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RHIZOTOXICITY OF THE INVASIVE SPECIES *MELIA AZEDARACH*: IMPLICATION OF PHENOLS ON ITS HERBICIDE POTENTIAL

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ABSTRACT

A biological invasion of non-native plants is spreading into our natural areas and rights-of way. In the present work, *Melia azedarach* (Meliaceae) known for its high allelopathic potential is used to investigate its bio-pesticide capacity on the common model used in bioassays: *Raphanus sativus* (*R. sativus*). Exposure of *R. sativus* to *M. azedarach* (aqueous/ethanolic) leaves extracts result on toxicity exclusively toward roots. The roots inhibition was dose dependent and more pronounced for ethanolic extract. Allelochemicals of *M. azedarach* ethanolic extracts induced an oxidative stress in radish focalized by a decrease on enzymatic anti-oxidant defense such superoxide dismutase (SOD) activity. However, the stimulation of phenyl-alanine ammonialyase (PAL) activity and the subsequent increase in phenols content failed to alleviate *R. Sativus* roots oxidative stress. Importantly, the result of *M. azedarach* leaves extracts inducing oxidative imbalance in roots seemed to be harmful *in vivo* on *R. sativus* but exceeded its antioxidant abilities *in vitro*. *M. azedarach* invasive species might be valorized, in Tunisia, by developing "root killers" bio-herbicides and enhancing biological proprieties of received plant.

Keywords: *Melia azedarach*, *raphanus sativus*, rhizotoxicity, oxidative stress and bio-herbicide.

INTRODUCTION

Weeds have been proved to be of a great harm to crops. They reduce crop yield and threaten food security (Roy *et al.,* 2006). It is true that the use of chemical herbicides increased agricultural production but they had several side effects on human health and environment (Wilson and Tisdell, 2001). In addition, the excessive use of chemicals in the same areas became inefficient and induces resistance development (Prather *et al.,* 2000).

The alternative of using synthetic agrochemicals products is the use of botanical herbicides, like those derived from plants, in the form of bioactive compounds or allelochemicals (Singh *et al.,* 2003). These are generally secondary metabolites from diverse chemical groups that are involved in plants reproduction and defence. Among them, phenolic compounds are the best studied, and the water soluble phenolic acids are responsive to a large portion of their allelochemical potential (Li *et al.,* 2010). Allelochemical's effects on the receiver plant are mostly negative affecting its growth and development (Niakan and Saberi, 2009). Several modes of action have been suggested, such as the inhibition of photosynthesis, ion uptake, respiration and reactive oxygen species (ROS)-mediated mechanism (Inderjit and Duke, 2003). Allelopathic stress causes a secondary oxidative stress (Omezzine *et al*, 2014). The plant antioxidant defense toward ROS accumulation involves both enzymatic detoxifying (superoxide dismutase *vs.* $O_2^{\cdot}$, and peroxidase, catalase *vs*. H_2O_2) and secondary metabolites (phenols). These later might also behave as oxidant, depending on their concentration, physiochemical property, structure (Chu *et al.,* 2000) and availability of oxygen and metal ion (Dai and Mumper, 2012).

Allelopathy has been proposed as a mechanism of defense used by invasive plants toward native species through the production of chemical substances (Callay *et al.,* 2000; Qin *et al.,* 2006). *M. azedarach*, for example, native of Asia and southern Australia (Chiffelle *et al.,* 2009) has been introduced and naturalized in many Arabic and African countries, particularly in Tunisia (Khan *et al.,* 2008). *M. azedarch* is an important plant used for a wide variety of medical applications with therapeutic (Szewczuk *et al.,* 2006; Ahmed *et al.,* 2008; Munir *et al.,* 2012; Khan *et al.,* 2014) and pesticidal (Schmutterer, 2002; Ardakani, 2012; Abdel Aty and Abdel Megeed, 2015) potentials but little is known about its allelopathic properties.

Therefore, we reportin this study, the effects of *M. azedarach* mature leaves aqueous and ethanolic extracts on the early developmental stages and onthe oxidative status (photoreceptors content, malondialdehyde MDA, superoxide dismutase activity SOD, phenyl-ammaonia-lyase PAL activity and phenols' content).*in vivo* of *Raphanus sativus (R.sativus)***.** We focused our interest to study the impact of *M. azedarach* leaves extracts on *R.sativus* antioxidant abilities *in vitro* (ROS scavenging capacities). Our aim is to contribute to the understanding of mode of action of *M. azedarach* allelochemicals, potential bio-herbicides, as a preliminary step of valorization of this invasive species.

MATERIALS AND METHODS

Plant Material: Mature leaves of *M.azedarach* L. were collected at fruiting stage in July-August from North of Tunisia Lat. (37.27 N, Long. 9.87 E) and air dried and pulverized.

Extraction procedures: Aqueous extracts: The leaves powder was macerated in distilled water (80g/1L/ 24 h) at room temperature and then centrifuged (1500 rpm/15min). The supernatant was freeze-dried using lyophilizer and kept at 4°C until use (Yield: 5%). Dilutions at 2, 4 and 6 % were prepared.

Ethanolic extracts: The leaves powder was extracted three times by maceration in ethanol (95%) (80g/L/24h) at room temperature. The solvent was evaporated in a vacuum rotary evaporator (Buchi Labortech AG, model 1, R-215) at 45-50 °C (Yield= 8.75%). The obtained residues were preserved at 4°C until use (Akacha *et al.*, 2016)

Effects of *M. azedarach* aqueous and ethanolic leaf extracts *in vivo* on *Raphanus sativus*

Germination and Growth: *Raphanus sativus* seeds were surface sterilized with 1% sodium hypochlorite for 10 min then rinsed three times with deionized water. Germination has been performed in Petri dishes in dark on filter paper imbibed with aqueous or ethanolic extracts (2, 4 and 8 %) with their respective control water and ethanol (95%). Filter papers placed in Petri dishes were soaked with 5 mL of distilled water or ethanol. Solvent was evaporated for 24 h at 24 °C, then 5 mL distilled water was added and 13 seeds per dish (6 dishes per treatment) were placed to germinate. The number of germinated seeds was counted each day. The plantlet growth occurred in the Petri dishes, whose upper filter paper has been removed at the third day after the germination beginning to allow the cotyledons photosynthesis. In the radish plantlets 10 days old, shoot and root length were measured, then different organs are placed in a vacuum oven at 78°C during 72 h to determine organs dry weight. In following experiments, we compared the concentrations of aqueous (4%) and ethanolic (2%) *M. azedarach* leaves extracts that induced a rhizotoxicity in the same range, on the dry biomass basis of the receiver plant (radish).

Oxidative status

MDA content: The level of lipid peroxidation was expressed as 2-thiobarbituric acid reactive substances (TBA-rm) (Buege and Aust, 1978). TBA-rm in samples was assayed according to the modified method of Heath and Packer (1968). Fresh tissue was ground in 10% TCA (10ml /1 g fresh weight) with a mortar and pestle and a small amount of sand. TBARS were added to TCA before heating of the samples. After heating at 95°C for 30 min, the mixture was quickly cooled in an ice-bath and centrifuged at 10000 g for 10 min. The absorbance of the supernatant at 532 nm was read and corrected for unspecific turbidity by subtracting the value at 600 nm. The blank was 0.25% TBA in 10% TCA. The concentration of MDA was calculated using an extinction coefficient of $155 mM^{-1}cm^{-1}$.

