Original article

First report of triterpenes pathway in *Calotropis procera* revealed to accumulate beta-amyrin

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A R T I C L E   I N F O

A B S T R A C T

The major reports on *Calotropis procera* (*C. procera*) indicated the importance of this plant as a resource of pharmaceutically active ingredients as well as its medical advantages. β-amyrin (BA) is a significant substance in this plant and has a pharmacological effects in some frameworks, like focal and fringe sensory system, digestive and immune systems. In this study, the impact of sunlight before and after irrigation on the BA production in *C. procera* is studied its pathway with involved eight key enzymes. The eight enzymes' genes were characterized and successfully submitted to NCBI; *AAS* (acc.no. KU997645) for α-amyrin synthase, *BAS* (acc.no. MW976955) for β-amyrin synthase, *SE* (acc.no. MW976956) for squalene epoxidase, *SS* (acc.no. MW976957) for squalene synthase, GPPS, (acc.no. MW976958) for geranyl pyrophosphate synthase, *FPPS* (acc.no. MW976959) for farnasyl pyrophosphate synthase, *CAS1*, (acc.no. MZ00598) for cycloartenol synthase1 and *LS* (acc.no. MZ005982) for lupeol synthase. qRT-PCR analysis revealed high expression levels of *GPPS, FPPS, SS, SE*, and *BAS* genes at all times specially midday. Otherwise, *CAS1, LS* and *BAS* expression levels were very low at all daylight periods. The UPLC β-amyrin data are in accordance with qRT-PCR results. This indicates that triterpenes biosynthetic pathway in *C. procera* is going to β-amyrin accumulation with the highest level at midday.

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1. Introduction

Triterpenes are a type of organic chemicals that composed of three terpene units with C30H48 as molecular formula. Animals, plants and fungi are all producers for triterpenes, including Amyrins (produced only by plants). Amyrins are the precursor of ursolic and oleanolic acids (Saimaru et al., 2007; Babalola and Shode, 2013).

Amyrins are closely related natural chemical compounds that classified into α-amyrin as ursane skeleton (Davis and Croteau, 2000), β-amyrin as oleanane skeleton (Tansakul et al., 2006) and δ-amyrin, all of them are C30H50O pentacyclic triterpenols.

A variety of plants have all the mentioned amyrins including *Solanum lycopersicum* (Yasumoto et al., 2017), *Protium heptaphyllum* (Da Silva et al., 2019), *C. procera* (Mali et al., 2019) and *Myrcianthes pungens* (Karen Cardoso et al., 2020)

Since its biological activities, α and β-amyrins have provoked clinical interest (Sheng and Sun, 2011). They are well-known as natural mixture of isomeric triterpenes exhibiting long lasting antinociceptive, gastroprotective (Oliveira et al., 2004), inflammation reduction via the activation of CB1 and CB2 cannabinoid receptors and inhibition of pro-inflammatory cytokines (Oliveira et al., 2004; Bandeira et al., 2007; Holanda et al., 2008), antitumor (Barros et al., 2011), anxiolytic (Aragão et al., 2006) and hepatoprotective (Oliveira et al., 2004).

In Saudi Arabia, the Apocynaceae plant *Calotropis procera* is known as Ashar. Wound-healing, antihyperglycemic, painkiller, anti-fever, Cholinesterase–blocking, laxative, and anti-cancer qualities are all thought to be present in its bark and leaves. The phytochemical analysis of the plant revealed the presence of anthocyanins, β-sitosterol, cardenolides, cardiac triterpenoid,

flavonoids, flavanols, triterpenoids, lupeol, mudarine, resins, α-amyrin and β-amyrin, proteolytic enzymes like calotropin, and calactin (Mali et al., 2019; Ramadan et al., 2019). Triterpenoid pathway may be going to accumulate different types of secondary metabolites like β-amyrin, lupeol, etc. (Mishra et al., 2016). Thus, the current study aimed to figure out triterpenoid pathway, especially β-amyrin, in C. procera and its relationship with environmental daylight periods before and after irrigation.

