

Exploring the Anti-inflammatory and Anti-Arthritic Potential of Malus peel Phenolics: A comprehensive comparative analysis of Royal delicious, Delicious, Golden delicious, and Maharaji Cultivars from Kashmir, India

Ashfaq Ahmad Shah

Graphic Era (Deemed to be University)

Neha Pandey

Graphic Era (Deemed to be University)

Rakesh Kumar Bachheti

Graphic Era Hill University, Clement Town

Nishant Rai

Graphic Era (Deemed to be University)

Amit Gupta

amitvsbt@gmail.com

University of Jammu

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Abstract

The aim of the current investigation was to reveal whether the peel phenolics of three red-colored ("Royal Delicious", "Delicious", "Maharaji") and one yellow-colored ("Golden Delicious") malus variety might have a differential tendency with regard to inflammation and arthritis, as these varieties indigenous to Kashmir have not yet been exploited for the same. The comparative assessment of the immunomodulatory properties of decoctures, prepared by amalgamating the extracts of different polar menstruums, was analyzed by subjecting them to *in vitro* assays of inflammation and arthritis. In the decoctures, the presence of carboxylic acids, poly-hydroxy compounds, aromatic rings, alcohols, alkenes, ethers, nitro compounds, phenols, hydrogen-bonded alcohols, and esters was demonstrated by FTIR. All the FTIR spectra correlate to the functionality of phenolic and polyphenolic compounds deemed to be present in malus varieties. All the peel decoctions showed significant inflammation suppressive and anti-arthritic efficacy, in a dose-dependent fashion. With the goal to treat oxidative stress-related, inflammatory, and arthritic illnesses, the present research assigns the greatest pharmacological precedence to the bioactive compounds extracted on a polarity premise from peels of *Malus domestica* var Maharaji.

Introduction

Among the most well-known and frequently grown fruit crops in temperate climates is *Malus × domestica* Brok (Rosaceae). The majority of an apple fruit's distinctive qualities, including flavor, scent, and hue, are closely tied to its metabolic makeup. Numerous bioactive substances may also be discovered at the same time as markers of a particular apple variety [1]. Apples generally have a wide and harmonious nutritional composition. Water accounts for more than 84% of the fruit's weight and is where trace minerals such as Se, F, Fe, Mo, Mn, Cu, B, Zn, etc. are disseminated [2]. Nearly all vitamins are abundant in them, with vitamin C and the B complex being the most prevalent. Due to their small amounts, proteins and lipids only make up a small portion of the nutrients in *Malus domestica* fruits. Apples have a lot of dietary fiber, which comprises intricate polysaccharides like celluloses, pectin, lignin, and hemicelluloses, compared to other fresh fruits. Apples have a lot of medicinal potential because of the possession of all these valuable constituents along with the presence of benign phenolic and polyphenolic secondary phytochemicals [2, 3]. Consequently, a lower risk of chronic diseases and more beneficial health effects have been associated with frequent apple eating. It is thus highly recommended to follow the proverb "An apple a day keeps the doctor away!" in order to be healthy and fit. The highest level of these compounds are being found in peels [4], indicating that apple peels may be biologically more functional than apple flesh in terms of promoting sound health. By and by, eating an apple with the skin on is strongly recommended. At the same time, phytoalexins and phytoanticipins, which play unique ecological roles in plants, are included in the repertory of plant defence amid such benign compounds [5]. The key groups of such organic substances that are prominently found in the many malus cultivars are flavonoids, hydroxycinnamic acids, anthocyanins, chlorogenic acids, quercetin conjugates, procyanidins, polyphenols, carotenoids, terpenes, and organic acids [3, 6]. The powerful free radical scavenging, immunomodulatory, and other human health-related bioactive capabilities of apples are mostly attributed

to their phenolic content, despite the fact that they additionally include a range of bioactive compounds. The presence of many double bonds and hydroxyl groups in the architecture of most of these compounds contributes to their antioxidant potential [7]. The basic families of polyphenolic compounds found in high concentrations almost in all varieties of apples include hydroxycinnamic acids, primarily chlorogenic acid, flavanols mainly distinct quercetin glycosides, flavan-3-ols, primarily (+)-catechin, (-)-epicatechin, dihydrochalcones such as phloridzin, anthocyanidins, and anthocyanins [3, 6, 7]. Among the most researched flavonoids/phenolics in various apple cultivars are procyanidin, (+)-catechin, (-)-epicatechin, chlorogenic acid, cyanidin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside, gallic acid, coumaric acid, and phloridzin (Fig. 1). According to studies, the peel of an apple contains 60% monomeric and polymeric flavan-3-ols, 18% hydroxycinnamic acids, 9% dihydrochalcones, 5% flavanols, and 5% anthocyanins in terms of the overall phenolic content [8]. Red-skinned apples are becoming more and more popular due to their high concentration of cyanidin glycosides (anthocyanins), which are primarily concentrated in the peel. The sheer quantity of anthocyanins in the phenolic category has been proposed to be the main cause of apple skin reddening, even if the pigments responsible for colour may differ. Apple anthocyanin concentrations and colour progression as the fruit ages are greatly influenced by temperature, light exposure, nutrition, and genetic variables [9]. Based on the type of soil and climate, planting technique, cultivar, phase of development and ripening, stressors from the environment, and other factors like storage period, after-harvest circumstances, etc., individual terpenoid, phenolic, S and N containing phytochemical amounts in apples and other fruits vary [10]. Therefore, it is difficult to juxtapose the findings of several studies in order to draw robust inferences.

The recognition that phenolic and polyphenolic secondary metabolites have therapeutic value has generated a lot of engrossments in pharmacognosy science over the past twenty years. The anti-microbial, inflammation reducing, cardioprotective, ROS scavenging, anti-carcinogenic, anti-hypertensive, anti-ageing, and immunomodulatory abilities of various phytoanticipins and phytoalexins have been uncovered and are becoming the subjects of nanotechnology that facilitates their specialized delivery to specific sites [10, 11]. They have also been shown to manipulate enzymatic operations, hinder cell division, aid in inducing apoptosis, and inhibit pathogen proliferation. Researchers are currently predicting their potential use as precursors for *in vivo* biological reactions, substrates for enzymatic reactions, ligands to antagonise or agonise cellular receptive proteins, etc. through utilising the concepts of bioinformatics and molecular docking [12]. The main motive of involving Phyto-active compounds into treatment options is to build advantageous connections with the physiological processes of the body while avoiding the undesired adverse reactions that pharmaceuticals are notorious for. Rheumatic and inflammatory illnesses, particularly various forms of hypersensitivity disorders and immunological exaggerations, are an important contributory factor of morbidity worldwide and have been referred to as the "King of Human Miseries." Pharmacologists from all around the world have been concentrating their efforts on developing more effective and safe inflammation suppressive medications. Non-steroidal anti-inflammatory medications (NSAIDs) and corticosteroids are the pharmaceuticals most frequently used to treat inflammatory and arthritic disorders [13]. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been shown to be effective in treating rheumatoid disorders and inflammation not only because

they are prostaglandins antagonists, but also because they have the ability to stabilize cell and lysosomal membranes as well as safeguard intrinsic proteins from denaturation, which can result in the formation of peptides that are thought to be autoantigens triggering rheumatism but the biggest drawback of the powerful synthetic corticosteroids and NSAIDs now on the market is their toxicity and recurrence of indications after stopping use [14]. Nowadays, organic products are seen as being safer than synthetic medications, which are viewed as being hazardous to both persons and the environment. So, in search of security and safety, people are going back to organic products in this day and age. In this view, the present investigation was conducted with the aim to assess the inflammation suppressive and anti-arthritic competence of the peel decoctures of various malus varieties native to Kashmir. This was the first research of its sort to reveal if the phenolic compounds from these malus varieties (Fig. 2) might be having differential expression towards inflammation and arthritis as no research has yet exploited these peel phenolics for the same. One of the goals of this study was to identify the malus variant that is most notable from a phytochemical and pharmacological standpoint. This study revealed that the peels of these malus species pack a big phytochemical punch that possesses anti-inflammatory properties. *Malus domestica* var Maharaji turned out to be superior in terms of the parameters analysed.

