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Chemical, Biological and Microbiological Studies on the Seeds and Peels of Annona Fruit

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ABSTRACT

The current investigation was carried out to examine the possible potential protective effects of ethanolic extracts of different parts of Annona fruit against trichloroacetic acid induced liver cancer. The chemical composition of the seeds and peel of Annona fruit were evaluated. After drying the seeds and peel, the bacterial, fungal and yeast count for a month. The total content of flavonoids, phenols, and antioxidants was estimated using DPPH, and HPLC conditions of phenols of all extracts. The biological experiment used thirty six albino rats. After the adaptation period, the rats were divided into six groups (6 rats each). One of them was considered as a negative control and another positive control (Trichloroacetic acid group). The remaining groups were divided into four groups consisting of extracts from seeds and peel at levels 100 and 150 mg / kg. Blood samples were collected to assays the levels of liver functions (ALT, AST, ALP, BIL, TP, ALB and GLB), some kidney functions (creatinine and urea), and oxidation stress (CAT, GPX and MDA). The study results were as the fallowing: all liver cancer protected groups marked a significant improvement in liver functions, some kidney functions, and oxidation stress, when compared them with the unprotected liver cancer group (+ve). Therefore, this study recommends that the insertion seeds and peels of Annona fruit in diets because it has anti-cancer and antioxidants effects.

Keywords: Annona fruit, Antioxidants, Cancer, Liver functions, Kidney, Trichloroacetic acid.

1. Introduction

Seeds and peels of Annona fruit had widely potent antioxidants such as ascorbic, quinic, caffeic, and ferulic acids, xanthoxylin, rutin, caffeoyltartaric acid, caffeoyl glucose and [quercetin +hexose + pentose H] (Silva *et al.,* 2013). The seeds and peels of Annona fruit contain phenolic compounds which showed very high antioxidant activities. Polyphenol compounds rich extracts, may be responsible for their apoptosis-dependent anticancer behavior against cancer (Shehata *et al.,* 2021). Annona seeds contain Acetogenins such as squafosacin B, squadiolin A and squadiolin B are well-known cytotoxic acetogenins. Hence, it was proved that squadiolin A and squadiolin B have a significant cytotoxic effect and also squafosacin B against HepG2 (Kumari *et al.,* 2022). Cancer is a major reason of death both in developed and developing countries. Among the various types of cancers, liver cancer represents about 4% of all cancers around world. Liver cancer is a major health problem worldwide which described as a complex and heterogeneous malignancy from liver tissues (Severi *et al.,* 2010; Abdelaal, 2019 and Sivakumari *et al.,* 2022). The global burden of cancer is projected to raise from 13.3 to 21.4 million incident cases from 2010 to 2030 due to demographic changes alone, which dominated by a growing burden in middle- and low-income countries (Subha, 2019). Liver cancer is the fourth in Egypt and the sixth common cancer in worldwide (Rashed *et al.,* 2020). Trichloroacetic acid (TCA) is a chemical carcinogen with the potential to cause experimental multistage hepatocarcinogenesis; it produces pre-neoplastic lesions after a short period in the form of dysplastic tissue changes, vascular congestion, hepatocyte ballooning, and liver cell foci with extensive vacuolation. (Abdel-Hamid and Morsy, 2010 and Alzergy *et al.,* 2018).

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2. Material and Methods

2.1. Material

2.1.1. Plant, chemicals, diet and microbiological environments

I. Plant: Annona fruit (*Annona crassiflora* Mart) was obtained from Local market, Mansoura, Egypt.

II. Chemicals: Ethanol alcohol and trichloroacetic acid (TCA) were brought from El-Gomhouria for trading chemicals and medical appliance, Mansoura, Egypt.

II. Microbiological environments: Nutrient Agar (NR) and Potato Dextrose Agar (PDA) were brought from El-Gomhouria, Mansoura, Egypt.

IV. Experimental rats: Thirty six healthy adult albino male rats (Sprague- Dawley strain.) were at the age of 2- 4 months. The weight of male rats was about 130± 10g and were purchased from the Agricultural Research Center, Giza, Egypt.

