Characterization of phenolics extracted from Amla (*Emblica officinalis*) and its processing waste and development of Amla-lemon based RTS drink

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

By

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February, 2014

Dedicated …

My son Aryan,

Husband and Parents

Characterization of phenolics extracted from Amla (*Emblica officinalis*) and its processing waste and development of Amla-lemon based RTS drink

ABSTRACT

The thesis includes extraction, characterization and isolation of phenolic compounds from Amla powder, Amla pomace powder and Amla seed coat powder. The present investigation deals with the efficacy of different solvents, temperature and pH on recovery of total phenolic content. It focuses on optimum conditions for highest recovery of phenolics from Amla and its processing waste. The study also evaluated the retention of antioxidant properties in isolated components from Amla pomace powder. The thesis describes the procedure for the development of Amla-lemon based RTS as a functional food.

The thesis is divided into eight chapters which are briefly discussed below.

Chapter-1 presents the general introduction about Amla, its production, morphology, composition, functional and medicinal properties. This chapter also reviews the bioactive components extracted from Amla till now. Different methods for the extraction of phenolics from plant sources are emphasised. It also focuses on commercially available Amla products. Finally the scope and objectives of the present investigation are included.

Chapter-2 deals with the physicochemical properties of different Amla varieties. Various physicochemical properties like size, shape, weight, proximate and total phenolic content of different Amla varieties were investigated. Amla variety selected on the basis of physicochemical properties and availability along with its seed and seed coat were investigated for their proximate composition, mineral analysis, functional properties (total phenolic content, water holding capacity, and swelling capacity). Total phenolic content was compared by both FCR (Folin-Ciocalteu reagent) and reverse phase high performance liquid chromatography (HPLC). The presence of functional groups in Amla fruit, seed and seed coat were also investigated by Fourier transform infrared spectroscopy (FTIR).

Chapter-3 reports the efficacy of different solvents on recovery of total phenolic content from Amla powder. The best optimum conditions for recovery of total phenolics with potent 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH*) scavenging activity from Amla powder through response surface methodology are reported. This chapter also deals with further purification of phenolics using different solvents and by column chromatography. Different fractions from different solvents and column chromatography of the ethyl acetate fraction were characterized by reverse phase HPLC and FTIR. The recovery and DPPH* scavenging activity of the different fractions of Amla powder were also studied.

Chapter-4 presents extraction, isolation, and characterization of phenolics and analyses of the antioxidant properties of Amla pomace powder. In this chapter total phenolics were extracted with a mixture of ethanol and water and the obtained extract was concentrated and freeze dried under vacuum. Obtained powder was partitioned with different solvents and each extract was freeze dried separately. Recovery, DPPH* scavenging activity, total phenolic content and characterization of phenolics (by HPLC and FTIR) in different solvents were studied. Powder of ethyl acetate fraction was further purified by column chromatography. Different fractions from column chromatography were investigated for their total phenolic content and recovery, and characterized by HPLC and FTIR. Phenolic components were isolated from selected fractions through preparatory HPLC. Isolated compounds were identified by HPLC, GC-MS and FTIR. DPPH and ABTS free radical scavenging activity of isolated components were also investigated.

Chapter-5 includes the optimization of extraction of total phenolic content from Amla seed coat powder using Response Surface Methodology. Further, extraction and purification of phenolics were carried out. The characterization of phenolics present in seed coat powder was carried out by HPLC and FTIR. Phenolics were identified by GC-MS after converting them into TMS (Tri methyl silyl) derivatives.

Chapter-6 discusses the effect of different concentrations of maltodextrin and inlet temperatures on physicochemical properties of spray dried Amla powder. Various parameters like moisture content, bulk density, water solubility index, hygroscopicity particle morphology using SEM and color value were investigated. Effect of inlet

temperature and maltodextrin level on total phenolic content and DPPH* scavenging activity of powder was also investigated.

Chapter-7 describes the effect of different concentration of maltodextrin and inlet temperature on physicochemical properties of spray dried lemon powder. Parameters like moisture content, bulk density, water solubility index, hygroscopicity, particle morphology using SEM and color value of spray dried lemon were investigated. It also deals with optimization of Amla-lemon based RTS (prepared from spray dried Amla and lemon powders) by response surface methodology. Compositional analysis, mineral analysis, color (*L*, *a* and *b*) values were also analyzed for the optimum product. Effect of storage on retention of color, phenolic content and DPPH* scavenging activity was also investigated. Amla-lemon based RTS was acceptable by the sensory panellists.

Chapter-8 presents the salient findings of the present investigation. It concludes that the major fraction of vital bioactive constituents of Amla is present in the Amla pomace powder which can be exploited after extraction of the juice. Seed coat powder has a huge potential for exploitation as a good source of antioxidant or bioactive components. However, solvent extraction method is not suitable for the extraction of phenolics from seed coat powder; hence alternative methods like supercritical fluid extraction or pressurized extraction need to be tried in future. Amla juice and lemon powder can be effectively spray dried by encapsulation with maltodextrin and may have lot of future as a commercial functional drink in the market.

DECLARATION BY THE CANDIDATE

The thesis entitled **"Characterization of phenolics extracted from Amla (*Emblica officinalis*) and its processing waste and development of Amla-lemon based RTS drink"** being submitted to School of Engineering, Tezpur University in partial fulfilment for the award of the degree of Doctor of Philosophy in the Department of Food Engineering and Technology is a record of bonafide research work accomplished by me under the supervision of **Prof. C.L. Mahanta.**

All helps from various sources have been duly acknowledged.

No part of the thesis has been submitted elsewhere for the award of any other degree.

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All helps received by her from various sources have been duly acknowledged.

No part of the thesis has been submitted elsewhere for award of any other degree.

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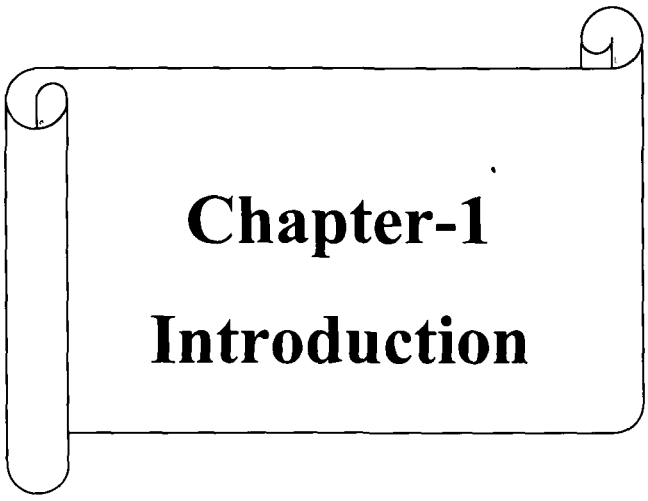
Abbreviation

AAS	Atomic absorption spectroscopy
ABTS	2, 2'-azino-bis, 3-ethylbenzo thiazoline-6-sulphonic acid
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BHA	Butylated hydroxyl anisole
BHT	Butylated hydroxyl toluene
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CAT	Catalase
CCRD	Central composite rotating design
DMDCS	Dimethyl dichloro silane
DPPH	2,2 diphenyl 1-picrylhydrazyl
FCR	Folin-Ciocalteu reagent
FTIR	Fourier transform infrared spectroscopy
GAE	Gallic acid equivalent
GC-MS	Gas chromatography mass spectroscopy
GPx	Glutathione peroxidase
HPLC	High performance liquid chromatography
ROS	Reactive oxygen species
RSM	Response surface methodology
RTS	Ready to serve
SEM	Scanning electron microscopy
SOD	Superoxide dismutases
TMS	Trimethyl silyl derivative
TPC	Total phenolic content
USDA	US Department of Agriculture
v/v	Volume by volume
v/w	Volume by weight
WSI	Water solubility index



Chapter-1

Introduction

1. Introduction

1.1. Amla (*Emblica officinalis*)

Amla (*Emblica officinalis*) (EO) has a hallowed position in Ayurveda- an Indian indigenous system of medicine[1]. According to belief in Indian mythology, Amla is the first tree to be created in the universe; which belongs to the family of Euphorbiaceae and is also known as *Phyllanthus emblica* or Indian gooseberry[1]. Amla is native to India and also grows in tropical and subtropical regions of Pakistan, Uzbekistan, Sri Lanka, South East Asia, China and Malaysia[1]. The fruits of Amla are widely used in the Ayurvedic preparation and are believed to increase defence against diseases[2]. It has a beneficial role in degenerative diseases like cancer, diabetes, liver treatment, ulcer, anaemia, heart trouble[1] and also is an important constituent in hepatoprotective formulas available[2].

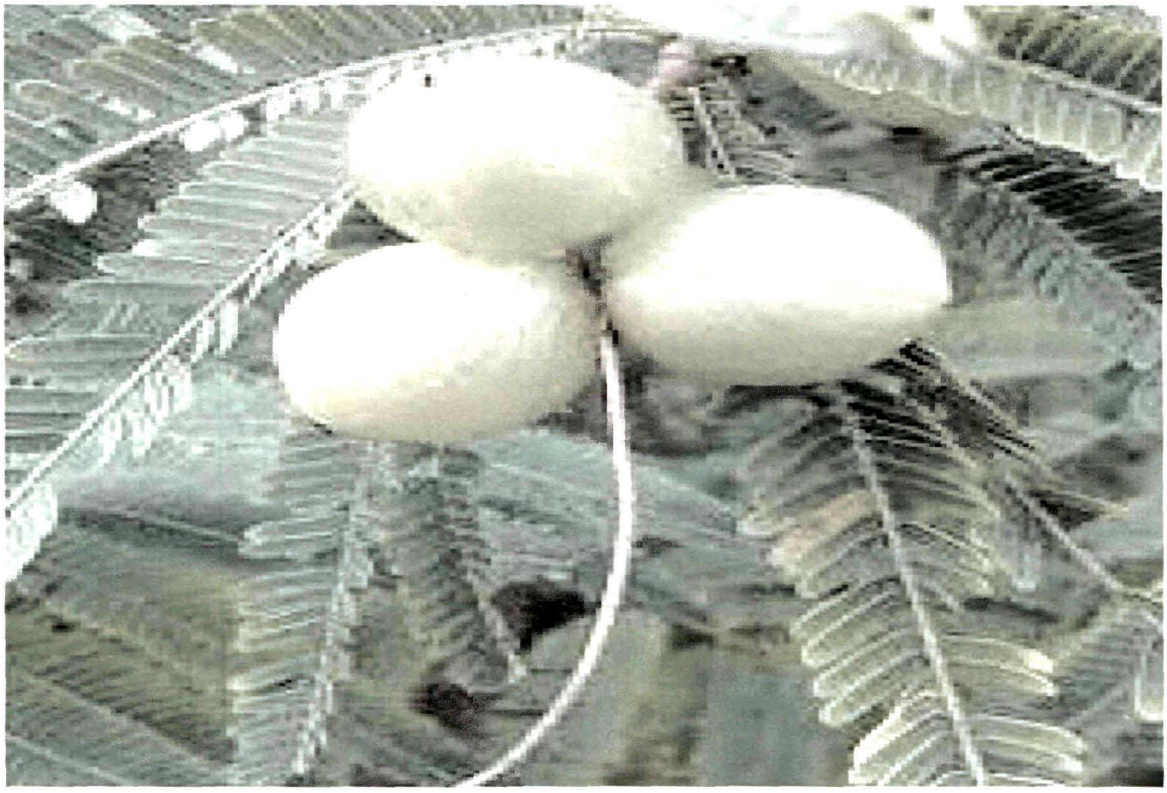
1.1.1. Morphology

Amla tree is a small to medium sized deciduous tree with an average height of 8-18 m, with thin light grey bark exfoliating in small thin irregular flakes, exposing the fresh surface of a different color underneath the older bark. The average girth of the main stem is 70 cm. In most cases, the main trunk is divided into 2 to 7 scaffolds very near to the base[3]. The image of Amla tree is shown in **Fig 1.1a**. Leaves are 10 -13 mm long, 3 mm wide, closely set in pinnate faishon[3] which makes the branches feathery in general appearance. After setting of the fruits leaves develop. Flowers are unisexual, 4 to 5 mm in length[4], pale green in color, borne in leaf axils in clusters of 6 to 10. Fruits are fleshy, almost depressed to globose shape, 2.1-2.4 cm in diameter, 5.3-5.7 g in weight, 4.5-5.0 mL in volume. The image of Amla fruit is given in **Fig 1.1b.** The stone of the fruit is 6 ribbed, splitting into three segments[4] each containing usually two seeds; seeds are 4-5 mm long and 2-3 mm wide, each weighing 572 to 590 mg[4-5].

1.1.2. The fruiting season of Amla

Amla is a deciduous tree and the emergence of new shoots starts in the beginning of April[7]. The fruiting season of Amla fruit is exceptionally long. The fruit become fit for harvesting in December which can be retained on the tree up to March without any significant loss in quality or yield. The picking of fruit is generally in January to March. Amla tree is a heavy bearer and the fruits also remain free from the attacks of birds and wild animals[8].

(a)



(b)

Fig. 1.1 Image of (a) Amla tree, and (b) Amla fruit [6].

1.1.3. Chemical composition of Amla

The Amla plant contains different classes of chemical constituents which are given in **Table 1.1.** Different environmental conditions can also affect the chemical constitutions of the Amla plant. Chemical constituents in different parts of the Amla plants are given below.

1.1.3.1 Amla Fruit

Physical characteristics of different varieties of Amla are given in **Table 1.2**. Size, shape and weight were found to vary among the different varieties of Amla[9]. The salient findings are given in **Table 1.3.** They also reported compositional differences in different varieties of Amla. In general the average composition of Amla fruits are: moisture 81.2%, protein 0.5%, fat 0.1%, carbohydrates 14.1%, mineral matter 0.7%, fiber 3.4%, Ca 0.05%, K 0.02%, Fe 1.2 mg/100g, nicotinic acid 0.2 mg/g, phyllemblin, phyllemblic acid, gallic acid, emblicol, quercetin, hydroxymethyl furfural, ellagic acid, pectin[10-11], putranjivan A,[12] two new hydrolysable tannins called emblicannin A and B, punigluconin and pendunculagin[13].

1.1.3.2. Seeds

A fixed oil, phosphatides and a small quantity of essential oil are present in seed. The fixed oil yield (16%) has the following physical and chemical properties : acid value 12.7, saponification value 185, acetyl value 2.03, iodine value 139.5, unsaponifiable matter 3.81%, sterol 2.70%, saturated fatty acids 7%, linolenic acid (8.78%), linoleic acid (44.0%), oleic acid (28.40%), stearic acid (2.15 %), palmitic acid (2.99%) and myristic acid (0.95%)[5]. Arora et al. (2011)[14] also reported that the seed oil is rich in unsaturated fatty acids like linoleic acid (18:2n-6) and oleic acid[14].

1.1.3.3. Leaves

Gallic acid, ellagic acid, chebulic acid, chebulagic acid, chebulinic acid, a gallotannins called amlic acid, alkaloids, phyllatidine and phyllantine are reported to be present in Amla tree leaves[15-16].

Table 1.1 The classes of chemical constituents reported in Amla plant[4]

Class	Compound
Alkaloid	Phyllantine
	phyllantidine
	zeatin
	zeatin nucleotide
	zeatin rioside
Benzoid	chebulic acid
	chebulinic acid
	chebulagic acid
	gallic acid
	ellagic acid
	amlaic acid
	3-6-di-O-galloyl-glucose
	ethyl gallate
	b-glucogallin
	1,6,di-O-galloyl-b-D-glucose
	putranjivan A
	digallic acid
	phyllemblic acid
	emlicol
	music (= galacteric acid)
Diterpene	gibberellin A-1
	gibberellin A-3
	gibberellin A-4
	giberellin A-9
Triterpene	lupeol
Flavonoid	leucodelphinidin
	kaempherol
	kaempherol-3-glucoside
	rutin
	quercetin
	kaempherol-3-O- β -D-glucoside
	quercetin -3-O- β -D glucoside
Furanolactone	Ascorbic acid
Sterol	β-sitosterol
Carbohydrate	glucose

1.1.3.4. Bark

Leucodephinidin, tannin and proanthoyanidin have been reported in the bark of Amla tree[15].

1.1.4. Amla cultivars

The cultivars of Amla are mostly known on the basis of their size color or name of the place[17-19]. *Francis*, *Chakaiya*, *Banarasi* and *Bansi Red* are the known Amla cultivars; and all these cultivars have their own merit and demerits[20]. *Banarasi* is timid bearing and prone to heavy fruit fall; *Francis* though productive has serious incidence of fruit necrosis. The most established cultivars i.e. *Chakaiya* has comparatively small sized fibrous fruits with a tendency to bear in alternate years[21-23]. Of the five cultivars introduced at Rahuri in Maharastra namely, *Krishna*, *Chakaiya*, *Kanchan*, *Francis* and *NA-7*; *Kanchan* gave highest yield (116 kg/tree)[24]. Fruit weight, length, diameter, pulp, seed content and polyphenols varied from variety to variety[22]. The physical characteristics and compositional analysis of Amla genotype are shown in **Table 1.2** and **1.3** [9].

On the basis of yield, potential and quality attributes, Pathak, (2003)[9] suggested that the *NA-10* and *Krishna* (early cultivars); *NA-7* and *NA-6* (medium maturity cultivars) and *Kanchan* and *Chakaiya* (late cultivars) can be recommended for commercial cultivation[9].

Pandey et al. (2003)[25] identified a new genotype *Luxmi-52* in Pratapgarh District in Uttar Pradesh. The tree of *Luxmi -52* has semi erect growth and branches do not drooping like its parent *Francis*. Fruit size is large weighing 40-60g of diameter 4.0-4.5 cm with 6 ridges. In early part of fruit growth, fruit color is light pink which disappears on full development[25]. Due to the large size and appealing color it fetches higher price in the market[25]. The fruit is found suitable for preparation of segments in syrup, candy and preserve[25].

Table 1.2 Physical characteristics of different varieties of Amla[9]

Cultivars	Fruit length (cm)	Fruit width (cm)	Fruit weight (g)	No of segments /fruit	Fruit size	Fruit shape
Banarasi	3.60	4.44	49.83	6	Large	Triangular
Chakaiya	3.40	4.10	30.66	6	Medium	Flattened round
Francis	3.91	4.29	42.73	6	Large	Flattened oval
NA-4	3.30	3.53	29.94	6-8	Small	Flattened oblong
NA-5	3.60	4.20	44.66	6-8	Large	Triangular
NA-6	3.60	4.03	33.33	6	Medium	Oval round
NA-10	3.40	4.15	41.50	6	Medium	Flattened round

Table 1.3 Fruit quality of different varieties of Amla[9]

Cultivars	Crude fiber (%)	Juice (%)	Pulp (g)	TSS (^{o}B)	Acidity (%)	Vitamin C (mg/100g)
Banarasi	2.32	47.00	39.80	13.00	2.19	484
Chakaiya	2.85	52.25	31.80	10.33	2.15	527
Francis	2.65	50.92	32.70	9.16	1.92	566
NA-4	2.95	42.92	22.17	11.00	2.47	504
NA-5	2.39	50.75	34.00	11.00	2.32	549
NA-6	2.86	47.00	31.87	9.50	2.11	422
NA-10	2.52	56.10	39.80	9.90	2.17	528

1.2. Functional properties of Amla

1.2.1 Antioxidant properties

The phenolic contents and antioxidant activities (by in vitro experiments using scavenging assays of 1,1-diphenyl-2-picrylhydrazyl (DPPH*) radicals, hydroxyl radicals, and superoxide anion radicals, chelating ability of ferrous ion, reducing power, and inhibition capability of Fe (II)-induced lipid peroxidation of methanolic extracts of Amla fruit from six regions in China were evaluated by Liu et al. (2008)[26].

Differences in total phenolic content (81.5 to 120.9 mg gallic acid equivalents (GAE)/g) and the flavonoid content (20.3 to 38.7 g quercetin equivalents /g)[26] were observed. They reported high correlation coefficient between the phenolic content and superoxide anion radical scavenging activity, but no significant correlation between the former and hydroxyl radical scavenging activity[26]. Methanolic extracts of Amla fruit from some selected regions exhibited stronger antioxidant activities compared to those of the commercial compounds i.e. quercetin and BHA[26]. The antioxidant property of phenolics is reported to increase with increase in number of hydroxyl groups[27].

The potent antioxidant properties of Amla has also been confirmed by Hazara et al. (2010)[28]. They compared the antioxidant properties of Amla with *Terminalia chebula*, and *Terminalia belerica*. Antioxidant and reactive oxygen species scavenging activities like DPPH*, hydroxyl, superoxide, nitric oxide, hydrogen peroxide, peroxynitrite, singlet oxygen, hypochlorous acid of methanolic extract of *Emblica officinalis* (Amla), *Terminalia chebula* and *Terminalia belerica* were evaluated[28]. The ability of the extracts of the fruits in exhibiting their antioxidative properties and flavonoid content follow the order: *T. chebula>E. officinalis>T. belerica*, whereas with respect to phenolic content the order was observed as *E. officinalis>T. belerica>T. chebula*[28]. The DPPH* and peroxynitrite radical scavenging activity of the extracts were observed in the order of *E. officinalis >T. belerica >T. chebula* whereas for superoxide and nitric oxide the order were as follows: *T. chebula >E. officinalis>T. belerica* [28].

Nampoothri et al. (2011)[29] also evaluated the free radical scavenging capacity and antioxidant potential of different solvent extracts (hexane, ethyl acetate, methanol, 70% methanol and water) of Amla fruits. Methanol extract of Amla fruits exhibited maximum scavenging activity against DPPH*, superoxide, hydroxyl and nitric oxide radicals[29]. The total antioxidant activity of aqueous extract of Amla is significantly higher than spirulina and wheat grass at 1mg /mL, the concentration in Amla, spirulina and wheat grass was 7.78, 1.33 and 0.28 mmol/l, respectively[30].

The antioxidant activity of Amla was 86.4% when evaluated by the β-carotene bleaching method[31]. The total phenolic content in of Amla as determined by the Folin–Ciocalteu method was 290.47 mg/g plant extract (in GAE)[31].

Anila and Vijayalakshmi, (2003)[32] studied the effect of oral administration of flavonoids from Amla at a dose of 10 mg /kg body wt in cholesterol-fed experimental

rats. Supplementation of Amla flavonoids increased the activities of free radical-scavenging enzymes and decreased the lipid peroxide content in flavonoid-treated hypercholesterolemic rats[32]. Chaterjee et al. (2011)[33] also reported the antioxidant properties in water extracted carbohydrates of Amla[33].

1.2.2. Antibacterial properties

The antimicrobial activities of Amla extract were investigated by Mayachiew & Devasahtin, (2008)[31] by two different methods (disc diffusion and agar dilution methods) against *Staphylococcus aureus*. The minimum inhibitory concentration (MIC) value of Amla was found to be 13.97 mg/mL and the minimum biocidal concentration (MBC) value was 13.97 mg/mL[31].

Aqueous infusion and decoction of *Emblica officinalis* exhibited potent antibacterial activity against *Escherichia coli* (270), *Klebsiella pneumoniae* (51), *K. ozaenae* (3), *Proteus mirabilis* (5), *Pseudomonas aeruginosa* (10), *Salmonella typhi* (1), *S. paratyphi* A (2), *S. paratyphi* B (1) and *Serratia marcescens* (2) but did not show any antibacterial activity against Gram negative urinary pathogens [34].

1.3. Medicinal properties of Amla

1.3.1. In cancer

Liu et al. (2012)[35] studied the anticancer potential of six phenolic compounds isolated from Amla fruit by in vitro proliferation assay. MTT method was used to study the effects of these compounds on splenocyte proliferation and the cytotoxicity to both human breast cancer cell (MCF-7) and human embryonic lung fibroblast cell (HELF)[35]. Their major findings are shown in **Fig 1.2.** Isocorilagin exhibited a strong cytotoxicity to HELF cell with IC_{50} of 51.4 g/mL whereas geraniin, quercetin, kaempferol and their glycosides showed weak cytotoxicity against HELF cells[35].

The significant activity of Amla extract against chemically induced liver tumour and sarcoma in animals and anticarcinogenic activity is due to the presence of phenolic constituents such as quercetin and ellagitannins[36]. Joy and Kuttan, (1995)[37] also reported that the anticarcinogenic activity of Amla extracts is due to the presence of the phenolic components[37].

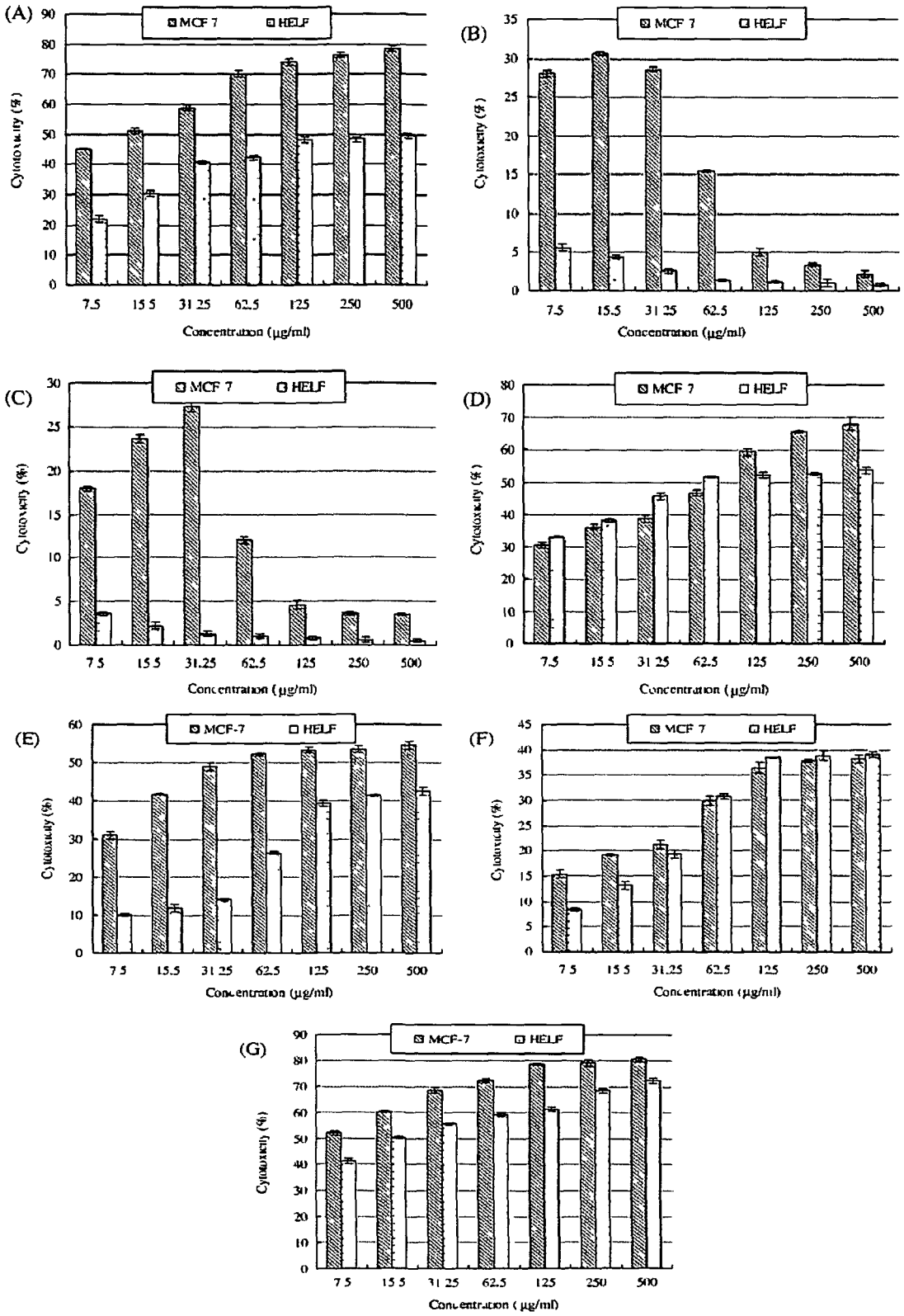


Fig.1.2 Cytotoxicities of (A), geraniin; (B), quercetin 3-β-d-glucopyranoside; (C), kaempferol 3-β-d-glucopyranoside; (D), isocorilagin; (E), quercetin; (F), kaempferol and (G), paclitaxel on proliferation of MCF-7 and HELF cells[35].

The antitumour activity of these phenolic compounds might be achieved by immunomodulatory properties which could partially be attributed to their antioxidant activity[35].

Many other studies indicated an interesting antiproliferative activity of phenolics[38], but mechanisms of action are not clearly determined yet. The possible mechanisms were explained by several scientists which include interference with the metabolite activation of promutagen[39], as blocking agents and adducts formation with ultimate mutagens, suppression of tumour cell invasiveness via the inhibition of matrix metalloproteinase-2/-9 activity and scavenging of free radicals[40]. Amla extract has ability to interfere with cell cycle regulation via the inhibition of cdc 25 phosphatase and partial inhibition of cdc 2 kinase activity which promotes the antitumor activity[41].

Effects of isolated phenolics from Amla on MCF-7 on tumor cells are shown in **Fig. 1.3**[42]. The positive role in suppressing activities of MCF-7 tumour cells of Amla is due to the good antioxidant activities of the major phenolics of Amla[42]. The tumor incidence, tumor yield, tumor burden and cumulative number of papillomas can be retarded by supplementation of Amla extract[43].

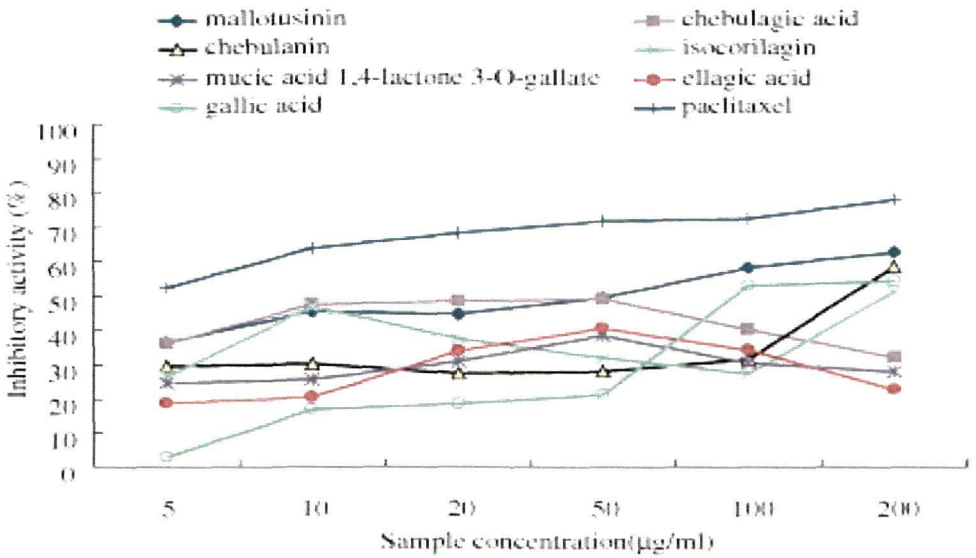


Fig 1.3 Effect of phenolics extracted from Amla and control on the growth of MCF-7 cancer cells[42].

1.3.2. Antitussive activity

The antitussive activity of Amla was investigated by Nosaloval et al. (2003)[44] in conscious cats by mechanical stimulation of the laryngopharyngeal and tracheobronchial mucous areas of airways in 2003. Dose of Amla of 50 mg/kg body weight perorally, showed unambiguous results; whereas at higher dose (200 mg/kg body wt) of this substance perorally was observed effective, especially in decreasing the number of cough efforts (NE) and frequency of cough (NE/min^{-1}), and the intensity of cough attacks in inspirium (IA^{+}) and expirium (IA^{-}) was more pronounced[44]. The antitussive activity of Amla is less effective than shown by the classical narcotic antitussive drug codeine, but more effective than the non-narcotic antitussive agent dropropizine[44].

1.3.3. In diabetes

Nampoothri et al. (2011)[29] reported that the methanolic extract of Amla can act as potent α-amylase and α-glucosidase inhibitor. Amla extract had also showed antiglycation activity which confirmed the therapeutic potential of Amla against diabetes[29]. Presence of ellagic acid and ascorbic acid in the extract was reported to be responsible for the antidiabetic activity of Amla[29]. Potent antioxidant and lipid peroxidation inhibiting activities of fresh juice and hydro alcoholic extract of Amla fruits is helpful to control the diabetes in rats[45].

1.3.4. In heart diseases

Ageing, as a multifactorial phenomenon, is related to many metabolic alterations, such as dyslipidaemia, atherosclerosis, obesity, type 2 diabetes, arthritis and neurodegenerative disease[46]. Yokozawa et al. (2007)[47] studied the effect of Amla on the lipid metabolism and protein expression involved in oxidative stress during the ageing process. Administration of Sun Amla (commercially available extract) and ethyl acetate extract of Amla at a dose of 40 or 10 mg/kg body weight per day for 100 days to young rats aged 2 months and aged rats of 10 months, decreased the lipid levels, such as cholesterol and triglycerides, in serum and liver (**Table 1.4**)[47]. Several scientists reported that the significant increase of lipid levels as ageing progresses is associated with a reduction in the expression and activity of PPARα, a nuclear transcription factor, in the liver of rats[48-49], which had reversed on oral administration of Amla extracts in aged rats (**Fig 1.4**).

According to Chan & Yu, (2004)[50] Bcl-2 family proteins that are anti-apoptotic proteins are effective in protecting the cell death from acute oxidative stress[50]. It has been also demonstrated that bcl-2 overexpression preserves the viability and diminishes lipid peroxidation in cells when exposed to oxidative stress[51]. Several workers have reported the efficiency of antioxidants to slow down or block the apoptotic process by stabilising mitochondrial functions[52-53]. Age associated diseases have increased due to chronic oxidative stress and inflammatory reactions and the ageing process was found to enhance the activation of NF-kB by down regulating IkB-a[54-55].

Supplementation of Amla decreased the level of bax significantly while increased the level of bcl-2 (**Fig 1.5**) as reported by Yokozawa et al. **(2007)[56].** They further reported that Amla extract reduced the iNOS and COX-2 expression levels by inhibiting NF-kB activation in aged rats (**Fig 1.6**) and finally concluded that Amla may prevent age-related hyperlipidaemia through attenuating oxidative stress in the ageing process[56].

Table. 1.4 Serum and hepatic cholesterol profiles[56]

Group	**Dose (mg/kg body weight per day)**	**Total cholesterol**	**Non esterified Cholesterol**	**Esterified cholesterol**
Serum cholesterol (mg/l)	-			
Young rats	-	703	492	211
Ageing rats	-			
Control		886	648	238
Sun amla	40	847	624	223
Ethyl acetate extract	10	795	583	212
Liver cholesterol (mg/g)	-			
Young rats		7.33	2.99	4.34
Ageing rats				
Control		10.63	4.29	6.34
Sun amla	40	8.74	3.58	5.16
Ethyl acetate extract	10	3.58	3.41	4.50

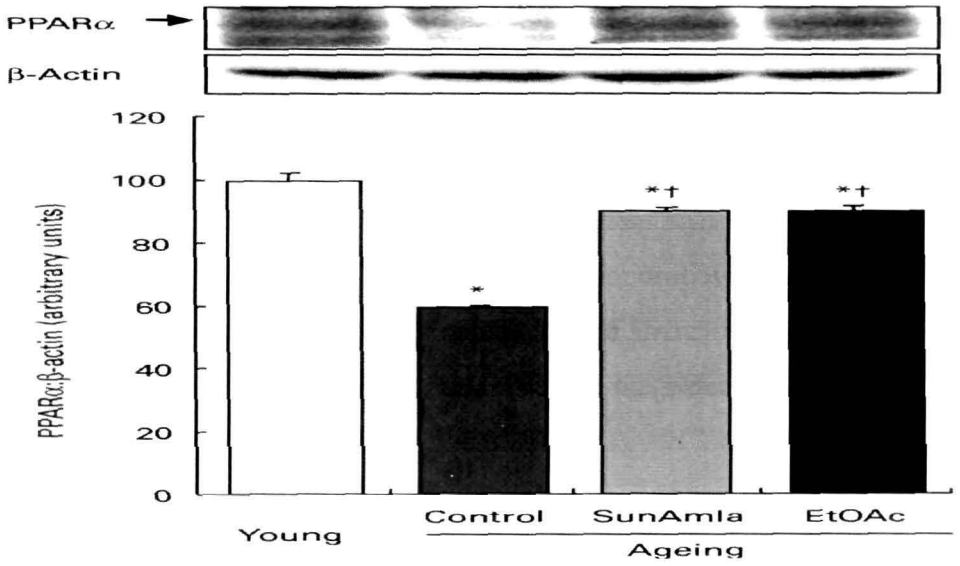


Fig. 1.4 PPARα protein in hepatic nucleus[56].

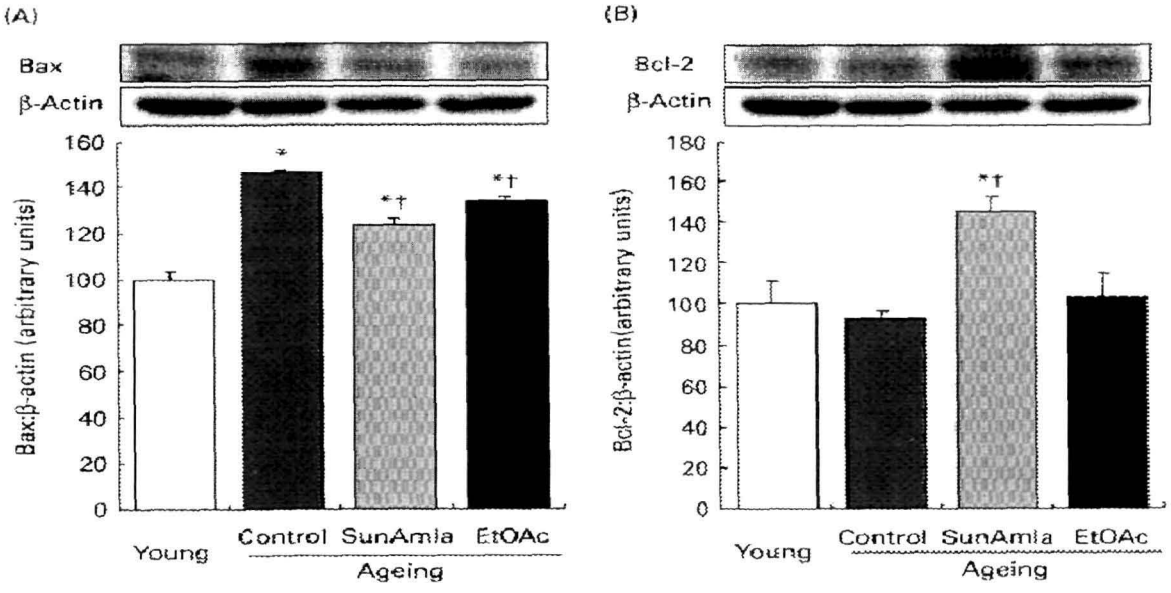


Fig. 1.5 (A) NF-kB, and (B) kB-a (IkB-a) inhibitor binding protein in hepatic nucleus and cytoplasm[56].

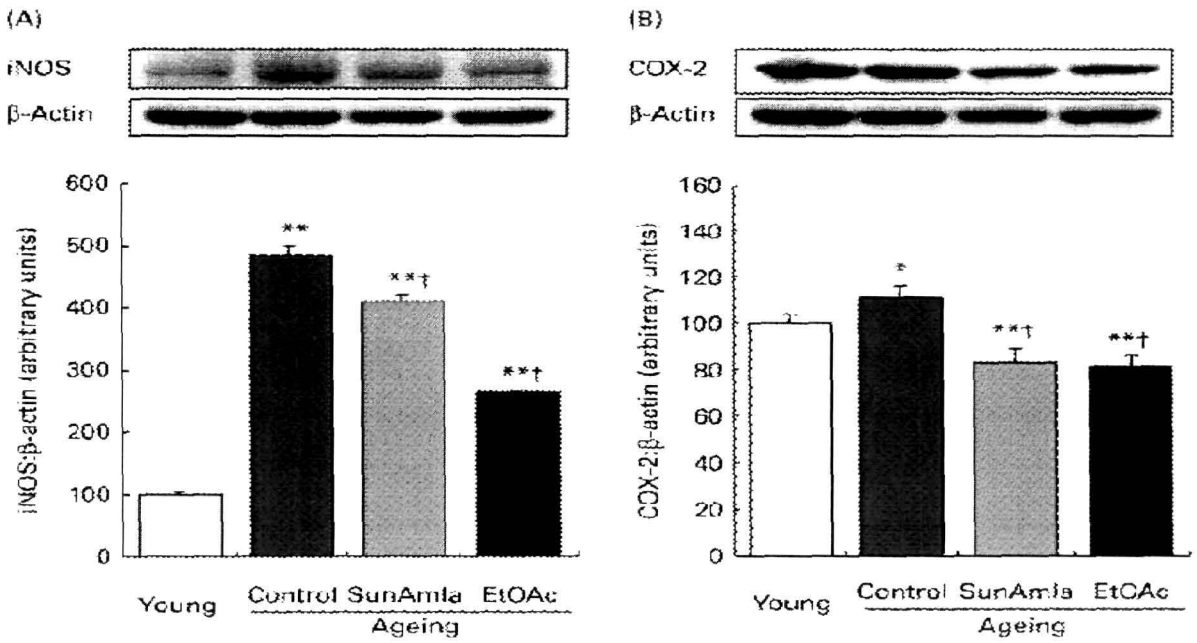


Fig. 1.6 (A) Inducible NO synthase (iNOS), and (B) cyclo-oxygenase-2 (COX-2) proteins in hepatic homogenate[56]

The supplementation of extract of Amla was observed to reduce the elevated levels of serum creatinine and urea nitrogen in the aged rats[56]. The systolic blood pressure was also found to be significantly reduced by the administration of different extracts of Amla[56]. Administration of Amla extracts also reduced the thiobarbituric acid-reactive substance levels of serum, renal homogenate, and mitochondria in aged rats which may also be effective in ameliorating oxidative stress due to aging[56].

1.3.5. Chondroprotection

Sumantran et al. (2007)[57] measured the chondroprotective potential of Amla fruits *in vitro*. Aqueous extracts of unprocessed Amla fruit powder (A) and the hot water extract of Amla powder (B) were examined by three different assay systems for : (i) effects of A and B powders on the activities of the enzymes hyaluronidase and collagenase type 2; (ii) *in vitro* model of cartilage degradation which was set-up with explant cultures of articular knee cartilage from osteoarthritis patients[57]. Damage of cartilage was measured by glycosaminoglycan release; and (iii) explant model of cartilage matrix damage. According to Sumantran et al. (2007)[57], the powder A induced a statistically significant, short-term chondroprotective activity in cartilage explants whereas powder B exhibited long term chondroprotective activity.

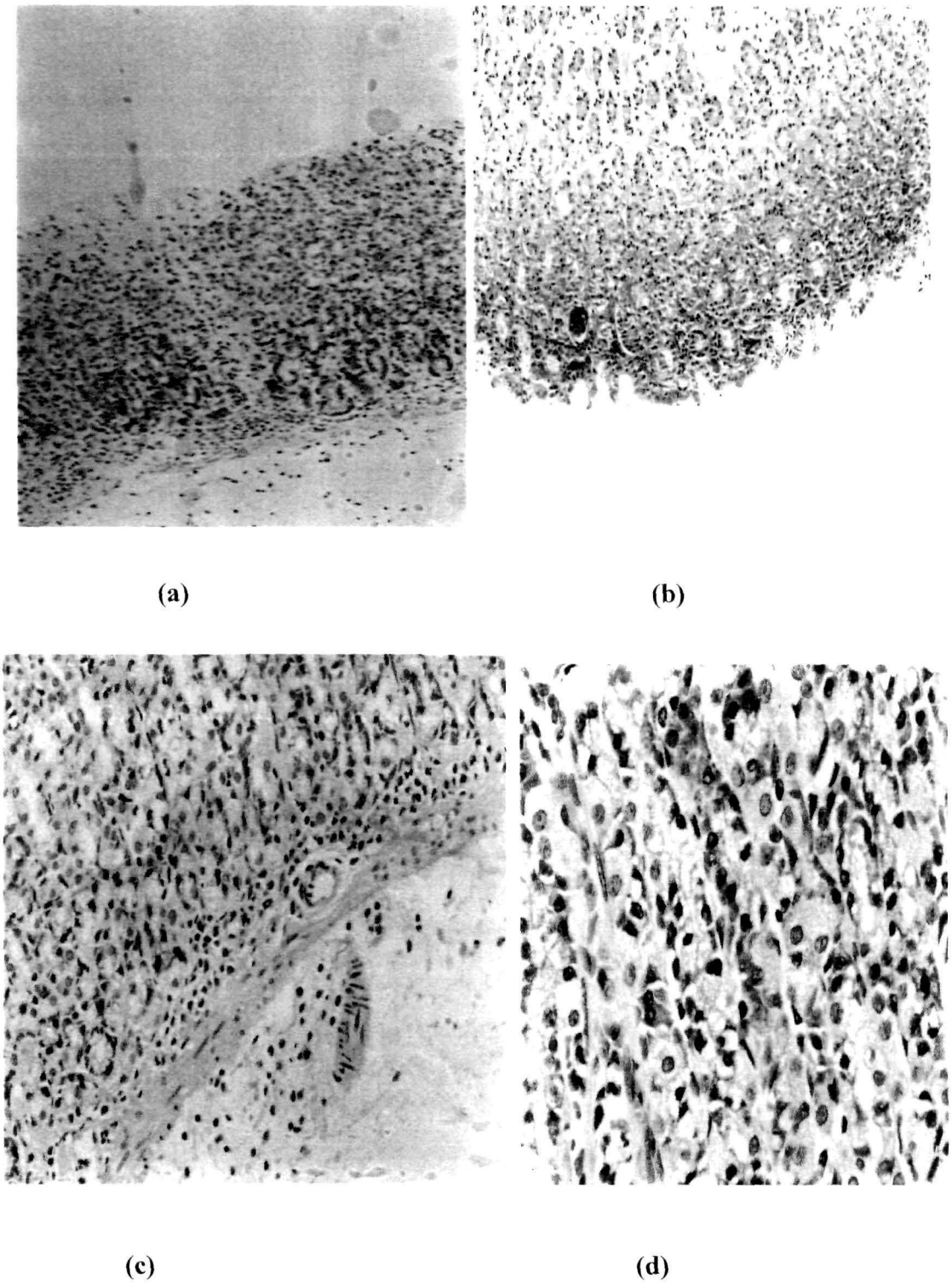
1.3.6. In hypolipidemia

According to Anila and Vijaya Lakshmi, (1996)[58] administration of Amla juice at a dose of 5 mL/kg body weight per rabbit per day for 60 days was effective to reduce the serum cholesterol, triglycerides (TG), phospholipid and low density lipoprotein (LDL) levels by 82%, 66%, 77% and 90%, respectively. Lipid levels were also decreased after administration of Amla juice. Mode of absorption was affected by the administration of Amla juice as treated rabbits excreted more cholesterol and phospholipids[58].

Flavonoids from Amla have a capability to reduce lipid levels in serum and tissues of rats induced with hyperlipidemia[59]. Administration of Amla inhibited the hepatic HMG (3-hydroxy-3-methyl-glutaryl) CoA reductase activity[59]. Lecithin-cholesterol acyltransferase (LCAT) level was also found to be increased after administration of Amla[59]. The degradation and elimination of cholesterol was highly enhanced in treated rats. The mechanism of hypolipidemic action is by the concerted action of inhibition of synthesis and enhancement of degradation[59].

1.3.7. In gastoprotection

Rehaily et al. (2002)[60] examined the antisecretory and antiulcer activities of Amla by different experimental models in rats[60]. Administration of Amla extracts orally at doses of 250 mg/kg and 500 mg/kg significantly inhibited the development of gastric lesions in all test models. The results on histopathological investigation are given in **Fig 1.7** which revealed the efficacy of Amla against ulceration[60]. Administration of Amla extracts also reduced the pyloric-ligation induced basal gastric secretion, titratable acidity and gastric mucosal injury[60]. Amla extract also offered the protection against ethanol-induced depletion of stomach wall mucus and reduction in nonprotein sulfhydryl concentration[60]. Brodie et al. (1962)[61] reported that increased acid output is associated with stress-induced lesions. Antisecretory drugs, like H_2-receptors antagonists such as ranitidine, cimetidine and famotidine markedly inhibit the stress induced ulceration[62]. Yesilada et al. (2000)[63] suggested that the inhibitory effect of Amla extract on stress-induced lesion may reduce the acid secretion[63].



