 Hz)	6.92 (1H, *dd, J*=8.4, 2.1 Hz)
7	2.68 (1H, *d, J*=14.1 Hz)	3.35 (1H, *d, J*=15.4 Hz)
8	6.3 (1H, *d, J*=15.4 Hz)	7.40 (1H, *d, J*=15.4 Hz)
1'	--	7.07 (2H, *d, J*=8.4 Hz)
2'	6.7 (*s*, 1H)	6.74 (2H, *d, J*=8.4 Hz
3'	6.35 (*d*, 1H)	--
4'	--	--
5'	6.35 (1H, *d, J*=8.4 Hz)	6.78 (1H, *d, J*=8.4 Hz)
6'	--	7.07 (2H, *d, J*=8.4 Hz)
7'	7.4 (*s*, 1H)	7.77 (*s*, 1H)
8'	3.5 (1H, *d, J*=6.7 Hz)	3.47 (1H, *t, J*=7.0 Hz)

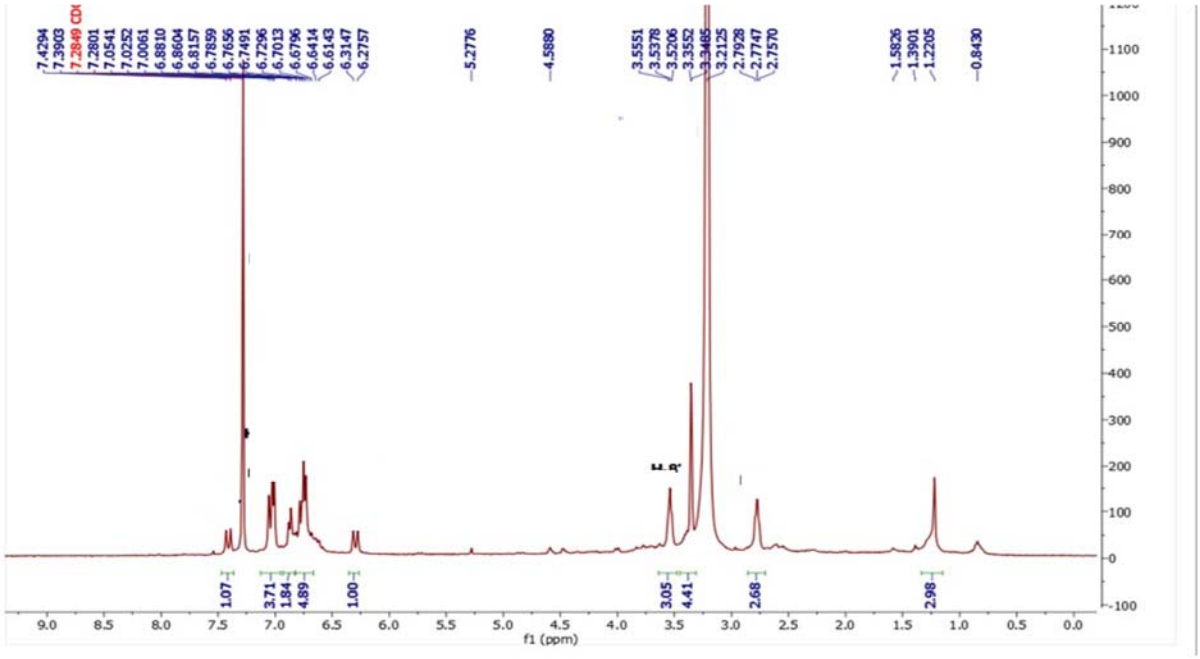


Figure 4.44: ^{1}H-NMR spectrum of compound 14 (*N*-trans-para-caffeoyl-tyramine).

4.4 Biological investigation of isolated compounds

4.4.1 Antioxidant effect of isolated components of *Stevia rebaudiana*

A promising antioxidant effects was observed by the compounds **1**, **3**, **4**, **7**, and **8** which were isolated from *Stevia rebaudiana* except compound **2**. Among them compounds **3** (IC_{50} values 8.926 µg/mL) revealed strong DPPH free radical scavenging properties. Besides compounds **1, 4**, **7** and **8** also gave good antioxidant activity and the percent of inhibition were 23.34, 21.53, 23.31 and 17.73 µg/mL respectively comparable to standard drug with IC_{50} 6.10 µg/mL (**Table 4.27**).

Table 4.27: Antioxidant properties of the isolated compounds (1, 2, 3, 4, 7 and 8) and the standard drug ascorbic acid with their IC_{50} (µg/mL) values.

Compound Code	R^2	IC_{50} (µg/mL)
1	0.9915	23.34
2	0.9856	174.627
3	0.9181	8.926
4	0.9248	21.535
7	0.9915	23.31
8	0.9781	17.73
Ascorbic acid	0.9132	6.10

4.4.2 Antibacterial effect of pure compounds 1, 2, 3, 4, 7 and 8 isolated from *Stevia rebaudiana*

Compounds **1, 2, 3, 4, 7** and **8** isolated from *Stevia rebaudiana* exhibited mild to moderate inhibitory effect toward both gram positive and gram-negative bacteria. Compound **1** exhibited highest antibacterial activity with 12-15mm zone of inhibition was observed while remaining components exerted mild to moderate antibacterial activity against both strains. However, the compounds were ineffective against fungus strain.

Table 4.28: Antimicrobial activity of the compounds (1, 2, 3 ,4,7 and 8) against gram positive and gram-negative bacteria.

Pathogens	Zone of Inhibition (mm)						
	1	**2**	**3**	**4**	**7**	**8**	Ciprofloxacin
Gram positive							
B. cereus	14.7±0.11	12.7±0.3	-	10.2±0.11	-	10.3±0.18	17.2±0.23
B. megaterium	15.3±0.48	12.4±0.23	10.0±0.66	11.1±0.32	-	10.8±0.21	16.8±0.14
B. subtilis	12.7±0.52	-	-	-	-	-	15.8±0.44
S. aureus	14.1±0.16	13.5±0.19	-	-	-	-	18.1±0.14
Sarcina lutea	13.2±0.12	12.5±0.29	11.5±0.44	-	-	-	20.0±0.18
Gram negative							
E. coli	15.8±0.33	12.6±0.65	-	12.0±0.52	-	-	21.2±0.23
P. aeruginosa	-	-	10.7±0.32	-	10.5±0.26	-	14.4±0.14
S. paratyphi	14.2±0.22	11.8±0.55	8.2±0.23	10.2±0.44	10.8±0.19	10.8±0.52	18.5±0.44
S. typhi	12.1±0.33	12.7±0.66	9.3±0.32	10.8±0.44	-	-	17.7±0.14
S. boydii	15.3±0.11	-	-	-	-	11.2±0.67	18±0.18
S. dysenteriae	14.4±0.26	11.5±0.45	-	-	-	-	19.5±0.33
V. mimicus	13.3±0.77	-	10±0.74	11.5±0.53	-	-	18.3±0.42
V. parahemolyticus	-	10.1±0.45	-	12.1±0.23	-	-	20.0±0.41

Values are expressed in Mean ± SEM. Dose of ciprofloxacin was 5 µg/disc whereas dose of components were 250µg/disc.

4.4.3 Evaluation of *In-vitro* anticancer property of the pure compounds 1, 2, 4, 7 and 8 from *Stevia rebaudiana* on HeLa cell line.

The *in-vitro* cytotoxicity of the five isolates against HeLa cells was accomplished by MTT assay. In this investigation firstly serial dilution was made and their percent of inhibition was executed against HeLa cell line which were illustrated in **Table 4.29**. The cell mortality at 500 mg/mL of tested compounds **1, 2, 4, 7** and **8** have been displayed in **Figure 4.45.** The regression line equation was calculated by plotting log concentration vs. cell mortality (in %) by which the IC_{50} values was computed for both selected components as well as reference drug (methotrexate). Among the

isolated phytochemicals (**1, 2, 4, 7**, and **8**), 5-*O*-caffeoyl quinic acid (**1**) demonstrated most appreciable anti-proliferative activity against HeLa cell and compound **2** exhibited 2nd highest activity on this cell and the IC_{50} value were 181.3 and 194.3 µg/mL respectively compared to 36.5 for methotrexate. Compounds **4, 7 and 8** also showed remarkable inhibition with IC_{50} values 252.14, 298.86 and 265.07 µg/mL respectively.

Table 4.29: *In-vitro* cytotoxic activity of isolated compounds (1, 2, 4, 7, and 8) on HeLa cell line.

Compound No.	Concentration (µg/ml))	% Of inhibition against in HeLa cell line	IC_{50} value (µg/ml)
Control (DMSO)	2.5	--	--
Methotrexate	62.4	30.3	36.5
	125	53.4	
	250	94.8	
1	62.5	21.45	181.3
	125	26.68	
	250	70.14	
	500	72.86	
2	62.5	5.28	194.4
	125	28.53	
	250	67.84	
	500	83.3	
4	62.5	3.38	252.14
	125	4.17	
	250	63.63	
	500	73.47	
7	62.5	1.88	298.86
	125	4.87	

	250	34.55	
	500	74.49	
8	62.5	1.58	265.07
	125	9.15	
	250	60.54	
	500	68.75	

Note: DMSO = Dimethyl sulfoxide

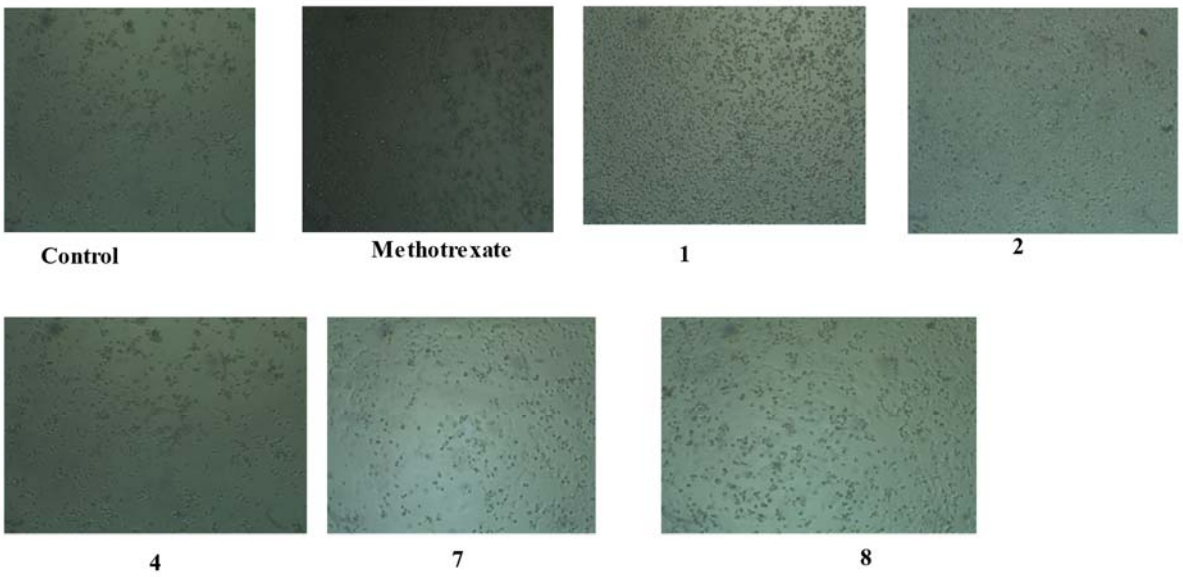


Figure 4.45: Cytotoxicity activity of control, standard and compounds (1, 2, 4, 7, and 8) at the concentration of 500 µg/ml.

4.4.4 Molecular docking studies of the isolated compounds (1, 2, 3, 4, 7 and 8) from *Stevia rebaudiana*.

4.4.4.1 Retardation of Dihydrofolate reductase (DHFR).

The molecular effects of the secondary metabolites on antimicrobial and anticancer targets, molecular docking which is a computer depiction investigation was executed by some appropriate program which revealed comprehensible regarding drug protein binding. DNA synthesis was performed by DHFR- protein which played key role for the bacterial or human cell development. In this regard, pharmacologically potential drug candidate/ligands were docked with DHFR. Therefore, it gave a feature about elevated binding affinity in comparison reference substance such as ciprofloxacin and methotrexate which was an incredible docking prediction to embrace antimicrobial and antiproliferative activities. In generally, higher binding strength with beneath binding energy has been considered. Besides, the extrapolated interaction profile having a null RMSD (root mean square deviation) value represents the best docking prediction.

A higher binding affinity with DHFR protein was observed by all components and the docking score -10.6 to -15.2 kcal/mol when compared with ciprofloxacin (-8.3 kcal/mol) and methotrexate (-9.1 kcal/mol). Among all the drugs involved in this investigation, the highest DHFR protein affinity (-15.2 kcal/mol) was found by 5-*O*-caffeoyl quinic acid **(Table 4.30)**. The sequence of the binding affinity could be as like 5-*O*-caffeoyl quinic acid > *trans*-syringin > apigenin > luteolin > jhanidiol > jhanol > methotrexate > ciprofloxacin (Table 4.26). The effective binding site of compound **1** (5-*O*-caffeoyl quinic acid) were ASP 21, GLY 20, GLY 17, ALA 9, GLU 30, LEU 22, THR 56, and SER 118, while compound **2** (*trans*-syringin) gave its affinity for GLU 30, ASN 64, ILE 16, TYR 16, THR 56, GLY 117, VAL 115, PHE 34 in chain A. Drugs binding with all active sites of DHFR is represented in **Figure 4.46 (a-l).**

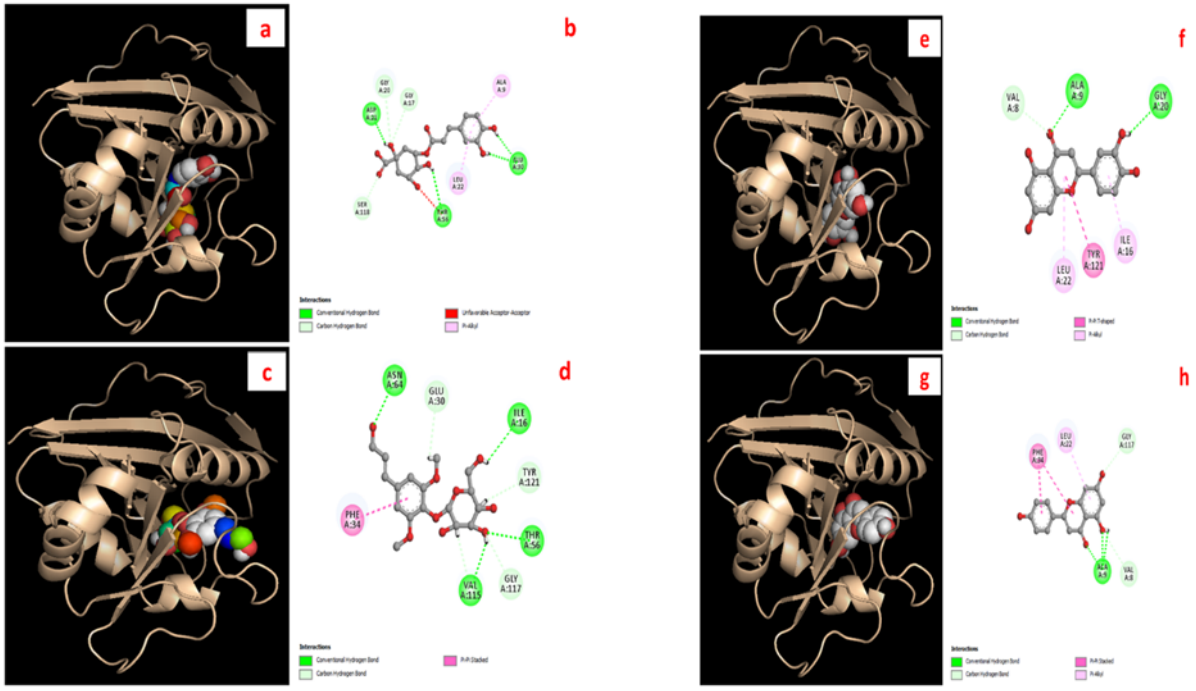


Figure 4.46: Three-dimensional binding feature of selected compounds with DHFR (DHFR; PDB ID: 4M6J). Where compound 1 = a and b, compound 2 = c and d, compound 3 = e and f, compound 4 = g and h, compound 7 =i and j, and compound 8 = k and l.

Table 4.30: Ligands interaction with proteins along with binding scores (kcal/mol) antibacterial, anticancer and antioxidant properties.

Compound No.	Compound Number	PubChem CID	Binding affinity (Kcal/mol)		
			4M6J	3GRS	IR4U
1	5-*O*-caffeoyl quinic acid	5280643	-15.4	-7.8	-7.8
2	*Trans*-Syringin	5316863	-13.8	-6.4	-7.4
3	Luteolin	5280447	-12.6	-7.8	-8.5
4	Apigenin	5280453	-12.8	-7.7	-8.4
5	Jhanol	13996314	-10.9	-7.2	-7.8
6	Jhanidiol	15226345	-10.10	-7.4	-7.4
Standard	Ciprofloxacin	2768	-8.6		
	Methotrexate	126931	-9.2		
	Ascorbic Acid	54672067		5.7	-5.4

Note: PDB ID 4M6J- Dihydrofolate reductase, PDB ID 4GRS- Glutathione reductase and PDB ID IR4U- Urase reductase.

4.4.4.2 Glutathione reductase and urase oxidase inhibition

The binding affinity of all the ligands was stronger towards glutathione reductase (-6.4 to -7.8 kcal/mol) and urase oxidase enzyme (-7.4 to -8.5 kcal/mol) than the ascorbic acid reference drug (-5.7 and -5.4 kcal/mol). The highest docking score was observed in compound **1** (-7.8 kcal/mol) and compound **3** (-7.8 kcal/mol) for glutathione reductase enzyme, on the other hand the greater binding affinity towards urase oxidase enzyme manifested by compounds **3** (-8.3 kcal/mol) and **4** (-8.1 kcal/mol). The most favorable active site for compound **1** in glutathione reductase were ARG 291, PHE 181, VAL 61, CYS 58, THR 339, GLU 201, CYS 63, VAL 370, THR 57, LEU 337, GLY 62, PRO 340, SER 177, ILE 198, ASP 331, THR 339, CYS 63, LEU 338, LYS 66, VAL 371, PHE 372, THR 339, GLU 201, LEU 337 and THR 369, PRO 340, LEU 338, THR 339, TYR 197, CYS 58, THR 57, ARG 291, VAL 61, PHE 181, SER 177, GLY 62, MET 202, GLU

201, LYS 66, ILE 198, CYS 63, PHE 372, VAL 371 for compound **3** (**Figure 4.47**). Compound **3** exerted most profound interaction at the active site of urease oxidase enzyme VAL 73, THR 74, PRO 76, PRO 75, ARG 128, TRP 208, TRP 106, THR 107, ARG 105, HIS 104, CYS 103, MET 32, GLU 31, TYR 30 (**Figure 4.48**). Similarly, graphical representation for all the compounds at most potential binding sites were given in **figure 4.48**.

