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Screening, Extraction, and Quantification of Melatonin in Waste of Some Plants

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Abstract:
Environmental pollution is one of the world's biggest problems, and plant waste is one of its causes. For this reason, we have tried in this work to take advantage of this waste and benefit from it instead of being one of the pollutants. Seven dry waste from different plants (bitter orange peels, pomegranate peels, bitter orange leaves, Ziziphus leaves, albizia leaves, waste of black tea, and zahidi date palm fibers) were tested as cheap source of melatonin(MLT), which is a very important indoleamine compound. Throughout the current study, this hormone was extracted from these plants' waste (which are considered as environmental pollutants) by applying different modified methods, whereby melatonin was identified and quantified by theHPLC-fluorescence system at The National Center for Drug Control and Researches (NCD). The results indicated the presence of different concentrations of melatonin in this waste. Bitter orange peels are a rich source of this hormone (868.868 µg melatonin /gm dried peel) in comparison to the other tested waste, followed by the waste of black tea (164.333 µg melatonin /gm waste). The results also showed the presence of trace concentrations of melatonin in Ziziphus leaves and Zahid date palms' fibers. This work provides a cheap source of MLT, it is a recycling method for plant waste.

Keywords: Melatonin, waste of plants, waste recycling, HPLC-Fluorescence detector.

التحري عن الميلاتونين واستخلاصه وتعيين كميته في مخلفات بعض النباتات

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الخلاصة
يعد التلوث البيئي من أكبر المشاكل التي يواجهها العالم وأحد مسبباته هي المخلفات النباتية. ولهذا السبب حاولنا في هذا العمل الأستفادة من هذه المخلفات بدلا من تركها لتكون أحد الملوثات . تم فحص سبع مخلفات نباتية جافة لسبعة نباتات مختلفة (قشور النارنج وقشورالرمان وأوراق النارنج و السدر والالبيزيا وبقايا الشاي الاسود والياف نخلة الزهدي) بوصفها مصدرا رخيصا لاحد مركبات الأندول أمين المهمة وهو الميلاتونين . خلال هذه الدراسة ، أستخلص هذا الهورمون من هذه المخلفات (والتي تعد ملوثات بيئية) بتطبيق ثلاث طرق مختلفة محورة ، وبعدها تم تعيين كميته بأستخدام جهاز كروموتوكرافيا السائل عالي الاداء المجهز بنظام فلورة الموجود في المركز الوطني للرقابة والبحوث الدوائية .أظهرت النتائج أن الميلاتونين موجود في معظم هذه المخلفات بكميات مختلفة. وأن قشور النارنج كانت المصدر الأغنى

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بالميلاتونين اذ أحتوت على اعلى تركيز (868,868 مايكروغرام ميلاتونين لكل غرام من القشرة المجففة) مقارنة بالمخلفات النباتية الاخرى تبعه بقايا الشاي الاسود (164,333 مايكروغرام ميلاتونين لكل غرام من البقايا) . أشارت نتائج الدراسة الحالية أيضا الى وجود كميات ضئيلة جدا من الميلاتونين في أوراق السدر وألياف النخيل الزهدي . يوفر هذا العمل مصدر رخيص للميلاتونين وهي طريقة لأعادة تدوير المخلفات النباتية.

Introduction:

Melatonin (N-acetyl-5-methoxy tryptamine) is a hormone found in all living creatures, from algae to humans. It was discovered in 1958 and **is**known as vertebrate pineal **secretory** product [1]**.** The presence of melatonin (MLT) in plants was demonstrated in 1995**,** and it has been proposed as a bioactive food component [2]. Generally, MLT has multiple physiological actions; wherein plants, phytomelatonin can act as a **a**growth promoter and regulator of plant reproductive physiology [2]. Due to its action as a highly efficient radical scavenger, it has been suggested to have **an**important role in **the**antioxidant **defence** system [3]. Furthermore, it is considered a friendly molecule to the environment because it assists plants to cope with harsh environmental stresses [1]-[4]. Melatonin has many physiological roles in human**it ;** can be taken orally as a supplement or drug **for** many conditions such as sleep disorders, depression, Parkinsonism, Alzheimer's disease and cancer. Moreover, exogenous MLT has a potential role in retardation of age-related oxidative events and a skin protectant against UV light besides its, as well as a skin protectant against UV light besides its topically use in cosmetics products [5], [6] This hormone possess both lipophilic and hydrophilic properties, so it gains access to every fluid and presumably every cell [7], [8]. Even though it may be not distributed in equal concentrations in all subcellular compartments, there is evidence that the nucleus and mitochondria have higher concentrations of MLT than other organelles [9], [10]. It has been studied extensively in different plants seeds, roots, stems, leaves, and flowers and medical herbs [10]-[12]. It is present in concentrations usually of a range of picograms to nanograms per gram of tissue [1], [13]. Previously different methods were applied for its extraction using either sodium carbonate or other buffers and solvents in the presence or absence of antioxidants, chelating agents, or protein precipitation agents. Among the methods that have been used for the measurement of MLT concentration in plants are Radioimmunoassay (RIA), enzyme-linked immunoabsorbent assay (ELISA), high-performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC-MS) [1], [2], [14].

