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Article

Phytochemical analysis of *Annona Reticulata* extract and an *in-vitro* study on its anti-proliferative effects

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SUMMARY

***Annona Reticulata* (Ramphal or custard apple) has been a plant of interest in the traditional medicinal system for the prevention and treatment of various inflammatory disease conditions, including cancer. But there has always been a requirement of continuous efforts for the efficient extraction of potential secondary metabolites that are used for treatment. This study is concerned with the anti-cancerous secondary metabolites present in the leaves of the *Annona Reticulata* plant (RL) and its anti-proliferative properties. Many extracts were tested on HeLa cells using a variety of solvents. The anti-proliferative effect of the combination of methanol dry extract and wet extract in acetone was observed to contain the greatest range of dissolved secondary metabolites. At concentrations over 2.5%, weight over volume, a sharp decrease in viability of below 50% for both 24 hours and 48 hour time duration. This result suggests that the leaves may have potent anti-proliferative and possibly anti-cancer properties.**

INTRODUCTION

In Ayurveda, the Ramphal or custard apple plant was often used to treat stomach ailments, fever, parasitic infections, hypertension and rheumatism (1). Other studies in the past have demonstrated that the Annona family has anti-carcinogenic effects (2). Thus this plant was chosen because it has been suggested to have anti-cancerous properties and it is present throughout the Indian subcontinent.

Secondary metabolites are organic compounds produced by bacteria, fungi, or plants which are not directly involved in the healthy growth, development, or reproduction but aid in the plants' long term survival (3). The metabolites were screened for following phytochemicals: saponins, polyphenols, flavonoids, alkaloids and tannins. Saponins are a group of plant glycosides, characterized by their strong foam-forming properties in aqueous solution; they are known to induce apoptotic pathways in cancer cells (4). Polyphenols are mildly acidic and used in chemical manufacture, and its dilute form as a disinfectant. They are also often found in spices and fruit, which are reported to have antioxidant, anti-inflammatory, spasmolytic, antidiarrheal, antimicrobial, and anti-carcinogenic activity (5). Flavonoids are a group of plant metabolites thought to provide health benefits through cell signalling pathways and antioxidant effects; they are demonstrated as anti-cancer agents, and have shown great potential as cytotoxic anti-cancer agents promoting apoptosis in cancer cells (6). Alkaloids are a class of nitrogenous organic compounds, including many drugs and poisons that affect cancer cells and prevent successful cellular divisions (7). Tannins are bitter-tasting natural substances present in some galls, barks, and other plant tissues, consisting of derivatives of gallic acid. They show powerful antioxidant properties and also show promise for the cancer chemoprevention (8). This study aims to understand how the possible the anti-proliferative property of *Annona Reticulata* leaves affect cancerous cells.

The plant, *Annona Reticulata*, is one that's found in abundance throughout the Indian Subcontinent, the birthplace of Ayurveda, a traditional Hindu system of medicine based on the principles of balance between bodily functions, incorporating diet, herbal treatment, and yogic breathing. This plant, even from ancient times, has always been held in high regard because of its inherent medicinal properties, and in India was named after the God Rama "Ramphal." Many studies have shown that the leaves of the plant contain anti-inflammatory and anti-ulcer compounds. For generations, the plant was made into salves and applied to infected wounds and even tumours.

To extract the many active compounds in the leaf, both a dry and wet extraction methods were employed. This allowed us to observe which extraction method would lead to a higher yield of secondary metabolites. Different solvents were also utilized to allow for a variety of different compounds to dissolve. Thus a range of polar, bipolar, and non-polar solvents was used: water (H_2O), very polar molecule to dissolve polar molecules; methanol (CH_3OH) another polar solvent; ethanol (C_2H_5OH) which has both hydrophobic and hydrophilic ends, allowing it to dissolve a wide range of solutes; chloroform ($CHCl_3$), another bipolar solvent; and, acetone (C_3H_6O), a non-polar molecule which readily dissolves non-polar molecules.

