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MORPHO-ANATOMICAL, HISTOCHEMICAL, PHYSICO-CHEMICAL AND THIN LAYER CHROMATOGRAPHY ANALYSIS ON *Annona muricata* L. SEEDS

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ABSTRACT

Annona muricata L. belongs to the family annonaceae commonly called as soursop, is an interesting plant known for its edible fleshy fruit resembling *Annona squamosa*. Fruits and seeds of A. *muricata* are used in the folk medicine for treatment of various diseases. The fruit is known to possess anti-cancer properties and it is believed that consumption of the fruit may prevent cancer. In present investigation, the anatomical structure, histochemical localization of storage chemicals, physicochemical analysis, powder drug analysis and thin layer chromatography were performed on the mature seed. The anatomical structure shows the presence of multilayered seed coat with three different layers of testa namely exo, meso and endo testa, the tegmen is found to be collapsed. The rumination extends into the endosperm and idioblast cells are seen on the either side of thick walled rumination. Histochemical staining revealed that the endosperm is rich in lipids and proteins. Idioblast cells show positive colouration with different stains and reagent indicating the presence of various biochemical substances like alkaloids, lipids. Physicochemical analysis results, especially extractive values show that the seed is rich in phytochemicals. Powder drug and fluorescence analysis revealed the potential chemical properties of seed powder for evaluation as drug. Thin layer chromatography result indicated the presence of phenolic substances.

KEYWORDS: *Annona muricata* L. seeds, Morpho-anatomical, Histochemical, Physico-chemical, Thin Layer Chromatography.

INTRODUCTION

Annona muricata L. belongs to the family Annonaceae, commonly known as Soursop. *Annona muricata* L. is the important medicinal plant which shows many medicinal properties

Fruit of *Annona muricata* L.

Fruit is dark green, prickly (or bristled) fruits are egg-shaped and can be up to 30 centimetres (12 inch) long, with a moderately firm texture. Pulp is juicy, acidic, whitish, aromatic and possess abundant seeds. The creamy and delectable flesh of the fruit consists of 80% water, 1% protein, 18% carbohydrates and fair amount of vitamins B and C, potassium and dietary fiber (PIER, 2008). The average weight of 1000 fresh seeds is 470 grams and had an average oil content of 24%. When dried for 3 days in 60 C (140F) the average seed weight was 322 grams and were tolerant of the moisture extraction; showing no problems for long-term storage under reasonable conditions (Royal Botanical Garden, 2005). Stephens (1936), Corner (1952), Martinez (1952) and studied the variations in the fruit size and shape of *Annona muricata* and stated that the soursops are more or less oval or heart-shaped, sometimes irregular, lopsided or curved due to improper carpel development or insect injury. The fruit is compound and covered with a reticulated, leathery-appearing but tender, inedible, bitter skin from which protrude few or many stubby or more elongated and curved, soft, pliable, spines. The tips break off easily when the fruit is fully ripe. The skin of the immature fruit is usually dark-green, becoming slightly yellowish-green (Zayas, 1944).

MATERIALS AND METHODS

Source of the seeds: Mature ripen fruits of *Annona muricata* L. were purchased from the Koyambedu fruit market, Chennai. Seeds were removed from the ripen fruits, air-dried, stored in the refrigerator and used for this investigation.

Morpho-anatomical Studies: The seed was macroscopically examined for its organo-leptic

characters such as colour, texture, surface, size, shape and weight adapting to standard procedures (Trease & Evans, 2002). Free-hand transverse sections were taken for anatomical description of seed. Transverse sections were made along the peripheral and central regions of the cotyledonary tissue and embryo and stained with safranin O and toluidine blue O.

Histochemical Studies: The seeds were subjected to histochemical analysis in order to understand the structural and storage biochemical constituents. A wide range of bright-field dyes and reagents were used to identify the storage components such as starch, lipids, proteins and secondary metabolites in the seeds of *Annona muricata* L.

Table 1: List of histochemical stains and reagents used in this investigation.

Histochemical Dyes/ Reagents	**Components**
Safranin O	General Structure & Lignin
Toluidine blue O	General Structure
Iodine potassium iodide (I_2KI)	Starch
Coomassie brilliant blue R250	Protein
Sudan III & IV	Lipid
Phloroglucinol	Lignin
Dragendorff's Reagent	Alkaloids
Flurochromes	**Compounds**
Coriphosphine	General Structure
Nile blue	Lipid
Cellofluor white	Cellulose

Physico-chemical Analysis: Physico-chemical properties such as moisture content (LOD), ash values viz., total ash, acid insoluble ash and extractive values viz., water soluble extractive, alcohol soluble extractive were determined (WHO, 1992, Kokate, 1997 & Harbone, 2005). The seed powder of *Annona muricata* is subjected to different physico-chemical parameters inorder to derive physico-chemical standards.

