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IN VITRO ANTI-OBESITY AND ANTI-CANCER ACTIVITIES OF DIFFERENT EXTRACTS OF *ANNONA SQUAMOSA* L. AND *FICUS RACEMOSA* L. LEAVES.

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ABSTRACT

Extracts of Annona squamosa and Ficus racemosa leaves were prepared by continuous soxhlet extraction using ethanol and hexane solvents and thereafter subjected for lipase inhibition assay to evaluate anti obesity activity and XTT assay against human lung cancer cell lines (A-549) and human metastatic breast cancer cell lines (MDA-MB-435S) to estimate anticancer property. All extracts showed their varying inhibition activities by dose dependent manner and the results were statistically significant with $p<0.05$ by using Dunnett's Multiple Comparison Test. The hexane extracts of *Annona Squamosa* and *Ficus racemosa* showed nearly 98.71 and 97.85% inhibition of lipase enzyme respectively at 100µg/mL tested dose. From the cytotoxic analysis it was found that the hexane extract of *Ficus racemosa* showed nearly 47.83% A-549 cell line inhibition at 200µg/mL tested dose and ethanolic extract of *Annona Squamosa* showed nearly 28.53% MDA-MB-435S cell line inhibition at 200µg/mL tested dose. Hexane extracts showed the best lipase inhibitory property which may attribute to anti obesity activity and the screening studies revealed that hexane extract of *Ficus racemosa* and ethanolic extract of *Annona Squamosa* were found to be active against A-549 and MDA-MB-435S cell lines respectively. Further investigation is needed to reveal the important biochemical constituents and their mechanism responsible for growth suppression and cell death of cancer cell lines.

KEYWORDS: *Annona Squamosa*; *Ficus racemosa*; XTT-assay; Anti Lipase; Anti cancer; Cytotoxic.

INTRODUCTION

Now-a-days cancer is among the leading causes of death worldwide and obesity is one of the major risk factors for a number of chronic diseases including diabetes, cardiovascular diseases and cancer.[1,2] As per WHO estimate 600 million people worldwide were obese in 2014 and 8.2 million people worldwide died from cancer in 2012. Of these 1.59 million deaths were due to lung cancer and 521,000 deaths, breast cancer.

Herbal medicine, as the major remedy in traditional medical systems, has been in medical practice for thousands of years. Considering the importance of plants as a source of medicine even today dried powdered leaves of *Annona squamosa* Linn[3,4] and *Ficus racemosa* Linn[5,6] have been chosen which are in use traditionally for the treatment of many ailments.[7,8] However, they have been less explored plants for their varying characteristic analysis, hence an effort has been made here to evaluate the invitro antiobesity activity by inhibiting porcine pancreatic lipase enzyme and anticancer activity by inhibiting human lung cancer cell lines (A-549) and human metastatic breast cancer cell lines (MDA-MB-435S) with XTT assay using ethanolic and hexane extracts of leaves of *Annona squamosa* Linn and *Ficus racemosa* Linn.

MATERIALS AND METHODS

Collection of plant sample

The fresh mature leaves of *Annona squamosa* L. and *Ficus racemosa* L. were collected from Indira Gandhi Zoological Park, Visakhapatnam, washed under tap water and shade dried for about 3-4 weeks at room temperature. The dried leaves were coarse powdered in a mixer grinder, sieved and stored in air tight container for study.

Preparation of extract

The powdered leaves were weighed, packed in the soxhlet column and extracted with ethanol and hexane at elevated temperatures by Soxhlet extraction method.[9] Then the extracted liquid was concentrated by using distillation apparatus for a period of 2-3 hours and then air dried over night at room temperature. The percentage yield of crude extracts of *Annona squamosa* L. was 23.08% and 07.59%, *Ficus racemosa* L. was 13.19% and 04.61% by using ethanol and hexane solvents respectively. The extracts were labeled, stored in stock vials and kept in refrigerator for further studies.

Pancreatic lipase inhibition assay

Inhibition of lipase by ethanolic and hexane extracts of dried powder leaves of *Annona squamosa* L. and *Ficus racemosa* L. was determined by using a modified assay method.[10,11] Triolein (1% v/v) was added to Tween 40 (1% v/v) in 0.1M phosphate buffer (pH8) to make a suspension and was then emulsified. The assay was performed by adding 800µL of the triolein emulsion to 200µL of porcine pancreatic lipase (0.5gm pancreatin in 15mL of 0.1M phosphate buffer, pH8) and 200µL of plant extract. The contents were mixed and absorbance measured immediately at 450nm and designated as T_0. The test tubes containing mixture were incubated at 37°C for 30 minutes and at the end of incubation, the absorbance was recorded at 450nm and designated as T_{30}. The variation in the absorbance = $A_{450}(T_0)$ - $A_{450}(T_{30})$ was calculated for both control and treatment. The percentage inhibition was calculated using the following formula.

