Molecular Biology Reports
https://doi.org/10.1007/s11033-019-05218-3

ORIGINAL ARTICLE

Lawsone, a 2-hydroxy-1,4-naphthoquinone from *Lawsonia inermis* (henna), produces mitochondrial dysfunctions and triggers mitophagy in *Saccharomyces cerevisiae*

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Received: 6 August 2019 / Revised: 28 November 2019 / Accepted: 30 November 2019
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Abstract

Lawsone is a natural naphthoquinone present in the henna leaf extract with several cytotoxic activities and used as precursor for synthesis of various pharmaceutical compounds. Its biological activities are thought to be the result of oxidative stress generated, although the hydroxy group at position C-2 in its structure tends to reduce its electrophilic potential. In view of lack of knowledge on its activity, the present work aimed to elucidate the biological effect of lawsone using the yeast *Saccharomyces cerevisiae*. In the model strain BY4741 it was defined 229 mmol/L as the minimal inhibitory concentration (MIC). Using 172 mmol/L as sub-MIC value it was observed that *yap1* deletion mutant was sensitive to lawsone independent the presence of oxygen. Lawsone affected yeast growth in glycerol, indicating interference in the respiratory metabolism. Intracellular content of thiol groups did not indicate intensive oxidative stress and the presence of the anti-oxidant *N*-acetylcysteine (NAC) exacerbated lawsone toxicity. By analysing the sensitivity of *atg* mutant strains and the localization of GFP-Atg8 fusion protein, it was concluded that lawsone primarily produces mitochondrial malfunctioning, leading to indirect oxidative stress. It triggers the autophagic response that ultimately induces mitophagy.

Keywords ATG genes · Henna · Mitochondrial dysfunction · Oxidative stress · Thiols

Introduction

Henna dye is one of the oldest natural dyes in the world (over 5000 years old in ancient Egypt), which is extracted from the henna plant (*Lawsonia inermis*). Its application includes from cosmetics to biomedicine, for treatment of hypoglycemia, hypertension, microbial infections, and as anti-parasite, immunostimulant, anti-inflammatory, analgesic and hepatic protector [40]. This extract is rich in quinones that is a group of molecules in which a benzyl ring contains two carbonyl groups in positions *ortho* or *para*. Their chemical structures enable these molecules to interfere in several biochemical processes regarding redox homeostasis and inhibition of electron transport [24], also including inhibition of DNA topoisomerase [38]. A specific type of quinones include the class of naphthoquinones, characterized by a quinonic groups supported by naphthalene rings, the most common quinone in nature. The biological actions of this class are mainly related to generation of reactive oxygen species (ROS) and to bioalkylations reactions of cellular components, like DNA and proteins [37]. Lawsone, a 2-hydroxy-1,4-naphthoquinone (Fig. 1), is the main active compound present in the henna leaf extract that was considered for biomedical uses due to its high cytotoxic activities against disease-causing bacteria, fungi and protozoa and by its antitumoral activity [40]. However, its nonspecific toxicity induced to a series of studies in which this molecule was used as precursor for the synthesis of various compounds with important biological activities, such as β-lapachone used for anti-cancer treatment [29, 34]. Despite all bioactive

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11033-019-05218-3) contains supplementary material, which is available to authorized users.

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Published online: 06 December 2019

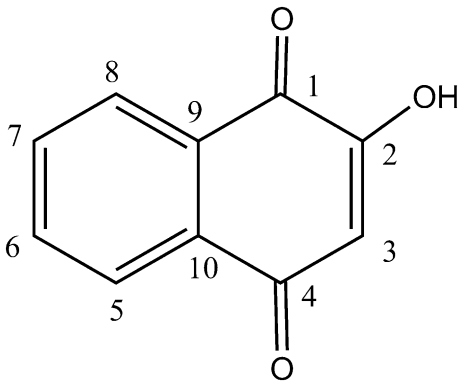

Fig. 1 Molecular structure of lawsone (2-Hydroxy-1,4-naphthoquinone). Numbers indicate carbon position in the molecule

potential reported, there is still not enough evidences of its mechanism of action at molecular level.

Naphthoquinones are thought to work as ROS generators as their main mechanism of biological action. Inside the cells, the redox cycle includes the transference of one electron from cytochrome P450 oxidoreductase to carbonyl group of quinonic ring to produce a semiquinonic radical, which ultimately transfers that electron to oxygen and produces superoxide radical [38]. However, the presence of the hydroxy group at position C-2 of lawsone (Fig. 1) tends to reduce its electrophilic potential and, consequently, affects its capacity to participate in redox cycles for generation of ROS [22]. Given this structure-function paradox, the present study aimed to uncover the major biological activity of lawsone that limits its potential biomedical applications. For this purpose, it was chosen the yeast *Saccharomyces cerevisiae* as biological model due to the vast knowledge on its biochemistry and genetics, and by the facility of cell manipulation. In addition, this organism shares 50% of the genes with homology to human genes, including those in which mutations are related to human diseases [10].

So far, there is only one report on the use of *S. cerevisiae* to study the toxicity lawsone and derivative molecules [1]. In that work, the authors concluded that lawsone was less biologically active than its derivatives. Moreover, at the concentration range tested, lawsone was not as so toxic to cells with deletion in the *YAP1* gene as its derivatives. This gene encodes a protein responsible for commanding the major oxidative stress response in yeast and *yap1* mutant cells are very sensitive to oxidant molecules [23]. This seems consistent with the above-mentioned assumption that the hydroxyl group at C-2 reduces its electrophilic potential. That report supported the assumption that lawsone does not cause oxidative stress, at least directly, which led us to investigate what should be the true mechanism of action of this molecule responsible for its toxicity. The results presented herein were not only helpful for the understanding of its activity but can also serve to guide the pharmaceutical industry in the synthesis of derivatives with more specific activities and controlled mode of action.

Material and methods

Plant material

Samples henna plant (*Lawsonia inermis*−) leaves were collected in the public garden at Mario Melo Avenue, Recife, Pernambuco, Brazil (latitude: − 8.05389022827148 longitude: − 34.8810997009277 err: ±18865 WGS84) and identified by Dr. Rita de Cassia Pereira, herbarium curator of the Agronomic Institute of Pernambuco (IPA). Exsicata of the biological material were deposited in the IPA herbarium under reference IPA30621. Sawdust was prepared from the leaves to fine powder and stored at *ca*. 28 °C protect from light.

Extraction and isolation

Lawsone (2-hydroxy-1,4-naphthoquinone) (Fig. 1) was extracted by soxhlet extractor method in ethanol [30]. Crude sawdust product was subjected to column chromatography in silica gel, eluted with ethanol-dichloromethane (0.2:9.8). Red-orange solid was physically analysed. TLC development was conducted on 0.25 mmol/L silica gel plates (60F254, Merck). Column chromatography was performed using silica gel 60 particle size 0.040–0.063 mm (230–400 mesh, Merck). Melting point was obtained using a BÜCHI-510 capillary apparatus and were uncorrected. IR spectra were measured on a PerkinElmer® (Spectrum 400) spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Varian-400-vnmrs400 spectrometer. High resolution mass spectra were recorded on a micrOTOF-Q apparatus. All chemical shifts were reported in parts per million (δ) downfield from tetramethylsilane as the internal standard. The compound exhibited physical and spectroscopic data consistent with literature values [30]. Spectral analysis and physical-chemical results are available in the supporting material (Fig. S1). These data indicated no contamination in the purified product, showing that red-orange solid consisted in pure preparation of lawsone.

