Journal of Animal & Plant Sciences, 33(4): 2023, Page: 971-977
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2023.4.0690

ISOLATION AND STRUCTURAL ELUCIDATION OF PHENOLIC COMPONENTS FROM *CALOTROPIS PROCERA* (AIT) AND EVALUATION OF INSECTICIDAL ACTIVITY

B. Nacira*[1], B. Mebarka[1], L. Yacine[2], H. Mohamed[2], H. Roukia[1] and H. M. Mahfoud[1].

[1]Biogeochemistry Laboratory of Desertic Environments, Kasdi Merbah Ouargla University, Ouargla 30000, Algeria.
[2]Center for Research and Physical-Chemical Analysis (CRAPC), Bou-Ismail, Tipaza, Algeria.
*Corresponding Author's Email: bellaouarnacira@gmail.com

ABSTRACT

The identification and characterization of two phenolic compounds from the ethyl acetate (EtAc) extract of *Calotropis procera* aerial parts were performed using chromatographic and spectroscopic methods namely Thin Layer Chromatography (TLC), Preparative Thin Layer Chromatography (PTLC), High-Performance Liquid Chromatography (HPLC), Infrared spectroscopy (FT-IR) and Nuclear Magnetic Resonance (^{1}H-NMR, ^{13}C-NMR).Two major compounds were isolated to be the 3, 3', 4', 5, 7-pentahydroxy flavones-3-rutinoside (Rutin) and 3,4,5-trihydroxybenzoic acid (Gallic acid), the results were confirmed by comparison with standards. In addition, the insecticidal activity of these isolated compounds was determined against *Parlatoria blanchardi targ* individuals, 71.73% and 59.44% mortality were recorded at 0.5 mg/ml of Rutin and Gallic acid respectively, after 24 h of treatment; whereas, the larval stages were the most sensitive to Rutin treatment (LD_{50} equal to 0.10 mg/ml for 1st larva and 0.18 mg/ml for 2nd larva).

Keywords: *Calotropis procera*, Rutin, Gallic acid, *Parlatoria blanchardi*.

Published first online April 15, 2023 Published final August 04, 2023

INTRODUCTION

Secondary metabolites play an important role in plants defense and protect from UV radiation, infection and atmospheric exposure like temperature (Cheynier, 2012; Tiago *et al*., 2017). During the last decade phenolic compounds have received widespread attention due to their numerous benefits for human health. For example, their direct and indirect antioxidant properties help in the precaution of oxidative stress-related to disorders such as cancer and cardiovascular diseases (Shahidi and Ambigaipalan, 2015; Bhuyan and Basu, 2017). Moreover, since their radical-scavenging properties are verified, polyphenols are associated during the metabolism with anti-carcinogenic and anti-inflammatory effects and other diseases caused by excessive oxidative stress such as atherosclerosis (Selby-Pham *et al*., 2017; Cheng *et al*., 2017)

C. procera (asclepaiadaceae) is one of flowering species widely distributed in West Africa and other parts of the tropics (Irvine, 1961). Previous phytochemical investigations on *C. procera* showed the presence of cardiac glycosides, flavonoids, phenolic compounds, terpenoides, which are well known by their pharmacological and therapeutic effects **(**Mueen Ahmed *et al*., 2005). *C. procera* organs showed variable phenolic amount according to seasonal variation; the highest content was recorded for samples of mature leaf, stem and apical bud, in winter (Akhtar and Malik, 1998). The present paper aimed to identify the phenolic constituents of *C. procera* ethyl acetate fraction since, to the best of our knowledge, no phytochemical investigation had been carried out previously in Algeria, and to evaluate their insecticidal activities towards *Parlatoria blanchardi targ* larvae and adult stages.

MATERIALS AND METHODS

Plant materials: *C. procera* aerial parts (leaves and stems) were collected in October 2019 from Djanet at Tasili N'Ajjer (Southern-East Algerian Sahara); they were washed thoroughly with tap water and shade drying for 40 days, then reduced to powder and stored for further studies. Plant identification was performed by Professor Ammar Eddoud, and insects were identified by Dr Hayet Saggou; both are from the department of agronomic science at the University of Ouargla.

Equipment and Chemicals: TLC plates (60 F254 silica gel plate on aluminum support) were from Merck (Darmstadt, Germany), HPLC (Agilent ChemStation), FTIR-8300 (Shimadzu), NMR (Bruker BioSpin GmbHAvance with TCI cyroprobe operating at 400MHz), Binocular magnifier (KARL KOLAB

Scientific Technical Supplies D-6072 Dreieich. West Germany).

All analytical grade solvents used were from BIOCHEM Chemopharma, except Methanol gradient HPLC grade, LiChrosolv Reag. Ph Eur, Germany.