2. Materials and methods

2.1. Plant material and environmental experment

The current experiment was carried out in a desert land of KSA, in Latitude 21°20′3.68 and Longitude 40°12′4.04. Three equal sized C. procera plants were carefully chosen for irrigating (25 L) and then leaf sampling (Ramadan et al., 2014). After water irrigation, plant samples used for metabolomic and RNA extraction were collected at dawn (iD), midday (iM), pre-dusk (iPD). Before irrigation, samples were collected one day before irrigating at the same assumed times {dawn (D), midday (M), pre-dusk (PD)}. All the taken samples stored at −80 °C.

Table 1
Primer sequences for the various genes.

Transcript	f. Primer	R. Primer
GPPS	5′TCTCCGGGAGGAGTGGAAAT3′	5′TCCTCCGGCAAGAAGAGAGT3′
FPPS	5′ TATCACCGATCTGCGAACCG3′	5′TCAATGACCGAGAGACCCCT3′
SS	5′ TTGGAGCTTGGCAGCAGTTA3′	5′AACCCCAATCCCACAAGTCC3′
SE,	5′ GTTCCACAGCTCCCAACTGA3′	5′TGGCGCATGTTGAAAGCATC3′
CAS1,	5′ GGGCTATGCTGGCTCTCATT 3′	5′ TCCTAGAGCCCAAATGGGGA3′
LS,	5′ GCCTGCGGTAAAACCTACCA3′	5′AAGCCCACGATGTCTGTACC3′
BAS	5′ TGGTGATCTCCTCTGGCGTA3′	5′GGAAAAAGAGAGGTCCGGCA3′
AAS	5′ ACCGATTCGACTGGCTGATG3′	5′ ACGCAATTAGCAGTGGTGGT3′
Actin	5′GCACACTGGTGTCATGGTTG3′	5′CCTCAGGAGCAACACGAAGT3′

Fig. 1. Triterpenoids biosynthetic pathway. Low and no regulated enzymes in red color, high and regulated enzymes in blue color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Triterpenoid extraction

70 °C was used to dry plant leaves until they reach a fixed weight. 0.1 g of dried plant leaves was grinded in Eppendorf tube using 1 ml of 80% methanol (HPLC grade, Fisher) and incubate with shaking at room temperature for overnight. Under cooling 4 °C centrifuge was used at 8000 rpm for 7 min. After that, supernatants were filtered using a 0.2-m syringe filter. Finally, 5 µl of filtrate were injected into chromatography machine.

2.3. LC-MS analysis

UPLC/MS/MS strategy was performed by Ramadan et al. (2019) who usedan Agilent 1290 Infinity LC framework furnished with an Agilent 6460 Series Triple Quadrupole UPLC framework (Agilent Technologies, California, USA), utilizing a Zorbax Eclipse in addition to C18 Rapid Resolution section (2.1 cm × 50mmID × 1.8 µm). Portable stages made of 0.1 rate formic corrosive/water (dissolvable A) and 0.1 rate formic/acetonitrile (dissolvable B). program conditions were 0.5 ml/min stream rate, the portable stage was angle as following: close to 100% An at min 1, direct inclination from close to 100% A to 65% at min 13, straight slope from 65% A to 30% An at min 14.5, straight angle from 30% A to 1% at min 15.5, held at 1% An until min 17, all-out time was 17.5 min for direct slope from 1% A to almost 100% A, and 4.5 min to re-equilibrate segment. Nitrogen was utilized as the drying gas, with a stream pace of 650 L each hour. Disintegration temperature of 350 °C and a fracture particle set at 135 V, the electrospray ionization source possibilities were set at a narrow voltage of 3.5KV and a cone voltage of 30 V. Using Nitrogen as impact gas, crash particle energy was optimized at 25 eV and location was in a positive way. The forerunner and daughter particles of BA were distinguished in the Multi Reaction Monitoring mode after mass filtering in the scope of 100–1000 to recognize all triterpenoids present in the control test.

2.4. Next generation sequencing

Publicly available RNA-seq data of C. procera paired-end (acc.no. SRR1554320) were used and de novo assembly was performed using trinityrna-seq r20131110 (Haas et al., 2013). Then, using a mix of alternative k-mer lengths and predicted coverage, putative unique transcripts are generated.

