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Extraction of *Nerium indicum* Mill, *Artocarpus heterophyllus* Lam, *Murraya koenigii* Linn, *Punica granatum* Linn: Qualitative and quantitative assessment

Renuka Shukla and Varsha Kashaw

Abstract
The objective of present work was to evaluate the qualitative and quantitative assessment of *Nerium Indicum* Mill, *Artocarpus Heterophyllus* Lam, *Murraya Koenigii* Linn. and *Punica Granatum* Linn extract for the natural product present in them. These plants were having the rich source of alkaloid, glycoside, tannins, carbohydrates, Saponin, flavonoids, proteins and amino acids and were used as anti-diabetic, antiulcer, anti-inflammatory, antitumor, anti-malarial and having wound healing potential. Extract of all four plants has been separated by the process of Soxhlet extraction by using 70% ethanol. The extract of *Nerium Indicum mill, Artocarpus Heterophyllus Lam, Murraya Koenigii Linn, and Punica Granatum Linn* was evaluated for qualitative and quantitative measurement of alkaloid, glycoside, tannins, carbohydrates, saponins, flavonoids, proteins and amino acids content. Different solvents was used includes petroleum ether, ethyl acetate, ethyl alcohol, distilled water and 70% ethanol for the extraction all four plants. Preliminary phyto-chemical screening was performed for extracts of *Nerium Indicum Mill* (NIL), *Artocarpus Heterophyllus Lam (AHP), Murraya Koenigii Linn (MKL) and Punica Granatum* Linn. *(PGB).* Identification test on extracts was shown the presence of alkaloid, glycoside, tannins, carbohydrates, Saponin, flavonoids, proteins, volatile oil, steroids and amino acids content. All results denote that extracts of these four Indian plants having a rich source of phyto-constituents of interest.

Keywords: *Nerium indicum* mill, *Artocarpus Heterophyllus* Lam, *Murraya Koenigii* Linn *and Punica Granatum* Linn, phytochemical screening

Introduction
Extraction processes (more precisely: solid-liquid extraction process) are used to separate soluble components from a solid sample. Examples: In Determine the fat content in foods, determine an impurity (e.g. PCB, abandoned armaments) in soil samples, examining the components of natural substances. Even brewing coffee is an extraction process. However, in the laboratory the focus is on completely dissolving the examined components from the sample under defined conditions and in a not unnecessarily diluted form. In the laboratory, solvents such as ligroin or hexane are used as extraction agents. The objective of all extraction processes is to dissolve as much of the soluble components as possible with a specific amount of solvent. This is achieved by constantly vaporising the solvent and allowing it to drip into the sample from a reflux condenser. The extracted component accumulates in the distillation flask. Typically, a Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a significant solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance [1].

***Nerium indicum* Mill:** *Nerium indicum mill*, also known as Kaner (Figure 1). It is evergreen shrub that grows up to 5m in height [2]. The white flower variety plant *Nerium indicum mill* is exclusively native to India, Bangladesh, Nepal, Myanmar, and China. It is most commonly known as oleander, from its superficial resemblance to the unrelated olive Olea [3].
Leaves and flowers are used to treat malaria and as traditional medicine it induces the termination of embryo. The root powder is an external remedy for hemorrhoids and ulcers around genitals. It is also used as antimalarial, anti-diuretic, emetic, expectorant, cardio tonic, anticancer and applied in the treatment of cardiac asthma.

***Artocarpus heterophyllus* Lam:** *Artocarpus heterophyllus* Lam (Figure 2) is belong to Moraceae family. It is also known as jackfruit (Eng.), Kathal (Hindi), Kanthal (Beng.). It is native to Western Ghats of India, Malaysia, central and eastern Africa [4].

Fig 1: Leaves of *Nerium Indicum* Mill. (Kaner)

Fig 2: *Fruit of Artocarpus heterophyllus Lam* (Jackfruit, Kathal**)**

Artocarpus heterophyllus Lam contains various chemical constituents such as flavones, colouring matters, morin, dihydromorin, cynomacurin, artocarpin, isoartocarpin, cyloartocarpin, artocarpesin, oxydihydro artocarpesin, artocarpetin, norartocarpetin, cycloartinone and artocarpanone [5]. *Artocarpus heterophyllus Lam has utilized for* fever, boils, wounds and skin diseases, astringent, and carminative, laxative, aphrodisiac, brain tonic, diuretic. The wood is nervine, anti-diabetic, antibacterial, sedative and useful in convulsions [6].

