ROS in *Emblica officinalis* (Amla) and its detection in *Emblica officinalis* juice (*pectinase* assisted) and *Emblica officinalis* probiotic drink

A Dissertation submitted in partial fulfillment of the requirement for the award of the degree of

MASTER OF TECHNOLOGY

IN

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By

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Certificate

This is to certify that the dissertation report entitled to **"ROS in *Emblica officinalis* (Amla) and its detection in *Emblica officinalis* juice (*pectinase* assisted) and *Emblica officinalis* probiotic drink"** submitted by Navreet Kaur Mann in the partial fulfillment of the requirement for the award of degree of the Master of Technology in Biotechnology, Department of Biotechnology, Thapar University, Patiala, is a record of Student's own work carried out by her under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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Candidate's Declaration

I hereby declare that the work presented in the dissertation entitled "**ROS in *Emblica officinalis* (Amla) and its detection in *Emblica officinalis* juice (*pectinase* assisted) and *Emblica officinalis* probiotic drink"**, in partial fulfillment of the requirement for the award of the degree of Master of Technology, Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work during the period of eleven months from July 2015 to June 2016, under the supervision of Dr. Jyoti Rani, Assistant Professor (FT), Department of Biotechnology, Thapar University. The thesis report has not been submitted for the award of any other degree or certificate in this or any other University.

Place: Patiala

Date: 24/8/16

Navreet Kaur Mann

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Navreet Kaur Mann

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ABSTRACT

The focus of this study was on the *Amla* and its processing into various useful products. Commercially the processed *amla* has been available in various purely or mixed (herbal plant extracts) products. Initiative has been carried out to standardize the process of juice extraction by using industrial enzymes or can be called as enzymatic method using 'pectinase enzyme' over mechanical (juicer) extraction method. *Amla* juice extracted from centrifugal juicer and using pectinase enzyme has been compared in juice extraction rate, overall antioxidant activity, vitamin C, tannins and flavonoids content, sensory characteristics and used for probiotic drink production. The mean values for the juice extraction rates in the case of mechanical and enzymatic methods were 42.63 percent and 78.54 percent, respectively and the pulp leftover was found maximum (35.09%) in case of mechanical method as compared to the enzymatic method (18.51%). The results observed in mechanical (juicer) (92.92 percent, 5.38mg/ml, 9.67mg/ml and 25.7mg/ml), enzymatic method (87.64 percent, 3.56mg/ml, 4.67mg/ml, 27.4mg/ml) and patanjali pure *amla* juice (94.27 percent, 8.02mg/ml, 10.03mg/ml, 23.5mg/ml) for the total antioxidant activity, vitamin C, tannins and flavonoids. All the three samples were analyzed for the antimicrobial activity against the *E.Coli, Bacillus subtilis, Lactobacillus* and *Saccharomyces cerevisae* and found the positive results.

An attempt has been made here to utilize the *amla* seed/stone waste (source for the growth of *Lactobacillus sporogenes*) in developing a 'Probiotic' drink using *amla* juice (juicer and enzymatic method) as a base for the drink along with 'Stevia' a sugar substitute. Among the different combinations made from mechanical and pectinase enzyme extracted juices the P2 (60:40; *amla* juce: probiotic culture in seed/stone powder) formulation of the drink was observed as the best for the antioxidant properties alongwith the alive probiotic culture and sensory characteristics. Keeping the concentration of stevia tablet (0.5mg/ml) constant in all the drinks the cfu/ml of *Lactobacillus sporogenes* in P2 observed was 2.61×10^{8} that meets the recommended cfu/ml (10^{7}-10^{9}) in the probiotic drinks. As a whole pectinase enzyme method was found the best with double juice extraction rate to mechanical (juicer) method in case of *amla* juice with good retention of flavonoids in comparison to other antioxidant properties. The 100 percent utilization can be possible of *amla* using pectinase enzyme for juice and pure *amla* based probiotic drink production as the waste generated would be least in percentage.

Keywords: *Amla* juice, Mechanical extracted juice, pectinase enzyme extracted *amla* juice, Antioxidant Activity, Vitamin C, Tannin and Flavonoid Content, Antimicrobial Activity, Probiotic Drinks, Stevia, HPLC.

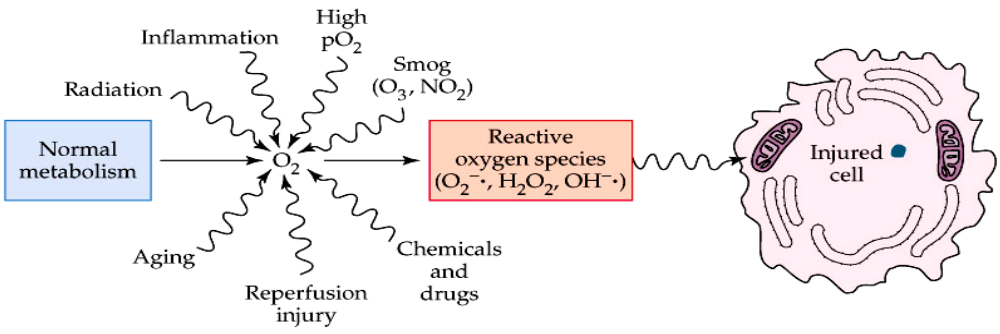
CHAPTER- I

Introduction:

Reactive oxygen species (ROS) are the most chemically reactive molecules or free radicals those are derived from the molecular oxygen like peroxides ($.O_2^{2-}$), superoxides ($.O^{2-}$), hydroxyl radicals ($.OH^-$) and hydrogen peroxide (H_2O_2). These kinds of products have been derived as a byproduct of the normal oxygen metabolism (Devasagayam *et al.,* 2004) and during the mitochondrial electron transport of aerobic respiration (Hancock *et al.,* 2001). Earlier, it has been reported that only phagocytic cells were responsible for ROS generation as a part of host cell defense mechanism but a recent study demonstrated that they impart an important role in cell signaling and homeostasis (Devasagayam *et al.,* 2004 and Hancock *et al.,* 2001). Obnoxious environmental factors also contribute to the production of the ROS in the human body like UV radiations, ionizing radiations, xenobiotics, tobacco smoke (Pinkus *et al.,* 1996 and Bartsche *et al.,* 2000). Normally in a cell metabolism, the oxygen derivatives produced were neutralized or eliminated by the enzymatic action of antioxidants (catalase, glutathione peroxidases and superoxide dismutase) as well as water or fat soluble non-enzymatic antioxidants or vitamins/minerals such as Vitamins E and C, glutathione and selenium (Malgorzata *et al.,* 2005). Ample of ROS are formed due to environmental disturbances, inflammation, aging, chemicals and drugs if the normal metabolism fails to work and these factors can damage the healthy cells. At this point the external antioxidants are required to combat the excess level of free radicals.

Antioxidants are the compounds that neutralize the effects of free radicals and protect the biological system against the harmful effects of the processes and reactions that are responsible for the excessive oxidation. Oxidation is the process in which loss of electrons occurs and due to this it produces free radicals (Bhattacharya *et al.,* 1999) as given in the figure (1).

Fig 1: Free Radical generation



Free radicals are generated as byproducts of normal metabolism like breathing, digestion, exercise, etc. These free radicals scavenge the dead cells and eliminated from the body. Sometimes these free radicals destroy the normal cells which can be called as free radical damage and causes many deadly diseases like cardiovascular, cancer, diabetes, premature aging and many more. Human body has its own antioxidant defense system to combat excess free radicals, but to effectively neutralize the free radical damage it requires dietary antioxidant (bioactive components in natural foods) (Halliwell, 1991). Gradually these free radicals initiate the chain reaction and damage the healthy cells. Termination of this chain reaction antioxidants are must in meals to maintain a healthy life (Sies *et al.,* 1997).

Natural antioxidants or bioactive components that are present in fruits and vegetables fight with these free radicals and protects the body from diseases (Kelly *et al.,* 1998). Most common antioxidants are vitamins, enzymes and phytochemicals found in fruits and vegetables. The vitamins cannot be synthesized in our body, so these needs to be supplemented. The main antioxidant vitamins are vitamin A, C and E (Mantena *et al.,* 2003). Vitamin C is a common antioxidant normally found in *Amla*, Lemon and citrus fruits etc. Other antioxidants are enzymes and they can be uniquely synthesized in the human body. These can be easily available in food that we eat on the daily basis. Food composed of proteins and minerals that are good source of enzymes as antioxidants (Vivek *et al.,* 2006) while others antioxidants are the phyto-chemicals normally present in the plants (cereals). Phyto-chemicals such as tannins, flavonoids and carotenoids etc. are present plant sources foods and having efficient antioxidant activity (Vivek *et al.,* 2006). Beyond three main categories several antioxidants role is played by selenium, manganese, copper and zinc (Shirwaikar *et al.,* 2004).

***Amla*:** It is the richest source of antioxidants for different types of antioxidants. English name of the *amla* is Indian gooseberry and the scientific name is *Emblica officinalis* (Dubois *et al.,* 1956) (Synonym is *Phyllanthus Emblica linn*. (satyajit *et al.,* 2012) and it belongs to the family of Euphorbiacea (Maurya *et al.,* 2011).

Fig 2: Fresh *amla* fruit

***Amla* phenotypic characteristics**: *Emblica officinalis* is a medium to large deciduous tree. Leaves are simple and linear-oblong blunt and 8-10mm long with greenish yellow color flowers. Fruits are small nearly globular or spherical in shape about 18-25mm wide and 15-20mm long that grows wild in the tropical forests of India, as well as in the tropical and subtropical areas of China and Myanmar (Krishnaveni *et al.,* 2011) (1). The best harvesting time for *amla* is in February when the fruits contain maximum ascorbic acid content while in the south India it is available throughout the year. A mature tree usually >10 years old will yield 50-70 kg of fruit (2).

Amla is the richest source of Vitamin C next to Barbados cherry and it is quoted out by Shankar *et al.,* 1969) and Rajesh *et al.,* 2001). Barbados cherry belongs to the family of Malpighiaceae. The scientific name of the Barbados cherry is *Malpighia emarginata*. The common names are acerola, Indian west cherry and it is extremely rich in vitamin C (Johnson *et al.,* 2003). Vitamin C is needed for human being to stay healthy. It is necessary for the synthesis of inter-cellular cementing substances that are very much responsible for binding the body cells together (Singh *et al.,* 2011). *Amla* has been proved as an excellent source of ascorbic acid or vitamin C, mare amino acids like glutamic acid, proline, aspartic acid, alanine, and lysine of the total amino acids (Krishnaveni *et al.,* 2010). It contains many bioactive components like tannins, alkaloids, flavonoids and phenols (Zhang *et al.,* 2003). Emblicol, linoleic acid, orilagin, phyllemblin and rutin are the minerals found along with various phytochemicals in *amla* (Ghorai *et al.,* 1996). Compounds which were isolated from Emblica fruit were gallic acid, chebulinic acid, chlorogenic acid, ellagic acid, chebulagic acid, quercetin, kaempferol (Zhang *et al.,* 2003). From all these compounds quercetin and ellagic acid were secondary metabolites which were the most biologically active and commonly used as dietary phenolic compounds to cure the risks of developing cancer, and cardiovascular diseases (Kamaraj *et al.,* 2007). The percentages of other chemical components present in the *amla* were protein (0.5%), fat (0.1%), carbohydrate (14.1%), fibre (3.4%) (Krishnaveni *et al.,* 2011). The major water soluble antioxidant occurs in the human body as Vitamin C (Sies *et al.,* 1995 and Levine *et al.,* 1995). Its main function was reported to lower down the blood pressure and cholesterol level (Rath, 1993). A study had shown that *amla* has 20 times more vitamin C as compared to orange juice. Many varieties were reported in India were Banarasi, Chakaiya, Krishna, Francis (Hathijhool), Kanchan (NA-4), NA-7, NA-6, Anand-1, 2, 3 (Goyal, 2008 and Singh, 2009). *Amla* has been processed into different products because

it is very difficult to consume directly due to its astringent and sour taste. The different processed products of *amla* are available in the market like preserves, juice, cheese, candy, jam, and powder (Tripathi *et al.,* 1988), chutney, ready-to-serve beverage, *amla* bar (Mishra *et al.,* 2011 & 2012). It has been found as a main constituent of many ayurvedic preparations like Chyawanprash and Triphla (Pant *et al.,* 2004; Goyal *et al.,* 2007 and Mishra *et al.,* 2009).

Amla is used to cure various ailments like anemia (Padma *et al.,* 2014), diarrhea, anomalies of urine, eye inflammation, leucorrhea, hyperacidity, liver complaints, jaundice and cough (Rastogi *et al.,* 1993). *Amla* also plays an important role in digestion and absorption of nutrients (Padma *et al.,* 2014). Apart from curing diseases it has a duty-bound role in curing different types of cancers like liver cancer, skin cancer etc. A few studies speculated preventive effects of *amla* against the liver cancer in chemo therapy form (Sultana *et al.,* 2008). In skin cancer, *amla* helps reducing the incidence of tumour, tumour yield, and cumulative number of papillomas (Sancheti *et al (*2005). It has been noticed that *amla* also owed a role in inhibition of heavy metal mutagenesis in mammals (Madhavi *et al.,* 2007). In an outcome the ethanolic extract of *amla* significantly enhanced the glutathione reductase, glutathione peroxidase, glutathione and significantly detoxified the enzyme glutathione-S-trasferase and also found to be to the antioxidant related because of its modulatory effects in enzymes detoxification (Banu *et al.,* 2004). Every part of *amla* plant exists with great medicinal and nutritional properties and because of this it was purposely used in Ayurveda preparations for animal as well as human therapeutics (Satyajit *et al.,* 2012). The leaves were used against the cold, anaemia, fever, dysentery, gravel, sores etc. (Nain *et al.,* 2012). It can be used as chemical free mouthwash for aphthaous mouth ulcers treatment (Treadway, 1994, Nadkarni *et al.,* 1999). *Amla* leaves combination with fenugreek seeds can be used to cure diarrhea (Jayaweera *et al.,* 1980). If, fresh green leaves mixed with the curd it showed a miracle to cure carminative and stomachic effect (Nadkarni *et al.,* 1999). Hence, *amla* had versatile medicinal applications due to its numerous phytochemical and pharmacological properties. It was considered as safe herbal medicine with no side effects (Krishnaveni *et al.,* 2011).

To maintain good human health, all plants have been used as valuable sources of bioactive compounds that act as antibacterial and antifungal (Nain *et al.,* 2012 and Satyajit *et al.,* 2012). Usage of the plant extracts and juices encountered with liable antibacterial or

antimicrobial properties having a great significance in therapeutic treatments. The maximum concentration of vitamin C, flavonoids, tannins in *amla* fruits serve as antioxidants in antagonisic action against disease (Drury, 1873). Many reports revealed that *amla* has proven in-vitro and in-vivo antibacterial or antimicrobial activities. Nowadays, various antibiotics and numerous synthetic antibacterial drugs were used to cure the bacterial diseases has many side effects. Drugs get accumulated in the body tissues and fluids which sometimes cause resistant strains development which was further more dangerous in several situations. So, the demand of the day is to resolve a safe and effective therapeutic to treat infectious diseases. Observing all these situations, presently scientists were focusing on natural products to overcome the problem of bacterial infections that has been found in *amla* with proven significance especially in antibacterial and antifungal properties (Nain *et al.,* 2012).

Food preservation is a method for better utilization of fruits and vegetables by avoiding the glut and utilizing the surplus during the off-season. So it is necessary to develop modern methods to enhance the storage life (Vidhya and Narain, 2011). One method is to preserve the fruits by converting into jams, jellies, fruit bars, murabbas, pickle etc. but the fruit juice is the best method to preserve the fruits like *amla* without changing the natural constituents. Productions of fruit and vegetable juices have importance for human health as well as from the commercial standpoint (Bhat, 2000). The traditional method for juice extraction was mechanical method commonly using juicer, belt press, screw press etc. Diffusion extraction, decanter centrifuge can also be used for juice extraction (Beveridge and Rao, 1997). The juice conversion of *amla* fruit commonly pursued by mechanical juicers or mechanical procedures industrially. But, now production of the juice with the help of enzymes had a great significance in juice industry (Kashyap *et al.,* 2001). But today, different enzymes have been produced from microbial sources from various food waste can be used for extraction of juices. The enzymatic treatment results in a better way as compared to the mechanical methods along with enhancing the juice recovery (Joshi *et al.,* 1991), processing efficiency and acceptability of final product in terms of appearance, color, flavor and taste with natural undisturbed disease fighting potency (Harsh *et al.,* 2014). The enzymatic method not only increased the yield but also ensured the good quality of end product (Kilara, 1982). It has also been claimed that enzymatic method offers a number of advantages over the mechanical method. The enzymes commonly exploited

for juice extraction were *pectinases* and *cellulases* that hydrolyze the cell wall and results in increase or juice extraction yield (Joshi *et al.,* 1991).

With new additions and innovations in food fermentations, popular terms are “Probiotic” and “Prebiotic”. The probiotic means “for life” which was coined by an Expert Committee of WHO as ‘‘live microorganisms which upon consuming in certain numbers exert health benefits beyond inherent general nutrition”. The probiotic foods are categorized under functional foods (FAO/WHO 2001). Probiotics are the live micro-organisms which can be reproduced into different type of products like drugs, foods, and dietary supplements and which when consumed in sufficient amount confers good health benefits on the host (WHO 20002). Probiotics has been used in fermented dairy products from centuries like commonly in curd, buttermilk, fermented drinks consumed with the micro-organisms like kefir etc. “Prebiotics” is the term related with the carbohydrates/sugars or dietary fibre required for the growth of probiotic bacteria in the human intestine like inulin, pentose sugars etc. If we combine both prebiotic and probiotic together in a food than the conditions should be such that the growth must not be hampered of the probiotic bacteria in the natural existence in the food will be called as Symbiotic foods. But recently, trends has been moved to agricultural and food applications of probiotics and resulting in various probiotic drinks and foods in the market. Nowadays, the selection of new probiotic strains and the development of new application have gained very much importance because of their proven sustainable effect on the human immune system.

The uses of probiotics showed many health benefits to the human and helped in normal digestion process and in maintaining the animal’s health (Krockel 2006). The health benefit of probiotics depends upon their concentration in foods, and ability to survive in adverse conditions of the gastrointestinal tract (Prado *et al.,* 2008, Marshall *et al.,* 1984). The other applications of probiotics in agricultural sectors with regard to animal, fish, and plants production have increased gradually (Krockel 2006). The bacterial strains that were most commonly used in probiotic foods and juices are *Bifidobacterium* and *Lactobacillus spp.* and some other species were *Escherichia coli* and *Bacillus spp*. are used as probiotic micro-organisms. Not only bacterial strains are used as probiotics but yeast strains can be used as probiotic such as *Saccharomyces cerevisiae* (Song *et al*., 2012)). Lactic acid bacteria (LAB) can serve dual function by acting as food fermenting agent and potentially health benefits provider. The

characteristics for probiotics must be like gram-positive rods with round ends, catalase-enzyme negative and paired occurrence, short or long chains (Von *et al.*, 2000). They should not be spore forming, non-flagellated, non-motile and intolerant to salt. Optimum temperature for the growth of probiotics is 37°C but some strains such as *L. casei* prefers 30°C. In a probiotic drink the recommended number of colonies must be 107cfu/ml at the end of the shelf life (Nualkaekul *et al.*, 2011).

