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Fractionation and extraction of functional compounds from rice bran wax

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Abstract
The increase in the concept of circular economy in agricultural and allied processing fields has increase by several folds in recent years. Use of agricultural by-products to produce beneficial materials for practical bio-valorisation applications has been sought by several researchers. Rice bran wax, a secondary metabolite from rice processing, is a subsidiary product separated from rice bran oil during refining. Rice bran wax is a very beneficial product, and has good phytochemical composition that exerts antimicrobial effect. Rice bran wax is an abundantly available by-product, due to large production and processing of paddy and rice in south-Asia. The chemical characterization of rice bran wax using FT-IR has revealed its chemical composition and orientation of the compounds. The FT-IR spectrum revealed the chemical orientations like O-H, C-O, C=C, CO-O-CO, N-H etc. corresponding to the chemical constituents present in the wax. The information about these chemical constituents can help in expanding the valorisation methods foe utilizing rice bran wax to its full potential.

Keywords: Rice bran wax, valorisation, FT-IR, chemical, characterization

1. Introduction
Plant oils, a source of lipid substances, are one of humans' most abundantly consumed food ingredients daily. They are generated from corn, gingelly, mustard, sunflower, groundnut, etc. Rice bran oil is also a plant oil generated from the by-product of rice milling, i.e., rice bran. Rice, the staple food for more than half of the world's population, is one of the most abundantly produced and processed food crops (Devraj *et al*., 2020; Lavanya *et al*., 2017) [11, 16]. Waxes from plant or animal sources are esters of carboxylic acids and mono-hydroxy alcohols of high molecular weights. These compounds are unique and are different from lipids or fats. Rice bran wax is a secondary by-product of rice processing (Modupalli *et al*., 2022; Thangaraju *et al*., 2020) [20, 25]. The rice bran oil extracted from bran will be in the crude form and require refining for culinary usage. This process includes winterization to eliminate cloudiness from the oil, which involves separating components of different melting and precipitation points. Generally, when rice bran oil is produced using the hexane dissolution method, it can contain about 2-3% wax components (Vali *et al*., 2005) [26]. The RBW has been effectively used in several food applications like forming oleogels and structured lipids and edible coating of food products (Dassanayake *et al*., 2009; Kodali, 2009) [7, 15].
The rice bran wax is a reddish brown, dark composite separated from rice bran oil and is known to have good nutritional properties. It is separated from rice bran, which has several health-benefiting components. It can be used as an efficient edible coating substance due to its excellent film-forming properties. The RBW has been effectively used in several food applications like forming oleogels, structured lipids, and edible coating of food products. However, the chemical and phytochemical characterization of RBW has a significant research gap. This research concentrates on evaluating the chemical constituents of the RBW and the properties of the bioactive chemicals. A detailed account of the biochemical activity of RBW can be helpful in further exploitation of the secondary metabolites from rice processing for food, cosmetic and pharmaceutical industries. This concept can be under the circular economy, which considers that a by-product for one process line can be utilized as a bio-resource in another. This research concentrates on the chemical characterization of rice bran wax and correlating it with phytochemical components present in the wax.

2. Materials and Methods
2.1 Rice bran wax
RBW was procured from SKM Porna industries, Erode (Erode district, Tamil Nadu, India),

where the crude RBW was separated and pressed to hard cakes. The procured wax was stored in air-tight condition at 4 ± 1 °C till the refining process.

2.2 Refining of Rice bran wax

The wax was de-oiled and refined using hexane and isopropanol as solvents in the laboratory. A 10 % sodium borohydride solution precipitated the crude resin, giving light yellow pure rice bran wax (Vali *et al*., 2005) [26]. The pure wax was dried, packed in air-tight condition, and stored away from direct sunlight till further investigation. All the investigations were performed in triplicates, and the results were reported as mean ± standard deviation.