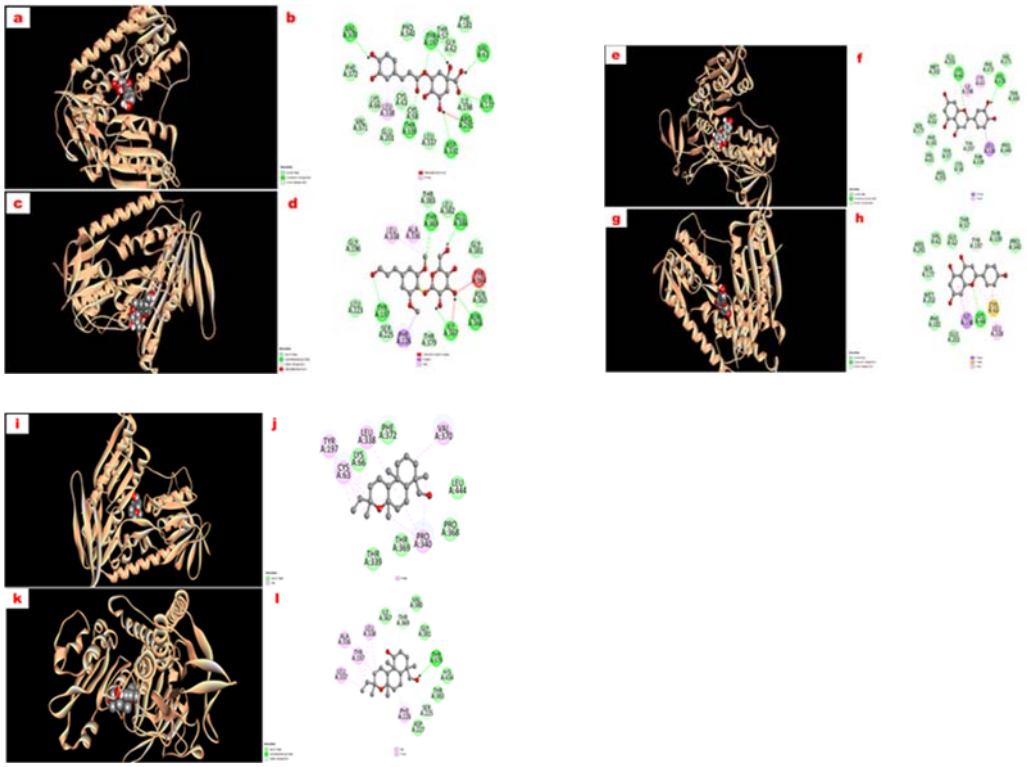


Figure 4.47: Three-dimensional binding feature of selected compounds with glutathione reductase (PDB ID 4GRS). Where compound 1 = a and b, compound 2 =c and d, compound 3 =e and f, compound 4 = g and h, compound 7 =i and j, and compound 8 = k and l.

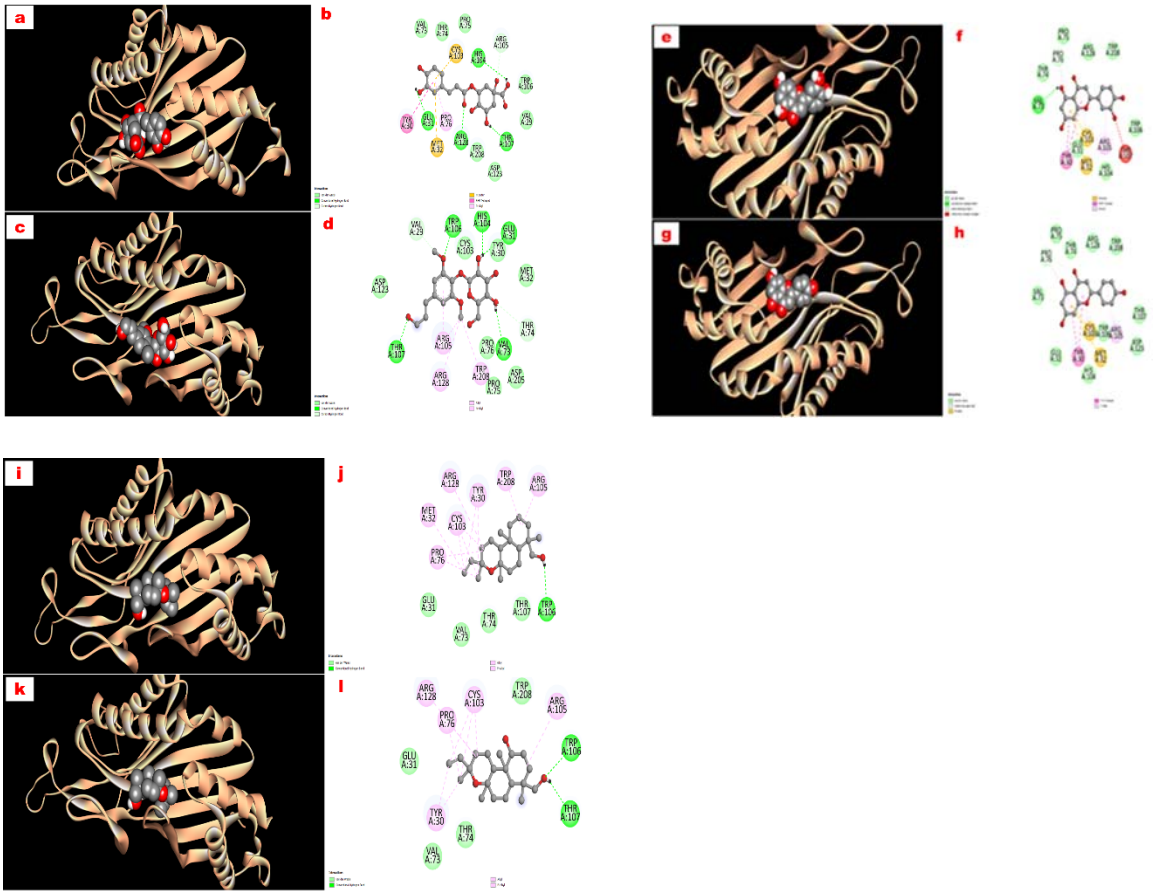


Figure 4.48: Three-dimensional binding feature of selected compounds with Urase oxidase (PDB ID: 1R4U). Where compound 1 =a and b, compound 2 = c and d, compound 3 = e and f, compound 4 =g and h, compound 7 = i and j, and compound 8 = k and l.

4.4.5 Evaluation of *In-vitro* anticancer property of the pure components 10, 11, and 13 from *Rhizophora mucronata* on EAC cell line.

The cytotoxicity activity of the drugs (**compound 10, 11** and **13**) by MTT assay against EAC cell has shown in **Table 4.31** which represented that compound **10, 11** and **13** exhibited most promising anticancer activity against cancerous cell (**Figure 4.49**) compared to standard drug bleomycin. At the concentration of 500 µg/ml compounds **10, 11** and **13** have shown highest antiproliferative activity and the percent of inhibition were 80.8%, 77.1% and 75.95% respectively. The lowest growth inhibition value of the compounds **10, 11** and **13** were found at the dose 62.5 µg/ml and the values were 36.5%, 44.8%, and 42.1% respectively. The IC_{50} value of the three compounds

10, 11 and **13** were 139.8, 112.01 and 144.92 µg/ml respectively compared to IC_{50} value of standard drug was 31.3 µg/ml which is given in **Table 4.31.**

Table 4.31: *In-vitro* cytotoxic activity of compound 10, 11 and 13 from RM on EAC cell line.

Compound No.	Concentration (µg/ml))	% Of inhibition against in EAC cell line	IC_{50} value (µg/ml)
Control (DMSO)	2.5	--	--
Bleomycin	62.5	43.7	31.3
	125	70.4	
	250	90.7	
10	62.5	36.5	139.8
	125	53.3	
	250	64.3	
	500	80.8	
11	62.5	44.8	112.01
	125	52.3	
	250	60.7	
	500	77.1	
13	62.5	42.1	144.92
	125	49.9	
	250	58.8	
	500	75.9	

Note: DMSO = Dimethyl sulfoxide

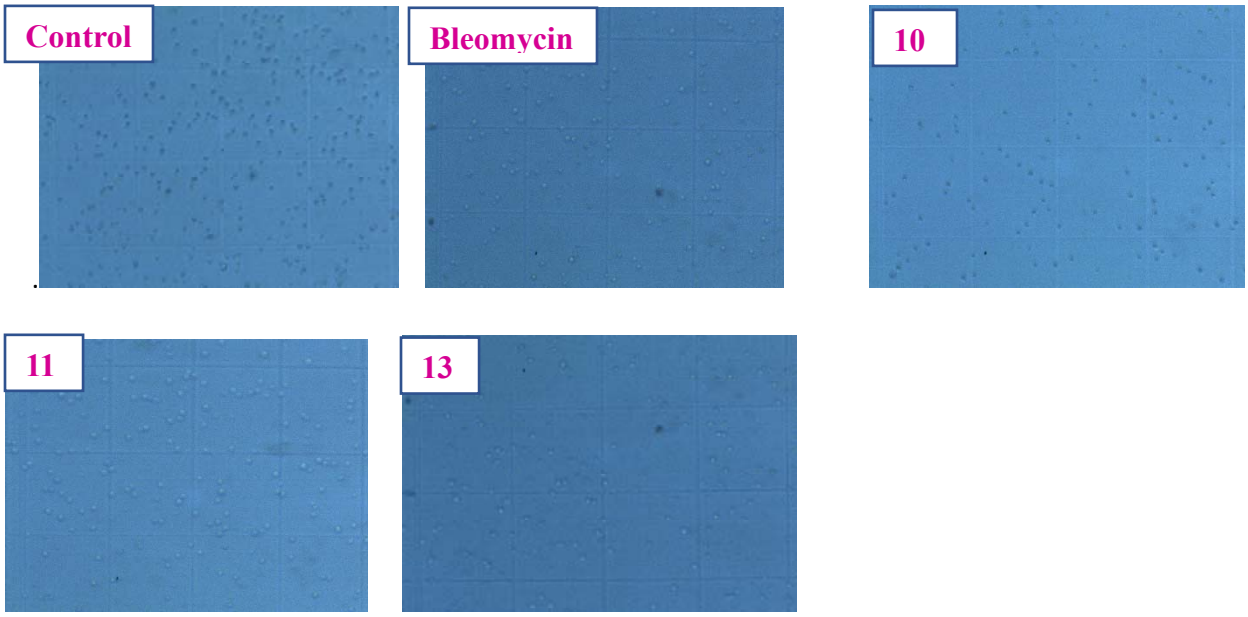


Figure 4.49: Cytotoxicity of control, standard and components (10, 11 and 13) at the concentration of 500 µg/ml observed by an advanced system of microscope.

4.4.6 *In-vivo* cell growth inhibition of compounds 10, 11 and 13

In vivo anticancer activity of the three compounds **10, 11 and 13** and standard drug bleomycin were examined with the help of hemocytometer is shown in **table 4.32** where cell growth inhibition was significantly confirmed. It has been shown that the maximum cell growth inhibition was given by the compounds **11** which was 58.7% and the compound **10** also had good EAC cell growth inhibition compared to standard drug bleomycin showed 84.83% cell growth inhibition whereas compound **13** did not gave significant inhibitory effect against cancer cell.

Table 4.32: *In vivo* cell growth inhibition of the compounds 10, 11 and 13 against EAC cell.

Group	Dose (mg/kg b.wt/day)	Viable EAC cell on day 5 after inoculation (x10^6cells/ml)	% of cell growth inhibition
Group I	**Control**	310± 31.2	-
Group II	**Bleomycin** (0.3 mg/kg)	47±22.5*	84.83
Group III	**10** (5 mg/kg)	180±30.2*	41.93
Group IV	**11** (5 mg/kg)	128±28.3*	58.7
Group V	**13** (5 mg/kg)	207±52.5	5.4

Bleomycin, compounds 10 and 11 significantly inhibit the growth of tumor cell at the doses of 0.3 and 5 mg/kg respectively. Data are expressed in mean ±SEM at significant value of p< 0.05 and compared with control group.

4.4.7 Apoptotic EAC cells quantification by DAPI staining.

To evaluate the morphological changes of the EAC cells, DAPI staining was done, and the cell was visualized by fluorescence and optical microscopy. In fluorescence microscope the cell of control group, nuclei of EAC cell were regular, round and gently stained as given in the **Figure 4.50**. While drug induced (**10, 11** and **13**) apoptosis occurred in treatment group where chromatin condensation, fragmented DNA and membrane abscess were observed. Hence, it suggested that drugs **10, 11** and **13** could have programmed cell death. In optical microscope, the number of cells were more and uniform in shape in control group (Group I) whereas drugs induced groups (Group II, Group III and Group IV) cell were decreased. The effect was more prominent in standard group as well as compound **11** induced group. This consequence let drop that compound invigorated apoptosis.

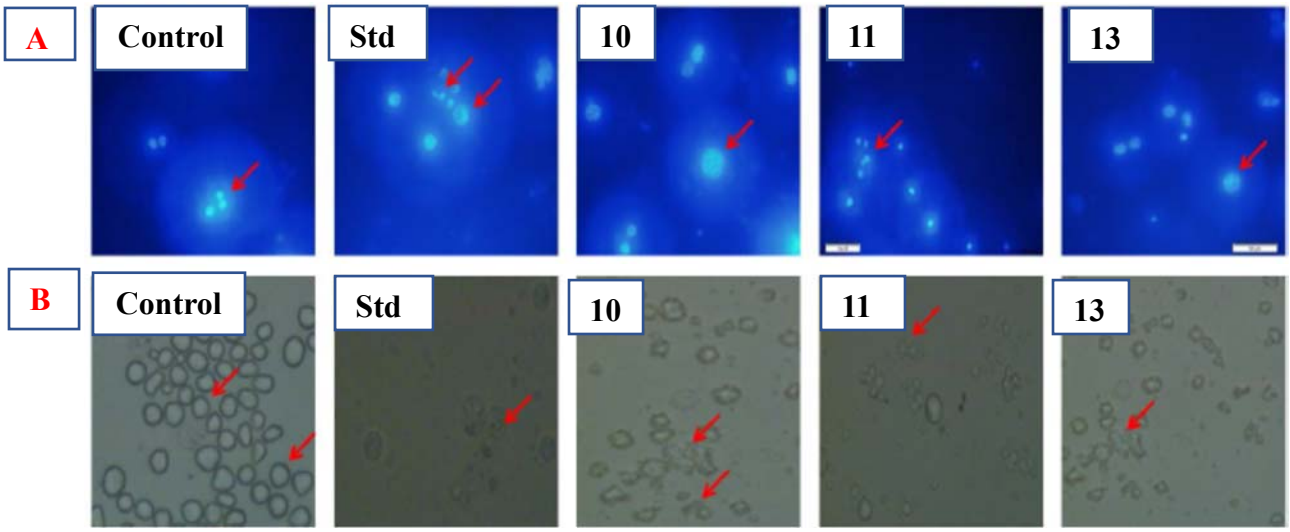


Figure 4.50: Displaying DAPI staining image of all group of mice in fluorescence microscope (A) and Optical microscope (B) on EAC cell. Both fluorescence microscope (A) and Optical microscope (B) represented compound 10, 11 and 13 induced apoptosis.

4.7.3.5 DNA fragmentation assay result.

Isolated DNA from control cell and components **10, 11, 13** and standard treated EAC cells was examined by electrophoresis pattern which is given in **figure 4.51**. Control group contained very distinct, uniform, and heavy molecular mass DNA was developed, while fragmented, smeared and ladder type of DNA were observed in compounds treated mice cells which revealed the point of character of apoptosis incitement.

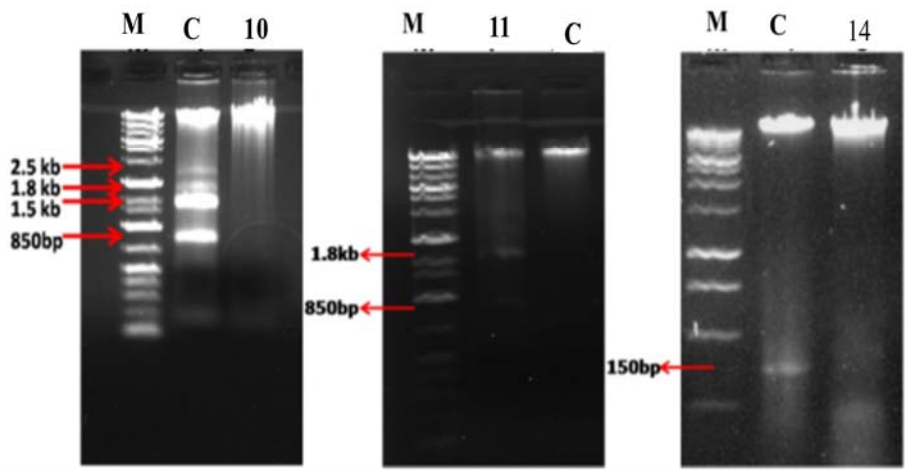


Figure 4.51: DNA fragmentation from both control and treated EAC cells. The line demonstrated as M-molecular marker, C-control, compound 10, 11and 13 treated cells respectively.

4.4.8 Molecular study of apoptotic genes

Mice was sacrificed after 5 days treatment with standard drug bleomycin (0.3 mg/kg) and compounds **10, 11**, and **13** (5 mg/kg) and both control and treated mice cell were collected. The RNA was extracted according to the manufacturer's guideline from both treatment group and control group mice cells and assessment was done by gel electrophoresis. Two distinct bands of 28s and 18s was found for both control and treated mice after gel electrophoresis of isolated RNA (**Figure 4.52**). Nanodrop was used to examine the quality or purity of RNA (**Table 4.33**).

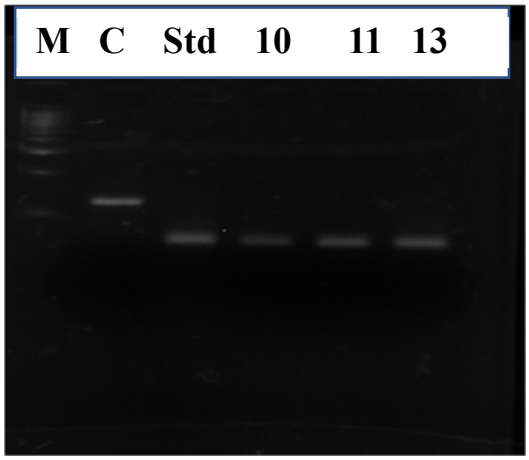


Figure 4.52: Gel electrophoresis band of molecular marker (M), Control (C), Std(bleomycin), Compound 10, 11, and 13 treated EAC cell.

Table 4.33: Optical density (OD) for RNA quality and purity evaluation.

Group	Absorbance Wavelength in nm			Concentration
	260	280	260/280	
Group I (Control group)	2.7	.77	3.2	120.5
Group II (Std. Bleomycin)	2.73	1.3	1.8	146.3
Group III (Compound**10**)	3.21	1.98	1.62	176.34
Group IV (Compound **11)**	1.97	.99	1.98	97.4
Group V (Compound **13)**	2.04	2.1	2.1	105.3

Two wavelengths 260 nm and 280 nm were used for checking the quality and purity of RNA. The sample containing nucleic acid was estimated by the absorbance 260 nm which was used to measure the concentration and conversion factor based on the extinction coefficient for nucleic acid (A_{260} of 1.0 = 40 µg/ml of RNA). At the wavelength 280 nm absorbs amino acid, so this wavelength was used to measure the amount of protein in the sample. If the ratio of the two absorbance (A_{260}/A_{280}) falls between 1.8-2.2, then the purity of nucleic acid is confirmed. After successfully completion of RNA extraction from both control and drug induced group reverse transcription was done from RNA to cDNA. To study the expression levels of several tumor related genes was done by reverse transcription PCR which was compared to the expression level of reference gene GAPDH. Hence, forward, and reverse primer of GAPDH was amplified on PCR which gave band around 350 bp region after gel electrophoresis for both control and compounds treated cDNA. The gene expression of Bcl-2 and NF-κB was high in control group (Group I) and low expression occurred in tumor suppressing gene like Bax, P^{53}, Cyt C, TNF-alpha, Cas-3, Cas-8, Cas-9. On the contrary, opposite situation was created by compounds **10, 11** and **13** (Group I, Group II and Group III) along with standard drug bleomycin which reveal that isolated pure compounds **10, 11** and **13** induced apoptosis when compared with control group.

4.4.9 Expression of pro and anti-apoptotic genes in compound 10 (β-amyrin)-treated EAC cell.

Semi-quantative RT-PCR was used to analyze expression of mRNA of antiapoptotic genes (Bcl-2, NF-κB) and apoptotic genes (Bax, P^{53}, Cas-3, Cas-8). Upregulation of proapoptotic gene Bax (higher than 500bp), P^{53} (500 bp) and down regulation of anti-apoptotic gene of Bcl-2, (150 bp) NF-κB (125bp) in treatment group was observed which is comparable to control group.

