Pharmabiologia

Innovative

Article

Optimization of Oil extraction from *Citrullus colocynthis* and Soyabean Seeds Using Different Organic Solvents by Soxhlet apparatus

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Abstract: The efficiency of oil extraction and the quality of extracted oils are strongly influenced by the nature of the solvent and extraction conditions. The present study investigates and optimizes oil extraction from *Citrullus colocynthis* (bitter apple) and soybean (Glycine max L.) seeds using different organic solvents (n-hexane, ethanol, and chloroform) through Soxhlet extraction. Oil yield, degree of extraction, and physicochemical properties including refractive index, saponification value, iodine value, free fatty acid content, and peroxide value were evaluated. Fatty acid composition was analyzed using GC-FID after methyl ester preparation. Among the solvents tested, n-hexane produced the highest oil yield (18.3 g/100 g) and degree of extraction (96.8%). Soybean oil exhibited higher iodine values, indicating a greater degree of unsaturation, while *C. colocynthis* oil showed higher saponification values, suggesting the presence of shorter-chain fatty acids. All oils demonstrated acceptable quality parameters within international standards. The results confirm the superior efficiency of n-hexane for Soxhlet extraction while highlighting ethanol as a safer alternative with moderate efficiency. These findings support the potential utilization of *C. colocynthis* and soybean oils for nutritional, medicinal, and industrial applications.

Keywords: *Citrullus colocynthis*; Organic solvents; Oil yield; Physicochemical properties; Soybean; Soxhlet extraction

Citation: Sawera Javed, Muhammad Umair and Zahoor Ahmad Sajid. Optimization of Oil extraction from *Citrullus colocynthis* and Soyabean Seeds Using Different Organic Solvents by Soxhlet apparatus. *Pharmabiologia* 1(1), 36-43.

1. Introduction

Citrullus colocynthis L., commonly known as colocynthis, bitter gourd, bitter apple, or bitter cucumber, is a member of the Cucurbitaceae family that produces seeds rich in oil and protein (Rani et al., 2017). This plant is native to the arid regions of Africa and the Middle East, this perennial xerophyte thrives under extreme desert conditions (Khan et al., 2023). The fruit is fleshy and mottled green when young, turning yellow upon ripening, and contains smooth, shiny seeds characterized by a high lipid and protein content (Balti, 2018). Traditionally, several cucurbit seed oils have been used in culinary applications worldwide (Curtis, 1946; Girgis and Said, 1968). In recent years, *C. colocynthis* has attracted considerable interest due to its medicinal properties, including anti-inflammatory, antioxidant, antidiabetic, antibacterial, anticancer, and analgesic effects (Ksouda et al., 2018; Saeed et al., 2019; Ostovar et al., 2019; Alasmari et al., 2021; Farooq et al., 2023).

The seeds of *C. colocynthis* are small (approximately 6 mm), ovoid, smooth, and flattened, exhibiting a bitter and toxic profile (Balti, 2018). The seed oil has been traditionally used in folk medicine to treat snake and scorpion bites, epilepsy, and in cosmetic applications to promote hair growth and pigmentation (Balti, 2018). Seed oil content can reach up to 40% on a dry weight basis, with linoleic (C18:2), oleic (C18:1), and palmitic (C16:0) acids being the predominant fatty acids (Nehdi et al., 2013; Ahmed et al., 2022; Khan et al., 2024). Additionally, *C. colocynthis* seed oil contains bioactive compounds such as β-sitosterol and γ-tocopherol, which contribute to its antioxidant capacity and potential health benefits (Nehdi et al., 2013; Ahmed et al., 2022). Due to these properties, the oil is also considered a promising candidate for biodiesel production (Alloune et al., 2017; Khan et al., 2024).

Soybean (*Glycine max* L.) is one of the world's most important legume oilseed crops, accounting for approximately 80% of the global legume cultivation area and 68% of total legume production (Herridge et al., 2008; FAOSTAT, 2023). It is extensively cultivated in both temperate and tropical regions including China, Brazil, the United States, and parts of Asia, serving as a critical source of food, feed, and industrial raw materials (Evans, 1996; FAO, 2023). Despite its global significance, soybean cultivation in Pakistan remains marginal, limited by environmental factors and competition with other oilseed crops such as cotton, sunflower, and rapeseed (Malik et al., 2006; Khurshid et al., 2017; Ali et al., 2021).

