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METHODS OF EXTRACTION AND FRACTIONATION OF BIO-ACTIVE COMPONENTS OF *MORINDA COREIA* FOR ANTI-TUBERCULAR AND ANTICANCER ACTIVITIES

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Keywords:

Morinda coreia, Anti-cancerous, Anti-tubercular, Method of extraction and fractionation

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ABSTRACT: In Asian countries, roots, barks, stems, leaves, and fruits of plants belonging to the Morinda species are considered as valuable traditional herbs. They are used in folk medicine for the treatment of many diseases, including diabetes, high blood pressure, and cancer. Anti-tubercular and cytotoxic activities of *Morinda citrifolia* (noni) have been reported, but *Morinda coreia* has not been explored for these two activities to date. In the present study, a systematic investigation was carried out with the air-dried leaves of *M. coreia* by bio-assay guided fractionation by three different extraction methods including conventional solvent extraction (SE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) methods to identify the best method for extraction for bioactive fractions responsible for anti-mycobacterium and anticancer activities. The n-hexane fraction of M. coreia was found to be most with MIC values ranging from 125 µg/L for SE and to 62.5 µg/mL for UAE against Mycobacterium H37Rv. Similar results were obtained against MCF-7 cells with IC_{50} values 33.78 µg/mL for SE and 22.22 µg/mL for UAE. In conclusion, the present study established that the most potential bioactive fraction of *M. coreia* can be best extracted with UAE.

INTRODUCTION: Medicinal plants are considered among the main sources of biologically active chemicals. These medicinal plants have been used extensively as a crude material or as pure compounds. However, out of the 250,000 - 500,000 plant species on earth, only 1–10% have been studied chemically and pharmacologically for their potential medicinal value [1].

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India is one of the few countries in the world which has a unique wealth of medicinal plants and vast traditional knowledge of the use of herbal medicine as a cure for various diseases [2].

Morinda coreia belongs to the family Rubiaceae which grows wildly and is distributed throughout Southeast Asia, commercially known as Nunaa and locally known as “Togaru”. *Morinda coreia* Buch.-Ham is an evergreen shrub or small tree that can grow up to 5 - 10 meters tall. The bark and wood of this plant are used to treat fever and also as an anti-malarial agent in north-eastern Thai (Isarn) traditional medicine [3]. In the Indian traditional system of medicine, leaves and roots of *Morinda*

coreia are used as astringent, emmenagogue (herbs which stimulate blood flow in the pelvic area and uterus; in some cases stimulate menstruation as well) and to relieve pain in gout [4]. There is a greater demand for fruit extract of Morinda species in treatment for different kinds of illnesses such as arthritis, cancer, gastric ulcer, and other heart diseases. *Morinda coreia* leaves are also reported to possess anticonvulsant, analgesic, anti-inflammatory, anti-oxidant activity and cyto-protective effects [5, 6]. The ashes of *Morinda coreia* leaves are also reported to act as bio-sorbents in controlling ammonia pollution in wastewaters. Though, *Morinda citrifolia* (noni) is reported to possess anti-tubercular and cytotoxic activity while *M. coreia* has not been explored for these two activities to date.

The phytochemical investigation of the plant have revealed the presence of nordamnacanthal, damna-canthal, soranjidiol, 1-methoxy-2-methyl-anthraquinone, anthragallol, 2-methoxy-6-methyl-1, 3, 5-trihydroxyanthraquinone, 1-hydroxy-5, 6-dimethoxy-2-methylanthraquinone as non-polar secondary metabolites. From the leaves and branches of the plant yopaaosides (A-C), 10-O-acetylmonotropein, 6-O-acetylscandoside was reported as additional polar compounds. The aqueous extract of the plant are reported to possess mainly iridoid, phenolic, anthraquinone, and secoiridoid based glycosides [7].

