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NEW ANTIOXIDATIVE PROCREATE FROM *CALOTROPIS PROCERA*

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Abstract

The plant *Calotropis procera* was screened to evaluate its phytochemical and antioxidant capabilities in an effort to find new, potent antioxidant agents. As a result, new glycosidic Procerate *((6E,8E)-7-methyl-10-oxo-10-((3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yl) oxy) deca-6,8-dienoic acid* **1** was isolated from the ethyl acetate soluble fraction of *Calotropis procera*, along with two known compounds *Octadecenoic acid* **2** and *Octadecadienoic acid* **3** reported for the first time from C. procera. Their structures were deduced by spectral studies such as NMR, IR, UV and EIMS spectrometry. Furthermore, the antioxidant potential of the new isolated compound Procerate**1** was also investigated, which showed dose-dependent antioxidant potential, where the 9 µg/mL concentration displayed highest (more than 80%) antioxidative activity in the DPPH assay. The antibacterial activity of the isolated compounds was also tested in comparison with ciprofloxacin standard against highly drug resistant bacteria *Escherichia coli* (*E. coli*) bacteria. The inhibition zones of the compounds (1-3) and ciprofloxacin are 19 ± (0.6) mm, 14± (0.1) mm, 12 ± (0.5) mm and 23 ± (0.7) mm respectively.

Keywords: *Calotropis procera*; Isolation; Glycoside; DPPH; Antioxidant Activity; Antibacterial activity

1. Introduction

Calotropis procera is associated to the family *Asclepiadaceae*. *Asclepiadaceae* is a large family comprises of about 240-350 genera and 2500-3100 species. *C. gigantea (Linn.)* and *C. procera (Ait.)* is a member of *Calotropis* genus, which is illustrated by the Sanskrit writers [1-3]. *C. procera* subsp. *Hamiltonii* and *C. procera* subsp. *procera*, are two subspecies of *C. procera*, which solely differ in their fruit traits. *C. procera* prefers climates with an annual rainfall range of 175–1200 mm and temperatures between 25–32 ºC. This plant has latex which is highly toxic and that's why it is not eaten by grazing animal [4-6]. The plant is used as an Ayurveda drug from earliest time against various diseases i.e. antihelmintic, expectorant, laxative, to treat bronchitis, asthma, leprosy, eczema, elephantiasis and others [7]. In Hindu culture the leaves and flowers of the Calotropis genus plants are used to present their Lord Ganesha, Hanuman and Shiva [8]. One of its common names is Akra due to its use in sacrificial rites. As *C.procera* is a shrub (Xerophytes perennial) that's why its growth is best in arid and semi-arid countries and inhibited to Asia, Africa, Latin America and Middle East [9-10]. Aiming potentially bioactive constituents from *C. procera*,

its ethyl acetate fraction was studied through standard isolation methods, modern spectroscopic characterization and DPPH scavenging assays. Info-graphically the current study is presented as below in Figure 1;