(a) **(b)**

(c) **(d)**

Fig 1.7 Section of gastric mucosa of rat (a) Control, (b) treated with 80% ethanol showing marked congestion, bleeding and odema. Hematoxylin and eosin 8.5, (c) treated with Amla (250 mg/kg) and 80% ethanol showing infiltration at the deep portion of the mucosa, (d) treated with Amla (500 mg/kg) and 80% ethanol showing no odema and no congestion[60].

1.3.8. Immunomodulation

Immunomodulatory effect of aqueous extracts of Amla was examined by Suja et al. (2009)[64] on Swiss Albino mice. Administration of extracted Amla powder increased the haemagglutination antibody titre, sheep red blood cells (sRBCs) in dose dependent manner and also induced the delayed type of hypersensitivity reaction, macrophage migration index, and respiratory burst activity of the peritoneal macrophages, total leukocyte count, percentage lymphocyte distribution, serum globulin and relative lymphoid organ weight[64].

1.3.9. Oxidative stress

Ischemia-reperfusion (IRI)-induced oxidative stress reduced the activities of cardiac superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and increased lipid peroxidation in rat[65]. Administration of Amla juice (50 -100 mg/kg body wt.) or vitamin E (200 mg/kg body wt) reduced the IRI-induced effects[65].

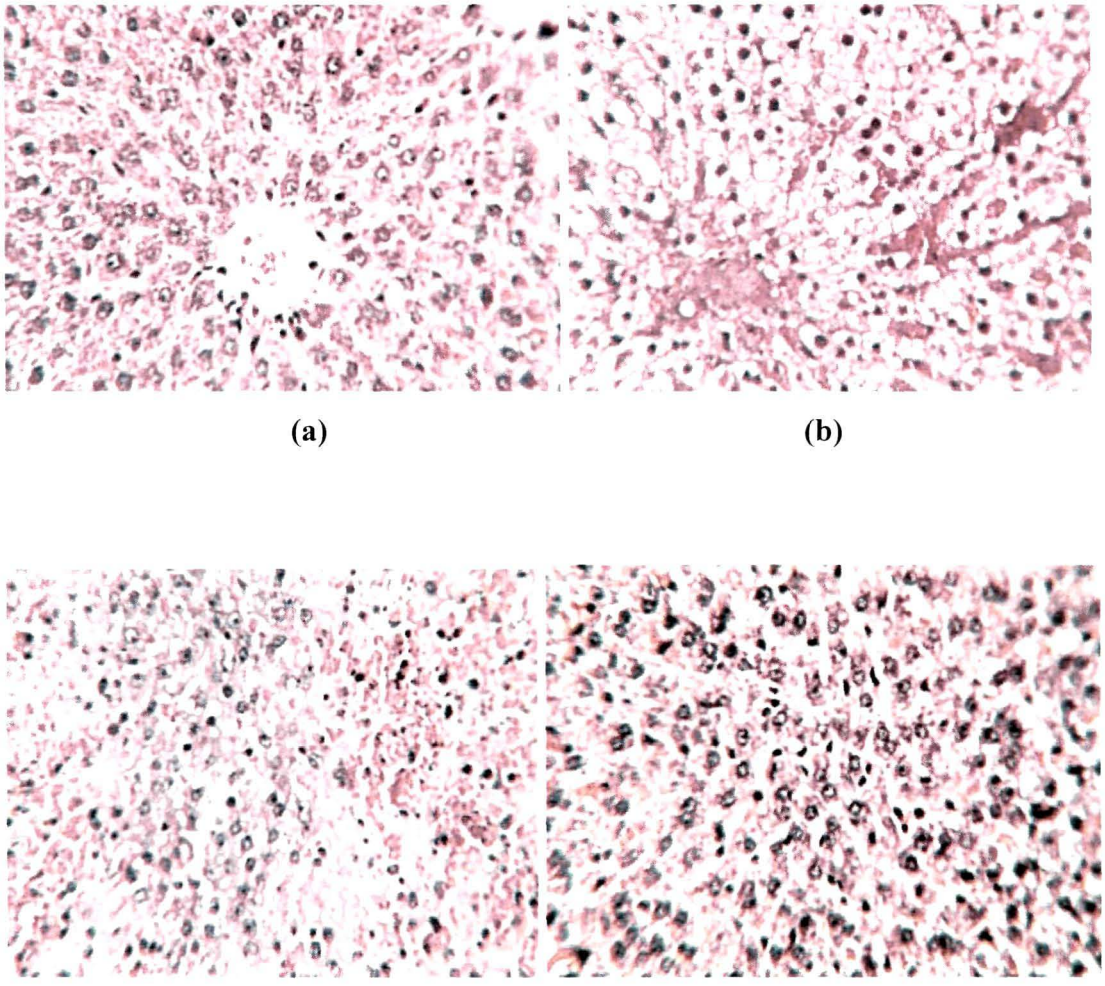
Amla fruit was also found to be effective against mercury induced oxidative stress in rat erythrocytes[66]. Mercury chloride oxidative stress decreased the glutathione (GSH), SOD and catalase activity, while increasing the lipid peroxidation (LPO)[66]. Amla extract @100 mg/kg was found to be effective in ameliorating oxidative stress significantly, while 50 mg/kg of Amla fruit extract could only reverse the values of LPO and catalase[66].

1.3.10. In alcohol induced damage

Chronic alcohol consumption may lead to tissue and organ damage, coronary heart diseases, alcohol liver disease and several other diseases[67-68]. Reddy et al. (2007)[69] administered 33% (v/v) alcohol (10 g/kg body weight) and aqueous extract of Amla powder (250 mg/kg body weight/day) once a day for 60 days. Aqueous Amla extract supplementation increased the GSH content and also the increased the activities of SOD, CAT and GPx [69].

1.3.11. Hepato protective effects

Vidhyamalar & Mettilda, (2009)[70] investigated the efficacy of the Amla against paracetamol induced hepatotoxicity in rats by the histopathological study of liver cells. The histoarchitecture obtained by the authors is shown in **Fig 1.8.** Treatment with aqueous extract of Amla fruits showed the appearance of normal hepatocytes, offset of necrosis and consequent appearance of leucocytes thus suggesting the hepatoprotective effect of Amla[70].



(a) **(b)**

(c) **(d)**

Fig 1.8 (a) Histology of liver (control group), (b) vacuolated liver with heavy bleeding (group 2), (c) A portion of liver with normal and affected area (group 3), and (d) area of liver with normal hepatocytes (group 4)[70].

1.3.12. Lipid damage

Membrane phospholipases, alteration of the membrane lipid packing and penetration of water molecule may induce formation by products of lipid oxidation[71-72]. The induced alterations in permeability of membranes, transport systems, and loss of membrane-bound enzymes can eventually lead to cell lysis and death under certain conditions[73]. The assessment of the efficiency of Amla to prevent lipid peroxidation was measured by thiobarbituric acid-reactive substrates, lipid hydroperoxide, conjugated diene and 4-hydroxynonenal[74]. Amla extracts have capability to afford excellent protection against iron-mediated lipid peroxidation that might also be useful in reducing photo-induced iron toxicity. The efficiency of Amla extracts to protect against lipid damage was correlated with phenolics[74].

Anila and Vijaya Lakshmi in 2003 reported that administration of Amla significantly reduced the lipid peroxide content in cholesterol fed rat[32].

1.3.13. Memory enhancing ability

Amla powder showed a dose-dependent improvement in memory scores of young and aged mice[75]. Supplementation of Amla reversed the amnesia induced by scopolamine (0.4 mg/kg, i.p.) and diazepam (1 mg/kg, i.p.); brain cholinesterase activity and total cholesterol levels were also reduced on administration of Amla powder for 15 days[75]. Their studies revealed that the Amla may be a useful remedy for the management of Alzheimer's disease on account of its multifarious beneficial effects such as memory improving property, cholesterol lowering property and anticholinesterase activity[75].

1.4. Extraction of phenolics

1.4.1. Solvent extraction

Conventionally, organic solvent i.e methanol, acetonitrile, ethyl acetate, ethanol etc. were used for the extraction of polyphenols from plant sources[76-78] and the extracts are evaporated under vacuum to remove the solvents. Nasis-Moragher et al. (1999)[79] reported the use of ethanol and boiling water for the extraction of phenolics. Use of water alone at high temperature and pressure results in the dissolution of undesired protein and polysaccharide[80].

Luo et al. (2009)[81] and Liu et al. (2008)[26] extracted the phenolics from Amla fruit by solvent extraction method. According to them ethyl acetate was found most effective to extract the phenolics with high 2,2 diphenyl picryl hydrazil (DPPH*) free radical scavenging activity.

Chetan and Malleshi, (2007)[82] reported the 1% HCl-methanol was very effective for extraction of the millet polyphenols. Polyphenol contents of the extract were stable to the changes in the temperature of the extract while alkaline pH affects the stability of the phenols[82]. Mativier et al. (1980)[83] also reported that methanol was 20% more effective than ethanol and 73% more effective than water[83].

1.4.2. Microwave assisted extraction

Factors like solvent composition, liquid to solid ratio and extraction time have significant effect on microwave assisted extraction[84-85]. Yinping et al. (2011)[86] developed microwave assisted extraction method for the extraction of polyphenols from grape seeds of *Vitis vinifera*. They reported that in comparison with other extraction methods, microwave assisted extraction provided comparable or better extraction within a very short duration of time[86].

1.4.3. Column chromatography separation

Sun et al. (2006)[87] separated polyphenols from aged wines by using combined techniques of solid-phase extraction and liquid chromatography. In the study, aged red wine polyphenols were separated into various distinct fractions including phenolic acid fraction, monomer flavanol fraction, oligomer procyanidin fraction, anthocyanin and its pyruvic acid derivative fraction, free or non-colored proanthocyanidin fraction, fraction of direct condensation products between anthocyanins and proanthocyanidins and fraction of other pigmented complexes[87]. Diagram of fractionation of wine is given in the **Fig. 1.9.** By this method the anthocyanins and their pyruvic derivatives were separated from other phenolic compounds, while free or non-pigmented polymer proanthocyanidins were separated from other pigmented complexes[87].

Wang et al. (2012)[88] loaded the tea powder into columns with 4-fold solvents and eluted through a cyclic column chromatography extraction. They extracted high-quality tea extracts with greater than 90% extraction efficiency for polyphenols[88]. The highly concentrated water extraction was further passed by the author through columns of polyamide, DM130 macroporous and 732 ion exchange resins and

obtained high-purity polyphenols (99%), caffeine (98%) and theanine (98%) after simple purification of the elutes from each column[88].

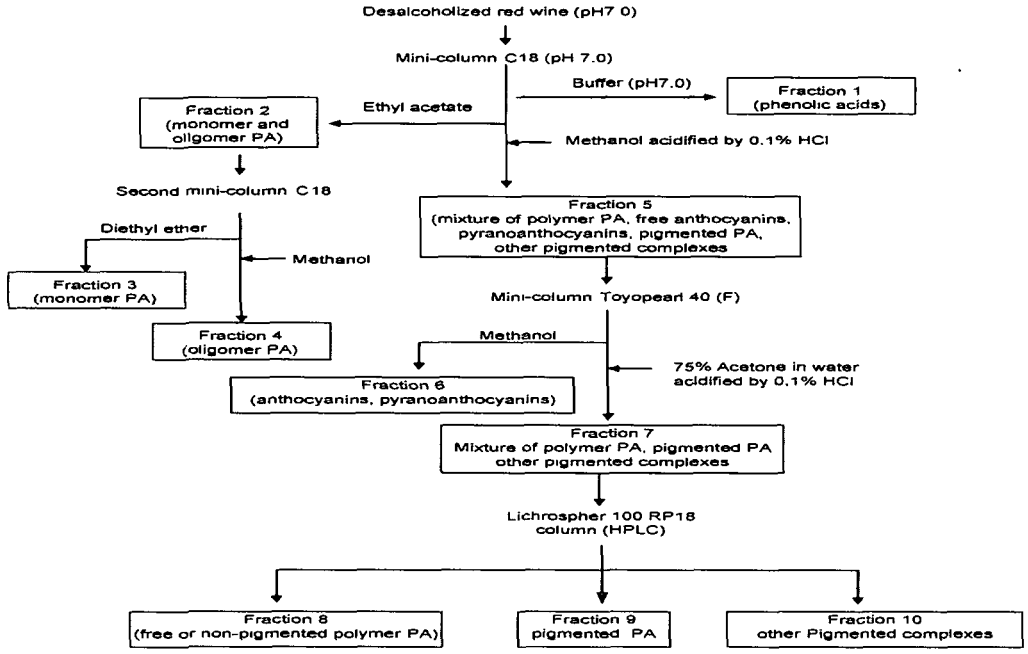


Fig. 1.9 Diagram for fractionation of red wine polyphenols. PA= proanthocyanidins[88].

1.4.4. Pressurised water extraction (PWE)

In pressurised water extraction (PWE) technique water is used as extractant at elevated pressure. PWE is used generally for extraction of thermally labile components[89] and naphtodianthrones from *Hypericum perforatum*[90]. Pressurised water extraction was investigated for the extraction of polyphenols from pomegranate peels[91]. In this study the effect of particle size, temperature, and static time on the recovery of phenolics from the pomegranate were investigated. The results indicated that pressurised water extraction was as comparable as conventional methanol extraction for the extraction of polyphenols from pomegranate peels. Other workers had also optimized the solvent concentration and the extraction temperature by pressurized liquid extraction method and reported it to be an ideal technology for high temperature short time extraction method[92].

1.4.5. Super critical extraction

Supercritical or subcritical CO_2 extraction with addition of organic solvents like ethanol and/or ethanol as a co-solvent has been applied to extract polyphenols from grape seeds[93-94], from wine industry and by products[95], from pistachio hulls[96], and from olive leaves[97]. Some of the phenolics like hydroxycinnamic acid (*p*-coumaric acid, caffeic acid and ferulic acid[98], and coumaric acid isomers (*o*-,*m*-, *p*-coumaric acids)[99] are slightly soluble in supercritical CO_2 hence cannot be extracted without addition of a co-solvent. Adil et al. (2007)[100] studied the effect of pressure (20-60 MPa), temperature (40-60°C), ethanol concentration (14-20 wt%) and extraction time (10–40 min) on subcritical (CO_2+ethanol) extraction of polyphenols from apple and peach pomaces. Total phenolic contents (TPC) and antiradical efficiencies of the extracts were increased by the variables[100].

1.4.6. Extraction through polymeric membranes

Luiz et al. (2008)[101] worked on the application of conventional (using ethanol) extraction with supercritical fluid, using ethanol or supercritical CO_2 ($scCO_2$) as a pure solvent and supercritical CO_2 with ethanol as a co-solvent to obtain polyphenols from cocoa seeds. The extracts obtained were retained by polymeric membranes. They found variation in the polyphenols extracted which depends on the amount of solvent and co-solvent added, the pressure and ethanol concentration used. Used membranes showed polyphenol rejections of between 80% and 95%, with good results for permeate flux and mechanical resistance to the transmembrane pressure applied[101].

1.4.7. High voltage electrical discharges (HVED)

Application of HVED on grape pomaces for 160s increased the yield of polyphenols up to 70%[102]. This yield represented more than twice the yield obtained after 240 min without HVED. HVED also increased the yield of polyphenols after 1 h of extraction compared to that obtained after 4 h of extraction without HVED. The yields of solutes and polyphenols increased with temperature.

1.5. Identification of bio-active compounds of Amla

Liu et al. (2008)[26] isolated the phenolic components from Amla powder by using solvent extraction method. Six components were isolated from ethyl acetate fraction of Amla which were as follows: geraniin, quercetin 3-β-D-glucopyranoside, kaempferol 3-β- D-glucopyranoside, isocorilagin, quercetin, and kaempferol. All the purified compounds shown in **Fig 1.10** had strong antioxidant and radical scavenging activities[26].

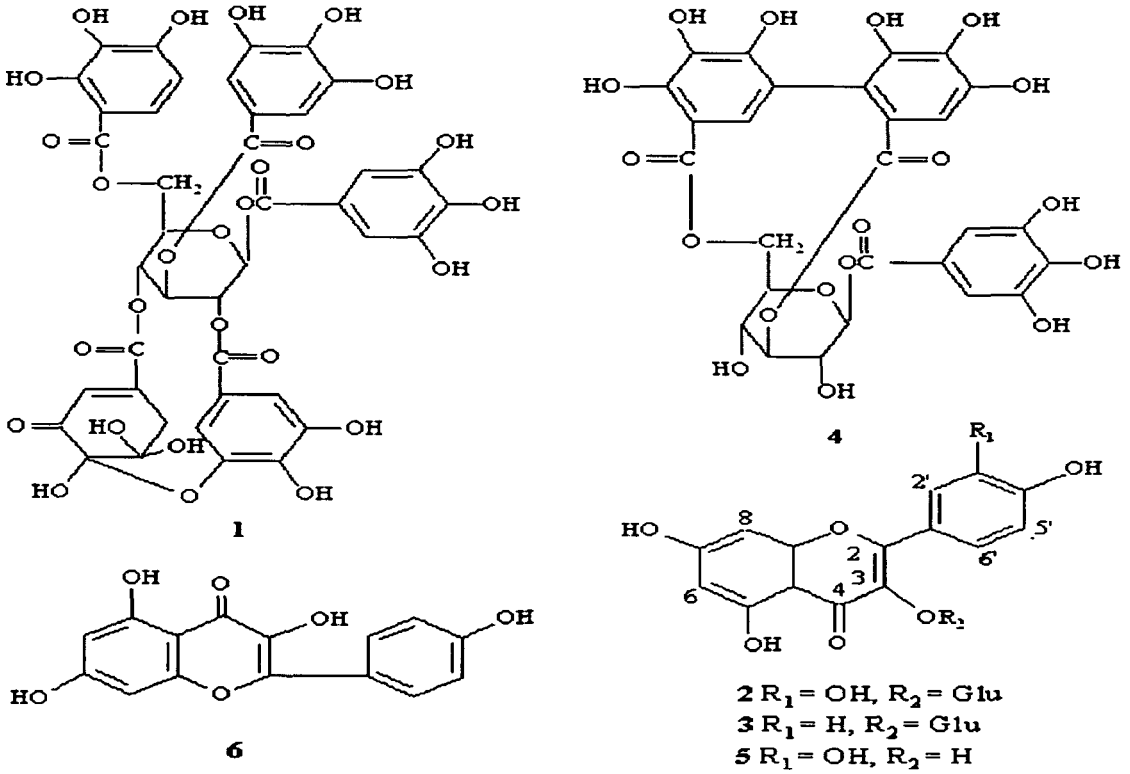


Fig. 1.10. Chemical structures of phenolic compounds isolated from emblica fruit. (1) gerannin, (4) quercetin, and (6) kaempeferol [26].

Luo et al. (2009)[81] isolated cinnamic acid, quercetin, 5-hydroxymethylfurfural, gallic acid, β-daucosterol, ellagic acid (C6) from Amla. All the isolated phenolics showed significant DPPH and $ABTS^+$ radical scavenging activity except for cinnamic acid. The structure of isolated compounds is given in **Fig 1.11.** Gallic acid showed the highest DPPH* scavenging activity while ellagic acid showed the highest $ABTS^+$ scavenging activity amongst all the compounds tested[81]. 1-O-galloyl-D glucose, chebulininc acid, chebulagic acid, mucic acid 1,4-lactone 3-O-gallate, isocorilagin,

chebulanin, mallotusinin and acylated apigenin glucoside compounds were also isolated by several other workers from the aqueous extract of Amla[103-105, 43].

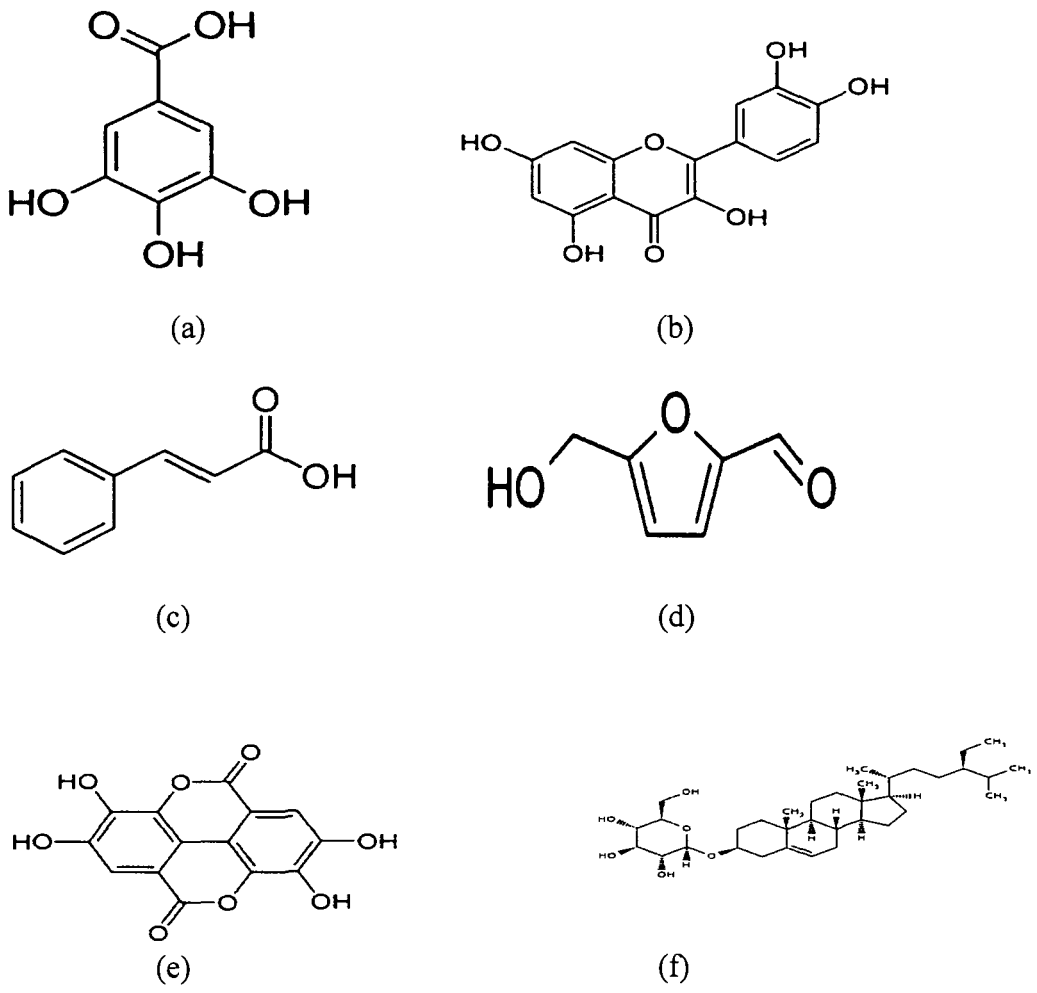


Fig 1.11 Phenolics isolated from Amla (a) gallic acid, (b) quercetin, (c) cinnamic acid, (d) hydroxyl methyl furfural, (e) ellagic acid, and (d) β-daucosterol[81].

1.6. Polyphenols : Biosynthesis

Plants contain a number of secondary metabolites which play a crucial role in plant physiology and in the interaction between the plant and its environment[7]. These metabolites take part in a variety of roles in plant life ranging from structural to protection. Phenolic compounds are secondary metabolites which spread throughout the plant kingdom[106]. Polyphenols accounts for majority of antioxidant properties when compared with other natural antioxidants like vitamin C[107-109]. Polyphenols are by products of metabolism of aromatic amino acids[110]. Being secondary metabolites

the syntheses of polyphenols varies depending on location, species, variety, environmental conditions, etc[110].

The biosynthesis of polyphenols occurs in number of stages. Firstly, formation of cinnamic acids occurs, then condensation of acyl group to build up the 'A' phenyl ring followed by transformation leading to various flavonoids. According to Gamborg, (1967)[111] cinnamic acid formation starts with the condensation of phosphoenol pyruvate with D-erythrose-4-phosphate to form a hepatulosonic acid. This can be cyclized, in the presence of divalent cobalt, yielding 5-dihydroquinic acid, an intermediate in the formation of both shikimic acid and quinic acid. In shikimic acid pathway phenylalanine is required as an intermediate component for the conversion of shikimic acid into cinnamic acid. Addition of malonyl groups to the C6: C3 position provides the carbon of the eventual 'A' phenyl ring. This condensation requires the participation of coenzyme A[112].

1.6.1. Significance of aromatic plant phenolics in human being

Several thousands of phenols are distributed widely throughout the plant kingdom[113-114]. Phenolic compounds exhibit wide diversity in their structures but they can be classified into two different classes, hydroxybenzoic acid (non flavonoids) and the hydroxycinnamic acid (flavonoid)[110].

Nonflavonoids are commonly represented by phenolic acids. Phenolic acids are widely present in fruits, although their distribution varies according to species, cultivar and physiological stage. Amla is one of the richest sources of phenolic acids. Several phenolics have been identified and isolated from Amla by several scientists[103-105]. Most naturally occurring antioxidants possess polyphenolic structure[115]. Phenolics can also act as an endogenous precursor for many of the phenolic molecules found in plants.

Flavonoids are characterized as molecules possessing two phenolics attached by a pyran (oxygen containg ring) structure. These phenolics have a large number of structures which differ in their number and position of hydroxyl (-OH) and methoxyl groups on the basic skeleton[112]. Flavonoids can be classified into flavonols, flavones, catechins, flavanones, anthocyanadins and isoflavonoids[7]. Flavonoids posses antiviral and endocrine effects, activity on mammalian enzymes, ability to modulate immune and inflammatory cell function, have effects on smooth muscles and have also effects on lipid peroxidation and oxyradical production[113, 116].

Natural phenolics play a key role in antioxidative defence mechanism in biological system. The human body is equipped with natural defense system to combat with the free radicals or ROS which includes various enzymes; high and low molecular weight antioxidants[7]. Antioxidants have a tendency to donate their own electrons and end the electron stealing reaction. After donating the electron they do not themselves become free radicals and they remain stable in either form. The defense system in the organism against free radical reactions (prooxidants and ROS) generated during normal cell aerobic respiration may be of endogenous (enzymatic or non-enzymatic) or dietary origin[117]. When natural defense systems are overwhelmed due to the oxidative stress, oxidative damage takes place because of the action of the free radicals, which can cause tissue injury[118-120] and affect immune system[121-122].

Antioxidants derived from dietary sources may help to maintain an adequate antioxidant status, defined as the balance between antioxidants and oxidants in living organism[123]. Earlier the protective effect of Amla has been attributed to vitamin C. In 1996, Ghosal et al. identified four vital components from Amla and claimed that the components responsible for antioxidant and functional properties of Amla is due to the presence of hydrolysable tannins and phenols instead of vitamin C[13]. The antioxidant properties of phenolic compounds are mainly due to their redox property which allows them to donate hydrogen, singlet oxygen quencher and metal chelator and to act as a reducing agent [124].

1.6.2. Major polyphenols from Amla

1.6.2.1. Tannins

Tannins are high molecular weight compounds[125] with many phenolics. Tannins can be classified into three different groups: (i) condensed tannins also called as proanthocyanidins; polyflavonoid tannin; pyrocatechollic type tannins; non hydrolysable tannin, which are oligomers of polyhydroxy flavan-3-ols units. They do not contain sugar residue[126]. Tannins are found throughout the plant kingdom as the most potent antioxidant. They are typically concentrated in the bark of the trees and in the outer seeds; (ii) Hydrolyzable tannins are simple phenolics consists of gallic acid esterified to a core polyol, (iii) third category of tannins are phlorotannin, a phloroglucinol based polymer found in marine [127].

1.6.2.2. Gallic acid

Gallic acid of molecular weight 188.14 with chemical formula $C_6H_2(OH)_3COOH(H_2O)$ is also known as 3,4,5 trihydroxybenzoic acid. Gallic acid and its derivatives are biologically active compounds which are widely present in plants[128-129]. Gallic acid is one of the major phenolics present in Amla[130]. Gallic acid and its derivatives are widely used in various industries as natural antioxidants, photographic developers, in tanning etc.[128-129, 131]. Cytotoxicity against cancer cells; antiinflammatory activity, antimutagenic, hepatoprotective and neuroprotective effects, antitumor effect and analgesic activity of gallic acid have been reported by several scientists [128-129, 132-138]. Pharmaceutical industries also use gallic acid as styptic agent and as a remote astringent in cases of hemorrhage[139].

1.6.3. Role of Amla phenolics as food additives

Autooxidation of fats and oils causes rancidity in processed foods which may be prevented by the use of oxidation inhibitors or antioxidants[140-141]. Synthetic antioxidants like butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) have been used as antioxidants for foods, since the beginning of this century[142-143], which are now being restricted because of their toxicity[144-145]. Natural plants have received much attention as sources of biologically active substances including antioxidants in prevention of lipid peroxidation [146-150]. Reddy et al. (2005)[151] studied the extracts of three different plant foods viz., Amla, drumstick leaves (*Moringa oleifera*) and raisins (*Vitis vinifera*) as sources of natural antioxidants[151]. All the three extracts exhibited a high percentage of antioxidant activity compared to synthetic antioxidants. The authors observed that the addition of Amla gave an excellent antioxidant effect on the biscuit compared with the effect of BHA; the % increase in both peroxide and acid values after 6 weeks were lower than that of the control and BHA treated samples. Extracts from drumstick leaves and Amla were more effective in controlling lipid oxidation during storage[151].

1.7. Amla products

Being very astringent in taste raw Amla is not as acceptable as other fruits hence processing becomes necessary. Several value added products have been reported from Amla[152]. Amla was used to prepare ready-to-serve beverage[153],

powder[154], Amla candy[155], ready to eat chutney[156], sauce[157] etc. The Amla preserve[158], slices, squash, juice, chyawanprash and *burfi* (Indian sweet) are some other major commercially available products of Amla. Flow charts of the some of the commercially available Amla products are given below[9]:

Pulp

↓

Mixing with syrup

↓

Addition of potassium metabisulfite

↓

Filling into clean sterilized bottle

↓

Capping

↓

Storage

Fig 1.12 Flow chart for preparation of Amla squash[9].

Amla fruits

↓

Washing

↓

Pricking

↓

Dipping in 2% salt solution for 24 h

↓

Washing

↓

Blanching in boiling water for 5 min

↓

Treatment with sugar syrup

Fig 1.13 Flow chart for preparation of Amla preserve[9].

(a) (b) (c)

Fig 1.14. Images of some of the commercially available Amla products (a) Amla mouth freshener, (b) Amla juice, and (c) Amla candy[159].

Pathak, (2003)[9] suggested that RTS blend can also be prepared by adding 2% ginger juice in pulp. Besides ginger it can also be mixed with carrot, lime etc. for improved nutrition and better palatability. The major problems associated with this product are the poor shelf life, limited marketability and discoloration during storage. Fruit juice powders have several benefits over the liquid counterparts of juice such as reduced volume or weight, reduced packaging, easier handling, transportation and longer shelf life[160]. Spray drying is a process widely used to convert fruit juices into powders[161-163]. Spray dried powders have also good reconstitutional characteristics, have low water activity and are suitable for longer shelf life. Spray drying technique is also appropriate for heat sensitive components. There is scope for making RTS using spray drying technique from Amla juice.

1.8. Lemon Juice

Citrus genus is one of the most important fruit crop in the world[164]. Lemon (*Citrus limon* (L.) Burm. f.) is the third most important crop of citrus genus[165]. Lemon fruit is a rich source of health promoting nutrients like vitamin C, minerals, citric acid, and flavonoids[165]. The health related properties of lemon have always been associated with their high vitamin C content. Studies have now confirmed that flavonoids have health promoting effects that includes anti-inflammatory, anti-allergic, anti-viral, antiproliferative, anti-mutagenic, anti-carcinogenic and anti-oxidant activities, anti cancer[167-168] effects due to the neutralization of free radicals, responsible of aging and oxidative stress in cells[169-175]. The most abundant flavonoids in lemon are hesperidin, eriotricin and diosmin. Hesperidin, has venotonic and vasoprotective properties (it reduces capillary permeability and enhances its resistance), analgesic, antioxidant and

anti-inflammatory properties[174,176-180]. Other notable flavonoids which have been identified in lemon are vicenin-2 (flavone), diosmin (flavone), quercetin and myricetin (flavonols) as well as other hydroxycinnamic acids[168, 181]. The food value of lemon is shown in **Table 1.5**. Addition and blending of lemon juice or lemon juice powder in foods may increase the functional and bioactive properties of the food.

Table 1.5 Nutritional value of per 100 g of fresh lemon fruit (without peel) (USDA National Nutrient data base) [182]

Principle	Nutrient value	Percentage of RDA
Energy	29 K cal	1.5%
Carbohydrate	9.32 g	7.0%
Protein	1.10 g	2%
Total fat	0.30g	1%
Cholesterol	0 mg	0%
Dietary fiber	2.80	7%
Folates	11µg	3%
Niacin	0.100 mg	1%
Pantothenic acid	0.190 mg	4%
Pyridoxine	0.08 mg	6%
Riboflavin	0.02mg	1.5%
Thiamin	0.04 mg	3.5%
Vitamin C	53 µg	.88%
Vitamin A	22 IU	1%
Sodium	2 mg	-
Potassium	138 mg	3%
Calcium	26 mg	3%
Copper	37 µg	4%
Iron	0.60 mg	7.5%
Magnesium	8 mg	2%
Zinc	0.06 mg	0.5%
β-Carotene	3 µg	....
α-Carotene	20 ug	....
Lutein-Zeaxanthin	11 µg	

1.9. Scope and objective of the present investigation

Recent research interest has been on the utilisation of the seeds of the fruits for their functional and nutraceutical properties. Amla, a fruit known for its medicinal value has seed that is approximately 16% by weight with 13.5% of fibrous portion (Amla pomace) that is discarded. Studies on the functional properties of seed coat and pomace have not been reported. Even though Amla fruit is beneficial for health it is not widely utilised and its limited acceptability is because of the astringent properties. An RTS powder with good taste, better shelf life and good reconstitubility offers a way for its utilisation. The present investigation aimed to study the antioxidant property of the Amla pomace, seed and seed coat, identify the phenolics present in them and develop an RTS drink from Amla and lemon juice powder.

The objectives of the present investigations are as follows:

- To examine the different varieties of Amla for their physical properties, proximate composition and total phenolic content
- To analyse the composition of Amla seed and Amla seed coat powder
- To optimize the solvent extraction methods for the extraction of phenolics from Amla fruit powder and Amla pomace (residue left after juice extraction) powder and characterise the phenolics
- To optimize the solvent extraction method for the extraction of polyphenols from Amla seed coat powder and characterise the phenolics
- To study the effect of maltodextrin and inlet temperature on physicochemical properties of spray dried Amla and lemon powders and optimization for development of Amla and lemon based RTS drink

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Chapter-2

Physicochemical analysis of different varieties of Amla and comparative analysis of functional and nutritive values of Amla fruit, seed and seed coat powder

2.1. Introduction

Amla (*Emblica officinalis* L.) as a Euphorbiaceous plant is widely distributed in subtropical and tropical areas of China, India, Indonesia and Malaysia[1]. The fruit is used as a major constituent in several Ayurvedic preparations for promotion of health and longevity[2]. It is known that Amla is a good source of polyphenols, flavones, tannins and other bioactive compounds[3]. These substances being strong antioxidants might contribute to the health effects of Amla. Several active compounds like gallic acid, ellagic acid, 1-O-galloyl-D glucose, chebulininc acid, quercetin, chebulagic acid, kaempferol, mucic acid 1,4-lactone 3-O-gallate, isocorilagin, chebulanin, mallotusinin and acylated apigenin glucoside compounds have been isolated from the aqueous extract of Amla[3,4,5,6]. These bioactive components have anticancer, hypolipidemic, expectorant, purgative, spasmolytic, antibacterial, hypoglycaemic[7,8] hepatoprotective, hypolipidemic activities and also can attenuate dyslipdaemia[9,10]. Though the functional properties of Amla have been reported, the seed and seed coat of Amla have never been investigated for their functional properties as well as compositional analysis. In this chapter the physicochemical properties of different varieties of Amla are presented. Further, Amla seed and seed coat of *Chakaiya* variety (major processing waste of Amla based industries) were separately analyzed for their proximate composition, antioxidant properties, total phenolic contents, major/micronutrients and fatty acid profile.

2.2. Materials and methods

2.2.1. Raw material

Amla of *Chakaiya*, *Francis*, *Kanchan*, *Nnarendra-7* and *Krishna* variety were procured from local market of Allahabad, India. The Amla fruits were cleaned thoroughly under tap water to remove adhering dust and wiped with muslin cloth. Fresh fruits of different varieties were evaluated for their physical, chemical and functional properties. The fleshy part of *Chakaiya* variety of Amla was grated and seed was separated manually from adhering Amla. Grated Amla shreds were dried in tray drier at 40°C. The dried Amla shreds were ground in laboratory grinder and passed through 0.5 mm screen sieve. Whole Amla seeds were dried in tray drier at 40°C. As the whole seeds dried they broke along the ridges with a crackling sound. The seed coat was separated from the brown seed from each of the broken units. Seed coat and seed were

separated manually and both were converted into powder separately as done for Amla shreds. The powder of fruit, seed and seed coat were stored at refrigerated temperature (4°C) for further analysis. **Fig 2.1** shows the image of whole seed, seed coat and seed of *Chakaiya* variety.

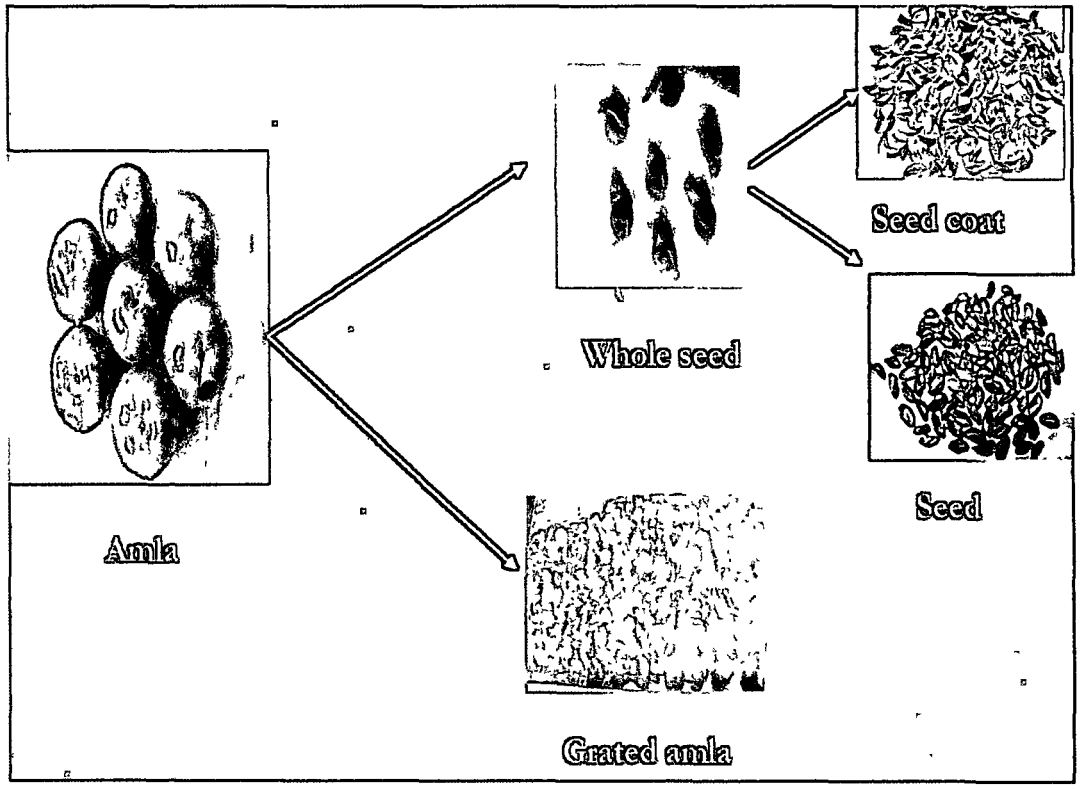


Fig. 2.1 Images of (a) Amla fruit of *Chakaiya* variety; whole seed; grated Amla; seed coat; and seed.

2.2.2. Methods

2.2.2.1. Analysis of physical properties of Amla

Different varieties of Amla were measured for their height and width with varnier callipers with least square of 0.02 cm. Number of fragments and shape were analyzed visually. Ten readings were taken for each physical property.

2.2.2.2. Proximate analysis of Amla

The moisture, crude fat, protein, crude fiber content of the samples were determined as per AOAC 18th edition, (2010)[11] procedures. Acidity and pectin were

determined by the method of Ranganna, 1986[12]. Available carbohydrate was calculated by balance method.

2.2.2.3. Vitamin C estimation

Sample solution equivalent to 0.2 mg ascorbic acid mL^{-1} was prepared in water containing 3% w/v metaphosphoric acid added to increase the stability of ascorbic acid. It was titrated against standard 2, 6 dichlorophenol indophenol (2,6 DCIP) solution of concentration 0.5 mg mL^{-1} until the pink color developed completely. The operation was repeated with a blank solution omitting the sample being examined. From the difference the ascorbic acid in each mg of sample was calculated from the ascorbic acid equivalent to DCIP[13].

2.2.2.4. Determination of hydration properties

The water retention property (WRC) and swelling capacity (SWC) of Amla seed and seed coat were analyzed by the methods given by Robertson et al. (2000) with slight modification[14]. For WRC, 1 g of sample was hydrated at room temperature in 30 mL of distilled water and centrifuged at 5000 rpm for 15 min and after 18 h of equilibration the supernatant was removed. The residue was dried at 105°C. The weight of residue was recorded both prior to drying (fresh weight) at 105°C and after drying until constant weight was obtained. WRC was calculated as the amount of water retained by the sample (g/g of dry weight)[14].

$$WRC = \frac{\text{Fresh weight of residue(g) - Dry weight of residue (g)}}{\text{Dry weight of residue (g)}}$$

For SWC determination, 0.1 g of sample was hydrated with 10 mL of distilled water in a calibrated cylinder (15 cm diameter) at 30°C temperature. After equilibration for 18 h, the bed volume was recorded and expressed as volume/g of original sample dry weight[14].

$$SWC = \frac{\text{Volume occupied by sample (mL)}}{\text{Original sample dry weight (g)}}$$

2.2.2.5.Diphenyl picryl hydrazil free radical (DPPH*) scavenging activity

The DPPH* scavenging activity of the extract was determined by the method of Luo et al. (2009) with slight modifications[15]. In brief, 250 mg of sample was extracted by 10 mL of methanol in incubator shaker at 25°C for 30 min. The mixture was centrifuged at 4°C for 10 min at 5687 *g* and 2 mL of extract was mixed with 2 mL methanolic solution containing 1mM DPPH. The mixture was shaken vigorously and then left to stand for 30 min in the dark. The absorbance was measured at 517 nm. The absorbance of control was obtained by replacing the sample with methanol.

$$\text{DPPH* scavenging activity (\%)} = \frac{\text{(1-absorbance of sample)x100}}{\text{absorbance of control}}$$

2.2.2.6. Total phenolic content estimation by high performance liquid chromatography (HPLC) analysis

The total phenolic content by HPLC was estimated by the method given by Seruga et al. (2011)[16] with some modifications. Gallic acid standard was used for total phenolic content estimation[16]. Calibration curves were made by diluting stock solutions with methanol to give concentration of the standards in the range of 1-100 mg/L of gallic acid. The components in the sample were separated by HPLC (Waters, model Breeze 2) column C_{18} binary system. For separation, 0.1% orthophosphoric acid as solvent A and 100% methanol (HPLC) grade as solvent B were used. The elution conditions were 0-30 min from 5% B to 80% B; 30-33 min 80% B; 33-35 min from 80% B to 5% B and 35 to 40 min 5 % B. Flow rate was 0.8 mL/min. The operating condition of temperature was 20°C and injection volume was 20 µL. The detection wavelength for gallic acid was 280 nm. The total phenolic was determined from the total area of RP-HPLC chromatogram at 280 nm and expressed as GAE/g of sample. For the sample preparation, 250 mg sample was extracted in mobile phase (HPLC grade methanol containing 0.1% of orthophosphoric acid) for 30 min. The mixture was centrifuged for 10 min at 8000 rpm and 4°C temperature. Supernatant was filtered through 0.45 µm filter paper. Supernatant (20 µL) was injected for HPLC analysis.

2.2.2.7. GC-MS analysis

Fatty acid profile analysis of the fat extracted from Amla seed and seed coat was analyzed by GC-MS (Perkin Elmer Clarus 600). For derivatization of fatty acid in the form of methyl esters, 40 mL of dried methanol was taken into a 500 mL round

bottom flask and cooled for 30 min on ice bath. During cooling, 14 mL of acetyl chloride was added to the dried methanol in a drop wise manner with constant shaking followed by addition of cold oil. To this, 20 mL of chloroform was further added to dissolve the oil completely. The whole mixture was refluxed for 2h, after which the mixture was cooled and subsequently 4.0 N NaOH was added to make the reaction mixture alkaline. Distilled water (100 mL) was added and the whole mixture was transferred to a 500 mL separating funnel to which diethyl ether (50 mL × 3) was added in order to extract fatty acid methyl esters (FAMES). The organic layer was dried with anhydrous sodium sulphate, filtered and solvent was evaporated in vacuum. A complete removal of solvent was achieved by flushing with nitrogen. The sample was analyzed by using GC-MS with capillary column Elite-5 (Ge column of 30 meter x 0.25 mm; phase thickness 0.25 μm) and the temperature was first held at 40°C (2 min) and then raised to 250°C (5 min) at a rate of 10°C/min[17]. Flow rate of helium was 1 mL/min. The compounds were confirmed by comparison of their retention time with that of the reference compounds of NIST libraries.

2.2.2.8. Mineral analysis

Major and micro minerals were analyzed by the method given by Food and Agriculture Organization of the United Nations, (1983) with slight modifications[18]. Briefly, 2 g of sample was placed in Kjeldahl tubes and 25 mL of freshly prepared nitric acid-sulphuric acid- perchloric acid mixture (3:1:1) was added. The sample as digested at 250°C for 2-3 h until a clear solution was obtained. After cooling the solution was diluted with 100 mL with deionized water and the residue was filtered through an ash less filter paper. The nutrient mineral content of the sample was determined by atomic absorption spectroscopy (AAS) (Thermo, ICE 2000) with air acetylene flame for Ca, P, Fe Zn, Mg, while graphite mode was used to analyze the Cu, Mn at ppb level[18].

2.2.2.9. Spectral analysis

To find out the functional characteristics the samples were scanned by Fourier transform infrared (FTIR) spectrophotometer (Perkin Elmer, Spectrum 100) in the range of 4000-600/cm with a resolution of 4/cm. Spectra were collected at ambient

temperature by averaging three scans and coupling the attenuated total reflection ATR accessory to an FTIR spectrometer[19].

2.2.3. Statistical Analysis

All the samples were analyzed in triplicate. Results are shown as mean±standard deviation. The significant difference was analyzed by using Analysis of variance.

2.3. Results and discussion

2.3.1. Physical and compositional properties of different varieties of Amla

The different varieties of Amla were evaluated for their height, width, weight and number of segments and the findings are shown in **Table 2.1**. In present study *Krishna* variety was found to be largest in fruit size (44.9 g/fruit) than the other varieties tested. Fruit length, fruit width and number of segments were observed to be maximum in *Krishna*. The weight of the varieties ranged from 29.9 to 44.9 g. The varieties also showed variation on the basis of height, width and shape. The present results support the finding of Pathak, (2003)[20].