2.2. Methods

I. Preparation of Annona fruit extracts:

The fruits were selected free from scratches and microbial infections or any color changes in preparation for making the ethanolic extract. Annona fruit was washed with tap water to remove surface dirt. The peels were removed from the fruit in slices shape then the seeds were manually removed from the pulp **(Arruda *et al.,* 2016)**.

II. Annona fruit powder (seed- peel):

Annona seed and peel were oven-dried at 45 °C. The dried seeds and peels were ground separately into powder by domestic electrical mill and stored at 4 °C until further use (Shehata *et al.,* 2021).

III. Extraction method:

The powder of seed and peel were weighed and soaked them separately in a containers with 95% ethanol was added at ratio (1:2 v/v) (Abd-Elrazek *et al.,* 2021). The soaking was done for three days with 95% ethanol (4 L) at room temperature (25 °C). This process was repeated 3 times. After filtration, ethanol was removed by using a rotary evaporator in a water bath at 40 °C (Justino *et al.,* 2019).

2.3. Chemical analysis:

I. The total content of moisture, protein, fat, ash and fiber in fresh seeds and peels of Annona fruit was estimated based on the method of AOAC, (2000). While carbohydrate was given by differences (Gul and Safdar, 2009).

II. Water activity: was determined in fresh seeds and peels of Annona fruit according to Trailer and Christian, (1978).

III. Total phenolic was measurement according to Slinkard and Singleton, (1977). While, The total flavonoid (Zhishen *et al.,* 1999). Determination of antioxidant by DPPH of Annona extracts was based on previous method of Nuengchamnong and Ingkaninan. (2010). HPLC was measurement according to Bataglion *et al.* (2015).

2.4. Microbiological analysis:

The serial dilution method was used in the total count of each of the bacteria, fungi and yeasts present in Annona powders (seed & peel) according to Blodgett, (2010).

2.5. Induction of liver cancer:

TCA was neutralized with NaOH to a final pH of 6.5 (Herren-Freund *et al.,* 1987). TCA had been given as a carcinogen after 28 days from experimental period at dose 500mg/kg orally once a day for 5 days consecutive according to Tao *et al.* (2000).

2.6. Experimental rats design:

The animals were housed in polypropylene cages under the standard laboratory condition (25 ± 2°C, humidity 60–70%, 12-h light/dark cycles). They were fed with standard commercial rat pellet diet and water was provided ad libitum. The rats were acclimatized to laboratory conditions for 7 days prior to the commencement of the experiment. After acclimatization period, the animals were divided into six groups (6 rats/ group). One of them healthy control group and five liver cancer groups (including one without protected and four groups protected with Annona extracts). The experiment continued for 47 days. The protected liver cancer groups received Annona extracts for 28 days. Then, TCA was given for 5 consecutive days at dose 500mg/kg orally (Tao *et al.,* 2000). After that, the extracts were given for 14 days. It was arranged as follows:

- **Group 1:** The animals fed on based diet as normal control group during the experiment period.
- **Group 2:** liver cancer group which the animals were subjected to chemo-induction of liver cancer through administration of trichloroacetic acid (TCA).
- **Group 3:** liver cancer group protected by Annona seed extract (ASE) at level 100mg/kg B.W. by oral stomach tube once daily.
- **Group 4:** liver cancer group protected by Annona seed extract (ASEX) at level 150mg/kg by oral stomach tube once daily.
- **Group 5:** liver cancer group protected by Annona peel extract (ALE) at level 100mg/kg by oral stomach tube once daily.
- **Group 6:** liver cancer group protected by Annona peel extract (ALEX) at level 150mg/kg by oral stomach tube once daily.