The massive amount of waste gives a significant economic potential for creative uses other than animal feed. Since environmental pollution is one of the biggest problems, the world faces, plant waste is one of its causes. Therefore the goal of this work is to screen the presence of MLT in the waste of some plants, including bitter orange peels, pomegranate peels, bitter orange leaves, Ziziphus leaves, albizia leaves, waste of black tea, and Zahdi date palm fibers to use them as a cheap source of this important compound, and at the same time get rid of these environment's pollutants.

Materials and Methods

Collection of plant samples

Different plants waste wasused in the present study are illustrated in Figure 1 and Table1.

Figure 1- The used plant's waste were: (1) bitter orange peels, (2) pomegranate peels, (3) bitter orange leaves, (4) Ziziphus leaves,(5) albizia leaves, (6) waste of black tea, and (7) Zahdi date palm fibers.

Table 1- The names and sources of the various plant waste used in this study.

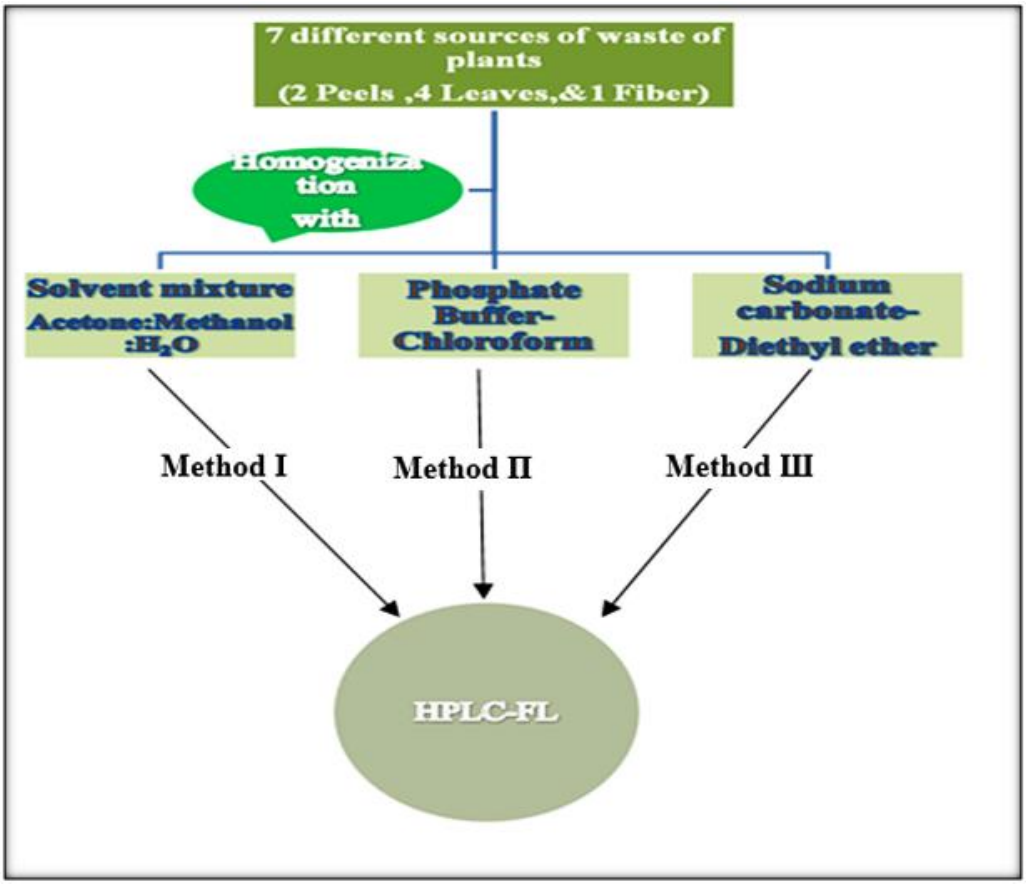
No.	Scientific name	Common Name	part used	Source
1.	*Citrus aurantium*	Bitter orange	Peels	Planted in Iraq
2.	*Punica granatum*	Pomegranate	Peels	Planted in Iraq
3.	*Citrus aurantium*	Bitter orange	fallen leaves	Planted in Iraq
4.	*Ziziphus Spina christi*	Ziziphus	fallen leaves	Planted in Iraq
5.	*Albizia julibrissin*	Albizia	fallen leaves	Planted in Iraq
6.	*Camellia sinensis*	Black tea	Leaves after preparation	Imported from Sri-lanka (Lipton yellow label)
7.	*Phoenix dactylifera*	Zahdi date palm	Fibers	Planted in Iraq