Conventional secondary metabolite extraction techniques from plants include solvent extraction, Soxhlet extraction, and blending. Although efficient extractions can be achieved using these techniques, they have certain limitations in terms of long extraction times and high solvent consumption. Over the last decade, new techniques have emerged that have superseded traditional extraction techniques. These include ultrasound, supercritical fluid, pressurized fluid, and microwave-assisted extractions. Microwave-assisted extraction (MAE) has been demonstrated to be a fast and efficient unconventional extraction method that was developed for extracting analytes from solid matrixes, in particular, secondary metabolites from plant material. Microwave energy is non-ionizing radiation that results in molecular movement by the migration of ions and rotation of molecules with permanent dipoles in liquids, without altering their molecular structures unless the temperature is too high. MAE greatly reduces solvent consumption and extraction times and improves extraction efficiency [8]. Ultrasound-assisted extraction (UAE) method has also been used as an alternative for solvent extraction for the extraction of secondary metabolites. UAE offers several advantages like simplicity, inexpensive equipment, and remarkable reduction in solvent amount, temperature, and time of extraction over conventional solvent extraction techniques [9]. These techniques are environment friendly in terms of solvent and energy consumption, and the yields are also comparable to conventional extraction, and in some cases, it is even higher.

In the present study, a systematic investigation was carried out with the air-dried leaves of *Morinda coreia* by bio-assay guided fractionation by three different extraction methods including conventional solvent extraction; microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) methods to identify the best method for extraction for bioactive fractions responsible for anti-mycobacterium and anticancer activities.

MATERIALS AND METHODS:

Plant Material: *Morinda coreia* was procured from Botanical Survey of India, Southern Regional Centre, Coimbatore, India. The plant material was authenticated by Dr. M. Palanisamy, Scientist 'C' In-charge, Botanical Survey of India, Coimbatore. A voucher specimen for the same was deposited at Amity Institute of Biotechnology, AUUP, Noidabearing the number AUUP/AIB/03/2014.

Extraction and Isolation:

Bioassay Guided Fractionation by Conventional Solvent Extraction (SE): Bioassay guided fractionation by conventional solvent extraction was performed as described by Mishra *et al.*, [10] The air-dried plant material (50 g) was crushed into powdered form, in a mechanical grinder. The material was then soaked thoroughly in 1:1 MeOH: H_2O (100 mL) overnight at room temperature. After 24 h the methanol extract was collected by filtration, and the residue was soaked again with MeOH: H_2O (50 mL) by following the same procedures. The method was repeated for five consecutive days, and the total volume of the collected MeOH-H_2O (9:1) extracts was 500 mL.

The plant residue was further extracted with distilled H_2O (200 mL) once. The aqueous extract was lyophilized in a lyophilizer, and the aqueous solvent was removed completely. The methanolic extract was evaporated to dryness at 50 °C under reduced pressure in a rotary vacuum evaporator.

The concentrated methanolic extract was resuspended in H_2O and subsequently partitioned with n-hexane (n-Hex). The process was repeated three times.

The total volume of the fraction collected was 100 mL. Thereafter, in a similar way, the extract was partitioned (3 times) with dichloromethane (DCM) to get the total volume of 150 mL of DCM fraction, followed by ethyl acetate (EtOAc) collecting 100 mL and residual H_2O fraction. The solvent from organic fractions was evaporated under reduced pressure below 50 ºC in a rotavapor followed by complete removal of the solvent under high vacuum and yields of each fraction were recorded. Each fraction and aqueous extract was screened for anti-mycobacterial and cytotoxic activity.

Bioassay Guided Fractionation by Microwave-Assisted Solvent Extraction (MAE): For MAE, 50 g of powdered plant material was mixed with 50 ml of 1:1 MeOH: H_2O in Pyrex beakers as described by Dhanani *et al.*, [11] Extraction was carried out by placing the beakers in the middle of the oven over a rotating dish and exposed to microwave radiation (LG Electronics, India, Model MG-556 P, 1350 W, 2450 MHZ) for 5, 10 and 20 min. At the end of heating, the beaker was left for temperature stabilization (1 min). The material was filtered, concentrated, and fractionated/partitioned with n-Hexane, DCM, and EtOAc, as described in the previous section. The yields for each treatment and fraction were recorded.

Bioassay Guided Fractionation by Ultrasound-Assisted Solvent Extraction (Uae): For UAE, 50 g of powdered plant material was mixed with 50 ml of 1:1 MeOH: H_2O and was sonicated in a Bandelin Sonorex, Germany, 480 W, 35 kHz ultrasonic bath at 35 °C for 5, 10 and 20 min [12]. The extract was collected, concentrated, and fractionated/partitioned with n-Hexane, DCM, and EtOAc, as described in the previous section. The yields for each treatment and fraction were recorded.

