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Der Pharmacia Lettre, 2010, 2 (2): 479-485
(http://scholarsresearchlibrary.com/archive.html)

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ISSN 0975-5071
USA CODEN: DPLEB4

Microwave facilitated extraction of Bixin from *Bixa orellana* and it's *in-vitro* antioxidant activity

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Abstract

Bixa orellana seeds are the only natural source of bixin, a carotenoid widely used in the food industry as colourant. The aim of this present work was to isolate bixin in a short duration by the use of facilitated microwave extraction. Several extraction parameters such as temperature, time, and amount of solvent were first optimized. The conventional heating for 90 min provided 8.231 % bixin and by microwave heating at 210 W for 18 min, the yield was 16.281 %. Microwave assisted extraction (MAE) was identified as the most effective extraction procedure for isolation of bixin. MAE is a green technology, alternative and superior to conventional extraction for the extraction of bixin. Both the isolated compounds were found to be same as evidence by spectroscopic and chromatographic studies. A comparative antioxidant study of both conventional and microwave isolated bixin were performed.

Key Words: *Bixa orellana*, Microwave Assisted Extraction**,** Bixin**,** Antioxidant activity.

INTRODUCTION

Microwave assisted extraction is a relatively novel method for extraction and it offers many advantages such as reduced energy consumption, smaller volume of chemical solvents, use of less toxic solvents and smaller quantity of waste products [1]. Recently, the use of microwave assisted extraction has been widely recognized as a simple, effective, and versatile extraction method. Extraction using microwave can result in an increased yield in shorter time, using less solvent [2]. Researches have been done for the extraction of biological compounds, such as

extraction of lycopene from tomatoes [3], flavonoids from *Radix Scutellariae* [4], total phenolic of Flaxseed [5], silymarin from milk thistle seeds [6], ferulic acid from *Radix Angelicae sinensis* [7], ginsenosides from ginseng root [8], and saponins from chickpea [9] etc. The recent availability of commercial microwave equipment that complies with the higher security standards and incorporates closed vessels in their protocols has enabled the extraction at high pressure and temperature, facilitating rapid and selective analyte desorption from complex matrices—all at a relatively moderate cost [10].

Bixin is the major pigment, accounting for 80% of the total carotenoid content present in *Bixa orellana* [Annatto] and are the only natural source of bixin [11]. The strong colouring power of carotenoids justifies the wide use of these compounds. Bixin have been used worldwide in the food industry due to its efficiency in physical and chemical quenching singlet oxygen and sensitizers [12], physical quenching involves the energy transferred to the carotenoid and further dissipation by rotational and vibrational interactions. The chemical process results in destruction of the chromophore and formation of oxidation products [13]. Besides, carotenoids have been extensively investigated due to their known biological functions, such as vitamin A activity, cancer-preventing effects, protective effect against cardio-vascular diseases and reducing risk of cataract and age-related macular degeneration [14, 15]. Bixa seeds are used as purgative, anti-pruritic and for buccal tumours [16]. As a colourant, annatto extracts are used to colour butter, cheese, bakery products, oils, ice creams, sausages, cereals and extruded products, and are relatively inexpensive when compared to other natural pigments. Among natural carotenoids, bixin is one of the more effective biological singlet molecular-oxygen quenchers and may contribute to the protection of cells and tissues against deleterious effects of free radicals [17]. Bixin has protective activity against clastogenic effects of antitumor agents [18] and chemo preventive [19].

The present study compares the extraction efficiencies of recently introduced microwave assisted extraction with the conventional method for extraction of bixin. In the conventional isolation of bixin from the fresh whole seeds of *Bixa orellana*, ethyl acetate was used as solvent. Since ethyl acetate is microwave transparent the procedure was modified by a mixture of ethyl acetate and water. Water absorbs microwaves and due to the heat generated, breaking of cells in the plant powder and release of the phytoconstituents takes place. Due to the many factors, that influence MAE, optimization of the extraction protocol is required.

