

***In vitro* Studies on Anti-Tumour activities of solvent extracts of leaves, fruit pulp and seeds of Graviola (Gishta) cultivar of Ethiopia, N. E. Africa.**

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ABSTRACT: Graviola/Gishta (*Annona* spp.) of Ethiopia is implicated in a variety of ethno-medicinal therapies ranging from the common parasitic infections to high blood pressure and cancer. The effect of this plant extracts for cytotoxicity study needs to be investigated. Thus, the present work was designed to access the lethality of the leaves, fruit pulp and seeds of Graviola cultivar of Ethiopia on the brine shrimps. The different fractions of solvent extracts of Graviola leaves, fruit pulp and seeds were subjected to cytotoxicity studies. The brine-shrimp lethality assay was analyzed using the Finney Probit method. The results showed that the different solvent extracts of the leaves, fruit pulp and seeds displayed LD₅₀ values which were dose-dependent, and also enhanced with increase in concentration. These findings indicate the potentials of Graviola (*Annona* spp.) of Ethiopia as panacea for infectious diseases and also reveal a novel potential in the fight against tumors in human.

KEYWORDS: Brine-shrimp lethality; Solvent extracts; Finney probit method.

I. INTRODUCTION

Cancers figure among the leading causes of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012. The number of new cases is expected to rise by about 70% over the next 2 decades. More than 60% of world's total new annual cases occur in Africa, Asia and Central and South America. These regions account for 70% of the world's cancer deaths. It is expected that annual cancer cases will rise from 14 million in 2012 to 22 within the next 2 decades [1]. Molecular targeted agents are currently being studied in all treatment settings including that of chemoprevention, which is defined as the use of natural or synthetic nonessential dietary agents to interrupt the process of carcinogenesis and to prevent or delay tumor growth [2, 3]. The available treatment methods include surgery, chemotherapy, and radiation [4]. The current available methods of treatment all induce significant side effects and therefore the need for alternate adjuvant therapies has arisen [5]. Natural products are extremely an important source of medicinal agents. The identification of novel antitumor agents that demonstrate efficacy is necessary to improve medical treatment based on new drugs. The pharma research sectors have been working together in this area for several years and trying to find novel compounds with good antitumor activity.

Brine shrimp (*Artemia salina*) larvae have been reported as an organism that can detect bioactive compounds. Since there is a good correlation between the toxicity on *Artemia salina* and antitumor activity [6, 7], this test is a good prescreening method for identifying new potential antitumor agents.

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II. REVIEW LITERATURE

Graviola is a small, upright evergreen tree, 5–6 m high, with large, glossy, dark green leaves. It produces a large, heart-shaped, edible fruit that is 15–20 cm in diameter and green in color, with white flesh inside. The leaves, fruit, seeds, bark, root and stem are used to make medicine[8]. Synonyms: *Annona bonplandiana*, *A. cearensis*, *A. cherimola*, *A. macrocarpa*, *A. muricata*, *Brazilian cherimoya* and *Guanabanus muricatus* [8].

Graviola is a fruit that generally grows in the rain forests of Africa, South America, and Southeast Asia. It has other names like thorny custard apple, cherimoya and brazilian pawpaw. In various languages, this fruit is referred as: Gishta (Ethiopia), guanabana (Spanish), corossol (French), aluguntugui (Ghana), sorsaka (Papiamento), adunu (Acholi), guyabano, guanavana, durian benggala, nangka blanda, sirsak, toge-banreisi, nangka londa, zuurzak, Brazilian Paw Paw, Corossol, Corossol Épineux, Corossolier, Durian Benggala, Guanabana, Guanavana, Nangka Blanda, Nangka Londa, Soursop, Sour Sop, Toge-Banreisi. guanaba, épineux, huanaba, toge-banreisi, durian benggala and cachiman épineux. In India, it is less known as shul-ram-fal and hanuman fal, and as mullaatha in Malayalam, whereas in Harar (Ethiopia) in Harari language known for centuries as *Amba Shoukh* (Thorny Mango or Thorny Fruit). "yebere lib" meaning heart of cow in Jimma, Ethiopia[9, 10].