Authentic standards: Standards were available for comparison: (+)-Rutin trihydrate, 97% was obtained from Alfa Aesar GmbH& Co KG (Germany), and Gallic acid (99%) from BIOCHEM Chemopharma (Quebec).

Extraction: The dried leaves and stems powder (400 g) of *C. procera* was macerated in methanol (3x2 L) at room temperature for 24 hours. The mixture was filtered, then dry concentrated to obtain (10.97 g) crude extract; it was fractionated successively with petroleum ether, chloroform, ethyl acetate, and n-butanol (500 ml ×3) for each. Each of the four organic phases were separately collected, filtered and finally evaporated to dryness under vacuum.

Phytochemical investigation: To detect the presence of phenolics, tests were performed according to the protocol described by (Khandelwal, 1995; Evans and Trease, 2002; Kokate *et al*., 2009; De *et al*., 2010).

Ellagic acid's test for phenols: EtAc fraction was treated with few drops of 5% glacial acetic acid followed by 5% Sodium nitrite solution. Appearance of muddy brown color indicates the presence of phenols.

Shinoda's test for flavonoids: Few drops of concentrate HCl were carefully added to EtAc fraction with some pieces of magnesium, appearance of red color indicates the presence of flavonoids.

Preparations of sample and standards: For TLC and HPLC analysis; EtAc fraction, standards Gallic acid and Rutin were prepared by dissolving 0.01 mg in 1 ml of Methanol (MeOH) HPLC grade and subjecting them to ultrasonication for 5 minutes, to ensure homogeneous mixtures.

TLC analysis: Drops of EtAc fraction and solutions of standards were put on TLC plate's baseline using different systems of solvents to obtain the best separation. Concerning the visualization of separated components, dried TLC plate's evaporated with Neu's reagent, heated at 100°C for 5 min then checked under UV light at 360 and 254 nm.

Isolation of EtAc compounds by PTLC: Using silica gel preparative TLC, two well-separated fluorescent spots were isolated: compound (1) and compound (2). They were scraped out and collected then extracted with MeOH for further experimental (HPLC, FT- IR, NMR) analysis.

HPLC analysis: in order to ensure the purity of the isolated compounds, samples of 20 µl of EtAc fraction, compound (1) and compound (2) were filtered through 0.45 µm filters then injected into analytical HPLC system using the following conditions : mobile phase composed of A: 1% acidified H_2O, C: MeOH; flow C= 5% to 95 % for 55 min then C= 5% for 3 min , the column was C18 (25 cm x 4.6 mm, 5 µm), at max column temperature 60˚C, the effluent was monitored at 254 nm by UV detector with flow rate 1.0 ml/min and P_{max} = 400 bar.

Spectroscopic analysis: KBr discs of compounds 1 and 2 were subjected to FT-IR analysis in the range of scanning 4000-400 cm^{-1} at a resolution of 8 cm^{-1}.^{1}H-NMR and ^{13}C-NMR analysis of both compounds used their solutions in MeOD, and as an internal standard Tetramethylsilane (TMS) was used. The chemical shift values (δ) and the coupling constants (*J*) were reported in ppm and Hz unit respectively.

Bioassays: Leaves from highly infested and non-infested date palm by *Parlatoria blanchardi targ* were collected from Metlili oasis at Ghardaia province- Algeria. Sufficient pieces of palm leaflets equal to 2 cm each were prepared for the treatment; after using a binocular magnifier, the number of living individuals (male, female, 1st and 2nd fixed larva) was determined.

The following five doses from each compound (isolated Rutin and Gallic acid): 0.5, 0.4, 0.3, 0.2, 0.1 mg/ml were tested. In every glass Petri dish was placed 3 pieces of leaflets on soaked cotton to keep them moist. As a negative control; distilled water was used. The biological variables of *P. blanchardi* adults and larvae were daily (24 h), each treatment was replicated 3 times. The LD_{50} values were determined using Excel software by Probit analysis method as described by Cavelier (1976).

RESULTS AND DISCUSSION

Phytochemical screening of *C. procera* aerial parts extract showed the presence of large number of compounds. Therefore, it was fractionated gradually with Petroleum ether, Chloroform, Ethyl acetate and n-Butanol to give the following fractions masses successively, (5.34 g), (3.13 g), (0.50 g), (1 g). Ellagic acid and Shinoda's tests indicated that EtAc fraction presented important quantities of phenol and flavonoids. In order to identify them, the fraction was subjected to further purification. In fact, previous studies reported the presence of phenols in *Calotropis* genus and especially, in the **EtAc** extract of *C. gigantea* root bark and *C. procera* leaves and bark (Mehmood *et al*., 2020; Hasballah *et al*., 2021).