Loss on Drying (LOD): Loss on drying is the loss in weight in % w/w determined by means of the procedure given below. It determines the amount of volatile matter of any kind (including water) that can be driven off under the condition specified (dessicator or hot air oven). If the sample is in the form of large crystals, then reduce the size by quickly crushing to a powder.

Ash Analysis: The ash remaining following the ignition of seed material is determined by three different methods which measure total ash, acid-insoluble ash and water soluble ash.

Total Ash: Place about 4g of the ground air-dried powder, accurately weighed, in a previously ignited and tared crucible. Spread the material in an even layer and ignite it by gradually increasing the heat to 500-600°C until it is white, indicating the absence of carbon. Cool in desiccators and weigh. If carbon-free ash cannot be obtained in this manner, cool the crucible and moisten the residue with about 2ml of water or a saturated solution of ammonium nitrate. Dry on a water bath, then on a hot plate and ignite to constant weight Allow the residue to cool in a suitable dessicator for 30 minutes, and then weight without delay.

Acid-insoluble Ash: To the crucible containing the total ash, add 25ml of 3N HCl, cover with a watch glass and boil gently for 5 minutes. Rinse the watch-glass with 5 ml of hot water and add this liquid to the crucible. Collect the insoluble matter on ashless filter paper and wash with hot water until the filtrate is neutral. Transfer the filter paper containing the insoluble matter to the original crucible dry on a hot plate and ignite to constant weight. Allow the residue to cool in suitable desiccators for 30 minutes, and then weigh without delay. Calculate the percentage reference to the weight of sample as determined under Total Ash.

Extractive values Alcohol soluble extractive value: 5 gm of accurately weighed powdered drug was taken in a stoppered conical flask and add 100 ml of 90% alcohol, and shake constantly for 6 hrs. in an electrical shakerand kept overnight for maceration and then filter carefully and filter was evaporated to dryness and weight of extractive was taken and percentage was calculated with reference to the air-dried drug.

Water soluble extractive value: 5 gm of accurately weighed powdered drug was taken in a stoppered conical flask and add 100 ml of chloroform water, and shake constantly for 6 hrs. in an electrical shaker and kept overnight for maceration and then filter carefully and filter was evaporated to dryness and weight of extractive was taken and percentage was calculated with reference to the air dried drug.

Powder drug and Fluorescence analysis: A small quantity of dry seed powder of *Annona muricata* L. is placed on watch glass and 1-2 drops of freshly prepared reagent solution is added, mixed by gentle and wait for few minutes. Then the slide is placed inside the UV

chamber for observation of colour change and the same is also observed in visible light. The colour observed by application of different reagents in visible light and UV light are recorded. Generally the colour change is noted in reagents like HCl, NaOH, HNO_3, H_2SO_4, Iodine, Acetic acid, $FeCl_2$, Methanol and NaOH+Methanol. Some constituents show fluorescence in the visible range in daylight. The ultra violet light produces fluorescence in many natural products which do not visibly fluorescence in daylight.

Thin-Layer Chromatography: 4 g of the seed powder of *Annona muricata* is soaked in 40 ml of chloroform kept it in overnight. The solution were boiled for 10 minutes and filtered. The filtrates were concentrated and made up to 10 ml in volumetric flask. 5µl, 10µl, 15 µl of the above solutions were applied on Merck Aluminium plate pre-coated with silica gel 60 F_{254} of 0.2 mm thickness using capillary tube. This is developed in *Toluene: Ethyl acetate* (7:3). No prominent spot was visualised in 254, 366 nm. The plate was dipped in vanillin-sulphuric acid and heated at 105^{o}C till the colour of the spots appeared

RESULTS AND DISCUSSION

Morphological characters of *Annona muricata* mature seed

In Table 2 gives a summary of the colour, texture, shape, surface, weight, length and width of the seed of *Annona muricata* in fresh and dry conditions. The shape, surface and texture of the Fresh and Dry seed is described as ovate, shiny and hard. The observed colour of the seeds when freshly removed from the pulp is black and the seed gradually turn brown after some time, until they attain a light brown colour. The weight of the seed with the seed coat of fresh is 0.44g and it will slightly increase in dry seed weight of 0.46 g . Then observed the length and width of the fresh seed is 1.59 and 0.73 cm and it differed in the dry seed as 1.73 and 0.87 cm. The freshly harvested shiny black seeds and change of colour from black to brown gradually.

