Apple Pomace; A source of Pharmaceuticals

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Abstract: Apple pomace is an abundant agro-industrial waste used as starting raw material for production of pectin. The prepared pectin was evaluated for its hypolipidemic and gastroprotective effects. Both the prepared pectin and apple marc left after pectin extraction were used as new fermentable substrates for production of citric acid employing *Aspergillus niger* solid state fermentation technique. The cultures were optimized to produce 37 % and 16.40 % citric acid from pectin and apple marc, respectively. Galacturonic acid was isolated from pectin hydrolysate in 61.5 % yield.

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1. Introduction

The vast development in industry, agriculture and the human civilization resulted in intensive production of a huge amount of agro-industrial wastes. In recent years, considerable interest has been developed in using agro-industrial wastes, including apple pomace (Hang and Woodams, 1984), grape pomace (Hang and Woodams, 1985) and kiwi fruit peel (Hang and Woodams, 1987) as substrates for citric acid production. Citric acid is one of the world's largest fermentation products (Berovic and Legisa , 2007). It has a wide variety of application due to high solubility, low toxicity and palatability in the food and beverage industry (70 %), in pharmaceuticals (12 %) and in other industrial applications (18 %) (Prescott and Dunn, 1982). It is produced commercially by submerged fermentation of sucrose or molasses based medium (Kapoor *et al.*, 1987). Recently, there have been an increasing number of reports on the use of solid state fermentation (SSF) processes as an alternative approach to submerged fermentation (Hang and Woodams, 1984). This is because of lower energy requirements, higher product yields, little risk of bacterial contamination, less waste water generation and environmental issues concerning the disposal of solid waste (Doelle *et al*., 1992). In Egypt, about 100,000 tons/year of under-utilized apple pomace are generated (according to an estimate prepared by the ministry of Agriculture 2008), the over accumulation causes a serious environmental and disposal problems as well as psychological hazards and other related health problems (Diomi *et al*., 2008). Apple pomace may serve as a renewable substrate for production of value-added pharmaceuticals such as pectin (Marcon *et al*., 2005). Pectin was prepared from apple pomace adapting a modified method to a yield of 20.2 % (El-Sharkawy *et al*, 2011). The prepared pectin was used for production of galacturonic acid in a yield of 82.6 %. The prepared pectin and apple marc remained after pectin extraction was used for the production of citric acids in high yield based on colorimetric assay. Pharmacologically, the hypolipidemic and gastroprotective activities of the prepared pectin were studied.

2. Material and Methods

A. Plant Material: Apple pomace (*Malus Domestica* Family Rosaceae) was collected from Beverage Company (BEST) (Nawasa, Mansoura, Egypt). One single batch was used in all the studies in order to minimize any possible interference due to variation in composition of residues. The entire fruit was identified by Prof. Dr. Ramadan A. Fouda, Prof. of Agricultural Plants, Faculty of agricultural, Mansoura University, Egypt.

B. Microorganisms: *Aspergillus flavipes* ATCC 16795, *Aspergillus flavus* ATCC 11013, *Aspergillus alliaceous* UI 315, *Bacillus cereus* NRLLB *14591*, *Debaryomyces polymorphus* ATCC 20280, *Rhodtorula rubra* ATCC 20129, *Mucor griseocynus* ATCC 1207, *Sacchromyces cerevisiae* ATCC 2366 and *Aspergillus niger* FS2008 isolated and identified by Prof. Dr. Yehia A. El-Lazeik, Prof. of Microbial Molecular Biology, Faculty of Science, Mansoura University, Egypt. The strains were maintained on potato dextrose agar slants at 4 ^{o}C and subcultured at intervals from 1 to 2 mounths (Sankpal *et al*., 2001) on PDA.

C. Experimental animals: Male albino rats (about 15 day old) weighing (70 ± 10 gm) were purchased from Abu-Rawash, El-Giza, Egypt, and housed in

metallic boxes, maintained under controlled light and temperature conditions and fed either a normal rat diet or a fatty diet (10 % fat) (Sudheesh and Vijayalakshmi, 2000) or pectin containing fatty diet (10 % fat, 5 gm /kg pectin) or pectin containing diet (5 gm /kg pectin) for 60 days with free access to tap water.

