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Isolation, Investigation and Antimicrobial activity of a dihydrochalcone from the leaves of the *Catharanthus roseus* L.

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Abstract

Dihyrochalcone is subclass of Flavonoids which are polyphenolic compounds that are widely found in the plant kingdom as secondary metabolites, they possess a variety of biological activities. The purpose of this study was to isolate and identify the Flavonoids from the leaves of *Catharanthus roseus* and testing it for anti-microbial activity. The plant was collected from Omdurman area, the powder was prepared from leaves and some chromatographic methods were applied to isolate compound I. The structures of this compound was elucidated based on spectroscopic methods as: IR,UV,MS and ^{1}HNMR spectroscopy, and also the microbial activity of Compound 1 gave activity against all organisms that were tested. The results of this study have provided scientific validity for use of this plant in traditional medicine to treat infectious diseases.

Keywords: *Catharanthus roseus,* dihydrochalcone, Flavonoids, anti-inflammatory, antimicrobial.

1. Introduction:

Dihydrochalcones are subgroups of Flavonoids have a basic C_6-C_3-C_6 skeleton chemical structure [1]. They have medicinal and pharmacological properties, including antithrombotic [2], antioxidants, antibacterial [3] neuroprotective, antidiabetics, antitumor, lipo-metabolism regulating, anti-inflammatory [4], antiviral [5], immunomodulatory [6] and estrogenic [7]. In folk medicine it is used for treatment of malaria,

yellow fever, dengue fever, hypertension, breast cancer, liver cirrhosis, gastroenteric, diabetes and inflammatory disorders [8,9]. *Catharanthus roseus* L. belongs to the family Apocynaceae and commonly known as Madagascar periwinkle. The synonyms of the plant name include *Vinca rosea, Ammocallisrosea, Lochnera rosea, Cape Periwinkle, Rose Periwinkle, Rosy Periwinkle and* Old maid [10]. The plant is found to be a species of *Catharanthus* in native to the Indian Ocean Island of Madagascar and in many tropical and subtropical regions worldwide [11]. It is an evergreen sub shrub or herbaceous plant growing up to 1 m tall. The leaves are oval to oblong, 2.5-9 cm long and 1-3.5 cm broad, glossy green, hairless, with a pale midrib and a short petiole 1-1.8 cm long, they are arranged in opposite pairs. The flowers are white to dark pink with a darker red center, with a basal tube 2.5-3 cm long and a corolla 2-5 cm diameter with five petal-like lobes. The fruit is a pair of follicles 2-4 cm long and 3 mm broad [12, 13].

The *Catharanthus roseus* (*Vinca rosea*) is an important medicinal plant. Alkaloids that are isolated from *C. roseus* are found to be hypertensive, sedative and possess tranquilizing and anti-cancerous properties. Traditionally, the plant used for relieving muscle pain, depression of the central nervous system and wasps stings. It is used in cases of nosebleed, bleeding gums, mouth ulcers and sore throats. It has also been used internally for the treatment of hypertension, cystitis, gastritis, enteritis, diarrhoea and the raised blood sugar Levels [14]. Its application ranges widely from the prevention of cancer, cancer treatment, and anti-diabetic, stomachic [15]. Also, it was used in treatment of Hodgkin's disease and childhood leukemia [16]. The plant has been used for the treatment of fever, malaria, throat infections, chest complaints, regulation of menstrual cycles and as aeuphoriant [17]. Asthma and menstrual problem [12]. This plant possesses known antimicrobial, antioxidant, anticancer activities [18]. The plant is considered due to its wide range of pharmacological activity like anti-inflammatory, antimalarial, antimitotic, antihypertensive, antifertility, anti-hypercholesterolemic, anti-mutagenic, antidiuretic, antifungal, antispasmodic, antiviral, cardio tonic, CNS depressant, antitumor, cytotoxic, anti-spermatogenic [13].

This study aimed to isolate and structure elucidate of the new compound from the leaves of *Catharanthus roseus* and testing it for anti-microbial activity.

