Isolation and Bioassay of a New Terminalone A from *Terminalia arjuna*

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Abstract

Terminalia arjuna possesses significant cardio protective, antidiabetic and antioxidant properties as these properties are described in Ayurveda. In the present study, three flavonoids were isolated through the separation and chromatographic purification of the whole plant material of T. arjuna. Spectroscopic characterization identified one of them as a new flavonoid "*Terminalone* A (1)" and two known flavonoids i.e. *6-hydroxy-2-(4-hydroxyphenyl)-7-methoxy-4H-chromen-4-one* (**2**) and *2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one* (**3**). The bioactivity studies showed considerable antibacterial and antioxidant (DPPH was used as a scavenger) potential for all the three compounds **1-3** where the compound **1** showed strong antibacterial and antioxidant activity.

Keywords: *Terminalia arjuna*; Terminalone A; flavonoids; antibacterial; antioxidants; reactive oxygen specie

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1.Introduction

The *Terminalia arjuna* (*Roxb.ex DC.*) Wight & Arn. plant belongs to the second biggest genus "*Terminalia*" of the *Combretaceae* possessing more than 200 species. It is commonly recognized as *Arjuna* [1]. Terminalia species are widely distributed in humid and hot Asian, Australian and African regions [2]. Height of the arjuna tree is about 60 to 80 feet. Among the various medicinally important Asian based species, *T. arjuna* and *T. chebula* are enormously reported in literature as well as recognized for great medicinal uses in Ayurvedic [3]. The plant usually develops in wide range of soil, however more suitable is sticky soil and red lateritic soil [4]. It is an evergreen tree and it develop new leaves usually in February to April [5]. The plant possesses shape of a spreading crown and have dropping branches therefore it is planted for ornamental and shade purposes [6].

The *T. arjuna* plant is locally named as "*Arjun*", famous as gardens' protector therefore also recognized as "baaghon ka muhafiz" in local community of Pakistan [7]. *T. Arjuna* is reported for its diverse range of bioactivities including antibacterial, antioxidant, anti-inflamatory and antitumor. It also possesses hypolipidemic, antimutagenic and hypocholesterolaemic effects [8]. Ayurvedic doctors recommend *T. arjuna* in treatment of three kinds of tumors i.e., Kapha, Vata and Pitta [2]. It has been reported that Saponin glycosides obtained from *T. Arjuna* are accounted for inotropic impacts. The plant also used for cancer treatment and as cardioprotective, where the flavonoids isolated from the plants is suggested to be responsible for these cardiotonic and anticancer properties [9]. Powder from the bark stem of *T. arjuna* has been to be used against coronary infection and hypercholesterolemia [10].

The *T. arjuna* plant is well recognized for having different classes of medicinally important natural products e.g. it contains high contents of polyphenolic compounds (60-70%) including ellagic acid, arjunolone, arjunone, gallic acid, oligomeric proanthocyanidins, and other [11]. It also contains triterpenoids, saponins, Tannins, Phytosterols and minerals like Ca, Zn, Mg and copper. Furthermore, the *T. arjuna* plant is rich in Amino acids like Tryptophan, Cysteine, Tyrosine and Histidine [9]. Among the various natural products flavonoids reported from this plant have been recognized for diverse biological activities including antioxidant and antibacterial [12]. Therefore, the present study describes the isolation of new flavonoids from *T. arjuna*, assessment

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of their antibacterial and antioxidant potential, and to study the mechanistic features of the bacterial inhibition.

1. Experimental

1.1. General procedure

Silica gel coated aluminum support based TLC cards (60 F_{254}, 0.2 mm thick; E. Merck, Germany) was applied to check the purity of the compounds. Silica gel (230 to 400 mesh, E. Merck) was used in chromatographic column for purification. Glass supported preparative thin layer chromatographic plates (20×20, 2mm) and coated with silica gel (0.5 mm thickness) were used to purify the semi pure compounds. Solutions of $Ce(SO_4)_2$ and $KMnO_4$ were used for visualization of the compounds on TLC plates. Ethanol was used in recording UV spectrum through UV spectrophotometer (Shimadzu, Japan, UV-2700) spectrophotometer. The TOF Mass spectrometer (Billerica, USA) in ESI mood was used for Mass spectral studies of the isolated compounds. The ^{1}H and ^{13}CNMR spectrums of the obtained compounds were recorded by using Bruker 300 MHz ^{1}H and 75 MHz ^{13}C NMR spectrometer where $CDCl_3$ was used as solvent·

1.2. Plant materials

The plant in whole was collected from the different areas of Gomal University. Then Professor Saddiq Khan from faculty of Agriculture (Gomal University) identified the plant.