Invitro anticancer activity

Human lung cancer cell lines (A-549) and human metastatic breast cancer cell lines (MDA-MB-435S) used in this study were procured from National Center for Cell Science, Pune. All cell lines were grown and maintained in a Thermo scientific sterile humidified incubator at 37°C and 5% CO_2 in a GIBCO's modified eagles medium (MEM, GIBCO), supplemented with 4.5g/L glucose, 2mM L-glutamine and 5% fetal bovine serum (FBS) together with the pH of 7.2.

The XTT cell proliferation assay was first described by 1988 by Scudiero., et.al as an effective method to measure cell growth and drug sensitivity in tumor cell lines.[12,13,14] It was modified and used to determine the inhibitory effects of the given extracts on cell growth in-vitro. The trypsinised cells from T-25 flask were seeded in each well of 96-well bottomed tissue culture plate at a density of $5x10^3$ cells/well in growth medium and cultured at 37°C in 5% CO_2 to adhere. After 24hr incubation, the supernatant was discarded and the cells were pretreated with growth medium and were subsequently mixed with different concentration of test compounds (12.5, 25, 50, 100 and 200 µg/mL) in triplicates to achieve a final volume of 100µL and then cultured for 48hr. The compound was prepared as 1.0mg/mL concentration stock solutions in DMSO. Culture medium and solvent were used as controls. Each well then received 50µL of fresh XTT (0.9% mg/mL in RPMI along with XTT activator reagent) followed by incubation for two hours at 37°C. At the end of the incubation was shacked the 96 micro well plate for fifteen seconds. The optical density of the culture plate was read at a

wavelength of 490nm (reference absorbance at a wavelength of 630nm) on an ELISA reader, Anthos 2020 spectrophotometer.

Statistical analysis

Results were expressed as Mean±SD, (n=3). Statistical analysis was carried out using Dunnett's Multiple Comparison Test in Graph pad Prism 5 software. All the results were statistically significant with $p<0.05$ compared with the standard drug.

RESULTS

Pancreatic lipase inhibitory activity

Ethanolic and hexane extracts of dried powder leaves of *Annona squamosa* L. and *Ficus racemosa* L. are tested for porcine pancreatic lipase inhibitory activity and the results have been tabulated in Table 1. The inhibitory property of the extracts are compared using Orlistat as the reference standard, the hexane extracts exhibited highest inhibitory activity and are found to be concentration dependent. (Figure 1)

Invitro anticancer activity

Screening of ethanolic and hexane extracts of dried powder leaves of *Annona squamosa* L. and *Ficus racemosa* L. with XTT assay resulted in moderate anticancer activities against A-549 and MDA-MB-435S cell lines and the results have been tabulated in table 2 and 3. The inhibitory properties of these extracts are compared with reference standard Cisplatin. Hexane extract of *Ficus racemosa* L. and ethanolic extract of *Annona squamosa* L. show better inhibition against A549 and MDA-MB-435S cell lines respectively and the results are found to be dose dependent. (Figure 2 & 3)

Table 1: Percentage inhibition of pancreatic lipase enzyme by ethanolic and hexane extracts of dried powder leaves of *Annona squamosa* L. and *Ficus racemosa* L.

% inhibition of Lipase enzyme					
		Ethanol extracts		Hexane extracts	
Conc. (µg/mL)	Std. drug (Orlistat)	*Annona squamosa* L	*Ficus racemosa* L	*Annona squamosa* L	*Ficus racemosa* L
3.125	19.93±1.10	45.92±0.77	8.52±0.59	51.15±0.81**	83.14±0.26**
6.25	30.13±1.62	52.22±1.11	9.15±0.22	82.14±1.12**	86.92±0.28**
12.5	49.00±1.00	60.51±0.24	18.79±0.37	89.95±0.28**	91.15±0.96**
25	58.63±1.18	62.80±0.41	40.04±0.72	91.80±0.62**	94.55±0.70**
50	65.10±0.85	66.33±0.99	57.79±0.85	98.37±0.15**	96.75±0.41**
100	77.56±1.43	80.16±0.88	66.09±0.15	98.71±0.26**	97.85±0.24**

Values expressed as Mean±SD (n=3). SD=Standard deviation, statistically significant with $^{**}p<0.01$ compared with standard drug.

Table 2: Percentage inhibition of A-549 cell lines by ethanolic and hexane extracts of dried powder leaves of *Annona squamosa* L. and *Ficus racemosa* L.

	% inhibition of A-549 cell lines				
		Ethanol extracts		Hexane extracts	
Conc. (µg/mL)	Std. drug (Cisplatin)	*Annona squamosa*.L	*Ficus racemosa*.L	*Annona squamosa*.L	*Ficus racemosa*.L
12.5	16.68±1.05	02.36±0.46***	04.29±0.34***	04.83±0.50***	09.92±0.50**
25	52.08±1.62	02.80±0.32***	08.82±0.25***	04.71±0.89***	13.42±0.37**
50	77.05±2.40	06.71±0.51***	13.20±0.32***	06.11±0.58***	14.38±0.51**
100	85.35±0.94	11.06±1.30***	13.79±0.53***	10.34±0.58***	15.45±0.45**
200	95.97±1.62	14.74±0.57***	18.60±0.66***	16.07±0.92***	47.83±0.27**