Effectively, analytical TLC of EtAc fraction were monitored under UV light at 366 and 254 nm using Chloroform: Ethyl acetate: Methanol: Formic acid (70: 14: 14: 10 v/v) as a mobile phase, it is considered one of the best solvent system used for phenolic compounds separation particularly flavonoids (Wagner and

Bladt,2008). Using standards, this qualitative result revealed the presence of Rutin (compound 1) and Gallic acid (compound 2). Once the TLC plates sprayed with Neu's reagent and then exposed to UV-Vis light at 366 nm, compound 1 (Rf: 0.18) was intensely orange and compound 2 (Rf: 0.62) was intensely blue). However, at 254 nm compound **(1)** presented an intensely yellow color but compound **(2)** showed a black color; in fact, they were the same colors observed for Gallic acid and Rutin standards, but in less intensity. By PTLC, isolated compounds were obtained in form of colored crystals; compound (1) ones were yellow and those of compound **(2)** were white. To confirm their purity, they were analyzed by HPLC. Figure 1 illustrates EtAc fraction profile which clearly shows that the EtAc part contains two compounds with 7.13 min (90.38%) and 29.72 min (9%) retention time.

As it is showed on Figure 2**,** both TLC and HPLC chromatograms show obviously one major peak with another less concentrated (Figure 2). The first compound refers to Rutin and the second one to Gallic acid that were purified from EtAc fraction of *C. procera* aerial parts. These results are consistent with Patel *et al*., (2014), who showed that *C. procera* has significant phenolics and flavonoids content in leaves, flower and root. The content of each compound could change according to geographical origin and the extraction method (Mehmood *et al*., 2020).

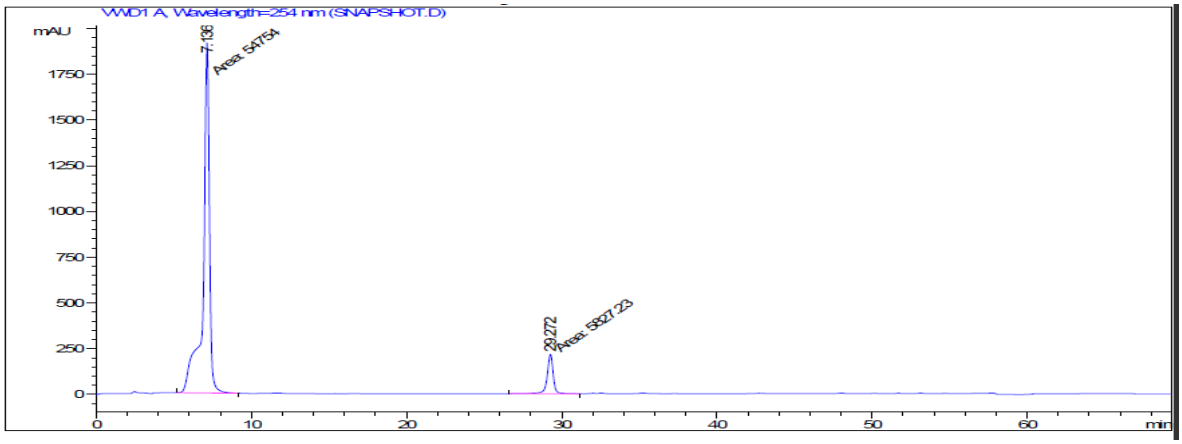

Fig.1. HPLC chromatogram of EtAc fraction at λ=254 nm.

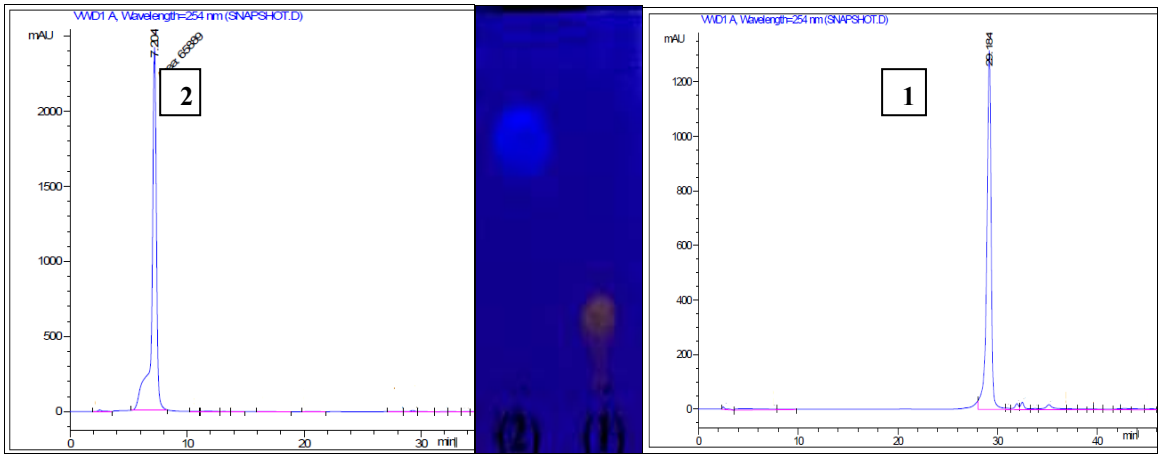

Fig.2. TLC and HPLC chromatograms of isolated compounds (1) and (2) at λ=254 nm.