Proper formulation of a drink requires sweetness. Though, table sugar provide sweetness, bulkiness, flavor enhancer and act as moisture binder in shelf life a beverage but provide calories that makes a drink unfit for most of the diabetic consumers. So, considering the above repercussions development of a composite drink of antioxidants, immunity booster with less of calories has been designed. For the same various non-caloric sugars are available for substitution like Acesulfame K, aspartame etc but to introduce a natural source of calorie less sugar substitute like stevia would be more beneficial over others. Stevia is natural herbal sweetener with botanical name *Stevia rebandiana*. Sweeteners can be categorized into two caloric and non-caloric that can be further categorized. From all of these stevia comes under non-caloric natural sweetener. The leaves and the stems of *Stevia rebandiana* produces steviol glycoside which has been reported 100-300 times more sweetener than table sugar or sucrose alongwith therapeutic benefits (Kylie *et al.*, 2015). If comparing the sweetness only 50 gram of stevia powder provide sweetness equal to 1kg of cane sugar. Beside as an intense sweetener it has antifungal and antibacterial activity. It can be safely used by the person who is suffering from phenylketonuria. Its leaves can be substituted to chocolates even candies to meet the craving of diabetic person for sweetness and deter tooth decay. Several studies acknowledged stevia as non-toxic, non-addictive and non-carcinogenic. Its usage has been increased in daily routine as well in beverage industry. Today, concoction of stevia leaves with tea and coffee ensured value addition simultaneously easing the preparation of the drink. Hence, the stevia has been used because of its wide applications and no side effects (Modi, 2012).

Keeping in view the exceptional therapeutic and antioxidants action against various chronic diseases in human beings made the *amla* to be utilized extensively in various forms like medicines and other processed products. In the similar way this study is also based on certain objectives.

1. Process standardization of *amla* juice production using pectinase enzyme in comparison to the established method of mechanical juicer.
2. 100 percent utilization of *amla* seed waste to use it as base for probiotic culture growth.
3. Application of pectinase extracted *amla* juice in combination with probiotic culture grown on *amla* seed waste and stevia a natural non-caloric sweetener to provide sweetness or can be called as ‘Development of Probiotic *Amla* drink’.

CHAPTER – II

2. Review of Literature:

2.1. About *Amla* and *amla* products

Amla can be called as the Indian Gooseberry, *Emblica officinalis* (scientific name) an important horticulture crop of Indian arid zone which is being grown in the India for last many centuries. It may be called that *Amla* belongs to Indian origin. The heterogeneity of *amla* has been reported in the North Eastern states (lower Assam, Mizoram, Meghalaya and Tripura) (Yadev *et al.,* 2001). It has been claimed that production of *amla* has been highest in the forests of Khasi and Gharo hills of Meghalaya and due to this also known as Sohmylleng (Pandey *et al (*1993). Hore, (1998) observed that Anolain, the natural population in Khasi hills has been declared as the gene sanctuary for *amla* species. The *amla* (*Emblica officinalis*) belongs to the family of Euphorbiaceae. It is a medium sized tree normally reaching to a height of 10-20 meters.

Amla or aonla has been recognized as an important fruits among other fruits especially in tropic and sub-tropic areas of India. In the several regions of India the Indian Gooseberry showing tremendous improvement to horticulture production, especially in Punjab's Kandi area where it is considered as a do able option to other horticultural and cereal crops. It is emerging as an important minor crop and achieving commercial status in the processing area. The main advantage of growing *amla* over other horticultural crops is that, it is easily adapted to xenophytic conditions which encounter recurrent crop failure and thereby economic crises. It has been a boon to marginal land owing farmers in terms of high remuneration. The cultivation area under *amla* is increasing every year because cheap inputs in its growing and very low establishment cost. Keeping in view the plantation of *amla* fruit is on hike in Indian Northern regions.

Amla has medicinal properties and therapeutic values which made it popular in the world. It can be used in either fully or partly. The seed and pulp has its own applications in the medical sector. Singh *et al* (2003) reported that the medicinal preparations from the *amla* has high potency in curing different diseases like anemia, common cold, fever, diarrhea, dysentery and

jaundice because of its acidic, cooling, diuretic and laxative properties of the *amla* fruit. A recent study had shown that anola or *amla* juice consist of antioxidants in the form of polyphenols and the ascorbic acid which helps in maintaining healthy cholesterol levels, thus providing a cardio-protective effect (Pathak *et al.*, 2003). The scientists have focused their attention to discover its nutritional and medicinal properties. Aman, 1969 worked on invaluable qualities of *amla* and characterized its beneficial uses for good health and longevity maintenance. In ayurvedic and unani system *amla* is a crucial herbal fruit indoctrinated medicine by virtue of its healing synergistic action and presence of antracin and phenol (Jain *et al.,* 1983).

Externally the berry is hard to touch so less damage during transportation. As a reason of high antioxidants concentration results into bitter and highly acidic nature of fruit causing less consumption by consumer as raw, that is why a variety of products were obtained after several types of processing and preservation methods. Due to a seasonal crop *amla* is very limited and only available in the month of October, November, December and January. After harvesting the most important is cold storage to protect it from mold growth and scalding on the surface. Different kinds of pre-treatments and storage conditions enhance the shelf-life of the fruit. As observed by research some *amla* fruits post-harvest life is short so it is necessary to be process it into different products for yearlong consumption. Even though, perishable *amla* berries can be utilized into variety of products and yield good returns from both internal and foreign markets (Gomez and Khurdiya, 2005). With globalization of beverages, cold beverages are going popular day by day and consumed almost throughout the year but mostly the synthetic beverages with low nutritive value (Kannan and Bannumathi, 2005). These synthetic drinks don't provide nutritional value except added calorific sugar, water and synthetic flavors in comparison to fruit based drinks or beverages contain dietary fibre, minerals and vitamins in addition to calories. Hence, the fruit based drinks possess more nutrition than the synthetic drinks. As lack of time in today's life made consumer more health conscious which results in more fruit drinks consumption as compared to the synthetic drinks; thereby increasing a great scope towards the production of the fruit based beverages. The Buvaneshwari and Gowda (2006) reported that due to *amla*'s acidic nature it is generally utilized for making blended beverages with other sweet fruits. Many researchers have explored its possibilities of utilizing in a different way and juice and beverages preservation. The Nath (1999) noticed that the *amla* beverages can be a base to increase the vitamin C content of other fruit based beverages. Vitamin C malnutrition can be

overcome by use of *amla* juice substituted drinks for vitamin C to replace the synthetic drinks. *Amla* taste is astringent affecting its consumption as a dessert fruit. Still there is tremendous scope for its processing into various fruit/juice based beverages like squashes, nectar, syrups and ready-to-serve (RTS) drinks because of its high tannins, vitamin C content, minerals and medicinal properties (Singh and Kumar, 1995).

2.1.1. Amla based products:

Presently in FMCG sector *amla* covers below 2 percent of products in the market. In this part of review of literature *amla* based products and changes in their nutritional qualities during processing and storage have been explained. Now various types of *amla* based products have been in the market in different categories like siddha, food and Ayurveda products. Dried & dehydrated using hot air (*amla* powder) and osmotic dehydrated products (preserves, candies) have been developed from *amla*. The description of such products is as below:

A) ***Amla*** **juice:**

One of best processed product from *amla* that can be easily consumed with high active antioxidants and vitamin C concentration is pure *amla* juice. Anup *et al* (2011) investigated the correlation of change in color and quality of *amla* juice during storage period. They extracted the juice and pasteurized at different temperatures such as 75°C, 89°C, 90°C and 95°C and further preserved with the sulphur dioxide solution in PET bottles and stored for 9 months under ambient conditions. As a result the Vitamin C and the polyphenols contents were decreased with the increase in storage time. But, an interesting fact was found that initially the gallic acid content in juice decreased but increased sharply as the storage period prolonged. Among different pasteurization temperatures 80°C was found the optimum for the preservation of the *amla* juice under the ambient conditions.

Nath (1999) carried out a study on the extraction of *Amla* pulp and emanated a method for *amla* pulp separation from fully matured fruits. In his designed method, washing of *amla* followed by blanching in boiling water for about 10 minutes to separate the segments from stone. After separation equal quantity of water was added to the segments and in the pulp, ended with properly mixing. To preserve the pulp, it should be pasteurized at 75°C and cooled at room

temperature. Preservative potassium meta-bisulphite (2g/kg of pulp) should be mixed properly and the pulp should be filled in clean sterilized bottles and then sealed.

2.1.2. Health benefits of *amla* juice:

Regular consumption of *amla* juice with honey twice a day proposes a number of medicinal benefits like against diabetes, cold blood purification and also cure weakness (4).

I) **Relieves Asthma And Bronchitis:** The chronic diseases like asthma and bronchitis can be easily cured by the regular consumption of *amla* juice with honey in morning and evening. Even it causes reduction in the chances of the tuberculosis, chronic cough and allergic asthma. It also relieves asthma and bronchitis complications and reduces the incidence of chronic cough, allergic asthma and tuberculosis (4).

II) **Treatment of Gastric Disorders:** *Amla* juice is also used for the treatment of gastric disorders as well as hyperchlorhydra (burning sensation in abdomen). *Amla* juice is very much effective for the treatment of peptic ulcers and acidity. The acidity problem can be reduced by consuming *amla* juice with pure ghee twice a day. It is good for the treatment of diarrhea and dysentery (4).

III) **Gout:** Gout is the inflammation of the big toe that is caused by defects in uric acid metabolism resulting in the acid deposit and salts in the blood and joints. This problem can be overcome by consuming *amla* juice with the old ghee which helps in softening of joints and helps in curing gout.

IV) **Piles:** It has been studied by Mirunalini *et al* (2013) that the piles problem can be cured by drinking *amla* juice half teaspoon of ghee and 1 teaspoon of honey 100g of milk after lunch.

B) ***Amla*** **squash:**

Nath (1999) reported another *amla* product that is *amla* squash. In which he cut the deseeded fruits into small, crushed further added with equal quantity of water leads to filtration. At the end to 1.86kg of each liter of extracts at 0.5 percent 30Kg of citric acid and at 0.18 percent 10g of KMS were added and then heated for some time. The color and flavor

at 100ppm per kg of the extract were added and poured into sterilized bottles and sealed (Bahadur *et al.*, 2010).

C) *Amla*-candy:

As reported by Tendon *et al.,* in the year 2003 that the blanching and lye peeling effects the vitamin C content during preparation of the *amla* candy which was found to be decreased in the blanched fruits. The best results were shown by Lakshmi-52, Kanchan and Chakaiya varieties in *amla* candy preparation. According to Agarwal and Chopra (2004) the changes in the total phenols and ascorbic acid content during the storage were different in various *amla* products. However, the *amla* shreds recorded with greater vitamin C concentration loss followed by jam, candy and squash. The candy showed the maximum loss in phenolic content followed by the shreds and squashes but jams showed the slight increase in total phenol content during the study. At the end the results observed that the squash retains highest total phenols percentage and vitamin C concentration.

C) ***Amla Preserves/Murrabba:***

Results showed by Goyal *et al.* in the year 2008 regarding various kinds or types preserves or murrabbas used for medicinal purpose and sensory enhancement that *amla* has been acclaimed to impart energy to brain, liver and heart. Even *amla* murabba stops diarrhea and act as a sustainable remedy for giddiness. Banarasi *amla* preserves when treated with salt and alum combination had proved to be the superior combination to retain maximum vitamin C content, total soluble sugars, reducing sugars, acidity and total sugars which can be stored at room temperature for 5 months (Sahu *et al.,* 2010). Earlier, Geetha *et al.* in 2005 revealed that the effect of the sugar content at different temperatures has a positive effect on the kinetics of the moisture loss, total soluble solids except ascorbic acid which showed loss in the *amla* preserve. It has been proclaimed that with the consumption of *amla* murabba every day in morning provides physical and mental strength to human body (Mirunalini *et al.,* 2013).

E) *Amla* shreds and *Amla* flakes:

Pragati *et al* in the year 2003 studied the effect of different methods of drying such as osmo-air drying, direct sun drying, indirect solar drying and oven drying on *amla* fruit and hence

observed that the osmo-air drying method among all methods was the best for *amla* drying with better retention of nutrition such as vitamin C, sugars and less acidity. The levels of anti-nutritional factors such as tannins content was found to be lower in osmo-air dried *amla* because of minimum leaching and browning. It can be stored for 90 days with undisturbed nutrition content. Further, Prajapati *et al.*, 2011) reported that the best quality *amla* shreds were obtained by using 0.1percent of KMS blanching followed by solar drying with the addition of 3 percent common salt. The most acceptable *amla* shreds had the composition of total sugar 24.0 percent, tannins 2.4 percent, reducing sugar 3.0 percent, non-reducing sugar 21.0 percent, ascorbic acid content 298.3mg per100g and acidity 2.6 percent.

F) *Amla* pickles:

The procedure of *amla* pickles for commercial production was claimed by Nath (1999). *Amla* fruit were cut into 4-6 pieces vertically after washing and deseeding. Then at 15percent per kg *amla* 150g of salt was added to the *amla* pieces and kept overnight. Several spices were grinded and added for taste such as 20g mustard seeds, 30g chilies, 10g turmeric powder and 40g fenugreek. After spices mixed properly the *amla* with spices was kept overnight followed by addition of 200ml heated oil/kg of *amla*. After that all the material was poured into jar or pouched and sealed.

G) *Amla* powder:

Thankitsunthorn *et al* (2009) standardized the method for production *amla* powder using spray drying method at $2.4x10^2$kPa pressure with inlet temperatures of 120°C and 160°C and the outlet temperature 80°C. The feed rate and the temperature of the drying was set to 1.2ml/min and 120°C respectively which was observed with more ascorbic acid content, with less water activity relatively which has been considered as stable for microbial growth. During storage *amla* powder there was no recordable change observed in the water activity when it was stored in air tight HDPE for one month and kept at cold temperature. The different methods used for the *amla* drying were like sun drying, spray drying, hot air drying, freeze drying and vacuum drying. Again, Mishra *et al* (2009) used these different methods for drying especially Chakiya variety of *amla* fruit and revealed that vacuum drying method gives more yield followed by solar dying, tunnel drying, spray drying and freeze drying. But the freeze dried powder exceptionally

observed with maximum vitamin C concentration followed by spray dried powder. Sun dried powder had the least vitamin C concentration.

2.1.3. Some health benefits of *amla* powder:

I) **Eye tonic:** Most commonly used Triphala powder (Hirda, behde and *amla* powder) if mixed with honey improves the eye sight good for the digestive system which has been reported by (Biswas *et al.,* 2001).

II) **Action on diabetes:** It was reported by Devalaraja *et al* (2011) that *amla* powder regulates the blood pressure. Triphala powder contains commonly three herbs *amla*, bihara and harada. Due to the alanine transaminase enzyme action which is present in liver the blood sugar level may be increased but it has been noticed that the action of the enzyme can be normalized by consuming one teaspoonful of mixture of equal quantity of the *amla*, jamun and bitter gourd powder once or twice a day. The chromium mineral boost the anti-diabetic effect of *amla* fruit which is present in it.

III) **To treat cardiac disease:** Cholesterol considered as the root cause for various diseases in human body is. As claimed by Bhattacharya *et al* (2012) that due to cholesterol body is prone to diabetes, Hypertension and heart disease. The unused cholesterol is collected into the blood vessels that lead to the high blood pressure which increases the chances of the heart attack. Dried *amla* powder mixture in combination with sugar candy decreased the cholesterol level. The neutralization of cholesterol level in blood was observed when one teaspoonful of this mixture was taken before meal or empty stomach in the morning with a glass of water. Ascorbic acid in *amla* causes enlargement of blood vessels and reduces blood pressure as reported by Anila *et al* (2002) and Kim *et al* (2005).

2.2. Composition of the *Amla:*

Amla said to be the richest source of ascorbic acid content (Shanker, 1969) alongwith the higher content for tannins was found responsible for the antioxidant properties. *Amla* pulp contains Vitamin C ranges from 200-900mg/100gm of the fresh fruit pulp as recorded by (Kalra, 1988). Again, the vitamin C concentration in the Indian gooseberry was found to be 160 times more than that in apple fruit as revealed by Barthakur and Arnold (1991). Goyal *et al* (2008)

made known that the *amla* consist of good amount of pectin and so, a good source to produce different products such as murraba, squash, jelly, pickles, candy and squash. Alongiwth this he also said that one serving of *amla* (100g) had moisture content of 81.8 percent, ascorbic acid (600mg), fibre (3.4 gm), carbohydrates (13.7gm), protein (0.5gm), fat (0.1gm), calcium (0.05 gm), niacin (0.2gm), iron (1.2mg), phosphorus (0.02gm), thiamine(0.03gm), carotene (9µg), riboflavin (0.01mg) and calories (58).

Amla fruits chemical composition is majorly influenced by environmental factors and the genetic factors too. For example Chakaiya variety of *amla* fruits was found to be a rich source of ascorbic acid (454.40mg/100g), good amount of total sugar content 7.53mg/100g), calcium (14.91mg/100g), Iron (0.62 mg/100g) and phosphorus (11.81mg/100g) (Dachiya *et al*., 2001). The banarasi variety *amla* fruits were observed with the moisture content of 84.36percent, pH 2.5, acidity 2.24percent, ascorbic acid 571mg/100g, total sugars 3.11percent, tannins 0.55percent and proteins 0.88 percent. Tripathi *et al* (1988) reported that the *amla* fruits were rich in ascorbic acid and tannins content. Further, noticed by the Kalra *et al* (1988) that the total sugars content among the various varieties of *amal* fruit ranged from 7 to 9.6 percent, reducing sugars from 1.04 to 4.09 percent and non-reducing sugars from 3.05 to 7.23percent. *Amla* has been cited as richest in vitamin C. The pulp of fresh *amla* berry contains approximate 200-900mg/100g of ascorbic acid in concentration. Khan (2009) described the different compositions of *amla* and thus concluded that it contains amino acids, carbohydrates, tannins, alkaloids and phenolic compounds. Chemical compositions composed of alkalods, Phenolic compounds, Amino acids, Carbohydrates, Vitamin C, Flavanoid, Ellagic acid, Chebulinic acid, Quercetin, Chebulagic acid in *amla* fruit.

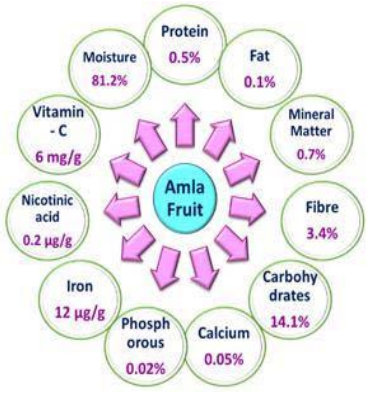


Fig 3: Composition of *amla*

2.3. Industrial Microbial Enzymes-*Pectinase*:

Enzyme pectinase that breaks down proto-pectin present in unripe fruits like guava, *amla* etc. It is exist in plant cell walls commonly in the plate (middle lamella), during cytokinesis it is the first part of the wall that is developed, following division of cell. Thus, it helps to break down the cell walls bound pectin that is proto-pectin. Indirectly, helps in increasing the juice volume obtained (percentage of yield), viscosity lowered of the juice (fluid characteristics), and cloudiness of the juice reduced, due to the suspended cell wall pieces.

At first enzymes to be used in homes were pectinases among some. For the first time that was used in commercial application in 1930 for wines and fruit juices production. During 1960s only, idea to explore the chemical plant tissues nature to become apparent with its usage, this knowledge provides a base to the scientists to use a wide range of enzymes more efficiently. So, now pectinases are one of the extensively used enzymes in commercial sector. Primarily, these enzymes cause degradation of the long and complex molecules called pectin (Kashyap *et al.*, 2001).

2.3.1. Structure of Pectin:

The pectic are very complex substances and are colloidal of acid polysaccharides. They are joined with the backbone of galacturonic acid residues by (1±4) linkages. L-rhamnose, xylose, galactose and arabinose are the side chains of the pectin molecules. The carboxyl groups that are present in the galacturonic acid are partially or completely neutralized by the potassium, sodium or ammonium ions and are partially esterified by methyl groups. Due to the changes in the backbone of pectic substances these can be further classified into pectic acid, pectinic acid, protopectin and pectin (Miller, 1986).

Pectic Acids: The pectic acids are galacturonans that contains very much less amount or negligible amount of methoxy groups. The term pectates are coined the normal or acid salts of the pectic acids.

Pectinic Acids: In this the various amount of methoxy group is present in the galacturonans. The pectinates are acid or normal salts of pectinic acids (Kilara, 1982). The pectinic acid has the unique property that it has the ability to forming a gel with sugar and acid

Protopectin: It is the main pectic substance and when hydrolysis occurs it produces pectin or pectinic acid. It is the term which is used to describe the water-insoluble pectic substances found in plant tissues and from which soluble pectic substances are produced (Kilara, 1982).