Materials and Methods

Sampling

All of the Malus varieties utilized in this investigation (*Malus × domestica* Delicious, Maharaji, Royal delicious, and Golden) were obtained in September 2021 from various apple gardens in Dodarkoot, a district in Kashmir's Kulgam region, which is situated at 33°38'24"N 75°01'12"E and has an average altitude of 1739 meters. Only healthy-looking fruits were selected via simple random sampling. The specimens were registered under the code CBT/KU/21 in the Kashmir University taxonomy department's "Herbarium," where a taxonomist verified them. Purchased from Hi-media, the chemicals and reagents used in this research were all of the analytical grades.

Modus operandi for extrication of polar compounds and concocting of decoctures

Peels from distinct Malus varieties were collected, scoured with distilled water, shade dried at room temperature for 20–25 days, and then pulverized aseptically. Polar menstruum systems of variable polarity [Methanol (P-0.762), Acetone (P-0.355), and Ethyl acetate (P-0.228)] were employed to extract the pulverized samples involving the aid of hot maceration and Soxhlet extraction. The rallied extracts were passed through muslin fabric followed by Whatman-1 filter paper prior to being concentrated in a rotary evaporator at a temperature lower than the menstruum's boiling point. Petroleum ether was utilised for processing each dried gooey exudate, eliminating sections that were rich in fatty components. After being refluxed with 6% H₂SO₄ for 2.5 hours to free them from linked sugars, the remaining fractions were filtered [15]. Once more, the filtrates were purified to neutrality using the appropriate solvents. The retrieved material was dried under a vacuum, and then FTIR analysis was used to look for the nature of

constituent compounds. The decocture of a particular peel was prepared by amalgamating its all the polarity-based extracts (500 mg each in 1ml DMSO) and was subjected to the anti-inflammatory and anti-arthritic potential at different concentrations

Preliminary screening of Secondary metabolites

All of the crude extracts underwent traditional assays of preliminary phytochemical screening [16] to screen for the presence or absence of phenolics\polyphenolics, terpenes, and other S/N-possessing metabolites. The nature of all these assays was qualitative relying on colour change and precipitation.

FT-IR Analysis

A certain amount of energy is transferred or ingested as an outcome of the impact that the infrared radiations have on the atomic vibrations of the molecules that exist in the specimen. A molecule's overall dipole momentum changes as a result of its rational or vibratory motions in order to absorb infrared light. The majority of compounds with covalent connections capture considerable infrared energy [17]. Diatomic molecules like oxygen, hydrogen, and nitrogen are the sole exceptions because they do not spin or pulsate enough just to lead to a net alteration in the dipole moment. The relative locations of atoms vary continuously as a result of different types of vibrational motions (stretching and bending) in molecules. Molecules consist of tiny springs called bonds that vibrate at a certain frequency. The electromagnetic spectrum's infrared region includes these frequency ranges. Every atomic group has distinct stretching frequencies seen in the IR spectrum [18]. As a result, each individual chemical compound's infrared spectrum serves as a distinctive indicator that sets it apart from the bands of other substances. This meant that by applying infrared light to the samples and seeing which frequency ranges were caught, it was possible to determine the types of chemical bonds present in the specimens.

Study of anti-inflammatory potential

In vitro tests using the human red blood cell membrane cell (HRBC) membrane stabilisation assay, proteinase suppressive activity, and anti-lipoxygenase performance were used to examine the retrieved decoctions' ability to reduce inflammation.

Human erythrocyte membrane stabilization assay

Membrane stabilization is a mechanism used by anti-inflammatory drugs to protect the intrinsic strength of cell membranes, particularly the lysosomal membrane, by stabilizing protein molecules in the membrane. Lysosomes contain a variety of enzymes which may be involved in the inflammatory response. Shortly after an inflammatory reaction, lysosomal enzymes are released into the cytosol, causing damage to the tissues nearby and, as a result, commencing inflammation. Most anti-inflammatory medications work by reducing the outflow of lysosomal enzymes, which helps to soothe the lysosomal membrane and decrease inflammation [19]. The basic idea herein is to stabilize the human RBC membrane (which is akin to the membranes of lysosome) to avoid hemolysis that can be triggered by hypotonicity, heat, or ROS (H_2O_2). If any compound stabilizes the HRBC membrane, it is deemed to stabilize the lysosomal membrane as well.

Prepping blood samples for analyses of membrane stabilization

Blood was supplied by a human volunteer in good health who gave consent to it, and the main exclusion criteria were the use of any NSAIDs or contraceptives during the two weeks' prior to the sample's collection. A vein pierce was used to acquire 10 mL of whole blood to be used for the erythrocyte suspension. An identical portion of Alsever's solution (2% $C_6H_{12}O_6$, 0.8% $C_6H_5O_7Na_3$, 0.5% $C_6H_8O_7$, and 0.42% NaCl) was combined with venous blood. The supernatant was evacuated after being spun at 3000 rpm (27° C) for 5 minutes. The emerging compacted mass of cells was centrifuged at 2500 rpm for 5 minutes after being cleaned with a sterile solution of normal saline (0.85% w/v NaCl). This process was performed again until the supernatant was transparent and finally, the total volume of packed cells was determined. The retrieved cellular component was employed in the experiment after being reconstituted to a 40% suspension in PBS (10 mM, pH 7.4).

Hypotonicity induced hemolysis

The assay brew was developed by blending a unit mL PBS (pH 7.4, 0.15M) and 0.5mL HRBC suspension (10% v/v) with 1 mL of each decoctures of various doses (100, 200, 400, 800 and 1600 $\mu\text{g/mL}$) and standard NSAID acetylsalicylic acid at same concentrations. Distilled water was incorporated as a hypotonic therapy for compacted RBCs to promote full hemolysis (anticipated 100% for the assay). An additional control that was deemed to cause 0% hemolysis was a blend comprising erythrocytes in an isotonic solution (154 mM NaCl in 10 mM sodium phosphate solution, pH 7.4). The combos were incubated for 30 minutes at 37°C before being spun at 3000rpm. The hemoglobin content in the supernatants of all test mixtures were estimated via spectrophotometer at 560nm, the release of which from erythrocytes is dependent on % hemolysis. When it is predicted that adding distilled water to erythrocytes will cause 100% hemolysis, anti-inflammatory activity was stated as the % hemolysis and % protection as follows [20].