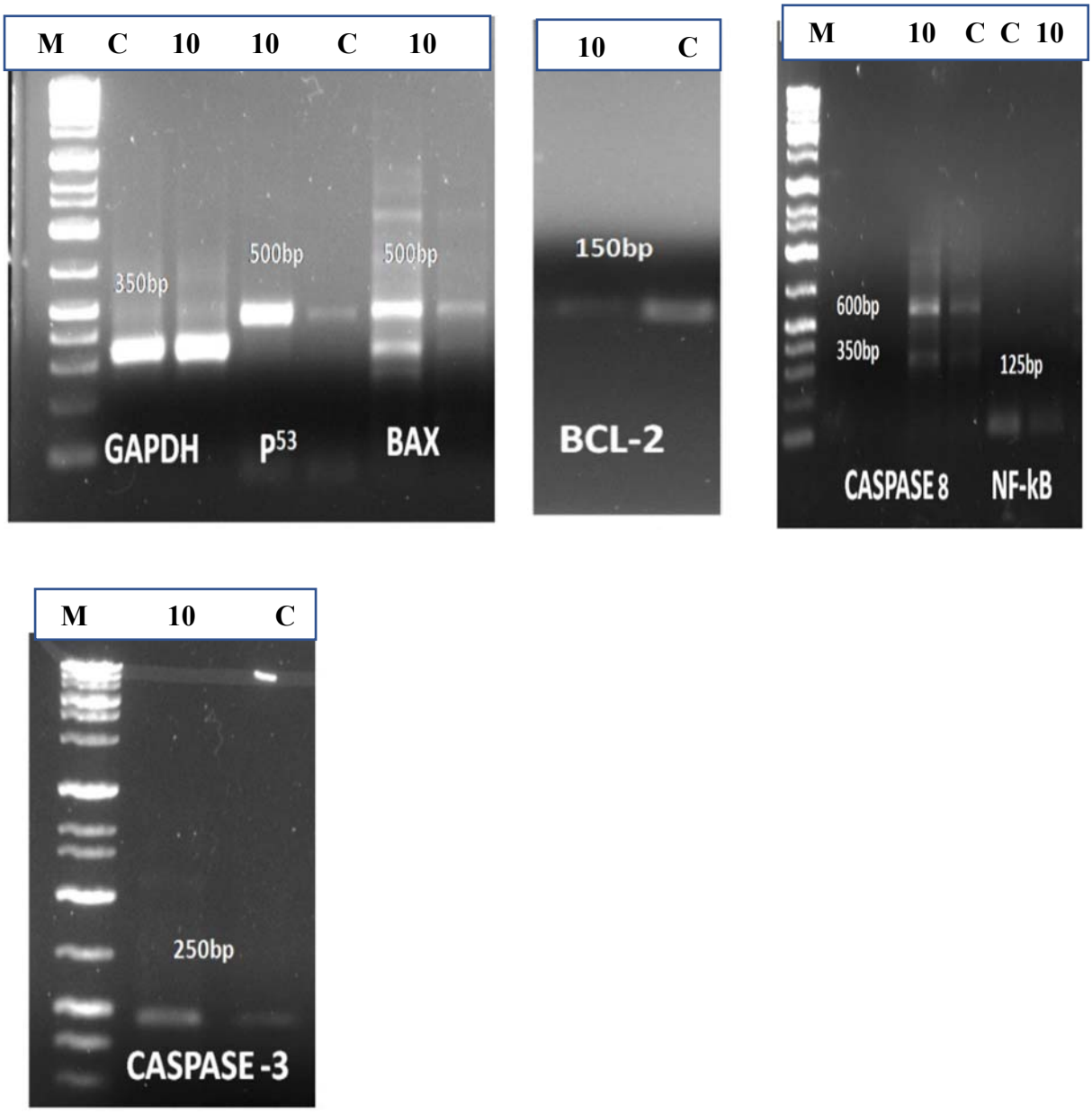


Figure 4.53: Pro and anti-apoptotic genes expression of compound 10 induced group (Group III), C (control group or Group I) and M (molecular marker) respectively. Here bp indicated the length and posture of bands.

In treatment group, initiator gene casepase-8 and effector gene casepase-3 expression was also high and prominent compared to control group which stimulate cell apoptosis representing in **Figure 4.53.**

4.4.10 Expression of pro and anti-apoptotic genes in compound 11 treated EAC cell:

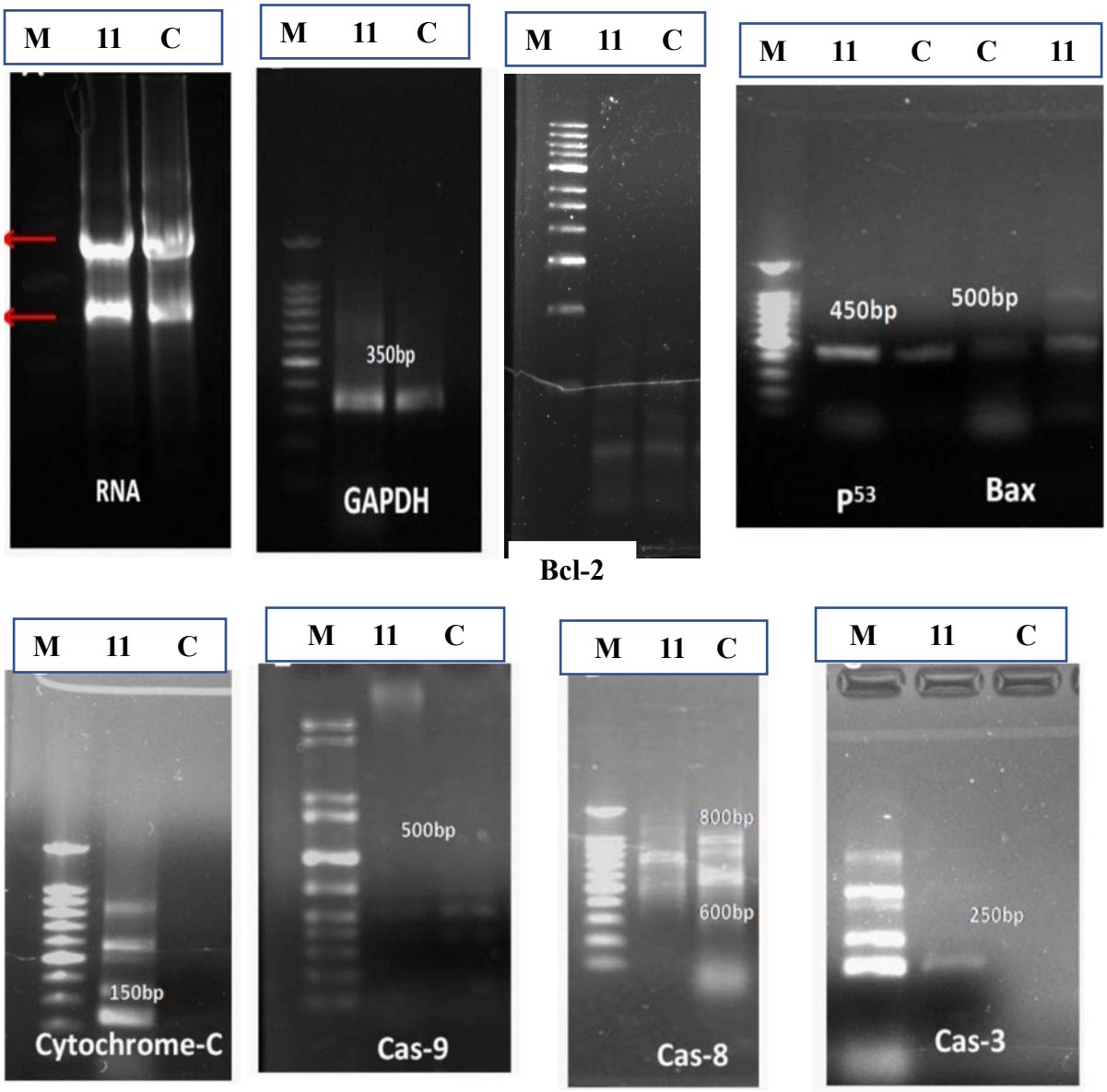


Figure 4.54: Pro and anti-apoptotic genes statement of compound 11 induced group (Group IV), C (control group or Group I) and M (molecular marker) respectively. Here bp indicated the length and posture of bands.

The **Figure 4.54** describe semi-quantative RT-PCR used to analyze expression of mRNA of antiapoptotic genes (Bcl-2) and apoptotic genes (Bax, P^{53}, Cyte-C, Cas-3, Cas-8, Cas-9). RNA was isolated from control and compound **11** ingested group which gave two distinct band at 28s and 18s for both control and treatment group representing in **Figure 4.54.** Housekeeping gene GAPDH expression was used as reference gene expression which was used to normalize the expression level of other gene and it was observed that similar level expression was given both control and compound **11** treated group. Increased expression of proapoptotic gene and decreased expression of anti-apoptotic gene in treatment group compared to control group indicate the installation of extrinsic and intrinsic pathway of apoptosis.

4.4.11 Expression of pro and anti-apoptotic genes in compound 13 (Group V) treated EAC cell

Apoptotic gene (Bax, P^{53}, Case-3) and antiapoptotic genes (Bcl-2) were enumerated by semi-quantitative RT-PCR to estimate the level of mRNA expression. The separated RNA from both control and treatment group gave similar band produced based on visualization because it gave two band at the region of 28s and 18s. Here also GAPDH was used as a reference gene expression which gave prominent band. But compound. **13** induced group gene expression of apoptotic gene like P^{53}, BAX and Case-3 as well as antiapoptotic gene Bcl-2 did not give any band on gel electrophoresis showing in **Figure 4.55**. Therefore, it indicates that compound **13** may be had no antiproliferative activity or it may be technical problem that occurred during working. So, further study is necessary.

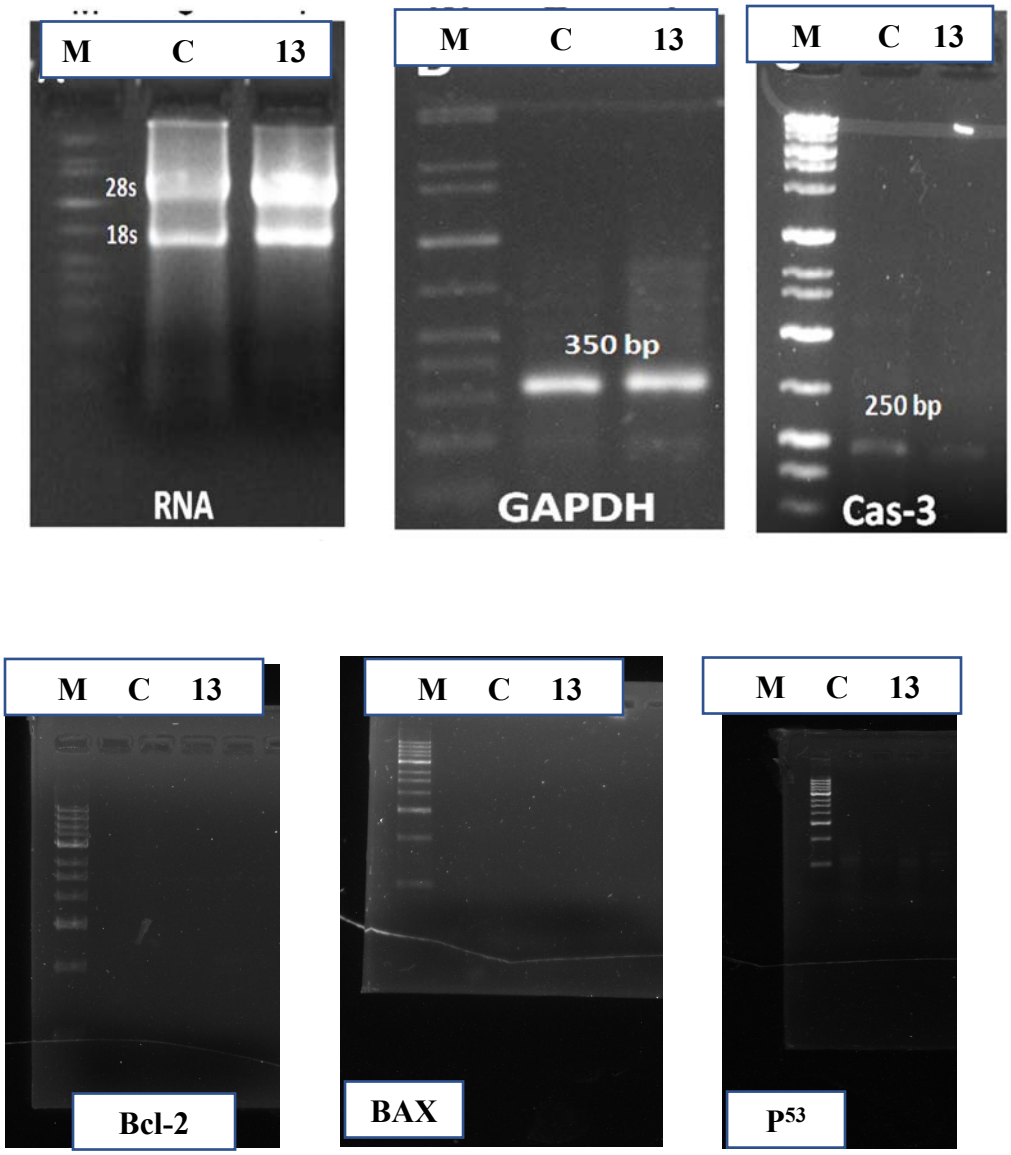


Figure 4.55: Pro and anti-apoptotic genes expression of compound 13 induced group (Group V), C (control group or Group II) and M (molecular marker) respectively. Here bp indicated the length and posture of bands.

4.4.12 Proposed model of Apoptosis

Based on gene expression analysis and DNA fragmentation of compound **10** and **11**, a design of apoptosis induction pathway has been put forward is given in **Figure 4.56**. In this model, intrinsic and extrinsic pathway was described. According to this model, cytochrome-c let out from mitochondria due to down expression of Bcl-2 and increase expression of Bax and P-53 genes because of this apoptosome is obtained which is responsible for stimulation of caspase-9. After that caspase-9 stimulates caspase-3 which is directly or incidentally accountable to cell death apoptosis. Actuation of trans-membrane receptor TNF-α is the prime step of apoptosis process in extrinsic pathway. This TNF-α activation binds assigned analogous compounds in existence their corresponding adaptor, death domain TRADD/FADD by the stimulation of NFκB. Then this complex activates the Casepase-8 which then activate the Caspase-3. Activation of Caspase-3 commences programmed cell death directly.

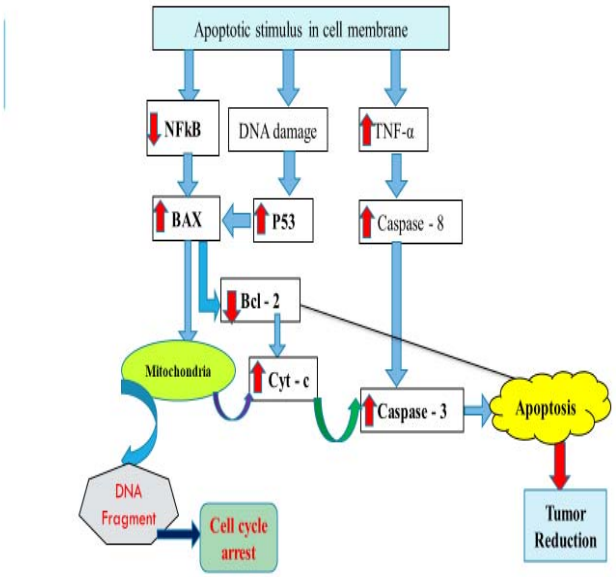


Figure 4.56: Compound 10 (β-amyrin) and 11 (2-oxo-14,15-bisnor-3,11 *E*-Kalavadien 10, 13, 17-triol) induced apoptosis.

4.4.13 Blood (RBC, WBC, and % Hb), liver and Kidney parameter check

All groups of mice (normal control group, control group and treatment group mice) were tested blood parameters have shown in **Table 4.34**. It has been shown that treatment and standard group go round to near normal scale after administration of pure compounds **10 (β-amyrin) and 11 (2-oxo-14,15-bisnor-3,11 *E*-Kalavadien-10, 13, 17-triol)** at the concentration of 5mg/kg b. wt/day compared with normal control group (Group I). In case of liver parameters such as ALT (ALT-Alanine Aminotransferase) AST (Aspartate aminotransferase) and ALP (ALP-Alkaline Phosphatase) analysis, dramatic change occurred in control group (Group II) like physiological worsening when compared with normal control group (Group I). Due to this decline physiological condition became to close normal levels in the treatment groups (Group IV & Group V) including the standard drug bleomycin inducing group (Group III). But there was no change occurred in bilirubin level for both control and treatment groups. Kidney toxicity test like urea, and creatinine volume checked from the drug induced groups compared with normal control group mice (**Table 4.34**). The results observed that there was no significant change occurred in kidney function to all the group that is, no deterioration of kidney parameters observed in control group and drug induced group.

Table 4.34: Effect of component10, 11 and 13 on blood parameters, liver, and kidney parameters in EAC cell bearing mice.

Treatment (dose mg/kg b. wt day $^{-1}$)	Hematological test (RBC and WBC in 10^6 cell mm^{-3}, Hb in gm d/L			Liver function				Kidney function mg/dL	
				U/L			Mg/dL		
	RBC	WBC	%Hb	ALT	AST	ALP	Bilirubin	Urea	Creatinine
Normal Control group or Group-I (without EAC cell)	50.5±2.1	5311.3±6.1	11.85±1.9	60	20	50	<10	18.6	0.6
Control Group-II (Containing EAC cell)	24±1.5	10234.44±2	6.1±2.1	94	50	157	<10	30.8	0.7
Group-III (Bleomycin 0.3 mg/kg	47.4±2.2	4459.8±4.3	10.7± 1.5	65	31	124	<10	22.4	0,6
Group-IV (Compound10, 5mg/kg)	43.7±1.5	5928.5±8.3	9.6 ±1.1	89	42	88	<10	28.8	0.6
Group-V (Compound 11, 5mg/kg)	47.3±1.4	6839.6±3.3	10.4±2.5	77	33	63	<10	24.3	0.6
Group-VI (Compound 13, 5mg/kg)	24.8±.9	6132.2± 5.4	8.4± 2.1	77	37	100	<10	27.8	0.6

The results are shown in mean±SEM. Hematological parameters were examined in 6 groups of mice, among them 4 groups were EAC cell containing mice treated with bleomycin and compounds 10, 11 and 13 for 5 days.

4.4.14 Evaluating compound induced hepatotoxicity and nephrotoxicity.

All group of mice hepatocyte demonstrated that histopathology of liver retained normal physiological architecture on the sixth day. Central vein and hepatic sinusoids did not contain any distention or obliteration. Therefore, the test compounds (**10,11,** and **13**) were not exhibited toxicity at the dose 5mg/kg b.wt. presenting in **Figure 4.57**.

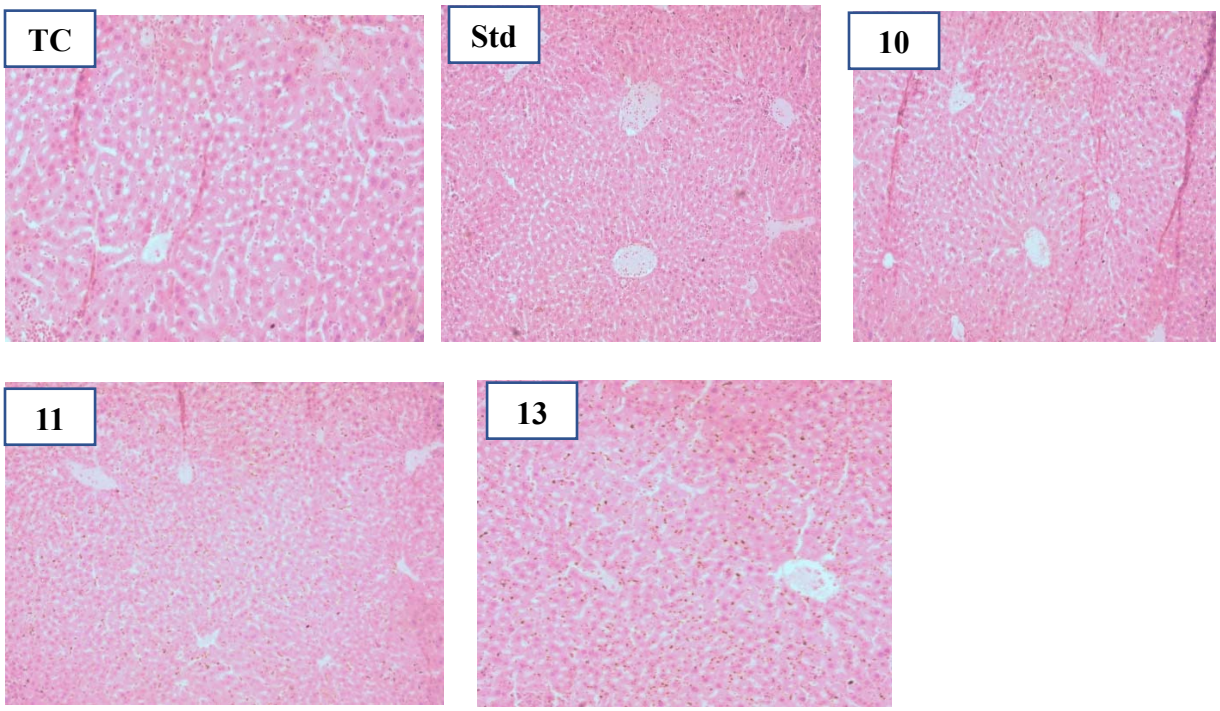


Figure 4.57: Microscopic views of H/E-stained slice of liver of mice. Control group, Standard group, and compound 10, 11 and 13 inducing group.