Punica granatum Linn

Punica granatum Linn (Figure 3) is a deep-rooted but slow-growing, spiny, deciduous shrub or small multipurpose tree, it is particularly valued for its edible fruit, but also has medicinal properties, is a good source of tannins and has many agro-forestry applications. Fruit–raw, Juicy and refreshing with a sub-acid flavor. The fruit is 5-13 cm in diameter; it has a hard, tough case that contains lots of seeds, each surrounded by a delicious juicy red flesh [7]. The fruit juice can be used in soups, sauces, jellies, ice cream, cakes etc. The fruit contains about 1.5% protein, 1.6% fat, 16.8% carbohydrate, 0.6% ash [8].

Fig 3: Fruit of *Punica Granatum Linn* (Anardana)

The whole plant, but in particular the bark, is having antibacterial, antifungal [20], antiviral, anticancer [9] and astringent properties. The flowers are used in the treatment of dysentery, stomach ache and cough. The juice of the flowers is used to treat nose bleeds. It is also used as anti-diabetic [10] and having antioxidant, anti-inflammatory [11], and anti-proliferation properties. A decoction of the seed is used to treat syphilis. The fruit is a mild astringent. Juice of the fruit is used to treat jaundice and diarrhea. It is a specific remedy for tapeworm infestation.

***Murraya koenigii* Linn.**

Murraya koenigii Linn, called curry leaf (Figure 4), is a small, tropical to sub-tropical tree or shrub that typically grows to 6-15’ tall and is noted for its pungent, aromatic. Curry leaves are an important flavoring used in Indian/Asian cuisine. This tree is native to moist forests in India and Sri Lanka. Fragrant white flowers (each to 5/16" across) in many flowered panicles (*Terminal cymes*) bloom irregularly throughout the year. Curry leaves are often added to vegetable dishes. They add subtle flavors to many other dishes, including meat, seafood, chutneys, coconut sauces, relishes, marinades and omelets [12].

Fig 4: Leaf of *Murraya koenigii* Linn. (Curry leaf)

The oil obtained from the leaves used as antioxidant, anthelmintic and antibacterial properties [13]. It has been shown that the leaves increase digestive secretions; relieve nausea, indigestion and vomiting. The leaves can be used internally in treating constipation, colic, antimicrobial and diarrhea. The leaves are used in the treatment of diarrhea and dysentery. The leaves can be applied externally as a poultice to treat burns and wounds [14].

The objective of the present paper was to evaluate the active constituent and secondary metabolities present in *Nerium Indicum Mill*, *Artocarpus Heterophyllus Lam*, *Murraya Koenigii Linn and Punica Granatum Linn* extract. Preliminary test has been performed for the measurement of the constituents present in all four Indian plants. Test for alkaloids, glycoside, tannins, carbohydrates, saponins, flavonoids, proteins and amino acids were performed. Total flavanoid content and total phenolic content present in the plants also evaluated.

Material and Method

Drugs are collected from wild or cultivated plants. The season in which the drug is collected plays an important role in determining the quality of drug. Organoleptic characters,

morphological characters and microscopically examination would help in identifying crude drug. Generally, three methods are employed in the extraction of plant materials as (1) Maceration (2) Percolation (3) Soxhlet extraction. Maceration and percolation may be employed in extraction of thermo labile constituents. Soxhlet extraction is rapid and continuous and may be employed in extraction of sparingly soluble constituents due to repeated extraction, which cannot be done by either maceration or percolation methods. Soxhlet extraction process was use for present study [15].

Plant material collection and authentication

The leaves of *Nerium indicum* Linn were collected at in the month of June, 2016 from local area of Shubham Nursery, Bhopal, and Madhya Pradesh. Pulp of *Atrocarpus Heterophyllus* Linn and Whole plant of *Murraya Koenigii* Linn procured from local market of Barkheda and bark of *Punica Granatum* Linn was procured from vidisha district, Madhya Pradesh. The specimens were submitted and identified as *leaves* of *Nerium indicum Linn*, pulp of *Atrocarpus heterophyllus Lam*, bark of *Punica granatum Linn*, whole plant of *Murraya koenigii Linn* authenticated by Dr. Zia ul Hassan, Department of Botany, Saifia Science College, Bhopal, Madhya Pradesh. The appession no. for the specimen is 498/BS/saifia/NI_MK_PG_AH/04/16/07 has been preserved for future identification. The samples were shade dried so as to protect its chemical constituents not to get degrade at high temp.

Physicochemical Characterization

These characterization parameters give the idea of the physical characteristics and the chemical correlation of constituents with it, present in the herbal drugs. They involve the determination of ash values, foreign matter, extractable matter and volatile oil content of the preparations or individual drugs [16].

Physical Evaluation

Determination of Foreign Matter

Hundred (100) grams of the sample was weighed and spread on a white tile uniformly without overlapping. Then the sample was inspected by means of 5x lens and the foreign organic matter was separated. After complete separation the matter was weighed and percentage w/w was determined

Determination of Solvent Extractive Values

Solvent Extractive Values

In this water soluble and alcohol soluble extractive value has been identified by followed procedure.