2.Materials and Methods:

2.1. Plant Material:

The leaves of *Catharanthus roseus* were collected in November 2015 from Khartoum State - Sudan. The plant was kindly authenticated by Department of Botany, University of Khartoum. The dried leaves were ground into powder by the electric

grinder and the powder was stored in an airtight bottle, until extract preparations and analyses.

2.2. Instruments:

The UV spectra were recorded on a Shimadzu 1601 Spectrophotometer and UV lamp was used for the localization of fluorescent spots on TLC. The IR spectrum was recorded as KBr disks, using Shimadzu IR-8400 Spectrophotometer. All the IR spectra were obtained in the range 4000–500 cm−1 with a spectral resolution of 4 cm^{-1}. Nuclear Magnetic Resonance spectra were run on a JEOL DELTA ESP-400MHZ HNMR Spectrophotometer. Mass spectra were measured on a Varian G-C450-MS-240 Spectrometer.

2.3. Phytochemical Screening:

Phytochemical screening of *Catharanthus roseus* leaves extract to detect the presence of secondary metabolites such as flavonoids, steroids, alkaloids, tannins, anthraquinones, saponins and glycosides was carried out according to standard procedures as reported by Sofowora [19].

2.4. Extraction and Isolation of Dihydrochalcone from *Catharanthus roseus*:

Powdered shade-dried leaves (1 Kg) were exhaustively percolated with 80% methanol (5L) at ambient temperature for 72hr. The solvent was evaporated under reduced pressure leaving a 50 g residue. The residue was suspended in 300 ml of water and successively extracted with petroleum ether, n-hexane, ethyl acetate and n-butanol. Removal of the solvent under reduced pressure gave crude products which were manipulated further by chromatographic techniques.

The n-butanol fraction (9.0 g) was subjected to column chromatography using a Merck silica gel with particle size 60 μm (70-230 mesh) as a stationary phase, Successive elution with methanol: chloroform in increasing order of polarity gave four fractions (100ml each). Fraction (3) showed two major spots on TLC. It was further subjected to flash column chromatography using methanol: chloroform (2:3) to afford compound I. For more purification compound I was chromatographed on a Sephadex LH-20 column (60 x1.5 cm) using methanol as an eluent. Compound I was isolated in chromatographically pure form as a yellow powder.

2.5. Antimicrobial Assay:

The isolated compound of n- butanol fractions from *Catharanthus roseus*, were screened for their antimicrobial activity against six standard human pathogens (*Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Aspergillus Niger* and *candida albicans).*

2.5.1. Preparation of Bacterial Suspensions:

One ml aliquots of 24 hours broth culture of the test organism were aseptically distributed onto agar slopes and incubated at 37ºC for 24 hours. The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce a suspension containing about 10^8-10^4 colony

forming units per ml. the average number of viable organisms per ml of saline suspension was determined by using the surface counting technique. Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volumes (0-.02ml) of the appropriate dilutions were transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drop to dry and then incubated at 37ºC for 24 hours [20].

2.5.2. Preparation of Fungal Suspensions:

Fungal cultures were maintained on saturated dextrose agar and incubated at 28ºC for four days. The fungal growth was harvested and washed with sterile normal saline, and the suspension was stored in the refrigerator until used.

2.5.3. Testing for Antibacterial Activity:

The cup-plate agar diffusion method was adopted with some minor modification, to assess the antibacterial activity. (20ml) Aliquots of the inoculated nutrient agar were distributed into sterile Petri dishes; the agar was left to settle in each of these plates which were divided into two halves. Two cups in each half (10 mm in diameter) were cut using sterile cork borer (No. 4). Each of the halves was designed for one of the test solutions. Separate Petri dishes were designed for standard antimicrobial chemotherapeutic (gentamycin and Clotrimazole). The agar discs were removed and cups were filled with 0.1ml sample of each extract and pure compound using adjustable volume microliter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37ºC for 24 hours. The above procedure was repeated for different concentration of the test solutions and standard antibacterial chemotherapeutics. After incubation the diameters of the resulting growth inhibition zones were measured in triplicates and averaged [20]. The antimicrobial sensitivity of Compound I was tested using a sample concentration of 100µg per disc. Standard disc of Gentamycin (40, 20, 10 mg per disc).