All parts of the Graviola (*Annona* spp.) tree have been used in traditional folkloric medicine in various cultures for various ailments and complaints. The leaves and seeds of the tree have long been used by native people of various cultures for an astounding variety of ailments, ranging from parasitic infections (the seeds) to high blood pressure and cancer. Seeds are emetic, Pulverized seeds and seed oil are effective against head lice. Flowers are regarded to be antispasmodic and pectoral and used to alleviate catarrh. Leaf Infusions have been used as emetic, sudorific, antispasmodic, antipyretic, insecticidal, eczema, skin eruptions, swollen feet and compresses for inflammation. Ripened fruit is reported as antiscorbutic and is also used as an anthelmintic. Pulp of soursop has been used as diuretic, hematuria and urethritis. The unripe and dried fruit and seeds used as astringent, used in diarrhea and dysentery. The fruit juice is used medicinally to treat illness ranging from stomach ailments to worms. The bark is used in powdered form for diarrhoea and dysentery. The fruit is also used as bait in fish traps. The bark has been used in tanning and the wood is a potential source of paper pulp. Recent studies have supported many of soursop's traditional medicinal uses and also showed that various parts of the tree contain potential bioactive compounds (acetogenins), which have been shown to be responsible for its myriad array of its medicinal attributes. Acetogenins exhibit a broad range of potent biological activities (antimalarial, antiprotozoal, antispasmodic, antiviral, antimicrobial, immunosuppressant, antifeedant, anthelmintic, pesticidal etc.,) [11].

To our knowledge Graviola (*Annona* spp.) of Ethiopia has not been investigated for its biological activities. The principal aim of this work was to study the cytotoxicity of Graviola (*Annona* spp.) leaves extracts in different solvents such as ethanol, chloroform, methanol and petroleum ether against Brine shrimp (*Artemia salina*) larvae.

In the experimental study, different solvent extracts of Graviola (*Annona* spp.) leaves of Ethiopia have been investigated at different concentrations.

III. MATERIALS AND METHODS

Plant collection, identification and authentication

Graviola/Gishta (*Annona* spp.) of Ethiopia was collected from Oromeo region, Ethiopia, in January 2014. The plant was identified, authenticated and supplied by Prof Behailu Etana Disasa of Natural Resource Management, College of Agriculture and Veterinary Medicine, Jimma University, Jimma, Ethiopia (Fig. 1).

Extract Preparation

Fresh plant materials (leaves, fruit pulp and seeds) were dried at room temperature ($28\pm 3^{\circ}\text{C}$) by exposing to atmosphere under aseptic conditions. The dried materials were pulverized into a very fine powder. Ten grams of each sample was Soxhlet (Fig. 2) extracted sequentially using petroleum ether, chloroform [12], methanol and ethanol at 60°C temperature. The crude extract was vacuum dried, redissolved in Dimethyl Sulfoxide (DMSO) and finally sterilized by filtration (Whatman $0.2\mu\text{m}$).

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Chemicals used

Ethanol, chloroform, methanol and petroleum ether (SD Fine Chemicals, India) were used during experimental protocol.

Other materials

Brine shrimp (*Artemia salina*) eggs were collected from pet shop and hatched for two days to get matured Nauplii [13].

Toxicity assay using brine shrimps, *Artemia salina* Leach

Cytotoxicity of organic solvent extracts of Graviola/Gishta (*Annona* spp.) leaves, fruit pulp and seeds were evaluated in triplicate using *Artemia salina* Leach. Brine shrimp eggs were hatched in artificial sea water [14]. After 48 hours the phototrophic nauplii were collected and a suspension of about 30–50 nauplii in 100 μ l were placed into bijoux bottles containing 900 μ l of extract in artificial sea water. Eight final concentrations of the extract ranging from 1–128 μ g/ml, at two-fold serial increments were used in the assay. The control bijoux bottle contained the nauplii in 1000 μ l artificial sea water but devoid of the extract. After 24 hours of incubation, the dead nauplii were counted under a dissecting microscope. Subsequently 200 μ l of methanol was added to each bottle and 60 minutes later the total numbers of nauplii were counted. The brine-shrimp lethality assay was analyzed using the Finney probit method.



Figure 1: Graviola (Gishta) Leaves and Fruits of Ethiopia.

IV. RESULTS AND DISCUSSION

The solvent extracts of different concentrations of Graviola (*Annona* spp.) plant materials (leaves, fruit pulp and seeds) were tested for toxic activities using brine shrimp lethality bioassay (Graph. 1, 2 and 3). The results are presented in Table 1, 2 and 3.