Determination of Water Soluble Extractive Value

Five grams of powdered drug was macerated with 100 ml of water closed flask for 24hr and was occasionally shaked with 6h time period and was allowed to stand for 18hr. After filtration the 25 ml of the filterate evaporated to dryness in a tared flat bottomed shallow dish. Dry at 105ºC and weighed. Percentage of water soluble extractive value was calculated with reference to the air dried drug.

Determination of Alcohol Soluble Extractive Value

Five grams of powdered drug was macerated with 100ml of ethanol closed flask for 24hr and was occasionally shaking for 6h time period and was allowed to stand for 18hr. After filtration the 25ml of the filtrate evaporated to dryness in a tared flat bottomed shallow dish. Dry at 105ºC and weighed. Percentage of ethanol soluble extractive value was calculated with reference to the air dried drug.

Determination of Ash Value

Determination of Total Ash

Total ash was determined by weighing 2-3gm of the air dried crude drug in the tared platinum or silica dish and incinerated at a temperature not exceeding 450ºC until free from carbon and then was cooled and weighed.

Determination of Acid Insoluble Ash

Ash insoluble in HCI is the residue obtained after extracting the sulfated or total ash with HCI and calculated with reference to 100gm of drug. The ash obtained from the previous process was boiled with 25ml of 2M HCI for 5min. and the insoluble matter was collected on ash-less filter paper and was washed with hot water, ignited, cooled in a dessicator and weighed. Percentage of acid insoluble ash was calculated with reference to the air dried drug.

Determination of Water Soluble Ash

The ash was boiled with 25ml of water for 5 min. and the insoluble matter was collected on ash-less filter paper and was washed with hot water, ignited for 15min. at a temperature not exceeding 50ºC. The weight of the insoluble matter was subtracted from the weight of the ash and this represents the water soluble ash. Percentage of water soluble ash was calculated with reference to the air dried drug.

Extraction

The leaves of NI, pulp of AH, bark of PG and leaves of MK were separated from the fresh and dried on filter paper sheets under shade at room temperature until with changing of color of filter papers. The shade-dried, coarsely powdered materials (500 g) were defatted by petroleum ether (45^{0}C). The defatted marc was then subjected to soxhlet extraction with 70% ethanol to obtain hydroalcoholic extract. The hydro alcoholic extracts were evaporated under reduced pressure at low temperature (30ºC) to dryness to yield different extracts, stored in an airtight container in refrigerator for further experimental studies.

Preliminary phytochemical Screening

Qualitative Test Analysis

In order to detect the various constituents present in the different extracts, those were subjected to the tests [17]. Phytochemical screening was performed for the detection of Alkaloids, Glycosides, Carbohydrates, Tannins, Resins, Flavanoids, Steroids, Proteins and Amino acids. Those were subjected to the tests as per methods [18-20].

Determination of Carbohydrates

Molish test: 2-3 ml. extract +few drops of α- naphthol solution (20% in ethyl alcohol) + 1 ml. conc. H_2SO_4 added along the side of the test tubes. Violet ring was formed at the junction of two liquids.

Fehling's Test: Extract heated with dil. HCl +NaOH + Fehling's solution A & B: Brick red precipitate was formed.

Benedict's Test: Extract +equal volume of Benedict's reagent. Heat for 5 min: Solution appears Green, Yellow or Red.

Determination of Proteins and Amino-acids

Biuret Test: 3 ml of extract + 4% NaOH + 2-3 drops of 1% copper sulphate solution: presence of red/violet coloration

Million's test: 3 ml of extract + millon's reagent: Presence of red coloration
Xanthoprotic test: Extract + conc. nitric acid: Yellowish orange color
Ninhydrin Test: Extract + ninhydrin reagent in boiling water bath for 10 min: Violet color appeared.

Determination of Steroids
Libermann- Burchard Test: 2 ml. extract + Chloroform + 1-2ml. acetic acid + 2 drops H_2SO_4 from the side of the test tube: First red, then blue and finally green color appeared.
Salkowski Reaction: 2 ml. of extract +2 ml. chloroform + 2 ml. conc. H2SO4. Shake well. Chloroform layer appeared red color and acid layer shows greenish fluorescence.

Determination of Glycosides
Raymond's test: Test solution + 1 ml of 50% ethanol + 0.1% solution of dinitrobenzene in ethanol + 2-3 drops of 20% sodium hydroxide solution. Appearance of violet color confirm the presence of glycosides.
Killer Killani test: 2 ml of extract + glacial acetic acid + one drop of 5% FeCl3 + conc. H_2SO_4. Reddish brown color appeared at the junction of the two liquid layers and upper layer appeared bluish green.
Foam test: Test solution containing extract is vigorously shaking with water. Formation of foam indicates the presence of glycosides.