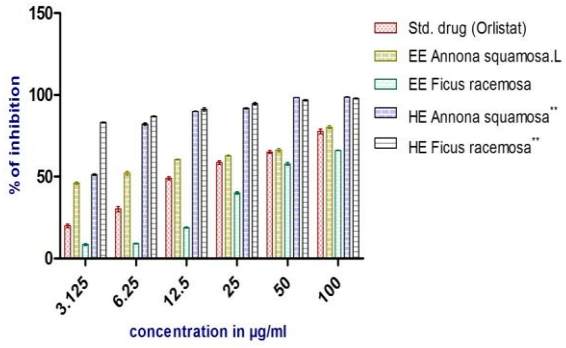
Values expressed as Mean±SD (n=3). SD=Standard deviation, statistically significant with $^{**}p<0.01$, $^{***}p<0.001$ compared with standard drug.

Table 3: Percentage inhibition of MDA-MB-435S cell lines by ethanolic and hexane extracts of dried powder leaves of *Annona squamosa* L. and *Ficus racemosa* L.

	% inhibition of MDA-MB-435S cell lines				
		Ethanol extracts		Hexane extracts	
Conc. (µg/mL)	Std. drug (Cisplatin)	*Annona squamosa*.L	*Ficus racemosa*.L	*Annona squamosa*.L	*Ficus racemosa*.L
12.5	07.77±0.14	15.25±0.65	06.46±0.36**	04.69±0.32**	06.26±0.21**
25	24.64±0.40	21.72±0.39	10.68±0.49**	08.86±0.41**	09.14±0.49**
50	37.70±0.45	24.31±0.48	12.10±0.28**	10.00±0.38**	12.71±0.65**
100	47.77±0.47	28.82±0.60	14.67±0.47**	14.70±0.39**	15.28±0.29**
200	60.45±0.66	28.53±0.35	16.94±0.23**	16.60±0.53**	18.79±0.51**

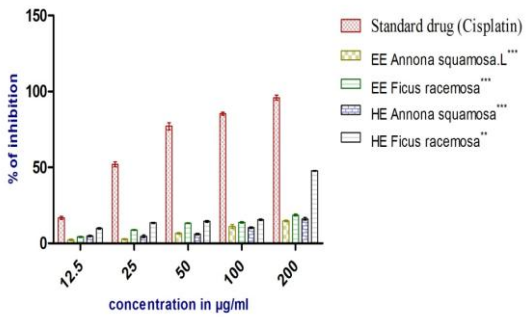
Values expressed as Mean±SD (n=3). SD=Standard deviation, statistically significant with $^{**}p<0.01$ compared with standard drug.

Fig. 1: Effect of plant extracts on lipase enzyme



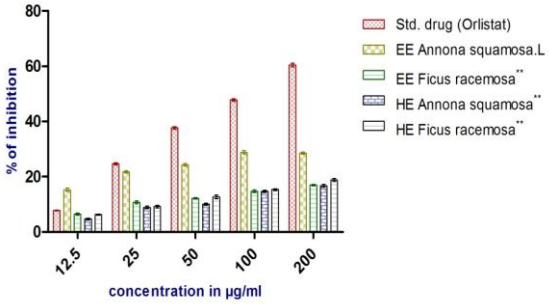
EE - Ethanol Extract, HE - Hexane Extract

Fig. 2: Effect of plant extracts on A-549 cell lines



EE - Ethanol Extract, HE - Hexane Extract

Fig. 3: Effect of plant extracts on MDA-MB-435S cell lines



EE - Ethanol Extract, HE - Hexane Extract

DISCUSSION

Traditionally *Annona squamosa* L. and *Ficus racemosa* L. has been used in the treatment of many diseases[7,8] and contain a vast amount of phytochemical constituents.[4] An effort has been made here to investigate the potential uses of ethanol and hexane extracts of leaves of these plants.

Estimation of percentage inhibition of lipase enzyme using standard method by conducting study at different concentrations (3.125, 6.25, 12.5, 25, 50, and 100µg/mL) shows that hexane extracts of both the plant leaves have very high lipase inhibition property to that of the standard drug compared at same concentrations. Cytotoxic analysis was conducted at different concentrations (12.5, 25, 50, 100, and 200 µg/mL) and the results shows that ethanol extract of *Annona squamosa* L. has anticancer property against Human metastatic breast cancer cell lines (MDA-MB-435S) and hexane extracts of *Ficus racemosa* L. shows high anticancer property against Human lung cancer cell lines (A-549).

CONCLUSION

In this study it has been concluded that the hexane extracts of dried powder leaves of *Annona squamosa* L. and *Ficus racemosa* L. possess lipase inhibitory property which may attribute to anti-obesity activity and the screening studies revealed that the hexane extract of *Ficus racemosa* L. and ethanolic extract of *Annona squamosa* L. are found to be active against A-549 and MDA-MB-435S cell lines respectively. Further investigation is needed to reveal the important chemical constituents and their mechanism responsible for growth suppression and cell death of cancer cell lines. The present findings of this study support that these plants have potential activities which can be used in the treatment of many ailments.

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