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Identification of Bioactive Compounds and Protein Inhibitory Activity by Using Different Solvent Extractions of *Annona Muricata*

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Abstract
The traditional medical methods, especially the use of medicinal plants still play a major role in the developing countries. The history of the use of herbal medicine may be as old as the history of mankind. Many of the herbs and spices used by humans as food which yields useful medicinal compounds. *Annona muricata* L. leaves are traditionally used to treat diabetes. People have been consuming raw leaves of *Annona muricata* L. to control blood glucose levels. Many compounds have been reported from the various parts of the plant. In the present study, acetone and methanol extract of the leaves of the plant was subjected to thin layer Chromatography and protein inhibition activity. Several phytoconstituents were identified used thin layer Chromatography and these compounds showed significant protein inhibition activity.

Keywords
Annona muricata, Extraction, TLC and Protein inhibition

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INTRODUCTION
Natural products are extremely an important source of medicinal agents. Although there are some new approaches to drug discovery, such as combinatorial chemistry and computer based molecular modeling design, none of them can replace the importance of natural products in drug discovery and development [1, 2]. Many synthetic drugs cause severe side effects that are not acceptable except as treatments of last resort for terminal diseases such as cancer and the metabolites discovered in medicinal plants may avoid the side effect of synthetic drugs [3]. Plant used in treating diseases is as old as civilization and traditional medicines are still a major part of habitual treatments of different maladies [4, 5, 6]. *Annona muricata* (*A. muricata*) is widely used in the

traditional treatment of cancer in many countries. *A. muricata* commonly known as Graviola or soursop belongs to the family of Annonaceae and is the most tropical semi deciduous tree with the largest fruits of the Annona genus [7, 8]. Ancient herbal medicines may have some advantages over single purified chemicals [9 10].

Plant have been used for medicinal purposes long before recorded history. Primitive men observed and appreciated the great diversity of plants available to them. Plants provide foods, clothing, shelter,and medicine. Much of the medicinal use of plants seems to be developed through observations of wild animals, and by trial and error, as time went on, each tribe added the medicinal power of herbs in their area to its knowledge base. They methodically collected information on herbs and developed well-defined herbal pharmacopoeias.

Protein de-naturation results in the disorganization and unfolding of the protein secondary and tertiary structure without breaking or hydrolysis of peptide bonds. De-naturation may under ideal condition, be reversible, and its original native structure will retain by refolding when the denaturating agent is removed. However, most protein, once de-natured, remains permanently disordered. Denatured proteins are often insoluble and therefore precipitate which increases the activity of macrophase in the protein de-naturation site within the tissue leading some neurodegenerative disease and inflammatory disease. The management of protein de-naturation related diseases is a big challenge to the medical practitioner as there are huge side effects for the long-term consumption of conventional drug. For the remedy of this serious problem, clinician tries to believe in some alternative or herbal medicine. Nature provides huge medical agents for thousands of years and a significant number of modern human drug are isolated from natural resources. So, in future, development of new plant-based drug with better bioactive potential and without or less side effects is the principal objective to the researcher.

Annona muricata

Annona muricata is a member of the Annonaceae family and is a fruit tree with a long history of traditional use. *A. muricata*, also known as soursop, graviola and guanabana, is an evergreen plant that is mostly distributed in tropical and subtropical regions of the world. The fruits of *A. muricata*are extensively used to prepare syrups, candies, beverages, ice creams and shakes. A wide array of ethnomedicinal activities is contributed to different parts of *A. muricata*, and indigenous communities in Africa and South America extensively use this plant in their folk medicine. This article summarizes external morphology of the plant including leaves, fruit and seeds. Numerous investigations have substantiated these activities, including anticancer, anticonvulsant, anti-arthritic, antiparasitic, antimalarial, hepatoprotective and antidiabetic, analgesic hypotensive, antiinflammatory, and immune enhancing effects. Phytochemical studies reveal that annonaceousacetogenins are the major constituents of *A. muricata*. More than 100 annonaceousacetogenins have been isolated from leaves, barks, seeds, roots and fruits of *A. muricata*. In view of the immense studies on *A. muricata*, this review strives to unite available information regarding its phytochemistry, traditional uses and biological activities.

Annona muricata is a member of the annonaceae family and is a fruit tree with a long history of traditional use annona muricata, also known as soursop, graviola, andgunabana, is an evergreen plant that is mostly distributed in tropical and subtropical region of the word. The fruits of *annona muricata* are extensively used to prepare syrups, candies, beverages, icecream sand shakes. A wide array of ethnomedical activities is contributed to different parts of annona muricata and indigenous communities in Africa and south Africa extensively use this plant in their folk medicine natural products especially those derived from plants, have been helped to use mankind sustain its health since the dawn of medicine. The importance of active ingredients of plants in agriculture and medicine has stimulated significant interest in the biological activities of these substances. The name soursop is due to sweet flavor and sour of its l arge.