2.5. Phylogenetic relationships and NCBI submissions

The bootstrap analysis was performed using the CLC program, which constructed a dendrogram using the maximum likelihood method. To designate the confidence level, the bootstrap esteem is appended to each branch. The resulted sequences were submitted to NCBI with following accession numbers: α-amyrin synthase (AAS, acc.no. KU997645), β-amyrin synthase (BAS, acc.no. MW976955), squalene epoxidase (SE, acc.no. MW976956), squalene synthase (SS, acc.no. MW976957), geranyl Pyrophosphate synthase (GPPS, acc.no. MW976958), farnasyl pyrophosphate synthase (FPPS, acc.no. MW976959), cycloartenol synthase (CAS1, acc.no. MZ005981), lupeol synthase (LS, acc.no. MZ005982).

2.6. Total RNA isolation and qRT-PCR

Total RNA was isolated from C. procera leaves by GeneJET Kit (Thermo Scientific, cat. no. K0801). To remove DNA, 3 µl of DNase (Thermo Scientific™ cat. no. EN0531) was added to samples, and incubated for 15 min at 30 °C. The concentration of the RNA was measured using NanoDrop™ 2000 (Thermo Fisher Scientific, cat. no. ND-2000) at optical density o 260 nm.

The relative expression (RE) of C. procera GPPS, FPPS, SS, SE, CAS1, LS, AAS and BAS genes was detected using RNA samples extracted from leaves. Reverse Transcriptase (Thermo Scientific™ catalogue number: EP0451) was used to make cDNA from 2 µg total RNA with oligo dT as a primer for each sample. The primers sequences for all mentioned genes in addition to actin as reference
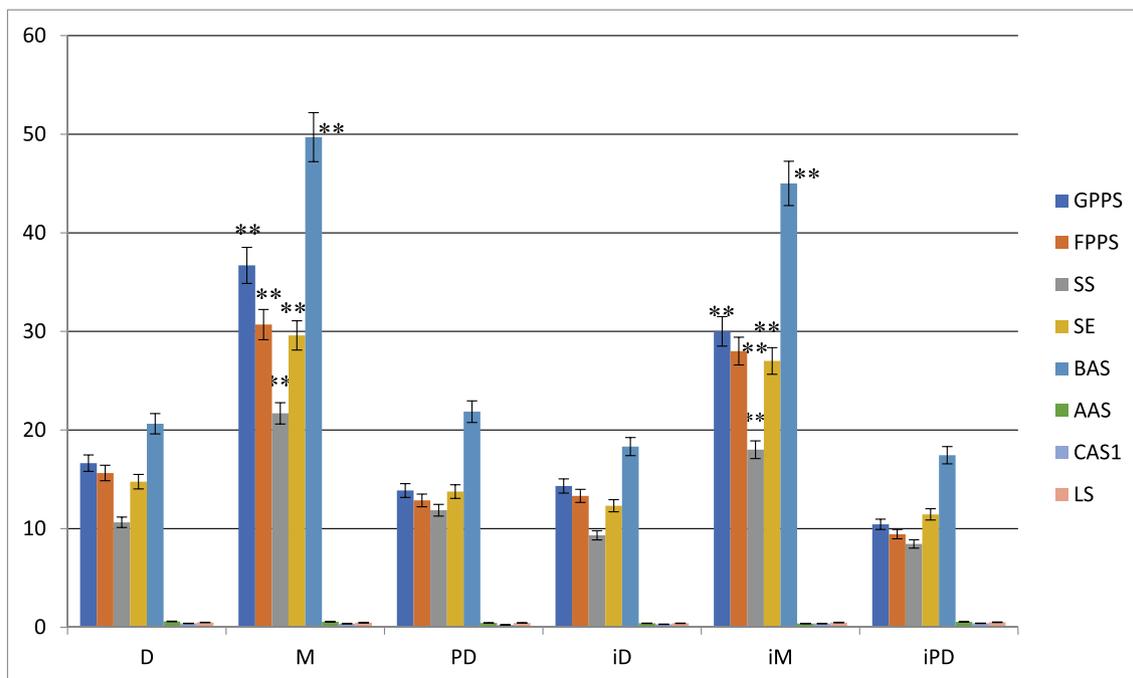
Fig. 2. The relative expression of C. procera GPPS, FPPS, SS, SE, BAS, AAS, CAS1 and LS genes before and after irrigation under various light periods (D, iD, M, iM, PD and iPD). Data are stated as means ± SD (black bars) for three different replicates. **Indicate for the significant difference between different treatments P < 0.01.

