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*Full Length Research Paper*

# Cactus (*Opuntia ficus indica*) extract improves endoplasmic reticulum stress in *Drosophila melanogaster*

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**We reported in this paper the requirement of the extract of cactus (*Opuntia ficus indica*) for regulating unfolded protein response (UPR) target genes and maintaining endoplasmic reticulum (ER) homeostasis. The endoplasmic reticulum (ER) is a subcellular organelle where many proteins are synthesized and sorted to various subcellular destinations. Accumulation of unfolded protein in ER can induce a stress. The ER responds to stress by initiating a cascade of events known as the "unfolded protein response" (UPR). The UPR pathway is triggered by sensors located at the ER membrane. Here, we analyzed a branch of the UPR mediated by ire-*1/xbp-1* in *Drosophila* to establish its regulation by aqueous extract from fruits and cladode of cactus "*Opuntia ficus indica*". In *Drosophila*, the *Dxbp-1* transcript was submitted to an "unconventional" splicing induced by IRE1 endoribonucease that generated a processed *Dxbp-1s* transcript encoding a *Dxbp-1* protein isoform. The *Dxbp-1s* is a key regulator in UPR to activate genes involved in folding and degradation to restore ER function. Here, we investigated the interaction between ER stress and bimolecular extract from cactus *O. ficus indica* which is a xerophyte that play multiple functions in medicine with anti-ulcer, anti-diabetic and anti-oxidants properties. We showed that the aqueous extract from fruit and cladode of *O. ficus indica* regulated ER stress mediated by the modulation of the unconventional splicing of mRNA *xbp-1* and for transcription of *bip* RNA. A good correlation was found between ire-*1/xbp-1* expression and *bip* expression.**

**Key words:** ER stress, unfolded protein response (UPR), *Drosophila*, *Dxbp-1* cactus, cladodes, fruit.

## INTRODUCTION

The unfolded protein response (UPR) is a mechanism which eukaryotic cells use to cope with stresses initiated in endoplasmic reticulum (ER) (Schroder et al., 2005). The UPR is essential for cell survival during ER stress at the same time to enhance protein folding capacity in the ER to regain homeostasis (Schroder and Kaufman, 2005). The primary targets of the UPR are genes encoding molecular chaperone and folding enzymes (Travers, 2000).

For protein folding, homeostasis is the physiological condition in which the protein folding demand is balanced with cellular protein folding capacity. Deviation from homeostasis threatens cell survival and causes endoplasmic reticulum (ER) stress. The unfolded protein response (UPR) maintains this balance or if this is not possible, it induces apoptosis to eliminate unhealthy cells from an organism or a population. UPR pathway is conserved through evolution from the yeast (Cox, 1996; Sidrauski, 1997) to mammals (Hase, 1999). In a

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particular branch of *Ire1/xbp-1*, ER stress, activated *Ire1* initiates an unconventional splicing of *xbp-1* mRNA. The spliced *xbp-1* mRNA produced an active trabscription factor to upregulate those UPR responsive genes. In mammal, UPR coordinates multiple mechanisms such as *Ire1/Xbp1*, ATF6 and PERK, the UPR in *Drosophila* S2 cells appears simpler by relying mainly on Ire1/Xbp1 pathway (Plonthongkum, 2007).

The cactus *Opuntia ficus indica* is a xerophyte producing member of the Cactaceae family. It is widely distributed in Mexico, Africa, Australia and the Mediterranean basin (Piga, 2004). It covers about 30% of the world's continental surface. This is due to its particular adaptation to water scarcity and sun irradiation (Magloire Feugang et al., 2006).

Since the mid 1970s, the interest in cactus pads has been increasing. Recent data revealed that the high content of some chemical constituents can give added value to this fruit (Piga,et al 2003) whose composition varies depending on the edaphic factors at the cultivation site, the season and the age of the plant. The total protein and fiber content decreases with age. Because a large number of studies published demonstrate that this plant has multiple functional properties in medicine with anti-ulcer (Trovato et al., 2000; Galati et al., 2001), anti inflammatory (Galati et al., 2000) and antioxidant properties (Hfaiedh et al., 2008; Zourgui et al., 2008; Ncibi et al., 2008), we undertook the study of the biological activity of the lopping fruits and cladodes.

