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Identification and characterization of chemical constituents of *Buxus sempervirens* and study of their larvicidal activity against *Galleria mellonella*

Hakima Oulebsir-Mohandkaci*, Sabrina Ait Kaki- Ait Slimane, Kaissa Boudiab, Farida Tihar-Benzina and Intissar Ghaffar

Laboratory of Valorization and Conservation of Biological Resources, Faculty of Sciences, University M'hamed Bougara of Boumerdes, 35000 Boumerdes, **Algeria**

***Correspondence:** h.mohandkaci@univ-boumerdes.dz **Received 11-02-2021, Revised: 22-04-2021, Accepted: 24-04-2021 e-Published: 01-05-2021**

The purpose of the present research work is exploiting of ornamental plant *Buxus sempervirens* in the biological control of the wax moth *Galleria mellonella*. Indeed, after Phytochemical Screening of aerial plant parts, we proceeded to the preparation of aqueous and methanolic extracts and alkaloids of boxwood. This was followed by the determination of total phenol contents (TPC) with the study of certain physicochemical parameters of the plants. The characterization and identification of the chemical constituents of boxwood were carried out by High-performance liquid chromatography (HPLC). Finally, *in vitro* and *in vivo* tests were carried out to determine the toxicity of the phytocompounds prepared against *G. mellonella* larvae.As results; the aerial parts of the Boxwood are rich in phytocompounds such as alkaloid, tannins and flavonoids. The methanolic extract of the polyphenols gave a yield of 29%, with 17% for aqueous extract. For alkaloids, the yield was 2.36%. HPLC allowed identifying several bioactive molecules such as catechin, rutin and gallic acid. The bioactive substances studied have a high larvicidal activity against wax moth. For the methanolic extract, an increase in mortality was recorded in larvae treated with all four doses. The alkaloids caused a highest mortality. In parallel, a significant increase in the concentration of proteins and lipids and a decrease in the concentration of carbohydrates in the haemolymph, were recorded after the injection of the aqueous extract of boxwood. As a result, the tested boxwood extracts cause a disruption of the biochemical metabolism and the physiology of the insect.

Keywords: Boxwood, wax moth, phytocompound, HPLC, biological control.

INTRODUCTION

The *Buxaceae* are a botanical family with five genera and 120 species, the largest genus *Buxus* has a disjunct intercontinental distribution, originally described as genus *Tricera* (Scherber, 1791) (EgonKöhler, 2005). This genus is comprised of about 70 different species (Saleem et al. 2020). Among the different species of boxwood, *Buxus sempervirens* is one of the most popular and commonly grown types in Algeria (Ait kaki-Ait Slimane et al. 2015). It is an evergreen shrub often used in urban plantings intended for forming the trimmed hedges, as well as for creating geometrical shapes in built landscapes and historic gardens (Batdorf 2004; Kurzawińska et al. 2019).They also have significant economic value, so, these plants represent a greatest proportion of sales among broadleaf evergreens in several countries in the world (Leblanc et al. 2018). Boxwood also plays an important role in forestry, where it is used to shore up unstable and stony soils. The production of its wood is of some economic interest (Eickermann et al. 2019; Naneli et al. 2020). Boxwood is also used as a medicinal plant due to the various substances contained in its leaves and bark (Eickermann et al.2019). In the world, extractions obtained especially from the leaf and bark parts started to be used in traditional treatments many years ago. Some active compounds are extracted from this plant and used in formulation of the drugs used in diseases in humans. It contains also alkaloids mainly buxine, which give it a toxicity against insects (Nedelec, 1993).This finding suggest that this plant could be the source of botanical biopesticides conception.

On the other hand, the great wax moth *Galleria mellonella* (Lepidoptera- Pyralidae) is an insect widely frequent in the world, causing serious problems in beekeeping such as the wax destruction and pathogens transmission to bees (Oulebsir-Mohandkaci et al. 2018). To protect the bee colony and hive products against this pest, it is appropriate to use a biological control agents like phytocompounds. Natural plant insecticides have several advantages over synthetic compounds because of their rapid biodegradation and the reduction of risks to the environment (Park, 2003). Among the botanical biomolecules already used against wax moth, we cite, extracts of coriander (*Coriandrum sativum*) and false fennel (*Aneth graveolens*) (Lalita et al. 2018; Oulebsir-Mohandkaci et al. 2018; Akkus et al. 2019, Kaya and Demir, 2020).