gene are designed by Primer-BLAST tool and shown in Table 1. In 25 µl, qRT-PCR analysis was performed in triplicates with 1 µl of cDNA, 500 nM of each primer and 12.5 µl of SYBR® Green qPCR (Sigma-Alderch cat. No. KCQS02), and. The reactions were carried out on a Stratagene Mx3005PQPCR System with one cycle at 94 °C for 4 min; 38 cycles at 94 °C for 30 s, at 60 °C for 60 s, at 72 °C for 20 s; and at 72 °C (infinite) as the last cycle. Melting curve analysis was used to examine the PCR products. As a reference gene, the levels of actin expression were measured. The response take-off points (cycle number) are compared using this software. To determine the RE for each gene, the ΔCT for each sample was calculated using the equation $\Delta CT = CT$ target gene $- CT$ reference gene.

2.7. Statistical analysis

Using the SPSS program, data were analyzed by the analysis of variance test (ANOVA). Several comparisons were conducted using Tukey's HSD (Tukey 1949).

3. Results

3.1. Gene characterization

C. procera paired-end RNA-seq short sequence reads were analyzed and eight genes involved in triterpenoid pathway were successfully identified (Fig. 1) and submitted to NCBI. To execute multi-sequence alignment, the best alignment hits were selected (Figs. S1–S9; Tables S1–S7). These genes are; *AAS* (acc.no. KU997645) for α-amyrin synthase, *BAS* (acc.no. MW976955) for β-amyrin synthase, *SE* (acc.no. MW976956) for squalene epoxi-

dase, *SS* (acc.no. MW976957) for squalene synthase, GPPS, (acc. no. MW976958) for geranyl pyrophosphate synthase, *FPPS* (acc. no. MW976959) for farnasyl pyrophosphate synthase, *CAS1*, (acc. no. MZ00598) for cycloartenol synthase1 and *LS* (acc.no. MZ005982) for lupeol synthase.