All these waste (Except the imported black tea) were collected during November from the above-mentioned local planted trees in Al- Jadria and Palestine Street in Baghdad City). The waste was pooled, washed thoroughly, dried on air, stored until being analyzed, and grinded to a powder just before the experiment. The tea was prepared by adding boiling water (7 gm

of tea was poured into 500 mL of boiling water) and left for 10 min without heating. After decantation, the remaining tea leaves were pooled, dried, and treated.

Extraction of MLT from plant waste:

Three extraction methods were applied that are based on using either acetone-methanol mixture (acetone:methanol: water 89:10:1) (method I) [15], phosphate buffer (50 mM, pH 8.0)(method II) or sodium carbonate(10%) (method III) for homogenization, then followed by using either chloroform or diethyl ether for extraction of MLT from the aqueous solutions in methods I and II, respectively [16], [17] as summarized in Scheme 1 with some modifications.



Scheme 1-Processing scheme for the MLT extraction using three different extraction methods (Method I, II, and III, respectively).

Determination of MLT concentration:

After completing the extraction methods, the presence of melatonin in each extract was detected, and its concentration was measured by the HPLC-fluorescence system (Knauer Company) as described below**:**

HPLC Analysis of Melatonin using HPLC-fluorescence system (HPLC-FL.):

The used apparatus was composed of A Knauer chromatographic system Plus a fluorescence detector. Separations were carried out using a Knauer C8 Eurospher column (250 mm, 4.6 mm id, 5 µm), and the mobile phase was composed of a mixture of ACN (25%, v/v) and an aqueous phosphate buffer (50 mM, pH 8.0) containing 0.2% triethylamine the mobile phase was filtered before use through a Phenomenex membrane filter (nylon, 47 mm membrane, 0.2 µm) and degassed by an ultrasonic bath. The used flow rate was 1 mL/min, and the injections

were carried out through a 100 µL loop. The fluorescence intensity was monitored at 386 nm while exciting at 298 nm. A 521 WTW pH meter was used [18].

Standard preparation:

Stock MLT solution (1mg/mL) was prepared by dissolving 10 mg of MLT standard in 10 mL of mobile phase and stored at -20^0C. Working standard solutions (0.01, 0.03, 0.05, 0.1, 1, 3, 5, and 7 µg/ml) were obtained by diluting the stock solution with the mobile phase and prepared freshly every day. Then these standard solutions were applied to the **(HPLC-Fl) System,** and MLT calibration curve was prepared by plotting MLT concentrations against their area under peaks from which the following equation was obtained: Y==132953.132X+2063.7241, R^2=0.999. The above equation was used to calculate the concentration of MLT extracted from each studied sample. It is worth mentioning that MLT is known to be subjected to oxidation by free radicals, so in all steps of any used extraction procedure, samples must be protected from light, an action that is necessary to avoid photo-oxidation [19].

Results and Discussion

Determination of the retention time of melatonin using the HPLC-fluorescence system

In this work, the extraction of MLT from different plants' waste was carried out using other methods. And to detect the presence of MLT in these extracts, an HPLC-fluorescence system was used to measure the retention time of this compound using standard MLT. The result is presented in Figure 2 . It is clear from this chromatogram that the retention time of MLT under the used experimental conditions is equal to 16 minutes.

