Extraction and Characterization of Bioactive Compounds from Kinnow Seeds *(Citrus reticulata)*

A thesis submitted in partial fulfilment of the degree of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

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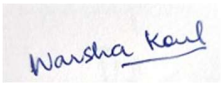
DECLARATION

I, hereby declare that the experimental work introduced in this dissertation entitled "Extraction and Characterization of Bioactive compounds from Kinnow Seeds *(Citrus reticulata)*" for the degree of Master of Technology in Biotechnology award is an authentic record of my work. The work has been performed under the supervision of Dr. Ovais Shafiq Qadri, assistant professor at the Department of Biotechnology at Thapar Institute of Engineering and Technology (Patiala).



Place: Patiala, Punjab

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CERTIFICATE

I certify that the project entitled "Extraction and Characterization of Bioactive Compounds from Kinnow Seeds *(Citrus reticulata)*" was submitted by Warsha Koul, Roll No. 602104018, for the fulfilment of the requirement for the award of the degree of Master of Technology in Biotechnology. The work has been carried out under my supervision. It is also certified that the work of the present thesis or any part of this has not been submitted to this university or any other university for the award of any other degree or diploma.

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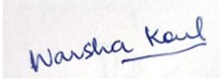

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Symbols	Abbreviations
%	Percent
min	Minutes
RSM	Response surface methodology
°C	Degree centigrade
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Ferric reducing antioxidant power
TPC	Total phenolic content
UAEE	Ultrasound assisted enzymatic extractions
TSS	Total soluble solids
cm	centimeter
ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
mg	milligrams
kg	kilograms
rpm	Revolutions per minute
NA	Nutrient agar
LA	Luria agar
EY	Extraction yield
SET	Transfer of single electrons
CO2	Carbon dioxide
NaOH	Sodium hydroxide
GC-MS	Gas chromatography mass spectrometry

HCL	Hydrochloric acid
ml	milliliter
gm	grams
hrs	hours
AT	Acid treatment
BT	Base treatment
F	Conversion factor
N	Normality
M	Molarity
FAME	Fatty acid methyl ester molecule
KOH	Potassium hydroxide
rt	Retention time
µl	microlitre
µg	microgram
µM	micromolar
A	absorbance
nm	nanometer
mm	micrometer
DMSO	Dimethyl sulfoxide
PDB	Potato dextrose broth

ABSTRACT

During the present study, a study on Kinnow seeds was conducted on their extraction, optimization, and characterization. Kinnow seeds were given three treatments for the extraction of pomace acid treatment, base treatment, and vacuum drying. The three treatment combinations were analyzed for the seeds' moisture, fat, protein, and nitrogen content. On analysis, the acid treatment proved to be an effective method among the three treatment combinations. For estimation of kinnow seed oil extraction yield, the suitability of ultrasound pretreatment in n-hexane solvent as well as enzymatic treatment with cellulase enzyme to extract oil from kinnow seed powders. To further optimization, the effects of ultrasonic time (30–90 min), cellulase enzyme concentration (1–2%) and ultrasound power (20-40%) were investigated using response surface methodology (RSM). The optimum conditions of ultrasound-assisted enzymatic extraction using n-hexane as solvent (UAEE) were found to be 49 min of ultrasonic pretreatment time, cellulase concentration of 1.66%, and ultrasound power of 38% before the incubation process at a temperature of 56 °C for 120 min. The Kinnow seed extracts were evaluated for their total phenolic content (TPC), antioxidant properties with two antioxidant assays DPPH (2, 2 diphenyl-1-picryl hydroxyl) and FRAP (ferric reducing antioxidant power) and antimicrobial (antibacterial and antifungal) properties using different microbial strains.

CHAPTER 1 - INTRODUCTION

Kinnow is a citrus fruit crop which comes in the mandarin group and is widely grown in India and Pakistan. Kinnow was initially developed at the University of California Citrus Experiment Station in the year 1935 and was introduced in India during the early 1940s. It is a hybrid of two citrus cultivators, namely 'King' (Citrus nobilis) and 'Willow Leaf' mandarin (Citrus deliciosa). In India, Kinnow is grown mainly in the parts of Punjab, Rajasthan, Himachal Pradesh, Haryana, Uttarakhand, and Jammu & Kashmir. The prevailing climatic condition during winters helps for this ample production in these states, which further helps enhance the sweetness index and distinct taste. Punjab is India's leading producer of kinnow, with 29% of total national production. Fazilka district of Punjab covers 55% area of cultivation and contributes 58% of total production(Mahawar et al., 2020a).Globally, the annual production of citrus fruits is 124.73 million metric tons; in India, the annual production of kinnow is 10.48 million tonnes.

- Domain: Eukaryota
- Kingdom: Plantae
- Phylum: Magnoliophyta
- Class: Magnoliopsida
- Subclass: Rosidae
- Order: Sapindales
- Family: Rutaceae

- Genus: Citrus

Large-scale kinnow mandarin cultivation occurs in northern India. Due to limited market opportunities and improper post-harvest practices, the fruit has high post-harvest losses, amounting to 25–30% of its original value. Most post-harvest losses occur on many levels, including those of orchards, transportation, wholesaler marketing, and retailers. Premature fruit drop occurs in orchards because of physiological and pathological reasons. Second, truckloads of fruit are frequently produced simultaneously and cumulatively from several farms, and after sorting, only the best fruit is sent for processing and fresh consumption, leaving the low-quality kinnow behind as waste. Furthermore, kinnow being a perishable good, several pack house procedures (sorting, waxing, and packaging) are carried out to make its use viable for both domestic and export marketing (Aggarwal et al., 2022).

Fig1. Kinnow fruit

- Colour: Golden yellow (peel), deep yellowish orange (pomace)
- TSS/acid ratio: 12:1–14:1
- Size: 5.0–9.7 cm

- Shape: Moderate to oblate; both base and apex flattened or slightly depressed
- Appearance: Very smooth, glossy, and sometimes faintly pitted
- Firmness: Firm, not soft and easily peelable

Medium-sized Kinnow mandarins have a diameter of 7 to 8 cm and a round to oblate form with a little flattened top and bottom. The mandarin's rind is paper-thin, glossy, and smooth, with a few small pits and recessed oil glands. The peeling process reveals that the orange part is separated in 9 to 10 sections by fragile membranes behind the thick but comparatively simple-to-peel dark orange skin. One fruit can have between 15 and 30 seeds, and its interior is soft, sensitive, and watery. The flavor of Kinnow mandarins is brilliant, strong, profoundly sweet, tart, and acidic with fruity and flowery undertones, despite being seedy. The kinnow juice processing industry produces peel and pomace waste in significant numbers, accounting for 55–60% of the fresh fruit weight, where around 30% of citrus fruits are processed to make juice(Singla et al., 2021). A considerable quantity of by-products comprising peel (30−40%), pomace (juice sac residue), rag (membranes and cores), and seeds are produced during citrus juice processing(Sharma et al., 2017).Like other horticultural crops, kinnow also requires specific conditions for their growth where soil types may range from clay loam and sandy loam to acidic soils with a pH range of 5.5–7.5. Kinnow, a member of citrus fruits, is a small to medium-sized, cadmium-yellow-colored fruit and the world's second most beneficial crop, after grapes, grown in nearly 125 countries (Purewal et al., 2022). Kinnow fruit processing industry produces about 60% of fruit wastes and by-products like pomace, seeds, and peel, with peels accounting for about 40–55% of the content. Kinnow Peels are rich in polyphenols, organic acids, and enzymes, making them a good alternative for developing functional food products with health-benefitting properties such as antibacterial, anticarcinogenic, antimutagenic, cardio-preventive, and immune-boosting activities (Rafiq et al., 2022).The naturally occurring compounds found in kinnow

peels, including polyphenols, flavonoids, carotenoids, limonoids, and tocopherol, have powerful antioxidant capabilities. The flavonoids found in Kinnow peels, such as naringin, hesperidin, nobiletin, limonin, narirutin, tangeretin, and eriocitrin, possess several health advantages that include antioxidant, anti-inflammatory, anti-cancer, anti-diabetic, anti-ulcer, and antimutagenic effects, while naringin (40,5,7-thrihydroxyflavanone-7-rhamnoglucoside) is the key flavonoid found in Kinnow peels with high biological activity. However, one of the most prevalent bitter compounds in Kinnow peels, considered to be the primary cause of their bitterness, is the naringin flavanone. As a result, the use and supplementation of the bioactive compounds of Kinnow peels in the food industry are impacted (Suri et al., 2022). Kinnow is a prevalent fruit crop nutritionally rich in vitamin C, vitamin B, b-carotene, calcium, phosphorous and other health-benefiting compounds. The primary limiting factor to the exploitation of fruit is its availability during the off-season which is very limited due to its poor shelf life of 8–10 days (Mahawar et al., 2020a).Kinnow fruit seeds are planted between August and October, and harvesting starts when the fruit's external color becomes orange, from December to February. Kinnow is a non-climacteric fruit that does not ripen after harvest. Therefore, the maturity of this fruit does not depend on the respiration rate and ethylene production deceptively; hence, fruits obtain optimum maturity on the tree within 8 to 9 months having TSS (11%), TSS/acid ratio (13.70) and acidity (0.8%)with their maximum juice content (Aggarwal et al., 2022).As mentioned earlier, the presence and activation of bitter flavanones (naringinin and limonin) cause kinnow juice to become bitter during processing. There lies another limiting factor that the citrus juice processing industry deal with is delayed bitterness brought on by limonin production. This accounts for a significant barrier to gaining the approval of consumers and the industrial processing of kinnow, thus resulting in only 5% of fruits being processed, with the remaining 95% still being served a fresh. Taking steps to lessen the bitterness of kinnow juice gives

manufacturers a chance to produce processed goods in the off-season (Aggarwal et al., 2022). During maturation, the color of the fruit changes from green to yellow or orange according to its genetic character and growing climatic conditions (Saini et al., 2022). Kinnow industry by-products, i.e., peels, are enriched with essential oils exhibiting antimicrobial activity. Kinnow pomace residue waste is generally rich in cellulose (40%), hemicellulose (10%), pectin (2%) and antioxidants (15%), with physical properties of ash, crude fat, and protein (Singla et al., 2021).However, the main issue with fully utilizing kinnow mandarin and its byproducts is the development of bitterness, which has an impact on consumer acceptability. Additionally, kinnow pomace quickly degrades because of the high moisture and sugar content, making it easy for molds and yeast to grow quickly. Byproducts from the kinnow juice industry have strong antioxidant activity, which aids in lowering cholesterol and blood sugar levels (Singla et al., 2021).Kinnow being a great source of vitamin C, and has an immune system-boosting activity, thus helping in the reduction of inflammation. It is also a good source of fibre, which supports the functioning of the digestive system. Kinnow could be consumed in raw as well as processed form. The fruits also include smaller quantities of copper, folate, calcium, phosphorus, iron, and copper, as well as antioxidants to prevent cell damage from free radicals, potassium to regulate fluid levels inside the body, vitamin A to maintain proper organ functioning, and folate. The pharmacological importance of kinnow is not only limited to its edible parts but also to non-edible parts like seeds and peel, which are also rich sources of bioactive constituents with health-benefiting properties. Kinnow juice, seeds and peel have received much attention, particularly due to the presence of antioxidant, antimicrobial, and anticancerous properties. Kinnow is a cheap industrial fruit crop which is easily available in the market (Purewal & Sandhu, 2020).