2.7. Biological analysis:

At the end of the experiment period, rats were anesthetized di-by using ethyl ether. Blood samples were collected from the inner canthus of the rats eye using heparinized capillary tubes, then the serum were obtained after centrifugation at 3000 rpm for 10 minutes. The blood samples were preserved in a deep freezer at -20 ˚C until be used for liver functions, kidney functions, MDA, CAT, and GPX analyzes according to Bull *et al.* (1993); Vidya *et al.* (2021). All experimental animals in this study were managed according to the guidelines for the Care and Use of Laboratory Animals in Neuroscience and Behavioral Research and were approved by the Research Ethics Committee, Home Economics Department, nutrition and food science, Mansoura University, Egypt, under animal protocol code No (R/35).

2.8. Determination of liver functions in blood:

ALT (alanine aminotransferase) and AST (aspartate aminotransferase) enzymes activity were measured accordance to the method of Hafkenscheid and Dijt, (1979). ALP (alkaline phosphatase activity) enzymes was measured accordance to the method of Tietz *et al.* (1983). Serum total bilirubin was measured accordance to the method of Doumas *et al.* (1985). Total protein was measured accordance to the method of Schneditz *et al.* (1989). Serum albumin was determined accordance to the method of Doumas *et al.* (1972). Serum globulin was determined accordance to the method of Fernandez *et al.,* (1966).

2.9. Determination of kidney function in blood:

Serum creatinine was determined according to the method of Houot (1985). Urea concentration in serum was determined by NED Dye method (colorimetric Fix Time test) (Patton and Crouch, 1977).

2.10. Determination of oxidation stress:

Malondialdehyde (MDA) was measured by the method of Stocks and Donnandy, (1971). GPX: glutathione peroxides GSH-PX activity in plasma was measured by the method of Tappel, (1978). CAT: catalase in plasma was measured by the method of Aebi, (1984).

2.11. Statistical analysis:

The gained data were statistically analyzed by SPSS computer software according to Artimage and Berry, (1987). The calculation accrued by analysis of variance ANOVA & follow up LSD (SPSS) Computer program variation.

3. Results and Discussion

3.1. Chemical results on seeds and peels of Annona fruit:

Data presented in Table (1) showed the chemical composition of seed and peel of fresh Annona fruit. These results noticed that, seed had the highest content of moisture, fats, ash and water activity when compared to peel. While the peel had the highest content of protein, fiber and carbohydrate when compared to seed. Our results agreed with Chimbevo and Essuman, (2019) who reported that, the peel of Annona fruit contained moisture, carbohydrate, protein, fat and fiber with values 42.38, 36.62, 4.36, 4.08 and 52.20% respectively. While, Shehata *et al.* (2021) who stated that the chemical composition of Annona seed contained fats, ash, protein and moisture% at concentrations of 29.21, 32.64, 1.90, 2.25 and 3.92 g/100g dry weight, respectively. Junior *et al.* (2020) who noticed that the moisture is related to the water activity, so the high water activity due to the high of moisture.

Table 1: Chemical composition of seed and peel of fresh Annona fruit.

Sample	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Seed	a 47.84 ±0.12	b 5.76 ±0.07	a29.11±0.04	a3.31 ±0.03
Peel	b42.10 ±0.08	a 9.51 ±0.04	c4.11 ±0.07	b2.35±0.05
Sample	**Fiber (%)**	**Carbohydrates (%)**	**Water activity**	
Seed	a21.93±0.04	c13.98 ±0.26	a0.86 ±0.02	
Peel	b34.63±0.06	a41.93 ±0.010	a0.85 ±0.03	

Data presented in Table (2) showed the phytochemical screen in total phenol, total flavonoid, and total antioxidant of Annona extracts (seed and peel). These results noticed that, the highest content of total phenols and total flavonoids were noticed in seed extract compared to peel extract. While the highest content of antioxidant activity was noticed in peel extract compared to seed extract. Our results agreed with Roesler *et al.* (2006) who stated that, the peel extract of Annona fruit had antioxidant activity by DPPH% 48.82 mg/mL, while the seed extract had 31.14 mg/mL. While, Arruda *et al.* (2019) who cleared that, seed and peel of Annona fruit is a good source of phenolic compounds.

Table 2: The phytochemical screen in Total phenol, Total flavonoid, and Total antioxidant of Annona extracts (seed and peel).