1.3. Extraction and Isolation of the compounds

The *T. arjuna* whole plant material (15 kg) was subjected to methanolic (3×50L of MeOH) extraction at room temperature. The extract gummy residue of about 600 g upon evaporation under reduced pressure. The obtained residue was then suspended in H_2O and further extracted with different solvents such as, n-hexane, dichloromethane, EtOAc, and MeOH to get 50g, 10 g, 38 g and 65 g of the corresponding fractions respectively. The ethyl acetate soluble fraction was then passed through silica gel packed column using different eluting systems in order of increasing polarity i.e. *n*-hexane (non-polar), *n*-hexane-DCM (less polar), DCM-ethyl acetate (polar), and highly polar EtOAc-MeOH system. The sub fraction AF displayed two spots on the TLC card with significant R_f value, therefore preparative TLC method was adopted to purify the compounds in each spot using *n*-hexane: EtOAc (8:2–3:7) as eluting solvents. The bands of the corresponding spots were scratched from the TLC plates and were filtered

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where fine brown solids were obtained that were latterly recognized as compound **1** and **2**. The sub fraction ASL on keeping under fuming hood rapidly changed into fine brown solid with *n*-hexane: EtOAc (4:6). This solid material was then identified as compound **3** with the help of spectroscopic techniques. The pure constituents were screened for antibacterial and antioxidant activity using standard protocols.

1.4. Antibacterial activity of compounds 1-3

Agar well diffusion procedure was followed to determine the antibacterial efficacy of compounds **1-3**. Two potential drug resistant *E. coli* (ATCC 6538) & *S. aureus* (ATCC 6633) bacteria were selected for this study. The said bacteria were cultured in broth media and incubated at 37ºC for 15 hrs. The inocula of each bacterium were splashed on the agar media. Sterile cork borer made two wells of 6 mm. About 60 µL of 0.8 mg/mL of each compound were poured in the wells. Afterward, the discs were incubated for 24 h at 37 ^{o}C. Streptomycin was used as a standard.

1.4.1. MIC of Compounds 1, 2, 3 and streptomycin

MIC of all the compounds was investigated by already reported serial dilution method [13]. Various sterile test tubes having 1 mL of the said bacterial solution and different concentrations or dilutions (15-120 µg/mL) of compounds were taken and incubated for 24 hours in shaking incubator at 37 ˚C. The test tube having only bacterial solution was taken as control.

1.4.2. Test for reactive O-species (ROS) in the presence of Compound 1

2,7-dichloro dihydrofluorescein diacetate colour was used to check the production of reactive O-species.This important hue provides a very accurate assessment of ROS in bacterial cells. Specific concentration of compound **1** and *E.coli* were incubated for three hours at 300 rpm . After appropriate incubation, the *E. coli* strain suspension was pricipitated for eight minutes at 7000 rpm then the obtained pellet was rinsed with saline buffer of phosphate. The phosphate buffer saline including pellet was accordingly blended in with 1 mL of 15 mM of 2,7-dichloro dihydro fluorescein diacetate color for sixty (60) minutes. The color treated cells were subsequently washed with phosphate buffer saline in order to eliminate the dye from the surface of the cells. The fluorescence magnifying instrument was utilized to capture fluorescence picture at excitation & emission frequencies ie. at 488 nm and at 535 nm separately [14].

