Extraction of total phenolics from different waste biomass

DISSERTATION

Submitted by

Kanika Kalra

302001019

in the partial fulfilment of the degree of

Master of Science in Biotechnology

Under the guidance of

Prof (Dr.) Dinesh Goyal

Department of Biotechnology

Department of Biotechnology

Thapar Institute of Engineering & Technology, Patiala

July 2022

CERTIFICATE

This is certified that the thesis entitled **"Extraction of total phenolics from different waste biomass"** submitted by **Ms. Kanika Kalra** (302001019), in partial fulfilment of requirement for the award of degree of Masters of Science in Biochemistry at Thapar Institute of Engineering & Technology (TIET), Deemed to be University, Patiala is a record of student's bonafide work carried out under my supervision and guidance. The matter embodied in the thesis has not been submitted in part or full to any other university or institute for award of any other degree.

Dr. Dinesh Goyal
(Supervisor)
Professor
Department of Biotechnology
Thapar Institute of Engineering & Technology
Patiala

DECLARATION

I hereby declare that the work presented in the dissertation entitled **"Extraction of total phenolics from different waste biomass"** in partial fulfilment of requirement for the award of degree of Masters of Science in Biochemistry at Thapar Institute of Engineering & Technology (TIET), Deemed to be University, Patiala is an authentic record of my own work during the period from January 2022 to July 2022, under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology, TIET. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

Date: 28/7/22
Place: TIET, Patiala

Kanika Kalra

ACKNOWLEDGEMENT

This dissertation/ project report would have never been successfully completed without the assistance of so many people. I cannot go ahead without mentioning the names of those who have constantly supported and encouraged me throughout the completion of this project report.

First and foremost, I would like to express my heartfelt gratitude to Prof (Dr.) Dinesh Goyal, my supervisor. He, like a true mentor, did show me the right path to walk on. I am grateful to my guide for providing me with his constant support and guidance during this project and enabling me to complete the thesis in his laboratory. I would like to thank Ms. Harmeet Kaur for her kind cooperation, moral support, and valuable instructions regarding my work. Without her inspiration and guidance, it would have been like a ship in the sea-facing a deadly storm. She was the anchor of my ship. Apart from it she was also a true friend who was there for me unconditionally. I am grateful to my fellow lab mates Mrs. Simarpreet Kaur, Mrs. Pawandeep Kaur, Mr. Divyanshu Aggarwal, and Mr. Nirmalya Halder for their helpful cooperation and I will always carry the learning and life lessons they gave me. They always treated me like their very own. Also a part of credit goes to my batch mate, Ms. Ravneet Kaur who like a true friend encouraged me to keep up my work and enhance my creativity.

My warm and heartful thanks goes to my family for their tremendous support and hope they had given me. Lastly, I had like to show my gratitude towards God for always giving me the strength and courage to fulfil my duties with utmost focus and dedication.

I thank the above-mentioned people and many more whose names couldn't be written but they hold special place for their contribution in this project from the bottom of my heart.

Date: 28/7/22
Place: TIET, Patiala

Kanika Kalra

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LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
B.subtilis	*Bacillus subtilis*
C=O	Carbonyl group
CCC	Counter-current chromatography
C-O-H	Aldehyde group
COOH	Carboxylic acid group
CVD	Cardiovascular
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
E.coli	*Escherichia coli*
EtOH	Ethanol
FTIR	Fourier transform infrared
GAE	Gallic acid equivalent
GLUT	Glucose transporter
H_2SO_4	Sulphuric acid
H_2SO_4	Sulfuric acid
HHPE	High hydrostatic pressure extraction
M.roseus	*Micrococcus roseus*
MAE	Microwave assisted extraction
MAE	Microwave assisted extraction
MeOH	Methanol
MSPD	Matrix solid-phase dispersion
Na_2CO_3	Sodium carbonate
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
ND	Not detected
OD	Optical density
OH	Hydroxyl group
PAH	Polycyclic aromatic hydrocarbons
PCDF	Polychlorinated dibenzofurans
PLE	Pressurized liquid extraction
ROS	Reactive oxygen species
RPM	Revolutions per minute
S.aureus	*Staphylococcus aureus*
SFE	Subcritical fluid extraction
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
SSLLE	Solid supported liquid-liquid extraction
SWE	Subcritical water extraction
TPC	Total phenolic content

UAE	Ultrasounds assisted extraction
UV-VIS	UV-visible
ZOI	Zone of inhibition

LIST OF SYMBOLS

mg	Milligram
g	Gram
%	Percentage
˚C	Degree celsius
K	Kelvin
kg	Kilogram
w	Weight
µg/ml	Microgram per milliliter
µl	Microliter
ml	Milliliter
mg g^{-1}	Milligram per gram
mg ml^{-1}	Milligram per milliliter
nm	Nanometer
cm^{-1}	Per centimeter

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ABSTRACT

Total phenolics were extracted from different waste biomass such as sugarcane bagasse, waste black tea leaves, orange peels, wheat straw and rice straw by solvent extraction and alkali hydrolysis method. Most efficient solvents for extracting phenolic compounds from waste biomass were methanol (60%) > dimethyl sulfoxide > ethanol (60%) > distilled water. The extraction yields were significantly impacted by solvents (ethanol, methanol, and dimethyl sulfoxide) due to varying polarity and concentrations. Extraction of phenolics using 60% methanol yielded highest phenolics (in terms of gallic acid equivalent (GAE) per gram of biomass) in orange peels followed by wheat straw, rice straw, waste black tea leaves and sugarcane bagasse. Alkali hydrolysed extract from orange peels contained 7.58±0.33 mg GAE g^{-1}. By using the solvent extraction technique, it was observed that 60% methanol is comparatively the best-suited solvent for extracting polyphenolic compounds and gave the maximum yield of 4.68 ± 0.47 mg GAE g^{-1} in orange peels extract. In addition, DPPH radical scavenging activity and reducing power of orange peels extract was checked, where 60% methanolic extract showed the highest antioxidant activity 85.50±0.009% for DPPH and dimethyl sulfoxide (DMSO) extract gave the highest value yield 1.75±0.01% for reducing power ability of the orange peels extract. Further, the solvent extracts and the alkali hydrolysed extract were evaluated for their antibacterial activity using agar well diffusion method against Gram-positive *Bacillus subtilis* MTCC441 and Gram-negative *Escherichia coli* MTCC729. Methanolic extract showed the diameter of around 16.33±0.47 mm at 300µl concentration against *Bacillus subtilis. Escherichia coli* gave the negative result by using solvent extracts. Further, using broth based turbidometric assay, the antibacterial effect of different volumes of orange peel extracts was determined against *Escherichia coli.* The maximum antibacterial effect was observed when 200 µl of extract was used. Characterisation of the polyphenolic compounds was done by using Fourier transformation infrared (FTIR) spectroscopy.

Keywords: Orange peels, solvents, alkali hydrolysis, total phenolic content, antioxidant, antibacterial, turbidometric assay

INTRODUCTION

Waste biomass comprises of agro-residues, crop-residues, or harvest wastes, consist of carbon-based components including wastes generated from food process and herbal industries as well as stalks, leaves, and straw left in the field after harvesting, are classified as primary residues, whereas secondary residues are the remains left after processing into a useful resource. Husks, seeds, rhizome, molasses, bagasse, and other process wastes can be either used as organic fertilizer after composting or as energy source as well as animal fodder. The global dry matter production of agricultural leftovers is predicted to reach 3.8 billion. Crop residue production in India is estimated between 500 to 550 million tonnes per year (Ginni G *et al.*, 2021). Crop residue burning is one of the major sources of pollution in the atmosphere which has detrimental effect on the soil's nutrient budget since carbon, nitrogen, and sulphur are totally lost into the atmosphere. Crop stubble burning in open fields releases carbon monoxide, NO_2, SO_2, CH_4, particulate matter, and hydrocarbons which are hazardous in nature.

When crop stubble is burned openly, hazardous substances such as polychlorinated dibenzo-p-dioxins, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated dibenzofurans (PCDFs) are also released which are toxic. Constant burning is not a sustainable agricultural practice (Kumar and Joshi, 2013) and therefore it is mandated to develop alternative and renewable bioenergy supplies.

Biofuel generation, citric acid production, enzyme manufacturing, pigment production, agricultural composting, extraction of bioactive compounds, food flavoring and preservative chemicals, and agricultural composting are all examples of agro-industrial waste recycled value-added uses (Yusuf 2017). Various studies have found that pomegranate peels, lemon peels, and green walnut husks may all be utilized as natural antimicrobials (Sadh *et al.*, 2018). Major constituents of various types of biomasses are cellulose, hemicellulose, lignin and some minor constituents such as polyphenols and flavonoids. 8,000 distinct types of polyphenols and their compounds have been reported in biomasses. Polyphenols are mostly found in biomass leaves, vascular tissues, bark, young fruits, seed coat, and disinfected tissues (Yan *et al.,* 2021). They are also found in a wide range of foods, including grains, fruits, drinks, and some wines. Most prevalent polyphenols found in everyday foods such as gallic catechins in green tea, resveratrol in red wine, anthocyanins in colored fruits, procyanidins in grapes, caffeic acid in coffee, wine and cucurmin in turmeric (Zhang et al., 2021). Polyphenols can also be found in fruits and vegetables (apples, cherry, citrus fruits,

pomegranate, strawberry, grapes), legumes (beans, sprouts), dry fruits (almonds, flax seeds, chestnuts, walnuts), cereals (rye, wheat, oats), herbs and spices (clove, cinnamon, cumin, basil, thyme), and beverages (wine, tea, coffee) among others.