% Hemolysis = $[(\text{OD}_{540} \text{ of Test sample} / \text{OD}_{540} \text{ of lysis triggering control}) \times 100]$

% Protection = $100 - [(\text{OD}_{540} \text{ of Test sample} / \text{OD}_{540} \text{ of lysis triggering control} \times 100)]$

Proteinase inhibitory activity

Neutrophils contain a large number of proteinases, especially serine proteinases in their lysosomal granules. Leukocyte proteinase has been implicated in the onset of tissue injury during inflammatory responses and arthritis. Proteinase suppressors have been shown to give a considerable amount of resistance [21]. Protease antagonist effectiveness was assessed using a slightly modified version of the Bijina *et al.*, 2011 [22] technique. 1 mL (0.1 mg/mL made in 0.1 M phosphate buffer pH 7) aliquot of trypsin [EC 3.4.21.4, 1000 units/mg] was pre-incubated with distinct doses of peel decoctures and positive control (100, 200, 400, 800 and 1600 $\mu\text{g/mL}$) at 36° C for 20 min. To this blend 2 mL of 1% Hammerstein casein (made in 0.1 M phosphate buffer) was added and again incubated at the same

temperature for 25 min. By adding 2.5 mL of 0.44 M trichloroacetic acid (TCA) solution, the reaction was stopped. The precipitated protein was eliminated by centrifuging the reaction mix at 10,000 rpm for 15 minutes after it was delivered to a centrifuge tube. Spectrophotometry was utilized to evaluate the cleared supernatant's absorbance at 280 nm in comparison to acceptable blanks. TCA-dispersed casein amino acids produced by trypsin in the presence and absence of the antagonist (tyrosine in casein without inhibitor) were measured. The expulsion of 1 g of tyrosine per ml of the reaction mix every minute under the test settings was regarded as one unit of trypsin action. The reduction in absorbance of the TCA soluble casein hydrolysis byproduct released by trypsin operation assessed at 280 nm every minute within the test settings by one unit was used to quantify antagonist activity. Inhibition percentages were used to convey the protease antagonist efficacy. The test was also accompanied by relevant blanks for the enzyme, inhibitor, and substrate.

Caseinolytic plate assay

Casein protein may be broken down by proteolytic exoenzymes. Casein agar plates, which are made of nutritional agar enriched with milk that includes the protein base casein, were utilised to demonstrate this action. Milk protein imparts the medium in question its colour and porosity since it reflects light instead of transmitting it [23]. On the agar plate, wells were drilled utilizing a metal plunger. Following inoculation with Trypsin (T positive control – 15µL, 1000 units/ml) alone and in combination with the decoctures (1.6mg/ml, 200µL). For one night, plate was placed for incubation at 37°C. TCA was added to inhibit the reaction following an incubation period overnight. The diminution of opacity, which occurred after overnight incubation, was used to deduce the results as the outcome of a hydrolytic process that produced soluble, non-colloidal amino acids. The reduction in the ZOIs produced by trypsin was taken as positive inhibitory activity of decoctures.

Lipoxygenase (LOX) antagonistic activity

Polyunsaturated fatty acids like arachidonic acid and linoleic acid are oxygenated by lipoxygenases (LOXs). The oxygenated lipids either start future biological events, trigger cellular signaling pathways through certain surface receptors on cells or are progressively metabolized into strong lipid mediators that play a role in inflammatory and immunological responses [24]. Leukotrienes (LTs) that had a significant role in a number of inflammation-related disorders, including arthritis, cancer, asthma, and fatal hypersensitivities are primarily produced by lipoxygenase. Inflammatory ailments like Crohn's disease, rheumatoid arthritis, ulcerative colitis, asthma, psoriasis, and allergic rhinitis exhibit elevated levels of LTs [25]. Hence is believed that blocking the LOX route can limit the synthesis of LTs and that using LOX antagonists for managing a variety of such disorders is a prospective therapeutic strategy. Linoleic acid was incorporated as the substrate and lipoxidase as its acting enzyme in the study of the LOX antagonistic activity of decoctures. In 0.25 mL borate buffer (of 2 M, pH 9), the decoctures (100, 200, 400, 800, and 1600 g/mL) were homogenized. 0.25 mL of lipoxidase enzyme (20,000 units/mL) was thereafter added. At 27°C, this blend was left to incubate for 10 min. It was followed by the addition of 1.0 mL of a 0.6 mM linoleic acid solution and absorbance was determined at 234 nm. As an acceptable

standard, meso-nordihydroguaiaretic acid (masoprocol) was employed. Three copies of the experiment were run, and the mean was taken. The following equation was used to compute the % inhibition:

$$\% \text{ inhibition} = ([\text{Abs}_{234} \text{ of control} - \text{Abs}_{234} \text{ of test sample}] / \text{Abs}_{234} \text{ of control}) \times 100$$

Study of Anti-arthritic potential of decoctures via Inhibition of heat-induced albumin denaturation

In some arthritic conditions, the formation of auto-antigens may be triggered by denaturation of tissue proteins. Therefore, tissue protein denaturation can be used as a diagnostic indicator for arthritic conditions [26]. Thus, a potential possibility for anti-rheumatic drug discovery would be compounds that may impede protein denaturation. Analysis of the ability of decoctures to prevent albumin denaturation provided insight into their anti-arthritic efficacy. A slightly modified version of the BSA test described by Yesmin *et al.* 2020 [27] was used to assess the same. In Tris Buffered Saline (0.05M Tris and 0.15M NaCl), BSA solution (0.5%, w/v) was made. Glacial acetic acid was used to bring the pH level down to 6.4. Bovine serum albumin (0.5%) was homogenized with 1 ml of the previously made decoctures and diclofenac sodium (positive reference) at different doses (100, 200, 400, 800, and 1600 g/ml) before being left to incubate at 30°C for 15 minutes. D H₂O and BSA constitute the monitoring tube. The proteins were denaturated by immersing the blend in a water bath at 70°C for ten minutes. Upon reducing the specimens to ambient temperature, the cloudiness of the solutions (amount of protein precipitation) was assessed using a spectrophotometer at 660 nm. The studies were carried out three times, and mean absorbance readings were kept. On a percentage basis, the proportion of blockage of precipitation (protein denaturation) was established.

Statistical analysis

At the very least, all measurements were recorded in triplicate and approximated. The 95% confidence interval (P 0.05) was used in this investigation. To reveal all data, the mean standard deviation of three replicates has been used. Using SPSS software, the analysis of variance (ANOVA) approach was applied to assess the results.

Results

FTIR Spectrums

Fig. 3-6 depict the FTIR spectra in the 4000-400 cm⁻¹ range along with the wave numbers (cm⁻¹) of the observable peaks discovered in the methanol decoctions of all the varieties. Spectra of all other decoctions can be found in the supplementary data associated with this manuscript. The characteristic peaks that serve as the signatures for specific functional groups and bond types are listed (Table 1).

Table 1
FTIR spectral peak broad ranges and anticipated functional groups

Wave number cm ⁻¹	Characteristic Functional group	Anticipated class
3650 – 3200	O-H stretch (symmetric) Hydroxy group, H-bonded	Poly Hydroxy compounds/Alcohols
3000 – 2800	C-H stretching (asymmetric), Aliphatic	Alkanes
3000 – 2840	N-H stretching	Amine
2900 – 2800	C-H stretch (vibration)	Methoxy methyl ether compounds
3400 – 2400	O-H stretch, (stretching vibration) Carboxylic group	Carboxylic acids
2380 – 2349	O = C = O stretching	Carbon compound
2140 – 1990	N = C = S stretching	Isothiocyanate
2000 – 1650	C-H bending	Aromatic compound
2000 – 1650	C-H bending	Aromatic compound
2000 – 1650	C-H bending	Aromatic compound
1580 – 1490	N-H deformation vibrations	Secondary amine
1580 – 1490	C-H bending	Methyl/methylene group
1440 – 1395	O-H bending	Carboxylic acid
1250 – 1020	C-N stretching	Amine
1275 – 1200	C-O stretching / C-N stretching	Alky ether/aryl ether
1150 – 1085	C-O stretching	Aliphatic ether
1155 – 1015	CH ₃ rocking vibration	P-O-C ₂ H ₅ ; Organic phosphorus compound

Results of Anti-inflammatory Potential

The anti-hemolytic capacity of the fractions via HRBC membrane stabilization

All peel decoctures and standard NSAID Diclofenac sodium were tested for membrane stabilization at doses of 100, 200, 400, 800 and 1600 µg/mL. The significant level of HBRC membrane stabilization was shown by the Maharaji peel decocture (MPD) followed by delicious peel decocture (DPD). MPD turned out to be potent in inhibiting the hypotonicity-triggered hemolysis of HRBC (71.72% at a dose of 1600 mcg/ml) when compared with standard NSAID showing 96.93% inhibition at the same concentration. At

1600 mg/ml, the other extracts also exhibited low to moderate activity (Fig. 7). The stabilization activity was of the order MPD ($IC_{50}=1.085\text{mg/ml}$) DPD ($IC_{50}=1.517\text{mg/ml}$) RdPD ($IC_{50}=1.537\text{mg/ml}$) GPD ($IC_{50}=1.93\text{mg/ml}$). NSAID revealed an IC_{50} of 0.768mg/ml . The membrane hemolysis is lowered, and the membrane stabilization is enhanced with increasing concentration of the samples. Consequently, the anti-hemolytic or membrane-stabilizing action of the decoctures will be said to be dose-dependent.

