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Sirisha Deepthi Sornapudi
Department of Textile and Apparel Designing, College of Community and Applied Sciences, MPUAT, Udaipur, Rajasthan, India

Meenu Srivastava
Department of Textile and Apparel Designing, College of Community and Applied Sciences, MPUAT, Udaipur, Rajasthan, India

Corresponding Author
Sirisha Deepthi Sornapudi
Department of Textile and Apparel Designing, College of Community and Applied Sciences, MPUAT, Udaipur, Rajasthan, India

Quantitative estimation of phytochemicals in different leaf extracts

Sirisha Deepthi Sornapudi and Meenu Srivastava

Abstract
Phytochemical analysis of leaf extracts provides the concentrations of various phytochemicals present in the leaf sample. In the present study, the different leaf extract made from guava, lemon, vitex, moringa and custard apple were used for quantitative estimation of phytochemicals like phenols, flavonoids, tannins and terpenoids. Three different leaves extracts like methanol, ethanol and aqueous were prepared from the five selected leaves. The highest phenol content was observed in ethanol extract of vitex leaves 26.9±0.56 (mg GAE/100 g) and lowest was observed in Moringa methanol extract 10±0.45 (mg GAE/100 g). The Flavonoid content was observed highest in methanol extract of guava leaves 63.8±3.51 (mg RE/100 g) and lowest in moringa aqueous extract 7.8±0.75 mg RE/100g. The tannin content was highest in aqueous extract of guava leaves 150.05±4.76 % and lowest in ethanol extract of lemon leaves 4.8±0.00%. The highest terpenoid content was seen in aqueous extract of guava leaves and methanol of guava leaves.

Keywords: Quantitative estimation, phytochemicals, leaf extracts, guava, lemon, vitex, Moringa and custard apple

Introduction
Phytochemicals have a high antioxidant capacity and are of great interest because of their positive effects on human health. They also provide significant health benefits to consumers. Approximately 20% of known plants have been used in pharmacological investigations, having a favourable impact on the healthcare system by treating cancer and other diseases. (Naczk and Shahidi, 2006) [14]. Plants are capable of producing a wide range of bioactive chemicals. Fruits and vegetables build high levels of phytochemicals, which may protect against free radical damage. Plants that possess beneficial phytochemicals may supplement the human needs by functioning as natural antioxidants. (Altemimi *et al.*, 2017) [1].

Phytochemicals are compounds found naturally in medicinal plants, leaves, vegetables, and roots that have a defined mechanism and protect against a variety of ailments (Muthukumar and Gurupriya, 2019) [13]. Alkaloids, amines, betalains, vitamins, terpenoids, phenolic acids, lignins, stilbenes, and tannins, as well as other secondary metabolites with significant antioxidant activity, are among the many free radical scavenging compounds found in plants. Phytochemicals, particularly polyphenols such as flavonoids, phenolic acids, tannins and anthocyanins, are known to have anti-oxidant and free radical scavenging properties (Farag *et al*., 2020) [7].

The guava leaf extract was found to possess analgesic, anti-inflammatory properties, hepatoprotective, antimicrobial, and antioxidant activities. In addition, many pharmaceutical preparations use the leaf extract as a cough sedative (Metwally *et al*., 2010) [12].

Vitex agnus-castus (Verbenaceae) is popularly used in folk medicine to treat ovarian insufficiency, uterine bleeding, premenstrual syndrome, fibroid cysts, infertility and acne in teenagers. It has also been traditionally used as a sedative, digestive aid and anti-infective. It includes iridoid glycosides, flavonoids, progestins, alkaloids, and essential fatty acids. Folk remedies to treat diarrhoea, gastro intestinal affections, malaria, colds and cough spells (Arokiyaraj *et al*., 2009) [3].

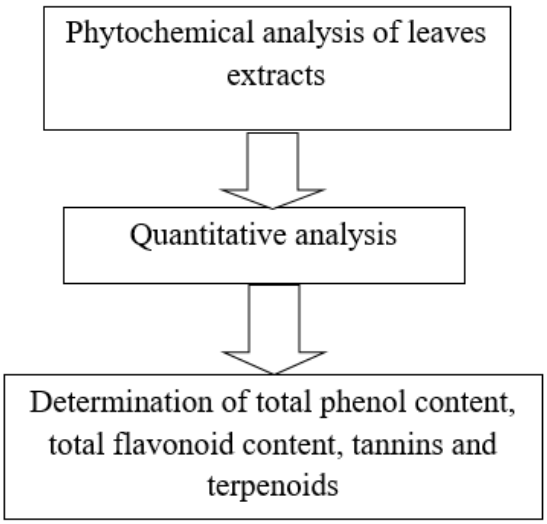
The leaves are used for extracting oil that has nutritional and medicinal properties such as antibacterial, antifungal and anti-inflammatory activities, in addition to its great importance as natural preservative agents (Ehiobu *et al*., 2021) [5].