Clinical data shows that polyphenols are useful in the prevention and treatment of a variety of chronic diseases, including degenerative diseases, type 2 diabetes, cancer, anti-inflammatory, cardiovascular (CVD), immune-related, osteoporosis, and other neurological disorders (Kumar and Joshi, 2013). Polyphenols have antimicrobial, antibacterial, antioxidant, and virucidal properties and promote formation of beneficial gut flora, prevent blood clots, inhibit tumour growth, and boost immunity (Kumar and Joshi, 2013). Citrus peels contain a wide range of bioactive compounds, including flavonoids and phenolic acids. Orange accounts for over 60% of the global citrus population. Peels account for 50 to 65% of total fruit weight and are the principal byproduct. Many active phytochemicals, such as vitamins, flavonoids, terpenoids, carotenoids, cumarins, and oxidation, contribute to the plant material's antioxidant properties. Citrus fruits are high in bioactive substances such ascorbic acid, flavonoids, phenolic compounds, and pectins, all of which are beneficial for human health. Citrus fruit contains three forms of flavanoids: flavanoids, flavones, and flavanols. The pharmacological action of these compounds as radical scavengers has piqued curiosity (Hegazy and Ibrahium, 2012). Citrus peel extract has a stronger antioxidant activity than synthetic antioxidants, as well as substantial inhibitory effects on lipid oxidation, according to recent research. Citrus peels include phenolic chemicals, limonoids, flavonoids, and polysaccharides, which function as antioxidants by scavenging single oxygen, hydroxyl radicals, and lipid peroxyl radicals (Shehata *et al.*, 2021).

In the present study total polyphenols from different agricultural and waste residues such as waste black tea leaves, sugarcane bagasse, orange peels, rice straw and wheat straw were extracted using different solvents. Phenolics obtained from orange peel waste were also checked for its antibacterial property against *Escherichia coli* and *Bacillus subtilis*.

REVIEW OF LITERATURE

Polyphenols are a class of physiologically active compounds found in plant such as fruits, vegetables, grains, and coffee and are common components of human diet (Abbas *et al.*, 2016). Phenolic compounds are widely available in low-cost sources such as agro-food processing waste, which has sparked renewed interest in their recovery and further utilization (Panzella *et al.*, 2020). Research is mainly focused on uses of biomass derived polyphenols in food and medicine, with development of protocols on preparation, purification, structural identifications and their biological activity. Secondary metabolites such as biomass polyphenols are produced predominantly through the shikimic acid and phenyl propane pathways. Numerous studies have shown that biomass polyphenols include multiple phenolic hydroxyl groups, which have been linked to important physiological processes such free radical scavenging and radical sequestration (Yan *et al.*, 2021).

The phenolic ring is the fundamental monomer in polyphenols, which contains phenolic acids and phenolic alcohols. Many fruits and vegetables, such as kale, onions, and broccoli, contain phenolic acids as a key polyphenol. Pomegranate juice, tea extracts, and grape extracts have all been linked to fewer atherosclerotic plaques. Polyphenols have a well-known protective role in health and disease, and they're abundant in antioxidants and phytochemicals (Abbas *et al.*, 2016).

2.1 Structure of Polyphenols

Polyphenols are naturally existing chemical compounds made up of multiple phenol units and are secondary plant metabolites that have industrial and medicinal uses. They are made up of phenolic rings and structural elements that bind them together. Polyphenols are macromolecules with a molecular weight of around 800 Daltons that may cross the cell membrane and gain space in intracellular places as pigments or phytochemicals.

As per literature 8000 different types of polyphenols have been identified (Yan *et al.*, 2021). The single or double aromatic rings are coupled with one or more hydroxyl groups in the fundamental structure of these polyphenols (-OH). The chemical structure, sugar rings, configuration, and synthesis routes of these secondary metabolites are used to classify them. For example, phenolic compounds are made up of simple sugars that include the benzene ring, oxygen, and hydrogen (Prabhu *et al.*, 2021). Furthermore, the polyphenols family is quite diverse, with various subgroups ranging from relatively simple substances like phenolic

acids and stilbenes to complex polymerized structures like tannins generated from the simpler compounds. Polyphenols in nature are usually found conjugated, with one or more sugar residues linked to a hydroxyl group, however direct attachment of the sugar unit to an aromatic carbon atom can sometimes occur. Associative sugars can be monosaccharides, disaccharides, or oligosaccharides (Cutrim and Cortez, 2018). Plants include simple phenolic acids and flavonoids in soluble free, soluble esterified, and insoluble-bound forms. Phenolics are covalently bonded to cell wall structural components such as pectin, cellulose, hemicellulose, and lignin in their bound state (Prabhu *et al.*, 2021).

2.2 Classification of Polyphenols

There are various types of polyphenols, including phenolic acids, stilbenes, flavonoids, tannins, and lignans.

1. Phenolic acids

Phenolic acids are also known as hydroxybenzoic acids. These acids are represented by aromatic rings with one carboxylic acid group (-COOH) as shown in **Fig.1**. Phenolic acid is found mostly in plant-based foods such as seeds, fruits, and green vegetables. These phenolic acids are used in a variety of cosmetics, food, and medicinal products (Prabhu *et al.*, 2021). Cinnamic acid and benzoic acid derivatives contain C1-C6 and C3-C6 backbones, respectively. Some phenolic acids are present in free form in vegetables and fruits, whereas phenolic acids in bound form are found in hull, bran, and seed. Acid, alkali, and enzymatic hydrolysis liberate bound phenolic acids from bran, hull, and seed (Abbas *et al.*, 2016).

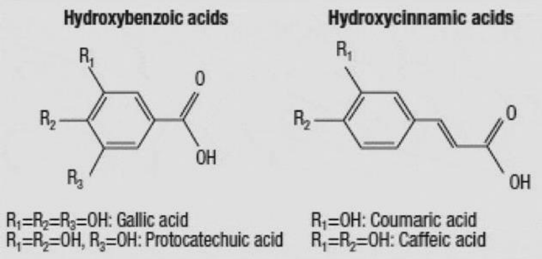


Fig. 1: Structural representation of Phenolic acids (Abbas *et al.*, 2016)

2. Flavonoids

Flavonoids include flavones, flavonols, isoflavones, flavanones and anthocyanins **(Fig.2)**. Flavonoids have a backbone structure of C6-C3-C6, with two phenolic units (C6). Daidzein and genistein, along with glycetein, are two main isoflavones present in soy. This type of compound can also be found in red clovers. The most frequent types of isoflavone-aglycones

are 7-O-glucosides and 6"-O-malonyl-7-O-glucosides. Dalbergin is the most abundant neo-flavonoid found in plant diets. Anthocyanins are water-soluble pigments that give red and blue colour to plants and fruits. Fruit covering is the primary source of anthocyanins, which are contained in the form of anthocyanidins and a moiety of sugar at C3 or at the 5, 7-position of the A-ring (Abbas *et al.*, 2016). Apigenin, luteolin is present in parsley and chilli, quercetin, rutin is present in apple, onions and other vegetables also naringenin and nobiletin is present in citrus fruits. *Staphylococcus aureus* infection is controlled when flavonoids are coupled with medicinal medicines. Flavonoids have been shown to have antibacterial properties against *Staphylococcus aureus* (Yan *et al.*, 2021).

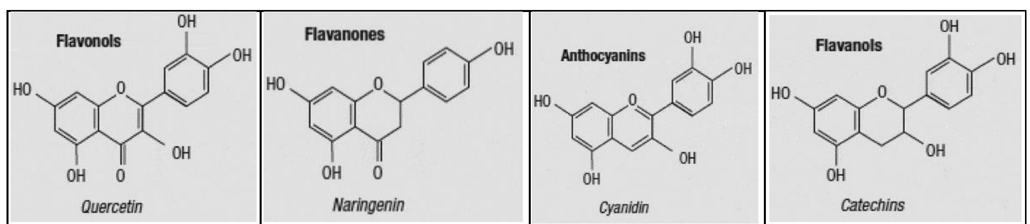


Fig. 2: Structural representation of Flavonoids (Abbas *et al.*, 2016)

3. Other Polyphenols

Other polyphenols, such as stilbenes (resveratrol, piceatannol), lignans (sesamol, pinoresinol, sinol, enterrodiol), tannins (hydrolysable, non-hydrolysable, and condensed tannins), and lignins, have a wide variety of medicinal and industrial applications based on their type of action (Prabhu *et al.*, 2021).

4. Stilbenes

The E isomer of stilbenes has the most common chemical structure **(Fig. 3)**, which consists of two benzene rings joined by a double bond. Grapes, almonds, beans, blueberries, bilberries, peanuts, vines, cranberries, mulberries, plums, and wine have all been observed to contain stilbenes. Consumption of stilbenes has been linked to lower all-cause mortality and a lower risk of hypertension development (Durazzo *et al.*, 2019).

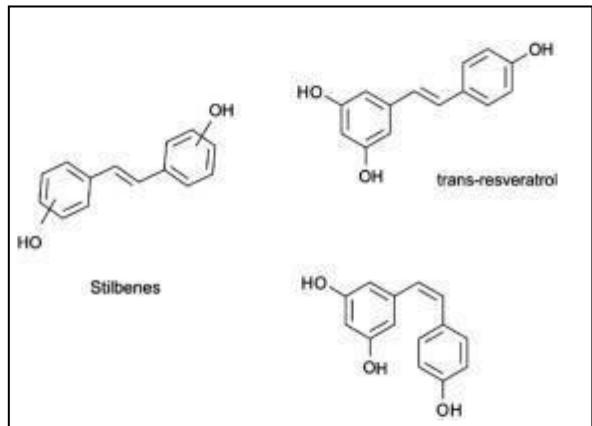


Fig. 3: Structural representation of Stilbenes (Singla *et al*., 2019)

5. Lignans

Lignans are a group of secondary metabolites formed by the oxidative dimerization of two or more phenylpropanoid units **(Fig.4)**. They have a wide structural variety despite their similar biosynthetic beginnings. This family of chemicals has also been shown to have a wide range of biological functions (Barker, 2019).