Chlorophylls and carotenoids contents: The cotyledons of 10 old radish plantlets were placed in acetone (80%) to extract photosynthetic pigments. The absorbance of the extract was read at 663, 645 and 480 nm. Chlorophyll a, b and carotenoids quantities were calculated in according to Arnon (1949).

SOD activity: Superoxide dismutase activity (SOD, EC 1.15.1.1) in samples was determined by the protocol of Misra and Fridovich (1972) at alkaline pH based on the superoxide dismutase competition with the autoxidation of epinephrine to adrenochrome, thus decelerating the adrenochrome formation. SOD activity was calculated by the formula: A (U) = (β/β')-1 where β and β' are respectively oxidation's gradients of epinephrine and both epinephrine and extract. For SOD isoforms activities, assays were performed in the presence of selective inhibitors; KCN (3 mM) inhibited only CuZn SOD. H_2O_2 (5 mM) inhibited both CuZn SOD and Fe SOD, Mn SOD was not inhibited by KCN nor H_2O_2 (Lee *et al*., 2001).

PAL activity: Phenylalanine ammonia-lyase (PAL) was extracted following the Takayoshi and Kawamura (1964) method with slight modifications. Fresh organs (cotyledons, hypocotyls or radicles) were ground at 4ºC in 0.1 M sodium borate buffer (pH 8.8). The homogenates were centrifuged at 12,000 x g for 15 min and the supernatant was used as enzyme preparation.

For the PAL activity assay (PAL; EC 4.3.1.5), the reaction mixture (sodium borate buffer and a suitable amount of enzyme extract in a final volume of 2 ml) was incubated at 40ºC for 5 min, started by the addition of L-phenylalanine (50 mM) and stopped after 90 min of incubation by the addition of 50 µl of 5 N HCl.

Absorbance of the samples as well as of the control (without L-phenylalanine) was read at 270 nm. PAL activity was expressed as µmol t-cinnamate $min^{-1}g^{-1}FW$ (U g^{-1} FW).

Phytochemical study

Total phenolic content: Total phenolic contents of *M. azedarach* leaves extract and *R. sativus* were determined using Folin-Ciocalteu reagent; according to the method of Falleh *et al.* (2011).Gallic acid was used to calibrate the standard curve. The extract was analyzed in triplicate and the results were expressed in milligrams of gallic acid equivalents per gram of dry weight (mg GAE /g).

Total flavonoid content: Total flavonoids content of *M. azedarch* leaves extractand *R. sativus*were estimated as Falleh *et al.* (2011). Absorbance was determined at 510 nm and flavonoid concentration was calculated according to the equation obtained from (+) catechin graph and was expressed as mg catechin equivalents per gram dry weight (mg CE g^{-1} DW).

Proanthocyanidines contents: The proanthocyanidines content of *M. azedarch* leaves extract and *R. sativus* were determined according to the method of Falleh *et al.* (2011). 50 µL AIE were mixed with 3 mL vanillin (4%; w/v). Then, 1.5 mL 12M hydrochloric acid was added to the mixture. After incubation for 15 min at 25°C, absorbance was measured at 500 nm. Results were expressed as mg Catechin/g of dry weight (mg C/g DW).

Evaluation of antioxidant capacities in vitro of M. azedarach and R. sativus: *2.6.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH)scavenging activity*

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay employed here is as described by Braca *et al.* (2001). The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. Ascorbic acid (AA) is the reagent used as standard. Experimentally, various dilutions of extracts or standard (0.01-1 mg/ml, in triplicate) were added to DPPH solution (0.035 mg/mL). The absorbance of the reaction mixture was read at 517 nm with ethanol as blank. A control sample with no added test compounds was also analysed. Radical scavenging activity was expressed as a percentage and calculated using the formula: % Scavenging = $[(A_{cont} - A_{test}) / A_{cont}] \times 100$, where A_{cont} is the absorbance of the control, and A_{test} is the absorbance of the sample in the presence of test-extract. The result was presented as IC_{50} (the concentration of test extract required for scavenging 50% of the DPPH radical).

H_2O_2 scavenging activity: H_2O_2, reactive ROS is harmful to biomolecules when reacting with metal ions as Fe^{2+} producing OH^-via the Fenton reaction (Halliwell, 1991). Scavenging of H_2O_2 by extract is partly attributed to phenolics (Ebrahimzadeh *et al.* 2010). The ability to scavenge hydrogen peroxide was determined according to the method of Yen *et al.* (1995). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4) then a solution of the test-extract or standard at various concentrations (0.01–1mg/mL) was added. Absorbance of hydrogen peroxide at 230 nm was determined 19 minutes later against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging was calculated from the equation: % Scavenging = $[(A_{cont} - A_{test}) / A_{cont}] \times 100$, where A_{cont} is the absorbance of the control, and A_{test} is the absorbance of the sample in the presence of test – extract or standard. The result was presented as IC_{50} (the concentration of test extract required for scavenging 50% of the H_2O_2 stable ROS).

$OH^\bullet$ scavenging activity: It was measured by the deoxyribose method **(**Halliwell *et al.*, 1987) and compared with that of ascorbic acid. The $OH^\bullet$ scavenging activity of different extracts as well as that of radish organs was carried out by measuring the competition between deoxyribose and the compounds that generate hydroxyl radicals from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. Attack of the hydroxyl radicals on deoxyribose led to formation of thiobarbituric acid-reactive substances (TBARS) which were measured by the method of Ohkawa *et al.* (1979). The $OH^\bullet$ scavenging activity was calculated from the equation: $OH^\bullet$ scavenged (%) = ((Acont –Atest) / A cont)* 100. Where Acont is the absorbance of the control reaction and Atest is the absorbance in the presence of the sample. The result was presented as IC_{50} (the concentration of test extract required for scavenging 50% of the $OH^\bullet$ radical).

Ferrous ion chelating ability (FIC): The FIC ability of different extracts was determined according to the method of Singh and Rajini (2004) and compared with that of ascorbic acid. Extracts or standard at various concentrations (0.01-1 mg/mL) were added to $FeSO_4$ (0.1 mM) and ferrozine (0.25 mM). The tubes were shaken well and left to stand for 10 min. The absorbance was measured at 562 nm. The ability of each sample to chelate ferrous ions was calculated relative to the control consisting of only iron ferrozine, using the following formula: % FIC = $[(A_{cont} - A_{test}) / A_{cont}] \times 100$, where A_{cont} is the absorbance of the control, and A_{test} is the absorbance of the sample in the presence of test compound. The result was presented as IC_{50} (the concentration of test extract required for chelate 50% of the iron ion).