Proteinase inhibitory activity

Royal delicious peel decocture (RdPD) revealed comparatively significant proteinase inhibitory activity (27.35% inhibition at the concentration of 1.6mg/ml followed by Maharaji peel decocture (MPD). The inhibitory activity was of the order RdPD ($IC_{50}=1.961\text{mg/ml}$) MPD ($IC_{50}=2.504\text{mg/ml}$) DPD ($IC_{50}=2.944\text{mg/ml}$) GPD ($IC_{50}=3.337\text{mg/ml}$). (Fig. 8). Caseinolytic plate assay was also employed to support the spectrophotometric results. Trypsin ($T-200\mu\text{L}$) alone was showing a visible zone of diameter corresponding to 19mm . Among the decoctures, royal delicious peel decocture (RdPD- 1600mcg/ml) to some extent inhibited the proteinase activity by reducing the zone ($ZOI = 15\text{mm}$ as compared to 19mm), thus showing inhibitory activity (Fig. 9).

Anti-lipoxygenase (LOX) activity

It is observed that the Maharaji peel decocture (MPD) comparatively showed the highest LOX inhibitory activity of 51.24% at $1600\mu\text{g/mL}$ concentration, whereas GPD, RdPD, AND DPD showed 42.31% , 38.51% , and 31.25% inhibition at the same concentration, respectively when compared with positive control Nordihydroguaiaretic acid (NDGA) which revealed 98.43% of inhibition at $800\mu\text{g/mL}$ ($IC_{50}=0.43\text{mg/ml}$). The inhibitory activity of the decoctures was of the order MPD ($IC_{50}=1.59\text{mg/ml}$) GPD ($IC_{50}=1.90\text{mg/ml}$) RdPD ($IC_{50}=2.06\text{mg/ml}$) DPD ($IC_{50}=2.63\text{mg/ml}$). Figure 10 shows the results. The results indicate that the bioactive compounds possessed by the decoctures had potent LOX inhibitory activities.

Anti-arthritic potential: inhibition of heat-induced albumin denaturation

All polar decoctures were able to inhibit albumin denaturation in a concentration-dependent manner, and the inhibitory effect was more as concentration was increased. Maharaji peel decocture revealed a comparatively significant inhibitory potential of 61.03% at the concentration of 1.6mg/ml when compared with positive control NSAID that shows an inhibition of 95.93% at the same concentration ($IC_{50}=0.738\text{mg/ml}$). The inhibitory potential of DPD, GPD, and RdPD was respectively 51.52% , 48.46% , and 52.35% at the highest concentration taken. The inhibitory activity was of the order MPD ($IC_{50}=1.26\text{mg/ml}$) RdPD ($IC_{50}=1.47\text{mg/ml}$) DPD ($IC_{50}=1.53\text{mg/ml}$) GPD ($IC_{50}=1.61\text{mg/ml}$). (Fig. 11).

Discussion

The goal of the current research investigation was to compare the phenolic metabolites of the peels of three Malus cultivars with red skin ("Delicious," "Maharaji," and "Royal delicious") and one with yellow skin

("Golden Delicious") along with the comparative assessment of the bioactive compounds retrieved in the decoctures for their anti-inflammatory and anti-arthritic potential. Apples are a significant source of phenolics, the buildup of which in the fruit's various portions is contingent upon a number of internal and external conditions. Distinctive phenolic and polyphenolic phytochemical ratios in apples are influenced by a variety of parameters, including fruit variety, fruit development phase, farming scheme, type of soil, stressors from the environment, meteorological variables, and other considerations including storage duration and after-harvest scenarios [28]. Each of these elements affects a fruit's total phenolic content, antioxidant value, and ability to fend against infections. As a result, it is difficult to draw conclusions from data from the initial study that was conducted on a range of cultivars across a variety of circumstances. The key parameters influencing the recovery of distinct classes of phytochemicals within the same temperature and time conditions are the type of solvent systems utilized and the physical/chemical properties of the specimens. The kind of menstruum utilized, and the process employed to create the concoction also have a considerable impact on the infusion of phytochemical content [29]. Using several menstrual systems with varying polarity, the Soxhlet and hot maceration techniques were used to obtain constituent metabolites, which are often polar in origin, from peels. The current study found that methanolic, a highly polar solvent, had the highest extraction rate and had a greater amount of phenolic makeup. This conclusion is consistent with Sultana *et al.*, 2009 [30], who discovered that methanolic extract had a greater extractive yield than other solvents. It is probably for this reason that the the highest number of bands indicating the amount of recoverable chemicals was found in the FTIR spectra of all the methanolic lysates. Flavonoids and polyphenols were found in all four apple species peels, but anthraquinones and emodins were not found in any of them, according to the present study. Except for Golden, all types have anthocyanins. This conclusion is consistent with Vasile *et al.*, 2021 [31], who discovered that anthocyanins are exclusively detected in red-skinned apples and are strongly linked to apple reddening. Other phytochemicals like saponins, glycosides, terpenoids, coumarins, anthocyanins, alkaloids, phytosterols, phlobatannins, quinones, phenols, chalcones, and tannins that were tested for were present in some extracts while absent in others. Some extracts showed their relatively abundant quantity while some extracts contain them moderately. The specimens were examined using infrared radiations with wavelengths of mid-range 4000 cm^{-1} to 400 cm^{-1} , with some radiation being absorbed and some being transmitted through. The sample's molecules transform the radiation they absorb into spinning and/or vibratory energy. The degree of functionality that exists in the sample was reflected in the absorbance severity. The resultant output at the detector appeared as a spectrum, denoting the sample's molecular signature. Each chemical arrangement or molecule generated a distinct spectral imprint. In almost peels of all apple varieties, our FTIR analysis revealed the existence of phenolics, carboxylic acids, poly-hydroxy compounds, aromatic rings, nitro compounds, alkenes, alcohols, ethers, esters, and hydrogen-bonded alcohols. The functional groups identified by FT-IR analysis in the malus peels correspond to previously published values in the scientific literature [32] as follows. The broad range frequency between 4000 and 3000 cm^{-1} includes the characteristic signature for different classes of functional groups. In this broad range, our results fall in different specific ranges like that of 3700 – 3584 cm^{-1} that portray O-H bond stretching of alcohols, with medium and sharp appearance. Absorptions between 3550 – 3200 cm^{-1} is the range for O-H bond stretching of

intermolecular bonded alcohols, with strong and broad appearance. It includes N-H stretching of primary amines (3500 cm^{-1}), the O-H bond vibration of carboxylic acids (3400 cm^{-1}), aliphatic primary amine N-H stretching (around 3300 cm^{-1}). Intermolecular bonded alcohols and amine salts usually occurs anywhere from around $3200 - 2800\text{ cm}^{-1}$. The characteristic broad range between $3000 - 2500\text{ cm}^{-1}$ portrays C-H stretching (sharp/medium) in various groups like alkyne, alkene, alkane, aldehyde, and S-H stretching (weak) in thiol. The broad range between $2400 - 2100\text{ cm}^{-1}$ portrays mainly the double and triple bonded groups. Absorptions around 2349 cm^{-1} end up causing the strong $\text{O}=\text{C}=\text{O}$ stretching in carbon compounds, between $2275 - 2250\text{ cm}^{-1}$, it gave signature for strong and board $\text{N}=\text{C}=\text{O}$ stretching in isocyanates, between $2260 - 2222\text{ cm}^{-1}$, $\text{C}\equiv\text{N}$ stretching in nitriles. Stretching of the C-C bond occurs in aromatic rings is as a result of absorption bands between 1600 and 1585 cm^{-1} and 1500 and 1400 cm^{-1} .