Sample	Total phenol (mg/g)	Total flavonoid (mg/g)	Antioxidant activity (DPPH %)
Seed	a2.18±0.05	a0.26±0.04	b74.18±0.57
Peel	b1.64±0.06	a0.21±0.03	a81.11±0.46

Data presented in Table (3) showed the HPLC conditions of phenols of Annona extracts (seed and peel). These results showed that, the seed extract contains Naringenin, Daidzein, Cinnamic acid, and Apigenin more than the peel extract. While, the peel extract contains Gallic acid, Chlorogenic acid, Catechin, Methyl gallate, Coffeic acid, Syringic acid, Pyro catechol, Ellagic acid, and Hesperetin more than the ethanoic seed extract. On the other hand, the seed extract doesn't have Catechin, Methyl gallate, Ellagic acid, Ferulic acid, and Hesperetin. While the peel extract doesn't have Vanillin, Ferulic acid, Naringenin, and Querectin, Cinnamic acid, and Apigenin. Our results agreed with Roesler *et al.,* (2007) who cleared that Annona extracts (peels and seeds) included bioactive components as potent antioxidants such as caffeic acid, ferulic acid. While, Menezes *et al.* (2019) mentioned that Annona seed had Gallic acid (135.6 mg kg-1 d.b.) more than Coffeic acid (40.5), Chlorogenic acid (14.7), and 57 Vanillin (3.1). While it didn't have Coumaric acid.

Table 3: The HPLC conditions of phenols of Annona extracts (seed and peel).

Sample	Seed		Peel	
Phenolic acids	**Conc. (µg/ml)**	**Conc. (µg/g)**	**Conc. (µg/ml)**	**Conc. (µg/g)**
Gallic acid	3.52	70.49	7.81	156.15
Chlorogenic acid	1.77	35.48	11.58	231.66
Catechin	0.00	0.00	18.90	377.93
Methyl gallate	0.00	0.00	3.07	61.48
Coffeic acid	0.61	12.17	2.56	51.28
Syringic acid	0.43	8.63	0.62	12.35
Pyro catechol	0.76	15.28	0.87	17.41
Ellagic acid	0.00	0.00	0.48	9.55
Vanillin	0.07	1.37	0.00	0.00
Naringenin	1.56	31.23	0.00	0.00
Daidzein	2.39	47.83	0.30	5.91
Querectin	0.28	5.59	0.00	0.00
Cinnamic acid	0.38	7.53	0.00	0.00
Apigenin	0.13	2.59	0.00	0.00
Hesperetin	0.00	0.00	0.92	18.37

3.2. Effect of storing seeds and peels powders at room temperature (25 ° C) on the total count of bacteria (TCB), fungi and yeasts:

The data contained in Table (4) showed the total count of bacteria (TCP), fungi and yeasts for the seed powder during its storage starting from zero time and for a period of 28 days per week. During storage, starting from zero time until the third week, Annona seed powder was the lowest in TCP compared to peel powder.

Table 4: Effect of storing seed, peel powder at room temperature (25 ° C) on the total count of bacteria (TCB), fungi and yeasts.

Sample		Storage period time/ day				
		Zero time X101 (cfu/g)	**7 days X103 (cfu/g)**	**14 days X105 (cfu/g)**	**21 days X106 (cfu/g)**	**28 days X108 (cfu/g)**
Seed powder	Bacteria	2.7	1.02	1.98	1.17	2.30
	Fungi and yeasts	11.7	1.13	3.97	2.01	1.05
Peel powder	Bacteria	12.1	3.43	3.20	2.63	2.23
	Fungi and yeasts	13.7	2.83	4.02	3.12	6.16

However, during the fourth week, Annona peel powder was the lowest in TCP compared to seed powder. While, seed powder was the lowest in total count of fungi and yeasts compared to peel powder

during storing period. Our results agree with Tavares *et al.* (2014); Chen *et al.* (2018); Nzogong *et al.* (2018); Kuan *et al.* (2019) who stated that the antimicrobial activity of Annona extracts due to the presence of bioactive compounds such as phenolic compounds and alkaloids that are potent antimicrobial agents. Based on Table (4), it was found that phenolic compounds and flavonoids were to a greater extent in the seeds compared to the peels, and this explains the higher microbial count in the peels compared to the seeds.