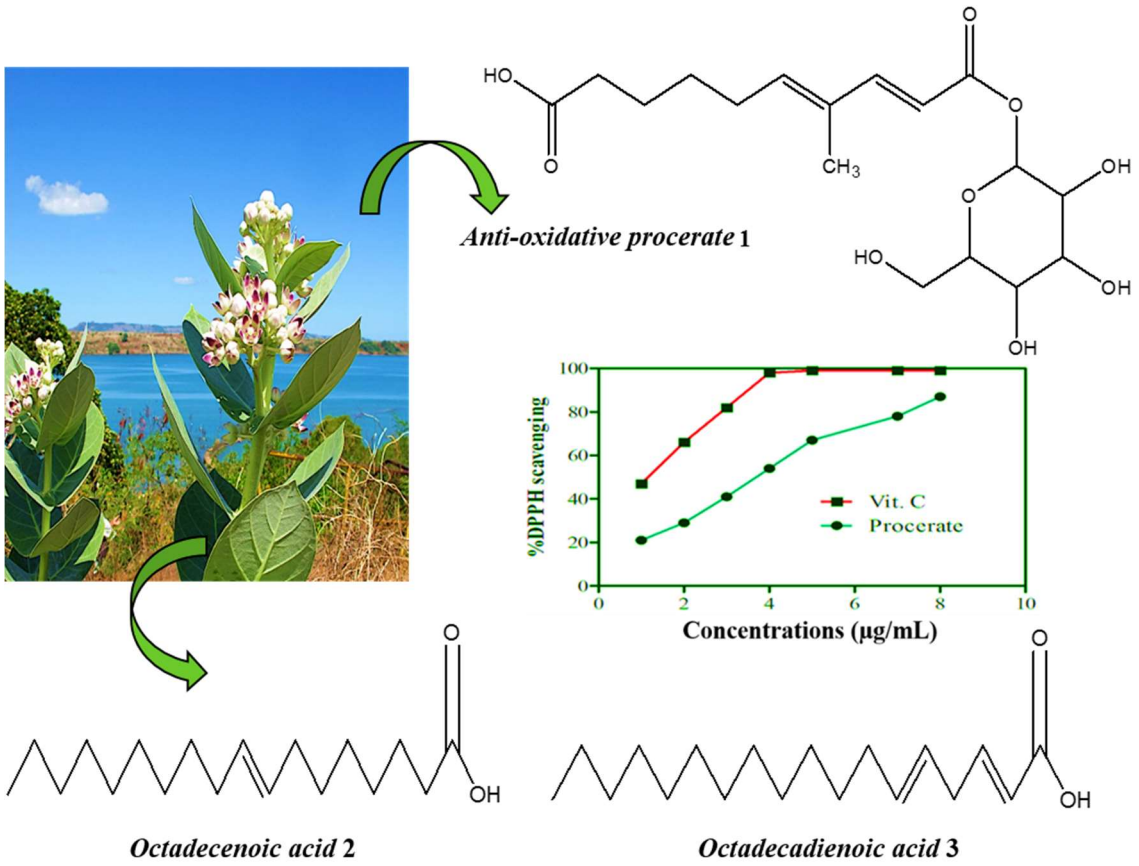

Figure:1 Graphical representation of the phytochemical and antioxidant study of *C. procera*

2. Experimental

2.1. General procedure

A silica gel (70-300 mesh) Column-Chromatographic method was used for purification. The UV spectra of the pure metabolites were obtained using a Perkin Elmer, U 35 spectrophotometer. P. In order to get the IR spectra of the isolated constituents, a Shimadzu-460 spectrometer was used. Mass spectrums for the three isolated compounds were obtained by using LCQ-thermo-Finnegan-spectrophotometer having an attached PDP11/34PC-system. The Bruker ARX400-spectrophotometer was used to record the NMR spectra of the isolated compounds (300 MHz for ^{1}H) & (75 MHz for ^{13}C).

2.2. Extraction, isolation and purification

The *Calotropis procera* plant was collected from Paharpur, D.I. Khan, KPK, Pakistan and was identified by Dr. Sadiq Khan Assistant Professor, Faculty of Agriculture, Gomal University D.I. Khan. The stem, leaves, flowers and fruits of the plant (*C.procera*) were dried under shade

separately for fifty days and converted to fine powder material. About 10Kg of the fine material was then subjected to extraction with methanol for 3 times. Subsequently, the solvent was removed via reduced pressure by using rotary evaporator which resulted into a greenish and gummy crude of about 540 g. Following fractionation, the crude was separated into various fractions of n-hexane (M1, 90 g), dichloromethane (M2, 105 g), ethylacetate (M3, 120 g), and methanol (M4, 200 g).

To get natural products, the EtOAc-soluble M3 fraction was further processed using a column-chromatography approach over silica and organic solvents with an increasing polarity, including n-hexane, CH_2Cl_2, EtOAc, and MeOH.Consequently, the purity of the compounds was also checked by TLC. The EtOAc fraction (M3 120 g) was loaded over silica gel packed column, eluting with increasing polarity of the mobile phase starting from n-hexane, following through n-hexane- EtOAc (with increase in 10% polarity in each step), ethyl acetate, ethylacetate-methanol (with increase in 5% polarity stepwise) and finally washed with MeOH (E). Various fractions were obtained and the fractions that afforded similar sots on TLC were combined that resulted into five fractions A-E.