CH_3O HO CH_2OH CH_2OH *Secoisolariciresinol* OCH_3 OH

Fig. 4 Structural representation of Lignans (Cutrim and Cortez, 2018)

2.3 Therapeutic applications of polyphenols

Wide range of biological activities has been due to diverse nature of biomass derived polyphenols. Polyphenols possess antioxidant activity, anti-bacterial, cardio-protective activity, anti-cancerous, enhances the immunity, protects from UV, neuro-protective activity and so on (Yan *et al*., 2021).

a) **Antioxidant activity:** In living organisms, redox reactions are an important class of metabolic reactions. When the electron flow is disrupted, harmful free radicals are produced, which has negative consequences. These responses, in turn, contribute to a variety of cell

abnormalities. The majority of the damage can be repaired, but if the free radical interacts with an antioxidant in the cell, the complete process can be averted. Antioxidants are essential for reducing the detrimental build-up of reactive oxygen species by blocking molecular oxidation processes (Yan *et al*., 2021). Polyphenols are the most common source of dietary antioxidants, and they are absorbed easily in the gut. Phenolic acids, a subclass of plant phenolics, have a phenol moiety and a resonance stabilised structure, resulting in H-atom donation and antioxidant properties via a radical scavenging mechanism. As a result, the antioxidant activities of free, esterified, glycosylated and non-glycosylated phenolic acids have been discovered (Kumar and Goel, 2019).

b) Antidiabetic activity: Diabetes is an oxidative stress illness caused by a mismatch between the generation of free radicals and the ability of an individual to oxidize them. Oxidative stress is strongly linked to organ damage caused by reactive oxygen species (ROS) that are not effectively neutralized by antioxidants, resulting in inflammation and a range of metabolic diseases. The action of glucose and insulin receptors is influenced by phenolic acids (have a crucial role in diabetes). Through the PI3K/Akt and AMP activated protein kinase pathways, they increase the expression of the glucose transporter GLUT2 in pancreatic -cells (which create insulin) and boost the translocation of GLUT4. Both chlorogenic and ferulic acids have been shown to stimulate transporters and serve as antidiabetic drugs (Kumar and Goel, 2019).

c) Cardio protective activity: Increased polyphenol consumption was linked to lower levels of inflammatory biomarkers, as evaluated by urine total polyphenol excretion (i.e., vascular cell adhesion molecule, intercellular adhesion molecule, interleukin, tumour necrosis factor alpha, and monocyte chemotactic protein). Furthermore, a high polyphenol diet lowered cardiovascular risk factors and improved blood pressure and lipid profile. Clinical data shows that consumption of polyphenols can lower the risk of cardiovascular disease. Although the exact mechanism by which polyphenols exert their protective benefits is unknown, it is thought that regulation of nitric oxide generation and stimulation of antioxidant defenses play a role (Durazzo *et al*., 2019).

d) Antibacterial activity: Due to the after effects of overuse of synthetic antibiotics, consumers are increasingly intending to use natural extracts and other substances as possible antibiotics to limit the growth of infective bacteria. Polyphenols are one of the most intriguing natural extracts for their ability to inhibit bacterial growth and proliferation through a variety of mechanisms, including altering bacterial membrane permeabilization, inhibiting

bacterial DNA gyrase, interfering with energy metabolism, and disrupting bacterial porin functions. Various sources of polyphenolic compounds with their target microorganisms are described in **Table 1**.Tea polyphenols acts as an antibacterial agent that inhibits a wide range of harmful microorganisms, including *Proteus vulgaris, Staphylococcus epidermidis, and Staphylococcus aureus*. Furthermore, apple polyphenol extract inhibits the development of *Bacillus* aerobics, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*. Pomegranate pulp contains eight distinct polyphenol chemicals, all of which have potent antibacterial properties against *Salmonella and Escherichia coli* (Yan *et al*., 2021).

Table 1: Sources of polyphenolic compounds with their target microorganisms

Source of Polyphenols	Compound present	Target bacteria	References
Bergamot ***(Citrus bergamia Risso)***	Flavonoid	*E. coli* *Pseudomonas putida*	Mandalari *et al*., 2007
DnaB helicase	Flavonols galangin, myricetin, quercetin, kaempferol	*Klebsiella pneumoniae*	Chen and Huang., 2011
Grape seed	Gallic acid (+) - catechin (-) - epicatechin	*Lactobacillus spp.*	Cueva *et al*., 2013
Sesame honey ***(Sesamum indicum L.)***	Apigenin, quercetin, rutin, ferulic acid, sesamin, myricetin	*Lactobacillus spp.*	Das *et al*., 2014
Barlett pears	Gallic acid, p-coumaric acid, quercetin derivatives, caffeic acid	*Lactobacillus helveticus*	Sarkar *et al*., 2015
Syrian propolis	Phenolic acid, phenolic aldehydes, flavonoids, quinones	*S. aureus* *Acitenobacter baumannii* *E. coli* *Pseudomonas aeruginosa*	Harfouch *et al*., 2016
Native Herb ***(Matricaria aurea)***	Phenols, phenolic acids	*B. subtilis* *S. aureus* *Klebsiella pneumoniae* *Colleotrichium gleosporides*	Rizwana *et al*., 2016
Neem ***(Azadirachta indica)***	Flavonoids, saponins, anthocyanins	*B. subtilis* *S. aureus* *M. roseus*	Othman *et al*., 2019

e) **Anti-cancerous activity:** Cancer refers to a category of disorders marked by a disruption in the regulation of cell development and metabolism. Unbalanced cell proliferation is a major hallmark of malignant cells and therefore any substance that may suppress cancerous cell growth could be employed as a chemo preventive drug. Green tea flavanols have been shown to have powerful anti-cancer effects in human cell lines and in human intervention

trials. Polyphenols and bioactive substances found in tea, red wine, chocolate, fruits, fruit juices, and olive oil have been shown to impact tumour growth and carcinogenesis at the cellular level. Nutrients, reactive metabolites, activated carcinogens, and mutagens, for example, can interact with them. It can also alter the expression of several cancer-related genes by modulating the activity of critical proteins involved in cell cycle progression regulation. Green tea use has also been linked to a lower incidence of cancers of the bile duct, bladder, breast, and colon (Abbas *et al.*, 2016).

2.4 Extraction methods of polyphenols

Extraction is the key step and presently, there is no established method for recovering all phenolics or those belonging to a specific category from plant materials. It is necessary to develop an optimal procedure for recovering phenolic chemicals from plant materials that take into account following factors (Alara *et al.*, 2021).

The type of sample and the compounds that are being targeted, such as total phenolics, a specific phenolic, and others;

1. The subject of the analysis, which is being employed for quantification or structural elucidation;
2. Techniques available at that very time.

Drying, homogenization, filtering, and grinding are some of the main sample preparation procedures used before extraction. In most situations, a hydrolysis step is also included to make it easier to liberate chemicals from the sample matrix. However, aided extraction techniques, such as those that include the use of ultrasounds, microwaves, and pressurized/supercritical fluids, have been widely employed to extract phenolic chemicals from plant material (Alara *et al.*, 2021). Various extraction methods used for different biomasses are mentioned in **Table 2**.

Extraction is crucial in the extraction and purification of numerous bioactive components present in biomass. To assess the polyphenol recovery from biomass, researchers looked at a variety of extraction procedures, ranging from traditional to contemporary. Soxhlet extraction, maceration, ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, high-voltage electric discharge, pulse electric field extraction, and enzyme-assisted extraction are some of the most widely used procedures for extraction (Sridhar *et al.*, 2021).

Pressurized liquid extraction (PLE), subcritical water extraction (SWE), supercritical fluid extraction (SFE), microwave assisted extraction (MAE), solid phase extraction (SPE), ultrasounds assisted extraction (UAE), high hydrostatic pressure extraction (HHPE), solid-supported liquid-liquid extraction (SSLLE), matrix solid-phase dispersion (MSPD), and counter-current chromatography (CCC) are some examples of conventional methods (Alara *et al.*, 2021).

Conventional methods

Extraction techniques are required since polyphenolic chemicals are often found in minute amounts within various foods, drinks, and plant matrices. Depending on the samples, pre-treatment methods such as drying, crushing, and grinding may be necessary. The active chemicals are recovered via extraction, separation, and purification. Percolation, decoction, heat reflux extraction, Soxhlet extraction, and maceration are the most common extraction procedures. These differ based on the food samples, composition and features. Traditional techniques are simple to use, have drawbacks such as long extraction times, significant energy usage, and solvent waste (Sridhar *et al.*, 2021).

Soxhlet extraction

Soxhlet extraction, which involves concentration of analyte and separation of bioactive constituents from natural products, has always been the most widely used method for extraction purposes. The most important step is sample preparation, which can be accomplished in a number of ways. Soxhlet extraction is one of the significant procedures when it comes to ecologically friendly methods. Solvents which are generally used in this technique are methanol, ethanol, ethyl acetate, water and hexane.

A dry sample is put in a thimble for Soxhlet extraction. The thimble is then placed in a distillation flask with the solvent, which is heated to evaporate the solvent. The condensate and reflux return to the thimble-holder until they reach an overflow level, at which point a syphon can aspirate them. The chemicals are removed and dissolved in a bulk liquid. The solvent is constantly refluxed and the compounds stay in the flask as the flask is continuously heated. The extraction procedure is done multiple times until it is complete. The procedure is then repeated until it achieves saturation. Polyphenol extraction takes roughly 24–50 hours, and more than half of the solvent is utilised for extraction research (Sridhar *et al.*, 2021).

However, because phenolic compounds are extracted utilising longer extraction durations and higher temperatures, the bioactivity of the extracts is reduced due to compound degradation (Tobón, 2020).

Maceration: One of the most used procedures for determining polyphenolic compounds is maceration. The two most important characteristic to consider in this procedure are the agitation speed and time. When the speed of the magnetic stirrer is changed, a vortex may emerge, causing turbulence, mass transfer rate is also increased (Sridhar *et al*., 2021).

This procedure is carried out in a closed vessel with the addition of a suitable solvent (menstruum). The solvent is then strained out, and the solid residue of the extraction process, called “marc," is pressed to recover the maximum amount of occluded solution. The acquired pushed- out liquid and the strained solvent are combined and filtered to remove any undesirable elements. Extraction is aided by frequent agitation during maceration by two processes: (1) diffusion (2) separation of concentrated solution from the sample surface by introducing additional solvent to the menstruum to increase extraction yield.

It's possible that it'll be utilised to extract thermolabile components. This is a straightforward extraction procedure with the disadvantages of a long extraction time and low extraction efficiency. Arvindekar *et al*., 2015 effectively recovered anthraquinone, a phenolic compound, from Rheum emodi (culinary plant) using the maceration process. Using ethanol as the extraction solvent, the extraction procedure was completed in 24 hours (Jha *et al*., 2021).

Green Extraction methods of Polyphenols

Green extraction methods are being developed using contemporary technologies that utilise fewer or no organic solvents to reduce environmental and health risks while increasing the yield of targeted polyphenols through selective extraction. With a dedication to developing green technology, novel extraction techniques for recovering polyphenols are emerging. Modern scientific progress is influencing development by supplying technologies such as microwave ovens and ultrasound probes, among other things. Microwave, ultrasound, pulsed electric field, and enzyme-aided extractions are potential ways to extract polyphenols from plant components including roots, leaves, and fruits. The key parameters regulating the mass transfer and solubility of polyphenol include temperature, pressure, solvent to feed ratio,

sample particle size, pH of a solution, extraction time, microwave power (MAE), ultrasonic power, frequency (UAE), electric field strength, and pulse length (PEF) (Panja, 2018).