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1.5. Antioxidant activity

Antioxidant potential of the isolated compounds 1-3was examined according to reported method [15]. Initially, 2 mM of DPPH in combination with various concentrations of the isolated compounds (0.1-0.8 mg / mL) were constantly stirred. These were then kept in dark for about 30 minutes. After that the absorbance was studied by UV-Vis Spectrophotometer at 517 nm. Vitamin C was used as a control. The % inhibition was examined by the following formula.

$$\% \text{ inhibition} = \frac{(\text{Absorbance by the control}) - (\text{Absorbance by the sample compounds})}{(\text{Absorbance by the control})}$$

2. Results and discussion

In the present work, compounds **1-3** were initially isolated from EtOAc soluble fraction of methanolic extract of *T. Arjuna*, then structural elucidation of the obtained compounds were carried out using modern spectroscopic techniques and finally the compounds were evaluated for their anti-oxidant and anti-bacterial potential.

2.1. Characterization of the isolated compounds

Repeated column chromatographic techniques was applied to the selected ethyl acetate soluble fraction of the *Terminalia Arjuna* plant material which provided compound **1-3** as brown solids. Their structureswere established by using modern spectroscopic techniques such as Mass spectrometry, IR, ^{1}H and ^{13}C NMR. The isolated compounds **2** and **3** were known compounds and were identified as *6-hydroxy-2-(4-hydroxyphenyl)-7-methoxy-4H-chromen-4-one* (**2)** [16] and *2-(3, 4- dihydroxyphenyl) -5,7-dihydroxy-4 H-chromen -4- one* **(3)** (see Figure 1) [17]. The new compound *terminalone* A (**1**) was obtained as a sticky brown solid. The FTIR absorption peaks observed at 3320-3310 cm^{-1} and 1605 cm^{-1} suggested that hydroxyl moiety and benzene ring are present in the compound **1**. Its molecular formula was established through HREI-MS as $C_{28}H_{36}O_{16}$ that displayed $[M+H]^{+}$ peak at *m/z* 629.2072 (calcd. for $C_{28}H_{36}O_{17}$ as 629.2076) indicated eleven degree of unsaturation, which represents the presence of five rings including two benzene rings. Additionally, the Linked scanning on molecular ion (M^{+}) indicated that the ions with m/z 465, 287, 195 arose directly from it corresponding to the fragments M-$C_6H_{11}O_5$ (M-163) with the loss of one glucose unit, M-$C_{12}H_{21}O_{11}$ (M-241) with loss of two glucose units and M-$C_{18}H_{25}O_{12}$ (M-433) with the loss of two glucose units and along with the ring B. The ^{1}HNMR spectra of compound **1** showed a

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singlet at δ 8.35 ppm (s) confirming the presence of methin proton at position 6 of ring A. A pair of doublets (d) at δ 7.25 ppm (J = 7.3 Hz) and δ 6.61 ppm (J = 1.7 Hz) and a doublet of doublet signal at δ 6.59 (J = 7.3, 1.7) of ring B, confirming 1', 3', 4' trii-substituted B-ring (Table-1, Note: See the table at the end of this document). A singlet at δ 2.50 ppm is attributed to methyl proton of benzene (a). The four-hydroxyl moiety of (a), (b) and (c) were resonated at δ 3.35-3.64 ppm as a singlet. The eight oxymethines resonated at around 3.57 to 3.22 ppm, two oxomethylenes signals at δ 4.56 dd (J = 1.8, 12.2, H_b) /3.78 dd (5.2, 12.2, H_a) and 4.31 (dd, J = 2.2, 12.0 Hz, H_b)/3.61 (dd, J = 5.0, 12.0 Hz, H_a) and the two anomeric peaks at δ 4.96 (d, J = 7.2) and 4.90 (d, J = 7.6 Hz, H-1$''$) suggested the rhamnose moiety [18]. The ^{13}C NMR spectra with the help of DEPT (distortion less enhancement via polarization transfer) indicated signals for 1 CH_3, 3 –CH_2-, 16 –CH- and 8 Quaternary carbons (Table 1, Note: See the table at the end of this document). The highly downfield peaks at δ 132.5, 130.0 and 127.7 ppm were assigned to the phenolic carbon, while δ 115.03 ppm and δ 132.5 ppm were attributed to benzene carbon (C-4a to C-8a). Other signals appearing at δ 97.3 ppm and δ 95.6 ppm correspond to anomeric Carbon-1″ and Carbon-1‴, while the peaks for other carbons of glucose moiety were resonated in the chemical shift region from 83.2-64.3 ppm, respectively. The obtained ^{1}HNMR and ^{13}CNMR data for compound **1** is given in table 1 and Figure (S1-4).