RESULTS

The aim of this study was to first quantify the amount of secondary metabolites by performing qualitative and quantitative analysis of the plant leaves' extracts and secondly to assess the cytotoxicity of the relevant plant solvent extracts by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The MTT assay is a test which measures NADPH-dependent cellular activity, showing the number of

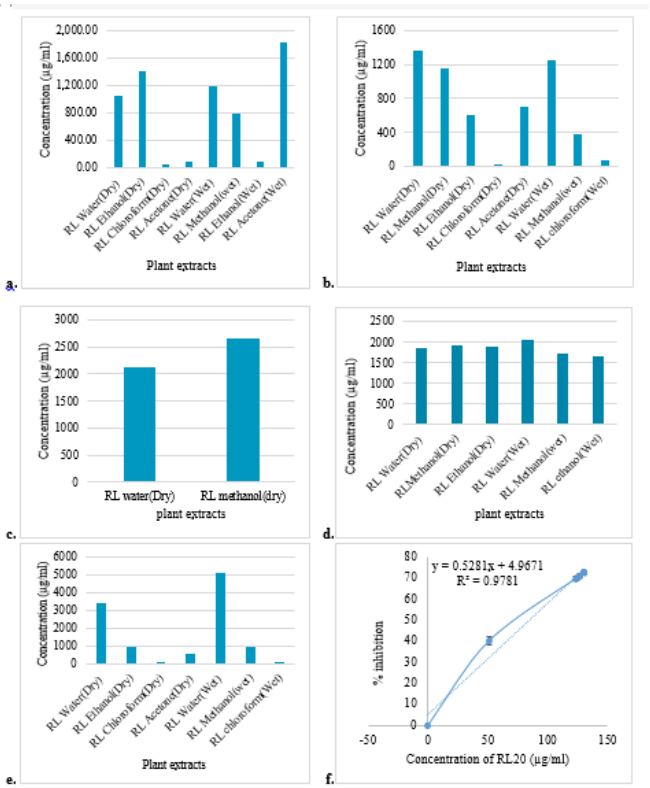

Figure 1: Graphs depicting the amount of phytochemicals present in different extracts of dry and wet RL leaves (a-e) and DPPH activity of RL'20 (f) (N=3, mean±SD). a. total saponins, b. total polyphenols, c. total flavonoids, d. total alkaloids, e. total tannins.

viable cells present (9).

Qualitative and quantitative analysis of phytochemicals in various extracts of RL

From the phytochemical analysis of the *Annona Reticulata* plant, we found that the plant contains a large variety of secondary metabolites and consists of over a thousand µg/ml of various secondary metabolites (**Table 1**). Qualitative data of dry extract of RL showed the presence of flavonoids, polyphenols and alkaloids in methanol and water extracts. Although a slight presence of polyphenols was observed in acetone extract, the flavonoids and alkaloids were completely absent. Saponins and tannins were scarcely seen in the acetone and chloroform extract of dry RL, whereas ethanol extract was lacking flavonoids (**Table 1**). Polyphenols and alkaloids were present in water, and methanol extract of wet RL along with the strong presence of tannins and saponins, as depicted in **Table 1**. Acetone and ethanol extracts of wet RL showed a strong indication of the presence of saponins and alkaloids, respectively. Moreover, the presence of phytochemicals was not evident in its chloroform extract. Methanol dry extract contained high concentrations of polyphenols, flavonoids and alkaloids, containing 1158.27 µg/ml, 2664.00 µg/ml and 1922.79 µg/ml respectively (**Figure 1b, c and d**). Meanwhile, the acetone wet extract contained a very high concentration of saponins, containing 1821.12 µg/ml of saponins (**Figure 1a**). The groups containing a high concentration of tannins were not considered for the preparation of the formulation as they were already a subset of the polyphenol group (**Figure 1e**). Thus, to include a high range of all the tested secondary metabolites, a combination of a wet and dry extract was chosen to create the extract that would be used for the MTT assay.

Thus, it was clear that this plant not only contains a large variety of these phytochemicals but also in large quantities. This abundance of available secondary metabolites indicated that it may potentially have significant anti-cancer properties. The DPPH assay is a test for the antioxidant properties of a substance, by the process of free radicle scavenging, the methanol extract was tested using the aforementioned assay

The methanol dry extract and acetone wet extract were weighed after drying for 72 h. Each dried extract was dissolved in DMSO at 0.001 g/mL concentration. These extracts were subjected to MTT assay. Based on the IC50 values, the extracts were then used to form RL'20 and demonstrate a high DPPH activity of 255.81 µg/ml. This indicates RL'20 is a potent antioxidant (**Figure 1f**).

Cytotoxicity effect of RL'20 on HeLa (cervical cancer) cells

The MTT Assay was carried out using these extracts separately and in combination to determine the cytotoxicity effects after 24 and 48 hours. These results are particularly exciting as we see an herbal extract having potent anti-proliferative impacts on the HeLa cells. The high antioxidant properties are also very exciting as this extract could potentially even reduce the chances of cancer in nearby cells by neutralising free radicles which could lead to catastrophic DNA damage, possibly reducing the risk of cancer.