Extracts from *Annona squamosa* plant such as the bark, roots, stem, leaves, peel, fruit and seeds, have been used in traditional pharmaceutical applications to treat a number of ailments, including diarrhoea, epilepsy, haemorrhage, fever, and tumours, in many countries.

Annona squamosa leaves (ASLs) possess extensive pharmacological characteristics and biological activities, such as antioxidant, antidiabetic, antibacterial, anticancer, antiviral, and hepatoprotective activity (Kumar *et al*., 2021) [9].

Moringa oleifera has long been used to treat a various disease in India, Pakistan, and Uganda like diabetes, hysteria, obesity, scurvy, and even cancer. Flavonoids, saponins, alkaloids, saccharides, tannins, phenolic acids, and nitrile glycosides are among the phytoconstituents found in Moringa oleifera. These natural complex phytochemicals contribute to its various pharmacological properties (Xu *et al*., 2019) [21].

In the present study, the extraction and quantitative identification of phenols, flavonoids, Tannins and terpenoids were estimated from selected leaves sources (vitex, guava, lemon moringa and custard apple). The solvent used for extraction are methanol, ethanol and aqueous.



Materials and Methods

1. **Collection of plant material:** Fresh leaves of vitex guava, custard, lemon, and moringa were collected near the Osmania University, Hyderabad, Telangana. The collected leaves were washed with tap water, shade dried and ground to fine powder. The powdered leaves were sieved and stored in air tight container for ethanol, aqueous and Methanolic extraction.
2. **Chemicals required:** Folin-Denis reagent, saturated sodium carbonate, Tannic acid, Gallic acid, Folin-Ciocalteu reagent, sodium carbonate, Rutin standard, sodium nitrite, Aluminium chloride, Sodium hydroxide and petroleum ether, methanol, ethanol.
3. **Preparation of sample extract:** All methanol, ethanol and aqueous extractions were prepared 1:15 ratio i.e., 1 part of leaf powder and 15 parts of solvent. Soxhlet method was used for the methanolic extraction of leaves. The five dried leaf powders (27 gm each) were packed in a muslin cloth each for placing in the Soxhlet thimble, which is placed inside the Soxhlet extractor. Methanol (400 mL) was taken in the round bottom flask of the Soxhlet apparatus. The solvent is heated through Isomantle. Upon heating, methanol evaporates, moves through the apparatus to the condenser. The condensate drips into the extractor. When the solvent level reaches the siphon, it then falls into the flask. This is one cycle of extraction. Extraction was done for 8 hours. The feed to solvent ratio was kept at 1:15 and was same for all the five leaf powders used in the study. The extract obtained was in dark greenish colour and was stored in a glass bottle in a refrigerator. This extract was utilised for subsequent analysis.

For ethanol extraction, the leaf powders were steeped in ethanol for 48 hours at room temperature and covered properly to prevent evaporation for ethanol extraction. The mixture was thoroughly stirred before being filtered using Whatmann filter paper and stored for further analysis.

The aqueous extraction was made with a slight modification done by (Alapati and Sulthana, 2015) [22]. The leaf powders were boiled for 15-20 minutes in conical flasks with distilled water at a 1:15 feed-to-solvent ratio. The flasks were then removed, cooled, and filtered with Whatmann filter paper. The filtrate was stored and utilised to do further analysis.

Materials and Methods

Total flavonoid: Total flavonoid content (TFC) was determined with slight modification of Pandey *et al*., 2020 [17]. A small quantity of the sample (0.5 ml) was combined with 4 ml distilled water and 0.3 ml of 5% $NaNO_2$ (Sodium Nitrite) solution. After 5 minutes, 0.3 ml of 10% $AlCl_3$ (Aluminium Chloride) was added and after 6 minutes 2 ml of 1M NaOH (Sodium Hydroxide) was added. After 30 minutes, the absorbance was measured at 510 nm against a blank solution. TFC was calculated using a quercetin calibration curve and represented as mg of Quercetin equivalents (QE/g) of extracts.

Total phenols: Total Flavonoid content was determined by Folin Ciocalteu method (Slinkard and Slingleton, 1997) [20]. Using distilled water, a known aliquot of samples was made up to 1.5 ml. After that, 0.5 ml Folin-Ciocalteu reagent was added and then 10 ml Sodium Carbonate, was added after which the solution was incubated for 1 hour at 37 °C. The absorbance values were read at 750 nm. Total phenolic content was expressed as mg gallic acid equivalent (GAE)/g of extracts by using the calibration curve of gallic acid.