Peaks were seen in the spectra for the axial C-O bond bending in phenols ($1300 - 1000\text{ cm}^{-1}$), the axial C-O bond deformation in $-\text{COOH}$ ($1320 - 1210\text{ cm}^{-1}$), and the angular deformation in the plane of the aromatic rings' C-H bonds ($1300 - 1000\text{ cm}^{-1}$). A stretched N-H peak (3309.85 cm^{-1}) is found and is caused by molecules that include nitrogen, such as amines and amides. Alkynes have been confirmed as existing based on the emergence of a peak of absorption for C-C-H at $3224.98\text{--}2987.87\text{ cm}^{-1}$. In carbonyl compounds, the mid-intense signal at $1735.93\text{--}1555.86\text{ cm}^{-1}$ is caused by the $\text{C}=\text{O}$ bond. The presence of unsaturated alkenes is the reason for the stretched band at $1635.64 - 1550\text{ cm}^{-1}$. The findings are consistent with those of Mallampati et al., 2013 [33], who found well-neigh identical peaks in the red apple variety's FT-IR study. The body's reaction to the deterioration of tissue homeostasis is inflammation. Inflammation may be brought on by a virus, a wound, an autoimmune condition, or a toxic substance. Leukocyte motility and enhanced permeability of the vascular system are the first signs of the immediate inflammatory reaction, which tries to get rid of harmful organisms by releasing ROS, RNS, proteases and other immunomodulators by neutrophils, luring macrophages, and other WBCs at the point of inflammation and escalating the inflammatory response [34]. Traditional anti-inflammatory medications work well to reduce inflammation but using them can have negative side effects that range from moderate to substantial. The potential of biopharmaceuticals, biologics and organic medicines with animal or plant origins to treat inflammation has prompted researchers to thoroughly examine their anti-inflammatory properties. These properties might be examined *in vivo* in the context of various illnesses or characterized *in vitro*. In the current study, four such *in vitro* assays were used to analyze the malus peel bioactive compounds for their inflammation suppressive and anti-arthritis competence. Erythrocyte membrane stabilization assay via inhibition of hypotonicity-induced hemolysis was done to analyze the cell membrane stabilizing properties of bioactive-rich decoctures. The primary concept at work here is to stabilize the erythrocyte membrane (which is akin to the membrane of lysosomes) to prevent lysis triggered by hypotonicity. It implies that if the HRBC is stabilized, the ejection of degradative enzymes from lysosomes is stabilized as well. The Maharaji peel decocture (MPD) demonstrated the highest amount of HBRC membrane stabilization, followed by the delicious peel decocture (DPD). MPD was shown to be more effective at suppressing hypotonicity-induced hemolysis of HRBC (71.72% at a dosage of 1600 mcg/ml) when juxtaposed with typical NSAID showing 96.93% suppression at a comparable dose. The

remaining decoctures had low to modest efficacy at 1600 mg/ml as well. Our study is in harmony with the reports revealing that saponins and flavonoids have a dramatic stabilizing impact on lysosomal membranes. Saponins, flavonoids and tannins and have the potential to attach cations, stabilizing the membranes of erythrocytes and other cellular macromolecules [35, 36]. Additional investigations have shown that phenolics effectively scavenge free radicals by directly modifying the physical characteristics of membranes, in part by blocking the lipid oxidation cycles. At the lipid-water boundary of membranes, the more hydrophilic phenolics form hydrogen bonds with the polar head groups to interact. This kind of engagement may offer some amount of defence against internal and external irritants (like oxidants) for the bilayer, helping to maintain the makeup and smooth operation of biological membranes [37]. Arthritic responses have been linked to proteinases. Proteinases especially serine proteinases are known to be abundant in neutrophils, which contain a large number of them in their lysosomal granules. Leukocyte proteinase has been implicated in the onset of tissue destruction following inflammatory responses, and proteinase antagonists have been shown to give a considerable amount of security. Protease inhibitors have to be benign and able to block all of the main intestinal proteases, like chymotrypsin, pancreatic trypsin, and elastase, in order for them to be utilised in people. For the purpose of preventing protease-induced hypersensitivities, a number of potentially innocuous protease inhibitors, primarily of bacterial or vegetative origin, have been isolated from cabbage leaves, barley seeds, and *Streptomyces* [38]. Royal delicious peel decocture (RdPD) has a comparatively higher proteinase inhibitory action (29.35% suppression at 1.6mg/ml), which is followed by Maharaji peel decocture (MPD). The inhibiting effect was of the order RdPD > MPD > DPD > GPD. Our study is content with the investigation by Naz *et al.*, 2017 [39] wherein the researchers revealed that the Methanol extracts of *O. corniculata* (having abundant phenolics) demonstrate significant anti-proteinase activity. A significant correlation between coefficient values with the total phenolic titer and the proteinase suppressing capacity of the chosen plant polar preparations was discovered in this investigation. Leukotrienes, lipoxins, and neuroprotectins are just a few of the eicosanoids and docosanoids that are produced as a result of the breakdown of arachidonic and docosahexaenoic acid by a group of enzymes known as lipoxygenases (LOXs). These enzymes are either involved in oxidative stress and cell death pathways or in cell defence, relying on the redox condition of the cells and other ambient factors. Lipoxygenases are lipid peroxidising enzymes that may be targeted for pharmaceutical intervention since they are involved in the pathophysiology of inflammatory and hyperproliferative disorders [40]. The greatest inhibitor of lipoxygenase, in accordance with the study of Sroka *et al.*, 2017 [41] was kaempferol, which has one hydroxyl group located at the 4' site in the B ring. This reinforces our study wherein malus decoctures that are deemed to possess such bioactive compounds hinder the activity of the LOX enzyme. When contrasted to the positive control Nordihydroguaiaretic acid (NDGA), the Maharaji peel decocture (MPD) indicated a maximum LOX inhibitory action of 51.24% at 1600 µg/mL concentration, whereas GPD, RdPD, AND DPD exhibited 42.31%, 38.51%, and 31.25% suppression respectively at the identical dosage. Denaturation of proteins in tissues could be the source of auto-antigen formation in some arthritic disorders. As a result, tissue protein degradation may be considered a hallmark of proinflammatory and arthritic disorders. Compounds that inhibit protein denaturation might thus be candidates for anti-arthritic medication formulation. The action of nonsteroidal anti-inflammatory medications (NSAIDs) in swelling and

rheumatic illnesses is related to more than only the reduction of prostaglandin synthesis. It is claimed that these medicines may also preserve endogenous proteins from denaturation [42]. The *in vitro* assay was performed as a preliminary screen to check the presence of the anti-arthritic property of decoctures. All polar decoctures were competent to inhibit albumin denaturation in a dose-dependent pattern according to our study. Maharaji peel decocture revealed a comparatively significant inhibitory potential of 61.03% at the concentration of 1.6 mg/ml. The inhibitory activity was of the order MPD > RdPD > DPD > GPD. According to numerous studies, plant bioactive compounds, especially secondary metabolites, are well-documented agents that inhibit protein denaturation to a significant degree [43, 44]. Decocture of *Malus domestica* var Maharaji outperformed the other species' peel decoctures in the context of all the assays analyzed possibly by synergistic effects of tota bioactive compounds. So, with regard to oxidative stress-related, inflammatory, and arthritic illnesses, our present study assigns the greatest medicinal emphasis to the bioactive components extracted from peels of this variety. Thus, supplements like dried peel formulations from the cultivar's peels which are pharmacologically striking are highly recommended.

