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EVALUATION OF *SYZYGIUM CUMINI* LINN. SEED FORMULATIONS AVAILABLE IN THE MARKET USING SPECTROPHOTOMETRIC AND CHROMATOGRAPHIC TECHNIQUES

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ABSTRACT

Most of the plant parts of *Syzygium cumini* are documented in the traditional systems of medicine in India for their key usage as a hypoglycemic in the management of diabetes. Many such formulations of Jamun fruits/seed powder are available in the market. Hence, in the present investigation, the detailed pharmacognostic evaluation of *Syzygium cumini* seed powders has been carried out to develop a fast, easy and efficient protocol for the authentication of the formulations of Jamun available in the market. Also, an effort has been made to quantitate the gallic acid component of tannins using different chromatographic and spectrophotometric techniques.

INTRODUCTION: The definition of pharmacognosy implies a particular knowledge of methods of identification and evaluation of drugs. *Syzygium cumini* (L.) synonyms such as *Syzygium cumini* (L.) Druce, *Eugenia jambolana* Lam., *Syzygium jambolanum* DC, belonging to the family Myrtaceae, is a large evergreen tree up to 30 m in height and a girth of 3.6 m with a bole upto 15 m found throughout India upto an altitude of 1,800 m [1]. It has been valued in Ayurveda and Unani system of medication for possessing variety of therapeutic properties.

According to Ayurveda, its bark is acrid, sweet, digestive and astringent to the bowels, Antihelmentic, good for sore throat, bronchitis, asthma, thirst, dysentery, blood impurities and to cure ulcers [2]. In Unani medicine system the ash of leaves is used to strengthen the teeth and the gums, the seeds are astringent, diuretic, stop urinary discharge and are a remedy for diabetes and the barks show good wound healing properties. *Syzygium cumini* is a medicinal plant, whose parts were pharmacologically proved to possess hypoglycemic, antibacterial, anti-HIV activity, anti-diarrhea effects and anti-inflammatory activity [1].

The use of herbal medicine due to toxicity and side effects of allopathic medicines, has led to sudden increase in the number of herbal drug manufacturers. The proper authentication of herbal raw materials is critically important to the safety and efficacy of herbal medicines. WHO has emphasized the need to ensure quality control of medicinal plant products by using modern techniques and by applying suitable parameters and standards.

Herb authentication is a quality assurance process which ensures that correct plant species and plant parts are used as raw materials for herbal medicines. India can emerge as the major country and play the lead role in the production of standardized, therapeutically effective ayurvedic formulations.

This can be achieved only if the herbal products are evaluated and analyzed using sophisticated modern techniques of standardization such as UV- visible, TLC, HPLC, HPTLC, GC-MS, spectrofluorimetric and other methods. Hence, in the present investigation, the detailed pharmacognostic evaluation of *Syzygium cumini* seed is carried out to develop a fast, easy and efficient protocol for the authentication of the formulations of Jamun available in the market. Also, an effort has been made to quantitate the gallic acid component of tannins using different chromatographic and spectrophotometric techniques [3, 4].

MATERIALS AND METHODS:

Sample: Four formulations of *Syzygium* were procured from the market. Sample A is Jamun seed powder available from a local manufacturer. Sample B is a polyherbal formulation containing Jamun seed powder. Sample C and Sample D are Jamun seed powder formulations from 2 different manufacturers.

Physical and Microscopic Studies: Physical characteristics of powdered formulations like color, odor, texture and taste were studied. Microscopic observation of the seed powder was performed for screening anatomical characteristics using safranin and iodine. Anatomical features were documented using the Magnus live camera.

Proximate analysis: Various Standardization parameters of the formulations were studied as per standard references [2, 5, 6, 7] which include pH of 1% solution, Moisture content (3 hrs at 105±2^{o}C), Ash Content (550±5^{o}C in muffle furnace till constant weight), Acid insoluble ash, Water Insoluble Ash, Water, Ethanol and Methanol Extractive values.

Phytochemical Screening: All four market formulations were extracted with Ethanol using the Soxhlet extractor (10 gm of powder was extracted with 120 mL of ethanol for 48 hrs). For few tests methanolic and water extracts were used. The extracts were screened for the presence of acidic compounds, tannins, saponins, alkaloids, glycosides, steroids, proteins and amino acids, carbohydrates and triterpenoids using various qualitative tests. All tests were performed as per the standard procedures [2, 6, 7, 8].