Fraction A gave two major spots on TLC with some minor impurity spots, which were purified by preparative TLC using n-Hex: EtOAc (4:1) as mobile phase to produce two pure compounds compound **2** (12 mg) [10] and compound **3** (9 mg) [11].

Fraction D, which produced three spots on TLC along with impurity spots was then purified via silica gel packed chromatographic column using elution system of n-Hexane and EtOAc (with increase in 5% polarity stepwise) which finally furnished one new compound **1** (14 mg) in a purified form.

2.3. Antioxidant assay

Literature revealed that flavonoids or extracts enriched in flavonoids are usually evaluated for their inhibitory potential of free radicals through the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) scavenging protocol [12]. Accordingly, a stock solution of about 1 mg/ml of the isolated new compound "Procerate **1**" and ascorbic acid (standard) was prepared in methanol. Then the dilutions at 1-10 µg/mL concentrations were performed. Briefly, about 120 µL of the different concentration working solutions of the procreated **1** (test compound) and the standard (ascorbic acid) were added to the 900 µL (20 µg/mL) DPPH solution in MeOH (methanol). The mixtures were then kept on room temperature for a 30 minutes and then the absorbance was examined at 515 nm using a UV-visible spectrophotometer against a blank. The blank solution was made through mixing of 1.0 mL of the DPPH solution (20 µg/ mL) with 1 mL of MeOH and the absorbance was measured at the same wavelength. The following formula was applied to determine the % antioxidant potential:

(%) activity = [(Absorbance of the control – Absorbance of the tested compound)/Absorbance of control x 100

The control includes all reagents except the test component. The IC_{50} values were determined by plotting the %age inhibition verses concentration.

2.4. Antibacterial activity of Compounds 1-3

The antibacterial activity of the isolated compounds (1-3) was studied against highly drug resistant bacteria (i.e. *E. coli*). The pathogenic bacterial strain was obtained from the Department of Biotechnology, Gomal University, D. I. Khan (Pakistan). On Mueller Hinton agar plates using the agar well diffusion method, about 60 µg/mL of 0.8 mg/mL concentrations of the isolated compounds were tested against a bacterial strain. Ciprofloxacin (10 µg/mL) antibiotic was used as a standard. The inoculated plates were then incubated for 24hrs at 37 ºC. The inhibition zones were measured and tabulated.

2.4.1. MIC of Compounds 1, 2, 3 and Ciprofloxacin

MIC is the smallest concentration of a compound that inhibits the growth of the bacteria. By using the previously described serial dilution approach, the MIC of all the compounds was assessed [13]. Different sterilized test tubes were used, and they were cultured for 24 hours at 37 °C in a shaking incubator with 1 mL of the prescribed bacterial solution and various concentrations/dilutions (10-100 µg/ mL) of the isolated compounds. The test tube having only bacterial solution was considered as control.

2.4.2. Production of reactive O-species (ROS)

In the presence of 2, 7-dichlorodihydrofluorescein diacetate dye, the generation of ROS was investigated. This significant dye is very effective for determining ROS in bacterial cells. A fixed amount of the isolated Procerate1 was treated with an *E. coli* strain for 3 hrs at 300 rpm. The *E. coli* cells suspension were properly incubated, precipitated (7000 rpm, 8 min), and the precipitated pellet washed in phosphate buffered saline (PBS). After that, 1 mL of 15mM 2, 7-dichlorodihydrofluorescein diacetate dye was added to the PBS-containing pellet and was stirred for 60 minutes. The dye-treated cells were then rinsed with PBS to get rid of it from the cell's surface. The fluorescence microscope image was taken at two different wavelengths i.e. excitation and emission at 488 nm and 535 nm respectively [14].