Conclusion

Amidst their function in plant defense, secondary metabolites also benefit human health. Utilizing cutting-edge dimensions of nanotechnology, secondary metabolites are now being used in the management of refractory diseases and play a pivotal part in avoiding the onset of chronic illnesses and the promotion of good health. Secondary phytochemical analysis of peel extracts of different malus cultivars (both red- and yellow-skinned) occurring in Kashmir was investigated in this study. The malus peel decoctures were revealed to be high in phenolic and polyphenolic compounds and have the ability to neutralize reactive oxygen species by acting as antioxidants as well as potent anti-inflammatory and anti-arthritic activity. According to the findings of the current study, there is a correlation between the chemical makeup of decoctures, particularly polar compounds that in plants are mostly phenolics, and their strong anti-inflammatory activities. According to the findings of the current investigation, it can be asserted that varietal variations may be responsible for the significant deviations in the phytochemical composition and immunomodulatory potential of malus peel bioactive decoctures. Anti-inflammatory and anti-arthritic activity were overall found to be higher in the peels of *Malus x domestica* var Maharaji. Eventually, it may be established that this variety has a striking pharmaceutical profile. This study offers fresh perspectives on how apple genetic diversity could be beneficial to produce specific kinds of benign bioactive molecules that may be incorporated in therapeutics to treat hypersensitivity, inflammatory, arthritic and oxidative stress-induced diseases.

Declarations

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Conflict of interest

During the research, there were no connections that could be viewed as having a potential conflict of interest in terms of business or finances.

Author Contribution

AAS prepared the draft manuscript, NP reviewed the draft and figures, RKB, AG, NR reviewed the manuscript, NR checked the similarity index and all authors final reviewed the whole manuscript.

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Figures

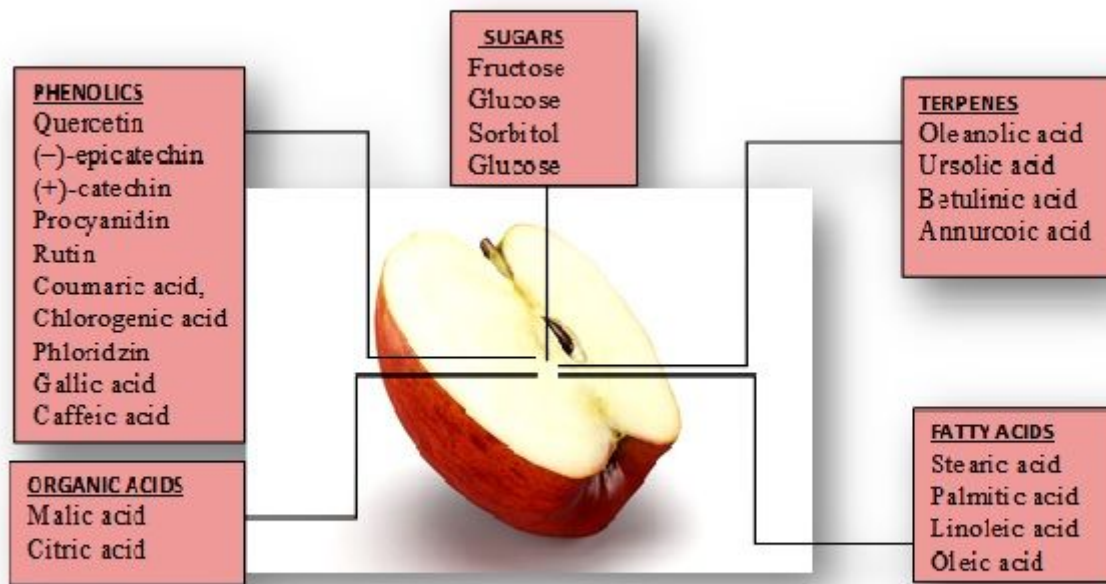


Figure 1

Some significant phenolics and other compounds connected to the physiological and nutraceutical benefits of apples



Figure 2

Apple varieties analysed in this study; 1 Maharaji; 2 Delicious; 3 Golden; 4 Royal Delicious

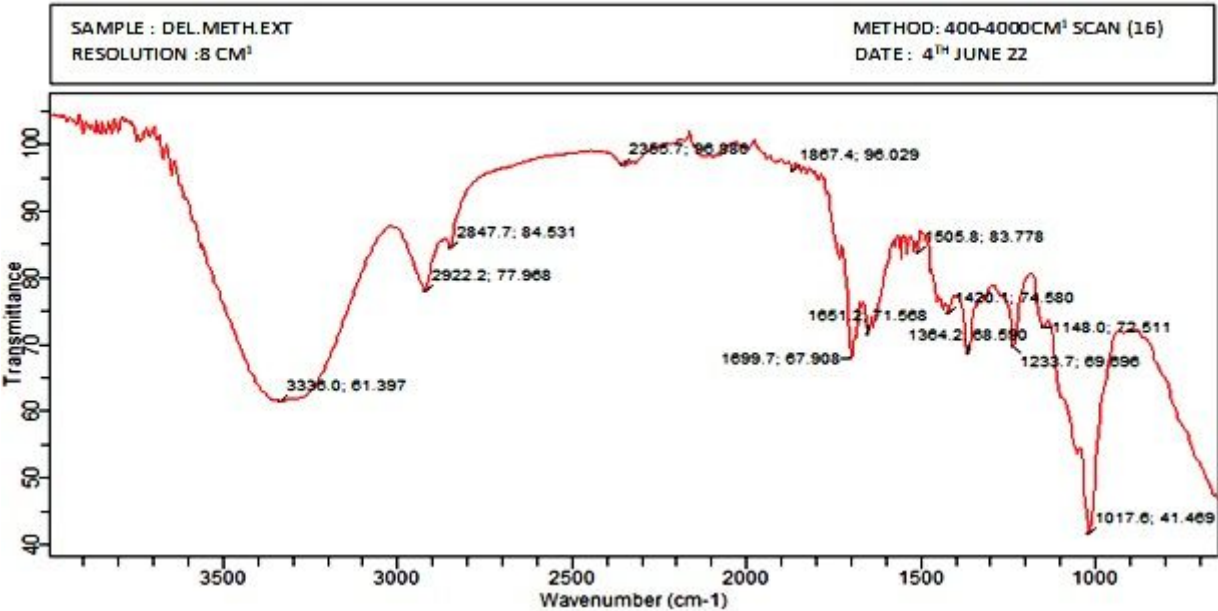


Figure 3

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FT-IR spectrum of Delicious peel methanol decoction