3.2. Gene expression profile

To see how the expression profile of identified transcripts in the triterpenoid biosynthetic pathway changed according to light and water availability, the abundance of *GPPS*, *FPPS*, *SS*, *SE*, CAS1, LS, *BAS*, and *AAS* transcripts was evaluated using qRT-PCR (Fig. 2). In both dehydrated and irrigated plants, the expression levels of *GPPS*,
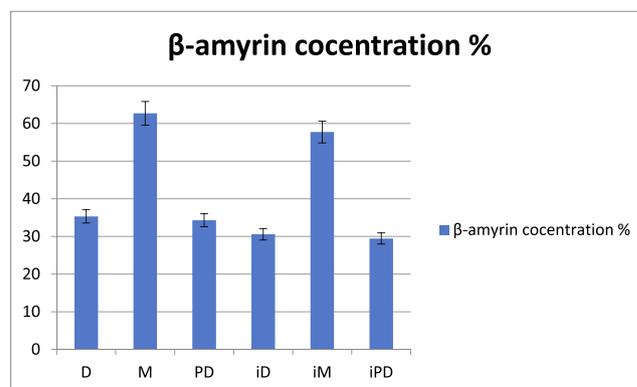
Fig. 4. The β-amyrin as percentages from the total glycosides before and after irrigation under various light periods (D, iD, M, iM, PD and iPD).
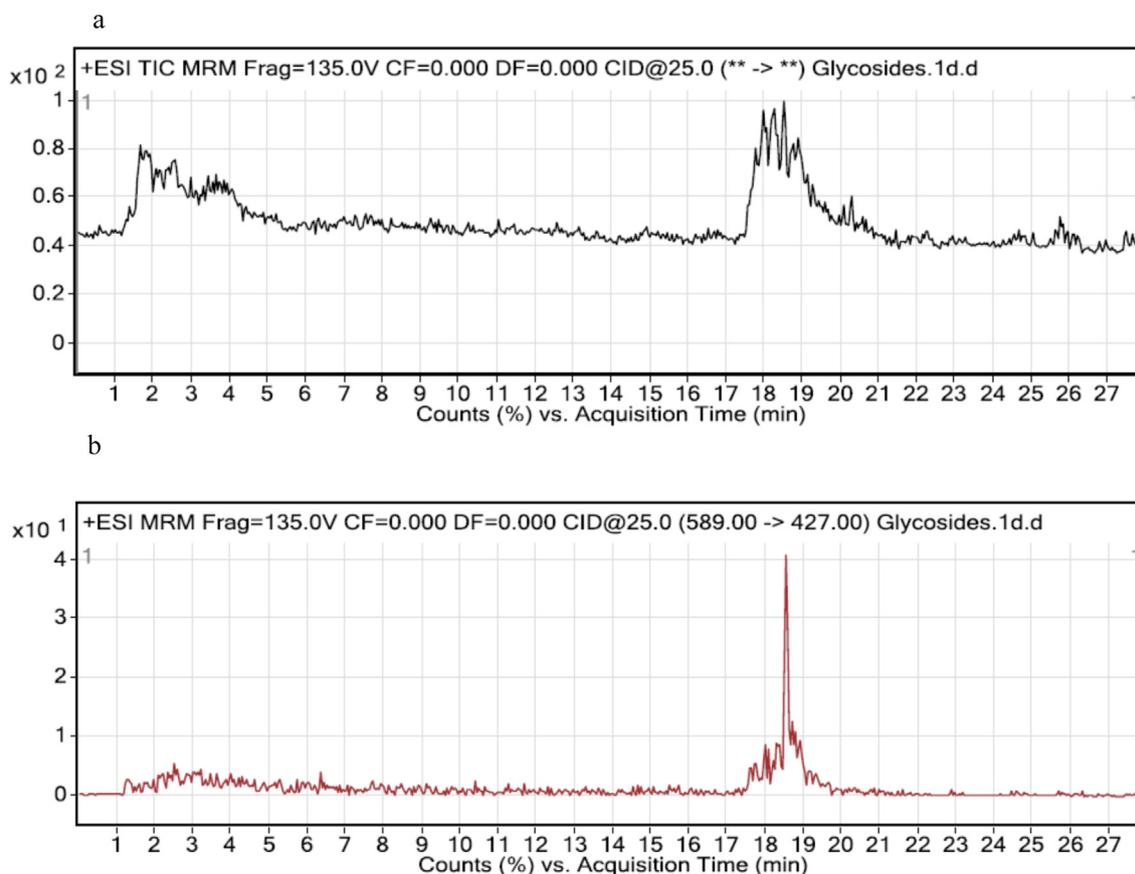
Fig. 3. The triterpenoid total ion chromatogram (TIC) evaluated by the scan mode of LC/MS/MS (a) represents two groups triterpenoid and MRM (b) represents alpha amyrin (Rt. 18.6, precursor 589, daughter ion 427).

FPPS, SS, SE and *BAS* found to be high across daylight periods, but it is significantly increased at midday. However, the expression levels were decreased considerably at dawn and pre-dusk after irrigation. On the other hands, the expression levels of *CAS1, AAM* and LS were extremely low across all daylight periods (Fig. 1).