2.5.4. Testing for Antifungal Activity:

The above-mentioned method was adopted for antifungal activity, but instead of nutrient agar saturated dextrose agar was used. Samples were used here by the same concentration used above except the standard disc of Clotrimazole (concentration 30, 15, 7.5 µg per disc).

3. Results and Discussion:

3.1 Preliminary Phytochemical Screening:

The Phytochemical screening of bioactive compounds from *Catharanthus roseus* leaves extract were explained the presence of flavonoids, steroids, alkaloids, tannins, anthraquinones, saponins but glycosides were not detected and the results are depicted in the table (1). The bioactive compounds provided the medicinal and pharmaceutical properties of the plant, Flavonoids

are anticancer, antiallergic, antioxidant, antiviral, and anti-inflammatory effects [21]. Tannins are compounds with antioxidant, antiseptic, anticancer, antidiarrheal, and heavy metals precipitation activities [22]. Alkaloids are applicable as painkillers, and to treat certain cancer [21]. Anthraquinones are widely used in medicine such as laxative, cathartic, anti-inflammatory, anticancer, and vasorelaxant actions [23]. Saponins have health benefits on blood cholesterol level, cancer, and bone and immune system trigger [21]. Biochemically, glycosides are natural cardioactive metabolites used in the treatment of congestive heart failure, and cardiac arrhythmia. Steroids are important for reducing cholesterol levels, regulating immune responses, and immune enhancement [24]. These benefits make it a very important medicinal plant.

Table 1: Phytochemical screening of *Catharanthus roseus* leaf extract

Compounds	*Catharanthus roseus* result
Flavonoids	+ve
Tannins	+ve
Saponins	+ve
Alkaloids	+ve
Steroids	+ve
Triterpenes	+ve
Glycoside	- ve
Anthraquinones	+ve

1.2. Identification of Compound I:

Compound I was isolated as a yellow powder from *Catharanthus roseus* leaves by silica gel TLC using BAW (4:1:4) as solvent. The IR spectrum (Fig.1) of compound I showed ν(KBr), 622,649.887(C-H , Ar.) ,1043 (C-O) ,1417,1577 (C=C , Ar) 1688 (C = O) , 2954 cm^{-1} (C- H, aliphatic).