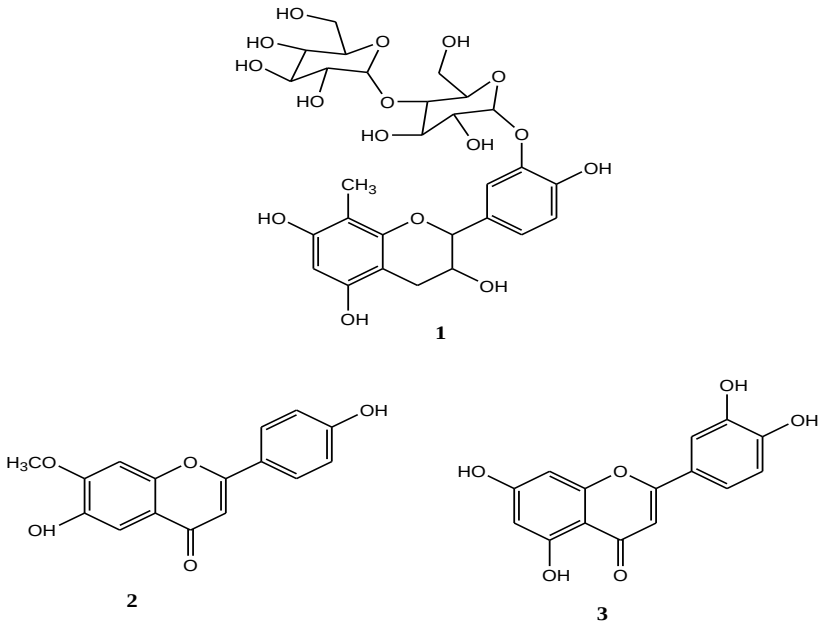

Figure 1. Structures of Isolated Compounds (**1-3**)

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Table 1. ^{1}HNMR and ^{13}CNMR of compound **1** ($CDCl_3$, δ ppm)

H/C	**^{1}H NMR** (δ ppm)	**^{13}C NMR** (δ ppm)
2 (CH)	5.38 (d, *J* = 6.7 Hz)	69.7
3 (CH)	4.89 (m)	63.5
4 (CH_2)	2.51 (dd, *J* = 6.4, 15.2 Hz) / 2.63 (dd, *J* = 2.1, 15.2 Hz)	39.56
4a	Q	115.03
5	Q	127.7
6 (CH)	8.35 s	98.4
7	Q	130.0
8	Q	104.6
8-Me	2.50 s	29.4
8a	Q	132.5
1′	Q	102.4
2′ (CH)	6.61 (d, *J* = 1.7))	111.3
3′	Q	129.2
4′	Q	120.01
5′ (CH)	7.25 (d, *J* = 7.3)	103.4
6′ (CH)	6.59 (dd, *J* = 7.3, 1.7 Hz)	100.0
1″ (CH)	4.96 (d, *J* = 7.2 Hz)	97.3
2″ (CH)	3.24 (dd, *J* = 7.2, 9.0 Hz)	76.1
3″ (CH)	3.35 (dd, *J* = 9.0, 7.3 Hz)	83.2
4″ (CH)	3.40 (dd, *J* = 8.6, 7.3 Hz)	73.4
5″ (CH)	3.57 (m)	79.2
6″ (CH_2)	4.56 (dd, *J* = 1.8, 12.2, H_b) /3.78 (dd, *J* = 5.2, 12.2, H_a)	65.6
1‴ (CH)	4.90 (d, *J* = 7.6 Hz)	95.6
2‴ (CH)	3.22 (dd, *J* = 7.6, 8.6 Hz)	75.0
3‴ (CH)	3.27 (dd, *J* = 8.6, 7.2 Hz)	81.9
4‴ (CH)	3.38 (t, *J* = 8.0, 7.2 Hz)	70.1
5‴ (CH)	3.50 (m)	77.2
6‴ (CH_2)	4.31 (dd, *J* = 2.2, 12.0 Hz, Hb)/3.61 (dd, *J* = 5.0, 12.0 Hz, Ha)	64.3

2.2. Antibacterial property of compounds 1, 2 and 3

The three isolated natural products **1-3**were tested against *E. coli* and *S. aureus*. These bacteria are very toxic and spread infection in different parts of Human and animals. The results of all the three compounds are shown in Table 2.