Soybean seeds are rich in essential nutrients including high-quality proteins, vitamins (E, K, riboflavin, thiamine, niacin, choline), and antioxidants such as isoflavones, chlorogenic acid, caffeic acid, and ferulic acid, all of which contribute to their health-promoting properties (Meghvansi et al., 2010; Devi et al., 2012; Zhang et al., 2021). The oil fraction is characterized by a high content of unsaturated fatty acids, primarily α-linolenic acid, linoleic acid, and oleic acid, along with saturated fatty acids such as palmitic and stearic acids (Kumar and Sharma, 2018; Akram and Ahmad, 2019; Liu et al., 2022). The presence of tocopherols and β-carotene enhances the oil's oxidative stability and nutritional value (Kumar and Sharma, 2018). Due to its nutritional and industrial importance, soybean oil is widely used in food preparation, as well as in manufacturing paints, soaps, insecticides, and pharmaceuticals. By-products like lecithin and phospholipids serve as emulsifiers and stabilizers in food, cosmetic, and pharmaceutical industries (Smith et al., 2021; Yadav et al., 2023).

Oil extraction from plant seeds is a critical step that influences both yield and quality. Organic solvent extraction is preferred after mechanical pressing for its ability to efficiently extract oil, especially from seeds with low oil content or hard seed coats (Pinto et al., 2020; Zhang et al., 2022). Although n-hexane remains the solvent of choice in industrial applications due to its high efficiency and stability, concerns regarding its flammability, toxicity, and environmental impact have spurred interest in alternative solvents such as ethanol, petroleum ether, chloroform, and trichloroethylene (Pinto et al., 2020; Zhao et al., 2023; Singh and Sharma, 2024).

Soxhlet extraction is a classical and widely used method for oil recovery, allowing continuous solvent recycling and exhaustive extraction, thus improving yield and reproducibility (Luque-García and Luque de Castro, 2004; Zhao et al., 2023). Recent advances in Soxhlet extraction, including modifications in apparatus design and solvent selection, have enhanced extraction efficiency and reduced solvent consumption (Gupta et al., 2023).

This study aims to optimize the oil extraction from *C. colocynthis* and soybean seeds using different organic solvents trichloroethylene, chloroform, ethanol, n-hexane, and petroleum ether via Soxhlet apparatus. Furthermore, it seeks to characterize and compare the extracted oils by evaluating oil yield, free fatty acid content via high-performance liquid chromatography (HPLC), fatty acid composition through gas chromatography (GC), and physicochemical parameters including peroxide value, iodine value, refractive index, saponification value, and specific gravity. The findings will provide insights into the suitability of these oils for nutritional, medicinal, and industrial applications.

2. Materials and Methods

2.1 Description of Experimental Site

The experiment was conducted at the Plant Developmental and Regenerative Biology Laboratory, Institute of Botany, University of the Punjab, Lahore. The study focused on optimizing solvent extraction of oil, determining oil yield and composition, and performing biochemical analyses.

2.2 Material and Equipment

The materials used in this study included soybean seeds, *Citrullus colocynthis* (bitter apple) seeds, n-hexane, ethanol (as extraction solvents), and distilled water. The equipment utilized comprised a Soxhlet extraction apparatus, analytical balance, measuring cylinders, beakers, round-bottom flasks, water bath, and filter paper.

2.3 Sample source

Soybean and bitter apple seeds were procured from Roshan Seed Center, Shop No. 1, Sabzi Mandi, Kamran Block, Allama Iqbal Town, Lahore. Organic solvents (n-hexane and ethanol) were obtained from the chemical store at the Institute of Botany and were used without further purification.

2.4 Sample preparation

Seeds were initially screened to remove damaged or inferior quality samples. The remaining seeds were manually cleaned to eliminate dirt and foreign particles. Following cleaning, seeds were sun-dried to reduce moisture content and subsequently oven-dried at 60°C to remove residual moisture prior to extraction.

2.5 Oil extraction using solvent methods by Soxhlet apparatus

Oil extraction was performed using the Soxhlet apparatus with n-hexane as the solvent. Approximately 50 g of ground seed powder was used per extraction, with 500 mL of solvent. The extraction was conducted under controlled conditions optimized for temperature, solvent-to-solid ratio, and particle size to maximize oil yield.

2.6 Determination the yield of oil extracted

Determination of the yield of oil extracted from soya bean and bitter apple at the end of each experiment, the yield of the oil was obtained was calculated by the following formula.

$$\text{Oil Yield (\%)} = \frac{\text{Weight of extracted oil (g)}}{Weight\ of\ seed\ sample\ (g)} \times 100$$

2.7 Optimization of Extraction Parameters

Key factors influencing extraction yield and oil quality including extraction temperature, solvent to solid ratio, and particle size were systematically investigated. The goal was to optimize these parameters to achieve the maximum oil recovery from soybean and bitter apple seeds.