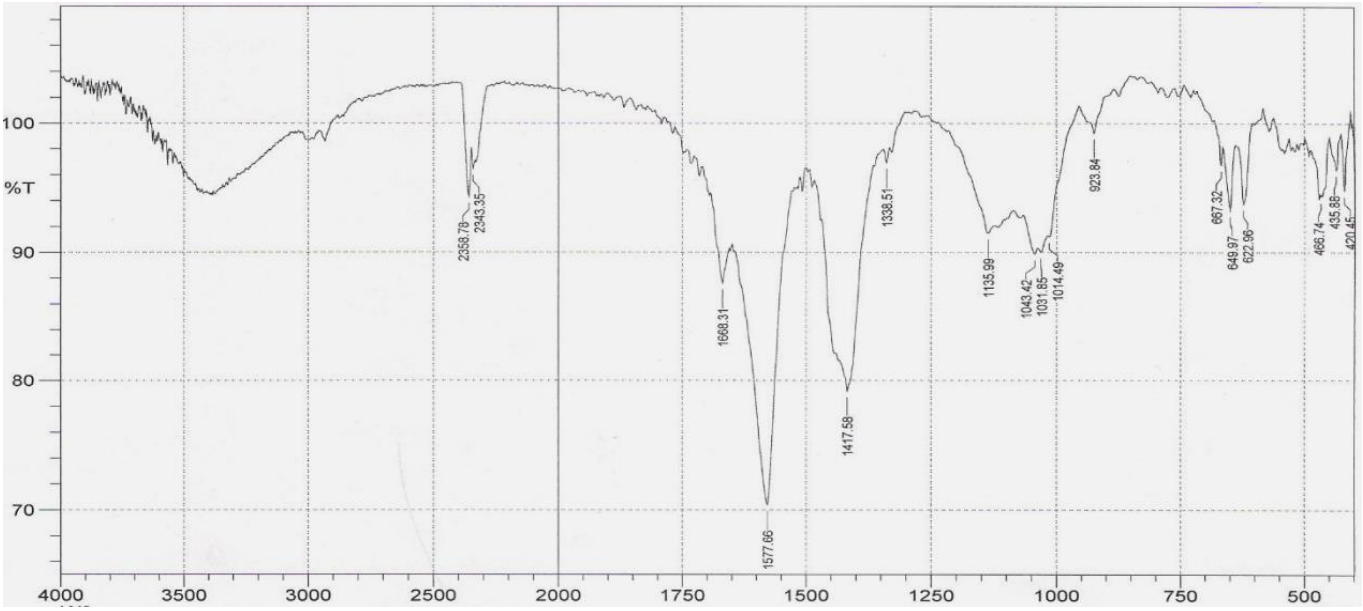


Fig.1: IR spectrum of compound I

The UV spectrum (Fig.2) showed λ_{max} (MeOH) 285nm. Since the spectrum revealed only band II then the isolate is probably a dihydroflavonol, Dihydrochalcone, flavonone and an isoflavone [25].

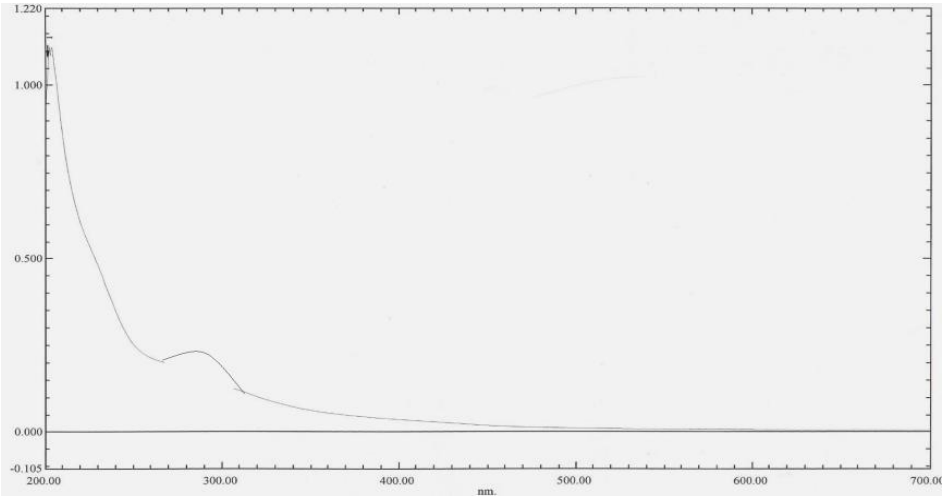
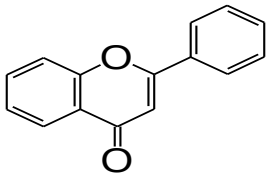
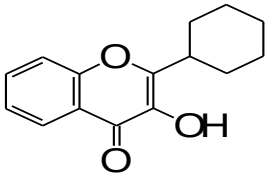


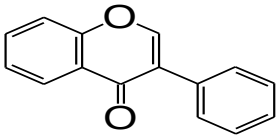
Fig.2: UV spectrum of compound I



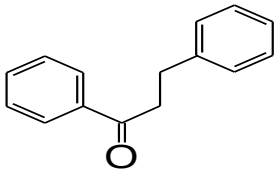
Flavone



Flavonol



Isoflavone



dihydrochalcone

Among these classes only dihydroflavonols are characterized by a 3-OH function which is detected by the UV shift reagent [26]: sodium methoxide. But the sodium methoxide spectrum did not show any bathochromic shift indicative of a 3-OH function (Fig.3). Also no shoulder in the range of 300-340 nm was observed in the methanolic spectrum of compound I. Such findings are flavones which usually exhibit such a shoulder. No double multipletes at δ2.8 and 5.2 ppm characteristic of flavanones were observed in the NMR spectrum (Fig.7). Such data limits the class to Dihydrochalcones.