Preliminary Phytochemical Screening: Phyto-chemical analysis for the presence of alkaloids, flavonoids, steroids, reducing sugars, cardiac glycosides, terpenoids, anthraquinones, tannins, phlorotannins, and saponins were conducted with each fraction by using standard protocol [13].

Anti-tubercular Activity:

Anti-mycobacterial Susceptibility Test: Rapid Identification and Drug Susceptibility Testing of Mycobacterium tuberculosis was performed using Colorimetric Redox Indicator Assay [14]. It is also known as Resazurin Microplate Assay. For this study, a fluorometric bioassay based on the reduction of resazurin was envisaged both for initial screening and bioassay-guided fractionation of plant extracts, because it has been reported to be a simple, accurate and reliable bioassay for evaluation of mycobacterial growth [15]. The resazurin microplate assay is commonly used to evaluate natural products and synthetic compounds for anti-mycobacterial activity.

Microbial Strain: The pan sensitive strain of *M. tuberculosis* H37Rv TMC-102 was procured from National JALMA Institute of Leprosy and Other Mycobacterial Diseases, Agra, India and was grown in Mycobacterium growth indicator tubes (BBLTM MGITTM, Becton-Dickinson, and Company) in the growth medium supplied within the tubes [casein peptone (8.8 mg) and modified Middlebrook 7H9 broth base (41.3 mg) in deionized water (7.0 mL)] with the addition of 280 µg PANTATM antibiotic mixture(Becton, Dickinson, and Company) in 800 µl OADC enrichment and Tween 80 (4 µL). Cultures were incubated at 37°C and 5% CO_2 in a humid environment (> 95% relative humidity) for 2 weeks and diluted to a turbidity equivalent to a McFarland 1.0 standard. The resulting mycobacterial suspensions (1.5 mL) were cryogenically stored at 70 °C for up to 4 weeks, thawed and diluted fivefold with MGITTM growth medium to obtain a concentration of 2.0×10^6 CFU/ml immediately prior to use in the bioassay.

Determination of Minimum Inhibitory Concentration (MIC): The evaluation of anti-mycobacterial activity was carried out using Resazurin micro-plate Assay [16]. Briefly, the extract/fractions were first dissolved in Dimethyl

Sulfoxide (DMSO) and then diluted in Middle brook 7H9 broth across a 96-well plate in a two-fold serial in duplicate. The bacterial suspension (1.5×10^6 cells/ml) was added to each well, and the last two wells were left with no drugs to serve as controls for the growth of inoculum. The plates were incubated at 37 ºC for 7 days. On 7th day, 50 µl of the resazurin dye was added to each well and the plate was incubated at 37 ºC for overnight. The MIC was interpreted visually by observing the change in color, and the results were further confirmed by reading absorbance at 570 nm and correlated with the growth control. Minimal inhibitory concentration (MIC) of each drug (fractions) was interpreted as the lowest concentration of the antibiotic that prevents the change in color of the dye. Extracts were considered active if they gave MIC ≤ 200 µg/mL.

Anticancer Activity on MCF-7:

Cell Line: Breast cancer cell line (MCF-7) was a cell line that was obtained from National Centre for Cell Sciences (NCCS) Pune, India. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), (100 U) 20 µg/ml penicillin and 100 µg/ml streptomycin. Cells were seeded in 96-well microtiter plate with a tested concentration of isolated compounds, and cell viability was determined using the tetrazolium dye assay.