2.3.2. Biotechnological Applications of Microbial Pectinases:

Pectinases have the wide application in the various areas such as textile, tea, coffee, oil extraction, plant fiber processing, containing pertinacious material and treatment of industrial wastewater etc. Salazar and Jayasinghe (1999) reported that pectinase have also works on purification of the viruses and in making of paper (Reid and Richard 2004 and Viikari *et al.*, 2001).

Among other enzymes the pectinase is one of the most important industrial enzyme. The biotechnological potential of pectinolytic enzymes isolated from microorganisms has diverted the attention of the researchers in the current biotechnological arena with wide ranging applications (protopectin) in different sectors. The pectinolytic enzymes are used to mineralize pectic substances that are present in the environment. The microbial alkaline pectinases have the wide application in environmental sectors and it is revealing their underestimated potential.

2.3.3. Fruit Juice Extraction:

Blanco *et al* (1999) reported that the pectinase has the largest industrial application in juice extraction and clarification. The combination of the pectinase and the amylase have been found effective in clarifying the fruit juice. They also reported that it decreases filtration upto 50 percent. Again Kaur and Kumar (2004) showed that the apple, grapes and banana juice volume increases when it was treated with the pectinase enzyme. The pectinase can combine with other enzymes like arabinases, xylanases and cellulases to increase the pressing efficiency for juice extraction of the fruits (Gailing *et al*., 2000). Vacuum infusion of pectinase is the technique used to soften the peel of citrus fruits and this technique may be popular in the future to replace hand cutting for the production of canned segments (Baker and Wicker, 1996). Infusion of free stone peaches with pectin methyl esterase and calcium increases the firmness of the fruits for four times. It can be applied to the processing of pickle for softening during fermentation and storage (Baker and Wicker, 1996).

2.3.4. Industrial Applications:

In the overall manufacturing of enzyme preparations the pectinase production occupies 10 percent of the total preparations. These enzymes are mostly used in the food sector in the production of the fruit drinks, wines and juices (Semenova *et al*., 2006).

2.3.4.1. Acid Pectinases

The acid pectinase enzyme can be isolated from the fungi especially *Aspergillus niger* and it was used in the removal of the pectin in the fruit juices, used for clarification and in maceration of the vegetables to produce the pastes. The pectin layer of the fruits stays linked with the fruits and hindering the juice extraction process by pressing. It increases the viscosity of the juice. The addition of the pectinase enzyme to extraction process degrades the pectin layer and thus, improves the fruit juice yield with decrease in juice viscosity, improved concentration capacity. It was reported that the combination of the pectinase and cellulases improved the juice extraction yields by 100 percent (Alkorta *et al*., 2005 & Kashyap *et al*., 2001). Rombouts *et al* (1980) reported that the extraction of fruit juices by enzymatic maceration can enhances the juice yield by more than 90 percent as compared to the mechanical method of the juice extraction. The juices that are extracted with the help of enzymes also improves the organoleptic (color and flavor) and the nutritional properties also improves the ease of filtering. It is reported that the combination of the pectinase and cellulases improves the juice extraction yields by 100 percent. Alvarez *et al* (1998) reported that the enzymatic treatment helps to decrease the apple juice viscosity by 62 percent. While ultrafilteration, it was found that the permeate flux is much higher in depectinized apple juice as compared to the undepectinized juice.

Table 1.Pectin content of fruits and juices

Sources	**Pectin**	**Esterification (%)**
Lemon	32	50-65
Apple	0.5-1.6	70-90
Grape juice	0.01- 0.09	-
Apple juice	0.2	-
Grapes	0.2-1.0	10-65
Orange (juice sacs)	16	-
Sugar beet (pulp)	30	-

2.4. Antioxidant properties of *amla*

2.4.1. Antioxidant Activity:

The antioxidants are the molecules individual or in combinations which can inhibit or delay the lipid oxidation as well as of other molecules by blocking oxidative chain reaction commencing (Tachakittirungrod *et al.,* 2007). This effect can be performed by different mechanisms like metal chelation, activity of antioxidative enzymes, reducing power, inhibition of scavenging activity against reactive oxygen species (Pinkus *et al.,* 1996). In recent studies, it was reported that plants contains a wide variety of free radical scavenging molecules like flavonoids, polyphenols and vitamins which were reported as excellent in antioxidant activity (Miller *et al.,* 2000).The antioxidant compounds found in food was found to play an effective role as protecting agent. It has been scientifically proved that the antioxidant reduces the risk of the chronic diseases. The whole grains, vegetables and fruits are primary source of naturally occurring antioxidants. Klimczak *et al.* (2007) concluded that vitamin C as the most valuable water-soluble antioxidant. Mostly the fruits and vegetables supply more than 90 percent of vitamin C to the human body which is synthesized in the body as L-ascorbate in phylogenetically higher animals. Ascorbic acid is the main biologically active form in our body but the oxidized form of ascorbic acid is L-dehydroascorbic acid also exhibits biological activity. It has been reported that the ascorbic acid act as an antioxidant which reduces or inhibits the risk of cardiovascular diseases, arteriosclerosis and cancer (Sarkar *et al.,*2009 and lee *et al.,* 2000). Kaur and Kapoor (2001) observed that in most biological systems vitamin C protects the compounds in intracellular and extracellular spaces also inhibits the tocopherol radicals back to their functional form at the cellular membranes.

Khopde *et al* (2001) postulated that *amla* extract showed antioxidant performance against gamma radiation-induced lipid peroxidation in microsomes and inhibits damage to superoxide dismutase in mitochondria of rat liver but its action was found to be both dose and concentration dependent. Moreover, the extract was more water-soluble that may scavenge the free radicals responsible for initiating lipid peroxidation. In *amla* amount of ascorbic acid was standardized by HPLC and titrimetric methods resulted in 3.25 to 4.5 percent w/w. However, inhibition in LPO was not observed in microsomes containing above mentioned composition of pure ascorbic acid alone. To estimate the ascorbic acid equivalents, cyclic voltammetry of the *amla* extract was

conducted which was observed with 9.4 percent w/w of *amla*. Compared with the reactivity of both *amla* and ascorbic acid towards ABTS assay radical, a stable free-radical 9.4 percent value was found to be acceptable. As a consequence it can be stated that is a more potent antioxidant than vitamin C.

Determining the antioxidant activity through different methodology was conducted by Vasudha *et al* (2009) and postulated that *amla* extract in aqueous has the maximum antioxidant activity as compared to *spirulina* and wheat grass and it was 7.78mg/ml. To ponder between the antioxidant activity of *amla* extract and alcoholic extract showed reductions in antioxidant activity approximate 6.67mg/ml. The methods used were titration for vitamin C concentration and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) for antioxidant activity.

In 2012, Vinita *et al* showed that fresh *amla* endures maximum vitamin C concentration and antioxidant activity to the processed *amla.* The methods used for the determination were DPPH (2,2-diphenyl-1-picrylhydrazyl) percent scavenging activity and titration method with 2, 6 DCIP (2,6- Dichlorophenolindophenol), respectively. The antioxidant activity shown by the fresh *amla* was 83.24 percent and the Vitamin C concentration was close to above study i.e. 680mg/100g.

Madhu *et al* (2012) also determined the antioxidant activity of *amla* by using phosphormolybdenum assay and reported as butylated hydroxyl toluene equivalent (BHTe) per gram of the fruit and the value obtained was 13.132mg of BHT/2.5 gm of *amla.* Alongwith *amla* lemon peel and cucumber peel was also studied for their antioxidant activity. Among these three types of sample *amla* expressed the maximum antioxidant activity while processed products discovered with lower antioxidant activity as compared to fresh *amla* and it was due to thermally processing of *amla.*

Again in an outcome, antioxidant components in *amla* fresh and processed products was ascertained with total antioxidant activity in fresh fruit of 2763.95 mg AAEAA (Ascorbic acid equivalent antioxidant activity)/100g and in *amla* juice, *amla* RTS, *amla* squash and candy was found out to be 2239.37 mg/100g, 1151.67 mg/100g, 1385.45mg/100g and 1663.70 mg/100g respectively (Karpagavalli *et al.,* 2014). Parveen *et al* (2005) worked on selected varieties of *alma* like Banarasi, Chakaiya, Kanchan, NA-7 and Desi and reported ascorbic acid concentration

of 3.15, 2.85, 1.93, 2.26, 3.29 mg/100g respectively by using AOAC method for determination of ascorbic acid content. Highest content of ascorbic acid was observed in desi variety of *amla*.

Table 2.Comparison of Antioxidant Activity of *amla* with other fruits

Sr. No.	Fruit	Type	Method used	Antioxidant activity	Description	References
1	*Amla*	Juice (untreated, thermally treated, PEF treated	DPPH assay	92.16 percent, 89.42 percent, 94.83 percent respectively	Results shown that Pulse Electric Field had maximum antioxidant activity. Untreated was recorded with lower value which was also reported in Hazra, 2010.	Vasudha *et al* (2014), Klimczak *et al* (2010).
2	Orange	Juice	DPPH assay	47.5-49.2 percent	Orange juices were stored for six months at three temperatures $18^{o}C$, $28^{o}C$, and $38^{o}C$. Results in terms of antioxidant activity were half in orange juice after storage.	Klimczak *et al* (2006), Malecka *et al* (2003)
3	Orange	Juice	FRAP assay	7.1-7.5mM of $FeSo_4$	It was similar to the above study except that here different method for detection was used.	Klimczak *et al* (2006)
4	Pomegranate	Peel extract	DPPH assay	81.0 percent	They used different solvents for extraction and showed that methanol extracts give maximum values.	Chidambara *et al* (2002)
5	Pomegranate	Peel extract	ABTS assay	85.0 percent	Observed that blending of solvents gives highest value as compared to extractions from individual solvent. Blending of ethanol to water in the	Li *et al* (2006)

					ration 70:30 recorded with maximum value	
6	Lemon *Amla*	Juice Juice	DPPH assay DPPH assay	42.31mg of Trolox/100 mg 1577 mg of trolox/100mg	Study results found that *amla* juice had much higher value of antioxidant activity to the lemon juice which was in value of trolox /100mg	Ali *et al* (2010) Mannan *et al* (2014)
7	Lemon grass	Fresh and dried grass	DPPH assay	63.3 percent and 43.3 percent respectively	Observed that the fresh plant has higher value to the dried plant. Again, it was depicted that when fresh plant prepared by decoction (68.8%) has higher antioxidant activity as compared to preparation with infusion (57.7%).	Cheel *et al* (2005), Mizpah, (2015)
8	Tinospora cordifolia	Methanol Stem extract	DPPH assay	10-50 percent	It was notice here that as the concentration of the extract increases the percentage of the radical scavenging activity increases. The maximum value showed was at the concentration of 100ppm.	Preshita *et al* (2011)

2.4.2. Vitamin C concentration

Amla berry naturally has the highest percentage of ascorbic acid due to this it had tremendous medicinal applications. Tripathi *et al* (1988) reported that *amla* pulp contains 500-1500mg of ascorbic acid per 100gm and confirmed that vitamin C content in comparison to other fruits is much more than guava, citrus and tomato fruits. It is explicitly effective free-radical scavenger so has been found to have good anti-inflammatory, antimutagenic properties and good antioxidant activity too. Nisha *et al* (1991) and Gopalan et *al* (1991) both concluded that *amla*

has the most concentrated form of Vitamin C existing in the plant kingdom. Further found that using whole fruit the vitamin C was easily assimilated in the body rather than an active ingredient. Also In whole fruit of *amla* the vitamin C exists in a bonded form with tannins which protects it from being destroyed by heat or light.

Karpagavalli *et al* (2014) determine the vitamin C percentage of fresh *amla* and freshly prepared products (*Amla* juice, *amla* ready to serve (RTS) beverage, *amla squash* and *amla* candy) and reported that fresh *amla* showed maximum vitamin C concentration i.e. 526.80mg/100g. In a group of processed products *amla* juice showed the highest value of vitamin C concentration and the amount was 456.17 mg/100g. Bhattacharya *et al* (1999) confirmed that vitamin C contents get degraded with the heat. Again, when its pieces were subjected to blanching with the 0.1percent KMS, the ascorbic acid content was undisturbed in the *amla* pieces (Alam *et al (*2010). The beneficial effect of blanching with KMS was also observed by many researchers and concluded that the vitamin C concentration was not degraded in the dried *amla* pieces, may be due to the inactivation of the peroxidase enzyme (Rao *et al (*1985 and Sethi *et al (*1986). It was noticed by the Verma *et al* (2004) that the ascorbic acid content was preserved in the sulphur treated fruits to non-sulhphur treated ones during storage period. While during the production of the dried *amla* shreds, the addition of ginger juice and black salt to blanched *amla* pieces helps in the retention of the vitamin C concentration. The vitamin C content of dried *amla* products are very much effected by drying method and the vitamin C concentration degrades during drying involves the oxidation and hydrolysis process occurs during drying process which may result in oxidation of ascorbic acid and its conversion to dehydroascorbic acid, further followed by hydrolysis to 2, 3-diketogulonic acid. Its further oxidation and polymerization forms other nutritionally inactive products (Gregory III, 2008). In comparison to other drying methods, indirect solar drying method gave the better results than direct solar drying methods in terms of nutritional value and retention of the antioxidant properties in dried *amla* fruit (Prajapati *et al (*2011 and Singh *et al (*2006).

Table 3.Comparison of Vitamin C concentration of *amla* with other fruits

Sr. No.	Fruit	Type	Method used	Vitamin C concentration	Description	References
1	*Orange*	Juice	HPLC column	408-333 mg/L at 18^{o}C 336-283 mg/L at 28^{o}C 265-83 mg/L at 38^{o}C	It showed direct correlation with the temperature increase the vitamin C concentration reduced. The highest value was observed in first month of storage and the lowest value was at the 6th month of storage and results were similar to Gliszczynska *et al.* (2004).	Klimczak *et al (*2006 Gliszczynska *et al (*2004
2	*Citrus limon*	Fruit	Volumetric method by DCIP	10.60g/100ml in ripe and 12.70g/100mlin unripe	The researcher the observed that the unripe fruit showed more vitamin concentration to the ripe fruit	Rekha *et al (*2012)
3	Citrus reticulata	Fruit	Volumetric method by DCIP	6.34g/100ml in ripe and 7.41g/100mlin unripe	Same results were observed as above in the case of Citrus reticulata	Rekha *et al* (2012)
4	Sweet potato	Leaf	2,4-DNPH	42.671mg/100 g	The edible part of the fruit contains less vitamin C content as compared to *amla* juice	Rahman *et al* (2007)
5	Mint	Leaf	2,4-DNPH	14.524mg/100 g	The edible part of the fruit contains less vitamin C content as compared to *amla* juice	Rahman *et al* (2007)
6	*Amla*	Leaf extract	DPPH radical and	40.24µg/ml 34.51µg/ml	Study concluded that the	Nain *et al* (2011)

			Hydrogen Peroxide scavenging		concentration of ascorbic acid compared with HMLEO* which shows maximum concentration.	

HMLEO*- Hydro-methanol (20:80) extract of leaves of *Emblica officinalis*

2.4.3. Moisture content:

Moisture content may be defined as it is the ratio of the mass of the water in the sample to the mass of the solids in the sample. It is also called as water content (ASTM, 2001). Drying of the particular fruit and vegetable has been established method for their preservation, in which the moisture content decreases to that level so that the product remains chemically stable. It also enhances the shelf life of the product (Prakash *et al (*2004). Moisture content can be determined on the dry basis and wet basis as well (Sajith *et al (*2013).

From different studies the reported moisture content of the *amla* ranged from 80 percent to 84 percent. The variation in the moisture content may be due to changes in the detection method used by different researcher. There are different methods of drying which were used in the determination of moisture content like hybrid photovoltaic thermal drying, oven drying (Sajith *et al (*2013), solar drying, vacuum drying, spray drying, tunnel drying and freeze drying (Poonam *et al (*2009). Poonam *et al (*2009 reported in her research that the moisture percent varies as the drying method changes. In this research the different methods were used like freeze, sun, vacuum, spray and tunnel drying and within all these methods freeze dried showed the maximum moisture percent and spray dried showed the minimum moisture content. Vinita *et al* (2012) postulated that the moisture content of *amla* was found to be 80.20 percent when it was measured by the AOAC method. Similarly, Karpagavalli *et al* (2014) reported that the moisture content of the *amla* was 81.80 percent and she used the AOAC method for detection too. In another study by Nikhil *et al* (2013), the moisture content of the sample was estimated by using the Karl-Fischer titration apparatus using standard KFR (Karl Fisher reagent- it consists iodine, sulfur dioxide a base and alcohol as solvent) and observed that the moisture content was 81.1 percent. In 2014, Swetha *et al* again revised the moisture content by Karl-Fischer titration method and observed that it was 81.2 percent. They summarize the research done on *amla* from past 5 years and found that there was no significant difference in the moisture content of the *Amla.* Moisture content also varied with the variation in varieties of *amla*. Parveen *et al* (2015)

compares different varieties of *amla* for the moisture content and detected with the closeness in the readings to each other. The different varieties selected were Banarasi, Chakaiya, Kanchan, NA-7 and Desi and their moisture content was 82.5percent, 84.24percent, 83.36percent, 84.65percent and 81.26 observed respectively. In comparative analysis of physicochemical, functional and nutritive values in different varieties of *amla* (fruit, seed and seed coat powder) showed that the varieties of *amla* which were from local market of Allahabad (Chakaiya, Francis, Kanchan, Narendra-7, Narendra-10 and Krishna variety) observed with different moisture contents ranged from 79 percent to 83 percent. The individual moisture content observed for the varieties were Francis (83.12percent), Chakaiya (83.81), Kanchan (81.18%), Narendra-7 (80.03%), Narendra-10 (80.12%) and Krishna (79.56%). Interestingly, Tonon *et al* (2008), Kha *et al* (2010) and Abadio *et al* (2004) used 10 to 30 percent (w/v) of maltodextrin to reduce the moisture content of the finished *amla* powder but in the effect of maltodextrin concentration and inlet temperature during spray drying on physicochemical and antioxidant properties of *Amla* (Emblica officinalis) juice powder showed that as the maltodextrin concentration increases from 5 to 9 percent the moisture content of the *amla* powder reduced 5.6 to 3.8 percent. Further, Umayal *et al* (2013) depicts that the variation in the air flow rate also affects the moisture content of the *amla*. The different air flow rates set in the designed drier were 4 m/s, 4.2m/s and 4.5m/s. The initial variation in the moisture content was ranged from 83.6 percent to 84.3 percent whereas the final moisture content ranged from the 0.1 percent to 0.6 percent. Observation in comparison to the natural sun drying as reference showed that the sun drying takes the 13 to14 hours of drying time to reach the equilibrium moisture content whereas the different air flow rates takes only 5 to 7 hours which is two time faster than the sun drying.

2.4.4. Tannins:

The *amla* fruit contains 28 percent of the total tannins which were distributed in the whole plant. Several active tannoid principles have been identified which showed the various health benefits and these were Punigluconin, Pedunculagin, Emblicannin A and Emblicannin B (Rastogi *et al (*1993). Among these specially two were the hydrolysable tannins Emblicannin A and B. During the process of hydrolysis one gives gallic acid, ellagic acid and glucose whereas the other produces ellagic acid and glucose. It was found that the *amla* fruit contains phyllemblin

(Wealth of Asia, 1998 and Ghosal *et al (*1996). Further it was reported that *amla* fruit alone contains 0.55 percent tannins along with phyllemblin (Rao *et al (*1985). It was studied by the Muhammed *et* al (2009) that the hydrolyzable tannins emblicanins A and B apart from the ascorbic acid content contributes to the antioxidant activity of the *amla.* The tannin acid was compared in different processed *amla* products as compared to the fresh *amla* fruit by the Karpagavalli *et al.,* 2014 and found that *amla* juice found with the highest value of tannins as compared to the *amla* RTS, *amla s*quash and *amla* candy. The values were 1.81, 0.35, 0.49, 0.52 gm of TAE/100g of sample. But the fresh *amla* fruit showed the maximum tannin content (2.14 gm of TAE/ 100g). The retention of the tannins found highest in the unblanched dried fruit of *amla* than other blanched fruits (Sethi *et al* (1986). Pant *et al* (2004) postulated that the tannins lost during blanching process if done in hot water and retention of tannins is possible in blanching with KMS (Prajapati *et al* (2011). Sagar *et al* (2006) compared retention of tannins in the drying processes and found that the tannins content in osmo-air dried *amla* was less as compared to oven drying. The tannin content was less in the indirect method of drying because of the leaching of tannins during osmosis. It was found that the total tannin content of *amla* decreased significantly during storage may be because of enzyme polyphenoloxidase which might have converted tannins into other products (Shrivastava *et al (*2007).