Ferrous reducing ability (FRA): The reducing ability of different extracts (ethanolic and aqueous) as well as that of radish organswas determined according to the method of Oyaizu (Oyaizu, 1986). Extracts or standard (1 ml) at various concentrations (0.01-1 mg/mL) were mixed with 2.5 mL of phosphate buffer (0.2 M) and 2.5 ml of 1% potassium ferricyanide and incubated at 50 °C for 20 min. To this mixture, 2.5 ml of 10% trichloroacetic acid was added and the mixture was centrifuged at 3000 rpm for 20 min. The upper layer (2.5 mL) was mixed with 2.5 ml of deionised water and 0.5 mL of 0.1% Ferric chloride and the UV absorbance was measured at 700 nm using a spectrophotometer. Increase of absorbance of the reaction mixture indicates increase in reducing ability. The percentage increase in reducing ability was calculated relative to the control prepared without adding test compound, using the following formula: Increase in reducing ability (%) = $[(A_{test} - A_{cont}) / A_{cont}] \times 100$, where A_{test} is the absorbance of the sample and A_{cont} is the absorbance

of the control. Index = IC_{50} (mg/mL) reducing ability/ IC_{50} (mg/mL) DPPH scavenging assay.

Evaluation of ProAntidex of *M. azedarach* and *R. sativus*: In plants, pro-oxydant and antioxidant effects are due to the balance of free radical scavenging activity and reducing ability of iron ions which might drive the Fenton reaction. In this reaction, Fe^{2+} react with H_2O_2 resulting in production of $OH^{\bullet}$ that is the most harmful ROS. The predominance of reducing ability over the free radical scavenging activity results in the prooxidant effect (Lahbib *et al.*, 2015). More the index is lower more the extract is antioxidant than prooxidant.

Statistical analysis: Results are expressed as the mean ± standard error of the mean (SEM). Statistical differences were evaluated by one-way analysis of variance (ANOVA). All analyses were performed using STATISTICA version 5.00 (Stat Soft- France, Tulsa, OK, USA) for Windows, *p* value <0.05 was considered significant.

RESULTS

Effects of M. azedarach aqueous and ethanolic leaf extracts in vivo on Raphanus sativus: *M. azedarach* leaf extracts reduced significantly the germination rate of radish seeds.($P<0.05$) and in a dose dependant manner. All non-treated seeds have been germinated on the third day. Meanwhile, the aqueous extract reduced the radish germination rate by approximately 20, 34 and 52 % for respectively 2, 4 and 8%. Concerning the ethanolic extract, the decrease was approximately 40 and 57 % for respectively 2 and 4%, this extract being lethal at 8% for radish seeds and consequently plantlets (**Figure 1**).

***M. azedarach* extracts action on 10 old plantlets would vary according to the organ and kind of the extracts:** The roots' growth was reduced due to*M. azedarach* leaves extracts, whereas that of hypocotyl was increased. Observed effects depended on the dose taken. The inhibitions of radish root growth at 2, 4 and 8 % of aqueous extract were respectively 14, 20 and 51 %.They were 44 and 51 % at respectively the concentrations 2 and 4% of ethanolic extract. The hypocotyl length was stimulated by about 11 and 39% at 2 and 4% concentration of aqueous extract. In the same line, the increase was by 14.5 and 38% for 2 and 4% concentration of ethanolic extract(Figure 2).

As observed for length, the *M. azedarach* extracts effects on dry biomass were inhibitor for the roots but stimulating for hypocotyls. The inhibitor effects on roots were at the same range for the aqueous extract at 4% (24.85 %) and ethanolic at 2% (23.32%), idem at A 8% (37.65 %) and E (33.63) at 4 %.

The hypocotyl dry biomass production was stimulated by the aqueous extract at 2, 4 and 8 % by 24, 42 and 11 % respectively. The stimulation was only observed for the ethanolic extract at the concentration 2% whereas that of 4% did not significantly induce modification by comparison to control. In radish cotyledons, the *M. azedarach* extracts affected reserves mobilization that support embryo growth and as a consequence, the dry biomass of radish cotyledons was higher to that of control when submitted to aqueous extract at 4 %, and not modified at 8 %, the ethanolic extract did not induce any modification of cotyledons dry biomass at 2 and 4 % **(Figure 3).**

Oxidative status of *R. sativus* treated with *M. azedarach* leaf extracts: The concentrations 4% of the aqueous extract and 2% of ethanolic one were chosen to realize the following experiments since they induced the same range of inhibition on root growth (respectively -24.85 % and -23.32%) and this based on the dry weight.

Allelochemicals contained in *M. azedarach* leaf extracts induced an oxidative stress, as shown by an increase in MDA content in rootsby approximately 33 % for aqueous extract (4 %) and 43% for the ethanolic one (2 %). In cotyledons and hypocotyls, the aqueous extract caused stronger changes in MDA content in cotyledons and hypocotyls ranging from 33 to 49% (Figure 4).

In addition, *M. azedarach* leaf extracts reduced the cotyledons assimilatory pigments content (chlorophylls and carotenoids) in a more pronounced way for the ethanolic extract than for the aqueous one (Figure 5).

The activities of SODs , first enzymatic of defence line against ROS, were affected by *M. azedarach* allelochemicals and in a more pronounced way by ethanolic extract than by the aqueous with the exception of the Fe-SOD isoform in growing organs (hypocotyls and roots) (Figure7).

The allelopathic stress induced in radish a stimulation of PAL activity in the same range for the two kinds of extracts particularly in growing organs and in a more pronounced way in radicles (Figure 7)

The ethanolic leaves extracts of *M. azedarach* show higher phenols (Figure 8A) -and in particular- flavonoids contents than the aqueous one (Figure 8B).However, tannins contents were in the same range for the two kinds of extracts (Figure 8C). The introducing of *M. azedarach* extracts in radish plantlets, stimulates PAL activity that leads to an increased phenols content particularly in roots by 42.22 % and 59.75 % respectively for the aqueous and ethanolic extracts (Figure 8A')) and in particular that of flavonoids (A: 38.95 % and E: 58.19 % (Figure 8B').Similarly, tannins content was also modified (A: 65.21 % and E: 72.41 %) compared with control (Figure 8C').

Effects of *M. azedarach* aqueous and ethanolic leaf extracts on *R. sativus* antioxidant capacities in vitro: DPPH scavenging activity of *M. azedarach*

aqueous extract (IC_{50}: 0.66 mg/ml) was higher than that of the ethanolic one (IC_{50}: 1.00 mg/ml) (Figure 9A). Activities of both extracts have been lower than that of ascorbic acid (IC_{50}: 0.0396 mg/ml).In radish, *M. azedarach* allelochemicals did not modify DPPH scavenging activity significantly of cotyledons whereas it increased that of radicle by 33.96 % and 32.47 % respectively for aqueous and ethanolic extracts by comparison to control. In hypocotyls, the aqueous extract increased the DPPHscavenging activity (8.76 %) whereas ethanolic extract decreased it (-13.06 %) (Figure 9A'). All tested extracts activities were compared with that of ascorbic acid (IC_{50}: 0.0396 mg/mL).