Tannins: Tannin content was determined by using Folin-Denis reagent (AOAC 2005) [2]. Known aliquot of sample was added to a volumetric flask containing 75 ml of water in a 100 ml volumetric flask. Later 5 ml of Folin Denis reagent and 10 Na_2CO_3 solution was added. After 30 minutes, the colour at 760 nm was measured against an experimental blank calibrated to 0 absorbency. Tannic acid percentage was determined by mg tannic acid from standard curve.

Terpenoids: 100 mg (initial weight-wi) dried plant extract was steeped in 9 ml ethanol for 24 hours and then filtered using Whatmann filter paper. Using a separating funnel, the filtrate was extracted with 10 ml of petroleum ether. The ether extract was separated and dried completely in pre-weighed glass vials (final weight-wf). Ether was evaporated and the yield (%) of total terpenoids was calculated by using the formula. (Wi-wf/wi x 100) (Malik *et al*., 2017) [11].

Statistical analysis

Data are expressed as mean ± standard deviation (SD) of triplicates.

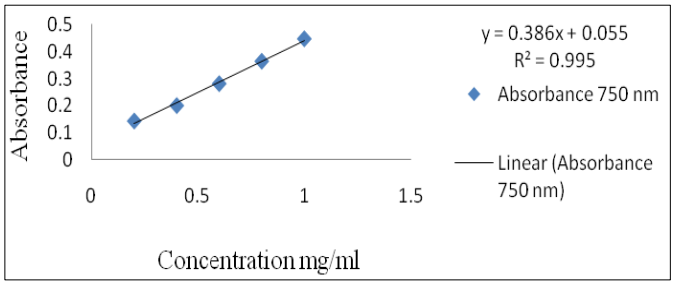


Fig 1: Gallic acid standard curve

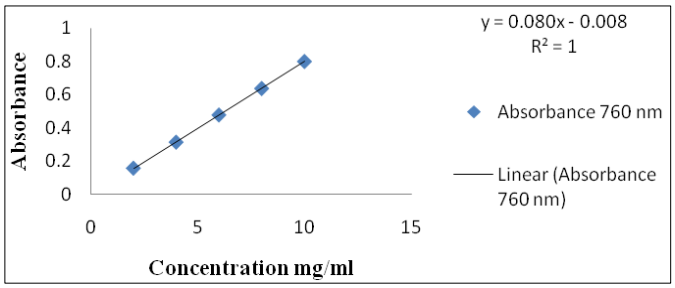


Fig 2: Tannin acid standard curve

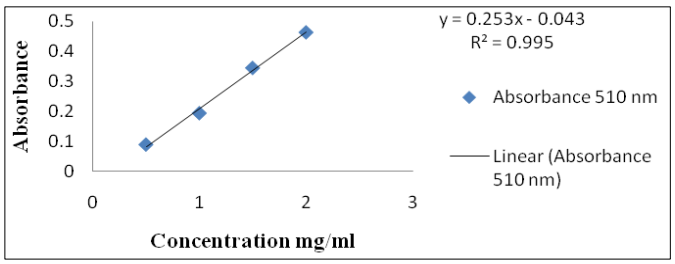


Fig 3: Quercetin standard curve

Results and Discussion

The quantitative estimation of phytochemical analysis of selected leaves of vitex, guava, lemon, custard apple and moringa are given in table 1. The results indicate a similar phenolic content (mg GAE/100 g) in methanolic extracts of guava 14.7±0.68 and lemon 14.3±3.55 leaves and ethanol extracts of vitex 26.9±0.56 and custard apple 26.05±2.62. No terpenoids content was observed in the ethanol extract of guava and lemon leaves extracts.

Phenols: The high phenolic content was observed in ethanol leaves extract of vitex, guava, custard apple and moringa except lemon leaves. In lemon leaves high phenolic content was observed in methanolic extract. Sankhalkar and Vernekar (2016) [18] reported the total phenolic content and flavonoid content in methanolic extract of moringa leaves as 2.28±0.02 (mg/ml) and 4.44±0.0045 (mg/ml). Nguyen *et al*., 2020 [15] reported the phenolic content as 242.88±6.13 mg GAE/g in *Annona squamosa* leaves. Kumar *et al*., 2013 [10] reported the phenols as 8.1±0.1 mg/g in methanolic extract of vitex leaves.