The pericarp (peel) and endocarp are the two main parts of the fruit, with the pericarp branching into epicarp (flavedo) and mesocarp (albedo). This mid-season cultivar has few seeds that are adhered to

9–10 segments, silky peel that becomes deep orange when ripe, and flavorful juice. The peak harvesting season of kinnow in different states varies from November (Jammu & Kashmir), January (Himachal Pradesh), December–January (Haryana), January (Rajasthan) and January–February (Punjab), respectively (Mahawar et al., 2020a).

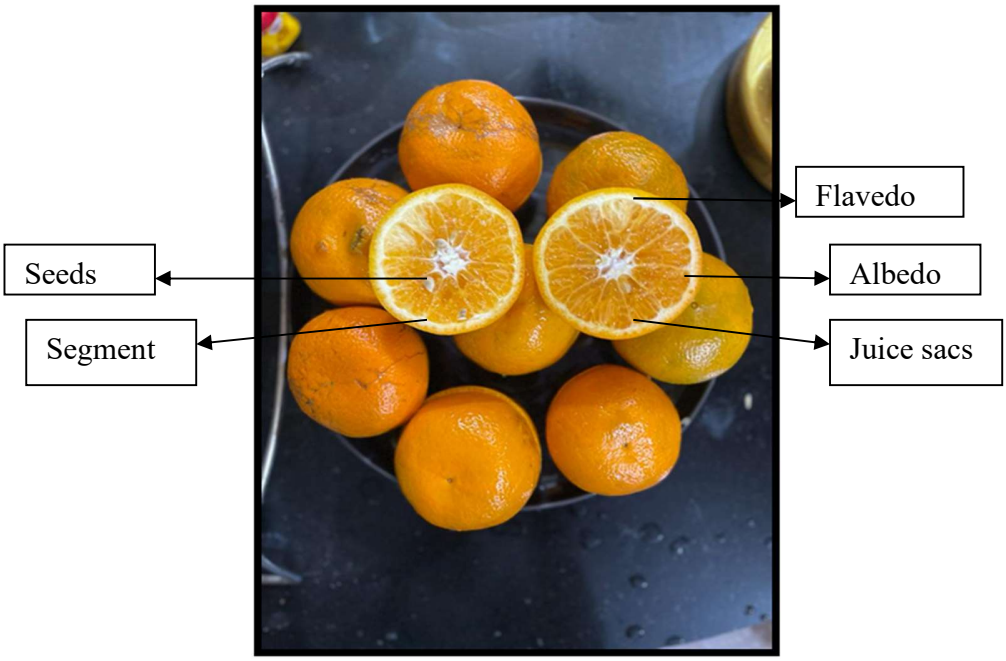


Fig2. Labelled diagram of kinnow

CHAPTER 2 - OBJECTIVES

- To design a process to extract seeds from kinnow pomace and analyze the physical properties of kinnow seeds.
- To extract different bioactive compounds from kinnow seeds.
- To characterize the extracts for antioxidant and antimicrobial properties.

CHAPTER 3 - REVIEW OF LITERATURE

A significant amount of waste is produced during the processing of fruits in the form of pomace remains, skin, and seeds. These remaining parts, which are typically discarded and treated as waste, provide industrial benefits since they may be put to a variety of different ends. Citrus fruits are among the fruits that are frequently used to boost immunity and are a significant source of minerals and vitamins. Citrus fruits contain significant quantities of seeds, which are often wasted following fruit processing. Suri et al. (2022) demonstrated that seeds of citrus fruits possess a good amount of protein and essential oil. According to reports, kinnow seeds contain important phenolics, limonoids, and carotenoids. These components make seeds potentially helpful in the medicinal, cosmetic, and agricultural sectors. Examples include the widespread use of fruit seeds as a source of essential oils, seed flour in sausages, burgers, and pancakes, antibacterial properties in skin care products, and the production of biodiesel (Purewal et al., 2022).

Bioactive compounds in kinnow

Natural resources like fruits and vegetables include bioactive substances, including a specific family of phytochemicals with pharmacological and antioxidant activities. These naturally occurring bioactive substances are a significant component of our daily diet. Kinnow peels have a comparatively rich number of beneficial components to kinnow juice (Purewal & Sandhu, 2020). The presence of primary and secondary metabolites such as amino acids, minerals, and vitamins, including bioactive substances like flavonoids, phenolics, carotenoids, and limonoids, as well as primary metabolites, makes Kinnow mandarins a distinctly important fruit (Aggarwal et al., 2022).Those bioactive compounds exhibit a significant nutritional value which is essential to

medicinal, food processing, and biofuel generation industries. Applying various high-efficiency procedures, the bioactive compounds may be isolated and utilized for a variety of purposes (Godara et al., 2020). The foremost fruit wastes, viz. peel and seeds, can be highly utilized for the extraction of aromatic compounds, essential oils and low-methoxyl pectin (Mahawar et al., 2020a).

Table 1. Types of bioactive compounds present in by-products of kinnow.

By-product	Bioactive compound	Extraction methods	References
Peel	Polyphenols	Solvent extraction methods. Ultrasound-Assisted extraction	Mahawar et al. (2020b)
Peel	Pectin	Sequential microwave assisted solvent extraction	Kaur et al. (2022)
Peel	Lutein	Ultrasonication technique	Saini et al. (2021)
Pomace/Pomace	Limonene	Gas chromatography	Mahawar et al. (2020b)
Seed flour	Polyphenols	Folin-Ciocalteu assay. TPC of seed extracts was quantified using gallic acid.	Mahawar et al. (2020b)
Seed extracts	Antioxidant properties	ABTS test	Singh et al. (2021)

Essential Oils

The potent antibacterial, antioxidant, and anti-inflammatory effects of essential oils found in kinnow wastes are of great advantage to medicines, cosmeceuticals, food additives, and preservatives in

preventing spoiling (Mahawar et al., 2020a). The kinnow peel consists of two tissues: albedo (inner layer) and flavedo (outer layer). Flavedo constitutes essential oils which are useful for the flavor and fragrance industry. The essential oils present in the flavedo layer are rich in carotenoids, terpenes, and linalool (Godara et al., 2020). These kinnow peels also contain large amounts of essential oils, which are composed of certain major volatile compounds, viz. limonene (71.80%), β-myrcene (4.55%), sabinene (1.39%), linalool (3.89%), and α-pinene (1.17%) (Suri, Singh, &Nema, 2022). The essential oils present in kinnow wastes have also been tested to have various therapeutic effects like antibacterial, antifungal, antiviral, anticancer, and anti-malarial effects (Kaur et al., 2023). In kinnow peels, the maximum amount of oil was obtained from oven-dried (0.50%) followed by ambient dried (0.48%) and fresh (0.30%) peel samples. Kinnow seeds have a great potential for being an oil source attributed to their fatty acid composition and important tocopherols present in them. It consists of 98.6% of fatty acid, which includes palmitic acid (21.9%), stearic acid (4.0%), oleic acid (21.3%), cis-vaccenic acid (2.0%), linoleic acid (43.7%), linolenic acid (5.0%), arachidic acid (0.4%), eicosenoic acid (0.1%), behenic acid (0.2%) and 13.7 mg/ kg of tocopherol content, which includes a-Tocopherol (7.1 mg/kg), c-Tocopherol (6.6 mg/kg) (Mahawar et al., 2020a).

Extraction of Seeds

Seed extraction techniques often used are alkali treatments, acid treatments and drying. Each technique has advantages and disadvantages depending on the application period, concentration, and temperature. For example, the advantages of acid treatments are efficient breakdown of the gelatinous coating and quick cleaning, eradication of bacterial canker, inactivation of tomato mosaic virus and producing bright-looking seed coat. On the other hand, it can be deteriorative to seed quality when concentration and application period are not appropriate (Degwale et al., 2023).

Optimization

Mechanical pressing and solvent extraction (often using hexane) are the conventional industrial methods for obtaining oil from citrus seeds. High-quality oil is produced using mechanical presses, although the process often consumes a lot of energy and results in low oil recovery (40–60%). The recovery rate of solvent extraction is between 90 and 98%, but it requires a significant upfront investment, a lot of energy, a high temperature, a long extraction period, and a lot of organic solvents, which may be dangerous for people and the environment. Additionally, the oil meal produced with this process has poor protein quality (Haji Heidari &Taghian Dinani, 2018).

Enzymatic and ultrasonic extractions, which give efficient and precise oil extraction procedures, are two enhanced alternative approaches that are both desirable and promising. An innovative and successful method for extracting oils from seeds is enzymatic extraction. This approach involved the addition of certain enzymes to the sample at a regulated pH level, followed by incubating the combination at a specific temperature, time, and shaking rate. The benefits of this oil extraction technique are excellent meal quality, affordability, health benefits, and environmental friendliness (Wei et al., 2022).