Unfortunately, scanty research has been carried out on the bioactivity of Phytocompounds of different parts of boxwood against insects pests especially wax moth. So, the present work has been focused on qualitative and quantitative analysis of various bioactive compounds of boxwood as well as their larvicidal activity on *Galleria mellonella*.

MATERIALS AND METHODS

Plant material, collection and processing

Fresh and healthy aerial plant parts (leaf lamina, stem and petiole) of boxwood were collected in 2020 on the territory of the garden of the faculty of sciences of Boumerdes (region of Northern Algeria; latitude: 36° 46′ 00″ North, longitude: 3° 28′ 0″ East, altitude 02) (Fig.1). Plant parts were cleaned cut into small pieces and shade dried at room temperature and grounded to fine powder to store in air tight bags until used (Paris and Moyse, 1976).

Figure 1: Plant material; a: boxwood plant in the sampling site; b: leaves of *B. sempervirens*

Phytochemical Screening

This qualitative test allows to determine the various chemical compounds present in the plant by coloring and precipitation reactions. It can be carried out on the powder or on the infused at 20%. The researched molecules are total tannins, gallic tannins, catechin tannins, alkaloids, starch, flavonoids, anthocyanins, leucoanthocyanins, saponosides, mucilages, glucosides (Tona et al. 1998; Longaga et al.2000, Shafi et al. 2021).

Extracts preparation

Aqueous extraction

Aqueous extract of dried boxwood leaves was made in the distilled water. 50g of the plant leaf powder were stored in a 500 ml conical flask and 300 ml of distilled water was added. The mixture was shaken and left to macerate at room temperature for 24 hours. The extract was filtered through filter paper then the solvent from the extract was removed by using rotary vacuum evaporator. The recovered filtrate (aqueous extract) was transferred into shaded glass pill boxes (Bruneton, 1999).

Methanol extraction

The extraction of phenolic compounds from the plant passes through the steps described by Revilla et al. (2001) and Ojeil et al. (2010) After maceration in methanol, filtration is carried out followed by Rotavapor extraction at 70 °C for 20 minutes to remove the methanol (Nowsheen et al. 2020).

Yield calculation

After extraction, we calculate the yield by the following formula:

$R\% = (M-M0 / Mt) X100$

Such that: R%: rate of the extracted material - M: mass of the balloon with the extract M0: mass of the empty balloon - Mt mass of the balloon with the extract after drying.

Extraction of alkaloids

The maceration took place in 10% H_2SO_4 for 24 h at room temperature, after filtration and alkalinization at pH = 9 with 30% NaOH, a liquid-liquid extraction was carried out with chloroform. The combined organic phases were dried over anhydrous sodium sulphate and then filtered and the solvent of the remaining solution was evaporated. Finally alkaloids were recovered using a spatula and then they were transferred into shaded pillboxes, glass and they were then stored in a refrigerator at 4 °C. The yield of alkaloids in the methanolic extract was deduced by the formula previously described (Bfaye, 1998; Huber 1998, Vercautern 2001).

Total Phenol contents (TPC)

TPCs of methanolic extracts were determined by colorimetric assay, using Folin-Ciocalteu according to the method described by Singleton and Rossi's (1965). 200µl of each previously diluted extracts (methanolic and aqueous extract individually) was mixed with 1 ml of Folin Ciocalteu diluted 10 times and 0.8 ml of 7.5% sodium carbonate. The test tubes were then shaken and kept for 30 minutes in the dark. The absorbance was read at 765 nm using a spectrophotometer (Maqbool et al. 2021).

High-performance liquid chromatography HPLC

UV HPLC detection was carried out at the Center for Research and Techniques for Physical and Chemical Analysis CRAPC, Algeria. The aqueous extract obtained is then centrifuged at 1000 rpm for 10 minutes to get rid of plant debris. The aqueous phase of the macerate was then filtered through filter paper. The resulting filtrate is then placed in tubes and stored for analysis HPLC (Bruneton, 1999). The analysis was performed by a liquid chromatograph of the type YL 9100 HPLC Ultra-violet detector. The column type is Agilent C 18 post grafted of 150 mm length and the diameter of 4.6 mm/5 microm. , the flow of both mobile phases is 1 ml/min, the injection volume is 20 µl, the column temperature was maintained at 25°C. UV-detection at 254 nm /280 nm (Mobile phases: phase A- Acetic acid 1% in water, phase B- methanol 5% grade HPLC) (Hossain et al. 2014).