Here, we tested whether the aqueous extract from the fruits and cladode of cactus *O. ficus indica* present a biochemical interactions with ER stress in *Drosophila*. By testing Tunicamycin drugs (Tm), we showed that UPR is activated by exogenous stress. When we combined the drugs with aqueous extract from the fruit or cladode, we observed that the ER stress was positively regulated, which suggested that homeostasis was rescued. Detailed expression analysis of *ire1*, *xbp-1* and *bip* transcripts genes confirmed that cactus *O. ficus indica* regulates UPR pathway. We proposed that the aqueous extract from the fruit and cladode possess a high anti-ER stress capacity.

## MATERIALS AND METHODS

### Cactus product

Prickly pears and cladodes were used for this study. The fruit was matured 3 months after flowering which was in April. Cladodes were present all year round, but we used younger cladodes because they show higher protein and water contents. In this study, we used cladodes; it was collected in the area of Gafsa which exhibit arid zones in southern Tunisia.

The cactus pear extract was purified from mature cactus fruit by blending. The cactus pear solution contained both the fruit and the seeds and was centrifuged at 3,500 rpm for 30 min, and then 15 ml aliquot was collected and stored at -20°C. The aqueous solution of cladode was centrifuged at 3,500 rpm for 30 min and then 15 ml aliquot was collected and stored at -20°C.

### *Drosophila* stocks

*Drosophila* melanogaster stocks were raised on standard cornmeal, yeast and agar medium at 25°C. The *lio-Gal4* driver line were kindly provided by Alain Debec (Bobinnec et al., 2003) and Jean-Maurice Dura (Taillebourg et al., 2005). The *UAS-Dxbp-1s* and *UAS-Dxbp-1:GFP* transgenic lines was generated previously, using a *w1118* strain as a recipient stock (Rubin and Spradling, 1982; Souid et al., 2007).

### Total RNA isolation and RT-PCR

#### *RT-PCR of xbp1*

Total RNA was isolated using the Rneasy kit, for RT-PCR experiments. 10 µg of total RNA were reverse transcribed with random hexamers and the first-strand cDNA synthesis kit for RT-PCR. To visualize the processing 23 bp introns, we carried out PCR amplifications of the newly synthesized complementary cDNA templates using the forward primer 5′-CAGATGCATCAGCCA ATC CAAC-3′ and the backward primer 5-GAGTGA GACCTTTCAACAC 3′ that were predicted to amplify the fragments of 191 bp (*Dxbp-1u* RNA) or 168 bp (the *Dxbp-1s* RNA) and the backward primer that were predicted to amplify the fragments of *Dxbp*-1u or *Dxbp*-1s. PCR products were separated by electrophoresis in 3% metaphor gels. All RNA samples were tested for a possible contamination by genomic DNA through RT-PCR, using a couple of primers flanking the 64 bp conventional intron common to both *Dxbp-1* RNA transcripts.

#### *RT-PCR of ire-1, bip and actin*

RT-PCR of Bip was performed using specific primers; BiP-F 5GCTGGTGTTATTGCCGGTCTGC3' and BiP-R 5'GATGCC TCGG GATGGTTCCTTGC3'. RT-PCR of *ire-1* was performed using specific primers IRE-1F 5' GAGATCAGCTTCTGCTCGCCTC CGCTCTTTGTGGCATCTG3' and IRE-1R 5'GGG GCTCGA GTCA CAGATCCTCCTCAGAGATCAGCTTCTGCTC3'. The actin level served as internal control amplified using Actin-F 50TT AGTTA TCGACAACGGATCGGGC30 and Actin-R 50CATGGTGGT ACCG CCGGACAG C30. All PCR amplification was performed using Taq DNA polymerase (Promega).

## RESULTS

### Spliced *xbp-1* is accentuated during ER stress

We showed that the *xbp-1* gene was essential for the development in *Drosophila* (Souid et al., 2007) and that the spliced form of transcript *xbp-1s* was present during the development of egg until the adults stage. In addition, Plonthongkum et al. (2007), showed that the unconventional splicing of *xbp-1* mRNA could be accentuated by an exogenic agent like Tunicamycine (Tm) drugs and the DTT in S2 cells. In order to know if the drugs used can induce the ER stress at the level of the adult, we treated the *Drosophila* with the these drugs. We found that the level of transcript mRNA *xbp-1s* increased on the level of the adults treated with the Tm. This was compared with standard condition where mRNA *xbp-1s* was present in low level (Figure 1).