Determination of Flavanoids and phenolic compounds
Lead acetate test: Filter paper strip was dipped in the alcoholic solution of extract. Addition of Ammonia solution. Color changed from white to orange.
Shinoda Test: Extract + 5 ml. 95% alcohol + few drops of conc. HCl + 0.5 g magnesium turning. Pink color observed

Determination of Alkaloids
Mayer's Test: Test solution + Mayer reagent (Potassium mercuric iodide solution). White or yellow precipitate was obtained.
Dragendorff's Test: Test solution + Dragendorff's reagent (Potassium iodide + bismuth nitrate). Showed orange red precipitate indicates the presence of alkaloids.
Wagner's Test: Test solution + Wagner's reagent (iodine solution): Brown or reddish brown precipitate.
Hager's Test: Test solution + Hager's reagent (saturated solution of picric acid). Gives characteristic-crystalline ppt.

Determination of Volatile oil
Determination of Tannins
Vanillin- HCl Test: Extract+ vanillin-HCl reagent (1 g vanillin + 10 ml. alcohol + 10 ml. conc. HCl): Formation of pink or red color.
Gelatin Test: Extract solution + aqueous solution of gelatin: White buff color precipitate was formed.

Quantitative Analysis
Determination of total phenolic content (TPC)
The total phenolic content of all the extracts was determined by using Folin- Ciocalteu method. A standard gallic acid curve was constructed by preparing the dilutions of (0.8, 1.6, 3.12, 6.25, 12.5 and 25 µg/ml) in methanol from standard solution of gallic acid. 100 µl of each of these dilutions were mixed with 500µl of water and then with 100µl of Folin-Ciocalteu reagent and allowed to stand for 6 min. Then 1ml of 7% sodium carbonate and 500µl of distilled water were added to the reaction mixture. The absorbance was recorded after 90 min at 760 nm UV/visible spectrophotometrically. The same procedure was repeated with extracts. The total phenolic content of the extracts was calculated as gallic acid equivalents (mgGAE/g). All the experiments were performed in duplicate. All experimental measurements were carried out in duplicate and are expressed as average of two analyses. The magnitude of correlation between variables was done using a Microsoft excel.

Determination of Total flavonoids content (TFC)
Quercetin was used as standard and flavonoid content was determined as quercetin equivalent. A calibration curve for quercetin was drawn for this purpose. From the standard quercetin solution the dilutions of (10, 20, 30, 40 and 50 µg/ml) concentrations were prepared in methanol. 100 µl of each of the quercetin dilution was mixed with 500 µl of distilled water and then with 100 µl of 5% Sodium nitrate and allowed to stand for 6 minutes. Then 150 µl of 10% Aluminum chloride solution was added and allowed to stand for 5 minutes after which 200 µl solution of 1M Sodium hydroxide was added sequentially. The absorbance of this reaction mixture was recorded at 510 nm on UV/ visible spectrophotometer. The same procedure was repeated with the extracts and total flavonoid content was calculated as quercetin equivalents (mgQE/g). All the procedures were performed in duplicate. All experimental measurements were carried out in duplicate and are expressed as average of two analyses.

Result and Discussion
Physicochemical characterization
Temperature and types of solvent can give impact on the quantity of extractable matter of a plant. The extractive capacity (measured as extractive value) increases with the amount of extractive matter produced under a particular condition. The herbal monograph specified that the limits for water soluble extractive values for NIL, AHP, PGB and MKL are not less than 20%, 5%, 15% and 5%, respectively for water and 10, 5%, 10% and 5%alcohol soluble extractive values. All specimens were found similar than the standard specifications. From this study, higher temperature and using water as solvent exhibited a better extractive capacity than in room temperature and alcohol based solvent. The water soluble extractive value indicated the presence of sugar, acids and inorganic compounds; the water soluble extractive value found to be 22.15±0.19, 6.98±0.42 and 18.14±0.03% and 8.79±0.01%w/w for NIL, AHP, PGB and MKL respectively. The alcohol soluble extractive values indicated the presence of polar constituents like phenols, alkaloids, steroids, glycosides, flavonoids. The alcohol soluble extractive value was found to be 12.78±0.23, 9.43±0.14, 12.70±0.05 and 7.24 ±0.02%w/w for NIL, AHP, PGB and MKL respectively which signify the nature of the phyto constituents present in plant.