First of all, the isolated compounds were analyzed by IR (KBr). IR spectrum of compound (1) displayed the following vibrations V*max* (cm^{-1}): 3409 and 3321(O-H stretching), 2935.5 (aromatic CH_2), 2661.6 and 2720 (aromatic CH bonding), 1631.7 (C=O), 1596.9 (C=C Vibration of aromatic groups), 1462 (C=O), 1384.8 (C-OH), 1114.8 (C-O).

Regarding the IR (KBr) data recorded for compound (2) it show the presence of a band at 3232.5 cm^{-1} indicating hydroxyl groups (O-H), at 2426 cm^{-1} for (CH) bond, 1631.7 cm^{-1} for carbonyl groups (C=O), The absorption bands 1608.5, 1446.5, 1384.8, 1238.2, 1029.9 cm^{-1} respectively indicating the presence of benzene cycle attached with three –O-aryls directly, 717.5 cm^{-1} confirmed substituted benzene. However, for a further

investigation of isolated compounds structures, that should be identified using ^{1}H-NMR, ^{13}C-NMR process on the basis of their NMR data as well as by comparison with literature.

In ^{1}H-NMR (400 MHz in MeOD, ppm) spectrum of isolated compound (1) all resonances of hydroxylic aromatic groups OH (C-4'), OH (C-3'), OH (C-7) and OH (C-5) were visible from 8 to 12.5 ppm; aromatic CH moieties (C-6', C-5', C-2', C-6 and C-8) appeared from 6 to 8 ppm; the aliphatic hydroxylic groups of the sugar units could be observed from 4 to 6 ppm, rutinose CH signals were detected from 3 to 4 ppm in particular, positions H-(C-6''), H-(C1''), H-(C-1''') were well-separated as double signals appearing at 5.23, 3.93, and 1.26 ppm. The assignment of the resonances was performed according to the literature (Jeon *et al*., 2014; Pivec *et al*., 2019).

Whereas, in ^{13}C-NMR (MeOD, 400 MHz, δ ppm) spectrum was present the following signals 18.74 (C-6'''), 69.40 (C-6''), 70.56 (C-5'''), 72.25 (C-2'''), 72.96 (C-3'''), 73.09 (C- 4''), 74.78 (C-4'''), 76.58 (C-2''), 78.08 (C-5''), 79.04(C-3''), 95.71 (C-8), 100.79 (C-6), 103.27 (C-1'''), 105.57 (C1''), 106.48 (C-10), 116.90 (C-2'), 118.54(C-5'), 123.79 (C-6'), 124.40 (C-1'), 136.48 (C-3), 146.70 (C-3'), 150.67 (C-4'), 159.37 (C-9), 160.19 (C-2), 163.85 (C-5),166.90 (C-7), 180.28 (C-4). ^{13}C-NMR data of isolated compound (1) in comparison with ^{13}C-NMR of Rutin obtained from the references (Olennikov and Partilkhaev, 2012; Selvaraj *et al*., 2013) was confirmed it as 3, 3', 4', 5, 7-pentahydroxy flavones-3-rutinoside (Rutin $C_{27}H_{30}O_{16}$), illustrates in Figure 3.

As for the elucidation of compound (2); ^{1}H-NMR and ^{13}C-NMR spectrums (400 MHz in MeOD, ppm) were employed, where its ^{1}H-NMR spectrum showed the presence of an aromatic singlet at δH 7.08 (2H, s), at δH 4.89 (3H, s) a broad singlet confirmed the presence of three OH-aryls. Table 1 illustrates the obtained data (compound 2) compared with those of Gallic acid isolated from chloroform fraction of syzygium litorale stem bark (Tukiran *et al*., 2016). It was characterized as 3, 4, 5-trihydroxybenzoic acid (Gallic acid). Figure 4 illustrates its structure.