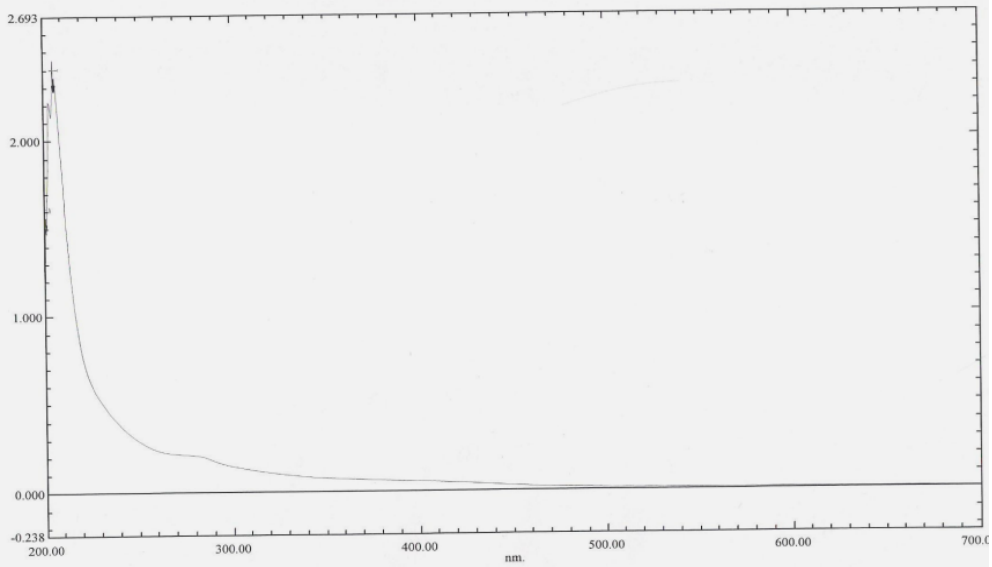


Fig.3: Sodium methoxide spectrum of compound I

The UV shift reagents were employed to investigate the hydroxylation pattern of this isolate. Sodium acetate is a shift reagent that is a weaker base than sodium methoxide and as such ionizes only the more acidic hydroxyl group in flavonoids i.e. 3-,7- and 4-' hydroxyl groups. Since ionization of the 7-hydroxyl group mainly affects band II, sodium acetate is a particularly useful diagnostic reagent for the specific detection of 7-hydroxy function [26]. However, no bathochromic shift was detected in the sodium acetate spectrum of compound I (Fig.4).

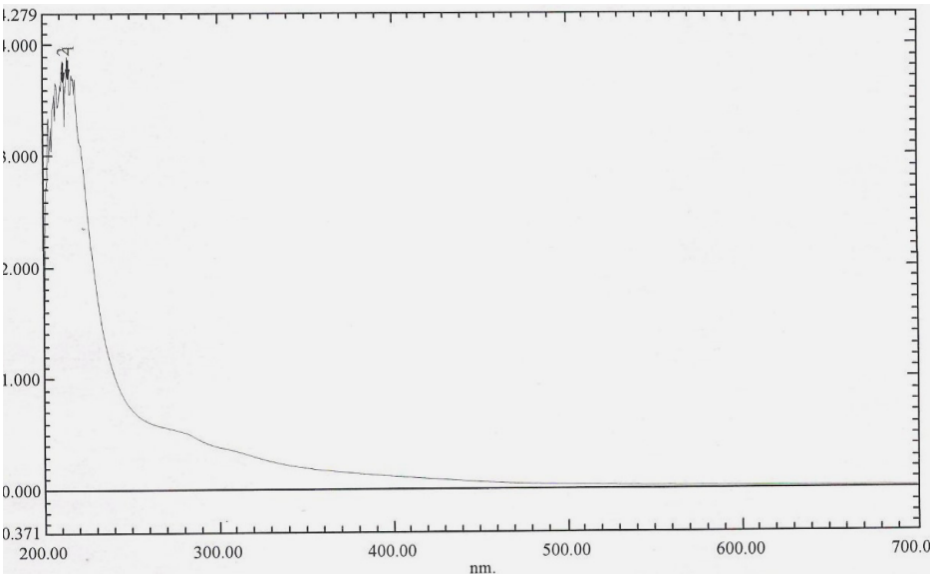


Fig.4: Sodium acetate spectrum of compound I

Aluminum chloride chelates with functional groups such as the 5-hydroxy-4-keto-, 3-hydroxy-4-keto and orthodihydroxyl systems and this are evidenced by bathochromic shifts of one or both bands in the spectrum. $AlCl_3$ complex between the C-4 keto function and either 3- or 5 -Hydroxyl groups are stable in presence of HCl acid. However catechols yield acid-labile complexes [26].