Table 2: Different extraction methods of polyphenols from waste biomasses

Agricultural waste biomass	Extraction method used	Optimum extraction conditions	Extracted bioactive compounds	References
Bamboo leaves	Ultrasound assisted extraction (UAE)	10g of material was combined with 200 mL of 60% ethanol-water solution for 40 min at 250W of ultrasonic power	isoorientin, orientin, and vitexin	Li *et al.*, 2013
Rice straw	Alkali hydrolysis	2% NaOH (w/w) in a solid-to-liquid ratio of 1:10 (w/v) autoclaved for 20 minutes at 120 °C	p-coumaric acid, ferulic acid	Chen *et al.*, 2017
Grape pomace	Microwave-assisted extraction (MAE)	10 minutes at 1000 W with 2% citric acid	Anthocyanins	Rocha *et al.*, 2019
Pomegranate peel	Hot Infusion	100 ml hot water (temperature: 70°C) was poured over 5 gm's of dried PP in a conical flask, extraction time 12 hours	gallic acid and hydrolysable tannins	Ghosh *et al.*, 2019
Spent black tea	Subcritical solvent extraction	At 125°C and 0.3 MPa, SBT was treated with ethanol-water (50% w/w) as solvent	Gallic acid malic acid epigallocatechin-gallate epitheaflagalline-3-gallate quercetin-3-O-rutinoside epicatechin-gallate Theaflavin-3,3′-digallate	Rajapaksha *et al.*, 2022
Coffee husk	Supercritical CO2 extraction	At 373 K and 300 pressure, the mass ratio of solvent to raw material: 197 kg CO2/kg husks	Caffeine	Capanoglu *et al.*, 2022
Tomato processing waste	Ultrasound-assisted extraction	Solvent used hexane: acetone: ethanol (2:1:1 v/v/v) along with 0.05% (w/v) butylated hydroxy toluene at 15°C, 90W for 30 min	Lycopene and β-carotene	Capanoglu *et al.*, 2022

MATERIALS AND METHODS

3.1 Collection and processing of waste biomass

Sugarcane bagasse, waste black tea leaves, orange peels were collected from the campus of Thapar Institute of Engineering and Technology (TIET), Patiala, Punjab, and rice straw and wheat straw were collected from the outskirts of Patiala, Punjab as shown in **Fig. 5**. After collection, the samples were dried using the oven at 60˚C whereas orange peels were sun-dried. The dried samples were grinded using a mixer grinder. The biomass was grounded into fine particles of size 0.5 mm and was kept in air-tight containers in dark at room temperature.

Fig. 5: A. Sugarcane bagasse, **B.** Waste black tea leaves, **C.** Orange peels, **D.** Rice straw, **E.** Wheat straw

3.2 Extraction of polyphenols from waste biomass

The extraction of phenolic compounds is a crucial stage in this process. Waste black tea leaves, sugarcane bagasse, orange peels, wheat straw and rice straw were extracted using an organic solvent, its aqueous formulation and, water. 1g of biomass was taken in 10 ml of solvent. The solvents used for extracting the phenolic compounds were either 60% MeOH (methanol), 60% EtOH (ethanol), ethyl acetate, water and DMSO (dimethyl sulfoxide). Extraction was carried out for 6 hours at room temperature at 120 rpm using an orbital shaker. The extracted liquid samples were centrifuged at 8000 rpm for 10 minutes, to allow the complete extraction with another round of centrifugation. Before analysis, the extracted samples were filtered through a Whatman No.2 filter paper to remove any leftover residue.

3.3 Extraction of polyphenols from orange peels by maceration using solvents (Safdar et *al*., 2016)

1. 5g of grounded and sieved orange peels were suspended in 50 ml of 60% Methanol, distilled water, dimethyl sulfoxide and ethyl acetate.
2. The Erlenmeyer flasks were properly capped and kept in water bath for at 80º C for 2 hours.
3. After the extraction the samples were centrifuged at 8000 rpm for 10 minutes.
4. The supernatant was dried in hot air oven at 40º C.
5. The dried extract was further dissolved in dimethyl sulfoxide for further studies.

3.4 Extraction of polyphenols from orange peels by alkali hydrolysis (Vadivel et al., 2017)

1. 5 g of grounded and sieved orange peels were suspended in 50 ml of 2% NaOH at 100ºC and kept in water bath for 30 minutes. The experiment was performed in triplicates.
2. After the extraction, the samples were centrifuged at 8000 rpm for 10 minutes.
3. The supernatant was checked for its pH and was adjusted to 2 with 6 molar H_2SO_4.
4. The mixture was centrifuged again to separate the precipitates after pH adjustment.
5. The supernatant was mixed with an equal volume of ethyl acetate following which the mixture was shaken at 120 rpm on an orbital shaker.
6. The organic phase was pooled and dried at 40º C.
7. The dry extract was dissolved in DMSO for further studies.

3.5 Estimation of Total phenolic content (Singleton *et al.*, 1999)

1. Using the Folin-Ciocalteu reagent (mixture of phosphomolybdate and phosphotungstate) the total phenolic content of the extracts was determined.
2. 50 µl of the extracted sample, 450 µl of distilled water, 2.5 ml of Folin-Ciocalteu reagent (10-fold diluted) and 2 ml of sodium carbonate (Na_2CO_3) solution (7.5% v/v) was added to test tubes and vortexed.
3. The mixture was incubated in dark for 90 minutes. The absorbance was measured at 760 nm using UV-VIS spectrophotometer.
4. The total phenolic content was determined by the calibration curve of gallic acid at the concentration range from 10-60 µg/ml as mentioned in **Fig. 6**. The total phenolic content can be calculated as the equivalent of a natural compound, gallic acid (GAE: Gallic acid equivalent).

   Total phenolic content $= C \times V/M$

T is the total phenolic content of the extracts as GAE in mg g^{-1}, C is the gallic acid concentration determined from the calibration curve in mg ml^{-1}, V is the volume of the extract solution in ml, and M is the weight of the extract in g.

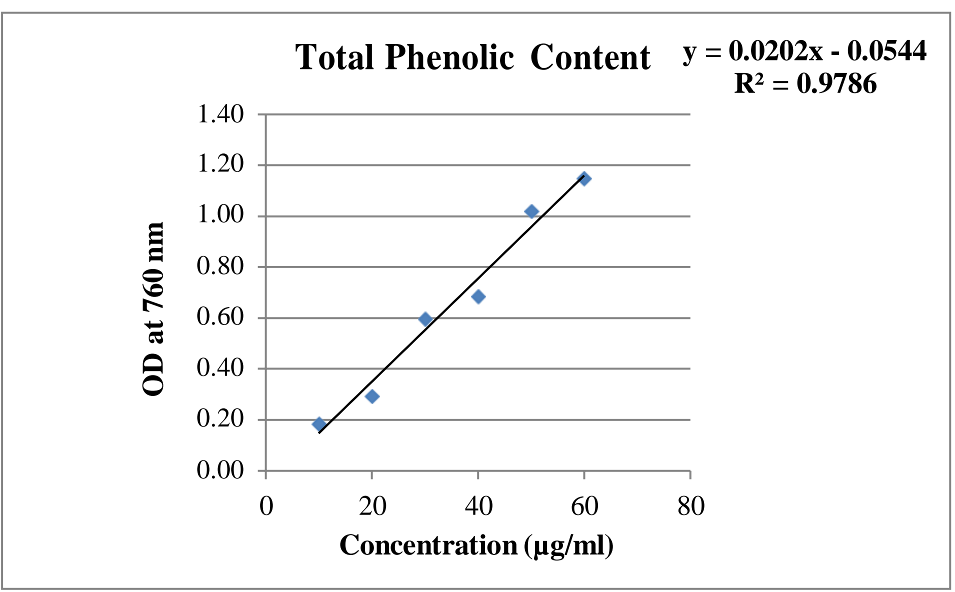
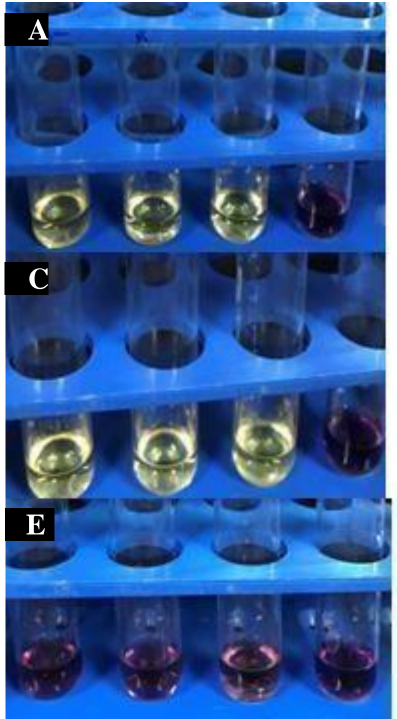


Fig. 6: Standard curve of gallic acid for Total Phenolic Content

3.6 Estimation of antioxidant activity using DPPH assay (Haya *et al.*, 2018)

1. 4 mg of DPPH was dissolved in 100 ml of pure methanol. The solution was made fresh each time before use and was stored in dark bottle at room temperature.
2. 1950 µl of DPPH solution was mixed with 50 µl of each extract (in DMSO) and incubated for 30 minutes in dark at room temperature. The control was prepared without any extract as shown in **Fig. 7**.
3. The O.D. was taken at 517 nm. The radical scavenging activity (%) was calculated by the formula, (Abs of control -Abs of sample)/ Abs of control* 100.

   Abs of control is the absorbance of DPPH solution without extract and Abs of sample is the absorbance with DPPH.