D. Chemicals: Authentic Galacturonic, Citric acid and Phosphate buffer (Sigma Co.), Pectin prepared from apple pomace (El-Sharkawy, 2011), Cellulose for column (E. Merk, Germany), Silica for TLC (Merk and Machery-Nagel, Germany), Serum cholesterol kit, Serum triglycerids kit and Serum HDL chlosterol kit: (Roche Diagnostic, Gmb H,D-68298 Mannheim distribution in USA, Indianapolis, IN.), Gastrofait, an antiulcer drug (Eipico Co., Egypt), Solvent for extraction (El-Nasr Company for Pharmaceutical Chemicals, Egypt) were of reagent grade, purified by distillation following the standard procedures (Vogel, 1974).

E. Instruments: Hitachi 902-Automatic analyzer, Hitachi ltd. 1996, Japan, HPLC Shimadzu 10A equipped with shim-pack SCR-101N reversed phase column (Shimadzu 7.9 mm × 30 cm) and maintained at 40 ^{o}C. Separation was achieved by pumping water through the column at 0.5 ml/ min for 25 min. monitoring was performed by measuring changes in the refractive index (RI). Identification and quantitative determination was done using external standard (El-Sayed *et al*., 2005), Horizontal laminar flow (Holten TL2448, Denmark), Shaker Incubator (New Brunswick Scientific Co, USA), Microtome (Leica, RM 2125, Germany).

Statistical Analysis:

Statistical analysis was carried out using one way analysis of variance (ANOVA) and Student t- test. Statistical calculations were carried out using Prism computer program (Graphpad software Inc., V4, San Diego, CA, USA).Data are expressed as mean ± standard error of the mean, S.E.M. (significance was calculated at p less than 0.001).

Experimental

1. Isolation of galacturonic acid:

The prepared pectin was hydrolyzed by formic acid (Radhakrishnamurthya and Berensona, 1963). The hydrolysate (1gm) was applied on the top of a cellulose column (30 gram), isocratically eluted with a mixture of benzene-butanol-pyridine-water (4:3:3:1, v/v) (upper phase). Fractions of 20 ml were collected, monitored by paper chromatography using the same composition of elution mobile phase and spots were visualized by spraying with aniline phthalate reagent**.** Similar fractions (at Rf 0.15 on PC) were pooled, dried under vacuum to yield 615 mg galacturonic acid as confirmed by HPLC analysis (Rt 11 min.).

2. Production of citric acid:

Two-stage fermentation protocol was adopted (Smith and Rosazza, 1975 and Rosazza, 1982) using 250 ml-flasks containing 50 ml of fermentation media. The flasks containing pectin and apple marc were sterilized at 121 ^{o}C for 20 min. and cooled. Stage-1-culture started by inoculation with the screened microorganisms (Lee and Yum, 1999). Stage-2- culture started by transfer of 1 ml of stage-1-culture to fresh medium. The cultures were sampled (2 ml) at 2-3 days intervals for the estimation of citric acid. The cultures were optimized and repeated using hydrolyzed pectin and apple marc [1 M Sulfuric acid at 121^{o}C for 60 min. (Tada *et al.,* 2004)] and neutralized with 1 M NaOH. Citric acid identification was done by HPLC, special chemical test and TLC Co-chromatography with authentic on precoated silica plate using NH_4OH-methanol (70:30, v/v), and methylene blue as spray reagent (Stahl**,** 1969). The Quantitative analysis was done conducting the acetic anhydride and pyridine (Marier and Boulet, 1958 and Dhillon *et al*., 2010).