Gupta *et al* (2014) used HPLC method for the detection of the gallic acid and the tannic acid and observed the positive results. They compared the tannic acid in the different extracts and found that the tannic acid concentration in the *amla* extract was 6.172 percent (w/w) and the peak retention time was 25.976 min.

2.4.5. Flavonoid:

The reported flavonoid components in the *amla* fruit and the leaves were quercetin and Kaempherol (Swetha *et al* (2014 and El-Mekkawy *et al* (1995). Bansal *et al* (2014) found while distinguishing between the quercetin content in three different juices that (1: untreated fresh *amla* juice, 2: thermally treated juice, 3: the pulse electric field treated juice) the higher level of the extraction of the quercetin was observed in the pulse electric field processed *amla* juice. The chromatogram of the quercetin was detected at the wavelength of 370nm had retention time of 14.8 minutes. It was also found that the pulse electric field exhibit as effective method to retain the antioxidant activity. Karpagavalli *et al* (2014) also studied on the flavonoid content of the

fresh *amla* juice and the processed products. They observed that the flavonoid content in the *amla* fruit (369.46 mg of QE/100g) was maximum and followed by the fresh *amla* juice (268.41 mg of QE/100g), *amla* candy (212.76 mg of QE/100g), *amla* squash (198.48 mg of QE/100g) and *amla* RTS (192.12 mg of QE/100g). Agarwal *et al* (2012) researched upon the flavonoid content like quercetin in three different peels of fruits: *amla*, lemon and cucumber peel and found that the flavonoid content in three samples was comparable except the maximum value of *amla* peel. The flavonoid contents were found to be increased as the power concentration increased in the 50 percent of methanol. Liu *et al* (2008) compared the flavonoid content of *amla* at six regions in china and found quercetin as flavonoid content in *emblica* extracts, the Chuxiong region sample showed the highest content of flavonoids (38.7 mg QE/g), wheras the the lowest content of the flavonoid was shown by Haikou sample (20.3mgQE/g).

2.4.6. HPLC method of detection:

HPLC technique has been vested as best method to detect the inseparable, compounds present in traces and their components in the food products. It is liquid chromatography analysis technique used to detect, separate and quantify single and a group of components present in the solution. There are two phases: mobile phase and stationary phase. The samples separated and differentiated on the basis of their interaction the stationary and mobile phases. The column is most commonly used in the HPLC which work as stationary phase (Tom *et al.*, 2004). The flow sheet of the HPLC as follows:

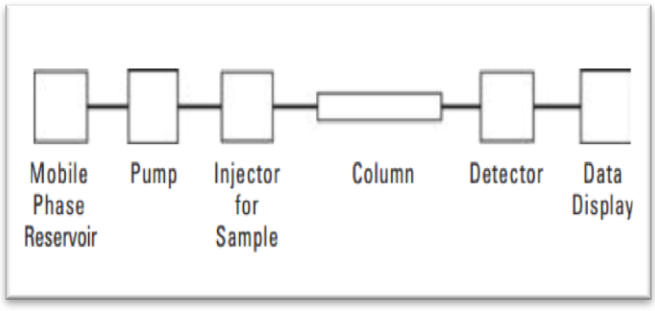


Fig 4: The flow sheet of HPLC method

2.4.7. Antimicrobial activity of *amla*:

The antimicrobial properties of the plant extracts and phytochemicals had a great importance in the therapeutic treatments. Especially in the Latin America researchers have been investigated on antimicrobial properties (Gislene *et al* (2000). The different studies on the medicinal plant showed that the *amla* plant possess antibacterial (Hossain *et al.*, 2012, Philip *et al.*, 2012 and Usha *et al.*, 2012) and antifungal (Mehmood *et al (*1999 and Hossain *et al (*2012|) properties. It was entrenched that the *E. officinalis* had in-vivo and in-vitro antibacterial potency. Further, it was proven experimentally that the fruits of *E. officinalis* showed inhibition in the colonization of *micro-oirganism*s in lungs when there was induction of pneumonia with *Klebsiella pneumoniae* in mice (Saini *et al* (2008). The plant contains the various types of free radicals scavenging molecules including phenols, flavonoids, vitamins, beta-carotene and terpenoids are rich in antioxidant activity which further shows the antimicrobial activity (Cai *et al* (2003).

The different studies expressed different results regarding the antibacterial and antifungal activity in *amla*. Nain *et al* (2011) worked on the different strains of gram positive and gram negative bacteria *spp*. The different strains taken were *Bacillus subtilis*, *Salmonella typhi*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli*. They used *E. officinalis* leaves extract in their studies and found that all the strains showed a particular zone of inhibition between 19.75±1.65 to 30.91±1.00mm when gentamycin and gatifloxcin used as a positive control and DMSO (Dimethyl sulfoxide) used as negative control. From all the strains *E.Coli* showed the maximum zone of inhibition. The method exploited was well diffusion method. Again, Satyajit *et al* (2012) used two strains *S. aureus* and *E. coli* using different method tube dilution method with fruit extract. They found that acetone fruit extract showed maximum activity against *E. coli* and the methanol extract had shown the highest antibacterial activity against *S. aureus*.

Javale *et al* (2010) concluded that the antibacterial activity shown by the *E. officinalis* could be due to the presence of bioactive components namely phenols, flavonoids, saponins, tannins in the fruit extract. Among all the active compounds saponins showed potential bactericidal potency. The fruit also contains tannins Emblicanin A and B, which were known to have potential antimicrobial properties too (Jyothi *et al* (2011). In 2012, Gupta and other

researchers used the *amla* seed extract to check the antibacterial activity on different strains of microbes such as *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* and *Enterococcus species*. They noticed that all the strains had shown antimicrobial activity. They used different concentrations from 50mg/ml to 200mg/ml of methanolic seed extract and noticed that as the concentration of the seed extract increases the zone of inhibition also increases. Among all the species maximum results was shown by *Staphylococcus aureus* at 200mg/ml concentration with the highest relative percentage of inhibition (91.11%). Jain *et al* (2015) experimented on six plant extracts such Aloe vera, *Amla*, Garlic, Ginger, Neem and Tulsi to check the antimicrobial activity against the most common bacterial oral pathogeni.e. *Streptococcus mutans*. In all plant extracts specially the crude extract of the garlic showed the maximum activity against the *Streptococcus mutans* followed by *amla*. The range of the zone of inhibition varied from 6 to 25mm at a volume of 100µl.

2.5. Methods of Juice extraction:

There are two methods of the juice extraction one is mechanical method and the other is enzymatic method. The traditional methods used for juice extraction includes juicer, belt press, screw press, decanter centrifuge, diffusion extraction (Beveridge and Rao, 1997). But the extraction through the means of enzymes like pectinase is one of the upcoming enzymatic methods for food and textile industry (Kashyap *et al* (2001). Kaur *et al* (2008) concluded that the enzyme concentration was very important for the extraction of juice. The enzyme concentration used by them was ranged between 0.16-0.84mg/100g in the guava pulp and the set incubation period was 0.95–11hr and incubation temperature was 36.6–53.4 °C on juice yield. Trappey *et* al (2008) described the process of the juice extraction as followed like the after fruit washed properly and cut into small pieces, the pretreatments were given like cooling, heating, streaming and all of these increased the juice extraction. Will *et al* (2000) reported that using hot water as extraction medium with addition of enzyme in apple pomace and combination of pectinases and cellulases lead to 37 percent increase in juice yield. They also compared the enzymatic and mechanical methods of the juices extraction in case of banana and found that the mechanical method showed better results as compared to the enzymatic method. They also noticed that the mechanical method offers advantages over enzymatic as there was no any usage of additives and not so much time consuming. After those Vaidya *et al* (2009) use the enzymatic extraction

method on the Kiwi fruit and used the combination of enzymes to extract the juice from kiwi include pectinase (0.025gm/kg), amylase (0.025gm/kg) and mash enzyme (0.05gm/kg) and macerated pulp for 2hr at 50 degree celcius. They observed that juice extraction rate was 78.46 percent more as compared to the control (58.44%) without any effects on the physiochemical parameters.

2.6. Probiotic drink:

The term Probiotic means "for life" and it was coined by an Expert Committee as ''live microorganisms which upon consuming in certain numbers exert health benefits beyond inherent general nutrition". The Probiotic foods were categorized under functional foods (FAO/WHO 2001). The concept of Probiotic came into focus in early 1900's but the term "Probiotic" was suggested by Lilly and Stillwell. The Probiotic drinks has shown many health benefits includes reduces the chances of cancer, resistance to enteric pathogens, reduction of serum cholesterol, alleviation of lactose intolerance and many more (Klaenhammer, 1998). The functional beverages includes Probiotic drinks, drinking yoghurts, dairy drinks functional and functionally fortified soft drinks, juices, energy drinks, sports drinks, functional waters and ready-to-drink tea. The interest of people regarding probiotic drinks are increases day by day due to its health benefits (Yadav *et al.,* 2010).

In 2013, Shukla and the other researchers prepared a probiotic beverage by using whey and pineapple juice and used *Lactobacillus acidophilus* as the probiotic *micro-organism*. The different combinations were prepared by using whey-pineapple juice with stable 10 percent sugar level. The different formulations were A (80w:20P), B (75w:25P), C (70w:30P) and D (65w:35P). 1percent of the inoculum of the *Lactobacillus acidophilus* was used. Post sensory analysis it was found that the sample D showed the best results with highest percentage of the overall acceptability. The viable counts after the10hr of fermentation were 4.7×107 cfu.ml^{-1}. After two years same procedure followed by Sasi *et al* (2015) and developed a probiotic beverage by using whey, aloe-vera juice and the *Bifidobacterium bifidus* (BB) strain was used as probiotic. He prepared the different combinations using whey and aloe-vera juice. The various formulation made were A (65wy:35Av), B (70wy:30Av), C (75wy:25Av) and D (80wy:20Av). The results of sensory analysis on the various formulations showed that the sample B scored highest in color, flavor, appearance and consistency. The data was analyzed and proved that the

B sample found to be good in quality as probiotic beverage. In this drink 1 percent of the inoculum was used with a shelf life of 30 days at 4^{o}C.

The different categories of the sweeteners showed the variation in the relative sweetness of the different sweeteners to sucrose. The stevia was used as sugar substitute the natural nonb-caloric sweetener in the probiotic drink and found that the sweetness in the leaves was due to the presence of the sweetening agent namely stevioside which was reported 300 times more sweeter than the sucrose (Modi H A, 2012). It was stable at high temperature and over a pH range 3-9. The best brands of the stevia are 100 percent pure and it fulfills the customer's choice of purity. Moreover, it provides and helped in zero caloric product production. Stevia showed no effect on the blood sugar as it has very low glycemic index. The very less amount of the stevia is sufficient to provide relative sweetness instead of using big amount of sucrose.

Table 4.The different sweeteners as discussed below:

Type	Sweetener	Relative sweetness to Sucrose
Caloric sweetener	Fructose	1.2
	Glucose	0.7
	Sucrose	1.0
	Caramel	0.9
Low-caloric sweetener	Maltitol	0.8
	Lactitol	0.5
	Sorbitol	0.5
Non caloric synthetic sweetener	Aspartame	200
	Sucralose	600
	Dulcin	250
	Cyclamate	30
Non caloric natural sweetener	Steviosides	100-300
	Phylloduclin	400
	Glycyrrhizin	50

Apart from the all sweeteners it showed many health benefits as compared to the others because of its wide applications and less of chemical interactions. Hence it can be used in the probiotic drink to provide sweetness.

2.7. Applications of *amla*:

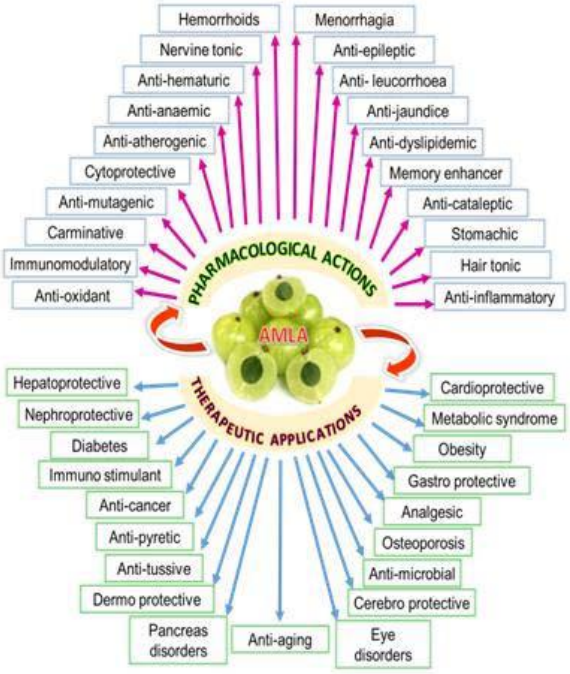


Fig 5: Pharmaceutical and Therapeutic application of *amla*

I. **Fortifies the liver:** It has been noticed by many researchers that the *amla* fruit helps in purifying the blood and the nutrient fluid and hence supporting in the functions of the liver. It also helps in the elimination of the toxins from the body (Tasduq *et al* (2005), Sultana *et al* (2004) and Haque *et al* (2000).

II. **Nourishes the brain and mental functioning:** It has been noticed that *amla* is also good for the brain. It nourishes the brain and increases the coordination among the retention, recall and the acquisition. It also enhances the mental amd intellectual functioning and supports the nervous system (Reddy *et al*, 2011 and Vasudevan *et al* (2007).

III. **Supports the heart:** Williamson, 2002 reported that the *amla* act as the best cardiac stimulant and it also the supports the cardiovascular system. It also lowers the cholesterol level in the human body and protects from the various heart diseases. (Kim *et al*; 2005, and Yokozawa *et al* (2007).

IV. **Helps the urinary system:** Tsarong, 1994 studied that the *amla* berry enhances the digestive fires and also supports the urinary system and eliminate the problem of the

burning sensation while urinating. In addition it also helps in elimination of the waste from the body.

V. **Flushes out toxins:** Singh *et al* (2011) studied on the *amla* helps in the toxins elimination from the body. He noticed that the individual who has been consuming junk food and preservatives and additives get accumulated in the liver. *Amla* helps in the removal of the chemicals and toxins from the body.

Table 5.Mechanisms reported for the pharmacological activities of *amla* fruit

Sr. No.	Pharmacological activities/Health benefits	Action	Mechanism	References
1.	**Cardio protective**	Due to the in vitro and in vivo antioxidant effect of Emblicanin A, B like ascorbic acid	Prevents ischemia-reperfusion, induced oxidative stress	Yi-Fei *et al* (2009) and Chatterjee *et al* (2010)
		Mutations in genes and chromosomal abnormalities inhibited by Ellagic acid	By inhibited the oxidation of the DNA	Pandey *et al* (2011)
		Inhibition of the Coxsackie virus B3 and myocarditis by Phyllaemblicin B	Due to its antiviral activity	Yi-Fei *et al* (2009)
2.	**Anti-cancer**	DPPH, ABTS, Superoxide scavenging; Iron chelation; anti-proliferative activity against MCF-7 tumor cells; MTT assay	Due to antioxidant and anti-proliferative activitiesof Mallotusinin and mucic acid 1,4-lactone 3-Ogallate	Luo *et al* (2011)
		Inhibition of *in vitro* cell proliferation in human tumor cell lines like T-lymphoid, B-lymphoid, erythrocyte leukemic HEL cell lines	Anti-proliferative and antioxidant property	Luo *et al* (2011)
		Induction of apoptosis in CeHa cell lines and DNA-Topoisomerase I and cdc25 tyrosine phosphatase in *Saccharomyces cervisiae*	Expression of mutant genes are inhibited	Rajesh *et al* (2003)

		Reduction of Ascites and solid tumors induced by Dalton's lymphoma as cites cells in mice; Increased lifespan of mice	Natural Killer (NK) cell activity enhances	Madhuri *et al* (2008) and Suresh *et al* (1994)
3.	**Immuno-modulatory**	Prevents side effects of Cyclophosphamide treatment when used in combination	it prevents the mutagenicity and Hemato and immuno protective	Haque *et al* (2001)
		Prevents chromium induced oxidative damageand immunosuppression	Immunomodulatory and Cytoprotective	Liu *et al* (2012)
4.	**Anti-diarrheal**	Inhibition of castor oil - induced diarrhea and the intestinal fluid accumulation in vivo and in vitro in isolated rabbit jejunum and guinea pig ileum	blockade of muscarinic receptors and Ca^{2+} channels	Mehmood *et al* (2011)
5.	**Anti-microbial**	Interferance with the adhesion of C. albicans to buccal epithelial cells and denatunation of acrylic	By inhibiting adhesion	Thaweboon *et al* (2011), Kamal *et al* (2012)
6.	**Anti-viral**	Pentagalloylglucose can inhibit Hemagglutination inhibition, Plaque-forming unit assay and inhibition of replication.	By Prevention of virus adsorption and suppression of virus release	Liu *et al* (2011)

CHAPTER – III

3 MATERIALS AND METHODS

3.1 Pre-requisites required for the study:

3.1.1 Collection of the *Amla* (Indian gooseberry) fruit samples:

It was obtained within the campus from *amla* trees in fully matured form which is managed by Horticulture Unit, Thapar University, Patiala and also from the local market, Patiala several times during the season of 2015-16. Fresh plucking or procuring from the local market of *amla* fruits was done at various intervals during the season like in the month of November, December and January 2016 and brought for research and experiment purpose to the laboratory of Department of Biotechnology, TU, Patiala.

3.1.2 Sugar substitute: Non-caloric natural sugar - Slimmer's Stevia (VLCC Brand, India)

The prescribed brand was purchased from the local market, Patiala. Sugar substitute especially stevia has been used to overcome the bitter after taste due to over concentration of ascorbic acid in *amla* juice. The main purpose of this sugar substitute is to provide the sweetness (200-300 times sweeter to sucrose) without changing the texture, color and other sensory characteristics of the *amla* juice. The date of manufacture was August, 2014 and the batch number was 14002SS.

3.1.3. *Bacillus coagulans* or *Lactobacillus* sporogenes: Sporolac (Manufacturer: Uni Sankyo):

It was purchased from the local medical shop of Patiala. It was in the form of sachet. It was pure white in color powder. The one sachet contains 1gm of powder. Selective media MRS was used to isolate pure culture of the *Lactobacillus spp.* and after biochemical tests confirmation used as a pure of probiotic culture in formulation.

3.1.4. Pectinase enzyme:

Pectinase enzyme was used for the extraction of the juice from the *amla* fruit. It was of Hi-Media brand and the enzyme activity was 8000-12000 U/g. It was made available by the Department of Biotechnology, TU, Patiala.

3.1.5. Juicer:

The Kalsi juicer was used for the extraction of *amla* juice by mechanical means. The type of the juicer was centrifugal and situated in the STEP of Thapar University**,** Patiala.

3.1.6. Muslin cloth:

Muslin cloth was used in the extraction of the juice. Basically it is used for the squeezing and to remove the pulp of the *amla* fruit. Filtration of the juice was done with the muslin cloth which is made available by the Department of Biotechnology, TU, Patiala having pore size varied from 0.7mm 0.22mm.