Bioactive compounds from plants

Plants are vast repository of compounds with wide range of biological activities such as antinociceptive, anti-inflammation, antidiabetic, anticancer, antimicrobial and antioxidant etc. These properties are due to bioactive principles including polyphenols, flavonoids, alkaloids, terpenoids, carotenoids, coumarins, curcumines, etc. which have also been confirmed by modern analytical techniques. The use of herbal extracts and nutritional supplements either as an alternative or as a complimentary medicine to conventional chemotherapy for the treatment of inflammatory diseases is well documented in Ayurveda, which is an alternative medicinal system that has been practiced primarily in the Indian subcontinent for 5000 years. Flavonoids are examples of herbal compounds normally reported

for their antioxidant and anti-inflammatory properties. They are polyphenolic compounds that occur ubiquitously in foods of plant origin. Over 4000 different flavonoids have been described, and they are categorized into flavanols, flavones, catechins, flavanones, anthocyanidins and isoflavonoids. Flavonoids are normal constituents of the human diet and are known for their variety of biological activities. In India, the leaves of *Annona muricata* plants are still used for the treatment of inflammation and diabetes mellitus.

Alkaloids

Alkaloids are often toxic to man and many have dramatic physiological activities; hence their wide use in medicine. They are usually colourless, often optically active substances; most are crystalline but a few (e.g. nicotine) are liquids at room temperatures. The most common precursors of alkaloids are amino acids. Many alkaloids are terpenoid in nature (Harborne, 1998).

The functions of alkaloids in plants are still largely obscure, although individual substances have been reported to be involved as growth regulators or as insect repellents or attractants. The theory that they act as a form of nitrogen storage in the plant is not now generally accepted (Harborne, 1998).

Phenolics

Phenolic phytochemicals are the largest category of phytochemicals and the most widely distributed in the plant kingdom. The three most important groups of dietary phenolics are flavonoids, phenolic acids, and polyphenols. Phenolic are hydroxyl group (OH) containing class of chemical compounds where the (-OH) bonded directly to an aromatic hydrocarbon group. Phenol (C_6H_5OH) is considered the simplest class of this group of natural compounds. Phenolic compounds are a large and complex group of chemical constituents found in plants. They are plant secondary metabolites, and they have an important role as defence compounds. phenolics exhibit several properties beneficial to humans and its antioxidant properties are important in determining their role as protecting agents against free radical-mediated disease processes. Flavonoids are the largest group of plant phenols and the most studied. Phenolic acids form a diverse group that includes the widely distributed hydroxybenzoic and hydroxycinnamic acids. Phenolic polymers, commonly known as tannins, are compounds of high molecular weight that are divided into two classes: hydrolysable and condensed tannins.

Phenolic Acids

The term “phenolic acids”, in general, designates phenols that possess one carboxylic acid functional group. Naturally occurring phenolic acids contain two distinctive carbon frameworks: the hydroxycinnamic and hydroxybenzoic structures. Hydroxycinnamic acid compounds are produced as simple esters with glucose or hydroxy carboxylic acids. Plant phenolic compounds are different in molecular structure and are characterized by hydroxylated aromatic rings. These compounds have been studied mainly for their properties against oxidative damage leading to various degenerative diseases, such as cardiovascular diseases, inflammation and cancer. Indeed, tumour cells, including leukaemia cells, typically have higher levels of reactive oxygen species (ROS) than normal cells so that they are particularly sensitive to oxidative stress. Many papers and reviews describe studies on bioavailability of phenolic acids, emphasizing both the direct intake through food consumption and the indirect bioavailability deriving by gastric, intestinal and hepatic metabolism.

In addition, Phenolic acid compounds and functions have been the subject of a great number of agricultural, biological, chemical and medical studies. In recent years, the importance of antioxidant activities of phenolic compounds and their potential usage in processed foods as a natural antioxidant compounds have reached a new level and some evidence suggests that the biological actions of these compounds are related to their antioxidant activity.

Flavonoids

Flavonoids are one of the most numerous and widespread group of phenolics found in plant. They are subdivided into several other groups including flavone, flavonol, flavanone, and isoflavones. Isoflavonoids differ from other flavonoids by having ring B attached to C-3 position of ring C. Flavonoids are mainly water-soluble compounds. Flavonoids are phenolic and hence change in colour when treated with base or with ammonia; thus, they are easily detected on chromatogram or in solution. Flavonoids contain conjugated aromatic systems and thus show intense absorption bands in the UV and visible regions of the spectrum. Flavonoids are present in plants as mixtures and it is very rare to find only a single flavonoid component in a plant tissue. Finally, the flavonoids can be separated by chromatographic procedures and the individual components identified by chromatographic and spectral comparison with known markers. Novel compounds discovered during surveys require more detailed chemical and spectral examination.

In plants, they are especially important one in guarding against oxidant damage, and they provide to the plant the colour that attracts pollinators and repels attacks by insects and microbes. Recent research suggests that in humans, these plant polyphenols provide important health benefits related to metabolic syndrome, cancer, brain health and the immune system. While many of these effects are of interest in youth, they are even of greater interest for treating the fastest growing sector of the population, i.e., aging adults. The relatively low toxicity and potential efficacy of most of these agents make them attractive to a large sector of the population. Further, there has been a growing public openness to non-traditional therapies such as botanical supplements. Evidence also suggests that the pharmacological effects of flavonoids are correlated with their antioxidant activities.