3.3. LC/MS/MS analysis

Chromatography data revealed two groups of triterpenoids (Fig. 3). BA was plainly apparent at Rt (retention time) 18.6 min. BA buildup was extremely high before irrigation (M) and increased
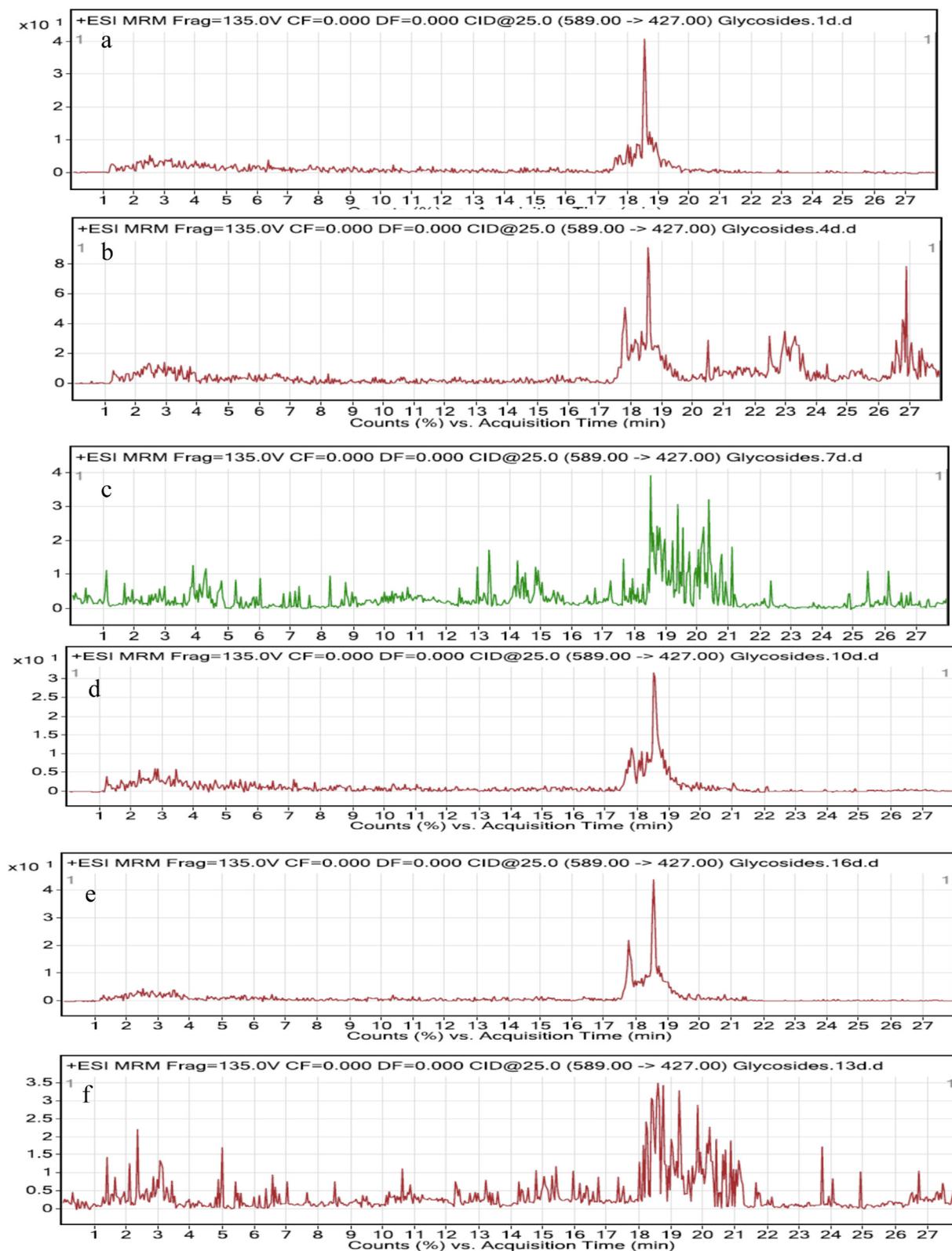
Fig. 5. The α-amyrene (Rt. 18.6, precursor 589, daughter ion 427) before irrigation at Dawn (a), Midday (b), pre-dusk (c) and after irrigation at Dawn (d), Midday (e) and Pre-dusk (f).

marginally after irrigation (iM) (Figs. 4 and 5). The expression level of the *BAS* gene is primarily linked to the accumulation of BA (Fig. 1).