Toxicity tests were carried out in triplicates using *A. salina*. Using the method given above, petroleum ether extracts proved to be the most toxic of graviola (*Annona* spp.). Leaves extract tested $LC_{50} = 6.43 \mu\text{g/ml}$, followed by chloroform extract ($5.20 \mu\text{g/ml}$), methanol extract ($24.09 \mu\text{g/ml}$) and ethanol extract ($24.84 \mu\text{g/ml}$). The extracts were effective at low concentrations, LC_{50} at $6.43 \mu\text{g/ml}$ indicated toxicity relatively at low concentration. Fruit pulp extract tested $LC_{50} = 1.27 \mu\text{g/ml}$, followed by chloroform extract ($5.48 \mu\text{g/ml}$), methanol extract ($15.595 \mu\text{g/ml}$) and ethanol extract ($30.33 \mu\text{g/ml}$). The extracts were effective at low concentrations, LC_{50} at $1.27 \mu\text{g/ml}$ indicated toxicity relatively at low concentration. Seeds extract tested $LC_{50} = 1.28 \mu\text{g/ml}$, followed by petroleum ether extract ($1.72 \mu\text{g/ml}$), methanol extract ($65.15 \mu\text{g/ml}$) and ethanol extract ($67.03 \mu\text{g/ml}$). The extracts were effective at low concentrations, LC_{50} at $1.28 \mu\text{g/ml}$ indicated toxicity relatively at low concentration. These results may undermine its usefulness. One study showed that acetogenins isolated from *Annona* spp., were selectively cytotoxic to colon adenocarcinoma cells, with a potency 10,000 times that of adriamycin [15]. Different parts of the plant extracts showed significantly selective in-vitro cytotoxicities toward the human hepatoma cell lines Hep G(2) and 2, 2, 15 [16]; shown cytotoxic in KB and VERO cell lines [17]; studied cytotoxicities against the human A-549 lung tumour cell line [18]; reported cytotoxicities against the A-549 human lung and the MCF-7 human breast solid tumour cell lines [19, 20]; showed significant inhibitory effects on different human tumour cell lines such as the prostate (PC-3) and pancreatic (PACA-2) cell lines [21]; showed significant cytotoxicities against prostate adenocarcinoma (PC-3) and pancreatic carcinoma (PACA-2) cell lines [22, 23]; Acetogenins isolated from seed extracts were found to be most potent against the growth of adriamycin

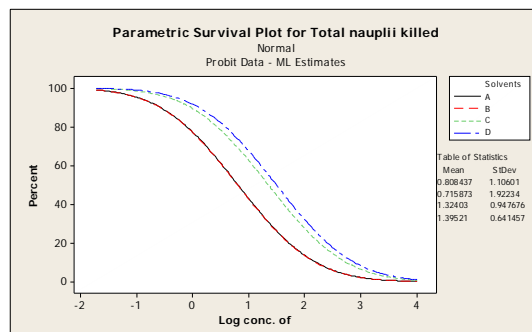
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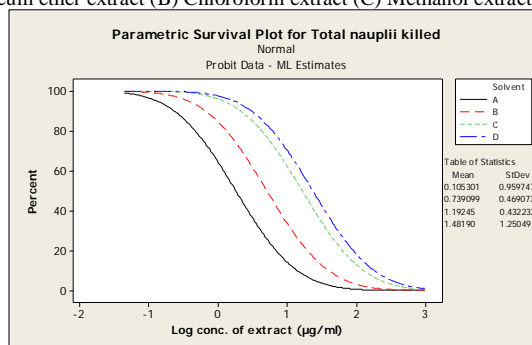
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resistant human mammary adenocarcinoma (MCF-7/Adr) cells with as much as 250 times the potency of adriamycin [24] and also reported to show cytotoxic activity against human tumour cell lines with potency from 105 to 106 times that of adriamycin [25]. These research studies confirmed significant anti-tumour, anticancer and selective toxicity against several different types of cancer cells which they studied. Toxicity of test samples has been shown to be dependent on the concentration of specific compound(s) in the sample [26]. The level of toxicity observed in this study may have resulted from presence of multiple compounds in the crude extracts, acting in synergy or independently. Thus, isolation and testing of the purecomponent may produce lower levels of toxicity.