Enzymatic treatment also uses mild conditions, which helps to protect the value of extracted components. Additionally, the presence of appropriate enzymes during extraction damages oil bodies and cell walls that promote oil release. As cellulose comprises quite a significant amount of the polysaccharides in plant cell walls, cellulase enzyme is favorable for the objective of destroying the cell walls to boost the yield of oil extraction. However, aqueous enzymatic oil extraction has some drawbacks, including a longer extraction time, lower oil recovery than conventional processes, higher enzyme costs, a need for a lot of water for de-emulsification, high operating costs, high greenhouse gas emissions, particularly CO_2 emissions, acute toxicity of the NaOH used to adjust

pH, a need for a lot of energy for de-emulsification and water removal from oil, and larger effluent generation. In the present study, the enzymatic extraction technique using n-hexane solvent was applied for the first time to address these disadvantages (Haji Heidari&Taghian Dinani, 2018).The oil recovery was reported to be increased by introducing hexane to the enzyme and oil seed powder slurry during the enzymatic hydrolysis. It is important to note that hexane-based processes have been used in industrial extraction processes for edible oil from oilseeds for a long time because it is possible to accomplish significantly higher oil yields and higher solvent recovery with significantly higher energy efficiency and lower greenhouse gas emissions (Zayed et al., 2021).

A recent innovation in the oil industry is the ultrasonic extraction technique. The implosion of cavitation bubbles, which results in localized pressure and high-shear gradients, is the fundamental mechanism of amplifying mass transfer phenomena by the ultrasonic process. These occurrences cause the breakdown of cell walls, disturb the flow of the solvent, allow the solvent to effectively enter the cells, and speed up the diffusion of the oil into the solvent. Another advantage of ultrasonic extraction is that it protects more of the extracts' bioactive components by functioning at lower temperatures (Haji Heidari &Taghian Dinani, 2018).

Characterization

Analyses were made of seed extracts (SWE, SEE, SME, SE50%E, SM50%E). In the current study, five distinct extraction phases—water, methanol, methanol 50%, ethanol, and ethanol—each performed a particular function in extracting phenolic compounds from seeds. Any botanical resource's ability to recover phenolic compounds is substantially impacted by the kind of extraction medium used. Since TPC directly contributes to the provision of antioxidant potential to any extract, the antioxidant qualities may differ depending on the kind of extractant utilized. The TPC value for kinnow seed extracts (SWE, SEE, SME, SE50%E, SM50%E) ranged from 2.14 to 8.95 mg GAE/g.

SEE had the lowest TPC value (2.14 mg GAE/g), while SM50%E had a higher TPC value (8.95 mg GAE/g) (Safdar et al., 2017). Following ethanol (at 50%), water, and other solvents as effective extraction phases, followed by ethanol (50%), water, methanol, and ethanol, respectively. A higher number of bioactive metabolites were used in the aqueous phase compared to the absolute solvent, agreeing with the observations reported by (Safdar et al., 2017).Purewal & Sandhu (2020) demonstrated that secondary metabolites (phenolic compounds) not only function as primary antioxidants (i.e., donate hydrogen atoms or electrons), but they additionally function as secondary antioxidants on extraction.

The highest percentage of DPPH inhibition found in seed extracts was 60.78% for SM50%E, followed by 49.25% for SE50%E, 41.26% for SWE, 37.05% for SME, and 31.7% for SEE(Safdar et al., 2017). The extracts prepared using aqueous methanol (50%) had the highest percentage of DPPH inhibition (60.78%), whereas the extracts prepared using ethanol as the extraction medium had the lowest percentage of DPPH inhibition (31.7%). (Safdar et al., 2017) studied the DPPH test to study the antioxidant capabilities of Kinnow peel extracts. The DPPH inhibition activity of extracts made in methanol (100%) was 55.61%, whereas that of extracts prepared in methanol (50%) was 60.67% (Safdar et al., 2017).

The FRAP values for seed extracts produced with different solvents were 20.23 mM/100g (SM50%E), 14.33 mM/100g (SE50%E), 12.46 mM/100g (SWE), 8.23 mM/100g (SME), and 5.87 mM/100g (SEE), respectively (Purewal & Sandhu, 2020). Furthermore, SM50%E's FRAP value was higher than SEE (Purewal & Sandhu, 2020). These findings show that aqueous methanol has a larger potential (50%) for extracting phenolic compounds from Kinnow seeds. Ghafar et al. (2010) studied that citrus species (*C. aurantifolia, C. sinensis, C. microcarpa*, and *C. hystrix*) were utilized to make the extracts. The comparison of the FRAP values of the studied extracts revealed that *C. hystrix* had

a higher value (89 mol Fe^{2+} equivalent/100 mL), whereas *C. microcarpa* had a lower value (48.18 mol Fe2+ equivalent/100 mL).

Table 2. Different extraction processes on citrus fruits, their optimization &characterization.

Fruit	**Extraction process**	**Chemicals and instruments used**	**Optimization and Characterization**
Mitha (Citruslimetta), Grapefruit (Citrus paradisi), Mussami (Citrussinensis), and Kinnow (Citrus reticulata)	Soxhlet extraction	n-hexane, rotary evaporator	Tocopherol content, Degumming of Oil, GC/MS Fatty Acid Composition, Analysis of Oilseed Residues. (Anwar et al., 2008a)
Citrus fruits	Solvent extraction i) Soxhlet extractor (non-polar solvents) ii) Polar solvents Ultrasound-assisted extraction Cold pressing	n-hexane, ethyl ether and petroleum ether, methanol, ethanol ethanol, methanol Hexane, methanol	Antioxidant, antibacterial, tocopherols phenolic compounds, carotenoids, Phytosterols, fatty acids, phenolic compounds, food packaging, biodiesel production, functional foods, preservative (Zayed et al., 2021)
Citrus fruits	Soxhlet apparatus	di-ethyl ether	Mosquito rearing, bioassay (Marabuto & Rebelo, 2018)
Citrus fruits	Soxhlet apparatus	di-ethyl ether, steam distillation method	Bioassay (Nasir et al., 2016)

Lemon seeds	Cold pressing Solvent extraction	Nitrogen, centrifuge. Hexane, centrifuge, nitrogen	Physiochemical analysis, fatty acid, sterol, tocopherol composition, thermal analysis (Yilmaz &Güneşer, 2017)
Citrus fruits	Soxhlet apparatus	Hexane, rotator evaporator	Fatty acid composition, chemical composition, physical properties, oil quality, stability, antioxidant activity, antimicrobial activity. (Ndayishimiye et al., 2017)
Citrus seeds	Soxhlet apparatus	Hexane	Antimicrobial, physical characteristics (Jorge et al., 2016)

CHAPTER 4 - MATERIALS AND METHODS

4.1 Preparation of sample

Fresh Kinnow pomace was procured from the juice vendor of the local market of Patiala, Punjab, India. The experimental samples were collected by extracting Kinnow pomace (fruit waste after collecting kinnow juice), which was stored in the refrigerator at -18°C for further use. Different treatments were performed on the pomace for collecting seeds from the kinnow pomace.

Fig3.Processing of kinnow fruit and by-product obtained.

4.2 Extraction of seeds

For extracting seeds from kinnow pomace, different treatments were performed through which seeds were recovered i.e., acid treatment, base treatment, and vacuum drying. Acid treatment was done using 2% HCl (35.45%) i.e., 5.64ml HCl and 94.36ml water, and base treatment was done using 2% NaOH [1:5], i.e., 100ml water and 4gm NaOH. These treatments were performed for 10 minutes at room temperature (Degwale et al., 2023). At the end of the treatment, the seeds were washed in running tap water. Drying treatment was given by drying the kinnow pomace in a vacuum oven for 12 hours, and then the seeds were collected from the dried sample (Purewal et al., 2022). After collecting the seeds from all the treatments, the moisture content present in the seeds was higher. After that, the seeds were dried in a vacuum oven for 2-3 hrs till the final seed moisture content was

determined by moisture balance, ensuring the moisture content was lower than 10% (Purewal et al., 2022).