Yeast strains and maintenance

Yeast strains used in this work were described in Table 1. The cells were maintained in YPD solid medium containing yeast extract (10 g/L), peptone (20 g/L), glucose (20 g/L) and bacto-agar (20 g/L). For selection of mutant cells, geneticin G-418 was added to 200 µg/mL. Cultivations used synthetic defined (SD) medium containing YNB (1.7 g/L),

Table 1 List of *Saccharomyces cerevisiae* strains used in this work

Strain	Genotype	Reference
JP1	*MATa/α*	[7]
CEN.PK2	*MAT a/α, ura3-52/ura3-52, leu2-3,112/leu2-3,112, trp1-289/trp1-289, his3D1/his3D1*	[12]
BY4741	*MATa his3Δ leu2Δ met15Δ ura3Δ*	Invitrogen Co.
yap1Δ	BY4741 *yap1*::KanMX	Invitrogen Co.
BY4742	*MATα his3Δ leu2Δ lys2Δ ura3Δ*	D. Klionsky (Univ. Michigan, USA)
atg1Δ	BY4742 *atg1*::KanMX	D. Klionsky (Univ. Michigan, USA)
SEY6210	*MATa leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 lys2-801*	D. Klionsky (Univ. Michigan, USA)
atg32Δ	SEY6210 *atg32*::*LEU2*	D. Klionsky (Univ. Michigan, USA)
atg41Δ	SEY6210 *atg41*::*HIS3*	D. Klionsky (Univ. Michigan, USA)
GFP-Atg8	BY4741 pRS406(*GFP-ATG8)LEU2*	D. Klionsky (Univ. Michigan, USA)

glucose (20 g/L), ammonium sulphate (5 g/L) supplemented with amino acids and nitrogen bases according to the auxotrophy of each strain. Galactose (20 g/L) or glycerol (10 g/L) replaced glucose as carbon source whenever necessary. Seed cultures were prepared by inoculating SD media with the cells and incubating at 30 °C for 24 h under constant agitation of 160 rpm.

Minimal inhibitory concentration (MIC)

MIC for lawsone was determined by adding different concentrations of the agent in the cultivation medium. Seed cells were used to inoculate 1.5 mL of lawsone-containing SD media to initial cell density of 0.1 units of absorbance at 600 nm (A600) in sterile 48-wells flowerplates® and the cultivations were performed in Biolector NA microfermenter device (m2p-Labs, Germany) at 800 rpm (equivalent to 180 rpm in rotatory shaker), 30 °C, 85% humidity and constant sterile air flushing. Automatic measurements of light scattering variation were taken and converted to A600 nm by the off-line defined calibration factor. Growth curves were prepared by plotting A600 vs time and maximal growth rates were calculated as the slope of the most linear portion in the exponential growth phase of the using Microsoft® Excel® 2010 worksheet. Mean values (± SD) were calculated from two biological experiments and three technical replicates each. MIC was defined as the concentration that impaired yeast growth and the sub-MIC concentration (172 mmol/L) was defined as the last concentration before MIC.

Oxidative stress assays

Three experiments were performed to evaluate the potential of lawsone in generating oxidative stress to the yeast cells. The first included the aerobic growth as defined above by comparing the growth of parental strain with its isogenic mutant *yap1*Δ, which is sensitive to oxidative stresses, in the presence of sub-MIC dose of lawsone. Whenever indicated, *N*-acetylcysteine (NAC) dissolved in sterile deionised water was added to the media at 5 mmol/L for testing protective potential against any direct or indirect oxidant products of lawsone. The second experiment was performed by inoculating 150 ∝ L of SD medium containing 172 mmol/L lawsone with seed cells to 0.1 A600 in sterile microtiter plates. The plates were incubated in anaerobic jars in the presence of Anaerocult™ to create an anoxic condition. In parallel, plates were incubated in aerobiosis. Absorbance values were taken at the beginning and after 24 h of incubation at 30 °C in multireader device (BioTek Instruments). Results were expressed as mean of ΔA600 (± SD). The naphthoquinone β-lapachone (247 mmol/L) was used as positive control as generator of oxidative stress as previously defined [38]. The third experiment involved the determination of sulfhydryl groups by 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) reagent in the extract of the yeast cells as reported [7]. Briefly, yeast seed cells in stationary phase of growth were collected by centrifugation and suspended to 0.5 A600 in SD medium containing lawsone at different concentrations and incubated for four hours at 30 °C and 160 rpm. Cells were collected by centrifugation, washed in saline solution (NaCl at 0.85% w/v) and resuspended in the same solution. Cell concentration was determined by direct counting in microscope using Neubauer chamber. A total of 10^9 cells were taken by centrifugation in microtubes at 4 °C, suspended in 400 µl of cold EDTA 20 mmol/L (pH 4.7) solution and mixed with 0.5 mg of glass beads (400–600 nm, Sigma-Aldrich Co.). Cell lysis was performed by six cycles by intercalating vortexing for 30 s with ice-bath incubation for 30 s. The lysates were transferred to new tubes and centrifuged at 14,000 rpm for 20 min. Each cell-free supernatant was collected and split to two tubes. The first tube was used to measure the total content of thiol groups by mixing 100 µl of the lysate with 390 µl of 200 mmol/L Tris–HCl (pH 8.2) and 10 ∝ L of DTNB solution. The mixture was incubated for 30 min in the dark and the absorbance was measured at 412 nm. The second tube was used for protein precipitation by mixing 225 µl of

the lysate with 11.25 µl of trichloracetic acid solution (10% v/v) and incubating on ice-bath for 30 min. The mixtures were centrifuged at 14,000 rpm for 5 min and 400 µl of the protein-free supernatant were mixed with 450 µl of 400 mmol/L Tris–HCl (pH 8.9) and 13 ∝ L of DTNB solution. The mixtures with incubated for five min in the dark and the absorbance was measured at 412 nm. The first tube defined the content of total thiols in the lysates while the second tube defined the amount of non-protein thiols. The difference was referred as the amount of protein thiols in the lysates [7].

Mitophagy induction assays

Induction of mitophagy was tested by two experimental procedures. First, seed cells from mutant strains with deletion in the main genes involved in autophagy induction were prepared and used to inoculate 150 ∝ L SD media containing glucose or galactose as carbon source and lawsone at 172 mmol/L to 0.1 A600 in 96-wells sterile microtiter plates. Growth experiments were performed in Biotek Synerg HT multireader at 30 °C and maximal agitation. Cell concentration was automatically recorded at A600 and growth curves were prepared as described above. All experiments were performed as two biological replicates with three technical replicates each and expressed as mean values (± SD). In the second experiment, induction of autophagic mechanism was monitored by fusion green fluorescent protein (GFP) at N-terminal portion of Atg8 protein in the recombinant BY4741 strain [41]. Cells were prepared as above-mentioned for thiols determination in the presence of 172 mmol/L lawsone, and in the presence or absence of 5 mmol/L NAC. As positive control of mitophagy induction, yeast cells were incubated in the presence of rapamycin at 20 ng/mL [28]. After four hours of incubation, cells were collected, washed twice in saline solution and resuspended in saline to 1.0 A600. Dyeing of DNA-containing structures was done by adding 10 µl of DAPI (4′,6-diamidino-2-phenylindol) solution. The labelled cells were visualized under fluorescence microscope Leica DM 5500B and the images were captured using Leica Las-AF digital camera, and were analysed with the Leica IM500 Image Manager software (Leica, Bensheim, Germany).