Phytochemical Estimation: Total alkaloids were estimated by the Harborne's method. Estimation of tannins is based upon the measurement of intensity of blue color formed by the reduction of phosphotungstomolybdic acid by tannins in alkaline medium [6,7,8,9]. Folin Denis reagent was used for development of color and Gallic acid was used as a standard (5ppm). Method validation parameters such as specificity, linearity and recovery were performed for the tannin estimation protocol.

For linearity, a series of 2ppm, 4 ppm, 6 ppm, 8 ppm 10 ppm standard Gallic acid was prepared and estimated. To study the specificity of the method, 1 ppm of Gallic acid standard was spiked with 1ppm each of quinine, bovine serum albumin and digoxin respectively. For recovery studies, Sample A was spiked with 1ppm, 2 ppm, 3 ppm of standard Gallic acid. All Spectrophotometric estimations were carried out on a UV-Visible Spectrophotometer**,** Shimadzu UV-1700.

Estimation of total proteins in the seed formulations was carried out by the Lowry's method. Bovine Serum Albumin was used as the standard. 0.1 gram of seed powder was used to precipitate proteins by the TCA:Cold acetone method. Obtained proteins were reconstituted in 100 ml of 100mM Tris buffer pH 8. Appropriate dilutions were used for quantitation [10].

Estimation of total soluble sugars was carried out by the Anthrone method. 0.1 g of each formulation was boiled with 5 mL of 2.5N HCl for 3 hours on water bath. The mixture was then cooled, neutralized with Na_2CO_3, volume made up to 10 mL, centrifuged, diluted (1:20) and used for analysis [8, 11].

HPTLC Fingerprint for detection of essential oils: 1gm of Powder was extracted with 10mL of ethanol overnight, filtered and used for HPTLC analysis.

- Spotting Volume: 15 µL,
- Mobile Phase: Toluene: Ethyl Acetate (9:1v/v),
- Chamber conditions: Double Saturation,
- Derivatization: Vanillin Sulphuric Acid (Essential Oils)
- Iodine vapors (conjugated double bonded compounds)

Quantitation of Gallic Acid using HPTLC: Method development was carried out for the optimization of the HPTLC parameters. 10 µL of various concentrations of standard gallic acid (10 ppm to 500 ppm) was spotted to detect the optimum concentration of gallic acid. 5µL, 10µL and 15µL of 100 ppm standard gallic acid were spotted to detect optimum volume. Various mobile phases were tried for separation and resolution of tannins; enlisted in **Table 1** [12, 13].

TABLE 1: MOBILE PHASES FOR RESOLUTION OF TANNINS

Composition of mobile phase	Ratio of components
Ethyl Acetate-Water-Formic Acid	(9:0.5:0.5 v/v/v)
Toluene-Acetone-Formic Acid	(2:7:1 v/v/v)
Chloroform: Methanol: Glacial Acetic Acid	(6.5:3.5:1 v/v/v)
Ethyl Acetate: Acetic Acid	(2:0.4 v/v)
Ethyl Acetate : Methanol	(5.8 : 0.2 v/v)
Hexane: Ethyl Acetate: Methanol	(4:4:1 v/v/v)
Toluene: Ethyl Acetate: Formic Acid: Methanol	(3:3:0.8:0.2 v/v/v/v)
Toluene: Ethyl Acetate: Water: Acetic Acid	(7:3:1: 0.1 v/v/v/v)
Ethyl Acetate/Formic Acid/Glacial Acetic Acid/Water	(10:1:1:2 v/v/v/v)
Toluene :Ethyl Acetate: Formic Acid	(2:7:1 v/v/v)

- 0.1g of powder was extracted overnight in 5mL of methanol, filtered and used for spotting
- Spotting Volume: 10µL
- Chamber conditions: Double Saturation
- Standard: 0.02g Gallic acid per mL of methanol)
- Mobile Phase: Toluene: Ethyl Acetate: Formic Acid (2:7:1 v/v/v)
- Derivatization: 1% Alcoholic Iron(III) Chloride

Antimicrobial Activity of Ethanolic extracts: 10 g of each formulation was extracted with 120 ml of ethanol for 48 hours using the Soxhlet Extractor [14]. The extract obtained was filtered and ethanol was evaporated using Rota evaporator. Semisolid Extract obtained was then diluted with sterile deionised water (1:10 & 1:20). Antimicrobial activity of 0.2 ml of the aqueous dilutions was tested in duplicates against *E. coli, S. typhi, C. albicans, K. pneumonia, S. aureus*. Inhibition zone sizes were measured in mm after incubation for 24 hours at 37°C. Sterile distilled water (processed using entirely the same procedure) was run along with the samples as a control.