Table.1. ^{1}H- NMR and ^{13}C-NMR spectral data of isolated Gallic acid compared with reference Gallic acid

Position	Compound (2)		Gallic acid (Tukiran *et al*,2016)	
	δC (400 MHz)	δH (400 MHz)	δC (400 MHz)	δH (600 MHz)
2(6)	111. 16	7.08(s)	110.37	7.06 (s)
1	122.80		122.04	
4	140.43		139.63	
3(5)	147.23		146.44	
7	171.24		170.45	

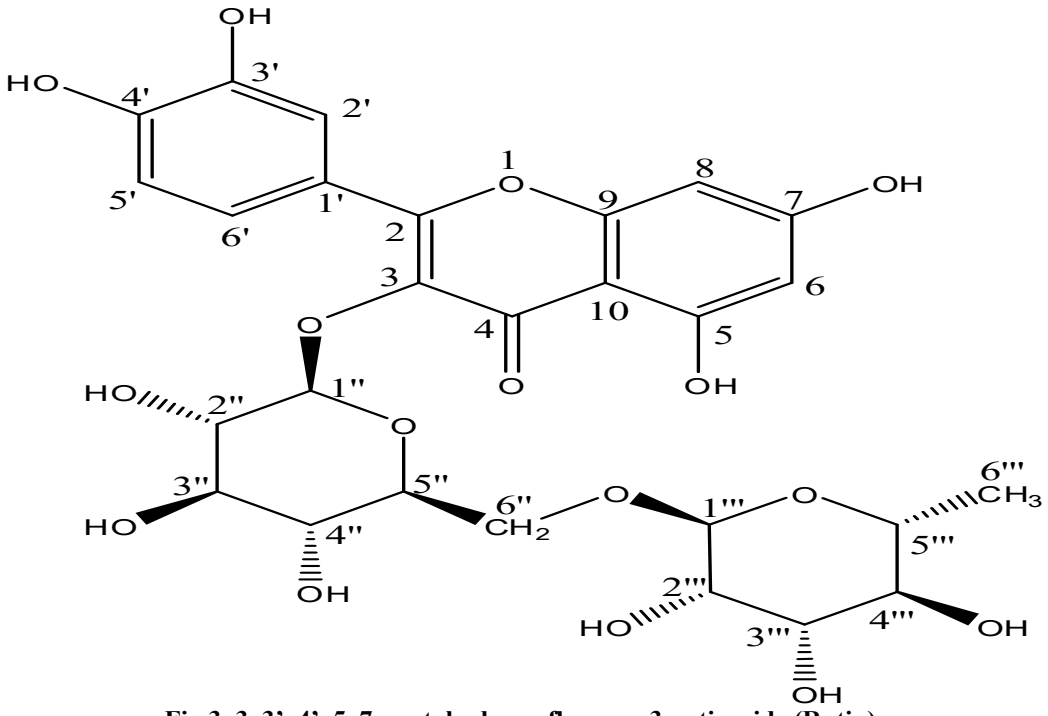

Fig.3. 3, 3', 4', 5, 7-pentahydroxy flavones-3-rutinoside (Rutin).

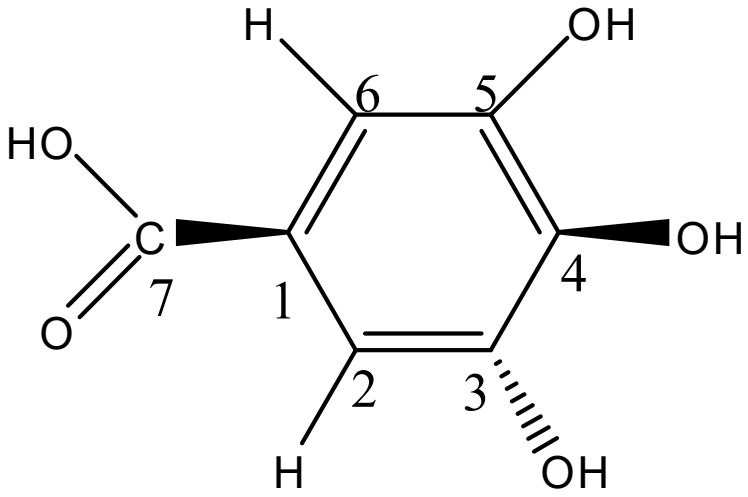

Fig.4. 3,4,5-trihydroxybenzoic acid structure (Gallic acid).

The present study provides a detailed report on the isolation and structure elucidation of 3, 3', 4', 5, 7-pentahydroxy flavones-3-rutinoside (Rutin) and 3,4,5-trihydroxybenzoic acid (Gallic acid) from ethyl acetate fraction of *C. procera* aerial parts from Algeria. These results were consistent with those found by Oraibi and Hamad (2018), who separated and purified Rutin, Quercetin and Kaempferol from *C. procera* ethyl acetate extract in Iraq. Furthermore, the comparison study conducted by Gholamshahi *et al*., (2014), in Jiroft and Bam regions of Kerman, Iran, which analyzed total phenols in *C. procera* leaves and latex extracts, showed that Gallic acid content were high in leaves extracts with 9.72 and 9.02 mg Gallic acid/g dry weight (in Bam and Jiroft plants, respectively).