Table 2: Organoleptic characters of mature seed of *Annona muricata.*

S. No.	Characters	Fresh Seed	Dry Seed
1	Colour	Black	Brown
2	Texture	Hard	Hard
3	Shape	Ovate	Ovate
4	Surface	Shiny & Hard	Shiny & Hard
5	Weight of seed with seed coat	0.44 g	0.46 g
6	Weight of seed without seed coat	0.32 g	0.29 g
7	Seed Length	1`.59cm	1.73cm
8	Seed Width	0.73cm	0.87cm

Structure of *Annona muricata* seed: *Annona muricata* seed is an albuminous seed with endosperm containing the major reserve storage biochemical substances. Seed contains seed coat having multi-layered testa, hilum, endosperm, and embryo. Safranin O and toluidine blue O stains are used for the observation of the structural details of seed. Fig.1 shows the structural details of seed stained with toluidine Blue O. Microscopic observation of the seed of the *Annona muricata* reveals the the seed coat derived from different integment composed of exotesta, endotesta and mesotesta and the inner integment or tegmen. The outermost layer referred to as exotesta is uniseriate layer comprises of cuboid cells with thin walls. The mesotesta had two layers of lignified fibres, one or two layers of hypodermic cells. The endotesta consists of multi layered of thickened walls, but less packed than the mestotesta. One inner integment composed of a single-layer tegmen with a collapsed appearance, arranged after the endotesta. The rumination interface between the collapsed tegmen and endosperm found to be extended into the endosperm. Spherical idioblast structures are seen on the either side of the rumination of the endosperm. Fig.2 is cross-sectional view of the seed taken close to the embryo showing more number of ruminations. Fig.3 shows the safranin stained cross section showing deep red colouration in the peripheral region of the endosperm compared to its central region.

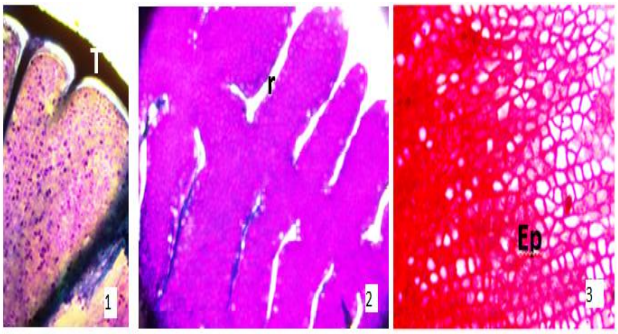


Fig. 1: Shows the structure of seed stained with toluidine Blue O.
Fig. 2: Is cross-sectional view of the seed taken close to the embryo showing more number of ruminations.
Fig. 3: Shows the safranin stained cross section showing deep red colouration in the peripheral region of the endosperm compared to its central region.

Histochemical studies on *Annona muricata* seeds: In this study, the various types of seed tissues are subjected to histochemical analysis for the localization and identification of biochemical and phytochemical constituents. Using bright-field stains and selected