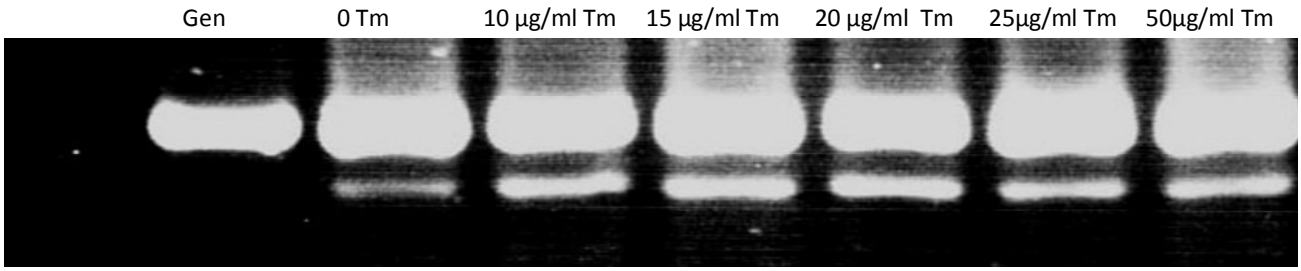


**Figure 1.** RT-PCR analysis of the expression of the *Dxbp-1u* and *Dxbp-1s* transcripts in standard and in stress conditions. ER stress increased the processing of unconventional splicing. PCR amplifications were performed using genomic DNA (Gen) as contol of the reverse-transcribed polyA+ extracted from adult.

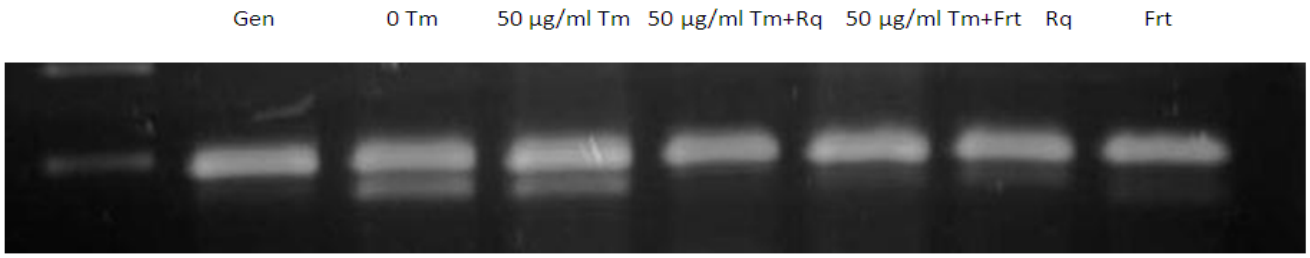


**Figure 2.** RT-PCR analysis of the expression of the *Dxbp-1u* and *Dxbp-1s* transcripts in standard, stress condition and stress condition in which the extract of cladodes and fruit wer added. Extract of cladodes and fruits decreased the processing of unconventional splicing of *Dxbp-1* until ER-stress. PCR amplifications were performed using genomic DNA (Gen) as contol of the reverse-transcribed polyA+ extracted from adult.