Tannins

Tannins occur widely in vascular plants, their occurrence in the angiosperms being associated with woody tissues. Industrially, tannins are substances of plant origin which because of their ability to cross-link with protein are capable of transforming raw animal skins into leather. Plant tissues high in tannin are, in fact, largely avoided by most feeders, because of the astringent taste they impart. One of the major functions of tannins in plants is thought to be as a barrier to herbivory.

Chemically, there are two main types of tannin, which are distributed unevenly throughout the plant kingdom. The condensed tannins occur almost universally in ferns and gymnosperms and are wide spread among the angiosperms, especially in woody species. By contrast, hydrolysable tannins are limited to dicotyledonous plants and here are only found in a relatively few families. Both types of tannin, however, can occur together in the same plant, as they do in oak bark and leaf.

Terpenoids

An enormous range of plant substances are covered by the word ‘terpenoids’, a term which is used to indicate that all such substances have a common biosynthetic origin. Chemically, terpenoids are generally lipid-soluble and are located in the cytoplasm of the plant cell. Essential oils sometimes occur in special glandular cells on the leaf surface, whilst carotenoids are especially associated with chloroplasts in the leaf and with chromoplasts in the petal. Terpenoids are normally extracted from plant tissues with light petroleum, ether or chloroform and can be separated by chromatography on silica gel or alumina using the same solvents.

Carotenoids

These are the natural compounds with lipophilic properties. About 500 different carotenoids have been identified, among them β-carotene is the most important. It can act as an antioxidant under low partial pressure of O_2. β-carotene usually functions in association with vitamin C and E. Lycopene, a fat-soluble pigment is a carotenoid. It is responsible for colour of certain fruits and vegetables (e.g. tomato). Lycopene possesses antioxidant property. Lutein and Zeaxanthin are also carotenoid pigments that impart yellow or green colour to fruits and vegetables. These pigments can also serve as antioxidants.

MATERIALS AND METHODS

Collection and identification of plant material

The leaves of *Annona muricata* were collected from the outskirts of Hosur, Krishnagiri district of Tamil Nadu, India. The type specimen was identified and authenticated by Dr. P.Balaji, HOD, PG and Research Centre in Biotechnology, MGR College, Hosur, Tamil Nadu, India. The collected leaf materials were cleaned shade dried and powdered for further extraction and analysis.

Preparation of extracts.

Preparation of Methanol Extract

The methanol extract of leaves of *Annona muricata* was prepared by refluxion technique. 50 gm of dried leaves of plant was added to 1 lit round bottom flask and extracted with methanol in batches. After complete extraction, the methanol extract was filtered and concentrated under reduced pressure by using rotary vacuum evaporator. The methanol extract was dried in vacuum dryer and stored at room temperature until used.

Preparation of Acetone Extract

The acetone extract of leaves of *Annona muricata* was prepared by refluxion technique. 50 gm of dried leaves of plant was added to 1 lit round bottom flask and extracted with acetone in batches. After complete extraction, the acetone extract was filtered and concentrated under reduced pressure by using rotary vacuum evaporator. The acetone extract was dried in vacuum dryer and stored at room temperature until used.

Extract Recovery Percent

The extract recovery in different solvents was expressed as grams of the dry matter. The amount of crude extracts recovered after extraction were weighed and interpreted.

Thin Layer Chromatographic Studies

TLC was performed on silica gel, G (Himedia, India) coated on the glass plate. Aliquots of extracts were spotted onto the TLC plate approximately 2 cm from

the bottom of the plate. The spots were dried with a warm current of air and then the plates were developed in a saturated glass TLC tank using the appropriate solvent system. The chromatogram was visualized under UV-light (366nm). The Rf values of the coloured spots were recorded.

Detection of metabolites using thin layer chromatography

Identification of the compounds in the Methanol, Acetone *Annona muricata* leaves were performed on TLC plates that had been spotted with extract and allowed to develop. For each detection reagent, two identical plates were prepared alongside each other. The first plate was used as the reference whilst the second plate was subjected to spray reagents for detection.

Detection of compounds

1. Visualization under UV light (366nm) (This will be done before reagent spray)
2. Spraying the TLC plates with reagents and observing under light.

Phytochemical Screening

Phytochemical screening was performed on TLC plates that had been spotted with extract and allowed to develop.

Detection Reagents

Vanillin Sulphuric Acid (VS)

VS reagent was used for the unspecific detection of essential oils, bitter and pungent principles, and saponin drugs. This reagent consisted of two parts.

a) A 5%(w/v) Ethanolic H_2SO_4 solution,
b) A 1% (w/v) Ethanolic vanillin solution.

The TLC plate was sprayed with 10ml of (A) followed by 5 to 10ml of (b), warmed at 100°C for 5 to 10 minutes and evaluated under visible light.