Besides, the insecticidal study has evaluated five treatments containing: 0.5, 0.4, 0.3, 0.2, 0.1mg/ml of isolated Rutin and Gallic acid, as Figure 5 present.

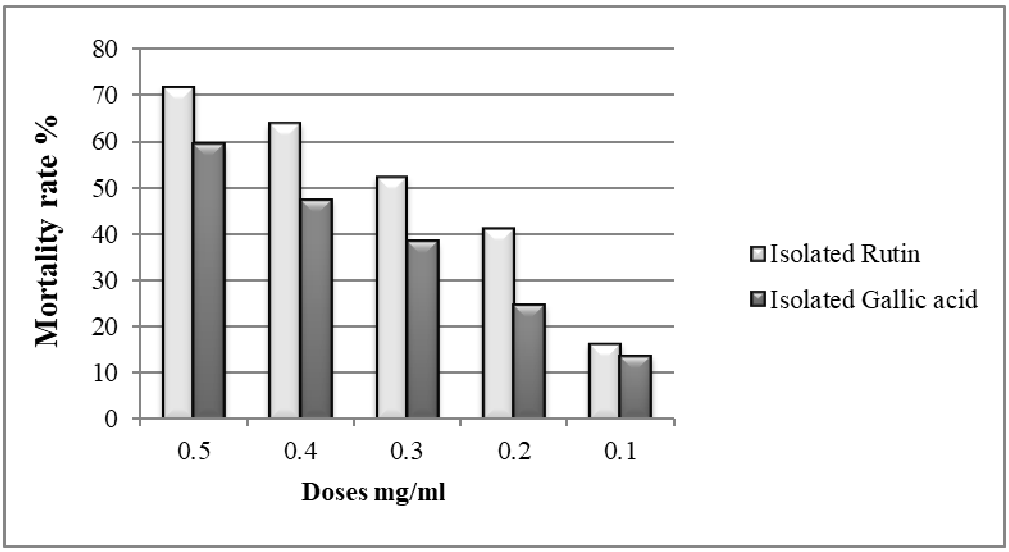

Fig.5. The toxicity effect of isolated Rutin, Gallic acid against *P. blanchardi*.

Effectively, the mortalities at 5 mg/ml vary between 71.73% and 13.59%; isolated Rutin provided the highest mortality rate on *P. blanchardi* stages evaluated to 71.73± 3.13% while 59.44 ± 3.82% was recorded for Gallic acid at the same concentration; the lowest percentages (16.46 ± 1.38%) and (13.59 ± 0.89%) were presented by Rutin and Gallic acid respectively at 0.1 mg/ml. Probably, oxygen radicals formed from phenolic compounds oxidation were the cause of the compounds toxicity; in fact, these radicals disrupt the ability of membrane integrity and the metabolism in the gut epithelium, precipitating proteins from feeding deterrence, digestion inhibition, digestive enzymes (Appel,1993). In particular, even at low doses, flavonoids can be feeding deterrents for phytophagous insects (Batista Pereira *et al*., 2002). Overall, an acceptable

toxicity effect was recorded for isolated rutin and gallic acid on *P. blanchardi* stages. Nevertheless, it is interesting to note that isolated rutin was more toxic than gallic acid towards all fixed larval stages; but, larvae were more susceptible than adults.

Regarding the sensitivity of male individuals, a similar effect was recorded for treatment with Rutin and Gallic acid with an LD50 equal to 0.33 mg/ml and 0.36 mg/ml respectively. Whereas the female stages were more resistant to treatment with gallic acid than with rutin with an LD50 equal to 0.5 mg/ml (Figure 6).