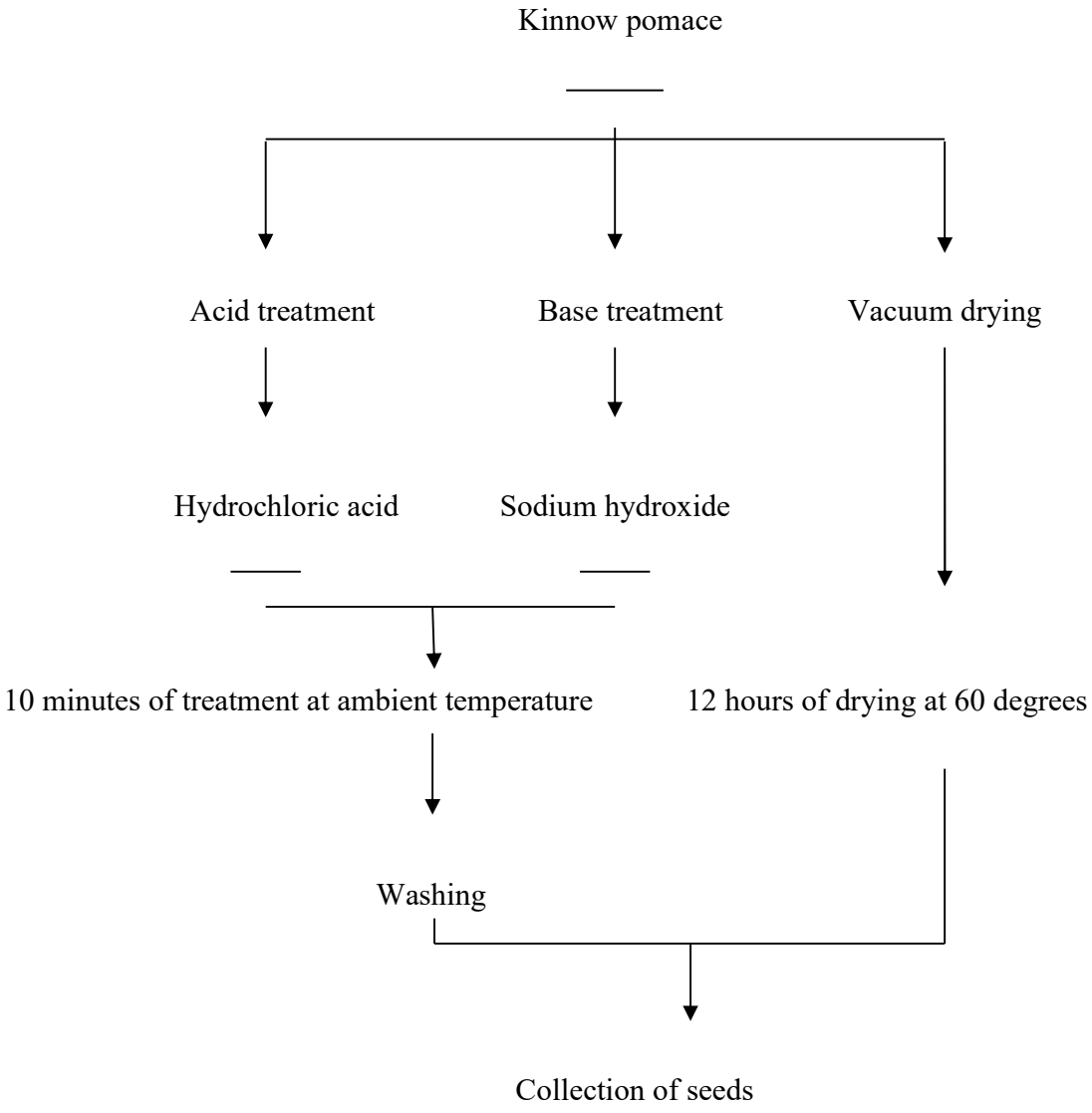


Fig4. HCl (Acid) treatment for seed extraction and removal of the pomace.

Fig5. NaOH (Alkali) treatment for seed extraction and removal of the pomace.

Fig6. Vacuum oven (Drying) treatment and manual removal of seeds.

4.2.1 Proximate analysis

4.2.1.1 Moisture content

This experiment used moisture analyzer apparatus (Aczet brand model MB 50). Seeds were placed in the moisture plate. The results were directly displayed on completion of the run. So, for further experimental design, the final moisture content of the seed samples should be lower than 10% (Degwale et al., 2023).

Fig7. Seeds obtained after AT, BT, and vacuum drying.

4.2.1.2 Fat content

The fat content was analyzed using the Soxhlet apparatus (modal SCS 4 E: SOCS PLUS). By using this method, fats, waxes, and oils may be precisely and quickly removed from food samples. Take 2 g of the sample into the thimble and place the thimble in the solvent holder with the help of a thimble supporting ring. Now add petroleum ether, a non-polar solvent, through a thimble to the solvent holder to submerge the sample. Connect the solvent holder to the condenser and insert it into the apparatus, i.e., on the heating plate. Make sure the water supply to the condenser is on before turning the device on. Run the automatic system for two hours. Only the extracted fat or oil is left in the solvent holder when the procedure is finished, and the greatest amount of solvent is preserved in the

condenser. Extracts (the extraction cups) were dried in a hot-air oven with an adjusted temperature of 100℃ and weighed to calculate crude fat content(Anwar et al., 2008a; Ndayishimiye et al., 2017).

$$\text{Crude Fat (\%)} = [\frac{W2 - W1}{S}] \times 100$$

Where,

- W2 = Weight of the beaker after extraction.
- W1 = Weight of the empty beaker before extraction.
- S = Weight of sample taken.

Fig8. Automatic Soxhlet extraction for determining fat content of seed samples.

4.2.1.2 Protein content

The estimation of protein content was conducted using the Kjeldahl method. Johan G.C.T. Kjeldahl, a Danish chemist, designed this technique to measure the nitrogen concentration of organic and inorganic compounds. The protein content of food is also estimated using this approach (Anwar et al., 2008a; Park et al., 2021). The Kjeldahl technique does not directly assess the protein content; rather, a conversion factor (F) is required to convert the nitrogen concentration that was measured into a protein concentration.

Digestion

The digestion tube containing the seed flour sample (1 g) was filled with 25 ml of sulfuric acid, 10 g of potassium sulfate, and 1 g of copper sulfate. The digestion tube was then heated for 3hrs at 350^{o}C(Mahawar et al., 2020a).

Fig9. Kjeldahl nitrogen estimation apparatus for protein content determination of the seed sample.

Distillation

The machine was shut off after three hours, and 90ml of 40% NaOH and 50ml of distilled water were then poured into the tube. In a flask, a few drops of mixed indicator and 4% boric acid were then added. Run the distillation apparatus for 20 min. Finally, the green color was observed, which was our nitrogen content recovered from the sample and contains ammonia.(Mahawar et al., 2020a).

Fig10. Determination of Nitrogen content present in the sample.

Titration

The resultant solution after distillation was titrated with O.1 N HCl until the endpoint (pink color) was reached.

Fig11. Sample after titration.

As the Kjeldahl apparatus determines the nitrogen percentage present in the test sample, the titre values are utilized for the estimation.

$$\text{Nitrogen (\%)} = \frac{\text{Sample titre} - \text{Blank titre}}{\text{Wx100}} \times 14 \times \text{N} \times 1000$$

Where,

- N = Normality of the standard HCL solution.
- W = Weight of sample taken.

The protein content was determined by multiplying a conversion factor (5.04) with the amount of nitrogen present in the test sample.

$$\text{Protein (\%)} = \text{Nitrogen \%} \times 5.04$$

4.3 Optimization

To evaluate the efficiency of the UAEE method, optimal UAEE treatment using n-hexane as solvent was given. The parameters included ultrasonic time of 30-90 min, cellulase concentration of 1-2% and amplitude of 20-40% before the incubation process. They were optimized using RSM. The incubation was carried out at a temperature of 56°C for 120 min (Haji Heidari & Taghian Dinani, 2018).

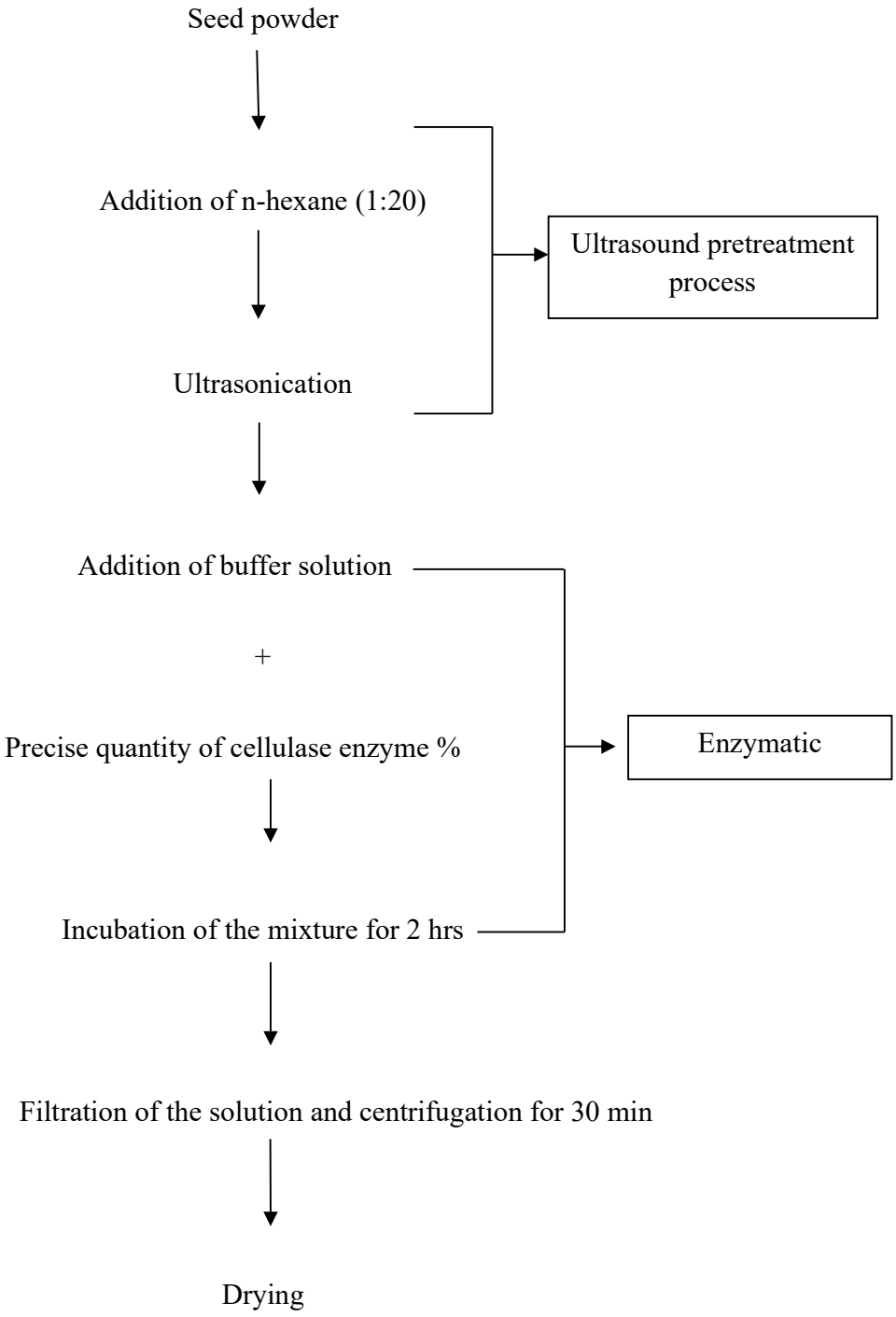
4.3.1Extraction of oil% using different solvents

In this experiment, 1g of seed sample was added to 25 ml of solvent (1:25) and incubated in an incubator shaker at 37℃ for 12hrs. After that, the mixture was filtered, and the remaining solvent was dried in a hot-air oven (*50*°C). Lastly, the yield of the seed sample was calculated (Zayed et al., 2021).

$$\text{Yield } = [\frac{\text{W2} - \text{W1}}{\text{Ws}}] \times 100$$

- W2=weight of beaker after extraction.
- W1=weight of beaker before extraction.
- Ws= weight of the sample.