3.1.7. Whatman filter paper:

Whatman filterpaper was used for the purification of the final juice. The whatman filter paper no. 4 was used and having pore size 20-25µm. GE healthcare provides the whatman filter papers.

3.1.8. Wide mouth sterilized bottles:

The borosil glass bottles of the 50ml, 250ml and 500ml was used for the practical work. It was used for the preparation for the media and for the experimental assays.

3.1.9. Aluminum foil:

Hindalco freshwrapp aluminum foil was used for wrapping the juice bottles to avoid the direct contact with the light and to avoid the oxidation of the juices.

3.1.10. Water bath:

Serological water bath was used for the extraction of the juice with the pectinase. The pectinase enzyme required 37°C for its activation.

3.1.11. Referigerators and Deep freezers:

The LG refrigerator was used for the storage of the samples, juices and for the storage of the mother cultures of the strains in the solid or in the liquid form.

3.1.12. Spectrophotometer: manufacture company and usage.

Hitachi spectrophotometer was used for measured the wavelength of the different samples. The tannin, flavonoid, vitamin content and antioxidant activity was measured with the help of the spectrometric method.

3.1.13. HPLC: manufacture company and usage

HPLC was used to determine and quantify the tannin content and the quercetin content the samples. The company of the HPLC was Shimadzu. The C-18 column was used as the stationary phase in the HPLC.

Fig 6: A) water bath B) Sectrophotometer C) HPLC D) Juicer

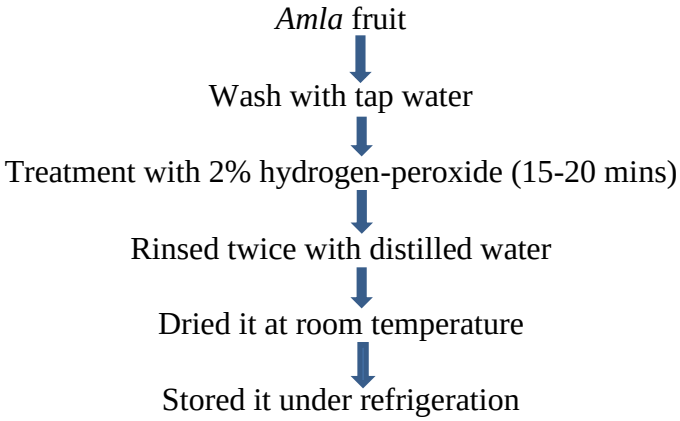
3.1.14. Patanjali *amla* juice (Pure)

It was purchased from Patanjali proprietary shop from local market, Patiala. It is used for the comparison study of tannins, flavonoids, antioxidant activity and Vitamin C to the experimental samples of *amla* juice produced by mechanical method and by enzymatic method.

3.2 Experimental Planning:

3.2.1 Processing and treatment of *amla* fruit

First, *amla* fruits were cleaned to remove dust, soil matter, and surface microflora washing under running tap water. Further, dipped into 2 % hydrogen-peroxide for 15-20 minutes to remove blemishes and stains on the surface of the *amla* fruit. Rinsed twice with distilled water to remove traces of hydrogen-peroxide and then kept at room temperature.



3.2.2 Process standardization of *amla* juice production by centrifugal juicer: Kalsi (Mechanical method) and by Pectinase (Enzymatic method).

3.2.2.1 Extraction of juice by mechanical method:

Amla fruits were cut into discs and the seeds were removed. Centrifugal juicer was used for the extraction of juice. The juicer was washed with distilled water and then rinsed with alcohol to create sterile conditions, than wait till alcohol evaporates in juicer. Extraction of juice was carried by putting discs of the *amla* fruit into the running (dried or sterilized) juicer and as a byproduct pulp is obtained from the waste outlet of the juicer. Further obtained juice was filtered through muslin cloth to remove froth and other dispersed particles. For final storage it was filtered through whatman filter paper and kept in vacuum tight sterilized bottles under sterilized conditions and then stored at 0 to 6^{o}C.

3.2.2.2 Extraction of juice by enzymatic method

Pectinase enzyme was used for the extraction of *amla* juice. Firstly, pre-weighed diced *amla* without seeds was macerated using pestle and mortar and then on the wet weight basis pectinase (powder) enzyme used at different levels as shown in the table (6) in a sterilized beaker. After mixing of the enzyme in the *amla* coarse paste, the beaker was placed in water bath at 37^{o}C. The time for juice extraction varied accordingly to the weight of the sample used. After few (approximate 5-6hrs) hours the beaker with *amla* paste was removed from the water bath and squeezed through muslin cloth and juice was extracted followed by filtration through whatmann filter paper number 4 and size was 20-25µm to make it clear juice. Extracted clear juice was stored at 0-6^{o}C in the vacuum tight bottles and covered with foil to retain the antioxidant activity present in the juice. All the steps were performed under sterile conditions i.e. in laminar.

Table 6. Standardization of pectinase enzyme in *amla* pulp.

Sr.No.	Weight of *amla* pieces (gm)	Pectinase enzyme used (gm)
1.	10	0.001
2.	10	0.01
3.	10	0.1
4.	400	0.4
5.	800	0.8

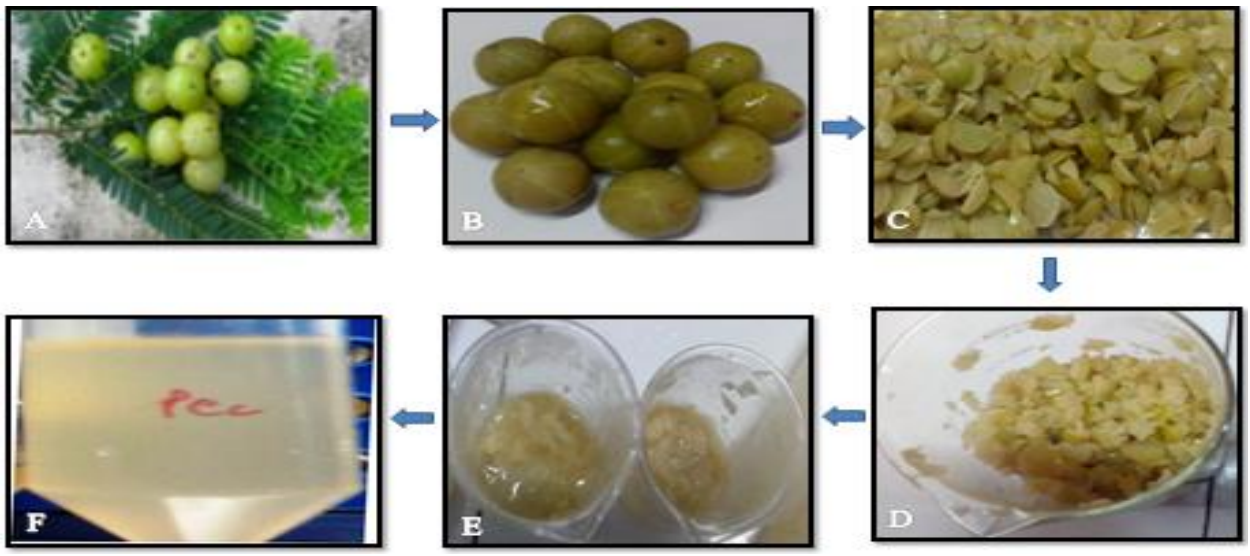


Fig 7: Pictorial representation of *amla* juice extraction from pectinase enzyme in laboratory A) Fresh *amla* fruit B) Treated *amla* fruit C) Pieces of *amla* fruit D) Pectinase + *amla* pieces E) *Amla* juice extracted with pectinase with pulp F) Filtered and clear *amla* juice

3.3.3. Formulation of the Probiotic *amla* drink:

The formulation of the probiotic drink is followed as given by Shukla *et al* (2013). Probiotic culture used after isolation was *Lactobacillus sporogenes or Bacillus coagulans.* Initially the ratio of *amla* juice from two methods (mechanical and enzymatic method) were mixed to the probiotic culture made from *amla* seed waste 50:50 percent, 60:40 percent, 75:25 percent and 85:15 percent respectively as shown in table (7). On the basis of sensory analysis only two formulations were selected for further analyses that were 50-50 percent and 60-40

percent for both juices. The stevia sugar substitute was added 0.5 percent to the volume of *amla* juice in all probiotic drinks while a control of both juices.

Table 7.Formulations of the *amla* juice with the probiotic culture and Stevia

Sr. No.	Juice added (µL)	Culture added (µL)	Stevia added (mg)
1.	50	50	0.5
2.	60	40	0.5
3.	75	25	0.5
4.	85	15	0.5

3.3.3.1. Isolation of the *Lactobacillus sporogenes*

The *Lactobacillus sporogenes* was isolated from the sporolac sachet (powder) which is used as medicine to overcome diarrhea in children. Stock solution of 1 percent *Lactobacillus sporogenes* prepared (dissolving1gm of sporolac powder in 9ml of saline solution) and then serial diluted from 10^{-2} to 10^{-9} concentration of bacteria in saline solution. Any three selected concentrations were spread on the MRS media for selective growth of *Lactobacillus sporogenes*. After that plates were incubated at 37^{o}C for 48 hours to check the selective growth of *Lactobacillus sporogenes* on MRS media plates. Selected colonies were streaked further on MRS media plates for enumeration. Nutrient broth was used to produce a pure culture of *Lactobacillus sporogenes* by incubating in shaker for 24 hours at 37^{o}C and for further tests of the species.

3.3.3.2. Gram staining

Differential staining has been the best method to distinguish different types of bacteria. The gram stain is the most useful and widely employed differential stain in bacteriology. It divides bacteria into two groups: Gram Positive and Gram Negative. The whole procedure is given below:

Prepared the smear on the slide of the culture and heat fix it

↓

Flood the smear with crystal violet (stand for 1 min)

↓

Rinsed with water for 5 seconds

↓

Flood with gram's iodine mordant (stand for 2 min)

↓

Rinsed with water for 5 seconds

↓

Decolorized it with 70 percent ethanol for 15 seconds

↓

Rinsed with water for 5 seconds

↓

Counterstained it saffranin for 30 seconds

↓

Rinsed with water for 5 seconds

3.3.3.3. Biochemical test: CO_2 gas production:

This test is used to check that the selected strain i.e. *Lactobacillus sporogenes* was gas producing or not as mostly *Lactobacillus spp.* are gas producing. The inverted Durham tube was inserted into the test tube containing nutrient broth and autoclaved to make it sterile and then inoculated with the bacterial culture. The test tubes were kept at 37°C for 24 hours. If the bubble comes in the Durham tube means the strain is gas producing otherwise not.

3.3.3.4. Enumeration of *Lactobacillus sporogenes* in dried *amla* seed powder and nutrient broth:

The two flasks were prepared in which one had 0.4 percent of dry *amla* seed powder and other had nutrient broth. After sterilization and cooling both flasks were inoculated with 10µL of the freshly prepared culture *of Lactobacillus sporogenes* which has been isolated from the sporolac. To observe the growth behavior of the culture after every hour the growth medium was observed in spectrophotometer at 760 nm wavelength. The readings were taken up to 9 hours. After that the growth curve was drawn to compare the growth.

3.3.3.5 Relative sweetness of stevia

The relative sweetness of stevia was assessed in comparison to the table sugar. The weight of one tablet of Stevia (VLCC) was 100mg, dissolved in 250ml of normal RO water. To the similar concentration of Stevia 100mg of table sugar was dissolved in 250ml of water and sensory analyzed. The Stevia tablets had intense sweeteness than table sugar.

3.3.3.6. Formulation of the probiotic drink

The probiotic drink acquires the base of *amla* juice obtained from two methods: juicer and enzymatic along with a market produce patanjali juice. The *Lactobacillus* culture in *amla* seed powder was used as a source of probiotic solution and the stevia was used as substitute to table sugar. Two controls formulated were: C1- Juice extracted by mechanical method& C2 - Juice extracted by enzymatic method while for comparison with the market produce sample was D3 –Patanjali *amla* juice. As described earlier the four formulations in ratios (50:50 percent, 60:40 percent, 75:25 percent and 85:15 percent) as shown in table (8). Among these only two formulations were carried on for further analysis were 50-50 percent and 60-40 percent for both juice extraction methods and their formulation as given below in table 3.

Table 8.Selected combinations sensory analysis

Sr. No.	*Amla* Juices extraction methods	Sample code	*Amla* juice added (ml)	Culture added (ml)	Stevia added (mg)
1	Mechanical	M1	50	50	50
2	Mechanical	M2	60	40	50
3	Pectinase	P1	50	50	50
4	Pectinase	P2	60	40	50

3.3.3.7. Sensory analysis:

The juice extracted from two methods: mechanical method and pectinase enzyme method was the base for the probiotic drinks. So, the sensory analysis of the selected samples i.e. M1, M2, P2, P2 along with two C1 and C2 was done in comparison to the D3- Patanjali juice sample by Hedonic scale (score 0-9) by the method given by (Singh *et al*., 1998).

3.4. Chemcial analysis of the *amla* fruit:

Several chemical compositional and other antioxidants' tests were done and mentioned in detail below:

3.4.1. Moisture content of *amla* fruit:

Moisture content of the *amla* fruit was estimated by oven drying method that is given in Sajith *et al*., 2013; AOAC (2000). To check the moisture content of the *amla* fruit drawn into a representative sample from all the four sides of the *amla*. Pre-weighed and dried petri plate is placed on the balance, than the approximate 5g weight of the macerated sample of *amla* was placed and noted down the readings of petri-plate and petri-plate with sample. The petri-plate with sample placed in hot air oven at 105^{o}C for three hours or so until the sample gets dried. Three consecutive readings of the dried sample with petri-dish were taken after cooling in dessicator.

Calculation of Moisture Content on wet basis (%)

$$M_{wb} = \frac{Wi - Wf}{Wi} \times 100$$

Wi =initial weight of the sample without petri-plate

Wf = final weight of the sample without petri-plate

3.4.2. Total solids in *amla* juice:

To determine the total solids it was mandatory to determine the moisture content. The moisture content was determined according to the AOAC (2000) method. The total solids were determined by subtracting the moisture content from 100 and expressed as percent of total solids.

Total solids (%) = 100 – moisture content (%)

3.5. Antioxidant activity of *amla* juices:

The Antioxidant activity of juices was determined by comparing and evaluating the free radical scavenging activity of DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay to the sample as given in method by Elez *et al*., 2007. The samples of juices were centrifuged at 6000 rpm for 15 minutes at 4^{o}C. The 100µl supernatant was taken followed by the addition 3.9ml of freshly prepared DPPH. The DPPH was prepared in the 0.1mM of methanol. The mixture was properly mixed with the help of vortex and then kept in dark for 30 minutes for uniform mixing. The absorbance of the sample was measured at 515nm wavelength against the blank (Methanol) after 30 minutes at room temperature in dark condition. The first reading of DPPH without sample was taken as control. The antioxidant activity calculated as the percentage of the inhibition of DPPH radical.

$$\text{Percent DPPH} = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100$$

Abs control = Absorbance value of control

Abs Sample = Absorbance value of sample

3.6. Determination of Vitamin C Concentration in *amla* juices (Vit. C):

Titration method was used to estimate vitamin C content in juices according to the method used by the Alagar *et al*., 2014. The 1mg/ml stock solution made from ascorbic acid tablet. Different concentrations 0.1 to 1.0 mg/ml were prepared from the stock solution. To 1ml of *amla* juice add freshly boiled and cooled water to make it to 10ml volume. Add 10 drops of one percent starch solution to it as indicator. Then titration was done against 0.05M iodine solution and observation of end point judged by yellow to blue color change and it was indicated that vitamin C was present in the sample. Note down the volume of the iodine solution used. A standard graph was drawn between the concentration of ascorbic acid and ml of iodine solution used and calculated the value of the ascorbic acid present in the sample.

3.7. Determination of tannin content in *amla* juices:

The colorimetric method used for the determination of tannin content in *amla* juices was estimated as evaluated by Vasundhara *et al*., 2013. The different samples of juices were: a) juice extracted by mechanical method b) juice extracted by enzymatic method c) Patanjali *amla* juice.

3.7.1. Preparation of standard curve:

The tannin content in the sample was determined by using Folin-Denis method. The stock solution of 0.1mg/ml of tannic acid was prepared using 100ml of distilled water. 1-10ml of the aliquot was taken in properly washed 100ml flasks containing 75ml water. 5ml of Folin-Denis reagent was added and after 5 minutes 10ml of Sodium Carbonate was added to each flask. Then distilled water was added to make up final volume 100ml. All the constituents in each flask mixed properly and kept undisturbed for 30 minutes. The absorbance was measured at wavelength of 760nm after 30 minutes against the blank (Distilled water+folin reagent+sodium carbonate).

3.7.2. Preparation of the sample:

5ml of juice was taken and 95ml of distilled water was added to make up final volume 100ml. To 5ml of sample from the above mixture add 5ml of Folin- Denis reagent followed by addition of 10ml of sodium carbonate. Rest of the volume was made up by using distilled water to 100ml and vortexed. The test tubes were kept undisturbed for 30 minutes and observed at wavelength of 760nm against the blank (Distilled water+folin reagent+sodium carbonate). The reading of the sample was compared with the standard graph and concentration of the sample was determined. The Total tannin content was measured as milligram of tannic acid equivalent/ 100ml of sample.

3.8. Determination of Flavonoid content in *amla* juices:

The method used for the determination of Flavonoid content in *amla* juices was estimated as evaluated by Kalita *et al*., 2013. The different juice samples were: a) juice extracted by mechanical method b) juice extracted by enzymatic method c) Patanjali *amla* juice.

3.8.1. Preparation of standard curve:

The Flavonoid content in the sample was determined by modified calorimetric method as followed by Jia *et al*., 1999. The stock solution of 1mg of Quercetin was prepared in the 1ml of methanol. The different concentration ranging between 100ppm to 600ppm was prepared in the test tubes using standard stock solution. Further 60µL of 5 percent sodium nitrite was added and

kept undisturbed for 5 minutes in the test tubes. To these test tubes 60µL of 10 percent aluminum chloride was added and after 6 minutes 400 µL of 1M sodium hydroxide was added too. At the end distilled water was added to make up the volume up to 2ml and vortexed. Absorbance was measured at wavelength 510nm against the blank (Methanol+sodium nitrite+aluminum chloride+sodium hydroxide). Using the observation reading Standard graph was drawn between concentration and O.D.

3.8.2. Preparation of the sample:

200µL of sample juice was taken in the test tube with addition of 800µL deionized water followed by 60µL of 5 percent sodium nitrite and kept undisturbed for 5 minutes. Then 60µL of 10 percent aluminum chloride was added and after 6 minutes 400µL of 1M sodium hydroxide was added and at the end distilled water was added to make up volume 2ml. All the constituents were mixed properly with the help of vortex. The absorbance was measured at wavelength of 510nm against the blank (Methanol+sodium nitrite+aluminum chloride+sodium hydroxide). The Total flavonoid content was expressed as ppm of quercetin equivalent (QE)/ µL of sample.

3.8.3. Estimation of quercetin in the samples by HPLC method:

3.8.3.1. Preparation of the standard sample:

The whole procedure of HPLC method was followed as given by Bansal *et al* (2014).The standard stock solution 1mg/ml of quercetin and tannic acid was prepared in the methanol. 10-50 ppm concentration was further prepared from the stock solution. All the samples were filtered before use.

3.8.3.2. Preparation of the tested samples:

The mechanical and enzymatic juice was extracted out. 2ml of the sample were taken into the ependorff and centrifuged at 10,000rpm for 10 minutes. Supernatant was taken and filtered out with the help of syringe filter having PTFE membrane.

3.8.3.3. Preparation of the solvents:

0.1 percent Orthophosphoric acid and acetonitrile two solvents were used for the HPLC method. Both the solvents were HPLC grade. 0.1 percent Orthophosphoric acid was prepared in the water (v/v).