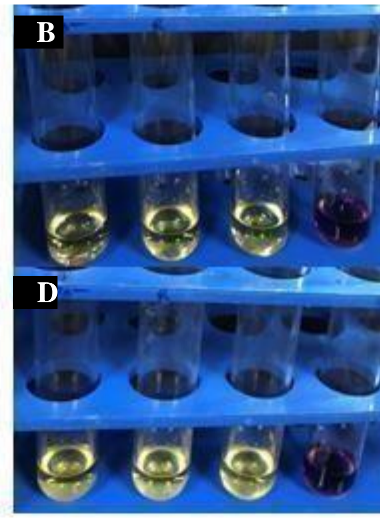


Fig. 7: A. 60% Methanol (MeOH), **B.** Alkali hydrolysis, **C.** Distilled water, **D.** Dimethyl sulfoxide (DMSO), **E.** Ethyl acetate

3.7 Estimation of reducing power of extracts (Irakli *et al*., 2018)

1. 0.5 ml of each extract was dissolved in 1.25 ml of sodium phosphate buffer (pH 6.6) and 1.25 ml of 1% potassium ferricyanide.
2. The mixture was vortexed and kept in water bath at 50° C for 20 minutes.
3. 1.25 ml of 10% trichloroacetic acid was added to the mixture.
4. From this, 1.25 ml of mixture was pipetted out in different set of test tubes followed by the addition of equal amount of distilled water.
5. To this, 0.15 ml of 0.1% ferric chloride was added.
6. The absorbance of the reaction mixture was recorded at 700 nm.

3.8 Characterisation of the polyphenolic profile of orange peel extracts by Fourier transform infrared (FTIR) spectroscopy (Zapata *et al*., 2009)

FTIR analysis is an excellent method for identifying chemical components of phenolics because each molecule or chemical structure will provide a distinct spectral fingerprint. The functional groups present on the surface of orange peels were observed using the FTIR spectroscopy technique. The FTIR analysis method scans test specimens and examines chemical characteristics using infrared light. Infrared radiation between 10,000

and 100 cm^{-1} is sent through a sample i.e. orange peels powder, Methanol (60%) alkali hydrolysed extract using the FTIR instrument, with some radiation being absorbed and some being sent through. The sample molecules of phenolics transform the absorbed radiation into rotational and/or vibrational energy. The resulting signal, which appears as a spectrum at the detector and typically ranges from 4000 cm^{-1} to 400 cm^{-1}, represents the sample's molecular fingerprint.

3.9 Estimation of antibacterial activity using agar well diffusion method (Othman et al., 2011)

The antibacterial activity of orange peel extracts was confirmed using the agar well diffusion method by testing them against *Bacillus subtilis* MTCC441 and *Escherichia coli* MTCC729. The bacteria were grown overnight at 37˚C. 100 µl of the bacterial culture was poured onto the petri plate. The bacterial culture was spread using L-shaped spreader by the spread plate method. On the cultured plate, wells were made using a cork borer of diameter 8mm ± 0.2 mm. The agar plate was divided into four parts in which one was control and three different concentration of extracts was added i.e., 100 µl, 200 µl, and 300 µl. DMSO was taken as a control. 100 µl of DMSO was introduced into each plate as a control. Liquid extracts of the Solvent extraction method and Alkali hydrolysis were used for the antibacterial screening. The inhibition zone was measured using HiMedia antibiotic zone scale. All assays were performed in triplicates.

3.10 Antibacterial effect of orange peels extract using broth based turbidometricassay (TB)

Antibacterial effect of orange peels extract was studied on *E. coli* MTCC729 by inoculating 100 µl of overnight grown bacterial culture in 10 ml of nutrient broth in presence of different volumes of orange peel extract (0, 100, 150, 200 and 250 µl) and then kept under incubation at 37˚C with shaking at 120 rpm. At varying time intervals *i.e.* after 2 hours, 4 hours, 6 hours, 8 hours and 24 hours the optical density at 600 nm was measured and the experiment was performed in triplicates.

RESULTS AND DISCUSSION

4.1 Total phenolic content in waste biomass

Table 3 summarizes the total phenolic content present in waste biomass obtained from macerated biomass using methanol (60%), ethanol (60%), distilled water and dimethyl sulfoxide (DMSO). The highest yield was obtained in methanol (60%) for orange peels extract 2.68 ± 0.37 mg GAE g^{-1} of biomass while the lowest yield was observed in Dimethyl sulfoxide (DMSO) waste black tea leaves extract 0.10 ± 0.01 mg GAE g^{-1} of biomass.

Most efficient solvents for extracting phenolic compounds from waste biomass were Methanol (60%) > Dimethyl sulphoxide > Ethanol (60%) > Distilled water as illustrated in the **Fig. 8**. For waste black tea leaves, the highest phenolic content was observed using the extractant distilled water 1.28±0.10 mg GAE g^{-1} of biomass, for orange peels the highest phenolic content was observed using the extractant methanol (60%) 2.68±0.37 mg GAE g^{-1} of biomass, for sugarcane bagasse the highest phenolic content was observed using the extractant distilled water 0.26±0.05 mg GAE g^{-1} of biomass, for wheat straw and rice straw the highest phenolic content was observed using the extractant ethanol (60%) 1.23±0.26 mg GAE g^{-1} of biomass in case of wheat straw and using rice straw 1.08±0.17 mg GAE g^{-1} of biomass was found.

Green tea was taken as a reference and compared well with the literature. The highest total phenolic content in green tea was observed by using the extractant 60% ethanol 22.68 ± 0.47 mg GAE g^{-1} of biomass.

Table 3: Total phenolic content in different waste biomass (mg GAE g^{-1} of biomass)

Total phenolic content (mg GAE g^{-1} of biomass)				
Biomass	**60% MeOH**	**60% EtOH**	**Distilled water**	**DMSO**
Waste black tea leaves	0.75±0.04	0.38±0.04	1.28±0.10	0.10±0.01
Orange peels	2.68±0.17	1.38±0.03	1.16±0.25	1.99±0.37
Sugarcane bagasse	0.19±0.05	0.23±0.08	0.26±0.05	0.07±0.01
Wheat straw	1.20±0.13	1.23±0.26	0.37±0.04	0.35±0.03
Rice straw	0.97±0.04	1.08±0.17	0.39±0.01	0.36±0.01

MeOH: Methanol, EtOH: Ethanol, DMSO: dimethyl sulfoxide, values are average of three replications±SE

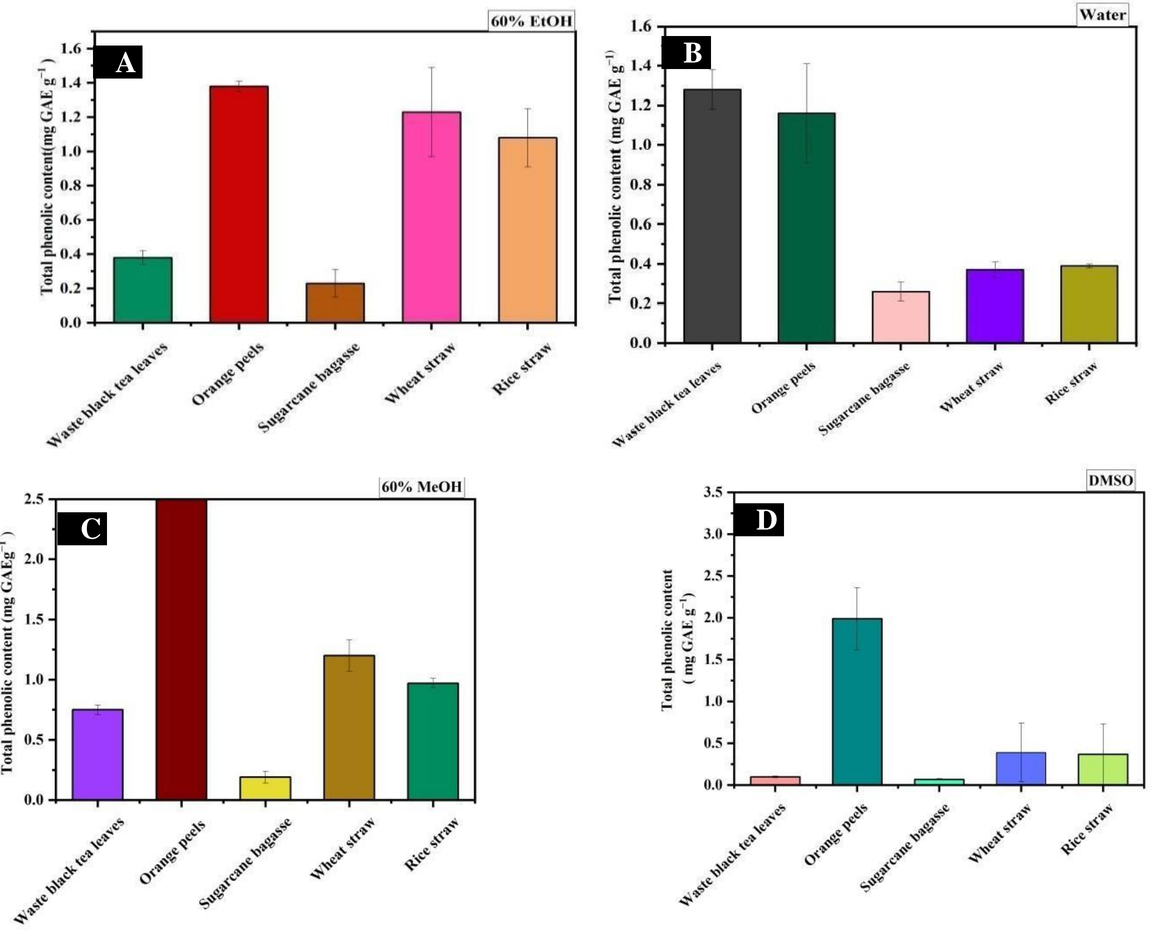


Fig. 8: Total phenolic content (TPC) in different waste biomasses using different extractants

A. 60% Ethanol (EtOH), **B.** Distilled water, **C.** 60% Methanol (MeOH), **D.** Dimethyl sulfoxide (DMSO)

4.2 Total phenolic content in orange peels by using solvents and alkali hydrolysis

The values for total phenolic content in orange peels performed ranged from 0.60 ± 0.21 mg GAE g^{-1} of biomass to 7.58 ± 0.33 mg GAE g^{-1} of biomass. The results given in the **Table 4** showed that the alkali hydrolysed extract contains the highest phenolic content 7.58 ± 0.33 mg GAE g^{-1} of biomass and the lowest phenolic content was observed in ethyl acetate fraction 0.60 ± 0.21 mg GAE g^{-1} of biomass.