Water content analysis

Three batches of fresh leaves were taken in order to measure the water content of the leaves. Each batch was subjected to drying at 105 °C in an oven. Let Wf be the weight of the fresh sample and Wd the weight of the sample after drying, the water content of the fresh leaves was given by the formula:

Water content (%) = (Wf-Wd) / Wf * 100

Chlorophyll content

The concentrations of chlorophyll a, chlorophyll b, chlorophyll a+b and carotenoids were measured according to the protocol of Mackiney (1941).

During this analysis, first 100 mg of fresh plant material (the leaves) were ground in 10 ml of 80% acetone. Centrifugation at 3000rpm was followed by reading the absorbance of the supernatant at three different wavelengths: 470nm, 645nm and 663nm. The concentrations of the different pigments are given by the following formulas:

Chlorophyll a = 12 * DO663-2.67 * DO645.

Chlorophyll b = 22.5 * DO645-4.68 * DO663.

Chlorophyll a + Chlorophyll b = 7.15 * DO663 + 18.71 * DO645.

Carotenoids = (1000 * DO470- (1.82 * DO645) - (85.02 * DO663)) / 198.

Susceptibility of *G. mellonella* larvae to the phytocompounds of Boxwood

Dose-mortality effect of Boxwood against G. mellonella- In Vitro essay

Three extracts were prepared; aqueous extract, methanolic extract and alkaloids with four doses for each (D1 = 15µl / ml, D2 = 30 µl / ml, D3 = 60µl / ml and D4= 120 µl / ml). After this, we proceeded to the application of biological treatments of wax moth larvae of 5th stage. A positive control was used; it is the marketed biopesticide B401 formulated with the spores of *Bacillus thuringiensis* berliner.

The daily mortality rate was evaluated in controls and treated individuals of greater wax moth during 10 days for polyphenols and aqueous extract treatments and 5 days only for alkaloids treatment. After this, the observed mortality were corrected using the formula of Abbot (1925) and LT50 with LD50 were calculated.

Insecticidal effect under semi-controlled conditions- In Vivo essay

To perform this test, the LD90 obtained in the In Vitro tests were applied. To do this, beaten squares from healthy hives were infested with larvae of the same age and sprayed with the various extracts previously adjusted to the desired doses. Some boxes are reserved for treatment with B401 and others for controls.

Study of the biochemical composition of the haemolymph

The larvae received a volume of 20 µl of extract by injection into their haemolymph, the latter was taken, 2h, 4h and 8h after injection.

For quantitative determination of proteins, the Bradford method was applied by the use of a dye, Coomassie brilliant blue G-250, which forms with the protein a colored complex, having a maximum of absorption at 595 nm. (Bradford, 1976).

In order to carry out quantitative determination of carbohydrates, 0.15 g of anthrone must be dissolved in 75 ml of sulfuric acid, then 25 ml of distilled water were added. The Glucose (1 g / l) was used as standard (Bachelier and Gavinelli, 1966). Finally, the haemolymphatic lipid assay was performed according to Goldsworthy et al. (1972) using the sulfophosphovanillin reagent.

Statistical Analysis

All the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The differences between the different extracts were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's significant difference post hoc test and value $p < 0.05$ was considered significant. In addition, boxplot is a method witch they display variation in samples of a statistical population. Outliers may be plotted as individual points. This treatment was carried out using SPSS v. 25.0 programs.

RESULTS

Phytochemical characterization

The aerial parts of the Boxwood are rich in alkaloid, but also in tannins, mucilages, saponosides and glucosides. They are moderately rich in flavonoids with low anthocyanin content. As, it is also starch-free.

The presence of tannins was determined with a dilute 5% solution of ferric chloride which reacts with these compounds to give a dark blue color (gallic tannin) or red color (catechin tannin). The sample analyzed has a high content of alkaloids, the latter were investigated using the Dragendorff reagent on the aqueous solution of sulfuric acid.The detection of saponosides was carried out by the formation of a white precipitate after the addition to 2 ml of infuse a few drops of lead acetate (Table 1).