Cell Viability Assay: MTT assay was used to determine the inhibition of cancer cell proliferation by extract and fractions of *M. coreia*. Exponentially growing MCF-7 cells were seeded into 96-well plates (104 cells/well in 100 µL of media) and allowed to attach for 24 h. Test fractions were prepared in 0.5% DMSO and serially diluted with media to obtain appropriate concentrations. Cells were treated with different concentrations of fractions and incubated for 48 h. Cells in the control group received only media containing 0.5% DMSO. The fractions containing media was removed and washed with 200 µL of PBS followed by addition of 20 µL of MTT [3-(4, 5-dimethylthiazol -2-yl)-2, 5-di-phenyltetrazolium bromide] reagent (5 mg/mL MTT in PBS) and incubated for 4 h at 37 °C. The formazan dye crystals formed were solubilized in DMSO, and the plate was incubated at room temperature for 1 h. The absorbance was measured at 570 nm with a reference wavelength of 620 nm [17] in an ELISA microplate reader (Biotek, New York, USA) followed by the calculation of percentage viability.

$$\text{Percentage cell viability} = 100 - [(A_o - A_t)/A_o \times 100]$$

Where A_o = Absorbance of cells treated with 0.5% DMSO medium, A_t = Absorbance of cells treated with fractions.

0.1% (v/v) DMSO in the medium was used as a negative control. Each treatment was performed in duplicate, and cytotoxicity for each fraction was expressed as IC_{50} values.

Cell Cytotoxicity Assay: The toxicity of various fractions was determined against Chick embryo fibroblast cells, as described by Kakad and Dhembare [18]. In brief, fibroblast cells obtained from chick embryo were cultured in DMEM medium supplemented with Fetal Bovine Serum (FBS) and gentamicin. The cell suspension (2 mL) was treated with a sample solution at MIC concentration and twice the concentration of MIC. The microtitre plate was incubated aseptically in CO_2 incubator for 24 hours at 37 °C. After incubation, cells were disaggregated using trypsin (0.25%), and cell viability (%) was determined by MTT assay.

Statistical Analysis: All experiments were carried out in triplicates, and statistical analysis was carried out using Graph pad Prism version 4.00 for Windows, GraphPad Software, San Diego California, USA. Data has been presented as means ± S.D.

RESULTS AND DISCUSSION: The present study investigated the effect of different extraction methods and conditions on the recovery yield and extraction of bioactive constituents for anti-myco-bacterial and anticancer activities by bioassay-guided fractionation.

A good extraction technique is expected to be less time consuming and offer a high yield of active compounds without compromising on the function or role of the bioactive ingredients. The present study examined the efficiency of microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) as compared to the conventional extraction methods (SE).

Extraction and Fractionation: Extraction yield (mass of extract/ mass of dry matter) was used as an indicator of the effects of the extraction conditions. Extract yield of *M. coreia* prepared by SE, UAE, and MAE methods using 1:1 methanol: water are summarized in **Table 1**.

TABLE 1: YIELD OF FRACTIONS OF *M. COREIA* USING DIFFERENT EXTRACTION METHODS

Extraction method	Treatment Time (min)	Fraction	Yield (%)
Solvent Extraction(SE)		*n*- Hex	0.45
		DCM	1.96
		EtOAc	0.82
		Aq.	1.24
Microwave assisted extraction(MAE)	5	n- Hex	0.51
		DCM	1.98
		EtOAc	0.91
		Aq.	1.32
	10	n- Hex	0.46
		DCM	1.94
		EtOAc	0.81
		Aq.	1.26
	20	n- Hex	0.32
		DCM	1.02
		EtOAc	0.91
		Aq.	1.12
Ultrasound assisted extraction(UAE)	5	n- Hex	0.49
		DCM	1.98
		EtOAc	0.83
		Aq.	1.28
	10	n- Hex	0.61
		DCM	2.04
		EtOAc	0.89
		Aq.	1.38
	20	n- Hex	0.78
		DCM	2.16
		EtOAc	1.01
		Aq.	1.56

In conventional SE, the yield of DCM fraction was found to be maximum (1.96%). For UAE and MAE, after a treatment of 5, 10, and 20 min the trend was similar. Here also, the maximum yield was observed with the DCM fraction. The solvent from organic fractions was evaporated under reduced pressure below 50 ºC in a rotavapor followed by complete removal of the solvent under high vacuum to obtain 0.1 g of n-Hexane fraction, 0.5 g of DCM fraction, 0.2 g of EtOAc fractions.