2.5. Identification of the compound (Procreate 1):

White color crystals (Purity>99%); FTIR spectrum (cm^{-1}): 3300, 2800, 1610 & 1450; EMIS m/z 374.160 [M-1] +, (for $C_{18}H_{34}O_2$); ^{1}HNMR in DSMO, 300 MHz, (δ in ppm): 10.46 (1H, br.s), 6.88 (1 H,d, H-2), 7.51 (1 H,d, H-3), 6.90 (1 H,t, H-5), 1.09 (2 H, m, H-6), 0.84 (2 H, m,H-7), 0.82 (2 H, m, H-8), 1.11 (2 H, t, H-9), 1.6 (3 H, s, H-11), 5.54 (1 H, d, H1'), 4.81 (1H, t, H2'), 4.18 (1H, t. H3'), 4.20 (1H, t,H4'), 4.23 (1H, m,H5'), 4.72 (2H, t, H6'). ^{13}CNMR in DSMO, 100MHz (δ in ppm): 167.2 (C-1), 125.9 (C-2), 127.1 (C-3), 127.5 (C-4), 128.5 (C-5), 14.4 (C-6), 22.0 (C-7), 22.0 (C-8), 25.6 (C-9), 175.8 (C-10), 29.4 (C-11), 63.4 (C-1′), 70.0 (C-2′), 71.0 (C-3′), 71.0 (C-4′), 72.9 (C-5′), 77.0 (C-6′).

3. Results and discussion

3.1. Isolation and spectral characterization of the compounds 1-3

The ethyl acetate fraction of the whole plant material of *Calotropis procera* provided compound **1-3** after using standard isolation protocol (Figure 2).