Table 1: Phytochemical characterization of leaf powder of *Buxus sempervirens*

Compound sought	Reaction	Obtained results
Total tannins	Dark blue coloration	+
Gallic tannins	Deep blue coloration	+
Catechin tannins	Red coloration	+
Anthocyanins	Red coloration	+
Leucoanth ocyanins	Purplish red coloration	-
Saponisides	White precipitation	+
Irridoïdes	Blue coloration	+
Alkaloids	Red precipitation	+
Flavonoids	Red orange coloration	+
Senosides	Purplish red coloration	-
Quinines	Red coloration	-
Coumarins	Formation of a trouble(flutter)	+
Mucilage	Flaky precipitation	+
Glycosides	brick-red coloration , then violet	+
Starch	Violet blue coloration	-

(-): absence of substances; (+): presence of substance

Yield Extraction

The methanolic extract of the polyphenols from the powder of the leaves of boxwood gave a yield of 29 % by cons the aqueous extract gave 17 %. For alkaloids, the yield of aqueous extract is 2.36%. This richness is in direct relation with the nature of the soil, the climate, the age of the plant, the period of harvest and the nature of the solvent extraction.

Total phenolic content

TPCs of the aqueous extract from the boxwood revealed an absorbance of 0.050 which corresponds to a low concentration of polyphenols with 0.23 mg / ml eqgallic acid.

Results of HPLC

Identification of the compounds of the the aqueous extract was carried out using the chromatogramme of chemical standards that have been analysed at the same operatory conditions.

The compounds identified with the retention time (RT) compared to those of the standards, peak area are shown in the figure 2. This extract is mainly composed of trans 2.4 dimethoxy cinnamic acid (16%), catechin (12.5%), rutin (13.3%), Berberine (6.3%), Ferulic acid (08%), Trans 2.4 dimetoxycinnamique acid (6.7%), gallic acid (4.9%) and isovalinic acid (3.3%).

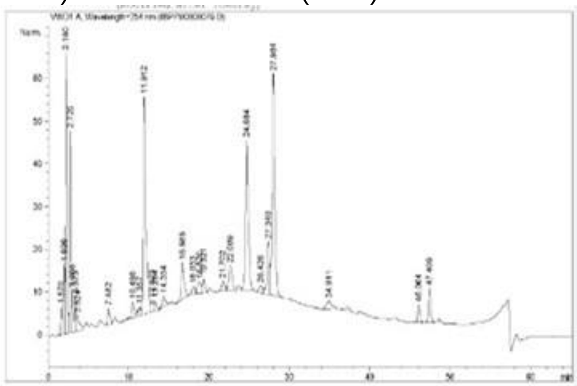

Figure 2: HPLC Chromatogram obtained of aqueous extract of Boxwood

Physicochemical characterization of Boxwood

Water content in leaves

60% is the water content of the fresh matter of Boxwood. To interpret this result, one must take into account the anatomy, physiology, and root-aerial part interaction of the plant.

Chlorophyll content

Indeed, the main fraction of pigments is chlorophyll A followed by chlorophyll B. Carotenoids represent the lowest fraction of the content of assimilating pigments in boxwood (Fig.3).

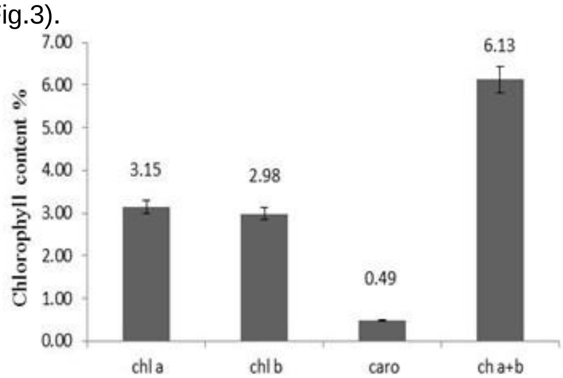

Figure 3: Rate of assimilating pigments in the different samples of boxwood (chl a = chlorophyll a, chl b = chlorophyll, caro = Carotenoids, chl a + chl b = chlorophyll a + chlorophyll b).