Table 1: Physical characteristics

S. No	Name of the drug	Values	Foreign organic matter	Total Ash value	Acid insoluble ash value	Water soluble ash value
1.	Nerium indicum Linn. leaves	Theoretical	<1%	<12%	<7%	<2%
		Observed	0.02±0.007%	10.14±0.027%	6.28±0.037%	1.17±0.005%
2.	Artrocarpus pulp	Theoretical	<1%	<0.5%	<0.2%	-
		Observed	0.08±0.002%	0.42±0.031%	0.01±0.007%	0.04±0.001%
3.	*Punica granatum* Linn. Bark	Theoretical	<1%	<3%	<0.5%	<15
		Observed	0.92±0.012%	2.32±0.047%	0.22±0.064%	10.27±0.054%
4.	Murraya koenigii whole plant	Theoretical	<2%	<12%	<7%	<2.5
		Observed	1.45±0.030%	11.22±0.015%	5.24±0.042%	1.86±0.034%

Evaluation of crude drug ensures the identity of drug and determines the quality and purity of drugs. The main reason behind the need for the evaluation of crude drug is biochemical variation in the drug, effect of treatment, storage of drug, adulteration and substitutions [21-22].

The results of the physicochemical parameters of fruit powder lie within the limit and shown in Table 1. The results of foreign organic matter denote presence of any organism, part or product of an organism, other than that named in the specification and description of the herbal material concerned[23] which was found to be 0.02±0.007, 0.08±0.002, 0.92±0.012 and 1.45±0.03% w/w for NIL, AHP, PGB and MKL respectively. A high ash value is indicative of contamination, substitution, adulteration, or carelessness in preparing the drug or drug combinations for marketing. All the individual drugs were found to have total ash values in the range from 2.32 to 11.22% w/w. The NIL, AHP, PGB and MKL respectively have 10.14±0.027, 0.42±0.031, 2.32±0.047 and 11.22±0.015% w/w total ash values.

These values were found to be reasonably low indicating low contamination. Total ash values of the of the individual drugs match up the standard values. Water-soluble ash is the part of the total ash content, which is soluble in water. It is a good indicator of either previous extraction of water-soluble salts in the drug or incorrect preparation. Thus, it is the difference in weight between the total ash and the residue obtained after treatment of total ash with water. The water-soluble ash values of the individual drugs were in the range of 0.04 to 10.27% w/w. This shows a normal quality of the drugs. Water-soluble ash values of the NIL, AHP, PGB and MKL respectivelywere found to be in the range of 0.04 to 10.279% w/w. These values match with the standard average water-soluble extractive values of individual drugs also.

Limit of total ash and acid insoluble ash value for a crude drug, with respect to any species, should be a maximum of 14% and 2%, respectively [24]. However, the various Herbal Monographs stated the different limits for individual drugs as per their region of production. High ash value is an indication that specimens were contaminated by extraneous matter in a great extent. Acid insoluble ash value is to further confirm that the high total ash value is derived from physiological or non-physiological ash. A plant of good quality should not contain high non-physiological ash, which is the residue of the extraneous matter such as sand and soil. Results from this study were in fact comparable to the standard ash values. The acid-insoluble ash values of the individual drugs ranges from 0.01 to 6.28, and is below 7.0% for all the drugs (Table 1). These values match with the average acid-insoluble extractive values of individual drugs. This also signifies that the marketed products also match as for as the acid-insoluble extractive values are concerned. The ash value determinations are important parameter to standardize the herbal drugs.

Extraction

The extraction was done by continuous hot percolation method i.e Soxhlet apparatus. The dried and pulverized drug was defatted with petroleum ether. The obtained marc was then extracted with 70% Hydroalcoholic solution. The drying of extract containing solvent (70% hydroalcholic solution) was done by rota vacuum evaporator. The percentage yield of the hydroalcoholic extract from different drugs was tabulated in Table 2.

Table 2: Percentage Yield of Hydroalcoholic Extracts from Different Drugs

S. No.	Parts	Extract colour	Yield (in g)	% Yield w/w
1.	NIL	Greenish brown	20.43	26.28
2.	AHP	Yellowish	8.24	10.05
3.	PGB	Dark brown	8.97	11.34
4.	MKL	Greenish brown	20.76	26.11

Initially 80 g of crude drug was taken. Where NIL- Hydroalcoholic Extract of Neium indicum leaves, AHP-Hydroalcoholic Extract of Artrocarpus heterophyllus pulp, PGB- Hydroalcoholic Extract of Punica granatum Linn. Barkand MKL- Hydroalcoholic Extract of Murraya Koenigi Linn. whole plant