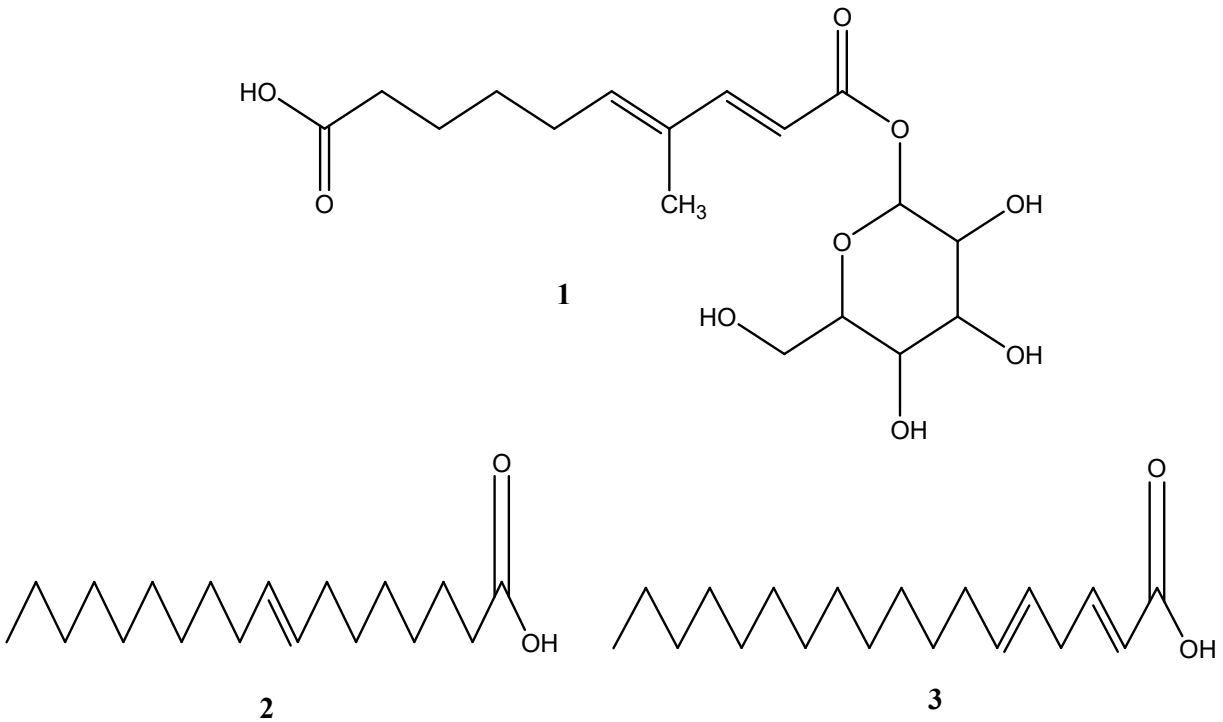

Figure: 2 Structure of the compound **1-3**

The obtained compounds were characterized through modern spectroscopic techniques. Compound **1** (Procerate) was identified as a new compound while compound **2** (Octadecenoic acid) [11] and compound **3** (Octadecedienoic acid) [15] were known compounds.

The new compound Procerate-**1** was isolated as white crystals. The FTIR absorptions at 3300 cm^{-1}, 2800 cm^{-1}, 1610 and 1450 cm^{-1} revealed the presence of –OH, sp^3/sp^2 –CH, carbonyl functional group and C=C bond, respectively. The proton NMR spectrum of compound **1** (Figure 3) displayed broad singlet at δ 10.46ppm corresponding to the proton of –OH of –COOH group. The signals observed in a range of 6.88 -7.51ppm was attributed to carbon carbon double bond conjugation, while the anomeric proton exhibited a singlet peak at δ 5.54 ppm. The signals about δ 4.18-4.81 ppm suggested the presence of glucose moiety. The CH_3 group was resonated at δ1.6 ppm as a singlet peak. Additional peaks for the alkyl group protons were observed in the spectrum with a chemical shift ranging from δ 0.82 to 1.11ppm.

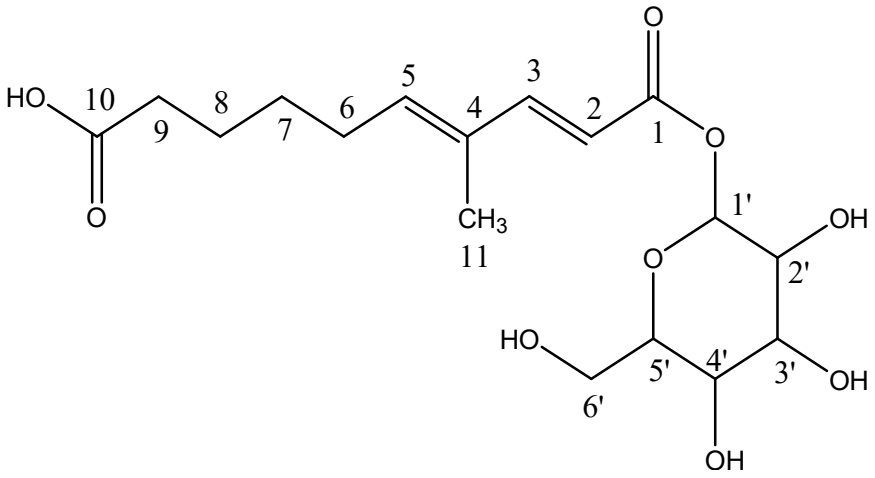

Figure: 3 Structure elucidation of compound **1**

The structural formula for the Procerate**1** was further confirmed with the help of ^{13}CNMR spectrum. The signal at δ175.8 ppm was corresponded to the carboxylic functionality. The C-signal appeared at 167.2 was assigned to ester moiety. Some peaks were also appeared down fielded at around δ 125.96 to 128.59 ppm that were attributed to the C=C functionalities. The signals appeared from δ 63.44 to 77.05ppm were attributed to glucose moiety. Also methyl showed peak at δ 29.45ppm while the carbons of alkyl chain were resonated at about δ14.43 to 25.65ppm. The ^{1}H &^{13}CNMR spectral data of the isolated Procerate**1** is given in Table 1 and Figure (S 1,2).