2.3 Sequential extraction of RBW

The sequential extraction of RBW was conducted to analyze the chemical fractionation of the compounds present in it using different solvents in increasing order of polarity (Ling *et al*., 2020) [17]. The wax samples were extracted using the solvents in the order of lowest to highest polarity, i.e., hexane (S1), ethyl acetate (S2), dichloromethane (S3), isopropanol (S4), methanol (S5), and water (S6). The sample and solvent were taken in a ratio of 1:10 in a conical flask and placed in a rotary shaker for 2 h to facilitate maximum extraction. The wax-solvent mixture was centrifuged at 6000 X g for 30 minutes at 10 °C. The supernatant was separated, and the process was repeated with the same solvent. The supernatants of both extractions were pooled and stored for further analysis. The residue was subsequently extracted using the next solvent, twice with each solvent for maximum extraction rate, until water extraction. All the supernatants from the same solvent were pooled and concentrated using vacuum evaporation and then completely dried using nitrogen flushing. The water extract was freeze-dried to obtain the dry extract. The phytochemical screening analysis and anti-microbial activity analysis of the extracts were performed and reported elsewhere (Modupalli *et al*., 2022) [20]. All the residues were dried under nitrogen flushing and analysed for chemical characterization using FT-IR spectroscopy.

2.4 FT-IR chemical characterization of RBW sequential extracts

The chemical orientations of the precipitates from sequential extraction were analyzed in FT-IR (Shimadzu IR Affinity-1S, Shimadzu Analytical and Measuring instruments, Japan) spectroscopic method (Tanner & Lichtenberg-Kraag, 2019) [24]. The spectroscopic scan was carried out in mid-infrared ranging from 5000 to 400 cm^{-1} with a 4 cm^{-1} resolution in KBr beam splitter and 45 scans per measurement. The precipitate samples were homogenized and applied to the crystal of the FTIR unit, and the samples were scanned at room temperature. The background spectrum was recorded as a graph for all six samples (SXP1 to SXP6) to compare the wavelengths and peak intensities. The spectral data was detected using a lithium tantalate ($LiTaO_3$) mid-IR detector, and the signal-to-noise ratio was 9300:1. The spectra of precipitates were correlated with the previous data on chemical characterization and phytochemical screening analysis reported by Modupalli *et al*., (2022) [20].

3. Results and Discussion

3.1 Extraction and yield of rice bran wax

With the increase in bio-valorisation and circular economy, using biological origin by-products for several extractions and other processes has been gaining much attention. This has acted as the driving force for the industries and researchers to find a use for several by-products of biological origin (Cruz *et al*., 2020) [6]. Rice, being one of the most grown crops in the world, can generate a substantial amount of rice bran. Processing rice bran to oil form can give rise to a comparatively large amount of rice bran wax, which can be utilized in several processes, food and non-food applications (Abhirami *et al*., 2019) [21]. Rice bran wax is a naturally occurring wax ester with good physico-chemical and melting properties. It has been previously reported that the melting point of rice bran wax is more than plant-based carnauba wax and lower than animal-origin beeswax. It has been previously reported that rice bran wax, a lipidic complex, is a good source of several phytochemicals like octacosanol, policosanol etc., (Chen *et al*., 2005; Chen *et al*., 2007) [4, 5]. The biochemical activity of the rice bran wax has shown mild to good anti-microbial activity against a few known microorganisms (Modupalli *et al*., 2022) [20].

During the de-oiling process, the crude rice bran wax was extracted using hexane in the Soxhlet extraction apparatus. This step removes the oil content in the crude wax, as the wax procured was separated from the oil using the sedimentation process. This eliminates about 90% of the oil from the crude wax, and the rest will be separated in the second extraction. The second step of extracting the de-oiled wax again using isopropanol as solvent ensures the removal of any residual oil content in up to 98% of the lipidic fats (Vali *et al*., 2005) [26]. This step also helps to reduce the free fatty acid contents of the wax composites along with iodine value and unsaturated lipid contents, helping to improve its shelf-life. In the final step, the de-oiled and defatted wax composite was extracted with isopropanol by adding a 10% (w/v) solution of Sodium borohydride drop-wise. This precipitates the crude resinous matter from the composites, forming brown-coloured sand-like residues. The wax is then filtered to remove the residues and dried to remove the solvent. The resulting wax would be in light yellow, very low-density powder form. The yield in this method of laboratory refining process was found to be 39.57±4.22%. This is a quite low yield and can be improved by scaling up the process and using larger seeping tanks to carry out the extraction (Abhirami *et al*., 2020; Abhirami *et al*., 2019) [1, 21].