Table 4: Total phenolic content (mg GAE g–[1] of orange peels)

Extractants	Total phenolic content (mg/ml)	Total phenolic content (mg GAE g^{-1} ofbiomass)
60% MeOH	2.009	4.68 ± 0.47
DMSO	1.28	2.83 ± 0.19
Ethyl acetate	0.2	0.60 ± 0.21
Water	2.3	2.76 ± 0.31
Alkali hydrolysed	7.5	7.58 ± 0.33

MeOH: Methanol, DMSO: dimethyl sulfoxide, values are average of three replications±SE

For the recovery of polyphenolic compounds using hot water extraction i.e. 5g of sample of orange peels powder in 50ml of solvent, temperature: 80˚C, extraction time: 2 hours, the maximum yield of phenolic compounds was observed. By using hot water (m/v: 1:20; 30 min; 100 °C; two extraction cycles), (Xu *et al*., 2007) evaluated the extraction of phenolic components from mandarin citrus peel. According to the authors, the total phenolic content of the hot water extract was comparable to that produced using methanol as the extraction solvent (M'hiri *et al*., 2014) According to the findings, heat treatment altered the distribution of phenolic acids due to the cleaving of esterified and glycosylated bonds. There are two types of phenolic acids one which is solvent extractable i.e. free phenolics and the other one is bound phenolics which are covalently linked to the plant matrix, they cannot be recovered using water or aqueous/organic solvents. The content of phenolic acids varied in different fractions *i.e.* using alkali hydrolysis extract 7.58 ± 0.33 mg GAE g^{-1} > in methanolic extract (60%) 4.68 ± 0.47 mg GAE g^{-1} > in dimethyl sulfoxide (DMSO) extract 2.83 ± 0.19 mg GAE g^{-1} of biomass > in distilled water 2.76 ± 0.31 mg GAE g^{-1} of biomass > in ethyl acetate extract 0.60 ± 0.21 mg GAE g^{-1} of biomass as demonstrated in the **Fig. 9**. From the percentage yield of orange peels extract it can be concluded that the type of solvent used plays a major role in the extraction of polyphenolic compounds. There are still questions about the best solvent for the extraction of polyphenols, although phytochemicals are often extracted using organic solvent and its aqueous formulation (Alara *et al*., 2020). For instance, acetone has been shown to be more effective than methanol, water, and ethanol at extracting polyphenols from lychee flowers (Liu *et al*., 2009).

Water was found to be better extractant for extracting polyphenols from walnut green husks (Fernández-Agulló *et al*., 2012). Aqueous and organic solvents have superior extraction efficiency than pure organic solvents (Amir *et al*., 2015). Since polyphenols are soluble in polar solvents, it is generally accepted that these solvents frequently perform better in terms of extracting polyphenols. The following criteria must be taken into consideration when

choosing a solvent for extraction: solvent power, solvent polarity, boiling temperature, solvent reactivity, viscosity, stability, safety considerations (Alara *et al*., 2020). Researchers claim that mixing water with organic solvents makes the mixture more polar, which may make it easier to extract compounds that are soluble in both water and/or organic solvents (Do *et al*. 2014; Meneses *et al*., 2013) (Socaci *et al*., 2018).

Based on the results it can be interpreted that as we increase the temperature the phenolic content also increases. A rapid mass transfer between the two phases is what causes the increase in phenolic content at 80 ˚C or above (Haya *et al*., 2018). Solubility increases of the phenolic components in the solvent as the temperature increases. For the recovery of bioactive compounds *i.e.* phenolics, extraction time is a crucial factor as optimum temperature should be provided for better results. According to studies, extraction performance steadily increases with time up to 100 -120 minutes (Otzurk *et al*., 2018). So, the extraction time was set at 120 minutes at which the maximum phenolic content was observed.

Other factors such as agitation speed, type of solvent used, effect of particle size and pH are important, while extracting phenolic compounds. Particle size is one of the main factor, the dried orange peels were grinded and sieved into fine powder to increase the solubility and for better extraction. pH affects the solubility and ionization state of the solid and is a critical factor in determining how the solid and aqueous phases separate (Haya *et al*., 2018). Type of solvent plays a crucial role while extracting polyphenolic components, as in the table it was observed that methanol (60%) showed the maximum phenolic content (4.68 ± 0.47 mg GAE g^{-1}) compared to other solvents. It can be concluded that aqueous mixture of solvent, specifically methanol (60%) can be used for the recovery of phenolic compounds.

Based on the experiment performed it was seen that alkali hydrolysed extract gave the highest yield of the phenolic compounds, referring to table 7.58 ± 0.33 mg GAE g^{-1}. According to literature chemical method is one of the best methods for extracting the bioactive compounds. Sodium hydroxide (NaOH) for hydrolysis can recover bound phenolics (ester/ ether linked). The optimum conditions for recovering the bound phenolics were 2.5-3.5 molar NaOH at 80˚C for 120 minutes (Irakli *et al*., 2018).

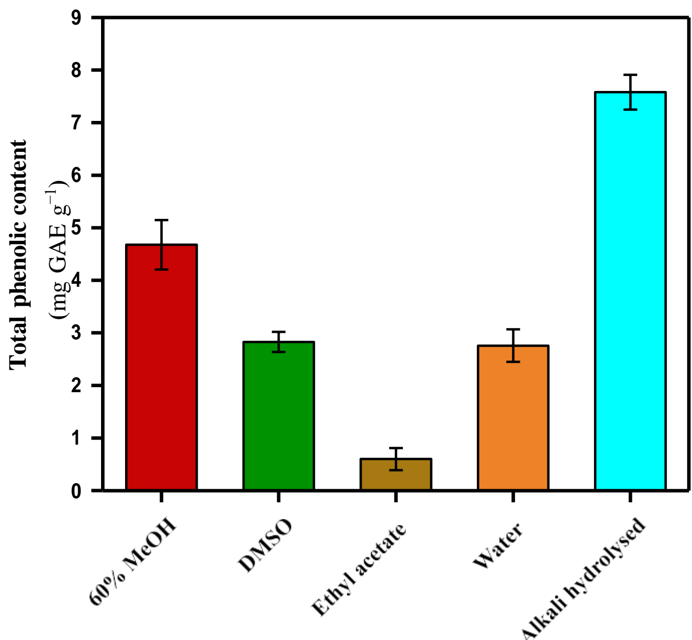


Fig. 9: Total phenolic content in orange peels using solvents and alkali hydrolysed extract

4.3 2, 2-diphenyl-1- picrylhydrazyl radical (DPPH) inhibition by orange peel extracts

The antioxidant activity of orange peel extract was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. Because of their capacity to delocalize uncoupled electrons, which can scavenge free radicals, chelate metal ions, and block oxidase activity, biomass polyphenols have considerable antioxidant activity (Yan *et al.,* 2021). Lower the absorbance value of the reaction mixture, higher the antioxidant activity.

As illustrated in the **Table 5**, the studied solvents produced different yield percentages for orange peel extracts. The highest percentage of DPPH radical was observed in the alkali hydrolysed extract 85.93±0.001% and the lowest percentage was seen in the ethyl acetate extract 31.71±0.012%. The percentage of free radical species were observed in the order with different solvents and alkali hydrolysed extract 31.71±0.012% > 72.58±0.067% > 84.46±0.010% > 85.50±0.009% > 85.93±0.001% with reference to **Fig. 10**. The reduction in DPPH radical absorbance is due to the interaction of an antioxidant and scavenging is accomplished by an antioxidant molecule and radical through hydrogen donation of the radical (Hegazy and Ibrahim, 2012). It can also be concluded that as methanol have the highest polarity, therefore it contains the maximum antioxidant activity in comparison to other solvents.

Table 5: DPPH radical scavenging of orange peels extracts using different solvents

DMSO: Dimethyl sulfoxide, 60% MeOH: 60% Methanol, values are average of three replications±SE

Extractant	DPPH radical scavenging activity (%)
Ethyl acetate	31.71±0.012
DMSO	72.58±0.067
Distilled water	84.46±0.010
Alkali hydrolysed	85.93±0.001
60% MeOH	85.50±0.009

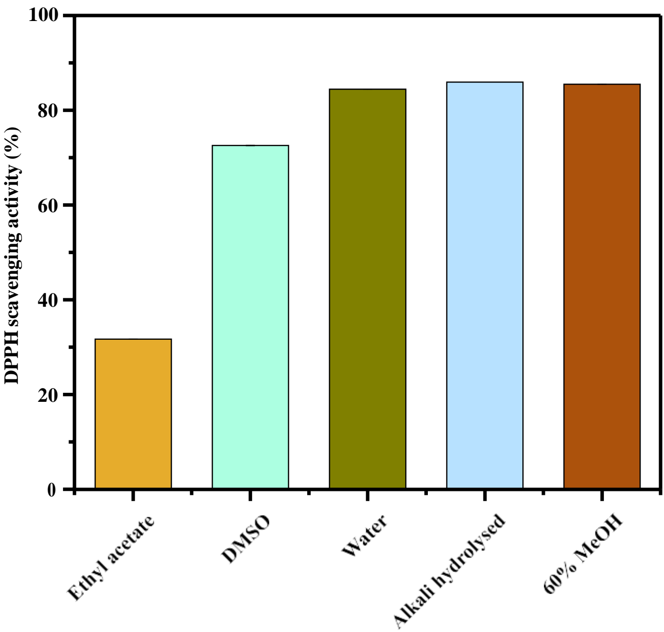


Fig. 10: DPPH radical scavenging activity of orange peel

4.4 Reducing power of orange peel extracts

Antioxidants were measured in the extracts from orange peels. The reducing power of orange peel extracts using different solvents such as methanol (60%), dimethyl sulfoxide (DMSO), distilled water, ethyl acetate, and alkali hydrolysed extract was seen in this order of 0.70±0.040% > 1.42±0.017% > 1.51±0.015% > 1.56±0.03% > 1.75±0.01% respectively (**Table 6**). Using the reducing power assay it was found that dimethyl sulfoxide have the maximum antioxidant potential. The graphical representation of reducing power ability of orange peel extracts is illustrated in **Fig. 11**. Orange peels contains high amount of antioxidant activity have a great importance from industrial point of view.