Liebermann-Burchard Reagent (LBr)

Freshly prepared LBr reagent was used for the detection of triterpenes, steroids (saponins and bitter principles) and sterols (cholesterol and esters). The reagent was prepared carefully by adding 5ml of acetic anhydride and 5ml of conc.H_2SO_4 to 50ml of absolute ethanol on ice. The TLC plate was sprayed with 5 to 10 ml of LBr reagent, warmed at 100°C for 5 to 10 minutes and evaluated under visible light.

Potassium hydroxide (KOH)

Freshly prepared KOH reagent was used for the detection of anthraquinones, anthrones and coumarins and arbutin drugs. The TLC plates was sprayed with 10ml of a 10% (w/v) ethanolic KOH solution, dried and then observed visible light.

Natural products-Polyethylene Glycol (NP-PEG)

NP-PEG was used for the detection of anthracene derivatives, coumarins, arbutin drugs, bitter principles and flavonoids, 10ml of NP 1%(w/v) methanolic diphenyl boryloxy ethyl amine) poly ethylene glycol-4000) was sprayed on to the TLC plate. The plate was then observed under visible light.

Fast Blue Salt (FBS)

250 mg of fast blue salt B (o-dianisidine bis(diazotized) zinc double salt) dissolved in 10 mL of water and mixed with 25 mL of methanol and 15 mL of dichloromethane. 0.5 g of iodine was placed in a glass bottle and dissolved in 100 mL of ethanol. Stored in a dark place. The TLC plate was sprayed with 5 to 10ml and then evaluated in visible light for the presence of blue zones.

Folin-Ciocaltue Reagent

Folin-Ciocalteu reagent was used for the detection of phenolic compounds and was purchased ready made from Merck (Darmstadt, Germany). The TLC plate was sprayed with 5 to 10ml and then evaluated in visible light for the presence of blue zones.

Dragendroff Reagent

Freshly prepared Dragendroff reagent was used for the detection of alkaloids. The reagent was prepared by dissolving 8g of KI in 20ml of H_2O. This solution was then added to a second solution containing 0.85g of basic bismuth nitrate in 40ml of H_2O and 10ml of acetic acid. The TLC plate was sprayed with 10ml and observed under visible light for the presence of yellow zones.

Aluminium chloride ($AlCl_3$)

$AlCl_3$ was used for the detection of flavanoids. A TLC plate was sprayed with 5 to 10 ml of a 1%(w/v) ethanolic $AlCl_3$ solution and evaluated visible light.

Protein denaturation method

Inhibition of protein denaturation method

The reaction mixture (0.5ml), consisted of 0.45ml bovine serum albumin (5% aqueous solutions) and 0.05ml of *Annona muricata* at different concentration. The samples were incubated at 37 °C for 30 min. After cooling the samples 2.5 ml of phosphate buffer saline (pH6.3) was added to do each tube. Turbidity was measured spectrophotometrically at 660 nm for control test 0.05ml distilled water was used instead of extracts while products control test lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated as follows

Percent inhibition = [{Abs Control-Abs sample}]/Abs control]×100

Inhibition of albumin denaturation method

The 5ml of reaction mixture was comprised of 0.2 ml of egg albumin (from hens' egg), 2.8 ml of phosphate buffer saline (PBS, Ph 6.4) and 2ml of varying concentration of extracts. Similar volume of double distilled water served a control. Then the mixture was incubated at 37°C for 15 minutes and then heated at 70°C for 5 mins. After cooling, their absorbance was measured at 660 nm by using pure blank. Diclofinac sodium (standard drug) was used as a reference drug and treated as such for determination of absorbance. The percentage inhibition of protein denaturation was calculated mentioned in membrane stabilized assay.

Proteinase inhibitory activity

The reaction mixture (2ml) containing 0.06 mg trypsin, 1ml 20Mm tris HCL buffer (pH 7.4) and 1 ml of test sample of different concentrations. The mixture was incubated at 37°C for 5 minutes and then 1ml of 0.8% (w/v) casein was added. The mixture was incubated for additional 20 minutes. 2ml 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged, and the absorbance of the supernatant was read at 210 nm against the blank. The experiment was performed in triplicate to perform the reproductivity of the result. The percentage of proteinase inhibitory activity was calculated follows

% of inhibition= optical density of test solution/optical density of control $\times$ **100**

RESULTS AND DISCUSSION

Detection of components in the different extracts by thin layer chromatography

TLC was performed as described in the above. TLC plates were spotted with methanol and acetone extracts. The solvent system used was Petroleum Ether: Ethyl acetate in 90:10 ratio. The TLC plates were placed in the solvent saturated TLC tanks, developed in the solvent system and then observed for the separation of components by visual colours. UV florescence or absorption at 366 nm and different spray reagents. Several spots on the TLC plates showed faint purple or blue fluorescence in the presence of UV 366 nm light

Spray Reagent Detection of Active Components

Vanillin Sulphuric Acid (VS)

VS reagent was used for the unspecific detection of essential oils, bitter and pungent principles, and saponins drugs. This reagent consisted of two parts.

a) A5% (w/v) Ethanolic H_2SO_4 solution,

b) A 1% (w/v) Ethanolic vanillin solution.