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Table 2. Anti-bacterial activity of the isolated natural products **1**, **2**, **3** and streptomycin

Bacteria	**Antibacterial activity** (Inhibition zone in mm)			
	Compound 1	**Compound 2**	**Compound 3**	**Streptomycin**
E. coli	18 (±0.3)	13 (±0.4)	11 (±0.4)	23 (±0.3)
S. aureus	21 (±0.4)	15 (±0.3)	14 (±0.5)	26 (±0.4)

The results illustrated that compound **1** has much higher inhibition against both bacteria as compared to compounds **2** and **3**. The efficacy of compound **1** against *S. aureus* is much higher than *E. coli*, which may be due strong and complex cell membrane of *E. coli*. The zone of inhibition (mm) of compounds **1-3**against *E. coli* and *S. aureus* were 18 (±0.3), 13 (±0.4), 11 (±0.4) and 21 (±0.4), 15 (±0.3), 14 (±0.5) respectively. Streptomycin was also applied against the both bacteria as standard drug. The result showed that the standard drug has much high inhibition efficiency against both the bacteria. The inhibition zone of the said standard drug against *E. coli* and *S. aureus was* 23 (±0.3), 26 (±0.4) respectively. In presence of compound **1**, the damage to *E. coli* was also examined through scanning electron microscopy as shown in Figure (2C). It is clear from the result that the morphology of *E. coli* is completely damaged. Most of the bacteria are shrunk and cell membrane was completely ruptured.

2.2.1. Minimum inhibitory concentrations (MIC) of compounds 1, 2, 3 and streptomycin

MIC is the lowest concentration of compound which inhibits the growth of bacteria. MIC of compounds **1-3** and streptomycin was examined in this assay. Different concentrations for all the compounds (15-120 µg/mL) were examined against *E. coli and S. aureus*. In all these compounds streptomycin showed lowest MIC value against both bacteria. In all the compounds **(1-3)**, Compound **1** showed better MIC value against *E. coli and S. aureus*. The MIC values for all the three compounds against *E. coli* were found 60, 75, 105 µg/mL and against *S. aureus* were 45, 75 and 75 µg/mL respectively. The MICs of streptomycin standard and compounds 1-3 are presented in table 3.

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Table 3. MIC of standard drug and compound 1-3 against *E. coli* and *S. aureus*

Compound	**MIC (µg/mL)**	
	E. coli	*S. aureus*
Standard (streptomycin)	30	15<
Compound 1	60	45
Compound 2	75	75
Compound 3	105	75

2.2.2. Mechanism of inhibition of the selected pathogens and examination of reactive O-species (ROS)

Considering mechanistic aspects of bacterial inhibition, the formation of reactive oxygen species can be correlated to the anti-microbial impact of compound **1** in the microbial cell. The reactive O-species such as $\bullet O_2^-$, •OH and H_2O_2 delivered because of energized electrons of OH bunch in the compound **1**, which advance the age of ROS with in the *E. coli* cell. Such reactive species are considered to be very harmful to DNA & Protein of *E. coli*. Furthermore, it is revealed that within the sight of ROS, the 2, 7-dichloro fluorescein diacetate color oxidized into its dichlorofluorescin derivatives. Additionally, green fluorescence was analyzed upon excitation at about 488nm within the sight of compound **1** as displayed in Figure 2(B). Without the said compound **1**, no fluorescence is noticed as shown in Figure 1(A). This outcome unmistakably affirms that the reactive O-species are delivered in the bacteria cells within the sight of compound **1** and is liable for their restraint. Subsequently, the got results propose that compound **1** has association to *E. coli* cells' surface which convinces the formation of intracellular reactive O-species and spillage of cytoplasm as illustrated in Figure 2(C).