Table 1: ^{1}H and ^{13}CNMR values for **Procerate 1**

Position of Hydrogen and Carbon	**δ (Chemical shift) in ppm**	
H/C	H signal	C Signal
1 (Quaternary C)	-	167.2
2 (Methine)	6.88 d	125.9
3 (Methine)	7.51 d	127.1
4 (Quaternary C)	-	127.5
5 (Methine)	6.90 t	128.5
6 (Methylene)	1.09 m	14.4
7 (Methylene)	0.84 m	22.0
8 (Methylene)	0.82 m	22.0
9 (Methylene)	1.11 t	25.6
10 (Quaternary C)	-	175.8
10-OH	10.46 br. S	
11 (Methyl)	1.60 s	29.4
1′ (Methine)	5.54 d	63.4
2′ (Methine)	4.81 t	70.0
3′ (Methine)	4.18 t	71.0

4′ (Methine)	4.20 t	71.0
5′ (Methine)	4.23 m	72.9
6′ (Methylene)	4.72 t	77.0

3.2. Antioxidant assay

The antioxidant potential of the Procerate**1** was also examined through a reported method [16]. Accordingly, the compound **1** (Procerate) was examined through DPPH scavenging method where ascorbic acid (Vit. C) was used a standard (Figure 4). The DPPH used in the study was selected due it radical stability as its structure is conjugated. The obtained results describe that the radical conversion potency of compound **1**which increased with enhancing its concentration. It inferred that the scavenging potential of Procerate**1** is due to the presence of many hydroxyl (-OH) groups in compound **1** (Procerate). The OH group is capable to donate electrons (e^-) and to stabilize radicals. The results indicated that Procerate**1** at a concentration of 9 µg/mL enabled higher than 80% scavenging.

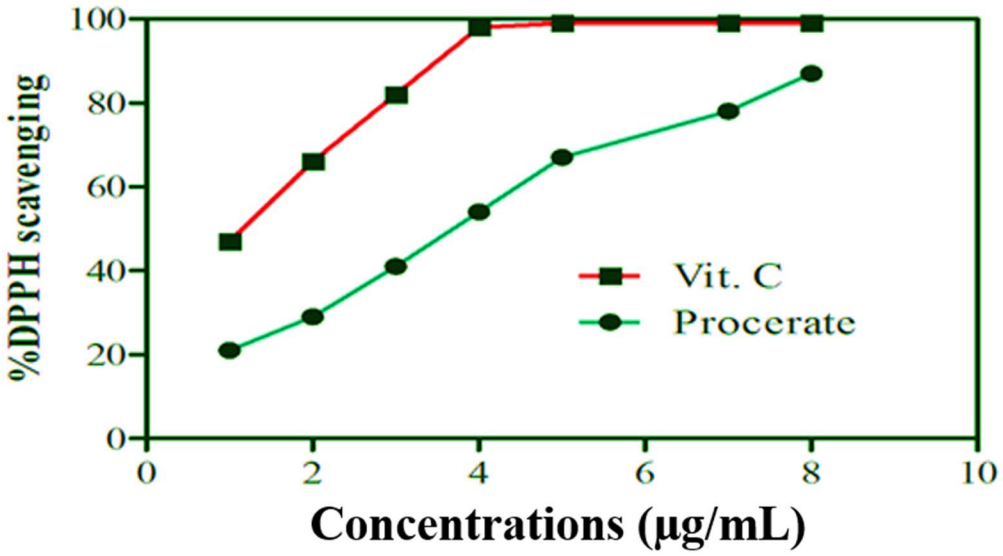

Figure: 4 DPPH scavenging activity of **Procerate 1**

3.3. Antibacterial activity

The most important human bacterial pathogen, *E. coli*, was used to investigate the antibacterial efficacy of the three isolated natural compounds, 1-3. The results obtained were compared with the Ciprofloxacin antibiotic standard. The three isolated compounds have inhibition zones of 19 ± (0.6) mm, 14± (0.1) mm, and 12 ± (0.5) mm respectively as given in Table 2. The results showed that compound 1 is less effective than ciprofloxacin but has a substantially greater inhibition against *E. coli* than compound 2 and 3. The efficacy of compound **1** against *E. coli* is much higher than the other two compounds, which may be due to its complex structure with more OH groups. The aforementioned standard drug has an inhibition zone of 23 ± (0.7) mm against *E. coli*. Figure 5(CD) illustrates the scanning electron microscopy analysis of the damage to *E. coli* in the presence

of compound 1. The outcome makes it quite evident that *E. coli's* morphology was considerably damaged. Most bacteria have shrunk and their cell membranes were entirely disrupted.