The H_2O_2 scavenging activities of aqueous and ethanolic *M. azedarach* leaves extracts were in the same range (IC_{50}: 0.462 mg/ml and 0.465 mg/ml respectively) (Figure 9B) and lower than that of ascorbic acid (IC_{50}: 0.06 mg/ml). The radish cotyledon treated by aqueous extract showed the highest H_2O_2scavenging activity with IC_{50} at 0.055 mg/ml. In growing organs (H, R), *M.azedarach* extract reduced the H_2O_2scavenging activity, in a more pronounced way by the ethanolic ones and in radicle (IC_{50}: 0.064 mg/mL) (Figure 9B'). All tested extracts showed a high activity compared with that of ascorbic acid (IC_{50}: 0.06 mg/mL).

Aqueous *M. azedarach* leaves extract $OH^{\bullet}$ scavenging activity (0.51 mg/ml) was higher than that of ethanolic one (1.21 mg/ml) (Fig. 9C): The radish cotyledon treated by aqueous extract showed the highest $OH^{\bullet}$ scavenging activity with IC_{50}= 0.058 mg/mL. In growing organs (H, R),*M.azedarach* extracts -notably ethanolic one- reduced the $OH^{\bullet}$ scavenging activity especially in radicle (IC_{50}: from 0.061in control to 0.054 mg/mL in treated radicles) (Figure 9C').

The Metal chelating activities of *M. azedarach* aqueous leaves extract (0.99 mg/ml) was higher than that of the ethanolic one (1.15 mg/ml) in comparison withascorbic acid (IC_{50}: 0.057 mg/ml) (Figure 9D).In radish, *M. azedarach* extracts reduced this activity in cotyledons but increased it in hypocotyls and radicles. The results of these experiments are summarized in Figure 9'D. It was found that aqueous extracthave the highest hydroxyl radical scavenging activityin hypocotyls with an IC_{50} at 0.052 mg/mL followed by ethanolic extract (IC_{50} at 0.053 mg/mL in hypocotyls) when compared with other compounds as well as standard (IC_{50}: 0.0062 mg/mL).

The ethanolic extract was more pro-oxidant than the aqueous one: *M. aezdarach* extracts exhibited a lower reducing ability than that of ascorbic acid (IC_{50} 0.003 mg/mL). Reducing ability of ethanolic extract (IC_{50} 0.150 mg/mL) was approximately twice as that of the aqueous one (IC_{50} 0.072 mg/mL).The ethanolic extract was more pro-oxidant than the aqueous one as the index of this later was lower than those of the first one (Table 1).

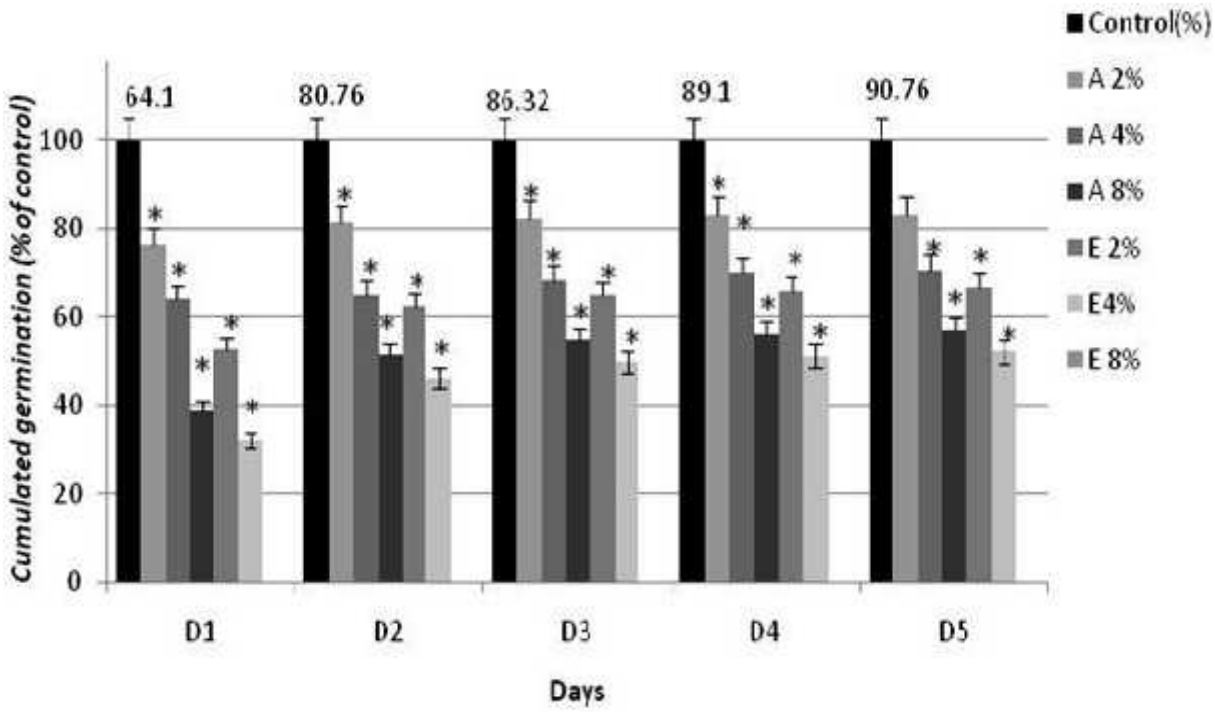


Figure.1. Cumulated germination (% of control) of radish seeds treated with aqueous and ethanolic *Melia azedarach* leaves extracts. All values represent means ± SEM. Data labels are added for control (mg) and asteriks indicate significant differences compared to control p<0.05.

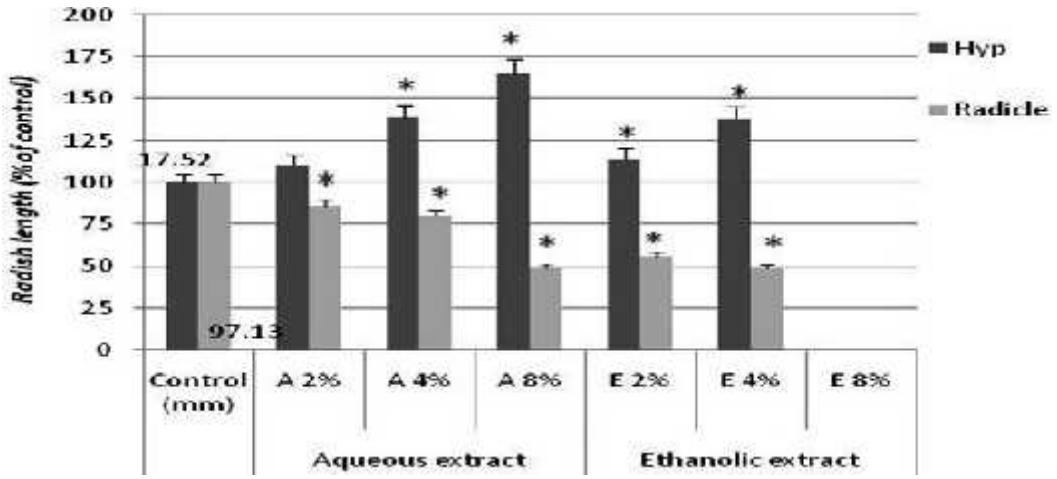


Figure.2.Radish length (% of control) of radish organs (hypocotyls and radicle) treated with aqueous and ethanolic *Melia azedarach* leaves extracts. All values represent means ± SEM. Data labels are added for control (mm) and asteriks indicate significant differences compared to control p<0.05.