4. Discussion

Many secondary metabolites pathways were reported in *Calotropis procera* like indole alkaloids and steroids pathways (Ramadan et al., 2019; Shaker et al., 2010). Triterpene's biosynthesis is a very important pathway to produce a lot of natural pharmaceutical compounds like β-amyrin, lupeol and withanolides (Mishra et al., 2016). All products of this pathway are not necessary to be found in same plant, but every plant can be marked by one or more of these products such as *Withania somnifera,* which marked by withanoloids (Sangwan et al., 2013). In the triterpenes biosynthesis pathways, the activity of *BAM*, *LS*, *LAS* (lanosterol synthase), AAS and CAS enzymes detect the end product of this pathway (Madina et al., 2007). These enzymes are competing in 2–3 oxidosqualene (Fig. 1), so the highly accumulated enzyme will define the destination of these pathway. In this study, β-amyrin found to be the end product of this pathway in *C. procera* plant leaves.

The Triterpenes pathway is started when isopentenyl pyrophosphate is accumulated in the cell as a product of mevalonate in cytosol and MEP (2-C-methyl-D-erythritol 4-phosphate) in plastid pathways (Mishra et al., 2016). The core enzymes of this pathway are GPPS, FPPS, SS and SE to accumulate 2,3-oxidosqualene. After 2, 3-oxidosqualene accumulation, the pathway forward to accumulate two groups of secondary metabolites (sterols and non-sterols) depend on the activity of the previously mentioned five enzymes (BAM, LS, LAS, AAS and CAS) (Sawai and Saito, 2011). In the current study, high expression of triterpenes pathway core enzymes (GPPS, FPPS, SS and SE) in *Calotropis procera* at all daylight period times was observed. These enzymes revealed significantly high activities at midday compared to other daylight periods (Fig. 2), meaning that the triterpenes pathway is induced in *C. procera*. The critical point here is, to what product the pathway will go due to intense competition of on the produced substrate (2, 3-oxidosqualene) from the core enzymes. So, we characterized and tested *BAM, LS, LAS, AAS* and *CAS* genes. Actually, we failed to characterize *LAS* either on DNA or RNA levels, which revealed the absence of lanosterol synthase activity of this plant and the competition would be between the other four genes. We successfully detected the expression levels of the other four genes; *AAS* (Alpha amyrin synthase, acc.no. KU997645), *BAS* (beta amyrin synthase, acc.no. MW976955), *CAS1* (cycloartenol synthase acc.no. MZ005981), *LS* (lupeol synthase acc.no. MZ005982) using qRT-PCR. The expression level of *BAM* gene was significantly high at all daylight periods, with the highest level at midday (Fig. 2). Otherwise, the other three genes revealed very low expression levels at all daylight periods. This indicates that the pathway is on the way to accumulate β-amyrin. The UPLC data indicate that β-amyrin is accumulated at all daylight periods, but it found to be significantly high at midday (Figs. 3, 4 and 5). Otherwise, the irrigation is negatively affected α-amyrin accumulation at midday (Figs. 2, 3, 4 and 5). Although our result indicated that the triterpenes pathway is induced in *C. procera* leaves to accumulate β-amyrin at different daylight periods (either with or without irrigation), but we thought a lot of further investigations to know whether if there are other conditions may redirect the triterpenes pathway to accumulate another secondary metabolite or not?

5. Conclusion

In *C. procera*, characterization of eight genes (acc.no. KU997645 for α-amyrin synthase, acc.no. MW976955 for β-amyrin synthase, acc.no. MW976956 for squalene epoxidase, acc.no. MW976957 for

squalene synthase, acc.no. MW976958 for geranyl pyrophosphate synthase, acc.no. MW976959 for farnasyl pyrophosphate synthase and acc.no. MZ00598) involved in triterpenoid pathway are helped to rich knowledge about this pathway. The triterpenoid pathway is induced in *C. procera* leaves under different daylight periods with significantly high at midday before and after irrigation and low in other light times. Irrigation is kindly negatively affected in β-amyrine accumulation. Identify the highly accumulation time of β-amyrine will help pharmacists to isolate this substance with high concentration from *C. procera*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2022.02.055.

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