3. Hypolipidemic activity:

A total of 36 male albino rats two weeks old (85-90 gm each) were randomly divided into four groups (9 rats per group) and received the following treatments: GA. received a normal diet (negative control), GB. received10 % fat containing diet (induction of hyper lipidemia, positive control) (Sudheesh and Vijayalakshmi, 2000), GC. fed a 10 % fat containing diet + pectin 5 gm / kg/ day (treated group) and GD. Received diet containing pectin 5 gm / kg daily for 60 days followed by a 10 % fatty diet for another 60 days (prophylactic group). At the end of the experiment period, blood was collected in clean dry heparinized tubes, immediately centrifuged at 3000 rpm for 10 minutes, and serum was collected and stored at -20 ^{o}C until ready for total cholesterol, triglyceride, LDL and HDL determination (Table 1, Wijesinghe and Ahmed, 1998). Rat's livers were rinsed with ice-cold saline and a small cross-section of the liver was obtained. Liver specimens were fixed in 10 % neutral buffered formalin, embedded in paraffin, sectioned (4 to 5 µm) and stained with hematoxylin and eosin (H & E) (Duvnjak *et al*., 2007). At least two different sections were examined per liver sample. The tissues were examined under a microscope in a random order and the pathologist was blind to the used animals and the treatment groups when assessing the histology (Figure 1).

Gastroprotective activity:

A total number of 20 male albino rats (three month old - weighing about 200 gm) were randomly divided into four equal groups, starved for 24 hours and treated as follows: GA. group of healthy rats (negative control), GB.ulcer induced group (0.2 ml of indomethacin IP as positive control), GC. Group received pectin 25 mg/kg orally followed by 0.2 ml of indomethacin IP and GD. Group received 2 ml of gastrofait orally (0.1 gm) followed by indomethacin (0.2 ml, IP) after 15 minutes**.** At the end of the experiment, the animals were sacrificed and their stomachs were opened, rinsed with saline and fixed on slabs. The specimens were examined for ulcers number and size (Figure 2 and Table 2). Stomach samples were rinsed with ice-cold saline, a small cross-sections were obtained, fixed in 10 % neutral buffered formalin, embedded in paraffin, sectioned (4 to 5 µm) and stained with hematoxylin and eosin (H & E) (Duvnjak *et al*., 2007). Different sections were examined under a microscope in a random order and the pathologist was blind to the used and treated animals groups.

3. Results

Citric acid production employing the catalytic activity of *A. niger* utilizing pectin and apple marc as substrates. The best citric acid yield was obtained in culture condition of 1 % pectin, initial pH 6 and at temperature 30 ^{o}C (18.22 %) after 10 days and 10 % apple pomace (1.2 %) after 14 days. However, the hydrolysis of pectin and apple pomace increased the yield to 37.6 % and 16.4 % and reduced the time to 7 and 9 days, respectively. Galacturonic acid was recovered efficiently from pectin (82.5 %) using liquid solid chromatography technique. Upon feeding the rats with fat rich diet for about 60 days, it showed an increase in cholesterol, triglyceride, LDL and body weight and decrease in HDL (Table 1) (Sudheesh and Vijayalakshmi, 1999 and Wang *et al*., 2002) and resulted in a significant pathological change in the liver and accumulation of fat droplets in hepatocytes (steatosis) as in Figure 1-B. The administration of pectin (5 gm/kg body weight) for 60 days caused a significant decrease in all patterns of plasma lipids studied, a moderated increase in HDL (Table 1) and no steatosis was observed in the histopathological examination of the treatment group (Figure 1-C). While induction of hyperlipidemia by fat rich diet after pectin administration for 60 days was slowly and little pathological changes were observed. The prepared pectin showed gastroprotective activity against indomethacin induced ulcer (Table 2, Figure 2).

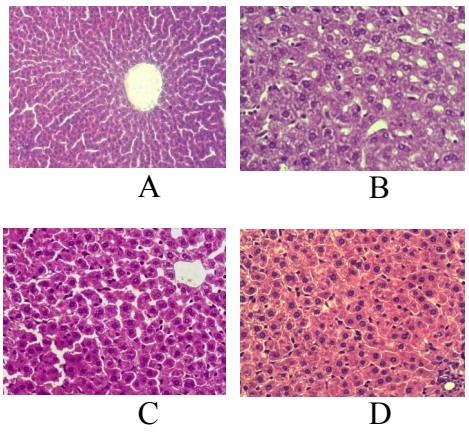

Figure (1): Photomicrograph of livers sections of male albino rats (A: negative control, B: positive control, C: treated group, D: prophylaxis).