3.2 Sequential extraction of RBW

Phytochemicals like polyphenolic and flavonoid compounds of dietary and non-dietary origin have been on focus of extensive research in the recent past, owing to their protective effects against certain lifestyle diseases. Also, the consumer shift toward natural products has created quite a stir in the pharmaceutical and nutraceutical industries and pushed toward using natural or synthetic products from natural sources. The variety of bioactive molecules present in the plant sources can be considered an immense solution to the problem (Sukanya *et al*., 2009) [23]. Several studies have stated that the phytochemical constituents of rice have health-promoting effects. The effects of rice bran constituents (containing policosanol, octacosanol, oryzanol etc.) on lipid metabolism in human intestines have also been studied to be highly beneficial (Seetharamaiah & Chandrasekhara, 1989) [22]. Previous studies have also stated that rice bran supplementation in the diet improved glucose and lipid metabolisms and blood lipid profile (Ardiansyah *et al*., 2006) [2].

The sequential extraction process involves using different solvents, preferably in order of polarity index lower to higher, and helps in accurately separating the compounds present in the substrate. Certain compounds of plant origin are solvent-specific and require the tenable solvent to be extracted and fractionated. Fractionation of the compounds specifically helps in understanding the number of different compounds from similar chemical families present in the product and will be useful in the identification of bioactive chemicals along with extracting the maximum concentration of the targeted compounds (Brannon *et al*., 1976; Kaasalainen & Yli-Halla, 2003; Martin *et al*., 1987) [3, 14, 18]. The solvent extraction has been performed using hexane, ethyl acetate, dichloromethane, isopropanol, methanol, and water, named S1 to S6 in serial order of extraction. As reported in previous publications, the extracted and dried compounds were screened for phytochemical presence using GC-MS/MS (Modupalli *et al*., 2022) [20]. It was mentioned that the compounds like octacosanol, policosanol, ferulic compounds, stigmasterol and campesterol occurred in different solvent fractions, along with other minor compounds. It has also been stated that the extracted fractions have been shown to have antibacterial activity against certainly known microorganisms.

3.3 Chemical characterization of precipitates from sequential extraction

The FT-IR spectra of the precipitates from sequential extraction have revealed variations between the precipitates. Though the variations were perceived to be small, they can give the spatial arrangement of spectral data that can give the information of the functional groups in the fractions. The FT-IR spectra of the precipitates given in Figure 1 show the details of the functional groups at different wavelengths. The spectral graphs have been juxtaposed and trimmed at different wavelengths to give a clearer picture of the changes in precipitates using solvents.

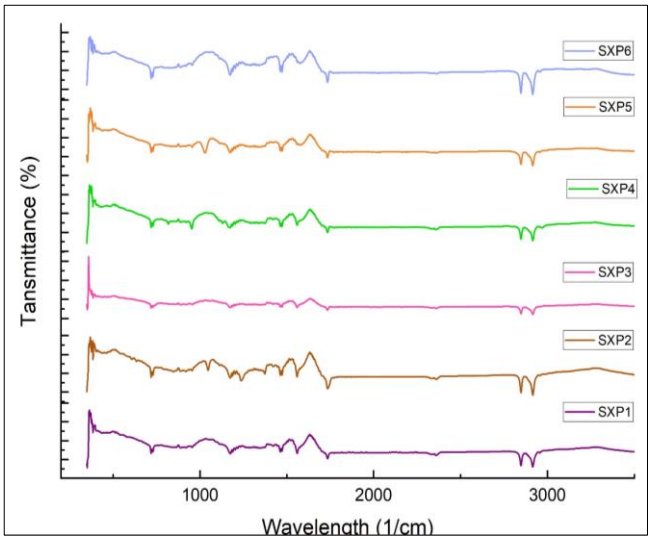

Fig 1: FT-IR spectra of precipitates (SXP1 to SXP6)