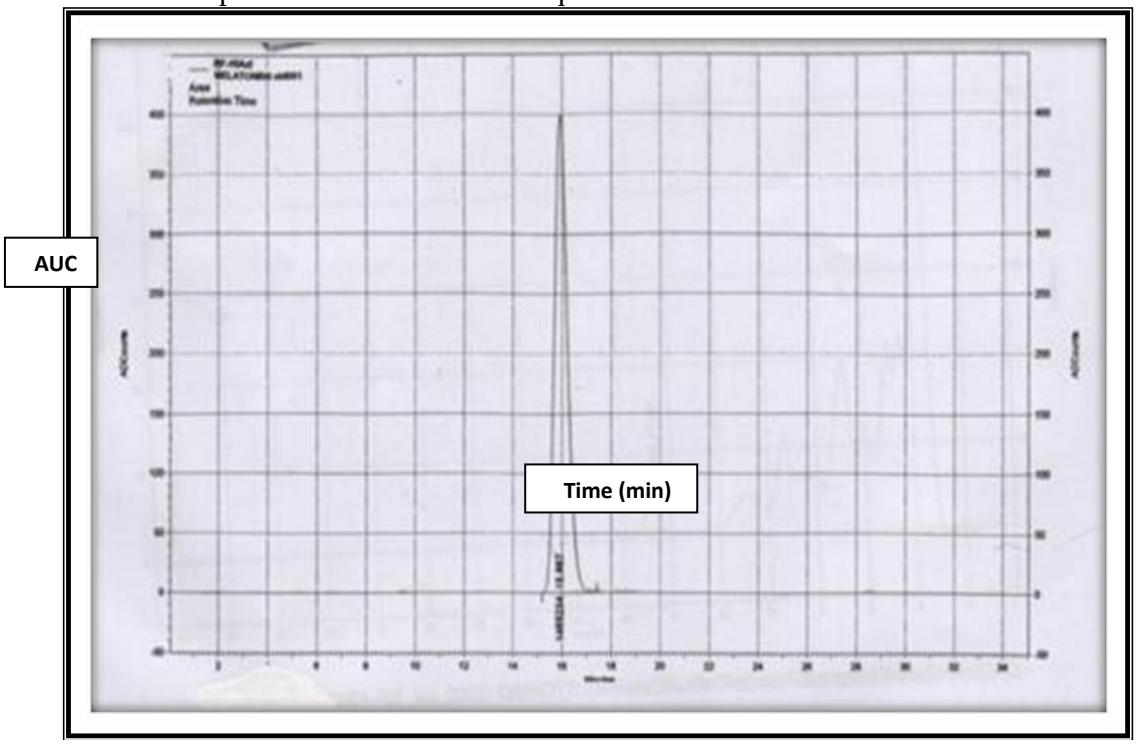


Figure 2-HPLC of MLT [0.1 µg/ml , retention time =16 min].The used chromatographic conditions were as follows: mobile phase was a mixture of ACN (25%, v/v)and an aqueous phosphate buffer (50 mM , pH 3.0)containing triethylamine 0.2% . The flow rate was 1 mL/min.

Determination of melatonin concentration in the different plant waste

The concentrations of MLT in the different obtained extracts using the three extraction methods were calculated using the equation derived from the MLT calibration curve, and the obtained results are listed in Table 2.

Table 2-Melatonin concentrations in the extracts of the studied plants waste by three different methods.

(µg Melatonin /gm plant) Waste of plants	Method I	Method II	Method III
Bitter orange peels.	**868.868**	**3.896**	**138.877**
Pomegranate peels.	**11.4560**	**-**	**-**
Bitter orange leaves.	**7.426**	**1.377**	**-**
Ziziphus Spina-christi leaves.	**-**	**-**	**-**
Albizia leaves.	**-**	**-**	**6.228**
Black tea's waste.	**1.835**	**-**	**164.333**
Zahidi Date palm fibors.	**-**	**-**	**-**

The results in Table 2 indicate that method I is the best method for MLT extraction from bitter orange and pomegranate peels, as well as from bitter orange leaves. While the most suitable method for MLT extraction from albizia leaves and black tea waste is when method III was applied.

Figure 3(A) shows the chromatogram of the extract obtained from bitter orange peels using an acetone-methanol mixture, while Figure 3 (B) indicates the chromatogram of the extract of black tea's waste; using carbonate-diethyl ether (method III).