Table 2. Antibacterial activity of the isolated natural products 1, 2, 3 and Ciprofloxacin

Bacteria	*Antibacterial activity (Inhibition zone in mm)*			
	Compound 1	***Compound 2***	***Compound 3***	***Ciprofloxacin***
E. coli	***19 (±0.6)***	***14 (±0.1)***	***12 (±0.5)***	***23 ±0.7)***

3.3.1. Minimum inhibitory concentrations (MIC) of compounds 1, 2, 3 and Ciprofloxacin

The MIC is the lowest concentration of a compound that prevents bacterial growth. In this test, the MIC of compounds 1-3 and ciprofloxacin was investigated. All of the compounds were tested against *E. coli* at varying concentration (10–100 µg/mL). Ciprofloxacin exhibited the lowest MIC value against bacteria among all of tested compounds. Compound 1 showed the best MIC value against E. coli among all of the compounds (1-3). The MIC values for all the three compounds against *E. coli* were found 40, 55, and 60 µg/mL respectively. Table 3 summarizes the MICs for the ciprofloxacin standard and compounds 1-3.

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

Compound	**MIC (µg/mL)**
Compound 1	40
Compound 2	55
Compound 3	60
Ciprofloxacin (standard)	30

3.3.2. Mechanism of bacterial inhibition and examination of reactive O-species (ROS)

Reactive oxygen species production and the antibacterial actions of compound 1 in the bacteria can be associated by considering the mechanistic aspects of bacterial inhibition. Reactive O-species such as $\bullet O_2^-$, •OH, and H_2O_2 are released as a result of energetic OH groups in compound 1, which increases the ROS production in the *E. coli* cell. Such reactive species are considered to be highly harmful to *E. coli* protein & DNA. Additionally, it is shown that the 2, 7-dichlorofluorescein diacetate dye oxidised into its dichlorofluorescin derivatives when exposed to ROS. Figure 5(B) shows the analysis of green fluorescence that was observed when compound 1 was excited at a wavelength of about 488 nm. Figure 5(A) illustrates the lack of fluorescence in the absence of the aforementioned compound 1. The result clearly demonstrated that the ROS was delivered in the bacterial cell within the region of compound 1 which is responsible for their inhibition. The obtained results thus suggest that compound 1 is associated with the surface of *E.*

coli cells, which is consistent with the development of intracellular ROS and leakage of cytoplasm, as seen in Figure 5 (CD).