3.3. Biological results

Data presented in Table (5) showed the mean values of Alanine transaminase (ALT), Aspartate transaminase (AST), alkaline phosphatase (ALP), Bilirubin (BiL), Globulin (GLb), total protein (TP), and Albumin (Alb) of normal control and liver cancer groups.

Table 5: Effect of ethanolic extracts (at levels 100 &150 mg/ kg) of the seed and peel of Annona fruit on liver function in experimental rats.

Variable	Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
Non- protected Groups	**Normal control**	d 24.75 ±2.50	d 68.25 ±3.40	e192.75±4.50
	Positive (+ve)	a55.00 ±2.94	a244.75 ±10.21	a367.75±10.21
Liver cancer groups protected with	**ASE**	b42.25 ±1.26	b 155.00 ±7.87	b279.00±10.42
	ASEX	c36.25 ±1.26	c 106.75 ±5.80	d227.00 ±8.98
	ALE	b45.00 ±1.63	b177.75±13.48	b295.75±10.08
	ALEX	b39.25±1.26	c 133.75 ±9.74	c252.75 ±9.03

Table 5: Continued

Variable	Groups	BiL (mg/dl)	TP (g/dl)	ALb (g/dl)	GLb (g/dl)
Non- protected Groups	**Normal control**	d 0.21 ±0.02	a 7.53 ±0.06	a 4.49 ±0.02	a 2.37 ±0.04
	Positive (+ve)	a 0.66 ±0.06	d 5.83 ±0.09	d 2.94 ±0.08	d 0.84 ±0.09
Liver cancer groups protected with	**ASE**	b 0.35 ±0.03	c 6.45 ±0.09	c 3.66 ±0.07	c 1.37 ±0.06
	ASEX	c 0.27 ±0.01	b 7.12 ±0.10	b 4.19 ±0.02	b 1.87 ±0.08
	ALE	b 0.42 ±0.05	c 6.29 ±0.10	c3.42 ±0.16	c 1.28 ±0.09
	ALEX	c 0.29 ±0.02	c 6.71 ±0.12	c3.96 ±0.11	b 1.61 ±0.08

Values (mean± SD, n= 4). Means in within the same column sharing the different superscript are significantly different (P> 0.05). ALT: Alanine transaminase, AST: Aspartate transaminase, ALP: Alkaline phosphatase, BiL: Bilirubin, GLb: Globulin, TP: Total protein, Alb: Albumin, ASE: Seed extract at level 100 mg/ kg, ASEX: Seed extract at level 150 mg/ kg, ALE: Peel extract at level 100 mg/ kg, ALEX: Peel extract at level 150 mg/ kg.

The obtained results showed that, non-protected group of rats with liver cancer showed a significant raise in ALT, AST, ALP and BiL, but correspondingly a significant fall in TP, Alb and GLb compared with the normal control (-ve). Liver cancer group protected by ASEX showed a significant lower in ALT, AST, ALP and BiL but on the other hand showed a significant higher in TP, GLb and Alb levels, compared to the positive group Followed by ALEX. But protection by ASE and ALE showed a slight improvement, compared to the positive group. Our results agreed with the results of Abdel-Hamid *et al.* (2011) who indicated that taking TCA at level 500 mg/kg of body weight for 5 consecutive days due to rise in ALT, AST, ALP, and BiL levels. While, Zahid *et al.* (2020) who stated that given of Annona seed extract at level 200 and 400 mg/kg orally daily for 8 consecutive days achieved hepatoprotective activity by raising albumin and total protein levels in rats with alcohol-

induced liver injury.