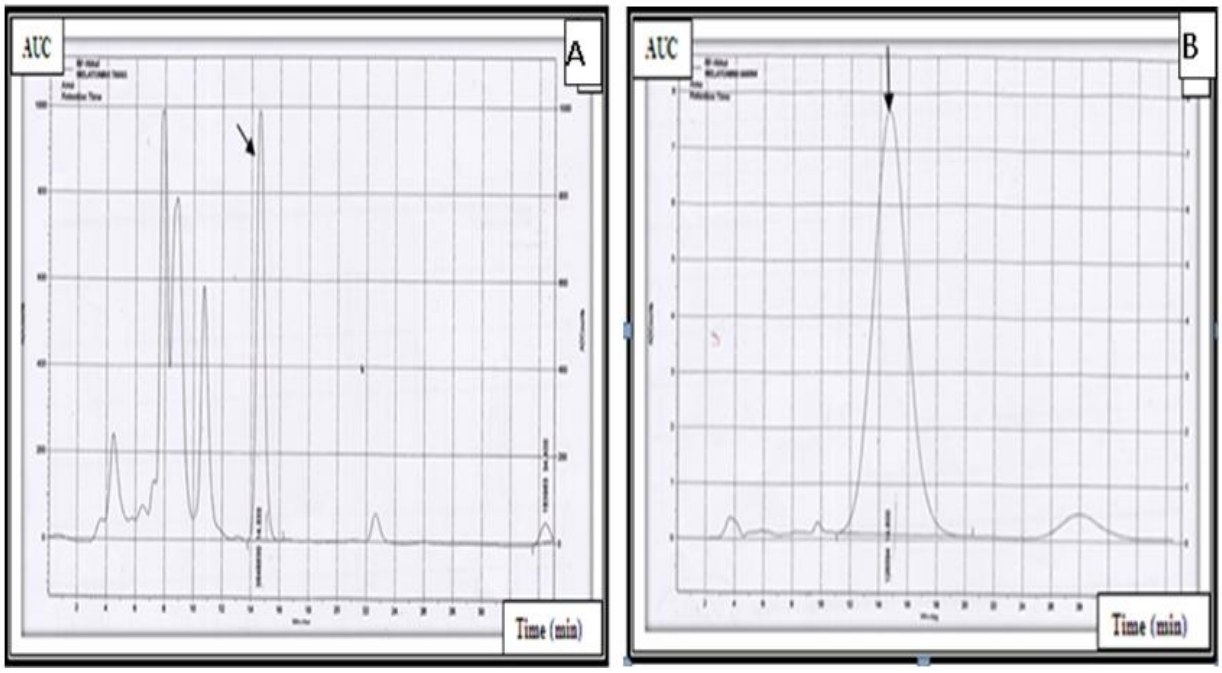


Figure 3-HPLC spectra of extracted MLT from: (A) bitter orange peels using an acetone-methanol mixture (method I). (B) black tea using carbonate-ether (method III).

Melatonin concentrations differ not only from species to species but also among varieties of the same species [1]-[4] as well as between various plant parts, in which the most pronounced being among seeds [20]. Banana provides an interesting case of the variability of indoleamine content in different parts and at different times. The highest concentration was in its peel, reaching its maximum level when the fruit was overripe [21].

Murch and his team [22] showed the effect of the drying process on MLT by comparing the MLT content of fresh feverfew leaves (*Tanacetum parthenium (L.) Schultz-Bip.*) with freeze-dried and oven-dried leaves. Their results indicated that the percentage of MLT lost during the drying process using the above drying methods was 15% and 30%, respectively. Such results demonstrate the relatively high stability of MLT within the tissue [21].

The presence of MLT in the waste of these plants was also confirmed by co-elution with standard MLT (Figure 5). Significant differences in MLT concentrations from sample to sample and from one method to was another for the same waste sample were observed. The reliability of the obtained data may be attributed to the efficiency of the used method related to the chemical properties of the extraction solvent and homogenate buffer, such as the polarity or radical scavenging properties [22]. These must be chosen to suit MLT physiochemical properties and its location in the cell, i.e., the efficiency of the used method is related to the increase of MLT solubility, as well as its protection from the effect of free radical presence concerning plant species and the part of studied plants. Using method I (Table 2), bitter orange peel was found to be a rich source of MLT (868.868 µg MLT /gm waste of plant) while MLT concentrations in the other waste extracts were lower with large gap. This may be explained by the chemical nature of bitter orange peel as it is oily and rich in biogenic amines. Furthermore, in this used method, acetone was the main organic solvent that present in the extraction mixture, and since MLT was reported to be present in all compartments [2], therefore MLT may be extracted from the membranes of the waste cells and their organelles by this solvent. Moreover, acetone is a radical trapping solvent that could protect against hydroxyl radical-mediated MLT degradation during the extraction step [23], [24].