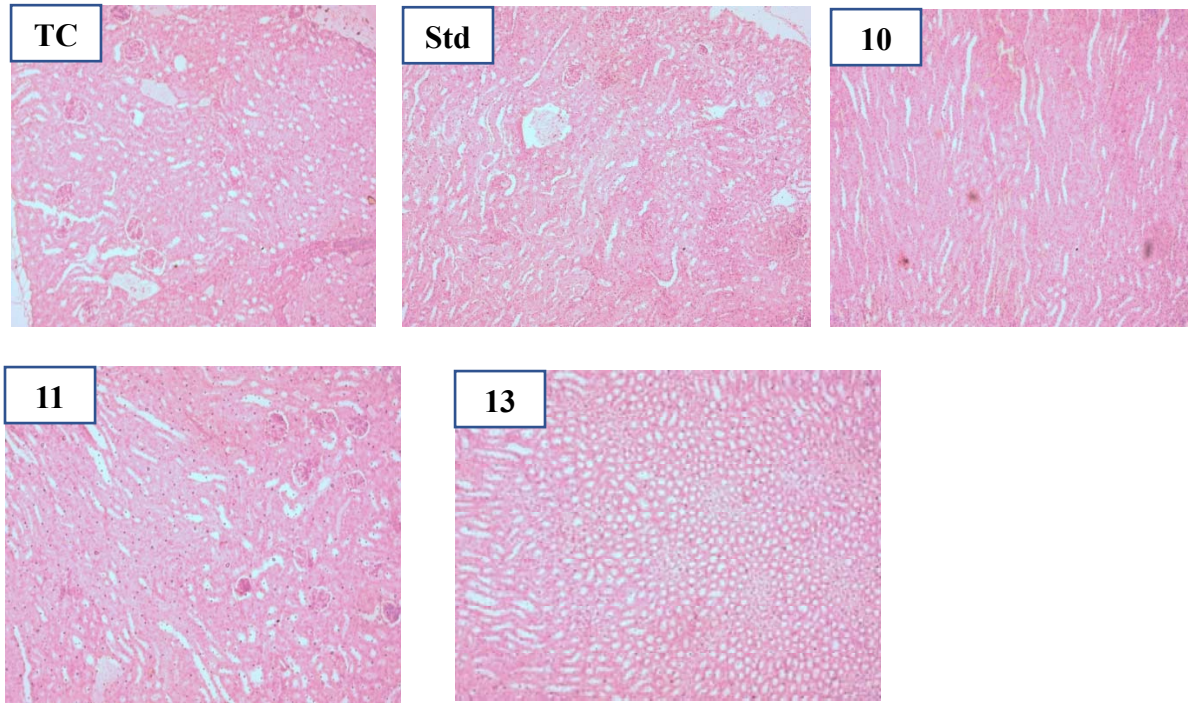


Figure 4.58: Microscopic views of H/E-stained slice of kidney of mice. Control group, Standard group, and compound 10, 11 and 13 inducing group.

The histological feature of both control and treated group showed normal photomicrograph showing in the **Figure 4.58**. The intact Bowmans capsule and glomerulus, regular shape of convoluted tubules and medullary ray was in the cortex of kidney. All the components like the Henles loop and the collecting tubules in the inner medulla region of the kidney was normal. Thus, the compounds (**10,11** and **13**) at this dose did not reveal nephrotoxicity.

5. Discussion

A large number of potential efforts have been conducted to identify the novel scaffolds from the plants that may help alleviate the global threat of infectious disease. Plant contains a variety of secondary metabolites among which phenolic compounds are considered of great interests for their wide applications. Phenolics such as coumaric acid, caffeic acid, ferulic acid, sinapic acid, tannins, flavonoids, glycosides etc. are synthesized from the phenylpropanoid pathway in plants and exhibited antibacterial activities [95-97]. Different type glycosides have been isolated from *Stevia rebaudiana* such as stevioside (5-13%), rebaudioside A (2-4%), and rebaudioside C (1-2%) which play great role for sweetening activities [98]. Other than glycosides, 5 volatile oils, sterebins, chlorogenic acids, triterpenes are also found in this plant [99]. In the current study some important secondary metabolites are also included to this plant. A phenylpropanoid glycoside *trans*-syringin (**2**) and jhanidiol (**6**) containing diterpene framework which is the first report from this plant to the best of our knowledge. About 25 to 65 mg/g gallic acid is equivalent to total phenolic concentration of leave of 80% ethanolic crude extract of *S. rebaudiana* which is near close to our findings regarding total phenolic constituents [100]. In this current investigation, HSR, DSR and ESR fractions were prepared from the methanolic extract of SR which contain crucial chemical constituents that could play great role in pharmacological activities. All the fractions have free radical scavenging activity and to estimate the antioxidant properties DPPH stable free radical method is a conventional procedure for the plants [101-102]. Due to deposition of free radicals for long time several life-threatening neurodegenerative diseases like asthma cancer and AIDS are produced and plant base antioxidant are gaining popularity which scavenge free radicals along with fewer side effects to control the diseases. In comparison to standard ascorbic acid, HSR and ESR imparted profound DPPH scavenging activity among the three fractions. DPPH scavenging ability of *S. rebaudiana* extracts might be attributed to the presence of phenolic flavonoids as well as alkaloid constituents published in several literature [103-104]. It is predicted that redox activity is accredited because of adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [105]. It is reported that *S. rebaudiana* is widely used as a human diet to enhance antioxidant activity [106]. Flavonoids and phenolic components are most common for both edible and non-edible plants due to this reason, they exert strong antioxidant activity. In this study, we have observed that ESR fraction gave better antioxidant activity among

others which could be due to existence of flavonoids and anthraquinone derivatives (compound **1** and **2**) separated from this fraction [107].

In the current study apigenin exhibited highest free radical inhibitory effects (IC_{50} = 8.93) than other components which is predicted due to occupancy of many hydroxyls group published initially study [108]. Besides, 5- *O* -caffeol quinic acid, *trans*-syringin and luteolin (compound **1**, **2**, and **4**) also have good inhibition against DPPH mediated free radicals previously reported- [109-110]. *Trans*-syringin gave moderate type antioxidant effect on comparison to published features, therefore our present study regenerated the earlier data [111]. On the other hand, 5-*O*-caffeol-quinic acid gave potent free radical scavenging properties compared to the previous result (IC_{50} 23.33 vs. 68) [112]. The antioxidant effect of jhanol and jhanidiol was also prominent which is first report as antioxidant effect for these two components. Docking study with glutathione reductase and urase oxidase enzymes of the isolated phytochemicals was performed to support *in vitro* antioxidant activity representing in **Figure 4.47 and 4.48.** Besides, the hydroxyl groups either at meta or para position in anthraquinone derivatives gave prominent antioxidant activities. It was also mentioned that carbonyl group containing anthraquinone skeleton other than hydroxyl group, also accountable for the antioxidant potential [113].

The fraction HSR, DSR and ESR have significant antidiabetic effect representing in the **Table 4.7** Among the three fractions, DSR and ESR (Group IV and group V) exerted 58.08% and 55.24% glucose lowering effect respectively (at the dose 200 mg/kg body weight) when compared with diabetic control group. The anti-hyperglycemic effect of the crude extracts may be due to restoring ß-cell for the secretion of insulin which is the prime factor to control diabetes. Another reason to decrease blood glucose by enhancing metabolism and glucose uptake by cell or obstruction of hepatic gluconeogenesis because of presence of phenolic component, flavonoid, alkaloid [114]. The present study explained that the crude extracts (HSR, DSR and ESR) has prominent analgesic potential which were 62.21%, 67.32% and 65.34% of inhibition of writhing respectively in mice model compared to control group (Group-I). In acetic acid-induced writhing experiments, acetic acid stimulated phospholipids by the action of phospholipase A2 and acyl hydrolase which secrete arachidonic acid. This acid produces peripheral pain [115] and another component prostaglandins release due to stimulation of prostacyclin and prostaglandin-E by acetic acid which causes pain sensation. The fraction of SR significantly reduces pain by inhibiting prostaglandin synthesis

[116]. The feedback is arbitrated by peritoneal mast cells, acid sensing ion channels and the prostaglandin pathway [117].

A prototype of drug obtains from plant source which is more effective, safe and less side effect medicaments against pathogenic infection [118]. It is surprising to mention that a very few studies regarding antimicrobial activity of *Stevia rebaudiana* have accomplished. Another two components such as quercitrin, a proposed compound name 1,8-dihydroxy-3,6-dimethylanthracene-9,10-dione were also isolated from *Stevia rebaudiana* and potent antioxidant and antibacterial activities were noticed from the fraction from where these two components isolated. It was reported that quercitrin obtained from *Dendrophthoe pentandra* (L.) leaves, has also dose dependent antimicrobial activities [119]. Anthraquinone quantified from plants have lots of published data which described the antibacterial and antifungal properties. Emodin and physcion derived from *Vintilago madraspatana* two potent anthraquinones exhibited antimicrobial effects against three species of Bacillus [120]. Hence, *in-vitro* antibacterial activity of *S. rebaudiana* is due to presence of anthraquonone, alkaloids, flavonoids, tanins and triterpenoids which is previously established [121].

It has been shown that in MTT cell viability assay the inhibitions of growth on HeLa cells of compounds **1, 2, 4, 5**, and **6** depend on the concentration of drug. Compound **1** gave prominent antiproliferative activity among the isolated compounds against HeLa cell. However, the activity is less than that of methotrexate. To develop a better drug, structural modifications are necessary. It was reported that diterpene glycosides and synthetic component of them, aglycone part steviol, isosteviol as well as methanolic extract of *Stevia rebaudiana* exhibited profound antibacterial and antitubercular properties [122-124]. In our current investigation, all the isolated components (**1**-**6**) manifested a significant inhibitory effect against microorganism which supported the previous results. The activity in the current study might be due to existence of six isolated components from SR. Molecular docking study was accomplished for the six components which confirmed the exact mechanistic pathway as well as the observed pharmacological effect. A significant binding affinity of all the components were observed against folate synthesis enzyme DHFR.

Compound **1** (5-*O*-caffeoylquinic acid) also exhibited promising anticancer and antibacterial activities. We have already mentioned that in the highest prohibition of growth on *invitro* study against the HeLa cell line (IC_{50} value = 181.3 µg/mL) was examined by compound **1.** Moreover, in molecular modeling study, compound **1** indicated highest binding affinity (binding energy = -15.3 kcal/mol) against DHFR which revealed strong anticancer and antimicrobial activities. Dipole-dipole and H-bond interaction with amino acids of targeted protein DHFR would be the reason of such type of potent activities. It may again authenticate this hypothesis by the ribbon model of protein with compound **1** bound intensely inside the pocket of DHFR, which indicated the mechanism of anticancer and antimicrobial activities across the anti-metabolite pathway. Intercalating of the drug at the binding site occurred by transferring charge due to interaction of non-covalent H-bond and Pi-sigma bond (Pi-alkyl). This output recommended that compound **1** has secure structural firmness and strong prohibition properties [125].

It is the first report of *trans*-syringin (**2**) isolated from SR whereas this phenylpropanoid glycoside isolated previously from several plants [126-127]. A phenomenal antibacterial and cytotoxic activities (IC_{50} =194.40µg/mL, **Table 4.29**) was exhibited by *trans*-syringin (**2**) which was further confirmed by *in silico* approach (binding affinity -13.8 kcal/mol with DHFR). In earlier study, it has been shown that a remarkable *in vitro* anticancer activity was manifested by *trans*-syringin isolated from *Foeniculum vulgare* against HeLa cell line [128]. Based on the computational study, three conventional H-bonds and two pi-alkyl interactions was found at the binding site of DHFR where *trans*-syringin bound firmly which is explained in **Figure 4.46.** In molecular modeling study, it has been shown that luteolin was containing two non-covalent H bonds, while three H-bonds, two Pi-alkyl bonds and one T shaped Pi-Pi stacking interaction was observed **(Figure 4.46**). Although the docking score in this investigation of compound **4** (apigenin) and compound **3** (luteolin) nearly equal (-12.6 kcal/mol and -12.7 kcal/mol respectively) but binding affinity of compound **4** with DHFR was greater than compound **3**. Due to great binding affinity and intense binding vacuum accredited that compound compound **4** are more potent than compound **3** [129].

In-vitro cytotoxicity and antimicrobial studies of compound **5** (jhanol) which is a labdane-type hydroxy diterpene has been shown to be mild inhibitory effects isolated from the flower part of *Stevia rebaudiana* [130]. From computational studies of jhanol, it was predicted that it contained

non-covalent bond with Pi-Pi interaction. Therefore, it gave -10.9 kal/mol was binding force for jhanol with DHFR. For the time being, the free binding energy of compound **6** was -10.10 kal/mol, due to this adequate binding energy which is responsible for antibacterial and cytotoxic activities representing in **Table 4.28 and 4.29**. Moreover, because of their structural similarity of compound **5** and **6** displayed almost same binding energy as well as both compounds bound to the same binding pocket of enzyme DHFR (**Figure 4.46**).

This study also explores new bioactive constituents from mangrove plants and *Rhizophora mucronata* is one of the flora found in mangrove in Sundarbans coastal area [131]. This investigation is concentrated on the separation and identification of medicinally important components from dichloromethane and ethyl acetate fraction of *Rhizophora mucronata*. In this study about six compounds have been isolated and purified from this plant and compounds are β-amyrin (10), 2-oxo-14,15-bisnor-3,11 *E*-Kalavadien-10, 13, 17-triol (**11**), β-sitosterol (**12**), rutin (**13**), N-trans-para-caffeoyl-tyramine (**14**). Among them 2-oxo-14,15-bisnor-3,11 *E*-Kalavadion 10, 13, 17-triol (**11**) is a new compound for the first time isolated and structure was determined from plant source. Besides N-trans-para-caffeoyl-tyramine (**14**) were first time identified from this plant.

The different fractions of RM (HRM, DRM and ERM) have demonstrated potential DPPH scavenging activity, among them DRM and ERM fractions have shown high free radical scavenging properties (IC_{50} value were 11.7 and 10.38 µg/mL) compared to reference one ascorbic acid (6.05 µg/mL, **Table 4.4)**. The intensity of antioxidant activity was quantified by the reducing ability with the decrease in its absorbance at 517nm. In this investigation, we have isolated flavonoid type new compound 2-oxo-14,15-bisnor-3,11 *E*-Kalavadien 10, 13, 17-triol (**11**), alkaloid *N-trans*-para-caffeoyl-tyramine (**14**) and glycoside rutin (**13**). The active constituents of RM were previously reported which indicates that the presence of these compound might be responsible for antioxidant activity [132-134].

About 14 million global deaths occur annually due to infectious disease and due to multidrug resistant to microbial strains reduces susceptibility to antibiotic [135]. Antibiotic and chemicals

are only used to solve the solution. However, bacterial pathogen is getting resistance day by day by antibiotic and chemotherapy which demands for new drug from plant source for their antimicrobial activity [136]. It was evaluated that the extracts did not show significant activity which discouraged us to assess the antibacterial test of pure compound.

The *in-vitro* cytotoxicity of crude extracts HRM, DRM, and ERM from RM revealed good inhibitory effect against HeLa cell with respectable IC_{50} value of 326.30%, 89.09% and 127.6%. This activity might be due to presence of some highly bioactive compounds. Based on this result, *in-vitro* cytotoxicity activity and *in-vivo* anticancer activity of some isolated pure components (**10, 11 and 13**) was accomplished against EAC cell line. The current study is a continuing examination of our above consequence to dictate whether the antioxidant enriched RM acquire antiproliferative modes at the molecular level in EAC cell. To measure the cytotoxicity activities of the isolated pure components from RM was performed by MTT colorimetric technique. The IC_{50} value of this components were 139.8, 112.01 and 144.92 µg/ml respectively whereas the IC_{50} value of standard drug bleomycin was 31.3 µg/ml. β-amyrin also active to another cancer cell line such as isolation of β-amyrin from *Bombax ceiba* displayed 58% and 57% cytotoxicity effect on breast (MDA-MB-231) cancer and colon cancer (BT-549,) respectively [137]. Previously it was reported that inhibitory activity of compound β-amyrin (**10**) displayed significant effect against liver cancer with IC_{50} values of 25 µM. β-amyrin induced apoptosis and inhibited G2/M cycle in a dose dependent manner accredited to anticancer activity [138]. Among three components, the new compound **11** exhibited highest *in-vitro* (112.01%) and *in-vivo* (58.7%) inhibitory effect and compound **13** (rutin) displayed minimum inhibitory effect in both *in-vitro* (144.92%) and *in-vivo* (5.4%) tests against EAC cell compared to standard drug bleomycin (IC_{50} value 31.3 µg/ml *in-vitro* and 84.83% cell growth inhibition). Earlier it was reported that rutin is bioflavonoid and have mild cytotoxic effect against renal cell carcinoma (786-O) due to low water solubility and pharmacokinetic but when it was combined with two choline amino acid ILs, it gave potential inhibitory effect against renal cancer cell as well as safe for Vero kidney cell [139]. However, this is first report of the anticancer effect of rutin against EAC cell line. The activity of rutin might be enhanced by increasing solubility or combined to other agent, so further study is necessary.

Morphologically cell contraction, membrane abscess, chromosomal consolidation is cellular signaling pathway which regulate cell apoptosis, is a cell suicidal mechanism [140]. DAPI dye was used to stain treatment and control group and observed under fluorescence microscope which indicated various morphological changes. A well uniform and regular shape cell in control group mice. It is clearer in bleomycin treated group and compound **11** treated group because this group gave promising antiproliferative activity. In DNA fragmentation assay, prominent and high molecular weight band observed for control group but in compound induced group splodge type band observed in 1% agarose gel electrophoresis. It might be due to the existence of protein known as DNA fragmentation factor (DFF) [141]. The principal biochemical endorsement of programmed cell death is the Ca^{2+}/Mg^{2+}-dependent endonuclease stimulation [142]. Nuclear fragmentation and chromatin condensation is the major fact for this type of apoptosis which firmly demonstrate that compounds **10 and 11** exhibited the cytotoxicity activity via apoptosis of the cancer cell.

There is different type of genes that involve regulating the cell cycle. The upregulation and downregulation of gene can help in recovering the cancer cell cycle which is the responsible to rein tumor growth and programmed cell death. The compounds **10, 11** and **13** inducing EAC cell gene expression of Bax, P^{53}, Cyt C, Bcl-2, NF-κB TNF-alpha, Cas-3, Cas-8, and Cas-9 has shown and the outcomes of our examination strongly proposed that the compounds **10** and **11** enhance the expression of Bax, P^{53}, Cyt C, TNF-alpha, Cas-3, Cas-8, and Cas-9 and decrease expression of Bcl-2, NF-κB gene explained in **Figure 4.53 and 4.54.** Compounds induced EAC cells revealed the apoptosis due to activation of tumor suppressor gene. Besides, decrease regulation of BcL-2 gene causes upregulation of Bax, which carry signal to mitochondria aiming to cell death [143].