Analysis of metals by AAS: Concentrations of Zinc, Iron and Copper were determined from the seed formulations. 1 g of each formulation was placed in a porcelain crucible and kept for ashing at 550º±5 C in a muffle furnace till constant weight was obtained. The ash was dissolved in 5ml of 20% HCl by warming, transferred to standard volumetric flask by filtering through Whatman filter paper no. 41,volume was made up to 50 mL with deionized water. This sample was then subjected to analysis using Perkin Elmer Analyst 700 as per the ideal conditions required for the metal [15].

- Software: Winlab32
- Mode of Atomization: Flame
- Flame: Air-Acetylene
- Flow rate: Air- Acetylene (17.0 M^3/min-2.0M^3/min)

RESULTS AND DISCUSSION:

Physical and Microscopic Studies: All the formulations studied were brown (various shades observed) in color, had an aromatic odor, were bitter to taste and had a coarse texture. The monoherbal formulations A, C and D showed the presence of Parenchyma cells deposited with pectin and numerous oval Starch grains isolated or in clusters of 4-5 grains. In addition to these, Polyherbal formulation B also showed the presence of some annular and spiral vessels.

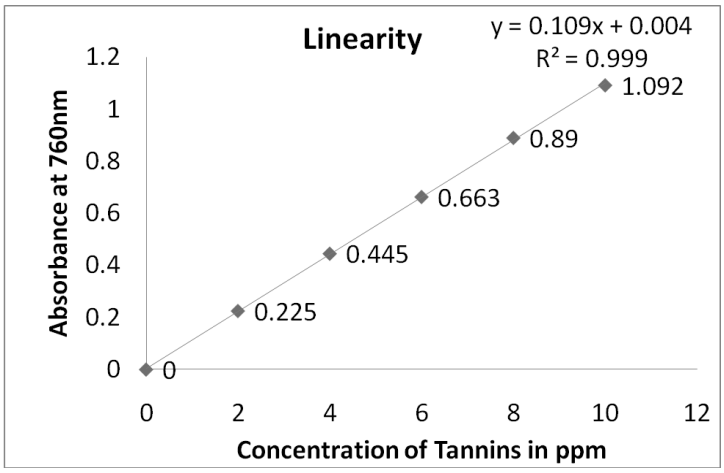
Proximate analysis: Proximate analysis yielded results as per **Table 2**.

TABLE 2: PROXIMATE ANALYSIS OF FORMULATIONS

PARAMETERS	A	B	C	D
pH of 1% w/v solution	4.49	4.8	4.4	5.0
Moisture Content	2.97%	2%	7%	13%
EXTRACTIVE VALUE				
Alcohol Soluble Extractive	45 %	45 %	45 %	35 %
Methanol Soluble Extractive	45 %	45 %	45 %	40 %
Water Soluble Extractive	20 %	45 %	55 %	40 %
ASH VALUES				
Total ash	5.78 %	6.79 %	3.24 %	4.71 %
Water insoluble ash value	2.08%	1.93%	0.77%	1.78%
Acid-insoluble ash value	4.53%	6.42%	0.87%	0.41%

Phytochemical Screening: All the formulations showed the presence of acidic compounds, tannins, alkaloids, glycosides, proteins and amino acids, carbohydrates and triterpenoids using various qualitative tests. Steroids and saponins were found to be absent in all the formulations.

Phytochemical Estimation: The formulations A, B, C and D contained 53.6, 68, 62.2, 63.2 mg of tannins per gram of powder respectively. Method validation of the above protocol demonstrated that the method is linear with R^2 of 0.999. The method is specific as negligible increase in absorbance was observed on spiking with quinine, BSA and digoxin (**Graph 1 and Table 3**).