Results

Minimal inhibitory concentration of lawsone

Minimal inhibitory concentration was defined by testing the growth of parental yeast cells in synthetic defined (SD) medium in the presence of different concentration of lawsone (Fig. 2a). The results showed just a slight interference in yeast growth up to 115 mmol/L of lawsone, while no cell growth was detected from 230 mmol/L. Proportional reduction in yeast growth was observed from 115 to 230 mmol/L, with the concentration of 172 mmol/L reducing the final biomass by 80% of the reference condition (Fig. 2a). It was made possible to predict the cytostatic effect of lawsone when plotting its concentration against final yeast biomass, refereed as percentage of final growth relative to reference condition (Fig. 2b). In this case, 140 mmol/L was defined as the dosage that limited cell growth to 50% of the reference condition. Therefore, it was defined 172 mmol/L as sub-MIC doses of this compound for BY4741 yeast strain for experiments in which cell growth still necessary, and 230 mmol/L as MIC dose for experiments in which complete inhibitory activity was required. Concentrations above MIC were also used whenever necessary.

It was also tested the effect of lawsone in strains other than BY4741 to discard any influence of the yeast genetic background in its toxicity. To this purpose, the laboratory strain CEN.PK2 and the industrial strain JP1 were cultivated in the presence of two sub-MIC and the MIC doses of lawsone and the final biomass achieved after 24 h of incubation was exactly the same observed for BY4741 (Fig. 2c). Therefore, the natural mutation in *HAP1* gene described for BY4741 [12] had no influence whatsoever in the cellular response to the toxic effects of lawsone.

Lawsone does not cause direct oxidative stress in yeast

First, we tested whether lawsone generates oxidative stress by exposing cells of *yap1*Δ mutant, an isogenic variant of BY4741 with deletion in the *YAP1* gene, to sub-MIC dose of the compound. This gene encodes the major protein that regulates the oxidative stress response in *S. cerevisiae* as well as in other microorganisms [44]. The results showed that parental and mutant strains grew very similar in SD-glucose medium (Fig. 3a), which promotes respiro-fermentative metabolism in this yeast. Lawsone reduced the growth of the parental strain as expected and even more for *yap1*Δ mutant (Fig. 3a). This growth reduction was first consequence of the extension of the lag growth phase (approx. 12 h) of mutant cell population (Fig. 3a), followed by the reduced growth rate to 0.14 h^{-1} in the exponential growth phase when compared to 0.18 h^{-1} calculated for parental strain. However, the final biomass achieved by the mutant strain was practically the same when the cultivation lasted for long time (data not shown). This result indicated that *yap1*Δ mutant cells were capable of growing in the presence of lawsone as soon they adapted to its presence in the medium. Afterwards, yeast cells were cultivated in synthetic medium containing glycerol as carbon source, which induced exclusive respiratory metabolism. Again, the exponential growth of both strains was similar in the absence of lawsone, with the difference

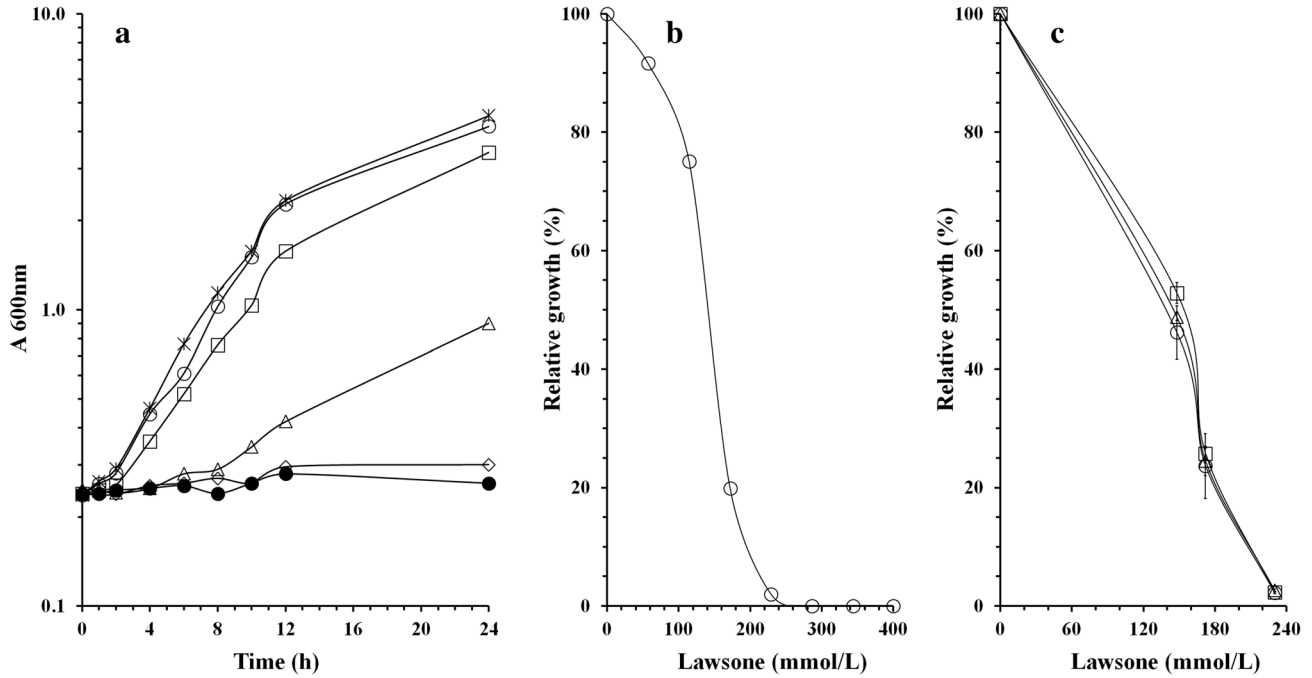

Fig. 2 Growth profile of Saccharomyces cerevisiae in synthetic defined medium containing different concentrations of lawsone. (panel a) Cells of BY4741 laboratory strain were cultivated in the presence of 57 mmol/L (open circle), 115 mmol/L (open square), 172 mmol/L (open triangle), 230 mmol/L (open diamond) and 345 mmol/L (filled circle) of lawsone. Reference condition without lawsone (asterisk) was shown. (panel b) Percentage of the final biomass of BY4741 (open circle) at the end of cultivations in the presence of different lawsone concentrations relative to reference condition. (panel c) Percentage of the final biomass of the laboratory strains BY4741 (open circle) and CEN.PK2 (open square) and the industrial strain JP1 (open triangle) in the presence of different lawsone concentrations relative to reference condition