Phytochemical Screening

Preliminary Phytochemical screening was performed for each hydroalcoholic extract of *Nerium indicum* Linn. Leaves (NIL), *Punica granatum* Linn bark (PGB), *Murraya Koenigi Linn* whole plant (MKL) and *Atrocarpus heterophyllus* Linn pulp (AHP). It was noted that NIL contains flavanoids, phenolic compounds, carbohydrates, proteins, glycosides, alkaloids and glycosides. The PGB contains flavanoids, phenolic compounds, carbohydrates, proteins, aminoacids, glycosides, alkaloid, and tannins. The MKL showed the presence of flavanoids, phenolic compounds, carbohydrates, proteins, amino acid, glycosides, and alkaloids. The AHP has shown the presence of phenolic compounds, flavanoids, carbohydrates, proteins, amino acids, terpenoids, glycosides, alkaloids and tannins. Qualitative analysis of hydro-alcoholic extract of *Nerium indicum* Linn. Leaves, *Punica granatum* Linn bark, *Murraya Koenigi* Linn *whole plant* and *Artocarpus pulp* was tabulated in Table 3.

Qualitative Analysis

Dried extracts were taken for the chemical test for detection of the phyto constituents like alkaloids, flavonoid, tannins, sterols, phenolic compounds, terpenoids, carbohydrates etc. In order to detect the various constituents, present in the different extracts of NIL, PGB, MKL, and AHP. Those were subjected to the tests as per identification and chemical test.

Table 3: Qualitative analysis of hydro-alcoholic extract of *Nerium indicum* Linn. Leaves, *Punica granatum* Linn bark, *Murraya Koenigi* Linn *whole plant* and *Artocarpus pulp*

S. No.	Chemical Test	NIL	PGB	MKL	AHP
1.	**Carbohydrate**				
a.	Molish test	+	+	+	+
b.	Fehling test	-	+	+	+
c.	Benedicts test	-	+	+	-
2.	**Protein**				
a.	Biuret test	+	+	+	-
b.	Millon's test	-	+	-	+
c.	Xanthoprotic test	+	+	+	+
3.	**Amino Acid**				
a.	Nihydrin test	-	+	+	+
4.	**Fats And Oils**				
a.	Filter paper test	-	-	-	-
5.	**Steroid (Tri Terpenoid)**				
a.	Salkowski reaction	-	-	+	+
b.	Libermann-Burchard reaction	-	-	+	+
6.	**Glycosides**				
a.	Raymonds test	+	+	+	+
b.	Keller-Killani test	+	+	+	+
c.	Foam test	+	+	+	+
7	**Flavanoids and Phenolic Compounds**				
a.	Shinoda test	+	+	+	+
b.	Lead acetate test	+	+	+	+
c.	5% $FeCl_3$ solution	+	+	+	+
7.	**Alkaloids**				
a.	Dragendorff's test	+	+	+	+
b.	Mayer's test	+	+	+	+
c.	Wagner's test	+	+	+	+
d.	Hager's test	+	+	+	+
8.	**Phenolic Compounds**				
a.	5% $FeCl_3$ solution	+	+	+	+
b.	Lead acetate test	+	+	+	+
9.	**Volatile Oil**				
a.	Sudan red	-	-	-	-
10.	**Tannins**				
a.	Vanilline HCL	-	+	-	+
b.	Gelatin	-	+	-	+

(-ve = absent& +ve =Present)

Quantitative Analysis

Determination of Total Phenolic Content

The concentration of total phenols in various plant extracts was determined using spectrophotometric method with Folin-Ciocalteu reagent. The content of total phenols was expressed in terms of gallic acid equivalent (the standard curve equation: y = 0.006x-0.041, r2 = 0.9991), mgGAE/g of extract (Table 4, 5 and 6). The concentration of total phenols in different plant extracts is in following ascending order: NIL< MKL< PGB< AHP.

Table 4: Standard Curve of Gallic Acid

Concentration (µg/ml)	Absorbance (Mean)
0	0
0.8	0.0453±0.024
1.6	0.0848±0.051
3.12	0.1575±0.038
6.25	0.3793±0.044
12.5	0.6041±0.058
25	1.1942±0.061

The hydroalcoholic extract of *Punica granatum* Linn.bark (PGB) has shown highest amount of total phenolic content (64.167 mgGAE/g) as compared to others. The bark of PG have more total phenolic content about 62% as compared to leaves of different plant extracts (e.g. NIL). Different concentrations (200, 500 and 1000 µg/ml) has been taken to calculate the total phenolic content in different extracts obtained from different plants. Concentration dependent total phenolic content variation has validated the analytical methodology e.g. spectroscopy. The total phenolic content was increased with the increase in concentration of extracts.