4.3.2Extraction of oil% using ultrasound-assisted solvent enzymatic extraction



In this experiment, cellulase enzyme was purchased.

4.3.2.1 Ultrasound pretreatment process

The ultrasonic pretreatment was given to the mixture of kinnow seed powder (2g) and n-hexane (20 ml) at a solid-to-liquid ratio of 1:20. The ultrasound extraction procedure was carried out from 30 to 90 minutes of ultrasonic time and amplitude of 20% to 40%. Following ultrasonic pretreatment, the sample and solvent mixture was removed from the beaker and put through an enzymatic extraction procedure (Wei et al., 2022).

Fig12. n-hexane addition to the seed sample after ultrasonication.

4.3.2.2 Enzymatic extraction process

After ultrasonic pretreatment, a buffer solution of 0.2 M aqueous disodium hydrogen phosphate and 0.1M aqueous citric acid with the correct pH and a precise quantity of cellulase enzyme 1% to 2% was dissolved. It was then mixed with the mixture of enzyme and buffer solution. The conical flask's mixture was incubated for 120 minutes at 56°C in a shaking incubator and filtered through Whatman No. 1 filter paper. The filtered sample was centrifuged at 6000 rpm for 30 min to separate water and solvent. The n-hexane solvent was removed from the extracted oil by heating it in an oven at 60°C for 2 h (Wei et al., 2022).

Fig13. Different steps for extraction of oil from seed sample.

Table 3. Different extraction parameters used to calculate the yield of the sample.

Std	Enzyme conc.	Time	Amplitude	Extraction yield
1	1.50%	90	30	20.65
2	1.50%	90	30	19.83
3	2.00%	30	40	23.85
4	2.00%	30	40	22.74
5	1.50%	60	20	21.25
6	1.50%	60	20	21.98
7	1.00%	30	40	20.81
8	1.00%	30	40	20.55
9	1.00%	90	40	22.65
10	1.00%	90	40	21.13
11	2.00%	90	20	23.11
12	2.00%	90	20	22.64
13	1.00%	90	20	24.43
14	1.00%	90	20	23.93
15	2.00%	90	40	23.91
16	2.00%	90	40	22.82
17	1.00%	60	30	26.72

18	1.00%	60	30	26.65
19	2.00%	30	20	25.77
20	2.00%	30	20	25.12
21	1.50%	30	30	26.46
22	1.50%	30	30	25.91
23	1.50%	60	30	24.51
24	1.50%	60	30	25.63
25	1.50%	60	30	24.75
26	1.50%	60	30	25.15
27	1.50%	60	30	24.65
28	2.00%	60	30	25.35
29	2.00%	60	30	25.95
30	1.50%	60	40	26.65
31	1.50%	60	40	26.11
32	1.00%	30	20	24.75
33	1.00%	30	20	23.81

4.4 Characterization

4.4.1 Total phenolic content

In this experiment, 500μL of Folin& Ciocalteu's phenolic reagent and 100 μL of seed extracts were put into a reaction vial (sample tube) to begin a reaction. Next, 1500μL of a 20% aqueous sodium carbonate solution was added. The final amount (10 mL) was then made using distilled water (7.9 mL). To prepare the standard curve, a gallic acid standard stock (mg/ml) was prepared and further diluted (100 g/ml, 200 g/ml, 300 g/ml, 400 g/ml, 500 g/ml, 600 g/ml, and 700 g/ml). The gallic acid standard curve equation was used to calculate the TPC of seed extracts, and the findings were given as mg GAE (Gallic acid equivalent)/g (Al Juhaimi et al., 2018; Purewal et al., 2022).

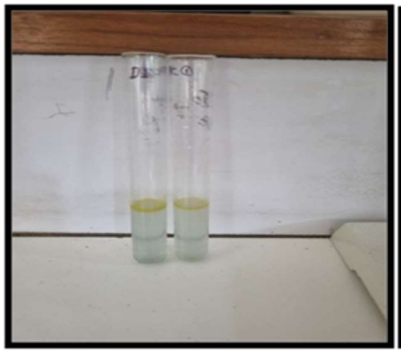


Fig14. Seed extract and reagent mixture **Fig15. Gallic acid standard**

4.4.2Fatty acid composition

Gas chromatography (GC-MS with library search)

It was done via GC-MS with a NIST library search. For the GC-MS analysis, the fatty acid sample was further converted into fatty acid methyl ester molecules (FAME). The prepared sample was sent to the Sophisticated Analytical Instrumental Faculty, which is in IIT Madras, Chennai, where the GC-MS with library search was carried out.

The preparation of the fatty acid methyl ester process was carried out in 5 steps:

Step 1: Preparation of reagent: 0.5 M methanolic Potassium hydroxide (KOH: 2.8 g of potassium hydroxide dissolved in 100 ml of methanol). After shaking, the mixture was stored in a dark and cool place.

Methanolic HCl: To prepare methanolic HCl, stock concentration was 4:1, i.e., HCl: Methanol respectively. 5 ml of Methanol was dissolved into the 20 ml of HCl and shaken till the solution was mixed properly.

Step 2: Preparation of sample: A dry saponification glass was taken, and the weighing balance was tared. 200 microliter of oil sample was added into the flask, and the weight was noted. 4 ml of 0.5

methanolic potassium hydroxide was added into the flask, and mixed the content properly on shaking.

Step 3: Saponification: The condenser was attached to the flask and heated to boiling point in the water bath. Heating was carried out with the periodic shaking of the flask. After 15 minutes of boiling, 1.6 ml of methanolic HCl solution was added to the flask and boiled for 25 minutes. After 25 minutes, the condenser was detached and cooled the flask at room temperature.

Step 4: Fatty acid methyl ester molecules (FAME) extraction: 8 ml of deionized water was added and mixed properly. 6 ml of n-Hexane was added and shaken well for one minute. Transferred the mixture into the clean glass tube and gave a quick spin. After the spin, two layers were observed. In the upper layer, hexane was separated. Collected the upper hexane layer into another clean glass vial. Removed the lower layer in the flask, added 5 ml of hexane and rotated properly. Again, two distinct layers were observed, and the upper layer was extracted into the glass vial. The lower layer was added to the flask again, adding 4 ml of hexane, and rotated properly. Again, two distinct layers were observed, and the upper layer was extracted into the glass vial. Stored the vial with the sample at 4°C before analysis.

Step 5: Filtration of FAME extraction: The sodium sulfate was taken up in the syringe and attached to the 0.4-micrometer syringe filter. Poured 3 ml of extracted FAME molecules into the syringe. Discarded the few drops. The filtered sample was collected in a GC vial.

Note: 2 ml of the sample was sent for the GC-MS with library search analysis.

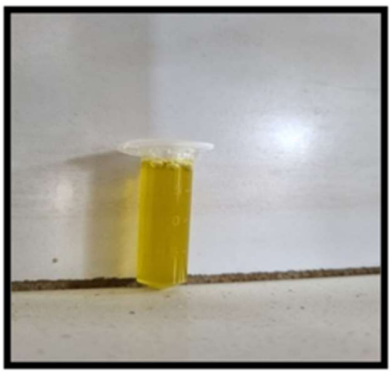


Fig16. Seed oil sample

4.4.2Antioxidant activity

4.4.2.1DPPH (2, 2’ Diphenyl-1’ picrylhydrazyl) radical scavenging assay

Seed extracts were evaluated for % DPPH inhibition using the DPPH assay described by (Purewal, Kaur, et al., 2022).100 μL of seed extract was added in a reaction vial (sample tube); thereafter, DPPH reagent (3 mL, 100 μM) was added, and the resulting mixture was then incubated at room temperature under dark conditions (30 min). Absorbance (A) of the colored extracts-reagent mixture was noted (517 nm) at 0 and 30 min against blank (Zayed et al., 2021). DPPH inhibition activity (%) was calculated using the formula mentioned below:

$$\% \text{ Inhibition} = \frac{\{\text{A (Extract t} = 0) - \text{ A (Extract t} = 30)\}}{\text{A (Extract t} = 0)} \times 100$$

Fig17.The reaction of seed extract with DPPH reagent

4.4.2.2FRAP

FRAP value of extracts was determined using the reported method with minor variations (Purewal et al., 2022). 100 μL of seed extract was added to the sample tube and allowed to react for 10 minutes in the dark with a freshly made FRAP reagent (3 mL, straw-colored). The combination of seed extracts and reagents (ferrous tripyridyltriazine complex) was measured for absorbance (A) at 595 nm (Park et al., 2021).

Fig18. FRAP reagent and reaction mixture.

4.4.3 Antimicrobial activity

4.4.3.1 Antibacterial activity

As test cultures, the following bacteria were used: *Escherichia coli; Lactobacillus*. The sensitivity of microorganisms was determined by the diffusion test. 20 µL of extracts were introduced into wells. The diameters of the inhibition zones were measured in millimetres, including the diameter of the well. The antimicrobial effect was assessed by the presence of a growth inhibition zone. Based on the literature, a positive control, ampicillin and for the negative control, DMSO was used (Salamon et al., 2021; Zayed et al., 2021).

Fig19. Luria broth and Nutrient broth

4.4.3.2 Antifungal activity

As test cultures, the following fungi were used: *Collectotrichuim gloeosporioids* 3801 ITCC Delhi; *Collectotrichuim gloeosporioids* 6152 ITCC Delhi, M.S Toshi; *Fusarium latertum* 4533 ITCC Jammu. The sensitivity of microorganisms was determined by the agar diffusion test. 20 µL of extracts were introduced into wells. The diameters of the inhibition zones were measured in millimeters, including the well's diameter. The antifungal effect was assessed by the presence of a growth inhibition zone (Jorge et al., 2016; Majhenič et al., 2007).