Study of the toxicity of *B. sempervirens* on the larvae of *G. mellonella*

The mortality rates obtained after treatment of *G. mellonella* larvae with boxwood polyphenols and alkaloids are shown below. The figure 4 indicates for the aqueous extract, the mortality rates were staggered in time but the dose-response relationship was not well manifested, the highest rate was obtained with D2, the tenth day with 90%. For the methanolic extract, a slight increase in mortality was recorded in individuals treated with all four doses during the first two days. Then this rate increases to reach 100% for D4 120µl/ml in 5 days against 95% recorded the same day with doses D2 and D3. Finally, the D4 alkaloids caused a high mortality from the first day and after only 4 days more than 90% mortality was already recorded for the 4 doses tested.

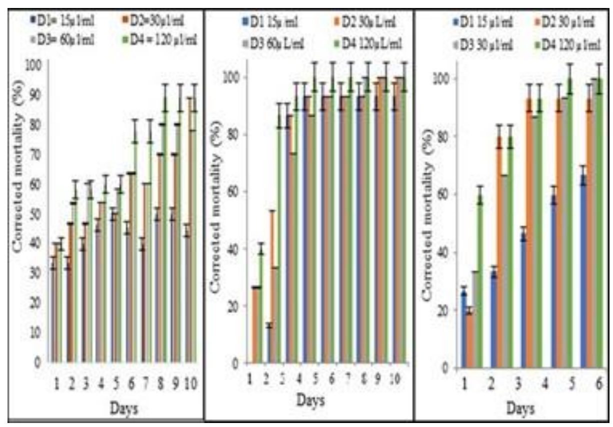

Figure 4: Corrected mortality rate in individuals of *G. mellonella* treated with: a. aqueous extracts, b. methanolic extracts and c. alkaloid extracts of boxwood (P < 0.05)

Alkaloids have shown a rapid and significant effect and the different components of these batches treated with the different doses are very homogeneous (fig. 5).

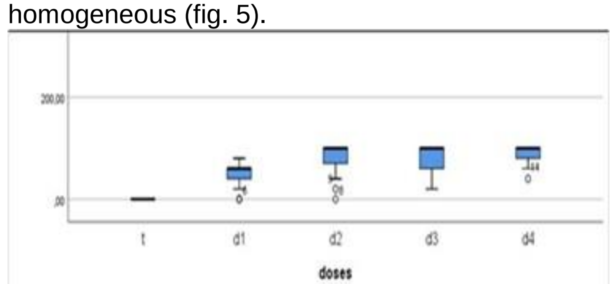

Figure 5: Boxplot of mortality rate obtained after treatment of *G. mellonella* larvae with Boxwood Alkaloids

From the results, it appears that the TL 50 varies between 9 and 1 day depending on the extract and dose tested. Alkaloids showed the lowest TL50 (1.04 to 2.95 days) followed by methanolic extracts and the highest value (9.35 days) was obtained with the aqueous extract (table 2).

Table 2: TL50 values (days) in *G.mellonella* treated larvae treated with boxwood extracts

DOSES	Aqueous extract	Methanolic extract	Alcaloids
D1 15 µl /ml	9.35	4.08	2.95
D2 30µl/ml	2.64	1,5	1.42
D3 60 µl/ml	2.06	1.94	1.44
D4 120 µl/ml	2	1.44	1.04

Lower lethal doses are obtained with the methanolic extract with an LD50 of only 5.62 µl/ml after 4 days (table 3).

Table 3: LD 50 and LD 90 values in *G.mellonella* treated larvae of the three boxwood extracts

Aqueous extract		Méthanolic extract		Alcaloids	
DL50	DL 90	DL50	DL 90	DL50	DL 90
18,92 µl/ml	68 µl/ml	5.62 µl/ml	57.54µl/ml	10.45µl/ml	54.56µl/ml

LD50s are calculated for results obtained 4 days after treatment.

against *G. mellonella* compared to B401.

The calculation of LD 90 for each applied extract was followed by its application to the larvae of the wax moth under semi-controlled conditions. Daily mortality counts were performed for 6 days and the results were compared to those obtained after treatment with the reference product B401. From the results obtained we noticed that boxwood marked its effectiveness with a mortality rate higher than that of B 401 (80% for alkaloids and 70% for methanolic extract) (fig. 6).