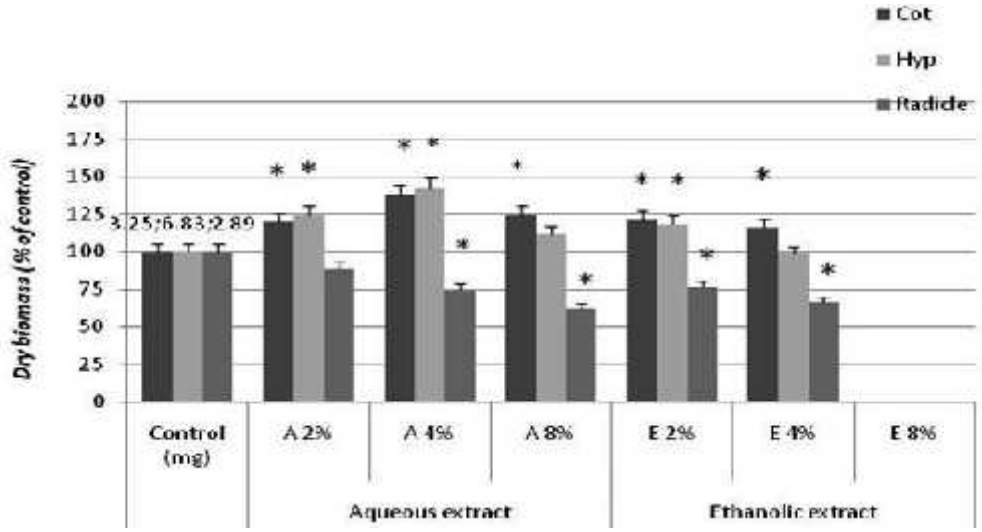


Figure.3. Dry biomass (% of control) of radish organs (cotyledons, hypocotyls and radicle) treated with aqueous and ethanolic *Melia azedarach* leaf extracts. All values represent means ± SEM. Data labels are added for control (mg) and asteriks indicate significant differences compared to control p<0.05.

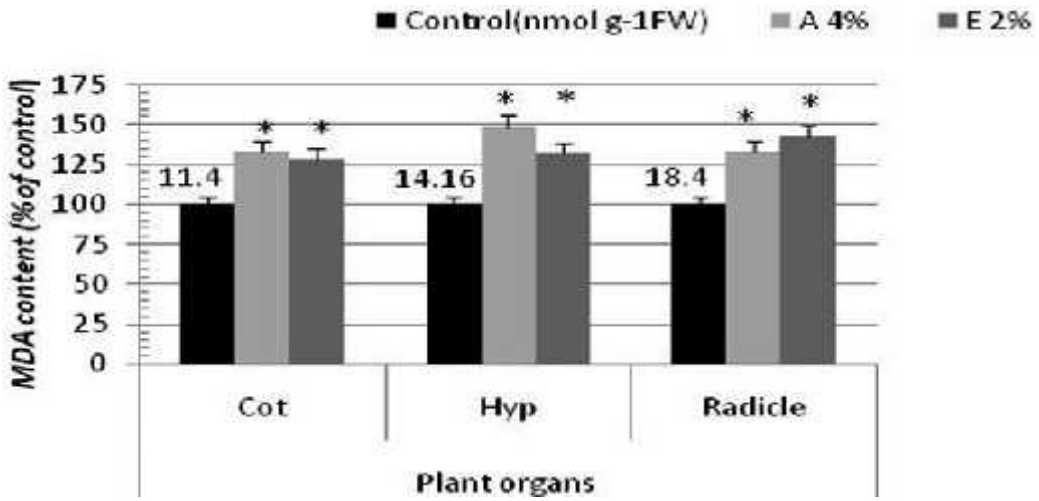


Figure.4. Malonedialdehyde MDA content (% of control) of radish organs (cotyledons, hypocotyls and radicle) treated or not (Control) with aqueous and ethanolic *Melia azedarach* leaf extracts. All values represent means ± SEM. Data labels are added for control (nmol g-1FW) and asteriks indicate significant differences compared to control p<0.05.

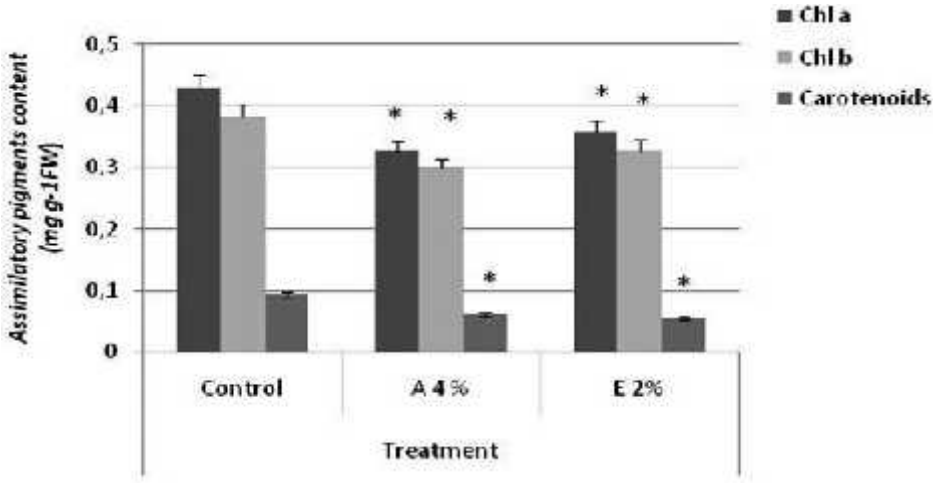


Figure. 5. Assimilatory pigments content (mg g^{-1}FW) of radish cotyledons, treated or not (Control) with aqueous and ethanolic *Melia azedarach* leaf extracts. All values represent means ± SEM. Asteriks indicate significant differences compared to control p<0.05.