The TLC plate was sprayed with 10ml of (A) followed by 5 to 10ml of (b), warmed at 100°C for 5 to 10 minutes and evaluated under visible light.

All active components exhibited colour violet in the visible light indicating the presence of terpenoids. The colour reaction with this reagent is strong, suggesting that the active components were terpenoid type.

Liebermann-Burchard Reagent (LBr)

Freshly prepared LBr reagent was used for the detection of triterpenes, steroids (saponins and bitter principles) and sterols (cholesterol and esters). The reagent was prepared carefully by adding 5ml of acetic anhydride and 5ml of conc.H_2SO_4 to 50ml of absolute ethanol on ice. The TLC plate was sprayed with 5 to 10 ml of LBr reagent, warmed at 100°C for 5 to 10 minutes and evaluated under visible light. All active components exhibited colour brown in the visible light indicating the presence of terpenoids. The colour reaction with this reagent is strong, suggesting that the active components were terpenoids type.

Potassium hydroxide (KOH)

Freshly prepared KOH reagent was used for the detection of anthraquinones, anthrones and coumarins and arbutin drugs. The TLC plates were sprayed with 10ml of a 10% (w/v) ethanolic KOH solution dried and then observed under visible light. With this detection reagent, there exhibited no colour by the active components indicating they were not anthracene derivatives and coumarins.

Natural products-Polyethylene Glycol (NP-PEG)

NP-PEG was used for the detection of anthracene derivatives, coumarins, arbutin drugs, bitter principles and flavonoids,10ml of NP (1% (w/v) methanolic diphenyl boryloxy ethyl amine) polyethyleneglycol-4000) was sprayed onto the TLC plate. There exhibited no colour by the active components indicating the absence of anthracene.

Fast Blue Salt (FBS)

Fast blue salt was used for the detection of flavonids and phenolic water, dried and then observed under visible light for the presence of red to brown zones. The presence red-brown spots after spraying indicated the presence of phenolic compounds.

Folin-Ciocaltue Reagent

Folin-Ciocalteu reagent was used for the detection of phenolic compounds and was purchased ready made from Merck (Darmstadt, Germany). The TLC plate was sprayed with 5 to 10ml and then evaluated in visible light for the presence of blue zones. The presence of blue spots after spraying indicated the presence of phenolic compounds.

Dragendroff Reagent

Freshly prepared Dragendroff reagent was used for the detection of alkaloids. The reagent was prepared by dissolving 8g of KI in 20ml of H_2O. This solution was then added to a second solution containing 0.85g of basic bismuth nitrate in 40ml of H_2O and 10ml of acetic acid. The TLC plate was sprayed with 10ml and observed under visible light for the presence of yellow zones. The appearance of yellowish orange colour spots indicated the presence of alkaloids.

Aluminium chloride ($AlCl_3$)

$AlCl_3$ was used for the detection of flavanoids. A TLC plate was sprayed with 5 to 10 ml of a 1%(w/v) ethanolic $AlCl_3$ solution and evaluated under light. The active components did not react with this detection reagent indicating that they are neither coumarin nor flavonoid type compounds.

Detection of Classes of Compounds

TLC plates run in the mobile phase Petroleum ether: Ethyl acetate (90:10) resulted in the separation of nine components in the methanol and acetone extracts of *Annona muricata* leaves. The spots were numbered from the origin. The spots 1, 2, 4, 5 and 7 were seen as Pink colour zones. Spots 3, 6 and fluoresced as blue colour at UV 366 nm and the spot 8 quenched fluorescence at UV 366 nm.

The spot number 1 was pink colour with Rf value 13.3, 14, 7 and 13 respectively in methanol and extracts of *Annona muricata* leaves. It tested positive for VS and LBr reagents indicating the prescence of terpenoids. It also tested positive for DR, Folin's, FBS reagent but tested negative for $AlCl_3$ reagent, NP-PEG and KOH. This indicates the presence of Phenols and Alkaloids.

The second spot was also pink in colour and had Rf values 20, 20, 13.3 and 20 respectively for methanol and acetone extracts. Its dark brown in UV 254 nm. It tests positive for VS and LBr and turned blue when sprayed with Folin's reagent and turned yellow brown when sprayed with DR. The spot turned brown colour was observed in the spots at chromatogram when sprayed with Fast blue salt reagent. It tests negative for $AlCl_3$, KOH and NP-PEG. Thus, the component was identified for alkaloid and phenols.

The third spot with Rf value 33.3, 33, 20 and 33 for methanol and acetone extracts appeared blue colour in UV 366 nm. It tests positive for VS and LBr reagent. The spots turned blue in the presence of Folin's and brown in the presence of fast blue salt reagent. It tests negative results when observed in $AlCl_3$, KOH and NP-PEG reagent. This component was identified as terpenoids and alkaloids.

The spot number four had Rf value of 40, 40, 27 and 40 respectively for all methanol and acetone extracts, appeared pink in colour and turned purple brown in response to for VS reagent. It turned brown in colour after spraying with LBr reagent. It tested negative for NP-PEG, KOH and $AlCl_3$reagent.It tested positive for Folins reagent and FBS reagent. This compound may belong to the terpenoids and alkaloids group.