Table 2.2 presents the chemical composition of different varieties of Amla. Significant changes in the composition of different varieties of Amla were observed. Moisture percent ranged from 79.56 to 83.81% with *Chakaiya* containing maximum amount of moisture. Vitamin C content of different Amla varieties ranged from 570.12 to 725.12 mg/100 g which is in agreement with Sankar, (1969)[21] that Amla is one of the richest sources of vitamin C known and can range upto 950 mg/100 g. *Krishna* showed comparatively higher amount of crude fiber (3.71%). Since *Chakaiya* variety had second highest total phenolic content after *Krishna* variety and due to the less availability of the *Krishna* variety, *Chakaiya* (commonly used variety by the manufacturing industries) was selected for rest of the studies.

Table 2.1 Physical characteristics of different varieties of Amla genotype

Varieties	Fruit height (cm)	Fruit width (cm)	Fruit weight (g)	No. of segments/fruit	Fruit shape
Chakaiya	3.4	4.1	42.7	6	Flattened, round
Francis	3.3	4.0	30.7	6	Flattened, oval
Kanchan	3.3	3.5	29.9	6-8	Flattened, oblong
Krishna	3.9	4.3	44.9	6-8	Triangular
Narendra-7	3.6	4.2	43.1	6	Flattened, oval

All observations are the average of 10 individual observations

Table. 2.2 Compositional analyses of different varieties of Amla

Sample	*Francis*	*Chakaiya*	*Narendra-10*	*Narendra-7*	*Krishna*	*Kanchan*
Crude fiber (%) (fwb)	2.42 ± 0.1^{a}	$2.45\pm0.0.1^{a}$	1.98 ± 0.2^{b}	2.99 ± 0.1^{c}	3.71 ± 0.1^{d}	2.02 ± 0.1^{b}
Moisture (%) (fwb)	83.12 ± 0.8^{a}	83.81 ± 0.9^{a}	80.12 ± 0.8^{b}	80.03 ± 0.9^{b}	79.56 ± 1.0^{b}	81.18 ± 1.0^{b}
Fat (%) (fwb)	0.03 ± 0.01^{a}	0.05 ± 0.01^{a}	0.04 ± 0.01^{a}	0.05 ± 0.02^{a}	0.05 ± 0.01^{a}	0.03 ± 0.01^{a}
Protein (%)	0.38 ± 0.1^{a}	0.45 ± 0.1^{b}	0.30 ± 0.1^{c}	0.37 ± 0.0^{a}	0.39 ± 0.1^{a}	0.39 ± 0.0^{a}
Carbohydrate (%) (fwb) (available)	13.69 ± 0.2^{a}	12.75 ± 0.4^{b}	17.01 ± 0.4^{c}	15.84 ± 0.3^{d}	15.75 ± 0.3^{d}	15.95 ± 0.4^{d}
Acidity (%)	3.20 ± 0.1^{a}	3.31 ± 0.1^{a}	3.77 ± 0.2^{b}	2.81 ± 0.1^{c}	3.22 ± 0.1^{a}	2.42 ± 0.2^{d}
Total phenols (g/100g) (db)	18.36 ± 0.8^{a}	24.50 ± 0.4^{b}	19.81 ± 0.4^{c}	21.32 ± 0.6^{c}	25.62 ± 0.9^{b}	21.09 ± 1.0^{c}
% Ash (fwb)	0.36 ± 0.0	0.49 ± 0.0	0.55 ± 0.0	0.72 ± 0.1	0.54 ± 0.0	0.42 ± 0.0
Vitamin C (mg/100g)	630.31 ± 3.6^{a}	592.32 ± 2.8^{b}	600.61 ± 1.7^{c}	570.12 ± 2.3^{d}	725.12 ± 3.4^{e}	610 ± 3.9^{f}

fwb means fresh weight basis; db is dry basis

Value = mean $\pm$ standard deviation

2.3.2. Proximate analysis, pectin, vitamin C and functional properties of Amla seed and seed coat powder

The Amla fruit, seed and seed coat powder were analyzed for proximate content. The results are presented in **Table 2.3**. The protein content of seed was significantly higher than fruit powder and seed coat powder at 5% probability level (**Table 2.3**). High % of protein in seed powder suggests that the level of carbohydrates in seed powder is less than that in Amla fruit powder and seed coat powder.

Fat content was high in seed with a mean value of 8.84%. The present finding does not match with findings of earlier workers who have reported 18% of fat in Amla[22]. Seed coat had comparatively less amount of ash than fruit powder and seed powder. Seed powder and Amla fruit powder showed higher concentration of ash with a mean value of 3.81% and 3.54% respectively whereas seed coat powder showed only 1.8% of ash (**Table 2.3**). The low % of ash in seed coat powder can be justified from **Table 2.4** also, which shows that the major minerals like Ca, P, K were significantly low in the seed coat powder than the fruit powder and seed powder. The % crude fiber was comparatively higher in seed coat powder than other samples tested. Water retention capacity is the quantity of the water that remains present in bound form along with hydrated fiber after application of an external force (pressure or centrifugation) [23]. The water retention capacity and swelling capacity of both seed and seed coat are presented in **Table 2.3**. Seed coat powder showed higher water holding capacity and swelling capacity with mean value of 9.5 g water/g dwb and 12.86 mL water/g dwb, respectively than the seed powder that were significantly different at 5% probability level. The high water retention capacity of seed coat may be related to the high dietary fiber content[24]. High pectic substances in seed coat might account for its high water holding capacity and swelling capacity. The same trend was observed during the analysis of the swelling capacity of seed and seed coat powder. While Amla seed coat and Amla powder showed 1.85% and 1.88 % DPPH* scavenging activity respectively, seed powder had only 0.74 % of DPPH* scavenging activity.

Table 2.3 Chemical composition and functional properties of Amla fruit, seed and seed coat powders

Particulars	Amla fruit powder	Amla seed powder	Amla seed coat powder
Ash (%)	3.54 ± 0.2^a	3.81 ± 0.1^b	1.48 ± 0.1^c
Fat (%)	0.51 ± 0.1^a	8.84 ± 0.2^b	2.45 ± 0.1^c
Protein (%)	6.04 ± 0.1^a	14.03 ± 0.4^b	7.04 ± 0.2^c
Crude fiber (%)	2.78 ± 0.1^a	3.42 ± 0.1^b	4.47 ± 0.2^c
Carbohydrates (%)	82.91 ± 1.3^a	73.35 ± 1.5^b	81.94 ± 1.1^a
Pectic substances (%)	ND	1.51 ± 0.1^a	3.56 ± 0.1^b
WRC (g water/g dwb)	ND	1.21 ± 0.2^a	9.50 ± 0.2^b
SWC (mL water/g dwb)	ND	2.21 ± 0.1^a	12.86 ± 0.1^b
% DPPH* scavenging activity/mg of powder	1.88 ± 0.0^a	0.74 ± 0.1^b	1.85 ± 0.2^a

Values in the same row with different superscripts differ significantly at 5 % probability level

WRC = water retention capacity; SWC = swelling water capacity; ND: not detected

2.3.3. Mineral analysis of Amla fruit, seed and seed coat powders

Minerals are inorganic nutrients which may be present both as single atom and in singlet form [25]. Processing wastes of Amla were analyzed for some of the major and micro minerals and results are shown in **Table 2.4**. High value of Ca was obtained in Amla fruit powder (129.77 mg/100 g) followed by seed coat and seed powder. Seed powder and Amla fruit powder contained higher amount of P with mean value of 395.44 mg/100 g and 159.02 mg/100 g, respectively whereas in seed coat the mean value of P was found to be 89.61 mg/100 g. All three samples showed higher concentration of K with mean values of 2543.70 mg/100 g, 1314 mg/100 g and 2542.50 mg/100 g in fruit powder, seed coat and seed powder, respectively. Magnesium levels in all three samples were lower than the recommended RDA (420 mg/100 g)[25]. Seed powder and Amla fruit powder had comparatively higher value of Mg with mean value of 53.25 mg/100 g and 43.6 mg/100 g than seed coat (23.60 mg/100g). The

concentration of Mn obtained in seed powder was maximum with content of 41.98 µg/100 g. The studies also revealed that Mn was the most abundant trace mineral among the tested micro minerals followed by Fe, Co and Cr. K is the most abundant intracellular cation which is known to activate number of enzymes which are responsible to catalyze the transfer of phosphoryl groups or elimination reactions[26]. According to RDA, 2000 mg of potassium is required [25]. It thus implies that 20 g of Amla fruit powder or seed powder will satisfy one fourth requirement of K whereas 38.5 g of seed coat will be required to fulfill one fourth RDA of K. Phosphorous is involved in energy transfers during cellular metabolism. A number of enzymes and vitamin B becomes activated in presence of phosphate group[25]. Calcium is important for bone formation. The mean concentration of Ca of Amla powder and seed coat powder shows that less than 50 g of powder may complete the RDA of Ca. Amla fruit powder, seed powder and seed coat powder had good amount of iron. Iron can bind a variety of ligands including cyanide, carbon monoxide, oxygen binding proteins such as haemoglobin, myoglobin and cytochrome oxidase etc[27]. But the form of iron in fruits and vegetables is of non heme form and the absorption of this form is low[28]. Co is the mineral in Vit B_{12} and its deficiency is rare. Cr aids the metabolism of carbohydrates by increasing the insulin function and recommended RDA of Cr is 35 µg and no toxicity of Cr is reported when consumed in amount greater than recommended value[29]. Consumption of 5g of Amla fruit powder or seed coat powder will satisfy the RDA of Cr whereas 7 g of seed will fulfill the requirement of Cr. Cu is responsible for hemoglobin and melanin production, electron transport, phospholipids synthesis, collagen synthesis and helps to maintain healthy bones, nerves and immune system. The RDA of Cu is 2-3 mg and too much Cu is poisonous and can lead to nausea, vomiting etc[30]. All three samples tested had very low level of Cu and consumption of 100 g of seed coat powder will only give 0.19 mg of Cu hence no toxicity of Cu will be experienced after consumption of large quantity of seed coat and seed powder. Zn is a very crucial micronutrient and is the mineral compound in a number of enzymes. Zn is also involved in protein and CO_2 metabolism and also involved in healing of cuts and wounds. The RDA of Zn is 15 mg[30]. Intake of Amla fruit, seed coat and seed powder will provide substantial amount of Zn.

Table 2.4 Comparative mineral analysis of Amla fruit, seed and seed coat powders (mg/100 g)

Minerals	Amla powder	Seed coat powder	Seed powder
Ca	129.78±1.21[a]	73.68±1.43[b]	23.60±1.56[c]
P	159.02±4.44[a]	89.61±2.53[b]	395.44±9.63[c]
K	2544.00±1.24[a]	1314±3.40[b]	2542.50±2.50[a]
Fe	11.70±0.08[a]	14.00±0.08[b]	11.30±0.09[a]
Mg	46.30±0.65[a]	23.60±0.65[b]	53.25±0.30[c]
Zn	0.23±0.02[a]	0.14±0.01[b]	0.20±0.01[a]
Cr	0.82±0.01[a]	0.86± 0.01[a]	0.56±0.01[b]
Co	0.69±0.01[a]	1.46±0.01[b]	1.42±0.01[b]
Mn	0.19± 0.01[a]	0.21±0.01[a]	0.42± 0.01[b]
Cu	0.22±0.01[a]	0.19±0.01[a]	0.14±0.00[b]

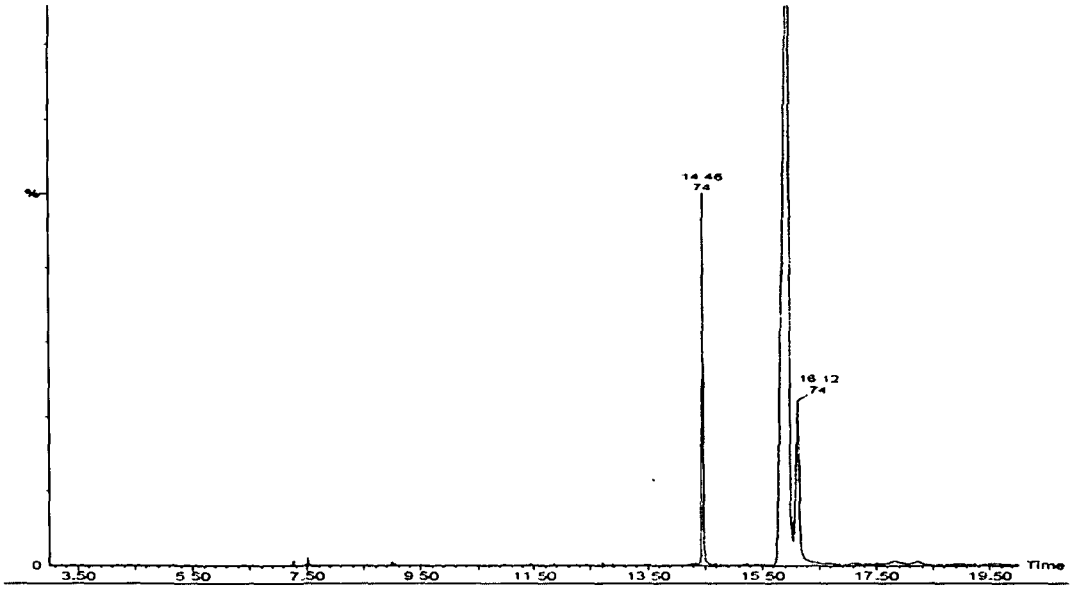
Value= mean ± sd

Values in the same row with different superscripts differ significantly at 5 % probability level

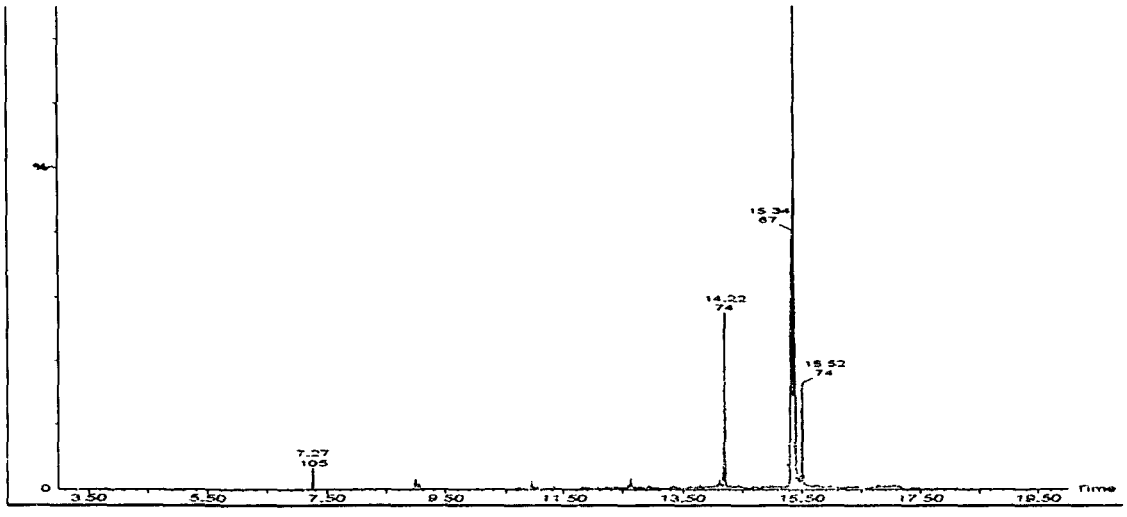
2.3.4. Fatty acid profile analysis of Amla seed and seed coat

Through GC-MS it was revealed that the major components in seed coat oil were 9,12,15 octadecatrienoic acid, tetradecanoic acid and linoleates, whereas benzoic acid, 6 tetradecansulfonic acid, hydroquinone, dodecane 1-fluoro, phthalic acid 2-cyclohexylethyl iso butyl ester were the minor components (**Fig 2.2a, Table 2.5**). The major fatty acids in seed oil of Amla were 9,12, 15, octadecatrienoic acid (z,z,z) and tetradecanoic acid while benzoic acid, octenoic acid, 6 tetradedecane sulfonic acid, octadecanoic acid, 11 methyl ester, hydroquinone, octadecane 1, 1 dimethoxy were present in very minute quantities. (**Fig 2.2b, Table 2.6**). In seed coat each component had different retention time except for tetradecanoic acid 10,13 dimethyl which was detected at two different retention times i.e. 14.22 and 15.52 min. Tetradecanoic acid was common in both seed and seed coat and this acid has antioxidant activity whereas 9,12, 15, octadecatrienoic acid (z,z,z), the major component of seed oils have anti-

inflammatory and antiarthritic properties[31,32]. Omega-3 fatty acid is essential for normal growth and may also be important in preventing coronary artery diseases. Omega-3 fatty acid is the major component of seed oil and the present finding was in agreement with the finding of the Arora et al. (2011)[22]. Dodecane and phthalic acid were present in very minute quantities but they are reported to have antibacterial properties as reported by Adeleye et al. (2011)[33].



(a)



(b)

Fig 2.2. Fatty acid profile analysis by GC-MS (a) Amla seed powder, and (b) Amla seed coat powder.

Table 2.5 Fatty acid profile analysis of Amla seed coat by GC-MS

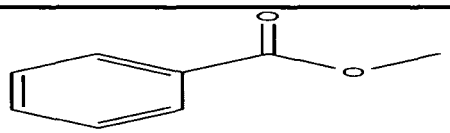
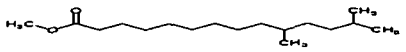
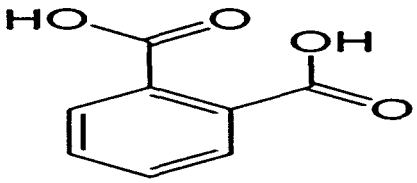
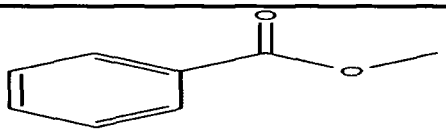
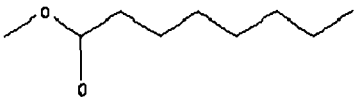
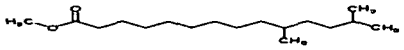
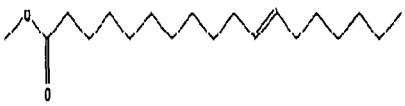
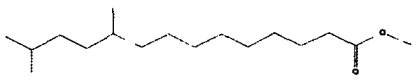
Compounds	Retention time	Molecular weight	Molecular structure
Benzoic acid methyl ester	7.270	136	
2 Chloroethyl linoleate	15.344	342	
9,12,15 Octadecatrienoic acid methyl ester	15.399	292	
Tetradecanoic acid 10, 13 dimethyl methyl ester	14.218, 15.52	270	
Dodecane, 1-fluoro	9.056	188	
Phthalic acid, 2-cyclohexylethyl iso butyl ester	13.803	332	

Table 2.6 Fatty acid profile analysis of Amla seed by GC-MS

Compounds	Retention time (min)	Molecular weight	Molecular structure
Benzoic acid, methyl ester	7.260	136	
Octenoic acid methyl ester	7.510	158	
Tetradecanoic acid 10, 13 dimethyl methyl ester	14.463	270	
Octadecanoic acid, 11 methyl , methyl ester	11.44	312	
9,12,15 Octadecatrienoic acid methyl ester	15.964	292	
Dodecane, 1-fluoro	9.056	188	
Octadecan 1, 1, dimethoxy	11.847	384	

2.3.5. HPLC analysis

HPLC analysis of Amla fruit, seed coat and seed for total phenolic content, and gallic acid content were carried out. UV detector with wavelength 280 nm was used to detect gallic acid. By comparing the spectra of the standard gallic acid was found to be one of the major phenolics in all three samples tested; the present finding was in agreement with the Kumar et al. (2006)[34] that gallic acid and tannic acids are the major phenolic acids of Amla[34]. Total phenolic content and gallic acid content in Amla fruit powder were 8738.00 mg GAE/100 g and 3000 mg/100 g (**Fig 2.3**) respectively. Total phenolic content and gallic acid content in Amla fruit powder was approximately 15 times more than that in seed coat powder (**Fig 2.4a, Table 2.7**). On the other hand, seed powder had very negligible levels of total phenolic content and gallic acid (**Table 2.7, Fig 2.4b**). Kumaran and Karunakaran, (2006)[35] also reported that gallic acid is the major component of ethyl acetate extract of Amla. These results do not agree with the findings of Matachiew and Devahastin, (2008)[36] who observed that gallic acid is not the main component of Amla.

From the HPLC analysis, it might be inferred that though the total phenolic content of seed coat powder is significantly less than Amla fruit powder, DPPH* scavenging activity of the seed coat powder is comparable with fruit powder which suggested that seed coat powder has very good potential in terms of bioactive properties. The lower value of total phenolic content in seed and seed coat may be because of the presence of high ratio of bound phenolics whereas in Amla fruit the major portion of phenolics is present in free form[34].

Table 2.7 Analysis of total phenolic content by HPLC

Sample powder	Total phenolic content GAE mg/100 g	Gallic acid mg /100 g
Amla fruit	8738.00 ± 5.38^{a}	3000.00 ± 2.1^{a}
Seed coat	593.06 ± 3.98^{b}	240.00 ± 1.21^{b}
Seed	51.53 ± 4.10^{c}	17.03 ± 0.37^{c}

Values in same column with different superscript differ significantly

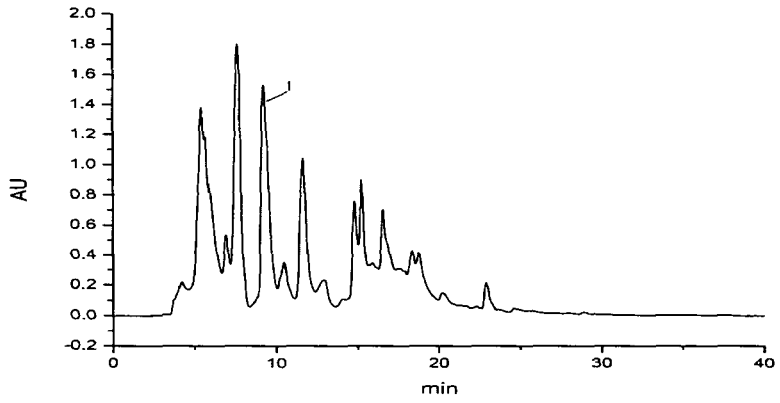
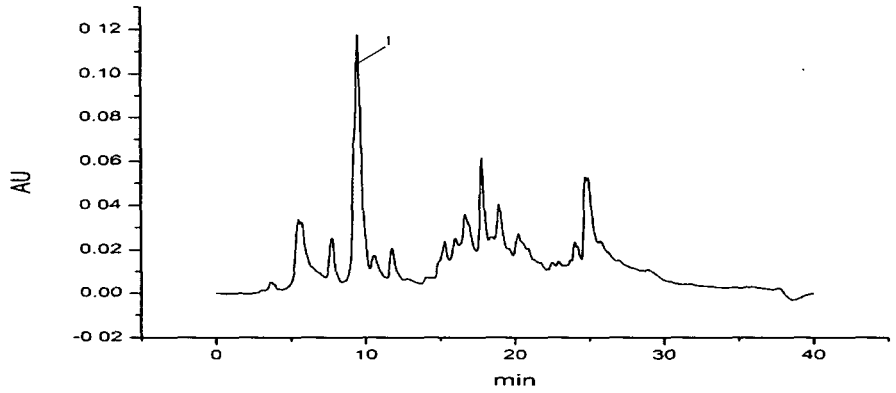
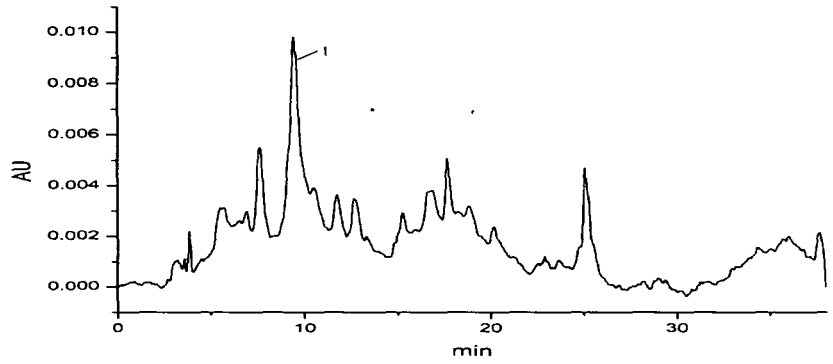


Fig.2.3 HPLC chromatogram of Amla fruit powder at 280 nm; (1) : gallic acid.



(a)



(b)

Fig. 2.4. HPLC chromatogram of (a) seed coat at 280 nm, and (b) seed powder at 280 nm. (1) Gallic acid.

2.3.6. FTIR analysis

FTIR spectroscopy was performed to obtain the finger prints of all three samples (**Fig 2.5**). On comparison the IR spectra of Amla fruit, seed coat and seed powder it was found that the seed coat and seed powders showed drop in relative peak intensities of 2924 cm^{-1}, 2854 cm-^{1}, 1461.71 and 1162.85 cm^{-1}. Seed coat and seed powder showed no relative peak at 666.26 cm^{-1} and 509.09cm^{-1} which were present in fruit powder. Broad signal in the range 3200-3420 cm^{-1} indicates the presence of O-H. The presence of C-O stretching in the range of 1200-1000 cm^{-1} in all three samples confirms the presence of phenolic content. Presence of two weak bands in the range of 1230-1030 cm^{-1} may also indicate the presence of tertiary amines in all three samples tested. A significant drop in the peak and peak intensity below 1000 cm^{-1} region were observed in both seed and seed coat powder. The presence of peak below 1000cm^{-1} region in amla powder may suggest the presence of monosubstitutes or disubstitutes at ortho, meta and para positions in benzene ring. The peak intensity of the seed powder was significantly less than seed coat and Amla fruit powder which is supported by the poor DPPH* radical scavenging activity and HPLC analysis.

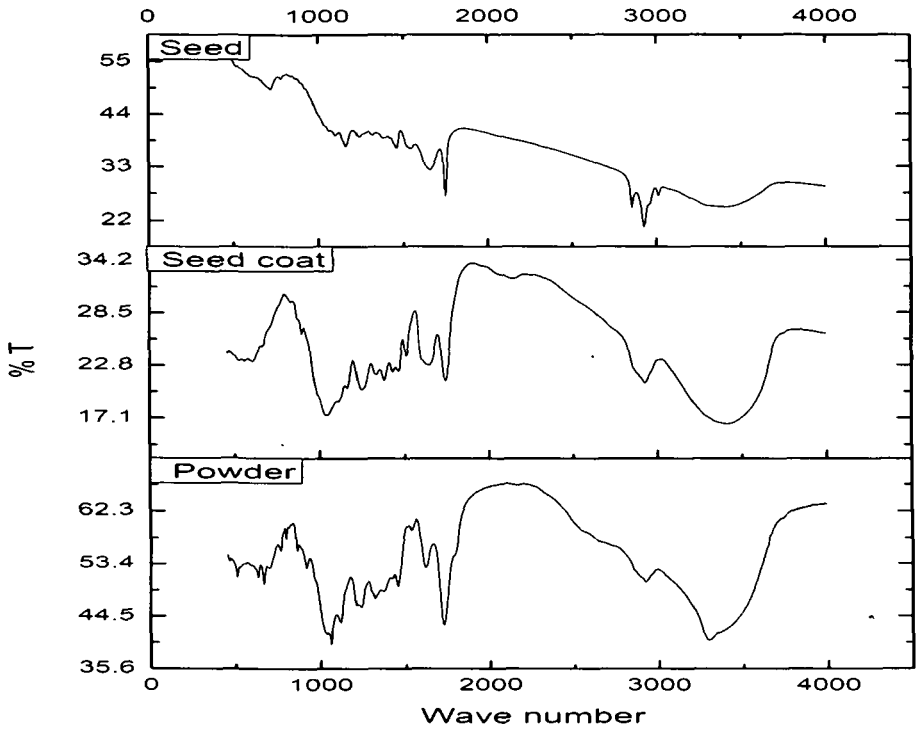


Fig 2.5 FTIR spectra of Amla fruit powder, seed, and seed coat powder.

2.4. Conclusion

The compositional analysis of different varieties of Amla was performed. *Krishna* and *Chakaiya* variety were found to be superior in terms of total phenolic contents. The fruit, seed coat and seed of *Chakaiya* variety of Amla were analyzed for their functional characteristics by GC-MS and HPLC and major and micro mineral analyses were conducted by AAS. Seed coat powder was found comparatively more potent than seed powder in terms of hydration property. The % DPPH* radical scavenging activity of seed coat was observed to be similar to fruit powder. Both seed and seed coat had good amount of major and micro minerals. Seed powder had very good amount of P, K, Mn, and Co whereas seed coat was a good source of Ca, Cr, Co and Fe but comparatively poor source of P and K when compared to fruit powder. Fatty acid profile of seed and seed coat showed that the major portion of fatty acid is unsaturated in nature and ω-3 fatty acid is the major fatty acid of the seed. So it can be concluded from the present study that seed coat may be a good source of antioxidants and may be used for value addition of products alone or in combination with seed. Seeds being very good source of protein, minerals, ω-3, ω-6 fatty acids can be used to enrich foods. Combined utilization of Amla seed and seed coat with better hydration and water retention properties and higher P, Cr, Co, Fe, Mn and ω-3 and ω-6 fatty acids levels than fruit powder will be more fruitful.

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Chapter-3

Process optimization for extraction of total phenolic content from Amla (*Emblica officinalis*) using response surface methodology and characterization of phenolics

3.1. Introduction

Medicinal plants are sources of important therapeutic aids for alleviating human ailments. Medicinal herbs are known to contain a variety of antioxidants[1-3]. The plant of genus *Emblica officinalis* belonging to the family of Euphorbiaceae are widely distributed in most tropical and subtropical countries and have long been used in folk medicines in India and most other countries for thousands of years in treatment of broad spectrum of diseases such as disturbances of kidney and urinary bladder, intestinal infections, diabetes and hepatitis B[4-5]. The major chemical constituents of Amla are phenolics[6]. Phenolics are secondary metabolites that include a wide range of compounds including flavonols, anthocyanins, ellagic acid and its derivatives[7].

In this chapter the extraction conditions for the maximum recovery of phenolics were optimized by using response surface methodology. Further the phenolics of Amla powder were also characterized.

3.2. Materials and methods

3.2.1. Raw material

Amla of *Chakaiya* variety was procured from local market of Allahabad, India. Fresh fruits were washed and wiped. Cleaned fruits were grated and dried at 40°C. Dried Amla shreds were ground in laboratory mill to pass through 0.5 mm sieve. The collected powder was packed in air tight container and stored at refrigerated temperature for further analysis. The image of air dried Amla powder is given in **Fig 3.1.**

3.2.2. Solvents and reagents

All solvents and reagents from various suppliers used for the present work were of the highest purity. Butanol, ethyl acetate, ethanol, Folin-Ciocalteu reagent, sodium carbonate, orthophosphoric acid and HPLC grade methanol were purchased from Merck. Standards of gallic acid, catechin, syringic acid, caffeic acid, quercetin, chlorogenic acid, vanillic and *p*-coumaric acid were procured from Sigma Aldrich.

Fig 3.1 Image of hot air dried Amla powder.

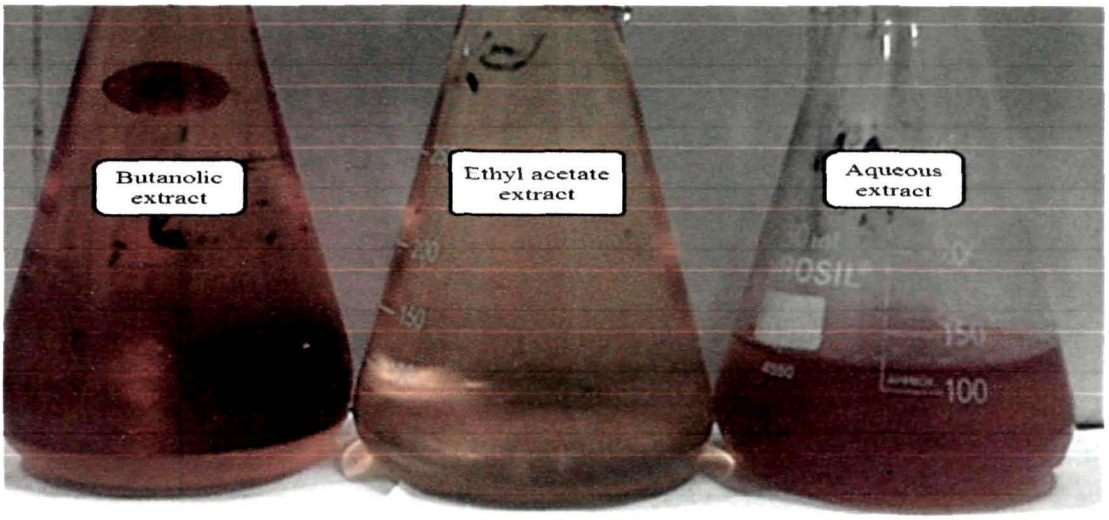
3.2.3. Optimization of extraction of total phenolics from Amla powder by response surface methodology

Response surface methodology was used to observe the effect of variables on total recovery of phenolic content and their antioxidant activities in the extract obtained from Amla powder. Temperature (x_1), ethanol (x_2) and pH (x_3) were selected as independent variables by means of preliminary experiments. The level of ethanol varied from 10-90%; temperature from 15 to 55° C and pH from 4 to 8. A central composite rotatable design was selected for designing the experimental data. A total of 20 experiments were included in the design for the extraction (**Table 3.1**). The CCRD design gave the negative values for ethanol. Ethanol concentrations of 117.27% and -14.27% were not achievable, hence, 100% ethanol was taken in place of 117.27% and distilled water was taken instead of -14.27% ethanol. Amla powder weighing 1 g was transferred into a 50 mL conical flask and 10 mL (constant volume) of an ethanol water mixture was added. **Table 3.1** presents the experiment design of response surface methodology for the extraction of Amla powder. The sample mixture was stirred in an incubator shaker at 160 rpm for 4 h at selected temperature. The sample mixture was centrifuged in refrigerated centrifuge for 10 min at 4°C temperature and 5678 *g*. The supernatant was carefully collected and stored at -20°C temperature for further analysis of total phenolic content and DPPH* radical scavenging activity. The significant terms present in the model were found by analysis of variance (ANOVA) for each response. Image of extract of Amla powder extracted under optimum conditions is shown in **Fig 3.2a**.

Table. 3.1 Central composite rotatable design with experimental values of response variables for extraction of total phenolics from Amla powder

Temperature (°C)	Ethanol (%)	pH	TPC (GAE mg/mL of extract)	% DPPH* scavenging activity/mL of extract
55.00	90.00	4.00	77.85	40.08
15.00	10.00	4.00	40.39	28.96
35.00	50.00	6.00	69.19	67.31
1.36	50.00	6.00	54.45	45.59
15.00	90.00	4.00	82.67	61.35
35.00	50.00	2.64	75.30	76.79
55.00	10.00	4.00	59.19	21.31
35.00	117.27	6.00	54.19	35.31
55.00	10.00	8.00	37.00	6.28
35.00	-17.27	6.00	17.59	8.29
15.00	90.00	8.00	35.60	36.67
68.64	50.00	6.00	95.40	26.68
35.00	50.00	6.00	69.19	67.31
35.00	50.00	9.36	27.87	15.53
55.00	90.00	8.00	52.50	21.32
35.00	50.00	6.00	69.19	67.31
35.00	50.00	6.00	69.19	67.31
35.00	50.00	6.00	83.11	67.31
15.00	10.00	8.00	25.78	9.71
35.00	50.00	6.00	69.19	67.31

(a)



(b)

Fig 3.2. Image of (a) tubes of ethanol extract, and (b) extracts partitioned with ethyl acetate, butanol, and water.

3.2.4. Purification of extracted total phenolics from Amla powder

For further purification of Amla powder the extract (extracted under optimum conditions i.e., with 78.0% ethanol:water mixture, 30.55°C temperature and 4.5 pH) of 30 g of Amla powder was centrifuged in refrigerated centrifuge at 4°C and 5687 *g* for 10 min. The residue was re-extracted under the optimum conditions and then centrifuged. The supernatants I and II were combined and evaporated in a rotary evaporator under reduced vacuum. The concentrated extract was further freeze dried (Lab Tech) under vacuum. Accurately weighed 13.35 g of ethanolic extract of Amla

powder was dissolved in 40 mL of water mixture and was partitioned with ethyl ether (150 mLx3), ethyl acetate (150 mLx3) and butanol (150 mLx3) successively. Image of extracts partitioned with ethyl acetate, butanol and water is shown in **Fig 3.2b**. All the four fractions were evaporated separately under reduced pressure; at 45°C for ethyl acetate and ethyl ether fraction and at 65°C for butanol and aqueous fraction as per the method of Liu et al. (2012)[8] and freeze dried under vacuum. Images of powder of ethyl acetate fraction, butanol and aqueous fractions are shown in **Fig 3.3**. Obtained fractions of ethyl ether, ethyl acetate, butanol and aqueous fraction were analyzed for their TPC content and % DPPH* scavenging activity. Ethyl acetate fraction was fractionated on a Sephadex G-25 (25-100 μm, Sigma Aldrich) column (450 x 25 mm) with water/methanol (100:0-100:0) as the eluent to obtain six fractions (I-VI). All six fractions were freeze dried and further evaluated for the characterization of the phenolic content through HPLC and FTIR. The images of different fractions obtained after column chromatography are given in **Fig 3.4**.

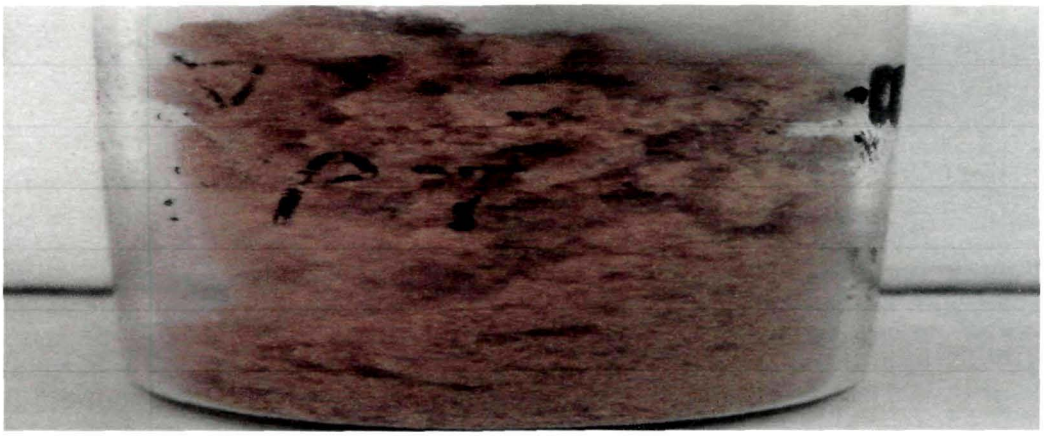
3.2.5. Fourier transform infrared spectroscopy (FTIR)

All freeze dried samples/dried samples were scanned in the range of 4000-650/cm with a resolution of 4/cm. Fourier transform infrared spectroscopic (ATR/FTIR) spectra were collected at room temperature coupling ATR accessory to an FTIR spectrometer (Perkin Elmer, Spectrum 100). The time required to obtain a complete background was 20 s. Time resolved experiments were collected by averaging of 4 scans[9].

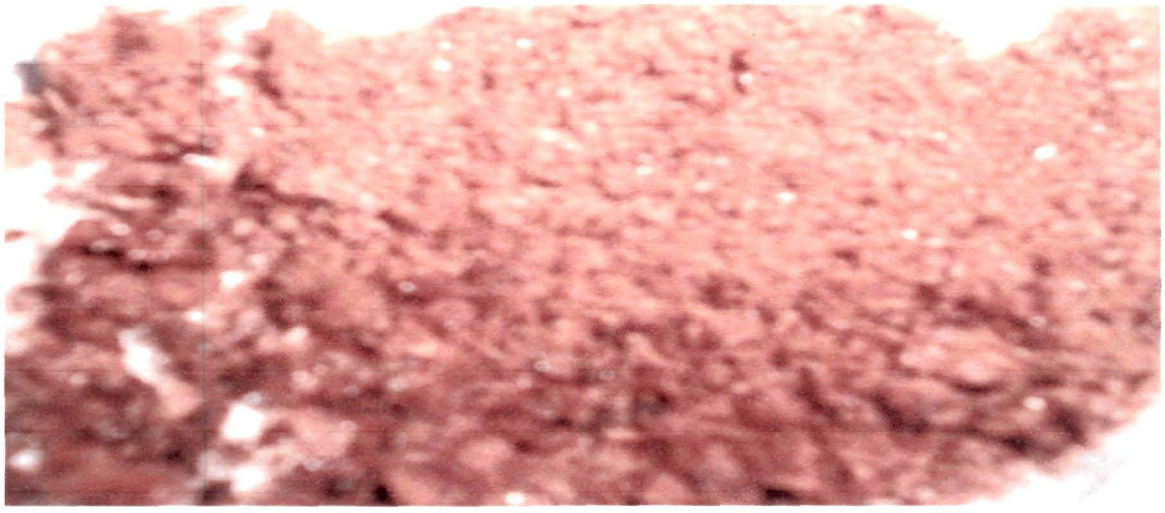
3.2.6. Total phenolic content (TPC)

Estimation of total phenolic content was performed by Folin-Ciocalteu method described by Liu et al. (2008)[10] with some modifications. Briefly, 60μL of extract, 300 μL of Folin-Ciocalteu reagent and 900 μL of 20% sodium carbonate in water were added in 4.75 mL of water. The mixture was allowed to stand for 30 min. The absorbance was carried out at 765 nm and the results were expressed as mg GAE. For estimation of total phenolic content of powder, 50 mg of powder was mixed with 5 mL methanol and the mixture was stirred for 30 min at 35°C. The stirred mixture was centrifuged and supernatant was analyzed for its polyphenolic content as described above.

(a)

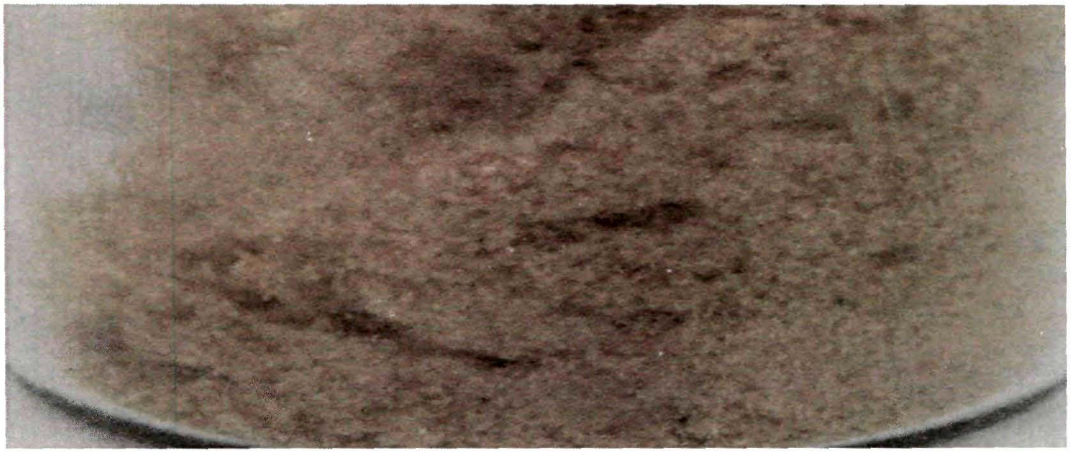


(b)



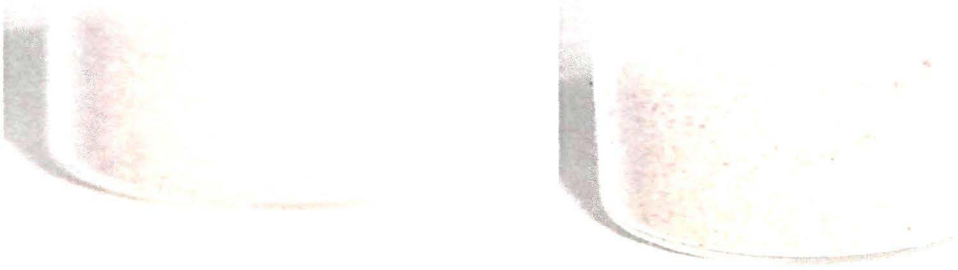
(c)

Fig. 3.3. Image of extracted Amla powder of (a) ethyl acetate extract, (b) water extract, and (c) butanol extract.



(a)

(b) **(c)**



(d) **(e)**

Fig. 3.4. Image of different fractions of Amla powder obtained after column chromatography (a) fraction I, (b) fraction II, (c) fraction III, (d) fraction IV, and (e) fraction V.

3.2.7. Analytical high performance liquid chromatography (HPLC)

For identification of phenolic content present in Amla powder, analytical HPLC (Waters, Breeze-2) was conducted by liquid chromatography fitted with a C-18, reversed phase (5 μm) column 7.2 x 300mm by the method of Seruga et al. (2012)[11] with slight modifications. In brief, orthophosphoric acid (0.1%) as solvent A and 100% methanol of HPLC grade was used as solvent B for the separation of phenolic content. The elution conditions were 0-30 min from 5% B to 80% B; 30-33 min 80% B; 33-35 min from 80 % B to 5 % B; 35-40 min at 5 % B. Flow rate, column temperature and injection volume were fixed at 0.8 mL/min, 20°C and 20 μL respectively. The detection wavelengths were 280 nm for gallic acid; 360 nm for quercetin; 280 nm for catechin; 320 nm for caffeic acid and p-coumaric acid.

3.2.8. DPPH free radical (DPPH*) scavenging activity

The DPPH* scavenging activity of extract was determined by the method of Luo et al. (2009)[12] with slight modifications. In brief, 2 mL of extract was mixed with 2 mL of methanolic solution containing 0.1mM DPPH. The mixture was shaken vigorously and then kept in dark for 30 min. The absorbance was measured at 517 nm in a double beam spectrophotometer (Evolution, Thermo scientific). The absorbance for control was measured by replacing the sample with methanol. For extracted powder 50 mg of the extracted powder was dissolved in 5 mL of methanol solution and extracted in an incubator shaker at 160 rpm at 25°C for 30 min and absorbance was taken at 517 nm.

$$\text{DPPH* scavenging activity (\%)} = \frac{\text{(1-absorbance of sample)x100}}{\text{absorbance of control}}$$

3.2.9. Statistical analysis

All experiments for extraction were carried out in duplicate however the analyses were carried out in triplicates and obtained mean values were analyzed by analysis of variance (ANOVA).