Abnormalities in blood formation, hepatotoxicity and renal toxicity are unwanted adverse effects that produce from most of the anticancer drug. Here, we examined blood, liver parameters (ALT, AST, ASP and bilirubin) and kidney parameters (Urea and creatinine) of both treated and un-treated mice. EAC bearing untreated mice have shown abnormalities such as number of RBC and percent of Hb was decreased and number of WBC was increased than normal level whereas treatment group turn the level near to normal range which was evident from the T**able 4.34.** Most of the conventional anticancer drug produce bone marrow depression as a result abnormalities in number of cells are found but, in this study, we have seen that the components come to blood

parameters to normal range. The liver parameters in normal control mice were 60 μ/L (ALP), 20 μ/L(ASP), 50 μ/L (ALP) and <10 $mgdL^{-1}$ (bilirubin) and the kidney function parameters were 18.6 $mgdL^{-1}$ (urea) and 0.6 $mgdL^{-1}$ (creatinine) whereas EAC cell bearing control mice exhibited abnormalities except creatinine levels in kidney was normal for all group mice. It might be because of short term EAC cell could not produce negative effect in creatinine level. This outcome revealed that the compounds isolated from RM especially compounds **10** and **11** have prominent anticancer activity.

Histopathology examination of liver and kidney was not found any severe damage to the host shown in **Figure 4.57** and **4.58.** The cellular structure was normal, and the hepatocytes of all group mice was normal and photomicrographs of renal retain normal architecture in both control and treatment group mice. It has been observed that anticancer drugs that used for breast cancer such as flulvestrant, palbociclib and erdafitinib produces liver and kidney dysfunction but the compounds **10**,**11** and **13** did not create any hepatotoxicity and nephrotoxicity. So, in conclusion the compounds **10**, **11** and **13** have promising anticancer activity without producing side effect and further study is necessary to synthesize newer drug which will be more effective with producing very mild side effects and cost effective for patient as well as effective to other cell line.

6. Conclusion

In conclusion, three fractions (HSR, DSR and ESR) from 70% methanolic extract of *S. rebaudiana* were evaluated for antioxidant, analgesic, antihyperglycemic and antimicrobial properties. Al fractions of the plants have demonstrated significant antioxidant, potent antidiabetic, and analgesic and moderate antibacterial activities. Compound **1** isolated from *Stevia rebaudiana* exhibited significant inhibitory activity against on HeLa cell and certain gram positive and gram-negative microorganisms. Remaining components such as compounds **2, 3, 4,7, and 8** that isolated from SR gave mild to moderate inhibitory effects against HeLa cell and the selected pathogens. In the present studies we have seen that compound **4** (apigenin) has prominent DPPH mediated free radical scavenging activity whereas average antioxidant activity was found in case compound **1** (5-*O*- caffeol quinic acid), **3**, (luteolin) and **6** (janidiol). In the current investigation, it has been seen those secondary metabolites (**1**, **2, 3, 4,7, and 8**) isolated from the plant SR imparted good binding affinity at the active site of target protein, DHFR, glutathione reductase and urase oxidase. Hence, due to this interaction, it is predicted that the isolated compounds (**1**, **2, 3, 4,7, and 8**) showed promising anticancer, antibacterial and antioxidant activities respectively.

Therefore, the findings in this investigation proved that the plant species is very predominant for anticancer, antibacterial, and free radical scavenging potential which is done for the first time. Further study is demanded to isolate new components or structural modification of the components or preparing synthetic components from the isolating phytochemicals to actuate new drug development.

In this experiment, we have isolated six compounds from 100% methanolic extract of *Rhizophora mucronata*. Among them 2-oxo-14,15-bisnor-3,11 *E*-Kalavadien-10, 13, 17-triol (**11**) was isolated as a new compound from the plant source and *N*-trans-para-caffeoyl-tyramine **(14)** isolated first time from this plant. This investigation explained that the three fraction HRM, DRM and ERM were exhibited potential antioxidant effect whereas these extracts showed mild analgesic and antimicrobial effect as well as it did not exert antihyperglycemic effect in streptozotocin induced diabetic mice. Besides, all the fractions gave promising antiproliferative activity on the HeLa cell and based on this result it was examined *in-vitro* and *in-vivo* anticancer activity of the isolated compounds beta-amyrin (**10**), 2-oxo-14,15-bisnor-3,11 *E*-Kalvadien -10, 13, 17-triol (**11**) and rutin (**13**) on EAC cell. Among them compounds **10** and **11** gave promising activity by inhibiting the

growth of tumor. In mice model, the isolated compounds (**10** and **11**) significantly induced apoptosis but rutin (**13**) exerted mild or very poor inhibitory effect on tumor cell and this study imparts evidence for the apoptosis inducing ability of the components **10, 11** and **13** for the first time. The apoptosis was associated with upregulation of Bax, P^{53}, Cyt C, TNF-alpha, Cas-3, Cas-8, and Cas-9 and down regulation of Bcl-2, NF-κB gene which indicate that the apoptosis induced by compounds was mediated by extrinsic and mitochondrial pathway. In the present study, compounds **10** and **11** have potential effect to recover the malformation due to cancer that indicate that compounds **10** and **11** have ability to gain healing process. Moreover, it has been examined that compounds β-amyrin (**10**) and 2-oxo-14,15-bisnor-3,11 *E*-Kalavadien -10, 13, 17-triol (**11**) and rutin (**13**) did not display toxicity on liver and kidney. Based on this study, we may assume that the isolated compounds might be the lead compounds for development of chemotherapy, free radical scavenging, and antibacterial therapeutic agents though the exact mechanism is not underlying yet and, the research should be carried for improvement of semi-synthetic derivatives or better drugs to combat cancer as well as various microbial infection.

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Phytochemical and Biological Investigation of *Stevia rebaudiana* (Bert.) Leaves Grown in Bangladesh

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ABSTRACT: Three fractions obtained by Kupchan partitioning of 70% methanol extract of leaves of *Stevia rebaudiana* with *n*-hexane, dichloromethane and ethyl acetate designated by HSR, DSR and ESR were investigated for their antioxidant and antimicrobial activities. HSR and ESR exhibited prominent free radical scavenging activity having IC_{50} value of 11.59 and 10.38 µg/ml compared to standard, ascorbic acid (IC_{50} value 6.05 µg/ml). HSR and ESR also demonstrated significant antibacterial activity by disc diffusion method on some Gram-positive and Gram-negative bacteria. Among the fractions ESR showed the highest antioxidant and antibacterial activity. No significant antifungal activity was observed for any fraction. Preliminary phytochemical screening of all fractions showed the presence of alkaloids, saponin, flavonoids, tannin, carbohydrate and triterpenoids. Total three compounds have been isolated from ESR fraction among which two known compounds (**1-2**) were characterized as quercitrin (**1**) and physcion (**2**) through 1D NMR spectroscopic technique and other one could be a 9,10-anthraquinone derivative which was not characterized yet. The compound physcion (**2**) is first time report from the plant *Stevia rebaudiana.*

Key words: Quercitrin, Physcion, Candy leaf, Antibacterial, Free radical, DPPH, and *Stevia rebaudiana.*

INTRODUCTION

Stevia rebaudiana (Bertoni) commonly known as “sugar leaf” or ‘candy leaf ‘belongs to the family Asteraceae.[1] This perennial sweet herb is distributed in humid and wet environment and is indigenous to Brazil and Paraguay.[2] Stevia is being used as a source of natural sweetener due to its high content in sweet diterpene glycosides, such as isosteviol, stevioside, rebaudiosides (A, B, C, D, E and F), steviolbioside and dulcoside A. About 100 or more compounds have been identified in *Stevia rebaudiana*, the best known of which are the steviol glycosides, particularly stevioside, rebaudioside A and C being the most abundant which are 250-300 times more sweet than sugar[3] and less common bioactive constituents are phenolic compounds and flavonoids like quercetin, luteolin, apegenin.[4,5] Beside glycosides, the leaves of stevia also contain other phytochemicals, such as flavonoids, phenolic acids, fatty acids, proteins and vitamins. Several studies have reported that stevia extracts strived significant antimicrobial, anti-hypertensive, anti-inflammatory, hepatoprotective and immunomodulatory activities, due to its high abundance in bioactive phyto-constituents.[6-8] Now-a-days, stevia is widely cultivated in many countries of South East Asian region including Bangladesh owing to its many health benefits. The phytochemical compositions and bioactivity of any plant and plant-derived products varies greatly with the change of climate and geographical conditions such as soil, water cultivation process etc. In the present time pathogenic and spoilage microorganisms has increased due to the increase outbreaks of several type of new microorganism.[9] To our concern a very few scientific trials on antimicrobial and the antioxidant capacity of

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S. rebaudiana cultivated in Bangladesh has been evaluated. The objective of the present study was to conduct a bioactivity directed phytochemical investigation on *S. rebaudiana*. In this study, antioxidant and antimicrobial activities of different fractions derived from leaves of *S. rebaudiana* will be investigated followed by isolation of active components from the different fractions of crude extract.

MATERIALS and METHODS

Collection of plant materials. The leaves of *Stevia rebaudiana* (SR) were collected from Brac Nursery located in Joydebpur, Gazipur in late March 2018. The exsiccated plant samples were identified by Mr. Sardar Nasir Uddin, Principal Scientific Officer, Bangladesh National Herbarium, Mirpur, Dhaka where a voucher specimen has been deposited for future reference (Accession number: DACB-38588).

Instruments and materials. Column chromatography was conducted on silica gel (60-120 mesh size, E-Merck) whereas thin layer chromatography (TLC) and preparative TLC were carried out on precoated silica gel 60 F_{254} (Aluminium sheets, E-Merk, Germany). ^{1}H and ^{13}C NMR spectral data were recorded on Bruker 400 and 100 MHz, respectively. Ascorbic acid and ciprofloxacin were obtained as gifts from Square Pharmaceuticals Ltd; Bangladesh and DPPH (1, 1-diphenyl-2- picrylhydrazyl) was purchased from Sigma Chemical Co. Ltd (St. Louis, MO, USA).

Extraction. The fresh leaves were shade dried and powdered (1 kg) which were extracted with 70% methanol at room temperature for 15 days. White cotton followed by Whatman filter paper number-1 was used to get clean filtrate. The filtrate was concentrated by using a rotary evaporator to obtain greenish black mass (100g). The crude extract was then fractioned by the modified Kupchan partitioning protocol[10] to yield n-hexane, dichlormethane and ethyl acetate soluble materials of *S. rebaudiana* designated as HSR, DSR and ESR having 8%, 10% and 15% w/w yield, respectively.

Phytochemical screening. Phytochemical analysis of the different extracts was performed with the standard methods outlined by Nayek and Pereira.[11]

Isolation of compounds from ESR fraction. ESR fraction (500 mg) was used to isolate pure components by using column chromatography. At first, the ESR fraction was dissolved in methanol and mixed with small amount of silica gel (mesh size 60-120, 1 g sample) to make dry sample for column chromatography (CC). After complete mixing with spatula, methanol was initially evaporated and finally it was completely dried in a vacuum desiccator. The fine adsorbed sample was then loaded on a clean and dry glass column packed with silica gel (60-120 mesh size) and eluted with ethyl acetate: acetic acid: water (9:0.5:0.5) in order of increasing polarities. Each column fraction (10ml) collected in test tubes was monitored by TLC. Fractions with identical TLC features were combined and a total 10 (A-J) fractions obtained. The pooled fractions were then re-chromatographed by preparative thin layer chromatography (PTLC) using ethyl acetate: acetic acid: water (6:2:2) system to afford yellowish crystals of compound **1** (8 mg), white powder of compound **2** (10 mg) and white crystals of compound **3** (6 mg) with R_f values of 0.3, 0.5 and 0.7 respectively. Structure of the isolates was characterized by ^{1}H-NMR and ^{13}C-NMR spectral data.

Properties of isolated compound. Quercitrin (1): Pale yellow needle crystals, ^{1}H NMR (400 MHz, CD_3OD): δ 7.35 (1H, d, *J*=1.5 Hz, H-2'), 7.30 (1H, dd, *J*=8.0 and 1.5 Hz, H-6'), 6.9 (1H, d, J= 7.5 Hz, H-5'), 6.37 (1H, d, *J*=1.5 Hz, H-8), 6.19 (1H, d, *J*=1.5 Hz, H-6), 5.34 (1H, br. s, H-1''), 0.82 (3H, d, *J*=3.5 Hz, H-6''). ^{13}C NMR (100 MHz, CD_3OD): δ 148.4(C-2), 134.8(C-3), 177.9(C-4), 161.8(C-5), 98.7(C-6), 164.6(C-7), 93.6(C-8), 157.8(C-9), 104.4(C-10), 121.4(C-1'), 115.6(C-2'), 157.2(C-3'), 145.1(C-4'), 115.1(C-5'), 121.6(C-6'), 102.1(C-1''), 70.6(C-1''), 70.7(C-1''), 71.9(C-1''), 70.5(C-5'') and 17.4(C-6'').

Physcion (2). White powder, ^{1}H NMR (400 MHz, CD_3OD): δ 7.75 (1H, m, H-7), 6.96 (1H, d, J= 2.7 Hz, H-5), 6.66 (1H, m, H-4), 6.21 (1H, m, H-2),

5.35 (2H, s, 1-OH and 8-OH), 4.12 (3H, s, H-3), 1.98 (3H, s, H-6).

Antioxidant activity. Antioxidant activity was evaluated by using stable free radical 0.04% DPPH (1,1-diphenyl-2-picrylhydrazyl). Suitably diluted stock solutions were spotted on stained silica gel TLC plate and the plate was kept in the chambers containing solvent according to different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extract. The plate was seared at room temperature and was sprayed with 0.02% DPPH in ethanol. Due to wash out by DPPH, resolved bands were observed for 10 minutes and the color changes (yellow on purple background) were observed.[12]

Stock solution of the crude drugs that means three fraction HSR, DSR and ESR were processed in methanol from which a serial dilution was executed to reach concentration of 1, 5, 10, 50, 100, 500 µg/ml. The new concentration of samples (each 2ml) were mixed with 2 ml of a 0.004% ethanol solution of DPPH and conceded to keep for 30 minutes for reaction to be found. The absorbance was examined at 517nm and from these values the respective percentage of inhibitions were figured. IC_{50} value was calculated from the graph, which was arranged by plotting % of inhibitions against log concentration. The experiment was done 3 times and average absorption was noted for each concentration.[13] Ascorbic acid was used as standard drug.

Antibacterial activity. Disc diffusion method [14] was used for the estimation of antibacterial activity of HSR, DSR and ESR materials against a number of Gram (-)ve *Bacillus cereus*, *B. megaterium*, *B. subtilis*, *Staphylococcus aureus*, *Sarcina lutea* and the Gram (-)ve *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *S. typhi*, *Shigella boydii*, *Sh. dysenteriae*, *Vibrio mimicus*, *V. parahemolyticus*, and three unicellular fungi *Candida albicans*, *Aspergillus niger*, *Sacharomyces cerevacae*. Test samples were prepared using sterile blank discs (6 mm) which were saturated with the test samples dissolved in methanol at concentration of 500µg/disc by a micropipette and used for the assay. After incubation as per the published method, the diameter of inhibitory zones formed around each discs were computed using digital slide calipers.[15] Ciprofloxacin (5µg/disc) and fluconazole (5µg/disc) were used as positive controls for antibacterial and antifungal activities, respectively.

RESULTS

Phytochemical screening. Phytochemical screening of the different fractions of the plant extract by the standard methods revealed the presence of several phytoconstituents such as alkaloids, flavonoids, saponins, tanins and terpneoids as shown in Table 1.

Table 1. Result of phytochemical screening of extracts.

Test sample	Alkaloids	Saponins	Flavonoids	Tannins	Triterpenoids
HSR	+	+	+	+	+
DSR	+	-	+	-	+
ESR	+	+	+	+	+

+ Presence, - Absence

Evaluation of antioxidant activity. Antioxidant activity of the tested fractions was evaluated according to the method specified above and the results are summarized in Table-2. Figure 1 depicts the percent inhibition of DPPH radicals upon increasing concentration of the compounds. The IC_{50} value of HSR, DSR, ESR and ascorbic acid were found to be 11.59, 44.7, 10.38 and 6.05 µg/ml, respectively. Among the extract, ESR showed the most prominent antioxidant property which is comparable to ascorbic acid. HSR and DSR also exhibited moderate antioxidant property.

Evaluation of antimicrobial activity. The antibacterial activity of HSR, DSR and ESR were evaluated at 500µg/ disc on the growth of pathogenic

bacteria and fungi by the disc diffusion method and the results are summarized in Table 3. The crude fractions designated as HSR, DSR and ESR exhibited antibacterial activity with an average zone of inhibitions 10.3-15.3 mm, 6.2 -8.5 mm and 10.2- 16.8 mm, respectively whereas standard ciprofloxacin (5µg/ml) showed 16-23 mm. The results indicated that at the dose of 500µg/ disc ESR and HSR accorded mild to moderate activity against both Gram (+)ve and Gram (-)ve strains than DSR fraction. However, ESR, HSR and DSR did not show any antifungal activity.

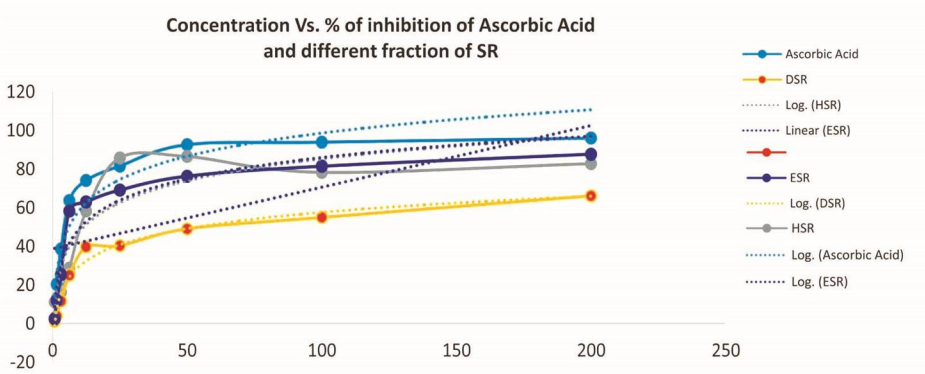

Figure 1. Concentration against % of inhibition of HSR, DSR, ESR extract and ascorbic acid

Table 2. IC_{50} values of HSR, DSR, ESR extract and ascorbic Acid.

Test sample	Regression line	R^2	IC_{50} µg/ml
Ascorbic acid	y = 17.353 In(x) + 18.713	0. 9142	6.05
HSR	y = 16.1336 In(x) + 9.905	0.8551	11.59
DSR	y = 12.092In(x) + 1.8883	0.9785	44.7
ESR	y = 15.92In(x) + 12.615	0.9197	10.38

*Ascorbic acid as standard

Table 3. Antimicrobial activity of the fractions against Gram positive and Gram negative bacteria.

Pathogens	Zone of Inhibition (mm)			
	HSR (500 µg/ml)	DSR (500 µg/ml)	ESR (500 µg/ml)	Ciprofloxacin (250 µg/ml)
Bacillus cereus	10.3 ± 0.21	-	14.2 ± 0.43	18 ± 0.11
B. megaterium	15.1 ± 0.33	-	15.1 ± 0.22	20 ± 0.32
B. subtilis	13.7 ± 0.22	7.1 ± 0.45	13.4 ± 0.33	19 ± 0.23
Staphylococcus aureus	11.1 ± 0.66	-	12.6 ± 0.44	17 ± 0.26
Sarcina lutea	14.2 ± 0.11	8.5 ± 0.29	12.4 ± 0.22	22 ± 0.32
E. coli	13.1 ± 0.23	-	10.5 ± 0.42	16 ± 0.52
P. aeruginosa	10.9 ± 0.43	6.2 ± 0.33	11.3 ± 0.34	17 ± 0.34
S. paratyphi	12.2 ± 0.21	7.4 ± 0.23	16.1 ± 0.21	23 ± 0.44
S. typhi	11.1 ± 0.31	-	10.2 ± 0.22	16 ± 0.44
Sh. boydii	13.3 ± 0.21	6.3 ± 0.23	16.8 ± 0.20	22 ± 0.74
Sh. dysenteriae	15.3 ± 0.66	6.5 ± 0.66	13.7 ± 0.13	18 ± 0.43
V. mimicus	14.7 ± 0.31	-	15.1 ± 0.22	21 ± 0.53
V. parahemolyticus	11.6 ± 0.11	-	13.7 ± 0.12	18 ± 0.23

*values mentioned here Mean ± standard deviation (n = 3)

Isolation of pure compounds from ESR extract. As the ESR fraction exhibited most prominent antioxidant and antibacterial properties, the fraction was subjected to isolation of active constituents. A total of three compounds (Figure 2) were isolated from ESR fraction of SR by column chromatography and PTLC. The high field NMR analysis was used for elucidation of structure of the isolated compounds and compared with published NMR data. The ^{1}H- NMR spectrum (400 MHz, CD_3OD) of compound **1** revealed a pair of meta-coupled aromatic doublet at δ 6.37 ppm and 6.19 ppm corresponding to H-6 and H-8 protons, respectively and an ortho-coupled double doublets at δ 7.30 ppm (J = 8.0 and 1.5 Hz) for H-6′ protons. Another meta-coupling was observed between the protons of H-2′ and H-6′ at 7.35 and 7.30 ppm, respectively. The compound **1** was composed of quercetin, a flavonoid which linked to rhamnose, in the position of C_3 via C-O-C bonding. The ^{13}C NMR spectrum of compound **1** displayed carbonyl group at 177.9 ppm and other signals between 93.6- 164.6 ppm for aromatic carbons. The ^{1}H NMR and ^{13}C NMR spectral data of compound **1** was compared to reference value of quercitrin and found to be identical.[16,17] Thus, compound **1** was identified as a flavonoid glycoside, quercitrin.