Fig20. Potato dextrose broth containing fungi cultures.

CHAPTER 5 - RESULTS AND DISCUSSION

In this study, extraction, optimization, and characterization of kinnow seeds are implemented. Firstly, Kinnow seeds are extracted from the kinnow pomace using different treatments. Afterwards, seeds are used to determine the fat and protein content present in the seeds. These seeds are then used to extract the oil from the seeds and determine the oil yield present in the kinnow seeds. UAEE is the method used to determine the yield. The oil extracted from the seeds is further used to characterize the antioxidant and antimicrobial activities.

5.1 Extraction of seeds

As shown in the below figures, different treatments were performed on kinnow pomace, i.e., acid treatment, base treatment, and vacuum drying. All three treatments show different effects on the physical properties of the seed. In acid treatment in which HCL was used, there was no change in the color of the seeds, and the pomace was easily removed, but in the case of base treatment in which NaOH has used, a slight change in color was seen, i.e. yellow colored coating appeared, and some of the pomace remained attached to the seed and in last treatment drying vacuum oven was used for drying in this treatment also change in color and there was difficulty in recovery of seeds.

Fig21. The color of seeds after acid treatment, base treatment, and vacuum drying

The number of seeds extracted from 100 gm of pomace ranged between 20 to 40. After doing treatments on seeds, different results were observed. As shown in the above picture, there was a change in color in both the base treatment and drying. In the case of acid treatment, the color of the seeds remained unchanged, and there was also ease in extracting seeds from the pomace. Whereas in base treatment, a small amount of pomace remains attached to the seeds, which requires 3-4 times more washing, and in vacuum drying, it takes much more time to extract seeds, as pomace remains attached to the seeds after the removal. As per a study by Degwale et al. (2023), the advantages of acid treatments are efficient breakdown of the gelatinous coating and quick cleaning, eradication of bacterial canker, inactivation of virus and producing bright-looking seed coat.

Also, different types of analysis were done on these seeds, shown in the table below. Acid treatment also shows slightly higher results than base treatment and drying. As discussed in Degwale et al. (2023), alkali treatment and drying decrease the seed quality and darkens the seeds' coating. On analyzing the results, acid treatment was selected to optimize and characterize seeds.

5.1.1 Proximate analysis

Table 4: Weight of seeds, moisture, fat, nitrogen, and protein content present in kinnow seeds (% value)

Treatment	Weight of seeds after treatment (100gm/gm)	Moisture content (%)	Fat content (%)	Nitrogen (%)	Protein (%)
Acid treatment	4.151 ± 0.11	5.717 ± 0.08	20.21 ± 0.02	2.142 ±0.01	10.72 ± 0.13
Base treatment	3.21 ± 0.04	5.68 ± 0.05	18.75 ± 0.08	1.83 ± 0.01	9.29 ± 0.02
Vacuum Drying	2.706 ± 0.19	4.56 ± 0.05	19.6 ± 0.16	1.972 ± 0.001	9.87 ± 0.06

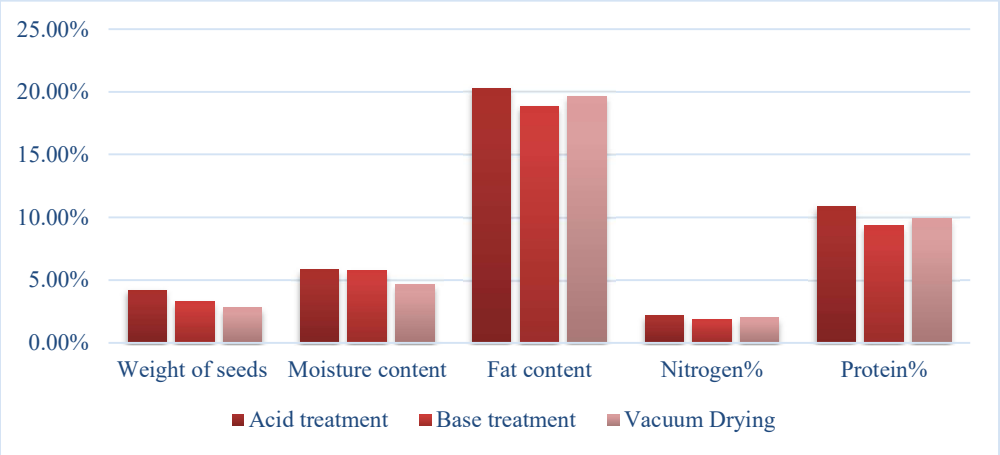


Fig 22. The comparative results of seed properties with different extraction treatments.

5.1.1.1 Moisture content

As shown in the below table, the moisture content was higher than 10% in seeds when given different treatments. To increase the quality and shelf stability of the seeds, the moisture content of the seeds should be less than 10%, so vacuum drying was done on the seeds for 2-3hrs, and the moisture content was decreased to less than 10% to make them more stable for longer periods (Degwale et al., 2023).

5.1.1.2 Fat content

The petroleum ether extracted fat content of kinnow seeds ranged from 18.75% - 20.21%, as shown in the table above. The fat content of all three treatments was calculated in which acid treatment showed a slightly higher amount than base and drying. As reported by Anwar et al. (2008a), a relatively low amount of oil content in kinnow seeds was observed in comparison to *C. sinensis*, *C. paradisi*, *C. aurantium*, *C. aurantifolia* which ranges from 24.1% - 32.4%. The oil concentration was highest in the seeds of Grapefruit (36.5%) and lowest in Mausami (20.12%). Juhaimi et al. (2018) reported that kinnow seeds are considered a potential oil source due to their fatty acid composition and important bioactive compound tocopherol.

Fig23. Oil remains after the fat extraction process while determining fat content.

5.1.1.3 Protein content

Proximate analysis of the kinnow oilseed residues revealed that the protein contents ranged from 10.72%-9.29%. The protein content of all three treatments was calculated in which acid treatment showed a slightly higher amount than base and drying. The Kinnow (*C. reticulata*) fruit exhibited the highest protein contents (9.7%), whereas, Grapefruit (*C. paradisi*) had the lowest protein level (3.9%) (Anwar et al., 2008a; Mahawar et al., 2020a). Anwar et al. (2008) also reported the protein contents of Egyptian citrus seeds, which ranged between 15.9–19.9% and 13.8–17.4%, respectively. The protein contents for sweet orange (*C. sinensis*) seeds were observed to be 3.1–3.2%. Anwar et al. (2008) evaluated the protein contents of sweet orange (*C. sinensis*) seeds to be 10.0%.

5.2 Optimization

5.2.1 Extraction of oil using different solvents

The solvent optimization was carried out using five different solvents for extracting oil from kinnow seeds. The selection criterion for the solvents was based on the extraction yield and their miscibility with water, and hexane was selected for the further process of extracting oil from seeds (Wei et al., 2022). For the further extraction process, one solvent was selected, i.e., hexane, because hexane

shows a slightly higher amount of yield after processing in an incubator shaker for 24hrs. As reported by (Wei et al., 2022), n-hexane was used as a solvent for its attributes, such as it shows simple recovery, non-polar nature, low latent heat of vaporization (330 kJ/kg) and high selectivity to solvents.

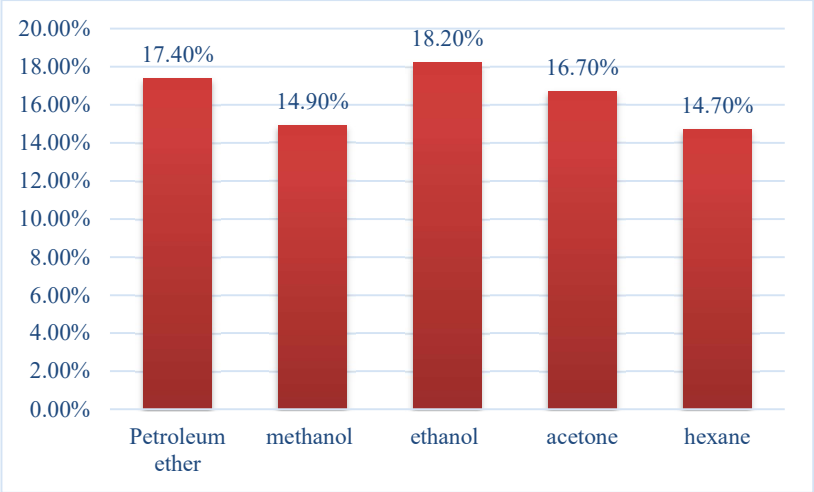


Fig24. Percentage oil obtained using various solvents for extraction.

5.2.2 Extraction of oil using ultrasound-assisted enzymatic extraction

To evaluate the efficiency of the UAEE method, optimal UAEE treatment using n-hexane solvent (ultrasonic time of 49 min, cellulase concentration of 1.66% and amplitude 38% before the incubation process at a temperature of 56°C for 120 min) shows the maximum amount of yield (Haji Heidari &Taghian Dinani, 2018). According to Hu et al. (2019), an increase in enzyme amount during the extraction process increases hydrolysis of the cell wall and thus improves seed oil release; however, a continuous increase in enzyme amount may result in high costs. Therefore, while the selection of the appropriate enzyme amount, the balance between cost and oil yield should be taken into consideration (Gai et al., 2013). In the seed oil extraction procedure involving ultrasound radiation, longer treatment time and higher ultrasound power may produce more bubbles, which can

induce a stronger shock wave and shear force in plant cells, causing them to form and burst instantaneously; this can result in the rupturing of plant cell walls, thus improving the plant seed oil yield. However, a lower ultrasonication time and power may result in incomplete extraction. The remaining variables—incubation time and solvent-to-solid ratio had no significant influence on the kinnow seed oil yield extracted using UAEE (Wei et al., 2022).