Table 6: Reducing power of orange peels extracts with different solvents and alkali hydrolysis

DMSO: Dimethyl sulfoxide, 60% MeOH: 60% Methanol, values are average of three replications±SE

Extract	*Reducing power*
Ethyl acetate	1.56±0.03
DMSO	1.75±0.01
Distilled water	0.70±0.040
Alkali hydrolysed	1.42±0.017
60% MeOH	1.51±0.015

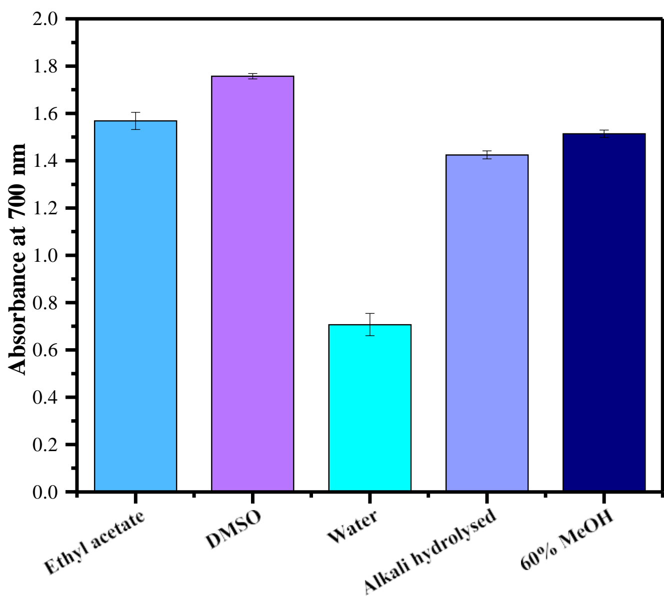


Fig. 11: Reducing power ability of orange peels

4.5 Characterisation of the polyphenolic profile of orange peel extracts by Fourier transformation infrared (FTIR)

The functional groups present on the orange peels surface were demonstrated using FTIR spectroscopy. The distribution of functional groups within the organic fractions is revealed by the FTIR analysis of the isolate samples, which also serves as a foundation for comparing compositional variations between isolates and within samples. **Fig.13** shows the spectra of orange peels. It was observed that phenolic acids contain many functional groups. The peak at 3278.61 cm^{-1}, 3271.43 cm^{-1} and 3285.79 cm^{-1} is due to –OH stretching and the peak at 2923.50 cm^{-1}, 2916.32 cm^{-1} and 2923.50 cm^{-1} is due to–CH stretching. At the range of 1582.07 cm^{-1}, 1589.26 cm^{-1} and 1602.82 cm^{-1} is due to (–C=O)

stretching (Kamsonlian *et al.*, 2011). Usually, complexation of the carboxyl oxygen causes changes in this band. As shown in the **Fig. 13** (C–O–H) bond was seen in the range of 999.54 cm^{-1}, 1013.90 cm^{-1} which confirms the presence of phenols in the tested samples. FTIR method is an excellent method for characterisation of phenolic compounds which shows the bond stretching and bending.

Table 7: FTIR spectra of orange peels wavenumber (cm^{-1})

Native orange peels (Wavelength cm^{-1})	**Alkali hydrolysed** (Wavelength cm^{-1})	**Methanol (60%)** (Wavelength cm^{-1})	**Band assignment** (Functional groups present)
3285.79	3278.61	3264.24	OH (stretching)
2901.96	2923.50	2909.14	CH (stretching)
1596.44	1582.07	1602.82	C=O (stretching)
1404.12	1390.56	1390.56	C=C (stretching)
1035.54	999.54	1035.45	C–O–H

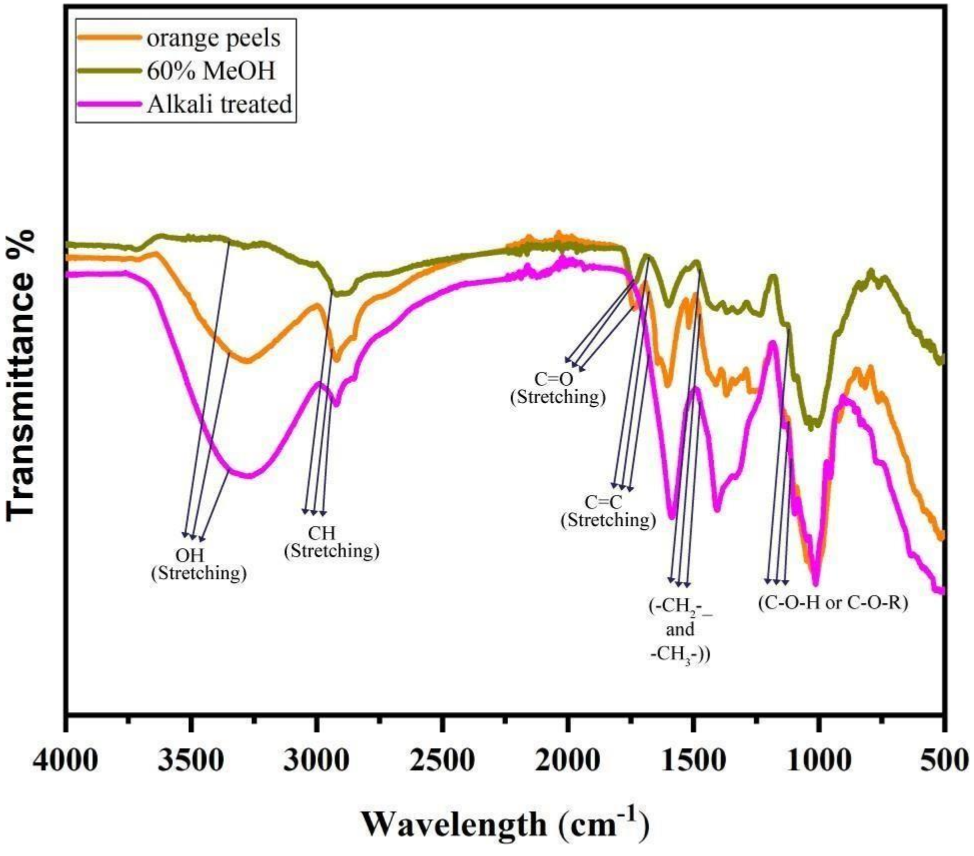


Fig. 12: shows the FTIR spectra of the orange peels

4.6 Antibacterial activity of orange peel extracts by agar well diffusion method

The results show that orange peel extracts possess antibacterial activity against Gram-positive bacteria *i.e. Bacillus subtilis* MTCC441. The widest zone of inhibition (ZOI) was observed in the alkali hydrolyzed extract 21.00±0.82 mm against *B. subtilis and E.coli* MTCC729*,* this *c*ould be possible because alkali hydrolysis extract gives the maximum yield of polyphenolic compounds and have a strong influence on *Bacillus subtilis* and *Escherichia coli*. Further, it was observed that *Escherichia coli*, Gram-negative bacteria gave the negative result by using different solvents. Because of the configuration of their external membranes and the composition of their cell walls, Gram-negative bacteria are thought to be able to withstand the bactericidal activity of polyphenols better. This resistance is due to periplasmic space present in Gram-negative bacteria structure, which is absent in Gram-positive bacteria (Radzik and Klecwicka, 2020). Additionally, the periplasmic region is packed with enzymes that can break down components with antibacterial potential and diffuse into the surrounding environment (Prochnow *et al*., 2016).

Table 8 shows that the alkali hydrolysis was the most effective, producing inhibition zone of 21.00±0.82 mm against *Bacillus subtilis and Escherichia coli.* Methanol (60%) showed the diameter of around 16.33±0.47 mm at 300µl concentration against *Bacillus subtilis*. In case of ethyl acetate extract at 100 µl concentration no zone was detected against gram-positive bacteria. Values were generally higher when 300µl of extract was used; this proved the fact that as we increased the amount/volume of the extracted sample, an increase in inhibition zone diameter was observed. For instance in case of distilled water the zone of inhibition (ZOI) at 100µl was 10.33±0.47 mm, 11.67±0.47 mm for 200µl and 13.00±0.82 mm for 300µl. Dimethyl sulfoxide was used as a control and it was observed that dimethyl sulfoxide do not have any antimicrobial activity on its own. Thus, it can be concluded that the growth of *Bacillus subtilis,* which is a Gram-positive bacteria was inhibited by using orange peels extract. **Fig. 13, 14, 15 and 16** demonstrates the antibacterial activity of orange peel extracts. Orange peels waste from industries can be potential source of naturally antibacterial compounds.

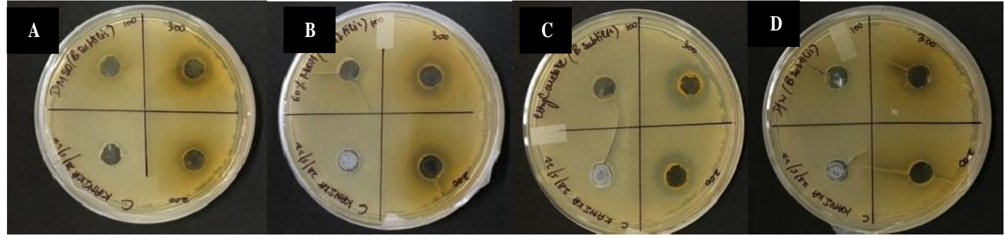


Fig. 13: Antibacterial activity of **A.** DMSO**, B.** 60% MeOH**, C.** Ethyl acetate**, D.** Water against *Bacillus subtilis*

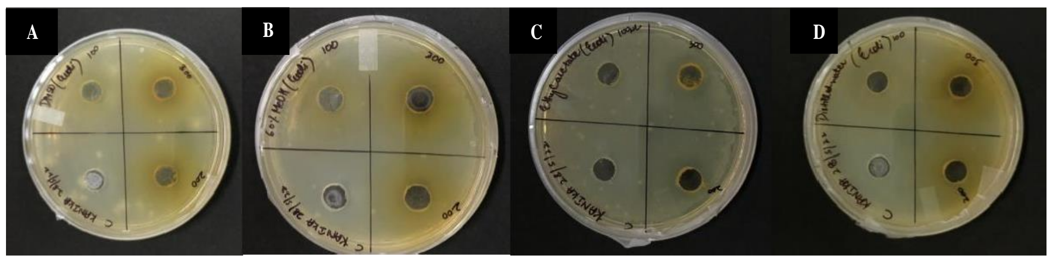
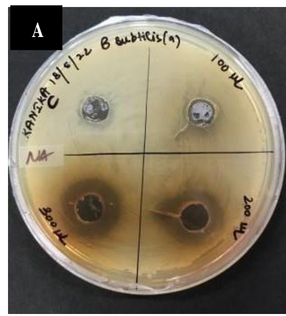