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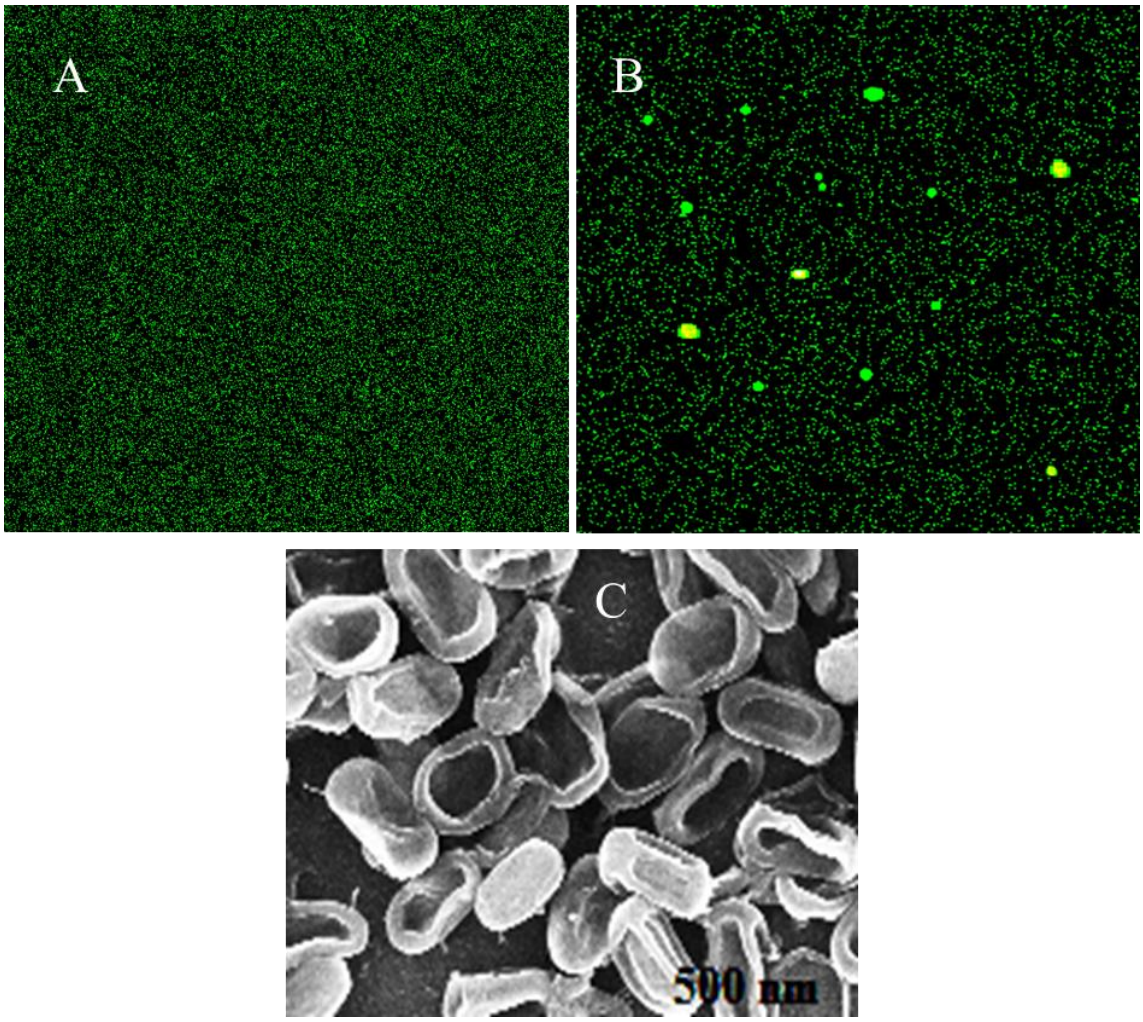

Figure 2. ROS examination (A) in the absence of compound **1**, (B) in the presence of compound **1** and (C) Scanning electron microscopic examination of *E. coli* in the presence of compound **1**.