fluorochromes attempt was made to localize the major storage biochemical substances.Starch is one of the major storage components of the seed found in the endosperm cells of cereal grains. Starch is a hetero-polysaccharide made up of amylose and amylo-pectin. Starch is revealed by staining with iodine potassium iodide (I_2KI) reagent as deep blue or black coloured bodies. The mature seed of *Annona muricata* does not show prominent colouration with I_2KI staining indicating absence of starch in the endosperm cells Fig.4. However the ruminations in the endosperm and idioblast cells surrounding the rumination show deep black colouration fig.5. Fig.6 is phloroglucinol stained cross-section of the endosperm region showing red colouration in the cell wall region indicating the presence of thickened cell wall and lignified nature of endosperm cells. Fig.7 is an unstained polarization photograph viewed under red plate showing distinct cell wall layers (testa) and red and blue colour pattern of the cellulose orientation in the cell wall of endosperm cells. The qualitative histochemical localization of protein in mature seed can be revealed by staining with Coomassie brilliant blue which shows blue colour for protein bodies. Fig8 shows the blue colouration in the endosperm cells. Fig.9 is the magnified view of endosperm cells showing blue coloured protein bodies whereas the idioblast cells are made up of thick-walled cells completely devoid of protein. The presence of lipids in mature seed can be revealed by staining with Nile blue and Sudan III. Nile blue is dual stain it can be used both as a bright-field stain and fluorochrome. Fig.10 staining gives deep blue colouration in the endosperm cells. Fig.11 is the magnified view of the idioblast structure showing positive blue colouration for lipids with nile blue stain. Figs.12&13 are photographs of Sudan III stained section showing intense red fluorescene indicating the presence of lipids. Fig.11 shows the red colouration of lipid droplets in the testa and fully packed in the endosperm cells. Histochemical staining for lipids reveal that *Annona muricata* seed endosperm is rich in lipids. In addition to the histochemical localization of major storage biochemical substances attempted was made to localize the alkaloids in mature seed using Dragendorff's reagent. Dragendorff's reagent stained section showing the presence of alkaloids as deep red colour in idioblast cells surrounding the ruminations in the endosperm. Cellofluor white staining and excitation with violet light show typical cell wall fluorescene of this fluorochrome the idioblast contents exhibit greenish fluorescence. Histochemical investigation on the mature seed of *Annona muricata* has revealed that lipids are the major storage biochemical reserve of this seed. Protein is the second abundant storage reserve of seed next to lipid. Starch is mostly absent in the endosperm whereas the ruminations show positive colouration to I_2KI. The idioblast structures seen surrounding the endosperm show positive colouration for Sudan III, Dragendorff's reagent, coriphosphine O, cellufluor and alizarin red indicating the presence of various biochemical substances like lipid, alkaloid. However, lipid is the main storage reserve of seed. In a recent study, Fabio Martinez (2013) also reported that idioblast contain oil substances.

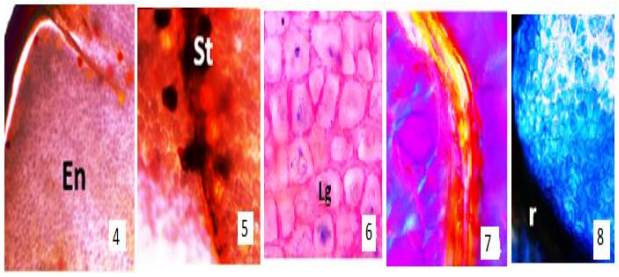


Fig. 4: I_2KI staining indicating absence of starch in the endosperm cells

Fig. 5: Shows idioblast cells surrounding the rumination show deep black colouration.

Fig. 6: Is phloroglucinol stained cross-section of the endosperm region showing red colouration in the cell wall region.

Fig. 7: Is an unstained polarization photograph viewed under red plate showing distinct cell wall layers (testa).

Fig. 8: Shows the blue colouration in the endosperm cells.

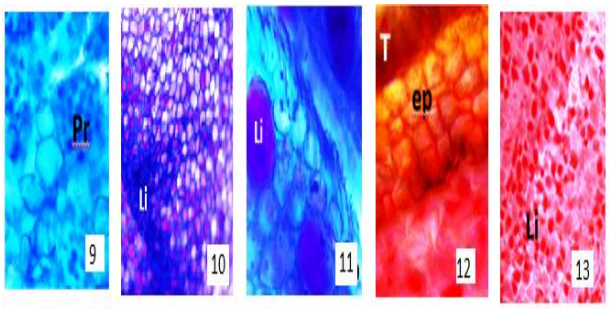


Fig. 9: Shows magnified view of endosperm cells showing blue coloured protein bodies.

Fig. 10: Staining gives deep blue colouration in the endosperm cells.

Fig. 11: Is the magnified view of the idioblast structure showing positive blue colouration for lipids with nile blue stain and the red colouration of lipid droplets in the testa and fully packed in the endosperm cells.

Figs. 12&13: Are photographs of Sudan III stained section showing intense red fluorescene indicating the presence of lipids.