Flavonoids

Flavonoid content was high in methanolic leaves extract of guava, vitex, aqueous leaf extract of lemon, ethanolic leaves extract of custard apple and moringa. Setufe *et al*., 2018 [19] reported the presence of flavonoids (0.60±0.40) % in aqueous extract and absent in the ethanolic extract in guava leaves. The results in the present study indicate the flavonoids presence in ethanolic extract of guava leaves which is opposite to the above study. Sapkota *et al*., 2020 [4] reported the citrus maxima total flavonoid content in ethanolic and aqueous extracts as 182.05±0.0057 µg QE/mg±SD), 68.55±0.003 µg QE/mg±SD). Nguyen *et al*., 2020 [15] reported the flavonoid content 82.61±0.82 mg QE/g dry weight ethanol extract in *Annona squamosa* leaves. Kumar *et al*., 2013 [10] reported the flavonoids as 9.5±0.1 mg/g in methanolic extract of vitex leaves.

Tannic acid

The tannic acid percentage was reported high in aqueous extract of guava, lemon, custard apple, moringa and high in methanolic extract of vitex. Kumar *et al*., 2013 [10] reported the tannins of vitex leaves as 3.9±0.8 mg/g in methanolic extract. Hossain *et al*., 2020 [8] reported the total tannin content as 10.22±1.11 mg GAE/100 g in 70 ^{0}C methanol extraction with 30 min of time. The tannin content extract was increased as per increase in temperature and extraction time.

Terpenoids

The terpenoids was reported high in aqueous extract of guava leaves, methanol extract of vitex leaves and aqueous extract of custard apple leaves. Sapkota *et al*., 2020 [4] reported the citrus maxima terpenoids as the highest yield value in ethanolic extract (32%) and aqueous extract possess the least yield value (7.4%) which is opposite to the study. Oncho *et al*., 2021 [16] reported that the terpenoid content of powder in Guava leaf extract was 105.00±8.66 mg extract/g.

The results reported in this study are different from that of other studies which may be due to the processing conditions like shade drying, pressure cooking methods employed for aqueous extraction and solubility rates. The plant, season, geographical location, time in the growing season may also contribute to the variation in the various phytochemical contents.

Table 1: Quantitative phytochemical estimation of different leaves extract

S. No	Leaves	Extracts	Phenols (mg GAE/100 g)	Flavonoids (mg RE/100 g)	Tannic acid %	Terpenoids %
1.	Guava	Methanol	14.7±0.68	63.8±3.51	122.95±5.43	96±0.57
		Ethanol	23.8±0.22	48.4±3.74	95.35±2.66	-
		Aqueous	23.55±1.43	54.35±1.44	150.05±4.76	97.6±0.57
2.	Vitex	Methanol	14.61±0.21	32±0.97	37.75±0.85	96.3±0.57
		Ethanol	26.9±0.56	28.35±0.39	18.65±0.17	95.3±0.57
		Aqueous	12.05±0.34	19.3±1.50	33.4±2.25	95±1.0
3.	Lemon	Methanol	14.3±3.55	17.1±1.76	23.15±0.31	97.6±0.57
		Ethanol	10.3±0.60	12.6±0.93	4.8±0.00	-
		Aqueous	10.75±1.06	18.3±0.15	26.55±0.15	97.3±1.15
4.	Custard Apple	Methanol	12.5±0.17	24.65±1.50	32.82±0.28	95.6±0.57

		Ethanol	26.05±2.62	32.35±1.12	17.35±0.17	95.6±0.57
		Aqueous	16.75±0.17	26.9±0.96	41.25±1.98	96.6±0.57
5.	Moringa	Methanol	10±0.45	15.25±1.80	10.22±0.24	96.6±0.57
		Ethanol	18.4±0.82	22.2±0.83	9.0±0.00	96.6±0.57
		Aqueous	14.2±0.22	7.8±0.75	23.3±0.08	95±1.0

Conclusion

Phytochemicals are naturally occurring chemicals in plants produced through primary and secondary metabolism. They have the ability to protect the plant from pathogens and predators. Analysis of phytochemicals can help to screen plant sources in many fields such as potential medicinal use, traditional medicinal formulations, antimicrobial, anti-oxidant activity, to name a few. The study aimed to quantify the four different types of phytochemicals from five leaf sources. Phenolic content of the four leaf extracts was found to be high in ethanolic extracts followed by aqueous extracts. The same cannot be said for lemon leaf extracts where the methanolic extracts showed higher phenol content. Flavonoid presence varied in five leaf extracts – highest seen in guava and vitex methanolic extract, followed by ethanol extract of custard apple, moringa and aqueous lemon extract. Interestingly, the tannic acid levels in all the aqueous leaf extracts were high when compared with the ethanol and methanol extracts. Terpenoid content seen in all types of extracts was almost same with very less differences in the values. It was noticed that ethanol extracts of guava and vitex showed no terpenoid content.

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