It was also found that the net yield of the extract was significantly higher for UAE treatment and the yield increased with treatment time. The maximum yield was observed with UAE treatment for 20 min (2.16%). Ultrasonic treatment uses a wave with a frequency higher than 20 kHz and can cause disruption of the cells with a release of bioactive compounds into the solvent at room temperature. UAE has been reported to be more efficient in keeping the phytochemicals from tea even at lower temperatures as compared to that of conventional techniques [19]. The technique is inexpensive, so it can be a good alternative to conventional extraction techniques.

In case of MAE extraction, a marginal increase in yield was observed for a treatment time of 5 min. However, increasing the treatment time resulted in decreased efficacy of extraction. Bioactive compounds in leaves are accumulated in vacuoles located inside the plant cell, surrounded by rigid cell wall [20]. High temperature and microwave energy may burst the cell wall and release the bioactive compounds into the extraction solvent mixture.

As a consequence of heating effect, the components in the cell dissolve into the solvent more effectively, in accordance with the disruption theory [21]. However, exposure to high temperatures for longer duration might have an adverse effect degraded the functionality of some bioactive compounds in the extract. As can be seen in **Table 1**, the recoveries are similar or better in both UAE and MAE as compared to conventional extraction. Reduced extraction time and solvent consumption are the additional advantages of UAE and MAE. These results indicate that the ultrasound and microwave-assisted solvent extraction methods can be a viable alternative for traditional extraction methods.

Phytochemical Analysis: Detailed phytochemical analysis was performed with n-Hexane, DCM, EtOAc, and Aq fractions of methanolic extract of *M. coreia* for the presence of various phyto-chemicals. The phytochemical analysis of the fractions showed the presence of alkaloids and saponins in all the fractions and flavonoids in EtOAc fraction and aqueous residue. Various plant secondary metabolites like flavonoids, saponins, cardiac glycosides, tannins, triterpenes, and alkaloids have been reported to possess biological activities and observed wide range of antimicrobial and antioxidant properties for the methanol extract and fractions can be explained by the presence of various groups of potentially active classes of these secondary metabolites [22].

TABLE 2: PHYTOCHEMICAL SCREENING OF METHANOL EXTRACT AND FRACTIONS OF THE PLANT *M. COREIA*

Group of chemical constituents	*n*-Hex Fraction	DCM Fraction	EtOAc Fraction	Aq. Residue
Alkaloids	++	++	++	++
Flavonoids	-	-	++	++
Steroid	-	-	-	-
Tannins	-	-	-	-
Reducing Sugars	-	-	-	+
Cardiac Glycosides	-	-	+	++
Triterpenoids	-	-	-	-
Anthraquinones	-	-	-	-
Phlobatanins	-	-	-	-

(+): Presence; (-): Absence

Anti-mycobacterial Activity: The anti-mycobacterial activity was tested against pan sensitive strain of *M. tuberculosis* H37Rv TMC-102. The activity was expressed as a minimum inhibitory concentration (MIC) in Resazurin microplate assay. The screening results of anti-tubercular activity are provided in **Table 3**. The results showed that n-hexane fraction of M. core possessed minimum inhibitory concentration (MIC) of 125 µg/mL.

In general, MIC value, less than 1 mg/mL is considered as active for crude plant extracts. Hence this fraction possesses the potential to be used against tuberculosis. The rest of the fractions, including DCM, EtOAc, and aqueous fractions, showed MIC values of greater than 250 µg/mL. For MAE, the anti-mycobacterial was similar to SE, but UAE demonstrated lower MIC values (62.5 µg/mL) for a treatment duration of 20 min. Ultrasound waves helped disrupt plant cell wall,s improved the solvent penetration, and enhanced mass transfer across the cell membrane [23]. This results in better extraction of bioactive molecules with potential anti-mycobacterial activity. Mycobacterium is characterized by a thicker cell wall rich in mycolic acids/mycolates. The hydrophobic mycolate layer and a peptidoglycan layer are held together by a polysaccharide, arabinogalactan. This particular composition of the cell wall makes Mycobacterium more resistant as compared to other bacterial species. A strong anti-mycobacterial activity demonstrated by *M. coreia* can be extended to other Gram-positive and Gram-negative bacteria also [24].