It has been observed from the spectra that a strong peak was found near the 2800-2900 wavelength region, which can depict the aldehyde group with medium C-H stretching. Similarly, profound peaks were found at 1300-1500 nm zone, which can be imperative of either C=C strong stretching, indicative of either ketones or alkenes. This can be correlated to the presence of 1-Hexacosene in phytochemical screening (Modupalli *et al*., 2022) [20]. The bending at 1400-1500 nm can indicate O-H bonding or medium stature, showing the presence of alcoholic compounds in the fractions. These O-H bonds can be due to the presence of traces of solvents in the fractions, as most of the fractions had alcohol traces in them. It can also be due to alcohol-containing phenolic compounds like campesterol, stigmasterol, octacosanol and policosanol (Devi *et al*., 2007; Devi & Arumughan, 2007a, 2007b; Ishaka *et al*., 2014; Maznah *et al*., 2010) [8, 9, 10, 13, 19].

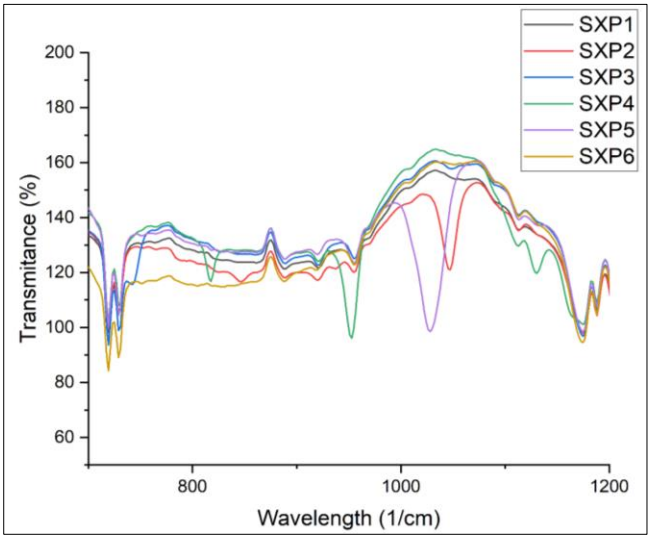

Fig 2(a): Juxtaposed FT-IR spectra between 600 and 1200 nm

Figure 2(a) represents the juxtaposed spectral data between 600 and 1200 nm. It has been observed that between 900 and 1100 nm, the precipitates SXP2, SXP4 and SXP5 showed distinguished as strong spectral signals. This can indicate the presence of either C=C or CO-O-CO or C-O stretching, indicating alkenes or ketones, anhydrides, or secondary alcohol groups. Similarly, SXP4 has shown mild peaks at 800 and 1100 nm, indicating strong C-O stretching, either aliphatic ether or secondary alcohols.