3.3. Results and discussion

3.3.1. Experiment design

The results of different runs for extraction of Amla powder are given in **Table 3.1.** *In the present investigation the temperature varied from 10° to 50°C as it is* important to keep the temperature relatively low (maximum temperature 50°C) to avoid possible degradation of the extract[13-14]. The total polyphenols was measured in the extracted material from the Amla powder by Folin-Ciocalteu method which involves

the oxidation of the phenolic compounds and reduction of the phosphomolybdic-phosphotungstic acid indicator[15]. Total polyphenols reported as gallic acid equivalent (GAE) ranged between 35.6 to 95.40 mg GAE/mL of extract and the DPPH* scavenging activity ranged from 6.28 to 76.79%. Run 6 (temperature 35°C, ethanol concentration 50% and pH 2.64) had the highest % DPPH* scavenging activity. The highest DPPH* scavenging activity at lowest pH suggested that the lower pH is effective in extracting the polyphenols which is having higher number of free hydroxyl groups. The present finding is in agreement with Remon et al. (2009)[16] that at higher pH the interaction of phenolic contents with protein and other components are high and subsequently poor recovery of phenolic content is obtained. The highest recovery of total phenolic content at 68.64°C temperature is supported by the findings of Mishra et al. (2013)[17] that at higher temperature the probable synthesis of phenolics may increase the total phenolic content. An analysis of variance was conducted to determine the significant effect of process variables on each response. Process variables, ethanol concentration, temperature and pH were found to be statistically significant at $p>0.05$ for recovery of total phenolic content and % DPPH* scavenging activity of the extract (**Table 3.2** and **Table 3.3**). For recovery of total phenolic content the model was found significant at 1% probability level (**Table. 3.2**); similarly for DPPH* scavenging activity the model was also found significant at 1% probability level (**Table. 3.3**)

3.3.2. Response surface

To visualize the combined effects of the two variables on the response, the response surface and contour plots were generated for each of the fitted models as function of two variables, while keeping other variable fixed at the central value. Fig. **3.5a** shows the effect of ethanol concentration and extraction temperature on total polyphenolic content of the extract in terms of gallic acid equivalent. Increasing the ethanol concentration increased the recovery of total phenolic content significantly ($p<0.001$). Especially, it was noted that when the ethanol concentration was increased from 10 to 80% the total phenolic per mL of the extract was increased. Higher ethanol concentration promoted better recovery of phenolics in the extract than higher temperature. The interactive effect of ethanol concentration and temperature was not found statistically significant for total recovery of phenolics (**Table. 3.2**). **Fig 3.5(b)** shows the interaction effect of ethanol concentration and temperature on DPPH* radical scavenging activity of per mL of extract. With an increase in the concentration of the

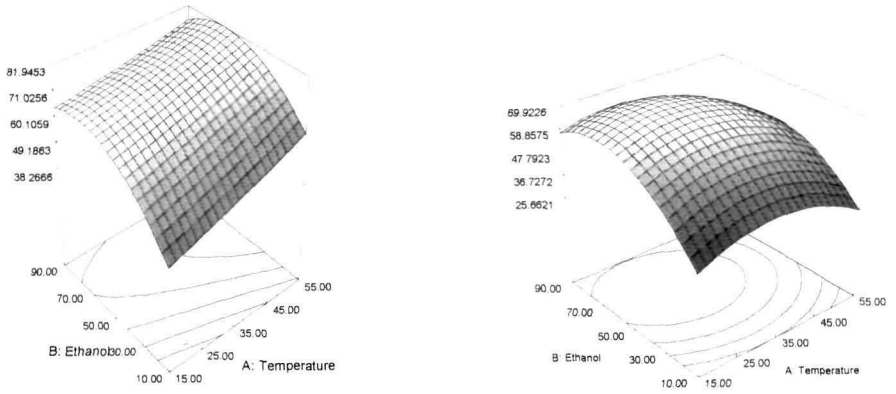
ethanol from 10 to 80%, a significant increase in the DPPH* radical scavenging activity of the extract was observed and beyond 80% concentration of the ethanol slight decrease in the DPPH* scavenging activity was observed. The DPPH* scavenging activity of the extract was increased with increase in extraction temperature up to 40° C but beyond that a reverse trend was observed (**Fig 3.5 b**). The present finding supports the findings of Yinping et al. (2011)[14] that ethanol as a solvent can be effectively used to extract the total phenolics from the plant sources. **Fig 3.6a** shows the effect of the pH and the temperature on the recovery of total phenolics from the Amla powder whereas ethanol concentration was kept constant at its middle level i.e. 50%. An increase in pH showed a negative effect on the total recovery of phenolics and the effect was more pronounced when the pH was increased beyond 6. The present finding supports the findings of Remon et al. (2009)[16]. The interactive effect of temperature and ethanol concentration was found statistically significant at 5% probability level (**Table 3.3**). **Fig 3.6b** presents the interactive effect of pH and temperature on the DPPH* scavenging activity of the extract. With increase in the pH value up to 6 a slight increase in the DPPH* scavenging activity was observed whereas further increase in the pH reduced the efficiency of the extracts to scavenge the DPPH*. The same trend could be observed while increasing the temperature. With the increase in ethanol concentration the total phenolic content significantly increased whereas as the pH value increased TPC content slightly reduced (**Fig 3.7 a**). **Fig 3.7b** depicts the 3 D plot, showing the effects of ethanol concentration and pH on % free radical scavenging activity of the extract. Increase in the concentration of ethanol at lower pH increased the % DPPH* scavenging activity of the extract significantly while high pH showed adverse effect on free radical scavenging activity of the extract because the interaction of phenolics with other constituents like protein, carbohydrate etc. increased at higher pH which hampered the extraction of phenolics and subsequently reduced the DPPH* scavenging activity of the extract. Considering all the responses obtained simultaneously, RSM was used to compromise the optimum conditions. Ethanol 78.0%, temperature 30.50°C and 4.5 pH were the optimized conditions. Triplicate samples were prepared using the optimum conditions and were analyzed for total phenolic content and % DPPH* scavenging activity of the extract; corresponding value under optimum conditions for TPC and % DPPH* scavenging activity were 73.56 mg GAE/ mL of extract and 68.45/mL of extract respectively, which shows an excellent agreement with the predicted responses and the actual value (**Table 3.4**).

Table 3.2 ANOVA table for total phenolic content

Model	Sum of squares	DF	Mean square	F Value	p>F
Model	8409.31	9	934.37	21.39	< 0.0001
A	901.69	1	901.69	20.64	0.0011
B	1599.85	1	1599.85	36.62	0.0001
C	2615.26	1	2615.26	59.86	< 0.0001
A^2	10.36	1	10.36	0.24	0.6368
B^2	2417.99	1	2417.99	55.35	< 0.0001
C^2	790.05	1	790.05	18.08	0.0017
AB	40.23	1	40.23	0.92	0.3599
AC	24.99	1	24.99	0.57	0.4669
BC	158.60	1	158.60	3.93	0.0859
R-squared	0.95				
Adj R-squared	0.90				
Pred R-squared	0.79				

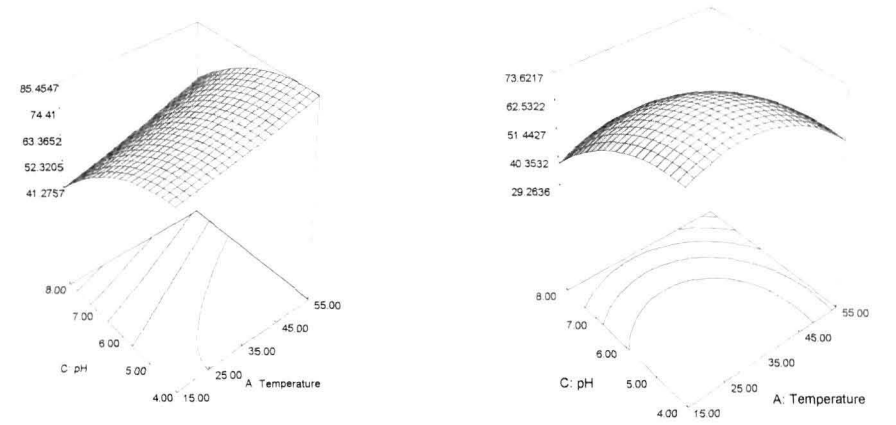
Table 3.3. ANOVA table for % DPPH* scavenging activity per mL of extract

Source	Squares	DF	Square	Value	p>F
Model	10585.67	9	1176.19	33.78	<0.0001
A	462.82	1	462.82	13.29	<0.0045
B	1406.66	1	1406.66	40.40	<0.0001
C	2392.16	1	2392.16	68.70	<0.0001
A^2	2066.16	1	2066.16	59.33	<0.0001
B^2	4185.45	1	4185.45	120.19	<0.0001
C^2	1023.99	1	1023.99	29.41	<0.0003
AB	81.54	1	81.54	3.34	<0.04
AC	12.85	1	12.85	0.37	<0.5570
BC	10.49	1	10.49	0.30	<0.0058
R-squared	0.97				
Adj R-squared	0.94				
Pred R-squared	0.82				



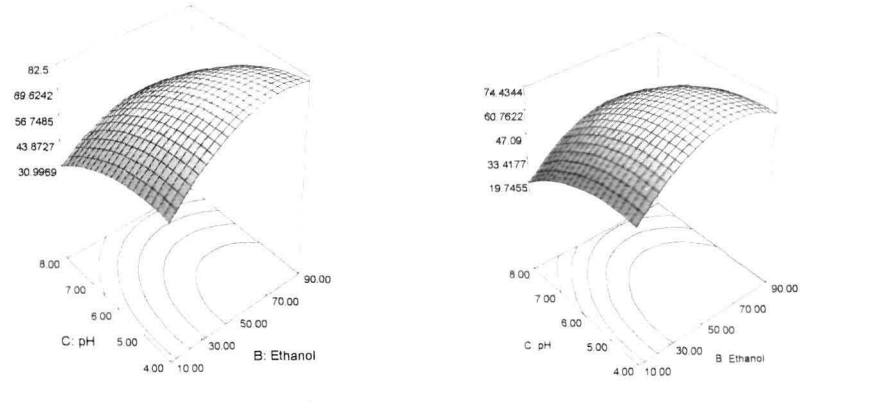
(a) (b)

Fig.3.5 Response surface and contour plots for (a) total phenolic content, and (b) DPPH* scavenging activity (at constant pH 6.0).



(a) (b)

Fig.3.6 Response surface and contour plots for (a) total phenolic content, and (b) DPPH* scavenging activity (at constant ethanol concentration 50.0).



(a) (b)

Fig.3.7 Response surface and contour plots for (a) total phenolic content, and (b) DPPH* scavenging activity (at constant temperature 35°C).

Table-3.4 Extraction of total phenols and % DPPH* scavenging activity under optimum conditions

Particular	Predicted	Optimum
% DPPH* scavenging activity /mL of extract	67.45	68.45
Total phenolic content as mg GAE/ mL of extract	71.56	73.56

3.3.3. Extraction of phenolics content

Amla powder extracted in ethanol under optimum conditions weighing 13.35 g was dissolved in 40 mL of water and then partitioned with ethyl ether, ethyl acetate and butanol. All four fractions were concentrated separately under vacuum and then lyophilized. Extracted powders were evaluated for their DPPH* scavenging activity. Diethyl ether fraction showed comparatively less DPPH* scavenging activity and therefore was discarded for further studies. The results support the findings of Roby et al. (2013)[18] that the solvents of low polarity have lower ability to extracting the phenolic compounds as compared to the polar solvents.

Table 3.5 shows the active constituents of ethyl acetate, butanol and aqueous fraction of Amla powder. The amount of total phenolic content varied in different extracts and ranged from 35.13 to 212.06 mg GAE/g (by HPLC method) and 78.98 to 685.32 mg GAE/g (by FCR method) of extracted powder (**Table 3.5).** One mg of ethyl acetate fraction showed 23.21% DPPH* scavenging activity, whereas butanol and aqueous fractions had 15.13% and 3.01% free radical scavenging activity, respectively for the same amount of the extracted powder (**Table 3.5**). The lower potency of free radical scavenging activity of aqueous fraction of Amla powder was also corroborated by the low amount of TPC as estimated by the HPLC method (**Fig 3.8a**) and FCR method (**Table 3.5**). The aqueous fraction of Amla powder had 35.13 mg/g GAE equivalent of total phenolic content by HPLC method (**Fig. 3.8 a**) and 78.98 mg/g GAE equivalent by FCR method (**Table 3.5**) whereas ethyl acetate fraction of Amla powder had 212.06 mg/g GAE equivalent by HPLC method (**Fig 3.8 b**) and 685.32mg/g GAE equivalent by FCR method (**Table 3.5**) and butanol fraction had 164.11 mg/g GAE equivalent by HPLC method and 321.03 mg/g GAE equivalent (**Table 3.5**) by FCR method.

Table 3.5 Bioactive components and % DPPH* scavenging activity of aqueous, ethyl acetate and butanol fractions of Amla powder

Particular	Aqueous fraction	Ethyl acetate fraction	Butanol fraction
% DPPH* scavenging activity /mg of powder	3.01±0.1[a]	23.21±0.2[b]	15.13±0.3[c]
TPC by FCR (mg/gm of GAE)	78.98±1.1[a]	685.32±2.5[b]	321.01±3.4[c]
TPC by HPLC (mg/g) GAE	35.13±2.9[a]	212.06±2.3[b]	164.11±2.2[c]
Gallic acid (mg/g)	ND	23.26±0.6	ND
Catechin (mg/g)	ND	28.18±0.8	ND
Caffeic acid (mg/g)	ND	1.17±0.1	ND
Quercetin (mg/g)	ND	0.50±0.1	ND
p-Coumaric acid (mg/g)	ND	0.29±0.1	ND
Syringic acid (mg/g)	ND	31.46±0.2	ND
Vanillic acid (mg/g)	ND	18.98±0.4	ND
Chlorogenic acid (mg/g)	141.73±1.4	ND	ND

ND means not detected
Observation with different superscript in same row differs significantly at 5% probability level

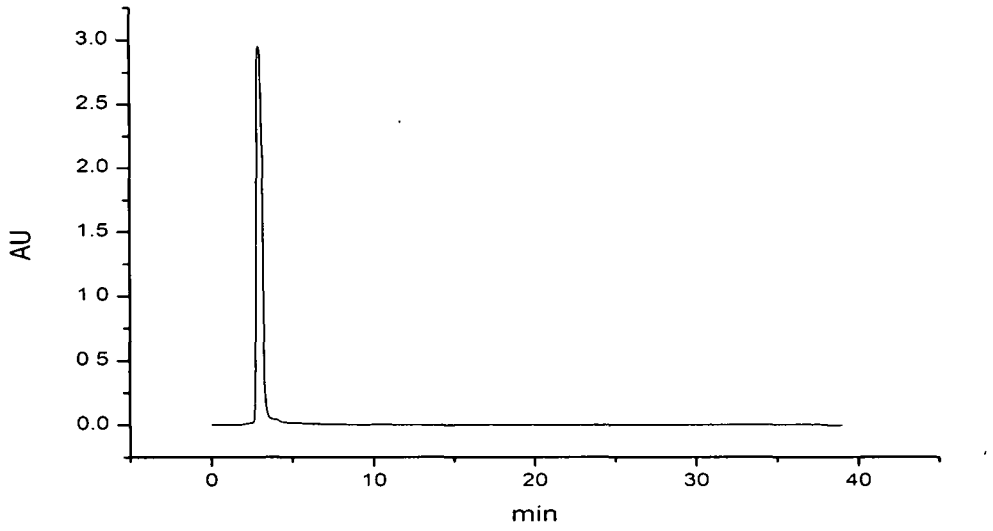
FCR method when compared with HPLC method was not found suitable for the total phenolic estimation because according to Ikawa et al. (2003)[19], FCR reagent reacts not only with phenolics but also with a number of non-phenolic reducing compounds such as tertiary aliphatic amines, tertiary amine-containing biological buffers, amino acids (tryptophan), hydroxylamine, hydrazine, certain purines, and other organic and inorganic reducing agents leading to overvaluation of the total phenolic content[19]. Vinson et al. (2002)[20] stated that different phenolics can react differently with the Folin-Ciocalteu's reagent, resulting in lower absorption that leads to an underestimation of various compounds[20]

The DPPH* scavenging activity of the butanolic extracts was comparatively lower than ethyl acetate extract fraction. One probable reason may be the high boiling point of butanol. The extract was exposed to higher temperature to remove the solvent which may adversely affect the recovery of total phenolics as well as DPPH* scavenging activity of the extracted powder. The low free radical scavenging activity and recovery of the phenolics of the Amla powder in the aqueous fraction may support the findings of Siddhuraju and Becker, (2003)[21] and Kequan and Liangli, (2006)[22] that organic solvents are better for extracting the phenolics compounds from the extracts due to their polarity and good solubility of the phenolic components from plant material[21-22]. So, during partitioning higher fractions of the phenolics went either in the ethyl acetate or butanol fraction. The present results suggested that the phenolic compounds in ethyl acetate fraction of amla powder had maximum DPPH* scavenging activity and therefore ethyl acetate fraction was subjected to further purification and identification.

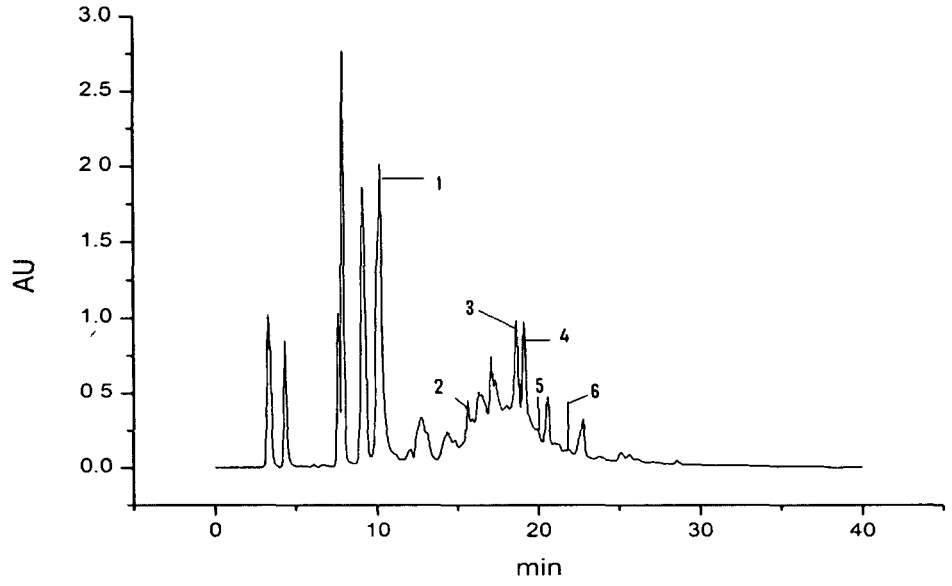
3.3.4. Identification of phenolics

3.3.4.1. Reverse phase HPLC

Amla fruit is known to contain a range of secondary metabolites such as polyphenols and flavonoids. Therefore ethyl acetate extract, butanolic extract, aqueous extract and different fractions of Amla powder obtained after Sephadex column chromatography of ethyl acetate fraction were qualitatively and quantitatively analyzed by reverse phase HPLC. HPLC chromatograms of ethyl acetate, and aqueous fractions are given in **Fig. 3.8. Table 3.5** shows that the major polyphenols contained in ethyl acetate fractions were gallic acid (42.32 mg/g), catechin (28.18 mg/g), caffeic acid (1.17 mg/g) and syringic acid (31.46 mg/g) and vanillic acid (18.98 mg/g). The main phenolic compounds in ethyl acetate fraction of Amla powder were gallic acid (42.34 mg/g) and syringic acid (18.98 mg/g) (**Table 3.5**). Quercetin was present in comparatively less quantity i.e 0.50 mg/g in ethyl acetate fraction. Presence of major portion of the phenolics in ethyl acetate fraction is in agreement with the finding of Liu et al. (2008)[10].



(a)



(b)

Fig 3.8 HPLC chromatogram of (a) aqueous fraction, and (b) ethyl acetate fraction. 1: Gallic acid, 2: catechin, 3: vanillic acid, 4 : syringic acid, 5: caffeic acid, and 6: p-coumaric acid.

Six different ethyl acetate fractions of Amla powder that were obtained from column chromatography were further lyophilized before analysis. The % yield of (in respect of initial weight of sample taken for column chromatography were 14.68% in fraction I, 48.39% in fraction II, 19.06% in fraction III, 7.14% in fraction IV, 8.36% in fraction V and 1.85% in fraction VI (**Table 3.6, F.g 3.9a**). It could be noticed from the results that the highest extract yield was obtained in fraction II followed by III, I, V, IV and VI. Inspite of having good yield, the recovery of total phenolics and % DPPH* radical scavenging activity was significantly less in fraction II than fraction III, IV and V (**Fig 3.9b**). The recovery of total phenolic content in fraction IV was highest followed by fractions IV, III, II and I. The poor DPPH* radical scavenging activity of fraction I and II may be corroborated with the TPC content of these fractions. Better recovery of phenolics with good DPPH* scavenging activity reconfirm our results that organic solvents like ethanol and methanol may be effectively used to extract the phenolics from the plant sources as compared to water or non polar solvents. **Fig 3.10 (a,b,c & d)** presents the HPLC chromatograms of fraction I, II, III and IV of Amla powder. It was found that catechin with mean value of 387.82 mg GAE/g and gallic acid 42.34 mg GAE/g were eluted in fraction III (**Table 5.6**; **Fig 3.10c**) and this may explain the potency of this fraction to scavenge the DPPH*. Vanillic acid (18.95 mg/g) and 24.58 mg/g of syringic acid was eluted in fraction IV. The high value of % DPPH* scavenging activity of fraction V may be due to the presence of some other phenolic contents which could not be identified due to the unavailability of standards of other phenolics during the present investigation.

3.3.4.2. FTIR analysis

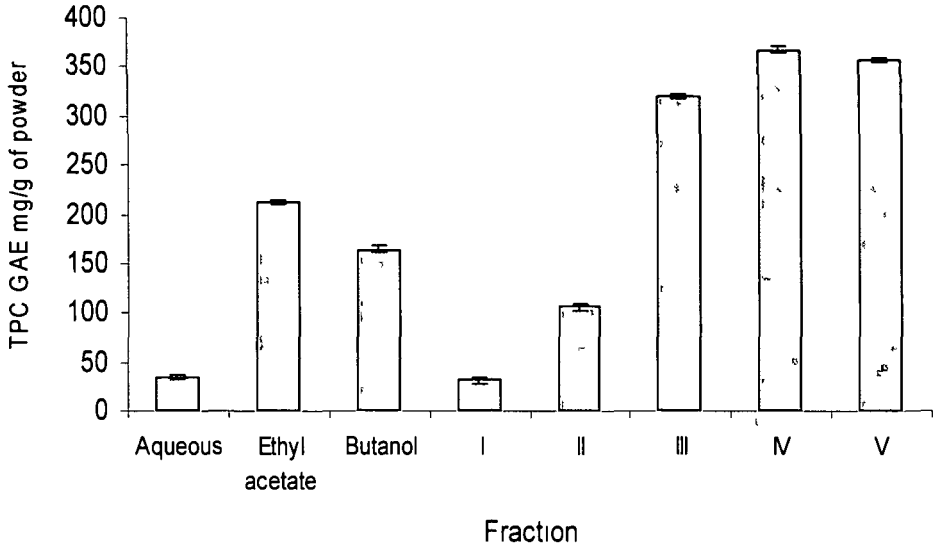
Fig 3.11 presents the FTIR spectra of different fractions of Amla powder obtained after column chromatography. It could be seen from **Fig 3.11** that intensity of peak of fraction I & II was comparatively less than fraction III & IV which may be correlated with poor recovery of phenolics in fraction I and II. Significant drop in functional groups in the position of 1300-1350 cm^{-1}, 1150-1220 cm^{-1} and 1020-1070 cm^{-1} were seen in fraction I. Noticeably the presence of gallic acid peak at 3377, 1703, 1617, 1539, 1254 cm^{-1}, 1100 cm^{-1} and 1025 cm^{-1} (Nirmaladevi et al., 2010)[3] were observed in fraction III at slightly different locations and confirms the presence of gallic acid in fraction III of Amla powder while in fraction II, peak in the rage of 1500

were absent. Presence of significant peaks in the region of 3900-3500 cm^{-1} in fractions V and VI showed the presence of monomeric alcohols and phenols. Fractions III and IV showed comparatively higher peak intensity which suggested the more polar character of bond which is further corroborated with high recovery of phenolics that are having good DPPH* radical scavenging activity. The comparatively poor recovery of phenolics in fraction VI suggested that mixture of methanol and water is more effective for extracting the phenolics as compared to 100% pure methanol.

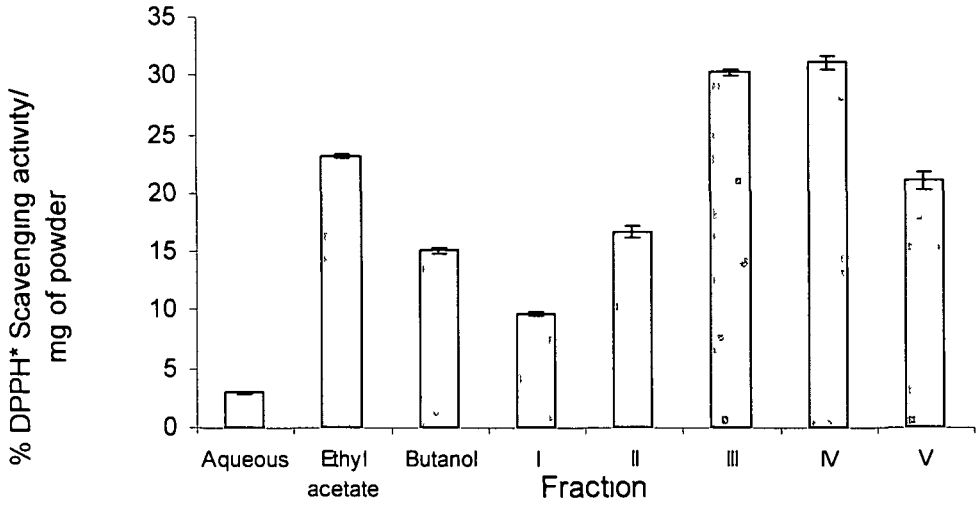
Table 3.6 DPPH* scavenging activity of different fractions obtained after column chromatography

Particular	I	II	III	IV	V	VI
% Recovery	14.68 ± 0.4^{a}	48.39 ± 1.0^{b}	19.06 ± 0.11^{c}	7.14 ± 0.2^{d}	8.36 ± 0.3^{b}	1.85 ± 0.1^{f}
TPC by HPLC (mg/g) GAE	32.19 ± 2.5^{a}	106.51 ± 2.9^{b}	320.91 ± 3.0^{c}	368.02 ± 4.2^{d}	357.86 ± 2.3^{e}	ND
% DPPH* scavenging activity/mg of powder	9.68 ± 0.2^{a}	16.68 ± 0.5^{b}	30.31 ± 3.0^{c}	31.18 ± 4.2^{d}	21.15 ± 0.8^{e}	3.96 ± 0.2^{f}
Gallic acid (mg/g)	ND	ND	42.34±0.2	ND	ND	ND
Catechin (mg/g)	ND	ND	387.82±1.3	ND	ND	ND
Syringic acid (mg/g)	ND	ND	ND	24.58±0.2	ND	ND
Vanillic acid (mg/g)	ND	ND	ND	14.53±0.2	ND	ND
p-Coumaric acid (mg/g)	ND	ND	ND	ND	18.23±0.4	ND
Quercetin (mg/g)	ND	ND	ND	0.55±0.1	ND	ND

ND means not detected
Observation with different superscript in same row differs significantly at 5% probability level

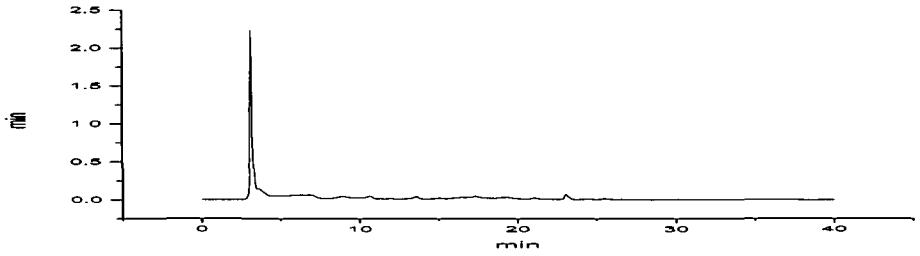


(a)

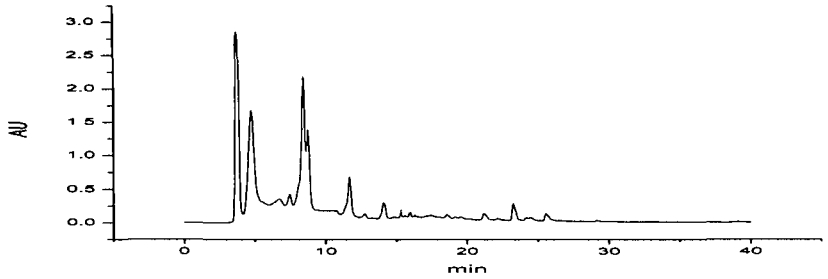


(b)

Fig 3.9 Bar diagram (a) TPC of different fractions of Amla powder, and (b) DPPH* scavenging activity.



(a)



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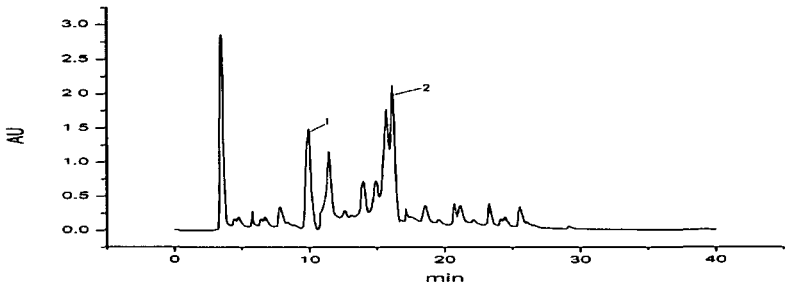

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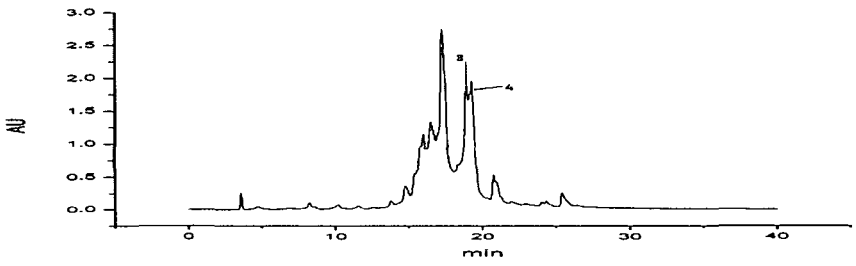

(d)

Fig 3.10 HPLC chromatogram of different fractions of Amla powder obtained after column chromatography (a) fraction I, (b) fraction II, (c) fraction III, and (d) fraction IV. (1) Gallic acid, (2) catechin, (3) vanillic acid, and (4) syringic acid.

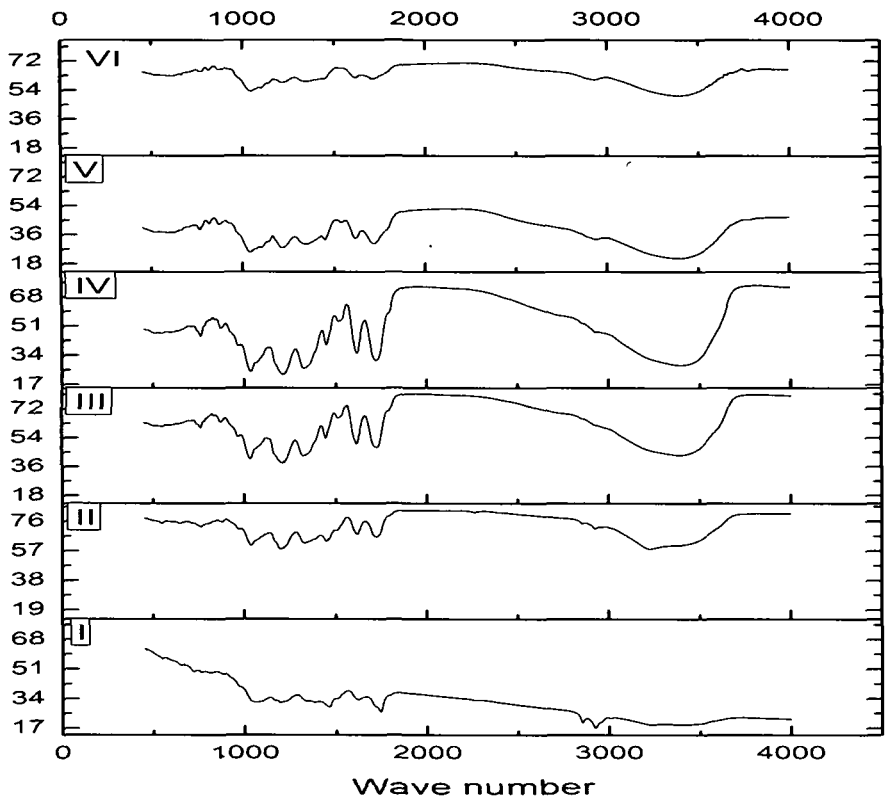

Fig 3.11 FTIR spectra of different fractions of Amla powder.

3.4. Conclusion

Conditions for extraction of total phenolic content from Amla powder were optimized by using response surface methodology. ANOVA showed that the process variables i.e. temperature, ethanol concentration and pH were found to be statistically significant for recovery of total phenolic content and % free DPPH* scavenging activity of the extract. Optimum operating conditions were found to be ethanol of 78.0%, temperature 30.50°C and 4.5 pH. At the optimum conditions, TPC content and % DPPH* scavenging activity of extract were found to be 71.56 mg/g of extract of GAE and 67.45% equivalent, respectively. Major polyphenols contained in ethyl acetate fraction were gallic acid, catechin, caffeic acid, vanillic acid and syringic acid. Quercetin was present in very less quantity only in ethyl acetate fraction. Ethyl acetate fraction extracted by methanol:water (80:20) showed the maximum DPPH* scavenging activity.

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Chapter-4

Extraction, identification and antioxidant properties of bioactive components of Amla (*Emblica officinalis*) pomace powder

4.1. Introduction

Oxidative stress, induced by oxygen radicals is one of the major causative factors of various degenerative diseases, such as cancer[1], atherosclerosis[2], gastric ulcer [3], and other conditions[4,5].

The strong antioxidant activity of the phenolics and their ability to protect cells against oxidative damage caused by free radicals are well established.[6] Due to the presence of conjugated ring structures the hydroxyl group of many phenolic compounds has the potential to function as singlet oxygen[7] and as antioxidants by scavenging superoxide anions[8] and lipid peroxy radicals[9].

Phenolics are the major chemical constituents of Amla[10] and these substances have strong antioxidant property and might contribute to the healthy affects of Amla. Several active compounds like ellagic acid, gallic acid, 1-O-galloyl-D glucose, chebulininc acid, quercetin, chebulagic acid, kaempferol, mucic acid 1,4-lactone 3-O-gallate, isocorilagin, chebulanin, mallotusinin, acylated apignin glucoside compounds etc. have been isolated from the aqueous extract of Amla[11-14]. Amla pomace is a major processing waste of Amla juice industries has never been investigated for its bioactive properties. The objective of present investigation is to characterize the polyphenols present in Amla pomace by analytical HPLC, GC-MS and FTIR.

4.2. Materials and methods

4.2.1. Raw material

Chakaiya variety of Amla was procured from local market of Allahabad, India. Grated Amla shreds were pulped in laboratory mixer and the juice was extracted by using double fold muslin cloth. Pomace left after the extraction of the juice was dried at 40°C and ground into powder in a laboratory mill by passing 0.5 mm sieve. The pomace powder was stored at refrigerated temperature for further extraction and purification of phenolics. On an average 0.58±0.02 kg of pomace powder was obtained from 5 kg of fresh Amla fruit. Image of Amla pomace powder is given in **Fig. 4.1**.

4.2.2. Solvents and reagents

Ethyl acetate, ethanol, butanol, Folin-Ciocalteu reagent, sodium carbonate, HPLC grade methanol and orthophosphoric acid were purchased from Merck.

Fig. 4.1 Image of Amla pomace powder.

Standards of caffeic acid, gallic acid, catechin, quercetin, syringic acid, chlorogenic acid, vanillic and *p*-coumaric acid, BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) and 5% DMDCS (dimethyldichlorosilane) in toluene were procured from Sigma Aldrich.

4.2.3. Purification and extraction of total phenolics from Amla pomace powder

The phenolics in 30g of Amla pomace powder was extracted (under optimised conditions of extraction from Amla powder as reported in Chapter-3) in 300 mL of ethanol (78%) by shaking in an incubator shaker for 4 h at 30.50°C and 4.5 pH. The image of ethanol extract of Amla pomace powder is shown in **Fig 4.2a.** The extract was centrifuged in refrigerated centrifuge at 4°C and 5687 *g* for 15 min and supernatant was collected. The residue was re-extracted and then further centrifuged. Both the collected supernatants were combined and evaporated in a rotary evaporator under reduced pressure followed by freeze drying under vacuum. Ethanolic extract of pomace powder (8.3 g) was dissolved in 40 mL of water and was partitioned with ethyl ether (100 mLx3), ethyl acetate (100 mLx3) and butanol (100 mLx3) successively. All four fractions were evaporated separately under reduced pressure at 45°C for ethyl acetate and ethyl ether fraction and at 65°C for butanol and aqueous

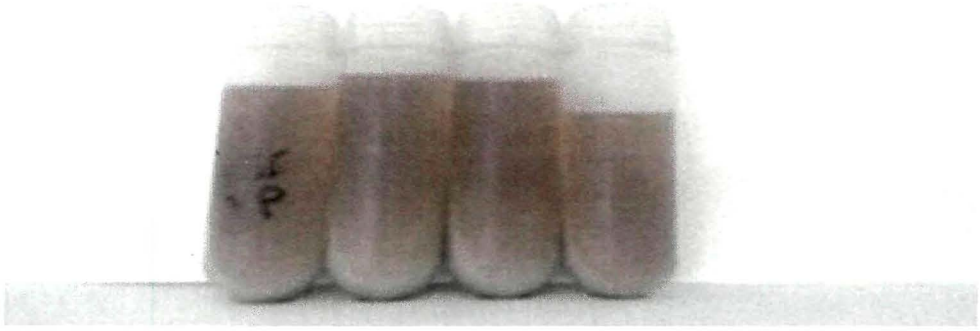
fraction as per the method of Liu et al. (2012)[15] and freeze dried under vacuum. Images of ethyl acetate extracted Amla pomace powder and butanol extracted powder are shown in **Fig 4.2b & c.** Ethyl acetate extract powder was light golden yellow color while butanolic extract powder was of light reddish yellow in color. The yields of ethyl ether, ethyl acetate, butanol and aqueous fractions were 0.25 g, 4.83 g, 3.45 g and 2.50 g, respectively. Ethyl acetate, butanol, aqueous and ethyl ether fractions were analyzed for their TPC content and %DPPH* scavenging activity. As ethyl acetate fraction showed highest DPPH* scavenging activity, it was further purified by fractionation on a Sephadex G-25 (25-100 μm, Sigma Aldrich) column (450 x 25 mm) with water/methanol (100:0-0:100) as the eluent and obtained six different fractions (I-VI). All six fractions were evaluated for characterization of the phenolics present by HPLC and FTIR.

4.2.4. Fourier Transform Infrared Spectroscopy (FTIR)

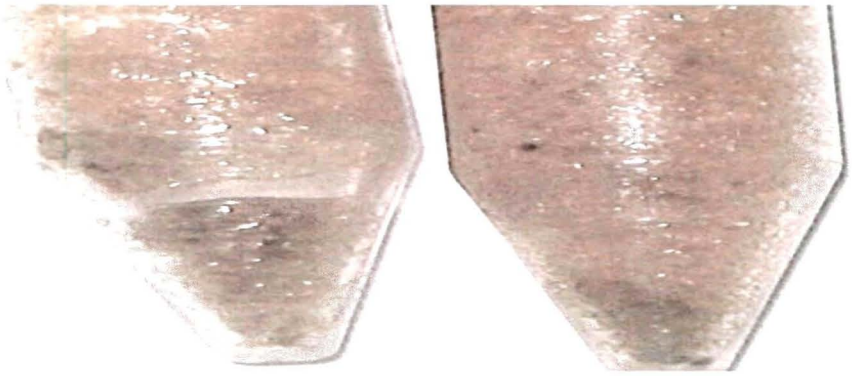
All extracted powders and different fractions obtained after column chromatography were scanned in the range of 4000-650 cm^{-1} with a resolution of 4/cm^{-1}. Attenuated total reflection/Fourier transform infrared spectroscopic (ATR/FTIR) spectra was collected at room temperature by coupling ATR accessory to an FTIR spectrometer (Perkin Elmer, Spectrum 100). The time required to complete background was 20 s. Average of 3 scans were collected[16].

4.2.5. Total phenolic content

Estimation of total phenolic content was performed by both Folin-Ciocalteu reagent (FCR) method and HPLC method. Estimation of FCR was conducted by the method of Liu et al. (2008)[17] with some modifications. Briefly, 60μL of extract (in methanol), 300 μL of Folin-Ciocalteu reagent and 900 μL of 20% sodium carbonate were added to 4.75 mL of water. The mixture was allowed to stand for 30 min at 37°C. The absorbance was noted at 765 nm and the results were expressed as mg of gallic acid equivalent (GAE). For estimation of total phenolic content of extracted powder, 50 mg of extracted powder was mixed with 5 mL of methanol and the mixture was stirred for 30 min at 25°C. The stirred mixture was centrifuged and supernatant was analyzed for its total phenolic content as described above.



(a)



(b)

(c)

Fig. 4.2 Images of extracted Amla pomace powder (a) ethanol extracted Amla pomace powder, (b) powder of Amla pomace powder partitioned with ethyl acetate, and (c) powder of Amla pomace powder partitioned with ethyl acetate.

Analytical HPLC was conducted on a Waters (Breeze-2) liquid chromatography fitted with a C-18, reversed phase (5 μm) column (7.2 x 300 mm) following the method of Seruga et al. (2012)[18] with slight modifications. In short, orthophosphoric acid (0.1%) was taken as solvent A and 100% HPLC grade methanol was used as solvent B for the separation of phenolics. The elution conditions used for the identification of phenolics were as follows: 0-30 min from 5% B to 80% B; 30-33 min 80% B; 33-35 min from 80% B to 5% B; 35-40 min at 5% B; flow rate 0.8 mL/min. Column temperature and injection volume were kept constant at 20°C and 20μL, respectively. The standards of gallic acid, quercetin, catechin, caffeic acid, syringic acid, chlorogenic acid, and p-coumaric acid were estimated at two different wavelengths, i.e. at 280 nm and 360 nm. Total area under curve at 280 nm was calculated as total phenolic content of the analyzed sample.

For identification of isolated components, 0.1 mg of the sample was dissolved in one mL of HPLC grade methanol and 20 μL of the sample was injected in HPLC with above prescribed method and obtained peaks were compared with the peak of standard components.

4.2.6. DPPH* scavenging activity

The DPPH* scavenging activity of extract of powder was determined by the method of Luo et al. (2009)[14] with slight modifications. Briefly, 50 mg of the extracted powder were dissolved in 5 mL of methanol solution and shaken in an incubator shaker at 150 rpm at 25°C for 30 min. The mixture was filtered and 2 mL of methanolic extract was mixed with 2 mL of methanolic solution containing 0.1 mM DPPH. The mixture was shaken vigorously and then left to stand for 30 min in the dark. The absorbance was taken at 517 nm. The absorbance of control was measured by replacing the sample with methanol.

$$\text{DPPH* radical scavenging activity (\%)} = \frac{\text{(1-absorbance of sample) x100}}{\text{absorbance of control}}$$

4.2.7. Assay of $ABTS^+$ scavenging activity

Antioxidant activity of extracted powder from Amla pomace was measured by the method of Cai et al. (2004)[19] and Re et al. (1999)[20]. The $ABTS^+$ cation ($ABTS^+$) solution was prepared by the reaction of 7 mM ABTS and 2.45 mM sodium persulphate, and kept for incubation at room temperature for 12 h in dark. The $ABTS^+$

solution was then diluted with methanol to obtain an absorbance in the range of 0.75±0.03 at 734 nm. For the experiment 1.5 mg of extracted powder was dissolved in 10 mL of methanol before analysis, 2mL of the dissolved sample or trolox standard (2 mL) were added to 2 mL of $ABTS^+$ solution and mixed vigorously. The reaction mixture was kept at room temperature for 6 min and the absorbance at 734 nm was immediately recorded. A standard curve was obtained using trolox standard solution at various concentrations (ranging from 0 to 20 µg/mL) in methanol. Antioxidant properties of samples were expressed as trolox equivalent antioxidant capacity (TEAC). The results were compared with the standard curve for calculation of antioxidant activity[21].

4.2.8. Preparatory HPLC

Fraction V had maximum DPPH* scavenging activity but recovery was very less hence, fraction III the second highest DPPH* radical scavenging activity was selected for further purification. Fraction III (170 mg) was further purified by reverse-phase HPLC (Waters 600E, Breeze-2, Milford, USA) on a C_{18} l-Bondàpak column (300 x 7.8 mm, flow rate = 1.5 mL/min) with methanol/water (40:60) for 40 min to yield pure compounds E1–E3.

4.2.9. UV–Vis spectrophotometric analysis

Each of isolated compounds, E1-E3 (1 mg) was dissolved in 10 mL of methanol. The sample solution was scanned from 200 to 750 nm, using a UV–Vis spectrophotometer (Evolution, Thermofisher, USA).

4.2.10. Derivatization for GC-MS

Isolated component E-1 could not be determined by HPLC hence isolated component was further derivatized for identification through GC-MS. The derivatization and identification by GC-MS was carried out by the method given by Proestos et al. (2006)[22] with slight modification. For the silylation procedure, 100 µL of BSTFA (N,O-Bis(trimethylsilyl) trifluoroacetamide) was added in 0.01 mg of sample (sensitivity of analytical balance 0.001 mg) and vortexed in screw-cap glass tubes (previously deactivated with 5% dimethyldichlorosilane in toluene, and rinsed twice with toluene and thrice with methanol), and consecutively placed in a water bath, at 80°C for 45 min. The silylated samples were injected into a GC-MS system of Perkin Elmer, model Clarus 600 gas chromatograph coupled 600C mass spectrometer

and the mass range scanned at m/z 25–500. A capillary column Elite 5 (30 m x 0.32 mm) was used for the identification. The injector and detector were set at 280°C and 290°C respectively. GC was performed in the split mode. The temperature programme was as follows : from 70° to 135°C at 10°C/min, from 135° to 220°C at 15°C/min, from 220° to 270°C at 10°C/ min and then held for 10 min. The flow rate of carrier gas (helium) was maintained at 1.9 mL/min. Identification of compound was achieved by comparing the spectral data obtained from the NIST libraries.

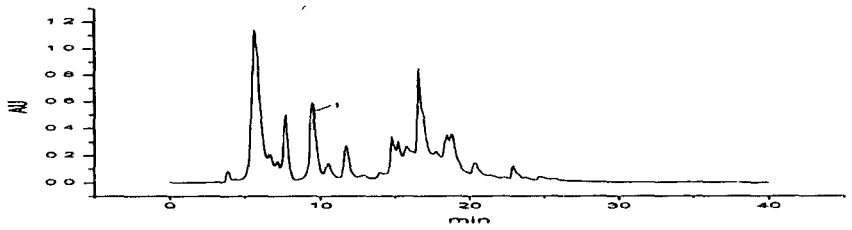
4.2.11. Statistical analysis

All experiments for extraction and purification were performed in duplicate however the analyses were performed in triplicates and the mean value and standard deviation were calculated using Excel version of 2003.

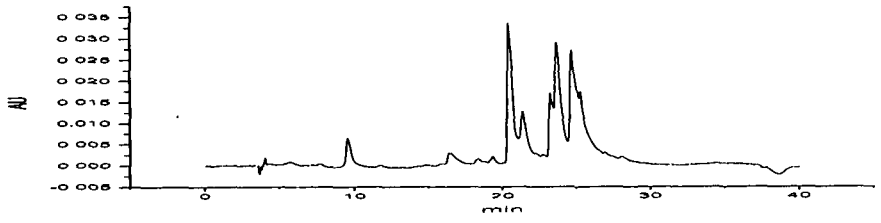
4.3. Results and discussion

4.3.1. Extraction of phenolics content

Fig 4.3 presents the HPLC chromatogram of Amla pomace powder at (a) 280 nm and (b) 360 nm which show the presence of significant amount of total phenolic content in pomace powder that were not extracted in juice. Some amount of gallic acid was found to be available in the pomace. The total phenolic content in Amla pomace powder was 95 mg/g and gallic acid was 9.77 mg/g (**Fig 4.3a).**



(a)



(b)

Fig 4.3 HPLC chromatogram of Amla pomace powder (a) at 280 nm, and (b) at 360 nm. (1) Gallic acid.

Ethanol extracted Amla pomace powder weighing 8.32 g was dissolved in 40 mL of water and then partitioned with ethyl ether, ethyl acetate and butanol. All four fractions were evaluated for their DPPH* scavenging activity. Diethyl ether fraction showed very poor scavenging activity and therefore was not taken for further studies. The present finding was in accordance with the observation of Roby et al. (2013)[23] that diethyl ether had much lower ability in extracting the phenolic compounds.