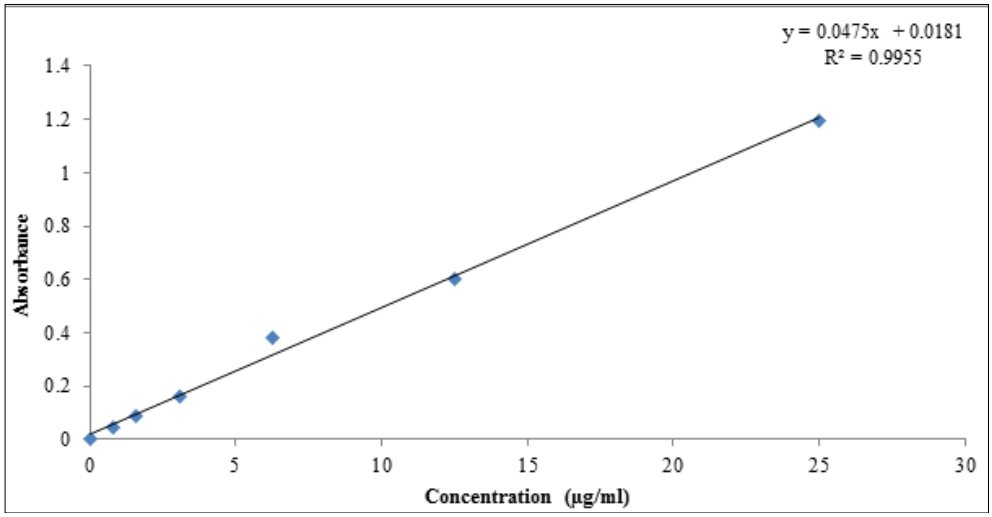

Fig 5: Standard Curve of Gallic Acid

Table 5: Total Phenolic Content in different Hydro-alcoholic extracts

S. No.		1	2	3	4
Concentration	**Extracts**	**NIL**	**MKL**	**PGB**	**AHP**
200 (µg/ml)	Absorbance	0.069	0.071	0.148	0.174
		0.073	0.075	0.152	0.096
	Conc. (µg/ml)	5	5.333	18.167	22.5
		5.667	6	18.833	9.5
	Avg. Conc.	5.333	5.667	18.5	16
500 (µg/ml)	Absorbance	0.083	0.099	0.205	0.194
		0.086	0.106	0.197	0.199
	Conc. (µg/ml)	7.333	10	27.667	25.833
		7.833	11.167	26.333	26.667
	Avg. Conc.	7.583	10.583	27	26.25
1000 (µg/ml)	Absorbance	0.185	0.149	0.318	0.217
		0.183	0.145	0.312	0.211
	Conc. (µg/ml)	24.3333	18.3333	46.5	29.6667
		24	17.6667	45.5	28.6667
	Avg. Conc.	24.167	18	46	29.167

Table 6: Gallic acid equivalent in mg/g extract

	200 (µg/ml)	Qty.1 Mg GAE/g	500 (µg/ml)	Qty.2 Mg GAE/g	1000 (µg/ml)	Qty.3 Mg GAE/g	Avg. Mg GAE/g
NIL	5.333	26.667	7.583	15.167	24.167	24.167	22.00
MKL	5.667	28.333	10.583	21.167	18.00	18.00	22.50
PGB	18.50	92.50	27.00	54.00	46.00	46.00	64.167
AHP	16.00	80.00	26.25	52.50	29.167	29.167	53.889

The total phenolic content was significantly more in the hydroalcoholic extract of PGB and AHP as compared to NIL and MKL e.g. [NIL+MKL(44.5mg GAE/g) < GB+AHP(118.06 mg GAE/g)] 73.56 mg GAE/g**.** The extract from pulp of *Atrocarpus heterophyllus* Linn has also more phenolic content about more than 2.4 times than the leaves of *Nerium indicum* Linn. And whole plant extract of *Murraya koenigii* Linn. It is evident from the data that the bark and fruit whole plant has more phenolic content than the leaves in the Hydroalcoholic extracts.

Determination of Total Flavanoid Content

The concentration of flavonoids in various plant extracts was determined using UV/ visible spectrophotometric method with aluminum chloride. The content of flavonoids was expressed in terms of quercetin equivalent (the standard curve equation: y = 0.0011x-0.0011, r2 = 0.996), mg of QE/g of extract (Table 7, 8 and 9). The concentration of flavonoids in different plant extracts is in following ascending order: NIL< MKL< AHP< PGB

Table 7: Standard Curve of Quercetin

Concentration (µg/ml)	Absorbance (Mean)
0	0
10	0.0085±0.037
20	0.0182±0.082
30	0.0257±0.077
40	0.0345±0.046
50	0.0443±0.087