## The cactus extracts regulation of the unfolded protein response in multiple stress condition

It has been shown that the extract of cactus *O. ficus indica* present a variety of biological effects, such as their therapeutic properties such as antioxidant activity (Park et Chun, 2001; Butera et al., 2002), anti-inflammatory activity (Wiese et al., 2004; Cho et al., 2006) correction of damage induced by toxin (Ncibi et al., 2008: Zourgui et al., 2008) and anti cancer (Galati et al., 2002; Lee et al., 2002; de La Barrera et Nobel, 2004). To test the effect of the cactus extract in active UPR that was induced by the drug Tm, we compared the levels of transcription of the branch *ire-1-xbp-1-bip*. RT-PCR analysis (Figure 2) indicated that the extract of cactus *O. ficus indica* (cladodes and fruits) displayed an inhibitor role in the unconventional splicing of ARNm *xbp-1.* This was marked by the absence of the spliced form of mRNA *xbp-1s* in the flies treated with the drug Tm combined on the one hand by the extract of cladodes and by the extract of the fruits. The spliced form of mRNA *xbp-1s* was present under the standard condition and it was accentuated at the time of the treatment with the drug (Figure 2). Flies treated only with the extract of cactus *O. ficus indica* (cladodes and fruits) presented a low level of mRNA *xbp-1s*. In order to analyze the branch *ire-1-xbp-1-bip* of the UPR pathway, we analyzed the levels of accumulation of transcript of the *ire-1* and *bip* under the same condition with that of the analysis of transcript *xbp-1*. The *ire-1* encodes an endoribonuclease, which is responsible with the unconventional splicing of *xbp-1* transcript (Sidrauski and Walter, 1997; Urano et al., 2000a; Yoshida et al., 2001; Lee et al., 2002 ; Souid et al., 2007). The strong accumulation of the form *xbp-1s* is correlated with a strong activation of the *ire-1* gene and *bip* gene (Bertolotti et al., 2000; Shen et al., 2001; Yamamoto et al., 2004). The level of accumulation of the transcript *ire-1* decreased in the flies treated with the drug and the extracts of cactus (Figure 2). The flies were elevated under standard conditions and the stress condition using the drugs showed an increased level in transcript *ire-1*. The flies treated only with the extract of cactus show a decreased in the level of transcript *ire-1*. This was found either with Tm (Figure 2) or the DTT (data not shown). In the analysis of the levels of transcript of *bip*, we obtained the same result which consisted or had a very clear reduction at the time of the treatment with the drug/extract compared with the massive increase in the transcription of *bip* in the standard conditions and in ER stress (Figure 3). For the positive control, we noticed that the extract of cactus cladode or fruits showed a reduction also of the level in accumulation of transcript *bip*. In order to see whether the found effect of the extract of cactus on the UPR pathway in particular connects it *ire-1/bip*, we analyzed the profile of accumulation of the transcript of actin which does not have anything with the UPR

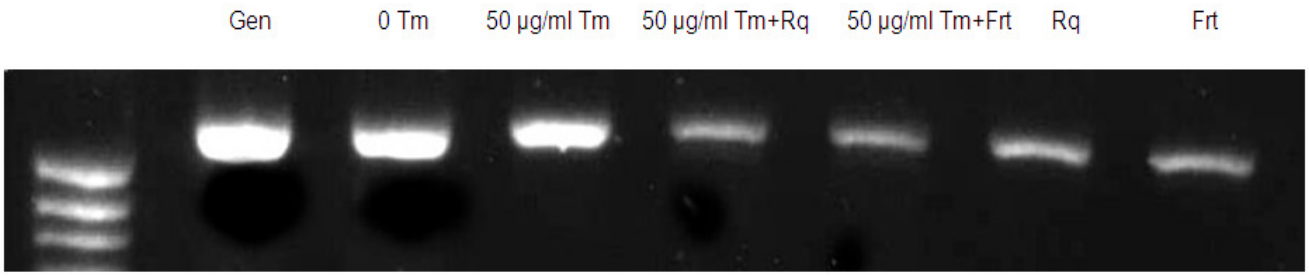


**Figure 3.** RT-PCR analysis of the expression of the *ire-1* transcripts in standard , stress condition and stress condition in which cladodes and fruits extraxts were added. Extract of cladodes and fruits decreased the processing of *ire-1* transcrips. PCR amplifications were performed using genomic DNA (Gen) as the contol of the reverse-transcribed polyA+ extracted from the adult.

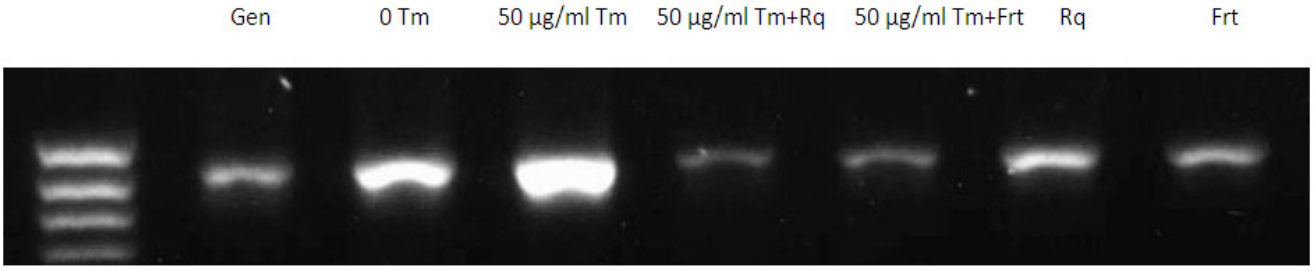


**Figure 4.** RT-PCR analysis of the expression of the *Grp 78-bip* transcripts in standard, stress condition and stress condition in which cladodes and fruits extracts were added. Extract of cladodes and fruits decreased the processing of *Grp78 (Bip)* transcripts. PCR amplifications were performed using genomic DNA (Gen) as the contol of the reverse-transcribed polyA+ extracted from the adult.