The aluminum chloride spectrum did not reveal any bathochromic shift (Fig.5) indicating the absence of 3- and 5-OH function as well as catechols systems. The absence of catechols systems was further evidenced by the absence of bathochromic shifts in the boric acid spectrum (Fig.6).

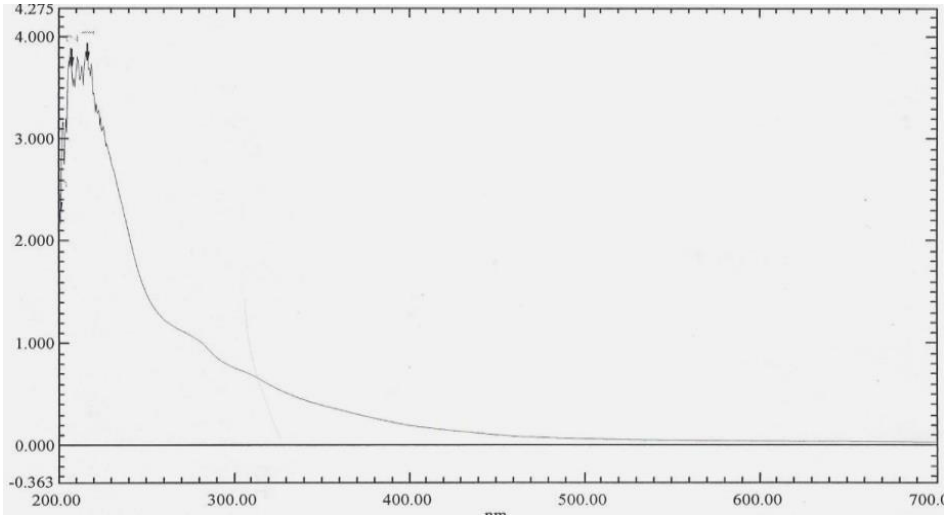


Fig.5: Aluminum chloride spectrum of compound I

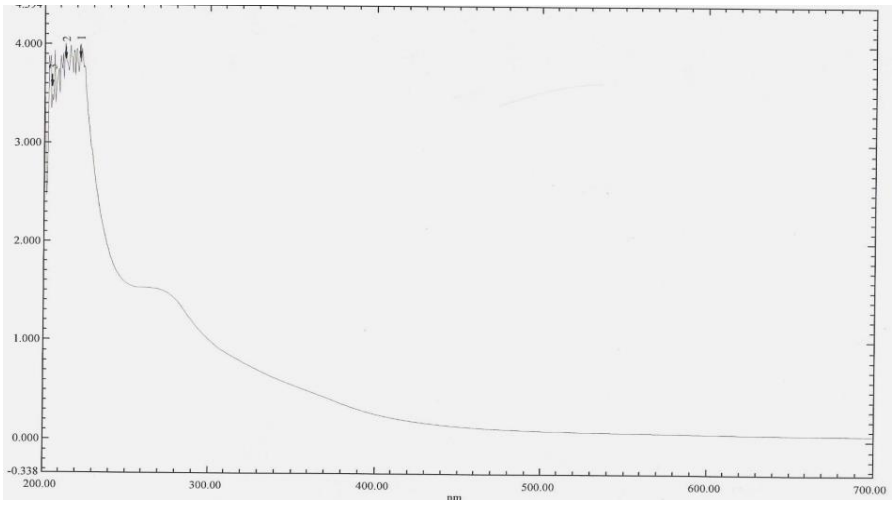


Fig.6: Boric acid spectrum of compound I

The ^{1}HNMR spectrum (Fig.7) showed signals for two methyl groups at ∂0.85(s, 3H) and ∂1.20ppm. The resonances at ∂1.80 (3H) accounts for an acetyl function, while the signal at ∂3.65ppm was attributed to a methoxyl group. The sugar protons appeared as a multiple at ∂4.2-4.8ppm. The C_6 and C_8 protons resonated as one proton signals at ∂6.25 and ∂6.80ppm respectively. The low field resonances at ∂7.05 and 7.25ppm correspond to aromatic protons.