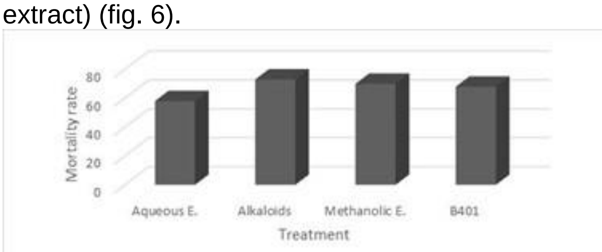

Figure 6: Mortality rate of boxwood polyphenols and alkaloids compared to B 401 (P < 0.05)

Effect of Boxwood on the haemolymphatic composition of G. mellonella larvae

The taking of the hemolymph was performed 2h, 4h and 8h after injection of the aqueous extract of Boxwood (D = 30µl / ml) to the larvae. The concentrations of proteins, carbohydrates and haemolymphatic lipids obtained in different in time in the control (T) and the treated individuals by the aqueous extract of boxwood, indicate that for protein, this concentration has increased over time and is higher in treated compared to controls. In contrast, for haemolymphatic carbohydrates, the levels decreased slightly compared with the control. Finally, the concentration of the haemolymphatic lipids was almost identical with the control (fig. 7).

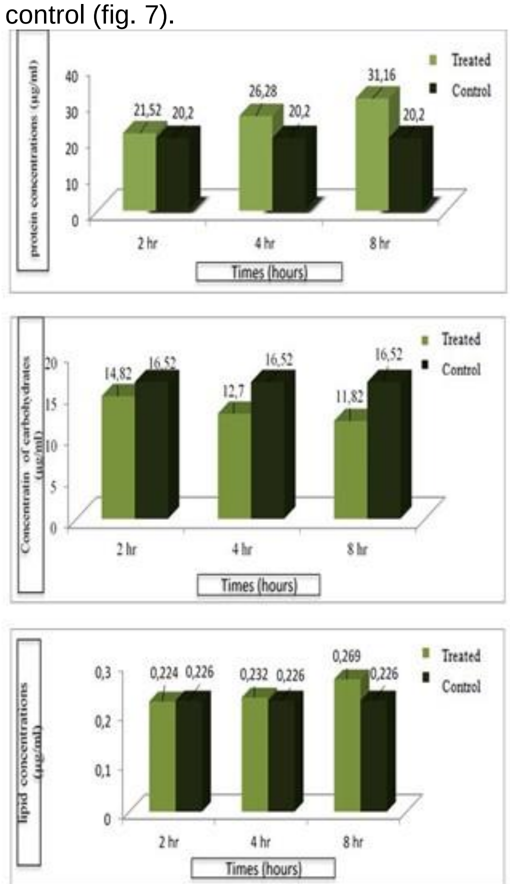

Figure 7: The biochemical composition of haemolymph in individuals treated with boxwood aqueous extract.

DISCUSSION

The boxwood can be found growing naturally in various areas in the world but is mainly cultivated as an ornamental plant (Kurzawińska et al., 2019). Many studies carried out about *Buxus sempervirens* (common boxwood), indicate that some active substances are identified and used in different applications (Ait slimane-Ait Kaki et al. 2015). The phytochemical screening performed shows the major presence of total tannins, saponoside, alkaloid, flavonoids and others constituents.

Indeed, the methanolic extract obtained from the powder of the boxwood gave a yield of 29 % of polyphenols by cons the aqueous extract gave 17 %. For alkaloids, the yield of aqueous extract is 2.36%. In the other hand, there is a low concentration of polyphenols despite the high yield of the extraction. This could be explained by the composition of the extract which contains, alongside the polyphenols, all the hydrophilic compounds of the plants analyzed, such as assimilating pigments and various sugars. The solvent shows a clear effect on the polyphenol extraction yield but does not seem to have a great influence on the polyphenol content of the extract (Ait slimane-Ait Kaki et al. 2015).

Concerning the physicochemical characterization of boxwood, the main fraction is chlorophyll A which is naturally the major assimilator pigment of higher plants. It should also be noted that chlorophyll B is an accessory pigment whose role is to broaden the absorption spectrum of chlorophyll A. Often, chlorophyll A can make up three-quarters of the total chlorophyll (Raven, 2014).