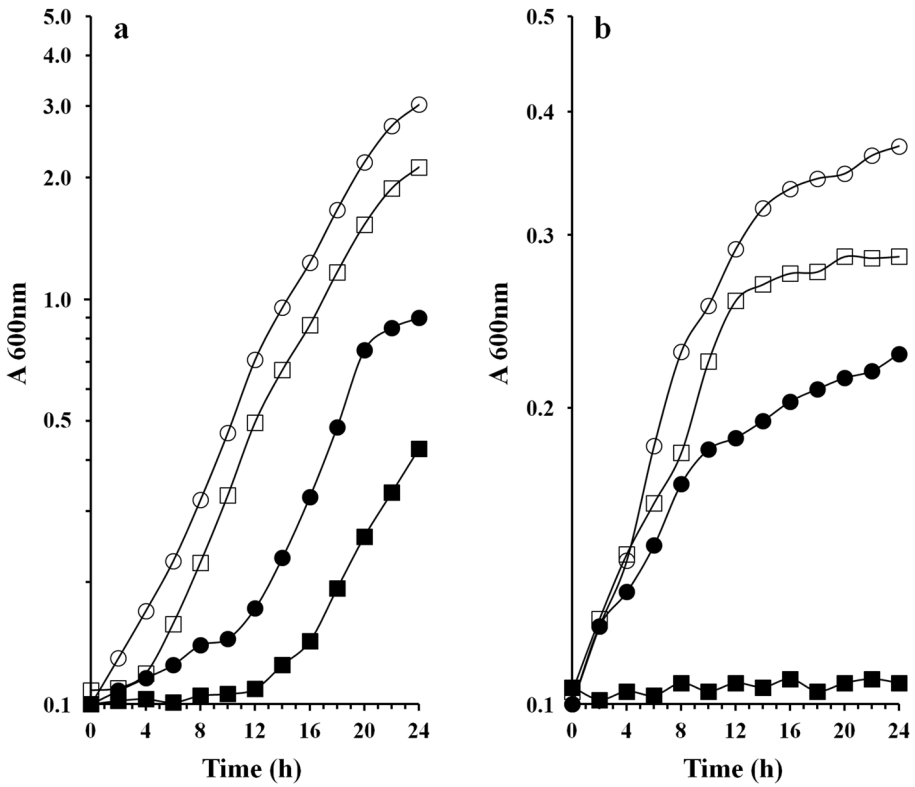

Fig. 3 Growth profile of Saccharomyces cerevisiae BY4741 strain (circle symbols) and its isogenic yap1Δ mutant (square symbols) in synthetic defined medium containing glucose (panel a) or glycerol (panel b) as carbon source in the absence (open symbols) or presence (closed symbols) of lawsone at sub-MIC dose of 172 mmol/L

of earlier entrance of *yap1*Δ mutant to stationary phase (Fig. 3b). This is an expected result by the fact that respiratory metabolism induced by glycerol lead to accumulation of ROS for which this mutant is very sensitive. Interestingly, lawsone induced different growth profile of parental strain according to the carbon source: (i) in glucose there was an initial period of slow growth followed by exponential growth and early entrance to stationary phase (Fig. 3a); (ii) in glycerol that slow growth period was observed later after the exponential phase (Fig. 3b). It can be supposed that glucose somehow repress a mechanism of cell adaptation to lawsone, which is promptly released in glycerol. Moreover, the growth deacceleration in glycerol indicated the induction of increasing level of biological damages caused by lawsone (Fig. 3b), which might be the same cause of the early entry of parental cells to the stationary phase in glucose (Fig. 3a). These physiological results in the presence of lawsone do not seem to fit the canonical pattern of oxidative stress generation. On the other hand, no growth of *yap1*Δ mutant was observed when respiring cells were incubated in the presence of lawsone (Fig. 3b). This was an indicative that lawsone could affect mitochondrial functions.

The intracellular content of sulfhydryl groups was measured in the yeast cells exposed to lawsone. These molecules, that includes glutathione as non-protein free-thiols and thioredoxins as protein-thiols, are involved in the detoxification of ROS though their -SH groups [15]. First, we compared untreated parental and mutant strains. The results showed that *yap1*Δ mutant already has 27% less free-thiols than parental yeast cells (Fig. 4a), expected by the recruitment of oxidative defence response during aerobic growth in glucose due to the absence of Yap1-dependent pathway [45]. However, it was observed an increasing by 45% in the protein-thiols in this mutant relative to its parental (Fig. 4a), which is the result of a putative compensatory defence mechanism by the reduction in free-thiols [6]. Regarding treatment with lawsone, no difference was observed in relation to untreated cells of both

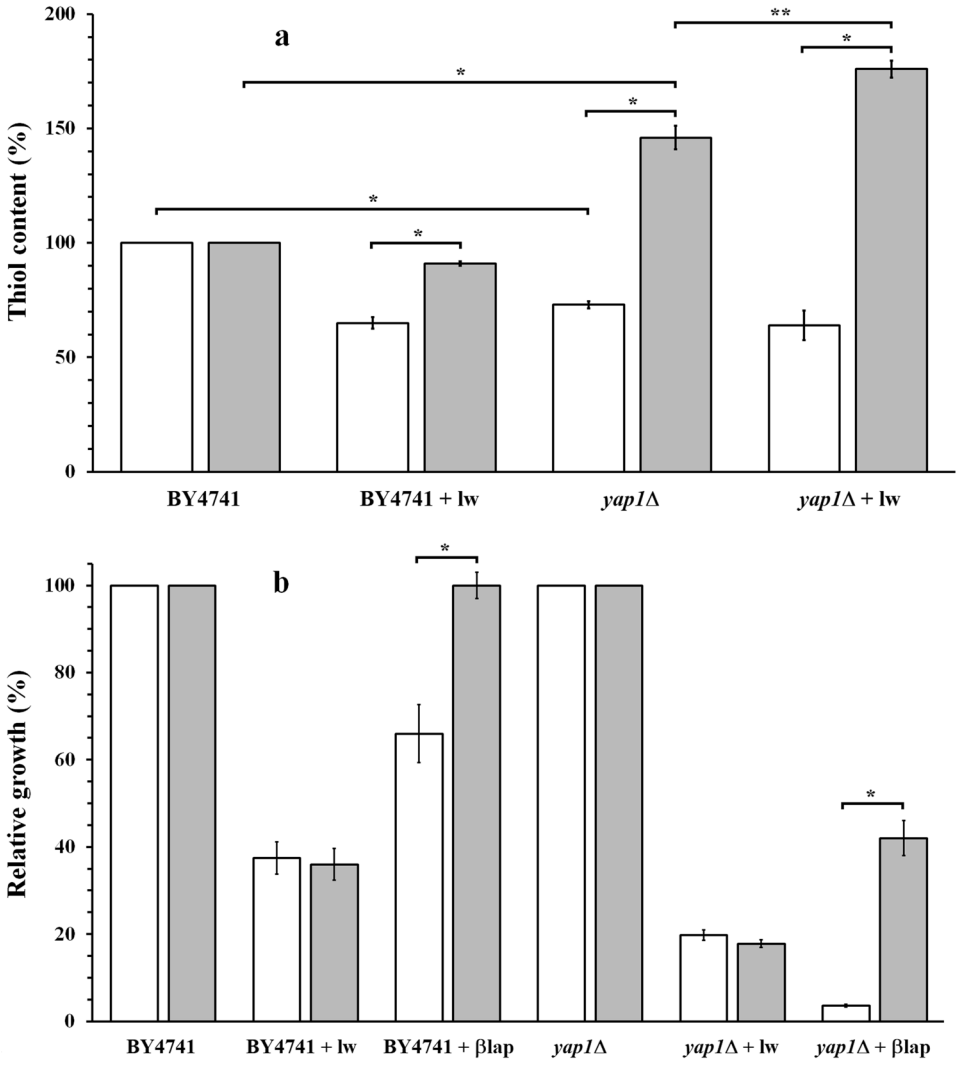

Fig. 4 Analysis of the potential of lawsone to produce oxidative stress by mean of intracellular sulfhydryl content and oxygen-dependent growth in Saccharomyces cerevisiae BY4741 and its isogenic yap1Δ mutant. (panel a) Content of free-thiols (white columns) and protein-thiols (grey columns) in the mid-exponential phase cells in the absence or presence of lawsone (lw) at 574 mmol/L. Levels were adjusted by the reference condition BY4741 without treatment (100%). (panel b) Relative growth in synthetic defined medium containing glucose in aerobiosis (white columns) or anaerobiosis (grey columns) in the presence of lawsone (172 mmol/L) or β-lapachone (247 mmol/L). Levels were adjusted by the reference condition for each strain without treatment (100%). Asterisks represent significant differences at $p < 0.01$(*) or at $p < 0.05$ (**)

strains in doses ≥ MIC (data not shown). It corroborated the initial assumption that lawsone did not cause direct oxidative stress. It was necessary to go far to 574 mmol/L in order to detect any mobilisation of thiol groups. In this case, free-thiol in parental cells was reduced by 35% in relation of untreated cells while no further significatively reduction was detected in mutant cells (Fig. 4a). Protein-thiol content of parental cells was not changed, while it increased by 20% above untreated mutant cells (Fig. 4a). Hence, in the absence of Yap1p the yeast cells increase the content of sulfhydryl-containing proteins in response to unrepaired dysfunctional mitochondria. In view of this assumption, the influence of oxygen on the toxicity of lawsone was evaluated by comparing cell growth in glucose in aerobiosis and anaerobiosis. The results on relative growth (final biomass of treated cells relative to untreated cells) showed that the presence of oxygen did not change the sensibility of both parental and mutant strains to lawsone at sub-MIC dose (Fig. 4b), indicating that any ROS was produced by lawsone. As reference, we used β-lapachone as example of ROS-generating agent. In this case, growth of the parental strains was completed restored while the growth of *yap1*Δ mutant was substantially increased by the absence of oxygen (Fig. 4b).