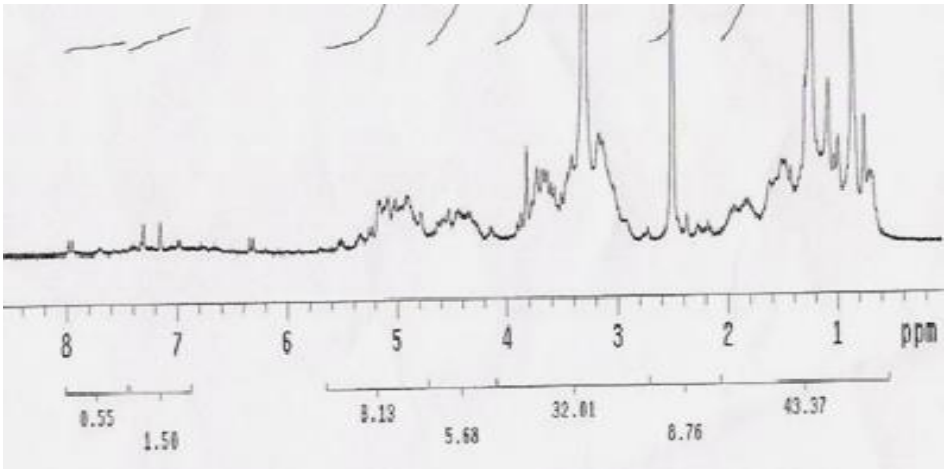
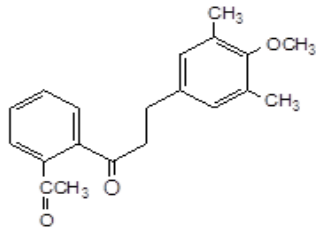


Fig.7 :^{1}HNMR spectrum of compound I

The mass spectrum (Fig.8) gave m/z 307 (M^{+}-3H) for aglycone. Fragments > m/z307 seem to originate from the glycoside which rarely afford a molecular ion peak .Based on the above spectral data, the following structure was assigned for aglycone of compound I.



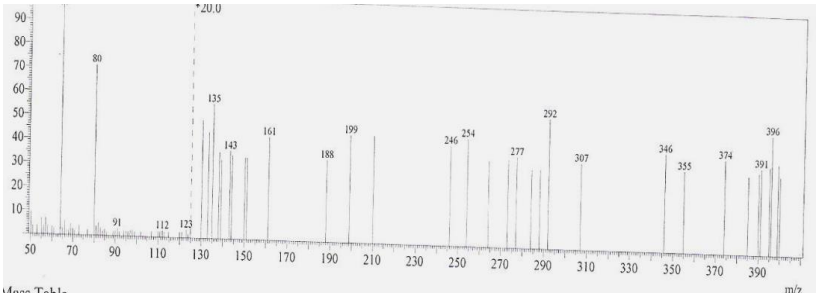
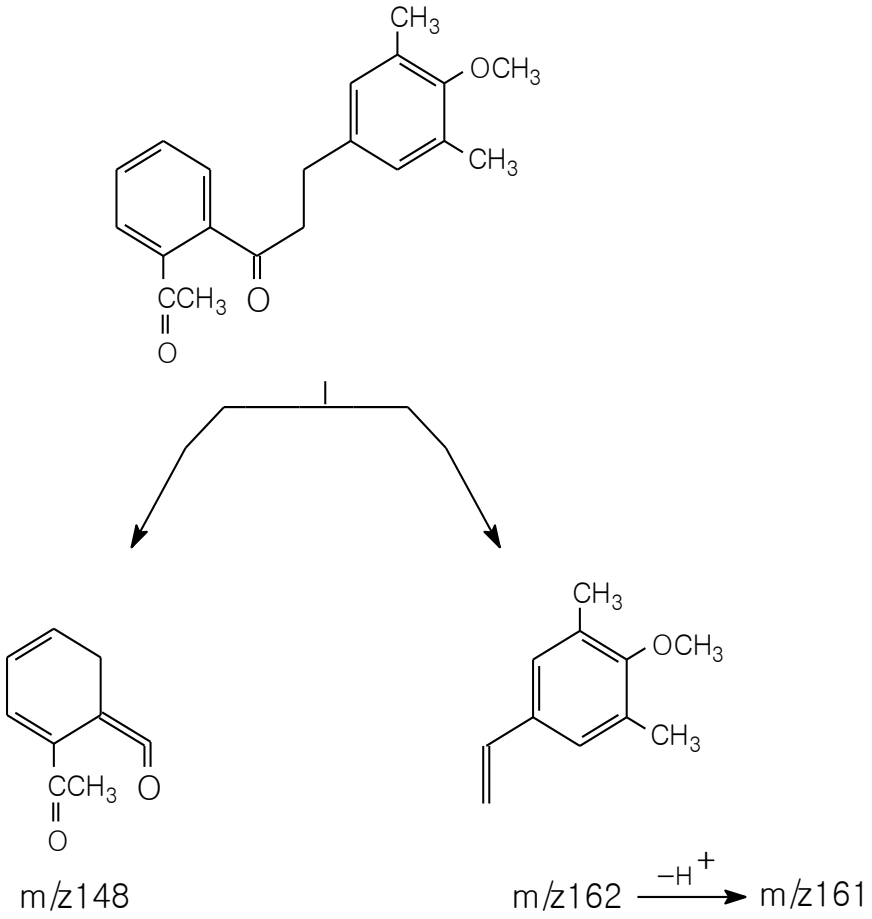