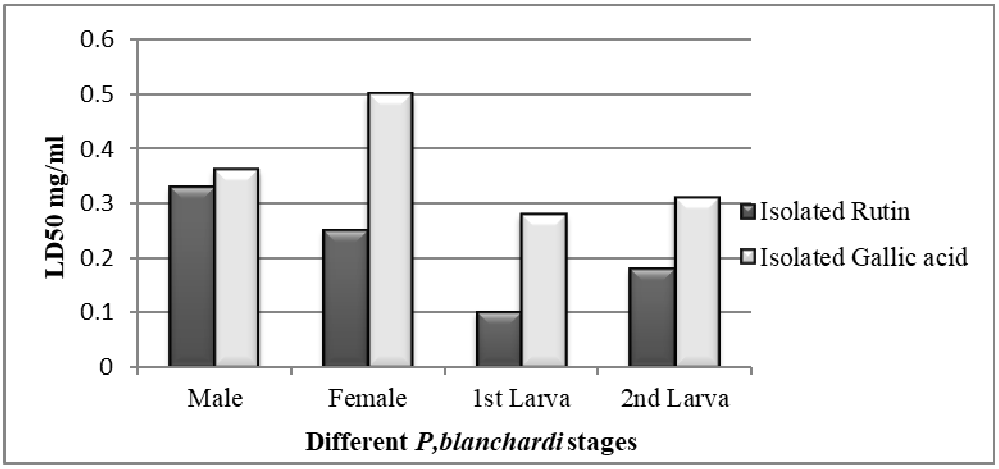

Fig.6. LD_{50} of isolated Rutin, Gallic acid against *P. blanchardi.*

The study carried out by Piubelli *et al* (2006) to evaluate the biological and physiological activity of rutin on populations of A. gemmatalis proved that Rutin have deleterious effects which reduced food intake by A. gemmatalis population, whereas, the larvae from the resistant population were more negatively influenced by Rutin even at low concentration of Rutin (0.65%) to the insect diet. The obtained results have demonstrated that ethyl acetate fraction of *C. procera* aerial parts and its flavonoids content had a potent insecticidal and larvicidal effect towards *P. blanchardi* adults and larvae, these selective compounds could be beneficial as naturally insecticidal and larvicidal compounds.

Conclusion: This work represents a detailed report on the phenolic composition of the ethyl acetate fraction of the aerial parts of *C. procera* from Algeria, as well as on its insecticidal potential. In fact, the analysis of the data provided by TLC, PTLC, HPLC, FT-IR, and NMR allowed the identification of two phenolic compounds, namely, 3, 3', 4', 5, 7-pentahydroxyflavones-3 -rutinoside (Rutin) and 3,4,5-trihydroxybenzoic acid (Gallic acid).While, the bioactivity study demonstrated the variable efficiency of these two phenolics against *P. blanchardi* stages.

Acknowledgment: The authors are grateful to the Algerian ministry of high education and scientific research. Special thanks to Pr. B. Hamdi-Aissa, the director of "BioGeochemistry of desertic environments" laboratory at the University of Ouargla, Algeria and all its members for the valuable guidance and support during the research work.

Conflict of Interest: The authors of this paper declare that there is no conflict of interest.

REFERENCES

Akhtar, N. and A. Malik (1992). Proceragenin, an antibacterial cardenolide from *Calotropis procera*. Phytochemistry. 31(8): 2821-2824. https://doi.org/10.1016/0031-9422(92)83639-G.

Appel, H.M. (1993). Phenolics in ecological interactions: the importance of oxidation. J. Chem. Ecol.19 (7):1521-1552. https://doi:10.1007/bf00984895

Batista Pereira, L. G., F. Petacci, J.B. Fernandes, A.G. Corrêa, P.C. Vieira, M.F.G.F. da Silva, and O. Malaspina (2002). Biological activity of astilbin from Dimorphandra mollis against Anticarsia gemmatalis and Spodoptera frugiperda. Pest. Manag Sci. 58(5):503-507. https://doi.org/10.1002/ps.478

Bhuyan, D.J. and A. Basu (2017). Utilisation of bioactive compounds derived from waste in the food industry. In Utilisation of Bioactive Compounds from Agricultural and Food Production Waste; Vuong, Q.V., Ed.; CRC Press: Boca Ratón, FL, USA.342–357 pp. https://doi.org/10.1201/9781315151540

Cavelier, A. (1976). Cours phytopharmacie. Ed. Inst.Nat.Agro.El-Harrach (Algeria) T.1,514 p.

Cheng, Y. C., J.M. Sheen, W.L. Hu and Y.C. Hung (2017). Polyphenols and oxidative stress in atherosclerosis-related ischemic heart disease

and stroke. Oxid. Med. Cell. Longev. 2017. https://doi.org/10.1155/2017/8526438

Cheynier, V. (2012). Phenolic compounds: from plants to foods. Phytochem Rev. 11(2): 153-177. https://doi.org/10.1007/s11101-012-9242-8

De, S., Y.N. Dey and A.K. Ghosh (2010). Phytochemical investigation and Chromatographic evaluation of the different extracts of tuber of *Amorphaphallus paeoniifolius* (Araceae). Int. J. Pharm & Biomedical Res. 1(5):150-157.

Evans, W.C and G.E. Trease (2002). pharmacognosy. W.B. Saunders, China. 193-407 pp.