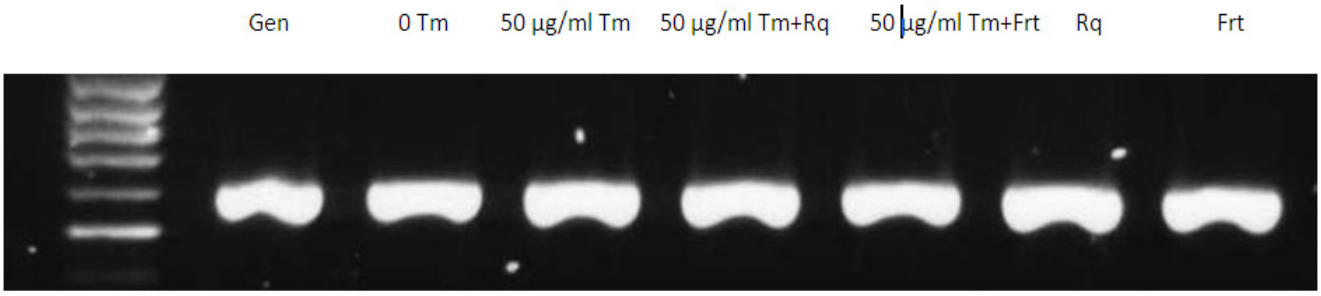


**Figure 5.** RT-PCR analysis of the expression of the actin transcripts in standard, stress condition and stress condition in which cladodes and fruits extracts were added. Extract of cladodes and fruits were not implicated in the processing of actin transcrips. PCR amplifications were performed using genomic DNA (Gen) as the contol of the reverse-transcribed polyA+ extracted from the adult.

pathway. In the same condition of the RT-PCR we realized with specific primers that the actin showed modification in the level of transcript product (Figure 4).

The activation of UPR pathway was also shown by the ignition of the GFP at the larva level of the lineages xbp-1:GFP (Souid et al., 2007). Indeed the unconventional splicing of *xbp-1* was detected by the presence of GFP, because transgenic flies were constructed in such a way that GFP was merged with *xbp-1* and what the unconventional splicing puts in phase *xbp-1* and GFP, while without stress the GFP is absent. We used this construction to visualize the activation of the UPR during the stress and to see if the extracts of cactus had an effect on the protein synthesis *Xbp-1*:GFP and inhibited the activation of UPR pathway.

Larva treated with Tm, showed activation on GFP in comparison to larvae without Tm treatment (Figure 5a, b). Larva treated with the drugs/extracts of cactus showed a significant decline of ignition of the GFP (Figure 5c) and this was the same for the extracts from the cladodes or fruits (Figure 5d, e). In an interesting way these larva presented a profile of ignition of the GFP similar to the

control which was not treated with the drugs. The larva treated only with the extract from cactus presented a profile of very weak ignition. This result indicated that extract of cladodes and fruit regulated the UPR.

To confirm these results, we performed Western blot using antibodies anti-GFP. A good correlation between them resulted from the fluorescence and the detection of the protein GFP and this showed that the inhibition of the synthesis of the protein GFP indicates the absence of the splicing of the *xbp-1* ARNm which will be translated into protein, and by comparison the detection of the actin protein was not modified.

## DISCUSSION

Transcriptional activation of *ire-1/xbp-1* genes and *bip* gene which encode molecular chaperones during ER stress is a major response in eukaryotic cells to mount stress for their survival. In this study, we analyzed the *ire-1-xbp-1-bip* branch for its expression in stress condition in which we added the extract of cladodes or fruits from *O. ficus indica*.

It has been shown that UPR signaling is activated in endogenesis stress (Souid et al., 2007) and *Ire-1* regulation by mRNA splicing is essential for the unfolded protein response (UPR) in *Drosophila melanogaster* ( Plongthongkum et al., 2007). The most conserved branch of UPR involves IRE-1, an ER transmembrane kinase and endoribonuclease that activate the transcription factor *xbp-1*. In standard condition, *xbp-1* mRNA encodes a transcription factor, *xbp-1u*. Upon stress *IRE-1* uses its endoribonuclease activity to excise and to relegate the *xbp-1*mRNA u for two sites separated by 26 nucleotides (nt) in vertebrates or 23 (nt) in *Drosophila* and *Caenorhabditis elegans* (Sidrauski et al., 1997; Yoshida et al., 2001; Lee et al., 2002; Souid et al., 2007; Plongthongkum et al., 2007).