The IC50 values of methanol dry extract were found to be 27.47 and 12.62 µg/ml at 24 and 48 h of treatment, respectively (**Figure 2a**). Acetone wet extract showed a higher IC50 value of 53.59 µg/ml at 24 h and was similar to that of methanol dry extract at 48 h (**Figure 2b**). IC50 of the formulation, RL'20, was found to be the lowest at 13.34 µg/ml and 5.96 µg/ml at 24 and 48 hours, respectively (**Figure 2c**). It was interesting to note that the 48 hour IC50 value for acetone wet extract was over four times less than the same extract at 24 h. In comparison, the 48 hour IC50 values for the methanol dry extract and combination drug were only around 2.25 and 2.6 times lesser than their respective 24 h IC50 values (**Figure 2**).

This study showed the presence of antioxidants and anti-cancer compounds (saponins, flavonoids, polyphenols, alkaloids, and tannins) in the plant extracts, which demonstrated a level of growth suppression and antioxidant properties. As **Figure 2** reveals, a combination of the extracts had the lowest IC50 value of just 5.69 µg/ml after 48 h treatment and was thus the most potent. This was the most exciting outcome considering the RL'20 contained all the secondary metabolites. The qualitative analysis

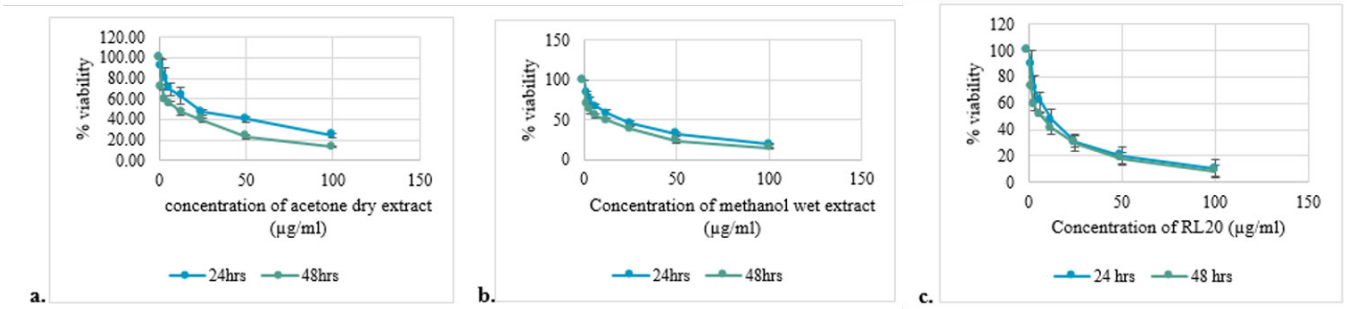

Figure 2: Graphs showing cytotoxicity effect of a. methanol dry extract, b. acetone wet extract and c. RL'20 at 24 and 48h. The data is the representative of three independent experiments performed in triplicates (N=3, mean±SD).

had determined RL'20 shows the most magnificent anti-proliferative properties, leaving only 8.61% of cells viable at its highest concentration after 48 h. The methanol extract also showed relatively high antioxidant properties, and thus probably has cancer prevention properties.

The results for the cytotoxicity assay were auspicious, with each of the three solvents having logarithmic curves for cell toxicity and cell viability (**Figure 2**). It indicated a strong trend, where after 24 h, even the least potent acetone wet extract showed less than 25% cell viability with its highest concentration (**Figure 1**b). At 48 h, the results were ever more prominent, where the methanol dry extract showed the highest cell viability of only around 14%, indicating the potency of the drug increases by time, which could be likely due to the time taken for the secondary metabolites to react on to the cells. Thus, it was clear that the compounds have an anti-proliferative impact on HeLa cells.

Our results demonstrated that saponins, in general, require a more extended period to act upon the cells but are especially potent, as the acetone wet extract and methanol dry extracts 48 h IC50 values were nearly identical even though the acetone extract only has a large amount of saponins and negligible amounts of the other phytochemicals. In contrast, the methanol dry extract has large quantities of polyphenols flavonoids and alkaloids (**Table 1** and **Figure 1**).