3.8.3.4. Procedure:

The reverse phase Zorbax SB RP C-18 was used and the pore size was 25×4.6×5 lm. The set solvent A (Orthophosphoric acid)and the solvent B (acetonitrile) was used as the mobile phase with the gradient of 95 percent of solvent A for 5 minutes and after that it was decreased to 50 percent for the next 15 minutes. Further the initial conditions were set as 95 percent of solvent A in last 5 minutes. After the conditions were set the 20µl of the sample was injected into the HPLC. The chromatograms were extracted at 370 nm for the quercetin and at 280nm for the tannic acid using PDA detector. The quercetin and the tannins in the samples were identified according to the absorbance spectrum and the retention time of the pure standards

3.9. Antimicrobial Activity of *amla* juices:

3.9.1. Test microorganisms:

A total three bacterial species including *Lactobacillus, E.Coli, Bacillus subtilis*, and one fungal strain *Saccharomyces cerevisae* was tested. All the species were taken from the department of Biotechnology, Thapar University, Patiala. All the cultures were freshly prepared in their respective media. *Lactobacillus, E.Coli, Bacillus subtilis* were grown in the nutrient broth and *Saccharromyces cerevisae* and *Yeast* were freshly grown in YPD media.

3.9.2. Estimation of antibacterial activity:

The in-vitro antibacterial activity of the different juice samples were studied against the bacterial and fungal strain by the Agar Well Diffusion method by Nair *et al*., 2005. The Gentamycin was used as positive control. Gentamycin injection of 80mg/2ml of Pfizer Company was procured from the local medical shop of the Patiala. Different concentrations of gentamycin 0.1mg/ml, 0.2mg/ml, 0.3mg/ml and 0.4mg/ml were prepared from the stock solution. Muller-Hinton agar was used as the growth medium. The plates were incubated overnight to check the contamination. The fresh 24 hours old culture was prepared in the respective media for the growth of microorganisms. On each plate the spreading was done with the respective culture. The plates were kept undisturbed for 15 minutes. A sterile cork-borer of 5 mm diameter was used to make wells on cultured plates. After wells were made, pour 100µl of the different concentration of samples in each well. At the same time in different plate pour the different concentration of juices in each well. The bacterial plates were incubated at 37^{o}C for 24 hours and fungal plates were incubated at 28^{o}C for 48 hours. After the completion of the incubation period

the diameter of the zone of inhibition was measured and the percentage of inhibition with respective to the control was calculated.

$$\text{Percent of inhibition} = \frac{Dia\ control - Dia\ samlpe}{Dia\ control} \times 100$$

Dia control = diameter of the gentamycin

Dia sample = diameter of sample

3.10. Sensory Evaluation:

The prepared probiotic drinks were evaluated by sensory analysis for Color, Appearance, Flavor or Aroma, Texture, Taste and Overall Acceptability from different subjects. The method was used according to the Larrmond 1970. The samples were rated on 9 point Hedonic scale as under:

Table 9.Hedonic scale

Sr.NO.	Scale	Sensory Score
1	Like extremely	9
2	Like very much	8
3	Like moderately	7
4	Like slightly	6
5	Neither like nor dislike	5
6	Dislike slightly	4
7	Dislike moderately	3
8	Dislike very much	2
9	Dislike extremely.	1

CHAPTER- IV

RESULTS AND DISCUSSIONS

Under the present study an attempt has been done to standardize the method of *amla* jucie production through the means of pectinase enzyme over the well-established mechanical juicer method with keeping in view maintaining the activity of antioxidants, vitamin C, tannins and other antibacterial activities. The effects of both methods on the various antioxidant components were read and expressed in this chapter as results. During *amla* juice production, seeds and left over shreds were released as waste but here a trial has been attempted to utilize the waste seed powder for the growth of isolated, pure probiotic culture i.e. *Bacillus coagulans* and its utilization in combination with *amla* juice and *stevia* sugar substitute to design a Probiotic drink. The growth of Probiotic bacteria in the waste seed powder has been monitored and various combinations of *amla* juice (both methods), *stevia* sugar substitute and Probiotic culture in waste seed powder have been further tested for antioxidants and sensory qualities. The results of the selected experiments have been discussed below with tables, growth curves and standard graphs of the antioxidant activity of *amla* juice, tannin content, flavonoid content and vitamin C concentration in *amla* juice under following heading and sub-headings.

4.1. Chemical analysis of *amla* fruit:

4.1.1 Physical characteristics of the raw material:

The fresh *amla* fruit was greenish in color. The fruits were washed out and stored it at room temperature for drying. When the juice was extracted out the color of the mechanically extracted juice was converted into the brown shade but in case of the pectinase enzyme obtained juicethe greenish color was changed into white. Both the juices were stored under refrigerator for further analysis.

4.1.2 Moisture and total solids analysis:

Initially the *amla* fruit was washed and treated with 2 percent hydrogen peroxide for 15-20 minutes to destroy the surface micro-flora, blemishes and stains. After rinsing twice the *amla* fruits were dried at room temperature in a sterilized environment i.e. in Laminar flow. After drying the *amla* shreds were separated from the seeds. To check the amount of total solids, the

moisture content has to be recorded. So, the results of moisture content estimated in *amla* shreds were recorded as 84.91 percent which was nearby to the findings of different researchers such as 81.80 percent by Karpagavalli *et al* (2014) and 80.20 percent by Puranik *et al* (2012)**.** The total solids were recorded maximum in Patanjali pure *amla* juice (16.18%). The least total solids were found in the mechanically obtained juice and the value was as shown in table (10).

Table 10: Observations of moisture content and total solids in different *amla* juice samples:

Sr. no.	Sample	Moisture content (%)	Total Solids (%)
1	Fresh fruit	84.91	15.09
2	Mechanically obtained juice	88.14	11.86
3	Pectinase enzyme obtained juice	84.30	15.70
4	Patanjali pure *amla* juice	83.82	16.18

4.2 Process standardization of *amla* juice by mechanical and the enzymatic methods:

First of all the standardization was done to exactly check the efficiency of the process for juice extraction methods used. Under different trials for the juice extraction from *amla* fruit, it was extracted out by mechanical method (centrifugal juicer) and enzymatic method. Initially the juice was extracted manually by crushing the *amla* fruit shreds and then straining through muslin cloth. The trials were performed thrice and the reading were recorded and it was observed that the mean values of the juice extracted mechanically were 4.1ml, 172.0ml and 439.0ml from the pieces of *amla* of 10g, 400g and 1000g, respectively as shown in table (11). Whereas the enzymatic method or the pectinase treated *amla* shreds were found double effective in case of juice extraction. As the 10g, 400g and 800g pieces of the *amla* shreds production was 8.1ml, 306.0ml and 625.0ml, respectively. As shown in table (12) it was also found that the percentage of leftover pulp was very less in the case of enzymatic method of juice extraction. The leftover may be due to pectinase enzyme action on the protopectin attached with the cell wall of the fruit as observed by Rombouts *et al* (1980). By using both the methods it was detected that the enzymatic method is very much effective as compared to the mechanical method. The rate of the extraction was also higher in the case of the enzymatic method. The extraction rate of the enzymatic method was ranged between 76.5 to 81.0 percent, whereas it was very much less in the case of the mechanical method as shown in fig. (9). The extraction rate of the mechanical

method ranged between 41.0 to 43.9 percent that is exactly half the volume of the juice as observed in enzymatic method. Vaidya *et al* (2009) also used enzymes for the extraction of the juice in case of the kiwi fruit and they also found that the enzyme enhanced the juice recovery as compared to that juice which was not treated with the enzyme. There was a significant color difference in both the juices. The pectinase enzyme obtained juicewas clear as compared to the mechanical extracted juice as shown in fig (8).

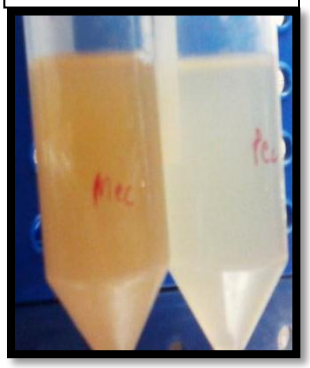
Table 11.Mechanical Method of Juice extraction

Sr. no.	Name of the sample	Original Weight of *amla* pieces (mg)	Mean values of juice extraction (ml)	Extraction rate (%)	Leftover (%) (Juice pulp + After straining)
1	*Amla* pieces	10	4.1	41	31.60
2	*Amla* pieces	400	172	43	32.60
3	*Amla* pieces	1000	439	43.9	41.06
4	Mean		205.03	42.63	35.09

Table 12.Enzymatic Method: *Pectinase* assisted Juice extraction

Sr. no.	Sample	Original weight of *amla* pieces (mg)	Enzyme Used (mg)	Volume of juice extracted (ml)	Extraction rate (%)	Leftover percent (Juice pulp + after straining)
1	*Amla* pieces	10	0.01	8.1	81.00	19.5
2	*Amla* pieces	400	0.1	306	76.5	18.82
3	*Amla* pieces	800	0.5	625	78.54	17.21
4	Mean			313.03	78.54	18.51

Fig 8: Juice extracted by mechanical and enzymatic method



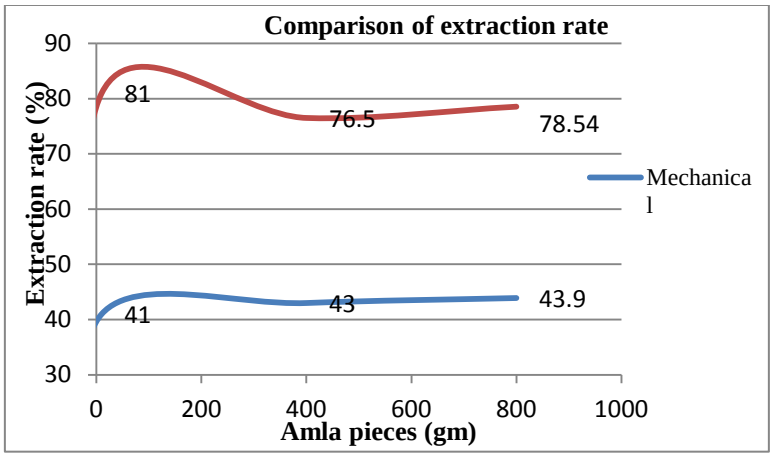


Fig 9: Comparison of extraction rate between the mechanically and enzyme assisted juice extraction.

4.3. Formulation of the probiotic drink:

4.3.1. Isolation of *Lactobacillus sporogenes* (Probiotic micro-organism)

The *Lactobacillus sporogenes* was isolated from the Sporolac© which is the probiotic drink for the children. The procedure followed as described in the materials and methods. The colonies of the *Lactobacillus sporogenes* was observed on the MRS plates and further isolated by the streaking method. The selected colonies were further grown in the nutrient broth and its 100µl was taken in MRS selective media and incubated for 24-48 hours and after the incubation period the growth was observed as shown in fig (10).

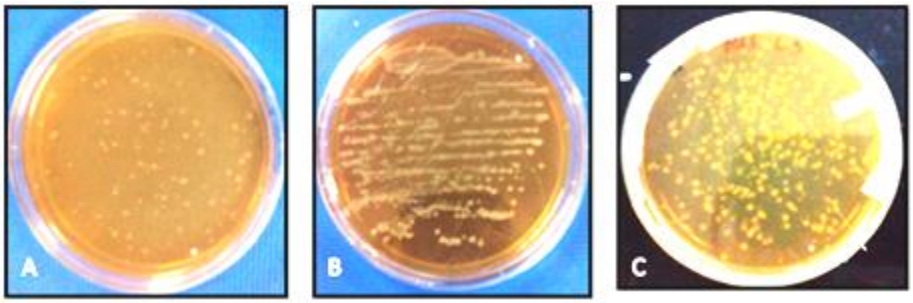


Fig 10: A) Isolated colony from *Sporolac*
B) Streaking to isolate single colony
C) Growth of *Lactobacillus sporogenes* on selective media

4.3.2 *Amla* fruit waste: Seed/Stone conversion to fine powder:

The effective media for the growth of any bacteria usually taken is the nutrient broth. The nutrient broth consists of all the nutrients that are required by the bacterial species to grow. But

during the research it was observed that the *Lactobacillus sporogenes* was growing effectively in the *amla* seed powder. The *amla* seeds were isolated and dried properly followed by crushing to convert into a powder. Then this powder was sieved under sterilized conditions as shown in the fig (11) given below. Then, 0.4 percent of this powder was taken as a growth medium for the *Lactobacillus sporogenes*.

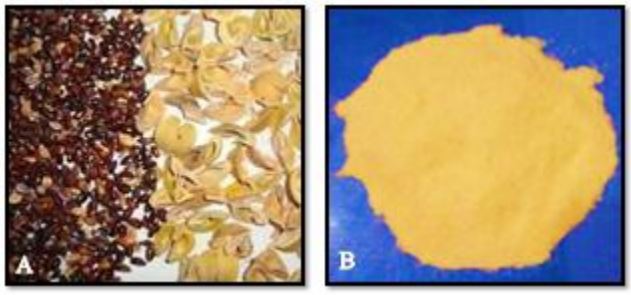


Fig 11: (A) Originally the *amla* seeds dried (B) *Amla* Seed Powder

4.3.3: Utilizing *Amla* seed powder as growing medium for *Lactobacillus sporogenes:* A base for Probiotic culture:

The *Lactobacillus sporogenes* has shown its growth in the dried *amla* seed powder and observed as under. The optical density (OD) was measured at the wavelength of 760nm after 1hr upto 10^{th} hr and the observation were given in the table (13). The *Lactobacillus sporogenes* was observed with changes in growth behavior due to changes in the pH as the bacterial growth increases the pH of the sample decreases and it was found that when the bacteria reached into the death phase the pH increases. The pH values were decreased from 3.1 to 2.92 upto 9 hours. The growth curve was shown as in fig (12). The *Lactobacillus sporogenes* was freshly grown in the dried seed powder of the *amla* for the preparation of the probiotic drink. The growth of the probiotic micro-organism in the freshly prepared culture was found to be 3.1×10^{6}*cfu*/ml. For further formulation this culture was used.

Table 13: Growth of the *Lactobacillus sporogenes* (probiotic micro-organism) in the *amla* seed powder.

Sr. no.	Hours	pH	*Lactobacillus Sporogenes* (in *amla* pulp powder) O.D at 760 nm
1	1	3.1	0.90
2	2	2.92	0.94
3	3	2.89	1.15
4	4	2.88	1.19
5	5	2.86	1.31

6	6	2.77	1.49
7	7	2.67	1.61
8	8	2.62	1.65
9	9	2.92	1.59
10	10	2.94	1.54

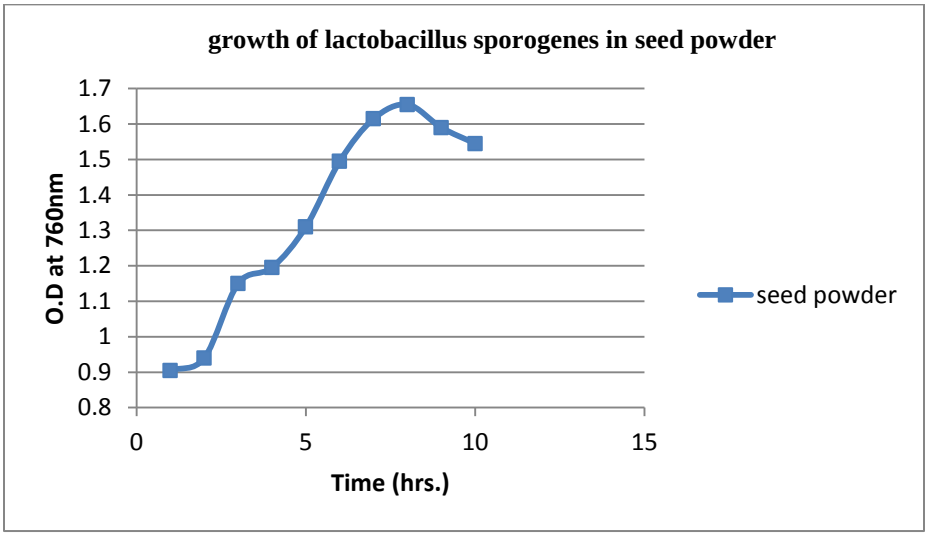


Fig 12: Growth of *Lactobacillus sporogenes* in seed powder

4.3.4. Growth comparison of the *Lactobacillus sporogenes* in Nutrient Broth and *Amla* seed powder:

While comparing the growth of the *Lactobacillus sporogenes* in the nutrient media and in the dried seed powder it was observed that the growth of the isolated species was almost same. The nutrient media was considered as reference to check the growth of the *Lactobacillus sporogenes.* The growth curve has been shown as in the fig. (13). The values of the wavelength and the effects of the pH on the growth of *Lactobacillus sporogenes* were given in the table **(14).**

Table 14.Growth comparison of the *Lactobacillus sporogenes* in Nutrient Broth

Sr. no.	Hours	pH	*Lactobacillus sporogenes* (in NB Media) O.D at 760 nm
1	1	5.3	0.007
2	2	5.3	0.012
3	3	5.28	0.016
4	4	5.26	0.24
5	5	5.24	0.79
6	6	5.10	1.35
7	7	4.89	1.80
8	8	4.79	1.94

9	9	4.81	1.94
10	10	4.89	1.50

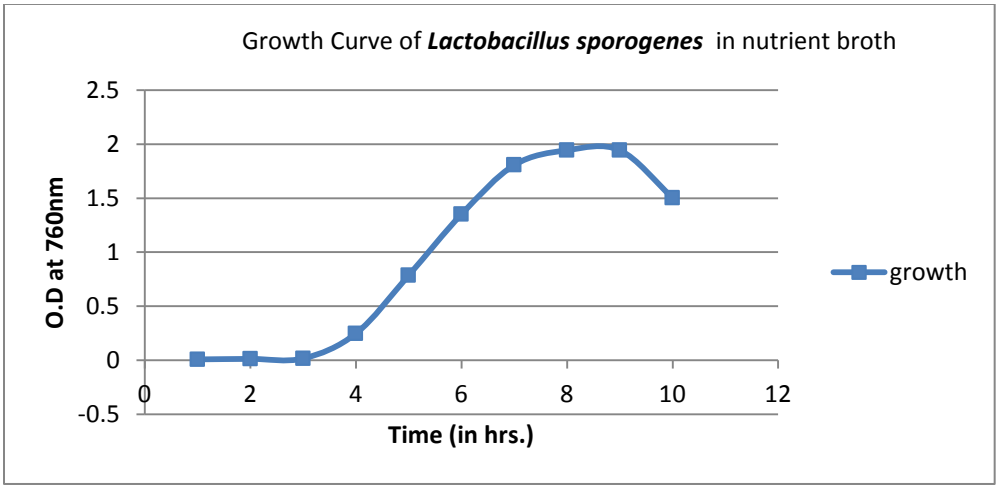


Fig 13: Growth Curve of *Lactobacillus sporogenes* in nutrient broth

The growth of the *Lactobacillus sporogenes* was also observed on the MRS selective media plates to cross check. The numbers of the colonies were more than 300 in number and it was uncountable as shown in the diagram. The growth records remain as such for the *Lactobacillus sporogenes* during first hour upto 6th hour.