It was previously demonstrated that ER stress activates *Drosophila xbp1*mRNA splicing in adult treated with tunicamycin; the same result was obtained in *Drosophila* S2 cells (Plongthongkum et al., 2007).

*xbp-1* is a critical transcriptional activator of the UPR that is responsible for the induction of a distinct set of target genes. The nonconventional splicing mechanism of *xbp-1* is a universal marker of ER stress and some pathology like tumor growth and neurodegenerative diseases (Feldman and Koong, 2007). For these rea-sons, it is judged necessary to find solutions against this situation. Within this context, nature can provide many substances of extract from medicinal plants that can attenuate this ER stress.

In this paper, the protective role of cactus in ER stress was investigated. Many studies have demonstrated the protective effect of cactus against chlorpyriphos (Ncibi et al., 2008), oxidative stress induced by mycotoxin zearalenone (Zourgui et al., 2008) and toxicity of Nickel (Hfaiedh et al., 2008). The extract of cladodes and fruits inhibited the UPR pathway by the absence of the unspliced transcript of *xbp-1*mRNA with regard to the control in which the unfolded protein response was induced and the spliced form of *xbp-1*mRNA was present. The same result was obtained when we used another drugs (DTT) (data not shown). To confirm this result, we repeated the same experimental procedure of RT-PCR, but we did not make the reverse transcriptase enzyme.

The levels of *ire-1* and *bip* were determined in the same condition of the study of the levels of spliced *xbp-1*. We found that the treatment of *Drosophila* adult with Tm combined with the extract of cladodes or of the fruits rescued ER homeostasis as indicated by lower levels of *ire-1* and *bip* transcript, reflecting resolution of basal level of ER stress. We speculated herein a possible mechanism underlying the rescuer of ER homeostasis. In our individual experiment, the absence of active spliced *xbp*-1 was associated with diminished levels of *ire-1* and *bip* transcript. The data presented here provide the evidence that maintenance of ER homeostasis in *Drosophila* does rely upon the UPR pathway, in accordance with those data generated in mice (Iwakoshi et al., 2003a; Reimold et al., 2001).

We also examined the unconventional splicing bye using a transgenic *Drosophila* UAS-*xbp-1*:GFP. This constrict was designed to reveal unconventional splicing through the production of a fluorescent *DXbp-1*:GFP fusion protein after the splicing of the 23 nt intron (Januscke et al., 2002; Souid et al., 2007). We tested specific drivers lio-Gal4 line (Taillebourg et al., 2005) to detect fluorescent in the salivary glands of the third-instar larvae, first with Tm drugs and second with the combination of Tm with aqueous extract of cladodes or fruits. The strong GFP fluorescent staining obtained in Tm treatment was reduced significantly in the context of Tm/extract of cladodes and Tm/extract of fruits (Figure 6). Our result clearly showed that the absence of spliced transcript of *xbp-1*mRNA in RT-PCR experiment was correlated with the spectacular decreased of GFP staining.

The results of this work showed that the cactus corrected different situations of stress of the endoplasmic reticulum, and by analysis of the profiles, it expressed the genes in UPR pathway.

The treatment with tunicamycine led strongly to the unconventional splicing and the accumulation of the shape splicing of the transcribed gene *xbp-1* b. In a very spectacular way, *Drosophila* treated by the tunicamycine showed clearly one transcriptional activation of the genes encoding molecular. In this study, we analyzed the effect of drug on UPR signaling. Our results suggest that the extract of cactus (fruits and cladodes) regulate UPR signaling. The aqueous extract from the fruits and cladodes of cactus contains many phenol compound, ascorbic acid, betalains, betacyanins, flavonoid fraction