Table 4.1 presents the bioactive properties of ethyl acetate, butanol and aqueous fractions. The amount of total phenolic content varied in different extracts and ranged from 43.20 to 119.50 mg GAE/g of extracted powder (**Table 4.1**). One mg of ethyl acetate fraction showed 14.29% DPPH* scavenging activity, whereas butanol and aqueous fractions had 8.85% and 4.55 %DPPH* radical scavenging activities, respectively for the same amount of the powder (**Table 4.1**). The less potency of free radical scavenging activity of aqueous fraction of Amla pomace powder is also corroborated by the low amount of TPC as estimated by the HPLC method (**Fig 4.4a**) and FCR method (**Table 4.1**). The aqueous fraction had 43.20 mg/g GAE equivalent by HPLC method (**Fig. 4.4a**) and 121.32 mg/g GAE equivalent by FCR method (**Table 4.1**). On the other hand, ethyl acetate fraction of Amla pomace powder had 98.80 mg/g GAE equivalent by HPLC method (**Fig 4.4b**) and 389.65mg/g GAE equivalent by FCR method (**Table 4.1**) and butanol fraction had 119.50 mg/g GAE equivalent by HPLC method (**Fig 4.4c**) and 264.33 mg/g GAE equivalent by FCR method (**Table 4.1**).

When analyzed by HPLC, butanolic fraction of Amla pomace powder had significantly higher amount of phenolic content than the ethyl acetate fraction and aqueous fraction but the reverse trend was observed in case of FCR method. FCR method used for determination of total phenolic content gave considerably high value of phenolics than determined by HPLC method (**Table 4.1**). FCR method was not found suitable for the total phenolics estimation because the FCR reagent reacts not only with phenolics but also with a number of non-phenolic reducing compounds such as tertiary aliphatic amines, tertiary amine-containing biological buffers, amino acids (tryptophan), hydroxylamine, hydrazine, certain purines, and other organic and inorganic reducing agents because of which the total phenolic content is overestimated[24]. Different phenolics can also react differently with the Folin-Ciocalteu's reagent, which results in lower absorption that underestimates the concentration of various compounds[25].

So, further modification and validation of FCR method is required to correct the error as it is widely used in routine analysis for the estimation of total phenolic contents.

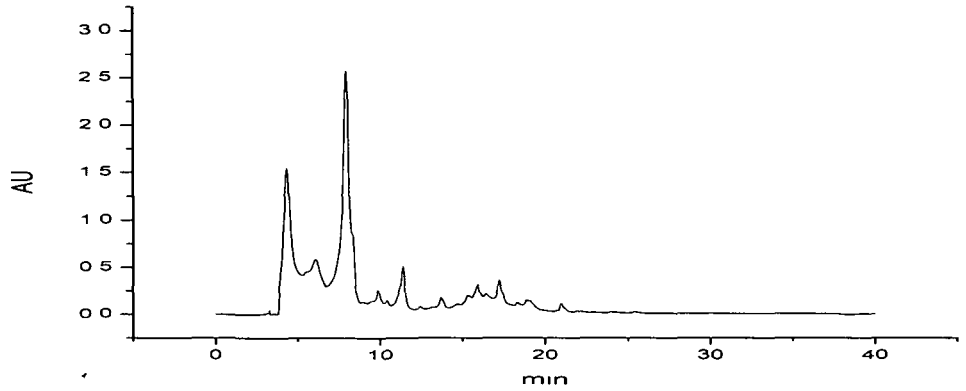
Inspite of having high amount of total phenolic content as determined by HPLC, the DPPH* scavenging activity of the butanolic extracts was comparatively lower than ethyl acetate extract fraction. One probable reason may be the high boiling point of butanol. The extract was exposed to higher temperature to remove the solvent which may adversely affect the availability of functional groups to form complexes with phosphomolybdate and hence butanolic extract showed less % DPPH* radical scavenging activity.

Table 4.1 Bioactive component and DPPH* scavenging activity of aqueous, ethyl acetate, and butanol fractions of Amla pomace powder

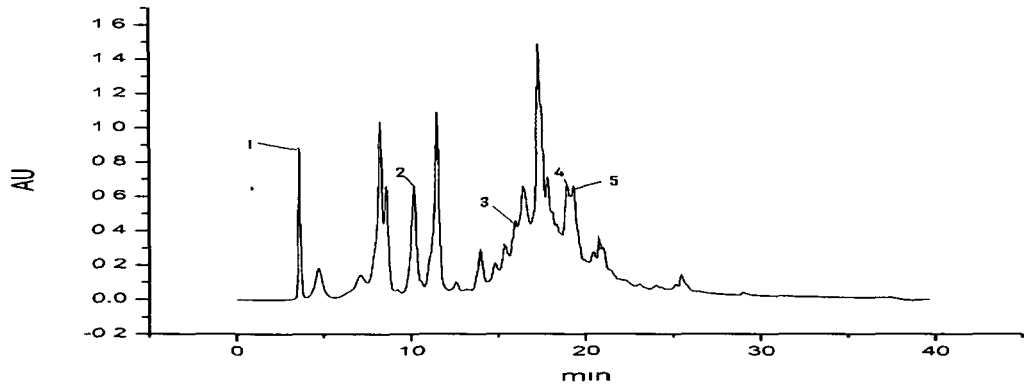
Particular	Aqueous fraction	Ethyl acetate fraction	Butanol fraction
% Yield	22.66 ± 1.6^{a}	43.78 ± 1.5^{b}	31.27 ± 1.8^{c}
% DPPH* scavenging activity/mg of powder	4.55 ± 0.3^{a}	14.29 ± 0.1^{b}	8.85 ± 0.2^{c}
TPC by FCR (mg/g of GAE)	121.32 ± 2.7^{a}	389.65 ± 2.2^{b}	264.33 ± 2.0^{c}
TPC by HPLC (mg/g) GAE	43.20 ± 0.4^{a}	98.80 ± 0.2^{b}	119.50 ± 0.3^{c}
Gallic acid (mg/g)	ND	8.81 ± 0.3	ND
Catechin (mg/g)	ND	9.70 ± 0.2	ND
Caffeic acid (mg/g)	ND	0.52 ± 0.1	ND
Quercetin (mg/g)	ND	ND	ND
p-Coumaric acid (mg/g)	ND	ND	ND
Syringic acid (mg/g)	ND	3.60 ± 0.1	15.60 ± 0.2
Vanillic acid (mg/g)	ND	ND	3.74 ± 0.2
Chlorogenic acid (mg/g)	ND	ND	ND

Mean ± standard deviation

Observation with different superscript in the same row differs significantly at 5% probability level



(a)



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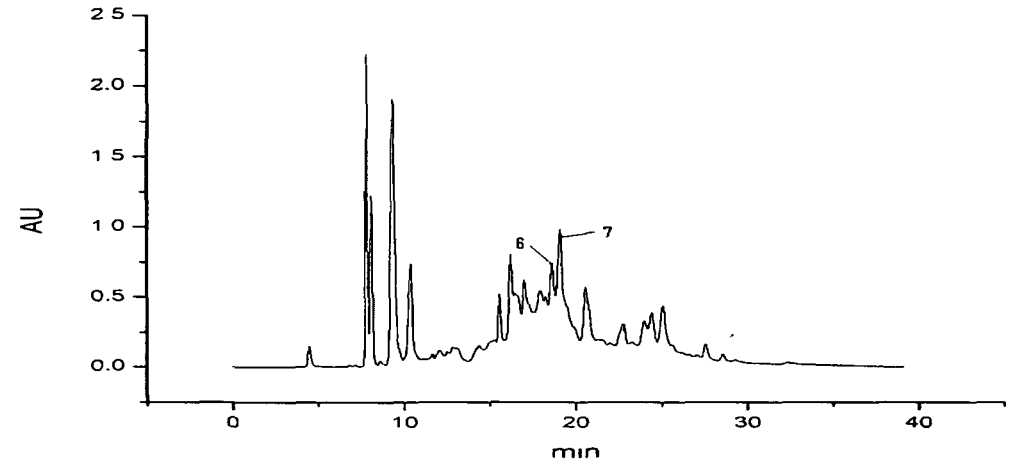


(c)

Fig. 4.4. HPLC chromatograms of (a) aqueous fraction, (b) ethyl acetate fraction, and (c) butanol fraction. (1) Unidentified, (2) gallic acid, (3) catechin, (4) syringic acid, (5) caffeic acid, (6) vanillic acid, and (7) syringic acid.

The low free radical scavenging activity of the aqueous extract of Amla pomace powder may be because of the phenolics left behind in the pomace after juice extraction were either present in bound form or had better affinity to solvents like ethyl acetate and butanol than water. So, during partitioning higher fractions of the phenolics went either in the ethyl acetate or butanol fraction. The present results suggested that the phenolic compounds in ethyl acetate fraction had maximum DPPH* radical scavenging activity and therefore ethyl acetate fraction was subjected to further purification and identification.

4.3.2. Identification of phenolics

4.3.2.1. Reverse phase high performance liquid chromatography (HPLC)

Amla fruit is known to contain a range of secondary metabolites such as polyphenols and flavonoids but the availability of phenolic constituents in Amla pomace powder have never been investigated. Therefore ethyl acetate extract, butanolic extract, aqueous extract and the different fractions of Amla pomace powder obtained after column chromatography of ethyl acetate fraction were qualitatively and quantitatively analyzed by reverse phase HPLC. The bioactive properties of HPLC chromatograms of above three fractions are given in **Fig 4.4**. Ethyl acetate fraction gave maximum recovery i.e. 43.78% (of initial amount of ethanol extracted powder taken for the partition) whereas diethyl fraction powder gave the lowest recovery. The present findings were in agreement with the findings of Liu et al. (2008)[17]. **Table 4.1** shows that the major polyphenols contained in ethyl acetate fractions were gallic acid (8.81 mg/g, rt 10.09), catechin (9.70 mg/g, rt 15.94 min), caffeic acid (rt 19.48 min, 0.52 mg/g) and syringic acid (3.6 mg/g, rt 19.01). Butanol fraction had syringic acid (15.6 mg/g, rt 19.01,) and vanillic acid (3.74 mg/g, rt 18.51). The main phenolic compounds in ethyl acetate fraction of pomace powder were catechin (9.70 mg/g) and gallic acid (8.81 mg/g) (**Table 4.1**). p-Coumaric acid and quercetin could not be detected in any of the three fractions tested. Presence of major portion of the phenolics in ethyl acetate fraction confirms the finding of Liu et al. (2008)[17] that the compounds with relatively high antioxidant activity and phenolics are present in ethyl acetate fraction. Six different ethyl acetate fractions obtained after column chromatography were further lyophilized before analysis. The % recovery of all six

fractions (in respect of initial weight of sample taken for Sephadex column chromatography) i.e. I, II, III, IV, V and VI were 20.59%, 40.37%, 27.04%, 12.20%, 1.48% and 1.09% respectively (**Table 4.2**). It could be noticed from the results that the highest extract yield was obtained in fraction II followed by III, I and IV, V and VI. Inspite of having good recovery of total phenolics in fraction II, the DPPH* scavenging activity of fraction II was significantly less than fraction III, IV and V suggesting the low availability of compounds having free radical scavenging activity low in that fraction (**Fig 4.5**). Fraction V showed exceptionally good free radical scavenging activity with a mean value of 149.86% but due to only 1.48% of recovery of powder it may not be economical for the extraction of the phenolics. **Fig 4.6** presents the HPLC chromatograms of fraction III, IV and V of pomace powder. It was found that catechin with 424.92 mg GAE/g and gallic acid with 17.15 mg GAE/g concentration were eluted in fraction III (**Table 4.2**; **Fig 4.6b**) and this may explain the potency of this fraction to scavenge the DPPH*. Syringic acid (18.95 mg/g) and caffeic acid (10.19 mg/g) (**Fig 4.6c**) were eluted in fraction IV (**Table 4.2**) whereas 4.24 mg/g of syringic acid was eluted in fraction V. The high value of % DPPH* scavenging activity of fraction V indicates the presence of some phenolic compounds which have not been identified. Since the extraction and purification were carried out in duplicate hence further investigation may be carried out in future to reconfirm our findings.

4.3.2.2. FTIR analysis

Fig 4.7 presents the FTIR spectra of different fractions of Amla pomace powder. It could be seen from **Fig 4.7** that peak intensity of aqueous fraction was less than ethyl acetate and butanol fractions. Significant drop in functional groups were also observed in aqueous fraction which may explain the low DPPH* scavenging activity of the aqueous fraction Noticeably the presence of gallic acid peak at 3377, 1703, 1617, 1539 and 1254 cm^{-1}, 1100 cm^{-1} and 1025 cm^{-1} (Nirmaladevi et al., 2010)[26] were observed in ethyl acetate fraction at slightly different locations and confirms the presence of gallic acid in ethyl acetate fraction of Amla pomace powder. In aqueous and butanolic fractions the significant drop in peaks below 750cm^{-1} region were observed. Presence of peak below 750cm^{-1} region in ethyl acetate fraction suggested the presence of disubstituted, meta, para or ortho hydrocarbons in ethyl acetate fraction. Significant drop in functional groups of fraction I was observed from **Fig 4.7** whereas fraction V showed comparatively higher peak intensity of the

functional groups which is substantiated by the potent ability of the fraction V to scavenge DPPH*. FTIR spectra also suggests the presence of some other phenolic compounds not yet detected in the present study. However, as the recovery of fraction V is very low it can not be recommended for further purification or extraction.

Table 4.2 Characterization of phenolics and DPPH* scavenging activity of different fractions obtained after column chromatography

Particular	I	II	III	IV	V	VI
% Recovery	20.59±2.2[a]	40.37±2.3[b]	27.04±2.5[c]	12.20±1.1[d]	1.48±0.8[e]	1.09±0.3[f]
% DPPH* scavenging activity/mg of powder	32.77±0.5[a]	17.27±0.3[b]	44.61±0.8[c]	29.91±1.1[d]	149.86±0.9[e]	13.1±0.7[f]
Gallic acid (mg/g)	ND	ND	17.15±1.1	ND	ND	ND
Catechin (mg/g)	ND	ND	424.92±1.4	ND	ND	ND
Syringic acid (mg/g)	ND	ND	ND	18.95±1.0	4.34±0.1	ND
Caffeic acid (mg/g)	ND	ND	ND	10.19± 0.8		ND

Mean ± standard deviation

Observation with different superscript in same row differs significantly at 5% probability level

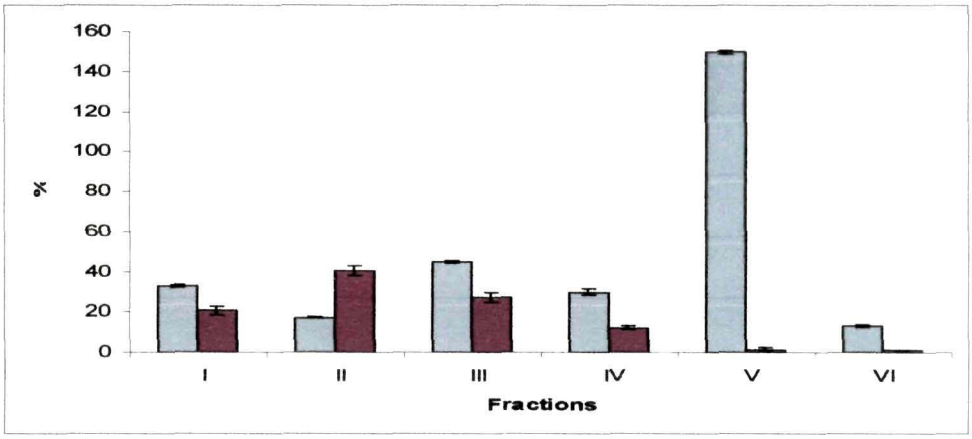
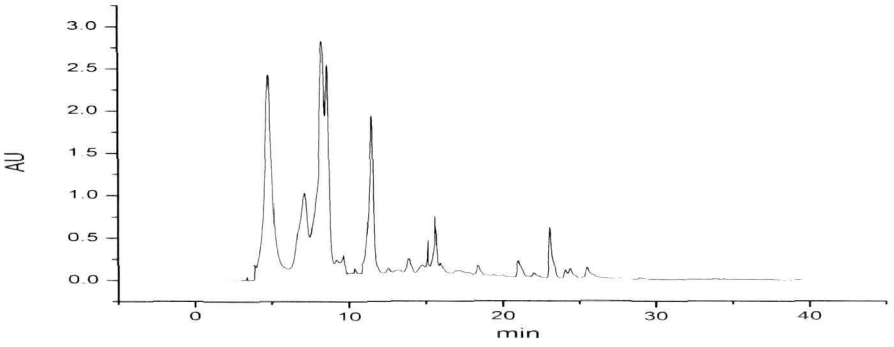
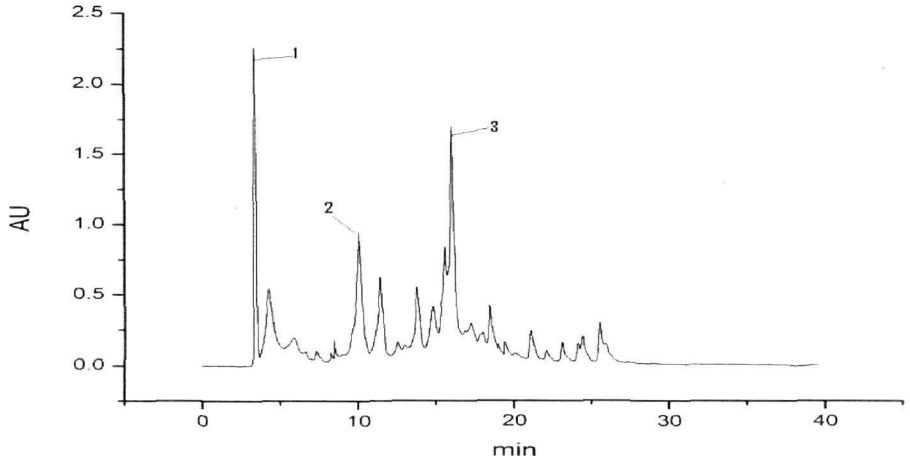


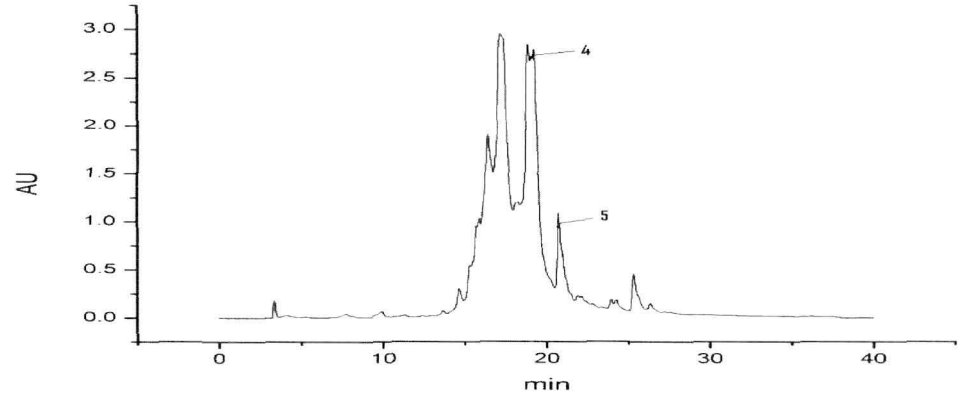
Fig. 4.5 % Yield and DPPH* scavenging activity of different fractions of Amla pomace powder.



(a)



(b)



(c)

Fig. 4.6 HPLC chromatograms of (a) fraction II, (b) fraction III, and (c) fraction IV. (1) Unidentified, (2) gallic acid, (3) catechin, (4) syringic acid, and (5) caffeic acid.

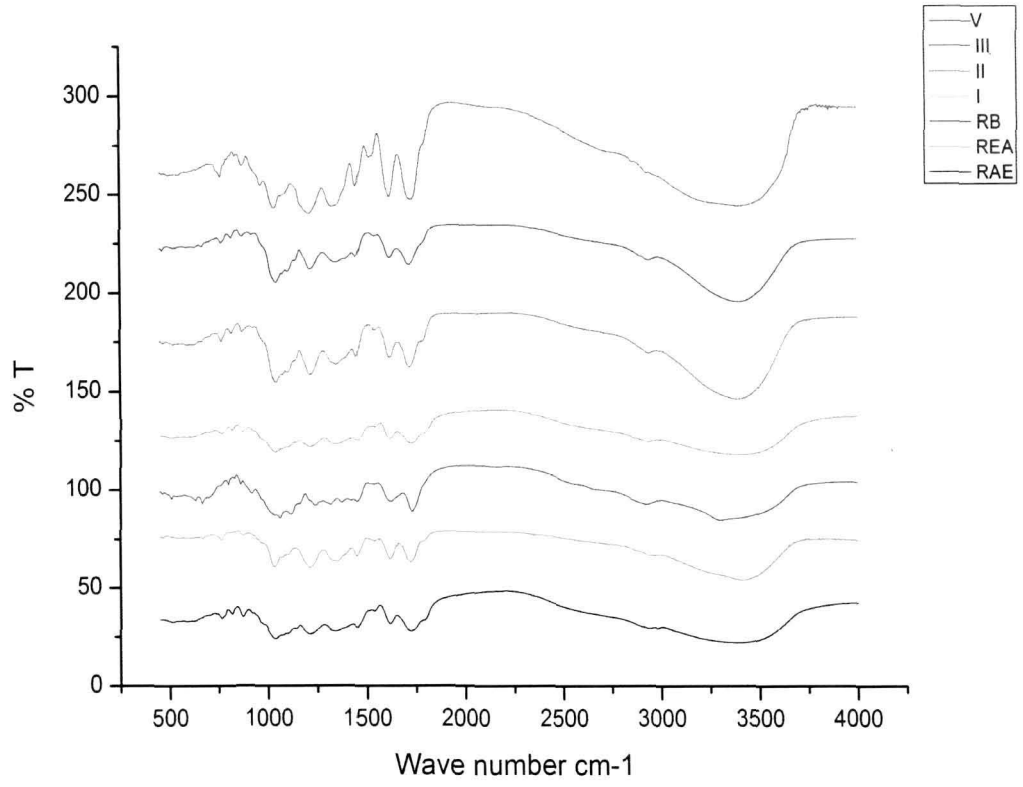


Fig. 4.7 FTIR spectra of different fractions of Amla pomace powder. REA: aqueous extract, REA: ethyl acetate extract, RB: butanolic extract, I: fraction I, II: fraction II, III: fraction III, and V: fraction V

4.3.2.3. Detection of isolated compounds

As seen from **Fig 4.5**, fraction V showed maximum DPPH* scavenging activity but its yield was low. On the other hand, fraction II with high yield showed poor DPPH* scavenging activity. Hence fraction III with moderate yield and moderate DPPH* scavenging activity was further purified by preparatory HPLC and three different components were isolated.

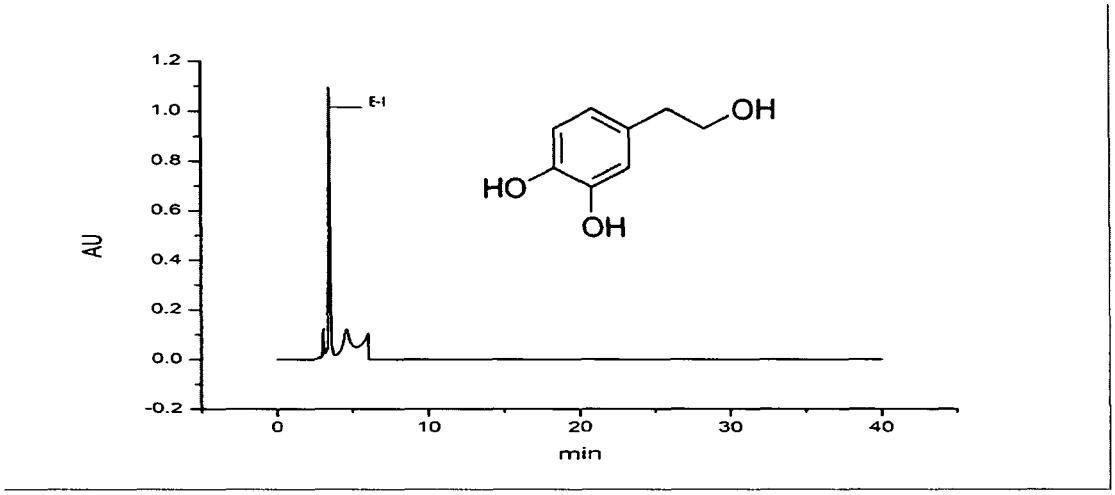
Isolated components, i.e E-1, E-2 and E-3 had maximum absorbance at 275 nm, 275 nm and 277 nm, respectively. Representative chromatograms of isolated components are shown in Fig **4.8**. By comparing HPLC chromatograms of E-2 and E-3 with standards it was observed that isolated component E-2 was gallic acid whereas E-3 was catechin. Oliver et al. (2010)[27] had also reported the maximum absorbance of catechin is 278 nm.

The present finding also supported the findings of Luo et al. (2009)[14] for the presence of gallic acid in Amla. Due to nonavailability of the standards, the

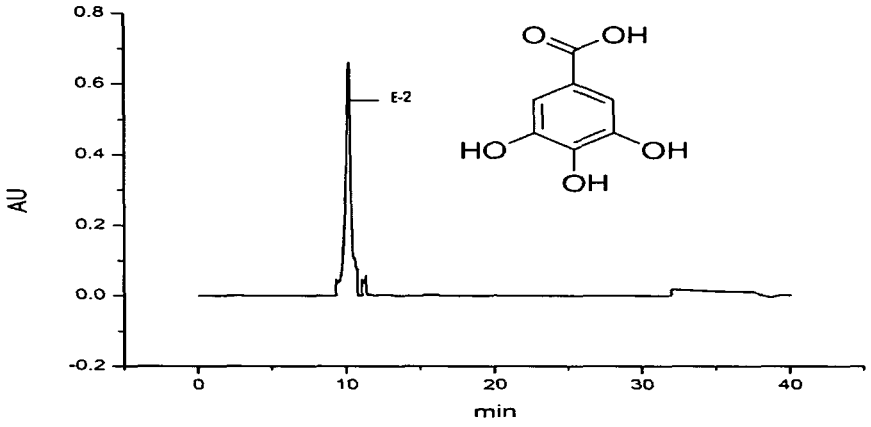
component of E-1 could not be identified by HPLC. E-1 was identified through silylation process, which is an ideal procedure for the GC analysis of non-volatile and thermolabile compounds. By comparing the mass spectra of E-1 by NIST library the compound was detected as hydroxytyrosol. The molecular weight (m/Z) of TMS derivative of hydroxyl tyrosol was 370 and major characteristic fragments of isolated compound were obtained at 73, 267, 193 and 179. The present finding was also in accordance with Proestos et al (2006)[22].

4.3.3. DPPH* scavenging activity

Ethyl acetate fraction showed significantly less total phenolic content than the butanolic fraction of the Amla pomace powder. Probably during evaporation of butanol solvent, some other phenolics may have been synthesized from the secondary metabolites which may increase the total phenolic content of the butanolic fraction. However inspite of having good amount of total phenolic content, the DPPH* scavenging activity was significantly less than the ethyl acetate fraction. Our present findings contradicted Piljac et al. (2007)[28] and Seruga et al. (2011)[18] for their observation that the DPPH* scavenging activity is directly proportional to the total phenolic content. The antioxidant activity and DPPH* scavenging activity not only depends on the total phenolic content but also depends on the position and availability of the free hydroxyl groups to show antioxidant activity or to scavenge the free radicals. Exposure of high temperature to evaporate the solvents from butanolic extracts adversely affects the availability of hydroxyl groups that reduces the functional properties of the phenolic compound present and hence was not found suitable for the extraction of bioactive components. Fraction V (**Table 4.2**) showed the highest DPPH* scavenging activity among all fractions tested but due to comparatively poor recovery of phenolics could not be recommended for the extraction of bioactive components. It can also be concluded from **Fig 4.7** that FTIR spectra of fraction V had comparatively higher intensity of functional peaks as compared to others. Luo and Foo (2001)[29] explained that such hydroxy-phenolic compounds can donate hydrogen atoms to DPPH* and can scavenge it. Fraction III with significant recovery of the phenolics and compatible DPPH* free radical scavenging activity may be suggested for the further extraction and purification of the components.



(a)



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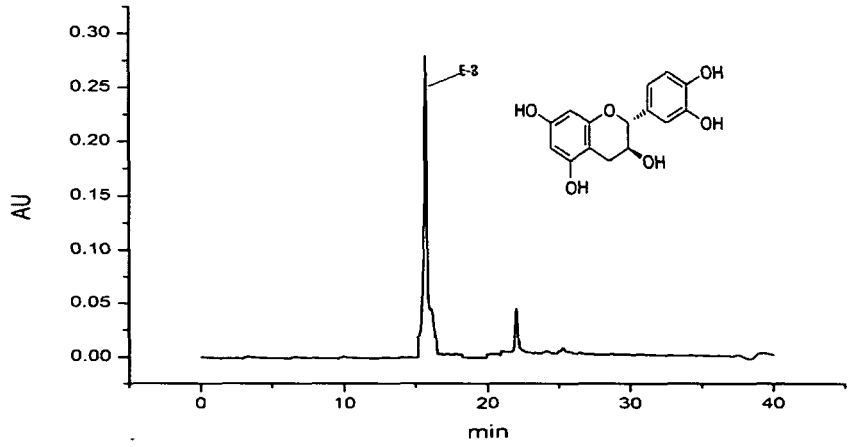

(c)

Fig. 4.8 HPLC chromatogram of (a) E-1 (hydroxytyrosol), (b) E-2 (gallic acid), and (c) E-3 (catechin) at 280 nm.

The DPPH* scavenging activities of compound E1-E3, and control are shown in **Fig 4.9**. It could be observed that all three compounds exhibited strong DPPH* scavenging activity in a dose dependent manner. The DPPH* scavenging activity was in decreasing order; gallic acid (E-2) > catechin (E-3) > hydroxytyrosol (E-1)> vitamin C > β carotene. As shown in **Fig 4.9**, the DPPH* scavenging activities of the isolated compounds were significantly higher than those of vitamin C and β-carotene.

Fig 4.10 presents the $ABTS^+$ scavenging activities of the isolated compounds and standards of vitamin C and β-carotene. Catechin, gallic acid and hydroxytyrosol exhibited strong antioxidant activities and these results were consistent with the report of Giedrius et al (2004)[30] and Luo et al. (2009)[14]. Moreover, catechin, gallic acid and hydroxytyrosol exhibited higher antioxidant activities than the vitamin C and β-carotene standards. The $ABTS^+$ scavenging activity in decreasing order was as follows: catechin > gallic acid > hydroxytyrosol > vitamin C > β carotene. This result confirmed the idea that a high scavenging activity can be attributed to the availability of their free hydroxyl groups, and the phenolic hydroxyl structural group in benzene ring contributes much to the free radical scavenging activity[31].

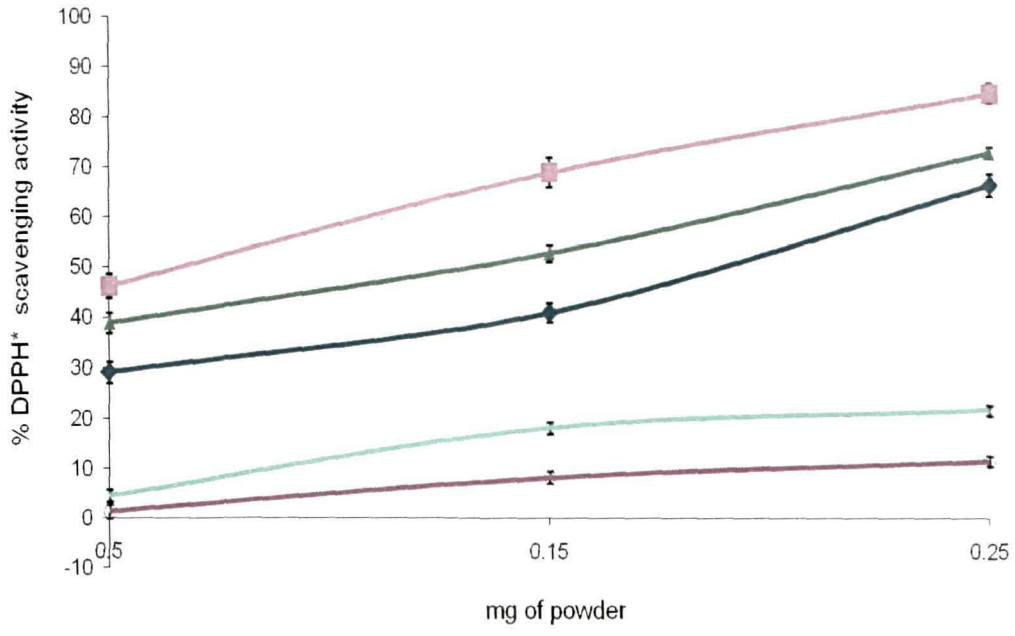

Fig. 4.9 DPPH* scavenging activity of isolated components (E-1 to E-3), standard vitamin C and β carotene.

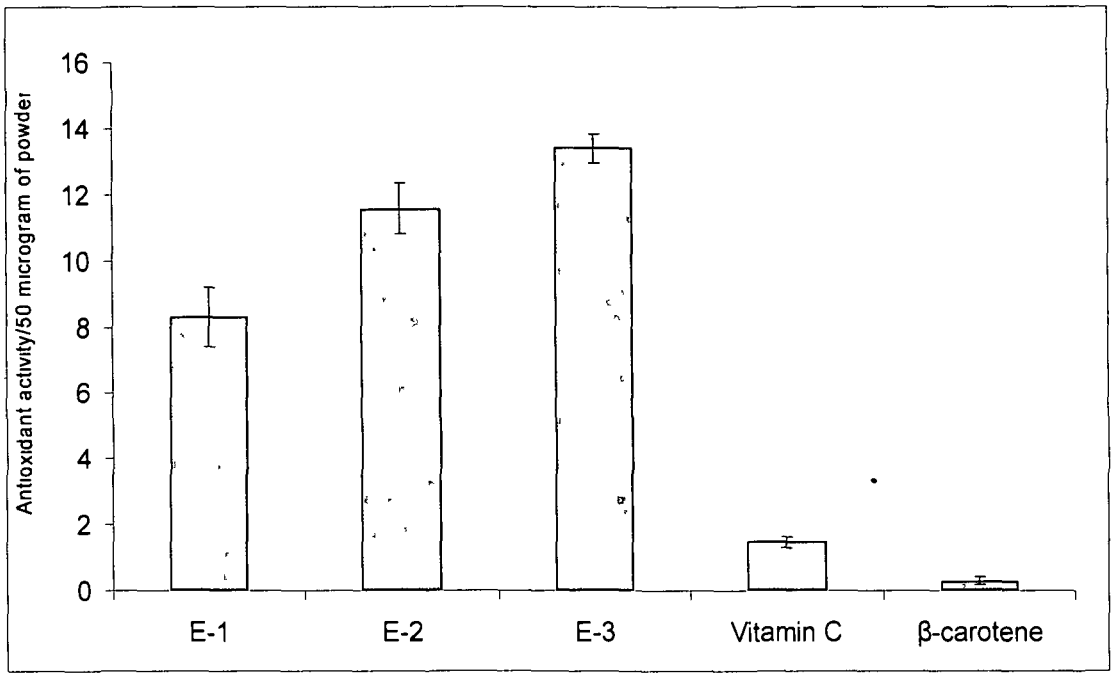

Fig. 4.10 ABTS$^+$ scavenging activity of isolated compounds (E-1 to E-3), standard vitamin C and standard β-carotene.

4.4. Conclusion

Amla pomace powder has never been investigated for optimization of extraction of total phenolic content. Major polyphenols contained in ethyl acetate fractions of pomace powder were gallic acid, catechin, caffeic acid and syringic acid. Ethyl acetate fraction extracted by methanol:water (80:20) showed the maximum DPPH* scavenging activity but due to very poor recovery it was not found suitable for the further purification. Catechin was the dominating acid present in fraction III. Isolated catechin, gallic acid and hydroxytyrosol exhibited stronger DPPH* free radical scavenging activity as compared to vitamin C and β carotene. Present study revealed that most of the major fractions of the crucial bioactive components of Amla are present in the pomace powder which can be exploited after extraction of juice from the fruit.

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Chapter-5

Partial extraction and identification of total phenolic content of Amla (*Emblica officinalis*) seed coat powder

5.1. Introduction

In recent decades consumer over the world are being attracted towards natural antioxidants due to their lower toxicity than synthetic antioxidants[1]. Many medicinal herbs having good antioxidant capacity have been employed as the source of natural antioxidants[2]. The Amla fruits are highly nutritious and contain phytochemicals of great interest to researchers, including vitamin C, tannins, phyllemblic acid, phyllemblin, rutin, curcuminodes and phenolic acids. The active constituents of Amla responsible for antioxidant activity have been investigated but the seed coat (a major processing waste) of Amla based industries have never been investigated for its bioactive properties. The objective of present investigation was to optimize the extraction condition of total phenolic content of seed coat by response surface methodology and characterize the available phenols by analytical HPLC and FTIR.

5.2. Materials and methods

5.2.1. Raw material

Amla of *Chakaiya* variety was procured from local market of Allahabad, India. Seeds were separated manually from Amla fruits and dried at 40°C temperature. The seeds on drying broke along the ridges. The seed coat was separated from the brown seed. The seed coat was ground in a laboratory mill by passing through 0.5 mm screen sieve. The powder was stored at refrigerated temperature for further extraction and characterization of phenolics.

5.2.2. Solvents and reagents

All solvents and reagents used were of the highest purity. Ethyl acetate, ethanol, butanol, Folin-Ciocalteu reagent and sodium carbonate were purchased from Merck.

5.2.3. Optimization of extraction of total phenolics from seed coat powder by response surface methodology

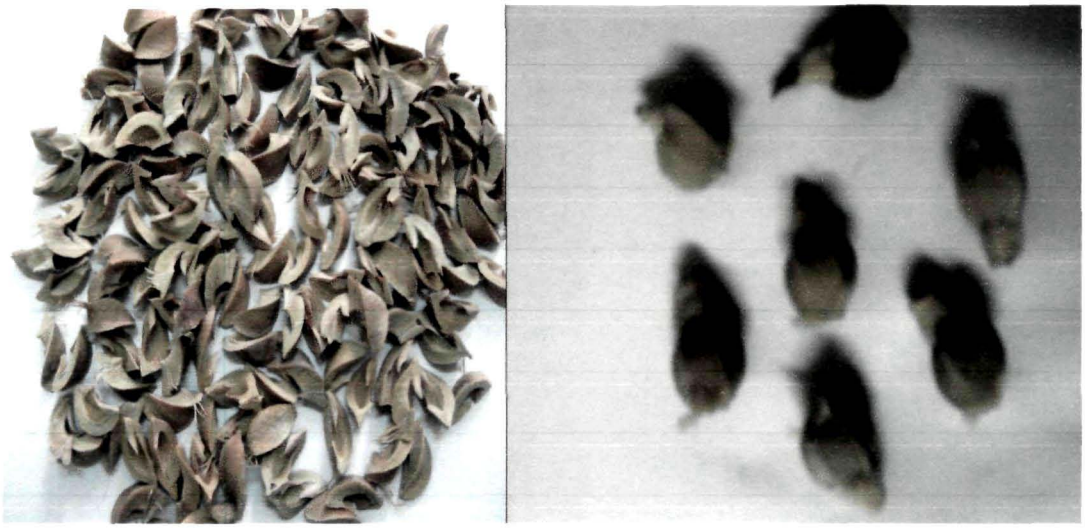
Response surface methodology was used to estimate the effect of variables on total recovery of polyphenols and their DPPH* scavenging activity in the extract obtained from Amla seed coat. Ethanol concentration (x_1), temperature (x_2) and pH

(x_3) were selected as independent variables by means of preliminary experiments. Level of ethanol varied from 10-90%; temperature from 15° to 55° C and pH varied from 4 to 8. A central composite rotatable design was used for designing the experimental data. The design included 20 experiments for the extraction. Seed coat powder weighing 1 g was transferred in to a 50 mL conical flask and 10 mL (constant volume) of an ethanol water mixture was added. The experimental design is given in **Table 5.1.** Ethanol concentrations of 117.27% and -14.27% were not achievable, hence, 100% ethanol was taken in place of 117.27% and distilled water was taken instead of -14.27% ethanol. The sample was stirred in an incubator shaker at 160 rpm at selected temperature for 4 h. The mixture was centrifuged in refrigerated centrifuge at 4°C temperature and 8000 rpm for 10 min. Supernatant was collected and stored at -20°C temperature for further analysis. The significant terms in the model were found by analysis of variance (ANOVA) for each response. Adequacy of model was checked by lack of fit test, R^2, predicted R^2, Adequacy precision and predicted residual error sum of squares (PRESS). Predicted R^2 comparable to adjusted R^2, non significant lack of fit, low PRESS and adequacy precision higher than 4, suggests that the model fitted is adequate for predicting[3-5]. Significance was judged from the F-statistics at 5% probability level. Response surfaces were generated to study the effect of ethanol concentration, temperature and pH on recovery of total phenolic content and % DPPH* scavenging activity.

5.2.4. Purification of extracted total phenolics from seed coat powder

For further purification, 100 g of Amla seed coat powder was extracted (under optimum conditions) in 1000 mL of ethanol (88.06%) by shaking in the incubator shaker for 4 h at 35.38°C and 4.41 pH. The extract was centrifuged in refrigerated centrifuge at 4°C and 5687 *g* for 15 min. The residue was re-extracted and then centrifuged. The supernatants were combined and evaporated in a rotary evaporator under reduced pressure. The concentrated extract was freeze dried under vacuum. Accurately weighed 4.49 g of ethanolic extract powder was dissolved in 40 mL of water mixture and was partitioned with ethyl ether (50 mLx3), ethyl acetate (50mLx3) and butanol (50 mLx3) successively. The fractions were evaporated under reduced pressure at 45°C for ethyl acetate and ethyl ether fractions and at 65°C for butanol and aqueous fractions as per the method of Liu et al. (2012)[6]. The yields of ethyl ether, ethyl acetate, butanol and aqueous fractions were 0.18 g, 2.55 g, 1.092 g and 0.67 g,

respectively. Ethyl acetate, butanol and aqueous fractions were analyzed for their TPC content and % DPPH* scavenging activity. Ethyl acetate fraction was fractionated on a Sephadex G-25 (25-100 µm, Sigma Aldrich) column (450 x 25 mm) with water/methanol (100:0-100:0) as the eluent to obtain six fractions (I-VI). All six fractions were freeze dried under vacuum and the freeze dried powder obtained were evaluated for the characterization of the phenolic content through HPLC and FTIR and GC -MS.



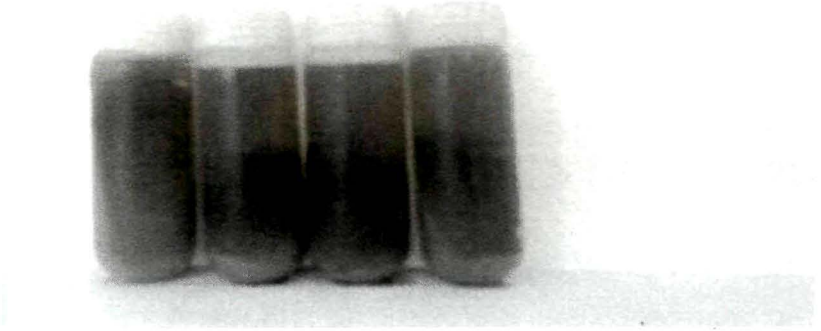
(a) **(b)**

Fig 5.1 Image of (a) Amla seed coat, and (b) Amla whole seed.

Fig. 5.2 Image of seed coat powder.

Table. 5.1 Central composite rotatable design with experimental values for response variables

Run	Temperature (°C)	Ethanol (%)	pH	% DPPH* scavenging activity/mL of extract	TPC (mg/mL of extract equivalent to GAE)
1	35.00	117.27	6.00	43.6	6.81
2	35.00	-17.27	6.00	2.43	0.28
3	55.00	90.00	8.00	28.92	2.97
4	15.00	10.00	4.00	18.6	1.28
5	15.00	90.00	4.00	48.52	7.52
6	35.00	50.00	6.00	40.67	5.55
7	35.00	50.00	6.00	40.69	5.55
8	15.00	10.00	8.00	12.15	0.98
9	35.00	50.00	2.64	46.99	8.62
10	35.00	50.00	6.00	40.69	5.55
11	35.00	50.00	6.00	40.69	5.55
12	55.00	90.00	4.00	46.12	7.83
13	55.00	10.00	4.00	17.92	2.5
14	35.00	50.00	9.36	11.09	2.24
15	15.00	90.00	8.00	30.17	2.09
16	35.00	50.00	6.00	40.69	5.55
17	35.00	50.00	6.00	40.69	5.55
18	1.36	50.00	6.00	28.33	2.73
19	68.64	50.00	6.00	15.01	5.81
20	55.00	10.00	8.00	7.01	2.00



(a)

(b)

(c)

Fig.5.3 Image of seed coat extracts (a) tubes of ethanol extract, (b) ethyl acetate extracted powder, and (c) butanol extracted powder.

5.2.5. Fourier transform infrared spectroscopy (FTIR)

All samples were scanned in the range of 4000-650 cm^{-1} with a resolution of 4/cm^{-1}. Attenuated total reflection/Fourier transform infrared spectroscopic (ATR/FTIR) spectra were collected at room temperature by coupling the ATR accessory to an FTIR spectrometer (Perkin Elmer, Spectrum 100). The time required to obtain a complete background was 20 s. Time resolved experiments were collected by averaging 3 scans[7].

5.2.6. Total phenolic content (TPC)

Estimation of total phenolic content was performed by Folin–Ciocalteu method described by Liu et al. (2008)[8] with some modifications. Briefly, 60µL of extract, 300 µL of Folin-Ciocalteu reagent and 900 µL of 20% sodium carbonate were added to 4.75 mL of water. The mixture was allowed to stand for 30 min. The absorbance was carried out at 765 nm and the results were expressed as mg GAE. For estimation of total phenolic content of powder, 50 mg of extract powder was mixed with 5 mL of methanol and the mixture was stirred for 30 min at 25°C. The stirred mixture was centrifuged and supernatant was analyzed for its polyphenolic content as described above.

5.2.7. 2,2, Diphenyl picryl hydrazil free radical (DPPH*) scavenging activity

The DPPH* scavenging activity of extract was determined by the method of Luo et al. (2009)[2] with slight modifications. Briefly, 2 mL of methanolic extract was mixed with 2 mL of methanolic solution containing 0.1 mM DPPH. The mixture was shaken vigorously and then left to stand for 30 min in the dark. The absorbance was taken at 517 nm. The absorbance of control was measured by replacing the sample with methanol.

$$\text{DPPH* scavenging activity (\%)} = \frac{\text{(1-absorbance of sample) x100}}{\text{absorbance of control}}$$

5.2.8. Analytical HPLC

Analytical HPLC was conducted on a Waters (Breeze-2) liquid chromatography fitted with a C-18, reverse phase (5 µm) column 7.2 x 300 mm by the method given by Seruga et al. (2012)[9] with slight modifications. In brief,

orthophosphoric acid (0.1%) as solvent A and 100% HPLC grade methanol was used as solvent B for the separation of phenolics. The elution conditions were 0-30 min from 5% B to 80% B; 30-33 min 80% B; 33-35 min from 80 % B to 5 % B; 35-40 min at 5% B; flow rate 0.8 mL/min. The operating conditions were: column temperature, 20°C; injection volume, 20 µL. The detection wavelengths were 280 nm for gallic acid; 360 nm for quercetin; 280 nm for catechin, 320 nm for caffeic acid and p-coumaric acid. Total area under curve at 280 nm was calculated as total phenolic content of the analyzed sample.

5.2.9. Derivatization for GC-MS

The derivatization and identification by GC-MS was carried out by the method given by Proestos et al. (2006)[10] with slight modification. For the silylation procedure, 100 µL of BSTFA was added in 0.01 mg of sample powder and vortexed in screw-cap glass tubes which was previously deactivated with 5% dimethyl dichlorosilane (DMDCS) in toluene, and rinsed twice with toluene and thrice with methanol, and consecutively placed in a water bath at 80°C for 45 min. Methanol (500 µL) was added in the silylated sample before injection. The silylated samples were injected into a GC-MS system (Perkin Elmer, model Clarus 600 gas chromatograph coupled 600C mass spectrometer and the mass range scanned at m/z 50–500) with Photo Multiplier Tube detector. A capillary column Elite 5 (30 m x 0.32 mm) was used for the identification of phenolics. The injector and detector were set at 280°C and 290°C, respectively. GC was performed in the split mode. The temperature programme was as follows: from 70 to 135°C at 10°C/min, from 135 to 220°C at 10°C /min, from 220 to 260°C at 7°C/ min and holding for 10 min. The flow rate of carrier gas (helium) was kept at 1.9 mL/min. Identification of compound was achieved by comparing the retention time of standards and spectral data obtained from the NIST libraries.