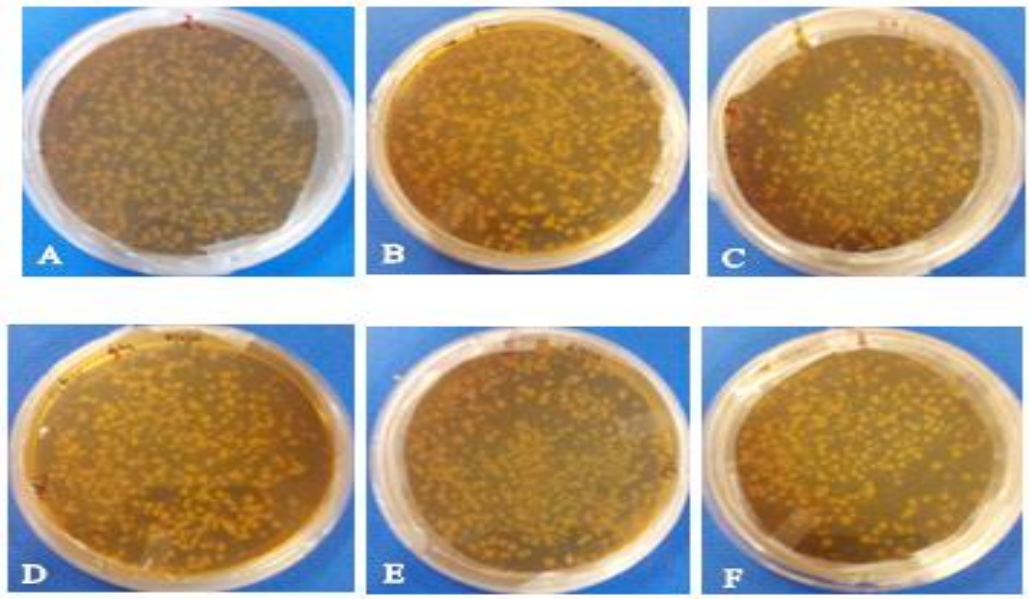


Fig 14: Petri-plates showing the *cfu* in the nutrient agar after every hour
A) After I hr, B) II hr, C) III hr D) IV hr E) V hr F) VI hr

4.3.5 Formulation of the probiotic drink:

The formulation of the probiotic drink was prepared taking *amla* juices from two different juice extraction methods (juicer and enzymatic) as base and the *Lactobacillus sporogenes* culture grown in *amla* seed powder. To the probiotic drink the stevia tablet was added @ 0.5g to the 100ml total volume of the drink. The pure Patanjali *amla* juice was used as sample to compare the sensory characteristic with the formulated juices. Firstly, four formulations were prepared as given in the materials and methods. Two formulations were selected on the basis of the taste, texture and the color. The 50:50 and 60:40 were the two formulations which were good in taste and the color also was very near to the original color of the *amla* juice. The other two formulations 75:25 and 85:15 were very bitter in taste and the color of those drinks was very different from the original color of the juice. In the selected formulations 10^6 to 10^7*cfu*/ml observed which was selected for further preparation of the probiotic drinks. It was reported by Nualkaekul *et al* (2011) that the recommended number of colonies should have 10^7*cfu*/ml at the end of shelf life. The other two formulations were not able to get the set number of *cfu*/ml. Hence, they were rejected for further sensory analysis.

4.4. Sensory evaluation of the selected formulations:

The 9-point Hedonic scale was used to assess the different parameters. The different parameters selected for the further sensory analysis (appearance, color, flavor or aroma, texture, taste and overall acceptability). The analysis data was given in table (15).

Table15.Sensory analysis of selected *Amla* drinks:

Sr. no.	Sample Code	Appearance	Color	Flavor or Aroma	Texture	Taste	Overall Acceptability	Average
1	**C1***	6.50±0.62	7.11±0.90	7.00±1.08	6.89±0.96	7.05±1.12	6.94±0.87	6.89
2	**C2***	7.17±1.38	7.05±1.21	7.11±1.08	6.78±0.88	6.39±1.65	6.67±1.28	6.86
3	**D***	6.28±0.89	7.00±0.77	6.94±0.80	6.83±0.78	7.11±0.76	7.17±0.70	6.89
4	**M1***	6.67±0.97	6.94±0.87	6.27±0.89	6.44±0.98	6.17±0.85	6.22±0.88	6.45
5	**M2***	6.72±1.17	6.94±1.16	6.781.43	6.33±1.37	6.28±1.32	6.70±1.21	6.64
6	**P1***	7.30±1.18	7.33±0.97	7.17±1.69	7.11±1.32	7.28±1.70	7.55±1.19	7.29
7	**P2***	8.00±0.94	7.78±1.13	8.00±1.25	7.44±1.34	7.78±1.39	8.17±0.96	7.86

Foot notes*: C1*-Control of Mechanical Juicer method, C2*- Control of Enzymatic method of juice extraction, D* Patanjali pure *amla* juice, M1*- 50:50 formulation with mechanical juice, M2*- 60:40 formulation with mechanical

juice, P1*- 50:50 formulation with pectinase enzyme obtained juice, P2*- 60:40 formulation with pectinase enzyme obtained juice

4.4.1. Appearance:

The data regarding the appearance score of the different samples have been tabulated in table (15). The mean values with the standard deviation of the appearance of all the drinks were ranged from 6.50 to 8.00. According to the hedonic scale the 6.50 was designated as the slightly liked and 8.0 as very much liked by the group of people. The highest mean value of the appearance was shown by the sample P2 (8.00±0.94) and the lowest value was shown by sample D (6.28±0.89) i.e. the Patanjali pure *amla* juice sample. The P2 has the highest appearance score due to the clarity of the juice and it was shinier as compared to the other samples. The other samples were also clear less than the P2 sample. The standard deviation showed variation may be the individuas tasted it for first time and the reviews varied huge personally. The data was compared with the Patanjali *amla* juice which is the monopoly producer at present in the Indian market. The appearance of the D was observed very much similar to the pectinase enzyme produced juice. But the appearance of the D was slightly liked by the individuals.

4.4.2. Color:

All the samples have their own color but the difference in the color of all the samples not very large. The data regarding the color of the samples was given in the table (15). The highest mean value of the color was shown by the sample P2 (7.78±1.13) and the least value was shown by M1 an M2 (6.94) that was made from *amla* juice obtained from the mechanical juicer. The overall acceptance of the color depends on the perception of different individuals. So, may be due to this there was a large variation in the standard deviation. The color of all the drinks was slight brown in color maybe it was bit difficult to rate. The color of the D was observed very much similar to the pectinase enzyme obtained juice. But the color of the D was moderately liked by the individuals. The color data ranged from 6.94 to 7.78. All the drinks were stored under refrigerator for 7 days. After 7 days the color of all the drinks were changed because the yeast growth was observed and spoiled.

4.4.3. Flavor or Aroma:

The highest mean flavor or aroma score 8.00 was recorded for P2 sample of *amla* drink. Among all the drinks while the lowest flavor or aroma mean score obtained was 6.27 for M1. The standard deviation varied from the 0.80 to the 1.69. The variation in the drinks flavor observed maybe because of variation in the individual acceptance. After 7 days the flavor of the drinks was spoiled.

4.4.4. Texture:

The viscosity or the texture of the P2 was observed best among all the drinks. The highest and lowest value of the texture was recorded as 7.44 and 6.33 respectively. The lowest value of the texture was shown by the M2. The sample D was thicker as compare to all other drinks as its total solids were observed more as compared to other samples.

4.4.5. Taste:

According to the hedonic scale the P2 found to have the best taste as compared to the other formulated drinks. The mean score observed was 7.78 and P2 was sweeter in taste as compared to all as shown in table (15). All other samples were slight bitter in taste but P2 was little bitter and sweeter and giving sweeter after taste. It gave the sweet taste at initial tasting and even after taste. M1 was very much bitter in taste and so, least scored (6.17) by the individuals. The taste of the D was similar to the pectinase enzyme obtained juice. But the taste of the D was moderately liked by the individuals. In P2 formulation, the base i.e. *amla* juice was taken from the pectinase or enzymatic method of juice extraction. So, the bitterness of obtained was less as compared to the mechanical method obtained juice. Hence, P2 was less bitter in taste. After the storage period of 7 days the taste of all samples were spoiled because of yeast growth and the taste became bitter.

4.4.6. Overall Acceptability:

In table (15) mean scores of overall acceptability for all the drinks have been tabulated. Highest overall acceptability was observed for the P2 (8.17) followed by the P1 (7.55). The least mean score was shown by the C2. In C2 the juice extracted out with the help of mechanical

method and in this case the juice was very much bitter in taste. The flavor of this formulation was not like proper juice but it was like juice that has been diluted with water.

4.4.7. Percentage of the overall acceptability:

The percentage of the overall acceptability was calculated on the basis of the sensory analysis. The data was given in the table (16).

Table 16: Percentage acceptability of the probiotic drink:

Sr. no.	Samples	Percentage of acceptability
1	C1	38.58
2	C2	37.03
3	D	39.81
4	M1	34.57
5	M2	37.65
6	P1	41.97
7	P2	45.37

The C1 was the pectinase enzyme obtained juice and used as the base for P1 and P2. On the other hand C2 was the mechanically obtained juice and used as the base for the M1 and M2. The D was the pure Patanjali *amla* juice sample and used for the comparison with the probiotic drinks and the juices that were extracted out mechanically and the enzymatically.

On the basis of sensory evaluation, P2 has shown the maximum percentage of the overall acceptability and it was recorded as 45.37 percent. The color, appearance, taste and flavor of the P2 was perfect. All the characteristics of P2 were in the range of 7-8 which was designated according to the hedonic scale as like moderately and like very much respectively.

Both the formulation which were prepared from the pectinase has more percentage acceptability as compared to the formulation were mechanical based. The least accepted drink was C2 due to its bitter taste and according to the average value of all characteristics was slightly liked by the individuals. According to the percentage acceptability the order of liking of the drink was P2>P1>D>C1>M2>C2>M1. After the analyses it was found that among all the drinks the P2 was the best one drink.

4.5. Antioxidant properties and antibacterial properties in the *amla* juice

4.5.1. Antioxidant activity:

The antioxidant activity of different samples was detected by the DPPH assay. After analysis it was found that the fresh *amla* has the higher antioxidant activity when extracted out manually as compared to the pectinase treated *amla* juice. It was found the manually extracted *amla* juice has 92.92percent and the pectinase treated *amla* juice has 87.64percent of the antioxidant activity reported as shown in the table (17). The patanjali *amla* juice was kept as the control which showed the highest (94.27%) antioxidant activity as compared to other two samples. The storage had a negative effect on the antioxidant activity. The antioxidant activity was degraded with time as shown in table (17) and found that it was decreased after the storage period of 15 days. The activity of Patanjali *amla* juice, freshly prepared and pectinase treated *amla* juice shown 90.96 percent, 89.69 percent and 76.75 percent respectively. Puranik *et al* (2012) also reported 83.24 percent of the antioxidant activity in fresh *amla* as compared to the processed *amla* products. The antioxidant activity of pectinase enzyme obtained juice was found to be less as compared to the fresh juice maybe due to the temperature for enzyme activity as discussed by the Karpagavalli *et al* (2014). Due to the thermal effects the percentage of the scavenging activity was reduced.

Table 17: Effect on the antioxidant activity in fresh *amla* juices and after storage.

Sr. no.	Subjects	Antioxidant Activity (%)	
		1^{st} day	15^{th} day
1	**A(Fresh)**	92.92	89.69
2	**B (Pectinase)**	87.64	76.75
3	**C (patanjali)**	94.27	90.96

4.5.2. Vitamin C Concentration in *amla* juices:

Vitamin C content was determined in the sample by titration method and it was found that the content of the vitamin C was very high in the tested samples. The test samples were compared with the Patanjali pure *amla* juice which was recorded with the highest amount of the vitamin C content i.e. 8.02mg/ml means 802mg/100ml as shown in the table (20). The pectinase enzyme obtained juice showed the least amount of vitamin C concentration as compared to the other samples. The fresh *amla* juice observed with 5.38mg/ml means 538mg/100ml content of

vitamin C and the similar result was quoted by the Karpagavalli *et al* (2014). They reported that the fresh *amla* juice has the 456.17mg/100g of the sample. The overall vitamin C concentration in the different samples was ranged from 3.56mg/ml to 8.02mg/ml as shown in the table (18). The values of the vitamin C concentration were calculated from the slope of the standard graph. The samples were stored under refrigeration for 15 days and found that the vitamin C concentration decreased. The vitamin C concentration was observed to be degraded with time. It was concluded that the vitamin C concentration decreased in the range of 0.5 to 0.7 percent. The reduction in the vitamin C content during the storage period might be due to the oxidation of ascorbic acid by trapped oxygen in the bottles or by ascorbic acid oxidative enzymes (Nagy, 1980). Due to the thermal affect the pectinase enzyme obtained juice showed the least vitamin C concentration. The incubation period was very long may be due to this effect the vitamin C concentration degraded in the pectinase enzyme obtained juice. The values of the vitamin C concentration after the storage period were shown in the table (20). The standard graph as shown in fig (16).

Table 18: Effect on vitamin C content in fresh and stored *amla* juices.

Sr. no.	Subjects	Vitamin C (mg/mL)	
1		**1st Day**	**15th Day**
	A (Frseh)	5.38	4.56
2	B (Pectinase)	3.56	2.86
3	C (Patanjali)	8.02	7.42

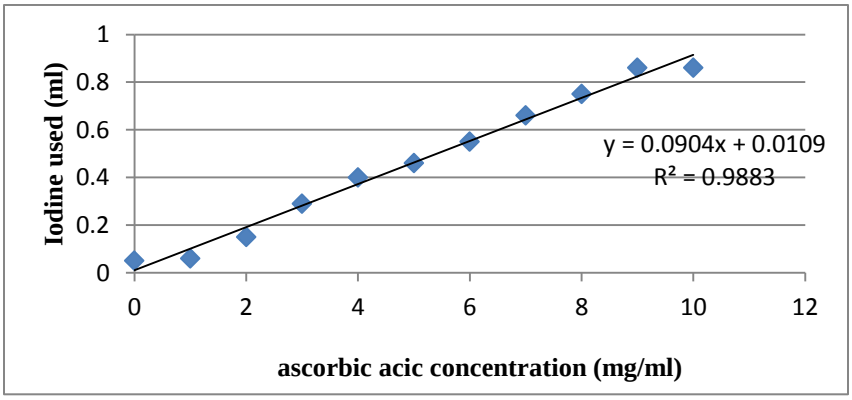


Fig 16: Standard curve for the determination of the vitamin C content

4.5.3. Tannin content in *amla* juices:

The total polyphenols was observed in the tested samples. The effect of storage on the tannin content of the *amla* juices: mechanically extracted *amla* juice, enzymatically extracted *amla* juice and the Patanjali pure *amla* juice have been shown in the table (19) and the standard curve as shown in the fig (17).

Table 19: Tannin content in fresh and stored *amla* juices.

Sr. no.	*Amla* juice samples	Tannins (mg/mL)	
		1st day	After 15th day
1	**A (fresh)**	9.67	8.84
2	**B (Pectinase)**	4.67	4.46
3	**C (Patanjali)**	10.03	8.43

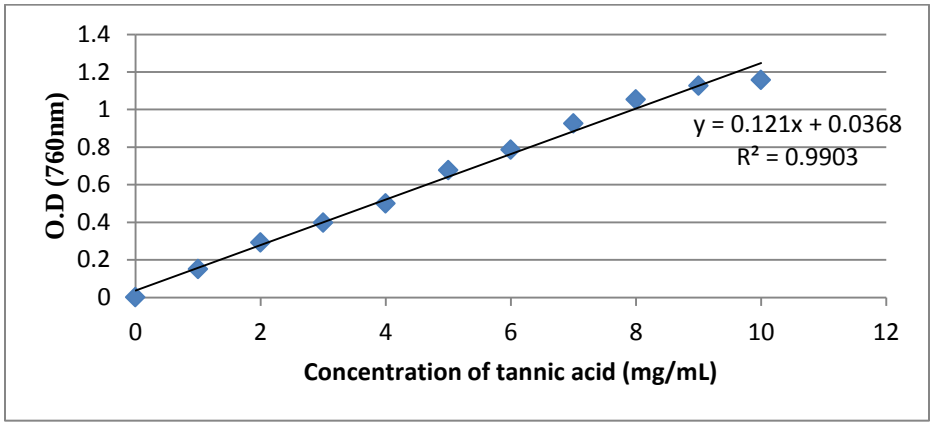


Fig 17: Standard curve for the determination of the tannin contents

The data revealed that the highest value of tannin content was found in the Patanjali pure *amla* juice. The concentration was recorded as 10.03mg/ml for pure Patanjali *amla* juice which was followed by mechanically treated juice having 9.67mg/ml of the tannin content. It was found that the pectinase treated *amla* juice had showed the least tannin content (4.67mg/ml) as compared to the others. The reduction in tannin content was recorded during the storage period after 15 days. All the samples have shown less tannin content after the 15 days as compared to the fresh. The tannin content was reduced to a range of 8.84mg/ml to 4.46mg/ml. The tannin content in mechanically obtained juice, pectinase enzyme obtained juice and the Patanjali pure *amla* juice was recorded with 8.84mg/ml, 4.46mg/ml and 8.43mg/ml respectively. Nayak *et al* (2011) recorded the loss of the tannin content in the aonla syrup from 0.51 to 0.39 percent after a storage period of 9 days. They concluded that the loss in the tannin content during the storage

period could be due to the oxidation of tannins or leaching of tannins into syrup and their condensation into coloured pigment (Fenemma, 1976).

4.5.4. Flavonoid content in *amla* juices:

The flavonoid content was assessed in all the samples of *amla* jucie. Against *Quercetin* used as the standard the total flavonoid was expressed as 'mg' of *Quercetin* equivalent in 100ml of juice sample and even observed that enzyme extracted juice showed the highest total flavonoid content as compared to the other samples. The concentration was calculated from the slope (y= 0.0021x + 0.0159) of the graph and the R2 value was 0.993. The concentration of the total flavonoid content was ranged from 23.5mg/ml to 27.4mg/ml as shown in the table (20). The values of the mechanically extracted juice, enzyme extracted juice and the Patanjali pure *amla* juices were detected with the total flavonoid concentration of 25.7mg/ml, 27.4mg/ml and 23.5mg/ml respectively. The relative data was discovered by the Karpagavalli *et al* (2014) and the value witnessed by them for fresh *amla* juice was 268.41mg Quercetin equivalent/100g.

Table 20: Effect on flavonoid content in *amla* juice in fresh and during storage:

Sr. no.	Subjects	Flavonoid (ppm)	Flavonoid (mg/ml)
1	A (fresh)	514.65	25.7
2	C (Pectinase)	548.619	27.4
3	B (Patanjali)	471.4762	23.5

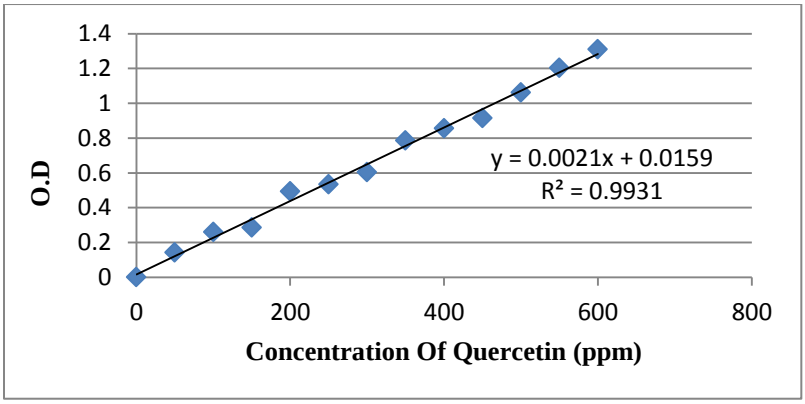


Fig 18: Standard curve for the determination of the flavonoid content.