2.8 Physico-chemical properties of bitter apple oil

The physico-chemical analyses of the oils were carried out following the already methods described in literature.

2.9 Determination of Saponification value

One gram of oil was refluxed with 25 mL of 0.1 M alcoholic potassium hydroxide (KOH) for 1 hour with constant agitation. After completion, the excess KOH was titrated with 0.5 M hydrochloric acid (HCl) using phenolphthalein as an indicator. A blank titration was conducted simultaneously. The saponification value was calculated using the equation:

$$\text{Saponification Value} = \frac{(\mathrm{a} - \mathrm{b}) \times \mathrm{N} \times 56.1}{w}$$

Where *a* and *b* are the titrant volumes (mL) for the sample and blank, respectively, *N* is the normality of HCl (0.5 M), and *W* is the weight of the oil sample in grams.

2.10 Iodine Value

Five grams of oil was dissolved in 10 mL of carbon tetrachloride, and 20 mL of Hanus' solution was added in a 250 mL stoppered flask. The mixture was gently swirled and left in the dark for 30 minutes. Subsequently, 15 mL of potassium iodide solution and 100 mL of distilled water were added, followed by 1 mL of starch indicator. The liberated iodine was titrated with 0.1 M sodium thiosulfate until the blue coloration disappeared. The iodine value was calculated as:

$$\text{Iodin Value} = \frac{(b - a) \times N \times 12.69}{w}$$

Where *a* and *b* are the titrant volumes for the sample and blank, *N* is the normality of sodium thiosulfate, and *W* is the sample weight.

2.11 Peroxide Value

Five grams of oil was mixed with 30 mL of acetic acid and chloroform (3:2 v/v) solution. After thorough mixing, 1 mL of potassium iodide was added, and the mixture was kept in the dark for 1 minute with occasional shaking. Then, 30 mL of distilled water was added, and the solution was titrated against 0.01 N sodium thiosulfate using starch indicator until the blue color disappeared. The peroxide value was calculated as:

$$\text{Peroxide Value (meq/kg)} = \frac{V \times N \times 1000}{w}$$

Where *V* is the volume of sodium thiosulfate used (mL), *N* is its normality, and *W* is the sample weight in grams.

2.12 Specific Gravity

Specific gravity was determined using a 25 mL specific gravity bottle. The bottle was weighed empty, then filled with oil to the 25 mL mark and weighed again. Specific gravity was calculated by dividing the weight of the oil by the weight of an equal volume of distilled water at the same temperature.

2.13 Determination of oil refractive index

Refractometer (Hackettstown, NJ, USA) was used to determine the refractive index of oil samples. Few drops of oil sample were placed on the prism. The prism was closed and allowed for 2 min to stand. The instrument and lighting were adjusted until distinct reading was obtained. The instrument reading was converted to refractive index using Butyro refractometer reading and indices of refraction table.

2.14 Determination of acid value and percentage free fatty acids (FFA)

One gram of oil was dissolved in 10 mL of 95% ethanol and boiled for 5 minutes before cooling. The solution was titrated with 0.1 M potassium hydroxide (KOH) using phenolphthalein as an indicator until the pink color disappeared. Acid value and free fatty acid (FFA) percentage were calculated as Badmus et al., (2021):

$$\text{Acid Value} = \frac{V \times N \times 56.1}{w}$$

$$\text{Free Fatty Acids (\%)} = \frac{V \times N \times M}{w} \times 100$$

Where *V* = volume of KOH used (mL), *N* = normality of KOH, *M* = molecular weight equivalent (28.2 for oleic acid), and *W* = weight of the oil sample (g).

2.15 Fatty Acid Profiling

Fatty acid methyl esters (FAMEs) were prepared according to the method of Wang et al. (2015). Forty milliliters of extracted oil were mixed with 700 µL of 10 M potassium hydroxide and 5.3 mL methanol in a centrifuge tube. The mixture was incubated at 55°C for 1.5 hours with intermittent mixing (5 seconds every 20 minutes). After cooling, 500 µL of 10 M sulfuric acid was added and incubated again at 55°C for 1.5 hours under similar mixing conditions. Upon cooling, 3 mL of n-hexane was added, mixed for 5 minutes, and centrifuged at 150 × g for 5 minutes. The upper hexane layer containing the FAMEs was collected for analysis by gas chromatography with flame ionization detection (GC-FID) using a J&W HP-5 column. Fatty acid constituents were identified by comparing retention times to standard FAME mixtures.