The fifth spot with Rf value 47,47,40 and 43 respectively for methanol and extracts appeared dull pink in UV 366 and reacted with Folin's reagent to give blue colour. The spot turned brown when sprayed with Fast blue salt reagent. It also tested positive for DR, VS and LBr reagents. It tested negative for $AlCl_3$, KOH and NP-PEG. This indicates that the components were alkaloids.

The sixth spot with Rf value 53.3, 53, 60 and 53 for methanol and acetone extracts respectively appeared blue in UV (366 nm). It tested negative for $AlCl_3$, NP-PEG and KOH. It tested positive showing yellow colour formation when sprayed with DR, blue colour formation when the plates were sprayed with folins reagent and blue violet in visible light with VS reagent and brown when sprayed with FBS reagent. This was identified as alkaloids and terpenoids.

Spot number seven with Rf value 60, 60, 67 and 60 for methanol and acetone extracts respectively appeared light pink in colour under UV 366 nm light. It tested negative for KOH and $AlCl_3$ and also for NP-PEG. A blue colour was formed when the spot received folins reagent spray. The spot turned purple violet in colour when TLC plates were sprayed with VS reagent and a brown colour was observed for LBr reagent. This component was identified as a terpenoid with a phenolic group.

The eighth spot with 67, 67, 73 and 67 for acetone and methanol extracts respectively. It quenched fluorescence at UV 366 nm. It reacted with folins to give blue colour. It tested positive for VS, LBr and FBS reagent. It tested negative for $AlCl_3$, NP-PEG and KOH reagents. The component in this spot has been identified as anthroquinone, alkaloids and terpenoids.

The spot number of nine and ten with Rf value 80 and 87 for acetone extract of *Annona muricate* quenched in UV 366 nm. It tested positive for folin, FBS, DR, VS and LBr by turning blue, brown, yellow, blue violet and brown in colour respectively. It tests negative after spraying with $AlCl_3$, KOH and NP-PEG reagent. This component was identified as anterpenoids and alkaloids.

Protein denaturation method

Protein Denaturation – Bovine Serum Albumin Assay

Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external source.

Inhibition of Albumin Denaturation Assay- Egg Albumin

Diclofinac sodium (standard drug) was used as a reference drug and treated as such for determination of absorbance. The percentage inhibition of protein denaturation was calculated mentioned in membrane stabilized assay.

Proteinase Inhibitory Activity

With the increasing the concentration of *Annona muricata* the protein inhibitory activity was found to be decreased and enhanced protection. Thus, the results reveal that plant sample were capable of inhibiting proteinase inhibitory activity.

Table 1: Identification of active components in the Acetone extract of *Annona muricata*

Identification of active components in the Acetone extract of *Annona muricata*										
Component	1	2	3	4	5	6	7	8	9	10
Rf x 100	7	13.3	20	27	40	60	67	73	80	87
UV-365nm	Pink	Blue	Pink	Light Pink	Light Blue	Pink	Pink	pink	Pink	Pink
VS	Violet	Violet	Violet	Violet	Violet Pink	Violet	Violet	Violet	Violet	Violet
LBr	Brown	Brown	Violet Brown	Brown	Brown	Brown	Brown	Brown	Brown	Brown
Folins	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
FBS	Brown	Brown	Brown	Brown	Brown	Brown	Brown	Brown	Brown	Brown
NP-PEG	-	-	-	-	-	-	-	-	-	-
KOH	-	-	-	-	-	-	-	-	-	-
AlCl3	-	-	-	-	-	-	-	-	-	-
Dragendroff	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange

Components in the fractions were separated using the mobile phase Petroleum ether: Methanol (90:10); Quenching of fluorescence; Reaction colours noted were observed in visible light; * Colour observed at UV 366 nm;

Table 2: Identification of active components in the Acetone extract of *Annona muricata*

Compounds	Reagents	Detection	Identification
Alkaloids	Dragendroff's Reagent	Yellow zones	+
Terpenoids	VS,Lbr Reagents	Violet/Purple,Brown	+
Saponins	VS	Violet	+
Phenolics	Folin's Reagent	Blue zones	+
Coumarins, Anthroquinones	KOH, NP-PEG	Blue to Blue violet zones	_
Flavonids	$AlCl_3$	Bright blue	_

Components in the fractions were separated using the mobile phase Petroleum ether: Ethyl acetate (90:10); +: Detected; -: Not detected.