Compound **2** is a natural pigment, gave intense benzoid absorption band on the UV region ranges between 240-260 nm.[18] Compound **2** displayed two pairs of meta-coupling proton at the position of H-7 & H-5 and H-2 & H-4, respectively. Additionally, it showed a singlet for methoxy group at C-3 and a singlet at 5.35 ppm for hydroxyl group corresponding to H-1 and H-8 which is resemblance to physcion and confirmed by comparison with previous report.[19] On the other hand, the unknown compound gave two peaks only among which a singlet at 4.98 ppm and another singlet at 1.91 with integrating 3H. All these features indicated that unknown compound may also be a 9,10-anthraquinone derivative which is still under investigation. Thus, the two isolated compounds were characterized as quercitrin (**1**) and physcion (**2**). This is the first report of isolation of compound **2** from this plant.

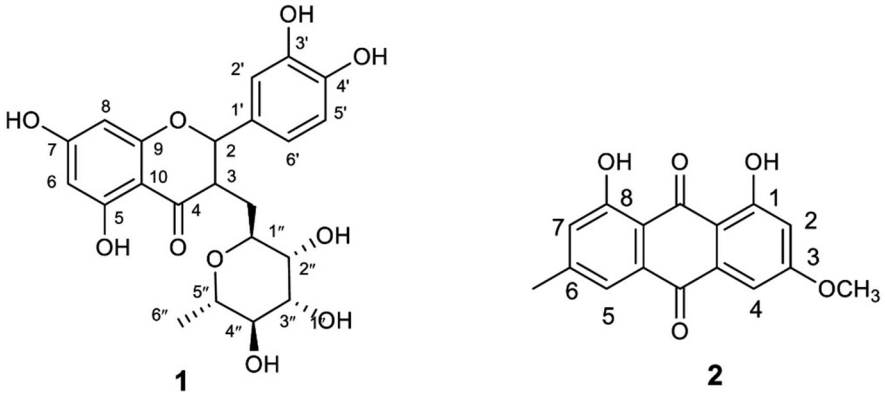

Figure 2. Structures of quercitrin (1) and physcion (2) isolated from *S. rebaudiana*

DISCUSSION

In this study, the plant extracts were fractionated into three major fractions namely HSR, DSR and ESR fractions. These fractions contained major phytoconstitutents which might be responsible for their activities. All the fractions exhibited antioxidant property. Antioxidant activity of the extracts depends on the ability to scavenge DPPH radical. DPPH stable free radical method is a most common method to determine the antioxidant activity of plant extracts.[20,21] Many disorders like neurodegenerative diseases, cancer and AIDS are associated with over activity of free radicals and it is accepted that antioxidants due to their scavenging properties are

useful for the management of those disease. Among the extracts, HSR and ESR provided promising antioxidant activity compared to standard ascorbic acid. The capability of *S. rebaudiana* extracts to scavenge DPPH could be accredited to the content of phenol, flavonoids as well as alkaloid as accentuated in several reports.[22, 23] Redox properties is mainly responsible for this activity which could be due to adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides.[24] *S. rebaudiana* extract could play a vital role as a human diet to improve antioxidant intake. [25] As it was found that ESR fraction showed most prominent antioxidant effect, it could be rationalized that the flavonoids and anthraquinone derivatives (compound **1-2**) isolated from the fraction might be responsible for antioxidant activity because antioxidant is the common biological effects of both edible and non-edible plants containing flavonoids and phenolic compounds.[26] Moreover, a study about the relationship of anthraquinone derivatives and their antioxidant activities concluded that hydroxyl groups either at meta or para position are responsible for the antioxidant activity.[27] It has also been reported that not only the hydroxyl group, but also the carbonyl group of anthraquinone skeleton is responsible for the antioxidant potential.[27]

The drug from plant sources act as a prototype to produce less toxic and more effective medicines against the growth of microorganism. [28] It is worthwhile to note that there are very few research works that have been done for antimicrobial activity of *Stevia rebaudiana*. In this study, we isolated three components quercitrin, physcion and an unidentified 9,10-anthraquinone derivatives from ESR fraction and this fraction showed prominent antioxidant and antibacterial activities than other fractions. It has been shown that the increase in concentration of quercitrin in culture media increase the linear value of inhibition zone. Quercitrin, a flavonoid, isolated from *Dendrophthoe pentandra* (L.) leaves is known to exhibit prominent antioxidant and antibacterial activities.[16] Many reports have already explained the antibacterial and antifungal activities of anthraquinone isolated from natural sources. Two anthraquinones identified as emodin and physcion isolated from *Vintilago madraspatana* revealed antibacterial activity against three species of *Bacillus*.[29] Therefore, It is well accepted that chemical compounds present in *S. rebaudiana* such as anthraquonone, alkaloids, flavonoids, tanins and triterpenoids play an important role in its antibacterial activity *in vitro*.[30] As the crude drugs are complex in nature, all components might be synergistically or cumulatively responsible for their antibacterial activity.

CONCLUSION

In conclusion, three fractions (HSR, DSR and ESR) from 70% methanolic extract of *S. rebaudiana* were evaluated for antioxidant and antimicrobial properties. HSR and ESR fractions of the plants have demonstrated significant antioxidant and moderate antibacterial activities. Phytochemical investigation of ESR fraction provided three compounds (**1, 2** and unidentified one). Among the three compounds, physcion is isolated first time from the plant *S. rebaudiana*. The plant material can be further studied extensively to isolate novel natural compounds by considering the potential bioactivity and scope which may persuade new drug development.

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Research article

Isolation of phytochemical constituents from *Stevia rebaudiana* (Bert.) and evaluation of their anticancer, antimicrobial and antioxidant properties via *in vitro* and *in silico* approaches

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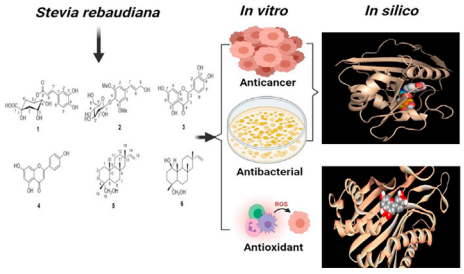
GRAPHICAL ABSTRACT



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ABSTRACT

The current study was designed to isolate and characterize some bioactive secondary metabolites by using repeated chromatographic and spectroscopic techniques, targeting their anticancer, antimicrobial, and antioxidant properties through *in vitro* and *in silico* approaches. Six compounds were isolated and analyzed by thin layer chromatographic technique and the compounds were identified as 5-*O*-caffeoyl quinic acid (**1**), syringin (**2**), luteolin (**3**), apigenin (**4**), jhanol (**5**), and jhanidiol (**6**) based on spectroscopic methods. The cytotoxic effect of each compound was dose-dependent, and compound **1** showed a higher anti-proliferative effect (IC_{50} = 181.3 μg/ml) than other compounds (compound **2**, **4**, **5**, and **6**). Besides, compound **1** showed the most promising antibacterial activity with a zone of inhibition ranges from 12–15 mm against different strains compared to ciprofloxacin (14–22 mm). In contrast, compound **3** exerted the highest scavenging property against DPPH free radical. Finally, the *in vitro* bioactivities were also supported by molecular docking studies. The computational study demonstrated that the isolated compounds exerted stronger affinity compared to the standard drugs towards the binding sites of dihydrofolate reductase (DHFR), glutathione reductase, and urase oxidase.

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1. Introduction

Since thousands of years ago, human civilization has been using various plants as therapeutic weapons to treat numerous diseases based on safe consumption. Nevertheless, screening of different medicinal plants and their application in the treatment of multiple illnesses increases with scientific development [1].

Stevia (*Stevia rebaudiana* Bertoni), a perennial shrub from the *Asteraceae* family, is native to South America. However, the plant is cultivated worldwide, including in Bangladesh [2]. This plant has drawn economic and scientific interest due to its natural, non-toxic, and nonmutagenic sweetener properties. Due to the presence of several steviol glycosides in its leaf, its sweetening potency is 200–300 times more than that of sucrose, worth of use as a sugar substitute in the food and drug industry [2, 3]. The leaves possess three main sweetening constituents namely stevioside, rebaudioside A and C; those have *ent*-kaurene diterpene steviol skeleton common in their structures [4, 5, 6]. Besides, some other glycosides such as staviolbioside, isostaviol, labdane-type diterpenes sterebins E, H, N and I were also found in this plant [7, 8]. In addition to glycosides, several phenolic compounds, the key components for antioxidant property, were also reported [9, 10]. The polyphenol compounds isolated from the plant leaves are chlorogenic acids, esters molecules of polyphenols, including hydroxycinnamic acids with quinic acid, which have excellent free radical scavenging properties, and other numerous health benefits [11]. Apart from its industrial applications, *stevia* has been found to be effective in the management of diabetes, obesity, cancer, hypertension, dental carries and infectious diseases [12, 13, 14]. Significant effect in alleviating kidney and liver damage was reported earlier along with increments of insulin level in Streptozotocin-induced diabetic rats [15]. A comprehensive review on the plant has been reported mentioning its role in blood pressure regulation, inflammatory bowel disease, renal function, glucoregulation, tumor and obesity management [16]. Although several bioactivities of *S. rebaudiana* extracts and some of the glycoside constituents have been reported, studies on antimicrobial and anticancer potentials are very limited. Only very few reports are found addressing antimicrobial and anticancer potentials of the crude extracts [17, 18, 19]. In this regard, we have also recently reported antimicrobial and antioxidant properties of different extracts of *Stevia rebaudiana* [20].

To the best of our knowledge, none of the above study has isolated the bioactive components that may exert these actions. Herein, we report the isolation and characterization of some bioactive secondary metabolites by using repeated chromatographic and spectroscopic techniques, respectively targeting their antimicrobial, anticancer, and antioxidant properties. Docking studies of the isolates were also performed with the active site of dihydrofolate reductase (DHFR), glutathione reductase, and urase oxidase to support the *in vitro* bioactivities.

2. Method and materials

2.1. Materials and instruments

HELA cell, DME medium, and 10% fetal bovine serum were purchased from authorized suppliers and methotrexate, ciprofloxacin, fluconazole, and ascorbic acid were obtained from Eskyf Pharmaceutical Limited, Bangladesh. Biological biosafety cabinet (NU-400E, Nuaire, USA), CO_2 incubator (Nuaire, USA), trinocular microscope with camera (Olympus, Japan), and hemocytometer (Nexcelom, USA) were used to execute the cytotoxicity test. A very safety environment was maintained to handle all instruments and chemicals. Fresh leave of *Stevia rebaudiana* were collected from BRAC cultivation center, Dhaka, Bangladesh in March 2018. The exsiccated plant samples were identified by Principal Scientific Officer, Bangladesh National Herbarium, Mirpur, Dhaka where a voucher specimen has been deposited for future reference (accession number: DACB-38588).

The ^{1}H and ^{13}C NMR spectra were measured on a Burker AMX-400 operating at 400 MHz and 100 MHz for ^{1}H and ^{13}C, respectively. Chemical shifts are expressed in δ (ppm) scale using tetramethylsilane as internal standard and coupling constants (*J*) are expressed in hertz (Hz). Analytical thin layer chromatography (TLC) was carried out on precoated (TLC Silica gel 60 F_{254}- MerkKGa) plates. TLC spots were screened under UV light (254 nm) and spraying with 10% H_2SO_4 and anisaldehyde reagents followed by heating. Column chromatography was carried out with powdered silica gel (Kieselgel 60, 230–400 mesh, Merck KGaA, Dermstadt, Germany) and primarily packed in a glass column with a dimension of 5 × 60 cm. Preparative TLC plates were prepared on a glass (20 × 20 cm) surface coated with slurry of silica gel.

2.2. Isolation of compounds

The dried and powdered fresh leaves (1 kg) were extracted with 70% methanol with occasional shaking and stirring. The whole mixer was then filtered through clean, white cotton followed by Whatman filter paper no. 1. The filtrate was concentrated with a rotary evaporator. An aliquot (100 g) of concentrated methanol extracts were fractionated by *n*-hexane, dichlormethane and ethyl acetate and the resultant fractions were evaporated to dryness to yield *n*-hexane *Stevia rebaudiana* (HSR), dichloromethane *Stevia rebaudiana* (DSR) and ethyl acetate *Stevia rebaudiana* (EtASR) soluble materials having 8%, 10% and 15% (w/w) yield, respectively. The dried residues were then stored in a refrigerator until further investigation. Based on the preliminary antimicrobial screening [20], EtASR was chosen for further isolation and purification. The EtASR fraction (3 g) was dissolved in methanol and mixed with small amount of silica gel (mesh size 60–120, 3 g) to make a dry sample for convenient application in column chromatography (CC). The dry sample was then loaded on a clean and dry glass column (3 × 40 cm) packed with silica gel (60–120 mesh size) and eluted with a mixture of solvents ethyl acetate: acetic acid: water (8:1:1) with increasing polarity and 10 ml elute was collected and monitored by thin layer chromatography (TLC). After TLC pooling, a total 15 fractions were obtained. Fractions 5–10 were mixed together and repeated preparative Thin Layer Chromatography (PTLC) was carried out to yield 5-*O*-caffeoyl quinic acid (**1**) 16 mg, syringin (**2**) 15 mg, luteolin (**3**) 8 mg and apigenin (**4**) 14 mg. Again, the *n*-hexane fraction (HSR) was subjected to silica column chromatography using the solvent system of *n*-hexane and ethyl acetate at a ratio 9:1 for elution. The test tubes number 46–53 gave same spot (**5**) and 60–72 numbers have identical spot (**6**) on TLC plate which were identified as jhanol (**5**) 30 mg and jhanidiol (**6**) 25 mg, respectively.

2.3. MTT cell viability assay

The cell proliferation studies of compounds **1, 2, 4, 5** and **6** were examined in Centre for Advanced Research in Sciences (CARS), Dhaka, Bangladesh against HeLa cell line where Methotrexate was used as a positive control with a series of concentration (62.5, 125 and 250 μL/mL), DMEN (Dulbeco's modified eagles' medium) containing 1% penicillin-streptomycin (1:1), 0.2% gentamycin, and 10% fetal bovine serum (FBS) were maintained for HeLa cell, a human cervical carcinoma cell line. HeLa cells were implanted onto a 96-well plate with 2×10^4 cells per well (100 μL) and incubated at 37 °C under a humidified atmosphere of 5% of CO_2 for 24 h. Therefore, the five components (**1, 2, 4, 5** and **6**) and positive control (methotrexate) were dissolved in 2.5% DMSO where DMSO was used as negative control. Components with a series of concentration (62.5, 125, 250 and 500 μL/mL) and positive control at the concentrations (25, 50 and 100 μL/mL) were added and incubated for 48 h. After incubation using cell counting Kit-8 (CCK-8), a non-radioactive colorimetric Cell Proliferation and Cytotoxicity assay kit (Sigma-Aldrich, USA) was used for measuring cell viability. Duplicate wells were used for each sample [21].

2.4. Antimicrobial assay

The initial screening of antimicrobial activity and minimum inhibitory concentration (MIC) determination for the tested compounds were

performed by using disc diffusion technique with different strains [22]. Thirteen bacterial standard strains and three fungal strains among which five Gram-positive and eight Gram-negative strains were obtained from the Biomedical research laboratory, University of Dhaka, Bangladesh. The gram (+)ve bacteria were *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus*, *Sarcina lutea* and the Gram (-)ve were *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Salmonella typhi*, *Shigella boydii*, *Shigella dysenteriae*, *Vibrio mimicus*, *Vibrio parahemolyticus*, and three unicellular fungi were *Candida albicans*, *Aspergillus niger*, *Sacharomycescerevacae* used. Test samples were prepared using sterile blank discs (6 mm) which were loaded with the test samples dissolved in methanol at concentration of 250 μg/disc by a micropipette. Ciprofloxacin (5 μg/disc) and fluconazole (5 μg/disc) were used as positive controls for antibacterial and antifungal activities, respectively. The experiment was carried out in thrice and the mean diameter was taken.

2.5. DPPH free radical scavenging assay

The antioxidant activity of the isolated compounds was assessed by utilizing the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay method explained by Brand-Williams et al. [23]. However, for the quantitative assay described by Sultana et al. [20], the diluted stock solutions were spotted on a TLC plate, which was then run in the proper solvent system for the appearance of the compounds at distinct positions on the plate. Then the plate was sprayed with 0.02% (w/v) DPPH in ethanol at room temperature. An apparent color change was observed as DPPH was cleared out by the effect of these compounds, detecting the potential antioxidant property of these compounds.

The spectroscopic method was further applied for quantitative measurement of antioxidant property of these compounds by utilizing DPPH free radical scavenging assay [20]. Initially, 2 ml of each sample (and ascorbic acid as standard) concentrations ranged from 500 μg/mL to 1 μg/mL by serial dilution (500, 100, 50, 10, 5, 1 μg/mL) were mixed with 2 ml freshly prepared DPPH solution (0.004% w/v). The resulting solutions were mixed in the test tubes carefully. After 30 min incubation in a dark place at room temperature, the UV absorbance of these mixes was recorded precisely against the blank solution (ethanol) at 517 nm wavelength. The percentage of DPPH scavenging or % inhibition was measured by using the following equation:

$$\%\ \text{Scavenging} = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100\% \quad (1)$$

Where A_{blank} = absorbance of the DPPH solution only prepared by methanol as solvent and A_{sample} = Absorbance of the plant extract (test sample) and ascorbic acid (standard) after reaction with DPPH solution. The measurement was done in triplicate for accuracy. The % scavenging of free radical was measured by utilizing equation number 1. A graph of inhibition percentages (I%) against sample concentrations was plotted. From the graph, 50% inhibitory concentration (IC_{50} value) was estimated for each sample.

2.6. Molecular docking study

The widely used software packages (PyRx, PyMoL 2.3, Discovery Studio 4.5, and Swiss PDB viewer) were utilized for computational docking of the six isolated compounds.

2.6.1. Target protein selection

Docking analysis of the isolated compounds were accomplished to understand the antibacterial, anticancer and antioxidant activities. For studies of antibacterial and anticancer activities, dihydrofolate reductase (DHFR) has been chosen as the target protein based on the biochemical pathways and previous evidence [24, 25]. The three-dimensional (3D) structure of the dihydrofolate reductase (DHFR) enzyme was retrieved from the protein data bank (https://www.rcsb.org) with PDB ID 4M6J. Besides, two other proteins with PDB ID 3GRS (Glutathione reductase) and PDB ID 1R4U (Urase oxidase) were downloaded from the same database (https://www.rcsb.org) for computational docking based on previous evidence to evaluate the potential antioxidant property [26]. All the proteins/receptors were saved in PDB format. The collected proteins were made water and any unwanted ligand/residue free by running in PyMoL 2.3. Then all the biomolecules were arranged by adding non-polar hydrogen atoms and kept to the lowest energy state by applying for an energy minimization program by Swiss PDB viewer for further analysis.