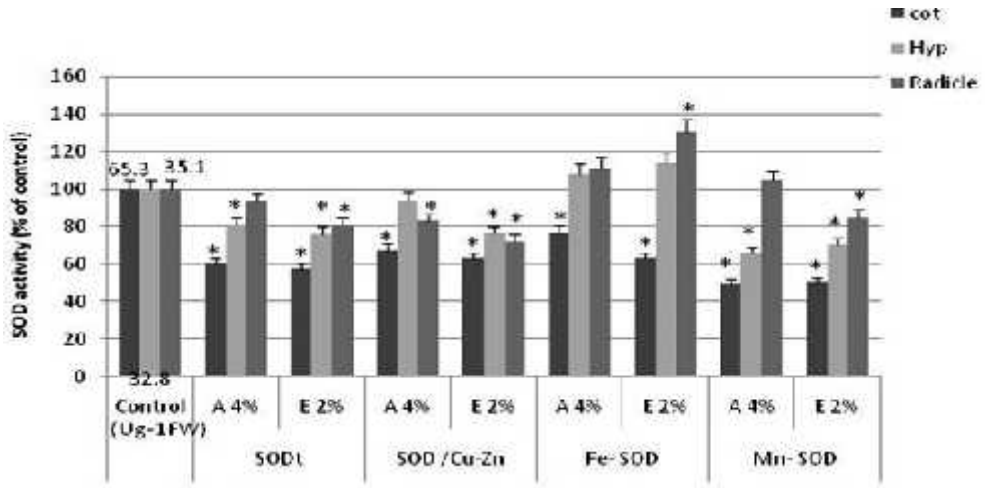


Figure.6. Total Superoxide dismutase activity SOD and its isoforms (% of control) of radish organs (cotyledons, hypocotyls and radicle) treated with aqueous and ethanolic *Melia azedarach* leaf extracts. All values represent means ± SEM. Data labels are added for control (mg) and asteriks indicate significant differences compared to control p<0.05.

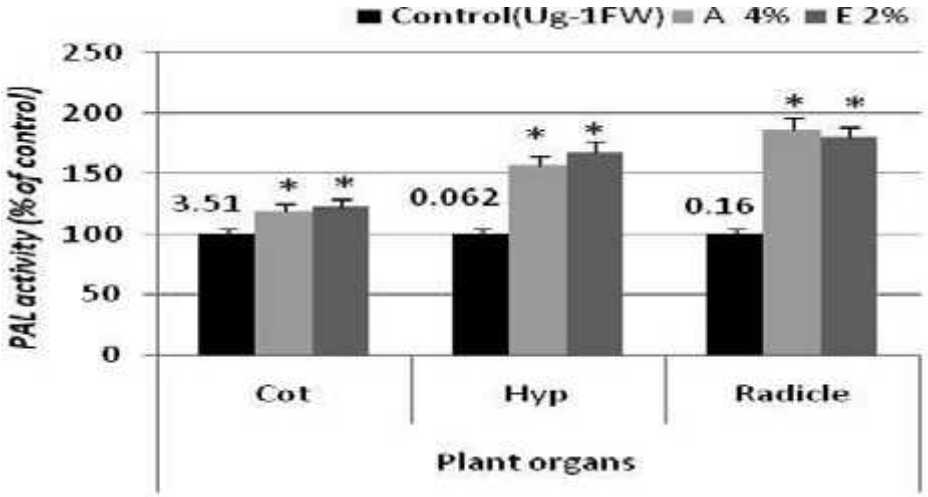
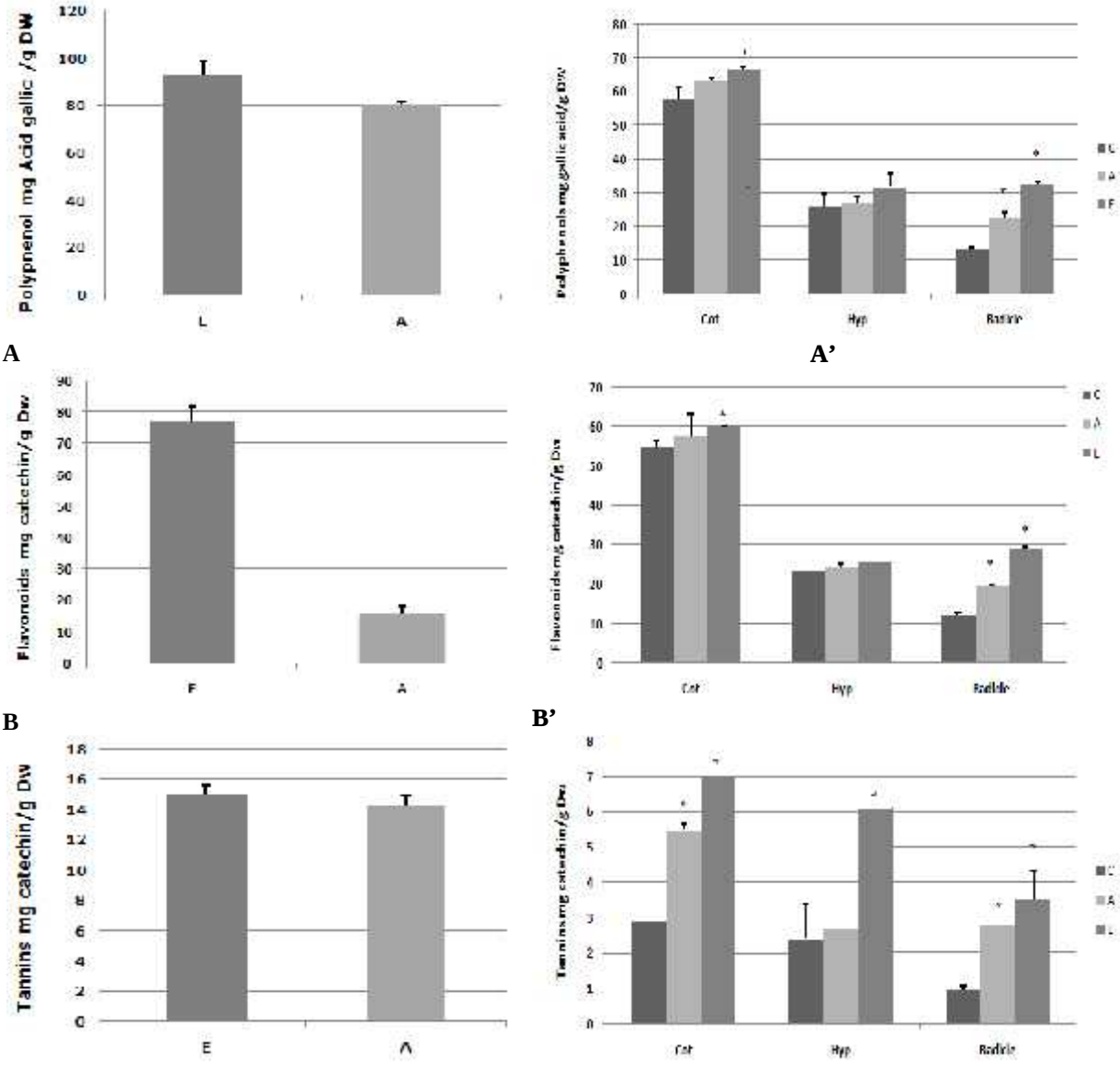


Figure.7. Phenyl-ammonia-lyase activity PAL (% of control) of radish organs (cotyledons, hypocotyls and radicle) treated with aqueous and ethanolic *Melia azedarach* leaf extracts. All values represent means ± SEM. Data labels are added for control (U g-1FW) and asteriks indicate significant differences compared to control p<0.05.