Table 3: Spray reagent detection of active components in the methanol extract of *Annona muricata*

Identification of active components in the Methanol extract of *Annona muricata*								
Component	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Rf x 100	13	20	33	40	43	53	60	67
UV-365nm	Pink	pink	Pink	Pink	Pink	Q	Q	Pink
VS	Violet	Violet	Violet	Violet	Violet Pink	Violet	Violet	Violet
LBr	Brown	Brown	Violet Brown	Brown	Brown	Brown	Brown	Brown
Folins	Blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
FBS	Brown	Brown	Brown	Brown	Brown	Brown	Brown	Brown
NP-PEG	-	-	-	-	-	-	-	-
KOH	-	-	-	-	-	-	-	-
AlCl3	-	-	-	-	-	-	-	-
Dragendroff	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange

Components in the fractions were separated using the mobile phase Petroleum ether: Ethyl acetate (90:10); quenching of fluorescence; Reaction colours noted were observed in visible light; * Colour observed at UV 366 nm;

Table 4: Identification of active components in the Methanolic extract of *Annona muricata*

Compounds	Reagents	Detection	Identification
Alkaloids	Dragendroff's Reagent	Yellow zones	+
Terpenoids	VS,Lbr Reagents	Violet/Purple,Brown	+
Saponins	VS	Violet	+
Phenolics	Folin's Reagent	Blue zones	+
Coumarins, Anthroquinones	KOH, NP-PEG	Blue to Blue violet zones	–
Flavonoids	$AlCl_3$	Bright blue	_

Components in the fractions were separated using the mobile phase Petroleum ether: Ethyl acetate (90:10); +: Detected; -: Not detected.

Table 5: Effect of Acetone extract of *Annona muricata* on bovine serum albumin denaturation assay

Extract	Concentration	Absorbance At 660nm	% Of Inhibition
Control	-	1.915	-
Acetone extract of *Annona muricata*	100	1.945	-1.56
	200	1.884	1.618
	300	1.690	11.74
	400	1.282	33.05
	500	1.093	42.92

Table 6: Effect of Methanolic Extract of Annona Muricata on Bovine Serum Albumin Denaturation Assay

Extract	Concentration	Absorbance at 660nm	% Of Inhibition
Control	-	1.915	-
Methanol extract of Annona muricata	100	1.403	26.73
	200	1.067	47.41l
	300	1.077	43.75
	400	1.068	44.22
	500	1.045	45.43

Table 7: Effect of acetone extract of *Annona muricata* On Egg Albumin Denaturation Assay

Extract	Concentration	Absorbance At 660 Nm	% Of Inhibition
Acetone Extract of Annona muricata	100	0.020	98.62
	200	0.024	98.35
	300	0.024	98.35
	400	0.025	98.28
	500	0.026	98.21
Control	-	1.457	-
Standard-Diclofinac Sodium	500	0.057	

Table 8: Effect of methanolic extract from *Annoan muricata* on egg albumin

Extract	Concentration	absorbance at 660 nm	% of inhibition
Methanolic extract of Annoan muricata	100	0.022	98.49
	200	0.026	98.21
	300	0.024	98.35
	400	0.027	98.14
	500	0.026	98.21
Control	-	1.457	-
Standard Diclofinac Sodium	500	0.057	-