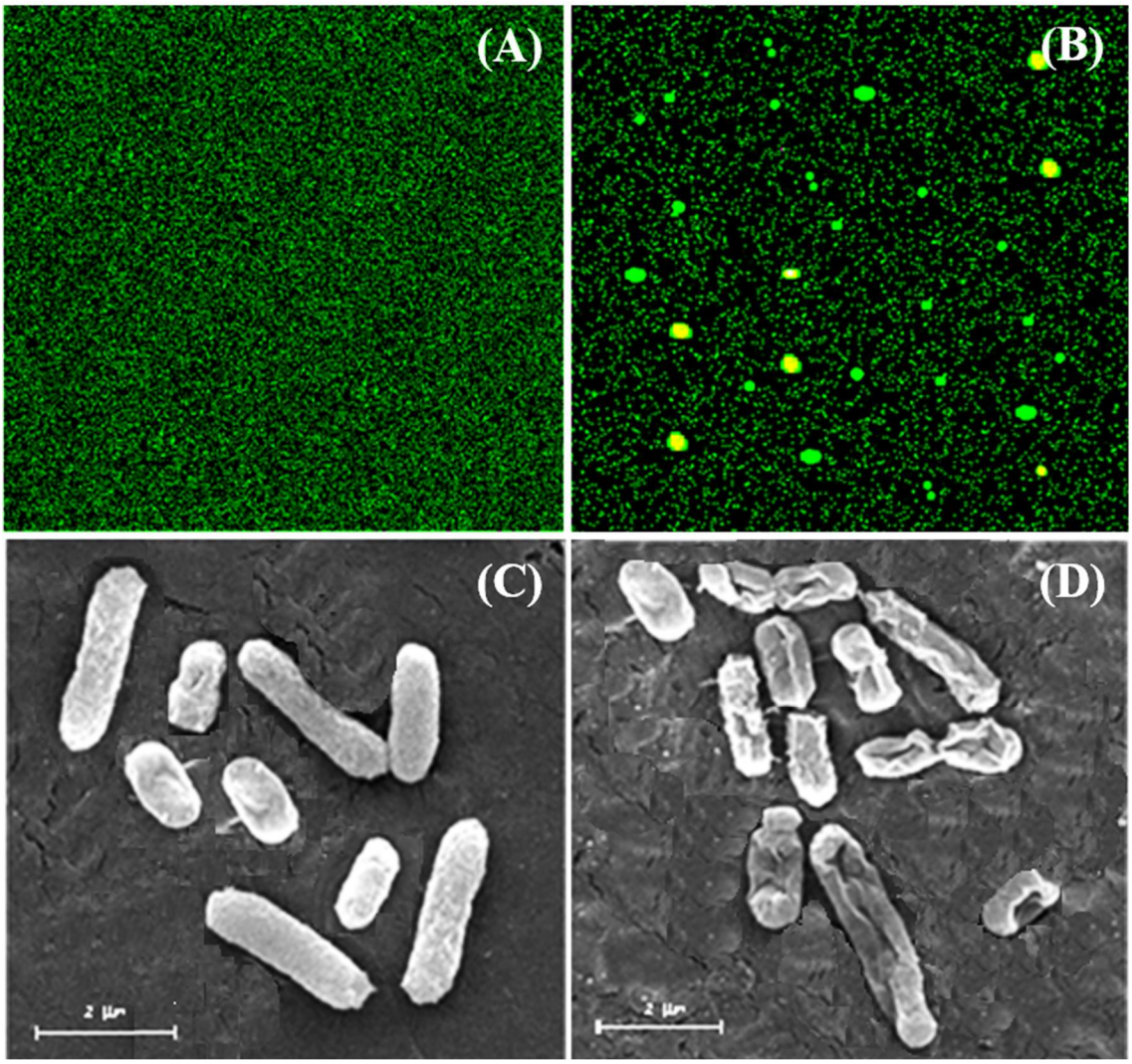

Figure: 5 ROS examination (A) in the absence of compound **1**, (B) in the presence of compound **1** and (CD) Scanning electron microscopy examination of *E. coli* in the absence and presence of compound **1** respectively

4. Conclusion

The present study resulted into isolation of one new glycosidicProcerate**1** along with two reported natural products (E)-octadec-8-enoic acid and (2E,4E)-octadeca-2,4-dienoic acid from ethyl acetate fraction of *Calotopis procera*. Further bioassay evaluation of the obtained products was recognized as significant antioxidants where the new one Procerate**1** exhibited highest potential. The isolated compounds were also showed remarkable antibacterial activities with inhibition zones of 19 ± (0.6) mm, 14± (0.1) mm, 12 ± (0.5) mm and 23 ± (0.7) mm respectively.

Author Contribution: Conceptualization**,** Maria Iqbal; methodology, Maria Iqbal, Dilfaraz Khan and Shafiullah Khan; validation, Maria Iqbal, Shafiullah Khan; writing—original draft preparation, Maria Iqbal, Dilfaraz Khan, and Shafiullah Khan; writing—review and editing, Maria Iqbal, and Hamid Ullah; supervision, Shafiullah Khan; methodology and funding acquisition, All authors have read and agreed to the published version of the manuscript.

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

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