2.3. DPPH Scavenging activity of compounds 1, 2, 3

DPPH scavenging assay of all the three compounds were examined and compared with vitamin C (Vit. C) as shown in Figure 3. DPPH was selected for this study because it is very stable radical due to resonance structure. Thus, very harsh conditions must be required for its stabilization. The results demonstrated that radical conversion efficacy of all the three compounds increased with enhancing their concentrations. The scavenging activity of compound **1** is more proficient than compounds **2** and **3** that may be due to lot of phenolic OH present in compound **1**. The phenolic OH has the ability to donate electrons and stabilize the radicals. The results showed that more than 90% scavenging was achieved in the presence of compound **1** at 0.8 mg/mL concentration.

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On the other hand, compounds **1** and **2** got DPPH scavenging 77% and 66% respectively at 0.8 mg/mL concentrations.

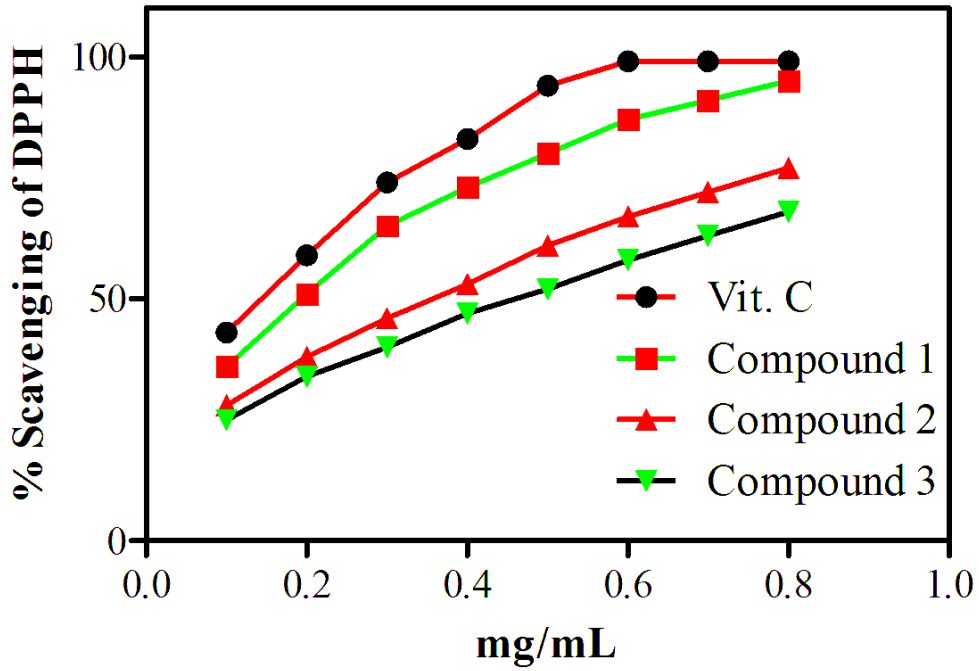

Figure 3. DPPH scavenging activities of compounds **1-3**

3. Conclusion

This study demonstrated that Terminalone A **1** as a new flavonoid along with two known flavonoids (6-hydroxy-2-(4-hydroxy phenyl)-7-methoxy-4H-chromen-4-one **2** and 2-(3,4-dihydroxy phenyl)-5,7-dihydroxy-4H-chromen-4-one **3** were attained from the EtOAc soluble fraction of *T. arjuna*. Further bioassay investigation of the isolated products recognized them as significant antibacterial and antioxidant agents where the new compound **1** showed highest activity such as 18 (±0.3) against *E. coli* and 21 (±0.4) against *S. aureus.* Overall the present research shows agreement with the reported ethnomedicinal importance of *T. arjuna* and exploited its hidden medicinal properties.

Author Contribution: Conceptualization**,** Bushra; methodology, Bushra, Khalil ur Rehman, Dilfaraz Khan and Shafiullah Khan; validation, Bushra, Shafiullah Khan; writing—original draft preparation, Bushra, Dilfaraz Khan, and Shafiullah Khan; writing—review and editing, Bushra, and Hamid Ullah; supervision, Khalil ur Rehman and Shafiullah Khan; funding acquisition, Abdulrahman A. Almehizia, Ahmed M. Naglah, Asma S. Al-Wasidi, Moamen S. Refat, Mohamed Y. El-Sayed. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest: The Authors declare that there is no conflict of interest among them.

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