Figure 1: Detection of components in acetone extracts of *Annona muricata*

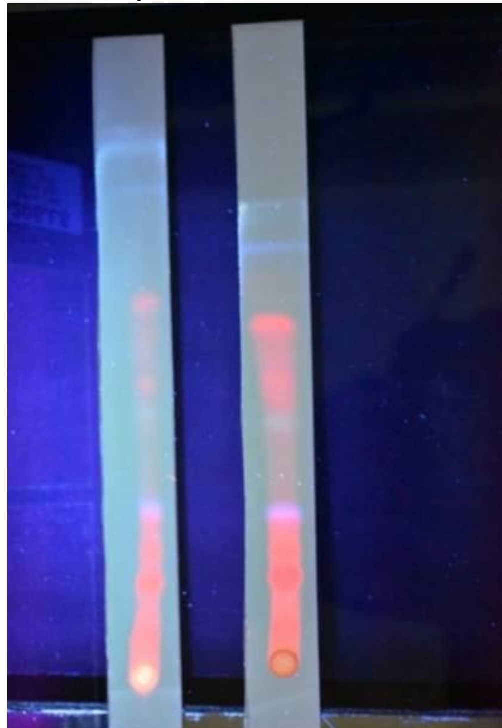


Figure 2: Detection of components in methanolic extracts of *Annona muricate*

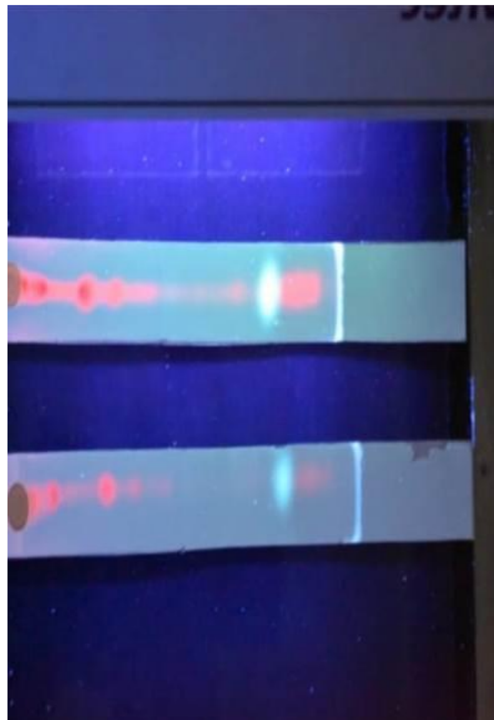
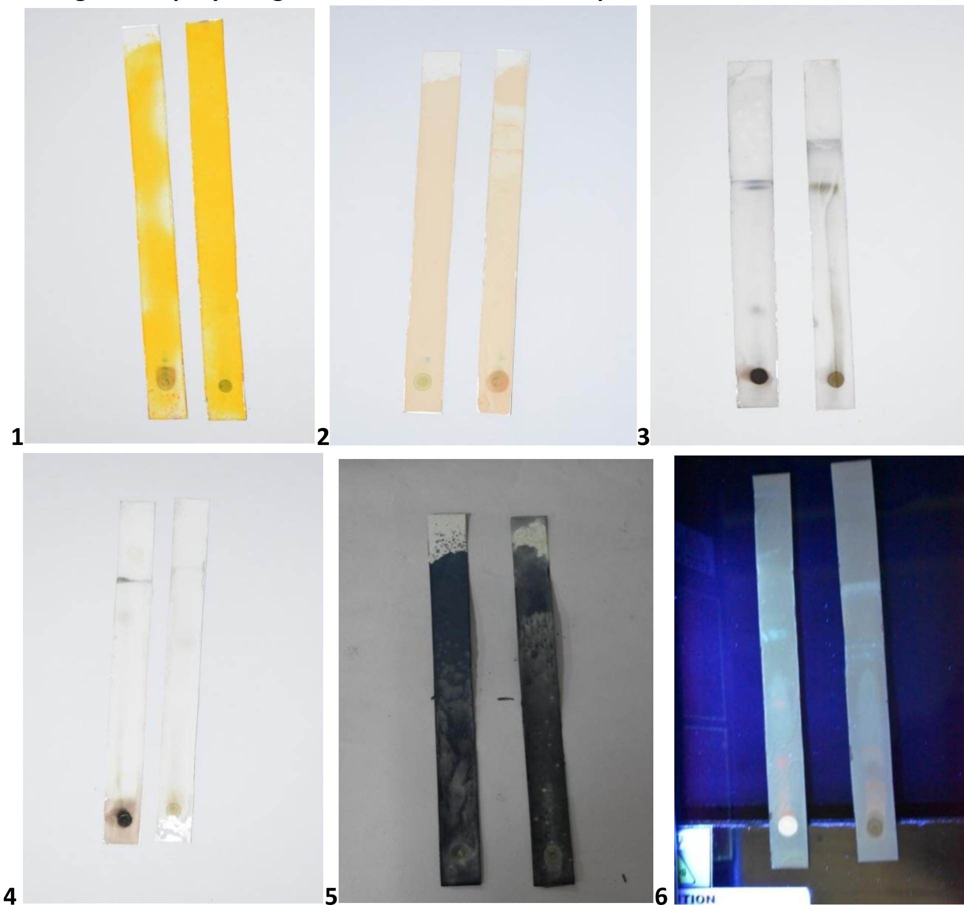
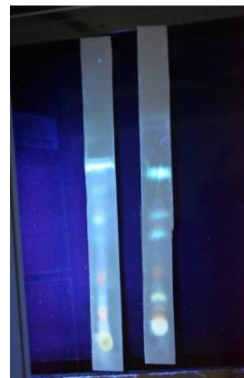


Figure 3: Spray Reagent Detection of active components in *Annona muricata*





1.Dragendroff's test
2.Fast Blue Salt test
3.VanillinSulphuric Acid test
4.LibermannBurchard test
5.Fc-reagent test
6.KOH Test
7.NP-PEG Test
8.$AlCl_3$ Test

CONCLUSION

Phytochemical screening conducted on leaves extracts of *A. muricata* revealed the presence of following classes of compounds: alkaloids, flavonoids, terpenoids, coumarins and lactones, anthraquinones, tannins, cardiac glycosides, phenols, phytosterols, and saponins. They were present in both the acetone and metanolic leaves extracts, but with noticeable differences in relative abundance ranging from low, average and high. These results are in line with earlier studies that carried out on the methanolic seeds extract of *A. muricata*, and the phytochemical tests showed that methanol soursop seeds extract contained secondary metabolites compounds: saponins, alkaloids and triterpenoids, flavonoids, anthraquinones, tannins, and cardiac glycosides. They are defense chemical compounds of plants produced in the plant tissue. Generally, presence of alkaloids, flavonoids, terpenoids, coumarins and lactones, anthraquinones, tannins, cardiac glycosides, phenols, phytosterols, and saponins confirms that *A. muricata* leaves extracts contain molecules known for extensive use in the medical field both traditionally and pharmaceutically. This would be an indication for its potential use in anti-inflammatory, anti-allergic, antibacterial, and antiviral, heart failure, antioxidant and anticancer activity among others. These findings emphasize the value of traditional knowledge in the use of plants for medicinal use as well as pharmaceutical development. The use of *A. muricata* in traditional medicine is validated by presence of these phytochemicals of known health benefits and thus further studies on this species are needed.

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