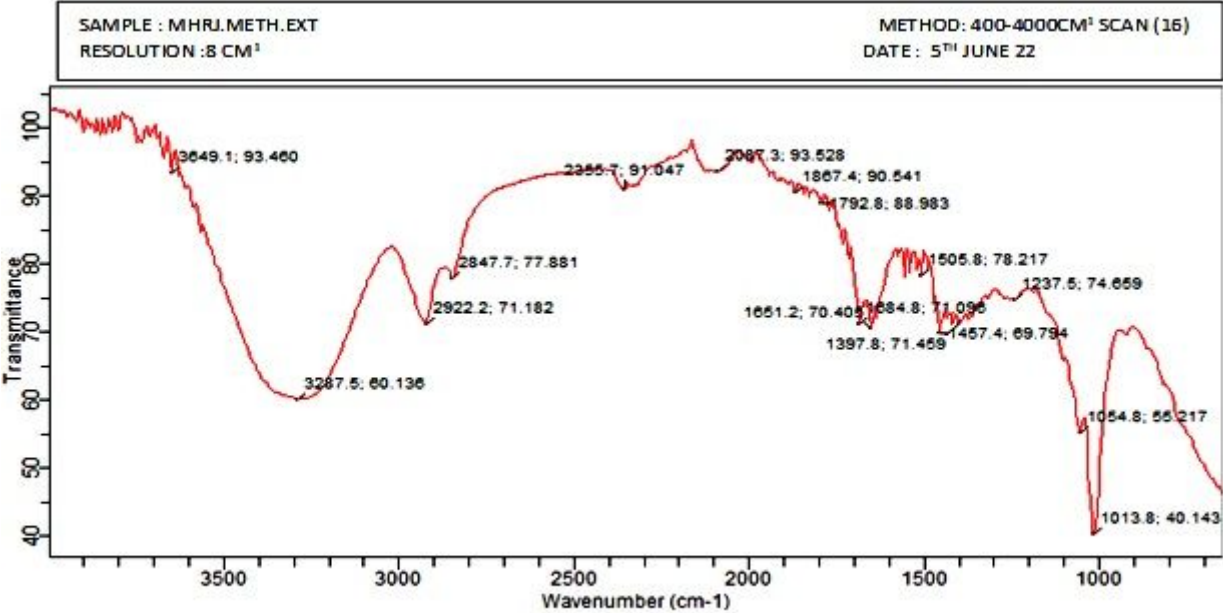


Figure 4

FT-IR spectrum of Maharaji peel methanol decoction

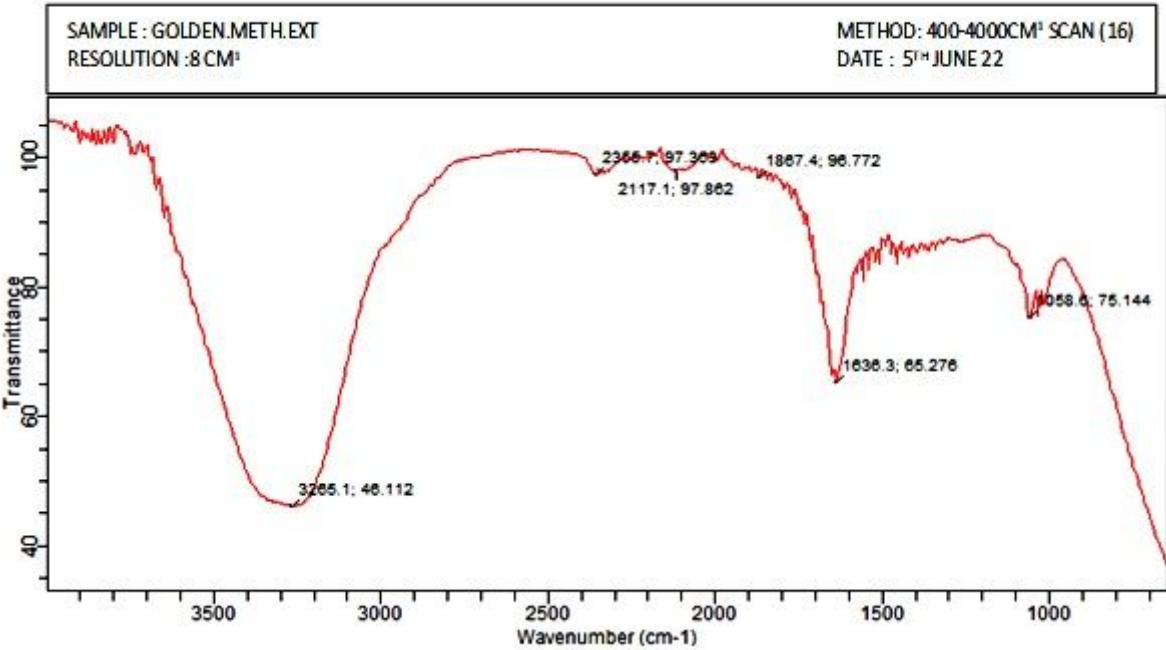


Figure 5

FT-IR spectrum of Golden peel methanol decoction

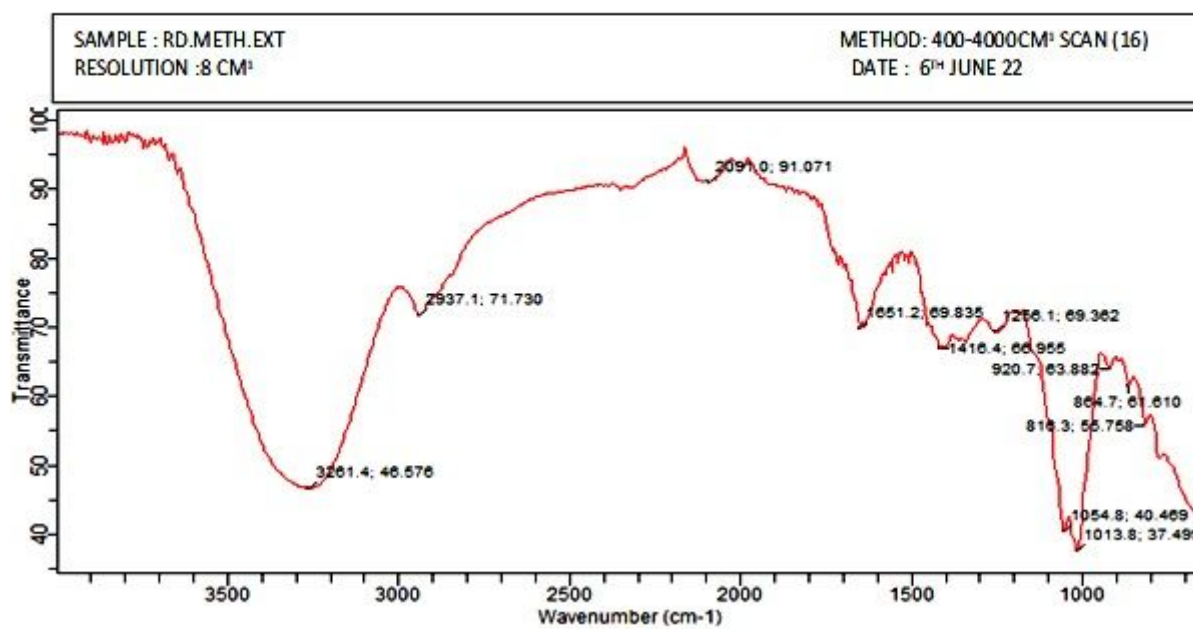


Figure 6

FT-IR spectrum of Royal delicious peel methanol decoction

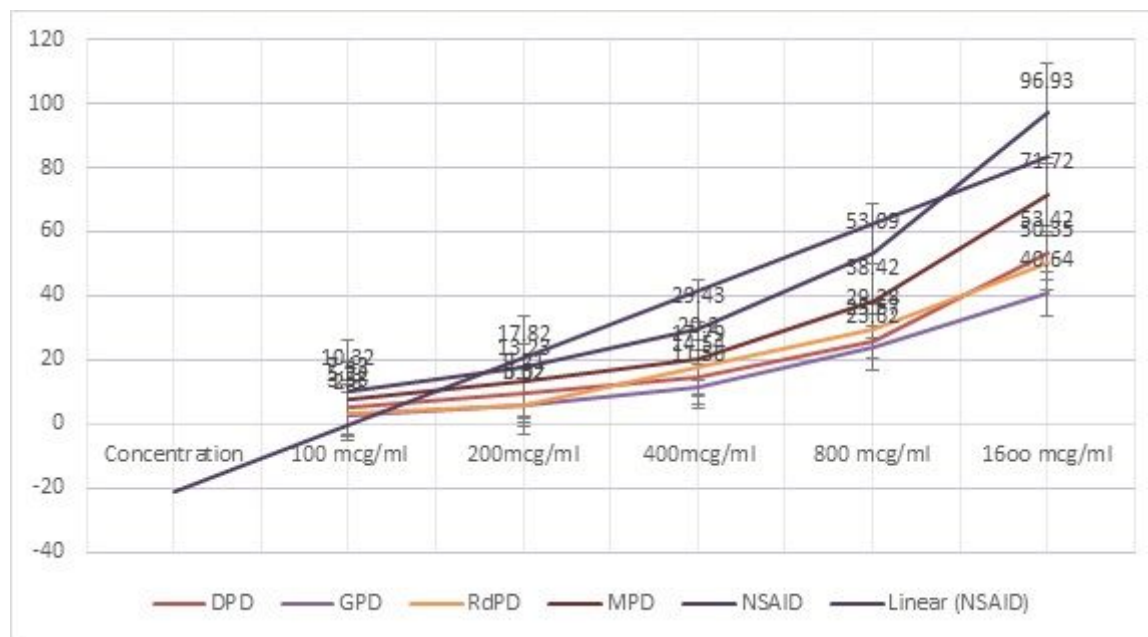


Figure 7

Graphical representation of Anti-hemolytic capacity of the fractions via HRBC membrane stabilization. All values are means of triplicate measurements of absorbances included in the % inhibition formula (n=3) , P<0.05 by ANOVA test.

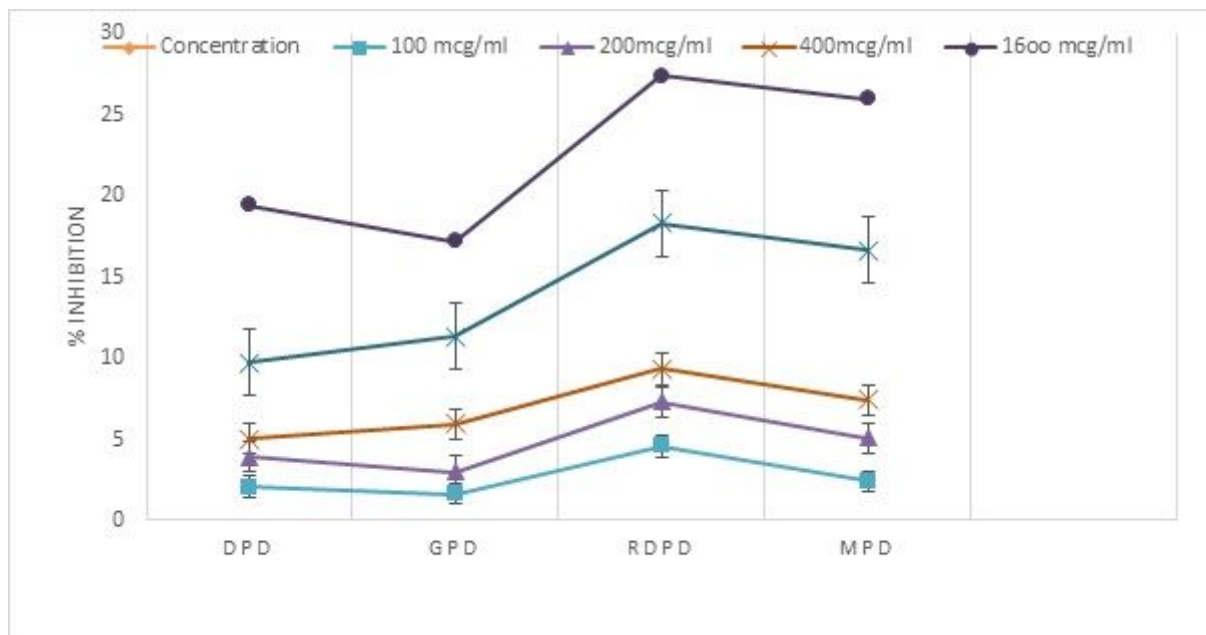


Figure 8