5.2.10. Statistical analysis

All experiments for extraction were carried out in duplicate however the analyses were carried out in triplicates. Obtained mean values were analyzed by analysis of variance (ANOVA). The graphs of mean value and standard error were calculated using Excel version of 2003.

5.3. Results and discussion

5.3.1. Experiment design

The results of different runs of extraction of seed coat are given in **Table 5.1**. It is important to keep the temperature range relatively low (maximum temperature 55°C) to avoid possible degradation of the extract[11,12] and therefore in the present investigation temperature was varied from 10 to 55°C. The total polyphenols was measured in the extracted material for optimization of extraction of total phenolic content from seed coat by Folin-Ciocalteu method which involves the oxidation of the phenolic compounds and reduction of the phosphomolybdic–phosphotungstic acid indicator[13]. Total polyphenols reported as gallic acid equivalent (GAE) ranged between 0.28 to 8.62 mg GAE/mL of extract and the DPPH* scavenging activity ranged from 2.43 to 48.52%. Process variables, ethanol concentration and pH were found to be statistically significant for recovery of total phenolic content and % DPPH* scavenging activity of the extract. Analysis of variance (ANOVA) was performed to determine the significant effects of variables on recovery of total phenolic content and % DPPH* scavenging activity of the extract (**Table 5.3** & **Table 5.4).** Model adequacy was checked by considering predicted R2, PRESS and adequate precision. The lack of fit was not found significant for all response surface models at 95% confidence level.

It can be observed from **Table 5.2** that the model was significant for DPPH* radical scavenging activity. The R^2 of model was 0.97, whereas the adjusted R^2 (0.95) and predicted R^2 (0.82) were comparable indicating that the model term was significant.

Table 5.3 presents the ANOVA table for the total phenolic content. The R^2 of model was 0.96, whereas the adjusted R^2 (0.93) and predicted R^2 (0.81) were comparable indicating that the model fitted provided appropriate approximation of the true findings. The adequacy of precision in both DPPH* scavenging activity and total phenolic content was greater than 4 which confirms that the model terms are fitted and adequate.

5.3.2. Response surface

To visualize the combined effects of the two factors on the response, the response surface and contour plots were generated for each of the fitted models as function of two variables, while keeping other variable at the central values.

Table 5.2 Analysis of variance (ANOVA) of fitted model for DPPH * scavenging activity of extracts of seed coat

Terms	Sum of squares	DF	Mean square	F value	p >F
Model	4260.60	9	473.40	43.44	< 0.0001*
A	65.34	1	65.34	5.99	0.0343*
B	2000.50	1	2000.50	183.55	< 0.0001*
C	1030.82	1	1030.82	94.58	< 0.0001*
A^2	592.53	1	592.53	54.37	< 0.0001*
B^2	507.90	1	507.90	46.60	< 0.0001*
C^2	171.82	1	171.82	15.76	0.0026*
AB	8.34	1	8.34	0.77	0.4021
AC	0.90	1	0.90	0.083	0.7792
BC	73.14	1	73.14	6.71	0.0269*
R squared	0.97				
Adj R^2 squared	0.95				
Pred R^2 squared	0.82				

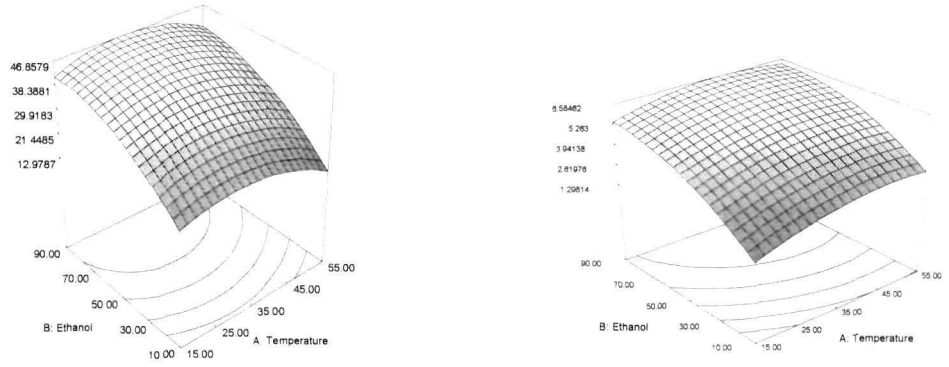
Table. 5.3 Analysis of variance (ANOVA) of fitted model for total phenolic content of extracts of seed coat

Terms	Sum of squares	DF	Mean square	F value	p >F
Model	117.50	9	13.06	29.38	< 0.0001*
A	5.18	1	5.18	11.66	0.0066*
B	47.36	1	47.36	106.59	< 0.0001*
C	35.81	1	35.81	80.60	< 0.0001*
A^2	6.26	1	6.26	14.08	0.0038*
B^2	12.07	1	12.07	27.17	0.0004*
C^2	0.55	1	0.55	1.24	0.2910
AB	0.43	1	0.43	0.96	0.3496
AC	0.17	1	0.17	0.39	0.5488
BC	11.74	1	11.74	26.42	0.0004*
R Squared	0.96				
Adj R-Squared	0.93				
Pred R Squared	0.81				

* Term significant at $p<0.05$

Fig. 5.4a shows the effect of ethanol concentration and extraction temperature on DPPH radical scavenging activity of total polyphenolic content of the extract. Increase in the ethanol concentration with temperature increased the DPPH* scavenging activity of the extract.

Especially, it was noted that higher ethanol concentration promoted better DPPH* scavenging activity in the extract than higher temperature. This interaction between ethanol concentration and temperature was in accordance with the results of analysis. **Fig 5.4(b)** shows the interaction effect of ethanol concentration and temperature on total recovery of polyphenolic content in extracts. Increase in the ethanol concentration increased the total recovery of polyphenols significantly, while temperature showed no significant effect on total recovery of polyphenols (**Fig 5.4b**). The obtained finding was in agreement with Li et al. (2011)[12]; where they reported that ethanol concentration is significantly effective for recovery of polyphenols from grape seed. **Fig 5.5a** shows the effect of pH and temperature on % DPPH* scavenging activity of the extract while ethanol concentration was kept constant at its middle level i.e. 50%. With the increase in the temperature of extraction, the DPPH radical scavenging activity was found to be significantly increased from 27.67% to 44.93%. As the pH value increased, the % DPPH* scavenging activity decreased slightly with increase in temperature and this effect was more pronounced after pH 7.0. This suggests that increasing the temperature at lower pH is more effective for better free radical scavenging activity of the extract. With the increase in ethanol concentration, the DPPH radical scavenging activity was significantly increased from 22.26% to 46.66%. As the pH value was increased the % radical scavenging activity decreased slightly (**Fig 5.5 a**). **Fig 5.5 (b)** depicts the 3 D plot, showing the effects of ethanol concentration and pH on total recovery of polyphenols. Increasing the concentration of ethanol at lower pH increased the total recovery of phenols significantly while pH showed negative effect on total recovery of phenols. To consider all the responses simultaneously for optimization, RSM was used to compromise the optimum conditions. Ethanol 88.06%, temperature 36.90°C and 4.17 pH were found optimum. Triplicate samples were prepared using the optimum conditions and were analyzed for % DPPH* scavenging activity and TPC; corresponding value for antioxidant and TPC was 42.69% and 7.74 mg/mL of extract of GAE equivalent respectively, which shows an excellent agreement with the predicted responses and the actual value (**Table. 5.4**).

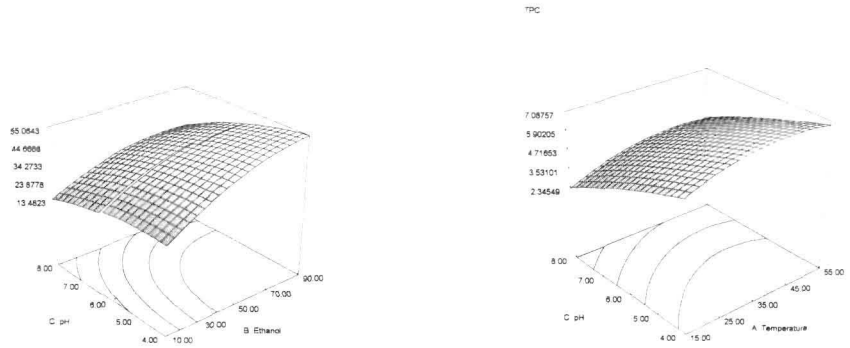


(a) (b)

Fig.5.4 Response surface and contour plots for (a) DPPH* radical scavenging activity, and (b) total phenolic content (at constant pH 6.0).

(a) (b)

Fig.5.5 Response surface and contour plots for (a) DPPH* radical scavenging activity, and (b) total phenolic content (at constant ethanol concentration 50.0).



(a) (b)

Fig.5.6 Response surface and contour plots for (a) DPPH* radical scavenging activity, and (b) total phenolic content (at constant temperature 35^{o}C).

Table-5.4 Extraction of total phenols and % DPPH* scavenging activity under optimized condition

Particular	Predicted	Optimum
% DPPH* scavenging activity /mL of extract	40.63	42.69
Total phenolic content mg/g of mL of extract GAE	7.68	7.74

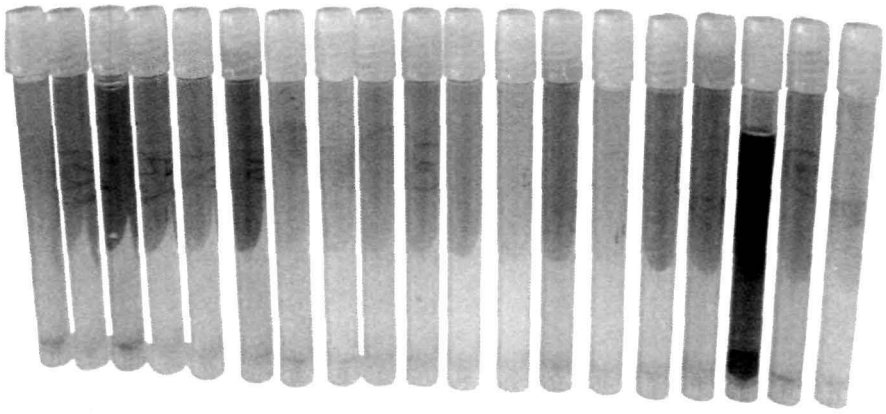


Fig. 5.7 Image of different extracts obtained from the experimental runs under central composite rotatable design.

5.3.3. Extraction of phenolics content

For further purification, 4.49 g of the seed coat extract extracted under optimum conditions were dissolved in 20 mL water and then partitioned with ethyl ether, ethyl acetate and butanol. One mg of ethyl acetate fraction showed 61.07% DPHH* scavenging activity, whereas butanol and aqueous fractions had only 12.99% and 0.098% DPPH* scavenging activity respectively for the same amount of the powder (**Table. 5.5**). The poor DPPH* scavenging activity of aqueous fraction of seed coat powder is also corroborated by the lowest amount of TPC as estimated by the HPLC method (**Fig 5.8a**) and FCR method. The aqueous fraction had 7.34 mg/g GAE equivalent by HPLC method (**Fig. 5.8a**) and 74.61 mg/g GAE equivalent by FCR method (**Table 5.5**) whereas ethyl acetate fraction had 64.80 mg/g GAE equivalent by HPLC method (Fig 2a.b) and 493.65 mg/g GAE equivalent by FCR method **(Table 5.5**) and butanol fraction had 156.40 mg/g GAE equivalent by HPLC

method (Fig 2a.c) and 311.55 mg/g GAE equivalent by FCR method (**Table 5.5**). Butanolic fraction had significantly higher amount of phenolic content (when analyzed by HPLC) than the ethyl acetate fraction but the reverse trend was observed in case of FCR method. Phenolics content determined by FCR method was considerably higher than that determined by HPLC method (**Table 5.5**). The reagent reacts not only with phenolics but also with a number of non-phenolic reducing compounds including tertiary aliphatic amines, tertiary amine-containing biological buffers, amino acids (tryptophan), hydroxylamine, hydrazine, certain purines, and other organic and inorganic reducing agents because of which FCR method overestimates the total phenolic content[14]. Furthermore, different phenolics can react differently with the Folin-Ciocalteu's reagent, resulting in lower absorption that leads to an underestimation of various compounds[15].

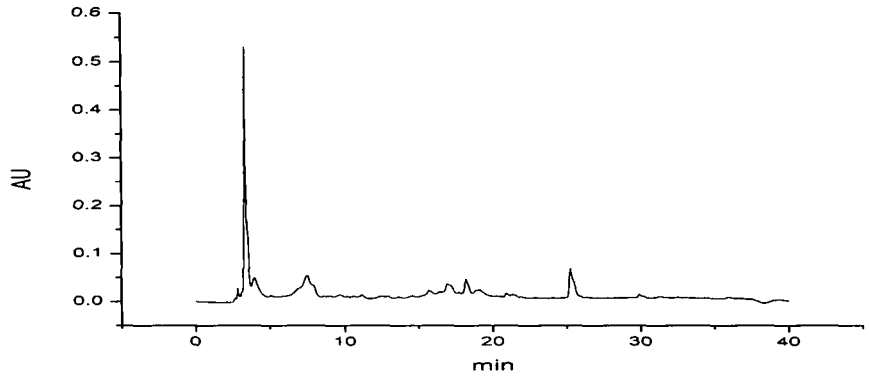
TPC content of butanolic extract estimated by FCR method was low probably because the extract was exposed to higher temperature while removing the solvent as butanol has high boiling point which may adversely affect the availability of functional groups to form complexes with phosphomolybdate and hence, gave lower TPC content as compared to ethyl acetate fraction and showed considerably less % DPPH* scavenging activity.

The reason for poor DPPH* scavenging activity of the aqueous extract of seed coat may be because the major portion of phenolics which are present in seed coat have poor solubility in water and hence could not be partitioned with water. The present results suggested that the compound with relatively higher antioxidant activity was in ethyl acetate fraction; therefore ethyl acetate fraction was subjected to further purification and identification.

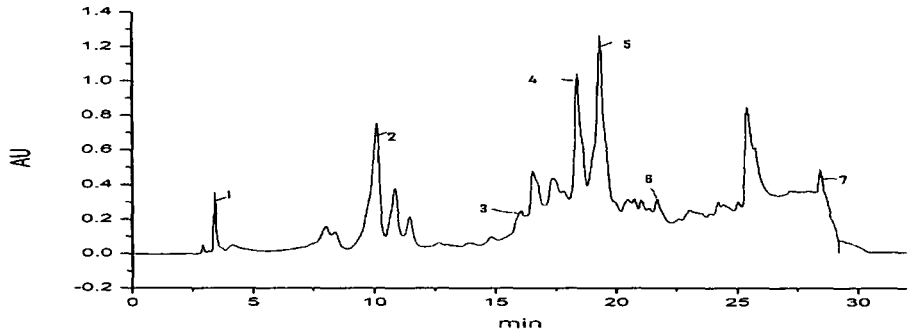
5.3.4. Identification of phenolics

5.3.4.1. Analytical HPLC

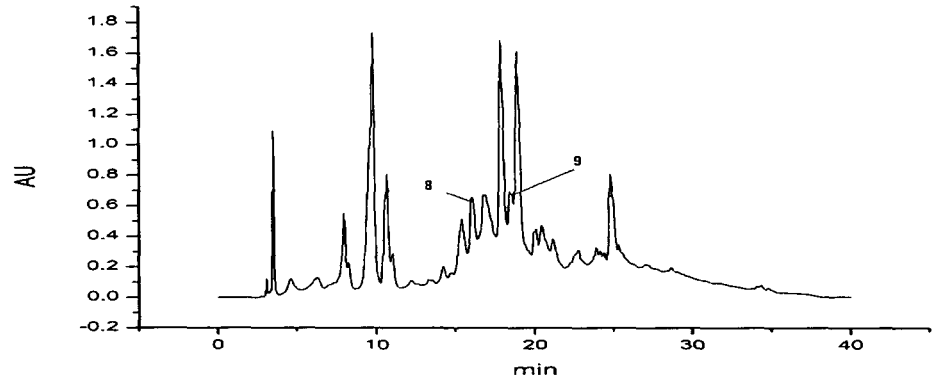
Amla fruit is known to contain a range of secondary metabolites such as polyphenols and flavonoids but Amla seed coat powder have never been investigated. Therefore qualitative and quantitative analysis of the ethyl acetate extract, butanolic extract, aqueous extract and different fractions of seed coat powder obtained after column chromatography of ethyl acetate fraction were investigated by analytical HPLC.



(a)



(b)



(c)

Fig. 5.8 HPLC chromatograms of (a) aqueous fraction, (b) ethyl acetate fraction, and (c) butanol fraction at 280 nm. (1) chlorogenic acid; (2) gallic acid; (3) catechin; (4) vanillic acid; (5) caffeic acid; (6) p -coumaric acid; (7) quercetin; (8) catechin; and (9) vanillic acid.

Table 5.5 Bioactive component and % DPPH* scavenging activity of aqueous, ethyl acetate, and butanol fractions of seed coat

Particular	Aqueous fraction	Ethyl acetate fraction	Butanol fraction
% Yield	14.88±1.1[a]	57.21±2.0[b]	24.31±1.2[c]
% DPPH* scavenging activity /mg of powder	0.098±0.9[a]	61.073±1.6[b]	12.99±2.3[c]
TPC by FCR (mg/gm of GAE)	74.61±2.2[a]	493.65± 1.7[b]	311.50±2.1[c]
TPC by HPLC (mg/g) GAE	7.34±1.0[a]	64.80±0.8[b]	156.34±0.9[c]
Gallic acid (mg/g)	ND	15.45±0.6	ND
Catechin (mg/g)	ND	25.16±0.1	97.43±0.3
Caffeic acid (mg/g)	ND	15.14±0.1	ND
Quercetin (mg/g)	ND	5.34±0.2	ND
p-Coumaric acid (mg/g)	ND	1.72±0.1	ND
Syringic acid (mg/g)	ND	ND	ND
Vanillic acid	ND	18.5±0.3	0.39±0.02
Chlorogenic acid	ND	9.85±0.1	ND

Mean ± standard deviation

Observation with different superscript in same row differs significantly ($p<0.05$ probability level)

Not detected

Table 5.5 presents the bioactive properties of ethyl acetate fraction, butanolic fraction and aqueous fraction. Representative chromatograms of above three fractions are given in **Fig.5.8**. Ethyl acetate fraction gave maximum recovery i.e. 57.21% (of initial amount of ethanol extracted powder taken for the partition) whereas aqueous fraction powder gave the lowest recovery. The present findings were in agreement with the findings of the Liu et al. (2008)[8]. **Table 5.6** shows that the major polyphenols contained in ethyl acetate fractions were gallic acid (15.45 mg/g, rt 10.09) ; catechin (rt 15.94 min, 25.16 mg/g), caffeic acid (15.14 mg/g, rt 19.48 min), quercetin (28.37 min, 5.34 mg/g), p-coumaric acid (rt 21.920, 1.72 mg/g), vanillic acid (rt 18.51, 18.5 mg/g) and chlorogenic acid (rt 3.06, 9.85 mg/g); however, butanol fraction also contained catechin. The predominant phenolics were catechin with a mean value of 25.16 mg/g followed by gallic acid (15.45 mg/g) and caffeic acid (15.14 mg/g) (**Table 5.5**). Syringic acid was not present in any of the extract tested. The results suggested that the compounds with relatively high antioxidant activity and phenolics were present in ethyl acetate fraction.

Six different ethyl acetate fractions obtained after column chromatography were further lyophilized. The % recovery of all six fractions (in respect of initial weight of sample taken for Sephadex column chromatography) i.e. I, II, III, IV, V and VI were 0%, 24.44%, 5.53%, 6.81%, 24.09% and 39.23%, respectively (**Table 5.6**). **Fig. 5.9** presents the HPLC chromatograms of different fractions of seed coat powder. The recovery of phenolics was comparatively less in fraction II as compared to the other fractions (**Fig. 5.9a**), however presence of quercetin in fraction II may explain the good DPPH* scavenging activity of the fraction II. Liu et al (2008)[8] also reported high DPPH* scavenging activity of quercetin in comparison to BHA and vitamin E. Caffeic acid and p-coumaric acid were eluted in fraction V whereas catechin could be recovered in fraction IV. Significant quantity of phenolics could be recovered in fraction III, IV, V and VI (**Fig 5.9b, c, d** and **e**). Recovery of gallic acid (7.34 mg/g) was maximum in fraction V whereas catechin (34.07 mg/g) was detected in fraction IV (**Table 5.5** and **Fig 5.9d**).

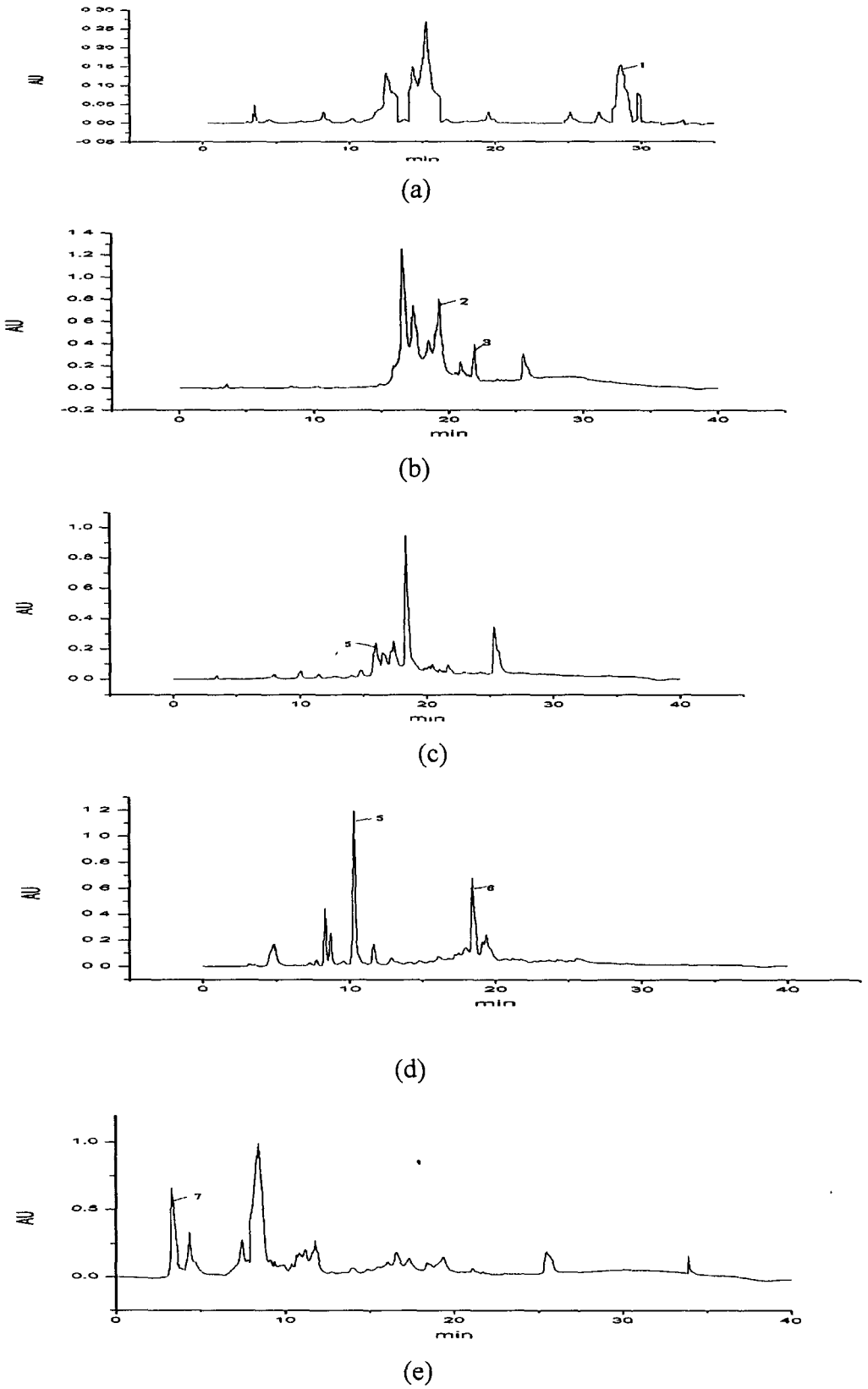


Fig. 5.9 HPLC chromatograms of fraction (a) II, (b) III, (c) IV, (d) V, and (e) VI at 280 nm. (1) quercetin; (2) caffeic acid; (3) p-coumaric acid; (4) catechin; (5) gallic acid; (6) vanillic acid; and (7) chlorogenic acid.

Table 5.6 Bioactive components and % DPPH* scavenging activity of different fractions of seed coat obtained after column chromatography

	Fractions					
Particular	**I**	**II**	**III**	**IV**	**V**	**VI**
% Recovery	0	24.44±1.1[a]	5.53±0.2[b]	6.81±0.5[c]	24.09±1.0[a]	39.23±1.0[d]
% DPPH* scavenging activity/mg of powder	ND	74.41±1.3[a]	110.64±2.1[b]	86.85±1.4[c]	168.64±2.1[d]	16.47±1.0[e]
Gallic acid (mg/g)	ND	ND	ND	ND	26.15±0.2	ND
Caffeic acid (mg/g)	ND	ND	21.32±0.2	ND	ND	ND
Catechin (mg/g)	ND	ND	ND	34.07±0.2	ND	ND
Quercetin (mg/g)	ND	7.34±0.1	ND	ND	ND	ND
p-Coumaric acid (mg/g)	ND	ND	4.27±0.1	ND	ND	ND
Chlorogenic acid (mg/g)	ND	ND	ND	ND	ND	23.31±0.2
Vanillic acid (mg/g)	ND	ND	ND	ND	19.32±0.3	ND

Mean ± standard deviation

Observation with different superscript in same row differs significantly ($p<0.05$)

Not detected

5.3.4.2. Gas chromatography mass spectroscopy

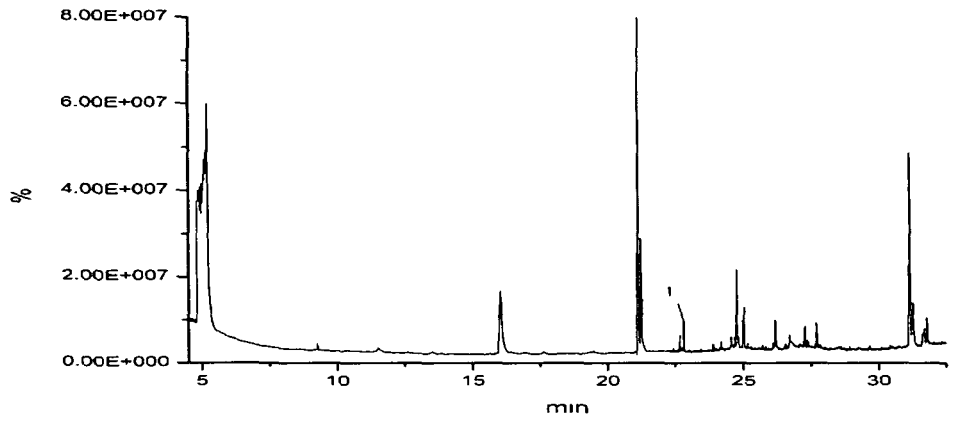
Silylation is an ideal procedure for the analysis of non-volatile and thermolabile compounds by gas chromatography. Compared to their original compounds, TMS derivatives are more volatile, less polar and more thermotolerant. In silylation procedure an active hydrogen present in –OH, –COOH, =NH, $-NH_2$ or –SH is replaced by a trimethylsilyl group[10]. In the present study reaction time and temperature was sufficient for the silylation of phenolic compounds. Since the mass scan rate for the present study was only upto 500 hence catechin and quercetin could not be determined by present method. Care was taken to ensure anhydrous conditions during the derivatization process due to the high sensitivity of trimethylsilyl (TMS) derivatives towards moisture. Anhydrous condition was maintained through the flushing of N_2 gas. Prior to employing GC-MS for the determination of phenolic compounds of different fractions of seed coat obtained after column chromatography, a standard mixture of all substances was tested after derivatization. **Fig 5.10** presents the chromatograms of different fractions of seed coat powder obtained after column chromatography. Retention time of silylated phenolic compounds in the examined extracted powder is presented in **Table 5.7**. The MS spectra of TMS derivative of gallic acid and gallic acid are given in **Fig 5.11a** and **Fig 5.11b** respectively. The fragmentation mechanism of simple phenols, such as gallic acid, has already been studied by other researchers[16]. The major fragmentation peak of derivatized gallic acid observed were : 179, 147, 280, 308 and 457 (Fig **5.11 a**) which is in accordance with Proestos et al. (2006). Phenolic compounds such as p-coumaric acid in fraction III (**Fig 5.10 b**); hydroxy caffeic acid, caffeic acid in fraction IV (**Fig 5.10 c**); vanillic acid, gallic acid and p-hydroxybenzoic acid in fraction V (**Fig 5.10 d**) were identified by their TMS derivative. Other components like tetracontane-1,40-diol in fraction I (**Fig 5.10 a**) and octadecanoic acid could also be identified based upon NIST library. The findings of GC-MS were in accordance with our HPLC analysis and prove the efficacy of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) as a derivatizing agent.

Table. 5.7 Retention time of TMS derivatives of different phenolics

Phenolics	II t_r (min)	III t_r (min)	IV t_r (min)	V t_r (min)	VI t_r (min)
Gallic acid	ND	ND	ND	24.79±0.2	
Quercetin	-	-	-	-	-
Caffeic acid	ND	ND	26.19±0.1	ND	ND
Syringic acid	ND	ND	ND	ND	ND
p-Coumaric acid	ND	24.19±0.2	ND	ND	ND
Vanillic acid	ND	ND	ND	9.43±0.3	ND
Chlorogenic acid	ND	ND	ND	ND	ND
Hydroxycaffeic acid	ND	ND	21.15±0.1	ND	ND
p-hydroxybenzoic acid	ND	ND	ND	31.48±0.2	ND
2,3-dihydroxypropyl elaidate	ND	ND	ND	ND	ND
Tetracontane-1,40-diol	22.83±0.2	ND	ND	ND	ND
Octadecanoic acid methyl ester	ND	26.34±0.1	ND	ND	26.33±0.2

Each value is the mean of retention time after two replications ± standard deviation.

ND: not detected.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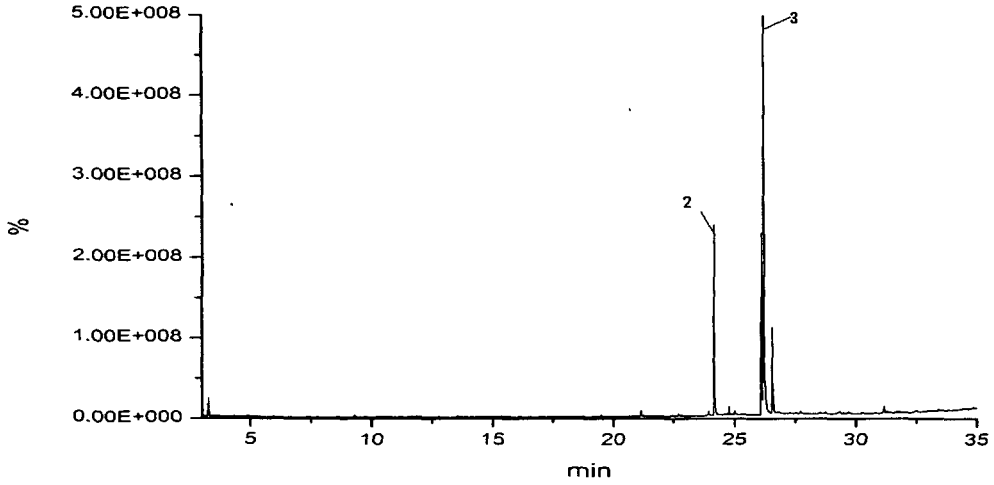
(a)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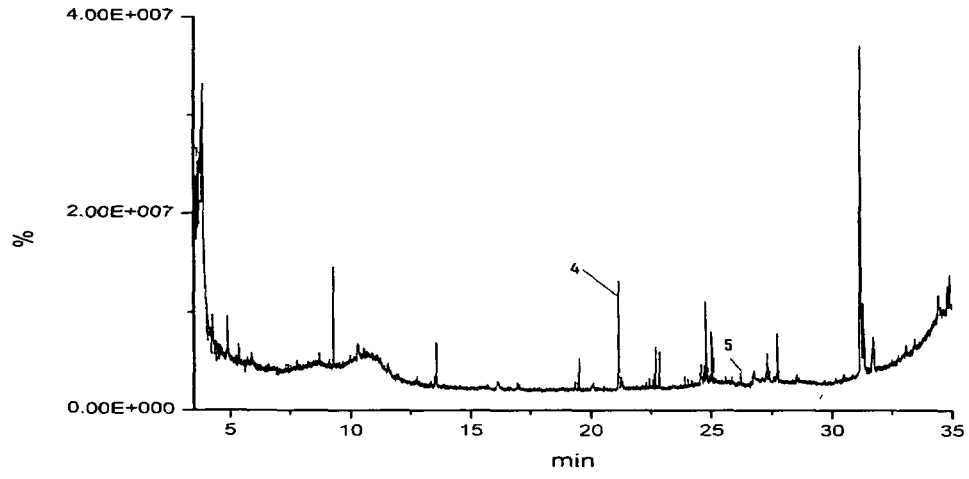
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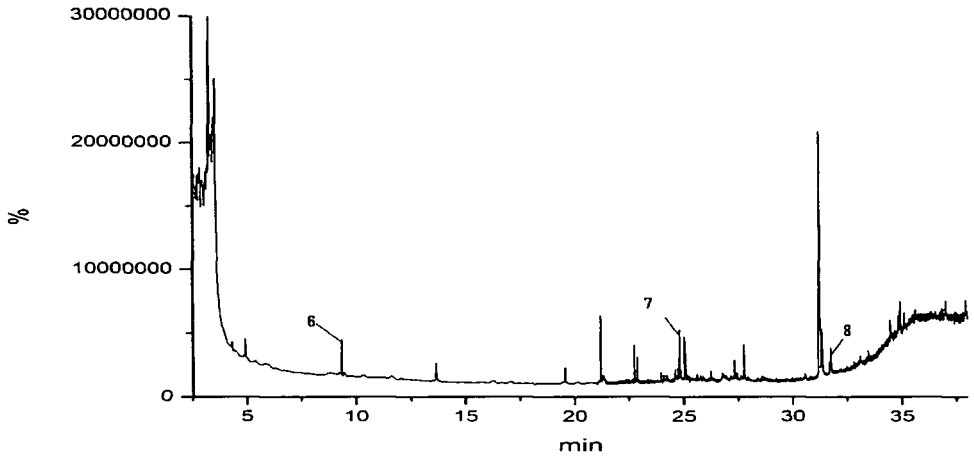

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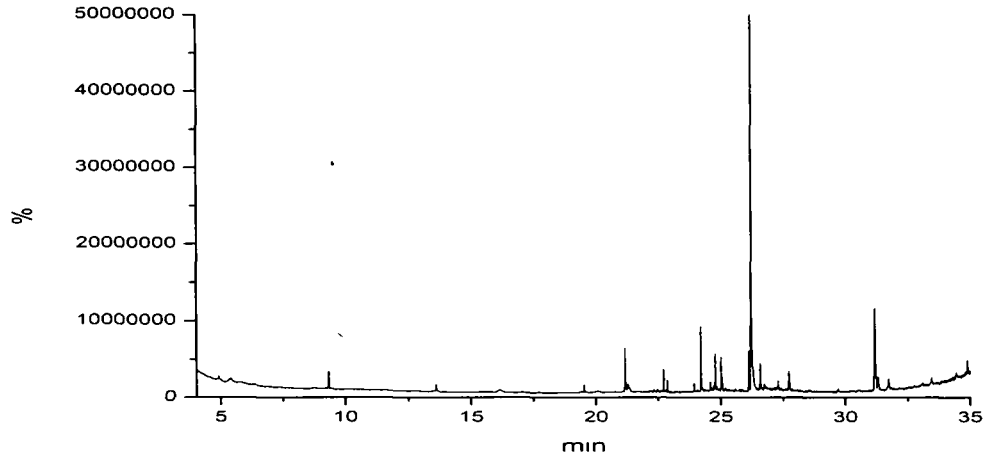
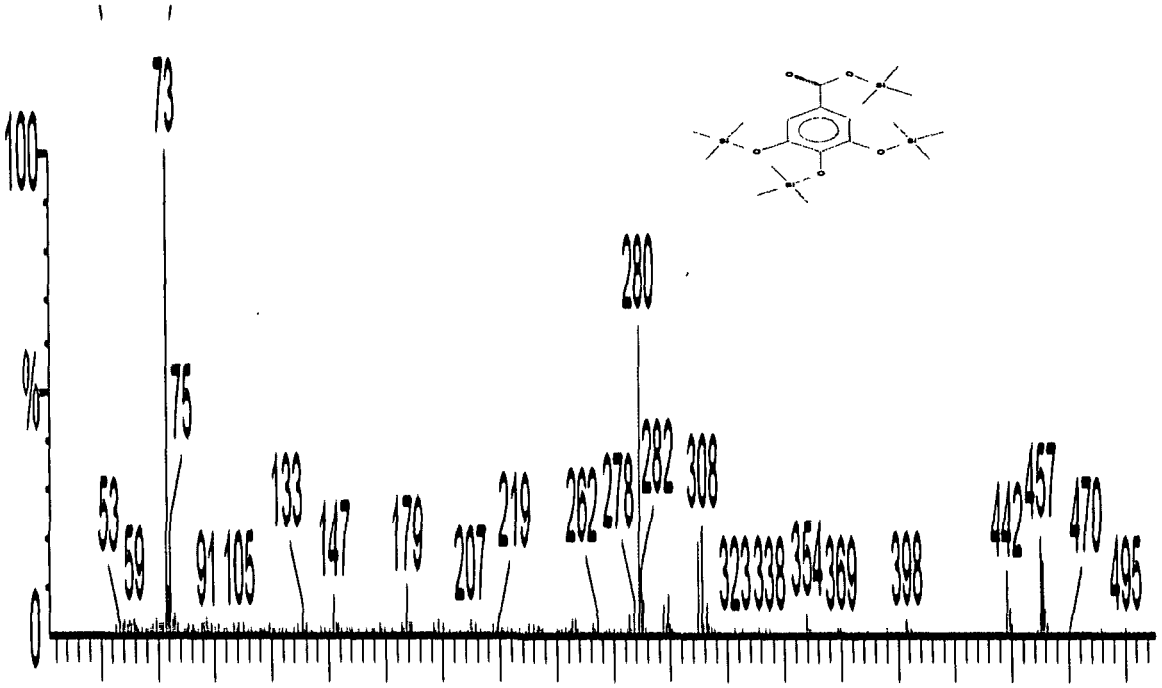
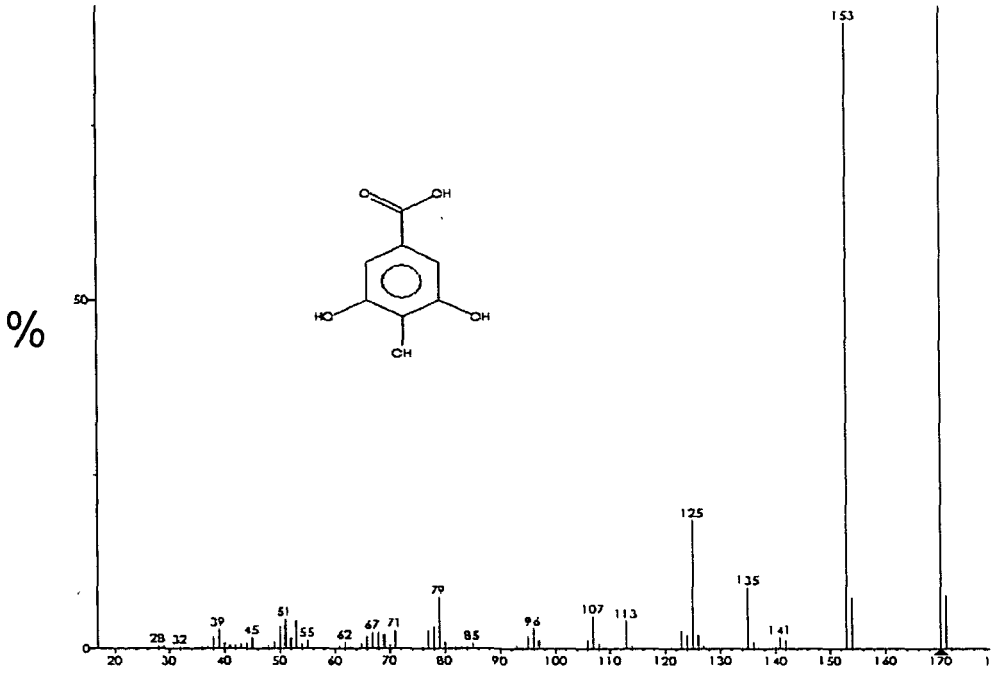


(e)

Fig. 5.10 Mass spectra of different fractions of seed coat powder (a) II; (b) III; (c) IV; (d) V; and (e) VI. (1) Tetracontane-1,40-diol; (2) p-coumaric acid; (3) Octadecanoic acid methyl ester (4) hydroxy caffeic acid; (5) caffeic acid; (6) vanillic acid; (7) gallic acid, and (8) p-hydroxybenzoic acid.



(a)



(b)

Fig. 5.11 Mass spectra of (a) silylated gallic acid, and (b) gallic acid.

5.3.4.3. FTIR analysis

Fig. 5.12 presents the FTIR spectra of different fractions of seed coat obtained after column chromatography. Noticeably, the presence of gallic acid peak at 3377, 1703, 1617, 1539 and 1254 cm^{-1}, 1100 cm^{-1}, 1025 cm^{-1} and 860 cm^{-1} (Nirmaladevi, & Kvitha, 2010)[17] were observed in ethyl acetate fraction of seed coat powder. The main bands present in ethyl acetate fraction were 1038 cm^{-1} (C-O alcohol) that appeared in slightly different location of 1046.98. The aromatic band of 1280 cm^{-1} (O-H aromatic) appeared in slightly different location of 1218.94 cm^{-1}. FTIR of quercetin as standard had band characteristics of -CO stretching at 1663 cm^{-1}. The aromatic bending and stretching around 1092 and 1663 cm^{-1} (B-G Heo et al., 2013)[18] were present at slightly different locations i.e 1614.17 cm^{-1} and 1031.25 cm^{-1} in ethyl acetate fraction which confirmed the presence of quercetin. Intensity of peak was comparatively less in aqueous fraction and fraction II (**Fig. 5.8**) which suggested that there was significant drop of functional group in these fractions. Significant drop of peak in aqueous fraction in the range of 1350-1000 cm^{-1} and below 1000 cm^{-1} region suggested the absence of C-O stretching of alcohols, ester, lactones and mono and disubstitution at ortho, meta, and para position in benzenes in aqueous fraction. Intensity of peak in the range of 3200-3500cm^{-1} were comparatively high in fraction V suggesting the more polar character of the bond which may be correlated with the presence of functional group (hydrogen bonded alcohols, hydrogen bonded acids or phenols) which is self-explanatory for the potent ability of the fraction V to scavenge DPPH*.

5.3.5. DPPH* scavenging activity

Free radicals which are involved in the process of lipid peroxidation are considered to play a major role in a number of chronic pathologies, such as cancer and cardiovascular diseases among others[19]. Ethyl acetate fraction showed significantly less total phenolic content than the butanolic fraction of the seed coat powder. However inspite of having good amount of total phenolic content, the DPPH* scavenging activity was significantly less than the ethyl acetate fraction. Our present findings contradicted the reported findings of Piljac et al. (2007)[20] and Seruga et al. (2011)[9]. The antioxidant activity and DPPH* scavenging activity not only depends on the total phenolic content but also depends on the position and availability of the free hydroxyl groups to scavenge the DPPH*. Exposure to high temperature to evaporate the solvents from butanolic extracts adversely affects the functional properties of the

total phenolic content and hence was not found suitable for the extraction of bioactive components. Fraction V (Table 4) showed the highest DPPH* scavenging activity among all fractions tested. The highest % DPPH* scavenging activity of fraction V is due to the presence of gallic acid, vanillic acid and some other phenolics which could not be detected in the present study (**Table 5.6** and **Fig. 5.9**). It can also be concluded from **Fig 5.12** that FTIR spectra of fraction V had comparatively higher intensity of functional peaks as compared to others. Luo and Foo (2001) explained that such hydroxy-phenolic compounds can donate hydrogen atoms to DPPH* and can scavenge it[21].

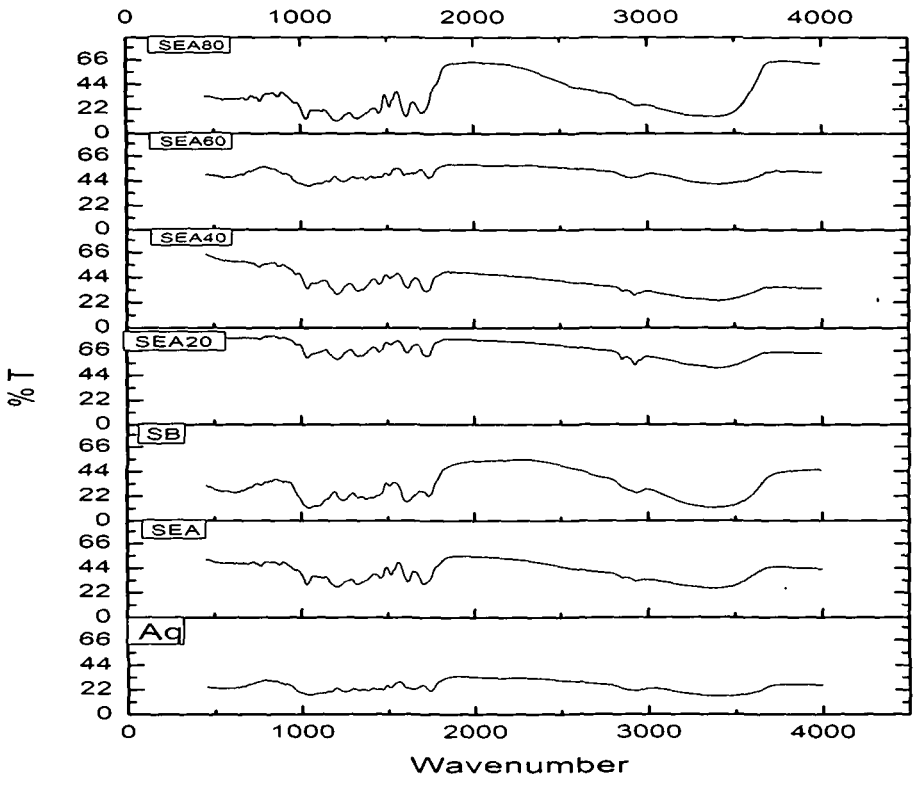


Fig. 5.12 FTIR spectra of different fractions of seed coat powder Aq: aqueous fraction of seed coat; SEA : ethyl acetate fraction of seed coat; SB: butanol fraction of seed coat; SEA 20: fraction II; SEA40: fraction III; SEA 60: fraction IV; SEA 80: fraction V.

5.4. Conclusion

Seed coat of Amla has never been investigated for optimization of extraction of total phenolic content. ANOVA shows that the process variables, ethanol concentration and pH were found to be statistically significant for recovery of total

phenolic content and % DPPH* scavenging activity of the extract. Optimum operating conditions were found to be ethanol of 82.98%, temperature 36.90° C and pH 4.17. At the optimum conditions, TPC content and % DPPH* scavenging activity of extract were found to be 6.74 mg/g of GAE and 42.69% equivalent, respectively. Major polyphenols contained in ethyl acetate fractions were gallic acid, catechin, caffeic acid, quercetin, p-coumaric acid, vanillic acid and chlorogenic acid. Ethyl acetate fraction extracted by methanol: water (80:20) showed the maximum DPPH* scavenging activity. Recovery of ethanol extracted powder was very less hence other methods like supercritical fluid extraction, ultrasonic or microwave assisted extraction may be explored to extract the vital bioactive constituents of seed coat powder.