TABLE 3: ANTI MYCOBACTERIAL AND ANTICANCER ACTIVITY OF *M. COREIA*

Extraction method	Treatment Time (min)	Fraction	MIC(µg/mL) against H37Rv	IC_{50}(µg/mL) against MCF-7
Solvent Extraction(SE)		*n*- Hex	125	33.78
		DCM	250	35.51
		EtOAc	250	38.98
		Aq.	1000	100
Microwave assisted extraction(MAE)	5	*n*- Hex	125	33.64
		DCM	250	35.56
		EtOAc	250	38.91
		Aq.	1000	100
	10	*n*- Hex	125	33.45
		DCM	250	35.64
		EtOAc	250	38.76
		Aq.	1000	100
	20	*n*- Hex	125	38.89
		DCM	250	45.21
		EtOAc	250	48.34
		Aq.	1000	100
Ultrasound assisted extraction(UAE)	5	*n*- Hex	125	33.58
		DCM	250	36.56
		EtOAc	250	38.46
		Aq.	1000	100
	10	*n*- Hex	125	33.45
		DCM	250	35.45
		EtOAc	250	38.21
		Aq.	1000	100
	20	*n*- Hex	62.5	25.22
		DCM	125	27.89
		EtOAc	125	35.21
		Aq.	1000	100

Anti-cancer Activity against MCF-7: Breast cancer cell line MCF-7 was used as the test system to study the anticancer activity of *M. coreia*. Though the cytotoxic screening was conducted only on MCF-7 cells; however, the result could be extrapolated to other breast cancer cells, for example, T47D. The MTT assay indicated that methanol extract of the plant exhibited significant cytotoxic effects on MCF-7. The results further indicate that the n-Hexane fraction is the most active with IC_{50} of 33.78 µg/mL. The DCM and the EtOAc fraction also had significant cytotoxic activity. The aqueous residue, however, did not exhibit a significant anticancer potential in **Table 3**.

For MAE, the activities were found to be similar for the treatment time of 5 and 10 min. For treatment time of 20 min, however, the anticancer potential was found to be lower as compared to the convention SE. Higher temperatures during micro-wave treatment may be causing damage to the bioactive molecules, thus lowering the biological activity of the extract. UAE for a treatment time of 5 to 10 min had comparable anticancer potential as SE. However, there was an increase in activity for the treatment time of 20 min for all the fractions with n-Hexane fraction recording lowest MIC value 25.22 µg/mL

The cell cytotoxicity was measured with the fractions against fibroblast cells at MIC and twice the MIC concentrations. The toxicity was found to be well within the permissible limit (less than 7%) [25]. This data is interesting as it suggests that *M. coreia* more toxic for cancer cells than on normal cells.

CONCLUSION: The investigation based on scientific evidence supports the claim of the local people of South India towards the use of leaves of *M. coreia* for the treatment of tuberculosis and breast cancer. Both the result established that n-Hexane fraction is the most active fraction constitution hydroxyanthraquinone based secondary metabolites as major constituents. Hence, the present study established that the extraction followed by the fractionation method could successfully concentrate biological activity in one fraction. As most active fraction is also the most nonpolar fraction, it suggests that the constituting compounds of the active fraction should be non-polar in nature. A study of the trend of biological activity across the fractions shows that activity of the fractions decreases as the polarity of the solvent increases. The phytochemical study reported a number of non-polar compounds *viz.*, anthrax-quinone, galloic acid derivatives and a steroidal class of compounds. Probably, phytochemical investigation of the n-hex fraction would result in the identification of such class of compounds. Hence, the study established that the n-Hex fraction could be used as chemo-preventive and anti-tubercular agent, subject to further *in-vivo* studies.

The application of ultrasonic-assisted extraction (UAE) is of interest for enhancing the extraction of components from plant and animal materials. It is a green and economically viable alternative to conventional extraction techniques. This study shows that UAE technology can potentially enhance extraction of bioactive components when used as a pre-treatment step in a unit process. The higher yield obtained in these UAE processes are of major interest from an industrial point of view also. This technology can be an “add on” step to the existing process with minimum alteration. UAE may result in reduction in solvent usage, and shortening the extraction time. The use of ultrasonic for extraction purposes is thus an economical alternative to traditional extraction processes, which is an industry demand for a sustainable development. In summary, the results from this study provide useful data regarding the effect of different extraction techniques on the recovery of extractable components from *M. coreia*.

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