Graphical representation proteinase inhibitory activity. All values are means of triplicate measurements of absorbances included in the % inhibition formula (n=3), P<0.05 by ANOVA test



Figure 9

Inhibition of the proteinase activity via Caseinolytic plate assay



Figure 10

Graphical representation of anti-lipoxygenase (LOX) activity. All values are means of triplicate measurements of absorbances included in the % inhibition formula (n=3), P<0.05 by ANOVA test.

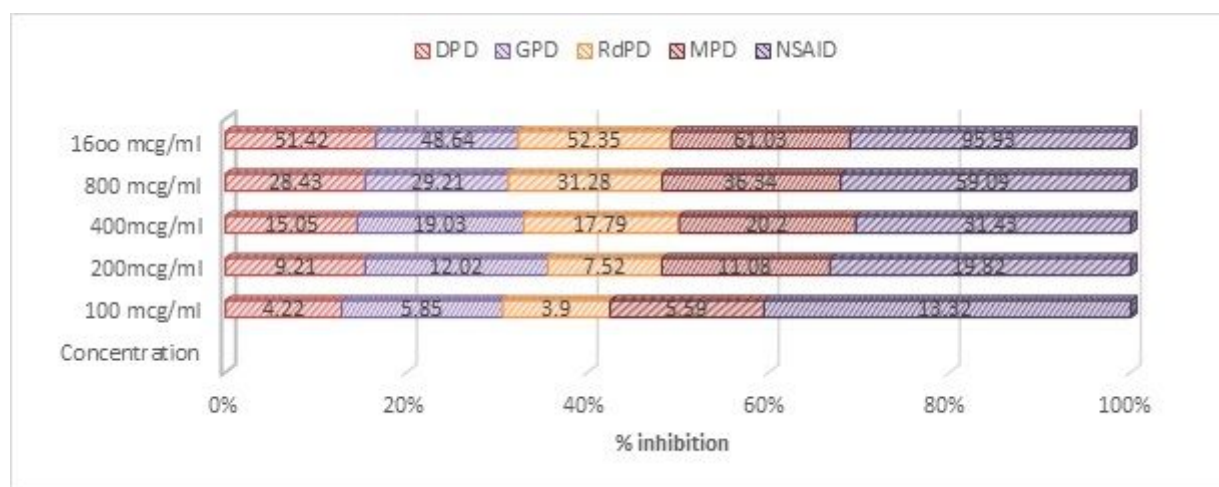


Figure 11

Graphical representation of anti-arthritic potential via inhibition of heat-induced albumin denaturation. All values are means of triplicate measurements of absorbances included in the % inhibition formula (n=3), P<0.05 by ANOVA test.

Supplementary Files

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