Data presented in Table (6) showed the mean values of in creatinine and urea of normal control and liver cancer groups. The non-protected group of rats with liver cancer (+ve) showed a significant higher of creatinine and urea, compared to the normal control (-ve). Liver cancer groups protected by ASEX showed a significant lower in creatinine and urea levels, compared to the positive control followed by ALEX. But protection by APE and ASE had slight lower, compared to the positive group (+ve). Our results are consistent with those of El Arem *et al.* (2014) who confirmed that creatinine in the serum increased significantly when mice were given trichloroacetic acid at a dose of 500 g/L in drinking water, compared to normal rats, from 0.20 ± 0.02 to 0.32 ± 0.01 mg/dl. While, Kang *et al.* (2002) reported that the elevation in creatinine and urea concentration in TCA-treated groups is indicative of renal injury. As for the increase in in urea concentrations in the plasma of animals may be associated with its effect on liver function, as urea is the end product of protein catabolism.

Table 6: Effect of ethanolic extracts (at levels 100 & 150 mg/ kg) of the seed and peel of Annona fruit on kidney function in experimental rats.

Variable	Groups	Creatinine (mg/dl)	Urea (mg/dl)
Non- protected Groups	**Normal control**	d0.42±0.03	d20.63±0.79
	Positive (+ve)	a1.19±0.02	a49.23±3.04
Liver cancer groups protected with	**ASE**	b0.82±0.03	b33.43±1.61
	ASEX	c0.59±0.02	c20.43±0.96
	ALE	b0.88±0.03	b38.68 ±3.48
	ALEX	b0.71±0.06	c26.20±2.19

Values (mean± SD, n= 4). Means in within the same column sharing the different superscript are significantly different (P> 0.05). ASE: Seed extract at level 100 mg/ kg, ASEX: Seed extract at level 150 mg/ kg, ALE: Peel extract at level 100 mg/ kg, ALEX: Peel extract at level 150 mg/ kg.

Data presented in Table (7) showed the mean values of malondialdehyde (MDA) free radical, glutathione peroxidase (GPX) and catalase (CAT) antioxidant enzymes of normal control and liver cancer groups. The obtained results showed that, non-protected group of rats with liver cancer showed a significant raise in MDA, but correspondingly a significant fall in GPX and CAT compared with the normal control (-ve).

Table 7: Effect of ethanolic extracts (at levels 100 & 150 mg/ kg) of the seed and peel of Annona fruit on oxidation in experimental rats.

Variable	Groups	MDA (nmol/ml)	GPX (mU/ml)	CAT (U/L)
Non- protected Groups	**Normal control**	e7.73±0.49	a127.23±2.82	a2.83±0.05
	Positive (+ve)	a34.10±1.31	e52.20±4.53	d0.91±0.07
Liver cancer groups protected with	**ASE**	c20.27±2.69	c81.08±5.56	c1.54±0.12
	ASEX	d11.58±1.13	b116.00±4.73	b2.45±0.11
	ALE	b24.48±2.21	d75.28±4.90	c1.33±0.08
	ALEX	c14.80±1.04	c96.23±6.14	b2.04±0.22

Values (mean± SD, n= 4). Means in within the same column sharing the different superscript are significantly different (P> 0.05). MDA: malondialdehyde, GPX: glutathione peroxidase, CAT: catalase, ASE: Seed extract at level 100 mg/ kg, ASEX: Seed extract at level 150 mg/ kg, ALE: Peel extract at level 100 mg/ kg, ALEX: Peel extract at level 150 mg/ kg.

Liver cancer group protected by ASEX showed a significant lower in MDA but on the other hand showed a significant higher in GPX and CAT levels, compared to the positive group followed by ALEX. But protection by ASE and ALE showed a slight improvement, compared to the positive group. Our results agreed with Fouad *et al.* (2013) who indicated that oral administration of TCA led to hepatocellular tumors in rats due to the carcinogenic effect of this chemical as a result of increased oxidative stress, lipid peroxidation and cellular proliferation. While, Chu *et al.* (2002) who reported that Polyphenols, especially flavonoids can protect cells against depletion of glutathione by increasing the capacity of antioxidant enzymes such as glutathione peroxide and catalase. El-Agroudy *et al.* (2016) who stated that GPX level had decreased in rats with liver cancer compared with normal control.

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