***Melia azedarach* extracts** ***Raphanus sativus* submitted to *Melia azedarach* extracts**



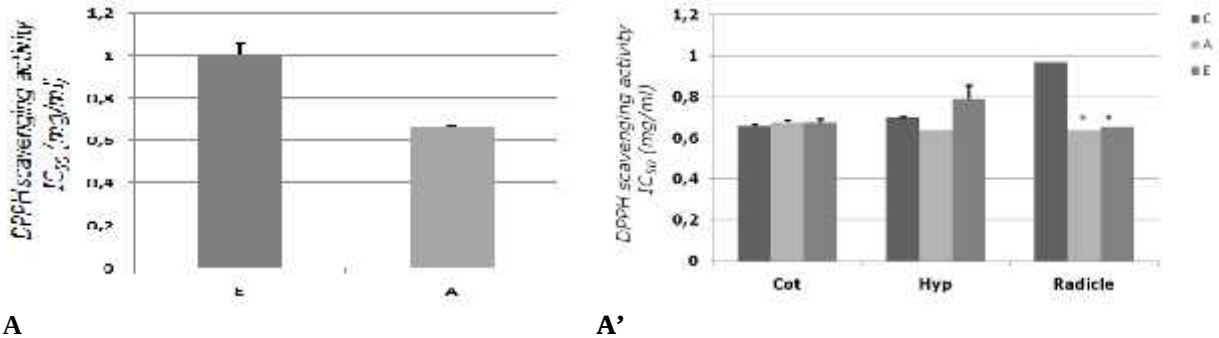
A **A'**

B **B'**

C **C'**

Figure.8.Total phenols (mg GA g^{-1} DW) (A,A'); flavonoids (mg C g^{-1} DW) (B, B') and tannins (mg C g^{-1} DW) (D,D') contents in *M. azedarach* extracts (A B, C,) and radish plantlets organs (A', B' C') (cotyledons, hypocotyls and radicle)(cotyledons C, hypocotyl H and radicle) treated with*M. azedarch* leaves extracts (Aqueous, A; ethalonic , E) or not(control, C). All values represent means ± SEM. Asteriks indicate significant differences compared to control p<0.05.

***Melia azedarach* extracts** ***Raphanus sativus* submitted to *Melia azedarach* extracts**



A **A'**

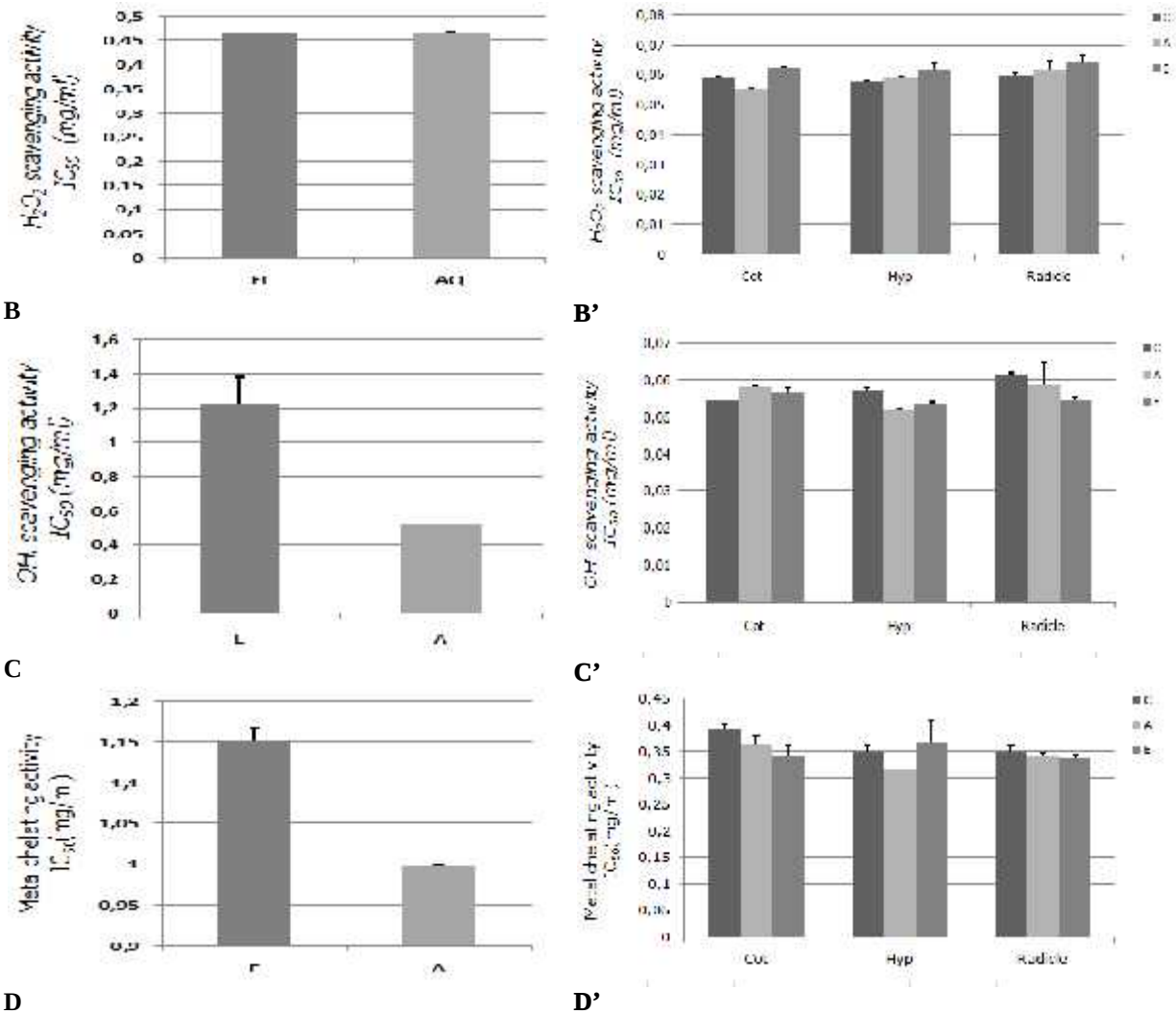


Figure.9. DDPH scavenging activity (A, A'), H_2O_2 scavenging activity (B,B'), OH^- scavenging activity (C, C') and metal chelating activity (D,D') (IC $_{50}$, mg/ml) of *M. azedarach* extracts A, B, C and D and radish plantlet (A', B', C' and D') different organs (cotyledons C, hypocotyl H and radicle) treated with *M. azedarch* extracts (Aqueous, A; ethalonic , E) or not (control, C). All values represent means ± SEM. Asteriks indicate significant differences compared to control p<0.05.

Table 1. Antioxidant (DPPH scavenging activity, IC $_{50}$, mg/ml) pro-oxidant (Reducing ability, IC $_{50}$, mg/ml) activities and Index (Pro-oxidant/antioxidant) of *M. azedarach* extracts (Aqueous, A; ethalonic , E) standards (ascorbic acid). ProAntidex was devised using the ratio of pro-oxidant to the antioxidant activities. All values represent means ± SEM.