The genus *Buxus* is known to contain many important classes of phytochemicals including steroids, alkaloids, terpenoids, among others (Saleem et al. 2020). These active substances of plants have a broad spectrum of action, they are recognized for their insecticidal power and do not spare non-target organisms (Barbouche et al. 2001), in addition, they do not cause the development of resistance in treated insects such as chemical insecticides, (Gestem et al, 2001, Park, 2003).

The presence of these secondary metabolites including tannins and alkaloids prompted us to study the insecticidal activity of the plant. The use of plant extracts as an insecticide has been known for a long time, as pyrethrum, nicotine and rotenone have already been used as insect control agents (Babaousmail, 2016). Currently, polyphenols as well as alkaloids are toxic metabolites used as insecticides (Raymond et al. 2011, Bouchelta et al. 2005).

The treatment of larvae of *Galleria mellonella* with increasing doses of polyphenols and alkaloids from boxwood resulted in high mortality rates especially with the high dose (120 µl / ml) with a rate of 100% after 7 days for polyphenols and 4 days for alkaloids. The toxicity of the extract is even higher when the doses are important. Similar results were found by Oulebsir-Mohandkaci et al. (2018), they showed that bioassays with polyphenolic extract of false fennel leaves and coriander gave 100% mortality in *G. mellonella* larvae within 3-4 days. The larvicidal activity of medicinal plant extracts has also been confirmed in the work of Elbehery et al. (2016). The present findings are in agreement with Surendra et al. (2010) who found that there was a variation in the larval mortality with the different concentrations of plant products used. They added that use of Neem seed extract resulted in the highest mortality rate between the tested *G. mellonella* larvae. Peppermint ethanolic extract and Chinese propolis show important toxicity against wax moth in 4th instar larvae (Fawzy et al. 2017). Neem oil, clove oil and peppermint oil present highly effects mortality in greater wax moth (*Galleria mellonella*) (Kalpana et al. 2017). Our study confirm the insecticidal efficacy of several phenolic compounds detected by HPLC, such as rutin, and gallic acid related by Harpal et al. (2014) et Abdelkhalek et al. (2020).

In addition, the injection of the aqueous extract into the haemolymph of the insect resulted in an increase in the protein concentration and a decrease in the level of haemolymph carbohydrates in the treated larvae compared to control larvae. which means that they have an impact on the biochemical metabolism of the insect.

Indeed, according to Benzina et al. (2017) who treated *G. mellonella* larvae with entomopathogenic bacteria; the comparative examination of the haemolymph test results shows that the injection of the bacteria into the larvae resulted in a significant increase in haemolymph protein and carbohydrate content as compared to controls. The author attributed this increase to the fact that the treated individual secretes substances of a protein nature called stress proteins. These stress proteins have been evidenced by Gourdon et al. (1998). The decrease in haemolyphatic carbohydrate concentration in treated larvae may be due to the energy expenditure of the treated individuals.

In the light of the present study, *B. sempervirens* is shown to have an insecticidal effect on *G. mellonella*, which has been statistically proven to be clearly correlated with dose and time.

CONCLUSION

The current experimental findings reveal that the boxwood contains natural substances (2.36% yield of alkaloids). HPLC allowed identifying very important bioactive molecules like catechin (12.5%), rutin (13.3%) and gallic acid (4.9%).

The application of the different extracts against wax moth larvae of 5th stage resulted in high mortality rates especially with the high dose (120 µl / ml) of methanolic extract and alkaloids. However, a significant increase in the concentration of proteins and a decrease in the concentration of carbohydrates in the haemolymph were recorded after the injection of the aqueous extract of boxwood to larvae. As a result, the tested boxwood extracts cause a disruption of the biochemical metabolism and the physiology of the insect. To conclude, boxwood is a plant species that could be further explored as a potential origin for the design of new biopesticides derived from natural molecules, as botanical pesticides are considered to be a safer and more environmentally friendly way to control insect pests such as *Galleria mellonella*.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

HOM designed the experiments and also wrote the manuscript.

SKA and KB participated in the experiments and data analysis.

FTB and IG participated in the document translation, scientific and reviewed the manuscript.

All authors read and approved the final version.

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