Fig. 14: Antibacterial activity of **A.** DMSO, **B.** 60% MeOH, **C.** Ethyl acetate, **D.** Water against *Escherichia coli*



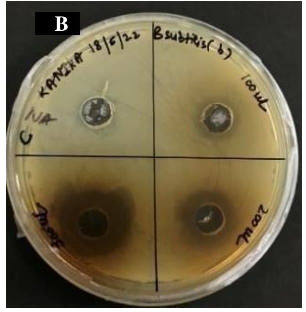
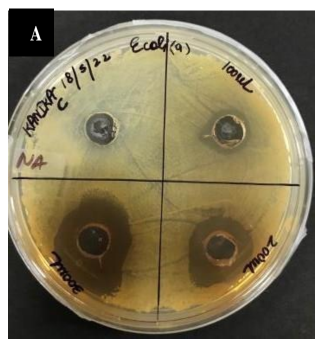


Fig. 15: Antibacterial activity against *Bacillus subtilis* using alkali hydrolysed extract



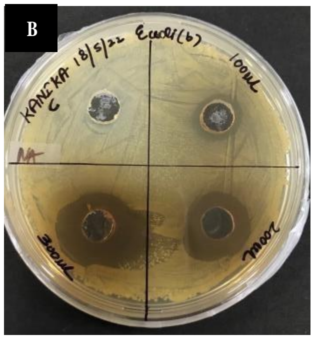


Fig. 16: Antibacterial activity against *Escherichia coli* using alkali hydrolysed extract

Table 8: Antibacterial activity evaluated through the inhibition zone test of polyphenol extracts of orange peels. Data are expressed in mm and the values are average of three replications±SE

Inhibition zone (mm) of orange peels extract				
Bacterial strains	**Extractants used**	**100 µl**	**200 µl**	**300 µl**
B. subtilis	60% MeOH	9.67±0.47	14.33±0.47	16.33±0.47
	DMSO	10.67±0.47	12.00±0.00	14.67±0.47
	Ethyl acetate	ND	17.33±0.47	18.33±0.47
	Distilled water	10.33±0.47	11.67±0.47	13.00±0.82
	Alkali hydrolysed	13.67±1.25	18.00±1.63	21.00±0.82
E. coli	60% MeOH	ND	ND	ND
	DMSO	ND	ND	ND
	Ethyl acetate	ND	ND	ND
	Distilled water	ND	ND	ND
	Alkali hydrolysed	13.33±0.47	17.67±1.25	21.00±0.82

ND: Not detected

4.7 Time course growth curve of *E.coli* by using broth based turbidometric assay (TB)

The effect of crude extract of orange peels was observed in broth, which showed that orange peels extract inhibits the growth of *E.coli* MTCC729 as compared to control. **Table 9** and **Fig. 17** shows antibacterial effect of orange peels extract on the growth curve of *Escherichia coli* MTCC729. The inhibitory effect of orange peels extract was seen at different concentration or dilutions of peel extract. In general, 200 µl extract was the most potent one against *E.coli* and showed the maximum inhibitory effect after 24 hours with absorbance at 600 nm was 0.22±0.001 whereas in control it was 1.73±0.105 **(Table 9, Fig. 17)**. After 6 hours the bacterial growth declined however there was less antibacterial activity against *E.coli* at lower concentrations and volumes. The results indicate bactericidal effect of orange peel extract on Gram-negative bacterium.

Table 9: Antibacterial effect of orange peel extracts on the growth curve of *E.coli* MTCC729 and the values are average of three replications±SE

Time (hours)	Volume (µl) 100 µl	150 µl	200 µl	250 µl	Control
0	0.06±0.001	0.07±0.009	0.07±0.004	0.07±0.002	0.06±0.003
2	0.01±0.0003	0.25±0.005	0.05±0.0009	0.25±0.028	0.19±0.001
4	0.03±0.003	0.26±0.003	0.12±0.004	0.27±0.025	1.39±0.002
6	0.08±0.001	0.28±0.003	0.12±0.0006	0.15±0.002	1.80±0.002
8	0.26±0.006	0.28±0.0005	0.12±0.005	0.22±0.002	1.85±0.033
24	1.46±0.018	0.31±0.006	0.22±0.001	0.34±0.0002	1.73±0.105

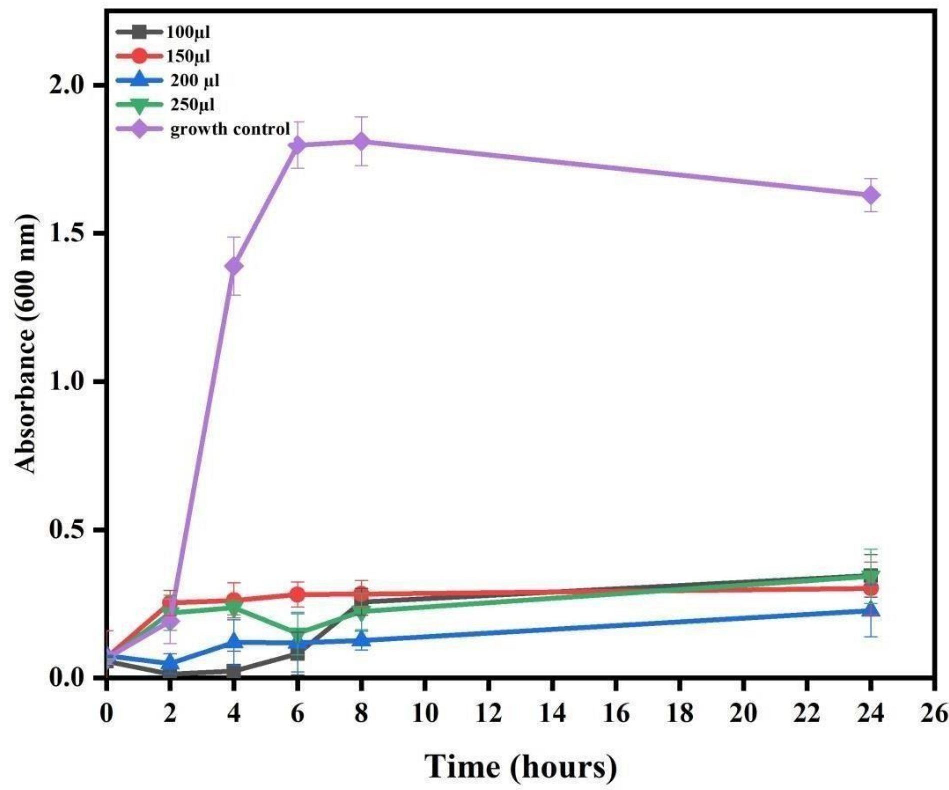


Fig.17: Antibacterial effect of orange peels extract on the growth curve of *Escherichia coli* MTCC729

SUMMARY

1. **1.** The main objective of the study was to extract the total phenolic content from different waste biomass using sugarcane bagasse, orange peels, waste black tea leaves, rice straw and wheat straw. The maximum total phenolic content was observed in methanolic fraction of orange peels extract 4.68 ± 0.47 mg GAE g^{-1} of biomass.
2. **2.** The extraction yields were significantly impacted by solvents (ethanol, methanol, and dimethyl sulfoxide) due to varying polarity and concentrations.
3. **3.** Antioxidant activity was assessed using the DPPH assay and reducing power assay method. Results showed that the highest percentage of DPPH radical was observed in the alkali hydrolyzed extract 85.93±0.001% and the ethyl acetate extract of orange peel contained the lowest DPPH radical 31.71±0.012%. Using the reducing power assay it was found that the dimethyl sulfoxide extract 1.75±0.01% have the maximum antioxidant potential.
4. **4.** FTIR analysis of orange peels showed the presence phenolic compounds.
5. **5.** Orange peel extracts showed antibacterial activity against Gram-positive bacteria i.e. *Bacillus subtilis*. The alkali hydrolysed extract has a 21.00±0.82 mm zone of inhibition (ZOI) against *Bacillus subtilis* whereas by using Gram-negative bacteria i.e. *Escherichia coli* no inhibitory zones were seen.
6. **6.** The effect of crude extract of orange peels was observed in broth, which showed that orange peels extract inhibits the growth of *E.coli* MTCC729.

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Introduction Agricultural residues, also known as agro-residues, crop-residues, or harvest wastes, consist of huge number of carbon-based components that are formed during the harvesting of agricultural crops. Primary residues, such as stalks, leaves, and straw left in the field after harvesting, are classified as primary residues, whereas secondary residues, which remain after processing into a useful resource, are classified as secondary residues. Residues such as husks, seeds, rhizome, molasses, bagasse, and other process wastes are used as fertilizer, energy, resources for animal fodder, soil enhancing, and other manufacturing operations. The global dry matter production of agricultural leftovers was predicted to reach 3.8 billion. Crop residue production in India is estimated to be between 500 and 550 million tonnes per year (Ginni G et al., 2021). Crop residue burning is one of the major sources of pollution in the atmosphere. This has a detrimental effect on the soil's nutrient budget. In the course of burning, carbon, nitrogen, and sulphur are totally burned and lost to the atmosphere. It produces smoke, which, when combined with other gases in the atmosphere like methane, nitrogen oxide, and ammonia, can generate severe pollution.

Crop stubble burning in open fields releases a variety of hazardous chemicals into the atmosphere, including carbon monoxide, NO2, SO2, CH4, particulate matter, and hydrocarbons. When crop stubble is burned openly, hazardous substances such as polychlorinated dibenzo-p-dioxins, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated dibenzofurans (PCDFs) are released. These air contaminants are toxicologically significant and may cause cancer. Furthermore, agricultural stubble burning releases carbon dioxide into the atmosphere, depleting the oxygen layer in the natural environment and generating the greenhouse effect. If done repeatedly, burning can degrade soil quality and make land more prone to erosion. Furthermore, constant burning is not an agricultural technique that can be sustained (Kumar and Joshi, 2013). As a result, it is now a global issue to mandate the development of alternative, cleaner, and renewable bioenergy supplies. These wastes create a significant disposal issue. For example, the juice industry generates a large amount of waste in the form of peels, while the coffee industry generates waste in the form of coffee pulp and cereal industries generate husks. Worldwide roughly 147.2 million metric tons of the fiber sources are found.