In the present work, extraction efficiency was defined as follows:

$$\text{Percentage extraction .w/w} = \frac{\text{Mass of bixin in extracted solution}}{\text{Mass of material } \textit{(Bixa seeds)}} \text{ x100}$$

In MAE heating rate plays an important role in extraction efficiency. In order to heat up rapidly under microwave radiation, the solvent must have high dielectric constant (which measures the efficiency in which the absorbed microwave energy can be converted into heat inside a material when an electric field is applied [20]. Due to the values of these constants, water results as the

best solvent for MAE and its addition can be exploited to increase polarity indices of other solvents that are commonly employed for the extraction of plant bioactive compounds

MATERIALS AND METHODS

Plant materials

The fresh whole seeds of *Bixa orellana* were collected from Nilgiri, Tamil Nadu and authenticated by Dr. S. Rajan, Medicinal Plants Survey and Collection Unit, Government Arts College, Ootacamund, Tamil Nadu, India, where a voucher specimen was preserved for further reference.

Equipments

Catalyst Scientific Microwave Oven (with variable power output ranging between 140 W and 700 W); The extraction system consist of a microwave extractor manufactured by Catalyst Systems (Pune, India) equipped with a magnetron of 2450MHz with a nominal maximum power of 700W, a reflux unit, 10 power levels, time controller, exhaust system, beam reflector and a stirring device), Conventional Soxhlet equipment; and Buchi Rotovapor.

Conventional extraction technique

Fresh and whole seeds of *Bixa orellana* (10 g) (without powdering) were boiled with 75 ml of ethyl acetate. The extract is then decanted and concentrated to less than half of its volume. The pure crystalline bixin separates out on cooling the concentrated extract [21]. The crystals were dried and recrystalized from dichloromethane/methanol (1:4). The crystals were completely dried, weighed and its spectroscopic and chromatographical studies were carried out.

Microwave assisted extraction

Fresh and whole seeds of *Bixa orellana* (10 g) (without powdering) were extracted with 75 ml of ethyl acetate and 10 ml distilled water using microwave power at 210W intensity for 18 min. The extract is then decanted and concentrated to less than half of its volume. The pure crystalline bixin separates out on cooling the concentrated extract. The crystals were dried and recrystalized from dichloromethane/methanol (1:4). The crystals were completely dried, weighed and its spectroscopic and chromatographical studies were carried out.

In Vitro Antioxidant Activity

The bixin isolated through conventional and microwave methods were tested for *in vitro* antioxidant activity using 5 standard methods. The final concentration of the sample and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25 and 15.625, 7.812, upto 0.025 µg/ml respectively. The absorbance was measured spectrophotometrically against the corresponding blank solution. The percentage inhibition was calculated using the following formula.

$$\text{Radical scavenging activity (\%)} = \frac{\text{OD control - OD sample}}{\text{OD control}} \times 100$$

IC_{50}, which is the concentration of the sample required to scavenge 50% of free radicals was calculated.

DPPH assay

The assay was carried out in 96 well microtitre plates. To 200 µl of DPPH (2, 2' diphenyl-1-picryl hydrazyl) solution, 10 µl of each of the sample or standard solution was added separately in wells of the microtitre plate. The plates were incubated at 37 ^{o}C for 30 min and the absorbance of each solution was measured at 490 nm [22] using ELISA reader.

Scavenging of ABTS radical cat-ion assay

Accurately weighed 54.8 mg of ABTS was taken and dissolved in 50 ml of distilled water (2 mM) and potassium persulpha (17 mM, 0.3 ml) was added. The reaction mixture was left to stand at room temperature overnight in dark before usage. To 0.2 ml of various concentrations of the sample or standard, add 1.0 ml of distilled DMSO (Dimethyl sulfoxide) and 0.16 ml of ABTS solution to make the final volume of 1.36 ml. Absorbance was measured after 20 min at 734 nm [23].

Scavenging of nitric oxide radical

The reaction mixture (6 ml) containing sodium nitroprusside (10 mM, 4 ml), phosphate buffer saline (PBS, pH 7.4, 1 ml) and sample or standard (1ml) in DMSO at various concentrations and it was incubated at 25 ^{o}C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite ion was removed, 1 ml of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 min for completion of diazotisation. Then, 1 ml of NEDD (Naphthyl ethylene diamine dihydrochloride) was added, mixed and allowed to stand for 30 min in diffused light. A pink colored chromophore was formed. The absorbance was measured at 540 nm [24].

Lipid per oxidation inhibitory activity

The reaction mixture containing (1ml) egg lecithin, (0.02 ml) ferric chloride, (0.02 ml) ascorbic acid and sample or standard (0.1 ml) in DMSO at various concentrations, was kept for incubation for 1 hour at 37^{0}C. After incubation, 2 ml of 15% TCA (trichloro acetic acid) and 2 ml of 0.375% TBA (thiobarbituric acid) were added. Then the reaction mixture was boiled for 15 min. Cooled, centrifuged and absorbance of supernatant was measured at 532 nm [25].