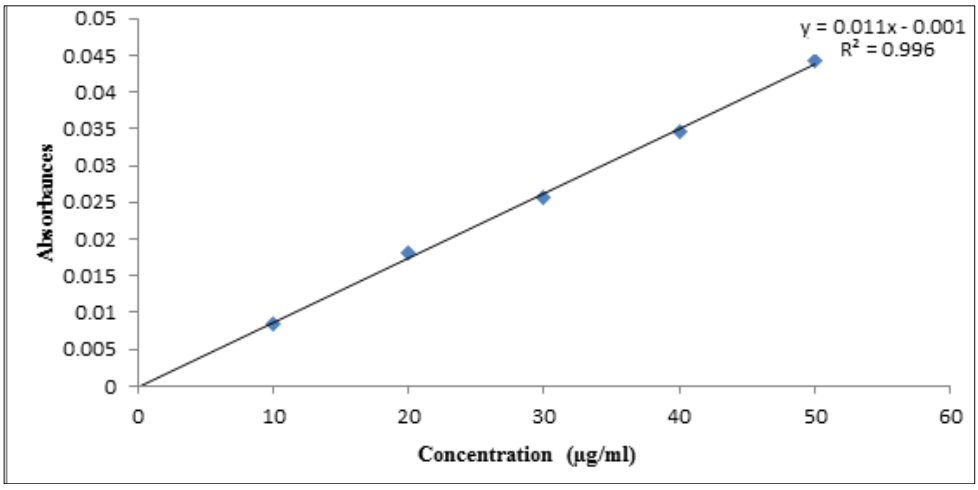

Figure 6: Standard Curve of Quercetin

Table 8: Total Flavanoid Content in different Hydro-alcoholic extracts

S. No.		1	2	3	4
Concentration	**Extracts**	**NIL**	**MKL**	**PGB**	**AHP**
200 (µg/ml)	Absorbances	0.187	0.185	0.265	0.197
		0.201	0.191	0.271	0.201
	Conc. (µg/ml)	170.909	169.091	241.818	180
		183.636	174.546	247.273	183.636
	Avg. Conc.	177.273	171.818	244.546	181.818
500 (µg/ml)	Absorbances	0.317	0.314	0.329	0.367
		0.323	0.318	0.332	0.362
	Conc. (µg/ml)	289.091	286.364	300	334.546
		294.546	290	302.728	330
	Avg. Conc.	291.819	288.182	301.364	332.273
1000 (µg/ml)	Absorbances	0.404	0.461	0.553	0.649
		0.398	0.457	0.549	0.654
	Conc. (µg/ml)	368.182	420	503.636	590.91
		362.727	416.364	500	595.455
	Avg. Conc.	365.455	418.182	501.819	593.182

Table 9: Quercetin equivalent in mg/g extract

	200 (µg/ml)	**Qty.1 mgQE/g**	**500 (µg/ml)**	**Qty.2 mgQE/g**	**1000 (µg/ml)**	**Qty.3 mgQE/g**	**Avg. mgQE/g**
NIL	177.273	886.364	291.818	583.636	365.455	365.455	611.818
MKL	171.818	859.091	288.182	576.364	418.182	418.182	617.879
PGB	244.546	1222.727	301.364	602.727	501.818	501.818	775.758
AHP	181.818	909.091	332.273	664.545	593.182	593.182	722.273

The hydroalcoholic extract of bark of *Punica granatum* Linn. has shown highest amount of total flavanoid content (775.76 mgQE/g) as compared to others. The bark has more flavanoidal content as compared to leaves of other plants. Different concentrations (200, 500 and 1000 µg/ml) has been taken to calculate the total flavanoidal content in different extracts obtained from different plant and parts of plants e.g. leaf, bark and whole plant. Table 7 shows the mean absorbance of various concentrations of quercetin while Figure 6 shows the standard quercetin curve and regression equation used for the calculation of total flavonoid content of the extracts [25].

Table 10: Total Flavanoid Content in different Hydroalcoholic Extracts

Extracts	**Conc. mgQE/g**
NIL	611.818
MKL	617.879
PGB	775.758
AHP	722.273

Concentration dependent flavanoidal content variation has validated the analytical methodology e.g. uv/ visible spectroscopy. The total flavanoidal content was more in the hydroalcoholic extract of PGB (775.76 mgQE/g) when compared to other concentrations (611.82, 617.88 and 722.27mgQE/g) (Table 10).

Conclusion

All four plants including *Nerium Indicum Mill*, *Artocarpus Heterophyllus Lam*, *Murraya Koenigii Linn*, *Punica Granatum Linn* extracted for the active constituent present. All the plant has the rich source of alkaloid, glycosides, tannins, amino acid, steroid, carbohydrate, protein, flavonoids and phenolic compounds. These all four plants has shown the Gallic acid and Quercetin as active constituents for flavanoid and phenolic content. These active constituents, further used for their wound healing potential and antioxidant properties on animal wound healing model.

Conflicts of interest

The author declares no conflicts of interest.

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