As shown in **Figure 2**, the methanol extract has an IC50 value of 87.25 µg/ml and thus has a moderate radical scavenging property. As it has antioxidant activity, which is often known to prevent or fight the onset of cancer (10), it can be hypothesized that this drug can not only fight the growth and spread of cancer, but also help in the prevention of cancer.

DISCUSSION

According to our conclusions, the leaf extracts showed a remarkable effect in both antioxidant and cell viability assays. This experiment therefore can conclude, based on the results of investigations, *Annona Reticulata* is a potent source of bioactive compounds and has a plethora of medicinal properties, such as growth suppressing and anti-proliferative

Solvents	Saponins	Polyphenols	Flavonoids	Alkaloids	Tannins
Water (Dry Extract)	+++	+++	++	+++	+++
Methanol (Dry Extract)	-	+++	+++	+++	-
Acetone (Dry Extract)	+	++	-	-	+
Chloroform (Dry Extract)	+	-	-	-	+
Ethanol (Dry Extract)	+++	++	-	+++	++
Water (Wet Extract)	+	+++	-	+++	+++
Methanol (Wet Extract)	+	++	-	+++	++
Acetone (Wet Extract)	+++	-	-	-	-
Chloroform (Wet Extract)	-	+	-	-	+
Ethanol (Wet Extract)	+	-	-	+++	-

Table 1: Qualitative analysis for the presence of different phytochemicals in various extracts of A. reticulata. '+', '++' and '+++' sign signifies the concentration range of each phytochemical as 1-500, 501-1000 and 1001-2000µg/ml, respectively. '-'signifies absence of the respective phytochemical.

properties. Nevertheless, the anti-cancer properties of the leaf extracts need to further proven. Furthermore, tests are needed to reveal whether the secondary metabolites target just cancerous cells or any dividing cell. The exact mechanism behind these properties are unknown. However, we hypothesized it may be related to anti-inflammatory processes.

The prevalence in Ayurveda has sparked many analyses of the plant; studies of Annona acetogenins isolated from the seeds of A. reticulata showed it caused significant cell death in various cancer lines and suggested potentially promising anti-cancer compounds. These studies on the promising nature of A. reticulata led to our experiment, in which the focus was on the leaves of the plant. According to our results, the plant shows excellent potential as an anti-cancer agent. The usage of this plant could lead to many discoveries in the field of oncology. However, more complex trials and experiments must be conducted on the efficacy of the plant.

MATERIALS AND METHODS

Extraction process: Dry extraction

Leaves were plucked and were shade dried for 48 hours. They were then crushed using a mortar and pestle to get a fine powder. A 1 g:10 ml w/v [1 g of ground dried leaf and 10 ml of solvent] ratio was utilized for extracting the phytochemicals from the all five aforementioned solvents. The leaves were kept in different solvents for 24 hours and the supernatant was centrifuged. The supernatant was collected and stored at 4˚C until use.

Extraction Process: Wet extraction

Leaves were plucked and mashed to a paste using a mortar and pestle. The pulp was left for shade dry for 48 hours. A 1 g:10 ml [1 g of the wet extract of the leaves and 10 ml of solvent] ratio was utilized to dissolve the paste. The leaves were mixed with each solvent and were centrifuged after 24 hours, the supernatant was stored at 4˚C until use.

Qualitative analysis to determine the presence of phytochemicals in each extract

Lambda maxing is a process that identifies the wavelengths at which absorbance is maximum. If there are many dissolved solutes, the lambda max graph may indicate many peaks. and the lambda max wavelength can also behave like a single qualitative parameter to compare the absorption range of different molecules (11). The lambda max curves showed promise as all extracts had shown over ten peaks, indicating the presence of a variety of different dissolved substances in each solvent. Thus, a qualitative analysis of the five secondary metabolites had to be performed on all of the solvents.

The olive oil test was performed to detect the presence of saponins, which utilized vigorous shaking of 0.5 ml of each extract and a few drops of olive oil [~2-3 drops]. A formation of the soluble emulsion after 5 minutes confirms the presence of saponins (12).

For polyphenols, the Folin and Caiocalteu's (FC) reagent test was utilized; ~4 drops of FC reagent and Na_2CO_3 was added to 0.5 ml of the sample and incubated at room temperature in the dark for 20 minutes. The appearance of the blue color shows the presence of polyphenols (13).

The test for flavonoids was performed using the Alkaline reagent test, where 5 drops of 5% NaOH was added to 0.5 ml of extract, which result in yellow color, then few drops of 2 M HCl were added to the solution. The solution turns colorless if flavonoids are present (14).