Scavenging of superoxide radical by alkaline DMSO method

To the reaction mixture containing 1 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml water) and 0.3 ml of the samples or standards in DMSO at various concentrations, add 0.1 ml of NBT (Nitro blue tetrazolium) (1 mg/ml) to give a final volume of 1.4 ml. The absorbance was measured at 560 nm [26].

RESULTS AND DISCUSSION

Study shows that microwave- assisted extraction has many advantages, such as shorter time, less solvent, higher extraction rate and better products with lower cost. Soxhlet method usually needs a few hours, even more than 20 h, while microwave-assisted extraction only need a few minutes [27, 28]. The use of microwave energy enables fast dissolution, drying, acidic digestion and extraction of organic compounds from complex environmental matrices; its main advantages are reduced solvent volume and time consumption [29].

Table-1: *In vitro* antioxidant activity of Bixin

Sample/ Standards	IC_{50} values ± SE (µg/ml) by methods*									
	DPPH		ABTS		Nitric oxide		Lipid per oxidation		Alkaline DMSO	
Sample	**C**	**M**	**C**	**M**	**C**	**M**	**C**	**M**	**C**	**M**
Bixin	2.72± 0.11	2.70± 0.09	0.38± 0.02	0.37± 0.02	>1000	>1000	>1000	>1000	282± 2.18	256± 2.18
Standard										
Ascorbic acid	11.25 ± 0.49		2.69 ± 0.05		--		--		>1000	
Rutin	0.51 ± 0.01		3.91 ± 0.10		65.40± 2.50		--		>1000	
BHA	--		24.88 ± 0.16		--		112.66 ± 1.32		>1000	
α–*Tocopherol*	--		--		--		91.66 ± 4.92		--	

**-Average of three determinants, C- Conventional, M- Microwave*

Isolation of bixin involving 90 min heating conventionally gave 8.231 % yield. A mixture of ethyl acetate and water was satisfactorily used in microwave extraction. The product was obtained successfully when 140 W and 210 W intensities were used. Yields higher than the conventional method were obtained at 210 W intensity and heating for a period of 10 to 40 min, and at 140 W for 50 min. The highest yield of 16.281 %, which was almost double than the conventional yield was obtained at 18 min. Hence a saving of 72 min (about $4/5^{th}$ of conventional heating) and 97.8 % increased yield. The present work has proved to be an alternative and time saving method for the isolation of bixin from *Bixa orellana*. An efficient MAE process has been developed for fast extraction of bixin. Under optimized extraction conditions of intensity, time and solvent a better yield was achieved.

The UV spectrum of standard solution of bixin obtained by conventional procedure showed λmax of 503 and 473 nm with absorbance 0.687 and 0.748 respectively. By microwave method the same solution showed λmax of 503 and 472 nm with absorbance 1.880 and 2.131 respectively. The HPTLC separation of bixin for both conventional and microwave method yields a single spot with R_f value 0.50 and 0.52 under the same conditions. ^{1}H NMR of conventional isolated bixin shows 7.96(2H, 8-H, 8'-H), 6.66(2H, 15-H, 15'-H), 5.91(2H, 7-H, 7'H), 3.787(s, 3H, $COOCH_3$), 1.99(s, 6H, 20-H, 20'-H).

The microwave isolated bixin shows 7.92(2H, 8-H, 8'-H), 6.66(2H, 15-H, 15'-H), 5.91(2H, 7-H, 7'H), 3.787(s, 3H, $COOCH_3$), 1.99(s, 6H, 20-H, 20'-H). Molecular formula of the isolated compound was found to be $C_{25}H_{30}O_4$.

The *invitro* antioxidant studies of conventional and microwave isolated bixin confirms that microwave procedure can be adapted for the extraction of phytoconstituents without losing its biological activity.

CONCLUSION

The present work has proved to be an alternative and time saving method for the isolation of bixin from *Bixa orellana*. An efficient MAE process has been developed for fast extraction of bixin. Under optimized extraction conditions of intensity, time, solvent and a better yield was achieved.

Acknowledgements
We are thankful to the management of J. S. S. College of Pharmacy, Tamil Nadu, Ooty, India for sponsoring this project and Sir C. R. Reddy College of Pharmaceuticals Sciences, Eluru, Andhra Pradesh, India for providing other facilities.

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