The potential of lawsone to generate oxidative was ultimately tested by adding the anti-oxidant agent *N*-acetylcysteine (NAC) in SD glucose medium. Reference conditions without NAC were repeated showing the toxic effect of the sub-MIC dose of lawsone in the parental cells, reducing but not impairing cell growth (Fig. 5a). Addition of NAC slightly increased the growth of parental cells as expected due to the capacity of NAC to quench ROS produced during aerobic growth (Fig. 5a). On the other hand, the presence of this anti-oxidant severely potentiated the toxic effect of lawsone by turning the dosage of 172 mmol/L from sub-MIC to MIC status in BY4741 parental strain (Fig. 5a). One hypothesis could be related to the possible chemical reaction between NAC and lawsone producing a more toxic adduct. It was reported a spontaneous reaction between NAC through its -SH group and one of 46 degradation products of glucose called 3,4-dideoxyglucosone-3-eno (3,4-DGE) to form a NAC-DGE adduct [27]. However, the presence of a hydroxyl group in C-2 position of lawsone would difficult such reaction. In order to reject this possibility, we performed several in vitro reactions mix lawsone and NAC and analysed the putative products. Only the individual substrates were recovered from in vitro reactions, as attested by the same analytical procedures described in Material and Methods used

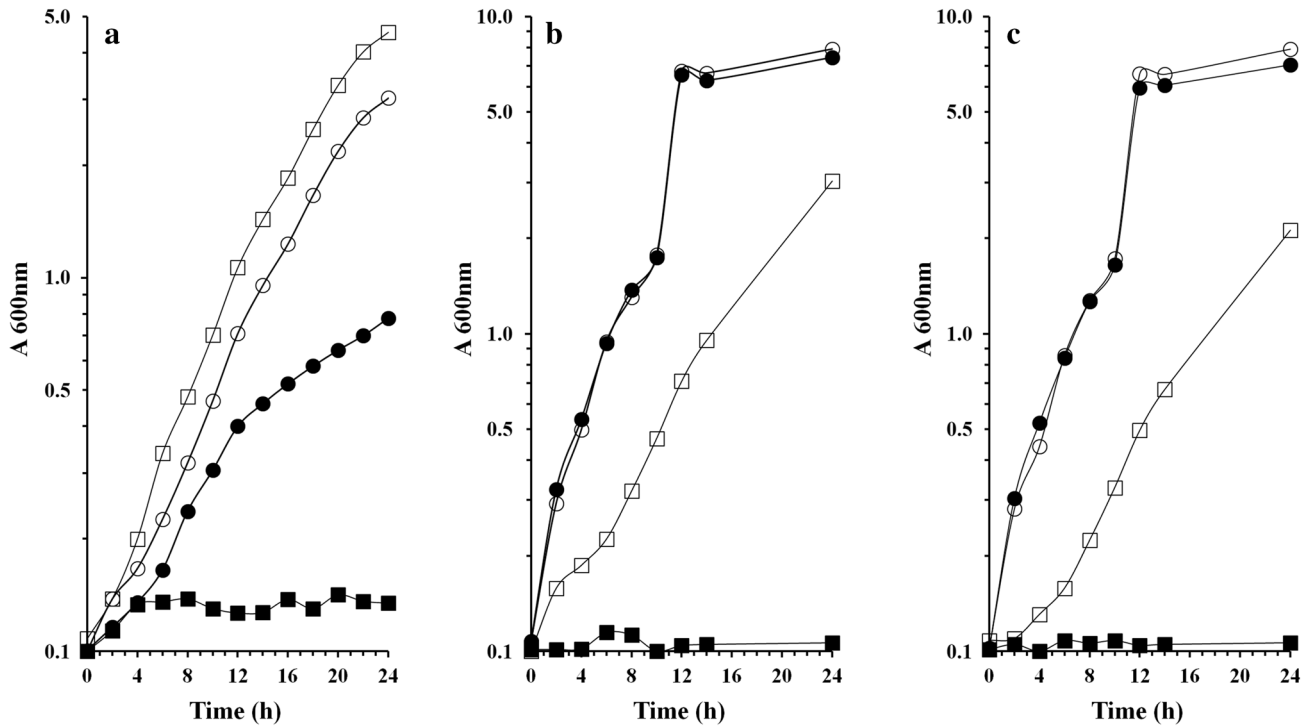

Fig. 5 Effect of medium composition on the toxicity of lawsone to *Saccharomyces cerevisiae* cells. (panel a) BY4741 parental strain cultivated in SD-glucose medium supplemented (square symbols) or not (circle symbols) with N-acetylcysteine (NAC) at 5 mmol/L in the absence (open symbols) or presence (closed on symbols) of lawsone at 172 mmol/L. (panel b) BY4741 parental strain cultivated in SD-glucose medium (square symbols) and in YPD (circle symbols) in the absence (open symbols) or presence (closed on symbols) of lawsone at 574 mmol/L. (panel c) yap1Δ mutant strain cultivated in the same conditions as in panel b

to characterise lawsone purified from henna leaves. Hence, the other possibility is that NAC might negatively interfere in some metabolic mechanism responsible for maintaining the viability of the yeast cells under lawsone treatment in SD medium. Moreover, we extended this analysis to test the influence of medium composition the toxic effect of lawsone using YPD complex medium, which contains a series of molecules with protective activities provided in yeast extract and peptone. When the parental yeast cells were cultivated in YPD medium absolutely no toxic effect of lawsone was observed, even when increasing lawsone concentration to 574 mmol/L (Fig. 5b). The same result was obtained when cultivating *yap1*Δ mutant cells in YPD (Fig. 5c). Hence, contrary to the presence of NAC, the presence of organic compounds in the complex medium completely supressed the biological activity of lawsone. Both YPD and YNB contain vitamins in their formulations, but YPD contains an excess of amino acids from peptone. Therefore, it was well stablished that NAC exacerbates while amino acids supressed the toxic effect of lawsone.

Autophagy induction by lawsone

To this point, the results indicated that lawsone induces biological damages that are oxygen-independent (Fig. 4) but are linked to the respiratory metabolism (Fig. 3). It pointed to possibility that the functioning of the mitochondria was weakened by lawsone. Altogether, the results led to the hypothesis that lawsone could specifically act in the activation mitophagy, a biological process responsible for recycling damaged mitochondria, by the fact that NAC was reported to block mitophagy process to go for a completion [5] while amino acids would act on the contrary [25]. For this purpose, we used a recombinant BY4741 strain harbouring the protein Atg8p fused at N-terminus with the green fluorescent protein (GFP-Atg8 construct) and incubated the cells in different conditions (Fig. 6). Slight and diffuse green fluorescent signal was observed in the cytoplasm of the cells collected at the exponential growth phase in SD glucose, with only faint dots accumulated in the vacuoles. It contrasted with the positive control of mitophagy induction in which cells were treated with rapamycin, resulting in intense green dot labelling inside the yeast cells. Incubation with lawsone also produced a strong signal of GFP-Atg8p dots typical of mitophagy induction, which was not observed for cells treated solely with NAC. It was noted that NAC neither stimulated mitophagy to be fired nor impaired its induction by lawsone. It provided the evidence that indeed lawsone produced functional damages to the mitochondria that lead to mitophagy induction, defining this compartment as a clear target of lawsone.