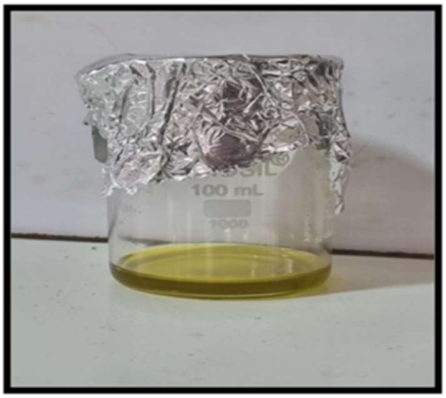


Fig 25. Extraction of oil using the UAEE method.

Extraction Yield

Table 5. ANOVA for Response Surface Cubic Model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	123.9361851	13	9.533552702	29.45570643	< 0.0001	significant
A-EnzymeCon	0.8649	1	0.8649	2.672271428	0.1186	
B-USTime	35.343025	1	35.343025	109.1989315	< 0.0001	
C-USAmplitude	22.705225	1	22.705225	70.15206848	< 0.0001	
AB	2.6569	1	2.6569	8.208992896	0.0099	
AC	5.2441	1	5.2441	16.20263452	0.0007	
BC	4.6225	1	4.6225	14.28208426	0.0013	
A^2	6.612524774	1	6.612524774	20.43064056	0.0002	
B^2	16.99838792	1	16.99838792	52.5197206	< 0.0001	
C^2	5.347045651	1	5.347045651	16.52070449	0.0007	
ABC	0.2401	1	0.2401	0.741834166	0.3998	
A^2B	24.28808	1	24.28808	75.04259708	< 0.0001	
A^2C	36.34208	1	36.34208	112.2857001	< 0.0001	
AB^2	2.67912	1	2.67912	8.277645771	0.0097	
AC^2	0	0				
B^2C	0	0				
BC^2	0	0				
A^3	0	0				
B^3	0	0				
C^3	0	0				
Residual	6.149487597	19	0.323657242			
Lack of Fit	0.014707597	1	0.014707597	0.043153421	0.8378	not significant
Pure Error	6.13478	18	0.340821111			
Cor Total	130.0856727	32				

Final Equation in Terms of Coded Factors:

A = Cellulase concentration; B =Ultrasonication time; C = Amplitude

Extraction Yield = +24.98 -0.46 * A -2.97 * B +2.38 * C -0.41 * A * B +0.57 * A * C +0.54 * B * C +1.11 * A^2 -1.79 * B^2 -1.00 * C^2 +0.12 * A * B * C +2.75 * A^2 * B -3.37 * A^2 * C +0.91 * A * B^2

The above equation shows the factors that affect the amount of yield in a positive or negative manner. When the factor A and B increases, it negatively affects the percentage of the yield. However, by increasing the factor C the positive effect was shown. The positive effects were shown on the extraction yield by multiplying all the factors, which shows an increase in oil extraction yield. The factors A, B, and C are responsible for the increase and decrease in oil extraction yield. The amplitude (C) shows the increase in the percentage of the yield, so by increasing the amplitude of the ultrasonicator, the extraction percentage of oil increases.

5.3 Characterization

5.3.1 Fatty acid composition

The crude fat's fatty acid content was determined by saponification, followed by the creation of their methyl esters. Later these fatty acid methyl esters were analyzed by the GCMS with a library search. Individual fatty acids were identified with the help of mass spectral deviation, as shown in Figure 28, and the compounds are listed below with different parameters.

The hexane extract of kinnow seed was methylated with diazomethane, and its volatile fraction was analyzed by gas chromatography-mass spectroscopy (GC-MS). Thirty-five compounds were detected, most of which were fatty acids (as their methyl esters), were seen in the below tables. γ-Sitosterol was by far the most dominant fatty acid, followed by ethyl iso-allocholate, stigmasterol,

and campesterol. Minor acids, found at levels between 3.05 and 4.21%, were Tetradecane, 2,6,10-trimethyl-, Dodecane,2,6,11-trimethyl-, Dodecane,2,6,10-trimethyl- and Hexadecane,2,6,11,15 trimethyl-. Several fatty acids present below 1% were also detected, and they are included in the below tables as trace components. The below tables show some fatty acids Cholest-8(14)-en-3-ol,4,4-dimethyl-, (3β,5α)- present in small amounts (0.78%) as compared to other fatty acids.

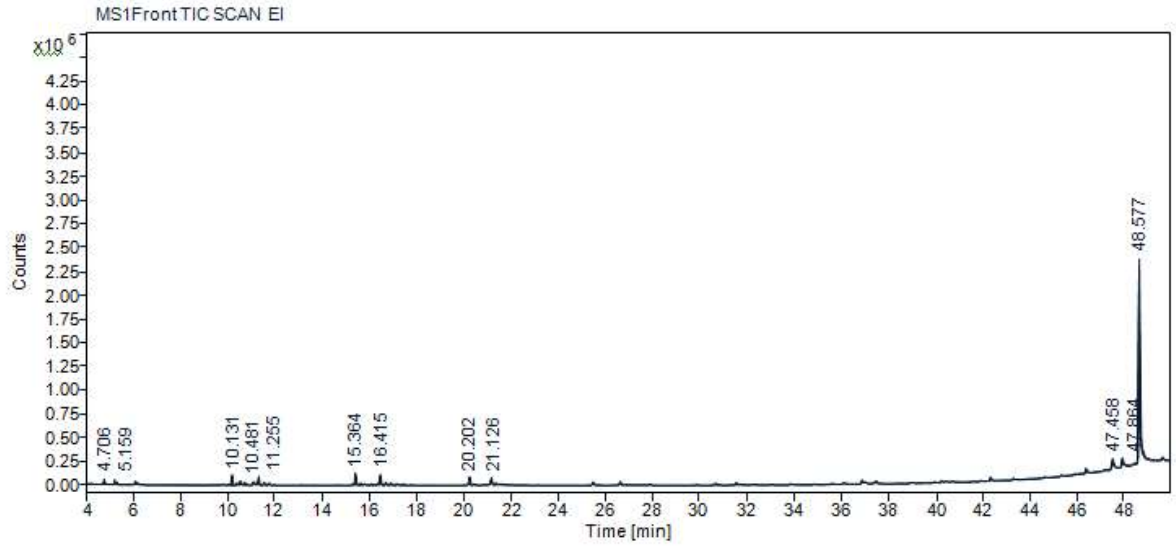


Fig 26. Various fatty acid compounds identified in kinnow

As shown in Figure 26, different fatty acid compounds are identified in the FAME molecules of kinnow. There are different peaks shown in Figure 26, each peak indicates the presence of different free fatty acid compounds with different concentrations.

Peak Results (AreaPercentatleast0%)

RT (min)	Signal Description	Width (min)	Area	Height	Area%
4.706	MS1FrontTICSCANEI	0.076	110427.1	59887.3	1.02
5.156	MS1FrontTICSCANEI	0.083	94680.3	53998.1	0.88
10.132	MS1FrontTICSCANEI	0.119	264090.7	113788.9	2.44
10.482	MS1FrontTICSCANEI	0.162	144795.8	49227.9	1.34

11.257	MS1FrontTICSCANEI		0.112	228934.2	97057.4	2.12
15.364	MS1FrontTICSCANEI		0.122	326827.7	134247.2	3.02
16.415	MS1FrontTICSCANEI		0.192	344035.3	118049.8	3.18
20.203	MS1FrontTICSCANEI		0.158	264486.3	90194.3	2.45
21.128	MS1FrontTICSCANEI		0.199	310603.4	83180.0	2.87
47.458	MS1FrontTICSCANEI		0.250	677505.9	118864.2	6.27
47.864	MS1FrontTICSCANEI		0.210	390309.0	94613.2	3.61
48.577	MS1FrontTICSCANEI		0.227	7648969.1	2106025.5	70.79
SumMS1FrontTICSCANEI					10805664.9	

Table 6: Different Fatty acid compounds identified in kinnow and their concentrations

S no.	Compound name	Concentration %
1.	Cyclohexene, 1-methyl-5- (1-methylethenyl)-, (R)-	10.14%
2.	Cyclohexene, 4-ethenyl1,4-dimethyl	7.36%
3.	Dihydrocarvyl acetate	6.3%
4.	Decane, 2,4,6-trimethyl-	15.8%
5.	Dodecane, 2,6,11- trimethyl	3.78%
6.	Tetradecane, 2,6,10- trimethyl	3.05%
7.	Pentadecane	11.79%
8.	Tetradecane, 2,6,10- trimethyl	5.72%
9.	Hexadecane	5.06%
10.	Dodecane, 2,6,10- trimethyl	7.12%
11.	Tetradecane, 2,6,10- trimethyl	5.73%
12.	5-Hydroxycyclooctane1,2-dione	4.84%
13.	Dodecane, 2,6,11- trimethyl	10.28%
14.	Hexadecane, 2,6,11,15- tetramethyl	8.29%

15.	Pentadecane	7.0%
16.	Eicosane, 2-methyl-	6.74%
17.	Hexadecane, 2,6,11,15- tetramethyl	6.48%
18.	Dodecane, 2,6,11- trimethyl	5.98%
19.	Nonadecane	4.38%
20.	Hexadecane	4.38%
21.	Hexadecane, 2,6,11,15- tetramethyl	4.21%
22.	Heptadecane, 2,6,10,15- tetramethyl	5.38%
23.	Eicosane, 2-methyl-	4.33%
24.	Dodecane, 2,6,10- trimethyl	3.99%
25.	Hexadecane, 2,6,11,15- tetramethyl	8.93%
26.	Tetradecane, 2,6,10- trimethyl	5.77%
27.	Tetradecane, 2-methyl-	4.65%
28.	Ethyl iso-allocholate	41.58%
29.	Campesterol	28.53%
30.	5-Cholestene-3-ol, 24- methyl	8.7%
31.	Stigmasterol	37.8%
32.	W-18	20.63%
33.	γ-Sitosterol	73.58%
34.	β-Sitosterol	19.66%
35.	Cholest-8(14)-en-3-ol,4,4-dimethyl-, (3β,5α)-	0.78%

5.3.2 Antioxidant activity

5.3.2.1 DPPH Assay

DPPH inhibition activity (%) shown by extracts is based on their capability to initiate color loss in solution or radical-like reagents. Researchers frequently employ the DPPH (radical scavenging test)

to identify the antioxidant capabilities of certain extracts. This is a color-based test in which the antioxidant potential of the extract is determined using hydrogen atoms or electron transfers. An antioxidant-rich seed extract was able to change the colour of DPPH from purple to mustard yellow. The DPPH assay's colour shift reveals the radical-scavenging capacity of seed extracts (Purewal et al., 2022). Per cent DPPH inhibition observed in seed extracts was observed at 60.78% for SM50%E, followed by 49.25% (SE50%E), 41.26% (SWE), 37.05% (SME) and 31.7% (SEE), respectively. Among selected extraction mediums, the extracts which were prepared using aqueous methanol (50%) showed higher % DPPH inhibition (60.78%), whereas the extracts prepared using ethanol as extraction medium showed the lowest value of % DPPH inhibition (31.7%). DPPH inhibition activity of seed extracts was found to be between 55.3% and 47.77%. The difference in activity can be attributed to the difference in the fruit part utilized to conduct the study, where they used peel as an experimental sample, whereas in the present study, Kinnow seeds were used (Safdar et al., 2017).