4.5.5. Estimation of the flavonoid content in mechanically and enzymatically treated juice by HPLC method:

High performance liquid chromatography has been discovered as the accurate method to analyze the antioxidant activity providing alkaloids or bioactive components. There have been various methods to isolate and detect a particular antioxidant component against the standard reference of that antioxidant. So, here an attempt was performed to detect the '*Quercetin*' alkaloid responsible for the antioxidant activity in the *amla* or Indian gooseberry. The standard solution of the '*Quercetin*' was prepared to check that whether it is present in the sample or not along with if present than how much concentration it is in *amla* juices obtained from different juice extraction methods as described in the materials and methods. The different graphs showing the concentrations of alkaloids present in *amla* juice obtained from Shimadzu-HPLC of the analysis as shown below:

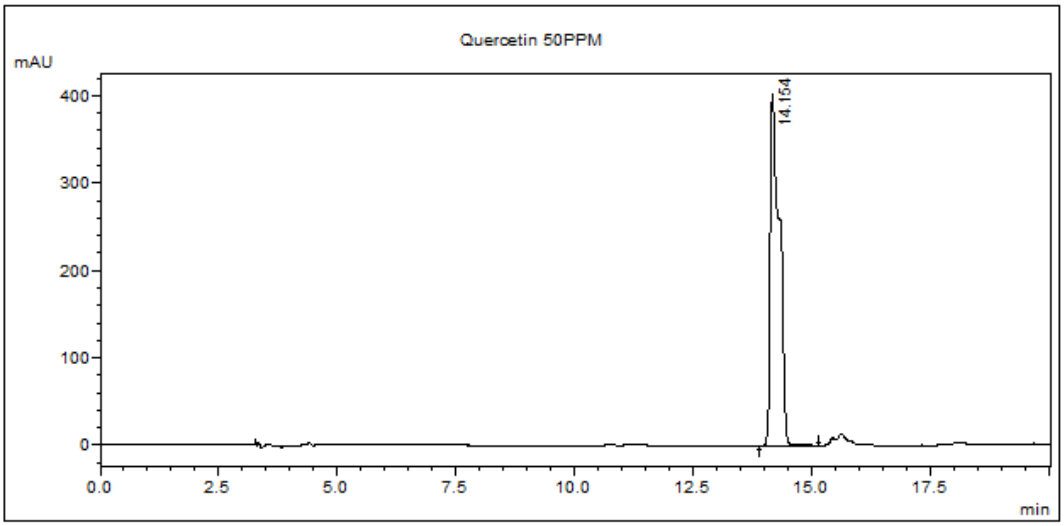


Fig 19: *Quercetin* Standard graph

The figure shows that the *Quercetin* retention time was 14.15 minutes at the wavelength of 370nm. The graph of the different samples was shown in figure (20 & 21).

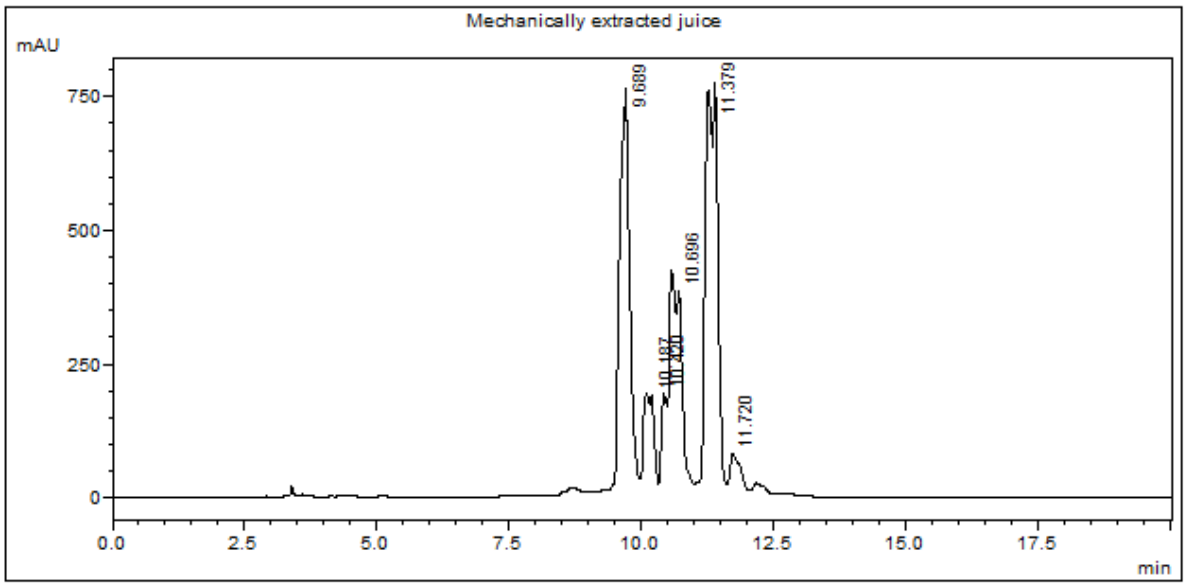


Fig 20: Estimation of the *Quercetin* in mechanically obtained juice

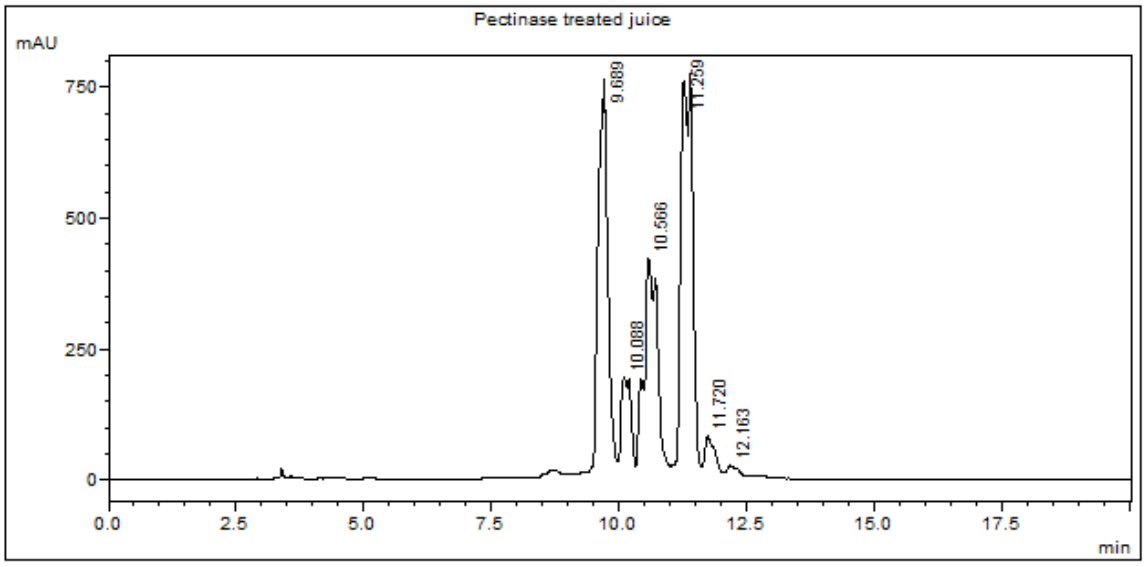


Fig 21: Estimation of the *Quercetin* in pectinase enzyme obtained juice

From the above figures, it was reported that the peak of the *Quercetin* was not shown at the retention time of 14.15 minutes it was in the standard graph. It can be concluded that the *Quercetin* may be present in the juice but it was unable to detect or maybe present in very least quantity as followed and observed by Bansal *et al* (2014).

4.6. Antibacterial Activity in *amla* juices:

The *amla* fruit singularity in terms of the capacity to kill the microbes those are harmful to our body has also been researched upon. It was reported by the Nain *et al* (2012) and Satyajit *et al* (2012) that the *amla* fruit and leaves showed the antimicrobial activity against the *Escherichia coli, Bacillus subtilis, Salmonella typhi* etc. The study was carried on different strains such as *Escherichia coli, Bacillus subtilis, Lactobacillus spp.* and *Saccharomyces cerevisae.* Again detected by Cai *et al* (2003) that the antimicrobial activity of the different strains because of the phytochemicals present in the fruit and witnessed with the positive results by different strains. The data regarding this mentioned in different tables as below.

4.6.1. Activity of *E. coli*:

The percentage of the antibacterial activity of the different juices was shown in the table (21). It was found that the mechanically extracted *amla* juice showed the highest antibacterial activity in all the concentrations. The activity varied from the 49.86 percent to 54.65 percent in case of the mechanically obtained juice and it may be due to the highest vitamin C and tannin concentrations as observed in mechanically obtained juice as shown in the table (21). The least activity was shown by the pectinase enzyme obtained juice ranged from 44.16 percent to 47.39 percent and it may be due to the least vitamin C and tannin concentrations as observed in mechanically obtained juice as shown in the table (21). As the concentration of the juices increased the percentage activity was also increased as similar results given in Nain *et al* (2011). The same trend was followed by the pectinase enzyme obtained juice and the Patanjali pure *amla* juice. The antibacterial activity was measured against the standard antibiotic named as *Gentamicin* having concentration 80mg/2ml.

Table 21: Antimicrobial activity of *E.Coli* in percentage:

Sr. no.	Concentration of sample (µl)	Percent of activity of *E. Coli*		
		Mechanically extracted juice (%)	Pectinase enzyme obtained juice (%)	Patanjali pure *amla* juice (%)
1	10	49.86	44.16	47.58
2	20	52.75	45.41	49.61
3	30	53.81	46.67	54.28
4	40	54.65	47.39	49.21

4.6.2. Activity of *B. subtilis*:

In the case of the *Bacillus subtilis* the pectinase enzyme obtained juice has shown the highest percentage antibacterial activity (37.50 -50.485) followed by mechanically extracted juice ranging from 30.55 percent to 36.19 percent and finally with least antimicrobial activity was recorded by Patanjali pure *amla* juice with the readings ranged between 29.86 percent to 40.00 percent. The reason may be because of antimicrobial alkaloids that responsible for the antimicrobial activity were least degraded in the pectinase enzyme obtained juice as compared to others. In case of the mechanically obtained juice these alkaloids may be disintegrated into their simpler inactive forms without antimicrobial activity. In this case it can be concluded that as the strain sensitivity increased in accordance with the increase in concentration as such the growth of the strain was inhibited.

Table 22: Antimicrobial Activity of *B.Subtilis*

Sr. no.	Concentration of sample (µl)	percent of Activity of *Bacillus subtilis*		
		Mechanically extracted juice	Pectinase enzyme obtained juice	Patanjali pure *amla* juice
1	10	30.55	37.50	29.86
2	20	32.71	42.68	33.33
3	30	35.96	49.34	44.09
4	40	36.19	50.48	40.00

4.6.3. Activity of *Lactobacillus spp.*:

Lactobacillus spp. also showed the positive response in case of antibacterial activity. The mechanically obtained juice showed the maximum antibacterial activity ranged from 48.48 percent to 54.54 percent as compared to the other juice samples. The reason may be due to highest vitamin C and tannin content as observed in the antioxidant properties shown in the table (23). The percentage values as mentioned below in table (23).

Table 23: Percentage of Antimicrobial Activity of *Lactobacillus*

Sr. no.	Concentration of sample (µl)	percent of Activity of *Lactobacillus*		
		Mechanically extracted juice	Pectinase enzyme obtained juice	Patanjali pure *amla* juice
1	10	48.48	28.79	31.81
2	20	50.00	36.18	35.52
3	30	54.40	48.18	43.01

4	40	54.54	47.98	51.51

4.6.4. Zone of inhibition (diameter) *Saccharomyces cerevisae*:

It was discovered that as the concentration of the sample increases the zone of inhibition in centimeters was also increases in case of *Saccharomyces cerevisae* as shown in tables (24). It can be concluded that as the zone of inhibition increases, it means the species sensitiveness towards that concentration is positive. It can be mentioned by observing the table that the pectinase enzyme obtained juice showed highest zone of inhibition in comparison to mechanically extracted juice that means the storage life of this juice would be more if experimented.

Table 24: Diameter of zone of inhibition

Sr. no.	Concentration of sample (µl)	Diameter of zone of inhibition of *Saccharomyces cerevisae* (cm)		
		Mechanically extracted juice	Pectinase enzyme obtained juice	Patanjali pure *amla* juice
1	10	1.37	1.45	1.37
2	20	1.45	1.60	1.60
3	30	1.58	1.85	1.70
4	40	1.83	1.90	1.85

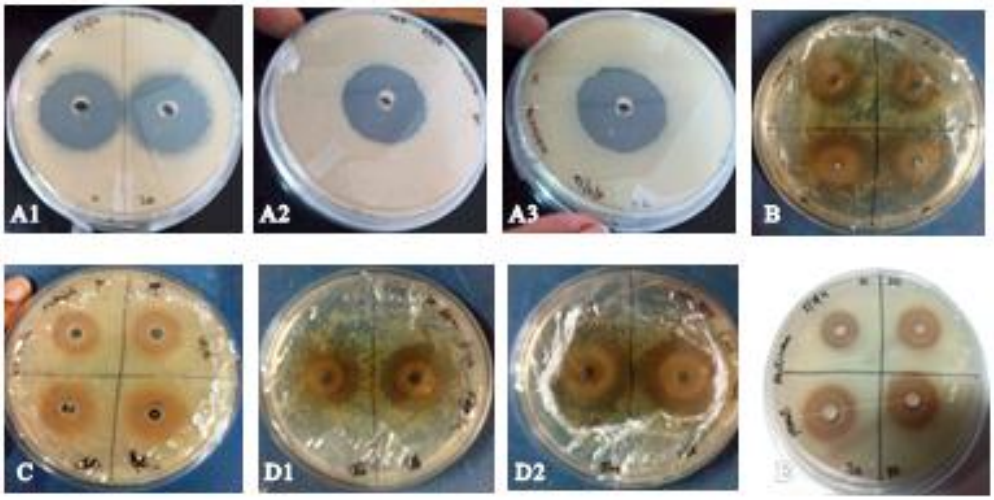


Fig 22: Zone of inhibition

(A1, A2 & A3) - Standard Gentamicin, (B) - *lactobacillus*, (C) - *Bacillus subtilis*, (D1 & D2) - *E.Coli* and (E) - *Saccharomyces cerevisae*

4.7. *Lactobacillus sporogenes cfu*/ml in the formulated Probiotic drinks:

The growth of the probiotic micro-organisms were detected on the MRS plates as shown in figure (15). The number of colonies those were counted on different samples as given in the table (25).

Table 25: The comparison of the number of colonies in probiotic drink

Sr. no.	Samples	*cfu*/ml
1	M1	2.38×10^{8}
2	M2	2.22×10^{8}
3	P1	1.88×10^{8}
4	P2	2.61×10^{8}

The P2 showed the maximum number of colonies (2.61×10^{8}*cfu*/ml) near to the results observed by Nualkaekul *et al* (2011) that recommended the number of colonies should have 10^{7}*cfu*/ml at the end of shelf life as compared to the other drinks. Hence it was also proved that the P2 formulation has shown the best results and it was best one out of all other drinks.

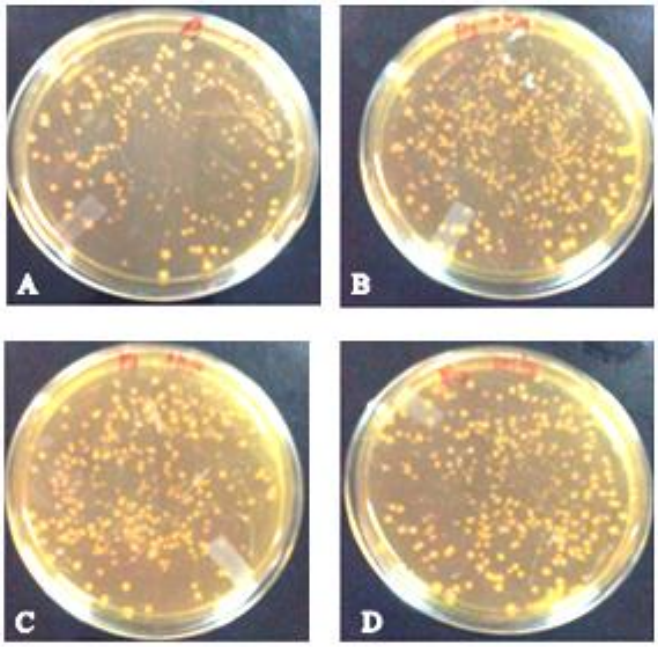


Fig 15: Growth of the *Lactobacillus sporogenes* in probiotic drinks
(A) P1 (B) P2 (C) M1 (D) M2

4.8. Antioxidant properties of the Probiotic drinks:

The different antioxidant properties were checked after the sensory evaluation to check whether the added components like *stevia* (sugar substitute), probiotic culture and the *amla* seed powder does cause any change in the reduction of the antioxidant activities. The antioxidant

properties were tabulated in table (26). The highest antioxidant activity was shown by the C1 (95.01%). C1 was used as the base for the M1 and M2 drinks. From the pectinase enzyme obtained juice the maximum antioxidant activity was shown by the P1 (89.79%). If we compare only in the case of probiotic drinks that were prepared from the *amla* juice obtained from two different methods, the maximum antioxidant activity was shown by the M1 and the least antioxidant activity was shown by the P2 (87.78%). Similar results were found in the case of tannin content in the drinks, the highest value was shown by the M1 (5.50mg/ml) and the least value was shown by the P2 (3.70mg/ml). In the case of mechanically extracted *amla* juice made probiotic drink the reason for the highest overall antioxidant activity and tannins because of highest vitamin c and tannins content as mentioned in the table (26). Vitamin C may get reduced during storage but tannins also sometimes contribute towards the overall antioxidant activity as detected by Ghosh *et al* (1982) However, in case of the flavonoid content the highest value was shown by the P2 (23.32mg/ml) and the least value was in the case of the M2 (18.48mg/ml). The reason for the highest flavonoid content observed in pectinase enzyme obtained *amla* juice may be because of lack of percentage of tannins as observed during the method of extraction as shown in the table (26). The different values for the different antioxidant properties were depicted in the table (26).

Table 26: Antioxidant properties of probiotic drink:

Sr. no.	Subjects	Antioxidant Activity (%)	Flavonoids (mg/ml)	Tannins (mg/mL)
1	C1	86.67	26.44	10.64
2	C2	95.01	25.09	5.83
3	D	93.32	24.91	9.32
4	M1	92.37	20.82	5.50
5	M2	91.99	18.48	5.02
6	P1	89.79	23.09	5.65
7	P2	87.78	23.32	3.70

CHAPTER-V

Conclusion:

The investigation was carried out to study the process standardization of the juice extraction by mechanical and enzymatic method and it was concluded that the enzymatic method is double effective method in the case of extraction rate, yield and in the purification process. It was also found that it reduces the wastage of the fruit as the pulp leftover is very much less as compared to the mechanical method of the juice extraction. The seed waste can be used for the growth of *Lactobacillus sporogenes* that can be further helpful in the preparation of the probiotic drink and hence, safe for the consumption. Interestingly, it was found that the growth of *Lactobacillus sporogenes* was observed similar in Nutrient Broth as well as in the dried *amla* powder. The different formulations were prepared with the help of the different *amla* juice and with the culture (*Lactobacillus sporogenes).* After the sensory analysis it was found that the P2 was the best probiotic drink as compared to all others. P2 was best in all the cases like appearance, color, flavor, taste, texture and overall acceptability. The P2 showed the highest value of overall acceptability (45.37%). It was also found that P2 had fulfilled the requirement for the recommended probiotic culture colonies in the drink. The stevia was added in the drink just to reduce the bitterness of the *amla* juice and it is 100-300 times sweeter than the sucrose without any side effect. At the end it can be concluded that there was 100 percent utilization of the *amla* fruit possible by the use pectinase enzyme for juice extraction and use of seed/stone of *amla* as a source for probiotic growth.

The different antioxidant properties of the different juices were compared like vitamin C concentration, Antioxidant activity, Tannin content and Flavonoid content with their respective methods. It was found that the antioxidant activity, vitamin C and the tannin content was foundmaximum in case of the Patanjali *amla* juice but if we compare the different extraction methods the mechanical extracted juice had the more antioxidant activity, vitamin C and the tannin content. The flavonoid content was found maximum in case of the pectinase treated juice. If we compare on the basis of the extraction rate yield and percentage of the leftover pectinase treated found best method. There was not very much difference in the antioxidant activity, vitamin C and tannin content, it can be neglected if the wastage reduces or extraction rate was

more. The *amla* juices have showed antimicrobial activity as the concentration of the juices increases the percentage activity also increases. The activity of the juices was checked against the *Bacillus subtilis, Lactobacillus, E.Coli* and *Saccharomyces cerevisae.* All the strains gave the positive results. The gentamicin was used as the standard antibiotic. It may be concluded that the *amla* juice can be taken in place of the antibiotics as it is safe for the health and having not any side effects.

At the end the HPLC method was used to quantify the '*Quercetin'* content in the juices that were extracted out by mechanical method and by enzymatic method. The '*Quercetin'* content was not able to found. The different peaks were observed that gave the idea that other flavonoid content may be present like kampherol etc.

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