Throughout the current work, no purification step was needed in contrast with the original method [25], which applied protein precipitation followed by solid-phase extraction for purification before estimation of MLT concentration; this may be one of the reasons that explain the obtained high MLT concentrations in the present study. Since usually, such purification steps involve the formation of a high level of oxidants, such as H_2O_2 and its deriving radicals that can easily lead to MLT destruction as a result of either its interactions with the free radicals or its photooxidation under the influence of photosensitizer [26].

Figure 4 shows a comparable series of MLT runs obtained from various waste using the three extraction methods. Applying method I gave the highest concentration of the extracted MLT from bitter orange peels (868.868 µg MLT /gm waste of plant). Therefore, method I was the best among the three used extraction methods for bitter orange peels and their leaves as well as for pomegranate peels. In contrast, the most suitable method for MLT extraction from albizia leaves and black tea waste was method III.

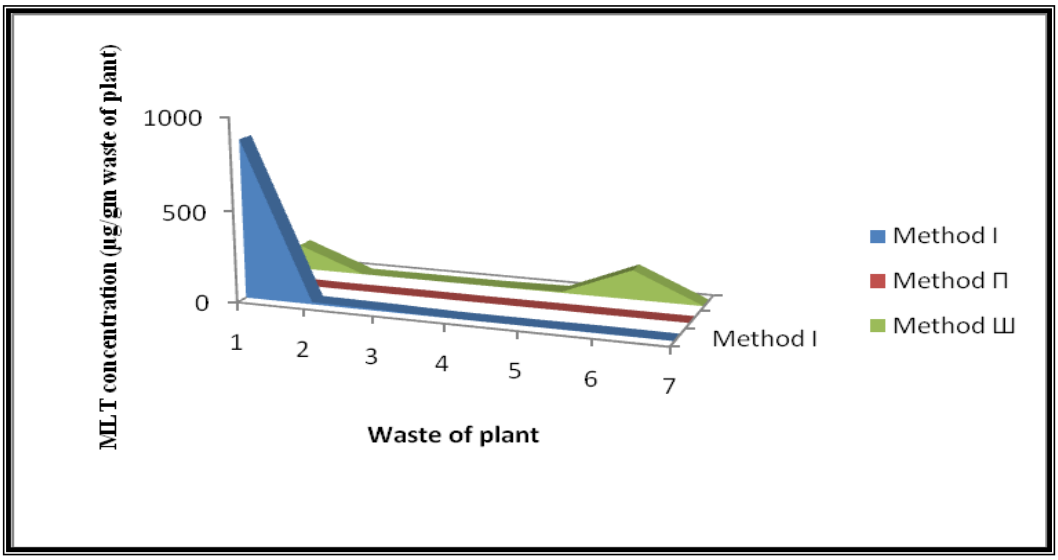


Figure 4-A comparable series of runs have been presented in the three methods (method I, II, and III, respectively) for the seven waste:1) bitter orange peels, 2) pomegranate peels, 3) bitter orange leaves, 4) Ziziphus leaves,5) albizia leaves, 6) waste of black tea, and 7) Zahdi date palm fibers.

Depending on the results of using the three extraction methods, MLT seems to be absent, or maybe is present in Ziziphus leaves and zahidi date palm fibers in a very low concentration that is out of the detection limit of the standard curve and the sensitivity of the used estimation method. The result of the MLT extracted from Ziziphus leaves using method I is illustrated in Figure 5.

The highest concentrations of MLT extracted using method II (Table 2) were from bitter orange peel and leaves (3.896 and 1.377 µg MLT /gm plant's waste), respectively. Meanwhile, using method III, a higher concentration was obtained from black tea leaves (164.333 µg MLT /gm plant's waste) followed by bitter orange peel (138.877µg MLT /gm plant's waste) as indicated in the same table.