To complete the analysis of mitophagy-dependent toxicity of lawsone, yeast mutant strains with deletions in the

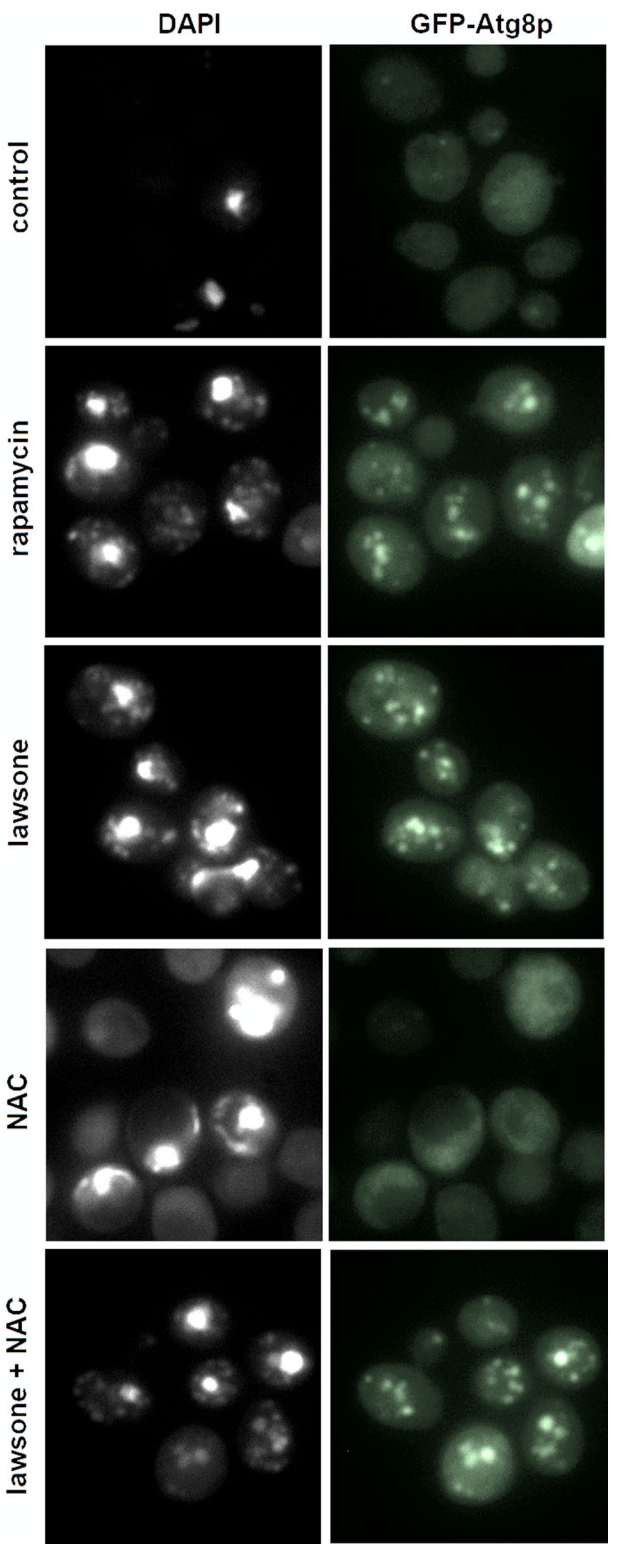

Fig. 6 Mitophagy induction in Saccharomyces cerevisiae BY4741 strain visualized by fluorescent microscopy using GFP-Atg8 protein fusion. Cells were cultivated in SD medium to mid exponential phase, collected by centrifugation and suspended in SD, SD + rapamycin (20 ng/mL), SD + lawsone (172 mmol/L), SD + *N*-acetylcysteine (NAC) (5 mmol/L) or SD + lawsone + NAC and incubated for four hours. Aliquots were mixed with DAPI for labelling DNA-containing compartments and visualised in DAPI filter and green filter for GFP localisation

key genes of the autophagic process were cultivated in SD media containing glucose for respiro-fermentative metabolism or galactose for mainly (but not exclusively) respiratory metabolism. Galactose was used because *atg* mutants are very deficient to grow in glycerol. There was no growth difference among the strains cultivated in SD-glucose (Fig. 7a), showing that deletion of these *ATG* gene did not affect respire-fermentative metabolism of the yeast cells. On the other hand, the growth of *atg1*Δ and *atg41*Δ mutants was severely affected while *atg32*Δ was only mildly affected by the presence of lawsone at sub-MIC dose in comparison to parental strain (Fig. 7b). It indicated that lawsone is more toxic to yeast cells when autophagic process cannot be initiated. However, it is worth noted that *atg1*Δ mutant initiated some slow growth after long time in lag phase. The growth of *atg32*Δ and *atg41*Δ mutants was delayed when galactose was used as carbon source, with their cell populations experiencing long lag phase before start growing at exponential rates close to parental and *atg1*Δ mutant (Fig. 7c). When lawsone was added to this medium, *atg1*Δ mutant was slightly affected while growth of other two mutants was severely affected (Fig. 7d). In summary, these data showed