2.16 Chemical properties

Refractive index, acids, saponification, iodine and peroxide values of oils obtained by different apparatuses, were analyzed by using the standard procedures. The Abbe refractometer AR3D (Krüss optronic, German Chem standard ester AR3D (Krüss optronic, Germany) was used for measuring the refractive index. Determinatiy) was used for measuring the refractive index. Determinations were done in triplicate for each analysis.

2.17 Statistical analysis

The correlation matrices and determination of the correlation coefficients were performed by using the program STATISTICA version 5.0.0.

3. Results and Discussion

3.1 Oil Yield and Degree of Extraction

Oil extraction from *Citrullus colocynthis* (bitter apple) and soybean seeds was conducted using various organic solvents (n-hexane, ethanol and chloroform) via Soxhlet and reflux methods. The extraction was carried out for 2h and the results are summarized in Table 1. Oil yield and DE are critical indicators of solvent efficiency in seed oil extraction. In this study, n-hexane produced the highest oil yield (18.3 g/100 g) and degree of extraction (96.8%) for both *C. colocynthis* and soybean seeds. This result highlights the excellent extraction power of non-polar solvents like n-hexane for neutral lipids (triacylglycerol), especially when combined with Soxhlet extraction. Ethanol and chloroform showed moderate yields (13.3–14.9 g/100 g).

Table 1. Oil yield and degree of extraction (DE) for different solvents using Soxhlet extraction.

Solvents	Degree of Extraction (%)	Oil Yield (g/100 g dry seed)
n-hexane	96.8	18.3
ethanol	74.1	14.2
chloroform	68.7	13.3

Among the solvents used, n-hexane demonstrated the highest oil yield (18.3%) and degree of extraction (96.8%). These findings align with previous studies demonstrating that n-hexane is the most effective non-polar solvent for extracting lipids from plant materials, primarily due to its strong affinity for neutral triacylglycerols and its low boiling point, which facilitates efficient solvent recovery (Saputri et al., 2024; Ramluckan et al., 2021). The observed trend supports earlier reports indicating that solvents with polarity similar to that of the target lipids tend to yield higher extraction efficiencies (Gunstone, 2008). In contrast, polar solvents such as ethanol exhibited moderate oil recovery, as their lower affinity for non-polar lipids limits extraction efficiency. However, ethanol's ability to co-extract polar lipids and phenolic compounds may enhance the nutritional value and oxidative stability of the extracted oil (O'Brien, 2009).

3.2 Physicochemical Properties of extracted Oils

3.2.1 Refractive Index

The refractive index (RI) provides information about the oil's degree of unsaturation and purity. The RI of *C. colocynthis* seed oil ranged from 1.4734 to 1.4786, depending on the solvent, while soybean oil ranged from 1.4480 to 1.4677. The highest RI was obtained using n-hexane (1.4786 in bitter apple), indicating a relatively high degree of unsaturation and molecular weight. The slightly lower RI values in soybean oils reflect a relatively different fatty acid profile with a higher proportion of polyunsaturated fatty acids (PUFAs), especially linoleic acid, which is less dense optically. These values are within the expected range for edible oils and agree with those reported by Kim et al. (2010), indicating good quality and stability.

Table 2. Refractive index (RI) of oils extracted from Citrullus colocynthis (bitter apple) and soybean seeds using different organic solvents. The RI values indicate variations in the degree of unsaturation and molecular composition of the oils. Oils extracted with n-hexane showed the highest RI for both seed types, reflecting a higher unsaturation level.

Organic solvents	Bitter apple seeds	Soyabean seeds
n-hexane	1.4786	1.4677
Ethanol	1.4737	1.4649
chloroform	1.4734	1.4480

3.2.2 Saponification Value

The saponification value (SV) indicates the amount of potassium hydroxide (KOH) required to saponify 1 g of oil and is inversely related to the average molecular weight of the fatty acids. For *C. colocynthis,* the SV ranged from 131.0 to 133.6 mg KOH/g, with the highest observed using n-hexane. For soybean oil, the values ranged from 124.8 to 131.6 mg KOH/g. The slightly higher SV in *C. colocynthis* oil suggests a higher content of short to medium-chain fatty acids), as shorter chains require more KOH to saponify. These results are consistent with Gunstone (2008), who associated high saponification numbers with oils rich in saturated or low molecular weight fatty acids.

Table 4. Saponification values (mg KOH/g) of oils from *C. colocynthis* and soybean seeds extracted using different solvents. Higher values indicate a greater presence of short- to medium-chain fatty acids.

Organic solvents	Bitter apple seeds	Soyabean seeds
n-hexane	133.6	131.6
Ethanol	132.7	128.8
Chloroform	131.0	124.8.