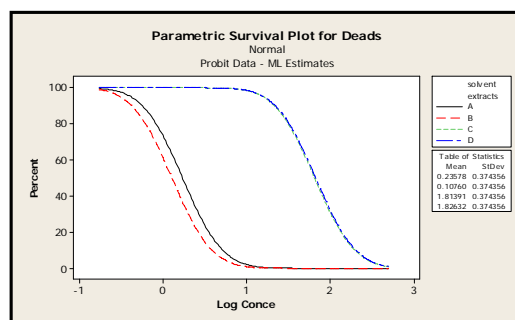
Graph 1: Log C versus average percentage of mortality of nauplii of solvent extracts of graviola (*Annona* spp.) leaves of Ethiopia.

(A) Petroleum ether extract (B) Chloroform extract (C) Methanol extract (D) Ethanol extract



Graph 2: Log C versus average percentage of mortality of nauplii of solvent extracts of graviola (*Annona* spp.) Fruit pulp of Ethiopia.

(A) Petroleum ether extract (B) Chloroform extract (C) Methanol extract (D) Ethanol extract



Graph 3: Log C versus average percentage of mortality of nauplii of solvent extracts of graviola (*Annona* spp.) seeds of Ethiopia.

(A) Petroleum ether extract (B) Chloroform extract (C) Methanol extract (D) Ethanol extract

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Table 1: Toxicity of solvent extracts of Graviola/Gishta (*Annona* spp.) leaves of Ethiopia against *Artemia salina* Leach.

Log conc. of extract (µg/ml)	Petroleum ether extract			Chloroform extract			Methanol extract			Ethanol extract		
	Total Nauplii	Total nauplii killed	% killed	Total nauplii	Total nauplii killed	% killed	Total nauplii	Total nauplii killed	% killed	Total Nauplii	Total nauplii killed	% killed
2.107	29	26	89.66	28	26	92.86	32	26	81.25	34	17	50.00
1.806	33	27	81.82	36	23	63.89	31	21	67.74	30	10	33.33
1.505	35	27	77.14	42	24	57.14	30	18	60.00	40	2	5.00
1.204	31	19	61.29	43	24	55.81	32	0	0.00	32	0	0.00
0.903	39	18	46.15	40	22	55.00	32	0	0.00	42	0	0.00
0.602	33	15	45.45	50	28	56.00	34	0	0.00	36	0	0.00
0.301	31	9	29.03	46	18	39.13	34	0	0.00	30	0	0.00
0.001	40	11	27.50	48	17	35.42	31	0	0.00	28	0	0.00

LC₅₀ leaves of Graviola cultivar of Ethiopia petroleum ether extract = 6.43 µg/ml; LC₅₀ leaves of Graviola cultivar of Ethiopia chloroform extract = 5.20 µg/ml; LC₅₀ leaves of Graviola cultivar of Ethiopia methanol extract = 24.09 µg/ml; LC₅₀ leaves of Graviola cultivar of Ethiopia ethanol extract = 24.84 µg/ml.

Table 2: Toxicity of solvent extracts of Graviola/Gishta (*Annona* spp.) Fruit pulp of Ethiopia against *Artemia salina* Leach.

Log conc. of extract (µg/ml)	Petroleum ether extract			Chloroform extract			Methanol extract			Ethanol extract		
	Total Nauplii	Total nauplii killed	% killed	Total Nauplii	Total nauplii killed	% killed	Total nauplii	Total nauplii killed	% killed	Total Nauplii	Total nauplii killed	% killed
2.107	27	20	74.07	28	20	71.43	34	19	55.88	37	20	54.05
1.806	31	23	74.19	37	26	70.27	33	14	42.42	32	13	40.63
1.505	32	24	75.00	42	29	69.05	32	3	9.38	39	3	7.69
1.204	29	21	72.41	44	30	68.18	34	0	0.00	31	0	0.00
0.903	37	24	64.86	39	26	66.67	33	0	0.00	45	0	0.00
0.602	36	16	44.44	50	31	62.00	36	0	0.00	35	0	0.00
0.301	29	13	44.83	46	21	45.65	37	0	0.00	29	0	0.00
0.001	42	11	26.19	48	14	29.17	33	0	0.00	31	0	0.00

LC₅₀ petroleum ether Fruit pulp extract of Graviola = 1.27 µg/ml; LC₅₀ Chloroform Fruit pulp extract of Graviola = 5.48 µg/ml; LC₅₀ Methanol Fruit pulp extract of Graviola = 15.595 µg/ml; LC₅₀ Ethanol Fruit pulp extract of Graviola = 30.33 µg/ml.