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Chapter-6

Effect of maltodextrin concentration and inlet temperature during spray drying on physicochemical and antioxidant properties of Amla (*Emblica officinalis*) juice powder

6.1. Introduction

Antioxidants are required to combat free radicals that are generated in the body as these free radicals accelerate hyper pigmentation syndrome, heart disease, stroke, atherosclerosis, diabetes and cancer[1,2]. Recently huge interest has been generated in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side effects like carcinogenicity, etc[3,4].

It is well established that Amla is a good source of total phenolic content, antioxidants, flavones, tannins and other bioactive compounds[5]; these substances may contribute to the health effects of Amla. However, being a seasonal fruit its availability as a fresh fruit is for a limited period. Hence spray drying of the fruit juice may be a good alternative to make its health promoting components available throughout the year.

Fruit juice powders have number of benefits over the liquid counterparts such as reduced volume or weight, reduced packaging, easier handling, transportation and longer shelf life[6]. Spray dried powders have good reconstitutional characteristics, have low water activity and are suitable for storage. Spray drying technique is also appropriate for heat sensitive components. Maltodextrin, gum arabic and gelatin are successfully used as drying aids to facilitate drying. Maltodextrin is one of the common drying aids for spray drying owing to its beneficial role as a carrier or an encapsulating agent. Workers have used carrier agents to protect heat sensitive components like vitamin C in camu-camu[7] and to increase stability of products in acerola powder[8]. However, there are no reported studies on spray drying of Amla juice powder to retain functional properties like total phenolic content and DPPH* scavenging activity.

A study was conducted wherein Amla juice was spray dried with different maltodextrin concentrations at different inlet temperatures. The effect of spray drying on the physicochemical properties, total phenolic content and DPPH* scavenging activity of spray dried Amla powder are reported in this chapter.

6.2. Materials and methods

6.2.1. Chemicals

Chemicals for present investigation i.e. Folin-Ciocalteu reagent (FCR), sodium carbonate, 2, 2, diphenyl picryl hydrazil (DPPH) and ethanol were purchased from Merck.

6.2.2. Raw materials

Amla of *Chakaiya* variety was procured from local market in Allahabad, India. The Amla was cleaned thoroughly under tap water to remove adhering dust and wiped with muslin cloth. The washed fruits were used for development of spray dried Amla juice powder.

6.2.3. Process standardization for development of spray dried Amla powder

Amla of *Chakaiya* variety containing 83.40±1.10% moisture content, 11.10±0.83% carbohydrates, 2.32±0.21% crude fibre on fresh wt basis and 26.5±1.12 g/100 g total phenolic content equivalent to gallic acid on dry wt basis were used. Amla fruits were cut into small pieces and pulped in a laboratory grinder. Juice was extracted by straining through double fold muslin cloth and concentrated upto 40% in rotary evaporator at 70°C temperature. Maltodextrin of varying concentration (5-9% w/v of initial juice) was added and stirred for 15 min in a stirrer. The juice was fed into spray dryer and spray dried at inlet temperatures ranging from 125° to 200°C. The spray dried powder was packed into laminates and stored at 4°C temperature for further analysis.

Preliminary spray drying trials showed that at maltodextrin level of 3% most of the material stuck on chamber wall, while at concentration above 9% a significant decrease in the DPPH* scavenging activity in finished spray dried Amla powder was observed. Hence, levels of maltodextrin used in the study varied between 5% and 9%. Feed material for all the formulations came from a single batch of Amla juice.

6.2.3.1 Spray drying conditions of fresh Amla juice powder

The feed comprising of maltodextrin and juice was spray dried in Lab plant LU 20 lab spray dryer (Labultima, Mumbai, India). The inlet temperatures/measured outlet temperatures were 125°C/81°C, 150°C/93.5°C, 175°C/103°C and 200°C/119°C. The compressor pressure, air flow rate and feed rate were constant at 0.12 MPa, 75±1.5 m^3/h and 13-15 mL/min, respectively. Drying conditions were selected on the basis of final moisture content in the finished spray dried Amla powder. All formulations for spray drying were carried out in duplicates.

6.2.4. Analytical methods

6.2.4.1. WSI (Water solubility index)

The WSI of the powder was determined using the method described by Anderson et al. (1969)[9]. Spray dried Amla juice powder (2.0 g) and distilled water (25 mL) were vigorously mixed in a 100 mL centrifuge tube, incubated in a 37°C water bath for 30 min and then centrifuged for 20 min at 7895 *g* (Sigma, 13 K, Germany). The supernatant was carefully collected in a pre-weighed beaker and oven dried at 103±2°C. The WSI (%) was calculated as the percentage of dried supernatant with respect to the amount of the original Amla powder.

6.2.4.2. Hygroscopicity

For hygroscopicity, 1.5 g of the powder was placed at 25°C in an airtight container containing saturated solution of sodium carbonate. Sample was weighed after 1 week and hygroscopicity was expressed as gram of adsorbed moisture per 100 g of powder[10].

6.2.4.3. Color characteristics of spray dried Amla powder

The color characteristics of the spray dried amla powder were analyzed by using Hunter Color Lab (Ultra scan VIS, USA) calibrated with white tiles. Obtained results were expressed as Hunter color values *L*, *a* and *b* where *L* denotes lightness and darkness, *a* redness and greenness and *b* yellowness and blueness. Powders were

packed in polyethylene pouches and were measured for color characteristics. The samples were analyzed in triplicates. Color intensity in terms of chroma was calculated by the formula $(a^2 + b^2)^{1/2}$, whereas hue angle (H^0) was calculated by the formula H^0 = arc tan (b/a). The hue values of 0°, 90°, 180° and 270° denote pure red, pure yellow, pure green and pure blue color respectively. The ratio of a/b was also estimated for color measurement of spray dried Amla powder [11].

6.2.4.4. Bulk density

Briefly, 2.0 g of Amla powder was added in 10 mL of graduated measuring cylinder and the mixture was vortexed for 1 min. Bulk density of the powder was calculated by measuring the ratio of mass of powder to the volume occupied by the powder [6].

6.2.4.5. Total phenolic content

Estimation of total phenolic content was performed by Folin–Ciocalteu method described by Liu et al. (2008)[12] with some modifications. Briefly, 250 mg of sample was mixed with 10 mL of 60% acetone and the mixture was stirred for 30 min at 30° C. Then 60 µL of supernatant, 300 µL of Folin - Ciocalteau reagent and 750 µL of 20% sodium carbonate in water were added in 4.75 mL of water. The mixture was allowed to stand for 30 min. The absorbance was measured at 765 nm using double beam spectrophotometer (Evolution 600, Thermoscientific) and the results are expressed as mg of GAE. A standard curve of absorbance vs concentration was plotted using gallic acid standard at various concentration (ranging from 50-500 mg/L).

6.2.4.6. 2,2, Diphenyl picryl hydrazil (DPPH*) radical scavenging activity

The DPPH* scavenging activity of extract was determined by the method of Luo et al. (2009)[13] with slight modifications. Briefly, 2 mL of extract were mixed with 2 mL methanolic solution containing 1mM DPPH. The mixture was shaken vigorously and then left to stand for 30 min in the dark. The absorbance was measured at 517 nm using double beam spectrophotometer (Evolution 600, Thermoscientific). The absorbance of control was obtained by replacing the sample with methanol. DPPH radical scavenging activity of the sample was calculated as follows:

$$\text{DPPH* scavenging activity (\%)} = \frac{(\text{absorbance of control} - \text{absorbance of sample}) \times 100}{\text{absorbance of control}}$$

6.2.5. Scanning electron micrograph (SEM)

Particle morphology was evaluated by SEM. Powders were attached to a double sided adhesive tape on SEM stubs, coated with 3-5 mA palladium under vacuum and examined with a JEOL scanning electron microscope (JSM-6390 LV, Japan, PN junction type, semi conducting detector). SEM was operated with 15 KV at magnifications of 1000X and 5500X.

6.2.6. Statistical analysis

Spray drying experiments were carried out in duplicate and analyses were carried out in triplicates. Obtained mean values were analyzed by analysis of variance (ANOVA). The graphs of mean value and error bar were created by using Excel version of 2003.

6.3. Results and discussion

6.3.1. Effects of spray drying conditions on physical properties of Amla powder

The effects of maltodextrin concentration, aspiration speed and different drying temperatures on the physical properties of the Amla juice powder are shown in Table 1. The levels of maltodextrin used for development of the Amla powder varied between 5 - 9% (w/v) which were less than 10-30% that were used by Tonon et al. (2008)[14], Abadio et al. (2004)[15], and Kha et al. (2010)[16]. Increase in the maltodextrin concentration resulted in a significant (5% probability level) decrease in moisture concentration in the finished powder. Moisture content of sample decreased from 5.6 to 3.8% as maltodextrin level increased from 5 to 9% (**Table 6.1**). Abadio et al. (2004)[15] there was also found a decrease in moisture content in final pineapple juice powder with increase in the level of maltodextrin from 10-15 % (w/v). Increased level of maltodextrin increased the level of feed solids and reduced the level of total moisture for evaporation[16-17].

Increase in drying temperature from 125°C to 200°C also resulted in significant decrease in the moisture content in amla powder from 6.3 to 3.8% (**Table 6.1**). Due to the increased rate of heat transfer into the particles at higher temperature, there was a greater driving force for moisture evaporation causing faster water removal[18,19]. The present findings were in agreement with the results obtained for

spray dried tomato powder[6], orange juice powder[20], cactus pear juice powder[21], black carrot powder[22] and gac juice powder[16].

Maltodextrin level had significant ($p<0.05$) effect on the hygroscopicity of the amla powder. Hygroscopicity was lowest when 9% maltodextrin was used for encapsulation. Rodriguez-Hernandez et al. (2005)[21] and Cai and Corke, (2000)[10] also observed a reduction in hygroscopicity with increasing maltodextrin concentrations in spray dried cactus pear juice powder and betacyanin pigments, respectively. Maltodextrin is a material having the property of low hygroscopicity and its utility as a carrier material for spray drying has been established[14]. Inlet temperature also influenced the hygroscopicity of the powder significantly. The highest hygroscopicity value of 56.32 g/100 g for Amla powder was obtained at 125°C inlet temperature. When inlet temperature of processing was increased the hygroscopicity of Amla powder was decreased. The present findings are in agreement with Moreira et al. (2009)[23] but contradicts the findings of Goula et al. (2004)[6] and Tonon et al. (2008)[14] in their work on spray drying of tomato pulp and acai juice powder, respectively. Amla powder showed greater tendency to adsorb moisture which may be due to the presence of higher level of carbohydrates in Amla fruit. Aspiration speed showed no significant effect on hygroscopicity of the powders.

Drying temperature did not show any significant effect on WSI of the Amla powder (**Table 6.1**) at 5% probability level (**Table 6.1**). Sousa et al. (2008)[24] also observed that drying conditions had no significant effect on WSI of tomato powder[24]. In the present study, WSI of Amla powder ranged from 91.34 to 94.98%. These values were higher when compared with 17.65-26.3% in spray dried tomato powder[24], 36.91-38.25% in gac powder[16] and 81.56% in pineapple juice powder[15]. The excellent WSI of the Amla juice powder could be due to the high content of free phenolics[25], the significant level of carbohydrates and the low level of fat (0.05% in fresh fruit) in the juice. According to Kumar et al. (2008)[25], 97.67% of total phenolics are present in free form in Amla fruit[25]. The dissolved solids in the Amla juice are highly water soluble. These soluble solids are mostly carbohydrates and free phenolics. Thus, free phenolics and soluble carbohydrates account for the high WSI in Amla juice powder. The high WSI observed in Amla powder makes it suitable for reconstitution. While maltodextrin level did not have significant effect on the bulk density of Amla powder

at 5% probability level, the drying temperature showed a significant effect (**Table 6.1**). A decrease in the density of the powder was observed with an increase in the inlet temperature. This finding is consistent with those by Walton and Mumford, (1999)[27], Cai and Corke, (2000)[10], Goula et al. (2004)[6] and Kha et al (2010)[16]. As explained by Jumah et al. (2000)[27], Walton, (2000)[28] and Chegini and Ghobadian, (2005)[20], the high rate of drying being rapid at very high temperatures meant that there was less droplet shrinkage, giving lower powder density. This explanation is supported by our findings. Aspiration speed had no significant effect on the bulk density of the powder.

Table. 6.1 Physicochemical properties of spray dried Amla powder

Particular		MC (%)	Hygroscopicity	WSI (%)	Bulk density (g/mL)
Maltodextrin concentration (MDC) (%)	3	ND	ND	ND	ND
	5	5.6 ±0.11^{a}	53.01±1.32^{a}	93.28±2.33^{a}	0.52±0.01^{a}
	7	4.07±0.15^{b}	47.74±1.04^{b}	94.11±1.87^{a}	0.49±0.03^{a}
	9	3.83±0.42^{c}	46.03±0.98^{c}	93.32±2.41^{a}	0.51±0.04^{a}
Inlet temperature (°C) (DT)	100	ND	ND	ND	ND
	125	6.32+0.31^{a}	56.32 ±1.22^{a}	94.98±1.98^{a}	0.61±0.02^{a}
	150	5.29±0.35^{b}	49.82 ±2.12^{b}	93.14±1.67^{a}	0.55±0.01^{b}
	175	4.54+0.28^{c}	47.02 ±1.98^{c}	92.36±2.03^{a}	0.52±0.01^{c}
	200	3.83±0.42^{d}	46.74 ±1.46^{d}	94.34±1.90^{a}	0.49±0.03^{d}
Aspiration speed (AS)	30	3.83±0.32^{a}	46.74±1.33^{a}	94.89±2.37^{a}	0.49±0.03^{a}
	40	4.01±0.29^{b}	47.08±2.09^{a}	93.28±2.01^{a}	0.52±0.18^{a}
	50	4.32±0.24^{c}	47.34±1.49^{a}	91.43±2.52^{a}	0.52±0.23^{a}
Significant interaction	MDC	**	***	NS	NS
	DT	***	**	NS	***
	MDCxDT	**	**	NS	

ND : not detected, NS : non significant; **, *** ; significant at p = 0.05 & p = 0.01 respectively. The values of the same column with different superscript differ significantly

6.3.2. Particle morphology

Fig. 6.1 shows the SEM micrographs of Amla juice powder produced with 9% maltodextrin at different inlet temperatures. It was observed that the number of particles in a given amount of the powder increased with an increase in inlet temperature. Similar findings were also reported by Tonon et al. (2008)[14]. Inlet temperature had no effect on the surface smoothness of the particles. This however contradicts the observation of Allamila- Beltran et al. (2005)[29], Nijdam and Langrish (2006)[30] and Tonon et al. (2008)[14]. SEM study (**Fig 6.1**) revealed that the average size of particles in the powder that was dried at higher inlet temperature was smaller than the particles in powder dried at lower inlet temperature. Similar finding was observed by Cai and Corke, (2000)[10] for spray drying of amaranthus betacyanin pigments. Probably, the particle size got fixed as large sized globules when there was more water in the material that was being dried. At higher inlet temperature, due to rapid rate of drying the particles got fixed as smaller sized globules. Image of spray dried Amla juice powder is shown in **Fig 6.2.**

6.3.3. Color characteristics of powder

Fig. 6.3 shows the effect of different concentrations of maltodextrin and inlet temperatures on color characteristics of spray dried powder. In general, the color attributes of the powder was significantly affected by maltodextrin and temperature. The lightness of the powders was significantly affected by maltodextrin concentration ($p<0.01$) when powders were produced at inlet temperatures below 200°C. Highest value of *a/b* and lowest value of hue angle were obtained with 5% concentration of maltodextrin at 200°C while no specific trend was observed for 7% and 9% levels of maltodextrin. No specific trend could be observed for chroma of the powder when produced at different inlet temperatures and maltodextrin levels. Spray drying temperature showed statistically significant effect on the color characteristics i.e lightness, *a/b*, chroma and hue value. A significant effect on lightness of the powder was observed when inlet temperature was increased from 125° to 200°C temperature.

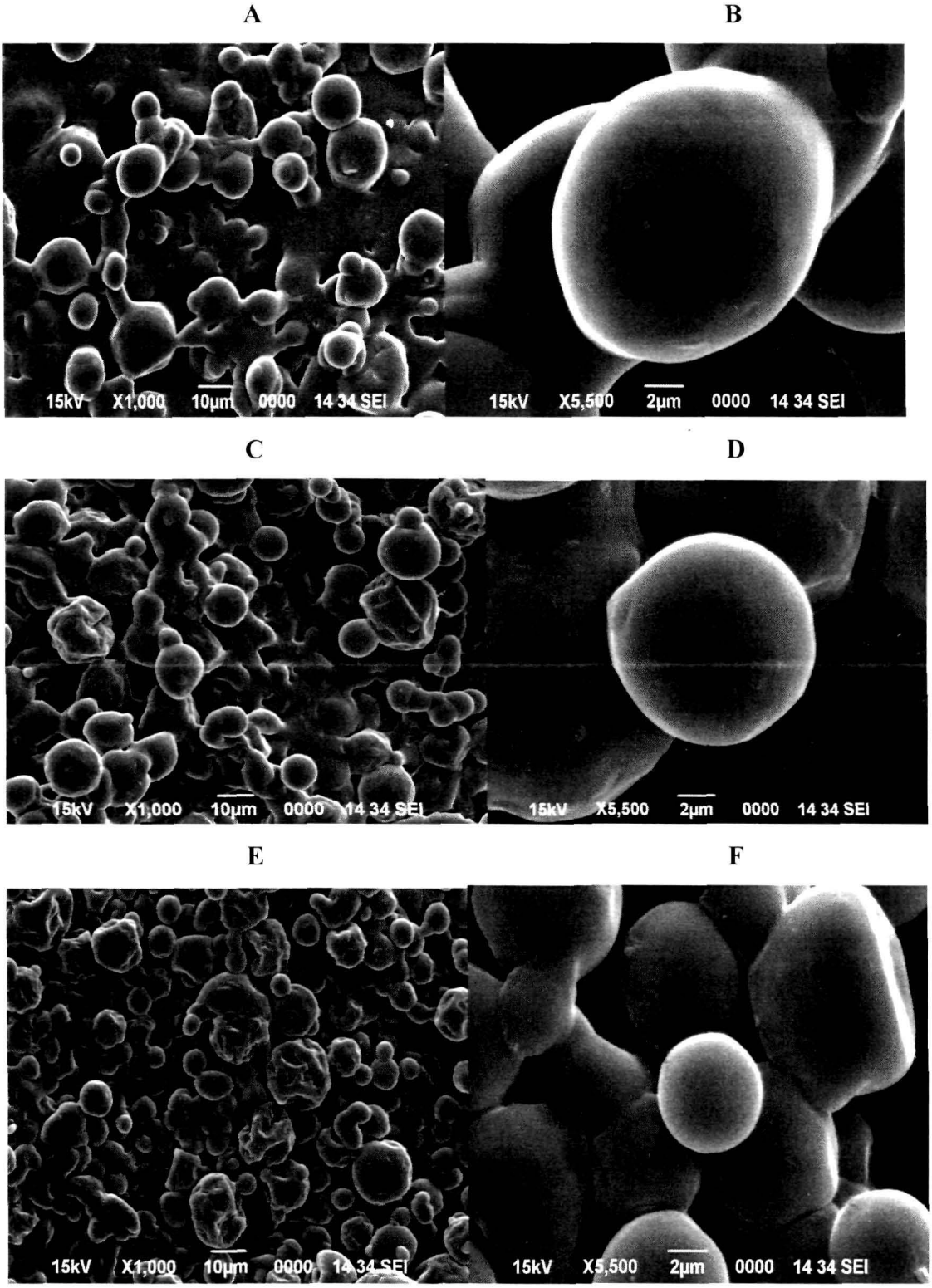


Fig 6.1 Micrographs of particles at different inlet temperature and constant maltodextrin level (9%) at magnifications of (a) 150°C, 1000 X, (b)150°C, 5500 X, (c) 175°C, 1000X, (d) 175°C, 5500 X, (e) 200°C, 1000X, and (f) 200°C, 5500 X.

An increase in inlet temperature produced significantly ($p<0.001$) lighter product than powder produced at lower inlet temperature. Greatest degree of lightness of spray dried Amla powder at highest inlet temperature indicates that the pigments had undergone oxidation. Similar results were observed by Sousa et al. (2008)[24] in spray dried tomato powders. The probable reason for higher degree of lightness of the Amla powder at higher inlet temperature may be attributed to the reduced rate of oxidation of the tannins. Tannins react slowly with iron in the absence of oxygen and form dark coloured complex. Probably, during the rapid rate of drying in a spray dryer, tannins get very little time to react with iron as availability of oxygen is reduced and therefore the powder colour had lessened.

Loss of redness of samples increased, resulting in low *a/b* value and high hue angle, when inlet temperature was increased from 125° to 175°C, however above 175°C temperature high *a/b* and low hue angle was found in the powder. Similar findings were reported by Graboswki et al. (2006)[17] for sweet potato powder, by Abadio et al. (2004)[15] for pineapple juice powder and by Kha et al. (2008)[16] for gac juice powder. Higher maltodextrin level and higher inlet temperature resulted in low *a/b* value and high hue angle which are in concurrence with Chen et al. (1995)[31]. The probable reason may be that spray drying increased the surface area causing rapid pigment oxidation (Desobry et al., 1997)[32]. Reduced formation of iron tannate complex at high inlet temperature is also speculated which needs further studies for confirmation.

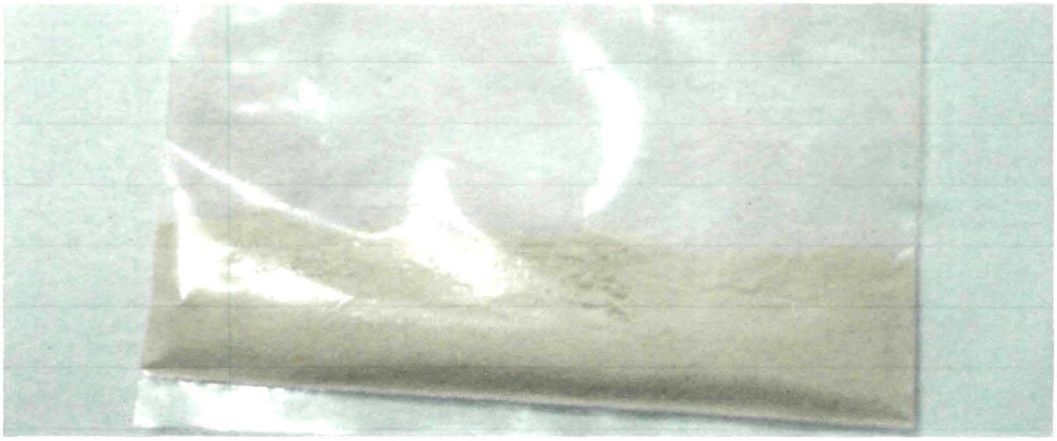


Fig 6.2 Image of spray dried Amla powder.

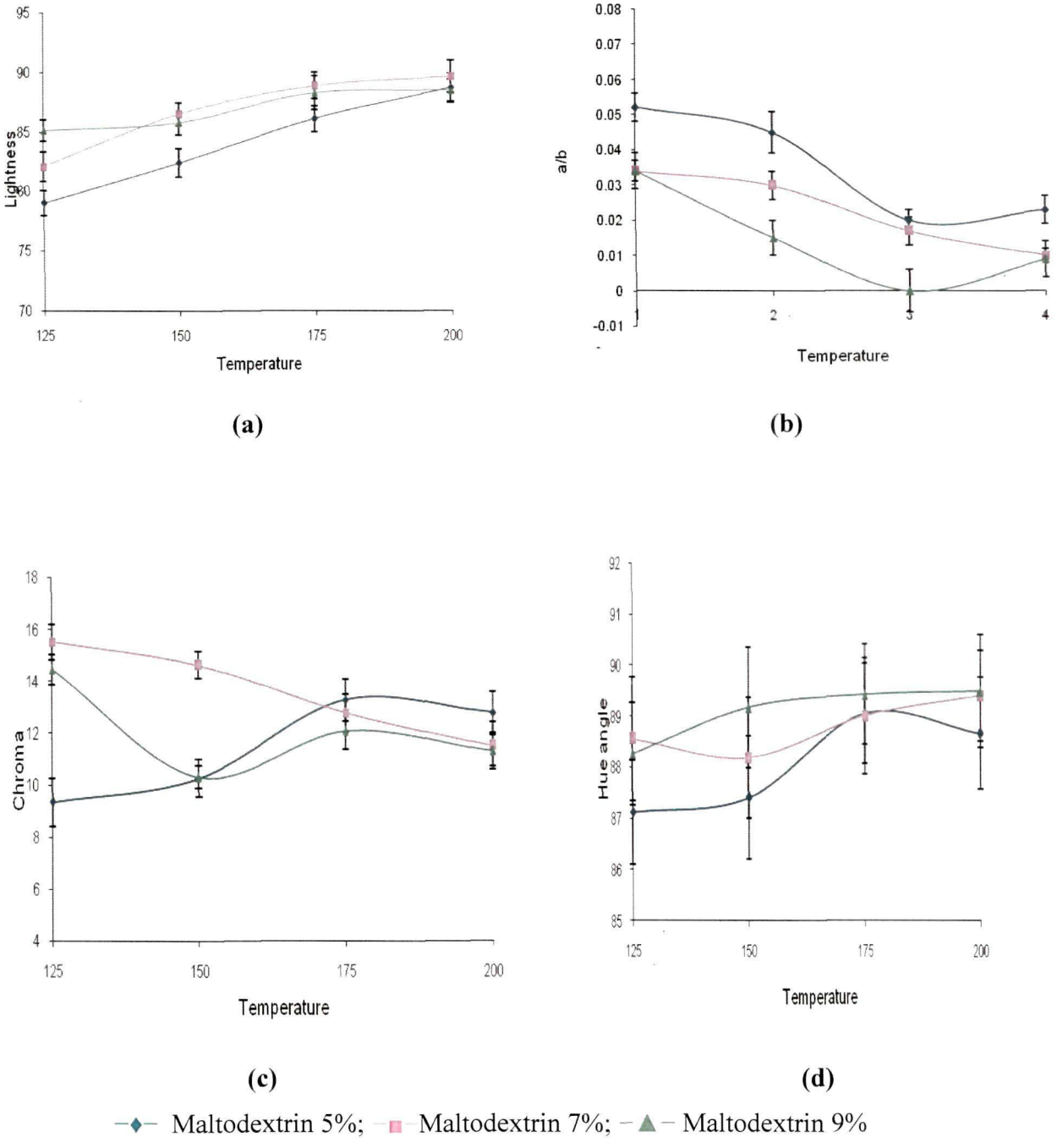
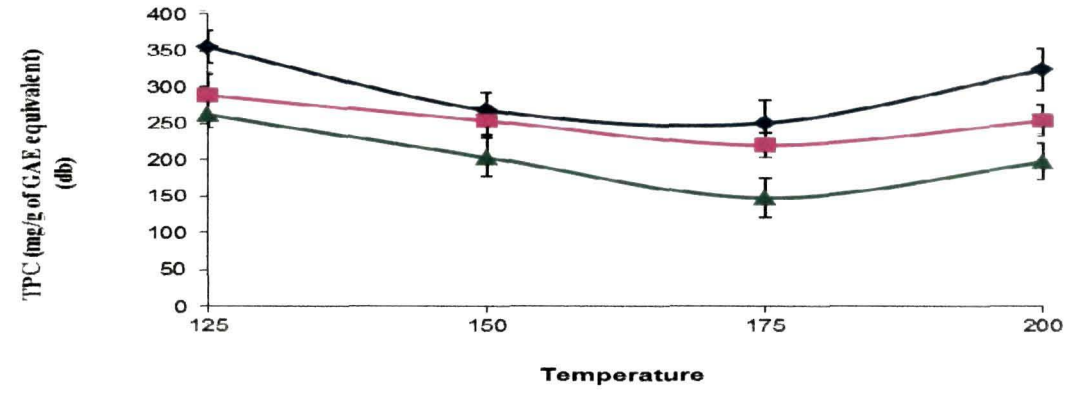


Fig. 6.3 The color characteristics of spray dried Amla powder (a) lightness, (b) ratio of *a/b*, (c) chroma, and (d) hue angle at different inlet temperatures and maltodextrin level.

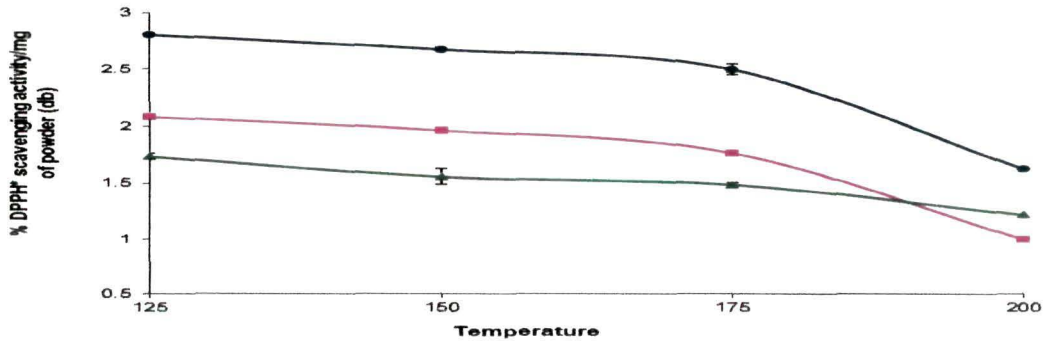
6.3.4. Effect of spray drying conditions on total phenolic content of Amla powder

Fig. 6.4a shows the effect of processing conditions on total phenolic content (TPC) of spray dried powder. Drying temperature and maltodextrin concentration showed significant effect on TPC of spray dried powder. TPC was significantly ($p<0.001$) reduced when inlet temperature was increased from 125° to 175°C temperature, however above 175°C there was a reverse trend. The reason for increased TPC content in the powder above 175°C may be because of the polymerisation as well as synthesis of polyphenols at 200°C which increases the total phenolic content of the powder.

TPC content of the powders was significantly reduced when the concentration of maltodextrin was increased from 5 to 9%. This can be explained to be due to the concentration effect of maltodextrin.



(a)



(b)

—♦— Maltodextrin 5%; —■— Maltodextrin 7%; —▲— Maltodextrin 9%

Fig. 6.4 Effect of spray drying conditions on (a) total phenolic content, and (b) DPPH* scavenging activity of Amla powder.

6.3.5. Effect of spray drying conditions on free radical scavenging activity of powder

Fig. 6.4b shows the effect of spray drying conditions on DPPH* scavenging activity of amla powder. Maltodextrin level and drying temperature showed statistically significant effect ($p<0.01$) on the DPPH* scavenging activity of the powder. DPPH* scavenging activity of the powder was significantly affected by increased inlet temperature. Overall, on increasing the inlet temperature from 125°C to 200°C a significant decrease in DPPH* scavenging activity was observed. Similar results were observed in spray dried gac juice powder by Kha et al. (2008)[16]. The possible explanation for the low free radical scavenging activity may be because of the exposure to higher temperatures which adversely affected the structure of phenolics causing its break down and/or synthesis into different forms.

Increase in the maltodextrin concentration which itself has no free radical scavenging activity, resulted in lower DPPH* scavenging activity. Powders containing 5% maltodextrin level showed significantly higher free radical scavenging activity (dry wt basis) than 7 and 9% of maltodextrin. This may be the dilution effect of maltodextrin when its concentration was raised. Obtained results contradicted the result of Kha et al. (2008)[16] who found that on varying the maltodextrin level from 10-20% there was no significant effect on total antioxidant activity of gac juice powder.

6.4. Conclusion

The effect of spray drying conditions on physicochemical properties of Amla powder was evaluated. Maltodextrin concentration (5-9%) and drying temperature (125°C to 200°C) significantly affected moisture content, bulk density, hygroscopicity, color attributes, TPC and DPPH* scavenging activity. However WSI was not significantly influenced by varying the concentration of maltodextrin or inlet temperature. The developed spray dried powder showed excellent water solubility that is essential for reconstitution. Amla juice powder dried at 175°C with 7% maltodextrin was adequately effective to produce powder with less hygroscopicity, acceptable color in terms of *L*, *a* and *b* and potent free radical scavenging activity. Spray dried Amla juice powder thus can be made into a health promoting reconstituted drink.

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Chapter-7
Process standardization for development of spray dried lemon powder and optimization of Amla-lemon based RTS drink using Response Surface Methodology

7. 1. Introduction

Fruit juice powders have number of benefits over their liquid counterparts i.e. reduced volume or weight, reduced packaging cost, easier handling and transportation and longer shelf life[1]. Spray dried powders have good reconstituting characteristics, low water activity and are suitable for storage. Spray drying technique is also an appropriate technique for heat sensitive components. Maltodextrin is one of the common drying aids for spray drying owing to its beneficial role as a carrier or as an encapsulating agent.

Being very astringent in nature the acceptability of Amla is comparatively less as a raw fruit than other commercially available fruits. Hence processing becomes necessary to enhance the palatability of the fruits. Several value added products like squash, preserve, candy etc. have been processed from Amla. But excessive processing reduces the valuable components from the Amla significantly. Lemon fruit is a rich source of nutrients, including vitamin C, minerals, citric acid and flavonoids which provide health benefits[2]. Being the third most important citrus crop, availability of good is lemon [2]. Spray dried lemon juice powder was used to enhance the acceptability and nutritive value of Amla based fruit concentrate. In the present study processing conditions for development of spray dried lemon juice powder were optimized. Spray dried Amla juice powder processed with optimized conditions (Chapter 6) and spray dried lemon juice powder were taken as raw materials for development of Amla-lemon based RTS using response surface methodology and the developed RTS was further investigated.

7.2. Materials and methods

7.2.1 Materials

Chemicals for present work i.e. Folin-ciocalteu reagent (FCR), sodium carbonate, 2, 2, diphenyl picryl hydrazil (DPPH) and ethanol were purchased from Merck.

7.2.1.1. Raw materials

Chakaiya variety of Amla fruits was procured from local market of Allahabad, India whereas lemon of *Kaji* variety was procured from local market of Assam, India.

The lemon was washed thoroughly to remove adhering dust and wiped with muslin cloth.

Lemon

↓

Cutting into two halves

↓

Extraction of juice using lemon squeezer

↓

Filtration of juice using double fold muslin cloth

↓

Concentration of juice to 40% in rotary evaporator (at 70°C)

↓

Addition of maltodextrin (10-20% w/v of initial juice)

↓

Stirring for 15 min

↓

Spray drying at desired inlet temperatures (125°-200°C)

↓

Packing in laminated packets and storing at 4°C

Fg 7.1 Flow chart for the preparation of spray dried lemon powder

7.2.2. Process standardization for development of spray dried lemon powder

Preliminary drying trials showed that when the concentration of maltodextrin was lower than 10% most of the material stuck to chamber wall and when the concentration was higher than 20%, there was a significant decrease in the free radical scavenging activity of powder. Lemon juice taken for present study was of 2.5 pH and 7.33°B. Feed material for all the formulations was taken from a single batch of lemon juice.

The feed mixtures containing maltodextrin and juice were spray dried in Lab plant LU 20 lab spray dryer (Labultima, Mumbai India). The inlet temperatures/measured outlet temperatures used were 125°C/83°C, 150°C/92.5°C, 175°C/101°C and 200°C/113°C. The compressor pressure, air flow rate and feed rate

were kept constant at 0.06 MPa, 65±2 m^3/h and 13-15 mL/min, respectively. All formulations for drying were carried out in duplicate.

7.2.3. Analysis of spray dried lemon powder

7.2.3.1. Water solubility index (WSI)

The WSI of the lemon powder was determined using the method described by Anderson et al. (1969)[3]. Spray dried lemon juice powder (2.0 g) and distilled water (25 mL) were vigorously mixed in a 100 mL centrifuge tube, incubated in a water bath at 37°C for 30 min and then centrifuged for 20 min at 7895 *g* (Sigma, 13 K, Germany). The supernatant was carefully collected in a pre-weighed beaker and oven dried at 103±2°C. The WSI (%) was calculated as the percentage of dried supernatant with respect to the amount of the original lemon powder.

7.2.3.2. Hygroscopicity

For hygroscopicity, 1.5 g of the lemon powder was placed at 25°C in an airtight container containing saturated solution of sodium carbonate. Sample was weighed after 1 week and hygroscopicity was expressed as gram of adsorbed moisture per 100 g of powder[4].

7.2.3.3. Color characteristics of spray dried lemon powder

The color characteristics of the spray dried lemon powder were analyzed by using Hunter Color Lab (Ultra scan VIS, USA) calibrated with white tiles. Obtained results were expressed as Hunter color values *L*, *a* and *b*. Powders were packed in polyethylene pouches and were measured for color characteristics. The samples were analyzed in triplicates. Color intensity in terms of chroma was calculated by the formula $(a^2+b^2)^{1/2}$, whereas hue angle (H°) was calculated by the formula H° = arc tan (*b*/*a*). The hue values of 0°, 90°, 180° and 270° denote pure red, pure yellow, pure green and pure blue color, respectively. The ratio of *a*/*b* was also estimated for color measurement of spray dried lemon powder[5].

7.2.3.4. Bulk density

Briefly, 2.0 g of lemon powder was added in 10 mL of graduated measuring cylinder and the mixture was vortexed for 1 min. Bulk density of the powder was

calculated by measuring the ratio of mass of powder to the volume occupied by the powder[1].

7.2.3.5. Total phenolic content

Estimation of total phenolic content was performed by Folin-Ciocalteu method described by Liu et al. (2008)[6] with some modifications. Briefly, 250 mg of sample was mixed with 10 mL of 60% acetone and the mixture was stirred for 30 min at 30° C. Then 60 µL of supernatant, 300 µL of Folin-Ciocalteau reagent and 750 µL of 20% sodium carbonate in water were added in 4.75 mL of water. The mixture was allowed to stand for 30 min. The absorbance was measured at 765 nm using double beam spectrophotometer (Evolution 600, Thermoscientific) and the results are expressed as mg of GAE.

7.2.3.6. 2,2, Diphenyl picryl hydrazil (DPPH*) radical scavenging activity

The DPPH* scavenging activity of extract was determined by the method of Luo et al. (2009)[7] with slight modifications. Briefly, 2 mL of extract was mixed with 2 mL methanolic solution containing 1mM DPPH. The mixture was shaken vigorously and then left to stand for 30 min in the dark. The absorbance was measured at 517 nm using double beam spectrophotometer (Evolution 600, Thermoscientific). The absorbance of control was obtained by replacing the sample with methanol. DPPH radical scavenging activity of the sample was calculated as follows:

$$\text{DPPH* scavenging activity (\%)} = \frac{(\text{absorbance of control} - \text{absorbance of sample}) \times 100}{\text{absorbance of control}}$$

7.2.4. Scanning electron microscopy (SEM)

Particle morphology was analyzed by scanning electron microscope (SEM). Powders were attached to a double sided adhesive tape on SEM stubs, coated with 3-5 mA palladium under vacuum and were examined with a JEOL scanning electron microscope (JSM-6390 LV, Japan, PN junction type, semi conducting detector). SEM was operated with 15 KV at magnification of 5500X and 6500X.

7.2.5. Optimization of Amla-lemon based RTS

A central composite rotatble design (CCRD) with three numerical factors was employed to design the experiments. The numerical factors were Amla powder, lemon

powder and citric acid. The minimum and maximum level of the variables were varied from 15-35%; 15-35% and 1-2% for Amla powder, lemon powder and citric acid, respectively. Amla powder 25%, lemon powder 25% and citric acid 1.5% were repeated five times as central point. The formulation was made up to 100% with glucose powder. A total of 20 experiments were performed (**Table 7.1**). The whole mixture was homogenized in a mixer. All twenty combinations were subjected for sensory quality evaluation by semi trained panellists. Proximate analysis of the optimized product was carried out by AOAC (18^{th} edition)[8].

7.2.5.1. Mineral analysis

Minerals were analyzed by the method given by FAO (1983)[9] with slight modifications. Briefly, 2 g of sample was placed in Kjeldahl tubes and freshly prepared nitric acid-sulphuric acid mixture (25 mL) in the ratio of 1.5:1 was added. The sample was digested at 250°C for 2-3 h or until a clear solution was obtained. After cooling the solution was diluted with 100 mL deionized water and the residue was filtered through an ashless filter paper. The mineral content of the sample was determined by atomic absorption spectroscopy (AAS) (Thermo, ICE 2000) with air acetylene flame for Ca, P, Fe, Na, K.

7.2.5.2. Sensory analysis

For sensory evaluation, 2.5 g of the prepared formulations and 15 g of sugar were mixed with 100 mL of water and kept at refrigerator temperature before serving to the panellists. A panel comprising of 15 trained panellists sat in individual booths and were asked to grade each sample on a 9 point hedonic scale with respect to colour, flavour and overall acceptability[10].

7.2.5.3. Storage study

The Amla-lemon based RTS was packed in laminates under two different conditions i.e (i) with N_2 flushing and (ii) without N_2 flushing and the packed samples were further stored at two different temperatures i.e. ambient and refrigeration (7°C) temperatures. The RH was kept constant at 75%. The samples were drawn an interval of 15 days and were analyzed for DPPH* scavenging activity, total phenolic content, moisture content and color characteristics.

Table 7.1 Central composite rotatable design with experimental values of response variables for optimization of Amla lemon based RTS

Amla powder (%)	Lemon powder (%)	Citric acid (%)	Flavor	Color	Overall acceptability	TPC (mg GAE/100g)
15.00	15.00	1.00	7.10	7.60	6.80	2.32
25.00	25.00	2.34	6.50	7.20	6.70	4.81
35.00	35.00	2.00	5.80	7.70	5.20	6.41
35.00	35.00	1.00	6.80	7.50	5.90	6.48
15.00	15.00	2.00	7.50	7.70	6.00	2.29
25.00	25.00	1.50	7.20	7.20	7.80	4.56
25.00	25.00	1.50	7.20	7.20	7.80	4.56
15.00	35.00	1.00	6.50	7.60	6.80	2.89
25.00	25.00	1.50	7.20	7.20	7.80	4.56
41.82	25.00	1.50	5.10	7.10	5.10	7.38
41.82	25.00	1.50	5.10	7.10	5.10	7.38
8.18	25.00	1.50	7.50	8.20	8.00	1.12
25.00	8.18	1.50	6.60	6.50	6.20	4.31
25.00	25.00	1.50	7.20	7.20	7.80	4.56
25.00	41.82	1.50	7.20	6.80	7.10	5.26
35.00	15.00	1.00	6.10	7.40	5.20	6.10
25.00	25.00	1.50	7.10	7.50	7.70	4.56
25.00	25.00	1.50	7.20	7.50	7.70	4.56
35.00	15.00	2.00	4.90	7.70	4.80	6.15
25.00	25.00	0.66	6.90	7.50	7.00	4.51
15.00	35.00	2.00	7.00	7.70	8.10	2.79

7.2.6. Statistical analysis

Spray drying experiments were carried out in duplicate and analyses were carried out in triplicates. Obtained mean values were analyzed by analysis of variance (ANOVA). The graphs of mean value and error bar were created by using Excel version of 2003.

7.3. Results and discussion

7.3.1. Effect of spray drying conditions on physical properties of lemon juice powder

The effect of maltodextrin concentration, aspiration speed and different drying temperatures on the physical properties of lemon juice powder is given in **Table 7.2**. Maltodextrin was taken at 10, 15 and 20% levels. Increase in the concentration of maltodextrin resulted in a decrease in moisture concentration in the finished powder from 4.59 to 3.57%, when produced at 175°C (**Table 7.2**). Abadio et al. (2004)[11] found that on increasing the level of maltodextrin from 10 -15 % (w/v), there was decrease in moisture content during spray drying of pineapple juice powder. Increased level of maltodextrin increased the level of feed solids which subsequently reduced the level of total moisture for evaporation[12-13].

A similar trend was found at inlet temperatures of 125°, 150°, 175° and 200°C. A significant decrease in moisture content from 7.03 to 3.89% was observed (**Table7.2**). This is due to the greater driving force for moisture evaporation at higher temperature[14-15]. The present findings were consistent with the results obtained for spray dried tomato powder[1], orange juice powder[16], cactus pear juice powder[17], black carrot powder[18] and gac juice powder[13].

Level of maltodextrin significantly ($p<0.05$) effected the hygroscopicity of the lemon powder; hygroscopicity was lowest with maximum maltodextrin concentration. Similar findings were reported by Cai and Corke, (2000)[4] and Rodriguez-Hernandez et al. (2005)[17] in spray dried betacyanin pigments and cactus juice powder, respectively. On the other hand, inlet temperature influenced the hygroscopicity of the powder positively. The highest hygroscopicity value of 19.44 g/100 g of lemon powder (with 10% maltodextrin) was obtained at 200°C inlet temperature.

Table7.2 Physicochemical properties of spray dried lemon powder

		MC (%)	Bulk density (g/mL)	Hygroscopicity (g/100g)	WSI (%)
Maltodextrin 10 %	125°C	7.03±0.6[a]	0.56±0.1[a]	16.69±0.3[a]	98.09±1.1[a]
	150°C	6.37±0.4[b]	0.51±0.1[b]	17.64±0.5[b]	97.44±1.0[a]
	175°C	4.59±0.3[c]	0.48±0.0[c]	17.89±0.4[c]	97.94±1.0[a]
Maltodextrin 15 %	125°C	6.58±0.3[a]	0.54±0.02[a]	14.44±0.3[a]	97.37±0.9[a]
	150°C	5.83±0.4[b]	0.50±0.03[b]	15.10±0.3[b]	94.42±1.4[a]
	175°C	4.12±0.3[c]	0.46±0.03[c]	15.70±0.2[c]	94.86± 1.5[a]
Maltodextrin 20 %	125°C	5.27±0.4[a]	0.55±0.04[a]	13.92±0.4[a]	97.90±1.5[a]
	150°C	4.20±0.3[b]	0.50±0.0[b]	14.28±0.3[b]	96.88±1.1[a]
	175°C	3.67±0.3[c]	0.47±0.0[c]	14.64±0.3[c]	96.52±1.6[a]

values = mean±sd

The values of the same column with different superscript differ significantly ($p<0.05$)

Tonon et al. (2008)[19] explained that at increased inlet temperature lower moisture content provides greater water concentration gradients between the products and surrounding air that increases the tendency of the powder to adsorb moisture. The present findings are in agreement with Goula et al. (2004)[1] and Tonon et al. (2008)[19] in their work on spray drying of tomato pulp and acai juice powder, respectively but contradicts the findings of Moreira et al. (2009)[20].

Drying temperature showed no significant effect on WSI of lemon powder (**Table 7.2**) at 5% probability level (**Table 7.2**). During drying of tomato powder Sousa et al., (2008)[20] also observed that drying conditions had no effect on WSI of powder. In the present study, WSI of lemon powder ranged from 94.34 to 98.08%. These values

were higher as compared to 17.65-26.3% in spray dried tomato powder[21], 36.91-38.25% in gac powder[13] and 81.56% in pineapple juice powder[11]. The excellent WSI of lemon powder indicates its suitability for reconstitution and for further product development.

Maltodextrin level had no effect on bulk density of lemon powder whereas inlet temperature significantly reduced the bulk density of powder (**Table 7.2**). This finding is in agreement with Walton & Mumford, (1999)[22], Cai & Corke, (2000)[4], Goula et al. (2004)[1] and Kha et al. (2010)[13].Several scientists explained that as rate of drying was rapid at very high temperature there was less shrinkage of droplets that resulted in lower density of the powder[23-24,5].

7.3.2. Particle morphology

Fig. 7.2 shows the SEM micrograph of the powders produced with 15% maltodextrin at different inlet temperatures. Inlet temperature showed no effect on the surface smoothness of the particles of the lemon powder which contradicts the observations of Allamila-Beltran et al. (2005)[25], Nijdam and Langrish, (2006)[26] and Tonon et al. (2008)[19], who reported that increased inlet temperature produced particles with smooth surface as compared to powder produced at low inlet temperature. It was observed that the average particle size of powder dried at higher inlet temperature was smaller than that of powder dried at lower inlet temperature and is in agreement with Cai & Corke, (2000)[4], but contradicts the findings of Nijdam & Langrish, (2006)[26] and Tonon et al. (2008)[19].