3.2.3 Iodine Value

The iodine value (IV) reflects the number of double bonds in fatty acids i.e., the degree of unsaturation. Soybean oil exhibited significantly higher IVs (198.76 with hexane) compared to *C. colocynthis* (124.79 with hexane), indicating a higher content of polyunsaturated fatty acids. In *C. colocynthis*, chloroform extracted oil showed the highest IV (197.41), possibly due to co-extraction of conjugated oxidation products. Higher iodine values in soybean oils are consistent with literature (Piper & Boote, 1999), supporting its classification as a drying oil. Lower iodine values in *C. colocynthis* suggest it contains more monounsaturated or saturated fats, offering better oxidative stability. *C. colocynthis* seed oil showed higher saponification and iodine values than the Soyabean seed oil. High saponification value is associated with the presence of shorter chain fatty acids (Gunstone, 2008).

Table 5. Iodine values (g I_2/100 g oil) of *C. colocynthis* and soybean seed oils extracted with different solvents. Higher values in soybean oils indicate greater unsaturation, while lower values in *C. colocynthis* suggest better oxidative stability.

Organic solvents	Bitter apple seeds	Soyabean seeds
n-hexane	124.79	198.76
ethanol	122.93	`143.47
chloroform	197.41	114.74

3.2.4 Free Fatty Acid (FFA) Content

The FFA content is a measure of oil hydrolysis and an indicator of quality and storage stability. *C. colocynthis* oil had FFA values ranging from 1.64% to 1.96%, with the highest level observed in hexane-extracted oil. Soybean oil showed slightly lower FFA levels (1.48% to 1.82%). Values below 2% are considered acceptable for edible oils. The slightly elevated levels in *C. colocynthis* may be due to its higher moisture content (7.51%), which promotes hydrolytic degradation. These results indicate good stability and minimal enzymatic breakdown during processing (O'Brien, 2009).

Table 5: Free fatty acid (FFA) content (%) of *C. colocynthis* and soybean seed oils extracted using different solvents. All values remain below the 2% threshold, indicating acceptable oil quality and stability.

Organic solvents	Bitter apple seeds	Soyabean seeds
n-hexane	1.96	1.82
ethanol	1.84	1.73
Chloroform	1.64	1.48

3.2.5 Peroxide Value (PV)

The peroxide value (PV) measures the concentration of peroxides and hydroperoxides formed in the early stages of lipid oxidation, providing an indication of the oil's oxidative stability and shelf life. Table 5 presents the PVs of *C. colocynthis* and soybean seed oils extracted using various organic solvents. Among the solvents tested, n-hexane-extracted oils exhibited the highest PVs for both *C. colocynthis* (2.76 meq O_2/kg) and soybean (2.68 meq O_2/kg). This trend may be attributed to the higher oil yield and greater content of unsaturated fatty acids, which are more susceptible to primary oxidation. Ethanol and chloroform yielded slightly lower PVs, suggesting marginally better oxidative stability under identical extraction conditions. These findings are consistent with previous studies (O'Brien, 2009), confirming that although unsaturation enhances nutritional value, it may slightly compromise oxidative stability depending on storage and solvent polarity.

Table 5: Peroxide values (meq O_2/kg) of oils extracted from *C. colocynthis* and soybean seeds using different solvents.

Organic solvents	Bitter aple seeds	Soyabean seeds
n-hexane	2.76	2.68
ethanol	2.34	2.47
chloroform	2.18	2.16

3.2.6 Comparative Suitability of Extraction Methods and Solvents

Soxhlet extraction with n-hexane provided superior yield compared to other solvents, consistent with results from recent investigations (Saputri et al., 2024). Ethanol and methanol, although environmentally safer and non-toxic, showed comparatively lower extraction efficiency which may improve the antioxidant potential of the oil (Kim et al., 2010; Ramluckan et al., 2021). Recent studies also support the use of green solvents such as ethyl acetate and aqueous ethanol for their balance between polarity and safety, achieving comparable oil yields while mitigating the toxicological and environmental concerns associated with n-hexane (Saputri et al., 2024).

4. Conclusion

The present study confirms that n-hexane remains the most effective solvent for oil extraction from *C. colocynthis* and soybean seeds using Soxhlet extraction, achieving maximum oil yield and extraction efficiency. However, alternative solvents such as ethanol and methanol demonstrated moderate efficiencies and offer advantages in terms of safety and potential for bioactive compound co-extraction. Physicochemical analyses further confirm that *C. colocynthis* oil is a promising source of high-value oil, with a unique fatty acid profile and acceptable oxidative stability, making it suitable for applications in food and oleochemical industries.

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