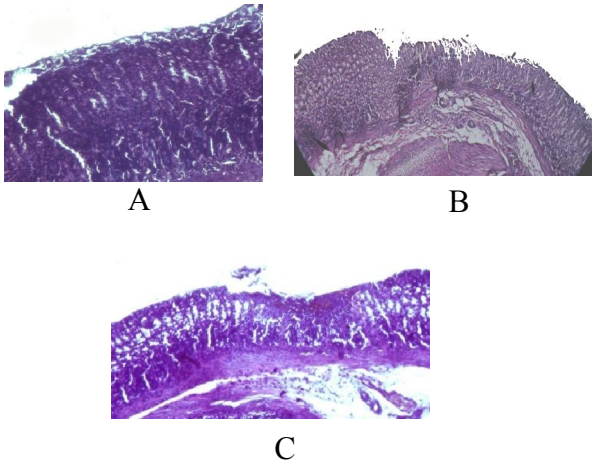

Figure (2): Photomicrograph of stomachs sections of male albino rats (A: negative control, B: positive control, C: pectin treated group).

Table (1): Hypolipidemic activity of the prepared pectin

Animal groups	Cholesterol mg/dl	Triglycerides mg/dl
A	88.8 ± 4.6#	47.7 ± 2.608#
B	178.3 ± 5*	146.5 ± 7.6*
C	86.3 ± 3.8#	47.3 ± 6.817#
D	80.2 ± 2#	43 ± 6.085#
D2	105±1.8#	106±1.3#

Table (1): Continued

HDL(mg/dl)	LDL(mg/dl)	Body weight (gram)
40.6 ± 1.9#	38.6 ± 3.5#	160.3 ± 6.9#
30.8 ± 1.2*	117.9 ± 4.5*	249.7 ± 9.4*
43 ± 1.6#	33.6 ± 4.3#	153.3 ± 6.7#
39.3+-1.2#	32.3 ± 1.9#	144.7 ± 7.8#
32.3 ± 2.2*	81 ± 3.3#	173 ± 5.9#

Table (2): Gastroprotective activity of the prepared pectin

Animal group	Ulcer number	Ulcer size (millimeter)
Group A	0.4 ± 0.54#	0.2 ± 0.27#
Group B	11 ± 1.3*	5.4 ± 0.9*
Group C	2 ± 1.6#	1 ± 0.35#
Group D	3 ± 1.8#	1.4 ± 0.41#

4. Discussions

Apple pomace is an abundantly available environmental waste used for pectin production employing a modified procedure. The pectin and remaining apple marc and their hydrolyzed forms were utilized to produce citric and galacturonic acids in high yield. Citric acid production employing the catalytic activity of *Asp. niger* utilizing pectin and apple marc as substrates. Similar results were reported (Shojaosadati and Babaeipour, 2002) at relatively lower yield (12.4%). This study demonstrates the capability of *Aspergillus niger* to use both pectin and apple pomace marc left after pectin extraction as substrates for production of citric acid for the first time. The present study investigated the curative and the prophylactic effect of apple pomace pectin on the hyperlipidemia, hypercholestremia and fatty liver induced by fat rich-diet on experimental rats. The administration of pectin caused a significant decrease in all patterns of plasma lipids and no steatosis was observed. The proposed mechanism is binding to bile acids and thus increasing cholesterol catabolism (Furda, 1979). Previous studies showed that pectin induces HMGCOA reductase activity and lowers cholesterol re-absorption in experimental animals (Patazy *et al*., 1991 and Kay and Truswell, 1977). The prepared pectin showed gastroprotective activity against indomethacin induced ulcer. These results are consistent with similar studies on rats (Galati *et al*., 2007and Khotimchenko *et al*., 2006) which reported that pectin suppress the formation of gastric mucosa lesions produced by indomethacin in rats. The pectin gastro- protective action was explained by the fact that it lines the stomach surface and improves the resistance of the gastroduodenal mucosa of experimental mice and rats to destructive effect of indomethacin-induced injuries to the gastric mucosa (Figure 2-B) (Krylova *et al*., 2006). In Conclusion; apple pomace pectin is a potential hypolipidemic and gastroprotective drug and an efficient source for citric and galacturonic acids production. Economically, 100,000 tons of apple pomace have the potential to produce 16,000 tons citric acid, 20, 000 tons pectin (each ton pectin can produce 640 kg galacturonic acid) which is enough to satisfy citric acid market demands in Egypt.

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