Physico-chemical analysis of *Annona muricata* seed powder

Based on the result of the ash analysis of the *Annona muricata* seed powder as shown in the Table 3, the LOD (moisture content) is 8.34% and the total ash content of the sample is 2.10%. The acid insoluble ash of the seed powder is 0.03%. The pH (10% aqueous solution) of the sample is the 3.38%. The water and alcohol extractive value are 7.00% and 36.40% respectively. Physico-chemical characteristics showed the possibility of producing good quality oil, with great potential for the fine oil market. However the presence of alkaloids in the

oil needs to be further studied. Kimbonguila *et al*, (2010) reported on the physicochemical analyses on the seed and extracted oil of *Annona muricata*. The oil extracts exhibited good physicochemical properties and could be useful as edible oils and for industrial applications. Mona *et al*., (2011) studied the pharmacognostic evaluation of the *Annona squamosa* in order to possibly help to differentiate the drug from its other species. Morton and Julia (2013) reported that the physical properties of the soursop seed determined at 4.20% (wb) moisture content. Ash values are used to determine quality and purity of crude drug. It indicates presence of various impurities like carbonate, oxalate and silicate. The water soluble ash is used to estimate the amount of inorganic compound present in drugs. The acid insoluble ash consist mainly silica and indicate contamination with earthy material. Moisture content (Loss on drying) of drugs should be at minimal level to discourage the growth of bacteria, yeast or fungi during storage. Extractive values determine the amount of the active constituents in a given amount of plant material when extracted with a particular solvent.

Table 3: Physico-chemical parameters of *Annona* seed powder.

S. No.	Parameters	Result
1	LOD (w/w)	8.34%
2	Total Ash (w/w)	2.10%
3	Acid insoluble ash (w/w)	0.03%
4	Water extractive value (w/w)	7.00%
5	Alcohol extractive value (w/w)	36.40%

Powder drug and fluorescence analysis of *Annona muricata* seed powder

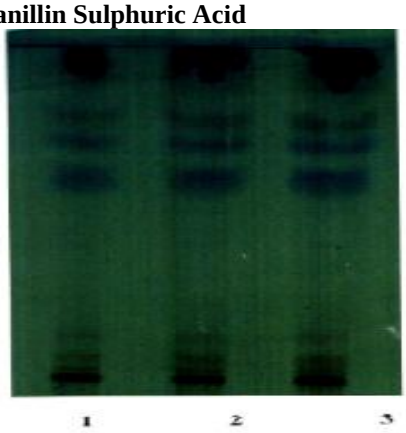
The seed powder of *Annona muricata* was subjected to powdered drug reactions by treatment with various chemicals and observing the same under UV light (220 nm) in Table.4.

Table 4: Powder drug and fluorescence analysis of A. muricata seed powder.

S. No.		White Light	UV Light
1	HCl	White	Black
2	NaOH	White	Brown
3	HNO_3	Yellow	Yellow
4	H_2SO_4	Reddish Black	Reddish Black
5	Iodine	Reddish Brown	Reddish Brown
6	Acetic Acid	Brown	Brown
7	$FeCl_2$	Yellow	Yellow
8	Methanol	White	White
9	NaOH + Methanol	Brown	Brown

Thin Layer Chromatography *Annona muricata* seed powder: TLC analysis provides an idea about the polarity of various chemical constituents, with respect to polar and non-polar solvents. Compounds with high Rf value show low polarity and with low Rf value have high polarity. The chloroform extract of the seed powder was prepared by cold percolation method and made up to 10 ml in volumetric flask. 5µl, 10µl, 15 µl concentrations and eluted in Toluene: Ethyl acetate (7:3) solvent system and derivatised with vanillin-sulphuric acid. A total of six spots were observed in all the three concentrations after spraying vanillin-sulphuric acid reagent of which three are prominent bluish grey coloured spots and other three are brown coloured spots. The colour of spots and respective Rf values are presented in the table 4. The result of TLC analysis indicates that the seed compound contain phenolic substances.

TLC - Vanillin Sulphuric Acid



Track – 1, 2, 3 Are Sample µ L, µ L Respectively. Solvent System: *Toluene: Ethyl Acetate* [7:3]

Table 5: TLC derivatised with Vanillin Sulphuric acid.

S. No	Colour	R_f Value
1	Brown	0.03
2	Brown	0.06

3	Brown	0.13
4	Bluish gray	0.60
5	Bluish gray	0.71
6	Bluish gray	0.78

CONCLUSION

This study on *Annona muricata* L. seed provides a comprehensive information on the seed structure, histochemical localization of storage components especially in the idioblasts, physico-chemical parameters, powder drug analysis and thin layer chromatographic analysis that may be used as pharmacognostic standards for identification of this seed material in the form of crude drug and also for scientific validation of its medicinal potential.

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