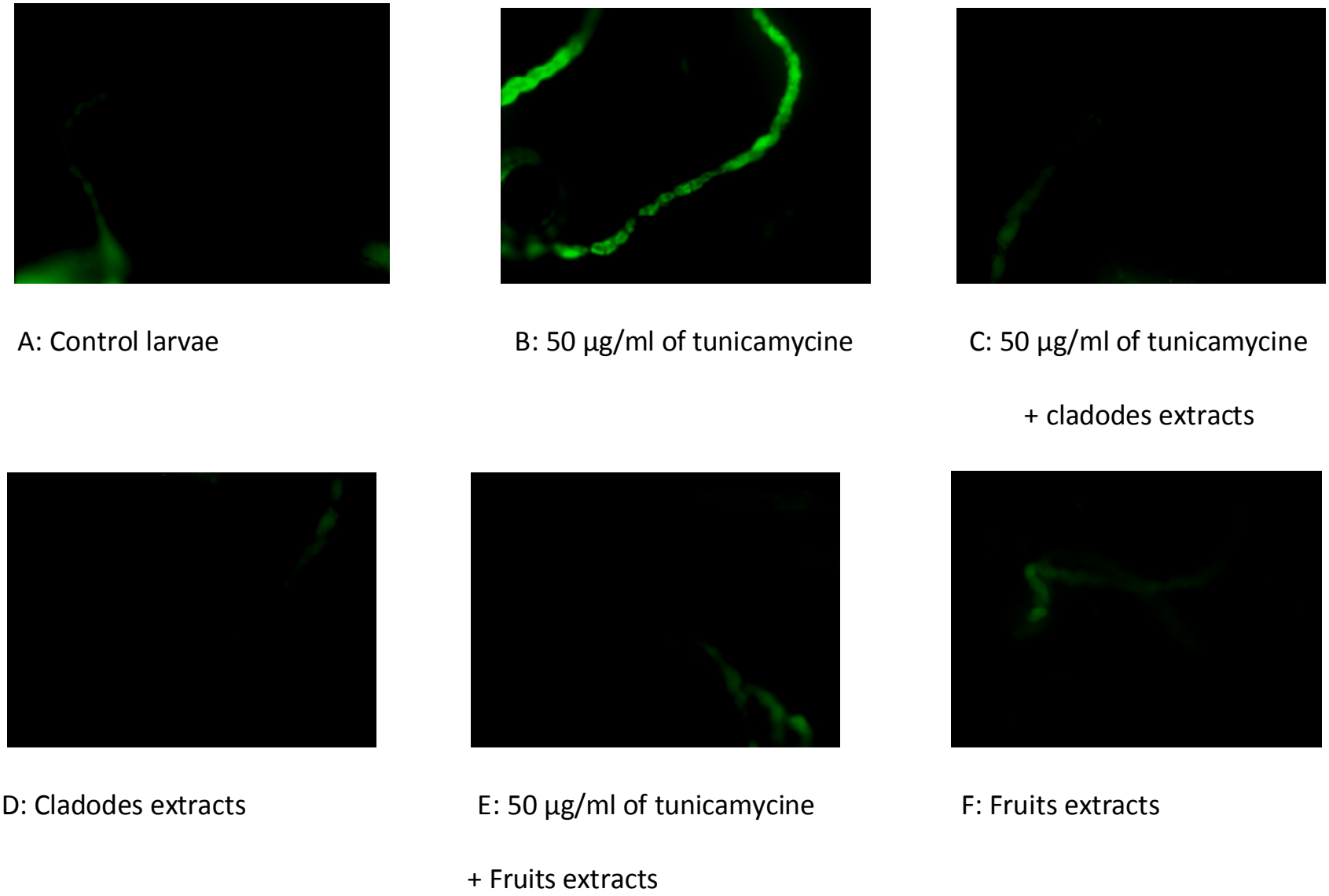


**Figure 6.** ER stress decreased the processing of *Dxbp-1*:GFP transcript until the use of cladodes extract and fruit extract. Tunicamycine (Tm) only increased the fluorescent labelling of salivary gland of Lio-Gal4; UAS-*Dxbp-1*:GFP larve but Tm + cladodes extracts and Tm + fruit extracts show a decreased fluoresecent labelling. All test were photographed in a single shot to fully visualize the staining of the salivary gland of lio-Gal4; UAS-*Dxbp-1*:GFP third instar larve. (A) contral larvae; (B) larvae fed with 50 µg/ml of tunicamycine; (C) larvae fed with 50 µg/ml of tunicamycine + exatract of cladodes; (D) larvae fed with 50 µg/ml of tunicamycine + extract of fruit; (E) larvae fed with cladodes extract; (F) larvae fed with fruits extract.

and vitamin C, which have biological activity. New experiments are under way to determine the precise, active biomolecule which has the correction effect on ER stress condition.

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