	DPPH scavenging activity (IC_{50}mg/mL)	Reducing ability (IC_{50} mg/mL)	Index (Pro-oxidant/ antioxidant)
***M.azedarach* ethanolic extract**	1.001±0.054	0.15±0.022	0.148±0.015
***M azedarach* aqueous extract**	0.0667±0.004	0.072±0.005	0.107±0.009
Ascorbic acid	0.039±0.002	0.003±0.000	0.09±0.005

DISCUSSION

The use of phytotoxicity of allelochemicals in the control of weeds needs identification of the responsive molecule (s) and sufficient information to a better understanding of their mode of action.

We used *M. azedarach* leaves extracts (aqueous, ethanolic) on the standard target specie, *Raphanus sativus* to precise the mode of action during early developmental stages.

***M. azedarach* leaf extracts toxicity on radish germination and seedling growth:** *M. azedarach* leaf extracts inhibited the germination of radish depending on the dose taken and more especially on the ethanolic extract than on the aqueous one. These observations were similar to those reported by Lungu

et al. (2011) using the aqueous and ethanolic extracts of *M. azedarach* leaves but lettuce as receptor plant. The aqueous extract of *M. azedarach* leaves was effective towards weeds (*Echinochloa crus-galli* and *Phaseolus lathyroides*), the germination and seedling growth inhibition was also dose dependent but it varied from one species to another (Phuwiwat *et al.* 2012). During germination, allelochemicals toxicity concern mainly reactivation of the seed metabolism by inhibiting enzymes implicated in the breakdown of the stored reserves (Kato-Noguchi and Marcia, 2005) and respiration (Muscolo *et al.*, 2001). In our assays, and in radish submitted to *M. azedarach* extracts, the reserve organ (cotyledons) dry weight was (according to extracts concentration) unmodified and even superior to that of control reflecting a reduced reserve mobilization. Aqueous extract of *M. azedarach* leaves inhibited alpha amylase activity in the weed *Phaseolus lathyroides* (Phuwiwat *et al.*, 2012); phenolic acids might be implicated (Baleroni *et al.*, 2000). In cotyledons, photosynthesis also contributes to seedling development (Zheng *et al.*, 2011). In our experiment, the *M. azedarach* allelochemicals might also affect the radish embryo growth through the observed reduction of cotyledons photoreceptors (chlorophylls and carotenoids) content. Photosynthesis rate had been shown to be affected by allelochemicals as phenolics (Li *et al.*, 2010). As a consequence, *M. azedarach* allelochemicals effects on cotyledons lead to a reduced organic nutriments flow to the radish embryo. In this later, the growing organs respond differently to allelopathic stress: the growth of roots was inhibited whereas that of hypocotyl was stimulated. Thus might involve interaction between allelochemicals and hormone, phenolics have been shown to remove the inhibition of hypocotyl growth of *Amaranthus caudatus* seedlings induced by abscisic acid (Ray *et al.*, 1984). This differential action of *M. azedarach* on growing organs might be partly explained by a reduced mitotic index of roots whereas that of shoot meristem is largely unaffected (Gatti *et al.*, 2010). This inhibition of root growth by allelochemicals has been in fact the subject of some studies (Itani *et al.*, 2013).

***M. azedarach* extracts induced rhizotoxicity and variations in oxidative status of radish plantlets:** Apart from their specific biological action (s) related with their chemical classes, allelochemicals present a common oxidative component by generating ROS (Darier and Tamman, 2012). These later are involved both in activating the antioxidant defence and phytotoxic action (Weir *et al.*, 2004). *M. azedarach* leaves extracts reduced, in radish organs, activity of SOD, the first line of enzymatic defense. In our experiment and in radish**,** allelopathic stress was probably deep as it inhibited SOD activity, thus led to the accumulation of $O_2^{\bullet-}$and subsequent damage as shown by the increased MDA content in radish organs plantlets. Allelochemicals effects on ROS detoxifying enzymes vary stress intensity (with concentration and/or exposure time.) In *Phaeodactylum tricornutum*, allelochemicals effect increased the activity of SOD, CAT, with the exposure concentration but decreased it with the exposure time prolongation (Yang *et al.*, 2011). In maize, CAT and GPX activities decrease at high concentration of aqueous extract of *Nicotiana plumbagini folia* (Singh *et al.*, 2009). On the other hand, *M. azedarach* leaf extracts induced**,** in the receiver plant, the stimulation of PAL and -as a consequence- an increased phenols' content that might contribute to the rise in DPPH scavenging capacity of radish plantlet**.** In plants, phenols act as antioxidant by donating electrons to peroxidase for the detoxification of H_2O_2 (Shakihama *et al.*, 2002). The stimulation of PAL activity has been observed by Herrig *et al.* (2002) in soybean submitted to ferulic acid and is thought to support an increase in lignin synthesis (Shann and Blum, 1987) and subsequently a reduction in the root growth as observed in radish submitted to *M. azedarach* extracts.

Moreover, phenols as gallic acid and catechin are respectively used by the invasive species *Phragmites australis* (Rudrappa *et al.*, 2007) and *Centaurea maculosa* (Bais *et al.*, 2003) to exert their rhizotoxicity. This ROS mediated allelochemicals action both trigger activation of gene implicated in cell death and oxidative stress. In addition, allelochemicals as catechin exhibit pro-oxidant activity that directly contributes to the death of roots **(**Kaushik *et al.*, 2010). This might occur in the studied invasive *M. azedarach* whose ethanolic extract- that exhibited higher rhizotoxicity in radish- was more pro-oxidant than the aqueous one. This might partly be due to a higher phenolic content implicated in -*M. azedarach* rhizotoxicity.

Conclusion: To conclude, our results showed that leaves of *M. azedarach* contain allelochemicals whose toxicity led to the inhibition of the seedlings roots growth of radish, the receiver species. Thus occurs partly via an induced oxidative stress whose intensity exceeded the antioxidant capacities of the radish as shown by the inhibition of SOD, the first line of enzymatic defense, thus leaded to $O_2^{\bullet-}$ accumulation and oxidative damage occurrence. The increased phenols content did not counteract the *M. azedarach* induced oxidative stress and might even contribute to its toxicity. *M. azedarach*, might be used as a "root killer" bioherbicide in the form of aqueous extract whose dose must be adjusted to field conditions. This very cheap bioherbicide will valorize, in Tunisia, this widespread invasive species that in spite of its deciduous character, product important leaves biomass. On the other hand, our study discusses treated *R. sativus* antioxidant effects *in vitro*. In fact, antioxidant potentialities of *M. azedarach* were investigated and proved by many earlier studies such as Nahak and Sahu (2010), Orhan

et al. (2012) and Akacha *et al.* (2016).Whereas; our data investigate for the first time, the antioxidant capacities of treated *R.sativus by M. azedarach*. Our results showed that *M. azedarach* treatment seemed to be harmful to the survival of the native plant (*Raphanus sativus* roots) by creating an oxidative stress *in vivo*; however, it has been advantageous on their therapeutic properties (as antioxidant) *in vitro*. *M.azedarach* treatment reinforced the antioxidant capacities of radish which may account for the mechanism of action of the extract as an antioxidant. Interestingly, invasive species could be used not only as bioherbicide by allelochemicals mechanism but also to enhance medicinal proprieties of natural received plants.

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