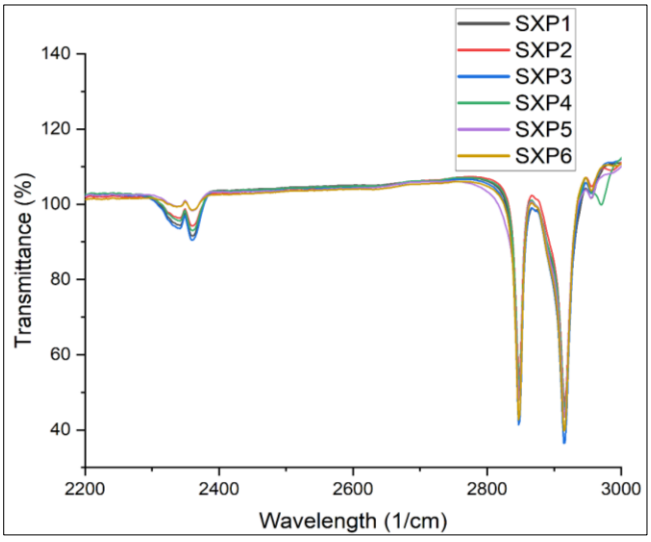

Fig 2(b): Juxtaposed FT-IR spectra between 2200 and 3000 nm

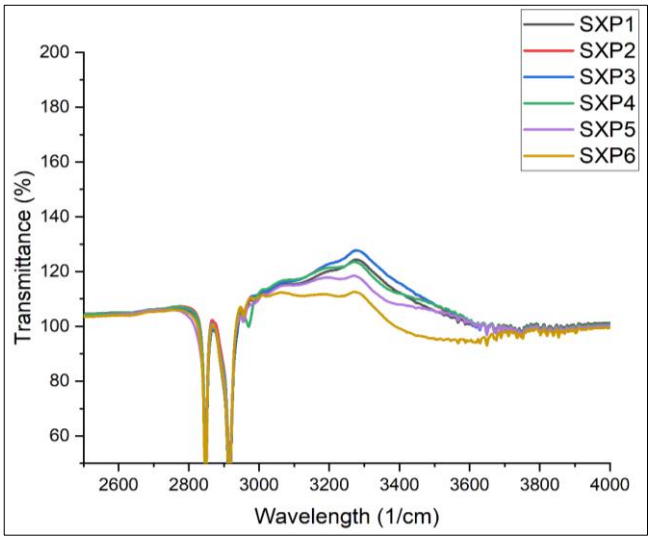

Fig 2(c): Juxtaposed FT-IR spectra between 3000 and 4000 nm

The spectral graphs of precipitates between 2200 to 3000 nm have shown no critical difference between the precipitates, except for the area under peak, which can be correlated to the intensity and concentration of the functional groups. The peaks were observed between 2300-2400 nm and 2800-3000 nm. The 2300-2400 nm peaks show a very strong intensity that can be distributed in alkyne, azide or nitrile groups. Even in the 3000-4000 nm zone, the spectral peaks in the entire graph coincide perfectly, except for the intensities of the concentration, showing very unclear disturbance after 3600 nm. This disturbance can be correlated to the intermolecular O-H bond, which is indicative of the presence of moisture in the sample. The stretching between 3200 and 3400 nm can also indicate the O-H bond of carboxylic nature or alcohol. It can also be presumed that this peak can also depict N-H bonding showing primary amines or secondary amines, which can be a component of rice bran protein fragments or residues trapped in the wax during extraction (Fabian & Ju, 2011; Wattanasiritham *et al*., 2016; Wu *et al*., 2020) [12, 27, 28].

The information about the chemical constituents and orientation of the rice bran wax can help the researchers to further analyze and extract the specific compounds from the wax matrix. Rice bran, an abundantly available by-product, especially in Asia, has been well exploited in recent years for rice bran oil production. Rice bran oil has been highly acceptable by consumers and has been incorporated into culinary trends. The better antioxidant and physico-chemical properties of rice bran oil, owing to its phytochemical array, have been well accepted. Similarly, the de-oiled rice bran and other by-products have also been finding a way for bio-valorisation in recent years. Rice bran wax, a by-product of the refining process of rice bran oil, is also an abundantly available secondary metabolite with beneficial properties.

4. Conclusion

Rice bran wax, an abundantly available by-product in the Southern part of Asia, has been one of the under-exploited products and has a great potential to be tapped. The phytochemical assay of RBW has shown the presence of some compounds with good anti-microbial activity against microorganisms, specifically gram-negative organisms. This can be useful in utilizing RBW for beneficial processes like extracting specific bioactive compounds and developing nutraceuticals and pharmaceutical compounds. However, the study of crude extracts has hindered the evaluation of compounds and their effect. Further studies on extracting and isolating specific bioactive compounds from RBW can elucidate information about its activity. RBW processing, which comes under by-product utilization, can contribute to the concept of the circular economy, where the waste from one bioprocess can become a treasure for another process.

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