GRAPH 1: LINEARITY PARAMETER FOR THE METHOD VALIDATION TANNIN ESTIMATION

TABLE 3: SPECIFICITY PARAMETER FOR THE METHOD VALIDATION OF TANNIN ESTIMATION

Parameters	1 ppm Gallic Acid (GA)	1 ppm GA + 1ppm Quinine	1 ppm GA + 1 ppm BSA	1 ppm GA+ 1 ppm Digoxin
Absorbance	0.111	0.115	0.115	0.118
Increase in Absorbance due to spiking	0	0.004	0.004	0.007

TABLE 4: RECOVERY PARAMETER FOR THE METHOD VALIDATION OF TANNIN ESTIMATION

Sample	Absorbance	Concentration (ppm)	% Recovery
Sample A	0.294	2.48	
Sample A+ 1ppm gallic acid	0.395	3.39	91
Sample A+ 2 ppm gallic acid	0.509	4.31	92.5
Sample A+ 3ppm gallic acid	0.637	5.48	97.33

Formula for % recovery=

$$\frac{\text{Strengthened concentration - Actual concentration}}{\text{Added concentration}} \times 100$$

The formulations A, B, C and D contain 25.33,18, 16.65 and 36 Gram % protein respectively. The total soluble sugars were found to be 41.24, 23.62, 19.63, 54.6 gram % respectively for samples A, B, C and D.

HPTLC Fingerprint for detection of essential oils: Out of 0.2, 0.3 and 0.95 marker peaks referenced in API only 0.2 and 0.3 were detected where as 0.95 was not detected in any of the formulations. Only sample A showed significant bands after derivatization with Iodine vapors for detection of conjugated double bond compounds.

Quantitation of Gallic acid using HPTLC: Gallic Acid was not detected below 80 ppm. No significant increase in AUC was observed after spotting concentration beyond 300 ppm, Therefore, the ideal working range was decided to be between 100 ppm to 300 ppm. Optimum volume was found to be 10µL. Best Suited Mobile phase was found to be Toluene: Ethyl Acetate: Formic Acid (2:7:1 v/v/v) amongst all those tried out.

HPTLC results showed similar R_f values (0.6$\pm$ 2) in market formulations and standard gallic acid (200 ppm). Concentration of gallic acid in formulations were found to be 0.68, 0.55, 0.35, 0.66 mg per gram of powder respectively in samples A, B, C and D.

Antimicrobial Activity of Ethanolic extracts: All formulations show appreciable activity against enteric pathogens as shown in **Table 5**. Sample A and C show very good activity against *Candida albicans*. Formulation A shows good antimicrobial profile against all the test organisms selected. No inhibition was observed in the well containing sterile distilled water which was processed using entirely the same procedure as the samples.

TABLE 5: ANTIMICROBIAL ACTIVITY OF THE ETHANOLIC EXTRACT OF JAMUN FORMULATIONS (1:10 dilution) AGAINST PATHOGENIC ISOLATES

Name of the Test Organism	Diameter of Zone of Inhibition (in mm)			
	A	B	C	D
Salmonella typhi	32	21	18	25
Escherichia coli	25	28	12	23
Staphylococcus aureus	16	14	20	26
Klebsiella pneumoniae	24	20	22	10
Candida albicans	35	21	37	22

Analysis of metals by AAS: Concentration of Zinc, Iron and Copper per gram of each formulation was detected as given in **Table 6**. There was considerable variation in concentration of Zinc among the formulations whereas concentrations of Iron and Copper were comparable.

TABLE 6: CONCENTRATION OF Cu, Fe AND Zn IN THE FORMULATIONS

Sample	Zn (mg)	Fe (mg)	Cu (mg)
A	0.452	20.93	0.304
B	0.55	19.36	0.270
C	0.342	22.61	0.313
D	0.305	20.04	0.280

CONCLUSION: Gallic Acid can be used as a marker peak for Jamun and its formulations. Concentration of Gallic acid was found to be relatively less in comparison with total tannin content in all formulations, indicating the complex nature of Tannins."Formulation A" was found to be the best among all those studied. All samples showed a good antimicrobial profile against pathogenic organisms. Formulations A and C show an appreciable activity against *Candida albicans*. It is evident from the present work that the authentication of the raw materials and finished product is essential and it should be made mandatory to publish all standardization parameters for the product.

Future Prospects: The detection and characterization of adulterants and pesticide residues which could be present in the formulations can be done. Bioavailability of Tannins and Essential metals from these formulations can also be studied. Moreover, the active ingredients contributing to the antimicrobial activity can be identified.

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