7.3.3. Color characteristics of powder

The color parameter of lightness and chroma were found to be influenced by increase in level of maltodextrin and inlet temperature (**Fig 7.3a & b**). Lightness increased with level of maltodextrin while a reverse trend was observed for chroma which may be attributed to the protective effect of maltodextrin.

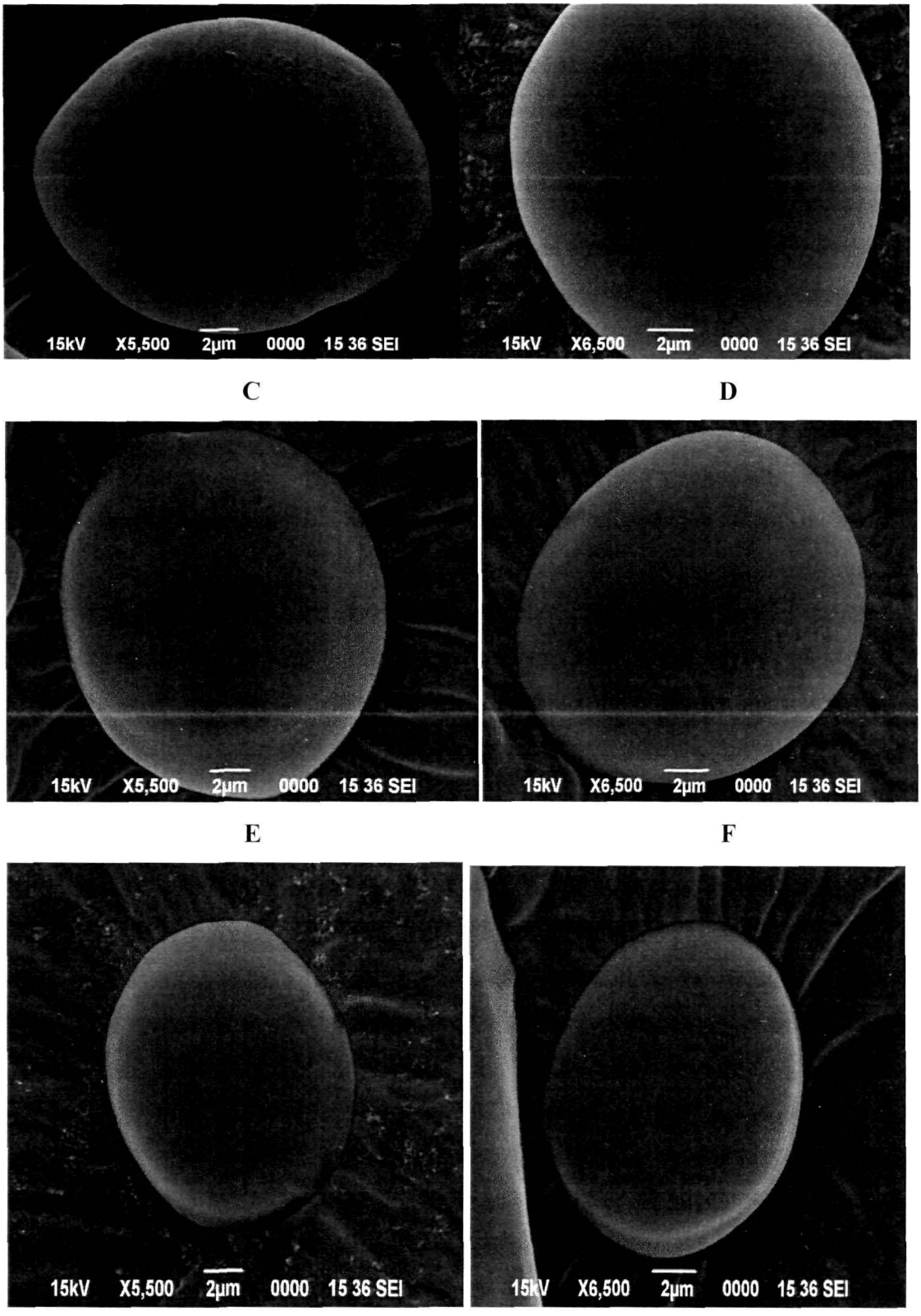


Fig 7.2. Micrographs of particles at different inlet temperature and constant maltodextrin level (15%) and magnifications (a) 150°C, 5500 X, (b)150°C, 6500 X, (c) 175°C, 5500X, (d) 175°C, 6500 X, (e) 200°C, 5500X, and (f) 200°C, 6500 X.

The 15% and 20% maltodextrin incorporated powders had closer lightness and chroma values at the different inlet temperatures studied. Interestingly, the a/b and hue angle values showed that the effect of spray drying with 20% maltodextrin level at temperatures of 125°, 150°, and 175°C was in between that of 10 % and 15% maltodextrin levels (**Fig 7.3c**). However at 200°C, the 20% maltodextrin level substantially decreased as compared to 15% maltodextrin level.

Higher maltodextrin level and higher inlet temperature, resulted in lower *a/b* value and lower hue angle which contradicts Chen et al. (1995)[27]. The probable reason may be that spray drying increased the surface area causing rapid pigment oxidation (Desobry et al., 1997)[28].

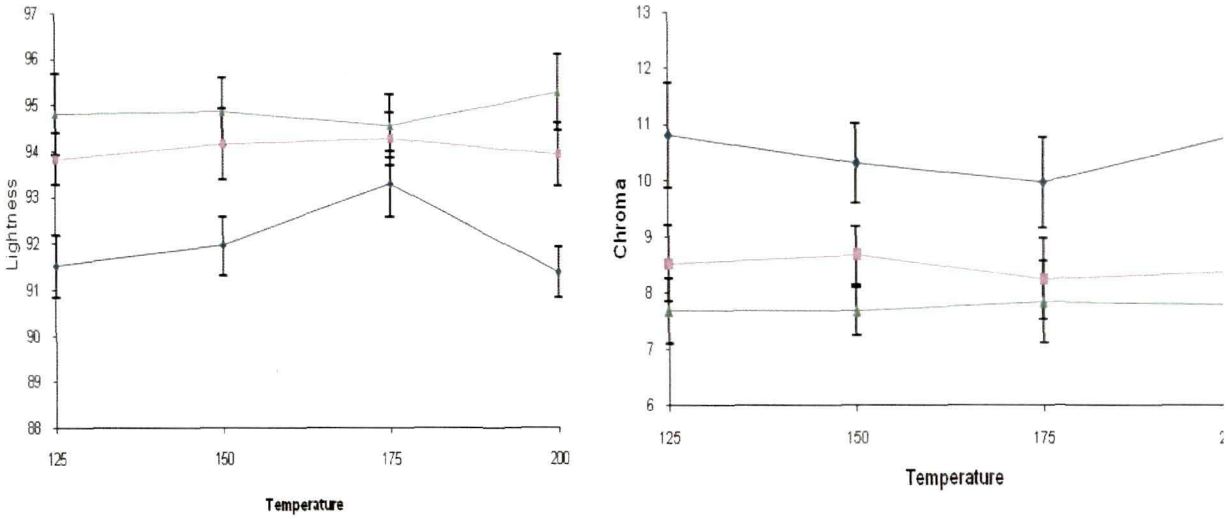
7.3.4. Effect of spray drying conditions on TPC of lemon powder

Fig. 7.4a shows the effect of processing conditions on total phenolic content (TPC) of spray dried lemon powder. TPC was significantly ($p<0.001$) reduced when inlet temperature was increased from 125°C to 200°C temperature indicating the thermal degradation of phenolics.

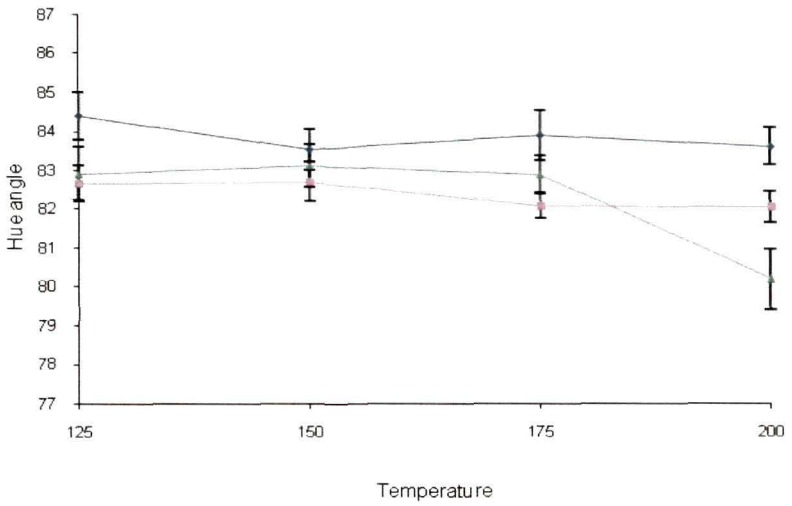
TPC content of the lemon powders was reduced when the concentration of the maltodextrin was increased from 10 to 20%. This can be explained to be due to the dilution effect of maltodextrin. No significant difference was observed in sample having 20% and 15 % of maltodextrin when produced at 200°C inlet temperature.

7.3.5. Effect of spray drying conditions on DPPH* scavenging activity of powder

Fig. 7.4b shows the effect of spray drying conditions on DPPH* scavenging activity of lemon powder. Maltodextrin level and drying temperature showed statistically significant effect ($p<0.05$) on the DPPH* scavenging activity of the powder. DPPH* scavenging activity of the lemon powder was significantly affected by increase in inlet temperature. Similar findings were reported by Kha et al. (2008)[13] in case of gac juice powder. The possible explanation for the low free radical scavenging activity may be due to the exposure to higher temperatures which adversely affected the structure of phenolics causing its break down and subsequent reduction in the antioxidant activity.



(a) **(b)**



(c)

–♦– Maltodextrin 10%; –■– Maltodextrin 15% ; –▲– Maltodextrin 20%

Fig. 7.3 Color characteristics of spray dried lemon powder.

Increase in maltodextrin concentration resulted in lower DPPH* scavenging activity in spray dried lemon powder which can be attributed to the dilution effects of maltodextrin. These results contradicted the result of Kha et al. (2008)[13] who did not find any significant effect of maltodextrin level from 10-20% on total antioxidant activity of gac juice powder.

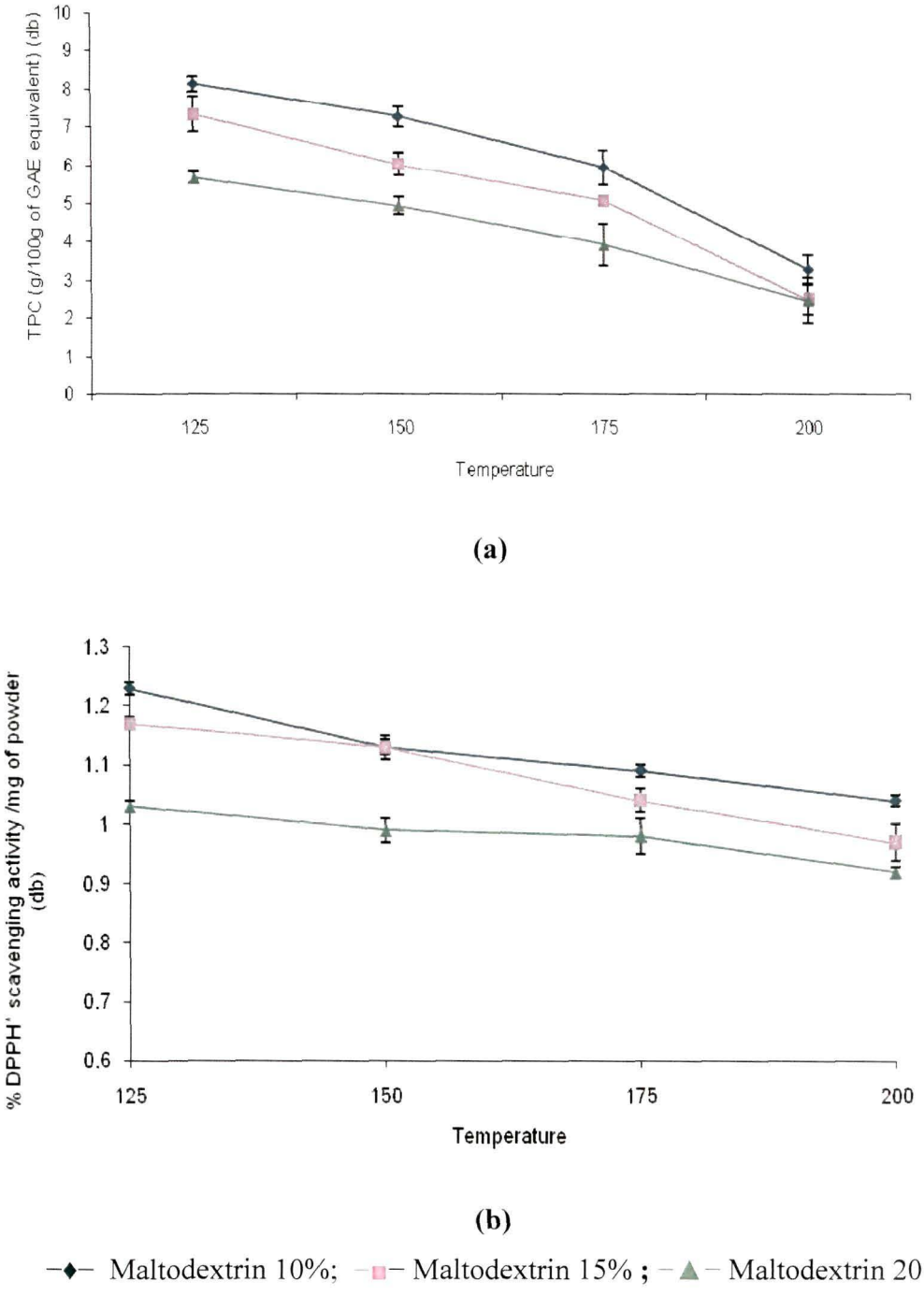


Fig. 7.4 Effect of spray drying conditions on (a) total phenolic content of lemon powder, and (b) DPPH* scavenging activity.

7.3.6. Amla –lemon based RTS

7.3.6.1. Experimental design for development of Amla-lemon based RTS

For Amla-lemon RTS, lemon juice was spray dried at 175°C inlet temperature with 10% maltodextrin and Amla juice was dried separately at 175°C inlet temperature with 7% maltodextrin. Design expert 6.1 was used for the optimization of level of variables. Significant terms in model were observed by analysis of variance (ANOVA). Model adequacy was checked by R^2, predicted R^2, adequacy precision and lack of fit test. Predicted R^2 comparable to fitted R^2, low PRESS and adequacy precision greater than 4[29-31] for flavour, overall acceptability and total phenolic content shows that model term were significant and fitted adequately for these attributes (**Table 7.3; Table 7.5 and Table 7.6**), whereas negative value of predicted R^2 for color shows that model terms were not fitted adequately for color value **(Table 7.4)**.

7.3.6.2. Response surface

Fig 7.5a shows the effect of lemon powder and Amla powder on flavor acceptability of the RTS. Increase in concentration of Amla powder was followed by decrease in the flavour acceptability of the product. Especially, higher concentration of Amla powder with lower concentration of lemon powder decreased the flavour acceptability of the product. For color the model was not found significant at 5% probability level; suggesting that the variation in levels of Amla powder, lemon powder and citric acid did not affect the color of the product as evident from **Fig 7.5(b), 7.6 (b)** and **7.7 (b).**

The surface response of overall acceptability as a function of lemon powder and Amla powder is given in **Fig 7.5 (c),** as the level of lemon was increased the product was found to be more acceptable by the panellists and the acceptability level had increased from 5.67 to 8.2 but when level of lemon powder was increased along with the Amla powder than the product secured less rating by the panellist which can be attributed to astringency in the Amla powder. The surface response of TPC as a function of Amla powder and lemon powder is shown in **Fig 7.5(d).** Amla powder had strong positive effect on TPC at 1% probability level. However interaction of lemon and Amla powder was not found significant at 5 % probability level. **Fig 7.6a** presents the surface response of flavour as function of citric acid and lemon powder.

Table. 7.3 ANOVA table for flavor acceptability of Amla-lemon based RTS

Terms	Sum of squares	DF	Mean Square	F value	Prob>F
Model	10.09	9	1.12	57.08	< 0.0001
A	5.34	1	5.34	271.80	< 0.0001
B	0.17	1	0.17	8.49	0.0154
C	0.28	1	0.28	14.52	0.0034
A^2	1.68	1	1.68	85.52	< 0.0001
B^2	0.24	1	0.24	12.25	0.0057
C^2	0.58	1	0.58	29.33	0.0003
AB	0.91	1	0.91	46.42	< 0.0001
AC	1.20	1	1.20	61.19	< 0.0001
BC	0.011	1	0.011	0.57	0.4665
R squared	0.9809				
Adj R squared	0.9637				
Pred R-squared	0.8601				

Table. 7.4 ANOVA table for color acceptability of Amla -lemon based RTS

Terms	Sum of squares	DF	Mean square	F value	Prob >F
Model	1.54	9	0.17	1.66	0.2210
A	0.34	1	0.34	3.27	0.1007
B	0.027	1	0.027	0.26	0.6222
C	2.798E-003	1	2.798E-003	0.027	0.8727
A^2	0.63	1	0.63	6.04	0.0338
B^2	0.30	1	0.30	2.94	0.1174
C^2	0.15	1	0.15	1.46	0.2553
AB	1.250E-003	1	1.250E-003	1.250E	003
AC	0.011	1	0.011	0.11	0.7485
BC	1.250E-003	1	1.250E-003	0.012	0.9147
R-squared	0.5987				
Adj R-squared	0.2376				
Pred R-squared	-1.7527				

Table. 7.5 ANOVA table for over all acceptability of Amla - lemon based RTS

Terms	Sum of squares	DF	Mean square	F value	Prob >F
Model	21.05	9	2.34	14.15	0.0001
A	9.65	1	9.65	58.37	< 0.0001
B	1.63	1	1.63	9.85	0.0105
C	0.089	1	0.089	0.54	0.4791
A^2	4.19	1	4.19	25.37	0.0005
B^2	3.66	1	3.66	22.16	0.0008
C^2	2.71	1	2.71	16.38	0.0023
AB	0.13	1	0.13	0.76	0.4048
AC	0.32	1	0.32	1.94	0.1942
BC	0.40	1	0.40	2.45	0.1485
R-squared	0.9272				
Adj R-squared	0.8617				
Pred R-squared	0.7426				

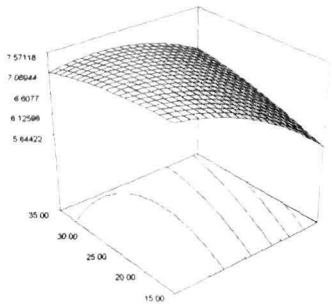
Table. 7.6 ANOVA table for total phenolic content of Amla-lemon based RTS

Terms	Sum of squares	DF	Mean square	F value	Prob >F
Model	48.34	9	5.37	470.37	< 0.0001
A	47.16	1	47.16	4130.03	< 0.0001
B	0.80	1	0.80	70.16	< 0.0001
C	9.204E-003	1	9.204E-003	0.81	0.3904
A^2	0.28	1	0.28	24.18	0.0006
B^2	0.037	1	0.037	3.25	0.1017
C^2	6.163E-004	1	6.163E-004	0.054	0.8210
AB	0.023	1	0.023	2.02	0.1853
AC	1.513E-003	1	1.513E-003	0.13	0.7235
BC	4.513E-003	1	4.513E-003	0.40	0.5437
R-squared	0.9976				
Adj R-squared	0.9955				
Pred R-squared	0.9821				

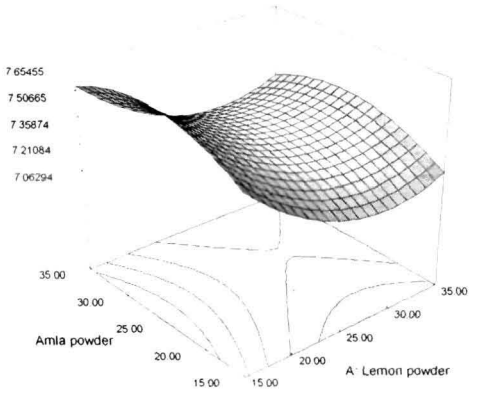
Lemon powder showed significance at 5% whereas citric acid was significant at 1% probability level. Interactive effect of lemon powder and citric acid was not found significant on flavour acceptability of the RTS. **Fig 7.6c** presents the surface response of overall acceptability as a function of lemon powder and citric acid. Lemon powder showed positive effect at 5% significance level whereas citric acid was not found effective at 5 % significance level. Interactive effect of lemon powder and citric acid was also not found significant at 5 % probability level. Fig **7.6 (d)** shows the interactive effect of citric acid and lemon powder on TPC of RTS in g/100 g of GAE. As the level of lemon powder increased the TPC was significantly increased from 2.56 to 4.86 g/100 g of GAE equivalent. Citric acid showed no significant effect on TPC of developed product. The interactive effect of citric acid and Amla powder on flavor acceptability of the RTS is given in **Fig 7.7a**. Both powder and citric acid showed positive effect on the flavor acceptability of the product but the positive effect was more pronounced when level of Amla powder was increased as compared to citric acid. The same inferences can be drawn for the overall acceptability of the product where amla powder was found more effective to increase the acceptability of the product ($p<0.01$) as compared to citric acid **Fig 7.7c**. When lemon powder was increased the TPC content was significantly increased but the increase in level was comparatively less than in case of increase in level of Amla powder, confirming the presence of significantly high level of phenolic content in Amla powder as compared to lemon powder. The composition of 25.94% Amla powder, 32.02% lemon powder and 1.72% citric acid and 40.32% glucose powder was found optimum. Triplicate samples were prepared using the optimum conditions and were analyzed for organoleptic quality and TPC; corresponding value for flavor, color and overall acceptability were 7.18, 7.28, 7.78 & 4.56 respectively which showed an excellent agreement with the predicted responses and the actual value (**Table 7.7**). Hence model was found satisfactory.

Table7.7 Optimum conditions for the development of Amla-lemon RTS

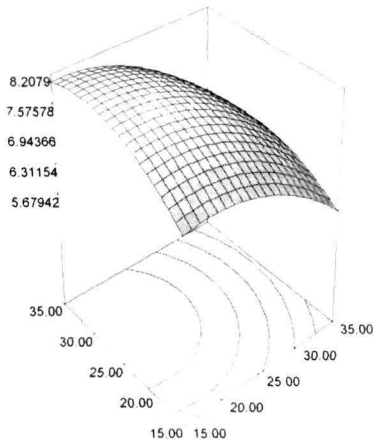
Particular	Predicted	Optimum
Flavor	7.18	7.32
Color	7.28	7.68
Overall acceptability	7.78	7.91
TPC mg/100g GAE	4.56	4.48



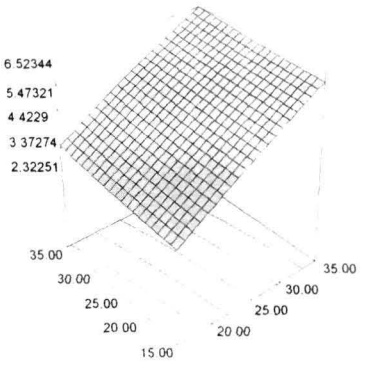
(a)



(b)

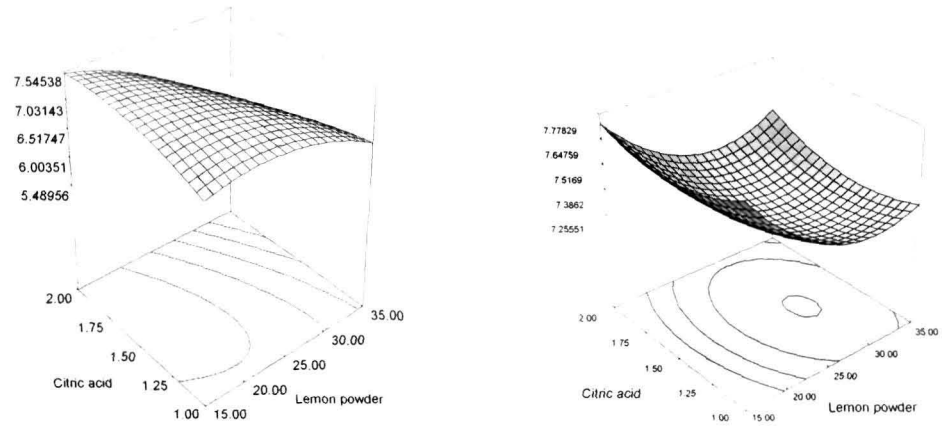


(c)

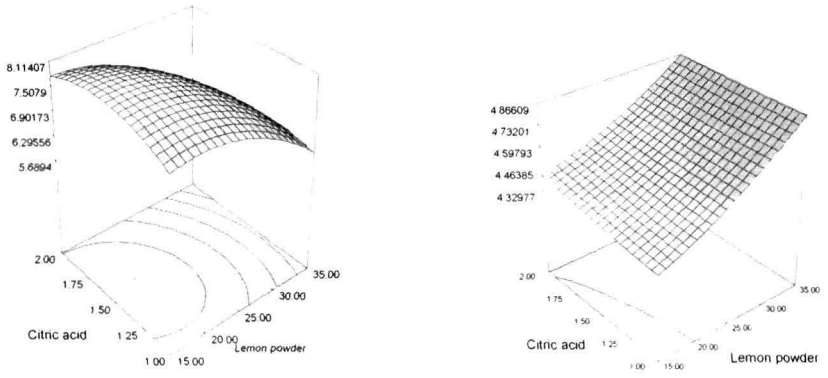


(d)

Fig.7.5 Response surface and contour plots for (a) flavor, (b) color, (c) Overall acceptability, and (d) TPC (at constant % of citric acid).

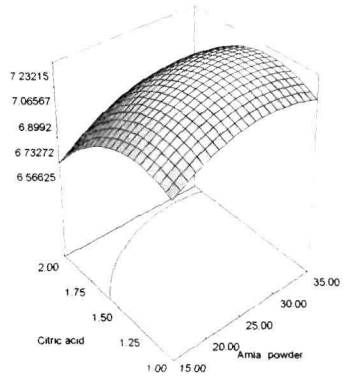


(a) **(b)**

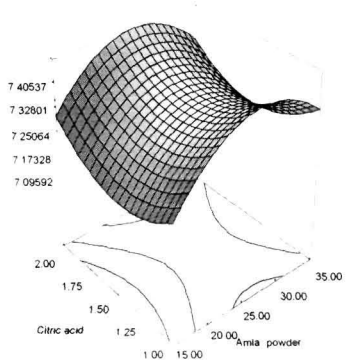


(c) **(d)**

Fig 7.6 Response surface and contour plots for (a) flavor, (b) color, (c) Overall acceptability, and (d) TPC (at constant % of Amla powder).



(a)



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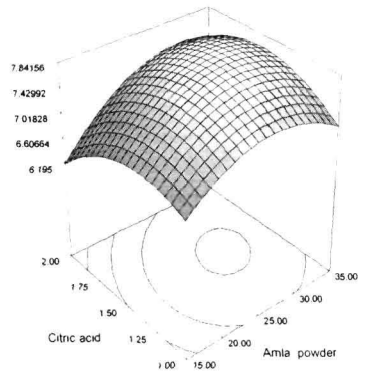
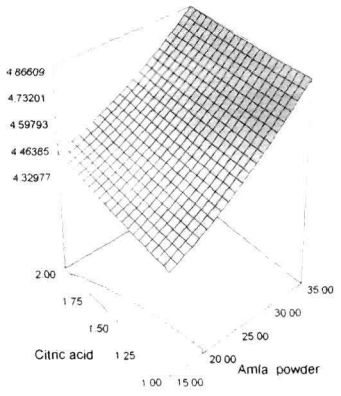


(c)



(d)

Fig 7.7 Response surface and contour plots for (a) flavor, (b) color, (c) Overall acceptability, and (d) TPC (at constant % of lemon powder at central point).

7.3.6.3. Chemical composition and storage stability of optimized Amla-lemon based RTS

Optimized Amla-lemon based RTS was analyzed for its nutritional quality and data are given in **Table 7.8**. Finished formulations had moisture content with mean value of 4.3% and contained good amount of K, Ca and P. The significant amount of total phenolic content and free radical scavenging activity makes it suitable as a health drink.

Fig 7.8 presents the color stability of the RTS during storage. Room temperature adversely affected the lightness of the stored product whereas the refrigerated product had better stability during the same period of storage (**Fig 7.8a**). During first thirty days of storage no statistically significant difference in overall color difference of the sample was observed in refrigerated samples, while in room temperature stored sample as the storage time progressed the overall color difference also increased simultaneously (**Fig 7.8b**). Increased rate of darkness and total color difference of sample at room temperature may be due to the increased rate of formation of iron tannate (present in amla powder) that causes increased degree of redness and darkness of the sample (a common phenomenon). The result is consistent with Mishra et al. (2010)[32] that sun dried Amla powder had significantly lower L value as compared to freeze dried or vacuum dried amla powder. N_2 flushing inside the package reduced the rate of degradation of color during storage at both ambient and refrigeration temperatures (**Fig 7.8a & b).** The reason for better retention of color in sample stored in the environment flushed with nitrogen may again be correlated with rate of formation of iron tannate. For the formation of iron tannate presence of oxygen is essential hence packing with N_2 flushing hampered the reaction of iron and tannic acid and so retained the color of the sample during the storage.

Stability of TPC in RTS during storage can be seen in **Fig 7.9a**. Storage temperature showed significant effect on retention of total phenolic content in the product. Sample stored at room temperature had increased rate of degradation of total phenolic content as compared to the sample stored at 7°C. Sample stored with N_2 flushing and stored at ambient temperature had better retention of phenolic content as compared to the sample stored at refrigeration temperature which suggested that the absence of oxygen is more important to retard the rate of degradation of phenolics as compared to refrigeration temperature. The rate of oxidation of total phenolic content

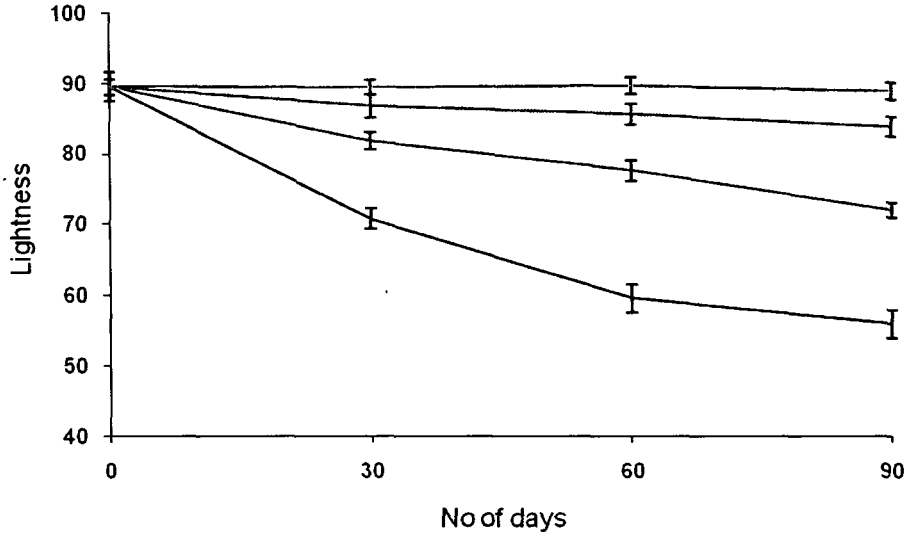
may be increased due to storage at room temperature which causes the reduction in overall total phenolic content. The lowering of total phenolic content at refrigeration temperature may be due to the precipitation of flavonones[33,2].

Fig 7.9b presents DPPH* scavenging activity of the Amla-lemon based RTS during storage. Sample stored at room temperature lost free radical scavenging activity at rapid rate as compared to refrigeration temperature which may be because of the degradation of the phenolics. Mutual degradation of total phenolic content and free radical scavenging activity has been broadly reported. Degradation rate of free radical scavenging activity was comparatively higher than TPC at both storage temperatures. Being secondary metabolites, phenolic compounds are probably synthesized or broken down into new components which adversely affected the free radical scavenging activity of RTS. **Fig 7.9** also showed that the sample stored with N_2 is effective to retain the bioactive properties like total phenolic content and DPPH* scavenging activity.

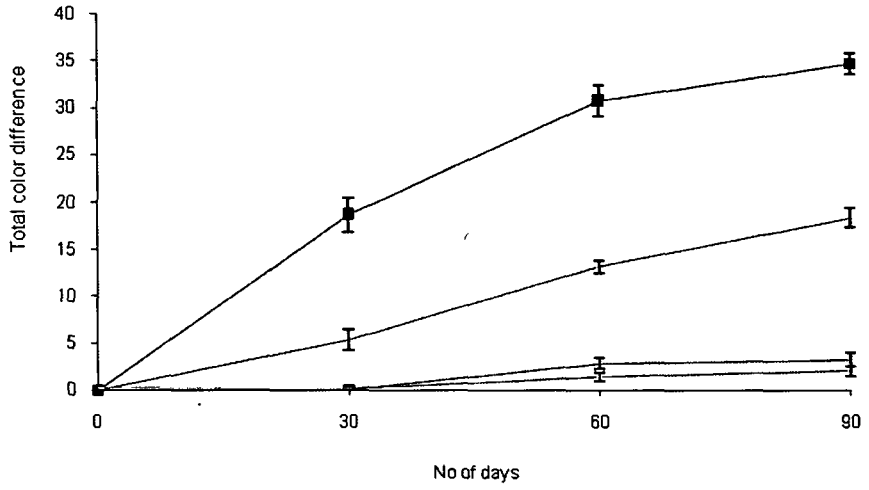
Table 7.8 Chemical composition of optimized Amla-lemon fruit RTS

Particular	Composition
Moisture (%)	4.63±0.3*
Protein (%)	0.04±0.1
Fat (%)	0.05±0.1
Carbohydrates (%)	95.64±0.8
Calcium (mg/100 g)	31.50±1.0
Phosphorous (mg/100 g)	22.40±1.2
Iron (mg/100 g)	2.45±0.6
Sodium (mg/100 g)	98± 1.7
Potassium (mg/100 g)	184±1.4
Total phenolic content (g/100 g) GAE	4.48±0.6
DPPH* scavenging activity (%)	87.64±1.5
L	89.56±2.1
a	-0.41±0.01
b	9.58±0.6
Kcal/100 g of powder	383.17±1.1

* mean ± standard deviation



(a)



(b)

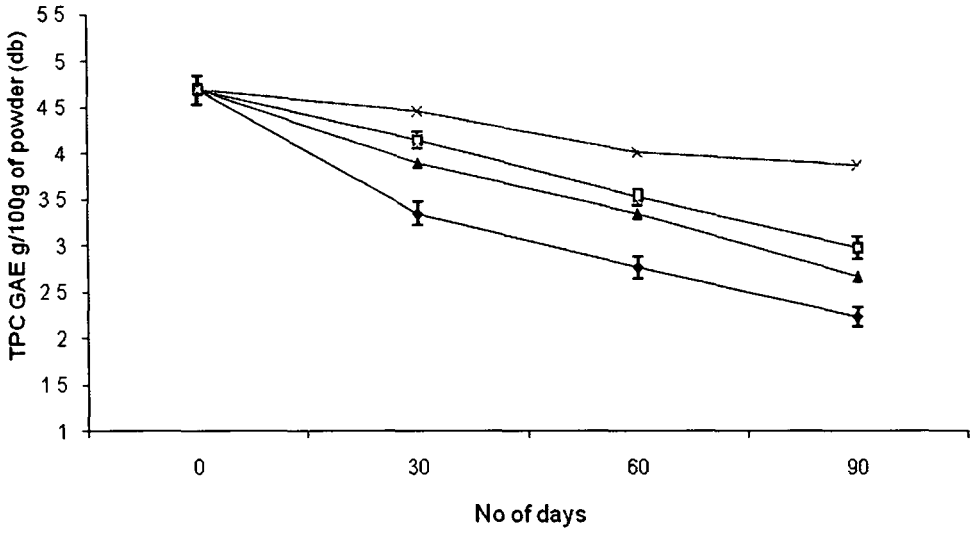
------- ambient temperature (N_2 flushing)

------- room temperature

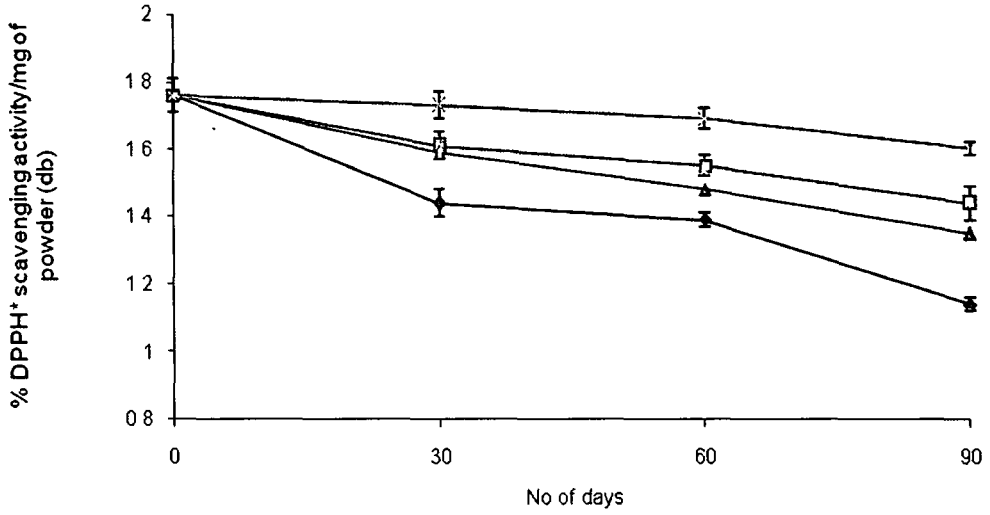
------- stored at refrigeration temperature

------- refrigeration temperature (N_2 flushing)

Fig. 7.8 Effect of storage temperature on color characteristics of Amla-lemon RTS (a) lightness, and (b) total color difference.



(a)



(b)

——— ambient temperature with N_2 flushing

——— room temperature

——— stored at refrigeration temperature

——— refrigeration temperature with N_2 flushing

Fig. 7.9 Effect of storage on (a) total phenolic content of RTS, and (b) DPPH* scavenging activity of RTS.

7.4. Conclusion

The effect of spray drying conditions on physicochemical properties of spray dried lemon powder was evaluated. Maltodextrin concentration (10-20%) and drying temperature (125°C to 200°C) significantly affected moisture content, bulk density, hygroscopicity, color attributes, TPC and DPPH* scavenging activity. However WSI was not significantly influenced by varying the concentration of maltodextrin or inlet temperature. The developed lemon powder showed excellent water solubility that is essential for reconstitution. The juice powder dried at 125°C temperature and maltodextrin concentration of 10% showed better retention of phenolic content and free radical scavenging activity but due to excessive stickiness can not be recommended as a product. Lemon juice dried at 175°C temperature and 10 % maltodextrin concentration is recommended. It was ascertained by RSM that 25.94% Amla powder, 32.02% lemon powder, 1.72% citric acid, and 40.32% glucose powder were found optimum for RTS development.

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Chapter-8

Conclusion and Future Scope of the Study

8.1. Conclusion

The present investigation was carried under five clearly focused objectives. Different varieties of Amla were evaluated for their nutritional and physicochemical properties and further, the processing wastes of Amla i.e seed and seed coat powder were evaluated for their chemical and nutritional potential. Extraction conditions for phenolics from Amla fruit powder and seed coat powder were optimized by response surface methodology and phenolics of Amla fruit powder, seed coat powder and pomace powder were characterized by analytical HPLC. Pure phenolic components were extracted from Amla pomace powder and extracted phenolics were further investigated for their antioxidant property like DPPH and ABTS free radical scavenging activity. Phenolics of seed coat powder were also characterized by GC-MS through silylation. Processing conditions were standardized on the basis of moisture content, WSI, bulk density, hygroscopicity, TPC and DPPH* scavenging activity for development of spray dried Amla juice and lemon juice powder. Spray dried lemon juice powder and spray dried Amla juice powder processed with optimized conditions were taken as raw materials for development of Amla-lemon based RTS using response surface methodology and the developed RTS was further investigated for its storage stability and the retention of TPC and DPPH* scavenging activity during storage.

The salient finding of thesis are summarized below.

I. Physicochemical analysis of different varieties of Amla and comparative analysis of functional and nutritive value of Amla fruit, seed and seed coat powder

- *Chakaiya* and *Krishna* variety had the highest total phenolic content as compared to other varieties.
- Amla varieties differed in composition of nutrients.
- Seed coat powder was found comparatively better than seed powder in terms of hydration properties, water holding capacities and functional properties.
- Seed powder had very good amount of P, K, Mn, and Co.
- Seed coat is a good source of protein, minerals, ω-3, ω-6 fatty acids. Seed and seed coat can be used to enrich food products.

- The bioactive constituents of seed powder were very low in comparison to fruit and seed coat powder.

II. Process optimization for extraction of total phenolic content from Amla (*Emblica officinalis*) using response surface methodology and characterization of phenolics

- Process variables i.e., ethanol concentration and pH have significant effect on recovery of total phenolic content and % DPPH* scavenging activity of the ethanolic extract of Amla powder.
- Optimum operating conditions were: ethanol 78.0%, temperature 30.50°C and pH 4.5.
- Major polyphenols present in ethyl acetate fractions were gallic acid, catechin, caffeic acid, vanillic acid and syringic acid.
- Butanol and diethyl ether as solvents are not recommended for the extraction of phenolics.

III. Extraction, identification and antioxidant properties of bioactive components of Amla (*Emblica officinalis*) pomace powder

- Major phenolics contained in ethyl acetate fractions of pomace powder were gallic acid, catechin, caffeic acid and syringic acid.
- Ethyl acetate fraction extracted by methanol:water (80:20) showed the maximum DPPH* scavenging activity.
- Due to very poor recovery, fraction V of ethyl acetate extract was not found suitable for the further purification of phenolics.
- Three phenolics i.e. gallic acid, hydroxytyrosol and catechin were isolated from fraction III. This is the first report of presence of hydroxytyrosol in the phenolics of Amla pomace powder.
- Isolated catechin, gallic acid and hydroxytyrosol exhibited stronger DPPH* radical scavenging activity than standard vitamin C and β-carotene.
- Amla pomace powder is, therefore a good source of bioactive phenolics.

IV Partial extraction and identification of total phenolic content of Amla (*Emblica officinalis*) seed coat powder

- Optimum operating conditions were found to be ethanol 82.06%, temperature 37°C and pH 4.17.
- Major polyphenols contained in ethyl acetate fractions were gallic acid, catechin, quercetin, p-coumaric acid, vanillic acid and chlorogenic acid.
- Fraction V showed maximum DPPH* scavenging activity.
- Silyl derivatives offer a very good alternative for the identification of phenolic compounds from seed coat powder.
- Recovery from ethanol extracted powder was very less hence other methods like supercritical fluid extraction, ultrasonic or microwave assisted extraction may be explored to extract the vital bioactive constituents of seed coat powder.

V Effect of maltodextrin concentration and inlet temperature during spray drying on physicochemical and antioxidant properties of Amla (*Emblica officinalis*) juice powder

- Maltodextrin concentration (5-9%) and drying temperature (125°C to 200°C) have significant effect on moisture content, bulk density, hygroscopicity, color attributes, and DPPH* scavenging activity.
- WSI was not influenced by varying the concentration of maltodextrin or inlet temperature.
- Amla juice powder dried at 125°C temperature and maltodextrin at 5% level had better retention of phenolic content and free radical scavenging activity but due to excessive stickiness of the powder cannot be recommended as optimum products.
- Amla juice dried at 175°C and 7% maltodextrin level was adequately effective to produce powder with less hygroscopicity, acceptable color in terms of *L*, *a* and *b* and potent free radical scavenging activity.

VI Process standardization for development of spray dried lemon powder and optimization of Amla-lemon based RTS drink using Response Surface Methodology

- Maltodextrin concentration (10-20%) and drying temperature (125° C to 200°C) significantly affected moisture content, bulk density, hygroscopicity, color attributes, TPC and DPPH* scavenging activity.
- Lemon juice dried at 175°C temperature and 10% maltodextrin concentration is recommended.
- Nitrogen flushing is effective to preserve the color and functional property of Amla lemon based RTS during storage.
- Refrigerated temperature is effective for storage of Amla-lemon based RTS drink.

8.2 Future scope of the present study

The results obtained from this thesis can form the basis for further studies on Amla and its processing wastes.

A few of the future scope are outlined below.

- To study the extraction of phenolics from seed coat and Amla residue powder by solvent extraction method.
- To identify all the constituents from seed coat powder and Amla residue powder by GC-MS.
- To study the role of extracted polyphenols as antioxidants and as preservatives in food industries.
- To develop some new Amla based functional and neutraceutical foods having export potential and explore their commercialisation.
- To investigate the effectiveness of the polyphenols in Amla juice, pomace powder, and seed powder on disease conditions like inflammation and degenerative diseases.

List of publications

1. Mishra, P., et al. Effect of maltodextrin concentration and inlet temperature during spray drying on physicochemical and antioxidant properties of Amla (*Emblica officinalis*) juice powder, *Food Bio Prod. Process.* **http://dx.doi.org/10.1016/j.fbp.2013.08.003**, 2013 (Available online).
2. Mishra P., et al. Physico-chemical properties of *Chakaiya* variety of Amla (*Emblica officinalis*) and effect of different dehydration. *African J. Food Sci.* **3**(10), 303-306, 2010.
3. Mishra, P., & Mahanta, C.L., Comparative analysis of functional properties and nutritive value of Amla (*Emblica officinalis*) and its processing wastes (seed and seed coat), *Am. J. Food Technol.* **9** (3), 151-161, 2014.
4. Mishra, P., et al. Process standardization for development of spray dried lemon powder and optimization of Amla-lemon based RTS by using Response Surface Methodology. *J. Food Process Pres.* (*Under revision*)
5. Mishra, P., et al. Extraction and characterization of total phenolic content of Amla (*Emblica officinalis*) seed coat powder, *Food Chem.* (*Under review*)
6. Mishra, P., & Mahanta, C.L., Extraction, isolation, characterization and antioxidant properties of bioactive components of Amla (*Emblica officinalis*) pomace powder. *Nat. Prod. Res.* (*Under review.)*
7. Mishra, P., & Mahanta, C.L., Extraction and identification of bioactive components and antioxidant properties of Amla (*Emblica officinalis*) powder, *Nat. Prod. Res.* (*Comm.)*

Patent published

S.No.	**Patent No.**	Patent Name	Patent Country	Publication number	**Patent Status**
1	Patent application no. 296/Del/2010	Amla-Lemon based (Polyphenol enriched) RTS	India	17/2012	Published

Conference presented

1. Mishra et al. Functional properties and nutritive value of Amla (*Emblica officinalis*) and its processing wastes (seed and seed coat) in International conference on Health Food at Jawaharlal Nehru University, 23-24th December 2013.
2. Mishra, et al. Neutraceutical properties of Amla & its processing wastes and microwave assisted extraction of polyphenols by using response surface methodology in International conference on Perspective and challenges in chemical and biological sciences: Innovation cross roads, at Gauwahati, 28-30th January, 2012.
3. Mishra et al. Study of release characteristics of antioxidants in Indian gooseberry (Amla) Extracts from encapsulated sodium alginate beads in International conference on Food and nutrition technology for public health care at Jawaharlal Nehru University, New Delhi, 4-5, 2012.
4. Mishra, P. et al. Nutraceutical properties and biochemical analysis of Indian gooseberry (Amla) and its products in National conference on Role of bioactive compounds in foods on human health (Bio Foods 2011) at Department of Food Engineering & Technology, Tezpur University, 14-16th November, 2011.
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