2.6.2. Ligand preparation

All the stated compounds (**1** to **6**) in Figure 1, namely, 5-*O*-caffeoyl quinic acid, syringin, luteolin, apigenin, jhanol, and jhanidiol, respectively, were searched and found in the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). Then the ligands with their corresponding standard drugs, including ciprofloxacin, methotrexate, and ascorbic acid, for studying the comparative binding affinity to the target macromolecules, were downloaded and saved in 3D SDF format. These ligands were loaded in the discovery studio 4.5 serially, including their PubChem CID. It is noted that all the phytochemicals were optimized via Pm6 semi-empirical method for enhancing the docking accuracy [27, 28].

2.6.3. Ligand-protein interaction

The current computer-aided ligand-protein interaction has been sketched for predicting the possible binding profiling of the isolated compounds with their binding affinities to the target molecules [28]. A highly advanced PyRxAutodock Vina was utilized for this molecular drug-protein linking process, where semiflexible modeling was applied for the molecular docking. Firstly, the protein has been loaded and formatted to target macromolecule, and the literature-based amino acids with their ID were selected for ascertaining target-specific binding of the ligands. For 4M6J target, Ala9, Ile16, Leu93, Ser92, Arg91, Arg77, Glu78, Ser76, Leu75, Lys54, Val120, Ser119, Lys55, Thr56, Ser 118, Gly117, and for 1R4U, Arg 176, Val 227, Gln 228, Asn254, His 256 amino acid were picked for site targeted docking [26, 29]. Besides, Val 102, Lys 127, Asn 129, Lys 143, Gly 148, Ser 145, Ser 147, Asp 183, Val 130, Gln 131, and Thr 185 were selected for site-specific docking of these isolated natural compounds with 3GRS protein for predicting antioxidant effects [26]. Secondly, all the PDB files of the ligands were imported and subsequently minimized into pdbqt format with Open Bable tool in the PyRxAutoDock Vina software to fit the best optimal hit during the docking against these chosen macromolecules. Thirdly, the grid box was generated by keeping the active binding sites of the protein within the center of the box where the grid mapping was set as center: X = 7.4850, Y = 7.3500, Z = −18.6398, and dimension: X = 43.8582, Y = 51.2793, Z = 48.8257 during docking with DHFR protein. Besides, the grid mapping was fixed center: X = 27.4790, Y = 50.2748, Z = 40.1613, and dimension: X = 31.7158, Y = 37.2601, and Z = 42.9295 for 1R4U protein while the grid map was kept for 3GRS center: X = 74.0693, Y = 51.4437, Z = 25.0217, and dimension: X = 24.7046, Y = 25.0, Z = 29.9240. The rest of the parameters were set to be default settings during the docking process. Then, the computer-aided molecular docking of the ligands was performed under maintaining all necessary conditions by utilizing AutoDock Vina (version 1.1.2) [29]. Finally, all the docking analysis for extrapolating the best-fitted figures with 2D and 3D configurations was conceived by using BIOVIA Discovery Studio version 4.5.

2.7. Statistical analysis

The *in-silico* experiments were done triplicate, and the obtained results in the study were presented and explained as mean ± standard error of the mean. The standard errors for all the analyses were below 1%. Besides, the data found from the *in vitro* antibacterial test were represented as mean ± standard deviation (SD). All the statistical analyses were performed in Microsoft Excel 2019 version.

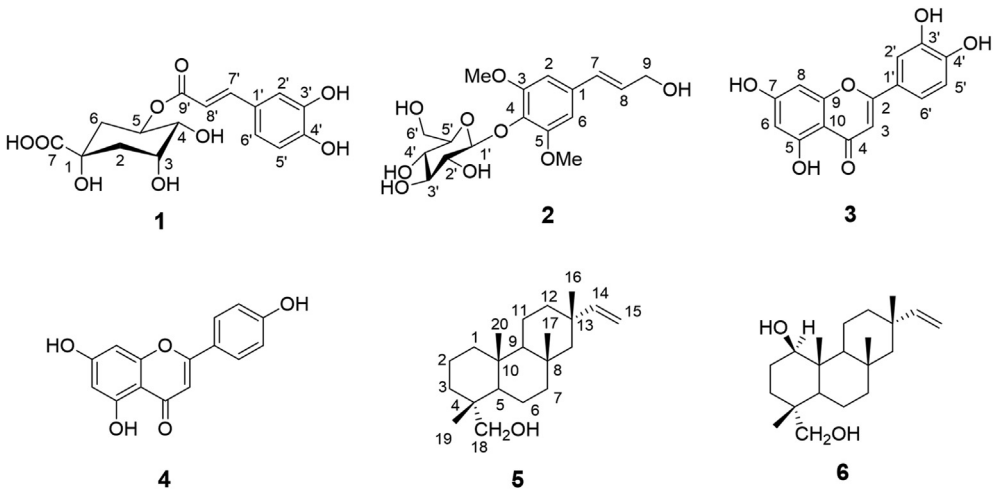

Figure 1. Isolated constituents from *Stevia rebaudiana* (**1–6**).

3. Results

3.1. Isolation and characterization of compounds

Dried powder leaves of *Stevia rebaudiana* was extracted with 70% methanol to isolate secondary metabolites having potential antimicrobial property. Significant antimicrobial activity was observed in the crude extract which led to the further purification through partitioning into different solvents (hexane, dichloromethane and ethylacetate) according to their polarities. Among the fractions, EtASR fraction showed prominent antibacterial activity in preliminary investigation which was subsequently purified. Repeated chromatographic techniques yielded total six compounds (Figure 1). The 1D and 2D spectral data of all the isolates were compared with established values and were identified as 5-*O*-caffeoyl quinic acid (**1**), syringin (**2**), luteolin (**3**), apigenin (**4**), jhanol (**5**) and jhanidiol (**6**) [30, 31, 32, 33]. The spectra obtained from NMR analysis of the compounds and the single spot purity check-in TLC plate with retardation factor (R_f) values were added in the supplementary file (Figure S1 and Table S1, respectively).

3.2. Spectral data

5-*O*-Caffeoyl quinic acid (**1**): White amorphous powder, ^{1}H NMR (400 MHz, CD_3OD): δ 7.58 (1H, d, J = 15.9 Hz, H-7′), 7.04 (1H, d, J = 2.0 Hz, H-2′), 6.92 (1H, dd, J = 8.0, 2.0 Hz, H-6′), 6.77 (1H, d, J = 8.0 Hz, H-5′), 6.24 (1H, d, J = 15.9 Hz, H-8′), 5.34 (1H, brs, OH), 3.74 (1H, ddd, J = 7.7, 2.9, 2.9 Hz, H-3), 3.66 (1H, dd, J = 6.9, 2.9 Hz, H-4), 2.05 (1H, m, H-2$_{ax}$), 2.01 (1H, m, H-6$_{ax}$), 1.96 (1H, m, H-6$_{eq}$), 1.90 (1H, m, H-2*eq*). ^{13}C NMR (100 MHz, CD_3OD): δ 173.8 (C-7), 167.0 (C-9′), 148.2 (C-4′), 145.7 (C-3′), 145.4 (C-7′), 126.2 (C-1′), 121.6 (C- 6′), 115.1 (C-5′), 114.3 (C-2′), 113.8 (C-8′), 71.2 (C-1), 70.7 (C-5), 69.3 (C-4), 67.7 (C-3), 36.4 (C-2), 34.7 (C-6).

Syringin (2): White crystalline solid, ^{1}H NMR (400 MHz, CD_3OD): δ 6.65 (2H, s, H-2 & 6), 6.42 (1H, d, J = 16.0 Hz, H-7), 6.23 (1H, dt, J = 16 and 6.0 Hz, H-8), 4.78 (1H, d, J = 8.0 Hz, H-1′), 4.11 (1 H, dd, J = 6.0 and 2.0 Hz, H-9), 3.75 (6H, s, OCH_3-3 &-5), 3.65 (1H, dd, J = 12 and 6.0 Hz, H-6′), 3.5 (1H, dd, J = 12 and 2.0 Hz, H-6′), 3.48 (1H, m, H-4′), 3.41 (1H, m, H-3′), 3.32 (1H, m, H-5′) and 3.1 (1H, m, H-2′).

Luteolin (**3**): Pale yellow needle like crystal, ^{1}H NMR (400 MHz, CD_3OD): δ 7.30 (1H, m, overlapped, H-6′), 7.30 (1H, m, overlapped, H-2′), 6.94 (1H, d, J = 7.5 Hz, H-5′), 6.68 (1H, s, H-3), 6.44 (1H, s, H-8), 6.11 (1H, s, H-6).

Apigenin (**4**): Pale yellow needle like crystal, ^{1}H NMR (400 MHz, CD_3OD): δ 7.30 (1H, m, overlapped, H-6′), 7.30 (1H, m, overlapped, H-2′), 6.94 (1H, d, J = 7.5 Hz, H-5′), 6.68 (1H, s, H-3), 6.44 (1H, s, H-8), 6.11 (1H, s, H-6).

Jhanol (**5**): Needle like solid, ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.91 (1H, dd, J = 17 and 12 Hz, H-14), 5.17 (1H, dd, J = 18 and 2 Hz, H-15), 4.93 (1H, q, J = 12 and 2 Hz, H-15), 3.43 (1H, d, J = 11, H-19), 3.13 (1H, d, J = 11, H-19), 2.34 (2H, m, H-12), 0.75 (3H, s, H-20), 0.85 (3H, s, H-18), 1.31 (3H, s, H-16), 1.35 (3H, s, H-17). ^{13}C NMR (400 MHz, $CDCl_3$): δ 37.6(C-1), 17.2(C-2), 36.9(C-3), 35.8(C-4), 49.9(C-5), 19.7(C-6), 42.4(C-7), 73.3(C-8), 55.6(C-9), 35.2(C-10), 17.8(C-11), 35.9(C-12), 72.0(C-13), 147.5(C-14), 110.4(C-15), 27.5(C-16), 25.5(C-17), 74.9(C-18), 15.8(C-19), 15.2(C-20).

Jhanidiol (**6**): Needle like solid, ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.95 (1H, dd, J = 17 and 12 Hz, H-14), 5.15 (1H, dd, J = 17 and 2 Hz, H-15), 4.95 (1H, q, J = 12 and 2 Hz, H-15), 4.2 (1H, t, J = 4.8, H-1), 3.45 (1H, d, J = 11, H-19), 3.14 (1H, d, J = 11, H-19), 1.35 (3H, s, H-16), 1.31 (3H, s, H-17), 0.88 (3H, s, H-20), 0.80 (3H, s, H-18). ^{13}C NMR (400 MHz, $CDCl_3$): δ 38.5(C-1), 17.2(C-2), 36.1(C-3), 36.8(C-4), 50.2(C-5), 20.03(C-6), 42.66(C-7), 75.8(C-8), 56.1(C-9), 36.9(C-10), 19.1(C-11), 35.9(C-12), 73.3(C-13), 148.7(C-14), 111.3(C-15), 77.8(C-16), 26.1(C-17), 66.9(C-18), 18.2(C-19), 15.7(C-20).

3.3. Evaluation of anticancer property

MTT assay was utilized to assess the anti-proliferative effect of the five compounds on HeLa cells. The serially diluted concentrations of the samples and the corresponding percentage of inhibitions against in HeLa cell line were tabulated in Table 1. As representative examples, the cell mortality at 500 mg/mL of each sample has been shown in Figure 2. The IC_{50} values of the compounds and standard drug (methotrexate) enumerated by using the regression line equation (log of concentration vs. percentage of cell mortality) were also enlisted in Table 1. Among the bioactive samples (**1, 2, 4, 5**, and **6**), compound **1** exhibited the highest *in vitro* cytotoxic effect (IC_{50} = 181.3 μg/mL) against the cervical cancer cell line followed by compound 2 (IC_{50} = 194.4 μg/mL). The IC_{50} values of compounds **4, 5, 6,** and methotrexate were found 252.14, 298.86, 265.07, and 36.50 μg/mL, respectively.

3.4. Evaluation of antimicrobial activity

All the compounds exhibited mild to moderate activity against both gram positive and gram-negative bacteria as compared with standard ciprofloxacin. Among the isolates, compound **1** displayed most promising result with a range of zone of inhibition 12–15 mm. Compound **2** also showed comparable antibacterial activity and other compound also exhibited mild to moderate antibacterial activity against some strains. However, no compound was effective against the fungus strains in comparison with standard fluconazole (18–28 mm) (Table 2).

Table 1. *In-vitro* anti-proliferative effects with IC_{50} values of the isolated compounds (**1, 2, 4, 5**, and **6**) on HeLa cell line.

Compound No.	Concentration (μg/mL)	% of inhibition against in HeLa cell line	IC_{50} value (μg/mL)
Methotrexate	62.5	30.3	36.5
	125	53.4	
	250	94.8	
1	62.5	21.45	181.3
	125	26.68	
	250	70.14	
	500	72.86	
2	62.5	5.28	194.4
	125	28.53	
	250	67.84	
	500	83.3	
4	62.5	3.38	252.14
	125	4.17	
	250	63.63	
	500	73.47	
5	62.5	1.88	298.86
	125	4.87	
	250	34.55	
	500	74.49	
6	62.5	1.58	265.07
	125	9.15	
	250	60.54	
	500	68.75	
Control (DMSO)	2.5	–	–

Note: DMSO = Dimethyl sulfoxide.

3.5. Evaluation of antioxidant property

All the phytochemicals except compound **2** showed significant antioxidant properties. Compounds **3**, **6,** and **4** exhibited the most promising antioxidant activities against DPPH free radical with IC_{50} values 8.93, 17.63, and 21.54 μg/mL, respectively, compared to the standard drug ascorbic acid with IC_{50} = 6.05 μg/mL (Table 3).

3.6. Molecular docking studies

3.6.1. Dihydrofolate reductase (DHFR) inhibition

For a clear and better understanding of the molecular effects of these isolated natural products on the above-mentioned biological target, the computer-designed molecular modeling study was performed by utilizing suitable tools. The DHFR protein has a significant role in DNA synthesis in the bacterial or human cell development process. To comprehend antibacterial and anticancer potentialities, we have docked these promising drug candidates/ligands with DHFR enzyme and found surprising docking profiles with high binding affinity compared to their respective standard molecules (ciprofloxacin and methotrexate). Apparently, the lower the binding energy, the higher the binding strength of the ligands has been postulated. Besides, the extrapolated interaction profile having a null RMSD (root mean square deviation) value represents the best docking prediction [29].

All the drug candidates displayed higher binding affinity to the DHFR protein with docking score −10.7 to −15.3 kcal/mol in comparison with ciprofloxacin (−8.4 kcal/mol) and methotrexate (−9.0 kcal/mol). Notably, 5-*O*-caffeoyl quinic acid exhibited the highest DHFR enzyme affinity (−15.3 kcal/mol) among all the ligands involved in the study. The order of the binding affinity might be noted here as like 5-*O*-caffeoyl quinic acid > syringin > apigenin > luteolin > jhanidiol > jhanol > methotrexate > ciprofloxacin (Table 4). The active binding sites of compound **1** (5-*O*-caffeoyl quinic acid) were ASP 21, GLY 20, GLY 17, ALA 9, GLU 30, LEU 22, THR 56, and SER 118, where compound **2** (syringin) showed its affinity towards GLU 30, ASN 64, ILE 16, TYR 16, THR 56, GLY 117, VAL 115, PHE 34 in chain A. Similarly, all the binding sites of the DHFR protein where these ligands interacted were shown in Figure 3 (a-l).

3.6.2. Glutathione reductase and urase oxidase inhibition

All the compounds exhibited much better binding affinity (−6.4 to −7.8 kcal/mol towards glutathione reductase and −7.3 to −8.3 kcal/mol towards urase oxidase enzyme) than the standard drug ascorbic acid (−5.7 and −5.3 kcal/mol towards glutathione reductase and urase oxidase enzyme, respectively). Compounds **1** (−7.8 kcal/mol) and **3** (−7.8 kcal/mol) showed the most binding affinity towards the glutathione reductase enzyme, while compounds **3** (−8.3 kcal/mol) and **4** (−8.1 kcal/mol) exhibited the highest docking score during interacting with urase oxidase enzyme. The active binding sites of the glutathione

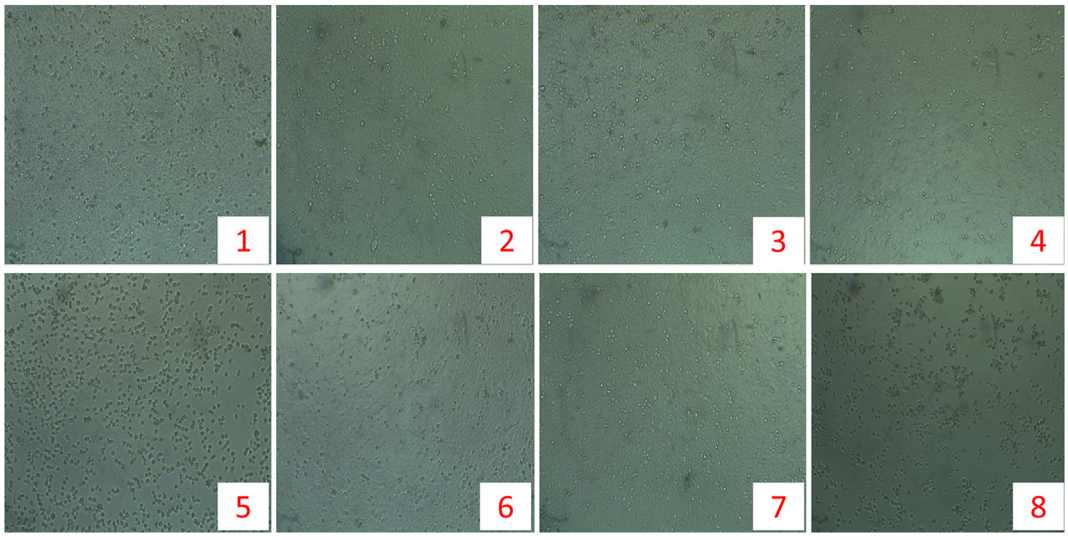

Figure 2. Anticancer potentialities of the isolated compounds (**1 to 6**) and the standard drug methotrexate under inverted light microscope at the dose of 500 (μg/ml).

Table 2. Antimicrobial activity of the compounds (**1–6**) against gram positive and gram negative bacteria.

Pathogens	Zone of Inhibition (mean ± SD; mm)						
	1	2	3	4	5	6	Ciprofloxacin
Gram positive							
Bacillus cereus	14.7 ± 0.11	12.7 ± 0.3	–	10.2 ± 0.11	–	10.3 ± 0.18	17.2 ± 0.23
Bacillus megaterium	15.3 ± 0.48	12.4 ± 0.23	10.0 ± 0.66	11.1 ± 0.32	–	10.8 ± 0.21	16.8 ± 0.14
Bacillus subtilis	12.7 ± 0.52	–	–	–	–	–	15.8 ± 0.44
Bacillus aureus	14.1 ± 0.16	13.5 ± 0.19	–	–	–	–	18.1 ± 0.14
Sarcina lutea	13.2 ± 0.12	12.5 ± 0.29	11.5 ± 0.44	–	–	–	20.0 ± 0.18
Gram negative							
Escherichia coli	15.8 ± 0.33	12.6 ± 0.65	–	12.0 ± 0.52	–	–	21.2 ± 0.23
Pseudomonas aeruginosa	–	–	10.7 ± 0.32	–	10.5 ± 0.26	–	14.4 ± 0.14
Salmonella paratyphi	14.2 ± 0.22	11.8 ± 0.55	8.2 ± 0.23	10.2 ± 0.44	10.8 ± 0.19	10.8 ± 0.52	18.5 ± 0.44
Salmonella typhi	12.1 ± 0.33	12.7 ± 0.66	9.3 ± 0.32	10.8 ± 0.44	–	–	17.7 ± 0.14
Shigella boydii	15.3 ± 0.11	–	–	–	–	11.2 ± 0.67	18 ± 0.18
Shigella dysenteriae	14.4 ± 0.26	11.5 ± 0.45	–	–	–	–	19.5 ± 0.33
Vibrio mimicus	13.3 ± 0.77	–	10 ± 0.74	11.5 ± 0.53	–	–	18.3 ± 0.42
Vibrio parahemolyticus	–	10.1 ± 0.45	–	12.1 ± 0.23	–	–	20.0 ± 0.41

Table 3. Antioxidant properties of the isolated compounds (**1** to **6**) and the standard drug ascorbic acid with their IC_{50} (μg/mL) values.