5.3.2.2 FRAP assay

The FRAP assay is a popular and widely used technique for identifying the presence of antioxidants in fruits and their corresponding fractions. Ghafar et al. (2010) demonstrated how bioactive compounds behave as reductants in colorimetric reactions (redox related) during the FRAP experiment. The FRAP assay is sensitive and reliable for identifying antioxidant potential. The reduction of the ferric ion (Fe^{3+})-ligand complex by antioxidants in an acidic media to the brightly blue ferrous (Fe^{2+}) complex is the mechanism behind the response during the FRAP test. Ghafar et al. (2010) studied extracts prepared from different citrus species (*C. aurantifolia*, *C. sinensis*, *C. microcarpa* and *C. hystrix*). The comparative assessment of the FRAP value of studied extracts showed a higher FRAP value in *C. hystrix* (89 µmol Fe^{2+} equivalent/100 mL) and a lower value in *C. microcarpa* extract (48.18 µmol Fe^{2+} equivalent/100 mL). The FRAP assay is conducted in an

acidic environment to keep the iron solubility and, more crucially, to promote electron transport. This will increase the redox potential, causing a shift in the dominant reaction mechanism. Higher absorbance during the FRAP assay represents higher FRAP values (Safdar et al., 2017).

$$\text{Antioxidant} + Fe^{3+} \rightleftarrows Fe^{2+} + \text{oxidized antioxidant}$$

Table 7. TPC and Antioxidant properties present in kinnow oil sample

	DPPH inhibition (%)	FRAP (mM/100g)	TPC (mg GAE/g)
Sample	51.535 ± 3.765	0.505 ± 0.0015	0.0572 ± 0.05

5.3.2.3 Total Phenolic Content

Folin-Ciocalteu assay is widely used by researchers to estimate total phenolic content by redox reaction (Prior et al., 2005) The principle behind this assay is based on the transfer of single electrons (SET) in an alkaline medium from phenolic compounds to molybdenum which ultimately forms a blue-colored complex that can be monitored using a spectrophotometer (765 nm) (Magalhães et al., 2008). As TPC directly contributes to providing the antioxidant potential to any extract, therefore, the antioxidant properties may vary with the extractant type used. Kinnow seed extracts (SWE, SEE, SME, SE50%E, SM50%E) showed TPC values ranging from 2.14 to 8.95 mg GAE/g. A higher TPC value was observed in SM50%E (8.95 mg GAE/g), whereas the lowest TPC value was shown by SEE (2.14 mg GAE/g). Methanol (50%) was observed as an efficient extraction phase, followed by ethanol (50%), water, methanol, and ethanol, respectively (Purewal et al., 2022).

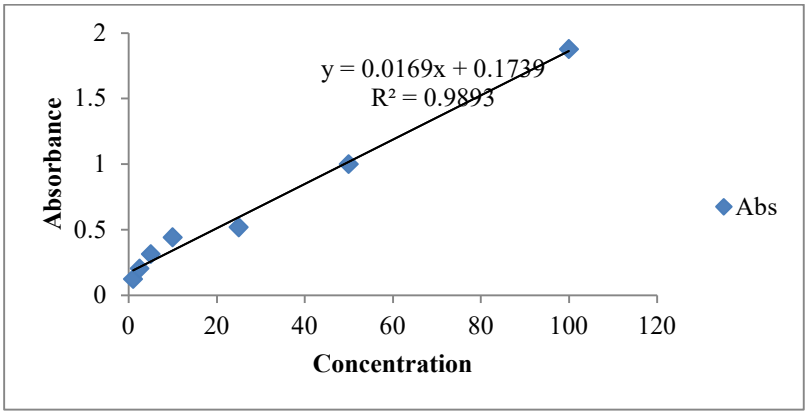


Fig27. A gallic acid standard curve

5.3.3 Anti-microbial activity

5.3.3.1 Anti-bacterial activity

Antibacterial activities of kinnow seed extracts against *Escherichia coli* and *Lactobacillus* are presented in Table 20. Our studies showed that plant extractions with organic solvents provide stronger antibacterial activity than those extracted with water (Majhenič et al., 2007). The research results show that the seed extracts display a small amount of antimicrobial activity against these strains. As a positive control, ampicillin displays antimicrobial activity against both *E. coli* and *Lactobacillus*. As a negative control, DMSO displays no activity against these strains (Salamon et al., 2021). As per reported by Özpolat (2019), the result of the antimicrobial susceptibility assay showed promising evidence for the antimicrobial effects of oil from lemon, lime, and bitter orange seeds against bacterial pathogens. Oil from bitter lemon showed maximum zone of inhibition (0.25mm), while oil from lemon seeds showed maximum zone of inhibition (0.20mm) and maximum zone of inhibition (0.15mm) against *E. coli* while Staphylococcus has the same zone of inhibition (0.10) against all tested oil samples.

Table 8. Inhibition zones in LA and NA plates (in millimetres)

	LA (mm)	NA (mm)

Positive control	22	14
	18	15
Negative control	0	0
	0	0
Sample	5	4
	6	9

Fig28. LA plates of E.coli

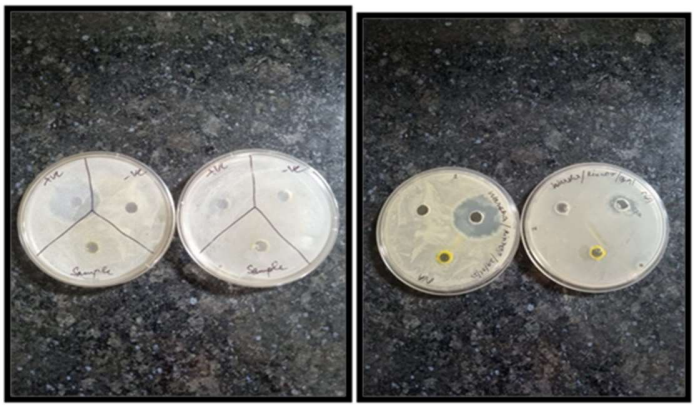


Fig29. NA plates of Lactobacillus

5.3.3.2 Anti-fungal activity

Antifungal activities of kinnow seed extracts against *Collectotrichuim gloeosporioids* 3801 ITCC Delhi; *Collectotrichuim gloeosporioids* 6152 ITCC Delhi, M.S Toshi; *Fusarium latertum* Jammu are presented in figure 76 displaying zero activity against these three fungal strains. So, no zone of

inhibition is shown against these fungi (Salamon et al., 2021). Few studies have examined the antifungal activity of citrus essential oils. Citrus essential oils are a complex mixture of volatile compounds that show, among other properties, antifungal activity by reducing or totally inhibiting fungal growth in a dose-response manner. This activity may be produced by a single major compound or by the synergistic or antagonistic effect of various. Several authors have attributed the antifungal capacity of citrus essential oils to the presence of components such as el D-limonene, linalool or citral (Viuda-Martos et al., 2008).

Fig 30. Different fungal plates showing no inhibition zones

CHAPTER 6 - CONCLUSION

Experimental data generated from the present investigation suggest that seed extracts of Kinnow are a source of bioactive compounds with antioxidant and antimicrobial properties. The kinnow seeds were efficiently extracted from the kinnow pomace using different treatments such as acid treatment, base treatment and drying. These treated seeds are then analyzed for their proximate analysis. By making a comparison between all the analyses, acid treatment was used for further oil extraction processes. Kinnow seed oil was efficiently extracted using the UAEE method. Cellulase was identified as an appropriate enzyme for enzymatic pretreatment. In this study, three independent parameters of ultrasonic time (30–90 min), cellulase enzyme concentration (1–2%), and ultrasound power (20-40%) in the UASEE technique were optimized to achieve high-performance extraction yield (EY) from kinnow seed powders using response surface methodology (RSM). The main conclusions can be summarized as follows: the optimal UASEE treatment obtained by RSM cellulase concentration of 1.66%, ultrasonic time of 49 min and ultrasound power of 38% using n-hexane solvent before incubation process at 56 °C for 120 min. From the results obtained, it can be suggested that from an economical and commercial point of view, the combination of emerging technologies of ultrasound and cellulase enzymatic extraction methods using n-hexane solvent could be considered as an industrial alternative method resulting in a higher extraction. For further analysis, kinnow seed oil was analyzed for its antioxidant properties, such as total phenolic content present in the kinnow oil, their percentage of DPPH inhibition and FRAP activities. According to this study, kinnow seed oil shows significant antioxidant activities, and it also shows antibacterial activity against the growth of food poisoning, spoilage bacteria, such as *E. coli* and *lactobacillus* but didn't show any antifungal activity specifically against fungal strains of *Collectotrichuim gloeosporioids, Collectotrichuim gloeosporioids, Fusarium latertum*.

CHAPTER 7–REFERENCES

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