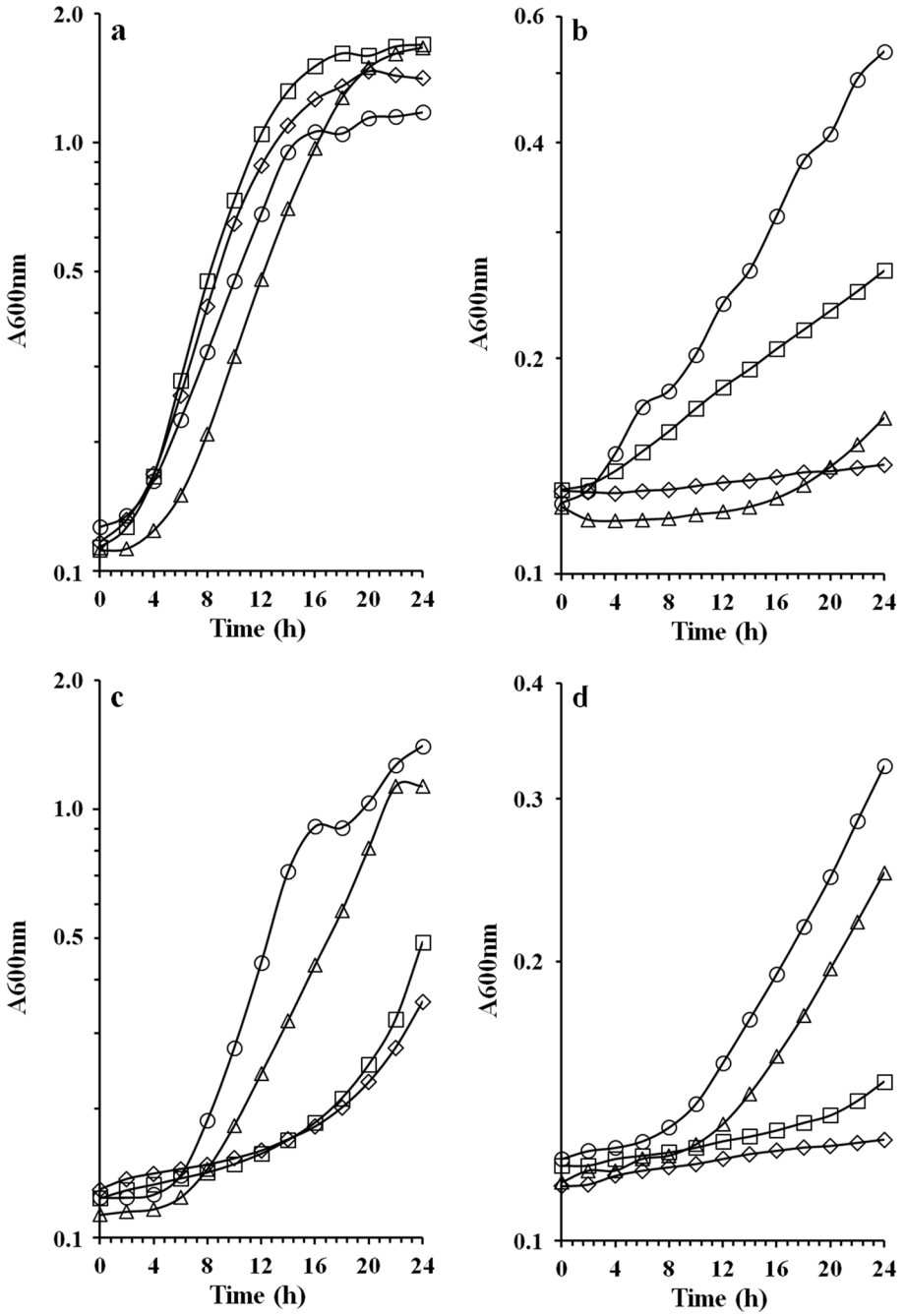

Fig. 7 Growth profile of Saccharomyces cerevisiae BY4741 parental strain (open circle) and its isogenic mutants atg1Δ (open triangle), atg32Δ (open square) and atg41Δ (open diamond) in synthetic defined medium containing glucose (panel a), glucose plus lawsone (panel b), galactose (panel c) or galactose plus lawsone (panel d) at sub-MIC dose of 172 mmol/L. Fig. S1. Chemical analysis of natural lawsone extracted from henna leaves

that Atg1p is important for the tolerance to lawsone in the more fermentative state while Atg32p is more relevant in the more oxidative metabolism. Atg41p is essential for the yeast cells to grow in the presence of lawsone.

Discussion

In the present work we aimed to determine the biological activity of lawsone by using the yeast *S. cerevisiae* as a model. The first aspect was the determination of MIC to use sub-MIC doses that still producing any toxicological without blocking cell growth. These values were defined as 230 and 172 mmol/L, respectively. Variation in MIC for lawsone can be found in the literature according to the type of fungi. Broad range of MIC values were reported for *Fusarium oxysporum* (68 mmol/L), *Aspergillus niger* (861 mmol/L), *A. flavus* (287 mmol/L) and *Candida albicans* (2.933 mmol/L) using commercially available synthetic lawsone [36, 37]. However, MIC value of 574 mmol/L was reported for *F. oxysporum* when testing natural lawsone extracted from henna leaves [4]. That eight-times difference in concentration for *F. oxysporum* was accredited by the authors to the source of lawsone used (maybe caused by impurities) as well as for genetic differences between the fungal strains. Previous reported indicated no interference on the growth of BY4741 cells when lawsone was used in concentrations up to 178 mmol/L [1], which contrasts to the present study showing that 172 mmol/L reduced the final biomass by three times (Fig. 2a). This difference can be explained by the source of lawsone as reported elsewhere, attesting the potency of the lawsone extracted from leaves in this work.

It is well-known that naphthoquinones exert their cytotoxicity through two main mechanisms: oxidative stress and/or arylation of cellular nucleophilic molecules [36]. So far, lawsone is currently included in the set of ROS-generating agents. However, it has not been well investigated and a carefully inspection in its chemical composition shows that the structure at C-2 position (Fig. 1) does not seems to fit this idea. This apparent paradox indicates that the toxic effect of lawsone still not yet elucidated. The direct production of ROS by naphthoquinones mediated by cytochrome P450 oxidoreductase is dependent upon the availability of molecular oxygen (O_2) [38]. In such reaction, P450 donate one electron to the quinone that is converted to a semiquinonic radical. Then, this radical transfer that electron to oxygen to form a superoxide radical. Cell protection against the toxic effect of this derivative depends on the action of Yap1p that control the most important mechanism of oxidative stress response in the yeast cells [1]. As it has been reported for long time, mutations that inactivate or delete *YAP1* gene turns the yeast cells very sensitive to ROS. Indeed, the results showed that *yap1*Δ mutant was sensitive to lawsone as it was reported [1]. However, lawsone was not dependent upon oxygen to exert its toxic effect since yeast cells were equally affected in aerobiosis and anaerobiosis. Therefore, we are indicating that the toxic effect of lawsone does not seems to be directly linked to ROS production, as we showed herein for its derivative β-lapachone (Fig. 4b) as positive control of the O_2-dependent direct ROS production [37]. This conclusion contrasts with the suggested effects of lawsone producing ROS in *F. oxysporum* [4]. Thus, the problem with *yap1*Δ mutant should be elsewhere in the yeast metabolism. Before continuing this analysis, it is necessary to take into account the fact that the BY4741 laboratory strain naturally carries an insertion of the Ty1 element in the 3 'region of the *HAP1* gene [12] and, consequently, its isogenic derivatives. This gene encodes a protein that regulates the expression of respiratory metabolism genes and this mutation should have some effect on respiratory metabolism and most likely affects the formation of ROS during aerobic growth [12]. Such a genetic background could mask the effect of lawsone. However, cell growth data showed that BY4741 behaved exactly as much as the CEN.PK2 laboratory strain as the JP1 industrial strain (Fig. 2c). Therefore, Hap1 protein is not related to cellular tolerance to lawsone.

In *S. cerevisiae*, Yap1 protein control the production of glutathione, an important intracellular anti-oxidant agent, by regulating under oxidative stress condition the expression of *GSH2* gene that encodes glutathione synthetase (Gsh2p) [44]. Despite the significant reduction of free thiols in BY4741 cells by lawsone, which mostly represent the intracellular glutathione content, these was no further mobilization of such agents in *yap1*Δ mutant. This scenario was not completely compatible with the assumption the lawsone cause direct oxidative stress, since a proportional reduction in free thiols would be expected in the *yap1*Δ mutant cells as observed for the parental cells. On the other hand, it was observed the increasing of protein thiols in lawsone-treated *yap1*Δ cells, but not in BY4741 cells. We recently reported that mutant yeast cells defective in mevalonate kinase activity contains higher level of protein thiols than BY4741 as consequence of the overexpression of sulphur amino acids metabolism genes [39]. Thus, this high content of SH-containing proteins would indicate that sulphur amino acids are being highly produced by the cells, supressing the toxic effects of lawsone. It might explain why *yap1*Δ cells are more sensitive to lawsone without necessarily including exacerbated ROS production. It was also reported that Gsh2p is dispensable for the protection against oxidative stress because glutathione functions in this response can be fulfilled by its precursor γ-glutamyl-cysteine [13]. Apparently, that observation was made by cultivating *gsh2* mutant in YPD medium, a rich complex medium which contains in its composition many molecules, including amino acids and glutathione itself, with protective activity against several

forms of stresses. In the present study, this assumption was turned very evident when it was shown that even extremely high concentration of lawsone does not affected cells growth in YPD, even for *yap1*Δ mutant (Fig. 5b, c). In this case, the presence of sulphur amino acids from peptone would help to counter-balance the toxic effects of lawsone.