Fig.8: mass spectrum of compound I

The pattern of substitution of the A ring was based on : i) the absence of a downfield field signal around 8 ppm(which is characteristic of C_5 –H) allows assignment of the acetyl function at this position,(ii) the fragmentation pattern(Scheme I) gave m/z 161 for intact A ring. The fragment m/z161 for intact B ring and the appearance of B ring protons as a single (δ7.25ppm) indicates their magnetic equivalence and supports the substitution pattern suggested for the B ring.



Scheme 1: Important fragments of compound I

3.3. Antimicrobial Activity of *Catharanthus Roseus*:

The result of antibacterial activity of Compound 1 showed in the table **(2)**. revealed that compound 1 possesses a broad spectrum of activity against gram-positive and gram-negative bacteria were tested and produced impressive antibacterial activity against the entire tested organism with zones of inhibition ranging between 10 - 15mm and 11 - 13mm antifungal. The activity of compound 1 is near the activity of standard drugs Gentamycin and Clotrimazole at concentrations of 10 and 7.5 mg/ml respectively.

Table (2): the antimicrobial activity for compound I

Type	Conc.(mg/ml)	Minimal inhibition concentration (µg mL^{-1})					
		Gram-positive		Gram-negative		Fungal species	
		Sa	Bs	Ec	Ps	Ca	An
Compound 1	100	10	15	11	13	15	8

Table (3): Antibacterial activity of standard chemotherapeutic agents:

Drug	Conc.(mg/ml)	Bs	Sa	Ec	Ps
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table (4): Antifungal activity of standard chemotherapeutic agents against standard fungi:

Drug	Conc.(mg/ml)	An	Ca
Clotrimazole	30	22	38
	15	17	31
	7.5	16	29

Key: Sa.*Staphylococcus aureus*, Ec.*Escherichia coli*, Pa.*Pseudomonas aeruginosa*, An.*Aspergillus niger*, Ca.*Candida albicans*, Bs.*Bacillus subtilis*

4. Conclusion:

Catharanthus roseus L Is an importance medicinal plant used to treatment hypertension, cystitis, gastritis, enteritis, diarrhea and the raised blood sugar levels, its application ranges widely from the prevention of cancer, cancer treatment, anti-diabetic and stomachic. The Dihydrochalcone compound was isolated and investigated from leaves it was possessed

antimicrobial activity that is support the researchers predict new compounds from this plant with pharmacological and medicinal properties.

5. Acknowledgments:

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