Table 3: Toxicity of solvent extracts of Graviola/ Gishta (*Annona* spp.) seeds of Ethiopia against *Artemia salina* Leach.

Log conc. of extract (µg/ml)	Petroleum ether extract			Chloroform extract			Methanol extract			Ethanol extract		
	Total Nauplii	Total nauplii killed	% killed	Total nauplii	Total nauplii killed	% killed	Total nauplii	Total nauplii killed	% killed	Total nauplii	Total nauplii killed	% killed
2.107	30	30	100.00	28	28	100.00	32	26	81.25	34	28	82.35
1.806	34	34	100.00	36	36	100.00	31	19	61.29	30	18	60.00
1.505	36	36	100.00	42	42	100.00	30	3	10.00	40	4	10.00
1.204	32	32	100.00	43	43	100.00	32	1	3.13	32	1	3.13
0.903	40	36	90.00	38	38	100.00	32	0	0.00	42	0	0.00
0.602	34	24	70.59	50	45	90.00	34	0	0.00	36	0	0.00
0.301	32	20	62.50	46	30	65.22	34	0	0.00	30	0	0.00
0.001	41	17	41.46	48	20	41.67	31	0	0.00	28	0	0.00

LC₅₀ seeds of Graviolachloroform extract = 1.28 µg/ml; LC₅₀ seeds of Graviola petroleum ether extract = 1.72 µg/ml; LC₅₀ seeds of Graviolamethanol extract = 65.15 µg/ml; LC₅₀ seeds of Graviolaethanol extract = 67.03 µg/ml

Research highlights

- Detected biologically active compounds of tested extracts of Graviola (Gishta) by measuring the activity of cellular enzymes based on cytotoxicity (loss of viable cells).
- Discovered anti-tumor potential or cancer therapeutics in the tested materials of Graviola (Gishta) Cultivar of Ethiopia.
- There was no research report on Graviola (Gishta) cultivar of Ethiopia in terms of anti-cancer potential.

Limitations

The following limitations were identified by the researchers in the present experimental study:

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The brine-shrimp lethality assay is time-consuming; this method was traditional method, so there was no real-time monitoring regarding cell death over time; maintaining permanent record of data is difficult.

Recommendations

After obtaining the results discussed above the answer to the problems presented in the anti-tumor potential of leaves, fruit pulp and seeds extracts of Graviola (Gishta) cultivar of Ethiopia against brine-shrimp, the researchers have arrived at the following recommendations to further improve the study:

- The study has focused only on the anti-tumor potential of leaves, fruit pulp and seeds extracts of Graviola (Gishta) cultivar of Ethiopia against brine-shrimp. In lined with this, the researchers highly recommend the phytochemical screening of tested extracts of Graviola (Gishta) cultivar of Ethiopia for the identification of its specific active bio ingredient(s).
- Future research can be encouraged to determine the effect of tested extracts of Graviola (Gishta) cultivar of Ethiopia, in the metabolism and other physiological function.
- In addition, future researchers are recommended to use the same plant sample but with different solvents. This study may compare more solvents, and identify which specific organic solvent is the best for specific plant material extractions of Graviola (Gishta) cultivar.
- Moreover, future researchers are recommended to use the same plant sample but with different and advanced bioassay method (new image-based technique). This study may provide the accurate potentiality of the plant extract.
- Lastly, for future studies, it is recommended to nanotechnology aspects to the leaves extracts of Graviola (Gishta) cultivar of Ethiopia and compares the anti-cancer potential of conventional and nanoparticles extracts of Gishta plant materials especially seed extracts.

V. CONCLUSION

These finding raises hope for the possibility of developing an antitumor agent(s) from Graviola (Gishta) tested extracts that can exhibit selective and specific action against *Artemia salina*. Among the tested extracts seeds extract were exhibited high potential activity followed by fruit pulp and leaves extract. Our results seem consistent with the correlation previously established between cytotoxicity and brine shrimp lethality in Graviola tested extracts. We suggest using this bioassay simultaneously to test natural products for pharmacological activity.

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