Gholamshahi, S., M. A. Vakili, F. Shahdadi and A. Salehi, (2014). Comparison of total phenols and antiradical activity of flower, leaf, fruit and latex extracts of milkweed (Calotropis procera) from Jiroft and Bam cities. Int. J. Biosci.4 (7):159-164. http://dx.doi.org/10.12692/ijb/4.7.159-164

Hasballah, K., M. Sarong, R. Rusly, H. Fitria, D.R. Maida and M. Iqhrammullah (2021). Antiproliferative activity of triterpenoid and steroid compounds from ethyl acetate extract of Calotropis gigantea root bark against P388 murine leukemia cell lines. Sci. Pharm. 89(2): 21. https://doi.org/10.3390/scipharm89020021

Irvine, F.R. (1961). Woody Plants of Ghana. Oxford University Press; London. 48 – 50 pp.

Jeon, J.K., J. Lee and J.Y. Imm (2014). Effects of laccase-catalyzed rutin polymer fraction on adipogenesis inhibition in 3T3-L1 adipocytes Process. Biochem. 49(7): 1189–1195. https://doi.org/10.1016/j.procbio.2014.04.005

Khandelwal, K.R. (1995). Practical Pharmacognosy. Nirali Prakashan. 149-155pp.

Kokate, C.K., A.P. Purohit and S.B. Gokhale (2009). Pharmacognosy. Nirali Prakashan. 6.16-6.17 pp.

Mehmood, T., H. Arshad, S. Nawaz, A. Ullah, A. Hafeez, F.Anwar and M. Iqbal (2020). Pharmaceutical potential and phenolics profiling of leaves and bark of Calotropis procera in relation to extraction solvents. Pharm. Chem. J. 54(6):631-641. https://doi.org/10.1007/s11094-020-02250-7.

Mueen A., K.K., A.C. Rana and V.K. Dixit (2005). Calotropis species (Ascelpediaceae) a comprehensive review. Pharmacognosy Magazine 1 (2): 48–52.

Olennikov, D and V. Partilkhaev (2012). Isolation and densitometric HPTLC analysis of rutin, narcissin, nicotiflorin, and isoquercitrin in Caragana spinosa shoots. JPC-J. Planar. Chromat-Modern TLC. 25(1): 30-35. https://doi.org/10.1556/jpc.25.2012.1.5

Oraibi, A.I. and M.N. Hamad (2018). Phytochemical investigation of flavanoid of Calotropis Procera in Iraq, Isolation and identification of rutin, quercitin and kampferol. J. Pharm. Sci. Res. 10(9):2407-2411.

Patel, H. V., J. D Patel and B. Patel (2014). Comparative efficacy of phytochemical analysis and antioxidant activity of methanolic extract of Calotropis gigantea and Calotropis procera. Int. J. Life. Sci. Biotechnol. Pharm. Res. 5(2): 107-13.

Piubelli, G. C., C. B. Hoffmann-Campo, F. Moscardi, S. H. Miyakubo and M. C. N. De Oliveira (2006). Baculovirus-resistant Anticarsia gemmatalis responds differently to dietary rutin. Entomol.Exp.Appl.119 (1):53-60. https://doi.org/10.1111/j.1570-7458.2006.00396.x

Pivec, T., R. Kargl, U. Maver, M. Bračič, T. Elschner, E. Žagar, L. Gradišnik, and S. Kleinschek (2019). Chemical structure–antioxidant activity relationship of water–based enzymatic polymerized Rutin and its wound healing potential. Polymers. 11(10):1566. https://doi.org/ 10.3390/ polym11101566

Selby-Pham, S.N., J.J. Cottrell, F.R. Dunshea, K. Ng, L.E. Bennett and K.S. Howell (2017). Dietary phytochemicals promote health by enhancing antioxidant defence in a pig model. Nutrients.9(7) : 758. https://doi.org/ 10.3390/nu9070758

Selvaraj, K., R. Chowdhury and C. Bhattacharjee (2013). Isolation and structural elucidation of flavonoids from aquatic fern Azolla microphylla and evaluation of free radical scavenging activity. Int. J. Pharm. Pharm. Sci. 5(3): 743-749.

Shahidi, F. and P. Ambigaipalan (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects-A review. J. Funct. Foods.18: 820–897. https://doi.org/10.1016/j.jff.2015.06.018

Tiago, O., N. Maicon, R.C. Ivan, N.F. Diego, J.S. Vincius, F. Mauricio and Q. D. S. Velci (2017). Plant secondary metabolites and its dynamical systems of induction in response to environmental factors: A review. Afr. J. Agric.Res.12 (2):71-84. https://doi.org/10.5897/AJAR2016.11677

Tukiran, T., F. Mahmudah, N. Hidayati and K. Shimizu (2016). Gallic acid: A phenolic acid and its antioxidant activity from stem bark of chloroform extracts of syzygium litorale (blume) amshoff (myrtaceae). Molekul.11(2):180-189. http://dx.doi.org/10.20884/1.jm.2016.11.2.215

Wagner, H. and Bladt, S. (2008). Plant Drug Analysis, London, New York.