Compound Code	R^2	IC_{50} (μg/mL)
1	0.9905	23.33
2	0.9859	174.16
3	0.9171	8.93
4	0.9258	21.54
5	0.9905	23.33
6	0.9791	17.63
Ascorbic acid	0.9142	6.05

reductase for compound **1** were VAL 370, PRO 340, TYR 197, THR 57, GLY 62, PHE 181, VAL 61, SER 177, ILE 198, ARG 291, ARG 291, ASP 331, LEU 337, CYS 58, THR 339, GLU 201, CYS 63, LEU 338, LYS 66, VAL 371, PHE 372 and for compound **3** were MET 202, GLU 201, LYS 66, ILE 198, CYS 63, PHE 372, VAL 371, THR 369, PRO 340, LEU 338, THR 339, TYR 197, CYS 58, THR 57, ARG 291, VAL 61, PHE 181, SER 177, GLY 62 (Figure 4). Similarly, the active binding sites of the glutathione reductase enzyme for all the compounds were shown in Figure 4(a-l). Moreover, the binding sites of the urase oxidase enzyme for the compound **3** were VAL 73, THR 74, PRO 76, PRO 75, ARG 128, TRP 208, TRP 106, THR 107, ARG 105, HIS 104, CYS 103, MET 32, GLU 31, TYR 30 (Figure 5). Likewise, the active binding sites of the urase oxidase enzyme for all the compounds were shown in Figure 5(a-l).

Table 4. Binding affinity or *in silico* docking scores (kcal/mol) of the isolated compounds (**1** to **6**) from *Stevia rebaudiana* (Bert.) and the corresponding standard drugs during molecular interaction with dihydrofolate reductase (PDB ID 4M6J), glutathione reductase (PDB: 3GRS), and urase oxidase (1R4U) enzymes for determining antibacterial, anticancer, and antioxidant potentialities.

Compound No.	Compound Name	PubChem CID	Binding affinity (kcal/mol)		
			4M6J	3GRS	1R4U
1	5-*O*-caffeoyl quinic acid	5280633	−15.3	−7.8	−7.7
2	Syringin	5316860	−13.6	−6.4	−7.3
3	Luteolin	5280445	−12.6	−7.8	−8.3
4	Apigenin	5280443	−12.7	−7.7	−8.1
5	Jhanol	13996014	−10.7	−7.2	−7.7
6	Jhanidiol	15226315	−11.2	−7.4	−7.3
Standard	Ciprofloxacin	2764	−8.4		
	Methotrexate	126941	−9.0		
	Ascorbic acid	54670067		−5.7	−5.3

4. Discussion

A large number of potential efforts have been conducted to identify the novel scaffolds from the plants that may help alleviate the global threat of infectious disease. Plant contains a variety of secondary metabolites among which phenolic compounds are considered of great interests for their wide applications. Phenolics such as coumaric acid, caffeic acid, ferulic acid, sinapic acid, tannins, flavonoids, glycosides etc. are synthesized from the phenylpropanoid pathway in plants and demonstrated antimicrobial properties [34, 35, 36]. *Stevia rebaudiana* contains a wide variety of diterpene glycosides among which stevioside (5–13%), rebaudioside A (2–4%), and rebaudioside C (1–2%) are the main constituents imparting sweetening properties [37]. Apart from these, minor constituents such as flavonoids, sterebins, chlorogenic acids, triterpenes, volatile oils etc. have also been reported [38]. Present study also added some valuable number of secondary metabolites in those lists. To the best of our knowledge, this is the first time reporting of a phenylpropanoid glycoside syringin (**2**) and jhanidiol (**6**) bearing diterpene skeleton from *Stevia* leaves. In terms of total phenolic constituents, the study finding is similar to a previous study where 80% ethanolic crude extracts of *S. rebaudiana* leaves contains total phenolic concentration with a range of 25–65 mg/g gallic acid equivalent [39].

In the cytotoxicity study (MTT cell viability assay), it has been indicated that the compounds **1, 2, 4, 5**, and **6** depicted growth inhibition against HeLa cells in a dose dependent manner. Among the isolated compounds, the compound **1** exhibited most profound effect on the HeLa cell viability indicating its anticancer activity. Although the effect is much lower compared to the standard drug Methotrexate, structural modifications might lead to development of more potent analogues. In some reported previous studies, aqueous and methanolic extracts of *Stevia rebaudiana* and the diterpene glycosides along with their synthetic congeners and their aglycone part steviol, isosteviol showed prominent antimicrobial and antitubercular properties [40, 41, 42]. In our study, all the isolates (**1**–**6**) displayed a significant zone of inhibition against a variety of pathogens, which postulates the findings observed in the previous studies. The present results further stipulate that the activity observed by the extracts in the previous studies might be due to the presence of the isolated constituents (**1–6**) from *Stevia*. To validate the resulting pharmacological effect and to discern the exact mechanistic pathway, molecular docking was also performed for the isolated

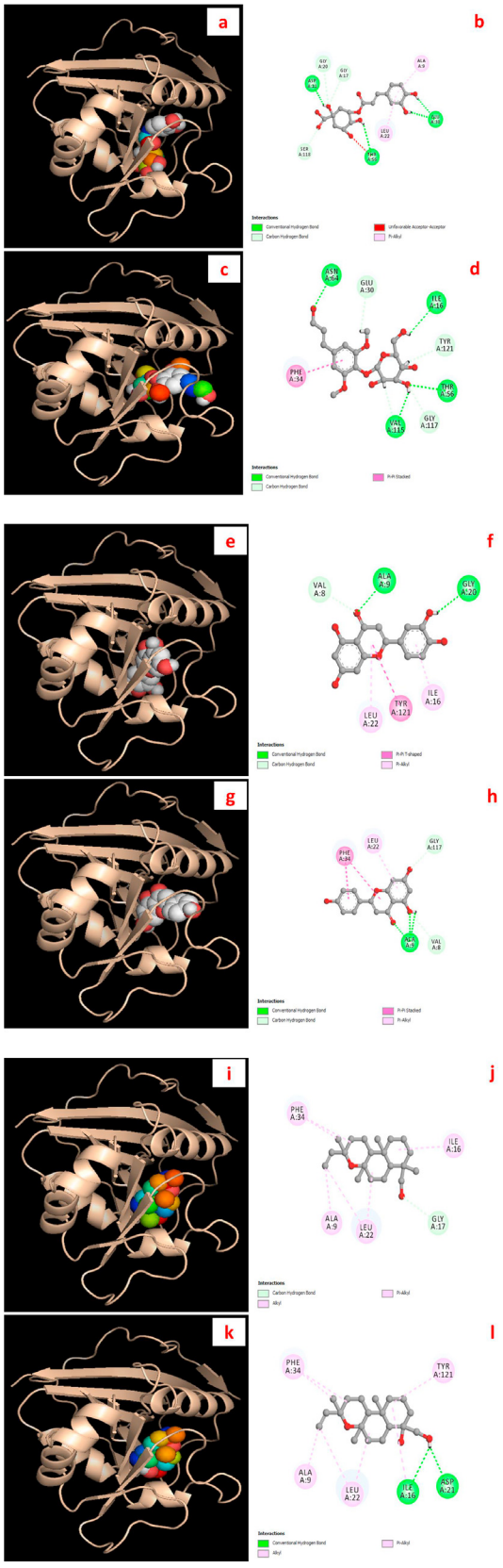

Figure 3. Graphical representation of the molecular interactions of the isolated compounds with dihydrofolate reductase (DHFR; PDB ID: 4M6J) enzyme with 3D and 2D visualization (compound **1** = a and b, compound **2** = c and d, compound **3** = e and f, compound **4** = g and h, compound **5** = i and j, and compound **6** = k and l). It is noted here that the docking visualization for the standard drugs were not displayed in the figure.

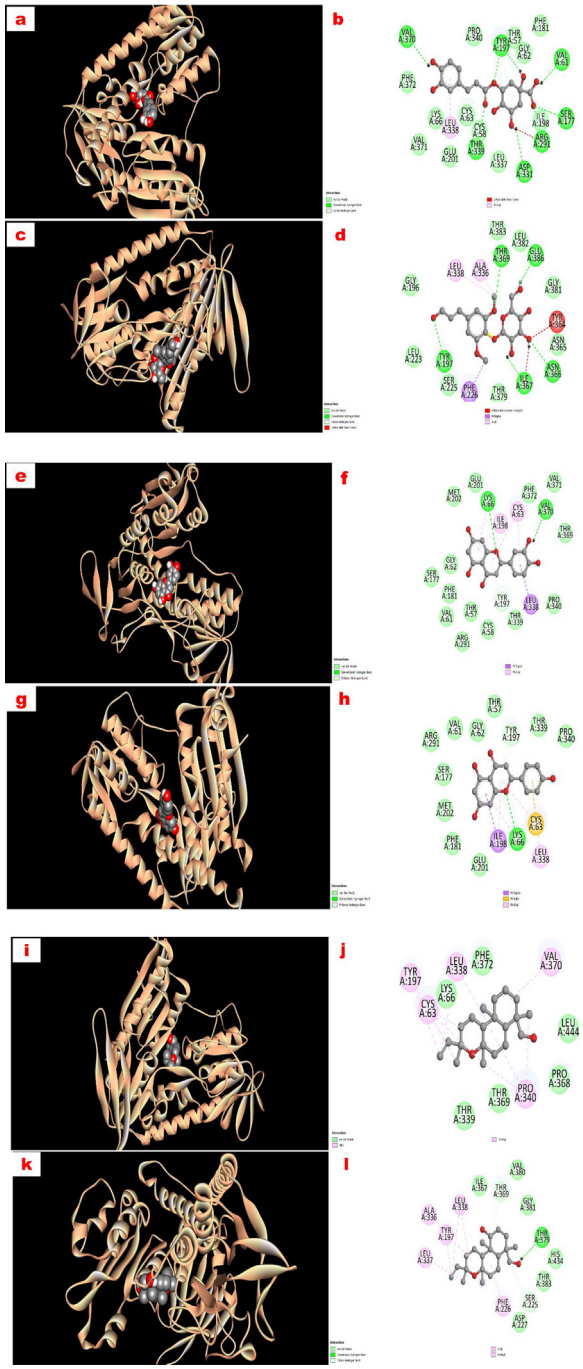

Figure 4. Graphical representation of the molecular interactions of the isolated compounds with glutathione reductase (PDB ID: 3GRS) enzyme with 3D and 2D visualization (compound **1** = a and b, compound **2** = c and d, compound **3** = e and f, compound **4** = g and h, compound **5** = i and j, and compound **6** = k and l). It is noted here that the docking visualization for the standard ascorbic acid was not displayed in the figure.

compounds. Interestingly, all the isolates showed significant binding affinity towards the folate synthesizing enzyme DHFR.

More than 24 different chlorogenic acid derivatives have been isolated from this plant [43]; among them 5-*O*-caffeoylquinic acid displayed significant anti-proliferative and antimicrobial activities. The *in vitro* study revealed that compound **1** showed the highest growth inhibition against the HeLa cell line (IC_{50} value = 181.3 μg/mL). Besides, compound **1** also exhibited maximum binding affinity in the molecular modeling study (binding energy = −15.3 kcal/mol) against DHFR, indicating potential anti-proliferative and antibacterial activity.

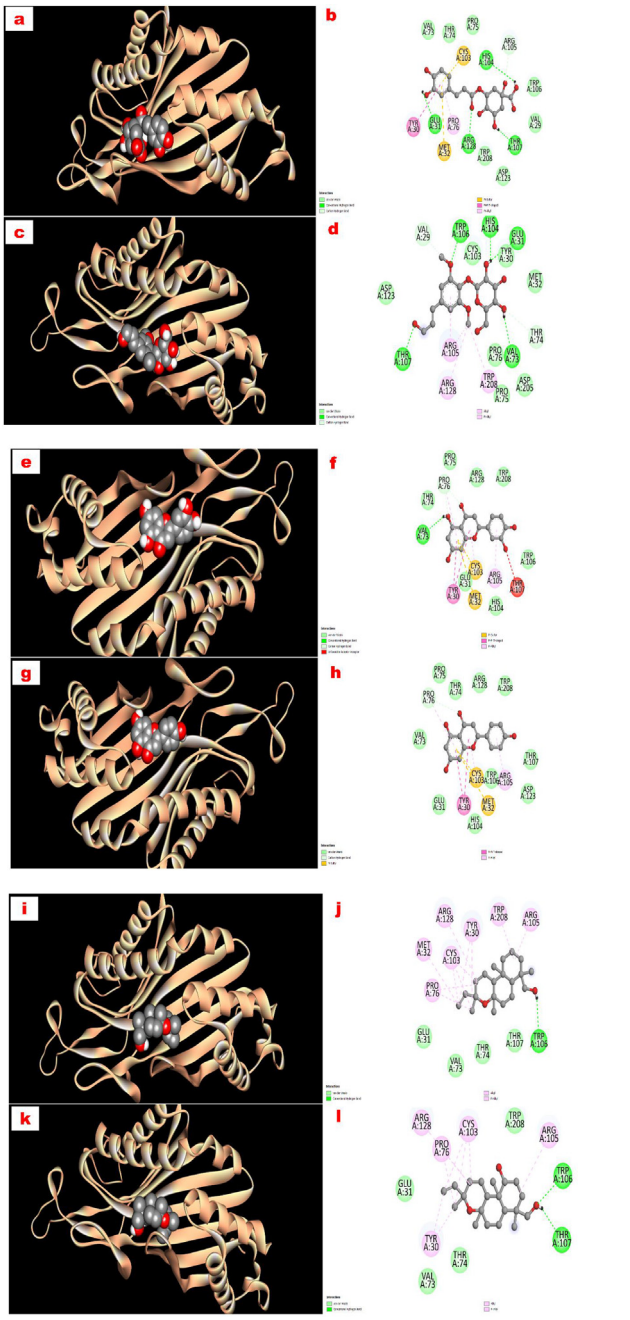

Figure 5. Graphical representation of the molecular interactions of the isolated compounds with urase oxidase (PDB ID: 1R4U) enzyme with 3D and 2D visualization (compound **1** = a and b, compound **2** = c and d, compound **3** = e and f, compound **4** = g and h, compound **5** = i and j, and compound **6** = k and l). It is noted here that the docking visualization for the standard ascorbic acid was not displayed in the figure.

Likewise, Sultana et al. [20] found promising antibacterial and antioxidant activities of this similar plant leaf's *n*-hexane and ethyl acetate extracts. The effects of the isolated compounds against bacteria, cancer cell lines and free radicals were consistent to the effects of the leaf extracts. This might be assumed and predicted by due to dipole-dipole and H-bond interaction with amino acids of targeted protein DHFR. It has been further confirmed by the ribbon model of protein where compound **1** bound deeply inside the pocket of DHFR, which depicted its anticancer and antimicrobial activities through the anti-metabolite pathway. A large number of non-covalent H-bond and Pi-sigma interactions (Pi-alkyl) which largely involves charge transfer help in intercalating the drug in the binding site. These findings suggest high structural stability and may lead to high inhibitory activity of compound **1** [44].

Syringin (**2**), a common phenylpropanoid glycoside was isolated earlier from different plants [45, 46] has been isolated from this plant for the first time. This compound exhibited remarkable *in vitro* anti-cancer effect (IC_{50} = 194.40 μg/mL, Table 1) and antimicrobial activity against a variety of pathogens which has been further evinced by *in silico* study (binding affinity −13.6 kcal/mol). In a previous report, it was found that syringin isolated from *Foeniculum vulgare* showed significant *in vitro* anti-proliferative effect against HeLa cell line [47]. As depicted in Figure 2, compound **2** interacted strongly with its binding sites through three conventional H-bonds and two pi-alkyl interactions.

Luteolin (**3**) and apigenin (**4**), have already exhibited antibacterial potentiality [48]. The luteolin (**3**) involved two non-covalent H bonds and two Pi bonds, while apigenin (**4**) has three H-bonds, two Pi-alkyl bonds and one T shaped Pi-Pi stacking interaction (Figure 2). This result indicates that apigenin (**4**) has more binding affinity towards DHFR than luteolin (**3**) though the docking score in case of both compounds were almost same (−12.6 kcal/mol and −12.7 kcal/mol for compound **3** and **4**, respectively). The higher inhibitory effect of compound **4** compared to compound **3** might be attributed due to the high binding affinity and deep binding pocket [49].

Jhanol (**5**), a labdane-type hydroxyditerpene was reported earlier from the flower part of *Stevia* plants [41] possessed mild cytotoxicity and antibacterial activity in *in vitro* studies. The binding energy towards DHFR was −10.7 kal/mol which could be due to non-covalent interaction like Pi-Pi interaction. Meanwhile, the binding affinity of compound **6** was satisfactory with an estimated free binding energy of −11.2 kal/mol (Figure 3) which might be responsible for its anticancer and antibacterial activity. Interestingly, labdane type diterpenes **5** and **6** bound to the same pocket of DHFR (Figure 3) and showed similar binding energy because of their structural similarity.

It has been shown that apigenin (**3**) gave most promising antioxidant effect (IC_{50} = 8.93) than other compounds. This might be due to the presence of multiple hydroxyl group as reported earlier in a previous study [50]. Some sort of antioxidant activity was reported by 5-*O*-caffeol quinic acid, luteolin and syringin (compound **1**, **2**, and **4**) in some previous studies [51, 52, 53]. Our results also re-establish the previous results; however, we found less potent antioxidant action of syringin by DPPH scavenging assay method compared to the reported data by Shen and co-authors [52]. Likewise, we found potent antioxidant effect against DPPH free radical in case of 5-*O*-caffeol-quinic acid compared to the previous result reported by Kim et al. (IC_{50} = 23.33 μg/mL vs. 37 μM) [53]. Jhanol and jhanidiol also showed potent antioxidant activity. This is the first DPPH scavenging report of these compounds. The capability to scavenge DPPH could be accredited to the content of polyphenols, flavonoids as well as alkaloids as accentuated in several reports [54, 55]. Redox properties are mainly responsible for this activity which could be due to adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [56]. The *in vitro* antioxidant activity was further supported by the docking study towards glutathione reductase and urase oxidase enzyme as stated in Table 4.

5. Conclusion

Among the isolated molecules, compound **1** showed significant anti-proliferative activity against HeLa cell and antibacterial activities against various gram positive and gram-negative microorganisms. Other isolated compounds also showed moderate to mild cytotoxicity and antibacterial effects. On the other hand, compound **4** (apigenin) exerted strong antioxidant effect compared to ascorbic acid. Compound **1** (5-*O*- caffeol quinic acid), 3 (luteolin) and 6 (janidiol) showed moderate free radical scavenging activity. This research work also demonstrated that the secondary metabolites isolated from this plant have different degrees of affinity towards binding sites of target protein, DHFR, glutathione reductase and urase oxidase responsible for anticancer and antimicrobial

actions and antioxidant effects, respectively. The results of *in-vitro* cytotoxicity and antibacterial activity were clearly justified by computational studies predicting plausible binding modes of the six compounds in the active site of DHFR. Therefore, this study has established the plant species as the potential source of anticancer, antibacterial and free radical scavenging agents for the first time. Based on this study, we may assume that the isolated compounds might be the lead compounds for development of chemotherapy and antibacterial therapeutic agents though the exact mechanism is not underlying yet and, the research should be carried for improvement of semi-synthetic derivatives or better drugs to combat cancer as well as various microbial infection.

Declarations

Author contribution statement

Most. Chand Sultana Khatun: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Md. Abdul Muhit: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Md. Jamal Hossain: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Muhammad Abdullah Al-Mansur: Performed the experiments; Contributed reagents, materials, analysis tools or data.

S. M. Abdur Rahman: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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Chapter -1

Introduction

Chapter -2

Plant Profile

Chapter -3

Methods and Materials

Chapter -4

Results

Chapter -5

Discussion

Chapter -6

Conclusion

References

Publications

Annexure-I	**Phytochemical and biological investigation of *Stevia rebaudiana* (Bert.) leaves grown in Bangladesh.**
Annexure-II	**Isolation of phytochemical constituents from Stevia rebaudiana (bert.) and evaluation of their anticancer, antimicrobial and antioxidant properties via *in-vitro* and *in silico* approaches.**