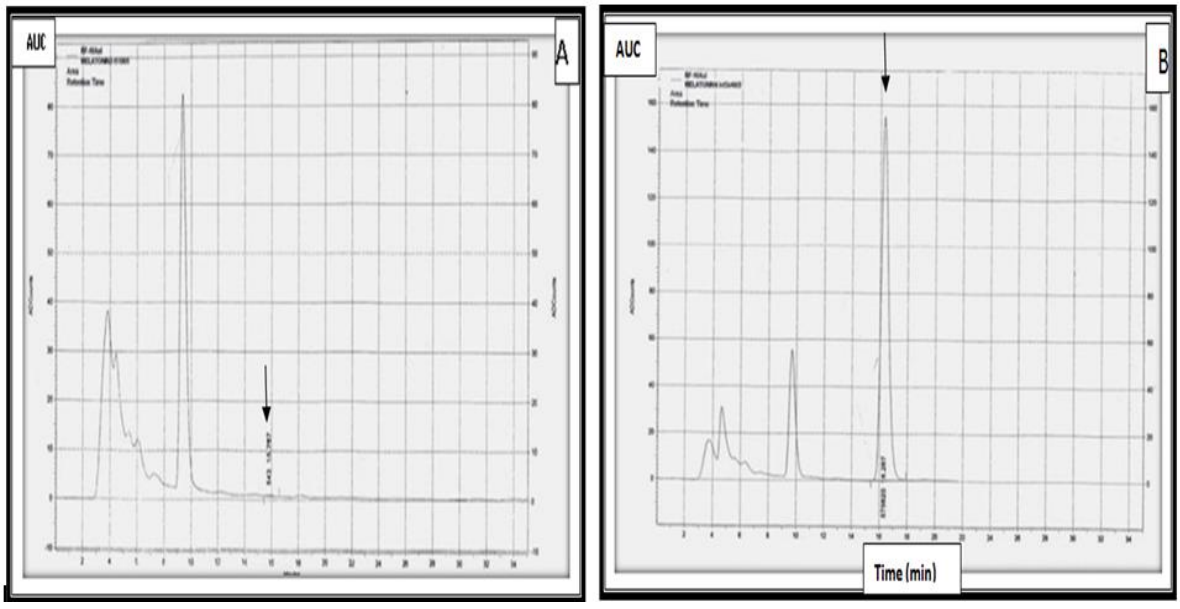


Figure 5- HPLC spectra of MLT (A): extracted from Ziziphus leaves using method I, and (B): the same extract co-eluted with standard melatonin.

The extraction of MLT using methods Π & Ш were done in aqueous solutions, which means that it is expected that only the cytosolic MLT was extracted only leaving the membrane's one behind. Furthermore, in both methods (Π & Ш), an additional step of liquid-liquid extraction was involved using an organic solvent (chloroform or diethyl ether in methods Π & Ш, respectively). This step was requested to employ an aqueous extraction solution into an organic solvent [27] meantime using such solvents induce phase separation, which leads to readily exposure of MLT to oxidants during the extraction step in the organic phase, the presence of such solvents may increase radical formation and facilities the propagation of radical reaction during the extraction step [28]. Using sodium hydroxide in method Ш was previously reported to affect the estimation of MLT concentration [6], while its presence removes possible cross-reacting indoles containing carboxyl and hydroxyl groups [29], [30].
Ansari *et al*, 2010 have reported that MLT concentration in dry plant powders (*T. disciforme)* was equal to 3.073 µg/g and 2.906 µg/g using HPLC, or ELISA techniques, respectively [31]. Throughout the current study using of HPLC-FL system gave a significant MLT peak where the retention time was equal to 16 mins. So this system is suitable method to estimate this compound especially since no destruction of MLT was observed during sample passage through the fluorescence U.V. light detectors in addition to the sensitivity of the technique [1],[2].
In addition to the several points mentioned above, in the present study, concentrations of MLT were relatively higher than that obtained in the previous studies dealing with different plants. This may be due to many factors such as the part of the plant used, geographic locations, storage conditions, plant species, degree of ripening and the extraction solvent used, and the sensitivity of the different quantification assays used previously.
To the best of our knowledge, this is the first report that points out the presence and measurement of MLT concentration in the waste of the plants including bitter orange peels, pomegranate peels, bitter orange leaves, Ziziphus leaves, albizia leaves, waste of black tea, and Zahidi date palm fibers. As conclusion, from the current study, one can consider bitter orange peels and waste of black tea as cheap sources of this indole compound, which has many applications in different aspects. In the meantime, this work provides a recycling method for this type of environmental pollutant. Further studies are being carried out in our laboratory to screen the waste of other plants for the presence of this compound.

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