The test for alkaloids was performed using Dragendorff's analysis, in which 0.5 ml of the extract was added to 0.2 ml of diluted HCl and 1 ml of dragendorff reagent. The presence of alkaloids would be confirmed by the presence of an orange-brown precipitate (15).

To test the presence of tannins, the Ferric Chloride test was performed as 0.5 ml of the extract was mixed with a few drops of 5% $FeCl_3$ and the formation of blue-green color shows the presence of tannins (16).

Quantitative analysis to determine the amount of phytochemicals present in various extracts of RL

Each solvent's absorbance was plotted against a standard absorbance curve to obtain the concentrations of different metabolites in each solvent.

Total Saponins

The Vanillin-Sulphuric acid method (17) was used to determine the total saponin content. 0.25 ml of the sample was added to 0.25 ml of 8% vanillin in ethanol and 2.5 ml of 72% Sulphuric acid. The solutions were incubated for 15 minutes at 60°C and the absorbance was recorded at 765 nm. A standard curve was prepared using Diosgenin.

Total Phenolic content

The total phenolics of the extract were determined using the FC reagent (18). The standard concentrations of gallic acid and samples were mixed with 0.2 ml of FC reagent. After 5 minutes, 1 ml 8% w/v of Na_2CO_3 was added to the solution and volume made up to 3ml with water. The absorbance of the bright blue color was measured after 30 minutes of incubation. The standard plot was prepared using gallic acid and was read at 765 nm.

Total Flavonoid content

Varying concentrations of green tea extracts were mixed with water to make up to 2.4 ml and 0.3 ml of 10% $AlCl_3$ was added. After 5 minutes, 2 ml of 1 M sodium hydroxide was added, the final volume was made up to 10 ml with distilled water and mixed well. The orange color was absorbed in 630 nm. The total flavonoid content was calculated as a green tea extract equivalent on the basis of a standard curve (19).

Total Alkaloid content

2 ml of Dragendorff's solution was added to samples

maintained at 2-2.5 pH with diluted HCl and was centrifuged for 10 min at 1500 rpm. 2 ml disodium sulfide solution was added to form a brown-black precipitate to which 2 ml of concentrated nitric acid was added and the volume was made to 10 ml with water. 1 ml solution was discarded and 5 ml of thiourea was added and the absorbance was recorded. The standard curve was obtained with bismuth nitrate pent-hydrate stock solution (20).

Total Tannin content

0.2 ml sample was added to 0.5 ml of FC reagent and 1 ml of 35% sodium carbonate and the final volume was made up to 10 ml using distilled water. The mixture was shaken and kept at room temperature for 30 minutes. A set of reference standard solutions of tannic acid was prepared using the same method and the absorbance was carried out at 700 nm (21).

Antioxidant assay by DPPH test

DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) assay is a method by which the antioxidant properties of a particular solution or molecule can be tested. This assay is based on the electron transfer between free radicals (22), a violet color form when oxidized and becomes colorless when reduced. 0.5 ml of the extract was added to 1M DPPH solution in methanol. The absorbance was measured at 517 nm after 30 minutes of incubation at room temperature.

Cytotoxicity assay using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) dye

This assay is a colorimetric assay that analyses the cellular metabolic activity (23); thus, the higher metabolic activity, the greater the cell viability. Therefore, the MTT Assay can be used to test the effectiveness of the plant extracts for their anti-proliferative and anti-cancer properties. Two different extracts and a combination of the two were chosen based on the qualitative and quantitative analysis and were tested using the MTT assay (Sigma). The formation of purple formazan crystals inside the mitochondrion indicates the presence of metabolic activity (24), thus higher the intensity of purple color, the higher the number of viable cells in that solution.

HeLa (cervical cancer) cells were obtained from NCCS, Pune. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Invitrogen) at 37˚C with 5% CO_2. 0.3 million cells were seeded in a 96 well plate and the extracts were added after 24 h of culture for 24- and 48 h. MTT was added to and dimethyl oxide was used to dissolve the formazan crystals. OD at 545 nm was taken (25) and the cell viability graphs were plotted.

Thus, the IC50 values of the extracts for cell viability could be tested for different times, this allows for even the slower acting metabolites to affect all the cells. IC50 values show the concentration at which there is precisely half the viability of the cells. It is commonly used as a measure of drug potency in pharmacological research. According to the FDA, IC50 represents the concentration of a drug that is required for 50% inhibition in vitro (26). It also allows one to understand how potent the drug is and helps in understanding the results.

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