As the final evidence for the lower importance of ROS as main cause of lawsone toxicity, we supplemented SD medium with NAC as wide used model of protective agent against oxidative stress and ROS generation [8]. Besides having direct action on detoxification of oxidant molecules, NAC can be deacetylated inside the cells to cysteine that also acts as oxidative protecting agent directly by its -SH group or by contributing to *de novo* biosynthesis of GSH [11, 14, 35, 42]. Therefore, the presence of NAC should, in principle, abolish or diminish the harmful oxidative effects of lawsone. On the contrary, NAC intensified the toxic effect of lawsone (Fig. 5a). Hence, all the points of the analyses including dependence of mitochondrial functions independent of oxygen, partial dependence of Yap1p together with the potentiating effect of NAC and the mitigating effect of amino acids led to the hypothesis that lawsone primary affects mitochondrial activities and the cells need the complete process of mitophagy to tolerate its toxic effects.

This first evidence that lawsone induces mitophagy came from the fact it induces overexpression of *ATG1* and *ATG8* genes in the filamentous fungus *F. oxysporum* [4]. Atg1 protein is responsible for the initiation of the autophagic process by recruiting other Atg proteins to the pre-autophagosomal structure (PAS) and Atg8 protein is the major constituent of the autophagosome [2, 18]. The autophagic process is important for cell survival and maintenance by recycling damaged proteins and organelles caused by nutrient starvation and/or environmental stresses [17]. All these clues led us to investigate whether lawsone is also involved in mitophagy induction in yeast. Hence, the production and processing of Atg8p fused to GFP (green fluorescent protein) maker was monitored to confirm that this mechanism is indeed being induced upon exposure to lawsone. Atg8p is linked at its C-terminal to a phophoethanolamine residue in the phagosome membrane and is released by hydrolyzation in the course of the autophagic process [31]. If Atg8p is fused at its N-terminal with GFP, the autophagy-induced proteolysis releases GFP maker that accumulates and fluoresces in the autophagic vacuole [31]. This picture was pretty much observed when lawsone was present in the medium (Fig. 6), similar to the effect of mitophagy-inducing molecule of rapamycin that inhibits TORC1 pathway and mimics nutrient starvation [16]. This definitively confirmed that lawsone causes mitochondrial injuries that triggers autophagy and ultimately mitophagy. Hence, increased concentrations of lawsone in the medium might proportionally impose mitochondrial dysfunctions to the point that no longer sustain cell cycle progression and growth, defining its MIC dosage. Previous work showed that NAC and cysteine [5] as well as methionine [26], both sulphur amino acid, are capable of inhibiting the mechanism of mitophagy [5]. This inhibition seems to be the consequence of the repression of *ATG32* gene by NAC, which encodes the major protein for the initiation of the mitophagy process [17, 33]. It is well-known that alterations in the redox equilibrium or in the oxidative metabolism of the cells triggers the general autophagic process as well as the specific mitophagy [20]. Hence, any perturbation that compromise the completion of this process would lead to loss of cellular homeostasis. Dysfunctions in mitochondria produce changes in a series of cytosolic factors, including energy charge (ATP/NTP ratio) and ROS content, that are detected by protein sensors such as Yap1p [25]. This protein, together with other sensors, transduces the message though the retrograde mechanism to trigger the expression of genes responsible for the replenishing of damaged mitochondria [25]. If the process is blocked, by chemical agents like NAC or genetic mutations in *YAP1* or *ATG* genes, damaged mitochondria increases ROS production and decreases energy charge that impairs yeast growth.

In summary, these data indicated that Atg proteins might have important rule on the yeast tolerance to lawsone. The physiological evidence of this hypothesis came from the use of strains carrying deletion in two important genes of the general autophagic process (*ATG1* and *ATG41*) and one gene specifically responsible for the mechanism of mitophagy (*ATG32*). All the three mutants were sensitive to lawsone at sub-MIC dose, like observed for cells with deletion of *YAP1* gene. The protein Atg1 is a serine/threonine kinase necessary for the initiation of autophagic process as well as the Cvt pathway, the *C*ytoplasm to *V*acuole *T*argeting structure that cargoes lytic enzymes to the autophagic vacuole [21]. This protein forms a complex together with Atg13p, Atg17p, Atg29p and Atg31p induced by inactivation of TORC1 complex caused by nitrogen starvation or by the presence of rapamycin [32]. The absence of Atg1p did not interfere in the recruitment of Atg8p to the complex, albeit compromising the extension of the autophagosome vesicle (PAS) and consequently affect the overall autophagic activity [3]. Therefore, cell tolerance to lawsone would depend on the functioning of this initiation complex. The high sensitivity of *atg1*Δ mutant to lawsone indicated that cell homeostasis is undone when autophagic process is not initiated. However, Atg1p is not so required for tolerance to lawsone when the cells are in more oxidative state in galactose, pointing that another Atg protein can fulfil this function that ends with mitophagy induction. This last process is pretty much dependent on the activity of Atg32p. It is located in the outer face of the mitochondrial membrane and initiates mitophagy by recruiting Atg11p. That complex cargoes damaged

mitochondria to the PAS by linking to Atg8p [9, 19]. The mutant *atg32*Δ showed intermediate sensitivity to lawsone between parental and *atg1*Δ in glucose, but was very sensitive in galactose. Therefore, we concluded that lawsone toxicity is more pronounced when cells in more oxidative state cannot initiate mitophagy.

Despite the few reports in the literature, there are growing evidences that Atg41p plays an essential function in the formation of autophagosome. *ATG41* gene is de-repressed and Atg41p is highly produced when the cells face nitrogen starvation or in the presence of rapamycin, and that its absence causes strong deficiency in the autophagic process [46]. Mutation in this gene completely inhibited the yeast growth sub-MIC dose of lawsone, being the most sensitive mutant tested under more fermentative or more respiratory state. It places Atg41p in a key position in the regulatory network that makes the narrow connection between autophagy and mitophagy processes.

Given the all the evidences above, it was proposed a mechanistic process for explaining the biological action of lawsone and the cellular response to the damages caused by this naphthoquinone. In this model, lawsone might cause mitochondrial malfunctioning that leads to the indirect production of ROS and/or oxidized molecules by damaged organelles, inducing oxidative stress and damaging other subcellular structures sensed through, but not exclusively by, the Yap1-dependent pathway. The retrograde response triggers the autophagosomal mechanism to the point that specific mitophagy process is activated to replenish dysfunctional mitochondria. Failure in this process, such those imposed by chemical (NAC) or biological (*atg* mutations) interferences might impede mitophagy to ending, impairing restoration of cell homeostasis and leading to severe negative effects on cell growth. Thus, this report stablished for the first time that lawsone did not cause oxidative stress directly, as its derivative β-lapachone does, but induces ROS production indirectly from its damages to mitochondrial functions. Moreover, it can be preconized that hard side effects detected when lawsone was tested in anti-cancer therapy might be associated to the indiscriminate induction of autophagy process in human cells by damages in mitochondrial functions. In this case, the association of lawsone therapy with NAC or any other molecules capable of interfering in the mitophagy completion would exacerbate those adverse reactions caused by lawsone in the patients.

Acknowledgements The authors are grateful to Prof. Daniel Klionsky, University of Michigan (USA), for kindly providing *atg* mutants and GFP-Atg8 fusion construction and to Prof. Andrea Harand, Federal University of Pernambuco (UFPE, Brazil) for the use of fluorescence microscopy. Chemical analyses were performed at the Analytical Centre of the Department of Fundamental Chemistry (UFPE, Brazil). This work was supported with grants from the Brazilian funding agencies FACEPE (project APQ-1452-2.01/10) and CNPq (project 472533/2013-4) and by the research support program of the Federal University of Pernambuco (project 23076.021846/2012-47). MRX and MGQ received master and PhD scholarship supports from CAPES agency and MMOL/LSS received PhD scholarship support from FACEPE agency.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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